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Influx of Extracellular Calcium Is Required for the Membrane Translocation of 5-Lipoxygenase and Leukotriene Synthesis

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ABSTRACT: Our studies assessed the effects of increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) on leukotriene synthesis and membrane translocation of 5-lipoxygenase (5LO). The calcium ionophore ionomycin and the tumor promoter thapsigargin stimulated leukotriene production and translocation of 5-lipoxygenase to the membrane. Both agents elicited prolonged rises in $[Ca^{2+}]_i$. Leukotriene C_4 production associated with $[Ca^{2+}]_i$ in cells stimulated with various concentrations of ionomycin and thapsigargin suggests that a threshold $[Ca^{2+}]_i$ level of approximately 300-400 nM is required. In the absence of extracellular Ca^{2+}, both the ionomycin- and thapsigargin-induced rises in $[Ca^{2+}]_i$ were transient, indicating that the prolonged $[Ca^{2+}]_i$ elevation is due to an influx of extracellular Ca^{2+}. Addition of EGTA to the external medium before, or at different times during, the treatment with ionomycin or thapsigargin instantaneously inhibited 5LO translocation and leukotriene synthesis, indicating that Ca^{2+} influx plays an essential role in 5LO membrane translocation and leukotriene synthesis. No leukotriene production was detected when cells were stimulated by a physiological stimulus of leukotriene D_4. The addition of 100 nM leukotriene D_4 triggered peak rises in $[Ca^{2+}]_i$ that were comparable to those achieved by the ionomycin and thapsigargin. However, the leukotriene D_4 induced rise was transient and rapidly declined to a lower but still elevated steady-state level, which was attributed to Ca^{2+} influx. Stimulation with 100 nM leukotriene D_4 for 15 s increased the cellular levels of 1,4,5-inositol triphosphate (IP_3), 1,3,4-IP_3, and 1,3,4,5-inositol tetraphosphate (IP_4). In contrast, 100 nM thapsigargin had no effect on generating inositol phosphate after 15, 60, or 300 s of treatment. These results argue against an essential role for inositol phosphates in leukotriene synthesis and indicate that the stimulation of 5-lipoxygenase membrane translocation and leukotriene synthesis is a consequence of a sustained increase in $[Ca^{2+}]_i$ resulting from an influx of external Ca^{2+}.

Studies with a variety of cell systems have led to the view that a rise in $[Ca^{2+}]_i$[1] is an obligatory step in the synthesis of leukotrienes. The main evidence for this conclusion is summarized as follows: First, leukotriene (LT) synthesis can be obtained after binding of agonists to cell surface receptors, which results in an increase of $[Ca^{2+}]_i$. For example, macrophages synthesize LTC_4 in response to several Ca^{2+}-mobilizing stimuli such as opsonized zymosan (Humes et al., 1982; Tripp et al., 1985) and immunoglobulin E complexed with antigen (Rouzer et al., 1982). Second, calcium ionophore A23187 induces leukotriene synthesis (Borgeat et al., 1979), presumably through a rise in $[Ca^{2+}]_i$. Third, at least two enzymes in the leukotriene synthesis pathway require Ca^{2+}: phospholipase A_2, for release of arachidonic acid (Wijkander & Sundler, 1989; Leslie et al., 1988), and 5-lipoxygenase (5LO), for synthesis of 5-hydroperoxyeicosatetraenoic acid and LTA_4 (Jakschik & Lee, 1980). In addition, Ca^{2+} regulates the membrane translocation of 5LO (Rouzer & Kargman, 1988; Wong et al., 1988). It has been hypothesized that a rise in $[Ca^{2+}]_i$ induces the binding of 5LO to a specific integral membrane protein, which might aid the enzyme in obtaining substrate released from membrane phospholipid stores (Rouzer et al., 1990; Dixon et al., 1990).

Despite these findings, some observations have cast doubt on the simplistic view that a rise in $[Ca^{2+}]_i$ is necessary or sufficient to trigger the synthesis of leukotrienes. Recent reports have demonstrated that some stimuli induce a rise in $[Ca^{2+}]_i$ that is insufficient for generating leukotrienes; for example, human leukocytes stimulated with [(*N*-formylmethionyl)leucinyl]phenylalanine or complement C_{5a} (Clancy et al., 1983; Haines et al., 1987) fail to generate a significant amount of leukotrienes. In order to understand the precise

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[1] Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium; AM, acetoxymethyl ester; IP_3, inositol triphosphate; IP_4, inositol tetraphosphate; LT, leukotriene; 5HETE, 5-hydroxytetraeicosanoic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH buffer, Krebs-Ringer-Henseleit buffer; 5LO, 5-lipoxygenase; RBL, rat basophilic leukemia; Tg, thapsigargin.

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role of $[Ca^{2+}]_i$ in leukotriene production, we have compared the abilities of three stimuli to induce leukotriene synthesis by rat basophilic leukemia cells (RBL-2H3): the calcium ionophore ionomycin, leukotriene D_4 (LTD_4), and the tumor promoter thapsigargin (Tg). Each stimulus elicits a different pattern of $[Ca^{2+}]_i$ response. Ionomycin forms stable complexes with Ca^{2+} and carries the divalent cation across the cell membrane (Kaufmann et al., 1980). Binding of LTD_4 to its receptors induces phosphoinositide turnover and mobilization of intracellular Ca^{2+} (Mong et al., 1988). Tg is a sesquiterpene lactone extracted from the umbelliferous plant *Thapsia garganica* (Rasmussen et al., 1978). Tg is not an ionophore (Ali et al., 1985). It inactivates the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1989, 1990) and releases Ca^{2+} from intracellular stores, which leads to a subsequent influx of extracellular Ca^{2+} (Takemura et al., 1989; Thastrup et al., 1990).

Using these stimuli, we have examined $[Ca^{2+}]_i$ changes resulting in intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx and have correlated these changes with the observed membrane translocation of 5LO and leukotriene production. Both ionomycin and Tg trigger the production of a substantial amount of leukotriene. However, stimulation with LTD_4 has no effect. Our results suggest that the common signaling element in leukotriene production in RBL-2H3 cells is a sustained influx of extracellular Ca^{2+}.

Materials and Methods

Materials

Thapsigargin was kindly supplied by Dr. S. Brøgger Christensen (Royal Danish School of Pharmacy, Copenhagen, Denmark). LTD_4 was supplied by the Department of Medicinal Chemistry, SmithKline Beecham. [1,2-^{3}H]*myo*-inositol (45–80 Ci/mmol) and ^{125}-labeled goat anti-rabbit IgG (100 μCi/mL) were purchased from NEN, Cambridge, MA. The RBL-2H3 subline was kindly supplied by Dr. R. P. Siraganian (Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health). Fetal calf serum was purchased from Hyclone Labs, Logan, UT. Arachidonic acid was from NuChek Prep. Inc., Elysian, MN. Calcium ionomycin and fura-2 acetoxymethyl ester were obtained from Calbiochem, La Jolla, CA. Sulfinpyrazone and all other chemicals were purchased from Sigma, St. Louis, MO. All solvents were HPLC grade and were obtained from J. T. Baker.

Methods

Cell Culture. RBL-2H3 cells were maintained as monolayer cultures in 75-cm^2 flasks in Eagle's essential medium supplemented with 20% fetal calf serum as described previously (Barsumian et al., 1981). The cells were harvested by trypsinization, diluted to (0.3–0.5) × 10^6 cells/mL with culture medium, and grown in spinner culture for 24 h.

