Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 496 (2004) 109–118

www.elsevier.com/locate/ejphar

Depletion of Ca^{2+} from intracellular stores potentiates spontaneous contractions of the rat portal vein

Richard P. Burt*

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK

Received 12 February 2004; received in revised form 2 April 2004; accepted 20 May 2004
Available online 8 July 2004

Abstract

Spontaneous contractions of the rat portal vein were potentiated in magnitude by phenylephrine, cyclopiazonic acid, ryanodine or caffeine. All these drugs can deplete Ca^{2+} from intracellular stores, which stimulates store-operated cation entry in some tissues. The possibility that depletion of Ca^{2+} from intracellular stores potentiates the spontaneous contractions was therefore investigated using functional experiments. Phenylephrine or cyclopiazonic acid was added to tissues in Ca^{2+}-free Krebs solution, followed by a 30-min washout. After addition of extracellular Ca^{2+}, the spontaneous contractions were potentiated. This showed the stimulus for potentiating the contractions remained so long as intracellular Ca^{2+} stores were depleted. Following phenylephrine washout in normal Krebs solution, potentiation of the spontaneous contractions was attenuated with time. This attenuation was abolished by the protein kinase C inhibitor calphostin C. These results show depletion of Ca^{2+} from intracellular stores potentiates spontaneous contractions of the portal vein. Protein kinase C may inhibit this mechanism.

Keywords: Portal vein, rat; Spontaneous contraction; Store-operated Ca^{2+} entry; Cyclopiazonic acid; Ryanodine; Protein kinase C

1. Introduction

Spontaneous phasic contractions are a characteristic of the rat isolated portal vein (Dacquet et al., 1987; Mikkelsen, 1985). These contractions are thought to result from depolarizations stimulated by spontaneous transient inward currents (STICS) and influx of Ca^{2+} through voltage-gated channels (Burt, 2003; Kirkup et al., 1996). STICS have been shown in portal vein cells to be produced by the opening of Ca^{2+} activated Cl^- channels (Pacaud et al., 1989; Wang et al., 1992). Influx of extracellular Ca^{2+} through voltage-gated channels following depolarization may then stimulate Ca^{2+} induced Ca^{2+} release from intracellular stores of the portal vein (Grégoire et al., 1993).

Spontaneous contractions of the portal vein can be potentiated in magnitude by low concentrations of the α_1-adrenoceptor phenylephrine (Schwietert et al., 1991). α_1-Adrenoceptors are usually linked to phospholipase C, resulting in a rise in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Minneman and Esbenshade, 1994). In rat portal vein cells stimulation of α_1-adrenoceptors has been shown to produce a rise in IP_3 (Leprêtre et al., 1994). IP_3 can release Ca^{2+} from intracellular stores by opening IP_3 channels on these stores (Berridge, 1993), while diacylglycerol activates protein kinase C (PKC) (Lee and Severson, 1994). The spontaneous contractions can also be potentiated by cyclopiazonic acid (Miwa et al., 1997). Cyclopiazonic acid is known to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase, which results in depletion Ca^{2+} from intracellular stores (Seidler et al., 1989).

In some cells depletion of Ca^{2+} from intracellular stores by agonists which produce a rise in IP_3 can stimulate an influx of extracellular Ca^{2+}, called store-operated Ca^{2+} entry or capacitative Ca^{2+} entry (Parekh and Penner, 1997; Putney, 1986). Agents such as cyclopiazonic acid and ryanodine, which deplete Ca^{2+} from intracellular stores without producing a rise in IP_3, can also stimulate store-operated Ca^{2+} entry (Burt et al., 1995; Ng and Gurney, 2001; Wayman et al., 1998; Zhang et al., 1994). Store-operated Ca^{2+} entry can result directly in a tonic contraction in some smooth muscle (Burt et al., 1995; Ng and Gurney, 2001; Noguera and D'Ocon, 1993; Ohta et al., 1995;

* Tel.: +44-20-7679-3750; fax: +44-20-7679-7298.
E-mail address: r.burt@ucl.ac.uk (R.P. Burt).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.05.032

Wallace et al., 1999). In other tissues store-operated Ca^{2+} entry does not directly stimulate contraction but may provide a source of Ca^{2+} to refill the intracellular stores (Flemming et al., 2002).

Store-operated Ca^{2+} entry was first shown in mast cells to be through specific non-voltage-gated Ca^{2+} channels (Hoth and Penner, 1992) and then in endothelial cells through non-selective cation channels (Zhang et al., 1994). In myocytes, store-operated Ca^{2+} entry has been shown to occur through non-selective cation channels (McDaniel et al., 2001; Trepakova et al., 2001; Wayman et al., 1996) including those of the rabbit portal vein (Albert and Large, 2002a). Some members of the transient receptor potential (*trp*) gene family have been proposed to encode for these cation channels activated by Ca^{2+} store depletion (for review see Hofmann et al., 2000).

Depletion of Ca^{2+} from intracellular stores in many tissues therefore actually results in store-operated cation entry through non-selective cation channels. Another effect of this is that it will to some extent depolarize cells (Scharff and Foder, 1996). This could lead to responses involving depolarization being enhanced. The possibility that depletion of Ca^{2+} from intracellular stores of the rat portal vein potentiates the spontaneous contractions was therefore investigated. Results of the present study using phenylephrine, cyclopiazonic acid, ryanodine and caffeine to deplete intracellular Ca^{2+} stores were found to be consistent with this.

2. Methods

All experimental protocols were approved by the institutional ethics committee. Male Sprague–Dawley rats between 350 and 450 g were stunned and killed by cervical dislocation. The portal vein was removed into Krebs solution and associated connective tissue was dissected away. The tissues (10–15 mm) were suspended longitudinally in 5 ml tissue baths containing Krebs solution (Krebs) of the following composition (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11, at 25 °C and bubbled with 95% O_2/5% CO_2. When Ca^{2+}-free Krebs was used, this always contained EGTA (1 mM) unless otherwise stated. The portal veins were placed under 0.5 g resting tension and equilibrated for 1 h. Changes in isometric tension were measured using Grass FT.03 transducers and recorded by Biopac Systems MP100WS for Windows. Experiments were carried out at 25 °C for consistency with the results of Burt (2003).

Phenylephrine (10^{-4} M, which produced a maximum response) was added to all tissues initially, with a recovery period of 45 min following washout. When drugs were tested on responses to phenylephrine (10^{-6} M), reproducible control responses were recorded at 45 min intervals before drug addition. Drugs were incubated for 30 min with tissues unless otherwise stated.

2.1. Potentiation of phasic contractions on the portal vein

Cumulative concentration–response curves to phenylephrine (10^{-8}–3×10^{-5} M) on the spontaneous contractions were measured in some tissues. The effect of cyclopiazonic acid (10^{-5} M), ryanodine (10^{-4} M) and caffeine (25 mM), following its washout, was also measured on the spontaneous contractions. The effect of cyclopiazonic acid (10^{-5} M) was recorded in the presence of either nifedipine (3×10^{-7} M) or niflumic acid (3×10^{-5} M).

