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A novel cholera toxin-sensitive G-protein (G_c) regulating receptor-mediated phosphoinositide signalling in human pituitary clonal cells

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Recent studies have implicated that a GTP-binding protein (G-protein) is involved in the coupling of both CCK-8 and muscarinic cholinergic receptors to phosphoinositidase C (PIC) in the human embryonic pituitary cell line, Flow 9000. Pretreatment of these cells with cholera toxin, but not pertussis toxin, inhibited the stimulation of [^{3}H]inositol phosphate production by CCK-8 and acetylcholine. These inhibitory effects of cholera toxin could not be reproduced by treating the cells with the B-subunit of cholera toxin or cAMP-generating agents such as forskolin. These data suggest the presence of a novel G_c protein which is responsible for receptor-PIC coupling in Flow 9000 cells.

Cholera toxin; G-protein; Inositol phosphate; Cholecystokinin; Acetylcholine; (Flow 9000 cell)

1. INTRODUCTION

It is now generally accepted that receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositidase C (PIC) is a common transmembrane signalling mechanism for calcium-mobilizing hormones and neurotransmitters [1,2]. Recent evidence also indicates that a GTP-binding protein (G-protein) is involved in the regulation of receptor-PIC coupling [3–6]. In the human embryonic pituitary cell line Flow 9000, we have reported the presence of functional cholecystokinin (CCK-8) and muscarinic cholinergic receptors. Activation of these receptors leads to the breakdown of PIP_2. In saponin-permeabilized Flow 9000 cells, the non-hydrolysable analogue of GTP, GTP[S], significantly potentiated the PIP_2 responses initiated by CCK-8 and muscarinic agonists implicating the mediation of a G-protein [7].

Reports have accumulated in the past 2 years that suggested at least two G-proteins participate in receptor-PIC coupling. In neutrophils [8], HL-60 cells [9] and human polymorphonuclear leukocytes [10], a pertussis toxin substrate G-protein has been implicated in coupling the chemotactic peptide receptor to PIC. Similar findings were also reported in compound 48/80-stimulated mast cells [11] and thrombin stimulation of PIP_2 hydrolysis in hamster fibroblastic CCL39 cells [12]. In contrast, a G-protein (G_{P1}) which is neither a substrate for pertussis toxin nor cholera toxin regulates PIP_2 responses activated by muscarinic receptors in astrocytoma cells [13] and in cardiac myocytes [14]; TRH receptors in GH_3 cells [15]; angiotensin, vasopressin and α_1-adrenergic recep-

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Abbreviations: G-protein, guanine nucleotide regulatory protein; PIC, phosphoinositidase C; [^{3}H]InsP, [^{3}H]-inositol phosphates; PIP_2, phosphatidylinositol 4,5-bisphosphate; CCK-8, the sulphated form of cholecystokinin octapeptide; GTP[S], guanosine 5′-*O*-(3-thiotriphosphate)

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tors in rat liver membranes [16]. Recently, evidence has accrued that a cholera toxin-sensitive G-protein is involved in receptor-PIC regulation [17,18]. Pike and Eakes [17] showed that cholera toxin was able to abolish the ability of EGF to stimulate an increase in PIP contents in A431 cells. In the T-cell cell line Jurkat, Imboden et al. [18] reported that CT inhibits T-cell antigen receptor-mediated stimulation in both inositol trisphosphate and cytosolic calcium contents.

Here, we have investigated the effects of cholera toxin on CCK-8 and muscarinic stimulation of inositol phosphate accumulation in the human embryonic pituitary cell line Flow 9000.

2. MATERIALS AND METHODS

The human embryonic pituitary cell line Flow 9000 was purchased from Flow Laboratories (Herts, England). Cells were cultured in Ham's F10 medium in the presence of sera [6]. Procedures for [^{3}H]inositol prelabelling and agonist stimulation of the cells as well as the extraction and separation of [^{3}H]inositol phosphates by anion-exchange chromatography were as described [19]. Cholera toxin was added only after the cells had been labelled with [^{3}H]inositol to isotopic equilibrium (48 h) for either 5 or 24 h. Cyclic AMP (cAMP) production induced by cholera toxin treatment with or without the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), was determined by radioimmunoassay using the ^{125}I-cAMP-RIA kit purchased from New England Nuclear. Labelled or pretreated cells were usually washed thoroughly with physiological buffer (Krebs bicarbonate buffer supplemented with glucose) before addition of drugs. CCK-8 (sulphated) was obtained from Bachem. GTP[S] and forskolin were purchased from Calbiochem. Cholera toxin and its A- and B-subunits as well as pertussis toxin were obtained from List Biologicals (Campbell, CA).

