0026-895X/94/051022-07$03.00/0

MOLECULAR PHARMACOLOGY, 45:1022-1028

Properties and Regulation of the Coupling to Adenylate Cyclase of Secretin Receptors Stably Transfected in Chinese Hamster Ovary Cells

J. P. VILARDAGA, E. CICCARELLI, C. DUBEAUX, P. DE NEEF, A. BOLLEN, and P. ROBBERECHT

Laboratory of Biochemistry and Nutrition, Faculty of Medicine (E.C., P.D.N., P.R.), and Laboratory of Applied Genetics, Faculty of Sciences (J.P.V., C.D., A.B.), Université Libre de Bruxelles, B-1070, Brussels, Belgium

Received November 23, 1993; Accepted February 9, 1994

SUMMARY

The binding properties, coupling to adenylate cyclase, and desensitization of secretin receptors stably expressed in transfected Chinese hamster ovary (CHO) cells were compared in two clones expressing high (CHO-SnR-c5, 450 ± 80 fmol/mg of protein) and low (CHO-SnR-c1, 40 ± 25 fmol/mg of protein) receptor densities. The K_d values for receptor occupancy by secretin, selected analogues, and fragments were identical in CHO-SnR-c1 and -c5 cells and identical to those described for native receptors from NG 108–15 cells. The K_{act} values for adenylate cyclase stimulation were identical to the K_d values in CHO-SnR-c1 cells but 5–10-fold higher than those in CHO-SnR-c5 cells. The K_{act} values in both CHO-SnR-c1 and -c5 cell lines were reduced in the presence of the nonhydrolyzable GTP derivative guanosine-5′-(β,γ-imido)triphosphate and after pretreatment of the cells with cholera toxin. Preincubation of both CHO-SnR-c1 and -c5 cell lines with secretin for 24 hr reduced their binding capacity and reduced secretin efficacy in CHO-SnR-c1 cells and secretin potency in CHO-SnR-c5 cells. These results suggest efficient coupling of the secretin receptor to the adenylate cyclase machinery and the existence of spare receptors in the clone expressing higher receptor density. Pretreatment of the two cell lines with the reducing agent dithiothreitol reduced the binding capacity and induced the appearance of a low affinity binding component. In both cell lines, dithiothreitol pretreatment decreased secretin potency but not secretin efficacy, suggesting the necessity of integrity of the disulfide bridges for optimal receptor recognition.

The molecular cloning of the rat secretin receptor from neuroglioma NG108–15 cells was achieved in 1991 by Ishihara *et al.* (1). Among the seven-helix receptors known at that time, the secretin receptor was related to the calcitonin (2) and parathormone receptors (3), defining a new subfamily of parent receptors. The subsequent cloning of the VIP (4), GRF (5), glucagon-like peptide 1 (6), and glucagon (7, 8) receptors confirmed the concept of a family of related receptors, the counterpart of the concept of a superfamily of peptide ligands. The availability of the secretin receptor sequence permits transfection of the DNA coding for the receptor and stable expression of the receptors in CHO cells that do not constitutively express secretin, VIP, GRF, or glucagon receptors. We used CHO cells transfected with the rat secretin receptor to analyze the structural requirements for binding of the ligand and coupling of the receptor to the adenylate cyclase system. Convenient models for studying the pharmacological and biological properties of the secretin receptor are rare. Secretin receptors are expressed at high density on pancreatic acinar cells (9) and on purified pancreatic membranes (10, 11), but the complex pattern of multiple classes of receptors for parent peptides makes interpretation of the data ambiguous. NG108–15 neuroglioma cells express a small number of secretin receptors (12); sodium butyrate increases that number, but the fatty acid may affect receptor glycosylation (13).

We first obtained a cell line that expressed a high receptor density and found that agonists were 4–10-fold more potent for adenylate cyclase activation than for receptor occupancy. This observation suggested the existence of an amplification process (existence of spare receptors) in the cell clone studied. This hypothesis was directly tested and proved by comparing binding data and adenylate cyclase activation for two CHO clones that expressed different concentrations of receptors and for cells pretreated with secretin.

We also studied the consequences of pretreatment with the reducing agent DTT, which was previously reported to decrease binding capacity and secretin adenylate cyclase activation on

This work was supported by Science Plan SC1*CT91–0632 from the Commission of the European Communities and by Grant 7.4587.92 from the Fonds de la Recherche Scientifique et Médicale, Belgium (Télévie).

ABBREVIATIONS: VIP, vasoactive intestinal peptide; K_{act}, concentration exerting half-maximal stimulation of adenylate cyclase; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; GRF, growth hormone release factor; DTT, dithiothreitol; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHO, Chinese hamster ovary.

pancreatic membranes (14). We finally explored the effect of permanent activation of G_s on the efficacy of coupling of the secretin receptor to the adenylate cyclase system.

Experimental Procedures

Construction of the expression plasmid, transfection, selection, and expression in CHO cells. The DNA coding for the rat secretin receptor was excised from the plasmid pCDM8 (kindly provided by Prof. S. Nagata, Osaka Bioscience Institute, Osaka, Japan) (1) by digestion with *Hind*III and *Not*I, treated with DNA polymerase I (Klenow fragment), and cloned into the *Hind*III and blunted *Xba*I sites of the mammalian expression vector pNIV187, a derivative of pRc/RSV (Invitrogen) that contains the selectable neomycin phosphotransferase gene and an expression cassette for dihydrofolate reductase, as described by Connors *et al.* (15). The resulting recombinant plasmid, pNIV1938, was transfected into the CHO cell line DG44 (16) by electroporation using a gene pulser. Approximately 10^7 cells were preincubated on ice for 30 min with 25 μg of DNA in 0.8 ml of 7 mM sodium phosphate buffer, pH 7.4, containing 272 mM sucrose and 1 mM $MgCl_2$. Electroporation was performed at 600 V and 3 μF. After electroporation, cells were kept on ice for 10 min, added to 10 ml of culture medium, and harvested in 96-well plates. Cells were maintained in α minimal essential medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin, under an atmosphere of 95% air/5% CO_2 at 37°.

