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Calphostin C-sensitive enhancements of force by lysophosphatidylinositol and diacylglycerols in mesenteric arteries from the rat

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1 A pharmacological characterization was made of the effects of lysophosphatidyl-inositol (lysoPI) and -ethanolamine (lysoPE) on the Ca^{2+}-sensitivity of contraction in α-toxin permeabilized rat mesenteric arteries. The effect of GTPγS (G-protein activator), diacylglycerols (DAGs, dioctanoyl glycerol (diC_8) and 1-stearoyl-2-arachidonoyl-sn-glycerol) and phorbol myristate acetate (PMA, protein kinase C (PKC) activator) on Ca^{2+}-sensitivity was also assessed.

2 LysoPI increased the Ca^{2+}-sensitivity, demonstrated by both an increase in tension induced by 1 μM $[Ca^{2+}]_{free}$ and an increase in the Ca^{2+}-sensitivity of Ca^{2+} concentration-tension curves. LysoPE did not enhance force or Ca^{2+}-sensitivity.

3 GTPγS enhanced force at constant Ca^{2+}, increased the Ca^{2+}-sensitivity, and increased force under Ca^{2+}-free conditions. PMA also increased force at constant Ca^{2+} and increased Ca^{2+}-sensitivity, but caused no force development under Ca^{2+}-free conditions.

4 DAGs, both diC_8 and the more physiological relevant DAG, 1-stearoyl-2-arachidonoyl-sn-glycerol, enhanced force at constant Ca^{2+} and increased the Ca^{2+}-sensitivity. DiC_8, in contrast to 1-stearoyl-2-arachidonoyl-*sn*-glycerol, caused force development under Ca^{2+}-free conditions and substantially enhanced force at maximal Ca^{2+}-induced contraction. GDP-β-S abolished the increased Ca^{2+}-sensitization induced by noradrenaline, but not that by DAGs.

5 The PKC inhibitor calphostin C completely abolished Ca^{2+}-sensitization induced by all of the Ca^{2+}-sensitizing agents.

6 These results show that lysoPI can increase the Ca^{2+}-sensitivity of smooth muscle contraction, and the Ca^{2+}-sensitization induced by DAGs was not completely G-protein mediated, because it was not inhibited by GDP-β-S. A central role for PKC in regulation of Ca^{2+}-sensitization in rat mesenteric small arteries was indicated by the abolishment of Ca^{2+}-sensitization by calphostin C.

Keywords: Calcium; smooth muscle; contraction; protein kinase C; noradrenaline; G-protein; phorbol ester

Introduction

Neurotransmitters, hormones and pharmacological agonists cannot only cause contraction of smooth muscles by increasing free cytosolic calcium, but also by increasing the Ca^{2+}-sensitivity of the contractile proteins (Somlyo & Somlyo, 1994).

Although the transmembrane and intracellular signalling pathways involved in the Ca^{2+}-sensitization are not known in detail, some potentially important steps have been identified. Binding of an agonist to its receptor is believed to cause activation of membrane-associated, trimeric G-proteins (Somlyo & Somlyo, 1994). The next step may be activation of the phospholipases C (PLC), D (PLD) or A_2 (PLA_2), which results in release of potential lipid messengers. Most focus has been on products of PLC and PLA_2 activity for involvement in Ca^{2+}-sensitization. Cleavage of membrane lipids by PLC causes release of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), and cleavage by PLA_2 releases arachidonic acid (AA) and lysophospholipids. IP_3 has long been recognized to be involved in release of Ca^{2+} from intracellular stores (Somlyo & Somlyo, 1994), but more direct evidence for contractile effects of the other metabolites has only recently been obtained. Addition of synthetic DAG (Masuo *et al.*, 1994; Sohn *et al.*, 1994), AA (Gong *et al.*, 1992) or some lysophosphatidylcholines (Jensen *et al.*, 1996) to vascular smooth muscle does indeed increase the Ca^{2+}-sensitivity.

The steps downstream from these messengers probably involve various mechanisms. AA has been suggested to inhibit directly the phosphatase that dephosphorylates the myosin light chains (MLC_{20}-phosphatase) (Gong *et al.*, 1992). DAG, AA and lysophospholipids can all activate protein kinase C in cell-free assays (Oishi *et al.*, 1988, see Stabel & Parker, 1991; Burns & Bell, 1992), and PKC activation has been suggested to be involved in agonist-induced Ca^{2+}-sensitization in smooth muscle (Nishimura *et al.*, 1988; Ruzycky & Morgan, 1989; Drenth *et al.*, 1989). However, none of these potential messengers has definitively been shown to be part of the physiological signalling pathways.

The aim of the present study was to characterize further the contractile effects of DAG and lysophospholipids, and to evaluate the role of PKC in Ca^{2+}-sensitization, by the use of putative inhibitors of PKC, in α-toxin permeabilized small mesenteric arteries from the rat. The results indicate that DAG and L-α-lysophosphatidylinositol (lysoPI), but not L-α-lysophosphatidylethanolamine (lysoPE), increased the Ca^{2+}-sensitivity. Furthermore, guanosine 5'-O-(3-thiotriphosphate) (GTPγS) and phorbol 12-myristate 13-acetate (PMA) also increased the Ca^{2+}-sensitivity. The PKC inhibitor calphostin C completely abolished the Ca^{2+}-sensitizing effect of DAG, lysophospholipids, GTPγS and phorbol esters.

Methods

Preparation

Small mesenteric arteries (i.d. ~200 μm) from rats killed with CO_2 were dissected and mounted in an isometric myograph (JP Trading, Denmark). The arteries were kept in physiological salt solution (PSS, composition see below) at room tempera-

ture during dissection and mounting. The arteries were stretched to near-optimal active force measurements. Before permeabilization, the arteries were stimulated twice with PSS containing high potassium (K-PSS, 145 mM K^+, composition see below) to ascertain the viability of the preparation.

