Activation Mechanism for CRAC Current and Store-operated Ca\(^{2+}\) Entry

CALCIUM INFLUX FACTOR AND Ca\(^{2+}\)-INDEPENDENT PHOSPHOLIPASE A\(_2\)B-MEDIATED PATHWAY*

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Here we tested the role of calcium influx factor (CIF) and calcium-independent phospholipase A\(_2\) (iPLA\(_2\)) in activation of Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels and store-operated Ca\(^{2+}\) entry in rat basophilic leukemia (RBL-2H3) cells. We demonstrate that 1) endogenous CIF production may be triggered by Ca\(^{2+}\) release (net loss) as well as by simple buffering of free Ca\(^{2+}\) within the stores, 2) a specific 82-kDa variant of iPLA\(_2\)B and its corresponding activity are present in membrane fraction of RBL cells, 3) exogenous CIF (extracted from other species) mimics the effects of endogenous CIF and activates iPLA\(_2\)B when applied to cell homogenates but not intact cells, 4) activation of I\(_{CRAC}\) can be triggered in resting RBL cells by dialysis with exogenous CIF, 5) molecular or functional inhibition of iPLA\(_2\)B prevents activation of I\(_{CRAC}\), which could be rescued by cell dialysis with a human recombinant iPLA\(_2\)B, 6) dependence of I\(_{CRAC}\) on intracellular pH strictly follows pH dependence of iPLA\(_2\)B activity, and 7) (S)-BEL, a chiral enantiomer of suicidal substrate specific for iPLA\(_2\)B, could be effectively used for pharmacological inhibition of I\(_{CRAC}\) and store-operated Ca\(^{2+}\) entry. These findings validate and significantly advance our understanding of the CIF-iPLA\(_2\)-dependent mechanism of activation of I\(_{CRAC}\) and store-operated Ca\(^{2+}\) entry.

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2 The abbreviations used are: SOC, store-operated channel; SOCE, store-operated Ca\(^{2+}\) entry; iPLA\(_2\), calcium-independent phospholipase A\(_2\); rec-iPLA\(_2\)B, recombinant iPLA\(_2\)B; CIF, Ca\(^{2+}\) influx factor; CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\)-conducting channel; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N\(\_N\)-tetracetic acid; BEL, bromoenol lactone (formula name, 6E-(bromomethylene)tetrahydroy-2-(1-naphthalenyl)-2H-pyran-2-one); CaM, calmodulin; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; RBL, rat basophilic leukemia; (R)-BEL, (R) enantiomer of bromoeno lactone; (S)-BEL, (S) enantiomer of bromoeno lactone; TG, thapsigargin; TPEN, N,N,N\(\_\)-tetrakis(2-pyridyldimethy)ethylendiamine; pF, picofarad(s).
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### Drugs and Treatments

Bromoenol lactone (BEL), thapsigargin (TG), N,N,N′,N′-tetraakis (2-pyridylmethyl)ethylendiamine (TPEN), and most other drugs were purchased from Sigma. Fura-2 and fura-2 AM were from Invitrogen. Anti-iPLA2β, a polyclonal antiserum against a 19-amino acid peptide (NQIHSKDPRYGASPLH-WAK) specific to the ankyrin region of iPLA2β (24), was a generous gift of Dr. R. W. Gross. Human recombinant iPLA2β (rec-iPLA2β) was expressed in and purified from Sf9 cells as previously described by Dr. Gross (21, 35). Briefly, Sf9 cells were infected with baculovirus harboring the human recombinant iPLA2βHis 

### MATERIALS AND METHODS

#### Cells

Rat basophilic leukemia RBL-2H3 cells were obtained from ATCC and maintained in minimum essential medium supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin (10,000 IU/10,000 μg/ml). Cells were passed every 3 days at a ratio of 1:5 and used for up to 12 passages. For Ca^{2+} imaging and patch clamp experiments, RBL cells were grown on small coverslips (~5 × 5 mm) placed into 6-well plates. For CIF preparations, Western blots, and iPLA2 activity measurements, RBL cells were grown in 10-cm tissue culture dishes.

#### Preparation of CIF Extracts

CIF extracts were purified from human platelets and from RBL-2H3 cells.

