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Differential Coupling of Glucagon and β-Adrenergic Receptors with the Small and Large Forms of the Stimulatory G Protein

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SUMMARY

Glucagon receptors (GRs) and β-adrenergic receptors (β-ARs) stimulate adenylate cyclase (AC) via G_s. The present study was performed to determine whether different cAMP-generating receptors share the same pool of G_s. In hepatocytes and liver plasma membranes from partially hepatectomized male rats, glucagon was more potent in stimulating AC than β-adrenergic agonists, but the effects of glucagon and β agonists on AC activity were not additive. This suggests that GRs and β-ARs share the same pathway. Glucagon lowered the affinity of β agonists for β-ARs in the presence of GTPγS, whereas β agonists had no effect on glucagon binding to GRs regardless of the presence or the absence of GTPγS. Therefore, the pool of G_s coupled to GRs appears to include that coupled to β-ARs. The α subunit of G_s ($G_{s\alpha}$) exists in small ($G_{s\alpha\text{-}S}$) and large ($G_{s\alpha\text{-}L}$) forms. Recently, with a new method that uses tryptic digestion, the G protein coupled to β-ARs was identified as $G_{s\text{-}L}$ in partially hepatectomized male rat livers because β-adrenergic agonists promoted trypsinization of $G_{s\alpha\text{-}L}$ but not of $G_{s\alpha\text{-}S}$. By contrast, the present study showed that glucagon enhanced the sensitivity of the two $G_{s\alpha}$ isoforms to trypsin in a concentration-dependent manner, indicating that GRs are coupled to both $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$. In conclusion, GRs share a common $G_{s\text{-}L}$ with β-ARs but are also coupled to another G_s, $G_{s\text{-}S}$, in partially hepatectomized male rat livers.

The liver plays a central role in homeostasis of blood glucose. Gluconeogenesis and glycogenolysis are stimulated by glucagon and catecholamines, which also stimulate AC via GRs (1) and β-ARs (2), respectively. This pathway is mediated by G_s (3). Because the enzyme activities induced by these two hormones are additive in liver homogenates from adult male rats, the hepatic AC system responding to glucagon has been suggested to differ from that responding to catecholamines (4, 5). However, the effects of glucagon and epinephrine on glycogenolysis are not additive in fetal rat hepatocytes (6). Thus, whether GRs and β-ARs share a common cAMP-generating pathway remains controversial.

G_s is the site at which signal transduction pathways from the two receptors converge. G_s is a heterotrimer consisting of α ($G_{s\alpha}$), β, and γ subunits (3). The most common approach to determine which G proteins are coupled with a given receptor has been to use purified receptors and G proteins to reconstitute receptor function in phospholipid vesicles (7, 8). However, this approach has generated conflicting results, presumably because of an unphysiological environment. For this reason, rat liver plasma membranes were used in the present study.

In adult male rat livers, glucagon is a potent stimulator of AC, whereas catecholamines are less potent (9, 10). However, partial hepatectomy enhances the hepatic AC in response to glucagon (11) and catecholamines (12, 13). During liver regeneration, the number of GRs is not altered (11), whereas that of β-ARs increases (12, 14). However, the basic binding parameters of ligands to these receptors remain unaltered during regeneration (11, 12). The coupling of β-ARs with G_s is enhanced after partial hepatectomy (15). The level of $G_{s\alpha}$ increases in regenerating livers (15–17), whereas the intrinsic AC activity is unaffected (12). Thus, liver plasma membranes from partially hepatectomized male rats appear to be suitable for a comparison of the coupling of GRs to G_s with that of β-ARs to G_s.

Because the affinity of a ligand to bind the receptor increases when the receptor is associated with its G protein, the radioligand binding assay can be used to assess the receptor/G protein interactions (18, 19). However, because of the existence of the small ($G_{s\alpha\text{-}S}$) and large ($G_{s\alpha\text{-}L}$) forms of $G_{s\alpha}$ (20, 21), another approach is required to determine which form of $G_{s\alpha}$ is coupled to a given receptor. The coupling of receptors to G_s can be evaluated via a newly developed tryptic digestion method (15). $G_{s\alpha}$ is associated with GDP in its inactive state. An appropriate agonist/receptor complex catalyzes the exchange from GDP to GTP on $G_{s\alpha}$ (3). The tryptic digestion method is based on the feature that the GTP-bound $G_{s\alpha}$ is more susceptible to trypsin than the GDP-bound $G_{s\alpha}$ in the initial stage of proteolysis (15). With this approach, the G

ABBREVIATIONS: AC, adenylate cyclase; GR, glucagon receptors; β-AR, β-adrenergic receptor; ICYP, iodocyanopindolol; IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

protein coupled to β-ARs was identified as $G_{s\text{-}L}$ in partially hepatectomized male rats (15). In this study, the radio binding assay and the tryptic digestion method were used to analyze the G protein coupling of GRs and β-ARs in partially hepatectomized male rat livers. The primary question was whether GRs share the common $G_{s\text{-}L}$ with β-ARs or have an independent pathway through the $G_{s\text{-}S}$.

