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Sphingosine 1-phosphate induces CREB activation in rat cerebral artery via a protein kinase C-mediated inhibition of voltage-gated K^+ channels

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Abstract

Sphingosine 1-phosphate (S1P) is a potential mitogenic stimulus for vascular smooth muscle. S1P promotes an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) in cerebral arteries, however S1P effects on regulation of gene expression are not known. Activation of the Ca^{2+}-dependent transcription factor, cAMP response element-binding protein (CREB), is associated with smooth muscle proliferation. The aim of this study was to examine the Ca^{2+}-dependent mechanisms involved in S1P-induced CREB activation in cerebral artery. Western blotting and immunofluorescence with a phospho-CREB antibody were used to detect CREB activation in Sprague–Dawley rat cerebral arteries. Whole-cell patch clamp recording and single cell imaging of $[Ca^{2+}]_i$ were performed on freshly isolated cerebral artery myocytes. S1P increased activation of CREB in the nucleus of cerebral arteries. This activation was mediated by Ca^{2+}/calmodulin-dependent protein kinase and was dependent on an increase in $[Ca^{2+}]_i$ via two mechanisms: (i) intracellular Ca^{2+} release via an inositol 1,4,5-trisphosphate ($InsP_3$)-dependent pathway and (ii) Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCC). Activation of the VDCC occurred through S1P-induced inhibition (approximately 50%) of the voltage-gated potassium (K^+) current. This inhibition was via a protein kinase C-mediated pathway resulting in tyrosine phosphorylation of at least one isoform of the Kv channel (Kv 1.2). These results demonstrate that S1P can activate the transcription factor CREB through different Ca^{2+}-dependent pathways including intracellular Ca^{2+} release and inhibition of voltage-gated K^+ channels leading to Ca^{2+} influx. Our findings suggest a potential role for S1P in regulation of gene expression in vascular smooth muscle.

Keywords: Vascular smooth muscle; Transcription factor; Sphingosine 1-phosphate; Calcium release; Potassium channels; Calcium channels

1. Introduction

Pathophysiological alterations in vascular structure and responsiveness are a major determinant of cardiovascular diseases. These alterations occur via a phenotypic modulation of vascular smooth muscle cells [1,2]. In atherosclerosis and also following vascular injury, a phenotypic modulation of vascular smooth muscle cells occurs from a differentiated contractile phenotype to a proliferative cell phenotype [3,4]. The processes that regulate this switch are not well understood. Studies of vascular smooth muscle proliferation have focussed on well-established growth factors for vascular smooth muscle cells such as platelet-derived growth factor (PDGF) [5,6]. Such factors stimulate a variety of mitogenic pathways that regulate transcription and lead to selective expression of genes required for vascular smooth muscle proliferation.

Sphingosine 1-phosphate (S1P) is a potentially important mitogenic factor which is found throughout the cardiovascular system [7]. It is a sphingolipid stored in high concentrations in platelets [8]. Activation of pro-thrombotic stimuli results in the release of S1P from platelets achieving high concentrations in serum (nmol/L to μmol/L range) [9,10]. Due to the lipophilic nature of

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Abbreviations: CREB, cAMP response element-binding protein; PDGF, platelet-derived growth factor; S1P, sphingosine 1-phosphate; VDCC, voltage-dependent calcium channel; PKC, protein kinase C; 2-APB, 2-aminoethoxydiphenylborate; Cam kinase, calcium/calmodulin-dependent protein kinase; InsP3, inositol 1,4,5-trisphosphate; 4-AP, 4-aminopyridine.

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S1P and the levels reached *in vivo*, vascular smooth muscle cells are exposed to relatively high concentrations in pathophysiological conditions. This sphingolipid may, therefore, have a pivotal role in the stimulation of vascular smooth muscle cell phenotypic modulation. It is now clear that S1P-induced intracellular effects occur predominantly through activation of selective S1P receptors on the plasma membrane [11]. These receptors belong to the G-protein-coupled receptor superfamily, originally known as endothelial differentiation gene (EDG) receptors. Several isoforms have now been cloned and $S1P_1$ [12], $S1P_2$ [13], $S1P_3$ [14], $S1P_4$ [15] and $S1P_5$ [16] have high affinities for S1P with EC_{50}s in the nmol/L range. Both cultured and freshly isolated vascular smooth muscle cells express at least $S1P_1$, $S1P_2$ and $S1P_3$ [17–19]. Recent studies have begun to reveal the possible physiological and pathophysiological roles of S1P in the cardiovascular system. A study with $S1P_1$ receptor knockout mice has demonstrated the essential involvement of this receptor for full blood vessel development [20]. In cultured vascular smooth muscle cells, S1P stimulates DNA synthesis [17,18] suggesting that S1P receptors could play important roles in regulating proliferation in vascular smooth muscle. We have recently demonstrated that S1P receptors are expressed in native vascular smooth muscles and can activate several intracellular pathways [19]. Interestingly, different vascular smooth muscles express different levels of the S1P receptor subtypes. For example, rat cerebral arteries express 4-fold higher levels of $S1P_2$ and $S1P_3$ compared to rat aorta [19]. This differential expression of S1P receptor subtypes translates into differences in the S1P-induced activation of intracellular pathways.

In the pathogenesis of vascular disease, the switch from a contractile to a proliferating phenotype may involve activation of S1P receptors [7]. It is, therefore, important to determine S1P-activated pathways in native contractile smooth muscle that may be involved in this process. This involves a number of key transcription factors that initiate a complex expression of specific genes and drive the proliferation process in vascular smooth muscle cells [21,22]. Studies using cultured vascular smooth muscle cells have implicated several transcription factors, including cyclic AMP response element-binding protein (CREB) [23,24]. Recent studies in native, contractile smooth muscle have demonstrated that this transcription factor associated with cellular proliferation is expressed and can be activated by agonists and growth factors [25–28]. Agonist-stimulated regulation of $[Ca^{2+}]_i$ can lead to activation of CREB [25–28], however in each case specific spatial and temporal characteristics of this Ca^{2+} signal are required. Although S1P can increase $[Ca^{2+}]_i$ as well as several other signalling pathways in vascular smooth muscles, its regulatory effects on transcription factors have not been determined.

