Phospholipase A_2 and protein kinase C contribute to myofilament sensitization to 5-HT in the rabbit mesenteric artery

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1. Calcium (Ca^{2+}, 0·1–100 μM) stimulated concentration-dependent contractions in small strips from the rabbit mesenteric artery in which the smooth muscle cells had been permeabilized with *Staphylococcus aureus* α-toxin.

2. 5-Hydroxytryptamine (5-HT) and phenylephrine, each in the presence of 10 μM guanosine 5′-triphosphate (GTP), concentration-dependently stimulated additional contractions in strips sub-maximally contracted by the presence of a buffered concentration of calcium (0·3 μM). All the additional contraction was abolished with the selective inhibitor of protein kinase C, Ro 31-8220 (10 μM).

3. Quinacrine (10–50 μM), an inhibitor of phospholipase A_2, selectively inhibited the sensitization to 5-HT, but did not alter the sensitization to either phenylephrine or GTP.

4. Myofilament sensitization to calcium was mimicked by exogenous arachidonic acid (300 μM, in the presence of indomethacin, miconazole and BW755c) and the stable analogue of arachidonic acid, 5,8,11,14-eicosatetrayonic acid (ETYA, 100 μM), and in both cases did not require the additional presence of GTP. Ro 31-8220, but not quinacrine, reduced the sensitization to arachidonic acid by around 30%.

5. These results indicate that G protein-linked myofilament sensitization to calcium in the mesenteric artery that follows the activation of 5-HT receptors, but not α_1-receptors, involves phospholipase A_2. The sensitization stimulated by each of these different receptors, and a component of the response to arachidonic acid, also appears to involve the activation of protein kinase C.

5-HT stimulated contraction in smooth muscle cells of the rabbit mesenteric artery permeabilized with *Staphylococcus aureus* α-toxin. The contraction appeared entirely to reflect a sensitization of the muscle myofilaments to cytoplasmic calcium, as 5-HT did not release calcium from internal storage sites (Seager, Murphy & Garland, 1994). 5-HT has also been shown to sensitize the smooth muscle myofilaments in rat coronary arteries, an effect which could be mimicked with GTPγS and which was augmented in vessels from stroke-prone spontaneously hypertensive rats (Satoh, Kreutz, Wilm, Ganten & Pfitzer, 1994). In the mesenteric artery, the non-selective protein kinase inhibitor H-7 blocked the 5-HT-induced calcium sensitization, and on this basis it was suggested that sensitization may reflect the activation of protein kinase C (Seager *et al.* 1994). The suggestion that protein kinase C can cause calcium sensitization in smooth muscle cells followed initially from the observation that phorbol esters, which directly activate the enzyme, can also induce sensitization (Jiang & Morgan, 1989).

However, some controversy exists over the precise role which protein kinase C plays in smooth muscle myofilament sensitization. Although the myofilament sensitization induced by a number of agonists is sensitive to protein kinase C inhibitors both *in vitro* and *in vivo* (Murray, Faraci & Heistad, 1992; Nishimura, Moreland, Ahn, Kawase, Moreland & van Breemen, 1992), the downregulation of protein kinase C, following prolonged exposure to phorbol ester, failed to block the sensitization evoked by noradrenaline, endothelin-1 or prostaglandin $F_{2\alpha}$ in the rat aorta (Hori, Satoh, Miyamoto, Ozaki & Karaki, 1993). These apparently conflicting observations may reflect the relatively non-selective action of the protein kinase inhibitors used or a tissue-based variation in the

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contribution made to sensitization by protein kinase C, for example in the extent of thin filament regulation (Rokoyla, Ahn, van Breemen & Moreland, 1994).

An alternative or additional mechanism for myofilament calcium sensitization may involve the synthesis of arachidonic acid (Somlyo & Somlyo, 1994). Arachidonic acid inhibited the activity of purified smooth muscle myosin light chain phosphatase, and also stimulated contraction in permeabilized smooth muscle cells at a constant calcium concentration, an action associated with a decrease in the rate of myosin light chain dephosphorylation (Gong *et al.* 1992). In addition, the constrictor agonists phenylephrine and U46619 have each been shown to stimulate a significant increase in cytoplasmic arachidonic acid (and diacylglycerol) concentration in vascular smooth muscle cells, an effect consistent with a role for arachidonic acid in sensitization (Gong, Somlyo & Somlyo, 1994).

