Chloride Channel Blockers Inhibit Ca^{2+} Uptake by the Smooth Muscle Sarcoplasmic Reticulum

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ABSTRACT Despite the fact that Ca^{2+} transport into the sarcoplasmic reticulum (SR) of muscle cells is electrogenic, a potential difference is not maintained across the SR membrane. To achieve electroneutrality, compensatory charge movement must occur during Ca^{2+} uptake. To examine the role of Cl^- in this charge movement in smooth muscle cells, Ca^{2+} transport into the SR of saponin-permeabilized smooth muscle cells was measured in the presence of various Cl^- channel blockers or when I^-, Br^-, or SO_4^{2-} was substituted for Cl^-. Calcium uptake was inhibited in a dose-dependent manner by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and by indanyloxyacetic acid 94 (R(+)-IAA-94), but not by niflumic acid or 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS). Smooth muscle SR Ca^{2+} uptake was also partially inhibited by the substitution of SO_4^{2-} for Cl^-, but not when Cl^- was replaced by I^- or Br^-. Neither NPPB nor R(+)-IAA-94 inhibited Ca^{2+} uptake into cardiac muscle SR vesicles at concentrations that maximally inhibited uptake in smooth muscle cells. These results indicate that Cl^- movement is important for charge compensation in smooth muscle cells and that the Cl^- channel or channels involved are different in smooth and cardiac muscle cells.

INTRODUCTION

In muscle cells, regulation of Ca^{2+} by the sarcoplasmic reticulum (SR) is essential for normal contractile function. The finding that the SR membrane in striated muscle is permeable to a number of ions has led to the conclusion that, even though a free-Ca^{2+} gradient of 10^3 or greater exists between the SR lumen and the cytoplasm in resting muscle (see Schatzmann, 1989; Somlyo and Himpens, 1989; Kargacin and Kargacin, 1995; Shannon and Bers, 1997), a membrane potential is unlikely to develop across the SR membrane (see, for example, Meissner and McKinley, 1982; Garcia and Miller, 1984). This conclusion is supported by the work of Russell et al. (1979a,b), who used voltage-sensitive probes but were unable to obtain clear evidence of a sustained potential across the SR membrane during Ca^{2+} uptake or release processes (Russell et al., 1979b). Other, more indirect findings also support this conclusion. It has been shown, for example, that reconstituted SR vesicles lacking ion channels are unable to take up Ca^{2+} unless ionophores allowing compensatory charge movement are also present in the membrane (Morimoto and Kasai, 1986; also see Beeler, 1980; Somlyo et al., 1981; Feher and Fabiato 1990; Tada and Kadoma, 1995; Kourie et al., 1996a,b). Work from a number of laboratories indicates that H^+ efflux from the SR is coupled to Ca^{2+} uptake (Chiesi and Inesi, 1980; Inesi and Hill, 1983; Yu et al., 1993; Zimniak and Racker, 1978; Beeler, 1980; Yamaguchi and Kanazawa, 1984, 1985; Levy et al., 1990; da Costa and Madeira, 1994; see also Hartung et al., 1997). Estimates of the stoichiometry of this process range from $1H^+$:$1Ca^{2+}$ (Chiesi and Inesi, 1980; Yu et al., 1993; da Costa and Madeira, 1994) to $3H^+$:$2Ca^{2+}$ (Levy et al., 1990) and indicate that the SR Ca^{2+} pump, acting in isolation, would transport net positive charge into the SR. The membrane potential (~50 mV, inside positive; Yu et al., 1993; also see Zimniak and Racker, 1978; Beeler, 1980; Morimoto and Kasai, 1986) that develops across the membrane of proteoliposomes that contain skeletal muscle SR Ca^{2+} pumps but are impermeant to other ions is consistent with this conclusion and with a stoichiometry of $1H^+$:$1Ca^{2+}$ for the pump (Yu et al., 1993). For the overall Ca^{2+} uptake process to be electrically neutral it would be necessary for additional charge movement to take place. This charge is likely to be provided by the movement of K^+ out of, and/or the movement of Cl^- into, the SR. The SR membranes in striated muscle have been shown to be permeable to Cl^- as well as H^+, Na^+ and K^+ (Meissner and McKinley, 1982; Fink and Stephenson, 1987; Yu et al., 1993; see also Kourie et al., 1996a,b), and a number of ion channels associated with striated muscle SR (reviewed in Kourie et al., 1996b) and the endoplasmic reticulum of nonmuscle cells (Clark et al., 1997) have been characterized electrophysiologically. Although the specific channels or channel types active during Ca^{2+} uptake have not been elucidated, measurements of the permeability of skeletal muscle SR vesicles to various ions indicated that Cl^- is ~50 times more permeable than K^+ (Kasai and Kometani, 1979). This suggests that Cl^- influx is more important than K^+ efflux in maintaining electroneutrality during SR Ca^{2+} uptake. This hypothesis is also consistent with the results of Fink and Stephenson (1987), who found that the K^+ channel inhibitors tetraethylammonium, 4-aminopyridine, procaine, and decamethonium increased, rather than decreased, the amount of Ca^{2+} that

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could be released from the SR of skinned skeletal muscle fibers (see discussion by Kourie et al., 1996a,b).

