Potentiation of Fcε Receptor I - activated Ca^{2+} Current (I_{CRAC}) by Cholera Toxin: Possible Mediation by ADP Ribosylation Factor

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Abstract. Antigen-evoked influx of extracellular Ca^{2+} into mast cells may occur via store-operated Ca^{2+} channels called calcium release–activated calcium (CRAC) channels. In mast cells of the rat basophilic leukemia cell line (RBL-2H3), cholester toxin (CT) potentiates antigen-driven uptake of 45Ca^{2+} through CaM P-independent means. Here, we have used perforated patch clamp recording at physiological temperature to test whether cholera toxin or its substrate, Gs, directly modulates the activity of CRAC channels. Cholera toxin dramatically amplified (two-to-fourfold) the Ca^{2+} release–activated Ca^{2+} current (I_{CRAC}) elicited by suboptimal concentrations of antigen, without itself inducing I_{CRAC}, and this enhancement was not mimicked by CaM P elevation. In contrast, cholester toxin did not affect the induction of I_{CRAC} by thapsigargin, an inhibitor of organelle Ca^{2+} pumps, or by intracellular dialysis with low Ca^{2+} pipette solutions. Thus, the activity of CRAC channels is not directly controlled by cholera toxin or Gs. Nor was the potentiation of I_{CRAC} due to enhancement of phosphoinositide hydrolysis or calcium release. Because Gs and the A subunit of cholera toxin bind to A DP ribosylation factor (ARF) and could modulate its activity, we tested the sensitivity of antigen-evoked I_{CRAC} to brefeldin A, an inhibitor of ARF-dependent functions, including vesicle transport. Brefeldin A blocked the enhancement of antigen-evoked I_{CRAC} without inhibiting A DP ribosylation of Gs, but it did not affect I_{CRAC} induced by suboptimal antigen or by thapsigargin. These data provide new evidence that CRAC channels are a major route for Fcε receptor I–triggered Ca^{2+} influx, and they suggest that ARF may modulate the induction of I_{CRAC} by antigen.

Key words: mast cells • patch clamp • Ca^{2+} imaging • Gs • brefeldin A

Introduction

Calcium influx is thought to be required for the secretion of inflammatory mediators, activation of transcription factors, and the elaboration of cytokines at rat mast cells stimulated through the high affinity receptor (FcεR1) for IgE. Cholera toxin (CT) markedly potentiates FcεR1-mediated uptake of 45Ca^{2+} (Narasimhan et al., 1988) and secretion of preformed mediators (McCloskey, 1986, 1990). Calcium currents associated with this pathway were first observed in Jurkat human T cells and rat peritoneal mast cells, in which they are called Ca^{2+} channels (Zhang and McCloskey, 1995). It is possible that the CT substrate, Gs, regulates Ca^{2+} entry by direct interaction with the presumed FcεR1-activated Ca^{2+} channel, as is thought to occur with voltage-gated Ca^{2+} channels in skeletal muscle (Hamilton et al., 1991). CT also might act indirectly by enhancing the driving force on Ca^{2+} influx via membrane hyperpolarization. Here, we test these hypotheses using perforated patch clamp recording at physiological temperature from intact RBL-2H3 mast cells (Zhang and McCloskey, 1995).

Experiments using radiotracer flux, Ca^{2+}-sensing fluorescent dyes, and patch clamping suggest that FcεR1-mediated Ca^{2+} influx in RBL-2H3 mast cells may occur largely by so-called store-operated or capacitative calcium entry (Ali et al., 1994; McCloskey, 1999). A corroboration to this scheme, proposed by J ames Putney to account for inositol trisphosphate (InsP_{3})-induced Ca^{2+} influx in exocrine cells, depletion of luminal Ca^{2+} from the ER activates a Ca^{2+} entry pathway in the plasma membrane (Putney, 1986, 1990). Calcium currents associated with this pathway were first observed in J urkat human T cells and rat peritoneal mast cells, in which they are called Ca^{2+} release–acti-
vated calcium currents, or I_{CRAC} (Lewis and Cahalan, 1989; Hoth and Penner, 1992; Zweifach and Lewis, 1993). Ca^{2+} store depletion is now known to elicit Ca^{2+} influx currents superficially related to I_{CRAC} in a variety of cell types (for reviews see Fasolato et al., 1994; Berridge, 1995; Fanger et al., 1995).

The mechanism that links Ca^{2+} store depletion to Ca^{2+} influx via calcium release-activated calcium (CRAC) channels has yet to be determined, and we do not address this issue here. A separate, unanswered question is whether I_{CRAC} can be elicited or amplified through means other than Ca^{2+} store depletion. That CT enhances antigen-evoked 45Ca^{2+} uptake into BRL-2H3 cells might suggest a role for the toxin substrate, Gs, in regulation of store-operated influx. This trimeric GTP-binding protein regulates Ca^{2+} transport in a number of different systems, through means in addition to CaMP-dependent phosphorylation. In skeletal and cardiac muscle cells, for example, direct binding of G_{s/-}GTP to voltage-gated Ca^{2+} channels is thought to increase the channel’s open probability (Yataki et al., 1987; Hamilton et al., 1991). Several other findings point to a more general involvement of Gs in cAMP-mediated processes (Kish et al., 1987; Hamilton et al., 1991). Several other findings point to a more general involvement of Gs in cAMP-mediated processes (Kish et al., 1987; Hamilton et al., 1991).

