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ABSTRACT

Neurons are a diverse cell type exhibiting hugely different morphologies and neurotransmitter specifications. Their distinctive phenotypes are established during differentiation from pluripotent precursor cells. The signalling pathways that specify the lineage down which neuronal precursor cells differentiate remain to be fully elucidated. Among the many signals that impinge on the differentiation of neuronal cells, cytosolic calcium (Ca^{2+}) has an important role. However, little is known about the nature of the Ca^{2+} signals involved in fate choice in neuronal precursor cells, or their sources. In this study, we show that activation of either muscarinic or platelet-derived growth factor (PDGF) receptors induces a biphasic increase in cytosolic Ca^{2+} that consists of release from intracellular stores followed by sustained entry across the plasma membrane. For both agonists, the prolonged Ca^{2+} entry occurred via a store-operated pathway that was pharmacologically indistinguishable from Ca^{2+} entry initiated by thapsigargin. However, muscarinic receptor-activated Ca^{2+} entry was inhibited by siRNA-mediated knockdown of TRPC6, whereas Ca^{2+} entry evoked by PDGF was not. These data provide evidence for agonist-specific activation of molecularly distinct store-operated Ca^{2+} entry pathways, and raise the possibility of privileged communication between these Ca^{2+} entry pathways and downstream processes.

Key terms: Ca^{2+}, PDGF, methacholine, TRPC6, SOCE, human neurosphere-derived cells

INTRODUCTION

The cells required for brain development and growth are generated through the proliferation and differentiation of a population of neuronal stem cells (Reynolds and Weiss, 1992). As well as being present in the developing brain, stem cells have recently been identified in several regions of the adult brain (Alvarez-Buylla and Temple, 1998; Cameron and McKay, 1998; Gage, 1998). Such neuronal stem cells may be responsible for the replacement of dying or damaged neurons (Armstrong and Svendsen, 2000; Gage, 1998; McKay, 2000; Reynolds and Weiss, 1992). These neuronal stem cells can be isolated and maintained in culture, and given the right cues they can differentiate into all the major cell types of the brain: astrocytes, oligodendrocytes and neurons (Gage, 1998; McKay, 2000; Svendsen et al., 1998). In addition, *in vitro* application of mitogens, such as epidermal growth factor, PDGF and fibroblast growth factor, cause these stem cells to self-renew, to form a three-dimensional structure called a neurosphere from which neuronal cells can

ABBREVIATIONS: platelet-derived growth factor, PDGF; methacholine, MCH; canonical transient receptor potential protein isoform 6, TRPC6; Store-operated Ca^{2+} entry, SOCE; NDC, human neurosphere-derived cells

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be obtained and studied (Caldwell et al., 2001; Doetsch et al., 2002).

Stem cells and their more committed daughter cells (neural precursor cells) have been cited as a possible source of tissue to replace dead, dying or damaged neurons in patients suffering from particular neurodegenerative disorders (Gage, 1998; McKay, 2000). For example, embryonic ventral mesencephalon cells have been successfully transplanted into the striatum of patients suffering from Parkinson's disease, producing a lasting symptomatic improvement (Dunnett et al., 2001). However, these primary cells are not readily available, whereas human neurosphere-derived cells (NDCs) can be expanded *in vitro* after dissection (Svendsen et al., 2001; Svendsen et al., 1998). NDCs therefore potentially represent an alternative source of neuronal cells without the ethical concerns associated with embryonic stem cells. Disappointingly, however, these cells have yet to show the same migration, differentiation and integration potential as their totipotent embryonic counterparts (Caldwell et al., 2001).

To provide insight into the mechanisms that control NDC fate, research has focussed on understanding the environmental factors and signalling pathways that control their proliferation and differentiation. It is apparent that diffusible factors within the developing brain create specific microenvironments that through defined signalling pathways direct stem cell fate choices (Spradling et al., 2001). These include growth factors, neurotrophins, cytokines, or more general neurotransmitters, such as those that are released by astrocytes at the site of brain injury (Ridet et al., 1997). It is likely that identification of the environmental cues and signalling pathways that specify the lineage down which neuronal precursor cells differentiate will have considerable clinical benefit (Armstrong and Svendsen, 2000).

Stimulation of neural stem cells with a range of physiological agonists, including neurotransmitters, such as acetylcholine, or growth factors including PDGF, promotes neural stem cell proliferation and/or differentiation (Erlandsson et al., 2001; Ma et al., 2000). Common to all of these agonists is the engagement of the phospholipase C (PLC) signalling cascade (Clapham, 1995). Upon activation, PLC cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). DAG remains in the plane of the membrane, whereas $InsP_3$ is water-soluble and diffuses through the cytosol to find its cognate receptor on the ER and stimulates Ca^{2+} release (Berridge et al., 2003). To replenish the depleted ER Ca^{2+} store, a plasma membrane Ca^{2+} influx pathway known as store-operated Ca^{2+} entry (SOCE) is activated (Parekh and Putney, 2005; Putney, 1986). In addition to activating SOCE, other downstream products of PLC activation, including DAG and arachidonic acid, have been demonstrated to stimulate non-store operated Ca^{2+} entry (Chyb et al., 1999; Moneer and Taylor, 2002; Peppiatt et al., 2004; Shuttleworth and Thompson, 1999). Ca^{2+} influx arising from these entry pathways serves to prolong the duration of the agonist-induced Ca^{2+} signals with long-term consequences for cell function (Parekh and Putney, 2005).

