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# Effects of inhibitors of the lipo-oxygenase family of enzymes on the store-operated calcium current $I_{CRAC}$ in rat basophilic leukaemia cells

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**In non-excitable cells, the major $Ca^{2+}$ entry pathway is the store-operated pathway in which emptying of intracellular $Ca^{2+}$ stores activates $Ca^{2+}$ channels in the plasma membrane. In many cell types, store-operated influx gives rise to a $Ca^{2+}$-selective current called $I_{CRAC}$ ($Ca^{2+}$ release-activated $Ca^{2+}$ current). Using both the whole-cell patch clamp technique to measure $I_{CRAC}$ directly and fluorescent $Ca^{2+}$ imaging, we have examined the role of the lipo-oxygenase pathway in the activation of store-operated $Ca^{2+}$ entry in the RBL-1 rat basophilic leukaemia cell-line. Pretreatment with a variety of structurally distinct lipo-oxygenase inhibitors all reduced the extent of $I_{CRAC}$, whereas inhibition of the cyclo-oxygenase enzymes was without effect. The inhibition was still seen in the presence of the broad protein kinase blocker staurosporine, or when $Na^+$ was used as the charge carrier through CRAC channels. The lipo-oxygenase blockers released $Ca^{2+}$ from intracellular stores but this was not associated with subsequent $Ca^{2+}$ entry. Lipo-oxygenase blockers also reduced both the amount of $Ca^{2+}$ that could subsequently be released by the combination of thapsigargin and ionomycin in $Ca^{2+}$-free solution and the $Ca^{2+}$ influx component that occurred when external $Ca^{2+}$ was re-admitted. The inhibitors were much less effective if applied after $I_{CRAC}$ had been activated. This inhibition of $I_{CRAC}$ could not be rescued by dialysis with 5(*S*)-hydroxyperoxyeicosa-6*E*,8*Z*,11*Z*,14*Z*,tetraenoic acid (5-HPETE), the first product of the 5-lipo-oxygenase pathway. Our findings indicate that exposure to pharmacological tools that inhibit the lipo-oxygenase enzymes all decrease the extent of activation of the current. Our results raise the possibility that a lipo-oxygenase might be involved in the activation of $I_{CRAC}$. Alternative explanations are also discussed.**

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In non-excitable cells, depletion of intracellular inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive $Ca^{2+}$ stores results in the activation of $Ca^{2+}$ influx through the store-operated $Ca^{2+}$ entry pathway (Putney, 1986; Parekh & Penner, 1997). Although several distinct store-operated $Ca^{2+}$ entry pathways have been described, the best characterized and most widely distributed is the $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$; Hoth & Penner, 1992). $Ca^{2+}$ entry through CRAC channels is necessary not only for refilling the $InsP_3$-sensitive $Ca^{2+}$ stores, but also provides the trigger $Ca^{2+}$ for driving a host of kinetically distinct processes including secretion, gene transcription and cell growth and proliferation (reviewed in Parekh & Penner, 1997).

The mechanism whereby CRAC channels, and indeed other types of store-operated $Ca^{2+}$ channels, are activated remains contentious. Three major models have been proposed: (i) conformational coupling in which $InsP_3$ receptors on the intracellular $Ca^{2+}$ stores directly interact with store-operated $Ca^{2+}$ channels in the plasma membrane (Irvine, 1990; Berridge, 1995; Rosado & Sage, 2000*a*), (ii) diffusible messengers that are released from the stores or generated/activated in the cytosol and which then open the channels (Randriamampita & Tsien, 1993; Csutora *et al.* 1999) and (iii) vesicular fusion in which the store-operated $Ca^{2+}$ channels are somehow inserted into the plasma membrane by regulated exocytosis following store depletion (Yao *et al.* 1999). In spite of intense research by many laboratories, there is no accepted mechanism for store-operated $Ca^{2+}$ influx.

In rat basophilic leukaemia (RBL) cells, a popular system for studying store-operated $Ca^{2+}$ entry, secretion is strongly dependent on $Ca^{2+}$ influx (Beaven *et al.* 1984; Mohr & Fewtrell, 1987). Using the capacitance technique to record vesicular fusion in single cells, we have found that quite marked store emptying is required for exocytosis to occur (Artalejo *et al.* 1998). In spite of robust $Ca^{2+}$ release, $InsP_3$ is unable to promote vesicular fusion unless store refilling is compromised by inhibiting the SERCA pumps. This is strikingly similar to the activation of $I_{CRAC}$ in weak intracellular $Ca^{2+}$ buffer. Here, $InsP_3$ generally fails to activate any detectable macroscopic current unless store refilling is suppressed by inhibition of the SERCA pumps (Broad *et al.*

1999; Fierro & Parekh, 2000). These findings lead us to speculate that there may be a link between RBL cell degranulation and the activation of CRAC channels. Some signal, associated with degranulation, might also increase the activity of CRAC channels such that a large macroscopic $Ca^{2+}$ current, required to drive exocytosis, develops.

Eicosanoids, produced by intracellular metabolism of arachidonic acid, are very important messengers from mast cells and basophils where they activate other cells of the immune system to release products that feedback to sustain mast cell and basophil degranulation (Samuelsson, 1983). The effects of eicosanoids on the properties of $I_{CRAC}$ have not been investigated. We have therefore examined the consequences of interfering with eicosanoid production on the activation of $I_{CRAC}$ in RBL-1 cells. We have found that inhibition of the lipo-oxygenase family of enzymes reduces the extent of $I_{CRAC}$, an effect not mimicked by interefering with the cyclo-oxygenase enzymes. Our results raise the possibility that the lipo-oxygenase pathway might be an important regulator of $Ca^{2+}$ influx in these non-excitable secretory cells.

## METHODS

Rat basophilic leukaemia cells (RBL-1) cells, which were bought from the Cell Bank at the Sir William Dunn School of Pathology, Oxford University, were cultured as previously described (Parekh *et al.* 1997; Fierro & Parekh, 2000).

Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–25 °C) (Hamill *et al.* 1981; Fierro & Parekh, 2000). Sylgard-coated, fire-polished pipettes had DC resistances of 2.8–4.0 MΩ when filled with standard internal solution that contained (mM): caesium glutamate 145, NaCl 8, $MgCl_2$ 1, EGTA 10, Hepes 10, adjusted to pH 7.2 with CsOH. In some experiments (described in text), cells were dialysed with a pipette solution in which $Ca^{2+}$ was strongly buffered at 120 nM (10 mM EGTA, 4.1 mM $CaCl_2$). A correction of +10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. Extracellular solution contained (mM): NaCl 145, KCl 2.8, $CaCl_2$ 10, $MgCl_2$ 2, CsCl 10, glucose 10, Hepes 10, adjusted to pH 7.4 with NaOH. Divalent ion-free external solution contained (mM): NaCl 155, KCl 2.8, CsCl 10, EDTA 2, glucose 10, Hepes 10, adjusted to pH 7.4 with NaOH. $I_{CRAC}$ was measured by applying voltage ramps (−100 to +100 mV in 50 ms) at 0.5 Hz from the holding potential of 0 mV as previously described (Fierro & Parekh, 2000). Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitized at 100 μs. Currents were normalized by dividing the amplitudes (measured from the voltage ramps at −80 mV) by the cell capacitance. Capacitative currents were compensated before each ramp by using the automatic compensation of the EPC 9-2 amplifier. All leak currents were subtracted by averaging the first one to three ramp currents, and then subtracting this from all subsequent currents. Data are presented as means ± S.E.M., and statistical evaluation was carried out using Student's unpaired *t* test.

$Ca^{2+}$ imaging experiments were carried out at room temperature using the IMAGO system from TILL Photonics (Bakowski, *et al.* 2001). Cells were alternately excited at 356 and 380 nm (30 ms exposures) and images were acquired using the TILLVision software once every 4 s. The images were analysed off-line using IGOR Pro for Windows (Wavemetrics, OR, USA). Cells were loaded with Fura 2-AM (1 μM) for 40 min at room temperature in external solution containing (mM): NaCl 145, KCl 2.8, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, Hepes 10, adjusted to pH 7.4 with NaOH, as previously described (Bakowski *et al.* 2001). After loading, cells were washed three times in the above solution and then left for 15 min to allow for further de-esterification. In some experiments, after loading, cells were pre-incubated with drugs in the presence of 10 mM $Ca^{2+}$ (described in text). Drugs were applied locally to the cell in $Ca^{2+}$-free external solution by means of an application pipette placed within 20 μm of the cell. Results are presented as $\Delta F/F_0$, where $F_0$ denotes the ratio (356 nm/380 nm) prior to stimulation (averaged over 10 s) and $\Delta F$ represents the ratio as a function of time. Each image was corrected for background fluorescence.

Nordihydroguaiaretic acid, indomethacin, 5,8,11,14-eicosatertaynoic acid (ETYA), cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) and gossypol were all dissolved in DMSO (50 mM stock solutions) and freshly prepared each day. Final DMSO concentration was < 0.05 % except when 50 μM NDGA was used (0.1 %). The stocks were stored at −20 °C and new aliquots were used for each coverslip. 5-HPETE was dissolved in ethanol as a 145 mM stock. All stock solutions (except 5-HPETE) were wrapped in aluminium foil and recordings were carried out with the microscope light intensity dimmed and using red light (patch clamp) or with the light switched off (fluorescence). Thapsigargin was from Alomone Laboratories. 5-HPETE, gossypol, ETYA and CDC were from Biomol (see text for full names). All other chemicals were purchased from Sigma.

