Capacitative Ca^{2+} Entry During Ca^{2+} Undershoot in Bovine Airway Smooth Muscle

Blanca Bazán-Perkins¹, Edgar Sánchez-Guerrero¹ and María G. Campos²

¹Departamento de Hiperreactividad Bronquial, Instituto Nacional de Enfermedades Respiratorias, Ismael Cosío Villegas. México DF, ²Unidad de Investigación Médica en Farmacología, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, México DF

Key Words

Ca^{2+} undershoot • Airway smooth muscle • Genistein • SKF-96365 • Econazole • Ni^{2+}

Abstract

In numerous cells, Ca^{2+} undershoot is commonly observed after withdrawing stimulus that release Ca^{2+} from intracellular stores. In airway smooth muscle (ASM), the fast intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) drop during undershoot is produced by sarcoplasmic reticulum (SR) reloading, but the mechanisms involved in the long lasting basal $[\text{Ca}^{2+}]_i$ recovery are unknown. We investigated the post-caffeine Ca^{2+} undershoot recovery in ASM isolated cells from bovine trachea. $[\text{Ca}^{2+}]_i$ determination was done by a ratiometric method by incubating cells with Fura-2/AM. After inducing a transient response, caffeine withdrawn generated a Ca^{2+} undershoot. SR- Ca^{2+} content during maximum undershoot drop was ~40% of SR caffeine-releasable Ca^{2+} (SR- Ca^{2+} load). Undershoot recovery rate increased in presence of cyclopiazonic acid (CPA, a SR- Ca^{2+} ATPase inhibitor), but SR- Ca^{2+} load was reduced. Genistein (a tyrosine kinase inhibitor) slowed down the Ca^{2+} undershoot drop and the SR- Ca^{2+} load but did not affect the

undershoot recovery rate. Ni^{2+} (a capacitative Ca^{2+} inhibitor), but neither SKF-96365 (a passive Ca^{2+} entry inhibitor) nor econazole (a capacitative Ca^{2+} inhibitor in non-excitable cells), inhibited Ca^{2+} undershoot recovery and SR- Ca^{2+} load. Our data suggest that capacitative Ca^{2+} entry is involved in bovine ASM Ca^{2+} undershoot recovery, and that changes in Ca^{2+} undershoot have an impact on SR- Ca^{2+} loading which might affect in turn ASM excitability.

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Introduction

Free calcium ion (Ca^{2+}) is a central second messenger in the regulation of airway smooth muscle tone. A main source of Ca^{2+} , besides the extracellular medium, is the sarcoplasmic reticulum (SR) [1-3]. In the airway smooth muscle, SR Ca^{2+} release takes place by the opening of inositol 1,4,5-trisphosphate and ryanodine Ca^{2+} channels [4]. SR- Ca^{2+} content can be restored by the activity of the sarco-endoplasmic Ca^{2+} ATPase (SERCA) [5]. A consequence of SERCA activity when SR- Ca^{2+} channels are closed is an abrupt reduction of the intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), process that

is compensated by an extracellular Ca^{2+} entry [6-10]. This phenomenon, named Ca^{2+} undershoot, has been scantily studied.

The Ca^{2+} entry pathways during Ca^{2+} undershoot have not been elucidated. In smooth muscle cells from guinea-pig urinary bladder, Ca^{2+} influx during undershoot occurs through L-type Ca^{2+} channels, while in bovine airway smooth muscle, voltage Ca^{2+} channels are not involved [1, 9]. In Ca^{2+} undershoot, the capacitative Ca^{2+} entry has not been evaluated as a potential Ca^{2+} entry pathway, in spite of being an important pathway activated by SR- Ca^{2+} depletion [7, 11]. Capacitative Ca^{2+} entry is known to be regulated by different molecules. For example, capacitative Ca^{2+} entry has been shown to be inhibited by econazole in human MG63 osteosarcoma cells and platelets [12, 13] and by genistein in guinea pig gallbladder smooth muscle. As far as we know, the role of econazole and genistein has not yet been evaluated in Ca^{2+} undershoot.

Added to this scenario, capacitative and passive Ca^{2+} entry have both been involved in SR- Ca^{2+} refilling in airway smooth muscle [1]. In this study, we have evaluated different mechanisms (genistein, econazole, passive and capacitative Ca^{2+} entry) that might be involved in Ca^{2+} undershoot induced by caffeine withdrawal in bovine tracheal smooth muscle cells.

