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ORIGINAL ARTICLE

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Role of sarcoplasmic reticulum Ca^{2+} content in Ca^{2+} entry of bovine airway smooth muscle cells

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Abstract Depletion of intracellular Ca^{2+} stores induces the opening of an unknown Ca^{2+} entry pathway to the cell. We measured the intracellular free-Ca^{2+} concentration ($[Ca^{2+}]i$) at different sarcoplasmic reticulum (SR) Ca^{2+} content in fura-2-loaded smooth muscle cells isolated from bovine tracheas. The absence of Ca^{2+} in the extracellular medium generated a time-dependent decrement in $[Ca^{2+}]i$ which was proportional to the reduction in the SR-Ca^{2+} content. This SR-Ca^{2+} level was indirectly determined by measuring the amount of Ca^{2+} released by caffeine. Ca^{2+} restoration at different times after Ca^{2+}-free incubation (2, 4, 6 and 10 min) induced an increment of $[Ca^{2+}]i$. This increase in $[Ca^{2+}]i$ was considered as Ca^{2+} entry to the cell. The rate of this entry was slow (~0.3 nM/s) when SR-Ca^{2+} content was higher than 50% (2 and 4 min in Ca^{2+}-free medium), and significantly ($p<0.01$) accelerated (>1.0 nM/s) when SR-Ca^{2+} content was lower than 50% (6 and 10 min in Ca^{2+}-free medium). Thapsigargin significantly induced a higher rate of this Ca^{2+} entry ($p<0.01$). Variations in Ca^{2+} influx after SR-Ca^{2+} depletion were estimated more directly by a Mn^{2+} quench approach. Ca^{2+} restoration to the medium 4 min after Ca^{2+} removal did not modify the Mn^{2+} influx. However, when Ca^{2+} was added after 10 min in Ca^{2+}-free medium, an increment of Mn^{2+} influx was observed, corroborating an increase in Ca^{2+} entry. The fast Ca^{2+} influx was Ni^{2+} sensitive but was not affected by other known capacitative Ca^{2+} entry blockers such as La^{3+}, Mg^{2+}, SKF 96365 and 2-APB. It was also not affected by the blockage of L-type Ca2^{+} channels with methoxyverapamil or by the sustained K^{+}-induced depolarisation. The slow Ca^{2+} influx was only sensitive to SKF 96365. In conclusion, our results indicate that in bovine airway smooth muscle cells Ca^{2+} influx after SR-Ca^{2+} depletion has two rates: A) The slow Ca^{2+} influx, which occurred in cells with more than 50% of their SR-Ca^{2+} content, is sensitive to SKF 96365 and appears to be a non-capacitative Ca^{2+} entry; and B) The fast Ca^{2+} influx, observed in cells with less than 50% of their SR-Ca^{2+} content, is probably a capacitative Ca^{2+} entry and was only Ni^{2+}-sensitive.

Keywords Airways smooth muscle · Capacitative Ca^{2+} entry · Passive Ca^{2+} entry · Sarcoplasmic reticulum Ca^{2+} content · SKF 96365, Ni^{2+}

Introduction

In airway smooth muscle (ASM) intracellular free calcium concentration ($[Ca^{2+}]i$) has an important role mediating excitation-contraction coupling. In this tissue two ways have been described to increase the $[Ca^{2+}]i$. One involves the extracellular Ca^{2+} entry through receptor-operated and voltage-sensitive (both L and T type) Ca^{2+} channels (Kajita and Yamaguchi 1993; Cuthbert et al. 1994; Montaño et al. 1996; Janssen 1997). The other mechanism requires intracellular Ca^{2+} release mainly from sarcoplasmic reticulum (SR) via inositol 1, 4, 5-trisphosphate- and ryanodine-sensitive channels (Baron et al. 1984; Madison et al. 1998; Bazán-Perkins et al. 1998, 2000).

Endoplasmic/sarcoplasmic reticulum Ca^{2+} depletion is associated to Ca^{2+} influx. This process, known as capacitative Ca^{2+} entry, was formerly observed in non-excitable cells by Putney (1986). In human ASM cells, Amrani and co-workers (1995) observed an increase of $[Ca^{2+}]i$ after SR-Ca^{2+} depletion suggesting a capacitative Ca^{2+} entry mechanism.

A direct relationship between the SR-Ca^{2+} content and the temporal course of Ca^{2+} entry has been demonstrated in non-excitable cells (Jacob 1990; Hofer et al. 1998; Sedova et al. 2000) and in excitable cells, such as pancreatic β-cells

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(Dyachok and Gylfe 2001). However, it is unknown if a similar relationship occurs in ASM.

Currently, there is no consensus regarding the efficiency and selectivity of capacitative channel blockers, but cations like La^{3+} and Mg^{2+} have been reported to block this Ca^{2+} entry in excitable cells (Yoshimura et al. 1997; Yang 1998). Ni^{2+}, an inorganic Ca^{2+} channel blocker, has also been used to inhibit the capacitative Ca^{2+} entry in non-excitable (Hoth and Penner 1993; Kerschbaum and Cahalan 1998) and excitable cells (Lee et al. 2002; McDaniel et al. 2001).