Experimental Procedures

Materials. [α-^{32}P]NAD (29.6 TBq/mmol), [^{125}I]glucagon (81.4 TBq/mmol), and [^{125}I]ICYP (8.14 TBq/mmol) were purchased from DuPont-New England Nuclear (Wilmington, DE). Isoproterenol bitartrate, glucagon, cholera toxin, bovine serum albumin, IBMX, *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). GTPγS and Gpp(NH)p were purchased from Boehringer Mannheim (Mannheim, Germany). The sources of all other chemicals were as described previously (22).

Animals and surgery. Male Slc:Wistar strain rats (9–10 weeks old), weighing 230–260 g, were obtained from Japan SLC (Hamamatsu, Japan). Partial hepatectomy (70%) was performed as described Higgins *et al.* (23). All surgical procedures were performed between 8:00 a.m. and 12:00 noon.

Measurement of AC activity in rat hepatocytes and liver plasma membranes. Rat hepatocytes and liver plasma membranes were prepared by the collagenase perfusion method (24) and the Percoll-centrifugation method (25), respectively. AC activity in isolated hepatocytes and liver plasma membranes was measured according to the method of Itoh *et al.* (26). The cell suspension (3×10^6 cells/ml) was first incubated for 15 min at 37° in HEPES-buffer medium composed of 10 mM HEPES, pH 7.4, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 5 mM $NaHCO_3$, in the presence of 400 μM IBMX under an atmosphere of 95% O_2/5% CO_2. After this preincubation, the resulting cell suspension (500 μl) was further incubated with 10 μl of various stimulants dissolved in HEPES-buffer medium at 37° for 2 min. After termination of the reaction by the addition of 50 μl of 1 N HCl, the cell suspension was homogenized for 10 min. The resulting supernatant was used for the measurement of cAMP. When plasma membrane was used for the assay of AC activity, the reaction buffer contained 25 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 400 μM IBMX, 500 μM ATP, 5 mM phosphocreatine, and 50 units/ml creatine phosphate kinase. The assay was started by the addition of plasma membrane (20 μg of protein) to 200 μl of the reaction mixture. After the incubation at 30° for 5 min with various stimulants, the reaction was terminated by the addition of 20 μl of 1 N HCl. The supernatant obtained by centrifugation at 1700 × *g* for 10 min was used for the determination of AC activity. cAMP generated from ATP in isolated hepatocytes or liver plasma membranes was measured with a Yamasa cAMP assay kit (Chiba, Japan) (27).

Assay of [^{125}I]ICYP binding. The binding assay was performed according to the method of Nakamura *et al.* (28) with a minor modification. The assay mixture (250 μl) contained 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM sodium ascorbate, 1 mM EGTA, 1 mM pyrocatechol, 50 kallikrein units/ml aprotinin, and liver plasma membranes (100 μg of protein). The mixture was incubated at 25° for 40 min in the presence or absence of GTPγS and glucagon with 100 pM [^{125}I]ICYP and various concentrations of isoproterenol. The reaction was terminated by rapid dilution with 5 ml of ice-cold 20 mM Tris·HCl, pH 7.5, containing 5 mM $MgCl_2$ and 100 mM NaCl. The dilute samples were immediately filtered under vacuum through Whatman GF/C glass fiber filters (2.4 cm; Kent, UK) that had been washed with the dilution buffer containing 1 mM sodium ascorbate and 1 mM pyrocatechol. The filters were promptly washed three times with 2.5 ml of ice-cold dilution buffer. The radioactivity on the filter was measured in an Aloka ARC 600. (Tokyo, Japan). Nonspecific binding was determined in the presence of 10 μM DL-propranolol, which was routinely <20%.

