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Increase by lysophosphatidylcholines of smooth muscle Ca^{2+} sensitivity in α-toxin-permeabilized small mesenteric artery from the rat

[1]Peter E. Jensen, *Jacqueline Ohanian, Brian Stausbøl-Grøn, Niels H. Buus & Christian Aalkjaer

Institute of Pharmacology and Danish Biomembrane Research Center, The Bartholin Building, University of Aarhus, 8000 Aarhus C, Denmark and *Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL

1 Pharmacological characterization of different lysophosphatidylcholines was performed based on their effect on the Ca^{2+} sensitivity of contraction in α-toxin-permeabilized rat mesenteric arteries. Furthermore, the effect of noradrenaline on [^{3}H]-myristate-labelled lysophosphatidylcholine levels was assessed, to investigate whether lysophosphatidylcholines could be second messengers.

2 Palmitoyl or myristoyl L-α-lysophosphatidylcholine increased the sensitivity to Ca^{2+}, whereas lysophosphatidylcholines containing other fatty acids had less or no effect.

3 L-α-phosphatidylcholine, L-α-glycerophosphorylcholine, palmitic acid, myristic acid and choline, potential metabolites of lysophosphatidylcholines, did not affect contractions.

4 Noradrenaline (GTP was required) and GTPγS increased the sensitivity to Ca^{2+}, and GDP-β-S inhibited the effect of noradrenaline. Lysophosphatidylcholines, however, had no requirement for GTP and caused sensitization in the presence of GDP-β-S.

5 Calphostin C, a relatively specific protein kinase C inhibitor, did not affect contraction induced by Ca^{2+}, but abolished the sensitizing effect of lysophosphatidylcholine.

6 Noradrenaline caused no measurable changes in the levels of [^{3}H]-myristate-labelled phosphatidylcholine and lysophosphatidylcholine at 30 s and 5 min stimulation.

7 These results suggest that lysophosphatidylcholines can increase Ca^{2+} sensitivity through a G-protein-independent, but a protein kinase C-dependent mechanism. However, the role for lysophosphatidylcholines as messengers causing Ca^{2+} sensitization during stimulation with noradrenaline remains uncertain because no increase in [^{3}H]-myristate labelled lysophosphatidylcholine could be measured during noradrenaline stimulation.

Keywords: Lysophosphatidylcholine; smooth muscle; rat mesenteric arteries; calcium sensitivity

Introduction

Elevations of extracellular and intracellular levels of lysophosphatidylcholine (lysoPC) have been suggested to play a role for muscle malfunction in conditions such as myocardial arrhythmias (DaTorre *et al.*, 1991), asthma and rhinitis (Mehta *et al.*, 1990) and atherosclerosis (Steinberg *et al.*, 1989). Thus one effect of lysoPC may be muscle hypercontractility. However, more dramatic changes in cell phenotype, for instance foam cell formation of macrophages in atherosclerosis, may also be the result of elevated lysoPC (Steinberg *et al.*, 1989).

Agonists, including endogenous neurotransmitters and hormones, contract smooth muscles by elevating intracellular Ca^{2+} and modulating the sensitivity to Ca^{2+} of the contractile proteins (Somlyo & Somlyo, 1994). The transmembrane and intracellular signalling pathways involved in modulating the Ca^{2+} sensitivity are not well defined. A recent study by Gong *et al.* (1992), suggested that arachidonic acid (AA) could be a messenger involved, and it was discussed whether phospholipase A_2 (PLA_2) activation by agonists could give an increase in AA levels. Other studies have shown that PLA_2 can be activated by agonists in smooth muscles (Yousufzai & Abdel-Latif, 1993; Lehman *et al.*, 1993; Resink *et al.*, 1993; Rao *et al.*, 1994). However, PLA_2 activity causes formation of two products, AA and lysophospholipids, and it is not known whether lysophospholipids affect Ca^{2+} sensitivity.

This was investigated in the present study by applying lysoPC to α-toxin-permeabilized rat mesenteric small arteries. Permeabilization allows intracellular calcium to be controlled, and changes in contraction at constant calcium can be taken as the result of a change in the Ca^{2+} sensitivity. Our results indicate that some lysoPCs but not others can increase the Ca^{2+} sensitivity, consistent with a potential messenger role for lysoPCs in modulating the Ca^{2+} sensitivity. On the other hand, no elevations in lysoPC levels were detected during stimulation with noradrenaline.

