Involvement of phospholipase D in store-operated calcium influx in vascular smooth muscle cells

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Abstract In non-excitable cells, sustained intracellular Ca^{2+} increase critically depends on influx of extracellular Ca^{2+}. Such Ca^{2+} influx is thought to occur by a 'store-operated' mechanism, i.e. the signal for Ca^{2+} entry is believed to result from the initial release of Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive intracellular stores. Here we show that the depletion of cellular Ca^{2+} stores by thapsigargin or bradykinin is functionally linked to a phosphoinositide-specific phospholipase D (PLD) activity in cultured vascular smooth muscle cells (VSMC), and that phosphatidic acid formed via PLD enhances sustained calcium entry in this cell type. These results suggest a regulatory role for PLD in store-operated Ca^{2+} entry in VSMC.

Key words: Calcium; Phospholipase D; Signal transduction; Thapsigargin; Bradykinin; Phosphatidylinositol; Phosphatidylcholine

1. Introduction

In excitable cells, increases in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) occur mainly via voltage-activated Ca^{2+} channels. In many non-excitable cells, by contrast, the stimulation of phosphoinositide-specific phospholipase C (PLC) subsequent to agonist–receptor binding increases the cytosolic inositol 1,4,5-trisphosphate ($InsP_3$) concentration, which releases Ca^{2+} from intracellular stores [1]. This initial rise of cellular Ca^{2+} is followed by entry of extracellular Ca^{2+}, which leads to a sustained Ca^{2+} increase and allows the cellular Ca^{2+} stores to refill [2]. The mechanism underlying the sustained Ca^{2+} influx is not entirely understood. Different messengers were proposed to be released upon depletion of Ca^{2+} stores. These include a small diffusible messenger [3], a small monomeric GTP-binding protein [4,5], cyclic GMP [6] and inositol phosphates [7]. Alternatively, a direct protein–protein interaction between a regulatory protein on the stores and a plasma membrane Ca^{2+} channel may exist [8]. Recently, several products of the Trp channel gene family were demonstrated to be Ca^{2+}-store-operated channels in invertebrate and mammalian cells [9,10]. These channels appear to be regulated by lipid mediators such as polyunsaturated fatty acids and diacylglycerols (DGs) [11,12]. Moreover, Yao and coworker demonstrated that SNAP-25, a protein involved in fusion of vesicles with the plasma membrane, plays an essential role in store-operated Ca^{2+} entry in *Xenopus* oocytes [13]. These authors hypothesized that the store-operated channels themselves or membrane-bound activator molecules may exocytotically be incorporated into the plasma membrane upon store depletion.

Phospholipase D (PLD), which hydrolyzes phospholipids to produce phosphatidic acid (PA) and the respective head group, is a major enzyme implicated in cell proliferation and membrane traffic [14,15]. PA seems to be an important co-factor for both protein and lipid secretion [15,16]. We therefore examined the possible involvement of this enzyme in store-operated calcium entry.

2. Materials and methods

2.1. Cell culture

All experiments were carried out using vascular smooth muscle cells (VSMC) derived from rat thoracic aortas from 6-month-old male normotensive Wistar-Kyoto rats. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Boehringer, Mannheim, Germany), 100 U/ml penicillin G, 100 μg/ml streptomycin and 2 mmol/l L-glutamine. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed initially after 24 h and then every 2–3 days. When cells had formed a confluent monolayer after about 8–10 days, they were harvested by addition of 0.05% trypsin, and the culture was continued up to eight passages. Separate experiments confirmed other reports that the resting cytosolic free calcium concentration ($[Ca^{2+}]_i$) in cultured VSMC was not significantly different in these passages [17]. To verify that cultured cells were VSMC, we carried out immunocytochemical localization of smooth muscle-specific α-actin using FITC-labeled monoclonal antibodies ASM-1 (Progen, Heidelberg, Germany). A viability of VSMC higher than 95% was observed by trypan blue exclusion. Cells were made quiescent by incubation in serum-free medium containing 0.1% bovine serum albumin, 100 U/ml penicillin and 100 μg/ml streptomycin for 48 h.

