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European Journal of Pharmacology 510 (2005) 97–105

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Depletion of Ca^{2+} from intracellular stores of the rat portal vein stimulates a tonic contraction

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Received 16 December 2004; accepted 13 January 2005

Abstract

The possibility that Ca^{2+} store depletion can stimulate contraction of the rat portal vein was investigated in functional experiments. Ca^{2+} stores were depleted with phenylephrine or cyclopiazonic acid in the absence of extracellular Ca^{2+} and then washed out for 30 min. Upon re-addition of extracellular Ca^{2+}, a tonic contraction was produced, showing the stimulus for contraction was Ca^{2+} store depletion. The contractions were abolished by niflumic acid and nifedipine however, indicating they were dependent on depolarization resulting from opening of Ca^{2+}-activated Cl^- channels and Ca^{2+} influx through voltage-gated channels. Cumulative additions of phenylephrine below 3×10^{-6} M did not produce tonic contractions but did in high K^+ Krebs solution, where levcromakalim had no effect. This showed the tonic contractions were initially prevented by K^+ channel opening. Increased Ca^{2+} entry through voltage-gated channels may therefore stimulate Ca^{2+}-activated Cl^- channels. Ca^{2+} store depletion could stimulate this by opening store-operated non-selective cation channels, resulting in depolarization.

Keywords: Rat portal vein; Capacitative Ca^{2+} entry; Store operated Ca^{2+} entry; Cyclopiazonic acid; Niflumic acid

1. Introduction

Depletion of Ca^{2+} from intracellular stores by inositol 1,4,5-trisphosphate (IP_3) generating agonists stimulates extracellular Ca^{2+} influx through non-voltage-gated channels in many cell types, known as capacitative or store-operated Ca^{2+} influx (Parekh and Penner, 1997; Putney, 1986). This can also be stimulated by cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Seidler et al., 1989). Capacitative Ca^{2+} influx was first shown in non-excitable cells to be mediated via Ca^{2+} selective channels (Hoth and Penner, 1992) and then through non-selective cation channels in endothelial cells (Zhang et al., 1994). In smooth muscle Ca^{2+} store depletion also stimulates non-selective cation channels (Trepakova et al., 2001; Wayman et al., 1996) including rabbit portal vein (Albert and Large, 2002). Capacitative Ca^{2+} influx in smooth muscle has been shown to result in a tonic contraction in tissues such as rat spleen (Burt et al., 1995) rat ileum (Ohta et al., 1995) mouse anococcygeus (Wayman et al., 1996) and rat pulmonary artey (McDaniel et al., 2001; Ng and Gurney, 2001). It has been proposed that some members of the transient receptor potential (*trp*) gene family encode for the cation channels activated by Ca^{2+} store depletion (for reviews see Hofmann et al., 2000; Montell et al., 2002).

The rat isolated portal vein displays spontaneous phasic contractions. These are dependent on depolarization and influx of Ca^{2+} through voltage-gated channels which stimulates release of Ca^{2+} from ryanodine stores (Burt, 2003; Grégoire et al., 1993). Stimulation of α_1-adrenoceptors in rat portal vein cells has been shown to produce a rise in IP_3 (Leprêtre et al., 1994a). In functional studies using the rat isolated portal vein it has been shown that depletion of Ca^{2+} from intracellular stores via α_1-adrenoceptor stimulation or by cyclopiazonic acid potentiated the spontaneous

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doi:10.1016/j.ejphar.2005.01.021

contractions without increasing resting tone (Burt, 2004). Ca^{2+} store depletion has been shown to stimulate Ca^{2+} influx (Pacaud et al., 1993) and non-selective cation channels (Albert and Large, 2002) in portal vein myocytes. Stimulation of non-selective cation channels by Ca^{2+} store depletion can also result in depolarization (Scharff and Foder, 1996). It is possible that depolarization of the portal vein stimulated in this way potentiates depolarizations associated with the spontaneous contractions. The results of this study show that greater depletion of Ca^{2+} from intracellular stores of the rat portal vein than required to potentiate the spontaneous contractions can also stimulate a tonic contraction. This involves depolarization via opening of Ca^{2+}-activated Cl^- channels, which may be stimulated by increased Ca^{2+} entry through voltage-gated channels.

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 (Krebs) and associated connective tissue was dissected away. The tissues (10–15 mm) were suspended longitudinally in 5 ml tissue baths containing Krebs solution 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. A modified high K^+ Krebs was sometimes used of the same composition except for an increase in K^+ to 50 mM and an equivalent decrease in Na^+ to 98.9 mM. 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 Inc. MP100WS for Windows.

Experiments were carried out at 25 °C so that the results were consistent with Burt (2003, 2004). Preliminary experiments have shown contractions of this tissue to be similar at 25 °C and 37 °C. Phenylephrine (10^{-4} M, which produced a maximum response) was added to all tissues initially, with a recovery period of 45 min following washout. Drugs were incubated for 30 min with tissues unless otherwise stated.

2.1. Experiments in normal Krebs

The response to a single high concentration of phenylephrine (10^{-4} M) and to cumulative additions of phenylephrine (10^{-8} M–3×10^{-5} M) was recorded. The effect of the protein kinase C (PKC) inhibitor calphostin C (10^{-6} M, 1 h incubation) or the K^+ channel opener levcromakalim (3×10^{-6} M) was measured on the tonic contraction to phenylephrine (10^{-6} M).

