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Dual Signaling Potential Is Common Among G_s-Coupled Receptors and Dependent on Receptor Density

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SUMMARY

We studied the relative ability to activate phospholipase C (PLC) of four G_s-coupled receptors expressed in L cells at different densities. Stable cell lines expressing various levels of the luteinizing hormone receptor (LHR), the type 2 vasopressin receptor (V2R), or the type 1 or type 2 β-adrenergic receptor (β_1- or β_2AR) were isolated. The PLC activity was assessed by the measurement of free intracellular Ca^{2+} concentrations and the accumulation of inositol phosphates. We previously reported that, at 24,000 sites/cell, the LHR in L cells stimulated adenylyl cyclase by 10-fold over basal levels and PLC by 50% over basal levels. The EC_{50} for stimulation was 20-fold higher for PLC than for adenylyl cyclase. We now report that LHR tends to stimulate PLC more at a higher receptor density and less at a lower density. EC_{50} values for accumulation of inositol phosphates remained unchanged. The human V2R and the human βARs are strong adenylyl cyclase stimulators, and their potential for dual signaling was unknown. Expressing the V2R at 100,000 sites/cell or more and the βARs at 300,000 sites/cell resulted in stimulation of PLC by these receptors. As with the LHR, higher concentrations of vasopressin or isoproterenol were needed to reach 50% stimulation of PLC, compared with that of adenylyl cyclase. The β_1AR was a stronger PLC stimulator than was the β_2AR. The orders of potency for isoproterenol, epinephrine, and norepinephrine to stimulate adenylyl cyclase and PLC were the same for each of the two βARs. These results indicate that the ability of G_s-coupled receptors to stimulate PLC is dependent on the levels of receptor expression, and they suggest that dual signaling potential is a common property of G_s-coupled receptors and possibly also of G_i-coupled receptors.

Many hormones and neurotransmitters exert their actions in target cells by binding to cell surface receptors, which in turn activate G proteins. A G protein is, in its resting state, a heterotrimer composed of α, β, and γ subunits. The activation of the G protein is thought to be associated with dissociation of the α subunit of the heterotrimer from the βγ dimer, allowing one or both subunits to modulate target effectors. To date, 16 G_α, four G_β, and seven G_γ subunits have been found in vertebrate cells and, according to the function and molecular structures of the α subunits, G proteins are categorized into several families. Among them, G_s proteins activate adenylyl cyclase, G_i proteins inhibit adenylyl cyclase, and members of the G_q family stimulate PLC. The modulation of these two enzymes regulates intracellular levels of second messengers, i.e., cAMP, diacylglycerol, inositol-1,4,5-trisphosphate, and Ca^{2+}, which induce cascades of signal transduction pathways (1).

It is common that a single ligand elicits changes in more than one second messenger pathway. Very often, this is mediated by multiple receptor types that couple to different G proteins. However, in recent years, through the expression of newly available cDNAs for some G protein-coupled receptors in mammalian cells lacking endogenous receptors, evidence has accumulated that a single receptor type can mediate multiple second messenger pathways. Since the first report on the M2 muscarinic receptor (2), many other G_i-coupled receptors have been found to be capable of stimulating PLC (3–6). The physiological significance of receptor dual coupling is not clear, because for many of the ligands there are corresponding G_q-coupled receptor subtypes that couple to PLC stimulation much better than do the G_i-coupled receptors.

Several G_s-coupled receptors have also been found to stimulate PLC. They are the D_1 dopamine receptor (7), two members of the glycoprotein hormone receptor family, namely the thyrotropin receptor (8) and the LHR (9), and several members of the secretin receptor family, including the receptors for parathyroid hormone and parathyroid hormone-related peptide

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ABBREVIATIONS: PLC, phosphatidylinositol-specific phospholipase C; AVP, arginine-vasopressin; AR, adrenergic receptor; CTX, cholera toxin; FSH, follicle-stimulating hormone; GLP-1, glucagon-like peptide 1; hCG, human chorionic gonadotropin; IP, inositol phosphate; LH, luteinizing hormone; LHR, luteinizing hormone receptor(s); PGE_1, prostaglandin E_1; V2R, type 2 vasopressin receptor(s); D-PBS, Dulbecco's phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl ester.

(10), calcitonin (11), pituitary adenylyl cyclase-activating polypeptide (12), glucagon (13), and GLP-1 (14). In contrast to the dual signaling by G_i-coupled receptors, the PLC-stimulating capability of these receptors has been implicated as the mechanism of dual signaling phenomena ascribed to some of these ligands in a variety of tissues, because no G_q-coupled receptor subtypes have been identified for them.

The common features of PLC coupling by the G_s- and G_i-coupled receptors are that it requires a relatively higher receptor density and higher agonist concentrations, compared with coupling to adenylyl cyclase, and the effect is rather weak even under optimal conditions. Nevertheless, when attempted, nearly all G_i-coupled receptors have been shown to be able to stimulate PLC, at least in some host cell lines. On the other hand, the situation for G_s-coupled receptors is much less clear. We previously reported that the murine LHR expressed in L cells was able to stimulate PLC, whereas other G_s-coupled receptors, i.e., the human V2R and hamster β_2AR, could not (9). Chabre *et al.* (11) compared the ability of the calcitonin receptor and the human LHR to stimulate PLC and found that only the former had dual signaling capacity. Thus, it seemed that the ability to stimulate PLC varied among different G_s-coupled receptors and some extremely high concentrations of receptor and agonist might be needed to reveal the dual coupling capability. To determine whether that dual signaling capacity was limited to certain types of G_s-coupled receptors and what role the receptor density played in the effectiveness of dual coupling, we compared the ability of four G_s-coupled receptors, i.e., murine LHR, human V2R, and human β_1AR and β_2AR, stably expressed in L cells at different densities, to stimulate PLC. We found that the coupling of the LHR to PLC was enhanced at a higher receptor density and vanished at a much lower receptor density. We also report that, when expressed at high receptor densities, V2R, β_1AR, and β_2AR are also capable of stimulating PLC.

Experimental Procedures

Materials

hCG, AVP, (−)-isoproterenol, (−)-epinephrine, (−)-norepinephrine, (−)-propranolol, and 8-bromo-cAMP were purchased from Sigma. [d(CH_2)$_5$,D-Ile^2,Ile^4,Arg^8]Vasopressin was obtained from Peninsula Laboratories. PGE_1 and forskolin were from Calbiochem. Bovine LH was obtained through the Hormone Distribution Program of the National Institutes of Health. Fura-2/AM was from Molecular Probes (Eugene, OR). CTX was from List Biological Laboratories (Campbell, CA). *myo*-[^{3}H]Inositol (20 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]AVP (60 Ci/mmol), [^{3}H]CGP-12177 (54 Ci/mmol), [^{3}H]IPs, and [^{3}H]phosphatidylinositol-4,5-bisphosphate were purchased from DuPont-New England Nuclear. ^{125}I-hCG (~4000 cpm/fmol) was synthesized according to the lactoperoxidase/H_2O_2 protocol described by Abramowitz *et al.* (15). All tissue culture reagents were purchased from GIBCO.

Cell Lines and Cell Culture Conditions

Cell lines expressing the murine LHR, human V2R, and human β_1AR and β_2AR were established as described previously (16–19). Cells were diluted twice weekly and cultured at 5% CO_2 in minimum essential medium α containing 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin and supplemented with either 400 μg/ml G418 or 100 μM hypoxanthine, 1 μM aminopterin, and 10 μM thymidine.

