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Effects of Ionomycin and Thapsigargin on Ion Currents in Oocytes of *Bufo arenarum*

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ABSTRACT In this study, two electrode voltage clamp technique was used to assess the ionic current of oocytes of the South American toad *Bufo arenarum* and to study the dependence of these currents on the extracellular and intracellular Ca^{2+} concentrations. Ca^{2+} chelators, ionomycin –a calcium ionophore- and thapsigargin, a blocker of the Ca^{2+} pump of the sarcoplasmic reticulum, were used. The main results were the following: Most oocytes showed a voltage activated rectifying conductance. Ionomycin (1 μM) increased inward and outward currents in control solution. The effect of ionomycin was blocked partially at negative potentials and was blocked completely at positive potentials in absence of extracellular Ca^{2+}. When the oocytes were treated with thapsigargin (2 μM) or BAPTA-am, a membrane-permeant intracellular chelator in control solution (10 μM), ionomycin did not increased either inward nor outward currents. The conclusion of our experiments is that there are two sources of Ca^{2+} for activation of the current induced by ionomycin, the cytoplasmic stores and the extracellular space. We believe ionomycin directly translocates Ca^{2+} from the SER into the cytoplasm but not from the extracellular medium. Ca^{2+} entry probably occurs through store-operated-Ca-channels. *J. Exp. Zool. 297A:130–137, 2003.*

INTRODUCTION

Ion channels predominant in *Xenopus laevis* and other types of frogs and toads include several types of Cl^- channels that are regulated by voltage, Ca^{2+}, and changes in medium osmolarity (Parker and Miledi, '88; Boton et al., '89; Yao and Parker, '93; Arellano et al., '95; Petersen and Berridge, '96; Gómez-Hernández et al., '97; Ivorra and Morales, '97; Zhang et al., '98; Machaca and Hartzell, '98; Weber, '99; Kuruma and Hartzell, '99; Faszewski and Kunkel, 2001). Recently we presented the basic electrical characteristics of the oocytes from the toad *Bufo arenarum* (Kotsias et al., 2002).

In *Xenopus laevis* there are various routes of Ca^{2+} entry into the cell. One is Ca^{2+} entry across the plasma membrane SOC channels (store operated calcium channels; Riccio et al., 2002), a pathway called capacitative calcium entry (CCE) (Putney, '90; Parekh and Penner, '97; Machaty et al., 2002). As Ca^{2+} stores are depleted experimentally by drugs such as ionomycin or thapsigargin, the entry by CCE is activated through a not known regulatory mechanism. When Ca^{2+} stores are replete the SOCCs are closed (Petersen and Berridge, '96; Parekh and Penner, '97; Weber, '99; Braun et al., 2001). Once in the cell, Ca^{2+} ions are sequestered in the sarcoplasmic/endoplasmic reticulum (SER) by a Ca-ATPase pump located in the reticulum membrane (SERCA) which plays an important role in maintaining Ca^{2+} levels in the cytosol. Clearance of Ca^{2+} from the cytosol is also regulated by uptake into mitochondria and efflux into the extracellular space. Ca^{2+} can also enter into the cell through an IP_3 activated pathway (Machaca and Hartzell, '98; Thurman et al., 2000; Braun et al., 2001).

Two types of drugs had been useful in studying calcium signals. One is thapsigargin, a plant derived drug, that produces an increase in intracellular Ca^{2+} via a direct liberation of ions from the SER as a consequence of the SERCA pump inhibition (Thastrup et al., '90; Machaca and Hartzell, '98). The second type of drugs are represented by ionophores, i.e., ionomycin, an antibiotic isolated from the *Streptomyces conglobatus*. This and other carboxylic ionophores such as A23187, form membrane-permeable complexes with divalent cations facilitating the transport of cations across the membrane (Liu et al., '78,

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Beeler et al., '79) this effect being dependent on membrane potential (Fasolato and Pozzan, '89; Morgan and Jacob, '94). The mechanism of Ca^{2+} release from intracellular stores was studied in *Xenopus laevis* oocytes. Pretreatment of oocytes with calcium ionophores resulted in a depletion of intracellular Ca^{2+} stores by stimulating SER with an increase in intracellular Ca^{2+} concentration and openings of Ca-activated Cl channels (Gillo et al., '87; Yoshida and Plant, '92; Machaca and Hartzell, '98; Thurman et al., 2000).