2.2. Radiolabeling and separation of radiolabeled hydrolysis products

To monitor phospholipid-derived 1,2-DG, PA and phosphatidylbutanol (PBut), cells were prelabeled with 0.5 μCi/ml [^{14}C]arachidonic acid (Amersham) for 24 h, or with 0.1 μCi/ml L-lyso-3-phosphatidylcholine (PC), [1-^{14}C]palmitoyl (56 mCi/mmol, Amersham), for 2 h, as previously described [16,18]. The cells were washed five times in phosphate-buffered saline (PBS) and stimulated with 500 nmol/l thapsigar-

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; DG, 1,2-diacylglycerol; $InsP_3$, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PBS, phosphate-buffered saline; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PLC, phospholipase C; PLD, phospholipase D; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; VSMC, vascular smooth muscle cells

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gin or with 100 nmol/l bradykinin or PBS (control). Thapsigargin or bradykinin were added at time 0. After termination of the incubation in a liquid nitrogen bath, cells were scraped from the dishes and radioactive lipids were extracted by the method of Folch et al. [19]. For transphosphatidylation experiments, 0.3% butanol was added 5 min before starting the incubation.

For lipid analysis, a double one-dimensional thin-layer chromatography as previously described [16,18] was used to separate phospholipids and neutral lipids of interest. After the plates had been dried thoroughly, autoradiography was performed by using Kodak X-OMAT film (Eastman Kodak) for 7–14 days. Radioactive bands were cut from the silica plates and quantitated by liquid scintillation counting using 10 ml Ultima-Gold scintillation fluid (Canberra-Packard). The identities of the labeled bands were determined based on R_f values obtained for authentic neutral lipids and phospholipids (from Sigma) visualized by iodine staining. [^{32}P]Phosphatidylinositol (PI), [^{32}P]phosphatidylinositol-monophosphate (PIP) and [^{32}P]phosphatidylinositol-bisphosphate (PIP_2) turnover were examined in VSMC prelabeled with [^{32}P]*ortho*-phosphoric acid and stimulated with thapsigargin or bradykinin. For this purpose, VSMC were incubated for 3 h at 37°C in phosphate-free DMEM–HEPES containing 0.2 mCi/ml of [^{32}P]*ortho*-phosphoric acid (NEN, Du Pont). The cells were washed five times and stimulated with 500 nmol/l thapsigargin or 100 nmol/l bradykinin. Extraction was performed with chloroform/methanol/HCl (100:200:2, per volume). [^{32}P]Phospholipids were developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7, per volume) using silica 60 plates (Merck) impregnated with 1% potassium oxalate, as previously described [20]. The amount of radioactivity in PA, DG, PBut, PI, PIP and PIP_2 was expressed as percentage of total lipid.