α-Toxin permeabilization

α-Toxin permeabilization was made as described previously (Jensen, 1994). Briefly, the arteries were incubated for 15–20 min in relaxing solution (for composition see below). The concentration of free calcium, $[Ca^{2+}]_{free}$, could be varied in this solution by adding calcium from a 0.1 M $CaCl_2$ stock solution, and readjusting pH to 7.10. When tension had stabilized, the relaxing solution was removed and the artery was incubated in 10 μl solution containing 1 μM $[Ca^{2+}]_{free}$ and 1000 u ml^{-1} α-toxin (GIBCO-BRL). Ten to 20 min later, when force had reached a stable level, the solution was replaced by relaxing solution. All experiments with permeabilized arteries were performed at room temperature (22–25°C). During experimental protocols with permeabilized arteries the resting arteries were kept in the calcium-free relaxing solution.

Solutions

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. K-PSS was made by equimolar substitution of NaCl with KCl. The relaxing solution contained (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 by KOH and the solution was gassed with 100% O_2.

Chemicals and drugs

Noradrenaline-HCl (NA), methane sulphonate, L-α-lysophosphatidylcholine (myristoyl, lysoPC), phorbol myristate acetate (PMA), phorbol 12,13-dibutyrate (PDBu) and GTPγS, guanosine 5′-triphosphate (GTP), lysoPI, lysoPE (palmitoyl) were from Sigma. KOH (suprapur) was obtained from Merck. Guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S), *sn*-1,2-dioctanoyl-glycerol (diC_8), 1-stearoyl-2-arachidonoyl-*sn*-glycerol and calphostin C were from Calbiochem. The drugs were dissolved in water, 96% alcohol or dimethylsulphoxide (DMSO). The final concentrations of alcohol or DMSO were kept below 0.5% with no effect on contractions.

Statistics

Data are shown as mean ± s.e.mean, n indicates number of arteries. Statistical difference was tested by paired or unpaired, two-tailed, Student's t-tests, and differences were considered significant when $P<0.05$. Concentration-tension curves were analyzed by curve fitting (GraphPad Inplot 4.01, GraphPad Software Inc.) to determine the half maximal effective concentrations (pD_2 or EC_{50}). Force is expressed as active wall tension, which is force divided by twice the artery segment length.

Results

Noradrenaline sensitization requires presence of GTP, and GDP-β-S inhibits the sensitization

To demonstrate a functional receptor coupling in the α-toxin permeabilized arteries, the effect of NA and GTP was assessed. As previously described, NA alone does not affect Ca^{2+}-induced contractions in the permeabilized arteries (Jensen, 1994; Jensen *et al.*, 1996). Furthermore, GTP in concentrations at or below 0.6 μM also had no effect on Ca^{2+}-sensitivity in permeabilized arteries. However, as shown in Figure 1, 100 μM NA added in the presence of 0.6 μM GTP caused an increase in tension in arteries activated by 1 μM $[Ca^{2+}]_{free}$. This increase was 0.60 ± 0.07 N m^{-1} ($n=10$, $P<0.0001$). The time to reach steady-state (maximal) increase (t_{max}) was 34 ± 2 min ($n=10$), and the time to half maximal effect ($t_{1/2}$) was 13 ± 1 min ($n=10$). NA and GTP added to arteries incubated in relaxing solution ($n=4$) caused no change in tension.

The effect of GDP-β-S was assessed in arteries activated with 1 μM $[Ca^{2+}]_{free}$ after preincubation with 10 μM NA and 0.6 μM GTP for 15 min. When the tension was stable 0.3 mM GDP-β-S was added. As shown in Figure 1b, GDP-β-S inhibited the tension to a level comparable to the tension in the absence of NA and GTP ($n=8$).

Direct stimulation of G-proteins causes sensitization

GTPγS (50 μM) added to steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$ caused a rapid and large increase in tension (Figure

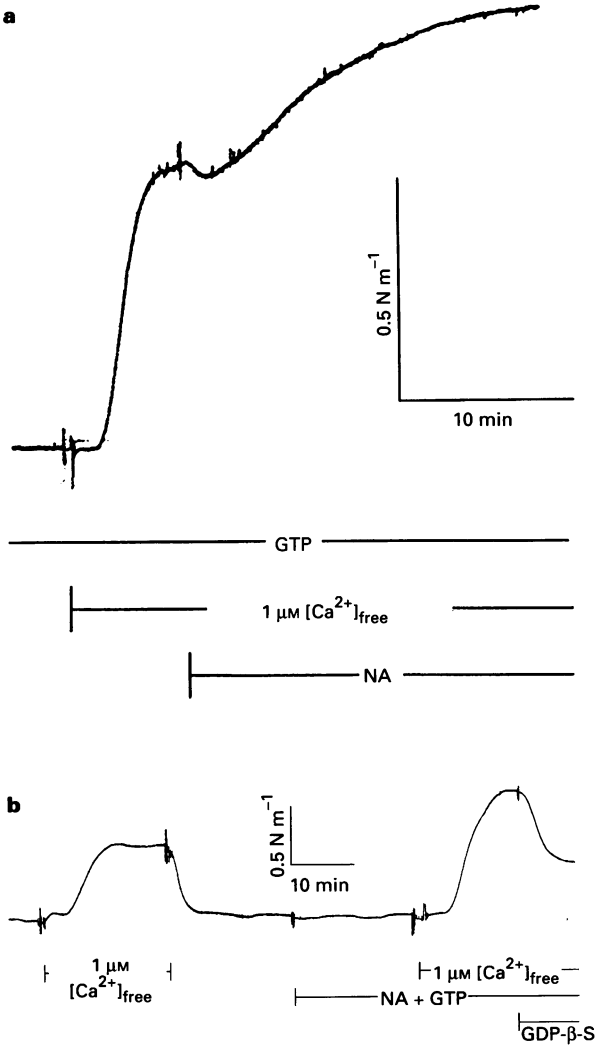

Figure 1 Enhancement of tension at constant $[Ca^{2+}]_{free}$ by noradrenaline (NA; a, $n=10$), and inhibition of the enhancement by GDP-β-S (b, $n=8$) in α-toxin permeabilized mesenteric arteries. In (a), contraction was induced by 1 μM $[Ca^{2+}]_{free}$ after incubation of the permeabilized muscle for ~15 min with 0.6 μM GTP. When tension stabilized 100 μM NA was added. In (b), a control contraction induced by 1 μM $[Ca^{2+}]_{free}$ was made first. Under relaxed conditions, the muscle was then incubated for ~15 min with 100 μM NA and 0.6 μM GTP, before contraction was induced again by 1 μM $[Ca^{2+}]_{free}$. When tension stabilized 0.3 mM GDP-β-S was added.

