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α-ADRENOCEPTOR ACTIVATION OF POLYPHOSPHOINOSITIDE HYDROLYSIS IN THE RAT TAIL ARTERY

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Abstract—α-Adrenoceptor coupling to polyphosphoinositide (PI) hydrolysis was studied in the rat tail artery. Inositol phosphate (IP) accumulation was stimulated by the non-selective α-adrenoceptor agonist norepinephrine and the α_1-adrenoceptor agonist phenylephrine. This stimulation was relatively dependent on extracellular Ca^{2+} and enhanced markedly in the presence of LiCl. In addition, norepinephrine- and phenylephrine-stimulated IP accumulation was relatively sensitive to blockade by prazosin, compared to rauwolscine. The putative α_2-adrenoceptor agonist UK 14304 also stimulated PI breakdown in a concentration-dependent manner, although this stimulation did not reach equilibrium at up to 10 mM and was relatively sensitive to prazosin, compared to rauwolscine, over the lower agonist concentrations. NaF stimulated IP accumulation independently of α-adrenoceptor activation. PI breakdown by α-adrenoceptor agonists and NaF was attenuated by *N*-ethylmaleimide but not pertussis toxin treatment. In addition, dithiothreitol blocked NaF-stimulated, but not α-adrenoceptor-mediated, PI breakdown. These results suggest the coupling of α_1-adrenoceptor, via phospholipase C, to PI hydrolysis in the rat tail artery. This study also provides evidence for the involvement of one or more non-G_i-like G-protein(s) in the signal transduction process.

That both α_1- and α_2-adrenoceptors are located postsynaptically in vascular smooth muscle and contribute to arterial vasoconstriction associated with sympathetic activation is now generally accepted [1]. Much of the evidence in favour of a dual role for α_1- and α_2-adrenoceptors is based upon blood pressure studies with pithed animals as, for instance, demonstrated by Drew and Whiting [2]. Supportive data from *in vitro* experiments are, however, sparse, at least for vascular smooth muscle from the arterial side of the circulation, and, with few exceptions, a true α_2-like response elicited by a selective α_2-agonist and unaffected by an α_1-antagonist, such as prazosin, has not been demonstrated. Thus, Agrawal *et al.* [3] concluded that their study did not clearly support the concept of pharmacologically distinct α_1- and α_2-sites; indeed, they suggested that their data could be interpreted as evidence for only one population of α-adrenoceptors with which the agonists, though not antagonists, non-selectively interact. Medgett and Langer [4] formed a somewhat similar conclusion and hypothesised that there were two subtypes of α_1-adrenoceptors.

A recent study by Cheung and Triggle [5] described distinct binding sites for [^{3}H]prazosin and [^{3}H]rauwolscine in vascular smooth muscle cell membranes from the rat tail artery. The [^{3}H]rauwolscine site demonstrated the expected α_2-adrenoceptor profile with agonist affinity reduced by Gpp(NH)p and Na^+ and, although agonist affinity for the [^{3}H]prazosin site was also reduced by Gpp(NH)p and Na^+, this modulation was clearly different from that at the [^{3}H]rauwolscine site. This initial study by Cheung and Triggle [5], however, did not address the question of whether the described α_1- and α_2-like sites were functionally coupled to smooth muscle contraction, the expectation (based upon the proposal of Fain and Garcia-Sainz [6]) being that stimulation of vascular α_1-adrenoceptors would lead to the activation of polyphosphoinositide metabolism whereas activation of α_2-adrenoceptors would lead to the inhibition of adenylate cyclase. There is considerable evidence in a variety of tissues for the coupling of the α_1-adrenoceptor to phospholipase C activation and subsequent inositol trisphosphate generation [7], although that for vascular tissue is less substantive.

The process of excitation–contraction coupling for postsynaptic α_2-adrenoceptors in vascular smooth muscle has remained obscure; however, based upon evidence with other tissues, α_2-adrenoceptor activation may be expected to couple, via the inhibitory G_i protein, to an attenuation of adenylate cyclase activity [8]. Stimulation of both postsynaptic α_1- and α_2-adrenoceptors in smooth muscle would seem to be linked to processes that result in the translocation of extracellular Ca^{2+} across the cell membrane. Some studies have suggested an apparently specific association between α_2-adrenoceptor activation and Ca^{2+} translocation through the voltage-operated calcium channel [9], although this view has aroused some controversies [10–12]. An association between the activation of an α_2-adrenoceptor, the Ca^{2+} mobilization, and a decrease in the activity of adenylate cyclase in modulating the tone of vascular smooth muscle has not been demonstrated.

