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Influence of bacterial toxins and forskolin upon vasopressin-induced inositol phosphate accumulation in WRK 1 cells

Gilles GUILLON,* Marie-Noelle BALESTRE,* Colette LOMBARD,* François RASSENDREN* and Christopher J. KIRK†

*Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, 34094, Montpellier Cedex, France, and †Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham, B15 2TT, U.K.

The accumulation of inositol phosphates in WRK 1 cells, stimulated with a range of vasopressin concentrations, was diminished by prior exposure to cholera toxin or forskolin, whilst that observed in the presence of maximal concentrations of the hormone was enhanced in pertussis-toxin-treated cells. In the presence of [^{32}P]NAD$^+$, both cholera toxin and pertussis toxin provoked the labelling of peptides with approximate M_rs of 45000 and 41000 respectively in the membranes of WRK 1 cells. Exposure to cholera toxin or forskolin for 15–18 h enhanced cyclic AMP accumulation in these cells. The concentrations of these agents which provoked half-maximal cyclic AMP accumulation were similar to those required to diminish receptor-mediated inositol phosphate accumulation by 50%. In contrast, half-maximal ADP-ribosylation of the 45000M_r peptide needed 100-fold greater concentrations of the toxin than were effective in provoking half-maximal inhibition of inositol phosphate accumulation. Cholera toxin or forskolin also reduced the maximal specific binding, to intact WRK 1 cells, of both [^{3}H][Arg^8]vasopressin and the V_{1a} antagonist [^{3}H][β-mercapto-β,β-cyclopentamethylenepropionic acid,*O*-methyl-Tyr^2,Arg^8]vasopressin. The kinetics for the loss of this binding capacity following cholera-toxin treatment were very similar to those describing the diminution of vasopressin-stimulated inositol phosphate accumulation in the same cells.

INTRODUCTION

Receptor-mediated PtdIns(4,5)P_2 hydrolysis is a widespread mechanism of stimulus–response coupling in eukaryote cells which use Ca^{2+} as an intracellular messenger (see [1,2] for reviews). In the rat mammary tumour cell line WRK 1, vasopressin and other extracellular ligands provoke a rapid degradation of this lipid to yield the intracellular messengers Ins(1,4,5)P_3 and diacylglycerol [3,4].

The molecular mechanisms which couple receptor occupation to the activation of PtdIns(4,5)P_2 phosphodiesterase ('phosphoinositidase') are not resolved. However, receptor-mediated activation of this enzyme in a number of cell-free systems, including WRK 1 cell membranes, shows an absolute requirement for guanine nucleotides and it may be mimicked by non-hydrolysable analogues of these compounds [5–9]. Hence, as in the adenylate cyclase system [10], it is probable that a GTP-binding protein (G-protein) is implicated in the coupling of Ca^{2+}-mobilizing receptors to inositol lipid hydrolysis at the plasma membrane.

A number of G-proteins have now been identified as playing a role in stimulus–response coupling at various biological membranes. All are thought to be $\alpha\beta\gamma$-heterotrimers in which the α-subunit confers specificity upon each of the proteins, which may share common β- and γ-subunits [10,11]. The role of G-proteins in the hormonal activation of adenylate cyclase has been partly elucidated with the help of bacterial toxins. Cholera toxin (CT) provokes the ADP-ribosylation of the α-subunit of the G_s protein that mediates the effects of various ligands which stimulate adenylate cyclase. This causes the persistent activation of the enzyme and the accumulation of cyclic AMP in the cell [10–14]. Pertussis toxin (PT) ADP-ribosylates and prevents the receptor-mediated activation of the α-subunits of a number of membrane-bound G-proteins including G_i, which is implicated in the hormonal inhibition of adenylate cyclase [10,11,14,15]. There are conflicting reports concerning the influence of these toxins upon receptor-mediated inositol lipid hydrolysis in stimulated cells [16–28] and little is known about the mechanisms which underlie those effects which have been reported. In this paper we report on the influence of the bacterial toxins and forskolin, another agent that activates adenylate cyclase, upon receptor-mediated inositol phosphate accumulation in WRK 1 cells.

MATERIALS AND METHODS

Cell culture and incubation

WRK 1 cells were established and cultured, with or without *myo*-[2-^{3}H]inositol as described previously [3,29]. Cells were plated, in 3.5 cm plastic dishes, at a density of (5–7) $\times 10^4$ cells in minimal essential medium containing Earle's salts, fetal calf serum (5%, v/v), rat serum (2%, v/v), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 mg/ml) and *myo*-[2-^{3}H]inositol (2 μCi/ml) as required. The medium was changed after 2 days and experiments were performed 3 days later, except where

Abbreviations used: CT, cholera toxin; PT, pertussis toxin ('islet-activating protein' from *Bordetella pertussis*); [^{3}H]AVP, [^{3}H][Arg^8]-vasopressin; cyclo-[^{3}H]AVP, [^{3}H][β-mercapto-β,β-cyclopentamethylenepropionic acid,*O*-methyl-Tyr^2,Arg^8]vasopressin; PBS, phosphate-buffered saline (for composition see [29]); TRH, thyrotropin-releasing hormone.

cells were to be incubated with bacterial toxins, in which case the culture medium was changed again at this stage for one containing the appropriate toxin, and incubation was maintained for a further 15–18 h.