In the present study, we have investigated the regulation of CREB activation by S1P in rat cerebral artery. S1P increases activation of CREB and is dependent on an increase in intracellular Ca^{2+}. This increase can occur via a release from intracellular stores, or via an influx through voltage-dependent Ca^{2+} channels (VDCC). Activation of these channels is the result of S1P-induced inhibition of the voltage-dependent K^+ current via a protein kinase C (PKC)-mediated mechanism.

2. Materials and methods

2.1. Tissue preparation

Sprague–Dawley rats were killed by inhalation of CO_2 followed by cervical dislocation. Cerebral arteries (middle, basilar) were placed into ice-cold HEPES-buffered Krebs solution. Arteries were cleaned of connective tissue and the endothelium was removed by gentle rubbing of the lumen. Arteries were stimulated with S1P *ex vivo* and immediately frozen. The investigation conforms with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Immunoblotting and immunoprecipitation

Frozen tissues were added to lysis buffer and homogenised at 4° in a Braun homogenising vessel as previously described [29]. In order to obtain sufficient tissue, the cerebral arteries from two to three rats were pooled for each sample. Protein was measured using a Lowry assay (Biorad) to ensure equal protein loading. In addition, samples were checked using Coomassie Blue stained gels, and membranes were stained with Ponceau Red to confirm protein loading. Whole-cell homogenates were used for immunoblotting with anti-CREB antibodies (phospho- and pan-) (New England Biolabs). Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Biorad) as previously described [29]. The membranes were immunoblotted with primary antibodies followed by detection with horseradish peroxydase-conjugated secondary antibodies. The immunoreactive bands were visualised using enhanced chemiluminescence and quantitated with an imaging densitometer (Biorad GS-690). Only blots that had entirely nonsaturated pixels (as determined using Multi-analyst software, Biorad) were analysed to ensure linearity of densitometric analysis.

For immunoprecipitation experiments, cerebral arteries were homogenised in lysis buffer (100 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 30 mmol/L sodium pyrophosphate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.5 mmol/L sodium orthovanadate, 20 mmol/L HEPES, pH 7.4). The primary antibody (anti-Kv 1.2 α-subunit, Upstate Biotechnology) was added to the tissue extract and gently agitated overnight at 4°. A protein G

agarose bead slurry was added and the extract incubated at 4° for a further 2 hr. The agarose beads were collected by a short centrifugation and the beads washed three times in ice-cold phosphate-buffered saline (PBS). The washed beads were boiled for 5 min in Laemmli sample buffer and subjected to SDS–PAGE followed by immunoblotting as above, with anti-phosphotyrosine antibody (Upstate Biotechnology).

2.3. Immunofluorescence

Cerebral arteries from rats were isolated and each artery was divided into five segments. Each segment was then treated at 37° with various agents and times as specified below. Comparisons could, therefore, be made for each treatment in the same artery. Different segments were pre-incubated, when indicated, with the following inhibitors: 2-aminoethoxydiphenylborate (2-APB; 30 μmol/L) for 40 min; and KN-93 (30 μmol/L) for 60 min or nifedipine (1 μmol/L) + 2-APB (30 μmol/L) for 40 min; before the addition of S1P. Cerebral artery segments were stimulated with 1 μmol/L S1P for 20 min at 37°. Following stimulation, segments were fixed in PBS containing 3% paraformaldehyde, embedded, and flash-frozen. Cerebral artery segments were cut in cross-section to a thickness of 10 μm, and blocked for 1 hr with 2% bovine serum albumin (BSA) in PBS. Primary antibody (rabbit anti-phospho-CREB (P-CREB) (Upstate Biotechnology) diluted 1:500 in 0.2% Triton-X/2% BSA/PBS) was applied overnight at 4°C. Secondary antibody (FITC–anti-rabbit IgG (Jackson Immunoresearch) 1:200 dilution in 2% BSA/PBS) was applied for 1 hr at 25°. The fluorescent nucleic acid dye BOBO-3 (1:5000 in PBS, Molecular probes) was used for identification of nuclei. Immunofluorescence was detected using a Biorad 1024 laser scanning confocal microscope and five to eight sections per treatment were analysed using standardised conditions. Specificity of immune staining was confirmed by the absence of fluorescence in the arteries incubated with secondary antibody alone.

2.4. Enzymatic dissociation of VSM myocytes

Single smooth muscle cells were obtained using methods previously described [19]. Briefly, cerebral arteries were transferred to a Ca^{2+}-free Hanks solution consisting (in mmol/L): 55 NaCl, 80 Na glutamate, 5.6 KCl, 2 $MgCl_2$, 10 glucose, 10 HEPES (pH 7.4). Arteries were incubated for 20 min in solution containing 0.3 mg/mL papain (Sigma), 1 mg/mL dithioerythritol (Sigma) and 1 mg/mL BSA. Tissues were further incubated for 5 min in Hanks solution (37°) supplemented with 1 mg/mL BSA, 0.8 mg/mL collagenase F (Sigma), and 100 μmol/L $CaCl_2$. After several washes, tissues were triturated. Isolated smooth muscle cells were stored on glass-bottom dishes maintained at 4° and used within 6 hr. Only cells that had an elongated morphology were used.