Recently, some substances as 2-aminoethyldiphenyl borate (2-APB) and the 1-(beta-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride (SKF 96365) have been used as capacitative Ca^{2+} entry blocker in vascular myocytes and mast cells, respectively (Franzius et al. 1994; Peppiatt et al. 2003; Wakabayashi et al. 2003). The aim of the present work was to study, in bovine ASM cells, the effect of different SR-Ca^{2+} content on Ca^{2+} entry of depolarised and non-depolarised cells, and to investigate the effect of 2-APB, SKF 96365, La^{3+}, Mg^{2+} and Ni^{2+} in this phenomenon.

Materials and methods

Preparation of tracheal smooth muscle

Bovine tracheas were obtained from a local slaughterhouse and transported to the laboratory in cold, oxygenated, physiological salt solution (PSS) with the following composition (mM): 118 NaCl, 25 $NaHCO_3$, 4.6 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$ and 11 glucose. Animals were sacrificed according the Mexican laws. Tracheal smooth muscle was dissected free of adhered fat and connective tissue using a stereoscopic microscope Nikon SWZ-10 (Tokyo, Japan), at room temperature in oxygenated PSS.

Tracheal myocytes isolation

Airway smooth muscle cells were obtained from bovine trachea as follows. Approximately 200 mg smooth muscle were minced, placed in 5 ml Hanks solution (Gibco BRL, Rockville, MD) containing 2 mg cysteine and 0.05 U ml^{-1} papaine, and incubated for 10 min at 37°C. The tissue was washed with Leibovitz's solution (Gibco) to remove the enzyme excess, and then placed in PSS containing 0.144 mg ml^{-1} of a highly purified collagenases and neutral proteases mixture (Liberase Blendzyme 2, Roche, Indianapolis, IN) during ~12 min until dispersed cells were observed. This procedure allowed us to obtain cells with consistent levels of resting [Ca^{2+}]i (Montaño et al. 2003). Then, cells were loaded with 0.5 µM fura 2/AM in low Ca^{2+} (0.1 mM) at room temperature (22–25°C). After 1 h, cells were allowed to settle into a heated perfusion chamber with a glass cover in the bottom. This chamber was mounted on a Nikon inverted microscope (Diaphot 200, Tokyo, Japan) and the cells adhered to the glass were continuously perfused at a rate of 2–2.5 ml/min with PSS (37°C, equilibrated with 95% O_2/ 5% CO_2, pH 7.4) and containing 2 mM Ca^{2+}.

[Ca^{2+}]i measurement

Cells loaded with fura 2 were exposed to alternating pulses of 340 and 380 nm excitation light, and emission light was collected at 510 nm using a PTI microphotometer (Photon Technology International, Princenton, NJ). Background fluorescence was automatically subtracted and determined by removing the cell from the field before starting the experiments. The fluorescence acquisition rate was 0.5 s. [Ca^{2+}]i was calculated according to the formula of Grynkiewicz and co-workers (1985). The K_d of fura 2 was assumed to be 386 nM (Kajita and Yamaguchi 1993). The mean 340/380 fluorescence ratios Rmax and Rmin were obtained by exposing the cells to 10 mM Ca^{2+} in presence of 10 µM ionomycin and in Ca^{2+}-free PSS with 1.11 mM EGTA, respectively. Rmax was 11.2 and Rmin 0.37. The fluorescence ratio at 380 nm light excitation in Ca^{2+}-free PSS and Ca^{2+} saturated cells (ß) was 5.2.

Experimental design

Bovine ASM cells stimulated with 10 mM caffeine or 10 µM carbachol (Cch) in PSS, were afterwards perfused with nominal Ca^{2+} free medium for about 10 min. During this period, caffeine was added at 2, 4, 6 or 10 min. In other set of experiments, following a similar protocol, Ca^{2+} was restored at the same intervals, 2, 4, 6 and 10 min. Cells used in these intervals are referred in the text as groups 2, 4, 6 and 10. In some cells of 10-min group, Ca^{2+}-free medium was supplemented with 1 µM thapsigargin. Other cells from groups 4 and 10 were previously depolarised by substitution of 118 mM NaCl with 122 mM KCl or incubated with 30 µM of methoxyverapamil hydrochloride (D-600), an L-type Ca^{2+} channel blocker. For each interval we used different cells.

Additionally, in groups 4 and 10, we explored the effect of 100 µM 2-APB, 20 µM SKF 96365, 4 mM Mg^{2+}, 0.2 mM La^{3+} or 1 mM Ni^{2+}, a well known capacitative Ca^{2+} entry blockers (Hoth and Penner 1993; Franzius et al. 1994; Yoshimura et al. 1997; Yang 1998; Bootman et al. 2002; Kawanabe et al. 2002; Peppiatt et al. 2003; Wakabayashi et al. 2003), during Ca^{2+} entry after SR-Ca^{2+} depletion. In La^{3+} and Mn^{2+} experiments the PSS had the following composition (mM): 138 NaCl, 5 HEPES, 4.6 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2 $CaCl_2$ and 11 glucose, the pH 7.4 was adjusted with NaOH (10 M).

