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Cholera-toxin and corticotropin modulation of inositol phosphate accumulation induced by vasopressin and angiotensin II in rat glomerulosa cells

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Vasopressin (VP) and angiotensin II (AT II) stimulate the production of inositol phosphates (IP) in rat glomerulosa cells. Guanosine 5'-[γ-thio]triphosphate (GTP[S]), but not VP or AT II, stimulates IP production in a *myo*-[^{3}H]inositol-prelabelled glomerulosa-cell membrane preparation. In combination with GTP[S], these hormones potentiate the response to GTP[S], indicating the existence of a G-protein involved in the coupling of the VP and AT II receptor with the phospholipase C. ADP-ribosylation with pertussis toxin (IAP) revealed the specific labelling of a single molecule of 41 kDa. No significant inhibition of VP- or AT II-stimulated IP accumulation was detected in intact cells when the whole 41 kDa molecule was endogenously ADP-ribosylated by IAP treatment. On the contrary, when glomerulosa cells were infected with cholera toxin (CT), both the VP- and AT II-stimulated IP accumulations were inhibited in a dose-dependent manner. Yet these effects were partial even at high concentrations of CT, and could not be related to the ADP-ribosylation of 'α_s' molecules. Similarly, when the cells were infected with 1 μg of CT/ml, the specific binding of VP and AT II decreased by 50–60 %. Such results may signify that the treatment primarily affects the densities of the hormone receptors. When glomerulosa cells were incubated for 15 h in the presence of 10 nM-corticotropin (ACTH), a condition in which the intracellular concentration of cyclic AMP was increased 3-fold, the maximum IP response to 0.1 μM-VP or -AT II was decreased by 50 %. When similar experiments were carried out only after a 15 min incubation period with the same concentration of ACTH, the increase in cyclic AMP was more pronounced, but no inhibition of hormone-induced IP accumulation was observed. Altogether, these results may suggest that CT exerts its action on the VP- or AT II-sensitive phospholipase C systems via a prolonged increase in intracellular cyclic AMP.

INTRODUCTION

Angiotensin II (AT II) and vasopressin (VP) are known to stimulate aldosterone secretion in rat glomerulosa cells [1–3]. Different groups have clearly demonstrated that AT II primarily acts via the stimulation of a specific phosphatidylinositol bisphosphate phosphodiesterase [4–6].

More recently, we [7] and others [8] have also demonstrated that VP induced a dose-dependent accumulation of inositol phosphates in these cells. Thus the intracellular messengers involved in the biological action of VP and AT II in glomerulosa cells seem to be inositol trisphosphate [I(1,4,5)P_3], which mobilizes free Ca^{2+} from endoplasmic-reticulum pools [9], diacylglycerol, which stimulates protein kinase C [4], and Ca^{2+}, which activates calmodulin kinase [10]. The mechanisms of coupling between the VP and the AT II receptors and the phospholipase C are largely unknown. Many experiments performed on membrane preparations derived from blowfly salivary glands [11], WRK1 cells [12], GH3 cells [13], broken or permeabilized cells (platelets [14] or neutrophils [15]) have clearly demonstrated that, for various types of receptors, GTP was absolutely necessary in order to observe a hormonal stimulation of IP accumulation. Such results mean that a G-protein seems to be involved in the coupling of the receptors which mobilize intracellular Ca^{2+} with the phospholipase C. The nature of this G-protein remains unclear. On neutrophils and mast cells, pertussis toxin (IAP) abolishes the IP accumulation induced by the chemotactic peptide fMet-Leu-Phe [16,17]. Similarly, IAP blocked the thrombin-induced phosphatidylinositol (PI) metabolism in fibroblasts [18]. Such results may imply that 'α_i' was probably involved in the transduction process. On the other hand, IAP was found to be ineffective in inhibiting hormonal stimulation of PI turnover in various systems [19–22], suggesting that 'α_i' or a 'α_i-like' protein was not involved in the stimulation of phospholipase C in these systems. Cholera toxin (CT) was described as partially inhibiting the TRH stimulation of IP accumulations in GH3 cells [23] or the VP

Abbreviations used: AT II, angiotensin II; VP, vasopressin; G-protein, guanine nucleotide-binding protein; IAP, pertussis toxin (islet-activating protein); CT, cholera toxin; IP, inositol phosphate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PI, phosphatidylinositol; GTP[S], guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, guanosine 5'-[γ-imido]triphosphate; ACTH, corticotropin; TRH, thyrotropin-releasing hormone (thyroliberin); 'α_i' and 'α_s', guanine nucleotide-binding regulatory proteins; PBS, phosphate-buffered saline.

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Table 1. Chemical structures and properties of labelled analogues of VP and AT II

Origins: (1) obtained from New England Nuclear; (2) Provided by Dr. E. Escher and prepared by the Iodogen method as previously described [30]. Dissociation constant (K_{bind}) was measured in intact glomerulosa cells as described in the Materials and methods section (G. Guillon, unpublished work). For biological properties, the ability of these peptides to stimulate phospholipase C was measured in glomerulosa cells as previously described [7]. For the antagonists, we have checked that they could inhibit completely the stimulation of PC that is induced by the agonist analogue (G. Guillon, unpublished work). Abbreviation: Sar, sarcosine.

Chemical structure	Origin	Abbreviation	Sp. radioactivity (Ci/mmol)	Dissociation constant (nM)	Biological properties
[^{3}H][Arg^8]vasopressin	(1)	[^{3}H]VP	70.0	1.8	Agonist
[β-Mercapto-$\beta\beta$-cyclopentamethylenepropionic acid,*O*-methyl-Tyr^2,Arg^8]-vasopressin	(1)	[^{3}H]Cyclo-VP	56.2	0.1	Antagonist
[^{125}I][Sar^1]angiotensin	(2)	[^{125}I]Sar AT II	470	0.5	Agonist
[^{125}I][Sar^1,Val^5,D-Phe^8]-angiotensin	(2)	[^{125}I]Sar D-Phe AT II	116.4	0.7	Antagonist

stimulation of PI turnover on WRK1 cells [22], possibly suggesting a role for 'α_s'.

More recently, Kikuchi *et al.* [24] showed the functional coupling between the chemotactic receptor from leukaemic cells and phospholipase C. Wakelam *et al.* [25] have also demonstrated that a 21 kDa protein originating from *ras* oncogene also increased the coupling of the bombesin receptor with phospholipase C.

Previous studies carried out on glomerulosa cells have indicated the presence of both 'α_i' and 'α_s' regulatory G-proteins, because AT II inhibits and corticotropin (ACTH) stimulates adenylate cyclase activity [26,27]. Enyedi *et al.* [28] and Kojima *et al.* [29] have postulated the existence of a G-protein involved in the stimulation of the phospholipase C. Their data indicated that this protein was not likely to be 'α_i', since IAP did not modify the stimulation of IP accumulation induced by AT II.