2.5. Imaging of $[Ca^{2+}]_i$

Individual VSM myocytes were loaded with 2 μmol/L fura-2 AM for 30 min in a solution containing (in mmol/L): 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 1.7 $CaCl_2$, 11 Glucose, 10 HEPES (pH 7.4) followed by a 20-min de-esterification period. A Zeiss Axiovert 200 inverted microscope, equipped with a cooled CCD camera (Photometrics) and a polychromatic illumination system (T.I.L.L. Photonics), was used to capture fluorescence images with excitations at 340 and 380 nm. The ratio of the fluorescence intensity between the pair of frames (FR340/380) was calculated after background subtraction. The Metafluor 4.6 software (Universal Imaging Corporation) controlled the illuminator and camera, and performed image ratioing and analysis. Results are expressed as F340/380 ratio. Experiments were carried out at room temperature (22–24°).

2.6. Membrane current measurements

Membrane currents were measured using the whole-cell patch clamp technique. Patch pipettes (resistances, 4–8 MΩ) were filled with solution containing (in mmol/L): 140 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 1.1 EGTA, 2 ATP (pH 7.2). Recordings were made in an extracellular solution containing (in mmol/L): 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 1.7 $CaCl_2$, 11 Glucose, 10 HEPES (pH 7.4). Whole-cell currents were measured with an Axoclamp 2A (Axon Instruments Inc) and stored on digital audiotape using a digital tape recorder (DTR 1200, Biologic). Data were analysed using a Tandon computer (Cambridge Electronic Design software, version 6.0). Whole-cell outward currents were elicited by applying step depolarisations in 10 mV steps from −60 to +130 mV from a holding potential of −30 mV. After steady state was reached, 1 μmol/L S1P (diluted in 0.02% DMSO) was externally applied by low-pressure ejection about 50–100 μm away from the cell being recorded. K^+ currents were not significantly altered by 0.02% DMSO alone, diluted in the extracellular solution (data not shown).

2.7. Analysis of data

Data are expressed as mean ± SEM. Significance was tested by means of Student's *t*-test or ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. S1P induces activation of the transcription factor CREB

To investigate whether S1P can phosphorylate CREB in rat cerebral arteries, we performed immunoblot analysis using an antibody that recognises the Ser133 phosphorylated

(activated) form of CREB. Rat cerebral arteries were stimulated with S1P *ex vivo*. Phosphorylation of CREB was increased by 10 ± 2.3-fold in S1P-stimulated cerebral arteries (1 μmol/L S1P; 20 min) compared with unstimulated arteries (Fig. 1A). Cross-sections (10 μm thick) of cerebral arteries were also analysed for P-CREB using immunostaining. Sections were double stained with the cyanine dye BOBO-3 to identify nuclei. Cerebral arteries treated with 1 μmol/L S1P exhibited a significant increase in the number of nuclei staining positive for P-CREB (Fig. 1B).

3.2. S1P-induced CREB activation involves Ca^{2+} release from the sarcoplasmic reticulum, extracellular Ca^{2+} influx and Ca^{2+}/calmodulin-dependent protein kinase

We examined the pathways responsible for S1P-induced phosphorylation of CREB. Recently, we reported that extracellular application of S1P-induced transient intracellular Ca^{2+} increases in single myocytes from cerebral artery [19]. Because Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) has been shown to play an important role in the signalling pathway leading to CREB phosphorylation, arteries were treated with KN-93 a CaM kinase inhibitor, followed by S1P application. Pre-incubation of cerebral arteries with KN-93, completely blocked the increase in P-CREB positive nuclei caused by S1P (Fig. 1C). We have also previously reported that the $[Ca^{2+}]_i$ was initially generated by intracellular Ca^{2+} release from the sarcoplasmic reticulum via an inositol 1,4,5-trisphosphate ($InsP_3$)-dependent pathway [19]. To clarify the role of $InsP_3$ in S1P-induced CREB activation, we used the cell-permeable $InsP_3$ channel inhibitor 2-APB (Fig. 1C). Treatment of cerebral arteries with 2-APB partially suppressed the S1P-induced increase levels of P-CREB staining. A combination of 2-APB and nifedipine decreased the percentage of S1P-induced P-CREB positive nuclei to control levels.

3.3. S1P promotes sarcoplasmic reticulum Ca^{2+} release and Ca^{2+} influx in rat cerebral artery

Intracellular Ca^{2+} was measured in isolated cerebral artery myocytes, with the fluorescent dye fura-2. S1P produces a biphasic rise in $[Ca^{2+}]_i$ characterised by an initial Ca^{2+} spike followed by a slowly declining phase as previously observed [19]. This second phase, which has not been reported, is somewhat variable between single myocytes. In approximately 50% of myocytes Ca^{2+} oscillations followed the initial Ca^{2+} signal induced by S1P. Fig. 2A, is a representative trace of S1P-induced repetitive Ca^{2+} signals. As shown in Fig. 2B and C, pre-incubation with 2-APB greatly decreased S1P-induced Ca^{2+} mobilisation (0.09 ± 0.02 ratio units, $N = 34$, $P < 0.05$) but did not completely abolish the response. The remaining 2-APB-insensitive Ca^{2+} rise was significantly delayed (Fig. 2D) and occurs over a longer timecourse. This was previously unreported by us [19] as it would normally be masked by the initial Ca^{2+} release event and subsequent oscillations. 2-APB not only blocks $InsP_3$ receptors and subsequent Ca^{2+} release from stores but also blocks store-operated Ca^{2+} entry activated in response to depletion of stores [30]. Application of S1P still produced a small Ca^{2+} rise in the continued presence of 2-APB indicating that this is not related to either $InsP_3$-induced Ca^{2+} release or store-operated Ca^{2+} entry. This slow component is likely due to L-type VDCC, as a combination of 2-APB and nifedipine (a dihydropyridine inhibitor of VDCC) completely abolished S1P-induced increases in $[Ca^{2+}]_i$ (Fig. 2C). These data confirm that Ca^{2+} release from the sarcoplasmic reticulum by an $InsP_3$-dependent pathway constitutes the main component of the S1P-induced Ca^{2+} rise. In addition to Ca^{2+} release via $InsP_3R$ activation, L-type VDCC contribute, at least partly, to the increased $[Ca^{2+}]_i$.

