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Evidence for receptor-mediated inhibition of intrinsic activity of GTP-binding protein, G_i1 and G_i2, but not G_0 in reconstitution experiments

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The receptor-mediated inhibition of intrinsic activities of GTP-binding proteins (G-proteins) was studied. Pertussis toxin (IAP)-substrate G-protein, G_i1, G_i2 or G_0, was prelabeled with [α-^{32}P]GDP and reconstituted with synaptic membranes of the guinea pig cerebellum in the presence of 0.02% of Chaps. Intrinsic activities of G-proteins were evaluated by the release of [α-^{32}P]GDP in exchange for added GppNHp or GDP in reconstituted preparations. U-50,488H (1 nM–10 μM), a specific κ-subtype of opioid receptor agonist, inhibited the [α-^{32}P]GDP release in exchange for added 1 μM GppNHp in G_i1-reconstituted preparations in a concentration-dependent manner. On the other hand, the κ-opioid agonist at 10 μM increases the K_m values of GppNHp, but not GDP in exchange for [α-^{32}P]GDP release in preparations reconstituted with G_i1 or G_i2, but not with G_0. These findings indicate that κ-opioid receptor is coupled to inhibition of intrinsic activities of G_i1 and G_i2, but not G_0, in guinea pig cerebellar membranes. In addition, it was revealed that the mode of action is mediated by a decrease in affinity of GTP (or its analog) for G-proteins, but not by a change in affinity of GDP.

κ-Opioid receptor; U-50,488H; GTP-binding protein; GDP-GTP exchange; Cerebellar membrane; Reconstituted preparation

1. INTRODUCTION

The guanine nucleotide-binding regulatory proteins (G-proteins) are transducers communicating between receptors and effectors involved in the formation of intracellular second messengers. According to current models of signal transduction between receptors and G-proteins [1], the receptors, when stimulated by their agonists, enhance the exchange of GDP bound to coupled G-proteins for GTP. The activation of G-proteins is terminated by the hydrolysis of GTP to GDP (or P_i release) due to the low-K_m (high affinity) GTPase activity inherent in the G-protein α-subunits. Thus, the agonist-mediated increase in low-K_m GTPase activity is accepted as a secondary event to increase in GDP–GTP exchange [2,3].

However, in the course of studying the functional coupling between opioid receptors and G-proteins by measuring low-K_m GTPase in synaptic membranes, we found an unexpected finding that an opioid agonist of κ-subtype receptor inhibited this activity in guinea pig cerebellar membranes [4]. Further study reveals that the G-protein involved in such mechanisms is pertussis toxin (IAP)-sensitive [5]. The GTPase reaction proceeds on G-proteins as a result of sequential repetition of the two processes, i.e. (i) the GDP–GTP exchange as a 'turn on' reaction in the signal transduction and (ii) the hydrolysis of GTP as a 'turn off' reaction. Therefore, it is an important question with regard to the physiological role in the signal transduction whether the κ-receptor-mediated inhibition of low-K_m GTPase activity is attributed to the inhibition of 'turn on' reaction or to inhibition of 'turn off' one.

The aims in the present study are to specify the kinds of IAP-substrate G-proteins relevant to, and to clarify the mode of action in, such inhibitory mechanisms of G-protein activities.

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2. MATERIALS AND METHODS

2.1. *Drugs*

U-50, 488H was a gift from Upjohn, Japan. [α-^{32}P]GTP was purchased from Dupont-New England Nuclear. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) was purchased from Dojindo Lab. (Kumamoto, Japan). Other reagents were of analytical grade and purchased from Sigma.

2.2. *Membrane preparation*

Male guinea pigs weighing 250–350 g were decapitated. The cerebellum was dissected out, homogenized in 10 vols of 0.32 M sucrose and centrifuged at 1000 × *g* for 10 min. The supernatant was mixed with 9 vols of 20 mM Tris-HCl buffer, pH 7.5 (buffer A), and centrifuged at 35 000 × *g* for 20 min at 4°C. The pellet was resuspended in buffer A containing 1 mM dithiothreitol (DTT) by use of Dounce-homogenizer to make a protein concentration of 0.5–0.8 mg/ml.

