The involvement of phosphatidylcholine-specific phospholipase C (PC-PLC) and D (PC-PLD) in the regulation of the thapsigargin-induced Ca\(^{2+}\) increase was investigated. Pretreatment of human lymphocytes with the PC-PLC inhibitors D609 or U73122 enhanced the thapsigargin-induced Ca\(^{2+}\) influx. By contrast, no effect was observed in the presence of phospholipase D inhibitor butanol. Addition of exogenous PC-PLC but not PC-PLD to lymphocytes prestimulated with thapsigargin led to a decrease of intracellular Ca\(^{2+}\). In addition, thapsigargin was shown to release diacylglycerol (DAG) from cellular phosphatidylcholine pools. The thapsigargin-induced DAG formation was inhibited by U73122 and D609 but not by butanol. Moreover, no formation of the PC-PLD activity marker phosphatidylbutanol was detected. Thapsigargin-induced DAG formation was dependent on the Ca\(^{2+}\) entry, as it was abolished in the absence of extracellular Ca\(^{2+}\) or in the presence of Ni\(^{2+}\). Further investigations demonstrated that the inhibition of the cellular DAG target, protein kinase C (PKC), enhanced thapsigargin-induced Ca\(^{2+}\) increase, whereas direct PKC activation had an inhibitory effect. Taken together, our results reveal the involvement of PC-PLC in the regulation of the thapsigargin-induced Ca\(^{2+}\) increase and point to the existence of a physiologic feed-back mechanism activated by Ca\(^{2+}\) influx and acting via consecutive activation of PC-PLC and PKC to limit the rise of intracellular Ca\(^{2+}\).

In human lymphocytes, as well as in other electrically non-excitable cells, receptor stimulation results in a transient increase in the cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)). [Ca\(^{2+}\)]\(_i\) increase primarily occurs due to the calcium release from inositol 1,4,5-trisphosphate-sensitive intracellular pools and is generally accompanied by an entry of extracellular calcium mediated by the activation of receptor-operated or second messenger-activated Ca\(^{2+}\) channels (1). In the hypothesis referred to as capacitative model, calcium influx is controlled by the filling state of intracellular calcium pools (2–4). The mechanisms by which capacitative calcium influx is elicited are not clear. Depletion of cellular calcium pools may trigger release of a diffusible cytoplasmic messenger which in turn opens transmembrane Ca\(^{2+}\) channels (5, 6).

One approach to study the role of intracellular Ca\(^{2+}\) pools for the Ca\(^{2+}\) entry utilizes inhibitors of the Ca\(^{2+}\)-ATPases in the store membrane. Thapsigargin has been shown to selectively inhibit Ca\(^{2+}\)-ATPases in the endoplasmic reticulum without affecting Ca\(^{2+}\)-ATPases in the plasma membrane (7, 8). In contrast to receptor agonists that initiate rapid formation of inositol 1,4,5-trisphosphate, thapsigargin depletes Ca\(^{2+}\) pools solely by preventing Ca\(^{2+}\) reuptake (9). In a variety of cells including human lymphocytes the thapsigargin-mediated depletion of calcium stores leads to a sustained elevation of [Ca\(^{2+}\)]\(_i\), supported by the entry of extracellular Ca\(^{2+}\) (10–15).

