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Short communication

Ca^{2+} release-activated channels in rat stomach smooth muscle cells

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Abstract

In rat stomach fundus, contractions induced by Ca^{2+} (1.8 mM) were strikingly potentiated by thapsigargin. This potentiation was partially inhibited by the blockers of Ca^{2+} release activated channels (CRACs), miconazole and SK&F96365 ({1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCL}) and slightly blocked by the antagonist of calcium voltage-operated channels (VOCs), isradipine. In dissociated cells in a 0Ca solution, thapsigargin potentiated the increase in intracellular calcium after reintroduction of Ca^{2+}. This potentiation was partially reduced by the CRAC blockers, but not by the VOC blockers. This data suggests that calcium influx increased due to the depletion of intracellular calcium by thapsigargin and that this influx occurs predominantly through CRACs. © 1998 Elsevier Science B.V.

Keywords: Stomach fundus, rat; Calcium store; Isradipine; Thapsigargin; SK&F96365; Miconazole

1. Introduction

Contractile responses to a number of agonists in several tissues, especially in smooth muscle, result from an elevation of the intracellular Ca^{2+} concentration. The source for this ion can be various intracellular Ca^{2+} storage compartments (Clapham, 1995), or the extracellular medium, by entrance of Ca^{2+} through different types of channels such as receptor-operated Ca^{2+} channels (ROCs), or voltage-operated Ca^{2+} channels (VOCs), which can be blocked, for instance, by nifedipine and isradipine (Smaili et al., 1991).

Recently, a phenomenon called capacitative Ca^{2+} entry, which is regulated by the concentration of Ca^{2+} in intracellular stores, was observed in some cells (Putney, 1990). Through this mechanism the withdrawal of Ca^{2+} activates a Ca^{2+} release activated channel (CRAC), leading to the entrance of extracellular Ca^{2+}. The entrance of Ca^{2+} through this channel can be triggered by a variety of pharmacological procedures or agents, such as caffeine and thapsigargin, that have in common the ability to release stored Ca^{2+} (Berridge, 1995). In addition, some drugs, such as miconazole (Clementi et al., 1992) and SK&F96365, can block CRACs.

This entry of Ca^{2+} through CRACs has been observed in several tissues (Putney, 1990; Hoth and Penner, 1992; Vaca and Kunze, 1994), but it has only recently been investigated in smooth muscle (Amrani et al., 1995; Ohta et al., 1995). Since there is no information about gastric smooth muscle, the aim of this study was to investigate whether CRACs are involved in Ca^{2+} influx, after emptying of the intracellular stores, in this preparation. With this purpose, the contractions of isolated stomach strips and calcium translocation in cell suspensions were measured.

2. Materials and methods

2.1. Contractile responses

Wistar rats (280–330 g) were killed with an ether overdose and the stomach fundus was removed (Smaili et al., 1991). Strips of longitudinal smooth muscle were prepared and mounted in 10-ml organ baths containing a Tyrode normal solution with 1.8 mM Ca^{2+} (NS solution)

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or with Ca^{2+}-free Tyrode (0Ca solution), for isotonic contraction experiments.

2.2. Measurements of intracellular Ca^{2+} in cell suspension

After removal of the stomach fundus, the longitudinal and circular muscle layers were freed of the mucosa, washed in a 0Ca solution and cut into fragments that were dissociated in a solution containing collagenase 0.5 mg/ml, pronase 0.3 mg/ml and bovine serum albumin 1% for 45 min at 37°C. Dissociated cells were prepared by gently passing the digested tissue through a fire-polished Pasteur pipette. These cells were then filtered through a nylon mesh, washed repeatedly with NS solution, suspended in NS containing bovine serum albumin (0.2 g%) for 1 h and centrifuged for 4 min (2000 rpm). The viability of the cells measured by Trypan blue exclusion test before loading with the fluorophore was higher than 90%. Aliquots of cell suspension in NS (10^6 cells/ml) were placed in quartz cuvettes and incubated with 4 μM fura-2 acetoxymethyl ester (fura-2/AM, or {1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′*N*′-tetraacetic acid pentaacetoxymethyl ester}) in the presence of 0.01% pluronic F-127 for 180 min at room temperature. After being loaded with fura-2, the cells were rinsed with NS solution, centrifuged and suspended in 2.5 ml of 0Ca solution for the experiments that were carried out in a fluorimeter (SPEX AR CM System). Cells were alternately illuminated at 340 and 380 nm and the intensity of fluorescence emission was measured at 505 nm. The ratio of fluorescence (R 340/380) was calculated from these values. The changes in fura-2/AM to fura-2 in the excitation spectra were monitored throughout the incubation time and showed a progressive shift of fluorescence to shorter wavelengths, indicating the entrance of fura-2/AM into intact and functional cells. The basal ratio was constant during the control period of 100 s. The auto-fluorescence was less than 10% and for this reason it was not necessary to correct the fluorescence values read at 340 and 380 nm. Previous stimulation with maximal doses of carbachol (300 μM) and substance P (50 μM) increased significantly the ratio 340/380, showing that the suspended cells were functional (Smaili et al., 1997). At the end of each experiment, the maximum ratio (R_{max}) was obtained after disruption of the cells with 0.1 mM digitonin, while the minimum (R_{min}) was obtained by using 2 mM $MnCl_2$ and 4 mM EGTA in the external medium. These values were used to estimate the intracellular Ca^{2+} concentration, according to the equation proposed by Grynkiewicz et al. (1985). Before the addition of EGTA the autofluorescence ratio was checked in the presence of 2 mM of $MnCl_2$ and 0.1 mM of digitonin, its value being similar to that obtained before fura-2 loading (Shimuta et al., 1993).