The SR Ca^{2+} pump in smooth muscle is closely related to the cardiac form of the enzyme (reviewed by Raeymaekers and Wuytack, 1995), and it is thought that Ca^{2+} regulation by the SR of smooth muscle is generally similar to that in striated muscle; however, the involvement of K^+ and Cl^- in charge compensation across the SR membrane of smooth muscle cells during Ca^{2+} uptake has received relatively little experimental attention. The work reported here was done using a saponin-skinned isolated cell preparation and was undertaken to determine if Cl^- acts as a compensatory ion during SR Ca^{2+} uptake in smooth muscle and to characterize the Cl^--permeant pathway. Our results show that SR Ca^{2+} uptake can be inhibited by the Cl^- channel blockers 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and indanyloxyacetic acid 94 (R(+)-IAA-94) and partially inhibited by the substitution of SO_4^{2-} for Cl^-. This is consistent with the hypothesis that Cl^- movement plays an important role in charge compensation in smooth muscle cells during SR Ca^{2+} uptake.

MATERIALS AND METHODS

Smooth muscle cell isolation

Stomachs were excised from rabbits sacrificed with an overdose of phenobarbital. Stomachs were immediately emptied of their contents and flushed with Hanks' balanced salt solution (HBSS). A small, healthy piece of tissue (identified by a mucosa of uniform pink color that loosely covered the underlying muscle) was cut to a piece ~5 cm in diameter. The mucosa was removed, and the gastric smooth muscle was secured on an O-ring with dissecting pins to ensure uniform exposure to the enzymatic solution during the digestion process. The tissue was incubated in a solution of 0.2% collagenase and 0.2% DNase in HBSS for 1 h at 37°C. The tissue was then triturated in HBSS and transferred to a second solution containing 0.2% protease and 0.2% DNase in HBSS and was incubated for 30 min at 37°C. The tissue was next cut into ~1 mm^2 pieces and triturated to release individual cells; the resulting cell suspension was filtered through cheesecloth to separate the isolated cells from undigested tissue. EGTA (final concentration, 2.5 mM) was added to the cell suspension, and the suspension was centrifuged at 10 × *g* in a benchtop centrifuge for 5 min. The supernatant was aspirated away, and the cells were resuspended in rigor buffer and skinned as described previously (Kargacin and Fay, 1987; Kargacin and Kargacin, 1995) with saponin (50 μg/ml) during a 5-min centrifugation at 10 × *g*. To wash away saponin, the resulting pellet was resuspended in rigor buffer, incubated for 5 min, then centrifuged (10 × *g*) for 5 min. After the wash in rigor buffer, the cells were washed three times in uptake buffer (see below). For each wash, buffer was added to the pellet from the previous centrifugation, and the resulting cell suspension was allowed to equilibrate on ice for 10 min before centrifugation (5 min at 10 × *g*), aspiration, and addition of fresh buffer for the next wash. After the final wash and centrifugation, the cells were resuspended in 500 μl to 1 ml of uptake buffer. This procedure selectively permeabilizes the plasma membrane and allows measurement of ATP-dependent Ca^{2+} uptake into the SR of smooth muscle cells (Kargacin and Kargacin, 1995).

Measurement of smooth muscle SR Ca^{2+} uptake

Ca^{2+} uptake into the sarcoplasmic reticulum of the isolated cells was measured as described previously (Kargacin and Kargacin, 1995). Briefly, 50 μl of saponin-permeabilized, isolated cells in uptake buffer was added to a small chamber on the stage of an inverted microscope, and background light scatter and fluorescence at 510 nm were measured. Fura-2 (final concentration 7.5 μM) was then added to the chamber. Uptake was initiated with the addition of (in final concentrations) 12 mM ATP, 12 mM creatine phosphate (CP), and 19 U/ml creatine phosphokinase (CPK). The contents of the chamber were continually stirred throughout an experiment with a small stirrer mounted above the chamber (see Kargacin and Kargacin, 1995). A fluorimeter (SPEX CMX model; Edison NJ), alternating between 340-nm and 380-nm excitation wavelengths, was used as a light source for the experiments. Emission was measured at 510 nm (through a 10-nm bandpass filter) with a photomultiplier. Ca^{2+} uptake by the SR of the skinned cells resulted in a decrease in the 340/380 fluorescence ratio over the duration of an experiment (see below).

Fura-2 calibration and determination of $[Ca^{2+}]_{free}$, $[Ca^{2+}]_{total}$, and uptake rate for smooth muscle experiments

The free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) in the uptake buffer at each time point was determined from the 340/380 ratio (R) according to the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_{free} = K_{D(Ca2+)} \cdot \beta \cdot (R - R_{min})/(R_{max} - R) \quad (1)$$