#### Human Platelets—Crude CIF extracts were obtained as described before (13). Briefly, resting (unstimulated) platelets (obtained from the local Red Cross) were kept at room temperature and used immediately for preparation of the control CIF extracts that contained no or very little CIF activity (control extract). Extracts containing high CIF activity (CIF) were prepared from platelets with depleted Ca^{2+} stores, which was achieved by exposing them to cold (4 °C) overnight, with subsequent application of TG (2 μM for 20 min). To prepare crude extracts, platelets (50 ml per platelet pack, about 10^{11} cells) were washed in Hanks’ balanced salt solution supplemented with 20 mM HEPES (20 ml) and resuspended in 0.85 ml of the same solution. The suspension was extracted with 0.2 ml of 1M NaOH, and BaCl_2 (10 mM) was added to precipitate compounds containing vicinal phosphates, including inositol 1,4,5-trisphosphate. After centrifugation the supernatant was lyophilized, and the
residue was extracted with methanol (0.8 ml) with continuous mixing for 15 min. The methanol extract was loaded on a Sep-Pak Vac C18 cartridge (Waters), and the cartridge was washed with methanol (0.8 ml). The combined methanol elutes were dried at 30 °C under N2 gas and resuspended in 200 µl of 100 mM acetic acid. The reconstituted extract was clarified by centrifugal ultrafiltration through a Ultrafree-MC 30-kDa filter (Millipore). To obtain fine extracts, the crude extracts were further subjected to anion exchange HPLC followed by reversed-phase HPLC. Unless specified, all the experiments in this study were done with crude CIF extracts.

RBL Cells—RBL cells grown in 10-cm culture dishes were washed with serum-free media and then treated with either 1 µM TG or 1 mM TPEN for 5 min at 37 °C to initiate CIF production. CIF was prepared from the cells exactly as described above for human platelets; however, the final dried methanol elutes were reconstituted in 50 µl of 100 mM acetic acid instead of the 200 µl used for platelets.

CIF Bioassay in Xenopus Oocytes

Stage V and VI oocytes were harvested from albino *Xenopus laevis* frogs (*Xenopus Express*) under anesthesia with 3-amino-benzoic acid ethyl ester (MS-222, Sigma). After defolliculation in 2 mg/ml collagenase A (Roche Applied Science), oocytes were maintained for up to 5 days in standard ND-96 oocyte bath solution to first test the oocytes for nonspecific activity. CIF was prepared from the cells exactly as described above (2). Briefly, after each experimental treatment, RBL cells were homogenized as described above. The activity of iPLA2 was determined in either total cell homogenate or in membrane and cytosol fractions, as specified in the figures. The iPLA2 activity was measured using a modified commercial assay kit originally designed for the cytosolic phospholipase A2 (Cayman). To detect the activity of iPLA2 instead of cytosolic phospholipase A2 (cPLA2), the assay buffers were modified to contain no Ca2++.

Molecular Inhibition of iPLA2β

RBL cells were transfected using Nucleofector II (Amaxa Biosystems). Batches of 1.5 × 106 cells were resuspended in 100 µl of Nucleofector solution R at room temperature followed by the addition of 2 µg of antisense or sense DNA together with 2 µg of green fluorescent protein. A 20-base-long antisense (5’-fluorescein-CTCCTTCACCCGGAATGGGT-3’) and sense (5’-fluorescein-ACCCATTCCGGGTGAAGGAG-3’) specific to iPLA2β was used as in our previous studies (3). Transfection was done at the T-20 setting of the Nucleofector II device. Immediately after transfection, the RBL cells were transferred to minimum essential medium and plated on the coverslips. Cells showing green protein fluorescence (excitation at 480 nm, emission at 515 nm) were used for experiments 38 ± 4 h after transfection.