Assay of [^{125}I]glucagon binding. The binding assay was carried out according to a modification of the method of Rodbell *et al.* (29). The incubation mixture (100 μl) contained 2.8% bovine serum albumin (fraction V), 1 mM EDTA, 50 mM Tris·HCl, pH 7.4, 300 pM [^{125}I]glucagon (approximately 1×10^5 dpm), and liver membranes (10 μg of protein). The binding reaction was terminated by the addition of 2.5 ml ice-cold 50 mM Tris·HCl, pH 7.4, and immediately followed by rapid filtration through a Whatman GF/C glass fiber filter (presoaked in 1% polyethyleneimine) under reduced pressure. The filter was then quickly washed five times with 2.5 ml ice-cold 50 mM Tris·HCl, pH 7.4. Specific binding was calculated by subtracting the nonspecific binding in the presence of 1 μM glucagon and was ~80% of the total binding.

Tryptic digestion of liver plasma membranes. Trypsin treatment of liver plasma membranes was performed essentially according to the method of Van Dop *et al.* (30). The mixture (20 μl), containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 500 μM dithiothreitol, 1 mM EGTA, 50 kallikrein units/ml aprotinin, and liver plasma membranes (20 μg of protein) prepared on the second day after partial hepatectomy, was preincubated at 30° for 20 min in the presence or absence of effectors. To the mixture we added 80 μl of the trypsin solution (12.5 mM MOPS, pH 7.5, 225 mM NaCl, 5 mM $MgCl_2$, 1.1 mM dithiothreitol, and 12.5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin), and the mixture was further incubated at 30°. At the indicated times, the reaction was terminated by the addition of trypsin inhibitor with a trypsin/trypsin inhibitor ratio of 1:10 (w/w). Membranes were washed with 10 mM MOPS, pH 7.5; ADP-ribosylated by cholera toxin; and subjected to SDS-PAGE.

ADP-ribosylation of liver plasma membranes. ADP-ribosylation by cholera toxin was carried out according to a modification of the method of Nakamura *et al.* (25). After tryptic digestion, membranes were incubated with cholera toxin (50 μg/ml) for 45 min at 30° in 100 μl of reaction mixture containing 100 μM potassium phosphate, pH 8.0, 12.5 mM thymidine, 100 μM GTP, 1 mM ATP, 1 mM $MgCl_2$, 500 μM EGTA, and 20 μM [^{32}P]NAD (1.85 MBq/ml). Cholera toxin was activated with 20 mM dithiothreitol at 30° for 20 min before use. After incubation, the membranes were collected by centrifugation at 7000 × *g* for 10 min, and the pellet was suspended in a 20-μl sample buffer (50 mM Tris·HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol), boiled for 3 min, and subjected to SDS-PAGE with 11% gels. After electrophoresis, the gel was soaked in 15% trichloroacetic acid, dried, and exposed to X-ray film with the use of an intensifying screen for 2–9 days at −80°. In addition, the gel was exposed on an imaging plate (Fuji Film Co., Ltd., Tokyo, Japan) for 3–6 hr, and the autoradiographic images were analyzed with a Bio-Imaging Analyzer (BAS-2000; Fuji Film Co. Ltd.) for measurement of the absorbance of bands of the [^{32}P]ADP-ribosylated $G_{s\alpha}$.

Results

Effects of glucagon and β-adrenergic agonists on hepatic AC activity. To determine whether the effects of glucagon and catecholamines on hepatic AC activity are additive, we examined glucagon- and epinephrine-induced cAMP accumulations in hepatocytes from partially hepatectomized male rats (Fig. 1). Both glucagon (Fig. 1A) and epinephrine (Fig. 1B) increased cAMP accumulation in a concentration-dependent manner. However, the maximal AC activity stimulated by glucagon was ~3-fold higher than that stimulated by epinephrine. Epinephrine added at the concentration at which it maximally stimulated hepatic AC activity (10 μM; Fig. 1B) enhanced the activity of AC responding to glucagon (0.1–10 nM), but the maximal AC activity stimulated by glu-

