Protein Kinase Involvement in Wild-Type and Mutant Calcium-Sensing Receptor Signalling

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Life Sciences

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ABRREVIATIONS

I.	PTH	Parathyroid hormone
II.	PTHrP	Parathyroid hormone-related protein
III.	$Ca^{2+}i$	Intracellular calcium concentration
IV.	$Ca^{2+}o$	Extracellular calcium concentration
V.	HEK	Human embryonic kidney
VI.	MDCK	Madin-Darby canine kidney
VII.	GPCR	G protein-coupled receptor
VIII.	CaR	Calcium-sensing receptor
IX.	mGluR	Metabotropic glutamate receptor
X.	IP_3R	Phosphatidylinositol 1,4,5-triphosphate receptor
XI.	PTH1R	Parathyroid hormone 1 receptor
XII.	IGF1R	Insulin-like growth factor 1 receptor
XIII.	mChR	Muscarinic receptor
XIV.	EGFR	Epidermal growth factor receptor
XV.	VFD	Venus fly-trap domain
XVI.	ICD	Intracellular domain
XVII.	ECD	Extracellular domain
XVIII.	TMD	Transmembrane domain
XIX.	ADH	Autosomal dominant hypercalcaemia
XX.	FHH	Familial hypercalcaemia hypocalciuria
XXI.	NSHPT	Neonatal severe hyperparathyroidism
XXII.	AH	Autoimmune hypoparathyroidism
XXIII.	PKC	Protein kinase C
XXIV.	PKA	Protein kinase A
XXV.	PLC	Phospholipase C
XXVI.	DAG	Diacylglycerol
XXVII.	PMA	Phorbol 12-myristate 13-acetate
XXVIII.	PIP_2	Phosphatidylinositol 4,5-biphosphate
XXIX.	AA	Arachidonic acid
XXX.	20-HETE	Hydroxyeicosatetranoic acid
XXXI.	ATP	Adenosine triphosphate

XXXII.	siRNA	Small interference ribonucleic acid
XXXIII.	PP2A	Protein phosphatase 2A
XXXIV.	PP1	Protein phosphatase 1
XXXV.	HEK-293	Human embryonic kidney 293
XXXVI.	PS	Phosphatidylserine
XXXVII.	nPKC	Novel protein kinase C
XXXVIII.	cPKC	Conventional protein kinase C
XXXIX.	aPKC	Atypical protein kinase C
XL.	PKM	Protein kinase M
XLI.	IP_3	Phosphatidylinositol 1,4,5-triphosphate
XLII.	P70S6K	P70 S6Kinase
XLIII.	AUC	Area under curve
XLIV.	pBF	Potential bias factor
XLV.	GFX	GF102803X
XLVI.	FSK	Forskolin
XLVII.	mRNA	Messenger ribonucleic acid
XLVIII.	ARE	Adenylate-uridylate-rich elements
XLIX.	AUF1	Adenylate-uridylate-rich binding factor 1
L.	UTR	Untranslated region
LI.	KSRP	K homology-type splicing regulatory protein
LII.	cAMP	Cyclic adenosine monophosphate
LIII.	ERK	Extracellular signal-regulated kinase
LIV.	MAPK	Mitogen-activated protein kinase
LV.	PAMs	Positive allosteric modulstors
LVI.	SNV	Single nucleotide variation
LVII.	AP2	Adapter protein 2
LVIII.	ADIS	Agonist-driven insertion signalling
LIX.	ROCK	Rho-associated protein kinase
LX.	DMSO	Dimethyl sulphoxide
LXI.	cTAL	Cortical thick ascending limb
LXII.	P1NP	Procollagen 1 intact N-terminal
LXIII.	PIP	Proximal Interphalangeal
LXIV.	CTX-1	C-terminal telopeptide type 1

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ABSTRACT

The calcium-sensing receptor (CaR) is a G-protein coupled receptor that controls mammalian extracellular calcium (Ca^{2+}_o) homeostasis. CaR downstream signalling involves intracellular calcium (Ca^{2+}_i) mobilisation which can be negatively modulated by protein kinase C (PKC)-mediated phosphorylation of CaR residue Thr-888 (CaR^{T888}). The nature of this regulation was investigated here using siRNA-based knockdown of individual PKC isotypes. Knocking down PKCα expression increased CaR-induced Ca^{2+}_i mobilisation in CaR-HEK cells, significantly lowering the EC_{50} for Ca^{2+}_o relative to control siRNA-transfected cells. In accordance, PKCα knockdown also decreased CaR^{T888} phosphorylation which also permitted the triggering of Ca^{2+}_i mobilisation in CaR-HEK cells at sub-threshold Ca^{2+}_o concentrations. Interestingly, PKCε knockdown attenuated CaR-induced Ca^{2+}_i mobilisation in CaR-HEK cells, significantly increasing the EC_{50} for Ca^{2+}_o. However, this knockdown was also also found to inhibit CaR^{T888} phosphorylation and this is the first time that CaR^{T888} phosphorylation has been shown to be dissociate from Ca^{2+}_i mobilisation. The results show the complexity of the interactions that potentially underlie the CaR's pleiotropic signalling and provides novel targets for examining signal bias.

Classically an increase in cAMP is known to trigger PTH secretion. The observation in this study shows that raising intracellular cAMP levels with forskolin also decreased CaR^{T888} phosphorylation permitting increased Ca^{2+}_i mobilisation. This suggests that cAMP may stimulate the phosphatase (most likely protein phosphatase 2A (PP2A)). Nevertheless, knocking down $G\alpha_{12}$, which has been shown to activate PP2A, resulted in increased CaR^{T888} phosphorylation and lower Ca^{2+}_i mobilisation (increased EC_{50} for Ca^{2+}_o). This suggests the possibility of CaR as a cAMP sensor that can detect an increase in intracellular cAMP in order to stop PTH serection.

Three novel CaR effectors, P70 ribosamal protein S6 kinase, insulin-like growth factor receptor-1 and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, were identified in CaR-HEK cells. It was shown that a) high Ca^{2+}_o stimulated the activation of these effectors and b) each effector was inhibited by knockdown of PKCα and $G\alpha_{12}$, which further confirmed the association of these signals with CaR. These data show that CaR also plays an important role outside Ca^{2+}_o homeostasis, such as growth and inflammation.

Finally, five CaR mutations associated with autosomal dominant hypocalcaemia (ADH) were found to increase Ca^{2+}_o-induced Ca^{2+}_i mobilisation, as well as ERK and $p38^{MAPK}$ activation, when transfected stably in HEK-293 cells. Cotreatment with the calcilytic NPSP795 inhibited ERK and $p38^{MAPK}$ phosphorylation in all 5 gain-of-function mutants and in the wild type CaR cells, with IC_{50}s for the compound in the nanomolar range. These data highlight the potential utility of CaR negative allosteric modulators in the treatment of gain-of-function CaR mutations. Together these data enhance our understanding of CaR^{T888} phosphorylation and CaR signalling.

DECLARATION

No portion of the work in this doctoral thesis has been submitted in any previous application of degree or other qualification at the University of Manchester or any other university or institute.

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CHAPTER 1

Introduction

1.0 INTRODUCTION

Calcium plays a fundamental signalling, functional and structural role in many physiological processes. Calcium is a messenger in complex intracellular systems that mediates a wide range of biological processes. It acts as a crucial intracellular messenger in the release of neurotransmitters from neurons, the contraction of cardiac, smooth and skeletal muscle and also in egg fertilisation and apoptosis. In addition, many enzymes require calcium as a cofactor; for example, tenase and pro-thrombinase in the blood clotting cascade both require calcium ions for activation. Furthermore, calcium also plays a crucial structural role in the body providing strength to bones and indeed 99% of the total human body content of calcium is present in bone mineral. Given calcium's physiological importance, it is clear therefore that the accurate maintenance of mammalian calcium homeostasis is a crucial process. Any change in blood calcium concentration, resulting in hypercalcaemia or hypocalcaemia, needs to be sensed and carefully returned back to normal. The concentration of free extracellular calcium ions (Ca^{2+}_o) must remain constant at ~1.2 mM whereas the cytosolic concentration is usually 10-100 nM (Brown *et al*., 1998). In order to regulate calcium homeostasis, the extracellular calcium-sensing receptor (CaR) is required to detect changes in Ca^{2+}_o concentration. In response to high Ca^{2+}_o concentration, this receptor couples with a series of Guanine nucleotide binding proteins (G-protein) to trigger downstream signals such as the formation of inositol trisphosphate (IP_3), induction of intracellular calcium (Ca^{2+}_i) mobilisation and activation of protein kinase C (PKC) so as to initiate the processes necessary to bring the Ca^{2+}_o concentration back to normal (Conigrave and Ward, 2013). This CaR is expressed endogenously in parathyroid cells that secrete the Ca^{2+}_o-regulating hormone, parathyroid hormone (PTH). The main role of PTH is to increase blood Ca^{2+}_o concentration by acting upon effector organs such as kidney, intestine, and bone. Under conditions of high Ca^{2+}_o concentration, PTH secretion is suppressed and PTH expression becomes downregulated by the cells. Such responses depend upon the Ca^{2+}_o-sensing ability of the parathyroid gland and thus the action of the CaR. However, the mechanism by which the CaR regulates the secretion of PTH remains unclear.

1.1 Parathyroid hormone

1.1.1 Discovery of parathyroid hormone

The existence of parathyroid gland in humans was first noted by Ivor Sandström in 1880 and their functional importance became clearer a decade later when their removal in cats, dogs and rodents was shown to cause death from tetany (Potts, 2005). Later, Jacob Erdheim noted that parathyroid gland removal from humans also caused tetany (Potts, 2005). MacCallum and co-workers then suggested that the function of parathyroid gland is to control blood calcium. They demonstrated that tetany could be prevented by infusing calcium into the blood or via oral administration of large doses of calcium lactate (Potts, 2005). However, this conclusion was initially controversial since parenterally-administered parathyroid gland extract did not reverse the tetany (Potts, 2005). However in 1925 Collip, using a new extraction method, demonstrated that an acid extract of parathyroid gland could relieve tetany and he established that parathyroid gland is an endocrine gland that secretes parathyroid hormone (Collip, 1925).

1.1.2 Structure of parathyroid hormone

The structure of PTH was determined in 1970, first for bovine (Niall *et al*., 1970; Brewer and Ronan, 1970) and then in 1974 for human PTH (Niall *et al*., 1974). Human PTH contains 84 amino acids in its mature form and has a molecular weight of 9.5 kDa (Torres, 2006). Its amino acid sequence is highly conserved amongst mammals, for example, the deduced amino acid of feline PTH is 84% identical with human, with 88% identity in the N-terminal region (Barber, 2004). There exist two functional fragments of PTH which are either N-terminal (amino acids 1-34) or C-terminal. Without the N-terminal region, C-PTH fragments had been thought of as non-functional peptides. However, this C-PTH fragment has been shown to be involved in the regulation of alkaline phosphatase, osteocalcin, collagen ∝1(I), and insulin growth factor binding protein-5 in rat and human osteoblast-like cells (Monier-Faugere *et al*., 2001).

Although secreted as an 84 amino acid polypeptide, PTH is actually formed as a 115 amino acid preproPTH peptide (Figure 1.1). Post-translational cleavage of this

preproPTH results in the mature, secreted PTH. Specifically, the 'pre' region is thought to be involved in transportation of the protein to the endoplasmic reticulum. The 25 amino acids in the N-terminal region are then cleaved in the endoplasmic reticulum to produce proPTH. Finally, this peptide is transported to the Golgi apparatus where the N-terminal hexapeptide of proPTH is removed, resulting in the formation of PTH (Habener *et al*., 1978). This hormone is then packed in constitutive vehicles or immature secretory granules to be transported outside the cells (Muresan and MacGregor, 1994).

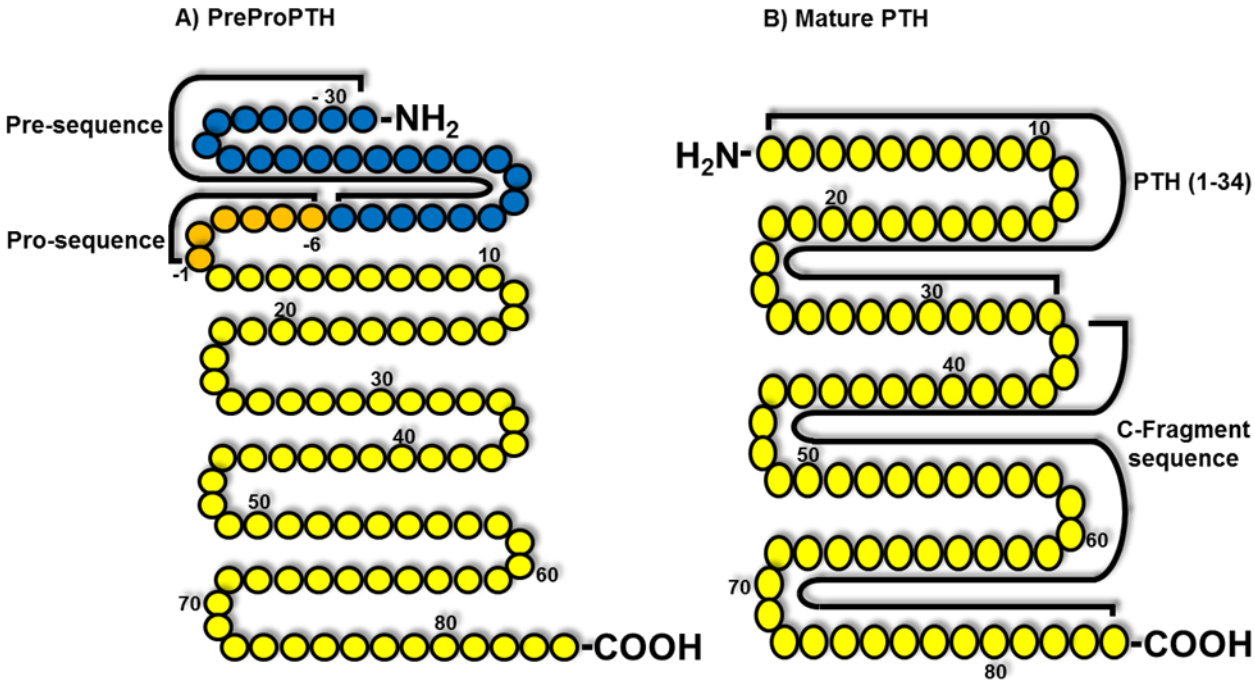

Figure 1.1: The primary structures of human prepro-parathyroid hormone and matured parathyroid hormone.

1.1.3 Function of parathyroid hormone

The main function of PTH is as a calciotropic hormone to maintain extracellular calcium homeostasis, primarily by increasing blood Ca^{2+}_{o} concentration during hypocalcaemia. Classically, the actions of this hormone involve the kidney and skeleton and are mediated through a G protein-coupled receptor, called PTH/PTHrP receptor (PTH1R) that is abundantly expressed in both major target tissues of PTH action (Saini *et al*., 2013).

In kidney, PTH regulates the renal handling of calcium, phosphate, sodium, and hydrogen ions (Bisello and Friedman, 2008) and was originally characterised as a phosphaturic substance. Approximately 90% of plasma phosphate is filtered by the glomerulus and 80% is actively reabsorbed primarily by the proximal convoluted tubules (Bisello and Friedman, 2008). PTH inhibits the entry of phosphate across the luminal membrane of proximal tubule cells which is the rate-limiting step in phosphate reabsorption. Next, PTH also stimulates renal calcium reabsorption by inducing transcellular calcium transport across epithelial cells in the cortical segment of the thick ascending loop of Henle and in the distal convoluted tubule (Bisello and Friedman, 2008). In the distal tubule cells, the stimulation of the PTH1R activates cAMP/protein kinase A (PKA) signalling and phospholipase D which then leads to the hyperpolarisation of membrane potential that stimulates both the entry of calcium across the apical membrane as well as its extrusion via sodium/calcium exchange across the basolateral membrane (Singh *et al*., 2005).

Moreover, PTH also regulates the conversion of 25-hydroxyvitamin vitamin D_3 ($25(OH)D_3$) to the active form of the hormone, $1,25(OH)_2D_3$ in kidney (Bisello and Friedman, 2008). This $1,25(OH)_2D_3$ stimulates calcium absorption from the gut and activates bone cell activity. PTH enhances $1,25(OH)_2D_3$ production by increasing 1α-hydroxylase activity via upregulation of the 1α-hydroxylase gene expression as well as by increasing enzymatic activity (Bisello and Friedman, 2008). Upregulation of the 1α-hydroxylase gene appears to be mediated via cAMP, while the enhanced enzyme activity may result from protein kinase C (PKC) signalling (Bisello and Friedman, 2008). Furthermore, PTH is also found to inhibit the degradation of $1,25(OH)_2D_3$ by decreasing 24-hydroxylase activity in the proximal tubule (Matsumoto *et al*., 1985). Apart from that,

sodium-hydrogen ion exchange in the proximal tubule is also modulated by PTH. This hormone inhibits the acid secretion and leads to a mild compensated hyperchloraemic acidosis (Seaton and Cohen, 1988). The activation of PTH1R by PTH induces the activation of PKA pathways, which then phosphorylates the sodium-hydrogen ion exchanger (Zhao *et al*., 1999).

In bone, PTH increases turnover, where the net effect of PTH depends upon the pattern of PTH administration. Continuous exposure of bone to PTH causes net bone catabolism whereas intermittent exposure to PTH causes net bone anabolism (Nissenson and Juppner, 2008). These effects of PTH on bone can be seen in states of hyperparathyroidism and in the therapeutic treatment of osteoporosis respectively. Bone resorption is due to an increase in the numbers and activity of osteoclasts, which can be stimulated by PTH (Nissenson and Juppner, 2008). However, these cells do not bear parathyroid receptor (PTHR1), thus their stimulation largely depends on the result of crosstalk between cells in the osteoblast lineage, which contains PTH1R, and osteoclasts.

1.1.4 Regulation of parathyroid hormone secretion

In the presence of decreased blood calcium levels, the parathyroid gland will respond in a manner that reflects the duration, rapidity and magnitude of the hypocalcaemic stress (Brown, 2000). For short duration hypocalcaemia, the parathyroid gland will release PTH stored in the secretory vesicles. This occurs rapidly i.e. within seconds, following the initiation of the hypocalcaemic stress and it can continue for 80-90 minutes until depletion of the vesicle stores (Brown, 2000). This PTH-Ca^{2+}_o interaction has been described by Brown (2000) as a steep, inverse sigmodial relationship (Figure 1.2). There are four notable components to the curve, namely the maximal PTH secretion at low Ca^{2+}_o concentration (*A*), the slope of the curve at PTH secretion's midpoint (*B*), the midpoint of the curve (*C*) and also the minimal PTH secretion at high Ca^{2+}_o concentration (*D*). Using this curve, the sensitivity of the parathyroid gland towards changes in Ca^{2+}_o concentration can be determined by the steepness of the slope and the position of the "calcium set point" (Caligara *et al*., 1996). The "set point" here is defined as the calcium concentration that produces half of the maximum inhibition of PTH secretion (Barber, 2004). An increase in the set point will shift the slope to the right and

reduce the steepness of the slope thus causing a reduction in parathyroid gland sensitivity to Ca^{2+}_o concentration (Valle *et al*., 2008). A decrease in the steepness of this slope value has been observed in patients suffering calcium-related diseases such as primary and secondary hyperparathyroidism (Malberti *et al*., 1999).

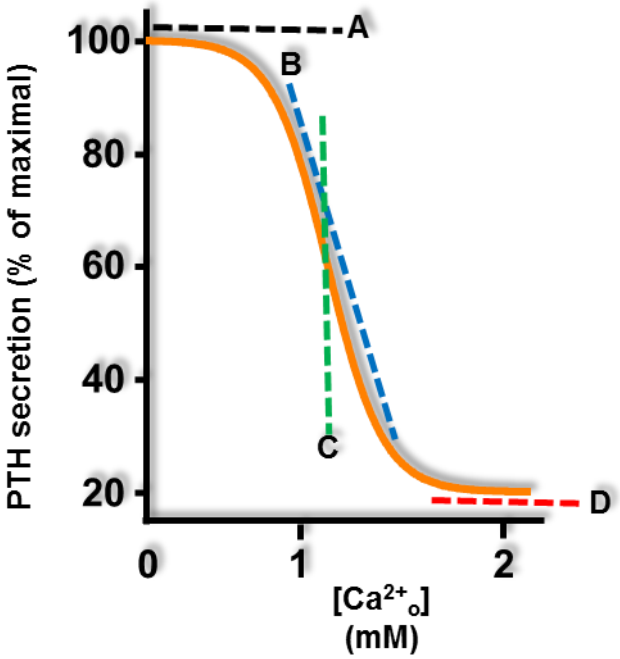

Figure 1.2: Four parameter model of the inverse sigmoidal curve relating Ca^{2+}_o and PTH release. (Adapted from Brown, 1983)

Besides Ca^{2+}_o concentration, the response of parathyroid gland also depends on the rate and direction of change of Ca^{2+}_o (De Chistofaro *et al*., 2001). A rapid drop in Ca^{2+}_o concentration will cause the parathyroid gland to secrete PTH more vigorously. This response is known as "rate-dependence" which allows the body to maintain calcium homeostasis when the Ca^{2+}_o concentration in the serum falls rapidly (Silverberg and Bilezikian, 2001). However, when there is a slow change in Ca^{2+}_o concentration, the PTH secretion is not dependent on the rate but Ca^{2+}_o concentration *per se* (Grant *et al*., 1990). In addition, the secretion of PTH also depends on the direction of change in light of the hysteresis concept, whereby the PTH level is higher as the Ca^{2+}_o concentration is falling but lower as it rises (Kwan *et al*., 1993). This response is important in Ca^{2+}_o homeostasis to avoid excess or undersecretion of PTH, since the gland can ensure higher PTH levels when it is needed (Brown, 2000).

Following a longer duration of hypocalcaemia (20-30 minutes), chief cells of the parathyroid gland will react by reducing the intracellular degradation of PTH resulting in the secretion of larger, intact PTH (1-84) (Brown, 2000). The degradation of PTH will result in the production of NH_2-truncated PTH peptide fragments that have been thought traditionally to be biologically inactive for maintaining Ca^{2+}_o homeostasis (Friedman and Goodman, 2006). More prolonged hypocalcaemia (several hours to a day) then will lead to the increase of production and stability of PTH mRNA in the cells. The high production of PTH mRNA is accompanied by the increase of the cell's biosynthetic capacity which means some modification to the cellular organelles such as an enlarged endoplasmic reticulum and Golgi apparatus (Brown, 2000). The high stability of mRNA can result from underactivation of the calcium-sensing receptor (Galitzer *et al*., 2009) which will be discussed later. If the hypocalcaemia lasts for more than a day, then cells will increase their proliferative activity to increase their number and thus subsequently produce higher amounts of PTH (Brown, 2000). Parathyroid cells normally exhibit a low level of mitosis but this can be increased under conditions of low Ca^{2+}_o concentration (Naveh-Many *et al*., 1995). The cells also have a slow rate of cell gain and cell loss and clinical problems arise when initiation of cell replication causes overproduction of PTH (Silver *et al*., 1991).

1.1.5 Control of parathyroid hormone secretion by the extracellular calcium-sensing receptor

In order to maintain Ca^{2+}_o homeostasis, secretion of PTH needs to be controlled and for this the calcium-sensing receptor (CaR) is employed. CaR has been shown to be expressed in the parathyroid gland (Brown *et al*., 1993) and also in other tissues not related to calcium homeostasis suggesting that the receptor plays roles in many cell systems (Kos *et al*., 2003). In parathyroid cells, the activation of CaR by an increase of Ca^{2+}_o concentration initiates a series of signalling pathways that lead to the suppression on PTH secretion (Drüeke, 2004). The way in which cells respond to increasing Ca^{2+}_o concentration vary with exposure time and the size of the increase. Following CaR activation, parathyroid cells react by suppressing PTH secretion, inhibiting transcription of the PTH gene, and ultimately, by decreasing cell proliferation (Brown, 2000).

The elevation of Ca^{2+}_o concentration activates CaR causing the activation of the coupled G-proteins, such as $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ (Ward, 2004). The coupling of CaR to $G\alpha_{q/11}$ induces the activation of phospholipase C (PLC) which then further activates protein kinase C (PKC). The activation of PKC sequentially activates the MAPK cascade that contains serine/threonine-specific protein kinases such as the extracellular signal-regulated kinases ERK1 and ERK2, the cJun NH2-terminal kinases and the p38-MAP kinases (Drüeke, 2004). Previously, ERK1 and ERK2 have been shown to activate cytoplasmic phospholipase A_2 ($cPLA_2$) in the membrane phospholipids to stimulate the release of arachidonic acid (AA) which can then be metabolised to hydroxyperoxyeicosatetranoic acid and hydroxyeicosatetranoic acid (20-HETE). These two active mediators can suppress the release of PTH (Bourdeau *et al*., 1998, Bourdeau *et al*., 1992). Apart from that, CaR also couples to $G\alpha_{i/o}$ (Tamir *et al*., 1996; Arthur *et al*., 1997) that inhibits adenyl cyclase which ordinarily converts intracellular ATP to cAMP (Lin and Chen, 1989). Intracellular cAMP drives the PTH secretion pathway in parathyroid cells (Chang *et al*., 1998), however, neither the source of the cAMP nor its downstream mechanism of action are fully understood.

Extracellular calcium also affects the post-transcriptional stability of PTH mRNA. High levels of PTH have been observed in hypocalcaemic rats due to a low rate of PTH mRNA degradation arising from the binding of *trans*-acting proteins to mRNA (Moallem *et al*., 1998). PTH cDNA consists of three exon regions which are exon 1, 2, and 3 that encode the 5'-untranslated region (5'-UTR), prepro region of PTH and the structural hormone together with the 3'-UTR respectively (Kemper, 1986). The 3'-UTR of PTH mRNA consists of 293 nucleotides out of 712 of the full length mRNA and is 42% conserved between humans and rats (Kemper, 1986). These 3'-UTR regions also contain *cis*-acting AU-rich elements (ARE) where the *trans*-acting proteins will interact to stabilise the RNA (Kilav *et al*., 2001). Two *trans*-acting proteins have been identified recently, namely AU-rich binding factor 1 (AUF1) and Upstream of N-*ras* (Unr) (Sela-Brown *et al*., 2000; Dinur *et al*., 2006). To study how this *trans*-acting protein regulates mRNA stability, Kilav *et al*. (2004) have studied the structure of 3'-UTR and the result showed that it has unstable secondary structure which can be stabilised by the binding of *trans*-acting proteins in the absence of calcium. However, the mechanism of RNA degradation (i.e. by exonucleolytic enzymes or self-degradation) in the absence of *trans*-

acting protein is still unclear (Kilav *et al*., 2004). In addition, the study by Galitzer *et al*. (2009), who examined HEK-293 cells cotransfected with CaR and PTH, showed that under conditions of high Ca^{2+}_o concentration, the binding of *trans*-acting protein to 3'-UTR of PTH was replaced by binding of a destabilising protein known as KH-type splicing regulatory protein (KSRP). How calcium acts to induce KSRP-mediated destabilisation of PTH mRNA remains to be explained. Galitzer *et al*. (2009) did at least confirm that the effect of Ca^{2+}_o (in the HEK cell model) is CaR-mediated since no effect of Ca^{2+}_o was detected in non-CaR expressing cells, while the CaR-selective calcimimetic agent R568 (section 1.2.3.2) mimicked the effect of Ca^{2+}_o in CaR-expressing cells.

The CaR has also been reported to be linked with modulation of parathyroid cell proliferation. A study on parathyroid adenoma from secondary hyperparathyroidism due to chronic kidney disease used immunohistochemical methods to show that in diseased tissue, CaR expression was reduced by 30 to 70% or more (Kifor *et al*., 1996). This data suggests a possible role for CaR in suppressing parathyroid cell proliferation such that the absence of the CaR results in increased gland size. However, the exact mechanism by which CaR regulates parathyroid cell proliferation remains controversial. Parathyroid hormone gene regulatory region-cyclin D1 (*PTH-cyclin D1*) has been suggested to possibly serve as a regulator of Ca^{2+}_o-dependent parathyroid cell proliferation (Bianchi *et al*., 1994). However, no expression of *PTH-cyclin D1* was detected in the parathyroid glands of patients with hyperparathyroidism secondary to chronic renal insufficiency (CRI) that have abnormally increased PT cell proliferation (hyperplasia) (Tominaga *et al*., 1996). Imanishi *et al*. (2001), using a mouse model of primary hyperparathyroidism showed that overexpression of *PTH-cyclin D1* oncogene can drive increased parathyroid cell proliferation. These authors also suggested that the overexpression of *PTH-cyclin D1* increased the calcium-PTH set point by decreasing the CaR expression in the parathyroid tissue of *PTH-cyclin D1* transgenic mice.

CaR is also important in regulating Ca^{2+} resorption in the kidney. CaR is abundantly expressed on the basolateral membrane in the Thick Ascending Limb of Henle's Loop (TAL) (Riccardi *et al*., 1998). The activation of CaR in TAL inhibits Ca^{2+} reabsorption by the renal outer medullary potassium K^+ (ROMK) channel thus attenuating the recycling of K^+ into the lumen of the TAL (Wang *et al*., 1996). This action dampens the

generation of the driving force for paracellular Ca^{2+} transport (Hebert, 1999). Apart from that, CaR is also present in the apical region of the proximal tubule (Riccardi *et al*., 1998). The experiment carried out on proximal tubule-derived cell line showed that the presence of high Ca^{2+} inhibited 1α-Hydrolase activity in the cells (Maiti and Beckman, 2007). This 1α-Hydrolase is crucial for the 1, 25(OH) $_{2D3}$ production in the kidney (Maiti and Beckman, 2007). In hypocalcaemic conditions, $1,25(OH)_2D_3$ enhances the Ca^{2+} resorption in intestine and Distal Covulated Tubule of the kidney (DCT) (Johnson and Kumar, 1994). In accordance with the above study, Egbuna *et al*. (2009) have shown that the ablation of CaR gene in murine model dampens the calcaemic response of $1,25(OH)_2D_3$ independently of PTH action. These show that CaR is also regulating the Ca^{2+} resorption by controlling $1,25(OH)_2D_3$ production.

1.2 Calcium-sensing receptor structure and pharmacology

1.2.1 Structure

CaR is expressed endogenously in all tissues that are involved in calcium homeostasis (Brown and McLeod, 2001). It is a G protein coupled receptor (GPCR) that belongs to family C. CaR has a high degree of amino acid sequence similarity among species, for example, CaR from human, rat, and rabbit are more than 90% identical to that of bovine CaR (Bai, 2004). The amino acid sequence of the receptor can be classified into three principal domains, namely the ~600 residues of the extracellular domain (ECD), ~250 residues of transmembrane domain (TMD) containing the ``serpentine", seven membrane-spanning helices of the CaR, and, the ~200 residues of the C-terminal tail or intracellular domain (ICD) (Garrett *et al*., 1995; Chattopadhyay, 2000; Brown *et al*., 2000; Figure 1.3).

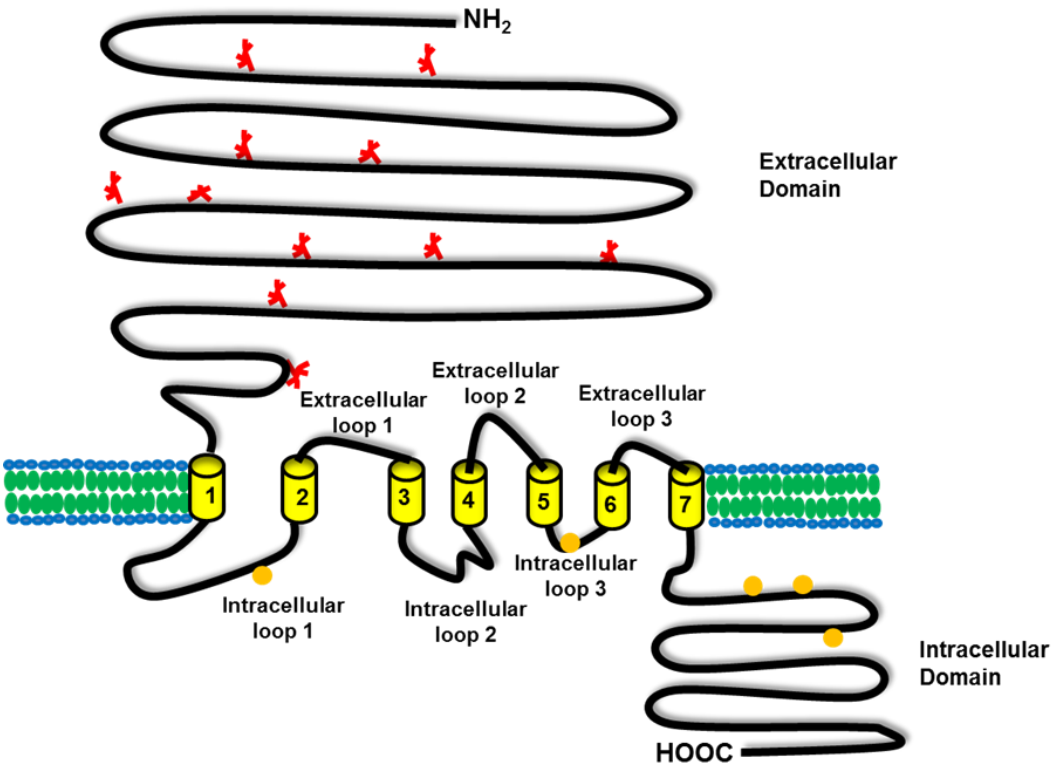

Figure 1.3. Schematic representation of the structure of the calcium-sensing receptor. 1–7, Membrane-spanning regions. The approximate locations of glycosylation (red) and phosphorylation (orange) sites are shown (Adapted from Brown, 2000).

The ECD of CaR contains sites that are important for Ca^{2+} binding, glycosylation and dimerisation (Bai, 2004). This ECD is thought to have a bilobed venus-flytrap structure similar to bacterial periplasmic binding proteins (Bai, 2004). It consists of two large lobes that are tethered by two peptides and each lobe contains Ca^{2+}-binding sites (Chang and Shoback, 2004; Silve *et al*., 2005; Huang *et al*., 2007). According to the Chang and Shoback (2004) CaR activation model, these lobes of ECD will be open in the absence of bound ligands resulting in stabilisation of the inactive state of the receptor. In such a state, the carboxyl (–COO−) groups of acidic residues and/or the hydroxyl (–OH) groups of serines and threonines in the CaR ECD sites are shielded by H_2O via hydrogen bonding. When the Ca^{2+}_o concentration increases, this ion will replace H_2O and chelate two or more electron donors at different sites on the receptor and close the lobes to activate receptor signalling. Previously, Huang *et al*. (2007) have predicted that three binding Ca^{2+} sites exist in ECD that consist of Glu224, Glu228, Glu229, Glu231 and Glu232 in binding site 1, Glu378, Glu379, Thr396, Asp398 and Glu399 in binding site 2 and Ser147, Ser170, Asp190, Tyr218 and Glu297 in binding site 3 (Huang *et al*., 2007). They performed site-directed mutagenesis on E224I and E228I/E229I in binding site 1, E378I/E379I and E398I/E399I in binding site 2 and E297I in binding site 3 and found that these mutations led to significant alterations in either maximal CaR response and/or sensitivity (Huang *et al*., 2007).

The CaR ECD also includes 20-40 kDa of either high mannose or complex carbohydrates (Ray *et al*., 1998). The detection of the CaR in lysates of CaR-transfected human embryonic kidney (HEK293) cells and parathyroid cells has revealed the presence of at least 3 CaR-specific immunoreactive bands between 120 and 200 kDa and additional bands of higher molecular mass (~350 kDa) (Bai *et al*., 1998a; Ward *et al*., 1998). The three major species of these bands are 120 kDa band representing non-glycosylated CaR (seen in kidney though rarely in CaR-HEK cells), and 140 and 160 kDa bands representing glycosylated CaR with high mannose (immature) or complex carbohydrate (mature) respectively. This glycosylation is believed to be important for cell surface expression of the CaR as opposed to its activity *per se* (Fan *et al*., 1997; Ray *et al*., 1998). A study using site-directed mutagenesis of the CaR, showed that 8 of 11 predicted N-glycosylation sites are efficiently glycosylated and that disruption of four to five of these sites reduced cell-surface expression by 50–90% (Hebert *et al*., 1997).

The CaR exists on the cell surface as a dimer. Apart from the three major bands mentioned above, CaR immunoreactivity on western blots often includes bands >200 kDa that represent the dimeric form of the receptor (Bai *et al*., 1998a; Ward *et al.*, 1998). This intermolecular binding of CaR monomers occurs at two locations: a) via covalent, disulphide binding of residues Cys-129 and Cys-131 in the ECD (Brown *et al*., 2001; Zhang *et al*., 2001) and b) via hydrophobic interactions in the TMD. In addition, it has been suggested that additional hydrophobic interactions may occur between Leu-112 and Leu-156 at the putative dimer interface (Bai, 2004). Receptor dimerisation has been suggested to have functional importance for CaR activity since mutation of Cys-129 and Cys-131, or other adjacent residues (either in transfected cells or naturally in humans), tend to result in gain-of-function changes to CaR (Bai, 2004; Hendy *et al*., 2009; see Section 1.3.2). This suggests that dimerisation, or at least the disulphide interactions necessary for dimerisation, may actually reduce the agonist sensitivity of the CaR in order to help achieve the physiological Ca^{2+}_o set point.

The heterodimerisation of a wild-type CaR monomer with a monomer harbouring an FHH mutation can interfere with the function of the resulting dimeric receptor (Tfelt-Hansen and Brown, 2005). Interestingly however, heterodimerisation with two CaR mutant monomers, where the mutation was in the ECD in one but in the ICD of the other monomer, permitted reconstitution of the CaR activity despite the inactivity of each mutant when transfected independently (Hebert *et al*., 2008). This result showed that the CaR requires at least one functional ECD and one functional ICD though but not necessarily on the same CaR monomer.

The ICD is pivotal for CaR signalling/efficacy since the amino acid residues in the intracellular loop 2 and 3 are key to determining the selectivity of CaR coupling to G-proteins (Tfelt-Hansen and Brown, 2005) in most GPCRs. To date, two amino acids residues within intracellular loop 2 (Phe-706 and Leu-703) and eight residues in intracellular loop 3 have been shown to be important for activating phospholipase C, the major pathway of CaR intracellular signalling (Chang *et al*., 2000). Among the eight residues at loop 3, Leu-797 and Phe-801 were shown to be most critical to the activation of PLC. Besides that, there are other sites in the ICD that are also important in mediating the effect of protein kinase C (PKC). PKC is known to inhibit CaR signalling by

phosphorylating one or more serine or threonine residues, with PKC consensus sequences existing in loop two (1), loop three (1) and at least three in the ICD (Brown, 2008). In contrast, protein phosphatase A (PP2A) has been shown to be able to reverse the inhibitory effect of PKC phosphorylation by dephosphorylating the amino acid residue Thr-888 (McCormick *et al*., 2010) and explained in detail in Section 1.6.3.

The ICD is embedded in the cytoplasm and begins with Lys-863 (Garrett *et al*., 1995). The amino acid sequence of the proximal ICD is well conserved between human, bovine, rat, rabbit, mouse and chicken, however, the amino acid residues in the C-tail beyond position 946 are quite diverse (Bai, 2004). This finding suggests that it is the amino acid residues between 863 and 946 that are of greatest functional importance for CaR. The studies by Bai *et al*. (1997) show that truncation of residues between this position caused CaR loss-of- function.

Notably there are three PKC sites in the ICD that appear to be responsible for mediating the action of PKC and these are Thr-888, Ser-895, and Ser-915 (Bai *et al*., 1998b) and mutation of these PKC sites blocks the inhibitory effect of PKC on CaR (Bai, 2004). However, it has been shown that Thr-888 in particular is the major site for the inhibition of CaR signalling mediated by PKC activation (Bai *et al*., 1998b). However, even after all five of the known PKC sites in CaR were mutated there still remained ~ 30% of the PKC-inhibitable activity remaining suggesting that there may be other PKC sites in the CaR (Bai, 2004). The significance of CaR phosphorylation will be discussed in detail later (section 1.6.3).

1.2.2 Orthosteric CaR agonists

The CaR is considered a promiscuous receptor since it can be activated by a variety of divalent, trivalent and polyvalent cations and other pharmacological compounds. CaR agonists can be divided into two types, 1 and 2. Type 1, orthosteric CaR agonists are thought to bind at the same site on the receptor as Ca^{2+} does and are sufficient to activate the CaR on their own. These compounds include organic polycations and other inorganic cations and activate the receptor in the absence of $Ca^{2+}{}_o$ (Ward and Riccardi, 2012). As for type 2 CaR agonists, they do not compete for the same binding site with Ca^{2+} and

they tend to act allosterically (Nemeth, 2004). This type of agonist includes L-aromatic amino acids and glutathione and the calcimimetic drugs (Ward and Riccardi, 2012; see section 1.2.3).

As mentioned previously, CaR is also able to bind various inorganic cations. These cations can be subdivided approximately into three groups; i) strong agonists (Gd^{3+}, La^{3+} and Yt^{3+}) that have EC_{50}s in the submicromolar to micromolar range, ii) medium-strength agonists (Ca^{2+}, Ba^{2+}, Sr^{2+}, Cd^{2+} and Pb^{2+}) that have EC_{50}s in the submillimolar to millimolar range, and iii) weak agonists (Mg^{2+}, Ga^{3+}, Fe^{3+} and Na^{+}) that have EC_{50}s in the high millimolar range (Chang and Shoback, 2004). It is suggested that the individual potencies of these cations depend on their charge number and ionic radius (see Table 1). Based on the CaR activation model discussed previously, even Na^{+} has a size similar to Ca^{2+}, however it appears to have a negative effect on CaR since the receptor requires multivalent cations to bind to two lobes of CaR in order to make the Venus flytrap close to activate the receptor. Increasing Na^{+}_{o} concentration inhibits Ca^{2+}_{o} binding to CaR, however, Na^{+} itself is not able to chelate electrons at different sites on the receptor and close the lobes. On the other hand, the small radius of Mg^{2+} and Ga^{2+} decreases their potencies in activating CaR even though they contain multivalent charges (Chang and Shoback, 2004). The smaller cations have slow association and dissociation rates when interacting with electrons in the receptor (Levine *et al*., 1982). However, the potencies of these ions activating CaR are predicted to be increased in higher concentration (Chang and Shoback, 2004).

Agonists	**La^{3+}**	**Gd^{3+}**	**Yt^{3+}**	**Ca^{2+}**	**Ba^{2+}**	**Sr^{2+}**	**Cd^{2+}**	**Pb^{2+}**	**Mg^{2+}**	**Ga^{2+}**	**Fe^{3+}**	**Na^{+}**
Potency	+++	+++	+++	++	++	++	++	++	+	+/–	+/–	–
Charge no.	**3**	**3**	**3**	**2**	**2**	**2**	**2**	**2**	**3**	**3**	**3**	**1**
Radius (Å)a	**1.06**	**0.94**	**0.90**	**0.99**	**1.34**	**1.12**	**0.97**	**0.84**	**0.66**	**0.62**	**0.64**	**0.97**

Table 1.1: Chemical properties and potency of ionic CaR agonists. (adapted from Chang and Shoback, 2004)**.**

In addition, many organic polycations have been shown to activate CaR such as simple polyamines (spermine and spermidine), aminoglycoside antibiotics (neomycin and gentamicin), polyamino acids (poly-lysine), protamine and arylamine spider toxin (araxin) (Nemeth, 2008). The potencies of these organic polycations in activating CaR also depend on their charges, with the greater charges resulting in higher potency (Chang and Shoback, 2004). For example, among polyamines, the potency order is as follows; spermine > spermidine ≥ putrescine, since they have 4, 3, and 2 free charge amino acids respectively (Quinn *et al*., 1997). Similarly, the rank order of potency for the aminoglycosides i.e. neomycin > gentamicin > kanamycin corresponds to the number of their amino groups, i.e. 6, 5 and 4 respectively (McLarnon *et al*., 2002; Ward and Riccardi, 2012).

1.2.3 Allosteric CaR modulators

CaR positive allosteric modulators (PAMs) are compounds that are able to potentiate the receptor's activity by binding to non-orthosteric sites i.e. allosteric sites on the CaR (Saidak *et al*., 2009). Such allosteric modulation requires the binding of an orthosteric agonist to the receptor to produce its stimulatory effect (Saidak *et al*., 2009). Endogenous PAMs for CaR include the L-aromatic amino acids however it is the synthetic calcimimetics that are of greatest note given that Cinacalcet was the very first GPCR PAM to reach the market (Nemeth *et al*., 1998; Conigrave *et al*., 2000). Because PAMs bind at an allosteric site on CaR, these compounds do not compete with the endogenous agonist(s) since they do not bind to the orthosteric site. Also these PAMs enhance the endogenous physiological response, but do not evoke supra-maximal ones and are presumed not to activate *de novo* signalling pathways. In addition, they affect CaR activity when the Ca^{2+}_o concentration is at its EC_{50} value, but not at maximal or minimal Ca^{2+}_o concentrations (Ward and Riccardi, 2012).

1.2.4 L-Aromatic amino acids

The expression of CaR in epithelial cells raises the possibility that amino acids acting as CaR PAMs may play a physiological role in regulating CaR activity, for example in the stomach and small intestine where the amino acids are liberated from ingested protein (Chang and Shoback, 2004). The potencies of these amino acids activating CaR are ranked according to the steric conformation of their side-chains and not to their charges as organic polycations and inorganic cations are (Chang and Shoback, 2004). This observation suggests that amino acids do not bind to the orthosteric site of CaR. Besides that, the fact that amino acids failed to activate CaR in absence of Ca^{2+} (Zhang *et al*., 2002) also supports the idea that these amino acids are allosteric activators and bind elsewhere to the orthosteric site. The binding site for amino acids in CaR is located in the ECD (Wang *et al*., 2006). The α amino and α carboxyl portions of the ligands will form interactions with highly-conserved residues in this binding site (Wang *et al*., 2006). The rank of amino acids potencies is as follow; aromatic > neutral > acidic > basic >branched chain amino acids. The actions of the amino acids are also stereoselective where L-amino acids have been shown to be several fold more potent compared to D-amino acids (Conigrave *et al*., 2000). The effects of amino acids on CaR activation are in the high micromolar to low millimolar range depending on the amino acid types as well as on the ambient calcium concentration (Wang *et al*., 2006).

Glutathione is a tripeptide that contains glutamate, cysteine, and glycine in its structure. Because it is derived from amino acids, this might explain the ability of glutathione to activate CaR. Similar to amino acids, glutathione also acts as allosteric modulator of CaR and binds at the same binding site (Wang *et al*., 2006). However, glutathione is more potent compared to amino acids. This agonist displays effects on CaR in the submicromolar range (Wang *et al*., 2006). In the glutathione structure, a γ carboxyl group instead of α carboxyl group of glutamate is used to form bonds with the adjacent cysteine. This would allow α carboxyl group of glutamate, together with α amino group of glycine, to play their role to bind to the CaR site (Wang *et al*., 2006).

1.2.5 Calcimimetics and calcilytics

Sustained abnormalities in plasma PTH levels can seriously disturb plasma calcium levels leading to functional and pathological changes. Because of this, over the past 20 years scientists have been looking for drugs to alleviate these problems. Since the secretion of PTH is mainly regulated by CaR, then compounds that can affect the activity of this receptor are being employed in order to treat PTH-related diseases. These compounds are termed calcimimetics and calcilytics and they act as positive and negative CaR modulators respectively (Ward and Riccardi, 2012).

Calcimimetics, also known as type-2 CaR agonists (Siadak *et al*., 2009) are believed to increase CaR activity by stabilising the active form of the Ca^{2+}-bound receptor (Ward and Riccardi, 2012) via binding to the TMD, rather than the ECD (Petrel *et al*., 2004; Nemeth 2008). These compounds were actually being studied by Nemeth and coworkers even before the CaR had been identified at the molecular level (Ward and Riccardi, 2012). The first class of calcimimetics were discovered serendipitously when using phenylalkylamines derived from the calcium channel blocker verapamil to try and block Ca^{2+}_o sensitivity in parathyroid cells; the compounds actually increased Ca^{2+}_o responsiveness in the cells (Nagano, 2006; Ward and Riccardi, 2012). This lead to the discovery of NPS-R467 and R568 and then the drug Cinacalcet (sold as Mimpara in the EU). These calcimimetics are stereoselective, where their R-enantiomers are 10-100 fold more potent as CaR PAMs than their S-enantiomers (Nemeth, 2008). Furthermore they are relatively selective for the CaR, since they do not affect the activity of mGluRs or various other GPCRs at concentrations that activate CaR maximally (Nemeth, 2008). However, NPS R-568 was found to have off target effects at high concentrations, including induction of β adrenergic-dependent second messenger generation, an action that can be mimicked by high Ca^{2+}_o concentration (> 1mM) (Ward and Riccardi, 2012). Other calcimimetics in use as either research tools or potential clinical agents include Calindol, and AC 265347 (Petrel *et al*., 2004; Ma *et al*., 2011) and more recently a type-1 CaR agonist (i.e. orthosteric agonist) has been discovered called AMG416 (Velcalcetide) (Walter *et al*., 2013). It has recently been suggested that the peptide

AMG416 acts by binding covalently to the free cysteine residue at position 482 of the CaR ECD (Alexander *et al*., 2015).

In contrast, compounds that reduce the potency of physiological ligands towards CaR, known as negative allosteric modulators (NAMs), have also been identified and these are termed calcilytics. Early in their development, Nemeth and colleagues screened compounds and identified a weak potential amino alcohol antagonist, finding that it had off-target effects on β-adrenoceptor, the cardiac human ether-go-go related gene (hHERG) and cytochrome P450 2D6 (Nemeth, 2001). Subsequent modification of this compound resulted in the synthesis of NPS-2143 which has better oral availability and greater potency and specificity for CaR (Nemeth, 2001). Currently, several calcilytic drugs are in use as research tools and potential clinical agents and these include NPS-2143, NPS-89636, NPSP795, SB-751689, Calhex 231 and JTT-305 (Nemeth 2008; Balan *et al.*, 2009). Since calcilytics can increase PTH secretion by inhibiting CaR, they offered a potential benefit in the treatment of osteoporosis (Steddon and Cunningham, 2005). However to date, no calcilytic has performed sufficiently well in clinical trials to become a proven treatment for osteoporosis (Gowen *et al*., 2000; Hodsman *et al*., 2002; Caltabiano *et al*., 2013; John *et al*., 2014). To work it is believed that the calcilytics would need to be short acting such that they would elicit intermittent PTH secretion i.e. with an anabolic effect on bone, whereas if they act for too long then instead they will produce more of a hyperparathyroid state with bone catabolism (Nemeth, 2002; Hodsman *et al*., 2002; Caltabiano *et al*., 2013; John *et al*., 2014). However, there is still the prospect of the calcilytics being repurposed for use in hypocalcaemic diseases such autosomal dominant hypocalcaemia (ADH) as detailed in the next section. At the molecular level, the calcilytics also appear to bind to the TMD, indeed their binding site partially overlaps with the calcimimetic binding site (Miedlich *et al*., 2004).

Interestingly, in addition to the use of calcilytics for the treatment of mineral diseases, there is very recent evidence that they may also represent a potential therapy in asthma and other inflammatory lung diseases (Yarova *et al*., 2015). It should be noted that as part of that study I contributed the signalling data shown in Figure 2*E-F* of that publication, however this will not be discussed further in this thesis (see appendix I). Furthermore, it is also worth noting that low extracellular pH represents another NAM of the CaR as shown originally by Quinn *et al*. (2004). Recently, we have shown that even

modest, pathophysiologic changes in extracellular pH can cause detectable changes in CaR signalling and PTH secretion (Campion *et al*. 2015) and I also contributed Ca^{2+}_i imaging data to that publication (Figure 1*C-D*) but again this will not be discussed further in this thesis (see appendix II).

1.3 Pathologies of calcium-sensing receptor mutation and dysfunction

The malfunctioning of the CaR can cause several diseases. Since homeostasis of serum calcium is controlled by PTH secretion, which is in turn regulated by CaR, then abnormal activity of this receptor can lead to either hypercalcaemic or hypocalcaemic disorders. Broadly, hypocalcaemia is caused by increased CaR activity while hypercalcaemia results from decreased CaR activity. Examples of such conditions are set out below.

1.3.1. Hypercalcaemic disorders of CaR dysfunction

Hypercalcaemic disorders of CaR dysfunction are usually caused by inactivating mutations of the CaR (or of proteins required for its function). Examples of hypercalcaemic CaR disorders include neonatal severe hyperparathyroidism (NSHPT) and familial hypocalciuric hypercalcaemia (FHH). FHH is an autosomal dominant disease requiring just one abnormal parental gene for the child to inherit the disease (Thakker, 1998). Patients that suffer from this disease can experience a lifelong hypercalcaemia, though often without any obvious symptoms (Marx *et al*., 1981, Paterson *et al*., 1981). Hypercalcaemia is usually mild-to-moderate (~10% above normal level), however in some patients, higher serum calcium concentration increases have been observed (Thakker, 2004). Apart from that, these patients are also usually observed having mild hypermagnesaemia, normal or mildly elevated serum PTH concentrations and an inappropriately low urinary calcium excretion (calcium clearance to creatinine clearance ratio <0.01) (Thakker, 1998). Although commonly asymptomatic, FHH can result in chondrocalcinosis and acute pancreatitis (Marx *et al*., 1981; Davies *et al*., 1981; Heath, 1989). Most FHH patients that have been investigated have heterozygous inactivating CaR mutations and these mutations occur in all 3 of the domains of the CaR but tend to concentrate in the N-terminal ECD and in the ICD (Thakker, 2012; and

curated at http://www.casrdb.mcgill.ca/). The remainder have an abnormality in the promoter of the gene or a mutation at one of FHH loci (Thakker, 1998). There are three FHH loci locations which have been identified which are at chromosome 3q21.1 (where the CaR gene located), 19p13.3 and 19q13 (Janicic *et al*., 1995; Heath *et al*., 1993; Lloyd *et al*., 1999). The identification of FHH loci locations other than 3q21.1 suggested the existence of other associated proteins crucial for the regulation of calcium homeostasis (Thakker, 2004). The analysis of single nucleotide variant (SNV) on 19q13 of FHH patients revealed one non-synonymous SNV in exon 2 of AP2S1 gene that encodes AP2σ2 (Nesbit *et al*., 2013). The adapter protein 2 (AP2) is one component of clathrin-coated vesicles, which facilitates the endocytosis of GPCRs (Ohno, 2006). Adapter protein 2 is a heterotetramer that consists of four subunits, named α, β, σ, and μ (Collin *et al*., 2002). This adapter protein connects clathrin to the vesicle membrane and binds to a tyrosine and dileucine motif within cargo proteins (Kelly *et al*., 2008). This SNV in FHH patients caused missense mutations including R15C, R15H and R15L. These mutations disrupt the AP2 diluecine motif and thus impair the binding of AP2 to cargo proteins thus negatively affecting CaR endocytosis and recycling which decrease CaR sensitivity to Ca^{2+}_{o} (Nesbit *et al*., 2013). In addition, FHH has also been shown to be caused by mutation of the G-protein $G\alpha_{11}$ (Nesbit *et al*., 2013). This $G\alpha_{11}$ is encoded by the GNA11 gene located at 19q13. DNA analysis of the GNA11 gene from a kindred of FHH patients who did not possess CaR or AP2S1 gene mutations, revealed an in frame deletion of $G\alpha_{11}$ at I200 (Nesbit *et al*., 2013). This defect in Gα11 will therefore disrupt the downstream signalling of the CaR.

Neonatal severe hyperparathyroidism (NSHPT) is a more serious hypercalcaemic disorder than FHH. Babies born with this condition can suffer from severe hypercalcaemia, failure to thrive, bone demineralisation, multiple fractures and rib cage deformity which can threaten their lives (Hillman *et al*., 1964; Marx *et al*., 1982; Steinmann *et al*., 1984). This disease is considered as the phenotype of homozygous abnormal gene inheritance from (often consanguineous) FHH parents (Heath *et al*., 1989). The study by Ho *et al*. (1995) showed that the homozygous deletion (-/-) of CaR in mice results in a phenotype similar to that of human NSHPT. However, sporadic NSHPT patients have also been reported to have *de novo* heterozygous CaR mutations

(Raisz, 2003). Early surgical removal of the parathyroid glands is often required to avoid neonatal death or impaired neuronal development (Kos *et al*., 2003).

1.3.2 Hypocalcaemic disorders of CaR dysfunction

Activating CaR abnormalities tend to associated with hypocalcaemic disorders such as autosomal dominant hypocalcaemia (ADH) and autoimmune hypoparathyroidism (AH). Autosomal dominant hypocalcaemia (ADH) is in effect the opposite of FHH and can be passed to children with just one mutated CaR gene from its parents (Thakker, 2012). Patients living with this disease are regularly reported as having mild hypocalcaemia that can be asymptomatic but can be associated with carpo-pedal spasm and seizures especially in children (Pearce *et al*., 1996; Egbuna and Brown, 2008). Other potential features of this disease are both elevated serum phosphate and lowered serum magnesium concentrations (Pearce *et al*., 1996; Hendy *et al*., 2000). The biochemical features (hypocalcaemia, hyperphosphataemia and hypomagnesaemia) are consistent with hypoparathyroidism and pseudohypoparathyroidism; however, these hypocalcaemia with hypercalciuria patients have serum PTH concentrations that are generally detectable though below the normal range (Finegold *et al*., 1994; Perry *et al*., 1994; Baron *et al*., 1996). In contrast, serum PTH concentrations in some hypoparathyroidism and pseudohypoparathyroidism patients can be virtually undetectable (Thakker, 2004). Yamamoto *et al*. (2000) have also reported that patients with gain-of-function CaR mutations have higher urinary calcium/creatinine ratio compared to those having idiopathic hypoparathyroidism. This is due to the presence of CaR in the renal tubules where it normally limits excess calcium reabsorption. Moreover, some activating mutations of CaR can be associated with Bartter's syndrome type V resulting in hypokalaemic alkalosis, renal salt wasting, hyper-reninaemic hyperaldosteronism, increased urinary prostaglandin excretion, hypercalciuria, hypocalcaemia and hypomagnesaemia (Egbuna and Brown, 2008; Thakker, 2012).

There are more than 80 activating (i.e. gain-of-function) CaR mutations associated with ADH (curated at http://www.casrdb.mcgill.ca/). Most of these are missense mutations but several deletion mutations have also been found in CaR's intracellular tail (Cole *et al*., 2009). Most of these mutations tend to be clustered in the N-terminal third of the

ECD (Cole *et al*., 2009). It is thought that these gain-of-function mutations stabilise the receptor in its active conformation (Brown, 2008). For example, Watanabe *et al*., 2002 showed that the CaR^{C131Y} mutation increased CaR's sensitivity towards $Ca^{2+}{}_o$. CaR^{C131} is an important site for the intermolecular disulphide linkages binding CaR's dimers which may actually reduce CaR sensitivity to that required physiologically (Hendy *et al*., 2009). In addition, CaR mutations may also increase CaR sensitivity by enhancing the surface expression of the receptor. It has been noted that the CaR's signalling is enhanced by the increase of CaR insertion into plasma membrane (Grant *et al*., 2011). The study by Leach *et al*. (2013) showed that the surface expression of the gain-of-function CaR^{V836L} mutation was higher than for wild-type CaR (CaR^{WT}).

Gain-of-function CaR mutations could also increase CaR responsiveness by blocking an intracellular constraint that would otherwise impair CaR activation. For example, CaR^{T888M} is a mutation associated with ADH and functional characterisation of it *in vitro* found that it likely impairs negative feedback phosphorylation of the CaR (Lazarus *et al*., 2011) as detailed in Section 1.6.

In the current study, I characterised functionally five apparently activating CaR mutations, namely C131Y, Q245R, E228K, E228A, and A840V. Four of these mutations have previously been reported to result in ADH while the fifth, CaR^{E228K} has been linked to ADH in unpublished work. CaR^{C131Y}, CaR^{Q245R}, CaR^{E228K} and CaR^{E228A} are all located in the CaR ECD, while CaR^{A840V} is located in the seventh span of the CaR TMD (Figure 1.4).

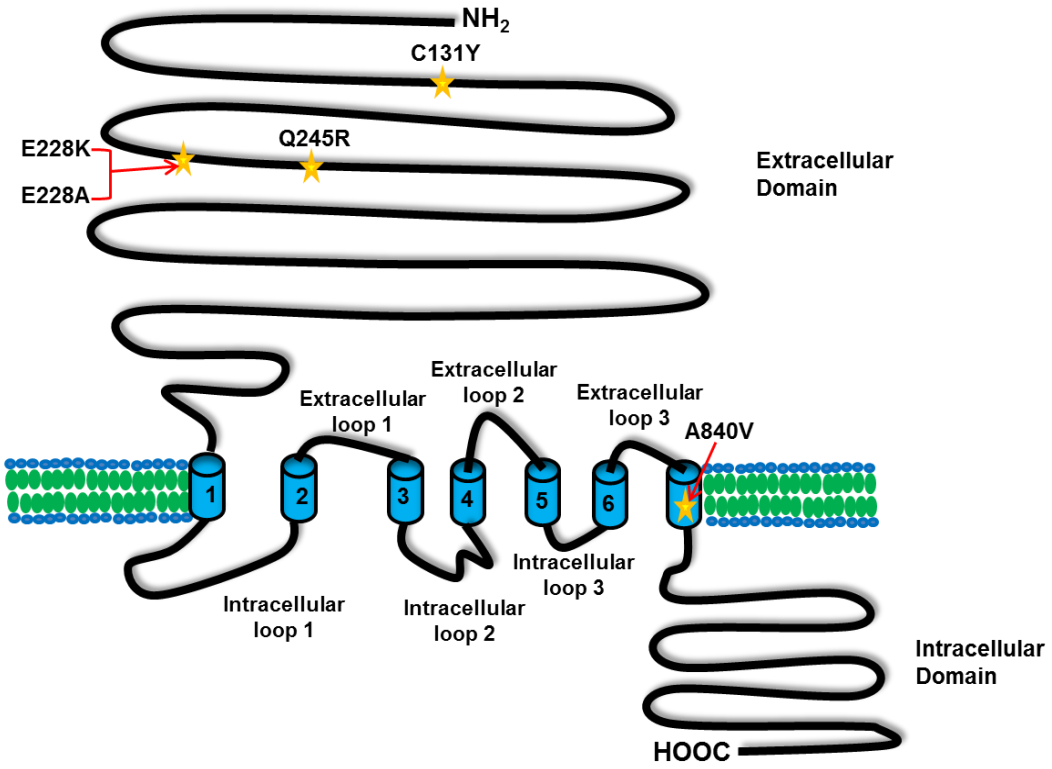

Figure 1.4. Locations of the CaR gain-of-function mutations studied here.