After incubation for 5 or 6 days as described above, the tissue culture medium was discarded and the cells were incubated for 30 min in a similar medium but without serum or *myo*-[2-^{3}H]inositol. Cells were then washed three times with phosphate-buffered saline containing 5.5 mM-glucose (PBS, see [29] for composition) and incubated at 37 °C for 15 min in PBS containing 10 mM-LiCl and 1 mg of bovine serum albumin/ml (fraction V). Cells were then used for the determination of vasopressin binding and inositol phosphate accumulation as described below.

Determination of [^{3}H]vasopressin binding

The hormone-binding reaction was initiated by rapidly aspirating the PBS (containing LiCl and bovine serum albumin) and adding, to each culture dish, 0.7 ml of PBS supplemented with LiCl (10 mM), bovine serum albumin (1 mg/ml), tyrosine (1 mM) and various amounts of [^{3}H]vasopressin [29]. Cells were incubated for 6 min at 37 °C and the binding reaction was stopped by aspiration of the incubation medium followed by the addition of 2 ml of ice-cold PBS. Cells were rapidly detached from the dishes by scraping with a rubber policeman and the cell suspension was layered on to the surface of a Gelman filter (Metrical membrane filter GA-3) under continuous aspiration. Culture dishes were rinsed with a further 2 ml of ice-cold PBS and filters were rinsed three times with 3 ml of ice-cold PBS. The radioactivity retained on the filters was measured by liquid-scintillation spectrometry. Non-specific binding was determined by incubating the cells in the presence of unlabelled vasopressin (10 μM). Specific binding was calculated as the difference between total and non-specific binding. The K_d (the concentration of vasopressin leading to half-maximal specific binding) and $B_{max.}$ (the maximum number of specific binding sites/cell) were determined by Scatchard analysis as described previously [29].

The accumulation of inositol phosphates and cyclic AMP in WRK 1 cells

The accumulation of [^{3}H]inositol phosphates was determined in pre-labelled WRK 1 cells incubated with 10 mM-Li^+ for 15 min prior to hormone stimulation [3]. Incubations were terminated with 5.0% (w/v) $HClO_4$, and neutralized, and inositol phosphates were separated by batch elution on Dowex columns (X-8, 100–200 mesh, formate form) as previously described [3,30]. This protocol generated fractions corresponding to InsP_1, InsP_2 and a mixture of InsP_3 and InsP_4. Elution characteristics were checked with the use of appropriate radioactive standards [31].

Adenylate cyclase activity was assessed by measuring the formation of cyclic [^{3}H]AMP from ^{3}H-labelled adenine nucleotide pools [32]. WRK 1 cells were incubated at 37 °C with [^{3}H]adenine (24 Ci/mmol) for 1 h, after which time the cells were washed and further incubated with isobutylmethylxanthine (1 mM) for 15 min. Incubations were terminated in 5% trichloroacetic acid, and [^{3}H]ATP and cyclic [^{3}H]AMP were determined as described previously [32].

ADP-ribosylation of WRK 1 cell membranes in the presence of PT or CT

Experiments were conducted as described by Ribeiro-Neto and collaborators [33] with minor modifications. WRK 1 membranes were prepared as described previously [5] except that, following the final centrifugation step, the pellet was resuspended in the appropriate buffer for the assay of ADP-ribosylation as described below. Bacterial toxins were pre-activated for 30 min at 35 °C in the presence of 30 mM-dithiothreitol. For ADP-ribosylation experiments with PT, WRK 1 membranes (40 μg) were incubated for 30 min at 35 °C in the presence of activated toxin (10 μg/ml), 10 mM-thymidine, 50 mM-potassium phosphate buffer (pH 7.5), 0.1 mM-GTP, 1.0 mM-ATP, 1.0 mM-EDTA, Lubrol PX (0.5%, v/v), and 0.3 μM-[^{32}P]NAD^+ (500 Ci/mmol). For ADP-ribosylation experiments with CT, 150 μg of WRK 1 membranes were incubated for 30 min at 32 °C in the presence of activated toxin (10 μg/ml), 10 mM-thymidine, 300 mM-potassium phosphate buffer (pH 7.5), 0.1 mM-GTP, 1.0 mM-ATP, 1.0 mM-EDTA, Lubrol PX (0.5%, v/v), 10 mM-$MgCl_2$ and 0.3 μM-[^{32}P]NAD^+ (500 Ci/mmol). Reactions were terminated by adding 1.0 ml of ice-cold trichloracetic acid (20%, v/v) and centrifuging the extracts for 30 min at 700 *g* and 4 °C. The pellets were washed twice by resuspension in 1.0 ml of diethyl ether followed by centrifugation for 20 min at 3000 *g* and 4 °C. The supernatants were discarded and the pellets were solubilized by addition of 50 μl of Laemmli's sample buffer [34]. Aliquots of these solubilized pellets were separated by SDS/polyacrylamide-gel electrophoresis (10% polyacrylamide gel). Electrophoresis was performed overnight at 3 V/cm. Gels were stained with Coomassie Blue, destained, dried and then autoradiographed using Kodak X Omat AR5 film, exposed for 6 h (PT) or 72 h (CT). The relative intensities of the labelled proteins with approximate M_rs of 41000 and 45000 were analysed with a Vernon spectrophotometer equipped with an Enica 10 integrator calculator (Delsi Instruments, Suresnes, France).