The effect of cyclopiazonic acid (10^{-5} M) or ryanodine (10^{-4} M) was measured on the phasic contractions to phenylephrine (10^{-6} M). The effect of cyclopiazonic acid (10^{-5} M) or ryanodine (10^{-4} M) was also measured on the phasic response to K^+ (20 mM).

2.2. The effect of depleting intracellular Ca^{2+} stores in Ca^{2+}-free Krebs on the spontaneous contractions

These experiments were designed to show if the stimulus for potentiation of the spontaneous contractions depended on whether or not the intracellular Ca^{2+} stores were depleted rather than the presence of the depleting agent. Phenylephrine (10^{-4} M) or cyclopiazonic acid (10^{-5} M) was added to some tissues in Ca^{2+}-free Krebs for 5 or 15 min, respectively. They were then washed out for 30 min still in Ca^{2+}-free Krebs, which was then changed to Ca^{2+}-free Krebs without EGTA. Total time in Ca^{2+}-free Krebs was 45 min. Ca^{2+} (2.5 mM) was then added to the tissues and responses following this measured. Control tissues were treated in the same way except no phenylephrine or cyclopiazonic acid was added.

2.3. The effect of inhibiting K^+ channels on ryanodine-induced potentiation

The effect of tetraethylammonium (TEA, 10^{-3}–3×10^{-3} M) was measured on the spontaneous contractions. The effect of ryanodine (10^{-4} M) on the spontaneous contractions in the presence of TEA (3×10^{-3} M) was also measured.

2.4. The effect of PKC inhibition on potentiation of the spontaneous contractions

Following washout of phenylephrine (10^{-6} M), the spontaneous contractions are potentiated when they return, compared with before addition of phenylephrine. This potentiation then declines with time. The effect of the selective PKC inhibitor calphostin C (10^{-6} M) was measured on this potentiation of the spontaneous contractions, following washout of phenylephrine (10^{-6} M).

2.5. Data analysis

All contractions were measured as a percentage response of that to phenylephrine (10^{-4} M) and calculated as the

mean from four separate experiments ($n=4$) unless otherwise stated. Calculation of the mean integral contraction (contraction × time) was performed using AcqKnowledge software (Biopac Systems). For measurement of some of the phasic contractions to phenylephrine, the time scale of the trace was expanded using the recording software so that the response was more distinct. Statistical significance of differences between control and test means was tested for on raw data using a paired *t*-test where a control and test value are given together and a non-paired *t*-test if given separately. A *P* value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using Prism (GraphPAD Software, San Diego, CA, USA).

2.6. Drugs and solutions

Phenylephrine hydrochloride, caffeine, nifedipine, niflumic acid and tetraethylammonium chloride were obtained from Sigma. Ryanodine, cyclopiazonic acid and calphostin C were obtained from Calbiochem. All stock solutions were made in distilled water and diluted to working concentrations in Krebs solution except nifedipine which was dissolved in ethanol and then diluted in distilled water, niflumic acid which was dissolved in dimethyl sulphoxide and further diluted in distilled water and cyclopiazonic acid and calphostin C which were dissolved and further diluted in dimethyl sulphoxide. Stock solutions were stored frozen except phenylephrine which was prepared fresh each day.

3. Results

3.1. Phenylephrine and cyclopiazonic acid potentiate spontaneous contractions of the portal vein

Phenylephrine (10^{-4} M) produced a phasic contraction of the portal vein followed by a larger tonic response (maximum response 1.78 ± 0.2 g, $n=12$) which abolished the spontaneous contractions of this tissue. When this was washed out the tonic contraction disappeared within a few minutes but the spontaneous contractions were potentiated when they returned and remained so for about 15 min (Fig. 1A). Cumulative additions of phenylephrine (10^{-8}–3×10^{-5} M) increased the magnitude of the spontaneous contractions from $36 \pm 5\%$ to $109 \pm 5\%$ ($P<0.05$) and their duration from 8 ± 1 to 32 ± 4 s ($P<0.05$) at 10^{-6} M (Fig. 1B).

Cyclopiazonic acid (10^{-5} M) increased the maximum response of the spontaneous contractions from $37 \pm 2\%$ to $124 \pm 5\%$ ($P<0.05$, Fig. 1C) and their duration from 9 ± 1 to 58 ± 4 s ($P<0.05$, Fig. 1C). The mean integral tension was increased from 0.18 ± 0.01 to 0.63 ± 0.11g s^{-1} ($P<0.05$) in the presence of cyclopiazonic acid. This showed that although the rate of contractions was slower in the presence of cyclopiazonic acid the total contractile response was greater. The spontaneous contractions are abolished by nifedipine or niflumic acid (Burt, 2003). Cyclopiazonic acid (10^{-5} M) did not produce any contraction in the presence of either nifedipine (3×10^{-7} M) or niflumic acid (3×10^{-5} M) (results not shown).

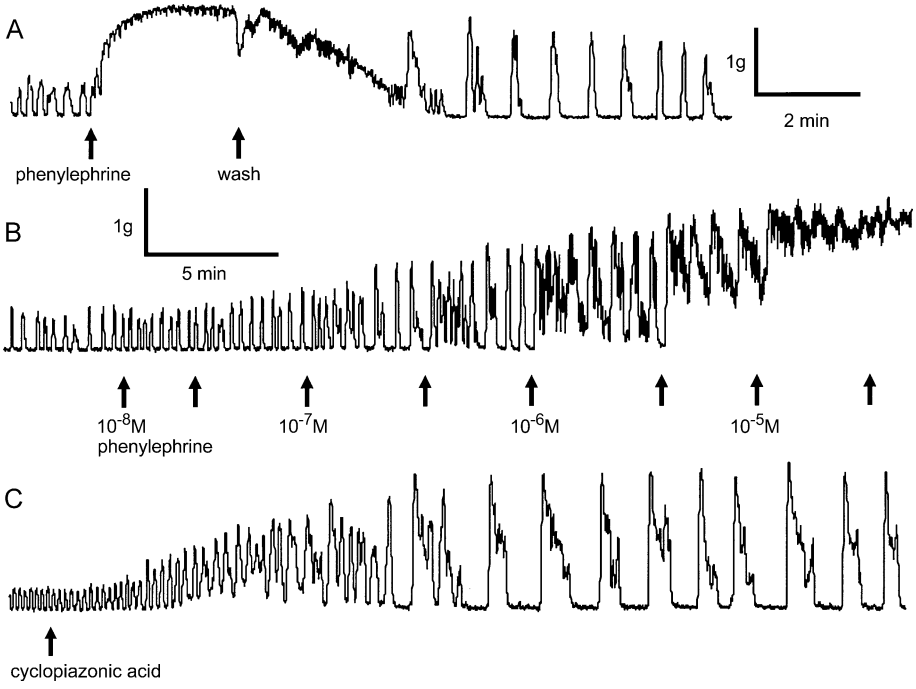

Fig. 1. (A) The tonic contraction to phenylephrine (10^{-4} M) of the rat portal vein returned to baseline within about 4 min following washout but the spontaneous contractions when they returned were potentiated. (B) Cumulative additions of phenylephrine on the rat portal vein, which potentiated the magnitude and duration of the spontaneous contractions. (C) Potentiation of the spontaneous contractions by cyclopiazonic acid (10^{-5} M).