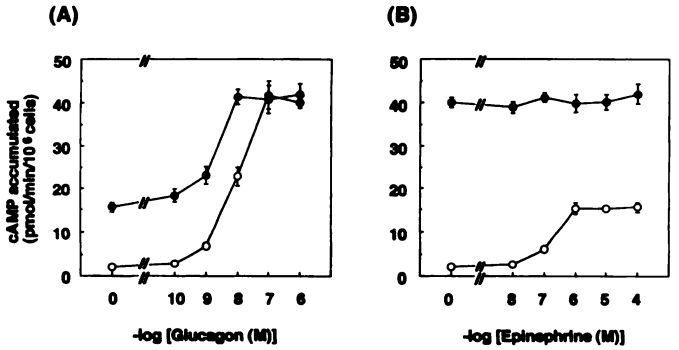

Fig. 1. Effects of glucagon and epinephrine on cAMP accumulation in rat hepatocytes. Hepatocytes were isolated from rat livers on second day after partial hepatectomy. Hepatocytes were preincubated with 400 μM IBMX for 15 min at 37°. A, Hepatocytes were incubated in the presence (●) or absence (○) of 10 μM epinephrine with indicated concentrations of glucagon for 2 min at 37°. B, Hepatocytes were incubated in the presence (●) or absence (○) of 1 μM glucagon with indicated concentrations of epinephrine for 2 min at 37°. Values are mean ± standard error for four separate experiments done in triplicate.

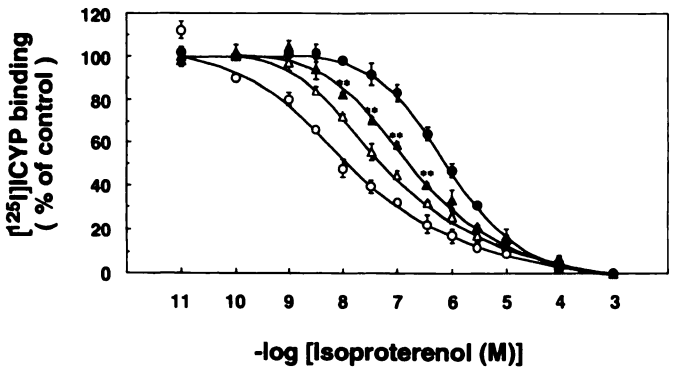

Fig. 2. Effects of GTPγS and glucagon on the displacement by isoproterenol of [^{125}I]ICYP binding to liver plasma membranes. Membranes prepared on second day after partial hepatectomy were incubated with 100 pM [^{125}I]ICYP and indicated concentrations of isoproterenol without (○) or with 10 nM GTPγS (△), 10 nM GTPγS plus 1 μM glucagon (▲), or 10 μM GTPγS (●) at 25° for 40 min. The control value of [^{125}I]ICYP binding was 123 ± 25 and 142 ± 18 fmol/mg protein, respectively, in the absence and presence of 10 μM GTPγS. Values are mean ± standard error for four separate experiments done in triplicate. **, $p < 0.01$ versus 10 nM GTPγS alone by *t* test.

cagon (0.1–1 μM) remained unaffected (Fig. 1A). On the other hand, the activity of AC responding to epinephrine was also enhanced (Fig. 1B) by glucagon added at the concentration at which it maximally stimulated hepatic AC activity (1 μM; Fig. 1A). However, the concentration-dependent effect of epinephrine on AC activity was lost (Fig. 1B). Replacement of epinephrine by isoproterenol gave the same results (data not shown).

Table 1 shows the AC activities examined in the liver plasma membranes prepared from partially hepatectomized male rats. In the presence of nonhydrolyzable GTP analogues (100 nM), AC activities responding to glucagon (1 μM) and isoproterenol (10 μM), a β-adrenergic agonist, showed ~4-fold and 1.5-fold increases, respectively. The concentrations of these agonists were sufficient to exert their maximal effects on their hepatic AC activity (11, 31). The addition of the two hormones induced together nearly the same enzyme activity as that induced by glucagon alone. Replacement of isoproterenol by epinephrine gave the same results (data not shown). These findings suggested that the effects produced by glucagon and β-adrenergic agonists are not additive.

TABLE 1

Effects of glucagon and isoproterenol on cAMP generation in liver plasma membranes

Liver plasma membranes prepared on second day after partial hepatectomy were incubated with various concentrations of hormones and/or drugs in the presence of 400 μM IBMX for 5 min at 30°. The concentrations of substances were 100 nM GTPγS, 100 nM Gpp(NH)P, 10 μM isoproterenol (ISO), 1 μM glucagon (GLU), 10 μM GTPγS*, and 10 μM forskolin (FSK). Values are mean ± standard error for four separate experiments done in triplicate.