It is well established that Ca^{2+} signals are central to neuronal physiology, controlling development, migration, synaptic transmission and memory consolidation (Berridge, 1998; Spitzer, 2002). Through regulation of the amplitude, frequency and location of Ca^{2+} signals, extracellular stimuli can specifically regulate diverse cellular functions (Berridge, 1997). In particular, hormones that signal through G-protein coupled receptors are known to evoke responses in fully differentiated neurons, and control many aspects of brain function. For example, the stimulation of muscarinic acetylcholine receptors has been linked to the modulation of synaptic plasticity (Yamamoto et al., 2002), in addition to the regulation of cholinergic and dopaminergic neurotransmission (Delmas et al., 2002; Lezcano and Bergson, 2002) and also the regulation of gene expression (Caulfield, 1993; Ma et al., 2000; Simpson et al., 1994; Zhao et al., 2003). Moreover, muscarinic receptors expressed by neural precursors transduce a growth-regulatory signal during neurogenesis via signalling pathways that

are known to evoke Ca^{2+} changes (Ma et al., 2000). Based on these observations, we set out to characterise the profile of the cellular Ca^{2+} signals generated following cellular stimulation by muscarinic receptor activation in NDCs, and compared them to the Ca^{2+} increases caused by the established mitogen PDGF.

METHODS

Neurosphere Culture

Human neural precursors (Clonetics¨, Cambrex, East Rutherford, NJ) were grown as neurospheres in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Poole, UK) /F12 (Invitrogen Life Technologies, Paisley, UK) 3:1; N2, 1% (Invitrogen Life Technologies); EGF, 20 ng/ml (Peprotech, London, UK) and LIF, 10 ng/ml (Chemicon, Hampshire, UK), as previously described (Richards et al., 2004; Richards et al., 2006). Neurospheres were passaged every two weeks by chopping into 250 μm cubes using a tissue chopper (Mickle Laboratory Engineering, Guildford, UK). Cells were prepared for plating 7-12 days post-chopping following dissociation with Accutase™ (1:5 HBSS, Innovative Cell Technologies, San Diego, CA). Briefly, neurospheres were washed three times in phosphate-buffered saline (PBS, Sigma-Aldrich), followed by the addition of 3 ml Accutase in HBSS (Invitrogen Life Technologies) and left on a shaker for 30 minutes at 37ºC. After addition of PBS, cells were dissociated by trituration with a 10 ml pipette. Cells were harvested by centrifugation at 150*g* for 5 minutes, and resuspended in standard plating medium (DMEM/F12, 3:1; transferrin, 50 μg/ml Merck Biosciences); insulin, 5 μg/ml; progesterone, 20 nM; putrescine, 100 μM; T3, 30 nM; selenium, 30 nM (all from Sigma-Aldrich) with further trituration. For microplate-based experiments, cells were plated at 21,000 cells per well in 96-well black-sided microtitre poly-D-lysine coated plates (Biocoat™ , BD Biosciences, Oxford, UK) that had also been coated with laminin (1 μg/ml (Sigma).

Cell-based Microplate Ca^{2+} Imaging

Cells were washed with Krebs/Hanks Buffer (KHB) three times and incubated with 5 μM Fluo-3-AM/0.004% pluronic acid (Molecular Probes) for 1 hour at room temperature. Following incubation, cells were washed 3 times in KHB, and transferred to a BD™ Pathway HT (BD Biosciences) plate imager for compound addition and image collection (Chan et al., 2005). Data was analysed using an in-house custom data analysis package to express data as change in fluorescence over basal or $\Delta F/F_0$.

ImmunoBlotting

NDCs were washed and scraped into ice-cold PBS. Cell suspensions were centrifuged at 1,000 rpm for 5 mins. Cells were lysed by incubation for 1 hr on ice in buffer containing: 10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1% Triton X-100, 5 mM NaF, 1 mM $NaPP_i$, 0.02 mM leupeptin, 0.2 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), as described previously (Kasri et al., 2004). Proteins were resolved by SDS-PAGE (10%) and electroblotted onto 0.2 μm nitrocellulose membranes. The membranes were incubated in blocking buffer (Tris-buffered saline (TBS) containing 0.1% Tween-20 (v/v) and 5% skimmed milk (w/v)) for 1 hour at room temperature, followed by overnight incubation at 4°C in the same buffer containing mouse anti-hTRPC6 (1:500; Alomone) or anti-beta actin (1:10,000; Sigma). Immunoreactive proteins were detected using horse radish peroxidase-(HRP-) conjugated anti-mouse IgG (1:10,000, Jackson Immunoresearch) and visualised with a chemiluminescent substrate for HRP (ECL, Pierce Chemicals).

siRNA Transfection of NDCs

Reduction in hTRPC6 expression was achieved by RNA interference (siRNA, Elbashir et al., 2001a; Elbashir et al., 2001b). SmartPool siRNAs consisting of four different siRNA oligonucleotides targeted against hTRPC6 were obtained from

Dharmacon. Efficient silencing was assessed by immunoblotting and immunofluorescence, using a primary antibody directed against hTRPC6 (Alamone). 0.1 μg siRNA duplex was transfected using JetSI (QBiogene) per well of a 96 well plate for imaging and immunofluorescence according to manufacturers instructions. 1 μg siRNA was used per well of a 6 well plate for Western blotting whit inmunoblotting.

Statistics

Statistical analysis of data was performed using Student's *t*-test in the case of two samples or One-Way ANOVA using Tukey's Multiple Comparison test for three or more samples. All experiments represent an average of 3 or more experiments, each using multiple wells of a 96 well plate, unless otherwise specified. In all experiments involving the ArrayScanII, multiple fields were recorded within each well this last sentencer. Statistical significance is denoted by: **$p<0.01$, ***$p<0.001$.

