Oxytocin receptors on cultured astroglial cells

Regulation by a guanine-nucleotide-binding protein and effect of Mg^{2+}

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Specific binding sites for the radio-iodinated oxytocin (OT) antagonist $d(CH_2)_5$ -[Tyr(Me)²,Thr⁴, Tyr-NH₂⁹]OVT ([¹²⁵I]OTA) have been characterized on cultured hypothalamic astroglial cell membranes. The rate of association of the ligand to OT-binding sites was identical in the presence and the absence of the non-hydrolysable GTP analogue guanosine 5'-[β -imido]triphosphate (Gpp[NH]p, 0.1 mM), whereas the monophasic dissociation reaction became biphasic in the presence of Gpp[NH]p. Scatchard analysis of equilibrium binding of [¹²⁵I]OTA resulted in a linear plot with a single class of binding sites (K_d 0.06 nM) which were insensitive to the addition of Gpp[NH]p. Unlabelled OT and [Arg⁸]vasopressin (AVP) bound to high- (H) and low- (L) affinity states with a dissociation constant ratio (K_L/K_H) of 100 for both hormones. Binding with both high and low affinity required the presence of Mg^{2+} in the incubation buffer, and the addition of Gpp[NH]p decreased the K_L/K_H ratio to 10 and increased the percentage of low-affinity binding sites. On the other hand, neither omission of Mg^{2+} from the buffer nor the addition of Gpp[NH]p altered the binding of either OT or V₁ AVP antagonists to OT receptors. In the presence of a G-protein inactivator (*N*-ethylmaleimide; 3 mM) during OT competition studies the affinities of the two OT-binding sites were unchanged, but 90 % of the high-affinity binding sites were converted into the low-affinity state. These results obtained with cultured hypothalamic astroglial cells provide further evidence for a coupling of OT receptors with a guanine-nucleotide-binding protein, with a requirement for Mg^{2+} .

INTRODUCTION

In the previous paper (Di Scala-Guenot & Strosser, 1992), OT-binding sites were pharmacologically characterized on astroglial cell cultures. From that study it appears that, in hypothalamic astroglial cells, both oxytocin (OT) and [Arg⁸]vasopressin (AVP) distinguished between two affinity states of the OT receptor labelled with a radioiodinated OT antagonist. This could reflect interaction of the OT receptor with a G-protein. There is increasing evidence to suggest that a G-protein is involved in the transduction of hormone-mediated physiological responses (see Litosch *et al.*, 1985). By analogy with adenylate cyclase-linked receptors, it has been suggested that receptors in various cell types whose activation mobilizes Ca^{2+} , when phosphoinositide turnover is involved, are coupled to a GTP-binding protein (Fain *et al.*, 1988) and thus are sensitive to guanine nucleotides. Evidence for coupling of receptors to G-proteins has been extensively studied and, among others, thyrotropin-releasing hormone (Aub *et al.*, 1987; Sullivan *et al.*, 1987), gonadotropin-releasing hormone (Perrin *et al.*, 1989), A₁ adenosine (Leung & Green, 1989; Leung *et al.*, 1990); histamine H₃ (Arrang *et al.*, 1990), growth hormone-releasing hormone (Struthers *et al.*, 1989) and Substance P (Luber-Narod *et al.*, 1990) receptors have been demonstrated to be coupled to G-proteins. The first evidence that vasopressin binding to V₁-subtype receptors is sensitive to guanine nucleotides was provided by Cantau *et al.* (1980) in work on rat hepatocytes and liver membranes, and this was confirmed by Fahrenholtz *et al.* (1984), Bojanic & Fain (1986) and Stassen *et al.* (1987). On the other hand, the mechanism of action of OT at the target tissues is not yet known, although OT-receptor-mediated stimulation of inositol phosphates has been demonstrated in ovine endometrium (Flint *et al.*, 1986), guinea pig myometrium (Marc *et al.*, 1986), rat mammary gland cells (Zhao & Gorewit, 1987), sensory nervous system (Horn &

Lightman, 1987), LLC-pK1 cells (Stassen *et al.*, 1988), human amnion cells (Moore *et al.*, 1988) and mammary and uterine slices (Pettibone *et al.*, 1990). So far, conflicting data have emerged concerning the interaction of the OT receptor with a GTP-binding protein. Ruzicky & Crankshaw (1987) and Marc *et al.* (1988) suggested involvement of a G-protein in the action of OT on myometrium, but Crankshaw *et al.* (1990) and Antoni & Chadio (1989) could not demonstrate an influence of guanine nucleotides on OT binding to myometrial cell membranes and to uterus rat membranes respectively.