Forty-eight hours after transfection, G418-resistant cells were selected by the addition of geneticin to a final concentration of 450 μg/ml. Selection was done in 96-well plates, with 2000–5000 cells/well. To produce sufficient amounts of cells for receptor characterization, the clones were then grown to confluency in six-well plates of 35-mm diameter.

The clones used in the present study, hereafter referred to as CHO-SnR-c1 and CHO-SnR-c5, were obtained without amplification and were selected on the basis of low and high binding capacity for ^{125}I-secretin and low and high secretin-stimulated adenylate cyclase activity, respectively. Geneticin at 0.5 mg/ml was maintained in the culture medium of the stock culture. Subcultures prepared for membrane purification were grown in medium without geneticin.

Membrane preparation, receptor identification, and measurement of adenylate cyclase activity. Cells were detached with a rubber policeman and pelleted by low speed centrifugation; the supernatant was discarded and the cells were lysed in 1 mM $NaHCO_3$ solution and immediately frozen in liquid nitrogen. After thawing, the lysate was centrifuged at 800 × *g* for 10 min and the supernatant was further centrifuged at 20,000 × *g* for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$, was used immediately as a crude membrane preparation.

^{125}I-Secretin was labeled by the chloramine T method, as described (12), and was purified by adsorption on a cellulose column eluted with BSA steps. Tracer specific radioactivity was 1.5 mCi/nmol. Binding of the tracer to the membranes was performed as described (12). Saturation curves were measured with increasing concentrations of iodosecretin and competition curves with a fixed tracer concentration (equal to the K_d of the tracer) and increasing concentrations of unlabeled peptides. In all cases the nonspecific binding was defined as the residual binding in the presence of 1 μM secretin. Binding was performed at 37° in 20 mM Tris-maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1% BSA, pH 7.4. Fifteen to 20 μg of protein were used in each assay. The bound radioactivity was separated from free radioactivity by filtration through GF/C glass fiber filters (which had been presoaked for 24 hr in 0.1% polyethyleneimine), which were rinsed three times with 20 mM phosphate buffer, pH 7.4, containing 1% BSA.

Adenylate cyclase activity was determined by the method of Salomon *et al.* (17). Membrane proteins (5–10 μg) were incubated in a total volume of 60 μl containing 0.5 mM [α-^{32}P]ATP, 10 μM GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho(enol) pyruvate, 30 μg/ml pyruvate kinase, and 30 mM Tris·HCl, at a final pH of 7.5. The reaction was initiated by addition of membranes and was terminated after a 12-min incubation at 37° by addition of 0.5 ml of cAMP and 20,000 cpm of [8-^{3}H]cAMP. cAMP was separated from ATP by two successive chromatographic steps on Dowex 50-WX8 and neutral alumina.

Pretreatment of cells. Pretreatment with secretin (for 24 hr) or with cholera toxin (for 2 hr) was done with confluent cells. Attached cells were washed twice with culture medium without fetal calf serum, harvested with a rubber policeman, and centrifuged at low speed. The cells were lysed with 1 mM $NaHCO_3$ and immediately frozen in liquid nitrogen. The lysate was stored at −80° until membrane preparation.

Materials. Restriction endonucleases, T4 DNA ligase, and *Escherichia coli* DNA polymerase I (Klenow) were purchased from Amersham, Boehringer-Mannheim, New England Biolabs, and GIBCO-BRL. Neomycin (Geneticin, G418), cell culture medium (α minimal essential medium), L-glutamine, penicillin, streptomycin, and fetal calf serum were from GIBCO-BRL. Secretin was a kind gift from Dr. L. Moroder (Max Planck Institute, München, Germany). All other peptides were synthesized in our laboratory by A. Vandermeers, M.-C. Vandermeers-Piret, and P. Gourlet, by solid-phase methodology using the 9-fluorenylmethoxycarbonyl strategy. The origins of all of the products used in the adenylate cyclase assay and in the binding assay were reported in previous publications (11, 12).

Results

Comparison of cell lines expressing large and small numbers of secretin receptors. As established by Scatchard analysis of saturation curves (Fig. 1A), clone 1 expressed a 10-fold lower secretin receptor density than did clone 5 (40 ± 15 fmol/mg of protein, compared with 450 ± 80 fmol/mg of protein, mean ± standard error of four determinations). Cellular growth rates of the two cell lines were identical. Adenylate cyclase activities were comparable (Table 1), although the

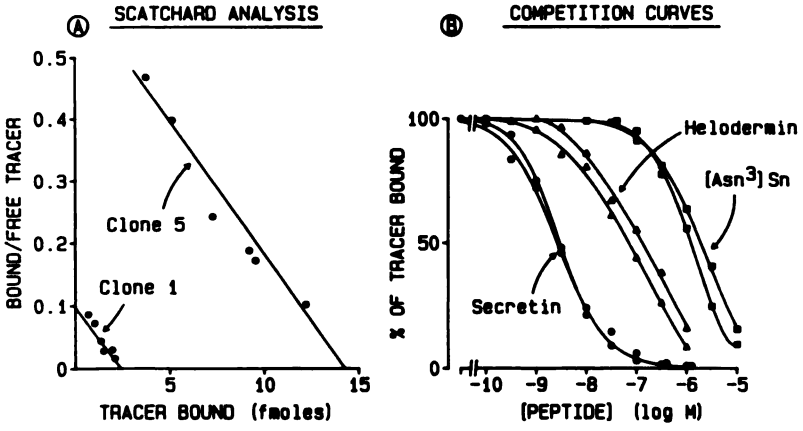

Fig. 1. A, Scatchard analysis of saturation curves obtained by incubating 35 μg of membrane protein from clone 1 or clone 5 in the presence of increasing concentrations of ^{125}I-secretin, in a total volume of 120 μl. Nonspecific binding was measured in the presence of 1 μM secretin. One representative experiment of four others is shown. B, Dose-dependent inhibition of ^{125}I-secretin binding (at a concentration 2-fold lower than the K_d) by unlabeled secretin (*circles*), helodermin (*triangles*) or [Asn^3]secretin (*squares*) with membranes from clone 1 (*open symbols*) or clone 5 (*closed symbols*). The results, expressed as percentage of tracer specifically bound, are the mean of at least three experiments.

