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European Journal of Pharmacology 313 (1996) 151–158

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Effects of external K^+ on depletion-induced Ca^{2+} entry in rat ileal smooth muscle

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Received 25 April 1996; revised 17 June 1996; accepted 2 July 1996

Abstract

The effects of K^+ on Ca^{2+} influx after transient depletion of Ca^{2+} stores with carbachol and long-lasting depletion with thapsigargin or ryanodine were examined in fura-2-loaded rat ileal smooth muscle. After transient depletion of Ca^{2+} stores, application of Ca^{2+} caused a rise in $[Ca^{2+}]_i$ and a contraction, both of which were increased with increasing K^+ applied simultaneously in the absence of methoxyverapamil, but were decreased in its presence. In tissues, long-lasting depletion of Ca^{2+} stores treated with thapsigarin or ryanodine, $[Ca^{2+}]_i$ and tension were dose dependently increased by the application of Ca^{2+} regardless of the absence or presence of methoxyverpamil. These responses were inhibited by K^+ replacement of Na^+ in a dose-dependent manner and the inhibitory action of K^+ was attenuated by increasing extracellular Ca^{2+}. The influx of Mn^{2+} was much greater in the tissues pretreated with thapsigargin or ryanodine than in untreated tissues. The enhanced Mn^{2+} influx was inhibited by the replacement of Na^+ with K^+. These results provide further evidence for the presence of a Ca^{2+} entry mechanism evoked by the depletion of Ca^{2+} stores in rat ileal smooth muscle, and suggest that there are two types of Ca^{2+} entry pathways to refill Ca^{2+} stores, one sensitive and the other insensitive to Ca^{2+} channel blockers. Ca^{2+} entry through the latter pathway is inhibited by increasing external K^+, perhaps due to a reduction of the electrochemical gradient for Ca^{2+} across the plasma membrane.

Keywords: Ca^{2+} store; Mn^{2+} influx; Ileum, rat; Ryanodine; Thapsigargin

1. Introduction

Ca^{2+} stores play an important role in regulating intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in various tissues. In smooth muscle cells, stored Ca^{2+} is released by inositol 1,4,5-trisphosphate (Somlyo et al., 1985; Hashimoto et al., 1986) and by Ca^{2+} itself (Iino, 1989). Increased Ca^{2+} is taken up into the stores through the activation of Ca^{2+}-pump ATPase (Van Breemen and Saida, 1989). Recently, Ca^{2+} stores have been shown to play a role in regulation of Ca^{2+} influx in some tissues. Putney (Putney, 1986, 1990) has proposed the 'capacitative Ca^{2+} entry hypothesis' in nonexcitable cells, postulating that a decrease in the Ca^{2+} content in Ca^{2+} stores triggers the influx of extracellular Ca^{2+}. A similar Ca^{2+} entry mechanism has been demonstrated in vascular (Gonzalez De La Fuente et al., 1995; Missiaen et al., 1990; Xuan et al., 1992; Noguera and D'Ocon, 1993; Pacaud et al., 1993) and urinary smooth muscles (Munro and Wendt, 1994).

In the visceral smooth muscle of the rat, we have found that depletion of Ca^{2+} stores induced by cyclopiazonic acid, an inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum, results in increases in Ca^{2+} influx insensitive to Ca^{2+} channel blockers and that the rise in $[Ca^{2+}]_i$ is decreased by increasing the extracellular concentration of K^+ (Ohta et al., 1995). The effect of K^+ is apparently incompatible with the increase in extracellular K^+ that causes depolarization, producing rises in $[Ca^{2+}]_i$ through voltage-dependent Ca^{2+} channels in smooth muscle cells (Bolton, 1979). Therefore, it is of interest to determine whether the effect of K^+ on Ca^{2+} influx is different when Ca^{2+} stores are depleted by Ca^{2+}-ATPase inhibition and by a commonly used agonist such as carbachol.

It has been reported that effects of cyclopiazonic acid are easily eliminated after its washout (Ohta et al., 1995), but that thapsigargin, another inhibitor of Ca^{2+}-ATPase in the sarcoplasmic reticulum (Jackson et al., 1988; Thastrup et al., 1990) and ryanodine, which open-locks Ca^{2+}-induced Ca^{2+} release channels (Fleischer et al., 1985; Iino et al., 1988) produce long-lasting effects. The aim of the present experiments was to obtain further evidence of

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PII S0014-2999(96)00517-1

capacitative Ca^{2+} entry with the help of thapsigargin and ryanodine in rat ileal smooth muscle and to compare the effects of extracellular K^+ on Ca^{2+} influx after transient depletion induced by carbachol and after long-lasting depletion of Ca^{2+} stores by thapsigarigin or ryanodine. For these purposes, we measured isometric tension and $[Ca^{2+}]_i$ simultaneously in muscle bundles loaded with fura-2, and used the Mn^{2+} quenching method for fura-2 fluorescence as an indicator of Ca^{2+} influx into intestinal smooth muscle cells of the rat.

