Regulation of inositol trisphosphate-induced membrane currents in *Xenopus* oocytes by a Jurkat cell calcium influx factor

David THOMAS, Hak Yong KIM and Michael R. HANLEY*

Department of Biological Chemistry, University of California, Davis School of Medicine, Davis, CA 95616-8635, U.S.A.

The functional interactions of a Jurkat cell-derived calcium influx factor (CIF) with $\text{Ins}(1,4,5)\text{P}_3$ were examined by microinjection and voltage-clamp recording of current responses in *Xenopus* oocytes. CIF, which stimulates Ca^{2+} entry directly on microinjection, was active at dilutions at which it had no direct effect by augmenting both initial rapid $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} discharge-activated currents and later sustained Ca^{2+} entry-activated currents. Augmented initial membrane currents were 3–5-fold greater in peak amplitude than currents evoked by injection of the same dose of $\text{Ins}(1,4,5)\text{P}_3$ alone. The augmented initial response was not decreased by removal of extracellular Ca^{2+} , suggesting that there is potentiation of $\text{Ins}(1,4,5)\text{P}_3$ -mediated discharge from intracellular Ca^{2+} stores. However, the augmentation of $\text{Ins}(1,4,5)\text{P}_3$ -mediated discharge cannot be due to an enhanced production of endogenous $\text{Ins}(1,4,5)\text{P}_3$ because maximal $\text{Ins}(1,4,5)\text{P}_3$ -activated currents saturate (approx. 500 nA) with supramaximal levels of $\text{Ins}(1,4,5)\text{P}_3$ (10–50 μM). Depletion of Ca^{2+} stores, by pretreatment with thapsigargin or by prior injection with the $\text{Ins}(1,4,5)\text{P}_3$ receptor antagonist heparin, abolished membrane currents elicited by $\text{Ins}(1,4,5)\text{P}_3$ /CIF co-injection, further suggesting that the $\text{Ins}(1,4,5)\text{P}_3$ receptor was the target for the initial-current-

potentiating actions of CIF. In this regard, CIF also induced augmented initial currents with co-injection of either $\text{Ins}(2,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$. The augmentation of $\text{Ins}(1,4,5)\text{P}_3$ -mediated currents by CIF was bell-shaped with regard to $\text{Ins}(1,4,5)\text{P}_3$ concentration, reminiscent of the regulatory influence of Ca^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ responses. Co-injection of $\text{Ins}(1,4,5)\text{P}_3$ and CIF also augmented (2–3-fold) later current responses arising from sustained Ca^{2+} entry. The augmented late-current responses were not due to enhanced Ca^{2+} store depletion because supramaximal levels of $\text{Ins}(1,4,5)\text{P}_3$ (50 μM) or injection of the poorly metabolized $\text{Ins}(1,4,5)\text{P}_3$ analogue, $\text{Ins}(2,4,5)\text{P}_3$, cannot activate the same magnitude of Ca^{2+} -entry-dependent currents. These results suggest that CIF at low levels interacts with $\text{Ins}(1,4,5)\text{P}_3$ to sensitize two pathways of Ca^{2+} signalling: initial discharge and later Ca^{2+} entry. Thus under physiological conditions CIF might be more potent as a co-messenger than as a direct Ca^{2+} entry signal and might provide a novel type of direct feedback regulation between the stores-activated influx pathway and the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Moreover these results suggest that CIF modulation of the receptor for $\text{Ins}(1,4,5)\text{P}_3$ may underlie control of both augmentation of discharge and Ca^{2+} entry, as has been predicted from the conformational coupling model of Ca^{2+} entry.

INTRODUCTION

Receptors that trigger the elevation of cytoplasmic free Ca^{2+} concentration are coupled to the intracellular release of Ca^{2+} from internal stores by the production of $\text{Ins}(1,4,5)\text{P}_3$ [1]. One important consequence of $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} discharge is the depletion of intracellular Ca^{2+} stores. Much evidence now reveals that Ca^{2+} store depletion constitutes the initiating signal for activating Ca^{2+} influx through several channels, including a biophysically unique class of voltage-insensitive plasma membrane Ca^{2+} channels, denoted as I_{CRAC} for Ca^{2+} -release activated current [2–5]. This mode of depletion-activated Ca^{2+} entry was first recognized by Putney and was termed ‘capacitative entry’ [6,7]. However, the mechanism by which stores depletion is coupled to activation of Ca^{2+} entry remains enigmatic.

Several hypotheses have been considered as potential coupling mechanisms between Ca^{2+} stores and Ca^{2+} -entry channels [1,8–12]. Two of these have attracted the greatest interest. The first major hypothesis is that the $\text{Ins}(1,4,5)\text{P}_3$ receptor is physically linked to the Ca^{2+} -entry channels and conformational coupling through this complex is triggered on store depletion, much as has been hypothesized to occur in muscle cells between ryanodine receptors and surface voltage-gated Ca^{2+} channels [13,14]. In

contrast, a major second hypothesis suggests that a diffusible messenger is mobilized in response to store depletion and gates Ca^{2+} influx in a manner analogous to $\text{Ins}(1,4,5)\text{P}_3$'s actions in gating Ca^{2+} release from internal stores [8,15–18]. We recently reported [17] and partly purified [18] a Ca^{2+} -influx activity present in extracts from Jurkat lymphocytes with properties consistent with a depletion-activated messenger's coupling store depletion to Ca^{2+} entry [16], but nothing is known of its interactions with $\text{Ins}(1,4,5)\text{P}_3$.