3.4. S1P inhibits voltage-gated K^+ currents

To determine whether activation of L-type VDCC by S1P reflects either a direct activation of a depolarising conductance or inhibition of voltage-gated K^+ currents, K^+ currents were measured in isolated myocytes using the whole-cell patch clamp technique. Membrane K^+ currents were recorded from a holding potential (V_h) of −30 mV on each cell before and after application of S1P. In voltage-clamped cerebral artery myocytes, steady-state outward current was significantly reduced ($63 \pm 10\%$ at +60 mV, $N = 6$) following extracellular application of 1 μmol/L S1P (Fig. 3A and B). The mean current after 5 min S1P treatment at +60 mV was 0.09 ± 0.03 nA, control 0.23 ± 0.04 nA ($N = 6$). This inhibition was maintained after S1P washout and no recovery was detected after 20 min.

3.5. S1P-induced inhibition of voltage-gated K^+ currents is independent of Ca^{2+} release from stores and involves protein kinase C activation

We examined the pathways responsible for S1P-induced inhibition of voltage-gated K^+ currents. To determine whether sarcoplasmic reticulum-mediated Ca^{2+} release is involved in S1P-induced voltage-gated K^+ channel inhibition, K^+ currents were recorded in the presence of thapsigargin (Fig. 3C). This treatment did not prevent the reduction of K^+ current induced by S1P ($49 \pm 14\%$ at +60 mV, $N = 4$), indicating that intracellular Ca^{2+} stores are not required for this effect. To determine if the S1P-induced inhibition of the voltage-gated K^+ channels involves PKC, myocytes were pre-incubated with either calphostin C or Ro 31-8220 (PKC inhibitors). Both these inhibitors completely blocked the S1P-induced inhibition of the K^+ current (Fig. 3C).

To further investigate the involvement of PKC in the inhibition of the voltage-gated K^+ current, the tyrosine phosphorylation of the α-subunit of Kv 1.2 was determined.