Although cellular phospholipases are crucial for the regulation of numerous physiological processes, their role in maintaining intracellular calcium homeostasis is little understood. The early phase of Ca\(^{2+}\) increase due to Ca\(^{2+}\) release from inositol 1,4,5-trisphosphate-sensitive stores is thought to involve activation of the receptor-coupled, phosphoinositide-specific phospholipase C. The role of this enzyme in producing the sustained phase of Ca\(^{2+}\) increase which is caused by the transmembrane Ca\(^{2+}\) influx is, however, less clear (16–18). There is also evidence pointing to the possible role of phospholipase A\(_2\) in modulating Ca\(^{2+}\) entry following depletion of intracellular Ca\(^{2+}\) stores (19, 20). To our knowledge, no data exist on the role of phosphatidylcholine-specific phospholipases in the regulation of intracellular Ca\(^{2+}\) homeostasis. Therefore, the aim of the present study was to investigate the involvement of phosphatidylcholine-specific phospholipase C (PC-PLC)\(^1\) and phosphatidylinositol-specific phospholipase D (PC-PLD)\(^1\) in the regulation of the calcium entry following depletion of Ca\(^{2+}\) stores with thapsigargin. Our results demonstrate that PC-PLC, but not PC-PLD, plays an important role in the down-regulation of Ca\(^{2+}\) influx in human lymphocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tricyclodecan-9-yl xantogenate (D609), Fura-3-AM, propranolol, and phospholipid standards were from Sigma, Deisenhofen, Germany. [3-1-3-(Amidinothio)propyl-1H-indolyl-3-yl]-3-(1-methyl-1H-indolyl-3-yl)maleimide methane sulfonate (Ro-31-8220), 1-(6-([17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl])aminoheptyl)-1H-pyrrrole-2,5-dione (U73122), phorbol myristate acetate (PMA), and thapsigargin were purchased from Calbiochem, Bad Soden, Germany. 4-n-PMA was obtained from Biomol, Hamburg, Germany. [1-14C]Arachidonic acid and 1-lyso-3-phosphatidylcholine [1-14C]-palmitoyl were from Amersham, Braunschweig, Germany, and [32P]orthophosphoric acid was from NEN Life Science Products, Dreieich, Germany. Phospholipase C (from *Bacillus cereus*) and phospholipase D (from *Streptomyces chromofuscus*) were purchased from Boehringer, Mannheim, Germany. Silica Gel 60 plates and solvents for thin layer chromatography were obtained from

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\(^{1}\) The abbreviations used are: PC-PLC, phosphatidylcholine-specific phospholipase C; PC-PLD, phosphatidylcholine-specific phospholipase D; DAG, diacylglycerol; PtdOH, phosphatidic acid; PtdInsP\(_2\), phosphatidylinositol bisphosphate; PtdInsP\(_3\), phosphatidylinositol monophosphate; PtdIns, phosphatidylinositol; PtdBut, phosphatidylbutanol; PtdChol, phosphatidycholine; PMA, phorbol myristate acetate.

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Merck, Darmstadt, Germany. Ficoll (Lymphoprep) was obtained from Nycomed, Uppsala, Sweden. Autoradiography was performed with Kodak X-Omat film (Eastman Kodak). Ultima GOLD scintillation mixture was provided by Packard, Frankfurt, Germany.

Assay of Lymphocytes—Lymphocytes were obtained from heparinized blood of 10- to 12-week-old donors according to previously described methods (21). Briefly, blood was centrifuged at 240 × g for 15 min, and the upper two-thirds of the supernatant were aspirated. The remaining blood was mixed 1:1 with Hanks’ balanced salt solution containing 136 mM NaCl, 5.4 mM KCl, 0.44 mM KH2PO4, 0.34 mM Na2HPO4, 1.0 mM CaCl2, 5.6 mM glucose, and 10 mM HEPES, pH 7.4. Lymphocytes were prepared by centrifugation of blood on a Ficoll gradient (Ficoll 5.6%, 1.5 ml) and resuspended in Hanks’ balanced salt solution. The lymphocyte viability was greater than 95% as determined by the trypan blue exclusion test.

Determination of Cytosolic Free Calcium Concentration ([Ca2+]i)—Intracellular calcium measurements were performed according to the established method (22). Lymphocytes were loaded with 2.5 μM Fura-2 AM for 45 min at 37 °C. The lymphocyte suspension was then washed twice (240 × g, 15 min) to remove unincorporated dye and adjusted to a final concentration of 1 × 106 cells/ml. Fluorescence was recorded at 510 nm (bandwidth 10 nm) with excitation wavelengths of 340 and 390 nm (bandwidth 10 nm) using a fluorescence spectrophotometer (model 2048PC; PerkinElmer, Waltham, MA). The intracellular calcium concentration was calculated as described previously (22). Briefly, the maximum fluorescence was obtained after addition of 1.0 mM digitonin. The minimum fluorescence was obtained after addition of 5 mM EGTA. The ratio out of the measured fluorescence values was calculated. ([Ca2+]i)max, was obtained according to Equation 1 by Grynkiewicz et al. (23).

$$[Ca^{2+}]_i = K_0 + (R - R_{min})(R_{max} - R)$$

(Eq. 1)

where Rmin stands for the ratio in calcium-free solution, Rmax for the ratio at calcium saturation, and K0 for K0 = Fmax/Fmin, the latter representing the fluorescence maximum and minimum at 380 excitation. K0 of Fura-2 was set to be 224 mmol/litter.