2.3. Measurement of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ were performed using the calcium-sensitive dye fura 2 according to the method of Grynkiewicz et al. [21], as described previously [22]. Monolayers of VSMC were grown on round coverslips with a diameter of 13 mm according to the method of Capponi et al. [23] and Okada et al. [24]. Briefly, VSMC were washed twice in physiological salt solution (mmol/l: NaCl, 135; KCl, 5; $CaCl_2$, 1; MgCl, 1; D-glucose, 5.5; HEPES, 10; pH 7.4) and then incubated for 60 min at 37°C with 0.5 μmol/l fura 2-AM. The fluorescence intensity of fura 2-loaded VSMC was measured at 37°C using a spectrofluorophotometer RF-5001 PC (Shimadzu, Tokyo, Japan) equipped with a thermostatically controlled cuvette holder and with intracellular calcium measurement software (Shimadzu, Düsseldorf, Germany). The fluorescence of fura 2 was measured using a data sampling interval of 0.5 s with alternate excitation wavelengths of 340 and 380 nm (bandwidth, 5 nm), and emission was collected at 510 nm (bandwidth, 5 nm). After the subtraction of autofluorescence for each wavelength, the ratio (R) of the measured fluorescence values at 340 and 380 nm excitation was calculated [21,22]. Digitonin (1 mmol/l) and EGTA (5 mmol/l) were sequentially added to determine the maximum (R_{max}) and minimum (R_{min}) of the 340/380 nm excitation ratio, respectively. $[Ca^{2+}]_i$ was calculated following the equation of Grynkiewicz et al. [20]: $[Ca^{2+}]_i = K \times (R_i - R_{min})/(R_{max} - R)$; K stands for $K_D \times F_{min380}/F_{max380}$, the latter representing the ratio of the fluorescence at 380 nm excitation measured in EGTA plus digitonin to that measured in 1 mmol/l external Ca^{2+} plus digitonin, and K_D represents the dissociation constant of fura 2 for Ca^{2+}, which was set to be 224 nmol/l [21]. For measurements, VSMC were preincubated with buffer alone for control or with 0.3% butanol for 5 min. The cells were stimulated with 500 nmol/l thapsigargin or 100 nmol/l bradykinin in the presence or absence of 1 mmol/l external Ca^{2+}.

2.4. Cell permeabilization

VSMC (10^6 cells) in 10 ml of ice-cold permeabilization solution (140 mM KCl, 10 mM glucose, 1 mM EGTA, 10 mM HEPES, pH 7.0) were twice subjected to a 1 kV/0.4 cm discharge from a 10 μF capacitator using a Bio-Rad Gene pulser [25]. At these conditions, >85% of the cells were permeabilized as detected by uptake of trypan blue. The permeabilization procedure was performed in the presence of 500 μM PA (L-α-PA, β-arachidonoyl-γ-stearoyl, from Sigma) dissolved in DMSO or in the presence of DMSO alone (control). Immediately, the cells were transferred to the spectrofluorophotometer, and $[Ca^{2+}]_i$ was monitored from 10 s subsequent to the permeabilization procedure onward. $CaCl_2$ (20 mmol/l) was added as indicated.

2.5. Statistics

Statistical analysis was performed by using the SPSS FOR WINDOWS 5.01 software package (SPSS Inc.). For statistical evaluation of the data, Friedman's two-way ANOVA was used, and two-tailed values of $P < 0.05$ were considered to be significant. The original Ca^{2+} tracings shown in the figures were superimposed by using the graphic software GRAPHPAD-INPLOT 4.03.

