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Metabotropic responses to acetylcholine and serotonin of *Xenopus* oocytes injected with rat brain mRNA are transduced by different G-protein subtypes

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To assign the GTP-binding protein (G-protein) subtype involved in the signal transduction from exogenous receptors to phospholipase C in the *Xenopus* oocyte translation system, antisense DNA complementary to rat G-protein α-subunit mRNA was designed and injected together with rat brain poly(A)$^+$ RNA. Current response of mRNA-injected oocytes to acetylcholine (ACh) was suppressed dose-dependently by a co-injection of G_{i1}α-antisense DNA, but response of the same oocytes to serotonin (5-HT) was not inhibited. In the oocytes co-injected with G_oα-antisense DNA, the 5-HT response was more effectively suppressed than the ACh response. These results suggest that G_oα but not G_{i1}α intermediates brain 5-HT_{1C} receptor function, and in contrast, muscarinic receptors derived from rat brain utilize G_{i1}α rather than G_oα to activate phospholipase C.

GTP-binding protein; Muscarinic receptor; 5-HT_{1C} receptor; Phospholipase C; Antisense DNA; *Xenopus* oocyte

1. INTRODUCTION

In voltage-clamped *Xenopus* oocytes several days after injection of rat brain poly(A)$^+$ RNA, acetylcholine (ACh) and serotonin (5-HT) evoke similar current responses mediated by a common sequential activation of pertussis toxin-sensitive GTP-binding protein (G-protein), phospholipase C, intracellular Ca^{2+} release oscillation, and calmodulin-dependent Cl^- channels [1–3]. It is proposed that both G_i and G_o proteins, newly-synthesized by rat brain mRAN, participate in the first step of the process, as well as *Xenopus* native G_i/G_o-like proteins [4]. Moriarty et al. [5] have shown that G_o participates in the signal transduction from native muscarinic receptors to phospholipase C; however, the G-protein subtype involved in the exogenous receptor-mediated response still remains unclear. To elucidate this point using the hybrid depletion technique, we designed oligodeoxynucleotides complementary to the α-subunit mRNAs for rat G_{i1} and G_o, and evaluated the effects of antisense DNA on the mRNA-induced expression of G-proteins and current responses by metabotropic agonists.

2. MATERIALS AND METHODS

2.1. *Oligomers*

The Gα-antisense DNAs used for hybrid arrest were 35 nucleotides long and complementary to the downstream region just beginning from the translation initiator codon ATG of rat G_{i1} [6] and G_o [7] cDNAs. The subtype G_{i1} was chosed since this type is predominantly expressed in the rat brain [6]. Sense DNAs were used as the control. The nucleotide sequence of synthetic oligomers is as follows:

G_{i1} sense (Gi-S),
5′-ATGGGCTGCACACTGAGCGCTGAGGACAAGGCGGC3′

G_{i1} antisense (Gi-AS),
5′-GCCGCCTTGTCCTCAGCGCTCAGTGTGCAGCCCAT-3′

G_o sense (Go-S),
5′-ATGGGATGTACTCTGAGCGCAGAGGAGAGAGCCGC-3′

G_o antisense (Go-AS),
5′-GCGGCTCTCTCCTCTGCGCTCAGAGTACATCCCAT-3′

In the corresponding 35 bases of rat G_{i1}, G_{i2}, G_{i3}, G_o, G_s [6], *Xenopus* G_{i1} [8] and *Xenopus* G_o [9], numbers of mismatch vs. Gi-S are 0, 2, 3, 8, 17, 6 and 5 bases, respectively; and those vs. Go-S are 8, 9, 6, 0, 23, 6 and 6 bases, respectively.

2.2. *Translation in oocytes*

Poly(A)$^+$ RNA was purified from whole brains of 4-week-old male Sprague–Dawley rats as described [1,4]. The stock RNA solution was mixed with various concentrations of synthetic DNA, and the mixture containing 16.7 mM NaCl, which is equivalent to the intracellular Na^+ concentration in *Xenopus* oocytes [10], was heated to 65°C for 10 min. After cooling to room temperature, the RNA–DNA solution was injected at a volume of 75 nl into *Xenopus* oocytes defolliculated with collagenase treatment. Oocytes were incubated at 23°C for 2–4 days.