1.4 Calcium-sensing receptor signalling

1.4.1 Coupling of CaR to G proteins

The conformation of the CaR changes after binding to Ca^{2+}_o causing the activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins) and the initiation of further downstream signalling pathways. These G proteins belong to the GTPase family and comprise both large, heterotrimeric G proteins and small, monomeric G-proteins (Takai *et al*., 2001). The heterotrimeric G proteins tend to be activated directly by GPCRs while monomeric G-proteins such as rho and ras can be activated indirectly by GPCRs and by other membrane receptors (such as tyrosine kinase receptors) and intracellular signals (McCudden *et al*., 2005; Tuteja, 2009). The heterotrimeric G proteins consist of an alpha (α) subunit and tightly bound beta (β) and gamma (γ) subunits. There exist a number of large G-protein subtypes including $G\alpha_s$ (G stimulatory), $G\alpha_i$ (G inhibitory), $G\alpha_o$ (G other), $G\alpha_{q/11}$ and $G\alpha_{12/13}$. Following GPCR stimulation, a guanine nucleotide exchange factor (GEF) swaps GDP for GTP on the G_α subunit. The binding of GTP instead of GDP causes the α subunit to dissociate from the βγ subunit and the GPCR. Both G_α and $G_{\beta\gamma}$ can then activate other signalling pathways respectively (Tuteja, 2009).

The CaR is known to stimulate the activation of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ in parathyroid cells (Conigrave and Ward, 2013). The activation of CaR elicits the $G\alpha_{q/11}$-mediated activation of phospholipase C (PLC) with the subsequent intracellular release of inositol trisphosphate (IP_3). The central role of $G\alpha_{q/11}$ signalling in the CaR-mediated regulation of PTH secretion has been most clearly demonstrated in two studies, one in mice (Wettschureck *et al*., 2007) and the other in man (Nesbit *et al*., 2013) which shows that altering activity of $G\alpha_{q/11}$ modulates PTH release and Ca^{2+}_o homeostasis. In the mouse study, a PT-specific knockout of $G\alpha_q$ on a $G\alpha_{11}$-null background resulted in increased PTH secretion and blood calcium levels (Wettschureck *et al*., 2007) similar to the effect of CaR knockout in mice (Ho *et al*., 1995; Chang *et al*., 2008). Then in humans, some loss-of-function Gα11 mutations produced a phenotype similar to FHH (CaR loss-of-function) while some gain-of-function $G\alpha_{11}$ mutations resulted in an ADH (CaR gain-of-function) phenotype (Nesbit *et al*., 2013).

In addition, CaR activation causes suppression of cAMP accumulation in the cells, suggesting the possible involvement of $G\alpha_{i/o}$, however the effect of pertussis toxin on this response in parathyroid cells remains controversial (Brown *et al*., 1992; Brown and McLeod, 2001). That is, it remains possible that the suppression of cAMP levels might occur by a mechanism independent of, or at least in addition to, $G\alpha_{i/o}$ activation.

It is clear, particularly from work in HEK-293 cells, that CaR can exert pleiotropic actions via multiple G-proteins, further including $G\alpha_{12/13}$ (Ward, 2004; Huang *et al*., 2004; Rey *et al*., 2005). The activation of $G\alpha_{12/13}$ is commonly associated with Rho (a monomeric G protein) activation though some studies have suggested that Rho can also be activated by $G\alpha_i$ and $G\alpha_q$ (Burridge and Wennerberg, 2004; Huang *et al*., 2004). A study by Huang *et al*. (2004) showed that stimulation of CaR by exposure to high Ca^{2+}_o concentrations causes the activation of Rho-mediated phospholipase D in CaR-transfected MDCK cells, which can be blocked by overexpression of p115RhoGEF-RGS, a GTPase-activating protein that is specific for $G\alpha_{12/13}$. In addition, a study by Rey *et al*. (2005) showed that $G\alpha_{12}$, Rho and filamin A are involved in generating a transient Ca^{2+}_i oscillation when CaR stimulated by L-aromatic amino acids in CaR-transfected MCDK cells. In contrast, Davies *et al*. (2006) found that inhibition of Rho kinase using Y-27623 failed to affect the Ca^{2+}_i oscillations induced by Ca^{2+}_o in CaR-HEK cells however this does not rule out a role for $G_{12/13}$ in modulating Ca^{2+}_i responses via a distinct i.e. rho-independent pathway. Thus, it would be interesting to determine whether the CaR-mediated generation of Ca^{2+}_i oscillations does involve $G\alpha_{12/13}$. For instance, might $G\alpha_{12}$ activate protein phosphatase 2A (PP2A) by direct interaction to its Aα subunit (Zhu *et al*., 2004; Zhu et al., 2007) which might affect CaR phosphorylation and thus activity. Apart from that, the activation of $G\alpha_{12/13}$ also triggers the stimulation of adenylyl cyclase isoform-7 (Jiang *et al*., 2008). Thus it would also be interesting to know whether CaR-mediated $G\alpha_{12/13}$ activation could regulate cAMP production in CaR expressing cells and thus modulate CaR action that way (Gerbino *et al*., 2005).

Finally, CaR has even been shown to stimulate $G\alpha_s$ in some cells such as breast cancer cells, which secrete PTH-related protein (PTHrP) (Mamillapalli *et al*., 2008). Interestingly, CaR was found to preferably couple to $G\alpha_i$ in the normal mammary epithelial cells thus suggesting that the receptor switches its G-protein preference in

breast cancer cells. As a result, the CaR is capable of activating all of the main G-protein families in different cells and in different contexts and this pleiotropy could have significant consequences for its physiological and indeed pathological function.

1.4.2 CaR–induced phospholipase C activation and intracellular Ca^{2+} mobilisation

CaR stimulation results in the activation of one or more isoforms of phospholipase C (PLC). To date, approximately 10 mammalian PLC isozymes have been identified. They can be classified into three types, namely β (4), γ (2), and δ (4) (Lee and Rhee, 1995). Among the PLC isozymes, PLC-δ has the smallest molecular weight (85 kDa) compared to the other two. The observation that PLC-δ exists in eukaryotes with PLC-β and PLC-γ also existing in higher eukaryotes suggests that the β and γ subtypes evolved from PLC-δ (Rhee and Bae, 1997). This enzyme hydrolyses the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two intracellular products, which are diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Rhee, 2001). DAG and IP_3 then promote the activation of protein kinase C and release Ca^{2+} from intracellular stores respectively.

PLC contains two catalytic domains in all of its PLC isozymes, structures that are located after its pleckstrin homology (PH) domain. A PH domain is located in the NH_2-terminal region, preceding the catalytic domains. Besides that, it also contains an EF-hand, after the PH domain and a C2 domain after the catalytic domains, that serve to mediate its Ca^{2+}-dependent binding to lipid vesicles (Rhee and Bae, 1997). Many signalling proteins such as PLC contain PH and SH domains as in their structures. The function of the PH domain is to mediate the interaction between the enzyme with membrane surface by binding to PIP_2, while the SH domain mediates the interaction of the protein to other proteins.

The α-subunits of $G\alpha_{q/11}$ activate PLC-β but not PLC-γ or PLC-δ (Noh *et al*., 1995). In addition, PLC-β is also activated by the βγ subunits but with lower sensitivity than for the α-subunit (Jiang *at al*., 1996). As for PLC-γ, the binding of polypeptide growth factors such as epidermal growth factor to their receptor will activate protein tyrosine kinases (PTK) and these enzymes will then activate PLC-γ via tyrosine phosphorylation

(Berridge, 1993). However, Godwin and Soltoff (2002) have shown CaR-mediated activation of PLC-γ downstream of PLC-β in MC3T3-E1 osteoblasts. In contrast, the mechanism of activation of PLC-δ, and its potential relevance to CaR action remains unclear (Rhee and Bae, 1997). Therefore PLC-β is currently presumed to be the PLC of most interest in CaR signalling.

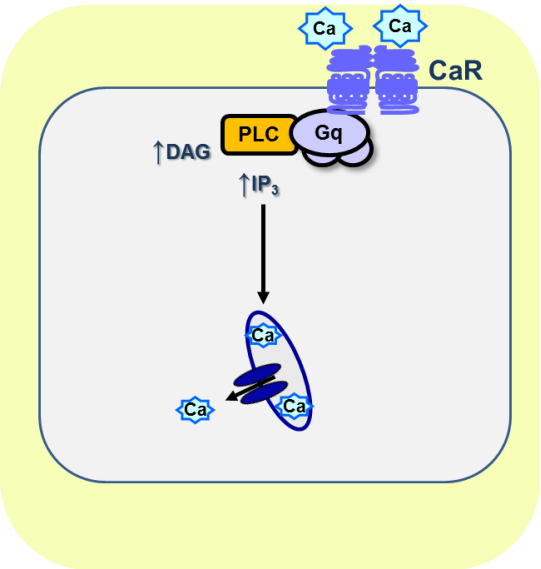

Figure 1.5: CaR–induced phospholipase C activation and intracellular Ca^{2+} mobilisation

1.4.3 Induction of intracellular calcium oscillations

The production of inositol trisphosphate (IP_3) by phospholipase C (PLC) triggers the release of calcium (Ca^{2+}_i) from the intracellular stores. IP_3, though produced at the plasma membrane, is highly diffusible and rapidly traverses the cytoplasm. This allows the IP_3 to diffuse to the cell interior that contains the IP_3 receptor on the sarco/endoplasmic reticulum (SR/ER). The binding of IP_3 to its receptor then causes the release of Ca^{2+}_i from stores into the cytoplasm (Bootman *et al.*, 2001). IP_3 is absolutely required for the IP_3 receptor to be opened, however, IP_3R activation is also affected by the cytoplasmic Ca^{2+} concentration. A modest increase in Ca^{2+}_i concentration (0.5-1 µM) will enhance IP_3R opening, whereas a higher concentration will inhibit it (Bootman *et al.*, 2001) and this is generally understood to represent the basis of Ca^{2+}_i oscillations. CaR-induced Ca^{2+}_i oscillations were first reported by Breitwieser and Gama (2001) using

CaR-HEK cells and this was confirmed by Young *et al*., (2002). The oscillation of Ca^{2+}_i is a potent activator for many pathways such as gene expression and MAPK pathways (Breitwieser, 2006). While the purpose of oscillatory Ca^{2+}_i signalling remains unclear, these oscillations are capable of providing more information to the cell than sustained plateau signals, since the magnitude and frequency of Ca^{2+}_i oscillations can encode more information than sustained Ca^{2+}_i (e.g. bone remodelling) (Berridge and Galione, 1988).

Besides the CaR driving IP_3–mediated Ca^{2+}_i mobilisation, it has also been suggested that the receptor can activate the influx of Ca^{2+} from outside of the cell through membrane-resident pathways (Breitwieser, 2006). Since basal Ca^{2+}_i levels can then be restored by the combined action of SERCA (Ca^{2+} reuptake to Ca^{2+}_i stores) and PMCA (Ca^{2+} extrusion to the cell exterior) then alternating influx and extrusion could also contribute to Ca^{2+}_i oscillation generation (Berridge *et al*., 2003).

Another mechanism proposed for explaining CaR-induced Ca^{2+}_i mobilisation concerns the phosphorylation status of the CaR ICD (Conigrave and Ward, 2013). Bai *et al* (1998b) first reported that the CaR contains five intracellular PKC sites (Thr^{646}, Ser^{794}, Thr^{888}, Ser^{895} and Ser^{915} as discussed in the previous chapter) and that PKC activation inhibits CaR-induced Ca^{2+}_i mobilisation while PKC inhibition tends to increase Ca^{2+}_i mobilisation. By mutating each of these five residues to non-phosphorylatable residues, Bai showed that CaR^{T888} plays the greatest role in modulating Ca^{2+}_i mobilisation since CaR^{T888V} gave the greatest left-shift in Ca^{2+}_o-induced Ca^{2+}_i mobilisation. In contrast, mutation of Thr^{646} and Ser^{794} was without effect. Young *et al* (2002) then showed that PKC inhibition tends to convert oscillatory Ca^{2+}_i mobilisation to sustained responses and that this effect can be mimicked by mutating CaR^{T888} to alanine. Data from the current laboratory then showed that CaR^{T888} can be rapidly phosphorylated following CaR activation and then rapidly dephosphorylated again by a calyculin-sensitive protein phosphatase, most likely PP2A (Davies *et al*., 2007; McCormick *et al*., 2010). Since these changes in CaR^{T888} phosphorylation can occur with a similar timescale to CaR-induced Ca^{2+}_i oscillations then there is the possibility that alternating cycles of CaR^{T888} phosphorylation and dephosphorylation may underlie CaR-induced Ca^{2+}_i oscillations. This mechanism will be described in greater detail in Section 1.6 as well as data demonstrating its functional relevance in whole body Ca^{2+} homeostasis.

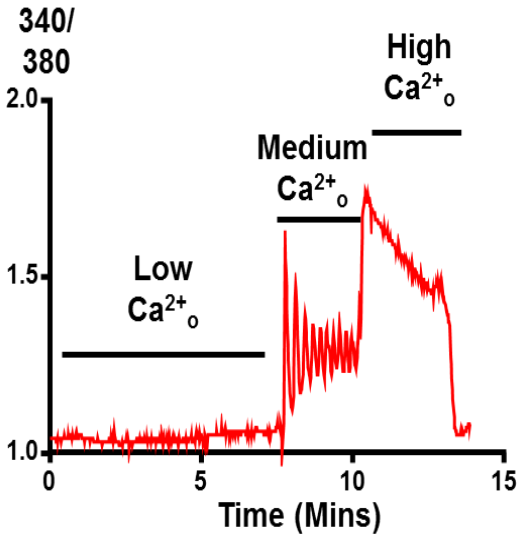

Figure 1.6: Typical CaR-induced intracellular mobilisation in CaR-HEK cells at low, medium and high Ca^{2+}_o.

1.4.4 CaR-mediated regulation of the actin cytoskeleton

CaR activation has been shown to alter the actin cytoskeleton of cells. Davies *et al.* (2006) showed that both the calcimimetic NPS R-467 and high Ca^{2+}_o or Mg^{2+}_o concentrations caused actin stress fibre assembly and cell process retraction. These effects were inhibited by blockade of Rho kinase signalling (with Y-27632 and H1152) and further unpublished data from this laboratory revealed that the Rho blocker C3 exoenzyme has the same effect on CaR-induced actin polymerisation. In addition to Rho, other proteins including β-arrestin, Arf nucleotide binding site opener (ARNO), ADP-ribosylating factor 6 (ARF6) and engulfment and cell motility protein (ELMO) have been implicated in inducing cytoskeletal re-organisation/membrane ruffling (Bouschet *et al*., 2007).

Interestingly, while inhibition of rho and/or rho kinase inhibits CaR-induced morphological change, these inhibitors do not affect CaR-induced Ca^{2+}_i mobilisation suggesting that the two pathways are separate. In addition, L-aromatic amino acids fail to alter CaR-HEK cell morphology, suggesting that they elicit distinct signalling from other CaR agonists (Davies *et al*., 2006). Similarly the L-aromatic amino acids only weakly potentiate CaR-induced ERK activation (Lee *et al*., 2007) despite having a much more

profound effect on CaR-induced Ca^{2+}_i mobilisation (Conigrave *et al*., 2000). Together, this is clear evidence of ligand bias whereby Ca^{2+}_o elicits robust Ca^{2+}_i mobilisation, ERK phosphorylation and morphological change in CaR-HEKs whereas L-aromatic amino acids are more selective in their responses (see Section 1.7 on signal "bias").

Regarding the possible functional significance of CaR-induced actin polymerisation, it has been suggested that this process might be important in inhibiting the exocytosis of PTH-containing vesicles. The exocytotic barrier function of conical actin has previously been demonstrated in endocrine cells such as adrenal chromaffin cells and pancreatic beta-cells and this mechanism can be regulated by extracellular signalling that causes actin polymerisation or depolymerisation (Burgoyne and Morgan, 2003). This exocytotic barrier function of conical actin was also suggested to exist in PT cells (Quinn *et al*., 2007). The filamentous actin-severing compounds, latrunculin and cytochalasin, were found to increase PTH secretion in dispersed PT cells while the actin polymerising agent, jasplakinolide, inhibited PTH secretion (Quinn *et al*., 2007). However, it should be noted that L-aromatic amino acids can suppress PTH secretion (Conigrave *et al*., 2004) and thus if they cannot induce CaR-mediated actin polymerisation (Davies *et al*., 2006) then this cytoskeletal exocytic blockade effect may not occur, at least not for all CaR ligands.

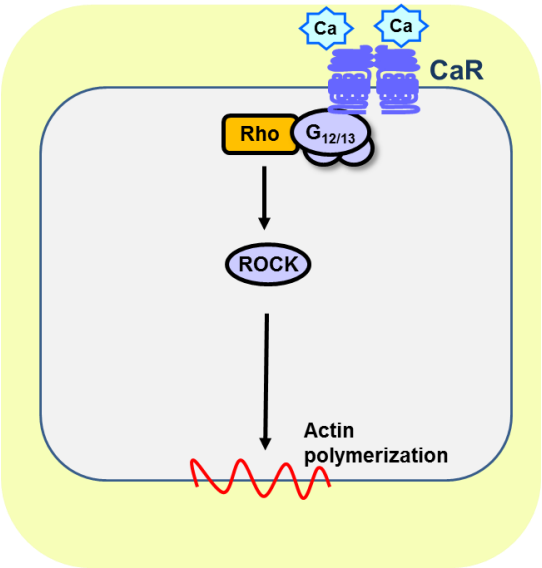

Figure 1.7: CaR-mediated regulation of the actin cytoskeleton

1.4.5 CaR-induced inhibition of adenylate cyclase

CaR activation is also associated with suppression of cAMP accumulation in parathyroid and CaR-HEK cells (Brown and Macleod, 2001). This is important since increased intracellular cAMP levels are associated with increased PTH secretion (Shoback *et al*., 1984). In parathyroid cells, the CaR-induced inhibition of cAMP accumulation can be attenuated by pertussis toxin in some laboratories which would suggest that the mechanism involves $G\alpha_{i/o}$-mediated inhibition of adenylate cyclase (Chen *et al*., 1989). However the action of pertussis toxin on PTH secretion remains controversial (Brown and Macleod, 2001). The inhibition of cAMP accumulation in tubules isolated from the medullary thick ascending limb (MTAL) of rat kidney is also pertussis toxin sensitive, but involves arachidonic acid production (Firsov *et al*., 1995). This shows that the inhibition of cAMP accumulation was through an indirect mechanism. It has been observed that the exposure of the tubules to arachidonic acid reduced the cAMP accumulation and that this response could be retarded by pertussis toxin (Firsov *et al*., 1995). Physiologically, the activation of CaR was also found to activate PLA_2 in PT cells, cTAL cells as well as CaR-HEK cells, which generates arachidonic acid production in cells (Ward *et al*., 2004). This suggests that these cells may also use indirect mechanisms to inhibit cAMP accumulation. However, the addition of arachidonic acid has no effect on the inhibition of cAMP accumulation by high Ca^{2+}_o thus suggesting that the inhibition involves $G\alpha_{i/o}$-mediated inhibition of adenylate cyclase (Brown and Macleod, 2001).

With regards a possible $G\alpha_{i/o}$-independent mechanism for suppressing PT cAMP levels, there is evidence that CaR may stimulate phosphodiesterase (PDE) isoform-1, which breaks down cAMP in response to elevated Ca^{2+}_i/calmodulin (Conigrave and Ward, 2013). Interestingly, as well as raised Ca^{2+}_i concentrations being associated with suppressed cAMP levels, there is evidence that agents that increase intracellular cAMP levels, such as forskolin and $G\alpha_s$-coupled GPCR agonists can enhance CaR-mediated Ca^{2+}_i mobilisation, at least in CaR-HEKs (Gerbino *et al*., 2005; Campion, 2013). These observations suggest crosstalk between these two signalling pathways. However, the mechanism by which cAMP enhances Ca^{2+}_i concentration is unclear. Clearly, cAMP

generation will lead to the activation of PKA (Petersen *et al*., 2008; Manni *et al*., 2008) and since the CaR contains two putative PKA sites at Ser-899 and Ser-900 (Garrett *et al*., 1995; Bai, 2004) then direct phosphorylation of the CaR by PKA may occur. However, unpublished data from this laboratory shows that the mutation of these two PKA sites to non-phosphorylatable residues fails to alter CaR-induced Ca^{2+}_i mobilisation, indeed PKA inhibitors also fail to act and thus cAMP's mechanism of action remains unknown and worthy of investigation.

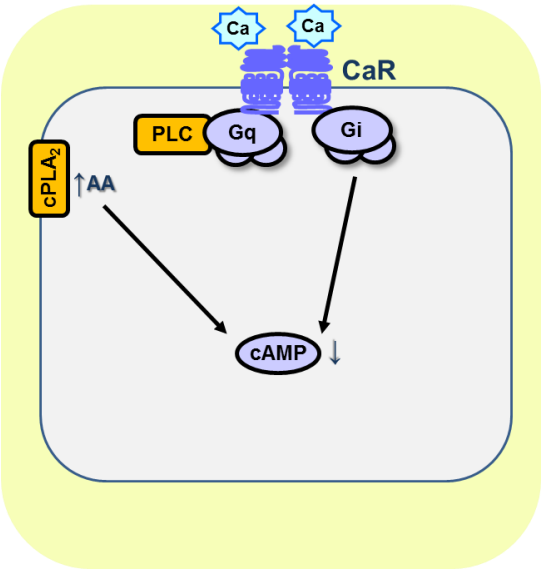

Figure 1.8: CaR-induced inhibition of adenylate cyclase

1.4.6 CaR-induced activation of mitogen-activated protein kinases

CaR activation is also known to trigger the activation of a number of mitogen-activated protein kinases (MAPKs) including the extracellular signal-regulated kinases (ERK1/2), p38 MAPK and the c-Jun NH2-terminal, stress-activated kinase (JNK) (Brennan and Conigrave, 2009). These MAPKs are activated through dual phosphorylation of their adjacent tyrosine and threonine residues (Brennan and Conigrave, 2009) and thus phospho-specific antibodies that detect such phosphorylation can be used as indirect activation assays.

Several mechanisms have been proposed for how the CaR induces ERK activation. The classic ERK activation pathway by receptor tyrosine kinases involves initial tyrosine phosphorylation followed by activation of the Ras-Raf-MEK-ERK pathway. In the case of CaR, the tyrosine kinase inhibitors, genistein and herbimycin inhibit CaR-mediated ERK activation in CaR-HEK cells (Kifor *et al*., 2001) and there is evidence that this may involve activation of the tyrosine kinase Src, at least in rat fibroblast (McNeil *et al*., 1998). Hobson *et al*. (2000) then showed that transfection of dominant negative Ras, Raf and MEK in rat ovarian surface epithelial cells each attenuate CaR-induced ERK1/2 activation, suggesting that CaR signals through the classic pathway Ras-Raf-MEK-ERK pathway. However, it have also been shown that the PLC pathway with its associated downstream PKC activation, may be involved in CaR-mediated ERK activation since treatment with the PLC inhibitor U-73122 and PKC inhibitor GF109203X each partially attenuate such a response while PKC stimulation with the phorbol PMA enhances ERK 1/2 activation in CaR-HEK cells (Kifor *et al*., 2001). Involvement of the PLC pathway would tend to implicate $G\alpha q_{/11}$ coupling, however pertussis toxin pretreatment also reduced ERK activation in CaR-HEK cells and together with U-73122 they produced a synergistic inhibitory effect (Kifor *et al*., 2001). These data suggest therefore that both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ contribute to CaR-mediated ERK activation and at some point such signals stimulate the Raf-MEK-ERK pathway. Intriguingly, ERK activation by CaR has also been linked to epidermal growth factor receptor (EGFR) activation (MacLeod *et al*., 2004). CaR activation was found to stimulate matrix metalloproteases on the plasma membrane which then cleaved pro-heparin bound EGF (HB-EGF) to release HB-EGF locally which then stimulates EGFR in an autocrine/paracrine manner. Treatment with either AG1478 (EGFR inhibitor) or GM6001 (matrix metalloproteases inhibitor) as well as antibodies of HB-EGF and EGFR inhibits, at least partially, CaR-induced stimulation of ERK (MacLeod *et al*., 2004). This recruitment of EGFR has been referred to as triple-pass signalling since the CaR signal enters the cell resulting in an extracellular signal which then re-enters the cell via EGFR.

Besides activating ERK, it is clear that CaR can also activate $p38^{MAPK}$. Indeed, not only does high $Ca^{2+}{}_o$ concentration elicit $p38^{MAPK}$ phosphorylation / activation in CaR-HEK cells but co treatment with the $p38^{MAPK}$ inhibitor SB-203580 attenuates high $Ca^{2+}{}_o$–induced PTHrP release from the cells (Tfelt-Hansen *et al*., 2003). Indeed CaR agonists

also increase $p38^{MAPK}$ phosphorylation in the mouse osteoblastic cell line, MC3T3-E1 (Yamaguchi *et al*., 2000), while Maiti *et al*. (2008) found that CaR activates $p38^{MAPK}$ to regulate vitamin D in proximal tubule human kidney epithelial cells. Next, CaR stimulation also triggers the activation of JNK as demonstrated in fibroblasts isolated from rat jaw cysts, which also resulted in enhanced cyclooxygenase-2 expression. This increase of CaR-mediated cyclooxygenase-2 expression could be attenuated by cotreatment with a JNK inhibitor (Otaga *et al*., 2006). In addition, Ca^{2+}_o-induced PTHrP secretion in H-500 cells has also been found to be partially inhibited by cotreatment with a JNK inhibitor (Tfelt-Hansen *et al*., 2003).

Finally, there are many hundreds of other protein kinases in the cell that may or may not be affected by CaR action. Previous unpublished experiments in this laboratory made use of a Bioplex® multiplex assay to examine the effect of CaR activity on multiple intracellular kinases in CaR-HEK cells. Those experiments provisionally identified three kinases that have not previously been reported to be downstream effectors of the CaR and they are P70 S6 kinase, IκBα and the IGF1R. The functions of these kinases will be detailed in Chapter 4.

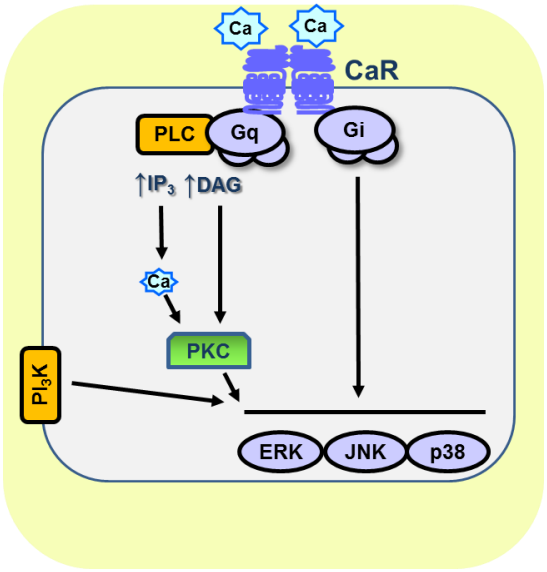

Figure 1.9: CaR-induced activation of mitogen-activated protein kinases

1.5 Protein kinase C

Protein kinase C (PKC) is a family of enzymes that regulates signal transduction cascades by phosphorylating the hydroxyl group of threonine and serine residues of other proteins (Newton, 1995). Protein kinase C is known to be important in modulating membrane structure, regulating transcription, immune responses, cell growth, and receptor desensitization (Newton, 1995). Protein kinase C is also a family of isomeric enzymes that contains three distinct subfamilies and where each isoform plays distinctive roles in cells. To date, at least 10 different PKC isoforms have been identified and they are classified into conventional (α, β, γ), novel (δ, ε, η, θ), and atypical (ζ, ι, λ) PKCs based on their structure and co-factor requirements (Martelli, *et al*., 2003). At least seven PKC isoforms are expressed in bovine PT cells and these include the conventional PKCs (cPKCs) α, β1, and β2; the novel PKCs (nPKC) ε and δ, and, the atypical PKCs (aPKCs) ξ and ι (Sakwe *et al*., 2004). The existence of this number of PKC isoforms represents a significant challenge to the understanding of PKC function (Mellor and Parker, 1999).

PKCs consist of an N-terminal regulatory domain and a C-terminal catalytic domain, separated by a hinge region (see Figure 1.5). Cleavage of the PKC hinge region by trypsin or Ca^{2+}-activated proteases (calpains) separates the regulatory fragment from the active catalytic fragment known as protein kinase M (PKM) (Kochs *et al*., 1993). Recently, one PKM isoform, PKM ζ, has been identified to play a critical role in the maintenance of long-term memory (Cai *et al*., 2011). The catalytic domains of the various PKC isoforms are very similar and thus, the classification of PKC isoforms is largely determined by the distinct structure of their regulatory domains. The regulatory domains of both cPKC and nPKC isoforms contain a conserved region, C1. C1 can be further subdivided to C1A and C1B motifs, each with 6 cysteines and 2 histidines that coordinate two Zn^{2+} ions and act as a binding site for 1,2 diacylglycerol (DAG) and the phorbol ester PMA (Mellor and Parker, 1999). The N-terminus of the C1 domains also contains a pseudosubstrate (alanine) at the predicted serine/threonine phosphorylation site for auto inhibition (House and Kemp, 1987). In contrast, atypical PKC (aPKC) contains a single zinc-finger motif making it unresponsive to DAG/PMA (Mellor and Parker, 1999). This happens due to greater homology of C1 motif of the aPKCs to the C1A motif than to the C1B (Mellor and Parker, 1999).

Apart from C1, both cPKC and nPKC also contain a C2 domain (Figure 1.5). The cPKC C2 represents the binding site for Ca^{2+} and also phosphatidylserine (PS) (Mellor and Parker, 1999). However, the nPKC's C2 lacks the acidic amino acids that co-ordinate Ca^{2+} binding (Ponting and Parker, 1996; Mellor and Parker, 1999). As for aPKC, they also lack a C2 domain making them insensitive to Ca^{2+} (Mellor and Parker, 1999).

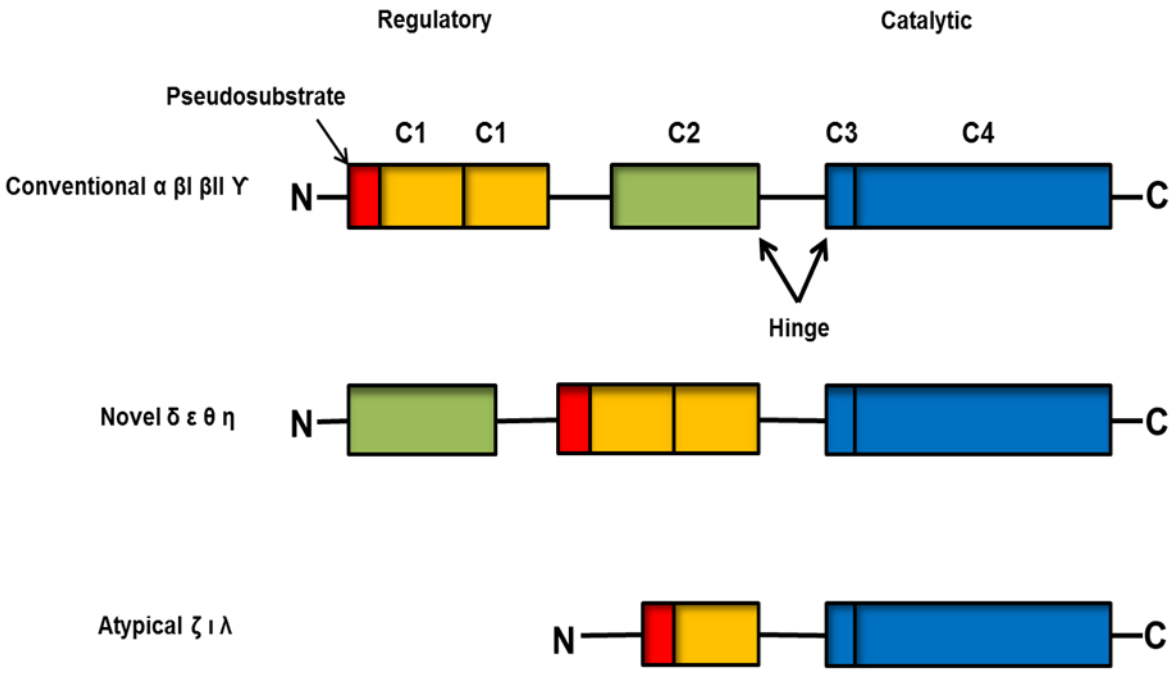

Figure 1.10. Primary structure of the protein kinase C (PKC) subfamilies. Figure depicts the catalytic domain in blue with the regulatory domain comprising differently coloured components. The amino and carboxyl termini are shown as N and C with different PKC regions labelled C1-C4. (adapted from Mellor and Parker, 1999)

1.6 Feedback phosphorylation of the CaR

The first report on the concept of feedback phosphorylation as a mechanism of class C GPCR regulation was on the metabotropic glutamate receptor mGluR5 as proposed by Nash *et al*. (2001; 2002). In light of the structural and sequence homology between CaR and the mGluR5 (Bai, 2004) and fact they both elicit oscillatory $Ca^{2+}{}_i$ signaling there existed the possibility that CaR may also exhibit feedback phosphorylation. The advance of single cell imaging and the ability to generate phosphospecific antibodies have allowed this possibility to be investigated. Of greatest functional importance is that, *in vivo*, the mutation of the CaR's key phosphorylation (CaR^{T888M}) represents a gain-of-function CaR mutation resulting in ADH (Lazarus *et al*., 2011) which suggests that CaR feedback phosphorylation is not just of academic interest but may play a functional role in regulating PTH secretion and thus whole body calcium homeostasis.

1.6.1 PKC-mediated CaR phosphorylation

The effect of PKC on the parathyroid $Ca^{2+}{}_o$-sensing mechanism was first observed in the mid-1980s when it was found that the phorbol ester, PMA (PKC activator) could inhibit the rise in $Ca^{2+}{}_i$ concentration and fall in PTH secretion that was elicited by high $Ca^{2+}{}_o$ concentrations in dispersed bovine parathyroid cells (Brown *et al*., 1984; Nemeth *et al*., 1986; Kobayashi *et al*., 1988). Later, Racke and Nemeth (1993) also demonstrated that the inhibition of the increase of $Ca^{2+}{}_i$ concentration in dispersed bovine parathyroid cells by PMA could be mimicked by other PKC-activating phorbol esters such as PDBu, but not by 4α-phorbol, (PMA's negative control), and, could be abolished by pretreating the cells with the protein kinase inhibitor staurosporine. Following the discovery of the CaR, Young *et al*., (2002) demonstrated that several PKC activators exert inhibitory effects on CaR-mediated $Ca^{2+}{}_i$ mobilisation, effects that could be blocked effectively by a PKC inhibitor which converted oscillatory Ca_i^{2+} responses to transient or non-oscillatory responses.

Thus PKC elicits an inhibitory effect on CaR signalling removes the inhibitory effect of high $Ca^{2+}{}_o$ concentration on PTH secretion, but precisely how this occurs is unknown. It would appear that PKC somehow uncouples CaR from its downstream effectors but

whether this occurs at the level of $G\alpha_{q/11}$ coupling or at some point further down the signal cascade, remains unclear (Ward and Riccardi, 2012). What is known is that preincubation of CaR-HEK and parathyroid cells for 10 minutes with PMA (to active PKC) inhibits high Ca^{2+}_o- evoked inositol phosphate (IP) accumulation which indicates that PLC is inhibited (Kifor *et al*., 1997). In support of this, another study has shown that PKC inhibition in CaR-stimulated CaR-HEK cells results in a five-fold enhancement of IP production (Bosel *et al*., 2003). Thus, regardless of any additional effect(s) that PKC may have downstream of PLC, for example on the IP_3 Receptor (IP_3R), it is clear that PKC can at least exert a substantial inhibitory effect on the initial IP_3 generation in response to CaR stimulation.

Sequencing of the human CaR revealed the presence of at least five predicted PKC phosphorylation sites in the receptor (Garrett *et al*., 1995). As already mentioned, mutagenesis of CaR^{T888}, CaR^{S895} and CaR^{S915} to non-phosphorylatable residues each elicit an increase in Ca^{2+}_o responsiveness but with CaR^{T888} exhibiting the greatest contributor to Ca^{2+}_o responsiveness by far (Bai *et al*., 1998b). As a result the greatest focus has been on understanding the dynamics and mechanism of CaR^{T888} phosphorylation and dephosphorylation, especially since it is this site that has been shown to be of functional relevance. In one study, CaR^{T888} was substituted with a variety of different residues which each had different functional consequences. For example, substitution of CaR^{T888} with hydrophobic amino acids (alanine, phenylalanine, and tryptophan) increased the sensitivity of CaR to its agonists, whereas the negatively charged amino acids (aspartate, glutamate, glycine) reduced sensitivity (Jiang *et al*., 2002). This latter effect is presumably because the negatively-charged residues are phosphomimetic. Interestingly, all of these mutated CaRs maintain partial sensitivity to PMA, suggesting the possibility of the presence of additional PKC sites or more downstream modulation by PMA (Jiang *et al*., 2002). It also should be noted though that that the PKC activating phorbols can also activate other C1 domain-containing molecules like chimaerins (a family of Rac GTPase-activating proteins), RasGRPs (exchange factors for Ras–Rap1), DAG kinase, and Munc13 isoforms (scaffolding proteins involved in exocytosis) (Sakwe *et al*., 2004).

To prove that CaR^{T888} is a substrate for PKC, Ward and colleagues developed a phospho-specific antibody to detect the phosphorylated CaR^{T888} site (Davies *et al*., 2007). Using this antibody, it was shown that elevated Ca^{2+}_o concentration and PMA treatment both elicited PKC-mediated phosphorylation of this site, with the former effect being abolished by cotreatment with either the calcilytic NPS-89636, the PKC inhibitor GF109203X, or by chronic PMA pretreatment (to downregulate the PMA-sensitive PKC isoforms). Furthermore, they also found that mutation of CaR^{T888} to a non-phosphorylatable residue (CaR^{T888A}) failed to completely abolish oscillatory CaR-induced Ca^{2+}_i mobilisation, which again supports the notion of additional PKC sites being present in CaR. It should be noted however that until recently no specific PKC isotype had been specifically linked to mediating CaR^{T888} phosphorylation or the modulation of CaR signalling.

The availability of the phospho-CaR^{T888} antibody also led to a potential explanation for the generation of CaR-induced Ca^{2+}_i oscillations in CaR-HEK cells and why Ca^{2+}_i mobilisation becomes sustained at higher Ca^{2+}_o concentrations. When CaR-HEKs are exposed to increasing Ca^{2+}_o concentrations between 0.5 and 2.5 mM there is a subsequent increase in CaR^{T888} whereas further increases in Ca^{2+}_o concentrations up to 5mM result in a decline in CaR^{T888} phosphorylation (McCormick *et al*., 2010; See the schematic representation of this in Figure 1.6). Interestingly, this biphasic (bell-shaped) relationship does not match the classic sigmoidal concentration dependence of CaR-induced Ca^{2+} mobilisation (Figure 1.6) (McCormick *et al*., 2010; Ward, and Riccardi, 2012). Indeed, moderate Ca^{2+}_o concentrations associated with the highest levels of sustained CaR^{T888} phosphorylation are also those that produce oscillatory Ca^{2+}_i mobilisation whereas higher Ca^{2+}_o concentrations can elicit more sustained Ca^{2+}_i responses by removing the inhibitory CaR^{T888} phosphorylation (Ward and Riccardi, 2012).

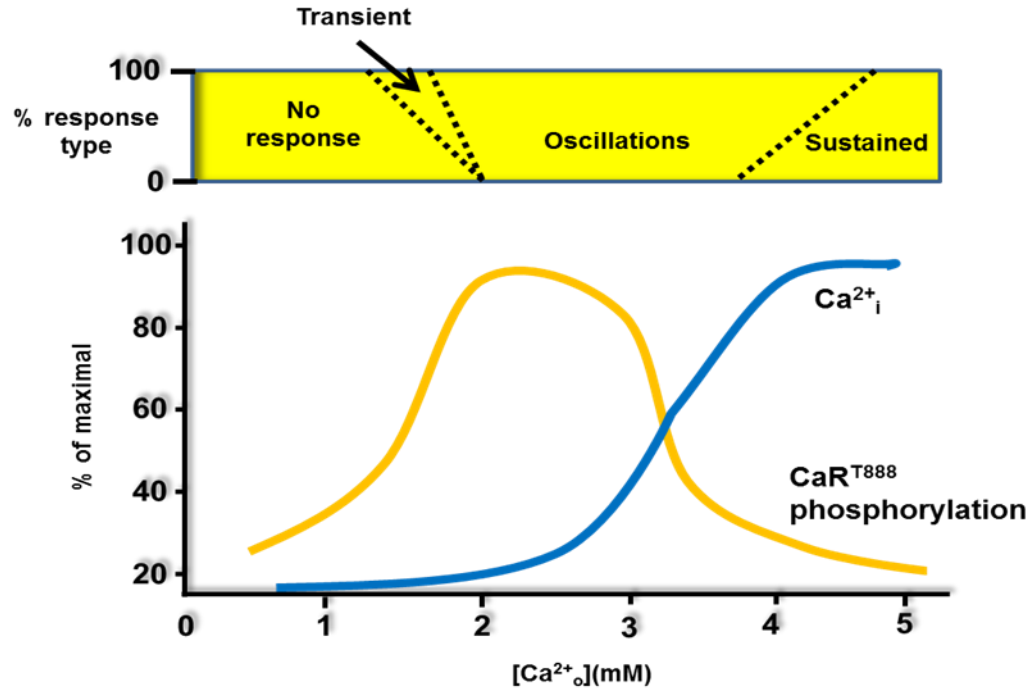

Figure 1.11: Schematic representation of the Ca^{2+}_o concentration dependence of CaR^{T888} phosphorylation and CaR-induced Ca^{2+}_i mobilisation (adapted from Ward, and Riccardi, 2012).

Grant *et al*. (2011) however have suggested that the increase in CaR signalling following exposure to high Ca^{2+}_o concentration is actually driven by an increase in the insertion rate of CaRs into the membrane from the endoplasmic reticulum. This mechanism they call *agonist-driven insertion* signalling (ADIS). This hypothesis is interesting since for most GPCRs, extended activation results in desensitisation through receptor internalisation (Lorenz *et al*., 2007) whereas the CaR is somehow able to avoid desensitisation (Breitwieser, 2012). This is physiologically important since the CaR is constantly exposed to moderate levels of Ca^{2+}_o that would partially activate it and thus it needs to maintain its membrane activity (Tfelt-Hansen and Brown, 2005). However, whether ADIS merely maintains a constant level of CaR on the membrane, or, it contributes to CaR cooperativity by driving increased membrane localisation at higher levels of Ca^{2+}_o remains to be determined. In either case it seems likely that receptor phosphorylation is central to the control of CaR activity whether by altering its effector coupling or perhaps contributing to the ADIS mechanism as well.

1.6.2 Protein phosphatase-mediated CaR dephosphorylation

That increasing Ca^{2+}_o concentrations from 2.5 - 5 mM actually decreases CaR^{T888} appears at first confusing since classic pharmacology might predict that more stimulation would drive more kinase activity and thus more phosphorylation, and thus more inhibition of function (*Davies et al., 2007; McCormick et al., 2010).* However, this ignores the effect of the CaR^{T888} phosphatase which Davies *et al* (2007) also showed was stimulated by CaR, particularly at Ca^{2+}_o concentrations above 2.5 mM. Thus, if there is more dephosphorylation at higher Ca^{2+}_o concentrations than phosphorylation then this would tend to produce more sustained Ca^{2+}_i mobilisation which is indeed what is seen experimentally (Davies *et al*., 2007; see Figure 1.6). It was further shown that both downregulation of PKC activity by chronic PMA pretreatment, and, PKC inhibition (GF109203X), increased the overall Ca^{2+}_o responsiveness of the CaR resulting in non-oscillatory, sustained responses in most CaR-HEK cells, mimicking the responses normally seen in high Ca^{2+}_o concentration. Thus, this suggests that at high Ca^{2+}_o concentration, the receptor is less phosphorylated and thus able to elicit either high frequency oscillations or even sustained Ca_i^{2+} mobilisation. In fact, 5 mM Ca^{2+}_o does briefly increase CaR^{T888} phosphorylation but this is quickly reversed by dephosphorylation permitting sustained Ca^{2+}_i mobilisation (McCormick *et al*., 2010).

Dephosphorylation of CaR^{T888} can be prevented by cotreatment with the PP1/PP2A inhibitor calyculin-A and this treatment also inhibits Ca^{2+}_i oscillations (Davies *et al*., 2007). In contrast, tautomycin (PP1 inhibitor) and FK506 (PP2B inhibitor) had little effect on CaR^{T888} dephosphorylation. Furthermore, protein phosphatase 2A activity has also been shown to be stimulated at 5 mM Ca^{2+}_o (*McCormick et al., 2010).* However, despite this preliminary data implicating PP2A as the CaR^{T888} phosphatase, the specific identity of the enzyme responsible remains to be properly established. But given that calyculin cotreatment (reversibly) abolishes Ca^{2+}_o-induced suppression of PTH secretion from human isolated parathyroid cells (McCormick *et al*., 2010) it is clear that this mechanism is of physiological importance.

1.6.3 CaR phosphorylation / dephosphorylation model

In light of the experiments detailed above, Ward and Riccardi (2012) proposed the CaR-induced Ca^{2+}_i oscillation model shown below in Figure 1.7. In the presence of ligands, CaR activates $G\alpha_{q/11}$ inducing the PLC-mediated release of Ca^{2+}_i. After that, PKC (isotype unknown) phosphorylates the receptor (at least at CaR^{T888}) uncoupling it somehow from its downstream effectors, thus preventing Ca^{2+}_i mobilisation, hence the downstroke. Next, a protein phosphatase (potentially PP2A) then dephosphorylates the receptor leading to renewed Ca^{2+}_i mobilisation and thus completion of the first Ca^{2+}_i oscillation cycle. Therefore, at moderate Ca^{2+}_o concentrations, PKC-mediated phosphorylation of CaR^{T888} matches its rate of dephosphorylation over time, albeit in an alternating manner so as to elicit stable oscillations. However, at higher Ca^{2+}_o concentrations, CaR preferentially elicits greater levels of phosphatase activity rendering the receptor less phosphorylated and thus able to elicit either high frequency oscillations or even sustained Ca^{2+}_i mobilisation (Ward and Riccardi, 2012). This model would also explain the suppressive effect of acute phorbol ester treatment and calyculin-A, as well as the stimulatory effects of PKC inhibition (GF109203X) and PMA downregulation (overnight PMA pretreatment) (Figure 1.7).

However there are a number of important questions still to be answered about this system. One such question concerns the Ca^{2+}_o threshold concentration and how inhibiting PKC gives a left-shift in the Ca^{2+}_o concentration-effect curve for Ca^{2+}_i mobilisation as this would imply that there is actually PKC activity in the cell even before there is any CaR-induced Ca^{2+}_i mobilisation. This question is consistent with the observation that CaR-induced actin polymerisation can occur at much lower Ca^{2+}_o concentrations than are capable of mobilising Ca^{2+}_i and this also raises the question therefore of whether $G\alpha_{12/13}$ may influence the shaping of the Ca^{2+}_i signals when they do occur.

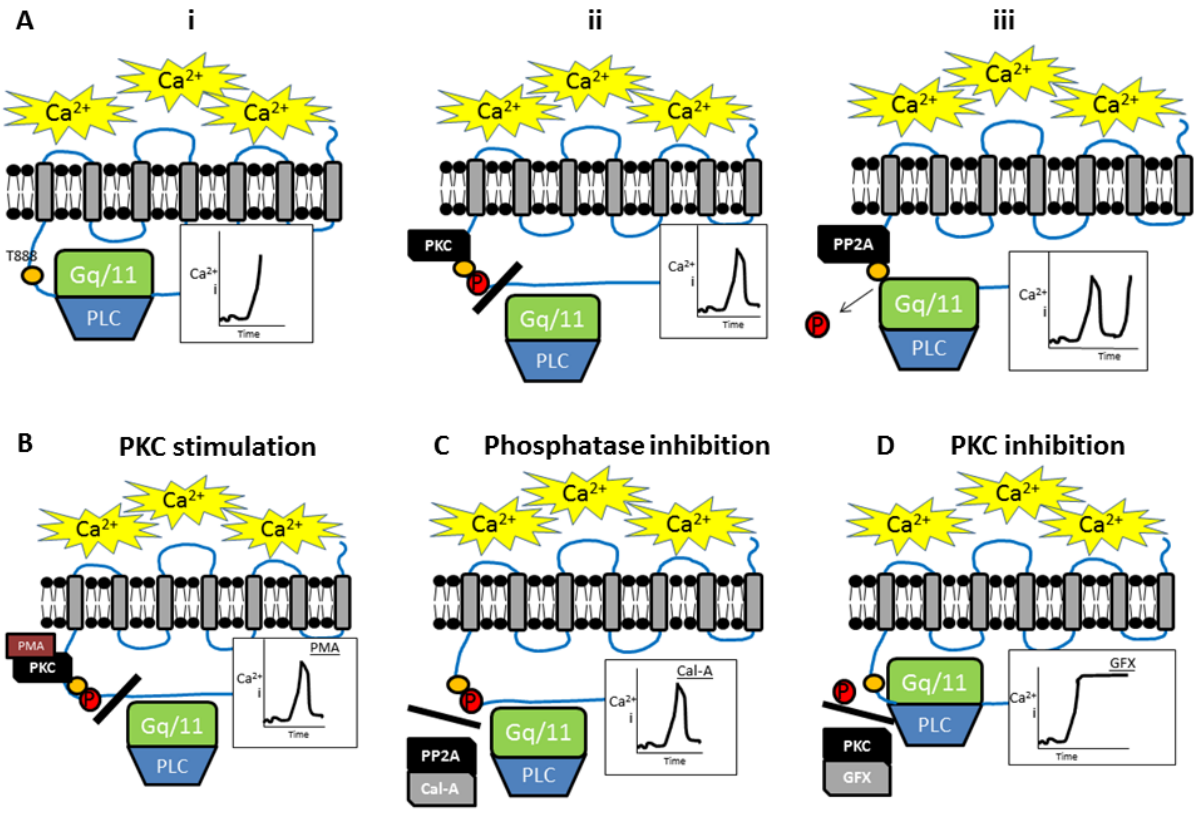

Figure 1.12: Schematic representation of the generation of CaR-induced Ca^{2+}_i oscillations based on dynamic phosphorylation of CaR^{T888}. Panel *A* indicates the three components of each oscillatory cycle including (i) CaR activation eliciting Ca^{2+}_i mobilisation, (ii) CaR^{T888} phosphorylation uncoupling the pathway components and (iii) CaR^{T888} dephosphorylation leading to further Ca^{2+}_i mobilisation. Panel *B*, *C*, and *D* show the effects of phorbol ester (PMA), calyculin-A (Cal-A) and GF109203X (GFX, Panel *D*) on CaR-induced Ca^{2+}_i mobilisation. (adapted from Ward, and Riccardi, 2012).

1.7 Signal bias and the CaR

Biased signalling is the ability of different ligands/modulator to favour distinct receptor conformations, triggering distinct downstream signalling pathways (Kenakin, 2011). Understanding biased signalling in GPCR action could be beneficial in drug discovery, since such information could help in producing a drug that specifically targets certain pathways over others (Conigrave and Ward, 2013; Leach *et al*., 2014). This might then lessen the off-target consequences of the new drug.

Since CaR is a) a promiscuous receptor that can be stimulated by numerous ligands, and b) a pleiotropic receptor that can couple to multiple G proteins and downstream signalling pathways, it is thus an ideal candidate for investigations of biased signalling. There are already several reported occurrences of biased signalling with the CaR. For example, Thompsen *et al*. (2012) reported that CaR preferentially couples to inhibition of cAMP production and stimulation of IP_3 accumulation over phosphorylation of ERK1/2 in CaR-HEK cells when stimulated by $Ca^{2+}{}_o$. In contrast, the stimulation of CaR by spermine favoured the phosphorylation of ERK1/2. Apart from that, L-Phe was also found to trigger lower frequency $Ca^{2+}{}_i$ oscillation in CaR-HEK cells compared to $Ca^{2+}{}_o$ (Rey *et al*., 2005). This group proposed that this may occur through a $G\alpha_{12/13}$-Rho-filamin A pathway, distinct from the classic $Ca^{2+}{}_o$-stimulated PLC-mediated $Ca^{2+}{}_i$ mobilisation pathway. Moreover, there is evidence of bias between CaR's allosteric modulators with cinacalcet and NPS2143 both reported to modulate $Ca^{2+}{}_i$ mobilisation with greater potency than for ERK1/2 phosphorylation (Leach *et al*., 2013).

In addition to ligand bias, there is also the possibility that CaR mutations could provide another route for bias. That is, the CaR mutation could change the CaR's conformation differently in each case and favour some intracellular pathways over others. For instance, the study by Leach *et al*. (2012) showed that CaR^{Q681H}, CaR^{E767K}, CaR^{L773R}, CaR^{S820F} and CaR^{F821L} increased the agonist sensitivity of the receptors for $Ca^{2+}{}_i$ mobilisation but with no significant effect on ERK1/2 phosphorylation. Thus, with the possibility of targeting the CaR pharmacologically in a variety of diseases it is important to consider how different CaR modulators may work on the same receptor, and also how the same compounds may work on different CaRs i.e. wild-type vs. mutants. Together this

information will aid in the development of improved CaR therapeutics for use in diseases of mineral homeostatic dysfunction.

CaR's Ligand	Bias Pathway
Ca^{2+}	**Preferentially towards cAMP inhibition and IP3 accumulation than ERK 1/2 activation in CaR-HEK cells.**
Spermine	**Preferentially towards ERK1/2 activation than cAMP inhibition and IP3 accumulation in CaR-HEK cells.**
NPS 2143	**Preferentially towards induction of $Ca^{2+}{}_i$ mobilisation than ERK1/2 activation in CaR-HEK cells.**

Table 1.2: Bias signalling of CaR stimulated by different ligands

1.7 Aims and Objectives

In light of a number of the unanswered questions discussed in this Chapter, it seems important to advance our understanding of CaR signalling *in vitro* in the first instance to inform future *in vivo* studies of CaR function and Ca^{2+} homeostasis. The specific research objectives in this study therefore are set out here:

Objective 1: To clarify the roles of protein kinase C (PKC) in CaR-dependent control of intracellular Ca^{2+} in HEK-293 cells. Specifically, to identify the specific isotype(s) responsible for CaR^{T888} phosphorylation and CaR signal modulation in CaR-expressing HEK-293 cells using gene-selective RNAi probes with the ultimate aim of identifying those proteins responsible for regulating PTH secretion *in vivo*. Knockdown of specific PKC isoforms genes will be silenced in CaR-HEK cells by RNA interference using siRNA.

Objective 2: To investigate the role of the PKC isoform(s) identified in Objective 1 on $Ca^{2+}{}_i$ mobilisation at sub-threshold $Ca^{2+}{}_o$ concentrations and on ERK phosphorylation.

Objective 3: To examine the effect of raising intracellular cAMP concentrations on the phosphorylation of CaR^{T888} and the effect of this on its resulting signalling.

Objective 4: To confirm that the three novel CaR readouts provisionally identified using the Bio-plex® platform, namely P70 S6Kinase, IGF1R and IκBα are indeed downstream effectors of CaR activation.

Objective 5: To functionally characterise five gain-of-function CaR mutations (C131Y, E228K, E228A, Q245R, and C131Y) in CaR-HEK cells by comparing their effects on $Ca^{2+}{}_i$ mobilisation, ERK 1/2 phosphorylation and $p38^{MAPK}$ phosphorylation to the same responses in wild-type CaR.

Objective 6: To determine whether the calcilytic NPSP795 inhibits these gain-of-function mutant CaRs and whether there is any evidence of signal bias between the mutants with regards to kinase signalling

CHAPTER 2

Materials and Methods

2.1 Materials

Fura 2/AM was obtained from Molecular Probes Inc. (Life Technologies Ltd, Paisley, UK). Bovine serum albumin was obtained from Melford Laboratories Ltd (Ipswich, UK). Polyclonal primary antibodies against PKC α, PKC δ, PKC ζ, PKC ε, β-actin, phospho ERK, phospho $p38^{MAPK}$, phospho IGF1R, phospho P70 S6kinase and phospho IκB α, as well as horse radish peroxidise-conjugated secondary anti-mouse and anti-rabbit antibodies were obtained from Cell Signalling Technology Inc. (New England Biolabs, Hitchin, UK). Polyclonal antibody for PKC βI and βII were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Monoclonal anti-CaR antibody (raised to amino acids 214 to 235 of the extracellular domain of human parathyroid CaR) was from Affinity Bioreagents (Fisher Scientific, Loughborough, UK). The custom-generated phospho-specific anti-CaR^{T888} antibody was from Sigma-Genosys (Haverhill, UK). The CaR^{C131Y} and CaR^{WT} plasmid DNAs and the calcilytic NPSP795 were gifts from Prof. D. Riccardi (University of Cardiff, UK). The calcilytic NPS2143, phorbol-12-myristate-13-acetate (PMA), PKC inhibitor GF109203X, Forskolin, and 1,9-Deoxyforskolin were purchased form Tocris Bioscience (Abigdon, UK). The calcimimetic NPS R568 was a kind gift of Dr. E. Nemeth (NPS Pharmaceuticals, Salt Lake City, US). The stable CaR-HEK cell-lines (CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R}, CaR^{C131Y} and CaR^{WT}) used in Chapter 6 were made and supplied by Dr S. Brennan and Prof D. Riccardi (University of Cardiff, UK). Glycine, Tris-base and sodium dodecyl sulphate (SDS) were from Fisher Scientific (Loughborough, UK). Unless otherwise stated, all other materials were obtained from Sigma-Aldrich (Gillingham, UK).

Activator	Concentration used in the studies	Target
NPSR568	1μM	CaR
PMA	1μM	PKC
Forskolin	1μM	Adenylate Cyclase
Inhibitor		
NPS2143	1μM	CaR
NPSP795	1nM-1μM	CaR
GF109203X	1μM	PKC

Table 2.1: The list of activators and inhbitors used in the studies

2.2 Cell culture

HEK-293 cells stably-transfected with human CaR (CaR-HEKs) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Ltd, Paisley, UK) supplemented with 10 % heat-inactivated foetal bovine serum (FBS) (Life Technologies Ltd, Paisley, UK). Cells used were split twice weekly at 80 % confluency, using trypsin (Life Technologies Ltd, Paisley, UK), and for no more than 30 passages from original transfection. HEK-293 cells stably transfected with human parathyroid CaR were gift from Dr E. F. Nemeth (NPS Pharmaceuticals, Bedminster Township, NJ, USA) were cultured in DMEM with 10 % FBS and 200 µg/ml hygromycin B (Boehringer-Ingelheim, Bracknell, UK).

2.3 Gene knockdown using siRNA

Small interfering RNAs (siRNA) are ~20 base pair double-stranded nucleotide RNA that interfere with the expression of specific genes (Carthew and Sontheimer, 2009; Rao *et al*., 2009). Naturally, siRNA is synthesised from double stranded RNA that is processed by the enzyme Dicer to produce short nucleotide pieces with 2-nucleotides overhangs on the 3′ ends (Kanasty *et al*., 2013). However, siRNA that mimics the structure of Dicer products can also be produced synthetically (Kanasty *et al*., 2013) and thus are used widely to alter gene and thus protein expression (Young *et al*., 2014; Lo *et al*., 2015; Kang *et al*., 2015). The synthetic siRNA can be introduced into the cells by using vehicles such as synthetic polymers, natural/biodegradable polymers and lipids (Rao *et al*., 2009). Here, the siRNA was delivered into the cells via the lipid base vehicle, Lipofectamine® RNAiMAX (Life Technologies Ltd, Paisley, UK). Lipofectamine is a cationic liposome that entraps the negatively charged siRNA and then delivers it into cells through membrane fusion (Dalby *et al*., 2004). Once inside the cell, the siRNA assembles with RNA-induced silencing complex (RISC) where the double-stranded RNA is unwound leaving the single-stranded RNA in the complex (Carthew and Sontheimer, 2009). The targeted RNA then recognises this single-stranded siRNA sequence through Watson-Crick base pairing and binds to the complex (Carthew and Sontheimer, 2009). The

catalytic component of RISC which is known as argonaute then cleaves the targeted RNA (Carthew and Sontheimer, 2009).

In this study, CaR-HEK cells were transfected with Silencer® Select (Life Technologies Ltd, Paisley, UK) by reverse transfection using Lipofectamine® RNAiMAX transfection reagent according to the manufacturer's protocol. Briefly, the siRNAs and transfection reagent were diluted in OptiMEM (Life Technologies Ltd, Paisley, UK) for 10 minutes prior the addition of cells in growth media. The cell number in media was adjusted to give 30-50 % confluency after 24 hours of plating. Control siRNA (Life Technologies Ltd, Paisley, UK) or mock transfection (without siRNA) was used for control experiments.

2.4 Plasmid DNA bacterial transformation

Plasmid DNAs were transformed into XL10-Gold® cells according to manufacturers' instructions. Briefly, 2 µl of plasmid DNAs were added to 40 µl of XL10-Gold® cells (Agilent Technologies, Cheadle, UK) and incubated on ice for 30 minutes. Cells were then heat shocked for 40 seconds in a 42^{o}C water-bath and incubated on ice for 2 minutes. The cells suspension was then added to 500 µl of warmed NZY broth (Fisher Scientific, Loughborough, UK) and the solution were shaken at 37^{o}C for 1 hour. The transformed bacteria were spread on agar plates containing ampicillin (100µg/ml) (Fisher Scientific, Loughborough, UK) and incubated overnight at 37^{o}C. Two colonies were then picked and grown in 3ml LB-broth for 8 hours. The growing cells were then transferred to 50 ml LB broth (Fisher Scientific, Loughborough, UK) containing ampicillin (200ug/ml) and left overnight at 37^{o}C shaking. Plasmid DNA in bacteria cells were harvested and purified using Hispeed Plasmid Midi Kits (Qiagen Limited, Manchester, UK) according to manufacturers' protocol.