2.3. Protocols

Organ strips were incubated in 0Ca solution for 30 min, followed by reintroduction of Ca^{2+} and registration of the corresponding Ca^{2+}-induced contraction. Under these experimental conditions, to be sure that the calcium stores were empty, we usually checked whether stimulation with 20 mM caffeine failed to cause a contractile response (data not shown).

For the fura-2 loaded cells, the incubation in 0Ca solution lasted 3 min, followed by reintroduction of Ca^{2+} and measurement of intracellular Ca^{2+} concentration. These procedures were repeated in the presence of thapsigargin (2 μM for 30 min) with or without isradipine (100 nM incubated for 15 min) or nifedipine (1 μM incubated for 30 min).

Contractions were also observed, after incubation with thapsigargin, miconazole or SK&F96365.

2.4. Drugs and solutions

Normal Tyrode (NS solution) contained (in mM): 136.8 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 0.4 NaH_2PO_4, 11.9

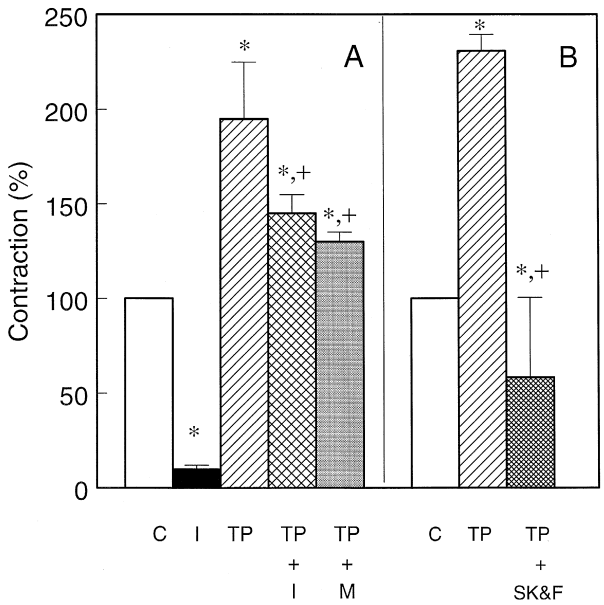

Fig. 1. Panel A: Histogram showing contractions induced in strips of rat stomach fundus by reintroduction of 1.8 mM Ca^{2+} after a 30-min incubation of the strips in 0Ca solution. C: In controls. I: After incubation with 100 nM isradipine for 15 min before reintroduction of Ca^{2+}, showing a reduction of the contraction. TP: After incubation with 2 μM thapsigargin for 30 min, showing a potentiation of the contraction. TP + I: After incubation with thapsigargin and isradipine, for 30 and 15 min, respectively, showing a partial reduction of the contractile response when compared with that produced by TP alone. TP + M: After incubation with thapsigargin and 10 μM miconazole for 30 min, showing a partial and greater reduction of the contraction when compared that produced by TP alone. Panel B: TP + SK&F. Similar experiment showing that incubation of TP with 100 μM SK&F96365 for 30 min caused a significant decrease of the response when compared with that produced by TP alone. Bars represent means ± S.E.M. of at least 6 (A) or 4 experiments (B). * Significantly different ($P < 0.05$) from control and + from TP.

$NaHCO_3$ and 5.6 glucose. The Tyrode Ca^{2+}-free solution (0Ca) had the same composition, except that $CaCl_2$ was not present.