TABLE 1

Adenylate cyclase activity of membranes from the cell lines CHO-SnR-c1 (clone 1) and CHO-SnR-c5 (clone 5)

The data were from untreated cells, from cells treated for 2 hr with 0.1 μg/ml cholera toxin (and their corresponding controls), and from membranes treated or not with 1 or 5 mM DTT. All values were expressed as pmol of cAMP produced/min/mg of protein and were given as mean ± standard error.

Cells and conditions	n[a]	Adenylate cyclase activity					
		Basal	Gpp(NH)p (10^{-5} M)	NaF (10 mM)	Forskolin (10^{-5} M)	GTP (10^{-5} M)	Secretin (10^{-6} M)
		pmol/min/mg					
Clone 1	10	5 ± 1	208 ± 8	220 ± 8	51 ± 4	9 ± 1	174 ± 6
Clone 5	10	4 ± 1	177 ± 7[b]	171 ± 8[b]	49 ± 5	9 ± 1	237 ± 8[b]
Clone 1							
Control	3	5 ± 2	170 ± 20	236 ± 15	37 ± 5	9 ± 3	160 ± 15
Cholera toxin	3	38 ± 5[c]	231 ± 20	219 ± 20	125 ± 15	196 ± 15[c]	340 ± 20[c]
Clone 5							
Control	3	4 ± 2	102 ± 13	166 ± 10	36 ± 5	8 ± 3	280 ± 20
Cholera toxin	3	34 ± 5[c]	130 ± 15	120 ± 10	123 ± 20	152 ± 10[c]	342 ± 30[c]
Clone 1							
Control	3	7 ± 2	291 ± 15	276 ± 20	65 ± 8	13 ± 4	282 ± 20
1 mM DTT	3	8 ± 2	369 ± 20[c]	403 ± 25[c]	59 ± 10	18 ± 3	342 ± 25
5 mM DTT	3	10 ± 2	438 ± 20[c]	466 ± 23[c]	52 ± 5[c]	21 ± 5	360 ± 20[c]
Clone 5							
Control	3	4 ± 2	200 ± 15	189 ± 15	52 ± 4	11 ± 2	290 ± 23
1 mM DTT	3	6 ± 2	189 ± 16	190 ± 16	47 ± 3	10 ± 2	322 ± 20
5 mM DTT	3	5 ± 2	191 ± 15	197 ± 15	39 ± 4	9 ± 2	298 ± 25

[a] n, number of experiments.
[b] Significant difference between clone 1 and clone 5.
[c] Difference between treated and untreated conditions.

maximum effect of secretin was weakly but significantly reduced in clone 1 (Fig. 2). The specificity and selectivity of the transfected secretin receptor were established by determination of the relative potencies of secretin, selected secretin analogues and fragments, and related peptides to inhibit ^{125}I-secretin binding (Table 2). With both clones, the IC_{50} for unlabeled secretin was 2 ± 0.2 nM (mean ± standard error of six experiments), corresponding, after the Cheng and Prusoff correction (18), to a K_d of 1.0 ± 0.1 nM; helodermin and VIP were 30- and 10,000-fold less potent, respectively, than secretin. Glucagon, truncated glucagon-like peptide 1, and GRF were inactive at 10 μM concentrations (data not shown).

A 10,000-fold increase of the IC_{50} was also observed after deletion of the amino-terminal histidine [secretin(2–27)]. Further deletions of amino acids 2, 3, 4, 5, and 6 did not markedly modify that pattern. Aspartate in position 3 was critical for receptor recognition, inasmuch as its substitution by asparagine or glutamic acid increased the IC_{50} by 1000- and 30-fold, respectively.

The concentrations required for 50% inhibition of tracer binding were not different in the two clones (Fig. 1B; Table 2). The peptide concentrations required for half-maximal stimulation of adenylate cyclase activity were lower in membranes from clone 5 than from clone 1 (Fig. 2; Table 2) for all analogues tested. The inhibitory constants for secretin fragment secretin(6–27) were identical in the two cell lines (Table 2). Thus, as summarized in Fig. 3, 50% of the receptors must be occupied in clone 1 to provoke half-maximal adenylate cyclase activation, whereas occupancy of 10% of the receptors is sufficient in clone 5.

Effects of secretin pretreatment on secretin receptors and their coupling to adenylate cyclase activity. Cells were preincubated for 24 hr with 0.1, 1.0, or 10 nM secretin, washed, and lysed. Membranes were prepared and tested for

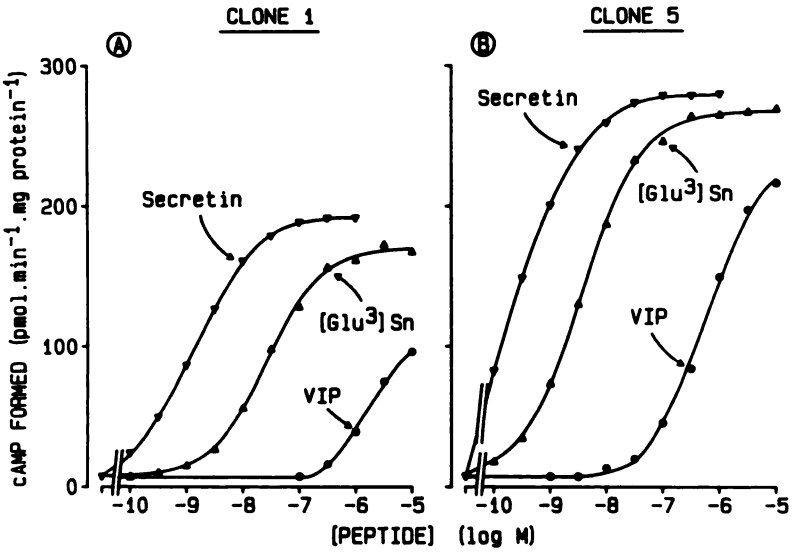

Fig. 2. Dose-effect curves for adenylate cyclase activation by secretin (*inverted triangles*), [Glu^3]secretin (*triangles*), or VIP (*circles*) of membranes from clone 1 (A) or clone 5 (B). The values, expressed as pmol of cAMP formed/min/mg of protein, are the mean of four experiments.