Chemicals

[^{3}H][Arg^8]vasopressin ([^{3}H]AVP) (70 Ci/mmol), [^{3}H]-[β-mercapto-β,β-cyclopentamethylenepropionic acid, *O*-methyl-Tyr^2,Arg^8]vasopressin (cyclo-[^{3}H]AVP) (60 Ci/mmol), [2-^{3}H]adenine (24 Ci/mmol), *myo*-[2-^{3}H]inositol (20 Ci/mmol) and [^{32}P]NAD^+ (700–900 Ci/mmol) were from New England Nuclear. CT was from Sigma Chemical Co. and PT was a generous gift from Dr. K. H. Jakobs (University of Heidelberg, Germany). All other chemicals were as described previously [3,5,29].

RESULTS

Influence of bacterial toxins on inositol phosphate accumulation in vasopressin-stimulated WRK 1 cells

As we have previously described [3] and is further illustrated in Fig. 1, vasopressin provokes a dose-dependent accumulation of InsP_1, InsP_2 and InsP_3 + InsP_4 in WRK 1 cells. When WRK 1 cells were incubated with CT (1 μg/ml) for 18 h before hormonal challenge, the accumulation of inositol phosphates was diminished in both control and hormone-stimulated cells. In five separate experiments, CT treatment reduced the accumu-

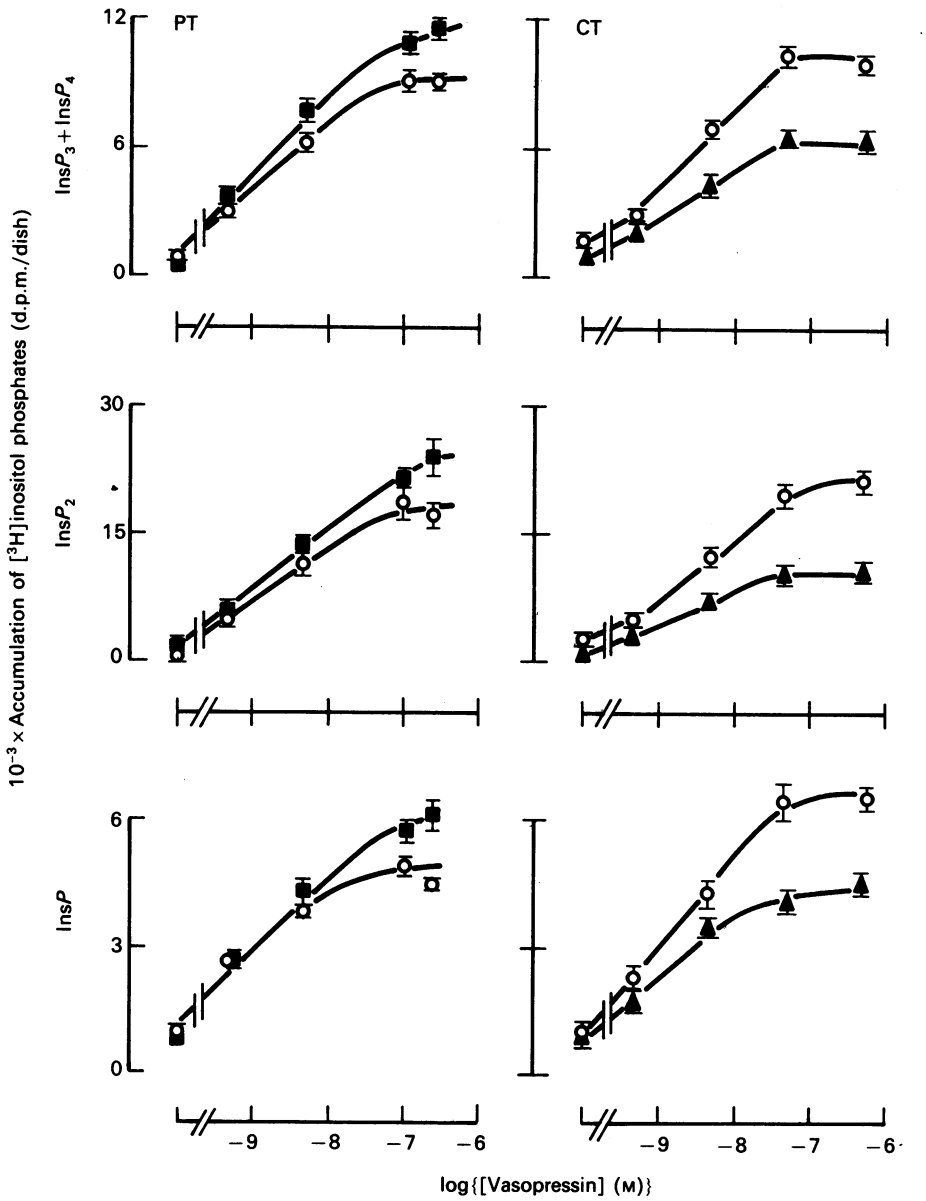

Fig. 1. Effect of CT and PT upon vasopressin-stimulated inositol phosphate accumulation in WRK 1 cells