The following drugs and reagents were used: thapsigargin, miconazole, nifedipine, collagenase, digitonin, bovine serum albumin and caffeine (from Sigma, USA), isradipine (PN-200 110, from Sandoz, Switzerland), pluronic F-127 and fura-2/AM (from Molecular Probes, USA), pronase (Boehringer Mannheim, Germany) and SK&F96365 ({1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCl}, from Calbiochem, USA). The solutions of thapsigargin, pluronic F-127 and Fura-2/AM were prepared by dilution with dimethylsulfoxide (DMSO). The concentration of DMSO in the final solution was less than 0.1% and did not affect the contraction or intracellular calcium levels. All chemicals were reagent grade from Merck.

2.5. Data presentation and statistics

Results were expressed as means ± S.E.M. Statistical analysis was performed by using the Student's *t*-test.

3. Results

When Ca^{2+} (1.8 mM) was reintroduced to a fundus strip, after the strip had been incubated in 0Ca for 30 min, a fast contraction was observed that reached its maximum after less than 30 s. The histogram on Fig. 1 shows that the L-type calcium channel blockers, isradipine (I) and nifedipine (not shown), antagonized this contraction, indicating the involvement of VOCs in this effect. However, when the initial 30 min incubation in 0Ca was carried out in the presence of the well-known blocker of Ca-ATPase from endoplasmic reticulum, thapsigargin, a striking potentiation of contraction was observed after reintroduction of Ca^{2+}. The latter response was blocked by about 20% by isradipine (Fig. 1A), but not by nifedipine (1 μM, results not shown), indicating that under these conditions Ca^{2+} entered predominantly through a channel type other than VOCs. In addition, the potentiation by thapsigargin was significantly antagonized by the CRAC blockers miconazole (Fig. 1A) and SK&F96365 (Fig. 1B), indicating the involvement of CRACs.

Fig. 2 shows the intracellular Ca^{2+} concentration (ratio 340/380) in cells incubated with 0Ca solution, followed by the addition of two concentrations of Ca^{2+} (respectively, 1.8 and 3.6 mM). It can be seen that cytosolic calcium rose from 227 in 0Ca to 413 and 740 nM, respectively (Fig. 2A). The incubation of cells in 0Ca in the presence of thapsigargin followed by the addition of Ca^{2+}, 1.8 and 3.6 mM (Fig. 2B), produced an increase in $[Ca^{2+}]_i$ that was significantly greater (518 and 1,126 nM) than that in the absence of thapsigargin. These observations corroborate the data shown in Fig. 1 for the func-

