# Studies on the phosphorylation of the $GABA_A$ receptor β subunits.

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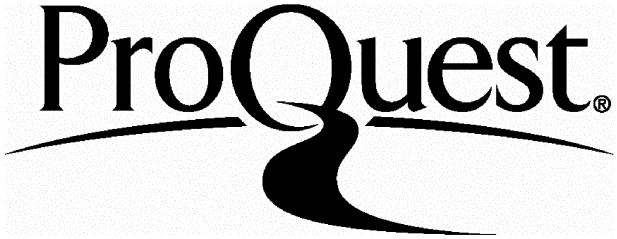

# ABSTRACT

$GABA_A$ receptor function has been shown to be modulated by protein serine/threonine and tyrosine kinases due to direct subunit phosphorylation. Phosphorylation sites within receptor subunits have been identified and their roles in receptor regulation have been determined. PKC phosphorylates S409 in the β1 subunit as well as S327 in the γ2S and both S327 and S343 in the γ2L subunit. Phosphorylation of any of these residues reduces GABA-induced currents through receptors containing α1, β1 and γ2 subunits. PKA only phosphorylates S409 of the β1 subunit in these receptors, reducing GABA-induced currents. This effect is abolished by mutation of S409 to alanine. Regulation of neuronal $GABA_A$ receptors is more complex, currents can be reduced, unaffected or enhanced by activation of PKA, depending on the neuronal preparation studied. The role of β subunit identity on receptor regulation was investigated as a possible mechanism for such differences in PKA regulation of receptor function.

Expression of subunit intracellular domains as GST-fusion proteins allowed identification of sites phosphorylated *in vitro* by protein serine/threonine kinases PKA, PKG, PKC and CamKII. This study extends earlier work on β1 and γ2 subunits and indicates that β2 and β3 subunits are phosphorylated at conserved sites (corresponding to β1-S409) by PKA, PKG, PKC and CamKII. Interestingly, PKC also phosphorylated S408 and CamKII phosphorylated S383 in the β3 subunit. The absence of S408 from both β1 and β2 subunits indicates potential for β subunit-dependent differential regulation of receptor function.

Purified GST-fusion proteins containing subunit intracellular domains were used to immunise rabbits and produce subunit-specific polyclonal antibodies. Antibodies were purified which specifically recognise β1 and β3 subunits by western blotting, immunocytochemistry and immunoprecipitation. These antibodies allowed study of receptor subunit phosphorylation in a heterologous expression system.

Receptor expression in HEK293 cells was used for biochemical and electrophysiological studies of β2 and β3 subunit phosphorylation by PKA. PKA did not phosphorylate the β2 subunit in these cells, accordingly PKA activation did not alter the function of receptors containing this subunit. The β3 subunit was highly phosphorylated by PKA on S408 and S409 and activation of PKA caused enhancement of GABA-induced currents through receptors containing this subunit. This enhancement was found to be critically dependent on both serine residues being phosphorylated within the same subunit. These results indicate a role for β subunit identity in the differential effects of PKA on $GABA_A$ receptor function in different neuronal cell types.

This thesis describes biochemical and electrophysiological studies of $GABA_A$ receptor regulation by protein phosphorylation. Using varied approaches, differential phosphorylation and regulation of receptor subtypes was investigated. The results presented identify a novel mechanism for the differential regulation of $GABA_A$ receptor subtypes in the brain. Furthermore, this work has resulted in the production of antibody reagents which should prove important in the investigation of $GABA_A$ receptor phosphorylation in the brain.

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Finally this thesis is dedicated to Tina Bridges for love and support in the demanding days of preparation and Joan McDonald whose support and belief made everything possible even in the darkest days.

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# ABBREVIATIONS

| | | |
|---|---|---|
| 3α-OH-DHP | - | 5α-pregnan-3α-ol-20-one |
| AKAP79 | - | 79kDa A-kinase anchoring protein |
| ATP | - | Adenosine 5'-triphosphate |
| BHK | - | Baby hamster kidney |
| BZD | - | Benzodiazepine |
| CamKII | - | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| cAMP | - | Cyclic adenosine 3':5'-monophosphate |
| cDNA | - | Complementary DNA |
| CNS | - | Central nervous system |
| DMSO | - | Dimethylsulphoxide |
| DNA | - | Deoxyribonucleic acid |
| EST | - | Expressed sequence tag |
| FITC | - | Fluoroscein isothiocyanate |
| GABA | - | γ-aminobutyric acid |
| $GABA_A$-R | - | Type A GABA receptor |
| $GABA_B$-R | - | Type B GABA receptor |
| $GABA_C$-R | - | Type C GABA receptor |
| Glu-R | - | Ionotropic glutamate receptor |
| Gly-R | - | Glycine receptor |
| GST | - | Glutathione-*S* -transferase |
| GTP | - | Guanosine 5'-triphosphate |
| HEK | - | Human embryonic kidney |
| $5HT_3$-R | - | 5-hydroxytryptamine type 3 receptor |
| IPTG | - | Isopropyl-β-D-thiogalactopyranoside |
| kDa | - | Kilodalton |
| MDCK | - | Madin Darby canine kidney |
| mRNA | - | Messenger RNA |
| MAP kinase | - | Mitogen activated protein kinase |

| | | |
|---|---|---|
| nAch-R | - | Nicotinic acetylcholine receptor |
| NMDA-R | - | N-methyl-D-aspartate receptor |
| PDBu | - | Phorbol 12:13-dibutyrate |
| PDGF-R | - | Platelet derived growth factor receptor |
| PKA | - | cAMP-dependent protein kinase |
| PKC | - | $Ca^{2+}$/phospholipid-dependent protein kinase (protein kinase C) |
| PKCI | - | PKC inhibitory peptide |
| PKG | - | cGMP-dependent protein kinase |
| PKIP | - | PKA inhibitory peptide |
| PKM | - | Trypsin-cleaved PKC (constitutively active) |
| $pp60^{c\text{-}src}$ | - | Cellular homolog of $pp60^{v\text{-}src}$ |
| $pp60^{v\text{-}src}$ | - | 60kDa transforming gene product of the Roux sarcoma virus |
| P2X-R | - | P2X receptor (ATP-gated ion channel) |
| RNA | - | Ribonucleic acid |
| SCG | - | Superior cervical ganglion |
| SDS | - | Sodium dodecyl sulphate |
| SDS-PAGE | - | SDS-polyacrylamide gel electrophoresis |
| SFV | - | Semliki forest virus |
| TM | - | Transmembrane domain |
| VIP | - | Vasoactive intestinal peptide |

# 1 INTRODUCTION

## 1.1 GABA is the major inhibitory neurotransmitter in the central nervous system

γ-Aminobutyric acid (GABA) has long been known to be present at high concentrations in the mammalian brain and in inhibitory neurons of crustacea (Roberts and Frankel, 1950; Awapara *et al.*, 1950; Kravitz, 1963). GABA was subsequently shown to inhibit electrical activity in the brain (Hayashi and Nagai, 1956), to mimic the postsynaptic effects of inhibitory axon stimulation in crustacean neuronal preparations (Kuffler and Edwards, 1958), to be taken up by a high affinity transporter (Neal and Iversen, 1969) and to be released in a calcium-dependent manner during inhibitory activity in the brain (Otsuka *et al.*, 1966). These observations together identified GABA as an inhibitory neurotransmitter, the later observation that voltage responses of cat cortical neurons to GABA application and inhibitory synaptic activity had similar reversal potentials (Krnjevic and Schwartz, 1967; Dreifuss *et al.*, 1969) indicated the importance of GABA in mediating central synaptic inhibition.

GABA is known to be the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Silvotti and Nistri, 1991) where it is synthesised from glutamic acid by the enzyme glutamic acid decarboxylase (Rando *et al.*, 1981). GABA has been shown to act at virtually all neurons of vertebrates (Dingledine *et al.*, 1988) and at 20-30% of all synapses (Bloom and Iversen, 1971). Central glial cells have also recently been shown to be sensitive to application of GABA (McVicar *et al.*, 1989; Barres, 1991) but the functional importance of this observation is not yet clear. GABA has also been shown to act as an inhibitory neurotransmitter in feline autonomic ganglia (De Groat,

1970) and also at some non-neuronal cells of the endocrine system (Rorsman *et al.*, 1989; Sorenson *et al.*, 1991). The enzymes involved in GABA synthesis and uptake are widely distributed in the autonomic nervous system and in many neuroendocrine, endocrine and exocrine cells (Erdö and Wolff, 1990). The existence of GABA-mediated links between the nervous and endocrine systems is suggested by the observation that some endocrine cells receive GABAergic innervation and show GABA responses similar to those seen in neurons of the CNS (Peters *et al.*, 1989).

Early studies identified the classical type A GABAergic ($GABA_A$) response which involves binding of two GABA molecules to a receptor complex (Sakmann *et al.*, 1983) leading to opening of an integral ion channel permeable to mainly chloride ions (Kuffler and Edwards, 1958). This current is activated by muscimol, blocked non-competitively by picrotoxin, antagonised by bicuculline and shows slow desensitisation in the continued presence of agonist (Nistri and Constanti, 1979). The $GABA_A$ response has an interesting and diverse pharmacology and is known to be allosterically modulated by a number of endogenous and exogenous compounds such as benzodiazepines, barbiturates and steroids. This GABA-gated $Cl^-$ current has a number of conductance levels between 10 and 30pS in rat and mouse central neurons, these include a main conductance level of 27-30pS and many subconductance levels (Kaila, 1994; Macdonald and Olsen, 1994). The measurement of single channel conductances has yielded various values in different cell types of the CNS suggesting varied receptor composition throughout the brain (Kaila, 1994). Molecular cloning studies have indicated that the $GABA_A$ receptor ($GABA_A$-R) is a hetero-oligomeric complex composed of numerous subunit polypeptides (Schofield *et al.*, 1987; Smith and Olsen, 1995). The identity of constituent subunits significantly

influences the pharmacological and physiological properties of the receptor (Smith and Olsen, 1995).

Later studies revealed the existence of a second, type B GABAergic ($GABA_B$) response in neurons. This response is insensitive to both bicuculline and muscimol and activated by baclofen. Activation of this response is caused by binding of GABA to a seven transmembrane spanning receptor which leads to activation of a heterotrimeric GTP-binding protein (G-protein) and modulation of the activity of adenylate cyclase. $GABA_B$ receptor ($GABA_B$-R) activation results in increased $K^+$ currents and/or reduced $Ca^{2+}$ currents causing hyperpolarisation of the membrane potential (Bowery *et al.*, 1980; Hill and Bowery, 1981; Dolphin, 1990; Gage, 1992). The $GABA_B$-R is widely expressed at very low levels within the CNS. Despite prolonged efforts this receptor has only recently been cloned by expression cloning, it is dissimilar to the classically studied seven transmembrane pass receptors such as the β-adrenergic receptor and rhodopsin but has been shown to share significant homology to metabotropic glutamate receptors (Kaupmann *et al.*, 1997).

Recently a third class of GABA response has been identified by molecular cloning (Enz *et al.*, 1995). These receptors were originally thought to contribute to $GABA_A$ responses but are now viewed as subunits of a separate class of GABA receptor. Unlike the $GABA_A$ response the $GABA_C$ receptors ($GABA_C$-Rs) are insensitive to bicuculline, are more sensitive to GABA than $GABA_A$-Rs and they show no desensitisation in its continued presence. $GABA_C$-R activation is characterised by opening of an integral chloride permeable ion channel which is similar to the $GABA_A$ response (Bormann and Feigenspan, 1995). These particular receptors are

believed to be homo-oligomers of ρ1 and ρ2 subunits which are expressed almost exclusively in the retina (Enz *et al.*, 1995; Enz and Bormann, 1995; Ogorusu *et al.*, 1995; Bormann and Feigenspan, 1995).

Together with performing a critical role in central synaptic inhibition in the adult animal, GABA has been shown to regulate the outgrowth of neurites, influence neuronal survival and act as a chemoattractant for migrating neurons in developing brain structures (Lipton and Kater, 1989; Meier *et al.*, 1991; Hansen *et al.*, 1987; Behar *et al.*, 1994). Independent studies have reported that $GABA_A$-R and NMDA-type glutamate receptor (NMDA-R) activition can regulate gene expression (Memo *et al.*, 1991; Fenelon and Herbison, 1996; Poulter *et al.*, 1997), however the mechanism underlying this effect is unclear. The inhibitory actions of type A GABAergic transmission are dependent on local chloride ion concentration gradients (reviewed by Kaila, 1994). Excitatory, depolarising $GABA_A$ currents have been reported in embryonic brain due to reversed chloride gradients and bicarbonate gradients (Staley *et al.*, 1995; Bonnet and Bingham, 1995) and these effects have been implicated in reduced DNA synthesis during neuronal differentiation (LoTurco *et al.*, 1995). In differentiating neurons depolarising type A GABAergic transmission has been shown to carry the majority of the excitatory current in developing neocortical structures. Such GABA-gated depolarisation could relieve voltage blockade of NMDA-Rs, allowing their activation followed by $Ca^{2+}$ influx, MAP kinase activation and regulation of gene transcription (Ben-Ari *et al.*, 1997; Xia *et al.*, 1996). These effects indicate that $GABA_A$ transmission may play an important role in nervous system development as well as synaptic transmission in the developed brain.

## 1.2 The $GABA_A$ response is subject to allosteric modulation

The concept of 'allosteric modulation' was adopted to explain the complex pharmacology of the $GABA_A$-R. This phenomenon involves the alteration of the $GABA_A$ response by simultaneous binding of another ligand at a separate site within the same receptor complex. The $GABA_A$ response is modulated in such a manner by many endogenous and exogenous ligands such as steroids (Majewska *et al.*, 1986), barbiturates (Study and Barker, 1981) and benzodiazepines (Haefely, 1984) as well as certain cations (Kaila, 1994) binding to distinct sites on the $GABA_A$-R (Puia *et al.*, 1990; Twyman and Macdonald, 1992).

Modulation of the $GABA_A$-R by benzodiazepines (BZDs) is of particular interest due to the widespread clinical use of compounds such as diazepam (valium) as sedatives and anxiolytic agents. Different classes of BZD have been shown to have different effects on $GABA_A$ currents. BZD 'agonists' (eg. diazepam) enhance the conductance of $Cl^-$ through activated receptors, 'inverse agonists' (eg. β-carbolines) reduce $Cl^-$ conductance and 'competitive antagonists' have no effect but block the effects of the other BZD classes (Richards *et al.*, 1986). None of these BZD types have any effects on the function of the $GABA_A$-R in the absence of GABA. The mechanism of diazepam modulation was thought to be due to an increase in receptor affinity for GABA at a low affinity binding site leading to an increase in the frequency of channel opening (Macdonald and Olsen, 1994). A recent report suggests that no increase in receptor affinity for GABA or frequency of opening occurs and that the current enhancing effect of diazepam is due to a large increase in the lowest conductance state of the activated receptor (Eghbali *et al.*, 1997).

Barbiturates (Study and Barker, 1981) and steroids such as 5α-pregnan-3α-ol-20-one (3α-OH-DHP) (Puia *et al.*, 1990; Hadingham *et al.*, 1993) have long been known to act at the $GABA_A$-R to enhance GABA-gated currents and have also been shown to directly activate receptors at high concentrations. Other steroid compounds have been shown to inhibit $GABA_A$ responses (Majewska *et al.*, 1990). The sedative and anaesthetic actions of barbiturates such as pentobarbital are thought to be mainly due to its actions at the $GABA_A$-R (Macdonald and Olsen, 1994). These compounds have been shown to enhance receptor function by causing an increase in channel open time with no change in the frequency of channel opening (Jackson *et al.*, 1982; Macdonald *et al.*, 1989; Porter *et al.*, 1992; Davies *et al.*, 1997a). Steroid enhancement of receptor function has been shown to be due to increased duration of channel opening together with an increase in the frequency of opening (Twyman and Macdonald, 1992).

Picrotoxin, a plant derived convulsant, is a non competitive antagonist of the $GABA_A$-R. This compound binds within the ion channel pore of the agonist bound receptor complex stabilizing the complex in its desensitised state (Smart and Constanti, 1986; Newland and Cull-Candy, 1992). The picrotoxin binding site has also been shown to be used by some γ-butyrolactone analogues which can have agonistic or antagonistic effects on the picrotoxin block without any intrinsic effects on $GABA_A$ currents (Whiting *et al.*, 1995).

Ethanol and other alcohols are known to regulate numerous ligand-gated ion channels and have been shown to potentiate the $GABA_A$ response (Dietrich *et al.*, 1989). Numerous general anaesthetic agents such as propofol, isoflurane, halothane and enflurane are also thought to potentiate $GABA_A$ responses at clinically relevant

concentrations (Harrison *et al.*, 1993; Franks and Lieb, 1994). The effects of ethanol in potentiating $GABA_A$ responses were thought to be due to an intracellular mechanism rather than direct binding to the receptor (Wafford *et al.*, 1991; Wafford and Whiting, 1992) while the effects of anaesthetic agents were believed to be due to a specific interaction with the receptor (Franks and Lieb, 1994). The argument that these effects may be due to direct interaction with the receptor is supported by a recent study which identified sites within $GABA_A$-R and glycine receptor (Gly-R) subunits which are required for functional modulation by ethanol and enflurane (Mihic *et al.*, 1997).

The function of $GABA_A$-Rs is also modulated by the action of other less well studied agents. The divalent zinc cation ($Zn^{2+}$) is concentrated by some central neurons and released during synaptic transmission leading to reduced $GABA_A$-R function (Smart, 1992; Smart and Constanti, 1990; Wooltorton *et al.*, 1997a). Loreclezole is an anticonvulsant drug which has been shown to potentiate the opening of $GABA_A$-Rs by binding to an unknown site within the receptor complex (Wafford *et al.*, 1994). A number of other neuroactive agents such as lanthanum ($La^{3+}$), the antihelminthic agent ivermectin, nonsteroidal anti-inflammatory drugs and penicillin are now also known to act at the $GABA_A$-R modulating its function (Whiting *et al.*, 1995).

## 1.3 Molecular cloning shows the $GABA_A$-R belongs to a superfamily of ligand-gated ion channels

The use of benzodiazepine-affinity chromatography to purify intact $GABA_A$-Rs to homogeneity from bovine cerebral cortex (Sigel and Barnard, 1984) allowed tryptic peptide micro-sequencing and cloning of cDNAs encoding α and β receptor subunits from a bovine brain cDNA library using degenerate oligonucleotides (Schofield *et al.*,

1987). The identity of these cDNAs as $GABA_A$-R subunits was confirmed by coexpression of *in vitro* transcribed mRNA in *Xenopus laevis* oocytes. This technique generated functional $GABA_A$-Rs with many of the characteristics of receptors which were observed on expression of total brain RNA in the same system (Schofield *et al.*, 1987). Importantly, benzodiazepine modulation was absent from recombinant receptors composed of these cloned subunits (Schofield *et al.*, 1987) indicating that a further component or subunit required to reconstitute the native receptor was still unidentified. No functional GABA-gated channels were observed on individiual expression of either subunit, indicating that the $GABA_A$-R is a hetero-oligomeric complex (Schofield *et al.*, 1987).

Sequence analysis of the cloned subunits indicated that they shared a common subunit structure including a signal sequence, a large extracellular amino-terminal domain, four putative transmembrane domains (TM1-TM4) and a large intracellular domain between the third and fourth transmembrane domains (Figure 1A). Similarity of sequence and structural determinants led to the proposal that the $GABA_A$-R and the nicotinic acetylcholine receptor (nAch-R) were members of a superfamily of ligand-gated ion channels (Barnard *et al.*, 1987). This superfamily is now believed to include the $GABA_A$-R, the nAch-R, the Gly-R and the 5-hydroxytryptamine type 3 receptor ($5HT_3$-R). The subunits of this receptor family have similar sequences and distributions of hydrophobic, membrane spanning domains and are thought to be structurally homologous. In addition they all contain two cysteine residues (Cys) which are 13 amino acids apart in their extracellular N-terminal domains which have been shown to form a loop by disulphide linkage in the nAch-R (Kao and Karlin, 1986). This feature distinguishes members of this family from other chemically-gated ion channels such as the ionotropic glutamate

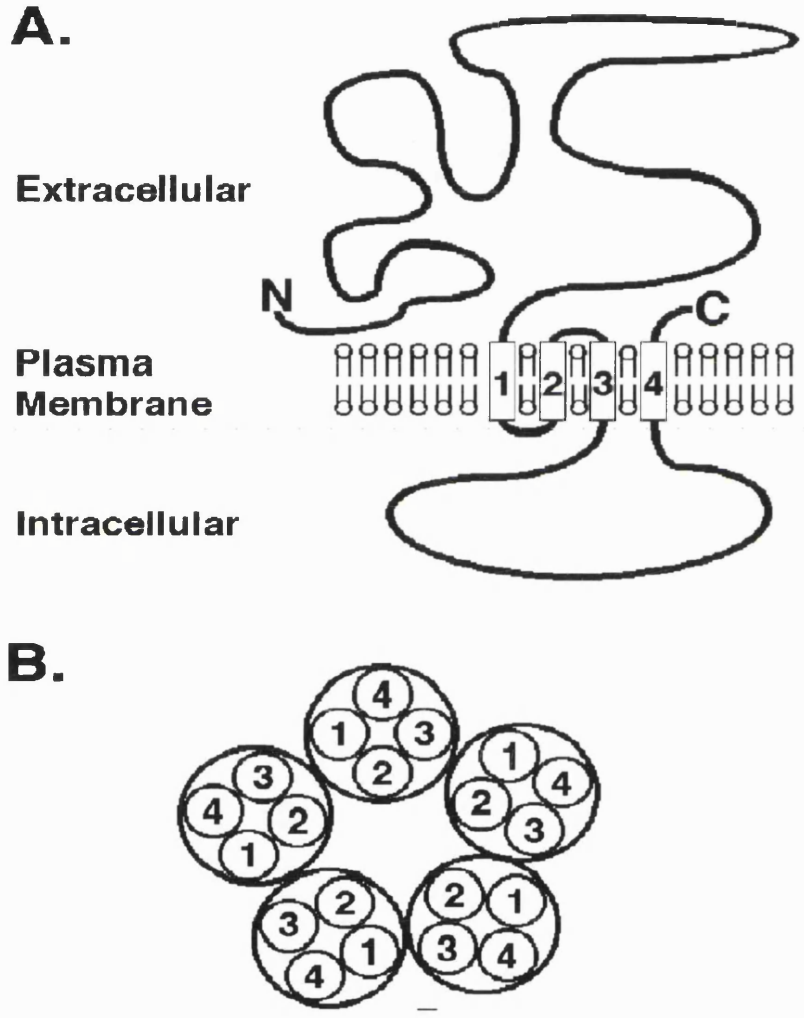
receptors (Glu-R), purinergic P2X receptors (P2X-R) or cyclic-nucleotide gated channels (Karlin and Akabas, 1995).

The prototypical member of this family is the muscle nAch-R which has been extensively studied. This receptor is present at the neuromuscular junction where it mediates depolarisation of the postsynaptic membrane on binding of acetylcholine. Due to its enrichment in the electric organs of the electric fish *Torpedo californica* and *Electrophorus electrica* , the nAch-R has been isolated and purified in semi-crystalline arrays which have permitted detailed structural characterisation of the receptor to a resolution of 9Å (Unwin, 1993; Unwin, 1995). The muscle nAch-R is a 250 kDa pentameric complex containing four different subunits in the stoichiometry $\alpha 2$-$\beta$-$\gamma$-$\delta$, arranged in specific order around a central aqueous pore through which permeant ions pass on receptor activation (Changeux, 1990).

Attempts to elucidate the structure of the $GABA_A$-R complex directly have been restricted due to their relatively low abundance and large heterogeneity in brain tissue. However, a similar structure has been implied for $GABA_A$-R and nAch-R subunits on the basis of sequence homology, as has the overall pentameric organisation of subunits (Figure 1B). Evidence supporting this direct comparison of subunit structure has come from many different studies of native and recombinant receptors. Ligand-binding studies in combination with site-directed mutagenesis and predictions of secondary structure indicate that ligand binding domains may be derived from structures conserved within the subunits of all superfamily members (Smith and Olsen, 1995). The extracellular location of subunit N-termini is supported by the presence of signal peptide sequences (Schofield *et al.*, 1987), identification of amino acids involved in extracellular

**Figure 1**

Proposed structure of the $GABA_A$ receptor




**A.** Subunit transmembrane topology including extracellular N-terminus, four putative transmembrane (TM) domains (1-4) and a large intracellular domain between TM3 and TM4. **B.** The proposed pentameric arrangement of subunits within the receptor complex indicating the central channel lumen lined by subunit TM2 domains.

ligand binding (Smith and Olsen, 1995), the presence of potential sites of *N*-glycosylation (Schofield *et al.*, 1987; Buller *et al.*, 1994) and the extracellular localisation of immunofluorescence staining in neurons and heterologous cell expression systems by antibodies directed against subunit N-terminal peptide sequences or N-terminal epitope tags (Benke *et al.*, 1994; Connolly *et al.*, 1996a; Connolly *et al.*, 1996b). Ion substitution experiments indicate that the aqueous pore of the $GABA_A$ , Gly and nAch receptors are of similar diameters (6Å, 5Å and 7-8Å respectively) (Karlin and Akabas, 1995; Bormann *et al.*, 1987). The use of noncompetitive receptor inhibitors or the substituted-cysteine accessibility method have identified residues within M2 as lining the channel pore of the nAch, Gly and $GABA_A$ receptors (Xu and Akabas 1993; Karlin and Akabas, 1995). The intracellular location of the domain between M3 and M4 has been shown directly by immunofluorescence using antibodies raised against recombinant protein fragments and indirectly by demonstration of phosphorylation within this domain by intracellular protein kinases (Moss and Smart, 1996). Heterologous expression of recombinant $GABA_A$-R subunits is currently being used to study the structure of the $GABA_A$-R in more detail.

## 1.4 Further cloning studies show $GABA_A$-R subunit heterogeneity

Original biochemical and pharmacological experiments provided evidence of significant $GABA_A$-R heterogeneity in the brain. Photoaffinity labelling experiments had indicated the presence of two types of subunit within the $GABA_A$-R, the α subunit (Mr ~53K) containing the BZD binding site and the β subunit (Mr ~57K) containing the GABA binding site (Casalotti *et al.*, 1986; Mamalaki *et*

*al.*, 1987). Further pharmacological studies of native $GABA_A$-Rs indicated that they were not a homogeneous population within the brain. BZD binding sites in brain could be classified as type I or type II based on their affinities for different BZD compounds (Squires *et al.*, 1979) indicating possible heterogeneity of receptor complexes. [$^3$H]-flunitrazepam photoaffinity labelling experiments followed by SDS-PAGE analysis showed several labelled polypeptides (Sieghart and Drexler, 1983) which could also be interpreted as indicating receptor subunit heterogeneity.

Further study of subunit cDNAs isolated by Schofield *et al.* identified a second α subunit (α2) which showed 79% identity to the first α subunit (α1) but only 34% identity to the β subunit (Levitan *et al.*, 1988). This provided the first direct evidence of subunit heterogeneity of the $GABA_A$-R. Since that time a large number of distinct receptor subunits (α1-6, β1-4, γ1-4, δ and ε) have been identified by traditional molecular cloning techniques (Stephenson, 1995) and by sequence similarity searching of expressed sequence tag (EST) databases (Davies *et al.*, 1997b). These subunits are classed together in subtypes (eg. α) on the basis of sequence homology, members of a subunit subtype share 70-80% sequence identity while members of different subtypes share only 30-40% sequence identity (Smith and Olsen, 1995). Of the 16 subunits cloned to date 14 are expressed in the mammalian CNS, the β4 and γ4 subunits expressed only in the chicken where they are thought to replace the β1 and γ3 subunits respectively.

Alternative splicing of $GABA_A$-R subunit mRNA has been shown to further increase receptor heterogeneity. Within the $GABA_A$-R gene family four subunit transcripts have been shown to be alternatively spliced to yield long and short subunit polypeptides. The α6 subunit

is alternatively spliced to produce a variant which has ten amino acids removed from its N-terminal domain and is unable to form functional receptors with other subunits (Korpi *et al.*, 1994). This variant results from splicing at a 3' splice site within an exon and has no known physiological role in $GABA_A$-R function. The chicken β2 subunit is expressed as a long or short form which differ by the inclusion of an additional exon. The presence of this exon results in inclusion of an extra 17 amino acids and a potential site for phosphorylation by protein kinase C (PKC) within the major intracellular loop of this subunit (Harvey *et al.*, 1994). Similarly, the human β2 subunit is expressed as long and short forms differing by the presence of an extra 38 amino acids in the intracellular loop (McKinley *et al.*, 1995). This alternatively spliced region contains concensus sequences for phosphorylation by cAMP-dependent protein kinase (PKA), $Ca^{2+}$/calmodulin-dependent protein kinase II (CamKII) and PKC (McKinley *et al.*, 1995). The chicken β4 subunit is also alternatively spliced resulting in an extra 4 amino acids within the major intracellular loop (Bateson *et al.*, 1991). The functional significance of this splice variant remains unknown. The γ2 subunit exists in two forms γ2-long (γ2L) and γ2-short (γ2S) resulting from inclusion of an extra exon in γ2L transcripts. This splice difference results in an eight amino acid insertion containing a potential PKC phosphorylation site, within the major intracellular loop of the γ2 L subunit (Whiting *et al.*, 1990; Kofuji *et al.*, 1991). The large number of identified subunits and alternatively spliced variants indicates great potential for receptor heterogeneity within the mammalian brain.

## 1.5 Analysis of subunit contributions to native $GABA_A$-R subtypes in the central nervous system

Given the diversity of receptor subunits found in brain an interesting question arises regarding the composition of native $GABA_A$-Rs. If a receptor complex is composed of a pentameric arrangement of subunits with no restriction on stoichiometry or interactions between subunits, then the 14 subunits (excluding the β4 and γ4 subunits found only in the chicken) have the potential to form $14^5$ (537,824) different receptor subtypes. Recombinant receptor studies have shown that at least one α, one β and one γ subunit are required for the production of functional recombinant receptors (Pritchett *et al.*, 1989b). Therefore the possible number of receptor combinations is reduced to 10,584 (6 α x 3 β x 3 γ x 14 any x 14 any). This number presumes that all subunits are present so that combinations are not ruled out due to restriction of subunit expression. Subunit specific contributions to the GABA (α and β) and benzodiazepine (α and γ) binding sites (Smith and Olsen, 1995) suggest that subunits occupy specific positions relative to each other in the receptor complex. It has also been shown that subunit expression varies between cell types with some neurons expressing most and some expressing very few subunits (Wisden *et al.*, 1992). Given these facts it seems likely that the potential number of receptor subtypes *in vivo* is much less than 10,584. Numerous studies have attempted to define and estimate receptor heterogeneity in the brain and there are now thought to be less than twenty major $GABA_A$-R subtypes present in the brain (Whiting *et al.*, 1995).

*In situ* hybridisation analysis has been used to define the repertoire of subunits expressed in brain regions and specific cell types. Expression of subunits has been shown to be regulated both spatially

and temporally in the developing rat brain (Wisden *et al.*, 1992; Laurie *et al.*, 1992a; Laurie *et al.*, 1992b). Expression of each α subunit is distinct, each being present at high levels in different brain regions (Wisden *et al.*, 1992). In a similar manner the expression of β and γ subunits each show unique profiles within the brain (Wisden *et al.*, 1992). The expression of individual subunits also changes dramatically during development (Laurie *et al.*, 1992b) indicating a changing role for $GABA_A$-Rs during neuronal maturation. These findings strongly support the idea of distinct receptor subtypes existing in specific brain regions and allow preliminary predictions of subtype composition in specific regions. However numerous subunits can be expressed within individual neuronal populations, for example the granule cells of rat dentate gyrus express all receptor subunits except α6 and ε (Wisden *et al.*, 1992; Davies *et al.*, 1997b). In this case the identification of expressed subunits provides little help in determining which receptor subunits can be incorporated into individual receptor complexes.

The relative abundance of individual subunits has been estimated by measuring the percentage of [$^3$H]-muscimol and [$^3$H]-benzodiazepine binding sites immunoprecipitated from whole brain and cerebellum by subunit-specific antisera. Such studies confirm the subunit expression patterns determined by *in situ* hybridisation (Whiting *et al.*, 1995). Immunoprecipitation followed by western blotting has been used to demonstrate interactions between subunits, thereby demonstrating their coexistence in individual solubilised receptor complexes. Using this approach the α subunit pairs α1-α2, α1-α3, α2-α3 and α1-α6 have been reported in single receptor complexes (Khan *et al.*, 1994; Whiting *et al.*, 1995). Using a similar experimental approach Li and DeBlas have recently inferred the coexistence of the β subunit pairs β1-β3 and β2-β3 in 19% and 33% respectively, of

receptors immunoprecipitated from rat cerebral cortex (Li and DeBlas, 1997). To date there have been no reports of homotypic interactions between γ subunits, they are however coprecipitated with both α and β subunits (Connolly *et al.*, 1996a; Tretter *et al.*, 1997). Certain neuronal cell types, such as cerebellar granule cells, express only a small number of subunits (Wisden *et al.*, 1992) which are believed to form only a small number of receptor subtypes (Thompson and Stephenson, 1994) and have therefore been subject to detailed investigation. These cells are believed to express receptors containing the α6-β2/3-γ2, α6-α1-β2/3-γ2 and α6-β2/3-δ subunit combinations (Whiting *et al.*, 1995) but the exact subunit combinations of these receptors remain unclear. Studies aiming to visualise subunit colocalisation by immunofluorescence or electron microscopy have shown specific subunits within defined neurons (Fritschy *et al.*, 1992; Nusser *et al.*, 1996), however this approach merely confirms the evidence provided by *in situ* hybridisation studies and provides no direct evidence of subunit interactions to form functional receptors. Despite numerous efforts combining many pharmacological and immunochemical techniques the exact subunit composition of a single native receptor population remains unknown.

## 1.6 Subunit composition of $GABA_A$-Rs as determined by the study of recombinant receptors

It has been reported that homomeric expression of α1 or β2 subunits in HEK293 cells cannot produce functional receptors (Connolly *et al.*, 1996a; Connolly *et al.*, 1996b), yet other investigators have seen functional channels on expression of α1 or β2 subunits alone (Blair *et al.*, 1988; Pritchett *et al.*, 1988). These discrepancies may be due to differences in the origin of the subunits or perhaps to endogenous receptor subunits expressed within the cells used to conduct these

studies (Ueno *et al.*, 1996). The β1 and β3 subunits however can form homomeric receptor channels at the surface of these cells (Sigel *et al.*, 1989; Connolly *et al.*, 1996b; Krishek *et al.*, 1996). However, electrophysiological and cell biological studies of receptor function have confirmed that any combination of α , β and γ subunits can give rise to functional receptors (Connolly *et al.*, 1996a; Pritchett *et al.*, 1989a), the ratios of subunits within the receptor complex however remains unclear. Given the technical difficulties experienced in the determination of subunit stoichiometry of native $GABA_A$-Rs, recombinant expression systems coupled to many other techniques have been used to study the subunit stoichiometry of $GABA_A$-Rs of known composition.