Cell Activation and Preparation of Subcellular Fractions. RBL-2H3 cells were grown to a cell density of (0.8–1.0) × 10^6 cells/mL. Cells were centrifuged (200*g*, 10 min) and resuspended in 5 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM glucose (buffer A) at 2.0 × 10^6 cells/mL. Cells were stimulated with various concentrations of ionomycin (0.01–1 μM), Tg (0.1–100 nM), or LTD_4 (0.01–1 μM) in a total volume of 5–8 mL. Agonists were applied to the test tubes before the cells were added, providing a good mixing of the samples, which is crucial for obtaining consistent stimulatory effects. In some experiments 2 mM EGTA was added to the cell suspension before it was mixed with the agonists. Drug treatments were carried out at 37 °C for 20 min. After incubation, 1.2 mM EDTA was added. The samples were centrifuged at 500*g* for 10 min. The supernatants were removed and assayed for the production of LTC_4 by radioimmunoassay or were mixed with an equal volume of ice-cold acetonitrile for assaying the release of 5LO metabolites by reverse-phase HPLC. The cell pellets were resuspended in homogenizing buffer (10 mM Hepes, pH 7.4, and 1 mM EDTA) at 20 × 10^6 cells/mL and incubated on ice for several minutes. The cells were lysed by vortexing vigorously two times, each for 15 s. The cell lysates were centrifuged at 35000*g* for 30 min. The pellets were resuspended in the original volume of the cell lysates with the homogenizing buffer. The 35000*g* supernatant and pellet obtained are referred to as the soluble and particulate fraction, respectively. The 5LO present in the soluble and particulate fractions was determined by Western blot.

Quantitation of 5LO Protein by Western Blot. In order to quantify the 5LO protein present in the soluble and particulate fractions, we added 33 μL of the sample buffer (8% SDS, 0.5 M Tris-HCl, pH 6.8, 40% glycerol, 5% β-mercaptoethanol, and 0.003% bromphenol blue) to the 100-μL samples. The samples were incubated at 70 °C for 5 min. Aliquots (5–10 μL) of the sample mixtures were subjected to SDS–PAGE, as described by Laemmli (1970). The gels were run at 35 mA/gel for 1.5 h and were transferred to nitrocellulose paper (0.35 A for 4 h, 4 °C). The nitrocellulose sheets were blocked with a solution of 5% nonfat dry milk in 50 mM Tris, pH 8, 80 mM NaCl, and 2 mM $CaCl_2$ (buffer C) containing 0.2% NP-40. The nitrocellulose sheets were incubated with a polyclonal antiserum raised against highly purified RBL-5LO (Wong et al., 1988) diluted in blocking solution (1:500) for 1 h at room temperature. Unbound antibody was washed from nitrocellulose sheets by three changes of buffer C containing 0.2% NP-40, for 5 min each. The nitrocellulose sheets were incubated with a solution of buffer C containing 2% NP-40, 0.2% SDS, and 4 μCi/mL ^{125}I-labeled goat anti-rabbit IgG for 45 min. The blots were washed three times with buffer C containing 0.2% NP-40, for 5 min each, and then dried and exposed to X-ray film for 12–14 h. Autoradiograms were scanned with a LKB-2202 Ultroscan laser densitometer (LKB Produktor, Bromma, Sweden).

Measurements of 5LO Products Released from RBL-2H3 Cells. PGB_2 (0.5 nmol) was added to the extracellular supernatants containing 5LO metabolites to serve as the internal standard. Samples were dried under vacuum overnight and then resuspended in 0.4 mL of 30% acetonitrile containing 30 mM ammonium acetate, pH 5.8. The samples were filtered to remove any insoluble material. Aliquots of 0.2 mL were loaded onto a Waters (Milford, MA) RCM Nova Pak C18 (100 × 8 mm) column with the starting mobile phase of 90% A (A = 10% acetonitrile buffered with 30 mM ammonium acetate, pH 5.8) and 10% B (B = 90% acetonitrile buffered with 30 mM ammonium acetate, pH 5.8). Flow rate was 2.5 mL/min. At 5 min, the concentration of B was increased to 27%. Between 12 and 17 min, the percentage of B was increased in a concave hyperbolic function (curve 9) to 40% and was then increased in a linear manner to 60% by 24 min. Under these developing conditions, the retention times for the eicosanoids were as follows: 20-hydroxy-LTB_4, 6.1 min; LTC_4, 8.2 min; LTD_4, 9.2 min; PGB_2, 10.8 min; Δ^6-*trans*-LTB_4, 12.6 min; LTB_4, 13.5 min; HHT, 17.3 min; 5,6-DiHETE, 18 min; 12HETE, 20.6 min; 5HETE, 21 min. The eicosanoids in the samples were verified by their retention times and their UV absorption spectra. The peaks were quantified with reference to a standard curve based on peak area for each metabolite,

and the amounts were corrected for recovery of the internal standard.

Radioimmunoassay of LTC_4. Aliquots of 0.1 mL of the extracellular supernatants were assayed for the amount of LTC_4 present. Radioimmunoassays were performed in polypropylene test tubes and assayed according to the manufacturer's instructions. Synthetic LTC_4 was detectable on a linear portion of the radioligand binding curve at concentrations ranging from 0.2 to 10 ng/mL.

Fura-2 Loading and $[Ca^{2+}]_i$ Measurements. Harvested RBL-2H3 cells were resuspended at 2×10^6 cells/mL in Krebs–Ringer–Henseleit buffer (KRH) which contained 118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1 mM KH_2PO_4, 11.1 mM glucose, 0.1% bovine serum albumin, 1.1 mM $MgSO_4$, 1 mM $CaCl_2$, and 5 mM Hepes, pH 7.4. Sulfinpyrazone and fura-2/AM were added to their final concentrations of 250 and 2 μM, respectively. The cells were incubated for 45 min at 37 °C. Leakage of fura-2 occurred fairly rapidly in RBL-2H3 cells; sulfinpyrazone, an organic anion transport inhibitor, was therefore included in all buffers to reduce leakage of fura-2 during and after fura-2 loading (Millard et al., 1989). Cells were pelleted by centrifugation at 2×10^6 cells/mL in fresh KRH buffer incubated at 37 °C for 20 min to ensure complete hydrolysis of entrapped ester. Cells were pelleted, washed once in buffer, and resuspended in cold buffer A. The cells were maintained on ice for up to 1 h prior to use for fluorescence measurements. No fura-2 leakage was detected during this period.

We determined $[Ca^{2+}]_i$ by measuring the fluorescence of the entrapped fura-2, using a fluorometer (designed by the Johnson Foundation Biomedical Instrumentation Groups) equipped with a temperature control unit and a magnetic stirrer under the cuvette holder, as described previously [see Saussy et al. (1989) for description]. All experiments were performed at 37 °C with wavelength settings a 339 nm for excitation and 499 nm for emission. The fura-2-loaded cells (2×10^6 cells/mL) were preincubated at 37 °C for 10 min. Fluorescence was recorded for 30 s to ensure a stable baseline before the addition of agonists. For experiments performed in the absence of extracellular Ca^{2+}, cells were resuspended in buffer A (with no Ca^{2+} added) containing sulfinpyrazone and 1 mM EGTA. Fluorescence was recorded continuously for 10 min after the addition of agonists. $[Ca^{2+}]_i$ was calculated as described in previous reports (Grynkiewicz et al., 1982; Dubyak & De Young, 1985).