2a). The increase in tension was 1.61 ± 0.27 N m^{-1} ($n=4$, $P=0.009$), and t_{max} and $t_{1/2}$ were 6 ± 1 min ($n=4$) and 1.5 ± 0.5 min ($n=4$), respectively. Cumulative concentration-tension curves, made by adding increasing concentrations of GTPγS to steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$, gave a pD_2 value of 5.70 ± 0.06 ($n=6$, mean $EC_{50}=2$ μM), and the maximal effect was reached by 10 μM. The effect of 100 μM GTPγS on Ca^{2+} sensitivity was assessed by performing two Ca^{2+} concentration-tension curves, the second after 15 min incubation with GTPγS. $[Ca^{2+}]_{free}$ was cumulatively increased from nominally calcium-free conditions to 13 μM. Each $[Ca^{2+}]_{free}$ was maintained until stable contractions developed or for at least 2 min. Treatment with GTPγS caused a significant increase in the Ca^{2+}-sensitivity (0.16 ± 0.02 ($n=4$, $P=0.004$) increase of pCa-EC_{50}). Addition of GTPγS to resting arteries caused a contraction of 0.35 ± 0.04 Nm^{-1} ($n=4$, $P=0.003$), and the maximal Ca^{2+}-induced contraction increased by 1.72 ± 0.05 N m^{-1} ($n=4$, $P<0.0001$).

Diacylglycerols can cause sensitization

The response to diC_8 in a permeabilized artery is shown in Figure 3. Contraction was induced by 1 μM $[Ca^{2+}]_{free}$, followed by addition of 100 μM diC_8. The increase in tension was 1.33 ± 0.17 N m^{-1} ($n=4$, $P=0.004$), and t_{max} and $t_{1/2}$ were 20 ± 3 min ($n=4$) and 5 ± 0.5 min ($n=4$), respectively. Concentration-tension curves made by cumulative addition of diC_8 on steady-state tension elicited by 1 μM $[Ca^{2+}]_{free}$ showed concentration-dependent enhancement of tension ($n=4$, Figure 3b). A concentration of 5 μM was effective, but the highest concentration used (100 μM) did not elicit maximal response. The effect of diC_8 on Ca^{2+} concentration-tension curves was assessed by adding 100 μM diC_8 for 15 min before repeating the Ca^{2+} concentration-tension curve (and maintaining it in the bath during the second curve). There was a significant increase in the Ca^{2+}-sensitivity (0.62 ± 0.01 ($n=4$, $P<0.0001$) increase in pCa-EC_{50} by diC_8, Figure 4b). DiC_8 induced con-

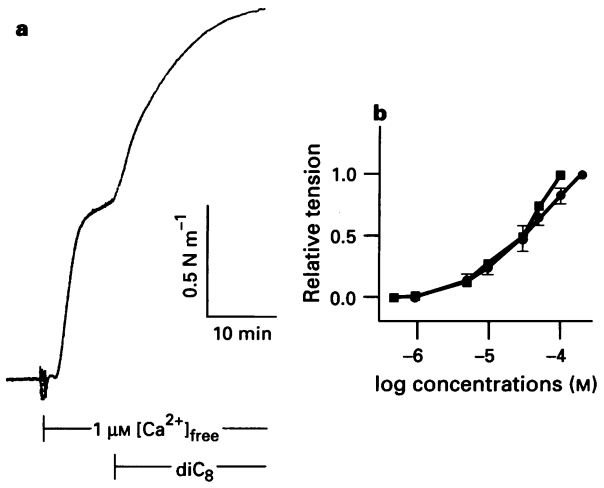

Figure 3 Enhancement of tension by dioctanoyl glycerol (diC_8; a, $n=4$) and cumulative concentration-tension curves for diC_8 ($n=4$) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol ($n=3$) (b). In (a), tension was induced by 1 μM $[Ca^{2+}]_{free}$ and when tension stabilized 100 μM diC_8 was added. In (b), summarized data from cumulative diC_8 (■) and 1-stearoyl-2-arachidonoyl-*sn*-glycerol (●) concentration-tension curves are shown. The arteries were contracted by 1 μM $[Ca^{2+}]_{free}$ and when tension stabilized either diC_8 or 1-stearoyl-2-arachidonoyl-*sn*-glycerol was added cumulatively up to concentrations of 100 and 200 μM, respectively.

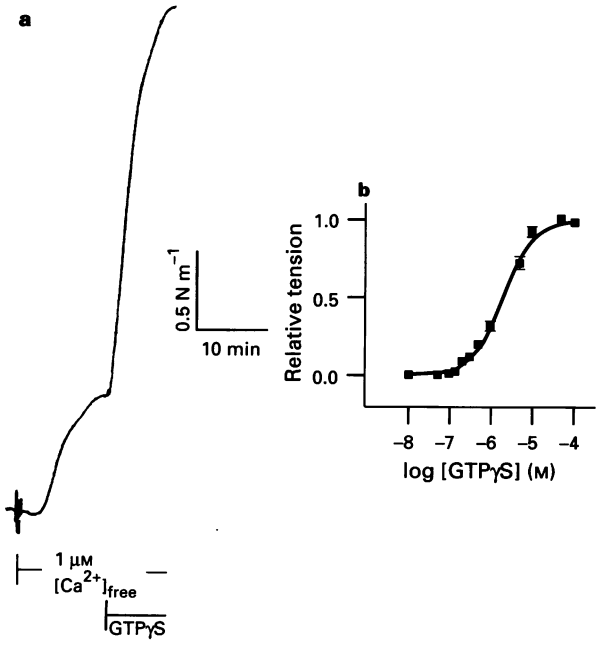

Figure 2 Enhancement of tension at constant $[Ca^{2+}]_{free}$ by GTPγS (a, $n=4$) and concentration-tension curve for GTPγS (b, $n=6$). In (a), contraction was induced by 1 μM $[Ca^{2+}]_{free}$, and when tension stabilized 50 μM GTPγS was added. In (b), summarized data from cumulative GTPγS concentration-tension curves are shown. The arteries were contracted by- 1 μM $[Ca^{2+}]_{free}$ and when tension stabilized GTPγS was added cumulatively up to a concentration of 100 μM.