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2.3. *Preparation of [α-^{32}P]GDP-bound G-proteins and measurement of [α-^{32}P]GDP release in exchange for GppNHp or GDP in reconstituted preparations*

Purified G_i1, G_i2 or G_o (50 pmol) derived from porcine brains [6] was incubated with [α-^{32}P]GTP (62.5 pmol) in 100 μl of buffer A containing 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.2% Chaps at 37°C for 60 min. The reaction mixture was applied on a Sephadex G-50 column (8 × 140 mm), which had been equilibrated in buffer A containing 1 mM DTT and 0.2% Chaps, in order to remove free [α-^{32}P]GTP/GDP. The materials obtained at void volume were concentrated to 100 μl (0.5 pmol/μl) by use of Centricon 30 (Amicon). Approximately 20% of each G-protein was labeled with [α-^{32}P]GDP/GTP. The radioactive guanine nucleotide released from the labeled G-protein by incubation with 100 μM GTPγS at 25°C for 10 min was analyzed with HPLC (Partisil 10 SAX, 25 cm × 4.6 mm, Whatman) system using a linear gradient (60 min) between water (Nano pure, Barnstead Co., Boston, MA) and 1.7 M ammonium formate (pH 3.7, adjusted with orthophosphoric acid), at a flow rate of 1 ml/min. More than 95% of radioactivity in any of the cases of G_i1, G_i2 or G_o was obtained at the retention time of GDP (22 min), while less than 1% at GTP (41 min).

In experiments for measurement of time-dependent release of [α-^{32}P]GDP, labeled G-protein (3 pmol) was incubated with 500 μg protein of cerebellar membranes in 60 μl of buffer A containing 1 mM DTT and 0.02% Chaps at 4°C for 90 min. The reconstituted preparation was incubated in the presence or absence of 10 μM U-50, 488H in buffer A containing 100 mM NaCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT (buffer B) at 25°C. After 2 min of incubation, 1 μM GppNHp was then added to the reaction mixture and aliquots (100 μl) of mixture were successively removed and rapidly filtrated on GF/B filter (Whatman) for measurement of [α-^{32}P]GDP release. The [α-^{32}P]GDP release in exchange for GppNHp (or unlabeled GDP) was calculated from the difference between radioactivities retained on the GF/B filter at 0 and at each time. The number of counts retained on the filter at 0 min incubation represents the amounts of the labeled G-protein incorporated into membranes. Approximately 60% of labeled G-protein (G_i1, G_i2 or G_o) was incorporated into membranes in the presence of 0.02% Chaps. The incorporation was not changed between 0.01% and 0.1% Chaps. In order to normalize the [α-^{32}P]GDP release from different experiments, results were evaluated as the fractional release, as follows: fractional release (%) = 100 × [α-^{32}P]GDP release/[α-^{32}P]GDP-labeled G-protein incorporated into membranes.

In experiments examining concentration-dependency of inhibitory effect of U-50, 488H on [α-^{32}P]GDP release, 3 min of incubation time and 1 μM GppNHp were adopted. In experiments testing concentration-dependent release by GppNHp in the presence or absence of 10 μM U-50, 488H, 3 min of incubation was adopted. In both experiments, 90 μl of reconstituted membranes (containing 0.3 pmol of G-protein and 50 μg protein of membranes) in buffer B in the presence or absence of U-50, 488H was incubated at 25°C for 2 min, and then added by 10 μl of GppNHp or GDP. The incubation was terminated by adding 600 μl of ice-cold buffer A and following by rapid filtration with GF/B.

3. RESULTS

In preparations of cerebellar membranes reconstituted with labeled G_i1, there was a [α-^{32}P]GDP release in exchange for 1 μM GppNHp, an unhydrolyzable analog of GTP, and the release increased with the incubation time, while there was no significant release in the absence of the guanine nucleotide. When 10 μM U-50, 488H, a specific agonist at κ-subtype of opioid receptor, was added 2 min prior to GppNHp addition, the release was inhibited (Fig. 1).

