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The effect of cholera toxin on the inhibition of vasopressin-stimulated inositol phospholipid hydrolysis is a cyclic AMP-mediated event at the level of receptor binding

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Incubation of L6 skeletal myoblasts for 16 h with cholera toxin but not with pertussis toxin, led to the inhibition of inositol phosphate generation induced by subsequent exposure to vasopressin. The effects of the toxin on inositol lipid metabolism were accompanied by the total ADP-ribosylation of the available cholera-toxin substrates within the cells. Immunological analysis demonstrated that the two polypeptides modified *in vivo* by cholera toxin were different forms of $G_s\alpha$ (α subunit of G_s). No novel cholera-toxin substrate(s) were detected. The cholera-toxin-mediated inhibition of vasopressin-stimulated inositol phosphate generation could be mimicked by both forskolin and dibutyryl cyclic AMP, but not by the separated subunits of the toxin. Receptor-binding studies demonstrated that the inhibition of agonist-stimulated inositol phosphate generation was accompanied by a decrease in cell-surface vasopressin-binding sites, with no effect on the affinity of these for the hormone. We suggest that the effect of cholera toxin and agents which increase intracellular cyclic AMP on vasopressin-stimulated inositol lipid hydrolysis is an effect on receptor number, and that there is no requirement to postulate a role for a novel G-protein, which is a substrate for cholera toxin, in the regulation of inositol phospholipid metabolism.

INTRODUCTION

Agonist-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] generates at least two second-messenger molecules, inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol (Downes & Michell, 1985; Berridge, 1987). This hydrolysis is catalysed by an inositide-specific phospholipase C whose activation is mediated via a guanine-nucleotide-binding regulatory protein (G-protein). The G-protein(s) (G_p) involved in the regulation of inositol phospholipid hydrolysis remain to be identified, although in certain cell types a pertussis-toxin-sensitive G-protein appears to be involved (see Cockcroft, 1987). Pretreatment of cells such as neutrophils (Verghese *et al.*, 1985) and CCL 39 fibroblasts (Paris & Pouyssegur, 1986) with pertussis toxin results in the ADP-ribosylation of a 40 kDa G-protein and the approx. 50% inhibition of agonist-stimulated PtdIns(4,5)P_2 hydrolysis. Such an effect of pertussis toxin on agonist-promoted PtdIns(4,5)P_2 hydrolysis is not, however, detected when other cell types such as A431 (Pike & Eakes, 1987) and adrenal glomerulosa cells (Enyedi *et al.*, 1986) are treated. These experiments have thus suggested the existence of at least two separate G_p species. It has been proposed that the pertussis-toxin-sensitive G_p might be identical with G_i2 (Backlund *et al.*, 1988), a 'G_i-like' G-protein initially identified in human neutrophils (Goldsmith *et al.*, 1987) and HL60 cells (Falloon *et al.*, 1986).

Lo & Hughes (1987) have shown that pretreatment of Flow 9000 cells with cholera toxin inhibits agonist-stimulated inositol phosphate generation. On the basis of this observation they have proposed the existence of G_c, a novel cholera-toxin-sensitive G-protein coupled to phospholipase C. In order to investigate the roles of various G-proteins in interacting with the inositol phospholipid pathway, we have examined the effects of both pertussis and cholera toxins on vasopressin-stimulated inositol phosphate generation in the L6 rat skeletal-muscle cell line. In this paper we demonstrate that the stimulation of inositol phosphate generation is insensitive to pertussis-toxin treatment of the cells, but that cholera-toxin treatment does lead to an inhibition of vasopressin-stimulated inositol phosphate generation. However, we demonstrate that this effect on inositol phospholipid metabolism is mediated by the ability of cyclic AMP to decrease the availability of vasopressin receptors rather than being due to the involvement of a novel cholera-toxin-sensitive G-protein. The effect of cholera toxin in the L6 cells is thus demonstrated to be via ADP-ribosylation of the α subunit(s) of G_s, which produces a permanent activation of adenylate cyclase and hence elevated intracellular concentrations of cyclic AMP.