The possibility of regulatory interactions between discharge and entry pathways has been addressed exclusively in terms of feedback effects of Ca^{2+} [4,19]. However, the concept of diffusible messengers in the regulation of Ca^{2+} entry raises the novel possibility of Ca^{2+} -independent feedback interactions between the $\text{Ins}(1,4,5)\text{P}_3$ receptor and the Ca^{2+} influx pathway. In this regard, the possibility of regulatory interactions between the sequential pathways of Ca^{2+} discharge and stores-depletion-activated entry is conceptually similar to earlier proposals as to how the potential co-messenger, $\text{Ins}(1,3,4,5)\text{P}_4$, might act together with $\text{Ins}(1,4,5)\text{P}_3$ on Ca^{2+} elevation in sea-urchin eggs [20]. To examine possible interactions, we adopted the strategy of single-cell functional analysis, using intracellular co-injection of $\text{Ins}(1,4,5)\text{P}_3$ and calcium influx factor (CIF) at low or sub-

Abbreviations used: BAPTA, bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; CIF, calcium influx factor; HBSS, Hanks balanced salt solution; TG, thapsigargin.

* To whom correspondence should be addressed.

threshold doses, in *Xenopus* oocytes. At subthreshold concentrations where CIF exhibited no direct activity on membrane currents, CIF was nevertheless highly active in synergistic augmentation of $\text{Ins}(1,4,5)P_3$ -mediated current responses. Both early (Ca^{2+} -discharge mediated) and late (Ca^{2+} -influx mediated) Ca^{2+} -dependent currents elicited by low doses of $\text{Ins}(1,4,5)P_3$ were enhanced. These results are discussed in terms of a novel form of feedback regulation of $\text{Ins}(1,4,5)P_3$ receptor by the stores-depletion signalling pathway, and in terms of the control of Ca^{2+} entry by conformational coupling.

MATERIALS AND METHODS

Materials

Thapsigargin (TG), $\text{Ins}(1,4,5)P_3$, okadaic acid, 1-norokadaone and calyculin A were from LC Services (Woburn, MA, U.S.A.). Genistein, $\text{Ins}(2,4,5)P_3$, $\text{Ins}(1,3,4,5)P_4$, guanosine 5' [γ -thio]triphosphate and guanosine 5' [β -thio]diphosphate were from Calbiochem. Niflumic acid, heparin (low molecular mass) and 1-(5-isoquinolylsulphonyl)-2-methyl-piperazine (H-7) were from Sigma. bis(*o*-Aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) was from Molecular Probes (Eugene, OR, U.S.A.). Staurosporine was from Kamiya Biomedicals. FK506 was a gift from Dr. Brett Premack (Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA, U.S.A.). The bastadin mixture was a gift from Dr. Isaac Pessah (Department of Molecular Biosciences, University of California, Davis School of Veterinary Medicine, Davis, CA, U.S.A.). Hanks balanced salt solution (HBSS) and L-15 medium were from Life Technologies (Grand Island, NY, U.S.A.). RPMI-1640 was from BioWhittaker (Walkersville, MD, U.S.A.). Bio-Gel P-2 gel filtration media was from Bio-Rad (Hercules, CA, U.S.A.). Microcon-30 filters were from Amicon (Beverly, MA, U.S.A.). Sep-Pak Cartridges (C18) were from Millipore (Milford, MA, U.S.A.).

Cell culture

Jurkat cells were maintained in suspension in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and penicillin (100 i.u./ml)/streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were passaged by 1:10 dilution every 4 days. NG115-401L neuronal cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin (100 i.u./ml)/streptomycin (100 $\mu\text{g}/\text{ml}$). NG115-401L cells were passaged at 1:10 ratios every 3 days by trypsinization.

Extraction and purification of CIF

Acid extracts were prepared from unstimulated and thapsigargin-stimulated Jurkat lymphocytes and NG115-401L cells in an identical fashion, as previously described [17,18]. Briefly, cells were washed three times and resuspended in HBSS containing 20 mM Hepes. Cells were stimulated with 1 μM TG for 15 min at 25 °C. After stimulation with TG, cells were centrifuged for 5 min at 200 *g* and the pellet was resuspended in 0.85 ml of HBSS containing 20 mM Hepes. The suspension was extracted with 0.15 ml of a 1 M HCl solution 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 the addition of 10 M NaOH. After neutralization, 10 mM BaCl_2 was added to the extract for precipitation of vicinal phosphate compounds including inositol polyphosphates. In control experiments we have shown that this treatment removes all inositol phosphates active on Ca^{2+} mobilization. Insoluble material was removed