TABLE 2

Characteristics of the secretin receptor expressed by CHO-SnR-c1 (clone 1) and CHO-SnR-c5 (clone 5) cell lines

The K_d of binding and the K_{act} for adenylate cyclase activation were derived from competition curves and dose-effect curves. The K_i of the secretin fragment secretin (6–27) was calculated from dose-effect curves for secretin in the presence of three concentrations of the antagonist (mean of three experiments).

Peptide tested	Clone 1		Clone 5	
	K_d	K_{act} or K_i	K_d	K_{act} or K_i
	nM	nM	nM	nM
Secretin	2	2	2	0.3
Helodermin	40	30	50	6
VIP	10,000	10,000	10,000	1,000
Secretin(2-27)	10,000	10,000	10,000	300
Secretin(6-27)	15,000	10,000	20,000	10,000
[Glu^3]Secretin	50	30	40	4
[Asn^3]Secretin	800	1,000	1,000	150

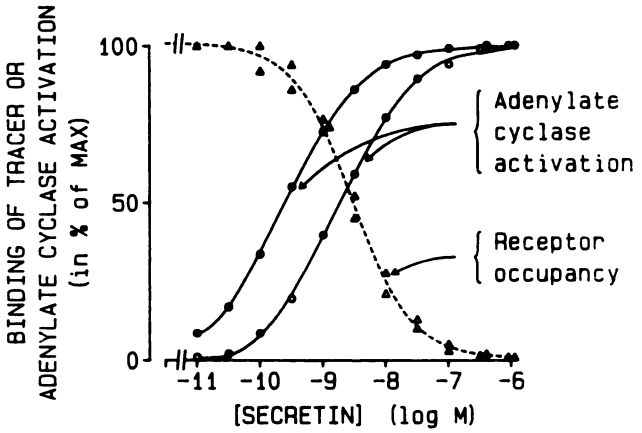

Fig. 3. Comparison between the ability of secretin to inhibit tracer binding (*triangles*) and to stimulate adenylate cyclase activity (*circles*). The results were derived from the data presented in Figs. 1 and 2. Adenylate cyclase activation curves were calculated as percentage of maximal stimulation after substraction of the basal value. *Open symbols*, clone 1; *closed symbols*, clone 5.

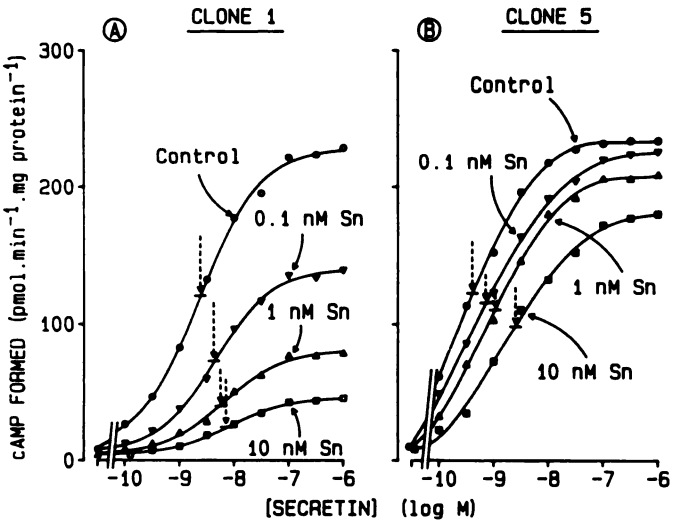

Fig. 4. Dose-effect curves for secretin-stimulated adenylate cyclase activity in membranes from clone 1 (A) and clone 5 (B) cells treated without (*circles*) or with 10^{-10} M (*inverted triangles*), 10^{-9} M (*triangles*), or 10^{-8} M (*squares*) secretin (*Sn*). *Dashed arrows*, K_{act} of secretin. The results are the mean of three experiments and are expressed as pmol of cAMP formed/min/mg of protein.

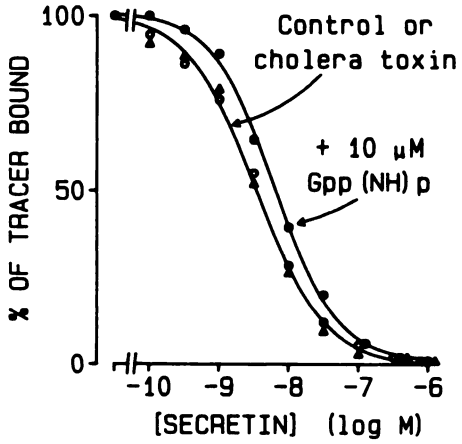

Fig. 5. Inhibition of ^{125}I-secretin binding by secretin in control membranes (○), in membranes in the presence of 10 μM Gpp(NH)p (●), or in membranes from cells treated for 2 hr with 0.1 μg/ml cholera toxin (△). The experiments were performed on clone 5 cell membranes. Results, expressed as percentage of tracer bound in the absence of unlabeled secretin, are the mean of three determinations.

their ability to bind ^{125}I-secretin and to stimulate adenylate cyclase activity. The binding capacity was evaluated for membranes from clone 5 by complete competition curves (which presented the same IC_{50} value) and for membranes from clone 1 by a single binding value in the absence of unlabeled peptide plus the corresponding nonspecific binding. The binding capacity, which can be considered as an evaluation of the number of high affinity receptors, was reduced in clone 5 to 60 ± 10, 40 ± 10, and 20 ± 8% and in clone 1 to 35 ± 10, 15 ± 8, and 5 ± 5% (mean ± standard error of three experiments) of the control value after pretreatment with 0.1, 1.0, or 10 nM secretin, respectively. Although it is difficult to be sure that after secretin pretreatment and washing there in no residual binding of secretin, it must be pointed out that after treatment there was no increase of the unstimulated adenylate cyclase activity (see below), suggesting that, if receptors were still occupied, their number was limited or they were uncoupled from the adenylate cyclase machinery.