Methods

Preparation

Arteries from the mesenteric bed of 12–16 week old male Wistar rats killed with CO_2 were mounted as ring preparations in a myograph (Mulvany & Halpern, 1977). Second or third order branches of the mesenteric artery, ~200 μm in internal diameter, were used. The vessels were stretched as previously described for optimal active isometric force measurements (Mulvany & Warshaw, 1979). The physiological salt solution (PSS) contained (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, EDTA 0.026 and glucose 5.5. The pH of this solution was 7.45–7.50 when gassed with 5% CO_2:95% O_2. PSS with high potassium concentration (K-PSS, 125 mM K^+) was made by equimolar substitution of NaCl with KCl.

In arteries, where the endothelium was removed by putting a hair through the lumen and rubbing gently, successful re-

[1] Author for correspondence.

moval was judged by the lack of relaxing effect of 1 μM acetylcholine on contraction induced with 5 μM noradrenaline. Before removal, acetylcholine caused almost complete relaxation.

α-Toxin permeabilization

α-Toxin permeabilization of rat mesenteric small arteries was carried out as previously described (Jensen, 1994). Briefly, the mounted rings were incubated for 15–20 min in relaxing solution containing (mM): EGTA 2, potassium methane sulphonate 130, $MgCl_2$ 4, Tris maleate 20, Na_2ATP 4, creatine phosphate 10 and creatine phosphokinase, 1 mg ml^{-1}. pH was adjusted to 7.10 with KOH and the solution was gassed with 100% O_2. The concentration of free calcium, $[Ca^{2+}]_{free}$, could be increased in this solution by adding calcium from a 0.1 M $CaCl_2$ stock solution, and readjusting pH to 7.10. Calculations of $[Ca^{2+}]_{free}$ based on association constants from Tsien & Pozzan (1989) and Vianna (1975) were made in accordance with the temperature and pH of the solution. Then the relaxing solution was removed and 10 μl solution containing 1 μM $[Ca^{2+}]_{free}$ and 1000 u ml^{-1} α-toxin (GIBCO-BRL) was put on the artery. Ten to 20 min later, force had reached a stable level and the 10 μl medium was removed and relaxing solution added.

Measurement of phospholipids

[^{3}H]-myristate labelling Rat mesenteric arteries < 300 μm in internal diameter were labelled with [^{3}H]-myristate as previously described (Ward *et al.*, 1995). Briefly, 10 μCi [^{3}H]-myristate (specific activity 33.5 mCi $mmol^{-1}$ NEN-Dupont) was dried under a stream of O_2-free N_2 gas and redissolved in 300 μl tissue culture medium (M199) by bath sonication for 5 min. Vessels were incubated in the [^{3}H]-myristate/M199 solution for 3 h at 37°C, washed twice in 5 ml of M199 and transferred to 300 μl M199 for 10 min. Tissues were either not stimulated, or stimulated by the addition of prewarmed vehicle (M199) containing noradrenaline (final concentration 100 μM) for 30 s or 5 min.

Phospholipid extraction Following stimulation the phospholipids were extracted by a modification of the method described by Shaikh (1994). The tissues were homogenized in 0.4 ml ice-cold chloroform/methanol (2:1 by volume) containing the following standards: 0.5 mg egg phosphatidylcholine (PC), 0.5 mg soybean lysoPC (Sigma) and 10 μl (approximately 20000 d.p.m.) lysoPC, 1-[1-^{14}C]-palmitoyl (specific activity 56 mCi $mmol^{-1}$, Amersham) and left on ice for 10 min, 0.7 ml chloroform/methanol (2:1) and 0.3 ml 0.9% NaCl was added. The mixture was mixed and centrifuged at 12000 *g* for 5 min. The lower lipid containing organic phase was transferred to a glass vial, the residual aqueous phase and protein layer was washed once with 0.5 ml chloroform/methanol/0.58% NaCl (86:14:1 by volume), the lower phases were pooled and dried under a stream of O_2-free N_2 gas prior to storage at −20°C in chloroform/methanol (2:1) layered with O_2-free N_2. The residual protein pellet was dried, dissolved in 2 M NaOH and the protein content was determined (Lowry *et al.*, 1951).

Phospholipid separation PC and lysoPC were separated by high performance liquid chromatography (h.p.l.c.) using the method of Gross & Sobel (1980). Briefly, the samples were dried under a stream of O_2-free N_2 gas, redissolved in 20 μl chloroform/methanol (2:1), injected onto a Partisil SCX column (25 cm × 4.6 mm, Technicol U.K.) and eluted with a mobile phase of acetonitrile/methanol/water (70/23/6.5%) at 2.5 ml min^{-1}. Ultraviolet absorbance was monitored at 206 nm and the eluant was fractionated and collected at 20 s intervals. One ml scintillant (Ultima-Flo M, Canberra Packard, U.K.) was added to each tube and radioactivity determined by liquid scintillation. The data were analyzed using FLO-ONE\beta A500 software (Canberra Packard U.K.), and the amount of ^{3}H-labelled PC and lysoPC calculated from the area under the peak, adjusted for losses occurring during sample extraction and h.p.l.c. separation estimated from the recovery of the [^{14}C]-lysoPC standard. Sample recovery was 78 ± 2% (n = 10). The data are expressed as d.p.m. mg^{-1} protein.