In view of these uncertainties concerning the coupling processes for postsynaptic α-adrenoceptors in

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smooth muscle, the present study was directed toward evaluating the role of α_1- and α_2-adrenoceptors in mediating the breakdown of polyphosphoinositide in the rat tail artery.

MATERIALS AND METHODS

Materials. *myo*-[2-^{3}H(N)]Inositol (14–20 Ci/mmol) was purchased from New England Nuclear. Norepinephrine bitartrate, phenylephrine hydrochloride *myo*-inositol, Dowex-1 resin, *dl*-dithiothreitol and *N*-ethylmaleimide were purchased from Sigma. Rauwolscine hydrochloride was obtained from Carl Roth (Karlsruhe, F.R.G.). UK14304 and prazosin hydrochloride were gifts from Pfizer, U.K.

Methods. Female rats obtained from Hybrid Farms of Nova Scotia, Canada, were killed by cervical dislocation, and rat tail arteries were dissected, cleaned and cut into segments approximately 1 cm long; typically 12–14 segments were obtained from each animal. The artery segments were then preincubated in Krebs-bicarbonate oxygenated buffer at 37° for 1 hr, during which buffer was changed three to four times. Subsequently, artery segments were incubated for 1.5 hr at 37° in fresh buffer containing approximately 25 μCi/mL of [^{3}H]*myo*-inositol. The incubation medium was oxygenated continuously. At the end of the incubation, [^{3}H]inositol-labelled artery segments were washed three times with ice-cold buffer before being added to individual assay tubes containing 10 mM LiCl in a total assay volume of 300 μL. Agonists were then added to yield the desired final concentrations.

In experiments in which antagonists were used, artery segments were pre-exposed to the antagonists for 20–30 min before the addition of agonists. Incubation was carried out at 37° for 45 min and was stopped by adding 0.3 mL of ice-cold trichloroacetic acid (1 M). The tubes were left in ice for 20–30 min. Afterwards the tube contents were vortexed, and 0.5-mL aliquots were transferred to clean assay tubes and washed with 2 vol. of water-saturated diethyl ether five times. After the final wash, $NaHCO_3$ (100 mM) was added to adjust the pH of the contents to 7–8. Then 0.4-mL aliquots were loaded on Dowex-1 (×8) ion-exchange columns (formate form, 100–200 mesh, 1 mL). The columns were washed initially with 10 mL *myo*-inositol (5 mM). Then tritium-labelled inositol phosphates were eluted with 8 mL of 0.1 M formic acid/1 M ammonium formate. Aliquots (2 mL) were counted for radioactivity in 20 mL scintillant (NEN Formula 963) using a liquid scintillation counter (Beckman LS3801).

In some experiments, anesthesized rats were injected, via the jugular vein, with 0.9% saline alone or 50 μg/kg pertussis toxin in saline. Five animals were treated with saline and five with pertussis toxin, and the animals were maintained for 48 hr before being killed. The tail artery was dissected, and the stimulation of the production of inositol phosphates (IP) was assayed using the procedure described above. In another series of experiments, intraperitoneal injections of pertussis toxin or saline were administered at a dose of 15 μg/kg and the animals again maintained for 48 hr.

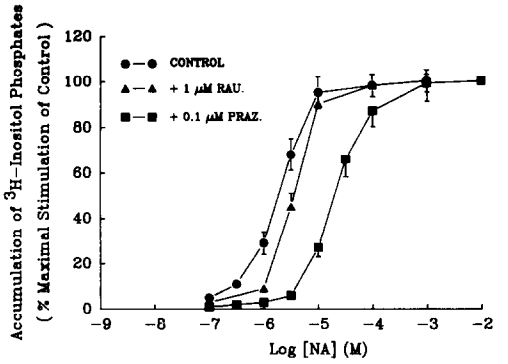

Fig. 1. Stimulation of the accumulation of inositol phosphates in the rat tail artery by norepinephrine (NA) in the absence or presence of rauwolscine and prazosin. The accumulation of [^{3}H]inositol phosphates was assayed as described in Methods. Results are expressed as a percentage of the maximal stimulation generated by the agonist alone. Maximal stimulation to NA was 20- to 40-fold above basal with basal levels ranging from 500 to 900 dpm/tissue. Each data point is the mean ± SE of three separate experiments.

In *in vitro* studies involving pertussis toxin, the toxin was first activated by incubating in buffer containing 50 mM dithiothreitol (DTT) (50 μg/mL) for 1 hr at 25°. Following pre-exposure to pertussis toxin for 2 hr, the [^{3}H]*myo*-inositol incorporated tail artery segments were stimulated in the absence or presence of agonist. The accumulation of inositol phosphates was assayed using the same procedure described before.