Hormones and drugs	cAMP generation
	pmol/min/mg protein
None	0.86 ± 0.10
GTPγS	6.00 ± 0.80
GTPγS + ISO	10.30 ± 0.95[a]
GTPγS + GLU	28.90 ± 1.91[a]
GTPγS + ISO + GLU	29.05 ± 1.43[b]
Gpp(NH)p	5.68 ± 0.24
Gpp(NH)p + ISO	8.32 ± 0.88[c]
Gpp(NH)p + GLU	21.21 ± 1.95[c]
Gpp(NH)p + ISO + GLU	23.28 ± 1.82[d]
GTPγS* + FSK	75.47 ± 7.61

[a] $p < 0.05$ versus GTPγS alone by *t* test.
[b] $p > 0.05$ versus GTPγS + GLU by *t* test.
[c] $p < 0.05$ versus Gpp(NH)p alone by *t* test.
[d] $p > 0.05$ versus Gpp(NH)p + GLU by *t* test.

Effects of glucagon on displacement by isoproterenol of [^{125}I]ICYP binding. To test the possibility that glucagon influences the coupling of β-AR with G_s, the effect of glucagon on isoproterenol competition in the binding of [^{125}I]ICYP was examined in liver plasma membranes prepared from partially hepatectomized male rats (Fig. 2). Isoproterenol inhibited the binding of [^{125}I]ICYP to β-ARs in a concentration-dependent manner (Fig. 2). The IC_{50} of isoproterenol for [^{125}I]ICYP was 18 ± 1 nM in the absence of GTP analogue. On the other hand, in the presence of GTPγS (10 μM), the isoproterenol competition curve for [^{125}I]ICYP binding was shifted to the right 30-fold (IC_{50} = 513 ± 21 nM). The competition curves in the presence of GTPγS (10 μM) had a Hill coefficient of ~1.0, suggesting a single site with low affinity. The complex of β-AR and GDP-bound G_s exhibits high affinity for agonists, whereas the receptor alone shows low affinity (18, 19). Thus, this suggests that the excess of GTPγS causes the exchange from GDP to GTPγS on G_s, the release of G_s from the receptors, and the resultant low affinity of free receptors for an agonist.

In the absence of GTPγS, the binding of [^{125}I]ICYP and isoproterenol to β-ARs was not affected by glucagon (data not shown). However, in the presence of GTPγS (10 nM), glucagon decreased significantly the apparent affinity of β-ARs for isoproterenol (Fig. 2). In the presence of 10 nM GTPγS alone, the IC_{50} of isoproterenol for [^{125}I]ICYP was 44 ± 5 nM. Glucagon (1 μM) increased the IC_{50} to 158 ± 13 nM. Similar findings were obtained when epinephrine and Gpp(NH)p were used instead of isoproterenol and GTPγS, respectively (data not shown). The high affinity agonist binding to receptors that is capable of being converted to the low affinity agonist binding by the addition of a GTP analogue reflects the coupling of the receptors with the G protein (18, 19). Thus, glucagon appears to uncouple β-ARs from G_s in the presence of GTP.

Effects of β-adrenergic agonists on glucagon binding to GRs. Because glucagon decreased the apparent affinity of

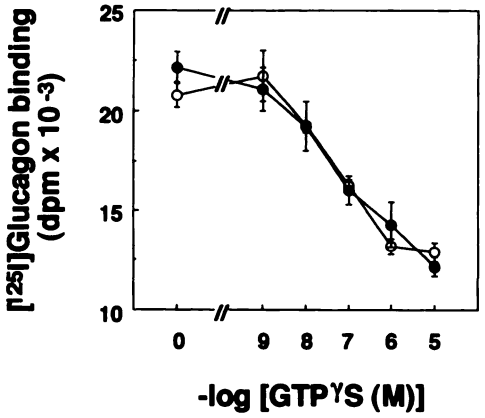

Fig. 3. Effect of isoproterenol on the inhibition by GTPγS of [^{125}I]glucagon binding to liver plasma membranes. Membranes prepared on second day after partial hepatectomy were incubated in the presence (●) or absence (○) of 10 μM isoproterenol with 300 pM [^{125}I] glucagon and indicated concentrations of GTPγS. Values are mean ± standard error for four separate experiments done in triplicate.

β-adrenergic agonists for β-ARs in the presence of GTP analogues, β-adrenergic agonists might also affect the guanine nucleotide-dependent binding of glucagon to its receptors. To test the possibility, the specific binding of [^{125}I]glucagon to liver plasma membranes was measured with various concentrations of GTPγS in the presence or absence of isoproterenol. The binding parameters of glucagon to GRs (B_{max} = 2000 ± 123 fmol/mg protein, K_d = 902 ± 103 pM) was not affected by isoproterenol (data not shown). As reported by Rojas *et al.* (32), GTPγS reduced the binding of [^{125}I]glucagon in a concentration-dependent manner (Fig. 3). Contrary to expectation, 10 μM isoproterenol did not affect the affinity of glucagon for GRs in the presence of GTPγS. The same results were obtained with the use of epinephrine and Gpp(NH)p instead of isoproterenol and GTPγS, respectively (data not shown).