Materials

Other drugs used were, noradrenaline-HCl, methane sulphonate, L-α-lysophosphatidylcholines (caproyl, decanoyl, lauroyl, myristoyl, palmitoyl, heptadecanoyl and stearoyl), L-α-phosphatidylcholine dipalmitoyl, L-α-glycerophosphorylcholine, guanosine 5′-triphosphate (GTP) and guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S) from Sigma. L-α-lysophosphatidylcholine (1-palmitoyl and 1-myristoyl) were obtained from Larodan (Sweden), and KOH (suprapur) from Merck. M199 tissue culture medium was from GIBCO-BRL, U.K., and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) and calphostin C were from Calbiochem.

Statistics

Results are shown as mean ± s.e.mean, n indicates number of artery segments, one artery segment per rat. For measurements of PC and lysoPC, larger samples of arteries were used, and in these experiments, n indicates number of rats. Statistical differences were tested by paired or unpaired, two-tailed, Student's t tests and were considered significant at $P < 0.05$. Contractile force is expressed as active wall tension, which is force divided by two times the artery segment length. Concentration-tension curves were analyzed by curve fitting (GraphPad Inplot 4.01, GraphPad Software Inc.) to determine the concentrations that elicited half maximal effects (pD_2 or EC_{50}).

Results

Characteristics of the Ca^{2+}-sensitizing effect of lysoPC in α-toxin-permeabilized arteries

Initial experiments showed that in α-toxin-permeabilized arteries maximal stimulation with 10 μM $[Ca^{2+}]_{free}$ elicited tension of 1.7 ± 0.1 N m^{-1} (n = 12). Stimulation with 1 μM $[Ca^{2+}]_{free}$ caused tension development of 0.7 ± 0.1 N m^{-1} (n = 12). On this submaximal tension elicited by 1 μM $[Ca^{2+}]_{free}$, the effect of lysoPC was assessed. First, palmitoyl or myristoyl lysoPC was tested. In Figure 1, a representative trace showing the effect of 50 μM palmitoyl lysoPC is depicted. This concentration elicited a near maximal effect (see concentration-tension curves described below). Palmitoyl and myristoyl lysoPC from two different sources (Sigma and Larodan) produced similar results. LysoPC had similar effects in arteries where the endothelium was removed prior to permeabilization. The increases in tension were 1.03 ± 0.11 N m^{-1} (n = 8, P = 0.00003) and 0.56 ± 0.08 N m^{-1} (n = 9, P = 0.0001) by palmitoyl and myristoyl lysoPC, respectively. The times to reach maximal effects (t_{max}) of palmitoyl and myristoyl lysoPC were 13 ± 3 min (n = 4) and 4 ± 0.5 min (n = 4), respectively. The times to reach half of the effect ($t_{1/2}$) were 3 ± 1 min and 2 ± 1 min, respectively.

Cumulative concentration-tension curves of palmitoyl and myristoyl lysoPC on submaximal contraction elicited by 1 μM $[Ca^{2+}]_{free}$ were obtained (Figure 2). The pD_2 values were 4.63 ± 0.06 (n = 6, mean EC_{50} = 24 μM) and 5.54 ± 0.03 (n = 4, mean EC_{50} = 3 μM), respectively. The effect of 50 μM palmitoyl and myristoyl lysoPC on calcium concentration-tension curves was also investigated (Figure 3). In control calcium concentration-tension curves pCa-EC_{50} was 6.10 ± 0.04 (n = 4, mean Ca^{2+}-EC_{50} = 794 nM). After 30 min treatment of relaxed arteries with the lysolipids, calcium concentration-tension curves were made. After treatment, pCa-EC_{50} values were

6.50 ± 0.05 ($n=4$, mean Ca^{2+}-$EC_{50}=314$ nM, $P=0.001$ compared to control) and 6.33 ± 0.05 ($n=4$, mean Ca^{2+}-$EC_{50}=470$ nM, $P=0.01$ compared to control) for palmitoyl and myristoyl lysoPC, respectively. The maximal contraction induced by Ca^{2+} was increased $70.0 \pm 7.3\%$ ($n=4$, $P=0.002$) and $57.0 \pm 8.3\%$ ($n=4$, $P=0.006$) in the presence of palmitoyl and myristoyl lysoPC, respectively.