Determination of Receptor Density

The density of receptors in each of the LHR- and V2R-expressing cell lines was determined by Scatchard analysis of the specific binding of ^{125}I-hCG or [^{3}H]AVP to intact monolayer cells in 12-well tissue culture plates, at cell densities of 0.5–1.0 × 10^6 cells/well. Binding conditions were as described (9).

To determine the receptor density for cells expressing either βAR, appropriate cells grown in 100-mm dishes were treated with trypsin/EDTA, collected by centrifugation at 400 × *g* for 5 min, washed once with ice-cold D-PBS, recentrifuged, and then resuspended at 2 × 10^6 cells/ml in an ice-cold binding solution composed of D-PBS supplemented with 2 mg/ml glucose and 10 mg/ml bovine serum albumin. Cells were transferred in 0.25-ml aliquots to 12- × 75-mm test tubes, and to each tube was added 0.25 ml of the binding solution containing appropriate dilutions of [^{3}H]CGP-12177 (16–2000 pM). The tubes were vortex mixed briefly and incubated in a shaking water-bath for 30 min at 37°. Nonspecific binding was determined under the same conditions in the presence of 3 μM (−)-propranolol. At the end of the incubation, 1.0 ml of ice-cold D-PBS was added to each tube. After brief vortex mixing, the tubes were allowed to remain in ice-water for 10 min and then centrifuged at 1000 × *g* for 15 min at 4°. The supernatant was carefully removed by aspiration and the cells were washed twice by resuspension in 1.0 ml of D-PBS plus 0.5 ml of the binding solution followed by centrifugation. The cell pellets were then lysed with 0.5 ml of 0.1 N NaOH at room temperature. Bound radioactivity was determined using a liquid scintillation counter, after the lysate was transferred to scintillation vials containing 4.0 ml of scintillation fluid.

Measurement of Adenylyl Cyclase Activity in Cell Homogenates

Cells grown to 90% confluence were rinsed, scraped off the plates, and homogenized, and adenylyl cyclase activities were measured as described (9, 17). The activity of adenylyl cyclase was expressed as picomoles of cAMP formed/minute/milligram of protein in the homogenate.

Assessment of PLC Activity

The ability of the G_s-coupled receptors to stimulate PLC was first tested by measuring their ability to mobilize intracellular Ca^{2+} in cells that had been preloaded with the fluorescent indicator fura-2 and was confirmed by measuring the accumulation of IPs in prelabeled intact cells in response to the appropriate ligands.

Measurement of intracellular Ca^{2+} concentrations. Cells grown to confluence in 150-mm dishes were trypsinized and collected in a 50-ml capped polypropylene tube. After centrifugation for 5 min at 400 × *g* and removal of supernatant, the cell pellets were resuspended at room temperature in an extracellular solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, 0.1% bovine serum albumin, and 15 mM HEPES, pH 7.4. Loading of cells with fura-2/AM, washing, and measurement of fura-2 fluorescence changes were performed as described by Liao *et al.* (20).

Measurement of accumulation of IPs. Cells were seeded in 12-well tissue culture plates with 1 ml of G418- or hypoxanthine/aminopterin/thymidine-containing selection medium supplemented with 2 μCi/ml *myo*-[^{3}H]inositol and were grown for 45 hr. Labeled cells were rinsed three times at room temperature with 1.0 ml of D-PBS supplemented with 5.5 mM glucose, 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$. After washing, the cells were incubated in 0.5 ml of the aforementioned solution at 37° for 30 min before 10 μl of 1 M LiCl were added to each well. The incubation was continued for 10 min, after which 0.5 ml of D-PBS with glucose, Ca^{2+}, and Mg^{2+}, containing dilutions of appropriate ligand, was added to the wells. The incubation was continued at 37° for another 30 min. At the end of the incubation the plates were placed on ice and, immediately after removal of the supernatant by aspiration, 0.75 ml of ice-cold 20 mM formic acid was added to each well. IPs produced were separated from *myo*-inositol according to a simplified Dowex chromatography procedure described by Hung *et al.*

(6), with minor modifications. Briefly, the cells were treated with 20 mM formic acid on ice for >1 hr and were mixed well by gentle swirling. The extracts were applied to Dowex columns (Bio-Rad AG 1-X8, 100–200 mesh, formate form, 0.6-cm diameter, 1.0-ml bed volume) that had been pretreated sequentially with 2 ml of 2 M ammonium formate/0.1 M formic acid, 4 ml of water, and 4 ml of 20 mM ammonium hydroxide, pH 9.0 (adjusted with formic acid). Immediately after the samples were loaded, 3 ml of 40 mM ammonium hydroxide, pH 9.0, were added to each column and eluates (~3.75 ml) were collected in scintillation vials containing 10 ml of scintillation fluid. When standard *myo*-[^{3}H]inositol was subjected to this procedure, 98% of the radioactivity was recovered in this eluate. The columns were then washed three times with 4 ml of 40 mM ammonium formate, and IPs were eluted with 5 ml of 2 M ammonium formate/0.1 M formic acid, into scintillation vials containing 15 ml of scintillation fluid. The recovery of radioactivity was 95% when a mixture of standard [^{3}H]IPs (including equal amounts of labeled inositol-1-phosphate, inositol-1,4-bisphosphate, and inositol-1,4,5-trisphosphate) was subjected to the procedure. The accumulation of IPs was normalized by dividing the counts for [^{3}H]IPs by the sum of the counts for *myo*-[^{3}H]inositol plus [^{3}H]IPs.

One concern with this procedure was whether some phosphoinositides would be coextracted by formic acid and, if they were, at which step of the chromatography they would be eluted. To test this, we extracted phospholipids from the formic acid extract and from cell membranes retained in the wells, according to the procedure of Bell *et al.* (21), and found that only 5% of phospholipid-associated radioactivity was in the formic acid extract. Phosphoinositides were retained in the column throughout the chromatography and eluted after sequential treatment with NaOH and HCl, as tested with standard [^{3}H]phosphatidylinositol-4,5-bisphosphate. Therefore, the formic acid extraction procedure extracted only a minor amount of phosphoinositides and this trace amount of labeled phosphoinositides did not appear in the eluate with the IPs.

The columns were regenerated by sequential addition of 20 ml each of water, 1 N NaOH, water, 1 N HCl, water, 1 N NaOH, and water, followed by 8 ml of 1 N formic acid and then 20 ml of water three times. The regeneration procedure hydrolyzed proteins and phosphoinositides, removed the residual radioactivity, and converted the resin back to the formate form.

Results

Establishment of Cell Lines

Stably transformed cell lines expressing different levels of murine LHR, human V2R, human β_1AR, or human β_2AR were identified by their ability to stimulate adenylyl cyclase activity in response to the appropriate agonists. The presence of receptor was confirmed by binding assays. Table 1 lists the names, receptor types, and receptor densities of these cell lines. The density of the receptors remained constant throughout this study.