from extracts by centrifugation for 5 min at 12000 *g*. The supernatant was freeze-dried and the residue extracted with methanol with continuous mixing for 15 min at 25 °C. The methanol extract (0.8 ml) was loaded on a Sep-Pak C18 Cartridge for the removal of hydrophobic material and was rinsed with methanol (0.8 ml, flow rate 0.25 ml/min). The methanol eluates were combined (1.6 ml), dried at 30 °C under N_2 gas and resuspended in 0.25 ml of 0.1 M acetic acid. The reconstituted extract was clarified by centrifugal ultrafiltration through a Microcon-30 filter. The filtrate is enriched for materials of molecular mass less than 30 kDa, whereas the non-diffusible material is of molecular mass over 30 kDa. For further purification, the filtrate was loaded on to a Bio-gel P-2 column (0.7 cm $\times$ 27 cm), equilibrated with 0.1 M acetic acid, and was eluted by the same buffer (7 ml/h); fractions of 0.5 ml were collected. Activity was assayed by measuring Ca^{2+} -dependent Cl^- current in *Xenopus* oocytes under voltage clamp as previously described [17,21]. Fraction 15 from the gel-filtration column (sometimes pooled with fraction 16) corresponded to activity enriched by thapsigargin stimulation [18] and constitutes the high-molecular-mass fraction, over approx. 600 Da, denoted as CIF in this report. It is important to emphasize that all of the observations reported here apply to CIF rigorously repurified through two cycles of high-performance TLC [17]. Thus both CIF purified by gel filtration and CIF repurified twice by high-performance TLC gave identical results in terms of $\text{Ins}(1,4,5)P_3$ augmentation. Moreover, the chemical properties of the factor augmenting $\text{Ins}(1,4,5)P_3$ responses were indistinguishable from CIF, suggesting that a common compound was responsible for both direct Ca^{2+} entry and $\text{Ins}(1,4,5)P_3$ -potentiating effects.

Oocyte procurement

Immature stage V and VI oocytes from *Xenopus laevis* were obtained by surgical removal of ovarian tissue. The animals were anaesthetized by hypothermia and several ovarian sacs were removed and minced with fine-tipped forceps. The follicular layer was removed by incubating the oocytes in type 1A collagenase (Sigma) in Ca^{2+} -free 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) for 2 h. Any remaining follicular cells on the oocytes were removed by rolling the oocytes on plastic Petri dishes with fine-tipped forceps. After removal and defolliculation, the oocytes were maintained in L-15 medium supplemented with 0.25% chicken egg ovalbumin, 2 mM L-glutamine, penicillin (50 i.u./ml)/streptomycin (50 $\mu\text{g}/\text{ml}$) solution, 50 $\mu\text{g}/\text{ml}$ gentamicin and 10 mM Hepes.

Data acquisition and current analysis

Two-electrode whole-cell voltage-clamp experiments with *Xenopus* oocytes were performed as described previously [17,21]. Briefly, stage V and VI oocytes were impaled with two micro-electrodes with resistances of 1 M Ω and back-filled with 3 M KCl. Oocytes were voltage-clamped at -60 mV in OR2 medium with a TEV-200 clamp amplifier (Dagan, MN, U.S.A.). For experiments requiring removal of extracellular Ca^{2+} , oocytes were perfused with Ca^{2+} -free OR2 medium (82 mM NaCl, 2.5 mM KCl, 5 mM MgCl_2 , 0.1 mM EGTA, 1 mM Na_2HPO_4 and 5 mM Hepes, pH 7.4). We have found, in agreement with others [22], that oocyte responses can be very unstable in Ca^{2+} -free buffers, but can be stabilized by adding a high concentration of magnesium. Currents were low-pass filtered at 50 kHz with an internal four-pole Bessel filter and digitized at sampling rates of 100 ms (10 Hz) or 500 ms (2 Hz) with a TL-1 analogue/digital

converter (Axon Instruments, Foster City, CA, U.S.A.) and the current analysis program SCAN (Dagan, Minneapolis, MN, U.S.A.).

For obtaining current–voltage relations, voltage ramps were run on the activated currents. The ramp I – V data were collected with pCLAMP software (version 5.5) for sampling current responses to potential changes at a rate of 4 kHz. Current output from the clamp amplifier was low-pass filtered at 50–100 kHz and digitized with the TL-1 interface board (Axon Instruments) at 12-bit resolution. The ramp protocol employed repeated episodes (every 5 s) of a -100 to $+60$ mV ramp (2 s duration ramp at a depolarization rate of 0.5 mV/ms) with an episode holding potential of -60 mV between episodes.

Oocyte microinjection

Microinjection was by the Picospritzer pressure injection apparatus (General Valve Corporation, Fairfield, NJ, U.S.A.). The volume delivered by pressure injection was calibrated by measuring the diameter of the droplet expelled in air with an eyepiece micrometer. With the exception of heparin and BAPTA injections, oocytes were injected with various drugs and other agents while under voltage clamp. For these experiments a third glass micropipette was inserted into the oocyte to serve as the injection pipette. The injection pipette was broken off to give a tip diameter of 20 μ m or less. The oocyte was impaled with the injection pipette first and then perfused in OR2 medium to let the cell stabilize from the insertion of the large injection pipette before inserting the two electrodes. Oocytes were routinely injected with 10 nl or less of compounds, which resulted in an approx. 100-fold dilution, assuming an oocyte cytoplasmic volume of approx. 1 μ l. The mere physical perturbation of pressure injection fails to elicit current responses when oocytes are microinjected with 10 nl of water or OR2. Compounds were injected by using N_2 gas and the pressure never exceeded 140 kPa. Membrane-permeant compounds such as thapsigargin, ionomycin and genistein were applied to oocytes by perfusion rather than by microinjection.