An electrophysiological study using mutated α3, β2 and γ2 subunits exhibiting outward rectification of GABA-induced currents provided support for the postulated pentameric assembly of subunits within the functional receptor and determined that the most likely subunit stoichiometries within these receptors was 2α1β2γ with the combinations 2α2β1γ and 1α2β2γ also being possible (Backus *et al.*, 1993). Backus *et al* also discounted the possibility of three copies of any subunit type being present in a functional receptor. Another study was done using expression of a tandem construct containing the α6 subunit joined at its C-terminus via a ten glutamine residue linker to the N-terminus of the β2 subunit. When expressed alone or in combination with single α6, β2 or γ2 subunits the authors concluded that only the combinations 2α2β1γ or 3α2β were possible (Im *et al.*, 1995). The importance of these observations remains unclear as the reciprocal tandem β2-α6 subunit construct has not been described and C terminal/N terminal subunit interactions may not reproduce the subunit interactions found in native receptors. A recent study using benzodiazepine affinity purification of α1β3γ2 and

$\alpha 1\beta 3$ receptors from HEK293 cells, followed by quantitative western blotting reports stoichiometries of $2\alpha 2\beta 1\gamma$ and $2\alpha 3\beta$ for these receptors respectively (Tretter *et al.*, 1997). Analysis of receptors composed of mutated $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits which exhibit reduced GABA sensitivities has also suggested the predominant stoichiometry to be $2\alpha 2\beta 1\gamma$ (Chang et al., 1996). Tretter *et al* also suggest the possibility that tetrameric receptors composed of $2\alpha$ and $2\beta$ subunits may exist. Interestingly, another study examining receptors composed of $\alpha 1$ and $\beta 1$ subunits using the semliki forest virus (SFV) expression system in baby hamster kidney (BHK) cells and rat superior cervical ganglion neurons indicated that these receptors may also exist as tetrameric complexes composed of $2\alpha$ and $2\beta$ subunits (Gorrie *et al.*, 1997). The different conclusions drawn by these investigators may be due to different assembly of the different subunit combinations used, to differences in the cell types used, to differences caused by expression systems or to some alteration of channel formation or function when subunits are expressed as linear multimers. A recent report has identified amino acid residues in the N-terminus of the $GABA_C$-R $\rho 1$ subunit which are required for homotypic subunit oligomerisation and receptor formation (Hackam *et al.*, 1997). Using different methodologies investigators have identified domains involved in oligomerisation of other ion channels such as the inwardly rectifying potassium channels (IRK1: Tinker *et al.*, 1996). The presence of such domains in $GABA_A$-R subunits remains speculative, however it seems likely that receptor subunits interact in a subunit specific manner. This view is supported by the elimination of $\delta$ subunit expression in cerebellar granule cells of mice containing a truncated, non-functional $\alpha 6$ subunit (Jones *et al.*, 1997). Identification of oligomerisation signals in $GABA_A$-R subunits would allow the prediction of subunit interactions which could then be

tested in recombinant systems. Given the wide range of subunits expressed in individual cell populations within the brain, the determination of the most favourable subunit combinations could prove important in the dissection of the receptor subtypes expressed by specific neurons.

## 1.7 Targeting of $GABA_A$-Rs to the cell surface is determined by subunit composition

The demonstration of the $GABA_A$-R as a hetero-oligomeric complex indicates that its varied biophysical characteristics and complicated pharmacology may be due to the subunit heterogeneity which has been revealed. It is now widely accepted that coexpression of $\alpha$, $\beta$ and $\gamma$ subunits are required for production of $GABA_A$-Rs with pharmacological and biophysical characteristics of native receptor subtypes found in the brain (Macdonald and Olsen, 1994; Barnard, 1995). However, cell surface expression of a $GABA_A$-R is a prerequisite for its regulation of neuronal excitability. Recent studies have aimed to identify subunit-specific determinants required for receptor cell surface expression and which regulate their targeting to specific locations of the cell surface.

Expression of single subunits in mammalian cell lines has produced contradictory reports of functional GABA-gated channels (Blair *et al.*, 1988; Pritchett *et al.*, 1988) or no channel function (Sigel *et al.*, 1990; Angelotti *et al.*, 1993a; Krishek *et al.*, 1994). Similarly, expression of $\alpha$-$\gamma$ or $\beta$-$\gamma$ subunit combinations have produced functional channels in some reports (Verdoorn *et al.*, 1990; Draguhn *et al.*, 1990) but not in others (Sigel *et al.*, 1990; Angelotti *et al.*, 1993a; Krishek *et al.*, 1994). Despite these differences it is widely accepted that expression of $\alpha$-$\beta$ subunit combinations produces GABA-gated channels at the cell

surface and that the presence of the γ2 or γ3 subunits render these channels sensitive to benzodiazepine modulation (Burt and Kamatchi, 1991). Recent work examining the subcellular distribution of α1, β2 and γ2L subunits expressed together and alone in HEK293 cells and utilising the 9E10 and FLAG epitope tags has shown that only α1-β2 and α1-β2-γ2L subunit combinations were observed at the cell surface (Connolly *et al.*, 1996a) leading to the conclusion that expression of α and β subunits is a minimal requirement for the efficient production of cell surface receptors. Another recent study has shown that insulin stimulation increases $GABA_A$-R number on the cell surface in a β subunit dependent manner (Wan *et al.*, 1997b). This suggests that neurons may contain intracellular pools of $GABA_A$-Rs which can be moved to the cell surface in response to certain stimuli.

Once the ability of a receptor to access the cell surface is determined the final localisation of $GABA_A$-Rs on the axonal or dendritic surface is likely to influence their role in neuronal function. A number of different systems have been used to investigate the role of subunit identity in determining the final localisation of functional receptors. One method has utilised the expression of recombinant subunits in the Madin Darby canine kidney (MDCK) cell line. MDCK cells are polarised epithelial cells whose surface is divided into distinct apical and basolateral domains. These cells are thought to serve as a model of neuronal polarity (Dotti *et al.*, 1991; De Hoop *et al.*, 1995). Contradictory results have been found using this system with one report showing that the β subunit identity determines targeting of the receptor (Connolly *et al.*, 1996b), while an earlier study in this same system had indicated that the α subunit identity fulfills this function (Perez-Velazquez and Angelides, 1993). However, the methods used to determine the final location of receptor subunits

within polarised cells in the latter report remains open to question as cells were permeabilised during detection of cell surface receptors (Perez-Velazquez and Angelides, 1993). Another approach has been the use of immuno-electron microscopic and electrophysiological analysis of serial sections through the rat hippocampus (Nusser *et al.*, 1996). This report indicated that within a single neuron $GABA_A$-Rs were differentially localised to postsynaptic sites innervated by distinct pathways in an α subunit dependent fashion. This report did not rule out the possibility that the different α subunits they identified could have been partnered by different β subunits in these cells.

## 1.8 $GABA_A$-R function is regulated by protein phosphorylation

Studies of native receptors as well as the newly cloned $GABA_A$-R subunits indicated that receptor function was regulated by unidentified 'phosphorylation factors' (Stelzer *et al.*, 1988; Gyenes *et al.*, 1988). Protein phosphorylation has been shown to be a major mechanism used for signalling in eukaryotic cells (Edelman *et al.*, 1987; Cohen, 1989). This process involves the transfer of a terminal phosphate group from adenosine triphosphate (ATP) to the hydroxyl group of serine, threonine or tyrosine residues in a substrate protein, causing modification of protein charge, conformation and function (Edelman *et al.*, 1987). This process is catalysed by a wide variety of enzymes called protein kinases whose activity is tightly regulated by numerous intracellular signalling mechanisms. These enzymes are divided on the basis of their substrate specificity into two broad classes the protein tyrosine kinases and the protein serine/threonine kinases (Edelman *et al.*, 1987; Hunter and Cooper, 1985).

Protein tyrosine kinases were first identified as viral gene products causing oncogenic transformation of eukaryotic cells. The observation of significant amounts of phosphotyrosine in untransformed cells indicated a role for these kinases in normal cellular function. These observations were followed by the identification of such kinases expressed in cells and elucidation of some roles in cellular function, particularly in cell proliferation and movement. Two broad classes of protein tyrosine kinases have been identified in mammalian cells, these are receptor tyrosine kinases and non-receptor tyrosine kinases (Fantl *et al.*, 1993; Hunter and Cooper, 1985). Members of each of these classes are present at high levels in adult mammalian neurons (Cotton and Brugge, 1983; Huganir, 1987) indicating their potential involvement in regulation of synaptic activity.

The protein serine/threonine kinases are also a diverse group of enzymes which can be subdivided into numerous types the best characterised of which are the second-messenger dependent protein kinases (Schulman, 1991). The activity of these enzymes is regulated by the local concentration of diffusible 'second-messenger' compounds. Second messengers are generated by specific signalling events initiated in many instances by the binding of hormone and neurotransmitter ligands to their receptors (Berridge, 1984; Gilman, 1984). The best studied of these kinases are cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), $Ca^{2+}$/phospholipid-dependent protein kinase (protein kinase C/PKC) and $Ca^{2+}$/calmodulin-dependent protein kinase II (CamKII). Binding of the appropriate second-messenger to a regulatory domain or subunit in each of these kinase complexes leads to activation of the protein kinase by dissociation of inhibitory regulatory subunits, by conformational change causing exposition of a catalytic domain and/or by stimulation of kinase autophosphorylation. Each of the

second messenger-activated serine/threonine kinases has been shown to be expressed abundantly in mammalian neurons (Schulman, 1991). Notably CamKII is highly concentrated in postsynaptic densities and is believed to be the most abundant kinase of its type in the brain where it can account for up to 0.4% of total protein in certain regions (Bennett *et al.*, 1983; McGuinness *et al.*, 1983). These observations suggest a role for protein kinases in regulation of synaptic activity.

The covalent modification of protein substrates by protein kinases is reversed by another group of important enzymes termed the protein phosphatases (Cohen, 1989). Intracellular signalling involving the transfer of phosphate groups to specific proteins is therefore a dynamic process involving a balance between phosphorylation (kinases) and dephosphorylation (phosphatases).

The effects of protein phosphorylation on native $GABA_A$-Rs are complex producing a wide variety of functional effects (Moss and Smart, 1996). Electrophysiological, biochemical and molecular biological analysis of recombinant receptors allows detailed study of these effects.

## 1.9 $GABA_A$-R subunits contain concensus sequences for protein phosphorylation

The determination of concensus sequences for protein kinase phosphoryation allows analysis of primary amino acid sequences to determine the likelihood of their being covalently modified on serine, threonine or tyrosine residues (Kennelly and Krebs, 1991; Songyang *et al.*, 1994; Songyang *et al.*, 1995). Analysis of $GABA_A$-R subunit sequences showed that they contain such concensus sequences for phosphorylation by a number of protein kinases within their major

intracellular domains (Table 1) (Schofield *et al.*, 1987; Moss and Smart, 1996). This approach is only useful as a guide as the presence of such sequences takes no account of protein secondary or tertiary structures which will influence the availability of candidate amino acid residues to protein kinases. Therefore sites phosphorylated by protein kinases must be determined experimentally.

Numerous studies have shown that affinity or immuno-purified $GABA_A$-R subunits from brain are phosphorylated *in vitro* by purified protein kinases. PKA and PKC both phosphorylate presumed β subunits with apparent molecular masses of 53-57 kDa as determined by SDS-PAGE (Kirkness *et al.*, 1989; Browning *et al.*, 1990; Tehrani and Barnes, 1994). Inhibition of phosphorylation by anti-β subunit antibodies indicates that this phosphorylation does occur mainly on β subunits (Browning *et al.*, 1993). A presumed α subunit (Mr ~51 kDa) copurifies with and is phosphorylated by an unknown protein kinase which is not stimulated by cyclic nucleotides or phorbol esters (Sweetnam *et al.*, 1988; Bureau and Laschet, 1995). Similarly, purified receptors are phosphorylated by the protein tyrosine kinase $pp60^{vsrc}$ on presumed β and γ subunits as determined by SDS-PAGE (Valenzuela *et al.*, 1995). Due to the heterogeneous nature of native receptor preparations and the relatively low abundance of $GABA_A$-Rs in the brain, the identity of subunits phosphorylated in these studies remains unclear.

## Table 1

Concensus sequences for protein phosphorylation within the intracellular domains of $GABA_A$-R subunits

| Subunit | Sequence* | Kinases$ |
|---|---|---|
| β1 | $^{405}$RRRA**S**QLK$^{412}$ | PKA, PKG, PKC, CamKII |
| β2 | $^{406}$RRRA**S**QLK$^{413}$ | PKA, PKG, PKC, CamKII |
| β3 | $^{405}$RRRS**S**QLK$^{412}$ | PKA, PKG, PKC, CamKII |
| γ1 | $^{369}$EDD**Y**G**Y**QC$^{377}$ | PDGF-R, *src* |
| γ2L/γ2S | $^{324}$RKP**S**KDKD$^{331}$ | PKA, PKC, CamKII |
| γ2L/γ2S @ | $^{352}$IRPR**S**ATI$^{359}$ | PKA, PKG, CamKII |
| γ2L/γ2S @ | $^{362}$DEE**Y**G**Y**EC$^{369}$ | PDGF-R, *src* |
| γ2L | $^{338}$LLRMF**S**FK$^{345}$ | PKA, PKC, CamKII |
| γ3 | $^{330}$RKP**T**IRKK$^{337}$ | PKA, PKG, PKC, CamKII |
| α6 | $^{371}$KKRI**SS**LT$^{378}$ | PKA,PKG,CamKII |

* While amino acids are numbered according to published sequences of rat subunits, these concensus sequences are conserved between human, rat, murine and bovine receptors. Putative phosphorylation sites are indicated in bold type and underlined.

@ Amino acid numbering refers to the published sequence for the murine γ2L subunit.

$ PKA: cAMP-dependent protein kinase; PKG: cGMP-dependent protein kinase; PKC: $Ca^{2+}$/phospholipid-dependent protein kinase (protein kinase C); CamKII: $Ca^{2+}$/Calmodulin-dependent protein kinase type II; PDGF-R: Platelet-derived growth factor receptor and src: $pp60^{vsrc}$ of Roux Sarcoma Virus.

## 1.10 Identification of phosphorylation sites within $GABA_A$-R subunits

Recombinant subunit proteins have been used to overcome some of the difficulties associated with identification of phosphorylation sites within native receptors. Receptor intracellular domains have been expressed as soluble glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* which allows their purification in large quantities (Smith and Johnson, 1988). These purified proteins have been used as *in vitro* kinase substrates to identify high affinity kinase phosphorylation sites within receptor subunits. Given the requirement of $\alpha$, $\beta$ and $\gamma$ subunits for the production of functional $GABA_A$-Rs, the best studied subunits to date are the $\alpha 1$, $\beta 1$ and $\gamma 2$.

Combining this approach with site-directed mutagenesis, the $\beta 1$ subunit has been shown to be phosphorylated by PKA, PKG, PKC and CamKII at Serine 409 [S409](Moss *et al.*, 1992a; McDonald and Moss, 1994) while a second high affinity site for CamKII phosphorylation within this subunit has been identified at S383 (McDonald and Moss, 1994). The $\beta 1$ subunit has also been shown to be phosphorylated by v-*src* by a similar method, however the sites of phosphorylation were not identified (Valenzuela *et al.*, 1995). Similar experimental approaches have shown the $\gamma 2$ subunit to be phosphorylated *in vitro* by PKC, CamKII and v-*src* . Both long and short versions of this subunit ($\gamma 2L$ and $\gamma 2S$ respectively) are phosphorylated by PKC on S327 and by CamKII on S348 and T350 (Moss *et al.*, 1992a; McDonald and Moss, 1994). The eight amino acid insertion contained in the $\gamma 2S$ subunit alone contains another high affinity site for protein phosphorylation. This site at S343 is phosphorylated by both PKC and CamKII and suggests that receptors containing $\gamma 2S$ and $\gamma 2L$ subunits may be regulated differently (Whiting *et al.*, 1990; Moss *et al.*, 1992a; Machu *et al.*, 1993a; McDonald and Moss, 1994). The $\gamma 2L$ subunit has

also been shown to be phosphorylated by v-*src in vitro*, however sites of phosphorylation were not identified in this study (Valenzuela *et al.*, 1995).

Experimental analysis has failed to show significant phosphorylation of the α1 or α6 subunits by PKA, PKG, PKC, CamKII or v-src (Moss *et al.*, 1992a; McDonald and Moss, 1994). These *in vitro* data suggest a role for the β1 and γ2 subunits in the regulation of $GABA_A$-R function by numerous intracellular signalling pathways. Heterologous expression studies of various receptor subunit combinations have been used to determine the *in vivo* importance of receptor phosphorylation. HEK293 cells transiently expressing wildtype and mutant receptor subunits were labelled with [$^{32}$P]-orthophosphoric acid and specific protein kinases are activated. Receptor complexes are then immunoprecipitated and phosphorylated subunits resolved by SDS-PAGE.

Receptors composed of α1β1 or α1β1γ2 subunit combinations are specifically phosphorylated by PKA on S409 of the β1 subunit (Moss *et al.*, 1992b). PKC phosphorylation of α1β1 and α1β1γ2 receptors also occurs specifically on S409 of the β1 subunit (Krishek *et al.*, 1994). The β1 subunit was also found to be phosphorylated specifically, but to a low level on Y383 and Y385 by coexpressed v-*src* in these cells (Moss *et al.*, 1995). Attempts to study phosphorylation of the γ2 subunits have been difficult due to the lack of reliable anti-γ2 subunit specific antibodies and the extreme susceptibility of the γ2 subunit to proteolysis. These problems have been largely overcome by tagging the N-terminus of the γ2 subunits with the myc epitope, this allows subunit specific immunoprecipitation using the 9E10 anti-myc monoclonal antibody (Connolly *et al.*, 1996b). Using subunits modified in this manner,

tyrosine phosphorylation of γ2 subunits by coexpressed tyrosine kinases has been examined in HEK293 cells (Moss *et al.*, 1995). These studies identified Y365 and Y367 within the γ2L subunit as being phosphorylated to high stoichiometry by coexpressed v-*src* in this cell line (Y357 and Y359 in γ2S: Moss *et al.*, 1995). In agreement with *in vitro* kinase assay experiments, no phosphorylation of α subunit subtypes has been observed in HEK293 cells (Moss *et al.*, 1992b; Krishek *et al.*, 1994; Moss *et al.*, 1995). These studies confirm the validity of the data obtained from *in vitro* experiments used to identify phosphorylation sites in GST-fusion proteins.

Another method which has been used involves the use of anti-phosphotyrosine antibodies to determine tyrosine phosphorylation of receptor subunits by western blotting. This method indicates a large increase in the level of phosphotyrosine in HEK293 cells on expression of v-*src* in combination with receptor subunits (Moss *et al.*, 1995). Immunoprecipitation of $GABA_A$-Rs from dorsal horn neurons followed by western blotting using anti-phosphotyrosine antibodies showed that β2 and/or β3 subunits are phosphorylated on tyrosine residues (Wan *et al.*, 1997a). This approach has so far failed to identify phosphorylation sites within receptor subunits and without specific mutants which can abolish phosphorylation these data remain open to question.

Combined *in vitro* and *in vivo* data suggests that protein phosphorylation of receptor subunits may represent a significant method for modulation of $GABA_A$-Rs *in situ*. Furthermore, these studies suggest that such a mechanism would be primarily mediated by receptor β and γ subunits. The biochemical study of receptor phosphorylation allows a more detailed examination of the functional effects of protein kinases on $GABA_A$-Rs.

## 1.11 Functional effects of $GABA_A$-R phosphorylation by PKA

The functional effects of PKA phosphorylation on native $GABA_A$-Rs have been determined in a number of experimental systems yielding complex and often contradictory results. Activation of endogenous PKA regulates receptor desensitisation and decreases receptor activation in cortical and spinal neurons and synaptosomal preparations from rat, mouse and chick (Harrison and Lambert, 1989; Heuschneider and Schwartz, 1989; Tehrani *et al.*, 1989; Porter *et al.*, 1990; Schwartz *et al.*, 1991; White *et al.*, 1992). Similarly, intracellular dialysis of the catalytic subunit of PKA produced reductions of $GABA_A$ responses in cultured neurons from superior cervical ganglia (SCG) and spinal cord and also in cultured cerebellar granule cells (Porter *et al.*, 1990; Moss *et al.*, 1992b; Robello *et al.*, 1993).

No effect of PKA activation was seen on the function of receptors in mouse spinal cord neurons (Ticku and Mehta, 1990). However, in rat retinal ganglion cells and cerebellar Purkinje neurons, activation of PKA by vasoactive intestinal peptide (VIP) and noradrenaline enhance $GABA_A$ responses (Veruki and Yeh, 1992; Veruki and Yeh, 1994; Parfitt *et al.*, 1990). Enhancement of $GABA_A$ responses on PKA activation has been reported in hippocampal dentate granule neurons (Kapur and Macdonald, 1996) as well as cerebellar Purkinje neurons where enhancement can be mimicked by cAMP analogs and blocked by the specific PKA inhibitor peptide (PKIP), indicating that this effect is indeed due to activation of PKA (Kano and Konnerth, 1992). Similarly intracellular dialysis of the catalytic subunit of PKA enhanced $GABA_A$ responses in rat retinal amacrine cells (Feigenspan and Bormann, 1994). However in the rabbit retina VIP treatment leads to reduced $GABA_A$ responses (Gillette and Dacheux, 1995; Gillette and Dacheux, 1996), indicating that differential effects of

kinase activation may be species dependent. Interestingly, dopamine but not noradrenaline enhanced $GABA_A$ responses in rat retina (Feigenspan and Bormann, 1994). As both of these neurotransmitters act to increase intracellular cAMP there may be a further restriction on PKA phosphorylation of receptor subunits within individual cellular compartments. It has recently been shown that targeting of PKA by protein-protein interaction regulates its ability to phosphorylate cardiac L-type $Ca^{2+}$ channels (Gao *et al.*, 1997), a similar mechanism may regulate $GABA_A$-R modulation by PKA in neurons.

These results suggest that PKA modulates receptor function in a cell type specific manner. This could be due to specific cell-type differences, receptor subunit heterogeneity, receptor differences between species examined or different experimental procedures used to activate protein kinases prior to measurement of receptor function. In order to address some of these questions the functional effects of PKA on heterologously expressed $GABA_A$-Rs in HEK 293 cells have been investigated. Such studies allow comparison of biochemical and electrophysiological effects of kinase activation on receptors composed of wildtype and mutant subunits.

Receptors composed of $\alpha1\beta1$ and $\alpha1\beta1\gamma2$ subunit combinations have been studied in HEK293 cells. Intracellular dialysis of cAMP or the catalytic subunit of PKA results in a time-dependent decrease in GABA-induced currents, the effect being greater for $\alpha1\beta1\gamma2$ receptors (Moss *et al.*, 1992b). Similar levels of inhibition were seen for peak and desensitised currents (Moss *et al.*, 1992b). The decreases in GABA-induced currents were similar to those seen in neuronal preparations and were prevented by mutation of S409 to alanine in the $\beta1$ subunit, this being the sole site of PKA phosphorylation within

these receptors (Moss *et al.*, 1992a; Moss *et al.*, 1992b). The mechanism of PKA phosphorylation induced receptor modulation has not been elucidated in detail. Analysis of dose-response data indicate that phosphorylation of S409 of the β1 subunit in α1β1 receptors leads to a increased $EC50_{GABA}$ and an decreased $I_{max}$ without any effect on single channel conductance (Krishek *et al.*, 1994). These effects are consistent with an decrease in receptor affinity for GABA and/or a decreased probability of channel opening.

It has also been reported that chronic PKA activity may regulate the assembly and surface expression of receptors composed of α1β1 and α1β1γ2 subunits (Angelotti *et al.*, 1993b). Using cell lines which had low, intermediate and high levels of cAMP-independent PKA activity, these workers found larger GABA responses for α1β1γ2 receptors expressed in cells with high levels of PKA activity. Curiously, this effect was not observed for α1β1 receptors but it could be abolished by mutation of S409 to alanine in the β1 subunit (Angelotti *et al.*, 1993b). The mechanism involved and the full significance of this finding have yet to be established.

The mechanisms underlying differential effects of PKA activation on $GABA_A$-R function remain unclear, however the use of recombinant expression systems offers the most convenient methods for addressing outstanding questions.

## 1.12 Functional effects of $GABA_A$-R phosphorylation by PKC

Measurement of $GABA_A$ responses from Xenopus laevis oocytes injected with rat or chick brain mRNA provided the first evidence for receptor modulation by PKC. The use of phorbol esters to activate PKC caused inhibition of GABA-induced currents (Sigel and Baur, 1988; Moran and Dascal, 1989). Later experiments showed that

similar activation of PKC inhibited GABA-induced currents in SCG neurons and cerebellar microsacs (Krishek *et al.*, 1994; Leidenheimer *et al.*, 1992). As was seen for PKA activation, no effect of PKC activation was seen on GABA-induced currents from spinal cord preparations (Ticku and Mehta, 1990). In agreement with these reports, inclusion of PKC inhibitory peptide (PKCI) enhances $GABA_A$-mediated inhibitory postsynaptic potentials in CA1 hippocampal neurons (Weiner *et al.*, 1994).

The effects of PKC activation have also been studied using heterologous expression of $GABA_A$-Rs composed of a range of subunits [$\alpha1$-$\alpha5$, $\beta1$-$\beta2$ and $\gamma2$]. Receptors composed of all subunit combinations tested were inhibited by phorbol ester activation of PKC and the modulation of $\alpha1\beta1\gamma2$ receptors was specifically blocked by PKCI (Sigel *et al.*, 1991; Leidenheimer *et al.*, 1992; Leidenheimer *et al.*, 1993). The role of specific subunits in the regulation of receptors by PKC has been examined using site-specific mutagenesis to remove phosphorylation sites from individual subunits. Receptors composed of $\alpha1\beta1$ and $\alpha1\beta2$ subunits in the presence or absence of $\gamma2S$ or $\gamma2L$ subunits are all inhibited by PKC (Kellenberger *et al.*, 1992; Krishek *et al.*, 1994). Inhibition occurs via multiple phosphorylation sites within the receptor complexes, these are S409 in the $\beta1$ subunit, S410 in the $\beta2$ subunit, S327 in the $\gamma2S$ and $\gamma2L$ subunits and S343 in the $\gamma2L$ subunit alone (Kellenberger *et al.*, 1992; Krishek *et al.*, 1994). The effects of phosphorylation are dependent on GABA concentration used, larger inhibition being seen for receptors containing the $\gamma2L$ subunit at high GABA concentrations (Krishek *et al.*, 1994). Analysis of mutant subunits indicated that phosphorylation of any one site could produce inhibition of receptor function, however single phosphorylation of S343 in the $\gamma2L$ subunit produced the largest effect and there was no linear relationship

between the number of sites phosphorylated and the degree of inhibition of the GABA-induced response (Krishek *et al.*, 1994). PKC also modulates rapid desensitisation of $\alpha 1\beta 1$ receptors in a manner previously described for PKA, this is not surprising as S409 in the $\beta 1$ subunit represents the sole phosphorylation site for both kinases in the receptor complex (Krishek *et al.*, 1994). As described earlier phosphorylation of $\alpha 1\beta 1$ receptors by PKC results in increased $EC50_{GABA}$ and reduced $I_{max}$ without any effect on single channel conductance (Krishek *et al.*, 1994). Mechanistically these effects are consistent with a decrease in receptor affinity for GABA and/or a decreased probability of channel opening. Intracellular dialysis of constitutively activated PKC (PKM) has also been used to modulate receptor phosphorylation and function. Application of 40nM PKM into L929 cells expressing receptors composed of $\alpha 1\beta 1\gamma 2L$ subunits caused significant enhancement of GABA-induced current (Lin *et al.*, 1994). This enhancement was blocked by PKCI and in a later report was shown to be blocked by mutation to alanines of either S409 ($\beta 1$ subunit), S327 ($\gamma 2S$ or $\gamma 2L$ subunit) or S343 ($\gamma 2L$ subunit) (Lin *et al.*, 1994, Lin *et al.*, 1996). While these mutations blocked the effects of PKM, phosphorylation of wildtype receptor subunits was not demonstrated biochemically.

These differences in the effects of PKC on receptor function could be due to differences between cell lines being used, differences between murine (Krishek *et al.*, 1994) and bovine subunit cDNAs (Lin *et al.*, 1994; Lin *et al.*, 1996), differences between phorbol ester activation of PKC and application of PKM or differences between recording protocols used to measure $GABA_A$ responses. While the mechanism underlying these differences have not been fully addressed it is worth noting that enhancement of $GABA_A$ responses by PKC activation in neurons has not yet been reported.

## 1.13 Functional effects of $GABA_A$-R phosphorylation by PKG and CamKII

While phosphorylation sites have been identified in $GABA_A$-R subunits *in vitro* (McDonald and Moss, 1994), the functional effects of PKG and CamKII are poorly understood. $GABA_A$ currents are reduced by cGMP or activation of metabotropic glutamate receptors in neurons of the tractus solitarius in the rat (Glaum and Miller, 1993). The mechanism underlying this inhibition however has not been demonstrated. Elevated intracellular $Ca^{2+}$ has been shown to enhance or reduce $GABA_A$ currents depending on the neuronal population studied (Stelzer and Shi, 1994). The role of CamKII in these different effects of $Ca^{2+}$ are however unclear. Constitutively activated CamKII has been shown to activate receptor-mediated currents in spinal cord neurons of the rat, however direct receptor phosphorylation by CamKII was not shown (Wang *et al.*, 1995). This effect is unexpected as both PKA and PKC which phosphorylate some of the same sites are known to have no effects on receptor function in these neurons (Ticku and Mehta, 1990). CamKII activation has recently been shown to be necessary for the establishment of 'rebound potentiation' in cerebellar Purkinje neurons (Kano *et al.*, 1996). This phenomenon involves a potentiation of $GABA_A$ responsiveness in Purkinje neurons in response to elevated intracellular $Ca^{2+}$ caused by glutamatergic stimulation (Kano *et al.*, 1992). Inhibition of CamKII function by treatment with specific kinase inhibitors prevents the establishment of this potentiation, however the role of receptor phosphorylation in this process has not been examined. Interestingly this process can be mimicked by treatment of these cells with cAMP analogs and inhibited by PKIP (Kano and Konnerth, 1992).

While evidence exists for modulation of $GABA_A$-R function by PKG and CamKII and *in vitro* kinase assays suggest that $GABA_A$-R

subunits contain high affinity sites for phosphorylation by both kinases, neither kinase has been shown to phosphorylate receptor subunits directly either in neurons or in heterologous expression systems.

## 1.14 Functional effects of $GABA_A$-R phosphorylation by protein tyrosine kinases

Recent investigations have focused attention on the effects of tyrosine phosphorylation on $GABA_A$-R function. Whole-cell $GABA_A$-responses recorded from SCG neurons are reduced by treatment with tyrosine kinase inhibitors and increased by tyrosine phosphatase inhibitors or intracellular dialysis of $pp60^{c\text{-}src}$ (Moss *et al.*, 1995). Single channel recordings suggest that receptor enhancement is due to increased probability of channel opening (Moss *et al.*, 1995). GABA-mediated chloride flux from brain microsacs was also found to be reduced by tyrosine kinase inhibitors (Valenzuela *et al.*, 1995). More recent work has shown suppression of $GABA_A$ responses in cultured spinal dorsal horn neurons after treatment with tyrosine kinase inhibitors, as expected intracellular dialysis of $pp60^{c\text{-}src}$ enhanced the $GABA_A$ responses in these cells (Wan *et al.*, 1997a). Tyrosine kinase inhibitors have been shown to reduce GABA-induced responses from *Xenopus* oocytes expressing receptors composed of $\alpha1\beta1$ and $\alpha1\beta1\gamma2L$ subunit combinations (Valenzuela *et al.*, 1995). Similarly, the functional effects of tyrosine phosphorylation on receptor function in SCG neurons have been reproduced in HEK293 cells expressing $\alpha1$, $\beta1$ and $\gamma2L$ subunits (Moss *et al.*, 1995). Tyrosine kinase mediated regulation of these receptors was abolished by mutation of Y365 and Y367 to phenylalanine in the $\gamma2L$ subunit, while phosphorylation of Y383 and Y385 in the $\beta1$ subunit had negligible effects on channel function (Moss *et al.*, 1995). Receptors

composed of $\alpha 1\beta 2$ or $\alpha 1\beta 2\gamma 2L$ subunits have each been shown to be negatively modulated by tyrosine kinase inhibitors (Wan *et al.*, 1997a). However this study has not shown convincing phosphorylation of any channel subunit and does not show elimination of modulation by site-directed mutagenesis (Wan *et al.*, 1997a).

There is overall agreement that tyrosine phosphorylation enhances $GABA_A$-R function, how this enhancement occurs however remains unclear. It seems likely that $\gamma 2$ subunit phosphorylation is crucial for this effect however the role of $\beta$ subunit phosphorylation in this process requires further investigation.

## 1.15 Questions to be addressed in this study

It is clear that $GABA_A$-R function is dynamically regulated by direct protein phosphorylation. This work aims to investigate mechanisms underlying the differential regulation of receptor function in various cell types of the brain. It is clear that protein kinases mediate different effects on the function of native receptors in different neurons. There are numerous potential explanations for this observation, kinase activation could trigger diverse downstream signalling events in different cell types, kinase subunit expression and/or targeting could be regulated in a cell type-specific manner or the ability of receptor complexes to be modulated could vary between cell types. This work focuses on the role of individual subunit subtypes in determining the functional effects of protein kinase activation on the $GABA_A$-R. Receptor $\beta$ subunits have been chosen for investigation as no PKA phosphorylation sites have been identified in $\alpha$ or $\gamma$ subunits and since PKA has been shown to differentially regulate receptor function in various neurons.

Using purified GST-fusion proteins containing intracellular domains of β2 and β3 subunits, high affinity phosphorylation sites will be identified in these subunits using *in vitro* kinase assays combined with phosphopeptide mapping, phosphoamino acid analysis and site-directed mutagenesis. GST-fusion proteins will also be used for immunisation of rabbits to produce anti-β subunit specific antisera. Resultant antibodies will be used in the biochemical analysis of receptor subunit complexes in HEK293 cells and brain regions. Heterologous expression of recombinant receptors followed by metabolic labelling and immunoprecipitation will be used to confirm the identity of phosphorylation sites identified *in vitro*. Electrophysiological analysis of recombinant receptor function cells will allow determination of the functional effects of phosphorylation at identified sites within different β subunits.

These studies will provide detailed information regarding the role of protein phosphorylation in receptor modulation. Generation of novel antibody reagents should also allow progress in the understanding of native receptor modulation at the biochemical level. Together these studies should provide clarification of some important unanswered questions concerning $GABA_A$-R regulation.

# 2 MATERIALS AND METHODS

## 2.1 cDNAs, vectors, bacterial strains and media used

The sequences of the murine α1, γ2S, β1, β2 and β3 subunit cDNAs and subunit cDNAs modified by the addition of the *myc* (9E10) epitope have been described previously (Evan *et al.*, 1985; Kamatchi *et al.*, 1995; Connolly *et al.*, 1996a). The cDNAs were cloned into the mammalian expression plasmid pGW1 (Moss *et al.*, 1990). Site-directed mutagenesis was performed in this vector as described below. pGW1 was used to express $GABA_A$ receptor subunits transiently in HEK 293 cells as described below. The major intracellular domains of $GABA_A$ receptor β subunits were subcloned into pGex-4T/3 (Pharmacia) to facilitate their expression and purification from *E. coli* as described below.

*E. coli* strain XL1-Blue [*F' :: Tn10 proA*$^+$*B*$^+$ *lacI*$^q$ *Δ(lacZ) M15 /recA1 endA1 gyrA96* (*Nal* $^r$) *thi hsdR17* ($r_K^-$ $m_K^+$) *supE44 relA1 lac* ] (Stratagene) was used for generation of plasmid stocks and for their maintenance as stocks in 20%(w/v) glycerol at -80°C. DNA was transformed into competent bacteria by electroporation in a BioRad gene-pulser II according to the manufacturer's instructions.

*E. coli* strain CJ236 [*F' cat* (pCJ105; $M13^s$ $Cm^r$)/ *dut ung1 thi-1 relA1 spoT1 mcrA*] (New England Biolabs) was used in conjunction with bacteriophage M13K07 to generate dUTP containing single-stranded DNA for site-directed mutagenesis as described below. *E. coli* strain BL21 [*F* $^-$ *ompT* [*lon* ] $hsdS_B$ ($r_B^-$ $m_B^-$; an E.coli B strain with DE3, a 1 prophage carrying the T7 RNA polymerase gene)] (Novagen) was used for production of GST-fusion proteins from the pGex-4T/3 vector as described below. DNA was transformed into $CaCl_2$

competent bacteria of both strains (BL21 and CJ236) as described previously (Sambrook *et al.*, 1989).

Bacteria were grown in culture in Luria broth (LB; 1%(w/v)bacto-tryptone (Difco), 0.5%(w/v) bacto-yeast extract (Difco), 10mg/ml NaCl, pH 7.0) which had been sterilised by autoclaving at 15lb/sq.inch for 20 minutes in a liquid cycle. Appropriate selection was applied by inclusion in the medium of Ampicillin (Amp; 50μg/ml; Sigma), Chloramphenicol (Chlor; 25μg/ml; Sigma) or Kanamycin (Kan; 50μg/ml; Sigma). LB agar plates were prepared from LB containing 1.5%(w/v) bacto-agar (Difco) which was sterilised by autoclaving as described above.

