Guanine nucleotide regulation of agonist binding to muscarinic cholinergic receptors

Relation to efficacy of agonists for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization

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The efficacies of a series of six muscarinic cholinergic receptor agonists for stimulation of phosphoinositide breakdown and unidirectional efflux of $^{45}\text{Ca}^{2+}$ in 1321N1 human astrocytoma cells were compared with the relative capacity of these agonists for formation of a GTP-sensitive high-affinity binding state in washed membranes. Carbachol and methacholine were 'full' agonists as regards phosphoinositide breakdown and Ca^{2+} mobilization, whereas bethanechol, arecoline and oxotremorine were 'partial' agonists for these two responses. Pilocarpine was the least efficacious of the six drugs tested. Except for pilocarpine, competition curves generated with the agonists and [^3H]quinuclidinyl benzilate did not follow the Law of Mass Action for ligand interaction at a single site. Non-linear regression analyses of these data indicated that the data significantly better fit a two-, rather than a single-, site model with a high- and a low-affinity binding component. Competition curves generated in the presence of GTP were shifted to the right, and the extent of receptors in the high-affinity agonist-binding state was decreased. The relative efficacies of the six agonists for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization were significantly correlated with the difference in affinities (K_L/K_H) between the two affinity states for each agonist. The relative efficacy of the agonists for stimulation of Ca^{2+} mobilization also was significantly correlated with the extent of receptors in the high-affinity state (% H) for each agonist. The results suggest that interaction with an as-yet unidentified guanine nucleotide regulatory protein is important in the mechanism whereby muscarinic receptors stimulate phosphoinositide breakdown in 1321N1 astrocytoma cells.

INTRODUCTION

Cellular responses to muscarinic cholinergic stimuli have been shown to be associated with both decreases in intracellular cyclic AMP (Murad *et al.*, 1962; Matsuzawa & Nirenberg, 1975) and increases in phosphoinositide turnover and Ca^{2+} mobilization (Michell, 1975; Michell *et al.*, 1976; Brown & Brown, 1984; Masters *et al.*, 1984). Probably the most clearly defined component of cholinergic action involves the mechanism whereby receptor stimulation results in an inhibition of adenylate cyclase. That is, a guanine nucleotide regulatory protein, N_i , has been shown to couple muscarinic and other inhibitory receptors to adenylate cyclase (Gilman, 1984). This guanine nucleotide regulatory protein can be labelled in the presence of pertussis toxin and [^{32}P]NAD $^+$ (Katada & Ui, 1982; Bokoch *et al.*, 1983), it has been purified to homogeneity (Bokoch *et al.*, 1984; Codina *et al.*, 1984), and putative sets of reactions involved in the interaction of its subunits with other components of the hormone-responsive adenylate cyclase have been proposed (Gilman, 1984; Katada *et al.*, 1984; Hildebrandt *et al.*, 1984).

Although much less is known about muscarinic-receptor-mediated regulation of Ca^{2+} mobilization, compelling evidence has been presented implicating the breakdown of phosphoinositides as a step in receptor-mediated elevation of cytoplasmic [Ca^{2+}] (Michell *et al.*, 1981; Berridge, 1984). The mechanisms whereby agonist-receptor interaction results in stimulation of the phospholipase C-mediated breakdown of phosphoinositides remain unknown.

Activation of muscarinic receptors on 1321N1 human astrocytoma cells attenuates cyclic AMP accumulation (Meeker & Harden, 1982). This response does not involve inhibition of adenylate cyclase (Meeker & Harden, 1982; Hughes *et al.*, 1984), but rather occurs as a consequence of activation of a Ca^{2+} -calmodulin-dependent phosphodiesterase (Tanner *et al.*, 1985). The ability of cholinergic agonists to stimulate phosphodiesterase apparently results from the stimulatory effect of cholinergic agonists on phosphoinositide breakdown and Ca^{2+} mobilization in this cell line (Masters *et al.*, 1984).

The binding of agonists to muscarinic receptors in washed membranes from 1321N1 cells is regulated by guanine nucleotides (Evans *et al.*, 1985). Guanine

Abbreviations used: N_i , the guanine nucleotide regulatory protein that couples inhibitory receptors to adenylate cyclase; N_s , the guanine nucleotide regulatory protein that couples stimulatory receptors to adenylate cyclase; QNB, quinuclidinyl benzilate; Ins1P, *myo*-inositol 1-phosphate; PSS, 118 mM-NaCl, 4.7 mM-KCl, 3.0 mM- CaCl_2 , 1.2 mM- MgSO_4 , 1.2 mM- KH_2PO_4 , 0.5 mM-EDTA, 10 mM-glucose, 20 mM-Hepes, pH 7.4.