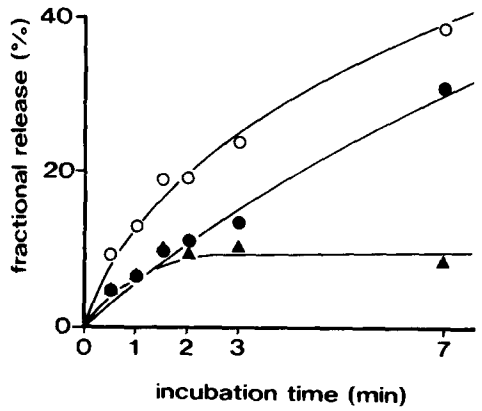

Fig. 1. Representative time-course profile of [α-^{32}P]GDP release in exchange for GppNHp in the presence or absence of the κ-agonist in membranes reconstituted with labeled G_i1. Results represent the fractional [α-^{32}P]GDP release in exchange for 1 μM GppNHp at each incubation time in the absence (○,*A*) or presence (●,*B*) of U-50, 488H at 10 μM. The fractional release represents the ratio in radioactivity of [α-^{32}P]GDP released at each indicated time to total one of labeled G_i1 incorporated into membranes (the amounts retained on the filter at 0 min incubation). (▲) the κ-agonist-mediated inhibition obtained from *A* minus *B*.

The inhibition reached a plateau at 2 min after the addition of GppNHp and lasted for at least 7 min.

In following experiments, the [α-^{32}P]GDP release at 3 min after the addition of unlabeled guanine nucleotide (GppNHp or GDP) was used for analysis of κ-agonist-induced inhibition. As shown in Fig. 2, the κ-agonist inhibited the [α-^{32}P]GDP release in exchange for 1 μM GppNHp in preparations reconstituted with labeled G_i1, in a concentration-dependent manner at a range from 1 nM to 10 μM. Similar inhibition was also observed with preparations reconstituted with G_i2, but not with G_o. Table I shows effects of the κ-agonist on

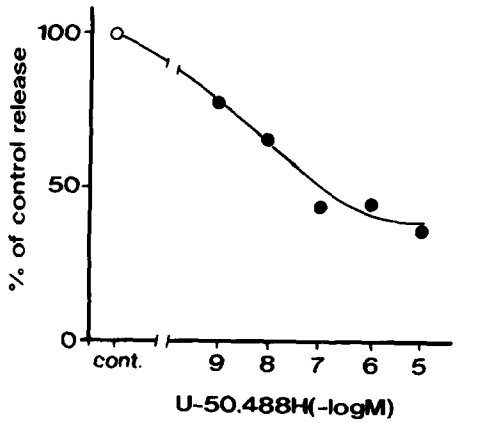

Fig. 2. Inhibition of [α-^{32}P]GDP release in exchange for GppNHp by various concentrations of U-50, 488H in membranes reconstituted with G_i1. Results represent the U-50, 488H-induced inhibition of [α-^{32}P]GDP release, measured at 3 m in after the addition of 1 μM GppNHp, as percentage of control (without κ-agonist) fractional release. The control fractional release in this experiment was 30%/3 min.

Table I

The κ-agonist-induced inhibition of [α-^{32}P]GDP release in exchange for GppNHp added in membranes reconstituted with various kinds of labeled G-proteins

	n	Control fractional release (%/3 min)[a]	Maximal % inhibition of control release[b]	IC_{50} (nM)[c]
G_i1	6	27.6 ± 4.0	44.6 ± 14.4[d]	1.4 ± 0.4
G_i2	3	17.6 ± 1.2	36.4 ± 5.9[d]	2.9 ± 0.9
G_o	5	24.9 ± 1.1	0	>1000

[a] The fractional release at 3 min after addition of 1 μM GppNHp. The release in G_i2-preparations was significantly lower than that in G_i1 or G_o ($P<0.05$), while there was no significant change between G_i1 or G_o (Student's t-test)

[b] The % inhibition of control fractional release by 10 μM U-50, 488H

[c] The concentration of U-50, 488H required for half-maximal inhibition of control fractional release at 3 min after the addition of 1 μM GppNHp. There was no significant change between G_i1- and G_i2-preparations

[d] The κ-agonist-mediated inhibition of [α-^{32}P]GDP release was significant ($P<0.05$, paired t-test)

[α-^{32}P]GDP release from these labeled G-proteins. The control fractional release in exchange for added GppNHp from G_i2 was significantly smaller than that from G_i1 or G_o. These findings suggest that the intrinsic GDP-GTP exchange activity in G_i2 is lower than those in the other two G-proteins. The IC_{50} of U-50, 488H in G_i1 was 2-fold lower than that in G_i2, but the difference was not statistically significant.