Reagents
Cholera holotoxin was from List Biological Laboratories. S-p-adenosine-3',5'-cyclic monophosphorothioate (Sp-cAMPS) was from Biomol Research Laboratories, Inc. BFA, EGTA, dibutyryl adenosine-3',5'-cyclic monophosphate, metyrosine, nystatin, probenecid, and thapsigargin were from Sigma Chemical Co. M-yradiol (2-[H]i-jositol (18 Ci/mmole) was from Sigma Chemical Co. [3H]-inositol (18 Ci/mmol) was from Amersham Life Sciences. 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) free acid was from Molecular Probes. BFA was used at a final concentration of 2 μM obtained by diluting 1,000-fold into growth medium at a holding potential of 0 mV and voltage ramp stimuli (−100 to +50 mV, 0.64 mV/ms) applied at 10-s intervals. A 140-ms conditioning pulse to −100 mV was applied before each ramp, in part to prevent rapid inactivation during the ramp from distorting the shape of the current-voltage (I-V) curve (Zhang and M c Closkey, 1995). The Ca^{2+} current induced by antigen during perforated patch recording decays more rapidly than does that elicited by thapsigargin or by high concentrations of intracellular BAPTA; hereafter, I_{Ca} refers to the peak Ca^{2+} current measured at −80 mV. Miceropipettes were pulled from A ccu-fill 90 M icropets (B-D) and heat polished to resistances of 2−4 M Ω when filled with cesium gluconate (see below).

Cell Culture
The rat basophilic leukemia (R B L-2H3) cell line (Barsumian et al., 1981) was obtained from Dr. Reuben Siraganian (National Institutes of Health, Bethesda, MD) and grown for up to 30 passages before starting fresh cultures from frozen cell suspensions. Monolayer cultures were maintained at 37°C, 5% CO_{2} in MEM (Earle’s salts) containing 15% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. Stock cultures were passaged by trypsinization at 4-d intervals. In two sets of experiments, Ca^{2+} currents were measured in R B L-2H3 cells obtained from the American Type Culture Collection. Ca^{2+} currents in these cells were larger than others measured in this study, whether the usual 4-d postpassage. Cells harvested from stock cultures were seeded onto 12-mm round glass coverslips contained in 24-plate wells (8 × 104 cells/well) and grown for 12-18 h in medium containing IgE before patch clamping. Monoclonal anti-TNP IgE, IGEL a2, (Rudolph et al., 1981) (TIB 142; A merican Type Culture Collection) partially purified from ascites was added to the culture medium at a protein concentration of 12 μg/ml just before the experiment, coverslips were rinsed in normal Ringer (see below) and placed into a recording chamber.

Materials and Methods

Electrical Recording

Except where noted otherwise, experiments were conducted on intact cells using nystatin perforated patch recording (Horn and M art y, 1988). Methods for conventional and perforated patch whole cell recording were as described previously (Fan and M c C loskey, 1994; Zhang and M c C loskey, 1995). Nystatin was used at a final concentration of 250 μg/ml, produced by a 200-fold dilution of a 50-mg/ml solution (in methyisulfisoxoide) into pipette solution. All experiments were conducted at 37°C, using a Peltier device to warm the sample (M edical Systems Corp.). For most experiments, cells were voltage-clamped at a holding potential of 0 mV, and voltage ramp stimuli (−100 to +50 mV, 0.64 mV/ms) applied at 10-s intervals. A 140-ms conditioning pulse to −100 mV was applied before each ramp, in part to prevent rapid inactivation during the ramp from distorting the shape of the current-voltage (I-V) curve (Zhang and Mc Closkey, 1995). The Ca^{2+} current induced by antigen during perforated patch recording decays more rapidly than does that elicited by thapsigargin or by high concentrations of intracellular BAPTA; hereafter, I_{Ca} refers to the peak Ca^{2+} current measured at −80 mV. Miceropipettes were pulled from A ccu-fill 90 M icropets (B-D) and heat polished to resistances of 2−4 M Ω when filled with cesium gluconate (see below).

Conductances induced by antigen or thapsigargin were determined by computer subtraction of average traces acquired before from those taken after induction of inward Ca^{2+} currents. This method was verified on a few cells by Ca^{2+} removal, which eliminated the inward current in standard tetraethylammonium (TEA) aspartate (see below). Due to the rapidity of induction by cytosolic BAPTA, I-V plots in these experiments were determined by subtraction of traces in 0 mM extracellular Ca^{2+} from those taken in 10 mM extracellular Ca^{2+}.

The experimental averages include cells from experiments conducted on multiple days. To minimize systematic errors, on each day we assayed at least three control cells and three cells from each treatment, where up to three treatments were carried out each day. A ll experimental values in this paper are presented as the average ± SE m, and statistical significance was determined using the t test. Differences were considered significant if P < 0.05, and all differences listed were significant unless stated otherwise.

