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Comparison of Membrane Currents in *Xenopus* Oocytes in Response to Injection of Calcium Influx Factor (CIF) and Depletion of Intracellular Calcium Stores

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The depletion of intracellular calcium stores by thapsigargin treatment evoked extracellular calcium-dependent membrane currents in *Xenopus laevis* oocytes. These currents have been compared to those evoked by microinjection of a calcium influx factor (CIF) purified from Jurkat T lymphocytes. The membrane currents elicited by thapsigargin treatment (peak current, 163 ± 60 nA) or CIF injection (peak current, 897 ± 188 nA) were both dependent on calcium entry, based on their eradication by the removal of extracellular calcium. The currents were, in both cases, attributed primarily to well-characterized Ca^{2+}-dependent Cl^- currents, based on their similar reversal potentials (-24 mV vs. -28 mV) and their inhibition by niflumic acid (a Cl^- channel blocker). Currents induced by either thapsigargin treatment or CIF injection exhibited an identical pattern of inhibitory sensitivity to a panel of lanthanides, suggesting that thapsigargin treatment or CIF injection evoked Cl^- currents by stimulating calcium influx through pharmacologically identical calcium channels. These results indicate that CIF acts on the same calcium entry pathway activated by the depletion of calcium stores and most lanthanides are novel pharmacological tools for the study of calcium entry in *Xenopus* oocytes.

Keywords: Calcium influx factor, Capacitative calcium entry, Lanthanide, Membrane currents, *Xenopus* oocytes.

Introduction

Cytosolic calcium is crucial in the regulation of cell responses in a wide variety of cells (Berridge, 1993; Park *et al.*, 1998; Oh, *et al.*, 1999; Song, 1999). The activation of receptors coupled to phosphoinositide hydrolysis releases cytosolic inositol 1,4,5-trisphosphate ($InsP_3$), which discharges calcium from intracellular stores (Berridge, 1993). The subsequent depletion of intracellular calcium stores by $InsP_3$ (Hoth and Penner, 1992; McDonald *et al.*, 1993), or alternatively loss of stores by inhibition of sarcoplasmic reticulum/endoplasmic reticulum (SERCA) type-Ca^{2+}-ATPases by selective inhibitors (Zweifach and Lewis, 1993; Petersen and Berridge, 1994; Yang and Lee, 1995) induces calcium influx through a novel calcium entry channel in the plasma membrane. This calcium influx pathway has been termed capacitative calcium entry (Putney, 1990). However, the mechanism, which couples depletion of the stores and opening of calcium entry channels, remains unclear.

Two major hypotheses are currently considered as potential signaling mechanisms underlying capacitative calcium entry. One model, termed conformational coupling, proposed that the depletion of calcium stores induced a conformational change of the $InsP_3$ receptor, leading to a direct interaction with the capacitative calcium entry channel in the plasma membrane (Berridge, 1990; Irvine, 1990; Berridge, 1995; Thomas *et al.*,1998a). The other model, termed diffusible messenger, proposed that depletion of calcium stores produced a novel intracellular messenger, denoted as a calcium influx factor (CIF), which then gated the capacitative calcium entry channel (Parekh *et al.*,1993; Randriamampita and Tsien, 1993; Berridge, 1995; Thomas *et al.*,1998a). Recent reports provided evidence that CIF activity may be detected upon calcium depletion in Jurkat cells (Randriamampita and Tsien, 1993; Kim *et al.*, 1995; Randriamampita and Tsien, 1995; Thomas and Hanley, 1995; Thomas *et al.*, 1998b; Thomas *et al.*, 1998c), neutrophils (Davies and Hallett, 1995), *Xenopus* oocytes (Kim and Hanley, 1999), and *Saccharomyces cerevisiae* (Csutora *et al.*, 1999).

Xenopus oocytes provided a powerful model system for

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studies of the capacitative calcium entry pathway (Parekh *et al.*,1993; Petersen and Berridge, 1994; Kim *et al.*, 1995; Thomas and Hanley, 1995; Kim *et al.*, 1996; Thomas *et al.*, 1998b; Thomas *et al.*, 1998c; Csutora *et al.*, 1999; Kim and Hanley, 1999). For example, activation of an endogenous lysophosphatidic acid (LPA) receptor coupled to the phosphatidylinositol signaling pathway produced initial calcium-dependent currents from calcium discharge (Ferguson and Hanley, 1992), but also activated a sustained capacitative calcium entry in *Xenopus* oocytes (Petersen and Berridge, 1994). Additionally, the depletion of intracellular calcium stores by thapsigargin (TG) (Petersen and Berridge, 1994), or injection of cytidine-5'-diphosphate-D-glucose (Kim *et al.*, 1996), has been shown to induce calcium influx in *Xenopus* oocytes.

