Cholera toxin modulation of angiotensin II-stimulated inositol phosphate production in cultured vascular smooth muscle cells

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Activation of phospholipase C by angiotensin II in vascular smooth muscle has been postulated to be mediated by an unidentified GTP-binding protein (G-protein). Using a permeabilized preparation of *myo*-[³H]inositol-labelled cultured vascular smooth muscle cells, we examined the ability of a non-hydrolysable analogue of GTP, guanosine 5'-[γ -thio]triphosphate (GTP[S]), to stimulate inositol phosphate formation. GTP[S] (5 min exposure) stimulated inositol polyphosphate release by up to 3.8-fold in a dose-dependent manner, with an EC₅₀ (concn. producing half-maximal stimulation) of approx. 50 μ M. Inositol bisphosphate (IP₂) and inositol trisphosphate (IP₃) accumulations were also stimulated by NaF (5–20 mM). Furthermore, angiotensin II-induced inositol phosphate formation could be potentiated by a submaximal concentration of GTP[S] (10 μ M), and this treatment appeared to interfere with the normal termination mechanism of the initial hormonal signal. The G-protein mediating angiotensin II-stimulated phospholipase C activation was insensitive to pertussis toxin at an exposure time and concentration which were sufficient to completely ADP-ribosylate all available substrate (100 ng/ml, 16 h). In contrast, a similar incubation with cholera toxin markedly inhibited angiotensin II-stimulated IP₂ and IP₃ release by 67 $\pm$ 6 % and 62 $\pm$ 6 % respectively. Cholera toxin appeared to inhibit angiotensin II stimulation of phospholipase C by a dual mechanism: it caused a 45 % decrease in angiotensin II receptor number, and also inhibited G-protein transduction as assessed by GTP[S]-stimulated IP₂ formation. This latter inhibition may be secondary to an increase in cyclic AMP, since it could be simulated by addition of dibutyryl cyclic AMP. Thus angiotensin II-stimulated inositol phosphate formation is cholera-toxin-sensitive, and is mediated by a pertussis-toxin-insensitive G-protein, which may be involved directly in termination of early signal generation.

INTRODUCTION

Many Ca²⁺-mobilizing vasoconstrictive agents activate a phosphatidylinositol (PI)-specific phospholipase C (PLC). The result of such activation is the hydrolysis of PI 4,5-bisphosphate (PIP₂) into two intracellular messengers: inositol trisphosphate (IP₃), a Ca²⁺-mobilizing agent, and diacylglycerol, an endogenous activator of the phospholipid- and Ca²⁺-dependent protein kinase C [1]. Although the correlation of polyphosphoinositide turnover and Ca²⁺ release has been widely demonstrated, in many cell types the coupling mechanisms linking receptor activation to biological responses remain to be identified.

By analogy with the adenylate cyclase system, a role for guanine-nucleotide-binding proteins in receptor-induced PLC activation has been proposed. GTP-binding proteins (G-proteins) are heterotrimers consisting of α -, β - and γ -subunits. The α -subunits bind and hydrolyse GTP, and it is the α -subunit-GTP complex that either activates or inhibits the target enzyme [2,3]. Non-hydrolysable GTP analogues such as guanosine 5'-[γ -thio]triphosphate (GTP[S]) or guanosine 5'-[β -imido]triphosphate (p[NH]ppG) irreversibly activate the α -subunit by virtue of their inability to be converted to GDP.

In systems utilizing isolated membranes [4–10] or permeabilized cells [11–16], these GTP analogues have been shown to activate PLC. In some cases this activation is inhibited by pertussis toxin [13,17–21], a compound which ADP-ribosylates several α -subunits [2]. In other systems, pertussis toxin has no effect on GTP-induced PLC activity [15]. Furthermore, some cell types exhibit a sensitivity to cholera toxin [10,22–26], whereas others are insensitive to either toxin [9,11,12,27–30].

The existence of a guanine-nucleotide-coupling mechanism for angiotensin II-induced activation of PLC in vascular smooth muscle cells (VSMC) remains inferential. Bruns & Marme [17] showed that pertussis toxin inhibited angiotensin II-stimulated Ca²⁺ release, but did not directly measure its effect on PLC-specific second messenger formation. Studies on other systems have suggested that not only does the G-protein serve to couple receptor occupation to PLC activation, but also that it plays an important role in regulating continued inositol phosphate production [31]. These observations raise the possibility that the normal pattern of intracellular signal generation seen in angiotensin II-stimulated VSMC may be in part dictated by activation of a G-protein.