Solutions Used for Electrical Recording

For perforated patch recording, the pipette solution contained 55 mM KCl, 70 mM K_{2}SO_{4}, 7 mM MgCl_{2}, 5 mM glucose, and 10 mM Hepes, pH 7.35. The Cs gluconate pipette solution used for conventional whole cell recording contained 150 mM gluconic acid, 8 mM NaCl, 10 mM BAPTA (H^1), 2.0 mM CaCl_{2}, 1.0 mM MgCl_{2}, 0.5 mM GTP, and 10 mM Hepes buffered to pH 7.20 with CsOH; the estimated free Ca^{2+} concentration in this solution was ~30 mM. The standard bath solution was TEA aspartate, which contained 10 mM CaCl_{2}, 1 mM MgCl_{2}, 88 mM NaOH, 152.5 mM aspartate acid, 64.3 mM tetraethylammonium hydroxide, 5.6 mM glucose, and 5 mM Hepes buffered to pH 7.4 with TEA hydroxide. This composition was chosen to eliminate outward Cl^− and inward K^+ currents, and to antagonize outward K^+ currents with tetraethylammonium ion. The zero Ca^{2+} external buffer was Ca^{2+}-free TEA aspartate containing 1 mM EGTA as well as 15 mM N-methyl-d-glucamine aspartate in place of CaCl_{2}. The solution used for Ba^{2+} substitution contained 10 mM Ba^{2+} in place of Ca^{2+}, and 1 mM EGTA was present to chelate Ca^{2+} remaining after solution exchange.

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**[32P]ADP Ribosylation**

A DP ribosylation was carried out as described previously (McCloskey, 1988), except that reactions were terminated by addition of 1 ml ice cold 10 mM Hepes, pH 7.3, 135 mM NaCl and the membranes pelleted by centrifugation for 10 min at 20,000 g. Radioactive bands in the dried gels were imaged and digitized using a PhosphorImager, the image labeled in Adobe Photoshop, and printed by photomechanical transfer.

**[3H]inositol Phosphates Production**

A nitogen-stimulated production of inositol phosphates was assayed on cell monolayers as described previously (Beaven et al., 1984), with the following modifications. Cells were cultured overnight under the same conditions used for setting up the patch clamp experiments. Cells were grown in three dram glass shell vials for 16–24 h before assay. Each vial was seeded with 2 × 10^6 cells in 0.5 ml medium containing 1.5 μg/ml anti-TNP IgE and 2 μCi/ml myo-[3H]inositol (18 Ci/mmol). The growth medium was as described above but containing 1% rather than 15% FBS.

**Calcium Imaging**

Digital imaging of fura-2 loaded mast cells was carried out essentially as described for J774 monocytes (Fan and McCloskey, 1994), except that cells were imaged at 33–34°C rather than room temperature. Cells were cultured overnight under the same conditions used for setting up the patch clamp experiments. Cells were loaded with 1 μM fura-2 AM for 30 min at 37°C, rinsed, and incubated for another 30 min at 37°C before imaging in a Ca2+-free external buffer containing 135 mM NaCl, 5 mM KCl, 2 mM MgCl2, 5.6 mM glucose, 2.5 mM probenecid, 1 mM EGTA, and 10 mM Hepes, pH 7.4. The Ca2+ signal from individual RBL-2H3 cells exhibits variable lag phases after antigen addition (Millard et al., 1988). To eliminate this variability analytically, we used an Excel program which detects the first time point at which F340/F380 crosses an arbitrarily set threshold, in this series of experiments defined as two SDs above the average resting F340/F380 before antigen addition. These points were then aligned for each cell and the average time course of F340/F380 calculated for the cells within the field of view.

**Results**

Enhancement of Antigen-induced Inward Current by CT

As observed previously (Zhang and McCloskey, 1995), addition of 50 ng/ml TNP-BSA to anti-TNP IgE-sensitized cells induced an inwardly rectifying current with a time-to-peak of 124 ± 70 s (~131 ± 9 s in the previous study). Decrease in antigen concentration lengthened the induction, the time-to-peak being ~200 s at 5 ng/ml and 255 s at 1 ng/ml TNP-BSA. A faster reaching a peak, the current normally decayed substantially within several minutes, this presumably reflecting in part the refilling of intracellular Ca2+ stores (Zeilfach and Lewis, 1995). Fig. 1A shows a series of I-V curves for the inward current induced by different concentrations of antigen. Each curve represents the average of measurements on multiple cells (see legend). Fig. 1B gives the peak inward current measured at ~80 mV as a function of antigen concentration. A graded increase in magnitude of the induced current was observed up to concentrations of TNP-BSA ~500 ng/ml, above which the response was saturated. At 50 ng/ml, TNP-BSA induced a peak current at ~80 mV of ~19.1 ± 2.8 pA (n = 16), similar to the value of I50 induced by 50 ng/ml TNP-BSA in a previous study (~25.7 ± 4.7 pA) that employed the same antibody-antigen combination (IGE a2 anti-TNP IgE and TNP15-BSA).