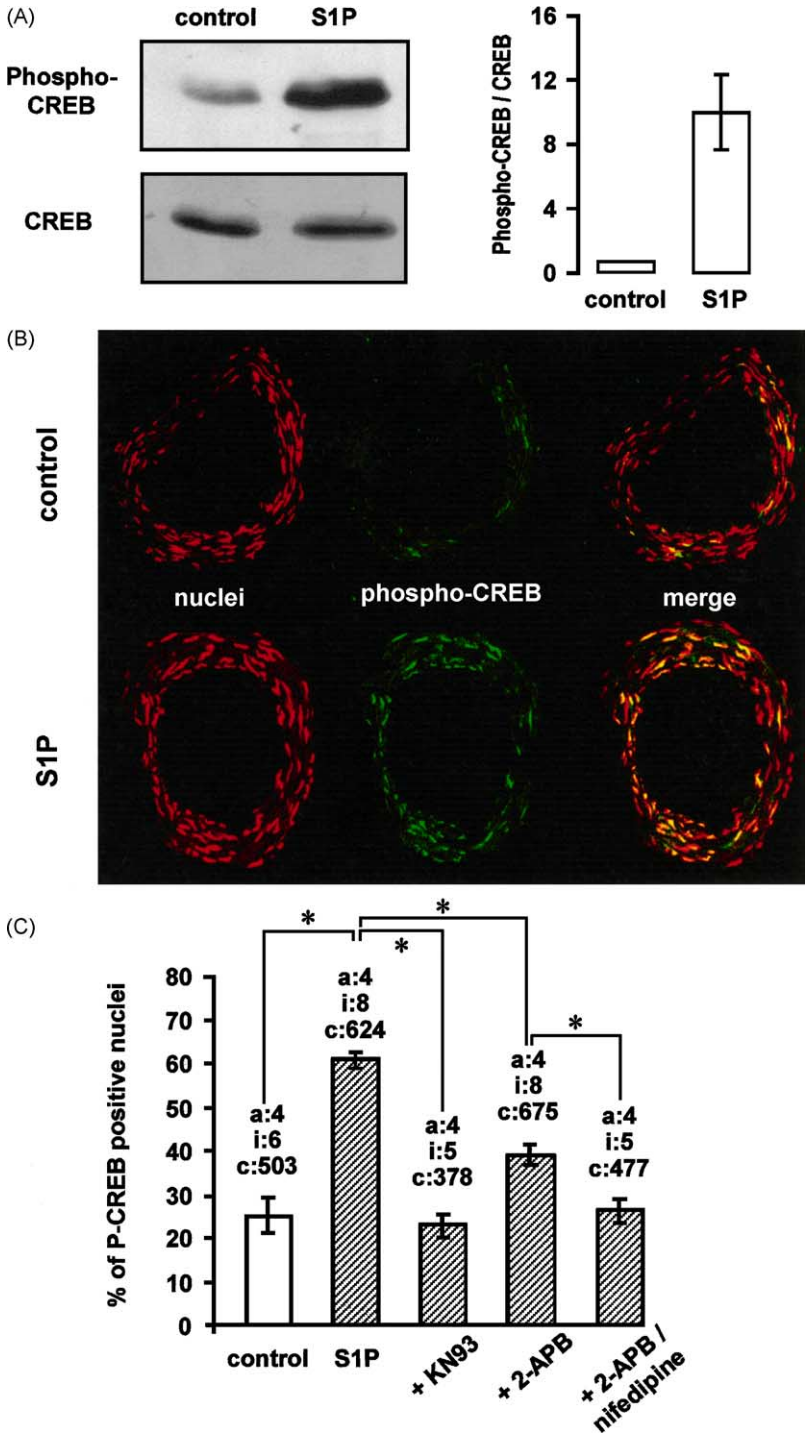

Fig. 1. Phosphorylation of CREB by S1P in cerebral arteries. (A) Phosphorylation of CREB was detected by immunoblot analysis using a phospho-specific CREB antibody. (Left panel) Representative immunoblots stained with a P-CREB antibody (upper panel) and with CREB antibody (lower panel). (Right panel) The density of the specific band was scanned and quantified with a densitometer. The ratio of phosphorylated CREB to total CREB is shown. The ratio of untreated arteries was designated as 1. S1P (1 μmol/L) increased the ratio of P-CREB/CREB 10 ± 2.3-fold in cerebral arteries after 20 min of stimulation (N = 4). (B) Arterial cross-sections were co-stained with anti-P-CREB antibody (green) and the nuclear dye BOBO-3 (red). Immunofluorescent images of control cerebral artery segments and S1P treated artery segments are shown. On the merged images, yellow indicates nuclear co-localisation of P-CREB (green) and BOBO-3 (red). (C) Bar graphs show the percentage of cells in arterial sections staining positive for nuclear P-CREB in response to S1P and various inhibitors. KN-93 (30 μmol/L), 2-APB (30 μmol/L) and nifedipine (1 μmol/L) were applied for 30–40 min prior to S1P (1 μmol/L) stimulation. (*a*: number of arteries, for a single artery each different condition was analysed and compared; *i*: analysed images, and *c*: number of cells counted). Comparisons of sections from segments treated as shown in panel C were made from the same artery ($a = 1$) and this was repeated four times to obtain mean data. $^{*}P < 0.05$ on comparisons as indicated.

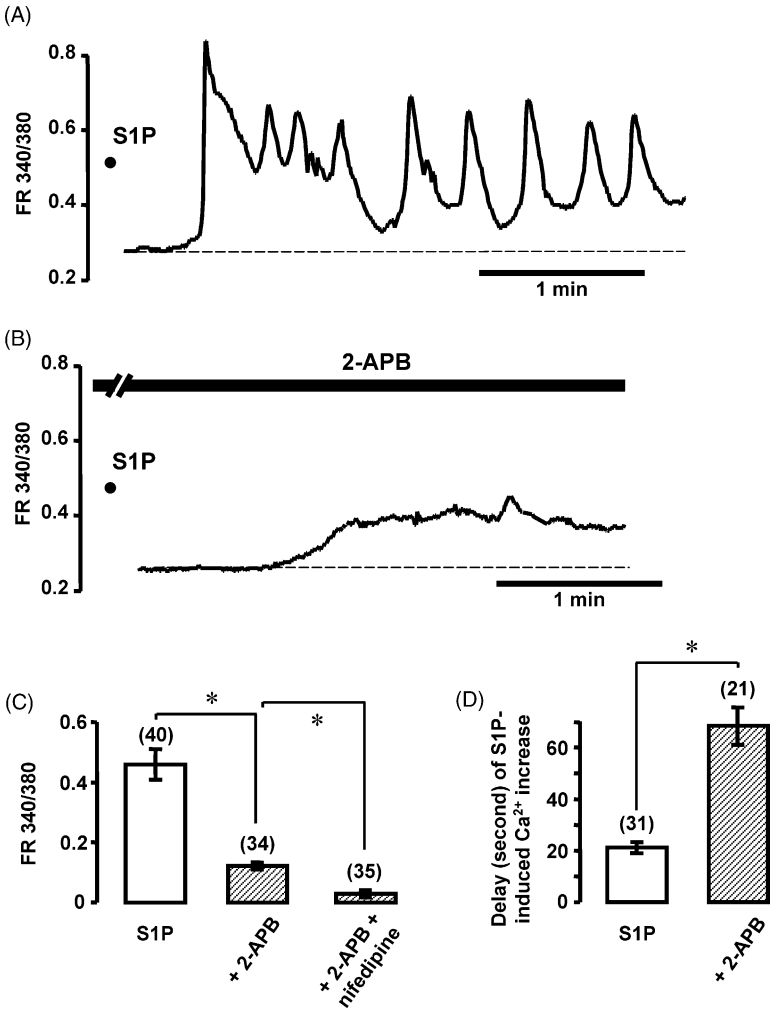

Fig. 2. Ca^{2+} spikes evoked by S1P in cerebral artery cerebral smooth muscle myocytes. (A) Increase in fluorescence ratio (FR340/380) induced by S1P (1 μmol/L) showing an initial Ca^{2+} transient followed by oscillating Ca^{2+} spikes in fura-2-loaded freshly isolated myocytes from cerebral artery. (B) Representative FR340/380 trace reflecting inhibition of S1P-induced rise in $[Ca^{2+}]_i$ by 2-APB. 2-APB (30 μmol/L) was applied 40 min prior to addition of S1P. Note that a small and delayed rise in $[Ca^{2+}]_i$ was not abolished by inhibition of InsP3Rs. Dots at the beginning of each trace represent time of addition of S1P. (C) Compiled data showing the relative amplitude of peak increases in $[Ca^{2+}]_i$ induced by S1P in control conditions and after the following treatments: incubation with 2-APB (30 μmol/L, 40 min); and in the presence of 2-APB + nifedipine (20 min). The relative amplitude of S1P-induced increases in $[Ca^{2+}]_i$ in control condition was calculated on the initial Ca^{2+} spike. (D) Bar graphs showing the delay (second) of S1P-induced Ca^{2+} increases in control conditions and after treatment with 2-APB (40 min). Data are mean ± SEM with number of cells in parentheses. Values significantly different from control conditions ($^*P < 0.05$).

Previous studies have shown that Kv 1.2 is highly expressed in vascular smooth muscles [31,32]. It has also been demonstrated that tyrosine phosphorylation of the α-subunit can result in an inhibition of the voltage-gated K^+ current [33,34]. Immunoprecipitation of Kv 1.2 from cerebral arteries, followed by immunoblotting for tyrosine phosphorylation, revealed an increase in phosphorylation following incubation with 1 μmol/L S1P for 10 min (Fig. 3D). This increase was inhibited by pre-incubation with Ro 31-8220.