Inositol Phosphate Accumulation. Harvested cells were resuspended at a concentration of 0.6×10^6 cells/mL of inositol-free RPMI-1640 media supplemented with 20% heat-inactivated dialyzed fetal calf serum. The cells were labeled with 1 μCi/mL [^{3}H]*myo*-inositol for 18 h. Cells were harvested by centrifugation at 500*g* for 10 min, washed three times in ice-cold KRH buffer, and resuspended at 10×10^6 cells/mL in cold KRH buffer containing 1 mM Ca^{2+}. An aliquot of the cell suspension (1 mL) was preincubated for 10 min at 37 °C in a shaking water bath. Agonist was added and the reaction was allowed to proceed for a predesignated time (15 s, 60 s, 5 min, or 30 min). In some experiments, 50 mM LiCl was added to inhibit the dephosphorylation of IP_1 (Berridge, 1984; Sherman et al., 1981). The reaction was stopped by adding 183 μL of 100% trichloroacetic acid (w/v), and the sample was placed on ice for 20 min prior to centrifugation (1750*g*, 10 min). The supernatant was saved, the pellet was washed once with 1 mL of water, and the wash supernatant was added to the original supernatant. Trichloroacetic acid was extracted from the supernatant with 3×5 mL washes with water-saturated diethyl ether. Excess ether was removed by evaporation under argon, and the sample was neutralized by adding 150 μL of 0.1 M Tris base. The sample was stored at −20 °C until it could be analyzed.

The inositol phosphate isomers present in the cell extracts were separated by HPLC. Separation was achieved with the anion-exchange system described by Winkler et al. (1988) with an ammonium formate gradient.

Preparation of Drugs. Stock solutions of ionomycin, ionophore, and Tg were prepared in absolute ethanol and added as 0.5% (v/v) in the cell suspensions. LTD_4 was prepared in aqueous solution. Concentrations of the ionomycin, ionophore, and LTD_4 stock solutions were quantitated spectrophotometrically before use.

Results

Increases in $[Ca^{2+}]_i$, Leukotriene Synthesis, and 5LO Translocation in Response to Stimuli in the Presence or Absence of Extracellular Ca^{2+}. (A) Effects of Ionomycin. The intrinsic fluorescence of the ionophore A23187 interfered with fura-2 measurements of $[Ca^{2+}]_i$; ionomycin was therefore used for fluorescence measurements. The $[Ca^{2+}]_i$ of unstimulated cells was 170 ± 10 nM (n = 14). Cells that were stimulated with 300 nM ionomycin showed an immediate rise in $[Ca^{2+}]_i$ to a peak level (1253 ± 165 nM, n = 3) at approximately 24 s; this was maintained for at least 10 min (duration of the experiment) (Figure 1A). Ionomycin caused a dose-dependent increase in $[Ca^{2+}]_i$ over the range of 0.01–1 μM (Figure 2A).

We examined the effect of ionomycin on $[Ca^{2+}]_i$ changes in RBL-2H3 cells in the absence of extracellular free Ca^{2+} (Figure 1B). EGTA (2 mM) was applied to the medium shortly before the addition of ionomycin. When EGTA was added, fura-2 fluorescence declined slightly to a new basal level, due to EGTA chelating the Ca^{2+} coupled to the extracellular fura-2 that had leaked out from cells. No further decline in fura-2 fluorescence could be detected after the immediate drop, suggesting that the permeation of the plasma membrane by EGTA in unstimulated cells was minimal. Figure 1B shows that adding EGTA attenuated the 300 nM ionomycin induced $[Ca^{2+}]_i$ increase by 85%. Moreover, the $[Ca^{2+}]_i$ returned to its resting level over 2 min, suggesting that the prolonged $[Ca^{2+}]_i$ increase is due to the influx of extracellular Ca^{2+}. A readdition of 2 mM $CaCl_2$ into the medium partially restored the $[Ca^{2+}]_i$ response (Figure 1B).

Although ionomycin and A23187 differ with respect to their counterion specificities (i.e., Ca^{2+}/Mg^{2+} exchange with A23187 and Ca^{2+}/H^+ exchange with ionomycin; DiVirgilio & Gomperts, 1983), both were equally active in stimulating leukotriene synthesis in RBL-2H3 cells (Table I). The majority of products (95% of the total) are leukotrienes, which include Δ^6-*trans*-LTB_4, LTB_4, peptidylleukotrienes (LTD_4 and LTC_4), and 5,6-DiHETE. 5HETE was detected in small amounts (no more than 6% of total product).

After the addition of 300 nM ionomycin, an increase in LTC_4 (measured by radioimmunoassay) was detected at 1 min; this continued for approximately 5 min (data not shown). Ten nanomolar ionomycin failed to stimulate LTC_4 production despite a moderate increase (250 nM) in $[Ca^{2+}]_i$ (Figure 2A). At ionomycin ≥ 20 nM where the $[Ca^{2+}]_i$ was 350 nM or higher, LTC_4 was synthesized in a dose-dependent fashion. Maximal increase was obtained at 200–500 nM ionomycin and was followed by a sharp decrease at 1 μM, probably due to the cytotoxic effect.

In conjunction with the increase in leukotriene synthesis, ionomycin caused a dose-dependent redistribution of immu-

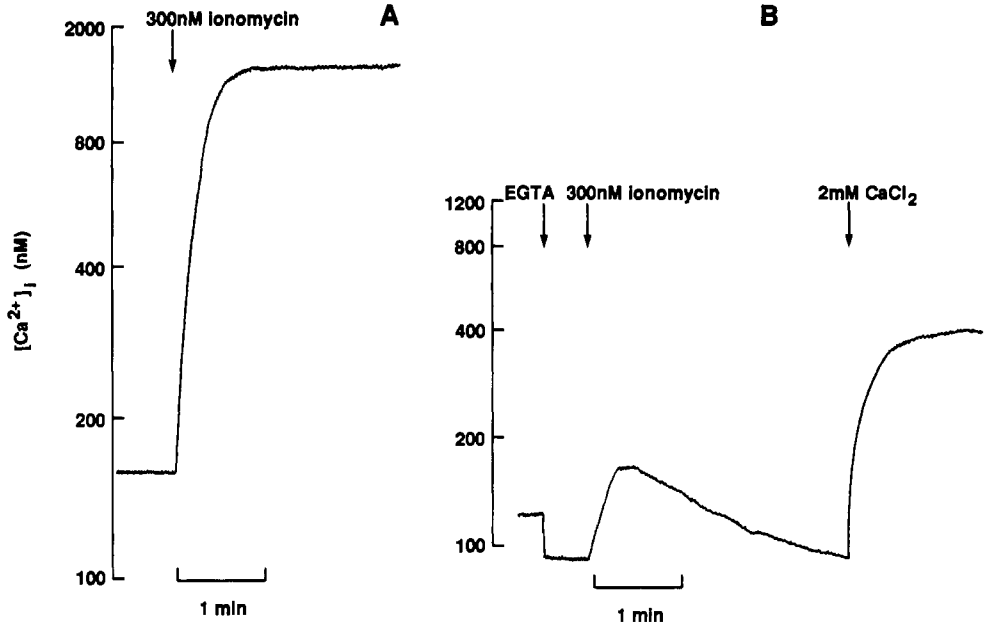

FIGURE 1: Effects of ionomycin on $[Ca^{2+}]_i$ in the presence and absence of extracellular Ca^{2+}. (A) Fura-2-loaded RBL-2H3 cells (2×10^6 cells in 1 mL of KRH buffer, 0.1 mg/mL bovine serum albumin, and 1 mM $CaCl_2$) were equilibrated at 37 °C as described in the Experimental Procedures section. Ionomycin (300 nM) was added to the cuvette where indicated. (B) EGTA (2 mM) was added prior to the addition of 300 nM ionomycin. $CaCl_2$ (2 mM) was added at the arrow.