Finally, using groups 4 and 10, variations in Ca^{2+} influx after SR-Ca^{2+} depletion were estimated more directly by Mn^{2+} (50 µM) quench approach. In this experiments 360 nm excitation light was used.

Drugs

Caffeine, fura 2/AM, EGTA, ionomycin, HEPES, methoxyverapamil hydrochloride, thapsigargin, lanthanum chloride heptahydrate, nickel chloride hexahydrate, magnesium chloride anhydrous and manganese chloride tetrahydrate were obtained from Sigma (St. Louis, MO). SKF 96365 was obtained from Biomol (Plymouth Meeting, PA). 2-APB was obtained from Calbiochem (La Jolla, CA). Liberase Blendzyme 2 was obtained from Roche (Indianapolis, IN). Ionomycin and fura 2/AM were dissolved in DMSO (final concentration 0.025%). In control experiments, use of DMSO alone had no effect.

Statistics

One-way analysis of variance and Tukey's test for multiple comparisons were used. In some experiments we used Student's t-test for paired data. Two-tailed p values <0.05 were considered statistically significant. Ca^{2+} entry rate was calculated for each experiment using straight linear regression and calculating the slope of each recording during Ca^{2+} restoration and expressed as nM/s. All values in the text and illustrations correspond to mean ± SEM.

Results

Effect of external Ca^{2+} removal on the [Ca^{2+}]i

The resting [Ca^{2+}]i in bovine ASM cells was 148±6 nM (n=90). Caffeine (10 mM) addition during 2 min resulted

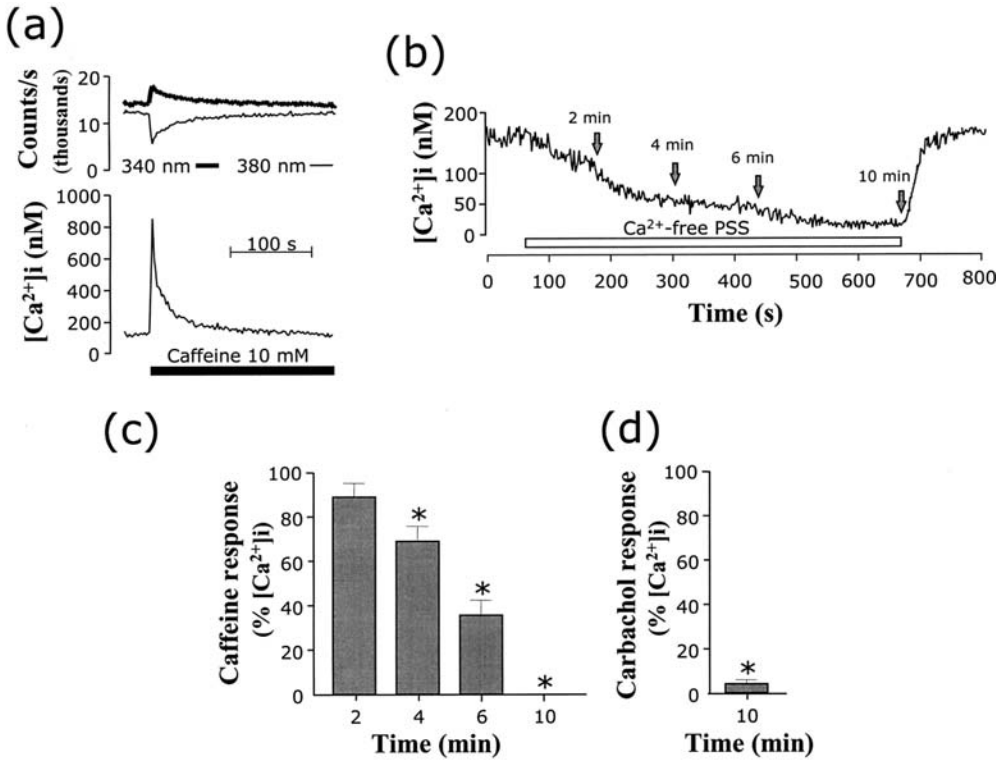

Fig. 1a–d External Ca^{2+} removal decreased the intracellular free-Ca^{2+} concentration ($[Ca^{2+}]i$) and the SR-Ca^{2+} content in isolated tracheal myocytes. (**a**) Original trace of caffeine-induced Ca^{2+} transient peak. (**b**) Typical recording from a myocyte incubated in Ca^{2+}-free PSS. Recovery of basal $[Ca^{2+}]i$, suggesting Ca^{2+} entry, was initiated after external Ca^{2+} (2 mM) was restored. (**c**) Ca^{2+} responses induced by caffeine (10 mM) after different incubation periods in Ca^{2+}-free medium. (**d**) Ca^{2+} response induced by carbachol (10 µM) after 10 min in Ca^{2+}-free medium. For details see text. *p<0.01