Abbreviations used: PI, phosphatidylinositol; PLC, phospholipase C; IP₃, inositol trisphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; G-protein, GTP-binding protein; VSMC, vascular smooth muscle cells; IP, inositol monophosphate; IP₂, inositol bisphosphate; EC₅₀, concn. causing 50 % of maximal stimulation.

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In the present study, we examined the ability of a non-hydrolysable analogue of GTP to stimulate PLC-mediated IP_3 formation in permeabilized cultured VSMC, and investigated the role of such a coupling mechanism in angiotensin II activation. We evaluated the toxin sensitivity of this pathway and assessed the possible role of GTP-stimulated activation of adenylate cyclase in mediating cholera toxin-induced effects. The data indicate that stimulation of vascular smooth muscle PLC by angiotensin II is mediated by a pertussis-toxin-insensitive G-protein, and that the signalling pathway stimulated by angiotensin II is sensitive to cholera toxin. The inhibition of the angiotensin II response by cholera toxin involves both direct inhibition of receptor binding and cyclic AMP-mediated modification of G-protein-PLC coupling. Furthermore, the normal termination mechanism for angiotensin II-stimulated polyphosphoinositide hydrolysis probably involves the G-protein.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium was obtained from Hazelton, Lenexa, KN, U.S.A. Calf serum, glutamine, penicillin and streptomycin were obtained from Gibco, Chagrin Falls, OH, U.S.A. *myo*-[3H]inositol (17.1 Ci/mmol) and [adenylate- ^{32}P]NAD $^+$ (800 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Pertussis toxin and cholera toxin were from List Biological Laboratories, Campbell, CA, U.S.A. GTP[S] was obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A.

Cell culture

VSMC were prepared from rat thoracic aorta as previously described [32,33]. The cells were maintained in monolayer cultures in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum, 2 mM-glutamine, 100 units of streptomycin/ml and 100 units of penicillin/ml. All experiments were carried out between passages 11 and 22.

Metabolic labelling, cell permeabilization and isolation of inositol phosphates

Monolayers of VSMC were grown in 35 mm dishes and labelled by addition of 15 μ Ci of *myo*-[3H]inositol/ml for 24–48 h. Unincorporated label was removed by washing with an iso-osmotic balanced salt solution (130 mM-NaCl, 5 mM-KCl, 1.5 mM-CaCl $_2$, 1.0 mM-MgCl $_2$ and 20 mM-Hepes/Tris, pH 7.4), and cells were permeabilized with saponin (30 μ g/ml) in a cytosol-like buffer (25 mM-NaCl, 120 mM-KCl, 1 mM-MgCl $_2$, 5 μ M-ATP and 15 mM-Hepes, pH 7.2) containing 13 mM-LiCl to inhibit breakdown of the inositol phosphates. The effectiveness of the permeabilization procedure was assessed by adding [3H]IP $_3$ and measuring subsequent metabolism to [3H]inositol biphosphate ([3H]IP $_2$) and [3H]inositol monophosphate ([3H]IP). After 10 min at 37 °C, the buffer was aspirated and replaced with 1 ml of fresh buffer containing the different agonists. The reactions were terminated at the indicated times with 400 μ l of 50% (w/v) trichloroacetic acid. The trichloroacetic acid solutions were then extracted with 3 $\times$ 8 ml of diethyl ether. The trichloroacetic acid-soluble material was resolved into IP, IP $_2$ and IP $_3$ by the method of Downes & Michell [34], as described previously [35].

Toxin treatments

Either pertussis toxin or cholera toxin (50–200 ng/ml) was added to *myo*-[3H]inositol-labelled VSMC monolayers 16 h before exposure of the cells to agonist.

Angiotensin II receptor binding

Angiotensin II receptor binding was performed on broken VSMC harvested from 100 mm culture dishes. After toxin (200 ng/ml) or dibutyryl cyclic AMP (0.5 mM) treatment (16 h), cells were washed four times with ice-cold Dulbecco's phosphate-buffered saline and incubated for 5 min at 4 °C in 3.0 ml of 50 mM-Tris/10 mM-MgCl $_2$ (pH 7.2 at 22 °C). Cells were then scraped from the plate, the dishes were rinsed, and the samples were transferred to a Dounce homogenizer (final volume 10.0 ml). Each sample was homogenized with 10 strokes at 4 °C, and 100 μ l aliquots were taken for assay.