As indicated in Fig. 1B, pretreatment of R BL-2H3 cells with cholera holotoxin potentiated the inward current induced by subsequent exposure to antigen. In these experiments CT was applied at a concentration (2 μg/ml) and for a time (1.5–2.5 h) shown previously to maximally enhance antigen-elicited 45Ca2+ uptake and secretion by RBL-2H3 cells (McCloskey, 1988; Narasimhan et al., 1988). Potentiation of the inward current was dependent upon antigen concentration, being quite strong at low antigen concentration and insignificant at an antigen concentration sufficient to saturate the induction. At a concentration of 1 ng/ml,
control cells exhibited an average current at −80 mV of ∼9 pA, and CT pretreatment nearly tripled this to a value of ∼24 pA, when all measurements are lumped in the averages. Table I summarizes the results of paired experiments conducted on different days (n = 2–23), where the enhancement each day was calculated from the average of three to six control and three to six CT-treated cells. Note that CT enhanced by nearly threefold the inward current induced by 1 ng/ml TNP-BSA, whereas the current induced by TNP-BSA at 500 ng/ml was not enhanced by CT. From these observations it appears that CT might amplify a step in the normal induction process that operates with submaximal efficiency at concentrations of TNP-BSA <500 ng/ml. Between 50 and 500 ng/ml TNP-BSA, this step has reached maximal efficiency, and potentiation by CT is not observed.

**Properties of CT-enhanced Ca\(^{2+}\) Current**

The ionic current elicited by antigen in CT-treated cells shared several features with that induced by antigen in control cells. For the sake of comparison, in Fig. 2A we show average I-V plots obtained from 12 control and 8 CT-treated cells, each stimulated with 1 ng/ml TNP-BSA. Fig. 2B gives average I-V plots obtained from 45 control and 33 CT-treated cells stimulated with 5 ng/ml TNP-BSA. The first point of similarity between the control and CT-enhanced currents is that the shape of their I-V curves was inwardly rectifying. In both cases the induced current had a highly positive reversal potential consistent with Ca\(^{2+}\) selectivity, and in fact Ca\(^{2+}\) is the only major permeant ion present in the TEA aspartate bath solution with such a high reversal potential. Moreover, removal of Ca\(^{2+}\) from the bath eliminated the inward current induced by antigen (data not shown). Fig. 3A shows the result of an ion substitution experiment carried out on a CT-treated cell. Note that the antigen-induced current was carried effectively by barium ions, and that the shape of the Ba\(^{2+}\) I-V plot was more steeply rectifying than the Ca\(^{2+}\) I-V plot. This behavior was demonstrated previously for I\(_{\text{CRAC}}\) in RBL-2H3 cells, whether I\(_{\text{CRAC}}\) was elicited by antigen (Zhang and McCloskey, 1995) or induced by intracellular dialysis with a solution buffered at very low free Ca\(^{2+}\) (Hoth, 1995). Together, these observations suggest that CT amplifies the same Ca\(^{2+}\) current (I\(_{\text{CRAC}}\)) as that activated by antigen alone.

The Ca\(^{2+}\) current through CRAC channels inactivates on two different time scales. Rapid but partial inactivation occurs after step changes of membrane potential from 0 mV to hyperpolarized voltages (Hoth and Penner, 1993; Zhang and McCloskey, 1995). Recovery from such voltage-dependent inactivation is complete within 2 s or less of returning the potential to 0 mV. Fig. 3B shows average traces of normalized membrane current obtained from three control and three CT-pretreated cells, in each of which the Ca\(^{2+}\) current was induced by 5 ng/ml TNP-BSA. In control cells, the Ca\(^{2+}\) current inactivated by 56 ± 6% (n = 3) within 100 ms of step hyperpolarization from 0 to −100 mV. This level of steady-state inactivation is essentially equal to that reported for the Ca\(^{2+}\) current induced by 50 ng/ml TNP-BSA, i.e., a 10-fold higher level of antigen (Zhang and McCloskey, 1995). The antigen-induced current inactivated to a similar extent (62 ± 6%; n = 3) in

### Table I. Cholera Toxin Enhances Antigen-induced I\(_{\text{CRAC}}\)

| TNP-BSA (ng/ml) | Enhancement factor* | Range | Mean †  |
|----------------|---------------------|-------|---------|
| 1              | 1.9–3.8             | 2.8 ± 0.4 (n = 5) |
| 5              | 1.2–3.3             | 2.2 ± 0.1 (n = 23) |
| 50             | 0.6–2.6             | 1.5 ± 0.3 (n = 6)  |
| 500            | 1.1                 | 1.1 (n = 2)        |

*Since I\(_{\text{CRAC}}\) varied significantly in different batches of cells, the CT enhancement of I\(_{\text{CRAC}}\) in control and CT-treated cells was compared on a day-by-day basis. Enhancement factor was calculated from results of paired experiments where antigen-induced Ca\(^{2+}\) current (at −80 mV) was measured in three to six control and three to six CT-treated cells each day.

†Mean enhancement ± SEM of experiments conducted on n different days.
because I due to reduced voltage-dependent inactivation. That is, ramp stimulation after a 140-ms conditioning pulse to McCloskey and Zhang control and CT-enhanced Ca\textsuperscript{2+} demonstrates that the enhancement of I\textsubscript{Ca} by CT was not due to diminished voltage-dependent inactivation in CT-treated cells.