Materials and Methods

Isolation of airway smooth muscle cells

Airway smooth muscle was dissected from bovine tracheas acquired in a local slaughterhouse, and placed in physiological saline solution (PSS, mM): 118 NaCl, 25 NaHCO_3 , 4.6 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 and 11 glucose. Smooth muscle was cut into strips (0.5 x 5 mm) weighing 200 mg total, and placed in 2.5 ml PSS containing collagenase type D (2.6 mg/ml) and elastase grade II (0.7 mg/ml) for 15 min at 37°C. Strips were then transferred to a similar PSS containing fresh enzymes plus desoxyribonuclease I (1 mg/ml) until dispersed cells were observed. This procedure allows us to obtain cells with consistent levels of resting $[\text{Ca}^{2+}]_i$ [1, 7]. The dispersed cells were loaded with 3 μM Fura-2 acetoxymethyl ester (AM) in low Ca^{2+} (0.1 mM) for 1 hour at room temperature (22-25°C); then, cells were allowed to settle into a perfusion chamber with a glass cover in the bottom. This chamber was mounted on a Nikon inverted microscope (Diaphot 200) and the cells adhered to the glass were continuously perfused at a rate of 2.5 ml/min with PSS (37°C, equilibrated with 95% O_2 , 5% CO_2 , pH 7.4) containing Ca^{2+} (2 mM).

Measurement of $[\text{Ca}^{2+}]_i$

Myocytes loaded with Fura-2/AM were excited by alternating pulses of 340 and 380 nm light, and emission was

collected at 510 nm using a PTI microphotometer (Photon Technology International, Princeton, NJ). Background fluorescence was automatically subtracted and determined by removing the cell from the field before beginning the experiments. The fluorescence acquisition rate was 0.5 s. The $[\text{Ca}^{2+}]_i$ was calculated according to the formula of Grynkiewicz and coworkers [15]. The K_d of Fura-2 was assumed to be 386 nM [16]. The mean 340/380 fluorescence ratios R_{max} and R_{min} 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 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), respectively. R_{max} was 11.7 and R_{min} 0.5. The fluorescence ratio at 380 nm light excitation in Ca^{2+} -free PSS and Ca^{2+} saturated cells (β) was 7.5.

Experimental design

Ca^{2+} undershoot in isolated bovine airway smooth muscle cells was obtained by withdrawing caffeine, a ryanodine receptor agonist. Previous to any pharmacological treatment, a post-caffeine Ca^{2+} undershoot control recording was obtained for each cell. Then, some cells were incubated with cyclopiazonic acid (CPA, 5 μM ; a specific SERCA inhibitor) [17] during maximum post-caffeine Ca^{2+} undershoot drop. Two other groups of cells were incubated with either 20 μM 1-(β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole (SKF 96365; an inhibitor of passive Ca^{2+} entry in airway smooth muscle [1]) or econazole (5 μM , a potent inhibitor of capacitative Ca^{2+} entry in osteosarcoma cells [12]) with caffeine administration. Two different cell groups were preincubated with either genistein (10 μM , a phytoestrogen with many biological effects, including the inhibition of tyrosine kinase activity), or daidzein (negative control for genistein) 20 min before caffeine administration. Another group of cells were incubated with 1 mM Ni^{2+} (an inhibitor of capacitative Ca^{2+} entry in airway smooth muscle) [1] for 60 s immediately prior to caffeine withdrawal. At the end of the study, cells were stimulated with caffeine once more in presence of the administered drugs.

Drugs

Fura-2/AM, caffeine, CPA, ionomycin, econazole, and nickel chloride hexahydrate were obtained from Sigma (St. Louis, MO). SKF 96365 was obtained from Biomol (Plymouth Meeting, PA). Genistein was purchased from Gibco BRL (Rockville, MD), daidzein from Biomol (Plymouth Meeting, PA) and collagenase, elastase and desoxyribonuclease I from Boehringer-Mannheim (Indianapolis, IN). Fura-2/AM, CPA, econazole, genistein and ionomycin were dissolved in dimethylsulfoxide (final concentration 0.025%). In control experiments, dimethylsulfoxide had no effect.

Statistical analysis

Paired Student's *t* test was used. One- and two-ways analyses of variance followed by Bonferroni's or Dunnett's multiple comparisons tests were also used. Statistical significance was set at two-tailed $P < 0.05$. Data in text, figures and tables are expressed as mean $\pm$ standard error (SE).