RESULTS

Properties of currents activated by microinjection of $\text{Ins}(1,4,5)P_3$ or CIF

By way of control experiments, characteristic current responses elicited by microinjection of $\text{Ins}(1,4,5)P_3$ into immature *Xenopus* oocytes were characterized extensively, and found to be indistinguishable from earlier reports [23–27]. Briefly, a standardized dose of $\text{Ins}(1,4,5)P_3$ (estimated to give a final intracellular concentration of 1 μ M) typically evoked an inward current of 100–400 nA peak amplitude (Figure 1, dotted traces). The amplitude of the current response was not significantly inhibited by the removal of extracellular Ca^{2+} , but rather the current decayed more rapidly in Ca^{2+} -free medium. In addition, the current was completely abolished by pre-injection of the fast Ca^{2+} chelator BAPTA (results not shown). The inward current was predominantly carried by Cl^- ions as revealed by the reversal potential of current responses (approx. -20 mV) [28,29]. In addition, the Cl^- current activated by $\text{Ins}(1,4,5)P_3$ injection exhibited a characteristic rectification at the greater hyperpolarized potentials, as previously noted [30]. A second injection of $\text{Ins}(1,4,5)P_3$ after the decay of the first current response failed to activate a second current response, in agreement with earlier work [24,25]. Lastly, the $\text{Ins}(1,4,5)P_3$ current response was blocked by pre-injection of the $\text{Ins}(1,4,5)P_3$ receptor antagonist heparin.

As a comparison, the direct current responses activated by CIF injection, as previously reported [17,18], were dependent on extracellular Ca^{2+} . The analysis of the I – V relationships of initial currents revealed that reversal potentials to CIF were slightly more negative than $\text{Ins}(1,4,5)P_3$ -induced currents, implying that CIF-induced initial currents might not be carried exclusively by Cl^- ions. Indeed, we have recently described currents activated by nucleotide derivatives that share several properties with CIF-evoked currents, including reversal potentials more negative than the expected Cl^- reversal potential [31]. This negative shift in the Cl^- reversal might reflect activation of K^+ currents, as has been reported in *Xenopus* oocytes for pool-depletion-activated currents [32]. The outward currents activated by CIF injections were abolished by the potassium channel blocker tetraethylammonium, and exhibited reversal potentials (-85 mV) consistent with this being primarily a K^+ current. The I – V curve also demonstrated a more linear current response at negative potentials than $\text{Ins}(1,4,5)P_3$ -activated Cl^- currents. However, CIF current responses were abolished by the Cl^- channel blocker niflumic acid as well as by intracellular pre-injection with BAPTA. In contrast with $\text{Ins}(1,4,5)P_3$ responses, CIF-induced currents were not sensitive to heparin pre-injection (results not shown).

Augmentation of rapid $\text{Ins}(1,4,5)P_3$ -activated discharge by co-injection with CIF

To investigate the effects of CIF on $\text{Ins}(1,4,5)P_3$ responses, CIF was diluted (1:4) to a subthreshold level that failed to activate inward currents (Figure 2a, left trace). However, when diluted CIF, prepared from thapsigargin-stimulated Jurkat lymphocytes, was co-injected with a submaximal dose of $\text{Ins}(1,4,5)P_3$ (final concentration 1 μ M) we observed a large potentiation (ranging from 500 to 2000 nA) of the peak amplitude current response compared with responses evoked by co-injection of $\text{Ins}(1,4,5)P_3$ with extracts prepared from unstimulated Jurkat lymphocytes (compare Figures 1b and 1a). Earlier we established that a cell line (e.g. NG115-401L cells) that lacked capacitative Ca^{2+} entry in response to thapsigargin [33] did not make CIF [17]. Significantly, extracts from thapsigargin-stimulated NG115-401L cells also did not show augmentation of $\text{Ins}(1,4,5)P_3$ responses (Figure 1c).

We have characterized this synergism elicited by purified Jurkat-cell CIF. Like $\text{Ins}(1,4,5)P_3$ injections alone, $\text{Ins}(1,4,5)P_3$ /CIF co-injections were strongly desensitizing to subsequent injections (Figure 2a, middle two traces; $n = 35$). The augmented peak current evoked by $\text{Ins}(1,4,5)P_3$ /CIF co-injection was not significantly decreased in Ca^{2+} -free OR2 (Figure 2a, right trace), suggesting synergism between CIF and $\text{Ins}(1,4,5)P_3$ on Ca^{2+} discharge. This interpretation is further supported by a complete blockade by heparin (Figure 2b). Moreover the response can be completely abolished by depleting stores with a 2 h thapsigargin incubation in Ca^{2+} -free OR2 (Figure 2c). Figure 2(d) shows that the ramp I – V curve for the CIF-augmented discharge current (●) resembles the I – V curve obtained by $\text{Ins}(1,4,5)P_3$ injection alone (▲).

Other inositol phosphates, $\text{Ins}(2,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$, have been shown to activate discharge from internal Ca^{2+} stores in *Xenopus* oocytes [23,34,35]. CIF co-injection augmented the magnitude of initial currents activated by $\text{Ins}(2,4,5)P_3$ ($n = 6$) or $\text{Ins}(1,3,4,5)P_4$ ($n = 8$) (Figures 3a and 3b). Moreover, these augmented currents exhibited the characteristic reversal potentials as observed for $\text{Ins}(1,4,5)P_3$ /CIF co-injection (results not shown). Because discharge through the $\text{Ins}(1,4,5)P_3$ receptor/channel can be potentiated by submillimolar concentrations of

Figure 1 Extracts from thapsigargin-treated Jurkat cells, but not thapsigargin-treated NG115-401L cells, potentiate Ins(1,4,5) P_3 responses