myo-[2-^{3}H]Inositol-labelled WRK 1 cells were incubated in 1 ml of full culture medium for 15 h in the absence (○) or presence of CT (▲, 1 μg/ml) or PT (■, 0.1 μg/ml). Cells were washed free of toxin, and incubated in PBS containing 10 mM-LiCl for 15 min prior to stimulation with the appropriate concentration of vasopressin. Hormone-stimulated inositol phosphate accumulation was determined after a further 6 min as described in the Materials and methods section. Results are means ± S.E.M. of three determinations from a single experiment in which there were 4×10^5 cells/dish. Where no error bars are shown, they are contained within the symbols. Similar results were obtained in four other experiments.

lation of $InsP$, $InsP_2$ and $InsP_3+InsP_4$ by 42±6%, 50±5% and 71±6% respectively in unstimulated cells and by 64±9%, 47±9% and 58±7% respectively in the presence of 0.5 μM-vasopressin (mean ± S.E.M.). Similar pretreatment of WRK 1 cells with PT (0.1 ng/ml) had no effect upon basal inositol phosphate accumulation, but the accumulation of inositol phosphates in the presence of a maximally-effective concentration of vasopressin was slightly enhanced in these cells (Fig. 1). In four independent experiments, PT treatment enhanced $InsP_3+InsP_4$ accumulation in the presence of 0.2 μM-vasopressin by 13.4±4.1% (P versus untreated cells < 0.02 by paired t test).

ADP-ribosylation of WRK 1 cell peptides in the presence of bacterial toxins

As has been observed in several other systems (e.g. [13]), when WRK 1 cell membranes were incubated with CT in the presence of [^{32}P]NAD$^+$ for 30 min, a number of peptides became labelled (Fig. 2). The labelling of one of these, a peptide with an approx. M_r of 45000, was specifically reduced when WRK 1 cells were preincubated with CT for 15 h in the absence of radioactive NAD$^+$ prior to the preparation of membranes (Fig. 2). ADP-ribosylation of this M_r-45000 peptide was sensitive to CT over a similar concentration range to that which inhibited

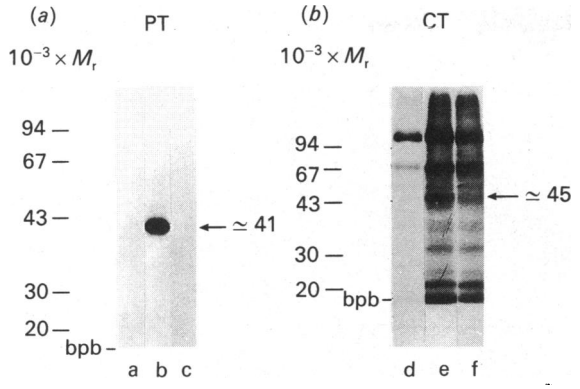

Fig. 2. ADP-ribosylation of WRK 1 cell membranes by CT and PT

(*a*) WRK 1 membranes (40 μg of protein/assay) were incubated with [^{32}P]NAD$^+$ alone (lane a) or [^{32}P]NAD$^+$ plus PT (10 μg/ml, lanes b and c) as described in the Materials and methods section. In lane c, membranes were prepared from WRK 1 cells which had been pre-incubated for 15 h with 0.1 μg of PT/ml in full culture medium. (*b*) WRK 1 membranes (150 μg of protein/assay) were incubated with [^{32}P]NAD$^+$ alone (lane d) or [^{32}P]NAD$^+$ plus CT (10 μg/ml, lanes e and f). In lane f, membranes were prepared from WRK 1 cells which had been preincubated for 15 h with 1.0 μg of CT/ml in full culture medium. After 30 min incubation with [^{32}P]NAD$^+$, membrane proteins were solubilized, separated on electrophoresis and autoradiographed as described in the Materials and methods section. The migration of protein markers of known molecular mass is indicated. Results are from a single experiment, typical of three. bpb, Bromophenol Blue.

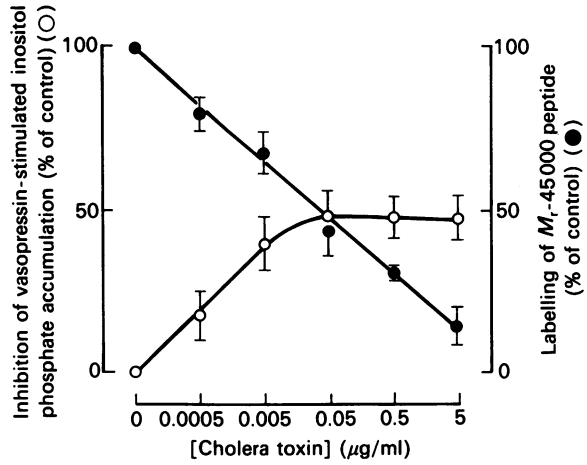

Fig. 3. Concentration-dependence of CT-induced inhibition of inositol phosphate accumulation and the labelling of an M_r-45000 peptide in WRK 1 cells