In principle, the magnitude of the peak Ca\textsuperscript{2+} current might reflect a balance between rates of activation and slow inactivation (Zweifach and Lewis, 1995). If so, CT could increase the peak I\textsubscript{Ca} by enhancing the rate of activation or reducing the rate of slow inactivation. But an increased rate of activation or a decreased rate of inactivation should reduce the average time-to-peak. The average time-to-peak was about the same in control and CT-treated cells. For example, at 5 ng/ml of TNP-BSA the average time-to-peak was 205 ± 29 s (n = 27) in control and 238 ± 24 s (n = 25) in CT-treated cells, an insignificant difference. That CT did not reduce the time-to-peak suggests that alteration of activation or inactivation rates does not cause the marked enhancement of I\textsubscript{Ca}.

We can also exclude the possibility that the large enhancement of Ca\textsuperscript{2+} influx currents by CT resulted from the induction of I\textsubscript{Ca} by CT itself. As noted in Materials and Methods, the I-V curves shown in Figs. 2 and 3, as well as others used to derive the data shown in Fig. 1 and Table I, were obtained by computer subtraction of averaged traces taken before antigen addition. Thus, the measured currents did not contain any contribution from I\textsubscript{Ca} that might have been induced by pretreatment with CT alone. It is still relevant to ask whether CT treatment, per se, induced I\textsubscript{Ca}. If it did, then by the time electrical recording was begun, the magnitude of any induced Ca\textsuperscript{2+} current was minuscule, much smaller than the extra ~15 pA of current observed at 1 or 5 ng/ml TNP-BSA (Fig. 1 and Table I). Thus, a difference I-V plot of average ramp currents obtained from 35 control cells subtracted from 20 CT-treated cells—all recorded before exposure to antigen—was linear through the origin (data not shown). The slope reflects a very small increase in nonspecific leak conductance in the CT-treated cells (<1 pA at −80 mV), rather than the induction of I\textsubscript{CRAC} by CT. The large enhancement of antigen-induced I\textsubscript{Ca} by CT was not caused by antigen-independent induction.

**Induction of I\textsubscript{Ca} Is Not Enhanced by Elevation of cAMP**

CT elevates cAMP levels in RBL-2H3 cells (McCloskey, 1988; Narasimhan et al., 1988), presumably through ADP ribosylation of G\textsubscript{s} and activation of adenylyl cyclase. If the enhancement of I\textsubscript{Ca} by CT is due to chronic elevation of cAMP, then cell-permeant cAMP mimetics should reproduce the effect of the toxin. To test this idea, cells were preincubated for 1.5-3 h with the cell-permeant and phosphatase-resistant cAMP analogue, Sp-cAMP PS (100 μM), and then permeabilized and subjected to voltage-clamp recording in the presence of this compound. Treatment with Sp-cAMP PS caused a modest but statistically insignificant increase in antigen-elicited inward Ca\textsuperscript{2+} current, considerably less than the enhancement caused by CT in the same experiments. The average Ca\textsuperscript{2+} current elicited by 5 ng/ml TNP-BSA was −14.0 ± 1.6 pA in control cells (n = 18), and −19.9 ± 2.5 pA in cells treated with Sp-cAMP PS (n = 141).
18). In these experiments, CT potentiated antigen-induced $I_{\text{Ca}}$ by 2.3-fold. We also tested the effect of another cell-permeant analogue of CA M P, dibutyryl CA M P, which at a concentration of 0.5 mM causes modest potentiation of antigen-induced secretion in RBL-2H3 cells (McCloskey, 1988). Dibutyryl CA M P at this concentration had no statistically significant effect on antigen-induced $I_{\text{Ca}}$. Thus, chronic elevation of CA M P does not mimic the enhancement of $I_{\text{Ca}}$ by CT. Although it is conceivable that CT could amplify a CA M P transient induced by antigen binding, and in this way affect $I_{\text{Ca}}$, previous studies have shown that cross-linkage of the FcεRI does not elevate CA M P in RBL-2H3 cells (Morita and Siraganian, 1981), and pretreatment with CT does not unmask a latent rise in CA M P (McCloskey, 1988). Thus, although elevation of CA M P may contribute, it is not the major factor in the large enhancement of antigen-elicted $I_{\text{Ca}}$ by CT.

