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Effect of Linoleic Acid Hydroperoxide on Endothelial Cell Calcium Homeostasis and Phospholipid Hydrolysis

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The relationship between intracellular free calcium ion concentrations ($[Ca^{2+}]_i$) and release of arachidonic acid from membrane phospholipids following peroxidation was examined in rabbit aortic endothelial cells treated with linoleic acid hydroperoxide (LOOH). LOOH (0.1–0.4) μmol/10^6 cells) caused a rapid and dose-dependent transient increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} that remained elevated over baseline for 15 to 30 s. In the absence of extracellular Ca^{2+}, LOOH also evoked a transient increase in $[Ca^{2+}]_i$ of lesser magnitude which immediately returned to basal (or below basal) levels. In this regard, the rise in intracellular Ca^{2+} after LOOH or vasopressin (AVP) treatments involved, at least in part, related intracellular pools that in each case was followed by influx of extracellular Ca^{2+}. The intracellular membrane sources of Ca^{2+} remain unidentified as common sources known to be affected by vasopressin were not directly involved. Most notably, the LOOH evoked rise in $[Ca^{2+}]_i$ was not associated with release of IP_3, suggesting that the source of intracellular Ca^{2+} is not IP_3-sensitive pools. However, pretreatment with LOOH strongly inhibited the rise in $[Ca^{2+}]_i$ upon subsequent addition of AVP or LOOH and the extent of such inhibition was dependent on the availability of free intracellular Ca^{2+} and presence of extracellular Ca^{2+}. These findings suggest that reuptake of Ca^{2+} into intracellular membrane pools is reduced in the presence of LOOH and/or the availability of Ca^{2+} from agonist-sensitive sites is inhibited by LOOH. An increase in free 20:4 levels was found after LOOH treatment that was only partly prevented using intracellular Ca^{2+} chelators which maintained $[Ca^{2+}]_i$ at basal levels after LOOH treatment. These findings suggest that LOOH induction of phospholipid hydrolysis proceeds following small transients in $[Ca^{2+}]_i$ that are considerably less than that evoked by agents such as AVP, approximating basal Ca^{2+} concentrations. Inhibition of LOOH-induced lipid peroxidation by vitamin E also prevented the rise in $[Ca^{2+}]_i$ and 20:4 release indicating that phospholipid hydrolysis is dependent, at least in part, on membrane lipid peroxidation. Inhibition of protein kinase C (PKC) completely blocked LOOH-induced release of 20:4 but had little effect on the LOOH-induced rise in $[Ca^{2+}]_i$, suggesting an indirect relationship between LOOH-induced membrane Ca^{2+} signalling events, with intervention via PKC-mediated induction of phospholipid hydrolysis. A rapid and progressive translocation of PKC to the membrane fraction was evident after LOOH addition over the time course corresponding to the maximal release of 20:4 which was also inhibited by vitamin E. The findings are discussed in terms of possible mechanisms underlying the stimulation of phospholipase(s), purportedly phospholipase A_2 (PLA_2) activity, by peroxidation of cell membrane phospholipids and enhancement of protein kinase activity.

Endothelial cell oxidant injury is thought to be involved in a number of pathological conditions (1–3). Exposure of endothelial cells to peroxides represents a common form of oxidative stress that causes lipid peroxidation (4), thiol depletion, and membrane disruption (5), where considerable attention has been directed to effects on intracellular free calcium ion concentrations ($[Ca^{2+}]_i$) as an early event in cell injury (6). It has been suggested that during early atherosclerosis, oxidatively modified LDL,[2] which contains various lipid peroxidation products, may be responsible for oxidative injury to the endothelium which is manifested, in part, by damage to cell membrane phospholipids (7). As such,

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[2] Abbreviations used: LOOH, linoleic acid hydroperoxide; REC, rabbit aortic endothelial cells; PLA_2, phospholipase A_2; PKC, protein kinase C; BAPTA, 1,2bis[2-aminophenoxy]-ethane-*N*,*N*,*N*,*N*-tetraacetic acid; BIM, bisindolylmaleimide; LDL, low density lipoprotein; PBS, phosphate-buffered saline; AVP, arginine-vasopressin.

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delivery of lipid peroxides by oxidized LDL may be a means for injury to the endothelium and underlying tissues that is manifested either by cell death or by sublethal events related to aberrant signaling.

A common product of cell lipid peroxidation is linoleic acid hydroperoxide (LOOH). This peroxide can decompose to form alkoxy- and peroxy-free radicals as well as aldehydes that are common products in the propagation of lipid peroxidation (8). LOOH reacts and oxidizes numerous cell components, particularly sulfhydryl rich proteins, and these oxidation reactions can lead to cell injury and death (5). Previous studies have shown that LOOH, and other inorganic and organic peroxides, can induce peroxidation of endothelial cell phospholipids which is associated with an enhanced hydrolysis of membrane phospholipids (9). Damage to cell phospholipids is likely a critical factor in peroxide-induced membrane injury, which is often manifested by leakage of ions and proteins (10). The increase in $[Ca^{2+}]_i$ is thought to be due to damaged membrane barrier functions or dysfunction of channels responsible for the transport and homeostatic regulation of Ca^{2+} and other ions (11). Elevations in $[Ca^{2+}]_i$ have been widely reported in the case of peroxide-induced cell phospholipid hydrolysis. Peroxidized phospholipids produce membrane structural perturbations and recent studies indicate that increases in intracellular Ca^{2+} levels and binding of Ca^{2+} to peroxidized membranes are effects consistent with enhanced phospholipase activity (12). In many of these studies, arachidonic acid (20:4) release was the most commonly used method for monitoring hydrolytic activity. The elevations in free 20:4 levels following treatments with peroxides have been attributed to stimulated phospholipase A_2 (PLA_2) activity through peroxidation of phospholipids and antioxidants, such as vitamin E, prevent the 20:4 release (13). A rationale for monitoring 20:4 release is based on its essential role as a precursor for eicosanoid synthesis, its susceptibility to peroxidation along with other polyunsaturated fatty acids and enhanced PLA_2 activity directed towards 20:4 when membrane phospholipids become peroxidized (12, 14, 15).

To further examine the biochemical mechanisms involved in oxidant-induced hydrolysis of cell phospholipids we focused our attention on the relationship between LOOH-induced changes in $[Ca^{2+}]_i$ and 20:4 release in endothelial cells. Use of LOOH to study peroxide-mediated phospholipid hydrolysis has been very limited in so far as cell culture studies are concerned. Previous studies have largely used agents such as H_2O_2 or tert-butyl-hydroperoxide, neither of which partition into cell membranes or display the reactivity of LOOH. Accordingly, both H_2O_2 and tert-butyl-hydroperoxide interact with numerous cell compartments and can oxidize cell constituents directly in addition to inducing lipid peroxidation. LOOH on the other hand is most representative of peroxidation products arising in cell membranes and offers a means for determining directly the hydrolytic response to peroxidation occurring within the confines of the membrane.

The following questions were addressed in this study: (1) To what extent is the 20:4 release from cell phospholipids calcium dependent, and specifically, are changes in $[Ca^{2+}]_i$ required for stimulated hydrolytic activity? (2) Does LOOH-induced phospholipid hydrolysis require lipid peroxidation and can membrane antioxidants inhibit the LOOH-induced 20:4 release? (3) Is the lipid peroxide-induced 20:4 release dependent on PKC activation, implicating signaling events mediating phospholipid hydrolysis?