(a) *Xenopus* oocytes were microinjected as described in the Materials and methods section with a 0.1 mM stock solution of Ins(1,4,5) P_3 (IP₃), yielding an estimated final concentration of 1 μ M in the oocyte. The dotted trace shows a typical current response to Ins(1,4,5) P_3 injection in oocyte Ringer's solution containing 1 mM Ca²⁺. The solid trace shows the Ins(1,4,5) P_3 response when extracts obtained from unstimulated Jurkat cells were co-injected into the oocyte. The Jurkat extracts were prepared as described in the Materials and methods section and diluted 1:4, where they fail to evoke current responses. (b) The dotted trace shows current responses evoked by Ins(1,4,5) P_3 injection alone. The solid trace shows the potentiation of Ins(1,4,5) P_3 -induced current responses by co-injection of 1 μ M Ins(1,4,5) P_3 with extracts prepared from thapsigargin-treated Jurkat cells. (c) The solid trace shows current responses evoked when extracts from thapsigargin-treated NG115-401L cells were co-injected with Ins(1,4,5) P_3 into oocytes, compared with responses elicited by Ins(1,4,5) P_3 injection alone (dotted trace). Extracts from unstimulated and thapsigargin-stimulated Jurkat cells and NG115-401L cells were prepared as described in the Materials and methods section. Extracts from NG115-401L cells failed to activate currents with direct injection into oocytes [17] and thus were not diluted in co-injection experiments with Ins(1,4,5) P_3 .

Figure 2 Co-injection of CIF potentiates Ins(1,4,5) P_3 -mediated discharge

(a) CIF diluted 1:4 fails to activate inward current (left), inward current triggered by 1 μ M Ins(1,4,5) P_3 (IP₃) injection alone (middle left), the potentiation of the Ins(1,4,5) P_3 -activated current by co-injecting CIF diluted 1:4 (middle right) and the effect of the removal of external Ca²⁺ on the potentiated Ins(1,4,5) P_3 current (right). Arrows indicate injection points and bars denote extracellular Ca²⁺ conditions (in mM). (b) Pretreatment with heparin blocks the potentiation of the current. Heparin (500 μ g/ml) was pre-injected for 30 min or more before co-injection of Ins(1,4,5) P_3 and CIF (1:4). (c) Pretreatment with thapsigargin blocks potentiation of current, but capacitive entry responses are unaffected. Oocytes were incubated with 1 μ M thapsigargin for over 2 h (see the Materials and methods section) before co-injection of Ins(1,4,5) P_3 and CIF (1:4). Arrows indicate injection points and bars indicate changes and durations of external Ca²⁺ conditions (in mM). (d) I-V curves of the current activated by Ins(1,4,5) P_3 injection (▲) and the potentiated Ins(1,4,5) P_3 -activated current (●).

ATP [36,37], ATP was tested by co-injection with Ins(1,4,5) P_3 . At ATP levels (50–100 μ M) reported to increase open times and conductances of the Ins(1,4,5) P_3 receptor maximally, there was

no effect of ATP co-injection. Ionomycin treatment was used to elevate cytosolic Ca²⁺ directly. Neither co-injection of ionomycin plus CIF nor external application of ionomycin with CIF

Figure 3 CIF potentiates other inositol polyphosphates that mediate Ca^{2+} discharge

(a) Current activated by microinjection of $1 \mu\text{M}$ $\text{Ins}(2,4,5)\text{P}_3$ ($2,4,5\text{-IP}_3$) (left) and potentiated current evoked by co-injection of $1 \mu\text{M}$ $\text{Ins}(2,4,5)\text{P}_3$ and CIF (1:4) (right). (b) Current activated by microinjection of $10 \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$ (left) and potentiated current evoked by co-injection of $10 \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$ and CIF (1:4) (right). Arrows indicate injection points and bars denote extracellular Ca^{2+} conditions (in mM).

injection resulted in currents larger than those activated by ionomycin alone (results not shown), further emphasizing that the potentiation we observe with CIF reflects an interaction with the $\text{Ins}(1,4,5)\text{P}_3$ receptor, not with Cl^- channels.

$\text{Ins}(1,4,5)\text{P}_3$ -activated Ca^{2+} discharge exhibits a bell-shaped dose-response curve to varying Ca^{2+} concentrations, which is thought to be crucial to Ca^{2+} signalling [38,39]. Decreasing the concentration of CIF by serial dilution at constant $\text{Ins}(1,4,5)\text{P}_3$ levels diminished currents (Figure 4). However, CIF-augmented $\text{Ins}(1,4,5)\text{P}_3$ responses exhibited a bell-shaped dose-response behaviour to varying $\text{Ins}(1,4,5)\text{P}_3$ concentrations, with maximum currents activated by co-injection of 200 nM $\text{Ins}(1,4,5)\text{P}_3$ /CIF (Figure 4, inset), but with reduced currents at more elevated levels of injected $\text{Ins}(1,4,5)\text{P}_3$.

Augmentation of stores-depletion-induced Ca^{2+} entry responses by CIF

Sustained membrane currents attributed to Ca^{2+} entry through depletion-activated pathways were also augmented by co-injection of $\text{Ins}(1,4,5)\text{P}_3$ and CIF (Figure 5a; compare left and middle traces). This increased Ca^{2+} permeability was strictly dependent on depletion of internal Ca^{2+} stores as it cannot be observed in unstimulated oocytes. This augmented depletion-activated current exhibited the same properties as the previously characterized stores-operated Ca^{2+} entry pathway in *Xenopus* oocytes [22,40] and in addition shared many of the same properties reported for the mammalian capacitative entry Ca^{2+} channels [2–5]. The late currents were blocked by perfusion with submillimolar concentrations of the inorganic bivalent ions Cd^{2+} , Co^{2+} and Ni^{2+} , as well as by La^{3+} , and were also blocked by the stores-operated channel inhibitor econazole (results not shown). The augmented depletion-activated current seems to retain the