In order to establish alternative systems in which ion channels, including those of *Xenopus laevis,* could be efficiently expressed (see Markovich and Regeer, '99) we initiated a functional characterization of the electrical properties of the oocytes of *Bufo arenarum*, a toad readily available in Argentina. This study had the objectives to see whether some of the ionic conductances found in *Xenopus* and other species are present in these cells and if they can be activated with Ca^{2+} ionophores. Here, we suggested that the main source of Ca^{2+} for the activation of current induced by ionomycin is from the cytoplasmic stores. An abstract with some of these results was presented elsewhere (Cavarra and Kotsias, 2001).

MATERIALS AND METHODS

Oocytes were surgically removed from the abdomen of *Bufo arenarum* adult females. Oocytes were defolliculated in 2 mg/ml collagenase Type 1A (Sigma, St Louis, MO) and equilibrated in standard Barth's solution (mM): NaCl 96; KCl 2.5; $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 5, pH 7.4 with NaOH.

The oocyte was immobilized in microwells, superfused continuosly with the standard solution and impaled with two microelectrodes. The microlectrodes were pulled from borosilicate glass capillaries by means of an horizontal puller and filled with 3 M KCl. They had a resistance of 1-3 MΩ in the standard solution. The bath was grounded with a silver-silver chloride reference electrode connected with the chamber via an agar bridge.

The oocyte was voltage-clamped using a homemade high output amplifier at a holding potential of −60 mV. This holding potential allows us to explore the depolarization and hyperpolarization-induced currents. A series of depolarizing and hyperpolarizing pulses were applied in steps of 20 mV and the current was measured at the end of the test pulse. Pulses were applied every 15 seconds. The response to different pulses was repeated in control solution 10 min after the first one to see if the currents remained stable. After that the test drug was added.

Ca^{2+} free solution was made by adding 200 μM BAPTA and 2 mM $MgCl_2$ to a nominally 0-Ca^{2+} solution. In other experiments 10 μM BAPTA-am was used in order to reduce intracellular Ca^{2+} concentration. Ionomycin and thapsigargin were dissolved in DMSO and added directly to solutions. All chemical were purchased from Sigma Chemical. Co. Membrane and voltage signals were recorded on a PC computer using a PClamp 5 software after sampling with a TL-1 A/D converter (Axon Instruments Co., Foster City, California).

Results were presented as means ± 1 SE with the level of significance determined using Wilcoxon test, two tails. For comparison between control and test solutions, the currents were normalized to the peak current in control solution measured at +60 mV ($I_{max\ control}$). Thus, the current at a particular voltage in control solution $= I_{control}/I_{max\ control} \times 100$ and in the presence of a test drug $= I_{drug}/I_{max\ control} \times 100$.

RESULTS

The oocytes of *Bufo arenarum* had a diameter of about 1 mm. The resting membrane potential values in the standard solution ranged from −20 to −40 mV.

Oocyte currents were obtained by applying 20 mV voltage steps between −100 and +60 mV from a holding potential of −60 mV (Fig. 1). Upward deflections correspond to, either anions entering into, or cations exiting the cell. Most oocytes showed a voltage activated outward rectifying conductance. As can be seen in Figure 1, after a delay, outward currents gradually rose to steady state at voltages more positive than 0 mV. Conductance values of 2.5 μS and 9.9 μS were estimated for the inward and outward currents respectively, indicating an outwardly rectification. Typical currents as shown in this figure were observed in both, freshly dissociated oocytes, and oocytes stored for up to three days at 18°C. After that, the resting membrane potential fell to about −10 mV or −15 mV, and the current necessary to voltage clamp the oocytes became much higher than in fresh oocytes.