MATERIALS AND METHODS

Chemicals and reagents. LOOH was prepared from linoleic acid (Nu-Chek Prep, Elysian, MN) as described previously (16) and then dissolved in absolute ethanol (USI Chemicals, Co., Tuscola, IL) and kept at −20°C under argon. LOOH purity was checked by HPLC. The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): $MnCl_2$, $CaCl_2$, EDTA, EGTA, bradykinin, digitonin, monensin, thapsigargin, Hepes buffer, and arginine [arg^8] vasopressin. A23187, bromo-A23187, Fluo3-AM, Fluo-3 and 1,2 bis[2-aminophenoxy]-ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA-AM) were obtained from Molecular Probes, Inc. (Eugene, OR). Vitamin E (*dl*-α-tocopherol) was obtained from United States Biochemical Corp. (Cleveland, OH). Bisindolylmaleimide (BIM) was purchased from Calbiochem Corp. (La Jolla, CA). Tritiated arachidonic acid, [5,6,8,9,11,12,14,15-^{3}H]20:4 (207 μCi/μmol) and [^{3}H]-inositol (102 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). All organic solvents were HPLC grade and purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Cell culture and conditions. Rabbit aortic endothelial cells (REC) were obtained from New Zealand albino rabbits and used between passages 9 and 13. Cells were grown to confluency on polymethacrylate fluorometer cuvettes (Sigma, St Louis, MO) and maintained in 80/20 DMEM/M199 with 15% fetal bovine serum (Gibco, NY), endothelial cell growth factor (5 μg/ml), and gentamicin (50 μg/ml)—this is otherwise referred to as complete medium. Conditioned medium was added at the time of medium change at a 1:4 ratio with fresh medium. The cells were characterized as endothelial on the basis of morphology and by measurement of angiotensin converting enzyme activity using commercially available radioassay kits. Stock cells were passaged using a one to three split ratio with mechanical disruption of the monolayer and cultures maintained with weekly media changes.

Distribution of cells from stock confluent cultures to various dishes or cuvettes used in the experiments described was accomplished by treatment with PBS containing 0.1% trypsin and 0.02% EDTA for 5 min at 37°C. For determinations of $[Ca^{2+}]_i$ the cell suspension was centrifuged and resuspended in 10 ml fresh complete medium ($\sim 3 \times 10^6$ cells/10 ml). This suspension was distributed as 1.0-ml aliquots into sterilized polymethacrylate fluorescence cuvettes which were placed into 100-mm Petri dishes (at a 15 to 30° inclination) and maintained in cell culture incubator until confluent. Confluent monolayers were obtained by 5 days on the inner surface of the cuvette.

Selected cultures were supplemented with vitamin E (*dl*-α-tocopherol) by incubating the cells with 10 μM vitamin E for 24 h. The cultures were then washed three times with complete medium before further treatment of the cells. The content of vitamin E was tripled under these conditions and verified by HPLC analysis of cell extracts as described previously (17).

Cell loading with fluorescent probe. The procedure of Tsien and Pozzan (18) was used, with minor modifications, to load REC with the acetomethoxy ester of the fluorescent probe fluo-3-AM. Five microliters of 1 mM fluo-3-AM in DMSO was added directly to cuvettes with 1 ml of complete media (buffered with 10 mM Hepes) resulting in a final dye concentration of 5 μM. After thorough mixing, the cuvettes were returned to Petri dishes (again supported at a 15 to 30° inclination) and left for 30 min to 1 h at 21°C. To test for cell uptake and completeness of ester hydrolysis, the fluorescence intensity signal was monitored over intervals up to 5 h. This served to establish the optimum cell loading time. The fluorescence signal was found to reach a maximum that stabilized in about 45 to 60 min after addition of the probe. Unincorporated probe was removed by two to three washings, first with fresh media and then with the Ca^{2+}-free Hepes buffer (10 to 20 mM). Additions of agents such as intracellular Ca^{2+} chelators or enzyme inhibitors was performed similarly to the addition of LOOH or fluo-3-AM. Thus, compounds were dissolved in either ethanol or PBS and added to the culture/treatment medium in 5- to 10-μl aliquots and usually added 10 min prior to LOOH. Specific details about treatment protocol are provided in the figure legends.

Measurement of cytosolic calcium concentration. The fluorescence signal was monitored using a Hitachi F-2000 fluorometer. A 0.1- to 2-s response time and a 150 W xenon lamp at 400 V was used to deliver a 506-nm excitation beam through a grating monochrometer to the sample compartment containing cells loaded with the fluorescent probe. The fluorescence emission signal at 526 nm was collected at a right angle from the excitation beam. The excitation and emission bandpass were set to 10 nm. LOOH and/or other agonist were added to the cuvettes while monitoring the fluorescence. After each experiment a calibration of the signal was performed. This fluorescence signal was converted to Ca^{2+} concentration using the procedure described below.

Calibration of the fluorescence signal was done according to the method of Tsien and Pozzan (18). Briefly this method involves determining the dye fluorescence at two points within the linear region of the calibration curve: F_{min} (the fluorescence of the unchelated dye) and F_{max} (the fluorescence of the fully chelated dye). Calibration was performed for each experiment, since the number of cells used and the amount of dye taken up by the cells varied between experiments. Determination of F_{Mn} was accomplished by saturating the dye with Mn (2 mM) after addition of the ionophores A23187 or ionomycin. The signal intensity observed, F_{Mn}, was then used to calculate F_{max} using the following equation:

$$F_{max} = 5 \times (F_{Mn} - F_{bkg}) + F_{bkg},$$

where F_{bkg} is the autofluorescence of the cells without dye which was measured in each instance before addition of the probe, in the absence and presence of LOOH. This background intensity, F_{bkg}, was also used to determine F_{min}, the minimum dye fluorescence intensity:

$$F_{min} = (F_{max} - F_{bkg})/40 + F_{bkg}.$$

Finally, the fluorescence intensity signal, F, was converted to cytosolic $[Ca^{2+}]_i$ by using the following equation:

$$[Ca^{2+}]_i = K_d\,(F - F_{min})/(F_{max} - F),$$

where K_d (400 nM) is the known dissociation constant of the fluo-3 dye/Ca^{2+} complex.

Measurement of IP_3 production in REC. REC were grown to confluence in the presence of 2.5 μCi/well (5×10^5 cells/well) [^{3}H]Inositol in M199 media + 10% fetal bovine serum, in 35-mm Corning 6-well tissue culture dishes. The monolayers were washed three times with a 2.5-ml solution of NaCl, 0.9% + 1 mM Ca^{2+} + 10 mM Hepes buffer + 0.1% albumin. Five milliliters of an albumin free buffer containing 10 mM LiCl was added to each well and after 15 min, 0.5% ethanol containing LOOH was added to specific wells to yield a final concentration of 20 μmol LOOH/10^5 REC. AVP (15 nM) was added to some wells as a positive control. The reaction was terminated at designated time points by aspiration of the medium and immediate addition of 5% PCA to the monolayer. After centrifugation of disrupted cells, the inositol phosphates were analyzed by neutralization of the supernatant using 2 M KOH + 2.4 M $KHCO_3$. The samples were again centrifuged at 1000 rpm for 10 min and the supernatants added to an equal volume of 25 mM tetrabutyl ammonium hydrogen sulfate 100 nM KH_2PO_4 (pH 5.5):acetonitrile [99:1, v/v]. Aliquots of this solution were analyzed for inositol phosphates using ion pair HPLC (19) and measurement of radioactivity in 1 ml eluent fractions by liquid scintillation spectrometry.