RESULTS

PDGF and MCH evoked biphasic Ca^{2+} responses

To explore the notion of Ca^{2+} as a signal downstream of G-protein-coupled receptors and growth factor receptors in NDCs, we compared the kinetics and profiles of the Ca^{2+} responses induced by a range of concentrations of MCH and PDGF (Fig. 1). Representative traces showing the response of the cells to 0.3, 3 and 30 ng/ml PDGF and 0.1, 10 and 100 μM MCH are shown in figure 1A. It is evident that both agonists evoked increases in cytosolic Ca^{2+} that comprised of a rapid rise, which typically reached a distinct peak, followed by a prolonged elevation in intracellular Ca^{2+} that persisted for the duration of stimulation. Extended dose response curves indicated that application of MCH or PDGF caused concentration-dependent effects. The peak amplitude (data not shown) and percentage of responding cells (Fig. 1B) increased in direct proportion to the concentration of agonist applied. Maximal concentrations of either MCH or PDGF evoked responses in almost all the cells imaged. The latency (time from addition of an agonist to the peak of the Ca^{2+} signal) declined as the agonist concentration was increased (Fig. 1C). It was evident that the latency was significantly longer over the full dose-response relationship for PDGF in comparison to MCH (Fig. 1C).

To determine the contributions of Ca^{2+} release and Ca^{2+} entry to the responses induced by PDGF and MCH, we examined the effects of the agonists in the presence or absence of extracellular Ca^{2+} (Fig. 2; concentrations of MCH and PDGF giving similar peak Ca^{2+} signals were used). The initial responses evoked by either agonist were not significantly affected by extracellular Ca^{2+} (see Ca^{2+} traces in Fig. 2A and 2C and summarised data the histograms in Fig. 2Bi and Di). However, the extended elevation in cytosolic Ca^{2+}, which occurred when either agonist was applied in the presence of extracellular Ca^{2+}, was abrogated in Ca^{2+}-free conditions (see Ca^{2+} traces in Fig. 2A and 2C and summarised data the histograms in Fig. 2Bii and Dii). In the absence of extracellular Ca^{2+} the cytosolic elevation generally returned to baseline levels within 150 - 200 seconds after initial stimulation ($n = 3$, $p<0.001$, figs 2Aii and Cii). These data indicate that the intracellular Ca^{2+} response to PDGF and MCH consisted of an initial spike, which represented Ca^{2+} release from intracellular stores and a sustained elevation that depended on extracellular Ca^{2+}. In many cells, the recovery of Ca^{2+} following stimulation in Ca^{2+}-free media did not follow a simple exponential decline. Rather, there were frequently one or two oscillations of the cytosolic Ca^{2+} concentration on the declining phase of the response.

Methacholine and PDGF activate a store-operated Ca^{2+} entry pathway in NDCs

Having established that Ca^{2+} influx contributed to the cytosolic Ca^{2+} transient induced by PDGF and MCH, we next