Results of this study are expressed mainly as the percentage of the control basal levels (in the absence of agonist or antagonist). In situations in which maximal stimulation could be established, results are expressed as the percentage of the maximal stimulation produced by the agonist. To minimize intra-assay variations, the radioactivity measured was further normalized to wet tissue weight. In most instances, a minimum of three separate experiments was performed with each data point for an individual experiment representing the result of quintuplicate measurements.

RESULTS

α-Adrenoceptor-mediated stimulation of polyphosphoinositide metabolism in rat tail artery. A number of membrane receptors are known to be coupled to inositol phospholipid metabolism in a variety of tissues, including vascular smooth muscle. In the rat tail artery, the production of inositol phosphates could be readily stimulated by α-adrenoceptor agonists. Figure 1 demonstrates the stimulation of total IP production by norepinephrine. The maximal stimulation was attained in approximately 45 min and was usually 20- to 40-fold above the basal level with basal levels being very similar in all protocols subsequently followed. As shown in Fig. 1, the stimulation of IP production by norepinephrine was relatively sensitive to prazosin, but insensitive to

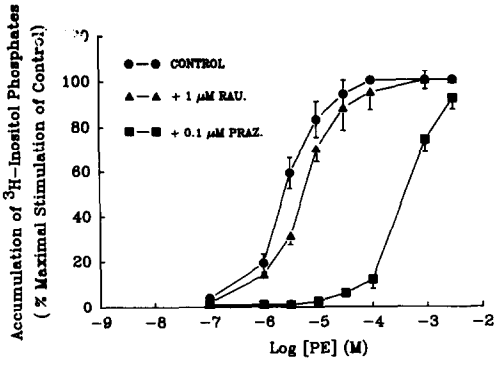

Fig. 2. Stimulation of the accumulation of inositol phosphates in rat tail artery by phenylephrine (PE) in the absence or presence of rauwolscine and prazosin. The accumulation of [^{3}H]inositol phosphates was assayed as described in Methods. Results are expressed as a percentage of the maximal stimulation produced by the agonist alone. Maximal stimulation to PE was 8- to 10-fold above basal with basal levels ranging from 650 to 750 dpm/tissue. Each data point is the mean ± SE of three separate experiments.

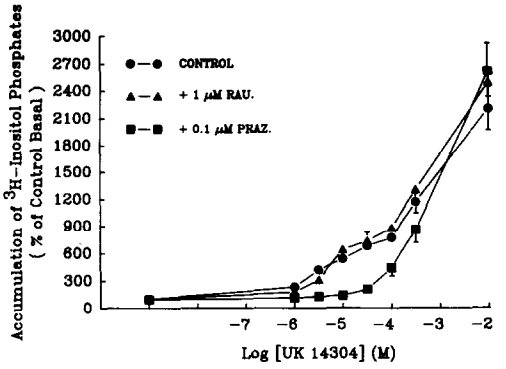

Fig. 3. Stimulation of the accumulation of inositol phosphates in rat tail artery by UK 14304. Stimulation was carried out at 37° in the absence and presence of rauwolscine and prazosin as described in Methods. Results are means ± SE from three separate experiments performed in quintuplicate, and are expressed as a percentage of the basal levels of the controls (i.e. in the absence of agonist and antagonist) which were 4950–5610 dpm/mg tissue weight.

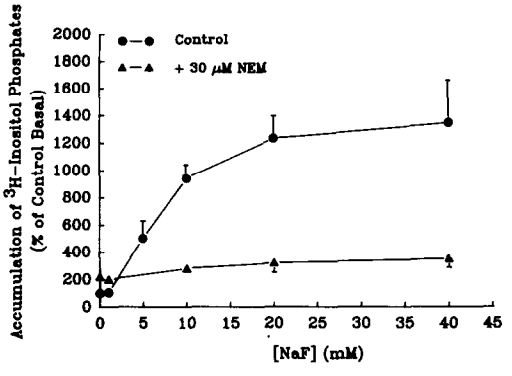

Fig. 4. Stimulation of the accumulation of inositol phosphates in rat tail artery by NaF in the absence and presence of NEM. Results are means ± SE from one experiment representative of two performed in quintuplicate. Results are expressed as a percentage of the basal level of control which was approximately 6630 dpm/mg tissue weight.

rauwolscine, indicating that it is an α_1-adrenoceptor-mediated phenomenon. In Fig. 2, stimulation of IP production by the selective agonist phenylephrine is illustrated. Again, the relative sensitivity of the response to prazosin, compared to rauwolscine, unequivocally confirms the coupling of α_1-adrenoceptor stimulation to membrane polyphosphoinositide metabolism in the rat tail artery.