In addition to the regulation of receptor binding by G-proteins, most receptor–ligand interactions are modulated by bivalent cations (Birnbaumer *et al.*, 1985). In particular, binding of AVP to both V₁ and V₂ receptors exhibits an absolute requirement for Mg^{2+} (Roy & Ausiello, 1981; Junig *et al.*, 1985), as does binding of OT to receptors in the mammary gland (Pearlmutter & Soloff, 1979) or in uterus membranes (Antoni & Chadio, 1989). Therefore the aim of the present paper was to determine whether a G-protein is involved in the coupling of OT receptors with the effector system on hypothalamic astroglial cell membranes in the presence and the absence of a non-hydrolysable analogue of GTP, guanosine 5'-[β -imido]triphosphate (Gpp[NH]p). Secondly, we examined the effect of Mg^{2+} on the OT-binding site affinity of OT and AVP agonist and antagonist.

MATERIALS AND METHODS

Cultures

Hypothalamic astroglial cell cultures were prepared as described in the previous paper (Di Scala-Guenot & Strosser, 1992).

Membrane preparation

Membranes were prepared from hypothalamic astroglial cell

Abbreviations used: OT, oxytocin; AVP, [Arg⁸]vasopressin; OTA, $d(CH_2)_5$ -[Tyr(Me)²,Thr⁴,Tyr-NH₂⁹]OVT; V₁ AVP antagonist, [$d(CH_2)_5$ -Tyr(Me)²]AVP; V₂ AVP agonist, 1-deamino[Val⁴,D-Arg⁸]vasopressin; Gpp[NH]p, guanosine 5'-[β -imido]triphosphate; NEM, *N*-ethylmaleimide.

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Fig. 1. Binding of [125 I]OTA to hypothalamic glial cell membranes

(a) Association at 37 °C of [125 I]OTA (0.03 nM) with hypothalamic glial cell membranes in the absence (●—●) or the presence (○—○) of Gpp[NH]p (0.1 mM). Inset: semilogarithmic plots of the data. (b) Dissociation of [125 I]OTA specific binding at 37 °C in the absence (●) or the presence (○) of Gpp[NH]p (0.1 mM). Inset: semilogarithmic plots of the data. Values of k_{app} and k_{-1} are given in Table 1. B_{eq} , [125 I]OTA bound at equilibrium; B_t , [125 I]OTA bound at a given time (d.p.m.).

cultures according to Cantau *et al.* (1990). After 12 days in culture, culture dishes were rinsed twice with Earle's Balanced Salt Solution (Gibco) without Ca^{2+} and Mg^{2+} . Cells were then scraped with a rubber policeman into 10 mM-Tris/HCl buffer (pH 7.4) containing 1 mM-EDTA, placed for 15 min on ice and homogenized using a Dounce homogenizer by 10 strokes of a loose-fitting glass pestle and a further 10 strokes of a tight-fitting glass pestle. The suspension was centrifuged for 10 min at 10 000 *g* at 4 °C and the pellet was resuspended in the binding buffer.

Binding assay

Binding was performed with freshly prepared membrane suspensions, since freezing the membranes at -80 °C led to a 50 % loss of binding. Binding was carried out in polypropylene microcentrifuge tubes in which 250 μ l membrane samples (corresponding to one culture dish or 600 μ g of protein) were incubated with 250 μ l of binding buffer (170 mM-Tris/HCl, pH 7.4, 5 mM- Mg^{2+} and 0.1 % BSA) containing 0.03 nM-d(CH₂)₅-[Tyr(Me)², Thr⁴, Tyr-NH₂⁹]OVT ([125 I]OTA). All incubations were allowed to proceed at 37 °C. After 90 min of incubation, the reaction was stopped by addition of 500 μ l of ice-cold binding buffer, the tubes were centrifuged at 20 000 *g* for 5 min and the pellet was rinsed twice with ice-cold binding buffer. The bottoms of the tubes containing the pellet were cut off and counted for radioactivity in a γ -radiation counter. *N*-Ethylmaleimide (NEM) pretreatment was carried out on an aliquot of the membrane suspension incubated for 15 min at 37 °C with 3 mM-NEM; the reaction was stopped by addition of ice-cold binding buffer, and the samples were centrifuged as above and washed once with binding buffer. The pellet was resuspended in binding buffer and processed for binding using the above procedure. In all experiments, each point was run in quadruplicate and results were analysed as described in the accompanying paper (Di Scala-Guenot & Strosser, 1992).