Measurement of fatty acid release from REC phospholipids. Tritiated arachidonic acid, [^{3}H]20:4, was added immediately before use to complete media (37°C) at a final level of 1 μCi/ml. Confluent REC were incubated in this radiolabeled medium for 24 h at 37°C prior to treatment under the conditions specified below. After labeling and pretreatment of cells, the media was removed and the cells washed once with media and then with 10 mM Hepes-buffered normal saline containing 2 mM EGTA. The cells were then washed with Hepes-buffered saline and treated with various concentrations of LOOH and/or other agonist which were added in ethanol or PBS as detailed in figure legends. The above conditions were used for treatments in Ca^{2+}-free medium, while treatments in Hepes-buffered saline containing 1 mM Ca^{2+} was used for treatments in Ca^{2+} sufficient medium. The cell monolayers were incubated in the presence of LOOH for 15 min under conditions previously described (20) to produce minimal toxicity and wherein rates of hydrolysis exceed rates of fatty acid reincorporation into phospholipids (21). Thereafter, the cultures were suspended in their media by trypsinization and 1-ml aliquot of the cell suspension was transferred to borosilicate test tubes containing 5 ml of chloroform-methanol (2:1) with 0.001% BHT. After mixing for 1 min the samples were centrifuged at 3000 rpm for 10 min, the organic layer was collected and saved while the aqueous layer was reextracted with 3 ml chloroform. The organic phases were pooled, dried under argon, and the lipid residues stored under argon at −20°C. The aqueous phase was collected and an aliquot measured for residual radioactivity which was usually less than 1% of the total counts added to the cell cultures.

Free fatty acids, representing those present in cells or released into the medium, were isolated from total lipids using aminopropyl "Bond Elute" solid phase extraction columns (Analytichem International, Harbor City, CA). The method described in detail by Kaluzny *et al.* (22) was modified slightly to isolate only the fatty acids from the total lipids. The dried lipids were dissolved in 1 ml chloroform while the columns were washed twice under vacuum with 2 ml hexane. The chloroform solutions were applied to the columns under vacuum and the solvent was collected until the columns were dry. The columns were then eluted with 4 ml of chloroform-isopropanol (2:1). This first eluate (I) containing neutral lipids (mono-, di-, and triglycerides, as well as cholesterol and cholesteryl esters) was collected, evaporated and an aliquot was taken for measurement of radioactivity. The columns were then eluted with 4 ml of 2% acetic acid in diethyl ether. This eluate (II) contained only free fatty acids which was dried and its radioactivity content was measured by transferring an aliquot in ethyl acetate to liquid scintillation cocktail which was counted in a liquid scintillation counter. Both free 20:4 and derived hydroperoxides and alcohols are eluted in this fraction. Finally the columns were eluted with 4 ml of methanol, which recovered all phospholipids. This eluate (III) was also evaporated and the radioactivity was measured in an aliquot as described above. The percentage hydrolysis was based on the level of free fatty acids (spe-

cifically free radiolabeled 20:4) relative to control cultures and was calculated as follows:

$$\% \text{ Hydrolysis} = \text{cpm in fraction II (fatty acids)/total cell cpm.}$$

The radioactivity in fraction I remained constant for each treatment, indicating that hydrolytic degradation of neutral lipids did not take place. Moreover, the hydrolytic activity measured was a result of fatty acid release from phospholipids and the content of radiolabel associated with free fatty acids in the neutral lipid fraction was consistently low (usually around 2% of the total radioactivity in cells). This procedure for lipid labeling results in more than 90% incorporation of [^{3}H]20:4 into phospholipids (17, 21).

Determination of PKC activity. PKC activity in REC was measured by means of H1 histone phosphorylation using a multiwell plate assay (23). Confluent cultures were kept in serum-free medium overnight and then treated with LOOH as described above in 100-mm petri dishes (2×10^6 cells/dish). The treated cells were washed with phosphate-buffered saline and homogenized in buffer consisting of 20 mM Tris–HCl, pH 7.4/1 mM EDTA/0.1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride. The soluble and membrane fractions were separated by centrifugation at 13,000*g* for 10 min. The membrane fraction was solubilized with 1% Nonidet P-40 detergent and both the soluble and detergent-solubilized membrane fractions were subjected to DEAE-cellulose chromatography as described previously (24). PKC activity was determined in the fraction of protein eluted from the DEAE-cellulose column with 0.1 M NaCl. The multiwell assay for PKC activity contained in each sample 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.33 mM $CaCl_2$, 0.1 mM ATP ([^{32}P]ATP at 120 cpm/pmol), 0.1 mg/ml histone H1, 0.04 μM leupeptin, and 25 μl of the PKC sample in a total volume of 125 μl. Samples were incubated at 30°C for 5 min in the wells fitted with Durapore membrane filtration discs. Histone H1 was precipitated with 25% TCA and the filters were punched into scintillation vials for measurement of radioactivity. PKC activity is expressed as units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H1 per min at 30°C.

All results are expressed as mean and standard error calculated from the results of three to five independent experiments, unless otherwise specified. Determination of statistical significance was calculated using the two-tailed Students *t* test, comparing a specific treatment condition to the corresponding experimental controls.

RESULTS

Characterization of Fluo-3 Assay for $[Ca^{2+}]_i$

A linear relationship was found between the fluorescence intensity of the fluo-3/Ca^{2+} complex vs Ca^{2+} concentration, encompassing the intracellular Ca^{2+} concentration range generally encountered in this study (20–120 nM). Above this range the relationship was curvilinear. Although it has been reported that the dye's fluorescence is quenched under acidic conditions, little difference in intensity was observed for the pH range (6.8–7.4) used in these experiments.

Effects of LOOH on $[Ca^{2+}]_i$

The concentrations of LOOH used for these experiments were found to be nontoxic according to methods previously described (20). At these concentrations the surviving fraction of cells was >80% after 15-min treatments in buffered saline, as determined by washing cultures immediately after treatment with complete medium and measuring the plating efficiency 24 h after treatment. Following LOOH treatment, an initial increase in $[Ca^{2+}]_i$ was observed (reaching a maximum level by 5 s), followed by a rapid decrease to approximately initial baseline levels by about 10 s (Fig. 1a). In the absence of fluo-3 no change in fluorescence intensity was found before or after addition of LOOH to cells. When cells were treated in Ca^{2+} containing medium (Fig. 1a), this decrease appeared as a nonlinear multicomponent decay following the initial rise. The nonlinear decay is referred to as the "second component" of the peak produced in the Ca^{2+} signal, which is interpreted as a sustained elevation of $[Ca^{2+}]_i$ associated with the influx of extracellular Ca^{2+}. This interpretation is based on the observation that when cells were treated in Ca^{2+}-free medium (Fig. 1b), the $[Ca^{2+}]_i$ returned to levels below the initial baseline by 5 s, followed by a slow rise back to the basal Ca^{2+} level. After a few minutes, the fluorescence signal returned to baseline levels in both Ca^{2+}-free and Ca^{2+}-containing medium. Moreover, the maximum increase in $[Ca^{2+}]_i$ was directly related to the LOOH treatment concentrations as shown in Fig. 2. Although considerable variability in the intensity of the fluorescence signal was found for different batches of cells, the quality and duration of the signals were highly reproducible. Addition of similar amounts of linoleic acid alcohol (LOH), derived by reduction of LOOH with $NaBH_4$ as described previously (16), produced no significant increases in $[Ca^{2+}]_i$ (Table I).