The optimal stimulation of IP production in the rat tail artery appears to be dependent on extracellular Ca^{2+}. The stimulation of IP production by norepinephrine was enhanced markedly by exogenous Ca^{2+} in a concentration-dependent manner. The maximal enhancement was achieved at approximately 5 mM Ca^{2+} with an EC_{50} of about 80 μM.

It is now known that Li^+ enhances the accumulation of inositol phosphates mainly by inhibiting the breakdown of inositol monophosphates. This phenomenon could also be demonstrated clearly in the rat tail artery. The pronounced modulatory effect of Li^+ was evident when compared to that of norepinephrine alone with an 8- to 12-fold maximal enhancement at a concentration of 10 mM and an EC_{50} of around 0.2 mM.

In separate studies, sequential elution of extract from rat tail artery through Dowex resin, according to the method of Berridge *et al.* [13], showed that IP_3/IP_4 were formed rapidly following agonist stimulation and reached a maximum in about 5 min (data not shown). However, the levels of these higher phosphates were relatively low. A longer time for agonist stimulation in the rat tail artery allowed an equilibrium accumulation of total inositol phosphates, of which the monophosphates represented the predominant component.

To evaluate the role of α_2-adrenoceptors in mediating the stimulation of inositol phospholipid metabolism in rat tail artery, the effect of UK 14304, a putative α_2-adrenoceptor selective agonist, was examined. As shown in Fig. 3, UK 14304 was able to stimulate a substantial IP production in the rat tail artery. However, the stimulation did not reach a maximum level even at a concentration of 10 mM. Moreover, the effect was relatively insensitive to blockade by rauwolscine, although susceptible to prazosin—at least over the lower agonist concentration range.

The stimulation of membrane inositol phospholipid breakdown is primarily dependent on phospholipase C. Recent evidence suggests that the mode of modulation of this enzyme by membrane receptors closely resembles that for adenylate cyclase. Thus, the role of guanine nucleotide binding protein(s) in coupling α-adrenoceptor to polyphosphoinositide breakdown in the rat tail artery was examined.

Effect of sodium fluoride (NaF). NaF markedly increased IP production in the rat tail artery independent of α-adrenoceptor agonist. This concentration-dependent stimulation attained a maximum between 15 and 20 mM (Fig. 4).

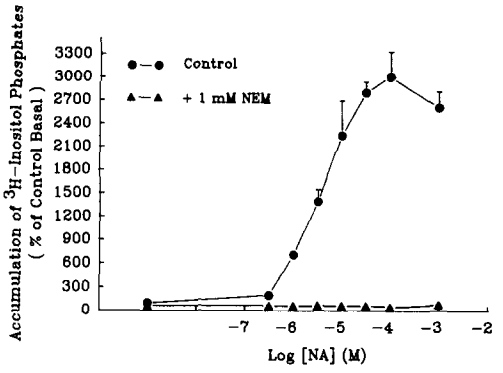

Fig. 5. Effect of NEM on norepinephrine (NA) stimulated accumulation of inositol phosphates in rat tail artery. Data points are means ± SE of five replicate determinations from one of two similar experiments. Results are expressed as a percentage of the basal level of control which was approximately 3720 dpm/mg tissue weight.

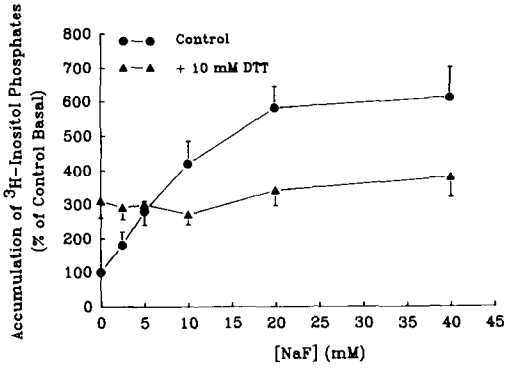

Fig. 6. Effect of DTT on NaF stimulation of the accumulation of inositol phosphates in rat tail artery. Following [^{3}H]*myo*-inositol incorporation, rat tail artery was stimulated with various concentrations of NaF in the absence or presence of 10 mM DTT. Tissue was preincubated in 10 mM DTT for 2 hr before the addition of NaF. Inositol phosphate production was assessed as described in Methods. Data points are means ± SE of three experiments. Results are expressed as a percentage of the basal levels of control which measured 5260–7410 dpm/mg tissue weight.