3.2. The effect of depleting intracellular Ca^{2+} stores in Ca^{2+}-free Krebs

Phenylephrine 10^{-4} M was added to tissues in Ca^{2+}-free Krebs for 5 min and then washed out for 30 min, followed by re-addition of Ca^{2+} (2.5 mM) to the Krebs. Upon re-addition of Ca^{2+} to the Krebs, a tonic contraction developed initially for a few minutes. Following the tonic contraction, the spontaneous contractions when they returned, were potentiated compared with before Ca^{2+}-free Krebs (control maximum response 30 ± 1%, duration 9 ± 1 s and following phenylephrine washout in Ca^{2+}-free Krebs, maximum response 83 ± 3% $P<0.05$, duration 25 ± 3 s $P<0.05$, Fig. 2B). The spontaneous contractions were not potentiated in control tissues where phenylephrine was not added during the period in Ca^{2+}-free Krebs (control 33 ± 2%, following Ca^{2+}-free Krebs 34 ± 2%, Fig. 2A). The inititial contraction also returned more quickly following re-addition of Ca^{2+} when phenylephrine had been added and washed out (10 ± 1 s, Fig. 2B) compared with following just Ca^{2+}-free Krebs (281 ± 5 s, Fig. 2A). When phenylephrine (10^{-4} M) was added in Ca^{2+}-free Krebs and washed out for 30 min, a second addition of phenylephrine in Ca^{2+}-free Krebs produced no response. This indicated that the intracellular Ca^{2+} stores remained depleted during this period (results not shown).

Cyclopiazonic acid 10^{-5} M was added to tissues in Ca^{2+}-free Krebs for 15 min and then washed out for 30 min, followed by re-addition of Ca^{2+} (2.5 mM) to the Krebs. In normal Krebs the maximum response to cyclopiazonic acid was reached within 15 min, the period that cyclopiazonic acid was added for in Ca^{2+}-free Krebs. A tonic contraction was stimulated initially on re-addition of Ca^{2+} to the Krebs. The spontaneous contractions, when they returned, were again potentiated compared with before the change to Ca^{2+}-free Krebs (control maximum response 31 ± 6% and duration 11 ± 1 s, following cyclopiazonic acid washout in Ca^{2+}-free Krebs maximum response 102 ± 11% $P<0.05$, and duration 33 ± 4 s $P<0.05$, Fig. 2C). The initial contractions were also produced more quickly (16 ± 2 s, Fig. 2C) compared with the return of the spontaneous contractions after 45 min in Ca^{2+}-free Krebs in the absence of cyclopiazonic acid (281 ± 5 s, Fig. 2A).

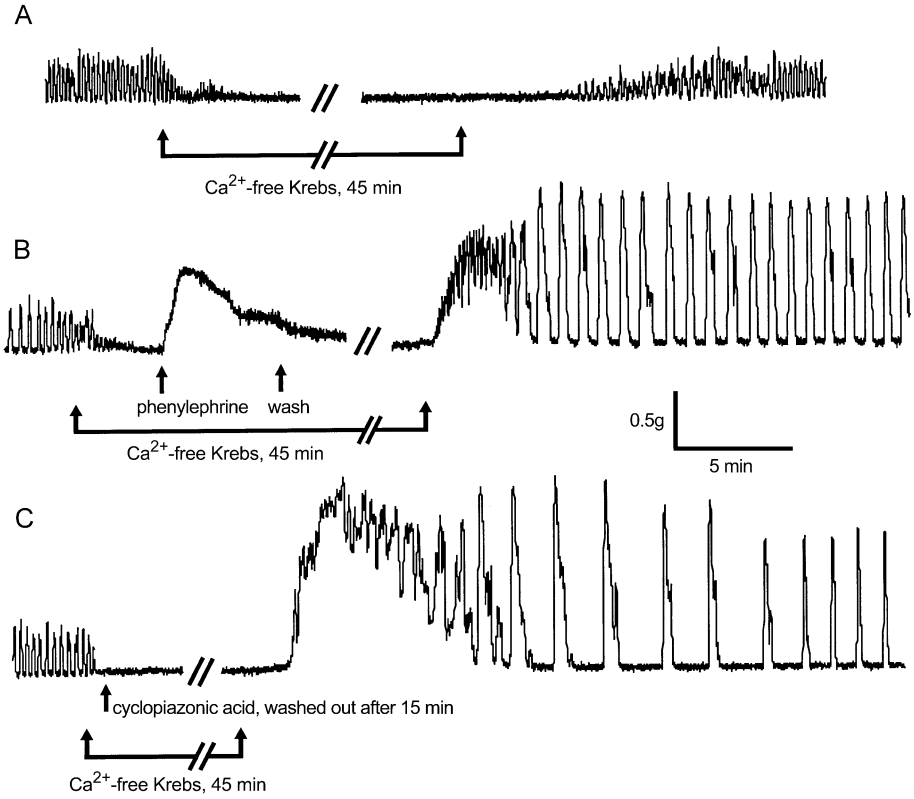

Fig. 2. Depletion of intracellular Ca^{2+} stores of the rat portal vein by phenylephrine or cyclopiazonic acid in Ca^{2+}-free Krebs, followed by their washout. These experiments showed the stimulus for potentiating the spontaneous contractions remained long after the depleting agent had been removed, provided the Ca^{2+} stores remained depleted. (A) When Ca^{2+} was removed from the Krebs for 45 min the spontaneous contractions, which returned after re-addition of Ca^{2+} (2.5 mM), were not potentiated compared with before Ca^{2+} removal. (B) Phenylephrine (10^{-4} M) was added in Ca^{2+}-free Krebs for 5 min and then washed out for 30 min (still in Ca^{2+}-free Krebs). Upon re-addition of Ca^{2+} (2.5 mM) to the Krebs, an initial tonic contraction was produced, followed by spontaneous contractions which were potentiated compared with before the Ca^{2+}-free Krebs. (C) Cyclopiazonic acid (10^{-5} M) was added to tissues in Ca^{2+}-free Krebs for 15 min and then washed out for 30 min (still in Ca^{2+}-free Krebs). Upon re-addition of Ca^{2+} (2.5 mM) to the Krebs, an initial tonic contraction was produced, followed by spontaneous contractions which were potentiated compared with before the Ca^{2+}-free Krebs.