We recently reported that thapsigargin-stimulated Jurkat cells produced a CIF activity, which could be assayed indirectly as a Ca^{2+}-dependent Cl^- current in *Xenopus* oocytes (Kim *et al.*, 1995; Thomas and Hanley, 1995; Kim *et al.*, 1996; Thomas *et al.*, 1998b; Thomas *et al.*, 1998c; Csutora *et al.*, 1999; Kim and Hanley, 1999). Using oocytes, we compared the properties of Ca^{2+}-dependent currents elicited by the thapsigargin-induced depletion of intracellular calcium stores to currents elicited by microinjection of CIF. As part of this comparison, we examined the relative sensitivities of thapsigargin- or CIF-induced currents to the inhibition by extracellular application of lanthanides as a pharmacological series of non-organic calcium entry channel blockers.

Materials and Methods

Cell culture Jurkat T lymphocytes were maintained as suspension cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 units)/ streptomycin (100 μg/ml). Jurkat cells were passaged by 1 : 10 dilution every 4 days.

***Xenopus* oocytes** *Xenopus* oocytes were obtained by ovarectomy as described previously (Kim *et al.*, 1995; Thomas and Hanley, 1995; Thomas *et al.*, 1998b; Thomas *et al.*, 1998c). Follicular cells were removed from oocytes by treating them with collagenase (2 mg/ml, 2 hrs, room temp.) followed by rolling oocytes on plastic Petri dishes. Defolliculated oocytes were maintained in a modified L-15 medium (diluted 1 : 1 with 30 mM HEPES, pH 7.4, containing 0.25% chicken ovalbumin, 1 mM L-glutamine, and 50 μg/ml gentamycin). For depletion of intracellular calcium stores, *Xenopus* oocytes were incubated (18°C, 2 hrs) with thapsigargin (1 μM) in calcium-free OR2 medium (82 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.4). Thapsigargin was dissolved in DMSO as a stock solution (1.53 mM) and stored at -20°C. The final concentration of DMSO was 0.065%.

Preparation of calcium influx factor (CIF) Highly purified CIF was prepared from thapsigargin-stimulated Jurkat cells by a sequence of purification steps; Sep-Pak reverse-phase column, Microcon-30 ultrafiltration, Bio-Gel P-2 gel filtration, and a high performance thin layer chromatography (HPTLC) as previously described (Kim *et al.*, 1995). Briefly, Jurkat cells were washed three times and resuspended in Hanks balanced salt solution (HBSS) containing 20 mM HEPES, cells were stimulated 1 μM thapsigargin for 15 min at 25°C. Cells were centrifuged for 5 min at 200 × g and the pellet was resuspended in HBSS containing 20 mM HEPES. The suspension was extracted with 0.15 M HCl and was incubated for 30 min at 25°C. The extract was clarified by centrifugation for 10 min at 400 × g and the supernatant was neutralized by addition of 10 M NaOH. After neutralization, 10 mM $BaCl_2$ was added to the extract and centrifuged for 5 min at 12,000 × g. The supernatant was lyophilized and the residue was extracted with methanol with continuous mixing for 15 min at 25°C. The methanol extract was loaded on the Sep-Pak C_{18} cartridge for removal of the hydrophobic material and was rinsed with methanol. The methanol eluents were combined, dried under N_2 gas, and resuspended in 0.1 M acetic acid. The reconstituted extract was clarified by centrifugal ultrafiltration through a Microcon-30 filter. The filtrate was loaded onto a Bio-Gel P-2 column (0.7 × 2.7 cm), equilibrated with 0.1 M acetic acid, and was eluted by the same solution, collecting fractions of 0.5 ml. Fraction 15, which has CIF activity as measured by Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes, was termed "Peak 1". Peak 1 was purified by HPTLC developed with an initial solvent system [chloroform/methanol/acetic acid/water (20/15/8/4, v/v)]. One HPTLC-purified band (R_F = 0.57) exhibited CIF activity, termed "HPTLC 1-4". HPTLC 1-4 was further purified on HPTLC plates developed with a second solvent system [1-butanol/pyridine/acetic acid/water (15/10/2/12, v/v)]. HPTLC 1-4 was resolved into at least three bands using UV detection. Each fraction was then reconstituted in a HEPES buffer (10 mM, pH 7.0, 20 μl) and the activity tested by microinjection. One HPTLC-repurified band (R_F = 0.14) exhibited CIF activity, termed "HPTLC 1-4-1". This was the material used for this study.