in a transient Ca^{2+} peak (630±10 nM, n=31; Fig. 1a). Cch (10 µM) incubation induced a similar transient response (472±96 nM, n=4). Removal of external Ca^{2+} lead to a continuous reduction in the $[Ca^{2+}]i$ until a steady state was reached after 10 min (22±6 nM, n=5; Fig. 1b). This decrement in $[Ca^{2+}]i$ was associated with a significant and proportional reduction of the caffeine Ca^{2+} transient responses observed at 2, 4 and 6 min in Ca^{2+} free PSS when compared with their respective control group in normal PSS (n=6; Fig. 1c). At 10 min, the response to caffeine was totally absent whilst Cch response was only 4% of its response in normal PSS (n=6 and 4 respectively, Fig. 1c, d). These results corroborated that the SR-Ca^{2+} stores were almost completely depleted after 10 min in Ca^{2+} free medium.

Restoration of extracellular Ca^{2+} (2 mM) produced, in all groups, a return to the initial $[Ca^{2+}]i$ basal levels (Fig. 2). This increase in $[Ca^{2+}]i$ was considered as a Ca^{2+} entry to the cell. The rate of Ca^{2+} entry in groups 6 and 10 min was significantly faster (p<0.01, fast Ca^{2+} entry) than those rates calculated at other times (slow Ca^{2+} entry). An additional group of 10 min that was incubated with 1 µM thapsigargin showed a faster Ca^{2+} entry rate (p<0.01) when compared with its control group (10 min, Fig. 2). The long lasting depolarisation of the ASM cells by substitution of NaCl by high potassium solution to induce inactivation of L and T-type Ca^{2+} channels and inversion of the Na^{+}/Ca^{2+} exchanger, did not appear to change Ca^{2+} entry rate, since this phenomenon was still faster after 10 min (1.48±0.21 nM/s, p<0.01, n=4) compared with the 4-min group (0.19± 0.06 nM/s, n=6). Indeed, we did not observe any statistical difference in Ca^{2+} entry in these groups when compared with their respective non-depolarised cells.

When Mn^{2+} (50 µM) was present in the PSS, Ca^{2+} restoration to the cells after 4-min incubation in Ca^{2+} free medium did not modify the fluorescence at 360 nm (n=5; Fig. 3a), i.e., we did not observe an additional Mn^{2+} influx. However, when Ca^{2+} was added after 10 min incubation in Ca^{2+}-free medium, we noticed an increment of Mn^{2+} influx (n=5, Fig. 3b), corroborating an increase in Ca^{2+} entry.

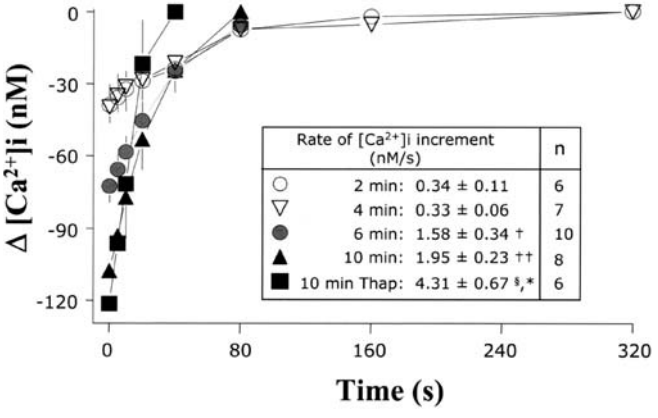

Fig. 2 Temporal course of $[Ca^{2+}]i$ increment after Ca^{2+} restoration of four different cell groups that have been incubated in Ca^{2+}-free medium during different periods. These values suggest the existence of two different mechanisms for Ca^{2+} entry. An additional group of 10 min was incubated with 1 µM thapsigargin (Thap, an SR-Ca^{2+} ATPase blocker). †,†† p<0.05, 0.01, respectively, between group 10, 6 vs. 2, 4 groups. *p<0.01 between 10, 6 and Thap groups. § p<0.01 between Thap group vs. all groups

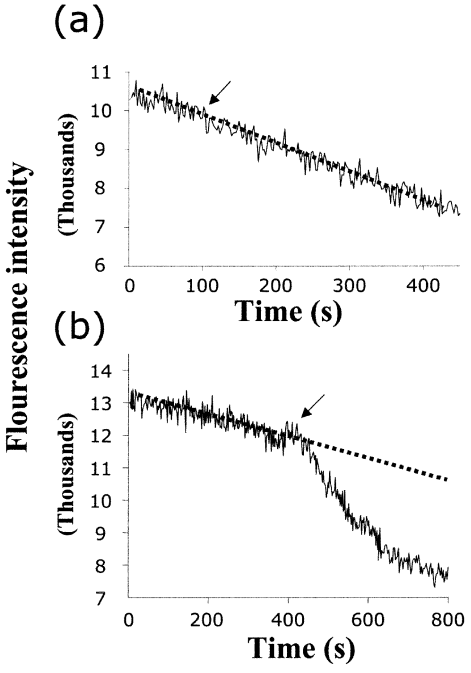

Fig. 3a, b Original recordings of Mn^{2+} quenching (50 µM) using 360 nm excitation light in cells perfused with Ca^{2+}-free medium during 4 (**a**) and 10 min (**b**). *Arrows* indicate when 2 mM Ca^{2+} was added in the perfusion medium. Ca^{2+} restoration to the medium modifies Mn^{2+} influx only 10 min after Ca^{2+} removal, suggesting the existence of a membrane Ca^{2+} pathway during the fast Ca^{2+} recovery