Stimulation of PLC by LHR expressed in L cells. In our previous report, a clonal LHR cell line expressing 24,000 receptor sites/cell was obtained by cotransfection of plasmid p91023(B) carrying the LHR cDNA and plasmid pHSV-106 carrying the thymidine kinase selection marker and selection in medium containing hypoxanthine/aminopterin/thymidine. This cell line stimulated adenylyl cyclase by 10-fold and PLC by 50% over the basal activities (9). For the present studies, we subcloned the LHR cDNA into plasmid pKNH, which carries the neomycin resistance marker, and transfected the plasmid into Ltk$^-$ cells. Transformed cell lines were selected by growth in medium containing G418. Cell lines expressing LHR at 500–40,000 binding sites/cell were used (Table 1). The basal adenylyl cyclase activities of the clonal cell lines varied from 5 to 25 pmol of cAMP formed/min/mg of protein, so that comparison of adenylyl cyclase coupling efficacy among the cell lines as the increase of activity over basal values was not informative. However, L cells express an endogenous G_s-coupled prostaglandin receptor and adenylyl cyclase activity stimulated by PGE_1 was relatively constant among different cell lines (72 ± 2 pmol of cAMP formed/min/mg) and not additive with LHR-stimulated activity. Therefore, stimulation of adenylyl cyclase by hCG in these cell lines was compared with the stimulation by PGE_1. The results shown in Table 2 indicate that, whereas LHR5/8 cells had much lower receptor density, their ability to activate adenylyl cyclase through the LHR was only 55% lower than that of cells expressing >40-fold more receptors (i.e., LHR5/9 and LHR20/20).

When the ability of the LHR to mobilize intracellular Ca^{2+} was tested for some of these cell lines by the fura-2 fluorescence method, it was found that application of 10 μg/ml hCG induced a Ca^{2+} signal in LHR15/24, LHR5/27, and LHR20/20 cells.

TABLE 1

Summary of stable L cell lines expressing different levels of four types of G_s-coupled receptors

The cDNAs for murine LHR, human V2R, and human β_1AR and β_2AR were subcloned into the eukaryotic expression vector pKNH and transfected into Ltk$^-$ cells by the calcium phosphate/glycerol shock method (9). Clonal cell lines were obtained by selecting G418-resistant transformants and testing *in situ* for acquisition of ligand-sensitive adenylyl cyclase activity.

DNA	Cell line	Vector	Selection marker	Sites/Cell[a]
Murine LHR	LHR5/8	pKNH	G418	500
	LHR15/24	pKNH	G418	4,000
	LHR5/27	pKNH	G418	12,000
	LHR5/9	pKNH	G418	24,000
	LHR20/20	pKNH	G418	40,000
Human V2R	HTB2	Genomic DNA[b]	HAT	20,000
	HKG4-4	pKNH	G418	100,000
	V2D4	Genomic DNA[b]	HAT	200,000
	V2E15	pKNH	G418	275,000
	V2E11	pKNH	G418	500,000
Human β_2AR	Lβ2/20	pKNH	G418	30,000
	Lβ2/5	pKNH	G418	120,000
	Lβ2/7	pKNH	G418	300,000
Human β_1AR	Lβ1/2	pKNH	G418	300,000

[a] Receptor numbers in intact monolayer cells were determined by Scatchard analysis using ^{125}I-hCG, [^{3}H]AVP, and [^{3}H]CGP-12177 for LHR, V2R, and βARs, respectively, as described in Experimental Procedures.

[b] These cell lines were established by cotransfection of human genomic DNA with plasmid pHSV-106 and were selected as described, except that hypoxanthine/aminopterin/thymidine (HAT) was used in place of G418 (18).

TABLE 2

hCG responsiveness of adenylyl cyclase in L cells expressing different levels of LHR

Receptor density for each cell line is listed in Table 1. Cell homogenates were incubated in the presence of [α-^{32}P]ATP and 10 μg/ml hCG or 10 μg/ml PGE_1 for 20 min at 32° and [^{32}P]cAMP formed was isolated as described in Experimental Procedures. Data show averages ± standard errors pooled from two to four experiments, each performed in duplicate. EC_{50} values were determined from dose-response curves for hCG. Data show averages ± 1/2 range from two experiments.

Cell line	hCG-stimulated adenylyl cyclase activity relative to PGE_1	EC_{50}
	%	*pM*
LHR5/8	40 ± 4	165 ± 2
LHR15/24	70 ± 2	208 ± 19
LHR5/27	84 ± 2	87 ± 1
LHR5/9	91 ± 3	42 ± 1
LHR20/20	89 ± 3	33 ± 1

Among these, the LHR20/20 cell line, which expressed the highest receptor density, also had the largest increase in Ca^{2+} concentration (Fig. 1, *upper*). The Ca^{2+} signal induced by hCG resulted from the release of Ca^{2+} from intracellular stores, because it was not inhibited by prior addition of 10 μM $LaCl_3$, a Ca^{2+} influx pathway blocker (data not shown). In contrast, LHR5/8 cells were not able to induce an intracellular Ca^{2+} increase in response to hCG even at a higher concentration (50 μg/ml). All of these cell lines displayed a constant increase in intracellular Ca^{2+} concentrations in response to 100 μM ATP, an agonist of endogenous P_2 purinergic receptors in L cells (Fig. 1, *upper*).

To confirm that the increase in intracellular Ca^{2+} levels was due to the stimulation of PLC by hCG in the LHR-expressing cell lines, we measured the accumulation of IPs in cells that had been prelabeled with *myo*-[^{3}H]inositol. As shown in Fig. 2a, the basal activity of PLC in these cells produced IPs ranging from 2.2 to 5.1% of the sum of IPs plus *myo*-inositol, and ATP stimulated accumulation of IPs by 100–170% over basal. Except for LHR5/8 cells, increases in accumulation of IPs stimulated by hCG were detected in all LHR-expressing cells, and the increment was found to be higher as the receptor density increased. In LHR20/20 cells (40,000 sites/cell), the accumulation of IPs increased by 2.3-fold over the basal value in response to hCG.

It has been frequently found that cells expressing higher densities of G_s-coupled receptors tend to have a leftward shift of the dose-response curve for stimulation of adenylyl cyclase activity by agonist, compared with cells expressing a lower receptor density (e.g., see Ref. 22). In other words, the increase in receptor density is associated with a decrease of the EC_{50} for the agonist. This effect was also seen in LHR cell lines expressing increasing numbers of LHR (Table 2). The EC_{50} values for hCG-stimulated adenylyl cyclase activity ranged from 33 pM to 208 pM hCG. However, when the dose-response curves for hCG-mediated stimulation of accumulation of IPs were compared for two LHR-expressing cell lines, LHR5/27 and LHR20/20 (Fig. 3), the EC_{50} for LHR5/27 (1.3 nM) was similar to, if not lower than, that for LHR20/20 (2.7 nM). These values are also similar to that previously reported by us for the LHR11/6 cell line (2.4 nM), which expressed 24,000 LHR sites/cell (9). Moreover, for LHR, bovine LH is a more potent adenylyl cyclase stimulator than is hCG, whereas the potencies of the two hormones to stimulate the accumulation of IPs were indistinguishable in both LHR5/27 and LHR20/20 cells, as shown by the overlapping dose-response curves (Fig. 3).