Fig. 1. Ca^{2+} content in SR and Ca^{2+} entry rate during post-caffeine Ca^{2+} undershoot in bovine isolated airway smooth muscle. A) Original recording showing a typical Ca^{2+} undershoot trace after caffeine washout in an isolated myocyte. B) Representative recording showing a caffeine peak during maximum Ca^{2+} undershoot drop. C) Ca^{2+} maximum response induced by caffeine during Ca^{2+} undershoot drop in relation to the immediately previous caffeine-induced Ca^{2+} peak. * $P < 0.01$ compared to caffeine peak before Ca^{2+} undershoot (paired Student's t test). $[\text{Ca}^{2+}]_i$ = intracellular free Ca^{2+} concentration. Values are mean $\pm$ SE.

Results

Ca^{2+} content during Ca^{2+} undershoot recovery

Basal $[\text{Ca}^{2+}]_i$ level during resting of isolated airway smooth muscle cells was 137 ± 8 nM ($n = 39$). Caffeine (10 mM) incubation during 2 min induced a transient increment of $[\text{Ca}^{2+}]_i$ (754 ± 35 nM) that lasted 52 ± 6 s to return to basal $[\text{Ca}^{2+}]_i$ ($n = 39$; Fig. 1A). Caffeine wash-out produced a fast drop of $[\text{Ca}^{2+}]_i$ (68 ± 6 nM), that lasted 27 ± 3 s to reach to the maximum decrease ($n = 39$). Immediately after this drop, a slow recovery that took 129 ± 8 s to reach $[\text{Ca}^{2+}]_i$ basal levels at a rate of 1.2 ± 0.3 nM/s was observed ($n = 39$; Fig. 1A).

Caffeine response during maximum Ca^{2+} undershoot drop was significantly lower than the first caffeine response ($P < 0.01$, $n = 6$; Fig. 1B and C).

Effect of the inhibition of SR- Ca^{2+} uptake during Ca^{2+} undershoot recovery

CPA (5 μM) administration during the maximum $[\text{Ca}^{2+}]_i$ undershoot drop induced a fast Ca^{2+} undershoot recovery (rate 2.4 ± 0.6 nM/s) in comparison with control (rate 1.3 ± 0.2 nM/s; $P < 0.05$, $n = 9$; Fig. 2). Subsequent application of caffeine in presence of CPA signifi-

Fig. 2. Temporal course of the effect of cyclopiazonic acid (CPA, open squares) on Ca^{2+} undershoot in isolated bovine smooth muscle cells. CPA was administrated when maximum Ca^{2+} undershoot drop was reached (see inset in figure). Closed squares represent the control recording previous to CPA administration. The bars in the inset represent the incubation time with CPA (gray bars) or caffeine (black bar). * $P < 0.05$ and ** $P < 0.01$ in comparison with a previous Ca^{2+} undershoot in the same cell. Two-way ANOVA with Bonferroni's multiple comparisons test. $\Delta[\text{Ca}^{2+}]_i = [\text{Ca}^{2+}]_i$ changes in relation to baseline. Values are mean $\pm$ SE.

Fig. 3. Temporal course of the effect of econazole, genistein and SKF-96365 (all in open squares) on post-caffeine Ca^{2+} undershoot in isolated bovine smooth muscle cells. Genistein (A) was administrated 20 min before and maintained during and after caffeine stimulus (see inset). Econazole (B) and SKF-96365 (C) were administrated simultaneously to caffeine (see respective insets). The bars in the inset represent the incubation time with genistein, econazole and SKF-96365, respectively (gray bars) or caffeine (black bar). In all figures closed squares represent a previous control recording with vehicle at the point in time when econazole, genistein and SKF-96365 were added, respectively. $**P < 0.01$ and $*P < 0.05$ respectively in comparison with a previous Ca^{2+} undershoot in the same cell. Two-way ANOVA with Bonferroni's multiple comparisons test. $\Delta[\text{Ca}^{2+}]_i = [\text{Ca}^{2+}]_i$ changes in relation to baseline. Values are mean $\pm$ SE.

cantly reduced in $86.8 \pm 2.8\%$ the Ca^{2+} peak in comparison with control recording obtained previously in the same cell ($P < 0.01$, $n = 9$).

Effect of genistein, econazole, SKF-96365 and Ni^{2+} administration during Ca^{2+} undershoot

Genistein (20 μM) preincubation reduced significantly in $27.8 \pm 9.8\%$ the caffeine maximum peak in relation to control caffeine peak obtained previously in the same cell ($P < 0.05$). Additionally, genistein inhibited significantly the maximum Ca^{2+} drop during undershoot ($P < 0.01$) and maintained significantly higher $[\text{Ca}^{2+}]_i$ levels during undershoot recovery in comparison with a previous control recording ($P < 0.05$, $n = 5$; Fig. 3A). Daidzein (20 μM) modified the Fura-2 fluorescence; for this reason daidzein was not assayed on $[\text{Ca}^{2+}]_i$ measurements.