Metabolic Radiolabeling and Extraction of Lipids—Phospholipid analysis was done as described previously (24, 25). For metabolic lipid radiolabeling the lymphocyte suspension was incubated at 37 °C for 90 min with the following compounds: 0.2 μCi/ml [1-14C]arachidonic acid, 0.2 μCi/ml [1-14C]lysophosphatidylcholine, and 100 μCi/ml [32P]orthophosphoric acid. In the latter case, phosphate was replaced by carbonate in the incubation buffer. The lymphocytes were then washed, adjusted to a final concentration of 1 × 106 cells/ml, and stimulated with the desired agonist. At different time points 0.5 μl aliquots were withdrawn and added to 1.5 ml of ice-cold chloroform/methanol (2:1, v/v) or chloroform/methanol/hydrochloric acid (2:1:0.01, v/v) in the case of 3P-labeling. The phases were split by adding 0.5 ml of chloroform and 0.5 ml of water. The samples were centrifuged at 3000 rpm for 5 min, and lipid phases were collected. Water-soluble phases were extracted once with chloroform. Lipid phases were then combined, dried under nitrogen, dissolved in 0.3 ml of hexane, and stored at −70 °C until analyzed.

Analysis of Radiolabeled Lipid Metabolites—In most experiments, a double one-dimensional TLC as described by Gruchalla et al. (26) was used to separate phospholipids and neutral lipids of interest. This approach, a series of samples was spotted 12 cm from the bottom of the plate. To resolve labeled neutral lipids (DAG, fatty acids, and triglycerides) from phospholipids that remained at the origin, the plates were twice developed in toluene/ether/ethanol/triethylamine (100:80:4:2, by volume). After they were developed a second time with the same solution to 16 cm. Plates were then twice developed in toluene/ether (23:18:12, by volume) using potassium oxalate-impregnated Silica 60 plates. Bands corresponding to PtdIns, PtdInsP, PtdInsP2, or PtdCho were cut out from the silica plates, placed in scintillation vials, and quantitated as described above. The identities of labeled bands were determined based on Rp values obtained for authentic neutral lipids and phospholipids.

Assay for Phospholipase D Activity—PC-PLD hydrolyzes phospholipids to yield the free polar head groups choline and PtdOH. In the presence of primary alcohols, however, PC-PLD catalyzes a phosphatidylglycerol transfer reaction producing phosphatidyl alcohol. Since the transphosphatidyltransferase is catalyzed solely by PC-PLD, the production of phosphatidyl alcohol is an unequivocal marker for involvement of this enzyme. For the examination of PC-PLD activity lymphocytes were labeled with 0.2 μCi/ml [1-14C]lysophosphatidylcholine. Butanol-1-ol was added 5 min prior to agonist addition. Radiolabeled phospholipids were extracted as described above and analyzed using Silica 60 plates developed with ethyl acetate/2,2,4-trimethylpentane/acetonic acid (9:2:1, v/v). As described by van der Meulen and Haslam (27) in some experiments the amount of PtdBut formed was examined using the double one-dimensional system as described above. The optimal concentration of butanol was determined by performing a butanol concentration curve using 1 μM PMA as an agonist. As shown in Fig. 1 the most effective PtdBut production was seen at butanol concentrations between 0.3 and 0.6% (v/v) and was accompanied by a decrease in PtdOH formation.

General Procedures—Each experiment was performed in duplicate and repeated 3–5 times, as indicated in appropriate legends. Unless otherwise indicated, data represent the mean from duplicate determination in a representative experiment. To avoid possible bias due to the variability of lymphocyte populations obtained from different donors, control and drug responses were determined in duplicate cell preparations. For calcium measurements data are presented as means ± S.E. The groups were compared with the non-parametric Wilcoxon-Mann-Whitney test using the computer software Instat 2.02 (GraphPAD). Two-tailed p values less than 0.05 were considered to be significant.