CT Does Not Potentiate $I_{\text{Ca}}$ Induced by Thapsigargin or BAPTA

The macroscopic Ca$^{2+}$ current, $I_{\text{Ca}}$, is directly proportional to the number of CA$^{2+}$ channels in the plasma membrane, their unitary conductance, and their probability of being open. Previous findings suggest that in RBL-2H3 cells, the CA$^{2+}$ currents associated with both antigen- and thapsigargin-induced CA$^{2+}$ influx (A Li et al., 1994) are carried by the same CA$^{2+}$ channel (Zhang and McCloskey, 1995). Thus, if CT were to increase the open probability or unitary conductance of this species, it should potentiate the macroscopic CA$^{2+}$ current induced by suboptimal concentrations of thapsigargin, as it does for the antigen-induced current. As demonstrated in Fig. 4, thapsigargin at 50 pM induced $I_{\text{CRAC}}$ equivalent to that induced by suboptimal antigen (1 ng/ml TNP-BSA). Whereas CT enhanced the antigen-induced current by ~2.2-fold at 1 ng/ml TNP-BSA, it did not affect the current induced by 50 pM thapsigargin. Indeed, CT did not significantly affect the CA$^{2+}$ currents induced by thapsigargin at any concentration tested. This suggests that neither CT nor its substrate Gs, modifies the unitary conductance or open probability of CRAC channels in RBL-2H3 cells.

Thapsigargin presumably activates $I_{\text{CRAC}}$ by inhibiting the CA$^{2+}$ pumps of the ER (Thastrup et al., 1990) and allowing passive leak of stored CA$^{2+}$ into the cytosol. In mast cells, $I_{\text{CRAC}}$ can also be induced by dialysis of the cell cytoplasm with low CA$^{2+}$ pipette solutions buffered with high concentrations of the calcium chelator BAPTA (Fasolato et al., 1993), conditions which prevent re-uptake of CA$^{2+}$ by the ER. We tested the effect of CT on $I_{\text{CRAC}}$ induced by dialysis with BAPTA. Cells were preincubated with 2 μg/ml CT for 1.5–3 h, then standard whole cell recording was performed at 37°C with a Cs glutamate pipette solution containing 10 mM BAPTA (~30 mM free CA$^{2+}$). CT failed to enhance the CA$^{2+}$ current induced by dialysis with BAPTA, just as it had failed to enhance the thapsigargin-induced current. The peak current at −80 mV was −30 ± 6 pA in control cells (n = 10) and −28 ± 5 pA in cells pretreated with CT (n = 10). These data provide further evidence that neither CT nor Gs acts directly on the CRAC channels, and they point to the intervention of CT at a step upstream of the channel itself.

Phosphoinositide Hydrolysis and Calcium Release

Because the CT target lies upstream of the CA$^{2+}$ channels, a logical candidate is the CA$^{2+}$-releasing messenger, InsP$_3$. A augmentation of InsP$_3$ formation should enhance $I_{\text{CRAC}}$ at low antigen levels, but as antigen concentration is increased, a point should be reached at which sufficient InsP$_3$ is generated to completely release the CA$^{2+}$ stores. No further effect of CT on $I_{\text{CRAC}}$ induction is expected beyond this concentration of antigen. In principle, this mechanism could explain why CT selectively amplifies antigen but not thapsigargin-induced $I_{\text{CRAC}}$, because thapsigargin releases stored CA$^{2+}$ independent of phosphoinositide hydrolysis. To test this hypothesis we measured antigen-stimulated production of [3H]inositol phosphates (InsP$_x$) in control cells and cells pretreated for 2 h with 2 μg/ml CT. InsP$_x$ were measured at 200 s after antigen addition, a time which corresponds to the peak CA$^{2+}$ current induced by 5 ng/ml TNP-BSA. As indicated in Fig. 5, at a concentration of TNP-BSA (5 ng/ml) for which CT amplified the induced current by 220%, CT did not significantly affect hydrolysis of [3H]labeled inositol phospholipids. For longer preincubations (5–6 h), CT caused a modest enhancement of antigen-stimulated InsP$_x$ production (McCloskey, 1988). Thus, it appears that CT does not potentiate $I_{\text{CRAC}}$ via enhancement of phosphoinositide hydrolysis.

To further test the hypothesis that CT potentiates $I_{\text{CRAC}}$ by accentuating antigen-induced CA$^{2+}$ release, we measured cytosolic free calcium after stimulation of IgE-sensitized RBL-2H3 cells with antigen. Cells were pretreated or not with 2 μg/ml CT for 1 h, loaded with 2 μM fura-2 AM for 30 min, kept for another 30 min at 37°C, and then stimulated with 5 ng/ml TNP-BSA on the stage of the microscope. Cells were plated at the same low density as during patch clamping, which limited the average number
per field to ~17. Nine control and nine CT-treated monolayers were examined over a 5-min period, during which 90.2 ± 4.8% of the control and 91.6 ± 3.6% of the CT-treated cells responded to antigen. Resting calcium levels were the same in the two populations, the fluorescence ratio F340/F380 being 0.18 ± 0.02 in control and 0.19 ± 0.01 in CT-treated cells. The average lag between antigen addition and the initial rise in [Ca\(^{2+}\)], was the same in control (1.92 ± 0.25 min) and CT-treated cells (1.80 ± 0.11 min), as was the maximum rate of rise of [Ca\(^{2+}\)]; (2.28 ± 0.15 min\(^{-1}\) in control vs. 2.19 ± 0.11 min\(^{-1}\) in CT-treated cells). Variability of lag phases was removed by thresholding, and the initial [Ca\(^{2+}\)] peaks were aligned as described in Materials and Methods. The corresponding plots as shown in Fig. 6 show no statistically significant difference between the peak heights or the rate of decline in [Ca\(^{2+}\)] in control and CT-treated cells. By these criteria, it does not appear that the ability of CT to double antigen-evoked \(I_{\text{CRAC}}\) is due to enhanced Ca\(^{2+}\) release from internal compartments.