Tryptic digestion of G_s protein. To determine which form of G_s is coupled to GRs, we examined the effect of glucagon on the trypsin sensitivity of G_s. Liver plasma membrane from partially hepatectomized male rats was preincubated with glucagon or isoproterenol in the presence of Gpp(NH)p, cleaved with trypsin, ADP-ribosylated by cholera toxin, and subjected to SDS-PAGE. Fig. 4A shows the autoradiogram of the gel and quantitative results. Cholera toxin specifically ADP-ribosylated 42-kDa ($G_{sα\text{-}S}$) and 47-kDa ($G_{sα\text{-}L}$) proteins in rat liver plasma membrane. The reliability of quantitative measurements by cholera toxin-catalyzed ADP-ribosylation is similar to that of the immunoblot method using antisera specific for $G_{sα}$ (17). The contents of $G_{sα\text{-}L}$ and $G_{sα\text{-}S}$ were estimated to be 460 ± 16 and 560 ± 18 fmol/mg protein by ADP-ribosylation, respectively. The possibility that trimmed $G_{sα\text{-}L}$ comigrates with $G_{sα\text{-}S}$ is excluded because the amino terminal domain of $G_{sα}$ required for cholera toxin-catalyzed ADP-ribosylation (33) is removed in the initial stage of tryptic digestion (34). Fig. 4B shows the time course of limited tryptic digestion of G_s. In the absence of GTP analogues, $G_{sα\text{-}L}$ was gradually trimmed by trypsin, whereas $G_{sα\text{-}S}$ was virtually insensitive, as previously reported (15, 34). However, Gpp(NH)p increased the sensitivities of both $G_{sα\text{-}S}$ and $G_{sα\text{-}L}$ to trypsin in a concentration-dependent manner. This effect of Gpp(NH)p on the tryptic digestion of $G_{sα}$ was significantly retarded by GDP (15). Preincubation of the plasma membranes with 100 nM Gpp(NH)p and 1 μM glucagon significantly enhanced the sensitivity of the two $G_{sα}$s to trypsin compared with the preincubation with 100 nM Gpp(NH)p alone. On the other hand, as described previously (15), 10 μM isoproterenol enhanced the sensitivity of $G_{sα\text{-}L}$ to trypsin but not that of $G_{sα\text{-}S}$. As shown in Fig. 5, each agonist increased the sensitivity of $G_{sα}$ to trypsin in a concentration-dependent manner.