## 2.2 DNA preparation

Supercoiled double-stranded DNA for transfection of HEK293 cells was prepared from XL1-Blue cells as previously described (Sambrook *et al.*, 1989). Briefly, DNA was obtained by alkaline lysis of bacteria followed by centrifugation at 100,000 x g through a 1.1g/ml CsCl gradient using a Beckman Optima™ TLX ultracentrifuge. Purified DNA was collected by ethanol precipitation and quantitated by measurement of $A_{260}$ in a Beckman DU-650 spectrophotometer using the folowing equation (Sambrook *et al.*, 1989):

$$[\text{DNA}]\text{mg/ml} = \frac{A_{260} \times D \times 50}{1000}$$

where D = the dilution factor of the sample measured.

Supercoiled DNA for analysis by double-stranded DNA sequencing was prepared from XL1-Blue cells as previously described (Sambrook *et al.*, 1989). Briefly, DNA was obtained by alkaline lysis of bacteria followed by extraction with phenol/chloroform (1:1). RNA

was removed by RNAase A (10μg/ml Calbiochem) digestion at 37°C for 30 minutes and DNA precipitated by addition of 0.6 volume 20% (w/v) $PEG_{6000}$/2.5M NaCl and incubation at 4°C for 1-16 hours. Purified DNA was resuspended in $H_2O$ and stored at -20°C until used.

Single-stranded DNA containing dUTP was prepared from *E. coli* CJ236 using M13K07 helper phage as described previously (Sambrook *et al.*, 1989). Phage particles containing the cDNA of interest were purified and DNA isolated by phenol/chloroform extraction followed by ethanol precipitation. DNA was checked by agarose gel electrophoresis as described below and stored at -20°C until use.

## 2.3 Site-directed mutagenesis

The following antisense oligonucleotides were synthesised by Cruachem Ltd. and used for site-directed mutagenesis of potential phosphorylation sites within the β1, β2 and β3 subunits as described in text (all oligos represent 5'->3' sequence):

| | | |
|---|---|---|
| A408S | (β1 subunit) | GAGCTGCGAGGAGCGCCTGCGG; |
| A408S & S409A | (β1 subunit) | CTTTGAGCTGCGCGGAGCGCCTG; |
| S410A | (β2 subunit) | CAGTTGAGCGGCACGTC; |
| S408A | (β3 subunit) | GAGCTGTGCAGACCTCC; |
| S409A | (β3 subunit) | CTGTGAAGCCCTCCTCC; |
| S408A & S409A | (β3 subunit) | GAGCTGTGCAGCCCTCCTCC; |
| S383A | (β3 subunit) | CTTGGGCATGGCCTGTTTCCTA. |

Crude lyophilised oligonucleotides were purified by electrophoresis through denaturing 14%(w/v) polyacrylamide gels containing 7M Urea/0.5xTBE (45mM Tris-Borate, 2mM EDTA) using 1xTBE (90mM Tris-Borate, 4mM EDTA) as running buffer in a vertical

electrophoresis apparatus (Protean II; BioRad). Oligonucleotides were visualised by UV shadowing on a fluorescent thin layer chromatography plate (Merck) using a hand held short wave UV lamp (Model UVG-54 Mineralight lamp; UVP). Oligonucleotides were removed as slices from the gels and eluted in 0.5M $NH_4OAc$. Eluted oligonucleotides were then desalted using a C-18 Sep-pak column (Waters), dried and resuspended in $H_2O$. Purified oligonucleotides were quantitated by measurement of $A_{260}$ in a Beckman DU-650 spectrophotometer using the folowing equation (Sambrook *et al.*, 1989):

$$[DNA]mg/ml = \frac{A_{260} \times D \times 30}{1000}$$

where D = the dilution factor of the sample measured.

Site-directed mutagenesis was carried out using the Muta-gene *in vitro* mutagenesis kit (BioRad) according to the supplier's instructions. Mutagenesis reactions were then transformed into *E. coli* strain XL1-Blue by electroporation and mutant constructs identified by colony hybridisation and DNA sequencing.

## 2.4 Mutant identification by colony hybridisation

200ng gel purified mutagenic oligonucleotide was phosphorylated at its 5' end using 15 units T4-Polynucleotide kinase (PNK) in the presence of 20μCi $\gamma$-$^{32}$P-ATP according to the supplier's instructions. The labelled oligonucleotide was purified from unincorporated label through a Sephadex G-50 column (Pharmacia).

The melting or annealing temperature (Tm) of oligonucleotide probes was calculated as described previously (Sambrook *et al.*, 1989)

$$Tm = 81.5 + 16.6 ( \log[Na^+] ) + 0.41 ( \%G+C ) - ( 600/N ) \ ^oC$$

where $\log[Na^+]$ is the logarithm to the base 10 of the $Na^+$ concentration of the buffer, %G+C is the percentage of the oligonucleotide composed of guanosine and cytosine and N is the total length of the oligonucleotide probe.

Colonies resulting from the transformation of the mutagenesis reaction into *E. coli* strain XL1-Blue, together with some negative control wildtype colonies were picked using sterile toothpicks and streaked in an asymmetric grid on an LB agar plate containing ampicillin and grown for 18 hours at 37°C. Colonies were replica plated onto a gridded nylon filter (Hybond-N; Amersham International) and the cells lysed by soaking the filter in 10%(w/v) SDS at room temperature for 5 minutes. The DNA contents of the lysed cells were then denatured and neutralised by soaking the filter at room temperature for 3 minutes in 0.5M NaOH/1.5M NaCl followed by 1M Tris-HCl pH7.5/1.5M NaCl. The filter was then washed in 2xSSC (300mM NaCl, 35mM sodium citrate pH7.0), air dried and the DNA fixed by baking at 80°C for 2 hours.

The baked filter was wet in 2xSSC, prewashed in 5xSSC/0.5%(w/v) SDS/1mM EDTA pH8.0 at 50°C for 30 minutes and prehybridised in 5xSSC/0.5%(w/v) SDS/5 x Denhardt's reagent (0.1%(w/v) Ficoll [type 400; Pharmacia], 0.1%(w/v) polyvinylpyrrolidone, 0.1%(w/v) BSA)/100µg/ml boiled denatured salmon sperm DNA for 2 hours at 20°C below the melting temperature of the probe. $^{32}P$ labelled oligonucleotide probe was added to a final concentration of 20ng/ml in the prehybridisation mixture with the filter and the incubation continued at the same temperature for 2 hours. Filters were washed 2 x 5 minutes in 0.1xSSC at room temperature followed by repeated washes in 0.1xSSC at 2°C below the Tm of the probe until an acceptable difference in bound probe was detectable between the

control colonies and the mutagenic reaction colonies. Mutant colonies were visualised by autoradiography on XAR-5 film (Kodak) at -70°C and regrown to allow DNA isolation. Mutations were confirmed by DNA sequencing as described below.

## 2.5 Double stranded DNA (ds-DNA) sequencing

DNA sequencing was carried out using the USB Sequenase Version 2.0 DNA Sequencing Kit (Amersham International) according to the manufacturers instructions. 5-10µg miniprep DNA were used per reaction. On completion, reactions were heated to 75°C for 2 minutes then subjected to electrophoresis at 70Watts (Power-pac 3000; BioRad) through a denaturing 6%(w/v) polyacrylamide gel containing 7M Urea, 0.5xTBE (45mM Tris-Borate, 2mM EDTA) using 1xTBE as running buffer in a vertical electrophoresis apparatus (Model S2; Life Technologies Inc.). Once electrophoresis was complete the gel was dried onto Whatman 3MM paper and visualised by autoradiography on XAR-5 film (Kodak).

## 2.6 Restriction digestion of DNA

5-50µg DNA were digested per reaction under conditions recommended by the supplier of restriction endonucleases (New England Biolabs).

Digestion reactions were stopped by addition of sample loading buffer (40%(w/v) Sucrose/0.25%(w/v) Bromophenol blue/0.25% (w/v) Xylene cyanol FF). Digestion products were separated by electrophoresis (60V; Power-pac 300; BioRad) through 0.8-2.0%(w/v) agarose gel (Electrophoresis grade; Gibco) in 1xTBE (90mM Tris-Borate, 4mM EDTA) containing ethidium bromide (5µg/ml) and visualised by ultraviolet transillumination.

## 2.7 Gel purification of DNA fragments from low melting point (LMP) agarose

Following electrophoresis through a suitable low melting point agarose (Nusieve; Flowgen) as described above, DNA fragments were excised as gel slices and their volumes estimated. Two gel volumes of $H_2O$, 0.1 gel volume 3M sodium acetate and 0.1 gel volume 10xTE were added, mixed and heated to 70°C for 5 minutes. The molten agarose solution was extracted once with an equal volume of phenol (pH 8.0) preheated to 55°C for 5 minutes, once with 0.5ml Phenol:Chloroform: Isoamyl alcohol (25:24:1) and once with an equal volume of Chloroform:Isoamyl alcohol (24:1). DNA was then ethanol precipitated and collected by centrifugation. Purified DNA was resuspended in $H_2O$, its purity reconfirmed by gel electrophoresis and stored at -20°C until use.

## 2.8 Subcloning of receptor subunit intracellular loops into pGex-4T/3

To facilitate the in-frame cloning of the fragments encoding the major intracellular loops of the β2 (amino acids 303-427) and β3 subunits (amino acids 305-427) these regions were amplified by the polymerase chain reaction (PCR) using oligonucleotide primers designed to introduce restriction sites into the reaction products. The following primers were used (represented 5'->3'):

| | | |
|---|---|---|
| β2 subunit | 5' | GTTCCGCGTGGATCCAATGCCAACAATGAGAAG |
| | and 3' | ACCATGAATTCCCCGATCAATGGCATTCAC |
| β3 subunit | 5' | GTTCCGCGTGGATCCAACTACATTTTCTTTGGA |
| | and 3' | GCGGCCGCTCGAGTCTGTCTATGGCATTCAC. |

250ng template DNA was amplified by 5U Taq polymerase (Promega) in 25µl PCR reactions containing 10mMTris-HCl pH8.0, 50mM KCl,

200μM dNTPs, 0.1%(v/v) Tween-20, 1.5mM $MgCl_2$ and 125ng each primer (5' and 3'). The reactions were placed in a thermocycler (Techne PHC-3) for 1 cycle at 95°C for 30 seconds; 30 cycles at 95°C for 30 seconds/50°C for 30 seconds/72°C for 1 minute and 1 cycle at 72°C for 10 minutes. The products of these reactions were digested using BamH1 and EcoR1 (β2) or BamH1 and Xho1 (β3). The expression plasmid pGex-4T/3 (Pharmacia) was similarly digested using BamH1/EcoR1 (β2) or BamH1/Xho1 (β3) and dephosphorylated by 1U Shrimp alkaline phosphatase (USB) as per the supplier's instructions. Following purification from LMP agarose gels, the insert and vector were mixed in a molar ratio of 10:1 in ligation reactions catalysed by 200U T4 DNA Ligase (New England Biologicals; $4x10^5$ units/ml) in 10μl 50mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP at 16°C for 12-16 hours.

Reactions were transformed into *E. coli* strain XL1-Blue and DNA prepared from resulting colonies was screened by restriction digestion to determine the presence of the PCR fragment insert. The fidelity of the final constructs was confirmed by ds-DNA sequencing.

## 2.9 Production and purification of intracellular loop GST-fusion proteins

pGex expression plasmids were transformed into *E. coli* strain BL21 as described and fusion protein production carried out by standard methods (McDonald and Moss, 1994, Moss *et al.*, 1992a, Smith and Johnson, 1988). Protein concentrations were determined by the Bradford assay (Biorad) following the manufacturer's instructions using bovine serum albumin (BSA) as the standard. Protein was analysed by SDS-PAGE as described below, aliquoted into suitable volumes and stored at -80°C until required.

## 2.10 Protein separation by SDS-PAGE

Proteins were separated by SDS-PAGE as described (Laemlli, 1970). Protein samples and molecular weight markers (Biorad) were solubilised in Sample buffer (2%(w/v) Sodium dodecyl sulphate (SDS), 100mM β-mercaptoethanol (BME), 50mM Tris-HCl pH6.8, 0.1%(w/v) Bromophenol blue, 10%(w/v) glycerol) and heated to 90°C for 5 minutes prior to electrophoresis. The running gel (8-10%(w/v) polyacrylamide) was prepared in 500mM Tris-HCl pH8.8, 0.2%(w/v) SDS and polymerised by addition of Ammonium persulphate (APS) to 0.1%(w/v) and N,N,N',N'-Tetramethylethylenediamine (TEMED) to 0.05%(v/v). Stacking gel (4%(w/v) polyacrylamide) was prepared in 155mM Tris-HCl pH6.8, 0.2%(w/v) SDS and polymerised by addition of APS and TEMED as described above. The gel was assembled in a vertical electrophoresis chamber (Protean II; Biorad) containing running buffer (25mM Tris-HCl pH8.8, 0.01%(w/v) SDS, 190mM Glycine) in both upper and lower buffer chambers. The preheated samples (1-100μg) were applied to the sample wells in the stacking gel and the gel run at 80V for 16 hours (Power-pac 300; Biorad). The gel was fixed and stained in 10%(v/v) acetic acid/50%(v/v) methanol/0.1% Coomassie brilliant blue then washed extensively in destain solution (10%(v/v) acetic acid/20%(v/v) methanol). Gels were dried onto cellophane and radiolabelled protein was visualised by autoradiography on Kodak XAR-5 film at -70°C.

## 2.11 cAMP-dependent protein kinase (PKA) phosphorylation of GST-fusion proteins

Fusion proteins (0.5-2.5μg) were phosphorylated in 25μl Hepes pH 7.0, 20mM $MgCl_2$ , 0.2mM ATP (specific activity 500-1000 cpm/pmol using [$\gamma$-$^{32}$P]-ATP (Amersham, 3000 Ci/mmol)), for 15 min at 30°C in the presence of 10-50ng kinase purified from bovine heart (Sigma).

The reaction was terminated by the addition of EDTA to 25mM. The extent of phosphorylation was analysed by SDS-PAGE after addition of sample buffer. Gels were stained in coomassie blue to ensure similar protein concentrations in all reactions. After visualisation by autoradiography the relevant bands were excised, [$^{32}$P]-incorporation was determined by Cerenkov counting and samples were analysed by phosphopeptide mapping and phosphoamino acid analysis.

## 2.12 cGMP-dependent protein kinase (PKG) phosphorylation of GST-fusion proteins

Fusion proteins (0.5-2.5µg) were phosphorylated in 25µl 40mM Tris-HCl pH 7.4, 20mM $MgCl_2$, 2mM cGMP, 0.2mM ATP (specific activity 500-1000 cpm/pmol using [$\gamma$-$^{32}$P]-ATP (Amersham, 3000 Ci/mmol)), for 15 min at 30°C in the presence of 10-50ng purified kinase (Sigma). The reaction was terminated by the addition of EDTA to 25mM. The extent of phosphorylation was analyzed by SDS-PAGE after addition of sample buffer. Gels were stained in coomassie blue to ensure similar protein concentrations in all reactions. After visualisation by autoradiography the relevant bands were excised, [$^{32}$P]-incorporation was determined by Cerenkov counting and samples were analysed by phosphopeptide mapping and phosphoamino acid analysis.

## 2.13 Protein kinase C (PKC) phosphorylation of GST-fusion proteins

Fusion proteins (0.5-2.5µg) were phosphorylated in 25µl 20mM Hepes, pH7.5, 10mM $MgCl_2$, 0.5mM $CaCl_2$, 40µg/ml phorbol-12,13-dibutyrate, 0.2mM ATP (specific activity 500-1000 cpm/pmol using [$\gamma$-$^{32}$P]-ATP (Amersham, 3000 Ci/mmol)), for 15 min at 30°C in the

presence of 10-50ng purified kinase. Assays were terminated by the addition of an equal volume of 2xSDS-PAGE sample buffer. The extent of phosphorylation was analyzed by SDS-PAGE. Gels were stained in coomassie blue to ensure similar protein concentrations in all reactions. After visualisation by autoradiography the relevant bands were excised, [$^{32}$P]-incorporation was determined by Cerenkov counting and samples were analysed by phosphopeptide mapping and phosphoamino acid analysis.

## 2.14 $Ca^{2+}$/calmodulin-dependent protein kinase type II (CamKII) phosphorylation of GST-fusion proteins

Purified rat brain CamKII (Schulman 1984) was a generous gift from Howard Schulman (Stanford University School of Medicine). Fusion proteins (0.5-2.5µg) were phosphorylated in 25µl 50mM Hepes pH 7.0, 10mM $MgCl_2$, 10µg/ml calmodulin, 0.2mM EGTA, 0.3 mM $CaCl_2$, 0.2mM ATP (specific activity 500-1000cpm/pmol using [$\gamma$-$^{32}$P]-ATP (Amersham, 3000 Ci/mmol)), for 15 min at 30°C in the presence of 10-50ng kinase. Assays were terminated by the addition of an equal volume of 2xSDS-PAGE sample buffer. The extent of phosphorylation was analyzed by SDS-PAGE. Gels were stained in coomassie blue to ensure similar protein concentrations in all reactions. After visualisation by autoradiography the relevant bands were excised, [$^{32}$P]-incorporation was determined by Cerenkov counting and samples were analysed by phosphopeptide mapping and phosphoamino acid analysis.

## 2.15 Phosphopeptide map analysis

Phosphopeptide map analysis was performed on excised gel slices essentially as described (Moss *et al.*, 1992a, McDonald and Moss,

1994). Phosphoproteins of interest were excised from dried polyacrylamide gels, washed extensively in destain solution, dried and incubated in 50mM $NH_4HCO_3$ containing 0.3mg/ml Trypsin (Sigma) at 37°C for 20 hours. Supernatants were dried in a speed-vac. Dried samples were resuspended thoroughly in 10µl $H_2O$, heated to 65°C for 1 minute and centrifuged at 13,000 x g for 15 minutes at room temperature. The required amount of each sample (20-100cps) was then applied to the middle of a chromatography plate over dye indicators basic fuchsin (1µl - 10mg/ml) and phenol red (1µl - 10mg/ml) and dried. Plates were then overlaid with peptide map buffer (17.4%(v/v) acetic acid, 0.92%(v/v) pyridine, pH 3.5) and subjected to electrophoresis in the x-dimension at 500V (Power-pac 3000; Biorad) until the dye indicators were within 4cm of the edges of the plate. Plates were removed from the chamber, allowed to dry at room temperature and subjected to thin layer chromatography using chromatography solvent buffer (37.5%(v/v) pyridine, 25%(v/v) n-butanol, 7.5%(v/v) acetic acid) in the y-dimension until the solvent front was 3cm from the top of the plate. Plates were then dried thoroughly, wrapped in Saran wrap and phosphopeptides visualised by autoradiography on Kodak XAR-5 film at -70°C.

## 2.16 Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described (Moss *et al.*, 1992a, McDonald and Moss, 1994). Unused trypsin digested samples from the phosphopeptide mapping procedure were dried in a speed-vac and resuspended in 100µl $H_2O$. The required amount of phosphoproteins (15-30 cps) were aliquoted into 1.5ml screw-top microfuge tubes, 1ml 6N HCl was added and air was excluded from the tube by gentle blowing of $N_2$ over the surface of the liquid. Tubes were incubated at 100°C for 1-2 hours to ensure complete hydrolysis

of peptides and samples were dried overnight in a speed-vac. Dried samples were resuspended in 10µl $H_2O$, heated to 65°C for 1 minute and spun at 13,000 xg for 15 minutes at room temperature. Samples were applied to a chromatography plate and dried together with 1µl phenol red (10mg/ml), 1µl phosphoserine (10mg/ml), 1µl phosphothreonine (10mg/ml), 1µl phosphotyrosine (10mg/ml). Plates were then overlaid with pAA buffer (10%(v/v) acetic acid, 1%(v/v) formic acid, pH 1.9) and subjected to electrophoresis at 500V (Power-pac 3000; Biorad) in the same buffer until the dye indicator had migrated 5cm toward the anode. Samples were then transferred to peptide map buffer (pH 3.5) and the electrophoresis continued at 500V until the dye indicator had migrated a further 7cm toward the anode. Plates was then dried and phosphoamino acid standards visualised by staining with 1%(w/v) Ninhydrin. Radiolabelled phosphoamino acids were then visualised by autoradiography on XAR-5 film at -70°C.

## 2.17 Electroblotting of protein from SDS-PAGE gel to nitrocellulose membrane (Western transfer)

Following separation by SDS-PAGE proteins were transferred by submerged western transfer in transfer buffer (12.5mM Tris-HCl pH8.0, 96mM Glycine, 20%(v/v) Methanol) in a Trans-blot cell (Biorad) according to the manufacturer's instructions. Transfer was carried out for 1600V/hours (Power-pac 300; Biorad), onto Hybond C nitrocellulose membrane (Amersham). Once transfer was complete the nitrocellulose sheets were stained with Ponceau S (0.5% in 10%(v/v) acetic acid) to check for efficient protein transfer, protein molecular weight standards were marked and membranes were washed extensively in PBS before being probed with antibody.

## 2.18 Immunodetection of electroblotted protein

Nitrocellulose membranes were blocked for 1 hour at room temperature in blocking buffer (Tris-Buffered Saline (TBS: 0.5M Tris-HCl pH7.4, 2M NaCl) containing 0.5%(w/v) Tween-20 and 5% Marvel™), then incubated for 1 hour at room temperature in blocking buffer containing the appropriate antibody at a suitable concentration. Unbound antibody was removed by washing 5 x 10 minutes at room temperature in blocking buffer. Antibody incubation was then repeated using a suitable Horse Radish Peroxidase (HRP)-conjugated secondary antibody at an appropriate concentration in blocking buffer. Unbound antibody was again removed by washing in blocking buffer. Membranes were then washed 2 x 5 minutes in TBS/0.5%(w/v) Tween-20 and excess buffer removed on tissue paper. HRP-conjugated secondary antibody was then detected using an enhanced chemiluminescent substrate (ECL: Amersham) as per the manufacturer's instructions.

When required membranes could be stripped of bound primary and secondary antibody by washing at 50-70°C for 20 minutes with shaking in 100mM β-mercaptoethanol, 2%(w/v) SDS, 62.5mM Tris-HCl pH6.7. The stripped membranes were then washed 3x10 minutes in large volumes of TBS/0.5%(w/v) Tween-20 at room temperature. The immunodetection procedure could then be repeated using different antibodies.

## 2.19 Maintenance of HEK293 cells

Human Embryonic Kidney 293 (HEK293) fibroblast cells were grown in an atmosphere containing 5% $CO_2$ at 37°C in Dulbecco's Modified Eagle Medium (DMEM; Gibco) containing 10%(v/v) Foetal Calf Serum (FCS; Gibco), 0.3mg/ml Glutamine (Gibco), 60µg/ml Penicillin (Sigma)

and 150µg/ml Streptomycin (Gibco). At 80-90% confluence on 90mm petri dishes (Falcon) the cells were passaged by treatment in prewarmed 0.05%(w/v) trypsin, 0.02%(w/v) EDTA in Modified Puck's Saline A (Sigma). Cells in suspension were diluted in prewarmed medium and seeded onto fresh 90mm petri dishes.

Frozen stocks of cells from early passages were prepared by washing in FCS followed by centrifugation at 800 xg for 2 minutes at room temperature and storage at -80°C in FCS containing 10%(v/v) dimethylsulphoxide (DMSO; Sigma). Frozen cells were transferred to liquid nitrogen for long term storage after 5 days at -80°C. When required frozen cells were thawed quickly, washed in 10ml prewarmed medium to remove DMSO, collected by centrifugation and plated at the required density in prewarmed medium.

## 2.20 Transient transfection of HEK293 cells

HEK293 cells were passaged as described and seeded on 90mm petri dishes at $5x10^5$ cells per dish. After 24 hours cells were transfected by electroporation using a BioRad gene-pulser II according to the manufacturers instructions. 10µg total DNA per transfection was electroporated into $5x10^5$ cells in 0.5ml Optimem. Cells were then plated on 60mm petri dishes (Falcon). Where necessary cells were plated on glass coverslips coated with fibronectin (5µg/ml; Sigma) and poly-L-lysine (10µg/ml; Sigma).

## 2.21 Detection of transiently expressed protein in HEK293 cells by immunofluorescence

Expression of protein in cells was assayed by immunofluorescence at 16-24 hours after transfection. Cells were washed in phosphate-buffered saline pH7.0 (PBS) and fixed in 4%(w/v) paraformaldehyde

in PBS for 20 minutes at room temperature. Fixing was quenched for 2x10 minutes in 50mM $NH_4Cl$ at room temperature. Cells were blocked for 2x15 minutes at room tempreature in PBS containing 0.5%(w/v) BSA, 10%(w/v) Horse serum (Gibco) ['block' buffer]. Cells were permeabilised if necessary by addition of 0.05%(w/v) Triton X-100 (Sigma) to 'block' buffer.

Cells were inverted onto 50µl 'block' buffer containing primary antibody/antibodies for 45 minutes at room temperature. Cells were washed 4x15 minutes in 'block' buffer and incubated for 1 hour on 50µl 'block' buffer containing secondary antibody. Cells were washed 4x15 minutes in 'block' buffer then rinsed in 5x1 ml PBS. Finally coverslips were inverted on 5µl n-propyl galate (Sigma) on microscope slides and examined for staining on an Axioskop fluorescence microscope (Zeiss). Images of fluorescence staining were recorded on TMax print film (ISO 400; Kodak) using a Micro100 spot camera attachment (Zeiss).

## 2.22 [$^{35}$S]-Methionine metabolic labelling of transiently transfected HEK293 cells

16-24 hours after transfection, cells were washed and starved of methionine for 30 minutes in prewarmed MEM without L-Methionine (Gibco) at 37°C and 5% $CO_2$. [$^{35}$S]-Met/[$^{35}$S]-Cys (EXPRE$^{35}$S protein labelling mix; ICN) was added to the starved cells to 250µCi/ml and incubation continued for 4 hours. After labelling, medium was removed and cells were washed repeatedly in PBS. Cell lysates were prepared and proteins then immunoprecipitated as described below.

## 2.23 [$^{32}$P]-orthophosphoric acid labelling of transiently transfected HEK293 cells

16-24 hours after transfection, cells were washed in 5ml prewarmed phosphate free DMEM (with glutamax and 25mM Hepes; Gibco). Cells were then labelled for 4 hours at 37°C/5% $CO_2$ in 1ml phosphate free DMEM containing 1mCi/ml $^{32}$P-orthophosphate ($^{32}$P-orthophosphate in dilute HCl; 10mCi/ml; Amersham). Protein kinases were then activated as described, $^{32}$P containing medium removed and the cells washed repeatedly with ice-cold PBS. Cell lysates were then prepared and proteins immunoprecipitated as described below.

## 2.24 Preparation of HEK293 cell lysates

Washed cells were resuspended in 750µl lysis buffer (20mM $NaH_2PO_4/Na_2HPO_4$ pH7.5, 5mM EDTA, 5mM EGTA, 50mM NaF, 10mM $Na_4P_2O_4$, 1mM $Na_3VO_4$, 5mM $NH_4MoO_3$, 0.1mM PMSF, 10µg/ml Pepstatin, 10µg/ml Leupeptin, 10µg/ml Antipain, 2%(w/v) NP-40, 0.5%(w/v) deoxycholic acid) and incubated on ice for 10 minutes. Nuclei were removed by centrifugation at 13,000 xg for 10 minutes at 4°C, supernatants were transferred to fresh tubes and proteins immunoprecipitated as described below.

## 2.25 Immunoprecipitation of $GABA_A$-R subunits

Cell lysates were precleared for 1 hour at 4°C with 20µg non-immune IgG from the same species as the antibody being used for immunoprecipitation. Protein G Agarose beads (Fast Flow; Pharmacia) were resuspended and washed in lysis buffer. Beads were then resuspended as a 1:1 slurry in lysis buffer containing 10mg/ml BSA. 50µl washed Protein G was added to the lysate/IgG solution and incubated for 1 hour at 4°C. Protein G was removed by centrifugation

at 13,000 xg for 2 minutes at 4°C. Lysates were transferred to fresh tubes containing the immunoprecipitating antibody and incubated for 1 hour at 4°C. 50µl washed Protein G was added and incubation continued a further 1 hour at 4°C. Protein G was collected by centrifugation at 13,000 xg for 2 minutes at 4°C. Beads were then washed extensively in wash buffer containing 0.5M NaCl followed by wash buffer. Following the final wash beads were resuspended in 50µl SDS-PAGE sample buffer, heated to 37°C for 5 minutes and immune complexes resolved by SDS-PAGE. Once dried radiolabelled proteins were visualised by autoradiography on Kodak XAR-5 film at -70°C.

## 2.26 Rabbit immunisation for production of polyclonal antibodies

Specific polyclonal antibodies were generated by Cocalico Biologicals Inc., Reamstown, PA., USA, using their standard immunisation protocol. Antibodies were raised by immunisation of rabbits or guinea pigs with GST-fusion proteins containing subunit major intracellular regions. Primary immunisations were administered in Complete Freund's adjuvant and all boosts in Incomplete Freund's adjuvant. All immunisations were administered intradermally at multiple sites in each animal and the immune response monitored by screening of preimmune and postimmune testbleed samples by dot-blot analysis.

## 2.27 Screening of immune response by dot-blot analysis

5µg immunising antigen was applied to nitrocellulose discs (0.5cm diameter) which were dried then blocked for 1 hour at room temperature in blocking buffer (Tris-Buffered Saline (TBS: 0.5M Tris-

HCl pH7.4, 2M NaCl) containing 0.5%(w/v) Tween-20 and 5% Marvel). Discs were incubated for 1 hour at room temperature in blocking buffer containing serum and unbound antibody removed by washing 5x10 minutes at room temperature in blocking buffer. Antibody incubation was repeated using Horse Radish Peroxidase (HRP)-conjugated secondary antibody in blocking buffer. Unbound antibody was removed by extensive washing in blocking buffer. Discs were washed 2x5 minutes in TBS/0.5%(w/v) Tween-20 and excess buffer removed. HRP-conjugated secondary antibody was then detected using an enhanced chemiluminescent substrate (ECL: Amersham) as per the manufacturer's instructions.

## 2.28 Affinity purification of polyclonal antibodies

Antigens were covalently coupled to an activated gel matrix (Affigel-15; Biorad) according to the manufacturer's instructions. Coupling of antigen was monitored by Bradford assay of unbound protein.

Columns were washed with 20 column volumes 10mM Tris-HCl pH 7.5, then 10 volumes 100mM glycine pH 2.8 followed by 10mM Tris-HCl pH 8.8 until the pH of column eluates reached 8.8. Columns were then washed with 10 volumes 100mM triethylamine pH 11.5 followed by 10mM Tris-HCl pH 7.5 until column eluates reached pH 7.5. 5ml crude sera were diluted 10-fold in 10mM Tris-HCl pH 7.5 and applied to the column 3-4 times. The column was washed with 20 volumes 10mM Tris-HCl pH 7.5 followed by 20 volumes 10mM Tris-HCl pH 7.5 containing 500mM NaCl. Bound antibody was eluted in 5 column volumes 100mM glycine pH 2.8 and collected in 0.5 column volume 1M Tris-HCl pH 8.0. Columns were then washed with 10mM Tris-HCl pH8.8 until column eluates reached pH 8.8 and antibodies were eluted in 5 column volumes 100mM triethylamine

pH 11.5 collected in 0.5 column volume 1M Tris-HCl pH 8.0. Antibody elution was monitored using the Bradford protein assay. Antibody containing fractions were combined and dialysed overnight against several changes of PBS at 4°C. Protein concentration was determined by measurement of $A_{280}$ (1.0 $OD_{280}$=0.8mg IgG/ml). Glycerol was added to 10%(w/v) and antibody solutions were stored at -80°C.

## 2.29 Electrophysiological determination of GABA-induced whole cell currents from HEK293 cells

Cells were transfected as described above (2.20) using combinations of cDNAs encoding receptor subunits and the S65T mutated jellyfish green fluorescent protein (GFP; Heim *et al.*, 1995). Transfected cells were identified by the expression of GFP fluorescence using a Nikon Diaphot 300 microscope with filter block B-2A.

24-72 hours post transfection single transfected cells were identified and membrane currents were measured using an Axopatch 200B amplifier (Axon Instruments). Patch pipettes (1-5MΩ) were fabricated from thin-walled borosilicate glass and filled with pipette electrolyte (140mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM Hepes, 11mM EGTA, 2mM ATP; pH 7.1). Cells were continuously superfused with Krebs solution (140mM NaCl, 4.7mM KCl, 1.2mM $MgCl_2$, 2.5mM $CaCl_2$, 10mM Hepes, 11mM Glucose; pH7.4). Membrane currents were filtered at 5-10kHz, displayed on a Gould 2200S ink-pen chart recorder and stored on a Racal Store 4 FM tape recorder for analysis.

Responses measured at a holding potential of -40mV, were evoked by fast application of 10μM GABA to transfected cells using a modified Y-tube positioned within 100-200μm of the recorded cell (Wooltorton *et al.*, 1997b).

Endogenous PKA activation was achieved by inclusion of 300mM cAMP in the pipette electrolyte. The time taken for cAMP to reach steady-state concentration within HEK293 cells was calculated assuming linear diffusion from the recording pipette throughout the cytoplasm and modelled using Fick's first law of diffusion:

$$\tau = V \,.\, R/\rho \,.\, D$$

where t is the time constant of diffusion, V is the volume of a HEK293 cell estimated as $10.47 \times 10^{-15}$ $m^3$, R the resistance of the patch pipette is 5MΩ, ρ the resistivity of the patch pipette electrolyte is 0.52 $\Omega \,.\, m^{-1}$ and D the diffusion coefficient for cAMP is approximately $3 \times 10^{-10}$ $m^2.s^{-1}$. The time constant, τ, calculated from these values was 33.56 s. Therefore allowing 3 minutes after formation of the whole-cell recording configuration was sufficient to enable cAMP to reach steady-state concentration within a single HEK293 cell.

## 2.30 Measurement of surface $GABA_A$ receptors in transiently transfected HEK293 cells

9E10 anti-*myc* antibody (Evan *et al.*, 1985) was labelled to a specific activity of 500 Ci/mMol using [$^{125}$I]-Bolton and Hunter reagent (Amersham International) according to the manufacturer's instructions.

Mock transfected HEK293 cells or cells expressing receptor subunits modified by N-terminal inclusion of the *myc*-9E10 epitope were plated at approximately $10^5$ cells/$cm^2$. Cells were cooled rapidly to 4°C and blocked in DMEM (Gibco-BRL) supplemented with 0.5% (w/v) BSA and 25mM Hepes; pH7.4 for 30 minutes. 10-15 μg/ml [$^{125}$I]-9E10 antibody was added to the medium and incubated a further 90

minutes. Cells were then washed extensively in PBS containing 0.5% (w/v) BSA. Cell surface bound [$^{125}$I]-9E10 anti-*myc* antibody was quantified by counting $\gamma$ emissions using a Wallac 1261 Multigamma gamma counter.

# 3 Identification of PKA, PKG, PKC and CamKII phosphorylation sites within the intracellular domains of $GABA_A$-R β2 and β3 subunits.

## 3.1 Introduction

Well characterised second messenger-regulated protein kinases can be activated by many neurotransmitter and hormone ligands binding their cognate receptors (Berridge, 1984; Gilman, 1984). Given the large amounts of such kinases found in brain it is not surprising that they can regulate the function of numerous proteins involved in synaptic transmission (Schulman, 1991; Raymond *et al.*, 1993). $GABA_A$-R function can be enhanced or reduced by activation of protein kinases in neuronal preparations (Raymond *et al.*, 1993; Moss and Smart, 1996). This diversity of effects of kinase activation has not been correlated with individual receptor subunit phosphorylation. In order to develop a complete understanding of receptor regulation it is necessary to determine which protein kinases phosphorylate individual receptor subunits.

