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European Journal of Pharmacology 277 (1995) 35–44

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Involvement of protein kinase C activation in α_2-adrenoceptor-mediated contractions of rabbit saphenous vein

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Received 18 July 1994; revised 18 January 1995; accepted 20 January 1995

Abstract

The role of protein kinase C α_2-adrenoceptor-induced contractions of rabbit saphenous vein was investigated. Contractions induced by the α_2-adrenoceptor-selective agonist 5-bromo-6-[2-imidazolin-2-ylamino]-quinoline (UK14304) were inhibited by prior treatment with pertussis toxin and by Ca^{2+} removal, confirming a G_i/G_o-dependent coupling pathway which was highly dependent upon Ca^{2+} influx. Protein kinase C inhibitors calphostin-C and staurosporine each caused a non-competitive inhibition of UK14304 response. Down-regulation of protein kinase C by pretreatment with tetradecanoylphorbol acetate reduced UK14304 response by almost 90% with no effect on contractions induced by elevated KCl. The ineffectiveness of L-type Ca^{2+} channel blockers and the absence of stimulated $^{45}Ca^{2+}$ uptake or efflux by UK14304 indicated that phospholipid-derived products were most likely responsible for protein kinase C activation. α_2-Adrenoceptor stimulation failed to increase [^{3}H]*myo*-inositol phosphate formation, but caused a significant increase in the formation of both [^{32}P]phosphatidic acid and diacylglycerol, indicating the possible activation of phospholipase D activity. These results suggest that protein kinase C is important for the vasoconstriction induced by α_2-adrenoceptors and that diacylglycerol derived from receptor-initiated phospholipase D activity may provide protein kinase C stimulation.

Keywords: α_2-Adrenoceptor; Protein kinase C; Smooth muscle, vascular; Phospholipase D

1. Introduction

Contraction of vascular smooth muscle can be accomplished by the activation of at least two distinct protein kinase-dependent pathways involving myosin light chain kinase and protein kinase C. The former pathway is predominant when cytoplasmic Ca^{2+} levels are elevated, allowing formation of the Ca^{2+}/calmodulin complex which activates myosin light chain kinase, providing for a close correlation between $[Ca^{2+}]_{cyt}$, myosin phosphorylation and developed tension (Hai and Murphy, 1988). The latter pathway predominates during the tonic phase of agonist-induced contractions such as those initiated by α_1-adrenoceptor stimulation, and is associated with lower cytoplasmic Ca^{2+} levels (Morgan and Morgan, 1982) and sustained phospholipid hydrolysis (Campbell et al., 1985). Diacylglycerol, provided by this sustained hydrolysis, helps to activate protein kinase C which is thought to phosphorylate a cytoskeletal element or myofilament regulatory protein (Khalil and Morgan, 1992; Nakamura et al., 1993). While Ca^{2+} can augment protein kinase C activation by diacylglycerol (Nishizuka, 1992), it has been shown that vascular contractions can be produced in the absence of extracellular Ca^{2+} at low cytoplasmic Ca^{2+} levels by phorbol esters which activate protein kinase C in a manner similar to diacylglycerol (Jiang and Morgan, 1991).

The existence of isoforms of protein kinase C has been documented which vary in their mode of regulation (Nishizuka, 1992). Ca^{2+} and diacylglycerol are the primary activating modulators while unsaturated free fatty acids such as arachidonate can greatly amplify enzyme activity, but only in the presence of sufficient diacylglycerol (Shinomura et al., 1991). Among protein kinase C isoforms are species which are either Ca^{2+}- or diacylglycerol-independent, leading to the possibility that varied combinations of regulatory substances (i.e. Ca^{2+}, diacylglycerol and free fatty acid) could provide differential activation of individual isoforms.

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Elsevier Science B.V.
SSDI 0014-2999(95)00054-2

The coupling pathway by which α_2-adrenoceptors cause vascular contraction is not well extablished. Contractions have been shown to be highly dependent upon extracellular Ca^{2+} (Van Meel et al., 1981a) and are inhibited by L-type Ca^{2+} channel blockers in several preparations (Van Meel et al., 1981b; Ruffolo and Nichols, 1988). This has led to the proposal that α_2-adrenoceptors cause contraction by increasing Ca^{2+} influx via voltage-dependent Ca^{2+} channels (Ruffolo and Nichols, 1988). However, electrophysiological studies have generally demonstrated that α_2-adrenoceptors cause a *decrease* in Ca^{2+} channel opening (Horn and McAfee, 1980; Dunlap and Fischbach, 1981) along with an increase in K^+ channel opening (Suprenant et al., 1992) which would also oppose Ca^{2+} channel opening. Thus it is unclear whether a dependence upon extracellular Ca^{2+} results from a direct Ca^{2+} influx-dependent coupling pathway or whether it reflects another mode of Ca^{2+} involvement.

Based upon their shared dependence on extracellular Ca^{2+}, it has been further proposed that the mechanism of α_2-adrenoceptor coupling in vascular tissues may be similar to the sustained phase of α_1-adrenoceptor-mediated contractile response (Ruffolo and Nichols, 1988). Since activation of protein kinase C has been shown to be important during sustained α_1-adrenoceptor response, we undertook the current studies of α_2-adrenoceptor responses in rabbit saphenous vein to determine whether protein kinase C plays a role in α_2-adrenoceptor-mediated contractions in this tissue and if so, how its activation may be achieved.

2. Materials and methods

Saphenous veins were excised from male New Zealand white rabbits (2--3 kg) and freed of connective tissue in a constantly gassed (5% CO_2, 95% O_2) bicarbonate buffer of the following composition (mM): NaCl 118; KCl 4.4; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_2$ 24.9; KH_2PO_4 1.2; glucose 11.1. Spiral strips were then made and cut into 2 cm long pieces.

2.1. Contraction studies

Isometric contraction experiments were carried out with spiral rabbit saphenous vein strips mounted with stainless steel hooks in a 20 ml organ bath at 37° C between a force transducer (connected to a physiograph recorder) and a fixed glass rod. An initial passive tension of 2 g was applied to the tissues for at least 60 min and experimental observations were begun only after successive agonist-induced contractions were reproducible within 10%. In some experiments, cumulative dose tissues were treated with drugs prior to agonist re-exposure for either single dose or dose-response studies. Contractile response in elevated (15 mM) or high potassium (60 mM) conditions was assessed using a buffer in which NaCl was replaced by KCl in an equimolar amount. In some studies rabbits were injected with 10 μg/kg pertussis toxin via the marginal ear vein and killed 40 h after the injection. In studies with added antagonists or inhibitors results are expressed as a percent of the pretreatment response unless otherwise indicated.