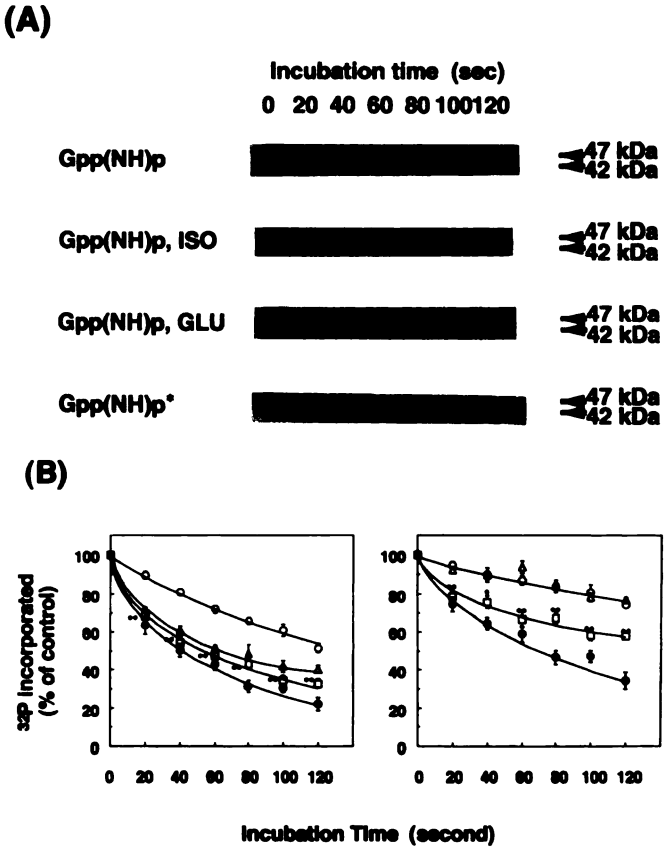

Fig. 4. Effect of glucagon (*GLU*) and isoproterenol (*ISO*) on trypsin sensitivity of $G_{sα}$. A, Autoradiogram of $G_{sα}$ [^{32}P]ADP-ribosylated after tryptic digestion. B, Quantitative data for $G_{sα\text{-}L}$ (*left*) and $G_{sα\text{-}S}$ (*right*) were obtained as described in Experimental Procedures. Membranes prepared on second day after partial hepatectomy were incubated at 30° with 100 nM Gpp(NH)p (○), 100 nM Gpp(NH)p plus 10 μM isoproterenol (△), or 100 nM Gpp(NH)p plus 1 μM glucagon (□) for 20 min or with 100 μM Gpp(NH)p* for 60 min (●). After treatment with trypsin, the membranes were ADP-ribosylated by cholera toxin and subjected to SDS-PAGE. Values are mean ± standard error for nine separate experiments. *, $p < 0.05$ or **, $p < 0.01$ versus 100 nM Gpp(NH)p alone by *t* test.

Discussion

Results of the present study showed that GRs share a common cAMP-generating pathway with β-ARs but also stimulate AC via the pathway independent of β-ARs in partially hepatectomized male rat livers.

The present findings confirm previous findings that glucagon induces glycogenolysis via the same pathway as that used by β agonists in fetal hepatocytes (6). The AC activity induced by the two hormones was not additive in hepatocytes (Fig. 1) or in liver plasma membranes (Table 1) from partially hepatectomized male rats. It might be argued that AC activity reaches a maximal level in response to glucagon or a β

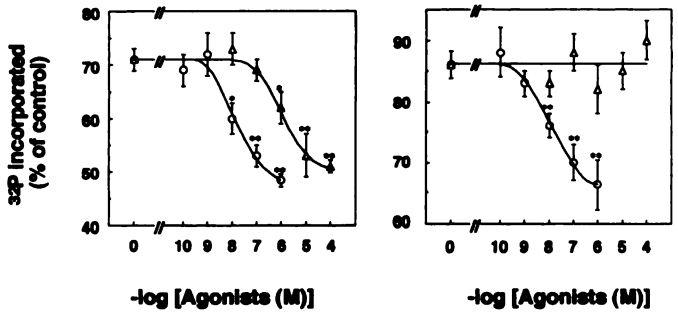

Fig. 5. Concentration dependence of glucagon and isoproterenol on trypsin sensitivity of $G_{s\alpha}$. Membranes prepared on second day after partial hepatectomy were incubated with indicated concentrations of glucagon (○) or isoproterenol (△) in the presence of 100 nM Gpp(NH)p for 20 min, cleaved with trypsin for 1 min, ADP-ribosylated by cholera toxin, and subjected to SDS-PAGE. *Left*, quantitative data for $G_{s\alpha\text{-}L}$. *Right*, quantitative data for $G_{s\alpha\text{-}S}$. Values are mean ± standard error for nine separate experiments. *, $p < 0.05$ or **, $p < 0.01$ versus 100 nM Gpp(NH)p alone by *t* test.

agonist alone. However, AC activity induced by a combination of forskolin and GTPγS was much higher than that induced by glucagon and/or β agonists (Fig. 2). Forskolin is known to stimulate AC directly (35) or indirectly via G_s (36). The activation of G_s is required for the full expression of forskolin-stimulated AC activity (36). The nonadditive actions of the two hormones are contradictory to the findings reported by Bitensky *et al.* (4, 5). It should be noted that these authors assayed the hepatic AC activity in liver homogenates without added GTP (4, 5) because the involvement of G proteins in cAMP production was not known at that time. With low levels of GTP, AC activation stimulated by glucagon or catecholamines is too low to make reliable quantification. In the present study, the enzyme activity was measured in the presence of nonhydrolyzable GTP analogues. Thus, the discrepancy between the present observations and the report of Bitensky *et al.* might be attributed to the effect of guanine nucleotides on hormone-induced AC activity. These findings suggest that GRs and β-ARs use, at least in part, the same signal transduction pathway.