Western Blots

RBL cells were homogenized on ice by sonication in the absence or presence of 1% Triton X-100 in the homogenization buffer containing 300 mM sucrose and 10 mM Tris-HCl (pH 7.0). The cell homogenate was centrifuged in an Eppendorf centrifuge at 14,000 × rpm for 10 min, and the supernatant was further centrifuged at 100,000 × g for 1 h to separate membrane and cytosol fractions. The membrane fraction was resuspended in the same homogenization buffer. The total protein amount in each sample was determined using the Bio-Rad protein dye reagent (Bradford method). The protein samples were incubated with Laemmli sample buffer at 95 °C for 2 min, and then one-dimensional protein gel electrophoresis was performed in 7.5% SDS-PAGE gels in a Mini-Protean system (Bio-Rad) with 30 µg of total protein loaded in each lane. Rec-iPLA2β of 84 kDa was used as a standard (0.5–1.0 ng) in all Western blots. Separated proteins were electrophotographically transferred overnight onto nitrocellulose membrane in a Mini Trans-blot system (Bio-Rad). Blots were incubated for 1 h with 5% (w/v) skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) to block residual protein binding sites. Blocked membranes were then incubated with primary anti-iPLA2β (1:2000 dilution) for 2 h at room temperature. The primary antibody was removed, and blots were washed 3 times for 10 min with milk/PBST. Then blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Cell Signaling) diluted 1:2000 in milk/PBST, washed 3 times in PBST, and treated with enhanced chemiluminescence reagents (Super ECL, Pierce) for 1 min. Blots were then exposed to photographic films, and the optical density was determined using the Un-scan-it analysis software (Silk Scientific).

Determination of iPLA2 Activity

The activity of iPLA2 was determined as previously described (2). Briefly, after each experimental treatment, RBL cells were homogenized as described above. The activity of iPLA2 was determined in either total cell homogenate or in membrane and cytosol fractions, as specified in the figures. The iPLA2 activity was measured using a modified commercial assay kit originally designed for the cytosolic phospholipase A2 (Cayman). To detect the activity of iPLA2 instead of cytosolic phospholipase A2 (cPLA2), the assay buffers were modified to contain no Ca2++.
EGTA, and 2 mg/ml bovine serum albumin (pH 7.4). The generated free thiols were visualized by the addition of 5,5'-dithiobis(2-nitrobenzoic acid) for 5 min, and the absorbance was determined at 405 nm using a standard microplate reader. The background iPLA₂-independent component of basal lipase activity was determined in control samples when all specific iPLA₂ activity was inhibited with (S)-BEL (10 μM for 5 min) and was subtracted from all the readings. The presence of 4 mM EGTA in the assay buffer (which was crucial for suppressing the contaminant Ca²⁺-dependent cPLA₂ activity) did not by itself cause any significant activation of Ca²⁺-independent BEL-sensitive iPLA₂. The specific activity of iPLA₂ was expressed in absorbance/mg of protein units.

**Measurement of Intracellular Ca²⁺**

RBL cells were transferred to minimum essential culture medium without serum and loaded with fura-2 AM (2 μM) for 30 min at 37°C. Then the cells were washed for 10 min and transferred to the bath solution of the following composition: 140 mM NaCl, 10 mM HEPES, 1 mM MgCl₂, 0.1 mM EGTA (pH 7.4). During the experiment, CaCl₂ (2 mM) was added to the cells to observe Ca²⁺ influx following different treatments as described under “Results.” Ca²⁺ measurements were done at 20–22°C. A dual-excitation fluorescence imaging system (Intracellular Imaging, see description above) was used for studies of individual RBL cells. The changes in intracellular Ca²⁺ were expressed as ΔRatio, which was calculated as the difference between the peak F₃₄₀/F₃₈₀ ratio after extracellular Ca²⁺ addition and its level right before Ca²⁺ addition. Summary data are shown without subtraction of the basal Ca²⁺ influx. Data were summarized from the large number of individual cells (20–40 cells tested each in 3–6 different experiments from at least 3 cell preparations).

**Electrophysiology**

Whole-cell currents were recorded in RBL cells using the standard whole-cell (dialysis) patch clamp technique as we pre-
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FIGURE 2. Activation of iPLA$_2$ in intact (A) and homogenized (B) RBL cells. A, summary data showing the activity of iPLA$_2$ (absorbance (Abs)/mg of protein) in samples that were obtained before (basal) or after intact RBL cells were treated with TG (1 mM for 5 min), TPEN (1 mM for 5 min), active (CIF), or inactive (control) extract from human platelets (at 1:10 volume ratio). Data are 4-9 measurements from 3 different cultures of RBL cells. Asterisks denote significant increases in activity in comparison with basal levels. B, same as in A but in the samples from untreated RBL cells that were first homogenized and then treated with TG, TPEN, CIF, control extract, and 10 mM EGTA (for 5 min). Data are 3-11 measurements from 3 cultures of RBL cells.

Statistical Analysis
Summary data are presented as mean $\pm$ S.E. Single or paired Student’s test was used to determine the statistical significance of the obtained data. The significance between multiple groups was evaluated using analysis of variance. Data were considered significant at $p < 0.01$.