RESULTS

Effect of D609, U73122, Butanol, and Propranolol on Thapsigargin-induced [Ca2+]i Mobilization—[Ca2+]i, was measured in intact human lymphocytes using the calcium-sensitive fluorescent dye Fura-2. The resting [Ca2+]i level in these cells averaged 94 ± 8 nM (n = 34). The resting [Ca2+]i level did not significantly change at least over 400 s (n = 23). As illustrated in Fig. 2, addition of 5 μM thapsigargin resulted in a time-dependent increase in intracellular calcium. Within 200 s [Ca2+]i increased to 1208 ± 27 nM (n = 34) over the resting level. Next, the effects of the PC-PLC-inhibitor D609, the unspecific PLC inhibitor U73122, the indirect PLD inhibitor butanol, and phosphatidic acid phosphohydrolase inhibitor propranolol on the thapsigargin-triggered [Ca2+]i elevation were tested. In the presence of 20 μM D609 or 10 μM U73122 the thapsigargin-induced [Ca2+]i increases were significantly enhanced and increased 377 ± 54 nM (n = 19; p < 0.02) and 517 ± 99 nM (n = 16; p < 0.01) over the resting level, respectively (Fig. 2). By con-
were then exposed to 5 mM, mean (control), D609, U73122, butanol, and propranolol. Data represent mined. 

\[ \text{Mn}^{2+} \] treated in Fig. 3, addition of 5 

propranolol, or vehiculum. Cells were then exposed to MnCl_2 (100 

μM), and the fluorescence was recorded as described under “Experimental Procedures.” A, original tracings obtained from one representative experiment were superimposed for comparison. B, bar graph showing thapsigargin-induced intracellular calcium increases (Δ[Ca^{2+}]) in the presence of vehiculum (control), D609, U73122, butanol, and propranolol. Data represent mean ± S.E. from 34, 19, 16, 10, and 6 determinations, respectively. * p < 0.02; ** p < 0.01. C, concentration-dependent effect of D609 and U73122 on the thapsigargin-induced [Ca^{2+}], increase (Δ[Ca^{2+}]). Each point represents the mean ± S.E. from at least five determinations.

trast, preincubation of cells with 0.3% butanol or 50 μM propranolol did not affect thapsigargin-induced [Ca^{2+}], elevation (Fig. 2). Under these experimental conditions [Ca^{2+}], rose by 188 ± 24 nM (n = 10; not significant versus thapsigargin alone) and 171 ± 15 nM (n = 6; not significant versus thapsigargin alone). The potentiating effects of D609 and U73122 on the thapsigargin-induced [Ca^{2+}], elevation were concentration-dependent (Fig. 2). For both compounds the significant enhancement of [Ca^{2+}], increases was seen at a concentration of about 10 μM.

Effect of D609 and U73122 on Thapsigargin-induced Calcium Influx—To test whether potentiating effects of U73122 and D609 on thapsigargin-stimulated intracellular calcium elevation occur as a consequence of increased Ca^{2+}, entry, the unidirectional uptake of Mn^{2+}, a Ca^{2+}, surrogate, was determined. Mn^{2+}, which quenches Fura-2 fluorescence, enters cells through physiological pathways, yet is not readily extruded at an appreciable rate. Thus, the rate of fluorescence decrease provides a relative measure of the divalent ion entry. As illustrated in Fig. 3, addition of 5 μM thapsigargin was found to accelerate the rate of Fura-2 quenching, indicating activation of the direct cation permeability pathway at the plasma membrane. When lymphocytes were first treated for 5 min with 20 μM D609 or 10 μM U73122, and subsequently stimulated with thapsigargin, the rate of Fura-2 quenching was considerably more pronounced (Fig. 3). These findings suggest that U73122 and D609 exert their potentiating effects on the thapsigargin-

induced [Ca^{2+}], elevation by increasing trans-plasma membrane calcium influx.