In the present study, we have confirmed and extended the results of Enyedi *et al.* [28] on rat glomerulosa cells. Moreover, for the first time, we have clearly demonstrated that in this system (1) a 'α_i-like' protein was not the G-protein involved in the coupling between the PC and the VP receptor, and (2) CT and ACTH inhibit the hormonal stimulation of IP accumulation.

MATERIALS AND METHODS

Chemicals

The chemicals used in the present study were obtained from the following sources: *myo*-[^{3}H]inositol (10–20 Ci/mmol) from NEN; collagenase from Worthington; ACTH-(1–24)-peptide (Cortrosyn) from Organon; ATP, DNAase, CT and forskolin from Sigma; Minimum Essential Medium (Eagle's medium) from Gibco; [8-arginine]VP and AT II from Bachem; GTP[S] and p[NH]ppG from Boehringer; Dowex 1X8 (100–200 mesh; Cl^- form) from Fluka; IAP from List Biological Laboratories. Other chemicals were of A-grade purity.

Labelled hormonal peptides

Two VP and two AT analogues were used in this study. Their properties are summarized in Table 1.

Cell culture

The glomerulosa cells were taken from the adrenal glands of Long–Evans rats (body wt. 200–250 g). Isolated cells were obtained by a slight modification of the method previously described [7]. After collagenase and DNAase digestion in Eagle's Minimum Essential Medium, the cells were plated at a density of about 5×10^4 cells per tissue-culture dish (35 mm diameter) and grown in the Eagle's medium containing 10% (v/v) fetal-calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. The culture medium was changed 24 h after seeding, and the cells were used after 3 days of culture. By then, the cell density was about $(1–3) \times 10^5$ cells/dish, as counted with a haemocytometer.

Toxin treatment

The protocol used for cell infection with the bacterial toxins was as follows: 57 h after seeding the cells, the culture medium was removed by aspiration and replaced by fresh medium alone (control cells) or by fresh medium with 0.1 μg of IAP/ml or with 1 μg of CT/ml. The cells were then further incubated for 15 h at 37 °C in the presence of toxins. For the experiments in which IP accumulation was measured, *myo*-[^{3}H]inositol was always present in the culture medium even during the toxin-treatment period. Controls experiments revealed that such treatments did not affect the growth of the cells (results not shown).

Measurement of PI breakdown in intact cells

For these experiments, cells were isolated as described previously and grown for 2 days in a culture medium supplemented with 2 μCi of *myo*-[^{3}H]inositol/ml as described previously [7,31]. Before the experiment, the radioactive medium was discarded and the cells were incubated for 30 min at 37 °C in a growth medium with no serum and no *myo*-[^{3}H]inositol. The cells were then washed with 2 ml of a PBS/Li^+ medium, containing 0.44 mM-$CaCl_2$, 2.7 mM-KCl, 0.5 mM-$MgCl_2$, 138 mM-NaCl, 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4 (pH 7.4), bovine serum albumin (1 mg/ml), 10 mM-LiCl, 1 mM-tyrosine and glucose (1 g/l), and further incubated for

15 min at 37 °C. The medium was then removed and the cells were incubated for a further 15 min at 37 °C. The medium was then removed and the cells were incubated for a further 15 min at 37 °C with 700 μl of fresh PBS/Li^+ medium and various amounts of the drugs to be tested. The incubation ended with the aspiration of the medium and the addition of 1 ml of 5% (v/v) $HClO_4$ and 200 μl of albumin (20 mg/ml). The cells were scraped from the Petri dish with a rubber policeman. This acid extract was centrifuged at 2000 *g* for 5 min and the pellet stored for lipid extraction. The supernatant was neutralized by 1.5 M-KOH containing 75 mM-Hepes in the presence of a universal indicator. $KClO_4$ was precipitated at 0 °C and removed by a short centrifugation. The neutralized acid extracts were diluted to 12 ml with 5 mM-$Na_2B_4O_7$/0.5 mM-EDTA, and the inositol phosphates were separated by ion-exchange chromatography on Dowex 1X8 (100–200 mesh; formate form) as described previously [7]. The radioactivity found in the IP_1, IP_2 and IP_3 fractions was determined by scintillation counting in gel phase. All the results were corrected for quenching and are expressed in d.p.m.

The $HClO_4$-precipitated protein pellet was washed with 1 ml of water, and the lipids were extracted as described previously [32]. The lipid extracts were dried, resuspended in 0.5 ml of chloroform and deacylated by alkaline hydrolysis. The ^3H-labelled water-soluble products of this deacylation were separated by anion-exchange chromatography as described previously [32].

Measurement of PI breakdown in glomerulosa-cell membrane preparations

The experimental procedure used was identical with that previously described for a WRK1-cell membrane preparation [12]. In short, glomerulosa cells were grown for 3 days in the presence of *myo*-[^3H]inositol (4 μCi/ml), incubated for 30 min at 37 °C in a culture medium lacking serum and labelled inositol, and washed three times with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}. The cells were then scraped off with a rubber policeman, and resuspended in an ice-cold medium containing 0.1 mM-ATP, 10 mM-Tris/HCl (pH 8.0), 10 mM-LiCl and 0.5 mM-EDTA. They were homogenized at 4 °C in a Dounce Potter homogenizer equipped with a loose pestle (15 strokes). The cell extract was centrifuged for 5 min at 700 *g* at 0 °C. The pellet was discarded and the supernatant was subjected to a second centrifugation (15 min, 30000 *g*, 0 °C). The pellet was resuspended in the ice-cold medium homogenizer with a Teflon Potter homogenizer. The membrane suspension was used at once. Electron-microscopic analysis revealed that this preparation lacked intact or partially lysed cells. It consisted of glomerulosa plasma membranes with few Golgi elements, mitochondria or endoplasmic reticulum (results not shown).

myo-[^3H]Inositol-prelabelled membranes (30–60 μg per assay) were incubated for 10 min at 37 °C in 150 μl of a medium containing 0.1 mM-ATP, 5 mM-$MgCl_2$, 0.25 mM-EDTA, 10 mM-Tris/HCl (pH 8.0), 1 μM-$CaCl_2$, 10 mM-LiCl and the effector to be tested. The reaction was stopped by the addition of 150 μl of 10% $HClO_4$ and 100 μl of albumin (20 mg/ml). The labelled inositol phosphates that accumulated during the incubation and that were present in the water-soluble phase were separated as described above for intact cells.