RESULTS
Endogenous CIF Production by RBL Cells—RBL-2H3 cells were used to test if the decrease in free Ca$^{2+}$ concentration in the ER may trigger production of CIF and if it would cause the same or different effects compared to physical depletion (net loss) of Ca$^{2+}$ from the stores. To produce a physical depletion of the stores, TG was used to inhibit sarcoplasmic/ endoplasmic reticulum Ca$^{2+}$ ATPase, prevent Ca$^{2+}$ back-sequestration, and allow the passive leak of Ca$^{2+}$ ions from the stores. To change free Ca$^{2+}$ concentration in the ER without its release, we used TPEN, a membrane-permeable low affinity Ca$^{2+}$ buffer that is known to accumulate and decrease free Ca$^{2+}$ in the ER (16).

Physical depletion of the stores with TG (1 mM for 5 min) triggered activation of a classical SOCE (Fig. 1A), which did not develop when iPLA$_2$ was irreversibly inhibited by its suicidal substrate BEL (25 mM for 30 min). Endogenously produced CIF was extracted from TG-treated RBL cells, and its biological activity was measured by the ability of these extracts to activate SOCE when injected into Xenopus oocytes, a model bioassay system for CIF studies (12). Fig. 1B shows that CIF extracted from TG-treated, but not quiescent (control) cells produced dramatic activation of SOCE when injected into oocytes. These results confirmed that CIF is readily produced by RBL cells when their stores are getting depleted.
When RBL-2H3 cells were treated with 1 mM TPEN for 5 min, Ca\(^{2+}\) influx was activated (Fig. 1C), which was identical to SOCE produced by TG (Fig. 1A). Similar to TG-induced SOCE, it was dependent on the functional activity of iPLA\(_2\), and did not develop in BEL-treated cells. We also found that CIF extracts from TPEN-treated RBL cells were indistinguishable from those produced by TG-treated cells; when injected into oocytes they activated identical SOCE (Fig. 1D). Fig. 1E shows the summary data for TG- and TPEN-induced SOCE in RBL cells, and Fig. 1F summarizes the data for Ca\(^{2+}\) influx in oocytes triggered by injection of CIF extracted from TG and TPEN-treated RBL cells.

In an additional series of experiments we demonstrated that CIF production is independent of iPLA\(_2\) and is not affected by BEL (Fig. 1G). BEL-induced inhibition of iPLA\(_2\) in SOCE pathway (Figs. 1, A and C) happens downstream from CIF.

**iPLA\(_2\,\beta\), Plasma Membrane Localization and Activation in Intact Cells and Cell Homogenates**—Fig. 2A shows that TG-induced depletion or TPEN-induced reduction of free Ca\(^{2+}\) in the stores of intact RBL cells produced similar activation of iPLA\(_2\), which was consistent with both treatments causing identical CIF production and biological effects (Fig. 1). As an additional control, we confirmed that iPLA\(_2\) could not be activated by TG in cell homogenates, in which Ca\(^{2+}\) stores were disintegrated and CIF could not be produced (Fig. 2B). We also found that iPLA\(_2\) remained totally functional in cell homogenates; after cell disruption it could be activated by application of exogenous CIF extracted from the cells with depleted stores (Fig. 2B) but not by extract from control cells. Exogenous CIF extract could originate from RBL cells or from other species and cell types (for example human platelets). Fig. 2B also demonstrates that iPLA\(_2\) in cell homogenates could be activated by a prolonged (5–10 min) treatment with 10 mM EGTA. Strong Ca\(^{2+}\) buffering and longer time seemed to be needed to displace inhibitory CaM from iPLA\(_2\), mimicking the physiological effect of CIF. Interestingly, buffering capacity of 4 mM EGTA (which was used in assay buffers to ensure that only the activity of Ca\(^{2+}\)-independent PLA\(_2\) is measured) was not enough to produce iPLA\(_2\) activation in live RBL cell homogenates; the basal activity (in 4 mM EGTA) was 0.046 ± 0.047 Abs/mg of protein, whereas 10 mM BAPTA or exogenous CIF increased it more than 18-fold (Fig. 2B). Another set of experiments demonstrated that extracellular application of exogenous CIF extract to intact cells was without any effect (Fig. 2A), consistent with CIF being membrane-impermeable and iPLA\(_2\) working at the intracellular leaflet of the plasma membrane.