where R_{max} is the fura-2 340/380 fluorescence ratio measured in saturating Ca^{2+} buffer (containing 95 mM KCl, 20 mM HEPES-K, 2.5 mM $CaCl_2$, and 10 mM $MgCl_2$; pH 7.0); R_{min} is the 340/380 ratio for fura-2 measured in a Ca^{2+}-free solution (25 mM EGTA in uptake buffer); and β is the ratio of 380-nm fluorescence intensity measured in Ca^{2+}-free solution to the 380-nm fluorescence intensity in saturating Ca^{2+} buffer. $K_{d(Ca2+)}$ was 200 nM (Williams et al., 1987). R_{min} and R_{max} calibrations were made each day of experimentation to correct for any changes in the relative outputs of 340-nm and 380-nm light from the excitation light source; β was determined for each lot of fura-2. The total Ca^{2+} concentration ($[Ca^{2+}]_{total}$) in the chamber at each time point was calculated from $[Ca^{2+}]_{free}$, as described in Kargacin and Kargacin (1995), using a set of simultaneous equations that included constants for the binding of Ca^{2+}, Mg^{2+}, and H^+ by fura-2, ATP, and CP. Values for the binding constants were taken from Smith and Martell (1975), Fabiato (1981), and Martell and Smith (1982). For each experiment, the maximum Ca^{2+} uptake rate (pmol-Ca^{2+}/s) was determined from the negative slope of the $[Ca^{2+}]_{total}$ versus time curve:

$$\text{Velocity} = (\text{volume in chamber}) \times -\Delta[Ca^{2+}_{total}]/\Delta t \quad (2)$$

To eliminate errors due to noise inherent in the fluorescence measurements, the slope was determined from 10–50 data points on the steepest part of an uptake curve. Fig. 1 illustrates the calculation of maximum uptake rate for a typical control experiment. Fig. 1 *A* shows the raw fluorescence data after correction for background fluorescence and light scatter (recorded as a decline in 340/380 fluorescence ratio), Fig. 1 *B* shows the decline in $[Ca^{2+}]_{free}$ in the buffer as a function of time determined from the curve in Fig. 1 *A*, using Eq. 1, and Fig. 1 *C* shows the decline in $[Ca^{2+}]_{total}$ in the buffer during the experiment. The larger open circles in Fig. 1 *C* show the data points used to determine the maximum uptake rate for the experiment. For this experiment, the maximum uptake rate was 7.44 pmol/s. The maximum rate of SR uptake was dependent upon the amount of SR in a particular cell preparation (a function of the cell density of the preparation), but, for control samples, generally fell between 15 and 40 pmole-Ca^{2+}/s·mg protein when expressed relative to the amount of protein in a cell preparation. In the work presented below, the results for each separate cell preparation were expressed as a percentage of the mean rate of uptake in the control experiments for that preparation, so that results obtained from different cell preparations could be combined. Unless noted otherwise, statistical significance was defined as $p \leq 0.01$, using Student's *t*-test. Results are given as ±1 SD. All experiments were conducted at 22°C.

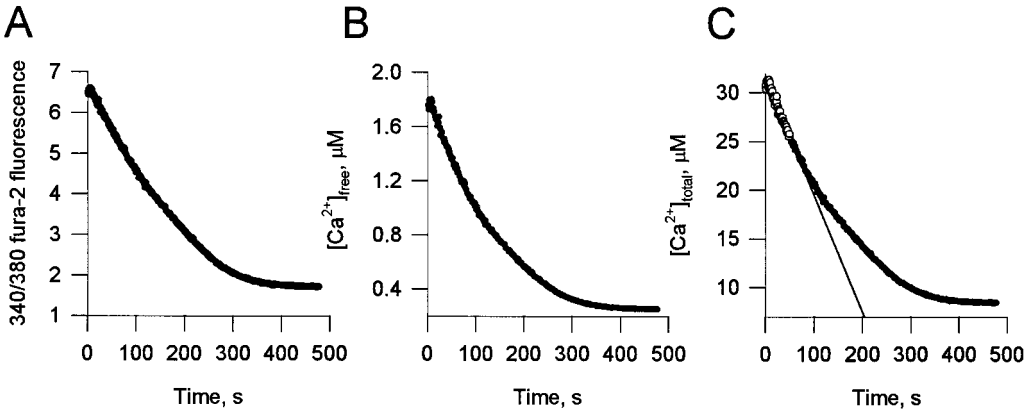

FIGURE 1 Determination of Ca^{2+} uptake rates for isolated cell experiments. (*A*) 340/380 fluorescence ratio versus time, after correction for background fluorescence and light scatter. (*B*) Ca^{2+}_{free} versus time, determined from Eq. 1. (*C*) Ca^{2+}_{total} versus time, determined from the curve in *B* and the binding of Ca^{2+} to various buffer components (see Materials and Methods). The points shown by the larger open symbols were used to determine the maximum rate of change in $[Ca^{2+}]_{total}$ for the experiment. For the experiment shown, the slope was −0.120 μM/s, and the volume in the chamber was 62 μl, giving a maximum uptake rate of 7.44 pmol/s.

Solutions for smooth muscle experiments

HBSS contained (in mM) 5 KCl, 0.3 KH_2PO_4, 138 NaCl, 5.6 D-glucose, 12.5 taurine, and 4 $NaHCO_3$ (pH 7.0). Rigor buffer contained (in mM) 150 K-methanesulfonate, 1 Mg-methanesulfonate, 5 EGTA, 20 piperazine-N,N′-bis(2-ethanesulfonic acid (pH 7.0). Uptake buffer was made using ultrapure chemicals and double-distilled water and contained (in mM) 100 KX, 10 MgX_2, 20 HEPES (pH 7.0) (where X represents Cl^-, I^-, or Br^-). For experiments in SO_4^{2-} buffer, KCl and $MgCl_2$ were replaced by 30 mM K_2SO_4 and 10 mM $MgSO_4$ (to maintain ionic strength). ATP (K_2 salt) and CP (Na salt) were dissolved in H_2O, and the solution was brought to a pH of 7.0 with KOH. Creatine phosphokinase was then added. Fura-2 was dissolved in double-distilled water. NPPB, R(+)-IAA-94, and niflumic acid were dissolved in 95% ethanol/5% H_2O. The final concentration of ethanol in the experiments with NPPB or R(+)-IAA-94 did not exceed 1.5%. This concentration of ethanol had no effect on the excitation spectra of fura-2 or on uptake rates in control experiments. 4,4′-Dinitrostilbene-2,2′-disulfonic acid (DNDS) was dissolved in uptake buffer.