MATERIALS AND METHODS

L6 skeletal myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, U.K.) containing 10% (v/v) heat-inactivated foetal-calf serum (Gibco) as previously described (Wakelam *et al.*, 1987). In a number of cases the cells were treated with either pertussis toxin (25 ng/ml) (Porton Products, Porton Down, Wiltshire, U.K.) or

Abbreviations used: G_p, the G-protein mediating activation of phosphoinositidase C; G_s, the G-protein mediating activation of adenylate cyclase; G_i, the G-protein mediating inhibition of adenylate cyclase; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium.

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cholera toxin (100 ng/ml) (Sigma) for 16 h before use. For inositol phospholipid experiments, cells were seeded into 24-well plates, and 18 h before use the medium was changed to inositol-free DMEM (Gibco) containing 10% (v/v) dialysed foetal-calf serum and [^{3}H]inositol (Amersham International, Amersham, Bucks., U.K.). The radioactive concentration was 1 μCi/ml for total phosphate experiments and 10 μCi/ml where the individual phosphates were separated. Cells were used when approx. 95% confluent. After the labelling period, the medium was removed and the monolayers were washed by three 5 min incubations with 1 ml of Hanks buffered saline containing 10 mM-glucose and 1% (w/v) bovine serum albumin (HBG); these and the subsequent incubations were performed with prewarmed buffers at 37 °C. They were then incubated with 1 ml of HBG containing 10 mM-LiCl for 10 min. This buffer was removed and replaced with 250 μl of HBG containing 10 mM-LiCl and the additions stated in the Results section. Incubations were terminated by rapid aspiration of the buffer, followed by addition of 100 μl of ice-cold 10% (v/v) $HClO_4$. After a 15 min incubation on ice, the precipitated cell extract was scraped out of the wells and mixed with a water wash of the well. The cellular debris was removed by centrifugation (14000 *g* for 5 min), the supernatant neutralized by addition of 2M-KOH/0.5 mM-Hepes and the precipitated $KClO_4$ removed by centrifugation. Chromatography on Dowex 1 × 8 was used to separate either the total inositol phosphates or the inositol mono-, bis- and tris-phosphate fractions by the methods of Berridge *et al.* (1982, 1983) as previously described by Wakelam *et al.* (1986).

The binding of vasopressin to cells was performed by using cells grown to confluence on 6-well plates. The culture medium was removed and the cells were incubated for 1 h with Hanks buffered saline containing 1% bovine serum albumin at 37 °C. This medium was removed and the cells were washed with Hanks saline containing 0.2% bovine serum albumin and 10 mM-Hepes, pH 7.3. The monolayers were then incubated on ice with the binding incubation medium (0.8 ml), but with no ligand, for 15 min. The incubation medium contained Hanks buffered saline, 0.05% bovine serum albumin, 10 mM-Hepes, pH 7.3, 10 mM-glucose, 2 mM-bacitracin, 2 mM-glutamine and DMEM amino acids (Gibco). This medium was removed and replaced with complete incubation medium (0.8 ml) containing ligand (0-15 nM) with or without antagonist (3 μM), as described in the Results section. After incubation for 1 h on ice, the medium was removed and the monolayers were washed four times with ice-cold Hanks saline containing 0.1% bovine serum albumin and 10 mM-Hepes, pH 7.3. The cells were then solubilized by addition of 0.5 ml of 0.5 M-NaOH/1% SDS/2% Na_2CO_3. A 0.45 ml portion of this solution was transferred to a scintillation vial containing 0.45 ml of 0.5 M-HCl. Scintillant (Ecoscint; National Diagnostics) was added and the radioactivity associated with the cells determined by liquid-scintillation counting. Cell number was determined by using one well per plate which was treated in the same way, except that no ligand or antagonist was added, and after the final wash the cells were removed from the dishes with trypsin and counted in a Coulter counter. [8-Arginine][*Phe*-3,4,5-^{3}H(n)]vasopressin was obtained from NEN Research Products, Darmstadt, Germany and had a specific radioactivity of 67 Ci/mmol. The vasopressin antagonist [β-mercapto-$\beta\beta$-cyclopentamethylenepropionic acid^1,-*O*-methyl-Tyr^2,Arg^8]vasopressin ([Pmp^1,*O*-Me-Tyr^2,-Arg^8]vasopressin) was obtained from Peninsula Laboratories (St. Helens, U.K.)