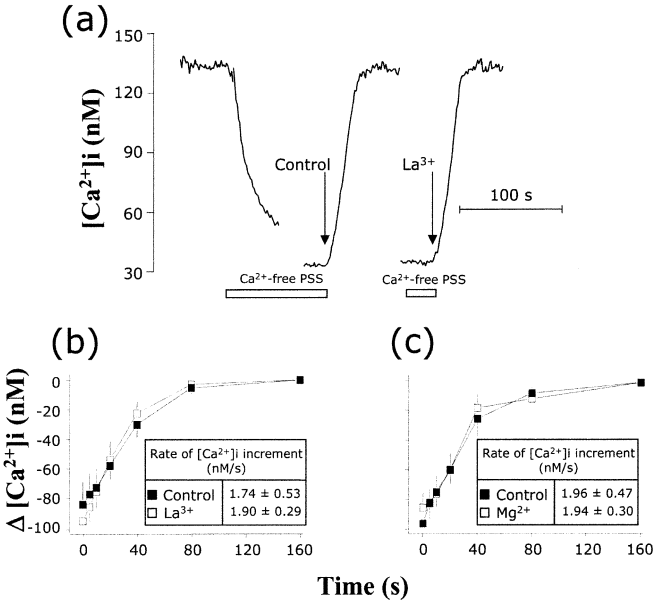

Fig. 4a–c La^{3+} (0.2 mM, n=9) and Mg^{2+} (4 mM, n=4) did not affect the fast Ca^{2+} entry observed in bovine airway smooth muscle cells (group of 10 min). (**a**) Original recording showing the lack of effect of La^{3+} on the Ca^{2+} entry. (**b, c**) Average data of the temporal course of Ca^{2+} entry during Ca^{2+} restoration in two different experimental conditions

Effect of La^{3+} and Mg^{2+} in Ca^{2+} entry

In non-stimulated myocytes, incubation with La^{3+} (0.2 mM) or Mg^{2+} (4 mM) during 10 min did not modify the $[Ca^{2+}]i$ basal levels. Some cells were incubated in Ca^{2+} free PSS during either 4 or 10 min (as described above) and the Ca^{2+} entry rate was determined by restoring this ion in the external medium in the presence of these two cations. Figure 4 shows the results of the 10-min group (n=9, 5, respectively) and it can be observed that Ca^{2+} entry rates were not modified when cells were incubated with the capacitative Ca^{2+} blockers. Similar lack of effect of La^{3+} and Mg^{2+} was observed in the 4-min group, which had an average value of 0.37±0.14 and 0.38±0.13 nM/s, n=6, 4, respectively, when compared with its corresponding control group: 0.30±0.10 and 0.38±0.09 nM/s, respectively.

Effect of Ni^{2+} in Ca^{2+} entry

Ni^{2+} (1 mM) addition to PSS induced a slow $[Ca^{2+}]i$ decrement in non-stimulated myocytes, and after 5 min perfusion, a new steady state of $[Ca^{2+}]i$ was observed (79±11 nM, n=6; Fig. 5a–c). Ni^{2+} removal restored Ca^{2+} basal levels even when cells were submitted to Ca^{2+}-free medium (Fig. 5a, b). Under the new steady state induced by Ni^{2+}, two groups of cells were incubated in Ca^{2+} free PSS during either 4 or 10 min and the Ca^{2+} entry rate was determined by restoring this ion in the external medium (n=5 each group; Fig. 5c, d). In the first group a rate of 0.30± 0.11 nM/s was obtained, which was not different than the rate values of the control group of 4 min (without Ni^{2+}, see Fig. 2). The calculated rate of the second group was slower (0.16±0.03 nM/s, n= 6) than that of the control group of 10 min (Fig. 2).

It has been proposed that Ni^{2+} induces fura 2 quenching (Merritt et al. 1989). In Fig. 5a we can observe that the signal from 340 and 380 nm during Ni^{2+} perfusion was not modified i.e., Ni^{2+} did not enter to the cell.