## RESULTS

### Inhibition of the lipo-oxygenase enzymes with nordihydroguaiaretic acid (NDGA) prevents the activation of $I_{CRAC}$

Figure 1*A* plots the amplitude of $I_{CRAC}$ (normalized to cell capacitance) against the time after the onset of whole-cell recording. The current was evoked by dialysing the cell with 30 μM Ins$P_3$ and 10 mM EGTA (Fig. 1*A*i, open circles). These concentrations activate $I_{CRAC}$ to its maximal extent (Parekh *et al.* 1997; Glitsch & Parekh, 2000). Within 2 s of breaking into the cell, $I_{CRAC}$ started to activate and developed with a mono-exponential time course (time constant of 26 s for this cell) peaking after almost 100 s. Figure 1*B* shows the *I–V* relationship, once the current amplitude had reached steady state. The key features of $I_{CRAC}$ are readily apparent (voltage independent, inwardly rectifying, reversal potential > +70 mV). Mean kinetic properties of the current are summarized in Fig. 1*C–E* (10 cells).

To examine the effects of interfering with the lipo-oxygenase pathway on $I_{CRAC}$, we pre-incubated cells for at least 15 min with different concentrations of the membrane-permeable lipo-oxygenase inhibitor nordihydroguaiaretic acid (NDGA). Three different lipo-oxygenases are known: the 5-, 12- and 15-enzymes, which oxidize polyunsaturated fatty acids that contain a 1,4-*cis*, *cis*-pentadiene system to generate a 1, hydroperoxy-2, 4-

*trans*, *cis*-pentadiene product (Needleman *et al.* 1986). These enzymes are expressed in RBL cells (Van der Donk *et al.* 1991; Wong *et al.* 1992) and all three enzymes are inhibited by NDGA, albeit with slightly different potencies (Hope *et al.* 1983; Salari *et al.* 1984). Pretreatment with a low concentration of NDGA (2 $\mu$M) reduced the amplitude of $I_{CRAC}$, when the latter was triggered by Ins$P_3$ and 10 mM EGTA (trace ii, filled circles in Fig. 1*A* and *B*; mean amplitude from 8 cells plotted in Fig. 1*C*, $P < 0.05$ relative to controls). However, neither the delay before the current activated (Fig. 1*D*) nor the time to peak (Fig. 1*E*) were significantly affected by this concentration of NDGA ($P > 0.3$ for both parameters). The time constant of activation of $I_{CRAC}$ was also similar between NDGA-treated cells and the controls (29.1 ± 4.3 *versus* 24.2 ± 2.8 s, respectively; $P > 0.3$). Raising the NDGA concentration to 20 $\mu$M had a stronger effect on $I_{CRAC}$ in that the amplitude of the current was substantially reduced (trace iii, open triangles in Fig. 1*A* and *B*; mean data from 12 cells shown in Fig. 1*C*, $P < 0.01$ compared with controls) and the delay before the current activated was increased almost 5-fold (Fig. 1*D*, $P < 0.01$). However, once the current started to activate, its time to peak was not significantly different from the controls (Fig. 1*E*). In the presence of 50 $\mu$M NDGA, $I_{CRAC}$ could not be detected at all (Fig. 1*A*–*C*; 4 cells).

## Inhibition of the cyclo-oxygenase class of enzymes does not affect the activation of $I_{CRAC}$

The lipo-oxygenase enzymes compete with the prostaglandin endoperoxide synthase complex for the same substrate, namely arachidonic acid. Inhibition of the lipo-oxygenase pathway could therefore lead to the build-up of arachidonic acid, and it is possible that this compound is responsible for the inhibition of $I_{CRAC}$ observed above. If this is true, then it predicts that inhibition of any pathway that metabolizes arachidonic acid should prevent $I_{CRAC}$ from activating, since the net effect would be a build-up in arachidonic acid levels. The prostaglandin endoperoxide synthase complex is composed of two distinct activities: the cyclo-oxygenase enzymes which oxidize arachidonic acid to form prostaglandin (PG)$G_2$ and then a peroxidase which reduces $PGG_2$ to $PGH_2$ (Needleman *et al.* 1986). We blocked the prostaglandin endoperoxide synthase by inhibiting the cyclo-oxygenase enzyme with indomethacin (at maximally effective concentrations of either 20 or 50 $\mu$M). However, this failed to reduce the size of $I_{CRAC}$ compared with control recordings (Fig. 2*A* and *B*). Similarly, neither the time constant for activation of $I_{CRAC}$ (Fig. 2*C*) nor the time to peak (Fig. 2*D*) were affected by indomethacin compared with control recordings.