Figure 4 Dose effect of $\text{Ins}(1,4,5)\text{P}_3$ and CIF on potentiated $\text{Ins}(1,4,5)\text{P}_3$ responses

The effect of varying CIF levels with constant $\text{Ins}(1,4,5)\text{P}_3$ (IP_3 ; 200 nM) on the potentiation of the $\text{Ins}(1,4,5)\text{P}_3$ -activated discharge current is shown (●). The current response elicited by injection of 200 nM $\text{Ins}(1,4,5)\text{P}_3$ alone is also shown (○). Inset: the effect of varying $\text{Ins}(1,4,5)\text{P}_3$ concentrations from 5 nM to $1 \mu\text{M}$ on the potentiation of the discharge current elicited by co-injection of $\text{Ins}(1,4,5)\text{P}_3$ with a fixed amount of CIF (1:4) (open bars), compared with current responses elicited by $\text{Ins}(1,4,5)\text{P}_3$ injection alone, at the same concentrations (shaded bars).

high selectivity for Ca^{2+} permeation, as currents were rapidly abolished with Ca^{2+} -free perfusion and were enhanced when external Ca^{2+} was increased from 2 to 10 mM (Figure 5a, left trace). There was no current attenuation with a perfusion medium in which Na^+ was replaced by the impermeant cation *N*-methyl *D*-glucamine (90 mM NMDGCl, 2 mM CaCl_2 , 5 mM HEPES, $\text{pH } 7.4$). The I - V behaviour of the augmented Ca^{2+} influx-dependent current was found to be the same as the influx current activated by $\text{Ins}(1,4,5)\text{P}_3$ and thapsigargin stimulation, and on the basis of the observed reversal potential (-25 mV) seemed predominantly to be the Ca^{2+} -activated Cl^- current (Figure 5b). The reversal potential for the influx current is slightly more negative than for the $\text{Ins}(1,4,5)\text{P}_3$ -activated discharge current and might reflect initial activation of the outward K^+ current. Thus the augmented late current elicited by co-injection of $\text{Ins}(1,4,5)\text{P}_3$ and CIF was very similar to that elicited by direct stores depletion by thapsigargin (Figure 5c). Direct injection of the undiluted CIF fraction failed to evoke current responses in oocytes depleted of Ca^{2+} stores by 2 h of thapsigargin pretreatment, even though the Ca^{2+} entry channels were still open as revealed by increasing extracellular Ca^{2+} to 10 mM (Figure 5d).

The potentiated Ca^{2+} influx response was not likely to be due exclusively to enhanced depletion, owing to CIF augmentation of the discharge response, because in some experiments the initial potentiation of the $\text{Ins}(1,4,5)\text{P}_3$ response was poor, yet we still observed enhanced Ca^{2+} influx responses (Figure 5a, right trace). Moreover we found that injecting as much as $50 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ failed to trigger influx currents of the magnitude activated by $\text{Ins}(1,4,5)\text{P}_3$ /CIF co-injection, even though these responses were

Figure 5 Co-injection of CIF augments $Ins(1,4,5)P_3$ -activated Ca^{2+} entry responses

(a) Ca^{2+} influx current activated by $Ins(1,4,5)P_3$ (IP_3) injection is a long-lived current sensitive to extracellular Ca^{2+} . Arrows indicate injection points and bars denote changes and durations of external Ca^{2+} conditions (in mM). The left trace shows that the current can be reversibly inactivated by perfusion of Ca^{2+} -free oocyte Ringer's solution. Re-addition of high Ca^{2+} (10 mM) activates subsequent inward currents that are rapidly inactivated by removal of extracellular Ca^{2+} , and are larger currents than those triggered by 2 mM external Ca^{2+} . The middle trace shows that after the potentiated discharge response (arrow) and the removal of extracellular Ca^{2+} , adding back 10 mM Ca^{2+} results in a potentiated influx current compared with Ca^{2+} influx activated by $Ins(1,4,5)P_3$ alone (left trace). The right trace shows that even when potentiation of the discharge response is small (note arrow denoting weak discharge potentiation), co-injection of $Ins(1,4,5)P_3$ and CIF activates an augmented Ca^{2+} entry response when adding back 10 mM Ca^{2+} . (b) The $I-V$ curve of the current activated by stepping to 10 mM Ca^{2+} , showing a reversal potential of approx. -25 mV. (c) Pretreatment with thapsigargin (2 h) activates a Ca^{2+} influx current with similar properties to the $Ins(1,4,5)P_3$ -activated current. The bar indicates changes and durations of extracellular Ca^{2+} conditions (in mM). After 2 h of incubation with thapsigargin the oocyte was voltage-clamped in 0 Ca^{2+} and perfused with 10 mM Ca^{2+} . (d) Direct injection (arrow) of undiluted CIF fraction fails to elicit current responses in oocytes pretreated with thapsigargin as described in (c). The thapsigargin-induced current was sensitive to external Ca^{2+} , was long-lived and exhibited an $I-V$ relationship similar to the response shown in (b).

matched for peak-amplitude discharge responses (compare Figures 6a and 6c). Indeed, the discharge current and the influx current activated by $Ins(1,4,5)P_3$ seem to saturate at approx. 500 nA and 100–150 nA respectively (Figures 6b and 6c). Previous reports have shown that injecting $Ins(2,4,5)P_3$ [a poorly metabolized $Ins(1,4,5)P_3$ analogue] into *Xenopus* oocytes can potentially stimulate capacitative Ca^{2+} entry, presumably by causing greater store depletion [34,35]. We observed, however, that microinjection of $Ins(2,4,5)P_3$ failed to trigger Ca^{2+} entry responses to the same extent as co-injection of $Ins(1,4,5)P_3$ and CIF (compare Figures 6a and 6d). Further, we diluted the $Ins(1,4,5)P_3$ /CIF mixture to match the peak amplitude discharge currents activated by 1 μ M $Ins(1,4,5)P_3$ alone, using this response as a rough index of the extent of store emptying, and found that these dilutions could still stimulate greater influx currents than $Ins(1,4,5)P_3$ by itself stimulates (results not shown).