3.6. Membrane depolarisation stimulates Ca^{2+} influx and CREB phosphorylation

Membrane depolarisation with 4-aminopyridine (4-AP) (5 mmol/L), which blocks voltage-gated K^+ channels, resulted in a rise in $[Ca^{2+}]_i$ (0.48 ± 0.03 ratio units, $N = 50$; Fig. 4A). The 4-AP response was sensitive to nifedipine (0.03 ± 0.01 ratio units, $N = 39$, $P < 0.05$) suggesting that the observed Ca^{2+} rise occurred through opening of L-type VDCC (Fig. 4B). To determine whether Ca^{2+} influx through L-type VDCC is a sufficient stimulus for induction of CREB phosphorylation, we used two complementary approaches. First, we activated VDCC by depolarising concentrations of K^+ (140 mmol/L). We also indirectly increased VDCC activity using 4-AP. Treatment with either high-K^+ depolarisation buffer or 4-AP-induced phosphorylation of CREB (9.6 ± 2.7- and 7.5 ± 1.4-fold over control, respectively) in rat cerebral artery (Fig. 4C). These data confirm that increased Ca^{2+} influx associated with membrane depolarisation is a potential pathway leading to CREB activation in VSM cells.

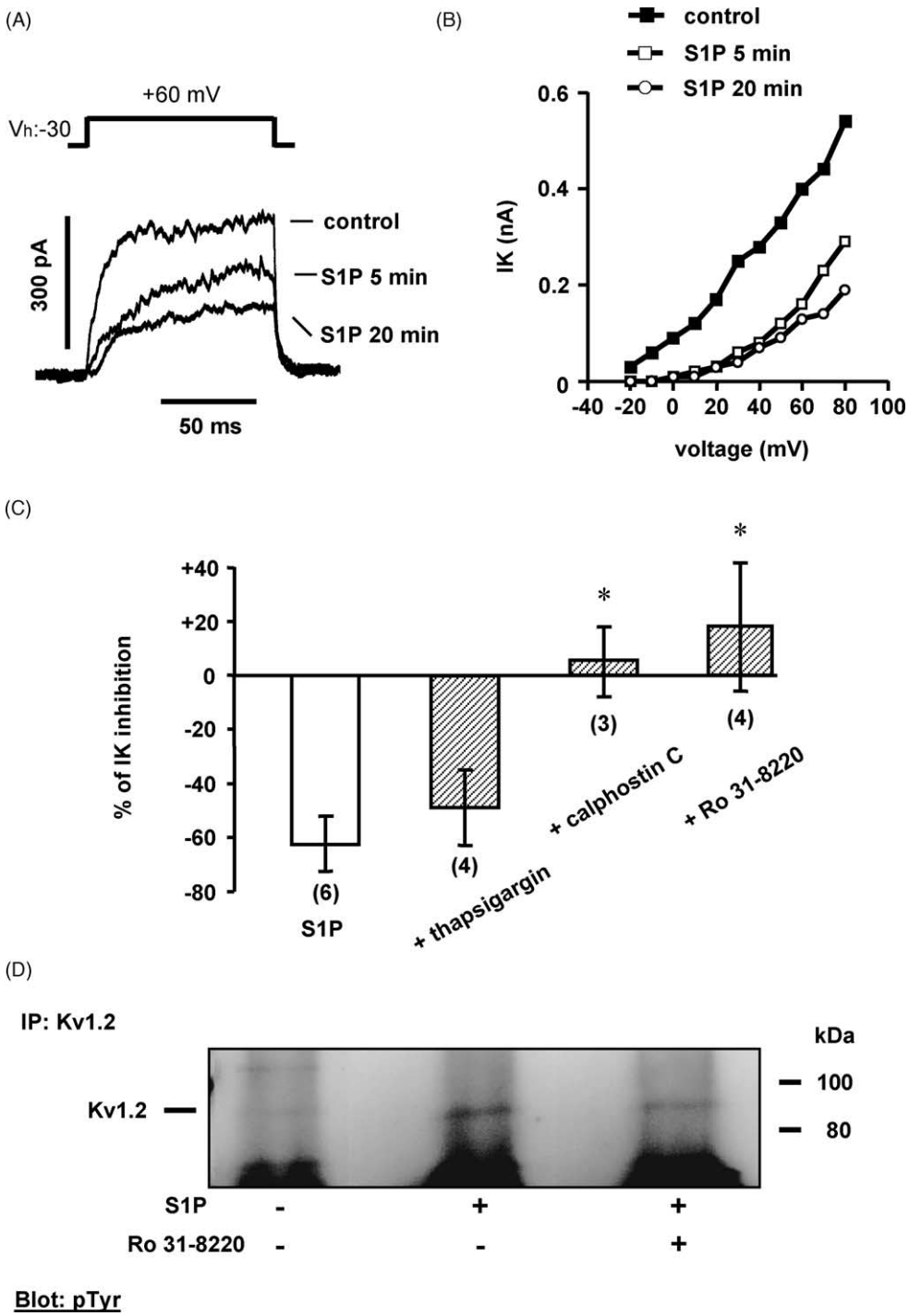

Fig. 3. Effects of extracellular application of S1P on voltage-gated K^+ currents. (A) Typical outward current elicited by depolarisation to +60 mV from a holding potential of −30 mV before (control conditions), after (test conditions) 5 min application of 1 μmol/L S1P and 20 min after removal of the perfusion pipette containing S1P. (B) Representative current–voltage relationships obtained from a holding potential of −30 mV in a cell recorded before and after treatment with 1 μmol/L S1P. S1P application resulted in inhibition of the K^+ current. No recovery was detected after 20 min washout. (C) Compiled data showing the percentage of inhibition of the K^+ currents induced by S1P compared to control conditions. Results are expressed as a fraction of the maximal current obtained on the same cell before application of 1 μmol/L S1P. Data are given as mean ± SEM for four to six cells. Steady-state outward current was reduced by 63 ± 10% at +60 mV in presence of S1P ($P < 0.05$). Some myocytes were also pretreated with 1 μmol/L thapsigargin for 20 min before application of 1 μmol/L S1P. In presence of thapsigargin, S1P reduced the K^+ current by 49 ± 14% at +60 mV ($P < 0.05$). In myocytes pretreated with either 250 nmol/L calphostin C or 10 μmol/L Ro 31-8220, protein kinase C inhibitors, for 20 min, S1P did not induce an inhibition of the K^+ current (N = 3–4). (D) Cerebral arteries incubated with 1 μmol/L S1P revealed an increase in tyrosine phosphorylation of the Kv 1.2 as assessed by immunoblotting. Pre-incubation of arteries with the PKC inhibitor, Ro 31-8220, for 1 hr inhibited the increased phosphorylation.