Different lysoPCs were tested on submaximal tension elicited by 1 μM $[Ca^{2+}]_{free}$, and compared to the effect of palmitoyl and myristoyl lysoPC (Figure 4). The effect of caproyl, decanoyl, lauroyl, heptadecanoyl and stearoyl lysoPC was assessed. A concentration of 50 μM was used for all the lipids, because this concentration gave near maximal effect (see above for palmitoyl and myristoyl lysoPC, data not shown for the other lipids). As seen in Figure 4, palmitoyl and myristoyl lysoPC caused the largest increases in tension.

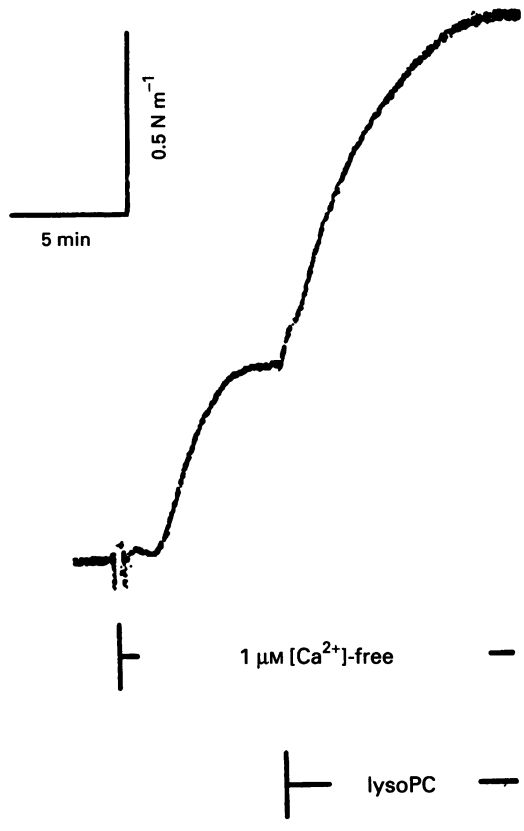

Figure 1 LysoPC enhances contraction at constant Ca^{2+} in α-toxin-permeabilized muscle: contraction was induced by increasing $[Ca^{2+}]_{free}$ to 1 μM. When tension was stable, 50 μM palmitoyl lysoPC was added.

Ca^{2+}-sensitization by lysoPC is not mediated by metabolic products of lysoPC

It is possible that the effect of lysoPC could be due to a metabolite of the added lipid. LysoPC may be converted to phosphatidylcholine (PC), or lysoPC could be degraded through several processes, and an active metabolite formed. Release of the free fatty acid would give palmitic or myristic acid if palmitoyl or myristoyl lysoPC was added. When the free fatty acid is released L-α-glycerophosphorylcholine would be formed. There is also the possibility that choline might be formed. There was no effect of 50 or 100 μM PC (L-α-phosphatidylcholine dipalmitoyl), palmitic or myristic acid, L-α-glycerophosphorylcholine or choline on tension elicited by 1 μM $[Ca^{2+}]_{free}$ ($n=3-4$, data not shown).

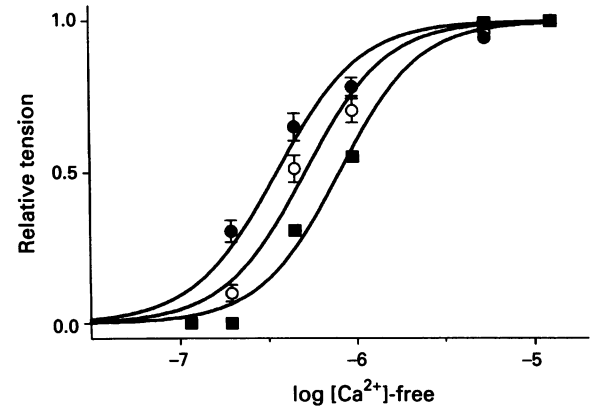

Figure 3 Effect of palmitoyl and myristoyl lysoPC on Ca^{2+} concentration-tension curves. Control Ca^{2+} concentration-tension curve (■) and curves in the presence of palmitoyl (●) or myristoyl (○) lysoPC are shown. $[Ca^{2+}]_{free}$ was increased cumulatively. Each pCa_{free} was maintained for 2 min if below contractile threshold or until a stable contraction was developed. Values are mean with s.e.mean, where they exceed the size of the symbol. Sigmoid curves are fitted using GraphPad Inplot 4.01, GraphPad software Inc.