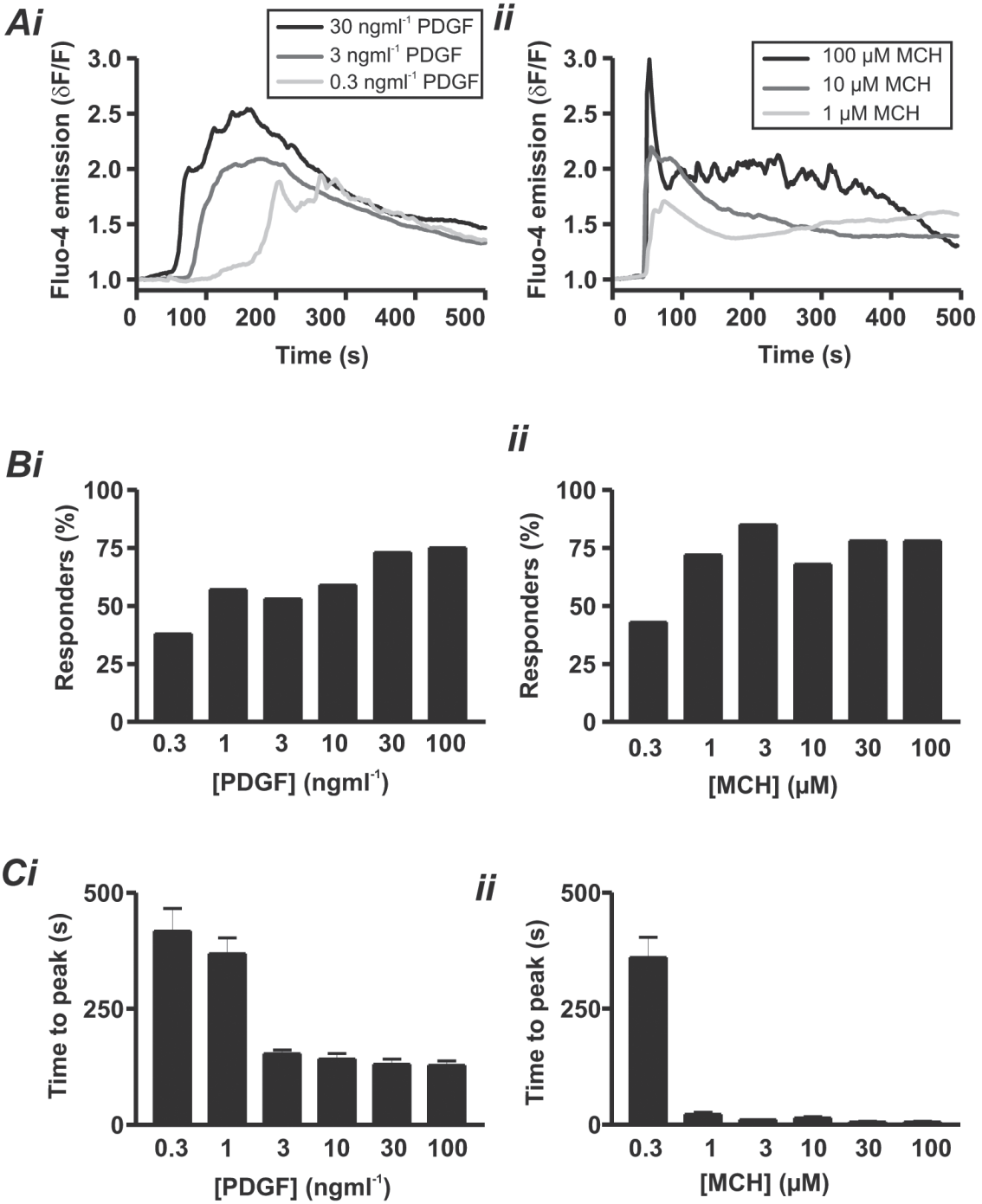

Figure 1: Characterisation of MCH- and PDGF-induced Ca^{2+} signals in NDCs. **A**, Representative traces of the Ca^{2+} responses induced by the indicated agonist concentrations. **B**, concentration-dependent effect of MCH and PDGF on the proportion of responsive cells. A change in fluorescence signal ($\Delta F/F_0$) >1.1 was classified as a response. **C**, averaged data (Mean ± SEM, *n* > 1000 cells for each condition) indicating the latency (time from agonist addition to the peak of the response) for MCH- and PDGF-stimulated Ca^{2+} signals.

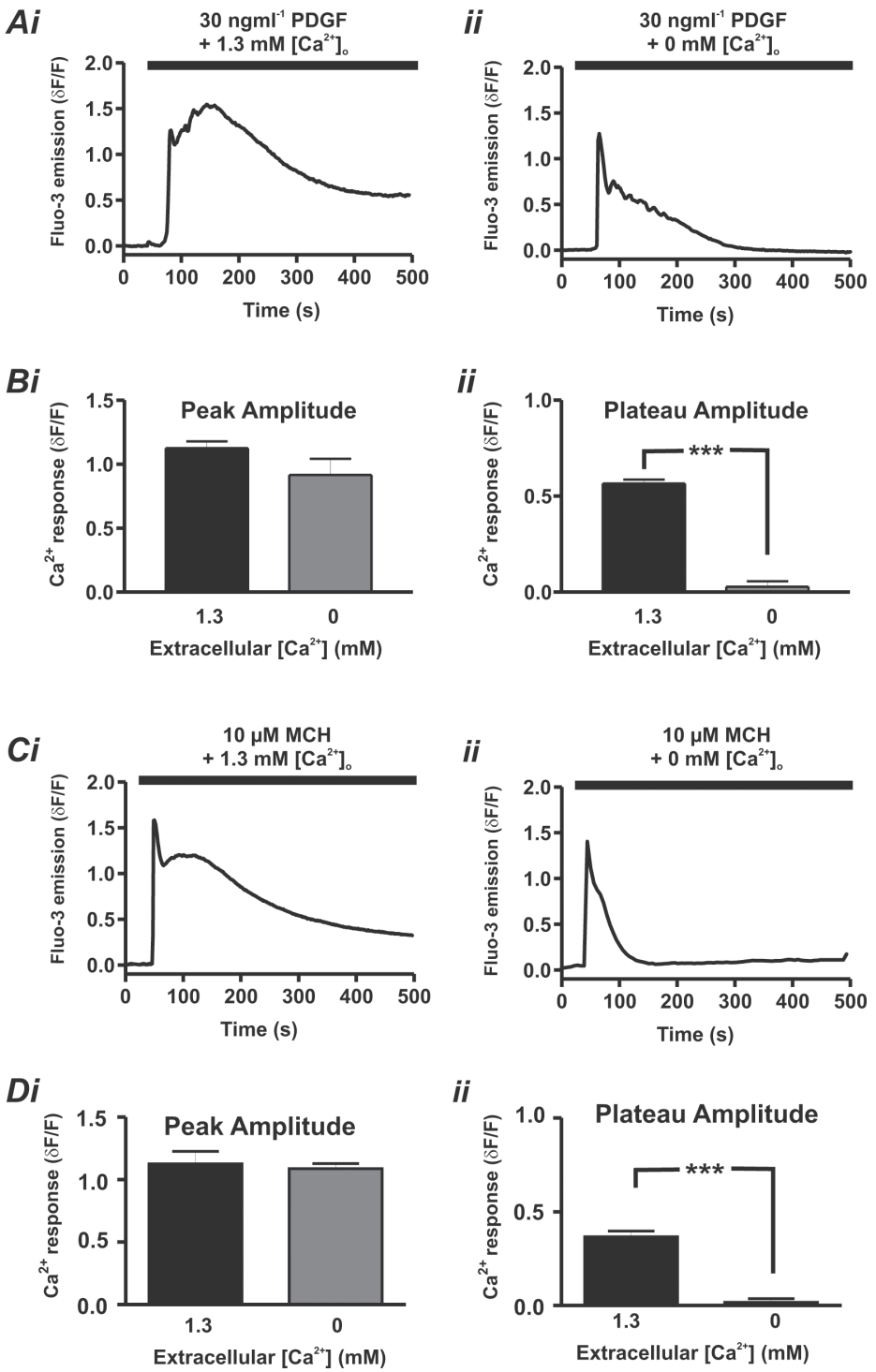

Figure 2: PDGF and MCH induce Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane. A and **C**, representative responses from single NDCs stimulated with the indicated agonist either in (**i**) Ca^{2+}-containing or (**ii**) Ca^{2+}-free medium. **B** and **D**, averaged data (Mean ± SEM, $n > 1000$ cells for each condition) summarising: (**i**) the peak Ca^{2+} release amplitude (measured 50 – 100 s after agonist addition), and (**ii**) amplitude of the Ca^{2+} (entry) signal measured at 300 s post addition of the agonist. *** indicates significance ($p < 0.001$), and 'ns' indicates lack of significant difference.

investigated the pharmacological profile of the pathway responsible for the entry of Ca^{2+}. In particular, we employed the established Ca^{2+} entry antagonists SKF-96365 and gadolinium (Gd^{3+}). The former compound has been used in many studies to probe SOCE (Baba et al., 2003; Flemming et al., 2003; Vostal and Shafer, 1996). Differential sensitivity to high (100 μM) or low (1 μM) concentration of lanthanides, such as Gd^{3+}, has been used previously to investigate whether Ca^{2+} entry is arising through a store-operated or non store-operated Ca^{2+} entry pathway (Broad et al., 1999).