3. RESULTS

Initial experiments established that cholera toxin used in the present study was biologically active as shown by its ability to stimulate cAMP production in intact Flow 9000 cells (table 1). The phosphodiesterase inhibitor IBMX (0.5 mM) greatly amplified cAMP formation elicited by cholera toxin treatment (50 μg/ml, 5 h). These results indicate that at the concentration used here, cholera toxin is able to stimulate cAMP accumulation presumably through ADP-ribosylating the G_s-protein of the adenylate cyclase pathway. It is also worth pointing out that cholera toxin pretreatment (5 or 24 h) had no effect on the morphology (at light-microscopic level) and the protein content of the cells.

Table 1

Effects of cholera toxin treatment on cAMP accumulation in Flow 9000 cells

	cAMP (pmol/10^6 cells)	
	− IBMX	+ IBMX
Control	12.1 ± 0.3	47.4 ± 2.5
Cholera toxin	45.9 ± 2.3	774.9 ± 29.6

Flow 9000 cells were incubated with cholera toxin (50 μg/ml) for 5 h and then 0.5 mM IBMX was added to one set of control and cholera toxin-treated incubations for another 30 min. Reaction was terminated by ice-cold trichloroacetic acid (6%) followed by ether extraction and lyophilization to remove the acid. Radioimmunoassay was used to determine the cAMP content. Data are means ± SE of three experiments performed in triplicate

However, pretreatment of the Flow 9000 cells with 50 μg/ml cholera toxin for just 5 h significantly inhibited accumulation of total [^{3}H]-inositol phosphate ([^{3}H]InsP) induced by CCK-8 and acetylcholine but had no effect on basal levels (table 2). This inhibition of cholera toxin on agonist-mediated PIP_2 breakdown was dose-dependent and the IC_{50} value of cholera toxin on CCK-8 stimulation of [^{3}H]InsP production was 2.8 ± 0.9 μg/ml. It is significant that even after 24 h incubation, the maximal inhibition of cholera toxin on agonist-induced PIP_2 turnover was only 65%.

Pertussis toxin did not affect CCK-8 and muscarinic agonist stimulation of [^{3}H]InsP in these cells (table 3). However, at a similar concentration, pertussis toxin (50 μg/ml, 24 h) significantly blocked muscarinic inhibition of prostaglandin E_2 (PGE_2) stimulation of adenylate cyclase activity in the same cell line (not shown). In addition, pertussis toxin did not alter GTP[S]-induced [^{3}H]-

Table 2

Effects of cholera toxin on agonist-induced [^{3}H]inositol phosphate accumulation in Flow 9000 cells

	[^{3}H]InsP (dpm/assay)	
	Control cells	CT-treated cells
Control	1113 ± 28	1005 ± 48
CCK-8 (1 nM)	4159 ± 50	2018 ± 29[a]
Acetylcholine (30 μM)	5254 ± 65	2978 ± 38[a]

[a] $P<0.05$ vs agonist stimulation in control cells

[^{3}H]Inositol-prelabelled Flow 9000 cells were treated with cholera toxin (CT) at 50 μg/ml for 5 h. After thorough washings, cells were stimulated with agonists for 30 min and total [^{3}H]inositol phosphates ([^{3}H]InsP) were extracted as described in section 2. Data are means ± SE of 3 independent experiments

InsP formation in saponin-permeabilized Flow 9000 cells (table 3).

As mentioned above, at cholera toxin concentrations that inhibited agonist-mediated PIP_2 hydrolysis, there was a concomitant increase in accumulation of cAMP in Flow 9000 cells. Accordingly, we have tested the ability of the cAMP-generating agents, noradrenaline and forskolin, to modify agonist-induced [^{3}H]InsP production. Using a concentration of these agents which stimulated cAMP formation maximally, neither of these agents showed any effect on CCK-8 or acetylcholine stimulation of PIP_2 hydrolysis (table 4). Table 4 also shows that neither the A- nor B-subunit of cholera toxin affects receptor-mediated PIC activation.