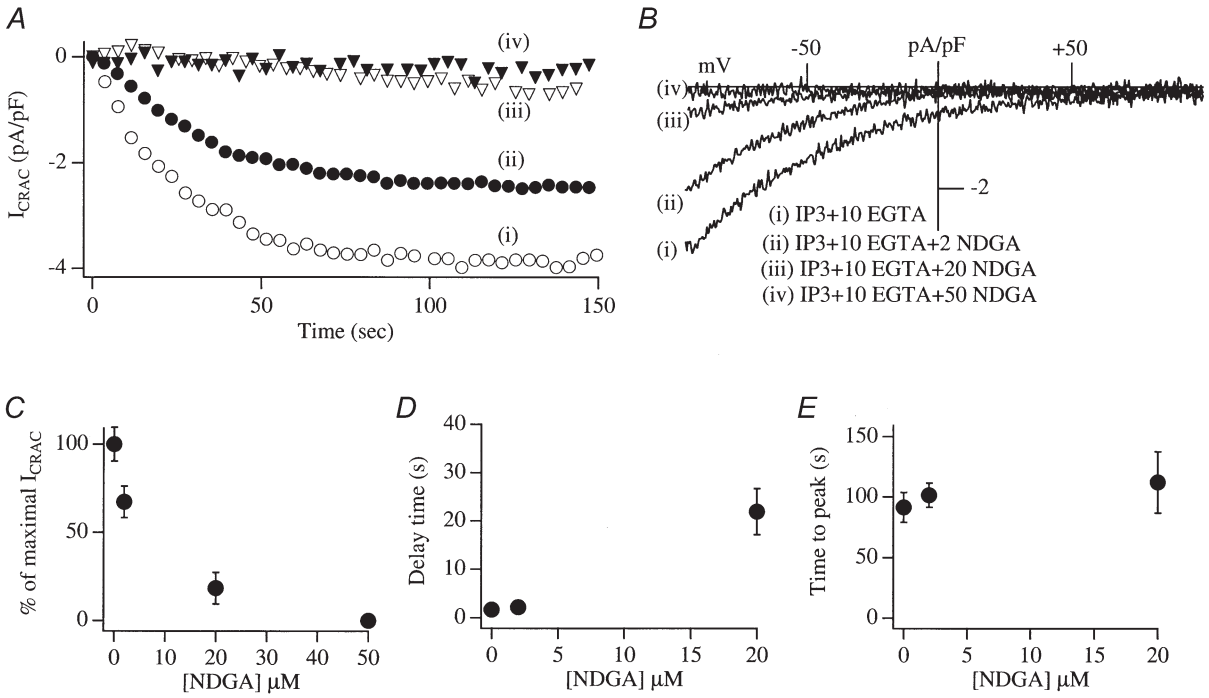


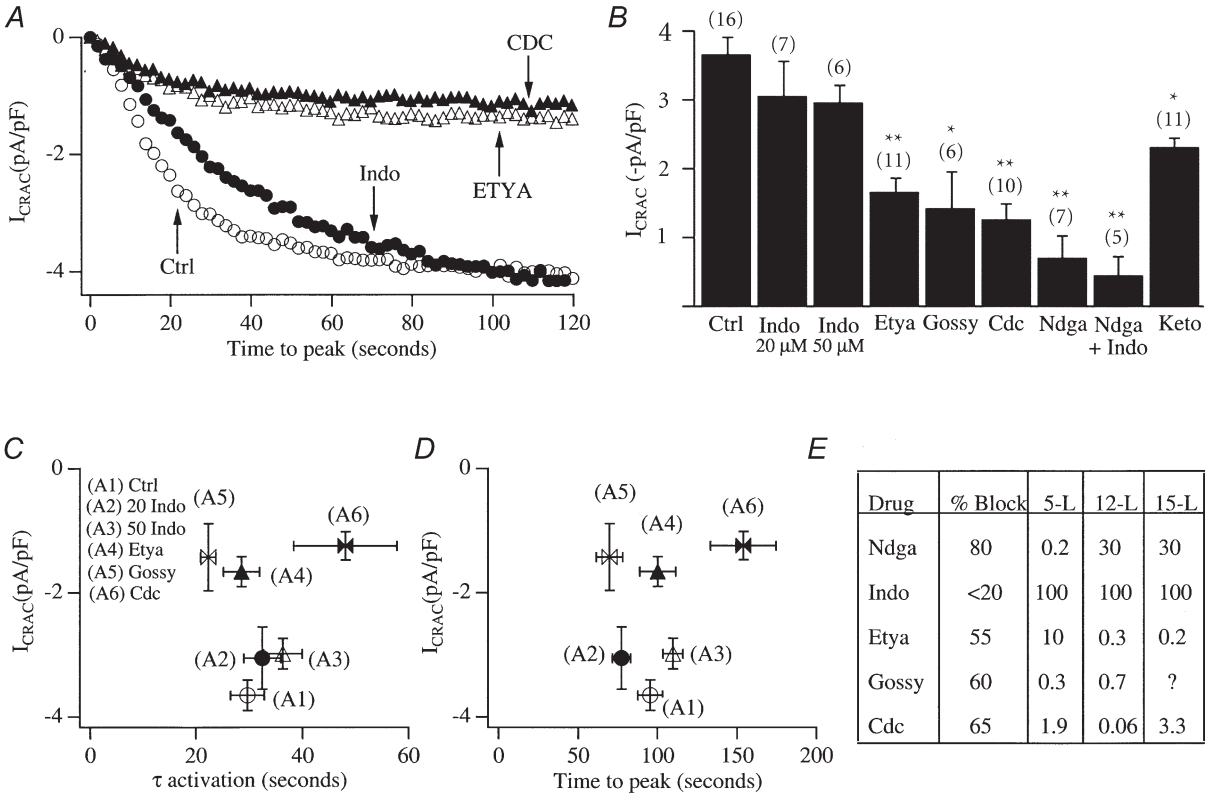

**Figure 1. The lipo-oxygenase inhibitor NDGA interferes with the activity of $I_{CRAC}$**

*A*, the development of $I_{CRAC}$ (measured at −80 mV from the voltage ramps) in control conditions (trace i, open circles) and then following pre-incubation (> 15 min) with different concentrations of NDGA. *B*, *I*–*V* relationships taken when the currents had reached steady state (80–100 s). Stores were emptied by dialysis with Ins$P_3$ + 10 mM EGTA. *C*, the relationship between the concentration of NDGA and inhibition of $I_{CRAC}$ is shown. *D*, the delay before $I_{CRAC}$ activates *versus* concentration of NDGA. *E*, time to peak *versus* NDGA concentration. In this, and all subsequent figures, the data points are means ± S.E.M.

We considered the further possibility that a product of the cyclo-oxygenase pathway was still responsible for inhibiting $I_{CRAC}$, but the flux through the pathway was modest under resting conditions. Hence interference with the cyclo-oxygenase alone would have no effect on $I_{CRAC}$. However, block of the lipo-oxygenase pathway by NDGA would increase arachidonic acid flux through the cyclo-oxygenase pathway, and this might then result in generation of an inhibitory factor. If this scenario is true, then simultaneous block of the cyclo-oxygenase and lipo-oxygenase enzymes should prevent the loss of $I_{CRAC}$ that is seen with inhibition of the lipo-oxygenase pathway alone. To test this, we treated cells with both indomethacin (20 $\mu$M) and NDGA (20 $\mu$M). However, $I_{CRAC}$ was still impeded, and to an extent not significantly different from that seen in the presence of NDGA alone (Fig. 2*B*, marked Ndga+Indo). Taken together, these results indicate that the inhibition of $I_{CRAC}$ is specific to NDGA and therefore presumably to the lipo-oxygenase pathway, rather than being a secondary effect due to increased substrate flux through the cyclo-oxygenase pathway.

## Effects of NDGA are mimicked by other lipo-oxygenase inhibitors

If the inhibition of the activation of $I_{CRAC}$ by NDGA were due to an action on the lipo-oxygenase family of enzymes, then one would expect that other drugs which share this property should also reduce the extent of the current. Specific inhibitors of the lipo-oxygenase enzymes are lacking and NDGA is in fact considered the most potent and diagnostic. However, 5,8,11,14-eicosatertaynoic acid (ETYA), and cinnamyl-3,4-dihydroxy- $\alpha$-cyanocinnamate (CDC) both interfere with lipo-oxygenase activity, although with less potency and selectivity than NDGA. Gossypol has also been found to be an effective 5- and 12-lipo-oxygenase inhibitor in RBL-1 cells (Hamaski & Tai, 1985). We found that these structurally distinct inhibitors were all able to significantly reduce the size of $I_{CRAC}$ compared with control cells (Fig. 2*A* and *B*). ETYA (20 $\mu$M) and gossypol (5 $\mu$M) did not affect either the time to peak (Fig. 2*D*) or the time constant of activation of $I_{CRAC}$ (Fig. 2*C*), whereas both were increased slightly, but significantly, by CDC (20 $\mu$M).




| Drug | % Block | 5-L | 12-L | 15-L |
|---|---|---|---|---|
| Ndga | 80 | 0.2 | 30 | 30 |
| Indo | <20 | 100 | 100 | 100 |
| Etya | 55 | 10 | 0.3 | 0.2 |
| Gossy | 60 | 0.3 | 0.7 | ? |
| Cdc | 65 | 1.9 | 0.06 | 3.3 |

**Figure 2. Structurally distinct lipo-oxygenase blockers all reduce the extent of $I_{CRAC}$**

*A*, the effect of various inhibitors on the activation of $I_{CRAC}$. Ctrl (open circles) denotes control (0.1 % DMSO-treated cells), Indo (filled circles) denotes indomethacin (50 $\mu$M). *B*, the histogram summarizes mean data from several cells. Gossy denotes gossypol, Keto denotes ketoconazole. 20 and 50 Indo represent 20 and 50 $\mu$M Indo, respectively. All inhibitors were tested at a concentration of 20 $\mu$M (except gossypol which was used at 5 $\mu$M), and pre-incubated for at least 15 min. $^{*}P < 0.05$ and $^{**}P < 0.01$. *C*, amplitude of $I_{CRAC}$ against activation time constant. *D*, amplitude of $I_{CRAC}$ against time to peak. *E*, table summarizing the percentage block of $I_{CRAC}$ for the various inhibitors together with reported $IC_{50}$ values for the different lipo-oxygenases, taken from the literature. Data for Ndga are from Salari *et al.* (1984) and Hope *et al.* (1983); data for Indo are from Salari *et al.* (1984) and Shen & Winter (1977); data for Etya are from Salari *et al.* (1984), Taylor *et al.* (1985) and Bokoch & Reed, 1981; data for Gossy were from Hamasaki & Tai (1985); and data for Cdc were from Cho *et al.* (1991).

At high concentrations (100 $\mu$M), NDGA has been reported to inhibit cytochrome P-450, an enzyme initially suggested to be involved in the regulation of store-operated $Ca^{2+}$ influx (Alvarez *et al.* 1991; Graier *et al.* 1995). Although a lower concentration of NDGA was effective in interfering with $I_{CRAC}$ (Fig. 1), we nevertheless tested the effects of ketoconazole (20 $\mu$M), a more potent and specific inhibitor of cytochrome P-450. $I_{CRAC}$ was reduced slightly following pre-incubation with ketoconazole, but to an extent significantly less than that observed in the presence of NDGA or CDC (Fig. 2*B*).

Figure 2*E* summarizes the extent of block of $I_{CRAC}$ for the inhibitors used (all at 20 $\mu$M except gossypol which was used at 5 $\mu$M) as well as the known $IC_{50}$ values of these inhibitors for the 5-, 12- and 15-lipo-oxygenases. Although it is hard to relate our results to these $IC_{50}$ values because the latter often involve studies on the purified enzyme, the pharmacological profile of the 5-lipo-oxygenase enzyme would appear most compatible with our data.

### The inhibition of $I_{CRAC}$ is not rescued by thapsigargin, intracellular ATP or staurosporine

We entertained the possibility that pre-incubation with the lipo-oxygenase blockers altered the activity of either Ins$P_3$ receptors or SERCA pumps such that it became harder for Ins$P_3$ to deplete the stores sufficiently for $I_{CRAC}$ to activate. However, because dialysis of RBL cells with 10 mM EGTA alone depletes the stores to such an extent that $I_{CRAC}$ is activated maximally (Fierro & Parekh, 1999*a*; Bakowski *et al.* 2001), and that this passive depletion is independent of Ins$P_3$-mediated $Ca^{2+}$ release (Fierro & Parekh, 1999*a*; Bakowski *et al.* 2001), we feel it unlikely that the combination of both Ins$P_3$ and 10 mM EGTA should fail to deplete the stores in the presence of the inhibitors. Nevertheless, after exposure to either 20 $\mu$M NDGA or CDC, we compared the size of $I_{CRAC}$ in cells dialysed with Ins$P_3$ together with 10 mM EGTA and 2 $\mu$M thapsigargin (a sarco-endoplasmic reticulum $Ca^{2+}$-ATPase) *versus* that seen in Ins$P_3$ and 10 mM EGTA alone. Despite the inclusion of thapsigargin, the current was still substantially reduced with both inhibitors (Fig. 3*A* and *B*).

In some experiments, we included 2 mM Mg-ATP in the recording pipette but $I_{CRAC}$ was still reduced following pretreatment with either 20 $\mu$M NDGA or CDC compared with controls (Fig. 3*A* and *B*).

Protein kinase C accelerates the inactivation of $I_{CRAC}$ (Parekh & Penner, 1995). In case the lipo-oxygenase blockers stimulated this enzyme, which would explain the reduction in $I_{CRAC}$, we examined whether the broad kinase blocker staurosporine could prevent the inhibition of the current evoked by CDC. Staurosporine suppresses the ability of protein kinase C to inactivate $I_{CRAC}$ (Parekh & Penner, 1995). However, CDC still reduced the current even in the presence of staurosporine (Fig. 3*B*).