myo-[2-^{3}H]Inositol-labelled and unlabelled WRK 1 cells were incubated for 15 h in full culture medium containing various concentrations of CT. Unlabelled cells were washed free of toxin before the preparation of membranes and the determination of ADP-ribosylation with [^{32}P]-NAD$^+$ as described for Fig. 2 and in the Materials and methods section (●). *myo*-[2-^{3}H]Inositol-labelled cells were washed free of toxin, and incubated for a further 15 min in PBS containing 10 mM-LiCl before the addition of 0.2 μM-vasopressin. Accumulation of total inositol phosphates (Ins$P$ − InsP_4) was determined after a further 6 min as described in the Materials and methods section (○). Results are expressed as a percentage of those observed without toxin treatment, and are means ± S.E.M. from three separate experiments.

InsP_3 + InsP_4 accumulation in WRK 1 cells. However, maximal inhibition of InsP_3 + InsP_4 accumulation was observed at a 100-fold lower concentration of the toxin than was required to provoke maximum ADP/ribosylation (Fig. 3). The persistence of some labelling in the vicinity of the M_r-45000 peptide, even after pre-incubation of the cells with CT, suggests that other peptides of approximately this molecular mass may become labelled with [^{32}P]NAD$^+$ in a CT-insensitive manner in WRK 1 cells.

Incubation of WRK 1 cell membranes with PT and [^{32}P]NAD$^+$ for 30 min resulted in the labelling of a single peptide band, separable on electrophoresis, and having an M_r of approx. 41000. As with the experiments described above, the labelling of this peptide was specifically reduced when the intact cells were pre-incubated with PT for 15 h prior to the preparation of cell membranes. Furthermore, GTPγS, which provokes dissociation of G_i into α and $\beta\gamma$ subunits, and reduces its ADP-ribosylation by PT [10,11,15], also reduced the labelling of the M_r-41000 peptide in the present study (results not shown). In contrast with our results with CT-treated cells, we observed no other labelled peptides in PT-treated membranes. This presumably reflects the use of a lower concentration of membranes and a briefer autoradiographic exposure in the experiments with PT.

Influence of CT and forskolin upon the accumulation of cyclic AMP and inositol phosphates in WRK 1 cells

As shown in Fig. 4, CT treatment for 15 h provokes the activation of adenylate cyclase in WRK 1 cells over

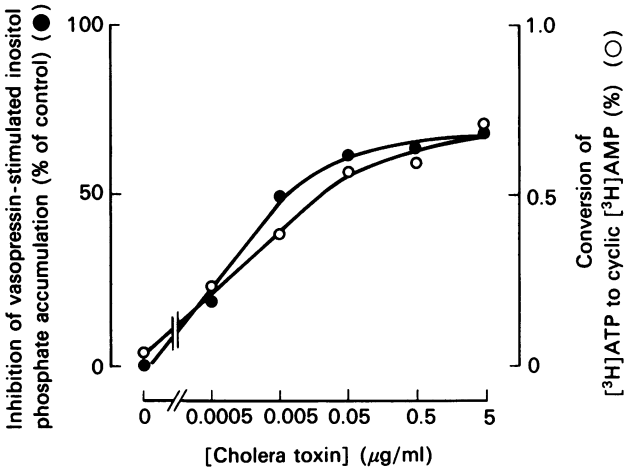

Fig. 4. Concentration-dependence of CT-induced cyclic AMP formation and the inhibition of inositol phosphate accumulation in WRK 1 cells

WRK 1 cells were incubated with various concentrations of CT for 15 h, and total inositol phosphate accumulation was determined as described in Fig. 3 legend (●). Cyclic AMP formation was measured by the incorporation of [^{3}H]adenine into cyclic AMP as described in the Materials and methods section (○). Results are expressed as percentages of [^{3}H]ATP converted to cyclic [^{3}H]AMP and are the means of duplicate determinations from a single experiment, representative of three.