Table I: Effects of Various Stimuli on Formation of 5-Lipoxygenase Products by RBL-2H3 Cells[a]

	5LO products (pmol/5 × 10^6 cells)				
stimulus	Δ^6-*trans*-LTB_4	LTB_4	LTC_4 + LTD_4	DiHETE	5HETE
A23187 (100 nM)	727 ± 14	95 ± 4	62 ± 2	148 ± 7	0
ionomycin (300 nM)	706 ± 42	126 ± 17	46 ± 1	150 ± 40	62 ± 20
thapsigargin (30 nM)	530 ● 48	142 ± 15	142 ± 8	123 ± 25	75 ± 10
LTD_4 (100 nM)	0	0	0	0	0

[a] RBL-2H3 cells (5×10^6 cells in a total volume of 5 mL) were incubated for 10 min at 37 °C with the appropriate stimulus. Cells were harvested by centrifugation (500*g*, 10 min). 5LO products released into the extracellular medium were measured by reverse-phase HPLC as described in the Experimental Procedures section. Results are expressed as picomoles per 5×10^6 cells ± SEM ($n = 3$).

noreactive 5LO between the soluble and particulate fractions (Figure 2B). No decrease in soluble 5LO was observed at ionomycin ≤ 20 nM, despite moderate production of LTC_4 (15 ng/mL). A 25% decrease of soluble 5LO was detected at 30–50 nM ionomycin. A maximal decrease (near 100%) was obtained at 1 μM. The soluble 5LO was recovered inactive in the particulate fraction. There was no change in the total amount of enzyme in RBL-2H3 cells after stimulation of ionomycin ≤ 500 nM.

(*B*) *Effects of Thapsigargin.* Stimulating cells with an optimal dose (30 nM) of Tg resulted in an increase in $[Ca^{2+}]_i$ in both the presence and absence of external Ca^{2+} (Figure 3). In both cases, there was a delay of 5–8 s before $[Ca^{2+}]_i$ began to rise. Peak $[Ca^{2+}]_i$ levels were reached after 100 and 50 s in the presence or absence of external Ca^{2+}, respectively. This was considerably slower than the rise in $[Ca^{2+}]_i$ seen with ionomycin and LTD_4 stimulation, which reached maximal $[Ca^{2+}]_i$ levels without measurable delay. In the presence of external Ca^{2+} (Figure 3A), the peak $[Ca^{2+}]_i$ level induced by Tg (1060 ± 56 nM, $n = 3$) was maintained for at least 10 min (the duration of the experiment). Tg induced a dose-dependent increase in $[Ca^{2+}]_i$. The half-maximum increase was obtained at 3 nM Tg (Figure 4A). In the absence of extracellular Ca^{2+}, the peak $[Ca^{2+}]_i$ (220 nM) returned to its resting level after 4 min (Figure 3B). The late phase response was restored when 2 mM $CaCl_2$ was added, suggesting that the steady-state $[Ca^{2+}]_i$ response is due to an influx of external Ca^{2+}.

After incubation for 20 min at 37 °C with an optimal concentration of Tg (30 nM), RBL-2H3 cells produced Δ^6-*trans*-LTB_4, LTB_4, 5,6-DiHETE, LTC_4, LTD_4, and a small quantity of 5HETE (Table I). Thus, Tg-induced 5LO products exhibit profiles similar to those induced by A23187 and ionomycin. Interestingly, the production of peptidyl-leukotrienes ($LTC_4 + LTD_4$) was slightly higher in Tg-treated cells.

Stimulating RBL-2H3 cells with Tg at concentrations of 3 nM (the $[Ca^{2+}]_i$ was 400 nM) or higher resulted in a concentration-dependent increase in LTC_4 production (Figure 4A). Maximal production was obtained at 10 nM Tg. The synthesis of LTC_4 was accompanied by the binding of the soluble 5LO to the particulate fraction. The effects of Tg on membrane association of 5LO were also dose-dependent and corresponded to LTC_4 production (Figure 4B). After being incubated with 30 nM Tg for 10 min, RBL-2H3 cells exhibited a 60% loss of 5LO in the soluble fraction. The total amount of enzyme in the cells remained unchanged after treatment with Tg.

(*C*) *Effects of Leukotriene D_4.* Marked increases in fura-2 fluorescence were observed in cells stimulated by 100 nM LTD_4 (Figure 5A). The LTD_4-stimulated rise in $[Ca^{2+}]_i$ occurred in two phases: an initial rise that peaked (1030 ± 192 nM, $n = 3$) after about 7 s, which then declined to a lower but still elevated level (280 ± 16 nM, $n = 3$) that was maintained throughout the experiment (>10 min). In the absence of extracellular Ca^{2+} (Figure 5B), the initial rise in $[Ca^{2+}]_i$ was attenuated by 60%, suggesting that part of the initial $[Ca^{2+}]_i$ change is due to release from intracellular stores. The steady-state $[Ca^{2+}]_i$ increase was completely abolished. Adding 2 mM $CaCl_2$ back into the Ca^{2+}-depleted medium resulted in a small overshot and restoration of the "late" $[Ca^{2+}]_i$ response (Figure 5B). This suggests that the late phase of elevated $[Ca^{2+}]_i$ is due to an influx of extracellular Ca^{2+}.