2.5 Transient cell transfection

Transient transfections were performed using TransIT-LT1 (Geneflow Ltd, Lichfield, UK) reagents according to the manufacturers' instructions. Briefly, 3µl transfection

reagent was added to serum-free Opti-Mem (Life Technologies Ltd, Paisley, UK) and incubated at room temperature for 5 minutes. 1μg DNA vector was then added and left for a further 15-30 minutes. The solution was then added to the (approximately) 60% confluent cells drop-wise. Cells were used experimentally within 72 hours of transfection.

2.6 Cell lysis and phospho CaR^{T888} assay

CaR-HEKs were grown to ~80% confluence in 35 mm culture dishes and the phosphorylation status of CaR^{T888} was assayed as described previously (Davies *et al*., 2007) using Experimental Buffer [(mM) 20 HEPES (pH 7.4), 125 NaCl, 4 KCl, 0.5 $CaCl_2$, 0.5 $MgCl_2$ and 5.5 glucose]. Briefly, cells were washed in experimental buffer to remove culture medium and then equilibrated in it at 37ºC for 20-mins prior to their exposure to various treatments (10 minutes at 37ºC) followed by lysis on ice. Cells were lysed in a RIPA-like buffer supplemented with protease and phosphatase inhibitors (Davies *et al*., 2007). Specifically, the ice-cold lysis buffer contained 12 mM HEPES (pH 7.6), 300 mM mannitol, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton-X 100, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1 mM NaF, 250 μM sodium pyrophosphate, 100 μM sodium orthovanadate, 1.25 μM pepstatin, 4 μM leupeptin and 4.8 μM PMSF. When lysing cells for CaR immunoblotting, 1 mM N-ethylmaleimide (NEM) was added to the RIPA buffer to prevent oxidation of free sulphhydryl residues. Cell lysates were then centrifuged at 10,000 xg for 10-mins and the supernatant stored at -80ºC until use.

2.7 Phosphospho ERK 1/2, $p38^{MAPK}$, P70 S6kinase, IGF1R and IκBα assays

CaR-HEKs were grown to ~80% confluence in 35 mm culture dishes and washed in experimental buffer to remove culture medium. The cells were then equilibrated in experimental buffer at 37ºC for 20-mins prior to their exposure to experimental buffer with various Ca^{2+} concentrations (10 minutes at 37ºC). The phosphorylations of ERK 1/2, P70 S6kinase, IGF1R and IκBα were stimulated by 5.0 mM Ca^{2+}. As for the determination of calcilytic NPSP795 IC_{50}, the wild type CaR-HEK cells were incubated in 3.4 mM of Ca^{2+} while the mutant CaR-HEK cells in 2.4 mM of Ca^{2+}. Cells were then lysed and subjected to immunoblotting.

2.8 Immunoblotting

Cell lysates were resolved by immunoblotting as follows. The lysis supernatant was diluted with 5X Laemmli buffer (320 mM Tris base [pH 6.8], 5% (w/v) sodium dodecyl sulphate, 25% (v/v) glycerol, 1% (w/v) bromophenol blue in the presence of 5% (v/v) β-Mercaptoethanol reducing agent) before heating to 65°C for three minutes. Samples were then resolved on 5% SDS-PAGE gels (for CaR; 8-10% gels for other proteins) using Running Buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and then transferred to nitrocellulose membrane (0.45 μM) for one hour at 200 milliAmps in Blotting Buffer (25 mM Tris, 200 mM glycine, 15% (v/v) methanol). Non-specific binding sites on the nitrocellulose membrane were blocked for 30-mins using a Tween-TBS buffer (15 mM Tris [pH 8], 150 mM NaCl, 0.1% (v/v) Tween 20) supplemented with 1% bovine serum albumin (BSA). Membrane was then probed with primary antibody (1/2000 – 1/5000 dilution) for 1- hour and washed for 20 minutes. These and subsequent steps were performed using Tween-TBS buffer. The membrane was then incubated for a further hour in secondary antibody (1/5000 dilution) conjugated to horse radish peroxidase and then washed for a final time (20-min). Immunoreactivity was then visualised using enhanced chemiluminescence solution (ECL) (GeneFlow, Lichfield, UK) and Kodak ECL film.

2.9 Immunoblot stripping

Bound antibody was stripped from the nitrocellulose membrane using Restore™ PLUS Western Blot Stripping Buffer (Fisher Scientific, Loughborough, UK) according to the manufacturers' instructions. Briefly, the membranes were incubated in appropriate volume of stripping buffer for at 37ºC for 15 minutes with occasional agitation. The stripping buffer was then removed and the membranes were washed extensively with Tween-TBS buffer six times for 5 minutes each. The membranes then were reblocked with 1% BSA and reprobed with the next antibody.

2.10 Intracellular Ca^{2+} measurement

Cells were cultured on glass coverslips to approximately 60 % confluency and loaded with Fura-2/AM (1µM for 1-2 h) at room temperature in the dark in a buffer (pH 7.4) containing 20 mM HEPES, 125 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose and 0.1 % BSA. Free fura 2/AM is excited at 380 nm while $Ca^{2+}{}_i$ bound fura 2/AM excited at 340 nm. The ratio of 340/380 nm excition allows accurate measurement of $Ca^{2+}{}_i$ concentration. Cells were washed to remove unabsorbed Fura-2/AM and equilibrated for 5 minutes in experimental buffer containing 0.5 mM $CaCl_2$. Dual-excitation wavelength microscopy was then carried out using a Nikon Diaphot inverted microscope. The cells were mounted in a perfusion chamber (Warmer Instruments, Hamden, CT) and observed through a x40 oil-immersion lens objective microscope. Experiments were performed in buffer (pH 7.4) containing 20 mM HEPES, 125 mM NaCl, 4 mM KCl, 0.5-10 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose at room temperature. The concentration of NaCl was reduced accordingly in experiments where $CaCl_2$ concentration was increased to normalise osmolarity. Cells were imaged using MetaFluor Software. The response of the cells were determined by measuring the area under curve (AUC) of the resulting graph that correlated to the 340/380 excitation ratio using GraphPad Prism software. The single cell response was determined by selecting at least 20 individual cells while the "Global response" was determined by selecting all cells in the field view area. The AUC of "Global response" was used to measure the effect of various treatments on CaR-HEK cells.

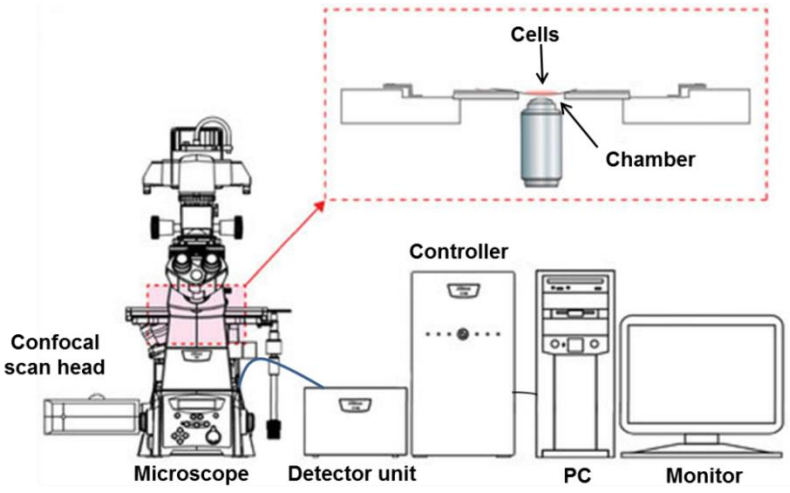

Figure 2.1: The diagram live of cell imaging set up

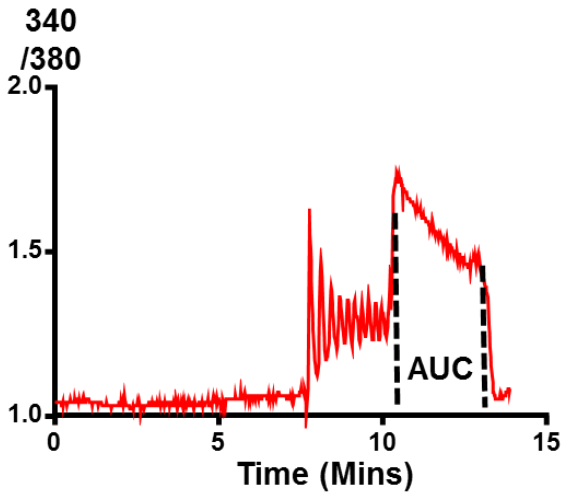

Figure 2.2: The area under curve (AUC)

2.11 Statistical analysis

Data were analysed using Microsoft Excel followed by GraphPad Prism (V6). To determine relative changes in Fura-2 ratios, the area under the curve of the "global" (all cells in field of view) traces were quantified using GraphPad Prism software. For experimental reasons the first minute of each experiment was excluded. All the data were transformed into logarithm to increase the change of passing the normality test. The normality of data was determined using D'Agostino-Pearson normality test. Whenever the data followed a normal Gaussian distribution, parametric tests (t-test, One-way ANOVA, or Repeated Measure ANOVA) were employed to test the statistical difference between data, while non-parametic tests (Mann–Whitney U or Kruskal–Wallis one-way analysis of variance) were used for data that did not follow a normal Gaussian distribution. Apart from that, for the data that have less than 5 replicates, non-paramtric tests were employed for the statistical analysis. Data are presented as means ± S.E.M unless otherwise stated. P values < 0.05 were considered statistically significant.

Chapter 3

Effect of selective PKC isotype knockdown on CaR negative feedback phosphorylation and function

3.1 INTRODUCTION

It has been shown previously that PKC is responsible for negative feedback of the CaR via phosphorylation of the Thr-888 site in its intracellular tail (Bai *et al*., 1998b). However, since the PKC family consists of a number of isotypes, it was therefore necessary to identify which specific PKC isotype(s) is/are involved in modulating CaR signalling. Several studies have attempted this previously (Young *et al*., 2002; Sakwe *et al*., 2004; Young *et al*., 2014), however, they tended to look at single PKC isotypes only, as well as $Ca^{2+}{}_i$ oscillation frequency alone rather than on the totality of $Ca^{2+}{}_i$ dynamics (i.e. including signal threshold and total $Ca^{2+}{}_i$ mobilisation), and did so without looking at CaR^{T888} phosphorylation itself. In the current study, a more holistic approach was employed whereby 5 different PKC isotypes were focussed on, particularly those of the conventional and novel PKC sub-families since chronic phorbol ester pretreatment appears to abolish negative feedback phosphorylation in our hands (Davies *et al*., 2007). This would suggest that it was the phorbol ester-sensitive PKCs that were the most likely candidates to be the predominant CaR signal modulator(s).

In addition, most CaR studies examining PKC action on CaR signalling use intracellular Ca^{2+} mobilisation, or MAP kinase (ERK and $p38^{MAPK}$) phosphorylation as the readout of CaR activation or inhibition (Young *et al*., 2002; Tfelt-Hansen *et al*., 2003; Holstein *et al*., 2004). However, none of these studies have determined directly the actual phosphorylation state of the receptor so as to be able to properly interpret whether CaR negative feedback phosphorylation has occurred or not. In contrast, two studies from this laboratory (Davies *et al*., 2007; McCormick *et al*., 2010) have shown the actual phosphorylation state of CaR^{T888} under various conditions, using a custom-generated phospho-specific anti-CaR^{T888} antibody. However, to date there still lacks a holistic investigation into the action of specific PKC isotypes in CaR^{T888} negative feedback phosphorylation. Thus, my first aim was to determine which PKC isotype(s) is / are directly responsible for controlling CaR phosphorylation / activity via the use of PKC isotype-specific siRNAs to selectively knockdown the individual PKC isotypes in the CaR-HEK cells. The cellular outcomes measured included single-cell microfluorometry ($Ca^{2+}{}_i$ imaging) and CaR^{T888} phosphorylation.

3.2 METHODS

CaR-HEK cells were cultured as detailed in (Chapter 2) section 2.2. Intracellular Ca^{2+} mobilisation in CaR-HEK cells was measured using single-cell microfluorometry as described in 2.9. The selective knockdown of individual PKC isotypes was achieved using PKC isotype-specific siRNAs as described in 2.3, using 50 nM siRNA in the presence of 50 µl/ml transfection reagent. The cells were lysed as described in 2.6 and (phospho)-proteins immunoblotted as described in 2.7.

3.2.1 Optimisation of siRNA-mediated gene knockdown conditions in CaR-HEK cells

The optimisation studies were done in the 24 wells plates. In the optimisation of siRNA concentration and transfection reagent volume studies, 10-50 nM of siRNA and 1.5-3.0 µl of transfection reagent were used respectively. The cells were lysed at Day-3 post transfection, using RIPA buffer prior to immunoblotting.

3.2.2 PMA chronic pretreatment

Cells were grown in growth media supplemented with 1 µM PMA overnight, resulting in downregulation of the conventional and novel PKC isotypes prior to immunoblotting.

3.3 RESULTS

3.3.1 Investigation of the effect of chronic PMA pretreatment on PKC isotype expression in CaR-HEK cells

Chronic phorbol ester treatment was previously shown to increase the CaR sensitivity of CaR-HEK cells presumably by preventing the negative feedback phosphorylation of the receptor (Davies *et al*., 2007). Thus, in order to determine the specific PKC isotype(s) responsible for regulating CaR phosphorylation and function, I began by examining how the protein abundance of each of seven PKC isotypes were affected by overnight treatment with phorbol ester. For this, CaR-HEKs were cultured overnight in the presence of either phorbol 12-myristate 13-acetate (PMA; 1 μM) or vehicle control DMSO (0.1 % v/v) then lysed and the protein abundance of each isotype examined by western blotting. This treatment was expected to downregulate the DAG-activated PKC isotypes and indeed, chronic PMA pre-treatment caused a substantial reduction in the expression of the conventional PKCs including PKCα, β I and β II (Figure 3.1). Protein kinase C γ, which is another conventional PKC, was excluded from the studies since it is generally considered to be expressed neuronally (Saito and Shirai, 2002), plus microarray data from this laboratory tends to confirm its lack of expression in CaR-HEK and parathyroid cells (data not shown). Similar PMA-induced downregulation was also observed for the novel PKC isotypes δ, ε and θ (Figure 3.1). However, perhaps surprisingly I observed no change in the abundance of the novel PKCη. The treatment also failed to reduce the expression of atypical PKCζ in the CaR-HEK cells which is unsurprising as it lacks one of the DAG-sensitive zinc fingers (Steinberg, 2008). Thus, based on this study I predicted that the isotype(s) responsible for regulating CaR phosphorylation would be one or more from PKCα, PKCβI, PKCβII, PKCδ, PKCε, and PKCθ and thus these became my targets for siRNA-mediated knockdown.

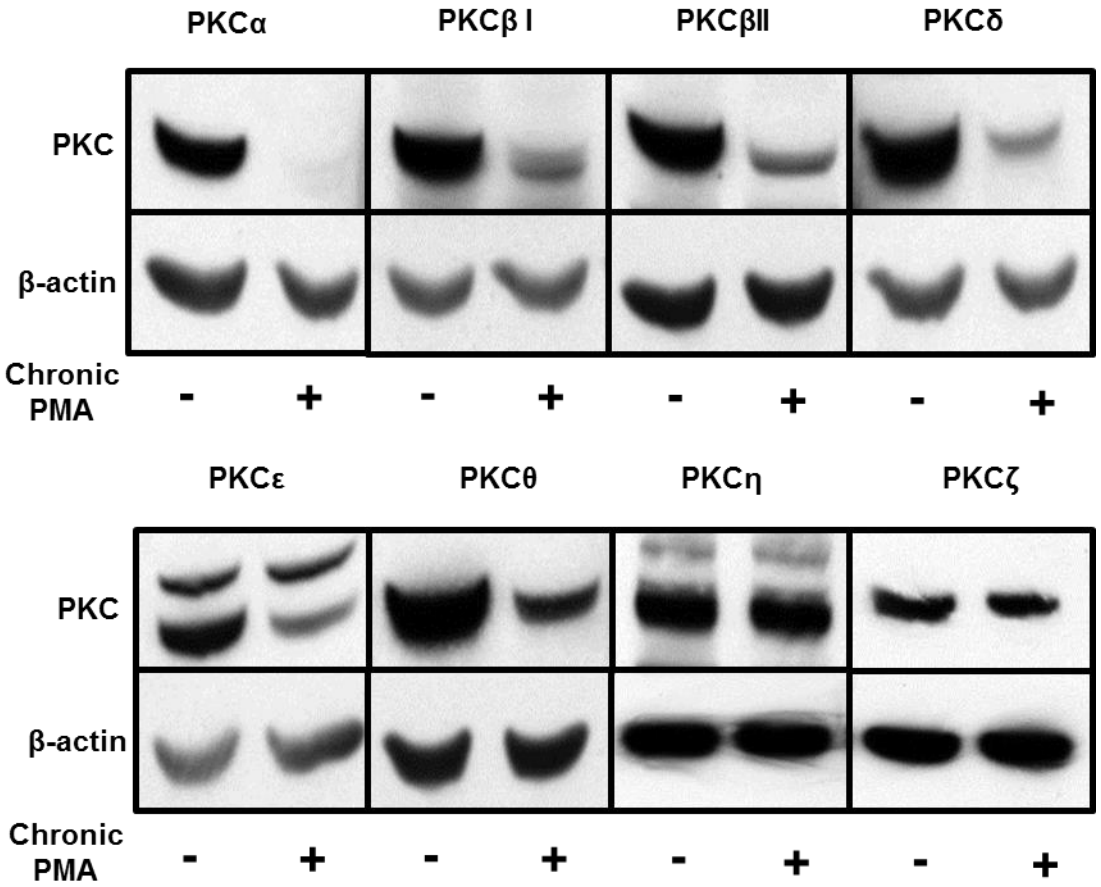

Figure 3.1: Western blot analysis examining the expression of PKCα, βI, βII, δ, ε, θ, η and ζ in chronic PMA-pretreated and vehicle-pretreated CaR-HEK cells. Anti-PKC immunoblots show that the protein expression of PKCα, βI, BII, δ, ε and θ were substantially downregulated by chronic PMA pretreatment whereas the abundances of PKCη and ζ were unchanged. The result from a single experiment confirms previous findings (Tsutsumi *et al*., 1993; Shih *et al*., 1999; Ahnadi *et al*., 2000); except for the PKCη western which is representative of three independent experiments. The protein equivalence in each lane ± PMA was confirmed using β-actin antibody.

3.3.2 Optimisation of siRNA and transfection reagent concentrations

In order to achieve the best conditions for gene knockdown**,** the concentrations of the siRNAs and transfection reagent were first optimised. The cells were transfected with anti-PKCα siRNA in Lipofectamine® RNAiMAX for three days prior to western blot analysis using anti-PKCα antibody. As shown in Figure 3.2*A*, in the presence of 50 μl/ml lipofectamine, there was an siRNA concentration-dependent decrease in PKCα expression that was quite marked in 25 nM siRNA, substantially greater in 50 nM siRNA but not decreased any further in 75 nM siRNA. Hence, this result suggested that the lowest siRNA concentration that maximally reduced PKCα expression was 50 nM. Similarly, in the presence of 50 nM siRNA, there was a lipofectamine concentration-dependent decrease in PKCα expression with little effect seen when using 12.5 μl/ml lipofectamine, but substantial downregulation observed using 25-50 μl/ml concentrations of RNAiMAX (Figure 3.2*B*). No additional decrease in PKCα expression was seen using 75 μl/ml lipofectamine suggesting that the lowest lipofectamine concentration necessary to achieve maximal PKCα knockdown was 50 μl/ml. It should be noted that in a number of initial experiments (not shown), siRNAs and transfection detergents from several different suppliers were compared with varying success. In addition, there was no change in the β-actin abundance under any of the conditions tested suggesting that these conditions were not toxic to the cells or able to cause any gross changes in cell-fate. Therefore, in light of the data shown in Figure 3.2, 50 nM siRNA (Life Technologies) and 50 μl/ml Lipofectamine® RNAiMAX (Life Technologies) were determined to be the optimal conditions for the CaR-HEKs in this study.

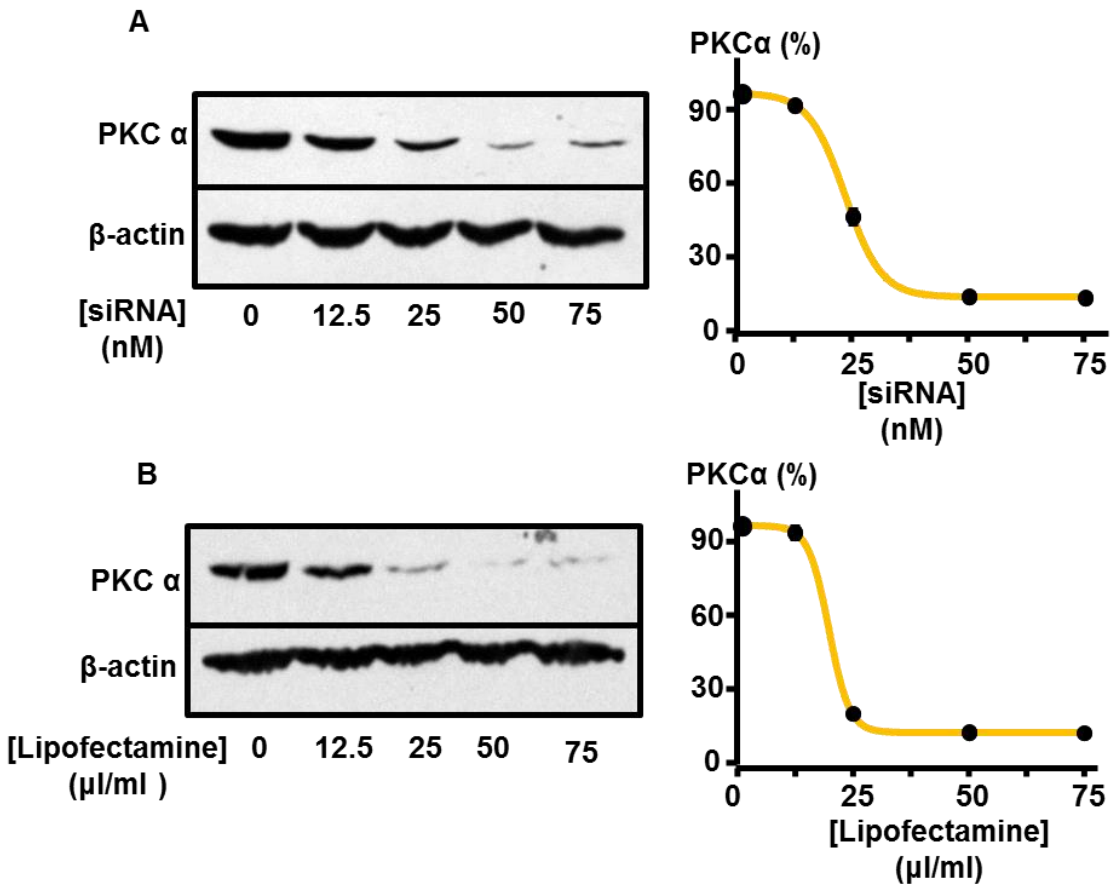

Figure 3.2: The optimisation of siRNA and transfection reagent concentrations for PKC knockdown. *A*) The western blot reveals that the minimum siRNA concentration necessary to achieve maximal PKCα knockdown was 50 nM. *B*) The next immunoblot then shows that the minimum transfection reagent concentration necessary to achieve maximal PKCα knockdown was 50 µl/ml. Protein equivalence in each lane was confirmed using a β-actin antibody. Results are representative of two independent experiments (n = 2).

3.3.3 Examination of possible off-target siRNA effects using the optimised PKC siRNA transfection conditions

Having optimised the siRNA and lipofectamine concentrations to achieve maximal PKCα knockdown with the least possible reagent, the other PKC isotype-specific siRNAs were then tested using the same conditions. Figure 3.3 shows a panel of multiple anti-PKC isotype western blots performed using CaR-HEK cells incubated in a variety of PKC isotype-specific siRNAs. By doing this it was possible to determine firstly that the siRNAs did downregulate the specific isotype they were supposed to, but also that they did not have any off-target effects such as downregulating one of the other PKC isotypes, or, cause a compensatory increase in the expression of another isotype. As shown in Figure 3.3, the siRNAs for PKCα, β, δ, ε and θ all achieved substantial downregulation of those isotypes (with PKCβ1 and βII equally affected as they derive from the same gene). There was no evidence that any siRNA altered the expression of any other isotype other than that it was targeted against. It should also be noted that two separate controls were used, one termed "mock" which included lipofectamine only, but not any siRNA, and "control" which included a control siRNA (Life Technologies) together with the lipofectamine. These suggest that the presence of siRNA *per se* had no non-specific effect on PKC expression levels either. Together, these results confirm that optimised siRNA transfection conditions were indeed resulting in selective knockdown of specific PKC isotypes in the CaR-HEK cells.

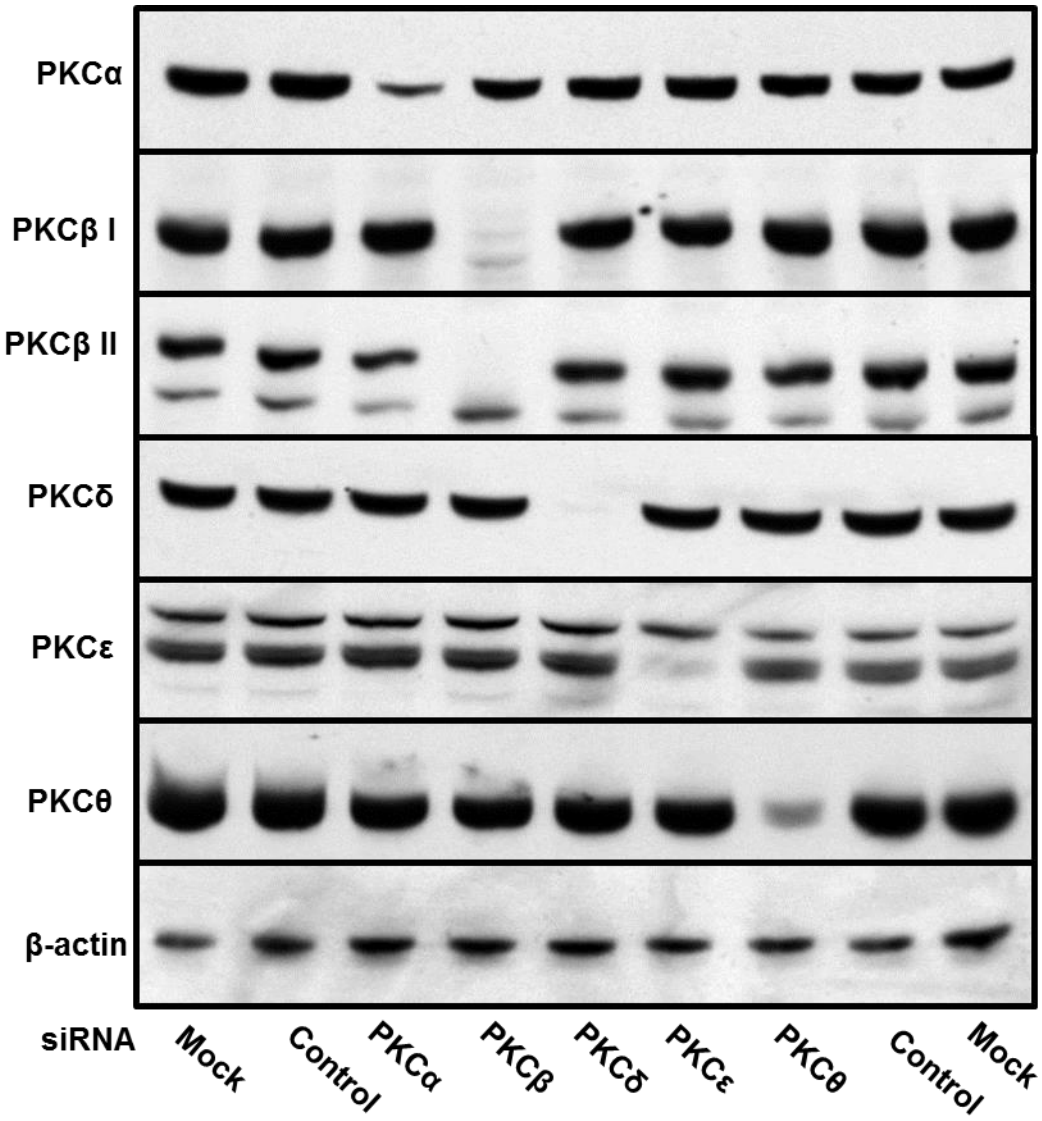

Figure 3.3: Demonstration of the specific knockdown of individual PKC proteins using isotype-specific siRNAs. The result shows that each isotype-specific PKC siRNA successfully knocked down the isotype it was targeted towards but without resulting in off-target downregulation, or a compensatory increase other PKC isotypes. In addition, the presence or absence of a control siRNA had no effect on expression of any of the isotypes tested. Protein equivalence between lanes was confirmed using β-actin immunoblotting.

3.3.4 Investigation of the effect of PKCα downregulation on CaR-induced Ca^{2+}_i mobilisation

Having successfully established the siRNA protocol for selective PKC isotype downregulation, this was first applied to test the effect of knocking down specific PKC isotypes on CaR-induced Ca^{2+}_i mobilisation, with the focus on PKCα in the first instance. The working assumption was that by knocking down the PKC isotype(s) responsible for CaR phosphorylation, Ca^{2+}_i mobilisation in CaR-HEK cells would be increased in response to moderate concentrations of extracellular Ca^{2+} (Ca^{2+}_o). In the experiments shown in Figures 3.4-3.9 the control cells were mock transfected using lipofectamine alone (the side-by-side comparison of mock-transfected controls vs control siRNA vs PKCα siRNA is shown later in Figure 3.10).

Figure 3.4 shows the Ca^{2+}_o concentration-dependent changes in Ca^{2+}_i levels in CaR-HEK cells previously exposed for 3-days to either mock transfection or PKCα siRNA. In the controls, the cells exhibited low or transient Ca^{2+}_i mobilisation in response to 2 mM Ca^{2+}_o with an increase in Ca^{2+}_o concentration to 2.5 mM eliciting more oscillatory Ca^{2+}_i mobilisation. Higher frequency oscillations were observed at 3mM Ca^{2+}_o concentration and then either even higher frequency oscillations or sustained responses were observed with 5 mM Ca^{2+}_o. Indeed virtually every cell exhibited sustained, maximal Ca^{2+}_i mobilisation in 10 mM Ca^{2+}_o and together this range of CaR-HEK responses would be considered typical in this (Davies *et al*., 2007; McCormick *et al*., 2010) and other (Miedlich *et al* 2002; Rey *et al*., 2005) CaR laboratories. In contrast, in cells in which the PKCα had been knocked down, oscillatory Ca^{2+}_i mobilisation was already exhibited in response to 2 mM Ca^{2+}_o. Indeed, in response to 2.5 and 3 mM Ca^{2+}_o the cells exhibited less oscillatory i.e. more sustained Ca^{2+}_i mobilisation than for controls. As for controls, the PKCα downregulated cells exhibited mostly sustained Ca^{2+}_i mobilisation in response to 5 and 10 mM Ca^{2+}_o (Figure 3.4*A*). From the results obtained from these microfluorometry experiments, the responses were quantified by measuring the area under the curve at each concentration for the "global" response (i.e. average response of all cells in the microscope's field of view) and Ca^{2+}_o concentration effect curves drawn up and EC_{50} values calculated accordingly (as detailed in 2.9). The resulting curves are shown in Figure 3.4 panel *B*, and these show that the Ca^{2+}_o concentration effect curve

was left-shifted following knockdown of PKCα in the cells. This result indicates that knocking down PKC α made the cells more sensitive to Ca^{2+}_o as was confirmed by statistical comparison of the Ca^{2+}_o EC_{50}s. That is, the EC_{50} for Ca^{2+}_o in the PKCα knockdown cells was significantly lower compared to the mock-transfected control cells (3.10 ± 0.1 mM vs 3.73 ± 0.2 mM; $P < 0.01$). These data suggest therefore that specifically knocking down PKCα in the cells increased CaR sensitivity in CaR-HEK cells.

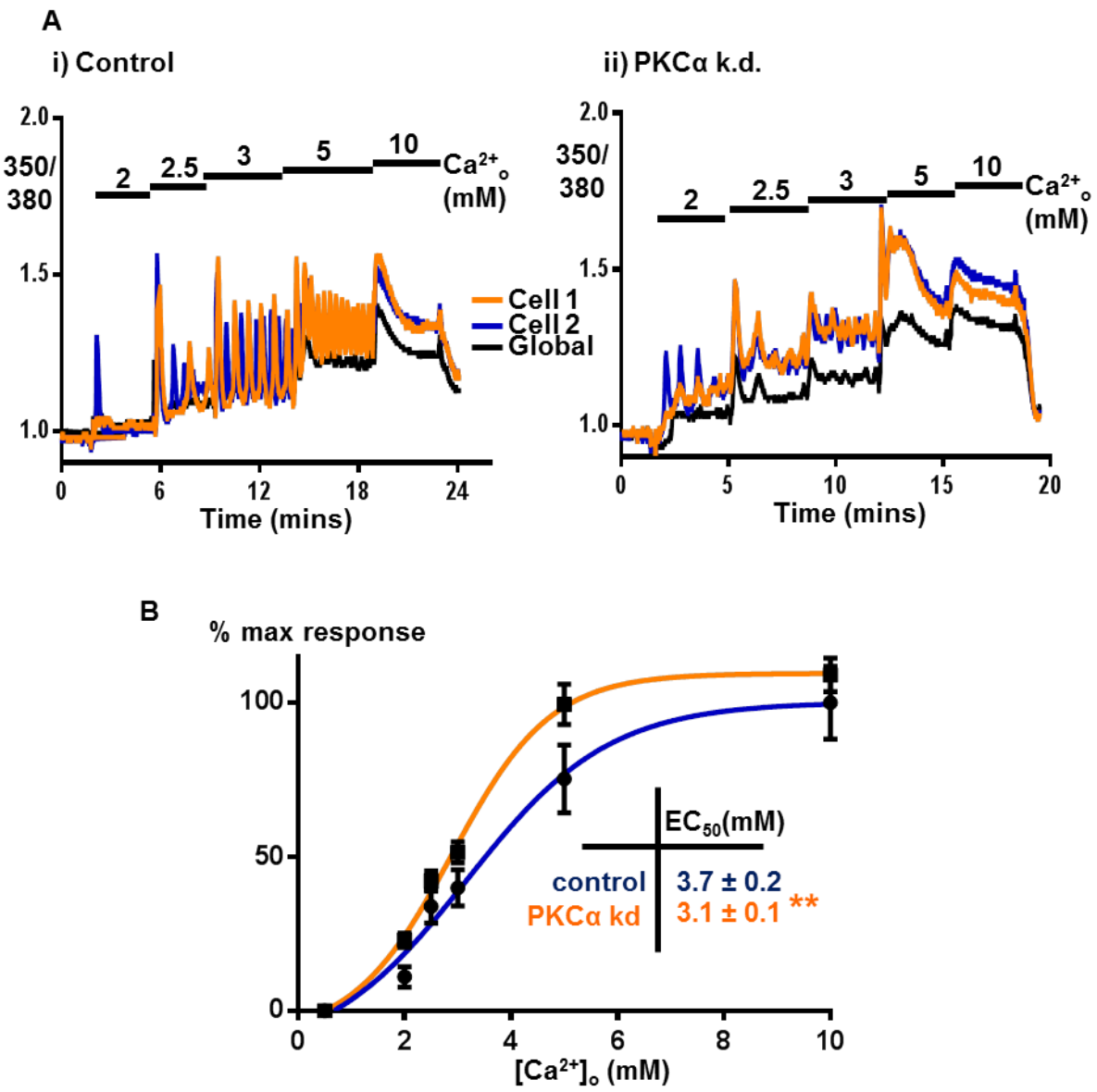

Figure 3.4: PKCα knockdown enhances CaR-responsiveness in CaR-HEK cells. CaR-HEK cells were cultured in media supplemented with 0.2% (v/v) OptiMem and 50 µl/ml (v/v) lipofectamine in the absence or presence of 50 nM PKC α-specific siRNA. *A*). Fura-2 loaded cells were then exposed to increasing concentrations of Ca^{2+}_o (0.5-10 mM) to determine the effect of PKCα knockdown on Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from 2 cells (shown in orange and blue) incubated with (ii) or without (i) the siRNA. The trace shown in black represents the combined responses of all the cells in the field of view ("global"). *B*) Concentration-effect curves showing the enhanced Ca^{2+}_o sensitivity (and significantly reduced EC_{50} for Ca^{2+}_o) in the cells exposed to PKCα siRNA. *P < 0.05 between log EC_{50}s vs control by unpaired t-test; n ≥ 13 coverslips from 4 independent transfections.

3.3.5 Investigation of the effect of PKCβ downregulation on CaR-induced Ca^{2+}_i mobilisation

Having identified in PKCα a kinase likely to limit PKC sensitivity under normal conditions, it was then necessary to determine whether other PKC isotypes also contribute to this effect. Thus, next the effect of knocking down another conventional isotype, PKCβ, on CaR-induced Ca^{2+}_i mobilisation was examined. The PKCβ siRNA used in this study caused the knockdown of both PKCβ splice variants, PKCβI and PKCβII (see Figure 3.3). However there was no obvious difference observed in Ca^{2+}_i mobilisation in the PKCβ knockdown cells compared to the controls (Figure 3.5*A*). Both sets of cells exhibited low or transient Ca^{2+}_i mobilisation in 2 mM Ca^{2+}_o with some cells showing Ca^{2+}_i oscillations in 2.5 mM Ca^{2+}_o and whose oscillation frequency increased in 3 mM Ca^{2+}_o. In 5 and then 10 mM Ca^{2+}_o, most/all cells exhibited sustained Ca^{2+}_i mobilisation. When quantified, there was no significant difference in the Ca^{2+}_o concentration effect curves observed between control and PKCβ knockdown cells (Figure 3.5*B*) such that the EC_{50}s for Ca^{2+}_o of each were not significantly different, with 3.9 ± 0.5 mM for PKCβ knockdown cells and 4.1 ± 0.7 mM for control ($P > 0.05$). From these data it appears that neither PKCβI nor βII are involved in regulating CaR phosphorylation or function.

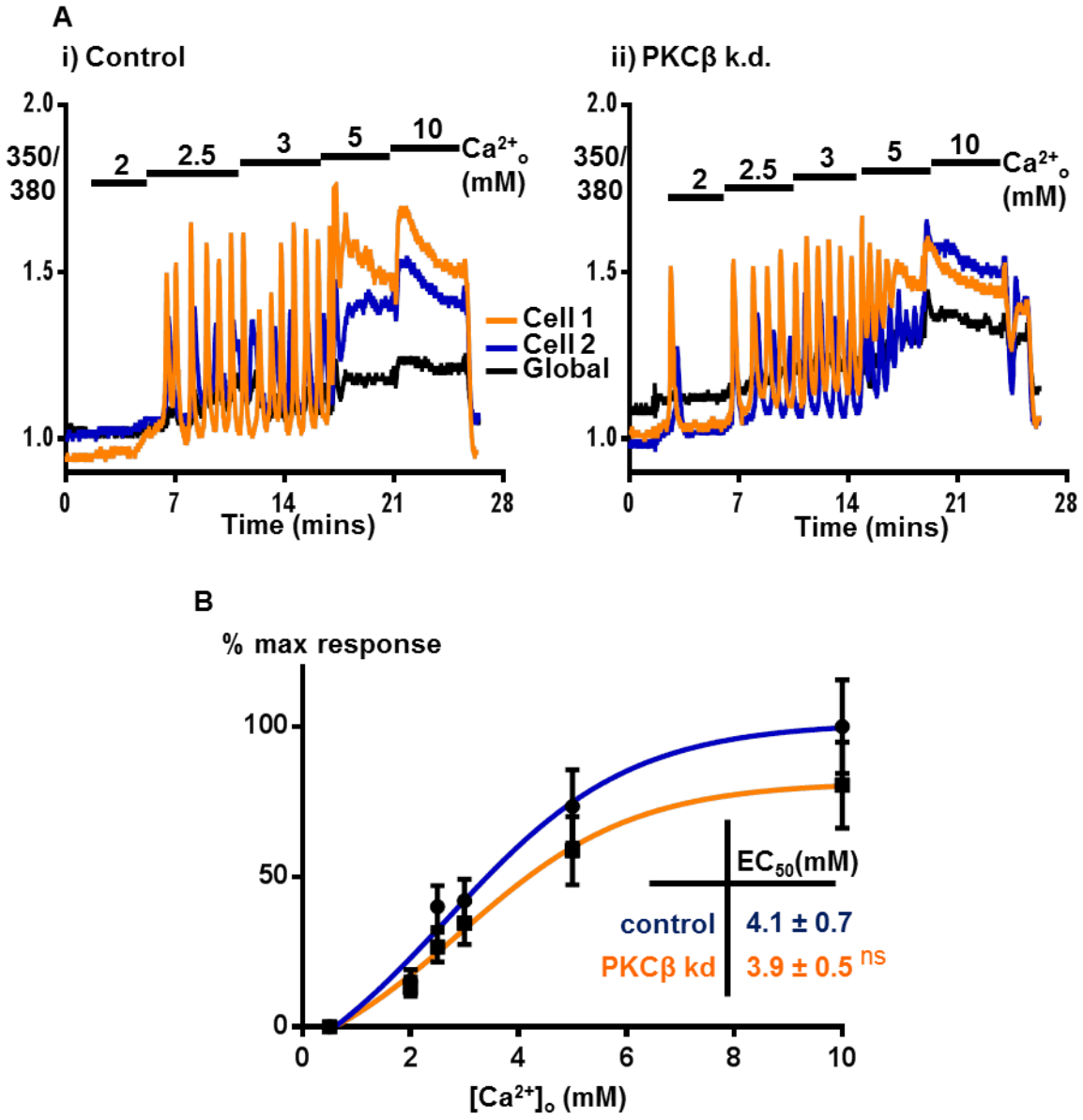

Figure 3.5: PKCβ knockdown is without effect on CaR responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCβ-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) As shown in the representative Ca^{2+}_i traces, PKCβ knockdown failed to alter Ca^{2+}_o responsiveness. *B*) Similarly, the resulting concentration-effect curves from these experiments show no significant different between EC_{50}s for Ca^{2+}_o in the cells incubated with or without PKCβ siRNA. $P > 0.05$ between log EC_{50}s vs control by unpaired t-test; $n \geq 8$ coverslips from 3 independent transfections.

3.3.6 Investigation of the effect of PKCδ downregulation on CaR-induced Ca^{2+}_i mobilisation

The consequence of knocking down the novel PKCδ on CaR-induced Ca^{2+}_i mobilisation was then investigated. Novel PKC isotypes are also activated by DAG (and thus phorbol ester) but they are not activated by calcium as they lack the calcium-binding domain necessary for this. The PKCδ siRNA successfully knocked down PKCδ expression as shown in Figure 3.3 however no functional effect of this on CaR-induced Ca^{2+}_i mobilisation was detected (Figure 3.6*A*). The single cell Ca^{2+}_i dynamic responses were the same in response to 2 - 10 mM Ca^{2+}_o as in control cells. Again, when quantified the EC_{50}s for Ca^{2+}_o for both sets of cells were not significantly different (Figure 3.6*B*; EC_{50} for Ca^{2+}_o, 4.5 ± 0.8 mM for PKCδ knockdown vs 4.2 ± 0.6 mM control; $P > 0.05$). Therefore these data suggest that PKCδ is not involved in CaR negative feedback phosphorylation or receptor function.

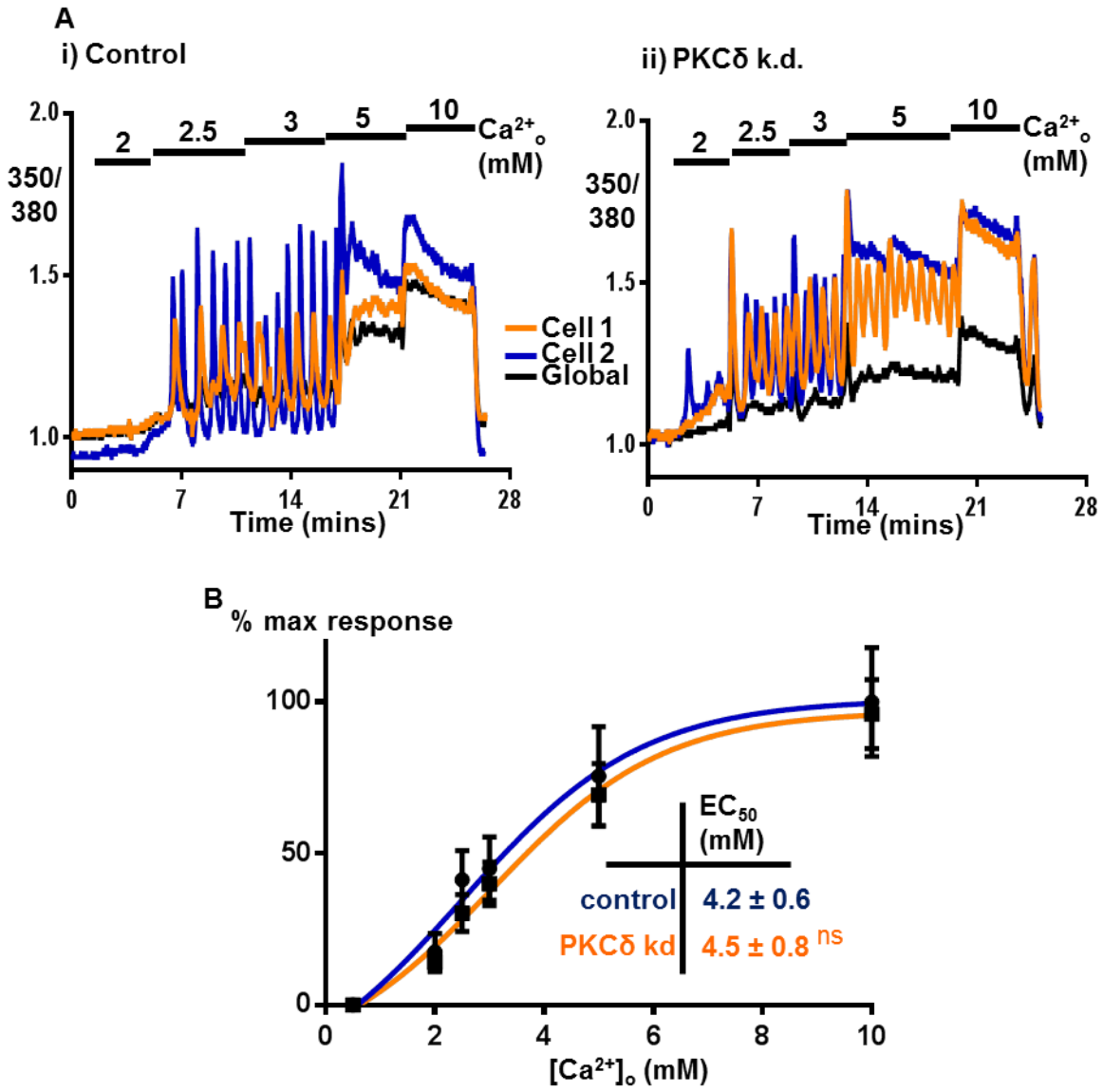

Figure 3.6: PKCδ knockdown does not affect CaR-responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCδ-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) As shown in the representative Ca^{2+}_i traces, PKCδ knockdown failed to alter Ca^{2+}_o responsiveness. *B*) Similarly, the resulting concentration-effect curves from these experiments show no significant difference between EC_{50}s for Ca^{2+}_o in the cells incubated with or without PKCδ siRNA. $P > 0.05$ between log EC_{50}s vs control by unpaired t-test; $n \geq 9$ coverslips from 3 independent transfections.

3.3.7 Investigation of the effect of PKCε downregulation on CaR-induced Ca^{2+}_i mobilisation

The next novel PKC isotype to be investigated was PKCε and the knockdown of PKCε using its isotype-specific siRNA is again shown in Figure 3.3. Perhaps surprisingly, knocking down PKCε did have an effect on Ca^{2+}_i mobilisation induced by CaR but in the opposite direction to that expected (Figure 3.7*A*). That is, the responses to 2.5 and to 3 mM Ca^{2+}_o in PKCε knockdown cells both appeared suppressed relative to those in control cells. In contrast, in response to 5 and then 10 mM Ca^{2+}_o the responses of PKCε knockdown cells were at least as great as those of the control cells and so any inhibition of Ca^{2+}_i mobilisation only occurred at moderate Ca^{2+}_o. Quantification of these responses confirms that in PKCε knockdown cells, the EC_{50} for Ca^{2+}_o was significantly higher (5.3 ± 0.4 mM) than for controls (3.4 ± 0.1 mM; $P < 0.05$) (Figure 3.7*B*). This apparent rightward shift in the concentration-effect curve for Ca^{2+}_o in PKCε knockdown cells indicates decreased sensitivity of the CaR and suggests that as well as inhibiting CaR activity, at least one PKC isotype may also provide a positive effect on CaR signalling that is normally masked by the greater inhibitory action of PKC.

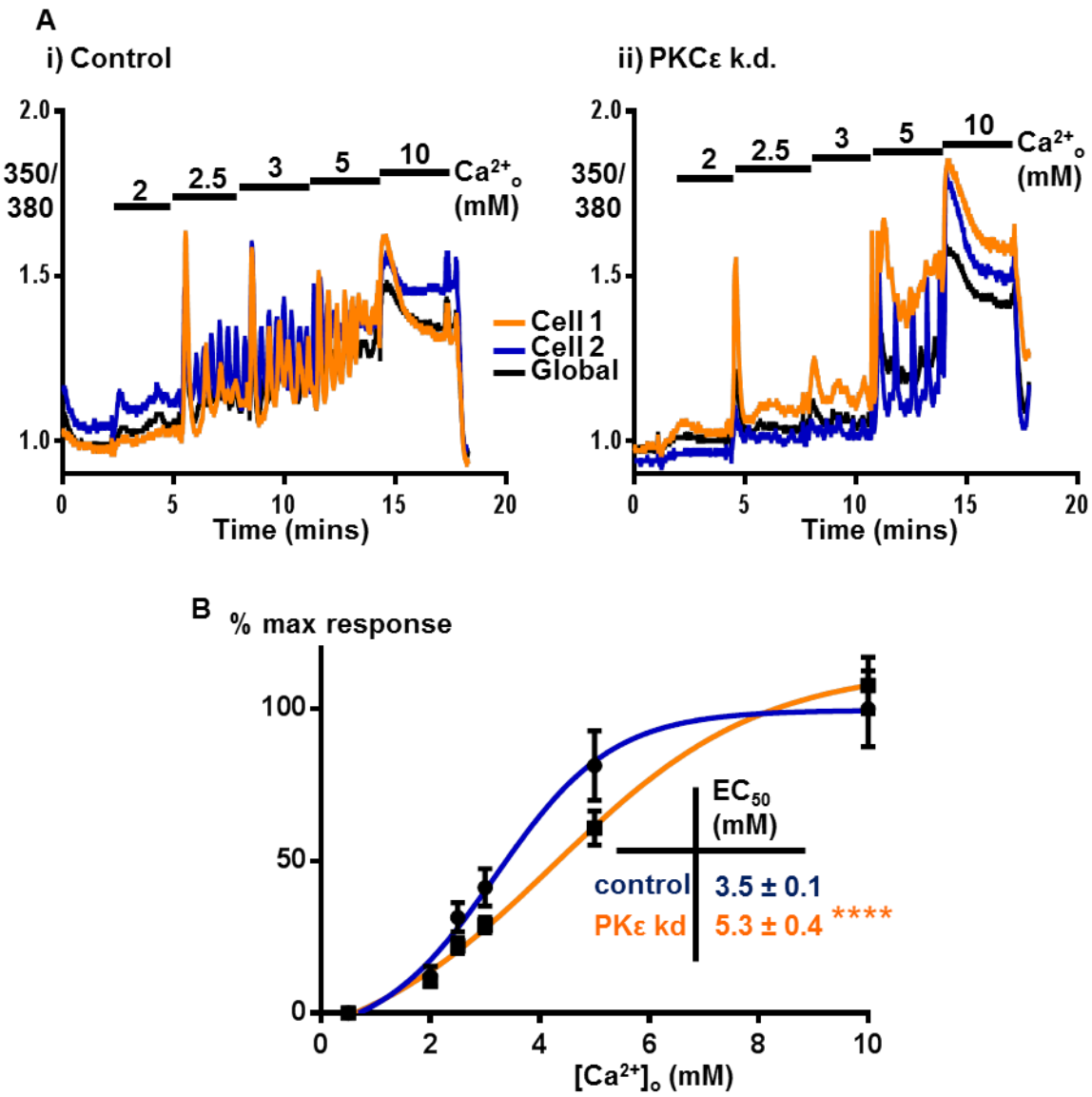

Figure 3.7: PKCε knockdown attenuates CaR-responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCε-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) As shown in the representative Ca^{2+}_i traces, PKCε knockdown failed to alter Ca^{2+}_o responsiveness. *B*) Concentration-effect curves showing the reduced Ca^{2+}_o sensitivity (and significantly reduced EC_{50} for Ca^{2+}_o) in the cells exposed to PKCε siRNA. ****P < 0.0001 between log EC_{50}s vs control by unpaired t-test; n ≥ 13 coverslips from 4 independent transfections.

3.3.8 Investigation of the effect of PKCθ downregulation on CaR-induced Ca^{2+}_i mobilisation

The next PKC to be investigated was the novel PKCθ and the selectivity of its knockdown is shown in Figure 3.3. However, knocking down PKCθ caused no obvious difference in the dynamics of CaR-induced Ca^{2+}_i mobilisation in the CaR-HEK cells at any of the Ca^{2+}_o concentrations tested (Figure 3.8*A*). In addition, quantification of the relative sensitivities for Ca^{2+}_o between the PKCθ and control cells did not show a significant difference between EC_{50} values (Figure 3.8*B*; EC_{50} for Ca^{2+}_o, 3.2 ± 0.1 mM, PKCθ knockdown vs. 3.3 ± 0.3 mM, control; $P > 0.05$;). Therefore, PKCθ is unlikely to regulate CaR-induced Ca^{2+}_i mobilisation.

3.3.9 Investigation of the effect of PKCη downregulation on CaR-induced Ca^{2+}_i mobilisation

Although chronic PMA pretreatment failed to alter (novel) PKCη expression in the CaR-HEK cells (Figure 3.1), in the interests of completeness it was decided to also test the consequence of knocking down PKCη on CaR-induced Ca^{2+}_i mobilisation. As shown in Figure 3.9*A*, anti-PKCη siRNA pretreatment did not cause any apparent change in Ca^{2+}_o-induced Ca^{2+}_i mobilisation in the cells. Successful knockdown of PKCη using siRNA was confirmed by western blot analysis as shown in Figure 3.9*C*, though off-target effects were not tested for in this case as there had been no change in CaR signalling in any case. Again, following quantification of the Ca^{2+}_i changes there was no significant difference between the EC_{50} vales for Ca^{2+}_o between PKCη knockdown and control cells (Figure 3.9*B*; EC_{50} for Ca^{2+}_o, 3.9 ± 0.0 mM vs 3.5 ± 0.1 mM respectively; $P > 0.05$).

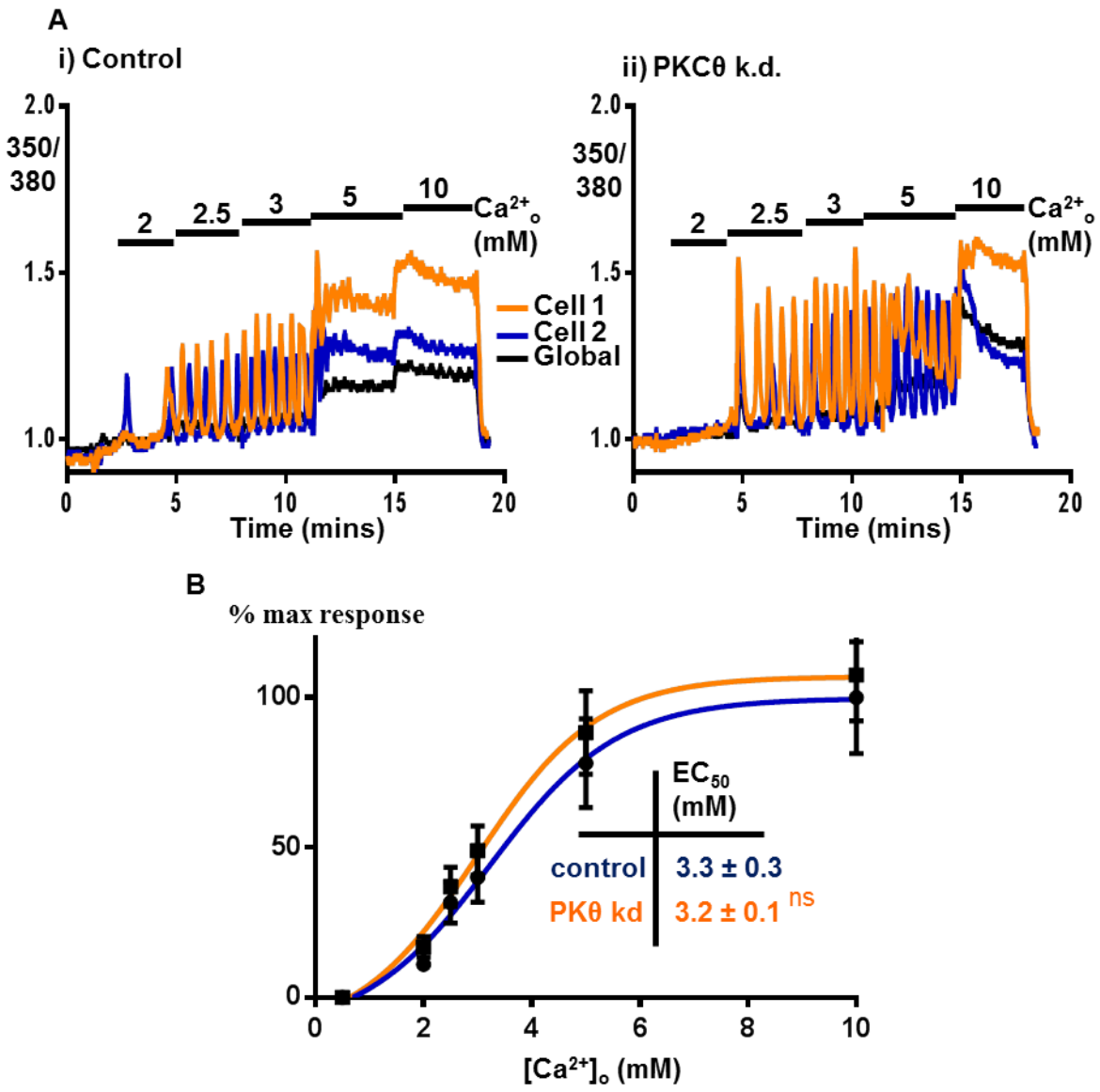

Figure 3.8: PKCθ knockdown does not affect CaR-responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCθ-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) As shown in the representative Ca^{2+}_i traces, PKCθ knockdown failed to alter Ca^{2+}_o responsiveness. *B*) Similarly, the resulting concentration-effect curves from these experiments show no significant difference between EC_{50}s for Ca^{2+}_o in the cells incubated with or without PKCθ siRNA. $P > 0.05$ between log EC_{50}s vs control by unpaired t-test; $n \geq 12$ coverslips from 3 independent transfections.

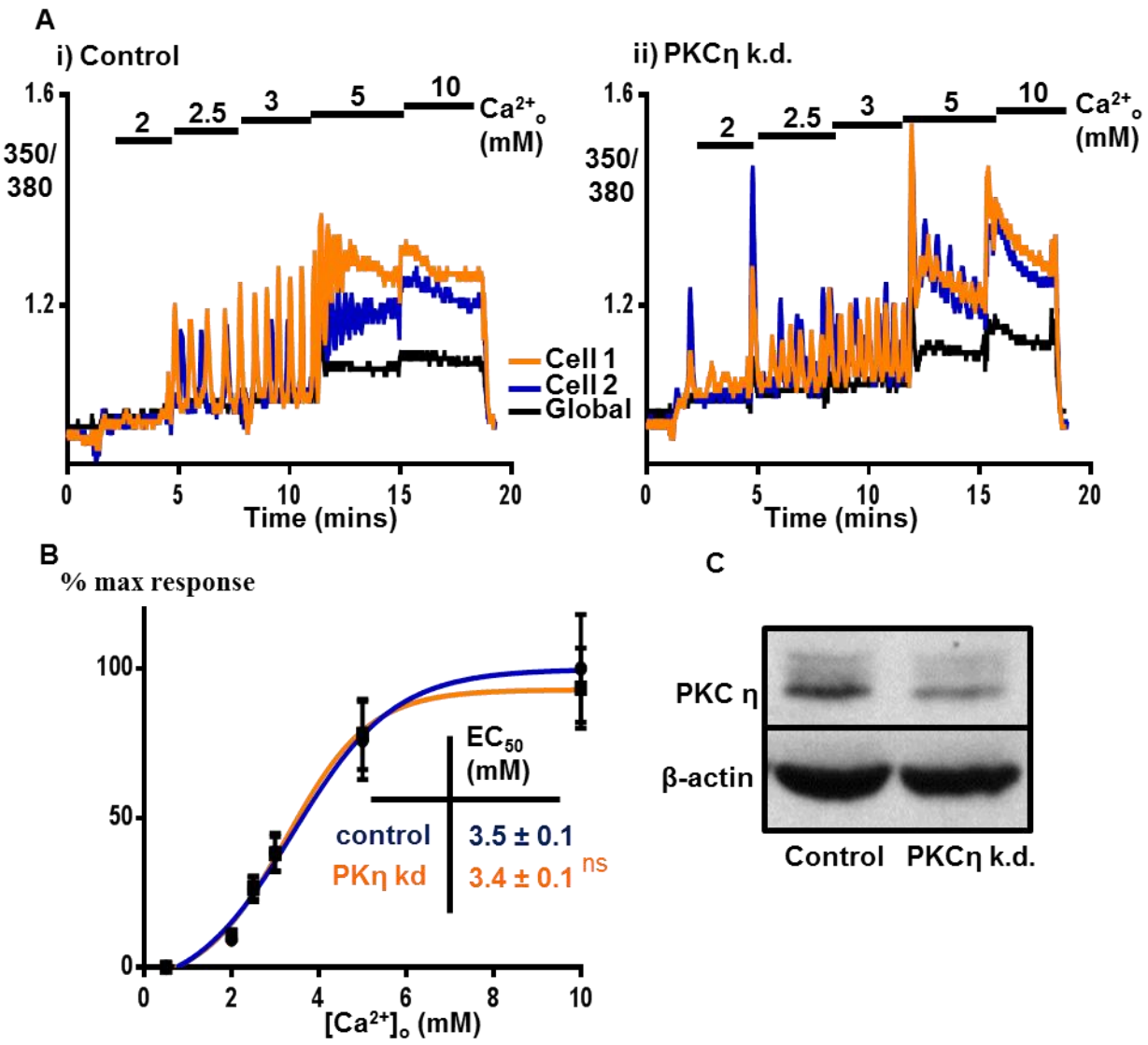

Figure 3.9: PKCη knockdown does not affect CaR-responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCη-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) As shown in the representative Ca^{2+}_i traces, PKCη knockdown failed to alter Ca^{2+}_o responsiveness. *B*) Similarly, the resulting concentration-effect curves from these experiments show no significant difference between EC_{50}s for Ca^{2+}_o in the cells incubated with or without PKCη siRNA. $P > 0.05$ between log EC_{50}s vs control by unpaired t-test; $n \geq 12$ coverslips from 3 independent transfections. *C*) Successful knockdown of PKCη by the siRNA was confirmed by immunoblotting using a specific antibody, with β-actin used as a loading control.