We have investigated directly the role of arachidonic acid in agonist-induced sensitization in the rabbit mesenteric artery, using the phospholipase A_2 inhibitor quinacrine in cells permeabilized with *Staphylococcus aureus* α-toxin (Blackwell, Flower, Nijamp & Vane, 1978; Seager *et al.* 1994). In addition, we have clarified further a role for protein kinase C, using a potent and selective inhibitor for this enzyme, Ro 31-8220 (Davis *et al.* 1989). Our results indicate that both the constrictor agonists 5-HT and phenylephrine act to induce myofilament sensitization through a pathway which in some way involves protein kinase C, and that the sensitization to 5-HT appears to involve the activation of phospholipase A_2.

METHODS

Preparation of arterial strips

Female New Zealand White rabbits (2·0–2·5 kg) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, I.V.) and killed by rapid exsanguination. A section of mesentery was removed and the third branch of the main mesenteric artery dissected free, cleared of adhering fat and connective tissue and placed in Krebs solution gassed with 95% O_2 and 5% CO_2. Arteries were cleared of the adventitia and the intimal surface gently rubbed with fine tungsten wire to remove the endothelium. Helical strips approximately 2 mm in length and 150–200 μm in diameter were cut and snared at each end with a loop of tungsten wire, each attached to a small stainless-steel tube, one coupled to an isometric force transducer (Harvard Instruments, USA) and the other to a micrometer. The tissue was then bathed either in Krebs solution or a mock intracellular solution (see below) and gassed with 95% O_2 and 5% CO_2 at room temperature (20–25 °C).

Permeabilization procedure

Tissue viability was assessed in each strip by inducing contraction with 10 mM caffeine dissolved in Krebs solution. Each viable preparation (contraction > 0·24 mN) was then placed in mock intracellular solution and incubated with *Staphylococcus aureus* α-toxin (2500 haemolytic units ml^{-1}) for 15–20 min. The extent of permeabilization was assessed by the ability of Ca^{2+} to stimulate contraction in concentrations (0·01–100 μM) which had no measurable effect before exposure to α-toxin. The free [Ca^{2+}] of the intracellular solution was controlled by mixing solutions containing 10 mM EGTA and 10 mM Ca-EGTA in variable proportions. The appropriate amounts of each solution were calculated with a program written by Dr G. L. Smith (University of Glasgow), which enabled the equilibrium concentrations of metal ions and affinity constants relating to the ionic strength and pH to be calculated (Smith & Miller, 1985). Affinity constants for H^+, Ca^{2+} and Mg^{2+} binding to EGTA were taken from that paper.

Solutions and drugs

The Krebs solution had the following composition (mM): 121·8 NaCl, 25·5 $NaHCO_3$, 5·2 KCl, 1·2 $MgSO_4$, 2·5 $CaCl_2$, 0·027 Na_2EGTA, 0·114 ascorbate and 9·4 D-glucose. The mock intracellular solution contained (mM): 120 potassium propionate, 7 $MgCl_2$, 5 Na_2ATP, 15 phosphocreatine and 25 Hepes, and was maintained at pH 7·00 by adding 1 M KOH.

Drugs used were: sodium pentobarbitone (60 mg kg^{-1}; May and Baker), potassium propionate and magnesium chloride (BDH), *Staphylococcus aureus* α-toxin and creatine phosphate sodium salt (Calbiochem, USA) and phenylephrine hydrochloride (Aldrich Chemical Company Inc.); caffeine, thapsigargin, arachidonic acid, indomethacin, 5,8,11,14-eicosatetrayonic acid (ETYA), miconazole, GTP (tris salt), Na_2ATP, 5-hydroxytryptamine (creatine sulphate complex), Hepes and EGTA were all from Sigma. Quinacrine was a generous gift from Dr A. Cowell (University of London), Ro 31-8220 was from Roche Pharmaceuticals Ltd and BW755c was from Wellcome Research Laboratories.

Drugs were dissolved in Krebs or mock intracellular solution except for thapsigargin, which was initially dissolved in dimethylsulphoxide (DMSO), arachidonic acid and indomethacin, which were initially dissolved in sodium carbonate, and miconazole, which was dissolved in 1% methanol.

Data analysis

The contractile force developed by arterial strips is expressed in millinewtons. Results are expressed as means ± S.E.M. and data are compared using Student's unpaired t test (C-stat, Cherwell Scientific, Oxford). Values of $P < 0·05$ were taken to indicate a significant difference between experimental groups.