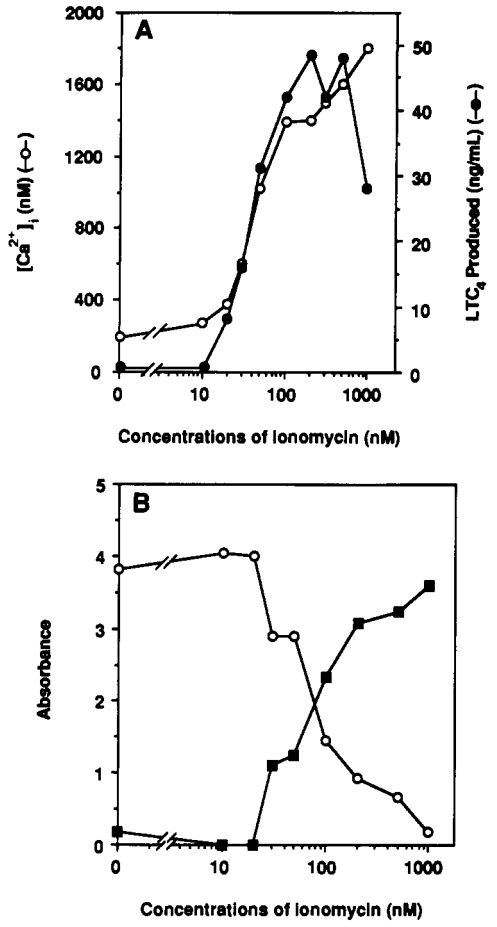

FIGURE 2: Concentration dependency of the ionomycin-stimulated $[Ca^{2+}]_i$ increase, LTC_4 production, and 5LO membrane translocation. (A) RBL-2H3 cells (2×10^6 cells/mL, a total of 6 mL) were incubated with various concentrations of ionomycin at 37 °C for 10 min in buffer A. After treatment, 1.2 mM EDTA was added. Cells were centrifuged and the extracellular media were assayed for the production of LTC_4 by radioimmunoassay. Experiments to examine ionomycin-induced $[Ca^{2+}]_i$ changes and LTC_4 production were performed side by side with the same cell preparation and ionomycin solution. (○) $[Ca^{2+}]_i$ changes; (●) LTC_4 produced. (B) The cell pellets were resuspended in 10 mM Hepes, pH 7.4, and 1 mM EDTA at 20×10^6 cells/mL. Cells were lysed by vortexing vigorously (20 s). The cell lysates were centrifuged at 35000*g* for 30 min. 5LO present in the soluble and particulate fractions was quantitated by Western blot. The blots were developed by using a rabbit polyclonal antibody against purified RBL-enzyme (1:500) and a second antibody of ^{125}I-labeled goat anti-rabbit IgG (0.4 μCi/mL). The autoradiographs were scanned by a laser densitometer as described in the Experimental Procedures section. (○) Soluble 5LO; (■) particulate 5LO. Values are the mean from triplicate samples. Similar experiment have been repeated three times.

HPLC analyses showed that no 5LO metabolite was generated by RBL-2H3 cells after stimulation by 100 nM LTD_4 (Table I). Moreover, no membrane association of 5LO was detected in the LTD_4-stimulated cells.

Mechanism of Thapsigargin-Induced $[Ca^{2+}]_i$ Changes. When RBL-2H3 cells were treated with a combination of 30 nM Tg and 100 nM LTD_4, there was an immediate rise of $[Ca^{2+}]_i$ (1028 nM), which declined slightly and then increased to reach a steady-state level of 1020 nM (Figure 6A). This "hybrid response" might be expected on the basis of the different mechanisms by which these agents mobilize intracellular Ca^{2+}: LTD_4 acts through the production of 1,4,5-IP_3, and Tg acts through the inhibition of Ca^{2+} uptake by the endoplasmic reticulum (Thastrup et al., 1990). The addition of LTD_4 together with Tg did not raise $[Ca^{2+}]_i$ to a level higher than that seen with Tg alone (Figure 6A). In another experiment,

Table II: Effects of LTD_4 and Thapsigargin on Production of Inositol Phosphates by RBL-2H3 Cells[a]

		total ^3H label in each fraction (dpm)		
treatment	time	[^3H]-1,3,4-IP_3	[^3H]-1,4,5-IP_3	[^3H]-1,3,4,5-IP_4
control		1349 ± 196	1301 ± 120	2350 ± 249
LTD_4	15 s	1964 ± 46	1685 ± 190	3135 ± 859
Tg	15 s	1250 ± 36	1280 ± 35	2310 ± 159
Tg	60 s	1004 ± 397	910 ± 316	1701 ± 699
Tg	300 s	1783 ± 380	1081 ± 263	2302 ± 653
control (+Li)	10 min	4979 ± 134	1533 ± 369	4528 ± 148
Tg (+Li)	10 min	4025 ± 126	1207 ± 235	4500 ± 106

[a] The RBL-2H3 cells were labeled with 1 μCi/mL [^3H]*myo*-inositol at 0.6×10^6 cells/mL for 18 h. Cells were harvested, washed, and resuspended at 10×10^6 cells/mL. Aliquots of the cells suspension (1 mL) were treated with either 2 μL of absolute ethanol (control), 100 nM LTD_4, or 100 nM Tg. Reactions were allowed to proceed for a predesignated time. In some experiments, LiCl (50 mM) was added. The reactions were stopped by adding trichloroacetic acid. Extraction and separation of the inositol phosphate isomers are described in the Experimental Procedures section. Data are means ± SEM (n = 3) from a representative experiment that was performed three times. Control values represent the means ± SEM (n = 9) for radioactivity of each inositol isomer after incubation for 15, 60, and 300 s.

LTD_4 was added to cells during the sustained Ca^{2+} entry caused by Tg treatment. No additional increase in $[Ca^{2+}]_i$ was detected (Figure 6B). If LTD_4 could activate Ca^{2+} entry by mechanisms other than those utilized by Tg, an additive sustained response might be expected in the presence of the two agonists. As this was not the case, it would appear that Tg and the LTD_4 receptor utilize the same Ca^{2+} entry mechanism.

Removal of External Ca^{2+} Blocked Leukotriene Synthesis. Removing Ca^{2+} from the medium before adding ionomycin or Tg completely abolished 5LO translocation and leukotriene synthesis. This suggests that the primary signal for the initiation of leukotriene synthesis is Ca^{2+} dependent. To provide evidence that removing Ca^{2+} from the medium inhibits leukotriene production by blocking the $[Ca^{2+}]_i$ response, we added Tg (30 nM) at time zero and applied 2 mM EGTA at various times (1, 3, 6, and 10 min) during the 20-min incubation. Figure 7 (inset) shows that fura-2 fluorescence declined rapidly in Tg-stimulated cells upon addition of EGTA. Cells that were harvested immediately or incubated for another 20 min generated the same amount of LTC_4. These results indicate that adding EGTA can inhibit any further leukotriene production even if maximum $[Ca^{2+}]_i$ has been generated, suggesting that leukotriene synthesis is dependent on extracellular Ca^{2+} throughout the response.

Quantitation of Relationship between $[Ca^{2+}]_i$ and Leukotriene Synthesis. We correlated leukotriene production with $[Ca^{2+}]_i$ levels in ionomycin- and Tg-stimulated cells. The plotting of leukotriene production versus $[Ca^{2+}]_i$ reveals that a threshold $[Ca^{2+}]_i$ level was required. At sustained $[Ca^{2+}]_i$ levels ≥300 nM, we obtained straight lines fitted by linear regression with correlation coefficients of 0.975 and 0.954, respectively, in ionomycin- and Tg-treated cells (Figure 8).