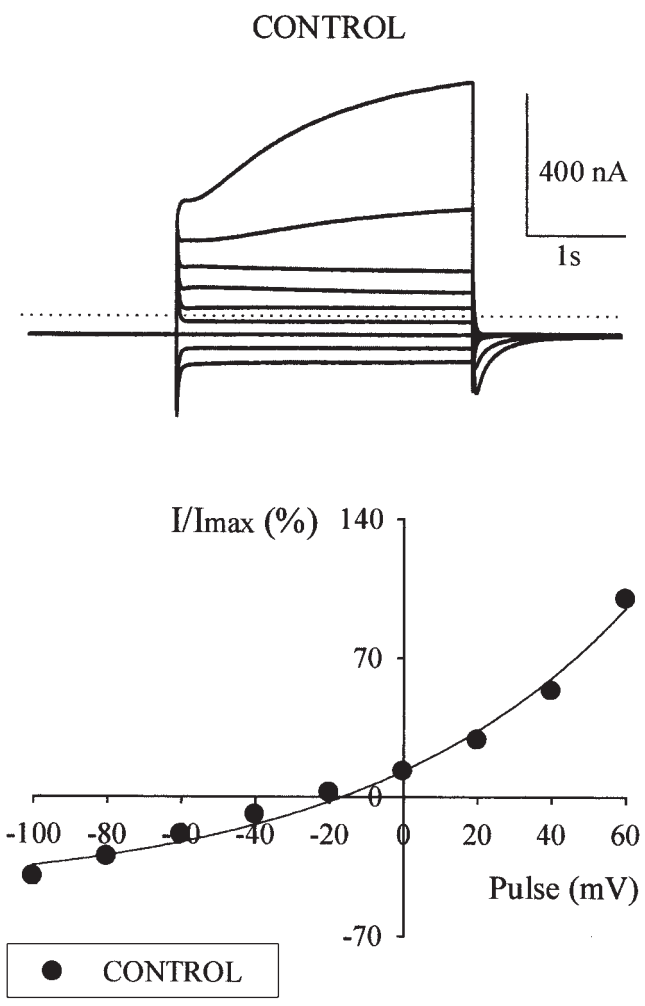

Fig. 1. Membrane currents of *Bufo arenarum* oocytes. Upper panel: common response obtained by applying 20 mV voltage steps between −100 and 60 mV in control solution. Upward deflections correspond to either anions entering into, or cations exiting the cell. This family of traces shows that at voltages more positive than 0 mV, outward currents rose gradually to steady state level, and displayed outward rectification. Dotted line represents zero current level. Bottom panel: Current-voltage (I–V) plots in control solution. In this as in the other figures, currents were measured at the end of the test pulse. Data are from 25 oocytes.

Effect of DMSO

DMSO alone, the vehicle used to dissolve the drugs employed in this work did not affect either inward or outward currents. Figure 2 shows the average results obtained in 7 experiments in which DMSO was added into the bath at a concentration similar to that used to dissolve the drugs. For comparison between control and test solutions, the currents were normalized to the peak current in control solution measured at +60 mV (see Methods).

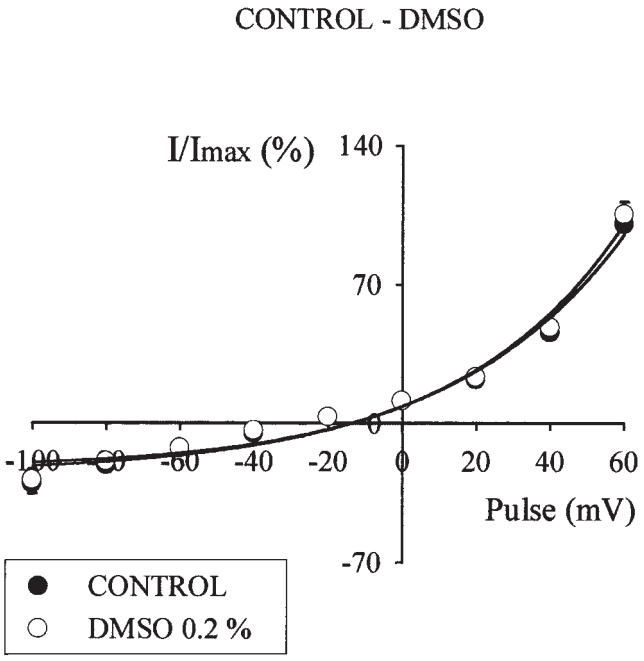

Fig. 2. I–V plot of normalized currents in control solution (filled symbols) and in a solution with 0.2% of DMSO, the maximum concentration employed to dissolve the drugs (empty circles, n=7). The differences between the two curves were not significant.

