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Lack of Coupling of D-2 Receptors to Adenylate Cyclase in GH-3 Cells Exposed to Epidermal Growth Factor

POSSIBLE ROLE OF A DIFFERENTIAL EXPRESSION OF G_i PROTEIN SUBTYPES*

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Exposure of GH-3 cells to epidermal growth factor for 4 consecutive days induced the expression of both D-2(415) and D-2(444) dopamine-receptor isoforms. Epidermal growth factor also promoted a remarkable increase in the content of G_i3 protein, which is responsible for receptor-induced activation of potassium channels in GH-3 cells. D-2 receptors in this model apparently activate a specific transducing pathway, leading to opening of potassium channels and inhibition of prolactin release by cAMP-independent mechanisms. This is shown by: 1) the selective D-2 agonist quinpirole, while inactive on vasoactive intestinal peptide-induced prolactin release, strongly inhibited the hormone secretion induced by neurotensin; 2) quinpirole, up to 100 μM, did not inhibit cAMP production evoked by vasoactive intestinal peptide both in intact cells and in broken cell membrane preparations; and 3) quinpirole and other D-2 agonists strongly potentiated Rb^+ efflux when measured in a nominally calcium-free reaction solution containing 100 mM potassium (voltage-dependent component), but did not modify Rb^+ efflux if measured in a reaction solution containing 1 mM calcium and 5 mM potassium (calcium-activated, cAMP-dependent component).

The clonal strain GH-3, which secretes both growth hormone (GH)[1] and prolactin (PRL) (1, 2) appears to provide an excellent model for studying the regulation of hormone secretion in anterior pituitary. PRL secretion from GH-3 cells responds, indeed, to a variety of neurotransmitters which physiologically regulate the hormone release by lactotrophs (3). The main difference between lactotrophs and the GH-3 clone is that the latter lacks receptors for dopamine (DA), the release of PRL being insensitive to DA agonist inhibition (4-8).

We have recently found that epidermal growth factor (EGF), in addition to changing the proliferation rate, morphology, and hormone production (9), induces the reexpression of DA D-2 receptors involved in the control of PRL secretion in the GH-3 cells, thus providing a pure cell clone endowed with D-2 DA receptors (8).

From a molecular point of view D-2 receptors have been characterized as two isoforms produced by alternative splicing of primary transcripts of the same gene (10-13).

Multiple transmembrane signaling pathways are activated by D-2 receptors both in the brain and pituitary (14-20). All of the effects of DA on the different signaling systems involve the activation of the G_o/G_i family of guanine nucleotide-binding proteins (G proteins); at present, three forms of $G_i\alpha$ subunits have been isolated which are distinct gene products (21-26) and which couple with D-2 receptors with different affinities (27).

In the present study we examined the expression of the D-2 receptor isoforms in response to EGF in the GH-3 clone and investigated the intracellular pathways involved in D-2 receptor activation and the final biological effect. For this purpose, inhibition of cAMP formation (14, 20), activation of calcium-activated, cAMP-dependent potassium channels (18), and inhibition of vasoactive intestinal peptide (VIP)-induced PRL release (15, 28) have been measured as indices for D-2 receptors associated with inhibition of adenylate cyclase. On the other hand, activation of voltage-dependent potassium channels (16-18) and inhibition of neurotensin (NT)-stimulated PRL secretion (15) have been taken as paradigms for adenylate cyclase-independent D-2 receptors.

The results suggested that both the "long" and the "short" receptor forms are induced by EGF in the GH-3 clone and that both are apparently associated with the activation of voltage-dependent potassium channels and with the inhibition of PRL secretion by cAMP-independent mechanisms. A EGF-directed change in G_i protein subtype content appears to be responsible for the selective D-2 activation of the potassium channel-coupled transmembrane signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—The GH-3 cell clone used in this study was derived from the rat MtT/W5 tumor (1, 2) and was obtained by the American Type Culture Collection (Rockville, MD).

The cells from a single donor culture were plated and grown at 37 °C under a humidified atmosphere of 5% CO_2, 95% O_2 in Ham's F-10 medium (GIBCO, Milano, Italy) supplemented with 15% horse serum (GIBCO, Milano, Italy), 2.5% fetal calf serum (GIBCO, Milano, Italy), 40 mM glutamine and 100 units/ml penstrepto ($F\text{-}10^+$) as previously described (8).

Cells were grown for 4 days in the presence or in the absence of

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[1] The abbreviations used are: GH, growth hormone; PRL, prolactin; NT, neurotensin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EBSS: Earle's balanced salt solution; PCR, polymerase chain reaction; VIP, vasoactive intestinal peptide; DA, dopamine; HEPES, 4-(2-hyroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; $F\text{-}10^+$, Ham's F-10 medium; G_i1, G_i2, G_i3, G_o, guanine nucleotide binding proteins named according to the nomenclature proposed by Gilman (49).

0.5 nM EGF as previously described (8). On the 5th day cells were used for biochemical experiments.

Measurement of PRL Release—Cells were rinsed twice at 37 °C for 10 min with 500 μl Earle's balanced salt solution containing 0.2% bovine serum albumin (EBSS) and 10 units/ml penstrepto. Cells were then incubated at 37 °C for 10 min with 500 μl EBSS for basal PRL release; the supernatants were collected and transferred into plastic tubes. The same cells were then incubated at 37 °C for 10 min in EBSS containing quinpirole or other dopaminergic drugs (0.1–100 nM); VIP (1–100 nM) or NT (0.1–100 nM) were then added to plates, cells were incubated at 37 °C for an additional 10 min, and the supernatants were collected. After this, cells were rinsed (10 min at 37 °C) with EBSS and then incubated for 4 h at 37 °C in F-10$^+$ either with or without 0.5 nM EGF. After this period, cells were washed twice at 37 °C for 10 min with 500 μl EBSS and then incubated at 37 °C for 10 min for basal PRL release. The cell preparations were then added with VIP (1–100 nM) or NT (0.1–100 nM) and incubated at 37 °C for 10 min; the supernatants were collected for VIP- or NT-stimulated PRL release.

The amount of PRL in the supernatants was determined by a double antibody radioimmunoassay method using materials and protocols kindly supplied by NIADDK rat pituitary hormone program. The radioimmunoassay standard was rat PRL (NIADDK-rPRL-RP-3) and was used over the range of 0.5–32 ng. The variation coefficient was less than 5% and the assay sensitivity was 0.3 ng.