SKF-96365 (20 μM , $n = 5$) and econazole (5 μM , $n = 5$) incubation had no effect on the post-caffeine Ca^{2+} undershoot (Fig. 3B-C).

Incubation with Ni^{2+} (1mM) reduced progressively the $[\text{Ca}^{2+}]_i$ levels reaching a new $[\text{Ca}^{2+}]_i$ basal in 62 ± 5 s ($n = 4$, data not shown). The basal reduction induced by Ni^{2+} was similar with or without caffeine ($n = 4$; Fig. 4). Caffeine withdrawal during Ni^{2+} incubation induced a long lasting Ca^{2+} undershoot recovery when compare with control ($n = 9$; Fig. 4). Ni^{2+} had no effect on the $\text{Na}^+/\text{Ca}^{2+}$ exchanger reversal response induced by substituting NaCl for choline chloride in equimolar amounts ($n = 4$; data not shown). Additionally, substitution of 118 mM NaCl in PSS for 122.6 mM KCl, to revert the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, did not modify the Ca^{2+} undershoot in either absence ($n = 5$) or presence of Ni^{2+}

Fig. 4. Temporal course of the effect of Ni^{2+} incubation on post-caffeine Ca^{2+} undershoot in isolated bovine smooth muscle cells. Open squares represent $\Delta[\text{Ca}^{2+}]_i$ in presence of Ni^{2+} . Closed squares represent control Ca^{2+} undershoot obtained previously in the same cell. Arrows represent the beginning of Ca^{2+} undershoot. Inset displays a sample tracing detailing time points when either caffeine or Ni^{2+} were added or withdrawn. Values are mean $\pm$ SE.

	Ca ²⁺ undershoot recovery ratio	SR-Ca ²⁺ content ratio
Control (<i>n</i> = 9)	1.30 ± 0.15	96.7 ± 4.2
Econazole (<i>n</i> = 5)	0.98 ± 0.27	95.1 ± 8.8
Genistein (<i>n</i> = 5)	1.22 ± 0.33	71.4 ± 8.2*
SKF-96365 (<i>n</i> = 5)	0.93 ± 0.18	96.6 ± 6.8
Ni ²⁺ (<i>n</i> = 7)	0.14 ± 0.10*	31.3 ± 5.4**

Table 1. Effect of different drugs on post-caffeine Ca²⁺ undershoot recovery and the SR-Ca²⁺ loading in bovine smooth muscle cells. Ca²⁺ undershoot recovery ratio = Ca²⁺ undershoot recovery rate during drug administration/Ca²⁺ undershoot recovery rate in the control recording obtained previously in the same cell. SR-Ca²⁺ content ratio = caffeine peak during drug administration /caffeine peak obtained in the control recording obtained previously in the same cell. ***P* < 0.01, **P* < 0.05 in comparison with control group (one way ANOVA with Dunnett's multiple comparisons test).

(*n* = 5), when compared to a previous undershoot in the same cell and similar conditions (data not shown).

Ca²⁺ undershoot recovery and SR-Ca²⁺ content

A second caffeine stimulation after Ca²⁺ undershoot induced a Ca²⁺ peak (SR-Ca²⁺ content) as well as an undershoot similar to the preceding ones (Table 1). Caffeine peaks during genistein and Ni²⁺ administration were significantly lower than the previous caffeine peak (*P* < 0.01 for Ni²⁺, *n* = 7; *P* < 0.05 for genistein, *n* = 5; Table 1). Ni²⁺ incubation significantly reduced the Ca²⁺ undershoot recovery rate in comparison with the control group (*P* < 0.05, *n* = 7 for Ni²⁺ and *n* = 9 for control groups; Table 1). Genistein, SKF-96365 and econazole did not modify the Ca²⁺ undershoot recovery rate. SKF-96365 and econazole incubation did not affect the SR-Ca²⁺ content (Table 1).