3.3.10 Treatment of CaR-HEK cells with control siRNA failed to alter CaR-induced Ca^{2+}_i mobilisation.

Having already shown that control siRNA treatment versus mock transfection (i.e. lipofectamine only) had no effect on the expression of any PKC isotypes in CaR-HEK cells (Figure 3.3), I next confirmed that the presence or absence of control siRNA was without a baseline effect on CaR-induced Ca^{2+}_i mobilisation. As a positive control I also retested the effect of anti-PKCα siRNA to confirm the positive modulatory effect of PKCα knockdown. As shown in Figure 3.10*A*, there was no apparent difference in CaR-induced Ca^{2+}_i mobilisation between the cells exposed to the control siRNA and the mock-transfected cells at any concentration between 2 and 10 mM Ca^{2+}_o. In contrast, PKCα knockdown elicited greater Ca^{2+}_i mobilisation responses to 2-3 mM Ca^{2+}_o, as before (Figure 3.4). This was confirmed statistically following the quantification of the concentration-effect responses (Figure 3.10*B*) with virtually identical Ca^{2+}_o concentration EC_{50} values between control siRNA and mock-transfected cells (3.1 ± 0.1 mM and 3.2 ± 0.1 mM respectively; $P > 0.05$) but with PKCα knockdown cells exhibiting greater sensitivity to Ca^{2+}_o (2.7 ± 0.1 mM; $P < 0.01$ vs control siRNA; $P < 0.05$ vs mock). Therefore, no molecular or functional differences were detected between mock-transfected and control siRNA-transfected cells and thus the effect of the anti-PKCα siRNA is most likely due to the loss of PKCα protein rather than an off-target effect of siRNA molecules.

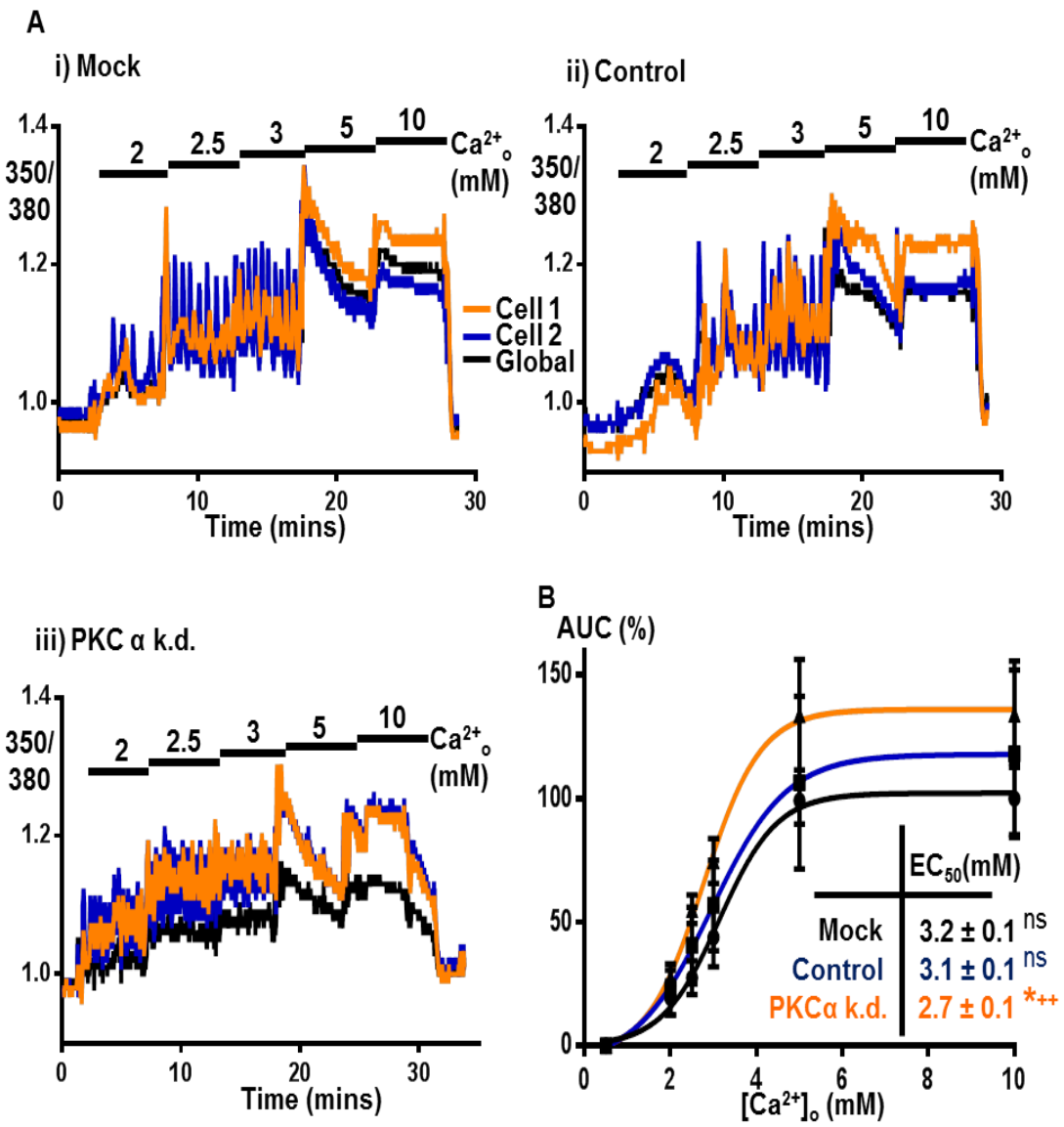

Figure 3.10: Control siRNA and mock transfection resulted in similar CaR responsiveness in CaR-HEK cells while PKCα knockdown enhanced CaR-induced Ca^{2+}_i mobilisation. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to either 50 µl/ml transfection reagent alone (mock), 50 nM control siRNA or 50 nM PKCα-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) Fura2-loaded cells were then exposed to increasing concentrations of Ca^{2+}_o (0.5-10 mM) to determine the effect of the pretreatments on CaR-induced Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from cells from the treatments. *B*) The concentration-effect curves showing no significant difference between log EC_{50}s for Ca^{2+}_o in the control and mock-transfected cells, but a significantly lower EC_{50} for Ca^{2+}_o in the PKCα knockdown cells. *P < 0.05 vs mock transfection;$^{++}$ P<0.01 vs control siRNA; ns P > 0.05 between log EC_{50}s of control vs mock by Kruskal–Wallis test followed by Mann–Whitney test with the adjustment for multiple comparison; n ≥ 3 coverslips from 2 independent transfections.

3.3.11 Determination of the relative effects of PKC inhibition on muscarinic receptor and CaR-induced Ca^{2+}_i mobilisation

The CaR-HEK cells used in the current study endogenously express other $G_{q/11}$-coupled GPCRs including a carbachol-sensitive muscarinic receptor. Therefore, here I made use of this endogenous activation pathway to test whether the effect of modulating PKC activity is specific to the CaR in these cells, or, a more general effect on Ca^{2+}_i mobilisation in the cell, perhaps by altering the phosphorylation of the IP_3 receptor for example. For this, I employed the same readout for both receptors, namely Ca^{2+}_i mobilisation.

The cells were first exposed to 3mM Ca^{2+}_o to elicit oscillatory Ca^{2+}_i mobilisation and then 1 μM of the general PKC inhibitor, GF102903X added. As expected, GF102903X increased the response of CaR-HEK cells to Ca^{2+}_o, converting the oscillations into sustained Ca^{2+}_i mobilisation (Figure 3.11*A*). In contrast, when 10 μM carbachol was instead used to induce Ca^{2+}_i oscillations, the addition of the same concentration of GF102903X failed to alter the carbachol-induced Ca^{2+}_i responses of the cells. The lack of effect of GF109203X on the carbachol response was confirmed by determining the AUC of the Ca^{2+}_o- induced responses which showed the Ca^{2+}_i mobilisation was not significantly altered by GF102903X (Figure 3.11*B* ; $P > 0.05$). This study suggests that the effect of PKC inhibition on oscillatory Ca^{2+}_i mobilisation is not a general effect and therefore might be relatively specific to the CaR.

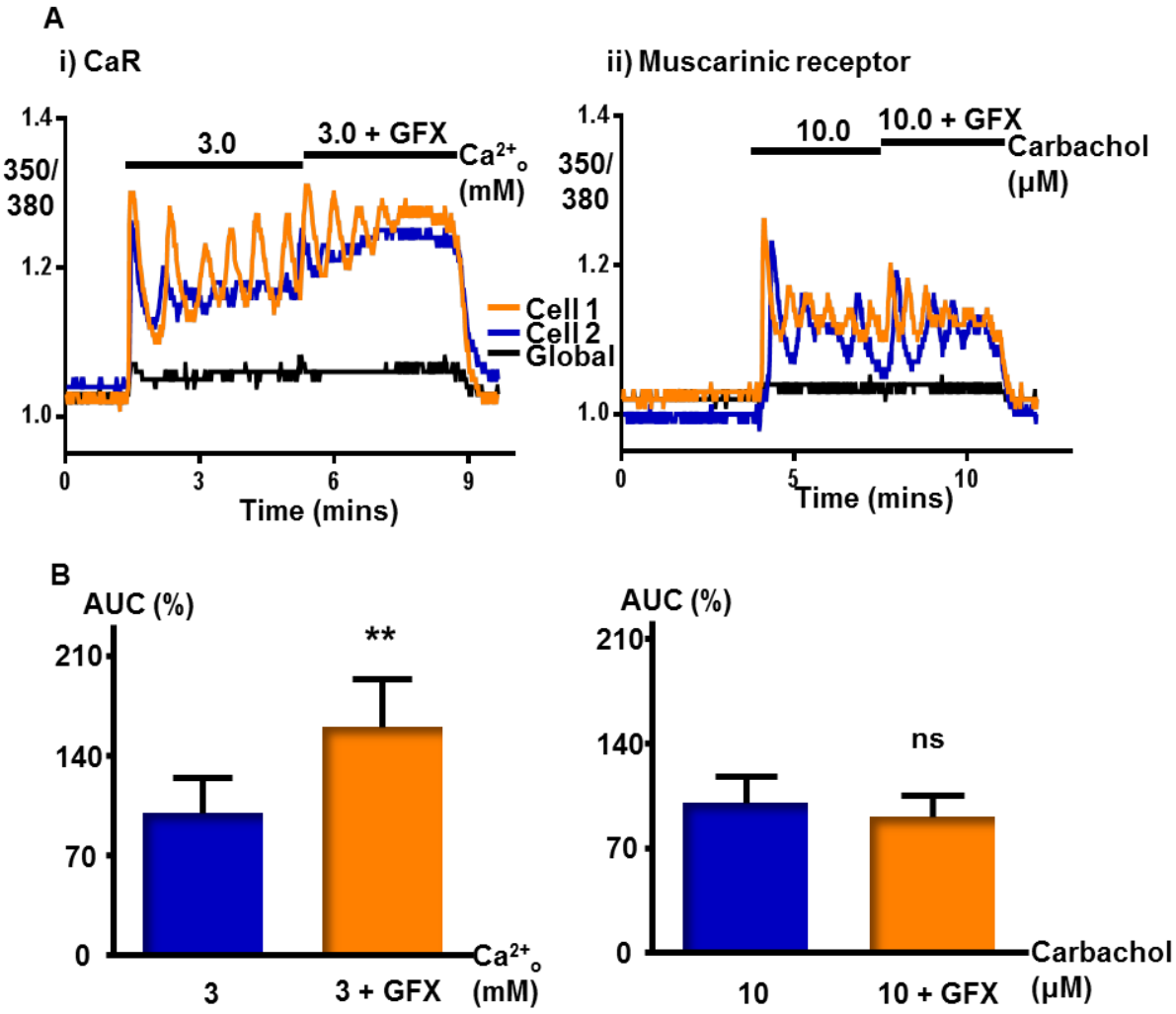

Figure 3.11: PKC inhibition by GF102903X enhances CaR but not muscarinic receptor responsiveness in CaR-HEK. *A*) Fura-2 loaded CaR-HEK cells were exposed to 3 mM Ca^{2+}_o or 10 μM carbachol with or without 1 μM GF102903X to determine the PKC inhibition effect on Ca^{2+}_i mobilisation induced by CaR and muscarinic receptor stimulation. Shown are representative Ca^{2+}_i traces from the cells. *B*) Quantification of the change in the percentage of area under curve/minutes (AUC %) normalised to the response to 3.0 mM Ca^{2+}_o and 10μM carbachol in untreated cells. GF102903X treatment significantly increased. The percentage AUC in cells exposed to Ca^{2+}_o but not to carbachol. **P < 0.01 on the pre-normalised data for before and during GF102903X treatment by paired t-test; n = 6 coverslips from 2 independent transfections.

3.3.12 Investigation of the effect of PKCα downregulation on muscarinic receptor-induced Ca^{2+}_i mobilisation.

To further investigate whether the effects of knocking down PKCα and PKCε on Ca^{2+}_i mobilisation are CaR-specific, PKCα and ε expression were decreased as before using siRNA and carbachol-induced Ca^{2+}_i mobilisation examined. The objective was to see whether carbachol responses would also be altered by modifying PKCα or ε expression. It was found that knocking down PKCα had no effect on muscarinic receptor-induced Ca^{2+}_i mobilisation (Figure 3.12*A*). In this experiment, a range of carbachol concentrations were tested (0.1-100 μM), with 1 μM carbachol giving detectable Ca^{2+}_i mobilisation, 5 μM giving robust oscillations and 10 μM carbachol giving largely maximal responses. However, the carbachol concentration-effect curves generated for control and PKCα knockdown cells were very similar (Figure 3.12*B*) and the carbachol EC_{50} values for both cells were not significantly different (control, 3.1 ± 0.7 μM, vs PKCα knockdown, 3.9 ± 0.8 μM; $P > 0.05$). This result confirms that while PKCα knockdown may enhance Ca^{2+}_o responsiveness in CaR-HEK cells, it is without effect on the responses to carbachol.

3.3.13 Investigation of the effect of PKCε downregulation on muscarinic receptor-induced Ca^{2+}_i mobilisation

Next, the effect of PKCε downregulation on muscarinic receptor-induced Ca^{2+}_i mobilisation was tested. The experiment was performed exactly as described in 3.3.12 except using anti-PKCε siRNA, which had been shown to knockdown PKCε expression in Figure 3.3. Again, carbachol elicited a concentration-dependent increase in Ca^{2+}_i mobilisation (Figure 3.13*A*) and this response was not altered by knocking down PKCε expression. Indeed, the EC_{50} for both sets of cells were not significantly different (Figure 3.13*B;* control, 3.0 ± 0.6 μM; PKC ε, 5.0 ± 1.0 μM; $P > 0.05$). This demonstrates that the apparent inhibitory action of PKCε knockdown on Ca^{2+}_o-induced Ca^{2+}_i mobilisation was not seen for muscarinic receptor signalling.

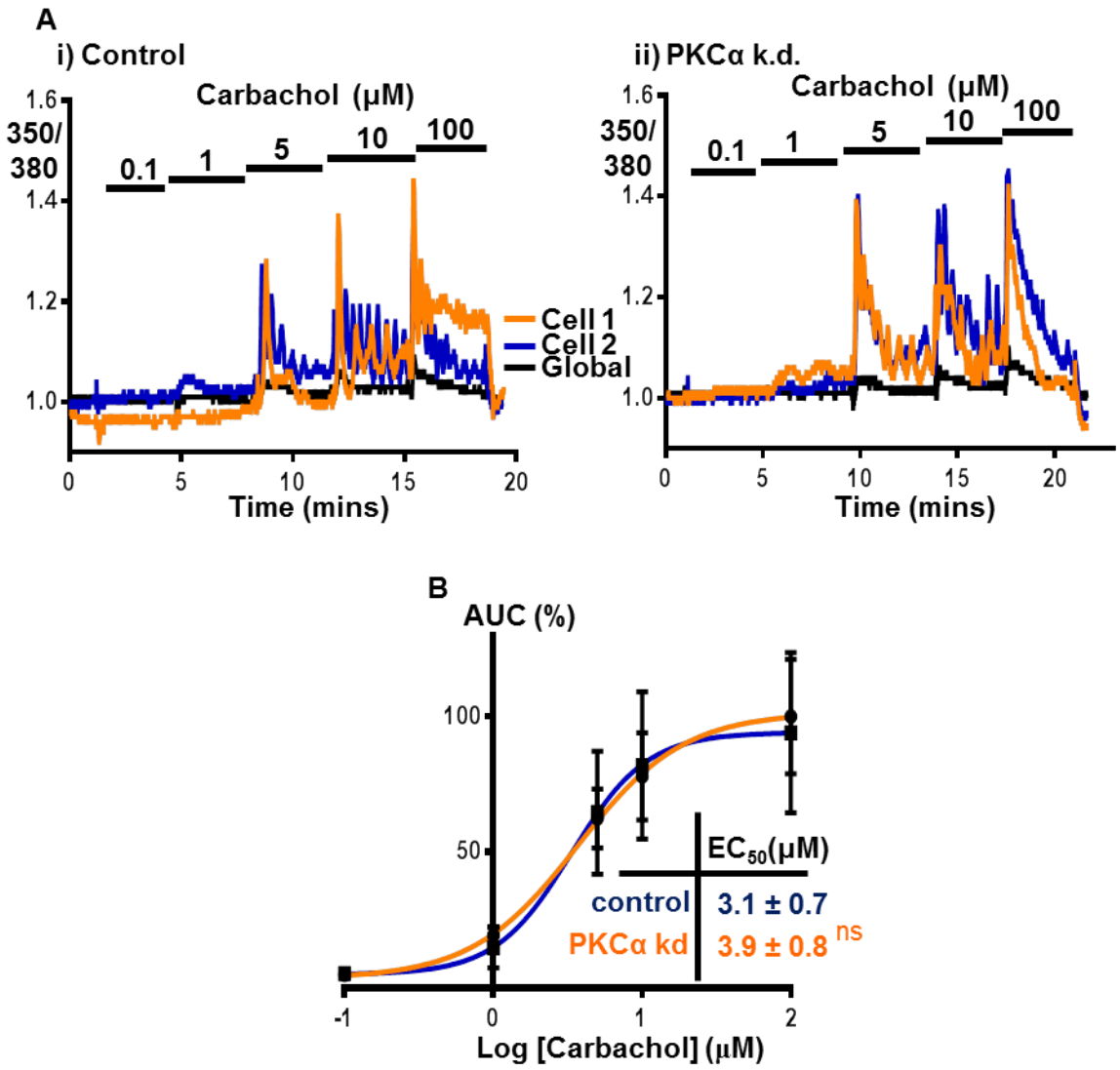

Figure 3.12: PKCα knockdown does not affect muscarinic receptor responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCη-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) Fura-2 loaded cells were then exposed to increasing concentrations of carbachol (0.1-100 μM) to determine the effect of PKCα knockdown on Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from cells incubated with or without the siRNA. *B*) The concentration-effect curves showing no significant different between EC_{50}s for carbachol in the cells expressing less PKCα and control. $P > 0.05$ between log EC_{50}s vs control by unpaired t-test; n = 6 coverslips from 2 independent transfections.

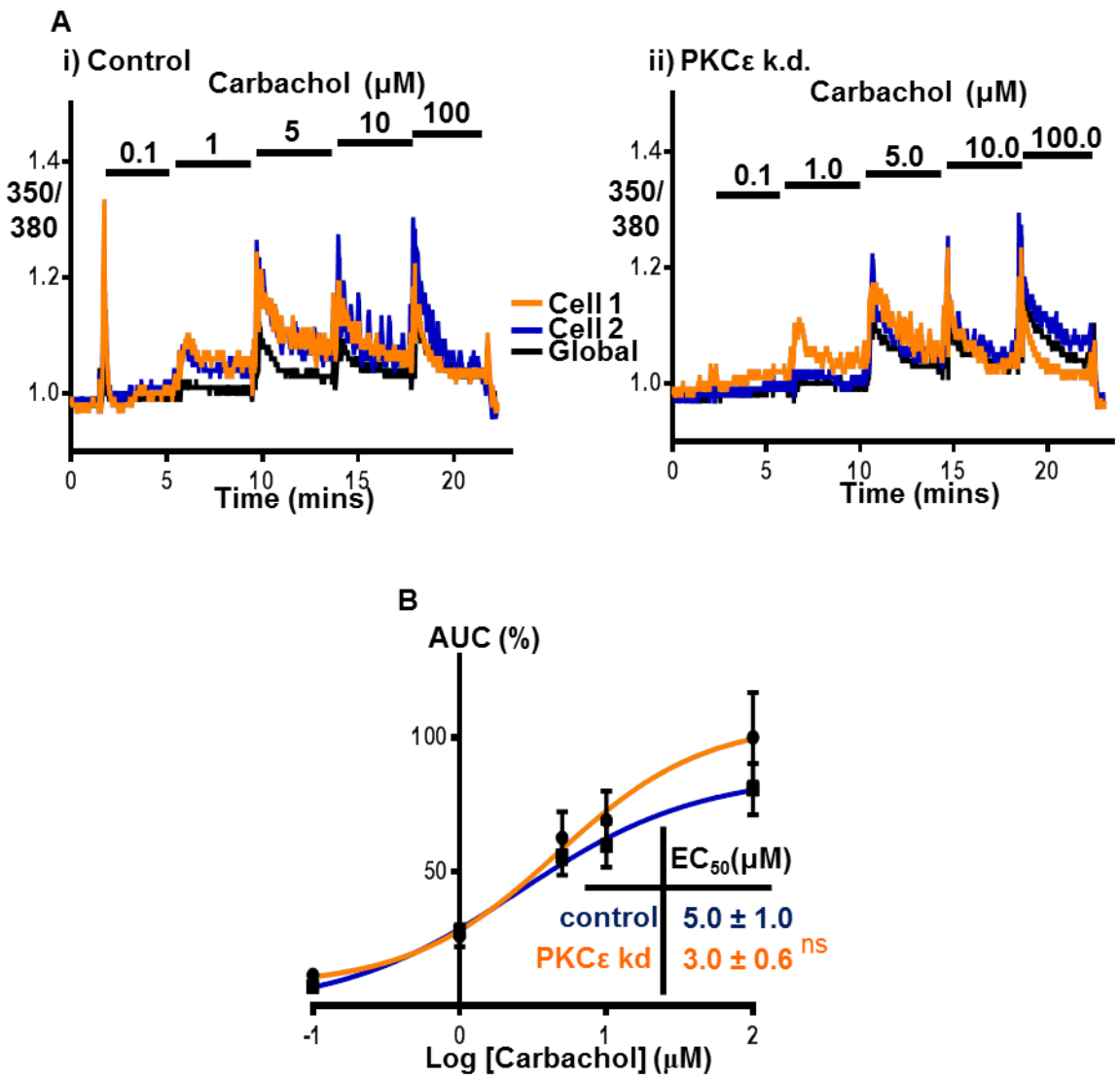

Figure 3.13: PKCε knockdown does not affect muscarinic receptor responsiveness in CaR-HEK cells. The Ca^{2+}_o sensitivity of CaR-HEK cells exposed to 50 nM PKCε-specific siRNA was performed as described in the Legend to Figure 3.4. *A*) Fura-2 loaded cells were then exposed to increasing concentrations of carbachol (0.1-100 μM) to determine the effect of PKCε knockdown on Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from cells incubated with or without the siRNA. *B*) The concentration-effect curves showing no significant different between EC_{50}s for carbachol in the cells expressing less PKCα and control. $P > 0.05$ between log EC_{50}s vs control by unpaired t-test; n = 6 coverslips from 2 independent transfections.

3.3.14 Investigation of the effect of PKCα downregulation on CaR^{T888} phosphorylation

Having found that downregulation of PKCα and ε affects Ca^{2+}_o- but not carbachol-induced Ca^{2+}_i mobilisation in CaR-HEK cells, next I investigated whether levels of CaR^{T888} phosphorylation were affected by PKC knockdown. The phosphorylation of CaR^{T888} acts as a negative regulator of CaR and is associated with attenuation of Ca^{2+}_i mobilisation and suppression of PTH secretion (McCormick *et al*., 2010, Lazarus *et al*., 2011). Therefore, next the CaR-HEK cells were treated with or without PKCα siRNA and then incubated in 0.5-2.5 mM Ca^{2+}_o with their phospho-CaR^{T888} contents then analysed by immunoblotting (Figure 3.14). The resulting western blot analysis shows that the antiphospho-CaR^{T888} antibody detected two CaR bands at 140 kDa and 160 kDa as we have reported previously (Davies *et al*., 2007). The 160 kDa band represents the mature glycosylated form of CaR, while the 140 kDa band is the high mannose, immature CaR that is presumed to be an intracellular species. In this study I focused on the 160 kDa CaR band as it represents the active mature CaR located at least in part on the cell surface. The phosphorylation of 160 kDa CaR was barely detected following incubation (10-mins at 37ºC) in 0.5 mM Ca^{2+}_o (Figure 3.14*A*) but was increased upon exposure to 2.5 mM Ca^{2+}_o. However, in cells in which PKCα had been first knocked down, CaR^{T888} phosphorylation in 2.5 mM Ca^{2+}_o was lower than for control cells. The same observation was also made for the 140 kDa CaR band. The successful knockdown of PKCα was confirmed by western blot as was the consistent expression of total CaR in each sample. In addition, β-actin levels were also equivalent in each lane. The level of 160 kDa CaR^{T888} phosphorylation in each sample was quantified by densitometry, corrected for total CaR content and then normalised to the 2.5 mM Ca^{2+}_o control. By this it was confirmed both that 2.5mM Ca^{2+}_o significantly increased CaR^{T888} phosphorylation relative to 0.5mM (Figure 3.14*B*; $P < 0.0001$), and, that PKCα knockdown significantly inhibited the response to 2.5m Ca^{2+}_o (Figure 3.14*B*; $P < 0.01$). This result confirms that PKCα is most likely the kinase responsible for CaR^{T888} phosphorylation in CaR-HEK cells.

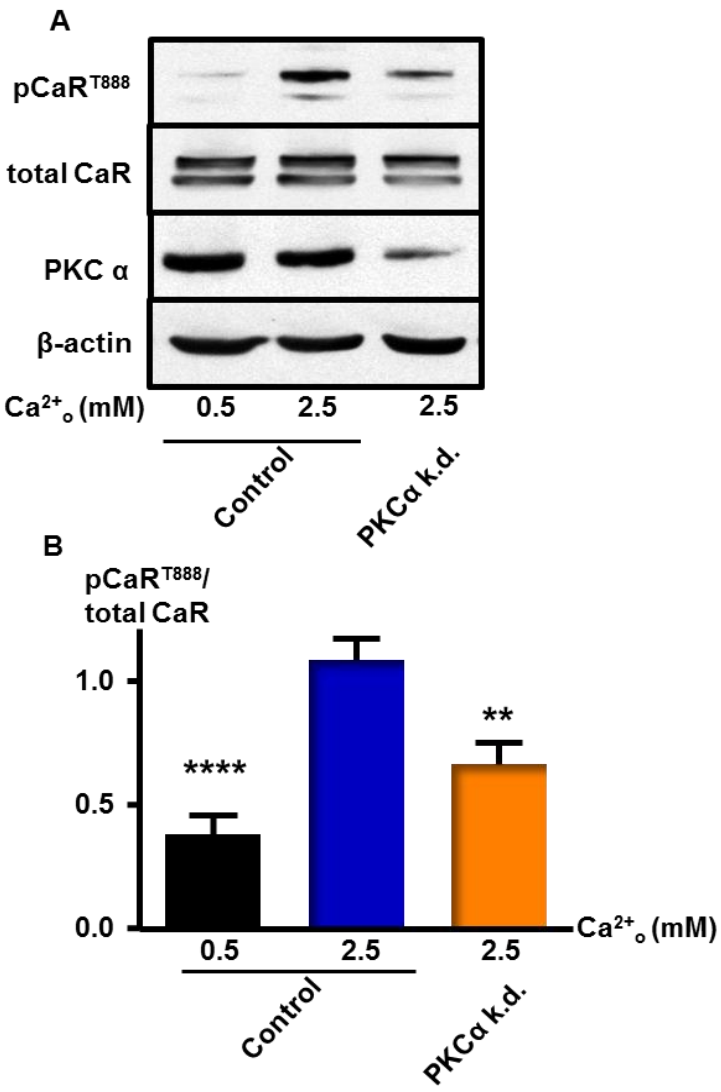

Figure 3.14: PKCα knockdown inhibits CaR^{T888} phosphorylation in CaR-HEK cells. CaR-HEK cells were treated with either 50 nM control siRNA or PKCα-specific siRNA as described in the Legend to Figure 3.4 and then exposed to 0.5 or 2.5 mM Ca^{2+}_o–containing buffer. The cells were then lysed using RIPA buffer and processed for western blotting. *A*) The phosphorylation of CaR^{T888} was increased in 2.5 mM Ca^{2+}_o but this was inhibited in cells with reduced PKCα abundance. Successful PKCα knockdown is shown in the PKCα panel. *B*) Immunoreactivity quantification showing the mean changes in CaR^{T888} phosphorylation, corrected for total CaR expression and normalised to the 2.5 mM Ca^{2+}_o control cell response. ****P < 0.0001, **P < 0.01 vs 2.5 mM Ca^{2+}_o by One-Way ANOVA with Dunnett's post-test on the pre-normalised data; n = 9 replicates from 3 independent transfections.

3.3.15 Investigation of the effect of PKCε downregulation on CaR^{T888} phosphorylation

Given that PKCε knockdown caused a significant suppression of Ca^{2+}_i mobilisation (Figure 3.7) next it was examined whether PKCε downregulation could also affect CaR^{T888} phosphorylation, for example by increasing it and thus inhibiting the receptor. The experiment was performed exactly as done in 3.3.14 except using PKCε-specific siRNA. As before, 2.5mM Ca^{2+}_o significantly increased CaR^{T888} phosphorylation (Figure 3.15*B*; $P < 0.001$). However, in the PKCε knockdown cells instead of increasing the CaR^{T888} phosphorylation, 2.5mM Ca^{2+}_o actually elicited significantly less CaR^{T888} phosphorylation than in the control siRNA-treated cells (Figure 3.15*B*; $P < 0.05$). Also, a similar observation was made for the 140 kDa immature CaR band. It was confirmed by immunoblotting that PKCε had been knocked down in the study and that total CaR and β-actin levels were consistent in each lane.

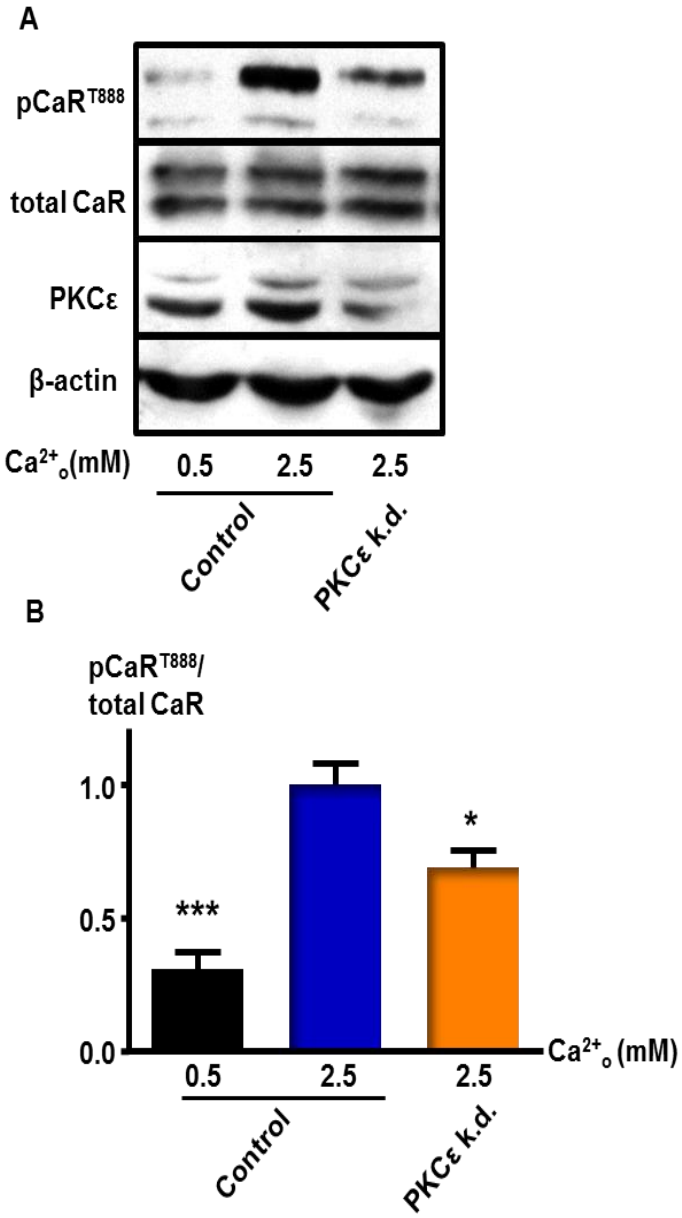

Figure 3.15: PKCε knockdown inhibits CaR^{T888} phosphorylation in CaR-HEK cells. CaR-HEK cells were exposed to 50 nM control or PKCε-specific siRNAs (as described in the Legend to Figure 3.4) and exposed to 0.5 or 2.5 mM Ca^{2+}_o. Cells were then lysed using RIPA buffer and then processed for western blotting. *A*) The phosphorylation of CaR^{T888} was increased in 2.5 mM Ca^{2+}_o but this was inhibited in cells with lowered PKCε abundance. Successful PKCε knockdown is shown in the PKCε immunoblot. *B*) Immunoreactivity quantification showing the mean changes in CaR^{T888} phosphorylation, corrected for total CaR expression and normalised to the 2.5 mM Ca^{2+}_o control cell response. ***P < 0.001, *P < 0.05 vs 2.5 mM Ca^{2+}_o by one-way ANOVA with Dunnett's post-test on the pre-normalised data; n = 8 replicates from 3 independent transfections.

3.4 DISCUSSION

3.4.1 Use of HEK-293 cells to model calcium-sensing receptor behaviour

The cells used in all studies here were HEK-293 cells stably transfected with the human CaR (CaR-HEK). Although ultimately it is cells that express the CaR endogenously that are the best models with which to study CaR signalling, the current lack of immortal CaR-expressing cell lines, particularly of parathyroid origin make CaR-transfected HEK-293 cells the best-used model in the CaR field. Nevertheless, CaR-HEK cells have proven a reasonably reliable model to date with numerous examples of CaR behaviour in HEK-293 cells also being observed in cells of calciotropic origin. As described in McCormick *et al* (2010), the activation of CaR in both HEK-293 and parathyroid cells triggered similar responses including IP_3-generated (oscillatory) Ca^{2+}_i mobilisation, decreased cAMP levels and activation of MAP kinases. Neither cell type shows electrical excitability in response to raised extracellular K^+ and, similarly to parathyroid cells, activation of CaR significantly increases the PTH mRNA transcript instability in HEK-293 cells that had been co-transfected with PTH gene (Galitzer *et al*., 2009). There was also a similar attenuation of CaR-induced Ca^{2+}_i mobilisation by both PKC activation and PP1/PP2A inhibition in HEK-293 and parathyroid cells, while PKC activation causes CaR^{T888} phosphorylation in both cell-types (McCormick *et al*., 2010). Finally, the gain-of-function signalling seen with CaR^{T888A} *in vitro* in this laboratory (Davies *et al*., 2007) was confirmed in a clinical collaboration in which a renal patient with a CaR^{T888M} mutation exhibited chronically suppressed PTH secretion i.e. ADH (Lazarus *et al*., 2011).

Microarray analysis of our CaR-HEK cells (unpublished data) suggests they express all conventional and novel PKCs except for PKC γ, which is known to be involved in memory signalling and is highly expressed in neurons (Saito and Shirai, 2002). The potential expression profile of the various PKCs detected by microarray was confirmed by western blot analysis (Figure 3.1). It should be noted that HEK-293 cells were first generated in the 1970s (Graham *et al*., 1977) through immortalisation of human embryonic kidney cells using sheared fragments of adenovirus type-5 DNA and represent one of the most commonly used human cell lines in bioscientific research.

Although derived from human embryonic kidney tissue there is some evidence that their transcriptomic profile is more neuronal than renal (Shaw, 2012) but they are not excitable, and do not express PKCγ as shown here and therefore were considered suitable for use in the current study as the best available model for parathyroid signalling. When a reliable parathyroid cell model eventually emerges, then it will be important to have these findings validated using it.

3.4.2 Effect of chronic PMA pre-treatment on PKC expression in CaR-HEK

It is known that PKC activity is responsible for the negative feedback phosphorylation of CaR (Young, *et al*., 2002; Davies *et al*., 2007; Young *et al*., 2014). Mutagenesis of the main PKC site in CaR, the threonine at position 888, causes the potentiation of CaR responsiveness towards its stimulants (Bai *et al*., 1998b). In addition, downregulation of PKC by chronic PMA treatment in CaR-expressing cells was also found to increase the response of the receptor functionally by converting CaR-induced Ca^{2+}_i oscillatory signalling into sustained Ca^{2+}_i mobilisation in CaR-HEKs (Davies *et al*., 2007). This treatment affects conventional and novel PKC isotypes which are naturally activated by DAG. Phorbol esters, such as PMA, have sufficient structural similarities to DAG, that they possess a similar ability to activate conventional and novel PKCs. This chronic PMA pretreatment causes overactivation of the conventional and novel PKC isotypes leading to their depletion over a number of hours (Matta and Mobasheri, 2014). Because of this PMA characteristic, it can be used to distinguish between the effects of DAG-activated PKC (conventional and novel) and non DAG-activated PKC (atypical) cell signalling. Since chronic PMA pretreatment increases CaR activity, the downregulation of conventional and novel PKC isotypes by this chronic treatment was studied first in order to help identify potential PKC isotypes that may contribute to CaR phosphorylation / regulation. The potentiation of CaR activity shown in Figure 3.11 (and previously in Davies *et al*., 2007) by this treatment is therefore most likely due to the effect of chronic PMA-induced downregulation of one or more of the PKC isotypes. As the CaR-HEK cells were exposed to PMA overnight, the expression of PKCα, βI and II, δ, ε, and θ in the cells were all reduced, but not PKCη and ζ (Figure 3.1). Similarly, Sakwe *et al*. (2004) also found that chronic PMA treatment in CaR-HEK cells reduced the expression of PKCα and ε. In contrast, chronic PMA treatment failed to downregulate PKCε in

hERG-HEK cells (Cockerill *et al*., 2007). Moreover, this hERG-HEK cell has also shown a lack of PKCη expression, contrary to what was found here. Although expressed in our cells, chronic PMA treatment failed to alter the expression of PKCη even though it is a novel PKC isotype and is known to be sensitive to PMA. For example, PKCη can be activated by PMA in glioblastoma cells where its activation caused the induction of mammalian target of rapamycin (mTOR) and Akt signalling leading to increased cell proliferation (Aeder *et al*., 2004). PMA has also been reported to induce SOCS-3 gene induction by activating PKCη in HUVEC cells (Wiejak *et al*., 2102). Thus it is unclear whether the lack of PKCη downregulation in the current study is due to a cell-specific lack of PMA sensitivity, or, to a particularly rapid replacement mechanism that prevents its depletion. In contrast, the failure of chronic PMA pretreatment to alter the expression of PKCζ was entirely as expected as this isotype is PMA-insensitive. Indeed similar observations have been reported in U937, HL-60, COS, and HeLa cells (Ways *et al*., 1992).

3.4.3 Optimisation of siRNA-based PKC knockdown

In order to identify the PKC isotype(s) responsible for phosphorylation of CaR, an siRNA-based gene knockdown technique was employed. There are other possible techniques that can be used to identify a protein's role in a biological pathway, such as dominant positive or negative gene transfection (Young *et al*., 2002; Zhou *et al*., 2003) and shRNA-based gene knockdown (Paddison *et al*., 2002). However, the siRNA-based approach was chosen here as it is more specific than a dominant-negative approach and lower cost than several other techniques, but in our hands just as effective. The siRNA-based gene knockdown works by causing the breakdown of targeted mRNA after its transcription in cells thus decreasing the targeted protein's abundance and thus total activity (Aggrawal *et al*., 2003). In contrast, dominant positive and negative gene transfection works in the opposite manner by overexpressing the active or inactive forms (respectively) of the targeted protein in cells to either enhance or dilute the normal targeted proteins functions. However, this technique carries a significant risk when attempting to identify the function of a particular PKC isotype since it would be very difficult to know whether the overexpressed mutant was acting specifically or not (Steinberg, 2008). So for example, Young *et al*. (2002) found that a dominant-negative

PKCβ1 mutant affected the frequency of CaR-induced Ca^{2+}_i oscillations however we saw no evidence of a role for PKCβ using an siRNA approach. With regards shRNA, these cause gene knockdown by a mechanism similar in principal to the siRNA technique i.e. by interfering with the targeted RNA. This technique involves vector transfection (infection if carried by virus) to express the shRNA in cells. Since the shRNA is then expressed stably in the cells, then it has the benefit of a low rate degradation (Roa *et al*., 2009). While shRNA may be associated with longer-lasting gene knockdown than for siRNA, this technique is more expensive than siRNA technique and is usually used in cell lines resistant to transfected by siRNA, such as human or animal primary cell lines (Moore *et al*., 2010).

Having selected an siRNA-based approach it was necessary to carefully optimise the protocol to ensure optimal gene knockdown but with the least possible impact on cell viability. High concentrations of siRNA and/or transfection reagent can be toxic to the cells (Carralot *et al*., 2009; Ki *et al*., 2010) and in any case the optimal conditions for its use might vary from cell to cell, requiring different cell culture conditions as well as reagent concentration and exposure times. The PKCα siRNA was used in this optimisation study and the optimal conditions obtained were assumed also to be optimal for the other PKC-specific isotype siRNA. In preliminary experiments (data not shown) it was found that better PKC knockdown was obtained when the cells were reversely transfected with siRNA, that is, as they are adhering to the plastic rather than afterwards. Thus, I applied this method to all knockdown experiments. The lowest concentration of siRNA to achieve maximal PKCα knockdown was 50 nM and so this was used throughout the study (Figure 3.2*A*). In a previous study, Zhu *et al*. (2011) used the same concentration of siRNA (50 nM) to achieve PKCα and β knockdowns in HEK cells. This group also used the same siRNA and transfection reagents from Invitrogen. In another study done by Gwak *et al*. (2006), lower concentrations of custom-designed siRNA, 20 and 40 nM, were applied to HEK cells to knockdown PKCα. Finally a third study used 250 nM of a custom-designed siRNA to knockdown PKCα in HEK cells (Irie *et al*., 2002) and therefore the conditions used in the current study were in the middle of that range. Then regarding transfection reagent concentration, it was found that 50 μl/ml was the lowest concentration that maximally reduced PKC expression and so this was used in all knockdown studies. With regards cell survival, it was noted that these siRNA and

detergent concentrations did not elicit substantial cell death with ~80 % of cells still alive following transfection (data not shown).

The reagent concentrations and protocol conditions that were obtained from the optimisation study also worked for the other PKC isotypes where knockdown of at least 70% was achieved in every case (Figure 3.3). In addition, neither mock transfection (i.e. detergent only) nor control siRNA treatment (i.e. plus detergent) caused a detectable change in the expression of the PKC isotypes examined. In addition, the control siRNA also had no effect on β-actin expression in cells compared to mock transfection suggesting no gross effect on cell-death or proliferation. In addition, another experiment demonstrated that control siRNA had no effect on CaR-induced $Ca^{2+}{}_i$ mobilisation relative to mock-transfected cells and thus together it was clear than the control siRNA conditions had no detectable molecular or functional effect on the cells and therefore any effects detected using PKC isotype-specific siRNAs are likely to be due to the loss of that particular enzyme. Finally, there was no evidence that any of the PKC-specific isotype siRNAs exerted off-target effects on any of the other PKC isotypes tested (Figure 3.3).

3.4.4 Effect of the downregulation of specific PKC isotypes on CaR-induced $Ca^{2+}{}_i$ mobilisation

In order to identify the PKC isotype(s) responsible for the CaR's negative feedback phosphorylation, cells had their expression of individual PKC isotypes knocked down followed by examination of CaR-induced $Ca^{2+}{}_i$ mobilisation. It has been shown previously that the phosphorylation of CaR^{T888} directly influenced the $Ca^{2+}{}_i$ mobilisation in CaR-HEK cells (McCormick *et al*., 2010). The phosphorylation state of CaR can increase or decrease the $Ca^{2+}{}_i$ released from the calcium store into the cytoplasm and changes the dynamic pattern of $Ca^{2+}{}_i$ mobilisation. This action is due to the phosphorylation of CaR controlling the capability of the receptor to activate the $G_{q/11}$ / PLC pathway and thus affecting the activity of the IP_3 receptor and the release of stored Ca^{2+} (Ward, 2004). McCormick *et al*. (2010) discovered that the CaR was less phosphorylated at high $Ca^{2+}{}_o$ concentration (i.e 5 mM) where the cells exhibited sustained $Ca^{2+}{}_i$ mobilisation. High activity of PP2A was also observed at high $Ca^{2+}{}_o$

concentration suggesting the CaR phosphorylation was reversed by PP2A activity. The model proposed by Ward and Riccardi (2012) suggests that the Ca^{2+}_i oscillation induced by CaR is the result of dynamic phosphorylation and dephosphorylation of the receptor. The shift of CaR phosphorylation or dephosphorylation state might change the Ca^{2+}_i mobilisation either into low/no mobilisation or sustained mobilisation. In support of this idea, impaired CaR phosphorylation due to a CaR^{T888A} mutation substantially inhibits oscillatory Ca^{2+}_i mobilisation, turning the CaR responses into sustained Ca^{2+}_i mobilisation (Young *et al*., 2002, Davies *et al*., 2007, McCormick *et al*., 2010). Moreover, the addition of PKC inhibitors in the CaR-HEK cells, also change their responses from oscillatory to sustained Ca^{2+}_i mobilisation (Davies *et al*., 2007). Similarly, the naturally-occurring mutation CaR^{T888M} also exhibits sustained Ca^{2+}_i mobilisation in CaR-HEK cells when exposed to 3mM Ca^{2+}_o and is associated with suppressed PTH secretion in the patient (Lazarus *et al*., 2011) demonstrating the pathophysiological importance of this CaR^{T888} phosphorylation-dependent mechanism.

The knocking down of PKCα was shown to increase CaR responsiveness by lowering the EC_{50} for Ca^{2+}_o in the CaR-HEK cells (Figure 3.4*B*). This observation seems likely due to the inhibition of CaR phosphorylation caused by the knockdown of PKCα (Figure 3.14) Together these data suggest that PKCα is the kinase responsible for controlling CaR phosphorylation. It should be noted however that the PKCα knockdown observed did not entirely abolish the inhibition of CaR, that is some cells still exhibited Ca^{2+}_i oscillations. It should be further noted though that while substantial downregulation was observed for each PKC isotype, complete abolition of PKCα expression was never achieved in the cells. There was still some PKC α detected in the cells and this may have been able to provide some phosphorylation of the receptor. It should also be noted that while PKCα knockdown has recently been shown to turn CaR responses from oscillatory into sustained Ca^{2+}_i mobilisation in CaR-HEK cells (Young *et al*., 2014), that study did not report the effect of PKCα knockdown on Ca^{2+}_o concentration dependency or provide molecular evidence for a change in CaR phosphorylation. Indeed it did not investigate the possible role of any other PKC isotype. In contrast, here I have shown a significant decrease in the EC_{50} for Ca^{2+}_o indicating that PKCα is central to the control of CaR signalling in CaR-HEK cells, inhibiting downstream Ca^{2+}_i mobilisation or at least entraining oscillatory rather than sustained Ca^{2+}_i mobilisation. Interestingly in this

regard, PKCα has also been shown to mediate negative feedback phosphorylation of other GPCRs such as the μ-opioid receptor (MOR) (Bailey *et al*., 2009). The desensitisation of MOR by high agonist concentration was found to be mediated by phosphorylation (Zhang *et al*., 1998) via either PKC or G-protein coupled receptor kinase (GRK), depending on the agonist (Bailey *et al*., 2009). The MOR desensitisation-induced by morphine was found to be mediated by PKCα in rat coeruleus neuron. It has been shown that the treatment of PMA enhanced the MOR desensitisation in these neurons from wild-type mice but not in those from PKCα knockout mice (Bailey *et al*., 2009).

In contrast to the finding that PKCα knockdown increases CaR responsiveness, knocking down PKCε was found to reduce CaR responsiveness towards $Ca^{2+}_{o,}$ as seen with a significantly higher EC_{50} for Ca^{2+}_{o} compared to control (Figure 3.5*B*). The PKCε knockdown cells exhibited less Ca^{2+}_{i} mobilisation at moderate Ca^{2+}_{o} concentrations (Figure 3.5*A*). This might happen due to the inhibition of PKCε which attenuates the increase of Ca^{2+}_{i} through the action of Archadonic acids (AA). It has been reported that PKC activation has positive effects on cPLA2 and increases the production of AA in RAW 264.7 cells (Lim and Chen, 1998). Apart from that, the increase of AA level was also found to enhance the Ca^{2+}_{i} in erythrocytes (Soldati *et al*., 2002). Interestingly, cPLA2 was found to be downstream of the CaR signalling (Ward, 2004), and thus it might utilise the similar PKC-induced cPLA mechanism. If this true, it might explain the lack of Ca^{2+}_{i} mobilisation in PKCε knockdown cells in this study. On the other hand, this finding also contradicts the observation where chronic PMA treatment, which down-regulated PKCε (Figure 3.1), was shown to increase Ca^{2+}_{i} mobilisation in CaR-HEK cells (Davies *et al*., 2007). This might be explained by the greater positive effect of PKCα knockdown that overshadowed the negative effect of PKCε knockdown on CaR-induced Ca^{2+}_{i} mobilisation. Previously, Young *et al*. (2002) showed that overexpression of a constitutively active PKCε mutant in CaR-HEK cells caused a reduction in CaR-induced Ca^{2+}_{i} oscillation frequency. They suggested that PKCε plays role in CaR feedback phosphorylation and that the additional effect of this PKC active mutant impaired rather than stimulated CaR activity. However, the difference between the two studies might be explained by differences in how PKCε activity was altered, that is between dominant positive transfection (Young *et al*., 2002) rather that siRNA

knockdown used here. As mentioned before, dominant positive transfection could cause off-target PKC effects arising due to over-expression.

In this study, I also found that knocking down PKCβI, βII, δ, θ, and η had no effect on CaR-induced Ca^{2+}_i mobilisation. In contrast, Young *et al*. (2002) found previously that PKCβI active mutant overexpression reduced the frequency of Ca^{2+}_i oscillation in CaR-HEK cells, indicating the inhibition of CaR activity. This finding is contrary with my observation since the knockdown of PKCβ (I and II) did not alter the CaR responses to Ca^{2+}_o. The result in Figure 3.3 confirmed that the application of PKCβ siRNA in this study was able to knockdown both PKCβ slice variants, namely PKCβI and βII. This different finding might be accounted for by the similar explanation as for PKCε above. That is, use of dominant active over-expression is not necessarily the opposite of isotype-specific silencing. Furthermore, Young *et al* (2002) measured the frequency of Ca^{2+}_i oscillations produced per minute but ignored the amplitude of each oscillation and total area under the curve and therefore it is not always clear whether such responses were actually larger or lesser than normal, they were merely reported in terms of how oscillatory they were. In comparison to other family C GPCR family members, PKCδ was reported to phosphorylate $mGluR5^{T840}$ and blocked the Ca^{2+}_i oscillations in HEK-mGlur5 cells (Ucino *et al*., 2004). In contrast, Bradley and Challiss (2011) found that PKCε (and not PKCδ) knockdown could convert glutamate-induced Ca^{2+}_i oscillations into sustained responses in astrocytes.

This study on the effect of PKC's isotypes on CaR provides an insight into how these different isotypes regulate the receptor. These findings might be useful in explaining the diverse role of CaR in cell physiology. However, further study on different endogenous CaR expressing cells needs to be conducted to further confirm the findings. The understanding of the PKC singnalling in CaR regulation might eventually lead to the production of a therapeutic drug that has fewer off-target effects.

3.4.5 Comparison of the effect of control siRNA and mock treatments on CaR-induced Ca^{2+}_i mobilisation

The process of gene silencing with isotype-specific siRNAs involves the application of a detergent as well as an siRNA molecule and therefore it is important to show that the act of transfecting a cell with any siRNA does not cause a non-specific effect beyond anything caused by the specific loss of the protein being targeted (Baum *et al*., 2010). It was shown that, relative to mock transfection (i.e. detergent only), transfection with control siRNA had no effect on the expression of PKCs in CaR-HEK cells (Figure 3.3) nor did it alter CaR responsiveness (Figure 3.10*A*). This was important to confirm since in Figures 3.4-3.9, treatment with the PKC isotype-specific siRNAs were compared to mock transfection rather than control siRNA. That said, it could be argued that the non-effect of treating with siRNAs specific for PKCβ, δ, θ, and η also acts as a series of siRNA controls, but nevertheless it was considered important to prove this point with a commercial control siRNA.

In contrast, PKCα knockdown increased CaR-induced Ca^{2+}_i mobilisation (Figure 3.10) with a significantly decreased EC_{50} for Ca^{2+}_o reconfirming this effect. It should be noted that the EC_{50} for Ca^{2+}_o following PKCα knockdown in this study was a little lower than found in the earlier study (Figure 3.4; 2.7 mM vs 3.2 mM mock control). This might be due to the different cell batches being used in those experiments or just random variation around the true average CaR responsiveness. However, this small difference did not change the fact that regardless of the precise EC_{50} for Ca^{2+}_o calculated, PKCα downregulation increases CaR responsiveness, at least with regards to Ca^{2+}_i mobilisation.

Furthermore, there is no evidence that control siRNA transfection on its own has any effect on PKC isotype expression or CaR-induced Ca^{2+}_i mobilisation or that mock transfection only is inferior as a control and has been used as such in other studies (Anitei *et al*., 2014) to identify genes that regulate mannose 6-phosphate receptor trafficking for example.

3.4.6 Effect of PKC downregulation on muscarinic receptor-induced Ca^{2+}_i mobilisation

Next, I examined the effect of PKC downregulation on mAChR-induced Ca^{2+}_i mobilisation in order to determine whether the downregulation of PKC affects CaR activity at or close to the receptor level, or, much further downstream. That is, if the effects of PKC downregulation on CaR activity were also observed for mAChR activity, then it would suggest that the effect of PKC downregulation occurred at a common effector downstream of the two receptors. Specifically, mAChR is also a GPCR and exists as several subtypes M1, M2, M3, M4, and M5 (Stehno-Bittel *et al*., 1995). For instance, M1, M3 and M5 have been found to bind predominantly to G_q while M2 and M4 bind to G_i (Stehno-Bittel *et al*., 1995). It has been reported previously that M3 mAChR is expressed abundantly in HEK cells (Atwood *et al*., 2011). This subtype predominantly bind to G_q upon its activation, thus triggering phospholipase C-induced Ca^{2+}_i mobilisation (Qin *et al*., 2011). Here it was confirmed that carbachol (mAChR agonist) does elicit Ca^{2+}_i mobilisation, but that acute treatment with the PKC inhibitor GF102903X was without effect on carbachol-induced Ca^{2+}_i mobilisation induced by carbachol and thus its potentiation of the Ca^{2+}_o effect was specific, at least with regards to mAChR (Figure 3.11). This was further tested by examining the effect of PKCα and ε knockdown on mAChR-induced Ca^{2+}_i mobilisation but again the carbachol responsiveness was not affected by either PKCα or ε downregulation (Figure 13.12*A* and 13.13*A*). Again, these data suggest that the effect of either PKC inhibition or PKCα / PKCε knockdown elicit effects that are relatively specific to the CaR rather than being general effects on cell signalling. That said it should be noted that the downregulation of PKCα by irradiation has been reported to impair Ca^{2+}_i mobilisation induced by M3-mAChR in parotid acinar cells (Coppes *et al*., 2005) while in rat myocardial cells, PKCε translocation from cytosolic to membrane fraction was required to phosphorylate M3-mAChR (Hang *et al*., 2009). Whether these differences are cell-specific or readout-specific was not further investigated.

3.4.7 Effect of PKCα and ε downregulation on CaR^{T888} phosphorylation

Having established that PKCα and ε knockdown affects CaR-induced Ca^{2+}_i mobilisation in CaR-HEK cells, I then examined the effect of these treatments on CaR^{T888} phosphorylation. The phospho-CaR^{T888} antibody that was used in this study reveals two immunoreactive bands at 140 and 160 kDa equivalent to those reported previously in this laboratory (Davies *et al*., 2007; McCormick *et al*., 2010). Indeed the validity of this antibody to detect just phosphorylated CaR^{T888} was confirmed in the Davies *et al* (2007) study, as the antibody detected wild-type CaR but not CaR^{T888A} following PMA treatment and this signal could be peptide blocked using the phospho-peptide but not the nonphospho-peptide. In this study, the phosphorylation of CaR^{T888} following exposure to 2.5 mM Ca^{2+}_o was compared between PKCα and ε knocked down cells and control. This Ca^{2+}_o concentration was chosen based on a previous optimisation study which revealed that it gave optimal CaR^{T888} phosphorylation (McCormick *et al*., 2010). To monitor this though, the phosphorylation of CaR^{T888} at 0.5 mM Ca^{2+}_o was also tested to confirm that little CaR^{T888} phosphorylation was observed until the CaR was partially activated.

Thus the consequence of knocking down PKCα was to significantly reduce the phosphorylation of 160 kDa CaR^{T888} (Figure 3.14), a result consistent with our finding that PKCα downregulation increases Ca^{2+}_i mobilisation. The result tends to confirm that PKCα is the enzyme responsible for CaR^{T888} phosphorylation though the possibility that the PKCα stimulates another kinase to phosphorylate the CaR cannot be ruled out. A technical point to note is that Ca^{2+}_o-induced CaR^{T888} phosphorylation is biphasic with moderate Ca^{2+}_o concentrations (~2.5 mM) causing sustained increases in CaR^{T888} phosphorylation whereas high Ca^{2+}_o concentration (5 mM) causes rapid CaR^{T888} phosphorylation but that is rapidly dephosphorylated thus permitting sustained Ca^{2+}_i mobilisation (McCormick *et al*., 2010). The issue here is that because of the biphasic response, one cannot be sure whether a decrease in the phosphorylation seen is due to an inhibition of the receptor (and less PKCα activation) or an activation of it (and thus more PP2A-mediated dephosphorylation of CaR^{T888} (McCormick *et al*., 2010). Nevertheless despite these caveats, the simplest explanation of the current data is that PKCα is the CaR^{T888} kinase. Indeed, this finding supports a broad range of *in vitro, ex vivo* and *in vivo* studies that show that a) PKC activation (phorbol ester) suppresses Ca^{2+}_i mobilisation in

CaR-HEK and PT cells, and permits PTH secretion (Brown *et al*., 1984; Nemeth *et al*., 1986; Kobayashi *et al*., 1988; Morrissey, 1988; Bai *et al*., 1998b; Young *et al*., 2002; Davies *et al*., 2007), b) PKC inhibition enhances Ca^{2+}_i mobilisation in CaR-HEK and PT cells and suppresses PTH secretion (Clarke *et al*., 1993 ; Young *et al*., 2002; Davies *et al*. 2007), c) mutation of CaR^{T888} to a non-phosphorylatable residue enhances Ca^{2+}_i mobilisation in CaR-HEK cells (Bai *et al*., 1998b; Young *et al*., 2002; Davies *et al*., 2007) and suppresses PTH secretion (Lazarus *et al*., 2011), d) mutation of CaR^{T888} to a phosphomimetic residue suppresses Ca^{2+}_i mobilisation in CaR-HEK cells (Bai *et al*., 1998b) and finally e) inhibition of PP2A with calyculin suppresses Ca^{2+}_i mobilisation in CaR-HEK and PT cells, and permits PTH secretion (Davies *et al*., 2007; McCormick *et al*., 2010).

Another interesting finding is that despite antagonising CaR-induced Ca^{2+}_i mobilisation, PKCε knockdown actually reduced 160 kDa CaR^{T888} phosphorylation (Figure 3.15). To date, this is the first observation of an experimental challenge that can decrease CaR^{T888} phosphorylation and Ca^{2+}_i mobilisation simultaneously. Since knocking down PKCε impaired Ca^{2+}_i mobilisation in CaR-HEK cells, it had been expected that knockdown of this PKC would increase CaR^{T888} phosphorylation at moderate Ca^{2+}_o concentrations. It might be the case therefore that PKCε mediates a more complex series of intracellular effects. If PKCε was simply a positive modulator of Ca^{2+}_i mobilisation in general then we would have expected to see a suppression of the carbachol response as well, following PKCε knockdown, however this did not occur. Therefore, whatever role PKCε plays is relatively specific to CaR signalling and appears uniquely capable of uncoupling CaR^{T888} phosphorylation and Ca^{2+}_i mobilisation by an unknown mechanism. Nevertheless it will be important to address this in future work since this is currently the only piece of data that otherwise contradicts the idea of PKC-mediated CaR^{T888} phosphorylation providing negative feedback phosphorylation and suppression of PTH secretion, and thus might open a new avenue of investigation.