Effect of D-600, 2-APB and SKF-96365 in Ca^{2+} entry

Incubation of the myocytes with D-600 (30 µM, n=4), 2-APB (100 µM, n=5) or SKF 96365 (20 µM, n=5) during 10 min did not modify the $[Ca^{2+}]i$ basal levels. In the 10-min group, the fast Ca^{2+} entry was not modify by D-600, 2-APB or SKF 96365 (Figs. 6 and 7a). Similar results were observed in the 4-min group where the slow Ca^{2+} entry (0.38±0.09 nM/s, *n=4*) was not modified by D-600 (0.30±0.12 nM/s), or 2-APB (0.72±0.21 vs. control 0.39± 0.09 nM/s, n=5). Contrary to this last effect, SKF 96365 significantly reduced the Ca^{2+} entry rates and the $[Ca^{2+}]i$ basal level was not restored, i.e., the slow Ca^{2+} entry was blocked in the 4-min group (Fig. 7b, n=6).

Discussion

External Ca^{2+} removal in bovine ASM cells induced a slow and continuous $[Ca^{2+}]i$ decrement until a new baseline

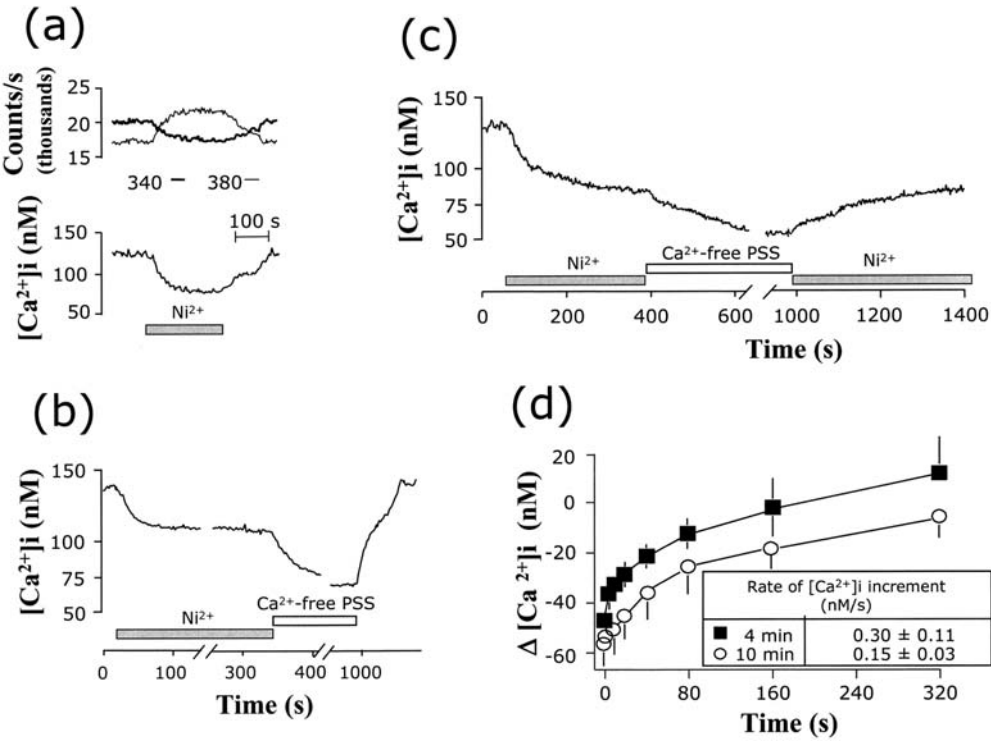

Fig. 5a–d Ni^{2+} (1 mM) decreased the basal $[Ca^{2+}]i$ and slowed down the fast Ca^{2+} entry triggered by Ca^{2+} restoration in bovine airway smooth muscle cells. Ca^{2+} addition restored $[Ca^{2+}]i$ basal levels in cells that were previously incubated with Ni^{2+} in normal (**a**) and Ca^{2+}-free PSS (**b**). (**c**) Original recording showing the effect of Ni^{2+} in Ca^{2+} entry after Ca^{2+} restoration. (**d**) Time course of Ca^{2+} entry during Ni^{2+} incubation in cells with slow (4 min) and fast (10 min) Ca^{2+} influx

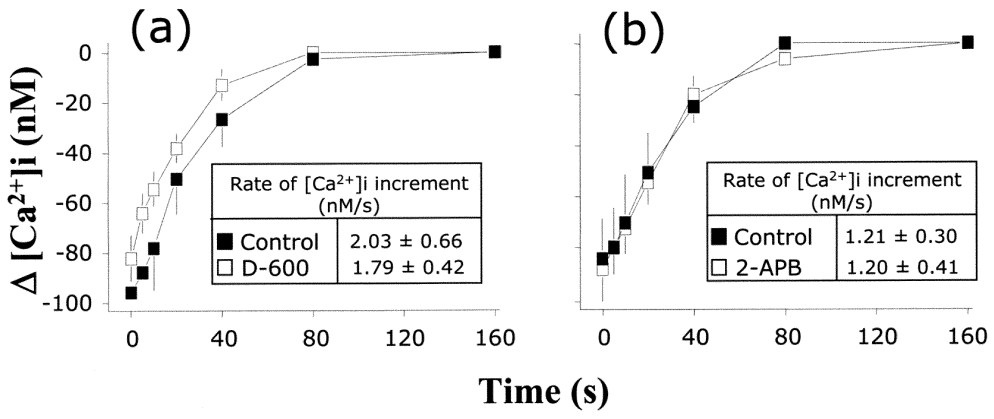

Fig. 6a, b Effect of D-600 (30 µM) and 2-APB (100 µM, a capacitative Ca^{2+} entry blocker) in the fast Ca^{2+} entry observed in bovine airway smooth muscle cells (group of 10 min). Both drugs, D-600 (**a**) and 2-APB (**b**) did not modify the Ca^{2+} entry rate when compared with their respective control groups