3. Results

3.1. Thapsigargin- and bradykinin-induced activation of PLD in VSMC

VSMC were prelabeled with [^{14}C]arachidonic acid and then

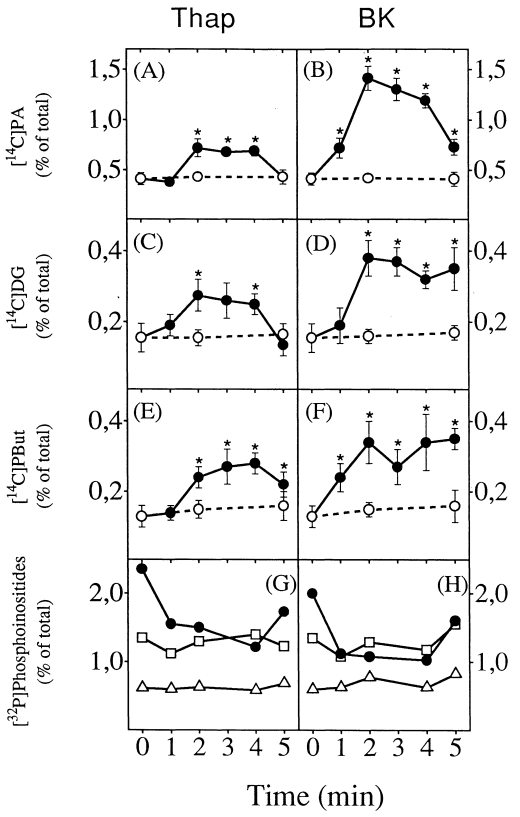

Fig. 1. Thapsigargin- and bradykinin-induced activation of PLD in VSMC. Time courses of [^{14}C]PA (A and B), (^{14}C]DG (C and D) and [^{14}C]PBut (E and F) formation were monitored in VSMC prelabeled with [^{14}C]arachidonic acid and stimulated with thapsigargin, bradykinin or PBS. Cells were prelabeled with 0.5 μCi/ml [^{14}C]arachidonic acid (Amersham) for 24 h, and stimulated with 500 nmol/l thapsigargin (● in A,C,E) or with 100 nmol/l bradykinin (● in B,D,F) or PBS (○ in A–F). Thapsigargin (Thap) or bradykinin (BK) were added at time 0. To examine the breakdown of phosphoinositides, the time courses of [^{32}P]PI (●), [^{32}P]PIP (□) and [^{32}P]PIP_2 (△) turnover were monitored in VSMC prelabeled with 0.2 mCi/ml of [^{32}P]*ortho*-phosphoric acid and stimulated with 500 nmol/l thapsigargin (G) or 100 nmol/l bradykinin (H). The amount of radioactivity in PA, DG, PBut, PI, PIP and PIP_2 was expressed as percentage of total lipid. The total amount of radioactivity incorporated into cellular lipids ranged between 30 000 and 60 000 dpm. In (A–F), each value represents the mean ± S.D. for three or four determinations from a typical experiment out of three. * indicates $P < 0.01$ versus time 0 according to Student's t-test. In (G) and (H), each point represents the mean of duplicate determinations. Similar results were obtained in four other independent experiments.

exposed to thapsigargin (500 nmol/l) or bradykinin (100 nmol/l). Thapsigargin and bradykinin were chosen as agonists since these substances release $InsP_3$-sensitive Ca^{2+} stores by different mechanisms. Bradykinin induces Ca^{2+} release by rapid $InsP_3$ formation [26], whereas thapsigargin inhibits the Ca^{2+}-ATPase responsible for refilling of intracellular Ca^{2+} stores [27]. The agonists were used at concentrations which exert maximal effects on $[Ca^{2+}]_i$ [28]. Both agents caused a transient increase in formation of [^{14}C]PA and [^{14}C]DG up to 1.5–2.5-fold of basal values within 2–4 min (Fig. 1A–D).

The thapsigargin- and bradykinin-induced increase of PA (the direct product of PLD) was the first indication for activation of PLD. However, PA and DG (the direct product of PLC) can rapidly be interconverted by hydrolases and phosphatases. We therefore examined phospholipid breakdown in the presence of *n*-butanol, which traps PA moieties such as PBut. In the presence of butanol, PLD catalyzes a transphosphatidylation reaction, and PBut instead of PA is formed. Butanol inhibited the thapsigargin- and bradykinin-induced PA and DG increases in a concentration-dependent manner (up to 80%). The inhibitory effect was maximal with 0.3% butanol, and was paralleled by the formation of [^{14}C]PBut (Fig. 1E,F). PBut formation unequivocally demonstrated activation of PLD.