2.3. *Quantitation of G-proteins*

Fifty oocytes were homogenized at 4°C with 0.5 ml of 250 mM phosphate buffer, pH 7.5, containing 10 mM dithiothreitol (DTT) and 0.1 mg/ml bovine serum albumin, and the homogenate was centrifuged at 10,000 × *g* for 3 min. The precipitate was washed twice, resuspended in the homogenization buffer, and incubated at 4°C for 60 min with 1% sodium cholate. The solubilized membranes were incubated at 30°C for 30 min with 10 μg/ml pre-activated *pertussis* toxin, 2.4 μM

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adenylate-[^{32}P]NAD, 0.4 mM ATP, 0.4 mM GTP, 10 mM thymidine, 10 mM DTT and 1 mM EDTA in 100 mM TRIS–HCl, pH 8.0. Aliquots from 30 oocytes were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the gels were stained with Coomassie blue, dried and exposed to X-ray film at −80°C.

2.4. Electrophysiological measurements

Detection of transmembrane current was done by the two-electrode voltage-clamp method as described [1,4]. Intracellular application of 5'-(3-*O*-thio)trisphosphate (GTPγS; Boehringer, Mannheim) and inositol-1,4,5-trisphosphate (IP_3; Sigma) was made by a pressure injection [4].

3. RESULTS AND DISCUSSION

In autoradiograms of SDS-PAGE gels loaded with oocyte membranes, a major 40 kDa and minor 32 kDa band was identified (Fig. 1a). Both bands were also detected previously [4] as well as in [^{32}P]ADP-ribosylated rat brain membranes (Fig. 1a); however, the origin of the minor signal is not known. Regarding the 40 kDa signal of pertussis toxin substrate as that derived from $G_i\alpha$ and $G_o\alpha$, both proteins in the oocytes injected with rat brain mRNA were increased to 159±41% (S.E.M. n=8) of those of non-injected, control oocytes (Fig. 1b). Co-injection of Gi-AS, Go-AS, or both with rat brain mRNA dose-dependently decreased the 40 kDa band intensity to the minimum 26% of non-injected oocytes at a dose of 12 ng Gi-AS+12ng Go-AS, indicating that not only translation of rat brain $G_i\alpha$ and $G_o\alpha$ mRNAs but also expression of oocyte-native G-proteins were arrested by antisense DNAs. Although the signals from $G_i\alpha$ and $G_o\alpha$ were not separated on the gel, effects of Gi-AS and Go-AS were additive.

Depletion of functional G-protein in the phosphoinositide pathway was evaluated by the magnitude of GTPγS-evoked current responses (Fig. 2) since an injection of GTPγS (3–50 pmol/oocyte) triggers a slow-onset Cl^- current fluctuation by directly activating phospholipase C [2,4]. In the oocytes injected with rat brain mRNA alone, the maximum deflection of fluctuating current by 35 pmol GTPγS was incresed to 147±25% (n=21) of that of non-injected oocytes (Fig. 2b), which corresponded to the increase in the [^{32}P] incorporation into 40 kDa pertussis toxin substrate (cf. Fig. 1b). Co-injection of Gi-AS, Go-AS, or both, dose-dependently decreased the amplitude of GTPγS response without changing fluctuating frequency (Fig. 2a). The inhibitory effects of Gi-AS and Go-AS on the GTPγS-evoked current response were additive, and the dose-dependency was also parallel to those on the intensity of 40 kDa bands, suggesting that quantity of pertussis toxin-sensitive Gα is parallel to the amount of cellular signal transduced from G-proteins to phosphoinositide breakdown. These results also suggest that both $G_i\alpha$ and $G_o\alpha$ of rat and *Xenopus* origins are all involved in the oocyte metabotropic response.

Injection of antisense DNA itself did not affect the signal transduction from IP_3 receptor to native Cl^- channels. In the oocytes injected with 12 ng Gi-AS+12 ng Go-AS+rat brain mRNA, intracellular injection of IP_3 (35 pmol) gave average currents of 305±51 nA (n=7), which is equivalent to those of rat brain mRNA-injected oocytes (287±55 nA, n=7) and non-injected oocytes (340±66 nA, n=7).

The effects of antisense DNAs on the metabotropic receptor-mediated current responses of brain mRNA-injected oocytes were first examined at a maximum dose