To define the effects of protein kinase activation on $GABA_A$-R function, previous investigations have used recombinant receptor subunits expressed in heterologous expression systems. Receptors composed of α, β and γ subunits have been widely studied as this receptor combination has been shown to reconstitute many of the properties of neuronal receptors (Pritchett *et al.*, 1989b). Studies have been carried out using *Xenopus laevis* oocytes and mammalian fibroblasts (eg. HEK293 cells) as model expression systems. Recombinant receptors have consistently been shown to be regulated by protein kinase activation in these systems (Moss and Smart, 1996). The function of these receptors can be enhanced or reduced on activation or inhibition of PKA, PKC or tyrosine kinases (Sigel *et*

*al.*, 1991; Leidenheimer *et al.*, 1992; Moss *et al.*, 1992b; Angelotti *et al.*, 1993b; Leidenheimer *et al.*, 1993; Krishek *et al.*, 1994; Lin *et al.*, 1994; Moss *et al.*, 1995; Kapur and Macdonald, 1996; Lin *et al.*, 1996; Wan *et al.*, 1997a). Regulation of recombinant receptors reproduces many but not all of the features of neuronal receptor regulation, discrepancies between neuronal and recombinant receptors may be due to subunit heterogeneity in the brain.

Analysis of deduced protein sequences from receptor subunit cDNAs has indicated that certain $GABA_A$-R subunits contain concensus sequences for protein phosphorylation within their putative intracellular regions (Table 1). The γ2 subunit for example contains potential sites for phosphorylation by PKC and CamKII (Whiting *et al.*, 1990; Harvey *et al.*, 1994). Similarly, all mammalian β subunits contain a concensus site for phosphorylation by cAMP-dependent protein kinase (PKA). This site is contained within the sequence RRRXSQLK where X=A (β1 and β2) or X=S (β3) (Moss and Smart, 1996). This conserved site within the β1 subunit (S409) has been shown to be phosphorylated *in vitro* by PKA, PKC, PKG and CamKII (Moss *et al.*, 1992a; McDonald and Moss, 1994).

Identification of phosphorylation sites has been difficult due to heterogeneity and low abundance of $GABA_A$-Rs in brain. To circumvent these technical problems recombinant methods have been used to identify the individual subunits and amino acid residues which are phosphorylated by various protein kinases. One useful technique which has assisted in this analysis has involved the expression and purification of subunit intracellular regions as soluble glutathione-*S*-transferase (GST) gene fusions in *E. coli* (Smith and Johnson, 1988).

*In vitro* phosphorylation of these proteins has been used as a method for the preliminary identification of protein kinases which can phosphorylate receptor subunits. Phosphopeptide mapping and phosphoamino acid analysis of phosphorylated GST-fusion proteins allows biochemical characterisation of the charge distribution surrounding phosphorylation sites, determination of the spatial distribution of these sites and identification of phosphorylated residues within the protein. Combined with site-directed mutagenesis these techniques have proven succesful in identifying a number of phosphorylation sites in the $GABA_A$-R β1 and γ2 subunits (Moss *et al.*, 1992a; Moss *et al.*, 1992b; McDonald and Moss, 1994; Krishek *et al.*, 1994 and Moss *et al.*, 1995).

Previous studies employing these techniques have demonstrated PKC, CamKII and c-*src* phosphorylation of the intracellular region of the γ2 subunit and PKA, PKG, PKC and CamKII phosphorylation of the β1 subunit. These studies have also identified the sites phosphorylated by each kinase within these subunits (Moss *et al.*, 1992a; McDonald and Moss, 1994; Moss *et al.*, 1995; Valenzuela *et al.*, 1995). Phosphorylation of recombinant subunits expressed in HEK293 cells by PKA, PKC and $pp60^{v\text{-}src}$ have been used to verify these sites, indicating the fidelity of the *in vitro* method for phosphorylation site identification (Moss *et al.*, 1992b; Krishek *et al.*, 1994; Moss *et al.*, 1995).

To date all studies of recombinant $GABA_A$-R regulation by protein kinases have used receptors containing the β1 subunit (Moss and Smart, 1996). Differential regulation of neuronal $GABA_A$-R function on kinase activation may be due to functional differences due to receptor subunit heterogeneity. In particular, receptor β subunits are differentially expressed in various regions of the brain. Therefore it

is necessary to identify protein kinases which phosphorylate all mammalian β subunits and to determine the sites at which such phosphorylation can occur. Given the previous success of this method, *in vitro* phosphorylation of GST-fusion proteins was used to identify sites phosphorylated within murine β2 and β3 subunits by purified PKA, PKC, PKG and CamKII.

## 3.2 Expression and purification of subunit intracellular loop GST-fusion proteins

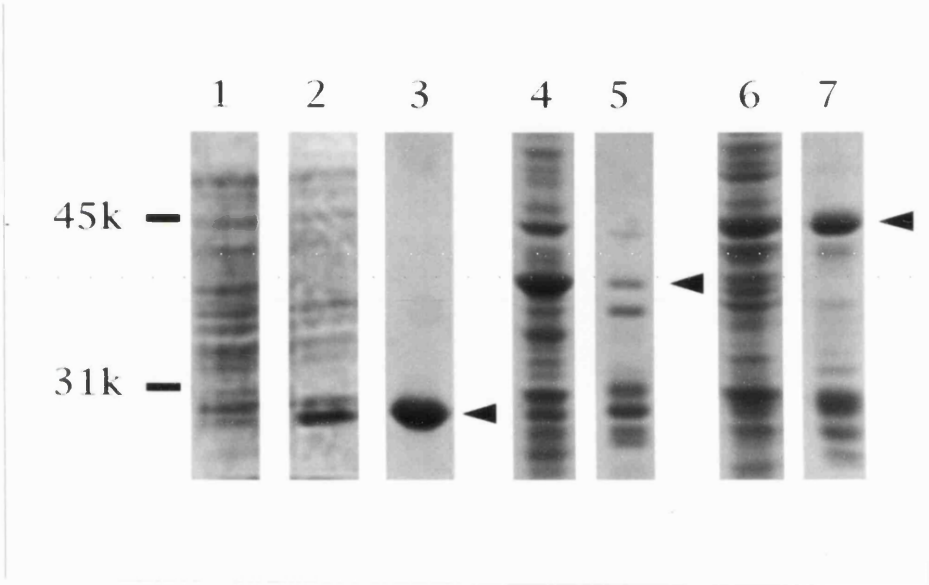
Biochemical characterisation of subunit phosphorylation sites *in vitro* required production of large amounts of highly purified subunit intracellular loop proteins. To achieve this level of expression and purity, the β2 and β3 subunit intracellular domains were subcloned in the correct reading frame into the pGex-4T/3 bacterial expression vector and expressed as GST-fusion proteins in *E. coli* as described (Smith and Johnson, 1988). Expressed proteins were affinity purified to near homogeneity on glutathione-agarose beads yielding approximately 3mg protein per litre induced bacterial culture. Purified proteins were separated by SDS-PAGE and visualised by staining with coomassie blue (Figure 2).

Solubilised protein products with apparent relative molecular masses of ~28kD (GST alone), ~42kD (GST-β2) and ~45kD (GST-β3) were observed by SDS-PAGE (Figure 2). These observed relative molecular masses are in agreement with those predicted from primary amino acid sequences of these proteins. Lower molecular weight bands were also seen in the preparations of purified GST-β2 and GST-β3 (Figure 2: lanes 5 & 7), however they were not seen in affinity purified GST (Figure 2: lane 3). These proteins of lower molecular mass probably represent degradation products of the full length fusion proteins as previously described (McDonald and Moss, 1994; Moss *et al.*, 1992a).

The solubility, purity and high yields of these proteins allowed their inclusion in *in vitro* kinase assays as potential substrates for phosphorylation by purified protein kinases.

## Figure 2

Expression and purification of the β2 and β3 subunit major intracellular domains as GST-fusion proteins




**1**- Uninduced *E. coli* cell lysate; **2**- IPTG induced *E. coli* cell lysate expressing GST alone; **3**- affinity purified GST; **4**- IPTG induced *E. coli* cell lysate expressing GST-β2; **5**- affinity purified GST-β2; **6**- IPTG induced *E. coli* cell lysate expressing GST-β3; **7**- affinity purified GST-β3. Moleclar weight standards are indicated in kilodaltons (k).

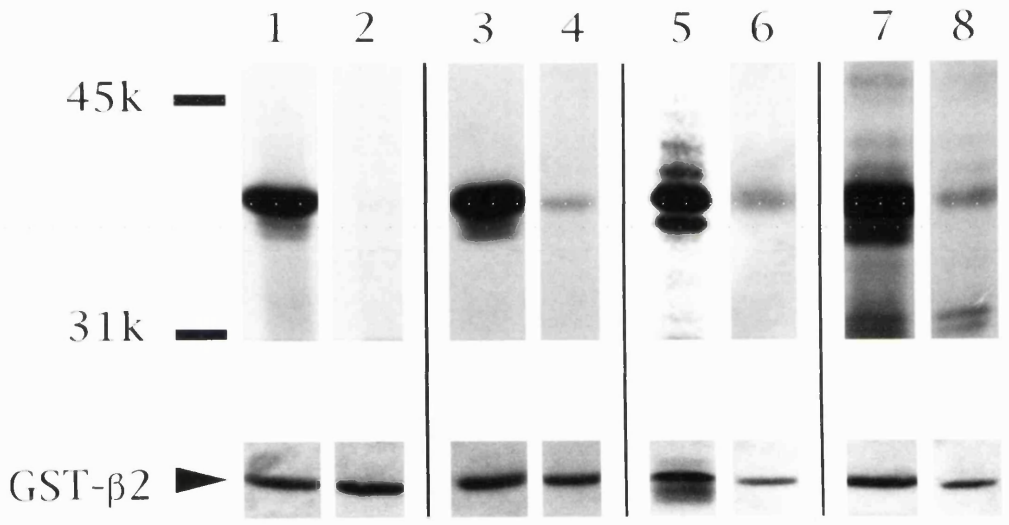
## 3.3 Phosphorylation of purified GST-β2 by PKA, PKG, PKC and CamKII

GST-β2 fusion protein included in *in vitro* kinase assays was rapidly phosphorylated to high stoichiometry at low kinase concentrations (~0.4ng/ul) by PKA (0.1-0.2pmol $PO_4$/pmol protein), PKG (0.1-0.2pmol $PO_4$/pmol protein), PKC (0.3pmol $PO_4$/pmol protein) and CamKII (0.3pmol $PO_4$/pmol protein) (Table 2 and Figure 3A: lanes 1, 3, 5 & 7). These stoichiometries were not increased at higher kinase concentrations or by lengthening the duration of the assay to 30 minutes. None of the kinases studied caused any detectable phosphorylation of the GST backbone alone (Moss *et al.*, 1992a; McDonald and Moss, 1994).

Phosphorylated GST-β2 was analysed by phosphoamino acid analysis (Figure 4) and phosphopeptide mapping (Figure 5). Phosphoamino acid analysis showed GST-β2 was phosphorylated exclusively on serine residues by all four kinases studied (Figure 4). Tryptic phosphopeptide mapping indicated the generation of two major positively charged phosphopeptides in PKA, PKG and CamKII phosphorylated GST-β2 or three positively charged phosphopeptides in PKC phosphorylated GST-β2 (Figure 5). These major phosphopeptides were seen to vary in intensity suggesting that they may be the result of incomplete trypsin digestion of the phosphorylated protein. Phosphorylation of GST-β2 on serine residues within the same tryptic phosphopeptides indicated that a single site may be phosphorylated by all four kinases used in this study. The previously identified PKA and PKC phosphorylation site at S409 in the β1 subunit (RRRA<u>S</u>$^{409}$QLK) is strongly conserved at S410 in the β2 subunit (RRRA<u>S</u>$^{410}$QLK) (Moss *et al.*, 1992a). This amino acid residue was therefore an obvious candidate site for phosphorylation of the β2 subunit and was modified by site-directed

**Figure 3**

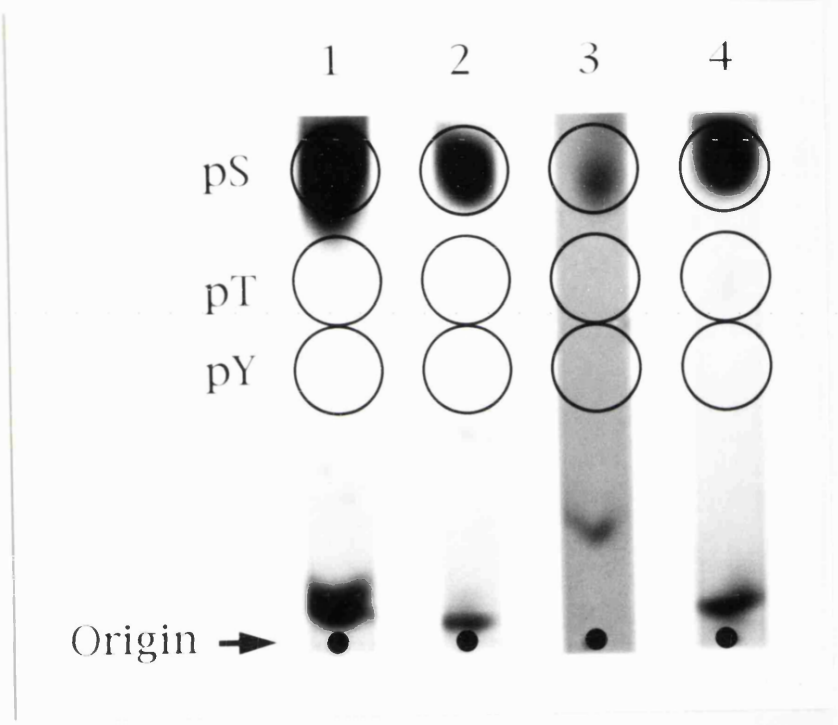
Phosphorylation of the β2 subunit major intracellular loop by PKA, PKG, PKC and CamKII




**Upper panel**- GST-β2 was phosphorylated by PKA (lane1), PKG (3), PKC (5) and CamKII (7). GST-β2S410A was not phosphorylated by PKA (2), PKG (4), PKC (6) or CamKII (8). Reaction products were separated by SDS-PAGE and visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k). **Lower panel**- Following separation by SDS-PAGE proteins were visualised by staining with coomassie brilliant blue to ensure equal protein loading.

**Figure 4**

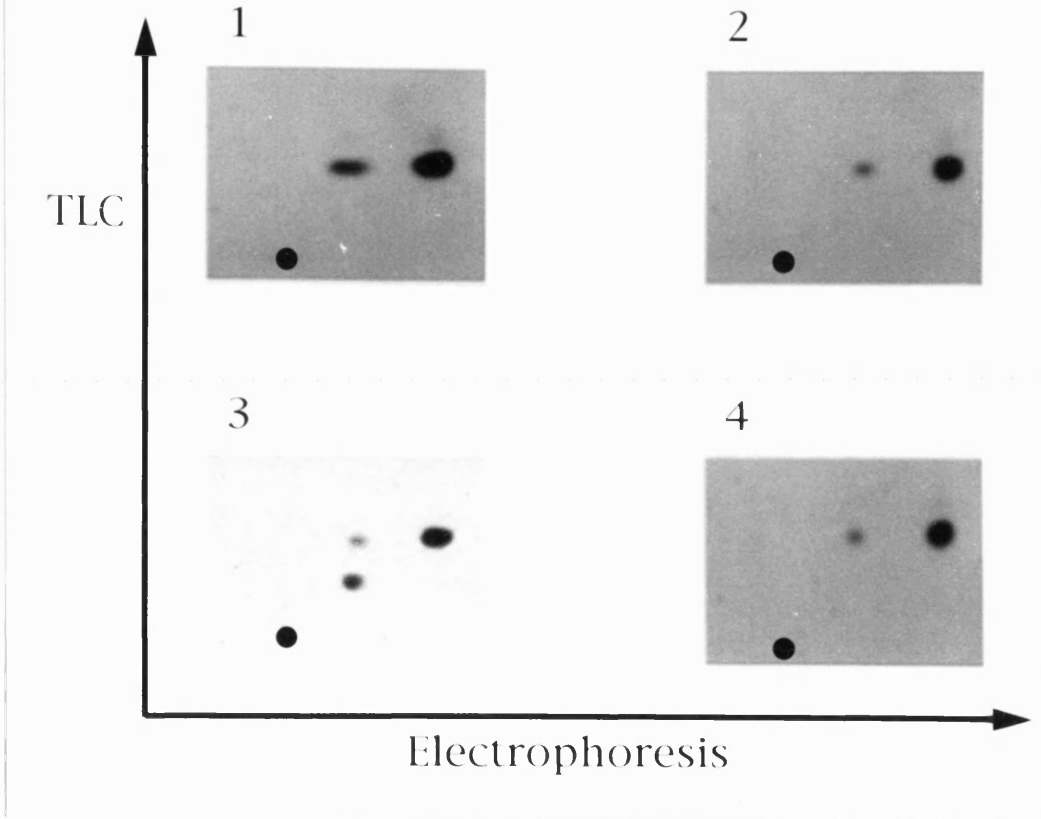
Phosphoamino acid analysis of GST-β2 following phosphorylation by PKA, PKG, PKC and CamKII




GST-β2 phosphorylated by PKA (1), PKG (2), PKC (3) or CamKII (4) was purified by SDS-PAGE and examined by phosphoamino acid analysis. Phosphoprotein hydrolysate was subjected to electrophoresis from the origin (•). Migration of phosphoamino acid standards (pS, pT, pY) was visualised by ninhydrin staining as indicated.

**Figure 5**

Phosphopeptide map analysis of GST-β2 following phosphorylation by PKA, PKG, PKC and CamKII




Phosphopeptide map analysis of SDS-PAGE purified GST-β2 after phosphorylation by PKA (1), PKG (2), PKC (3) or CamKII (4). Tryptic phosphoprotein digests were separated from the origin (•) by electrophoresis in the first dimension (arrowheads) followed by thin layer chromatography in the second dimension. Phosphopeptides were visualised by autoradiography.

mutagenesis.

Conversion of S410 to alanine within the major intracellular loop of the β2 subunit allowed production of a mutant fusion protein (GST-β2S410A). This mutated fusion protein was not phosphorylated to a significant level by PKA, PKG, PKC or CamKII (Table 2; Figure 3A: lanes 2, 4, 6 & 8). Inclusion of similar amounts of wildtype and mutated proteins in kinase assays was demonstrated by staining with coomassie brilliant blue (Figure 3B).

This abolition of phosphorylation suggests that S410 (RRRA**S**$^{410}$QLK) is the principal site of phosphorylation for each of these protein kinases within the β2 subunit *in vitro*.

### 3.4 Phosphorylation of purified GST-β3 by PKA, PKG, PKC and CamKII.

GST-β3 fusion protein included in *in vitro* kinase assays was rapidly phosphorylated to high stoichiometry at low kinase concentrations (~0.4ng/ul) by PKA (0.2-0.4pmol $PO_4$/pmol protein; Figure 6A: lane 1), PKG (0.1-0.3pmol $PO_4$/pmol protein; Figure 6A: lane 4), PKC (0.3pmol $PO_4$/pmol protein; Figure 9A: lane 1) and CamKII (0.4pmol $PO_4$/pmol protein; Figure 12A: lane 1) (Table 2). Stoichiometries were not increased at higher kinase concentrations or by lengthening the duration of the assay to 30 minutes.

Phosphoamino acid analysis (Figure 7) showed that both PKA and PKG caused phosphorylation of GST-β3 on serine residues only and by phosphopeptide map analysis it was seen that this produced two major positively charged tryptic phosphopeptides (Figure 8). The major phosphopeptides produced by trypsin digestion of PKA and

PKG phosphorylated GST-β3 were similar to those seen within GST-β2 (Figure 5). The phosphorylation sites at S409 in the β1 subunit (RRRA**S**$^{409}$QLK) and S410 in the β2 subunit (RRRA**S**$^{410}$QLK) is conserved with a small modification at S409 of the β3 subunit (RRRS**S**$^{409}$QLK). For these reasons both S408 and S409 in GST-β3 were candidate sites for PKA and PKG phosphorylation and were modified by site-directed mutagenesis.

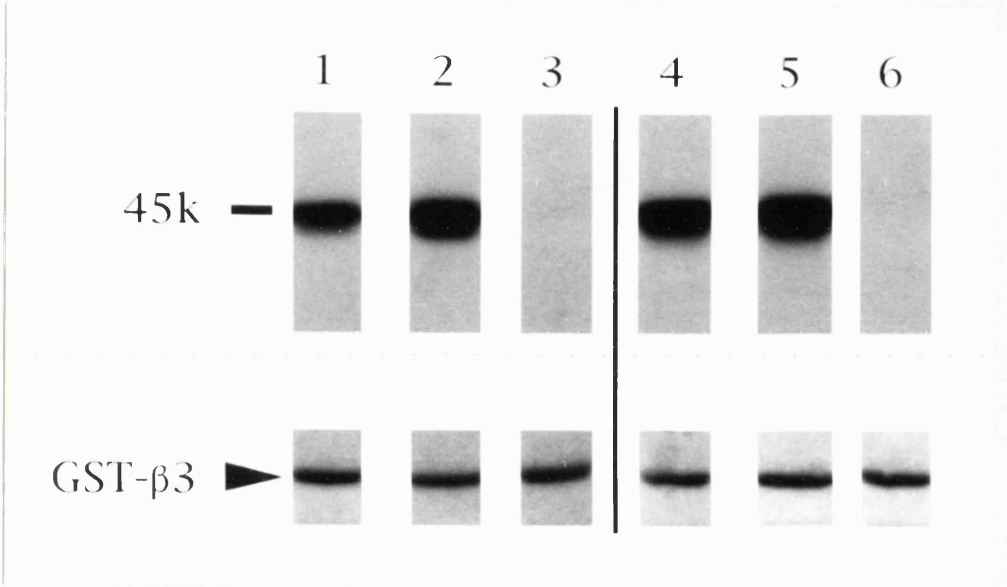
Mutation of S408 or S409 to alanine within the β3 subunit allowed production of two singly-mutated intracellular loop fusion proteins termed GST-β3S408A and GST-β3S409A. GST-β3S408A was phosphorylated to high stoichiometry by PKA and PKG (Figure 6A: lanes 2 & 5), however GST-β3S409A was not significantly phosphorylated by either kinase (Figure 6A: lanes 3 & 6). Coomassie brilliant blue staining was used to ensure that similar amounts of wildtype and mutated proteins were included in kinase assays (Figure 6B). The abolition of GST-β3 phosphorylation identifies S409 (RRRS**S**$^{409}$QLK) as the sole site of PKA and PKG phosphorylation in the major intracellular domain of the β3 subunit.

Both GST-β3S408A and GST-β3S409A were rapidly phosphorylated to intermediate stoichiometries by PKC (0.1pmol $PO_4$/pmol protein) (Table 2, Figure 9A: lanes 2 & 3). Therefore after individual conversion of S408 or S409 to alanine, at least one other site of phosphorylation by PKC remained.

Phosphopeptide map analysis indicated that PKC phosphorylated residues within the same tryptic phosphopeptides in GST-β3, GST-β3S408A and GST-β3S409A (Figure 11).

**Figure 6**

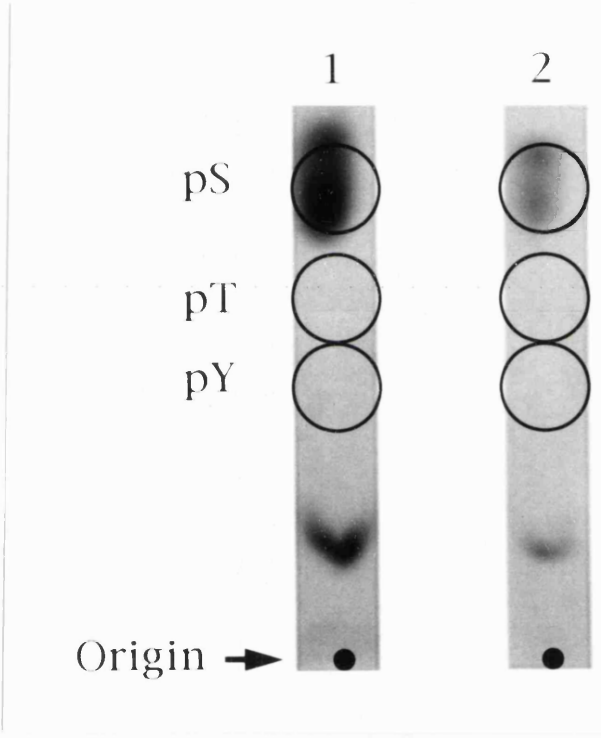
Phosphorylation of the β3 subunit major intracellular loop by PKA and PKG




**Upper panel**- GST-β3 was phosphorylated by PKA (lane1) and PKG (4), GST-β3S408A was also phosphorylated by both PKA (2) and PKG (5) however GST-β3S409A was not phosphorylated either by PKA (3) or PKG (6). Reaction products were separated by SDS-PAGE and visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k). **Lower panel**- Following separation by SDS-PAGE proteins were visualised by staining with coomassie brilliant blue to ensure equal protein loading.

**Figure 7**

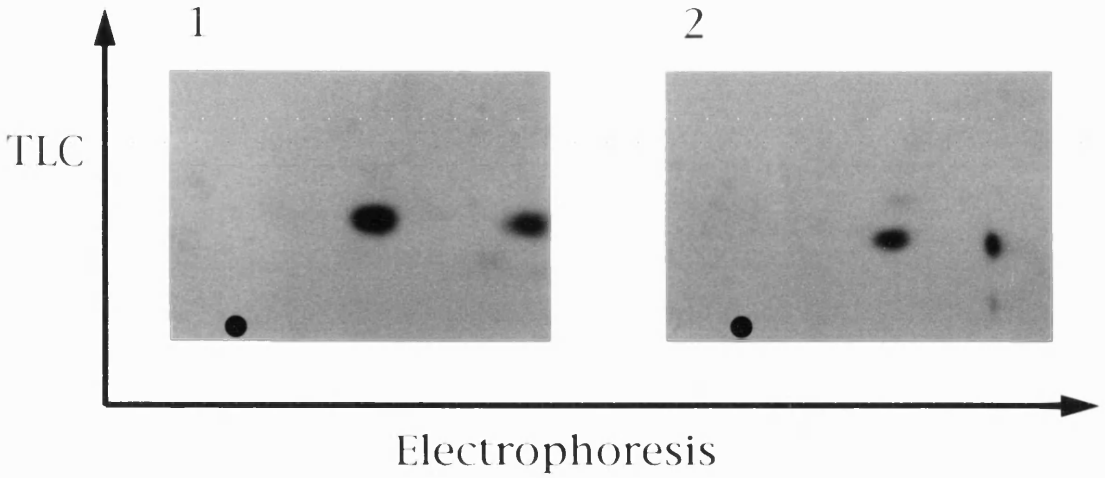
Phosphoamino acid analysis of GST-β3 following phosphorylation by PKA and PKG




GST-β3 phosphorylated by PKA (1) and PKG (2) was purified by SDS-PAGE and examined by phosphoamino acid analysis. Phosphoprotein hydrolysate was subjected to electrophoresis from the origin (•). Migration of phosphoamino acid standards (pS, pT, pY) was visualised by ninhydrin staining as indicated.

**Figure 8**

Phosphopeptide map analysis of GST-β3 following phosphorylation by PKA and PKG




Phosphopeptide map analysis of SDS-PAGE purified GST-β3 after phosphorylation by PKA (1) or PKG (2). Tryptic phosphoprotein digests were separated from the origin (•) by electrophoresis in the first dimension (arrowheads) followed by thin layer chromatography in the second dimension. Phosphopeptides were visualised by autoradiography.

As S408 is the closest potential phosphoacceptor residue to S409, it would be the most likely phosphorylation site that could occur in the same phosphopeptides as S409. Therefore both S408 and S409 were modified by site-directed mutagenesis within a single β3 subunit.

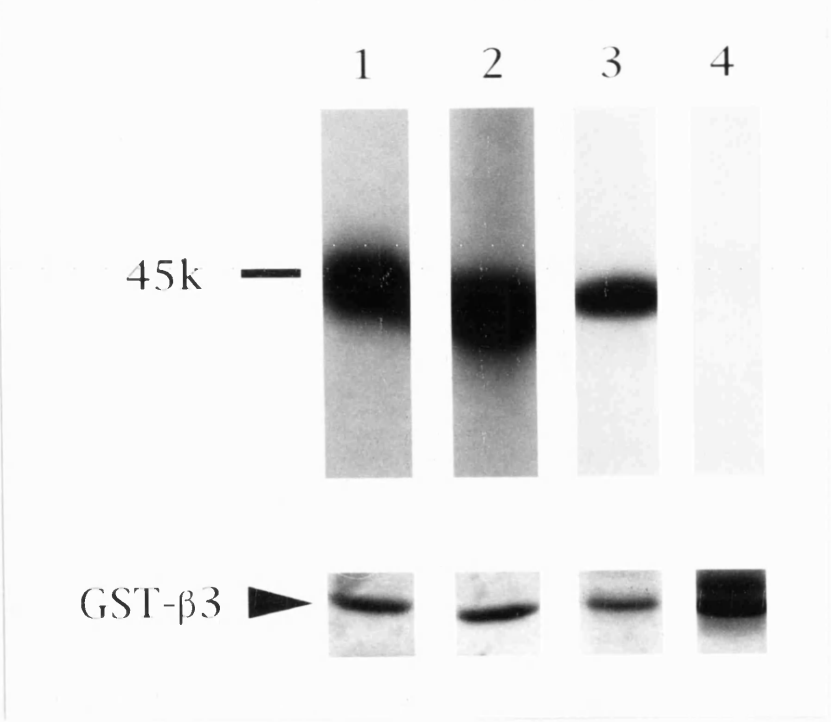
Conversion of S408 and S409 to alanine within the same β3 subunit allowed production of a double mutant intracellular domain fusion protein (GST-β3S408;409A). PKC did not phosphorylate GST-β3S408;409A to a significant level (Figure 9A: lane 4). Similar protein loadings were demonstrated by protein staining following separation by SDS-PAGE (Figure 9B).

A small amount of phosphothreonine (pT) was seen by phosphoamino acid analysis after PKC phosphorylation of GST-β3 (Figure 10), suggesting the presence of another site of PKC phosphorylation. Phosphorylation of this site however represents a very small fraction of total PKC phosphorylation by comparison to that on S408 and S409 (Table 2). Together these data suggest that S408 and S409 (RRR$\underline{S}^{408}\underline{S}^{409}$QLK) represent the major sites of PKC phosphorylation within the intracellular loop of the β3 subunit.

Both GST-β3S408A and GST-β3S409A were phosphorylated by CamKII as was the double mutant GST-β3S408;409A (Figure 12A: lanes 2, 3 & 4). Phosphoamino acid analysis indicated that CamKII phosphorylation of GST-β3 took place predominantly on Serine residues (Figure 13) while phosphopeptide map analysis indicated the generation of a novel neutral tryptic phosphopeptide after CamKII phosphorylation of GST-β3 (Figure 14). Together these data indicate the presence of at least one other site for CamKII phosphorylation apart from S408/S409.

**Figure 9**

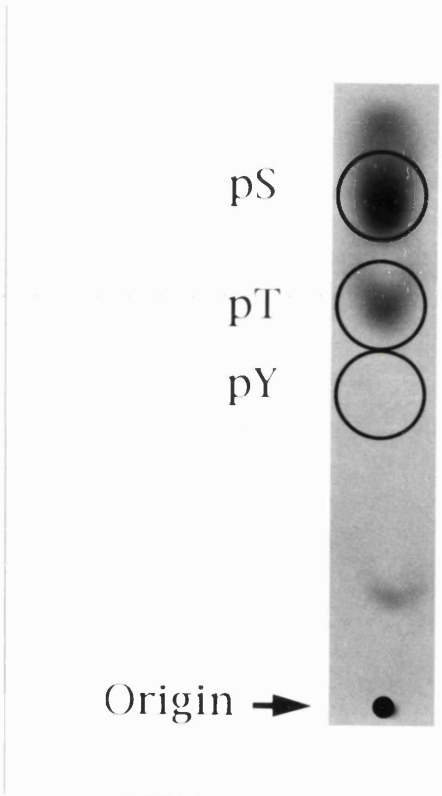
Phosphorylation of the β3 subunit major intracellular loop by PKC




**Upper panel**- PKC phosphorylated GST-β3 (lane1), GST-β3S408A (2) and GST-β3S409A (3) but not GST-β3S408;409A (lane 4). Reaction products were separated by SDS-PAGE and visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k). **Lower panel**- Following separation by SDS-PAGE proteins were visualised by staining with coomassie brilliant blue to ensure equal protein loading.

**Figure 10**

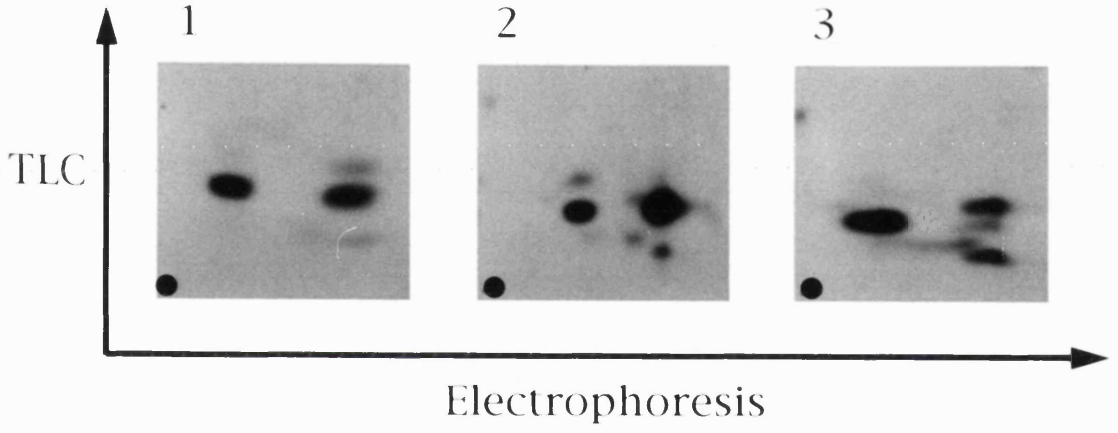
Phosphoamino acid analysis of GST-β3 following phosphorylation by PKC




PKC phosphorylated GST-β3 was purified by SDS-PAGE and examined by phosphoamino acid analysis. Phosphoprotein hydrolysate was subjected to electrophoresis from the origin (•). Migration of phosphoamino acid standards (pS, pT, pY) was visualised by ninhydrin staining as indicated.

**Figure 11**

Phosphopeptide map analysis of GST-β3 following phosphorylation by PKC



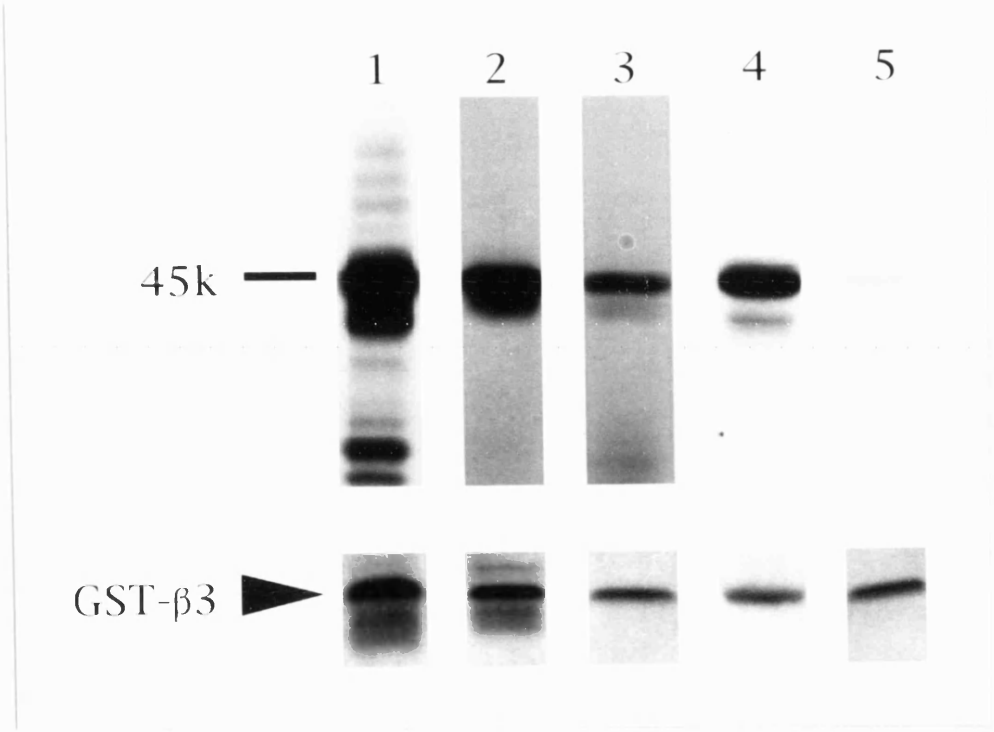

Phosphopeptide map analysis of SDS-PAGE purified GST-β3 (1), GST-β3S408A (2) and GST-β3S409A (3) after phosphorylation by PKC. Tryptic phosphoprotein digests were separated from the origin (•) by electrophoresis in the first dimension (arrowheads) followed by thin layer chromatography in the second dimension. Phosphopeptides were visualised by autoradiography.