RESULTS

Contractile responses of permeabilized artery strips to exogenous Ca^{2+}

Small strips of mesenteric artery incubated in *Staphylococcus aureus* α-toxin contracted when subsequently exposed to a range of Ca^{2+} concentrations reflecting those normally encountered in the cytoplasm of activated smooth muscle cells. This indicated that the majority of smooth muscle cells in each strip had been successfully permeabilized. Figure 1*A* illustrates a typical response obtained by increasing [Ca^{2+}] from 0·03 to 100 μM in the presence of 10 mM EGTA. The mean responses to Ca^{2+} from a series of these experiments are shown in Fig. 1*B*. The threshold concentration of Ca^{2+} required to initiate contraction was around 0·1–0·3 μM, and maximal contraction followed the addition of 10 μM Ca^{2+} (0·47 ± 0·03 mN; $n = 10$). The same range of Ca^{2+} concentrations failed to evoke smooth muscle contraction in non-permeabilized strips.

Effect of 5-HT and phenylephrine on permeabilized artery strips activated with Ca^{2+}

Permeabilized artery strips were sub-maximally contracted in the presence of 0·3 μM Ca^{2+} (buffered by 10 mM EGTA), and 1 μM thapsigargin to prevent any release of calcium from intracellular stores. Additional contractions were stimulated by the subsequent addition of 10 μM GTP and then either 5-HT or phenylephrine. Both agonists stimulated concentration-dependent contractions in the presence of GTP, reflecting a sensitization of the muscle myofilaments that is summarized in Fig. 2*A*. Neither agonist had an effect in the absence of GTP. From these concentration–response curves 1 μM, which represented approximately the EC_{60} concentration for each agonist, was selected for subsequent experiments. The mean sub-maximal force induced by 0·3 μM Ca^{2+} was 0·15 ± 0·01 mN ($n = 30$), which was around 30% of the maximal force developed in the presence of 100 μM Ca^{2+}. The addition of 10 μM GTP increased force by a further 0·1 ± 0·01 mN and the subsequent application of 1 μM 5-HT increased force by a further 0·12 ± 0·02 mN ($n = 12$). This represented a total increase in the Ca^{2+}-induced contraction of 152·2 ± 14·7% (Fig. 2*B*). Phenylephrine (1 μM) stimulated a similar increase in force of 0·1 ± 0·01 mN ($n = 10$; total increase, 135·6 ± 14·0%; Fig. 2*C*).

Effect of the protein kinase C inhibitor Ro 31-8220 on 5-HT- and phenylephrine-induced force

Ro 31-8220 (10 μM) markedly inhibited both the GTP- and 5-HT-induced increase in force (by 93·5 ± 6·8%; $n = 8$, $P < 0.001$) and the increase induced by GTP and phenylephrine (by 96·9 ± 2·6%; $n = 6$, $P < 0.001$, Fig. 2*C*).

Effect of the phospholipase A_2 inhibitor quinacrine on 5-HT- and phenylephrine-induced force

Quinacrine (10 μM) selectively and completely inhibited the increase in force evoked by 5-HT, but did not reduce the initial contraction to either GTP or Ca^{2+} ($n = 4$; Fig. 3*A*). In contrast, quinacrine (10–50 μM) did not inhibit the contraction induced with phenylephrine, so that a total increase in contraction of 148·1 ± 15·1% was still obtained with this agonist in the presence of GTP and Ca^{2+} ($n = 4$, $P > 0.05$; Fig. 3*B*). In this series of experiments, the subsequent application of 10 μM Ro 31-8220 inhibited both the GTP- and phenylephrine-induced contraction that remained after quinacrine, by 96·4 ± 3·2% ($n = 3$, $P < 0.05$).

The effect of exogenous arachidonic acid on permeabilized artery strips

Arachidonic acid (300 μM) increased the contraction induced by 0·3 μM Ca^{2+} by a similar extent to 5-HT and phenylephrine (0·092 ± 0·016 mN, $n = 5$; Fig. 4). The increase in force with arachidonic acid was reduced by the presence of Ro 31-8220 (by 30·6 ± 2·8%; $n = 5$, $P < 0.05$). All these experiments were carried out in the presence of 3 μM indomethacin, 100 μM miconazole and 10 μM BW755c to prevent the metabolism of arachidonic acid. The stable arachidonic acid analogue, ETYA (100 μM), stimulated a similar-sized contraction of 0·090 ± 0·008 mN ($n = 4$).