Binding assays were initiated by adding 100 μ l aliquots of tissue to 100 μ l of assay mixture. The assay mixture consisted of 50 mM-Tris, 5 mM-MgCl $_2$, 100 mM-NaCl and 0.5% BSA, pH 7.2, at 22 °C. Each tube also contained various concentrations of angiotensin II (0.1–5 nM) diluted in 50 mM-phosphate buffer (pH 7.5) containing 0.25% BSA. A total of 30% of the angiotensin II in each tube was [^{125}I]-labelled angiotensin II; the remainder was unlabelled. Assay tubes were incubated for 45 min at 25 °C. The reaction was terminated by addition of 4.5 ml of ice-cold 0.9% NaCl to each tube. Bound and free radioactivity were separated by rapid vacuum filtration through glass-fibre filters (Whatman GF/F) prewetted with 50 mM-Tris/5 mM-MgCl $_2$ /100 mM-NaCl/0.25% BSA (pH 7.2 at 22 °C). Each tube and filter was rinsed $\times$ 2 with an additional 4.5 ml of 0.9% NaCl. Radioactivity trapped on the filter was counted in a γ -counter. Non-specific binding was measured in the presence of 1 μ M-unlabelled angiotensin II and was 2–4% of the total binding. K_d and B_{max} (maximum number of binding sites) were determined by Scatchard analysis.

ADP-ribosylation assays

The transfer of [^{32}P]ADP-ribose from NAD $^+$ catalysed by both toxins was assayed with VSMC homogenates. VSMC monolayers (100 mm dishes), either untreated or previously treated with the toxins, were washed twice with balanced salt solution at 4 °C. Cells were then scraped from the dish and homogenized by freezing and thawing twice in a solution containing 50 mM-Tris/HCl, pH 7.4, 1 μ g of leupeptin/ml, 1 μ g of soybean trypsin inhibitor/ml and 1 mM-benzamidine. The ADP-ribosylation assays used the methods of Neer *et al.* [36] for pertussis toxin and Lotersztajn *et al.* [6] for cholera toxin. In brief, ADP-ribosylation of pertussis toxin was estimated by incubating 30 μ l of a homogenized sample (60 μ g of protein) in 0.1% Triton X-100/5 μ M-NAD $^+$ /12 mM-isonicotinic acid hydrazide/10 mM-thymidine/0.1 mM-GTP/134 ng of creatine kinase/98 ng of phosphocreatine/3 μ M-ATP for 40 min at 37 °C. The reaction was catalysed by addition of pertussis toxin (250 ng) in the presence of [^{32}P]NAD $^+$ (sp. radioactivity 60 μ Ci/nmol). The incubation was stopped by addition of 30 μ l of modified Laemmli's dissociating buffer [37]: 250 mM-Tris/HCl, pH 6.8, 10 mM- β -mercaptoethanol, 60% (w/v) sucrose, 3% (w/v) SDS and 0.005% Bromophenol Blue. The samples were then boiled for 5 min. Cholera-toxin-catalysed [^{32}P]ADP-ribose transfer was

carried out by incubating the samples with 50 mM-Tris/HCl (pH 7.4)/1 mM-ATP/3 mM-dithiothreitol/0.1 mM-GTP[S]/10 μ M-NAD⁺ in a final volume of 60 μ l. The cholera toxin (2.5 μ g) was preactivated with 37.5 mM-dithiothreitol (15 min at 37 °C), and added simultaneously with [³²P]NAD⁺ (35 μ Ci/nmol). After a 30 min incubation at 37 °C, the reactions were terminated as described above for the pertussis toxin assays.

Polyacrylamide-gel electrophoresis

Gel electrophoresis in 11 % polyacrylamide gels was carried out using the protocol described by Laemmli [37]. The samples were boiled in dissociating solution, loaded on to the gel and run for 4 h at 30 mA (constant current). The gel was stained with 0.5 % Coomassie Brilliant Blue in methanol/acetic acid/water (25:10:65, by vol) for 15 min, and then destained with methanol/acetic acid/water (10:10:80, by vol.). Autoradiography of the dried gels was performed by exposing them to Kodak XAR-5 film at -70 °C.

Measurement of intracellular Ca²⁺

Changes in intracellular Ca²⁺ concentrations were measured using the calcium-sensitive dye fura-2, as described previously [38].

RESULTS

GTP[S]- and NaF-induced inositol phosphate formation

Fig. 1 shows the dose-dependence of the GTP[S]-induced release of inositol phosphates from permeabilized VSMC. IP₃ and IP₂ release increased with increasing concentration of the analogue, whereas IP₁ levels did not consistently change. IP₃ levels increased by up to 1.5-fold, and IP₂ levels increased by 3.8-fold. The EC₅₀ (concn. producing half-maximal stimulation) was approx. 50 μ M. This effect of GTP[S] was not seen with guanosine 5'-[β -thio]diphosphate (500 μ M) (results not shown).