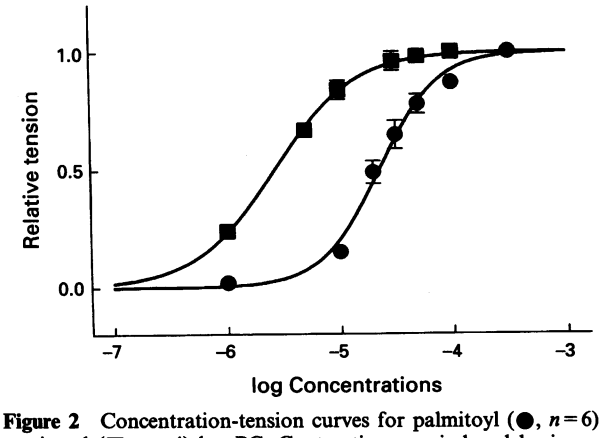

Figure 2 Concentration-tension curves for palmitoyl (●, $n=6$) and myristoyl (■, $n=4$) lysoPC. Contraction was induced by increasing $[Ca^{2+}]_{free}$ to 1 μM, and when tension stabilized, lysoPC concentrations were increased cumulatively. Values are mean with s.e.mean, where they exceed the size of the symbol.

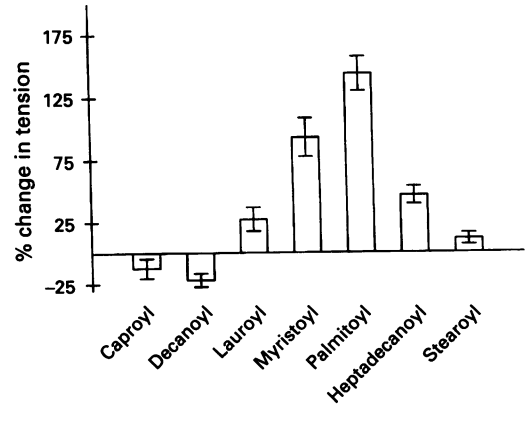

Figure 4 Effect of different lysoPCs on contraction induced by 1 μM $[Ca^{2+}]_{free}$. Protocol for experiments was as shown in Figure 1, and a concentration of 50 μM of all the lysoPCs was used. The saturated fatty acids in lysoPC are indicated. Columns show mean ± s.e.mean, $n=4-9$.

Ca^{2+}-sensitization by lysoPC is not caused by activation of G-proteins

To show that there were functional G-proteins and functional receptor coupling involving G-proteins in the α-toxin-permeabilized rat mesenteric arteries, the effect of GTPγS and of noradrenaline and GTP was assessed. Addition of 50 μM GTPγS on tension elicited by 1 μM $[Ca^{2+}]_{free}$ caused a 145±11% ($n=4$, $P=0.0009$) increase in tension. Noradrenaline concentrations of 10 and 100 μM had no effect on calcium concentration-tension curves. Preliminary experiments, where increasing concentrations of GTP were added to tension elicited by 1 μM $[Ca^{2+}]_{free}$, showed that GTP concentrations below 0.6 μM had no effect but higher concentrations increased tension. When relaxed arteries were treated with 10 μM noradrenaline and 0.6 μM GTP for 10–15 min followed by calcium concentration-tension curves, there was an increase in Ca^{2+} sensitivity (0.12±0.01, $n=4$, $P=0.001$, increase of pCa-EC_{50} in the presence of noradrenaline and GTP compared to controls). There was also a 60.4±5.2% ($n=4$, $P=0.001$) increase in maximal contraction. Eight arteries were treated with noradrenaline and GTP followed by stimulation with 1 μM $[Ca^{2+}]_{free}$. The steady-state tension elicited was 1.5±0.1 N m^{-1}. Addition of 0.3 mM GDP-β-S caused a decrease in tension to 0.8±0.1 N m^{-1} ($P=0.0007$). A representative recording is shown in Figure 5. The effect of GTPγS, noradrenaline and GTP and GDP-β-S indicated that there was functional G-proteins and functional receptor coupling involving G-proteins. However, as seen in Figure 5, myristoyl lysoPC, when added after the inhibition with GDP-β-S, still increased tension. A similar effect was found with palmitoyl lysoPC. However, the time course of the enhancement of force was slower than observed when adding lysoPC directly to a Ca^{2+}-induced contraction. The increases in tension were 2.1±0.1 N m^{-1} ($n=4$, $P=0.0006$) and 1.2±0.1 N m^{-1} ($n=4$, $P=0.001$) for palmitoyl and myristoyl lysoPC, respectively.