Cyclic AMP determination

Intracellular cyclic AMP was determined by measuring the formation of cyclic [^3H]AMP from [^3H]adenine nucleotide pools as described previously [33]. On day 3 of culture, the cells were washed and incubated for 37 °C with culture media containing 2 μCi of [^3H]adenine/ml. After 1 h, the cultures were washed and incubated with PBS plus 1 mM-isobutylmethylxanthine for 15 min at 37 °C. Then the drugs were added to the incubation media for a further 15 min incubation period at 37 °C. The reaction was ended by aspiration of the media and addition of 1 ml of ice-cold 5% (v/v) trichloroacetic acid. The cells were scraped with a rubber policeman, and 100 μl of cold 5 mM-ATP/5 mM-cyclic AMP was added to the mixture. The cellular extracts were centrifuged at 5000 *g* for 5 min, and the supernatant was eluted through sequential chromatography on Dowex and alumina columns, which allowed the separation of [^3H]adenine nucleotide precursors (primarily [^3H]ATP) and cyclic [^3H]AMP. Cyclic AMP formation is expressed as follows:

$$\text{Conversion } (\%) = \frac{\text{cyclic } [^3H]\text{AMP}}{\text{cyclic } [^3H]\text{AMP} + [^3H]\text{ATP}} \times 100$$

Hormone-binding assay

Hormone binding was assayed by using cells which were attached to a plastic substratum as described previously [31], with minor modifications. In short, as with PI measurements, the cells were rinsed with 2 ml of PBS/Li^+ medium and incubated for 15 min at 37 °C in this medium. The hormone-binding reaction was initiated by the quick aspiration of the PBS/Li^+ medium and the addition to each Petri dish of 0.8 ml of PBS/Li^+ medium containing the labelled peptide to be tested.

The cells were incubated for 15 min at 37 °C. The reaction was stopped by the aspiration of the incubation medium and the addition of 2 ml of ice-cold PBS/Li^+. The cells were rapidly detached by scraping them with a rubber policeman. The cell suspension was layered on to the surface of a Gelman Metriciel Membrane Filter (GA3) (1.2 μm pore size) under continuous aspiration. Then 2 ml of ice-cold PBS/Li^+ was added to rinse the Petri dishes and transferred on to the filter, which was washed with 3 × 3 ml of ice-cold PBS/Li^+. The whole procedure, including the cell suspension and the filtration, did not exceed 50 s. The radioactivity retained on the filter was measured by liquid-scintillation spectrometry. All the determinations were performed in triplicate and were corrected for non-specific binding, determined by incubating the cells in the presence of the labelled hormone plus an excess (200-fold) of the corresponding unlabelled hormone.

ADP-ribosylation of glomerulosa-cell membranes in the presence of toxins

The experiments were conducted as described by Ribeiro-Neto *et al.* [34], with minor modification. Glomerulosa-cell membranes were prepared as described above except that, after the final centrifugation step, the pellet was resuspended in the buffer used for the ADP-ribosylation described below. IAP and CT were pre-activated for 30 min at 35 °C in the presence of 30 mM-dithiothreitol. For ADP-ribosylation with IAP, 10–20 μg of glomerulosa membrane proteins was incubated for 30 min at 35 °C in the presence of activated IAP (10 μg/

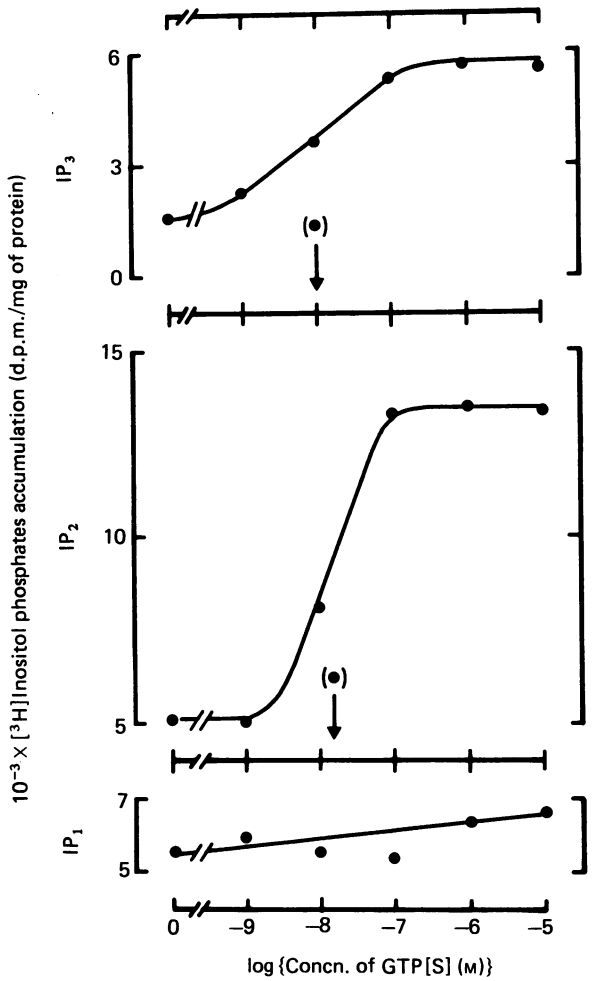

Fig. 1. Phospholipase C stimulation induced by GTP[S] on a glomerulosa-cell plasma-membrane preparation

The plasma membranes derived from glomerulosa cells grown in the presence of *myo*-[^{3}H]inositol were incubated for 10 min at 37 °C in the presence of increasing amounts of GTP[S]. The labelled IP_1, IP_2 and IP_3 were separated on Dowex columns (see the Materials and methods section). The results shown are from representative experiments performed in duplicate, which were repeated four times. The arrows represent the concentration of GTP[S] giving half-maximal stimulation (EC_{50}).

ml), 10 mM-thymidine, 50 mM-potassium phosphate buffer (pH 7.5), 0.1 mM-GTP, 1 mM-ATP, 1 mM-EDTA, Lubrol PX (0.5%, v/v), 0.3 μM-NAD$^+$ (labelled + unlabelled) and [^{32}P]NAD$^+$ [(2–4) × 10^6 d.p.m./assay]. For ADP-ribosylation with CT, 40–70 μg of glomerulosa membrane proteins was incubated for 30 min at 32 °C in the presence of activated toxin (50 μg/ml), 10 mM-thymidine, 100 mM-potassium phosphate buffer (pH 7.5), 1 mM-GTP, 1 mM-ATP, 1 mM-EDTA, 10 mM-$MgCl_2$, Lubrol PX (0.5%), 0.3 μM-NAD$^+$ (labelled + unlabelled) and [^{32}P]NAD$^+$ [(4–8) × 10^6 d.p.m./assay]. Reactions were stopped by adding 1.0 ml of ice-cold trichloroacetic acid (20%) and centrifuging the extracts at 700 *g* for 30 min at 4 °C. The pellets were washed with 2 × 1.0 ml of diethyl ether and centrifuged at 4 °C (20 min, 3000 *g*). The supernatant was discarded and the pellet solubilized by the addition of 50 μl of Laemmli sample buffer. Portions of the solubilized pellet were separated by SDS/polyacrylamide-gel electrophoresis in 10%-acrylamide gel. Electrophoresis was performed overnight at 50 V/cm. Gels were stained with Coomassie Blue, destained, dried and then autoradiographed by using Kodak X-Omat AR5 film, exposed for 3–10 days.