The next step was to determine which variant of iPLA\(_2\) may be present and responsible for CIF-induced phospholipase A\(_2\) activity in RBL cells.
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activity in plasma membrane of RBL cells. Membrane and cytosol fractions of RBL cells were separated by ultracentrifugation and used for Western blot analysis and iPLA$_2$ activity measurements. Fig. 3A demonstrates that an antibody directed against a unique ankyrin repeat region in iPLA$_2$$\beta$ recognized an 84-kDa human recombinant iPLA$_2$$\beta$ (24) that we used as a control in all Western blots and an ~82-kDa band that is predominantly located in the membrane but not in the cytosol fraction of RBL cells. Importantly, membrane localization was confirmed by iPLA$_2$ activity measurements; more than 87% of the BEL-sensitive iPLA$_2$ activity was found in the membrane fraction (Fig. 3B). As expected, inclusion of 1% Triton in the homogenization buffer shifted iPLA$_2$$\beta$ protein and the corresponding activity from membrane to cytosolic fraction (Fig. 3). These results demonstrate that an 82-kDa iPLA$_2$$\beta$ is present and functionally active in the plasma membrane of RBL cells. It is very similar to a human recombinant iPLA$_2$$\beta$; it has a close molecular weight and is recognized by the same antibody specific to the unique ankyrin repeats.

**CIF-induced Activation of $I_{\text{CRAC}}$**—After demonstrating that CIF is produced in RBL cells and activates specific iPLA$_2$$\beta$ that is associated with plasma membrane, we tested if intracellular application of exogenous CIF could activate CRAC channels in resting RBL cells, when endogenous production of CIF and $I_{\text{CRAC}}$ are not triggered. We already showed (Fig. 1) that resting RBL cells do not produce CIF. Furthermore, we confirmed that $I_{\text{CRAC}}$ did not develop during a 5-min dialysis with 0.1–1 mM BAPTA (inward current remained $-0.16 \pm 0.04$ pA/pF ($n=6$)) could be activated not only by CIF endogenously produced upon store depletion but also by CIF extracted from other cell species and applied into cytosol of the resting cells with full stores.

**iPLA$_2$$\beta$ Is Required and Sufficient for Activation of $I_{\text{CRAC}}$**—To test if a full-length iPLA$_2$$\beta$ is not only required but also sufficient for CRAC channel activation, two approaches were used. First, we tested if its molecular down-regulation could prevent $I_{\text{CRAC}}$ development and, second, if exogenous iPLA$_2$$\beta$ could restore $I_{\text{CRAC}}$ when the endogenous enzyme is irreversibly blocked. Figs. 4, C and D, show that antisense-induced knock down of iPLA$_2$$\beta$ protein (shown in the inset) impaired BAPTA-induced development of $I_{\text{CRAC}}$ consistent with this enzyme being required for CRAC channel activation. Furthermore, CIF dialysis of RBL cells transfected with antisense to iPLA$_2$$\beta$ also failed to activate $I_{\text{CRAC}}$; the maximum inward current (at $-80$mV) was $-0.14 \pm 0.04$ pA/pF ($n=6$) in cells transfected with antisense to iPLA$_2$$\beta$ compared with $-1.56 \pm 0.17$ pA/pF ($n=8$) in control cells. (Figs. 4, E and F).