**Effects of BFA on Ca\(^{2+}\) Current**

In addition to their cell surface localization, some heterotrimeric G proteins, including Gs, are located on intracellular membranes, where they regulate vesicle trafficking (Helms, 1995). CT enhances transcytosis of vesicles containing the poly Ig receptor as well as the apical transport of influenza hemagglutinin (Bomsel and Mostov, 1993; Pimplikar and Simons, 1993). Conceivably, CT affects the trafficking of vesicles, including those bearing CRAC channels, to or from the plasma membrane of RBL-2H3 cells. To test this hypothesis, we examined the effects of BFA on CT-enhanced \(I_{\text{Ca}}\). BFA is a fungal metabolite that inhibits certain vesicle transport and fusion steps by inhibiting GTP/guanosine diphosphate exchange on ARF proteins, thereby blocking their association with membranes (Klausner et al., 1992; Randazzo et al., 1993). Cells were preincubated with BFA, CT, or BFA plus CT for 1.5–3 h and voltage-clamp measurements performed after patch permeabilization. BFA was present throughout the nystatin permeabilization and recording periods. At a concentration (2 \(\mu\)g/ml) that had no significant effect on \(I_{\text{Ca}}\) induced by suboptimal antigen (5 ng/ml TNP-BSA), BFA reduced by 84% the enhancement of \(I_{\text{Ca}}\) by CT (Fig. 7). This implicates the involvement of ARF in the enhancement of \(I_{\text{CRAC}}\) by CT.

We next determined whether BFA reduced the enhancement of \(I_{\text{Ca}}\) through blocking the ADP ribosylation of G\(\alpha_{s}\), rather than by modulating a function of Gs so modified. This is an important question because BFA prevents the membrane association of ARF proteins, which can enhance ADP ribosylation of G\(\alpha_{s}\) in vitro (Kahn and Gilman, 1986). We examined the effect of BFA on ADP ribosylation of endogenous G\(\alpha_{s}\) by assaying the CT-mediated \(^{32}\)P ADP ribosylation of Gs in membrane preparations. Cells were pretreated with 2 \(\mu\)g/ml CT in the presence or the absence of 2 \(\mu\)g/ml BFA. Membranes from control and CT-treated cells were then isolated and treated with activated CT and \(^{32}\)P-NA D. Fig. 8 shows that pretreatment of cells with CT (Fig. 8, lane D) prevented the subsequent transfer of \(^{32}\)P ADP ribosyl moieties to G\(\alpha_{s}\), presumably because the acceptor arginine residue in G\(\alpha_{s}\) was already substituted with nonradioactive ADP ribose from endogenous NAD. If BFA were to prevent this reaction in intact cells, then incubation of cells with both CT and BFA before membrane isolation should cause the reappearance of a radioactive band in the gel after in vitro treatment with radioactive NAD and activated CT.
BFA at 2 μg/ml did not interfere with ADP ribosylation either in vitro (Fig. 8, lane C) or in intact cells (Fig. 8, lane E). Thus, the inhibition by BFA of CT-enhanced IC\textsubscript{CRAC} is not an artifact of reduced ADP ribosylation of the CT substrate, G\textsubscript{s}.

Could the differential effect of CT at low vs. high antigen levels indicate a progressively greater contribution of an ARF-mediated event with increase in antigen concentration? At a concentration of TNP-BSA (500 ng/ml) that induced the maximal Ca\textsuperscript{2+} current, BFA substantially inhibited the induction. In these experiments cells were pre-incubated with 2 μg/ml BFA for 1 h at 37°C before patch clamping, and they were also exposed to the drug during the permeabilization and induction periods. In measurements performed on 5-d cultures, the magnitude of IC\textsubscript{Ca} was $-61.7 \pm 6.6$ pA (n = 12), whereas in BFA-treated cells, the peak IC\textsubscript{Ca} was $-45.6 \pm 4.9$ pA (n = 14). In 4-d cultures, 500 ng/ml TNP-BSA induced IC\textsubscript{Ca} of $-46.8 \pm 2.2$ pA (n = 5) in control, and $-35.8 \pm 4.3$ pA (n = 5) in BFA-treated cells. Thus, BFA inhibited the induction of CRAC currents ~30% for both 4- and 5-d cultures, although at this sample size the difference is barely significant at P = 0.05.