### NDGA pretreatment reduces store-operated $Ca^{2+}$ influx in intact cells

Although NDGA inhibited the activation of $I_{CRAC}$ in whole-cell recording, it was necessary to confirm that this was also the case for intact cells. To this end, we loaded cells with the fluorescent calcium dye fura-2 (see Methods). Stores were depleted by local application of thapsigargin (2 $\mu$M) together with the calcium ionophore ionomycin (100 nM) in $Ca^{2+}$-free external solution (containing 0.2 mM EGTA; Bakowski *et al.* 2001). Note that, with these low concentrations of ionomycin, $Ca^{2+}$ influx is entirely due to store depletion and not to calcium transport into the cell directly by the ionophore (Morgan & Jacob, 1994). A typical control recording is shown in Fig. 4*A*. The combination of thapsigargin and ionomycin resulted in robust, but transient, $Ca^{2+}$ release due to store emptying. The mean increase in cytosolic $Ca^{2+}$ due to release from the stores, is summarized in Fig. 4*C*. Readmission of external $Ca^{2+}$ now resulted in further elevation of intracellular $Ca^{2+}$ since $Ca^{2+}$ influx occurred through the open store-operated $Ca^{2+}$ channels (Fig. 4*A*). Although the size of this response is not

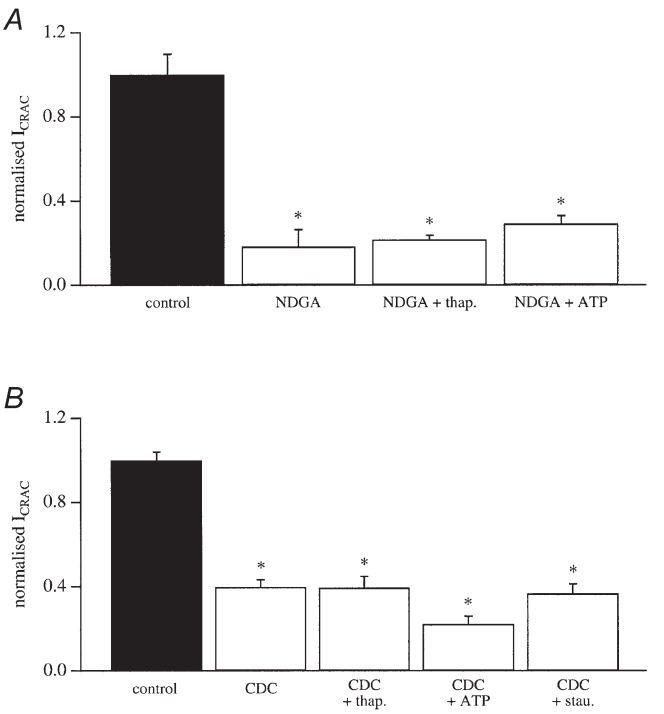



**Figure 3. Lipo-oxygenase inhibition of $I_{CRAC}$ persists in the presence of thapsigargin, ATP and staurosporine**

*A*, the size of $I_{CRAC}$ in control cells, in those pre-exposed to 20 $\mu$M NDGA, and then after either 2 mM Mg-ATP or 2 $\mu$M thapsigargin have been included in the pipette solution following pre-exposure to NDGA. *B*, the size of $I_{CRAC}$ in control cells, after exposure to 20 $\mu$M CDC, and then after inclusion of either Mg-ATP or thapsigargin in the pipette solution following CDC pretreatment. Also included are data taken from cells first exposed to staurosporine and then CDC. Note that CDC still reduces $I_{CRAC}$ even in the presence of the kinase blocker. Each bar is the mean of 4–7 cells.

an accurate assessment of the extent of $Ca^{2+}$ influx (since the activity of other $Ca^{2+}$ removal mechanisms also contribute), it is nevertheless a rough estimate of the amount of store-operated $Ca^{2+}$ influx. We therefore measured the peak amplitude of this signal, as well as the time to peak. Pooled data for $Ca^{2+}$ influx from 13 control cells is summarized in Fig. 4*D* and *E*. The pattern of the $Ca^{2+}$ signal was dramatically altered by pre-exposure to NDGA (20–50 $\mu$M). Now, the combination of ionomycin and thapsigargin was generally unable to trigger any detectable $Ca^{2+}$ release (Fig. 4*B* and *D*) and the subsequent $Ca^{2+}$ entry was substantially reduced and developed at a very slow rate (Fig. 4*B* and *E*, 10 cells). These results are consistent with our whole-cell patch clamp recordings, and indicate that NDGA pretreatment can reduce store-operated $Ca^{2+}$ influx in intact cells. However, the observation that NDGA pretreatment suppressed calcium release to the combination of ionomycin and thapsigargin indicated that NDGA itself was depleting the intracellular $Ca^{2+}$ stores.

## NDGA releases $Ca^{2+}$ in a concentration-dependent manner

To test whether NDGA mobilized intracellular $Ca^{2+}$, we applied NDGA in $Ca^{2+}$-free solution and monitored the $Ca^{2+}$ response. We found that NDGA concentrations as low as 5 $\mu$M were able to generate a $Ca^{2+}$ signal (14/15 cells, Fig. 5*Aa*) and, because no external $Ca^{2+}$ was present, this reflected $Ca^{2+}$ release from intracellular stores. We applied different concentrations of NDGA in $Ca^{2+}$-free solution and constructed a dose–response curve (Fig. 5*C*, open circles), where the response was obtained by integrating the entire $Ca^{2+}$ signal over a defined time window (20–300 s). To see whether NDGA was also able to trigger $Ca^{2+}$ influx, we constructed a dose–response curve but now in the continuous presence of 2 mM external $Ca^{2+}$. A typical recording in external $Ca^{2+}$ is shown in Fig. 5*Ab*, where 5 $\mu$M NDGA was applied, and pooled data is included in Fig. 5*C* (filled circles). The dose–response curves overlapped for all concentrations tested, indicating that NDGA-induced $Ca^{2+}$ release was not associated with

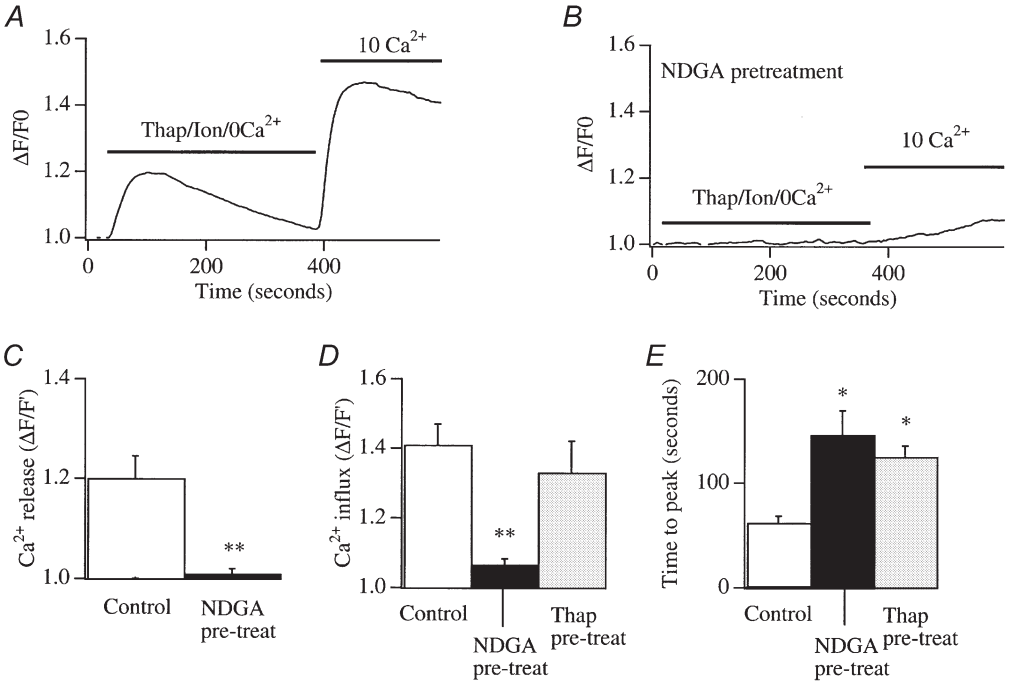



**Figure 4. NDGA reduces store-operated $Ca^{2+}$ influx to thapsigargin and ionomycin**

*A*, $Ca^{2+}$ release by the combination of ionomycin (100 nM) and thapsigargin (2 $\mu$M) is followed by store-operated $Ca^{2+}$ influx when external $Ca^{2+}$ is readmitted. *B*, pretreatment with NDGA (> 20 min) abolishes $Ca^{2+}$ release to ionomycin/thapsigargin and subsequent $Ca^{2+}$ entry is substantially reduced. *C*, pooled data for the extent of $Ca^{2+}$ release for control (i.e. where thapsigargin and ionomycin were applied) cells and for those pre-exposed to NDGA. *D*, the extent of store-operated $Ca^{2+}$ entry for control cells and after pre-treatment with NDGA. Included in the bar chart are the results from cell pre-exposed to thapsigargin for 15 min, perfused with $Ca^{2+}$-free solution, and then external $Ca^{2+}$ was re-admitted (see text for details). *E*, the time to peak of the store-operated $Ca^{2+}$ entry signal for the different conditions indicated. The Thap pre-treatment data are discussed in a later section.