RESULTS

Stimulation of phospholipase C activity present in *myo*-[^{3}H]inositol-prelabelled glomerulosa-cell membrane preparations

In partially purified preparation of plasma membranes derived from glomerulosa cells grown in the presence of *myo*-[^{3}H]inositol, GTP[S] was able to stimulate the accumulation of IP_2 and IP_3 (Fig. 1). This effect was dose-dependent and reached a plateau at 0.1 μM-GTP[S]. In these conditions, IP_2 and IP_3 accumulations were increased approx. 2-fold (see Table 2). The concentration of GTP[S] leading to half-maximum stimulation was 0.01 μM for both IP_2 and IP_3 accumulations.

As previously described for other membrane systems [12], GTP[S] only slightly affected the accumulation of IP_1. This probably means that the IP_2 phosphatase present in intact cells was lost during the membrane preparation steps. This is the reason why only IP_3 and IP_2 accumulations were measured in the following experiments on membrane preparations.

As illustrated in Table 2, the other guanine nucleotides, GTP and p[NH]ppG, also stimulated the phospholipase C activity, but GTP[S] was found to be the most potent activator. NaF, as in other similar systems [35], also stimulated IP_2 and IP_3 accumulations, but was less potent than GTP[S] and p[NH]ppG.

When AT II or VP was added to prelabelled glomerulosa-cell membrane preparations, no significant stimulation of IP accumulation could be observed whatever the concentration of hormone used (up to 1 μM; Table 2). However, if a maximal dose of hormone (1 μM) was added along with GTP[S], a small potentiation of $IP_2 + IP_3$ accumulation was observed. As shown in Fig. 2, this effect shifted the dose–response curve to the left. Such a potentiation was weak but significant. The concentration of GTP[S] leading to the half-maximum response was 20 ± 6 and 4 ± 1 nM (means ± S.E.M. for three distinct experiments) for respectively GTP[S] alone or GTP[S] plus AT II (1 μM). Similarly, VP alone (0.1 μM) did not stimulate IP accumulation (see Table 2), but potentiated the accumulation of IP_2 and IP_3 induced by 1 μM-GTP[S] by 23 ± 7% (means ± S.E.M. for three distinct experiments).

Effect of treatment with bacterial toxin on the hormonal stimulation of IP production in glomerulosa cells

As previously described and further illustrated in Fig. 3, VP and AT II stimulated the PI breakdown in *myo*-[^{3}H]inositol-prelabelled glomerulosa cells. Both hormones were found to be able to stimulate total IP accumulation about 4–6-fold when used at maximal doses (1 μM). The concentrations of VP at AT II leading to the half-maximum effect ($K_{act.}$) were close to 1 nM for both hormones. When similar experiments were performed on *myo*-[^{3}H]inositol-prelabelled glomerulosa cells that were treated for 15 h before the beginning of the experiment with 0.1 μg of IAP/ml, no statistically

Table 2. Effect of hormones and guanine nucleotides on stimulation of IP accumulation on glomerulosa-cell membrane preparations

myo-[^{3}H]Inositol-prelabelled glomerulosa-cell membrane preparations were incubated for 10 min at 37 °C as described in the legends of Figs. 1 and 2 with various effectors. The inositol phosphates which accumulated were measured. The results are expressed as means ± S.E.M. (as % of corresponding control values), derived from three to six distinct experiments: 100% corresponds to 4100 ± 400, 3700 ± 400 and 1300 ± 200 d.p.m./mg of protein for IP_1, IP_2 and IP_3 respectively.

Effector tested	IP accumulation (%)		
	IP_1	IP_2	IP_3
None (control)	100	100	100
GTP (0.1 mM)	99 ± 8 (5)	138 ± 7 (5)	141 ± 15 (5)
p[NH]ppG (0.1 mM)	102 ± 3 (3)	143 ± 6 (3)	167 ± 20 (3)
GTP[S] (0.1 mM)	91 ± 6 (5)	198 ± 14 (6)	198 ± 7 (5)
AT II (1 μM)	89 ± 4 (6)	106 ± 5 (6)	104 ± 8 (7)
VP (1 μM)	100 ± 11 (4)	110 ± 5 (6)	94 ± 16 (6)
NaF (10 mM)	99 ± 8 (3)	158 ± 20 (3)	151 ± 19 (3)

significant difference was observed compared with untreated cells (Fig. 3). The values obtained from four or five distinct experiments indicate that the maximum hormonal responses obtained on IAP-treated cells were 87 ± 3% and 97 ± 4% of those of control cells for respectively 0.1 μM-VP or 0.1 μM-AT II. Moreover, as shown in Fig. 3, the $K_{act.}$ values of both VP or AT II for IP accumulation were not modified by IAP treatment.

In contrast with IAP, CT treatment of labelled glomerulosa cells (15 h at 37 °C with 1 μg of CT/ml) produced an inhibition of both the VP- and AT II-stimulated IP accumulation (Fig. 4, upper panels). Statistical values derived from three distinct experiments indicate that the maximum inhibitions induced by CT were 63 ± 10% and 68 ± 9% for respectively 0.1 μM-VP and -AT II. This treatment modifies only the maximal effect of either VP or AT II to stimulate IP accumulation, since the concentrations of either VP or AT II required to elicit the half-maximum IP accumulation were unchanged. Fig. 4 (lower panels) clearly demonstrates that the effect of CT on the hormone-stimulated IP production is dose-dependent. Increasing the amounts of CT present during the cell infection period led to a progressive inhibition of both the VP- and the AT II-stimulated IP accumulation. Maximum effects were observed after 15 h incubation in the presence of 0.1 μg of CT/ml. Moreover, CT did not modify the basal value of IP accumulation whatever the CT concentration used.

Control experiments demonstrate that neither CT nor IAP treatment affects the size of the labelled inositol lipid pools present in glomerulosa cell membranes (results not shown).

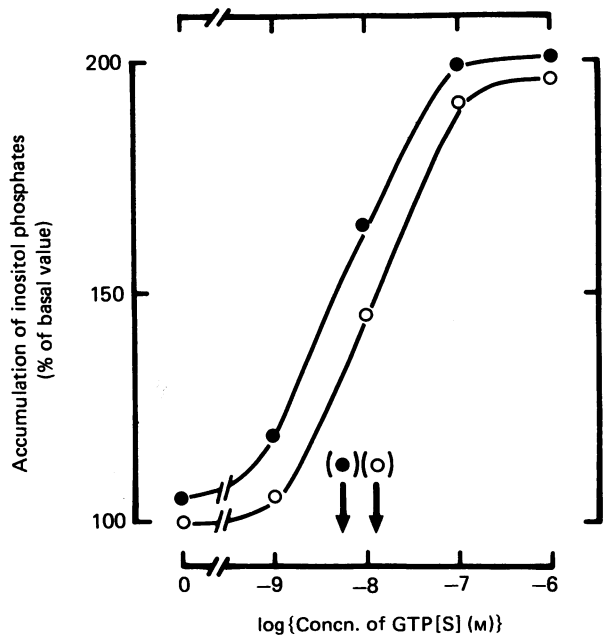

Fig. 2. Hormonal potentiation of phospholipase C stimulation induced by GTP[S]

myo[^{3}H]Inositol-prelabelled membranes were incubated as described in Fig. 1 with an increasing amount of GTP[S] in the presence (●) or in the absence (○) of 1 μM-AT II. The accumulation of $IP_2 + IP_3$ was measured. The arrows in the graphs indicate the concentration of GTP[S] giving half-maximum stimulation (EC_{50}). The results shown are from representative experiments performed in duplicate, which were repeated four times.