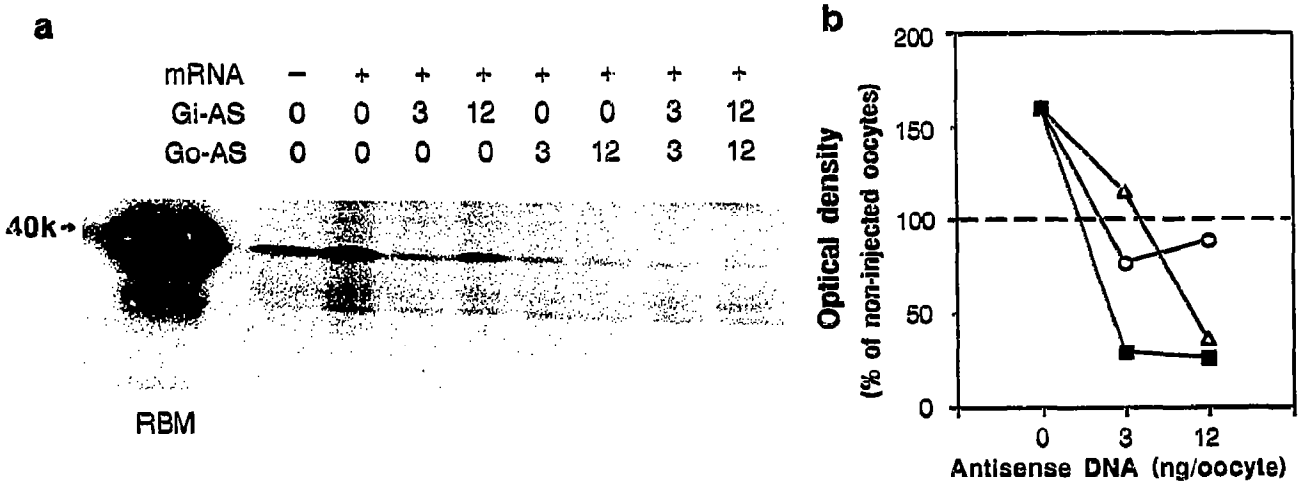

Fig. 1. Hybrid depletion of Gα by antisense DNA injected together with rat brain mRNA into *Xenopus* oocytes. (a) Autoradiogram of an SDS-PAGE gel loaded with oocyte membranes [^{32}P]ADP-ribosylated in the presence of pertussis toxin. Rat brain mRNA (50 ng) was mixed with the indicated amount (in ng) of antisense DNA and heated to 65°C for 10 min. Injected oocytes were cultured for 3 days and the membranes were incubated with [^{32}P]NAD and pertussis toxin. A constant amount of membrane protein was subjected to SDS-PAGE and the dried gel was exposed to Fuji X-ray film for 48 h. The lane RBM was loaded with rat brain membranes treated in the same way. (b) Densitometric analysis of ^{32}P-labelled Gα. The data are from oocytes injected with Gi-AS (○), Go-AS (△) or both (■) together with rat brain mRNA (50 ng). The optical density of the 40 kDa band on the X-ray film was scanned by a flatbed densitometer and normalized to the value of non-injected oocytes as 100%. Each point represents the mean from 8 separate experiments.

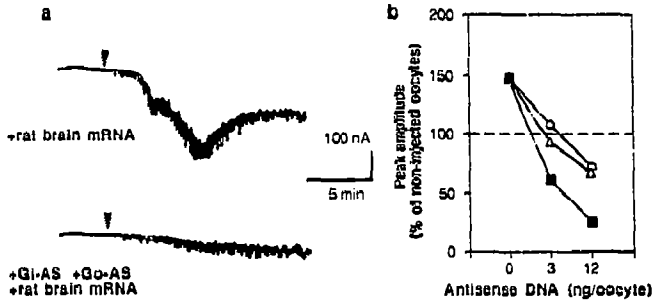

Fig. 2. Inhibitory effect of Gα-depletion on GTPγS-evoked response in *Xenopus* oocytes. GTPγS (35 pmol) was applied by a brief pressure-pulse into oocytes held at −60 mV. (a) A difference in the GTPγS-evoked current between an oocyte injected with 50 ng rat brain mRNA alone and an oocyte injected with 12 ng Gi-AS, 12 ng Go-AS and rat brain mRNA. (b) Dose-dependent effect of Gi-AS (○), Go-AS (△), or both (■), injected together with rat brain mRNA (50 ng constant) on the GTPγS-evoked current. Data are means from 7 experiments and normalized to the value of non-injected oocytes.

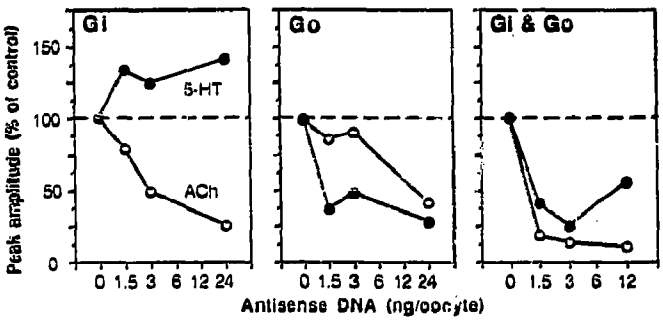

Fig. 4. Dose-dependent effects of Gi-AS and Go-AS on ACh and 5-HT responses expressed in *Xenopus* oocytes by rat brain mRNA. Indicated amount of antisense DNAs were mixed with 50 ng rat brain mRNA and injected. Current responses to ACh (1 mM, ○) and 5-HT (1 μM, ●) were recorded at −60 mV and the magnitude in 5–11 oocytes were averaged and normalized to that of oocytes injected with rat brain mRNA alone.