Adenylate cyclase activation was determined with the same membrane preparations. Basal and Gpp(NH)p-, NaF-, GTP-, and forskolin-stimulated activities were not modified by secretin treatment. The secretin stimulation was altered, as presented in Fig. 4. In clone 1, preincubation reduced the maximum effect of secretin, with only a small increase in the K_{act}. In clone 5, there was a progressive increase in the K_{act} and a significant decrease in the maximum response was observed after preincubation with 10 nM secretin.

Effects of forskolin, Gpp(NH)p, or cholera toxin pretreatment on secretin receptors and their coupling to adenylate cyclase activity. In clone 5, the binding properties of secretin were unchanged in the presence of forskolin (data not shown) or after an efficient 2-hr pretreatment of the cells with 0.1 μg/ml cholera toxin (Fig. 5; Table 1). In the presence of 10 μM Gpp(NH)p, tracer binding was reduced by 30 ± 8% (mean ± standard error of three experiments) and the curve for inhibition of tracer binding by unlabeled secretin was moderately but significantly shifted to the right (from an IC_{50} of 3.0 ± 0.3 nM to 6.2 ± 0.5 nM, mean ± standard error of three experiments, $p < 0.05$ for paired values) (Fig. 5). These parameters were not evaluated for clone 1. Forskolin (in the presence of GTP) potentiated the effect of secretin in clones 1 and 5 (i.e., the combined effect of the two stimuli was larger than the sum of the individual effects) but did not significantly change

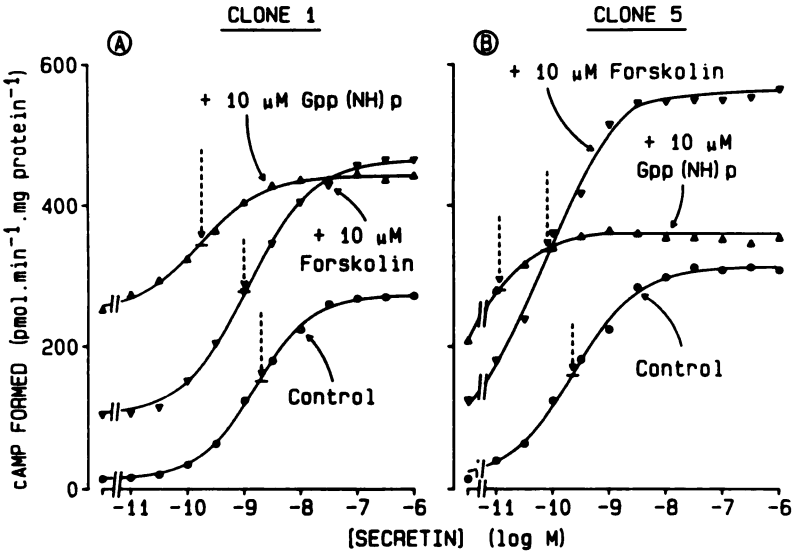

Fig. 6. Dose-effect curves for secretin in the presence of 10^{-5} M GTP (control) (*circles*) or in the combined presence of 10^{-5} M GTP and 10^{-5} M forskolin (*inverted triangles*) or 10^{-5} M Gpp(NH)p (*triangles*), with membranes from clone 1 (A) or clone 5 (B) cells. *Dashed arrows*, K_{act} for secretin. The results, expressed as pmol of cAMP formed/min/mg of protein, are the mean of three experiments.

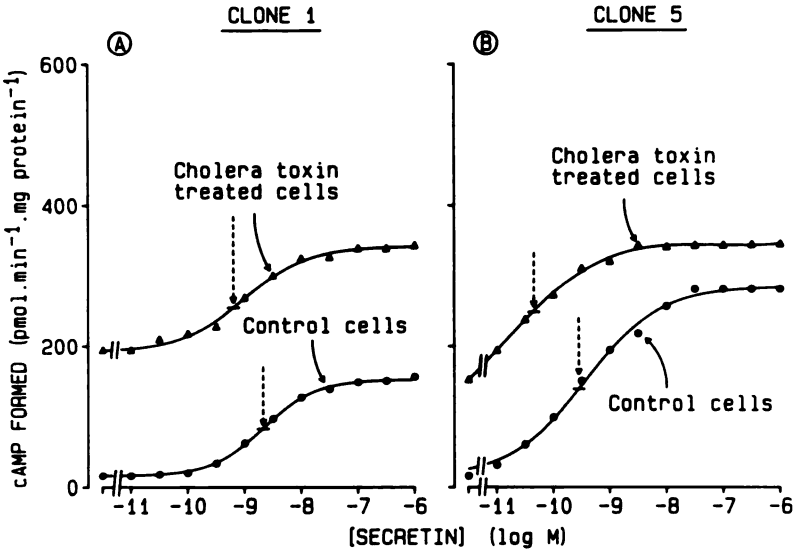

Fig. 7. Dose-effect curves for secretin with membranes from control cells (*circles*) or cells treated for 2 hr with 0.1 μg/ml cholera toxin (*triangles*). A, Clone 1; B, clone 5. Values are mean of three experiments.

the K_{act} of secretin (Fig. 6). In the presence of Gpp(NH)p, the amplitude of the secretin response was increased but the K_{act} was reduced 10-fold in clone 1 and 15-fold in clone 5. In clone 5, in the presence of Gpp(NH)p the K_{act} of secretin was thus as low as 0.01 nM (Fig. 6). Dose-effect curves for secretin in the presence of GTP in membranes prepared from cells treated with cholera toxin were similar to those observed in the presence of Gpp(NH)p with control cell membranes (Fig. 7), although the K_{act} of secretin was less reduced (4-fold reduction for clone 1 and 8-fold reduction for clone 5).