We previously reported that treatment of REC with LOOH under the conditions described above resulted in peroxidation and enhanced turnover of phospholipids (21). These effects were inhibited when vitamin E-supplemented cells were challenged with LOOH. To test whether the elevations in $[Ca^{2+}]_i$ are dependent on LOOH-induced lipid peroxidation, vitamin E-supplemented cells were treated with LOOH. A substantial attenuation of the $[Ca^{2+}]_i$ peak was found after LOOH addition to vitamin E-supplemented cells as compared to cells not pretreated with the antioxidant (Table I). In vitamin E-supplemented cultures a significant reduction in endothelial cell lipid peroxidation takes place after LOOH addition (20), indicating that inhibition of lipid peroxidation is associated with a reduction in the LOOH-induced rise in $[Ca^{2+}]_i$. As shown in Fig. 3a, monensin did not affect the LOOH-induced Ca^{2+} release nor did LOOH pretreatment inhibit the monensin-induced rise in $[Ca^{2+}]_i$ (Fig. 3b). This suggests that LOOH does not act upon Ca^{2+} stores released via stimulation of Na^+/H^+ exchange (25). The collapse of transmembrane pH gradients with monensin and release of Ca^{2+} may be attributed to lysosomal (25) or mitochondrial stores (26). As reported previously (16), LOOH has been shown to cause depolarization primarily of the plasma membrane with minimal, if any, effects on

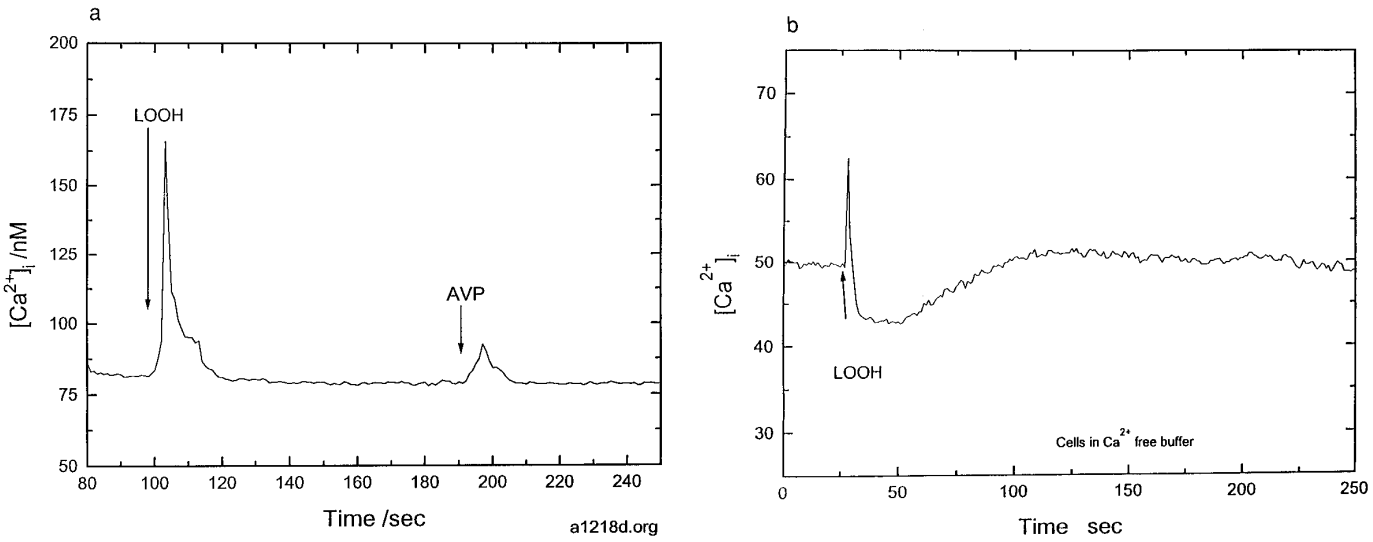

FIG. 1. The cytosolic Ca^{2+} concentration following LOOH treatment of REC in 10 mM Hepes/0.9% NaCl (a) with extracellular Ca^{2+} (2 mM) and (b) in Ca^{2+}-free buffer. Confluent monolayers composed of 1×10^5 cells were loaded with fluo-3, AM by pretreating cultures in 1 ml complete media with the fluorescent dye (5 μM) for 30 min, followed by rinsing twice with buffer. The basal fluorescence signal was monitored prior to addition of LOOH at a treatment concentration of 20 nmol/10^5 cells. The times of addition for LOOH and AVP are indicated by arrows.

mitochondrion under treatment conditions similar to those used in the present study.

Pretreatment with AVP inhibited the initial sharp rise in $[Ca^{2+}]_i$ following LOOH addition (Fig. 4). Similarly, LOOH pretreatment inhibited the AVP-induced increase in $[Ca^{2+}]_i$ (Fig. 1a). Notably, pretreatment of cells with AVP attenuated the increase in $[Ca^{2+}]_i$ upon LOOH addition; however, if sufficient time was allowed before LOOH was added, the refractory behavior of the cells resolved and much greater amounts of Ca^{2+} could be discharged by addition of LOOH (Fig. 4). Bradykinin (BK) treatment of endothelial cells has been previously shown to release the low K_m Ca^{2+}-stores (27) and pretreatments with this agonist produced the same inhibitory effect on the LOOH-induced $[Ca^{2+}]_i$ rise as found with AVP (Data not shown). These results indicate that the source(s) of Ca^{2+} affected by LOOH and AVP (or BK) appear to overlap in so far as Ca^{2+}-mediated signalling events, which are detailed further below, are concerned.

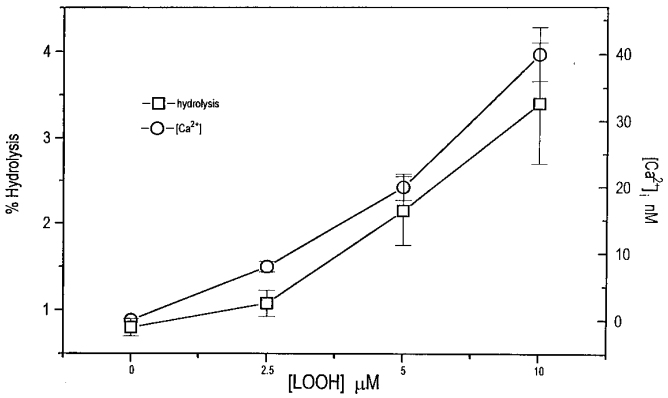

FIG. 2. LOOH-induced hydrolysis of REC phospholipids, expressed as percentage labeled free fatty acids of total labeled cell lipids after addition of the indicated concentrations of LOOH. A 10 μM treatment dose as shown in the figure corresponds to the addition of 20 nmol LOOH/10^5 cells. Treatments with LOOH were made at room temperature for 15 min in 10 mM Hepes/0.9% NaCl buffer. LOOH was added in ethanol vehicle (indicated by arrow) at a final volume less than 0.5% of the media volume. Treatment conditions were otherwise as described in the legend of Fig. 1.

Treatment of cells with thapsigargin is reported to increase $[Ca^{2+}]_i$ derived from IP_3-sensitive, ATP-dependent stores such as those found in the endoplasmic reticulum (28), largely by inhibiting Ca^{2+} reuptake. A marked increase in $[Ca^{2+}]_i$ was produced upon thapsigargin treatment of REC and subsequent addition of LOOH also stimulated a rise in $[Ca^{2+}]_i$. This effect was seen in either Ca^{2+}-free medium (Fig. 5) or Ca^{2+}-containing medium (data not shown) and indicates that thapsigargin does not act upon the same Ca^{2+} pools as LOOH.