We previously reported that a mutant LHR whose carboxyl terminus was deleted from position 628 was able to activate adenylyl cyclase and was desensitized normally (19). When

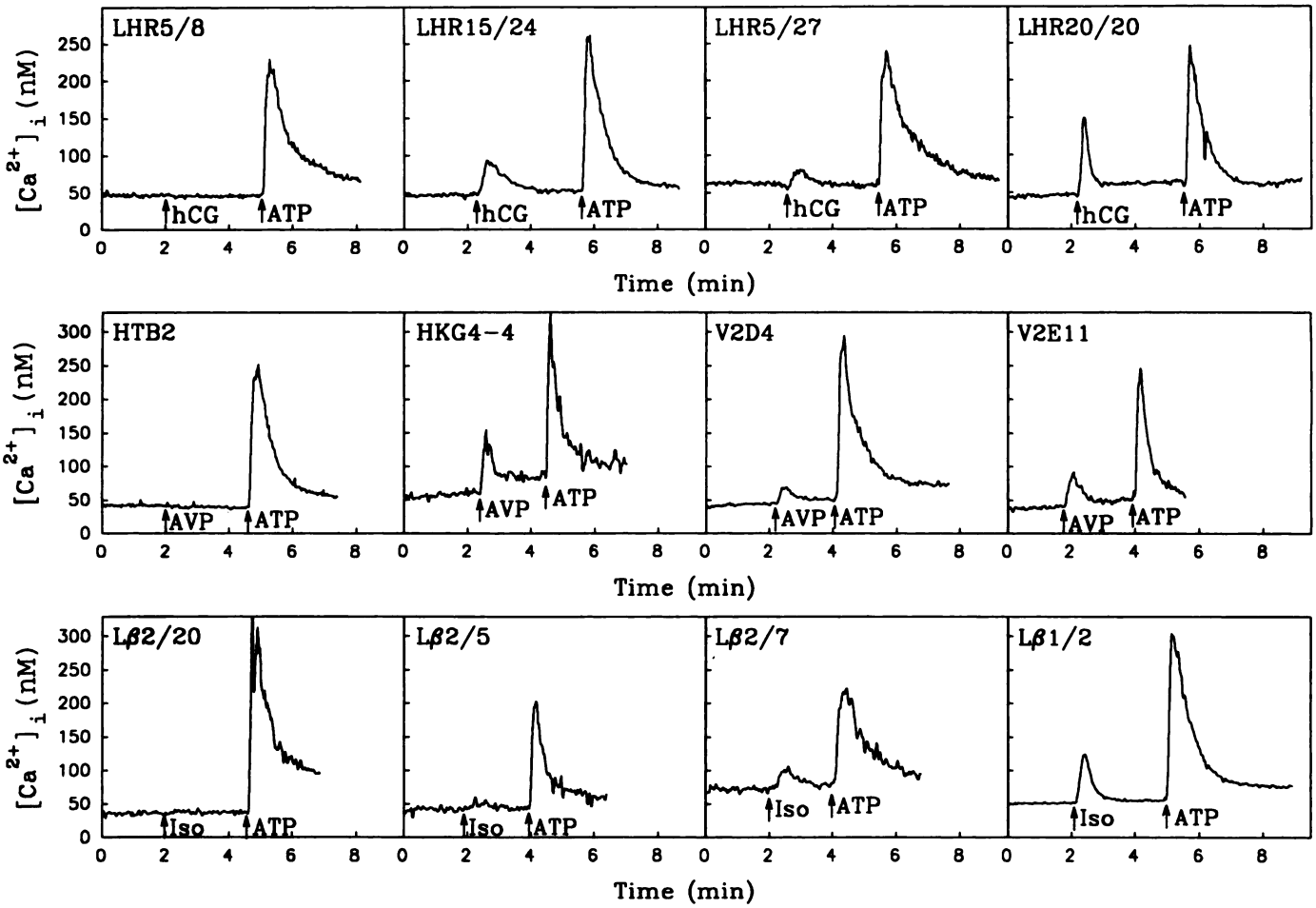

Fig. 1. Agonist-induced mobilization of intracellular Ca^{2+} in L cells expressing different levels of the G_s-coupled receptors LHR (*upper*), V2R (*middle*), and βARs (*lower*). Receptor density for each cell line is listed in Table 1. Cells were loaded with fura-2 and the intracellular Ca^{2+} concentration was monitored as described in Experimental Procedures. *Arrows*, time at which agonists were added, to give a final concentration of 10 μg/ml hCG (or 50 μg/ml hCG for LHR5/8), 10 μM AVP, 10 μM isoproterenol (*Iso*), or 100 μM ATP.

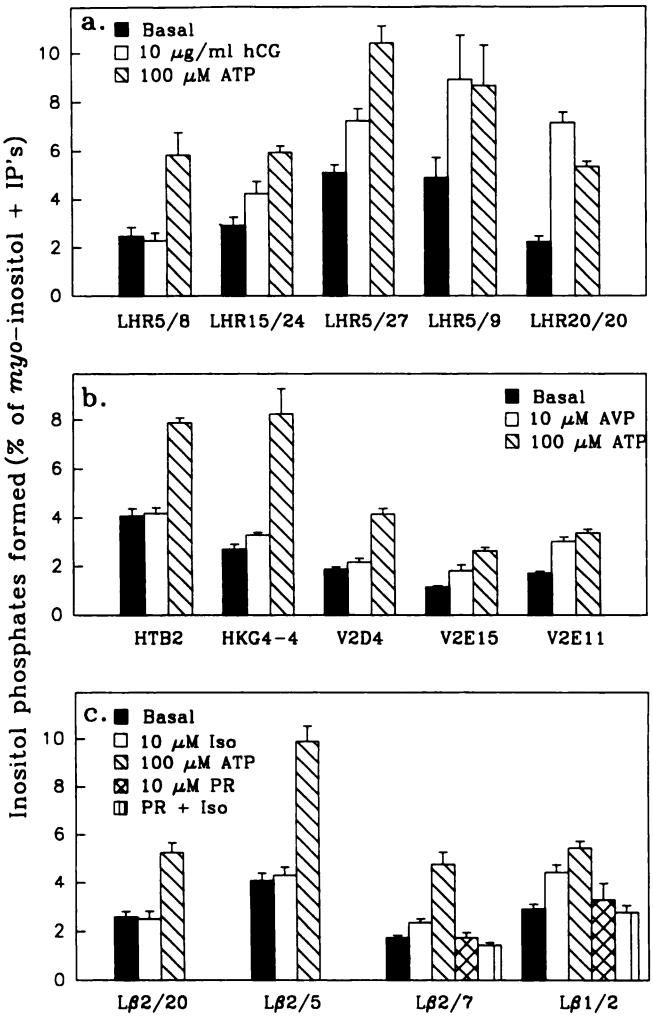

Fig. 2. Agonist-stimulated accumulation of IPs in L cells expressing different levels of the G_s-coupled receptors LHR (a), V2R (b), and βARs (c). Receptor density for each cell line is listed in Table 1. Cells were seeded in 12-well plates and cultured in the presence of 2 μCi/ml *myo*-[^{3}H]inositol for 45 hr. After a 10-min treatment with 20 mM LiCl, the cells were treated with appropriate agonists for 30 min. The production of IPs was determined as described in Experimental Procedures. Data are average ± standard error pooled from at least two experiments, each performed in triplicate. Basal IP levels were determined from wells without agonist treatment. *Iso*, isoproterenol; *PR*, (−)-propranolol.