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

These experiments were designed to show if the stimulus for the tonic contraction depended on the intracellular Ca^{2+} stores being depleted rather than the presence of the depleting agent. Phenylephrine (10^{-4} M) or cyclopiazonic acid (10^{-5} M) was added to tissues in Ca^{2+}-free Krebs for 10 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. For some tissues, following the washout of phenylephrine or cyclopiazonic acid, nifedipine (3×10^{-7} M) or niflumic acid (3×10^{-5} M) was added before the addition of Ca^{2+} (2.5 mM).

2.2. Experiments in high K^+ Krebs

These experiments were designed to show the possible effect of K^+ channels on contractions of the portal vein. It has been shown previously that high K^+ Krebs can abolish the effect of K^+ channel opening (Cook et al., 1988). Following a contraction to phenylephrine (10^{-4} M) in normal Krebs, some tissues were placed in the modified high K^+ (50 mM) Krebs. Experiments were then performed once the contraction to high K^+ Krebs had returned to baseline or close to it (after about 40 min).

The effect of the K^+ channel opener levcromakalim (3×10^{-6} M) was measured on the response to phenylephrine (10^{-6} M). The effect of the K^+ channel blocker tetraethylammonium (10^{-3} M–3×10^{-3} M) was also measured in high K^+ Krebs.

A cumulative concentration–response curve to phenylephrine was recorded and the effect of nifedipine (3×10^{-7} M) or niflumic acid (3×10^{-5} M) measured on this. The response to cyclopiazonic acid (10^{-5} M) was recorded and the effect of removing extracellular Ca^{2+} or niflumic acid (3×10^{-5} M) on this response was measured.

2.2.1. The effect of depleting intracellular Ca^{2+} stores in high K^+, Ca^{2+}-free Krebs

Phenylephrine (10^{-4} M) was added to tissues in high K^+, Ca^{2+}-free Krebs for 5 min. It was then washed out for 30 min, still in high K^+, Ca^{2+}-free Krebs, which was then changed to high K^+, Ca^{2+}-free Krebs without EGTA. Ca^{2+} (2.5 mM) was then added to the tissues and the following response measured. Control tissues were treated in the same way except no phenylephrine was added.

2.3. Data analysis

All contractions were measured as a percentage response of that to phenylephrine (10^{-4} M) and calculated as the mean from 4 separate experiments (n=4) unless otherwise

stated. 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).

2.4. Drugs and solutions

Phenylephrine hydrochloride, nifedipine, niflumic acid, and tetraethylammonium chloride were obtained from Sigma. Cyclopiazonic acid and calphostin C were obtained from Calbiochem and levcromakalim from Tocris. 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 (DMSO) and further diluted in distilled water and cyclopiazonic acid and calphostin C which were dissolved and further diluted in DMSO. Stock solutions were stored frozen except phenylephrine, which was prepared fresh each day.

3. Results

3.1. Results in normal Krebs

A high concentration of phenylephrine (10^{-4} M) produced a well maintained tonic contraction of the portal vein (maximum response 1.62±0.1 g, Fig. 1A). When phenylephrine was added in cumulative additions, low concentrations only potentiated the spontaneous contractions. At a concentration of 3×10^{-6} M (n=2) or 10^{-5} M (n=2), a maximal tonic contraction was then produced (maximum response 102±3%, Fig. 1B). During the cumulative additions, phenylephrine (10^{-6} M), only potentiated the spontaneous contractions but a single dose of phenylephrine (10^{-6} M) could stimulate a tonic contraction (compare Fig. 1B with Fig. 4A). The reason for this is given in Section 4.2. The PKC inhibitor calphostin C (10^{-6} M) did not significantly affect the tonic contraction to phenylephrine (10^{-6} M) (control 82±2%, +calphostin C 80±2%, results not shown). The K^+ channel opener levcromakalim (3×10^{-6} M) abolished the tonic contraction to phenylephrine 10^{-6} M (Fig. 4A). Some phasic contractions were stimulated (after about 1 min) by phenylephrine (10^{-6} M) in the presence of levcromakalim, maximum response after 5 min 42±3% (Fig. 4A).

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

When phenylephrine 10^{-4} M was added to tissues in Ca^{2+}-free Krebs and then washed out for 30 min, upon re-addition of Ca^{2+} (2.5 mM) to the Krebs a tonic contraction developed