[^{14}C]Arachidonic acid is mainly incorporated into phosphoinositides. We could not detect a significant activation of PLD in cells labeled with [^{14}C]lyso-PC, which is nearly completely converted to PC under the used experimental conditions (data not shown). These results suggested that a phosphoinositide-specific PLD activity plays the major role in thapsigargin- and bradykinin-induced PLD activation. To further strengthen this hypothesis, we examined the effect of bradykinin and thapsigargin on phosphoinositide metabolism by [^{32}P]*ortho*-phosphoric acid labeling. Addition of 500 nmol/l thapsigargin (100 nmol/l bradykinin) caused a decrease in endogenous PI to 55 ± 13 (50 ± 19)% after 4 min ($n = 8$), which paralleled the increase of PA, PBut and DG described in Fig. 1A–F. Thereafter, the level of PI rose and reached a level of 77 ± 13 (80 ± 9)% of that in unstimulated cells at 5 min ($n = 8$). Concomitantly, PIP_2 and PIP levels did not change or increased slightly at longer incubation times, indicating de novo synthesis of phosphoinositides to replenish pools of depleted inositol phospholipids (Fig. 1G,H). [^{32}P]PA and [^{32}P]PBut formation showed time kinetics similar to those observed in [^{14}C]arachidonic acid-labeled cells.

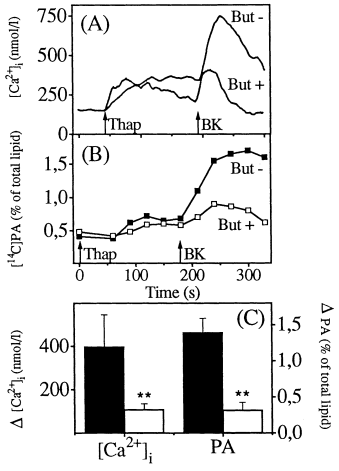

Fig. 3. Effect of depletion of $InsP_3$-sensitive intracellular calcium stores on the bradykinin-induced $[Ca^{2+}]_i$ response and PLD activity. Representative tracings of $[Ca^{2+}]_i$ (A) and PA (B), in absence (■) or presence (□) of 0.3% butanol, and summary data (C) giving the bradykinin-induced Ca^{2+} increase ($\Delta[Ca^{2+}]_i$) and PA (ΔPA) formation in the absence (black bars) and presence (open bars) of 0.3% butanol (But). 100 nmol/l bradykinin was added subsequent to depletion of $InsP_3$-sensitive Ca^{2+} stores. Depletion was achieved by pretreatment of VSMC with thapsigargin (500 nmol/l) in zero-Ca^{2+}/EGTA buffer. Thapsigargin (Thap) and bradykinin (BK) were added at the times indicated by the arrows. PA was determined in [^{14}C]arachidonic acid-labeled cells treated as described in Fig. 1. The amount of [^{14}C] radioactivity in PA was expressed as percentage of total lipid. In (C), each value represents the mean (± S.E.M.) of $n = 25$ experiments.

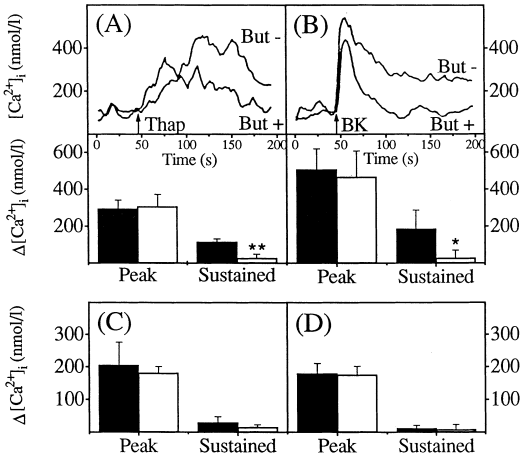

Fig. 2. Effect of inhibition of PA formation on the thapsigargin- and bradykinin-induced $[Ca^{2+}]_i$ increase in VSMC. $[Ca^{2+}]_i$ was measured in VSMC grown on coverslips using fura 2. VSMC were preincubated with buffer alone for control (But −) or with 0.3% butanol (But +) for 5 min. The cells were stimulated with 500 nmol/l thapsigargin (Thap) (A and C) or 100 nmol/l bradykinin (BK) (B and D) in the presence (A and B) or absence (C and D) of 1 mmol/l external calcium. At the top of (A) and (B), representative original tracings of $[Ca^{2+}]_i$ in normal medium are depicted. The peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) and the sustained increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) after 2.5 min in the presence of extracellular Ca^{2+} (A and B) as well as in Ca^{2+}-free medium (C and D) are shown. Each value represents the mean (± S.E.M.) of $n = 18$–26 experiments. Black bars: absence, open bars: presence of 0.3% butanol. * indicates $P < 0.05$, ** indicates $P < 0.01$ compared to the appropriate control value (no butanol pretreatment).