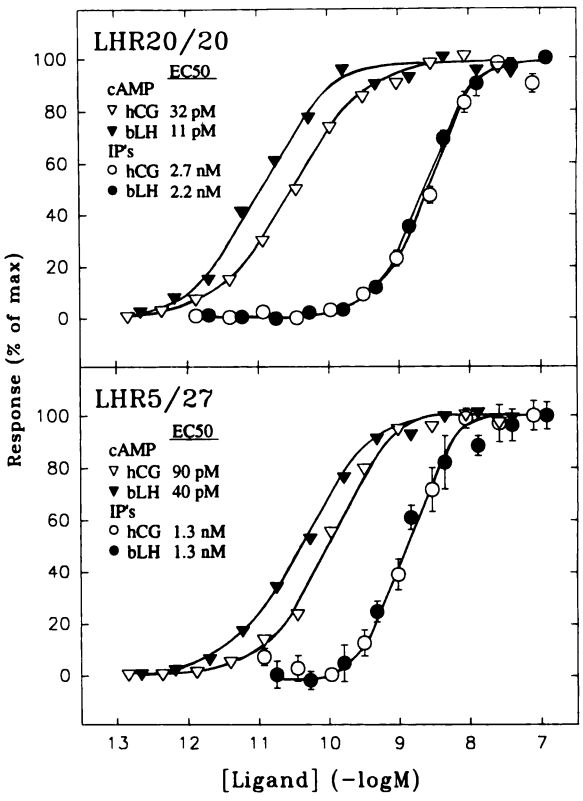

Fig. 3. Dose dependence of stimulation by hCG and bovine LH (*bLH*) of the formation of cAMP and IPs in the LHR20/20 (*upper*) and LHR5/27 (*lower*) cell lines, expressing LHR at 40,000 and 12,000 sites/cell, respectively. For cAMP formation, cell homogenates were incubated in the presence of [α-^{32}P]ATP and appropriate dilutions of hormones for 20 min at 32° and [^{32}P]cAMP formed was isolated as described in Experimental Procedures. Data show averages of duplicate determinations from a representative experiment. For the formation of IPs, intact cells were labeled with *myo*-[^{3}H]inositol for 45 hr, washed, and treated with appropriate dilutions of hormone for 30 min at 37°. The production of IPs was determined as described in Experimental Procedures. Data are average ± standard error pooled from at least two experiments, in which each hormone dilution was tested in triplicate.

expressed in L cells at 10,000 sites/cell, the truncated receptor was also able to stimulate PLC (accumulation of IPs, 73 ± 7% over basal at 10 μg/ml hCG; results from three experiments).

Stimulation of PLC by V2R expressed in L cells. Vasopressin receptors belong to a subfamily of G protein-coupled receptors that are activated by the hypothalamic peptides vasopressin and oxytocin. Two subtypes of pharmacologically distinct vasopressin receptors have been found, namely type 1 and type 2, and their molecular sequences have been reported (16, 23). The type 1 receptor appears to couple to G proteins from the G_q family and stimulates PLC, whereas the V2R couples to the G_s protein and stimulates adenylyl cyclase. We previously reported that the human V2R in L cells, obtained either by cotransfection of human genomic DNA with the plasmid pHSV-106 or by transfection of the plasmid pKNH carrying the V2R cDNA, couples positively to adenylyl cyclase and that in such cells stimulation by AVP is comparable to stimulation by PGE_1. We also reported that some of our V2R-expressing cell lines did not couple to the stimulation of Ca^{2+} release in response to 0.1 μM AVP (9), a saturating concentration for stimulation of adenylyl cyclase by the agonist. In the present studies, we re-examined the possibility that the V2R might affect PLC, by testing some newly selected V2R-expressing cell lines (Table 1) and using higher agonist concentrations.

Fig. 1, *middle*, shows the results of fura-2 fluorescence measurements for V2R-expressing cell lines in response to AVP and ATP. At 10 μM AVP, cells expressing >100,000 V2R sites/cell released intracellular Ca^{2+}, whereas HTB2 cells, which express 20,000 receptor sites/cell, failed to respond to the addition of AVP. All of the V2R-expressing cells had a normal response to ATP.

The basal PLC activity of the V2R-expressing cell lines, as measured by accumulation of IPs, ranged from 1.1 to 4.1% of the sum of IPs plus *myo*-inositol, and ATP stimulated accumulation of IPs by 90–140% over basal levels (Fig. 2b). Except for HTB2 cells, increases in the accumulation of IPs stimulated by AVP were detected in all V2R-expressing cells and the increment was found to be greater with greater receptor density. The V2E11 cells (500,000 sites/cell) had an 86% increase over basal levels for accumulation of IPs stimulated by AVP, and

this increase was inhibited by preincubation of the cells with 1 μM [d(CH_2)$_5$,D-Ile^2,Ile^4,Arg^8]vasopressin, a V2R-specific antagonist (data not shown). A dose-response analysis for the V2E11 cell line showed a 35-fold rightward shift for activation of PLC by AVP, compared with activation of adenylyl cyclase (Fig. 4).

Stimulation of PLC by β_1AR and β_2AR expressed in L cells. Numerous studies have characterized ARs. Pharmacologically, three distinct subtypes have been defined. The α_1 subtype couples to G_q proteins to stimulate PLC. The α_2 subtype couples to pertussis toxin-sensitive G_i and G_o proteins to inhibit adenylyl cyclase, stimulate potassium channels, and inhibit calcium channels. The β subtypes couple to G_s and stimulate adenylyl cyclase. The two principal βARs, β_1AR and β_2AR, are pharmacologically defined by well established relative orders of potencies for interaction with both agonists and antagonists. In vertebrate tissue or cells, the α_1-, α_2-, β_1-, and β_2ARs can exist singly or in any combination. As seen often with transfection of the cDNAs into reporter cells, a dual signaling capacity has been established for the α_1- and α_2-ARs (4, 24). Whether mammalian βARs have a dual signaling capacity in intact cells was unknown.

We have expressed the human β_1AR and β_2AR in L cells at different densities. We found that when the density of β_2ARs was increased from 0.4 to 3.2 pmol/mg of membrane protein the stimulation of adenylyl cyclase by a saturating concentration of isoproterenol changed very little, increasing by about 20%, compared with the stimulation by PGE_1. At equal receptor densities, i.e., 3.2 pmol/mg, the β_2AR stimulated adenylyl cyclase about 20% more than did the β_1AR (17). In the present studies, we investigated the possibility of stimulating PLC through these receptors.

The ability of 10 μM isoproterenol to mobilize intracellular Ca^{2+} in the βAR-expressing cell lines was tested by fura-2 fluorescence measurements and compared with that of ATP. As shown in Fig. 1, *lower*, Lβ1/2 and Lβ2/7 cells, expressing 300,000 β_1AR and 300,000 β_2AR sites/cell, respectively, released Ca^{2+} in response to isoproterenol. The response was not affected by 10 μM $LaCl_3$ but was inhibited by 10 μM levels of the βAR antagonist (−)-propranolol, added 1 min before the agonist (data not shown). The Lβ2/5 and Lβ2/20 cells, expressing 120,000 and 30,000 β_2AR sites/cell, respectively, failed to mobilize Ca^{2+} in response to isoproterenol, although the response to ATP was normal for both cell lines.

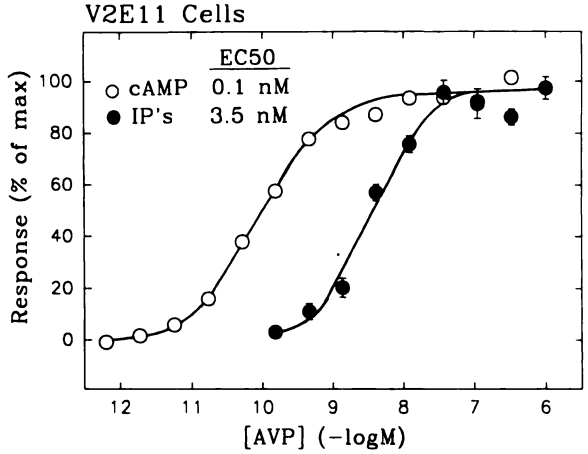

Fig. 4. Dose dependence of stimulation by AVP of the formation of cAMP and IPs in the V2E11 cell line, expressing 500,000 V2R sites/cell. For more details, see the legend to Fig. 3 and Experimental Procedures.