significant $Ca^{2+}$ influx. To monitor $Ca^{2+}$ entry arising from maximal store depletion, we applied the combination of ionomycin and thapsigargin in 2 mM external $Ca^{2+}$. A typical recording is shown in Fig. 5*B* and the integrated fluorescence signals obtained following exposure to ionomycin and thapsigargin in either 0 (open triangles) or 2 mM external $Ca^{2+}$ (filled triangles) are included in the graph of Fig. 5*C*. The integrated signal in $Ca^{2+}$-free solution to ionomycin and thapsigargin was similar to that evoked by 50 $\mu$M NDGA, consistent with the notion that NDGA can deplete the intracellular $Ca^{2+}$ stores. The integrated signal in external $Ca^{2+}$ elicited by ionomycin and thapsigargin (Fig. 5*C*, filled triangle) was substantially larger than that evoked by high NDGA concentrations in external $Ca^{2+}$ (open circles, Fig. 5*C*), or by ionomycin and thapsigargin in $Ca^{2+}$-free solution (open triangle, Fig. 5*C*; $P < 0.01$), the difference reflecting store-operated $Ca^{2+}$ entry.

We also tried a different approach, namely to readmit $Ca^{2+}$ to cells that had been exposed to NDGA in $Ca^{2+}$-free solution. However, results with this were much more variable. Some cells showed a clear increase in intracellular $Ca^{2+}$ upon $Ca^{2+}$ readmission whereas others showed no increase at all. Unexpectedly, we found that simply perfusing cells with $Ca^{2+}$-free solution alone for a similar time was sufficient to elicit a calcium response when external $Ca^{2+}$ was readmitted (7/7 cells), although its size was quite variable. This would lead us to overestimate the extent of NDGA-induced $Ca^{2+}$ entry and we therefore abandoned this approach.

Application of indomethacin failed to evoke any consistent increase in intracellular $Ca^{2+}$ (data not shown).

## Release of intracellular $Ca^{2+}$ by other lipo-oxygenase blockers

To see whether the other lipo-oxygenase blockers were also able to release $Ca^{2+}$, we repeated the above experiments but applied different inhibitors. Results are summarized in Fig. 6. Unlike NDGA, ETYA was much less effective in mobilizing $Ca^{2+}$ (Fig. 6*Aa* and *b*) and again, there was no significant difference between the integrated signals in the absence and presence of external $Ca^{2+}$ (Fig. 6*Ac*). CDC and gossypol both triggered a rapid decrease in the fluorescence ratio (Fig. 6*B*), associated with a change in the 356 nm signal. These drugs are brightly coloured and probably interfere directly with the fluorophore.

## $Ca^{2+}$ release by lipo-oxygenase blockers does not prevent $I_{CRAC}$ from activating

Because $Ca^{2+}$-dependent inactivation of $I_{CRAC}$ channels has been extensively described (Zweifach & Lewis, 1995; Parekh, 1998; Fierro & Parekh, 1999*b*), we considered that the increase in intracellular $Ca^{2+}$ following the release of stored $Ca^{2+}$ by the lipo-oxygenase blockers might inactivate CRAC channels such that $Ca^{2+}$ entry does not occur when whole-cell recordings are started. However, the following arguments can be raised against this. First, we have recently succeeded in recording $I_{CRAC}$ even when cells are dialysed with very weak $Ca^{2+}$ buffer and high cytosolic $Ca^{2+}$ (Fierro & Parekh, 2000). Although $Ca^{2+}$-dependent inactivation can reduce the overall extent of the current, it does not prevent the current from being detected. Second, we pre-incubated cells with thapsigargin (2 $\mu$M) in 10 mM external $Ca^{2+}$ (conditions which gave a very large increase in intracellular $Ca^{2+}$ concentration) and then broke in with a pipette solution containing $InsP_3$ and

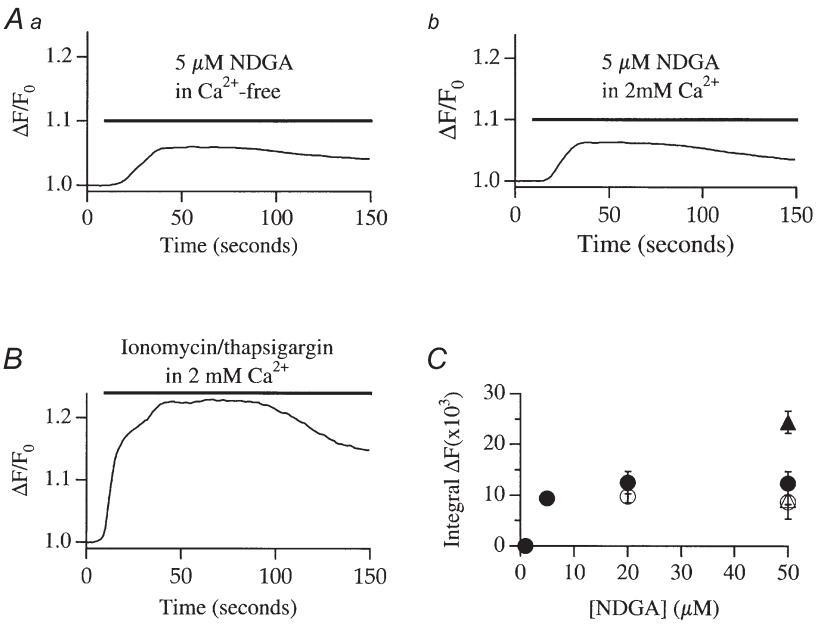



**Figure 5. NDGA releases $Ca^{2+}$ in a concentration-dependent manner**

*Aa*, the time course of $Ca^{2+}$ release from a cell exposed to 5 $\mu$M NDGA. *b* depicts a similar experiment but now in the presence of external $Ca^{2+}$. *B*, time course of the $Ca^{2+}$ response in a cell in which stores were fully depleted (combination of ionomycin and thapsigargin) in the presence of external $Ca^{2+}$. *C*, dose–response curve to NDGA in the absence (open circles) and presence (filled circles) of external $Ca^{2+}$. Included in the graph are the responses to ionomycin+ thapsigargin in the absence (open triangle) and presence (filled triangle) of external $Ca^{2+}$. These latter two points are included in the graph for comparative purposes, but the cells were not exposed to NDGA.

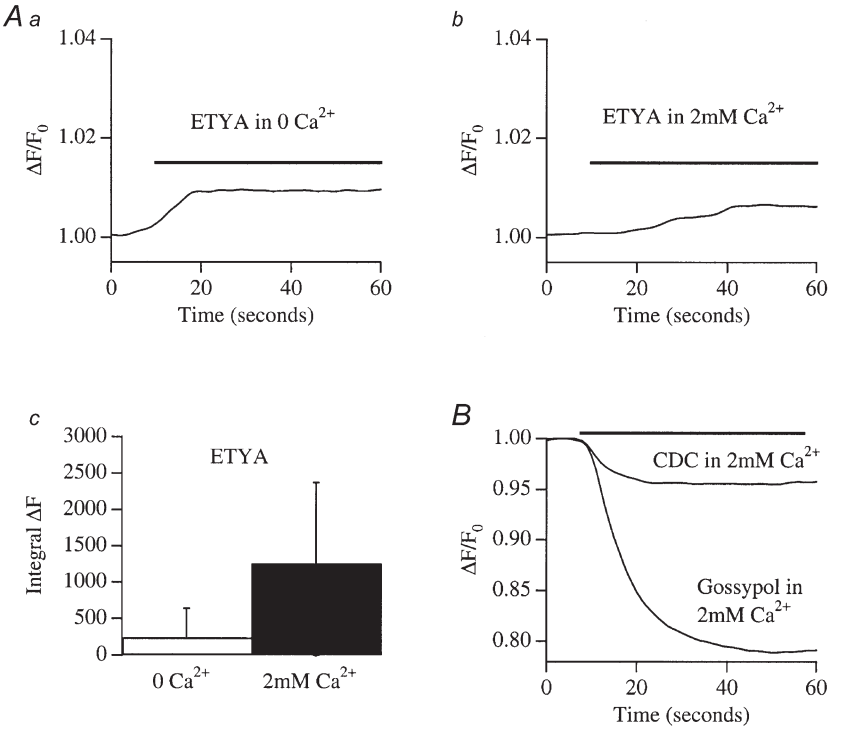



**Figure 6. Effects of other lipo-oxygenase blockers on $Ca^{2+}$ release**

*A*, ETYA (20 $\mu$M) evoked a small $Ca^{2+}$ increase both in the absence (*a*) and presence (*b*) of external $Ca^{2+}$. The integrated signals were not significantly different (*c*). *B*, CDC and gossypol both caused a fall in the fluorescence ratio, due to a decrease in the 356 nm signal.