3.2. PLD activity and capacitative calcium entry in VSMC

As there is no commercially available specific PLD inhibitor, we used a primary alcohol to evaluate the possible involvement of PA in capacitative Ca^{2+} entry. This method has previously been used to show the involvement of PA in secretion-like processes in mast cells, fibroblasts and CHO cells [16,29,30]. In practice, we studied the consequences of butanol-induced inhibition of PA formation on cellular Ca^{2+} handling.

In the presence and absence of extracellular Ca^{2+}, pretreatment of cells with butanol did not significantly influence the early $[Ca^{2+}]_i$ response (Fig. 2A–D). Thus, the release of $InsP_3$-sensitive Ca^{2+} stores was apparently not influenced by PLD. By contrast, the sustained thapsigargin- and bradykinin-induced $[Ca^{2+}]_i$ response after 2.5 min was inhibited by butanol

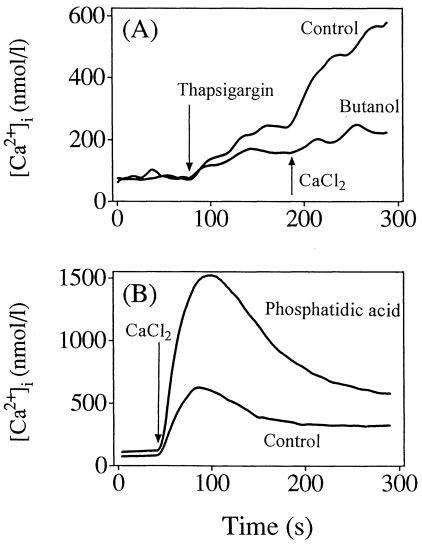

Fig. 4. Influence of PLD and PA on capacitative Ca^{2+} influx. (A) In the absence of extracellular Ca^{2+}, the $InsP_3$-sensitive Ca^{2+} stores of VSMC were depleted by addition of thapsigargin (500 nmol/l). Subsequently, extracellular Ca^{2+} was increased by adding $CaCl_2$ (20 mmol/l). $[Ca^{2+}]_i$ was measured with fura 2 in absence or presence of 0.3% butanol, as described in Fig. 2. (B) VSMC (10^6 cells) in 10 ml of ice-cold permeabilization solution were twice subjected to a 1 kV/0.4 cm discharge from a 10 μF capacitator using a Bio-Rad Gene pulser. The permeabilization procedure was performed in the presence of 500 μM PA (L-α-PA, β-arachidonoyl-γ-stearoyl) dissolved in DMSO or in the presence of DMSO alone (control). Immediately, the cells were transferred to the spectrofluorophotometer, and $[Ca^{2+}]_i$ was monitored. The addition of $CaCl_2$ (20 mmol/l) is indicated by the arrow.

in Ca^{2+}-containing medium (Fig. 2A,B). Maximal inhibition was observed at a butanol concentration of 0.3%. In Ca^{2+}-free medium, the late Ca^{2+} response was not detectable either in the presence or absence of butanol (Fig. 2C,D). These data demonstrated that a transmembrane Ca^{2+} flux is the main cause of the late Ca^{2+} response, and that inhibition of PA formation is associated with inhibition of Ca^{2+} entry.