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nucleotides also regulate agonist binding to α_1 -adrenergic (Boyer *et al.*, 1984), thyroliberin (Hinkle & Kinsella, 1984) and fMet-Leu-Phe (Snyderman, 1984) receptors, although these receptors, like the cholinergic receptors of 1321N1 cells, act through mechanisms other than regulation of adenylate cyclase. In addition, a role for GTP has been proposed in regulation of Ca^{2+} -dependent effects (Gomperts, 1983; Haslam & Davidson, 1984) and more recently for receptor-mediated breakdown of phosphoinositides (Litosch *et al.*, 1985; Cockcroft & Gomperts, 1985). We show in the current work that the relative capacity of cholinergic agonists to form a GTP-sensitive high-affinity binding state is related to their efficacy for stimulation of phosphoinositide breakdown and $^{45}\text{Ca}^{2+}$ mobilization. The work implies that a guanine nucleotide regulatory protein subserves a function in coupling muscarinic receptors to phosphoinositide breakdown, and hence to increases in diacylglycerol concentrations and the cytoplasmic concentration of Ca^{2+} .

MATERIALS AND METHODS

Materials

Drugs, culture medium and supplies were obtained from sources previously described (Evans *et al.*, 1984, 1985).

Muscarinic cholinergic receptor assay

Competition binding experiments, with [^3H]QNB (20–25 pM) as the radioligand, were performed essentially as previously described (Evans *et al.*, 1985). Assays were in 10 ml of 10 mM-Hepes (pH 7.5)/5 mM- MgCl_2 , and incubations were carried out at 37 °C for 90 min. Binding reactions were initiated by the addition of washed membranes and terminated by filtration through Schleicher and Schuell no. 30 glass-fibre filters. Each filter was washed with 10 ml of 10 mM-Tris (pH 7.5)/145 mM-NaCl. Non-specific binding was defined as the amount of radioligand bound in the presence of 1 μM -atropine and represented < 5% of the total radioligand retained by the filters. Conditions were modified so that < 15% of the total added radioligand was bound in all experiments. All assays were performed in triplicate. Agonist-binding curves were analysed by computerized non-linear least-squares-regression analysis as previously described (Martin *et al.*, 1984). %H is the percentage of receptors found in a high-affinity binding state for agonists; K_H and K_L respectively refer to the affinity of an agonist for the high- and low-affinity component of binding curves resolved by computer analysis of curves that better fit a two-site model.

Phosphoinositide assays

Phosphoinositide hydrolysis was monitored by measuring the accumulation of the phosphoinositide metabolite [^3H]Ins1P, in the presence of LiCl, an inhibitor of the enzyme that converts Ins1P into inositol (Berridge *et al.*, 1982). Phosphoinositides were radio-labelled by incubating 1321N1 cell monolayers with 1.0 ml of growth medium containing 1.0 μCi of *myo*-[2- ^3H]inositol (New England Nuclear)/ml for 18–20 h. Just before the assay, the monolayers were washed with PSS, and assays were initiated by the addition of warmed PSS containing LiCl (10 mM) and drugs. To terminate the incubation (10 min), the drug

solution was rapidly removed by aspiration, the monolayer was washed with 3×1.5 ml of PSS, and 0.5 ml of cold methanol was added. Cells were removed from the dishes by scraping and each dish was rinsed with an additional 0.5 ml of cold methanol. Then 500 μl of chloroform and 0.4 ml of water were added to the pooled methanol washes to give a final solution of chloroform/methanol/water (5:10:4, by vol.), and the samples were sonicated for 15 s with a Kontes cell disrupter set at the maximal power setting. An additional 0.5 ml each of chloroform and water were added, and the resulting two-phase solution was clarified by centrifugation at 1500 g for 15 min at 4 °C.

Ins1P was determined as described previously (Brown & Brown, 1983). Briefly, 1.6 ml of the upper (aqueous) phase was transferred to a column containing approx. 175 mg of anion-exchange resin (Bio-Rad AG 1-X8; 100–200 mesh; formate form). Columns were washed with 10 ml of water to remove [^3H]inositol. Labelled Ins1P was eluted with 8 ml of 200 mM-ammonium formate/100 mM-formic acid and was counted for radioactivity in an equal volume of Liquiscint (National Diagnostics).