D-Glucose, taurine, collagenase IV, protease, EGTA, methanesulfonic acid, PIPES dipotassium salt, saponin, ATP, CPK, and CP were obtained from Sigma Chemical Co. (St. Louis, MO). DNase was obtained from Boehringer-Mannheim (Laval, QC). To minimize Ca^{2+} contamination, uptake buffers were made from AnalaR grade KCl, and KBr and suprapur KI obtained from BDH (Toronto, ON) and puriss $MgSO_4 \cdot 7H_2O$, microselect $MgCl_2 \cdot 6H_2O$, $MgBr_2 \cdot 6H_2O$, and HEPES-K^+ salt and MgI_2 obtained from Fluka (Ronkonkoma, NY). Analytical reagent grade KH_2PO_4, $NaHCO_3$, NaCl, and $Mg(OH)_2$, and aristar grade KOH and H_2SO_4 were obtained from BDH (Toronto, ON). Fura-2 free acid and DNDS were obtained from Molecular Probes (Eugene, OR). NPPB was obtained from ICN (Montreal, QC), and R(+)-IAA-94 was obtained from RBI (Natick, MA).

Preparation of cardiac SR vesicles and measurement of cardiac SR uptake

Canine cardiac SR vesicles were prepared using the method of Chamberlain et al. (1983) as described previously (Kargacin and Kargacin, 1994). Vesicles were stored at −80°C in a storage buffer containing 300 mM sucrose, 100 mM KCl, 5 mM histidine, and 0.5 mM dithiothreitol (pH 7.1). The protein concentrations of the vesicle samples used in the experiments were determined with the Bradford protein assay. Standard curves were obtained with known concentrations of bovine serum albumin (BSA).

Ca^{2+} uptake into the cardiac SR vesicles was measured as described previously (Kargacin and Kargacin, 1994) in 3-ml cuvettes in the sample compartment of a SPEX fluorimeter. Vesicles (~275 μg total protein) were added to a cuvette containing 2 ml of vesicle uptake buffer (100 mM KCl, 4 mM $MgCl_2$, and 20 mM HEPES, pH 7.0). After K_2ATP (final concentration 1.8 mM), CP (final concentration 1.8 mM), CPK (final concentration 3.1 U/ml), and fura-2 (final concentration 2.9 μM) were added to the cuvette. Uptake was initiated by the addition of Ca^{2+}. For the experiments with NPPB or R(+)-IAA-94, the blockers were added to the cuvette before the addition of the vesicles. $[Ca^{2+}]_{free}$ was determined from Eq. 1 with values for R_{max}, R_{min}, and β obtained with 0-Ca^{2+} (vesicle uptake buffer containing 25 mM EGTA) and high-Ca^{2+} (vesicle uptake buffer containing 2.5 mM Ca^{2+}) buffers. Vesicle experiments were carried out at 22°C.

RESULTS

Excitation spectra measurements with NPPB, R(+)-IAA-94, and DNDS

The excitation spectra of the uptake buffers containing high (mM), zero, or intermediate Ca^{2+} concentrations were measured in the presence and absence of NPPB, R(+)-IAA-94, niflumic acid, or DNDS to determine if these agents were fluorescent. Excitation light was scanned from 300 nm to 400 nm, and emission was measured at 510 nm. Fig. 2 *A* shows that NPPB did not contribute to background fluorescence. For comparison, the fluorescence intensity from 2.9 μM fura-2 was typically 500,000–700,000 cps. Neither R(+)-IAA-94 nor niflumic acid detectably altered the background fluorescence in the absence of fura-2 (result not shown). DNDS (50 μM) caused a small increase in background fluorescence that was greater at 380-nm excitation than at 340 nm. At 380-nm excitation, the background increased from ~700 cps in the absence of DNDS to ~2500 cps in the presence of DNDS.