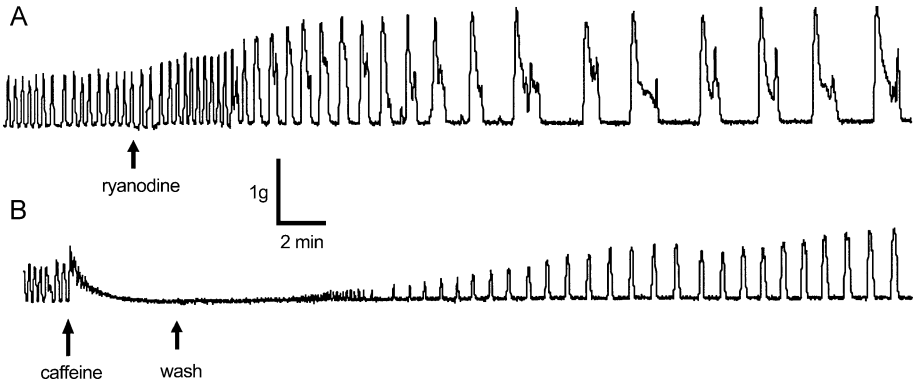

Fig. 3. Potentiation of rat portal vein spontaneous contractions by (A) ryanodine (10^{-4} M) and (B) caffeine (25 mM) following its washout (spontaneous contractions were abolished in the presence of caffeine). These results showed that other agents which can deplete Ca^{2+} from intracellular stores also potentiate the spontaneous contractions.

The above experiments showed that depleting intracellular Ca^{2+} stores in Ca^{2+}-free Krebs could result in potentiation of the spontaneous contractions. This occurred long after the depleting agent had been removed, so long as the Ca^{2+} stores remained depleted.

3.3. The effect of ryanodine and caffeine on the spontaneous contractions

Ryanodine (10^{-4} M) increased the maximum response of the spontaneous contractions from $39 \pm 5\%$ to $83 \pm 8\%$ ($P < 0.05$, Fig. 3A) and their duration from 9 ± 1 to 51 ± 3 s ($P < 0.05$, Fig. 3A). Following the contraction to caffeine (25 mM) the spontaneous contractions were abolished but returned after it was washed out with an increased maximum response from $31 \pm 2\%$ to $58 \pm 4\%$ ($P < 0.05$, Fig. 3B) and their duration increased from 10 ± 1 to 15 ± 1 s ($P < 0.05$, Fig. 3B). These results showed that other agents which can deplete intracellular Ca^{2+} stores were also able to potentiate the spontaneous contractions.

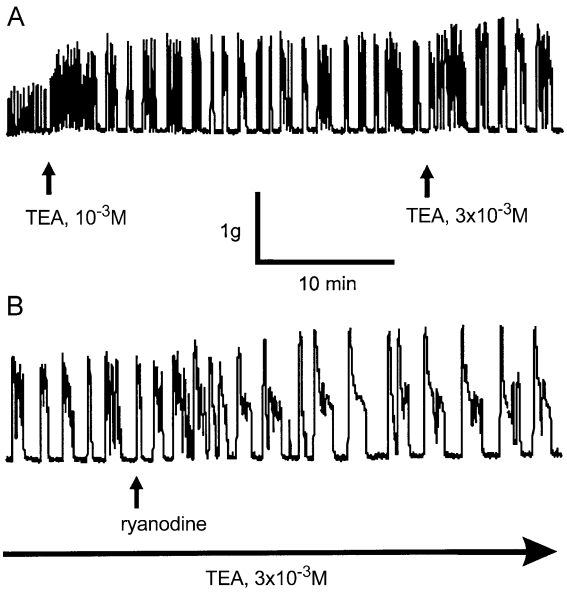

Fig. 4. Potentiation of spontaneous contractions of the rat portal vein by (A) cumulative additions of TEA (10^{-3}–3×10^{-3} M) and by (B) ryanodine (10^{-4} M) in the presence of TEA (3×10^{-3} M). These results showed that potentiation of the spontaneous contractions by ryanodine was not due to inhibition of K^+ channels.

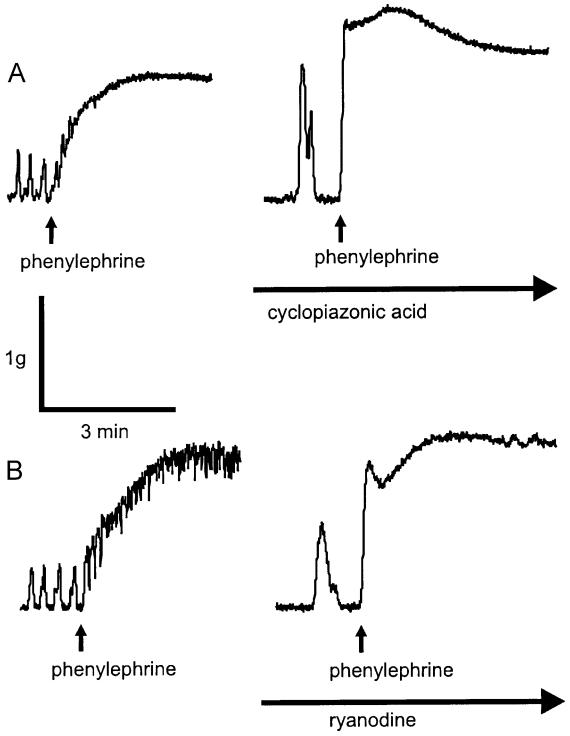

Fig. 5. The phasic contraction to phenylephrine (10^{-6} M) was potentiated in the presence of (A) cyclopiazonic acid (10^{-5} M) and (B) ryanodine (10^{-4} M). These results showed that the phasic contraction to phenylephrine, which is dependent on depolarization, could be potentiated by Ca^{2+} store depletion. In the presence of cyclopiazonic acid, there is only a small decrease in contraction from the peak of the phasic response before the large tonic contraction. The peak of the phasic response can still be seen however.

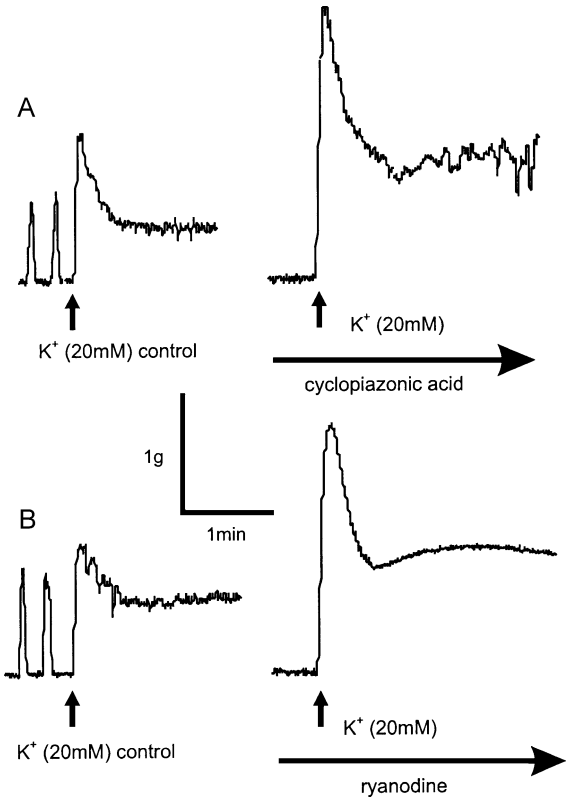

Fig. 6. The phasic contraction K^+ (20 mM) was potentiated in the presence of (A) cyclopiazonic acid (10^{-5} M) and (B) ryanodine (10^{-4} M). These results showed that the phasic contraction to K^+, which is dependent on depolarization, could be potentiated by Ca^{2+} store depletion.