NaF has been shown to activate G-proteins by activating the α -subunit. Fig. 2 shows the effect of NaF on inositol phosphate release from permeabilized VSMC. As with GTP[S], IP release changed very little with NaF addition. In contrast, IP₂ and IP₃ formation increased continually, even at the highest NaF concentration used (3-fold increase in IP₂ and 1.5-fold increase in IP₃ at 20 mM-NaF). The addition of AlCl₃ did not augment the effect observed with NaF.

Potentiation of angiotensin II-induced inositol phosphate release by GTP[S]

To determine whether the GTP-sensitive pool of inositol phospholipids corresponds with that hydrolysed in angiotensin II-stimulated cells, permeabilized VSMC were challenged with angiotensin II in the presence or absence of a sub-maximal concentration of GTP[S]. As shown in Fig. 3, angiotensin II induces a transient increase in IP₃ and IP₂ release in saponin-treated cells, which is maximal at 30 s and returns to baseline values by 2 min. However, preincubation of these cells with 10 μ M-GTP[S], which did not by itself stimulate inositol phosphate formation, potentiated the angiotensin II-stimulated release of both inositol polyphosphates, and prevented the return to baseline. These observations

Fig. 1. Effect of GTP[S] on the release of inositol phosphates from permeabilized VSMC

VSMC were labelled with *myo*-[³H]inositol (15 μ Ci/ml) for 24–48 h, washed, and permeabilized in a cytosol-like buffer containing saponin (30 μ g/ml) for 10 min at 37 °C. The buffer was then replaced with fresh buffer containing GTP[S] (0–500 μ M). The reactions were terminated after 5 min with 400 μ l of 50 % trichloroacetic acid, and the inositol phosphates were resolved by ion-exchange chromatography and quantified by liquid scintillation spectroscopy. Each point represents the mean $\pm$ S.E.M. of duplicate determinations from three to five experiments. (a) IP₃, (b) IP₂, (c) IP₁.

suggest that angiotensin II and GTP[S] primarily hydrolyse the same pool of inositol phospholipids.

The experiments shown in Fig. 3 also address the issue of how termination of the initial hormonal signal is regulated. We have shown previously that angiotensin II causes a transient increase in the inositol polyphosphates ([35,38], see also Fig. 3), and that this signal appears to be terminated by activation of protein kinase C. The present experiments suggest that the G-protein may be involved in this termination mechanism, since irreversible activation of the G-protein prevented the return of angiotensin II-stimulated IP₂ and IP₃ levels towards baseline.

Fig. 2. Effect of NaF on the release of inositol phosphates from permeabilized VSMC

VSMC were labelled and permeabilized as described in the legend to Fig. 1, and then exposed to increasing concentrations of NaF (0–20 mM). The reactions were terminated after 5 min with 50 % trichloroacetic acid, and the inositol phosphates were resolved by ion-exchange chromatography and quantified by liquid scintillation spectroscopy. Each point represents the mean $\pm$ S.E.M. of duplicate determinations from three to five experiments. (a) IP_3 , (b) IP_2 , (c) IP.

Fig. 3. Synergism between angiotensin II and suboptimal concentrations of GTP[S] in permeabilized VSMC

VSMC were labelled and permeabilized as described in the legend to Fig. 1. One group of cells was challenged with angiotensin II ($1 \mu M$, $\square$); a second group was exposed to GTP[S] ($10 \mu M$, $\bullet$); and the third group was preincubated with GTP[S] ($10 \mu M$, 5 min) and then exposed to GTP[S] and angiotensin II simultaneously ($\blacksquare$). Reactions were terminated after various times with 50 % trichloroacetic acid, and the inositol phosphates were resolved by ion-exchange chromatography. Each point represents the mean $\pm$ S.E.M. of duplicate determinations from three or four experiments. (a) IP_3 , (b) IP_2 .

Effect of pertussis toxin and cholera toxin on angiotensin II-stimulated inositol phosphate formation and Ca^{2+} mobilization

To gain insight into the identity of the coupling G-protein, VSMC were preincubated with pertussis toxin

Table 1. Effect of pertussis toxin on angiotensin II-induced inositol phosphate release in VSMC

VSMC were prelabelled with *myo*-[3H]inositol ($15 \mu Ci/ml$) for 24–48 h and pretreated with pertussis toxin ($200 ng/ml$) for 16 h. Cells were then exposed to angiotensin II ($100 nM$) for 15 s. Values are means $\pm$ S.E.M. for triplicate determinations from a representative experiment.