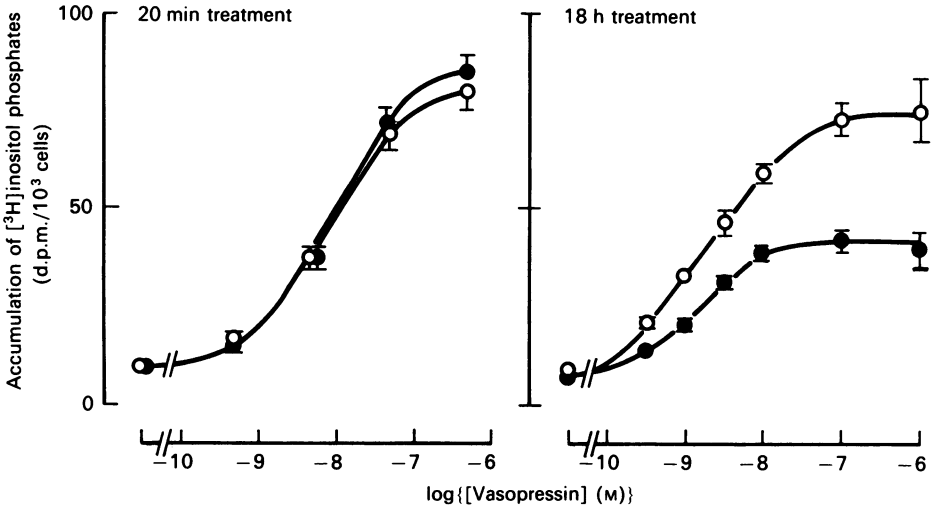

Fig. 5. Influence of forskolin upon vasopressin-stimulated inositol phosphate accumulation in WRK 1 cells

myo-[2-^{3}H]Inositol-labelled WRK 1 cells were incubated in full culture medium with (●) or without (○) 10^{-5} M-forskolin for 20 min or 18 h as indicated. Cells were then washed in PBS and incubated, in the continued presence of forskolin where appropriate, with 10 mM-LiCl for 15 min before the determination of vasopressin-stimulated accumulation of total inositol phosphates as described in Fig. 3 legend. Results are means ± S.E.M. of three determinations from a single experiment, typical of three.

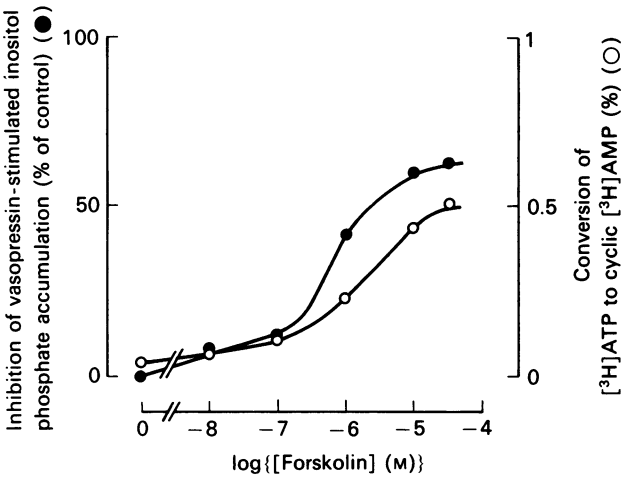

Fig. 6. Concentration-dependence of forskolin-induced cyclic AMP formation and the inhibition of inositol phosphate accumulation in WRK 1 cells

WRK 1 cells were incubated with various concentrations of forskolin for 15 h as described for Fig. 5 legend. Vasopressin-stimulated accumulation of total inositol phosphates (●) and of cyclic AMP (○) were determined as described for Figs. 3 and 4 respectively, both in the continued presence of forskolin as appropriate.

the same concentration range as is required to diminish vasopressin-stimulated inositol phosphate accumulation. As with the latter effect, CT induced maximal cyclic AMP accumulation at a concentration which was 100-fold lower than that required to cause maximal ADP-ribosylation of the M_r-45000 peptide (compare Figs. 3 and 4).

In view of the ability of CT to diminish vasopressin-stimulated inositol phosphate accumulation over a range of concentrations similar to those required to activate adenylate cyclase in WRK 1 cells, we have investigated the influence of forskolin upon these two parameters. Forskolin treatment for 18 h, but not for 20 min (Fig. 5), diminished the accumulation of inositol phosphates in vasopressin-stimulated cells. By contrast, this agent was effective in enhancing cyclic AMP accumulation over either treatment period (results not shown). As with CT, similar concentrations of forskolin were required to exert half-maximal effects upon the accumulation of both cyclic AMP and the inositol phosphates (Fig. 6).

Influence of CT and forskolin upon V_{1a} receptor binding

The treatment of WRK 1 cells with CT or forskolin for 18 h caused a significant reduction in the density of [^{3}H]AVP binding sites detected by Scatchard analysis of binding experiments performed on intact cells (Table 1). The apparent loss of vasopressin binding sites following treatment with CT (34%) or forskolin (47%) was broadly similar in magnitude to the inhibition by these agents of vasopressin-stimulated inositol phosphate production in these cells (45 and 49% respectively). CT treatment also diminished the number of binding sites detected with the V_{1a} antagonist cyclo-[^{3}H]AVP as a ligand. The apparent affinity of the V_{1a} receptor for either [^{3}H]AVP or cyclo-[^{3}H]AVP was not affected by prior treatment of the cells with either CT or forskolin in any of the conditions tested (Table 1).