ADP-ribosylation of G-protein present in glomerulosa cell membranes

When glomerulosa cell membranes were ADP-ribosylated with IAP in the presence of [^{32}P]NAD^+, only one molecule was specifically labelled (Fig. 5, lanes *a* and *b*). Its molecular mass was about 41 kDa. As depicted in Fig. 6, the labelling of this molecule was sensitive to the presence of guanine nucleotide. Increasing the concentration of GTP[S] in the ADP-ribosylation medium led to a progressive decrease in the labelling of the 41 kDa band. When similar experiments were carried out on membranes derived from glomerulosa cells previously incubated for 15 h with 0.1 μg of IAP/ml, no labelling could be observed (Fig. 5, lane *c*). This may signify that the IAP treatment leads to a complete endogenous ADP-ribosylation of 'α_i' or of a similar molecule present in the cells.

Similarly, glomerulosa cell membranes were ADP-ribosylated with CT. As illustrated in Fig. 5, the autoradiogram (lanes *d* and *e*) revealed the existence of many bands. Yet only two molecules were specifically labelled in the presence of CT (molecular masses 46 and 52 kDa). These molecules probably correspond to the

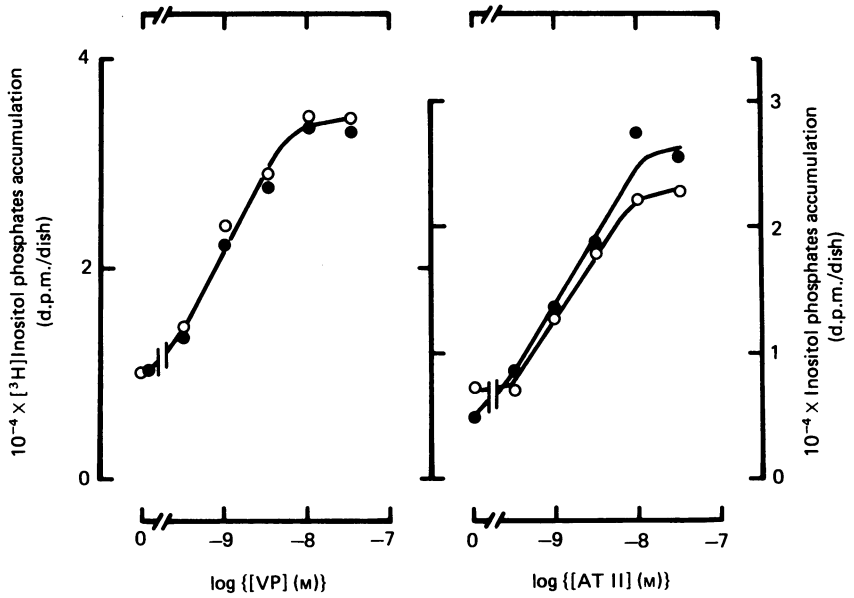

Fig. 3. Effect of IAP treatment on the activity of the hormone-sensitive phospholipase C present in glomerulosa cells

Glomerulosa cells grown in the presence of *myo*-[^{3}H]inositol were incubated for 15 h at 37 °C in the presence (●) or in the absence (○) of 0.1 μg of IAP/ml. They were incubated for a further 15 min at 37 °C in the presence of an increasing amount of either VP or AT II as described in the Materials and methods section. The accumulation of inositol phosphates (IP_1 + IP_2 + IP_3) was measured. The results shown are from representative experiments in duplicate, which were repeated three times.

'α_s' proteins present in this tissue, since immunoblotting experiments using antisera raised against purified 'α_s' molecules specifically precipitated two bands in bovine adrenal medulla (45 and 52 kDa) [35]. As illustrated in Fig. 5, the labelling of these two molecules was always seen on membrane derived from glomerulosa cells previously incubated for 15 h with 1 μg of CT/ml. Such results indicate that this CT treatment did not allow their complete endogenous ADP-ribosylation.

Effect of CT treatment on binding of VP and AT II

The glomerulosa cells were treated with a concentration of CT which induced a maximum inhibition of hormone-stimulated phospholipase C activity (1 μg/ml; 15 h). In these conditions, the specific binding of [^{3}H]VP (agonist) or [^{3}H]cyclo-VP (antagonist) in intact cells was considerably decreased as compared with untreated cells (Table 3). Given the affinities of the VP analogues for their receptors (Table 1), the concentration tested allowed different degrees of receptor occupancy (53% and 90% for respectively 2 nM- and 15 nM-VP and 67% and 98% for respectively 0.2 nM- and 6 nM-cyclo-VP). Similar inhibitions were observed whatever the concentration of the labelled peptides used. These results probably mean that CT mainly affects the maximum density of VP-binding sites more than the affinity of this receptor. Similar results were obtained for the AT II receptor (Table 3).

Effect of ACTH and CT on the intracellular concentration of cyclic AMP

As previously described and further illustrated in Fig. 7 (upper panel), ACTH and CT stimulated the cyclic AMP accumulation in glomerulosa cells. These effects were dose-dependent and reached a plateau at a concentration of 1 μM-ACTH and 1 μg of CT/ml. For these concentrations, the cyclic AMP formation was increased 23- and 13-fold respectively for ACTH and CT. As shown in Fig. 7 (lower panel), the kinetics of cyclic AMP accumulation were different: ACTH (10 nM) stimulates a maximal cyclic AMP accumulation after a 15 min incubation (7-fold increase). There follows a decrease in the stimulation after a prolonged exposure with the peptide (3-fold increase after 15 h of treatment). On the contrary, a 3 h incubation with CT was necessary to obtain maximal stimulation.

Effect of ACTH on the hormonal stimulation of IP accumulation

As seen in Table 4, when glomerulosa cells were incubated for 15 min with 10 nM-ACTH, before stimulation with maximal doses of VP or AT II, no inhibition of IP accumulation was observed. On the contrary, when glomerulosa cells were incubated for 15 h before the experiments with 10 nM-ACTH, a significant inhibition (about 50%) of hormone-stimulated IP accumulation was observed.