2. Materials and methods

Male Wistar rats (200–300 g) were stunned and bled to death. The ileum was isolated and luminal contents were removed by washing with physiological salt solution (PSS). The longitudinal smooth muscle layer was peeled from the circular muscle layer and dissected into small muscle strips (1 mm in width, 5 mm in length). They were then incubated with 20 mM fura-2 acetoxymethyl ester (fura-2/AM) and 0.02–0.04% cremphore EL for more than 3 h at room temperature for measurement of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The fura-2 method used in this experiment was similar to that described in a previous paper (Ohta et al., 1995). In brief, using a fluorimeter (CAF-110, Japan Spectroscopic), the muscle bundles were alternatively illuminated by 340 nm and 380 nm light at a frequency of 128 Hz and the fluorescent intensity at 500 nm was measured at room temperature (22–25°C). The calculated ratio of the fluorescence due to excitation at 340 nm to that at 380 nm (F340/F380) was considered to be an index of $[Ca^{2+}]_i$. Mn^{2+} influx was measured by monitoring the 500 nm wavelength fluorescent signals excited at 360 nm, isosbestic points of fura-2, before and during the addition of $MnCl_2$ (0.1 mM) to the bathing solution.

Normal PSS was of the following composition (mM): NaCl 144, KCl 5.8, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.1, Hepes 5 (pH 7.4 with NaOH). In the Ca^{2+}-free solution, $CaCl_2$ was omitted and 2 mM EGTA was added. When concentrations of KCl, LiCl or TrisCl were increased, corresponding amounts of NaCl were removed.

The following chemicals were used: caffeine, methoxyverapamil and nifedipine (Wako Pure Chem., Japan), carbachol, cremophore EL and thapsigargin (Sigma, USA), EGTA, fura-2/AM and Hepes (Dojindo, Japan) and ryanodine (Agrisystem Int., USA).

Results of the experiments were expressed as the means ± S.E.M. Student's *t*-test was used for statistical analysis of the results and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Changes in $[Ca^{2+}]_i$ and contraction induced by application of Ca^{2+} and K^+

All these experiments were carried out after a depletion of Ca^{2+} stores by stimulation with carbachol (0.1 mM) in the absence of extracellular Ca^{2+}. To examine the effects of external K^+ on contraction and changes in $[Ca^{2+}]_i$ induced by Ca^{2+}, we applied 2.5 mM Ca^{2+} together with various concentrations of K^+ for 3 min to muscle bundles

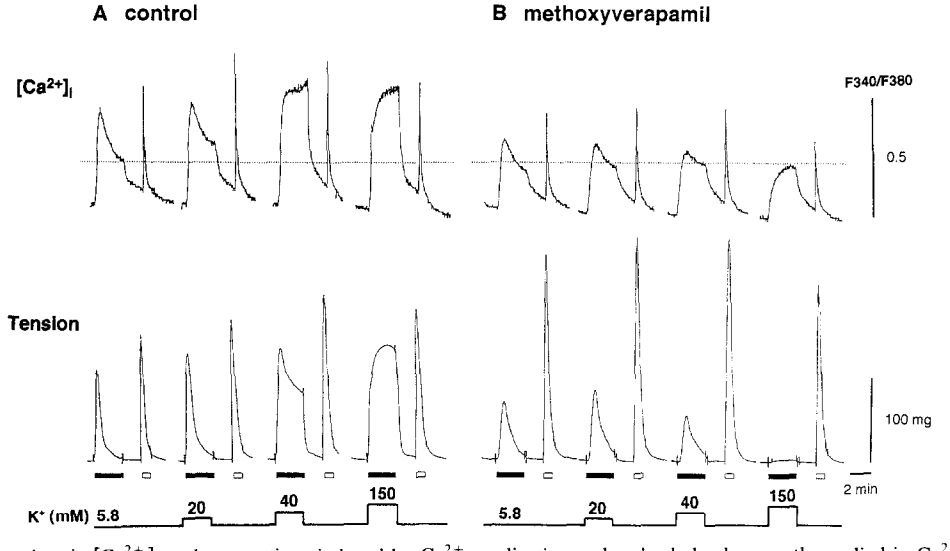

Fig. 1. Effects of K^+ on rises in $[Ca^{2+}]_i$ and contractions induced by Ca^{2+} application and carbachol subsequently applied in Ca^{2+}-free solution in the absence (A) and presence (B) of methoxyverapamil (10 μM). Upper traces represent the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the lower ones, tension. After depletion of Ca^{2+} stores by stimulation with carbachol in Ca^{2+}-free solution containing 2 mM EGTA, the tissue was exposed to 2.5 mM Ca^{2+} (filled bar) together with various concentrations of K^+ for 3 min (shown in the lowest panel). The tissue was then washed out for 2 min with Ca^{2+}-free solution containing 2 mM EGTA and was stimulated by carbachol for 1 min (open bar). The ratio of fluorescent signals excited by 340 and 380 nm (F340/F380) indicates $[Ca^{2+}]_i$. Dotted line indicates the resting $[Ca^{2+}]_i$ level in normal physiological salt solution containing 2.5 mM Ca^{2+}. The results (A) and (B) were obtained from different preparations. In (B), carbachol responses in 5.8 mM K^+ before treatment with methoxyverapamil were much greater than those in (A) (data not shown).