Effects of Leukotriene D_4 Receptor Activation and Thapsigargin on Generation of Inositol Phosphates. We examined the abilities of Tg and LTD_4 to increase metabolism of phosphatidylinositides in RBL-2H3 cells. Table II shows that treatment with 100 nM LTD_4 for 15 s increased the cellular level of [^3H]-1,4,5-IP_3, [^3H]-1,3,4-IP_3, and [^3H]-1,3,4,5-IP_4 approximately 40%, 53%, and 21%, respectively. In contrast, 100 nM Tg inhibited the accumulation of [^3H]-1,4,5-IP_3, [^3H]-1,3,4-IP_3, and [^3H]-1,3,4,5-IP_4 at 15 and 60 s of treatment (Table II). Treatment with Tg for 10 min in the presence

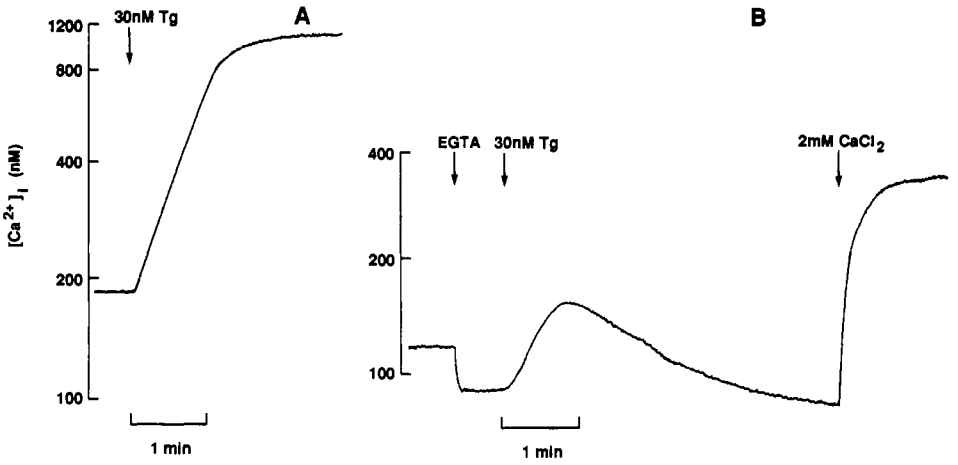

FIGURE 3: Effects of thapsigargin on $[Ca^{2+}]_i$ in the presence and absence of Ca^{2+}. (A) Fura-2-loaded RBL-2H3 cells (2×10^6) were equilibrated at 37 °C. Tg (30 nM) was added where indicated. (B) EGTA (2 mM) was added to cuvette prior to the addition of 30 nM Tg. $CaCl_2$ (2 mM) was added at the arrow.

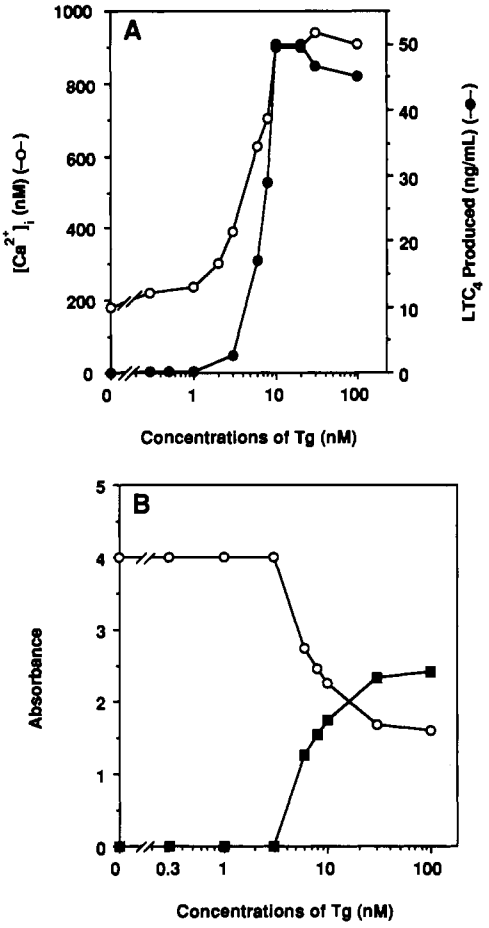

FIGURE 4: Concentration dependency of thapsigargin in changes of $[Ca^{2+}]_i$, LTC_4 production, and 5LO membrane translocation. (A) RBL-2H3 cells (2×10^6 cells/mL, a total of 6 mL) were incubated with various concentrations of Tg at 37 °C for 20 min. After treatment, 1.2 mM EDTA was added to the samples and cells were harvested by centrifugation. LTC_4 released into extracellular media was measured by radioimmunoassay as described in the Experimental Procedures section. (○) $[Ca^{2+}]_i$ changes; (●) LTC_4 produced. (B) Cell pellets were lysed at 20×10^6 cells/mL and the cell homogenates were centrifuged at 35000*g* for 30 min. 5LO enzyme present in the soluble and particulate fractions was quantitated by Western blot followed by scanning of the autoradiographs. (○) Soluble 5LO; (■) particulate 5LO. Values are the mean from triplicate samples. Similar experiments have been repeated three times.

of LiCl also caused a slight decrease in the production of inositol phosphates.

Discussion

Signaling for the synthesis of leukotrienes is believed to involve an increase in $[Ca^{2+}]_i$. The study presented here compares the effects of three agonists, ionomycin, Tg, and LTD_4, on the stimulation of leukotriene synthesis, with each increasing $[Ca^{2+}]_i$ via a specific mechanism. Ionomycin and Tg produced substantial amount of leukotrienes, whereas LTD_4 had no effect.

Optimal concentrations of the three stimuli (300 nM ionomycin, 30 nM Tg, and 100 nM LTD_4) triggered comparable maximal increase in $[Ca^{2+}]_i$. However, duration of the $[Ca^{2+}]_i$ signal varied. The initial increase in $[Ca^{2+}]_i$ was transient in LTD_4-activated cells. In contrast, the peak $[Ca^{2+}]_i$ produced by ionomycin and Tg was persistent, suggesting that leukotriene synthesis in RBL-2H3 cells requires prolonged $[Ca^{2+}]_i$ elevation.

To examine the source of Ca^{2+} that caused the changes in $[Ca^{2+}]_i$, we measured the $[Ca^{2+}]_i$ changes triggered by the three agonists in the absence of extracellular Ca^{2+}. Results showed that removing extracellular Ca^{2+} attenuated the peak $[Ca^{2+}]_i$ increases induced by the ionomycin and Tg by 85%. The $[Ca^{2+}]_i$ signals became transient, and 5LO translocation, together with leukotriene synthesis, was inhibited. In cells stimulated with LTD_4, the initial peak of $[Ca^{2+}]_i$ was attenuated by 60%. In addition, the steady-state $[Ca^{2+}]_i$ signal was abolished. These data indicate that the sustained elevation of $[Ca^{2+}]_i$ induced by the three agonists is generated as a consequence of an influx of extracellular Ca^{2+} that is crucial for leukotriene synthesis. The essential role of Ca^{2+} influx is further supported by the studies in which EGTA was added at the $[Ca^{2+}]_i$ sustained phase during ionomycin and Tg treatments. This resulted in a rapid decrease of $[Ca^{2+}]_i$ to basal levels, which was accomplished by the inhibition of any further 5LO translocation and leukotriene production.