DISCUSSION

As illustrated in this study, guanine nucleotides stimulate the production of IP_2 and IP_3 by plasma membranes derived from glomerulosa cells. Such results suggest the existence of a GTP-binding protein involved in the activation of phospholipase C. Moreover, this interpretation is confirmed by the fact that NaF, which is known to interact with the 'GDP forms' of G-protein (transducin and α_s) [36,37], also produces a stimulation of IP accumulation. In view of these results, NaF seems to be a general activator of phosphatidylinositol

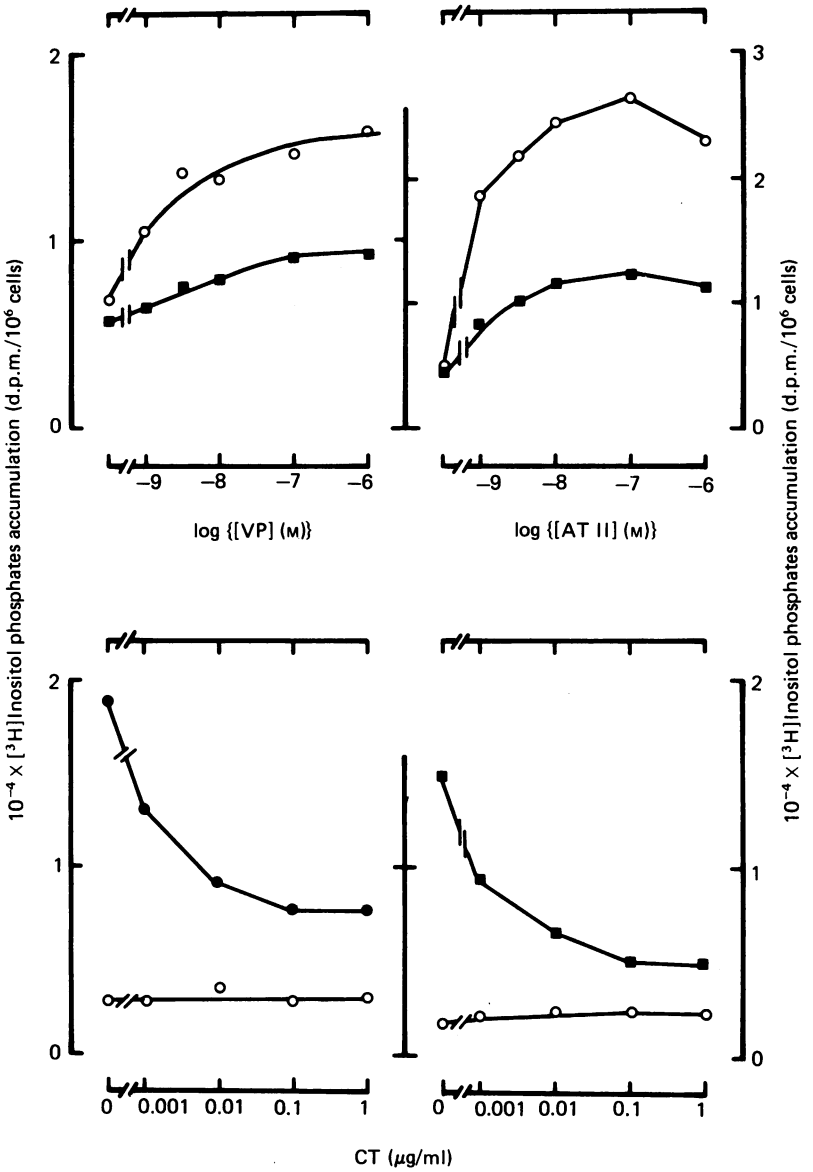

Fig. 4. Effect of CT treatment on the activity of the hormone-sensitive phospholipase C present in glomerulosa cells

Upper panel: *myo*-[^{3}H]inositol-prelabelled glomerulosa cells were incubated for 15 h at 37 °C with (■) or without (○) 1 μg of CT/ml. They were incubated for a further 15 min at 37 °C with an increasing amount of either AT II or VP. The inositol phosphates ($IP_1+IP_2+IP_3$) that accumulated were determined. Lower panel: glomerulosa cells grown in the presence of *myo*-[^{3}H]inositol were incubated for 15 h at 37 °C with increasing amounts of CT. They were incubated for a further 15 min at 37 °C with 1 μM-VP (●), 1 μM-AT II (■), or without hormone (○). The accumulation of inositol phosphates ($IP_1+IP_2+IP_3$) was determined. The results shown are from representative experiments in duplicate, which were repeated three times.

bisphosphate phospholipase C, since its action is now described in numerous systems [38–40].

VP or AT II alone was found to be ineffective in stimulating IP production in this system despite their action on PI metabolism in intact cells [7]. But, as previously observed in other systems [11,12], guanine nucleotides were found to be absolutely necessary in order to observe a hormonal stimulation. With glomerulosa-cell membrane preparations, the potentiating effect of hormones on GTP[S] stimulations was weak but significant as compared with other systems [11,12]. Such results may signify that the hormonal receptors present in glomerulosa cells were coupled to a phospholipase C via a G-protein.

In order to determine the nature of the G-protein involved in these coupling mechanisms, we have used (1) CT, which is known to ADP-ribosylate the α subunit of transducin (T) and of the G protein involved in the positive coupling between receptor and adenylate cyclase (α_s), and (2) IAP, which ADP-ribosylates the α subunit of G_i, the G-protein involved in the negative coupling between receptor and adenylate cyclase, of G_0, a G-protein found in the brain, and of T (for review see [41]).

Control experiments revealed the existence of a single 41 kDa molecule selectively ADP-ribosylated in the presence of IAP (Fig. 5). This protein probably represents 'α_i' or a 'α_i-like' G-protein, since, as described by

Nakamura & Ui [16] and illustrated in Fig. 5, GTP[S] selectively inhibits the labelling of the 41 kDa molecule. The labelling of this molecule could be completely blocked by incubating glomerulosa cells for 15 h at 37 °C in the presence of 0.1 μl of IAP/ml before ADP-ribosylation experiments. This probably means that this treatment allowed a complete endogenous ADP-ribosylation of the 'α_i-like' molecule present in these cells. In such conditions, the IP accumulation induced by VP or AT II was not modified as compared with untreated cells (Fig. 3). Such negative results could not be explained by a modification of the labelling of the glomerulosa cells' phosphoinositide pools. They probably mean that the G-protein involved in the coupling between the VP and AT II receptor to phospholipase C was neither 'α_i' nor an 'α_i-like' G-protein. Yet we cannot exclude the possibility that an