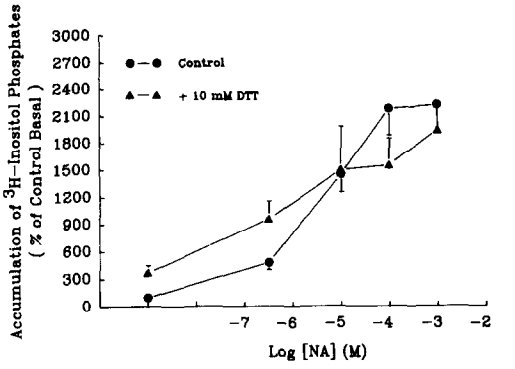

Fig. 7. Effect of DTT on norepinephrine stimulation of the accumulation of inositol phosphates in rat tail artery. Following [^{3}H]*myo*-inositol incorporation, rat tail artery preincubated with or without 10 mM DTT for 2 hr was stimulated with various concentrations of norepinephrine, and IP production was assessed as described in Methods. Data points are means ± SE of three separate experiments. Results are expressed as a percentage of the basal levels of control which ranged from 5600 to 7130 dpm/mg tissue weight.

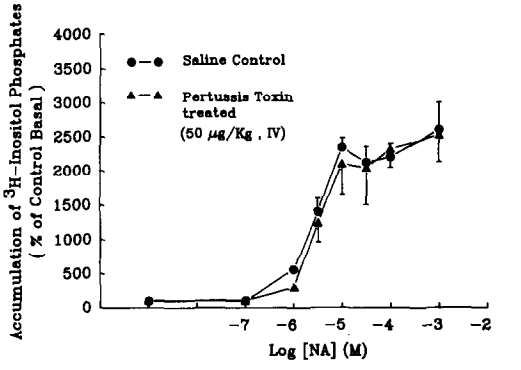

Fig. 8. Effect of *in vivo* treatment with pertussis toxin (PT) on norepinephrine-stimulated accumulation of inositol phosphates in rat tail artery. Rats were pretreated with PT for 48 hr, and IP accumulation in tail artery was assessed as described in Methods. Data points are means ± SE of five replicate determinations from a single experiment which itself is representative of two such experiments demonstrating identical results. Results are expressed as a percentage of basal level of the saline control which measured approximately 5010 dpm/mg tissue weight.

Effect of N-*ethylmalimide (NEM).* Pretreatment of rat tail artery with the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM) resulted in a drastic reduction of the NaF stimulation (Fig. 4). NEM treatment also caused pronounced attenuation of the stimulation mediated by the α_1-adrenoceptor agonist norepinephrine (Fig. 5).

Effect of dithiothreitol (DTT). In vitro pretreatment of rat tail artery for 2 hr with dithiothreitol, a —SH group protective agent, markedly enhanced the basal IP level by 3- to 4-fold. At 10 mM, DTT essentially abolished the stimulatory effect of NaF (Fig. 6). However, at such a concentration, DTT did not alter significantly the maximal stimulation of IP accumulation generated by norepinephrine (Fig. 7).

Effect of pertussis toxin. To evaluate further the nature of the G-protein involved in the coupling process, the effect of pertussis toxin was examined. *In vitro* treatment of rat tail artery with pertussis toxin in the presence of DTT did not affect norepinephrine and NaF stimulation of IP production any differently from that with DTT alone (data not shown). Furthermore, the treatment of rat tail artery with pertussis toxin alone did not change the basal IP level or the maximal stimulation generated by norepinephrine and NaF. Likewise, norepinephrine stimulated IP production was not attenuated significantly in the tail artery of rats pretreated intravenously with pertussis toxin for 48 hr (Fig. 8). The effects of the intraperitoneal administration of per-

tussis toxin were also studied (data not shown) and, again, norepinephrine-stimulated IP production was not affected significantly.

DISCUSSION

In vascular tissue, α-adrenoceptor activation is associated with tone development which is dependent, to varying degrees, on both extracellular Ca^{2+} (Ca^{2+}_e) and intracellular Ca^{2+} (Ca^{2+}_i) with Ca^{2+}_e entry occurring via receptor-operated Ca^{2+} channels (ROC). Receptor-coupled Ca^{2+}_i release is thought to be mediated through phospholipase C (PLC) activity on polyphosphoinositide (PI) hydrolysis and the subsequent generation of inositol trisphosphate [14], and PI hydrolysis has been suggested as a primary step in α-adrenergic stimulus–response coupling [15]. In part, based upon studies with other tissue types, it is assumed that it is the α_1-adrenoceptor subtype that is coupled to PLC, whereas the α_2-subtype is coupled in a negative manner to adenylate cyclase activity [6, 16].