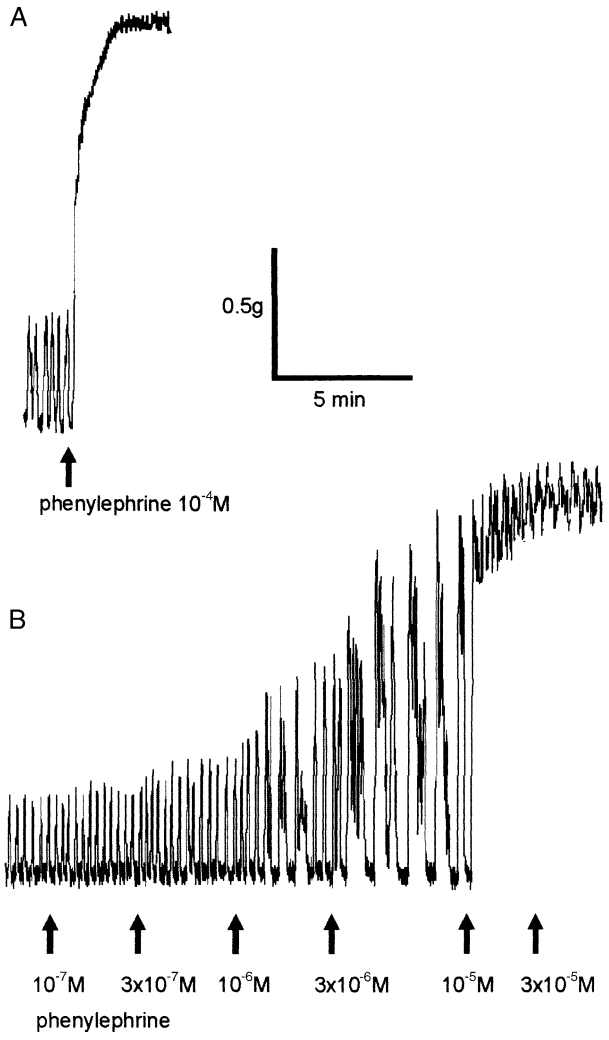

Fig. 1. (A) Tonic contraction to phenylephrine (10^{-4} M) and (B) the maximal tonic contraction to phenylephrine 3×10^{-6} M or 10^{-5} M, produced during cumulative additions. Lower concentrations of phenylephrine only potentiated the spontaneous contractions of the portal vein.

(maximum response 68±2%, duration 3.1±0.1 min, Fig. 2B). This contraction was abolished by the addition of either niflumic acid (3×10^{-5} M, Fig. 2C) or nifedipine (3×10^{-7} M, results not shown) before the re-addition of Ca^{2+} (2.5 mM) to the Krebs. No tonic contraction was observed in control tissues where phenylephrine was not added during the period in Ca^{2+}-free Krebs (Fig. 2A).

When cyclopiazonic acid (10^{-5} M) was added to tissues in Ca^{2+}-free Krebs for 15 min and then washed out for 30 min, upon re-addition of Ca^{2+} to the Krebs a tonic contraction was stimulated for 6.1±0.2 min (maximum response 95±4%, Fig. 3A). This contraction was also abolished by the addition of either niflumic acid (3×10^{-5} M, Fig. 3B) or nifedipine (3×10^{-7} M, results not shown) before the re-addition of Ca^{2+} (2.5 mM) to the Krebs.

These experiments showed that the contraction depended on the intracellar Ca^{2+} stores being depleted and not the presence of the depleting agent. They also indicated the contractions depended on opening of Ca^{2+}-