The basal PLC activity of the β_1AR and β_2AR cell lines, as measured by the accumulation of IPs, ranged from 1.8 to 4.1% of the sum of IPs plus *myo*-inositol, and ATP stimulated the accumulation of IPs by 80–180% over basal (Fig. 2c). For Lβ1/2 and Lβ2/7 cells, 10 μM isoproterenol stimulated accumulation of IPs by 58% and 34% over basal levels, respectively, and this stimulation was inhibited by 10 μM (−)-propranolol. The Lβ2/5 and Lβ2/20 cells were not stimulated by isoproterenol to accumulate IPs. Adenylyl cyclase assays of Lβ1/2 and Lβ2/7 cell homogenates showed the expected dose-response relationships for isoproterenol, epinephrine, and norepinephrine (Fig. 5). These relationships were retained in the PLC assays, as measured by the accumulation of IPs. As was the case with other G_s-coupled receptors, dose-response curves for PLC stimulation in cells expressing βARs were shifted rightward with respect to stimulation of adenylyl cyclase. However, this shift was larger for the β_2AR (100-fold) than for the β_1AR (25-fold). This indicates that, although when expressed at high densities both β_1AR and β_2AR are able to stimulate PLC, the β_1 subtype tends to stimulate the enzyme better than the β_2 subtype.

Effect of enhanced cAMP/protein kinase A pathway on the stimulation of PLC. In this study, all cell lines that expressed the G_s-coupled receptors displayed enhanced adenylyl cyclase activities in response to the corresponding agonists. However, only cells that expressed these receptors at high densities showed receptor-mediated stimulation of PLC. This excludes the possibility that the stimulation of PLC resulted from the enhanced cAMP/protein kinase A pathway. Indeed, when 8-bromo-cAMP (1 mM) or forskolin (100 μM) was added to the cells, no increase of the accumulation of IPs was detected (data not shown).

CTX permanently activates G_s protein by inhibiting its GTPase activity through ADP-ribosylation. In an effort to determine whether activation of the G_s protein is involved in the stimulation of PLC by its receptors, we tested the effect of CTX on the accumulation of IPs in LHR20/20 cells. As shown in Fig. 6a, preincubation with 1 μg/ml CTX for 16 hr caused an 8-fold increase of basal adenylyl cyclase activity and >90% occlusion of hCG- and PGE_1-stimulated adenylyl cyclase activity. On the other hand, treatment with either 1 or 10 μg/ml toxin did not affect the basal level of IP accumulation but resulted in approximately 50% inhibition of both hCG- and ATP-stimulated activities (Fig. 6b). Because ATP does not activate G_s in these cells, the inhibition of agonist-stimulated PLC activities by CTX seems to be a rather general effect not related specifically to the G_s-coupled receptors. In another experiment, addition of 2 μg/ml CTX simultaneously with the agonists caused slight increases of the basal and hCG- or ATP-stimulated PLC activities (Fig. 6c). Therefore, CTX tends to mimic the G_s-coupled receptors in the stimulation of PLC, but the effect by CTX is very small and the mechanism may not be the same. It has been shown that CTX does not induce the release of intracellular Ca^{2+} in L cells (7).

Discussion

For this study, four types of G_s-coupled receptors were stably transfected into L cells and clonal cell lines were established based on the acquisition of adenylyl cyclase activation in response to the corresponding agonists. Receptor expression was

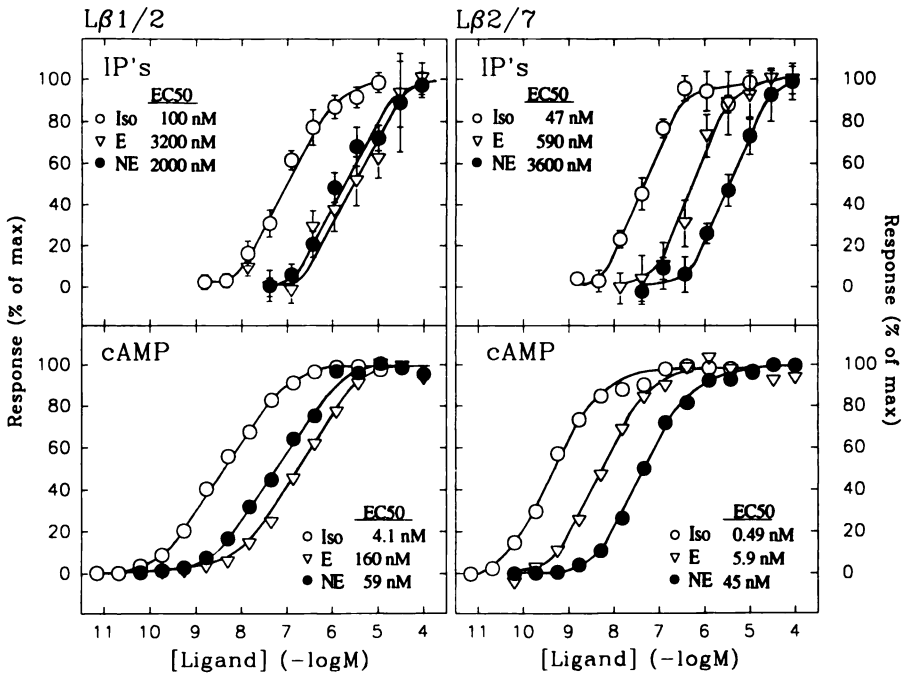

Fig. 5. Dose dependence of stimulation of the formation of cAMP (*lower*) and IPs (*upper*) by isoproterenol (*Iso*), epinephrine (*E*), and norepinephrine (*NE*) in Lβ1/2 (*left*) and Lβ2/7 (*right*) cells, expressing 300,000 β_1AR or β_2AR sites/cell, respectively. For more details, see the legend to Fig. 3 and Experimental Procedures.

confirmed by radioligand binding. In our experiments, although all clones gained the property of expressing the receptors at certain densities that varied because of unknown reasons, the receptor expression level in each clonal cell line stayed constant throughout the period of the study. However, because the processes that allowed the receptors to be expressed in the host cells, for example the site of integration of the foreign DNA, were different, other changes also occurred to varying degrees. These included differences in morphological appearance and in basal activities of both adenylyl cyclase and PLC. The variation in basal activities for stable clones was also observed by others (25). Therefore, we used ATP and PGE_1 to stimulate the endogenous receptors for these ligands, to normalize differences caused by the variation in basal activities. Unfortunately, this could not overcome all the unknown changes that resulted from each transfection event. Thus, a V2R-expressing cell line, HKG4–4, consistently showed an approximately 2-fold increase in intracellular Ca^{2+} concentration but only a very small increase in the production of IPs in response to AVP (Figs. 1 and 2). For this reason, both the rise in intracellular Ca^{2+} concentration and the accumulation of IPs were used to demonstrate the ability of a receptor to stimulate PLC in each cell line.