The [α-^{32}P]GDP release from G_i1-preparations increased with concentrations of GppNHp in control (Fig. 3A, open circle). The K_m and maximal release were calculated to be 0.20 ± 0.08 μM and 35.4 ± 4.8%/3 min, respectively, from double reciprocal plot analysis (Table II). When U-50, 488H at 10 μM was added, the release was significantly inhibited in various concentrations of GppNHp (Fig. 3A, closed circle). From the kinetic analysis, it was revealed that the K_m was increased approximately 3-fold, while there was no significant change in maximal release. Similar [α-^{32}P]GDP release was also observed with the addition of various concentrations of unlabeled GDP, while there was no inhibition by U-50, 488H at 10 μM (Fig. 3B).

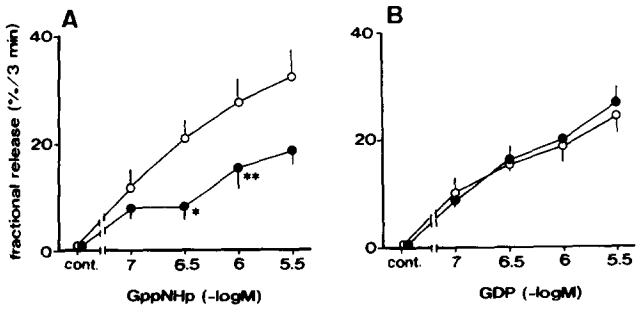

Fig. 3. Effects of the κ-agonist on the [α-^{32}P]GDP release in exchange for GppNHp or GDP in membranes reconstituted with G_i1. Results represent the effects of U-50, 488H at 10 μM on fractional release for 3 min in exchange for various concentrations of guanine nucleotide, GppNHp (A) or GDP (B). Data represent the mean ± SE from 6 separate experiments. *$P<0.05$, **$P<0.01$ (paired t-test), compared to control (without U-50, 488H). Other details are given in the legend of Fig. 1.

As shown in Table II, in preparations reconstituted with G_i2, [α-^{32}P]GDP was also released in exchange for added GppNHp or unlabeled GDP in control, and the κ-agonist selectively increased the K_m value of GppNHp, but not the maximal fractional release. However, U-50, 488H showed no significant change in the release in exchange for GppNHp or unlabeled GDP.

4. DISCUSSION

Reconstitution experiments are a well accepted means to clarify the molecular basis of coupling between receptors and G-proteins. Previously we have reported that the functional reconstitution experiments using purified G-protein and receptors in membranes pretreated with IAP or *N*-ethylmaleimide (NEM), both known to inactivate G-proteins, such as G_i1, G_i2 and G_o by ADP-ribosylation or alkylation of their α-subunits [7,8]. In these previous studies, the inactivation of naturally occurring G-proteins with IAP or NEM is definitely required, in order to eliminate the contribution of unspecified G-proteins naturally occurring in membranes. However, there is some risk of receptors coupling to G-proteins devoid of physiological relevancy when only one kind of G-protein is reconstituted in the assay system after inactivation of G-proteins by IAP or NEM, as the primary structures of IAP-substrate G-proteins, G_i1, G_i2 and G_o are very similar to each other [9]. The present study is sharply in contrast with such reports in that membrane preparations were used without any pretreatment. Such membrane preparations without inactivation of naturally occurring G-proteins therein (G_i1, G_i2 and G_o) seem to have an advantage in detecting selective coupling between membrane-bound receptors and labeled G-proteins with [α-^{32}P]GDP, since the [α-^{32}P]GDP release in exchange for unlabeled guanine nucleotide, is definitely from the reconstituted G-protein.