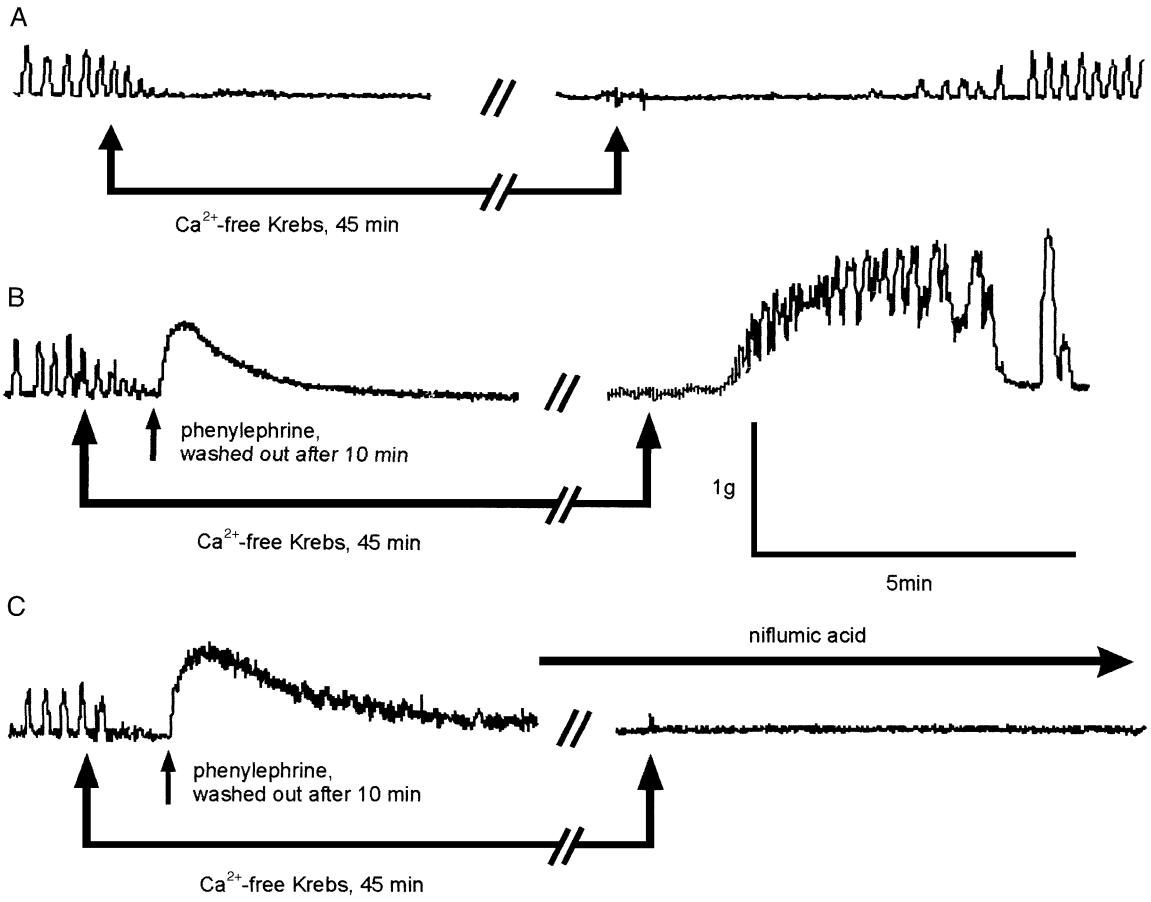

Fig. 2. (A) When Ca^{2+} was removed from the Krebs for 45 min without the addition of phenylephrine, no tonic contraction was produced upon re-addition of extracellular Ca^{2+} (2.5 mM). (B) When phenylephrine (10^{-5}M) was added to tissues in Ca^{2+}-free Krebs for 10 min and then washed out for 30 min, upon re-addition of Ca^{2+} (2.5 mM) to the Krebs a large tonic contraction developed. This showed the stimulus for contraction was the intracellular Ca^{2+} stores being depleted because this lasted so long as the stores remained depleted in Ca^{2+}-free Krebs. The contraction did not require the presence of phenylephrine, which was washed out before the re-addition of extracellular Ca^{2+}. (C) When phenylephrine (10^{-5} M) was added to tissues in Ca^{2+}-free Krebs for 5 min and then washed out for 30 min, the addition of niflumic acid (3×10^{-5} M) before the re-addition of Ca^{2+} (2.5 mM) to the Krebs abolished the tonic contraction which had been stimulated in (B). This indicated the contraction also involved opening of Ca^{2+}-activated Cl^- channels.

activated Cl^- channels and Ca^{2+} influx through voltage-gated channels.

3.2. Results in high K^+ Krebs

Following the initial contraction to phenylephrine (10^{-4} M) in normal Krebs, when tissues were placed in the modified high K^+ (50 mM) Krebs, a tonic contraction was stimulated. The following experiments were performed once the contraction to high K^+ Krebs had returned to baseline or close to it (after about 40 min).

3.2.1. The function of K^+ channels in high K^+ Krebs

The K^+ channel opener levcromakalim (3×10^{-6} M) did not significantly affect the tonic contraction to phenylephrine (10^{-6} M) in high K^+ Krebs (control response 85±2%, +levcromakalim 83±2%, Fig. 4B). Tetraethylammonium (10^{-3} M) did not produce any response in high K^+ Krebs (results not shown). This indicated that K^+ channels did not functionally affect responses after equilibration of tissues in high K^+ Krebs.

3.2.2. The effect of depleting intracellular Ca^{2+} stores in high K^+, Ca^{2+}-free Krebs

When phenylephrine 10^{-4} M was added to tissues in high K^+, Ca^{2+}-free Krebs and then washed out for 30 min, upon re-addition of Ca^{2+} (2.5 mM) to the high K^+ Krebs, a tonic contraction developed initially for a few minutes (maximum response 68±2%, duration 3.1±0.1 min, Fig. 5B). No tonic contraction was observed in control tissues where phenylephrine was not added during the period in Ca^{2+}-free Krebs (Fig. 5A).

3.2.3. Phenylephrine and cyclopiazonic acid always produce tonic contractions in high K^+ Krebs

Cumulative additions of phenylephrine (10^{-7} M–3×10^{-5} M) in high K^+ Krebs always resulted in a tonic contraction (no phasic response) of the portal vein (maximum response 102±3%, Fig. 6A). Nifedipine 3×10^{-7} M (results not shown) or niflumic acid 3×10^{-5} M (Fig. 6B), both abolished the phenylephrine contractions. Cyclopiazonic acid (10^{-5} M) produced a well maintained tonic contraction in high K^+ Krebs (maximum response 34±1%,