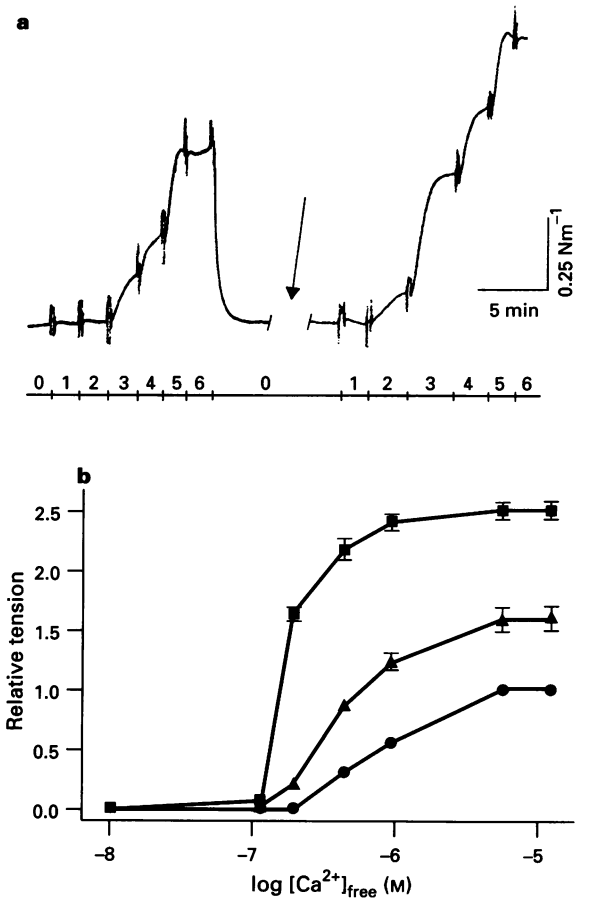

Figure 4 1-Stearoyl-2-arachidonoyl-*sn*-glycerol increased the Ca^{2+}-sensitivity (a, $n=4$) and effects of 1-stearoyl-2-arachidonoyl-sn-glycerol (▲, $n=4$) and diC_8 (■, $n=4$) on Ca^{2+} concentration-tension curves (b). In (a), a control Ca^{2+} concentration-tension was made first by cumulative increases in $[Ca^{2+}]_{free}$. The artery was then relaxed by low $[Ca^{2+}]_{free}$, and 100 μM 1-stearoyl-2-arachidonoyl-*sn*-glycerol was added for 15 min (arrow). In the continuous presence of 1-stearoyl-2-arachidonoyl-*sn*-glycerol, the cumulative Ca^{2+} concentration-tension curve was repeated. In (b), the data from identical experiments with 1-stearoyl-2-arachidonoyl-*sn*-glycerol and diC_8 (100 μM) are shown in comparison to the control Ca^{2+} concentration-tension curves (●, $n=8$).

tractions in resting arteries, (0.17 ± 0.05 N m^{-1} ($n=8$, $P=0.01$)), and the maximal Ca^{2+}-induced tension increased by 2.13 ± 0.07 N m^{-1} ($n=4$, P = 0.001).

Experiments were also made with another diacylglycerol, 1-stearoyl-2-arachidonoyl-*sn*-glycerol. Cumulative addition of 1-stearoyl-2-arachidonoyl-*sn*-glycerol to steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$ caused a concentration-dependent enhancement of tension (Figure 3b). As with diC_8, tension was affected by concentrations from 5 μM and the highest concentration used (200 μM) did not elicit maximal responses. The steady state increase in tension induced by 100 μM was 0.42 ± 0.06 N m^{-1} ($n=3$, $P=0.02$). The effect of 100 μM 1-stearoyl-2-arachidonoyl-sn-glycerol on the Ca^{2+} concentration-tension curve was assessed as described above for diC_8 (Figure 4a). 1-Stearoyl-2-arachidonoyl-*sn*-glycerol caused a significant increase in the Ca^{2+}-sensitivity (0.27 ± 0.01 ($n=4$, $P<0.0001$) increase in pCa-EC_{50} by the presence of 1-stearoyl-2-arachidonoyl-sn-glycerol, Figure 4b). This increase was significantly smaller than that to 100 μM diC_8 ($P<0.0001$). There were no significant contractions induced by 1-stearoyl-2-arachidonoyl-*sn*-glycerol in resting arteries (0.03 ± 0.01 N m^{-1} ($n=4$, $P=0.06$)). The maximal Ca^{2+}-induced tension increased by 0.60 ± 0.13 N m^{-1} ($n=4$, $P=0.02$), which was significantly less than the increase to diC_8 ($P<0.0001$).

The ability of GDP-β-S to affect the sensitization induced by diC_8 was also assessed (Figure 5). The initial part of the protocol for these experiments was identical to that in Figure 1b. Firstly, 100 μM NA and 0.6 μM GTP were added for 10 min and then $[Ca^{2+}]_{free}$ was elevated to 1 μM. When this agonist-sensitized response was stable, 0.3 mM GDP-β-S was added. This caused a decrease in tension, and when the tension was stable 100 μM diC_8 was added. The increase in tension induced by diC_8 was 2.67 ± 0.16 N m^{-1} ($n=3$, $P=0.0001$). This increase was greater than that obtained above in the absence of GDP-β-S ($P=0.0001$).