Pharmacology of the augmented early Ca^{2+} -discharge- and late Ca^{2+} -influx-dependent currents

The effects of pharmacological agents previously reported to modulate stores-operated channel pathways were evaluated on

the CIF-augmented discharge and entry currents (Table 1) [4,15,32,34,41–46]. None of the reagents tested exhibited any difference between $Ins(1,4,5)P_3$ -induced or CIF-augmented $Ins(1,4,5)P_3$ -induced currents. These results argue against several potential mechanisms for the synergism of $Ins(1,4,5)P_3$ and CIF and support the possibility of direct modulation of an $Ins(1,4,5)P_3$ receptor/ Ca^{2+} entry pathway complex.

DISCUSSION

Using *Xenopus* oocytes under voltage clamp as a bioassay we have identified novel small-molecule Ca^{2+} regulatory factors from activated lymphocytes and have focused on a stores-depletion-induced signal (here termed CIF) that can stimulate Ca^{2+} entry directly on microinjection [17,18]. However, it is important to note that this direct effect of CIF might not be the most physiologically relevant. Indeed, under physiological conditions CIF would be mobilized secondarily to $Ins(1,4,5)P_3$ production, as a result of depletion of Ca^{2+} stores. This consideration suggests that $Ins(1,4,5)P_3$ and CIF might act effectively as 'co-messengers'. Such an interaction was suggested earlier for $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ as a possible model for how

Figure 6 Augmentation of Ca^{2+} -entry response is not dependent on the extent of store depletion

(a) Co-injection of $\text{Ins}(1,4,5)\text{P}_3$ (IP_3 ; $1 \mu\text{M}$) and CIF (1:4) evokes a potentiated discharge current accompanied by an augmented Ca^{2+} influx response, revealed by perfusion of 10 mM Ca^{2+} . Arrows indicate injection points and bars denote changes and durations of exposure to different external Ca^{2+} conditions (in mM). (b) Response elicited by $10 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ with the same protocol as in (a), i.e. injection in 1 mM external Ca^{2+} , removal of extracellular Ca^{2+} , followed by perfusion with high Ca^{2+} (10 mM). (c) Response elicited by injection of $50 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$. (d) Response elicited by injection of $2 \mu\text{M}$ $\text{Ins}(2,4,5)\text{P}_3$ ($2,4,5\text{-IP}_3$).

$\text{Ins}(1,3,4,5)\text{P}_4$ might enhance $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} elevation [20]. We have evaluated the possibility of functional interactions of $\text{Ins}(1,4,5)\text{P}_3$ with CIF by examining well-characterized Ca^{2+} -dependent current responses in oocytes on co-injection.

By using dilutions of CIF that have no direct Ca^{2+} entry activity, $\text{Ins}(1,4,5)\text{P}_3$ -induced initial Ca^{2+} discharge- and late Ca^{2+} -entry-dependent membrane currents were augmented to levels beyond that achievable by the highest doses of $\text{Ins}(1,4,5)\text{P}_3$ injection. This might explain a recurring observation that

receptor-generated Ca^{2+} currents are invariably larger than those elicited by $\text{Ins}(1,4,5)\text{P}_3$ injection alone [24,40]. Thus a missing factor might be needed to reconstruct the full dynamic range of Ca^{2+} discharge by endogenously produced $\text{Ins}(1,4,5)\text{P}_3$. Although how this would be achieved is not clear, one possibility is that CIF interacts directly with the $\text{Ins}(1,4,5)\text{P}_3$ receptor. This is an appealing hypothesis because preliminary evidence has suggested that CIF might be a nucleotide derivative [31], and there are two identified 'nucleotide binding sites' on $\text{Ins}(1,4,5)\text{P}_3$ receptors [36,37]. One of these sites seems to have stringent recognition requirements, preferring adenine nucleotides [37]. CIF is unlikely to be acting at this site because of its recognition specificity. Moreover, ATP co-injection with $\text{Ins}(1,4,5)\text{P}_3$ had no effect and did not therefore mimic the synergism of CIF with $\text{Ins}(1,4,5)\text{P}_3$. This raises the possibility that the second site, a 'non-selective nucleotide site', on the $\text{Ins}(1,4,5)\text{P}_3$ receptor is a candidate site for CIF modulation, a possibility that can be investigated directly by targeted mutation of $\text{Ins}(1,4,5)\text{P}_3$ receptor.