of 30 ng DNA/oocyte with the control experiment using sense DNAs (Fig. 3). Average peak amplitudes of ACh (1 mM) and 5-HT (1 μM) responses in the sense DNA-injected oocytes were not significantly different from those of oocytes injected with rat brain mRNA alone (usually 200–800 nA). In the oocytes injected with Gi-AS, amplitude of ACh response was 28% of that of Gi-S-injected oocytes. In contrast, the amplitude of 5-HT response was not different between the groups of Gi-S and Gi-AS. In oocytes injected with Go-AS, both ACh and 5-HT responses were decreased to 53% and 43%, respectively, of Go-S-injected oocytes. These effects were not caused by a decrease in the receptor affinity because no shift in the dose–response curves of

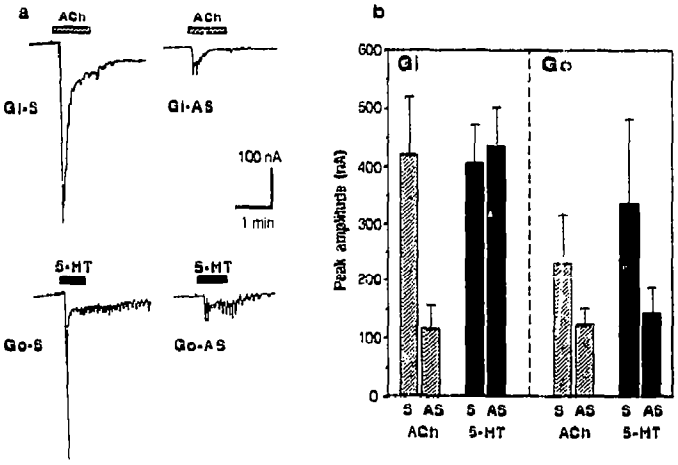

Fig. 3. Effects of Gα sense (Gi-S and Go-S) and antisense (Gi-AS and Go-AS) DNAs on oocyte responses evoked by metabotropic receptor stimulation. Sense or antisense DNA (30 ng) was mixed with rat brain mRNA (50 ng) and injected. Current responses to ACh (1 mM) and 5-HT (1 μM) were recorded at −60 mV. Several typical traces are shown in panel a. In b, bars are means ± S.E.M. of 6 experiments.

receptor agonists were found in these oocytes (data not shown).

In a dose–response analysis (Fig. 4), 5-HT response was not affected by Gi-AS in a range of 1,5–24 ng; however, the response was markedly decreased by as little as 1.5 ng Go-AS. Since the inhibition curve of 5-HT response by Gi-AS+Go-AS was similar to that of Go-AS, it is concluded that signals from 5-HT_{1C} receptors mediating 5-HT-evoked metabotropic response [11] are mediated by Go-AS-sensitive G-proteins. On the other hand, reduction in ACh response was caused by Gi-AS more apparently than by Go-AS at doses of 1.5 and 3 ng, and their effects were dose-dependent and additive (Fig. 4). Since non-injected oocytes often elicited small fluctuations in response to ACh perfusion, the remaining muscarinic responsiveness of *Xenopus* origin may be somewhat contaminated in the ACh response of brain mRNA-injected oocytes. Although it was shown that the native muscarinic receptors couple the phosphoinositide pathway via $G_o\alpha$ [5], our results suggest that rat brain muscarinic receptors of the pirenzepine-sensitive M_1 type [1] prefer $G_{i1}\alpha$ rather than $G_o\alpha$ to activate phospholipase C.

The present study was based on the structural differences found in the coding-start region of various Gα mRNAs. Since the differences are not so striking we can not assign the amount of every G-protein variants or their contribution in transducing metabotropic signals. However, it is clear that comparable ACh- and 5-HT-evoked responses of *Xenopus* oocytes injected with rat brain mRNA are transduced by different G-protein subtypes. Recently, applying similar antisense approach to rat pituitary GH_3 cells, Kleuss et al. [12] have shown that voltage-dependent L-type Ca^{2+} channels are inhibited by muscarinic and somatostatin receptors via $G_{o1}\alpha$ and $G_{o2}\alpha$, respectively. The results are consistent with our observations that a specific Gα subtype is in-

volved in the signal linkage from a G-protein-coupled receptor to a post-G-protein effector.

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