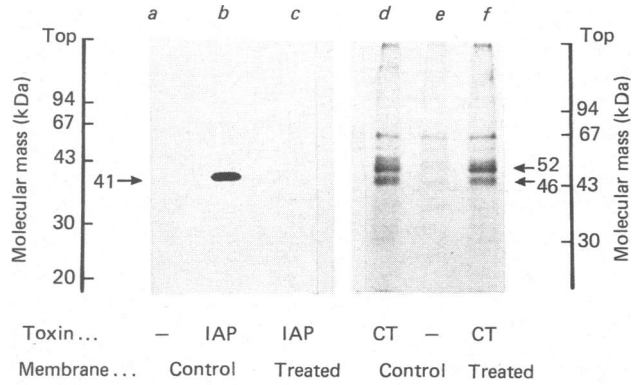

Fig. 5. ADP-ribosylation of the G-proteins present in glomerulosa cells

The glomerulosa cells were either grown in the absence of IAP (control) or incubated for 15 h at 37 °C in the presence of either 0.1 μg of IAP/ml or 1 μg of CT/ml. The plasma membranes derived from these three batches were ADP-ribosylated in the presence of [^{32}P]NAD$^+$ with or without pre-activated toxins. The membranes were then solubilized and subjected to electrophoresis with standard proteins of known molecular mass. An autoradiograph of the gels is shown and is representative of two or three distinct experiments. Lanes: *a*, membrane derived from control cells and ADP-ribosylated without IAP; *b*, membrane derived from control cells and ADP-ribosylated with IAP; *c*, membrane derived from IAP-treated cells and ADP-ribosylated with IAP; *d*, membrane derived from control cells and ADP-ribosylated with CT; *e*, membrane derived from control cells and ADP-ribosylated without CT; *f*, membrane derived from CT-treated cells and ADP-ribosylated with CT.

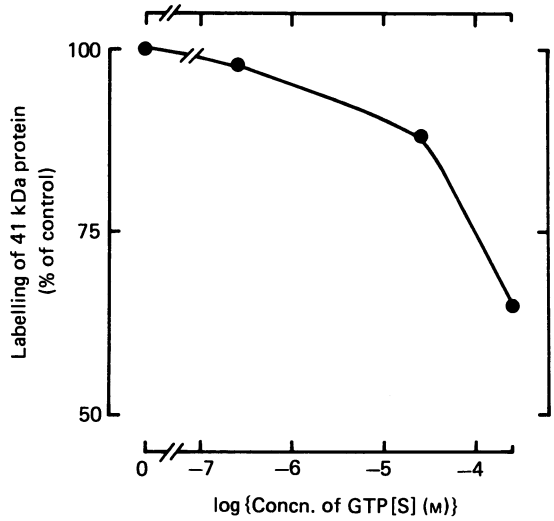

Fig. 6. Effect of GTP[S] on the ADP-ribosylation of the 41 kDa molecule

Glomerulosa-cell membranes were ADP-ribosylated in the presence of IAP with or without (control) increasing amounts of GTP[S] as described in the Materials and methods section. The membranes were solubilized, subjected to gel electrophoresis and autoradiographed. The relative intensities of the 41 kDa protein were measured with a spectrophotometer equipped with an integrated calculator. The results are expressed as percentages of the labelling of the 41 kDa protein found in control membranes.

Table 3. Effect of CT treatment on the specific binding of VP or AT agonists or antagonists

Adrenal glomerulosa cells were treated for 15 h with (CT-treated cells) or without (control) 1 μg of CT/ml. The specific binding of the analogues was measured as described in the Materials and methods section. The results are corrected for non-specific binding and are expressed in fmol specifically bound per 10^6 cells; they are means ± S.E.M. of three experiments, each in triplicate.

Labelled hormone used	Concn. (nM)	Hormone-specific binding (fmol/10^6 cells)		Inhibition of binding (%)
		Control cells	CT-treated cells	
[^{3}H]VP (agonist)	5	7.5±1.6	3.3±1.2	56
	15	9.1±1.7	3.0±1.3	67
[^{3}H]cyclo-VP (antagonist)	0.2	5.4±0.7	2.0±0.5	65
	6	8.8±1.5	3.1±0.7	65
[^{125}I]Sar AT II (agonist)	0.4	23.3±4.1	8.6±1.5	63
	10	26.6±1.0	6.0±0.5	77
[^{125}I]Sar D-Phe AT II (antagonist)	0.1	8.1±0.6	3.8±0.4	53
	0.6	37.2±6.6	16.3±3.1	56

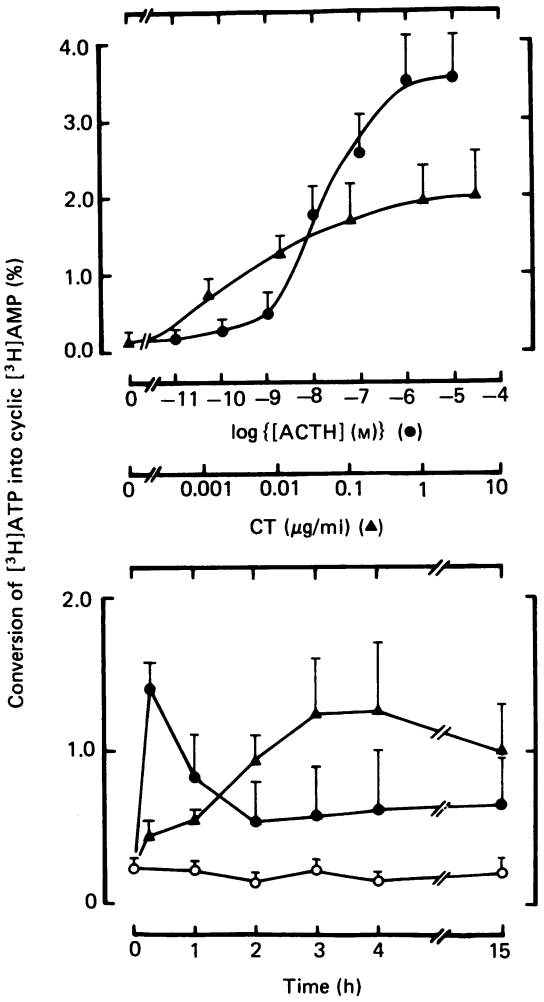

Fig. 7. Effects of ACTH and CT on the intracellular concentrations of cyclic AMP in glomerulosa cells

Upper panel: glomerulosa cells, prelabelled with [^{3}H]-adenine (see the Materials and methods section), were incubated for 15 min at 37 °C with increasing amounts of ACTH (●), and the production of cyclic AMP was measured. For CT treatment (▲), cells were infected with increasing amounts of CT for 4 h at 37 °C and then prelabelled with [^{3}H]adenine, and the content of cyclic AMP was measured. The results are expressed as percentage of ATP converted into cyclic AMP. Lower panel: glomerulosa cells were incubated without toxin (○), with 1 μg of CT/ml (▲) or with 10 nM-ACTH (●), for different periods of time at 37 °C in a controlled atmosphere. Just before the cyclic AMP measurements, the cells were pre-equilibrated with [^{3}H]adenine, and the ratio of [^{3}H]ATP to cyclic [^{3}H]AMP was measured (see the Materials and methods section). The results are means ± S.E.M. for three distinct experiments each done in triplicate.