2.2. $^{45}Ca^{2+}$ uptake studies

Strips of rabbit saphenous vein were preincubated at 37° C in $^{45}Ca^{2+}$ containing (1 μCi/ml) bicarbonate buffer for 30 min with or without adrenoceptor antagonists before 10 min of UK14304 or noradrenaline exposure. To remove extracellular $^{45}Ca^{2+}$ after the uptake period, groups of tissues were quickly rinsed in ice-cold bicarbonate buffer containing 10 μM EGTA and 11.5 mM $CaCl_2$ and then transferred twice to 50 ml of the same buffer for 20 min for a total washout period of 40 min. After determination of wet weight, strips were placed in a 10 mM EGTA solution overnight at room temperature to release intracellular $^{45}Ca^{2+}$ and $^{45}Ca^{2+}$ content was determined by liquid scintillation spectrophotometry.

2.3. $^{45}Ca^{2+}$ efflux studies

Strips of rabbit saphenous vein were preincubated at 37° C in $^{45}Ca^{2+}$-containing (5 μCi/ml) buffer for 3 h and then briefly rinsed (5 s) before being transferred at 5 min intervals to successive vials containing 5 ml of gassed bicarbonate buffer containing 10 mM EGTA and 11.5 mM $CaCl_2$ at 37° C. The total washout period was 70 min and either UK14304 (10 μM) or caffeine (10 mM) was added after 40 min of washout. A 40 min washout was determined in previous studies with this tissue to be appropriate to remove the majority of extracellularly bound $^{45}Ca^{2+}$ while most of the cellular content of $^{45}Ca^{2+}$ was still retained. $^{45}Ca^{2+}$ was then measured in the washout solutions by liquid scintillation spectrophotometry.

2.4. [^{32}P]Phospholipid hydrolysis

Strips of rabbit saphenous vein prepared as described above were incubated for 1 h at 37° C in ^{32}P-containing (25 μCi/ml) bicarbonate buffer. Agonists and/or antagonists were added for the last portion of the labeling period after which tissues were removed, blotted on filter paper and the wet weight recorded. Tissues were then homogenized in 3 ml of $CHCl_3$: CH_3OH : HCl (200 : 200 : 1) and the homogenate centrifuged at 2000 rpm for 20 min. The supernatant was removed and saved while the pellet

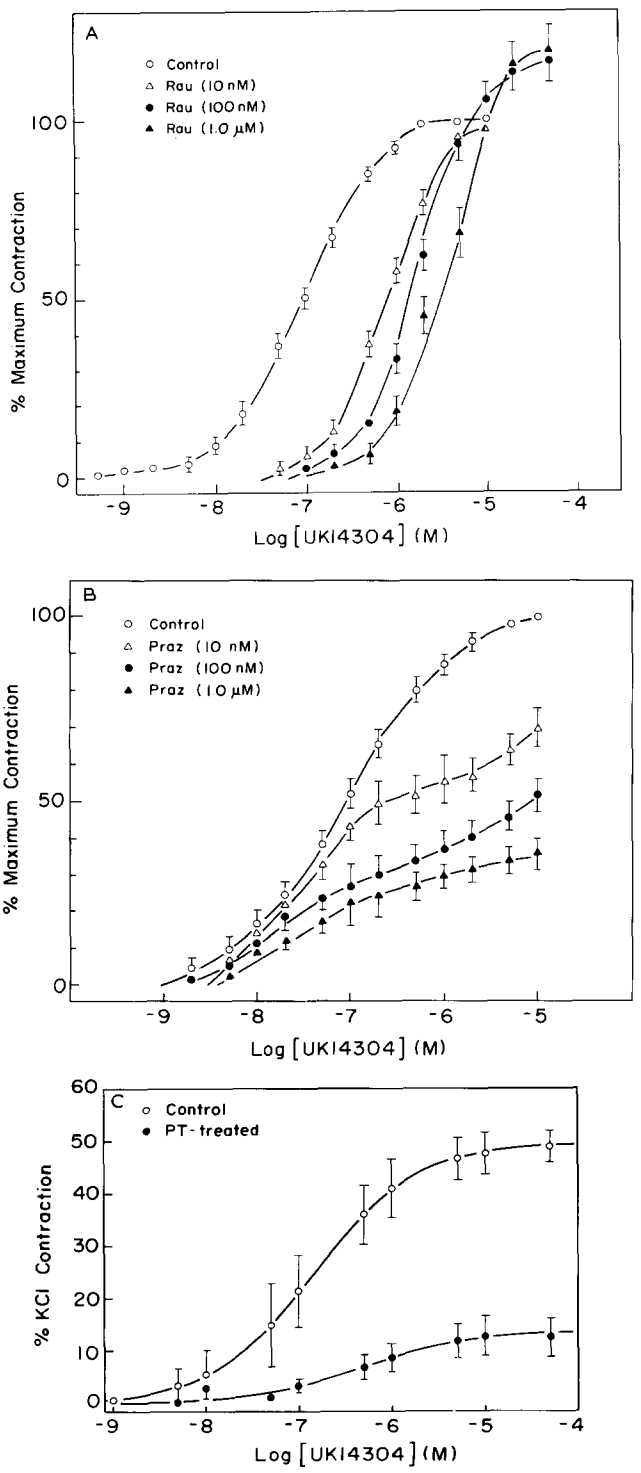

Fig. 1. Inhibition of rabbit saphenous vein α_2-adrenoceptor responses by rauwolscine, prazosin or pertussis toxin pretreatment. A and B: Dose-response curves for UK14304 were obtained in the absence (Control) or presence of various concentrations of rauwolscine (A) or prazosin (B) after a 20 min incubation period. C: Dose-response curves for UK14304 were obtained for veins from rabbits treated with 10 μg/kg pertussis toxin (PT) for 24 h or from untreated animals (Control). Contractile responses were normalized to the 60 mM KCl response in each tissue. Each data point is the mean ± S.E.M.