Discussion

Our results suggest that in bovine tracheal smooth muscle capacitative Ca²⁺ entry is involved in Ca²⁺ undershoot recovery. Ca²⁺ undershoot is a common phenomenon in many cells, such as cardiac cells [6], smooth muscle cells [6, 7, 9, 10], endothelial cells [19], and neurons [8, 20]. This phenomenon can be triggered not only by caffeine but by noradrenaline [6], spontaneous Ca²⁺ transients, ATP, acetylcholine [10] and electrical stimu-

lation [6]. It is widely recognized that initial [Ca²⁺]_i drop during Ca²⁺ undershoot is a consequence of SR-Ca²⁺ refilling [6-10, 19, 20]. In this study we observed that when Ca²⁺ undershoot has reached the maximum drop, SR has stored around ~40% of its total Ca²⁺ content. Then, SR continues reloading Ca²⁺ during undershoot recovery. Additionally, the blockade of SERCA activity with CPA during undershoot drop induced a rapid increase of [Ca²⁺]_i; hence, SR Ca²⁺ reloading is likely to regulate the rate of Ca²⁺ undershoot recovery.

The deficit of intracellular Ca²⁺ produced by the refilling of Ca²⁺ in the SR is filled by external Ca²⁺ entry. Some mechanisms described in bovine airway smooth muscle SR-Ca²⁺ refilling are the reverse mode of Na⁺/Ca²⁺ exchanger [21], L-type channels [21, 22], passive [1] and capacitative Ca²⁺ entry [1, 23]. In a previous study in bovine airway smooth muscle, we discarded the participation of Na⁺/Ca²⁺ exchanger and L-type channels in SR-Ca²⁺ refilling during Ca²⁺ undershoot [7]. However, capacitative Ca²⁺ entry has not been evaluated in Ca²⁺ undershoot.

Capacitative Ca²⁺ entry is an important mechanism involved in SR-Ca²⁺ reloading and provides extracellular Ca²⁺ by opening plasma membrane Ca²⁺ channels through a signal activated by the empty SR [24, 25]. In bovine tracheal smooth muscle, CPA has been described to induce capacitative Ca²⁺ entry via a Gd³⁺- and La³⁺-sensitive, non-selective cation channel [23]. Moreover, in this tissue it has been observed that capacitative Ca²⁺ entry depends on the Ca²⁺ emptiness of SR [1, 24]. In this context, when SR has less than 50% of stored Ca²⁺, the main mechanism of Ca²⁺ entry is capacitative and sensitive to Ni²⁺ but unaffected by La³⁺, Mg²⁺, 2-APB, SKF-96365, methoxyverapamil or sustained K⁺-induced depolarisation. In fact, SKF-96365 mainly affected passive Ca²⁺ entry in those cells [1, 24]. In bovine airway smooth muscle cells, we observed that SR Ca²⁺ content was less than 50% at the beginning of Ca²⁺ undershoot recovery, and that Ni²⁺, but not SKF-96365, inhibited Ca²⁺ undershoot recovery. These findings suggest that capacitative Ca²⁺ entry might be involved in the recovery of basal Ca²⁺ levels during post-caffeine Ca²⁺ undershoot in bovine smooth muscle cells.

Econazole and genistein are known as capacitative Ca²⁺ entry inhibitors [13, 14, 26]. Nevertheless, econazole did not affect Ca²⁺ undershoot or SR-Ca²⁺ loading. Additionally, genistein did not induce any change in Ca²⁺ undershoot recovery, but affected both, Ca²⁺ undershoot drop and SR-Ca²⁺ refilling. This is likely to occur by an inhibition of SERCA activity by genistein as it has been

suggested previously [27]. Therefore, genistein and econazole are not involved in capacitative Ca^{2+} entry in bovine airway smooth muscle.

The role of SR-Ca^{2+} load in smooth muscle excitability is crucial. Low Ca^{2+} content in SR modifies muscle excitability in guinea pig ureteric smooth muscle. Additionally, one of the mechanisms involved in LTD_4 -induced airway hyperresponsiveness in bovine airway smooth muscle is by increasing SERCA activity [28]. We observed that Ni^{2+} and genistein induced Ca^{2+} under-

shoot alteration, either drop or recovery, and this change is associated with SR-Ca^{2+} load. Then, changes in Ca^{2+} undershoot have an impact on SR-Ca^{2+} loading which might affect in turn airway smooth muscle excitability.

In conclusion, our data suggest that the capacitative Ca^{2+} entry is involved in the post-caffeine Ca^{2+} undershoot recovery, and that it is regulated by SR-Ca^{2+} loading in bovine airway smooth muscle. Additionally, Ca^{2+} undershoot alterations are associated to changes in SR-Ca^{2+} loading.

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