ADP-ribosylation

Cholera-toxin-catalysed ADP-ribosylation of membranes of both untreated and cholera-toxin-pretreated L6 cells was performed with [^{32}P]NAD$^+$ (NEN/Dupont), in the absence of GTP, as has previously been described for other cells (Milligan, 1987). Samples were resolved by SDS/PAGE [10% (w/v) acrylamide]. The gels were then dried and autoradiographed for 48 h by using Kodak X O-Mat X-ray film. Cells were harvested from tissue-culture and stored as cell pastes at −80 °C before disruption in 10 mM-Tris/HCl (pH 7.5)/0.1 mM-EDTA (buffer A) with Teflon/glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant from this original centrifugation for a further 10 min at 40000 *g*. The membrane pellet from the second centrifugation was then resuspended in buffer A and stored at −80 °C before use. Protein was assessed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Immunological analysis

Confirmation or denial of the identity of polypeptides which were ADP-ribosylated in response to cholera toxin as the α subunit(s) of G_s was performed by immunoblotting SDS/PAGE-resolved L6-cell membranes with an anti-peptide antiserum, CS1, which was raised against a synthetic peptide corresponding to the *C*-terminal decapeptide (RMHLRQYELL) of the α subunit of G_s. The immunoblotting protocols used have been reported previously (Milligan *et al.*, 1987, McKenzie *et al.*, 1988).

RESULTS

An overnight incubation of L6 cells with pertussis toxin (25 ng/ml) had no significant effect on the stimulation of inositol phosphate generation by vasopressin (Fig. 1). This lack of effect of the toxin was despite the apparent complete ADP-ribosylation of all of the pertussis-toxin substrates within the cells, as assessed by the inability of fresh pertussis toxin in the presence of [^{32}P]NAD$^+$ to incorporate radioactivity into a 40 kDa polypeptide in membranes derived from these cells (results not shown). By contrast, overnight treatment of L6 cells with cholera toxin (100 ng/ml) resulted in a significant decrease in vasopressin-mediated stimulation of inositol phosphate generation. Fig. 1 shows that this inhibition was observed at all vasopressin concentrations examined and that there was no change in the apparent EC_{50} (concn. giving half-maximal stimulation) for vasopressin-stimulated inositol phosphate generation (2.5 nM). The stimulation of inositol trisphosphate generation by vasopressin was also decreased in cells treated with cholera toxin (Fig. 2), demonstrating that the toxin treatment brought about a decrease in vasopressin-stimulated PtdInsP_2 hydrolysis.

Figs. 1 and 2 show that the inhibition of vasopressin-stimulated inositol phosphate generation by cholera-toxin treatment was not complete. When cholera-toxin-catalysed ADP-ribosylation was performed, in the absence of exogenously added guanine nucleotides, on membranes of untreated L6 cells, then three polypeptides