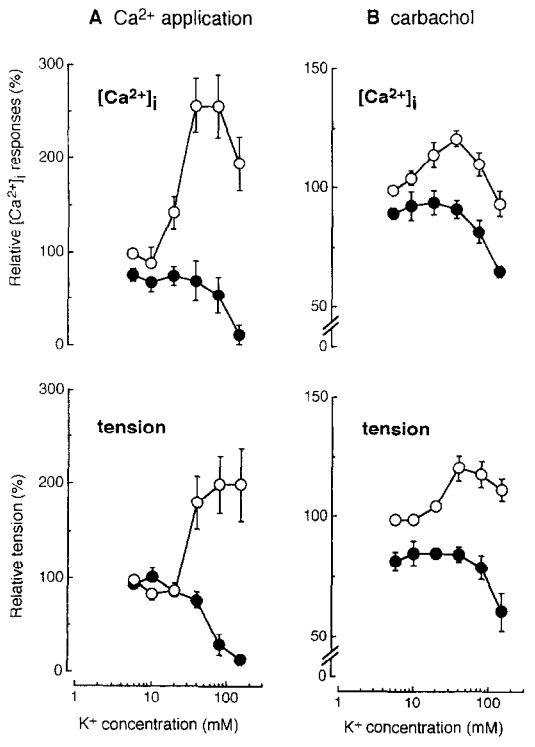

Fig. 2. Relationships between K^+ concentration and $[Ca^{2+}]_i$ or contraction in response to Ca^{2+} application (A) and carbachol (B) in the presence (●) or absence (○) of methoxyverapamil (10 μM). In experiments similar to those shown in Fig. 1, the area of the $[Ca^{2+}]_i$ response above the resting level in normal PSS, that of the contractile response induced by Ca^{2+} application and the amplitudes of carbachol-induced responses were plotted against concentrations of K^+ as a percentage of those obtained in the presence of 5.8 mM K^+ before treatment with methoxyverapamil. Symbols show the mean values (and, if vertical lines are present, S.E.M.) obtained from 5 (filled) and 4 (open) experiments.

pretreated with Ca^{2+}-free solution containing 2 mM EGTA. Then the preparation was washed with the Ca^{2+}-free solution for 2 min and carbachol (0.1 mM) was subsequently applied for 1 min under the Ca^{2+}-free conditions to measure the amount of Ca^{2+} refilled into the Ca^{2+} stores. Actual chart records of contractions and changes in $[Ca^{2+}]_i$ induced by application of Ca^{2+} (2.5 mM) in the presence of various concentrations of K^+ and subsequently applied carbachol are shown in Fig. 1A. When applied with a normal K^+ concentration (5.8 mM), Ca^{2+} caused a transient contraction and rise in $[Ca^{2+}]_i$ which declined and returned nearly to the original level in normal PSS within 3 min. Application of Ca^{2+} together with 20 mM K^+ caused a slight increase in peak amplitude and a subsequent sustained level without changes in the shapes of responses. However, increases in the K^+ concentration to 40 mM or more caused sustained increases in $[Ca^{2+}]_i$ and contractions in response to Ca^{2+} application.

The relationships between the K^+ concentration and the increase in $[Ca^{2+}]_i$ and contractions in response to Ca^{2+} application, and those to carbachol are shown in Fig. 2A,B, respectively. Both $[Ca^{2+}]_i$ and contractile responses to Ca^{2+} and carbachol were maximal at around 40 mM K^+. Over 40 mM K^+, the amplitude of the responses to Ca^{2+} was sustained in size, but the responses to carbachol tended to be decreased. Both $[Ca^{2+}]_i$ and contractile responses to carbachol at 150 mM K^+ were significantly smaller in amplitude than those at 40 mM K^+.

3.2. The effects of methoxyverapamil on changes in $[Ca^{2+}]_i$ and contraction in response to Ca^{2+} and carbachol

The application of Ca^{2+} together with excess K^+ is expected to increase Ca^{2+} influx through voltage-depen-