Rubidium Efflux Measurement—The F-10$^+$ medium was replaced with 10 ml of the "loading solution," the cells were scraped out from the plates and incubated at 37 °C for 1 h. After this, cells were centrifuged at 2000 rpm for 10 min and the pellet was resuspended in the "loading solution" containing tracer $^{86}Rb^+$ (100–200 Ci/ml) and 0.1 mM unlabeled RbCl to give 10^6 cells/ml. The suspension was incubated for 60 min at 30 °C. $^{86}Rb^+$ efflux was measured as previously described (18) according to the manual quench method of Bartschat and Blaustein (29). Briefly, aliquots of $^{86}Rb^+$ loaded cells (5×10^5 cells/sample) were pipetted into wells of a filtration apparatus (Hoeffer Scientific Apparatus) containing 2 ml of a "wash solution" and vacuum was immediately applied. The filters (Whatman GF/B) were then washed five times with 4 ml of the same solution. When the effects of drugs were tested, the appropriate drug concentration was added to the wash solution to facilitate equilibrium of the drug with the cells. Measurement of $^{86}Rb^+$ efflux was started 10 min later at addition of 2 ml of the "reaction solution" to the filters followed by the rapid addition of 2 ml of the "stopping solution" 5 s later. Vacuum was then applied, and the filtrate was collected into plastic vials. The amount of $^{86}Rb^+$ in both filter and filtrate was determined by scintillation spectroscopy. $^{86}Rb^+$ efflux was expressed as the percentage of the total $^{86}Rb^+$ appearing in the filtrate from the following equation.

$$\text{Percentage } Rb^+ \text{ efflux} = \frac{Rb^+ \text{ in filtrate}}{Rb^+ \text{ in filtrate} + Rb^+ \text{ in filter}} \times 100$$

The "loading solution" was 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.02 mM $CaCl_2$, 0.1 mM RbCl, 10 mM glucose, 10 mM HEPES buffer titrated to pH 7.4 with NaOH. The "wash solution" was 145 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.1 mM RbCl, 10 mM glucose, 10 mM HEPES, pH 7.4. The "reaction solution" was 0–145 mM NaCl, 5–150 mM KCl (NaCl + KCl = 150 mM), 2 mM $MgCl_2$, 0.1 mM RbCl, 10 mM glucose, 10 mM HEPES, pH 7.4. In some experiments Ca^{2+} was substituted mole for mole for Mg^{2+}. The "stopping solution" was: 145 mM tetraethylammonium, 1 mM tetrabutylammonium, 5 mM RbCl, 5 mM $MgCl_2$, 10 mM $NiCl_2$, 20 mM HEPES, pH 7.4.

Adenylate Cyclase Activity—The culture medium was aspirated and cells were washed twice with EBSS. Cells were then scraped from the plates with 10 ml of EBSS and lysed by suspension and vortex mixing in 5 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ for 20 min at 4 °C. Aliquots of the suspension (5×10^5 original cells/sample) were incubated for 20 min at 30 °C in a 500-μl reaction mixture containing 80 mM Tris-HCl, 16 mM $MgSO_4$, 0.5 mM 3-isobutyl-1-methylxanthine, 0.6 mM EGTA, 0.02% ascorbic acid, pH 7.4, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 53 μM GTP, 2 mM ATP, 100 nM VIP, or 10 μM forskolin with or without various concentrations (1–100 μM) of quinpirole. The reaction was stopped by placing samples in boiling water for 5 min and then in ice. Samples were centrifuged at 3000 rpm for 10 min and the cAMP in the supernatant was measured by radioimmunoassay using the commercial kit provided by Du Pont-New England Nuclear (Firenze, Italy).

For the determination of intracellular cAMP cells were scraped out from the plates, gently suspended in EBSS, and incubated at 37 °C for 20 min. Aliquots of the suspension (5×10^5 cells/sample) were incubated for 10 min at 37 °C with 100 nM VIP or 10 μM forskolin with or without various concentrations (0.1–100 μM) of quinpirole. The reaction was stopped by placing samples in boiling water for 5 min and then in ice. Samples were then sonicated at 50 watts for 30 s and centrifuged at 3000 rpm for 10 min. The cAMP in the supernatant was measured as previously described.

Membrane Preparation, SDS-Polyacrylamide Gel Electrophoresis, and Immunoblotting—Control and EGF-treated GH-3 cells were washed with 10 ml of ice-cold PBS and scraped into 2 ml of 40 mM Tris-HCl pH 7.5 containing 10 mM EDTA and 150 mM NaCl. After low speed centrifugation at 1500 rpm for 5 min, cells were resuspended in 1 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin and broken with a tight fitting homogenizer (15 strokes). Nuclei and debris were removed by centrifugation at 6500 rpm for 5 min in a Sorvall SS34 rotor. The supernatant was centrifuged at 35,000 rpm for 30 min at 4 °C in a Beckman T150 rotor. The pelleted membranes were resuspended in 10 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride and frozen at −80 °C.

Protein content of the membrane fractions were determined as described by Bradford (30). Membranes were dissociated in the loading buffer containing 62.5 mM Tris-HCl, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromphenol blue, pH 7.5, and subjected to electrophoresis in a 10% SDS-polyacrylamide gel (31). The proteins were then transferred electrophoretically onto Hybond nylon membranes (Amersham). The blotted proteins were immunoreacted with antibodies directed to various G protein α subunits (Du Pont-New England Nuclear) at 1:200 dilution for 2 h using standard conditions. Immunoreaction was revealed by 2-h incubation with 0.5 μCi/ml of iodinated protein A (Amersham Corp.). Autoradiography was performed on Kodak X-OMAT AR films with intensifier screen at −80 °C.

RNA Extraction, Polymerase Chain Reaction, and Hybridization Conditions—Total RNA was isolated (32) from control and EGF-treated GH-3 cells and 1 μg of each sample was transcribed into cDNA by standard methods (33) using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and oligo$(dT)_{18}$ as primer. Of each cDNA, 5 ng were used as template in a PCR (34) (50 μl final volume, 250 μM of each deoxinucleoside trisphosphate, 50 mM KCl, 10 mM Tris-HCl, pH 3.0, 2.5 mM $MgCl_2$, 0.01% gelatin) with 40 pmol each of oligos PCR I (5′-TCCTGCCCACTGCTCTTCGGACT C-3′ encoding rat D-2 receptor residues SCPLLFGL within TM4) and PCR II (5′-GAGAGTGAGCTGGTGGTGACTGGG-3′, complementary to a sequence encoding residues PSHHQLTL located in the rat D-2 receptor C-terminal to exon 5) (13) as primers and 2.5 units of Ampli-Taq (Cetus, Emeryville, CA). Reactions were started at 94 °C and performed for 30 cycles (94 °C, 0.5 min; 60 °C, 0.5 min; 72 °C, 1 min) in a thermocycler (Techne Corp., Princeton, NJ), within the linear range of amplification.