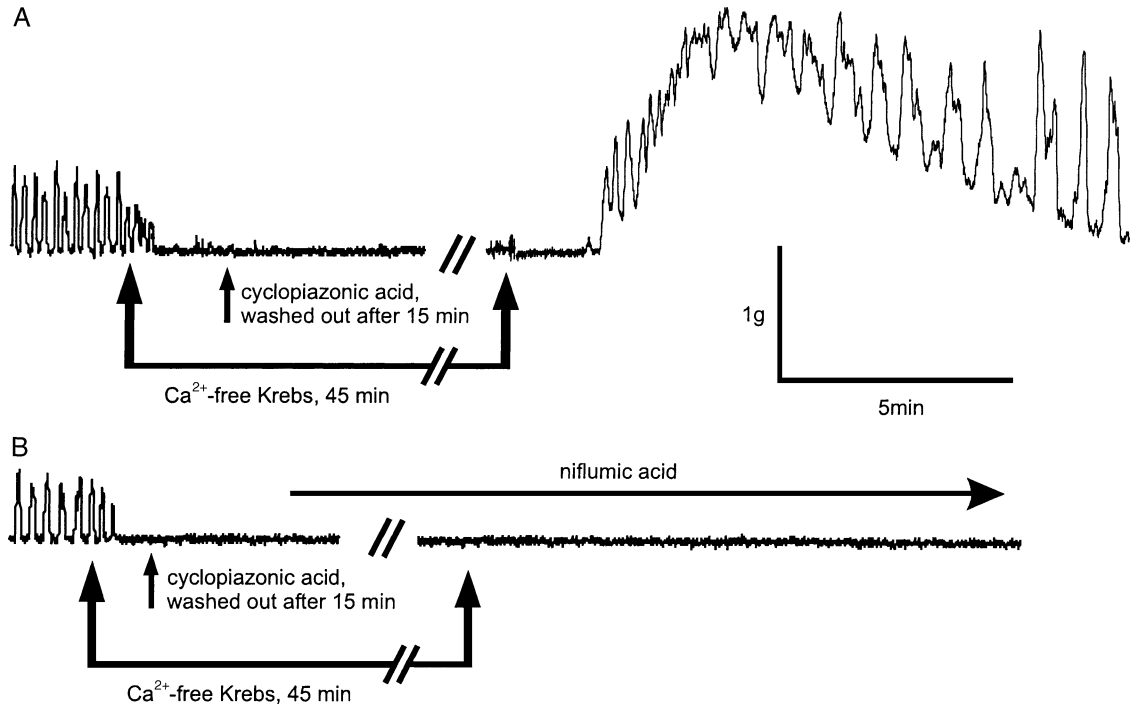

Fig. 3. (A) When cyclopiazonic acid (10^{-5} M) was added to tissues in Ca^{2+}-free Krebs for 15 min and then washed out for 30 min, upon re-addition of Ca^{2+} (2.5 mM) to the Krebs a large tonic contraction developed. This showed that the contraction was stimulated by the intracellular Ca^{2+} stores being depleted by cyclopiazonic acid and then remaining depleted until the re-addition of extracellular Ca^{2+}. The contraction did not require the presence of cyclopiazonic acid, which was washed out before the re-addition of extracellular Ca^{2+}. (B) When cyclopiazonic acid (10^{-5} M) was added to tissues in Ca^{2+}-free Krebs for 15 min and then washed out for 30 min, the addition of niflumic acid (3×10^{-5} M) before the re-addition of Ca^{2+} (2.5 mM) to the Krebs abolished the tonic contraction which had been stimulated in (A). This indicated the contraction also involved opening of Ca^{2+}-activated Cl^- channels.

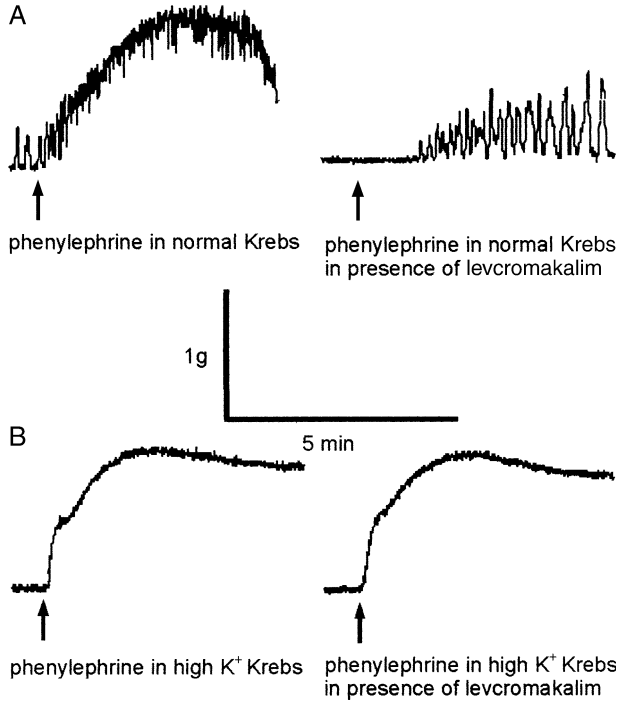

Fig. 4. The K^+ channel opener levcromakalim inhibits contractions to phenylephrine in normal Krebs but not in high K^+ Krebs. This indicated that opening of K^+ channels does not hyperpolarize tissues in high K^+ Krebs. (A) The effect of levcromakalim (3×10^{-6} M) on the response to phenylephrine (10^{-6} M) in normal Krebs. (B) The effect of levcromakalim (3×10^{-6} M) on the response to phenylephrine (10^{-6} M) in high K^+ (50 mM) Krebs.

Fig. 6C). The contraction to cyclopiazonic acid (10^{-5} M) was abolished by removal of Ca^{2+} from the high K^+ Krebs (results not shown) or niflumic acid, 3×10^{-5} M (Fig. 6D).

4. Discussion

The α_1-adrenoceptor agonist phenylephrine stimulates a tonic contraction of the rat portal vein. Previously it was shown that depletion of Ca^{2+} from intracellular stores by low concentations of phenylephrine potentiated the spontaneous contractions of this tissue (Burt, 2004). At concentrations of 3×10^{-6} M or 10^{-5} M phenylephrine during cumulative additions, a maximal tonic contraction was then produced (see Fig. 1B). Results of the present study show that greater depletion of Ca^{2+} from intracellular stores by phenylephrine than required to potentiate the spontaneous contractions, produces a tonic contraction of the portal vein.

4.1. Depletion of Ca^{2+} from intracellular stores of the portal vein stimulates a tonic contraction

Depleting intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+} has been shown to result in a tonic contraction in smooth muscle upon re-addition of extracellular Ca^{2+} (Burt et al., 1995). The agonist is washed out for a long period before the re-addition of extracellular