10 mM EGTA (Fig. 7*A*). In all cells we were able to record a clear $I_{CRAC}$ (around −2.8 pA $pF^{-1}$), although the current did have a slightly reduced amplitude (by 25 %) compared with controls from the same preparations and developed more slowly (Fig. 7*B* and *C*). Third, we carried out identical fluorescence experiments to those used for the lipo-oxygenase blockers, but now with thapsigargin instead. After loading with fura-2, cells were exposed to thapsigargin in the presence of 10 mM external $Ca^{2+}$ for 15 min. We then briefly perfused the cells with $Ca^{2+}$-free solution and then readmitted external $Ca^{2+}$. Robust $Ca^{2+}$ increases were seen (Fig. 4*D* and *E*, columns marked Thap pre-treat).

## $Na^+$ permeation through CRAC channels is suppressed by NDGA and CDC

In the absence of external divalent cations, CRAC channels become permeable to $Na^+$ (Hoth & Penner, 1993) and large inward $Na^+$ currents can be detected in RBL-1 cells

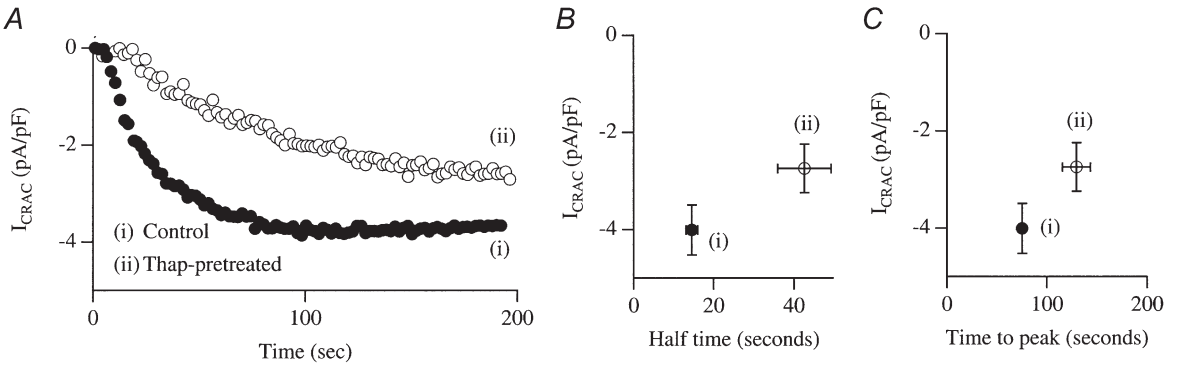



**Figure 7. Pretreatment with thapsigargin does not prevent $I_{CRAC}$ from developing**

*A*, recordings taken from a control cell and from a cell that had been exposed to 2 $\mu$M thapsigargin in 10 mM external $Ca^{2+}$ for 20 min. *B*, size of $I_{CRAC}$ against half-time for control and thapsigargin-treated cells. *C*, size of the current against the time to peak for the two conditions.

(Fierro *et al.* 2000). Under these conditions, increases in intracellular $Ca^{2+}$ which arise from $Ca^{2+}$ influx clearly do not occur. Hence both $Ca^{2+}$ entry-dependent fast and slow inactivation of CRAC channels (Zweifach & Lewis, 1995; Parekh, 1998; Fierro & Parekh, 1999*b*) would be suppressed when $Na^+$ is the conducting cation. If the actions of the lipo-oxygenase blockers were due to an acceleration of $Ca^{2+}$ entry-dependent inactivation, then one would predict that the $Na^+$ current should not be affected by pretreatment with these drugs. Figure 8*Aa* and *Ba* compare the time course of the $Na^+$ current through CRAC channels from control cells (filled circles) and from one pre-exposed to 20 $\mu$M NDGA or CDC, respectively. The $Na^+$ current was largely suppressed by pretreatment with either inhibitor. Corresponding ramp *I–V*s are shown in Fig. 8*Ab* and *Bb*. Bar charts summarizing mean data from four control and four NDGA-pretreated cells is shown in Fig. 8*Ac* and the corresponding one for CDC is shown in Fig. 8*Bc*. The $Na^+$ current through CRAC channels was almost abolished by either NDGA or CDC, indicating that the underlying inhibition does not arise from $Ca^{2+}$-dependent inactivation of CRAC channels.

## Do the lipo-oxygenase inhibitors directly block CRAC channels?

One possible explanation for the ability of the lipo-oxygenase blockers to inhibit activation of $I_{CRAC}$ is that the drugs block CRAC channels directly. To test this, we activated $I_{CRAC}$ by dialysis with Ins$P_3$ and 10 mM EGTA and, once the current had reached its maximum extent and was stable, we then applied the inhibitors to the cell. For more than 150 s of NDGA application, very little decline in the current was observed in 6/9 cells (a representative recording is given in Fig. 9*Ai*). The ramp *I–V* for this cell is shown in the right panel. The lack of effect of NDGA over this time frame is unlikely to reflect a diffusion problem because local application of $La^{3+}$ or 2-aminoethoxydiphenyl borate (2-APB) block $I_{CRAC}$ with time constants of 10–20 s under similar conditions (Bakowski *et al.* 2001). In three other cells, however, a rapid decline was seen when NDGA was applied and this had a time course similar to that observed previously with 2-APB (Fig. 9*Aii*; ramp *I–V* shown in right panel). We compared the extent of inhibition of $I_{CRAC}$ when cells were either pretreated with NDGA or when the drug was applied after the current had

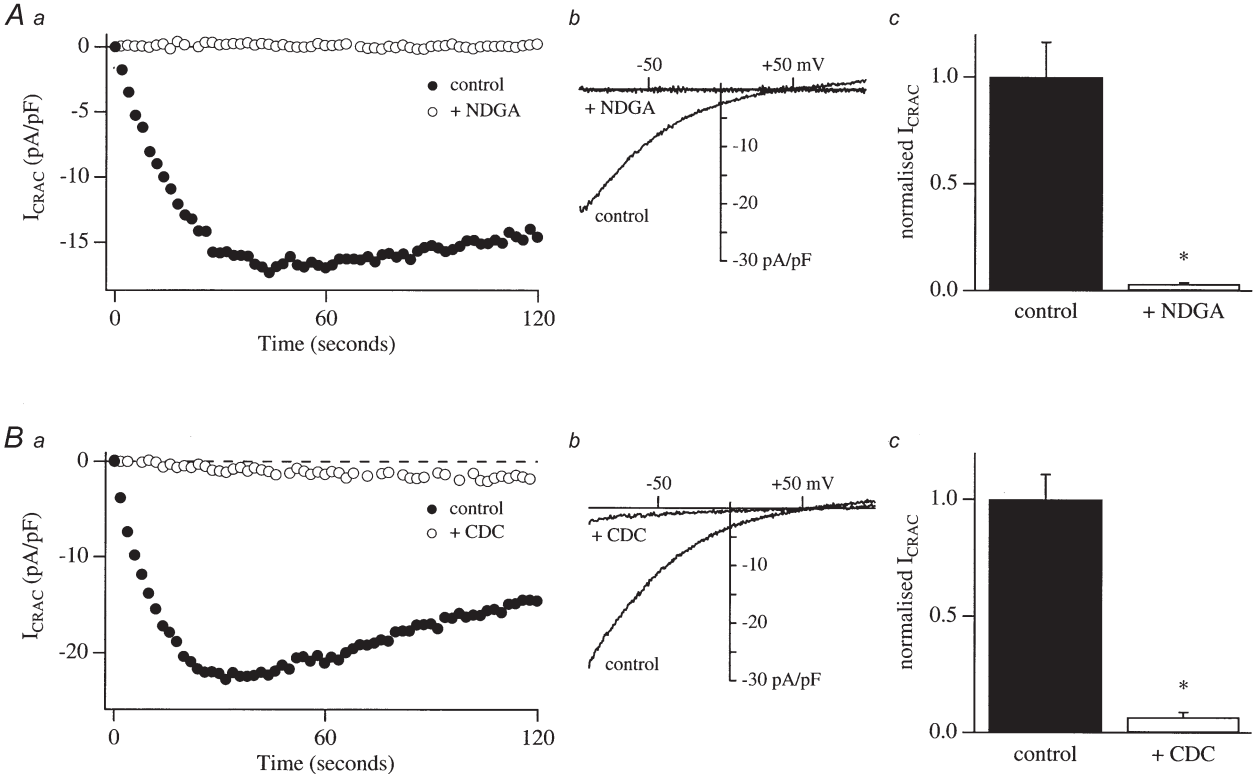



**Figure 8. Pretreatment with either NDGA or CDC suppresses the $Na^+$ current through CRAC channels in divalent ion-free external solution**

*Aa*, comparison of the time course of the monovalent $Na^+$ current for a control cell and that for a cell which had been pre-incubated with 20 $\mu$M NDGA for around 15 min. *b*, the ramp *I–V* relationships are shown for the cells in *a*, taken at 60 s for both conditions. *c*, mean data from four control cells and four cells pre-exposed to NDGA. *Ba*, time course of a control cell and a cell pre-incubated with 20 $\mu$M CDC. *b*, corresponding ramp *I–V*. *c*, mean data from four control cells and four cells pre-exposed to CDC prior to break-in. Data have been normalized to the amplitude of the controls.

developed. Pooled data for the two conditions are plotted in Fig. 9*D*. The block by NDGA was significantly less when applied after $I_{CRAC}$ had activated, but there was considerable variability in the extent of block (all 9 cells to which NDGA was applied have been included). Application of ETYA after $I_{CRAC}$ had developed (Fig. 9*Bi*) resulted in a significantly smaller block than was seen when cells were first exposed to the drug prior to store depletion (Fig. 9*Bii*). Mean data are summarized in Fig. 9*D*. The greatest difference was seen with CDC. Here, application of the drug had little effect on $I_{CRAC}$ once it had activated (Fig. 9*Ci*), but strongly reduced the development of the current if applied prior to the emptying of the stores (Fig. 9*Cii*; mean data in Fig. 9*D*). Hence these inhibitors are less effective if applied after $I_{CRAC}$ has developed.