Chemicals

OTA was a gift from Dr. M. Manning (Medical College of Ohio, Toledo, OH, U.S.A.). OT and AVP were purchased from Bachem (Torrance, CA, U.S.A.). [d(CH₂)₅-Tyr(Me)²]AVP (V₁ AVP antagonist) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.) and 1-deamino[Val⁴, D-Arg⁸]vasopressin (V₂ AVP agonist) was from Ferring A.B. (Malmö, Sweden). Gpp[NH]p was purchased from Boehringer (Mannheim, Germany), and NEM was from Sigma.

RESULTS

Kinetic studies

[125 I]OTA binding to cultured astroglial cell membranes proceeded rapidly and reached equilibrium after 60 min of incubation. Receptor-bound [125 I]OTA appeared stable for at least 120 min at 37 °C (Fig. 1a). Therefore 90 min incubations were performed in all subsequent studies. The semilogarithmic plot of the time course of association was linear (Fig. 1a, inset) and allowed us to calculate an apparent rate constant of association (k_{app}) of 0.0342 min⁻¹ (Table 1). Addition of Gpp[NH]p (0.1 mM) during the association experiment did not affect the rate of association, and the k_{app} determined under these conditions was 0.0352 min⁻¹. Dissociation of bound [125 I]OTA, initiated at binding equilibrium by addition of an excess of unlabelled OT, proceeded slowly (Fig. 1b), with a time constant (k_{-1}) of 0.00625 min⁻¹ and a half-life of 110 min. The same experiment when repeated in presence of Gpp[NH]p resulted in a biphasic dissociation, with a rapid loss of the bound ligand ($t_{1/2}$ 2.15 min; k_{-1} 0.117 min⁻¹) followed by a slow loss of the remaining bound ligand ($t_{1/2}$ 39.5 min; k_{-1} 0.00625 min⁻¹). The slowly dissociating component was indistinguishable from the dissociation rate in the absence of nucleotide. From values of k_{app} and k_{-1} in control conditions, the rate constant of association (k_{+1}) was calculated

Table 1. Effect of Gpp[NH]p (0.1 mM) on kinetic parameters measured during association, dissociation and saturation experiments

k_{app} , and k_{-1} are the linear regressions in association and dissociation experiments, and correspond to the apparent association and dissociation rate constants respectively. k_{+1} is the second-order rate constant of association, and is equal to $(k_{app} - k_{-1})/F$, where F is the free labelled ligand concentration (0.03 nM). K_d from kinetic data is equal to k_{-1}/k_{+1} .

	Control	+ Gpp[NH]p
k_{app} , (min ⁻¹)	0.0342	0.0352
k_{-1} (min ⁻¹)	0.00625	0.011
k_{+1} (nM ⁻¹ ·min ⁻¹)	0.93	0.806
K_d from kinetic data (nM)	0.062	0.014
K_d from saturation data (nM)	0.068	0.068
B_{max} , (fmol/mg of protein)	37.5	39.6

Fig. 2. Saturation binding with increasing concentrations of the labelled ligand, in the absence or the presence of Gpp[NH]p (0.1 mM)

Main panel: Scatchard plots of saturation studies performed without (●—●) or with (○—○) Gpp[NH]p. Inset: saturation isotherms of specific binding. K_d and B_{max} values are given in Table 1, and results are from one experiment with four determinations.

to be 0.93 nM⁻¹·min⁻¹, yielding an equilibrium dissociation constant (K_d) of 0.062 nM (Table 1).

Saturation studies

Specific [¹²⁵I]OTA binding to cell membranes at equilibrium was saturable and of high affinity, and the curves obtained in the presence and the absence of Gpp[NH]p were superimposable (Fig. 2). The Scatchard representations of the saturation experiments as shown in Fig. 2 were linear over the range of concentrations tested, giving a K_d of 0.06 nM and a B_{max} of 40 fmol/mg of protein. The data demonstrated that only a single class of binding sites is labelled with the radioiodinated OT

antagonist, and that the antagonist binding characteristics were insensitive to exogenous guanine nucleotide.