Calphostin C can inhibit Ca^{2+}-sensitization by lysoPC

To assess the effect of calphostin C, a relatively specific protein kinase C (PKC) inhibitor, on the sensitization induced by lysophosphatidylcholine, arteries were first contracted with 1 μM $[Ca^{2+}]_{free}$. When steady state tension was reached, arteries were incubated in relaxing solution. Then they were treated for 1 h with 1 μM calphostin C, and contracted again with 1 μM $[Ca^{2+}]_{free}$. There was no difference between the contractions before and after treatment with calphostin C (−0.44±0.31 N m^{-1}, $n=7$, $P=0.20$). After this treatment with calphostin C, the effect of lysoPC was assessed in 4 arteries; 50 μM myristoyl or palmitoyl lysoPC caused no increase in force, when added to the steady state contraction induced by 1 μM $[Ca^{2+}]_{free}$ (a 0.03±0.01 N m^{-1} change in contraction, $P=0.09$).

The effect of noradrenaline on [^{3}H]-lysophosphatidylcholine levels

[^{3}H]-myristate was used to radiolabel preferentially PC in intact rat mesenteric arteries (Ward *et al.*, 1995). Separation of the labelled compounds by h.p.l.c. revealed a major peak of radioactivity eluting with egg PC standard. The elution time of egg-PC was 7.5–15 min and of [^{3}H]-PC was 9.7–16.7 min with a similar difference in peak retention time, egg-PC 11 min and [^{3}H]-PC 13.7 min, reflecting differences in the fatty acid side-chains of the PC species. Two peaks of radioactivity were detected eluting with the soybean lysoPC standard. The later peak eluted immediately after the [^{14}C]-palmitic-lysoPC standard, retention time of the ^{14}C standard 23.3 min and of the [^{3}H]-myristate containing peak 25 min demonstrating slight differences in the retention times of these species of lysoPC. The earlier peak of ^{3}H eluted immediately after the minor peak of activity present in the ^{14}C standard (retention times ^{14}C 21.3 min and ^{3}H 22 min) identifying this peak as the lysoPC isomer 2-[^{3}H]-myristate-lysoPC. [^{3}H]-myristate incorporation into 1-lysoPC was approximately 3 fold greater than into 2-lysoPC (Table 1), suggesting that this is the major isomer of lysoPC in intact vascular tissue. To confirm this identification, small arteries were radiolabelled with [^{3}H]-palmitic acid and the phospholipids separated by h.p.l.c. Again, two peaks of radioactivity were detected coeluting with the soybean lysoPC standard and also coeluting with the [^{14}C]-palmitic-lysoPC standard (retention time of peak 1: ^{14}C 21.3 min and ^{3}H 21 min; and peak 2: ^{14}C 23.3 min and ^{3}H 23 min).

The effect of maximal stimulation with noradrenaline on [^{3}H]-PC and [^{3}H]-lysoPC was assessed. The data are summarized in Table 1. There was no significant change in the levels of the [^{3}H]-phospholipids during 30 s or 5 min stimulation.

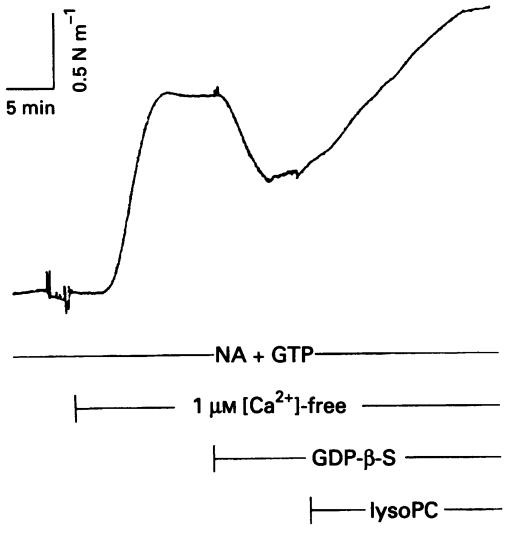

Figure 5 GDP-β-S inhibits Ca^{2+} sensitization by noradrenaline but not by lysoPC. The artery was pretreated with 10 μM noradrenaline and 0.6 μM GTP for 15 min, and $[Ca^{2+}]_{free}$ was increased to 1 μM in the continuous presence of noradrenaline and GTP. When tension stabilized, 0.3 mM GDP-β-S was added, and when the decrease in tension stabilized, 50 μM myristoyl lysoPC was added.

Discussion

The main finding of our study is that some lysoPCs enhanced smooth muscle force at constant calcium and increased the sensitivity of contraction to calcium. Both of these results will be referred to as an increase in Ca^{2+} sensitivity of contraction. This Ca^{2+} sensitizing effect of lysoPC is consistent with a messenger role for lysoPC.