Unidirectional $^{45}\text{Ca}^{2+}$ efflux

Muscarinic-receptor-mediated Ca^{2+} mobilization was measured as previously described (Masters *et al.*, 1984). Cells were equilibrated with $^{45}\text{Ca}^{2+}$ by incubation with 1.0 ml of growth medium containing 5 μCi of $^{45}\text{CaCl}_2$ (Amersham)/ml for 18–20 h. This period of labelling allowed the cellular $^{45}\text{Ca}^{2+}$ to reach a steady state (achieved by 16 h). To initiate the efflux assay, the growth medium containing extracellular $^{45}\text{Ca}^{2+}$ was removed by aspiration, monolayers were quickly (< 8 s) washed three times with Hepes (20 mM, pH 7.5)-buffered Eagle's medium (37 °C) without $^{45}\text{Ca}^{2+}$, and Hepes-buffered Eagle's medium (37 °C) containing cholinergic agonists was added. Incubations were terminated after 1 min by rapidly washing the monolayers four times with 3 ml of a solution containing 5 mM- LaCl_3 , 150 mM-NaCl and 1.5% (w/v) bovine serum albumin. Cells were solubilized in 1.0 ml of 0.1 M-NaOH, the samples were neutralized with 1.0 ml of 0.1 M-HCl, and radioactivity was determined by scintillation counting in 6 ml of Budget-Solve (RPI). Under the conditions used for measurement of effects of muscarinic receptor agonists, $^{45}\text{Ca}^{2+}$ efflux approximated a first-order exponential with only a single kinetic component (Masters *et al.*, 1984).

RESULTS

Previous work with chick heart cells (Brown & Brown, 1984), NG108-15 neuroblastoma $\times$ glioma cells (Evans *et al.*, 1984) and other tissues (Jakobs *et al.*, 1979; Olinas *et al.*, 1983) has indicated that the muscarinic receptor agonists oxotremorine and carbachol are equally efficacious as inhibitors of adenylate cyclase activity. In contrast, carbachol is much more efficacious than oxotremorine for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization (Fisher *et al.*, 1983; Masters *et al.*, 1984; Brown & Brown, 1984). Concentration-effect curves were generated for six cholinergic agonists to determine their relative capacities for stimulation of Ins1P formation and $^{45}\text{Ca}^{2+}$ mobilization in 1321N1 cells (results not shown). As illustrated in Table 1, a wide range of relative efficacies was observed when maximally

Table 1. Relative efficacies of muscarinic-receptor agonists for stimulation of phosphoinositide breakdown and $^{45}\text{Ca}^{2+}$ efflux

Phosphoinositide breakdown and $^{45}\text{Ca}^{2+}$ efflux were measured in response to a maximally effective concentration (0.1–1.0 mM) of the indicated agonists. The responses to agonists in each experiment were compared with that observed with carbachol (100%). The data are means $\pm$ S.E.M. for three experiments.

Agonist	Phosphoinositide breakdown (%)	$^{45}\text{Ca}^{2+}$ efflux (%)
Carbachol	100	100
Methacholine	86 ± 1	88 ± 5
Bethanecol	49 ± 2	75 ± 2
Arecoline	26 ± 2	64 ± 2
Oxotremorine	11 ± 1	45 ± 1
Pilocarpine	2 ± 1	13 ± 5

effective concentrations of these agonists were examined for their capacity to stimulate phosphoinositide breakdown. Methacholine and carbachol exhibited similar efficacies, whereas arecoline and bethanecol, like oxotremorine, were partial agonists compared with carbachol. Pilocarpine did not measurably increase InsIP accumulation.

The relative efficacy of these six agonists for stimulation of $^{45}\text{Ca}^{2+}$ efflux also was measured (Table 1) in 1321N1 cells. Again, carbachol and methacholine were

the most efficacious agonists, whereas oxotremorine, bethanecol, arecoline and pilocarpine were partial agonists for stimulation of Ca^{2+} efflux.