was resuspended in another 2 ml of $CHCl_3$: CH_3OH : HCl (400 : 200 : 5) and centrifuged at 1000 rpm for 10 min. Supernatants were then combined and evaporated to dryness under nitrogen. The residue was dissolved in 3 ml of $CHCl_3$ and washed 3 times with 1 ml of 0.1 N HCl, added to the resuspended extract, vortexed and centrifuged at 1000 rpm for approximately 3 min, after which the upper acid layer was removed and saved. 3 ml of chloroform was added to the combined upper phases, which were vortexed and centrifuged at 1500 rpm for 15 min after which the lower chloroform layer was added to the original chloroform phase and evaporated to dryness under nitrogen. The phospholipid residue was resuspended in a small volume of chloroform (50 μl), spotted on a heat-activated silica gel 60 TLC plate (10 cm) and separated using two-dimensional thin layer chromatography. First dimension: $CHCl_3 : CH_3OH : NH_4\text{-}OH : H_2O$ (1.0 : 1.0 : 0.07 : 0.22); second dimension: $C_4H_9OH : CH_3COOH : H_2O$ (6 : 1 : 1). An autoradiograph was made to identify the pattern of phospholipid migration after which the TLC plates were exposed to iodine vapor until the phospholipids were visualized. Individual phospholipids were identified by comparison with known standards and were scraped into scintillation vials and quantified by liquid scintillation spectrometry.

2.5. [^{3}H]myo-Inositol phosphate formation

Strips of rabbit saphenous vein were labeled overnight at room temperature in [^{3}H]*myo*-inositol (100 μCi/ml) containing bicarbonate buffer in sealed vials. Parallel studies confirmed the vitality of the tissues by contraction studies after overnight incubation under the same conditions. Tissues were then transferred to 5 ml of a 10 μM LiCl buffer at 37° C for 30 min during which 10 μM UK14304 or noradrenaline was or was not present. Tissues were then homogenized in 1 ml of 10% perchloric acid, neutralized to pH 6–8 with 30% KOH and centrifuged. The supernatant was filtered, spiked with 100 μl of a mixture of 0.1 mM ATP/ADP (as internal standards) and injected onto a strong anion exchange HPLC column. Inositol phosphates were eluted with a stepped gradient of ammonium formate in distilled water starting with 0% for 8 min, then 33% and 50% for 12 min each and finally to 100% for the last 12 min. Standards of ^{3}H-labeled inositol phosphates were run in parallel to determine the retention time of individual inositol phosphates and the size of each peak was quantitated by a radioactivity flow detector.

2.6. Diacylglycerol measurement

After appropriate treatment and determination of wet weight, strips of rabbit saphenous vein were trans-

ferred to 3 ml of chloroform/methanol (1 : 2 v/v) mixed with 0.5 ml of 0.2% sodium dodecylsulfate containing 1 M NaCl and left on ice as a one-phase solution for 2–3 h. Subsequent addition of 1 ml each of CH_3Cl_3 and 1 M NaCl yielded a 2-phase solution. The lower chloroform phase containing neutral lipids was removed and evaporated to dryness under a stream of nitrogen and then stored on ice. A commercially available kit based upon the diacylglycerol kinase reaction was used for diacylglycerol determination.

2.7. Data analysis

Grouped data were compared for significant differences using Student's t-test, with a value of $P < 0.05$ taken as the limit for significance.

2.8. Drugs

Drugs used during these investigations were obtained from the following sources: UK14304 was provided by Research Biochemicals International as a part of the Chemical Synthesis Program of the National Institute of Mental Health; prazosin (Pfizer, Groton, CT, USA); rauwolscine (Thomae, Biberach, Germany); pertussis toxin (Calbiochem, LaJolla, CA, USA); staurosporine (Kamiya Biomedical, Thousand Oaks, CA, USA); nifedipine (Miles Pharmaceuticals, North Haven, CT, USA); tetradecanoylphorbol acetate (LC Services, Woburn, MA, USA); methoxamine (Research Biochemicals, Natick, MA, USA); noradrenaline, mepacrine, verapamil, caffeine (Sigma, St. Louis, MO, USA).

3. Results

3.1. α_2-Adrenoceptor-mediated contractile response of rabbit saphenous vein

The α_2-adrenoceptor-selective agonist UK14304 caused dose-dependent contractions of rabbit saphenous vein which were shifted to the right in a parallel manner by the α_2-adrenoceptor-selective antagonist rauwolscine (Fig. 1A) verifying their α_2-adrenoceptor origin. In the presence of 100 nM and 1 μM rauwolscine, the maximum response to UK14304 was approximately 20% above the level of untreated control tissues. Maximal UK14304 contractions reached only 45% of the contractile response to 60 mM KCl, but 88% of the response to the non-selective α-adrenceptor agonist norepinephrine (data not shown). The α_1-adrenoceptor-selective agonist methoxamine failed to produce contraction in most vessels and its overall maximal response was less than 10% of that for UK14304. Thus α_2-adrenoceptors appear to provide the major source of UK14304-induced activation in rabbit saphenous vein, as previously reported (Alabaster et al., 1985). However, in accord with earlier observations (Daly et al., 1988; Shimamoto et al., 1992), we found that the α_1-adrenoceptor antagonist prazosin caused a non-competitive type inhibition of UK14304 response (Fig. 1B). While the underlying mechanism is unclear, this effect may reflect a dependence of α_2-adrenoceptor efficacy upon costimulation of α_1-adrenoceptors.

In order to identify the type of G-protein involved in the α_2-adrenoceptor response of rabbit saphenous vein, some animals were treated with pertussis toxin (10