Effects of ionomycin, a calcium ionophore, upon the inward and outward currents

Figure 3 shows I–V plots of steady state currents elicited in the same oocytes in control solution and 5 minutes after the addition of 1 μM ionomycin (empty circles). As in all experiments, before adding ionomycin, we confirmed that the currents in control solution were stable. For this, another family of currents was repeated in control solution 10 min after the first one; after that the test drug was added into the bath. It is evident that ionomycin increases the inward and outward currents in comparison with the control currents with a range between 18 and 33%, with the largest effect at negative potentials (see Discussion).

Effects of thapsigargin, a sarcoplasmic reticulum/endoplasmic reticulum calcium pump inhibitor

The effect of ionomycin was tested in oocytes preincubated with 2 μM thapsigargin for 2 hours before the experiment. This time was enough for normalization of the currents because thapsigargin by itself increased transiently the ion currents in control solution. Figure 4 (upper panel) shows

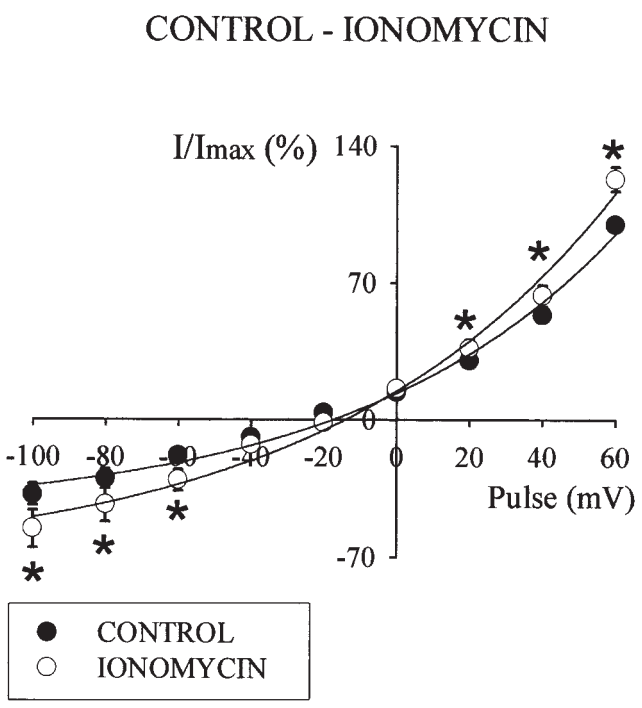

Fig. 3. I–V plots of steady state currents in control solution (filled circles) and ionomycin (1 μM, empty circles, n=10). Asterisks indicate that the differences between the two curves were significant ($p<0.05$).

the I–V plot obtained 45 minutes after introduction of thapsigargin where it is evident the increment in the outward currents. The graphs also show the normalization of the currents after 2 hours of the introduction of thapsigargin into the bath (n=7, not significant). When oocytes treated for two hours with thapsigargin were exposed to ionomycin, currents in the test solution were not different from those recorded in the absence of the ionophore (Figure 4, lower panel).