To assess whether the effect of lysoPC was direct or mediated by a cellular modified product of lysoPC, PC and some possible metabolites were tested. However, there were no effects of PC, or of the other possible metabolites assessed. This indicates that lysoPC itself may mediate the effect.

Both noradrenaline and lysoPC could further increase the maximal Ca^{2+}-induced contractions in the rat mesenteric small arteries. A similar ability to enhance maximal Ca^{2+}-induced contractions has been reported in phasic rabbit smooth muscles, but not in tonic muscles (Kitazawa *et al.*, 1991), and it was suggested that there may be a difference in the regulatory and contractile properties, probably a different MLC_{20} kinase/phosphatase ratio, in the two type of muscles. However, it is not known how the rat mesenteric arteries compare with the rabbit muscles, but the results indicate that they function more like the phasic muscles with respect to Ca^{2+} sensitization of maximal Ca^{2+}-induced force.

It is generally accepted that the increase in Ca^{2+}-sensitivity

Table 1 The effect of noradrenaline on [^{3}H]-myristate-lysophosphatidylcholine in rat small arteries

Treatment	*[^{3}H]-PC*	*1-[^{3}H]-lysoPC*	*2-[^{3}H]-lysoPC*
Basal	$4.2 \times 10^6 \pm 0.3 \times 10^6$	11379 ± 511	4113 ± 266
Noradrenaline 30 s	$4.5 \times 10^6 \pm 0.6 \times 10^6$ ($P=0.73$)	9532 ± 1253 ($P=0.76$)	3915 ± 479 ($P=0.36$)
Noradrenaline 5 min	$5.6 \times 10^6 \pm 0.8 \times 10^6$ ($P=0.25$)	12147 ± 1651 ($P=0.84$)	4325 ± 371 ($P=0.74$)

[^{3}H]-myristate-labelled arteries were stimulated with 100 μM noradrenaline for 30 s or 5 min. Phospholipids were extracted from the tissue and [^{3}H]-PC and [^{3}H]-lysoPC were separated by h.p.l.c. as described in Methods. Results are expressed as d.p.m. mg^{-1} protein and are mean ± s.e.mean. For measurements of basal levels of PC and lysoPCs n was 4, and n was 3 for all the measurements in the presence of noradrenaline. P values for t tests of stimulated vs basal levels are shown in parentheses.

caused by agonists in smooth muscle involves activation of G-proteins (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1991; Somlyo & Somlyo, 1994). The evidence for this is that agonists need the presence of GTP to have a sensitizing effect in permeabilized muscles, and the sensitizing effect can be antagonized by GDP-β-S. Furthermore, GTPγS causes increase in Ca^{2+} sensitivity presumably through activation of G-proteins (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1991). The results in the present study are compatible with a receptor and G-protein coupled increase in Ca^{2+} sensitivity during stimulation with noradrenaline. LysoPC might also act through a membrane receptor and G-proteins. However, the Ca^{2+} sensitizing effect of LysoPC did not require the presence of GTP, and in the presence of GDB-β-S lysoPC could still induce an enhancement of force at constant Ca^{2+}. These results indicate that lysoPC was not acting through a G-protein-dependent mechanism. It was noted that the lysoPCs caused greater sensitization in the presence of GDB-β-S, but there is no obvious explanation for this. A speculative hypothesis may be that in addition to the sensitizing effect, lysoPCs may activate a G-protein coupled desensitizing mechanism, which is eliminated by GDP-β-S allowing development of the maximal sensitizing response.

LysoPC has in cell-free assays been shown to cause activation of protein kinase C (PKC) (Oishi *et al.*, 1988), and it has been implied that activation of PKC is involved in agonist-induced Ca^{2+} sensitization in smooth muscle (Drenth *et al.*, 1989; Ruzycky & Morgan, 1989). Furthermore, lysoPC has been suggested to cause T-lymphocyte activation through an increase in PKC activity (Asaoka *et al.*, 1992). Thus, it is possible that lysoPC has a similar ability to activate PKC in smooth muscle. That calphostin C abolished the tension development induced by lysoPC in the permeabilized arteries is consistent with activation of PKC being involved in the sensitization.

In smooth and skeletal muscle, about 50% of the phospholipids are PC (Pearce *et al.*, 1981; Ford & Gross, 1989), and it is generally accepted that PC in position 1 normally contains a saturated fatty acid and in position 2 an unsaturated. The most abundant saturated fatty acid in muscle PC is palmitic acid, with stearic acid also being a significant constituent (Pearce *et al.*, 1981; Ford & Gross, 1989). Thus, PLA_2 cleavage of PC is expected to give lysoPC containing primarily palmitic or stearic acid. This expectation has been confirmed by treating smooth muscle PC with PLA_2 and analyzing the lysoPC produced (Ford & Gross, 1989). Furthermore, quantitation of endogenous lysoPC species in cardiac muscle also showed that lysoPCs containing palmitic or stearic acid were the most abundant (Vesterquist *et al.*, 1992). In the present study, the lysoPC causing the largest contraction contained palmitic acid, with smaller effects of lysoPC containing myristic or stearic acid. Thus, the lysoPCs having effect are physiological relevant species.