Labeled G-proteins are easily incorporated into membranes simply by incubation at 4°C for 90 min in the presence of Chaps, a detergent, at concentrations more than 0.01%. The incorporation of G-proteins in mem-

Table II

Kinetic analysis of the κ-agonist-mediated inhibition of [α-^{32}P]GDP release in exchange for GppNHp or GDP in membranes reconstituted with various kinds of labeled G-proteins

	K_m (μM)		Maximal fractional release (%/3min)	
	GppNHp	GDP	GppNHp	GDP
G_i1				
control	0.20 ± 0.08	0.24 ± 0.04	35.4 ± 4.8	27.0 ± 2.1
U-50, 488H[a]	0.58 ± 0.14[b]	0.27 ± 0.09	29.9 ± 5.2	28.1 ± 2.0
G_i2				
control	0.45 ± 0.15	0.14 ± 0.03	31.1 ± 5.5	24.7 ± 1.7
U-50, 488H[a]	1.26 ± 0.24[b]	0.17 ± 0.05	27.9 ± 2.2	24.7 ± 1.1
G_o				
control	0.22 ± 0.03	0.34 ± 0.15	32.8 ± 1.0	29.7 ± 1.6
U-50, 488H[a]	0.26 ± 0.07	0.34 ± 0.12	30.7 ± 1.2	33.7 ± 3.5

Results represent the K_m and maximal fractional release (%/3 min) calculated from double reciprocal plot analysis from at least 6 separate experiments.

[a] The concentration was 10 μM

[b] The difference is statistically significant ($P<0.05$, paired t-test), compared to each control. Other details are given in the legend of Fig. 1

branes was approximately 60% when Chaps was used in concentrations between 0.01% and 0.1%. On the other hand, a selective κ-agonist, U-50, 488H, produced no significant change in [α-^{32}P]GDP release in preparations with G_i1 or G_i2, when the concentration of Chaps was more than 0.04% (unpublished data). It is likely that various kinds of membrane proteins are partially solubilized in the presence of higher concentrations of detergent and interfere the functional coupling between receptors and G-proteins. Accordingly, we chose 0.02% of Chaps for incorporation of G-proteins into membranes.

In the present study, we provided direct evidence of receptor-coupled inhibition of intrinsic (GDP–GTP exchange) activity of G-protein ('turn on' reaction). However, we have not yet obtained data about the κ-receptor-mediated changes in GTPase activity per se ('turn off' reaction). Taking into account that most of G-protein-coupled receptors mediate the stimulation of the intrinsic activities of G-protein [1–3], the present finding may be the first evidence for receptor-mediated inhibition of G-protein activity in signal transduction mechanisms through plasma membranes. Experimental support for this conclusion is as follows. First, the agonist-induced inhibition of [α-^{32}P]GDP release in exchange for GppNHp was observed when membranes were reconstituted with labeled G_i1 or G_i2, but not with G_o. Second, the mode of inhibition is related to the decrease in affinity for GppNHp (as a GTP analog) in GDP–GTP exchange reaction, but not to the increase in affinity for GDP.

The G_i1 or G_i2 inhibited via κ-opioid receptors will lead to inhibition of certain effector system(s) in the cell membrane. Here, adenylate cyclase is an unlikely candidate, since we obtained no significant changes to it by the κ-agonist in cerebellar membranes of the guinea pig (unpublished data). The lack of effect on adenylate cyclase by the κ-agonist was supported by other investigators [10]. However, the possibility remains that κ-agonist blocks the inhibition of adenylate cyclase mediated through activation of G_i1 or G_i2 by other receptors. Phospholipase C seems to be a more likely candidate. There are some reports that IAP-sensitive inhibition of phospholipase C via stimulation of dopamine D_2- or adenosine A_1-receptors was observed [11]. We also have data that the κ-opioid agonist inhibits GTP-stimulated phospholipase C activity [12], in an IAP-sensitive manner [13]. Thus, it is plausible that the inhibitory regulation of G_i (G_i1 and/or G_i2) activity by receptor stimulations leads to inhibition of phospholipase C activity, respectively.

The present communication may be the first to report the direct demonstration of receptor-mediated inhibition of G-protein activity in cell membranes; intrinsic G_i1 or G_i2 activity was inactivated via opioid receptors of the κ-subtype in the guinea pig cerebellum.

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