The previously identified CamKII phosphorylation site S384 in the β1 subunit (RKPL**S**$^{384}$SREG; McDonald and Moss, 1994) is conserved at S383 in the β3 subunit (RKQ**S**$^{383}$MPK). Therefore S383 was a candidate for CamKII phosphorylation in GST-β3 and was modified by site-directed mutagenesis. S383 was converted to alanine within the mutant subunit β3S408;S409A. This allowed production of the mutant fusion protein GST-β3S383;408;409A which was not phosphorylated to a detectable level by CamKII (Figure 12A: lane 6). Coomassie brilliant blue staining following SDS-PAGE indicated the presence of equal amounts of wildtype and mutated proteins in each kinase assay (Figure 12B). This abolition of phosphorylation identifies S383 as a site for phosphorylation of GST-β3 by CamKII. Phosphopeptide map analysis of GST-β3S409A after CamKII phosphorylation shows no phosphorylation on the tryptic peptides containing S408 and S409 (Figure 14), this indicates that S408 in GST-β3 is not phosphorylated by CamKII under these conditions.

Together these data identify S383 (RKQ**S**$^{383}$MPK) and S409 (RRRS**S**$^{409}$QLK) as the major sites of CamKII phosphorylation within the β3 subunit.

**Figure 12**

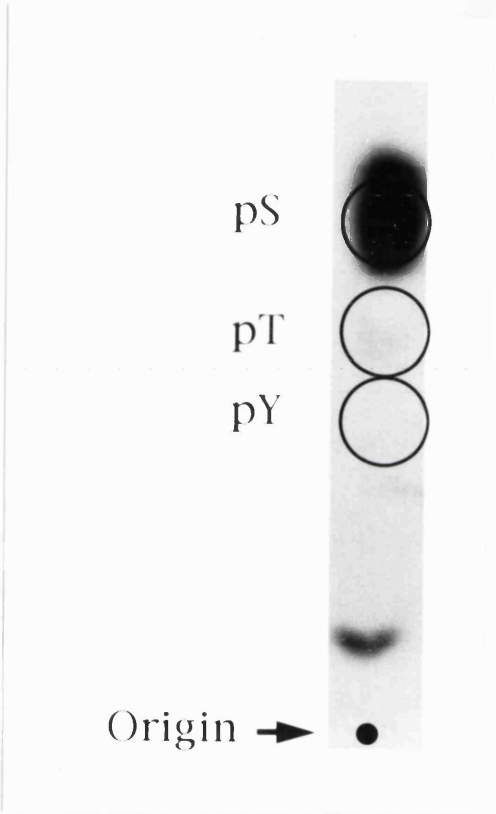
Phosphorylation of the β3 subunit major intracellular loop by CamKII




**Upper panel**- CamKII phosphorylated GST-β3 (lane 1), GST-β3S408A (2), GST-β3S409A (3), GST-β3S408;409A (4) but not GST-β3S383;408;409A (5). Reaction products were separated by SDS-PAGE and visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k). **Lower panel**- Following separation by SDS-PAGE proteins were visualised by staining with coomassie brilliant blue to ensure equal protein loading.

**Figure 13**

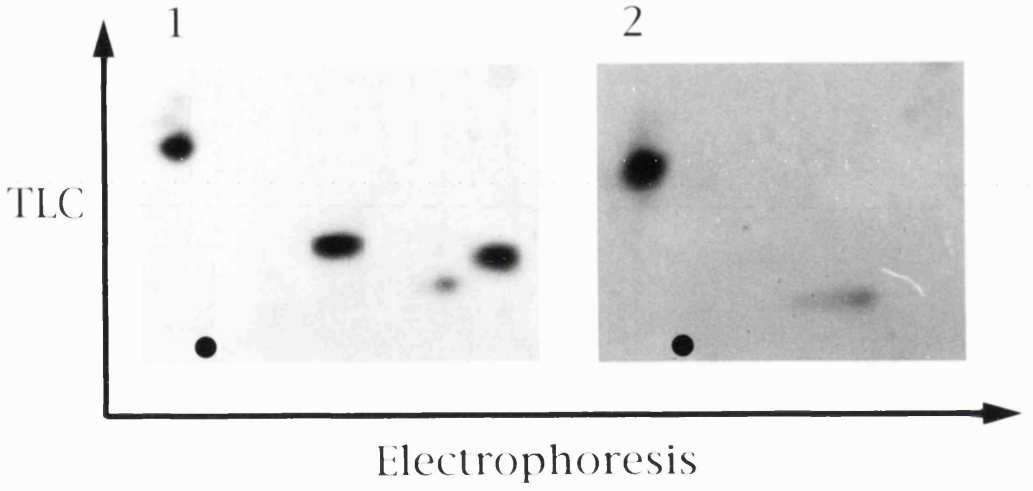
Phosphoamino acid analysis of GST-β3 following phosphorylation by CamKII




CamKII phosphorylated GST-β3 was purified by SDS-PAGE and examined by phosphoamino acid analysis. Phosphoprotein hydrolysate was subjected to electrophoresis from the origin (•). Migration of phosphoamino acid standards (pS, pT, pY) was visualised by ninhydrin staining as indicated.

**Figure 14**

Phosphopeptide map analysis of GST-β3 following phosphorylation by CamKII




Phosphopeptide map analysis of SDS-PAGE purified GST-β3 (1) and GST-β3S409A (2) after phosphorylation by CamKII. Tryptic phosphoprotein digests were separated from the origin (•) by electrophoresis in the first dimension (arrowheads) followed by thin layer chromatography in the second dimension. Phosphopeptides were visualised by autoradiography.

## 3.5 Conclusions

Protein phosphorylation is widely regarded as an important mechanism for modulating the function of many ligand-gated ion channels including the $GABA_A$-Rs (Raymond *et al.*, 1993; Moss and Smart, 1996). Biochemical analysis of phosphorylation of the ligand-gated ion channels, with the notable exception of the AChR (Swope *et al.*, 1992), has proven difficult. In the case of the $GABA_A$-Rs this is largely due to receptor heterogeneity and low abundance. In these experiments soluble *E. coli* fusion proteins containing the intracellular domains of $GABA_A$-R β subunits were used to study subunit phosphorylation by a number of second messenger activated protein kinases. This method requires that fusion proteins *in vitro* contain similar determinants for protein phosphorylation as do the native receptor subunits *in vivo*. To date this approach to phosphorylation site identification within other $GABA_A$-R subunits has proven succesful (Krishek *et al.*, 1994; Moss *et al.*, 1992a; Moss *et al.*, 1992b and Moss *et al.*, 1995). Therefore it would seem that fusion protein phosphorylation *in vitro* is a suitable model for preliminary studies of receptor subunit phosphorylation.

These data show that the major intracellular domain of the β2 subunit is phosphorylated to high stoichiometry by PKA, PKG, PKC and CamKII and that S410 (RRRA$\underline{S}^{410}$QLK) is the sole site of phosphorylation by each of these protein kinases. Similarly the β3 subunit intracellular domain was also phosphorylated to high stoichiometry by each of these protein kinases. S409 (RRRS$\underline{S}^{409}$QLK) is identified as the sole site of phosphorylation by PKA and PKG, and one of the sites of phosphorylation by PKC and CamKII within the β3 subunit intracellular domain.

**Table 2**

Stoichiometry of PKA, PKG, PKC and CamKII phosphorylation of GST fusion proteins containing the β2 and β3 subunit major intracellular loops

| | PKA | PKG | PKC | CamKII |
|---|---|---|---|---|
| β2 | 0.18±0.05 | 0.16±0.07 | 0.31±0.04 | 0.30±0.10 |
| β2S410A | - | - | 0.03±0.01 | - |
| β3 | 0.34±0.08 | 0.27±0.07 | 0.34±0.04 | 0.42±0.12 |
| β3S408A | - | - | 0.15±0.03 | 0.23±0.08 |
| β3S409A | - | - | 0.12±0.03 | 0.18±0.07 |
| β3S408;409A | nd | nd | - | 0.16±0.09 |
| β3S383;408;409A | nd | nd | nd | - |

GST-fusion protein were phosphorylated as described, separated by SDS-PAGE and visualised by autoradiography. Phosphoprotein bands were excised and $^{32}P$ incorporation quantified by Cerenkov counting. Stoichiometries are calculated in pmol PO4/pmol protein. Values indicate mean ± s.e.m. and n=3. nd ; not determined. - ; not detectable.

Previous studies have shown the conserved equivalent residue S409 in the β1 subunit to be phosphorylated by these same four protein kinases *in vitro* (Moss *et al.*, 1992a; McDonald and Moss, 1994) and by PKA and PKC in HEK293 cells (Moss *et al.*, 1992b; Krishek *et al.*, 1994). Identification of this conserved serine residue as a site of protein phosphorylation by PKA, PKC, PKG and CamKII in all mammalian $GABA_A$-R β subunits suggests a critical role for the phosphorylation of β subunits in $GABA_A$-R regulation *in vivo*.

In addition the intracellular domain of the β3 subunit is also shown to be phosphorylated on S408 (RRR**S**$^{408}$SQLK) by PKC and on S383 (RKQ**S**$^{383}$MPK) by CamKII. S408 of the β3 subunit is not present in the other β subunits, it therefore represents a unique site for phosphorylation within this subunit. While absent from β2, S383 of the β3 subunit is found in the β1 subunit (S384) within the sequence RKPL**S**$^{384}$SREG where it is also phosphorylated by CamKII *in vitro* (McDonald and Moss, 1994). Therefore these data indicate a potential role for differential β subunit phosphorylation in the regulation of receptor function.

There is considerable evidence that phosphorylation can modulate $GABA_A$ receptor function and the effects of protein kinase activation on receptor function are complex. In neurons PKA, PKG, PKC or CamKII activation can enhance or inhibit $GABA_A$ receptor function depending on the preparation studied (Macdonald and Olsen, 1994; Moss and Smart, 1996; Kapur and Macdonald, 1996; Raymond *et al.*, 1993). These different functional effects are not well understood but may be due to heterogeneity of $GABA_A$-R structure or subunit composition. It is tempting to speculate that differential regulation of receptor function by protein kinases may be determined by β subunit identity. Such a role for receptor β subunits is possible given

their unique phosphorylation profiles determined by these experiments.

Whether the sites identified from these *in vitro* experiments, within the β2 and β3 subunits are phosphorylated *in vivo* remains to be established. The differences observed in the stoichiometries of phosphorylation by PKA for GST-β2 (0.18±0.05 pmol $PO_4$/pmol protein) and GST-β3 (0.34±0.08 pmol $PO_4$/pmol protein) may reflect the suitability of full length β2 and β3 subunits as PKA substrates *in vivo*. It will therefore be of interest to determine the functional effects of phosphorylation on $GABA_A$-Rs containing β2 or β3 subunits coexpressed with defined α and γ subunits. Such experiments should reveal whether phosphorylation of distinct β subunits can differentially modulate $GABA_A$-R function.

# 4 Production of antibodies specific for $GABA_A$-R β subunits.

## 4.1 Introduction

Characterisation of $GABA_A$-R subunit phosphorylation in the cellular environment requires specific purification of subunit polypeptides following metabolic labelling of intracellular ATP with $^{32}P$ and activation of specific protein kinases. Phosphorylated subunits may then be separated according to their size by SDS-PAGE and visualised by autoradiography. Biochemical subunit analysis allows correlation of specific phosphorylation events with specific effects on receptor function.

The preferred method for such biochemical analysis of protein phosphorylation involves the use of specific antibodies to purify proteins by immunoprecipitation. The use of this technique in $GABA_A$-R subunit purification has many advantages over purification by affinity chromatography. Most importantly, the use of antibodies allows purification of higher yields of receptor subunits from brain membranes or heterologous cell expression systems. Antibodies also precipitate specific subunits regardless of receptor subunit combination, thereby allowing comparison of subunits without complications arising from receptor subtype diversity in neurons. Previous investigations using this approach have confirmed the identity of phosphorylation sites within β1 and γ2 subunits (Moss *et al.*, 1992b; Krishek *et al.*, 1994; Moss *et al.*, 1995).

Large scale purification of $GABA_A$-R complexes by affinity chromatography on benzodiazepine affinity-columns initiated the cloning of the first cDNAs encoding receptor subunits (Sigel and Barnard, 1984; Schofield *et al.*, 1987). Other receptor subunit cDNAs

were then cloned and their primary amino acid sequences have been described (Stephenson, 1995; Davies *et al.*, 1997b). This information has allowed the design of subunit sequence specific peptides which have been used to raise a variety of subunit specific antibodies. Using peptides derived from N-terminal amino acid sequences, polyclonal and monoclonal antibodies have been raised which specifically recognise receptor α, β, γ and δ subunit isoforms (Schoch *et al.*, 1985; Sato and Neale, 1989; Stephenson *et al.*, 1989; Benke *et al.*, 1990; Stephenson *et al.*, 1990; Buchstaller *et al.*, 1991; Pollard *et al.*, 1991; Endo and Olsen, 1992; Machu *et al.*, 1993b).

Due to the high degree of sequence homology between β subunit isoforms, the development of β subunit-specific antibodies has proven difficult (Ymer *et al.*, 1989). The best described and most widely used anti-β subunit antibody is bd17, a monoclonal antibody which was raised against purified $GABA_A$-R subunits. Epitope mapping has shown that bd17 recognises the N-terminal 15 amino acid residues of the β2 subunit (Ewert *et al.*, 1990), an epitope common to both β2 and β3 subunits. bd17 is therefore unsuitable for studies requiring differentiation of these two β subunits.

To circumvent the problems of multiple subunit specificity, sequence specific peptides from β subunit intracellular domains have recently been used to produce polyclonal antibodies which recognise receptor β subunits (Endo and Olsen, 1992; Machu *et al.*, 1993b; Benke *et al.*, 1994). These antibodies have allowed identification of individual receptor β subunits in neurons by immunoprecipitation, western blotting and immunofluorescence.

A previous report of $GABA_A$-R β1 subunit phosphorylation described production of an antibody specific for this subunit (Moss *et al.*, 1992b). This polyclonal antibody was raised by immunisation of

rabbits with the intracellular domain of the β1 subunit expressed and purified as a GST-fusion protein from *E. coli* (Moss *et al.*, 1992a). Subunit specific antibodies affinity-purified from immune serum were shown to immunoprecipitate β1 subunit polypeptides specifically from HEK293 cells after metabolic labelling. Use of these antibodies was succesful in the characterisation of β1 subunit phosphorylation by PKA and PKC in HEK293 cells (Moss *et al.*, 1992b; Krishek *et al.*, 1994).

In order to produce antibodies specific for $GABA_A$-R β subunit isoforms it was decided to raise subunit specific polyclonal antibodies directed against the intracellular domains of $GABA_A$-R β subunits expressed as GST-fusion proteins. Polyclonal antibodies raised against such a large immunogen do not depend on recognition of a single epitope for immunoprecipitation and therefore are ideal for the study of receptor subunit phosphorylation which may alter individual intracellular epitopes. The production of such antibodies would allow study of recombinant receptor phosphorylation in HEK293 cells and could also facilitate immunoprecipitation of receptor subunits from neuronal preparations and whole brain lysates. Rabbits were immunised with GST-fusion proteins containing the intracellular domains of receptor β1, β2 and β3 subunits. Immune serum was screened for the ability to recognise receptor domains expressed as GST-fusion proteins and specific antibodies were affinity purified. Subunit specificity of purified antibodies was then examined by western blotting, immuno-fluorescence and immunoprecipitation of full length receptor subunits expressed in HEK293 cells.

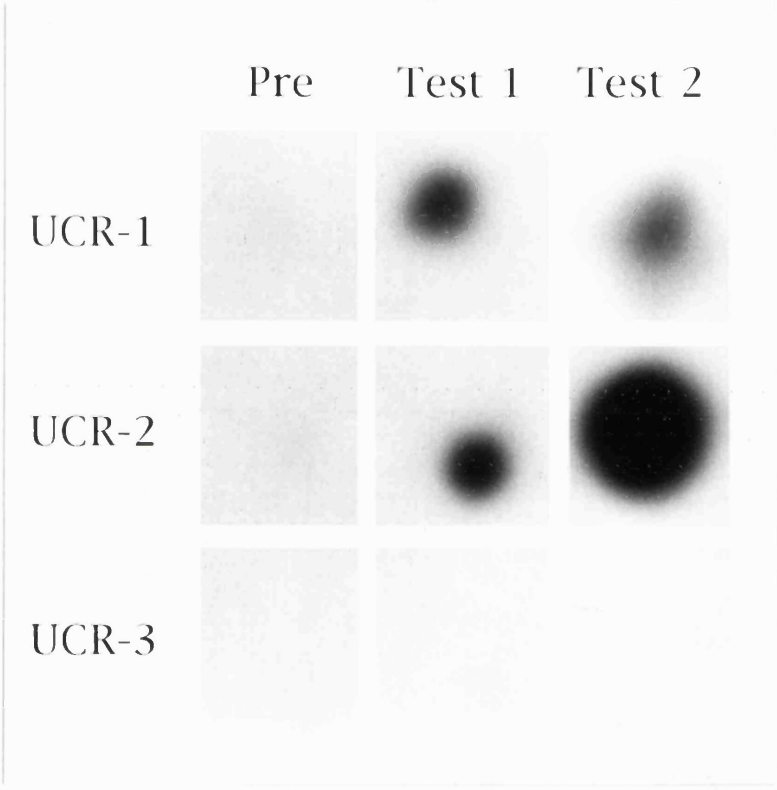
## 4.2 Rabbit immunisation with fusion proteins containing intracellular domains of $GABA_A$-R β subunits

GST-fusion proteins containing intracellular domains of receptor β1, β2 and β3 subunits were expressed and purified from *E. coli* as described (Moss *et al.*, 1992a; Figure 2). These proteins purified to near homogeneity, were used as antigens for immunisation of rabbits and production of polyclonal antisera. Prior to immunisation serum samples were taken (prebleed) to establish baseline immunoreactivity levels. Primary immunisations were carried out by subcutaneous injection of protein (100μg) at numerous sites in Freund's complete adjuvant. Boost immunisations of 50μg protein in Freund's incomplete adjuvant were administered 14, 21 and 49 days post immunisation. Test serum samples were obtained at days 35 and 56 post immunisation to allow monitoring of immune responses (Figure 15).

GST-β1, GST-β2 and GST-β3 were each used to raise immune responses in two rabbits as described. Rabbit immune responses were monitored by dot-blot assay using dilute serum to detect affinity purified GST-fusion proteins immobilised on nitrocellulose (Figure 15). This screening indicated the production of antibodies in one of six rabbits (UCR-2) immunised with intracellular domains of receptor β subunits (Figure 15). None of the six rabbits showed immunoreactivity toward GST-fusion proteins before immunisation and one rabbit (UCR-3) failed to develop a significant immune response even after repeated immunisation with GST-β2 (Figure 16). All other rabbits developed a low level immune response in test bleed 1, taken at day 35 post immunisation. This response did not increase significantly by day 56 post immunisation (eg. UCR-1) and was therefore not deemed suitable for further study (Figure 15).

**Figure 15**

Immune response in rabbit UCR-2 immunised with GST-β1




5µg GST-β1 immobilised on nitrocellulose circles was detected by crude serum from rabbit UCR-2 (1/100).

Progression of the immune response was monitored by screening with preimmune serum (Pre), serum obtained 35 days post immunisation (Test 1) and serum obtained 56 days post immunisation (Test 2).

Rabbit UCR-2 showed a significant response to immunisation with GST-β1 which increased significantly between days 35 and 56 post immunisation (Figure 15). Given the robust nature of this immune response serum from rabbit UCR-2 was further studied in an attempt to produce antibodies specific for $GABA_A$-R β subunits.

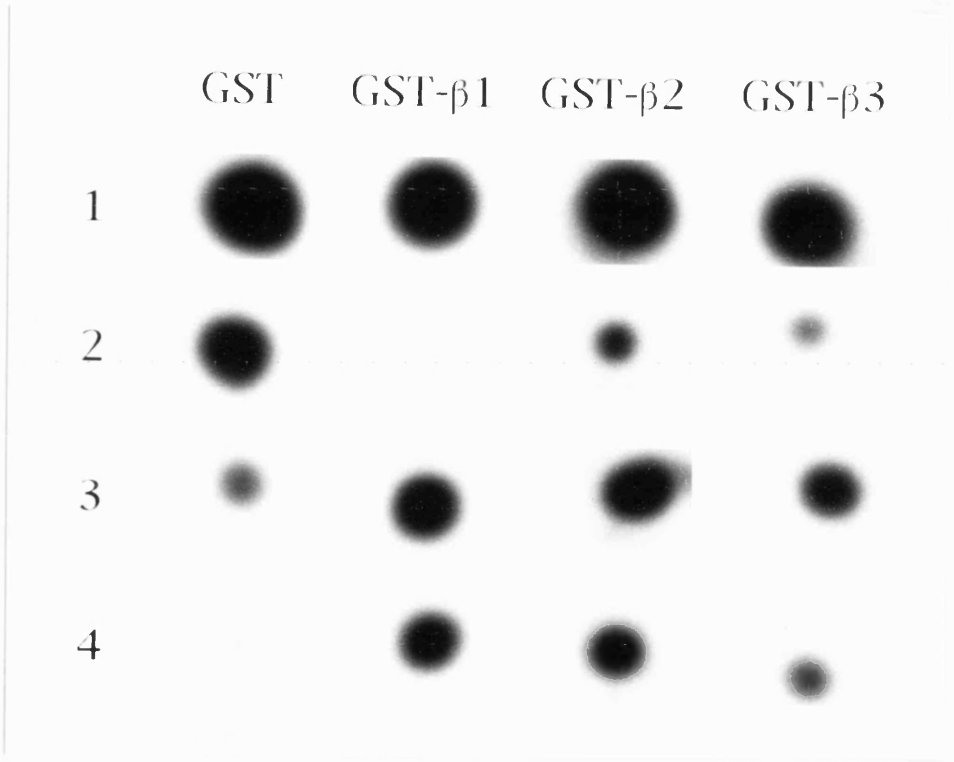
## 4.3 Affinity purification of anti-β subunit antibodies from rabbit serum

Having identified a robust immune response in rabbit UCR-2 after immunisation with GST-β1 (Figure 15), antibodies were affinity purified from immune serum to allow their characterisation. Subunit specific antibodies were purified by binding to GST-β1 covalently linked to an affigel matrix.

Affinity purified UCR-2 antibodies were tested for the ability to recognise GST-fusion proteins containing intracellular domains of $GABA_A$-R β subunits by dot blot assay (Figure 16). Fusion proteins (5μg) which had been quantitated using the Bradford assay and checked by SDS-PAGE (Figure 2), were immobilised on nitrocellulose then detected using crude serum (1/100) from rabbits showing high (UCR-2) and low (UCR-1) immune responses to GST-β1, affinity purified UCR-2 antibodies (5μg/ml) and UCR-2 antibodies (5μg/ml) blocked by preincubation with GST (50μg/ml). Serum from rabbit UCR-2 diluted 1/100 showed high reactivity with GST, GST-β1, GST-β2 and GST-β3 (Figure 16; row 1) while serum from rabbit UCR-1 (1/100) showed reactivity to GST alone (Figure 16; row 2). Affinity purified UCR-2 antibodies (5μg/ml) showed low reactivity to GST alone, but high reactivity with GST-β1, GST-β2 and GST-β3 (Figure 16; row 3).

**Figure 16**

Dot-blot detection of β1, β2 and β3 subunit major intracellular loops by rabbit UCR-2 serum




5μg GST, GST-β1, GST-β2 or GST-β3 immobilised on nitrocellulose (indicated by columns) were detected by crude serum from rabbit UCR-2 (1/100; row 1), crude serum from rabbit UCR-1 (1/100; row 2), antibodies (5μg/ml) affinity purified from UCR-2 using GST-β1 (row 3) or antibodies (5μg/ml) affinity purified from UCR-2 preincubated with GST (50μg/ml; row 4).

Preincubation of UCR-2 antibodies (5μg/ml) with GST (50μg/ml) eliminated GST reactivity but had no significant effect on reactivity with GST-β1, GST-β2 or GST-β3 (Figure 16; row 4). Together these data suggested the purification of specific antibodies which reacted with the intracellular domains of all three mammalian β subunits.

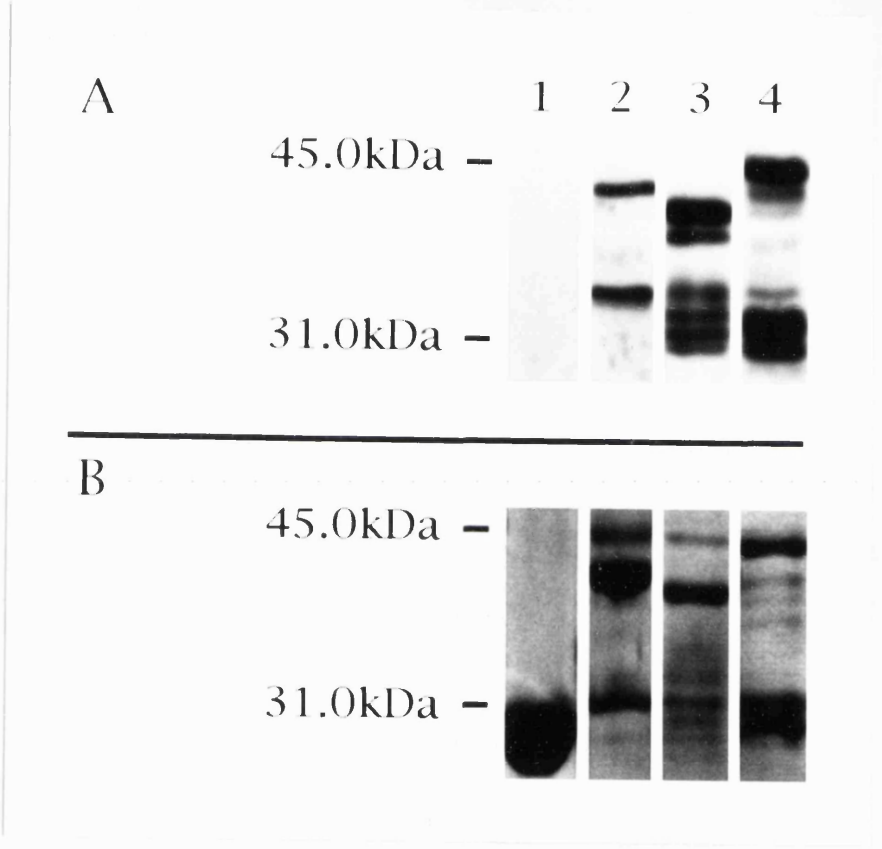
In order to further test the specificity of affinity purified UCR-2 antibodies, their ability to detect GST-fusion proteins separated by SDS-PAGE was determined by western blotting (Figure 17). UCR-2 antibodies (5μg/ml) preincubated with GST (50μg/ml) did not recognise GST alone (Figure 17A; lane 1) they did however specifically recognise full length GST-β1 (Mr 43kDa; Figure 17A; lane 2), GST-β2 (Mr 42kDa; Figure 17A; lane 3) and GST-β3 (Mr 45kDa; Figure 17A; lane 4). The molecular weights of these proteins are in agreement with those predicted from this and previous studies (Figure 2 and Moss *et al.*, 1992a). UCR-2 antibodies also recognised lower molecular weight bands which probably represent degradation products of these fusion proteins (Figure 17A; lanes 2-4).

In order to ensure approximately equal protein loading in each well, proteins separated by SDS-PAGE were visualised by staining with coomassie brilliant blue (Figure 17B). Protein staining shows similar loading in each lane and indicates that proteins detected by UCR-2 antibodies are of similar molecular weights to affinity purified GST-fusion proteins.

Interestingly, while GST-β1 was used as the immunogen for rabbit UCR-2, polyclonal antibodies produced by this immune response seem to specifically recognise the intracellular domains of all mammalian $GABA_A$-R β subunits.

**Figure 17**

Western blot detection of β subunit major intracellular loops by affinity purified UCR-2 antibody




**A**. GST-fusion proteins separated by SDS-PAGE, transferred to nitrocellulose and detected by purified rabbit UCR-2 antibody (5μg/ml) preincubated with GST (50μg/ml). **1**- affinity purified GST (5μg); **2**- affinity purified GST-β1 (5μg); **3**- affinity purified GST-β2 (5μg); **4**- affinity purified GST-β3(5μg).

**B**. GST-fusion proteins separated by SDS-PAGE were visualised by staining with coomassie brilliant blue. **1**- affinity purified GST (5μg); **2**- affinity purified GST-β1 (5μg); **3**- affinity purified GST-β2 (5μg); **4**- affinity purified GST-β3(5μg).

Molecular weight standards are indicated in kilodaltons (kDa).

## 4.4 Subunit specificity of affinity purified UCR-2 antibodies determined by immunofluorescence

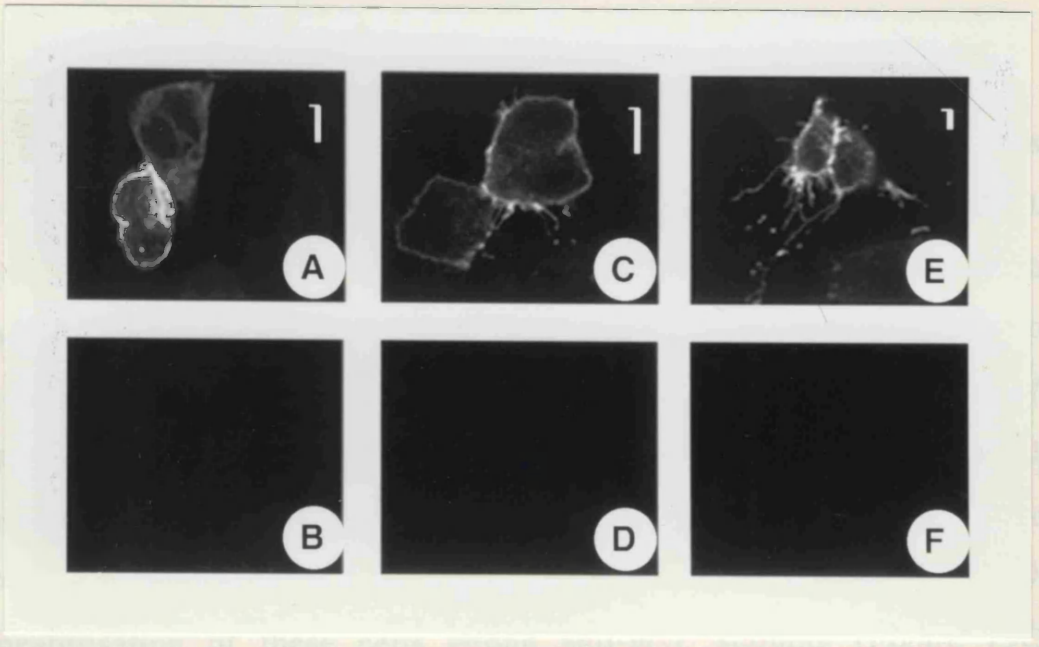
In order to confirm their specificity, affinity purified UCR-2 antibodies were examined for the ability to recognise full length receptor subunit polypeptides by immunofluorescence analysis of recombinant receptors expressed in HEK293 cells (Figures 18, 19 and 20). Cells were transiently transfected with the mammalian expression vector pGW1 containing cDNAs encoding $GABA_A$-R subunits and processed for immunofluorescence staining.

Cells expressing the α1 subunit alone containing a *myc* epitope tag near the amino terminus show no surface staining by the 9E10 anti-*myc* antibody (Connolly *et al.*, 1996a). Permeabilisation of these cells allows visualisation of α1 subunit expression by strong anti-*myc* staining which shows a distribution resembling the endoplasmic reticulum (Figure 18A). UCR-2 antibody (5μg/ml) preincubated with GST (50μg/ml) did not detect any protein in these permeabilised α1 subunit expressing cells (Figure 18B). Similar anti-*myc* staining of cells expressing the $\gamma 2L^{myc}$ subunit was seen after permeabilisation, but no protein was detected by GST-preincubated UCR-2 antibody (data not shown). These data indicate that UCR-2 antibody does not recognise the full length α1 or γ2L subunit polypeptides.

Coexpression of the β2 subunit with $\alpha 1^{myc}$ allows $\alpha 1^{myc}$-β2 receptor complexes to access the cell surface (Connolly *et al.*, 1996a). The presence of these receptor complexes at the cell surface was detected as strong anti-*myc* staining of $\alpha 1^{myc}$-β2 transfected cells without permeabilisation (Figure 18C). On permeabilisation of these cells affinity purified UCR-2 antibody did not detect the presence of the β2 subunit polypeptide (Figure 18D).

## Figure 18

Immunofluorescent detection of α1 and β2 subunit polypeptides by UCR-2 antibody



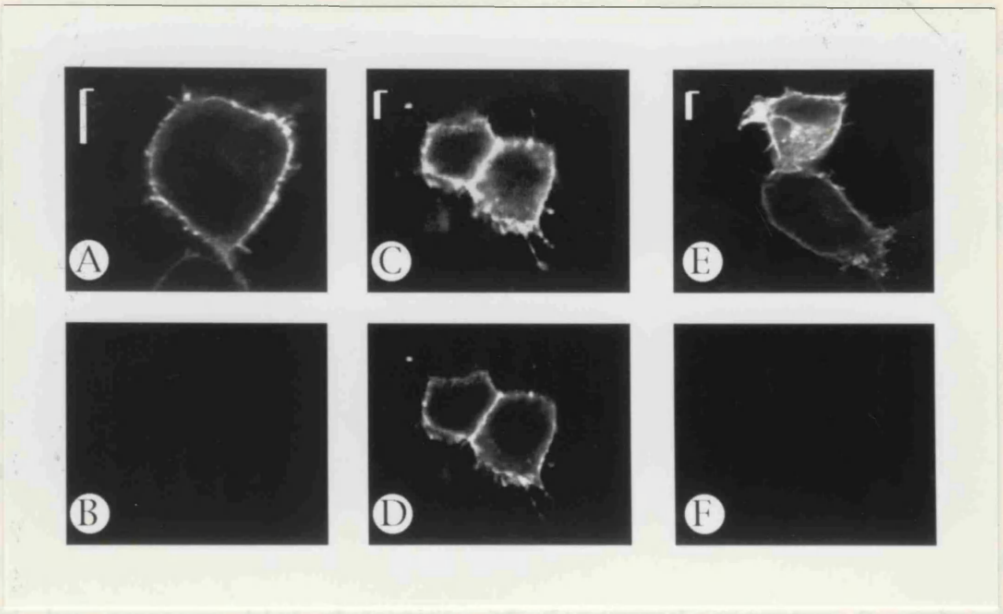

HEK293 cells expressing the $\alpha 1^{myc}$ subunit alone (**A** and **B**) or $\alpha 1^{myc}$ and β2 subunits (**C**, **D**, **E** and **F**) were processed for immunofluorescence staining with mouse 9E10 anti-*myc* antibody (5μg/ml; **A** and **C**- FITC), mouse anti-β2/β3 subunit antibody bd17 (5μg/ml; **E**- FITC) or UCR-2 antibody (5μg/ml) preincubated with 50μg/ml GST (**B**, **D** and **F**- Rhodamine). Staining was carried out in the presence of 0.05%(w/v) Triton X-100. Scale bars = 5μm.