The kinetics of CT-induced inhibition of [^{3}H]-vasopressin binding and inositol phosphate accumulation were investigated by varying the period of incubation with CT prior to the determination of these parameters (Fig. 7). Although the reduction in [^{3}H]vasopressin binding following 1 h exposure to CT (16 ± 4%) was greater than the inhibition of inositol phosphate accumulation at this time (4 ± 3%), the

Table 1. The influence of CT and forskolin upon ligand binding to V_{1a} receptors on WRK 1 cells

WRK 1 cells were incubated at 37 °C with CT or forskolin in 1 ml of full culture medium for the times indicated. The culture medium was then replaced by one containing no serum [29] and receptor binding was assessed after 6 min incubation with the appropriate radioligand in the continued presence of CT or forskolin as described in the Materials and methods section. K_d (the concentration of peptide leading to half-maximal specific binding) and $B_{max.}$ (the maximum number of specific binding sites) were determined by Scatchard analysis. In control cells, $B_{max.}$ for [^{3}H]AVP binding was $(5.6 \pm 0.9) \times 10^4$ sites/cell and that for cyclo-[^{3}H]AVP binding was $(7.5 \pm 1.1) \times 10^4$ sites/cell. Results are means ± S.E.M. from the number of separate experiments indicated in parentheses. N.D., not determined.

	[^{3}H]AVP binding:		Cyclo-[^{3}H]AVP binding:	
Pretreatment	K_d (nM)	$B_{max.}$ (% of control)	K_d (nM)	$B_{max.}$ (% of control)
None	2.0 ± 0.3 (4)	100 (4)	1.1 ± 0.2 (7)	100 (4)
CT (1 μg/ml, 18 h)	1.4 ± 0.3 (4)	66 ± 5 (4)	1.0 ± 0.2 (7)	73 ± 7 (7)
Forskolin (10 μM, 18 h)	1.6 ± 0.4 (3)	53 ± 2 (3)	N.D.	N.D.

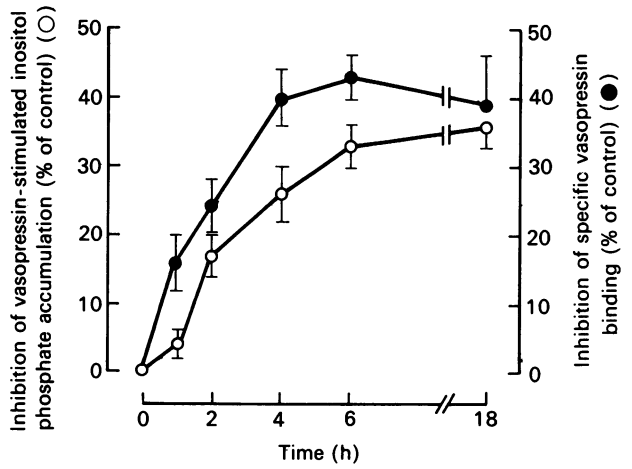

Fig. 7. Kinetics of cholera-toxin-induced inhibition of specific vasopressin binding and of vasopressin-stimulated inositol phosphate accumulation in WRK 1 cells

myo-[2-^{3}H]Inositol-labelled or unlabelled WRK 1 cells were incubated in full culture medium with or without 1 μg of CT/ml for the times indicated. Total inositol phosphate accumulation in the presence of 0.2 μM-vasopressin was determined as described in Fig. 3 legend (○). Specific vasopressin binding was determined in unlabelled cells incubated in the presence of 10 nM-[^{3}H]vasopressin as described in the Materials and methods section (●). Both parameters are expressed as a percentage of values measured in cells which had not been exposed to toxin (vasopressin binding in control cells was 26.7 ± 5.0 fmol/10^6 cells). Results are means ± S.E.M. for three determinations from a single experiment, typical of three.

difference between these two parameters was not significant ($0.05 < P < 0.1$). In all other respects, the loss of binding capacity and the inhibition of vasopressin-stimulated inositol phosphate accumulation followed broadly similar kinetics. Maximum inhibition of both parameters was observed after 4–6 h treatment with CT.

DISCUSSION

The accumulation of inositol phosphates in vasopressin-stimulated WRK 1 cells is a consequence of receptor-mediated degradation of PtdIns(4,5)P_2 [3]. Evidence from a variety of tissues indicates that guanine-nucleotide-dependent transducing proteins are implicated in stimulus–response coupling at these receptors [5–9]. The influence of CT and PT upon cyclic-AMP-mediated signal transduction has been shown to be mediated via the ADP-ribosylation of such G-proteins [10–15]. In the present study, vasopressin-stimulated inositol phosphate accumulation was diminished following treatment with CT but was slightly enhanced in PT-treated cells, at least in the presence of maximally-effective concentrations of the hormone.

Previous studies with CT and PT have revealed qualitatively different effects upon receptor-mediated inositol lipid degradation in individual tissues. In agreement with the present study, CT treatment diminished this process in HL 60 cells [16], hepatocytes [17], glomerulosa cells [18] and GH_3 cells [19], but had no effect in exocrine pancreas [20]. PT treatment has been reported to inhibit ligand-stimulated inositol lipid breakdown in neutrophils [21–23], mast cells [24], HL 60 cells [16] and rat myocytes [25], but to have no effect in a variety of other tissues including the exocrine pancreas [20], 3T3 cells [26], permeabilized GH_3 cells [27], chick heart muscle [28], a human astrocytoma cell line [28] and rat glomerulosa cells [18]. In agreement with our results, treatment with PT has also been reported to enhance accumulation of inositol phosphates in glucagon-stimulated hepatocytes [17].