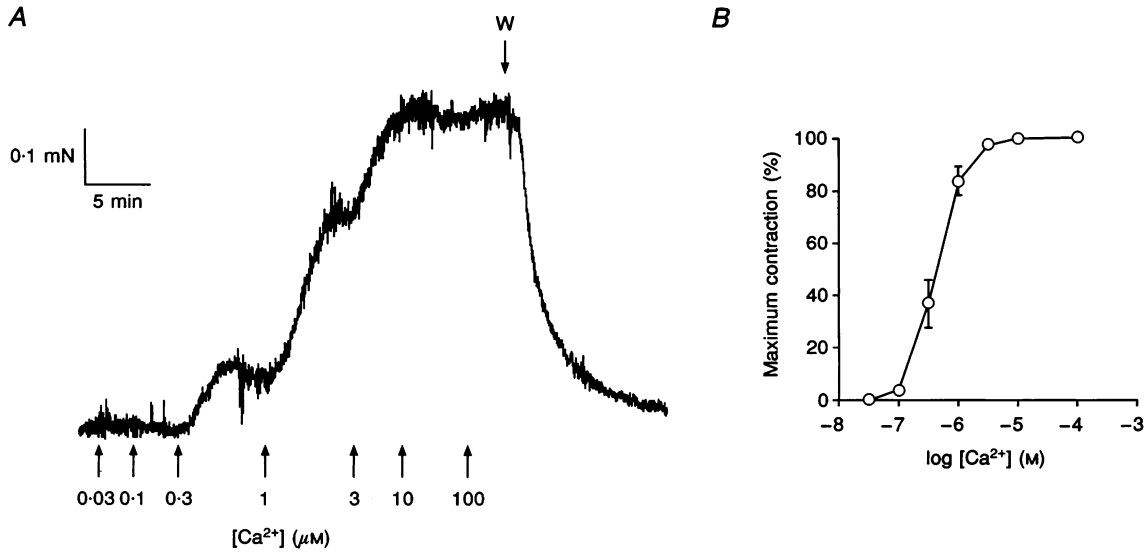

Figure 1. Concentration-dependent smooth muscle contractions in permeabilized artery strips with calcium

A and *B* show the effect of increasing concentrations of calcium in strips of rabbit mesenteric artery permeabilized with *Staphylococcus aureus* α-toxin. *A*, representative trace from one experiment; *B*, mean concentration–response curve ($n = 10$). Each point is mean ± S.E.M. W, washout.