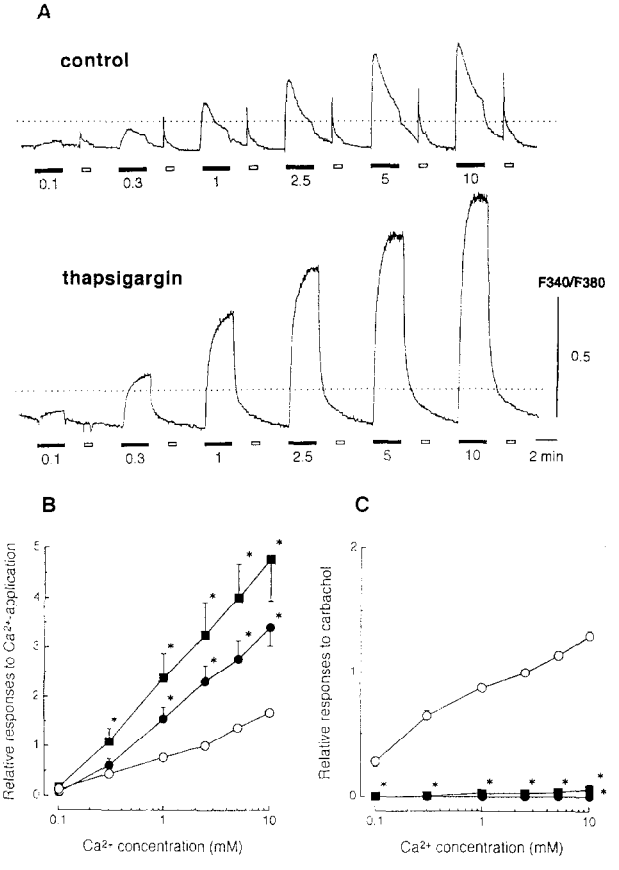

Fig. 3. Effects of thapsigargin and ryanodine on the rise in $[Ca^{2+}]_i$ in response to Ca^{2+} application and to carbachol in Ca^{2+}-free solution. Actual chart records are shown (A). Under normal K^+ concentration (5.8 mM), tissues were exposed to various concentrations of Ca^{2+} for 3 min (filled bar) and then carbachol (0.1 mM) was applied in Ca^{2+}-free solution containing 2 mM EGTA for 1 min (open bar) without (upper trace) and with (lower trace) treatment with thapsigargin (10 μM) for 30 min in the absence of Ca^{2+}. Methoxyverapamil (10 mM) was present throughout the experiment. The ratio of fluorescent signals excited by 340 and 380 nm (F340/F380) indicates $[Ca^{2+}]_i$. The dotted line indicates the resting $[Ca^{2+}]_i$ level in normal PSS containing 2.5 mM Ca^{2+}. Experiments were carried out according to a protocol similar to that shown in Fig. 1 except that the concentration of Ca^{2+} applied was varied. Summarized data were obtained from the control tissues (○, $n = 5$), the tissues treated with thapsigargin (10 μM, ●, $n = 4$), and ryanodine (0.1 mM) plus caffeine (30 mM) (■, $n = 4$) for 30 min. The area of $[Ca^{2+}]_i$ responses to Ca^{2+} above the level of the Ca^{2+}-free condition (B) and the amplitudes of carbachol-induced rises in $[Ca^{2+}]_i$ (C) are plotted against the Ca^{2+} concentration applied as a ratio of those obtained in the presence of 2.5 mM Ca^{2+}. Data show the mean values (and, if vertical lines are present, S.E.M.). * Significantly different from the control value at each concentration of Ca^{2+} at $P < 0.05$.

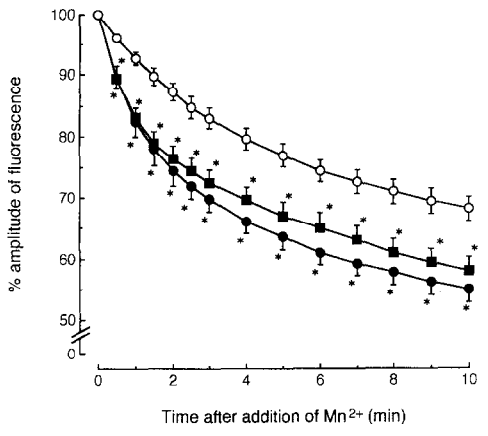

Fig. 4. Effects of thapsigargin and ryanodine on Mn^{2+} influx. The quenching of fura-2 fluorescence excited at 360 nm (isosbetic point for fura-2) by Mn^{2+} influx was monitored after the addition of Mn^{2+} (0.1 mM) to the nominal Ca^{2+}-free solution. Symbols show the mean values (and, if vertical lines are present, S.E.M.) obtained from the tissues which were not treated with drugs (○, $n = 7$), pretreated with thapsigargin (10 μM, ●, $n = 7$), or with ryanodine (0.1 mM) plus caffeine (30 mM) (■, $n = 7$). Methoxyverapamil (10 μM) was added to all solutions. Fura-2 fluorescence after Mn^{2+} application is expressed as a percentage of that before Mn^{2+} application. * Significantly different from the control value at $P < 0.01$.

dent Ca^{2+} channels. Therefore, the relationships between the K^+ concentration and the responses to Ca^{2+} or carbachol were re-examined in the presence of methoxyverapamil to inhibit these Ca^{2+} channels. Actual chart records are shown in Fig. 1B, which was obtained from a different preparation from that in Fig. 1A, and data are summarized in Fig. 2. With excess K^+ lower than 40 mM, the application of Ca^{2+} caused rises in $[Ca^{2+}]_i$ and contraction, the shapes of which were similar to those obtained in the absence of methoxyverapamil. With the concentrations over 40 mM K^+, however, both responses decreased with the increase in the concentration of K^+ in the presence of methoxyverapamil. In particular, when Ca^{2+} was applied together with 150 mM K^+, the decrease in the responses was marked, and no contraction was evoked by Ca^{2+} application in some preparations (Fig. 1B). It should be noted that the $[Ca^{2+}]_i$ level remained much lower than the level before the removal of external Ca^{2+} and carbachol could elicit a rise in $[Ca^{2+}]_i$ and contraction under these conditions. Regardless of the presence or absence of methoxyverapamil, the amplitude of responses to carbachol was reduced after the application of Ca^{2+} together with higher concentrations of K^+. Ca^{2+} stores seemed to

