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# Inositol phospholipid metabolism in *Xenopus* oocytes mediated by endogenous $G_o$ and $G_i$ proteins

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**Abstract** To characterize G-proteins which mediate the signal transduction from ligand stimulated receptor to phospholipase C (PLC), we injected antisense DNAs complementary to *Xenopus* $G_o\alpha$ or $G_{i\text{-}1}\alpha$ to suppress these endogenous G-proteins, together with the mRNAs encoding metabotropic glutamate receptor 1 (mGluR1), 5 (mGluR5) or with M1 type muscarinic receptor into oocytes. Receptor-stimulated chloride current responses were reduced by the suppression of *Xenopus* $G_o\alpha$ regardless of the types of receptors. However, injection of $G_{i\text{-}1}$ antisense DNA resulted in the reduction of M1-stimulated responses but not mGluR-stimulated responses. These results suggested that all these receptors could use $G_o\alpha$, and M1 receptors, but not mGluRs, could also use $G_{i\text{-}1}$ proteins, to activate PLC in *Xenopus* oocytes.

*Key words:* G-protein; Antisense DNA; Phospholipase C; Metabotropic glutamate receptor; M1 muscarinic acetylcholine receptor; *Xenopus laevis* oocyte

## 1. Introduction

The signal transduction pathway from cell surface receptors to phospholipase C (PLC), one of the major cellular effectors, involves the heterotrimeric GTP binding proteins (G-proteins) [1], and this pathway could be categorized by sensitivity to pertussis toxin (PTX) [2]. The PTX-insensitive pathway involves $G_q$ class G-protein [3], and its $\alpha$ subunit is known to interact with PLC-$\beta$1 isoform specifically [4,5]. As for the PTX-sensitive G-proteins, recent reports suggest the possibility that the $\beta\gamma$ subunits of G-protein may also play an important role, because they could activate PLC-$\beta$2 and $\beta$3 isoforms specifically [6,7].

*Xenopus* oocytes have its own PLC signal transduction pathway which is, at least partly, sensitive to PTX [8]. Using this expression system, many G-protein-coupled receptors have been cloned, but it is not clear which types of G-proteins could interact with these receptors to activate PLC, although several subtypes of G-proteins were cloned in oocytes [9,10]. To clarify this point, we designed the antisense DNAs for *Xenopus* $G_o\alpha$ and $G_{i\text{-}1}\alpha$, and injected them into oocyte together with mRNAs for metabotropic glutamate receptor 1 (mGluR1) [11,12], 5 (mGluR5) [13,14], or M1-type muscarinic acetylcholine (ACh) receptor [15], and examined the peak amplitude of the chloride current which was evoked by L-glutamate (Glu) or ACh stimulation.

## 2. Materials and methods

### 2.1. Preparation of DNAs and mRNAs

The sense and antisense DNAs were designed based on the reported sequences of *Xenopus* oocyte $G_o\alpha$ [9] or $G_{i\text{-}1}\alpha$ [10] mRNA. They are 35 bases long, and correspond to the initial parts of the coding regions starting at the initiation codon:

Sense $G_o$:
5′-ATGGGCTGCACACTGAGCGCGGAGGAAAGAGCAGC-3′
Antisense $G_o$:
5′-GCTGCTCTTTCCTCCGCGCTCAGTGTGCAGCCCAT-3′
Sense $G_{i\text{-}1}$:
5′-ATGGGATGTACTCTGAGCGCCGAAGACAAGGCAGC-3′
Antisense $G_{i\text{-}1}$:
5′-GCTGCCTTGTCTTCGGCGCTCAGAGTACATCCCAT-3′

The number of mismatch between sense $G_o$ and sense $G_{i\text{-}1}$ is 8. In the corresponding 35 bases of *Xenopus* $G_q$ (Genbank, L05540), the number of mismatch vs. sense $G_o$ or sense $G_{i\text{-}1}$ is 27 or 30, respectively. These oligodeoxynucleotides were synthesized and stored at −20°C until use.