Effect of D609, U73122, and Butanol on Thapsigargin-induced [14C]DAG Formation—Since diacylglycerol is the principal product released upon activation of PLC, we next investigated whether it is formed following stimulation of lymphocytes with 5 μM thapsigargin. The lymphocytes were labeled for 2 h with 0.2 μCi/ml [1-14C]arachidonic acid. Upon this treatment phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdChol) incorporated 42 and 34% of the total phospholipid radioactivity, respectively. As demonstrated in Fig. 4, the [14C]DAG level increased by 98 ± 15% (n = 5) within 120 s following addition of 10 μM thapsigargin. Thereafter, [14C]DAG remained elevated over the basal level for at least 300 s. Preincubation of lymphocytes with 20 μM D609 for 5 min reduced the thapsigargin-induced [14C]DAG accumulation to 48 ± 7% (n = 3) at 120 s after stimulation (Fig. 4). Similarly, the reduction of the thapsigargin-induced [14C]DAG formation to 37 ± 6% (n = 3) at 120 s after stimulation was observed in lymphocytes pretreated with 10 μM U73122 (Fig. 4). In contrast to D609 and U73122, no effect on the thapsigargin-induced [14C]DAG formation was noted in lymphocytes pretreated with 0.3% butanol (Fig. 4).

Effect of Thapsigargin on [32P]Phosphatidylinositol and [32P]Phosphatidylcholine Breakdown—Due to the uniform radioactivity incorporation in all major phospholipids, the experiments with [1-14C]arachidonic acid labeling do not allow us to conclude whether [14C]DAG is primarily derived from PtdIns or PtdChol. To elucidate the phospholipid substrate for thapsigargin-stimulated phospholipase activation, we labeled lymphocytes with 0.1 mCi/ml [32P]orthophosphoric acid. As shown in Table I, the radioactivity associated with PtdInsP_2, PtdInsP, and PtdIns was not significantly altered following stimulation of lymphocytes with 5 μM thapsigargin. By contrast, 5 μM thapsigargin induced decrease of the radioactivity associated with PtdChol (Fig. 5). This effect was abolished in the presence of 20 μM D609. These results suggest that phosphatidylcholine rather than phosphatidylinositol is the main source for the thapsigargin-induced DAG production.

Effect of D609, U73122, Butanol, and Propranolol on Thapsigargin-induced Phosphatidylinositol-derived [14C]DAG Formation—To investigate further which phospholipid pool serves as a source for DAG during lymphocyte stimulation with thapsigargin, cells were labeled for 2 h with 0.2 μCi/ml [1-14C]lysophosphatidylinositol. Under this condition, the majority of the radioactivity incorporated into phospholipids was found in PtdChol with less than 2% incorporated into PtdIns. Addition of 5 μM thapsigargin to the lymphocyte suspension resulted in a substantial increase in [14C]DAG. The maximum response
The amount of [14C]DAG then declined within the next 5 min. At indicated times aliquots of the cell suspension were withdrawn and analyzed for [14C]DAG as described under "Experimental Procedures." Results are given as a percentage of total radioactivity in the lipid extract and represent mean ± S.E. from three to five separate experiments, each in duplicate.

### Table I

| Time (min) | [32P]PtdInsP <sub>2</sub> (dpm/ml/10<sup>6</sup> cells) | [32P]PtdInsP <sub>3</sub> (dpm/ml/10<sup>6</sup> cells) |
|------------|---------------------------------------------------|---------------------------------------------------|
| 0          | 2084 ± 171                                       | 2507 ± 205                                        |
| 1          | 2290 ± 205                                       | 2831 ± 315                                        |
| 2          | 2203 ± 254                                       | 2804 ± 301                                        |
| 5          | 1935 ± 98                                        | 2292 ± 237                                        |

(74 ± 4.1% over the basal level (n = 6)) was attained 60 s after stimulation. The [14C]DAG level remained elevated above the basal value from 60 s onwards (Fig. 6A). In the presence of 0.3% butanol or 50 μM propranolol, [14C]DAG increased by 54 ± 7.6% (n = 3) and 71 ± 11.3%, respectively, within 120 s after stimulation. The amount of [14C]DAG then declined within the next 180 s but remained elevated above the basal level. Preincubation of lymphocytes with 20 μM D609 or 10 μM U73122 virtually abolished the thapsigargin-induced accumulation of [14C]DAG. As shown in Fig. 6B, the inhibition of the thapsigargin-stimulated [14C]DAG formation by D609 and U73122 was concentration-dependent. For both compounds the inhibitory effect was noted at concentrations above 10 μM.