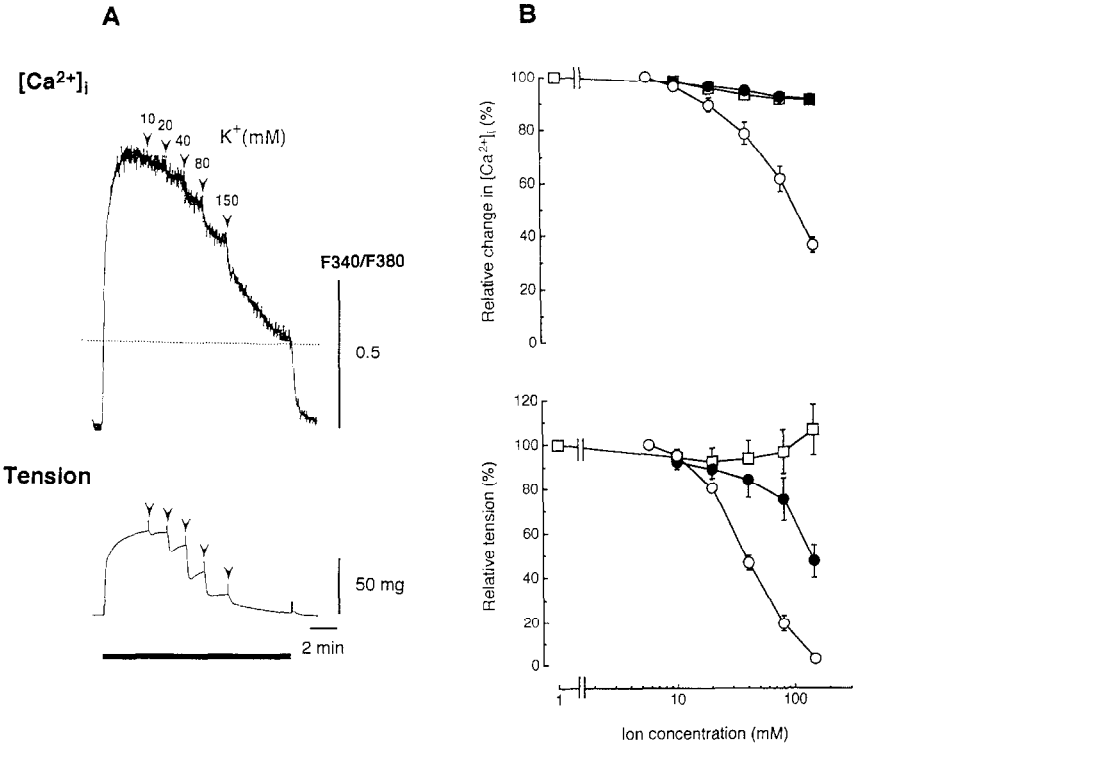

Fig. 5. Effects of increasing K^+ on the contraction and rises in $[Ca^{2+}]_i$ in response to application of Ca^{2+} to the tissues pretreated with thapsigargin (10 μM) in Ca^{2+}-free solution. Methoxyverapamil (10 μM) was present throughout the experiment. In (A), after the response to Ca^{2+} application (2.5 mM) attained a maximum, external K^+ was progressively increased from 5.8 mM to 150 mM by replacing Na^+ isosmotically. The ratio of fluorescent signals excited by 340 and 380 nm (F340/F380) indicates $[Ca^{2+}]_i$. The dotted line indicates the resting $[Ca^{2+}]_i$ level in the presence of 2.5 mM before treatment with thapsigargin. In (B), concentration-response relationships for K^+ (○, $n = 4$), Li^+ (●, $n = 4$) and $Tris^+$ (□, $n = 4$), which replaced Na^+ are shown as a percentage of contractile and $[Ca^{2+}]_i$ responses to 2.5 mM Ca^{2+} in the presence of 144 mM Na^+.

be refilled with Ca^{2+} via Ca^{2+} entry pathways insensitive to Ca^{2+} channel blockers.

3.3. Effects of thapsigargin and ryanodine on changes in $[Ca^{2+}]_i$ and contraction in response to Ca^{2+} and carbachol

The effects of thapsigargin and ryanodine were examined to see the effects of long-lasting depletion of Ca^{2+} in the stores. Experiments were carried out in the presence of methoxyverapamil (10 mM) or nifedipine (1 μM). The muscle bundles were stimulated with various concentrations (0.1–10 mM) of Ca^{2+} in the presence of 5.8 mM K^+ and then challenged by carbachol (0.1 mM) under Ca^{2+}-free conditions with the same protocol as used in Fig. 1. As shown in Fig. 3A, in the control muscle bundles, application of Ca^{2+} caused a dose-dependent increase in $[Ca^{2+}]_i$ and subsequent carbachol-induced responses. On the other hand, after the muscle bundles were treated with thapsigargin (10 μM) for 30 min in the absence of extracellular Ca^{2+}, the rise in $[Ca^{2+}]_i$ in response to application of Ca^{2+} markedly increased and maintained its level during Ca^{2+} application, but the subsequent carbachol application failed to produce any responses. Similar results were obtained with tissues treated with ryanodine (0.1 mM) and caffeine (30 mM) in the Ca^{2+}-free solution. The failure of carbachol to release Ca^{2+} lasted for more than 2 h even after the removal of thapsigargin and ryanodine. In the absence of methoxyverapamil, thapsigargin produced $[Ca^{2+}]_i$ responses to Ca^{2+} application similar to those in its presence (data not shown).