### Effects of 5-HPETE on $I_{CRAC}$

If the lipo-oxygenase blockers interfere with the activation of $I_{CRAC}$ through a mechanism involving 5-lipo-oxygenase (as suggested by the pharmacological profile of the inhibitors summarized in Fig. 2*E*), one might expect 5(*S*)-hydroxyperoxyeicosa-6*E*,8*Z*,11*Z*,14*Z*,tetraenoic acid (5-HPETE), the first product of the 5-lipo-oxygenase pathway, to regulate $I_{CRAC}$.

To test this, we dialysed control cells with 1 $\mu$M 5-HPETE in the presence of buffered $Ca^{2+}$ and ATP (in order to prevent passive depletion of stores). As shown in Fig. 10*A*, 5-HPETE did not activate any inward current (4/4 cells). After 180 s of dialysis, we applied thapsigargin to see whether $I_{CRAC}$ could still be activated in the presence of intracellular 5-HPETE. The current developed normally and reached

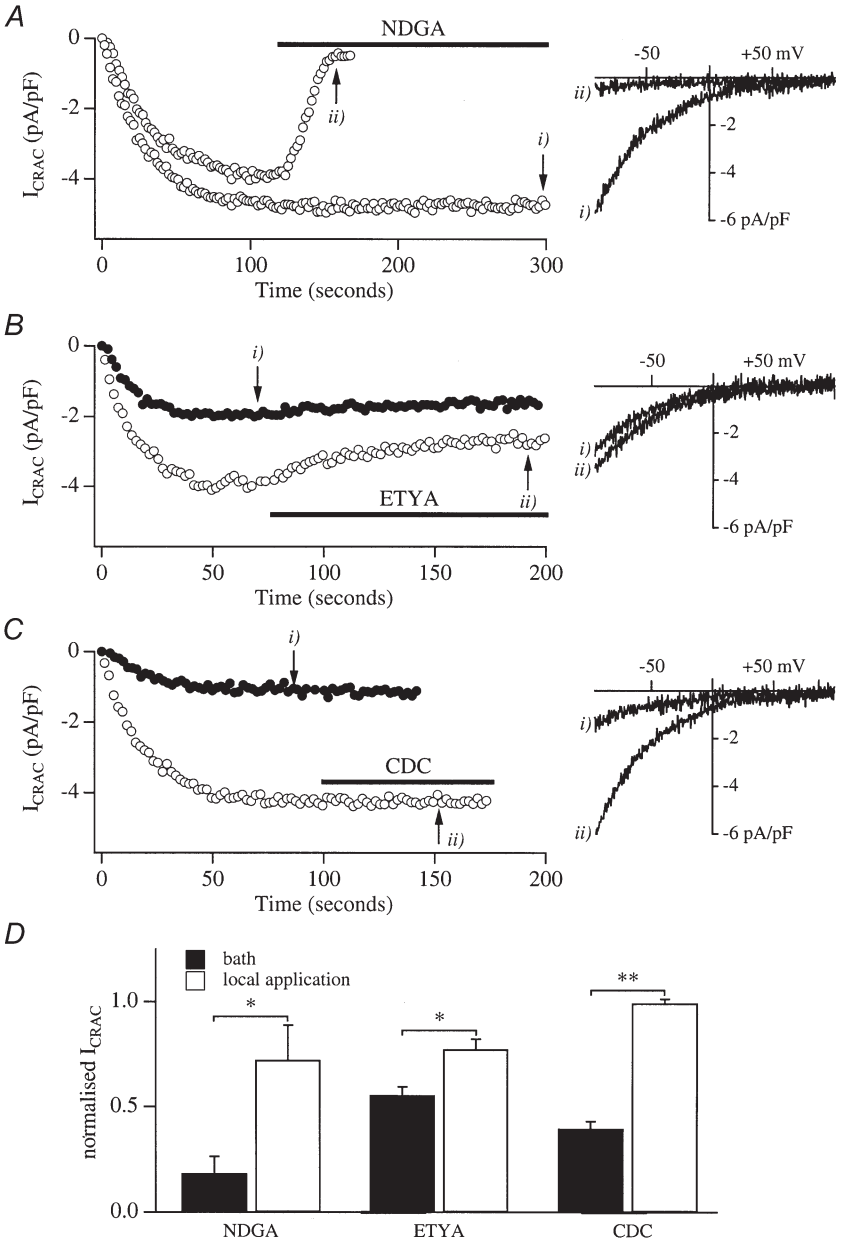



**Figure 9. Effect of application of the lipo-oxygenase inhibitors after $I_{CRAC}$ had developed**

*A*, the effects of applying 50 $\mu$M NDGA after $I_{CRAC}$ had developed in two cells. Ramp *I–V* relations are shown on the right. *B*, the effects of applying ETYA either before (filled circles) or after (open circles) $I_{CRAC}$ had activated. *C*, comparison of the ability of CDC to interfere with $I_{CRAC}$ when applied before (filled circles) or after (open circles) $I_{CRAC}$ had activated. *D*, summary of the effects of the three inhibitors on $I_{CRAC}$. The filled columns represent the inhibition seen when cells were pre-incubated with each drug and the open columns reflect the inhibition seen following exposure to each drug after $I_{CRAC}$ had developed. For cells pre-exposed to inhibitors, recordings were normalized to controls taken from the same preparations in the absence of inhibitors. For those experiments where drugs were applied after $I_{CRAC}$ had developed, the steady-state level in the presence of inhibitors was normalized to the peak amplitude reached before the inhibitors were applied. These latter currents were not significantly different from the controls used for normalizing the currents obtained following pretreatment with the lipo-oxygenase blockers. Each column represents the mean of 6–11 cells. In all cases, inhibition was significantly less if the drug was applied after store depletion than before.

an amplitude similar to that evoked by thapsigargin in control cells (Fig. 10*A* and *B*). Hence 5-HPETE did not activate $I_{CRAC}$ itself nor did it seem to interfere with the ability of thapsigargin to evoke the current.

We then examined whether 5-HPETE could rescue $I_{CRAC}$ in cells pre-exposed to CDC. A typical experiment is depicted in Fig. 10*C*. We first obtained control recordings for each preparation, evoked by dialysing cells with Ins$P_3$+ thapsigargin+10 mM EGTA (shown as filled circles). We then incubated cells in 20 $\mu$M CDC and confirmed that $I_{CRAC}$ was reduced (filled triangles in Fig. 10*C*). We then examined whether inclusion of 5-HPETE in the pipette (together with Ins$P_3$+thapsigargin+10 mM EGTA) could rescue $I_{CRAC}$ in CDC-treated cells. However, in spite of the presence of 5-HPETE, $I_{CRAC}$ could not be increased in size (Fig. 10*C*, filled circles; mean data in Fig. 10*D*). 5-HPETE also could not rescue $I_{CRAC}$ following exposure to NDGA (data not shown).

5-HPETE is an unstable compound. Although we used 5-HPETE shortly after its arrival and followed the procedures described by the supplier, we do not have a positive control that it was indeed active. We were also unable to test 12- and 15-HPETEs, alone or in combination with 5-HPETE, because of the cost of these compounds.

## DISCUSSION

Our central finding is that a variety of structurally distinct lipo-oxygenase blockers all share the ability to reduce the activation of $I_{CRAC}$. The key question is whether this reflects a requirement for the lipo-oxygenase enzymes in the elusive activation mechanism of $I_{CRAC}$ or whether it involves an action on either an inhibitory pathway or on a step not related to lipo-oxygenase block.