3.4. The effect of inhibiting k^+ channels on potentiation of spontaneous contractions by ryanodine

TEA potentiated the spontaneous contractions (control maximum response $43 \pm 3\%$, +TEA (10^{-3} M) $72 \pm 2\%$ and +TEA (3×10^{-3} M) $80 \pm 1\%$, $P < 0.05$, Fig. 4A). Ryanodine (10^{-4} M) further potentiated the spontaneous contractions in the presence of TEA 3×10^{-3} M ($106 \pm 4\%$ $P < 0.05$, Fig. 4B). These results showed the effect of ryanodine was not due to inhibition of Ca^{2+}-activated K^+ channels.

3.5. The effect of cyclopiazonic acid and ryanodine on phasic contractions to phenylephrine or K^+

A single high dose of phenylephrine produces a phasic and tonic contraction. The phasic contraction to phenylephrine (10^{-6} M) was increased from $43 \pm 2\%$ to $134 \pm 5\%$ in the presence of cyclopiazonic acid 10^{-5} M ($P < 0.05$, Fig. 5A) and ryanodine (10^{-4} M) increased this phasic response from $39 \pm 3\%$ to $72 \pm 9\%$ ($P < 0.05$, Fig. 5B). Cyclopiazonic acid (10^{-5} M) and ryanodine (10^{-4} M) both increased the maximum phasic response to K^+, 20 mM (control $48 \pm 3\%$, +cyclopiazonic acid $115 \pm 3\%$, $P < 0.05$ Fig. 6A, and control, $36 \pm 3\%$, +ryanodine, $73 \pm 5\%$, $P < 0.05$ Fig. 6B). These experiments showed that other phasic contractions dependent on depolarization could be potentiated by cyclopiazonic acid and ryanodine.

3.6. The effect of inhibiting PKC on potentiation of spontaneous contractions

When phenylephrine (10^{-6} M) was added to tissues and then washed out, the spontaneous contractions which returned after the tonic contraction were potentiated (Fig. 7A). This potentiation was attenuated to control levels within 10 min. The PKC inhibitor calphostin C (10^{-6} M) increased the maximum potentiation of spontaneous contractions produced following washout of phenylephrine 10^{-6} M (control potentiation $76 \pm 4\%$, +calphostin C $123 \pm 5\%$, Fig. 7B). Calphostin C (10^{-6} M) also abolished the decline in this potentiation with time (Fig. 7B). This showed that PKC activity may be involved in attenuating

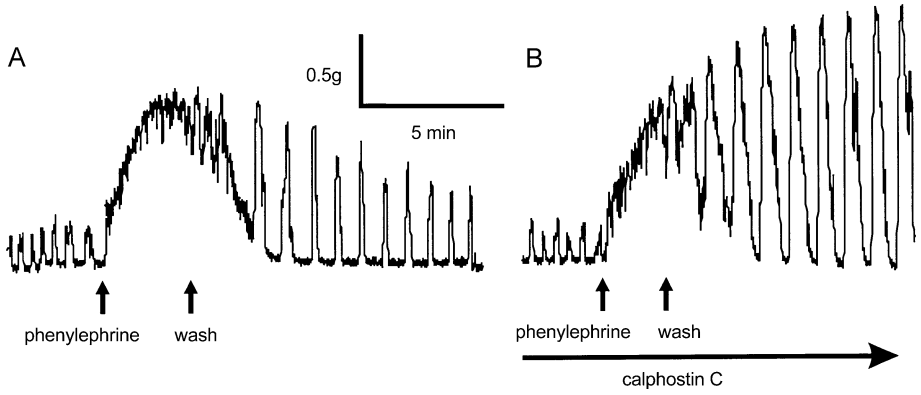

Fig. 7. The effect of the protein kinase C inhibitor calphostin C on the potentiation of the spontaneous contractions, following phenylephrine washout. (A) Following washout of phenylephrine (10^{-6} M), the spontaneous contractions when they return, are potentiated compared with before addition of phenylephrine. This potentiation then declines with time. (B) In the presence of calphostin C (10^{-6} M), the magnitude of the potentiated spontaneous contractions following washout of phenylephrine was even greater. The decline with time in potentiation of the contractions was also abolished by calphostin C. This result showed that PKC activity may be involved in attenuating the potentiating effect of Ca^{2+} store depletion on the spontaneous contractions.

the potentiating effect of phenylephrine on the spontaneous contractions.

4. Discussion

Spontaneous contractions of the rat portal vein were potentiated by phenylephrine and cyclopiazonic acid. Although cyclopiazonic acid slowed the rate of the contractions the mean integral tension was still greatly increased. Stimulation of α_1-adrenoceptors on portal vein cells has been shown to produce a rise in IP_3 (Leprêtre et al., 1994), while cyclopiazonic acid is an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Seidler et al., 1989). Both phenylephrine and cyclopiazonic acid are able therefore to deplete Ca^{2+} from intracellular stores. In some cells depletion of Ca^{2+} from intracellular stores can stimulate influx of extracellular Ca^{2+}, known as store-operated Ca^{2+} entry. This Ca^{2+} entry occurs through non-selective cation channels in smooth muscle (Albert and Large, 2002a; McDaniel et al., 2001; Trepakova et al., 2001; Wayman et al., 1996). The opening of non-selective cation channels can also result in some depolarization (Scharff and Foder, 1996) and so another effect of Ca^{2+} store depletion could be to enhance responses involving depolarization. Spontaneous contractions of the portal vein are dependent on depolarization (Burt, 2003; Kirkup et al., 1996). The possibility that depletion of Ca^{2+} from intracellular stores of the portal vein results in potentiation of the spontaneous contractions was therefore investigated.