Fig. 3B,C show the relationships between the Ca^{2+} concentration and rises in $[Ca^{2+}]_i$ induced by Ca^{2+} application and by carbachol in control and thapsigargin- and ryanodine-treated tissues. Pretreatment with thapsigargin or ryanodine shifted the Ca^{2+} dose-response curve upward. Thapsigargin and ryanodine increased the rise in $[Ca^{2+}]_i$ by about 2 to 2.5 times in most Ca^{2+} concentrations tested. Carbachol also caused rises in $[Ca^{2+}]_i$ dependent on the concentrations of Ca^{2+} in the control muscle bundles. However, in the muscle bundle pretreated with ryanodine or thapsigargin, the response to carbachol disappeared even when higher concentrations of Ca^{2+} were applied. Similar relationships were obtained for the contractile response. These results indicated that thapsigargin and ryanodine give rise to the depletion of Ca^{2+} stores.

3.4. Effects of thapsigargin and ryanodine on Mn^{2+} influx

Mn^{2+} is known to be able to pass through most Ca^{2+} permeable channels (Sage et al., 1989; Missiaen et al., 1990) and to eliminate fura-2 fluorescence (Hallam et al., 1988). We investigated the effect of thapsigargin and that of ryanodine on Mn^{2+} influx by observing the quenching of fura-2 fluorescence in the presence of methoxyverapamil (10 mM). Tissues treated with or without thapsigargin (10 μM) or ryanodine (0.1 mM) plus caffeine (30 mM) for 30 min were exposed to nominal Ca^{2+}-free solution and then the external solution was replaced with the solution containing Mn^{2+} (0.1 mM) in the presence of methoxyverapamil. The time courses of the quenching of fura-2 fluorescence induced by Mn^{2+} under various conditions are shown in Fig. 4. Application of Mn^{2+} caused a gradual decrease in the fluorescent intensity in control tissues with intact Ca^{2+} stores. However, in tissues in which Ca^{2+} stores were depleted by thapsigargin or ryanodine, Mn^{2+} significantly increased the rate of fura-2 quenching. These results suggested that Ca^{2+} permeability of the plasma membrane was increased by these drugs.

3.5. Effects of K^+ on the contractile and $[Ca^{2+}]_i$ responses to Ca^{2+} and Mn^{2+} influx after long-lasting depletion of Ca^{2+} stores

After the contractile and $[Ca^{2+}]_i$ responses had been evoked by Ca^{2+} (2.5 mM) in tissues pretreated with

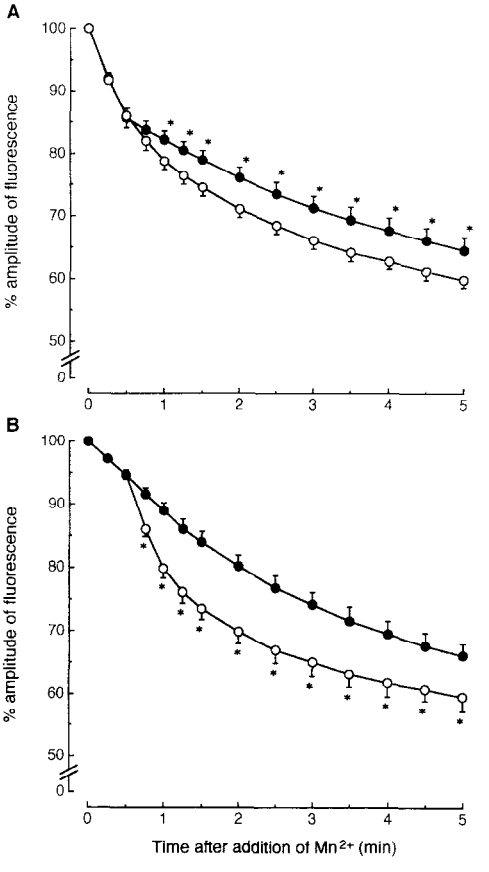

Fig. 6. Effect of K^+ on Mn^{2+} influx after treatment with thapsigargin (10 μM). Mn^{2+} (0.1 mM) was applied to nominal Ca^{2+}-free solution in the presence of 5.8 mM K^+ (A, ○, $n = 7$) and 150 mM K^+ (B, ●, $n = 5$), the concentration of which was respectively changed to 150 mM (A, ●, $n = 7$) and 5.8 mM (B, ○, $n = 4$) 30 s after the addition of Mn^{2+}. Symbols show the mean values (and, if vertical lines are present, S.E.M.). * Significantly different from the control value at $P < 0.05$. Methoxyverapamil (10 μM) was present throughout the experiment.