Aliquots (10 μl) of these reactions were resolved on 1.5% agarose gels (Sigma) and the gel-alkali blotted onto Hybond membrane (Amersham). Blots were then hybridized with oligo A (5′-GTTCAC TGGGAAACTCCCATTAGACTTCATGATAACGGTGCAGAG-3′ complementary to a sequence encoded by exon 5 of the rat D-2 gene) or a cDNA probe encoding for the complete D-2(415) receptor (10).

Oligo(A) was 5′ end ^{32}P-labeled to a specific activity of 5×10^7 cpm/μg using T4 polynucleotide kinase (Amersham) according to Maniatis *et al.* (33). Filter hybridization was in 30% formamide, 5 × SSC at 37 °C and excess probe washed from membranes in 2 × SSC for 15 min at 65 °C.

The D-2(415) receptor cDNA probe was labeled with ^{32}P to a specific activity of at least 5×10^8 cpm/μg DNA using a multiprime labeling system (Amersham). Hybridization was performed in 50% formamide, 5 × SSC at 42 °C, and filters were washed to a stringency of 0.1 × SSC, 0.1% SDS at 55 °C. All blots were then exposed to Kodak X-Omat films and autoradiograms scanned using an ultrascan laser densitometer (LKB 2202).

Amplification of the cyclophilin fragment was obtained by 25 PCR cycles under conditions analogous to those described for the D-2 receptor utilizing 40 pmol of oligo PCR III (5′-TGCCATCCAGCCA CTCAGTCTTGG-3′ encoding rat cyclophilin residues MVNPTVFF) and oligo PCR IV (5′-TGCCATCCAGCCACTCAGTCTTGG-3′ complementary to rat cyclophilin residues AKTEWLDG) (35). Blotting, labeling, and hybridization conditions of the cyclophilin cDNA probe were as described for the D-2(415) receptor cDNA probe.

Statistical Analysis—The analysis of variance followed by a two-tailed Student's *t* test was used for statistical evaluation of the data.

Materials—Epidermal growth factor, neurotensin, VIP, 3-isobutyl-1-methylxanthine, forskolin, ATP, GTP, EGTA, EDTA, and phenylmethylsulfonyl fluoride were from Sigma; phosphocreatine and creatine phosphokinase were from Boehringer Mannheim; quinpirole, haloperidol, dopamine, D-butaclamol, L-butaclamol were from Research Biomedicals Inc. (Wayland, MA); bromcriptine was from Sandoz (Milano, Italy); (+)- and (−)-sulpiride were from Ravizza (Muggió, Italy); oligo(A) and the cDNA probe for D-2 receptors were kindly provided by Dr. P. Seeburg (Heidelberg, Germany); antibodies to various G protein α subunits, $^{86}Rb^{+}$, and ^{125}I-PRL were from Dupont-New England Nuclear (Firenze, Italy).

RESULTS

Effect of D-2-Receptor Activation on PRL Release—The effects of VIP and NT on PRL release were studied in both GH-3 cells and EGF-treated GH-3 cells. As shown in Fig. 1*A* VIP stimulated PRL secretion from GH-3 cells and from the cell population exposed to 0.5 nM EGF for 4 days by the same extent (+316%) and with the same potency (ED_{50} = 2.5 ± 0.41 nM in GH-3 cells and ED_{50} = 2.0 ± 0.44 nM in EGF-treated GH-3 cells). Similar results were obtained with NT. The effect of NT on PRL secretion was dose-dependent in both cell populations approaching maximum at 10 nM (+275% in GH-3 cells exposed to EGF and +250% in GH-3 cells). The NT half-maximal stimulatory concentrations were 0.8 ± 0.24 nM in GH-3 cells and 0.5 ± 0.16 nM in EGF-treated GH-3 cells (Fig. 1*B*).

The D-2 agonist quinpirole (100 nM) did not inhibit PRL secretion induced by both 100 nM VIP and 100 nM NT in control GH-3 cells (VIP: 70 ± 6.5 ng of PRL/2 × 10^5 cells, VIP + quinpirole: 64 ± 4.3 ng of PRL/2 × 10^5 cells; NT: 65 ± 5.1 ng of PRL/2 × 10^5 cells, NT + quinpirole: 55 ± 3.3 ng of PRL/2 × 10^5 cells).

As shown in Fig. 2*A*, quinpirole over the range of 0.1 nM to 100 nM did not inhibit VIP-stimulated PRL release in EGF-treated GH-3 cells; the dose-response curves for VIP-elicited PRL secretion measured in the absence or in the presence of 100 nM quinpirole were also superimposable (Fig. 2*B*). The same results were obtained with GH-3 cells grown for an additional 4 days in the presence of 0.5 nM EGF (data not shown).

Fig. 3*A* shows that 100 nM quinpirole strongly prevented PRL release induced by increasing concentrations of NT in EGF-treated GH-3 cells. The dose-response curve for quinpirole-induced inhibition of NT-stimulated PRL release is reported in Fig. 3*B*; the drug, over the range of 0.1 to 100 nM, dose-dependently inhibited the hormone release induced by 100 nM NT with half-maximal inhibitory concentration of 1.8 ± 0.25 nM.

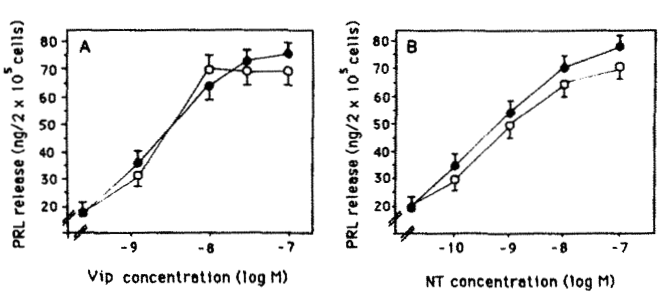

FIG. 1. **Effects of VIP and neurotensin on prolactin release in GH-3 cells grown in the absence or in the presence of EGF.** The data are the means ± S.E. of three independent experiments with each point measured in quadruplicate. GH-3 (○); EGF-treated GH-3 (●). The calculated ED_{50} values were 2.5 ± 0.41 nM and 2 ± 0.44 nM for VIP effects in control and EGF-treated GH-3 cells, respectively, and 0.8 ± 0.24 nM and 0.5 ± 0.16 nM for NT effects in the two cell preparations.