Fura-2 excitation spectra were also measured in the presence and absence of NPPB, R(+)-IAA-94, niflumic acid, or

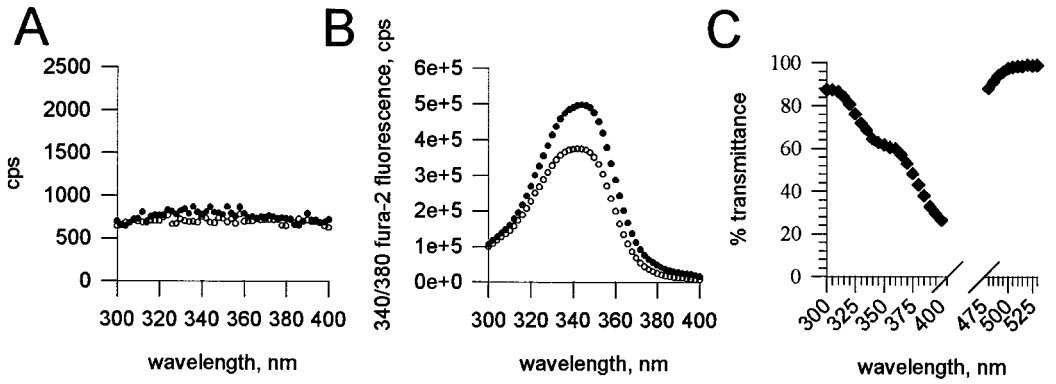

FIGURE 2 (*A*) Effect of 100 μM NPPB on background fluorescence over excitation wavelengths between 300nm and 400 nm. ●, Background fluorescence in uptake buffer alone. ○, Background fluorescence in uptake buffer containing 100 μM NPPB. (*B*) Excitation spectra of 3.0 μM fura-2 with and without 100 μM NPPB. ●, Fura-2 fluorescence in uptake buffer. ○, Fura-2 fluorescence when 100 μM NPPB was added to uptake buffer. (*C*) Absorbance spectrum of 100 μM NPPB in uptake buffer at excitation wavelengths of 300–400 nm and 480–530 nm. In *A* and *B* emission was measured at 510 nm through a 10-nm bandpass filter.

DNDS to determine if these compounds altered fura-2 fluorescence. As can be seen in Fig. 2 *B*, NPPB (100 μM) changed the fura-2 excitation spectra in uptake buffer. This effect was further analyzed by examining the absorbance spectrum of NPPB. Fig. 2 *C* shows that NPPB absorbed more strongly at 380 nm than at 340 nm. Compared to buffer alone, percentage transmittance was decreased by ~60% at 380 nm; at 340 nm, percentage transmittance was decreased by ~35% compared to buffer alone (Fig. 2 *C*). To correct for the difference in absorbance of NPPB at 340 nm compared to 380 nm, R_{min} and R_{max} (see Materials and Methods) were measured in the presence of NPPB for each NPPB concentration used. The effect of 75 μM R(+)-IAA-94 on the excitation spectrum of 3 μM fura-2 in uptake buffer is shown in Fig. 3. Although there was a significant change in fluorescence intensity at wavelengths below ~330 nm, R(+)-IAA-94 only slightly altered fura-2 fluorescence at 340 nm. Both niflumic acid and DNDS absorbed more strongly at 340 nm than at 380 nm (results not shown). Therefore, as was the case with NPPB (see above), R_{min} and R_{max} were measured in the presence of each of the blockers at each concentration used.

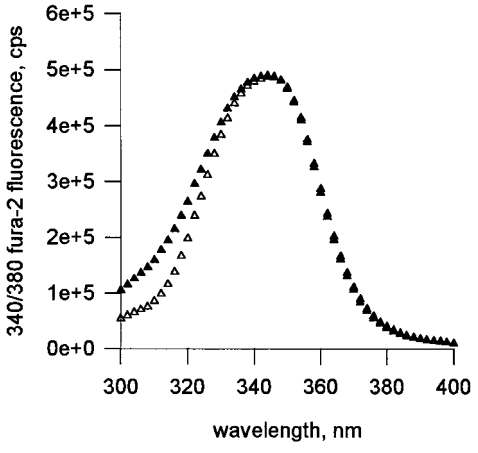

FIGURE 3 Excitation spectra of 2.9 μM fura-2 with and without 75 μM R(+)-IAA-94. ▲, Fura-2 fluorescence in uptake buffer. △, Fura-2 fluorescence when 75 μM R(+)-IAA-94 was present in the uptake buffer. Emission was measured at 510 nm through a 10-nm bandpass filter.

Effect of NPPB on smooth muscle SR Ca^{2+} uptake

NPPB has been used to block sarcolemmal Cl^- channels in a variety of tissues (see, for example, Lukacs et al., 1991; Sorota, 1994). The K_i's for Cl^- channel inhibition range from 0.1 μM to 100 μM (Lukacs et al., 1991). To examine the effects of NPPB on smooth muscle SR Ca^{2+} uptake, NPPB concentrations ranging from 6 μM to 100 μM were used. NPPB was added and mixed with the cell suspension 1 min before ATP was added to initiate Ca^{2+} uptake.

Fig. 4 *A* shows typical traces of $[Ca^{2+}]_{free}$ versus time for a control experiment and an experiment done in the presence of 20 μM NPPB. Uptake rate in the presence of NPPB was 24% of that measured in the control experiment. In the control experiment, maximum Ca^{2+} uptake rate was 7.6 pmol/s; in the presence of 20 μM NPPB, the maximum uptake rate was 1.9 pmol/s. Fig. 4 *B* shows the results from an experiment on one cell preparation in which the maximum rate of Ca^{2+} uptake was determined in the presence of various concentrations of NPPB. For this experiment, uptake rate was half-maximum in the presence of 10 μM NPPB. When results from five similar experiments were combined, the uptake rate was half-maximum with 15 μM NPPB. The uptake rate was reduced to 12 ± 7% (n = 4) of control at NPPB concentrations ≥ 75 μM (see Table 1).