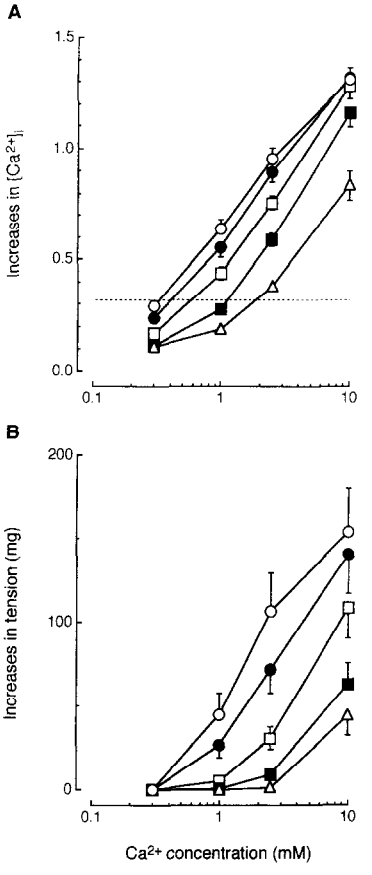

Fig. 7. Effects of the replacement of Na^+ with K^+ on the contractile and $[Ca^{2+}]_i$ responses to Ca^{2+} at various concentrations in tissues pretreated with thapsigargin (10 μM). Experiments were carried out according to a protocol similar to that shown in Fig. 5A except that the concentration of Ca^{2+} applied was varied. Methoxyverapamil (10 μM) was present throughout the experiment. The increase in $[Ca^{2+}]_i$ from the level of the Ca^{2+}-free condition (A) and contraction (B) in response to Ca^{2+} application are plotted against the log concentration of Ca^{2+} in the presence of the various concentrations of K^+ applied: 5.8 mM (○); 20 mM (●); 40 mM (□); 80 mM (■); 150 mM (△). Symbols show the mean values (and, if vertical lines are present, S.E.M.) from 7 experiments. The dotted line indicates the resting level of $[Ca^{2+}]_i$ in 2.5 mM Ca^{2+} in normal tissues.

thapsigargin (10 μM) for 30 min, the concentration of KCl was gradually increased by replacing it with isosmotical NaCl in the presence of 10 μM methoxyverapamil (Fig. 5). K^+ caused a concentration-dependent decrease in the tension and $[Ca^{2+}]_i$ level increased by Ca^{2+} application. The replacement of NaCl with TrisCl had no effect on the developed tension and slightly potentiated the $[Ca^{2+}]_i$ response. In contrast, the substitution of LiCl for NaCl decreased the tension without affecting the $[Ca^{2+}]_i$ level. Next, it was determined whether K^+ affected Ca^{2+} permeability after depletion of Ca^{2+} stores using Mn^{2+} quenching. In thapsigargin-treated tissues, the concentration of KCl was switched to 150 and 5.8 mM at 30 s after the application of Mn^{2+} in the presence of 5.8 mM and 150 mM KCl, respectively. Application of Mn^{2+} caused a rapid decrease in fura-2 fluorescence in the presence of 5.8 mM KCl and the rate of this decrease was significantly slowed down by increasing the concentration of KCl to 150 mM during its descending course (Fig. 6A). When Mn^{2+} was applied in the presence of 150 mM KCl, which was switched to 5.8 mM KCl, the reverse relation was true (Fig. 6B). These results suggested that Ca^{2+} permeability after the depletion of Ca^{2+} stores was decreased with increasing concentrations of KCl.

3.6. Effect of K^+ on the contractile and $[Ca^{2+}]_i$ responses induced by various concentrations of Ca^{2+}

The effect of K^+ on the responses to various concentrations of Ca^{2+} after depletion of Ca^{2+} stores was examined in a way similar to those shown in Fig. 5. The contractile and $[Ca^{2+}]_i$ responses were evoked by the application of various concentrations of Ca^{2+} in the presence of 5.8 mM KCl, which was gradually increased from 10 to 150 mM by replacing NaCl isosmotically. The results are summarized in Fig. 7, in which the increase in the ratio of F340 to F380 is plotted against the concentration of Ca^{2+} applied. Application of Ca^{2+} caused a dose-dependent increase in $[Ca^{2+}]_i$ in the presence of 5.8 mM KCl. The dose-response curve for Ca^{2+} was shifted to the right by increases in the K^+ concentration. The lower the concentration of Ca^{2+}, the greater the inhibition by excess K^+, suggesting that the Ca^{2+} gradient across the plasma membrane was associated with inhibition of the rise in $[Ca^{2+}]_i$ by excess K^+.

4. Discussion

The present results indicated that there were two types of Ca^{2+} entry pathways to refill intracellular stores: one sensitive and the other insensitive to methoxyverapamil in rat intestinal smooth muscles. Carbachol seemed to cause a transient depletion of Ca^{2+} stores and activate both pathways. On the other hand, either thapsigargin or ryanodine caused long-lasting or irreversible depletion of Ca^{2+} stores and resulted in a great increase in Ca^{2+} influx mainly through the methoxyverapamil-insensitive pathway.