Next, we examined PA formation and capacitative Ca^{2+} influx after sequential addition of thapsigargin and bradykinin. As shown in Fig. 3B, the depletion of Ca^{2+} stores by thapsigargin resulted in enhancement and prolongation of subsequent bradykinin-induced PA formation. Despite the depletion of $InsP_3$-sensitive Ca^{2+} stores by thapsigargin, bradykinin still induced a substantial increase of $[Ca^{2+}]_i$ (Fig. 3A). This increase was only slightly smaller than that produced by bradykinin in untreated VSMC. Thus, the failure to release Ca^{2+} from $InsP_3$-sensitive stores seems to be compensated for by an increased Ca^{2+} influx. Moreover, the bradykinin-induced $[Ca^{2+}]_i$ response in thapsigargin-treated cells depended even more on PLD than in untreated cells. The increases in $[Ca^{2+}]_i$ and PA were inhibited by 60–80% in the presence of butanol (Fig. 3A–C).

To monitor the store-operated Ca^{2+} influx and its PLD-dependence more directly, we performed an experiment in which Ca^{2+} was added after thapsigargin-induced depletion of $InsP_3$-sensitive Ca^{2+} stores in Ca^{2+}-free medium. As shown in Fig. 4A, the resulting increase of cellular Ca^{2+} was markedly inhibited in the presence of butanol, further supporting the suspicion that the PLD-dependent increase in $[Ca^{2+}]_i$ originates mainly from the influx of extracellular Ca^{2+}.

We also examined the influence of exogenously added PA on Ca^{2+} entry. The addition of PA dissolved in DMSO did not induce Ca^{2+} entry. Vigorous sonication just before addition to the cells induced a weak response. Direct introduction of PA into electropermeabilized cells, however, induced a significant Ca^{2+} influx (Fig. 4B). Electroporation per se prior to the addition of Ca^{2+} resulted in a slight increase of the cellular Ca^{2+} concentration ('control' in Fig. 4B). However, the increase of $[Ca^{2+}]_i$ was several fold higher, when the electroporation was performed in the presence of PA. The rapid increase of $[Ca^{2+}]_i$ in electropermeabilized cells peaked 50–60 s after the addition of $CaCl_2$, and it decreased at longer incubation times.

4. Discussion

We have shown that two agonists which induce the release of $InsP_3$-sensitive Ca^{2+} stores by different mechanisms activate PLD subsequent to the initial Ca^{2+} response in VSMC. In arachidonic acid-labeled cells, addition of bradykinin or thapsigargin stimulated a monophasic accumulation of [^{14}C]PA, within 2–4 min. The rise in PA preceded or paralleled that of DG which was inconsistent with PLC/DG kinase pathway being the source of PA and suggested the involvement of PLD. PLD activation was confirmed by the detection of bradykinin-/thapsigargin-mediated phosphatidyl transfer, producing [^{14}C]PBut instead of [^{14}C]PA in the presence of butanol.

PLD-mediated accumulation of PA and PBut was observed in arachidonic acid-labeled VSMC. Arachidonic acid is mainly incorporated into phosphoinositides, and we could not detect a significant activation of PLD in [^{14}C]lyso-PC-labeled cells. These data suggested that phosphoinositides are a major source for thapsigargin- and bradykinin-induced PA formation. This contention was further supported by the observation that PI hydrolysis in [^{32}P]*ortho*-phosphoric acid-labeled VSMC paralleled the increases of PA and PBut and DG.

It is generally assumed that the depletion of cellular Ca^{2+} stores by thapsigargin is not accompanied by the hydrolysis of PIP_2 [31,32]. This assumption is in accordance with the here described data. A slight increase of PIP_2 and PIP rather than hydrolysis of PIP_2 was registered 2–4 min after addition of thapsigargin or bradykinin. The thapsigargin- or bradykinin-induced hydrolysis of PI, however, has not been studied in detail in previous studies. Moreover, most studies focused on very early second messenger responses (within the first 30 s after stimulation), when PIP_2 is hydrolyzed by the action of PLC, triggering the formation of $InsP_3$ and the initial release of cellular Ca^{2+} stores.