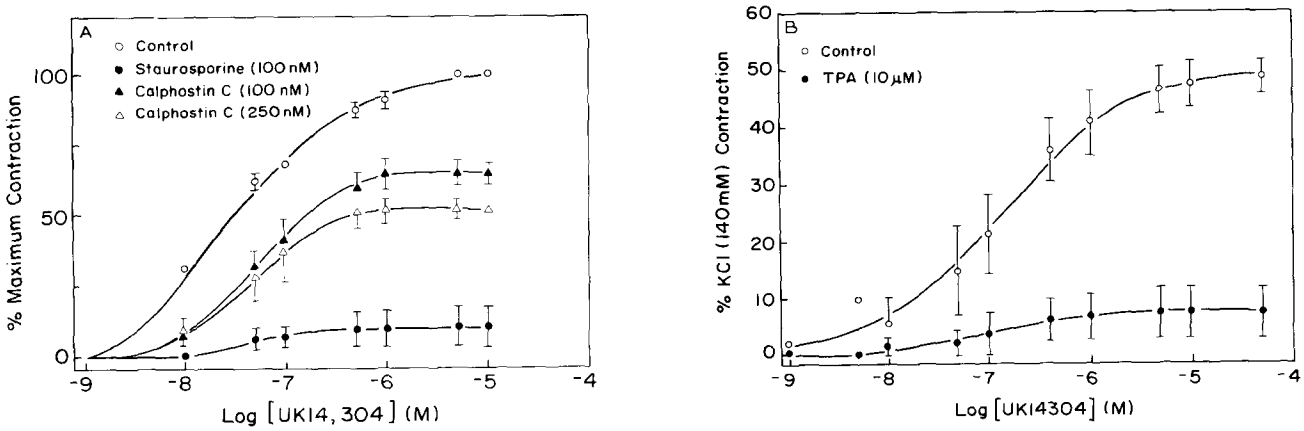

Fig. 2. Role of protein kinase C in α_2-adrenoceptor response in rabbit saphenous vein. A: Tissues were pretreated with either 100 nM staurosporine (●) or calphostin C (100 nM, ▲; 250 nM, △) or untreated (○) for 30 min prior to eliciting a UK14304 dose-response curve. B: Tissues were incubated for 20 h in buffer with (●) or without (○) 10 mM tetradecanoylphorbol acetate (TPA) prior to eliciting a UK14304 dose-response curve. Each data point is the mean ± S.E.M. of six observations.

μg/kg) for 24 h prior to tissue preparation and contraction studies. As shown in Fig. 1C, this treatment caused a marked reduction (86%) in the maximum response to UK14304 with no apparent rightward shift of the dose-response curve. Results were normalized to the tissue response to 60 mM KCl in order to correct for any 'non-specific' reduction of tissue contractility. This decrease is consistent with a role for either G_i or G_o in transducing α_2-adrenoceptor responses in rabbit saphenous vein.

3.2. Role of protein kinase C in α_2-adrenoceptor-mediated contractions of rabbit saphenous vein

To assess the role of protein kinase C in α_2-adrenoceptor-mediated contractions, tissues were incubated with protein kinase C-selective inhibitors (staurosporine or calphostin-C) for 30 min prior to eliciting a UK14304 dose-response curve. As shown in Fig. 2A, both inhibitors caused a significant non-competitive inhibition of UK14304 response. Staurosporine was more effective and more potent than calphostin-C, producing a 90% inhibition of maximal UK14304 response at 100 nM, while 100 nM and 250 nM calphostin-C produced 38% and 49% inhibition respectively. UK14304 potency was largely unaffected by either inhibitor, consistent with their interference with signal transmission events rather than receptor binding.

In order to further identify a role for protein kinase C, some tissues were incubated 20 h at room temperature in aerated buffer containing 10 μM of the phorbol ester tetradecanoylphorbol acetate in order to down-regulate protein kinase C activity. UK14304 responses and responses to 60 mM KCl were then compared in phorbol-treated and untreated groups. As shown in Fig. 2B, protein kinase C down-regulation reduced maximal UK14304 response to 12% of the level in untreated controls, verifying a critical role for protein kinase C in α_2-adrenoceptor response. KCl-induced contractions were not significantly affected by this phorbol ester treatment.

3.3. Role of Ca^{2+} in α_2-adrenoceptor-mediated contractions of rabbit saphenous vein

To investigate Ca^{2+} as a possible source of protein kinase C activation, tissues were incubated in a Ca^{2+}-free buffer for 3 min prior to the start of and during a UK14304 dose-response procedure. As shown in Fig. 3A, maximal response to UK14304 was inhibited by 91% after Ca^{2+} removal, and the small remaining response was shifted to the right, occurring between 1 and 10 μM. This confirms the well-documented dependence of α_2-adrenoceptor contractions on extracellular Ca^{2+} and suggests that the residual contraction may be a minor component of UK 14304-stimulated α_1-adrenoceptor response.

Despite their dependence on extracellular Ca^{2+}, α_2-adrenoceptor-induced contractions of rabbit saphenous vein were largely unaffected by blockers of L-type voltage-dependent Ca^{2+} channels. Thus verapamil and nifedipine, at a concentration of 1 μM, respectively produced 0% and 19% inhibition of maximal UK14304 contractile response (Fig. 3B). There was some evidence of greater inhibition by nifedipine at lower concentrations of UK14304 and a non-significant decrease in verapamil-treated tissues at lower concentrations.

In order to determine whether α_2-adrenoceptor activation caused an increase in either influx of extracellular Ca^{2+} or release of intracellular Ca^{2+}, we mea-

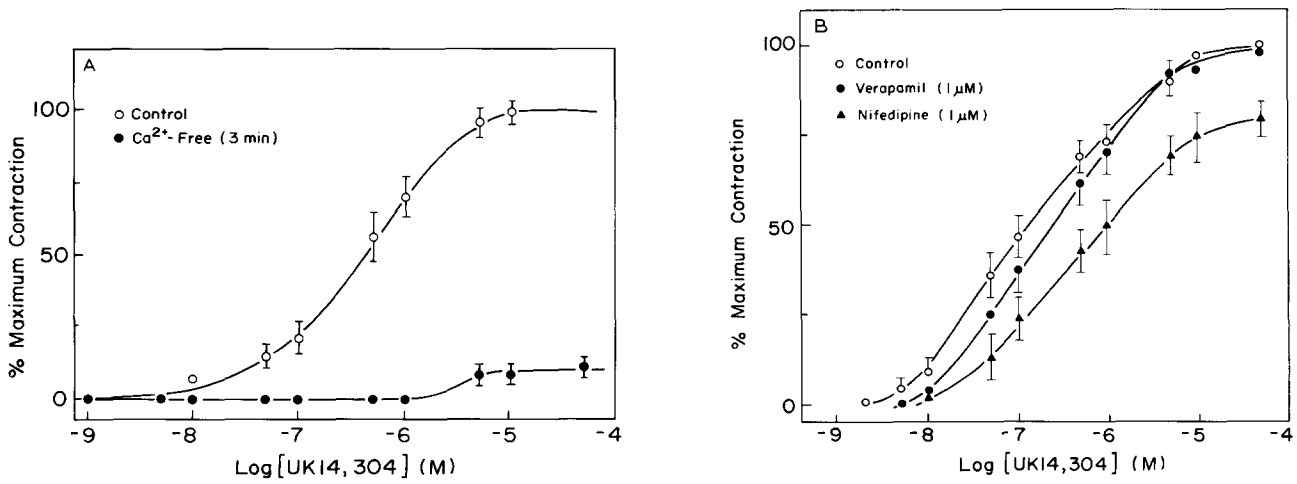

Fig. 3. Role of Ca^{2+} influx in the α_2-adrenoceptor response of the rabbit saphenous vein. A: Dose-response curves for UK14304 were obtained either in normal buffer (Control) or in Ca^{2+}-free buffer (Ca^{2+} omitted) after a 3 min incubation period. B: Dose-response curves for UK14304 were obtained after a 20 min incubation with either verapamil (1 μM) or nifedipine (1 μM) or normal buffer (Control). Each data point is the mean ± S.E.M. of six observations.