Specific mRNAs were obtained by an in vitro transcription from the cDNA clones of porcine M1 ACh receptor (the generous gift from Dr. Nukada [15]), rat mGluR1$\alpha$ [12], or human mGluR5a, 5b [13,14] using a mRNA capping kit (Stratagene), and stored at −80°C until use.

### 2.2. Microinjection and electrophysiological measurements

The oocytes of *Xenopus laevis* were prepared and examined as described [16,17]. The sense or antisense DNAs were mixed with the receptor mRNAs (mGluR1$\alpha$, mGluR5, or M1 receptor), and were injected into oocytes. The oocytes were then cultured at 20°C for 3 days and analyzed.

Electrophysiological experiments were performed under the voltage clamp conditions (−60 mV), as described [16,17]. For mGluR5, we examined both of the variants mGluR5a and mGluR5b, and obtained essentially the same results. Therefore we described here only the results of mGluR5a.

### 2.3. Binding assay

Thirty to fifty oocytes injected with the mRNA of M1 receptor (10 ng/oocyte) were homogenized at 4°C in 1 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulphonyl fluoridate (PMSF), 1 $\mu$g/ml pepstatin and 0.32 M sucrose. The homogenate was centrifuged at 28,000 × *g* for 15 min. The pellet was suspended in the same buffer, and used for binding assay. [$^3$H]quinuclidinyl benzilate ([$^3$H]QNB, 52.3 Ci/mmol, NEN) binding activity was determined at 1 nM [$^3$H]QNB using a glassfiber filter (Whatman GF/C) assay as previously described [15,18]. Non-specific binding was determined in the presence of 10 $\mu$M atropin.

### 2.4. Quantitation of G-proteins

Sixty oocytes were homogenized at 4°C in 1 ml of 250 mM potassium phosphate buffer (pH 7.5) containing 10 mM dithiothreitol (DTT), 0.1 mM PMSF, 0.1 mg/ml bovine serum albumin. The homogenate was centrifuged at 10,000 × *g* for 5 min. The precipitate was washed twice, and resuspended in the same buffer and used for reaction. Pertussis toxin (PTX, Seikagaku Kogyo) was preactivated as described [19]. The isolated membrane fraction was incubated at 30°C for 2 h with 200 $\mu$l of 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 10 mM thymidine, 2.5 mM $MgCl_2$, 0.4 mM ATP, 0.4 mM GTP, 0.24 $\mu$M nicotinamide adenine dinucleotide (NAD), 10 $\mu$g/ml preactivated PTX, and 0.0625 $\mu$M [$^{32}$P]NAD (800 Ci/mmol, NEN). The reaction was

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stopped by cooling the tube on ice, then the tube was centrifuged at 10,000 × g for 5 min. Precipitate was washed three times by 10 mM HEPES buffer (pH 7.9) containing 130 mM NaCl, 0.01% sodium azide, and 0.1 mM PMSF, and dissolved in Laemmli's sample buffer at 90°C for 5 min. Aliquots corresponding to 15 oocytes were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gels. Gels were analyzed by BAS-1000 Bio-Imaging Analyzer (Fuji Film).

## 3. Results and discussion

Three days after injection of specific mRNAs, the oocytes were examined electrophysiologically for chloride current responses to 100 μM Glu or 10 μM ACh (Fig. 1; controls). These responses involve the signal transduction from the activated receptor to G-protein and PLC, the production of inositol 1,4,5-trisphosphate ($IP_3$), intercellular $Ca^{2+}$ mobilization, and the opening of chloride channels. It has been shown that, under some conditions, native oocytes have endogenous muscarinic receptors which produce ACh-stimulated chloride current responses [20]. However, under our conditions, oocytes without mRNA-injection rarely produced such responses to ACh, and we could assume that ACh-evoked current responses were due to exogenously induced M1 receptors.