Next we tested if exogenous iPLA$_2$$\beta$ could substitute the endogenous iPLA$_2$$\beta$ and rescue activation of $I_{\text{CRAC}}$: RBL cells were transfected with antisense to iPLA$_2$$\beta$ (which decreased the amount of this protein to 16 ± 5% of the control; see the inset in Fig. 4C) and then dialyzed with a human recombinant iPLA$_2$$\beta$ (24), which has a CaM binding domain, ankyrin repeats, and can be recognized by the same antibody as endogenous iPLA$_2$$\beta$ (as seen in Fig. 3A). Inclusion of the recombinant iPLA$_2$$\beta$ (10 ng/μl) into the dialyzing pipette appeared to be enough to fully restore activation of $I_{\text{CRAC}}$ (Fig. 4, E and F) with 0.1 mM BAPTA and $-0.18 \pm 0.04$ pA/pF ($n=6$) with 1 mM BAPTA in the pipette). However, when exogenous CIF (HPLC-purified extract from TG-treated human platelets, 1:20 dilution) was included in the dialyzing buffer, a classical $I_{\text{CRAC}}$ readily developed (Figs. 4, A and B). Similar to $I_{\text{CRAC}}$ recorded upon passive depletion of the stores during cell dialysis with 10 mM BAPTA (Figs. 4, C and D), CIF-induced current had pronounced inward rectification and was inhibited by diethylstilbestrol (10 μM). The only noticeable differences were faster development and higher amplitude of the current upon CIF dialysis; at 100 s $I_{\text{CRAC}}$ reached 76 ± 8% of its maximum amplitude of $-1.56 \pm 0.17$ pA/pF ($n=8$) compared with 36 ± 10% of its maximum in 10 mM BAPTA ($-1.11 \pm 0.1$ pA/pF, $n=7$). When extract from resting platelets (with no significant CIF activity, as seen in Fig. 2B) was added to the dialyzing buffer, $I_{\text{CRAC}}$ did not develop (Fig. 4, A and B). Thus, $I_{\text{CRAC}}$ in RBL cells
iPLA₂ of iPLA₂ has been extensively studied. Recently, Gross and co-workers (22) demonstrated that racemic BEL (a commonly used mechanism-based suicidal substrate for all major isoforms of iPLA₂ (37)), is composed of two enantiomers, (S)-BEL and (R)-BEL, which has higher specificity to iPLA₂β and (R)-BEL, which is more specific to iPLA₂γ. Here we thought to test the effects of these two enantiomers on the activity of iPLA₂β, and the activation of I₇ CRAC and SOCE in RBL cells to determine whether (S)-BEL could be used as an advanced enantio-selective pharmacological tool for the studies of the role of iPLA₂β and store-operated channels in Ca²⁺ entry.

First, we purified BEL enantiomers (Fig. 6A) and showed that (S)- but not (R)-BEL inhibits TG-induced activation of I₇ CRAC (Fig. 6B), consistent with iPLA₂β but not iPLA₂γ being activated by depletion of Ca²⁺ stores in RBL cells. Next, we tested the effects of (S)- and (R)-BEL on I₇ CRAC and SOCE. Figs. 6, C

F) that was lost in the cells in which endogenous iPLA₂β was knocked down. Similar recovery of I₇ CRAC with recombinant iPLA₂β was also obtained in RBL cells in which functional activity of endogenous iPLA₂β was inhibited by (S)-BEL (this inhibitor will be described in details later); maximal I₇ CRAC was −0.54±0.19 pA/pF (n = 5) in the absence and −2.1±0.24 pA/pF (n = 5) in the presence of rec-iPLA₂β in the pipette when cells were pretreated with 20 μM (S)-BEL. These data provided solid proof that the presence and functional activity of iPLA₂β is required and sufficient for activation of I₇ CRAC in RBL cells.
and $D$, show that (S)- but not (R)-BEL inhibits BAPTA-induced $I_{\text{CRAC}}$ in RBL cells. TG-induced Ca$^{2+}$ influx was also effectively inhibited by (S)-BEL but not (R)-BEL (Fig. 6F). Dose-response profiles presented in Fig. 6F demonstrates that (S)-BEL inhibits capacitative Ca$^{2+}$ influx with IC$_{50}$ about 3 $\mu$M. In contrast, (R)-BEL produces very little or no effect at 10-fold higher concentrations. Identical inhibition of iPLA$_2$ activity and SOCE by (S)- and not (R)-BEL was also obtained in vascular smooth muscle cells (data not shown). These results demonstrate that (S) enantiomer of BEL could be used as a highly specific pharmacological tool to inhibit iPLA$_2$, $I_{\text{CRAC}}$, and SOCE.

**DISCUSSION**

This study carefully tested and fully confirmed the CIF- and iPLA$_2$-dependent mechanism of CRAC channel activation in RBL-2H3 cells. We obtained new important information on the stimulus for endogenous CIF production, the ubiquitous nature of CIF, and its interchangeable biological activity between different species, strict dependence of $I_{\text{CRAC}}$ activation on the presence and functional activity of a specific membrane-bound 82-kDa variant of iPLA$_2$,$\beta$, and new advanced pharmacological tools for SOCE studies.