	[³ H]inositol phosphate release (c.p.m.)				
	IP ₂		IP ₃		
	Angiotensin II...	—	+	—	+
Untreated		506 ± 13	3936 ± 352	318 ± 13	1541 ± 116
Pertussis toxin (100 ng/ml)		179 ± 4	3170 ± 118	128 ± 30	1387 ± 61

Table 2. Effect of cholera toxin and dibutyryl cyclic AMP on angiotensin II-induced inositol phosphate formation

VSMC monolayers were labelled with *myo*-[³H]inositol (15 μ Ci/ml) for 24–48 h and pretreated with either cholera toxin (100 ng/ml) or dibutyryl cyclic AMP (1 mM) for 16 h. Cells were then exposed to angiotensin II (100 nM) for 15 s. Values are given as a percentage of control (without angiotensin) and are expressed as means $\pm$ S.E.M. from (*n*) experiments.

	Inositol phosphate formation (% of control)	
	IP ₂	IP ₃
Untreated	203 $\pm$ 39 (3)	400 $\pm$ 80 (6)
Cholera toxin	130 $\pm$ 11 (3)	285 $\pm$ 57 (6)
Dibutyryl cyclic AMP	145 $\pm$ 18 (3)	–

or cholera toxin, and inositol phosphate formation in response to angiotensin II was measured. Pretreatment of cells with pertussis toxin (100 ng/ml, 16 h) had no effect on angiotensin II-induced IP₂ and IP₃ release at 15 s (Table 1). Conversely, incubation of VSMC with cholera toxin (100 ng/ml, 16 h) significantly inhibited angiotensin II-stimulated inositol phosphate formation (Table 2). The toxin-induced inhibition was clearly concentration-dependent (Fig. 4). In further experiments a dose of 100 ng/ml was used, since this dose caused a

Table 3. Effect of cholera toxin and dibutyryl cyclic AMP on angiotensin II binding

VSMC monolayers were pretreated for 16 h with either cholera toxin (200 ng/ml) or dibutyryl cyclic AMP (0.5 mM). Cells were then harvested and angiotensin II binding was determined by Scatchard analysis as described in the Materials and methods section. Values are means $\pm$ S.E.M. from three experiments.

	B_{max} (fmol/mg of protein)	K_d (nM)
Control	2033 $\pm$ 450	4.01 $\pm$ 0.49
Cholera toxin	1230 $\pm$ 563	3.22 $\pm$ 0.64
Dibutyryl cyclic AMP	1855 $\pm$ 387	4.05 $\pm$ 0.99

near-maximal inhibition. Angiotensin II-induced IP₃ formation at 15 s was inhibited by 62 $\pm$ 6 % by incubation with this concentration of cholera toxin, and IP₂ formation was inhibited by 67 $\pm$ 6 % (*n* = 3, means $\pm$ S.E.M.). This inhibition of IP₃ release was reflected by an inhibition of the angiotensin II-stimulated increase in intracellular Ca²⁺ in cholera-toxin-treated cells (control, 357 $\pm$ 16 nM; cholera-toxin-treated, 133 $\pm$ 10 nM; 68 % inhibition; *n* = 2).

Effect of cholera toxin on angiotensin II receptor binding

In other systems, cholera toxin has been shown to exert an effect by inhibiting binding of the hormone to its receptor [39–41]. To determine whether cholera toxin inhibits angiotensin II-stimulated inositol phosphate formation at the level of the receptor, we incubated VSMC for 16 h with cholera toxin (200 ng/ml) and measured angiotensin II receptor number and affinity using ¹²⁵I-angiotensin II. As shown in Table 3, cholera toxin caused a 45 % decrease in receptor number, but no change in affinity.

Effect of cholera toxin on GTP[S]-stimulated inositol phosphate formation

Cholera toxin has also been shown to inhibit intracellular signal generation at the level of PLC [22]. To determine whether the entire inhibitory effect of cholera toxin on angiotensin II signalling in VSMC occurs at the level of receptor binding, or whether cholera toxin also affects VSMC PLC activity, we investigated the effect of the toxin on direct activation of the G-protein by GTP[S] (300 μ M, 5 min). Pretreatment of VSMC with cholera toxin (100 nM, 16 h) reduced GTP[S]-induced IP₂ accumulation from 370 $\pm$ 86 % of control to 217 $\pm$ 30 % of control. Thus cholera toxin was as effective at inhibiting GTP[S]-induced IP₂ formation (51.6 $\pm$ 6 % inhibition, *n* = 5) as it was at inhibiting angiotensin II-stimulated inositol polyphosphate release and at decreasing angiotensin II receptor number. Cholera toxin-induced inhibition of GTP[S]-stimulated IP₃ formation was difficult to demonstrate because of the relatively small increase under control conditions; however, in three out of five experiments, IP₃ formation was clearly inhibited (results not shown).