Table 3

Effects of pertussis toxin (PT) pretreatment on [^{3}H]InsP formation in Flow 9000 cells

	[^{3}H]InsP (dpm/assay)	
	Control	PT-treated
Intact cells		
Control	1125 ± 90	1015 ± 98
CCK-8 (1 nM)	5880 ± 605	5775 ± 512
ACh (30 μM)	5525 ± 220	5254 ± 120
Permeabilized cells		
Control	1072 ± 90	1094 ± 75
GTP[S] (100 μM)	2130 ± 150	2075 ± 98

[^{3}H]Inositol-prelabelled Flow 9000 cells were preincubated with pertussis toxin (50 μg/ml) for 24 h. Cells were washed thoroughly and were then challenged with either CCK-8 (1 nM) or ACh (30 μM) for 30 min. Total [^{3}H]InsP were extracted and quantified as described in section 2. Results are means ± SE of four separate experiments performed in duplicate

Table 4

Effects of cholera toxin and various agents on agonist-induced [^{3}H]InsP formation in Flow 9000 cells

Pretreatment	[^{3}H]InsP (dpm/assay)		
	Control	CCK-8 (1 nM)	Acetylcholine (33 μM)
Control	1037 ± 38	4298 ± 99	5269 ± 76
Cholera toxin (50 μg/ml)	970 ± 99	2011 ± 23[a]	2998 ± 60[a]
A-Subunit (2.5 μg/ml)	1002 ± 34	4400 ± 45	5055 ± 79
B-Subunit (20 μg/ml)	1009 ± 59	4459 ± 89	5301 ± 78
Noradrenaline (10 μM)	999 ± 67	4502 ± 101	5405 ± 56
Forskolin (33 μM)	1012 ± 56	4499 ± 77	5098 ± 40

[a] $P<0.05$ vs agonist stimulation in control cells

[^{3}H]Inositol-prelabelled Flow 9000 cells were pretreated with various agents for 24 h. Procedures for agonist stimulation (30 min) and [^{3}H]inositol phosphate extraction were as described in section 2. Data are means ± SE of 4 separate experiments

Table 5

Effects of cholera toxin on phosphoinositide hydrolysis in saponin-permeabilized Flow 9000 cells

	[^{3}H]Insp (dpm/assay)	
	Control cells	CT-treated cells
Control	1069 ± 80	1001 ± 56
GTP[S] (100 μM)	2233 ± 54	2166 ± 98
CCK-8 (1 μM)	1234 ± 99	1188 ± 43
GTP[S] + CCK-8	4116 ± 105	2608 ± 150[a]

[a] $P<0.05$ vs agonist stimulation in control cells

[^{3}H]Inositol-labelled Flow 9000 cells were treated with cholera toxin (CT, 50 μg/ml) for 24 h. CT-treated cells were incubated with saponin (50 μg/ml) for 15 min. After thorough washings, CT-pretreated saponin-permeabilized cells were then subjected to agonist stimulation for 30 min and [^{3}H]inositol phosphates were extracted. Results are means ± SE of 3 separate experiments

To elucidate the site of action of cholera toxin on receptor-PIC coupling, we have investigated the effect of cholera toxin on GTP[S] stimulation of PIP_2 turnover as well as the potentiation of CCK-8-activated [^{3}H]InsP formation by GTP[S] in permeabilized Flow 9000 cells. Table 5 shows that cholera toxin (50 μg/ml, 24 h) did not affect GTP[S]-stimulated [^{3}H]InsP accumulation but significantly reduced the GTP[S]-potentiated CCK-8 response. These results suggest that the site of action of cholera toxin on the G_c protein is neither the GTP- nor PIC-binding site. Preliminary results showed that both the maximal binding capacity (B_{max}) and the apparent dissociation constant (K_d) of saturation binding of [^{3}H]QNB and ^{125}I-CCK-8 in intact Flow 9000 cells were not significantly altered by cholera toxin pretreatment (not shown) indicating that the primary effect of cholera toxin is not at the receptor level.