Lanthanides as blockers of Ca^{2+}-dependent currents All lanthanides were dissolved in water as 0.1 M stock solutions except $GdCl_3$ (0.05 M) and stored at room temperature. Lanthanides were added in an OR2 medium (82 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.4) containing either 2 mM $CaCl_2$ for CIF experiments, or an elevated concentration of 10 mM $CaCl_2$ for thapsigargin depletion experiments for incubation with *Xenopus* oocytes. The levels of peak currents were measured and compared with normal peak currents for determining the relative effectiveness of lanthanides to a block of calcium entry, as assessed by Ca^{2+}-dependent currents.

Data acquisition and current analysis Two-electrode whole cell voltage clamp experiments using *Xenopus* oocytes were performed as described previously (Ferguson and Hanley, 1992; Kim *et al.*, 1996). Briefly, oocytes were voltage-clamped at -60 mV in OR2 medium (82 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.4) with the use of a TEV-200 clamp amplifier (Dagan, MN). Currents were low-pass filtered at 50 kHz using the internal four-pole Bessel filter and digitized at sampling rates of 100 msec (10 Hz) or 500

Table 1. *Lanthanides block 10 mM $[Ca^{2+}]_o$-induced late currents but not LPA-induced early currents in Xenopus oocytes.*

Lanthanides	LPA-induced early currents		10 mM $[Ca^{2+}]_o$-induced late currents	
	nA	(%)	nA	(%)
Control	3340 ± 396	100	195 ± 41	100
La^{3+}	3798 ± 427	114	20 ± 12	10
Ce^{3+}	3254 ± 410	97	47 ± 25	24
Pr^{3+}	3745 ± 768	112	20 ± 28	10
Nd^{3+}	3466 ± 50	104	57 ± 5	29
Sm^{3+}	2853 ± 150	85	180 ± 28	92
Eu^{3+}	3497 ± 776	105	207 ± 34	106
Gd^{3+}	3267 ± 523	98	47 ± 9	24
Dy^{3+}	2912 ± 339	87	187 ± 52	96
Ho^{3+}	3524 ± 256	106	67 ± 19	34
Er^{3+}	3019 ± 735	90	67 ± 25	34
Tm^{3+}	3267 ± 586	98	27 ± 37	14
Yb^{3+}	2850 ± 114	105	70 ± 10	36
Lu^{3+}	3745 ± 747	112	133 ± 75	68

The concentrations of each lanthanide were 1 mM and 2 mM for 10 mM $[Ca^{2+}]_o$ -evoked Ca^{2+} entry and for LPA-evoked Ca^{2+} discharge, respectively. Values are means ± standard deviation of three or four independent determinations.

msec (2 Hz) with the use of the TL-1 analog/digital converter (Axon Instruments, Foster City, CA). To obtain current-voltage relations, voltage ramps were run on the activated currents. The ramp data were collected with the use of pCLAMP software (version 5.5) to sample current responses to potential changes at a rate of 4 kHz. The ramp protocol consisted of repeated episodes (every 5 sec) of a -100 mV to +60 mV ramp (2-sec duration ramp at a depolarization rate of 0.5 mV/msec) with an interepisode holding potential of -60 mV.

Results

When *Xenopus* oocytes were stimulated with LPA (1 μM, 20 s) in a calcium-free medium, there was an initial rapid increase in inward current (peak current, 3340 ± 396 nA, Table 1), which is recognized to be principally a chloride current activated by the calcium released from intracellular stores (Petersen and Berridge, 1994). LPA has no current responses in the thapsigargin treated oocytes (Data not shown. See Petersen and Berridge, 1994). Following treatment with LPA in a calcium-free medium, superfusion with a medium containing high external calcium (10 mM) activated sustained currents, as reported previously (Petersen and Berridge, 1994). These results suggest that the intracellular calcium stores are completely emptied by the thapsigargin treatment for 2 hrs, and that depletion of intracellular stores was invariably coupled to the calcium entry pathway.