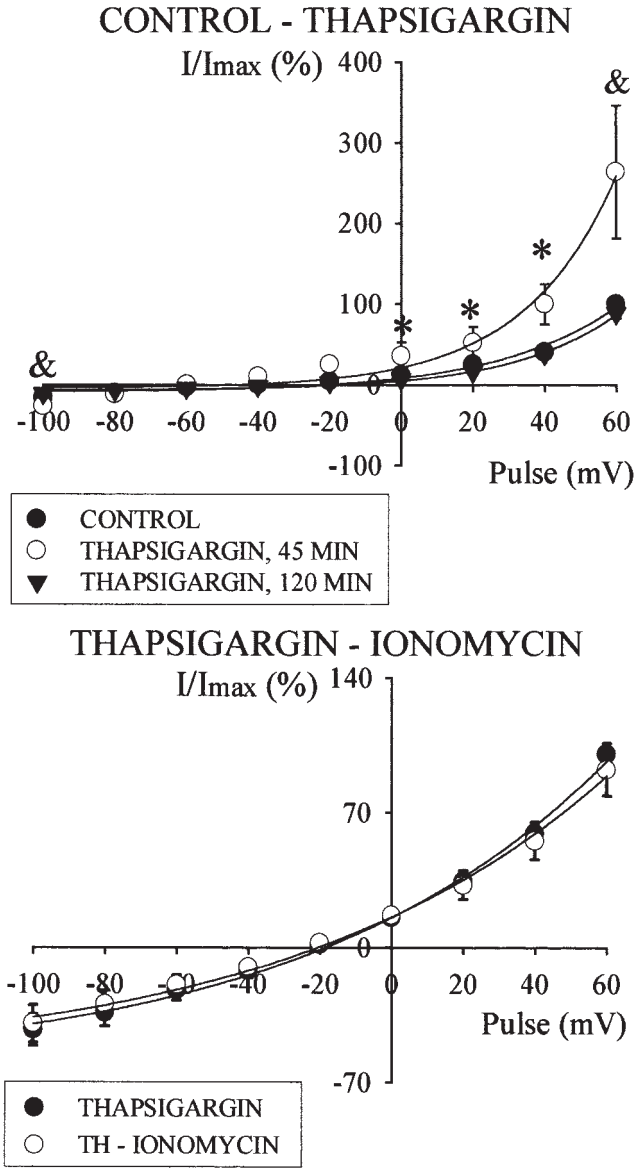

Fig. 4. Upper panel. I–V plot showing the increment in currents 45 minutes after introduction of 2 μM thapsigargin (*: $P<0.05$; $P<0.06$) and the normalization of the currents after two hours (n=7, not significant). Lower panel. I–V plot of normalized currents in oocytes pretreated with 2 μM thapsigargin (2 hours, filled symbols) and in the presence of 1 μM ionomycin (empty circles, n=5). The differences between the two curves were not significant.

Effects of ionomycin in BAPTA-am, a cytoplasmic Ca^{2+} buffer

The effect of ionomycin upon the currentes was pursued with another series of experiments in which the oocytes were preincubated in 10 μM BAPTA-am for about 1–3 hours before the experiment. BAPTA-am is a well known cytoplasmic Ca^{2+} buffer. BAPTA-am diffuses into the cells and suffers a subsequent cleavage to yield free acid. Inside the cell BAPTA chelates the Ca^{2+} and reduces its concentration to <10 nM (Albert and Large, 2002). The results obtained in 6 oocytes under these experimental conditions are shown in Figure 5 where it is evident that ionomycin did not affect either inward or outward currents.

Effects of ionomycin in 0-Ca^{2+} extracellular solution

Ca^{2+} free solution was made by the use of 200 μM BAPTA and 2 mM $MgCl_2$ in a nominally