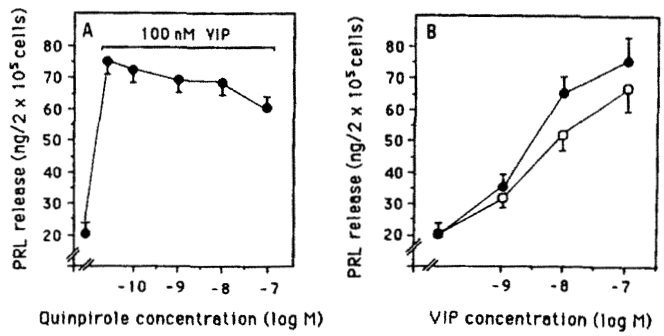

FIG. 2. **Effects of quinpirole on VIP-induced PRL release in GH-3 cells grown in the presence of EGF.** *A*, PRL release was measured with 100 nM VIP in the absence or in the presence of various concentrations (0.1–100 nM) of quinpirole. *B*, PRL release was measured with various concentrations (1–100 nM) of VIP in the absence (●) or in the presence (○) of 100 nM quinpirole. Values are the means ± S.E. of four independent experiments with each point measured in quadruplicate.

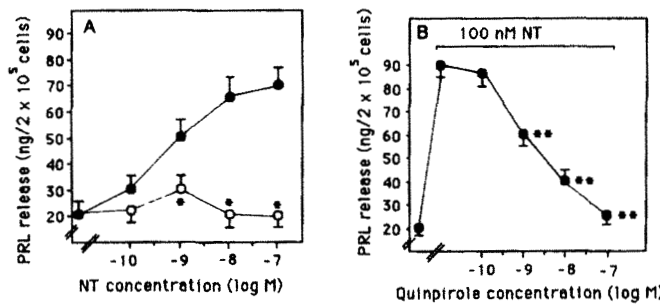

FIG. 3. **Effects of quinpirole on neurotensin-induced prolactin release in GH-3 cells exposed to EGF.** *A*, PRL release was measured with various concentrations (0.1–100 nM) of NT in the absence (●) or in the presence (○) of 100 nM quinpirole. *B*, PRL release was measured with 100 nM NT in the absence or in the presence of various concentrations (0.1–100 nM) of quinpirole. Values are the means ± S.E. of three independent experiments with each point measured in quadruplicate. *$p < 0.001$ *versus* the control value. The statistical analysis was applied to the points corresponding to individual NT concentrations. **$p < 0.001$ *versus* NT.

The pharmacological characterization of this effect with D-2 agonists and antagonists is given in Figs. 4 and 5. The dose-response curves for various DA agonists as inhibitors of NT-induced PRL secretion revealed an order of potency (bromocriptine > quinpirole > DA > RU 24213) which is typical of a D-2-mediated phenomenon. DA inhibited NT-induced PRL secretion with an IC_{50} value of 15 ± 2.8 nM; the IC_{50} values for the selective D-2 agonists bromocriptine, quinpirole, and RU 24213 were 0.4 ± 0.12 nM, 1.5 ± 0.21 nM, and 30 ± 5.2 nM, respectively. As reported in Fig. 5, D-2 receptor antagonists prevented the inhibitory effect of quinpirole on NT-induced PRL secretion in EGF-treated GH-3 cells. In particular, sulpiride antagonized the effects of 100 nM quinpirole stereospecifically with (−)-sulpiride very active and the pharmacologically inactive enantiomer (+)-sulpiride virtually uneffective. Similarly, haloperidol, at the concentration of 1 μM also prevented the effects of quinpirole almost completely.

D-2 Receptor Stimulation Does Not Inhibit cAMP Formation in EGF-treated GH-3 Cells—Fig. 6 and Table I show the data obtained by measuring cAMP formation in both membrane preparations and intact GH-3 cells exposed to EGF. As reported in Fig. 6, quinpirole up to 100 μM did not inhibit the formation of cAMP elicited either by 10 μM forskolin (Fig.

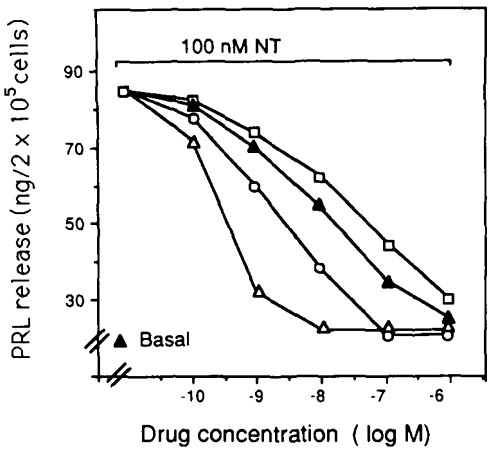

FIG. 4. **Effects of dopamine and various dopamine agonists on neurotensin-induced prolactin release in GH-3 cells grown in the presence of EGF.** Data are representative of one of three independent experiments with each point measured in quadruplicate. The standard error for each value was less than 10%. The calculated IC_{50} values were: 0.4 ± 0.12 nM for bromocriptine (△), 1.5 ± 0.21 nM for quinpirole (○), 15 ± 2.8 nM for dopamine (▲), and 30 ± 5.2 nM for RU 24213 (□).

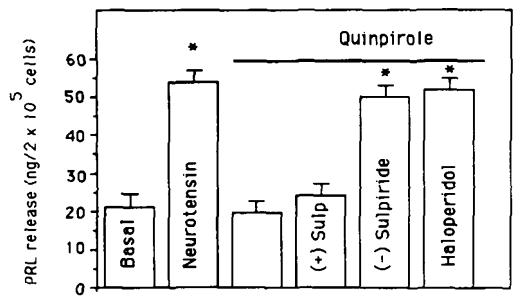

FIG. 5. **Effect of dopaminergic antagonists on quinpirole inhibtion of neurotensin-induced prolactin release in EGF-treated GH-3 cells.** Each *bar* is the mean ± S.E. of three independent experiments run in quadruplicate. *$p < 0.001$ *versus* basal.

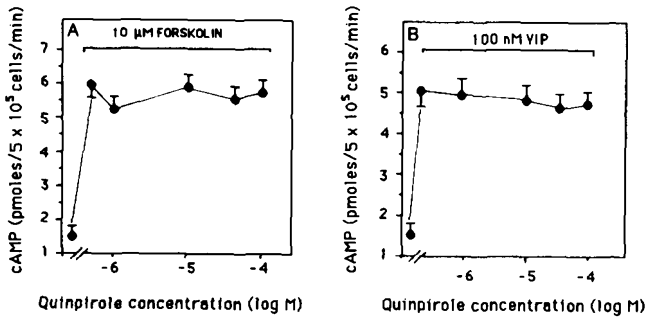

FIG. 6. **Effects of quinpirole on forskolin-induced (*A*) and VIP-induced (*B*) cAMP formation in membranes from EGF-treated GH-3 cells.** Data are the means ± S.E. of five independent experiments with each point measured in triplicate.