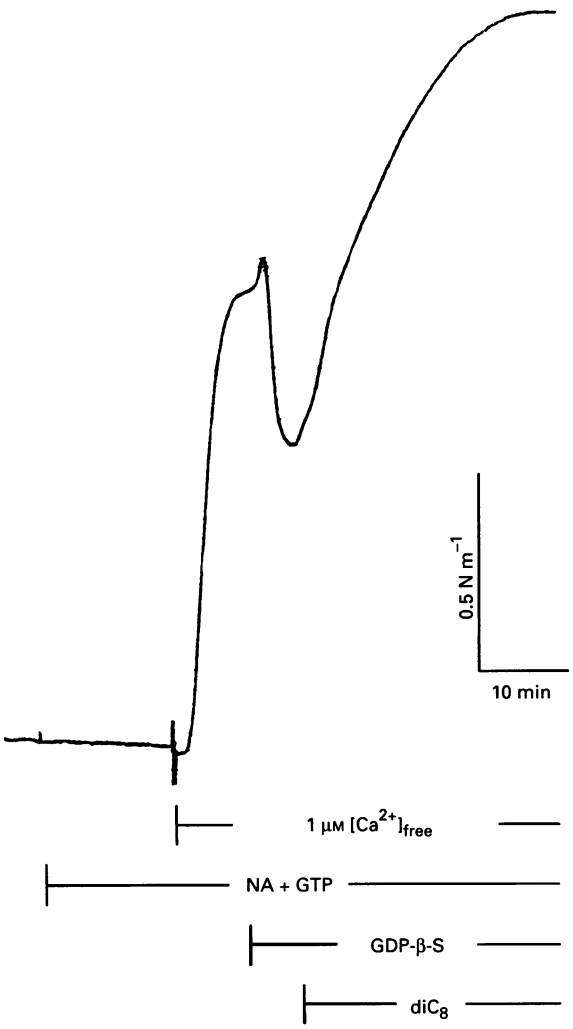

Figure 5 GDP-β-S does not inhibit Ca^{2+}-sensitization induced by diC_8 ($n=3$). The relaxed artery was incubated with noradrenaline (NA, 100 μM) and GTP (0.6 μM) for ~15 min, before contraction was induced by 1 μM $[Ca^{2+}]_{free}$. When tension stabilized 0.3 mM GDP-β-S was added. GDP-β-S induced a relaxation and when tension was stable 100 μM diC_8 was added.

LysoPI, but not LysoPE, can cause sensitization

We have previously demonstrated that certain lysoPCs can increase the Ca^{2+} sensitivity in small mesenteric arteries from the rat, but the effects of lysoPI or lysoPE were not assessed (Jensen *et al.*, 1996). Addition of 50, 100 or 200 μM lysoPE to steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$ did not enhance tension (for 50 μM lysoPE the effect was -0.01 ± 0.05 N m^{-1} ($n=3$, $P=0.9$)). Preliminary experiments where arteries were treated with 100 μM lysoPE for 15 min before the Ca^{2+} concentration-tension curves also showed that the Ca^{2+} sensitivity was unaffected by lysoPE (data not shown).

In contrast, lysoPI had obvious effects on the Ca^{2+} sensitivity. An example is shown in Figure 6a. The increase in tension induced by 100 μM lysoPI added to arteries preconstricted with 1 μM $[Ca^{2+}]_{free}$ was 1.12 ± 0.13 N m^{-1} ($n=4$, $P=0.003$); t_{max} and $t_{1/2}$ were 25 ± 4 min ($n=4$) and 9 ± 2 min ($n=4$), respectively. Concentration-tension curves made by cumulative addition of lysoPI to steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$ gave a pD_2 value of 4.89 ± 0.07 ($n=4$, mean $EC_{50}=13$ μM, Figure 6b). The effect of 100 μM lysoPI on Ca^{2+} concentration-tension curves was assessed by adding lysoPI 30 min before a second curve and keeping it in the bath during the second curve. LysoPI caused a significant increase in the Ca^{2+} sensitivity (0.27 ± 0.05 ($n=4$, $P=0.01$) increase in pCa-EC_{50} by lysoPI, Figure 6c). LysoPI caused contractions in resting arteries (0.11 ± 0.02 N m^{-1} ($n=8$, $P=0.0009$)), and lysoPI increased the maximal Ca^{2+}-induced contraction by 1.47 ± 0.15 N m^{-1} ($n=4$, $P=0.002$).

Phorbol ester (PMA) can cause sensitization

Addition of 1 μM PMA to steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$ enhanced the contraction (Figure 7a). The increase in tension was 1.05 ± 0.13 N m^{-1} ($n=4$, $P=0.004$), and $t_{½}$ and t_{max} were 28 ± 2 min ($n=4$) and 57 ± 2 min ($n=4$), respectively. The effect of PMA on the Ca^{2+}-sensitivity was assessed by addition of 1 μM PMA 30 min before a second Ca^{2+} concentration-tension curve. PMA increased the Ca^{2+}-sensitivity significantly (0.60 ± 0.02 ($n=4$, $P<0.0001$) increase in pCa-EC_{50} by PMA, Figure 7b). In resting arteries, PMA caused no contractions (0.03 ± 0.03 N m^{-1} ($n=4$, $P=0.004$)). PMA increased maximal Ca^{2+}-induced tension by 1.35 ± 0.17 N m^{-1} ($n=4$, $P=0.004$).

Calphostin C inhibits sensitization

To investigate the involvement of PKC in the Ca^{2+}-sensitization, putative PKC inhibitors were used. In preliminary experiments, it was shown that 100 nM staurosporine or 20 μM H7 (1-(5-isoquinoline sulfonyl)-2-methylpiperazine, HCl) abolished contractions induced by 1 μM $[Ca^{2+}]_{free}$ (data not shown). Furthermore, addition of increasing concentrations of H7 (from 1 μM to 100 μM) to arteries precontracted with 1 μM $[Ca^{2+}]$ in the absence or presence of 1 μM PMA or 50 μM GTPγS showed concentration-dependent relaxations with no difference between the effective concentrations on the Ca^{2+}-induced contractions and the PMA or GTPγS-sensitized contractions (data not shown). One μM H7 had no effect and 100 μM almost completely relaxed contractions. These inhibitors were therefore not used to investigate further the mechanisms of Ca^{2+}-sensitization.