Experiments were performed to test the relative sensitivities of PDGF and MCH-induced Ca^{2+} entry to 10 μM SKF-96365 and 1 μM Gd^{3+}. As a positive control, the effect of these inhibitors upon Ca^{2+} entry induced by thapsigargin was also tested. Thapsigargin is an irreversible inhibitor of the sarcoplasmic endoplasmic reticulum ATPase (SERCA), and it depletes the ER Ca^{2+} store resulting in robust and specific activation of SOCE (Holmes et al., 2007). To uncover the effect of SKF-96365 and Gd^{3+} on Ca^{2+} entry induced by MCH or PDGF, a protocol was used that involved store depletion by agonist application in the absence of extracellular Ca^{2+}, followed by activation of Ca^{2+} entry due to re-addition of Ca^{2+} to the extracellular medium. As shown in figures 3A and B, application of either MCH or PDGF to the cells in Ca^{2+}-free medium evoked an initial rise in intracellular Ca^{2+}, which was due to release from intracellular stores. After this increase in intracellular Ca^{2+} had returned to baseline levels, Ca^{2+} was added to the extracellular medium resulting in an elevation in intracellular Ca^{2+} due to influx across the plasma membrane. Application of 1 μM Gd^{3+} completely abrogated Ca^{2+} entry induced by both MCH and PDGF (Figs. 3A and B). This was manifest as a decrease in both the peak Ca^{2+} signal and integrated Ca^{2+} transient following Ca^{2+} re-addition (Figs. 3A and B). No effect of Gd^{3+} was seen on the MCH- or PDGF-induced Ca^{2+} release. Gd^{3+} exhibited a similarly complete degree of inhibition upon Ca^{2+} influx following store depletion with thapsigargin (Fig. 3C). SKF-96365 also significantly reduced the Ca^{2+} entry signal induced by both MCH and PDGF, without affecting Ca^{2+} release (Figs. 3A and B). The degree of inhibition of PDGF- and MCH-induced Ca^{2+} entry by SKF-96365 was not as great as that observed for Gd^{3+} (Fig. 3A and B). The reduced efficacy of SKF-96365, compared to Gd^{3+}, in inhibiting Ca^{2+} influx induced by MCH and PDGF was also mirrored by its lower potency in inhibiting thapsigargin-induced Ca^{2+} entry (Fig. 3C). These data indicate that Ca^{2+} signals induced by MCH, PDGF- and thapsigargin were similarly affected by Gd^{3+} and SKF-96365.

We also tested the possibility that MCH or PDGF could activate alternative, non-SOCE, Ca^{2+} entry pathways. This was examined by applying MCH or PDGF to cells during an on-going SOCE signal that had been activated by thapsigargin (Fig. 4). As a positive control, we also utilised calmidazolium (CMZ), which we have previously demonstrated to activate a non-SOCE pathway (Holmes et al., 2007; Peppiatt et al., 2004). Cells were treated with thapsigargin for 20 minutes to completely discharge the ER and activate SOCE. Subsequently, MCH, PDGF or calmidazolium were added. Of these agents, only calmidazolium triggered an additional Ca^{2+} influx. The similarity of the pharmacological profiles of both MCH-, PDGF- or thapsigargin-induced Ca^{2+} influx, and the inability of MCH or PDGF to induce further Ca^{2+} entry after thapsigargin-mediated SOCE activation, suggests that a common Ca^{2+} entry mechanism is activated by all three agents.

MCH-activated SOCE is dependent upon TRPC6 expression

TRP channels have been suggested to underlie store-operated Ca^{2+} entry in a number of cell types, and play important roles in neuronal physiology. Microarray analysis of our NDCs revealed that mRNAs for TRPC5 and TRPC6 were abundant (data not shown). We considered the possibility that TRPC5 might underlie the Ca^{2+} entry signals that we see. However, TRPC5 has been shown to negatively regulate neuronal