Several studies have provided evidence that receptor activation in vascular smooth muscle does lead to the stimulation of PI turnover. Thus, Villalobos-Molina *et al.* [17] have demonstrated that α-adrenoceptor agonists increased ^{32}PI labelling and contracted the rabbit aorta preparations, and they concluded that, based on both agonist profile and comparative prazosin and yohimbine antagonistic potency, these reactions were attributed to activation of an α_1-adrenoceptor. Berta *et al.* [18] have reported that the α_1-adrenoceptor agonist phenylephrine both contracts the isolated perfused rat tail artery and activates PI metabolism, and Fox and Friedman [19] reported that norepinephrine also activates PI metabolism in the rat tail artery and that the concentration–response curve could be shifted to the right with phenoxybenzamine; the non-selective α-adrenoceptor agonist, clonidine, is only a partial agonist and is non-competitively antagonized by phenoxybenzamine. Similarly, in the rat aorta, Rapoport [20] has reported that norepinephrine stimulation of PI metabolism is prazosin and nifedipine sensitive and dependent upon the presence of Ca^{2+}_e, thus suggesting that PI metabolism is occurring by a Ca^{2+}-dependent activation of phospholipase C via an α_1-adrenoceptor. Chiu *et al.* [21] also provide evidence for a prazosin-sensitive and yohimbine-resistant activation by norepinephrine of PI hydrolysis in the rat aorta. Furthermore, it was demonstrated that the α_1-adrenoceptor partial agonist Sgd 101/75 which produces muscle contraction via the entry of Ca^{2+}_e, was ineffective in stimulating PI metabolism. The latter data are suggestive that there may be at least two subtypes of α_1-adrenoceptors or that there is a single type of α_1-adrenoceptor capable of activating two separate pathways of Ca^{2+} metabolism [22].

Cheung and Triggle [5] have reported previously that the rat tail artery possesses pharmacologically separable binding sites for [^{3}H]prazosin and [^{3}H]rauwolscine and that these two sites are regulated in a distinct manner by GTP binding proteins. This latter study is indicative that there are distinct α_1- and α_2-adrenoceptors associated with rat tail artery vascular smooth muscle. This conclusion is supported by the recent observation by Cheung and Triggle [23] that the rat tail artery also possesses specific binding sites for the selective α_2-adrenoceptor agonist UK 14304, and this binding site was also modulated by both Na^+ and Gpp(NH)p (unpublished data). Overall, these results are indicative that both α_1- and α_2-adrenoceptor sites are present in vascular smooth muscle from the rat tail artery and support the functional data of Medgett and Langer [4]; however, this conclusion is not supported by some studies that have failed to demonstrate distinct functional α_2-receptors in the isolated perfused rat tail artery preparation [24, 25] except in the spontaneously hypertensive rat [26].

In the present study, we have investigated and compared the effects of the non-selective α-adrenoceptor agonist norepinephrine and the α_1-selective agonist phenylephrine, as well as the α_2-selective agonist UK 14304 on PI hydrolysis metabolism. We have also investigated, by studying the effects of pertussis toxin, the —SH alkylating agent *N*-ethylmaleimide (NEM), and NaF, the role of a GTP-dependent protein in coupling α-adrenoceptors to phospholipase C activation.

The results with the α-adrenoceptor agonists indicate that both norepinephrine and phenylephrine are potent activators of PI hydrolysis and that this response is mediated, as indicated by prazosin-sensitivity and rauwolscine-insensitivity, via α_1-adrenoceptors. Although the α_2-selective agonist UK 14304 also stimulates an increase in PI breakdown, it is not a potent activator and the response is relatively prazosin sensitive and rauwolscine insensitive, suggesting that the interaction does not involve α_2-adrenoceptors. At concentrations of UK 14304 greater than 1 mM, a prazosin-insensitive increase in PI metabolites was observed and this, perhaps, relates to the interaction of UK 14304 with atypical or non-adrenoceptor sites in the rat tail artery preparation [23]. In a comparison of the guinea pig aorta and human digital arteries, Moulds *et al.* [27] have reported that whereas the α_1-adrenoceptor agonist methoxamine stimulates PI hydrolysis in both vessel types, the α_2-adrenoceptor agonist, TL99, activates PI hydrolysis only in human digital arteries. The authors conclude that where both postsynaptic α_1- and α_2-adrenoceptors co-exist, they share the same coupling processes. However, the effects of selective antagonists were not determined.