Because the Ca^{2+}-sensitizing effect of lysoPC was consistent with a potential messenger role, and since stimulation of T-lymphocytes had shown that the level of lysoPC was increased (Asaoka *et al.*, 1992), a similar elevation by, for instance, noradrenaline in the small arteries used in the present study would substantiate a role for lysoPC as a messenger. To address this question, we used [^{3}H]-myristate to label the phospholipids. We have shown previously that in mesenteric small arteries [^{3}H]-myristate was preferentially incorporated into PC and that the pool of labelled PC is available for noradrenaline-stimulated phospholipase D hydrolysis (Ward *et al.*, 1995). Chromatographic separation showed that [^{3}H]-myristate was incorporated into both the 1 and 2 position on the glycerol backbone although 1-acyl-lysoPC was the predominant isomer present. Stimulation with noradrenaline for 30 s or 5 min did not alter the levels of [^{3}H]-lysoPC in the tissue extracts.

These results may imply that noradrenaline does not stimulate PLA_2-mediated hydrolysis of PC in rat mesenteric arteries. This observation is contrary to reports in Madin-Darby canine kidney (MDCK) cells and FRTL5 thyroid cells where α_1-adrenoceptor stimulation increased PLA_2 activity (Burch *et al.*, 1986; Weiss & Insel, 1991). These studies utilized arachidonic acid release as an indicator of PLA_2 activity and measurement of lysophospholipids in MDCK cells demonstrated an increase in lysophosphatidylethanolamine, but not lysoPC, following stimulation with adrenaline. However, bradykinin did increase lysoPC production in these cells (Weiss & Insel, 1991). Therefore, it is possible that noradrenaline activates PLA_2 with phosphatidylethanolamine, and also phosphatidylserine and phosphatidylinositol are possibilities, as substrates. Another explanation for our inability to detect a rise in [^{3}H]-lysoPC is that it could be released from the tissue into the surrounding medium. Indeed stimulation with thrombin has been reported to increase the release of lysoPC from endothelial cells (McHowatt & Corr, 1993). We were unable to detect reliably [^{3}H]-lysoPC in the medium bathing the tissue, most probably reflecting the low levels of this lipid in rat mesenteric small arteries, and therefore we could not explore this possibility further. Other vasoconstrictor hormones such as endothelin, vasopressin and angiotensin II have been shown to activate PLA_2 in smooth muscle (Yousufzai & Abdel-Latif, 1993; Lehman *et al.*, 1993; Resink *et al.*, 1993; Rao *et al.*, 1994), and it is possible that they also may do so in rat mesenteric small arteries, but this needs to be demonstrated.

As regards pathophysiology, the results in the present study indicate that an effect of increased levels of lysoPC could be muscle cell hypercontractility caused by the increase in Ca^{2+} sensitivity. Interestingly, muscle cell hypercontractility has been indicated in both atherosclerosis (Shimokawa *et al.*, 1983; Heistad *et al.*, 1984; Galle *et al.*, 1990) and asthma and rhinitis (Mehta *et al.*, 1990). LysoPC may also affect Ca^{2+} homeostasis in intact cells (Sachinidis *et al.*, 1989; Bochov *et al.*, 1992; Weisser *et al.*, 1993; Stoll & Spector, 1993), intracellular pH (Sachinidis *et al.*, 1989) and phosphoinositide catabolism (Bochov *et al.*, 1992; Resink *et al.*, 1989), which may all contribute to muscle cell hypercontractility. Another effect of lysoPC which has received much attention in several studies is

the ability to impair endothelial cell function in the vascular system (Yokoyama *et al.*, 1990; Kugiyama *et al.*, 1990). Thus, lysoPC seems to have effects on many essential parameters affecting muscle contractility indicating that a precise regulation of extracellular and intracellular levels of lysoPC is of crucial importance.

In summary, lysoPC could increase the Ca^{2+} sensitivity of contraction in α-toxin-permeabilized rat mesenteric small arteries through a mechanism that did not involve G-proteins but probably involves PKC. This is consistent with a potential role for lysoPC as a second messenger causing increases in the Ca^{2+} sensitivity of the contractile proteins during agonist stimulation. However, noradrenaline had no effects on lysoPC levels, which questions this potential messenger role.

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