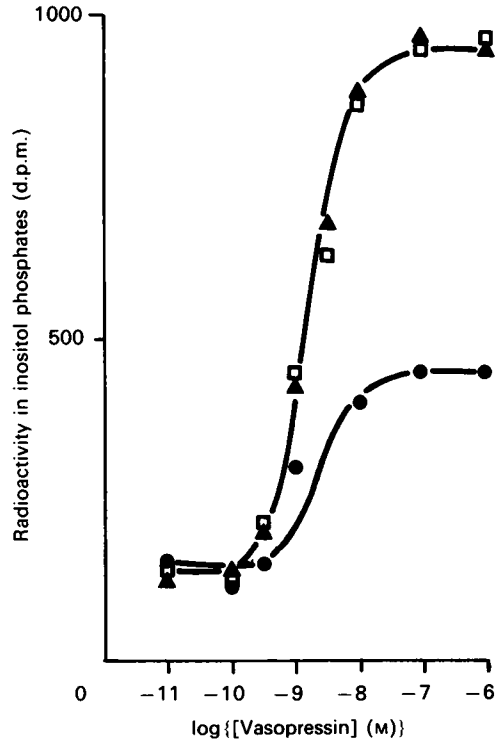

Fig. 1. Effect of pretreatment of L6 cells with either cholera toxin or pertussis toxin on vasopressin-stimulated inositol phosphate generation

The stimulation of total inositol phosphate generation in control, (□), cholera-toxin-treated (100 ng/ml, overnight) (●), and pertussis-toxin-treated (25 ng/ml, overnight) L6 cells (▲) was performed as described in the Materials and methods section. The results represent the means (d.p.m.) for each stimulation and are from one experiment typical of three others; error bars corresponding to the S.D. for each value have been omitted for clarity, but did not exceed 9% in any case.

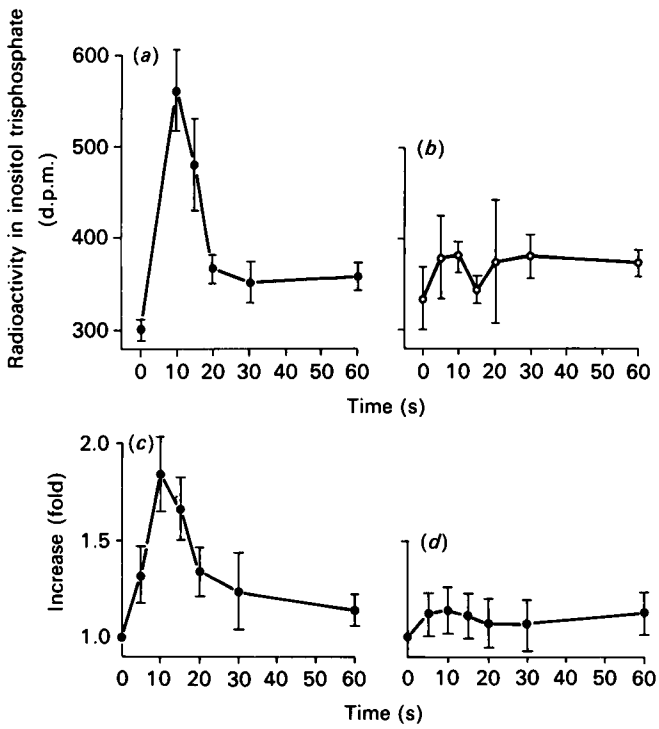

Fig. 2. Stimulation of inositol trisphosphate generation in control and cholera-toxin-treated L6 cells

The stimulation of inositol trisphosphate generation in response to 1 μM-vasopressin was determined as described in the Materials and methods section. The results are means ± S.D. and are from one experiment where $n = 4$ for (*a*) and (*b*). Two further experiments gave qualitatively similar results. Pooled data from all three experiments are presented as fold stimulation in (*c*) and (*d*). No attempt has been made to separate the isomeric forms, and the inositol trisphosphate fraction therefore contains both the 1,4,5 and the 1,3,4 isomers and probably some inositol tetrakisphosphate. (*a*) Control L6 cells; (*b*) L6 cells treated for 16 h with cholera toxin (100 ng/ml); (*c*) fold stimulation of control L6 cells; (*d*) fold stimulation of L6 cells treated for 16 h with cholera toxin (100 ng/ml).

of apparent molecular masses 44, 42 and 40 kDa were identified (Fig. 3). A similar experiment on membranes derived from cholera-toxin-pretreated L6 cells identified only the 40 kDa polypeptide, thus indicating that both the 44 and 42 kDa forms, but not the 40 kDa polypeptide, had been fully ADP-ribosylated *in vivo* with endogenous NAD^+ as substrate (Fig. 3). The 40 kDa band was only a substrate for cholera toxin *in vitro*, in the absence of GTP, and not *in vivo* (Fig. 3) and, as we have previously noted for other systems, this polypeptide represented the α subunit of a form of G_i (Milligan, 1989; Milligan & McKenzie, 1988) (results not shown).