Calphostin C, a PKC inhibitor, which does not affect Ca^{2+}-

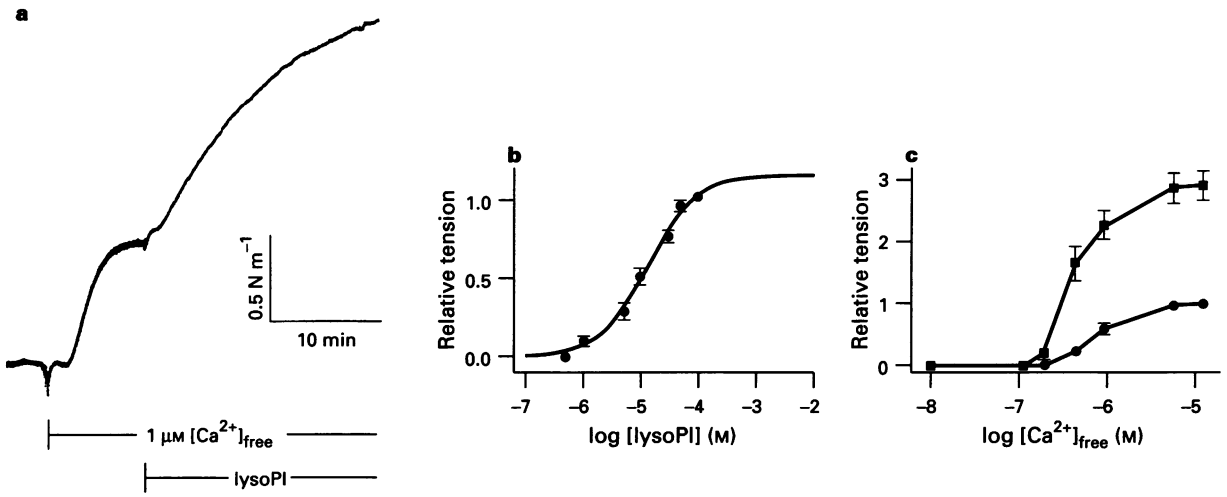

Figure 6 Enhancement of tension at constant $[Ca^{2+}]_{free}$ by lysoPI (a, $n=4$), concentration-tension curve for lysoPI (b, $n=4$), and effect of lysoPI on Ca^{2+} concentration-tension curves (c, $n=4$). In (a), tension was induced by 1 μM $[Ca^{2+}]_{free}$, and when it was stable 100 μM lysoPI was added. In (b), results from cumulative additions of lysoPI up to 100 μM to arteries contracted by 1 μM $[Ca^{2+}]_{free}$ are summarized. In (c), the summarized effect of 100 μM lysoPI (■) on cumulative Ca^{2+} concentration-tension curves is compared to control curves (●, $n=4$).

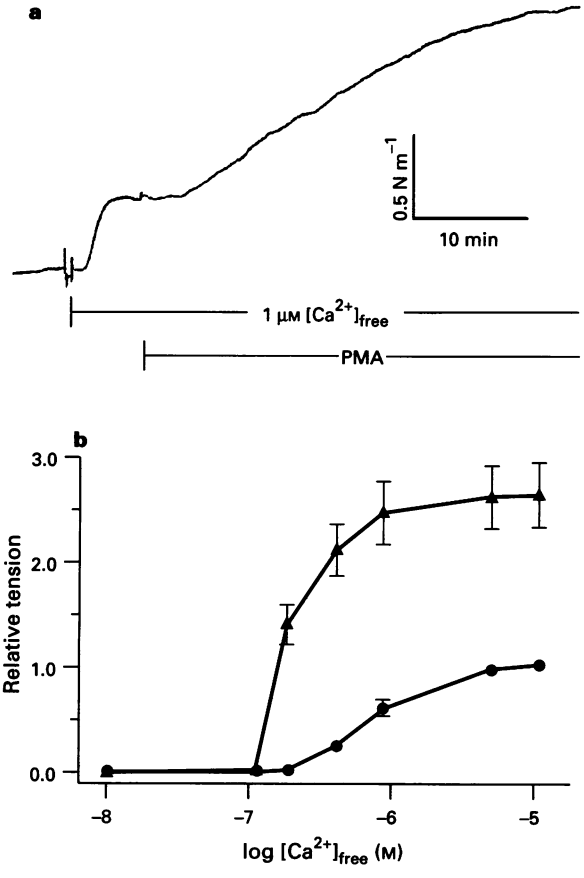

Figure 7 Enhancement of tension at constant $[Ca^{2+}]_{free}$ by phorbol-12-myristate 13-acetate (PMA; a, $n=4$) and effect of PMA on Ca^{2+} concentration-tension curves (b, $n=4$). In (a), tension was induced by 1 μM $[Ca^{2+}]_{free}$, and when it was stable 1 μM PMA was added. In (b), the summarized effect of 1 μM PMA (▲) on cumulative Ca^{2+}-concentration-tension curves is compared to control curves (●, $n=4$).

induced contractions in α-toxin permeabilized small mesenteric arteries from rats (Jensen *et al.*, 1996), was then used. Before the treatment with calphostin C, a control contraction with 1 μM $[Ca^{2+}]_{free}$ was made. After this, the arteries were treated for 1 h with 1 μM calphostin C. Then the arteries were stimulated again with 1 μM $[Ca^{2+}]_{free}$, and 100 μM GTPγS, 100 μM diC_8, 100 μM lysoPI, 100 μM lysoPC or 1 μM PMA were added when the Ca^{2+}-induced contractions were stable. After treatment with calphostin C no sensitization could be induced by GTPγS, diC_8, lysoPI, lysoPC or PMA. A representative recording of the Ca^{2+}-induced contraction and addition of PMA, myristoyl lysoPC and GTPγS is shown in Figure 8a. Figure 8b shows the addition of 1 μM PDBu, lysoPI, diC_8 and GTPγS to the steady-state tension elicited by 1 μM $[Ca^{2+}]_{free}$ after treatment with calphostin C. PDBu is a phorbol ester that has been shown to induce Ca^{2+}-sensitization in permeabilized smooth muscles (Chatterjee & Tejada, 1986; Masuo *et al.*, 1994).