Effects of membrane treatment with DTT on secretin receptors and their coupling to adenylate cyclase. Binding properties of the secretin receptor after DTT treatment were investigated in clone 5 only (Fig. 8). The Scatchard analysis was compatible with the presence of one class of binding sites; the apparent number of receptors was reduced by 45 ± 10 and 60 ± 5% (mean ± standard error of three experiments) after exposure to 1 and 5 mM DTT, respectively, and the affinity of the remaining receptors was reduced 1.5-fold.

Competition curves, however, revealed the appearance of a progressively increasing component with low affinity (Hill coefficient of 0.87 ± 0.3, 0.80 ± 0.3, and 0.77 ± 0.1 in control membranes and membranes pretreated with 1 and 5 mM DTT, respectively; the value obtained after 5 mM DTT treatment was significantly different from control values, $p < 0.05$). Adenylate cyclase determinations were performed on clones 1 and 5. Preincubation with 1 or 5 mM DTT significantly increased the maximal response to GTP, Gpp(NH)p, NaF, and secretin in clone 1, had no effect on these parameters in clone 5, and decreased the response to forskolin in both clones (Table 1). The K_{act} for secretin was increased 10-fold after 5 mM DTT incubation (Fig. 9).

Discussion

In studies on hormone receptor interactions, discrepancies between binding data and biological data (even when the measured parameter is close to the binding step) are frequent, particularly when peptide hormones are tested; the tracer is usually a high affinity agonist sensitive to the presence of guanyl nucleotides and its proteolytic degradation can be reduced but rarely abolished. Furthermore, receptor subclasses may coexist and cross-recognition of peptides by parent receptors may occur; in addition, each receptor may be differently coupled to the effector. The stable transfection and expression of one defined receptor in cells that do not constitutively

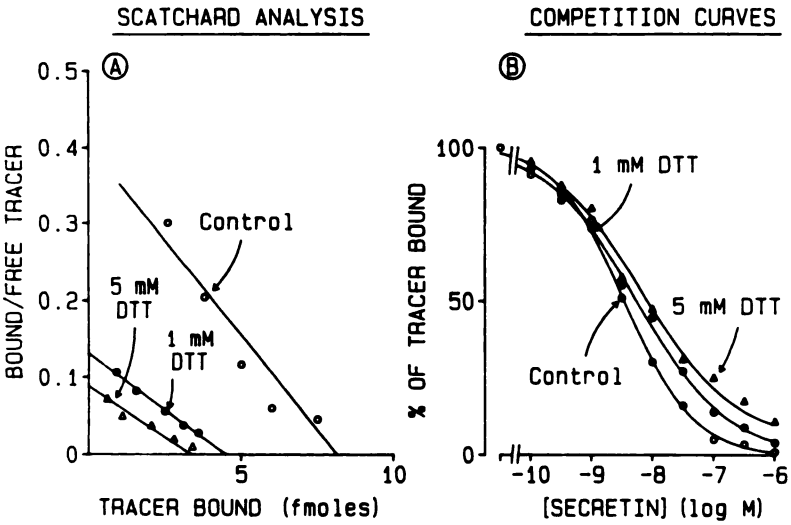

Fig. 8. A, Scatchard analysis of saturation curves for ^{125}I-secretin binding to membranes preincubated without (○) or with 1 mM (●) or 5 mM (△) DTT. The membranes were prepared from clone 5 cells and used in this representative experiment at a concentration of 32 μg of protein/assay. B, Inhibition of ^{125}I-secretin binding by unlabeled secretin with clone 5 membranes prepared as described for A. The results, expressed as percentage of tracer bound in the absence of competitor, are the mean of three experiments.

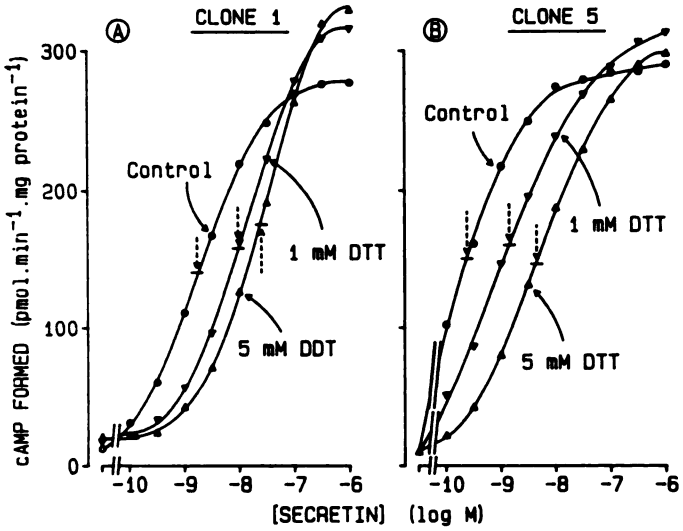

Fig. 9. Dose-effect curves for adenylate cyclase activation by secretin in membranes from clone 1 (A) and clone 5 (B) cells treated without (*circles*) or with 1 mM (*inverted triangles*) or 5 mM (*triangles*) DTT. *Dashed arrows*, K_{act}. The results, expressed as pmol of cAMP formed/min/mg of protein, are the mean of three experiments.

express the receptor or any other related receptor circumvent most of the problems; this was achieved in the present study by transfection of CHO cells with the recently cloned secretin receptor.