Competition studies

Competition binding studies were performed with both agonists and antagonists. With OT and AVP as agonists, the competition curves were shallow (Figs. 3a and 3b), with a pseudo-Hill coefficient (h) below unity (h is 0.59 for OT and 0.63 for AVP); this result is compatible with the presence of more than one form of binding site and heterologous affinities for OT and AVP (see Table 2). Addition of 0.1 mM-Gpp[NH]p caused a shift of the competition curves to the right, but the curves remained biphasic and h was below unity (0.7 for OT and 0.62 for AVP), again suggesting binding site heterogeneity. However, in the presence of the GTP analogue, the high-affinity form (10⁻¹⁰ M) was converted into a lower-affinity form (10⁻⁹ M) (see Table 2), with an increase in the percentage of the receptors present in the low-affinity state. Similarly, during an OT competition experiment with NEM-pretreated membranes the affinities of the two sites were unaffected, but NEM caused a loss in the number of high-affinity sites which markedly decreased from 81 % to 9 % due to conversion into the low-affinity state, with a final ratio of high-to-low-affinity sites of 9:91 (Fig. 4).

For the OT antagonist, the competition curves in the absence and in the presence of Gpp[NH]p were superimposable (Fig. 3c) and characterized by an h of 1. Analysis of the displacement curve could be adequately explained by a single class of receptor, and this result confirms the observation obtained with the saturation study performed with the labelled OT antagonist. Thus it appears that the OT antagonist binds uniformly to all OT receptor sites. For the V₁ AVP antagonist the competition data were better fitted by a two-site model, as suggested by an h as low as 0.55 and by LIGAND analysis. The binding affinity of the low-affinity site was slightly decreased in the presence of Gpp[NH]p, and the percentage of the low-affinity binding sites remained unchanged.

Modulation of [¹²⁵I]OTA binding by Mg²⁺

The effect of the bivalent cation Mg²⁺ has been investigated during competition studies with the unlabelled peptides OT, AVP, OT antagonist, and V₁ AVP antagonist. The absence of the cation in the binding buffer did not affect the binding affinity of either OT and V₁ AVP antagonists (Figs. 5c and 5d) to OT receptor sites; indeed, the two competition curves are superimposed. In contrast, competition with the agonists in Mg²⁺-free binding buffer showed that AVP, at all concentrations tested, was unable to displace [¹²⁵I]OTA binding (Fig. 5b), whereas OT displaced the tracer at high concentrations only (5 μM; Fig. 5a). From the competition data analysis, it was clear that all binding sites in the high-affinity form were converted into a low-affinity form. This result confirms the more complex nature of agonist binding to the OT receptor site shown with the competition studies.

DISCUSSION

The present paper reports evidence that, in brain cultured cells, OT receptors are coupled to a GTP-binding protein. This was previously suggested by the complex competition curves obtained on binding of OT and AVP agonists to intact cells (Di Scala-Guenot & Strosser, 1992). Our observations on the effects of Gpp(NH)p on the characteristics of [¹²⁵I]OTA binding to cultured hypothalamic astroglial cell membranes are consistent with such an interaction. Indeed, kinetic experiments clearly indicated the occurrence of a biphasic dissociation process upon addition of Gpp[NH]p, with both fast and slow dissociation states, and the increase in the rate of dissociation may reflect a

Fig. 3. Competitive displacement of specifically bound $[^{125}\text{I}]\text{OTA}$ by increasing concentrations of unlabelled OT (a), AVP (b), OT antagonist (c) and V_1 AVP antagonist (d) without (—) or with (----) Gpp[NH]p (0.1 mM)

Values of K_d calculated from LIGAND analysis are given in Table 2. Values are results from one or two experiments, with four determinations each.

Table 2. Potency of OT, AVP, OT and V_1 AVP antagonists for competition with $[^{125}\text{I}]\text{OTA}$ in the absence and in the presence of Gpp[NH]p (0.1 mM)

K_H and K_L are the dissociation constants of the high- and the low-affinity binding sites respectively, as determined by LIGAND analysis. H,L (%) is the percentage of receptors in the high (H) or low (L) affinity state, as appropriate. h is the pseudo-Hill coefficient.