To evaluate the possible role of IP_3-mediated release of Ca^{2+}, measurements of inositol phosphate levels were performed by HPLC analysis of REC extracts after LOOH addition. Addition of LOOH at 20 nmol/10^5 cells produced no measurable peaks corresponding to any of the known inositol phosphates while treatment with AVP caused a marked release of I[1,3,4]P_3 and I[1,4,5]P_3, as well as I[1,3,4,5]P_4. IP_3 and IP_4 levels

TABLE I

Treatment (In Hepes buffer with 2 mM Ca^{2+})	$\Delta_1[Ca^{2+}]_i$ (nM) (±5%)[a]	$\Delta_2[Ca^{2+}]_i$ (nM) (±5%)[b]	% Hydrolysis (±10%)
Control	0	5	1.2
Control in Ca^{2+} free buffer	−40	NA	1.0
Control[c] + BAPTA (100 μM)	−45	NA	0.4
LOH (10 μM)	10	0	0.8
LOOH (10 μM)	45	20	3.4
AVP (10 nM)	145	50	3.1
LOOH (10 μM) in Ca^{2+} free buffer	10	−5	1.9
LOOH (10 μM)[c] + BAPTA	−45	NA	2.0
AVP (10 nM) in Ca^{2+} free buffer	45	5	1.8
AVP (10 nM)[c] + BAPTA	10	5	1.2
LOOH + VIT E (α-tocopherol, 10 μM)	20	0	0.95

Note. NA, Measurement was not applicable or possible under these conditions.

[a] $\Delta_1[Ca^{2+}]_i$ represents the maximum increase (or decrease) in the initial peak representing the $[Ca^{2+}]_i$ (measured in terms of the nM concentration) after addition of the agents indicated above. This is done by subtracting the calculated basal $[Ca^{2+}]_i$ from the calculated $[Ca^{2+}]_i$ at the peak of the response.

[b] $\Delta_2[Ca^{2+}]_i$ represents the maximum rise (or decrease) over the basal $[Ca^{2+}]_i$ (measured in terms of the nM concentration) for the secondary (or prolonged) component of the calcium signal after addition of the agents indicated above. This is determined by calculating the maximum $[Ca^{2+}]_i$ at the shoulder immediately after the peak response and subtracting the calculated basal $[Ca^{2+}]_i$.

[c] Measurements made in calcium-free medium.

three- to fourfold greater than background were measured at a 10- to 20-s interval after addition. As I[1,3,4,5]P_4 is a relatively stable product of I[1,3,4,5]P_3 and I[1,4,5]P_3 under the analytical conditions employed, the absence of I[1,3,4,5]P_4 after LOOH treatment further suggests that no IP_3 production took place at time intervals between LOOH addition and inositol phosphate measurement. These findings strongly implicate an IP_3-independent mechanism for Ca^{2+} release.

Effects of LOOH on REC Phospholipid Hydrolysis

Using treatment procedures similar to those described above, a direct linear relationship was found between the LOOH treatment dose, the corresponding elevations in $[Ca^{2+}]_i$ and the increase in free fatty acid levels measured 15 min after LOOH addition (Fig. 2). Since over 90% of the ^{3}H-20:4 added to the cells was found to be incorporated into the sn-2 position of phospholipids, the rise in free 20:4 levels is likely due to

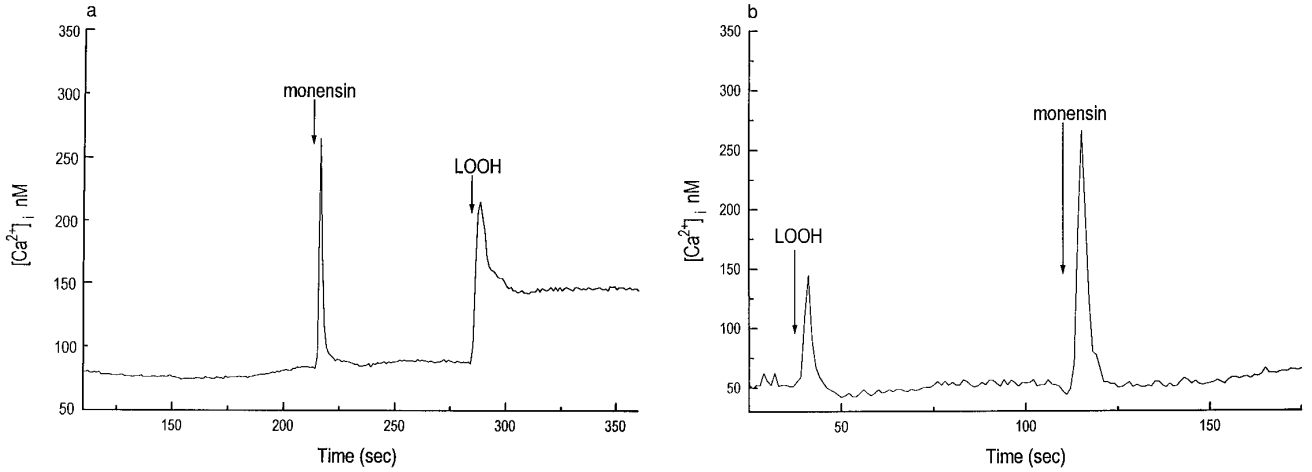

FIG. 3. $[Ca^{2+}]_i$ of REC following treatments with: (a) monensin (1 μM) followed by LOOH (20 nmol/10^5 cells); (b) LOOH followed by monensin (1 μM). LOOH and monensin were added in ethanol vehicle where the final ethanol content was less than 0.5% (v/v) of the 10 mM Hepes/0.9% NaCl, Ca^{2+}-free buffer. Cells were preloaded with fluo-3, AM as described in the legend of Fig. 1. LOOH treatment did not inhibit the subsequent $[Ca^{2+}]_i$ rise due to monensin and vice versa.

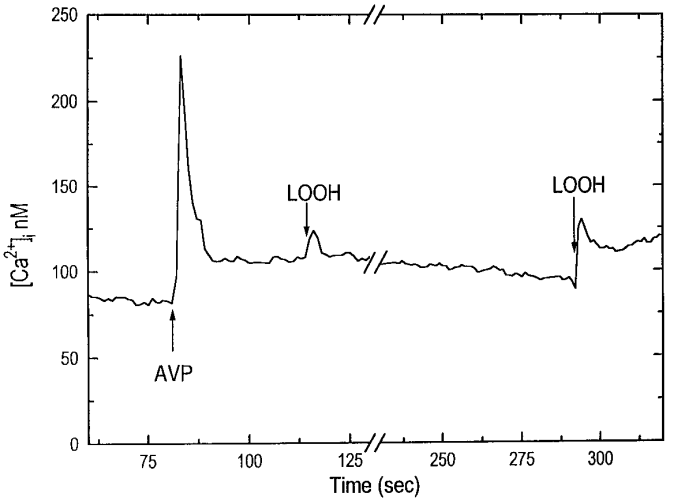

FIG. 4. $[Ca^{2+}]_i$ of REC following treatments with AVP (15 nM) followed by LOOH (20 nmol/10^5 cells). LOOH and AVP were added in ethanol vehicle or buffer, respectively, where the final ethanol content was less than 0.5% (v/v) of the 10 mM Hepes/0.9% NaCl buffer. Cells were preloaded with fluo-3, AM as described in the legend of Fig. 1. LOOH treatment inhibited the subsequent $[Ca^{2+}]_i$ rise upon AVP addition.