As we have previously reported (Evans *et al.*, 1984), carbachol inhibits $[^3\text{H}]\text{QNB}$ binding with high affinity in 1321N1 cell membranes. The competition curve for carbachol is steepened and markedly shifted to the right by the addition of $100\ \mu\text{M}$ -GTP (Fig. 1) or by guanosine 5'-[γ -thio]triphosphate, GDP or guanosine 5'-[$\beta\gamma$ -imido]-triphosphate (Evans *et al.*, 1985). Analogous data were obtained with the agonist methacholine (Fig. 1): that is, relatively flat competition curves were obtained in incubations carried out in the absence of GTP (Fig. 1). The addition of guanine nucleotide steepened and markedly shifted the methacholine competition curve to the right. In contrast with the results obtained with the full agonists methacholine and carbachol, very little regulation by guanine nucleotide of the binding of oxotremorine to 1321N1-cell muscarinic receptors occurred. No reproducible effect of guanine nucleotides was observed in competition curves carried out with pilocarpine (Fig. 1).

A large series of competition curves similar to those illustrated in Fig. 1 was analysed for six agonists in accordance with a two-site model as described in the Materials and methods section. Competition curves for carbachol, methacholine, arecoline, bethanecol and oxotremorine in the absence of GTP were significantly better fitted by a two-site model (Table 2). Pilocarpine competition curves were not better fitted to a two-site model. The presence of GTP markedly decreased the

Fig. 1. Regulation of agonist interaction with muscarinic receptors by GTP

Washed membranes from 1321N1 cells were incubated in the presence of $20\ \text{pM}$ - $[^3\text{H}]\text{QNB}$, the indicated concentration of agonists, and in the absence (●) or presence (■) of $100\ \mu\text{M}$ -GTP.

Table 2. Determination of agonist affinity states for muscarinic cholinergic receptors

Agonist competition curves were generated with 1321N1 cell membranes and [^3H]QNB (20–25 pM) in the presence or absence of GTP (100 μM) or guanosine 5'-[thio]triphosphate (1 μM). Competition curves (see Fig. 1) were analysed by non-linear least-squares curve fitting of the raw data. The Cheng & Prusoff (1973) approximation was utilized to convert IC_{50} values into K_{H} and K_{L} values. To determine if the data were fitted significantly better to a two-site model, the residual sums of squares of the respective fits were compared by a partial F test. %H and %L values are means $\pm$ S.E.M. ($n = 3-8$). Values for K_{H} and K_{L} are in μM . The ranges of experimental values are provided in parentheses.

Agonist	No GTP					+ GTP			
	K_{H}	%H	K_{L}	%L	$K_{\text{L}}/K_{\text{H}}$	K_{H}	%H	K_{L}	%L
Carbachol	0.15 (0.07–0.29)	41 ± 2	6.8 (3.6–9.9)	59 ± 2	44	0.83 (0.46–1.8)	16 ± 5	13 (8–14)	84 ± 5
Methacholine	0.10 (0.05–0.14)	43 ± 3	3.6 (3.0–5.2)	57 ± 2	37	0.59 (0.28–0.93)	18 ± 7	5.6 (3.3–8.7)	82 ± 7
Bethanecol	2.90 (1.2–4.5)	58 ± 3	106 (43–182)	42 ± 3	37	0.53 (0.33–0.73)	10 ± 7	114 (96–133)	90 ± 7
Arecoline	0.18 (0.13–0.24)	36 ± 2	4.4 (1.5–7.1)	64 ± 2	24	0.20 (0.05–0.35)	17 ± 5	8.6 (8.2–11)	83 ± 5
Oxotremorine	0.013 (0.009–0.022)	25 ± 8	0.22 (0.19–0.29)	75 ± 8	17	—	0	0.21 (0.15–0.28)	100
Pilocarpine	—	0	0.36 (0.31–0.45)	100	—	—	0	0.43 (0.36–0.56)	100

Fig. 2. Relationship between the ratio $K_{\text{L}}/K_{\text{H}}$ and efficacy of agonists for stimulation of Ca^{2+} efflux and phosphoinositide breakdown

The ratio of dissociation constants for low- and high-affinity binding states determined by non-linear-regression analysis of competition binding curves for the indicated agonists is plotted versus the efficacy of these agonists (relative to carbachol) for stimulation of Ca^{2+} efflux (a) or phosphoinositide breakdown (b). Least-squares linear-regression analysis was used to determine correlation between the ratio $K_{\text{L}}/K_{\text{H}}$ and biochemical responses. Abbreviations: CAR, carbachol; MET, methacholine; BET, bethanecol; ARE, arecoline; OXO, oxotremorine; PIL, pilocarpine.

percentage of receptors in the high-affinity form in the presence of carbachol, methacholine, bethanecol, arecoline or oxotremorine (Table 2); oxotremorine competition curves in the presence of GTP were no longer better fitted by a two-site model (Table 2).