The affinity of receptors for agonists reflects the coupling state of the receptor to G proteins (18, 19). When two different receptor classes compete for the same pool of G proteins, the addition of the agonist for one of the receptors will allow that receptor to catalyze the exchange from GDP to GTP on the G proteins and the dissociation of G_α from $G_{\beta\gamma}$. Because the dissociated G proteins are no longer associated with the receptors, the other class of receptors is prevented from binding to the G proteins, thereby showing low affinity for its agonists (37). This method was used to examine the possibility that different cAMP generating receptors share the same pool of G_s. Glucagon, through its interaction with the GRs, decreased the apparent affinity of β-ARs for β agonists (Fig. 2). We wondered whether this inhibitory effect of glucagon could be mediated by protein kinases activated by GRs. This possibility can be excluded because no ATP, a substrate of protein kinases, was added to liver plasma membrane preparations used for the binding assay. Thus, GRs appear to share the same G_s with β-ARs.

Interestingly, the binding parameters of [^{125}I]glucagon to GRs were not affected by activation of β-ARs (Fig. 3). Two alternative explanations are possible for these results. First, GRs may bind to their G proteins with higher affinity than β-ARs. Second, because the number of GRs (2000 ± 123 fmol/mg protein) is much higher than that of β-ARs (245 ± 21 fmol/mg protein), the pool of G_s available for GRs may be larger than that available for β-ARs. Which explanation is more likely remains unknown at present.

Receptor/G protein interaction can also be monitored by determining the receptor-mediated stimulation of [γ-^{35}S]GTPγS binding (38) and GTPase activity (39). However, in liver plasma membranes, these activities could not be detected in response to agonists. This failure may be due to the presence of other G proteins such as G_i (40, 41). To overcome this problem, we used a new method that used tryptic digestion. This method was developed based on the following results. The free α subunit of transducin ($G_{t\alpha}$) bound with GTPγS is digested by trypsin more rapidly than the $G_{t\alpha}$ associated with βγ subunits in the initial stage of proteolysis (42). Thus, GTP-bound active $G_{t\alpha}$ appears to be more sensitive to trypsin than the GDP-bound inactive $G_{t\alpha}$. $G_{t\alpha}$ is first cleaved by trypsin at Lys^{18} and then at Arg^{310} and Arg^{204}. In contrast, the presence of Gpp(NH)p or GTPγS enhanced cleavage at Lys^{18} in the amino terminal region and at Arg^{310} in the carboxyl terminal region but protected cleavage at Arg^{204} (42). In the case of $G_{s\alpha}$, the GTP analogue-bound form is also resistant to trypsin at Arg^{232}, which corresponds to Arg^{204} of $G_{t\alpha}$ (43). Furthermore, trypsinization of $G_{s\alpha}$ in plasma membranes from partially hepatectomized male rat livers was promoted by Gpp(NH)p in a concentration-dependent manner (15). This effect of Gpp(NH)p was abolished in the presence of GDP (15). Therefore, the acceleration of proteolysis of $G_{s\alpha}$ reflects the conversion of GDP-bound $G_{s\alpha}$ to GTP-bound $G_{s\alpha}$. $G_{s\alpha\text{-}L}$ was more susceptible to this protease in the presence of β-adrenergic agonists, and the effect of agonists was abrogated in the presence of a β-adrenergic antagonist (Fig. 4) (15). On the other hand, the sensitivity of $G_{s\alpha\text{-}S}$ to tryptic cleavage remained unchanged regardless of the presence or absence of the agonist and/or antagonist (Fig. 4) (15). These findings indicate that a β-adrenergic agonist/receptor complex catalyzes the exchange from GDP to GTP on $G_{s\alpha\text{-}L}$ but not on $G_{s\alpha\text{-}S}$. By this method, we evaluated the coupling of GRs to G_s (Figs. 4 and 5). In contrast to β-adrenergic agonists, glucagon enhanced not only the trypsinolysis of $G_{s\alpha\text{-}L}$ but also that of $G_{s\alpha\text{-}S}$ in a concentration-dependent manner. Thus, GRs appear to catalyze the exchange of guanine nucleotides on both $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$, whereas β-ARs do on $G_{s\alpha\text{-}L}$ preferentially.

In conclusion, assessment of receptor/G protein coupling with tryptic digestion proved to be useful in determining the specificity of receptor/G protein interaction, even if these proteins are difficult to be reconstituted. With this approach, we demonstrated that GRs share a common $G_{s\text{-}L}$ with β-ARs but are also coupled to another G_s, $G_{s\text{-}S}$, in partially hepatectomized male rat livers. This accounts for the high potency of glucagon in stimulating AC compared with catecholamines.

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