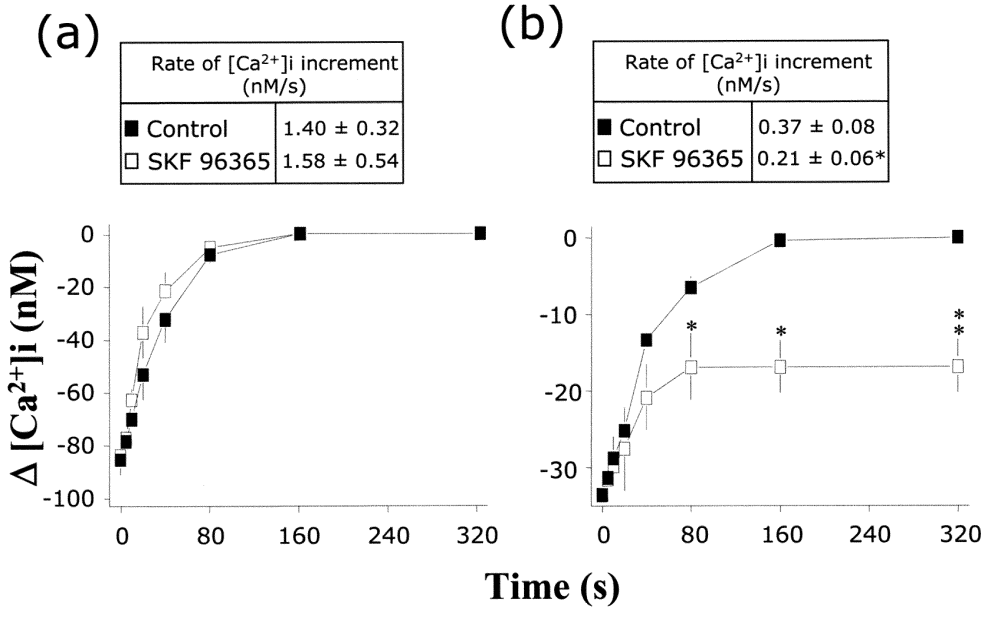

Fig. 7a, b Effect of SKF 96365 (20 µM) in the fast and slow Ca^{2+} entry observed in bovine airway smooth muscle cells. In (**a**) we can observe that SKF 96365 did not modify the fast Ca^{2+} entry, but significantly inhibited the slow Ca^{2+} entry (**b**)

was achieved. This slow $[Ca^{2+}]i$ fall was associated with a similar reduction in the SR-Ca^{2+} content. When external Ca^{2+} was restored, an increase in $[Ca^{2+}]i$ was observed, which would imply a Ca^{2+} entry to the cell. This Ca^{2+} entry had a faster rate (>1 nM/s) when the SR-Ca^{2+} content was less than 50% and was sensitive to Ni^{2+}. However, when the SR-Ca^{2+} content was above 50%, Ca^{2+} entry rate was slower (~0.3 nM/s) and sensitive to SKF 96365. Both influxes, the slow and the fast, were not blocked by 2-APB, La^{3+}, Mg^{2+} or voltage Ca^{2+} channels inhibition.

In non excitable cells such as endothelial (Jacob 1990, Sedova et al. 2000), RBL/1 cells, BHK/21 fibroblasts (Hofer et al. 1998) and excitable cells as mice pancreatic β cells (Dyachok and Gylfe 2001), it has been observed that capacitative Ca^{2+} entry is specifically controlled by the Ca^{2+} content of intracellular stores, i.e., the rate of Ca^{2+} entry varies inversely with the degree of fullness of these stores. Opposite to what happens in these cells, in ASM cells the rate of Ca^{2+} entry does not appear to vary with the degree of fullness of the SR-Ca^{2+}, since we found two patterns of Ca^{2+} entry, the slow and the fast influx. This would imply that ASM uses different mechanisms to regulate this Ca^{2+} entry than those used in previous mentioned cells. In this regard, it has been reported in RBL cells (non excitable cells) that opening of Ca^{2+}-release-activated Ca^{2+} (CRAC) channels occurs only when the endoplasmic reticulum Ca^{2+} content reaches a thershold level (Parekh et al. 1997). It is possible that in ASM cells capacitative Ca^{2+} influx signaling is also activated when SR-Ca^{2+} stores reach a threshold level, and this one seems to be when the SR-Ca^{2+} content was less than 50%.