4.1. Depletion of Ca^{2+} from intracellular stores potentiates the spontaneous contractions

Depleting intracellular Ca^{2+} stores with an IP_3 generating agonist in the absence of extracellular Ca^{2+} (followed by a long washout period) has previously been shown to stimulate store-operated Ca^{2+} entry, upon addition of extracellular Ca^{2+} (Burt et al., 1995; Jacob, 1990). These studies showed the stimulus for Ca^{2+} influx remains after IP_3 and $Ca^{2+}{}_i$ levels are no longer raised but when absence of extracellular Ca^{2+} meant intracellular Ca^{2+} stores remained depleted. So under these conditions the stimulus for Ca^{2+} influx must result from the Ca^{2+} stores being depleted. It was therefore investigated if this protocol could potentiate the spontaneous contractions of the portal vein. When phenylephrine was added in Ca^{2+}-free Krebs and then washed out for 30 min, following addition of extracellular Ca^{2+} a tonic contraction developed initially for a few minutes, followed by the return of the spontaneous contractions. In normal Krebs a washout period of 20 min for phenylephrine was long enough for the spontaneous contractions to return to their control magnitude. The spontaneous contractions were potentiated in magnitude however when they returned, compared with before the change to Ca^{2+}-free Krebs. This result was therefore consistent with phenylephrine potentiating the spontaneous contractions of the portal vein by depletion of Ca^{2+} from intracellular stores.

Cyclopiazonic acid was also added to tissues in Ca^{2+}-free Krebs and then washed out for 30 min before re-addition of extracellular Ca^{2+}. The effects of cyclopiazonic acid have been shown to be reversible (Ng and Gurney, 2001) and 30 min washout in normal Krebs for cyclopiazonic acid was long enough for the spontaneous contractions to return to their original magnitude (unpublished observation). When Ca^{2+} was added back to the Krebs, a tonic contraction was produced initially for several minutes. Following this contraction the spontaneous contractions were potentiated compared with before the change to Ca^{2+}-free Krebs. This again showed depletion of Ca^{2+} from intracellular stores could potentiate the spontaneous contractions, even after the depleting agent had been removed. Also, cyclopiazonic acid would not have activated other second messenger systems which might be stimulated by phenylephrine.

Potentiation of the spontaneous contractions by cyclopiazonic acid is in agreement with Miwa et al. (1997). They proposed that its effect was due to inhibition of the buffering capacity of the sarcoplasmic reticulum, which resulted in increased $[Ca^{2+}]_i$. The effect of cyclopiazonic acid on the spontaneous contractions when it was added and washed out in Ca^{2+}-free Krebs could not be explained by this however, as it would require the presence of cyclopiazonic acid.

Ryanodine and caffeine (following washout) also potentiated the spontaneous contractions of the rat portal vein. These agents also have the ability to deplete Ca^{2+} from intracellular stores but by different mechanisms. Ryanodine can either block or open ryanodine channels (Meissner, 1986). Ryanodine was shown previously to inhibit the phasic contraction to phenylephrine of the portal vein in Ca^{2+}-free Krebs (Burt, 2003), but did not inhibit this response in normal Krebs. If the effect of ryanodine was due to channel blockade it should also have abolished the phasic contraction to phenylephrine in normal Krebs. These effects of ryanodine on the phenylephrine contraction are also the same as those produced by cyclopiazonic acid (Burt, 2003), which depletes Ca^{2+} from intracellular stores. The effect of ryanodine in this tissue is therefore consistent with the opening of ryanodine channels. Caffeine also depletes Ca^{2+} from intracellular stores by the release of Ca^{2+} through ryanodine channels (Rousseau et al., 1988). Following the contraction to caffeine the spontaneous contractions were abolished until after washout of caffeine. This may be due to block of voltage-gated Ca^{2+} channels by caffeine (Zholos et al., 1991). After washout of caffeine, there was a progressive recovery of the spontaneous contractions, which after about 20 min were potentiated compared with before the addition of caffeine. Thus the effect of caffeine did not depend directly on its presence, or the Ca^{2+} it releases, but was a consequence of the depletion of Ca^{2+} from intracellular stores.

A functional link between Ca^{2+} dependent K^+ channels and ryanodine channels has been shown in arteries, where blocking ryanodine channels produced contraction (Knot et al., 1998). The non-selective K^+ channel blocker TEA potentiated the spontaneous contractions of the rat portal vein but ryanodine was able to further potentiate their maximum response in the presence of TEA. This suggests the effects of ryanodine were not mediated by inhibition of K^+ channels. The selective Ca^{2+} dependent K^+ channel blocker charybdotoxin has also been reported to be less effective than TEA at potentiating the spontaneous contractions of this tissue (Winquist et al., 1989). The spontaneous contractions were also potentiated by depleting intracellular Ca^{2+} stores in Ca^{2+}-free Krebs, after washout and re-addition of Ca^{2+}. This showed Ca^{2+} store depletion potentiated the spontaneous contractions even once the stores should have refilled and in the absence of a depleting agent. The potentiation therefore could not be due to inhibition of K^+ channels.

4.2. Potentiation of the phasic contractions to phenylephrine and K^+

The phasic contractions to a high concentration of phenylephrine or to K^+ are also dependent on depolarization, followed by release of intracellular Ca^{2+} (Burt, 2003; Pacaud et al., 1991). These contractions were also potentiated by cyclopiazonic acid and ryanodine. It may seem contradictory that depletion of Ca^{2+} from intracellular stores could result in potentiation of contractions, which are dependent on release of intracellular Ca^{2+}. This is possible however if the part of the Ca^{2+} stores from which depletion of Ca^{2+} results in potentiation is different from the part responsible for producing the phasic contraction, as results have indicated. Removal of extracellular Ca^{2+} was shown to abolish contractions to phenylephrine (Burt, 2003) but in the present study Ca^{2+}-free Krebs alone did not potentiate the spontaneous contractions, upon re-addition of Ca^{2+}. This showed only part of the Ca^{2+} stores was depleted in Ca^{2+}-free Krebs, from which Ca^{2+} release produces the phasic phenylephrine contraction, following depolarization. The part of the Ca^{2+} store most sensitive to IP_3 is not depleted by just Ca^{2+}-free Krebs but does result in potentiation of the phasic contractions when depleted.

4.3. Store-operated Ca^{2+} entry does not directly stimulate contraction of the portal vein

Noradrenaline, ryanodine and caffeine have been shown to stimulate Ca^{2+} influx through nifedipine-insensitive channels via depletion of intracellular Ca^{2+} stores in rat portal vein cells (Macrez-Leprêtre et al., 1997; Mironneau et al., 1996; Pacaud et al., 1992, 1993). Cyclopiazonic acid however did not produce any contraction of the portal vein in the presence of nifedipine or niflumic acid. Ca^{2+} influx through non-voltage-gated channels does not appear therefore to result directly in contraction. This may be due to the superficial buffer barrier in this tissue (Abe et al., 1995, 1996; van Breemen et al., 1995). When Ca^{2+} stores of the portal vein were depleted in Ca^{2+}-free Krebs however, contractions returned more quickly on re-addition of extracellular Ca^{2+}. This suggests Ca^{2+} influx through nifedipine-insensitive channels may be involved in refilling intracellular Ca^{2+} stores.

4.4. PKC activity may attenuate the effects of Ca^{2+} store depletion

When phenylephrine was added in Ca^{2+}-free Krebs, after addition of extracellular Ca^{2+}, the spontaneous contractions remained potentiated long after intracellular Ca^{2+} stores should have refilled. This indicated Ca^{2+} store depletion potentiates these responses by a mechanism that is not inhibited by refilling of the Ca^{2+} stores. A similar effect was observed with the rat spleen, where Ca^{2+} store depletion produces a tonic contraction (Burt et al., 1995).