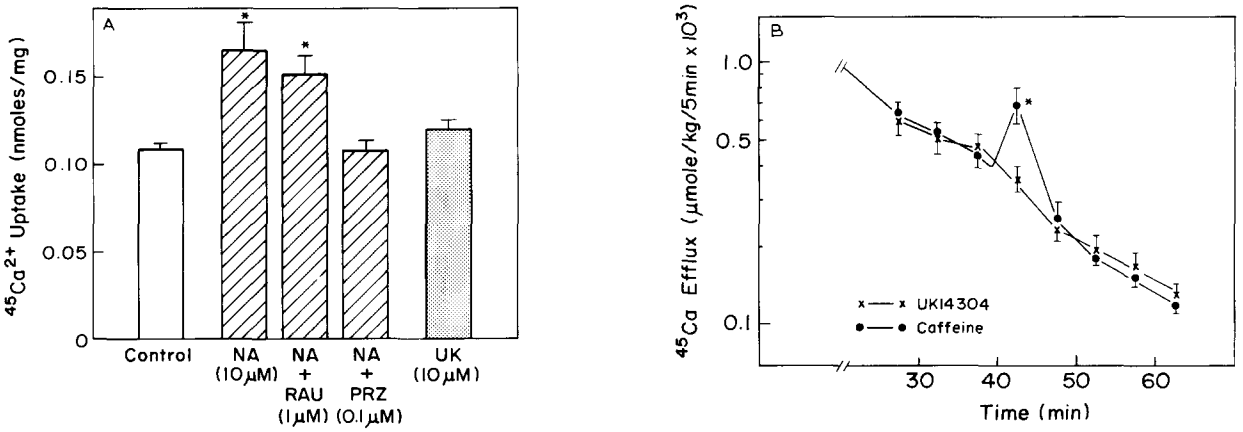

Fig. 4. Influence of α_2-adrenoceptor stimulation on $^{45}Ca^{2+}$ influx and efflux in rabbit saphenous vein. A: Uptake of $^{45}Ca^{2+}$ during a 40 min incubation during which agonist (10 μM UK14304 (UK) or 10 μM noradrenaline (NA)) was present for the final 10 min. Antagonists (1 μM rauwolscine (RAU) or 10 μM prazosin (PRZ)), when added, were present for the entire 40 min period. Asterisk (*) indicates significant increases above the unstimulated tissue value ($P < 0.05$; $n = 8$–10). B: $^{45}Ca^{2+}$ efflux from vein segments exposed to either UK14304 (10 μM) or caffeine (10 mM) starting after 40 min of washout. Asterisk (*) indicates an efflux rate significantly higher than the level prior to drug addition ($P < 0.05$; $n = 8$).

sured $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux from segments of rabbit saphenous vein during treatment with 10 μM UK14304. UK14304 failed to significantly augment $^{45}Ca^{2+}$ influx although noradrenaline (10 μM) caused a 43% increase above basal levels (Fig. 4A). The latter increase was not significantly reduced by pretreatment with the α_2-adrenoceptor antagonist rauwolscine, but was eliminated by the α_1-adrenoceptor antagonist prazosin. This indicates that stimulation of α_2-adrenoceptor does not increase basal Ca^{2+} entry in rabbit saphenous vein, consistent with the lack of influence of Ca^{2+} channel blockade on contraction. UK14304 failed to alter the rate of $^{45}Ca^{2+}$ efflux from prelabelled vein segments (Fig. 4B) although the release of intracellular $^{45}Ca^{2+}$ by caffeine (10 mM) could be readily detected.

Taken together, the above observations demonstrate that while α_2-adrenoceptor contractile response in rabbit saphenous vein is almost completely dependent upon the basal influx of extracellular Ca^{2+}, receptor activation does not augment Ca^{2+} entry or release and therefore protein kinase C activation does not result from an increased supply of Ca^{2+}. Furthermore, under our experimental conditions, basal Ca^{2+} influx via non-L-type Ca^{2+} channel pathways is sufficient to sustain all or close to all of the α_2-adrenoceptor response.

3.4. Role of phospholipid hydrolysis in α_2-adrenoceptor-mediated contractions of rabbit saphenous vein

We investigated the role of phospholipid hydrolysis by examining the effects of the phospholipase in-