Discussion

The main observations in the present study were; (1) lysoPI, but not lysoPE, caused Ca^{2+}-sensitization, (2) the physiological relevant DAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol, caused Ca^{2+}-sensitization, but was less effective than diC_8, and (3) calphostin C, a relatively specific inhibitor of PKC, completely abolished Ca^{2+} sensitization induced by GTPγS, DAGs, lysophospholipids and PMA.

The time course of the Ca^{2+}-sensitization induced by NA in permeabilized arteries was relatively slow compared to the contraction induced by NA in intact arteries (Jensen *et al.*, 1992), and the Ca^{2+}-sensitization induced by for instance GTPγS (Figure 2). The slow time course of Ca^{2+}-sensitization induced by NA seen here may indicate that the Ca^{2+}-sensitizing pathways activated by NA are slower than the other events initiating contraction (release of Ca^{2+} from intracellular stores and influx of Ca^{2+}). However, in intact arteries Ca^{2+}-sensitization induced by NA is maximal within 30 s (Jensen *et al.*, 1992), and thus faster than in the permeabilized arteries even when the difference in experimental temperatures (25 vs 37°C) is considered. A slow time course of Ca^{2+}-sensitization has been obtained for endothelin in human umbilical artery (Cricton *et al.*, 1993), but fast responses have been demonstrated in other studies on both vascular and other smooth muscles (Nishimura *et al.*, 1988; Drenth *et al.*, 1989; Kitazawa *et al.*, 1991). The slow time course observed in rat mesenteric arteries could indicate that even mild permeabilization with α-toxin affects the coupling from membrane receptors to downstream events (by the loss of essential components or other effects of α-toxin pores in membrane).

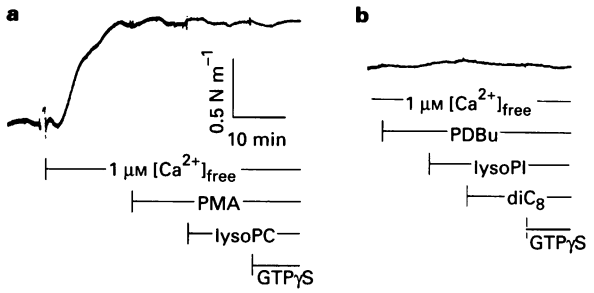

Figure 8 Effect of calphostin C on Ca^{2+}-sensitization in α-toxin permeabilized arteries. The arteries were treated with 1 μM calphostin C and tension induced by 1 μM $[Ca^{2+}]_{free}$. When this Ca^{2+}-induced contraction was stable, phorbol-12-myristate 13-acetate (PMA, 1 μM), lysoPC (100 μM) and GTPγS (100 μM) were added in (a). PMA, lysoPC and GTPγS were not added cumulatively, but fresh solution containing the respective agent was used. In (b), from another experiment, the effect of phorbol 12,13-dibutyrate (PDBu, 1 μM), lysoPI (100 μM), diC_8 (100 μM) and GTPγS (100 μM) on steady-state tension induced by 1 μM $[Ca^{2+}]_{free}$ in the presence of calphostin C is shown. Addition of the agents in different sequences gave identical results ($n=3$ for all agents).

Several of the sensitizing agents used here required long periods to reach maximal and half-maximal effects, but this does not exclude physiological importance. Tonic contractions are likely to result from the concerted action of several processes, some of which may be characterized by slow time courses. Furthermore, processes with a slow time course are also well suited for involvement in regulation of basal Ca^{2+}-sensitivity of tone.

The Ca^{2+}-sensitizing effect of GTPγS in rat mesenteric arteries is consistent with other studies on permeabilized smooth muscles (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1991). Furthermore, stimulation of intact rat mesenteric arteries with the G-protein activator AlF_4^- induces contraction (Boonen & De Mey, 1990; Jensen *et al.*, 1993). Thus studies on both intact and permeabilized rat mesenteric arteries indicate that activation of G-proteins is involved in regulation of contraction, and the present study of permeabilized arteries suggests specifically that G-proteins play a role for regulation of the Ca^{2+}-sensitivity of the contractile proteins. It is not known which type of G-proteins is involved, but heterotrimeric G-proteins are likely to be of importance because AlF_4^- primarily activates these G-proteins (Kahn, 1991), and AlF_4^- induces Ca^{2+}-sensitization in both intact (Jensen *et al.*, 1993) and permeabilized smooth muscles (Kawase & Van Breemen, 1992). Other studies have also suggested that smaller G-proteins like RhoA (Hirata *et al.*, 1992; Fujita *et al.*, 1995; Itagaki *et al.*, 1995) and ras (Satoh *et al.*, 1993) can be mediators in the regulation of Ca^{2+}-sensitization in smooth muscle. It is not known if both types of G-proteins are involved in the signalling cascade.

In rat mesenteric arteries GTPγS caused tension development in Ca^{2+}-free solution and also enhanced the maximal Ca^{2+}-induced contraction. It has previously been noted that GTPγS enhances maximal Ca^{2+}-induced contraction only in phasic smooth muscles like ileum and portal vein, whereas force development in Ca^{2+}-free solution was seen only in tonic smooth muscles like pulmonary artery and femoral artery smooth muscles (Kitazawa *et al.*, 1991). GTPγS induced both these effects in rat mesenteric arteries, consistent with this preparation being intermediate between phasic and tonic muscles.