Effect of R(+)-IAA-94 on smooth muscle SR Ca^{2+} uptake

Indanyloxyacetic acid (IAA) derivatives (at concentrations ranging from 1 μM to 200 μM) have been found to block

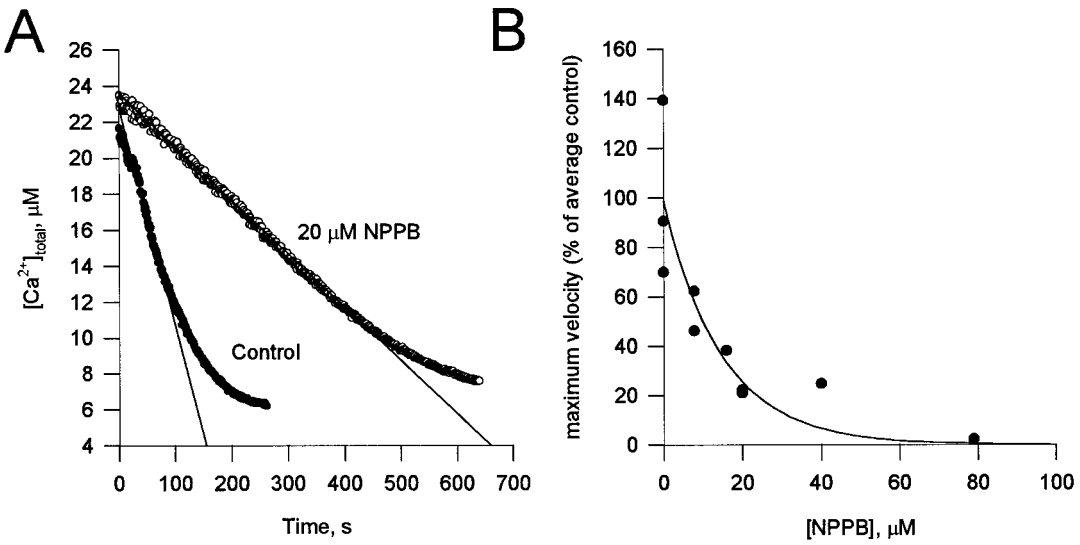

FIGURE 4 Inhibition of smooth muscle SR Ca^{2+} uptake by NPPB. (*A*) Comparison of uptake rates in control (●) and in the presence of 20 μM NPPB (○). The slopes of the lines are the maximum rates of uptake determined from the curves. The uptake rate in the control experiment was 7.6 pmol/s; the uptake rate in the presence of NPPB was 1.9 pmol/s. (*B*) Concentration dependence of the inhibition of SR Ca^{2+} uptake with NPPB for one cell preparation. The solid line is an exponential curve fit to the data points; half-maximal inhibition was at ~10 μM NPPB.

plasmalemmal and intracellular membrane Cl^- channels in a variety of tissues, including kidney, trachea, and heart (see, for example, Landry et al., 1989; Redhead et al., 1992; Weber-Schürholz et al., 1993; Reeves and Gurich, 1994; Sorota, 1994; Takenaka et al., 1996; Clark et al., 1997). These compounds appear not to have been tested on smooth muscle SR Cl^- channels, however. Experiments on saponin-permeabilized smooth muscle cells demonstrated a dose-dependent decrease in Ca^{2+} uptake with increasing concentrations of R(+)-IAA-94. Fig. 5 shows the results of experiments in which the rate of Ca^{2+} uptake was measured in the absence and the presence of R(+)-IAA-94. The maximum uptake rate in the control experiment was 7.0 pmol/s. In the presence of 47 μM R(+)-IAA-94 this rate was reduced to 2.0 pmol/s. The inhibitory effect of R(+)-IAA-94 was dose dependent. Combined results from six cell preparations showed that the uptake rate was half-maximum at [R(+)-IAA-94] equal to 46 μM; [R(+)-IAA-94] ≥ 190 μM reduced the uptake rate to 7 ± 9% (n = 9) of control (see Table 1).

SR Ca^{2+} uptake in smooth muscle in the presence of niflumic acid and DNDS

Niflumic acid and stilbene derivatives have been used to block Cl^- channels in a variety of tissues. Niflumic acid at a concentration of 30 μM had no detectable effect on SR Ca^{2+} uptake in smooth muscle cells (Table 1). The stilbene derivative DNDS was also without effect on SR Ca^{2+} uptake at concentrations as high as 300 μM and incubation times with the cells of up to 30 min. In control experiments, maximum uptake rates ranged from 4.45 to 4.8 pmol/s; in the presence of 300 μM DNDS, maximum uptake rates ranged between 4.55 and 4.65 pmol/s.

Smooth muscle SR Ca^{2+} uptake in the presence of I^-, Br^-, or SO_4^{2-}

Smooth muscle SR Ca^{2+} uptake was also examined when the Cl^- in the uptake buffer was completely replaced by I^-, Br^-, or SO_4^{2-} (results are summarized in Table 1). Uptake rates in I^- or Br^- buffers were not significantly different from those measured in Cl^- buffer. In I^- uptake buffer, the maximum uptake rate was 90 ± 16% (n = 4) of the mean maximum uptake rate in control experiments; in Br^- uptake buffer, the maximum uptake rate was 102 ± 20% (n = 4) of the mean maximum uptake rate in control experiments. The maximum rate of SR Ca^{2+} uptake was reduced when Cl^- in the uptake buffer was replaced with SO_4^{2-}. The maximum uptake rate in SO_4^{2-} buffer was 80 ± 11% (n = 5) of the mean maximum uptake rate in control experiments. This was significant at p = 0.03.