Mg^{2+} and La^{3+}, two capacitative Ca^{2+} entry blockers in vascular myocytes (Yoshimura et al. 1997) and mast cells (Hoth and Penner 1993), did not modify the Ca^{2+} entry rate in ASM cells with slow and fast Ca^{2+} entry. These results would imply that in ASM the channels involved in this Ca^{2+} entry are not sensitive to these blockers.

The fast Ca^{2+} entry appears to be sensitive to Ni^{2+}. In our experiments, we observed that Ni^{2+} produced a gradual $[Ca^{2+}]i$ decrement until a new steady state of $[Ca^{2+}]i$ was reached. This effect might be mediated by its known action as Ca^{2+} antagonist that involves the blockade of voltage-sensitive and receptor-operated Ca^{2+} channels, and the Na^{+}/Ca^{2+} exchange (Cuthbert et al. 1994; Hoya and Venosa 1995). The effect of Ni^{2+} on Ca^{2+} entry after SR depletion is, however, unlikely mediated by such an action, hypothesis that is supported by the following: 1) we found that the slow and fast Ca^{2+} entry, was not affected by prolonged depolarisation or incubation with D-600, suggesting that voltage-sensitive L- and T-type Ca^{2+} channels and the Na^{+}/Ca^{2+} exchanger are not important in this phenomenon; 2) receptor-operated Ca^{2+} channels require the action of an agonist to be opened and this is not the case.

Recently, in vascular smooth muscle from aorta and pulmonary artery, it was found that Ni^{2+} abolished the capacitative Ca^{2+} entry (Lee et al. 2002; McDaniel et al. 2001), suggesting that this cation is an appropriate antagonist for excitable cells. In our study, we observed that Ni^{2+} only abolished the fast Ca^{2+} entry. Thus, these observations support the hypothesis that Ni^{2+} is blocking the putative capacitative Ca^{2+} entry pathway, which is only present when the SR-Ca^{2+} content is lower than 50%. On the other hand, since Ni^{2+} did not block the slow Ca^{2+} entry, we could suggest that this is a non capacitative phenomenon, probably the so called passive influx. The results with 2-APB, recently described as a capacitative Ca^{2+} inhibitor (Prakriya and Lewis 2001), in the slow Ca^{2+} entry support our hypothesis that this Ca^{2+} influx is a non capacitative phenomenon since the drug did not block it. In this context, SKF 96365, a compound with several effects such as store-operated and receptor-operated Ca^{2+} channels blocker (Merritt et al. 1990; Prakriya and Lewis 2002), significantly reduced this slow Ca^{2+} entry. This effect of SKF 96365 on the passive Ca^{2+} entry was also previously reported in human leukemic HL-60 cells (Leung et al. 1996). In this last work SKF 96365 almost completely blocked (basal) Mn^{2+} entry. These results suggested the participation of Ca^{2+} channels in the slow Ca^{2+} entry, however further experiments are required to determine the type of Ca^{2+} channels involved.

In order to corroborate that during the fast Ca^{2+} entry an additional Ca^{2+} pathway was activated, we used the Mn^{2+} quench approach to confirm more directly the Ca^{2+} influx. We found an increase in Mn^{2+} influx when Ca^{2+} was restored in cells with SR-Ca^{2+} content lower than 50%, suggesting a change in Ca^{2+} entry that probably was due to a capacitative Ca^{2+} phenomenon. In cells with SR-Ca^{2+} content higher than 50%, Mn^{2+} quench did not change when Ca^{2+} was added to PSS. Thus, Ca^{2+} influx that contributes to restore the intracellular Ca^{2+} levels in these cells seems not to be related to the activation of an additional Ca^{2+} pathway, confirming that this slow Ca^{2+} entry is passive.

Our observations also indicate that in the fast Ca^{2+} entry, the rate of $[Ca^{2+}]i$ increase after reintroduction of extracellular Ca^{2+} could be influenced by sequestration of Ca^{2+} by the SR, because we found that the blockade of SR-Ca^{2+} pump with thapsigargin significantly accelerates the rate of $[Ca^{2+}]i$ increase. The SR might be playing a similar role in experimental cells with slow Ca^{2+} influx. Unfortunately, experiments with thapsigargin in these later groups would have been difficult to interpret because this maneuver also depletes the SR.

In conclusion, we observed that in bovine ASM cells Ca^{2+} influx after SR-Ca^{2+} depletion has two rates: A) The slow Ca^{2+} influx which occurred in cells with more than 50% of their SR-Ca^{2+} content, is sensitive to SKF 96365 and appears to be a non-capacitative Ca^{2+} entry (passive entry). B) The fast Ca^{2+} influx observed in cells with less than 50% of their SR-Ca^{2+} content, is probably a capacitative Ca^{2+} entry and was only Ni^{2+}-sensitive. Both mechanisms were not blocked by La^{3+}, Mg^{2+}, 2-APB, and voltage-Ca^{2+} channels were also not involved.

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