We confirmed the identity of the 44 and 42 kDa polypeptides as forms of the α subunit of G_s by immunoblotting membranes of L6 cells with an anti-peptide antiserum, CS1, which is directed against the *C*-terminal decapeptide of $G_s\alpha$ (Fig. 4).

Treatment of cells with cholera toxin could be envisaged to modulate agonist-stimulated inositol phosphate production by either a cyclic AMP-dependent or -independent mechanism. Table 1 shows the results of experiments designed to test these possibilities. The cholera-toxin effect could not be mimicked by either the isolated A or B subunits of the toxin, but could be by the addition of the diterpene forskolin or by the addition of dibutyryl cyclic AMP. Fig. 5 shows the dose-dependence of forskolin's effect on vasopressin-stimulated inositol phosphate generation, in which the maximal response was decreased by some 75%, but the apparent EC_{50} for the hormone was unaltered (results not shown).

The inhibition of vasopressin-stimulated inositol phosphate generation appears to be a consequence of a decrease in the availability of specific vasopressin-binding sites, therefore probably receptors, on L6 cells as a result of overnight treatment with holomeric cholera toxin, forskolin and dibutyryl cyclic AMP. Fig. 6 illustrates typical Scatchard plots obtained with control, cholera-toxin-treated and forskolin-treated L6 cells. These data show that untreated L6 cells express a single population of high-affinity vasopressin receptors with a K_d value which, between experiments, varied from 3.4 to 8.1 nM. This value is very similar to the EC_{50} value of 2.5 nM obtained for the stimulation of inositol phosphate generation in these cells (Fig. 1). Overnight treatment with either cholera toxin or forskolin had no significant effect on the receptor K_d values (Fig. 6). These treatments did, however, cause a significant decrease in the number of

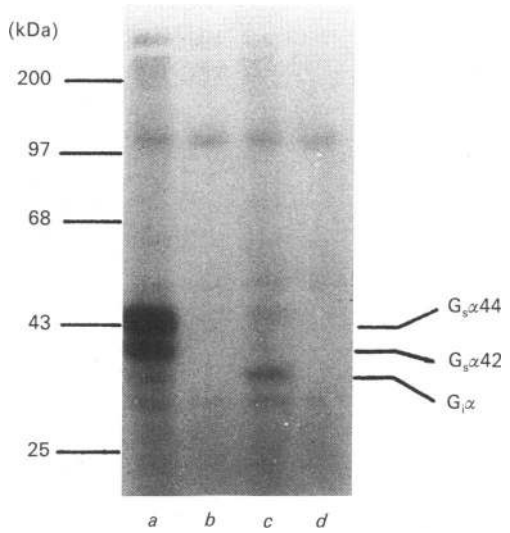

Fig. 3. Cholera-toxin-catalysed ADP-ribosylation of membranes of L6 cells

ADP-ribosylation of membranes of both untreated (*a*, *b*) and cholera-toxin-pretreated (*c*, *d*) L6 cells was performed in the presence (*a*, *c*) or absence (*b*, *d*) of cholera toxin as described in the Materials and methods section and in Milligan (1987). Three polypeptides, of 44,42 and 40 kDa, were specifically identified by the presence of cholera toxin in the untreated membranes (*a* versus *b*). Only the 40 kDa polypeptide was identified in the membranes of cholera-toxin-pretreated cells (*c* versus *d*). Two other polypeptides, of 100 and 30 kDa incorporated radioactivity, but in both cases this was not dependent on the presence of cholera toxin. The apparently greater incorporation of radioactivity into the 30 kDa band in the presence of cholera toxin compared with the absence of toxin (*a* and *c* versus *b* and *d*) is solely due to the darker background of lanes *a* and *c*.