In contrast, BFA did not affect IC\textsubscript{Ca} induced by thapsigargin. The average current induced by 100 nM thapsigargin was $-38.9 \pm 5.9$ pA (n = 10) in control cells, and $-38.9 \pm 5.5$ pA (n = 10) in cells pretreated for 1.5–2.5 h with 2 μg/ml BFA. These findings suggest that the FceRI activates IC\textsubscript{CRAC} through means in addition to Ca\textsuperscript{2+} store depletion, and that BFA and CT affect a step unique to the antigen-induced pathway to IC\textsubscript{CRAC}. But if so, why were the maximal Ca\textsuperscript{2+} currents induced by thapsigargin and antigen similar? One clue comes from preliminary experiments on the effect of thapsigargin added after maximal induction of IC\textsubscript{CRAC} by antigen. Thapsigargin induced a Ca\textsuperscript{2+} current of $-46 \pm 3$ pA (n = 6) in cells stimulated previously with optimal antigen (500 or 5000 ng/ml TNP-BSA), a 44% increase over the initial antigen-induced current in the same cells, and 21% greater than the thapsigargin-induced current in antigen-naive cells. Thus, the antigen-stimulated cells might contain a greater number of CRAC channels with a lower open probability than those in thapsigargin-stimulated cells. The latter would not be surprising, given that IC\textsubscript{CRAC} in RBL-2H3 cells is desensitized by protein kinase C-dependent phosphorylation (Penner et al., 1986), and this enzyme could be more active in antigen- than thapsigargin-treated cells. Moreover, thapsigargin irreversibly depletes the Ca\textsuperscript{2+} stores, whereas antigen causes an oscillatory Ca\textsuperscript{2+} signal that requires InsP\textsubscript{3}, to which the InsP\textsubscript{3} receptor becomes desensitized.

Discussion

Other than a role for Ca\textsuperscript{2+} store depletion, the molecular mechanisms that regulate antigen-stimulated Ca\textsuperscript{2+} influx into mast cells are not well-understood. The observation that CT dramatically enhances 45Ca\textsuperscript{2+} influx into RBL-2H3 cells suggests that this reagent might be a useful tool to study the Ca\textsuperscript{2+} entry pathway (Narasimhan et al., 1988). That CT amplifies both antigen-evoked IC\textsubscript{CRAC} and 45Ca\textsuperscript{2+} influx to a similar extent bolsters the idea that CRAC channels are a major pathway for FceRI-mediated Ca\textsuperscript{2+} uptake into RBL-2H3 mast cells (Zhang and McCloskey, 1995).

Two hypotheses to explain the effect of CT on 45Ca\textsuperscript{2+} influx are immediately testable by patch clamping. First, it is possible that CT activates Cl\textsuperscript{-} or K\textsuperscript{+} channels, and thereby increases the electrical force propelling Ca\textsuperscript{2+} entry. This indirect mechanism cannot explain the enhancement of Ca\textsuperscript{2+} influx currents that we observed, because voltage-clamp measurements eliminate any difference in membrane potential between control and CT-treated cells. Second, G\textsubscript{s} might bind directly to CRAC channels and increase their open probability, as occurs with voltage-

![Figure 7](image-url)
dependent Ca\textsuperscript{2+} channels (Hamilton et al., 1991). This mechanism is no longer tenable, as CT did not affect the CRAC currents elicited by BA PTA or thapsigargin (at concentrations inducing submaximal or maximal I\textsubscript{Ca}). A though the negative result with BA PTA could be due to loss of critical cytosolic factors during conventional whole cell recording, this is not true for the induction by thapsigargin during perforated-patch recording, nor can reduced rates of fast or slow inactivation explain the amplified I\textsubscript{CRAC}.

CT by itself does not provide all signals required to activate I\textsubscript{CRAC}. Rather, it appears to amplify a signal unique to the Fc\textsubscript{e}RI-initiated pathway for induction of I\textsubscript{CRAC}, somewhere upstream of the channels themselves. A obvious candidate for the site of intervention is the formation of Ca\textsuperscript{2+}-releasing second messengers. As shown in Fig. 5, at a concentration of antigen at which CT enhanced I\textsubscript{CRAC} by 2.2-fold, CT did not affect antigen-stimulated phosphoinositide hydrolysis. A observed previously, prolonged incubation (6 h) with CT significantly enhanced inositol phosphates production, but this preincubation was much longer than that required for I\textsubscript{CRAC} enhancement (McCloskey, 1988). In addition, others have reported that CT does not affect the Fc\textsubscript{e}RI-linked production of inositol-1,4,5-trisphosphate per se (Narasimhan et al., 1988). In any case, we found that neither the rate of Ca\textsuperscript{2+} release nor the peak Ca\textsuperscript{2+} rise was greater in CT-treated than control cells, suggesting that the ability of CT to double antigen-induced I\textsubscript{CRAC} is not due to enhanced Ca\textsuperscript{2+} release.

ARF is a monomeric GTPase that interacts with the CT-A subunit to enhance ADP ribosylation of G\textsubscript{s} (Kahn and Gilman, 1986). Six members of the ARF family are currently recognized, each of which reversibly associates with membranous organelles (Hosaka et al., 1996). In their GTP-bound state, ARF proteins activate phospholipase D (Brown et al., 1993; Cockcroft et al., 1994) and promote transcytosis of both constitutive secre-

The effect is restricted to antigen-induced I\textsubscript{CRAC}, and the site of intervention apparently lies upstream of the CRAC channels themselves. It appears to be independent of phosphoinositide hydrolysis or the rate of Ca\textsuperscript{2+} release. A though other interpretations are tenable, the data suggest that Fc\textsubscript{e}RI may act via ARF to enhance surface CRAC channel activity.

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