We demonstrated that CIF is produced and can be extracted from RBL cells after depletion of their stores, and its biological activity is identical to the activity of CIF purified from other cell types (14), which suggests that CIF is highly preserved and may be interchangeable between the species. When extracted from donor cells and dialyzed into acceptor cell, exogenous CIF worked identically to the endogenous CIF; it produced the same activation of iPLA$_2$,$\beta$ and $I_{\text{CRAC}}$. Furthermore, exogenous CIF was active only upon intracellular application; 1) it activated iPLA$_2$,$\beta$ when added to cell homogenates but not to intact cells, and 2) intracellular CIF dialysis was needed to activate $I_{\text{CRAC}}$. Also, exogenous CIF was able to activate $I_{\text{CRAC}}$ in resting cells in which Ca$^{2+}$ stores were not depleted, and $I_{\text{CRAC}}$ did not develop upon dialysis with control (inactive) extract. Importantly, CIF produced much faster activation of $I_{\text{CRAC}}$ than what is usually seen upon cell dialysis with BAPTA, exactly as one would expect from CIF directly activating iPLA$_2$,$\beta$ which is only one step away from activation of the channels, whereas BAPTA needs extra time to deplete the stores and trigger CIF production first. Inability of CIF to activate $I_{\text{CRAC}}$ in RBL cells in which iPLA$_2$,$\beta$ was abolished further confirmed that iPLA$_2$,$\beta$ is indeed a physiological target for CIF and an essential component of CRAC channel activation.

The studies in RBL cells brought new important information on a specific variant of iPLA$_2$,$\beta$ that is required and sufficient for activation of CRAC channels as well as its localization and intracellular CIF- and pH-dependent regulation. The existence of multiple splice variants of iPLA$_2$,$\beta$ (33) may play an important role in diversification of iPLA$_2$,$\beta$ functions and be one of the possible reasons for the variety of reports on its localization and role in cellular biology. Our results suggest that a full-size 82–85-kDa splice variant of iPLA$_2$,$\beta$ (featuring 7 ankyrin repeats in N terminus) may localize within specific signaling domains at, or in close proximity to plasma membrane, making it available for the SOCE pathway. Our previous studies showed that it is bound to the plasma membrane and could be extracted and retain its functional activity in membrane patches (13); CIF application to the intracellular side of inside-out membrane patches was able to activate single nonselective SOCs in smooth muscle cells, and CaM binding back to iPLA$_2$ in those patches could shut SOCs down. Here we demonstrated that, despite its seemingly tight association with plasma membrane, 82-kDa iPLA$_2$,$\beta$ could be detached by detergent and probably by other more physiological/pathological conditions, raising an attractive possibility that association of iPLA$_2$,$\beta$ with plasma membrane represents one of many ways of iPLA$_2$-dependent regulation of SOCE in living cells. Our results also suggest that completely detergent-free homogenization conditions are necessary to keep iPLA$_2$,$\beta$ in the membrane fraction after cell disruption and may explain why iPLA$_2$,$\beta$ was found in the cytosol in some previous studies.

Several lines of evidence in this study confirmed the specificity of BEL and the exact location of its target in the store-operated pathway. First, we demonstrated that treatment by BEL mimics the effects of molecular down-regulation of iPLA$_2$,$\beta$ on $I_{\text{CRAC}}$. Second, it does not affect CIF production, which is located upstream from iPLA$_2$,$\beta$, and does not impair activation of SOCE by lysophospholipids (2), downstream products of iPLA$_2$. Also, we demonstrated that only (S)-BEL (one of the two chiral enantiomers of BEL) inhibits TG-induced activation of iPLA$_2$,$\beta$, $I_{\text{CRAC}}$, and SOCE in RBL cells. Thus, (S)-BEL may be used as a highly specific pharmacological tool for broader studies of Ca$^{2+}$ homeostasis in cells and organs, in which molecular manipulations with iPLA$_2$,$\beta$ and SOC channels may pose considerable difficulties.

Our studies on the nature of the signal in ER that may trigger CIF machinery not only reflect the first attempts to peek into a “black box” of CIF production but also address the major question of what is a real signal for SOCE. In these studies we found that simple buffering of free Ca$^{2+}$ within the stores triggers CIF production and activation of SOCE in RBL cells, which was indistinguishable from that triggered by a physical Ca$^{2+}$ loss from ER. Thus, a drop in intraluminal free Ca$^{2+}$ concentration provides a physical stimulus for CIF production and SOCE activation.

Taken together our studies in RBL cells significantly extend our understanding of the crucial role of CIF and the specific membrane-bound variant of iPLA$_2$,$\beta$ in activation and physiological regulation of CRAC channels and SOCE.

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