Microinjection of HPTLC 1-4-1 elicited current responses (peak current, 897 ± 188 nA, solid traces in Fig. 1A), which were eliminated by removal of the extracellular calcium (peak current, 37 ± 8 nA, dotted traces in Fig. 1A) and were blocked by perfusion of 1 mM $LaCl_3$ (peak current, 87 ± 12 nA, dashed traces in Fig. 1A). Thus, the HPTLC 1-4-1 fraction is subsequently termed "CIF", since it contained the purified activity.

Xenopus oocytes, pretreated with thapsigargin (1 μM) in order to activate sustained calcium entry, exhibited membrane currents (peak current, 163 ± 60 nA, solid traces in Fig. 1B) that were enhanced and readily detected in elevated extracellular Ca^{2+} (10 mM), but not in normal oocytes (dotted

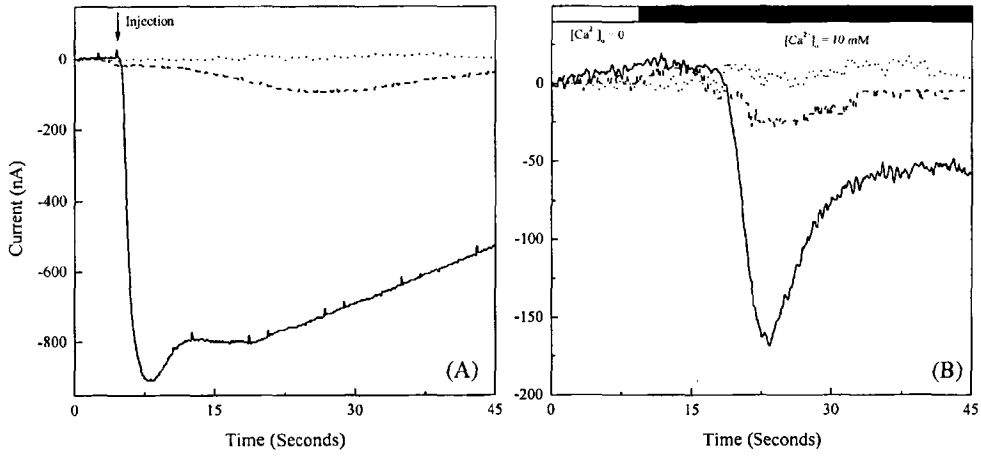

Fig. 1. CIF injection and thapsigargin treatment elicit membrane currents in *Xenopus* oocytes. A) Microinjection (10 nl) of HPTLC 1-4-1 (R_F = 0.14) evoked currents (897±188 nA, n = 13, solid traces). Removal of extracellular Ca^{2+} (dotted traces, 37 ± 8 nA, n = 7) and perfusion of 1 mM $LaCl_3$ (dashed traces, 87 ± 12 nA, n = 12) inhibited current response elicited by microinjection of HPTLC. B) External Ca^{2+} was removed ($[Ca^{2+}]_o$ = 0) and subsequently replaced with 10 mM Ca^{2+} ($[Ca^{2+}]_o$ = 10 mM) induces current responses in TG-treated oocytes (solid traces, 163 ± 60 nA, n = 26), but not in normal oocytes (dotted traces, n = 4). Perfusion of 1 mM $LaCl_3$ inhibited current response in TG-treated oocytes (dashed traces, 26 ± 9, n = 12)

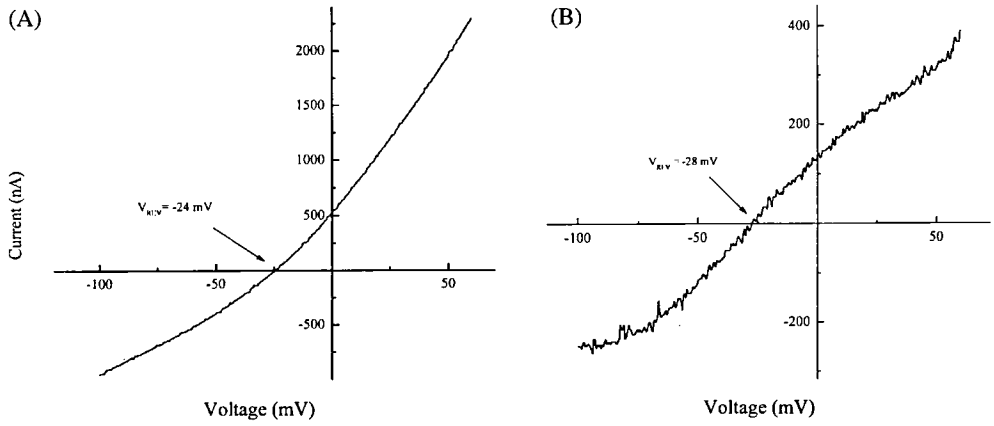

Fig. 2. The I-V curve of the currents activated by CIF injection (A) and thapsigargin treatment (B) in *Xenopus* oocytes. See Materials and Methods for experimental details. The reversal potentials were 24±0.4 mV for CIF injection and 28±0.4 mV for depletion of calcium stores. Results are representative of 6 independent experiments.