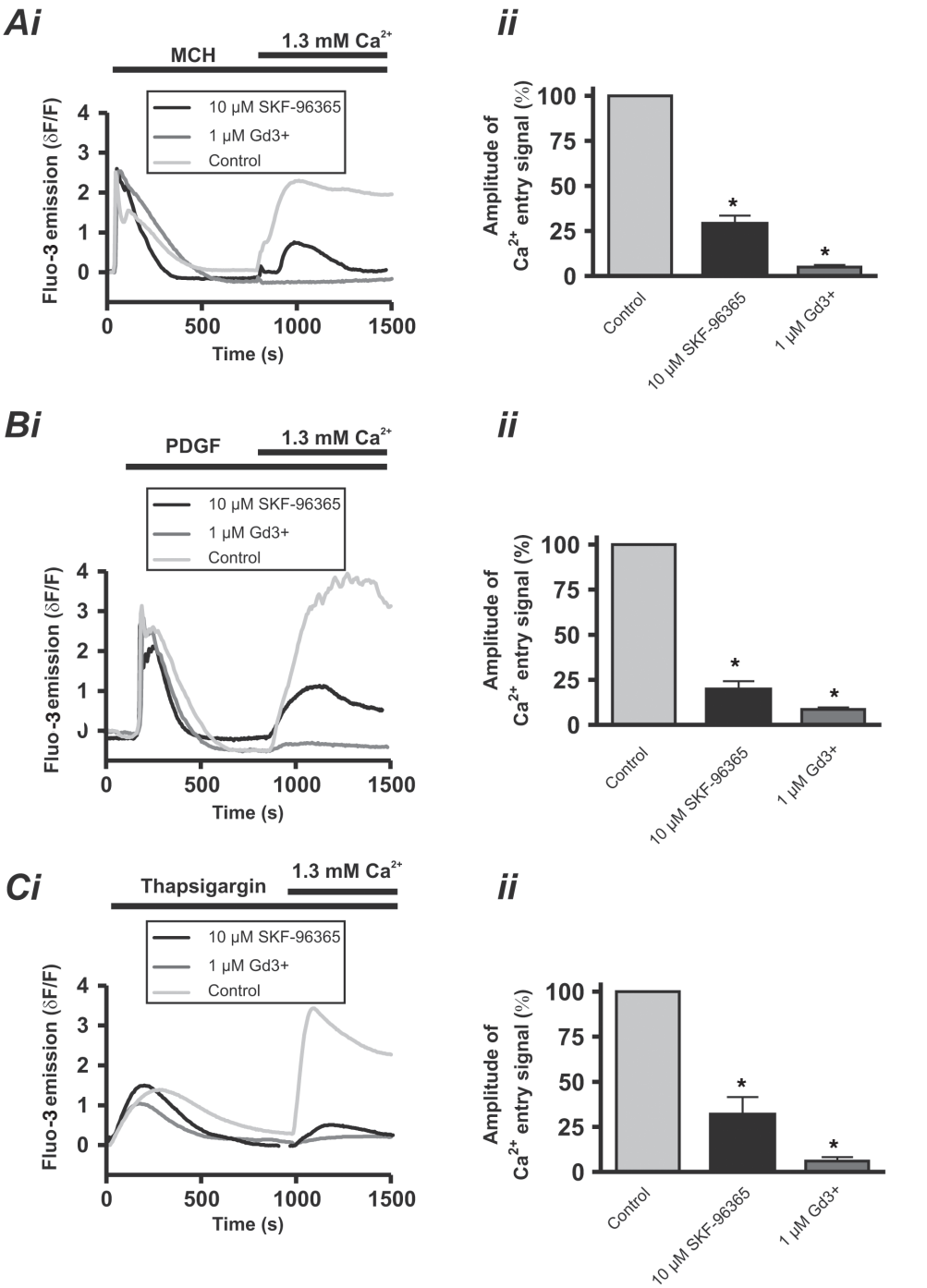

Figure 3: PDGF- and MCH-stimulated Ca^{2+} entry is pharmacologically similar to thapsigargin-activated SOCE. Ca^{2+} influx in response to cellular stimulation with MCH, PDGF and Thapsigargin was imaged in NDCs. Following depletion of the intracellular Ca^{2+} store, extracellular Ca^{2+} (1.3 mM) was added back to the cells in the presence or absence of Gd^{3+} (1 µM) or SKF-96365 (10 µM). **Ai** – **Ci**, representative traces showing responses to MCH, PDGF and thapsigargin, in the presence or absence of the Ca^{2+} entry inhibitors. **Aii** – **Cii**, averaged data (Mean ± SEM, $n > 1000$ cells for each condition) indicating the integrated Ca^{2+} signal (i.e. area under the Ca^{2+} trace) measured for 10 minutes following readdition of extracellular Ca^{2+}. * indicates statistically significance ($p < 0.05$).

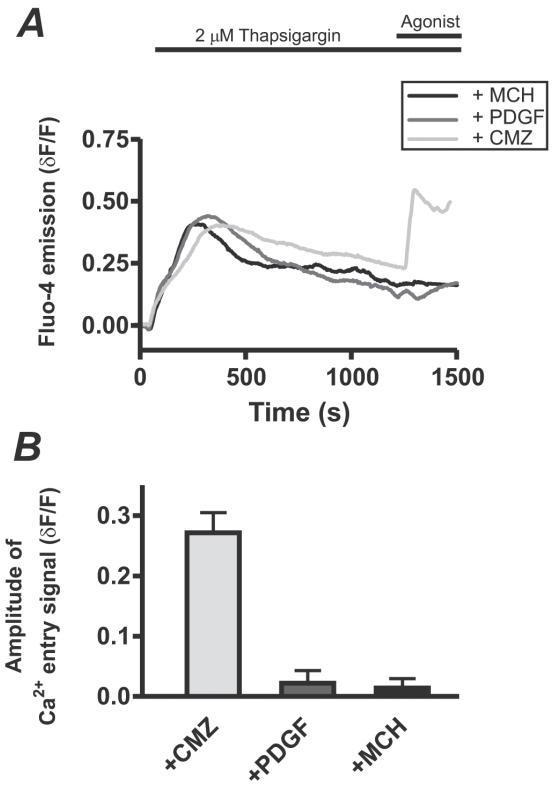

Figure 4: The Ca^{2+} influx stimulated by PDGF or MECH is not additive with thapsigargin-activated SOCE. A, representative traces showing the lack of effect of PDGF (30 ng/ml) or MCH (10 μM) during an on-going thapsigargin-evoked SOCE signal. Calmidazolium (10 μM) was used as a positive control, and did induce additional Ca^{2+} influx. **B**, averaged data (Mean ± SEM, $n > 100$ cells for each condition) indicating the net effect of calmodazolium, PDGF or MCH to the thapsigargin-evoked SOCE response.

outgrowth (Greka et al., 2003), but Ca^{2+} entry potentiates neurite outgrowth in our NDCs (unpublished observations). Furthermore, TRPC5 activity is potentiated by lanthanides (Clapham et al., 2001), whereas we observed an inhibition of Ca^{2+} entry by Gd^{3+}. For these reasons, TRPC5 was not considered further during this study. TRPC6 appeared an attractive candidate as it has previously been implicated in mediating SOCE in other cell types (Yuan et al., 2003).