TABLE I

Effects of quinpirole on forskolin- and VIP-induced cAMP formation in intact EGF-treated GH-3 cells

cAMP formation in intact cells was measured as described under "Experimental Procedures" with 10 μM forskolin or 100 nM VIP. Data are the means ± S.E. of three independent experiments with each point measured in triplicate. The basal cAMP formation was 2.3 ± 0.18 pmol/5 × 10^5 cells/min.

Drugs	cAMP	
	Forskolin (10 μM)	VIP (100 nM)
	pmol/5 × 10^5 cells/min	
None	9.3 ± 1.0	7.4 ± 0.81
Quinpirole		
100 nM	6.5 ± 0.70*	6.8 ± 0.63
1 μM	4.3 ± 0.51*	7.1 ± 0.78
10 μM	3.1 ± 0.28*	7.7 ± 0.80
100 μM	3.2 ± 0.33*	6.5 ± 0.72

* $p < 0.001$ *versus* forskolin.

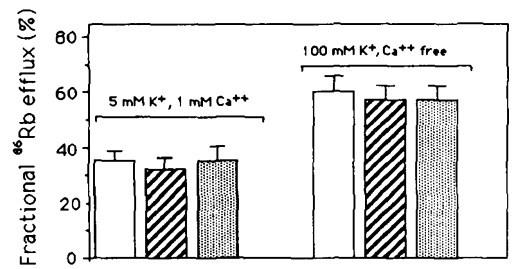

FIG. 7. **Effect of quinpirole on $^{86}Rb^+$ efflux in control GH-3 cells.** $^{86}Rb^+$ efflux was measured in GH-3 cells grown in F-10^+ as described under "Experimental Procedures." The calcium-dependent component was measured in a reaction solution containing 5 mM K^+ and 1 mM Ca^{2+} and the voltage-dependent one in a nominally calcium-free reaction solution containing 100 mM K^+. Cells were incubated for 5 s with buffer (□), 10 nM quinpirole (▨), and 100 nM quinpirole (▩). Each *bar* represents the mean ± S.E. of three independent experiments run in triplicate.

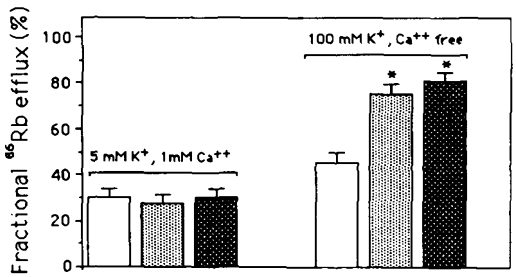

FIG. 8. **Effects of quinpirole on $^{86}Rb^+$ efflux in GH-3 cells grown in the presence of EGF.** The calcium-dependent component was measured in a reaction solution containing 5 mM K^+ and 1 mM Ca^{2+}, while the voltage-dependent Rb^+ efflux was defined in a nominally calcium-free reaction solution containing 100 mM K^+. Cells were incubated for 5 s with buffer (□), 10 nM quinpirole (□), and 100 nM quinpirole (■). Each *bar* represents the mean ± S.E. of three independent experiments run in triplicate. *$p < 0.001$ *versus* basal.

6*A*) or by 100 nM VIP (Fig. 6*B*) in broken cell membrane preparations. The results obtained with intact GH-3 cells are reported in Table I. 10 μM forskolin induced a 4-fold increase in intracellular cAMP accumulation, an effect which was inhibited by quinpirole over the range of 100 nM to 100 μM. In contrast, cAMP formation elicited by 100 nM VIP was not modified by up to 100 μM quinpirole.

Effects of D-2 Receptor Activation on Rubidium Fluxes—Two components of rubidium efflux, measured as an index of potassium channel activity (18, 29), were identified in GH-3 cells: one, calcium-dependent, which was measured in a reaction solution containing 1 mM Ca^{2+} and 5 mM K^+, and one, calcium-independent and voltage-sensitive, which was determined in a nominally calcium-free reaction solution containing 100 mM K^+; both components were insensitive to quinpirole, the fractional Rb^+ efflux being similar under basal conditions and in the presence of two different concentrations of the drug (10 and 100 nM) (Fig. 7). The data obtained by measuring Rb^+ efflux in GH-3 cells exposed for 4 days to 0.5 nM EGF are reported in Fig. 8. Quinpirole, up to 100 nM, did

not change the Rb^+ efflux measured in the presence of 5 mM K^+ and 1 mM Ca^{2+}; in contrast, quinpirole markedly potentiated Rb^+ efflux (45% without *versus* 80% with quinpirole) in a nominally calcium-free reaction solution containing 100 mM K^+. Pharmacological studies on the voltage-dependent component were performed with agonists selective for D-2 receptors. As reported in Fig. 9, bromocriptine and RU 24213 and DA itself induced a stimulation of Rb^+ efflux comparable to that produced by quinpirole. The involvement of a D-2 receptor was further confirmed by the results obtained with DA antagonists (Fig. 10). 1 μM (−)-sulpiride, unlike (+)-sulpiride, was able to completely prevent the effects of quinpirole on Rb^+ efflux; similarly, 1 μM haloperidol and 1 μM D-butaclamol reversed the Rb^+ efflux stimulation elicited by quinpirole.

EGF Selectively Changes G Protein α Subunit Content of GH-3 Plasma Membrane—The expression of G_o and G_i proteins in GH-3 cells was evaluated by Western blot analysis with specific antibodies to the α subunits of G_o, G_i1–2, and G_i3. As shown in Fig. 11, antibodies directed to the various α subunits recognize three major polypeptides with an apparent molecular mass of 42, 40, and 30 kDa (36).

Different amounts of each immunoreactive polypeptide species were present in naive GH-3 cell membranes with $G_i\alpha1$–2, $G_i\alpha3$ and $G_o\alpha$ present, respectively, at high, low, and undetectable levels. Exposure to EGF (0.5 nM) for 4 days did not modify the amount of G_o protein α subunit associated with GH-3 membranes. In contrast, G_i subunit subtypes were strongly and selectively changed by EGF treatment. As determined by optical densitometry of autoradiograms, antibodies anti-$G_i\alpha3$ detected a 9-fold increase of the 42-kDa polypeptide in GH-3 plasma membrane following EGF treatment. The relative amount of $G_i\alpha1$–2 was less affected by EGF treatment; a 50% decrease of the 42-kDa band was indeed observed (Fig. 11).