the activity of phospholipase(s). Treatments with LOH under the same conditions failed to produce a significant increase in hydrolysis (Table 1). In all cases 2–5% of the total 20:4 incorporated into cell phospholipids was recovered as free fatty acid from the cultures after 15 min treatment with LOOH. The low levels of free 20:4, especially in control cultures, reflect *net* release since albumin was not present in the medium to prevent reincorporation of fatty acids. Omission of albumin was essential since it also binds LOOH and prevents interaction (and oxidation) with cell phospholipids. Thus, the effects of LOOH are measured in terms of the net release of 20:4 under the influence of reactions that reincorporate it into phospholipids or other complex lipids. The extent of phospholipid hydrolysis was significantly greater for treatments in Ca^{2+} containing buffer as compared to calcium-free buffer (Table 1). Indeed, in Ca^{2+}-free medium the $[Ca^{2+}]_i$ were approximately 30 nM and no prolonged elevations in $[Ca^{2+}]_i$ were evident even after LOOH addition. The greater hydrolytic response found in Ca^{2+}-containing medium may be due to the prolonged secondary component associated with the rise in $[Ca^{2+}]_i$. Thus, in the absence extracellular Ca^{2+}, increased levels of free fatty acids were measurable immediately after LOOH addition (i.e., within 1 min), while no further increases were found over the remaining 15 min of incubation. This indicates that the induced hydrolysis is most pronounced in Ca^{2+} containing medium, suggesting that influx of Ca^{2+} is required for maximal 20:4 release. Nevertheless, increased net release of 20:4 took place in Ca^{2+}-free medium but by 15 min the free 20:4 content was not significantly different from the basal levels. The extent of phospholipid hydrolysis after AVP addition was also greater in a Ca^{2+}-containing medium than in a Ca^{2+}-free medium (Table 1).

To further study the Ca^{2+}-dependency for LOOH-induced phospholipid hydrolysis, cells were pretreated with BAPTA-AM. BAPTA has been used to chelate free intracellular Ca^{2+} and prevent the rise in $[Ca^{2+}]_i$ after addition of various agonists (29). Treatment of REC in the presence of BAPTA lowered the $[Ca^{2+}]_i$ to levels well below ambient concentrations and buffered the effects of LOOH on $[Ca^{2+}]_i$, maintaining levels at approximately ambient concentrations (~70 nM). Additionally, BAPTA inhibited phospholipid hydrolysis by approximately 50% in both control and LOOH-treated cells (Fig. 6). Treatments with BAPTA at concentrations greater than that shown in Fig. 6 (i.e., 100 μM or greater) did not decrease hydrolysis further but were also cytotoxic. Thus, although LOOH-induced phospholipid hydrolysis occurs in conjunction with transient elevations in $[Ca^{2+}]_i$, at least part of the stimulated hydrolysis takes place independently of changes in $[Ca^{2+}]_i$, or at $[Ca^{2+}]_i$ substantially lower than those following AVP treatment. By contrast, AVP-induced phospholipid hydrolysis was entirely Ca^{2+}-dependent since addition of AVP to cells pretreated with BAPTA completely blocked the stimulated phospholipid hydrolysis along with preventing the rise in $[Ca^{2+}]_i$ (Fig. 6). Also indicated in Fig. 6 is the finding that pretreatment of cells for 24 h with the protein kinase C inhibitor, bisindolylmaleimide (BIM) (30), completely blocked

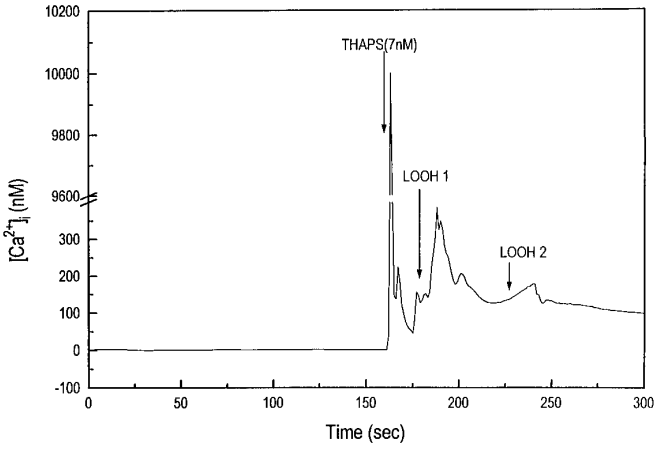

FIG. 5. $[Ca^{2+}]_i$ following LOOH treatment of REC in Ca^{2+}-free 10 mM Hepes/0.9% NaCl buffer with thapsigargin (7 nM) followed by LOOH (20 nmol/10^5 cells) added at 175 s and again at 225 s after starting of the analysis. LOOH and thapsigargin were added at the point shown in ethanol vehicle or buffer, respectively, where the final ethanol content was less than 0.5% (v/v) of the 10 mM Hepes/0.9% NaCl buffer. Cells were preloaded with fluo-3, AM as described in the legend of Fig. 1.

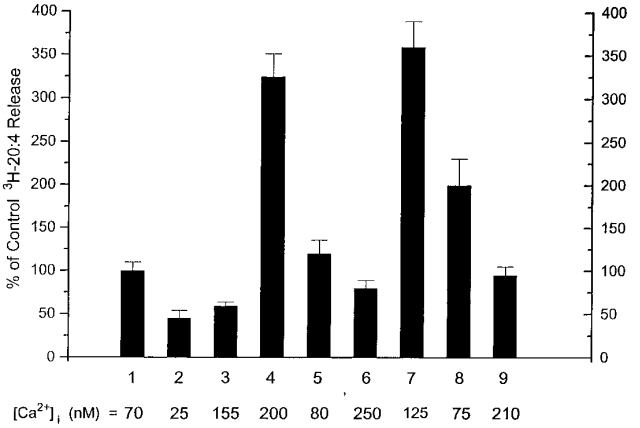

FIG. 6. LOOH (20 nmol/10^5 cells) and AVP (15 nM) induced hydrolysis of REC phospholipids in the absence and presence of BAPTA (50 μM) or bisindolymaleimide (BIM, 250 nM). LOOH and AVP were added to cultures at room temperature and hydrolysis measured after 15 min using 2.5×10^5 cells per assay. BAPTA or BIM were added to cultures in 10 mM Hepes/0.9% NaCl buffer 30 min (in Ca^{2+} free medium) and 24 h (treated in complete medium), respectively, prior to LOOH or AVP treatments. Numbers below each column are the key to treatment conditions, ie: 1, control; 2, control + BAPTA; 3, control + BIM; 4, AVP; 5, AVP + BAPTA; 6, AVP + BIM; 7, LOOH; 8, LOOH + BAPTA; 9, LOOH + BIM. Also shown along the bottom are the maximum $[Ca^{2+}]_i$ levels (nM) corresponding to each treatment.

LOOH-induced hydrolysis. Treatment with BIM alone caused marked elevations in $[Ca^{2+}]_i$, which were measured at the end of the pretreatment period, and increased further by combined treatments with LOOH and BIM. BIM also completely inhibited 20:4 release after AVP treatment of REC consistent with the reported PKC requirement for AVP-induced phospholipase activation (31) and completely inhibited LOOH-induced 20:4 release from phospholipids. The inhibitory effect of BIM was evident by 6 h of pretreatment (the earliest interval determined in terms of 20:4 release), but greater inhibition was found after a 24-h pretreatment interval (data not shown).