The role of TRPC6 in PDGF-, Thapsigargin- and MCH-induced Ca^{2+} entry into NDCs was examined by exploiting specifically-targeted siRNAs to reduce protein expression. NDCs were transfected with pooled siRNAs designed against four regions of TRPC6 mRNA for 24 hours, after which TRPC6 expression was investigated by immunoblotting (figure 5A). Parallel experiments using a fluorescent, Cy3-conjugated siRNA indicated that greater than 90% transfection efficiency was achieved using the transfection protocol (Fig. 5B). Endogenous TRPC6 in mouse brain lysates, and in lysates prepared from the untreated control NDCs was detected as two bands. These two bands are proposed to represent endogenous TRPC6 (~106 kDa) and a splice variant (~50 kDa). Following 24 hours TRPC6 siRNA transfection, TRPC6 protein levels were substantially reduced (the 106 kD band by 66%, and the 50 kD band by 50%).

For Ca^{2+} imaging experiments, NDCs were transfected with TRPC6 siRNA 24 hours prior to experimentation. Cy3-conjugated, luciferase-directed siRNAs were used for control. TRPC6 siRNA and Cy3-conjugated siRNA transfected cells were stimulated with MCH (10 μM), thapsigargin (2 μM), or PDGF (30 ng/ml) in the absence of extracellular Ca^{2+} to deplete the intracellular Ca^{2+} store. SOCE was subsequently initiated by addition of Ca^{2+} to the extracellular buffer (Fig. 5Ci-Ei). TRPC6 reduction had no effect upon the amplitude of the initial Ca^{2+} transients arising through release from intracellular Ca^{2+} stores. However, TRPC6 siRNA transfection had different effects upon SOCE induced by PDGF, MCH or thapsigargin. Specifically, MCH-induced Ca^{2+} entry was significantly reduced in cells transfected with TRPC6 siRNA when compared with control-transfected cells (Fig. 5Cii). Whereas, TRPC6 reduction did not affect thapsigargin- or PDGF-induced Ca^{2+} influx (Figure 5Dii and Eii).

DISCUSSION

It has been demonstrated that Ca^{2+} signals play a key role in neuronal development controlling differentiation, neurite