Then with regards to the high mannose, immature receptor, phosphorylation of 140 kDa CaR^{T888} was also found to be significantly reduced by the knocking down of PKCα (Figure 3.14; quantification not shown). Interestingly, McCormick *et al*. (2010) have previously shown that increases of Ca^{2+}_o concentration from 0.5 to 4.0 mM elicit a

sustained increase in the phosphorylation of 140 kDa CaR, suggesting that it is not subject to the same biphasic regulation that the mature 160kDa band is. The function of the phosphorylation of immature 140 kDa CaR is still unknown. It has been reported that the activation of CaR by its agonists has triggered the maturation and translocation of the receptor from endoplasmic reticulum to membrane (Grant *et al*., 2011). Thus, the previous observation by McCormick *et al*. (2010) of Ca^{2+}_o-induced 140 kDa CaR phosphorylation might represent part of the process of maturation and translocation of the receptor to the membrane at high Ca^{2+}_o concentrations. Whether such phosphorylation inhibits or stimulates forward trafficking, is unknown though potentially the knockdown of PKCα and/or ε in this study might have interfered with this mechanism also. However, forward trafficking and ADIS (Grant *et al*., 2011) was not further investigated here.

3.5 Conclusion

In conclusion, PKCα was found to be the kinase likely responsible for the phosphorylation of CaR^{T888} and thus contributes to the negative feedback of PKC on CaR-induced Ca^{2+}_i mobilisation. With the exception of PKCε, knockdown of the other PKC isotypes had no effect of CaR-mediated Ca^{2+}_i signalling. However, knockdown of PKCε had a paradoxical effect on CaR^{T888} and CaR-induced Ca^{2+}_i mobilisation which could not be adequately explained here. Nevertheless, the greater physiological effect of PKC still appears to be to phosphorylate CaR to inhibit it and thus permit PTH secretion.

Chapter 4

Modulation of calcium-sensing receptor signalling by protein kinase C, $G\alpha_{12}$ and cyclic-AMP

4.1 INTRODUCTION

Most GPCRs undergo repeated rounds of activation and inactivation resulting from episodic bursts of local or systemic agonist release. In contrast, the CaR is constantly exposed to levels of Ca^{2+}_o and other endogenous orthosteric and allosteric stimulators that may leave it partially activated most, if not all of the time (Riccardi and Brown, 2010). This provides the opportunity for tonic CaR activity to be regulated not only by extracellular changes but also by intracellular changes. That is, in a stable system in which extracellular CaR agonist concentrations are partially-activating and relatively constant, then the total CaR activity in the cell could be increased by altering the responsiveness of the CaRs in the cell and this could occur either by inserting more receptors in the cell membrane (ADIS; Grant *et al*, 2011), or by altering the phosphorylation status of the CaR's intracellular domains. Since plasma Ca^{2+} levels change very little from minute-to-minute in humans (Brown, 2013), this means that these latter two actions could have profound consequences in CaR biology. In this chapter, the role of PKC in regulating CaR-induced Ca^{2+}_i mobilisation at Ca^{2+}_o sub-threshold concentration will be examined. Then the function of $G\alpha_{12}$, which is more usually associated with actin polymerisation and cell morphology will be examined for its role in regulating CaR activity. The modulation of intracellular cAMP levels was previously shown in this laboratory (Campion, 2013) and elsewhere (Gerbino *et al*., 2005) to profoundly alter CaR-induced Ca^{2+}_i mobilisation. Since CaR phosphorylation has been found to shape Ca^{2+}_i mobilisation dynamics (Riccardi and Ward, 2012), the effect of this cAMP modulation on CaR phosphorylation will also be examined in this chapter. Finally, I will identify three novel phosphorylation signalling pathways activated downstream of the CaR and look at the effect of altering the expression of PKCα, PKCε, and $G\alpha_{12}$ on these CaR effectors.

4.2 METHODS

CaR-HEK cells were cultured as detailed in (Chapter 2) section 2.2. The selective knockdown of individual PKC isotypes and $G\alpha_{12}$ were achieved using PKC isotype and $G\alpha_{12}$-specific siRNAs, performed as described in section 2.3. Intracellular Ca^{2+} mobilisation in CaR-HEK cells were measured using single-cell microfluorometry as described in 2.9. The cells were lysed as described in 2.6 and (phospho) proteins immunoblotted as described in 2.7.

4.3 RESULTS

4.3.1 Effect of PKC inhibition on CaR-induced Ca^{2+}_i mobilisation at sub-threshold Ca^{2+}_o concentrations

It is well known that the CaR has multiple, downstream effector pathways many of which can be used as readouts of receptor activity. Interestingly however, some pathways appear to be activated at lower Ca^{2+}_o concentrations than others (Davies *et al*., 2006) raising the possibility that the CaR may be in an active conformation even when the less sensitive readouts appear to suggest that it is inactive. Therefore, this next experiment evaluated the effect of PKC inhibition on CaR-induced Ca^{2+}_i mobilisation at sub-threshold Ca^{2+}_o concentrations. The Ca^{2+}_o threshold concentration in this study was defined as the minimal Ca^{2+}_o concentration to initiate the Ca^{2+}_i mobilisation inside the cells. It was routinely observed using the CaR-HEK cells in our laboratory that 1.8–2 mM Ca^{2+}_o usually failed to exhibit Ca^{2+}_i mobilisation, despite these concentrations being sufficient to elicit robust CaR-induced actin polymerisation and process retraction in the same cells (Davies *et al*., 2006). Thus, 2 mM Ca^{2+}_o was used in this experiment as the sub-threshold (for Ca^{2+}_i) Ca^{2+}_o concentration. The lack of Ca^{2+}_i mobilisation at sub-threshold Ca^{2+}_o concentration was hypothesised therefore to be mediated by PKC-mediated CaR phosphorylation such that the removal of CaR phosphorylation by PKC inhibition could permit the otherwise active receptor to initiate Ca^{2+}_i mobilisation. To test this, the CaR-HEK cells were pre-treated for 10 minutes with or without the PKC inhibitor GF102903X (1 μM) prior to an increase in Ca^{2+}_o concentration from 0.5 to 2 mM. In the DMSO vehicle pre-treated cells, most cells remained unresponsive even in

the presence of 2 mM Ca^{2+}_o (Figure 4.1*A*) whereas in the GF102903X pre-treated cells, 2 mM Ca^{2+}_o induced low frequency Ca^{2+}_i oscillations. Quantification of the AUC of the "global" cell responses confirmed that the GF102903X had significantly increased the response to 2 mM Ca^{2+}_o (Figure 4.1*B*; $P < 0.05$). Therefore, this experiment suggests that PKC activity can inhibit Ca^{2+}_i mobilisation at sub-threshold Ca^{2+}_o concentration and thus that the CaR may be in active conformation at 2 mM Ca^{2+}_o but normally prevented from mobilising Ca^{2+}_i due to PKC-mediated phosphorylation in CaR-HEK cells.

4.3.2 Effect of PKCα knockdown on the Ca^{2+}_o concentration threshold for CaR-induced Ca^{2+}_i mobilisation

In this next experiment, instead of pre-exposing the CaR-HEK cells to a PKC inhibitor, the cells were instead pretreated with PKCα-selective siRNA to knockdown PKCα expression as performed in Section 3.3.4. Then the cells were exposed to 1.6, 1.8 and 2.0 mM Ca^{2+}_o, in turn, to test their effect on Ca^{2+}_i mobilisation. In the control cells, little or no change in Ca^{2+}_i mobilisation was observed at any of the Ca^{2+}_o concentrations tested (Figure 4.2*A*). However, in the PKCα knockdown cells, robust Ca^{2+}_i mobilisation was seen at 1.8 mM Ca^{2+}_o and this was heightened in 2 mM Ca^{2+}_o. The quantification of the cell responses (Figure 4.2*B*) confirms that the responses to 1.8 and 2.0 mM Ca^{2+}_o were both significantly greater (both $P < 0.05$) in cells in which PKCα had been knocked down. In contrast, PKCα knockdown did not significantly alter the response to 1.6 mM Ca^{2+}_o although a trend increase was observed. Together, these experiments show that knocking down PKCα in CaR-HEK cells lowers the Ca^{2+}_o threshold concentration to 1.8 mM of Ca^{2+}_o, suggesting that in control cells the CaR may already be in an active conformation in 1.8 mM Ca^{2+}_o but prevented from mobilising Ca^{2+}_i as a result of PKC activity.

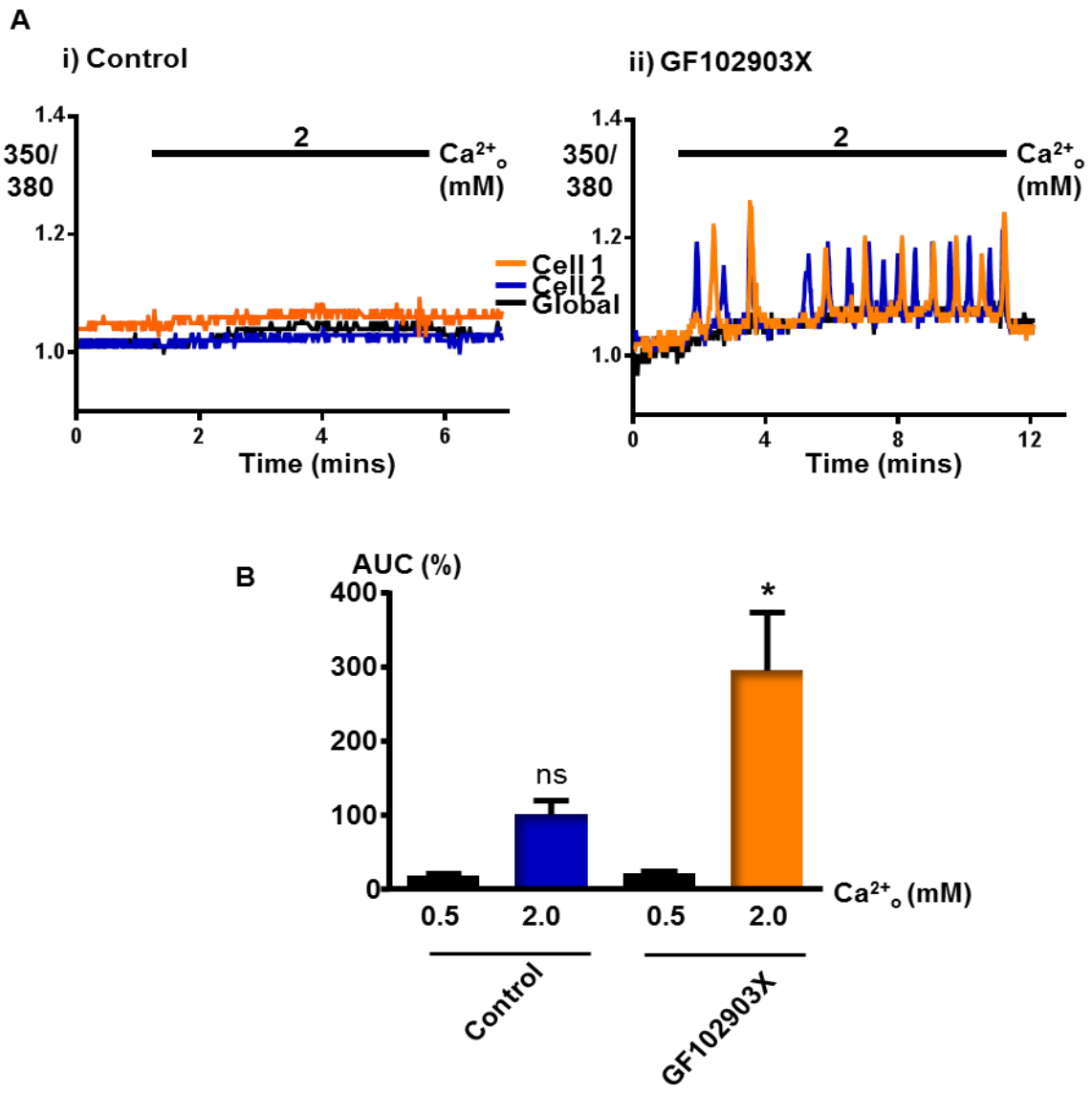

Figure 4.1: GF102903X-induced increase in Ca^{2+}_i mobilisation in sub-threshold Ca^{2+}_o concentration. *A*) Representative traces that showing Ca^{2+}_i changes in GF102903X (1 µM) pre-treated and non-pre-treated cells (control) at sub-threshold Ca^{2+}_o concentration. The cells were pre-treated with G102903X for 30 minutes in GF102903X pre-treatment prior to the imaging. The GF102903X was replaced by 0.01% (v/v) of DMSO in (vehicle) control cells. *B*) Quantification of the change in the percentage of area under curve/minutes (AUC %) compared to the response to 2.0 mM Ca^{2+}_o in untreated cells. The percentage AUC for non-pretreated cells was not significantly different at 0.5 mM and 2.0 mM of Ca^{2+}_o concentration (P = 0.48). In contrast, the percentage AUC for GF102903X pretreated cells was significantly higher at 2.0 mM compared to 0.5 mM of Ca^{2+}_o concentration (*P < 0.05; paired t-test on the pre-normalised data). n = 6 coverslips from two independent experiments.

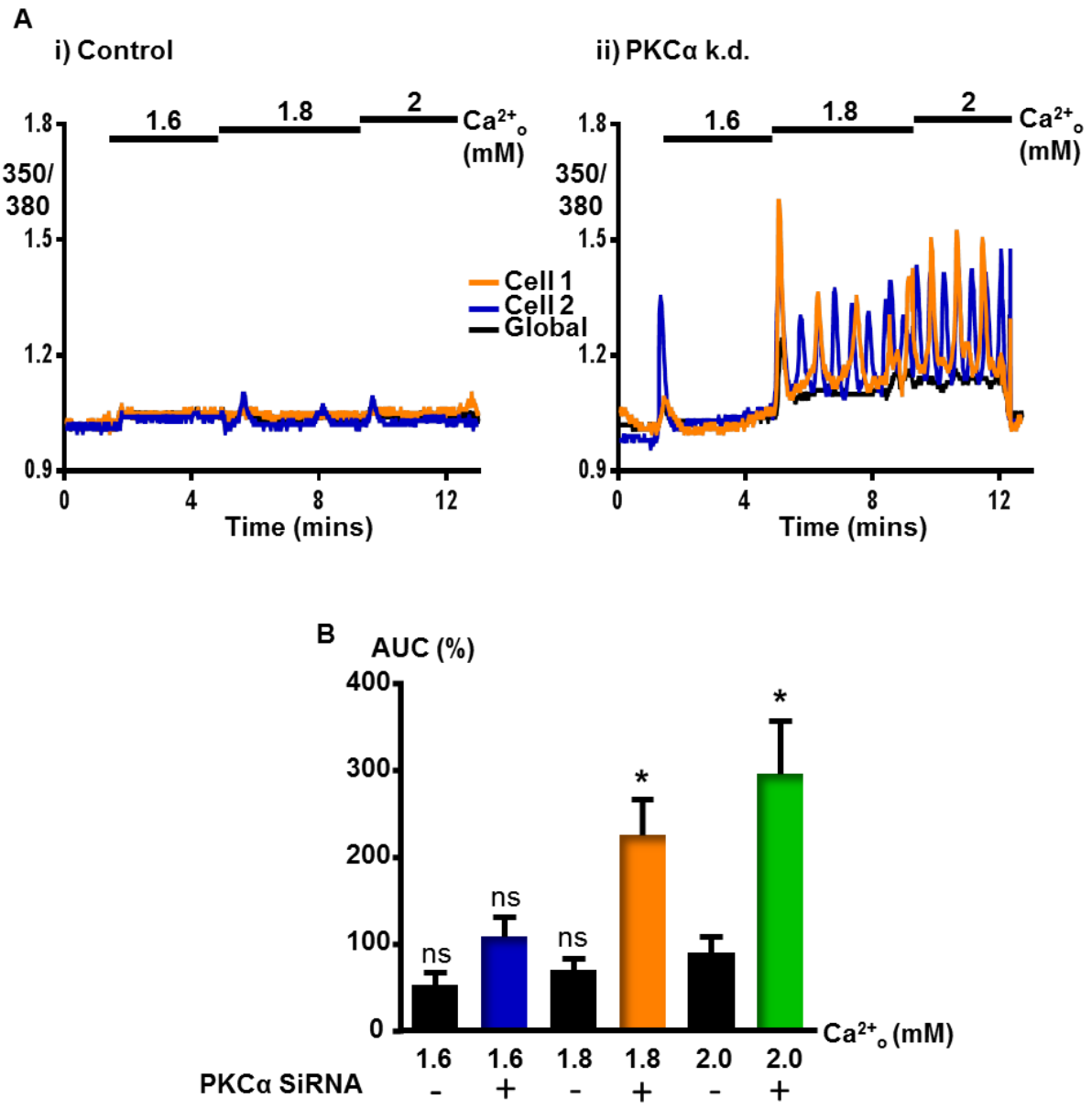

Figure 4.2: PKCα knockdown-induced increase in Ca^{2+}_i mobilisation in sub-threshold Ca^{2+}_o concentration. CaR-HEK cells were cultured in media supplemented with 0.2% (v/v) OptiMem and 50 µl/ml (v/v) lipofectamine in the absence or presence of 50 nM PKCα-specific siRNA. *A*). Fura-2 loaded cells were then exposed to increasing sub-threshold concentrations of Ca^{2+}_o (0.5- 2.0 mM) to determine the effect of PKCα knockdown on Ca^{2+}_i mobilisation at threshold Ca^{2+}_o concentration. Shown are representative Ca^{2+}_i traces from cells incubated with or without the siRNA. *B*) Quantification of the change shown as percentage area under the curve/minutes (AUC %) of PKCα knocked down cells compared to control at 2.0 mM Ca^{2+}_o concentration. The percentage AUC in PKCα knockdown cells was significantly higher at 1.8 mM and 2.0 mM of Ca^{2+}_o concentration compared to control (*P < 0.05 respectively). The analysis was done using 1-way ANOVA with Dunnett's post-test on the pre-normalised data. n ≥ 12 coverslips from three independent experiments.

4.3.3 Investigation of the role of $G\alpha_{12}$ activity in CaR-induced Ca^{2+}_i mobilisation

The CaR is known to bind and stimulate several heterotrimeric G-proteins such as $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ upon its activation (Ward, 2004). The coupling of CaR to $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ triggers different downstream signalling effects such as mobilisation of Ca^{2+}_i, inhibition of cAMP formation and actin polymerisation respectively. However, the extent to which these pathways are independent of each other or interactive, remains poorly understood. With Zhu *et al*., (2007) suggesting that $G\alpha_{12}$ may activate PP2A, we hypothesised that if $G\alpha_{12}$ does activate PP2A in CaR-HEK cells then by downregulating $G\alpha_{12}$ then we may see less CaR^{T888} dephosphorylation and therefore an inhibition of CaR-induced Ca^{2+}_i mobilisation. Thus to test this, CaR-HEK cells were treated with $G\alpha_{12}$-specific siRNA as described in section 2.3 and then exposed to increasing concentrations of Ca^{2+}_o to compare the CaR-induced Ca^{2+}_i mobilisation between control and $G\alpha_{12}$ knockdown cells. As shown in Figure 4.3*A*, $G\alpha_{12}$ knockdown impaired Ca^{2+}_i mobilisation at moderate Ca^{2+}_o concentrations resulting in a significantly elevated EC_{50} for Ca^{2+}_o (Figure 3.4*B*; control, 3.3 ± 0.3 mM; $G\alpha_{12}$ knockdown 4.8 ± 0.4 mM; $P<0.05$). The successful knockdown of $G\alpha_{12}$ protein was confirmed by western blotting as shown in Figure 4.3*C*.

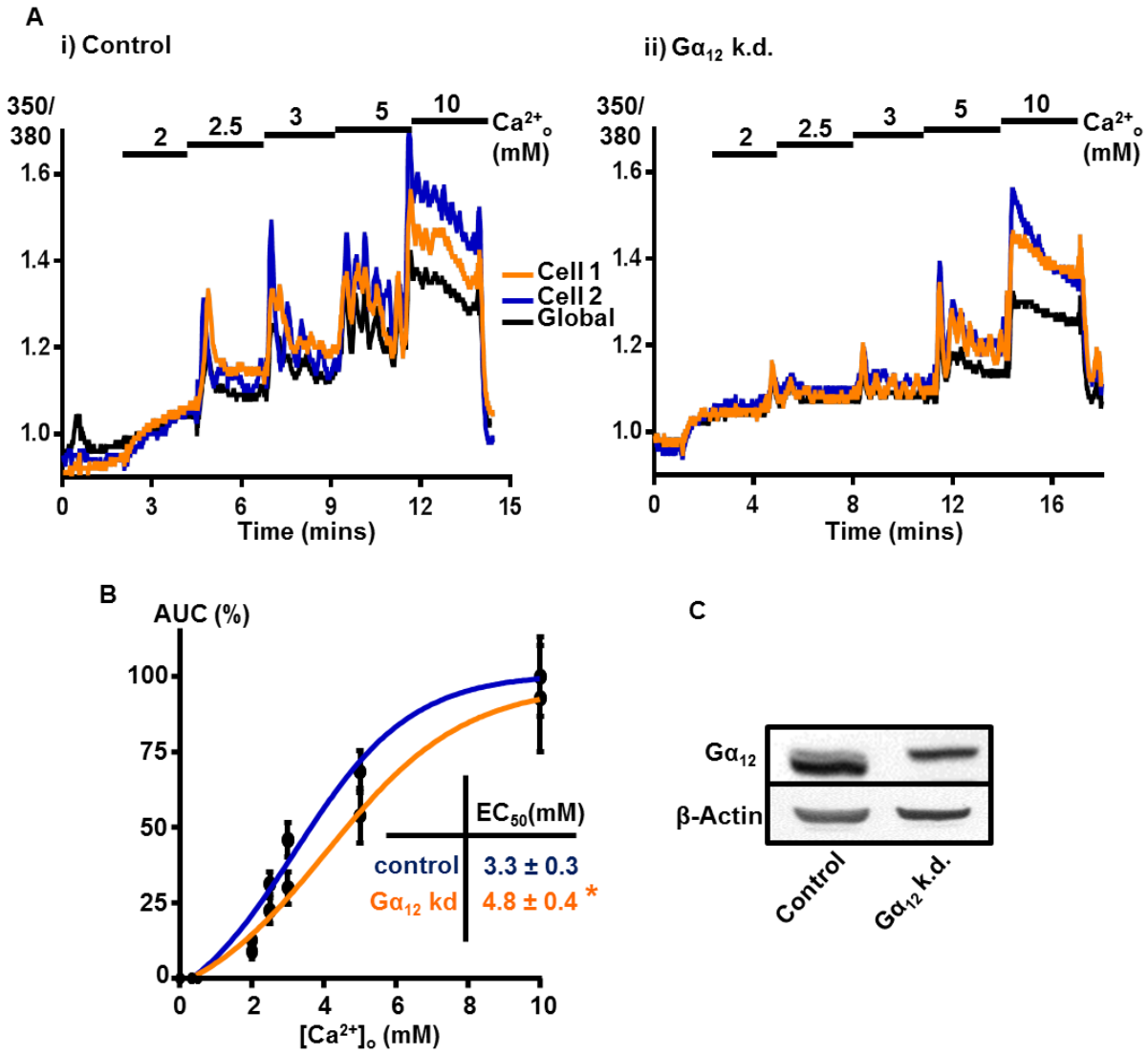

Figure 4.3: Gα12 knockdown reduced CaR-responsiveness in CaR-HEK cells. CaR-HEK cells were cultured in media supplemented with 0.2% (v/v) OptiMem and 50 µl/ml (v/v) lipofectamine in the absence or presence of 50 nM Gα12-specific siRNA. *A*). Fura-2 loaded cells were then exposed to increasing concentrations of Ca^{2+}_o (0.5-10 mM) to determine the effect of Gα12 knockdown on Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from cells incubated with or without the siRNA. *B*) The concentration-effect curves showing significant difference between EC_{50}s for Ca^{2+}_o in the cells expressing less Gα12 and control. $P < 0.05$ between log EC_{50}s vs control by unpaired t-test; $n \geq 13$ coverslips from 3 independent transfections. *C*) The representative western blot shows the reduction of G_{12} expression after application of Gα12-specific siRNA.

4.3.4 Effect of $G\alpha_{12}$ protein downregulation on mAChR-induced Ca^{2+}_i mobilisation

In order to confirm whether the negative effect of $G\alpha_{12}$ knockdown on Ca^{2+}_i mobilisation was CaR-specific or not, the effect of $G\alpha_{12}$ knockdown on mAChR-induced Ca^{2+}_i mobilisation was then studied. Cells were pretreated with control or $G\alpha_{12}$-specific siRNA as before and then exposed to increasing concentrations of carbachol. As can be seen in Figure 4.4*A*, both control and $G\alpha_{12}$ knockdown cells exhibited similar concentration-dependent responses to carbachol. Specifically, both sets of cells lacked responsiveness to 0.1 and 1 μM carbcahol but elicited Ca^{2+}_i oscillations in response to 5 and then 10 μM carbachol. The cells both exhibited sustained Ca^{2+}_i mobilisation at 100 μM of carbachol. The resulting EC_{50} values for carbachol were not significantly different (Figure 4.4*B*; control, 5.0 ± 0.9 μM; $G\alpha_{12}$ knockdown, 4.5 ± 0.8 μM; P = 0.34). This suggests that the inhibitory effect of $G\alpha_{12}$ knockdown is relatively specific to CaR activation

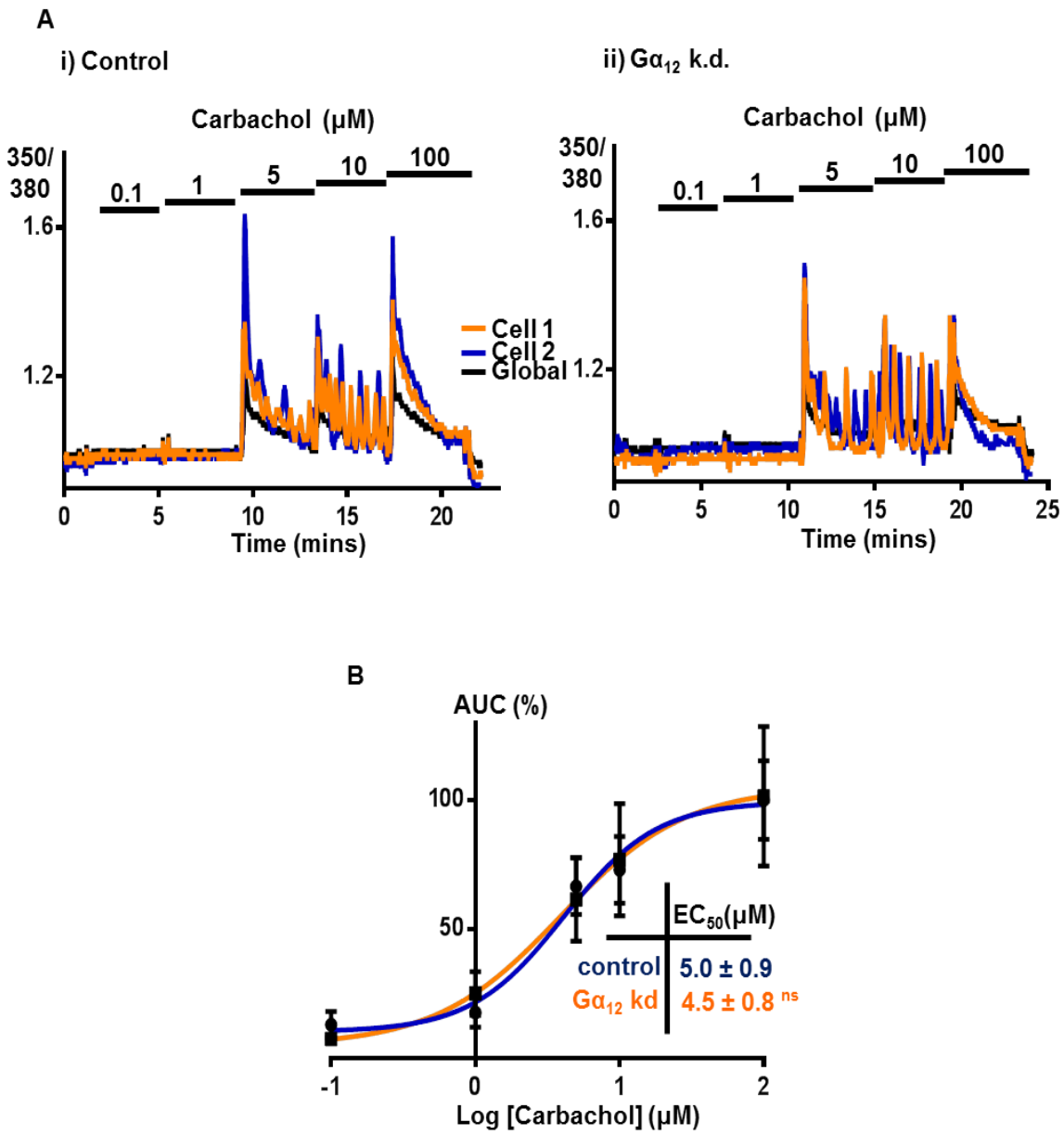

Figure 4.4: Gα12 knockdown does not affect muscarinic receptor responsiveness in CaR-HEK cells. CaR-HEK cells were cultured in media supplemented with 0.2% (v/v) OptiMem and 50 μl/ml (v/v) lipofectamine in the absence or presence of 50 nM Gα12-specific siRNA. *A*). Fura-2 loaded cells were then exposed to increasing concentrations of carbachol (0.1-100 μM) to determine the effect of Gα12 knockdown on Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from cells incubated with or without the siRNA. *B*) The concentration-effect curves showing no significant different between EC_{50}s for carbachol in the cells expressing less Gα12 and control. $P > 0.05$ ($P = 0.74$) between log EC_{50}s vs control by unpaired t-test; n = at least 5 coverslips from 2 independent transfections.

4.3.5 Effect of $G\alpha_{12}$ protein downregulation on CaR^{T888} phosphorylation

Having found that the downregulation of $G\alpha_{12}$ impairs Ca^{2+}_i mobilisation in CaR-HEK cells, elicited in response to Ca^{2+}_o though not to carbachol, it was next necessary to determine whether the loss of $G\alpha_{12}$ results in an increase in CaR^{T888} phosphorylation which could therefore explain this receptor-specific effect. Thus in this next experiment, 3 mM Ca^{2+}_o was used to stimulate CaR^{T888} phosphorylation and thus assess the effect of $G\alpha_{12}$ knockdown on this response. This Ca^{2+}_o concentration was chosen based on our previous observation that CaR^{T888} phosphorylation is maximal at ~ 2.5 mM Ca^{2+}_o and begins to decline slightly at 3 mM (McCormick *et al*., 2010). Since knocking down $G\alpha_{12}$ was expected to increase CaR phosphorylation, then this Ca^{2+}_o concentration was hoped to be optimal for detecting such an effect. Thus, CaR-HEK cells pre-exposed to control or $G\alpha_{12}$-specific siRNA were then incubated in either 0.5 or 3 mM Ca^{2+}_o and the resulting CaR^{T888} phosphorylation assayed. Figure 4.5*A* shows representative immunoblots from three independent experiments which indicate a consistent increase in 160 kDa CaR^{T888} phosphorylation in the $G\alpha_{12}$ knockdown cells in response to 3mM Ca^{2+}_o concentration compared to control cells. Densitometry confirmed that 160 kDa phospho-CaR^{T888} band intensity was significantly higher in the $G\alpha_{12}$ knockdown cells (Figure 4.5*B*; $P < 0.05$). CaR^{T888} phosphorylation is reported as a fraction of total 160 kDa CaR immunoreactivity. A similar effect for the (immature) 140 kDa phospho-CaR^{T888} band was seen in some experiments though the signal was barely detectable in others and therefore whether $G\alpha_{12}$ knockdown affected 140 kDa CaR^{T888} phosphorylation remains unclear. The knockdown of $G\alpha_{12}$ in this study was confirmed by western blot (Figure 4.5*C*) which shows decreased band intensity $G\alpha_{12}$ following treatment with specific siRNA, while the β-actin loading control indicates equivalent protein content in each lane. Together these data suggest that since loss of $G\alpha_{12}$ protein increases CaR^{T888} phosphorylation then under control conditions, $G\alpha_{12}$ activity may tend to cause decreased CaR^{T888} phosphorylation, potentially explaining why $G\alpha_{12}$ knockdown suppressed CaR-induced Ca^{2+}_i mobilisation reported in Section 4.3.4.

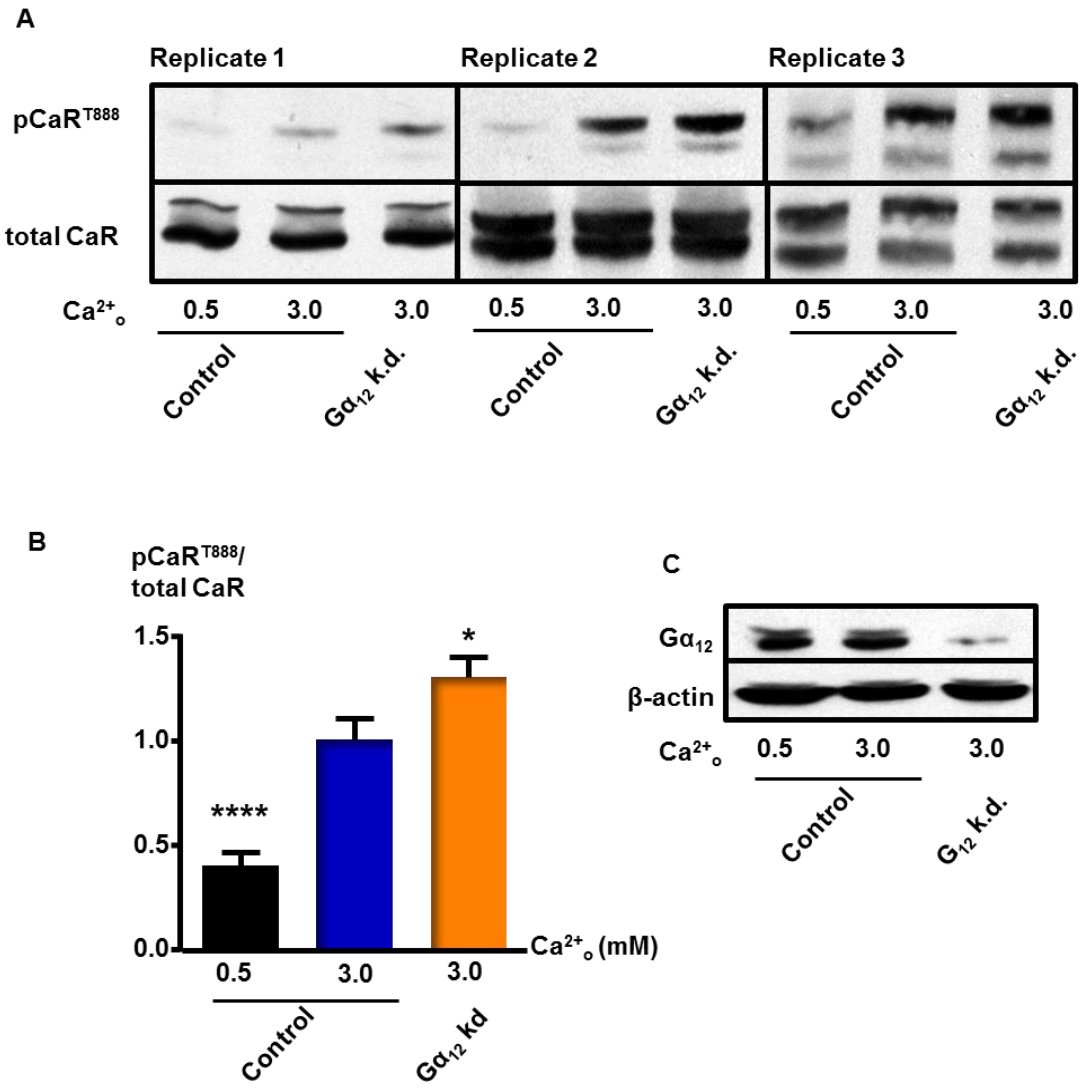

Figure 4.5: Gα$_{12}$ knockdown inhibits CaR^{T888} phosphorylation in CaR-HEK cells. CaR-HEK cells were cultured in media supplemented with 0.2% (v/v) OptiMem and 50 µl/ml (v/v) lipofectamine in the absence or presence of 50 nM Gα$_{12}$-specific siRNA and exposed to 0.5 or 3.0 mM Ca^{2+}_o concentration. The cells were then lysed using RIPA buffer prior performing western blot. *A*) The phosphorylation of CaR^{T888} increased with the raise of Ca^{2+}_o concentration from 0.5 to 3.0 mM but less CaR^{T888} phosphorylation was observed in cells express less Gα$_{12}$ at 3.0 mM Ca^{2+}_o compared to control. *B*) The quantification of band intensity shows the phosphorylation of CaR^{T888} in control cells was significantly higher at 3.0 mM Ca^{2+}_o compared to 0.5 mM Ca^{2+}_o and CaR^{T888} phosphorylation in cells expressing less Gα$_{12}$; ****P<0.0001, *P < 0.05 vs 3.0 mM Ca^{2+}_o by one-way ANOVA with Dunnett's post-test on the pre-normalised data. n = 27 replicates from 7 independent transfections. The mean changes in CaR^{T888} phosphorylation, corrected for total CaR expression and normalised to the 3.0 mM Ca^{2+}_o control cell response. *C*) The knocking down of Gα$_{12}$ was confirmed by its decreased immunoreactivity in the cells.

4.3.6 Effect of forskolin on CaR-induced Ca^{2+}_i mobilisation

It has been shown previously in our laboratory that exposure to the adenylate cyclase activator forskolin, which raises intracellular cAMP levels, also potentiates CaR-induced Ca^{2+}_i mobilisation in CaR-HEK cells (Campion, 2013). Therefore, the next question to ask was how increased cAMP levels cause this potentiation and whether such a mechanism may also involve changes in CaR^{T888} phosphorylation. Thus, firstly I exposed CaR-HEK cells to 3mM Ca^{2+}_o in the absence and then presence of forskolin (10 μM) to confirm that the forskolin does elicit this stimulatory effect in my hands. Indeed, in accordance with our previous finding, the addition of forskolin potentiated the Ca^{2+}_i mobilisation induced by 3mM Ca^{2+}_o in the CaR-HEK cells (Figure 4.6*A*) and that the effect was statistically significant $P < 0.01$ by paired t-test (Figure 4.6*B*).

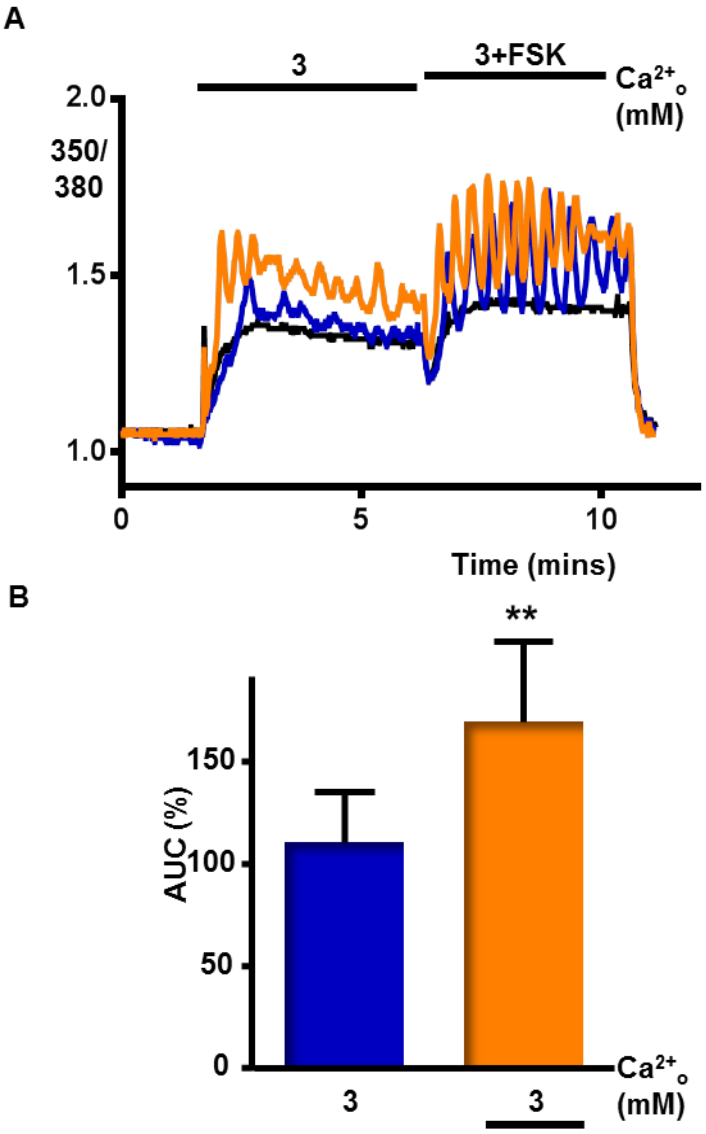

Figure 4.6: Forskolin-induced increase in Ca^{2+}_i mobilisation in CaR-HEK cells. *A*) Representative traces that showing Ca^{2+}_i changes in single cells (orange and blue) and global (black) before and after the exposure of 10 μM of forskolin at 3.0 mM of Ca^{2+}_o. *B*) Quantification of the change in the percentage of area under curve/minutes (AUC %) compared to the responses to 3.0 mM Ca^{2+} of untreated cells. The percentage of AUC is significantly higher following the forskolin treatment (**P < 0.01 by paired t-test on the pre-normalised data). n = 10 coverslips from one experiment.

4.3.7 Effect of forskolin on the inhibitory action of phorbol ester on CaR-induced Ca^{2+}_i mobilisation

Having confirmed that forskolin increases CaR-HEK responsiveness to Ca^{2+}_o, the effect of forskolin effect on phorbol ester-induced inhibition of Ca^{2+}_i mobilisation was next examined. The phorbol ester PMA inhibits Ca^{2+}_i mobilisation by stimulating PKC activity thus increasing CaR phosphorylation (Davies *et al*., 2007; McCormick *et al*., 2010). So in the current study, forskolin was used to see if it could rapidly reverse the inhibitory effect of PKC activation on CaR activity. The resulting observations of the cell responses are shown in Figure 4.7*A*. The cells exhibited oscillatory Ca^{2+}_i responses when exposed to 2.5 mM Ca^{2+}_o concentration and this was suppressed upon the addition of 1 μM PMA in the 2.5 Ca^{2+}_o buffer. Once suppressed, the buffer was replaced with 3 mM Ca^{2+}_o-containing buffer in the absence of presence of 10μM forskolin or 1,9-deoxyforskolin. 1,9-deoxyforskolin is an inactive forskolin analogue used here as a negative control. Exposure of the PMA-suppressed cells to 3 mM Ca^{2+}_o alone failed to produce substantial recovery of CaR-induced Ca^{2+}_i mobilisation. Similarly, the addition of 10 μM of 1,9-deoxyforskolin to the 3mM Ca^{2+}_o buffer also failed to substantially restore the CaR response. In both cases, CaR-induced Ca^{2+}_i mobilisation could then be restored by 5 mM Ca^{2+}_o as shown previously (Davies *et al*., 2007). In contrast, the addition of 10 μM forskolin did increase Ca^{2+}_i mobilisation in the cells following PMA removal, rapidly reversing the inhibitory effect of the phorbol. It should be noted that in preliminary experiments, 2.5mM Ca^{2+}_o plus forskolin failed to restore Ca^{2+}_i mobilisation (data not shown) and thus 3mM $Ca2+_o$ was required to achieve this response. Figure 4.7*B* shows the quantification of these responses and confirms that forskolin cotreatment resulted in a significantly higher response to 3 mM Ca^{2+}_o than either 1,9-deoxyforskolin or 3 mM Ca^{2+}_o alone ($P < 0.01$). These data suggest that forskolin accelerates the recovery of cellular CaR-induced Ca^{2+}_i mobilisation following PMA treatment.

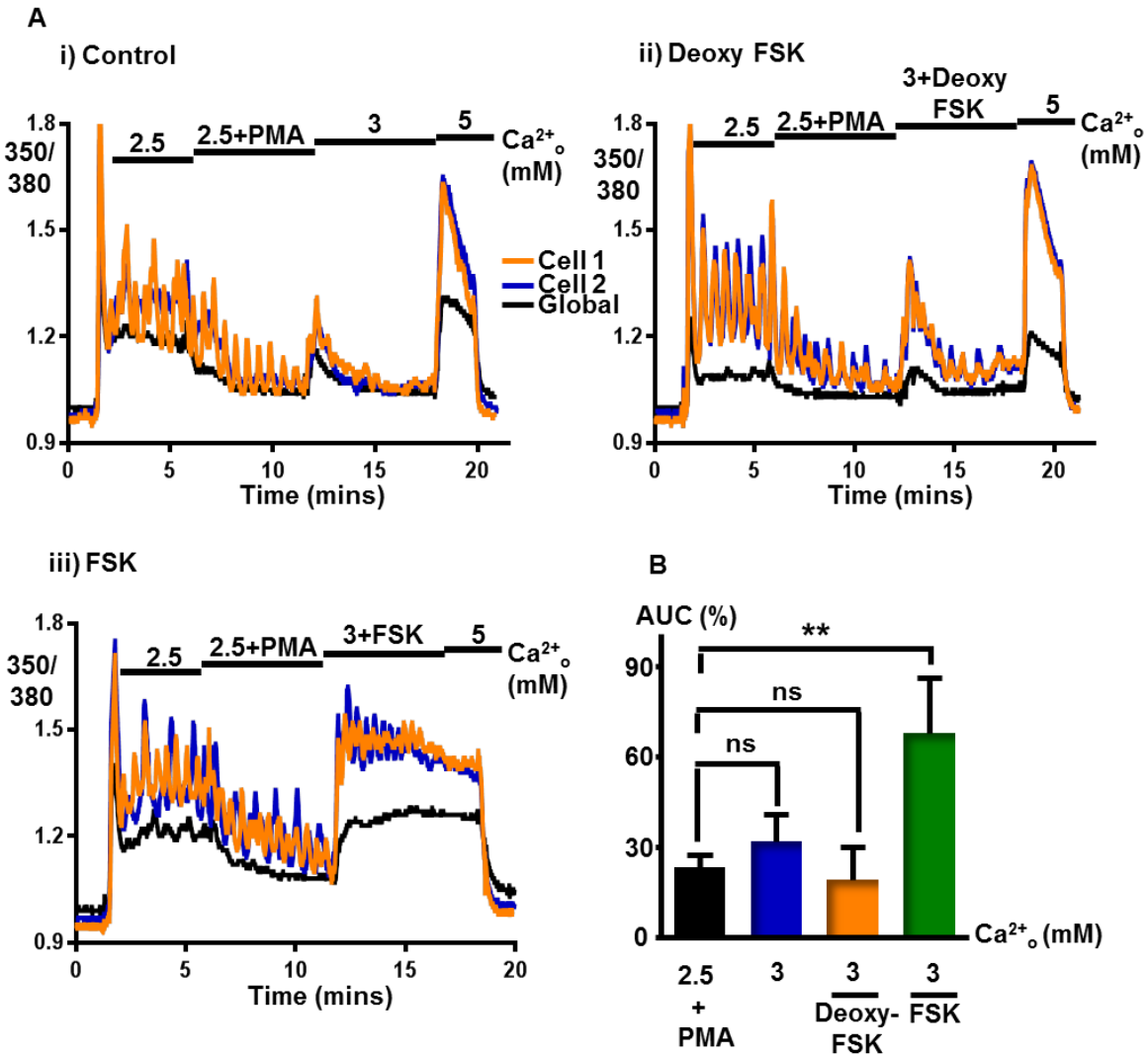

Figure 4.7: Forskolin reversed the inhibition effect of PMA on Ca^{2+}_i mobilisation in CaR-HEK cells. *A*) Representative traces that showing the changes in Ca^{2+}_i mobilisation in cells that exposed to 3.0 mM of Ca^{2+}_o alone, or with the addition of 1 μM of forskolin and 1,9-deoxyforskolin after PMA inhibition. *B*) Quantification of the change in area under curve for 350/380 shows that the response to 3 mM Ca^{2+}_o with or without 1 μM 1,9-deoxyforskolin was not significantly different compared to the PMA / 2.5 Ca^{2+}_o treatment. In contrast, the forskolin treatment was significantly higher compared to the PMA / 2.5 Ca^{2+}_o treatment. ** P< 0.01 vs prior response to PMA / 2.5 Ca^{2+}_o treatment. The quantification was based on the relative change of Ca^{2+}_i mobilisation as % of response at 2.5 mM of Ca^{2+}_o. The analysis was done using paired t-test. N ≥ 5 coverslips from two independent experiments.

4.3.8 Effect of forskolin on CaR^{T888} phosphorylation in CaR-HEK cells

Since forskolin appears to "release" CaR-HEKs more rapidly from PMA-induced inhibition of CaR-induced Ca^{2+}_i mobilisation, it is possible that the forskolin / cAMP acts by causing dephosphorylation of CaR^{T888}. Thus to test this, CaR-HEKs were incubated with 2.5 mM Ca^{2+}_o in the absence or presence of either 10 μM forskolin or 1,9-deoxyforskolin and then their CaR^{T888} phosphorylation levels determined. As shown in Figure 4.8*A*, 2.5 mM Ca^{2+}_o increased CaR^{T888} phosphorylation of the 160 kDa protein and this effect was inhibited by cotreatment with 10 μM forskolin, whereas 10 μM 1,9-deoxyforskolin was without effect. Similar observations were made for the 140 kDa immature CaR band. Quantification of the phospho-CaR^{T888} levels (corrected for total CaR abundance) confirm that the 2.5 mM Ca^{2+}_o response is significantly lower in the presence of forskolin (Figure 4.8*B*; $P < 0.001$). This result shows that forskolin treatment is associated with decreased CaR^{T888} phosphorylation and thus this might explain the enhancement of CaR-induced Ca^{2+}_i mobilisation seen with forskolin treatment as well as the enhanced recovery of CaR-induced Ca^{2+}_i mobilisation following acute PMA treatment.

4.3.9 Effect of forskolin on muscarinic receptor-induced Ca^{2+}_i mobilisation

With the previous finding suggesting that forskolin might potentiate CaR-induced Ca^{2+}_i mobilisation by causing CaR^{T888} phosphorylation, it was necessary to determine whether forskolin potentiates Ca^{2+}_i mobilisation for other $G_{q/11}$-coupled GPCRs. For this, CaR-HEKs were stimulated with 10 μM carbachol to which forskolin was then added. As shown in Figure 4.9, carbachol induced Ca^{2+}_i mobilisation as before but when 10 μM forskolin was further added, this caused additional Ca^{2+}_i mobilisation (Figure 4.9*A*). Quantification of these response confirmed that forskolin caused significant enhancement of the carbachol effect (Figure 4.9*B*; $P<0.05$). This result indicates that the positive modulatory effect of forskolin on Ca^{2+}_i mobilisation is not specific to the CaR and can be seen with muscarinic receptor activation as well.

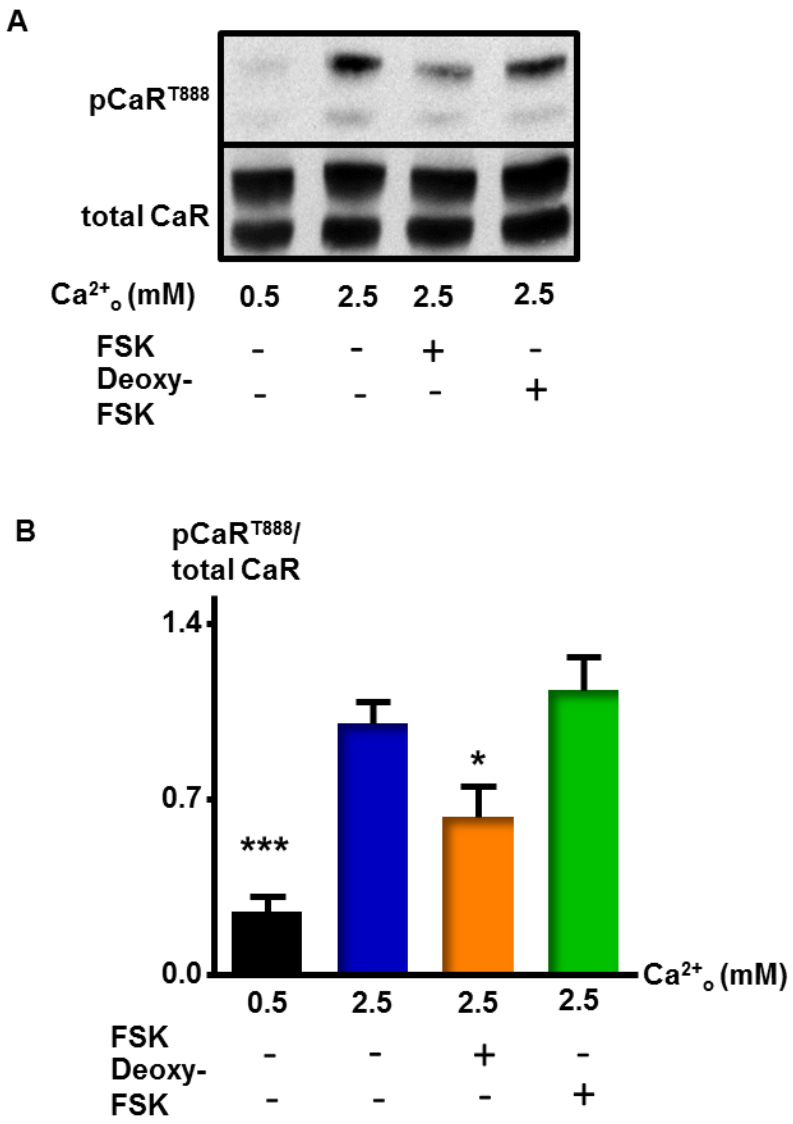

Figure 4.8: Forskolin inhibits CaR^{T888} phosphorylation in CaR-HEK cells. *A*) The phosphorylation of CaR^{T888} increased with the raise of Ca^{2+}_o concentration from 0.5 to 2.5 mM but less CaR^{T888} phosphorylation was observed in cells treated with 10 μM of forskolin at 2.5 mM Ca^{2+}_o compared to control and cells treated with 10 μM of 1,9-deoxyforskolin. *B*) The quantification of band intensity shows the phosphorylation of CaR^{T888} in control cells was significantly higher at 2.5 mM Ca^{2+}_o compared to 0.5 mM Ca^{2+}_o and CaR^{T888} phosphorylation in cells treated with forskolin but not CaR^{T888} phosphorylation in cells treated with 1,9-deoxyforskolin. ***P < 0.001 between pCaR^{T888} band intensity of control at 0.5 and 2.5 mM Ca^{2+}_o; *P < 0.05 between pCaR^{T888} band intensity of forskolin treated cells vs control at 2.5 mM Ca^{2+}_o; P > 0.05 between pCaR^{T888} band intensity of 1,9-deoxyforskolin treated cells vs control at 2.5 mM Ca^{2+}_o. The analysis was done using one-way ANOVA with Dunnett's post-test vs 2.5 Ca^{2+}_o on the pre-normalised data. N = 7 replicates from 3 independent experiments.

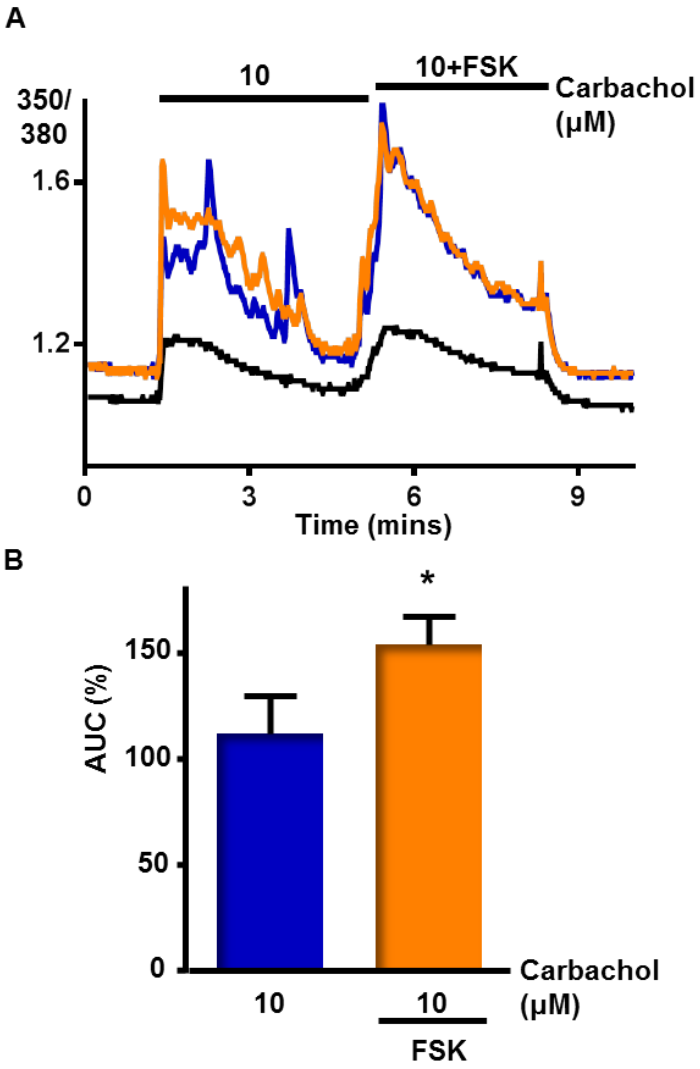

Figure 4.9: Forskolin affects muscarinic receptor-responsiveness in CaR-HEK cells. *A*). Fura-2 loaded cells were exposed to 10 μM of carbachol prior the addition of 10 μM of forskolin to determine the effect of forskolin on Ca^{2+}_i mobilisation. Shown are representative Ca^{2+}_i traces from cells incubated with or without forskolin. *B*) Quantification of percentage of Area under curve/ minutes (AUC %) showing the AUC % for forskolin treatment is significantly higher than carbachol treatment alone. The quantification was based on the relative change of Ca^{2+}_i mobilisation expressed as % of the response at 3.0 mM of Ca^{2+}_o. *P < 0.05 between Ca^{2+}_i mobilisation in forskolin treatment and carbachol treatment by paired t-test on the pre-normalised data. n = 6 coverslips from 2 independent transfections.

4.3.10 Effect of CaR activation on the phosphorylation / activation of ERK, IGF1R, IκBα, and P70 S6Kinase

In the next series of experiments, several signalling pathways not previously associated with CaR activation were examined to see whether they are in fact novel effector pathways of the receptor. CaR is well known to cause phosphorylation and activation of the MAP kinases ERK (p44 ERK-1 and p42 ERK-2) and $P38^{MAPK}$ (Kifor *et al*., 2001; Tfelt-Hansen *et al*., 2004; Davies *et al*., 2006). However, in order to investigate biased signalling of CaR then it will be helpful to identify more effector pathways to use as readouts of CaR activity. In this study, I examined the effect of CaR activation on the phosphorylation / activation of three kinases, namely IGF1R, IκB α, and P70 S6Kinase in CaR-HEK cells. These three kinases were selected based on the results of preliminary screening collected previously using a Bio-Plex® immunoassay (unpublished data, not shown). The phosphorylation of ERK was also included in this study as a positive control. In the first series of experiments, CaR was exposed to 5 mM of Ca^{2+}_o either in the absence or presence of 1 μM NPS2143 (calcilytic, negative CaR modulator) and then the phosphorylation levels of the various proteins determined by immunoblotting. As shown in Figure 4.10*A*, raising Ca^{2+}_o from 0.5 to 5 mM markedly increased the phosphorylation of all four proteins tested, namely ERK, IGF1R (Tyr1131) IκBα (Ser32) and P70 S6Kinase (Thr389). In addition, cotreatment with the calcilytic NPS2143 largely abolished each of these responses. Quantification of these changes demonstrated that the 5 mM Ca^{2+}_o-induced increase in protein phosphorylation was significant for each (at least $P<0.05$) and that the calcilytic-induced inhibition of this effect was also significant for each (Figure 4.10*B*; at least $P < 0.05$). These results suggest that the CaR stimulates the phosphorylation / activation not only of ERK, but also of IGF1R, IκBα and P70 S6Kinase.

Then, to further confirm the CaR activity elicits phosphorylation of ERK, IGF1R, IκBα, and P70 S6Kinase, the effect of the calcimimetic R568 on the phosphorylation of these kinases was also studied. In addition, it was also necessary to measure the concentration-dependency of the Ca^{2+}_o effect to see whether CaR stimulates some pathways with greater potency than others. For this, CaR-HEK cells were exposed to various Ca^{2+}_o concentrations from 0.5 to 10 mM, or, to 2mM Ca^{2+}_o in the presence of 1μM R568

(positive CaR modulator) and then assayed for their phosphoprotein content as before. The results are shown in Figure 4.11 and 4.12. Firstly, Figure 4.11 shows the Ca^{2+}_o-concentration dependent increase in ERK and IGF1R phosphorylation with EC_{50} values for Ca^{2+}_o of 2.1 ± 0.2 mM and 4.1 ± 0.5 mM respectively (Figure 4.11*B*). In both cases, cotreatment of cells exposed to 2 mM Ca^{2+}_o with 1 μM R568 caused a significant increase in phosphorylation (Figure 4.11*B*; $P < 0.001$, IGF1R; $P < 0.01$, ERK).

Then the same results for IκBα and P70 S6Kinase are shown in Figure 4.12. Again, Ca^{2+}_o caused a concentration-dependent increase in the phosphorylation of both kinases with EC_{50} values for Ca^{2+}_o of 3.8 ± 0.3 mM for IκBα and 2.6 ± 0.5 mM for P70 S6Kinase (Figure 4.12*B*). Furthermore, cotreatment with R568 caused a substantial and statistically significant increase in IκBα ($P < 0.01$) and P70 S6Kinase ($P < 0.001$) phosphorylation compared to treatment with 2 mM Ca^{2+}_o buffer alone (Figure 4.12*B*). Finally, to compare the relative potency of the CaR for each pathway, the log-EC_{50} values of each Ca^{2+}_o curve were compared by 1-way ANOVA with Dunnett's post-test which showed that the Ca^{2+}_o EC_{50} values for IGF1R and P70 S6Kinase were significantly lower than those for ERK (Figure 4.11*B* and 4.12*B*; $P < 0.01$ and $P < 0.05$). In contrast, there was no significant difference between the Ca^{2+}_o EC_{50} for IκBα and ERK (Figure 4.12*B*; $P > 0.05$). Together, these data confirm that CaR stimulation results in increased IGF1R, P70 S6Kinase and IκBα phosphorylation and further suggest that the first two are induced at lower concentrations of Ca^{2+}_o than the latter.