Induction of membrane lipid peroxidation appears to be a possible mechanism by which LOOH stimulated hydrolysis takes place. We noted in previous studies that addition of LOOH to REC produced a rapid peroxidation of PC and PI, accompanied by enhanced rates of hydrolysis and reacylation (21). LOOH-induced lipid peroxidation is known to take place via peroxidatic reactions that involve metal catalyzed decomposition of LOOH and its participation in subsequent propagation reactions (32). These peroxidation reactions are inhibited in vitamin E-supplemented cells as indicated by the data in Table 1, as is the rise in $[Ca^{2+}]_i$ and stimulated phospholipid hydrolysis. Thus, the induction of membrane phospholipid peroxidation represents a critical step in the LOOH-induced signalling process leading to phospholipid hydrolysis. The Ca^{2+} requirement for this process is evident from the reduction in 20:4 release in the presence of BAPTA, and the diminished extent of hydrolysis in Ca^{2+}-free medium.

The effect of LOOH on REC PKC activity is shown in Fig. 7. Addition of LOOH (10 μM) increased total PKC activity by 1 min after treatment. Most of the PKC activity was initially found in the cytosolic fraction (control), but rapidly became associated with the membrane fraction, indicating translocation. A progressive increase in membrane-associated PKC activity was found over the 30 min following LOOH addition. Vitamin E inhibited the activation and translocation induced by LOOH (Fig. 8); however, vitamin E alone had little effect on PKC activity. Indeed, there was a slight increase in PKC activity in vitamin E-supplemented cells and greater activity associated with cell membranes.

DISCUSSION

Previous studies involving treatment of cells with peroxides have largely used agents such as H_2O_2 or tert-butyl-hydroperoxide, neither of which partition selectively into cell membranes or display the reactivity of LOOH. H_2O_2 and tert-butyl-hydroperoxide interact with numerous cell compartments and can oxidize cell constituents directly in addition to inducing lipid peroxidation. LOOH on the other hand is more representa-

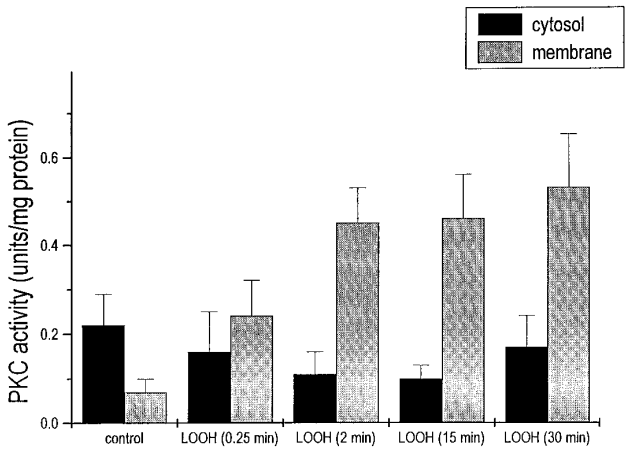

FIG. 7. PKC activity is shown at selected intervals following addition of 10 μM LOOH to REC. The conditions for REC treatment with LOOH and the PKC assay are described in the text. The values shown are the mean and standard error determined from three independent experiments where each treatment conditions was analyzed in duplicate. Measurements at 0.25 min represent the earliest time interval after LOOH addition where cell samples could be harvested reproducibly for analysis of cytosolic- and membrane-associated PKC activity. Limited measurements of PKC activity 60 min after LOOH addition were also performed where the activities associated with the cytosolic and membrane fractions returned to levels similar to that of controls (data not shown).

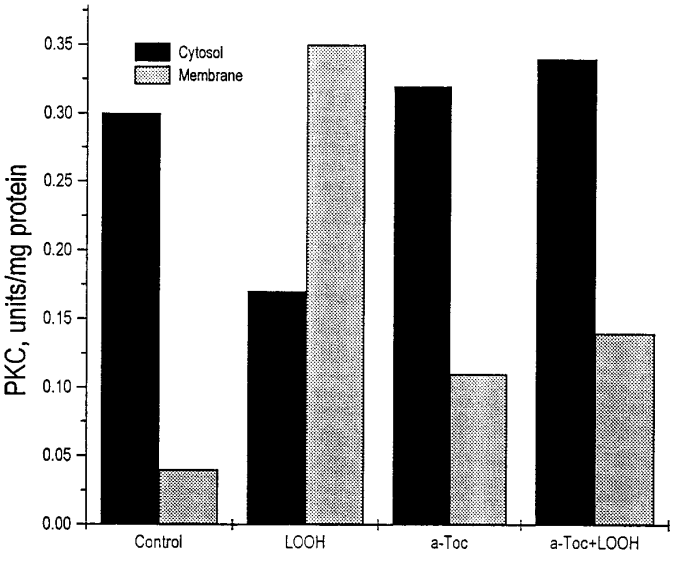

FIG. 8. The effects of 10 μM LOOH on REC PKC activities and the inhibitory action of 10 μM vitamin E are shown. Cells were supplemented for 24 h with 10 μM vitamin E as described under Materials and Methods and then incubated with LOOH for 1 min. Thereafter, all samples were immediately harvested and analyzed for PKC activities as described in the text. Values are the mean activities determined from separate cell cultures for each duplicate determination.

tive of peroxides formed in cell membranes and effectively induces cell lipid peroxidation within the confines of the membrane (21). Enhanced hydrolysis of membrane phospholipids following lipid peroxidation may be based on a combination of interrelated effects that include altered membrane structure and membrane Ca^{2+} interactions. Using unilamellar vesicles as model membranes, we reported that induction of lipid peroxidation or incorporation of phospholipid hydroperoxides into liposomes significantly increased the extent of Ca^{2+} binding and susceptibility to snake venom PLA_2 hydrolysis (12, 33). The increased PLA_2 activity appears to be due to the greater affinity of peroxidized membranes for Ca^{2+} which, in turn, facilitates enzyme binding and lowers the threshold concentration required for phospholipase activation (12). Enhanced kinetics are consistent with the reported preferential hydrolysis of oxidized phospholipids, as well as intact unsaturated phospholipids, following peroxidation of membranes (33). This may occur by activation of cellular PLA_2(s) in a manner analogous to extracellular PLA_2(s) acting on model membranes. Although lipolytic reactions other than catalyzed by PLA_2 could lead to the release of 20:4 from the *sn*-2 position of phospholipids, the Ca^{2+} dependence of this response and its analogous behavior to other well described agonists, such as AVP and bradykinin, reasonably accounts for a PLA_2-mediated process. Implication of PLA_2 is also based on the release of 20:4 from various phospholipids which were more than 90% labeled with 20:4 in the *sn*-2 position and the inhibitory effect of 30 μM mepacrine resulting in a 50% reduction in 20:4 release (Data not shown). Although higher concentrations of mepacrine were even more inhibitory, this effect may be confounded by significant toxicity since at doses exceeding 50 μM the surviving fraction of cells was less than 0.5 as determined by 24-h plating efficiency measurements. The differences found for LOOH vs AVP under conditions where $[Ca^{2+}]_i$ were buffered suggests that hydrolysis induction is only partly linked to changes in $[Ca^{2+}]_i$ in so far as LOOH is concerned, but the effects of AVP are fully linked to changes in $[Ca^{2+}]_i$.