In contrast to [14C]DAG, no increase in [14C]PtdOH was detected following stimulation of [14C]lyso-phosphatidylcholine-labeled lymphocytes with thapsigargin (not shown).

**Effect of Exogenously Added PC-PLC and PC-PLD on Thapsigargin-induced Calcium Influx**—To study further the role of PC-PLC and PC-PLD in the thapsigargin-stimulated DAG production, lymphocytes were stimulated with various concentrations of thapsigargin in the presence of 0.3% (v/v) butanol. Phosphatidylinositols were synthesized by PLD, synthesis of phosphatidyl alcohols is considered to be an unequivocal marker of PLD activation. To confirm the lack of PC-PLD involvement in the thapsigargin-stimulated DAG production, lymphocytes were stimulated with 10 μM thapsigargin at concentrations up to 10 μM (Fig. 7). By contrast, marked [14C]PtdInsP formation was observed in cells stimulated with thapsigargin at concentrations up to 10 μM (Fig. 7). By contrast, marked [14C]PtdInsP formation within 5 min after stimulation was observed in the same experiment when PMA was used instead of thapsigargin (Fig. 7). The latter compound stimulates PC-PLD via PKC activation.

**Effect of Exogenous Added PC-PLC and PC-PLD on Thapsigargin-induced Calcium Influx**—To study further the role of PC-PLC and PC-PLD in the thapsigargin-induced Ca<sup>2+</sup> influx, we examined the effects of exogenous PC-PLC and PC-PLD on the thapsigargin-induced Ca<sup>2+</sup> elevation. Fig. 8A demonstrates that the addition of 5 units/ml PC-PLC to lymphocytes resulted in a rapid formation of [14C]DAG, whereas the addition of PC-PLD led to the accumulation of [14C]PtdOH. As shown in Fig. 8B, depletion of intracellular Ca<sup>2+</sup> stores with 5 μM thapsigargin for 10 min resulted in an increase of [Ca<sup>2+</sup>], by 587 ± 77 nM (n = 24). Subsequent addition of 5 units/ml PC-PLC led to a significant decrease of [Ca<sup>2+</sup>], by 309 ± 48 nM (n = 14; p < 0.001 versus control) (Fig. 8, B and C). This effect was completely abolished in the presence of 20 μM D609 (Fig. 8, B and C) or when heat-treated PC-PLC was used instead of the
of PKC modulation on the thapsigargin-induced calcium Increase—

Since DAG liberated by PC-PLC is the main calcium entry. As shown in Fig. 9A. Under this experimental condition [Ca\(^{2+}\)]\(_{i}\) rose by 20 ± 4 nM (n = 18; p < 0.01). Similarly, in the presence of 5 mM Ni\(^{2+}\), which blocks divalent cation entry pathways, thapsigargin-induced [Ca\(^{2+}\)]\(_{i}\) elevation in lymphocytes resuspended in Ca\(^{2+}\)-free medium is shown in Fig. 9A. Under this experimental condition [Ca\(^{2+}\)]\(_{i}\), rose by 20 ± 4 nM (n = 14, p < 0.001).

We next investigated whether the thapsigargin-induced DAG formation depends on the store-operated calcium influx. [\(^{14}\)C]Arachidonic acid-labeled lymphocytes were suspended in Ca\(^{2+}\)-free medium or in Ca\(^{2+}\)-containing medium in the presence of 5 mM Ni\(^{2+}\) and stimulated with 5 μM thapsigargin. Fig. 9B demonstrates that under these experimental conditions the thapsigargin-triggered DAG formation was markedly reduced indicating that PC-PLC activation depends on the extracellular calcium entry.