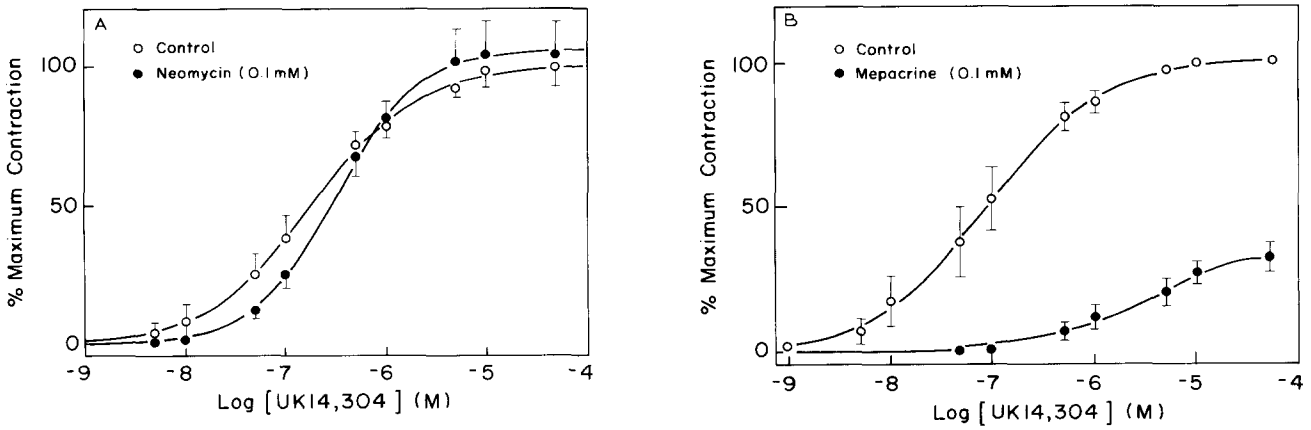

Fig. 5. Influence of phospholipase inhibitors on α_2-adrenoceptor response in rabbit saphenous vein. A: Tissues were pretreated with neomycin (0.1 mM) for 30 min (•) or were untreated (○) prior to eliciting a UK 14304 dose-response curve. B: Tissues were pretreated with mepacrine (0.1 mM) for 30 min (•) or were untreated (○) prior to eliciting a UK14304 dose-response curve. Each data point is the mean ± S.E.M. of six observations.

Table 1
Influence of UK14304 and noradrenaline on [^{3}H]*myo*-inositol phosphates (IPs) in rabbit saphenous vein

	cpm ± S.E.M.			
	IP_1	IP_2	IP_3	*n*
Control	3378 ± 518	2671 ± 434	743 ± 03	8
UK14304 (10 μM; 10 min)	2927 ± 671	2855 ± 601	693 ± 182	6
Noradrenaline (10 μM; 10 min)	7009 ± 1201 [a]	3792 ± 530 [a]	916 ± 274	4

[a] Significantly different from Control ($P < 0.05$).

hibitors neomycin and mepacrine as well as by measurements of [^{3}H]*myo*-inositol phosphate formation, ^{32}P-labelled phospholipid turnover and diacylglycerol formation.

Neomycin (0.1 mM), which inhibits phosphoinositide hydrolysis by phospholipase C, failed to significantly alter UK14304 dose-response curves (Fig. 5A). Mepacrine (0.1 mM), which is a non-selective inhibitor of phospholipases, caused a substantial reduction of 65% in the maximum UK14304 response along with a rightward shift (Fig. 5B). Indomethacin (1 μM) failed to alter UK14304 response, indicating that cyclooxygenase products did not play a role in the α_2-adrenoceptor-mediated contraction.

To further determine whether α_2-adrenoceptor stimulation affected phosphoinositide hydrolysis, vein segments were labelled with [^{3}H]*myo*-inositol then, after a brief washout, some groups of tissues were stimulated with UK14304 (10 μM) or noradrenaline (10 μM) for 10 min in a buffer supplemented with 10 μM LiCl while others (control) received no agonist. Subsequent HPLC analysis of ^{3}H-labelled inositol phosphates failed to reveal any difference between UK14304-stimulated and unstimulated groups (Table 1), confirming the absence of UK14304-stimulated phosphoinositide hydrolysis, as was suggested by its

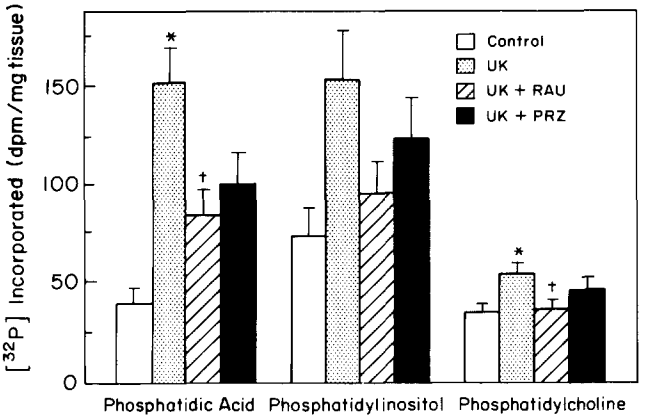

Fig. 6. Influence of UK 14304 on [^{32}P]phospholipid labeling in rabbit saphenous vein. Vein segments were labeled 1 h in ^{32}P-containing buffer and the effects of UK14304 (UK; 10 μM) on [^{32}P]phosphatidic acid (PA), [^{32}P]phosphatidylinositol (PI) and [^{32}P]phosphatidylcholine (PC) levels determined as described in Materials and methods. In some groups either rauwolscine (RAU; 1 μM) or prazosin (PRZ; 1 μM) was added 20 min before UK14304. Data shown are the mean ± S.E.M. of five tissues from a single representative experiment which was replicated in triplicate. An asterisk (*) indicates a significant increase above control group levels, while (†) indicates a significant decrease from the UK14304 only group ($P < 0.05$).

inability to cause intracellular Ca^{2+} release. Noradrenaline did cause a 110% increase in inositol monophosphate and a 42% increase in inositol diphosphate levels.

In other studies the phospholipids in rabbit saphenous vein segments were labelled by incubation with ^{32}P for 1 h and tissues were exposed to UK14304 (10 μM) or vehicle for the final 5 min of the incubation. Thin layer chromatographic analysis of the phospholipids showed that α_2-adrenoceptor stimulation caused a significant 297% increase in the level of ^{32}P-labelled phosphatidic acid, a smaller 115% increase in the labelling of phosphatidylinositol and a 51% increase in phosphatidylcholine labelling (Fig. 6). Incubation with