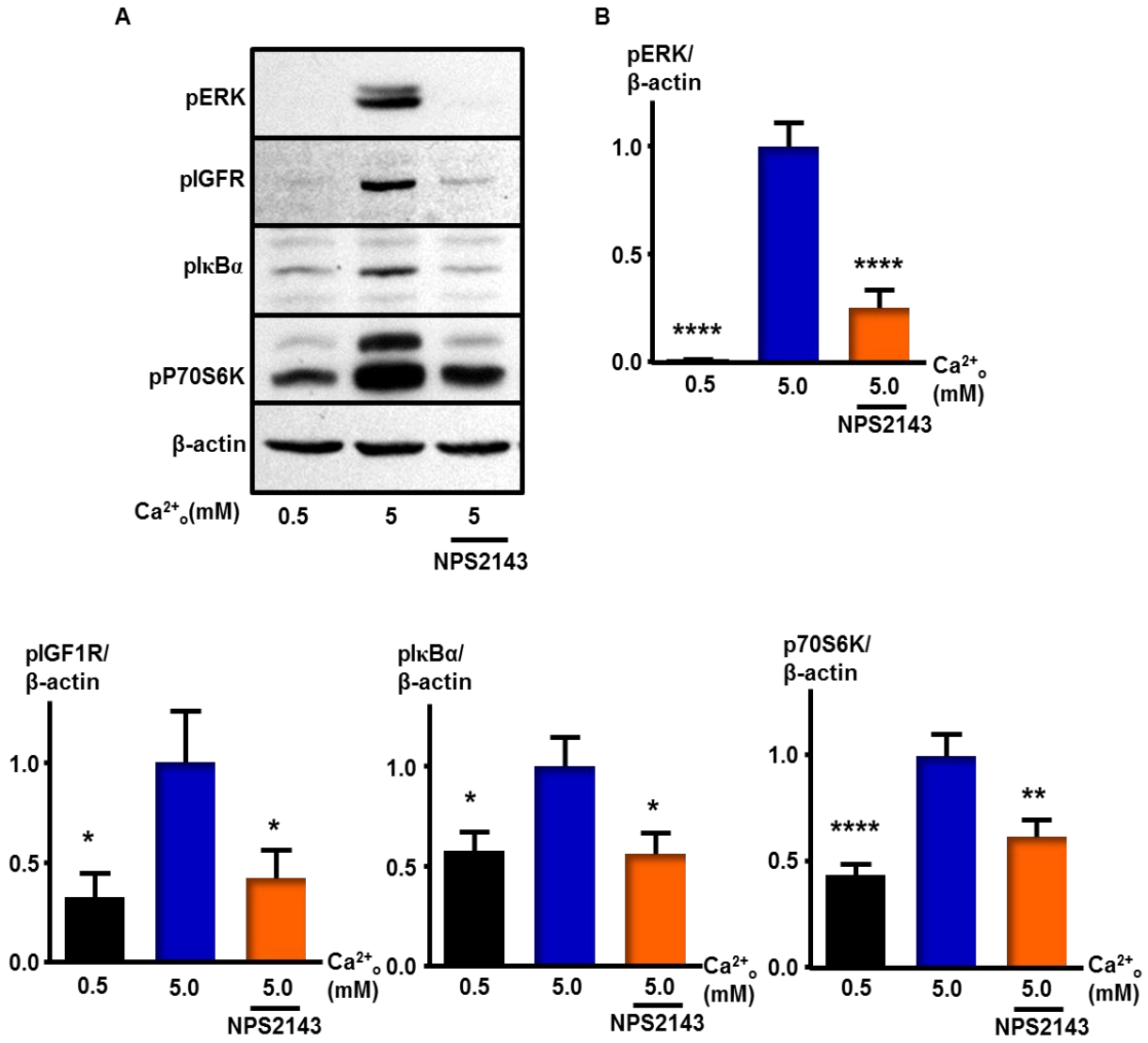

Figure 4.10: High Ca^{2+}_o concentration elicits phosphorylation of ERK, IGF1R, IκBα and p70S6kinase via a calcilytic-inhibitable mechanism. *A*) Representative immunoblots showing that phosphorylation of ERK, IGF1R, IκBα and P70 S6kinase was increased in response to 5 mM Ca^{2+}_o, whereas cotreatment with 1 μM NPS2143 (Cx; in 5 mM Ca^{2+}_o) largely ablated the response. β-actin is used a loading control. *B*) Bar graphs showing the quantification of phosphoprotein immunoreactivity relative to the loading control. **P < 0.01; ***P < 0.001, ****P < 0.0001 vs 5 mM Ca^{2+}_o only; one-way ANOVA with Dunnett's post-test on the pre-normalised data; n = 9 replicates from 3 independent experiments.

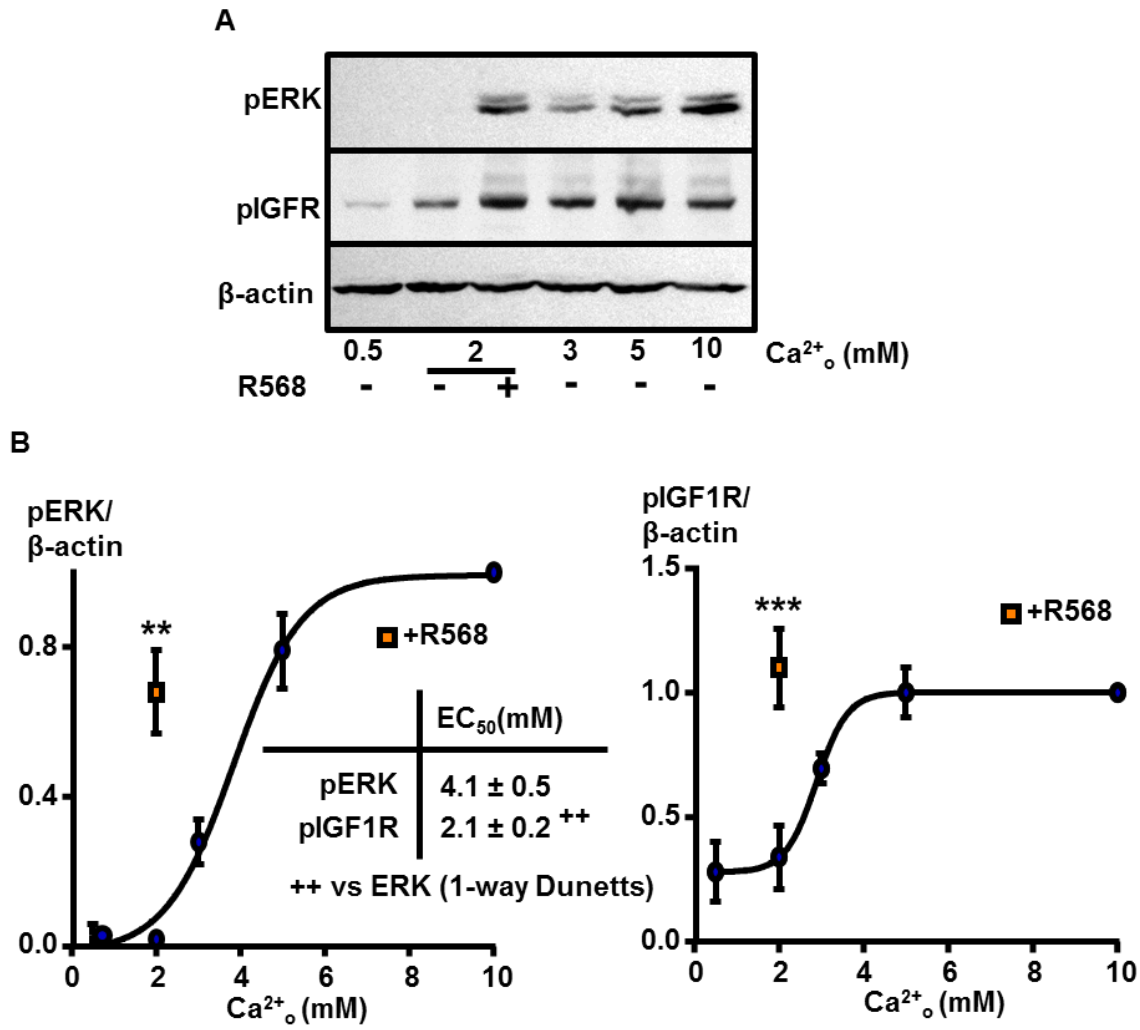

Figure 4.11: Ca^{2+}_o concentration-dependent increases in ERK and IGF1R phosphorylation and their potentiation by NPS-R568. *A*) The phosphorylation of ERK and IGF1R increased with the addition of 1 μM NPS R568 and/or by increasing Ca^{2+}_o concentration from 0.5 to 10.0 mM. *B*) Quantification of band intensity shows ERK and IGF1R phosphorylation to be significantly higher in the presence of NPS R568 than in 2.0 mM Ca^{2+}_o alone. The Ca^{2+}_o EC_{50} for IGF1R phosphorylation was also significantly lower compared to ERK phosphorylation. **P < 0.01, *** P < 0.001 NPS R568 in 2 mM Ca^{2+}_o vs 2 mM Ca^{2+}_o alone; $^{++}$P < 0.01 Log EC_{50} Ca^{2+}_o for IGF1R phosphorylation vs ERK phosphorylation. Both by unpaired t-test; n = 9 replicates from 3 independent experiments.

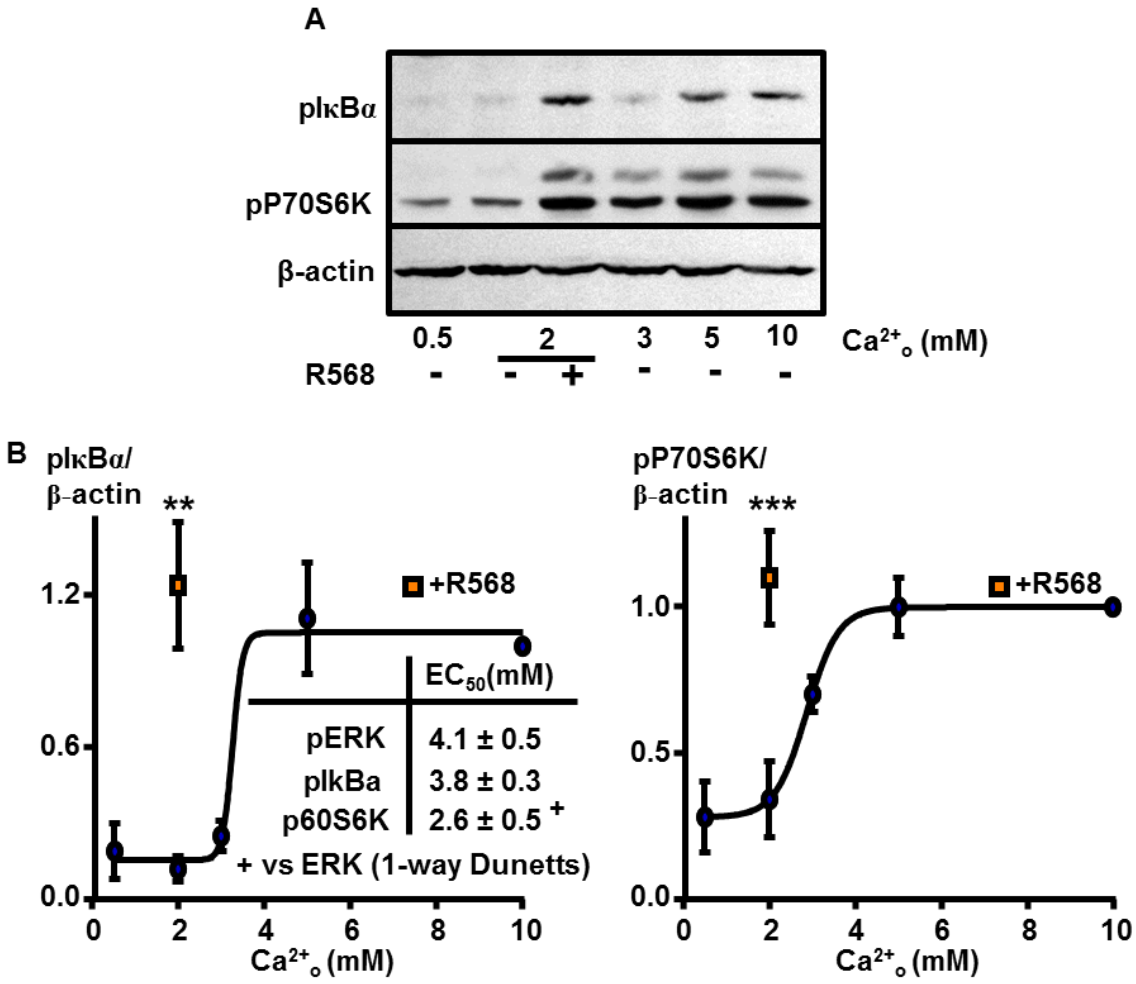

Figure 4.12: Ca^{2+}_o concentration-dependent increases in IκBα and P70 S6kinase phosphorylation and their potentiation by NPS-R568. *A*) The phosphorylation of IκBα and P70 S6kinase increased with the addition of 1 μM of NPS R-568 and by increasing of Ca^{2+}_o concentration from 0.5 to 10 mM. *B*) Quantification of band intensity shows that NPS R-568 increased IκBα and P70 S6kinase phosphorylation relative to treatment in 2.0 mM Ca^{2+}_o alone. The Ca^{2+}_o EC_{50} for P70 S6kinase phosphorylation was significantly lower than for ERK phosphorylation whereas the Ca^{2+}_o EC_{50} for IκBα phosphorylation was not different from ERK phosphorylation. **P < 0.01, ***P < 0.001 NPS R-568 in 2 mM Ca^{2+}_o vs 2 Ca alone by unpaired t-test; $^+$ P < 0.05 Log EC_{50} Ca^{2+}_o vs. ERK phosphorylation by 1-way ANOVA with Dunnett's post-test; n = 9 replicates from 3 independent experiments.

4.3.11 Effect of PKCα downregulation on the phosphorylation of ERK, IGF1R, and P70 S6Kinase

Having established in the previous chapter that downregulation of PKCα enhances CaR-induced Ca^{2+}_i mobilisation and then shown in 4.3.10 that CaR can also activate the IGF1R, P70 S6Kinase and IκBα pathways (and with different levels of sensitivity), it was necessary to next test the effect of PKCα downregulation on ERK, IGF1R, P70 S6Kinase and IκBα signalling. For this, CaR-HEKs pretreated with either control or PKCα siRNAs were stimulated with 2.5 mM Ca^{2+}_o and their phosphoprotein content examined. The results are shown in Figure 4.13*A* and it should be noted that IκBα phosphorylation was often undetectable below 5 mM Ca^{2+}_o (data not shown) and thus was not tested further. For the remaining kinases, phosphorylation of ERK, IGF1R, and P70 S6Kinase were all increased upon exposure to 2.5 mM Ca^{2+}_o but rather than potentiating the responses, knocking down of PKCα in fact partially inhibited the phosphorylation of all three kinases at 2.5 mM of Ca^{2+}_o. The protein equivalence of each sample was confirmed by the uniform β-actin protein expression on the western blots. The phosphoprotein levels were quantified by densitometry, corrected for β-actin signal and normalised to the response in 2.5 Ca^{2+}_o (Figure 4.13*B*). Statistical analysis of these changes by Repeated Measures ANOVA with Dunnett's post-test confirmed that PKCα knockdown did partially inhibit 2.5 mM Ca^{2+}_o-induced phosphorylation of ERK, IGF1R, and P70 S6Kinase, though by less than half in each case.

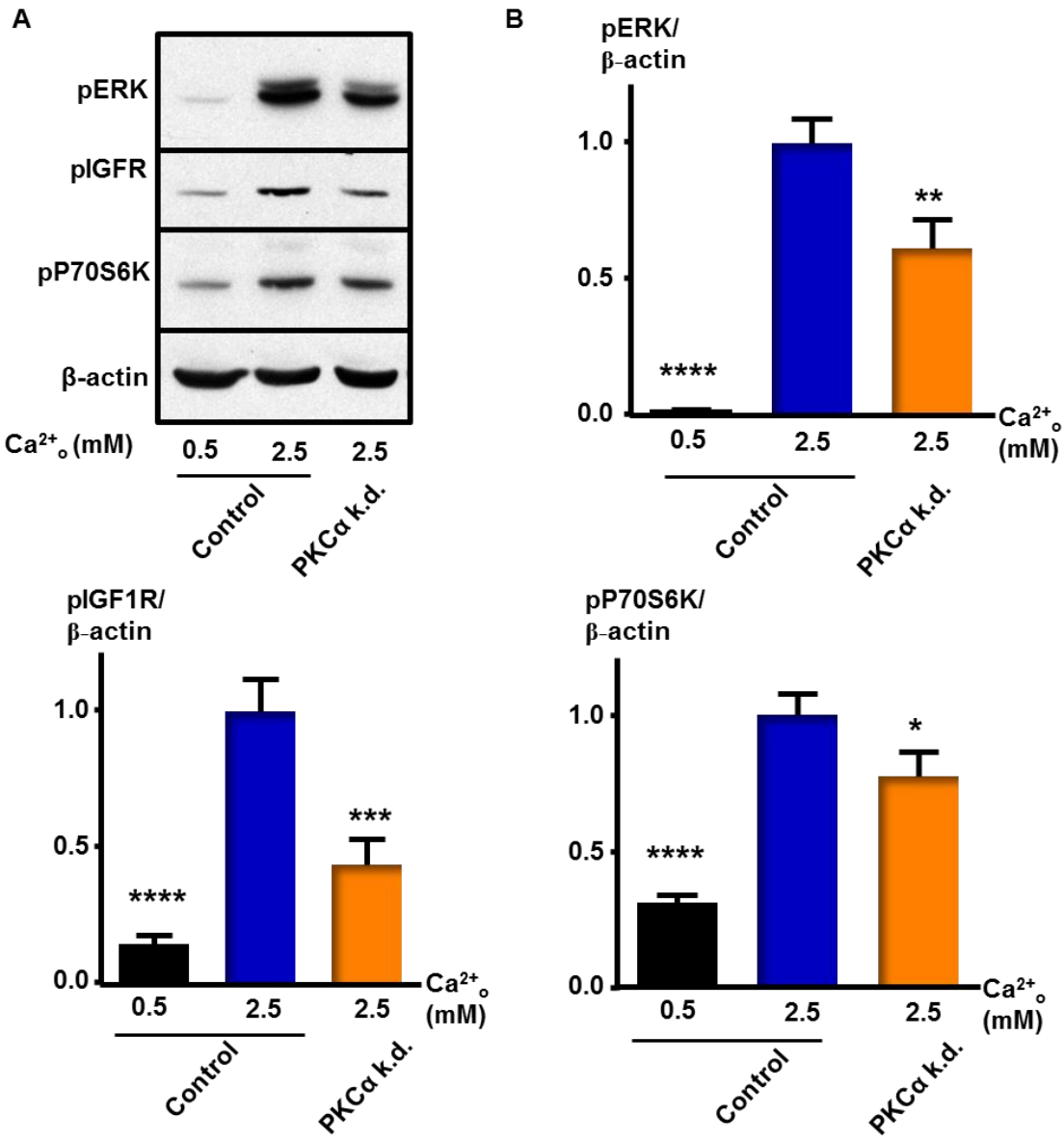

Figure 4.13: PKCα knockdown inhibits ERK, IGF1R, and P70 S6kinase phosphorylation. Cells were cultured in the presence or absence of control or PKCα-specific siRNA (as described in 2.3) and then exposed to either 0.5 or 2.5 mM Ca^{2+}_o concentration for 10-mins. Cells were then lysed as before and processed for immunoblotting. *A*) Representative immunoblot showing that the phosphorylation of ERK, IGF1R and P70 S6kinase phosphorylation increased in response to 2.5 mM Ca^{2+}_o concentration but that this effect was significantly lower in the PKCα knockdown cells. β-actin is used a loading control. *B*) Bar graphs showing the quantification of phosphoprotein immunoreactivity relative to the loading control. *P < 0.05, **P < 0.01, ****P < 0.0001 vs 2.5 mM Ca^{2+}_o by one-way ANOVA with Dunnett's post-test on the pre-normalised data. Data were normalised to the 2.5 mM Ca^{2+}_o control for the bar chart. n = 8 replicates from 3 independent transfections.

4.3.12 Effect of PKCε downregulation on the phosphorylation of ERK, IGF1R, and P70 S6 Kinase

Next, since PKCε knockdown had also been shown to affect CaR-induced Ca^{2+}_i mobilisation, negatively in this case (Section 3.3.7), the consequence of PKCε downregulation on the phosphorylation of ERK, IGF1R, and P70 S6Kinase was also tested. As before, 2.5 mM Ca^{2+}_o treatment increased phosphorylation of ERK, IGF1R, and P70 S6Kinase and this effect was inhibited in cells in which PKCε had been knocked down (Figure 4.14*A*), although the inhibition was less than 50% in each case. Protein loading was shown to be equivalent using β-actin immunoreactivity and kinase phosphorylation levels were corrected for β-actin abundance and then normalised to the response to 2.5 mM Ca^{2+}_o. By this it was shown that the inhibition of ERK, IGF1R, and P70 S6Kinase phosphorylation by PKCε knockdown was statistically significant ($P < 0.05$ in each case; Figure 4.14*B*).

4.3.13 Effect of $G\alpha_{12}$ downregulation on the phosphorylation of ERK, IGF1R and P70 S6Kinase

Finally, with $G\alpha_{12}$ knockdown having been shown to inhibit CaR-induced Ca^{2+}_i mobilisation in Section 4.3.4, here the effect of downregulating $G\alpha_{12}$ protein abundance was tested on the phosphorylation of ERK, IGF1R and P70 S6Kinase following stimulation with 2.5 mM Ca^{2+}_o. As shown in Figure 4.15*A*, 2.5 mM Ca^{2+}_o increased phosphorylation of ERK, IGF1R, and P70 S6Kinase in the control cells whereas in the $G\alpha_{12}$ knockdown cells, the responses were inhibited. Though significant, the inhibition of the ERK response was relatively small whereas the inhibition of the IGF1R and P70 S6Kinase responses were much more marked, indeed the S6kinase response was essentially ablated (Figure 4.15*B*).

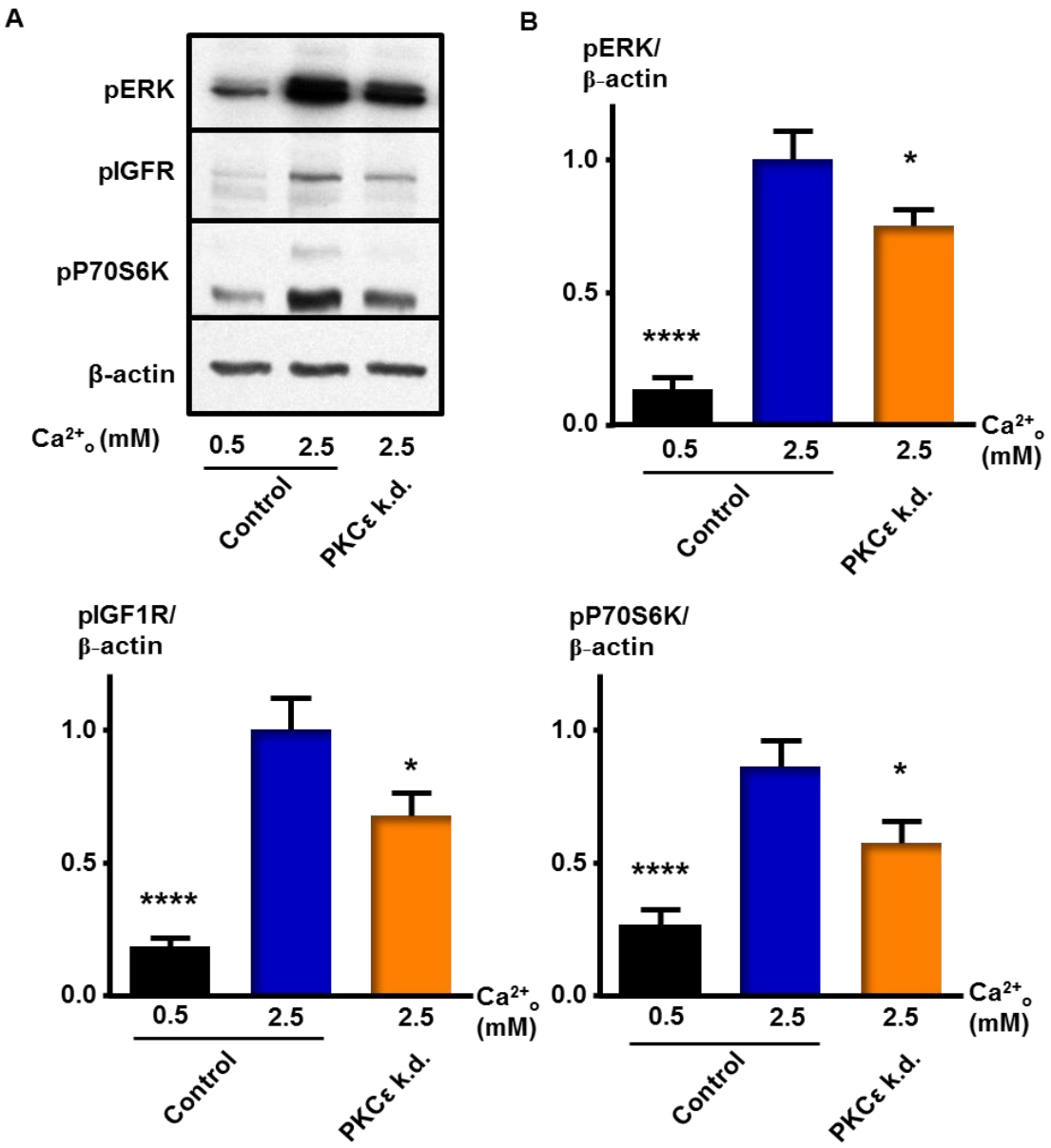

Figure 4.14: PKCε knockdown inhibits ERK, IGF1R and P70 S6kinase phosphorylation in CaR-HEK cells. Cells were cultured in the presence or absence of control or PKCε-specific siRNA (as described in 2.3) and then exposed to either 0.5 or 2.5 mM Ca^{2+}_o concentration for 10-mins. Cells were then lysed as before and processed for immunoblotting. *A*) Representative immunoblot showing that the phosphorylation of ERK, IGF1R and P70 S6kinase phosphorylation increased in response to 3 mM Ca^{2+}_o concentration but that this effect was significantly lower in the PKCε knockdown cells. β-actin is used a loading control. *B*) Bar graphs showing the quantification of phosphoprotein immunoreactivity relative to the loading control. *P < 0.05, ****P<0.0001 vs 2.5 mM Ca^{2+}_o by one-way ANOVA with Dunnett's post-test on the pre-normalised data. Changes in phosphorylation at 2.5 mM Ca^{2+}_o were normalised to 1. n = 8 replicates from 3 independent transfections.

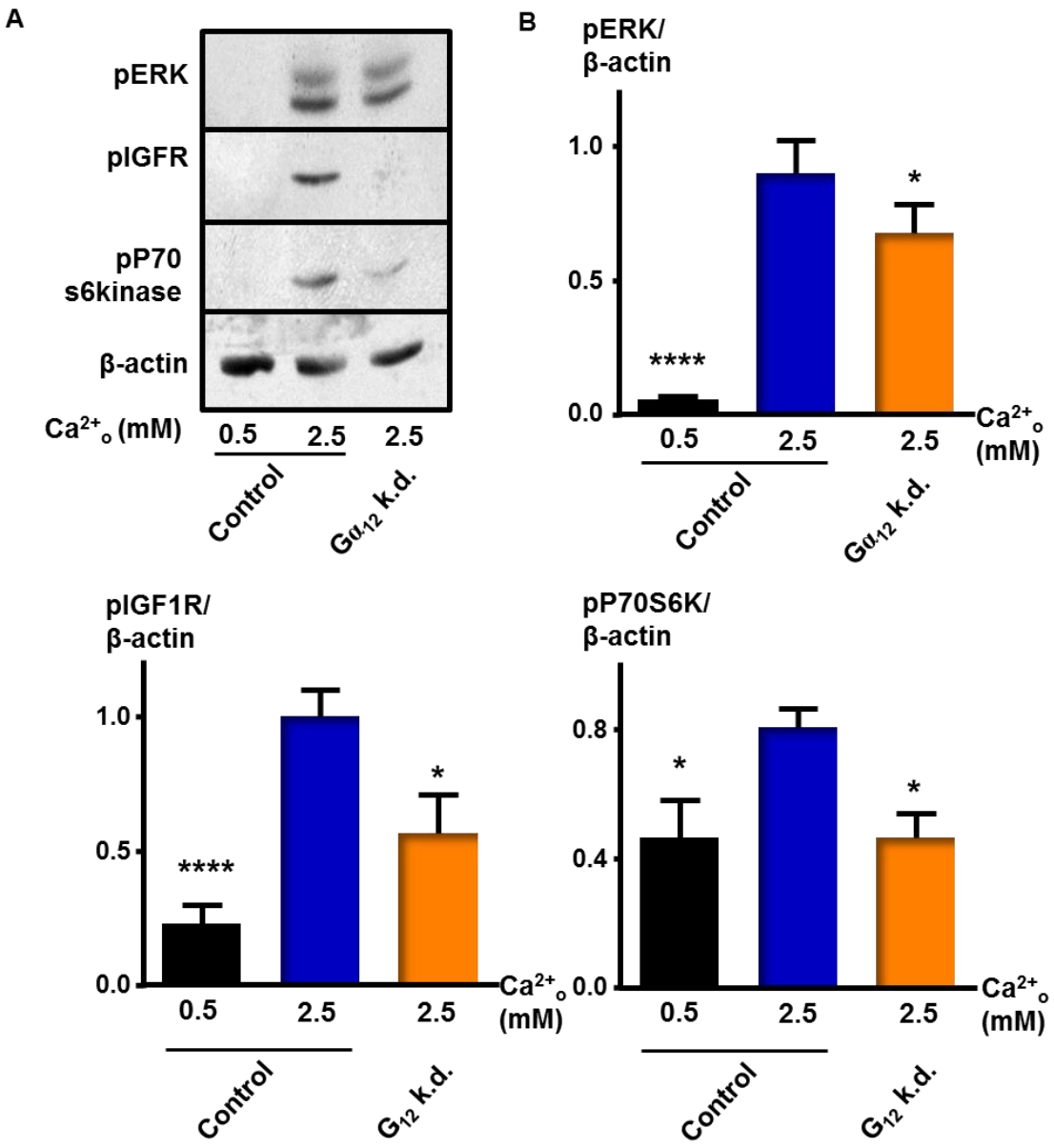

Figure 4.15: Gα$_{12}$ knockdown inhibits ERK, IGF1R, and P70 S6kinase phosphorylation in CaR-HEK cells. Cells were cultured in the presence or absence of control or Gα$_{12}$-specific siRNA (as described in 2.3) and then exposed to either 0.5 or 2.5 mM Ca^{2+}_o concentration for 10-mins. Cells were then lysed as before and processed for immunoblotting. *A*) Representative immunoblot showing that the phosphorylation of ERK, IGF1R and P70 S6kinase phosphorylation increased in response to 3 mM Ca^{2+}_o concentration but that this effect was significantly lower in the Gα$_{12}$ knockdown cells. β-actin is used a loading control. *B*) Bar graphs showing the quantification of phosphoprotein immunoreactivity relative to the loading control and then normalised to the response to 3 mM Ca^{2+}_o. *P < 0.05, ****P < 0.0001 vs 2.5 mM Ca^{2+}_o by one-way ANOVA with Dunnett's post-test on the pre-normalised data. n ≥ 10 replicates from 3 independent transfections.

4.4 DISCUSSION

4.4.1 The effect of PKC downregulation on CaR-induced Ca^{2+}_i mobilisation at sub-threshold Ca^{2+}_o concentration

The Ca^{2+}_o-threshold for Ca^{2+}_i mobilisation in CaR-HEK cells was found to be between 2 and 3 mM (Vargas-Poussou *et al*., 2002; Davies *et al*., 2007; McCormick *et al*., 2010). Below this Ca^{2+}_o concentration (sub-threshold), $G_{q/11}$/PLC-mediated Ca^{2+}_i mobilisation is either poorly detected or even undetectable. Interestingly however, CaR-induced actin polymerisation (believed to be $G_{12/13}$-mediated) occurs at lower Ca^{2+}_o concentrations (EC_{50}, 1.55mM; threshold ~1.2mM) i.e. Ca^{2+}_o concentrations that are sub-threshold for Ca^{2+}_i mobilisation (Davies *et al*., 2006). This suggests therefore that in CaR-HEK cells, the CaR may be in a largely active conformation in the presence of 1.8mM Ca^{2+}_o for instance, and thus able to cause robust actin polymerisation but yet be unable to mobilise Ca^{2+}_i. A similar Ca^{2+}_o sensitivity was seen for cAMP response element binding protein (CREB) phosphorylation by Avlani *et al* (2013), where the EC_{50} for Ca^{2+}_o was 1.53mM with a detection threshold close to 1mM. This CaR-inducd CREB phosphorylation in CaR-HEK cells was found to be mediated through PLC and PKC signalling (Avlani *et al*., 2013). Based on these observations it was hypothesised that Ca^{2+}_i mobilisation in Ca^{2+}_o concentrations between 1.6 and 2 mM was being inhibited by PKC-mediated CaR phosphorylation, including phosphorylation of CaR^{T888}, which acts as a brake in a system that contains an otherwise active CaR. CaR-HEK cells were exposed to a sub-threshold (with regards to Ca^{2+}_i mobilisation) Ca^{2+}_o concentration. Indeed, the inhibition of PKCs by GF102903X was found to trigger Ca^{2+}_i mobilisation in CaR-HEK cells at sub-threshold concentrations (Figure 4.1). Moreover, the PKCα knockdown, which was earlier shown to potentiate Ca^{2+}_i mobilisation and attenuate CaR^{T888}, triggered Ca^{2+}_i mobilisation at sub-threshold Ca^{2+}_o concentrations as low as 1.8 mM (Figure 4.2). Although 2.5 mM Ca^{2+}_o was used in Figure 3.14 to examine PKCα-mediated CaR^{T888} phosphorylation while 1.8 – 2 mM was used in Figure 4.2 to examine PKC-regulated Ca^{2+}_i mobilisation, McCormick *et al*. (2010) found no significant difference between the CaR^{T888} phosphorylation at 1.8 mM and 2.5 mM of Ca^{2+}_o.

The observations in this study suggest that PKCα-mediated CaR^{T888} phosphorylation might prevent Ca^{2+}_i mobilisation at apparently sub-threshold Ca^{2+}_o concentrations even though the receptor is sufficiently active to increase actin polymerisation (Davies *et al*., 2006) and CREB phosphorylation (Avlani *et al*., 2013), that is, be in an otherwise active conformation. Indeed it is even possible that one or more of the other CaR signals seen at Ca^{2+}_o concentrations sub-threshold for Ca^{2+}_i mobilisation (~1.8 mM) crosstalk with the PLC/IP_3 pathway to either sustain the suppressed Ca^{2+}_i release, or even to attempt to overcome the phosphoCaR^{T888}-mediated inhibition of Ca^{2+}_i mobilisation. This possibility will be examined next with regards to $G\alpha_{12}$ and then again later with regards to cAMP concentration.

4.4.2 The effect of $G\alpha_{12}$ downregulation on CaR-induced Ca^{2+}_i mobilisation

Upon its activation, CaR binds to and activates several different heterotrimeric G proteins. To date, CaR has been shown to couple to $G\alpha q_{/11}$, $G\alpha_{12/13}$, and $G\alpha_{i/o}$ to mediate Ca^{2+}_i mobilisation, actin polymerisation, and cAMP inhibition respectively (Davies *et al*., 2006; Davies *et al*., 2007; Saidak *et al*., 2009; McCormick *et al*., 2010; Davey *et al*., 2012). Interestingly, the knockdown of $G\alpha_{12}$ was found to attenuate the Ca^{2+}_i mobilisation in CaR-HEK cells and increase the Ca^{2+}_o EC_{50} suggesting the involvement of $G\alpha_{12}$ in Ca^{2+}_i mobilisation in cells (Figure 4.3). CaR-mediated activation of $G\alpha_{12}$ is associated with a subsequent increase in activity of the monomeric G protein Rho which leads in turn to induction of actin polymerisation via the Rho-kinase, ROCK (Awata *et al*., 2001; Hjläm *et al*., 2001; Davies *et al*., 2006). Interestingly the Rho-kinase inhibitors, Y-27632 and H1152 fail to attenuate Ca^{2+}_i mobilisation in CaR-HEK cells, despite inhibiting the cytoskeletal changes (Davies *et al*., 2006) suggesting that the $G\alpha_{12}$ effect may split to cause rho kinase-mediated cytoskeletal changes on the one hand, and rho kinase-independent modulation of Ca^{2+}_i mobilisation on the other. Rey *et al*. (2005) previously suggested that $G\alpha_{12}$ was involved in the CaR-induced Ca^{2+}_i mobilisation triggered by aromatic amino acids. The same laboratory then found that the aromatic amino acids-triggered Ca^{2+}_i mobilisation was mediated by TRPC1 cation channel activity and occurred independently of the PLC pathway (Rey *et al*., 2006). In contrast to my current findings, this laboratory also showed that G_{12} down-regulation had no effect on

CaR-induced Ca^{2+}_i mobilisation triggered by Ca^{2+}_o (Rey *et al*., 2005). However, it should be noted that they only tested the effect of $G\alpha_{12}$ knockdown at a single, high concentration of Ca^{2+}_o, namely 5 mM and even in our hands this maximal stimulation was large enough to overcome the inhibitory effect of $G\alpha_{12}$ knockdown. Indeed if $G\alpha_{12}$ knockdown does inhibit Ca^{2+}_i mobilisation by permitting higher CaR^{T888} phosphorylation then it is not surprising that the effect is not seen in 5 mM as this concentration has been shown previously to elicit potent CaR^{T888} dephosphorylation.

In contrast to its effect on CaR responsiveness, knocking down $G\alpha_{12}$ in CaR-HEK cells was found to have no effect on the Ca^{2+}_i mobilisation induced by mChR (which is most likely to be the $G_{q/11}$-coupled M3 mChR based on our unpublished microarray data; Figure 4.4). Interestingly, M3 mChR has also been reported previously to couple to $G\alpha_{12}$ in HEK cells upon its activation (Rümenapp *et al*., 2001). These data suggest therefore that the $G\alpha_{12}$ effect is relatively specific to CaR. Furthermore, if M3 can couple to $G\alpha_{12}$ in CaR-HEK cells but without appearing to affect Ca^{2+}_i mobilisation then this would also suggest a more CaR-specific explanation for how $G\alpha_{12}$ modulates Ca^{2+}_i mobilisation in our hands. It should be noted though that the effects of carbachol on actin polymerisation were not specifically tested for in the current study.

In the absence of an effect of $G\alpha_{12}$ knockdown on the carbachol response this suggests that the attenuation of CaR-induced Ca^{2+}_i mobilisation caused by $G\alpha_{12}$ knockdown is unlikely to result from altered IP_3 receptor as this should affect both responses similarly. However, one possible explanation for this difference is with the relative production of IP_3. Huang *et al*. (2002) demonstrated that CaR stimulates the activity of PI 4-kinase, the enzyme that converts phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI4-P) in the production of the PLC substrate phosphatidylinositol 4,5-bisphosphate (PIP_2). This CaR-induced pathway was proposed to support the highly sustained signalling particular to the CaR and was specifically shown to be Rho-dependent (Huang *et al*., 2002), suggesting the involvement of CaR-$G\alpha_{12}$ coupling. If true then without sufficient $G\alpha_{12}$-mediated rho activity, the CaR may tend to quickly deplete PIP_2 levels causing the Ca^{2+}_o concentration effect curve to become right-shifted. However, if this were also true then one might expect transient responses to 2 and 3 mM in the $G\alpha_{12}$ knockdown cells to

be quickly terminated whereas in fact these responses are smaller throughout the time-course. In addition, one might also expect the larger stimuli (5 and 10 mM) to be disproportionately affected as their responses should deplete the PIP_2 even faster, though again this was not observed. Thus, to specifically test these issues it would be necessary to specifically assay relative IP_3 levels induced by the two receptors in the presence and absence of $G\alpha_{12}$ knockdown. In the meantime, a simpler explanation for the inhibitory effect of $G\alpha_{12}$ knockdown on Ca^{2+}_i mobilisation is the increased CaR^{T888} phosphorylation observed in Figure 4.5. This could explain why the $G\alpha_{12}$ knockdown affected the Ca^{2+}_o but not carbachol responses (since the M3 receptor lacks the CaR^{T888} site). Indeed the reason $G\alpha_{12}$ knockdown was tested in this study was because it had been shown to mediate the activation of PP2A by binding to its regulatory subunit (Zhu *et al*., 2007). Since calyculin (PP1/PP2A inhibitor) impairs CaR-induced Ca^{2+}_i mobilisation in CaR-HEK and bovine parathyroid cells (Davies *et al*., 2007) and increases PTH secretion from human parathyroid cells (McCormick *et al*., 2010), we hypothesised that $G\alpha_{12}$ might stimulate PP2A, causing CaR dephosphorylation and increased Ca^{2+}_i mobilisation. Thus, the increase in CaR^{T888} phosphorylation following $G\alpha_{12}$ knockdown seen here might result from underactivation of PP2A at moderate concentrations of Ca^{2+}_o that can be overcome at higher Ca^{2+}_o concentrations. This could in turn explain the rightward-shift in the Ca^{2+}_o concentration-effect curve for Ca^{2+}_i mobilisation. It will be interesting to see therefore whether decreased $G\alpha_{12}$ expression or function in parathyroid cells either *in vitro* or *in vivo* will have a detectable effect on CaR signalling and PTH secretion.

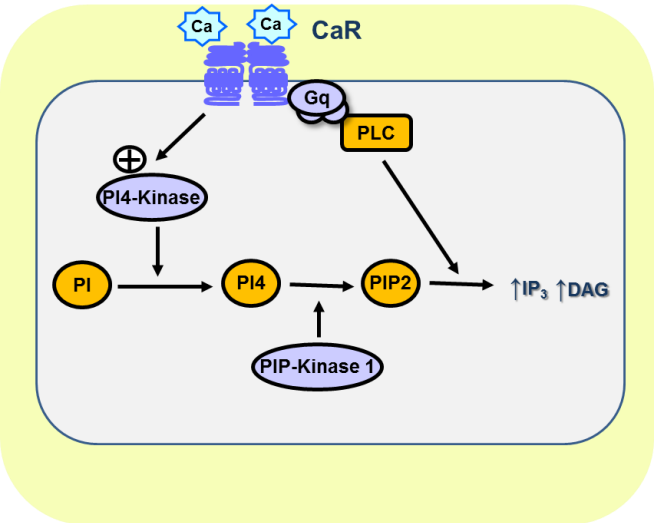

Figure 4.16: The mechanism of CaR induces PIP2 generation through the activation of PI4-Kinase

4.4.3 The effect of cAMP on CaR^{T888} phosphorylation

The generation of cAMP in parathyroid cells has long been associated with increased PTH secretion (Brown and MacLeod, 2001). Treatment of bovine parathyroid cells with either isoproterenol, dopamine or PGE_2 has been reported to enhance PTH secretion presumably via the activation of endogenously-expressed $G\alpha_s$-coupled GPCRs for those ligands (Brown *et al*., 1977a; Brown *et al*., 1977b; Gardner *et al*., 1978). The CaR is then believed to oppose this effect by lowering intracellular cAMP levels either via $G_{i/o}$ activation or via Ca^{2+}-inhibited adenylate cyclases, ultimately resulting in decreased PTH secretion (Brown and McLeod, 2001; Conigrave and Ward, 2013). This effect could be described as long-loop feedback where the PTH secreted in response to cAMP elicits a systemic effect, resulting in a rise in blood Ca^{2+} levels that then feeds back upon the parathyroid CaR. However, it is possible that these pathways might interact or crosstalk with each other within the cell in what could be considered a short-loop feedback, that is, that a rise in cAMP levels immediately affects CaR signalling to dampen any increase in PTH. This would help prevent an overshoot in blood Ca^{2+} levels occurring i.e. by beginning to limit the secretion even before the systemic change in Ca^{2+}_o concentration has occurred.

Such intracellular crosstalk between these pathways has been observed previously both here (Campion, 2013) and elsewhere (Gerbino *et al*., 2005) where a forskolin-induced rise in cAMP levels enhanced CaR-induced Ca^{2+}_i mobilisation in CaR-HEK cells. This finding was further confirmed in the study (Figure 4.6) however what is less clear is how the cAMP acts to enhance Ca^{2+}_i mobilisation and whether it is a general effect or a CaR-specific effect. One possibility is that the cAMP activates PKA (Petersen *et al*., 2008; Manni *et al*., 2008) which either phosphorylates the CaR directly (Garrett *et al*., 1995) or a signalling mediator such as the IP_3R (Baggaley *et al*., 2007). However, we have unpublished data showing that two different PKA inhibitors failed to alter CaR-induced Ca^{2+}_i mobilisation and that mutation of the two putative PKA sites failed to significantly alter Ca^{2+}_o responsiveness.

Thus, whether cAMP can affect CaR phosphorylation was investigated next and indeed it was found that forskolin caused an overall decrease in CaR^{T888} phosphorylation. Such a decrease could either be due to less phosphorylation e.g. PKC inhibition, or, more dephosphorylation e.g. via activation of PP2A activity. Davies *et al* (2007) showed that following exposure to PMA which caused suppression of CaR-induced Ca^{2+}_i mobilisation (in the presence of 2 mM Ca^{2+}_o), exposure to 5mM Ca^{2+}_o overcomes this PKC-mediated inhibition. Thus in the current study, it was found that following exposure to PMA in the presence of 2.5 mM Ca^{2+}_o, subsequent exposure to 3 mM Ca^{2+}_o fails to elicit sustained Ca^{2+}_i mobilisation whereas in the presence of forskolin, the 3 mM Ca^{2+}_o has a profound effect. It is difficult to conclude from this data whether the cAMP has inhibited PKC or stimulated phosphatase and both options remain viable. For example, exposure to 3mM Ca^{2+}_o (in the absence of forskolin, or, in the presence of the inactive forskolin analogue) does cause a small, transient rise in Ca^{2+}_i concentration which could result from an initial CaR dephosphorylation followed by a second phase of phosphorylation. Since forskolin cotreatment instead causes sustained Ca^{2+}_i mobilisation, then this might suggest that the PKC is inhibited in this case, however in individual cells, the Ca^{2+}_i responses remain oscillatory. The ability of cAMP to regulate PP2A activity has been reported in several studies previously. In the majority of cases, PKA activation is believed to be the mechanism of action (Davare *et al*., 2000; Ahn *et al*., 2007; Hong *et al*. 2008), but which is not the case in our laboratory (Campion, 2013). However, Feschenko *et al*. (2002) have also reported a PKA-independent mechanism of PP2A

activation by cAMP. Treatment with either forskolin or phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was found to enhance phosphatase activity in NRK-52E cells and completely dephosphorylated the elongation factor 2 (EF-2), which is a selective substrate of PP2A. Moreover, treatment with the PKA inhibitor, H89, in these cells failed to block the cAMP-induced dephosphorylation of EF-2 suggesting that this mechanism was PKA-independent.

Although further experiments would be required to settle this, on balance the current data tend to point more towards cAMP accelerating dephosphorylation, presumably via increased phosphatase activity, as opposed to PKC inhibition. However, it is the general finding that is more important and that is, that any signal that increases cAMP levels could potentially modulate CaR^{T888} phosphorylation and thus CaR signalling.

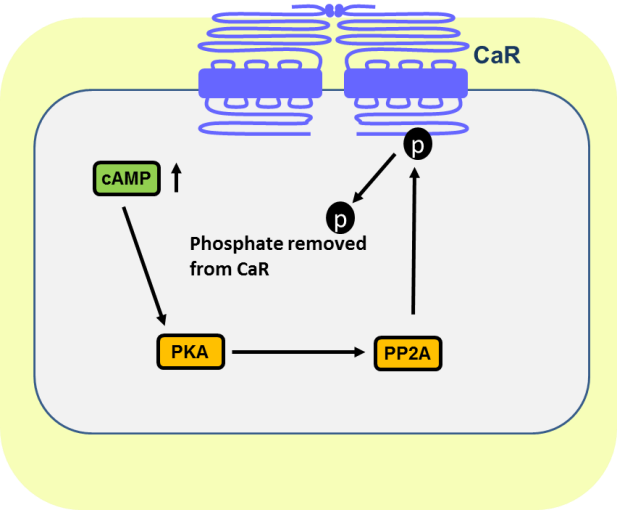

Figure 4.17: Possible mechanism cAMP potentiates CaR signalling

4.4.4 The effect of CaR activity on the phosphorylation of ERK, IGF1R, IκBα and P70 S6Kinase

The calcium-sensing receptor is a pleiotropic receptor that can be activated by various orthosteric agonists such as divalent and trivalent cations (Brown, 2008), aminoglycoside antibiotics (McLarnon *et al*., 2002), and polyamines (Cheng *et al*., 2004). Apart from that, CaR activity is also regulated by allosteric modulators such as aromatic L-amino acids (Conigrave *et al*., 2000) and several calcimimetics and calcilytics (Riccardi and

Ward, 2012). The concept of ligand biased signalling at GPCRs has attracted the interest of pharmacologists as it raises the possibility of producing better drugs that preferentially target one effector pathway over another with the potential to produce fewer off-target effects. Several studies have reported that the CaR can elicit ligand biased signalling. For instance, Davey *et al*. (2012) have shown that calcimimetics (NPS R-568 and cinacalcet) exhibit different potencies when triggering CaR-induced Ca^{2+}_i mobilisation, as opposed to ERK phosphorylation and actin polymerisation in CaR-HEK cells. In addition, the use of strontium as a CaR allosteric modulator has also shown to promote the CaR signalling that is biased towards ERK phosphorylation rather than $G\alpha_{q/11}$ signalling / Ca^{2+}_i mobilisation in rat medullary thyroid carcinoma 6-23 cells (Thomsen, *et al*., 2012). To further develop our understanding of ligand bias in CaR signalling, it would be helpful to identify more CaR effector pathways to act as readouts of receptor activity.

Currently, the vast majority of CaR studies examine either Ca^{2+}_i mobilisation or ERK activation as the main readouts of receptor activity, with $p38^{MAPK}$ activation, cAMP inhibition and actin polymerisation sometimes used (Chang *et a*l., 1998b; Tfelt-hansen *et al*., 2003; Davies *et al*., 2006; Davies *et al*., 2007). In this study, I have identified 3 novel downstream effector pathways of the CaR in HEK-293 cells which might at the very least represent useful additional activity readouts to allow for broader examinations of biased signalling. These three novel readouts are the phosphorylation of IGF1R, IκBα, and S6Kinase. These phosphoproteins were initially indicated using a Bio-Plex® assay (Bio-Rad) that can analyse the phosphorylation status of multiple proteins (~20) at the same time. Thus to prove that these 3 proteins become phosphorylated in response to CaR activation the phosphorylation status of each protein was investigated in elevated Ca^{2+}_o concentration in the absence and presence of the calcilytic, NPS-2143 (Figure 4.10) and the calcimimetic NPS R-568 (Figure 4.11 and 4.12). Together, these observations confirm the regulation of IGF1R, IκBα, and S6Kinase phosphorylation by CaR. Furthermore, based on the calculated EC_{50}s for Ca^{2+}_o for the phosphorylation of these kinases, it is clear that IGF1R and S6Kinase (though not IκBα) were phosphorylated with significantly greater Ca^{2+}_o sensitivity than for ERK (Figure 4.13 and 4.14) or indeed Ca^{2+}_i mobilisation (Figures 3.4 - 3.10). This greater sensitivity to Ca^{2+}_o more closely matched the responses of CREB phosphorylation (Avlani *et al*., 2013) and actin polymerisation (Davies *et al*., 2006; Davey *et al*., 2012). As mentioned earlier,

most CaR studies tend to use cytosolic Ca^{2+} or ERK phosphorylation as the standard CaR readouts but what the current data suggest is that these signals are relatively insensitive to Ca^{2+}_o compared to phosphorylation of IGF1R, CREB or S6Kinase, or to actin polymerisation. It would seem sensible in light of this to consider using additional, more Ca^{2+}_o-sensitive CaR readouts more widely.

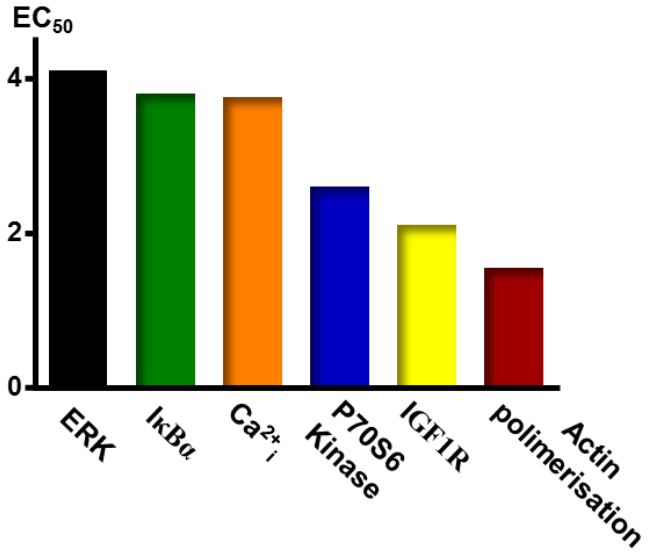

Figure 4.18: Summary of relative potencies of Ca^{2+} induced activation of ERK, IκBα, Ca^{2+}_i, P70 S6Kinase, IGF1R, and actin polimerisation in CaR-HEK cells

4.4.5 Potential cellular significance of CaR-induced IGF1R, IκBα and S6Kinase phosphorylation.

Insulin-like growth factor receptor-1 is a tyrosine kinase that phosphorylates its downstream effector proteins upon its activation. Upon activation by IGF, this receptor itself becomes phosphorylated on residues Y1131, Y1135 and Y1136 either by auto-phosphorylation or resulting from the action of other kinases (Maniar *et al*., 2005). In the current study, the phospho-specific antibody detected IGF1R phosphorylation on Y1131. Interestingly, my observation shows that even in the absence of exogenous IGF1, this receptor can become phosphorylated in response to CaR activation (Figure 4.16). Similarly, IGF1R in vascular smooth muscle cells (VSMC) was found to be activated by angiotensin II (Ang II) and thrombin which is an IGF1R substrate (Du *et al*., 1996). The

function of IGF1R in regulating Ca^{2+} homeostasis is still unclear. This receptor was previously found to be the cause of uncontrolled cell growth in PT carcinoma cells suggesting its involvement in stimulating PT cell growth (Tanaka *et al*., 1994). The increase of PT cells however will lead to more production of PTH. In contrast, my observation shows that IGF1R was activated by high Ca^{2+}_o concentration thus suggesting its activation might try to reduce the spike of Ca^{2+}_o. IGF1 has previously been found to enhance the store-operated influx of Ca^{2+} leading to increased Ca^{2+}_i levels (Ju *et al*., 2003). Thus, it is possible that the increase in IGF1R activity in high Ca^{2+}_o concentration might potentiate the Ca^{2+}_i mobilisation but this would need to be specifically tested with either molecular or pharmacological modulators of IGFR1 expression or function.

Next, S6kinase, is a serine/threonine kinase that is phosphorylated and activated by mammalian target of rapamycin 1 (mTORC1; Magnuson *et al*., 2012) and in turn then phosphorylates ribosomal protein s6 (rpS6) which is involved in the regulation of cell size, cell proliferation and glucose homeostasis (Figure 4.16; Magnuson *et al*., 2012). Apart from that, S6kinase is also known to be the downstream of IGF1R and ERK signalling (Snabaitis *et al*., 2005; Li *et al*., 2009). Thus, the CaR-induced increase in P70 S6kinase phosphorylation CaR-HEK cells might involve IGF1R or ERK activation although it occurs at lower Ca^{2+}_o concentrations than are able to activate ERK and thus this would seem unlikely. IκBα is an inhibitor of the transcription factor, NF-κB (Jacob and Harrison, 1998). In its inactive form, NF-κB binds to IκBα and forms an inactive complex. The phosphorylation of IκBα by IκB kinase then leads to the dissociation of this complex allowing the translocation of NF-κB to the nucleus (Gilmore, 2006). The translocation NF-κB to the nucleus has been reported to increase the expression of genes that mediate anti-apoptotic signals (Vega *et al*., 2009). Thus, the phosphorylation of IκBα in high Ca^{2+}_o concentration might important for the survival of the cells in stressed i.e. hyperstimulated conditions. In this regard, CaR-HEK cells exposed to the CaR-activating aminoglycoside gentamicin for 4-days show increased cell death via a mechanism that could be inhibited by the pan-specific caspase inhibitor Z-VAD (Ward *et al*., 2005).

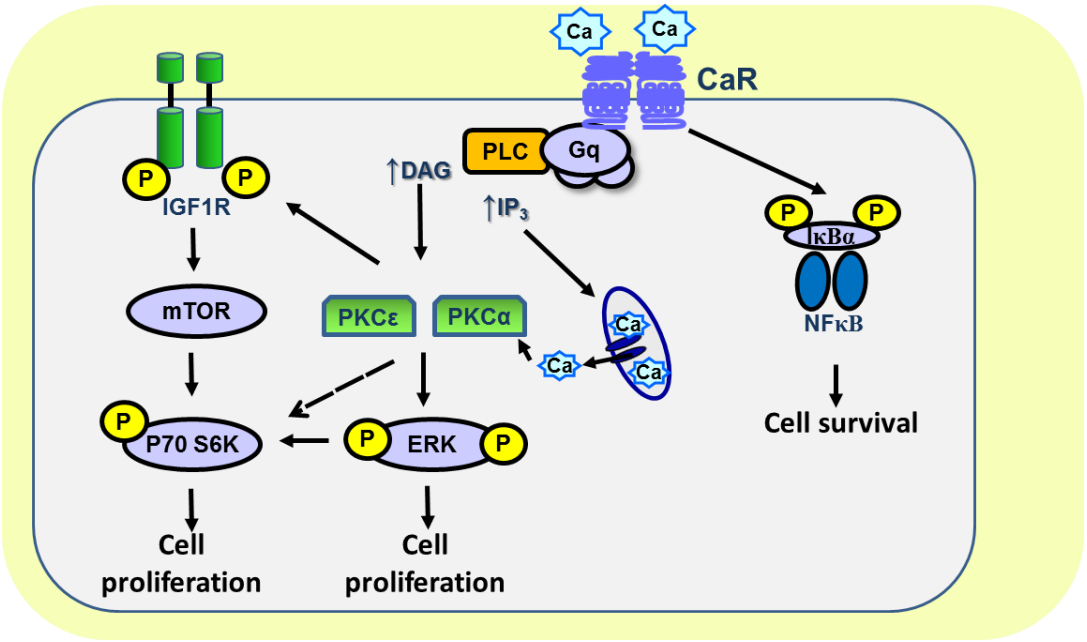

Figure 4.19. Schematic diagram of CaR-HEK signalling incorporating the three novel effectors IGF1R, P70 S6Kinase and IκBα and how they might interact.

4.4.6 The effect of PKCα, PKCε, and $G\alpha_{12}$ downregulation on the phosphorylation of ERK, IGF1R and P70 S6Kinase

Having shown in Chapter 3 that PKCα knockdown decreases CaR^{T888} phosphorylation and increases CaR-induced $Ca^{2+}{}_i$ mobilisation in CaR-HEK cells, it was then necessary to see whether it has a similar positive effect on ERK, IGF1R and P70 S6Kinase phosphorylation. Interestingly however, PKCα knockdown showed the opposite effect on the CaR-induced phosphorylation of ERK, IGF1R and P70 S6Kinase, that is by attenuating their phosphorylation (Figure 4.15). This observation shows firstly that CaR-induced $Ca^{2+}{}_i$ mobilisation can be dissociated from the phosphorylation of ERK, IGF1R and P70 S6Kinase upon PKCα knockdown. In contrast, Lazarus *et al*. (2011) showed that the potentiation of $Ca^{2+}{}_i$ mobilisation in CaR^{T888M} CaR-HEK cells was matched by an increase of ERK phosphorylation. If the sole action of PKCα was CaR^{T888} phosphorylation then one would expect ERK phosphorylation to be increased similarly to $Ca^{2+}{}_i$ mobilisation as shown by the CaR^{T888M} data. However since PKCα knockdown has the opposite effect on ERK activation to $Ca^{2+}{}_i$ mobilisation then this might suggest that PKCα has more cellular actions than just CaR^{T888} phosphorylation, for example to

also mediate downstream kinase signalling. For instance, PKCα was previously reported to mediate the phosphorylation of ERK in human embryonal rhabdomyosarcoma cells (Mauro *et al*., 2002), thus in my case, the knocking down of PKCα might also cause less phosphorylation/activation of ERK in CaR-HEK cells despite the enhanced the $Ca^{2+}{}_i$ mobilisation. The inhibition of CaR-induced ERK activity was also previously observed in dominant negative (DN) PKC α and ε co-expressed CaR-HEK cells (Sakwe *et al*., 2004). Moreover, the knockdown of PKCα was also previously reported to attenuate the activation of IGF1R in adult rat ventricular cardiomyocytes and the embryonic rat heart cell line, H9c2 (Maniar *et al*., 2005).

Next, having found that PKCε knockdown inhibited $Ca^{2+}{}_i$ mobilisation in CaR-HEK cells at moderate $Ca^{2+}{}_o$ concentrations, the effect of PKCε knockdown on the phosphorylation of ERK, IGF1R, and P70 S6Kinase was also evaluated. Similarly to its inhibitory effect on CaR-induced $Ca^{2+}{}_i$ mobilisation (Section 3.7), PKC ε knockdown also inhibited the phosphorylation of these proteins (Figure 4.16). However, whether this inhibition occurred because PKCε is a crucial activator of these pathways, or, because it had attenuated CaR activity was not clear. Bourbon *et al*., 2001 has found that the activation of PKCε in HEK cells by IGF1R caused ERK activation via an interaction with Raf-1, which is upstream of MEK / ERK signalling. As for IGF1R and S6kinase, no study has yet shown the involvement of PKCε in regulating these kinases and so the inhibition of CaR activity by PKCε knockdown might help explain this effect.