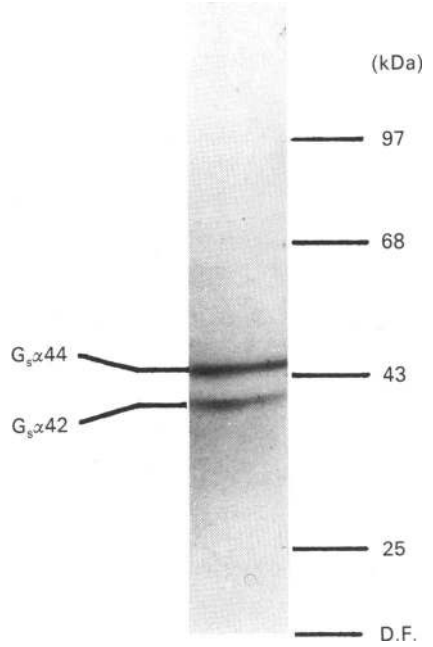

Fig. 4. Identification of two forms of the α subunit of G_s in membranes of L6 cells

A crude membrane preparation (500 μg) of broken L6 cells was resolved on SDS/PAGE [10% (w/v) acrylamide] and immunoblotted by using a 1:200 dilution of antiserum CS1 as primary antibody. The blot was treated and developed as described by McKenzie *et al.* (1988). The antiserum identified two polypeptides of 44 and 42 kDa. The ratios of these two polypeptides (44 > 42) was as noted for cholera-toxin-catalysed ADP-ribosylation (Fig. 3). Abbreviation: D.F., dye front.

available vasopressin-binding sites (71% and 83% decreases in forskolin-treated and cholera-toxin-treated cells respectively). Experiments performed to assess maximum binding using a single concentration of ligand (15 nM) after an overnight treatment with 10 mM-dibutyryl cyclic AMP also demonstrated a significant decrease in vasopressin binding (499 ± 22 d.p.m./10^6 cells in control, 94 ± 10 d.p.m./10^6 cells after dibutyryl cyclic AMP treatment).

DISCUSSION

The results presented in this paper clearly demonstrate that treatment of L6 cells with cholera toxin, but not with pertussis toxin, leads to a decrease in the generation

Table 1. Effect of cholera-toxin subunits and agents which increase intracellular cyclic AMP on vasopressin-stimulated inositol phosphate generation

Cells were treated as stated for 16 h before the stimulation of inositol phosphate generation was determined in response to 1 μM-vasopressin as described in the Materials and methods section. Results are means ± S.D. ($n = 4$), and are from one experiment typical of two others. The inhibition of vasopressin-stimulated inositol phosphate generation after treatment with cholera toxin, forskolin or dibutyryl cyclic AMP was statistically significant ($P < 0.01$). Although a similar trend towards inhibition was noted in all three experiments when the cells were pretreated with cholera toxin B subunit, this effect did not achieve statistical significance.

Treatment of cells	Radioactivity in control (d.p.m.)	Radioactivity with vasopressin (d.p.m.)	Stimulation (fold)
Control	2563 ± 667	10187 ± 1312	3.97
Cholera toxin (100 ng/ml)	3835 ± 770	5290 ± 150	1.38
Cholera toxin A subunit (100 ng/ml)	3410 ± 278	13508 ± 1425	3.96
Cholera toxin B subunit (100 ng/ml)	3400 ± 306	9564 ± 753	2.81
Forskolin (10 mM)	2542 ± 600	3243 ± 18	1.28
Dibutyryl cyclic AMP (10 mM)	2846 ± 182	3707 ± 402	1.30