Fig. 4. Concentration-dependence of cholera toxin inhibition of angiotensin II-induced inositol phosphate formation

VSMC were labelled with *myo*-[³H]inositol for 24–48 h and exposed to various concentrations of cholera toxin for 16 h. Cells were then washed and exposed to angiotensin II (100 nM) for 15 s. The reaction was terminated with trichloroacetic acid and samples were processed for determination of inositol phosphates. Data are expressed as the increase in radioactivity above control values induced by angiotensin II under each of the experimental conditions. The average control values were 630 c.p.m. for IP₃ (a), 753 c.p.m. for IP₂ (b) and 27950 c.p.m. for IP (c). Each point represents the mean of duplicate determinations from a representative experiment.

Fig. 5. Concentration-dependence of dibutyryl cyclic AMP inhibition of angiotensin II-induced IP₂ formation

VSMC were labelled with *myo*-[³H]inositol for 24–48 h and exposed to various concentrations of dibutyryl cyclic AMP for 16 h. Cells were then washed and exposed to angiotensin II (100 nM) for 15 s. The reaction was terminated with trichloroacetic acid, and IP₂ was resolved by column chromatography. Data are expressed as the increase in radioactivity above control values induced by angiotensin II at each concentration of cyclic AMP. The average control value was 1417 c.p.m.

Effect of cyclic AMP on angiotensin II-induced inositol phosphate release

Cholera toxin has been shown to elevate cyclic AMP in numerous cell types [42]. To determine whether the observed inhibition of PLC activity by cholera toxin was due only to an increase in intracellular cyclic AMP, VSMC were incubated for a comparable time (16 h) with various concentrations of dibutyryl cyclic AMP and then exposed to angiotensin II. The maximum effect of dibutyryl cyclic AMP in inhibiting angiotensin II-induced inositol phosphate release occurred at a concentration of 0.5 mM (Fig. 5). As shown in Table 2, the maximal inhibition of IP₂ release by dibutyryl cyclic AMP was only slightly less than the maximal inhibition obtained in cholera-toxin-treated cells. Treatment of VSMC with forskolin (25 μM, 4 h) also inhibited IP₂ and IP₃ formation, although to a lesser extent (results not shown).

Effect of cyclic AMP on angiotensin II receptor binding

The observed inhibition of angiotensin II-induced inositol phosphate formation by cyclic AMP could occur either at the level of the receptor or at the level of the G-protein. Binding studies performed on cells treated with dibutyryl cyclic AMP (0.5 mM) for 16 h showed only a small decrease in angiotensin II receptor number and no change in affinity (Table 3).

Effect of cyclic AMP on GTP[S]-induced inositol phosphate formation

Cyclic AMP also inhibited GTP[S]-induced inositol polyphosphate formation. Dibutyryl cyclic AMP (1 mM, 16 h) decreased GTP[S] (300 μM, 5 min)-stimulated IP₂ production from 370 ± 86 % of control to 252 ± 30 % of control (*n* = 4, 44 % inhibition). IP₃ formation was also

Fig. 6. ADP-ribosylation of the G-proteins

VSMC were pretreated with pertussis toxin (100 ng/ml, 16 h, lane 6), cholera toxin (100 ng/ml, 16 h, lane 2), or dibutyryl cyclic AMP (1 mM, 16 h, lane 3). Control and treated samples were then assayed for ADP-ribosylation of the remaining available substrates in the presence of [³²P]NAD⁺ and pertussis toxin (lanes 4–6) or cholera toxin (lanes 1–3) as described in the Materials and methods section. Lane 1, Control for cholera toxin assay; lane 4, control for pertussis toxin assay; lane 5, pertussis toxin (100 ng/ml) added at time 0.

inhibited, suggesting that cyclic AMP exerts an effect mainly at the level of G-protein–PLC coupling.

ADP-ribosylation of G-proteins

To characterize the alteration of α-subunits by pertussis toxin, cholera toxin and dibutyryl cyclic AMP, VSMC were incubated in the presence or absence of these compounds, and then assayed for ADP-ribosylation of any remaining unmodified G-proteins in the presence of [³²P]NAD⁺ (Fig. 6). Control cultures of VSMC showed a major substrate for pertussis toxin at 41 kDa, which was completely ribosylated under the experimental conditions employed here. Three major substrates for cholera toxin were evident: a doublet at 45 and 52 kDa and a 23 kDa species. Preincubation with cholera toxin for 16 h resulted in complete abolition of label incorporation into the doublet, but did not affect ADP-ribosylation of the 23 kDa band. Conversely, incubation with dibutyryl cyclic AMP inhibited subsequent ADP-ribosylation of the 23 kDa band, but had little effect on ribosylation of the doublet.