The following observations suggested the presence of two different Ca^{2+} entry pathways. After ileal smooth muscles were stimulated by carbachol in the absence of Ca^{2+} to deplete Ca^{2+} in the stores, application of Ca^{2+} together with various concentrations of K^+ caused a dose-dependent increase in $[Ca^{2+}]_i$ and contraction, both of which were inhibited by methoxyverapamil. In the presence of methoxyverapamil, however, application of Ca^{2+} together with K^+ still caused increases in $[Ca^{2+}]_i$ and contraction and, interestingly, these responses decreased with increases in the concentration of K^+. An inhibitory effect of K^+ on Ca^{2+}-induced responses and Mn^{2+} influx was demonstrated in tissues treated with thapsigargin for long-lasting depletion of Ca^{2+} stores.

The amount of Ca^{2+} stored in the sarcoplasmic reticu-

lum, which was estimated as an increase in $[Ca^{2+}]_i$ and contraction in response to carbachol after the removal of extracellular Ca^{2+}, increased with increasing K^+ until it attained a maximum at 40 mM and decreased with further increases in K^+. Although the content of stored Ca^{2+} was decreased in the presence of methoxyverapamil, carbachol still released Ca^{2+} in amounts sufficient to produce Ca^{2+} transients and contraction. These results also support the view that two different pathways, sensitive and insensitive to Ca^{2+} channel blockers, play a role in refilling the Ca^{2+} stores. The increase in stored Ca^{2+} induced by excess K^+ may have been due to an increase in uptake of Ca^{2+} passing through voltage-dependent Ca^{2+} channels as reported in dog tracheal smooth muscle (Bourreau, 1993). The carbachol-induced responses were reduced by pretreatment with higher concentrations of K^+ even in the presence of methoxyverapamil, indicating that the amount of Ca^{2+} in the stores decreased regardless of the presence or absence of methoxyverapamil at higher concentrations of K^+. On the other hand, excess K^+ failed to decrease the amount of Ca^{2+} in the stores associated with voltage-dependent Ca^{2+} channels, which was estimated by subtracting the rise in $[Ca^{2+}]_i$ caused by carbachol in the presence of methoxyverapamil from that in its absence. Excess K^+ has been shown to release Ca^{2+} from the stores in rat aortic smooth muscles (Kobayashi et al., 1986). Considering these facts together, it seems likely that the decrease in stored Ca^{2+} in the presence of excess K^+ was due to decreases in Ca^{2+} taken up in the stores or release of Ca^{2+} from the stores.

Long-lasting depletion of Ca^{2+} stores by treatment with thapsigargin or ryanodine greatly potentiated the increase in $[Ca^{2+}]_i$ in response to Ca^{2+} application and completely abolished responses to subsequently applied carbachol. The increase in $[Ca^{2+}]_i$ after the depletion of Ca^{2+} stores has been proposed to result from a decrease in the Ca^{2+}-buffering action of the store (Sturek et al., 1992; Van Breemen et al., 1995) or an increase in Ca^{2+} influx (Hoth and Penner, 1992; Vaca and Kunze, 1994; Ohta et al., 1995). The Ca^{2+} influx pathway after treatment with thapsigargin has been reported to be associated with voltage-dependent Ca^{2+} channels in rat aortic A10 smooth muscle cells (Xuan et al., 1992) and other unknown channels in pulmonary arterial smooth muscle (Gonzalez De La Fuente et al., 1995). In the present experiment, thapsigargin and ryanodine increased Mn^{2+} influx even in the presence of methoxyverapamil, supporting the view that the increase in Ca^{2+} influx after depletion of Ca^{2+} is due to the activation of pathways insensitive to Ca^{2+} channel blockers as reported previously (Ohta et al., 1995).

It has been reported that Ca^{2+} influx is decreased in the presence of high concentrations of K^+ after depletion of Ca^{2+} stores with thapsigargin in rat parotid acinar cells (Zhang and Melvin, 1993) and in rat pulmonary artery (Gonzalez De La Fuente et al., 1995), and is greatly affected by the concentration gradient for Ca^{2+} across the plasma membrane (Mohr and Fewtrell, 1987). This was the case in our experiment, that is, increases in $[Ca^{2+}]_i$ and contraction in response to the application of Ca^{2+} were decreased by the increasing K^+ concentration after depletion of Ca^{2+} stores. In addition, we found that Mn^{2+} influx was significantly reduced by increasing the concentration of K^+ and accelerated by decreasing it, and that the lower the concentration of Ca^{2+}, the greater the inhibitory effect of excess K^+ on Ca^{2+} influx. These phenomena can probably be explained by the decrease in the driving force for Ca^{2+}, because depolarization by increasing extracellular K^- decreases the electrochemical gradient for Ca^{2+} across the plasma membrane (Pacaud and Bolton, 1991; Pacaud et al., 1993). In addition to the decrease in the driving force for Ca^{2+}, it is also possible that a decrease in second messenger substances responsible for Ca^{2+} influx is involved in the inhibitory effect of excess K^+ on Ca^{2+} influx, because depolarization-induced Ca^{2+} influx has been shown to decrease production of the second messenger substances in HL-60 cells (Pittet et al., 1990). In any case, further studies are certainly required to evaluate the effect of K^+ on Ca^{2+} influx after depletion of Ca^{2+} stores.

Acknowledgements

This work was supported by a Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan and the Naito Foundation (Japan).

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