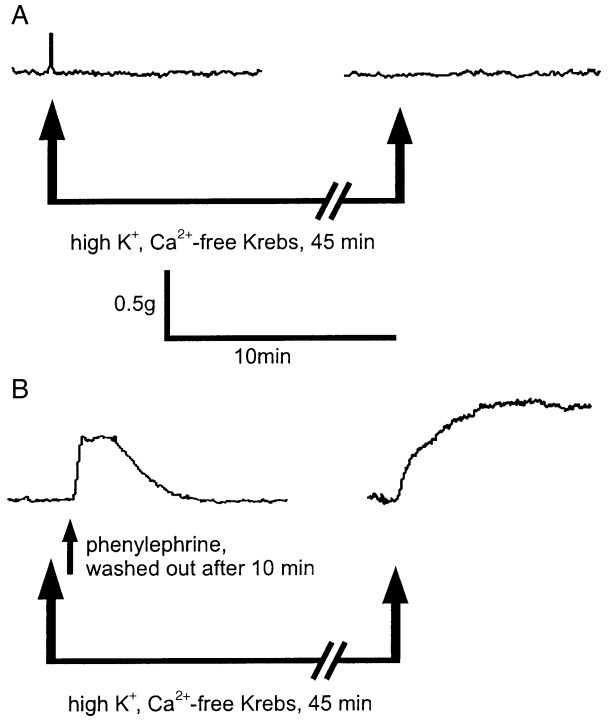

Fig. 5. (A) When Ca^{2+} was removed from the high K^+ Krebs for 45 min without the addition of phenylephrine, no tonic contraction was produced upon re-addition of extracellular Ca^{2+} (2.5 mM). (B) When phenylephrine (10^{-5} M) was added to tissues in high K^+, Ca^{2+}-free Krebs for 5 min and then washed out for 30 min, upon re-addition of Ca^{2+} (2.5 mM) to the high K^+ Krebs, a large tonic contraction developed. This showed that intracellular Ca^{2+} store depletion could stimulate a contraction in high K^+ Krebs, as in normal Krebs (see Fig. 2).

Ca^{2+}, during which time any rise in second messengers or $[Ca^{2+}]_i$ should have return to basal levels. The intracellular Ca^{2+} stores will however remain empty. Any response produced after re-addition of extracellular Ca^{2+} must therefore result from the intracellular Ca^{2+} stores being depleted. When intracellular Ca^{2+} stores of the portal vein were depleted by phenylephrine in Ca^{2+}-free Krebs, followed by 30 min washout in Ca^{2+}-free Krebs, a tonic contraction was produced after re-addition of extracellular Ca^{2+} (see Fig. 2B). This showed that depletion of intracellular Ca^{2+} stores could stimulate a tonic contraction of the portal vein. Simply placing tissues in Ca^{2+}-free Krebs for 45 min did not result in a tonic contraction upon addition of extracellular Ca^{2+}.

Cyclopiazonic acid also depletes Ca^{2+} from intracellular stores (Seidler et al., 1989) and potentiates the spontaneous contractions of the portal vein without increasing basal tone (Burt, 2004). This effect of cyclopiazonic acid disappears after 30 min washout, indicating that it was completely washed out after this time (unpublished observation). When cyclopiazonic acid was added to tissues in Ca^{2+}-free Krebs however, and then washed out for 30 min, a large tonic contraction was produced on re-addition of extracellular Ca^{2+}, which lasted several minutes (see Fig. 3A). This again showed depletion of Ca^{2+} from intracellular stores could stimulate a tonic contraction. The effect of cyclopiazonic acid here could not be due to reducing the buffering capacity of the sarcoplasmic reticulum which might allow more extracellular Ca^{2+} to enter the contractile compartment of portal vein cells or any other possible effects. This is because cyclopiazonic acid had already been washed out before the addition of extracellular Ca^{2+}. It also shows the reason why cyclopiazonic acid only potentiated the spontaneous contractions in normal Krebs (Burt, 2004) was because its ability to deplete the Ca^{2+} stores is limited in the presence of extracellular Ca^{2+}.

The tonic contractions produced by Ca^{2+} store depletion with either phenylephrine or cyclopiazonic acid in Ca^{2+}-free Krebs only lasted several minutes after the addition of extracellular Ca^{2+}. This suggests the stimulus resulting in a tonic contraction is inhibited by influx of extracellular Ca^{2+} or Ca^{2+} store refilling in the absence of continued store

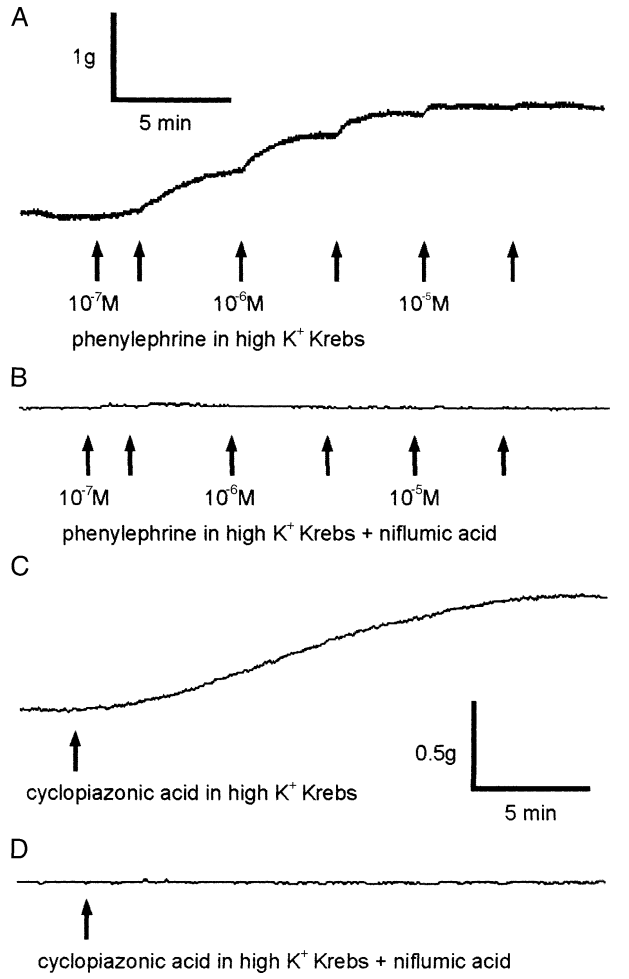

Fig. 6. (A) Cumulative additions of phenylephrine on the rat portal vein in high K^+ Krebs, which always produced a tonic contraction. Low concentrations of phenylephrine in normal Krebs only potentiated the spontaneous contractions (see Fig. 1B). (B) The effect of niflumic acid (3×10^{-5} M) on cumulative additions of phenylephrine in high K^+ Krebs. (C) Tonic contraction of the rat portal vein to cyclopiazonic acid (10^{-5} M) in high K^+ Krebs. (D) The effect of niflumic acid (3×10^{-5} M) on the tonic contraction to cyclopiazonic acid in high K^+ Krebs.

depletion. Potentiation of the spontaneous contractions of the portal vein by lower levels of Ca^{2+} store depletion (Burt, 2004), may be attenuated by PKC (Burt, 2004).