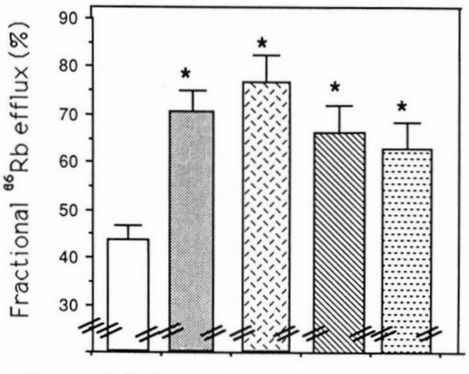

FIG. 9. **Effects of different dopamine agonists on the voltage-dependent component of $^{86}Rb^+$ efflux in EGF-treated GH-3 cells.** Rb^+ efflux was measured in a nominally calcium-free reaction solution containing 100 mM K^+. Cells were incubated for 5 s with buffer (□), 100 nM quinpirole (■), 100 nM bromocriptine (▨), 100 nM RU 24213 (▧), and 100 nM dopamine (□). Each *bar* is the mean ± S.E. of three independent experiments run in triplicate. *$p < 0.001$ *versus* basal.

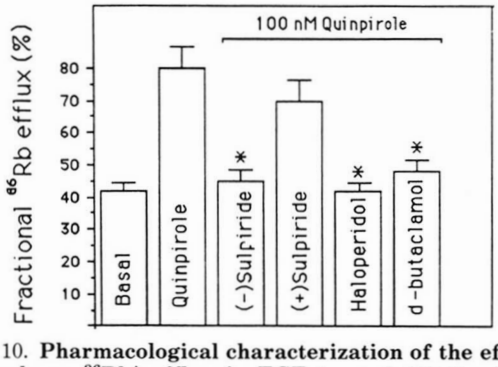

FIG. 10. **Pharmacological characterization of the effects of quinpirole on $^{86}Rb^+$ efflux in EGF-treated GH-3 cells with dopamine antagonists.** $^{86}Rb^+$ efflux was measured in a nominally calcium-free reaction solution containing 100 mM K^+. Cells were incubated for 5 s with buffer, 100 nM quinpirole, 100 nM quinpirole with 1 μM (−)-sulpiride, 1 μM (+)-sulpiride, 1 μM haloperidol, 1 μM D-butaclamol. Each *bar* represents the mean ± S.E. of three independent experiments run in triplicate. *$p < 0.001$ *versus* quinpirole.

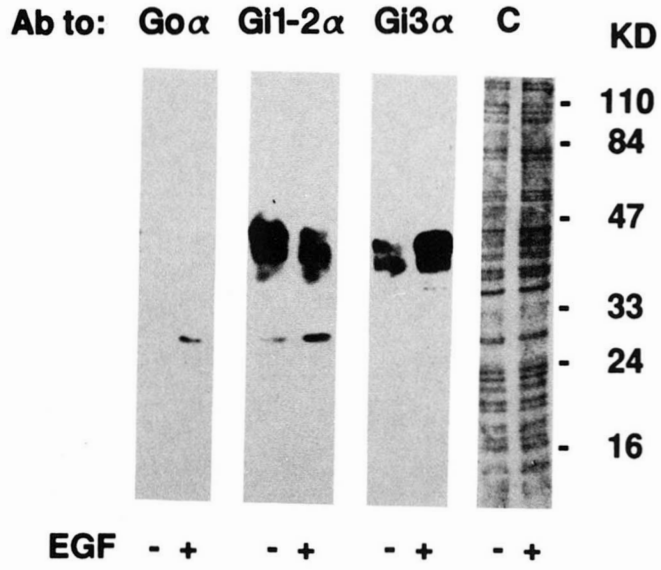

FIG. 11. **EGF-induced differences in G protein α subunit content of GH-3 cell membrane.** Membrane proteins from GH-3 cells grown for 4 days with (+) or without (−) EGF were loaded in quadruplicate on a 10% SDS-polyacrylamide gel (15 μg/lane) and separated electrophoretically. Western blot analysis of three sets of samples was performed using separately antibodies (*Ab*) to G_o subunit ($G_o\alpha$) and to G_i subtypes ($G_i\alpha1$–*2* and $G_i\alpha3$). One set of samples and the molecular mass markers that were stained with Coomassie Blue after electrophoresis, are shown on the *right side* of the figure as reference.

TABLE II

Induction of D-2-receptor isoforms by EGF in GH-3 cells

Quantification of D-2-receptor probe and cyclophilin hybridization bands in Fig. 12 by optic density measurement expressed in arbitrary units.

Cells	D-2(415)	D-2(444)	Cyclophilin
Controls	0.05	0.28	1.57
EGF-treated	0.32*	1.97*	1.62

* $p < 0.001$ *versus* controls.

Induction of D-2(444)- and D-2(415)-Receptor Isoforms by EGF—PCR products derived from both controls and EGF-treated GH-3 cells, resolved on agarose gel, comprised two DNA fragments of 336 and 423 base pairs in length consistent with two D-2 receptor mRNA species differing in a 87-nucleotide insert between TM5 and TM6. Both receptor forms were present in control GH-3 cells; as judged by the relative abundance of PCR-generated DNAs, the mRNA encoding the longer receptor form was expressed at approximately 6-fold higher levels than the mRNA for the shorter form (Table II). Both isoforms appeared to be highly induced (at least 7-fold) by EGF treatment as measured by the optical density of the hybridization bands using either oligo(A) specific for the D-2(444) form or the D-2(415) cDNA probe which hybridizes to both forms (Fig. 12 and Table II). The ratio D-2(444)/D-2(415) was not modified by the treatment. PCR amplification

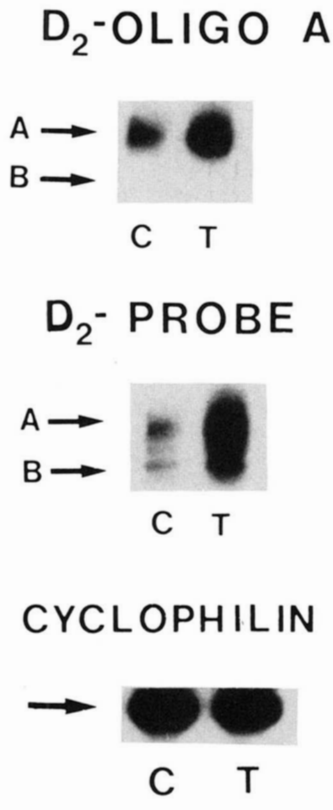

FIG. 12. **Induction of D-2-receptor isoforms by EGF in GH-3 cells. Cells were grown in F-10$^+$ containing 0.5 nM EGF for 4 days.** PCR fragments amplified with D-2-receptor specific primers (PCR I and PCR II) from either controls (*C*) or EGF-treated (*T*) GH-3 cells were blotted on nylon filters and hybridized with either D-2 oligo(A), specific for the D-2(444) isoform, or the D-2 probe, which recognizes both D-2(444) (*A*) and D-2(415) (*B*) isoforms. PCR fragments amplified with cyclophilin specific primers (PCR III and PCR IV) were hybridized with cyclophilin cDNA probe.

of control and EGF treated cells with cyclophilin primers (25 cycles) showed that the relative abundance of this mRNA was not significantly modified by EGF treatment (Fig. 12), indicating a differential pattern of transcriptional activation induced by the growth factor.