Expression of the β2 subunit was also shown directly by staining with the anti-β2/β3 subunit monoclonal antibody bd17 (Ewert *et al.*, 1990; Figure 18E). Permeabilised cells which were positively identified as expressing the β2 subunit using bd17 also showed no staining by UCR-2 antibody (Figure 18F). Therefore, while UCR-2 recognises the GST-β2 protein (Figure 17) it does not seem to react with the full length recombinant β2 subunit polypeptide.

The ability of UCR-2 to recognise the β1 subunit polypeptide was then examined in HEK293 cells expressing $\alpha 1^{myc}$ and β1 subunits (Figure 19). Anti-*myc* staining of these cells was evident without permeabilisation, indicating the presence of α-β subunit receptor complexes on the cell surface as previously described (Connolly *et al.*, 1996a; Figure 19A). Under the same conditions no UCR-2 antibody staining was evident (Figure 19B) indicating that UCR-2 does not recognise any extracellular epitopes of the β1 subunit. On permeabilisation of these cells strong anti-*myc* staining (Figure 19C) and strong UCR-2 staining (Figure 19D) were observed close to the plasma membrane. Antibody specificity was indicated by abolition of UCR-2 staining (Figure 19F) but not anti-*myc* staining (Figure 19E) on preincubation of antibodies with GST-β1 (50μg/ml). These data indicate that the UCR-2 antibody specifically recognises the major intracellular domain of the full length β1 subunit polypeptide in the cellular environment. UCR-2 antibody recognition of the β3 subunit polypeptide was studied in HEK293 cells coexpressing $\alpha 1^{myc}$ and β3 subunits (Figure 20). Without permeabilisation strong anti-*myc* surface staining was observed (Figure 20A) indicating expression of the β3 subunit protein and the presence of $\alpha 1^{myc}$-β3 subunit receptor complexes at the cell surface (Connolly *et al.*, 1996a).

## Figure 19

Immunofluorescent detection of full length β1 subunit polypeptides by UCR-2 antibody




HEK293 cells expressing $\alpha 1^{myc}$ and β1 subunits were processed for immunofluorescence staining with mouse 9E10 anti-*myc* antibody (5μg/ml; **A**, **C** and **E**- FITC) or UCR-2 antibody (5μg/ml) preincubated with 50μg/ml GST (**B**, **D** and **F**- Rhodamine). Staining was carried out in the presence (**C**, **D**, **E** and **F**) or absence (**A** and **B**) of 0.05%(w/v) Triton X-100. Preincubation of antibodies with 50μg/ml GST-β1 was carried out to demonstrate specificity of antibody binding (**E** and **F**). Scale bars = 5μm.

β3 subunit expression was also detected by permeabilised staining with the monoclonal antibody bd17 (Ewert *et al.*, 1990; Figure 20E). No UCR-2 staining was evident in unpermeabilised cells (Figure 20B), indicating that UCR-2 antibody did not react with extracellular epitopes of the β3 subunit. Strong anti-*myc* staining was evident near the plasma membrane and intracellularly, on permeabilisation of these cells (Figure 20C). Permeabilisation also allowed strong UCR-2 staining of the β3 subunit to be seen (Figure 20D). Specificity of antibody reactivity was demonstrated by abolition of UCR-2 staining (Figure 20G) but not anti-*myc* staining (Figure 20F) on preincubation of antibodies with GST-β3 (50μg/ml), indicating specific reactivity between UCR-2 antibody and the major intracellular loop of the full length β3 subunit protein.

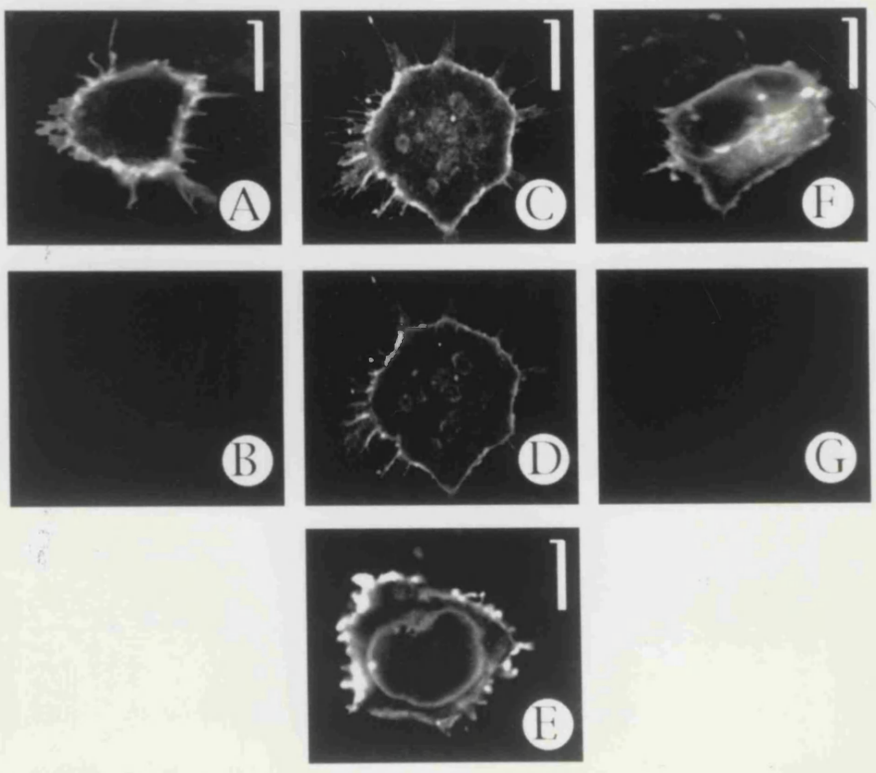
## 4.5 Subunit specificity of affinity purified UCR-2 antibodies determined by western blotting

Further confirmation of the specificity of UCR-2 antibodies was obtained by western blot detection of denatured receptor β subunit polypeptides separated by SDS-PAGE (Figure 21). HEK293 cells were transiently transfected with the mammalian expression vector pGW1 containing cDNAs encoding $\alpha 1^{myc}$ and γ2S subunits alone or in combination with β1, β2 or β3 subunits. Efficient transfection and expression of receptor subunits in transfected cells were determined by immunofluorescent detection using 9E10 anti-*myc*, bd17 anti-β2/β3 subunit and UCR-2 antibodies (Figures 18, 19 & 20).

Cell lysates were prepared from cerebellar granule cells and transiently transfected HEK293 cells. 50μg total protein from each lysate was separated by SDS-PAGE and transferred to nitrocellulose. Receptor subunit polypeptides were then detected by affinity

**Figure 20**

Immunofluorescent detection of full length β3 subunit polypeptides by UCR-2 antibody




HEK293 cells expressing $\alpha 1^{myc}$ and β3 subunits were processed for immunofluorescence staining with mouse 9E10 anti-*myc* antibody (5μg/ml; **A**, **C** and **F**- FITC), UCR-2 antibody (5μg/ml) preincubated with 50μg/ml GST (**B**, **D** and **G**- Rhodamine) or mouse bd17 anti-β2/β3 subunit antibody (5μg/ml; **E**- FITC). Staining was carried out in the presence (**C**, **D**, **E**, **F** and **G**) or absence (**A** and **B**) of 0.05%(w/v) Triton X-100. Preincubation of antibodies with 50μg/ml GST-β3 was carried out to demonstrate the specificity of antibody binding (**F** and **G**). Scale bars = 5μm.

purified UCR-2 antibody (5μg/ml) which had been preabsorbed with purified GST (50μg/ml). No polypeptide bands were detected in lysate prepared from HEK293 cells expressing $\alpha 1^{myc}$ and γ2 S subunits alone (Figure 21A; lane 1) confirming that these subunits are not recognised non-specifically in these cells. Multiple protein bands of relative molecular mass 55-60kDa approximately, were detected in lysate from cells expressing receptor $\alpha 1^{myc}$, γ2S and β3 subunits (Figure 21A; lane2). Similarly, a major protein band of approximately 58kDa was detected in lysate from cells expressing $\alpha 1^{myc}$, γ2S and β1 subunits (Figure 21A; lane 4). These molecular masses are in good agreement with predictions from the sequences of these proteins as well as previous reports from studies of receptor β subunits (Moss *et al.*, 1992b; Krishek *et al.*, 1994; Benke *et al.*, 1994). Interestingly, western blotting using affinity purified UCR-2 antibody did not detect β2 subunit polypeptides expressed in these cells (Figure 21A; lane 3).

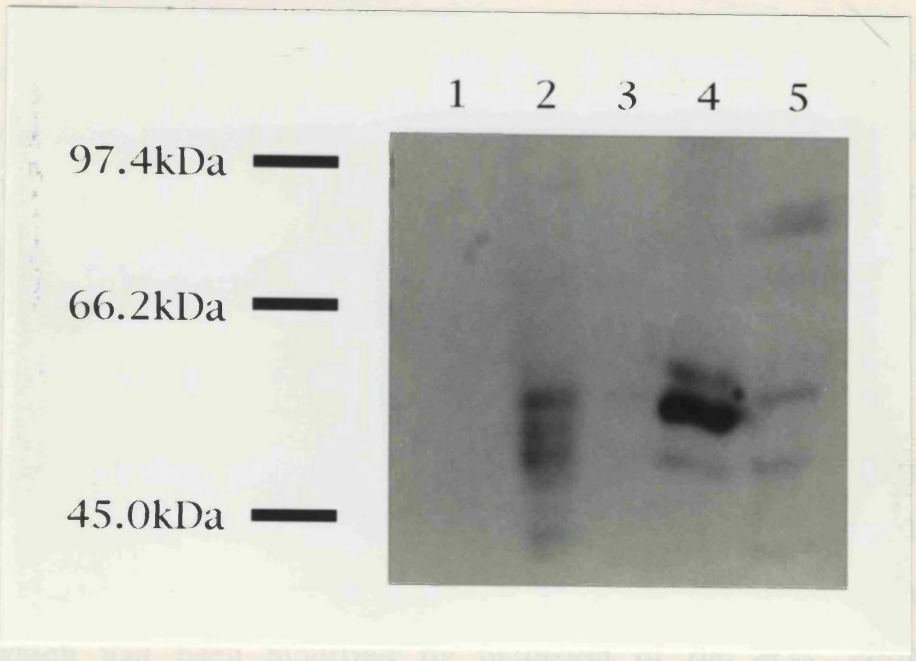
This antibody specificity observed by western blotting concurs precisely with the subunit specificity determined for UCR-2 antibodies by immunofluorescent staining of receptor β subunits in HEK293 cells (Figures 18, 19 & 20).

In cell lysates generated from cerebellar granule neurons two major polypeptides were detected with relative molecular masses of approximately 55 and 60kDa (Figure 21A; lane 5).

These bands correspond to two proteins detected in HEK cells lysates containing recombinant β3 subunits (Figure 21A; lane 2). The presence of β3 subunit polypeptides in these neurons is in agreement with previous studies of receptor subunit expression in the brain (Laurie *et al.*, 1992a).

**Figure 21**

Western detection of β subunit polypeptides by affinity purified UCR-2 antibody




Total cell lysate (50µg/lane) was separated by SDS-PAGE and transferred to nitrocellulose. **1**- HEK293 cells expressing $\alpha 1^{myc}$ and γ2S subunits; **2**- HEK293 cells expressing $\alpha 1^{myc}$, γ2S and β3 subunits; **3**- HEK293 cells expressing $\alpha 1^{myc}$, γ2S and β2 subunits; **4**- HEK293 cells expressing $\alpha 1^{myc}$, γ2S and β1 subunits; **5**- Rat (P15) cerebellar granule cell lysate. Molecular weight standards are indicated in kilodaltons (kDa). $GABA_A$-R β subunits were detected by UCR-2 anti-β subunit antibody (5µg/ml) preincubated with GST (50µg/ml).

The low level of β subunit detection in this neuronal lysate illustrates the relatively low abundance of $GABA_A$-Rs in these neurons and indicates the advantage of recombinant expression when studying these receptors biochemically.

## 4.6 Specific immunoprecipitation of receptor β subunits by affinity purified UCR-2 antibodies

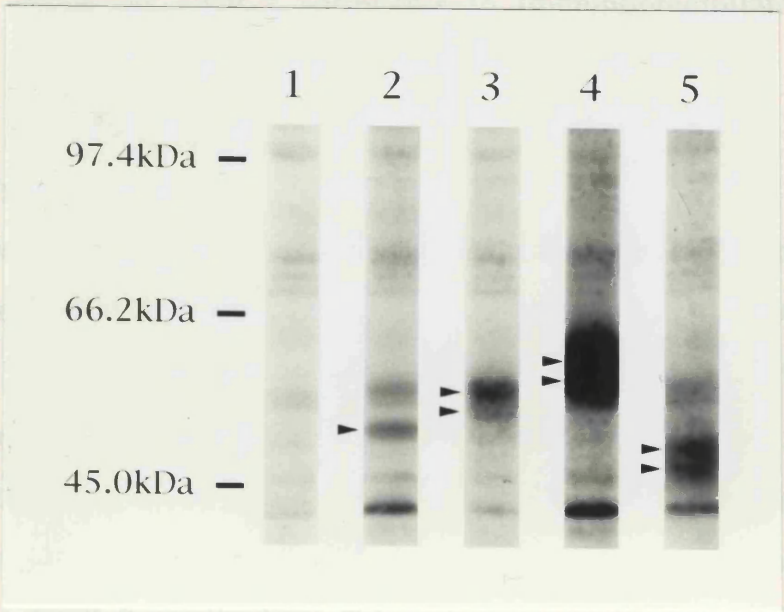
The use of affinity purified UCR-2 antibodies in the study of $GABA_A$-R phosphorylation requires that they are capable of efficiently and specifically immunoprecipitating receptor β subunits from cell lysates. Therefore, UCR-2 antibodies were examined for the ability to immunoprecipitate β subunit polypeptides from transiently transfected HEK293 cell lysates.

In order to determine individual subunit relative molecular masses HEK293 cells were transiently transfected with the mammalian expression vector pGW1 containing cDNAs encoding α1, β2, β3 or γ2S subunits which had been modified by inclusion of the *myc* epitope tag at their N-termini (Evan *et al.*, 1985; Connolly *et al.*, 1996a; Connolly *et al.*, 1996b). Efficient transfection and expression of receptor subunits in transfected cells were determined by immunofluorescent detection using 9E10 anti-*myc* antibodies as previously described (Connolly *et al.*, 1996a; Connolly *et al.*, 1996b).

Following metabolic labelling with [$^{35}$S]-methionine, solubilised receptor subunits were immunoprecipitated using 9E10 anti-*myc* antibody immobilised on protein G-sepharose and resolved by SDS-PAGE (Figure 22). 9E10 anti-*myc* antibody did not specifically immunoprecipitate polypeptides from mock transfected HEK293 cells (Figure 22; lane 1). Specific polypeptides were immunoprecipitated

## Figure 22

Immunoprecipitation of individual epitope tagged receptor subunit polypeptides from HEK293 cells by 9E10 anti-*myc*



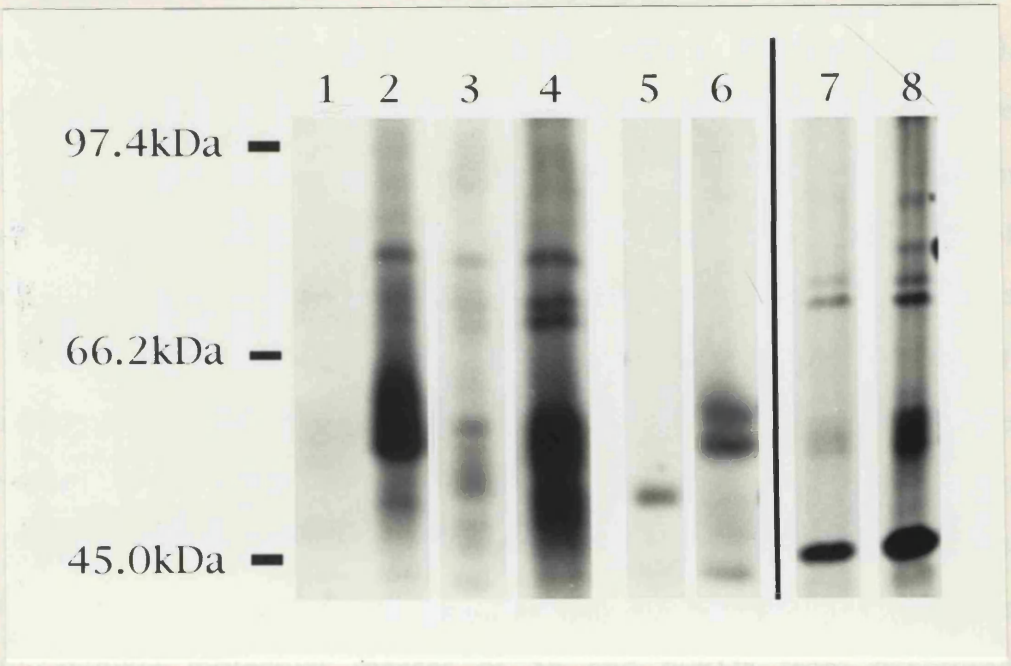

Mock transfected HEK293 cells (lane **1**) or cells expressing $\alpha 1^{myc}$ (lane **2**), $\beta 2^{myc}$ (lane **3**), $\beta 3^{myc}$ (lane **4**) or $\gamma 2S^{myc}$ subunits (lane **5**) were metabolically labelled with [$^{35}$S]-methionine. Cell lysates were prepared and receptor subunits precipitated using 9E10 anti-*myc* monoclonal antibody. Immune complexes were resolved by SDS-PAGE and visualised by fluorography. Molecular weight standards are indicated to the left in kilodaltons (kDa). Receptor subunits are indicated by arrowheads.

by 9E10 anti-*myc* antibody from cells expressing α1*myc* (50-52kDa: Figure 22; lane 2), β2*myc* (55-57kDa: Figure 22; lane 3), β3*myc* (56-60kDa: Figure 22; lane 4) and γ2S*myc* subunits (42-48kDa: Figure 22; lane 5). These values are in close agreement with those previously reported (Krishek *et al.*, 1994; Benke *et al.*, 1994; Connolly *et al.*, 1996a).

To test the ability of UCR-2 antibodies to immunoprecipitate receptor β subunits HEK293 cells were transiently transfected with the mammalian expression vector pGW1 containing cDNAs encoding α1$^{myc}$ and γ2S subunits in combination with β1, β2 or β3 subunits. Efficient transfection and subunit expression were determined by immunofluorescence staining using 9E10 anti-*myc*, bd17 anti β2/β3 subunit and UCR-2 antibodies as described (Figures 18, 19 & 20). Following metabolic labelling with [$^{35}$S]-methionine, solubilised $GABA_A$-R complexes were immunoprecipitated using UCR-2 antibodies, resolved by SDS-PAGE and visualised by autoradiography (Figure 23). UCR-2 antibodies did not precipitate any polypeptides from mock transfected HEK293 cells (Figure 23; lane 1), they did however specifically precipitate receptor complexes containing β1 and β3 subunits (Figure 23; lanes 2 & 4 respectively). As predicted from antibody specificity determinations by western blotting and immunofluorescence studies, UCR-2 antibodies did not efficiently immunoprecipitate β2 subunit containing receptor complexes (Figure 23; lane 3). To ensure that β2 subunit containing receptor complexes were efficiently solubilised prior to immunoprecipitation, a rabbit anti-β2 subunit specific antibody (Benke *et al.*, 1994) was used to precipitate these receptors. This antibody did not specifically precipitate polypeptides from mock transfected HEK293 cells (Figure 23; lane 7). It did efficiently precipitate receptors containing β2

**Figure 23**

Specific β subunit polypeptide immunoprecipitation by affinity purified UCR-2 antibody




Mock transfected HEK293 cells (lanes 1 and 7) or cells expressing $\alpha 1^{myc}$, β1 and γ2S (lane 2); $\alpha 1^{myc}$, β2 and γ2S (lanes 3 and 8) or $\alpha 1^{myc}$, β3 and γ2S subunits (lane 4) were labelled using [$^{35}$S]-methionine. Solubilised $GABA_A$-Rs were precipitated using UCR-2 antibody (15μg) preincubated with 50μg/ml GST (lanes 1-4) or rabbit anti-β2 subunit antibody (15μg; lanes 7 and 8). Immune complexes containing $\alpha 1^{myc}$, β3 and γ2S subunits (lane 4) were denatured in 1%(w/v) SDS and subunits precipitated using 9E10 anti-*myc* antibody (15μg; lane 5) or UCR-2 antibody (15μg) preincubated with 50μg/ml GST (lane 6). Immune complexes were resolved by SDS-PAGE and visualised by fluorography. Molecular weight standards are indicated to the left in kilodaltons (kDa).

subunits as previously described (Benke *et al.*, 1994) showing that failure of UCR-2 to precipitate these receptors was not due to inefficient receptor solubilisation (Figure 23; lane 8).

Receptor complexes precipitated by UCR-2 are seen as large ill-defined bands due to the fact that they contain $\alpha 1^{myc}$ and $\gamma 2$ S subunits together with $\beta 1$ and $\beta 3$ subunits (Figure 23; lanes 2 & 4). Immunoprecipitated receptor complexes containing $\beta 3$ subunits were reprecipitated after denaturation in order to show that precipitation of these complexes is due to specific UCR-2 antibody recognition of receptor $\beta 3$ subunits. Precipitation of denatured receptor complexes using 9E10 anti-*myc* antibody purified a single polypetide of approximate molecular mass 52kDa (Figure 23; lane 5). This size measurement is in close agreement to the predicted molecular mass of the $\alpha 1^{myc}$ subunit and is similar to that described earlier (Figure 22; lane 2). UCR-2 antibodies specifically precipitated polypeptides with approximate molecular masses of 56 and 60kDa from denatured $\beta 3$ subunit containing receptors (Figure 23; lane 6). The relative molecular mass values observed for these polypeptide products of UCR-2 immunoprecipitation agree with predicted values from sequence analysis of $\beta 3$ subunits (Ymer *et al.*, 1989), values reported in this study of these subunits (Figures 21 and 22) and previously reported results (Benke *et al.*, 1994).

## 4.7 Conclusions

The use of $GABA_A$-R subunit-specific antibodies has greatly facilitated the study of subunit distribution and subunit-subunit interactions in neurons. Subunit-specific antibodies allow individual subunits to be studied biochemically regardless of receptor subunit combination in neurons and heterologous expression systems. Production of antibodies which specifically recognise receptor β subunits has proven particularly troublesome due to the high degree of sequence homology between these subunits.

As the major intracellular domains contain the highest sequence divergence between the β subunits, these domains were used to immunise rabbits and produce polyclonal subunit specific antibodies. These data outline the production of polyclonal antibodies in rabbits by immunisation with the β1 subunit intracellular domain expressed and purified as a GST-fusion protein in *E. coli*. Interestingly, these antibodies reacted with the intracellular domains of β1, β2, and β3 subunits expressed as GST-fusion proteins indicating the presence of conserved epitopes between the β subunit intracellular domains. Immunisation with β2 and β3 subunit intracellular domains did not cause robust immune responses in any rabbits. The reasons for this failure remain unclear but it could be due to lower antigenicity of these proteins compared with the β1 subunit intracellular domain. Antibodies were affinity purified from rabbit serum and their subunit specificity examined. These antibodies recognised GST-fusion proteins containing the intracellular domains of all three mammalian β subunits as determined by dot-blot and western blot methodologies. This data indicated that purified polyclonal antibodies recognised epitopes which were common to all three β subunit GST-fusion proteins.

The ability of affinity purified UCR-2 antibodies to recognise full length recombinant receptor subunit polypeptides was examined by immunofluorescence. Expression of β1 and β3 subunits were specifically detected by these antibodies in HEK293 cells, however β2 subunits were not detected even though their expression was confirmed using the β2/β3 subunit specific antibody bd17. The specificity of β1 and β3 subunit detection was demonstrated by its elimination using the immunising protein.

The restricted subunit specificity of UCR-2 antibodies was confirmed by western blot detection and immunoprecipitation of β1 and β3 but not β2 subunits. The specificity of receptor β subunit immuno-precipitation was further demonstrated by purification of β3 subunit polypeptides from denatured receptor complexes and the elimination of precipitation by preincubation of UCR-2 antibodies with GST-β1 immobilised on agarose beads.

The ability of UCR-2 antibodies to indicate specific expression of receptor β1 and β3 subunits together with their ability to specifically immunoprecipitate these subunits will be of great value in the study of $GABA_A$-R phosphorylation *in vitro* and *in vivo*.

# 5 Differential regulation of $GABA_A$-R function by PKA can be mediated by phosphorylation of distinct receptor β subunits.

## 5.1 Introduction

Given the large number of neurotransmitter and neuropeptide receptors which activate or inhibit adenylate cyclases, it seems likely that PKA-mediated regulation will be of fundamental importance to neuronal $GABA_A$-R function (Moss and Smart, 1996). In order to examine the effects of PKA activation on $GABA_A$-R function, recombinant receptors have been expressed in mammalian cell lines. Previous reports have focused on regulation of receptors composed of α1 and β1 or α1, β1 and γ2 subunits. In HEK293 cells both receptor types produce robust GABA-gated channels and activation of PKA by intracellular dialysis of cAMP causes a time-dependent reduction of whole-cell GABA-induced currents (Moss *et al.*, 1992b). PKA activation causes a 30-40% reduction in current through α1β1 and α1β1γ2 receptors. The reduction in GABA-currents was shown to correspond to PKA phosphorylation of the receptor β1 subunit (Moss *et al.*, 1992b). Mutation of S409 to alanine within the β1 subunit abolished PKA phosphorylation of the β1 subunit and prevented PKA-mediated reduction of GABA-induced whole-cell currents (Moss *et al.*, 1992b). Chronic activation of PKA, in cell lines with different levels of cAMP-independent PKA activity, has also been reported to enhance GABA responses from cells expressing α1, β1 and γ2 S receptor subunits (Angelotti *et al.*, 1993b). The mechanism responsible for this enhancement remains unclear as no subunit phosphorylation was demonstrated biochemically. Although this effect could be blocked by mutation of S409 to alanine within the β1 subunit, the enhancements were not seen for receptors composed of

α1 and β1 subunits alone (Angelotti *et al.*, 1993b), indicating that this effect may not be due to direct receptor phosphorylation alone.

From the studies conducted to date it seems that activation of endogenous PKA reduces GABA responses in a similar manner to that seen in cultured neurons (Moss *et al.*, 1992b). However, PKA activation has diverse effects on $GABA_A$-R function in different neuronal cell types. These effects can vary between significant reductions and significant enhancements of GABA-induced currents. There are numerous possible explanations for these different effects of PKA activation. They could be due to differential signal transduction mechanisms or indirect effects not due to receptor phosphorylation. Other possibilities include differential targeting of receptors or protein kinases in different neuronal populations or differences in receptor composition between neuronal types.

Subunit composition could determine the effects of PKA activation in distinct ways. β subunits could assemble with different α and γ subunits leading to different responses to PKA phosphorylation. Alternatively, the β subunits could differ in their responses to phosphorylation at the conserved serine residue which is phosphorylated by PKA *in vitro*. The experiments described here were designed to investigate the role of subunit composition on receptor regulation by PKA phosphorylation.

$GABA_A$-R β subunits alone contain concensus sequences and identified sites for PKA phosphorylation within their intracellular regions (Moss *et al.*, 1992a; McDonald and Moss, 1997). In order to examine potential differences in β subunit phosphorylation, receptors containing β2 and β3 were expressed and studied in HEK293 cells. The experiments were carried out in the same manner, using the

same α and γ subunits as a previous report which showed PKA-mediated reduction of channel function (Moss *et al.*, 1992b).

Functional receptors composed of α1 or α1 and γ2S subunits in combination with wildtype or mutated β2 or β3 subunits were expressed in HEK293 cells. Cells expressing various receptor types were then used to investigate the phosphorylation state of β subunit subtypes by immunoprecipitation after radiolabelling with [$^{32}$P]-orthophosphate and PKA activation using Forskolin as described (Moss *et al.*, 1992b). Examination of wildtype and mutated phosphoproteins by peptide mapping and phosphoamino acid analysis allowed determination of sites phosphorylated by PKA within the β2 and β3 subunits. Measurement of whole-cell currents with and without PKA activation allowed determination of the functional effects of PKA phosphorylation at specific residues within the β2 and β3 subunits.

## 5.2 The $GABA_A$-R β3 subunit is phosphorylated on S408 and/or S409 by PKA in HEK293 cells

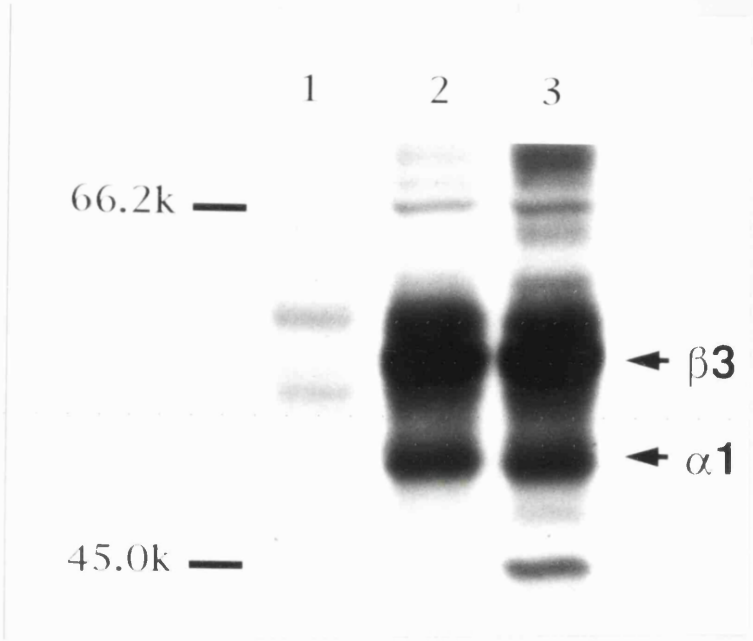
In order to begin examination of PKA-mediated regulation of receptors containing β3 subunits, the ability of PKA to phosphorylate the β3 subunit was examined in HEK293 cells. Experiments were carried out on receptors composed of α1, β3 and γ2S subunits.

Forskolin treatment of [$^{35}$S]-methionine labelled HEK293 cells did not alter β3 subunit immunoprecipitation using affinity purified UCR-2 antibodies (Figure 24). Immunoprecipitation of receptor complexes from [$^{32}$P]-orthophosphate labelled cells indicated that the β3 subunit was basally phosphorylated in these cells (Figure 25; lane 2). Stimulation of PKA by forskolin caused a large increase in subunit phosphorylation (Figure 25; lane 3). Forskolin stimulated phosphorylation of the β3 subunit was reduced to approximately basal levels by mutation of two serine residues S408 and S409 to alanines in the β3 subunit (Figure 25; lane 5). To further characterise this phosphorylation of the β3 subunit, phosphoproteins were removed from SDS-PAGE gels and subjected to phosphoamino acid analysis and tryptic phosphopeptide mapping. Analysis of β3 subunit polypeptides without (Figure 26; lane 1) or after forskolin treatment (Figure 26; lane 2) indicates that both basal and PKA phosphorylation occur predominantly on serine residues. It is also evident that PKA phosphorylation of the double mutant β3S408A/S409A subunit occurs predominantly on serine residues, indicating that the loss of these potential phosphorylation sites does not indirectly lead to phosphorylation of threonine or tyrosine residues (Figure 26; lane 3).

Tryptic peptide mapping of the β3 subunit after PKA phosphorylation indicated the generation of four individual phosphopeptides [a-d] (Figure 27; map 2). Double mutation of S408

## Figure 24

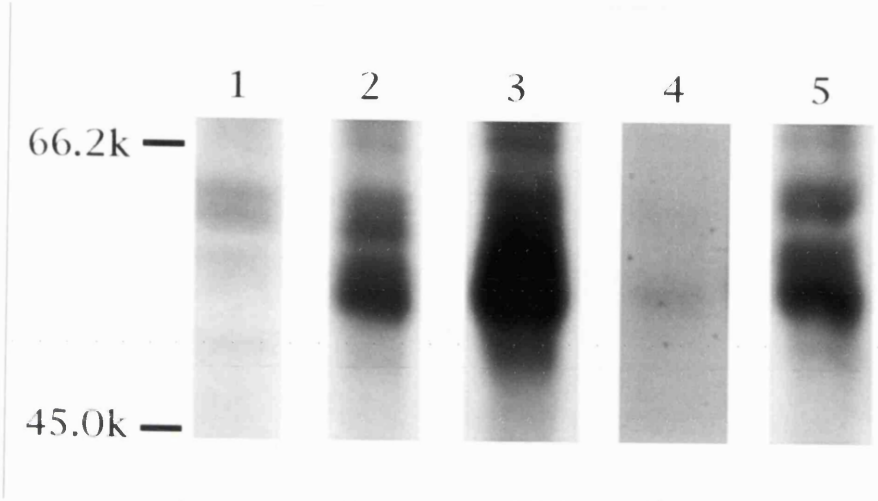
Forskolin treatment does not alter β3 subunit immunoprecipitation from HEK293 cells




Mock transfected HEK293 cells (lane 1) or cells expressing α1 and β3 subunits (lanes 2 and 3) were labelled with [$^{35}$S]-methionine then treated with forskolin (20μM; lanes 1 and 3) or vehicle alone (DMSO; lane 2) for 20 minutes. Cell lysates were prepared and $GABA_A$-R complexes immunoprecipitated using affinity purified UCR-2 antibody (10μg) preincubated with GST (50μg/ml). Receptor complexes were resolved by SDS-PAGE and visualised by fluorography. Molecular weight standards are indicated to the left in kilodaltons (k). Immunoprecipitated receptor subunits are indicated to the right.

**Figure 25**

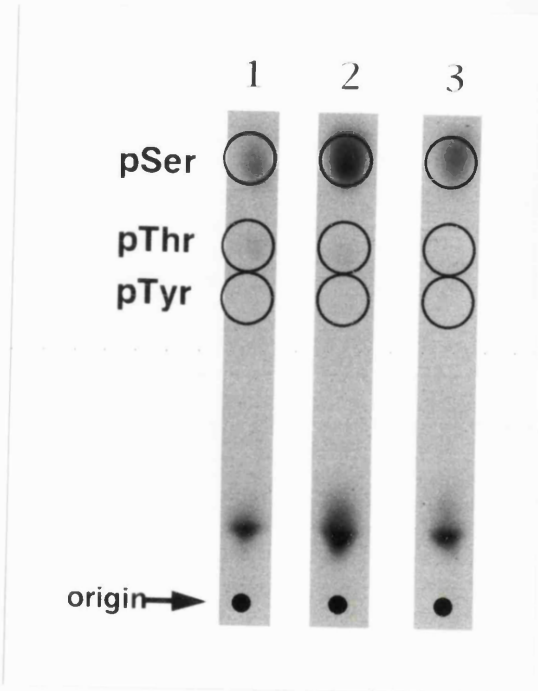
The $GABA_A$-R β3 subunit is phosphorylated by PKA in HEK293 cells




Mock transfected HEK293 cells (lane 1) and cells expressing α1, β3 and γ2S (lanes 2 and 3) or α1, β3S408A/S409A and γ2S subunits (lanes 4 and 5) were labelled with [$^{32}$P]-ortho-phosphoric acid then treated with forskolin (20μM; lanes 1, 3 and 5) or vehicle alone (DMSO; lanes 2 and 4) for 20 minutes. Cell lysates were prepared and $GABA_A$-R complexes immunoprecipitated using affinity purified UCR-2 antibody (10μg). Receptor complexes were resolved by SDS-PAGE and phosphoproteins visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k).