4.2. Store-operated Ca^{2+} entry stimulates tonic contractions via opening of Ca^{2+}-activated Cl^- channels

Results so far have shown that depletion of Ca^{2+} from intracellular stores of the portal vein produces a tonic contraction. In some smooth muscle tissues Ca^{2+} entry through store-operated channels directly stimulates contraction (Burt et al., 1995; Ng and Gurney, 2001; Noguera and D'Ocon, 1993; Ohta et al., 1995; Wallace et al., 1999). The tonic contractions stimulated by Ca^{2+} store depletion were abolished however by nifedipine and niflumic acid (see Figs. 2C and 3B). This indicated they depended on depolarization stimulated by opening of Ca^{2+}-activated Cl^- channels and influx of extracellular Ca^{2+} through voltage-gated channels. The effect of niflumic acid on contractions of the portal vein does not appear to be due to other possible actions. Niflumic acid (10^{-4} M) did not block store-operated Ca^{2+} entry in the rabbit portal vein, which was always included in the pipette solution (Albert and Large, 2002). In cells other than smooth muscle, where niflumic acid has been suggested to block store-operated Ca^{2+} entry, the IC_{50} value was above 10^{-4} M (Reinsprecht et al., 1995). Niflumic acid has also been shown not to inhibit α-adrenoceptors or voltage-gated Ca^{2+} channels (Hogg et al., 1994). Finally, the results in high K^+ solution of the present study, discussed in Section 4.3, show the effect of niflumic acid could not be due to opening of K^+ channels.

The reason why store-operated Ca^{2+} entry in the portal vein does not directly stimulate contraction is probably due to the presence of the superficial buffer barrier (Abe et al., 1995, 1996; Burt, 2003). This is formed by the sarcoplasmic reticulum, which acts as a buffer for Ca^{2+} entering the cell. When Ca^{2+} enters the cell it must therefore pass through a non-contractile compartment before reaching the deeper contractile compartment of the cell. Store-operated Ca^{2+} entry however may contribute to refilling of intracellular Ca^{2+} stores.

The contractions stimulated by Ca^{2+} store depletion in Ca^{2+}-free Krebs followed by re-addition of extracellular Ca^{2+} showed they were not stimulated by intracellular Ca^{2+} release. The tonic contractions produced during cumulative additions of phenylephrine were initiated by the potentiated spontaneous contractions, which turned into a maintained rather than a phasic response. The spontaneous contractions depend on depolarization and Ca^{2+} influx through voltage-gated channels. This suggests that increased Ca^{2+} influx through voltage-gated channels may stimulate Ca^{2+}-activated Cl^- channels to produce constant depolarization and Ca^{2+} influx. When Ca^{2+} stores were depleted in Ca^{2+}-free Krebs, there was a delay between addition of Ca^{2+} and the tonic contraction. This is probably because there is a latency in the return of the spontaneous contractions when extracellular Ca^{2+} is removed and then put back (see Fig. 2A) and a spontaneous contraction is required to initiate the tonic contraction (as seen with the cumulative additions of phenylephrine). It has been reported that PKC stimulates L-type Ca^{2+} channels in rat portal vein cells (Leprêtre et al., 1994b). The PKC inhibitor calphostin C (Burt et al., 1996; Kobayashi et al., 1989) however did not inhibit the tonic contraction to phenylephrine in portal vein tissues.

It has been shown in portal vein cells that Ca^{2+}-activated Cl^- channels can be stimulated by Ca^{2+} influx through voltage-gated channels. At more positive membrane potentials the Ca^{2+}-activated chloride current was found to be sustained due to constant influx of Ca^{2+} through non-inactivating voltage-gated channels (Greenwood and Large, 1996). In smooth muscle depletion of Ca^{2+} from intracellular stores stimulates non-selective cation channels (McDaniel et al., 2001; Trepakova et al., 2001; Wayman et al., 1996) including rabbit portal vein (Albert and Large, 2002), which can result in depolarization (Scharff and Foder, 1996). It is possible therefore that increasing Ca^{2+} store depletion eventually results in enough depolarization to open the non-inactivating voltage-gated channels and allow constant influx of Ca^{2+}. This could then stimulate a sustained depolarization via opening of Ca^{2+}-activated Cl^- channels, resulting in a tonic contraction.

Cyclopiazonic acid does not stimulate Ca^{2+} activated Cl^- channels by increasing subsarcolemmal $[Ca^{2+}]$, due to a reduced buffering capacity of the sarcoplasmic reticulum. This is because cyclopiazonic acid had already been washed out when it produced a contraction following its addition in Ca^{2+}-free Krebs. Work on voltage-clamped portal vein cells has also shown that cyclopiazonic acid does not affect $I_{Cl(Ca)}$ when stimulated by Ca^{2+} influx through voltage-gated channels (Greenwood et al., 1997).