4. DISCUSSION

Previous reports have indicated that there are at least two classes of G-proteins involved in receptor-PIC coupling. One is pertussis toxin-sensitive and therefore could be G_o, G_i or a G_o/G_i-like protein and is predominantly present in bone marrow-derived cells such as neutrophils. The other is not toxin-sensitive (G_{PI}) and is present in a great variety of tissues. Our studies suggest that a novel G-protein, which we tentatively named G_c (c - cholera) regulates agonist-induced PIP_2 hydrolysis in Flow 9000 cells. This G_c protein is pertussis toxin-insensitive. The mechanism of the inhibitory action of cholera toxin on receptor-PIC coupling is at present not clear. We are tempted to suggest that ADP-ribosylation of G_c is essential but experiments using [^{32}P]NAD have yet to be performed to show that cholera toxin ADP-ribosylates a substrate protein in these cells. In WRK-1 mammary tumour cells, vasopressin-activated PIP_2 hydrolysis is mediated via a G-protein [20]. Pretreatment of WRK-1 cells with cholera toxin, but not with pertussis toxin, produced a dose-dependent inhibition of vasopressin-stimulated inositol phosphate formation (Kirk, C. and Michell, R., personal communication). This finding further supports the role of a cholera toxin-sensitive G-protein in receptor-activated PIP_2 metabolism.

One intriguing finding was that cholera toxin treatment did not affect GTP[S]-stimulated PIP_2 hydrolysis in permeabilized cells (table 5). Thus, the site of ADP-ribosylation appears to be distal from both GTP- and PIC-binding sites. In contrast, the potentiating effect of GTP[S] on CCK-8 stimulation of [^{3}H]InsP production in permeabilized cells was abolished by cholera toxin (table 5). These results suggest that ADP-ribosylation of G_c by cholera toxin has in some way impaired the coupling between the receptor and G_c. Since cholera toxin does not completely block the PIP_2 response, it is unlikely that complete uncoupling between receptor and PIC occurs. Lotersztajn et al. [21] have observed that cholera toxin treatment blocked glucagon-mediated inhibition of (Ca^{2+}-Mg^{2+})-ATPase in liver plasma membranes, this being consistent with a possible role of cholera toxin-sensitive substrate in regulating transmembrane processes.

In conclusion, we propose that a novel G-protein (G_c) mediates receptor-PIC coupling in Flow 9000 cells. The wider implications of this observation require further study as does the exact mechanism of action of cholera toxin on the coupling process.

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REFERENCES

[1] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[2] Berridge, M.J. (1984) Biochem. J. 220, 345–360.
[3] Cockcroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
[4] Litosch, I., Wallis, C. and Fain, J.N. (1985) J. Biol. Chem. 260, 5464–5471.
[5] Lo, W.W.Y., Clark, C.R. and Hughes, J. (1986) Biochem. Soc. Trans. 14, 1135–1136.
[6] Taylor, C.W., Merritt, J.E., Putney, J.W. jr and Rubin, R.P. (1986) Biochem. Biophys. Res. Comm. 136, 362–368.
[7] Clark, C.R., Hughes, J. and Lo, W.W.Y. (1987) Br. J. Pharmacol. 90, 75P.
[8] Volpi, M., Naccache, P.H., Molski, T.F.P., Shefcyk, J., Huang, C.-K., Marsh, M.L., Munoz, J., Becker, E.L. and Sha'afi, R.I. (1985) Proc. Natl. Acad. Sci. USA 82, 2708–2712.

[9] Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558-11562.
[10] Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875-5878.
[11] Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584-3593.
[12] Paris, S. and Pouyssegur, J. (1986) EMBO J. 5, 55-60.
[13] Hepler, J.R. and Harden, T.K. (1986) Biochem. J. 239, 141-146.
[14] Masters, S.B., Martin, M.W., Harden, T.K. and Brown, J.H. (1985) Biochem. J. 227, 933-937.
[15] Straub, R.E. and Gershengorn, M.C. (1986) J. Biol. Chem. 261, 2712-2717.
[16] Lynch, C.J., Prpic, V., Blackmore, P.F. and Exton, J.H. (1986) J. Pharmacol. Exp. Ther. 29, 196-203.
[17] Pike, L.J. and Eakes, A.T. (1987) J. Biol. Chem. 262, 1644-1651.
[18] Imboden, J.B., Shoback, D.M., Pattison, G. and Stobo, J.D. (1986) Proc. Natl. Acad. Sci. USA 83, 5673-5677.
[19] Lo, W.W.Y., Clark, C.R. and Hughes, J. (1986) Biochem. Soc. Trans. 14, 1108-1109.
[20] Guillon, G., Balestre, M.N., Mouillae, B. and Devilliers, G. (1986) FEBS Lett. 196, 155-159.
[21] Lotersztajn, S., Pavoine, C., Mallat, A., Stengel, D., Insel, P.A. and Pecker, F. (1987) J. Biol. Chem. 262, 3114-3117.