A lipo-oxygenase-sensitive step could easily be accommodated into each of the three major mechanisms proposed to account for the activation of store-operated $Ca^{2+}$ influx. It could be important in maintaining close proximity of Ins$P_3$ receptors and CRAC channels, as required by the conformational-coupling model. Our recent results impose some constraints on this model in that, if it is applicable to RBL-1 cells, then the coupling must already be preformed prior to store depletion and involve a novel Ins$P_3$ receptor (Bakowski *et al.* 2001). Perhaps lipo-oxygenase enzymes maintain the integrity of this tightly formed complex. On the other hand, the lipo-oxygenase pathway might generate a diffusible metabolite which then activates CRAC channels. Finally, the lipo-oxygenase pathway might be required for fusion of vesicles containing CRAC channels with the plasma membrane upon store depletion. The fact that we could not rescue $I_{CRAC}$ with 5-HPETE in cells already exposed to lipo-oxygenase blockers is a major problem for a model that proposes that lipo-oxygenases are involved in the activation of $I_{CRAC}$. But interpretation of this result is not straightforward for several reasons. First, we do not have a positive control for 5-HPETE. Although we used it shortly after its arrival and ordered it twice, it is a relatively unstable compound and we cannot be sure that it was fully active. Second, the products arising from the activity of

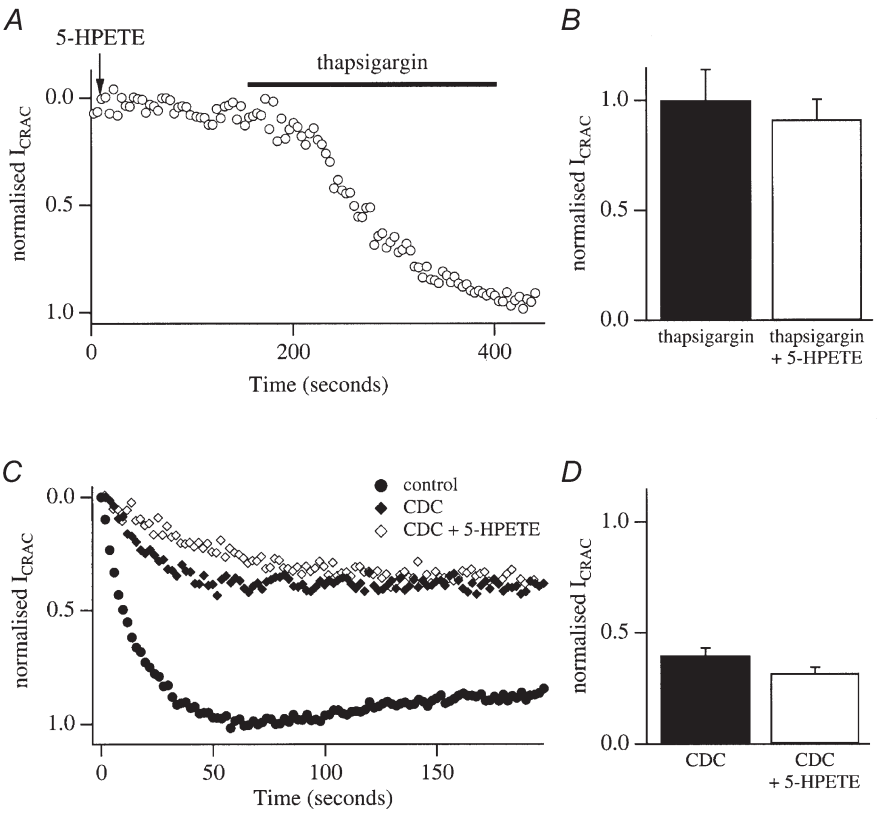



**Figure 10. Effects of 5-HPETE on $I_{CRAC}$**

*A*, representative whole-cell recording from a cell dialysed with 5-HPETE in 120 nM buffered $Ca^{2+}$. $I_{CRAC}$ did not activate. After 180 s, thapsigargin was applied and $I_{CRAC}$ subsequently developed. *B*, size of $I_{CRAC}$ following thapsigargin application in control cells, and in those dialysed with 5-HPETE. There was no significant difference. *C*, control recording (filled circles), recording taken after pre-exposure to CDC (diamonds) and recording after pre-exposure to CDC but with 5-HPETE in the pipette solution. The recording pipette contained Ins$P_3$ + 10 mM EGTA. Data have been normalized to control recordings. *D*, pooled data indicating that the reduction in $I_{CRAC}$ amplitude following pretreatment with CDC cannot be rescued by inclusion of 5-HPETE in the pipette.

one lipo-oxygenase enzyme can regulate fluxes through the others (Needleman *et al.* 1986). It is possible that a combination of metabolites are required rather than just 5-HPETE. Third, certain lipo-oxygenases translocate to the plasma membrane in RBL-1 cells upon stimulation. The site of arachidonic acid production might therefore be critical, and this may not be suitably mimicked by dialysing cells with 5-HPETE from a patch pipette. Finally, lipo-oxygenase activity might be involved in the activation of $I_{CRAC}$ but without the requirement for a subsequent metabolite. This would be similar to the putative role of phospholipase C, an enzyme which hydrolyses phosphatidylinositol 4,5-bisphosphate, in the activation of $I_{CRAC}$ in RBL-1 cells. Here, an inhibitor of the enzyme suppressed $I_{CRAC}$ but neither hydrolysis product ($InsP_3$ or diacylglycerol) could rescue the current (Broad *et al.* 2001).

It is unlikely that a known mechanism that inactivates $I_{CRAC}$ becomes more prominent in the presence of lipo-oxygenase block and that this accounts for the reduction in the current. In RBL cells, $I_{CRAC}$ can be inactivated by fast $Ca^{2+}$-dependent inactivation (reflecting a build-up of $Ca^{2+}$ in the vicinity of each open channel; Fierro & Parekh, 1999*b*) and $Ca^{2+}$-dependent slow inactivation (Parekh, 1998), which both arise from $Ca^{2+}$ influx, $Ca^{2+}$-dependent store refilling (Bakowski, Glitsch & Parekh, 2001) and protein kinase C (Parekh & Penner, 1995). Although some lipo-oxygenase blockers elevated intracellular $Ca^{2+}$, we do not think $Ca^{2+}$-dependent inactivation is involved because first NDGA and CDC were still effective in the absence of external $Ca^{2+}$ under conditions where $Na^+$ was the charge carrier, and second both store-operated $Ca^{2+}$ influx (using Fura-2 in intact cells) and $I_{CRAC}$ were still substantial in spite of pretreating cells with thapsigargin in external $Ca^{2+}$, a condition which elevates intracellular $Ca^{2+}$ levels significantly. Because inclusion of thapsigargin in the recording pipette failed to prevent the loss of $I_{CRAC}$ following exposure to the lipo-oxygenase blockers, it is unlikely that store refilling is the mechanism of inhibition. A role for protein kinase C in the inhibition of $I_{CRAC}$ by lipo-oxygenase blockers is also unlikely because staurosporine, which prevents kinase-mediated inactivation of the current (Parekh & Penner, 1995) was not able to suppress the inhibition brought about by CDC. However, we cannot rule out the possibility that a novel inhibitory mechanism exists, which is both $Ca^{2+}$ and protein kinase independent, and is activated following inhibition of the lipo-oxygenase pathway.

The lipo-oxygenase blockers could, in spite of their structural divergence, all share an action unrelated to lipo-oxygenase inhibition. This is always a concern when utilizing a pharmacological approach. The CRAC channels are possibly themselves one such site. In this scenario, the inhibitors would function as direct channel blockers. Indeed, it has been reported that NDGA can directly inhibit voltage-gated $Ca^{2+}$ channels independently of any actions on lipo-oxygenases (Korn & Horn, 1990) and NDGA evoked rapid inhibition of whole-cell $I_{CRAC}$ in some cells, which would be consistent with a direct channel block. But it is not clear why most cells were relatively resistant to NDGA application after $I_{CRAC}$ had activated. We do not know why local application of NDGA had such kinetically distinct effects. The $Ca^{2+}$ channels that are activated by epidermal growth factor stimulation of the 5-lipo-oxygenase pathway in human A431 and murine P19 embryonal carcinoma cells can be inhibited following only a 2 min pre-exposure to NDGA (Peppelenbosch *et al.* 1992). Moreover, application of NDGA to outside-out patches completely abolished channel activity within 60 s (Peppelenbosch *et al.* 1992). Hence inhibition of lipo-oxygenases can be fast and rapid kinetics of block in whole-cell recording (time constant of 20–35 s) does not necessarily demonstrate direct channel block. Interestingly, NDGA does not block the *drosophila* TRP protein (Chyb *et al.* 1999), and certain TRP homologues are thought to encode the CRAC channels (Zhu & Birnbaumer, 1998). Furthermore, direct CRAC channel block is not an entirely satisfactory explanation for the other drugs. ETYA and CDC in particular were much less effective when applied after $I_{CRAC}$ had activated than before. This kind of result is interpreted as evidence that first the drugs are not direct channel blockers and second, once activated, store-operated influx becomes independent of the initial signal (Rosado & Sage, 2000*b*; Broad *et al.* 2001). Our results could most easily be interpreted along these lines. However, it is possible that the inhibitors still block the CRAC channels directly but in a state-dependent manner. In this admittedly speculative scheme, CDC may bind to the resting but not open state of the CRAC channels and hence would be a more effective blocker if administered prior to store depletion. ETYA would bind significantly more favourably to the resting than the open state whereas NDGA would have to show only a modest preference for the resting state in some cells but bind very favourably to this state in other cells.

Hence we cannot rule out the possibility that the inhibitors interact with CRAC channels in a complex and state-dependent manner. One way to resolve this would be to record CRAC channel activity in an excised patch and then test whether the lipo-oxygenase blockers suppress the current and with what time course. Single-channel $Na^+$ currents, thought to reflect monovalent permeation through CRAC channels in jurkat T-lymphocytes, have already been reported (Kerschbaum & Cahalan, 1999). But we have consistently failed to see such single-channel currents in RBL-1 cells and the properties of the $Na^+$ current through CRAC channels differ substantially between the two cell types (Bakowski & Parekh, 2002).

In summary, our results show that lipo-oxygenase blockers all reduce the activation of $I_{CRAC}$ in RBL-1 cells. One possibility is that the activation step encompasses a lipo-oxygenase enzyme. Although we can eliminate other possible explanations, we cannot rule out a slow, state-dependent direct channel block by the inhibitors we have used. We are currently trying to resolve this using a molecular biological approach.

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