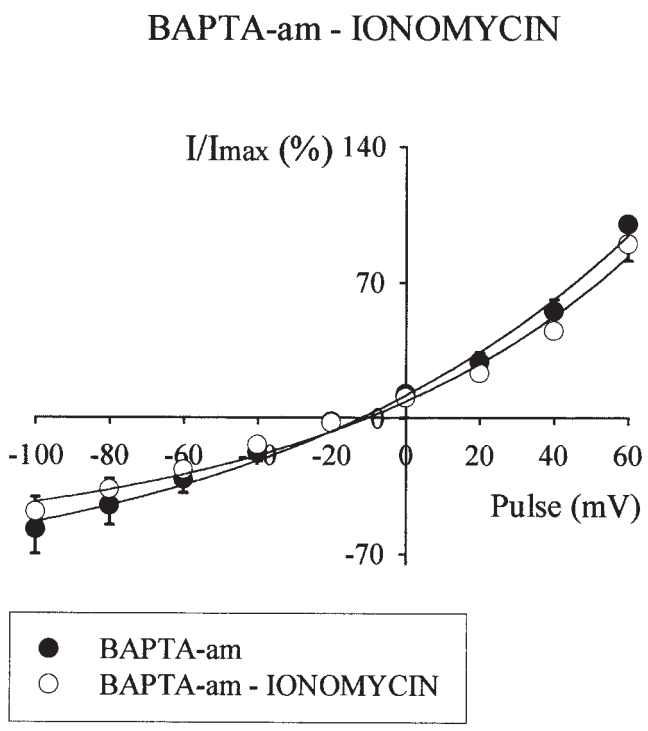

Fig. 5. IV plots before (filled circles) and after the addition of 1 μM ionomycin (open symbols) in oocytes incubated in 10 μM BAPTA-am. Average values obtained in 6 oocytes. The differences between the two curves were not significant.

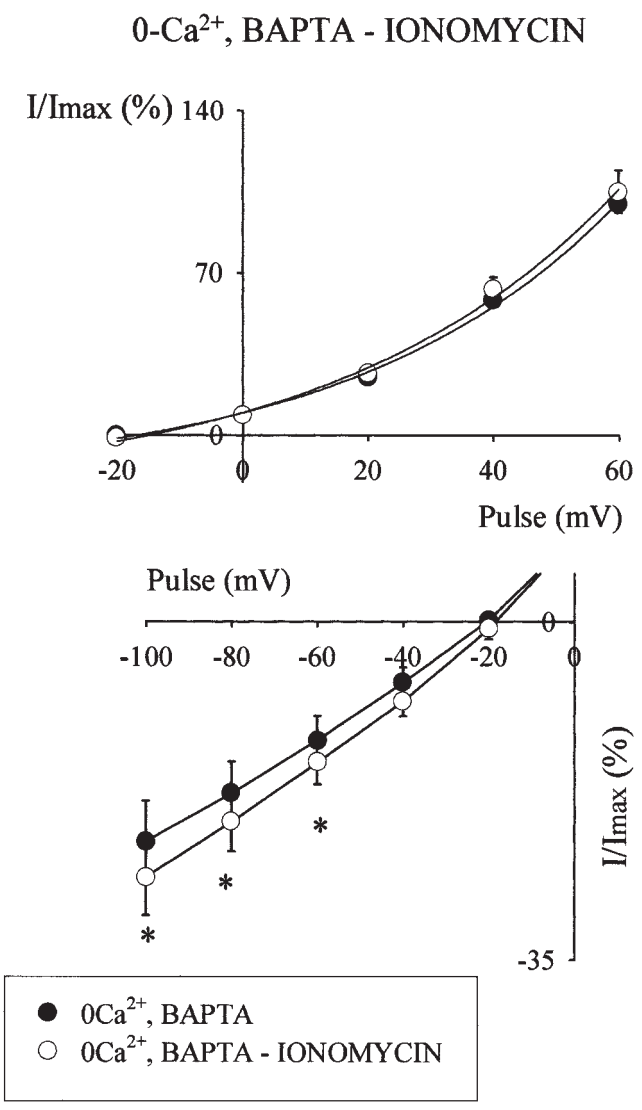

Fig. 6. I–V plots of normalized outward (upper panel) and inward currents (lower panel) in O Ca^{2+} solution in the absence (filled circles) and 5 min after the introduction of ionomycin (1 μM, empty circles, n=7). Oocytes were incubated for about 2 hours before the experiment in this solution and then challenged with 1 μM ionomycin. The absence of extracellular Ca^{2+} is evidenced in the effect of ionomycin only at the negative potentials where asterisks indicate that the differences between the two curves were statistically significant ($P<0.05$).

0-Ca^{2+} solution. The effect of BAPTA, a charged moiety that does not cross membranes, is due to chelation of extracellular Ca^{2+}. Adding Mg^{2+} to the solution (3 mM total concentration) was done to avoid the membrane harmful effect of Ca^{2+} free solution (Weber et al., 1995; Thurman et al., 2000). In 0-Ca^{2+} solution the effect of ionomycin is evidenced only with hyperpolarizing pulses that activated inward currents. Although this increment in the inward current was small, the differences with the control oocytes were still significant (Fig. 6).