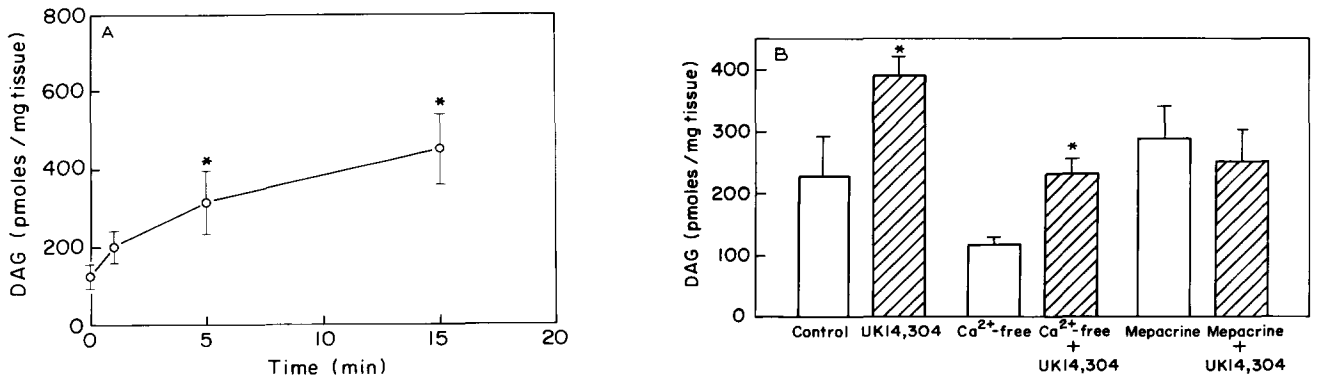

Fig. 7. Influence of UK14304 on diacylglycerol levels in rabbit saphenous vein. A: Tissues were treated with UK14304 (10 μM) for 0, 1, 5 or 15 min prior to determination of diacylglycerol content. B: Tissues were treated with UK14304 (10 μM) for 5 min in either normal buffer (Control) or after 5 min in a Ca^{2+}-free buffer ((−)Ca^{2+}) or after a 30 min pretreatment with mepacrine (0.1 mM). Each data point is the mean ± S.E.M. of 10–15 vein segments. Asterisk (*) indicates a significant increase above unstimulated levels ($P < 0.05$).

rauwolscine (1 μM) significantly reduced the level of phosphatidic acid labelling in UK14304-treated tissues, although not fully to control values. Prazosin (1 μM) also reduced the influence of UK14304 on phosphatidic acid labelling, but the difference did not reach statistical significance ($P = 0.085$). Rauwolscine also eliminated the UK14304-stimulated labelling of phosphatidylcholine while prazosin was less effective.

An α_2-adrenoceptor-induced increase of phosphatidic acid levels could lead to protein kinase C activation by the intermediate formation of diacylglycerol via the activity of phosphatidate phosphohydrolase. Measurement of total diacylglycerol levels in vein segments during 15 min of UK14304 exposure showed a gradual increase which reached significance ($P < 0.05$) at 5 and 15 min time points (Fig. 7A). The increase at 5 min amounted to 148% above control levels, which can be compared to the 297% increase of [^{32}P]phosphatidic acid noted above at the same time point. When Ca^{2+} was deleted from the tissue buffer, basal diacylglycerol levels were reduced to 50% of control; however, UK14304 was still able to increase diacylglycerol formation but only back to control levels (Fig. 7B). Thus extracellular Ca^{2+} availability can modulate diacylglycerol levels and α_2-adrenoceptor stimulation does not increase levels above basal in its absence. This could account for the absence of α_2-adrenoceptor contractile response under Ca^{2+}-free conditions. Mepacrine (0.1 mM) did not significantly alter basal diacylglycerol levels, but blocked the UK14304-induced increase (Fig. 7B).

4. Discussion

The coupling pathways utilized by α_2-adrenoceptor are diverse. While their initially described ability to inhibit adenylate cyclase may be the primary signalling pathway in a few tissues such as adipose tissue, other mechanisms appear to be involved in many tissues including contraction of vascular smooth muscle (Ruffolo and Nichols, 1988). Recognition of three α_2-adrenoceptor subtypes and their ability to couple to more than a single G-protein serves to emphasize the potential for a variety of coupling pathways. Our results provide evidence for the coupling of α_2-adrenoceptor to phospholipid hydrolysis and protein kinase C activation in the rabbit saphenous vein as a primary pathway leading to vasoconstriction.

Involvement of protein kinase C in the α_2-adrenoceptor contractile response is supported by both the substantial inhibition caused by the protein kinase C inhibitors calphostin-C and staurosporine (Fig. 2A) and by a loss of response following down-regulation of PKC with prolonged phorbol ester treatment (Fig. 2B). The greater effectiveness of staurosporine may result from its additional inhibitory effects on other kinases. Thus staurosporine's potency for protein kinase C inhibition is only slightly better than for inhibition of myosin light chain kinase (Sullivan et al., 1992), while calphostin-C is approximately 100-fold more selective for protein kinase C inhibition (Kobayashi et al., 1989). Down-regulation of protein kinase C activity by prolonged phorbol ester treatment was found to be more effective than calphostin-C in reducing α_2-adrenoceptor responses (cf. Figs. 2A and 2B), suggesting the possibility that calphostin-C may not be fully effective against all protein kinase C isoforms.

Isoforms of protein kinase C have been identified which vary in their requirement for activation by Ca^{2+}, diacylglycerol and free fatty acid (Nishizuka, 1992). Whereas intracellular Ca^{2+}-mobilizing receptors such as the α_1-adrenoceptor can provide both Ca^{2+} and diacylglycerol from phosphatidylinositol diphosphate (PIP_2) hydrolysis and resultant inositol trisphosphate (IP_3) formation, α_2-adrenoceptors have generally not been found to elevate inositol trisphosphate, or to cause intracellular Ca^{2+} release as confirmed in the data of Table 1. If protein kinase C is activated by α_2-adrenoceptors, Ca^{2+} elevation would therefore require either a novel Ca^{2+} release mechanism or an augmented influx of extracellular Ca^{2+}. While an augmented influx would be consistent with the recognized critical dependence of α_2-adrenoceptor responses on extracellular Ca^{2+}, we found that rabbit saphenous vein responses were largely unaffected by high concentrations of L-type channel blockers (Fig. 3B), and no augmentation of $^{45}Ca^{2+}$ uptake could be detected (Fig. 4A). Thus while basal extracellular Ca^{2+} influx is vital for its response, α_2-adrenoceptor stimulation in rabbit saphenous vein does not appear to activate protein kinase C via increased Ca^{2+} entry. The role of basal extracellular Ca^{2+} entry may be critical for permitting the coupling of α_2-adrenoceptor to other pathways.

In an earlier study in rabbit saphenous vein (Aburto et al., 1993), UK14304 (10 μM) was found to elevate intracellular Ca^{2+} and to cause a shift in the relationship between force and intracellular Ca^{2+} such that higher force developed at each Ca^{2+} level than was the case during K^+-induced depolarization. This increased force or 'Ca^{2+} sensitization' is similar to that previously identified with activation of protein kinase C by α_1-adrenoceptor agonists in other blood vessels (Nishimura et al., 1988), supporting a role for protein kinase C in α_2-adrenoceptor coupling in rabbit saphenous vein.