Finally, the knocking down of $G\alpha_{12}$ was also found to inhibit the phosphorylation of ERK, IGF1R, and P70 S6Kinase (Figure 4.17). Previously, it was reported that the thrombin receptor (GPCR member) required $G_{12/13}$ together with G_i and G_q signalling in order to significantly activate ERK in adult rat ventricular myocytes cells. In contrast, the activation of ERK by two other GPCRs, namely α1 adrenergic receptor and endothelin 1 receptor, in similar cells was found to be $G_{12/13}$-independent (Snabaitis *et al*., 2005). However, as for PKCε knockdown, one cannot be sure whether the attenuation of CaR-induced ERK activity by $G\alpha_{12}$ knockdown relates to the inhibition of CaR activity seen in the $Ca^{2+}{}_i$ mobilisation study, or, to the disruption of a potential $G\alpha_{12}$-induced ERK activation pathway. It can only be stated that there is no evidence to date that $G\alpha_{12}$

signalling is involved in IGF1R activation which could point more towards the attenuation of CaR signalling but this would need to be properly investigated. In either case, these data represent the first reported observation of $G\alpha_{12}$ involvement in CaR-induced kinase activation.

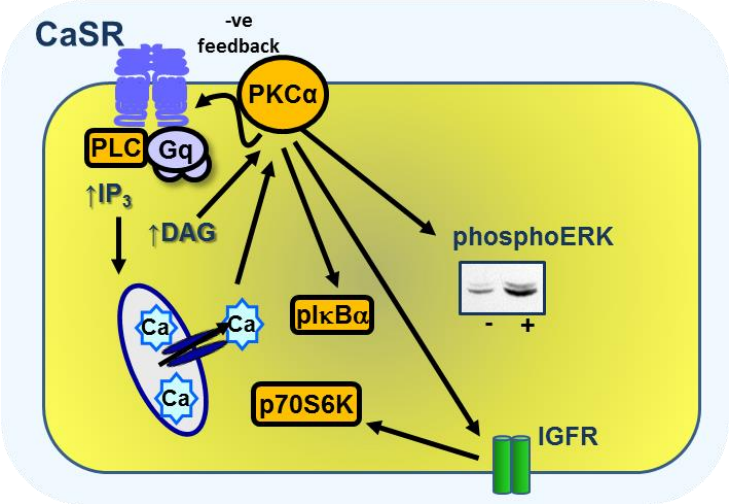

Figure 4.20: Summary of the effect of CaR-induced PKCα activation on ERK, IκBα, IGF1R, and p70S6Kinase activations

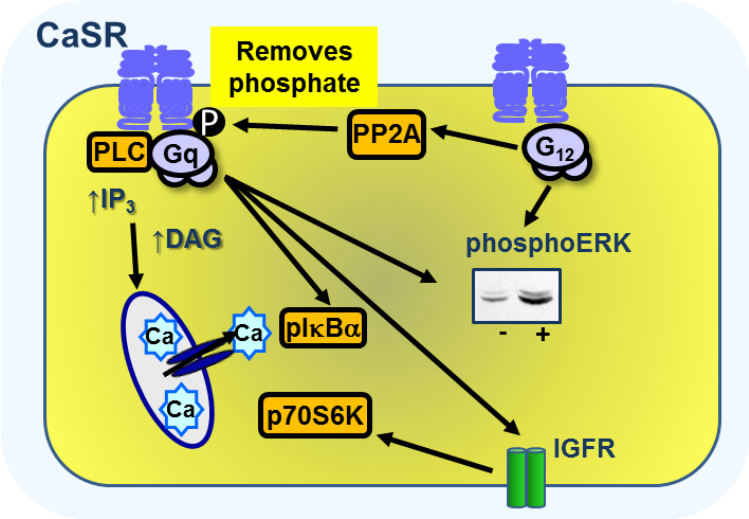

Figure 4.21: Summary of the effect of CaR-G_{12} coupling on ERK, IκBα, IGF1R, and p70S6Kinase activations

4.5 Conclusion

In conclusion, CaR-induced Ca^{2+}_i mobilisation at Ca^{2+}_o sub-threshold concentrations is ordinarily prevented by PKCα, apparently via CaR^{T888} phosphorylation such that downregulation of PKCα, or, PKC inhibition lowers the Ca^{2+}_o threshold. In addition, cAMP also potentiates CaR-induced Ca^{2+}_i mobilisation and reduces CaR^{T888} phosphorylation suggesting the potential for crosstalk between cAMP-generating receptors and the CaR in regulating cell signalling. However, the mechanism in which cAMP stimulating Ca^{2+}_i mobilisation was not entirely due to altered CaR phosphorylation since it also enhanced the Ca^{2+}_i mobilisation induced by mAChR.

Finally, this study has identified three novel, independent readouts for CaR signalling, namely IGF1R, IκBα and P70 S6Kinase. This was fully established using CaR-selective calcimimetics and calcilytics. When cells were exposed to the siRNAs for either PKCα, PKCε or $G\alpha_{12}$ the result in each case was a general inhibition of all kinases tested despite the fact PKCα potentiates CaR-induced Ca^{2+}_i mobilisation rather than inhibiting it. The results from this study give us a bigger picture of CaR's signalling. Previously, many studies on CaR's downstream signalling were focused on MAP kinases, and these new findings might help to expand the knowledge on the effect of CaR's activation on cell physiology. Besides that, this addition of CaR's readouts is also very helpful in determining bias in the CaR's signalling.

Chapter 5

Investigating the relative gain-of-function of five ADH mutations and their inhibition by the calcilytic NPSP795

5.1 INTRODUCTION

The calcium-sensing receptor maintains extracellular Ca^{2+} homeostasis in humans by controlling PTH secretion and urinary Ca^{2+} excretion (D'Souza-Li, 2006). Therefore, mutations in the CaR that lead to either gain or loss of its function are likely to have an impact on these parameters and thus risk disorders of low or high blood calcium levels respectively. Specifically, gain-of-function CaR mutations are associated with ADH while loss-of-function CaR mutations are associated with FHH and NSPHT (D'Souza-Li, 2006). More than 100 CaR mutations have been identified that cause ADH (http://www.casrdb.mcgill.ca/; Hannan and Thakker, 2013). This chapter will focus on five CaR mutations; C131Y, E228K, Q245R and A840V, known or believed to be associated with ADH in humans (Conley *et al*., 2000; Hendy *et al*., 2009; Cole *et al*., 2009; Winer *et al*., 2014) plus a novel mutation, E228A, appearing to result in ADH (data not shown). This study was done as part of a larger series of experiments undertaken in conjunction with Cardiff University where the mutations were made and stably-transfected into HEK-293 cells, and, NPS Pharmaceuticals who are testing the clinical utility of calcilytic drugs in ADH. It is necessary therefore to first demonstrate *in vitro* a) that these mutations are specifically gain-of-function for the CaR and b) that they can be allosterically inhibited by cotreatment with a calcilytic compound as it is for the wild-type receptor. Thus, in this study HEK-293 cells made to stably express each of the 5 mutant ADH CaRs were tested for their responsiveness to Ca^{2+}_o relative to the responses of cells expressing wild-type CaR. The responses in question are Ca^{2+}_i mobilisation, as well as ERK and $p38^{MAPK}$ phosphorylation.

Finally, the newly discovered CaR readouts IGF1R, IκBα and S6Kinase were also examined in some cases and compared to ERK responses to determine whether they show any gross indications of biased signalling between the CaR mutations. That is, is the gain-of-function for each mutation equivalent for all effector pathways or different and therefore potentially biased?

5.2 METHODS

CaR-HEK cells were cultured as detailed in (Chapter 2) section 2.2. The HEK-293 cells were transiently transfected with mutant and wild type CaR as described in section 2.9. Given the potentially stimulatory effect on the gain-of-function mutants of DMEM (containing 1.8 mM $CaCl_2$), the cells were routinely cultured in low calcium RPMI, so as to avoid cell death (Ward *et al*., 2005). The cells were lysed as described in 2.7 and phosphoproteins immunoblotted as described in 2.8.

5.2.1 Signal Bias Calculations

The possible existence of biased signalling between the mutant and wild type CaRs was determined using the method described by Thomsen *et al*. (2012). Briefly, the negative logarithm of the EC_{50} for $Ca^{2+}{}_o$ (pEC_{50}) was determined for each pathway (ERK, $p38^{MAPK}$ and P70 S6Kinase). Then, the negative logarithm bias factor (ppBF) between two pathways was calculated using this equation:

$$ppBF(pathway1:pathway2) = -Log\ (pEC_{50}(pathway1)/pEC_{50}(pathway2))$$
$$= pEC_{50}(pathway1) - pEC_{50}(pathway2)$$

The positive value of ppBF represents the tendency of the bias towards the first pathway while the negative value represents bias towards the second pathway. The ppBFs of each mutant CaR was then compared to wild type CaR with any significant difference between the ppBFs indicating biased signalling.

5.3 RESULTS

5.3.1 Characterisation of the effect of CaR^{A840V} mutation on Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK phosphorylation

In this study, the functional effect of five CaR point mutations reported to be associated with ADH (A840V, E228K, E228A, Q245R, and C131Y) were studied following their stable transfection into HEK cells using the Flp-In^{TM} system. These mutated CaR-HEK cells were generated by Sarah Brennan at the University of Cardiff who examined the effects of the ADH mutations on CaR membrane expression and Ca^{2+}_i mobilisation whereas their effects on ERK and $p38^{MAPK}$ were examined here. It should be noted that I also examined Ca^{2+}_i mobilisation here also to confirm the findings of the Cardiff work. For the investigation of the effect of A840V, E228K, E228A, and Q245R mutations on CaR-induced Ca^{2+}_i mobilisation, the equivalent wild-type CaR was used as a control since these experiments were done on the same days.

Figure 5.1*A* shows that the A840V mutation caused a leftward shift in the concentration-effect curve for Ca^{2+}_o-induced Ca^{2+}_i mobilisation indicating an increase in Ca^{2+}_o sensitivity in the cells. Both CaR^{WT} and CaR^{A840V} cells showed no Ca^{2+}_i mobilisation in 0.5 mM of Ca^{2+}_o suggesting that while the A840V mutation may be gain-of-function, it is not constitutively active. In 1.8 mM Ca^{2+}_o, the CaR^{A840V} cells did exhibit oscillatory Ca^{2+}_i mobilisation. In contrast, the Ca^{2+}_i mobilisation in wild-type CaR cells was barely observed at this Ca^{2+}_o concentration. As the Ca^{2+}_o concentration was increased to 2.3 mM, the wild-type CaR cells started to exhibit Ca^{2+}_i oscillations while A840V mutated cells exhibited higher frequency Ca^{2+}_i oscillations. The further rise in Ca^{2+}_o concentration to 3.3 mM produced higher frequency Ca^{2+}_i oscillations in CaR^{WT} cells while in CaR^{A840V} cells the Ca^{2+}_i mobilisation was sustained and close to maximal. Where ERK phosphorylation was the functional readout, little response was seen in either cell-type in 0.5 mM Ca^{2+}_o whereas in 2 mM Ca^{2+}_o the CaR^{A840V} cells exhibited substantially increased ERK phosphorylation, the response reaching a maximum at 3 mM Ca^{2+}_o. In contrast, in CaR^{WT} cells, there was only a very small ERK response in 2 mM while for the maximal ERK response to be achieved, at least 5 mM Ca^{2+}_o was required (Figure 5.1*B*). In accordance with the qualitative observation above, the EC_{50} for Ca^{2+}_o-induced

ERK phosphorylation was significantly lower in CaR^{A840V} cells than in CaR^{WT} cells (Figure 5.1*C*; 2.0 ± 0.1 mM vs 3.0 ± 0.2 mM respectively; $P < 0.05$). The CaR immunoreactivity exhibited by the two cell-lines was similar, with equal loading of the samples on the blot confirmed by uniform β-actin expression (Figure 5.1*D*). This indicates that the apparent gain-of-function observed by Ca^{2+}_i mobilisation and ERK activation did not result from higher expression of receptor in the CaR^{A840V} vs the CaR^{WT} cells and thus is most likely due to a real gain-of-function resulting from the mutation.

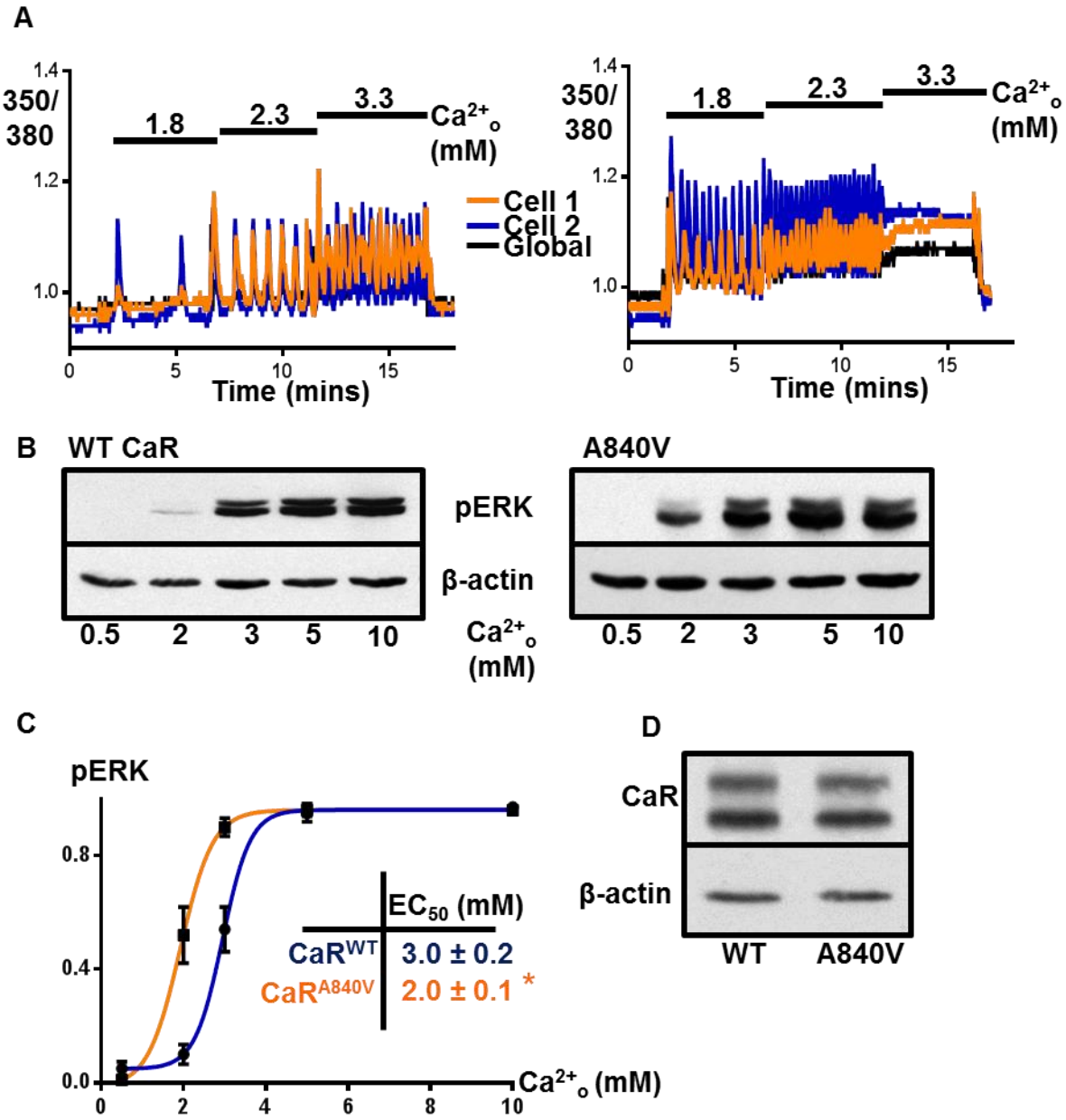

Figure 5.1: CaR^{A840V} exhibits increased Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK activation. *A*) Fura2-loaded HEK cells stably expressing CaR^{A840V} and CaR^{WT} were exposed to increasing Ca^{2+}_o concentrations (0.5-10mM). The representative traces show the Ca^{2+}_i changes (Fura-2) in two single cells (orange, blue) and in a 'global' cluster (black) of all cells in the field of view. *B*) Representative western blots showing ERK phosphorylation and β-actin immunoreactivity in the same cell-lines incubated for 10-mins in various Ca^{2+}_o concentrations from 0.5–10 mM. *C*) Concentration-effect curves showing the quantification of the changes in ERK phosphorylation with the resulting EC_{50} values shown as an inset. ERK responses are expressed as a fraction of the maximal response in each experiment. *P < 0.05 vs. CaR^{WT} log EC_{50} values by 1-way ANOVA with Dunnett's post-test, n ≥ 10 from 3 independent experiments. *D*) CaR immunoblots showing equivalent CaR^{WT} and CaR^{A840V} immunoreactivity in samples. Equivalent protein loading was confirmed by uniform β-actin expression.

5.3.2 Characterisation of the effect of CaR^{E228K} mutation on Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK phosphorylation

Next, a similar series of experiments as in 5.3.1 were performed on cells stably expressing the CaR^{E228K} mutant. Specifically, the effect of the mutant on CaR-induced Ca^{2+}_i mobilisation and ERK phosphorylation was examined (Figure 5.2). The CaR^{E228K}-expressing cells exhibited increased Ca^{2+}_i mobilisation at lower Ca^{2+}_o concentrations as compared to CaR^{WT}-expressing cells (Figure 5.2*A*). The CaR^{WT} Ca^{2+}_i mobilisation responses were as described in 5.3.1. CaR^{E228K} elicited no Ca^{2+}_i mobilisation or ERK response to 0.5 mM Ca^{2+}_o suggesting that the receptor is not constitutively active. The Ca^{2+}_i oscillations induced by CaR^{E228K} appeared different to those of CaR^{WT} in that they had a qualitatively higher amplitude.

The CaR^{E228K} cells also exhibited increased ERK activation compared to wild type CaR at modest Ca^{2+}_o concentrations (Figure 5.2*B*). In 0.5 mM Ca^{2+}_o, neither cell-type exhibited ERK phosphorylation. However, the ERK phosphorylation in the CaR^{E228K} cells was increased at 2 mM of Ca^{2+}_o. In contrast, ERK phosphorylation in CaR^{WT} cells was barely observed at this Ca^{2+}_o concentration. As the Ca^{2+}_o concentration was increased to 3 mM, the ERK phosphorylation in the CaR^{E228K} cells was increased further to a maximal response with no additional change in ERK phosphorylation observed at higher Ca^{2+}_o concentrations (5 and 10 mM Ca^{2+}_o). In contrast, a further increase in ERK phosphorylation was observed in CaR^{WT} cells at 3 mM Ca^{2+}_o but this response did not reach maximal until exposure to 5 mM of Ca^{2+}_o. Indeed, quantification of the EC_{50}s for Ca^{2+}_o on ERK phosphorylation in both cells confirms the significantly lower EC_{50} in CaR^{E228K} cells compared to CaR^{WT} cells (Figure 5.2*C*; 3.0 mM ± 0.2, CaR^{WT} vs 1.7 ± 0.3 mM, CaR^{E228K}; $P < 0.05$). As before, the CaR^{E228K} and CaR^{WT} cells appeared to express similar amounts of CaR (Figure 5.2*D*) with the uniform expression of β-actin on the immunoblots confirming the equal loading of the samples.

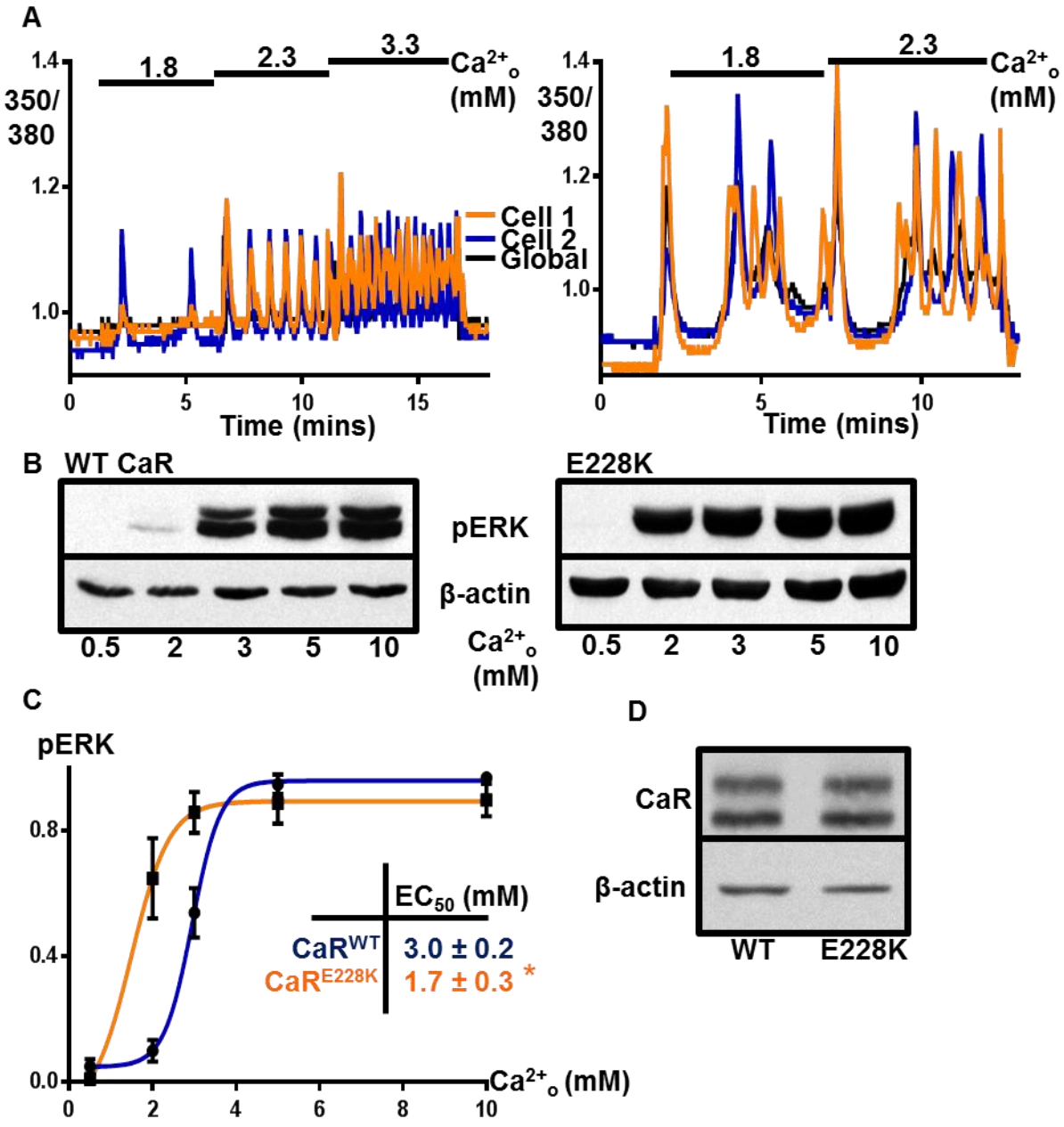

Figure 5.2: CaR^{E228K} mutation increases CaR-induced Ca^{2+}_i mobilisation and ERK activation. *A*) Fura2-loaded HEK cells were stably transfected with CaR^{E228K} and CaR^{WT} and then exposed to increasing Ca^{2+}_o concentrations. The representative traces show Ca^{2+}_i responses in two single cells and in a 'global' cluster of all cells in the field of view. *B*) Representative western blot showing ERK phosphorylation and β-actin immunoreactivity in HEK cells stably-transfected with CaR^{E228K} and CaR^{WT} following treatment with increasing Ca^{2+}_o concentrations. *C*) Quantification of the ERK phosphorylation in CaR^{E228K} and CaR^{WT}-transfected cells. The responses are expressed as percentage of maximal response in each cell-type.* $P < 0.05$ CaR^{E228K} versus CaR^{WT} log EC_{50} values by 1-way ANOVA with Dunnett's post-test, $n \geq 6$ from 2 independent experiments. *D*) CaR immunoblots show equivalent CaR^{WT} and CaR^{E228K} immunoreactivity in samples. The equivalent protein loading was confirmed by uniform β-actin expression.

5.3.3 Characterisation of the effect of CaR^{E228A} mutation on Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK phosphorylation

In this experiment, the effect of CaR^{E228A} mutation on CaR-induced Ca^{2+}_i mobilisation and ERK phosphorylation was evaluated as described in 5.3.1. Figure 5.3*A* shows that CaR^{E228A} appears to be gain-of-function for Ca^{2+}_i mobilisation. The Ca^{2+}_i mobilisation in CaR^{WT} cells was described previously in 5.3.1 with the CaR^{E228A} responses qualitatively similar to the CaR^{A840V} (5.3.1) and CaR^{E228A} (5.3.2) responses. That is, the CaR^{E228A} cells exhibited no Ca^{2+}_i responses in 0.5 mM but greater responses to 1.8 mM Ca^{2+}_o than for CaR^{WT} cells (Figure 5.3*A*). As before CaR^{E228A} cells exhibited higher frequency Ca^{2+}_i oscillations in 2.3 mM Ca^{2+}_o. In accordance with Ca^{2+}_i mobilisation, the E228A mutation also increased CaR-induced ERK activation (Figure 5.3*B*). In 0.5 mM Ca^{2+}_o, ERK phosphorylation was barely detected in either cell-type. In contrast, ERK phosphorylation in CaR^{E228A} cells was highly increased as the Ca^{2+}_o concentration was raised to 2 mM, but not in CaR^{WT} cells. The EC_{50} for Ca^{2+}_o on ERK phosphorylation in CaR^{E228A} cells was found to be significantly lower compared to CaR^{WT} cells (Figure 5.3*C*; 3.0 ± 0.2 mM, CaR^{WT}; 2.1 ± 0.1 mM, CaR^{E228K}; $P < 0.05$). As before, CaR^{E228A} and CaR^{WT} expression was similar between each cell-type (Figure 5.3*D*) with the uniform expression of β-actin on the immunoblots confirming equal loading of the samples.

5.3.4 Characterisation of the effect of CaR^{Q245R} mutation on Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK phosphorylation

Next was compared the effect of the CaR^{Q245R} mutation on CaR-induced Ca^{2+}_i mobilisation and ERK phosphorylation. The results are shown in Figure 5.4 with each broadly similar to the previous gain-of-function mutations described in 5.1 – 5.3. Briefly, the CaR^{Q245R} cells exhibited both increased Ca^{2+}_i mobilisation (Figure 5.4*A*) and ERK phosphorylation (Figure 5.4*B*) at moderate Ca^{2+}_o concentrations compared to CaR^{WT} cells. As before, the CaR^{Q245R} cells were unresponsive to 0.5 mM Ca^{2+}_o by either readout. Following densitometry, the Ca^{2+}_o-induced ERK response for the CaR^{Q245R} cells was found to be significantly left-shifted compared to CaR^{WT} cells (Figure 5.4*C*; 3.0 ± 0.2 mM, CaR^{WT}; 2.0 ± 0.0 mM CaR^{Q245R}; $P < 0.05$). Again, CaR^{Q245R} and CaR^{WT} expression

was similar between each cell-type (Figure 5.4*D*) with the uniform expression of β-actin on the immunoblots confirming equal loading of the samples.

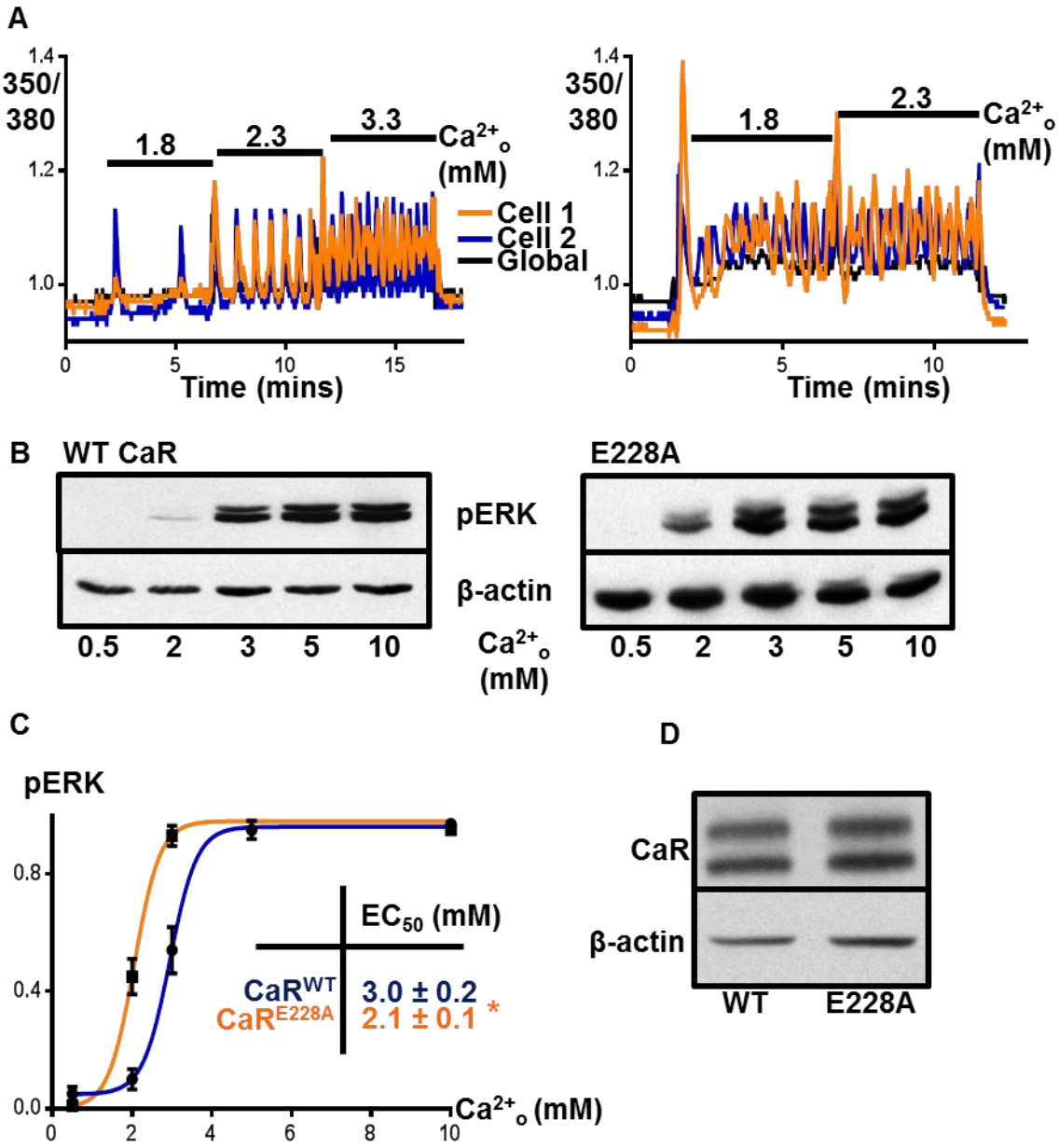

Figure 5.3: CaR^{E228A} mutation increases CaR-induced Ca^{2+}_i mobilisation and ERK activation. *A*) Fura2-loaded HEK cells were stably transfected with CaR^{E228A} and wild type CaR, then exposed to increasing Ca^{2+}_o concentrations. The representative traces show the Ca^{2+}_i in two single cells and a 'global' cluster of all cells in the field of view *B*) Representative western blot showing ERK phosphorylation and β-actin immunoreactivity in HEK cells stably-transfected with CaR^{E228A} and wild type CaR following the treatment with increasing Ca^{2+}_o concentrations. *C*) Quantification of ERK phosphorylation in CaR^{E228A} and wild type CaR-transfected cells. The responses are expressed as percentage of maximal response in each cell-type. *P < 0.05 CaR^{E228A} versus WT-CaR log EC_{50} values by 1-way ANOVA with Dunnett's post-test, n ≥ 7 from 2 independent experiments. *D*) CaR immunoblots show equivalent CaR^{WT} and CaR^{E228A} immunoreactivity in samples. The equivalent protein loading was confirmed by uniform β-actin expression.

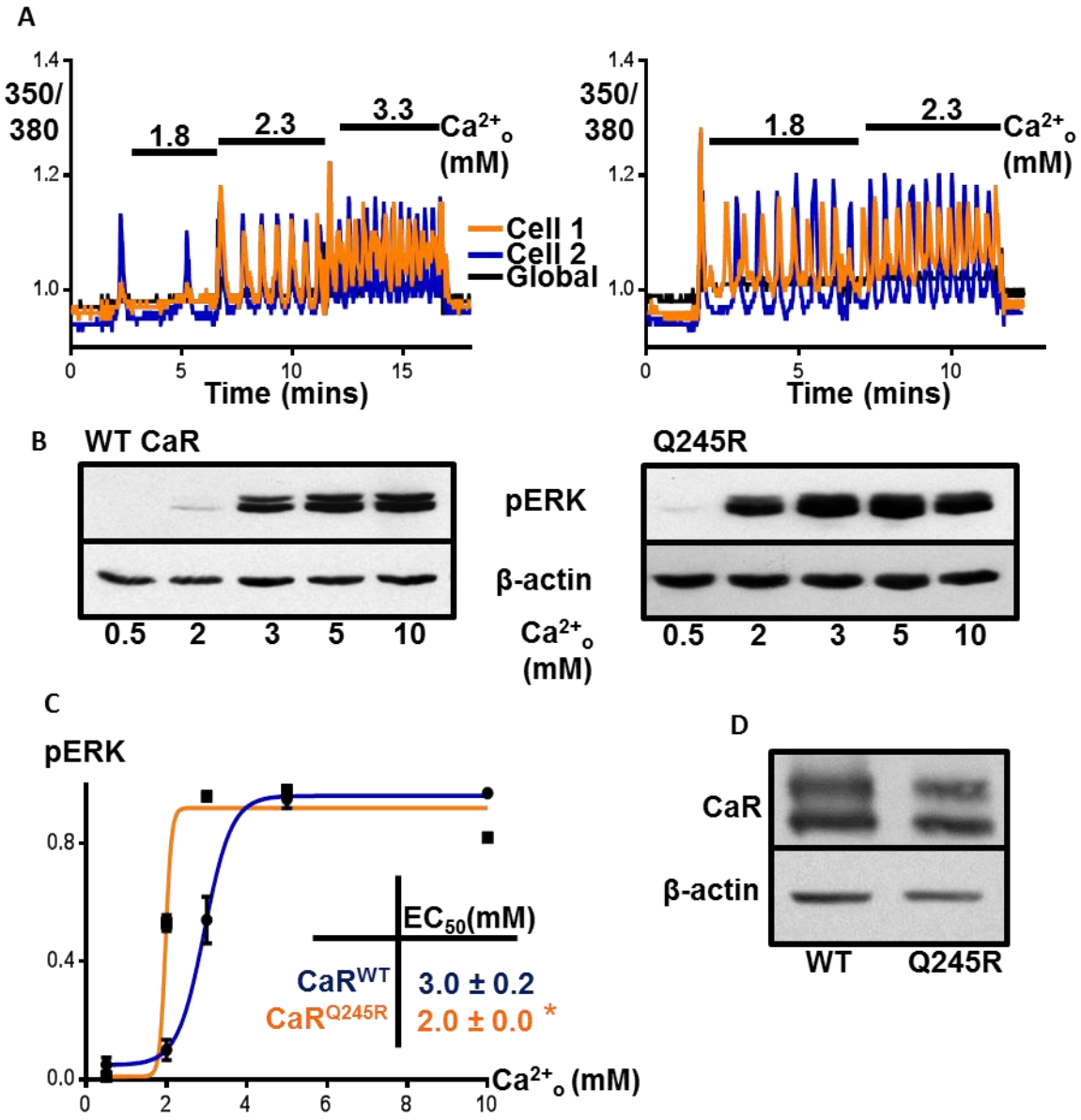

Figure 5.4: CaR^{Q245R} mutation increases CaR-induced Ca^{2+}_i mobilisation and ERK activation. *A*) Fura2-loaded HEK cells were stably transfected with CaR^{Q245R} and wild type CaR, then exposed to increasing Ca^{2+}_o concentrations. The representative traces show the Ca^{2+}_i in two single cells and a 'global' cluster of all cells in the field of view *B*) Representative western blot showing ERK phosphorylation and β-actin immunoreactivity in HEK cells stably-transfected with CaR^{Q245R} and wild type CaR following the treatment with increasing Ca^{2+}_o concentrations. *C*) Quantification of ERK phosphorylation in CaR^{Q245R} and wild type CaR-transfected cells. The responses are expressed as percentage of maximal response in each cell-type. * $P < 0.05$ CaR^{Q245RV} versus WT-CaR log EC_{50} values by 1-way ANOVA with Dunnett's post-test, $n \geq 9$ from 3 independent experiments. *D*) CaR immunoblots show equivalent WT-CaR and CaR^{Q245R} immunoreactivity in samples. The equivalent protein loading was confirmed by uniform β-actin expression.

5.3.5 Characterisation of the effect of CaR^{C131Y} mutation on Ca^{2+}_o-induced Ca^{2+}_i mobilisation and ERK phosphorylation

Finally, the effect of CaR^{C131Y} mutation on CaR-induced Ca^{2+}_i mobilisation and ERK phosphorylation was then investigated. The results are shown in Figure 5.5 and are broadly similar to the previous gain-of-function mutations described in 5.1 – 5.4. Briefly, the CaR^{C131Y} cells exhibited both increased Ca^{2+}_i mobilisation (Figure 5.5*A*) and ERK phosphorylation (Figure 5.5*B*) at moderate Ca^{2+}_o concentrations compared to CaR^{WT} cells. As in each case before, the CaR^{C131Y} cells were unresponsive to 0.5 mM Ca^{2+}_o by either readout. It should be noted that in the case of the CaR^{C131Y} cells, Ca^{2+}_o-induced ERK phosphorylation was examined in HEK-293 cells expressing the mutant receptor stably, whereas the Ca^{2+}_i mobilisation experiments and the calcilytic experiments described later employed HEK-293 cells expressing the mutant transiently. This was because the initial CaR^{C131Y} cell-line stopped proliferating during the study (in both Manchester and Cardiff) and so had to be remade. The new CaR^{C131Y} cell-line was not ready in time before the end of my project but has since been used by my supervisor to reconfirm the results reported here (data not shown).

The EC_{50} for Ca^{2+}_o-induced ERK phosphorylation was significantly lower in CaR^{C131Y} cells compared to CaR^{WT} cells (Figure 5.5*C*; 3.0 ± 0.2 mM, CaR^{WT}; 1.0 ± 0.2 mM, CaR^{C131Y}; $P < 0.05$) As before, CaR^{C131Y} and CaR^{WT} expression was similar between each cell-type (Figure 5.5*D*) with the uniform expression of β-actin on the immunoblots confirming equal loading of the samples.

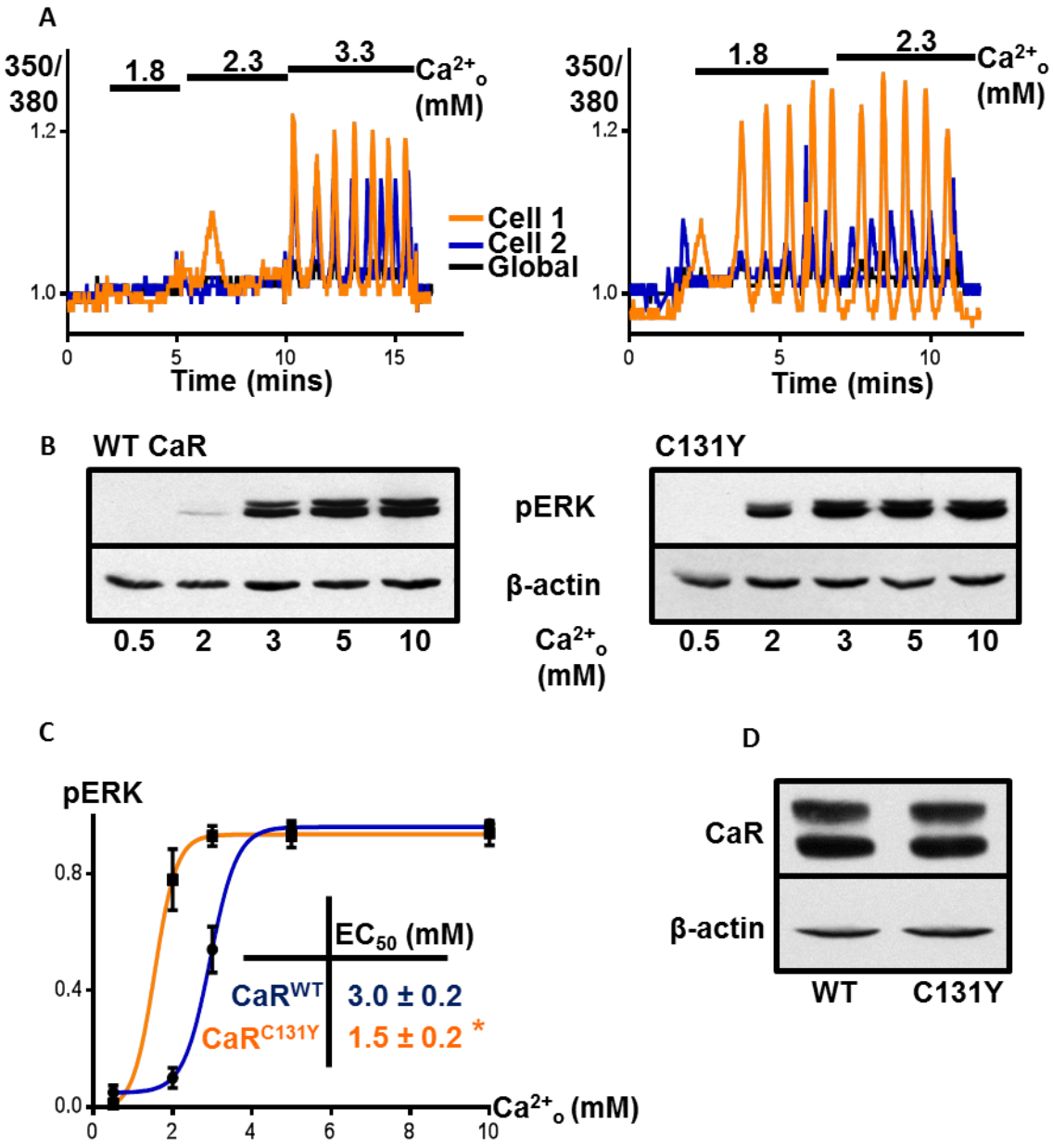

Figure 5.5: CaR^{C131Y} mutation increases CaR-induced Ca^{2+}_i mobilisation and ERK activation. *A*) Fura2-loaded HEK cells were transiently transfected with CaR^{C131Y} and wild type CaR, then exposed to increasing Ca^{2+}_o concentrations. The representative traces show the Ca^{2+}_i in two single cells and a 'global' cluster of all cells in the field of view. *B*) Representative western blots showing ERK phosphorylation and β-actin immunoreactivity in HEK cells transiently transfected with CaR^{C131Y} and CaR^{WT} following the treatment with increasing Ca^{2+}_o concentrations. *C*) Quantification of ERK phosphorylation in CaR^{C131Y} and CaR^{WT}-transfected cells. ERK responses are expressed as a percentage of the maximal response in each cell-type.* $P < 0.05$ CaR^{C131Y} versus WT-CaR log EC_{50} values by 1-way ANOVA with Dunnett's post-test, $n \geq 5$ from 2 independent experiments. *D*) CaR immunoblots show equivalent CaR^{WT} and CaR^{131Y} immunoreactivity in both sets of samples. Equivalent protein loading was confirmed by uniform β-actin expression.

5.3.6 Characterisation of the effect of CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{C131Y} mutations on Ca^{2+}_o-induced $p38^{MAPK}$ phosphorylation

Another important MAP kinase known to be activated by CaR is $p38^{MAPK}$ (Yamaguchi *et al*., 2000). In order to determine whether the gain-of-function effects seen were specific to ERK, or, more general, the immunoblots were stripped and reprobed with an antibody against the phosphorylated (i.e. active) form of $p38^{MAPK}$ (at Thr180 and Tyr 182). Thus, the effect of CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{CI31Y} mutations on CaR-induced $p38^{MAPK}$ phosphorylation was studied. All the HEK cells used in this study were stably transfected with either the wild-type or mutant CaRs. The results for CaR^{A840V}, CaR^{E228K} and CaR^{E228A} are shown in Figure 5.6*A*. The phosphorylation of $p38^{MAPK}$ was found to be very low or absent in each cell-type at 0.5 mM Ca^{2+}_o. Raising Ca^{2+}_o concentration to 2 mM did not stimulate the $p38^{MAPK}$ phosphorylation in CaR^{WT} cells but did initiate $p38^{MAPK}$ phosphorylation in CaR^{A840V}, CaR^{E228K} and CaR^{E228A} cells. In 3 mM Ca^{2+}_o, $p38^{MAPK}$ phosphorylation was detected in wild type CaR and further increased in CaR^{A840V}, CaR^{E228K}, and CaR^{E228A} cells. Raising the Ca^{2+}_o concentration further to 5mM substantially increased $p38^{MAPK}$ phosphorylation in CaR^{WT} cells but had only a marginal additional effect on the mutant cells. Finally, 10 mM Ca^{2+}_o had no further effect on $p38^{MAPK}$ phosphorylation in CaR^{A840V}, CaR^{E228K} and CaR^{E228A} cells but did increase it further in CaR^{WT} cells. In accordance with these observations, the EC_{50}s for Ca^{2+}_o-induced $p38^{MAPK}$ phosphorylation for CaR, CaR^{E228K} mutation was significantly lower than for CaR^{WT} cells (Figure 5.6*B*; 4.1 ± 0.3 mM, CaR^{WT};; 1.8 ± 0.1 mM, CaR^{E228K}; P < P < 0.01, vs CaR^{WT}). However, the EC_{50}s for CaR^{A840V} and CaR^{E228A} were not significantly lower than CaR^{WT} even there was trend of increase in their p38 phosphorylation compared to CaR^{WT}.

Next, Figure 5.7*A* shows the same Ca^{2+}_o-induced $p38^{MAPK}$ responses for the remaining two mutant CaR^{Q245R} and CaR^{C131Y} cells. As before, 0.5 mM Ca^{2+}_o was without effect on $p38^{MAPK}$ phosphorylation in either cell-type but the mutant cells exhibited heightened $p38^{MAPK}$ responsiveness to moderate Ca^{2+}_o concentrations relative to CaR^{WT} cells. The calculated EC_{50}s for Ca^{2+}_o-induced $p38^{MAPK}$ phosphorylation in CaR^{Q245R} cells were also found to be significantly lower than for CaR^{WT} cells but not in CaR^{C131Y} (Figure 5.7*B*; 4.1 ± 0.3 mM, CaR^{WT}; 1.8 ± 0.1 mM, CaR^{Q245R}; *P < 0.05 vs. CaR^{WT}).

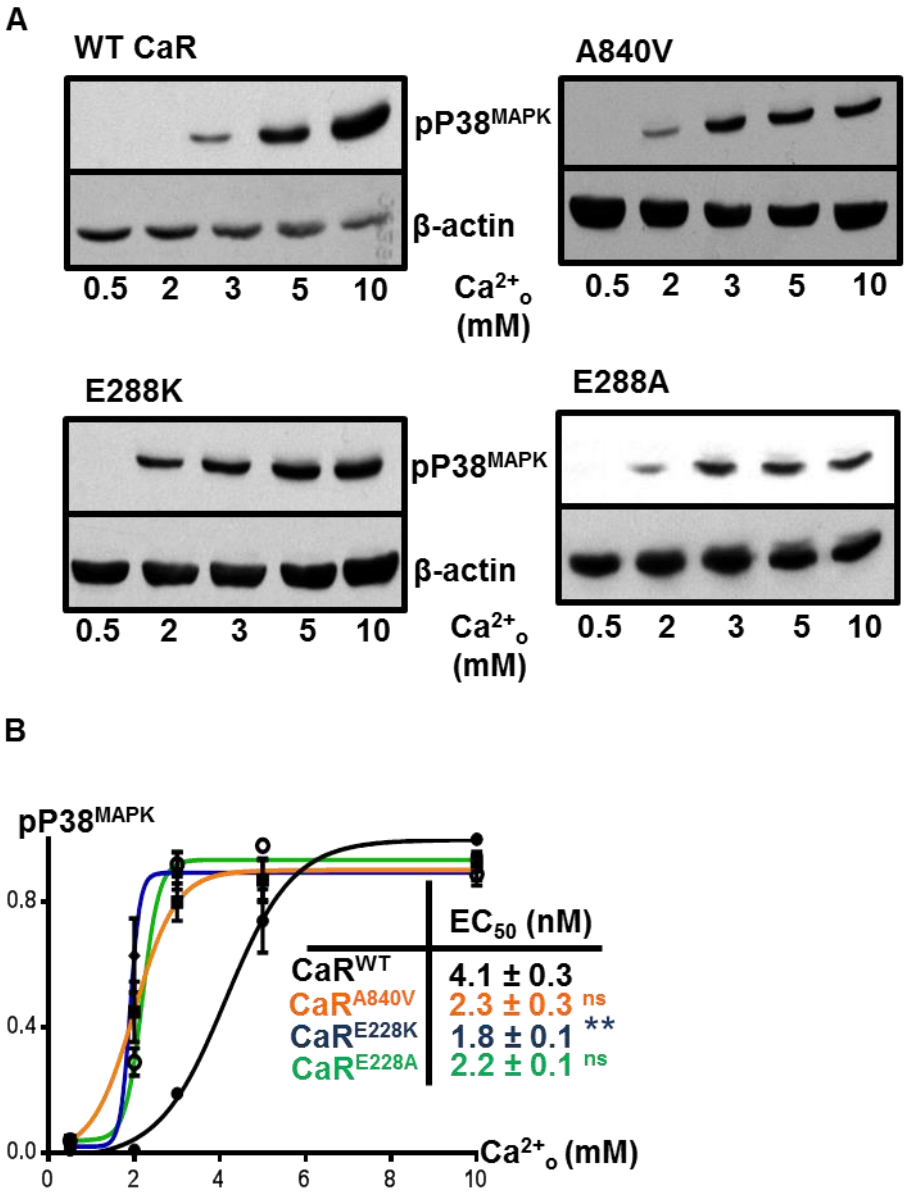

Figure 5.6: CaR^{A840V}, CaR^{E228K} and CaR^{E228A} mutation increases p38^{MAPK} responsiveness in HEK-293 cells. *A*) Representative western blots showing p38^{MAPK} phosphorylation and β-actin immunoreactivity in HEK cells stably transfected with CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT} following treatment with increasing Ca^{2+}_o concentrations. *B*) Quantification of p38^{MAPK} phosphorylation in CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT}-transfected cells. The responses were expressed as percentage of maximal response in each cells' sets. ** $P < 0.01$ CaR^{E228K} versus WT-CaR log EC_{50} values; $^{ns}P > 0.05$ vs. CaR^{WT} log EC_{50} values by Kruskal-Wallis with Dunnett's post-test. $n \geq 3$ from at least 2 independent experiments.

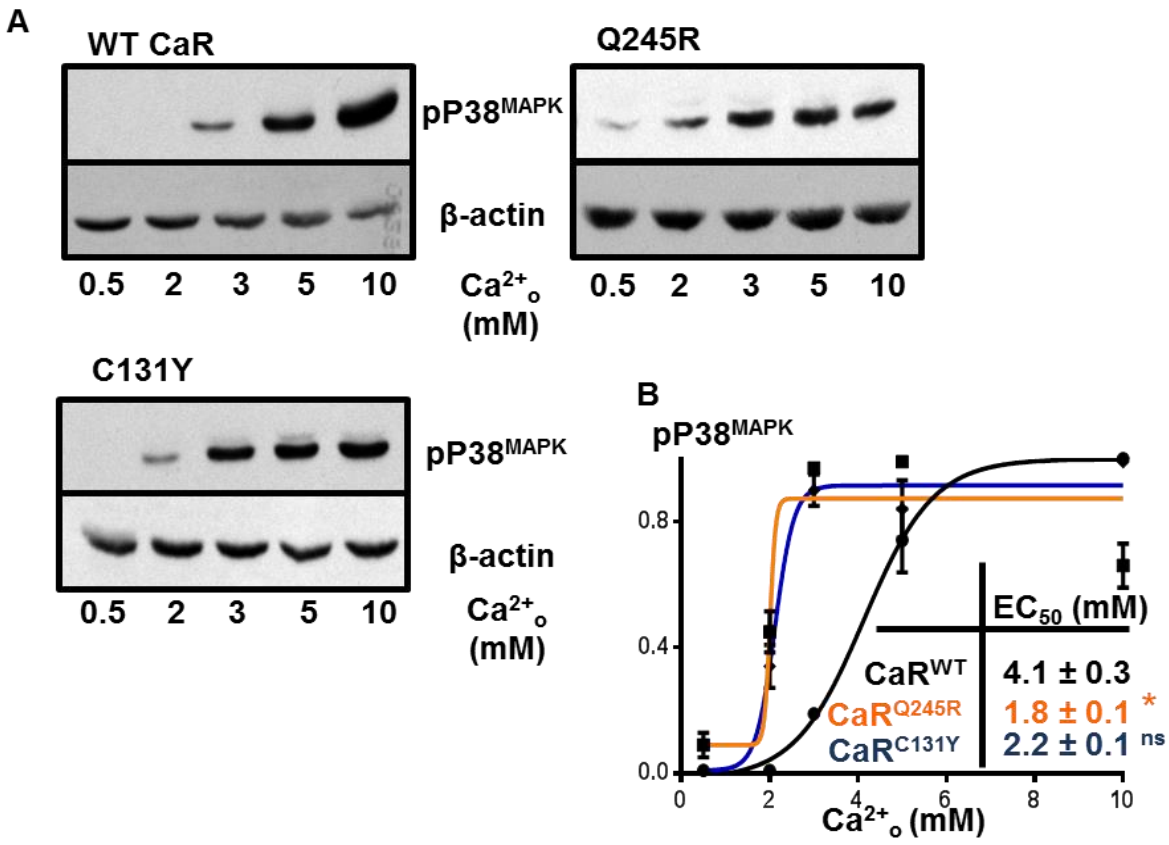

Figure 5.7: CaR^{Q245R} and CaR^{C131Y} mutation increases p38^{MAPK} responsiveness in HEK-293 cells. *A*) Representative western blots showing p38^{MAPK} phosphorylation and β-actin immunoreactivity in HEK cells stably transfected with CaR^{Q245R}, CaR^{C131Y} and CaR^{WT} following treatment with increasing Ca^{2+}_o concentrations. *B*) Quantification of p38^{MAPK} phosphorylation in CaR^{Q245R}, CaR^{C131Y} and CaR^{WT}-transfected cells. The responses are expressed as a percentage of the maximal response in each cell-type. *P < 0.05 vs. CaR^{WT} log EC_{50} values; ^{ns}P > 0.05 vs. CaR^{WT} log EC_{50} values by Kruskal-Wallis with Dunnett's post-test with Dunnett's post-test, n ≥ 3 from at least 2 independent experiments.

5.3.7 Determination of the effect of NPSP795 on CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{C131Y} -induced ERK phosphorylation

Having found that these five CaR's gain-of-function mutations (A840V, E228K, E228A, Q245R, C131Y) increased ERK and $p38^{MAPK}$ responsiveness, next the ability of the calcilytic, NPSP795 to inhibit ERK and $p38^{MAPK}$ activation in these mutants and wild type CaR was investigated. For CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{WT} the stably-transfected HEK cells were the same clones as those used in the earlier experiments while the CaR^{C131Y} (as well as its CaR^{WT} control) was transiently transfected into wild-type HEK cells in our laboratory. For this it was first necessary to determine the EC_{80} for $Ca^{2+}{}_o$-induced ERK and $p38^{MAPK}$ phosphorylation for each CaR so as to be able to employ that $Ca^{2+}{}_o$ concentration in the calcilytic experiments. The EC_{80} was specifically chosen as it is the concentration at the top of the linear portion of the $Ca^{2+}{}_o$ concentration-effect curve but that is not supra-maximal and therefore any inhibitory effect of the calcilytic should be easily observed. Table 5.1 shows a summary of the EC_{50} values for $Ca^{2+}{}_o$-induced ERK and $p38^{MAPK}$ phosphorylation reported earlier in the chapter together with the equivalent EC_{80} values.

	EC_{50} (mM) pERK	EC_{80} (mM) pERK	EC_{50} (mM) $p38^{MAPK}$	EC_{80} (mM) $p38^{MAPK}$
CaR^{WT}	3.0 ± 0.2	3.6 ± 0.3	4.2 ± 0.4	5.6 ± 0.8
CaR^{C131Y}	1.2 ± 0.3	1.6 ± 0.4	2.1 ± 0.1	2.4 ± 0.2
CaR^{A840V}	2.0 ± 0.1	2.7 ± 0.3	2.3 ± 0.2	2.9 ± 0.4
CaR^{E228K}	1.4 ± 0.4	1.8 ± 0.6	1.6 ± 0.3	2.1 ± 0.5
CaR^{Q245R}	2.0 ± 0.0	2.1 ± 0.1	2.0 ± 0.0	2.1 ± 0.1
CaR^{E228A}	2.1 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	2.5 ± 0.2

Table 5.1. Extracellular Ca^{2+} EC_{50} and EC_{80} values for the ADH CaR mutants ERK and $p38^{MAPK}$ responses.

Based on these data (Table 5.1) it was decided to stimulate CaR^{WT} with 3.4 mM Ca^{2+}_o and the ADH CaR mutants with 2.4 mM Ca^{2+}_o in order to have sufficient ERK and p38MAPK responsiveness to be able to test the antagonistic effect of the NPSP795. The vehicle concentration (DMSO) was also standardised in all NPSP795 treatments to eliminate the possibility of a confounding effect of the vehicle. Thus using these conditions it was found that NPSP795 was able to inhibit ERK phosphorylation in the mutant CaRs, in addition to the CaR^{WT} (Figure 5.8 and 5.9). The effect of NPSP795 on CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, and wild type CaR is shown first in Figure 5.8*A* with CaR^{Q245R} and CaR^{C131Y} shown after on Figure 5.9*A*. Cotreatment with 1000 nM NPSP795 was found to almost completely abolish the ERK phosphorylation in all cell-types tested. Using only 100nM NPSP795, there was also substantial inhibition of ERK phosphorylation in most cases whereas 1-10 nM NPSP795 exposure was largely without effect. Together, the calculated IC_{50}s of NPSP795 for ERK phosphorylation were 50.1 ± 9.3 nM for CaR^{A840V}, 76.4 ± 13.3 nM for CaR^{E228K}, 32.4 ± 14.1 nM for CaR^{E228A}, 51.5 ± 6.9 nM for CaR^{Q245R} and 96 ± 36.7 nM for wild type CaR (Figure 5.8*B* and 5.9*B*).

A similar effect was observed for cotreatment with NPSP795 in the cells transiently transfected with CaR^{C131Y} and CaR^{WT} (Figure 5.9*C*). That is, 1000 nM NPSP795 virtually abolished ERK phosphorylation in both sets of cells with 100 nM having a substantial effect but 1-10 nM NPSP 795 having little or no inhibitory effect. The IC_{50}s for NPSP795 on CaR^{C131Y} and CaR^{WT} were 79.2 ± 10.6 nM and 48.7 ± 8.2 nM respectively (Figure 5.9*D*).

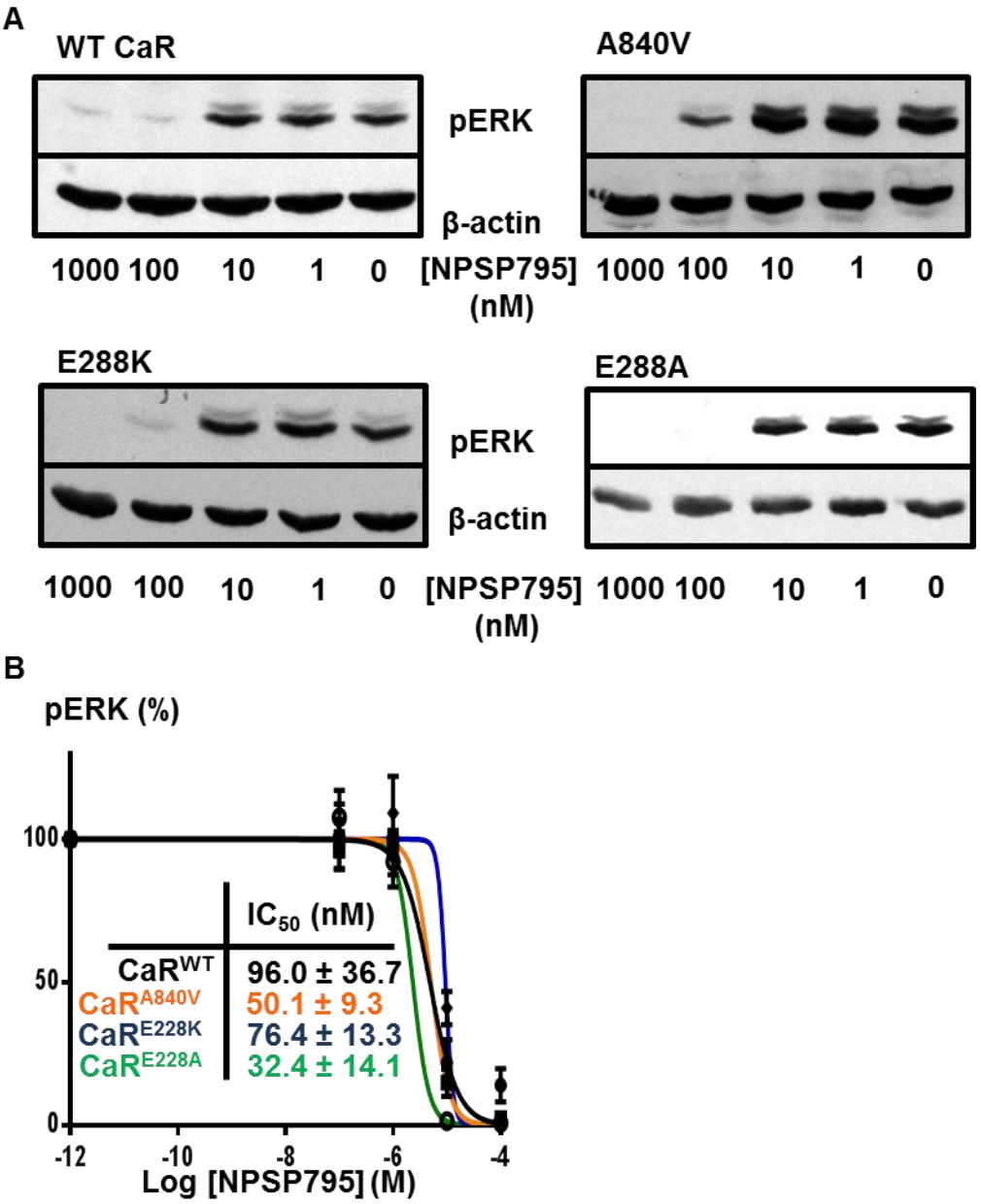

Figure 5.8: NPSP795 attenuates CaR-induced ERK activation in CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and wild-type CaR-HEK cells. *A*) Representative western blots showing ERK phosphorylation and β-actin immunoreactivity in HEK cells stably transfected with CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT} following treatment with increasing NPSP795 concentrations (0-1000 nM) at 3.4 mM Ca^{2+}_o for wild type CaR and 2.4 mM Ca^{2+}_o for mutant CaRs. *B*) Quantification of ERK phosphorylation in CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT}-transfected cells and the NPSP795 IC_{50} values in each cell-types. The responses were expressed as a percentage of the Ca^{2+}_o responses in the absence of calcilytic. $n \geq 4$ from at least 2 independent experiments.