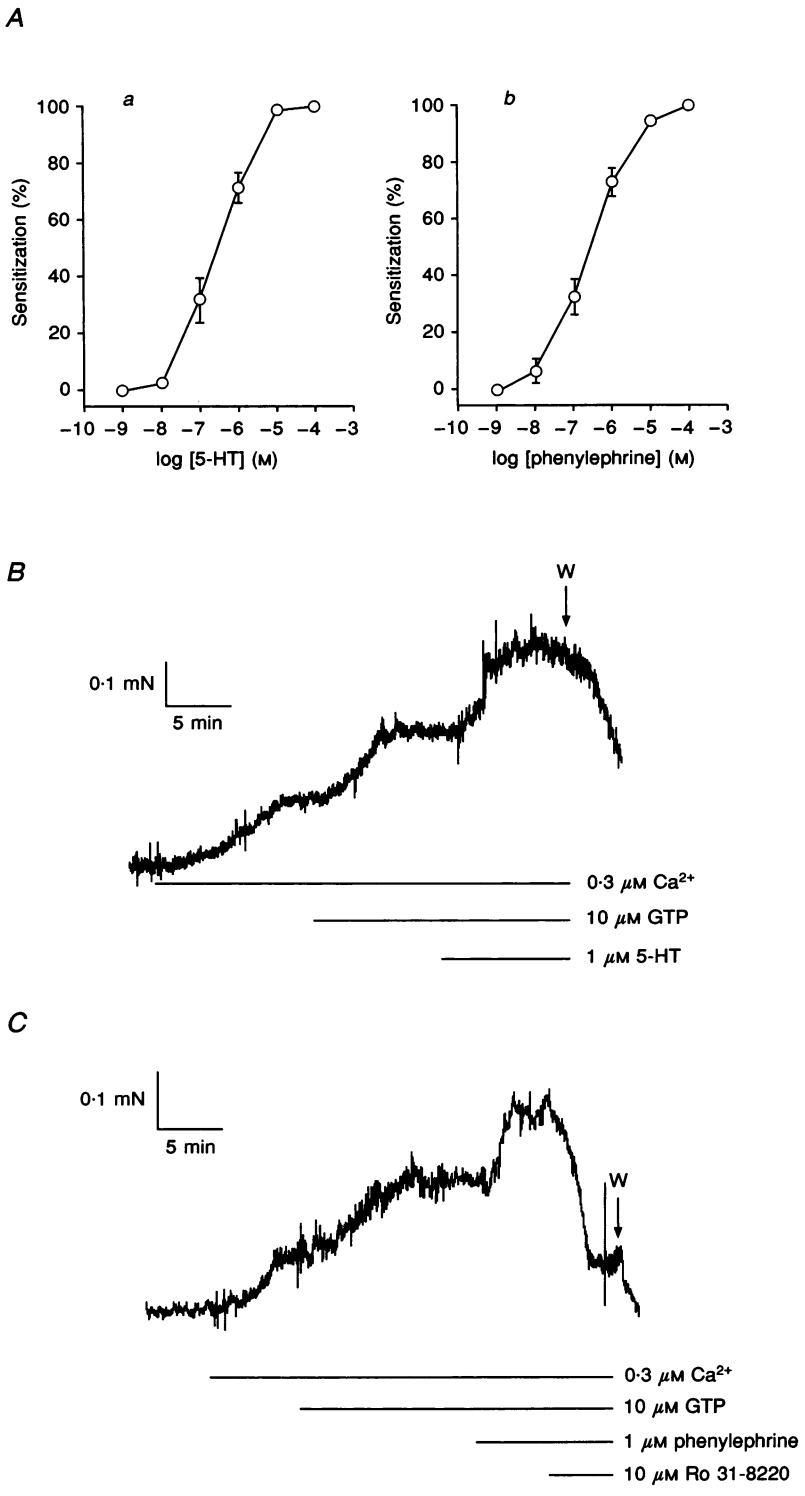

Figure 2. Phenylephrine and 5-HT sensitization to calcium

A, 5-HT (*a*) and phenylephrine (*b*) each induced concentration-dependent sensitization to calcium in the presence of 0·3 μM Ca^{2+} and 10 μM guanosine 5′ trisphosphate (GTP) ($n = 5$ and 4, respectively). Each point is the mean ± S.E.M. *B* and *C*, representative traces of individual arterial responses to Ca^{2+}, GTP and either 5-HT (*B*) or phenylephrine (*C*). The sensitization to GTP and phenylephrine was abolished by the protein kinase C inhibitor Ro 31-8220, after an initial drop in tension reflecting the addition of this inhibitor to the tissue chamber (*C*). The horizontal lines under the traces indicate the period of application for each agent. W, washout.