During cumulative additions, phenylephrine (10^{-6} M) only potentiated the spontaneous contractions of the portal vein but added as a single dose produced a tonic contraction (compare Fig. 1B with Fig. 4A). This suggests that release of intracellular Ca^{2+} by phenylephrine can also contribute to stimulating a tonic contraction, by opening Ca^{2+}-activated Cl^- channels. The combined effects of this and Ca^{2+} store depletion could produce enough Ca^{2+} influx to result in constant depolarization and a tonic contraction. Cumulative additions of phenylephrine gradually depletes Ca^{2+} from the intracellular stores, so that the rise in $[Ca^{2+}]_i$ produced by this is not enough to stimulate depolarization.

4.3. Ca^{2+} store depletion always stimulates a tonic contraction in high K^+ Krebs

Further evidence that depletion of Ca^{2+} from intracellular stores can stimulate a tonic contraction of the portal vein by increasing Ca^{2+} influx through voltage-gated channels was shown by experiments in high K^+ Krebs. The spontaneous contractions were abolished in high K^+ Krebs. The inhibitory

effect of the K^+ channel opener levcromakalim on contractions to phenylephrine was also abolished in high K^+ Krebs (see Fig. 4), in agreement with Cook et al. (1988). This indicated that K^+ channels do not affect the membrane potential under these conditions. Consistent with this, tetraethylammonium did not produce any response in high K^+ Krebs.

Depleting intracellular Ca^{2+} stores with phenylephrine in high K^+ Ca^{2+}-free Krebs again stimulated a tonic contraction upon re-addition of extracellular Ca^{2+} (see Fig. 5). This showed the contraction in high K^+ Krebs was still stimulated by depletion of intracellular Ca^{2+} stores. Cumulative additions of phenylephrine (see Fig. 6A), or cyclopiazonic acid (see Fig. 6C), always produced tonic contractions in high K^+ Krebs. The contraction to cyclopiazonic acid in high K^+ Krebs was smaller however than for phenylephrine. This may be due phenylephrine being more efficient at depleting Ca^{2+} from intracellular stores in the presence of extracellular Ca^{2+}, as was shown in normal Krebs.

The contractions in high K^+ Krebs were still inhibited by niflumic acid and nifedipine (see Fig. 6B and D). This indicated that depolarization stimulated by opening of Ca^{2+} activated Cl^- channels could still produce contraction in high K^+ Krebs, even though the tissues would be depolarized to some extent in this solution. A 10 fold increase in extracellular K^+ concentration has been shown to produce a maximum depolarization of about 43 mV in smooth muscle (Casteels and Kuriyama, 1966). The resting membrane potential of rat portal vein cells has been measured to range from −70 mV to −75 mV (Loirand et al., 1986). The membrane potential of the portal vein in high K^+ Krebs could therefore still be more negative than the equilibrium potential for chloride, which can be around −20 mV in smooth muscle cells (Aickin, 1990). This could therefore still allow depolarizations stimulated by opening of Ca^{2+} activated Cl^- channels in the high K^+ Krebs. In further support of this, the phasic contraction to a high concentration of phenylephrine is also dependent on depolarization stimulated by opening of Ca^{2+} activated Cl^- channels (Burt, 2003; Pacaud et al., 1991). The phasic contraction was not abolished in high K^+ Krebs but is abolished by niflumic acid (and nifedipine) in normal and high K^+ Krebs (Burt, 2003). This phasic response would not be inhibited by blocking store-operated Ca^{2+} influx and it was shown that niflumic acid did not block the release of intracellular Ca^{2+} stimulated by caffeine (Burt, 2003). Finally, the effect of niflumic acid in high K^+ Krebs could not be due to opening of K^+ channels.

In normal Krebs, concentrations of phenylephrine below 3×10^{-6} M, or cyclopiazonic acid, only increased the magnitude and duration of the spontaneous contractions (Burt, 2004). Cumulative additions of phenylephrine in normal Krebs only produced tonic contractions at higher concentrations, which were initiated by the spontaneous contractions. In high K^+ Krebs however, phenylephrine always produced a dose related tonic response. Cyclopiazonic acid also produced a tonic contraction. The reason why low levels of Ca^{2+} store depletion do not stimulate a tonic contraction in normal Krebs may therefore be due to stimulation of repolarizing K^+ channels. This would close voltage-gated Ca^{2+} channels and is consistent with increased Ca^{2+} entry through these channels during the spontaneous contractions eventually producing a maintained depolarization via stimulation of Ca^{2+} activated Cl^- channels. As discussed in Section 4.2, store-operated cation entry could result in increased Ca^{2+} influx through voltage-gated channels. In high K^+ Krebs, Ca^{2+} influx through voltage-gated channels is not inhibited by stimulation of repolarizing K^+ channels and so Ca^{2+} store depletion always produces a tonic contraction.

5. Conclusion

Depletion of Ca^{2+} from intracellular stores of the rat portal vein stimulated a tonic contraction. The contraction was not directly dependent on Ca^{2+} influx through store-operated cation channels but required the opening of Ca^{2+} activated Cl^- channels and Ca^{2+} influx through voltage-gated channels. Greater Ca^{2+} store depletion is required to stimulate the tonic contraction than is needed to potentiate the spontaneous contractions of this tissue. This is due to the repolarizing effect of K^+ channels and suggests it is increased Ca^{2+} influx through voltage-gated channels which stimulates Ca^{2+} activated Cl^- channels, producing a maintained depolarization and contraction. It is possible that the depolarizing effect of store-operated cation entry results in the increased Ca^{2+} influx through voltage-gated channels. The mechanism by which Ca^{2+} store depletion stimulated the tonic contraction may be attenuated by extracellular Ca^{2+} influx or refilling of the Ca^{2+} stores.

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