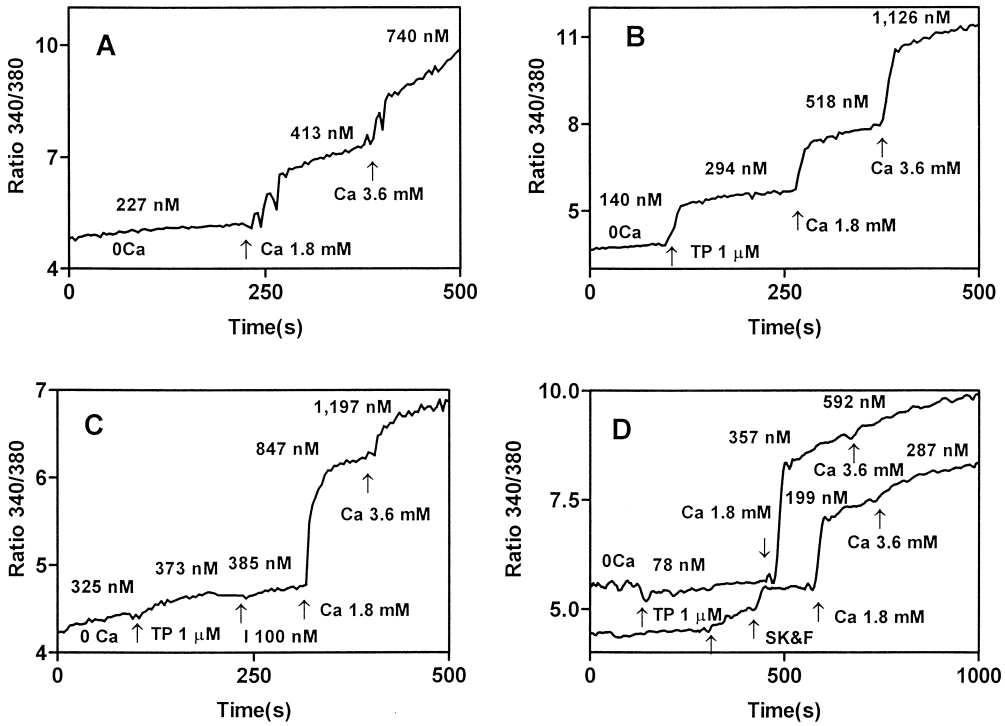

Fig. 2. Measurement of the intracellular Ca^{2+} concentration in freshly dispersed suspension of smooth muscle cells from rat stomach fundus (A). In the control experiment, cells were first incubated in 0Ca solution for 3 min followed by the addition of 1.8 mM of Ca^{2+} and plus 1.8 mM (total amount 3.6 mM). (B) Cells were incubated in 0Ca solution plus thapsigargin. (C) Cells were incubated in 0Ca solution plus thapsigargin and isradipine before the addition of 1.8 and 3.6 mM Ca^{2+}. (D) Cells were incubated in 0Ca solution plus thapsigargin and 100 μM SK&F96365. A control response, in the absence of SK&F96365, is also shown. Figures are representative records of 4 experiments. Arrows indicate drug additions.

tional responses in the presence of TP. In addition, we could not demonstrate any inhibition by isradipine or nifedipine (300 nM, result not shown) of the effect of thapsigargin (Fig. 2C). However, when SK&F96365 (30 μM) was used instead of isradipine, the increase in cytosolic calcium after TP was clearly reduced (Fig. 2D).

4. Discussion

Reintroduction of Ca^{2+} (1.8 mM) after incubation in 0Ca solution produced a contraction in rat fundus smooth muscle that was blocked by the dihydropyridines isradipine and nifedipine, indicating that the ion entered the cytoplasm through L-type voltage-dependent Ca^{2+} channels. When the experiment was repeated after depletion of intracellular Ca^{2+} pools by thapsigargin, the effects were potentiated and were not blocked by nifedipine, or only slightly blocked by isradipine, showing that when thapsigargin is present, Ca^{2+} enters the cytoplasm by an alternative mechanism. Similar effects were observed for Ca^{2+} measurements in cell suspensions: a significant increase in intracellular Ca^{2+} concentration was observed after reintroduction of Ca^{2+} in the presence of thapsigargin and this increase was not inhibited by isradipine or nifedipine.

One could explain our results by assuming that in 0Ca solution depolarization can open voltage-operated Ca^{2+} channels that can be blocked by isradipine or nifedipine. Thus, when 1.8 mM Ca^{2+} is added to the nutrient solution, it enters the cytoplasm through these channels. However, in the presence of thapsigargin, the depletion of internal pools of calcium opens the so-called release activated calcium channels (CRAC). In this case, when 1.8 mM Ca^{2+} is added to the nutrient solution it enters the cytoplasm through both types of channels, VOCs and CRACs, causing a larger effect compared with that in the absence of thapsigargin. This hypothesis was supported by the results of the Ca^{2+} measurements, which suggested the involvement of capacitative Ca^{2+} entry in these responses (Fig. 2). The fact that miconazole and SK&F96365 decreased the TP-induced potentiation of the contraction and Ca^{2+} translocation is an additional strong indication of the involvement of CRACs in this response.

The fact that the effects were not totally inhibited by miconazole and SK&F96365 shows that these responses are complex and that more than one mechanism of Ca^{2+} entry might be activated to maintain the concentration of the ion. Another possibility is that the block elicited by miconazole as well as by SK&F96365 was only partial, because of their low selectivity (Clementi et al., 1992). In fact, more selective inhibitors are being developed and tested (Clementi et al., 1995).

Capacitative calcium entry (Putney, 1990) has been observed in several types of cells and tissues (Berridge, 1995) and can be activated by agents such as thapsigargin. That the latter drug effectively empties Ca^{2+} stores is known, for instance, from experiments with PC_{12} cells (Clementi et al., 1992) and with vascular endothelium (Vaca and Kunze, 1994). Former studies indicated that CRAC channels were present only in non-excitable cells (Putney, 1990). However, these channels have recently been described in many kinds of cells, especially in smooth muscles (Amrani et al., 1995; Ohta et al., 1995). Several hypotheses have been put forward to explain the mechanism of CRAC activation, including the stimulation of phosphatases, G-proteins or regulation by a tyrosine kinase pathway, but not by a direct activation of an inositol 1,4,5-triphosphate-pathway (Berridge, 1995). We believe that the present results, being the first functional and biochemical indication of CRACs in rat stomach fundus, open new possibilities for the study of the capacitative entry of Ca^{2+} in smooth muscle tissue.

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