If the $\text{Ins}(1,4,5)\text{P}_3$ receptor were to be physiologically regulated by a stores-depletion-activated chemical messenger, CIF would be an additional component in the generation of complex spatio-temporal patterning of Ca^{2+} signalling, such as waves and oscillations [13,47]. CIF would, at low levels, act in two ways: regulation of $\text{Ins}(1,4,5)\text{P}_3$ receptor sensitivity, and subsequent modulation of its own production due to altered levels of stores depletion. In this regard it is intriguing that $\text{Ins}(1,4,5)\text{P}_3$ seems to have a bell-shaped response curve for its interaction with CIF, so at low levels of $\text{Ins}(1,4,5)\text{P}_3$ production Ca^{2+} discharge is enhanced whereas high levels of $\text{Ins}(1,4,5)\text{P}_3$ production lead to decreased discharge, thereby favouring Ca^{2+} store refilling. This has the appealing consequence that Ca^{2+} store replenishment would become protected under conditions of sustained or intense $\text{Ins}(1,4,5)\text{P}_3$ production whereas cytosolic Ca^{2+} elevation would be maintained by Ca^{2+} entry. These interactions are illustrated in Scheme 1. More generally, functional interactions of $\text{Ins}(1,4,5)\text{P}_3$ with cofactors might prove to be cell-specific and could be significant in certain cell types, such as T lymphocytes, that require prolonged phases of Ca^{2+} influx for activation in which the initial discharge response acts as a priming response to drive the sustained Ca^{2+} influx phase [2,5,19,48,49].

In addition to CIF-mediated potentiation of the $\text{Ins}(1,4,5)\text{P}_3$ -activated discharge response, we also observed potentiation of the stores-operated Ca^{2+} -entry-dependent current responses ini-

Table 1 The effects of pharmacological agents on the synergism of the early Ca^{2+} discharge current and on the augmented late Ca^{2+} entry responses were not different from effects on currents induced by $\text{Ins}(1,4,5)\text{P}_3$ alone

All drugs were microinjected into oocytes as described in the Materials and methods section. Drug concentrations were estimated as final intracellular concentrations based on an oocyte cytoplasmic volume of $1 \mu\text{l}$. The effects of the drugs on the discharge current and influx current were determined by comparison with control responses elicited by $\text{Ins}(1,4,5)\text{P}_3$ ($1 \mu\text{M}$) + CIF (1:4) co-injection in the absence of drug exposure. All drugs were pre-injected for 5 min before co-injection of $\text{Ins}(1,4,5)\text{P}_3$ and CIF. Abbreviations: GTP[S], guanosine 5'-[γ -thio]triphosphate and guanosine; GDP[S], 5'-[β -thio]diphosphate.

Drug	Concentration	Effect on early current	Effect on late current	Number of cells
Okadaic acid	100 nM	Prolongs entry phase	Potentiation	6
1-Norokadaone	100 nM	Prolongs entry phase	Potentiation	3
Calyculin A	10 nM	No effect	No effect	3
FK506	50 nM	No effect	No effect	6
Bastadin	10 μM	No effect	No effect	3
GTP[S]	200 μM	Weak potentiation	Inhibition	10
GDP[S]	200 M	No effect	No effect	3
Genistein	30 μM	No effect	Weak inhibition	6
Staurosporine	100 nM	No effect	No effect	3
H-7	10 μM	No effect	No effect	3

Scheme 1 Hypothetical scheme depicting possible feedback interactions of the depletion-activated messenger with the Ins(1,4,5) P_3 receptor

The hypothetical scheme illustrates that the store-depletion-generated messenger, produced by receptor activation and Ins(1,4,5) P_3 -dependent Ca^{2+} store depletion, might both positively and negatively regulate the Ins(1,4,5) P_3 receptor (broken arrow) as well as gating Ca^{2+} entry. Abbreviations: G, G-protein; PLC, phospholipase C; PIP_2 , PtdIns(4,5) P_2 ; IP_3 , Ins(1,4,5) P_3 ; IP_3R , Ins(1,4,5) P_3 receptor.

tiated by Ins(1,4,5) P_3 . Although the simplest interpretation of this result would be the enhancement of late currents due to an enhancement of depletion, this seems unlikely. Notably, we cannot activate a similar magnitude of Ca^{2+} -entry-dependent current responses with large doses of Ins(1,4,5) P_3 (10–50 μ M) or any other active inositol polyphosphate [e.g. the poorly metabolized analogue Ins(2,4,5) P_3 , which should deplete intracellular Ca^{2+} stores more extensively than the concentration of Ins(1,4,5) P_3 used in the co-injection design]. This suggests that CIF is facilitating the stores operated Ca^{2+} influx pathway at doses lower than its direct effects. The actions of CIF do not seem to be at the level of the Ca^{2+} -sensitive channels because activation of Ca^{2+} -dependent channels by ionomycin was unaffected by CIF injection. The use of a panel of pharmacological inhibitors did not reveal a potential mechanism for Ins(1,4,5) P_3 synergism with CIF. These observations are, however, consistent with a model of conformational coupling of Ins(1,4,5) P_3 receptors to Ca^{2+} entry [12].

In summary, CIF, a diffusible messenger produced by Ca^{2+} stores depletion, seems to act in at least two ways. At high doses CIF initiates Ca^{2+} entry but at lower doses it exhibits complex facilitating interactions with Ins(1,4,5) P_3 , reinforcing the notion that it might function as a physiological co-messenger in Ins(1,4,5) P_3 -receptor-mediated Ca^{2+} mobilization.

We thank Brett Premack (Molecular Pharmacology, Stanford University School of Medicine) for critical advice and his assistance with current analysis, Isaac Pessah for providing us with the bastadin mix, and Rich Nuccitelli for the use of the *X. laevis* colony. This research was supported in part by grants from the National Institutes of Health and the Council for Tobacco Research. D.T. was supported by an NIH training grant in Molecular and Cellular Biology.

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