Ligand	Control			+ Gpp[NH]p		
	K_d (nM)	h	H,L (%)	K_d (nM)	h	H,L (%)
OT K_H	0.4		81	1		58
OT K_L	12	0.59	19	20	0.7	42
AVP K_H	0.35		43	2.5		29
AVP K_L	20	0.63	57	50	0.62	71
OT antagonist	0.26	1.0		0.34	1.2	
V_1 antagonist K_H	1.4		62	1.2		40
V_1 antagonist K_L	27	0.55	38	30	0.40	60
OT+NEM K_H	0.2		9			
OT+NEM K_L	60	0.6	91			

shift of the receptor from a slowly dissociating high-affinity state to a rapidly dissociating low-affinity state.

OT antagonist bound only to a high-affinity form of the receptor which was insensitive to guanine nucleotides, whereas

Fig. 4. Inhibition of $[^{125}\text{I}]\text{OTA}$ binding by increasing concentrations of OT in the absence (●) or the presence (○) of NEM (3 mM)

OT and AVP agonist discriminated, among the sites labelled by the radioligand, both high- and low-affinity forms of the receptor. In addition, guanine nucleotides modulated the proportion of the sites with high affinity for agonists, as indicated by their ability to shift the agonist competition curves; indeed, the high-affinity form of the receptor represented 80% of the total binding in control conditions, but this decreased to 58% in the presence of the nucleotide. The lack of complete transition to the

Fig. 5. Effect of absence of Mg^{2+} in the incubation buffer on the ability of OT (a), AVP (b), OT antagonist (c) and V_1 AVP antagonist (d) to compete with $[^{125}I]OTA$.

---, Without Mg^{2+} ; —, control.

low-affinity form could suggest that a subpopulation of the receptors is insensitive to the nucleotide. A better demonstration of the coupling of the receptor with a G-protein was given in competition studies performed on membranes pretreated with NEM, a G-protein inactivator, since in this case all of the high-affinity binding sites (91%) were converted into the low-affinity state. The effect of Gpp[NH]p on agonist binding may be explained by an equilibrium between two interconvertible OT receptor states of high and low affinities, in which the high-affinity state is stabilized by interaction with a guanine nucleotide regulatory protein to form a ligand-receptor-G-protein ternary complex (De Lean *et al.*, 1980).

The ability of guanine nucleotides to shift G-protein-linked receptors from a high- to a low-affinity state has already been reported for a wide variety of receptors (Rodbell *et al.*, 1971; Zahniser & Molinoff, 1978; Berrie *et al.*, 1979; Stassen *et al.*, 1987; Laitinen *et al.*, 1990) and our results appear consistent with the hypothesis that OT receptors can exist in two forms of different affinities that are discriminated by agonists and modulated by guanine nucleotides. The lower affinity we observed for OT binding on intact cells compared with the affinity obtained on membranes could suggest that OT receptors are modulated by endogenous GTP (Cantau *et al.*, 1980). Also, puzzling data emerged from competition studies with the V_1 AVP antagonist, since in the accompanying paper (Di Scala-Guenot & Strosser, 1992), V_1 AVP antagonist bound on intact cells to one high-affinity OT receptor site, as expected for antagonist binding (Williams & Lefkowitz, 1977), whereas the data herein indicated the presence of two receptor affinity states on cell membranes.

No convincing explanation can be given, although Cantau *et al.* (1980) and Stassen *et al.* (1987) also reported expression of a second affinity state when AVP binding was performed with membrane preparations. However, for the V_1 AVP antagonist, the two forms were both insensitive to Gpp[NH]p.

For OT glial cell receptors, binding of antagonists was insensitive to the nucleotide, as already observed for β -adrenergic (Lefkowitz *et al.*, 1976), α -adrenergic (Hoffman *et al.*, 1980) and peptide (Crane *et al.*, 1982; De Lean *et al.*, 1984; Perrin *et al.*, 1989) receptors, whereas for A_1 adenosine (Yeung & Green, 1983; Ramkumar & Stiles, 1988; Stiles, 1988), muscarinic (Ehlert *et al.*, 1980; Burgisser *et al.*, 1982) and dopamine (De Lean *et al.*, 1982a,b) receptors the guanine nucleotide affected antagonist binding.