As illustrated in Fig. 2(a), there was excellent correlation ($P < 0.001$) between the ratio $K_{\text{L}}/K_{\text{H}}$ for six

agonists and their capacity to stimulate $^{45}\text{Ca}^{2+}$ efflux. Although the correlation was somewhat less striking, the efficacy for stimulation of phosphoinositide breakdown was also proportional ($P < 0.05$) to the value of $K_{\text{L}}/K_{\text{H}}$ (Fig. 2b). The correlation between the capacity of these agonists to form a high-affinity-binding complex (% H) and their capacity to stimulate Ca^{2+} mobilization and

Fig. 3. Relationship between the percentage of receptors in a high-affinity guanine-nucleotide-sensitive binding state and the efficacy of agonists for stimulation of Ca^{2+} efflux and phosphoinositide breakdown

The percentage of receptors in a high-affinity binding state (%H) was determined for a series of agonist competition curves as described in the Materials and methods section. This value is plotted versus the efficacy of the indicated agonists for stimulation of Ca^{2+} efflux (a) or phosphoinositide breakdown (b). Least-squares linear-regression analysis was used to determine correlation between %H and biochemical responses. For abbreviations, see Fig. 2 legend.

phosphoinositide breakdown was also examined (Fig. 3a). There was a significant correlation ($P < 0.05$) between %H and the capacity of agonists to stimulate $^{45}\text{Ca}^{2+}$ efflux, but not between %H and phosphoinositide breakdown (Fig. 3b).

DISCUSSION

Although there is evidence strongly implicating the breakdown of phosphoinositides and the generation of inositol 1,4,5-trisphosphate as crucial steps in receptor-mediated mobilization of intracellular Ca^{2+} , the molecular mechanisms involved in this process are largely unknown (Michell *et al.*, 1981; Berridge, 1984). This is particularly true for the steps in the plasma membrane that immediately result from the initial agonist-receptor interaction. Thus, whereas the molecular events involved in agonist-induced coupling of muscarinic receptors to adenylate cyclase at least tentatively can be assigned (Gilman, 1984), the events subserving coupling of these receptors to the phospholipase C involved in phosphoinositide metabolism are not yet understood.

The current work suggests that an important relationship exists between the efficacies of agonists for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization and the formation of a guanine-nucleotide-sensitive high-affinity-binding complex. The most convincing correlation was observed when the affinity ratios of the high- and low-affinity forms observed with individual agonists were compared with their efficacies for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization. In general the correlation was better between binding parameters and Ca^{2+} mobilization than between binding parameters and Ins1P formation. This may be related to the fact that the immediate product of phosphoinositide breakdown in these cells is inositol 1,4,5-trisphosphate (Masters *et al.*, 1985b), the second messenger for Ca^{2+} mobilization, whereas the inositol

phosphate (Ins1P) that actually was measured in the experiments is a metabolic product of the second messenger.

Although the molecular basis for the guanine-nucleotide-sensitive agonist-binding state of the muscarinic receptor in 1321N1 cells is not known, one interpretation of these data is that a guanine nucleotide regulatory protein is involved in coupling the agonist-occupied muscarinic receptor to the enzyme responsible for phosphoinositide breakdown. Speculation about these points obviously is influenced by our knowledge of the role of a high-affinity complex of agonist- β -adrenergic receptor- N_s in the activation of adenylate cyclase (Stadel *et al.*, 1982), and the fact that the values for K_L/K_H of a series of β -adrenergic-receptor agonists determined in competition-binding experiments correlated strongly with the efficacies of the same agonists for stimulation of adenylate cyclase (Kent *et al.*, 1980).