TABLE 1 Inhibition of smooth muscle SR Ca^{2+} uptake by Cl^- channel blockers or anion substitutions

Blocker or anion	NPPB (≥75 μM)	R(+)-IAA-94 (≥190 μM)	Niflumic acid (30 μM)	DNDS (300 μM)	I^-	Br^-	SO_4^{2-}
Uptake rate: % control	12	7	100	100	100	100	80

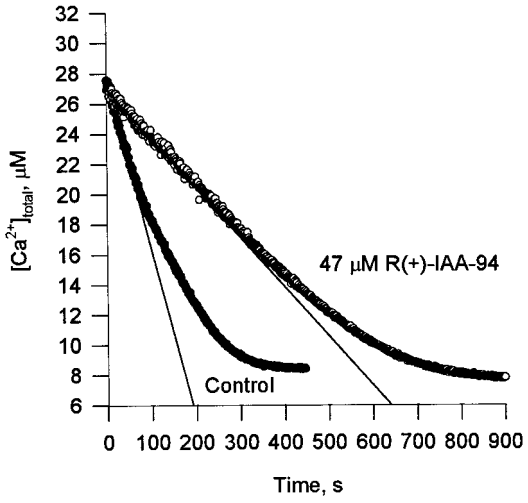

FIGURE 5 Inhibition of smooth muscle SR Ca^{2+} uptake by R(+)-IAA-94. (*A*) Comparison of uptake rates in control (●) and in the presence of 47 μM R(+)-IAA-94 (○). The slopes of the lines are the maximum rates of uptake determined from the curves. The uptake rate in the control experiment was 7.0 pmol/s; the uptake rate in the presence of R(+)-IAA-94 was 2.0 pmol/s.

The inhibitory effect of NPPB on SR Ca^{2+} uptake was also seen in buffers in which Cl^- was replaced with I^- or SO_4^{2-}. In one experiment, the uptake rate in SO_4^{2-} buffer containing 96 μM NPPB was 1.5% of the maximum uptake rate measured in SO_4^{2-} buffer without NPPB. This percentage inhibition was similar to that measured in Cl^- buffers (see Table 1). A similar result was obtained when NPPB was added to I^- uptake buffer.

Ca^{2+} uptake by canine cardiac SR vesicles in the presence of NPPB or R(+)-IAA-94

In previous electrophysiological studies, Townsend and Rosenberg (1995) showed that NPPB blocked an SR Cl^- channel from porcine cardiac myocytes. This block, however, appeared to be from the lumenal side of the SR membrane. Weber-Schürholz et al. (1993) reported that R(+)-IAA-94 inhibited a Cl^- channel that appeared to be present in the sarcolemmal but not the SR membrane of skeletal muscle. Because both NPPB and R(+)-IAA-94 inhibited Ca^{2+} uptake into smooth muscle SR in the functional studies reported here, the effects of these blockers on Ca^{2+} uptake into canine cardiac SR vesicles was examined. Neither of these agents, at concentrations that almost completely inhibited smooth muscle SR uptake, had a significant effect on the rate of Ca^{2+} uptake into cardiac SR vesicles. Ca^{2+} uptake in the presence of 100 μM NPPB was 94 $\pm$ 35% (n = 8) of the mean maximum uptake rate in control experiments. R(+)-IAA-94 also failed to inhibit cardiac SR Ca^{2+} uptake (in the presence of 226 μM R(+)-IAA-94, the maximum uptake rate was 98.3% of the control rate).

DISCUSSION

In previous work (Kargacin et al., 1988; Kargacin and Kargacin, 1994; 1995) it was shown that the methods employed in the present study could be used to study Ca^{2+} uptake into the SR of isolated, saponin-permeabilized smooth muscle cells and striated muscle SR vesicles. In the work on smooth muscle (Kargacin and Kargacin, 1995), it was shown that uptake was Ca^{2+} and ATP dependent and could be blocked by thapsigargin. Uptake was not inhibited by the mitochondrial blockers FCCP or azide.