**Figure 26**

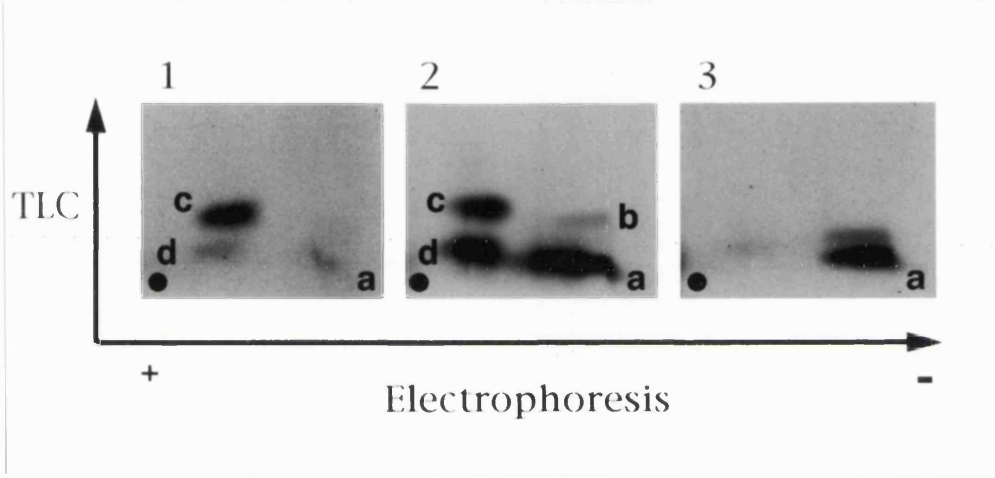
Phosphoamino acid analysis of β3 subunit phosphorylated by PKA in HEK293 cells




Phosphorylated β3 subunit polypeptides purified and visualised by SDS-PAGE were examined by phosphoamino acid analysis as described. Lane 1- β3 subunit from untreated cells; lane 2- β3 subunit from forskolin treated cells; lane 3- β3S408A/S409A subunit from forskolin treated cells. Phosphoprotein hydrolysates were subjected to electrophoresis from the origin (•). Migration of phosphoamino acid standards (pS, pT, pY) was visualised by ninhydrin staining as indicated.

**Figure 27**

Phosphopeptide map analysis of β3 subunit phosphorylated by PKA in HEK293 cells




Phosphopeptide map analysis of SDS-PAGE purified β3 subunit polypeptides. Map 1- β3 subunit purified from untreated HEK293 cells; map 2- β3 subunit purified from forskolin treated cells; map 3- β3S408A/S409A subunit purified from forskolin treated cells. Tryptic phosphoprotein digests were separated from the origin (•) by electrophoresis in the first dimension followed by thin layer chromatography in the second. Phosphopeptides were visualised by autoradiography.

and S409 to alanines abolished three of these phosphopeptides [b-d] (Figure 27; map 3), indicating that peptide 'a' probably contains unidentified residues phosphorylated by PKA. Peptide map analysis of the basally phosphorylated β3 subunit from HEK293 cells (Figure 27; map 1) indicates the presence of three [a, c and d] of the four phosphopeptides seen on activation of PKA.

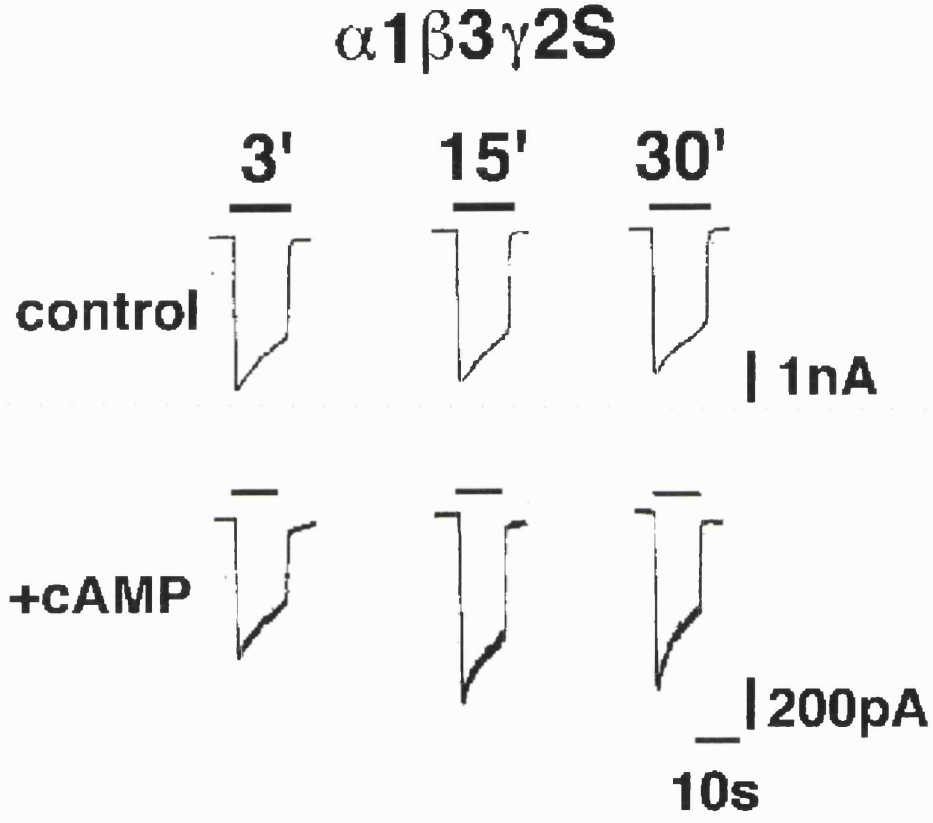
These data indicate that the β3 subunit polypeptide exists in a basally phosphorylated form and can become more highly phosphorylated on activation of PKA. Both basal and PKA-mediated phosphorylation seem to occur exclusively on serine residues. Mutation of S408 and S409 to alanine within the β3 subunit abolishes phosphorylation on two of three basally phosphorylated and three of four PKA phosphorylated tryptic phosphopeptides.

## 5.3 PKA activation leads to enhanced $GABA_A$-R function via phosphorylation of receptor β3 subunits

The functional effects of PKA activation on $GABA_A$-Rs containing β3 subunits were measured using whole cell patch clamp recording from HEK293 cells expressing α1, β3 and γ2S subunits. PKA activation was achieved by inclusion of 300μM cAMP in the patch pipette electrolyte. Cells expressing $GABA_A$-R subunits produced GABA-gated receptors which showed desensitising $Cl^-$ currents in the presence of GABA (Figure 28). In the absence of cAMP in the patch pipette electrolyte these $Cl^-$ currents were stable and showed no increase or rundown up to 30 minutes after patch formation (Figure 28). On inclusion of 300μM cAMP in the pipette electrolyte GABA-activated responses were increased by approximately 30% over initial values recorded 3 minutes after formation of the whole cell recording mode (Figure 28). Enhancement of GABA-induced current reached steady

**Figure 28**

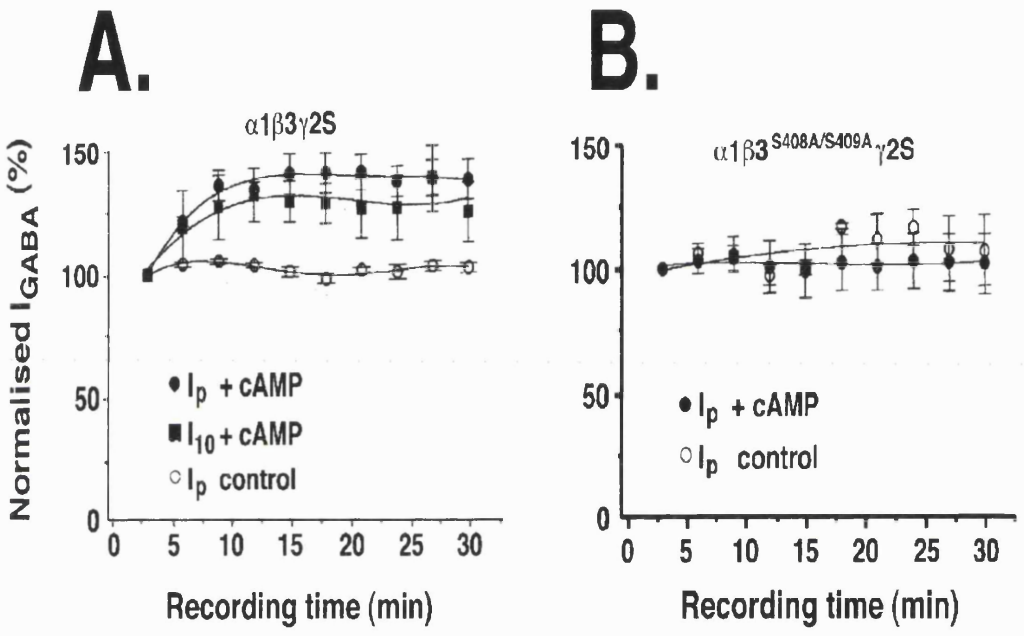
PKA activation enhances function of $GABA_A$-Rs containing β3 subunit in HEK293 cells




Membrane currents activated by rapid application of 10μM GABA were recorded from HEK293 cells expressing α1, β3 and γ2S subunits at a holding potential of -40mV at various times (minutes) after formation of the whole-cell recording mode (t=0). Cells were exposed to normal pipette electrolyte (control) or electrolyte supplemented with 300μM cAMP (+cAMP). The duration of GABA application is indicated by solid lines above traces.

## Figure 29

Timecourse of functional enhancement of $GABA_A$-Rs containing β3 subunit by PKA in HEK293 cells




Time dependence of the effect of cAMP on $GABA_A$-Rs composed of α1, β3 and γ2S (**A**) or α1, β3S408A/S409A and γ2S subunits (**B**) in HEK293 cells. GABA-activated membrane currents were normalised to the initial response to 10μM GABA (100%) recorded in each cell 3 minutes following formation of the whole cell recording mode either in the presence or absence of 300μM cAMP. All points are mean ± s.e.m. from 4-6 control and 5-8 cAMP treated cells. $I_P$- peak GABA current; $I_{10}$- current at 10 seconds following GABA application (steady state current).

state after approximately 15 minutes and was maintained during the recording period used (Figure 29A). PKA activation enhanced both peak and steady state currents elicited by application of GABA, indicating no preference for desensitised or nondesensitised states of the receptor complex (Figure 29A).

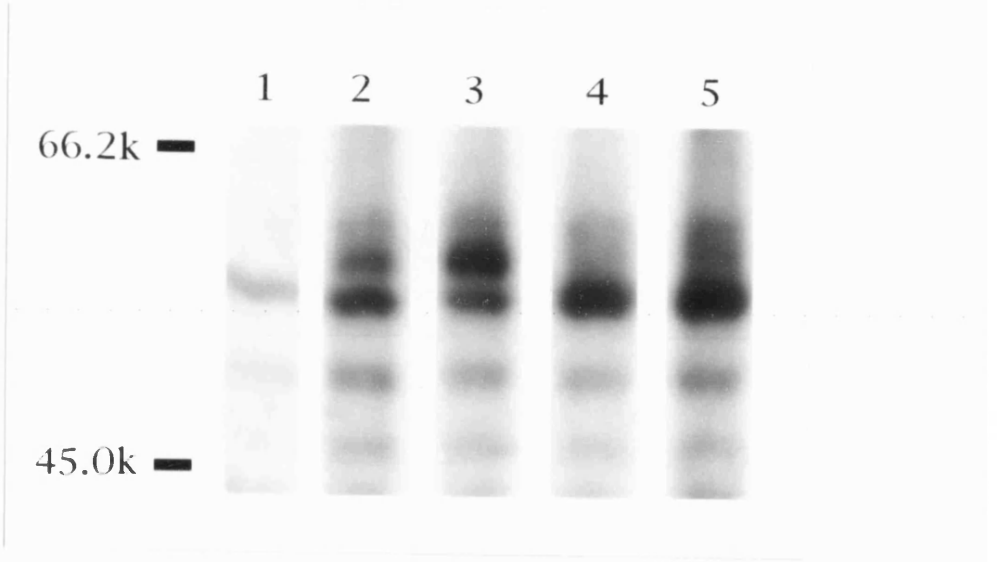
In order to examine the role of β3 subunit phosphorylation in PKA enhancement of receptor function, whole cell recordings were made from HEK293 cells expressing α1, β3S408A/S409A and γ2S subunits (Figure 29B). These cells produced GABA-gated receptors which showed desensitising $Cl^-$ currents in the presence of GABA, however they were unaffected by inclusion of 300mM cAMP in the patch electrolyte (Figure 29B). These data indicate that phosphorylation of S408 and/or S409 underlies the enhancement of receptor function by PKA.

## 5.4 Investigation of PKA phosphorylation of S408 and S409 in the $GABA_A$-R β3 subunit

To investigate the phosphorylation state of these serine residues single serine to alanine mutations were produced. Mutation of either S408 or S409 individually did not eliminate the forskolin induced increase in β3 subunit phosphorylation (Figure 30). The β3S408A subunit was basally phosphorylated (Figure 30; lane 2) however forskolin stimulated a significant increase in phosphorylation of this mutated subunit (Figure 30; lane 3). Similarly, the β3S409A subunit was basally phosphorylated in HEK293 cells (Figure 30; lane 4) and this phosphorylation was increased by forskolin treatment (Figure 30; lane 5).

## Figure 30

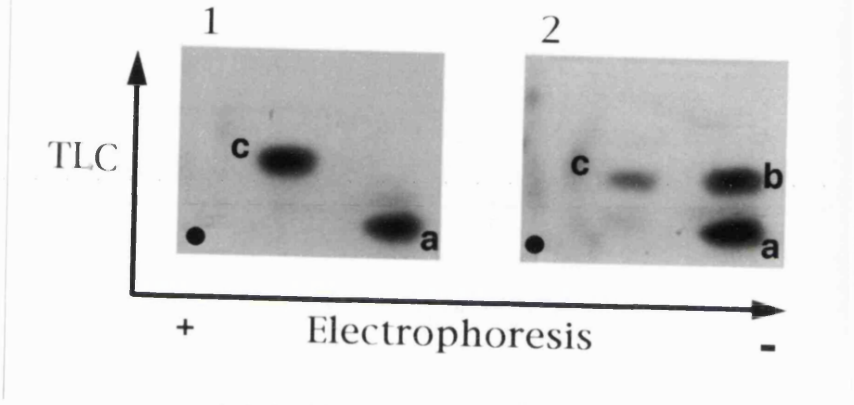
PKA phosphorylates both S408 and S409 within the $GABA_A$-R β3 subunit




Mock transfected HEK293 cells (lane 1) and cells expressing α1, β3S408A and γ2S (lanes 2 and 3) or α1, β3S409A and γ2S subunits (lanes 4 and 5) were labelled with [$^{32}$P]-ortho-phosphoric acid then treated with forskolin (20μM; lanes 1, 3 and 5) or vehicle alone (DMSO; lanes 2 and 4) for 20 minutes. Cell lysates were prepared and $GABA_A$-R complexes immunoprecipitated using affinity purified UCR-2 antibody (10μg). Receptor complexes were resolved by SDS-PAGE and phosphoproteins visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k).

## Figure 31

Phosphopeptide map analysis of $GABA_A$-R β3 subunit phosphorylated on S408 or S409 by PKA




Phosphopeptide map analysis of SDS-PAGE purified β3S408A and β3S409A subunit polypeptides following PKA phosphorylation. Map 1- $\beta3^{S408A}$ subunit purified from forskolin treated HEK293 cells; map 2- $\beta3^{S409A}$ subunit purified from forskolin treated cells. Tryptic phosphoprotein digests were separated from the origin (•) by electrophoresis in the first dimension followed by thin layer chromatography in the second dimension. Phosphopeptides were visualised by autoradiography.

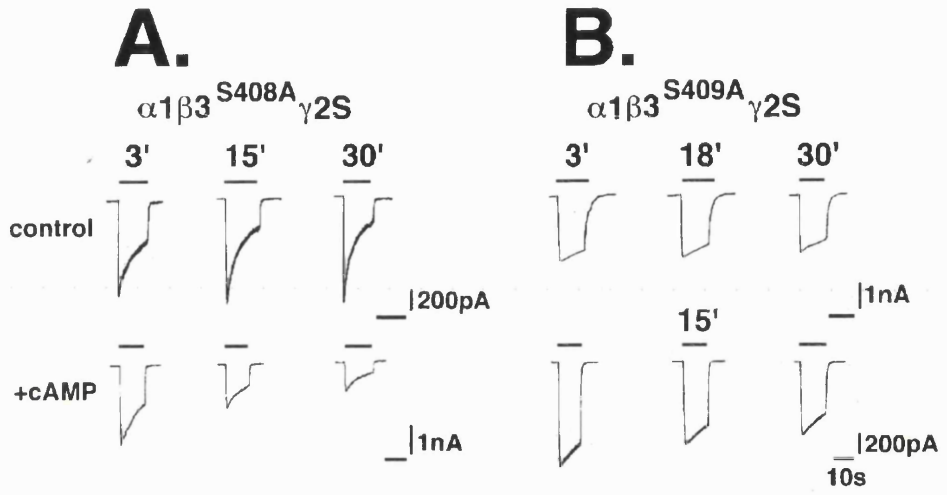
The increased phosphorylation of both subunits on PKA activation was further investigated by tryptic phosphopeptide mapping (Figure 31). Peptide mapping of the β3S408A subunit following PKA phosphorylation yields two positively charged tryptic peptides (Figure 31; map 1) corresponding to peptides 'a' and 'c' produced by trypsin digestion of wildtype β3 subunit (Figure 27). On trypsin digestion of the β3S409A subunit three positively charged phosphopeptides were detected (Figure 31; map 2), these correspond to peptides 'a', 'b' and 'c' produced from the β3 subunit (Figure 27). These mutations each specifically abolished the presence of peptide 'd' produced on trypsin digestion of the β3 subunit. This indicates that peptide 'd' is only generated when both S408 and S409 are present and strongly suggests that both residues can be phosphorylated within the same β3 subunit polypeptide molecule.

## 5.5 Investigation of the roles of S408 and S409 in PKA regulation of $GABA_A$-Rs containing the β3 subunit

To investigate the individual roles of these serine residues in PKA-mediated regulation of receptor function, GABA currents were measured using whole cell patch clamp recording from HEK293 cells expressing α1 and γ2S with either the β3S408A or β3S409A subunits. Cells expressing these receptor subunit combinations expressed robust $GABA_A$-Rs which did not show significant rundown during the course of experimental recording (Figure 33). Inclusion of 300μM cAMP in the patch electrolyte caused an approximately 50% reduction of GABA currents through receptors containing the β3S408A subunit (Figure 32A). This reduction in receptor function reached steady state after 15 minutes and lasted the entire duration of experimental recording (Figure 32A). In contrast, intracellular

## Figure 32

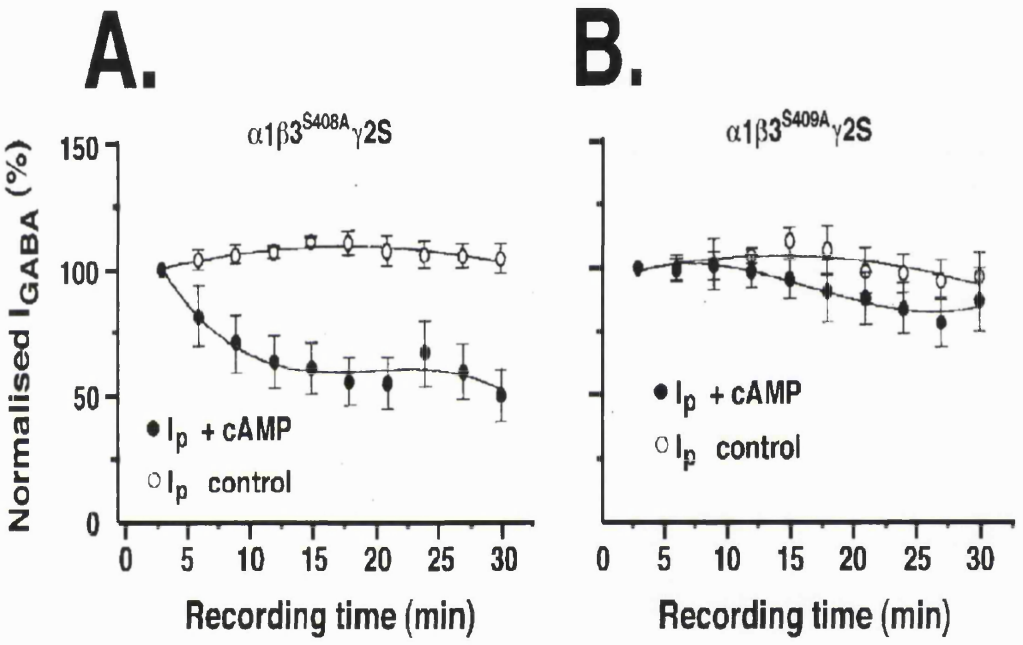
PKA enhancement of $GABA_A$-R function in HEK293 cells is determined by the presence of S408 and S409 in the β3 subunit




Membrane currents activated by rapid application of 10μM GABA were recorded from HEK293 cells expressing α1, β3S408A and γ2S (**A**) or α1, $\beta3^{S409A}$ and γ2S subunits (**B**) at a holding potential of -40mV at various times (minutes) after formation of the whole-cell recording mode (t=0). Cells were exposed to normal pipette electrolyte (control) or electrolyte supplemented with 300μM cAMP (+cAMP). The duration of GABA application is indicated by solid lines above traces.

## Figure 33

Timecourse of $GABA_A$-R regulation by PKA phosphorylation of S408 or S409 within the β3 subunit




Time dependence of the effect of cAMP on $GABA_A$-Rs composed of α1, β3S408A and γ2S (**A**) or α1, β3S409A and γ2S subunits (**B**) in HEK293 cells. GABA-activated membrane currents were normalised to the initial response to 10μM GABA (100%) recorded in each cell 3 minutes following formation of the whole cell recording mode either in the presence or absence of 300μM cAMP. All points are mean ± s.e.m. from 4-6 control and 5-8 cAMP treated cells.

dialysis of cAMP had no significant effect on the function of receptors containing the β3S409A subunit (Figures 32B and 33B).

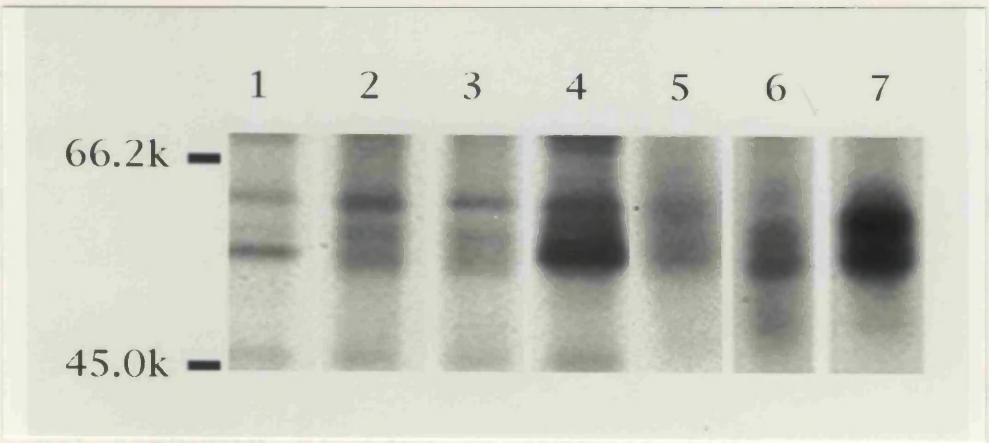
The data presented here suggest that PKA phosphorylation of the β3 subunit on S408 alone (RRRSAQLK) is functionally silent while phosphorylation on S409 alone (RRRASQLK) leads to reduction in receptor function. Interestingly, PKA phosphorylation of the $GABA_A$-R β1 subunit on S409 alone (RRRASQLK) also leads to significant reduction in receptor function (Moss *et al.*, 1992b).

## 5.6 The $GABA_A$-R β2 subunit is not phosphorylated by PKA in HEK293 cells

In order to study PKA-mediated regulation of receptors containing β2 subunits, phosphorylation of the β2 subunit in HEK293 cells was characterised biochemically (Figure 34). Phosphorylation of the β2 subunit was examined using a previously described affinity purified rabbit β2 subunit-specific antibody (Benke *et al.*, 1994). No basal phosphorylation of the β2 subunit was observed when expressed in combination with the α1 and γ2S subunits in these cells (Figure 34; lane 2). Forskolin-induced PKA activation did not lead to any increase in β2 subunit phosphorylation within these cells (Figure 34; lane 3). Expression of β2 subunit polypeptide was demonstrated by immunofluorescence in these cells prior to metabolic labelling. Furthermore, stimulation of protein kinase C by treatment of transfected cells with the phorbol ester phorbol-12,13-dibutyrate (PdBu) lead to significant phosphorylation of the β2 subunit polypeptide (Figure 34; lane 4) which was abolished by mutation of serine residue S410 to alanine within the β2 subunit (Figure 34; lane 5). This indicates the correct expression of β2 subunits and shows that their resistance to PKA phosphorylation is probably not due to

## Figure 34

The $GABA_A$-R β2 subunit is not phosphorylated by PKA in HEK293 cells




Mock transfected HEK293 cells (lane 1) and cells expressing α1 and β2 (lanes 2, 3 and 4), α1 and β2S410A (lane 5) or α1 and β3 subunits (lanes 6 and 7) were labelled with [$^{32}$P]-orthophosphoric acid then treated with forskolin (20μM; lanes 1, 3 and 7), PDBu (100nM; lanes 4 and 5) or vehicle alone (DMSO; lanes 2 and 6) for 20 minutes. Cell lysates were prepared and $GABA_A$-R complexes immunoprecipitated using rabbit anti-β2 subunit antibody (lanes 1-5) or affinity purified UCR-2 antibody (lanes 6 and 7). Receptor complexes were resolved by SDS-PAGE and phosphoproteins visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k).

incorrect protein folding.

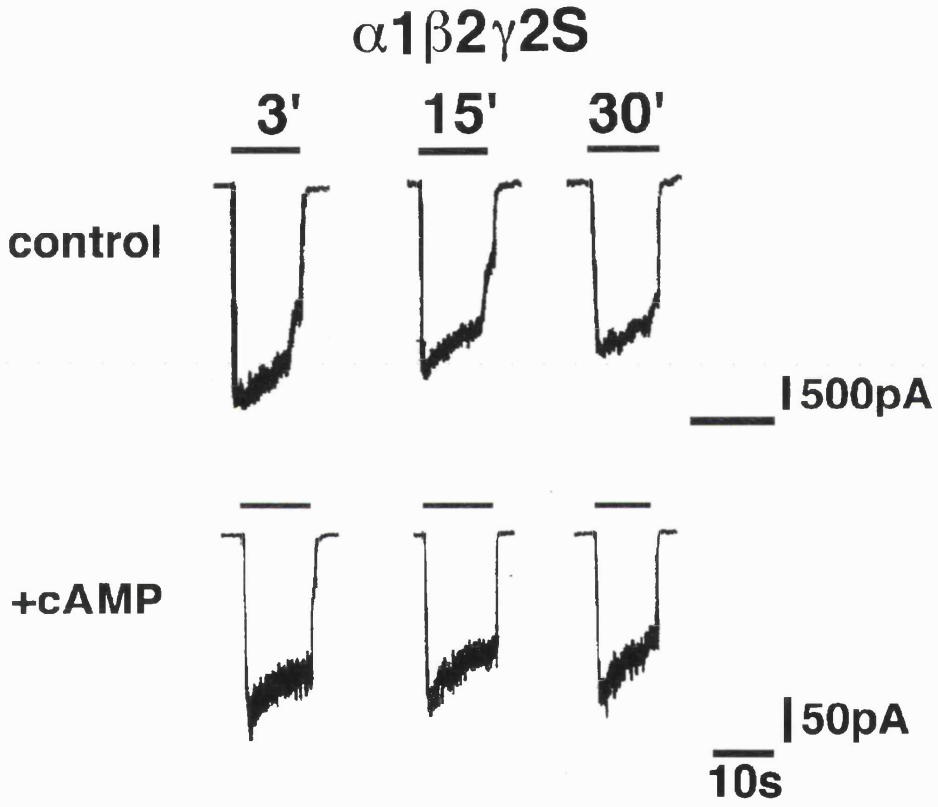
In order to test PKA activation by forskolin, cells expressing receptor β3 subunits were included as a positive control in this experiment. The β3 subunit was basally phosphorylated as previously described (Figure 34; lane 6) and forskolin stimulation of PKA caused a significant increase in phosphorylation of the β3 subunit (Figure 34; lane 7). These data suggest that these cells contain functional adenylate cyclase which was stimulated by forskolin, and that the ensuing increase in intracellular levels of cAMP caused correct PKA activation. Together these observations indicate that the $GABA_A$-R β2 subunit is not phosphorylated by PKA. It was necessary therefore to confirm the corollary of this finding, that PKA activation would not regulate the function of receptors containing the β2 subunit.

## 5.7 Function of $GABA_A$-Rs containing the β2 subunit is not regulated by PKA in HEK293 cells

The functional regulation of recombinant $GABA_A$-Rs containing β2 subunits by PKA was carried out in HEK293 cells expressing α1, β2 and γ2S subunits. Membrane currents induced by fast application of 10μM GABA were recorded from cells expressing robust GABA-induced $Cl^-$ currents (Figure 35). Inclusion of 300μM cAMP in the patch electrolyte had no effect on the magnitude of GABA currents from these cells (Figure 35). Control GABA currents exhibit a small rundown of approximately 10% over the experimental recording period (30 minutes). This rundown was not significantly altered by inclusion of cAMP in the patch electrode (Figure 36). Interestingly, activation of PKC in HEK293 cells expressing α1 and β2 subunits causes significant reduction in GABA-induced currents (T.G. Smart, unpublished observation).

## Figure 35

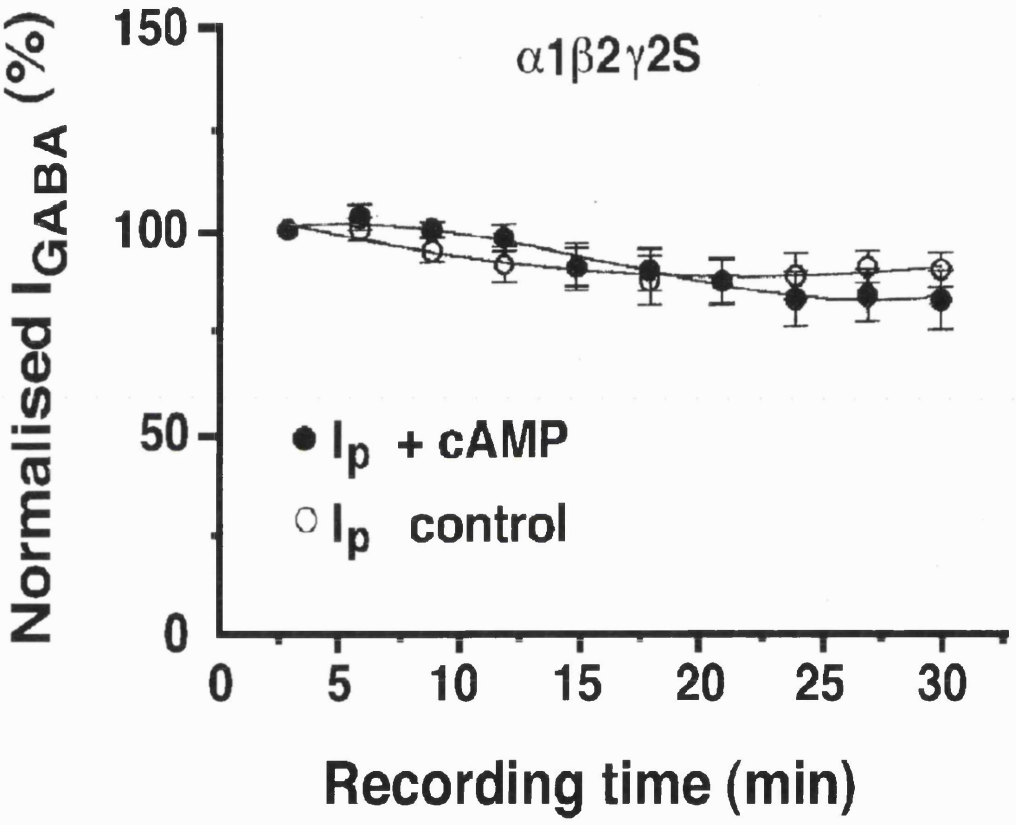
Activation of PKA does not modulate the function of $GABA_A$-Rs containing the β2 subunit in HEK293 cells




Membrane currents activated by rapid application of 10μM GABA were recorded from HEK293 cells expressing α1, β2 and γ2S subunits at a holding potential of -40mV at various times (minutes) after formation of the whole-cell recording mode (t=0). Cells were exposed to normal pipette electrolyte (control) or electrolyte supplemented with 300μM cAMP (+cAMP). The duration of GABA application is indicated by solid lines above traces.

**Figure 36**

Timecourse of PKA activation on $GABA_A$-Rs containing the β2 subunit in HEK293 cells




Time dependence of the effect of cAMP on $GABA_A$-Rs composed of α1, β2 and γ2S subunits in HEK293 cells. GABA-activated membrane currents were normalised to the initial response to 10μM GABA (100%) recorded in each cell 3 minutes following formation of the whole cell recording mode either in the presence or absence of 300μM cAMP. All points are mean ± s.e.m. from 4-6 control and 5-8 cAMP treated cells.

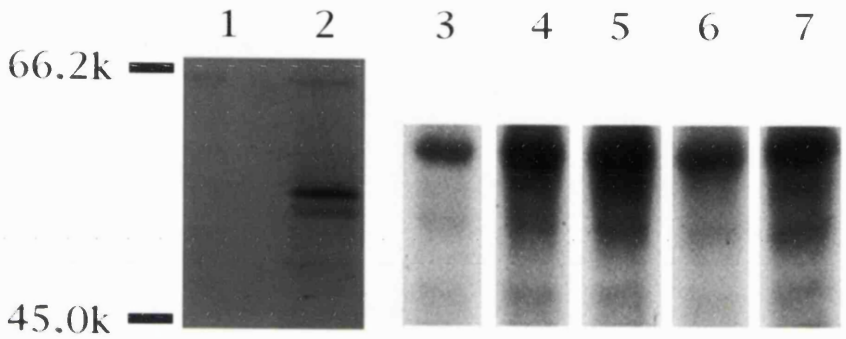
Together these data confirm the biochemical observation that PKA activation does not directly affect the phosphorylation state of the $GABA_A$-R β2 subunit in HEK293 cells. Reduced function of receptors containing β2 subunits phosphorylated on S410 (RRRA$\underline{S}^{410}$QLK) accords well with previous reports of functional regulation of receptors due to phosphorylation of S409 (RRRA$\underline{S}^{409}$QLK) of the β1 subunit (Moss *et al.*, 1992b; Krishek *et al.*, 1994).