It was also possible to select, after transfection, cells that stably expressed different receptor densities. We compared two clones that differed essentially, if not solely, in the density of secretin receptors.

Several converging arguments suggest that a relatively small number of secretin receptors is sufficient to provoke maximum stimulation of adenylate cyclase in CHO cells. (a) Adenylate cyclase activation by secretin and analogues in membranes from clone 5 (450 fmol of receptors/mg of protein) occurs at peptide concentrations 5–10 times lower than those needed for membranes from clone 1 (40 fmol of receptors/mg of protein). Furthermore, in clone 1, the maximum response was 80% of the response of clone 5. (b) Preincubation of clone 1 with secretin induces a decrease in the density of secretin receptors and a progressive decrease in the maximum response, with only a small change in peptide potency.

Preincubation of clone 5 with secretin also induces a decrease in the density of secretin receptors, characterized for adenylate cyclase activation first by a decrease in secretin potency without a significant change in peptide efficacy and then by a decrease in peptide efficacy. In rat gastric glands (19), preincubation with 1 nM secretin for 30 min induced a 50% decrease of the binding capacity and a 50% decrease of secretin-induced cAMP production without a change in the K_{act} or K_d of the peptide. We estimate that a density of approximately 100–150 fmol of secretin receptors/mg of protein represents the receptor density needed for "stoichiometric" coupling to adenylate cyclase in CHO cells. Thus, there is efficient coupling of the secretin receptor to the G_s-adenylate cyclase machinery, and, at least in CHO cells, increasing the number of receptors increases the sensitivity to secretin and analogues. If the concept of spare receptors is applicable to other systems (20–22), it could explain (a) the relatively low K_{act} value of secretin in heart membranes (23) and (b) the fact that secretin fragments had variable intrinsic activities in different systems (24–27).

The present results indicated also that preincubation of the cells for 24 hr with low secretin concentrations induced a marked reduction of available secretin receptors. The sensitivity of clone 1 (with a small number of receptors) was higher than that of clone 5 (with a large number of receptors). Although we have no explanation for that high sensitivity, we suggest first that secretin was stable in the medium, second that there was poor or slow recycling of the occupied receptors, and thus a low turnover rate of the receptors in the presence of secretin, and third that a possible excess of receptors on the Gα sites in clone 5 reduced the capability for receptor internalization or desensitization.

Blocking the GTPase activity of α_s with Gpp(NH)p, a stable analogue of GTP, or by cholera toxin-induced ADP-ribosylation further decreased the K_{act} of secretin in both clones. Considering that the concentration of secretin required for occupancy of the receptor is 2 nM (on the basis of both binding data and the K_{act} of secretin in the clone 1 cell line), there is a 200-fold increase in sensitivity produced by combining a high receptor density and the presence of Gpp(NH)p.

It thus appears, as suggested previously (28, 29), that blocking the GTPase activity increases the efficacy of coupling between the secretin receptor and adenylate cyclase. Forskolin poten-

tiates the response to secretin but does not significantly modify the K_{act} of the hormone.

We previously showed that secretin-activated adenylate cyclase of the rat pancreas was affected by pretreatment of membranes with the reducing agent DTT (14). Although no binding data were obtained at that time, the results suggested structural changes of the secretin receptor, on the basis of a marked decrease in the K_{act} value of secretin with only a limited reduction in peptide efficacy. The structure of the secretin receptor supports this view (1); indeed, seven cysteine residues are present in the amino-terminal extracellular domain of the receptor, with two and one cysteines in the first and second extracellular loops, respectively. Disulfide bridges have not yet been identified, but it is likely that a reducing agent markedly modifies the general conformation of the receptor. In clone 5 (clone 1 could not be studied due to the small number of receptors), pretreatment of the membranes with DTT decreases the apparent number of secretin receptors and reduces the affinity for the tracer, as judged by Scatchard analysis. A likely interpretation of these data is that a large proportion of the secretin receptors are transformed into low affinity receptors, which are difficult to detect in saturation experiments. The competition curves suggest that the homogeneous population of secretin receptors is transformed into a heterogeneous population of sites with lower affinity. If clones 1 and 5 behave similarly in that respect, then the data on adenylate cyclase activation also suggest a decrease in the ability of the receptors to recognize the peptide, rather than a decrease in the number of receptors; in both clones, DTT provokes an increase of the K_{act} of secretin without a change (or even with an increase) in the maximal response. Thus, DTT appears to impair secretin recognition but not the ability of the receptor, once occupied, to couple to G_s sites and adenylate cyclase.

Acknowledgments

We thank Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan) for the kind gift of the plasmid pCDM8 containing the rat secretin receptor.