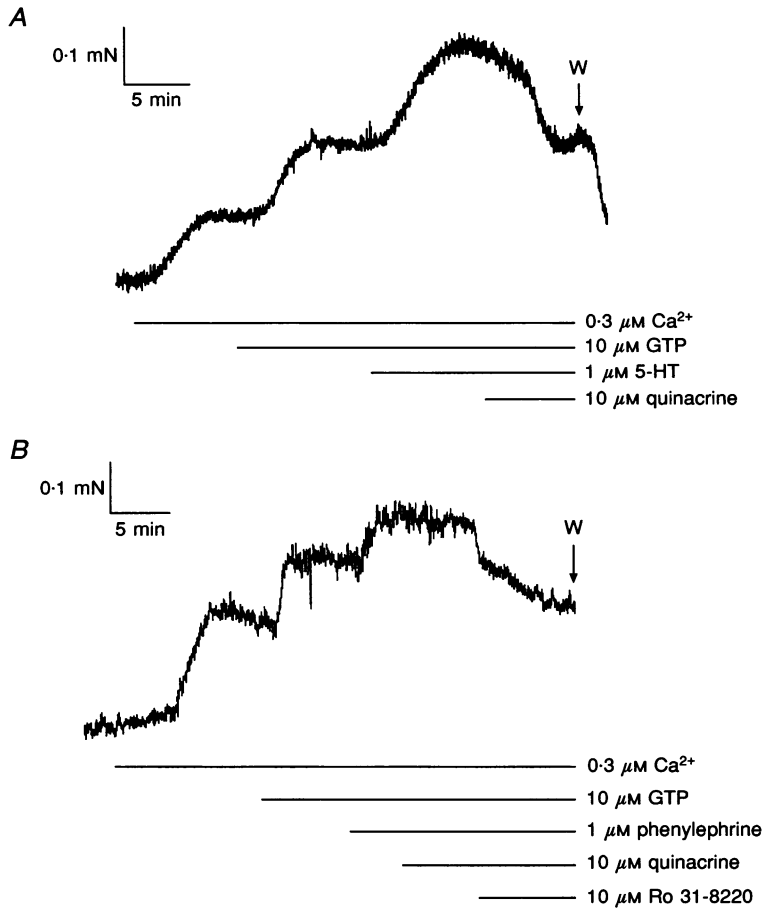

Figure 3. Quinacrine selectively inhibited 5-HT- but not phenylephrine-induced sensitization in the presence of Ca^{2+} and GTP

Representative traces of contraction of permeabilized mesenteric artery strips to Ca^{2+}, GTP and either 5-HT (*A*) or phenylephrine (*B*). Quinacrine inhibited sensitization to calcium induced by 5-HT, but not by GTP or phenylephrine in rabbit mesenteric artery myofilaments. The subsequent addition of the protein kinase C inhibitor Ro 31-8220 inhibited the quinacrine-insensitive phenylephrine-induced sensitization. The horizontal lines under the traces indicate the periods of exposure to each agent. W, washout.

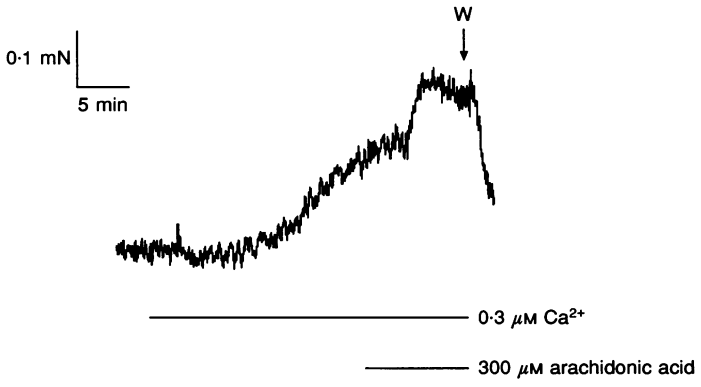

Figure 4. Exogenous arachidonic acid stimulates myofilament sensitization in the presence of Ca^{2+}

Representative trace showing contraction with 300 μM arachidonic acid in a permeabilized strip of mesenteric artery sub-maximally contracted in the presence of 0·3 μM Ca^{2+}, reflecting myofilament sensitization. The horizontal lines under the trace indicate the period of exposure. W, washout.

DISCUSSION

The ability of quinacrine to block myofilament sensitization to 5-HT, together with the individual ability of both arachidonic acid and its stable analogue, ETYA, to mimic sensitization, provides strong evidence that the link between 5-HT receptors and smooth muscle contraction, which reflects myofilament sensitization, involves the activation of phospholipase A_2 and the generation of arachidonic acid.

5-HT stimulates smooth muscle contraction in the rabbit mesenteric artery via 5-HT_{1D} receptors (Seager *et al.* 1994; Choppin & O'Connor, 1995). The only effect on secondary messenger systems so far ascribed to the activation of 5-HT_1 receptors, including 5-HT_{1D} receptors, is a reduction in cAMP (Schoeffter, Waeber, Palacios & Hoyer, 1988; Sumner & Humphrey, 1990). It is not known whether this reduction in cAMP is a direct or indirect result of receptor activation, and if the reduction is linked to smooth muscle contraction. In non-permeabilized smooth muscle of the mesenteric artery, 5-HT causes little or no contraction when applied on its own. However, large reproducible contractions can be obtained if another constrictor agent, such as U46619 or either noradrenaline or raised potassium, is present in concentrations around the threshold for smooth muscle contraction (Choppin & O'Connor, 1995; F. Plane, J. M. Seager & C. J. Garland, unpublished data). The fact that large contractions with 5-HT can be obtained in the presence of raised extracellular potassium levels (at concentrations which have little or no direct action) suggests that myofilament sensitization requires a slight elevation in cytoplasmic calcium and is a major route of contraction with 5-HT in this artery.