DISCUSSION

This study demonstrates that a pertussis-toxin-insensitive G-protein mediates angiotensin II-induced PLC activation in VSMC. This second-messenger pathway is, however, sensitive to cholera toxin, although the mechanism(s) by which cholera toxin inhibits hormone-stimulated PLC activity remains incompletely defined. Cholera toxin appears to inhibit angiotensin II binding directly by decreasing receptor number, and its effects on GTP[S]-stimulated inositol phosphate production may be secondary to a rise in cyclic AMP. Both of these effects may

play a role in determining the final response. Additionally, we have demonstrated that suboptimal concentrations of GTP[S] and a maximal concentration of angiotensin II synergize to stimulate inositol polyphosphate production, resulting in impairment of the normal termination of angiotensin II-stimulated IP_3 signalling. These latter observations suggest that the negative-feedback mechanism regulating this signal may occur at the level of a G-protein.

The existence of a GTP-sensitive pool in VSMC is shown by the ability of GTP[S] and NaF to stimulate inositol phosphate accumulation in a dose-dependent manner. Previous reports had suggested that hormonal signalling in VSMC was mediated by a G-protein based on observations that GTP analogues altered the affinity of the receptor for angiotensin II [43], and that pertussis toxin inhibited Ca^{2+} mobilization in angiotensin II [17] or noradrenaline [18]-stimulated cells. In contrast with the latter reports, no sensitivity of angiotensin II-induced IP_3 formation to pertussis toxin was observed here, although the concentration of pertussis toxin used in our study was sufficient to modify all of the available substrate (Fig. 6). Furthermore, in our system, pertussis toxin had no effect on angiotensin II-induced Ca^{2+} mobilization (T. A. Brock, R. W. Alexander & K. K. Griendling, unpublished work). The discrepancy between these studies may be explained by a non-specific effect of the high concentration of pertussis toxin used by Burns & Marme [17], or by a difference in VSMC line or passage level [18]. Nonetheless, all three studies strongly suggest the existence of a G-protein mediating PLC activation in VSMC.

In some systems, cyclic AMP has been shown to inhibit PLC directly. Imai & Gershengorn [22] reported that thyrotropin-releasing-hormone-induced IP_3 formation was inhibited by preincubation with non-hydrolysable cyclic AMP analogues. Lerner *et al.* [44] found that the inhibitory effect of cholera toxin on lectin- or antibody-stimulated PLC activity in mouse T-cells could be completely mimicked by elevation of intracellular cyclic AMP. Conversely, in other studies, the kinetics of cholera toxin activation of the adenylate cyclase system and inhibition of PLC are clearly diverse, suggesting that these two effects are mechanistically distinct. For example, cholera toxin stimulated cyclic AMP formation for a maximum of 30 min in rat pancreatic cells, whereas inhibition of IP_3 production was evident only after 1.5 h of cholera toxin incubation [24]. Similar kinetics for cholera-toxin-induced cyclic AMP formation have been reported in Jurkat T-cells, whereas inhibition of the antigen-stimulated increase in cytosolic free Ca^{2+} was evident only after 3 h of cholera toxin incubation [25]. Our own data suggest that only part of the effect of cholera toxin on angiotensin II signalling can be attributed to its ability to increase cyclic AMP, since dibutyryl cyclic AMP inhibited GTP[S]-induced IP_2 formation but did not affect angiotensin II binding.

A recent report on the G-protein coupling angiotensin II activation of adrenal glomerulosa cells suggested that cholera toxin exerted its effects via an inhibition of hormone-receptor binding [41]. Cholera toxin also inhibited angiotensin II binding in VSMC. However, this effect could not be mimicked by dibutyryl cyclic AMP, and thus may occur through a non-cyclic-AMP-dependent mechanism. It is possible that the angiotensin II receptor is down-regulated after toxin treatment. Cholera

toxin may regulate the receptor at the level of gene expression, as has recently been described for the thyrotropin-releasing hormone receptor [45]. This latter possibility is intriguing, since VSMC must be exposed to cholera toxin for 16 h before an inhibitory effect is observed (L. Socorro, R. W. Alexander & K. K. Griendling, unpublished work).