Results in Figure 6 suggest that Tg mediates Ca^{2+} influx by a mechanism shared by the LTD_4 receptors. Similar observations were made in parotid acinar cells, where Tg and methacholine stimulated the same Ca^{2+} entry mechanism (Takemura et al., 1989). RBL-2H3 cells do not possess voltage-sensitive Ca^{2+} channels (Mohr & Fewtrell, 1987), and very little is known about their pathway of Ca^{2+} entry. It is possible that RBL-2H3 cells, like many nonexcitable cells, mediate the agonist-dependent Ca^{2+} influx by the receptor-operated Ca^{2+} channels (Putney et al., 1989; Hallam & Rink, 1989), which are activated by second messengers. These include 1,4,5-IP_3 (Kuno & Gardner, 1987; Llano et al., 1987;

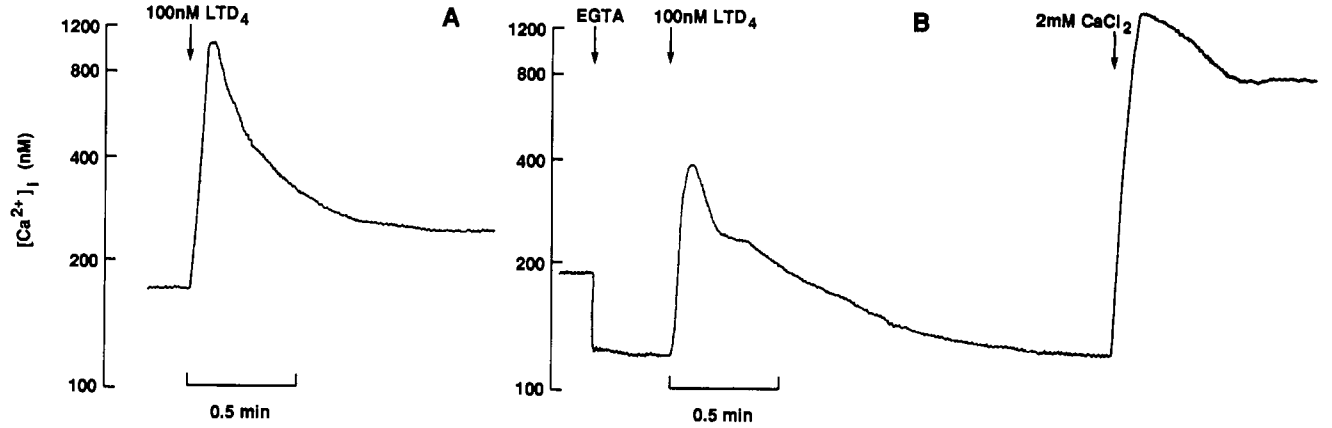

FIGURE 5: Effects of LTD_4 on changes of $[Ca^{2+}]_i$ in the presence or absence of extracellular Ca^{2+}. (A) Fura-2-loaded RBL-2H3 cells (2×10^6) were equilibrated at 37 °C. LTD_4 (100 nM) was added at the arrow. (B) EGTA (2 mM) was added prior to the addition of 100 nM LTD_4. $CaCl_2$ (2 mM) was added where indicated.

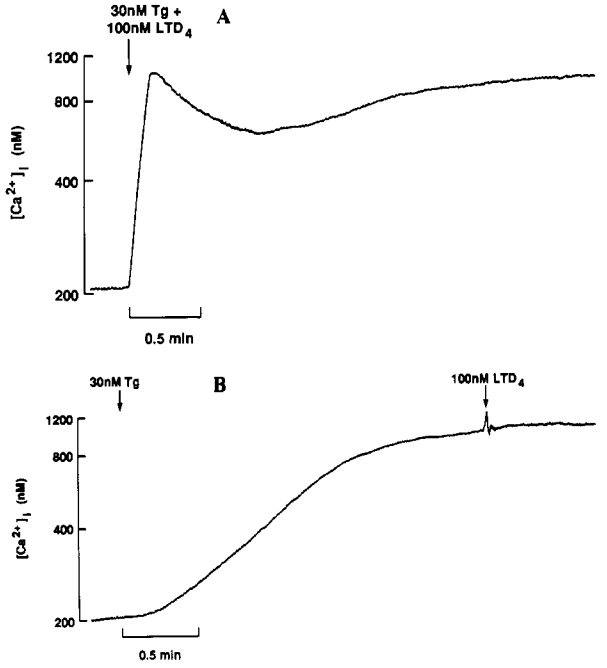

FIGURE 6: (A) Effects of the simultaneous addition of thapsigargin and LTD_4 on $[Ca^{2+}]_i$ in RBL-2H3 cells. A combination of 30 nM Tg and 100 nM LTD_4 was added at the arrow. (B) Effects of the sequential addition of thapsigargin and LTD_4 on $[Ca^{2+}]_i$ in RBL-2H3 cells. Tg (30 nM) and LTD_4 were added where indicated.

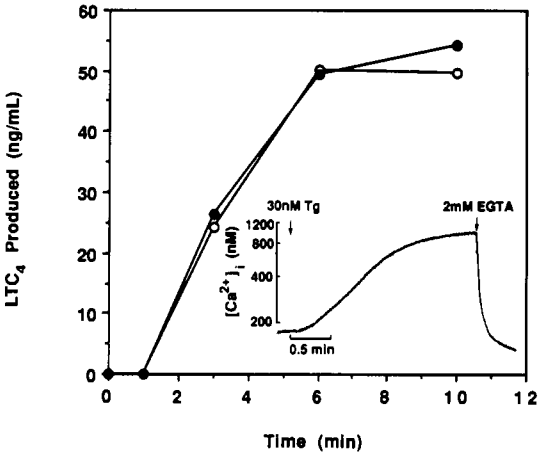

FIGURE 7: Effects of 2 mM EGTA on the $[Ca^{2+}]_i$ responses and leukotriene production in thapsigargin-stimulated cells. Tg (30 nM) was added at time zero and 2 mM EGTA at 1, 3, 6, and 10 min. Half of the cell suspension was removed from each sample and immediately centrifuged (800*g*, 10 min). The remaining samples were incubated at 37 °C for an additional 10 min before harvesting. The extracellular media were assayed for the release of LTC_4 by radioimmunoassay as described in the Experimental Procedures section. (○) Cells immediately centrifuged; (●) cells incubated for an additional 10 min.

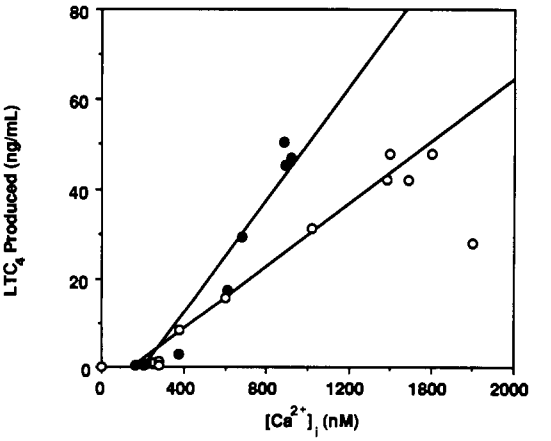

FIGURE 8: Quantitation relationship between $[Ca^{2+}]_i$ increases and leukotriene production in response to various concentrations of ionomycin and thapsigargin. (○) Ionomycin-treated cells; (●) Tg-treated cells.

Penner et al., 1988), 1,3,4,5-IP_4 (Morris et al., 1987), $[Ca^{2+}]_i$ itself (Poggioli et al., 1985; von Tscharner et al., 1986), and GTP-binding protein (Hughes & Barritt, 1987). In addition, emptying the endoplasmic reticulum Ca^{2+} pool also stimulates the Ca^{2+} entry (Takemura & Putney, 1989). Nevertheless, our results support the involvement of a receptor-mediated Ca^{2+} influx pathway in leukotriene synthesis. Our view is further strengthened by a recent study (Hatzelmann et al., 1990) demonstrating that sodium ethylmercurithiosalicylate enhances the [(*N*-formylmethionyl)leucinyl]phenylalanine-induced formation of leukotriene in human polymorphonuclear leukocytes by enhancing the agonist-induced influx of extracellular Ca^{2+}, indicating that Ca^{2+} influx is a general mechanism utilized by other types of cells.