In our experiments with permeabilized smooth muscle cells, neither 5-HT nor phenylephrine caused sensitization in the presence of calcium alone. Exogenous application of GTP was required in each case, with the contraction with both GTP and either 5-HT or phenylephrine susceptible to blockade with the protein kinase C inhibitor Ro 31-8220. The inhibitory action of Ro 31-8220 indicates that protein kinase C plays an essential role in the sensitization process. Myofilament sensitization induced in α-toxin-permeabilized smooth muscle by exogenous GTP appears to reflect the activation of protein kinase C (Nishimura, Moreland, Moreland & van Breemen, 1991). However, whether 5-HT and phenylephrine can stimulate an additional activation of protein kinase C is not clear. With phenylephrine this may well be the case, as the activation of α_1-receptors leads to the synthesis of both IP_3 and diacylglycerol (Berridge, 1990). However, 5-HT could activate protein kinase C indirectly through phospholipase A_2, as the products of this enzyme have been shown to interact synergistically with diacylglycerol and activate protein kinase C (Lester, Collin, Etcheberrigaray & Alkon, 1991; Shinomura, Asakoa, Oka, Yoshida & Nishizuka, 1991). This suggestion is supported by the 30% reduction in the observed contraction with exogenous arachidonic acid obtained in the presence of Ro 31-8220.

The phospholipase A_2 inhibitor quinacrine did not modify the contraction with either calcium or GTP, but it did abolish the component of myofilament sensitization specifically induced by 5-HT. The inhibitory action of quinacrine was selective for 5-HT, as equivalent responses to phenylephrine were unaffected. There are a number of ways that 5-HT might potentially activate phospholipase A_2. Firstly, the 5-HT_{1D} receptor may be directly linked to phospholipase A_2 through a G protein. Secondly, 5-HT could act through mitogen-activated protein kinase (MAP kinase). Available evidence indicates two possible routes for the latter possibility. Phospholipase A_2 can be activated by protein kinase C acting first on the low molecular weight GTP-binding protein Ras and the serine/threonine kinase Raf-1, which then stimulate MAP kinase kinase (MEK) before activating MAP kinase and finally phospholipase A_2 (Lee & Severson, 1994). Alternatively, the activation of MEK kinase has been suggested to be coupled to G protein-linked receptors, but whether or not these include 5-HT_{1D} receptors remains to be shown (Lange-Carter, Pleiman, Gardner, Blumer & Johnson, 1993). Which of these pathways actually operates in the mesenteric artery will clearly require further study.

Activation of phospholipase A_2 will form arachidonic acid, which, based on its ability to inhibit purified myosin light chain phosphatase, by dissociating the haloenzyme, and the demonstration that phosphatase inhibitors induce myofilament sensitization in permeabilized smooth muscle preparations, may represent a major signalling pathway for agonist-induced smooth muscle sensitization (Somlyo & Somlyo, 1994). In the case of 5-HT, data presented here support this suggestion, although a direct demonstration that myosin light chain phosphorylation is increased by 5-HT will be necessary to strengthen this possible mechanism as a route for myofilament calcium sensitization.

Phenylephrine might also induce sensitization through the formation of arachidonic acid, but independently of the activation of phospholipase A_2. Arachidonic acid can be generated by a number of different pathways, including the action of di- and monoglyceride lipases on diacylglycerol (Irvine, 1982) or the inhibition by protein kinase C of both arachidonyl-CoA synthase and arachidonyl-CoA lysophosphatidate acyltransferase, which are involved in the reacylation of arachidonic acid into phospholipids (Irvine, 1982; Fuse, Iwanaga & Tai, 1989; Naor, 1991).

In summary, 5-HT-induced contraction of smooth muscle cells in the rabbit mesenteric artery is mediated by 5-HT_{1D} receptors (Choppin & O'Connor, 1995) and myofilament sensitization to calcium which does not involve an increase in either IP_3 levels or the release of internal stores of calcium (Seager *et al.* 1994). We now provide evidence

which indicates that the sensitization to 5-HT operates via the activation of phospholipase A_2 and the subsequent generation of arachidonic acid. Arachidonic acid may then cause sensitization indirectly, by inhibiting myosin light chain phosphatase (Gong *et al.* 1992). Our data also indicate a crucial requirement for GTP and the activation of protein kinase C in the process of myofilament sensitization in the mesenteric artery to both 5-HT and phenylephrine, although the sensitization to phenylephrine did not appear to involve the activation of phospholipase A_2.

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