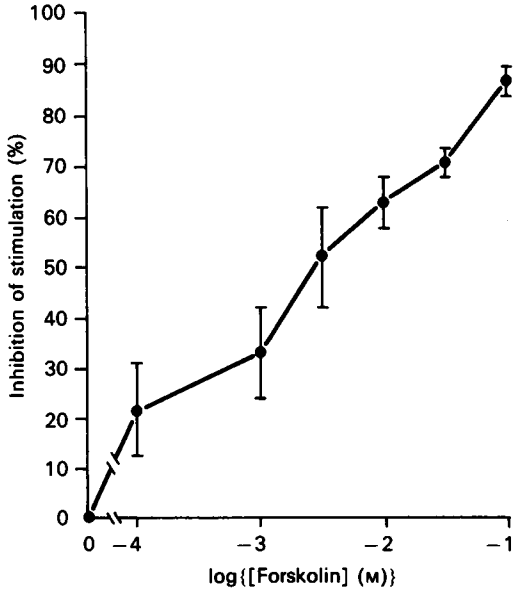

Fig. 5. Concentration-dependence of forskolin pretreatment in inhibiting vasopressin-stimulated inositol phosphate generation

L6 cells were treated for 16 h with increasing concentrations of forskolin. The stimulation of total inositol phosphate generation was determined as described in the Materials and methods section. The results are expressed as the percentage inhibition by each forskolin concentration of the generation of inositol phosphates in response to 1 μM-vasopressin compared to that observed in untreated cells. The results are means ± S.D. and are pooled from two experiments in which $n = 8$ in each case.

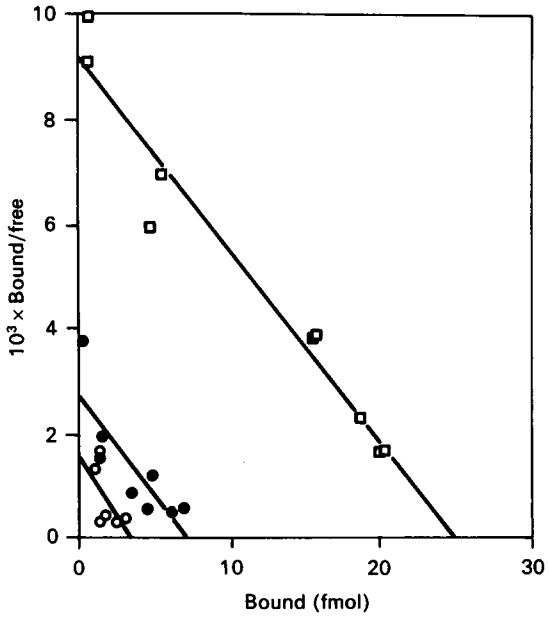

Fig. 6. Scatchard plots of vasopressin binding to L6 cells

The Figure shows the results from one experiment typical of five others. Binding was performed as described in the Materials and methods section. The lines were drawn and binding characteristics were determined by linear regression. Non-specific binding varied from 35% at 0.1 nM to 12% at 15 nM-vasopressin. Better Scatchard plots could not be obtained for cholera-toxin- or forskolin-treated cells, probably because of the extremely small extent of specific binding in these cells. □, Control cells (K_d = 3.4 nM, 16080 receptors/cell); ○, cells treated for 16 h with 100 ng of cholera toxin/ml (K_d = 2.6 nM, 2635 receptors/cell); ●, cells treated with 10 mM-forskolin for 16 h (K_d = 3.3 nM 4630 receptors/cell).

of inositol phosphates in response to vasopressin. The lack of effect of pertussis toxin, under conditions in which all of the available pertussis-toxin substrate(s) had been ADP-ribosylated *in vivo*, demonstrates that in this cell line the vasopressin-receptor-linked G_p is not a substrate for this toxin. The effect of cholera toxin in decreasing vasopressin stimulation of inositol phosphate generation was qualitatively similar to that reported for other agonists in Flow 9000 cells by Lo & Hughes (1987), who interpreted their findings as providing evidence for the existence of a novel cholera-toxin-sensitive G-protein, G_c, involved in the coupling of receptors to phospholipase C. The evidence given in the present paper demonstrates that, at least in the L6 cell line, there is no requirement to postulate a role for a novel G-protein to account for the observed effect of cholera toxin in the inhibition of hormone-stimulated inositol phosphate generation.