The existence of a PI-specific PLD has previously been shown in pancreas cells [33] and in neutrophils [34]. For the first time, the present study points to a possible physiological function of a cellular PI-PLD activity. The dose- and time-related decline in PA accumulation observed in the presence of butanol was similar to butanol-mediated inhibition of sustained Ca^{2+} entry, suggesting that PI-derived PA could be of importance in store-operated Ca^{2+} entry at longer stimulation times. Of course, it cannot entirely be excluded that butanol influenced the Ca^{2+} influx by unspecific mechanisms (e.g. by

others than inhibition of PA formation). For several reasons, however, this possibility is less likely. First, we found a close temporal relationship between the inhibitory effect of butanol on PA formation and $[Ca^{2+}]_i$ increase. Second, inhibition of PA formation and Ca^{2+} response were dependent on the butanol concentration used, showing maximal effects at identical concentrations in both cases. Third, the sequential application of thapsigargin and bradykinin enhanced both the PLD activation and the Ca^{2+} response. Fourth, only the sustained Ca^{2+} entry was inhibited by butanol; the early Ca^{2+} response, by contrast, was not influenced by this treatment. Fifth, the direct introduction of PA into permeabilized VSMC just prior to the extracellular addition of Ca^{2+} greatly enhanced Ca^{2+} entry.

The postulated involvement of PLD in sustained Ca^{2+} entry is in apparent contradiction with a previous study, which failed to detect thapsigargin-induced activation of PLD in human lymphocytes [35]. Similarly discrepant results were obtained regarding the involvement of PKC, which limits the rise of Ca^{2+} in lymphocytes [35], but appears to stimulate store-operated Ca^{2+} entry in pancreatic cells and VSMC [36,37]. Altogether, these observations give further support for the hypothesis, originally proposed by Louzao et al., that either subtypes of store-operated Ca^{2+} channels are expressed in different cells or that a single channel type may exist which is differentially regulated [38].

From the present study no firm conclusion can be drawn as to which mechanism underlies the putative role of PA in sustained Ca^{2+} entry. It is of interest that the putative 'calcium influx factor' postulated by Randriamampita and Tsien to mediate store-operated Ca^{2+} influx is a non-protein <500 D phosphorylated pH-stable anion [3], and thus shares some properties of PA. In this respect, it is noteworthy that PA itself was proposed to act as a ionophore [39–42], and thereby may stimulate Ca^{2+} influx by direct interaction with the plasma membrane. Alternatively, PA might serve as a substrate for PA-phosphohydrolases or PA-specific phospholipases. The DGs or fatty acids released by these reactions might then stimulate membrane channels, which mediate store-operated Ca^{2+} influx. Finally, it has recently been demonstrated that a protein involved in membrane fusion (SNAP-25) plays an essential role in store-operated Ca^{2+} entry in *Xenopus* oocytes [11]. The involvement of small G-proteins in store-operated Ca^{2+} entry has previously been suggested [4,5]. Fusion proteins, small G-proteins, PA and PIP_2 closely act together in membrane trafficking [15]. It was shown that small G-proteins are important activators of PLD [43,44], and that PA (together with PIP_2) is essential for the formation of certain types of transport vesicles [30,45]. Thus, PLD activation and PA liberation upon cellular depletion of intracellular Ca^{2+} stores could support vesicle formation and incorporation of Ca^{2+} channels into the plasma membrane, and thereby contribute to the sustained Ca^{2+} entry.

In summary, we have shown that the sustained Ca^{2+} influx in response to thapsigargin and bradykinin is associated with activation of a PLD activity in VSMC. PA formed via PLD may play a regulatory role in store-operated Ca^{2+} entry in VSMC, possibly by altering the biophysical properties of cellular membranes and interfering with a signal transduction and/or a secretion-like process.

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