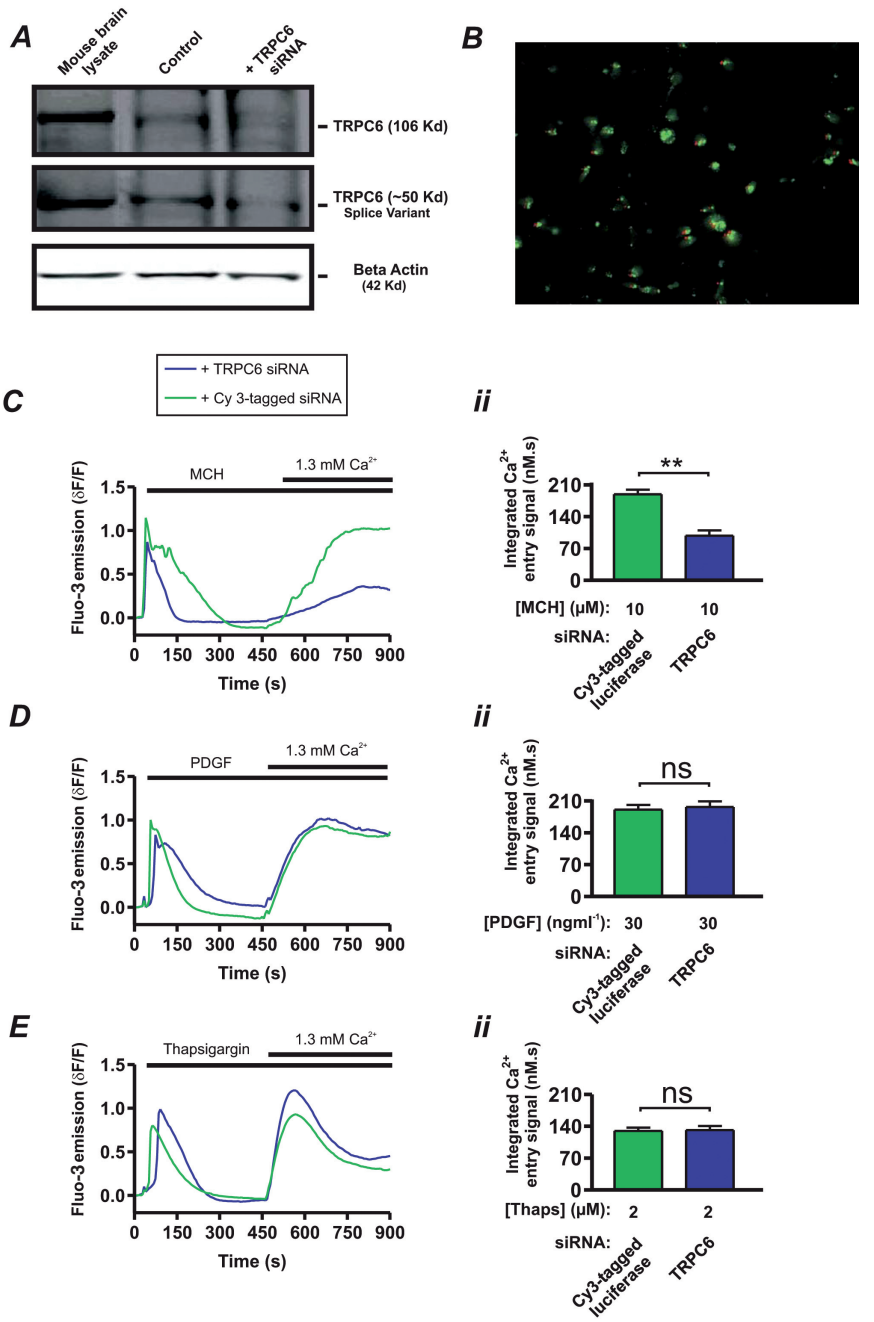

Figure 5: TRPC6 is a component of MCH-induced Ca^{2+} entry. **A**, upper panels, immunoblots showing the abundance of the 105 kDA and 50 kDa splice variants of TRPC6 in mouse brain lysate, and in lysates prepared from NDCs 24 h post-transfection with control Cy3-conjugated luciferase siRNA or TRPC6 siRNA. Lower panel, immunoblot showing the abundance of β-actin (used as a loading control) in the lysates described above. **B**, Representative overlay image of cells loaled with calcein (marker of cell viability, green) and Cy3-tagged luciferase siRNA (control siRNA, red). **Ci - Ei**, typical traces showing MCH-, PDGF- and thapsigargin-induced Ca^{2+} influx in NDCs 24 hours post transfection with TRPC6 siRNA or control Cy3-conjugated luciferase siRNA. Data is shown as mean ± SEM (n > 500 cells). **Cii - Eii**, averaged data (Mean ± SEM, n > 1000 cells for each condition) indicating the integrated Ca^{2+} signal (i.e. area under the Ca^{2+} trace) measured for 8 minutes following readdition of extracellular Ca^{2+}. * indicates statistically significance ($p < 0.01$).

outgrowth and pathfinding (Bezzerides et al., 2004; Shim et al., 2005). Indeed, we have previously shown that PDGF induces neurite outgrowth in NDCs in a Ca^{2+}-dependent manner (Richards et al., 2006). Stimulation of muscarinic receptors, on the other hand, promotes NDC proliferation without neurite outgrowth (unpublished observations). An aim of this study was to examine whether the differing biological outcomes (i.e. neurite outgrowth versus proliferation) caused by PDGF and muscarinic receptor stimulation was due to alternative patterns of Ca^{2+} signalling.

Ca^{2+} transients elicited by both agonists were comprised of Ca^{2+} release from finite intracellular stores, followed by a prolonged elevated level of intracellular Ca^{2+}, which was due to Ca^{2+} flux across the plasma membrane (Figs. 1 and 2). The pattern of Ca^{2+} signals evoked by PDGF and MCH in NDCs is typical of the response of many different cell types to $InsP_3$-generating agonists (Bootman et al., 1992; Trebak et al., 2002b). Ca^{2+} entry induced by both PDGF and MCH was pharmacologically similar to the SOCE pathway initiated by ER Ca^{2+} store depletion with thapsigargin (Fig. 3).

Although the amplitudes of the Ca^{2+} transients induced by either MCH or PDGF were similar at maximal concentrations, the time taken to reach the peak of the Ca^{2+} transient was typically greater for PDGF than MCH (Fig. 1). This increased latency became more pronounced with decreasing agonist concentrations. These differences can in part be ascribed to the different kinetics of $InsP_3$ generation downstream of PDGFR and mAChR activation. Stimulation by PDGF results in dimerisation of plasma membrane-localised receptors and phosphorylation of serine and tyrosine residues, which leads to downstream activation of PLCγ (Clapham, 1995). A recent study presented evidence for a lipase-independent role for PLCγ in the activation of Ca^{2+} entry (Patterson et al., 2002), suggesting that rather than Ca^{2+} release from intracellular stores driving Ca^{2+} entry, PDGFRs could couple directly to Ca^{2+} entry channels with which they co-localise in signalling microdomains (Ambudkar et al., 2004). Although our data cannot rule out such a mechanism for PDGF-evoked Ca^{2+} entry, we did not observe any additional Ca^{2+} influx when PDGF was applied to NDCs with an on-going SOCE response (Fig. 4). This would suggest that any store-independent Ca^{2+} entry activated by PDGF is less profound than SOCE.

In this study, PDGF and MCH were both found to activate SOCE. This conclusion was based upon the findings that they released Ca^{2+} from intracellular stores, and that the Ca^{2+} entry stimulated by both agonists was sensitive to Gd^{3+} and SKF-96365, which are known inhibitors of SOCE. The degree of inhibition of MCH- and PDGF-evoked Ca^{2+} influx by Gd^{3+} and SKF-96365 was similar to the reduction of SOCE when it was induced by thapsigargin. The incomplete inhibition of Ca^{2+} entry by SKF-96365 is typical of its effects seen in other systems (Merritt et al., 1990; Vassilopoulos et al., 2007). Although the Ca^{2+} entry pathways activated by PDGF, MCH and thapsigargin were pharmacologically indistinguishable from each other, we detected a differential sensitivity to reduction of TRPC6 (Fig. 5). siRNA-mediated knockdown of TRPC6 resulted in a profound decrease in the magnitude of MCH-induced Ca^{2+} entry. No effect on Ca^{2+} entry induced by PDGF or thapsigargin was however seen. Although TRPC6 was initially characterised as a DAG-activated cation channel (Hofmann et al., 1999), other reports suggest that it may mediate SOCE (Singh et al., 2007; Yu et al., 2003). In certain cellular contexts, TRPC3 and 7, other members of the TRPC subclass to which TRPC6 belongs, have also been reported to form store-operated channels. For example, when expressed at low levels, TRPC3 and 7, form channels that are store-operated, whereas when highly overexpressed they form channels that are non-store operated (Lievremont et al., 2004; Trebak et al., 2002a).

Given that Ca^{2+} is a profound regulator of neuronal behaviour, the channels that underlie its movement represent attractive targets for therapeutic intervention. We have previously found that stimulation of

NDCs with PDGF or MCH results in different biological outcomes. In our hands, the former agonist promotes NDC proliferation neurite outgrowth, whilst the latter stimulates proliferation but not neurite extension. It is likely that Ca^{2+} is a key signal in both of these activities. At a gross level, the Ca^{2+} signals evoked by MCH and PDGF are very similar. However, probing the molecular nature of the pathways responsible for Ca^{2+} influx reveals that MCH and PDGF do not employ the same channels. It is therefore possible that subtle differences in the spatial distribution of the Ca^{2+} channels recruited by MCH or PDGF, along with the engagement of other signalling elements, allow these two agonists to produce distinctive biological effects.

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