traces in Fig. 1B). The membrane currents were also blocked by the extracellular application of 1 mM $LaCl_3$ (peak current, 26 ± 9 nA, dashed trances in Fig. 1B). To establish the nature of the membrane currents activated by the thapsigargin treatment or CIF injection, we examined the I-V relationship (Fig. 2). The reversal potential for CIF (-24 ± 0.4 mV) suggested that the current was carried by chloride ions (Fig. 2A). For comparison, the reversal potential of a current elicited by the thapsigargin treatment was -28 ± 0.4 mV (Fig. 2B). The currents by both thapsigargin treatment and CIF injection were completely abolished by perfusion of niflumic acid (1 mM) and injection of 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*,-tetraacetic acid (BAPTA) (1 mM) (Data not shown. Refs. Kim *et al.*, 1995 and Kim *et al.*, 1996). These results suggest that the currents are, in both cases, carried by chloride ions. The injection of BAPTA eradicated all current activities, indicating that the observed responses were completely calcium-dependent.

The calcium entry that is elicited by the depletion of intracellular stores was completely blocked with the extracellular application of La^{3+} (1 mM) in *Xenopus* oocytes (Petersen and Berridge, 1994). As mentioned earlier, the extracellular perfusion of $LaCl_3$ (1 mM) inhibited current responses elicited by both CIF injection (Fig. 1A, dashed traces, 87 ± 12 nA) and thapsigargin treatment (Fig. 1B, dashed traces, 26 ± 9 nA). In addition, we extended the use of inorganic cations as pharmacological tools for studying calcium entry by focusing on the lanthanide ion series. This series of cations provides a novel pharmacological panel for characterizing the sensitivity of Ca^{2+}-dependent currents to the blockade of calcium entry induced by different stimuli. As shown in Fig. 3 and Table 2, most lanthanides in the series were similarly active in reducing currents elicited by either the thapsigargin treatment or CIF injection.

To identify whether lanthanides block the calcium entry channels on plasma membrane, we measured the effects of lanthanides on the LPA-induced early currents and 10 mM

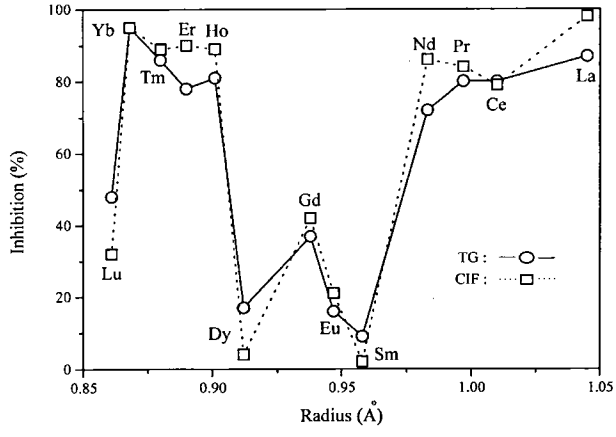

Fig. 3. Inhibition of Ca^{2+} entry by lanthanides. The efficiency of the lanthanides in inhibition of Ca^{2+} entry evoked by CIF injection (open squares), or thapsigargin treatment (open circles) in *Xenopus* oocytes as a function of their ionic radius. The concentrations of each lanthanide were 2 mM and 1 mM for thapsigargin treatment- and CIF injection-induced Ca^{2+} entry, respectively. Ionic radii were obtained from the Handbook of Chemistry and Physics (Lide, 1995). Results are representative of 4 to 8 independent experiments.