Effect of Ro31-8220 and PMA on Thapsigargin-induced Calcium Increase—Since DAG liberated by PC-PLC is the main physiological activator of PKC, we next investigated the effect of PKC modulation on the thapsigargin-induced Ca\(^{2+}\) increase. This was accomplished using the direct PKC activator, PMA, or the selective PKC inhibitor Ro31-8220. As shown in Fig. 10A, 10 μM PMA markedly inhibited Ca\(^{2+}\) increase induced by 5 μM thapsigargin. Under this experimental condition [Ca\(^{2+}\)]\(_{i}\), increased by 65 ± 18 nM (n = 6, p < 0.01). By contrast, in the presence of 10 μM Ro31-8220 the thapsigargin-induced [Ca\(^{2+}\)]\(_{i}\), increase was significantly enhanced and averaged 348 ± 41 nM (n = 12, p < 0.01). The effect of PKC activation on the Ca\(^{2+}\) elevation was further examined in cells pretreated for 10 min with 5 μM thapsigargin. Under this experimental condition, direct stimulation of PKC with 10 μM PMA led to a gradual decrease of [Ca\(^{2+}\)]\(_{i}\), by 202 ± 22 nM (n = 6, p < 0.01 versus control) (Fig. 10B). By contrast, inactive PMA analogue 4α-PMA (10 μM) failed to affect [Ca\(^{2+}\)]\(_{i}\), in cells pretreated with thapsigargin (Δ[Ca\(^{2+}\)]\(_{i}\)), 26 ± 29 nM, n = 4, not significant). Furthermore, the PC-PLC-induced decrease of [Ca\(^{2+}\)]\(_{i}\), was significantly inhibited by the PKC inhibitor Ro31-8220 (Fig. 10B).

Effect of thapsigargin or PMA on [\(^{14}\)C]PtdBut formation in human lymphocytes. Lymphocytes (1 × 10\(^6\) cells/ml) labeled with [\(^{1-14}\)C]lyso-3-phosphatidylcholine were stimulated with various concentrations of PMA or thapsigargin. After 5 min cells were extracted, and extracts were analyzed for [\(^{14}\)C]PtdBut as described under “Experimental Procedures.” Results are given as a percentage of total radioactivity in the lipid extract and represent means ± S.E. from three separate experiments.

Phospholipase C and Calcium Influx

DISCUSSION

In the present study we examined the role of phosphatidylcholine-specific phospholipases in the regulation of the store-operated calcium influx. For this purpose we utilized two structurally unrelated inhibitors, D609 and U73122. Whereas D609 is a PC-PLC inhibitor (28), the specificity of U73122 is less well defined, and additional effects distinct from inhibition of phospholipases have been observed in the presence of this agent (29, 30). The possible role of PC-PLD for [Ca\(^{2+}\)]\(_{i}\) regulation was studied with butanol. In the presence of butanol PC-PLD forms metabolically inactive phosphatidylbutanol instead of its physiologically relevant product PtdOH. In several experimental systems primary alcohols were shown to blunt PC-PLD-mediated physiological responses (31–35). Moreover, we noted de-
increased PMA-induced PtdOH production in the presence of butanol.

Our results demonstrate enhancement of thapsigargin-induced Ca^{2+} mobilization by D609 and U73122. The larger increases in [Ca^{2+}], in the presence of U73122 may point to additional, phospholipase C-independent effects of this compound. However, both structurally unrelated agents enhanced thapsigargin-induced Mn^{2+} quenching to a similar extent. By contrast, butanol failed to affect the thapsigargin-induced calcium mobilization. We interpret these findings to mean that PC-PLC but not PC-PLD is involved in the regulation of the phospholipase. Moreover, the involvement of phosphoinositide-specific phospholipase A_2 is consistent with the activation of a phosphatidylcholine-specific phospholipase. However, the involvement of phosphoinositide-specific phospholipases is unlikely, as no phosphatidylinositol breakdown was observed in lymphocytes treated with thapsigargin. The latter observation agrees with previous findings that depletion of intracellular calcium stores by thapsigargin is not accompanied by the hydrolysis of phosphatidylinositols (9-11).

Here we show for the first time that phosphatidylcholine breakdown and phosphatidylcholine-derived DAG formation occur in thapsigargin-stimulated lymphocytes. These findings are consistent with the activation of a phosphatidylcholine-specific phospholipase. Moreover, the involvement of phosphoinositide-specific phospholipases is unlikely, as no phosphatidylinositol breakdown was observed in lymphocytes treated with thapsigargin. The latter observation agrees with previous findings that depletion of intracellular calcium stores by thapsigargin is not accompanied by the hydrolysis of phosphatidylinositols (9-11).