Activation of heterotrimeric G-proteins could cause activation of PLC, and hence cleavage of membrane phospholipids with production of IP_3 and DAGs. In two recent studies Ca^{2+}-sensitization was induced by adding synthetic DAGs to permeabilized smooth muscles (Masuo *et al.*, 1994; Sohn *et al.*, 1994), and the results in mesenteric arteries here are consistent with these studies. However, a difference in the effect of diC_8 and the more physiologically relevant DAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol (not used in previous studies), was noted. DiC_8 enhanced tension at constant $[Ca^{2+}]_{free}$ and increased the Ca^{2+}-sensitivity more effectively than 1-stearoyl-2-arachidonoyl-*sn*-glycerol. This pharmacological difference indicates the importance of using the physiologically relevant species of DAG.

GDP-β-S did not inhibit the Ca^{2+}-sensitization induced by diC_8, which indicates that G-proteins do not completely mediate the effect of diC_8. It is generally known that DAGs activate PKC directly *in vitro*. That calphostin C inhibited the Ca^{2+}-sensitization induced by DAGs in the present study is consistent with a role for PKC in the effect of DAG. The Ca^{2+}-sensitizing effect of DAGs *in vivo* indicates a potential role for DAGs as physiological messengers. However, a study of measurements of DAGs in small arteries from the rat during stimulation with agonists does not support a role for DAGs as physiological messengers (Ohanian *et al.*, 1990).

G-protein mediated activation of PLA_2 is another pathway for production of second messengers. Cleavage of membrane phospholipids by PLA_2 causes production of AA and lysophospholipids. Both these products have been shown to be potential messengers because they induce Ca^{2+}-sensitization (Gong *et al.*, 1992; Jensen *et al.*, 1996). AA can directly inhibit the MLC_{20}-phosphatase without involvement of PKC (Gong *et al.*, 1992). The effect of lysoPC was inhibited by calphostin C (Jensen *et al.*, 1996), consistent with involvement of PKC, but measurements of the levels of lysoPC during stimulation with NA do not support a role for lysoPCs as messengers. However, PLA_2 may also cleave phosphatidyl-inositol or -ethanolamine, which produces lyso-PI and -PE. These metabolites were tested in this study, and lysoPI, but not lysoPE, caused Ca^{2+}-sensitization. This result indicates a potential role for lysoPI as a messenger, but measurements of lysoPI will be required to evaluate further a role for lysoPI as a messenger involved in the regulation of Ca^{2+}-sensitivity of the contractile proteins during stimulation with agonists.

PKC has been suggested to be involved in regulation of Ca^{2+}-sensitivity in smooth muscle (Nishimura *et al.*, 1988; 1992; Drenth *et al.*, 1989; Ruzycky & Morgan, 1989). The effect of PMA in α-toxin permeabilized rat mesenteric artery is consistent with a previous study (Nishimura *et al.*, 1990). To evaluate whether PKC is of importance for Ca^{2+}-sensitization induced by GTPγS, DAGs and lysoPI in rat mesenteric arteries the inhibitors H7, staurosporine and calphostin C were used. H7 and staurosporine caused inhibition of the contractions induced by Ca^{2+} in α-toxin permeabilized arteries, in contrast to previous studies (Nishimura *et al.*, 1988; 1992), but could not be used to study selectively effects on Ca^{2+}-sensitization. However, calphostin C has been shown to have no effect on tension induced by Ca^{2+} in α-toxin permeabilized rat mesenteric arteries (Jensen *et al.*, 1996). Treatment of the arteries with calphostin C completely abolished the Ca^{2+}-sensitization induced by GTPγS, DAGs, phorbol esters and lysoPI. The inhibition of the Ca^{2+}-sensitization induced by GTPγS is consistent with results from a previous study (Nishimura *et al.*, 1988), but the present results also showed that the Ca^{2+}-sensitization induced by DAGs and lysoPI was inhibited. This indicates a central role for PKC in regulation of the Ca^{2+}-sensitivity in rat mesenteric arteries. Thus a cascade starting with binding of the agonist to its receptor, followed by activation of G-protein(s) and production of DAGs or lysophospholipids leading to activation of PKC is a possibility in this preparation. However, as already mentioned, there are results which question the involvement of DAGs and lysophospholipids, and there are also studies suggesting that PKC is not involved in the regulation of agonist-induced Ca^{2+}-sensitization in smooth muscle (Hori *et al.*, 1993; Itoh *et al.*, 1994; Fujita *et al.*, 1995; Rapoport *et al.*, 1995). Further studies will clearly be needed to confirm these results and consider other possible signalling pathways. It has recently been shown that NA can activate PLD (Ward *et al.*, 1995), but it is not known if the metabolites have contractile effects and could function as messengers.

Several of the agents could induce contractions both in the presence and absence of calcium. Whether the contractions induced in the absence of calcium, reflect activation of a calcium-independent process such as a calcium-independent PKC (e.g. PKC-ε) should be considered. However, contractile responses in the absence of calcium are not unambiguous indicators for the involvement of specialized calcium-independent mechanisms. The force developed in smooth muscle is primarily determined by the ratio of the activities of MLC_{20} kinase/MLC_{20} phosphatase (Somlyo & Somlyo, 1994). Inhibition of MLC_{20} phosphatase can result in contraction even in the absence of calcium because there is some activity of the MLC_{20} kinase in the absence of calcium. This has been suggested to be the likely mechanism for the contractions induced by GTPγS in Ca^{2+}-free media in rabbit femoral and pulmonary arteries (Kitazawa *et al.*, 1991). It is thus possible that the same mechanism that elevates Ca^{2+}-sensitivity may be responsible for causing contractions in the absence of calcium.

In summary, both G-proteins, DAGs and lysoPI have the potential to be mediators in the regulation of Ca^{2+}-sensitization during stimulation with agonists in rat mesenteric arteries. The results also indicate a central role for PKC, because calphostin C completely inhibited the Ca^{2+}-sensitization induced by GTPγS, DAGs and lysoPI.

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