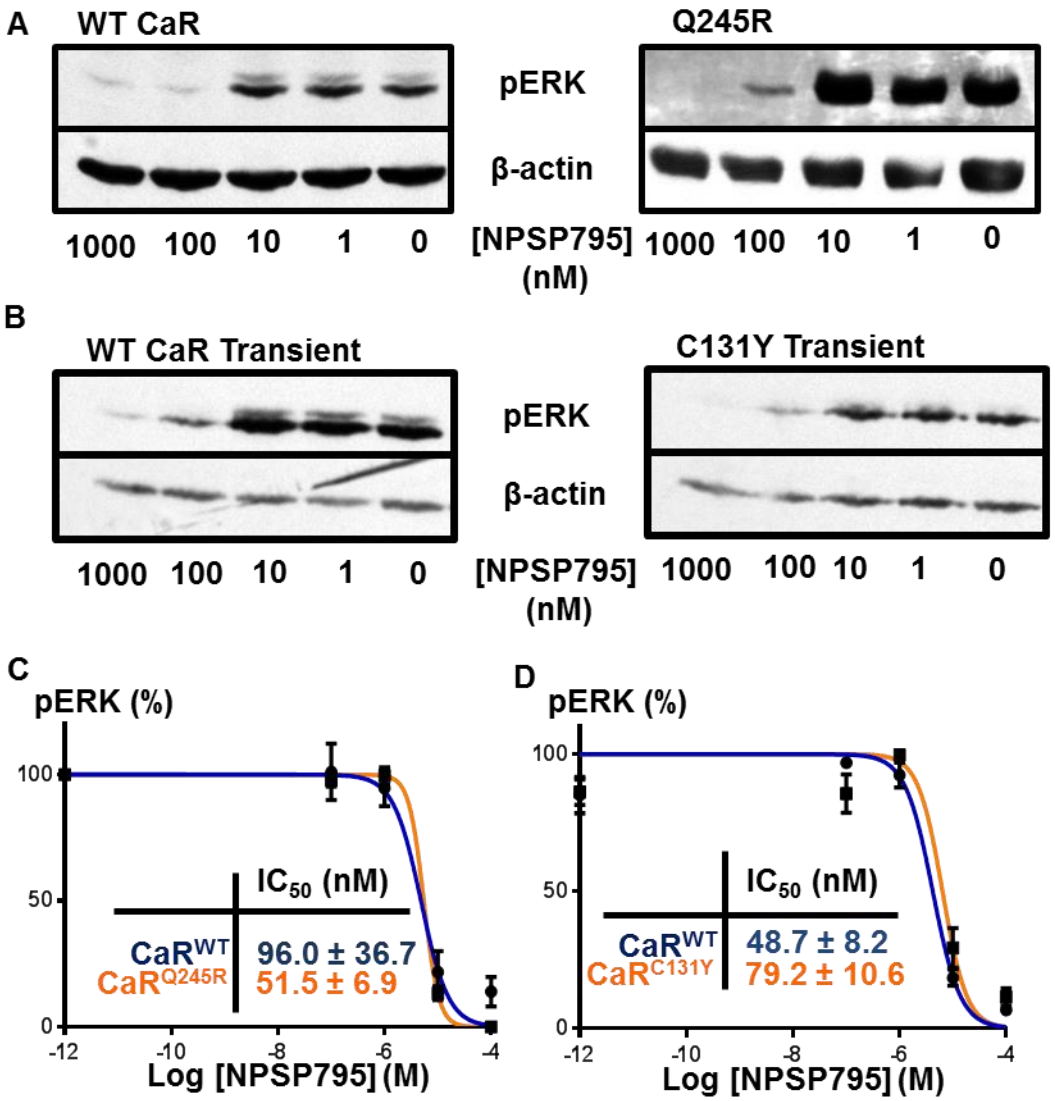

Figure 5.9: NPSP795 attenuates CaR-induced ERK activation in CaR^{Q245R}, CaR^{C131Y} and CaR^{WT} transfected HEK cells. HEK-293 cells transfected with CaR were treated with increasing NPSP795 concentrations (0-1000 nM) at 3.4 mM Ca^{2+}_o for wild type CaR and 2.4 mM Ca^{2+}_o for mutant CaRs. *A*) Representative western blots showing ERK phosphorylation and β-actin immunoreactivity in CaR^{Q245R} and CaR^{WT}-stably transfected cells. *B*) Representative western blots showing ERK phosphorylation and β-actin immunoreactivity in CaR^{C131Y} and CaR^{WT} transiently-transfected cells. *C*) Quantification of ERK phosphorylation in CaR^{Q245R}, and CaR^{WT} stably-transfected cells and the NPSP795 IC_{50} values in each cell-type. *D*) Quantification of ERK phosphorylation in CaR^{C131Y} and CaR^{WT} transiently-transfected cells and the NPSP795 IC_{50} values for each set of cells. The responses were expressed as percentage of the Ca^{2+}_o responses in the absence of calcilytic. n ≥ 5 from 2 independent experiments / transfections.

5.3.8 Determination of the effect of NPSP795 on CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{C131Y} -induced $p38^{MAPK}$ phosphorylation

Next, the effect of NPSP795 on CaR-induced $p38^{MAPK}$ activation was also evaluated in CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R}, CaR^{C131Y} and CaR^{WT}. In these experiments, the immunoblot membranes from the previous pERK experiments were stripped and reprobed with the phospho-$p38^{MAPK}$ specific antibody. The observations on stably transfected CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{WT} are summarised in Figure 5.10*A* and 5.11*A*. Similar to ERK phosphorylation, $p38^{MAPK}$ phosphorylation was substantially reduced / abolished in all cell-types in response to 1000 nM NPSP795 with 100 nM also exerting marked inhibition in most cases. However, 1 nM NPSP795 was without effect and 10 nM NPSP795 was only weakly inhibitory in most cases. The IC_{50}s for NPSP795 on $p38^{MAPK}$ were were 62.1 ± 7.7 nM for CaR^{A840V}, 48.2 ± 11.5 nM for CaR^{E228K}, 9.9 ± 5 nM for CaR^{E228A}, 57.7 ± 17.5 nM for CaR^{Q245R} and 44.3 ± 21.4 nM for CaR^{WT} (Figure 5.10*B* and 5.11*B*). In a single experiment in which the NPSP795 was tested on CaR^{WT}-HEK cells generated previously in this laboratory, the IC_{50} was 83.4 ± 43.6 nM which is consistent with the compound's effect in the new stable clone.

Next, the effect of NPSP795 on $p38^{MAPK}$ phosphorylation in HEK-293 cells transiently transfected with CaR^{C131Y} and CaR^{WT} was then tested (Figure 5.11*C*). The phosphorylation of $p38^{MAPK}$ in both sets of cells was largely abolished by 1000 nM NPSP795 with 100 nM of the compound having a partial inhibitory effect. In contrast, $Ca^{2+}{}_o$-induced $p38^{MAPK}$ phosphorylation was largely unaffected when cotreated with either 1 or 10 nM NPSP795 showing that $p38^{MAPK}$ phosphorylation was not inhibited at these lower NPSP795 concentrations. The IC_{50}s for NPSP795 on $Ca^{2+}{}_o$-induced $p38^{MAPK}$ phosphorylation on CaR^{C131Y} and CaR^{WT} were 108.9 ± 33.9 nM and 182.3 ± 80.9 nM respectively (Figure 5.11*D*).

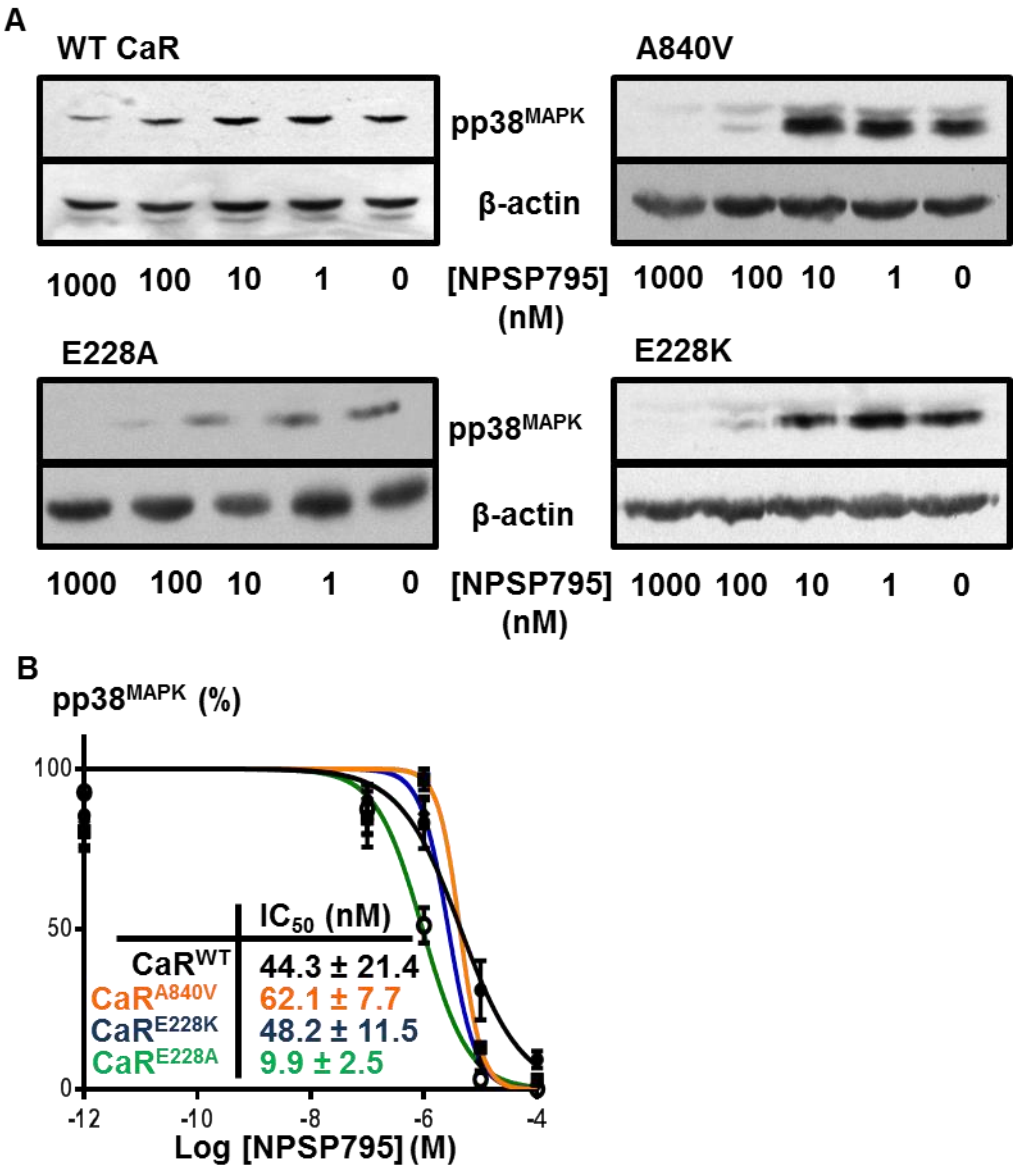

Figure 5.10: NPSP795 attenuates CaR-induced p38^{MAPK} activation in CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT}-HEK cells. *A*) Representative western blots showing p38^{MAPK} phosphorylation and β-actin immunoreactivity in HEK cells stably transfected with CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT} following treatment with increasing NPSP795 concentrations (0-1000 nM) at 3.4 mM Ca^{2+}_o for CaR^{WT} and 2.4 mM Ca^{2+}_o for mutant CaRs. *B*) Quantification of p38^{MAPK} phosphorylation in CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT}-transfected cells and the NPSP795 IC_{50} values for each cell-type. Responses are expressed as a percentage of the Ca^{2+}_o response in the absence of calcilytic. n ≥ 4 from a minimum of 2 independent experiments.

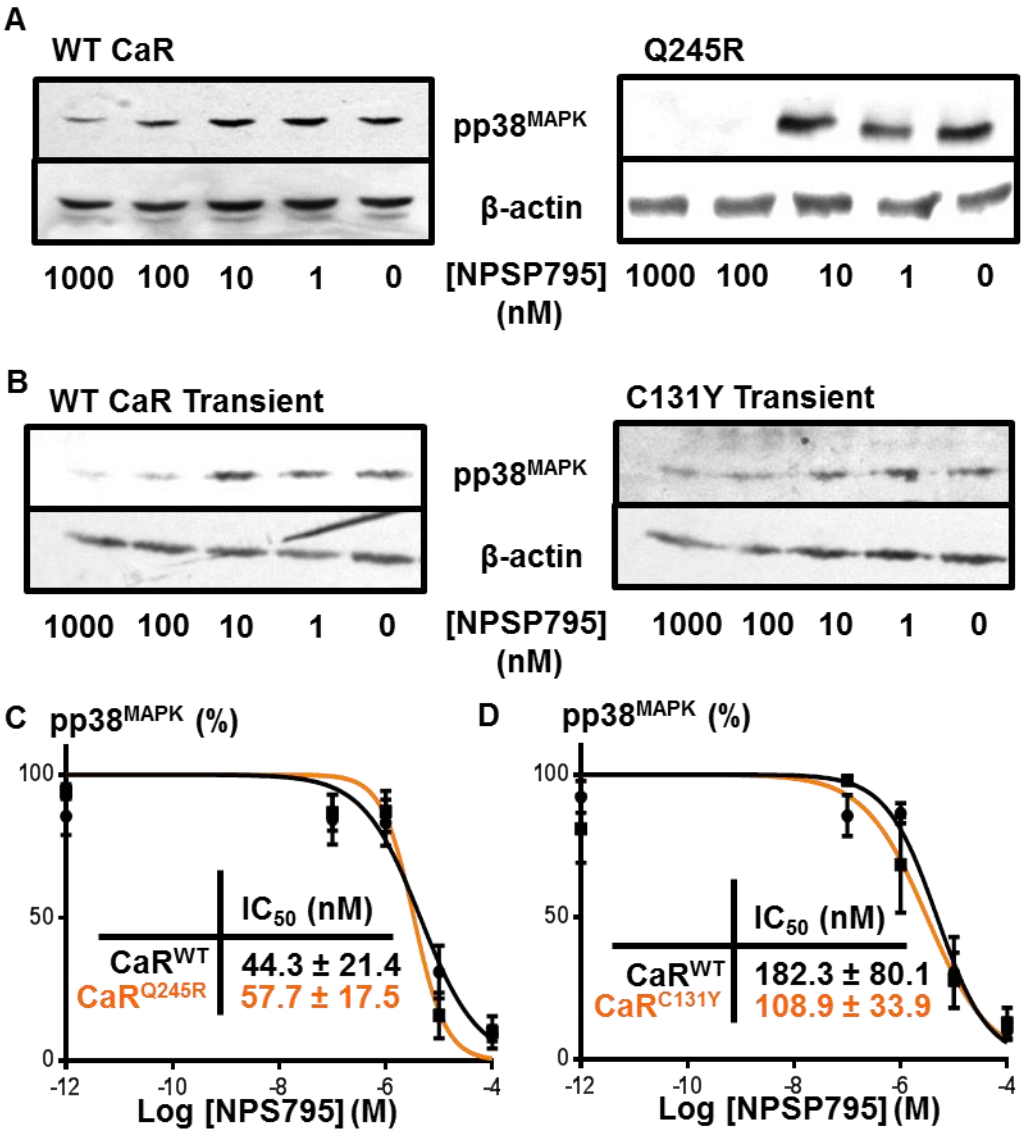

Figure 5.11: NPSP795 attenuates CaR-induced p38^{MAPK} activation in CaR^{Q245R}, CaR^{C131Y} and CaR^{WT}-HEK cells. HEK cells transfected with CaR were treated with increasing NPSP795 concentrations (0-1000 nM) at 3.4 mM Ca^{2+}_o for CaR^{WT} and 2.4 mM Ca^{2+}_o for mutant CaRs. *A*) Representatives western blot showing p38^{MAPK} phosphorylation and β-actin immunoreactivity in CaR^{Q245R} and CaR^{WT}-stably-transfected cells. *B*) Quantification of p38^{MAPK} phosphorylation in CaR^{Q245R} and CaR^{WT} stably-transfected cells with the NPSP795 IC_{50} values in each case. *C*) Representative western blots showing p38^{MAPK} phosphorylation and β-actin immunoreactivity in CaR^{C131Y} and CaR^{WT}-transiently-transfected cells. *D*) Quantification of p38^{MAPK} phosphorylation in CaR^{C131Y} and CaR^{WT} transiently-transfected cells and the NPSP795 IC_{50} values in each case. The responses were expressed as a percentage of the Ca^{2+}_o response in the absence of calcilytic. $n \geq 4$ from a minimum of 2 independent experiments.

5.3.9 Determination of the effect of CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{CI31Y} mutations on CaR-induced P70 S6Kinase phosphorylation

Having established earlier in the study that P70 S6Kinase is also a downstream effector of CaR and thus represents another CaR activity readout, the effects of CaR^{A840V}, CaR^{E228K} and CaR^{C131Y} on Ca^{2+}_o-induced P70 S6Kinase phosphorylation, were then tested by stripping the relevant blots and reprobing them with anti-phospho S6 kinase (Thr389) polyclonal antibody. The data in Figure 5.12*A* show that these CaR mutations potentiated Ca^{2+}_o-induced P70 S6Kinase phosphorylation, similar to their effect on ERK and $p38^{MAPK}$ phosphorylation. In 0.5 mM Ca^{2+}_o, a low level of P70 S6Kinase phosphorylation was observed in CaR^{A840V}, CaR^{E228K}, CaR^{C131Y} and CaR^{WT} cells. Raising the Ca^{2+}_o concentration to 2 mM increased P70 S6Kinase phosphorylation in the CaR^{A840V}, CaR^{E228K} and CaR^{C131Y} cells but was without effect on CaR^{WT}. Raising the Ca^{2+}_o concentration further to 3 mM elicited maximal or near to maximal P70 S6Kinase phosphorylation in the mutant CaR cells and initiated a response in the CaR^{WT} cells as well. For CaR^{WT}, 5-10mM Ca^{2+}_o were required to elicit maximal P70 S6Kinase phosphorylation. In accordance with the observations described above, the plotted Ca^{2+}_o-induced P70 S6Kinase phosphorylation concentration-effect curves for CaR^{A840V}, CaR^{E228K} and CaR^{C131Y} showed the trend to be left-shifted compared to CaR^{WT}-HEK cells (Figure 5.12*B*). However, it was just EC_{50} for Ca^{2+}_o for CaR^{E228K} mutant that significantly lower than for CaR^{WT} ($P < 0.05$).

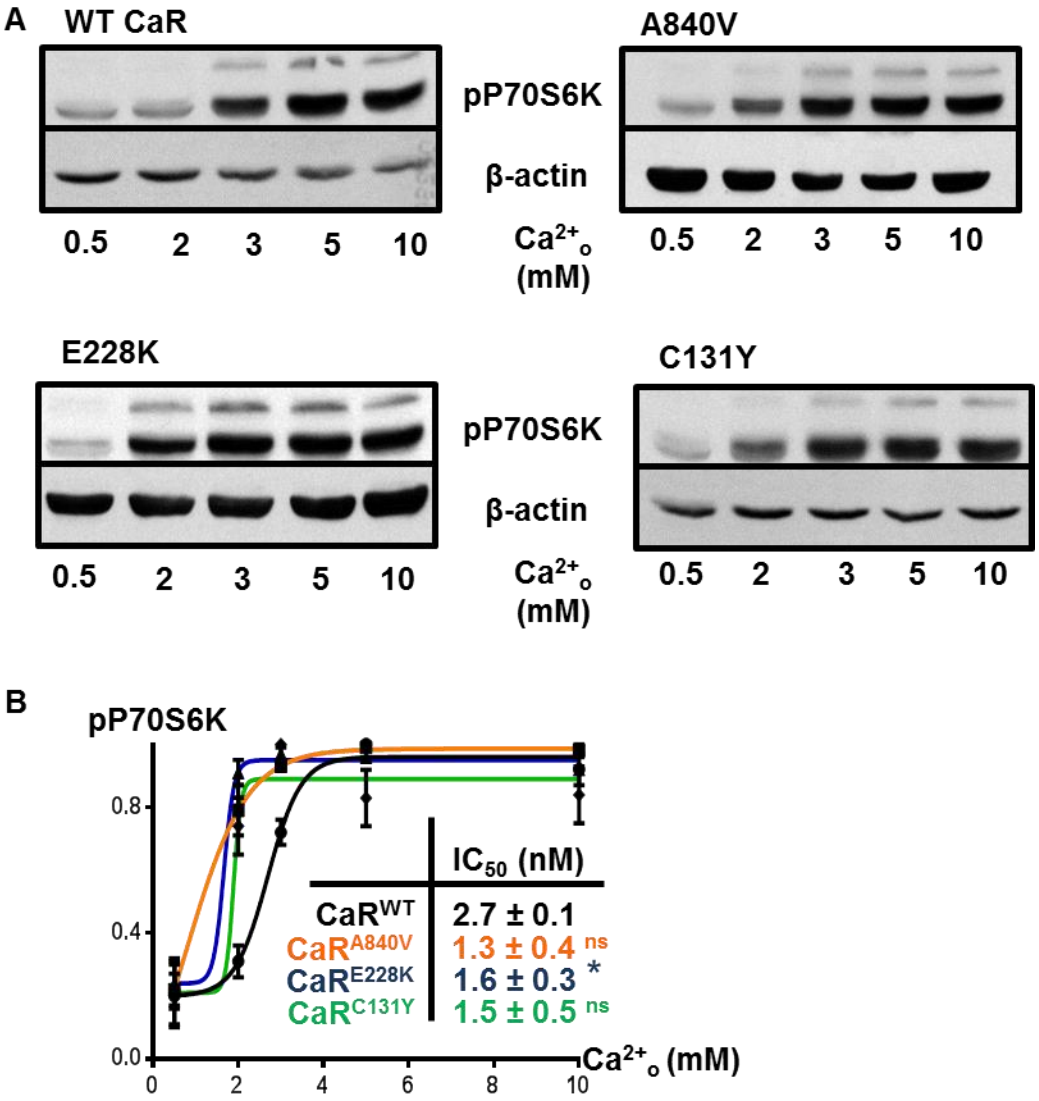

Figure 5.12: CaR^{A840V}, CaR^{E228K} and CaR^{C131Y} mutations increase CaR-induced P70 S6Kinase activation. *A*) Representative western blots showing P70 S6Kinase phosphorylation and β-actin immunoreactivity in HEK cells stably-transfected with CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{WT} following treatment with increasing Ca^{2+}_o concentrations (0.5-10mM). *B*) Quantification of P70 S6Kinase phosphorylation in CaR^{A840V}, CaR^{E228K}, CaR^{C131Y} and CaR^{WT}-transfected cells. Responses are expressed as a percentage of the maximal response in each cell set. *P < 0.05 vs CaR^{WT} log EC_{50} values; $^{ns}P > 0.05$ vs CaR^{WT} log EC_{50} values by Kruskal-Wallis with Dunnett's post-test. $n \geq 3$ from a minimum of 2 independent experiments.

5.3.10 Investigation of potential ERK, $p38^{MAPK}$ and p70 S6Kinase signal bias between the gain-of-function CaR mutants.

It has been reported that some CaR mutations change CaR conformation in such a way that it may not only increase or decrease the receptor's overall agonist sensitivity but that it may alter its signalling preference (Leach *et al*., 2013). To look for evidence of such signal bias among the gain-of-function CaR mutants investigated here, the ERK, $p38^{MAPK}$ and p70 S6Kinase data were compared using the equation detailed in 5.2. The results of this analysis are shown in Table 5.2. The five CaR mutations were found not to induce any significant bias towards ERK or $p38^{MAPK}$ as their ppBF (ERK: $p38^{MAPK}$) values were not significantly different compared to wild type CaR ($P > 0.05$ by 1-way ANOVA with Dunnett's). The ppBF (ERK:$p38^{MAPK}$) values for CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R}, CaR^{C131Y} and CaR^{WT} were 0.06 ± 0.04, 0.04 ± 0.09, 0.01 ± 0.02, -0.05 ± 0.05 and 0.14 ± 0.08 respectively. Similarly, there is no significant difference between ppBF (ERK:P70 S6Kinase) values for CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{C131Y} when compared to CaR^{WT} suggesting that these mutations did not cause bias towards ERK or P70 S6Kinase either. The ppBF (ERK:P70 S6Kinase) values for CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R}, CaR^{C131Y} and CaR^{WT} were -0.38 ± 0.36, -0.01 ± 0.08, 0.30 ± 0.26 and -0.04 ± 0.05 respectively. Finally, CaR^{A840V}, CaR^{E228K}, CaR^{E228A} and CaR^{C131Y} were found to cause no bias between p70 S6Kinase and $p38^{MAPK}$ signalling. The ppBF (p70 S6Kinase: $p38^{MAPK}$) values for CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{C131Y} and CaR^{WT} were 0.45 ± 0.28, 0.06 ± 0.1, 0.47 ± 0.23 and 0.18 ± 0.04 respectively and the comparison between these ppBFs shows no significance difference between mutants and CaR^{WT}.

Mutation	ppBF (ERK:p38)	ppBF (ERK:P70S6K)	ppBF (P70S6K:p38)
WT	**0.14 ± 0.08^{ns}**	**-0.04 ± 0.05^{ns}**	**0.18 ± 0.04^{ns}**
C131Y	**0.18 ± 0.07^{ns}**	**-0.12 ± 0.19^{ns}**	**0.23 ± 0.20^{ns}**
A840V	**0.06 ± 0.04^{ns}**	**-0.38 v 0.36^{ns}**	**0.45 ± 0.28^{ns}**
E228K	**0.04 ±0.09^{ns}**	**-0.01 ± 0.08^{ns}**	**0.06 ± 0.10^{ns}**
Q245R	**-0.05 ± 0.05^{ns}**	**ND**	**ND**
E228A	**0.011 ± 0.02^{ns}**	**ND**	**ND**

Table 5.2: Determination of ppBF values comparing the relative responsiveness of mutant and CaR^{WT} cells for ERK, p38^{MAPK} and p70 S6Kinase activation. The ppBF values were calculated as the negative algorithm ratio between the $Ca^{2+}{}_o$ EC_{50}s for each signalling pathway. Statistical comparisons between ppBFs of mutant CaRs and wild type CaR were performed by 1-way ANOVA with Dunnett's post-test. ns = no significant difference vs CaR^{WT}; ND = not determined due to insufficient data-points.

5.4 DISCUSSION

5.4.1 Determination of gain-of-function in five ADH CaR mutants with respect to Ca^{2+}_i mobilisation, ERK and $p38^{MAPK}$ phosphorylation

Mutations of the CaR gene are associated with dysregulation of calcium homeostasis which can lead in turn to a series of moderate-to-serious clinical problems. CaR mutations can be found in all three CaR domains (ECD, TMD, and ICL) (curated at http://data.mch.mcgill.ca/casrdb/) where they may exert different conformational modifications to alter receptor activity. The majority of these are missense mutations (though frame-shift and splice site mutations also occur) (Hendy *et al*., 2009) and can result in loss- or gain-of-function resulting most likely in hypercalcaemia or hypocalcaemia respectively.

CaR activation stimulates several G proteins to which it is coupled including $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ resulting in the activation of numerous downstream signals such as altered cAMP formation, IP_3 production, Ca^{2+}_i mobilisation and actin polymerisation as well as the activation of an array of protein kinase pathways, most notably mitogen-activated protein kinases such as ERK and $p38^{MAPK}$ (Kifor *et al*., 2001; Ward, 2004; Conigrave and Ward 2013). Assuming the absence of signal bias, then in theory any gain-of-function CaR mutation should increase each downstream signal equally. However, there is no reason to believe that any change in responsiveness should be equal for every signal since the mutation could cause a change in conformation that alters the pleiotropic interaction between receptor and G protein (or other signal mediator). That is, mutations located in different regions of the receptor, or, in the same location but with different resulting residues could affect the conformation differently and thus produce biased signalling. In this chapter, the effect of five ADH mutations on CaR-induced Ca^{2+}_i mobilisation, ERK and $p38^{MAPK}$ activation were examined. Firstly, we found that CaR^{A840V} enhanced Ca^{2+}_o-induced Ca^{2+}_i mobilisation, and ERK (Figure 5.1).

The A840V mutation occurs in the TMD region. CaR mutation within the TMD at V817I, causes CaR inactivation and it has been suggested that the replacement of valine with isoleucine at this site might stabilise an inactive CaR conformation (Pearce *et al*.,

1996). This shows that the TMD plays an important role in maintaining CaR stability. Moreover, it has also been suggested that the TMD is crucial for the maintenance of CaR dimerisation through hydrophobic interactions (Bai, 2004). That is, CaR dimers are still maintained even when Cys-129 and Cys-131, the residues responsible for disulphide linkage, are mutated (Zhang *et al.*, 2001; Bai, 2004). Thus, the A840V mutation might increase the intramolecular or even intermolecular stability of the CaR active conformation.

The proband for CaR^{A840V} was diagnosed with hypoparathyroidism having been hospitalised with severe hypocalcaemia and a family history revealing that the proband's father had similar clinical problems (Winer *et al*., 2014). The current data provides *in vitro* confirmation that CaR^{A840V} elicits enhanced receptor signalling which would explain the chronically inhibited PTH secretion and hypocalcaemia. In addition, the proband also had nephrolithiasis (kidney stones). CaR is expressed on the basolateral membrane of cortical thick ascending limb (cTAL) where it inhibits NaCl reabsorption and K^+ recycling and thus paracellular Ca^{2+} and Mg^{2+} reabsorption (Ward and Riccardi, 2012; Tyler-Miller, 2013). Therefore, in the current study it was shown that CaR^{A840V} represents a gain-of-function mutation, *in vitro*, by three different cellular readouts.

The other four mutations examined, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{C131Y} and these mutations were found to enhance $Ca^{2+}{}_i$ mobilisation and ERK activation in HEK-293 cells while CaR^{E228K} and CaR^{Q245R} were also found to increase $p38^{MAPK}$. All of these mutations occur within the ECD which is dominated by negatively-charged amino acids and all four are specifically located within the first ~500 amino acids that form the "venus flytrap structure" which is important for $Ca^{2+}{}_o$ binding (Ray *et al*., 1998). Thus, mutations in this region might positively or negatively affect the ability of CaR to sense $Ca^{2+}{}_o$. In particular, a number of gain-of-function mutations are located between residues 116 and 297 with a particular cluster at 116-131 (http://data.mch.mcgill.ca/casrdb/). As already mentioned, the cysteine residues at 129 and 131 are thought to mediate the intermolecular disulphide binding between two CaR subunits and their mutation increases CaR sensitivity to $Ca^{2+}{}_o$ *in vitro* (Zhang *et al*., 2001; Hendy *et al*., 2009). Also, it is worth noting a similarly positive effect on CaR activity was observed when ICD's loop two (amino acid 114-126) was deleted (Reyes-Cruz *et al*., 2001). Again, the current

study demonstrated that these four CaR mutants that lie in this proximal region of the ICD exhibit gain-of-function activity in HEK-293 cells as shown by increases in Ca^{2+}_i mobilisation, ERK activation or $p38^{MAPK}$ activation. One possible explanation for a CaR mutant exhibiting greater agonist responsiveness is increased receptor expression, particularly that localised in the plasma membrane. For example, Brennan *et al*. (2015) showed that increased CaR expression, as controlled by a tetracycline-inducible expression system, caused enhanced Ca^{2+}_i mobilisation in response to elevated Ca^{2+}_o, L-phenylalanine and cinacalcet exposure. However in the current study, the cell-lines (generated by Dr Brennan in Cardiff) were clonal and had been deliberately selected because of the equivalence of their CaR expression levels. The equivalent levels of CaR^{WT} and CaR^{mutant} expression were confirmed here by immunoblotting (Figures 5.1-5.5), but this was further confirmed at the membrane localisation level by Dr Brennan (data not shown). Thus, the potentiation of CaR activity with these mutations is unlikely to have been caused by increased CaR abundance in the cells.

In the current study, neither CaR^{WT} nor any of the CaR mutants elicited Ca^{2+}_i mobilisation, or marked ERK or $p38^{MAPK}$ phosphorylation at low Ca^{2+}_o concentration (0.5mM) demonstrating that they are not constitutively active. In contrast, CaR^{S820I} and CaR^{A843E} mutations were found to exhibit high MAPK activity at zero Ca^{2+}_o environment (Lia-Baldini *et al*., 2013) and thus constitutive CaR activation can occur, presumably by permanently stabilising the active CaR conformation even in the absence of agonist. Therefore, the gain-of-function mutations examined in the current study appear to stabilise the agonist-bound active state but not the unbound state.

Changes in Ca^{2+}_i concentration and ERK phosphorylation are the two most widely-used readouts of relative receptor activity in the functional characterisation of CaR mutations (Vargas-Poussou *et al*., 2002; Lazarus *et al*., 2011; Leach *et al*., 2012; Leach *et al*., 2013). Therefore in the current study, both of these experimental readouts were used together with $p38^{MAPK}$ and to a lesser extent P70 S6kinase principally to confirm the gain-of-function findings by more than one method but also to look for evidence of differences between the readouts i.e. potential bias. In this laboratory the work focused on MAPK assays since the Ca^{2+}_i assaying was extensively done in our collaborator's laboratory (University of Cardiff) although some similar experiments were repeated here

to reconfirm their findings. It should be noted that different signals can result in different functional responses with CaR-induced changes in ERK 1/2 and $p38^{MAPK}$ activation known to be associated with cellular proliferation (Tfelt-Hansen *et al*., 2004; Brennan *et al*., 2013). However, even acutely there may be different time courses between signalling pathways and therefore the precise time-point at which two signals are measured could result in variable differences between the two readouts. That is, if one signal is more sustained than the other then the later the two signals are measured then the bigger the apparent difference may be seen between the two and this could be confused for bias for example. Another potential issue that may affect concentration-effect measurements is whether the treatments are cumulative or not. In single cell (or cuvette) Ca^{2+}_i imaging, cumulative Ca^{2+}_o concentration effect curves are standard in this and other laboratories (Bai *et al*., 1998b; Davies *et al*., 2007; Conigrave *et al*., 2000). However, if ADIS (or desensitisation) is functionally important, then each subsequent Ca^{2+}_o response could be greater (or lesser, respectively) than had each Ca^{2+}_o concentration been tested following exposure to 0.5mM (baseline) Ca^{2+}_o, as would be the case where multiwell plate Ca^{2+}_i assays are performed (Grant *et al*., 2011), or indeed with the dishes used here for the kinase assays. Strictly speaking, non-cumulative assays provide a better measure of the specific response of a particular Ca^{2+}_o concentration since it is unaffected by what preceded it. That said, physiologically the CaR works more like a continuous, cumulative assay and therefore it may be advantageous to employ both methodologies side-by-side.

5.4.2 The effect of NPSP795 on Ca^{2+}_o–induced ERK and $p38^{MAPK}$ phosphorylation in five gain-of-function CaR mutants

The central role of the membrane-localised CaR in Ca^{2+} homeostatic regulation makes it an ideal potential drug target for diseases of Ca^{2+} homeostatic dysfunction. In this regard, cotreatment with the calcilytic NPSP795 inhibited the action of both CaR^{WT} and all five CaR mutants, with 1μM of the calcilytic largely abolishing the receptor's responsiveness to an ~EC_{80} concentration of Ca^{2+}_o (Figure 5.8-5.11). This indicates the compound's potential utility in treating the ADH of patients with their CaR mutation either in the venus fly trap domain or in the TMD.

It has long been hypothesised that inhibiting CaR could lead to increased PTH release to stimulate bone formation for the treatment of osteoporosis (Nemeth, 2002) since subcutaneous injection of rhPTH (1-34) causes bone formation (Caltabiano *et al*., 2013). To make an effective osteoporosis treatment it was reasoned that the calcilytic should act quite briefly and be rapidly metabolised from the body (Nemeth, 2002). This is because bone formation is stimulated by transient increases in PTH whereas prolonged rises in PTH secretion have catabolic effects on bone (Nemeth, 2002). The 3 to 4 fold increase of PTH that lasts for one to two hours is found to be required to stimulate bone formation (Hodsman *et al*., 2002). However, it was found clinically that Ronacaleret administration caused prolonged PTH secretion causing a net catabolic effect on bone (Caltabiano *et al*., 2013). Another clinical study showed that the administration of AXT914 failed to increase the bone formation markers such as Procollagen 1 intact N-terminal (P1NP), Proximal Interphalangeal (PIP), C-terminal telopeptide type 1 (CTX-1) and osteocalcin (John *et al*., 2014). Currently, a calcilytic that is able to treat osteoporosis has yet to be discovered.

However with regards to the treatment of ADH, it is still useful that previous clinical studies on calcilytics found that they did increase PTH secretion (Caltabiano *et al*., 2013; John *et al*., 2014). Ronacaleret was found to cause longer PTH secretion compared to rhPTH(1-34) (Caltabiano *et al*., 2013). As for AXT914, its administration caused similar PTH levels in blood as rhPTH (1-34) (John *et al*., 2014). These observations raise the possibility of using a calcilytic for ADH treatment. Currently, ADH tends to be treated similarly to other hypoparathyroid cases, such as with vitamin D analogues, high dose Ca^{2+} and rhPTH (1-34) (Tirumani and Saddala, 2014; Dong *et al*., 2015). These treatments however can cause hypercalciuria in ADH patients increasing the risk of kidney stones and nephrocalcinosis. A recent study by Hannan *et al*. (2015) showed that treatment with NPS-2143 was able to rectify hypocalcaemia in a mouse model that harbours a CaR^{L723Q} mutation (Hough *et al*., 2004). This calcilytic increased plasma Ca^{2+} and PTH concentrations in the mice without elevating urinary Ca^{2+} excretion. The associated *in vitro* study showed that NPS-2143 corrected the increase of Ca^{2+}_i mobilisation in HEK-293 cells transfected with CaR^{L723Q}. However, it should be noted that this calcilytic was previously found to be relatively poorly metabolised in humans and thus persistent in blood though whether this is advantageous in ADH is not clear

(Gowen *et al*., 2000). Apart from that, the *in vivo* study on the calcilytic JTT-305/MK-5442 also showed this calcilytic increased plasma Ca^{2+} and $P_{i,}$ and urinary cAMP excretion in mouse model harbouring CaR^{A843E} and CaR^{C129S} mutations (Dong *et al*., 2015). The mouse model used in that study was found to develop renal calcification since they were fed with high Ca^{2+} and vitamin D diet to normalise the plasma Ca^{2+}. Moreover, the treatment of JTT-305/MK-5442 was found to prevent renal calcification by normalising urinary Ca^{2+} excretion. This observation might occur due to increased renal Ca^{2+} resorption due to CaR inhibition by the calcilytic. Interestingly, this calcilytic also increased the bone mass of the mouse, thus making it a potential candidate for osteoporosis treatment. In the *in vitro* study, the treatment of this calcilytic was found to suppress the increased $Ca^{2+}{}_i$ mobilisation in HEK-293 cells transfected with CaR^{A843E}, CaR^{C129S}, CaR^{K47N}, CaR^{S820F}, CaR^{C131Y} and CaR^{F821L}. Together, these various studies indicate the potential usefulness of the calcilytics for the treatment of ADH and thus further clinical testing appears appropriate.

In this study, NPSP795 supressed CaR-induced ERK and $p38^{MAPK}$ activation in HEK-293 cells stably transfected with various gain-of-function CaR mutations. It was also found that the IC_{50}s of this calcilytic on ERK and $p38^{MAPK}$ activations in mutated CaR were similar to those for CaR^{WT} where each receptor was exposed to a $Ca^{2+}{}_o$ concentration at or close to its EC_{80}. Specifically, since the IC_{50}s of the mutants for $Ca^{2+}{}_o$-induced ERK and $p38^{MAPK}$ activation were $\leq$ ~100 nM then modest target plasma concentrations of the drug should be sufficient to inhibit the CaR *in vivo*. The advantage of this is that use of higher concentrations of a therapeutic agent risks off-target effects. In comparison, the IC_{50} of NPS53574 on $Ca^{2+}{}_i$ mobilisation in CaR-HEK cells is 3500 nM and the IC_{50} of Calhex-231 on IP_3 accumulation in CaR-CHO cells is 330 nM (Shcherbakova *et al*., 2005; Kessler *et al*., 2006).

5.4.3 The effect of five ADH CaR mutations on $Ca^{2+}{}_o$-induced P70 S6Kinase phosphorylation and the investigation of signalling bias by the mutants

Having established in Chapter 4 that P70 S6Kinase phosphorylation lies downstream of CaR^{WT} activation, this pathway was also investigated to test the consequence of the 5 ADH mutants on this pathway. It was shown that CaR^{E228K} increased P70 S6Kinase

phosphorylation relative to CaR^{WT}, thus shifting their Ca^{2+}_o concentration-effect curves to the left (Figure 5.12). The activation of P70 S6Kinase is known to be related to protein translation and cell growth, though no association with PTH secretion has been reported. Previously, it has been reported that calcimimetic treatment can attenuate parathyroid hyperplasia in rats with secondary hyperparathyroidism (Colloton *et al*., 2005; Miller *et al*., 2012). This observation shows that the activation of CaR tends to inhibit parathyroid cell proliferation. How such a functional effect relates to CaR-induced p70 S6Kinase activity or indeed the pro-proliferative kinase, ERK remains to be determined.

Next, I examined the possibility of biased signalling between ERK, $p38^{MAPK}$ and P70 S6Kinase induction by the gain–of-function mutations. The use of these three phospho assays offers the benefit that technical bias can be avoided as they use the same procedure and are indeed tested on the same cell lysates. The hypothesis is that different mutations might alter the conformational state of CaR differentially thus favouring certain signalling pathways over others (Kenakin, 2011). Previously, a few studies have been done to evaluate biased signalling on mutant CaRs. A study done by Vargas-Poussou *et al*. (2002) showed that the gain-of-function, CaR^{L125P} mutant induced a higher increase in Ca^{2+}_i mobilisation compared to ERK activation. Similarly, CaR^{Q681H}, CaR^{E767K}, CaR^{L773R}, CaR^{S820F} and CaR^{F821L} gain-of-function mutations caused increased Ca^{2+}_o-induced Ca^{2+}_i mobilisation but no/little change was observed in ERK activation, suggesting they were biased towards Ca^{2+}_i mobilisation (Leach *et al*., 2013). It has also been shown in CaR-HEK cells that the stimulation of CaR^{WT} by Ca^{2+}_o is already biased towards cAMP inhibition and IP_3 accumulation compared to ERK activation (Thomsen *et al*., 2012). Thus, the gain-of-function mutations above might further amplify this preferential signalling. However, in the current study, no specific bias was seen between ERK, $p38^{MAPK}$, and p70 S6Kinase phosphorylation. This study is the first attempt to evaluate relative responsiveness of ERK, $p38^{MAPK}$, and p70 S6Kinase between different gain-of-function CaR mutations. While it is possible that the experiments were insufficiently powered to observe bias, it might reasonably be concluded that even if signal bias between the mutants did occur it was relatively subtle at best. Otherwise, it is possible of course that kinase signal bias does not occur between CaR mutants though further studies would be needed to confirm this (Figure 5.13).

5.5 Conclusion

In conclusion, CaR^{A840V}, CaR^{E228K}, CaR^{E228A}, CaR^{Q245R} and CaR^{C131Y} mutations increase $Ca^{2+}{}_o$-induced $Ca^{2+}{}_i$ mobilisation, and, ERK and $p38^{MAPK}$ activations in CaR-HEK cells in which receptor expression levels were equivalent. In addition, these gain-of-function mutations were not constitutively active and their induction of ERK and $p38^{MAPK}$ activation could be reversed by cotreatment with the calcilytic NPSP795. Whether such calcilytics might prove a useful treatment for people with gain-of-function CaR mutations, will be clarified by clinical trials of such compounds in ADH patients. CaR^{A840V}, CaR^{E228K} and CaR^{C131Y} also exhibited gain-of-function for p70 S6Kinase activation however no statistically significant signal bias between ERK and $p38^{MAPK}$ (and p70S6Kinase where tested) was observed with the ADH mutants.

Chapter 6

General Discussion and Conclusion

6.1 General discussion

It has been known for some time that the activity of the CaR is negatively regulated by PKC-mediated phosphorylation at CaR^{T888} (Bai *et al*., 1998b) but there is little clarity about how this occurs or which specific kinases and phosphatases are involved. PKCα – specific siRNA was found to enhance CaR-induced Ca^{2+}_i mobilisation in CaR-HEK cells and inhibit CaR^{T888} phosphorylation consistent with the observation that chronic PMA pre-treatment (which down-regulates conventional and novel PKCs) had a similar positive effect while Young *et al* (2014) recently showed that PKCα siRNA decreased the proportion of cells exhibiting Ca^{2+}_i oscillations. Together, these data suggest that PKC α is the isoform responsible for mediating negative feedback phosphorylation of CaR and that its removal potentiates CaR's activity. Thus, having specifically determined that PKCα is the main isoform responsible for CaR^{T888} phosphorylation and for suppressing CaR activity in CaR-HEK cells, this identifies PKCα as the first isoform to potentially target *ex vivo* (in isolated parathyroid cells) or *in vivo* to study the functional relevance of PKC. Without such information, we cannot know for certain why the CaR^{T888M} mutation results in ADH.

While multiple PKC isotypes were also examined, it was only the knocking down of PKCε that also altered CaR signalling, though surprisingly its effect was to attenuate (as opposed to potentiate) CaR-induced Ca^{2+}_i mobilisation. This suggests therefore that PKCε exerts a positive effect on CaR signalling. Interestingly, the apparent negative effects of PKCα and positive effect of ε are specific to CaR since carbachol-induced Ca^{2+}_i mobilisation was unaffected by either siRNA. Furthermore, while PKCα and ε appear to exert opposite effects on CaR-induced Ca^{2+}_i mobilisation, knockdown of either caused similar inhibition of CaR^{T888} phosphorylation and of ERK and $p38^{MAPK}$ phosphorylation. These data indicate firstly that it is possible to a) simultaneously increase Ca^{2+}_i mobilisation while inhibiting the MAPK pathways (via PKCα knockdown), and, b) simultaneously inhibit both CaR^{T888} phosphorylation and Ca^{2+}_i mobilisation (via PKCε knockdown). While it was not possible to explain both of these observations, these data certainly indicate the complexity of the interactions that potentially underlie the CaR's pleiotropic signalling and provides novel targets for examining signal bias. However, this

complexity should be also taken into consideration in future studies to avoid false positive/negative results. Apart from that, it is also crucial to determine PKC signalling in other cells that endogenously express CaR. By understanding these PKC-mediated CaR regulations, the efficiency of CaR signalling in different cell types might be determined. However, while not discounting the paradoxical effect of PKCε, it is nevertheless the case that the effect of PKCα knockdown was entirely consistent with the inhibitory effects of phorbol ester (PKC activation) and calyculin (PP2A inhibition) and positive effects of PKC inhibition and downregulation.

The CaR can induce actin polymerisation / membrane ruffling and cAMP inhibition at Ca^{2+} concentrations below those required to induce Ca^{2+}_i mobilisation. This shows that the CaR can be in an active conformation yet uncoupled from the Ca^{2+}_i mobilising mechanism. As a result, I hypothesised that PKC α-mediated CaR phosphorylation blocks Ca^{2+}_i mobilisation at these sub-threshold Ca^{2+} concentrations. Indeed, by knocking down PKCα, the cells exhibited Ca^{2+}_i mobilisation at Ca^{2+} concentrations that elicit no Ca^{2+}_i mobilisation in control CaR-HEK cells. This further shows that detectable Ca^{2+}_i mobilisation is not required to activate PKC α and thus that it may be partially active even at apparent baseline Ca^{2+}_i levels.

With CaR-induced actin polymerisation occurring at Ca^{2+}_o concentrations sub-threshold for Ca^{2+}_i mobilisation this raised the question of whether $G\alpha_{12}$ might regulate CaR-induced Ca^{2+}_i mobilisation particularly at threshold Ca^{2+}_o concentrations. Indeed, knocking down $G\alpha_{12}$ caused a right-ward shift in the concentration-effect curve for Ca^{2+}_o, but was without effect on the response to carbachol. In parallel, $G\alpha_{12}$ knockdown also increased CaR^{T888} phosphorylation which could explain the CaR-specific attenuation of Ca^{2+}_i mobilisation observed. This suggests that $G\alpha_{12}$ activity may contribute to CaR-induced Ca^{2+}_i mobilisation.

Next, Gerbino *et al*., 2005 showed previously that agents that increase intracellular cAMP concentration also enhance CaR-induced Ca^{2+}_i mobilisation. This effect is not apparently PKA-mediated since it could not be overcome using PKA inhibitors (Campion, 2013). Thus, another possible explanation for this is if the cAMP can somehow decrease CaR^{T888} phosphorylation to permit increased Ca^{2+}_i mobilisation. Indeed, forskolin cotreatment

decreased CaR^{T888} phosphorylation and accelerated the response recovery to Ca^{2+}_o challenge following PMA-mediated attenuation of CaR-induced Ca^{2+}_i mobilisation. It should be noted though that forskolin did also enhance mAChR-mediated Ca^{2+}_i mobilisation, suggesting that whatever effect it has is not purely CaR-specific. Nevertheless, CaR^{T888} may contribute at least part of the mechanism explaining cAMP-mediated potentiation of CaR responsiveness. These observations also show the possibility of CaR to acts as cAMP sensor to inhibit PTH secretion. It is generally accepted that the increase of PTH triggers PTH secretion. Thus, the cells may employ an alternative mechanism to stop the raise of PTH level by detecting intracellular cAMP level. This provides a shorter route for the cells to react to the increase of Ca^{2+}_o which can avoid PTH over-secretion.

Next in this study I discovered new signal readouts for CaR activity namely P70 S6Kinase, IGF1R and IκBα phosphorylation. An increase in the number of known CaR effectors that can be used as potential readouts of receptor activity should be helpful in the search for biased signalling so that a much broader picture of signalling complexity can be monitored. Since the three signals were induced by calcimimetic cotreatment, inhibited by calcilytic cotreatment and exhibited sigmoidal pharmacology in response to increasing Ca^{2+}_o concentrations then it is highly likely that they are indeed CaR effectors, at least in CaR-HEK cells. Apart from that, these results also highlighted the possible role of CaR outside maintining Ca^{2+}_o hemeostasis. The activation of P70 S6Kinase, IGF1R are known to be associated with growth and proliferation, while IκBα is important for cell survival. However, it will be necessary to next examine these signals in other cells in which the CaR is expressed endogenously.

Interestingly, the phosphorylation of IGF1R and P70 S6Kinase occurred at lower Ca^{2+}_o concentrations than for ERK, $p38^{MAPK}$ or IκBα phosphorylation (by comparison of EC_{50}s) and thus whether the former help prime the latter, or indeed Ca^{2+}_i mobilisation might be worthy of further investigation. What was shown than knocking down PKC α, PKC ε or Gα12 all reduced IGF1R, P70 S6Kinase and ERK phosphorylation. This is interesting since PKCα knockdown was earlier shown to enhance CaR-induced Ca^{2+}_i mobilisation by removing CaR^{T888} phosphorylation, yet the same knockdown attenuated phosphorylation

of these kinases suggesting that their phosphorylation can be dissociated from Ca^{2+}_i mobilisation.

Finally, gain-of-function in 5 ADH-associated CaR mutants (CaR^{A840V}, CaR^{C131Y}, CaR^{E228K}, CaR^{E228A} and Q^{245R}) was demonstrated for Ca^{2+}_i mobilisation as well as for ERK and p38MAPK phosphorylation. Furthermore, the calcilytic NPSP795 attenuated this activity with IC_{50}s in the nanomolar range. These data represent *in vitro* validation of the potential utility of the calcilytics in inhibiting gain-of-function CaR activity and across a variety of mutations found throughout the CaR. One might expect therefore that calcilytics should permit increased PTH secretion, decreased urinary calcium secretion and thus a correction of blood calcium levels in patients with ADH.

With regards to the 5 CaR mutants tests, none exhibited constitutive activity, since no CaR activity was observed at low Ca^{2+}_o concentrations (0.5 mM). Then in all cases, the calcilytic NPSP795 was able to fully inhibit the protein kinase signals tested with IC_{50}s in the "druggable" range, that is, concentration of drugs that should be achievable in the plasma *in vivo*, and, with less risk of off-target effects. What were not detected however, were any obvious signs of biased signalling between the gain of function CaR mutations. It remains to be determined whether this apparent lack of bias was because the experiments were insufficiently powered to detect such differences, or, because bias will be more limited between related protein kinase pathways as opposed to the potentially larger differences between Ca^{2+}_i mobilisation, cAMP levels, ERK phosphorylation and actin polymerisation.

6.2 General conclusion

Together, the data reported in this thesis advance our understanding of the regulation of CaR^{T888} phosphorylation and its effect on CaR signalling *in vitro*. PKC α in particular has been identified as the key PKC isotype responsible for this task but with a contradictory involvement of PKC ε also being identified and this observation requires further investigation. The regulatory effects of PKC α and cAMP at Ca^{2+}_o concentrations sub-threshold for Ca^{2+}_i mobilisation indicate the potential role of signal crosstalk in controlling CaR responsiveness but also that it may be susceptible to regulation by other GPCRs that modulate these signals as well. This is especially so given that $G\alpha_{12}$, which is not usually

associated with Ca^{2+}_i mobilisation, was shown to be a positive modulator of CaR-induced Ca^{2+}_i signalling, most likely by somehow decreasing CaR^{T888} phosphorylation.

While little specific evidence of signal bias between mutant CaRs was observed here, this project nevertheless identified three novel CaR effectors, namely IGF1R, P70 S6kinase and IκBα that can now be used as additional pathways to examine as part of the broader search for pathway specific signalling. In the longer term it is hoped that such information will be useful in the search for improved therapeutics. Finally, gain-of-function for MAPK activation was demonstrated for 5 CaR mutations associated with ADH and cotreatment with the calcilytic NPSP795 substantially inhibited these responses confirming, *in vitro* at least, the potential utility of the calcilytics as a possible therapy in diseases of excess CaR activity.

6.3 Future studies

In future, it is important to determine PKCα, PKCε, and G_{12}, IGF1R, P70 S6Kinase and IκBα signalling in cells that endogenously express CaR. Apart from that, it is beneficial to study these signals in different cell types such as parathyroid cells and thick ascending limb cells to understand the different roles of this receptor in maintaining homeostasis. Moreover, the possible crosstalk between the receptor that increases cAMP levels in parathyroid cells and CaR should also be studied. Finally, the effectiveness of calcilytic NPSP795 on treating CaR-related diseases such as ADH should be clinically tested.

7.0 APPENDICES

Appendix I

RESEARCH ARTICLE

ASTHMA

Calcium-sensing receptor antagonists abrogate airway hyperresponsiveness and inflammation in allergic asthma

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Airway hyperresponsiveness and inflammation are fundamental hallmarks of allergic asthma that are accompanied by increases in certain polycations, such as eosinophil cationic protein. Levels of these cations in body fluids correlate with asthma severity. We show that polycations and elevated extracellular calcium activate the human recombinant and native calcium-sensing receptor (CaSR), leading to intracellular calcium mobilization, cyclic adenosine monophosphate breakdown, and p38 mitogen-activated protein kinase phosphorylation in airway smooth muscle (ASM) cells. These effects can be prevented by CaSR antagonists, termed calcilytics. Moreover, asthmatic patients and allergen-sensitized mice expressed more CaSR in ASMs than did their healthy counterparts. Indeed, polycations induced hyperreactivity in mouse bronchi, and this effect was prevented by calcilytics and absent in mice with CaSR ablation from ASM. Calcilytics also reduced airway hyperresponsiveness and inflammation in allergen-sensitized mice in vivo. These data show that a functional CaSR is up-regulated in asthmatic ASM and targeted by locally produced polycations to induce hyperresponsiveness and inflammation. Thus, calcilytics may represent effective asthma therapeutics.

INTRODUCTION

Despite substantial advances in our understanding of its pathophysiology and improved therapeutic regimens, asthma remains a tremendous worldwide health care burden with around 300 million individual sufferers. Although the symptoms of asthma are potentially controllable in most asthma sufferers using conventional therapy such as topical bronchodilators and corticosteroids, these are troublesome to administer efficiently and present unwanted side effects. There remains a significant minority of patients whose symptoms fail to be controlled with these approaches and who face chronically impaired quality of life with increased risk of hospital admission and even death, although in a minority such patients account for the major share of asthma health care costs. Accordingly, there is an urgent unmet need for identification of novel asthma therapies that target the root cause of the disease rather than its clinical sequelae.

Asthma is characterized by inflammation-driven exaggeration of airway narrowing in response to specific and nonspecific environmental stimuli [nonspecific airway hyperresponsiveness (AHR)], as well as chronic remodeling of the conducting airways (*1*). A number of mechanisms, many driven by inflammation, have been hypothesized to contribute to AHR and/or remodeling. Among these, there is increasing recognition that airway inflammation results in augmented local concentrations of polycations (*2*–*7*). The polycations eosinophil cationic protein (ECP) and major basic protein are well-established markers for asthma severity and stability, with some evidence that they may contribute directly to the pathogenesis of asthma (*6*, *8*–*10*). Furthermore, in asthma, increased arginase activity diverts L-arginine toward increased production of the polycations spermine, spermidine, and putrescine (*4*, *5*, *11*). Although in human peripheral blood monocytes spermine exhibits anti-inflammatory properties (*12*), associations between increases in polycations in the asthmatic airway mucosa and AHR/airway remodeling and inflammation (*4*, *5*, *13*) have long been apparent and ascribed to their positive charge (*9*). However, the cause-effect relationship remains hitherto unexplained. Here, we provide evidence that activation of the cell surface, G protein (heterotrimeric guanine nucleotide–binding protein)–coupled calcium-sensing receptor (CaSR) by polycations drives AHR and inflammation in allergic asthma.

The CaSR is the master controller of extracellular free ionized calcium ion (Ca^{2+}_o) concentration via the regulation of parathyroid hormone (PTH) secretion (*14*). Accordingly, CaSR-based therapeutics is used for the treatment of systemic disorders of mineral ion metabolism. Pharmacological activators of the CaSR (calcimimetics) are used to treat hyperparathyroidism, and negative allosteric modulators of the CaSR (calcilytics) are in clinical development for treating autosomal dominant hypocalcemia (*15*).

In addition to its pivotal role in divalent cation homeostasis, the CaSR is expressed in tissues not involved in mineral ion metabolism such as the blood vessels, breast, and placenta, where the CaSR regulates many fundamental processes including gene expression, ion channel activity, and cell fate (*16*). Furthermore, altered CaSR expression has also been associated with several pathological conditions including inflammation, vascular calcification, and certain cancers (*16*–*19*). In these noncalciotropic tissues, the CaSR responds to a range of stimuli including

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Appendix II

BASIC RESEARCH www.jasn.org

Pathophysiologic Changes in Extracellular pH Modulate Parathyroid Calcium-Sensing Receptor Activity and Secretion *via* a Histidine-Independent Mechanism

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ABSTRACT

The calcium-sensing receptor (CaR) modulates renal calcium reabsorption and parathyroid hormone (PTH) secretion and is involved in the etiology of secondary hyperparathyroidism in CKD. Supraphysiologic changes in extracellular pH (pH_o) modulate CaR responsiveness in HEK-293 (CaR-HEK) cells. Therefore, because acidosis and alkalosis are associated with altered PTH secretion *in vivo*, we examined whether pathophysiologic changes in pH_o can significantly alter CaR responsiveness in both heterologous and endogenous expression systems and whether this affects PTH secretion. In both CaR-HEK and isolated bovine parathyroid cells, decreasing pH_o from 7.4 to 7.2 rapidly inhibited CaR-induced intracellular calcium ($Ca^{2+}{}_i$) mobilization, whereas raising pH_o to 7.6 potentiated responsiveness to extracellular calcium ($Ca^{2+}{}_o$). Similar pH_o effects were observed for $Ca^{2+}{}_o$-induced extracellular signal-regulated kinase phosphorylation and actin polymerization and for L-Phe-induced $Ca^{2+}{}_i$ mobilization. Intracellular pH was unaffected by acute 0.4-unit pH_o changes, and the presence of physiologic albumin concentrations failed to attenuate the pH_o-mediated effects. None of the individual point mutations created at histidine or cysteine residues in the extracellular domain of CaR attenuated pH_o sensitivity. Finally, pathophysiologic pH_o elevation reversibly suppressed PTH secretion from perifused human parathyroid cells, and acidosis transiently increased PTH secretion. Therefore, pathophysiologic pH_o changes can modulate CaR responsiveness in HEK-293 and parathyroid cells independently of extracellular histidine residues. Specifically, pathophysiologic acidification inhibits CaR activity, thus permitting PTH secretion, whereas alkalinization potentiates CaR activity to suppress PTH secretion. These findings suggest that acid-base disturbances may affect the CaR-mediated control of parathyroid function and calcium metabolism *in vivo*.

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Mammalian calcium homeostasis is maintained by the regulated secretion of parathyroid hormone (PTH) and renal calcium reabsorption under the control of the calcium-sensing receptor (CaR).[1,2] Hypercalcemia stimulates the CaR to suppress PTH secretion, while chronic underactivation of CaR can contribute to pathologically elevated PTH secretion, such as in

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