In the present study, it was found that SR Ca^{2+} uptake in smooth muscle could be almost completely blocked by the Cl^- channel blockers NPPB and R-IAA-94. On the other hand, neither the stilbene derivative DNDS nor niflumic acid had any measurable effects on SR Ca^{2+} uptake. Maximum uptake rate was also partially reduced by the replacement of Cl^- by SO_4^{2-}, but not in I^- or Br^- uptake buffers. A functional assay such as the one used in the present study has the advantage that it can provide information about the properties of the anion-permeant pathway or pathways that are important for charge compensation in vivo that cannot be determined solely from the study of single channels in isolation. However, because the movement of Ca^{2+} rather than Cl^- is measured, one must consider the possibility that the ion channel inhibitors acted on the SR Ca^{2+} pump or at other sites on the SR membrane rather than on Cl^- channels. To our knowledge, there have been no reports of a direct action of NPPB or R(+)-IAA-94 on the SERCA Ca^{2+}-ATPases in any tissue. The SERCA2b Ca^{2+} pump found in smooth muscle is identical for most of its amino sequence to the SERCA2a Ca^{2+} pump of cardiac muscle. The two isoforms differ only in the C-terminal region, where a 4 amino acid segment of SERCA2a is replaced by a 49 amino acid hydrophobic segment in SERCA2b (reviewed in Raeymaekers and Wuytack, 1995). Because NPPB and R(+)-IAA-94 both inhibited Ca^{2+} uptake in smooth but not in cardiac muscle, it seems unlikely that the inhibitory actions of these agents were due to a direct inhibition of the Ca^{2+} pump, unless both agents act nonspecifically on the unique amino acid segment of the SR Ca^{2+}-ATPase of smooth muscle. It might also be argued that the different effects of NPPB and R(+)-IAA-94 on smooth and cardiac muscle SR Ca^{2+} uptake were the result of comparing experiments using SR vesicles with those using permeabilized isolated cells. In preliminary experiments (S. V. Phillips, G. J. Kargacin, and M. E. Kargacin) it was found that SR Ca^{2+} uptake in isolated rat cardiac myocytes was not inhibited by either of the Cl^- channel blockers, a result consistent with that obtained with cardiac SR vesicles. Lukacs et al. (1991) found that NPPB can act directly on membranes as a proton ionophore at concentrations (25 μM or greater) that have been used to block Cl^- channels, and high concentrations of IAA (500 μM to 1 mM) have been shown to open K^+ channels in smooth muscle (Toma et al., 1996). If the effect of NPPB on smooth muscle SR Ca^{2+} uptake were due to its protonphoric prop-

erties, we would have expected to see a similar effect on the cardiac muscle SR vesicles. This, however, was clearly not the case. The concentration of R(+)-IAA-94 (200 μM) that had a maximum inhibitory effect on SR Ca^{2+} uptake in smooth muscle was less than that (>500 μM) used by Toma et al., (1996) to open K^+ channels. Furthermore, if R(+)-IAA-94 had opened a K^+-permeant pathway in the SR, we would have expected to see an increase rather than a decrease in the rate of Ca^{2+} uptake in the smooth muscle cells. When the effects of both Cl^- channel blockers are taken together, our results appear to be most consistent with the interpretation that they acted on an anion channel in the smooth muscle SR.

Because smooth muscle SR Cl^- channels have received relatively little experimental attention, prior information was not readily available to assess the likely action of any of the Cl^- channel blockers on SR Ca^{2+} uptake in smooth muscle. As noted above, Townsend and Rosenberg (1995) showed that NPPB (10–50 μM; on the luminal side of the SR membrane) blocked an SR Cl^- channel from porcine cardiac myocytes. Luminal NPPB (but not NPPB applied to the cytoplasmic side of the SR membrane) decreased channel open probability with a K_i of 52.6 μM (Townsend and Rosenberg, 1995). This result is consistent with our finding that SR Ca^{2+} uptake in cardiac muscle was not blocked by NPPB applied to the outside (cytoplasmic side) of SR vesicles. If the Cl^- channels in the cardiac SR membrane are similar to those found in skeletal muscle, our results on cardiac muscle are in agreement with those of Weber-Schürholz et al. (1993), who did not find R(+)-IAA-94-sensitive channels in skeletal muscle SR membranes. Our results on smooth muscle are consistent with the presence of an anion channel in the SR membrane that is sensitive to both NPPB and R(+)-IAA-94. Although we cannot completely rule out the possibility that the properties of the Cl^--permeant pathway we have determined represent the combined properties of more than one anion channel, our results could be explained by the presence of a Cl^- channel similar to the channel recently described by Clark et al. (1997) that is found in the endoplasmic reticulum of rat brain. This channel was blocked by NPPB at concentrations ranging from 10 μM to 100 μM and by R(+)-IAA-94 over a concentration range between 25 μM and 200 μM (K_i for block = 35 μM). NPPB blocked from either side of the membrane; R(+)-IAA-94 blocked from the cytoplasmic side of the membrane. Consistent with our results, the channel was insensitive to niflumic acid and was permeant to Br^-. Unlike our result with DNDS, the rat brain channel was blocked by the stilbene derivative 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), however.

Because we were able to almost completely inhibit SR Ca^{2+} uptake in smooth muscle with either NPPB or R(+)-IAA-94, it seems reasonable to conclude that Cl^- movement plays a more important role than K^+ movement in charge compensation during Ca^{2+} uptake. This is consistent with the suggestion by Kourie et al. (1996a,b) that Cl^- movement is also more important in striated muscle. The rate at which Ca^{2+} must be removed from the cytoplasm after the contraction of striated muscle cells is much greater than that required in smooth muscle (reviewed by Raeymaekers and Wuytack, 1995), however. It therefore seems possible that the Cl^- channel or channels involved are different in the two muscle types. In this light, the fact that our results point to a Cl^- channel in smooth muscle SR that resembles one found in the endoplasmic reticulum makes it of interest to note that the isoforms of a number of proteins that are found in smooth muscle cells more closely resemble those found in nonmuscle cells than they do those found in the more highly specialized cells of cardiac and skeletal muscle.

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