$[Ca^{2+}]_o$-induced late currents. Following the extracellular perfusion of LPA (1 μM, 20s) in a free Ca^{2+} medium (peak current, 3340 ± 396 nA, Table 1), 10 mM Ca^{2+} medium was replaced. Replacement of the Ca^{2+}-free medium with 10 mM Ca^{2+} medium activated sustained currents (peak current, 195 ± 41 nA). Lanthanides (1 mM) did not block the LPA-elicited early currents. However, lanthanides (2 mM) blocked 10 mM $[Ca^{2+}]_o$-evoked late currents in *Xenopus* oocytes (Table 1). These results suggest that lanthanides might block calcium entry, but not calcium discharge. The lanthanides did not block Cl^- currents since they must block Ca^{2+}-dependent Cl^- currents regardless of the calcium sources (calcium discharge or calcium entry) if lanthanides blocked Cl^- currents. To

Table 2. Thapsigargin treatment-induced currents were blocked in response to lanthanide concentrations in *Xenopus* oocytes.

Lanthanides	IC_{50} (mM)
La^{3+}	0.58 ± 0.04
Ce^{3+}	0.84 ± 0.08
Pr^{3+}	1.31 ± 0.18
Nd^{3+}	1.72 ± 0.56
Sm^{3+}	5.11 ± 0.15
Eu^{3+}	2.64 ± 0.03
Gd^{3+}	2.22 ± 0.16
Dy^{3+}	2.88 ± 0.58
Ho^{3+}	0.81 ± 0.04
Er^{3+}	0.97 ± 0.06
Tm^{3+}	0.62 ± 0.02
Yb^{3+}	0.40 ± 0.02
Lu^{3+}	1.30 ± 0.07

For obtaining IC_{50}, we used 4 different concentrations; 1, 2, 3, and 6 mM for $SmCl_3$, 0.5, 1, 2, and 3 mM for $NdCl_3$ and $DyCl_3$, and 0.25, 0.5, 1, and 2 mM for other lanthanides. Values are means ± standard deviation of three independent determinations.

determine whether lanthanides are permeating blockers or not, we injected a variety of Ca^{2+} concentrations from micromolar to millimolar levels with or without lanthanides. Calcium injection elicited Cl^- currents and, expectantly, injection of calcium and lanthanide mixtures also induced Cl^- currents. This would indicate that injection of the lanthanides had no effect on the inhibition of calcium-dependent Cl^- currents in *Xenopus* oocytes (data not shown). These results imply that lanthanides act as non-permeating blockers of the calcium entry channels in *Xenopus* oocytes.

The most effective lanthanides in attenuating current responses were La^{3+}, Ce^{3+}, Pr^{3+}, Nd^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}. The relative rank order of potencies for inhibition of currents were very similar for currents elicited by either thapsigargin treatment or injection of CIF. As shown in Table 2, IC_{50} values of the lanthanide ion series, obtained by several different concentrations, were also similar to those of the thapsigargin treatment in the inhibitory pattern. This similarity in the inhibitory pattern of lanthanides suggests that both the thapsigargin treatment and CIF injection activate the same calcium entry channels in *Xenopus* oocytes.

Discussion

We previously demonstrated that partially purified CIF from thapsigargin-stimulated Jurkat cells induced calcium entry upon microinjection into *Xenopus* oocytes (Kim *et al.*, 1995; Thomas and Hanley, 1995; Thomas *et al.*, 1998b; Thomas *et al.*, 1998c). Pretreatment with thapsigargin, which depletes intracellular calcium stores in oocytes, also induced calcium entry (Petersen and Berridge, 1994; Kim *et al.*, 1996). To compare whether CIF is similar in its stimulation of currents to thapsigargin treatment, we used several approaches, including a pharmacological approach that is based on the relative inhibition of currents by the extracellular application of lanthanides. The effective lanthanides do not inhibit Cl^- currents, but block calcium entry channels in *Xenopus* oocytes (Table 1). We interpret these results as indicating an inhibition of calcium entry by lanthanides, which was very similar for both thapsigargin treatment- and CIF injection-induced current responses (Fig. 3 and Table 2). An additional point of interest is that the relative potency of the series of lanthanides indicated that members with a hydrated radii of 0.912 - 0.958 angstroms were relatively ineffective. We cannot say from the present results why these lanthanides are not effective in the inhibition of calcium entry channels.

Additionally, oocytes pre-treated with thapsigargin were shown to produce a CIF activity which was functionally identical to that derived from Jurkat cells (Kim and Hanley, 1999). This result, together with other aspects of the characterization of the responses, suggests that CIF is a plausible candidate for mediating the initiation of calcium entry following calcium stores depletion in oocytes, and also that lanthanides are novel pharmacological tools for the study of calcium entry channels in *Xenopus* oocytes.

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