Our data thus suggest that the effect of cholera toxin on angiotensin II signalling in VSMC is complex. There appear to be at least two separate mechanisms for the inhibitory effect of cholera toxin: a cyclic AMP-independent effect on angiotensin II receptor binding, and a cyclic AMP-dependent inhibition of G-protein-PLC coupling. It is significant that angiotensin II binding and GTP[S]-stimulated inositol phosphate formation were each inhibited by cholera toxin to about the same extent as was angiotensin II-induced inositol phosphate production. Since angiotensin II exerts its effects first by binding to the receptor and then by activating PLC via a G-protein, inhibition of either of these steps might be expected to be as limiting as inhibition of the intact signalling pathway. One explanation for this lack of additivity may be the existence of spare receptors in VSMC. If a portion of the receptors inactivated by cholera toxin are spare receptors and as such are not coupled to PLC, the effective inhibition of signal generation would be less than predicted from the decrease in receptor number. The remainder of the inhibitory effect of cholera toxin could then be postulated to occur at a step distal to hormone-receptor coupling; that is, at the G-protein which is activated by GTP[S].

The identity of the G-protein coupled to activation of PLC remains elusive. Although the present study suggests that the pertussis toxin substrate α_i (41 kDa) is not involved in second messenger formation in angiotensin II-stimulated VSMC, none of the cholera toxin substrates (45, 52 or 23 kDa) can unequivocally be demonstrated to be the coupling protein. Dibutyryl cyclic AMP mimicked the cholera toxin inhibition of angiotensin II-stimulated PLC activity, but not its effect on angiotensin II binding, and modified only the 23 kDa substrate, interfering with its subsequent ADP-ribosylation. Cholera toxin caused only a 50% inhibition of angiotensin II- or GTP[S]-induced IP_2 formation, and only the two larger of the three cholera toxin-sensitive substrates were ADP-ribosylated. It is possible that PLC activity in VSMC is regulated by both a G-protein-dependent and a G-protein-independent pathway, accounting for the incomplete inhibition seen at even maximal doses of cholera toxin. Whether this putative G-protein-independent pathway is sensitive to cyclic AMP, or whether the effects of cyclic AMP are due solely to the effects of cholera toxin in activating adenylate cyclase remains unclear. Recent reports indicate that there is a family of low-molecular-mass G-proteins, of 20–25 kDa, which share many of the properties of the α -subunits of the better characterized, higher-molecular-mass G-proteins [46]. No function has yet been demonstrated for these smaller proteins, but the current data imply that modulation of PLC activation is at least a possibility.

Preincubation with a suboptimal concentration of GTP[S] impairs the termination mechanism of the angiotensin II-induced inositol phosphate release, since incubation with both compounds resulted in a continuous increase in IP_3 and IP_2 accumulation. In the context of our previous data concerning the mechanisms controlling

secondary signalling in angiotensin II-stimulated VSMC, this observation provides insight into the precise biochemical site at which termination of the early response occurs. In VSMC, angiotensin II causes a biphasic response: an initial rapid increase in inositol polyphosphate and diacylglycerol accumulation which is over by 2 min, and a second, slowly developing sustained increase in diacylglycerol [35,38]. We have demonstrated previously that phorbol ester, an exogenous activator of protein kinase C, inhibits both IP_3 formation [47] and the early phase, but not the sustained phase, of diacylglycerol accumulation [38]. Based on these observations, we postulated that activation of protein kinase C by diacylglycerol feeds back to inhibit continuing polyphosphoinositide hydrolysis [38,47]. Whether this inhibition occurs at the level of the receptor, PLC or the G-protein remains uncertain. The present data raise the possibility that protein kinase C exerts its effect at the level of the G-protein. As shown in Fig. 3, in the presence of GTP[S], the generation of IP_2 and IP_3 in response to angiotensin II is prolonged, suggesting that irreversible activation of the G-protein interferes with the normal termination mechanism present in these cells. Orellana *et al.* [48] have made similar observations in astrocytoma membranes. In their cells, phorbol ester appeared to block IP_3 formation by inhibiting G-protein interaction with PLC. Alternatively in renal mesangial cells, Pfeilschifter & Bauer [49] have shown that phorbol ester interferes with the interaction of the angiotensin II receptor with the G-protein. Although our data do not permit us to distinguish between these two possibilities, they strongly suggest that the normal termination of polyphosphoinositide hydrolysis involves the coupling G-protein.

In summary, angiotensin II-stimulation of inositol phosphate formation in VSMC is mediated by a pertussis-toxin-insensitive G-protein. The signalling pathway invoked by angiotensin II is sensitive to cholera toxin, both at the level of the receptor and at the level of G-protein-PLC coupling. The data also suggest that the normal termination of initial signal generation in response to angiotensin II occurs at the level of the G-protein.

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