References

1. Ishihara, T., S. Nakamura, Y. Kaziro, T. Takahashi, K. Takahashi, and S. Nagata. Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J.* **10**:1635–1641 (1991).
2. Lin, H. Y., T. L. Harris, M. S. Flannery, A. Aruffo, E. H. Kaji, A. Gorn, L. F. Kolakowski, Jr., H. F. Lodish, and S. R. Goldring. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. *Science (Washington D. C.)* **254**:1022–1024 (1991).
3. Jüppner, H., A.-B. Abou-Samra, M. Freeman, X. F. Kong, E. Schipani, J. Richards, L. F. Kolakowski, Jr., J. Hock, J. T. Potts, Jr., H. M. Kronenberg, and G. V. Segre. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science (Washington D. C.)* **254**:1024–1026 (1991).
4. Ishihara, T., R. Shigemoto, K. Mori, K. Takahashi, and S. Nagata. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* **8**:811–819 (1992).
5. Lin, C., S.-C. Lin, C.-P. Chang, and M. G. Rosenfeld. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature (Lond.)* **360**:765–768 (1992).
6. Thorens, B. Expression cloning of the pancreatic cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proc. Natl. Acad. Sci. USA* **89**:8641–8645 (1992).
7. Jelinek, L. J., S. Lok, G. B. Rosenberg, R. A. Smith, F. J. Grant, S. Biggs, P. A. Bensch, J. L. Kuijper, P. O. Sheppard, C. A. Sprecher, P. J. O'Hara, D. Foster, K. M. Walker, L. H. J. Chen, P. A. McKernan, and W. Kindsvogel. Expression cloning and signaling properties of the rat glucagon receptor. *Science (Washington D. C.)* **259**:1614–1616 (1993).
8. Svoboda, M., E. Ciccarelli, M. Tastenoy, P. Robberecht, and J. Christophe. A cDNA construct allowing the expression of rat hepatic glucagon receptors. *Biochem. Biophys. Res. Commun.* **192**:135–142 (1993).
9. Jensen, R. T., C. G. Charlton, H. Adachi, S. W. Jones, T. L. O'Donohue, and J. D. Gardner. Use of ^{125}I-secretin to identify and characterize high-affinity secretin receptors on pancreatic acini. *Am. J. Physiol.* **245**:G186–G195 (1983).
10. Robberecht, P., M. Waelbroeck, M. Noyer, P. Chatelain, P. De Neef, W. König, and J. Christophe. Characterization of secretin and vasoactive intestinal peptide receptors in rat pancreatic plasma membranes using the native peptides, secretin-(7–27) and five secretin analogues. *Digestion* **23**:201–210 (1982).
11. Robberecht, P., D. H. Coy, P. De Neef, J.-C. Camus, A. Cauvin, M. Waelbroeck, and J. Christophe. [D-Phe^4]Peptide histidine-isoleucinamide ([D-Phe^4]PHI), a highly selective vasoactive-intestinal-peptide (VIP) agonist, discriminates VIP-preferring from secretin-preferring receptors in rat pancreatic membranes. *Eur. J. Biochem.* **165**:243–249 (1987).
12. Gossen, D., M. Tastenoy, P. Robberecht, and J. Christophe. Secretin receptors in the neuroglioma hybrid cell line NG108–15: characterization and regulation of their expression. *Eur. J. Biochem.* **193**:149–154 (1990).
13. Bloom, E. J., B. Siddiqui, J. W. Hicks, and Y. S. Kim. Effect of sodium butyrate, a differentiating agent, on cell surface glycoconjugates of a human pancreatic cell line. *Pancreas* **4**:59–64 (1989).
14. Robberecht, P., M. Waelbroeck, J.-C. Camus, P. De Neef, and J. Christophe. Importance of disulfide bonds in receptors for vasoactive intestinal peptide and secretin in rat pancreatic plasma membranes. *Biochim. Biophys. Acta* **773**:271–278 (1984).
15. Connors, R. W., R. W. Sweet, J. P. Noveral, D. S. Pfarr, J. J. Trill, R. J. Shebuski, B. A. Berkowitz, D. Williams, S. Franklin, and M. E. Reff. DHFR coamplification of t-Pa in DHFR$^+$ bovine endothelial cells: *in vitro* characterization of the purified serine protease. *DNA* **7**:651–661 (1988).
16. Urlaub, G., and L. A. Chasin. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. USA* **77**:4216–4220 (1980).
17. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
18. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
19. Bawab, W., E. Chastre, and C. Gespach. Functional and structural characterization of the secretin receptors in rat gastric glands: desensitization and glycoprotein nature. *Biosci. Rep.* **11**:33–42 (1991).
20. Ariëns, E. J., J. M. Van Rossum, and P. C. Koopman. Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested in isolated organs. *Arch. Int. Pharmacodyn.* **127**:459–478 (1960).
21. Van Rossum, J. M., and E. J. Ariëns. Receptor reserve and threshold phenomena. II. Theories on drug action and a quantitative approach to spare receptors and threshold values. *Arch. Int. Pharmacodyn.* **136**:385–413 (1962).
22. Kenakin, T. P. The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.* **36**:165–222 (1984).
23. Chatelain, P., P. Robberecht, P. De Neef, M. De Schodt- Lanckman, W. König, and J. Christophe. Secretin- and VIP-stimulated adenylate cyclase from rat heart. *Pflügers Arch.* **389**:21–27 (1980).
24. Gardner, J. D., A. J. Rottman, S. Natarajan, and M. Bodanszky. Interaction of secretin$_{5-27}$ and its analogues with hormone receptors on pancreatic acini. *Biochim. Biophys. Acta* **583**:491–503 (1979).
25. Makhlouf, G. M., M. Bodanszky, M. L. Fink, and M. Schebalin. Pancreatic secretory activity of secretin$_{5-27}$ and substituted analogues. *Gastroenterology* **75**:244–248 (1978).
26. Adler, M., M. Mestdagh, P. De Neef, W. König, J. Christophe, and P. Robberecht. *In vivo* effects of five secretin analogs on fluid and potassium secretion from the rat pancreas. *Life Sci.* **26**:1175–1181 (1980).
27. Robberecht, P., P. De Neef, M. Waelbroeck, J.-C. Camus, J.-L. Scemama, D. Fourmy, L. Pradayrol, N. Vaysse, and J. Christophe. Secretin receptors in human pancreatic membranes. *Pancreas* **3**:529–535 (1988).
28. Svoboda, M., P. Robberecht, J. Camus, M. Deschodt-Lanckman, and J. Christophe. Association of binding sites for guanine nucleotides with adenylate cyclase activation in rat pancreatic plasma membranes: interaction of gastrointestinal hormones. *Eur. J. Biochem.* **83**:287–297 (1978).
29. Olinger, E. J., and J. D. Gardner. Action of VIP and secretin on adenylate cyclase activity in acinar cells from guinea pig pancreas. *Gastroenterology* **77**:704–713 (1979).

Send reprint requests to: P. Robberecht, Laboratory of Biochemistry and Nutrition, Faculty of Medicine, Université Libre de Bruxelles, Bat. G/E, CP 611, 808 route de Lennik, B-1070 Brussels, Belgium.