Table 4. Effect of ACTH on the hormone-stimulated IP accumulation in glomerulosa cells

myo-[^{3}H]Inositol prelabelled glomerulosa cells were incubated for either 15 min or 15 h at 37 °C (with or without 10 nM-ACTH). The cells were then further incubated without any hormone (control) or with a maximum dose (0.5 μM) of VP or AT II, and the inositol phosphates that were liberated in the medium were measured as described in the Materials and methods section. ACTH was kept constant at 10 nM during IP-accumulation measurements for cells previously incubated with this hormone. The results are means ± S.E.M. for three separate experiments, each done in quadruplicate, and are expressed as percentages of values for basal untreated cells: 100% = 5400 ± 800 d.p.m./10^6 cells.

	IP accumulation (% of basal values)		
Cell treatment	Control	VP	AT II
None	100	488 ± 48	697 ± 120
15 min with 10 nM-ACTH	96 ± 10	420 ± 86	843 ± 100
15 h with 10 nM-ACTH	104 ± 4	210 ± 32	216 ± 21

ADP-ribosylated form of 'α_i' may be active in this system. Thus the VP and AT II receptors present in glomerulosa cells were different from the fMet-Leu-Phe and the thrombin receptors present in leucocytes, mast cells or fibroblasts [16–18] in terms of the G-protein involved in their coupling with the phospholipase C. Indeed, several authors have clearly demonstrated that, for these receptors, IAP blocked the hormonal stimulation of IP production. On the other hand, our results are similar to those obtained by different groups for other various receptors [19–22]. Altogether, these data reinforce the idea that, among the receptors which were coupled with PI metabolism, at least two categories exist: those in which the coupling G-protein is sensitive to IAP and those in which it is not.

CT, unlike IAP, inhibits both VP and AT II stimulations of IP accumulation in glomerulosa cells (Fig. 4). This effect is dose-dependent and specific to hormonal stimulation, since the basal IP accumulation is unaffected by CT treatment. It cannot be caused by: (1) the endogenous ADP ribosylation of the 46 and 52 kDa molecules, since, as illustrated in Fig. 5, under the experimental conditions used (15 h incubation with 1 μg of CT/ml) no complete endogenous ADP-ribosylation of these G-proteins was observed; (2) a defect of *myo*-[^{3}H]inositol incorporation into inositol-lipid pools; or (3) the possible cytotoxicity of such a treatment, since glomerulosa cells that were exposed for 18 h to CT (1 μg/ml), washed and further incubated for 2 h in a culture medium without toxin were always able to produce mineralocorticoids after ACTH stimulation (results not shown). Yet, on the basis on our ADP-ribosylation experiments, we cannot exclude the existence of a minor unidentified CT-sensitive G-protein involved in the coupling of the VP or AT II receptor with the phospholipase C.

As demonstrated in binding experiments, CT treatment also decreases the number of binding sites for VP and AT II on glomerulosa cells (Table 3). The percentage decrease in the number of binding sites was closely related to the maximum inhibition of IP accumulation induced by VP and AT II. Since it has been demonstrated, in glomerulosa cells [31] and in other similar systems [22,42], that a close relationship exists between receptor occupancy and phospholipase C activation, we can suggest that CT mainly acts at the level of the receptor.

Moreover, such an inhibition of VP or AT II specific binding cannot be accounted for by an action of CT, at the level of the G-protein coupled with the receptor, since the binding of both agonist and antagonist was similarly affected (Table 3). The mechanisms by which CT exerts its effect on receptors remained partially unclear. Thus the effect of ACTH treatment on the hormonal stimulation of PI metabolism seems to indicate a cyclic AMP process. But, as shown in the present study, this effect was observed only after a long-term incubation of glomerulosa cells with ACTH (Table 4). A 15 min incubation with ACTH, which causes a maximum increase in cyclic AMP, had no effect on the hormonal stimulation of PI metabolism induced by AT II or VP.

These results, together, demonstrate that CT strongly affects the mechanisms involved in the hormonal stimulation of phospholipase C in these cells. Different hypotheses can be postulated. (1) An unidentified G-protein, distinct from the well-known 'α_s' subunits, also ADP-ribosylated by CT, may be directly involved in the coupling of the VP or AT II receptors with the phospholipase C. The ADP-ribosylation of this G-protein may uncouple the VP or the AT II receptors from phospholipase C. These uncoupled forms of hormonal receptors might have a very low affinity for their respective hormones, and thus might not be detectable by the classical binding techniques used. (2) The coupling G-protein is not ADP-ribosylated by CT and represents a new coupling molecule called 'Gx'. CT modulates the hormone-sensitive PI response by affecting the receptor density. Two processes may be suggested. First, the increase in cyclic AMP by CT treatment stimulates a protein kinase A, which can phosphorylate the VP or AT II receptors, leading to a desensitized or uncoupled form of hormonal receptors. Second, the increase in cyclic AMP may affect the turnover of VP and AT II receptors (inhibition of synthesis and/or recycling). The first hypothesis seems improbable, since (1) CT did not block completely the VP and AT II stimulation of IP accumulation, even at high concentration; (2) the endogenous ADP-ribosylation of CT-sensitive molecules was not affected by a CT treatment which inhibits the VP- and AT II-stimulated IP accumulation; and (3) CT treatment affects similarly the specific binding of VP and AT II agonist and antagonist.

Recent work by Leeb-Lundenberg and co-workers [43,44] on purified α_1 receptors demonstrate that both kinase A (cyclic AMP-dependent) and kinase C (diacylglycerol-dependent) may phosphorylate this receptor. It has also been demonstrated in different transduction systems (rhodopsin/cyclic GMP phosphodiesterase, and β-adrenergic receptor/adenylate cyclase [45–47]) that a phosphorylated form of receptor may represent an inactive form (desensitized or uncoupled). Thus our results may imply that CT and ACTH primarily act via an increase in the intracellular cyclic AMP concentration, then leading to an activation of protein kinase A. The resulting phosphorylation of the hormone receptors may partially uncouple the transduction systems. Such a hypothesis is also favoured by the fact that bromo cyclic AMP and CT have also been described as decreasing the density of thyroid and insulin receptors in adipocytes [48,49], and of TRH receptors in GH3 cells [23]. As is also the case in our experiments, the effects of CT on TRH receptors were only observed after a chronic treatment [23]. The time needed to obtain such an effect (18 h) favoured an action of cyclic AMP on the synthesis or on the recycling of the hormone receptors. Yet we cannot exclude the possibility that only a rapid increase in the intracellular cyclic AMP concentration was sufficient to trigger the decline of the hormone-receptor densities several hours after.

There are conflicting reports in the literature concerning such an effect of CT on PI metabolism. For example, CT was found to be able to inhibit the IP accumulation induced by VP on WRK1 cells [22], that by glucagon on rat hepatocytes [50], that by monoclonal antibodies to antigen receptors on Jurkat cells [51], and that by thrombin on platelets [52]. On the contrary, CT treatment did not affect the PI turnover of hepatocytes when stimulated by vasopressin [19]. Additional experiments are now needed in order to understand better the process by which CT affects these coupling mechanisms, and to determine the nature of the G-protein involved in the coupling of the VP and AT II receptor with the phospholipase C in rat glomerulosa cells.

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