Following washout of phenylephrine in normal Krebs, the spontaneous contractions remained potentiated for about 10 min, during which time the Ca^{2+} stores again should have refilled. The selective PKC inhibitor calphostin C (Burt et al., 1996; Kobayashi et al., 1989) increased the potentiation of spontaneous contractions which occurred following washout of phenylephrine. Calphostin C also prevented the decline in this potentiation with time. Basal spontaneous contractions were previously shown not to be affected by calphostin C (Burt, 2003). Activation of PKC by phenylephrine (stimulated by diacylglycerol) may therefore attenuate the effects of Ca^{2+} store depletion in this tissue.

PKC activity has been shown to inactivate store-operated Ca^{2+} entry in some cells (Murphy et al., 1996; Nofer et al., 1997; Parekh and Penner, 1995), including smooth muscle (Horibe et al., 2001). Recently, however, PKC was shown to activate a store-operated non-selective cation channel in rabbit portal vein myocytes (Albert and Large, 2002b). The regulation of store-operated Ca^{2+} entry by PKC appears therefore to be complex. One possible explanation is that low level activation of PKC has been shown to potentiate store-operated Ca^{2+} entry, while higher levels of activation inhibit it (Petersen and Berridge, 1994).

5. Conclusion

Depletion of Ca^{2+} from intracellular stores potentiated the maximum response of the spontaneous contractions of the rat portal vein. This represents a new effect of Ca^{2+} store depletion in vascular smooth muscle, which may result from the depolarizing effect of store-operated cation entry. The mechanism by which Ca^{2+} store depletion potentiated the spontaneous contractions was attenuated by PKC activity. Spontaneous contractions are a characteristic of small

arteriole resistance vessels (Colantuoni et al., 1984), which may also be potentiated by Ca^{2+} store depletion. This would be stimulated in vivo by IP_3 generating hormones or neurotransmitters, such as noradrenaline.

References

Abe, F., Mitsui, M., Karaki, H., Endoh, M., 1995. Calcium compartments in vascular smooth muscle cells as detected by aequorin signal. Br. J. Pharmacol. 116, 3000–3004.

Abe, F., Karaki, H., Endoh, M., 1996. Effects of cyclopiazonic acid and ryanodine on cytosolic calcium and contraction in vascular smooth muscle. Br. J. Pharmacol. 118, 1711–1716.

Albert, A.P., Large, W.A., 2002a. A Ca^{2+}-permeable non-selective cation channel activated by depletion of internal Ca^{2+} stores in single rabbit portal vein myocytes. J. Physiol. 538, 717–728.

Albert, A.P., Large, W.A., 2002b. Activation of store-operated channels by noradrenaline via protein kinase C in rabbit portal vein myocytes. J. Physiol. 544, 113–125.

Berridge, M.J., 1993. Inositol triphosphate and calcium signalling. Nature 361, 315–325.

Burt, R.P., 2003. Phasic contractions of the rat portal vein depend on intracellular Ca^{2+} release stimulated by depolarization. Am. J. Physiol. 284, H1808–H1817.

Burt, R.P., Chapple, C.R., Marshall, I., 1995. The role of capacitative calcium influx in the α_{1B}-adrenoceptor mediated contraction to phenylephrine in the rat spleen. Br. J. Pharmacol. 116, 2327–2333.

Burt, R.P., Chapple, C.R., Marshall, I., 1996. The role of diacylglycerol and activation of protein kinase C in α_{1A}-adrenoceptor-mediated contraction to noradrenaline of rat isolated epididymal vas deferens. Br. J. Pharmacol. 117, 224–230.

Colantuoni, A., Bertuglia, S., Intaglietta, M., 1984. Quantitation of rhythmic diameter changes in arterial microcirculation. Am. J. Physiol. 246, H508–H517.

Dacquet, C., Mironneau, C., Mironneau, J., 1987. Effects of calcium entry blockers on calcium-dependent contractions of rat portal vein. Br. J. Pharmacol. 92, 203–211.

Flemming, R., Cheong, A., Dedman, A.M., Beech, D.J., 2002. Discrete store-operated calcium influx into an intracellular compartment in rabbit arteriolar smooth muscle. J. Physiol. 543, 455–464.

Grégoire, G., Loirand, G., Pacaud, P., 1993. Ca^{2+} and Sr^{2+} entry induced Ca^{2+} release from the intracellular Ca^{2+} store in smooth muscle cells of rat portal vein. J. Physiol. 472, 483–500.

Hofmann, T., Schaefer, M., Schultz, G., Gudermann, T., 2000. Transient receptor potential channels as molecular substrates of receptor-mediated cation entry. J. Mol. Med. 78, 14–25.

Horibe, M., Kondo, I., Damron, D.S., Murray, P.A., 2001. Propofol attenuates capacitative calcium entry in pulmonary artery smooth muscle cells. Anesthesiology 95, 681–688.

Hoth, M., Penner, R., 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355, 353–356.

Jacob, R., 1990. Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. J. Physiol. 421, 55–77.

Kirkup, A.J., Edwards, G., Green, M.E., Miller, M., Walker, S.D., Weston, A.H., 1996. Modulation of membrane currents and mechanical activity by niflumic acid in rat vascular smooth muscle. Eur. J. Pharmacol. 317, 165–174.

Knot, H.J., Standen, N.B., Nelson, M.T., 1998. Ryanodine receptors regulate arterial diameter and wall $[Ca^{2+}]$ in cerebral arteries of rat via Ca^{2+}-dependent K^+ channels. J. Physiol. 508, 211–221.

Kobayashi, E., Nakano, H., Morimoto, M., Tamaoki, T., 1989. Calphostin C (UCN-1028C), A novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 159, 548–553.

Lee, M.W., Severson, D.L., 1994. Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC action. Am. J. Physiol. 267, C659–C678.

Leprêtre, N., Mironneau, J., Arnaudeau, S., Tanfin, Z., Harbon, S., Guillon, G., Ibarrondo, J., 1994. Activation of α_{1A}-adrenoceptors mobilizes calcium from the intracellular stores in myocytes from rat portal vein. J. Pharmacol. Exp. Ther. 268, 167–174.

Macrez-Leprêtre, N., Kalkbrenner, F., Schultz, G., Mironneau, J., 1997. Distinct functions of G_q and G_{11} proteins in coupling α_{1A}-adrenoreceptors Ca^{2+} release and Ca^{2+} entry in rat portal vein. J. Biol. Chem. 272, 5261–5268.

McDaniel, S.S., Platoshyn, O., Wang, J., Yu, Y., Sweeney, M., Krick, S., Rubin, L.J., Yuan, J.X., 2001. Capacitative Ca^{2+} entry in agonist-induced pulmonary vasoconstriction. Am. J. Physiol. 280, L870–L880.