## 5.8 Distinct regulation of $GABA_A$-R function by PKA is interchangeable between receptor β subunits

It was decided to test the hypothesis that enhanced function of β3 subunit containing receptors is due to phosphorylation of adjacent serine residues S408 and S409 by PKA. The β1 subunit polypeptide contains a single PKA phosphorylation site at S409 within the sequence RRRA$\underline{S}$QLK and phosphorylation of this residue leads to significant reduction in GABA-gated $Cl^-$ currents (Moss *et al.*, 1992b). The β1 subunit was subject to site-directed mutagenesis in order to insert a serine residue in place of A408 in the presence of serine (wildtype) or alanine at position 409. This mutagenesis lead to the production of two β1 subunit mutants termed β1A408S (RRR$\underline{S}^{408}\underline{S}^{409}$QLK) and β1A408S/S409A (RRR$\underline{S}^{408}\underline{A}^{409}$QLK). The ability of PKA to phosphorylate these mutated β1 subunits was examined in HEK293 cells. When expressed together with the α1 and γ2S subunits, β1A408S was highly basally phosphorylated (Figure 37; lane 4) and PKA activation by forskolin caused a significant increase in phosphorylation of this subunit (Figure 37; lane 5). In contrast, β1A408S/S409A was not basally phosphorylated (Figure 37; lane 6) and forskolin treatment caused significant phosphorylation on this subunit (Figure 37; lane 7). These observations indicate that the

## Figure 37

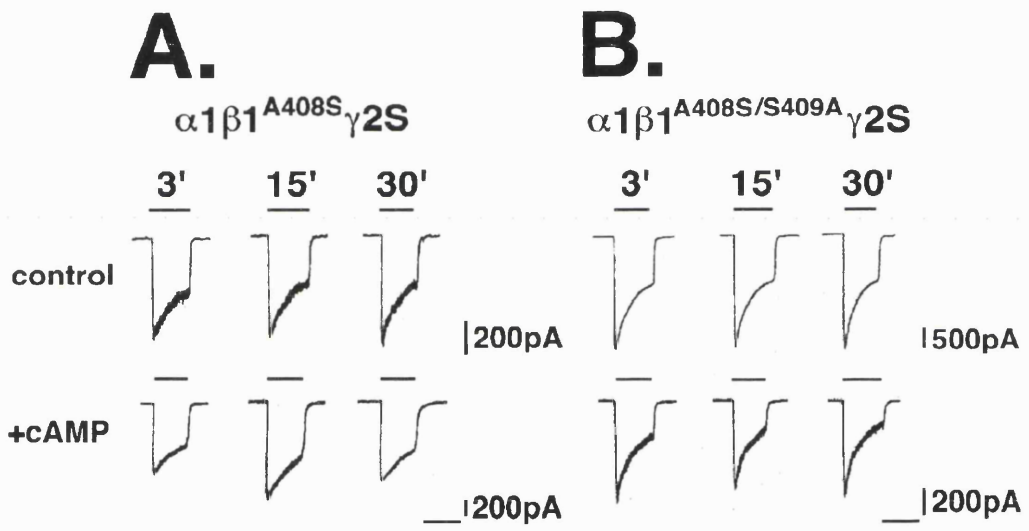
PKA phosphorylates mutant receptor subunits β1A408S and β1A408S/S409A in HEK293 cells




Mock transfected HEK293 cells (lane 1) and cells expressing α1 and β1A408S (lanes 2 and 3) or α1 and β3A408S/S409A subunits (lanes 4 and 5) were labelled with [$^{32}$P]-ortho-phosphoric acid then treated with forskolin (20μM; lanes 1, 3 and 5) or vehicle alone (DMSO; lanes 2 and 4) for 20 minutes. Cell lysates were prepared and $GABA_A$-R complexes immunoprecipitated using affinity purified UCR-2 antibody (10μg). Receptor complexes were resolved by SDS-PAGE and phosphoproteins visualised by autoradiography. Molecular weight standards are indicated in kilodaltons (k).

**Figure 38**

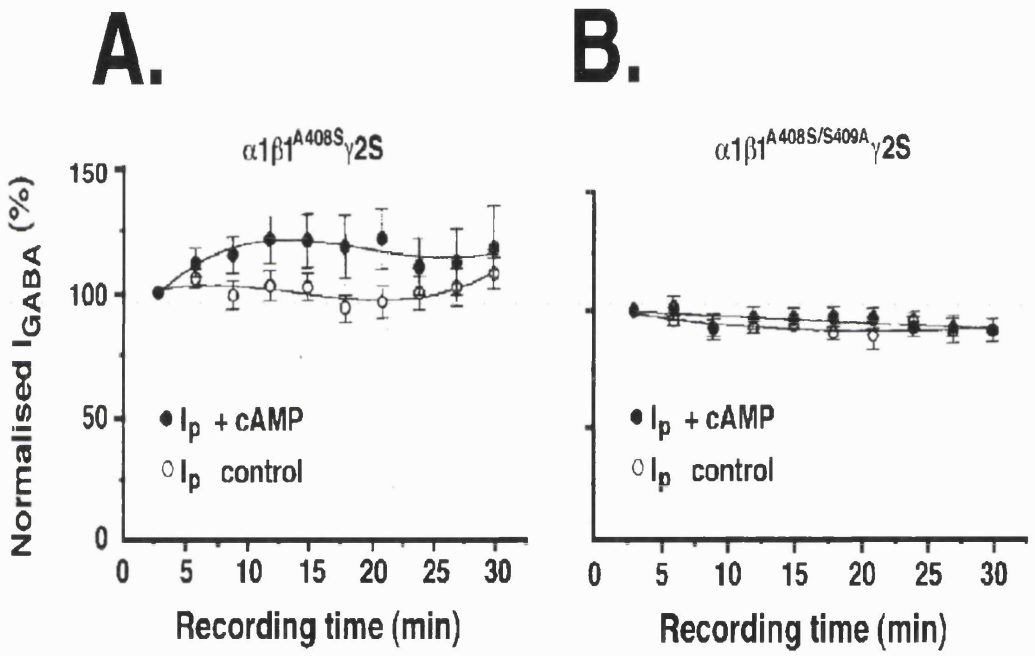
PKA modulation of $GABA_A$-Rs containing mutated β1A408S and β1A408S/S409A subunits in HEK293 cells




Membrane currents activated by rapid application of 10μM GABA were recorded from HEK293 cells expressing α1, β1A408S and γ2S or α1, β1A408S/S409A and γ2S subunits at a holding potential of -40mV at various times (minutes) after formation of the whole-cell recording mode (t=0). Cells were exposed to normal pipette electrolyte (control) or electrolyte supplemented with 300μM cAMP (+cAMP). The duration of GABA application is indicated by solid lines above traces.

**Figure 39**

Timecourse of PKA modulation of $GABA_A$-Rs containing mutated β1A408S and β1A408S/S409A subunits in HEK293 cells




Time dependence of the effect of cAMP on $GABA_A$-Rs composed of α1, β1A408S and γ2S (**A**) or α1, β1A408S/S409A and γ2S subunits (**B**) in HEK293 cells. GABA-activated membrane currents were normalised to the initial response to 10μM GABA (100%) recorded in each cell 3 minutes following formation of the whole cell recording mode either in the presence or absence of 300μM cAMP. All points are mean ± s.e.m. from 6-7 control and 5-8 cAMP treated cells.

mutated β1A408S and β1A408S/S409A subunits were phosphorylated by PKA. β1A408S/S409A is presumably phosphorylated at S408 as S409 the sole PKA phosphorylation site within the β1 subunit is no longer present (Moss *et al.*, 1992b). The high level of basal phosphorylation within the β1A408S subunit which is not seen in the β1 subunit indicates the likelihood that it too is phosphorylated at S408.

The functional effects of PKA on receptors containing the β1A408S and β1A408S/S409A subunits were determined by whole cell patch clamp recording from HEK293 cells expressing α1 and γ2S subunits together with β1A408S or β1A408S/S409A. Both receptor subunit combinations resulted in robust GABA-gated desensitising $Cl^-$ currents (Figure 38). Intracellular diaysis with 300μM cAMP in the patch electrolyte caused functional enhancement of receptors containing β1A408S while receptors containing β1A408S/S409A were unaffected by such treatment (Figure 38). The effects of PKA activation on β1A408S containing receptors reached a maximum of approximately 20% enhancement after 10-15 minutes (Figure 39A). Receptors containing the β1A408S/S409A subunit were resistant to PKA activation throughout the experimental recording period (Figure 39B).

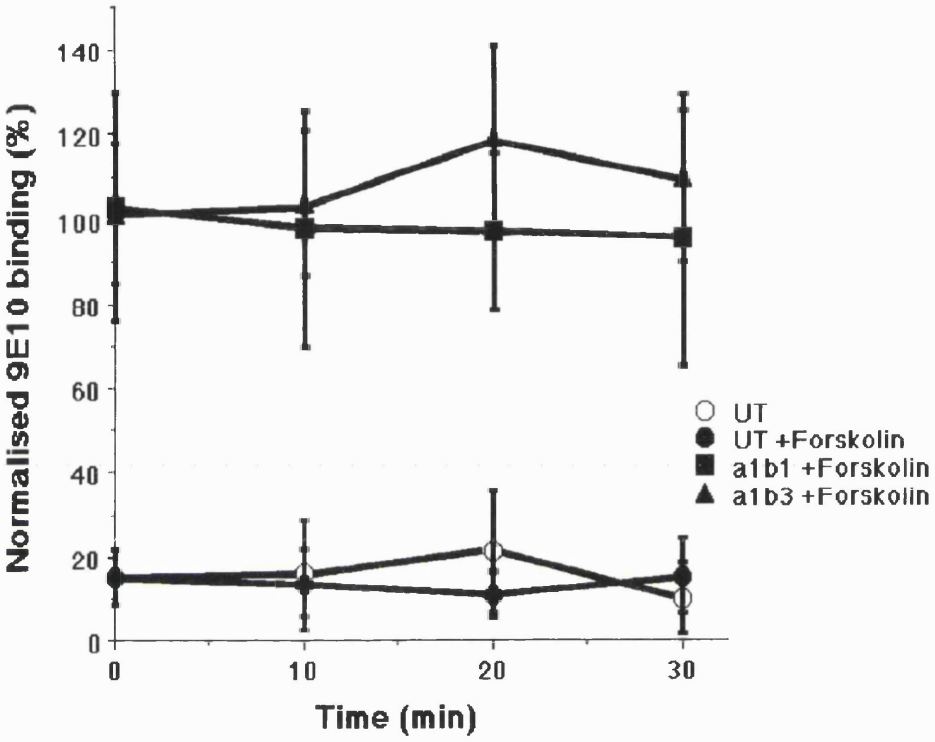
These observations of $GABA_A$-Rs containing mutated β1 subunits further support the observation that PKA phosphorylation of adjacent serine residues at positions 408 and 409 within the β3 subunit leads to enhanced receptor function. They also indicate that phosphorylation of a serine residue at position 408 alone is functionally silent, while an earlier report showed that phosphorylation of S409 alone reduced receptor function.

## 5.9 β1 or β3 subunit phosphorylation by PKA does not alter receptor cell surface levels

It has previously been suggested that enhancement or reduction of GABA-induced currents folowing PKA activation may be due to other factors distinct from receptor subunit phosphorylation (Angelotti *et al.*, 1993b). The most likely other factor which could lead to a changed GABA response would be an alteration in the relative abundance of receptors at the cell surface. HEK293 cells expressing $\alpha 1^{myc}$ and $\beta 1^{myc}$ or $\beta 3^{myc}$ subunit combinations were used to address this possibility. Measurement of the relative amounts of surface receptor were made using [$^{125}$I]-9E10 anti-*myc* antibody before and after treatment with forskolin. Forskolin treatment did not alter the relative number of $\alpha 1^{myc}$-$\beta 1^{myc}$ or $\alpha 1^{myc}$-$\beta 3^{myc}$ receptors at the surface of HEK293 cells nor did it affect the low level of non-specific 9E10 anti-*myc* binding to these cells (Figure 40). These data indicate that regulation of $GABA_A$-R function by PKA in HEK293 cells is not due to alterations in number of cell surface receptors.

## Figure 40

PKA activation does not alter cell surface distribution of $GABA_A$-Rs containing β1 and β3 subunits in HEK293 cells




Relative amounts of cell surface $GABA_A$-Rs composed of $\alpha 1^{myc}$ and $\beta 1^{myc}$ or $\alpha 1^{myc}$ and $\beta 3^{myc}$ subunits were quantified by binding of [$^{125}$I]-9E10 anti-*myc* antibody. HEK293 cells expressing $\alpha 1^{myc}$ and $\beta 1^{myc}$ or $\alpha 1^{myc}$ and $\beta 3^{myc}$ subunits were treated with forskolin (20μM) for 0, 10, 20 or 30 minutes. Mock transfected HEK293 cells were used to establish nonspecific binding of [$^{125}$I]-9E10 anti-*myc* antibody. Following treatment cells were cooled to 4$^{o}$C and incubated with saturating [$^{125}$I]-9E10 anti-*myc* antibody. After extensive washing labelling was determined by counting of cell associated γ emissions. Values represent mean + s.e.m. from 7 experiments.

## 5.10 Conclusions

Activation of PKA can be achieved by numerous neurotransmitter and neuropeptide receptors and therefore may be of great importance in the regulation of synaptic plasticity. Phosphorylation of ligand-gated ion channels could provide one mechanism for cAMP-dependent regulation of synaptic function (Moss and Smart, 1996). Among the multitude of $GABA_A$-R subunits the β1 subunit alone has been shown to be phosphorylated by PKA activation in a cellular environment. This phosphorylation at serine residue S409 leads to significantly reduced GABA-induced currents through receptors containing the β1 subunit. Mutation of S409 to alanine abolishes PKA phosphorylation of the β1 subunit and prevents PKA mediated reduction of receptor function (Moss *et al.*, 1992b). This reduction of GABA-induced currents after PKA activation in cells expressing recombinant receptors closely resembles the effects of PKA activation seen in many neuronal types (Moss and Smart, 1996). However, there have been numerous reports of PKA activation having no effect or increasing GABA-induced currents in different neuronal types (Moss and Smart, 1996). Subunit sequence analysis together with previous biochemical studies indicate that receptor β subunits alone are substrates for phosphorylation by PKA (Ymer *et al.* 1989; Moss *et al.*, 1992a; Moss *et al.*, 1992b; McDonald and Moss, 1997). As described earlier, *in vitro* phosphorylation using purified protein kinases indicated that both other β subunits (β2 and β3) were phosphorylated by PKA on the conserved phosphorylation site corresponding to S409 within the β1 subunit [RRRX$\underline{S}^{409}$QLK] (McDonald and Moss, 1997). Therefore heterologous expression of receptor subunits was used to examine the role of receptor β subunit heterogeneity in this differential regulation of receptor function by PKA.

When expressed in HEK293 cells with α1 or α1 and γ2S subunits the β2 subunit was not significantly phosphorylated by activation of PKA. This result was surprising, as the β2 subunit contains an identical concensus site for PKA phosphorylation as found in the β1 subunit where it is phosphorylated efficiently. PKC activation by the phorbol ester PdBu did cause significant phosphorylation of the β2 subunit, this phosphorylation was abolished by mutation of S410 to alanine. This finding indicates that incorrect subunit expression or folding was unlikely to underlie the inability of PKA to phosphorylate this protein.

As expected from the results of biochemical studies, activation of PKA by intracellular dialysis of 300μM cAMP had no effect on the function of receptors containing β2 subunits. In contrast, activation of PKC lead to significant reduction in function of these receptors (data not shown). This functional modulation, due to phosphorylation at the single serine residue S410, was similar to regulation of β1 subunit-containing receptors on activation of PKA or PKC (Moss *et al.*, 1992b; Krishek *et al.*, 1994).

In HEK293 cells the β3 subunit was basally phosphorylated and this phosphorylation was significantly increased on activation of adenylate cyclase by forskolin and phosphoamino acid analysis indicated that this phosphorylation took place entirely on serine residues. Mutation of either S408 or S409 alone to alanine reduced but did not abolish this phosphorylation, but combined mutation of both residues did reduce forskolin stimulated phosphorylation to basal levels. Phosphopeptide mapping of wildtype and mutated β3 subunit proteins indicated that S408 and S409 were basally phosphorylated and that this phosphorylation was significantly

increased by forskolin treatment. Importantly, it was also seen that both residues could be phosphorylated within a single polypeptide.

Functional regulation of β3 subunit-containing receptors was carried out using the same HEK293 cell expression system used for biochemical analysis. Activation of PKA by intracellular dialysis of 300μM cAMP lead to significantly increased GABA-induced currents in these cells with peak and steady state currents equally affected. This enhancement of receptor function was abolished by mutation of S408 and S409 to alanines within the β3 subunit, indicating the role of subunit phosphorylation in this regulation. Interestingly, receptors containing β3 subunits carrying single mutations of S408 or S409 were regulated in a distinct manner by PKA phosphorylation. β3 subunit phosphorylation at S409 alone lead to significant inhibition of receptor function similar to that previously reported for receptors containing the β1 subunit. In contrast, phosphorylation at S408 alone within the β3 subunit caused no significant alteration to channel function.

Together these data indicated that single phosphorylation on a conserved serine residue (S409[β1 or β3]/S410[β2]) caused reduction in receptor function. They also suggested that phosphorylation of S409 together with phosphorylation of the adjacent serine at S408 led to increased function of receptors containing the β3 subunit. In order to further confirm the distinct role of these serine residues the β1 subunit was mutated to include a serine residue at position 408 (β1A408S) or a serine residue at 408 together with an alanine at 409 (β1A408S/S409A).

When expressed in HEK293 cells with α1 and γ2S subunits the β1A408S subunit exhibited high basal phosphorylation which could be increased by treatment with forskolin. No basal phosphorylation

of the β1 subunit is observed in these cells indicating the likelihood that both S408 and S409 are phosphorylated within the mutated subunit. Intracellular dialysis of cAMP resulted in small but significantly increased GABA-induced currents through these receptors. The magnitude of this effect was less than that seen for the β3 subunit, perhaps due to the high basal phosphorylation seen on this subunit in these cells. However, the qualitative difference between β1 and β1A408S subunits indicated a decisive role for S408 in the PKA-mediated increase in receptor function.

The β1A408S/S409A subunit was not basally phosphorylated when expressed in combination with α1 and γ2S subunits. Forskolin treatment led to significant β1A408S/S409A phosphorylation indicating that the serine residue at position 408 was phosphorylated in the absence of S409. This phosphorylation caused no change in the function of these receptors again indicating that phosphorylation at position 408 alone is functionally silent.

Subunits containing a *myc* epitope tag together with [$^{125}$I]-9E10 anti-*myc* monoclonal antibodies were used to examine the distribution of $GABA_A$-Rs following their phosphorylation in HEK293 cells. Anti-*myc* binding was determined at 0 minutes without forskolin treatment and then at ten minute intervals in the presence of forskolin. Activation of PKA in such a manner was seen to have no significant effect on the relative amounts of β1 or β3 subunit-containing receptors at the cell surface. Similarly forskolin treatment caused no significant alteration in the amount of nonspecific anti-*myc* binding to untransfected HEK293 cells. Together these data indicate that PKA phosphorylation does not alter cell surface levels of β1 or β3 subunit-containing $GABA_A$-Rs. This would suggest that

regulation of receptor function by PKA is indeed due to direct alteration of channel properties following subunit phosphorylation.

The role of PKA in the regulation of $GABA_A$-R function has proven a controversial issue with increased and reduced channel function reported. One possible explanation for the different effects reported may be the distinct subunit compositions which most probably exist in different neuronal populations. The results reported here suggest that receptor β subunit identity is of central importance in the regulation of receptor function by PKA. Indeed receptor β subunits show distinct patterns of temporal and spatial expression in the brain (Laurie *et al.*, 1992a; Laurie *et al.*, 1992b; Wisden *et al.*, 1992) and varied effects of PKA activation could be due to phosphorylation of different β subunit isoforms in different neuronal preparations. The requirement of β subunits for production of functional $GABA_A$-Rs (Connolly *et al.*, 1996a) underlines the importance of β subunit identity in determining receptor regulation by PKA. The demonstration that all three mammalian β subunit isoforms are distinctly phosphorylated and regulated by PKA activation constitutes a novel mechanism for the regulation of receptor function by protein phosphorylation. Since $GABA_A$-Rs are critically involved in synaptic inhibition this mechanism is likely to be of profound importance in the regulation of neuronal excitability.

# 6 DISCUSSION

This thesis describes work carried out to examine the role of receptor β subunit identity in the differential regulation of $GABA_A$-R function by protein kinases. Studies carried out to date have focused exclusively on the roles of β1 and γ2 subunit phosphorylation. Evidence from numerous electrophysiological studies indicate that receptor β subunit identity may determine distinct regulation of receptor function by second messenger-regulated protein kinases. In order to address this possibility receptor subunit phosphorylation was studied using a variety of biochemical, molecular biological and electrophysiological techniques.

Preliminary investigation involved identification of potential phosphorylation sites within the major intracellular domains of the β2 and β3 subunits. Subunit intracellular domains were expressed in *E. coli* as GST-fusion proteins, purified to near homogeneity and included in *in vitro* phosphorylation assays with purified protein kinases. Sites phosphorylated *in vitro* were mapped using phosphoamino acid analysis and phosphopeptide mapping then identified using site-directed mutagenesis.

GST-β2 and GSTβ3 were phosphorylated to high stoichiometries *in vitro* by purified PKA, PKC, PKG and CamKII. Furthermore GST-β2 and GSTβ3 were phosphorylated at the conserved serine residue S410 (β2 subunit) or S409 (β3 subunit) by each of these protein kinases *in vitro* (McDonald and Moss, 1997). This data corresponds closely with previous reports of phosphorylation of the β1 subunit intracellular domain at S409 by these protein kinases *in vitro* (Moss et al., 1992a; McDonald and Moss, 1994). Interestingly, GST-β3 was also phosphorylated at S408 by PKC and at S384 by CamKII *in vitro* (McDonald and Moss, 1997). CamKII phosphorylation of GST-β1 at

S383 has previously been reported (McDonald and Moss, 1994). Therefore *in vitro* phosphorylation site identification indicates that receptor regulation by PKC may differ between receptors containing β1 or β2 subunits and those containing β3 subunits. Enhanced function of receptors composed of bovine α1, β1 and γ2L subunits expressed in L929 cells has been reported on administration of proteolytically-activated PKC (Lin *et al.*, 1994). This effect was blocked by serine to alanine mutation of S409 within the β1 subunit together with S327 and S343 in the γ2L subunit (Lin *et al.*, 1996). Other studies of PKC activation have been carried out using phorbol ester treatment to activate endogenous PKC. This method of PKC activation has invariably resulted in reduced murine $GABA_A$-R function in HEK 293 cells or *Xenopus laevis* oocytes (Sigel *et al.*,, 1991; Kellenberger *et al.*, 1992; Leidenheimer *et al.*, 1992; Leidenheimer *et al.*, 1993; Krishek *et al.*, 1994). PKC activation in neurons has been shown to reduce $GABA_A$-R function in SCG neurons (Krishek *et al.*, 1994) and cerebellar microsacs (Leidenheimer *et al.*, 1992), while PKC activation had no effect on $GABA_A$-R function in spinal cord preparations (Ticku and Mehta, 1990). These contrasting reports indicate that there may be species specific differences in $GABA_A$-R regulation by protein kinases or differential receptor modulation may be due to the distinct methods of PKC activation or administration used.

In contrast to PKC, the effects of PKA activation on $GABA_A$-R function in neurons have been extensively described. On activation of PKA $GABA_A$-R function can be reduced, enhanced or unchanged depending on the neuronal population studied (Moss and Smart, 1996). A conserved concensus site for PKA phosphorylation is found in all mammalian receptor β subunits (Ymer *et al.*, 1989) while α and γ subunits are not phosphorylated by PKA (Moss *et al.*, 1992a; Moss

*et al.*, 1992b). PKA phosphorylation of the conserved site (S409) in the murine β1 subunit has been shown to cause reduced receptor function in HEK293 cells (Moss *et al.*, 1992b). The major intracellular domain of the β1 subunit expressed as a GST-fusion protein (GST-β1) in *E. coli* was phosphorylated to high stoichiometry (0.48 pmol $PO_4$/pmol protein) on S409 by PKA *in vitro* ( Moss *et al.*, 1992a). The stoichiometries of PKA phosphorylation reported here for GST-β2 ($0.18\pm0.05$ pmol $PO_4$/pmol protein) and GST-β3 ($0.34\pm0.08$ pmol $PO_4$/pmol protein) differ significantly from each other and the value reported for GST-β1. These data suggest that β subunits may differ in their ability to be phosphorylated by PKA *in vivo*. Therefore it was decided to investigate the ability of PKA to phosphorylate receptor β2 and β3 subunits expressed in HEK293 cells.

In order to study the phosphorylation of full length receptor β2 and β3 subunits *in vivo* it was necessary to develop appropriate subunit-specific antibody reagents. Polyclonal antibodies were raised in rabbits by immunisation with GST-fuion proteins containing intracellular domains of receptor β subunits. These proteins were used as immunogens as they are likely to contain multiple epitopes which would be expected to increase the likelihood of raising a significant immune response in animals. They recognition of multiple epitopes within target proteins would also be expected to improve the likelihood of efficient subunit detection and immunoprecipitation. This strategy for antibody production has other technical advantages as hybridoma production or peptide coupling to carrier proteins was not required.

Immunisation of two rabbits each using GST-β2 and GST-β3 failed to produce useful immune responses. This failure may reflect poor immunogenic properties of these proteins, however it is possible that

enough animals were not immunised to guarantee production of at least one good response. GST-β1 however produced a robust immune response in one of two animals immunised. Immune serum from rabbit UCR-2 was screened against GST-β1, GST-β2 and GST-β3 to determine the subunit specificity of the immune response. Interestingly this serum reacted with all three β subunit intracellular domains, indicating that they shared at least a portion of the epitopes against which the immune response was directed. Antibodies affinity purified from UCR-2 serum also recognised intracellular regions of all three β subunits by dot-blot and western blot detection.

The ability of UCR-2 antibodies to detect full length β subunit polypeptides in HEK293 cells was examined. Immunofluorescent staining of transiently transfected cells indicated that these antibodies recognised β1 and β3 but not β2 subunits. Specificity of this detection was demonstrated by preincubation of antibodies with intracellular loop containing fusion proteins. This β subunit specificity of UCR-2 antibodies was confirmed by efficient western detection and immunoprecipitation of β1 and β3 subunit proteins in transfected HEK293 cell lysates. The inability of UCR-2 antibodies to detect native or denatured full length β2 subunit proteins was not examined closely however it is possible that β2 subunit polypeptide is folded differently to β1 and β3 subunits and that the three dimensional structure of β subunit intracellular domains may vary significantly.

The production of this antibody reagent enables efficient immunoprecipitation of the β3 subunit. This step is a prerequisite for the biochemical characterisation of β3 subunit phosphorylation by PKA *in vivo*. The production of an antibody specific for β1 and β3 subunits will also be of use in the study of $GABA_A$-R β subunits in

the brain. The most widely used anti-β subunit antibody is bd17, a monoclonal antibody which recognises both β2 and β3 subunits (Ewert *et al.*, 1990). The use of UCR-2 and bd17 antibodies together will allow more definite identification of receptor β subunits in the brain.

Phosphorylation of full length β2 and β3 subunit polypeptides by PKA was investigated in HEK293 cells expressing α1, β2 and γ2S or α1, β3 and γ2S subunits. In this recombinant system the β3 subunit was basally phosphorylated and this phosphorylation was significantly increased on activation of PKA by forskolin. Combined biochemical analysis and site-directed mutagenesis showed that PKA stimulated phosphorylation occurred predominantly on two adjacent serine residues S408 and S409. Electrophysiological analysis using whole-cell patch clamping of transfected cells indicated that PKA activation enhanced the function of receptors containing the β3 subunit and that this enhancement was abolished on mutation of S408 and S409 to alanines. These data indicated that receptor β subunit identity could indeed underlie differential receptor regulation by protein kinases.

Biochemical analysis indicated that PKA activation in HEK293 cells did not cause phosphorylation of the β2 subunit polypeptide, accordingly PKA activation did not regulate the function of receptors composed of α1, β2 and γ2S subunits. In the same cells PKC activation did cause phosphorylation of the β2 subunit on S410 leading to reduced receptor function. These observations indicate that the β2 subunit polypeptide is probably folded correctly in these cells and is immunoprecipitated efficiently. *In vitro* phosphorylation studies indicated that GST-β2 was phosphorylated on S410 by PKA and PKC to stoichiometries of $0.18\pm0.05$ and $0.31\pm0.04$ pmol $PO_4$/pmol protein

respectively. The significant differences between these stoichiometries of phosphorylation *in vitro* does reflect the suitability of the β2 subunit as an *in vivo* substrate for these two kinases. It is interesting to note that the stoichiometry of PKA phosphorylation of GST-β3 in vitro (0.34±0.08 pmol $PO_4$/pmol protein) resembles that of PKC phosphorylation of GST-β2, as these phosphorylation reactions were demonstrated to occur and regulate receptor function in the cellular environment.

There are a number of potential explanations for the inability of PKA to phosphorylate the β2 subunit while it readily phosphorylates the β1 and β3 subunits. One possibility is that the β2 subunit intracellular domain folds differently to those of the β1 and β3 subunits. Indirect evidence supporting this hypothesis comes from the different stoichiometries of PKA phosphorylation observed for GST-β1, GST-β2 and GST-β3 (Moss et al., 1992a; McDonald and Moss, 1997) and the β1/β3-subunit specificity of UCR-2 polyclonal rabbit antibodies raised by immunisation with GST-β1. These data suggest that the β2 subunit has a distinct structure to the β1 and β3 subunits, it is also possible however that PKA phosphorylation of the β2 subunit polypeptide requires an additional factor which is not present in HEK293 cells. A recent report has described the requirement of the PKA anchoring protein AKAP79 for regulation of the cardiac L-type $Ca^{2+}$ channel by PKA phosphorylation of the channel $\alpha_{1C}$ subunit (Gao *et al.*, 1997).

Phosphorylation of the murine β1 subunit on S409 by PKA (Moss *et al.*, 1992b) and PKC (Krishek *et al.*, 1994) has been shown to reduce receptor function. More recently inhibition of receptor function on PKC phosphorylation of the β2 subunit at S410 and enhanced receptor function on PKA phosphorylation of the β3 subunit at S408

and S409 were observed. These data indicated the potential importance of the adjacent serine residues S408 and S409 in the enhancement of receptor function by PKA. Accordingly both residues were individually mutated to alanines. Biochemical experiments indicated that each of these two serine residues were phosphorylated independently by PKA. Interestingly phosphorylation of S409 alone led to reduced receptor function which closely resembled receptor inhibition on PKA phosphorylation of the β1 subunit. Phosphorylation of S408 alone did not lead to any alteration in receptor function in HEK293 cells, indicating that the functional effects of these two phosphorylation sites were not equal. Together these data indicate that phosphorylation at S408 alone is functionally silent and phosphorylation of S409 alone inhibits channel function while phosphorylation of these two residues together enhances receptor function. The presence of adjacent PKA phosphorylation sites with such distinct functional significance represents a novel and sophisticated mechanism for the control of receptor regulation.

In order to confirm the importance of adjacent phosphorylation events in the determination of receptor regulation the β1 subunit was mutated to include a second PKA phosphorylation site at position 408 (β1A408S). A second β1 subunit mutant was produced which contained a PKA phosphorylation site at position 408 but not at position 409 (β1A408S/S409A). Mutated β1 subunits were expressed in HEK293 cells in combination with the α1 and γ2S subunits. β1A408S was highly basally phosphorylated and PKA activation caused a small but consistent increase in its phosphorylation. In contrast β1A408S/S409A was not significantly basally phosphorylated and forskolin treatment caused significantly increased phosphorylation at S408. Electrophysiological analysis showed that PKA phosphorylation at S408 alone caused no change in

receptor function while phosphorylation of S408 together with S409 led to a small but significant enhancement in receptor function. The magnitude of receptor enhancement on PKA activation was not comparable to that seen for the β3 subunit containing receptors, this could be due to the high level of basal phosphorylation on β1A408S. This possibility may be examined by intracellular dialysis of protein phosphatases and measurement of the effects of β1A408S dephosphorylation at S408 and S409. The qualitative similarity between regulation of receptors containing the β3 and β1A408S subunits lends further support to the observed importance of adjacent serine residues in determination of receptor regulation by PKA.

A previous report had suggested that PKA activity may influence $GABA_A$-R function by regulating levels of receptor expression at the cell surface (Angelotti *et al.*, 1993b). In order to examine this possibility [$^{125}$I]-9E10 anti-*myc* antibodies were used to measure changes in cell surface levels of receptors before and after PKA activation. PKA stimulation did not significantly alter the levels of receptors composed of *myc* epitope-tagged α and β subunits at the cell surface. This observation indicates that the effects of PKA phosphorylation on receptor function are probably due to alterations in channel function rather than changes in the number of receptors contributing to whole cell currents.

The mechanism by which a single phosphate covalently attached to the β subunit can reduce receptor function remains unclear. The local charge density due to the presence of the phosphate had been thought to underlie the effect. The ability of a second phosphate within the same region to reverse the functional effect indicates that the functional effects are more likely due to a conformational change

within the receptor subunit proteins. Conversion of receptor enhancement to inhibition or inhibition to enhancement indicates that the mechanism of PKA modulation is likely to be similar for β1 and β3 subunits. The distinct functional effects on phosphorylation of S408 and/or S409 within the β3 subunit will allow examination of functional regulation by PKA using site-directed mutagenesis of these residues.

Together these data indicate that receptor β subunit identity may underlie the reported differential regulation of $GABA_A$-R function by PKA. *In situ* hybridisation studies have been extensively used to examine subunit expression in many regions of the brain (Laurie *et al.*, 1992a; Laurie *et al.*, 1992b; Wisden *et al.*, 1992). Comparison of β subunit expression profiles with reports of receptor modulation by PKA indicate good correlation of β3 subunit expression with receptor enhancement in cerebellar purkinje neurons (Laurie *et al.*, 1992a; Parfitt *et al.*, 1990). Similarly, PKA enhancement of receptor function in rat retinal bipolar cells (Feigenspan and Bormann, 1994) and rat retinal ganglion cells (Veruki and Yeh, 1994) correlates well with immunocytochemical demonstration of β2 and/or β3 subunit expression in these cell types (Greferath *et al.*, 1995). PKA has also been reported to enhance GABA responses in hippocampal neurons (Kapur and Macdonald, 1996; Wang *et al.*, 1997) which are also believed to express predominantly β3 subunit polypeptides (Laurie *et al.*, 1992b; Wisden *et al.*, 1992).

PKA activation in rat cerebellar granule cells has been reported to reduce $GABA_A$ currents (Robello *et al.*, 1990). These cells express predominantly β2 subunits as detected at the mRNA level by *in situ* hybridisation (Laurie et al., 1992a; Laurie et al., 1992b). Immunochemical analysis of α6 subunit containing receptors in these

cells has recently shown that 18% of receptors contain two different β subunits while 51% contain only β2 subunits and β1 or β3 subunits are found alone in 10 and 21% of receptors respectively (Jechlinger *et al.*, 1998). While the effects of PKA phosphorylation on receptors containing multiple β subunits are unknown, the function of receptors containing β1 or β3 subunits alone would be expected to be inhibited or enhanced respectively. These cells could therefore contain populations of receptors which are unaffected, inhibited or enhanced by PKA. Distinct $GABA_A$-R subtypes have been shown to be differentially distributed in hippocampal neurons (Nusser *et al.*, 1996) and a polarised epithelial cell line (Connolly *et al.*, 1996b). Distribution and colocalisation of these numerous receptor subtypes with protein kinases and other signalling molecules could have important implications for their functional regulation in cerebellar granule cells, as has been recently demonstrated for cardiac L-type $Ca^{2+}$ channel modulation by PKA (Gao *et al.*, 1997). Further receptor colocalisation studies together with analysis of receptor trafficking should indicate the relative importance of such compartmentalisation in the regulation of the $GABA_A$-R.

While differential $GABA_A$-R regulation by PKA in neurons may be due to distinct β subunit expression between cell types, there still remains some controversy over the regulation of recombinant receptors by protein kinases. Receptors composed of bovine α1, β1 and γ2S subunits but not α1 and β1 subunits have been shown to be enhanced in mammalian cell lines with high levels of constitutive PKA activity (Angelotti *et al.*, 1993b). While mutation of S409 to alanine within the β1 subunit abolished this receptor regulation PKA phosphorylation of the receptor has not been demonstrated (Angelotti *et al.*, 1993b). In contrast, receptors composed of murine α1 and β1 or α1, β1 and γ2 subunits have been reported to be

inhibited on PKA phosphorylation of S409 within the β1 subunit (Moss *et al.*, 1992b).

The differences between the functional consequences reported in these studies may be due to alternative methods used for PKA activation or measurement of receptor function. Alternatively, these functional differences may reflect distinct receptor regulation between species. Comparison of bovine and murine β1 subunit sequences indicates that they share greater than 97% sequence identity (Schofield *et al.*, 1987; Ymer *et al.*, 1989). 10 of 12 amino acid differences between these subunits occur within the major intracellular domain. Site-directed mutagenesis studies should indicate the role of interspecific differences in the regulation of $GABA_A$-R function.

This work has involved the biochemical and electrophysiological study of $GABA_A$-R regulation by protein phosphorylation. Using a variety of approaches, differential phosphorylation and regulation of receptor subtypes was demonstrated. These data identify a novel mechanism for the differential regulation of $GABA_A$-R subtypes by PKA in the brain. The effects of PKA activation on receptors containing β1, β2 or β3 subunits indicate the distinct roles of $GABA_A$ receptor subtypes in the regulation of synaptic plasticity by protein phosphorylation.

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