Effects of gadolinium

The effect of ionomycin upon the currents was also tested in another series of oocytes preincubated in 50 μM gadolinium for about 20 minutes before the experiment. It has been shown that gadolinium, a lanthanide-ion, blocks the CCE in several types of cells (Dietl et al., '96; Jan et al., '99; Flemming et al., 2002). Figure 7 present the average results. Gadolinium has no effect upon the control currents (upper panel) but abolished the increment in inward and outward currents promoted by ionomycin (lower panel).

DISCUSSION

The aim of these experiments was to study the effects of extracellular and intracellular Ca^{2+} levels upon the currents of *Bufo arenarum* oocytes. For this we measured whole-cell currents

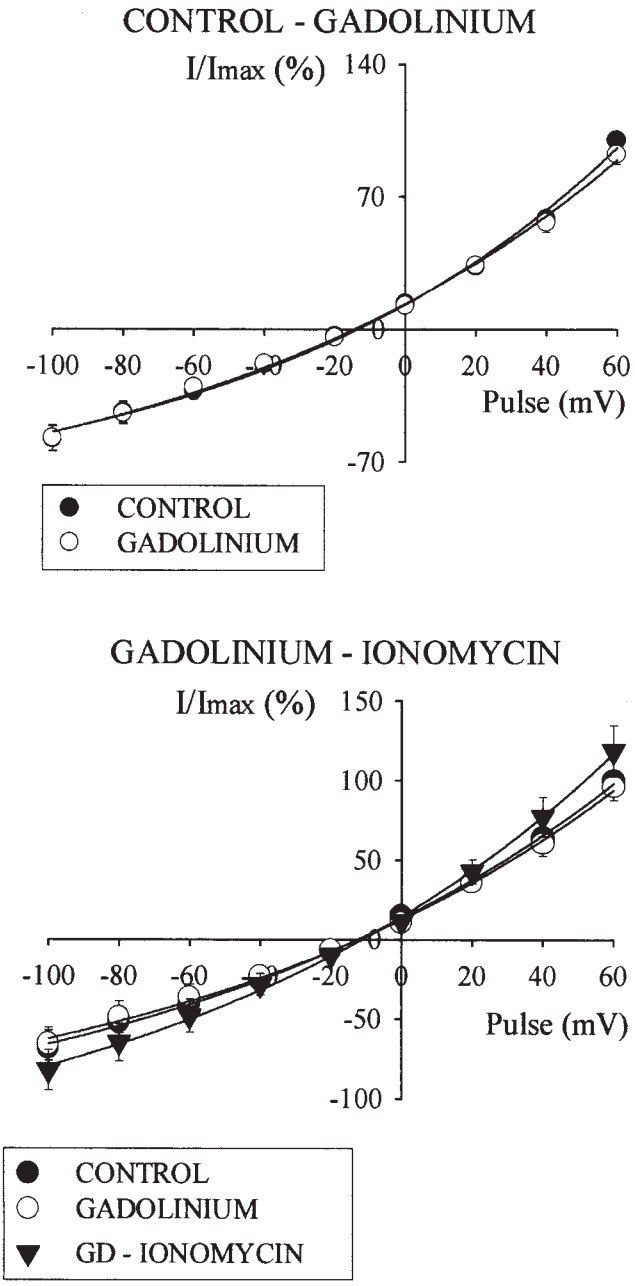

Fig. 7. Upper panel. I–V plot of normalized inward and outward currents in control solution in the absence (filled circles) and 20 min after the introduction of 50 μM gadolinium (empty circles, n=6). Lower panel. I–V plot of normalized inward and outward currents in control solution in the absence (filled circles) and presence of gadolinium (50 μM, empty circles) and 10 min after the introduction of 1 μM ionomycin (filled triangles, n=6).

using the two-electrode voltage-clamp method in the presence and absence of external Ca^{2+}, ionomycin, thapsigargin and gadolinium. The drug concentrations used in these experiments are in the range used in other works (see Introduction).