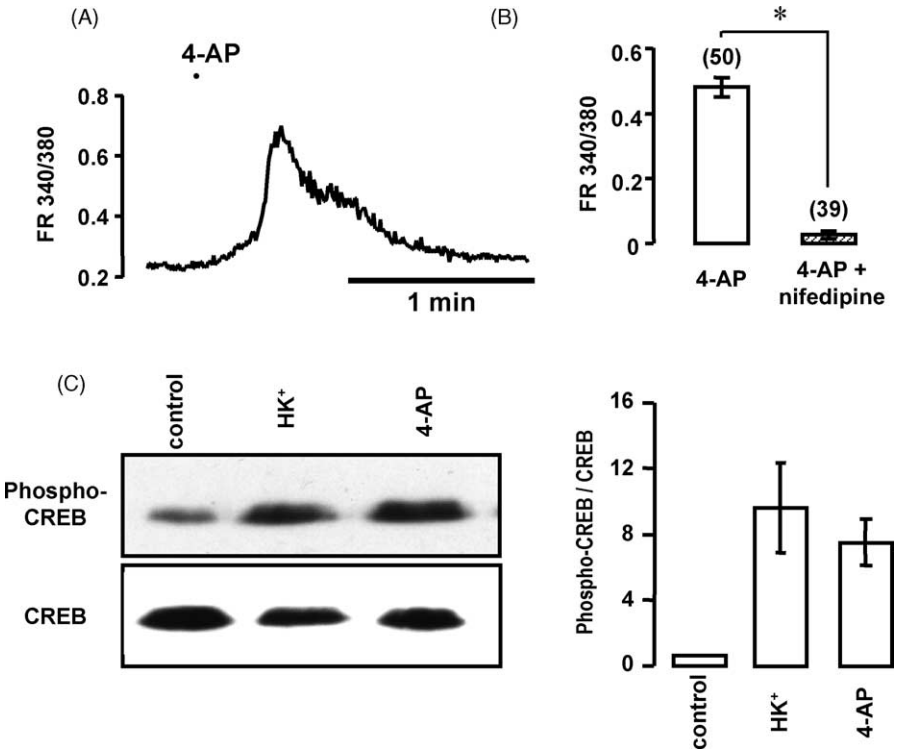

Fig. 4. Ca^{2+} influx induced by membrane depolarisation produce CREB phosphorylation. (A) Representative FR340/380 trace reflecting rise in $[Ca^{2+}]_i$ induced by application of 5 mmol/L 4-AP in fura-2-loaded freshly isolated myocytes from cerebral artery. (B) Bar graphs showing the relative amplitude of peak increases in $[Ca^{2+}]_i$ induced by 5 mmol/L 4-AP in presence or not of 1 μmol/L nifedipine. Data are mean ± SEM with number of cells in parentheses. Values significantly different from control conditions ($^*P < 0.05$). (C) (Left panel) Representative immunoblots stained with specific antibodies raised against CREB and phospho-CREB. (Right panel) High $[K^+]$ depolarisation buffer (140 mmol/L K^+) or 4-AP (5 mmol/L) increased the ratio of P-CREB/CREB 9.6 ± 2.7- and 7.5 ± 1.4-fold over control, respectively in rat cerebral artery (N = 3).

4. Discussion

The ability of vascular smooth muscle cells to switch phenotype from fully differentiated to a proliferative cell type is a feature of vascular disease [2]. This switch is dependent on external stimuli triggering appropriate transcription and gene expression. Several studies have now highlighted the importance of calcium homeostasis in the regulation of selective gene expression in various cell types [35,36]. In native vascular smooth muscle, activation of the transcription factors, CREB and the c3 isoform of nuclear factor of activated T-cells (NFATc3), can be manipulated by pharmacological intervention of Ca^{2+} homeostasis [25–28]. In this study, S1P, a potential mitogenic stimulus [17,18], was observed to activate CREB, a transcription factor that is involved in vascular smooth muscle proliferation [23,24]. This activation was dependent on an increase in the $[Ca^{2+}]_i$, via $InsP_3$-induced Ca^{2+} release from intracellular stores as well as Ca^{2+} influx. This study further describes a novel intracellular mechanism whereby S1P can regulate CREB activation through increasing $[Ca^{2+}]_i$. The addition of S1P to freshly isolated cerebral artery vascular smooth muscle cells produces a significant inhibition (approximately 50%) of voltage-gated K^+ channels. This leads to a depolarisation of the cell membrane and activation of VDCC.