The effects of bacterial toxins upon inositol phosphate accumulation in WRK 1 cells could arise in a number of ways. These include: (1) modulation of the lipid kinases and phosphomonoesterases which interconvert the inositol lipids, or of the phosphomonoesterases which catalyse inositol phosphate degradation; (2) direct modulation of the activity of the 'phosphoinositidase C' which catalyses PtdIns(4,5)P_2 hydrolysis or of its coupling to the activated receptor, perhaps via ADP-ribosylation of a G-protein; and (3) effects upon receptor density and/or affinity.

The labelling of all three inositol lipids was unaffected by toxin treatment (results not shown) and experiments were performed in the presence of 10 mM-Li^+, which effectively prevents the breakdown of inositol phosphates to free inositol. It is therefore unlikely that any of the enzymes listed in point (1) above are involved in

the effects of bacterial toxins upon inositol phosphate accumulation.

PT provoked the ADP-ribosylation of an M_r-41000 peptide in WRK 1 cells which may be the α_i subunit of G_i, the G-protein which is implicated in the hormonal inhibition of adenylate cyclase. ADP-ribosylation of G_i is thought to occur only when the G-protein is in the heterotrimeric state and to inhibit its subsequent dissociation into α and $\beta\gamma$ subunits [10,11,14,15]. Gilman and his colleagues have postulated that dissociation of G_i causes inhibition of adenylate cyclase by providing excess $\beta\gamma$ subunits to complex free α_s [15]. By analogy, it is possible that PT treatment enhances inositol phosphate accumulation in vasopressin-stimulated WRK 1 cells by reducing the dissociation of G_i and thus the availability of free $\beta\gamma$ to complex the α-subunit of the G-protein involved in receptor-mediated PtdIns(4,5)P_2 hydrolysis.

CT treatment of WRK 1 cells provoked the ADP-ribosylation of an M_r-45000 peptide, displaying the essential characteristics of α_s, the α subunit of the G-protein implicated in receptor-mediated activation of adenylate cyclase [10,11,13,14]. However, the concentration of CT necessary to provoke maximal ADP-ribosylation of this peptide was 100-fold greater than that which caused maximal inhibition of vasopressin-stimulated inositol phosphate accumulation. Hence it seems unlikely that α_s is directly implicated in the mechanism whereby CT diminishes vasopressin-induced inositol phosphate accumulation in WRK 1 cells. However, we cannot exclude the possibility that CT exerts this effect via a sub-population of an 'α_s-like' peptide, which co-migrates with α_s on SDS/polyacrylamide-gel electrophoresis and couples activated V_{1a} receptors to the phosphoinositidase C responsible for PtdIns(4,5)P_2 hydrolysis.

Prolonged treatment with forskolin, as with CT, diminished the inositol phosphate response to vasopressin. Since forskolin is thought to activate adenylate cyclase principally via a direct effect upon its catalytic subunit [35], it seems probable that the effects of both these agents upon receptor-mediated inositol lipid hydrolysis are mediated by cyclic AMP. In accordance with this suggestion, we have observed that the cell-permeant cyclic AMP analogue, dibutyryl cyclic AMP, inhibits vasopressin-stimulated inositol phosphate accumulation to a similar extent as that observed in the present study (C. J. Kirk, unpublished work). Similar effects of CT and cell-permeant cyclic AMP analogues have been reported in thyrotropin-releasing hormone (TRH)-stimulated GH_3 cells [19], and we have recently reported that prolonged treatment with CT or corticotropin diminished receptor-mediated inositol phosphate accumulation in rat glomerulosa cells, probably via an increase in intracellular cyclic AMP concentration [18]. Hence this phenomenon may represent a more widespread mechanism whereby agents which stimulate adenylate cyclase influence the inositol lipid signalling system.

The results of our binding studies indicate that long-term exposure of WRK 1 cells to cyclic AMP, as a result of CT or forskolin treatment, may influence vasopressin-stimulated inositol phosphate accumulation by reducing the density of V_{1a} receptors on the cell surface. Since PtdIns(4,5)P_2 hydrolysis is closely coupled to V_{1a} receptor occupation [3,9], reduced receptor density would be expected to lead directly to diminished inositol phosphate accumulation in these cells. Support for this view derives from the similar kinetics for V_{1a} receptor depletion and the diminution of the inositol phosphate response to vasopressin following addition of CT to WRK 1 cells (Fig. 7). Diminished inositol phosphate accumulation in TRH-stimulated GH_3 cells which have been treated with CT or cell-permeant cyclic AMP analogues was also accompanied by a reduction in the $B_{max.}$ of these cells for TRH [19].

As with other receptors, agonist binding to V_{1a} receptors preferentially requires receptor/G-protein interaction, whilst antagonists will bind to the free receptor ([36]; G. Guillon, unpublished work). The influence of cyclic AMP upon receptor binding in the present study is probably a direct effect upon the V_{1a} receptor since the binding of both agonist and antagonist were similarly affected. The effect of cyclic AMP on receptor binding could be a consequence of phosphorylation of the V_{1a} receptor protein. However it is interesting to note that cyclic AMP has recently been shown to 'down-regulate' TRH receptors on GH_3 cells by inhibiting the expression of the gene coding for the TRH receptor [37].

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