Treatment of endothelial cells with LOOH caused a rapid increase in $[Ca^{2+}]_i$ from membrane stores that are distinct from the IP_3-sensitive pools released after AVP or BK treatment. However, LOOH affected the release of intracellular Ca^{2+} by AVP or BK but did not affect Ca^{2+} released by thapsigargin, suggesting an indirect interaction with IP_3-sensitive intracellular pools. Alternatively, Ca^{2+} may be released from other undefined sources that indirectly influence IP_3-mediated Ca^{2+} release. Inhibition by LOOH of Ca^{2+} reuptake into IP_3-sensitive stores could deplete them and make the cell less responsive to AVP. Oxidants such as tert-butyl-hydroperoxide decrease the peak response to agonists such as AVP and BK by inhibition of agonist-stimulated Ca^{2+} influx, explained, in part, by membrane depolarization (34). Inhibition of receptor activated Ca^{2+} release from intracellular membrane stores is consistent with inhibition of Ca^{2+} pump activity and depletion of intracellular, agonist sensitive, stores (34). Increased $[Ca^{2+}]_i$ derived from either extracellular or intracellular Ca^{2+} sources appears to sustain hydrolytic activity, as measured by the levels of free [^{3}H]-20:4 produced after 15 min of LOOH/agonist treatment. However, LOOH is able to stimulate phospholipid hydrolysis even when $[Ca^{2+}]_i$ are low in contrast to AVP. This may take place by lowering the requirement for Ca^{2+} during membrane binding and phospholipid hydrolysis by PLA_2 (12). It is also apparent that most of phospholipid hydrolysis induction is associated with an influx of extracellular Ca^{2+} after treatments with either AVP or LOOH. This is evident from experiments involving treatments in Ca^{2+}-free medium (Table 1); however, even under these conditions the significant increase in hydrolysis over control levels suggests that part of the hydrolysis induction is linked to release of Ca^{2+} from intracellular membrane-associated sites. LOOH vs AVP induction of phospholipid hydrolysis may also be contrasted, in part, on the basis of their effects on phospholipid turnover. Under similar treatment conditions we showed previously that LOOH not only induced phospholipid hydrolysis but acutely impaired reincorporation of fatty acids into phospholipids (21). Inhibition of 20:4 reincorporation was over-

come considerably after the rapid induction of phospholipid hydrolysis (particularly of phosphatidylcholine). Thus, the net hydrolysis seen with LOOH may differ from that induced by AVP by virtue of an LOOH-mediated inhibition of phospholipid repair in the wake of enhanced hydrolysis. Nevertheless, the complete inhibition of LOOH-induced phospholipid hydrolysis by BIM (discussed further below) argues against the inhibition of 20:4 reincorporation as being a prominent mechanism distinguishing the effects of LOOH and agonists such as AVP.

The relationship between 20:4 release and $[Ca^{2+}]_i$ after LOOH addition must take into account that activation of PLA_2 (or related phospholipid hydrolytic reactions) may not require marked elevations in $[Ca^{2+}]_i$. By contrast, the increases in $[Ca^{2+}]_i$ evoked by receptor-mediated signaling are required for PLA_2 activation and are "constrained" by the flux of Ca^{2+} in and out of specific pools. The receptor-mediated hydrolysis of phospholipids is proposed to be circumvented by membrane lipid peroxidation as distinct stores appear to be released that are not subject to the regulation described for receptor-mediated processes. The LOOH-induced rise in $[Ca^{2+}]_i$ is assumed to manifest through "signalling" events following lipid peroxidation that are distinct from those induced by receptor-mediated signaling since no I[1,3,4,5]P_4 could be measured. Thus, no IP_3 production took place at time intervals between LOOH addition and inositol phosphate measurement despite the rapid rise in $[Ca^{2+}]_i$. These findings strongly implicate an IP_3-independent mechanism for Ca^{2+} release. A direct action of LOOH on signalling events leading to phospholipid hydrolysis is not likely since virtually no stimulation was found with vitamin E-supplemented cells, suggesting that further lipid peroxidation is required.

The complete inhibition of AVP- or LOOH-induced 20:4 release by BIM is consistent with the reported PKC requirement for phospholipase activation (31). Our findings also implicate PKC activity in LOOH-mediated release of 20:4 from cell phospholipids. In this respect LOOH-induced enhancement of PKC activity may activate PLA_2 under conditions that do not require marked elevations in $[Ca^{2+}]_i$ as described previously (35), indicating that elevations in $[Ca^{2+}]_i$ may be involved in PLA_2 activation via receptor-mediated signaling but not required after membrane lipid peroxidation. PKC activation by oxidants and lipid peroxides has been described previously (36) and oxidant-mediated activation altered the Ca^{2+} requirement of the enzyme, converting it to a Ca^{2+} independent form (37). It was suggested that fatty acid hydroperoxides activate PKC by satisfying the requirement for phosphatidylserine and that this effect occurred in the presence or absence of Ca^{2+} (38).

The lowered Ca^{2+} requirement for phospholipid hydrolysis following peroxide exposures is analogous to the effects of oxidants on PKC, where conversion to Ca^{2+}-independent catalytic activity has been reported (37). The effects of LOOH are consistent with a oxidant-mediated activation of PKC. Previous studies have shown that the lipid (membrane) bound form of PKC becomes active at low (physiological) Ca^{2+} concentrations (39). Recently, Gopalakrishna *et al.* (37) described the transient activation of PKC following addition of H_2O_2, altering the kinetic behavior of the enzyme to an isoform that is Ca^{2+}/PL-independent. Addition of LOOH to REC appears to evoke a similar response, either by direct reaction with PKC or secondarily by formation of lipid peroxides and derived species. LOOH or derived peroxidation products may directly oxidize a key vicinal thiol in PKC which is reported to affect the reversible activation/inactivation of the enzyme (23); however, past studies revealed that very high concentrations of peroxide were required for this effect (38). Alternately, the induction of membrane lipid peroxidation could amplify the stimulatory action on PKC. Activation of PKC may serve to phosphorylate and activate a high molecular weight cytosolic PLA_2 acting on 20:4-containing phospholipids and PKC-mediated activation of $cPLA_2$, as described in previous reports (40). Enhanced hydrolysis of phospholipids would conceivably arise through a combined effect on enzyme–enzyme/enzyme–membrane interaction. This process would also be more responsive to the inhibitory effects of antioxidants such as vitamin E and, in part, account for the previously reported inhibitory effect of vitamin E on PKC (41).

Lipid peroxides may function as another class of lipid activators for PKC (42). The inhibitory effect of vitamin E on LOOH-induced phospholipid hydrolysis and increased $[Ca^{2+}]_i$ shown in Table 1 provides evidence for the requirement of cellular lipid peroxidation to elicit the hydrolytic response to LOOH. It appears that low levels of LOOH may be relatively ineffective at stimulating phospholipid hydrolysis directly; however, the propagation of lipid peroxidation induces or amplifies the cellular response to LOOH. It should be noted that unsaturated fatty acids such as arachidonic acid also activate PKC (44). Although inhibition of lipid peroxidation is a plausible mechanism by which vitamin E inhibits LOOH-induced hydrolysis, we cannot exclude the other possible actions of vitamin E including direct inhibition of PKC activity or its translocation (41) as well as the direct inhibition of PLA_2 (43). The effect of vitamin E in our studies may be consistent with the alternate mechanisms noted above in that the simple inhibition of lipid peroxidation may modulate the effect of peroxide tone on PKC or PLA_2 activity.

ACKNOWLEDGMENT

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