For several reasons, the present study indicates that the activation of PC-PLD did not contribute to the thapsigargin-induced formation of PC-derived DAG. First, no substantial thapsigargin-induced increase of PtdOH, the primary PC-PLD product, was noted in lymphocytes labeled with lyso-phosphatidylcholine. Second, preincubation of lymphocytes with butanol should blunt PC-PLD-mediated DAG synthesis by shunting PtdOH to PtdBut. However, both in arachidonic acid-labeled...
and in lysophosphatidylcholine-labeled lymphocytes butanol failed to diminish the thapsigargin-stimulated DAG formation. Third, only a slight increase in PtdBut was observed in thapsigargin-treated cells, whereas stimulation of lymphocytes with the PKC activator PMA resulted in considerable accumulation of PtdBut. Fourth, the phosphatidic acid phosphohydrolase inhibitor propanolol failed to affect thapsigargin-induced DAG formation excluding PtdOH as a DAG precursor. In agreement with these results previous studies have failed to detect an effect of thapsigargin on PC-PLD in lymphocytes, mesangial cells, and airway smooth muscle cells (40–42).

Numerous investigations have shown that constitutive levels of [Ca2+]i may be necessary and sufficient for proper PC-PLD function (43, 44), that large increments in [Ca2+]i due to Ca2+ entry may not provide adequate stimuli for PC-PLD activation (42, 45–47), and that PC-PLD does not necessarily substantially contribute to PC-derived DAG formation (53, 54). In the present study, we have demonstrated that Ni2+ influx was shown to activate PC-PLC in intact cells and hence to stimulate PC-derived DAG formation (53, 54). The increase in [Ca2+]i was reported to increase the activity of partially purified PC-PLC (52). Furthermore, a transmembrane Ca2+ influx was shown to activate PC-PLC in intact cells and hence to stimulate PC-derived DAG formation (53, 54). The increase in [Ca2+]i has been demonstrated to be the primary and specific target of the phorbol ester-mediated inhibition. Moreover, in Jurkat T cells and in HPB-ALL T-lymphocytes phorbol esters and other PKC activators have been reported to impair anti-CD3 monoclonal antibody-induced Ca2+ fluxes, whereas PKC inhibitors exerted opposite effects (15, 57, 58), although the latter authors did not observe major potentiating effects of Ro31-8220 on thapsigargin-induced Ca2+ influx. Finally, PKC activation was shown to inhibit thapsigargin-induced Ca2+ entry in cells other than lymphocytes such as FRTL-5 cells and neutrophils (59, 60). Based on the present results it is not possible to conclude which mechanisms underlie the inhibitory effect of PKC. One plausible mechanism of action is through membrane depolarization. Previous studies demonstrated that calcium influx is strongly dependent on the membrane potential and that PKC induces depolarization in some cells (59, 61, 62).

The increase in [Ca2+]i during lymphocyte activation is thought to have important functional consequences for their proper function (63–65). The mechanism by which lymphocytes are able to maintain Ca2+ increase over prolonged periods is not yet clear. Inhibition of PC-PLC by U73122 and D609 and inhibition of PKC by Ro31-8220 potentiated the influx of extra-cellular Ca2+ triggered by depletion of intracellular Ca2+ stores. These observations together with the strong dependence of PC-PLC activation on extracellular calcium entry suggest that a physiological feedback mechanism exists, which is activated by Ca2+ influx and acts via consecutive activation of PC-PLC and PKC to limit the rise in [Ca2+]i. On the other hand, the Mn2+ quenching rates increased following thapsigargin addition and then decreased again implying that the influx rate is inhibited to some extent even in the presence of PLC blockers. Thus, additional mechanisms may exist by which thapsigargin-induced Ca2+ entry is modulated. In several cell types including lymphocytes the existence of the feedback inhibition of store-operated calcium increases responsible for maintaining the long term Ca2+ homeostasis was postulated (66–68). Furthermore, feedback regulation of the capacitative calcium entry appears to be heterogeneous (69). For the first time, the present study provides an explanation of how one of the putative feedback mechanisms might operate.

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