The optimal concentration of LiCl, necessary to inhibit the dephosphorylation of IP derived from (1,4,5)IP_3 [13] was determined to be 10 mM and was included henceforth in all PI assay procedures. Our studies also revealed the dependency upon extracellular Ca^{2+}_e of the PI response to norepinephrine, a relationship that may be expected based upon the stated universality of requirement of phospholipase C for Ca^{2+} [28]. It should also be noted, however, that a number of secretory responses have been described where, although secretion is inhibited by the removal of Ca^{2+}_e, the PI response remains unaffected [29, 30]. In addition, Rapoport [20] has reported that norepinephrine activation of PI hydrolysis in the rat aorta requires extracellular Ca^{2+}, whereas acetylcholine-induced PI hydrolysis

in the rat vas deferens is independent of Ca^{2+}_e [31]. Similarly, Berta *et al.* [18] report that although contractions of the rat tail artery to both 5-hydroxytryptamine (5-HT) and phenylephrine are dependent upon Ca^{2+}_e, the PI response to 5-HT, but not phenylephrine, was unaffected by Ca^{2+}_e removal. Berta *et al.* [18] conclude that the Ca^{2+}_e dependency of the phenylephrine response may be at the level of agonist binding to the α_1-adrenoceptor.

The elution profile from the Dowex columns revealed that the principal inositol phosphate formed following our experimental protocol was IP with significantly smaller amounts of IP_2 and low levels of higher phosphates. In our experimental protocol, an agonist exposure time of 45 min was followed; time–course studies of the generation of inositol phosphates in other smooth muscle preparations, namely the rat aorta and the guinea pig ileal longitudinal, reveal similar information [20, 32].

The activation of PLC by agonists in broken cells has been reported to be augmented by non-hydrolysable analogues of GTP [33], leading to the expectation that a G-protein couples receptor activation to the PLC-mediated hydrolysis of PIP_2. In a number of instances, but certainly not all, receptor activation of PLC has been inhibited with pertussis toxin [33]. Our studies indicated that neither *in vivo* nor *ex vivo* treatment of the vascular tissue with pertussis toxin prevented the activation of PI hydrolysis by α-agonists, thus suggesting that if a G-protein does regulate α-adrenoceptor to PLC activation, it is not analogous to the pertussis toxin sensitive G_i protein that is negatively coupled to adenylate cyclase.

Since, in the present study, the results with pertussis toxin were essentially negative, it may be questioned as to whether our *in vivo* or *in vitro* protocols for pertussis toxin treatment do result in the ADP ribosylation of sensitive G proteins. The *in vitro* procedure and the *in vivo* intraperitoneal protocol which we followed are essentially those of Anand-Srivastava *et al.* [34] who have reported that such treatments attenuate the atrial natriuretic factor (ANF)-mediated inhibition of basal, isoproterenol, and forskolin-stimulated adenylate cyclase activities in the rat aorta. Furthermore, we have used the jugular vein *in vivo* protocol in our laboratory [35] and recently demonstrated that pertussis toxin pretreatment selectively attenuates the pressor response to endothelin in the pithed rat, leaving the response to vasopressin unaffected. It, therefore, seems most likely that the pertussis toxin pretreatment procedures that we have followed should lead to the ADP ribosylation of sensitive proteins. Thus, the fact that the systems we examined were pertussis toxin insensitive clearly suggest that the G proteins, or proteins, that couple α-adrenoceptors and the NaF-mediated effect to phospholipase C in rat arterial smooth muscle are not substrates for this toxin.

It has been suggested [28] that it is a novel G protein, G_p, that regulates PLC activity and that this protein is pertussis toxin sensitive in some cell types, such as mast cells and neutrophils, but not in others such as liver, heart cells and pancreas. Alternatively, two or more different G proteins may regulate PLC and at least one of these proteins is ADP-ribosylated by pertussis toxin, although it should also be noted that ADP-ribosylation independent actions of pertussis toxin have been reported [36].

It is of interest to note that several studies have indicated that the responses mediated by postsynaptic vascular α_2-receptors are pertussis toxin sensitive, whereas responses mediated by α_1-adrenoceptors and presynaptic α_2 are not [37–41]. Thus, the insensitivity to pertussis toxin of PI hydrolysis stimulated by α-adrenoceptor agonists in the rat tail artery may also be interpreted as indicating that α_2-adrenoceptors are not involved in PI hydrolysis.