Firstly, we have been able to mimic the effects of cholera toxin both with forskolin, an agent which directly activates adenylate cyclase, and with cell-membrane-permeant dibutyryl cyclic AMP (Table 1). It can therefore be concluded that the effect of the toxin is unlikely to be directly on a G-protein involved in the stimulation of phospholipase C, but rather as a result of increased intracellular cyclic AMP. Secondly, cholera toxin is able to catalyse ADP-ribosylation of only two polypeptides, of 44 and 42 kDa, *in vivo* in these cells (Fig. 3). We have confirmed the identify of these two polypeptides as forms of the α subunit of G_s by immunoblotting membranes of the L6 cells with an anti-peptide antiserum raised against the *C*-terminal decapeptide of $G_s\alpha$ (Fig. 4). It is well established that at least two forms of the α subunit of G_s can be generated in a single tissue by differential splicing of transcripts derived from a single gene (Robishaw *et al.*, 1986). For the concept of a role for a novel cholera-toxin substrate involved in the inhibition of hormone-stimulated inositol phosphate generation to have credence, it is necessary that cholera toxin be demonstrated to catalyse the ADP-ribosylation *in vivo* of a polypeptide which is not $G_s\alpha$. A third polypeptide, of 40 kDa, can also be ADP-ribosylated by cholera toxin (Fig. 3), but, as this protein is still ADP-ribosylated by cholera toxin in membranes of cholera-toxin-pretreated cells, then it is not ADP-ribosylated *in vivo*, but only *in vitro*, under the assay conditions used, i.e. in the absence of exogenously added guanine nucleotides. As such it cannot be related to the observed effects of cholera toxin on inositol lipid metabolism. Further, we have previously demonstrated that this pattern of cholera-toxin-catalysed ADP-ribosylation of a 40 kDa polypeptide is characteristic of a form of G_i (Milligan, 1989; Milligan & McKenzie, 1988), and anti-peptide anti-G_i antisera identify a 40 kDa polypeptide in membranes of L6 cells (results not shown).

The results in Table 1 also demonstrate that the effect of cholera toxin is unlikely to be directly on the receptor, as the separated individual toxin subunits had little effect on vasopressin-stimulated inositol phosphate generation. We did note a small, non-significant, decrease in

vasopressin-stimulated inositol phosphate generation after treatment of the cells with cholera toxin B subunit (Table 1). We cannot define the mechanism of this effect, but it may reflect either that the B-subunit preparation was contaminated with a small proportion of the holomeric toxin or that the B subunit was able to hinder directly the access of the hormone to the receptor.

We believe that the effect of a raised intracellular cyclic AMP concentration on vasopressin-stimulated inositol phosphate generation is at the level of the vasopressin receptor. This inhibition noted in response to cholera toxin was not due to any change in receptor affinity for the hormone, as vasopressin displayed both a similar EC_{50} for inositol phosphate generation and a similar K_d as assessed from saturation-binding isotherms in both untreated and cholera-toxin-pretreated cells (Figs. 1 and 5). Further, Fig. 6 shows that, in cells treated either with cholera toxin or with other agents which increase intracellular cyclic AMP, there is a loss of high-affinity vasopressin binding. Although we suggest that is indeed due to a decrease in the number of cell-surface vasopressin receptors, it is possible that the loss of binding could be due to a substantial change in the binding characteristics of a proportion of the receptor population after phosphorylation by protein kinase A. The answer to this question awaits the generation of antibodies capable of immunoprecipitating the vasopressin receptor.

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