Several previous studies have shown that G_s- and G_i-coupled receptors are capable of stimulating PLC when expressed in reporter cells lacking the endogenous receptor, but those studies did not address the question of the role of receptor density in these dual responses. Using stably transfected cell lines expressing receptors at different levels, we have now demonstrated for the first time that the ability of several G_s-coupled receptors to stimulate PLC tends to increase as the expression level of the receptor increases. Moreover, the dual signaling capacity was found to exist in a broader spectrum of G_s-coupled receptor types than previously thought, as seen by the responses obtained with the receptors for a glycoprotein hormone (i.e., LHR), for a small peptide (i.e., V2R), and for the catecholamines epinephrine and norepinephrine (i.e., β_1AR and β_2AR). It is therefore predicted that more G_s-coupled receptors will be found to stimulate PLC at a suitable receptor density and agonist concentration. Furthermore, our results suggest that it may be necessary to re-evaluate the conclusion of others that two of the splice variants of the pituitary adenylyl cyclase-activating polypeptide receptor may not couple to PLC stimulation whereas the other variants do, because the receptor densities for these variants in the transfected cells were not known (12). It is also important to point out that most mutant receptors are not expressed efficiently in the plasma membrane; therefore, in the mutagenesis studies a loss of coupling to the PLC pathway by the mutant may not necessarily result from a loss of the ability of the mutant to couple to PLC, unless the expression levels in cells expressing the mutant are comparable to those of the wild-type receptor (26).

The biological significance of PLC stimulation by the G_s- and G_i-coupled receptors is not known. In most cases, the stimulation of PLC by these receptors is relatively inefficient and requires high receptor density. In our hands, a G_q-coupled receptor, i.e., the M5 muscarinic receptor, expressed in L cells (27) stimulates the accumulation of IPs by 16-fold over basal levels in response to carbachol, but the greatest PLC stimulation by a G_s-coupled receptor, LHR, was 2.3-fold. However, it is still possible that stimulation of PLC by some G_s- or G_i-coupled receptors occurs in a particular tissue at certain times, especially for receptors and hormones or neurotransmitters whose levels are strictly regulated. In luteal membranes from ovulating animals, LHR levels reach 400–700 fmol/mg of membrane protein (28, 29), a level capable of stimulating PLC. In sympathetic nerve endings, a high density of ARs can be expected. Because levels of norepinephrine in the synaptic cleft can also be very high, dual signaling by the βARs may be physiologically relevant.

One possible physiological effect of PLC stimulation by dual-signaling receptors is receptor desensitization and/or receptor down-regulation, which sometimes involves phosphorylation by protein kinase C. For example, in both opossum kidney cells (30) and human SaOS-2 osteoblast-like cells (31) the desensi-

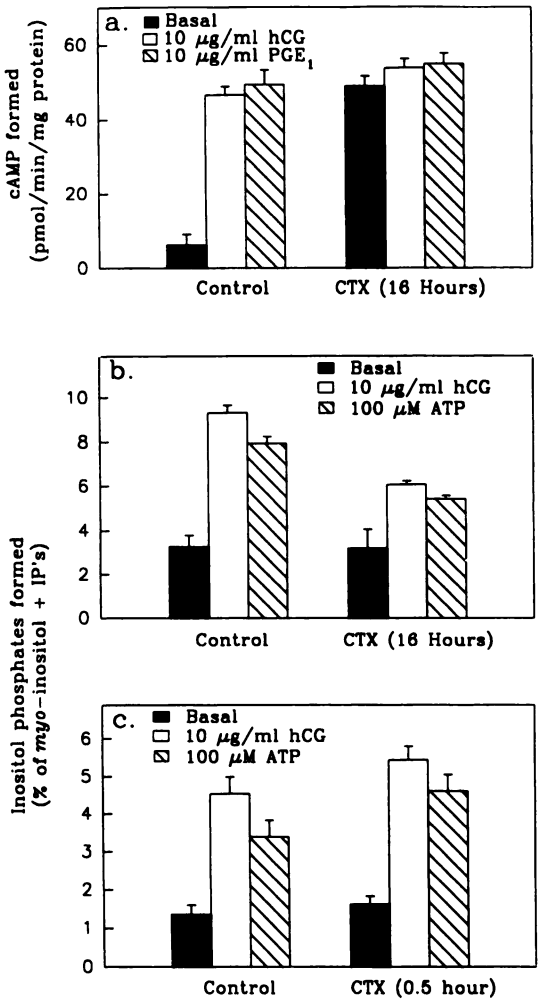

Fig. 6. Effect of CTX modification of G_s on the formation of cAMP (a) and the accumulation of IPs (b and c) in the LHR20/20 cell line. a and b, CTX was added to the cell culture medium to a final concentration of 1 μg/ml (a) or 10 μg/ml (b) 16 hr before the assays, which were performed in the absence of the toxin. c, The direct effects of CTX on the accumulation of IPs are shown. CTX was added alone (basal) or together with the agonists (hCG or ATP), at a final concentration of 2 μg/ml, to the wells for 30 min. IP production was measured as described in Experimental Procedures. Data are average ± standard deviation for triplicate determinations of a representative experiment.

tization of parathyroid hormone receptor stimulation of adenylyl cyclase caused by pretreatment with the hormone is mimicked by protein kinase C-activating phorbol esters and the protein kinase C inhibitor staurosporine prevents the desensitizing effect of parathyroid hormone.

Caution should be used when interpreting dual coupling results in physiological terms. Recent studies showed that the GLP-1 receptor expressed in COS-7 cells could activate both the adenylyl cyclase and PLC pathways (14). In a hamster β cell line, HIT cells, the activation of the endogenously expressed GLP-1 receptor also induced an intracellular Ca^{2+} increase. However, the rise of intracellular Ca^{2+} in HIT cells was found to result from a cAMP/protein kinase A-induced influx of extracellular Ca^{2+} through a voltage-dependent calcium channel and not from an inositol trisphosphate-mediated release of Ca^{2+} from internal stores (32). Given the fact that HIT and COS-7 cells had 2,000 and 260,000 receptor sites/cell, respectively, it would appear that the mobilization of intracellular Ca^{2+} in COS-7 cells is solely a result of receptor overexpression. Therefore, although the increase of intracellular Ca^{2+} levels plays a key role in GLP-1-induced insulin secretion by β cells (32), the activation of PLC does not appear to be required for the process, even given the potential of the receptor to activate the phospholipase.

The mechanism of PLC stimulation by G_s- and G_i-coupled receptors remains to be elucidated. Although it was recently found that the stimulation of adenylyl cyclase by some G_q-coupled receptors was secondary to the activation of the PLC pathway and resulted from the activation of protein kinase C, because the effect was mimicked by phorbol esters and prevented by down-regulation or inhibition of this kinase (33), the stimulation of PLC by G_s-coupled receptors is not a result of cAMP accumulation and protein kinase A activation, because neither forskolin nor 8-bromo-cAMP caused any increase in accumulation of IPs in our cell lines.

The fact that stimulation of PLC by G_s-coupled receptors is weaker than that by G_q-coupled receptors and in most cases requires higher agonist concentrations than does the activation of adenylyl cyclase suggests that the transducer and kinetics for the secondary coupling are different from those for the primary coupling. The involvement of an α subunit of the G_q family has long been suggested, ever since dual coupling by a single receptor type was first described. Evidence exists that a single type of receptor can interact with two G proteins. For example, Eason *et al.* (24) demonstrated by immunoprecipitation that agonist-treated α_2AR was directly associated with the $G_{\alpha s}$ protein, indicating that the receptor may interact with both $G_{\alpha i}$ and $G_{\alpha s}$. Cotransfection studies in COS and human embryonic kidney 293 cells have demonstrated that some G_i-associated receptors, i.e., the receptors for interleukin-8 and the peptide C5a, can stimulate PLC through coupling to α_{16}, a member of the G_q family (34–36). Therefore, it is possible that one or several G_q proteins are activated by the G_s-coupled receptors, especially at high levels of expression.