DISCUSSION

The study of D-2 receptor characteristics has been so far limited by the lack of a convenient experimental cell model; the present knowledge of D-2 receptor biochemistry and pharmacology derived, indeed, from studies carried out on pituitary, brain tissues, and primary culture preparations (14–20) which have the limitation of being not homogeneous cell systems.

The GH-3 cell line, which is widely used to study the regulation of PRL secretion, has the advantage of being a homogeneous cell population, but lacks D-2 receptors (4–8). Recently, we have developed a pure cell clone endowed with D-2 receptors; exposure to EGF for 4 days induced, indeed, the GH-3 cells to reexpress D-2 receptors as shown by the appearance of a high affinity binding for [^{3}H]spiroperidol and by the fact that selective D-2 agonists recovered the property to inhibit PRL secretion induced by depolarizing concentrations of potassium (8).

D-2 dopamine receptors are known to inhibit adenylate cyclase (14, 20, 28) as well as to activate potassium channels (16–19). The interpretation of these results, however, remains questionable since it has not been conclusively defined whether a single receptor entity activates different intracellular signals or multiple D-2 receptor subtypes are involved. Indeed, pharmacological studies have so far provided only preliminary evidence for D-2 receptor subtypes (37) and the molecular biology approach has revealed that two D-2 receptor isoforms may exist (10–13). These molecules, which appear to be generated by alternative splicing of primary transcripts of a single gene, differ from each other in the insertion of a stretch of 29 amino acids in the third cytoplasmic loop (10–13). Whether or not the "short" and the "long" molecular forms represent functionally distinct receptor subtypes remains to be determined.

In the present study we investigated the biochemical and molecular properties of D-2 receptors and their effector-coupling mechanisms in GH-3 cells treated with EGF. Both the D-2(444) and the D-2(415) receptor isoforms are induced severalfold by EGF, with no changes in the relative abundance ratio. It should be noted that, despite the absence of a measurable D-2 receptor binding, low levels of both D-2 receptor related mRNAs are detectable in naive GH-3 cells. Whether a defect in mRNA translation mechanisms in the GH-3 clone is involved, or whether the different sensitivities of the methods used to measure mRNA levels and the receptor biochemistry may be invoked to explain this finding, cannot be established from our present data.

Exposure of EGF-treated GH-3 cells to quinpirole blunted the secretion of PRL induced by NT. The detailed pharmacological analysis of this response was fully consistent with the involvement of a receptor indistinguishable from the dopamine D-2 receptor. Interestingly, the picture was completely different when quinpirole was tested on VIP-induced PRL release. The D-2 agonist was, indeed, virtually ineffective on this response, suggesting that EGF induced the expression of a D-2 receptor population which lacks coupling to the inhibition of cAMP-mediated responses. It is known, indeed, that VIP and NT stimulates PRL release by lactotrophs by activating different intracellular signals, the effects of VIP being cAMP-dependent (14, 20, 28) and those of NT independent of the adenylate cyclase system (15, 38).

D-2 receptors in pituitary lactotrophs appear to be directly associated with openings of voltage-dependent potassium channels and with openings of Ca^{2+}-activated potassium channels through inhibition of cAMP formation (16–19, 29). Electrophysiological studies demonstrated both voltage-dependent and Ca^{2+}-activated potassium currents in GH-3 cells (39, 40); similar results have been obtained with the neurochemical approach in the present study.

Quinpirole did not affect $^{86}Rb^+$ efflux occurring through Ca^{2+}-activated, cAMP-dependent potassium channels in EGF-treated GH-3 cells; in contrast, the D-2 agonist was very active in potentiating $^{86}Rb^+$ efflux through the voltage-dependent component. This effect was reproduced by other D-2 agonists and was prevented by selective D-2 antagonists, thus confirming the involvement of a D-2 receptor.

In line with these results are the data obtained by measuring cAMP formation in both intact cells and cell membrane preparations. Quinpirole failed indeed to inhibit adenylate cyclase activation induced by both forskolin and VIP in the membrane preparation obtained from EGF-treated GH-3 cells. Quinpirole did not modify cAMP formation elicited by VIP in intact cells as well; in contrast the drug did inhibit forskolin-induced activation of the cAMP generating system in the latter preparation. The simplest interpretation of this effect is that it is related to quinpirole-induced openings of potassium channels; it could be inferred that the cell hyper-

polarization impairs the capability of forskolin to stimulate adenylate cyclase.

Taken all together the data here reported are apparently at variance with those obtained with cDNA transfection experiments. Transfection of either the "short" or the "long" cDNA into human embryonic kidney 293 cells resulted, indeed, in the expression of D-2 receptors both associated with inhibition of adenylate cyclase (10). On the other hand, the characterization of the effector systems coupled to the short D-2 receptor transfected into the GH-3 related clone GH4C1 demonstrated that this molecule can transduce with similar efficiency at least two distinct responses: the inhibition of adenylate cyclase (41–43) and a K^+ channel-dependent hyperpolarization (44).

The data obtained by measuring G protein content in GH-3 cells after exposure to EGF may contribute to give insight into this matter. A marked increase in the amount of G_i3 associated with cell membranes was indeed found in EGF treated cell membranes; G_i1–2 immunoreactivity was less modified by the treatment causing a slight decrease in the 42-kDa band. G_i3 is responsible for muscarinic and somatostatin receptor-induced stimulation of potassium channels in GH-3 cells (45–48); purified D-2 receptors from bovine pituitary, on the other hand, couple with higher affinity to G_i2 than to G_i3, G_i3 being maximally activated at a 10-fold higher G protein/receptor ratio (27). The high increase in the amount of G_i3 in GH-3 cells exposed to EGF may raise the G_i3/D-2 receptor ratio to the appropriate value to promote the maximal activation of G_i3. The imbalance of the G_i2/G_i3 ratio produced by EGF in GH-3 cells could thus account for the preferential coupling of D-2 receptors with G_i3 leading to a selective activation of potassium channels.

In conclusion, EGF has two major effects on GH-3 cells: 1) it promotes the expression of both the short and the long D-2 receptor isoforms and 2) it markedly increases the amount of G_i3 subunit associated with the plasma membrane. These effects result in the appearance of a D-2 receptor protein which activates a specific transmembrane signaling system opening potassium channels and inhibiting PRL release by cAMP-independent mechanisms. The relative amounts of the different G_i proteins present in the cell membrane, rather than the specific subtype by itself, may thus influence the choice of the effector system by an individual D-2 receptor molecule.