As far as modulation of OT receptors by guanine nucleotides is concerned, conflicting data have been reported. Indeed, Ruzyky & Crankshaw (1987) and Marc *et al.* (1988) suggested a G-protein involvement in the action of OT on rat uterus, whereas Crankshaw *et al.* (1990) and Antoni & Chadio (1989) could not reproduce such a result. However, receptors for hormones mobilizing Ca^{2+} by hydrolysis of phosphoinositides (Litosch & Fain, 1986; Fain *et al.*, 1988), as well as those linked to adenylate cyclase (Lefkowitz *et al.*, 1976; De Lean *et al.*, 1982a; Koch & Schonbrunn, 1984; Leung & Green, 1989) may be coupled to a GTP-binding protein. As it is well established that OT and AVP stimulate phosphatidylinositol hydrolysis (Marc *et al.*, 1986; Schrey *et al.*, 1986; Soloff *et al.*, 1989; Pettibone *et al.*, 1990) and that G-proteins are the likely transducers of the AVP response (Uhing *et al.*, 1986; Guillon *et al.*, 1986; Bojanic & Fain, 1986;

Fitzgerald *et al.*, 1986; Aiyar *et al.*, 1989), it is likely that OT receptors are also regulated by a G-protein. Different regulatory G-proteins are activated by the agonist-membrane-associated receptor interaction, and the nature of the G-protein concerned in OT binding in our system can only be speculated upon on the basis of other reports. Studies on guinea pig myometrium have shown that OT receptors are coupled to a phospholipase C through a G-protein that is insensitive to pertussis toxin, and different from both G_s and G_i (Marc *et al.*, 1988). Likewise, AVP receptors on rat liver plasma membranes are coupled to a G-protein that is insensitive to both cholera and pertussis toxins (Fitzgerald *et al.*, 1986).

A question is raised concerning the coupling of the receptor to a G-protein in the absence of the ligand. Using the solubilization procedure it has been demonstrated that, in absence of agonists, the β -adrenergic (Limbird & Lefkowitz, 1978), D_2 dopamine (Kilpatrick & Caron, 1983) and α -adrenergic (Smith & Limbird, 1981; Michel *et al.*, 1981) receptors are not coupled to a G-protein, whereas A_1 adenosine receptors are (Leung & Green, 1989; Leung *et al.*, 1990). On the other hand, antagonists seemed to bind preferentially to free uncoupled receptors (Limbird & Lefkowitz, 1978; Smith & Limbird, 1981; Michel *et al.*, 1981; De Lean *et al.*, 1984; Wreggett & De Lean, 1984; Leung *et al.*, 1990). However, no conclusion could be drawn from our data about the OT receptor conformation in the presence of agonists and antagonists.

Direct cationic modulation of agonist binding to the OT receptor has been demonstrated in this paper. Omission of Mg^{2+} ions from the incubation buffer modified displacement of [125 I]OTA by agonists, but not by antagonists. A conversion of the high-affinity site into a low-affinity site in the absence of Mg^{2+} was likely for OT, while AVP was not able to displace the tracer at any concentration tested. The agonist-specific requirement for Mg^{2+} has already been shown for OT binding to its receptor (Antoni & Chadio, 1989; Ayad & Wathes, 1989) as well as for the modulation of OT binding affinity (Pearlmutter & Soloff, 1979). In addition the biological activity of OT *in vitro* is also enhanced by addition of Mg^{2+} (Soloff, 1985). This would suggest the involvement of Mg^{2+} in stabilization of the high-affinity form of the receptor, which is necessary for agonist binding, in association with the G-proteins.

In conclusion, binding of OT to its receptors in hypothalamic cultured astroglial cells is regulated by guanine nucleotides in a manner characteristic of other receptors coupled to a G-protein with modulation of the binding affinity by cationic bivalent ions. One would be tempted to propose a model of the mechanism of action of OT whereby coupling of the receptor to a GTP-binding protein in turn activates coupling to phospholipase C, thus initiating phosphoinositide hydrolysis. Such direct interactions need further investigations to be validated.

This work was supported by grants from the CNRS (URA 1446) and INSERM (88.6012). We are deeply indebted to Dr. M. Manning for the generous supply of OT antagonist. We also thank Mrs. M. Horrenberger and B. Waltisperger for their excellent technical assistance, and E. Wendling for typing the manuscript.

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Received 12 June 1991/18 December 1991; accepted 16 January 1992