On the other hand, the release of the free βγ dimer from the activated G proteins provides an alternative explanation for the dual signaling phenomenon. It has been shown in reconstitution assays that three PLC-β subtypes can be stimulated by the free βγ subunits of G proteins, with PLC-β_2 and PLC-β_3 being more sensitive to $G_{\beta\gamma}$ than is PLC-β_1 (37, 38). Simon and co-workers (34, 39) demonstrated by transient transfection in COS-7 cells not only that the coexpression of PLC-β_2, $G_{\beta 1}$, and $G_{\gamma 1}$ or $G_{\gamma 2}$ enhanced the PLC activity but also that the expression of these proteins together with $G_{\alpha i}$ plus either the M2 muscarinic receptor or the interleukin-8 receptor resulted in agonist-induced PLC activation in the transfected cells. As was the case when the M2 receptor was transfected into Chinese hamster ovary cells (2), or in neutrophils where the native interleukin-8 receptors are expressed, the stimulation of PLC by these receptors was inhibited by pertussis toxin, which catalyzes ADP-ribosylation of the α subunit of G_i and G_o and consequently uncouples these G proteins from their receptors. These results indicate that the pertussis toxin-sensitive PLC stimulation in blood cells and in the case of dual signaling by G_i-coupled receptors may be mediated by $G_{\beta\gamma}$ formed upon the activation of G_i proteins.

For the G_s-coupled receptors, it was shown that the dual signaling was not pertussis toxin sensitive (7, 9, 11), indicating that the stimulation of PLC does not result from the interaction

between G_i or G_o and these receptors. CTX modifies $G_{\alpha s}$ by ADP-ribosylation at a different site, which inhibits GTPase activity and causes permanent activation of the G protein, indicating that CTX is a potential agent to study the involvement of G_s in PLC activation. Our results with CTX-treated cells showed that, even if the toxin enhanced the accumulation of IPs, it could not activate the activity to the same extent as the receptors that are expressed at high levels in L cells. On the other hand, in contrast to what has been shown by others, that the stimulation of both adenylyl cyclase and PLC by the D_1 dopamine receptor was diminished after a 24-hr treatment with 2 μg/ml CTX (7), the G_s-coupled receptor-mediated stimulation of PLC activity in our cell lines was not preferentially inhibited by this toxin, because ATP-stimulated accumulation of IPs was also inhibited to the same extent whenever an inhibitory effect was seen. Therefore, the stimulation of PLC by the G_s-coupled receptors is neither completely mimicked nor inhibited by the permanent activation of G_s with CTX. However, because the kinetics of CTX activation of the G protein are different from those of the receptor-mediated event, we cannot completely exclude the involvement of the CTX-sensitive G protein, and hence the $\beta\gamma$ subunits of the G_s protein, in the mechanism of stimulation of PLC by these receptors.

The strict requirement for the coincidence of specific subtypes of effector enzymes and subunits of G protein for multiple signaling may explain some discrepancy among results obtained when receptors are expressed in different cell lines. An example is the D_2 dopamine receptor investigated by Vallar *et al.* (5). When expressed at the same density in L cells and pituitary GH_4C_1 cells, the D_2 receptor was not able to stimulate PLC in GH_4C_1 cells but was capable of doing so in L cells. Analogously to its G_i-coupled counterpart, the D_1 dopamine receptor expressed in L cells induced intracellular Ca^{2+} release through activation of PLC, but this receptor expressed in GH_4C_1 cells induced only a rise in intracellular Ca^{2+} concentrations through a voltage-dependent Ca^{2+} channel, a result secondary to the increase of cAMP due to G_s-coupled adenylyl cyclase activation (7). It would appear that the components for dual coupling, either the necessary α subunits of the G_q family or subtypes of PLC-β, are not expressed in these cells.

The intrinsic efficacies of G_s-coupled receptors to activate PLC are different. Among the four receptors tested in our study, the LHR is the strongest PLC stimulator. The coupling to PLC was clearly evident in LHR cells expressing 4000 sites/cell, whereas such coupling was not detected for V2R cells expressing 20,000 sites/cell or β_2AR cells expressing 120,000 sites/cell. Furthermore, the LHR cells expressing 40,000 sites/cell stimulated PLC by about 2-fold more than did the V2R cells expressing 500,000 sites/cell and β_1AR and β_2AR cells expressing 300,000 sites/cell. It has recently been hypothesized that the ability of some G_i-coupled receptors to couple to PLC may be related to the duration of receptor activation (40); for example, the thrombin receptor is persistently activated by cleavage of its amino terminus (6) and thus couples to PLC much better than does the α_2AR, which is activated as a result of a rapidly reversible, short-lived interaction with its agonist. In our examples with G_s-coupled receptors, hCG and LHR form a more stable hormone-receptor complex, and this results in longer receptor activation, compared the other ligands and their receptors. This may also explain our observation that bovine LH stimulated adenylyl cyclase but not PLC better than did hCG (Fig. 3), because it is believed that LH binding is more reversible than hCG binding (41). In addition, of the two βARs, the β_1 subtype coupled better to PLC and more weakly to adenylyl cyclase than did the β_2 subtype. Among the glycoprotein hormone receptors, the rat FSH receptor expressed in L cells at 10,000 sites/cell stimulated adenylyl cyclase in response to ovine, equine, or human FSH to 80% of PGE_1-stimulated activity, comparable to levels seen in response to hCG in LHR cells expressing a similar density of LHR, but did not stimulate PLC.[2] Others have failed to detect FSH-stimulated PLC activity in cultured Sertoli cells (42). These observations indicate that the dual signaling potential may depend on minor differences in individual receptors that define the nature of agonist-receptor and/or receptor-G protein interactions and should not be categorized simply according to the overall homology of the molecular structures.

The classical pharmacological model predicts that doubling of the receptor concentration is associated with reduction by half of the EC_{50} values of the dose-response curves. In cells stably transfected with G_s-coupled receptors, a general trend was observed for agonist-stimulated adenylyl cyclase activity, namely that EC_{50} tends to decrease as the receptor density increases. This is true for cells expressing LHR (Table 2), V2R, and βARs.[3] However, the change of the EC_{50} values does not fit the model precisely, which points to the fact that this is not an ideal system in which all components diffuse freely to interact according to the law of mass action. In addition, we have made no attempt to evaluate, on a statistical basis, the quantitative relation between receptor density and EC_{50} values. Due to cell-to-cell variability (25), such an evaluation was outside the scope of this study.

In conclusion, our results suggest that most G protein-coupled receptors have the potential to couple to multiple signaling pathways and the occurrence of an individual pathway is dependent on the amount of receptor and the concentration of agonist. The multiple pathways may play a very important role in regulating the effectiveness of the signals at different sites in the body. Depending on the expression level of the receptor and the local concentration of the signal, the final outcome of ligand binding may differ. The mechanism underlying the multiple signaling phenomenon requires further investigation.

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[2] X. Zhu and L. Birnbaumer, unpublished observations.
[3] M. Birnbaumer and F. O. Levy, unpublished observations.

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