REFERENCES

1. Tashjian, A. H., Bancroft, F. C., and Levine, L. (1970) *J. Cell Biol.* **47,** 61–70
2. Tashjian, A. H., Bancroft, F. C., Levine, L., Sato, G. H., and Parker, M. L. (1968) *Endocrinology* **82,** 342–352
3. Gourdij, D., Tougard, C., and Tixier-Vidal, A. (1982) *Front. Neuroendocrinol.* **7,** 317–357
4. Bang, S., and Gautvik, K. M. (1977) *Acta Pharmacol. Toxicol.* **41,** 317–327
5. Malarkey, W. B., Groshong, J. C., and Milo, G. E. (1977) *Nature* **266,** 640–641
6. Cronin, M. J., Faure, N., Martial, J. A., and Weiner, R. I. (1980) *Endocrinology* **106,** 718–723
7. Faure, N., Cronin, M. J., Martial, J. A., and Weiner, R. I. (1980) *Endocrinology* **107,** 1022–1026
8. Missale, C., Castelletti, L., Boroni, F., Memo, M., and Spano, P. F. (1991) *Endocrinology* **127,** 13–20
9. Schonbrunn, A., Krasnoff, M., Westendorf, J. M., and Tashjian, A. H. (1980) *J. Cell Biol.* **85,** 786–797
10. Dal Toso, R., Sommer, B., Ewert, M., Herb, A., Pritchett, D. B., Bach, A., Shivers, B. D., and Seeburg, P. H. (1989) *EMBO J.* **8,** 4025–4034
11. Giros, B., Sokoloff, P., Martres, M. P., Riou, J. F., Emorine, L. J., and Schwartz, J. C. (1989) *Nature* **342,** 923–926
12. Monsma, F. J., McVittie, L. D., Gerfen, C. R., Mahan, L. C., and Sibley, D. R. (1989) *Nature* **342,** 926–929
13. Grandy, D. K., Marchionni, M. A., Makam, H., Stofko, R. E., Alfano, M., Frothingham, L., Fischer, J. B., Burke Howie, K. J., Bunzow, J. R., Server, A. C., and Civelli, O. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9762–9766
14. De Camilli, P., Macconi, D., and Spada, A. (1979) *Nature* **278,** 252–254
15. Memo, M., Castelletti, L., Missale, C., Valerio, A., Carruba, M. O., and Spano, P. F. (1986) *J. Neurochem.* **47,** 1689–1695
16. Ingram, C. D., Bicknell, R. J., and Mason, W. T. (1986) *Endocrinology* **119,** 2508–2518
17. Israel, J. M., Kirk, C., and Vincent, D. (1987) *J. Physiol.* **390,** 1–22
18. Castelletti, L., Memo, M., Missale, C., Spano, P. F., and Valerio, A. (1989) *J. Physiol.* **410,** 251–265
19. Freedman, J. E., and Weight, F. F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3618–3622
20. Vallar, L., and Meldolesi, J. (1989) *Trends Pharmacol. Sci.* **10,** 74–77
21. Bray, P., Carter, A., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. M., and Nirenberg, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5115–5119
22. Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kowasaki, H., Suzuki, K., and Kaziro, Y. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 3776–3780
23. Jones, D. T., and Reed, R. R. (1987) *J. Biol. Chem.* **262,** 14241–14249
24. Michel, T., Winslow, J. W., Smith, J. A., Seidman, J. G., and Neer, E. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 7663–7667
25. Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Haga, T., Ichiyama, A., Kanagawa, K., Hiranga, M., and Matsuo, H. (1986) *FEBS Lett.* **197,** 305–310
26. Van Meurs, K. P., Angus, C. W., Lavu, S., Kung, H. F., Czarnecki, S. K., Moss, J., and Vaughan, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3107–3111
27. Senogles, S. E., Spiegel, A. M., Padrell, E., Iyengar, R., and Caron, M. G. (1990) *J. Biol. Chem.* **265,** 4507–4514
28. Onali, P. L., Schwartz, J. P., and Costa, E. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6531–6534
29. Bartschat, D. K., and Blaustein, M. P. (1985) *J. Physiol.* **361,** 419–440
30. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
31. Laemmli, U. K. (1970) *Nature* **277,** 680–685
32. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
33. Maniatis, T., Fritsch, and E. F., Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Mullis, K. B., and Faloona, F. (1987) *Methods Enzymol.* **155,** 335–340
35. Danielson, P. E., Forss Petter, S., Brow, M. A., Calavetta, L., Douglass, J., Milner, R. S., and Sutcliffe, G. J. (1988) *DNA* **7,** 261–267
36. Weiss, E. R., Kelleher, D. J., Woon, C. W., Soparkar, S., Osawa, S., Haesely, L. E., and Johnson, G. L. (1988) *FASEB J.* **2,** 2841–2848
37. Pizzi, M., Valerio, A., Benarese, M., Missale, C., Carruba, M. O., Memo, M., and Spano, P. F. (1990) *Mol. Neuropharmacol.* **1,** 37–42
38. Enjalbert, A., Arancibia, S., Ruberg, M., Priam, M., Bluet-Pajot, M. T., and Kordon, C. (1982) *Neuroendocrinology* **34,** 95–98
39. Dubinsky, J. M., and Oxford, G. S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4282–4286
40. Richtie, A. K. (1987) *J. Physiol.* **385,** 611–625
41. Albert, P. R., Neve, K. A., Bunzow, J. R., and Civelli, O. (1990) *J. Biol. Chem.* **265,** 2098–2104
42. Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M. D., Machida, C. A., Neve, K. A., and Civelli, O. (1988) *Nature* **336,** 22–29
43. Neve, K. A., Henningsen, R. A., Bunzow, J. R., and Civelli, O. (1989) *Mol. Pharmacol.* **36,** 446–451
44. Vallar, L., Muca, C., Magni, M., Albert, P. R., Bunzow Meldolesi, J., and Civelli, O. (1990) *J. Biol. Chem.* **265,** 10320–10326
45. Codina, J., Grenet, D., Yatani, A., Birnbaumer, L., and Brown, A. M. (1987) *FEBS Lett.* **385,** 611–625
46. Yatani, A., Codina, J., Sekura, R. D., Birnbaumer, L., and Brown, A. M. (1987) *Mol. Endocrinol.* **1,** 283–289
47. Mattera, R., Yatani, A., Kirsch, G. E., Graf, R., Okabe, K., Olate, J., Codina, J., Brown, A. M., and Birnbaumer, L. (1989) *J. Biol. Chem.* **264,** 465–471
48. Brown, A. H., and Birnbaumer, L. (1990) *Annu. Rev. Physiol.* **52,** 197–213
49. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649