Computer-based analyses of complex binding curves of muscarinic receptor agonists were first reported by Birdsall *et al.* (1978), although comparisons between these binding parameters and efficacies of cholinergic agonists for production of biochemical responses were not made. Fisher *et al.* (1983) have published data obtained with guinea-pig cerebral cortex that may be analogous to the present results. These workers illustrated that muscarinic receptor agonists, such as oxotremorine, that were 'partial' as regards stimulation of phosphoinositide turnover, inhibited $[^3\text{H}]\text{QNB}$ binding in washed membrane preparations according to Law-of-Mass-Action kinetics. In contrast, full agonists, such as carbachol, inhibited $[^3\text{H}]\text{QNB}$ binding with heterogeneous competition curves that fit a two-site model of high- and low-affinity forms of the receptor. Although the effect of guanine nucleotides was not studied by Fisher *et al.* (1983), results from the present study suggest that guanine nucleotides would have markedly shifted the competition curves of full agonists to the right, whereas

little regulation by guanine nucleotides of 'partial'-agonist binding would have occurred. In other words, the difference between full and partial agonists would be that a high-affinity guanine-nucleotide-sensitive state is formed in the presence of full agonists, but not with partial agonists.

The ideas put forth in the present paper relating the capacity of agonists to induce a GTP-sensitive high-affinity binding complex and the efficacy of these agonists for stimulation of phosphoinositide breakdown are not at odds with the suggestion that the low agonist affinity observed in competition curves most closely matched muscarinic-receptor-agonist affinity for stimulation of phosphoinositide turnover (Michell *et al.*, 1976, 1981). The initial step in agonist action indeed may be its binding to a low-affinity state of the receptor. We suggest here that, in addition, the capacity of the agonist-occupied receptor to interact with a guanine nucleotide regulatory protein (as evidenced by formation of the high-affinity GTP-sensitive state) is critical for translation of binding into response.

There are a number of direct observations with other receptors and tissues that are consistent with the idea that a guanine nucleotide regulatory protein is involved in phosphoinositide breakdown and/or Ca^{2+} mobilization. Of potential relevance is the report by Gomperts (1983) suggesting that guanine nucleotides potentiate the secretory response to Ca^{2+} in permeabilized mast cells, and that by Haslam & Davidson (1984) indicating that guanine nucleotides increase the secretory response of permeabilized platelets to thrombin. Other circumstantial evidence for a role of a guanine nucleotide regulatory protein in phosphoinositide breakdown can be marshalled from studies of *Limulus*. That is, intracellular application of either active GTP analogues (Fein & Corson, 1981) or inositol 1,4,5-trisphosphate (Fein *et al.*, 1984) produces the same physiological response of the photoreceptor cell as does light. Litosch *et al.* (1985) have shown that 5-hydroxytryptamine-receptor-mediated breakdown of phosphoinositides in homogenates and washed membranes of blowfly salivary gland is dependent on the presence of GTP or a GTP analogue. Guanine nucleotides also have been shown to stimulate the breakdown of phosphatidylinositol 4,5-bisphosphate in human neutrophil plasma membranes (Cockcroft & Gomperts, 1985).

The insensitivity to inactivation by pertussis toxin of muscarinic-receptor-mediated attenuation of cyclic AMP accumulation (Hughes *et al.*, 1984), muscarinic-receptor-mediated stimulation of phosphoinositide breakdown and Ca^{2+} mobilization (Masters *et al.*, 1985a), and GTP-sensitive binding of agonists to muscarinic receptors (Evans *et al.*, 1985), suggests that N_i is not involved in cholinergic action in 1321N1 cells. Likewise, muscarinic-receptor-mediated stimulation of inositol phosphate formation in chick heart cells is not sensitive to pertussis toxin (Masters *et al.*, 1985a). The lack of effect of pertussis toxin on GTP-sensitive agonist binding to α_1 -adrenergic receptors of rat kidney membranes also suggests that these receptors, which couple to the phosphoinositide system (Fain & Garcia-Sainz, 1980), act through a guanine nucleotide regulatory protein that is not N_i . In contrast, N_i or an N_i -like protein has been suggested to fulfil such a role, on the basis of studies with pertussis toxin in neutrophils (Bokoch & Gilman, 1984; Okajima *et al.*, 1985) and in mast cells (Nakamura & Ui, 1985).

In summary, hormones that modify cellular function through Ca^{2+} mobilization and/or diacylglycerol-mediated activation of protein kinase C may couple to their second-messenger system by a mechanism not dissimilar to that involved in hormone-receptor coupling to adenylate cyclase. An important goal of future studies will be to identify the putative guanine nucleotide regulatory protein involved in receptor-stimulated hydrolysis of phosphoinositides.

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