A recent study by Nichols *et al.* [41] of the effects of α_1- and α_2-adrenoceptor function in the cardiovascular system of the pithed rat have reported that pressor effects mediated by the full α_1-adrenoceptor agonist cirazoline was pertussis toxin insensitive; however, after removal of the receptor reserve by phenoxybenzamine, the response became pertussis toxin sensitive. Phenoxybenzamine treatment also results in an increased sensitivity of the α_1-adrenoceptor mediated pressor response to Ca^{2+} channel antagonists [42]. The α_2-adrenoceptor response is also both pertussis [41] and Ca^{2+} channel antagonist sensitive [42], leading Nichols *et al.* [41] to conclude that the α_1-adrenoceptor may be coupled to two distinct G proteins, one of which is pertussis toxin sensitive and couples the receptor to Ca^{2+} channels, and the other is toxin insensitive and is coupled to PLC and intracellular Ca^{2+} mobilization.

Our studies with the sulfhydryl alkylating agent NEM, however, are indeed suggestive that a G-protein of some type is involved in regulating PLC activity in the tail artery. Pretreatment with 1 mM NEM completely inhibited norepinephrine activation of PI hydrolysis. It is, of course, possible that the effects of NEM are a result of some non-selective actions on plasma membrane proteins, including for instance, receptor proteins. However, some studies do suggest a relatively selective action of NEM on the G-proteins [43, 44].

Further support of the role of a pertussis toxin insensitive, NEM sensitive G-protein in regulating PLC activity in vascular smooth muscle is provided by the ability of NaF to both initiate contraction and stimulate PI hydrolysis (Figs. 4 and 6), which could be inhibited by NEM. Fluoride, complexed with aluminum in the form of AlF_4^-, is thought to activate G-proteins by mimicking the action of the γ-phosphate of GTP at the guanine nucleotide site on the α-subunit [28, 45, 46]. Furthermore, the actions of NaF have been shown to demonstrate selectivity towards G_i vs G_s [47], although one study has also reported that NaF can inhibit agonist-induced formation of inositol trisphosphate with a G-protein negatively coupled to phospholipase C [48].

It is of interest to note that DTT produced an effect on NaF stimulation similar to that of NEM (Fig. 6), although this sulfhydryl group protective agent did not alter significantly the maximal stimulation generated by norepinephrine, suggesting that the G proteins activated by NaF versus norepinephrine may differ. It also appears evident that DTT significantly elevated the basal IP levels. It has been suggested that the ability of F^- to activate G-proteins depends on the direct effective linkage of

AlF_4^- to GDP normally residing at the guanine binding site with the axial and the two lateral oxygens of the β-phosphate of the guanine nucleotide being essential for binding to and interacting with G-proteins [46]. If the β-phosphate is absent, has its lateral oxygens substituted or is already bound to a γ-phosphate, AlF_4^- cannot bind. It is possible that the ability of NEM and DTT to inhibit NaF stimulation is attributed in part to some chemical modifications occurring to the β-phosphate or its binding domain, thus interfering with the interaction with AlF_4^-. Furthermore, the ability of DTT (and to a certain extent, NEM) to elevate the basal PI levels may also relate to a modulation of the hydrolysis of GTP on the G-protein by GTPase. In the event of receptor activation by residual endogenous agonists, intracellular GTP, upon catalysed exchange for GDP, may persistently pre-occupy a proportion of the guanine nucleotide sites, resulting in an elevated "basal" stimulation of PLC and a significant blockade to the subsequent access of AlF_4^- to the G-protein. As for the norepinephrine stimulation of PI hydrolysis, the complete blockade by NEM, compared to DTT, may reflect the additional inhibitory effect of NEM on the receptor–G-protein coupling, possibly at sites on the agonist binding domains and/or the coupling domains on the G-protein involving —SH interactions.

In conclusion, the current study has demonstrated that norepinephrine and putative α_1 and α_2 selective adrenoceptor agonists stimulate PI hydrolysis in the rat tail artery via an α_1-mediated increase in production of inositol phosphates which is dependent upon $Ca^{2+}{}_e$ and enhanced in the presence of LiCl. The coupling of α_1-adrenoceptor activation of PLC hydrolysis of phosphatidyl inositol biphosphate was insensitive to pertussis toxin but inhibited by NEM, suggesting a regulatory role for a non G_i-like G-protein. Further support for G-protein modulation of PLC activity was provided by the pertussis-insensitive, NEM- and DTT-sensitive, action of NaF to increase PI hydrolysis.

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