To date, the activation of transcription factors following S1P receptor activation has been examined only in cell lines [37–39]. The ability of S1P to activate transcription factors in vascular smooth muscle has not been examined. We have previously shown that S1P can activate p42/p44 mitogen-activated protein kinase [19], an upstream event often associated with transcription, in freshly isolated vascular smooth muscle. Other recent studies have also shown that the transcription factor, CREB can be activated by the growth factor, PDGF, and by membrane depolarisation in native vascular smooth muscle [25,26]. The present study is the first to demonstrate that S1P can stimulate phosphorylation (and therefore activation) of CREB, and provides a potential mechanism through which S1P can regulate the initiation of phenotypic modulation in fully differentiated vascular smooth muscle. Regulation of CREB is known to be Ca^{2+} dependent in many cell types. In mouse cerebral artery, CREB phosphorylation has previously been shown to require activation of CaM kinase when induced by membrane depolarisation [25]. This is also shown in the present study to be the case with S1P. The Ca^{2+} dependence of CREB phosphorylation was further demonstrated when S1P stimulation of CREB phosphorylation was partially inhibited by pretreatment with 2-APB, an $InsP_3R$ channel inhibitor, and completely inhibited by a combination of both 2-APB and nifedipine. As

2-APB alone could not completely inhibit S1P-induced CREB activation, this suggests that nifedipine-sensitive Ca^{2+} influx also plays an important role. These results correspond with S1P effects on the $[Ca^{2+}]_i$; initially an $InsP_3$-dependent Ca^{2+} release and a smaller, more prolonged increase in $[Ca^{2+}]_i$ via voltage-operated Ca^{2+} channels. Additionally, following the initial intracellular Ca^{2+} release, S1P produced regular oscillations in the $[Ca^{2+}]_i$ which were found to persist for several minutes. These oscillations are not involved in alterations in vascular tone (data not shown) but could play an important regulatory role in activation of selective transcription factors such as CREB although this remains to be determined.

The mechanisms of S1P-induced intracellular Ca^{2+} release have been explored previously [19]. However, the mechanism of activation of VDCC by S1P that could regulate CREB activation has not, until the present study, been investigated. In rat cerebral artery, S1P produced an approximately 50% inhibition of the voltage-gated K^+ current. This is a delayed rectifier-mediated current and is unlikely to be regulated by Ca^{2+}, as thapsigargin has no effect on the inhibition. This is the first report of stimulation with S1P leading to an inhibition of voltage-gated K^+ channels. Previous reports in other cell types have demonstrated that inhibition of the K^+-delayed rectifier channels can be mediated via tyrosine phosphorylation [33,34]. In cerebral arteries, S1P can induce tyrosine phosphorylation of Kv 1.2, one of the K^+-delayed rectifier channel isoforms predominantly expressed in vascular smooth muscle [31,32]. In addition, this phosphorylation (and the subsequent inhibition of the voltage-gated K^+ current) is dependent on PKC activation. Endothelin has also recently been shown to produce an inhibition of voltage-gated K^+ channels via a PKC-dependent mechanism [40], similar to that shown here. Previous studies have shown that PKC may activate a nonreceptor tyrosine kinase, PYK2, which can phosphorylate Kv 1.2 [33,34]. The S1P-induced inhibition will result in an increased depolarisation, leading to activation of VDCC (see Fig. 5). In support of this, an inhibitor

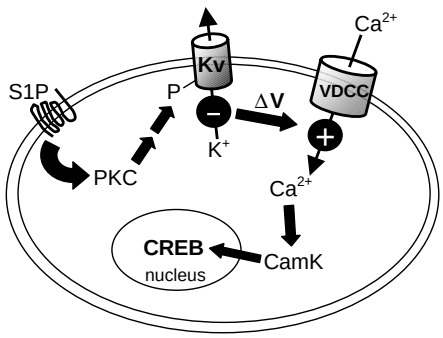

Fig. 5. Schematic diagram of S1P-induced CREB activation via an inhibition of the voltage-gated K^+ current. S1P activates one or more PKC isoforms, leading to tyrosine phosphorylation and inhibition of the Kv channels. This inhibition results in a depolarization, activating VDCC and an influx of Ca^{2+}. This influx activates CamK and subsequently results in the phosphorylation of CREB.

of voltage-gated K^+ channels, 4-AP, induced an increase in intracellular Ca^{2+} in rat cerebral artery myocytes that was nifedipine-sensitive. Importantly, 4-AP, in addition to depolarisation induced by high $[K^+]$, can produce CREB phosphorylation to a similar level (8–10-fold increase) as S1P. Taken together, these data demonstrate for the first time that S1P receptors can activate CREB via an inhibition of voltage-gated K^+ channels, and subsequent voltage-dependent Ca^{2+} influx in vascular smooth muscle. Although it is of importance to determine the S1P receptor subtype involved in this pathway, there are currently no S1P receptor subtype selective antagonists available.

As the regulation of the mitogenic effects of S1P in vascular smooth muscle may occur partly via this mechanism, this could have implications for the pathogenesis of vascular disease. There is good evidence that an inhibition of the voltage-gated K^+ current is associated with long term changes in vascular function. In a rat genetic model of experimental hypertension, cerebral arteries exhibited a tonic decrease in the voltage-gated K^+ current of approximately 50% compared to normotensive controls [41]. This decrease resulted in a membrane depolarisation of 15 mV, and an increase in $[Ca^{2+}]_i$. This was also associated with an increase in CREB activation. It is interesting to note that S1P also inhibited the voltage-dependent K^+ current by approximately 50%, and therefore mimics the effect observed in experimental hypertension. This also suggests that the inhibition produced by this S1P-induced intracellular pathway, leading to CREB activation, is in the pathophysiological range observed *in vivo*. This provides further evidence that this mechanism of CREB activation may have an important role in vascular disease.

In conclusion, this study has demonstrated that S1P can activate the transcription factor, CREB via an intracellular pathway that involves intracellular Ca^{2+} release and Ca^{2+} influx. Ca^{2+} influx occurs via VDCC activated indirectly by an inhibition of the voltage-gated K^+ current. The level of this inhibition is in the pathophysiological range and obtained with physiologically relevant concentrations of S1P. This pathway may, therefore, have important implications in the development of vascular disease.

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