Meissner, G., 1986. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. J. Biol. Chem. 261, 6300–6306.

Mikkelsen, E., 1985. Comparison of effects of a new dihydropyridine, Bay K 8644, and nifedipine on spontaneous mechanical activity in rat portal vein. Br. J. Pharmacol. 85, 383–385.

Minneman, K.P., Esbenshade, T.A., 1994. α_1-Adrenergic receptor subtypes. Annu. Rev. Pharmacol. Toxicol. 34, 117–133.

Mironneau, J., Arnaudeau, S., Macrez-Leprêtre, N., Boittin, F.X., 1996. Ca^{2+} sparks and Ca^{2+} waves activate different Ca^{2+}-dependent ion channels in single myocytes from rat portal vein. Cell Calcium 20, 153–160.

Miwa, T., Endou, M., Okumura, F., 1997. Prostaglandin E_1 potentiation of the spontaneous phasic contraction of rat isolated portal vein by a cyclopiazonic acid-sensitive mechanism. Br. J. Pharmacol. 120, 1419–1426.

Murphy, C.T., Bullock, A.J., Westwick, J., 1996. A role for protein phosphorylation in modulating Ca^{2+} elevation in rabbit platelets treated with thapsigargin. Biochem. J. 313, 83–89.

Ng, L.C., Gurney, A.M., 2001. Store-operated channels mediate Ca^{2+} influx and contraction in rat pulmonary artery. Circ. Res. 89, 923–929.

Nofer, J.R., Tepel, M., Walter, M., Seedorf, U., Assmann, G., Zidek, W., 1997. Phosphatidylcholine-specific phospholipase C regulates thapsigargin-induced calcium influx in human lymphocytes. J. Biol. Chem. 272, 32861–32868.

Noguera, M.A., D'Ocon, M.P., 1993. Evidence that depletion of internal calcium stores sensitive to noradrenaline elicits a contractile response dependent on extracellular calcium in rat aorta. Br. J. Pharmacol. 110, 861–867.

Ohta, T., Kawai, K., Ito, S., Nakazato, Y., 1995. Ca^{2+} entry activated by emptying of intracellular Ca^{2+} stores in ileal smooth muscle of the rat. Br. J. Pharmacol. 114, 1165–1170.

Pacaud, P., Loirand, G., Lavie, J.L., Mironneau, C., Mironneau, J., 1989. Calcium-activated chloride current in rat vascular smooth muscle cells in short-term primary culture. Pflugers Arch. 413, 629–636.

Pacaud, P., Loirand, G., Baron, A., Mironneau, C., Mironneau, J., 1991. Ca^{2+} channel activation and membrane depolarization mediated by Cl^- channels in response to noradrenaline in vascular myocytes. Br. J. Pharmacol. 104, 1000–1006.

Pacaud, P., Loirand, G., Bolton, T.B., Mironneau, C., Mironneau, J., 1992. Intracellular cations modulate noradrenaline-stimulated calcium entry into smooth muscle cells of rat portal vein. J. Physiol. 456, 541–556.

Pacaud, P., Loirand, G., Grégoire, G., Mironneau, C., Mironneau, J., 1993. Noradrenaline-activated heparin-sensitive Ca^{2+} entry after depletion of intracellular Ca^{2+} store in portal vein smooth muscle cells. J. Biol. Chem. 268, 3866–3872.

Parekh, A.B., Penner, R., 1995. Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. Proc. Natl. Acad. Sci. 92, 7907–7911.

Parekh, A.B., Penner, R., 1997. Store depletion and calcium influx. Physiol. Rev. 77, 901–930.

Petersen, C.C., Berridge, M.J., 1994. The regulation of capacitative calcium entry by calcium and protein kinase C in Xenopus oocytes. J. Biol. Chem. 269, 32246–32253.

Putney Jr., J.W., 1986. A model for receptor-regulated calcium entry. Cell Calcium 7, 1–12.

Rousseau, E., Ladine, J., Liu, Q.Y., Meissner, G., 1988. Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. Arch. Biochem. Biophys. 267, 75–86.

Scharff, O., Foder, B., 1996. Depletion of calcium stores by thapsigargin induces membrane depolarization by cation entry in human neutrophils. Cell Calcium 20, 31–41.

Schwietert, H.R., Gouw, M.A., Wilhelm, D., Wilffert, B., Van Zwieten, P.A., 1991. The role of α_1-adrenoceptor subtypes in the phasic and tonic responses to phenylephrine in the longitudinal smooth muscle of the rat portal vein. Naunyn-Schmiedeberg's Arch. Pharmacol. 343, 463–471.

Seidler, N.W., Jona, I., Vegh, M., Martonosi, A., 1989. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. J. Biol. Chem. 264, 17816.

Trepakova, E.S., Gericke, M., Hirakawa, Y., Weisbrod, R.M., Cohen, R.A., Bolotina, V.M., 2001. Properties of a native cation channel activated by Ca^{2+} store depletion in vascular smooth muscle cells. J. Biol. Chem. 276, 7782–7790.

van Breemen, C., Chen, Q., Laher, I., 1995. Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. Trends Pharmacol. Sci. 16, 98–105.

Wallace, P., Ayman, S., McFadzean, I., Gibson, A., 1999. Thapsigargin-induced tone and capacitative calcium influx in mouse anococcygeus smooth muscle cells. Naunyn Schmiedeberg's Arch. Pharmacol. 360, 368–375.

Wang, Q., Hogg, R.C., Large, W.A., 1992. Properties of spontaneous inward currents recorded in smooth muscle cells isolated from the rabbit portal vein. J. Physiol. 451, 525–537.

Wayman, C.P., McFadzean, I., Gibson, A., Tucker, J.F., 1996. Two distinct membrane currents activated by cyclopiazonic acid-induced calcium store depletion in single smooth muscle cells of the mouse anococcygeus. Br. J. Pharmacol. 117, 566–572.

Wayman, C.P., Gibson, A., McFadzean, I., 1998. Depletion of either ryanodine- or IP_3-sensitive calcium stores activates capacitative calcium entry in mouse anococcygeus smooth muscle cells. Pflugers Arch. 435, 231–239.

Winquist, R.J., Heaney, L.A., Wallace, A.A., Baskin, E.P., Stein, R.B., Garcia, M.L., Kaczorowski, G.J., 1989. Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulfate and diazoxide in vascular smooth muscle. J. Pharmacol. Exp. Ther. 248, 149–156.

Zhang, H., Inazu, M., Weir, B., Buchanan, M., Daniel, E., 1994. Cyclopiazonic acid stimulates Ca^{2+} influx through non-specific cation channels in endothelial cells. Eur. J. Pharmacol. 251, 119–125.

Zholos, A.V., Baidan, L.V., Shuba, M.F., 1991. The inhibitory action of caffeine on calcium currents in isolated intestinal smooth muscle cells. Pflugers Arch. 419, 267–273.