In control solution, after a delay, outward currents gradually rose to steady state at voltages more positive than 0 mV (Fig. 1). The instantaneous increase reflected a current due to the larger driving force through channels already open at −60 mV and the time-dependent component represented voltage-dependent opening of additional channels.

We found that DMSO added into the bath at a concentration similar to that used to dissolve the drugs did not cause increase in either inward or outward currents, thus the effects of other drugs on currents can be ascribable to their own properties. In regard to the effect of ionomycin, we demonstrated: a) that ionomycin increased the inward and outward currents measured with voltage clamp techniques in control solution, with no effect upon the reversal potential (Fig. 3). b) Ionomycin did not affect either inward nor outward current in oocytes preincubated whith BAPTA-am or thapsigargin in Ca^{2+} -solution (Figs. 4, 5). c) In the absence of extracellular Ca^{2+} ionomycin increased only the inward current (Fig. 6).

The ability of thapsigargin to interfere selectively with Ca^{2+} trapping systems, makes this drug a pharmacological probe for studying calcium signals. Here we showed that thapsigargin increased the outward current (upper panel Figure 4) probably due to an activation of channels in response to a high intracellular Ca^{2+} concentration (Lupu-Meiri et al., '93; Goudeau and Goudeau, '98; Machaca and Hartzell, '98; Jan et al., '99; Takahashi et al., 2000). This effect was transient because 2 hours after introduction of the drug the currents were returned to the basal level. It should be pointed out that currents were measured every 15 minutes. This effect could be related to the work of Lupu-Meiri et al. ('93) showing that thapsigargin caused a net increased in Ca^{2+} efflux from oocytes. Oocytes incubated in control solution and exposed to thapsigargin (2 μM) for two hours were then exposed to ionomycin. The lack of response to ionomycin in these oocytes (Fig. 4, lower panel) suggest that a) ionomycin has no effect on Ca translocation at the plasma membrane; b) ionomycin-sensitive Ca^{2+} stores were depleted after being exposed to thapsigargin.

If ionomycin forms complexes with Ca^{2+} facilitating the transport of this ion across the plasma membrane (Liu et al. '78, Beeler et al., '79) we

could expect the activation of Ca-activated-Cl-channels by the subplasmalemal accumulation of Ca^{2+} before being chelated by intracellular BAPTA in these oocytes in standard solution and pretreated with BAPTA-am. Moreover, ionomycin was applied during 5 min and the Ca^{2+} entry would be high enough to saturate the BAPTA buffer capacity, increasing the Ca^{2+} concentration. However, under our experimental conditions, ionomycin did not affect the currents in BAPTA-am pretreated oocytes in Ca^{2+} rich medium. Thus, it is evident that ionomycin does not translocate Ca^{2+} at the plasma membrane level.

Our results in Ca^{2+} free solution suggest that ionomycin acts upon the SER, increasing the intracellular Ca^{2+} level enough to open the Ca-activated-Cl-channels only at negative potentials. Fasolato and Pozzan ('89) reported that the effect of ionomycin at low concentrations ($<1\,\mu M$) was dependent on membrane potential, being smaller at depolarizing potentials. For two reasons this effect in unlikely to occur in our experiments. First, because we used $1\,\mu M$ ionomycin; the second is that in the presence of Ca^{2+} ionomycin increased both inward and outward currents. The additional effect of the drug on the outward and inward currents seen on control solution may reflect an increased intracellular Ca^{2+} level, due to Ca^{2+} entry from the extracellular space in addition to the Ca^{2+} translocated from the SER. Gadolinium abolished the increment in outward currents promoted by ionomycin, an effect similar to that obtained with O-Ca^{2+}, but it also blocked the increment in inward currents (Fig. 7). It has been shown that lanthanide-ions block the CCE in several types of cells (Diet et al., '96; Jan et al., '99; Flemming et al., 2002) but unfortunately it has other actions. Thus, gadolinium binds to the Ca^{2+}- transporting ATPase of the sarcoplasmic reticulum and is also generally regarded as a nonselective cation channel blocker (see Jan et al., '99 for references). Thus, at present we do not have a drug that can be used to test the CCE mechanism proposed for ionomycin, at least in our preparation.

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