The results with ionomycin and Tg shown in Figure 8 suggest that the modulation of leukotriene synthesis involves a threshold effect. Above this threshold level (300 nM) there is a high degree of concordance between the levels of $[Ca^{2+}]_i$ and leukotriene production in ionomycin- and Tg-treated cells. A critical $[Ca^{2+}]_i$ level may be required for activating 5LO and phospholipase A_2 and/or for generating other mediators that act alone or in concert with elevated $[Ca^{2+}]_i$ to evoke leukotriene synthesis. The partially purified RBL-5LO is inactive when assayed at Ca^{2+} concentrations below 100 nM;

enzyme activity is detected at 300 nM Ca^{2+} in the presence of phosphatidylcholine (P. Marshall, W. De Wolf, and A. Wong, unpublished observations). The partially purified phospholipase A_2 from macrophages (Wijkander & Sundler, 1989) and the purified phospholipase A_2 from human monocytic U937 cells (Clark et al., 1991) are activated at Ca^{2+} concentrations ranging from 100 nM to 1 μM. In addition, Ca^{2+} (between 100 nM and 1 μM) induces binding of arachidonyl-hydrolyzing phospholipase A_2 to membranes (Clark et al., 1991; Channon & Leslie, 1990). The identification of other Ca^{2+}-sensitive components in addition to the two enzymes is complicated by potential interactions among the signal transduction pathways.

A recent study (Wightman & Dallob, 1990) demonstrated that modulation of phosphoinositide breakdown resulted in a coordinate modulation of LTC_4 release when mouse peritoneal macrophages were stimulated by the calcium ionophore A23187 and zymosan. To examine whether inositol phosphate generation is essential for the induction of leukotriene synthesis, we compared the actions of Tg on the two responses. Tg, which increases $[Ca^{2+}]_i$ by inactivation of the endoplasmic reticulum Ca^{2+}-ATPase, stimulated Ca^{2+} influx and leukotriene synthesis without the production of inositol phosphates. Therefore, it appears that Ca^{2+} influx is quantitatively sufficient to account for the Tg-induced leukotriene production in RBL-2H3 cells. However, phosphoinositide metabolism and other signal transduction pathways might be involved during the receptor-stimulated leukotriene synthesis. We are currently examining the signal transduction pathways involved in leukotriene synthesis by the IgE receptor-activated cells.

In RBL-2H3 cells, 300 nM ionomycin induced the translocation of 80% of the soluble 5LO to membranes. On the other hand, 30 nM Tg, which produced the same maximal amount of LTC_4, induced the translocation of 60% of the soluble 5LO to membranes. Both ionomycin- and Tg-treated cells excluded trypan blue and did not release cytosolic lactate dehydrogenase, indicating that membrane binding of 5LO is not due to cytotoxicity (data not shown). We do not yet know what causes the discrepancy in the effects of the two agonists on 5LO translocation and LTC_4 production. One possibility is that ionomycin, which intercalates in membrane lipids (Brasseur et al., 1983), may change membrane properties and induce nonspecific binding of the enzyme.

In summary, using three stimuli that elevate $[Ca^{2+}]_i$ via different mechanisms, we provided evidence that a sustained influx of Ca^{2+} across the plasma membrane is essential for leukotriene synthesis. Consistent with the proposed model (Rouzer & Kargman, 1988), our data indicate that an elevation of $[Ca^{2+}]_i$ in RBL-2H3 cells would directly or indirectly cause cytosolic 5LO to bind to membranes. The activated, membrane-associated enzyme would then metabolize substrate released by the membrane phospholipases, which would also be activated by the increased $[Ca^{2+}]_i$ level. 5-Lipoxygenase would then undergo suicide inactivation, resulting in dead enzyme remaining bound to the membranes.

Acknowledgments

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Registry No. 5LO, 80619-02-9; LTC_4, 72025-60-6; LTD_4, 73836-78-9; 1,4,5-IP_3, 88269-39-0; 1,3,4-IP_3, 98102-63-7; IP_4, 102850-29-3; Ca, 7440-70-2.

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Nonclassical Hydrophobic Effect in Membrane Binding Equilibria†

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ABSTRACT: The enthalpy of transfer of four different amphiphilic molecules from the aqueous phase to the lipid membrane was determined by titration calorimetry. The four molecules investigated were the potential-sensitive dye 2-(*p*-toluidinyl)naphthalene-6-sulfonate (TNS), the membrane conductivity inducing anion tetraphenylborate (TPB), the Ca^{2+} channel blocker amlodipine [Bäuerle, H. D., & Seelig, J. (1991) *Biochemistry 30*, 7203-7211], and the positively charged local anesthetic dibucaine. All four amphiphiles penetrate into the hydrophobic part of the membrane, and their binding constants, after correcting for electrostatic effects, range between 600 M^{-1} for dibucaine and 60 000 M^{-1} for tetraphenylborate. The corresponding changes in free energy were about −6 to −9 kcal/mol. Binding of the amphiphiles to membrane vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was accompanied by exothermic heats of reaction for all four molecules. For TNS, TPB, and amlodipine, the enthalpies of transfer were almost identical and corresponded to $\Delta H \approx -9$ kcal/mol, essentially accounting for the total free energy change. Thus, the binding of these charged amphiphiles to the hydrophobic membrane was driven by enthalpy. This is in contrast to the classical hydrophobic effect, where the transfer is considered to be entropy driven. For dibucaine, the enthalpy of transfer was smaller with $\Delta H \approx -2$ kcal/mol but was still about one-third of the total free energy change. All enthalpies of transfer exhibited a distinct temperature dependence with molar heat capacities ΔC_p of −30 to −100 cal mol^{-1} K^{-1} for the transfer from water to the membrane. These molar heat capacities are quite large and correspond to those obtained for the partitioning of small organic molecules between water and a pure organic phase. The nonclassical hydrophobic binding of the amphiphiles is explained by van der Waals interactions between the nonpolar residues of the solute and the hydrophobic core of the lipid bilayer.

The interaction of nonpolar molecules such as hydrophobic peptides or drugs with the lipid membrane is usually ascribed to the *hydrophobic effect*. The hydrophobic residues of these molecules tend to avoid contact with the aqueous environment and penetrate into the hydrophobic core of the membrane. It is also common thinking to consider the hydrophobic effect as an *entropic* phenomenon, i.e., the driving force for the association is the release of water molecules from the nonpolar surface of the solute [cf. Tanford (1980)]. The entropic interpretation of the hydrophobic effect is based on measurements of the water solubility of small organic molecules such as benzene or hexane as a function of temperature. It was found that the entropy of transfer, ΔS, of these compounds from the pure liquid state to water was large and negative (at room temperature), whereas the corresponding enthalpy, ΔH, was approximately zero or only slightly negative. The low solubility of nonpolar molecules in the aqueous phase is thus caused by a negative excess entropy. This experimental finding has led to a specific molecular picture for the hydrophobic effect: the insertion of the nonpolar molecule in water is assumed to produce an ordering of the water molecules around the solute such that the perturbation of the hydrogen-bonding pattern of water is minimized. The nonpolar molecule and its hydration sphere constitute an "iceberg" swimming in the aqueous phase. The ordering of the water molecules by the nonpolar solvent explains the large negative entropy of transfer. The same picture also explains the second characteristic feature of the hydrophobic effect, namely, its strong dependence on temperature. For nonpolar substances, the heat capacity, ΔC_p,

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