Increased formation of [^{32}P]phosphatidic acid and diacylglycerol during α_2-adrenoceptor stimulation (Figs. 6 and 7) indicates that protein kinase C activation could be caused by diacylclycerol, provided by activation of a phospholipase. The absence of increased [^{3}H]*myo*-inositol phosphate formation, how-

ever, would seem to rule out phosphoinositide hydrolysis by phospholipase C and suggests involvement of other phospholipases. Phospholipase D activation leads to initial phosphatidic acid formation and subsequent diacylglycerol formation via the action of phosphatidate phosphohydrolase, which could account for the increase of both which we observed. The ability of mepacrine, a non-selective phospholipase inhibitor, to reduce both UK14304-induced contractions (Fig. 5B) and diacylglycerol formation (Fig. 7B) strongly indicates the involvement of a phospholipase in providing for protein kinase C activation.

While coupling of α_2-adrenoceptor to phospholipase D has not been previously described in vascular tissues, MacNulty et al. (1992) have demonstrated the ability of a cloned α_2-adrenoceptor to activate phospholipase D in rat fibroblasts. Several studies have shown the ability of norepinephrine to stimulate vascular smooth muscle phospholipase D activity in close association with tension development (Gu et al., 1992; Jones et al., 1993), although based upon its inhibition by prazosin (10 μM), the stimulation has been attributed to α_1-adrenoceptor activation. However, inhibition by prazosin could alternatively reflect a requirement for α_1-adrenoceptor costimulation rather than direct phospholipase D activation. The importance of costimulation of α_1-adrenoceptors for allowing α_2-adrenoceptor response has been amply demonstrated by others (Daly et al., 1988; Shimamoto et al., 1992) and we have likewise found that prazosin produces a non-competitive reduction in UK14304 responses of rabbit saphenous vein (Fig. 1B). α_1-Adrenoceptor activation presumably accounts for the augmented Ca^{2+} influx caused by noradrenaline (Fig. 4A) which may reflect refilling of released Ca^{2+} storage sites or receptor-operated channel activity. The permissive role of α_1-adrenoceptor stimulation for α_2-adrenoceptor responsiveness may derive from these or other coupling events, as suggested by the observations of Shimamoto et al. (1992).

The receptor-mediated activation of phospholipase D has been shown to require activation of a tyrosine kinase (Bourgoin and Grinstein, 1992; Uings et al., 1992). Recent studies in our laboratory have found that tyrosine kinase inhibitors are able to completly abolish the UK14304 contractile response in rabbit saphenous vein while α_1-adrenoceptor-mediated contractions of rabbit aorta and high K^+ contractions of rabbit saphenous vein are relatively unaffected (Jinsi and Deth, 1994). In addition, wortmannin, which inhibits receptor activation of phospholipase D and is also a kinase inhibitor, causes a preferential inhibition of α_2-adrenoceptor-stimulated contractions in rabbit saphenous vein (Waen-Safranchick and Deth, 1994). These observations further support a role for α_2-adrenoceptor-induced phospholipase D activation as a source of the increased diacylglycerol during UK14304-induced contractions of rabbit saphenous vein.

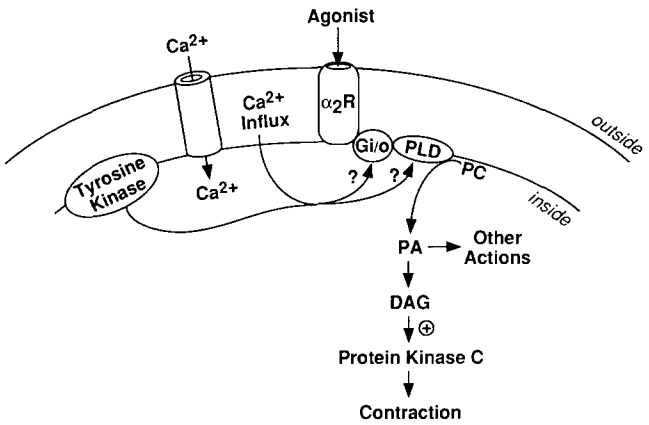

Fig. 8. Proposed coupling pathway for α_2-adrenoceptors in the rabbit saphenous vein. α_2-Adrenoceptor activation may be coupled via a pertussis toxin-sensitive G-protein (G_i) to phospholipid hydrolysis involving phospholipase D (PLD) and the formation of phosphatidic acid (PA). Once formed, PA may give rise to diacylglycerol (DAG) via the action of phosphatidate phosphohydrolase leading to an increase of protein kinase C (PKC) activity and contraction. Basal influx of extracellular Ca^{2+} and an as yet unidentified tyrosine kinase are critical regulators of α_2-adrenoceptor receptor coupling to phospholipase D.

Based upon our findings, the pathway for α_2-adrenoceptor-induced contraction of rabbit saphenous vein can be summarized as outlined in Fig. 8. According to this scheme, α_2-adrenoceptor agonists activate a pertussis toxin-sensitive G-protein (e.g. G_i/G_o) to provide increased activity of phospholipase D. Phosphatidic acid produced by phospholipase D can lead to augmented dyacylglycerol levels and subsequent activation of protein kinase C. The precise mechanism by which protein kinase C activity supports contraction remains obscure. The dependence of α_2-adrenoceptor contractile response upon extracellular Ca^{2+} may illustrate the importance of Ca^{2+} levels for supporting phospholipase D activity rather than representing the source of the receptor-dependent signal for contraction. A criticaly important tyrosine kinase activity may regulate the ability of the receptor to provide for phospholipase D activation. Verification of this suggested pathway will require additional studies.

While our studies indicate that increased phosphatidic acid formation is associated with increased diacylglycerol levels, it may also serve as a substrate for phospholipase A_2 activity leading to lysophosphatidic acid and arachidonic acid formation. Phosphatidic acid itself may also exert other effects including activation of protein kinase C (Oishi et al., 1988) and phosphatidylinositol-4-phosphate kinase (Moritz et al., 1992) so that the net α_2-adrenoceptor response may reflect the composite influence of several phospholipid-derived messenger substances.

In conclusion, we have provided evidence for the involvement of protein kinase C activation in the α_2-adrenoceptor-mediated contractile response of rabbit saphenous vein and the source of the activation is most likely an increase of diacylglycerol, produced by the action of a phospholipase D. Extracellular Ca^{2+} is critical for efficacy of the α_2-adrenoceptor although increased Ca^{2+} influx per se is not the primary receptor coupling pathway.

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