When antisense $G_o$ was co-injected with mGluR1, mGluR5 or M1 receptor mRNAs, peak amplitudes of both Glu-stimulated or ACh-stimulated current responses were reduced in a dose-dependent manner (Figs. 1 and 2). When antisense $G_{i-1}$ was co-injected, however, mGluR-evoked responses were not affected significantly, whereas M1-evoked responses were suppressed in a dose-dependent manner (Figs. 1 and 2). These results suggest that the antisense oligonucleotides used could distinguish and affect the corresponding G-proteins. Sense $G_o$ or sense $G_{i-1}$ used as the controls caused only nominal effects on these responses. At the highest dose, however, M1 receptor responses appeared to be suppressed to a small extent probably by non-specific effects (Fig. 2). Thus it cannot be excluded that the strong suppression by the highest dose of antisense DNA (Fig. 2) may include some contributions of such non-specific inhibitory effects.

Antisense $G_{i-1}$ we used showed a sequence complementarity to M1 receptor to some extent. The consecutive 9 nucleotides sequence (1st to 9th) was exactly complement to M1 receptor cDNA (2,024th to 2,032nd). Therefore it seemed possible that the suppressing effects of antisense $G_{i-1}$ might be exerted through the inhibition of the expression of M1 receptors. To examine this possibility, we carried out the binding assay of muscarinic receptors expressed in the oocytes using [$^3$H]QNB. In this experiment, we injected a larger amount (10 ng) of M1 mRNA, considering the detection limit of [$^3$H]QNB binding assay, together with 5 ng of sense or antisense DNAs. Electrophysiological measurements of ACh-stimulated current responses confirmed that, even under these conditions, the current response amplitudes were reduced by antisense $G_o$ or antisense $G_{i-1}$, while sense $G_o$ or sense $G_{i-1}$ had no significant effects (Fig. 3, open columns). Then the specific [$^3$H]QNB bindings of these oocytes were determined. None of the oligodeoxynucleotides examined (either sense or antisense, $G_o$ or $G_{i-1}$) was found to cause significant effects on the specific bindings (Fig. 3, stippled columns). Thus the reduction of current responses by antisense oligodeoxynucleotides was not due to the inhibition of receptor expression.

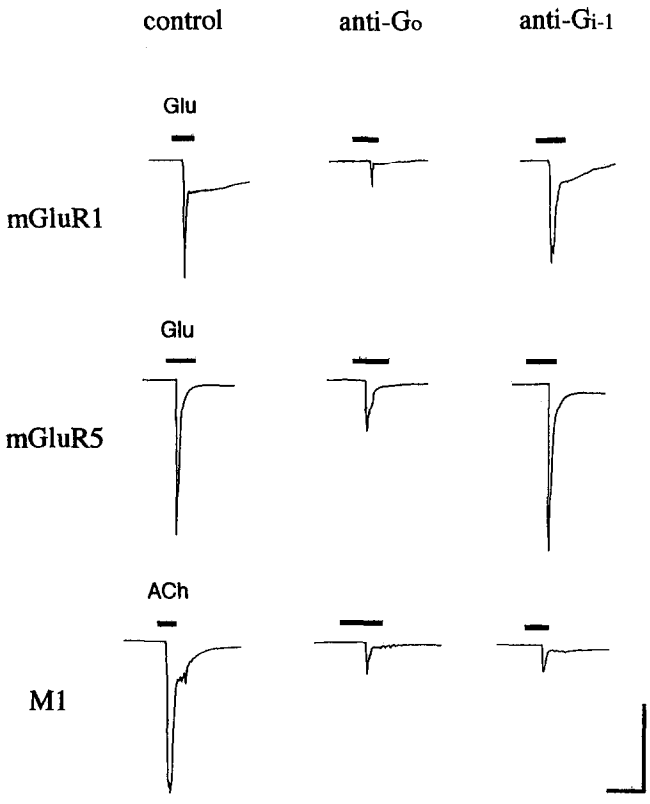


Fig. 1. Typical current responses of *Xenopus* oocytes injected mGluR1, mGluR5 or M1 mRNA together with antisense Gα DNA. Left, control oocytes in which only receptor mRNA were injected (100 pg of mGluR1 or M1 receptor, or 1 ng of mGluR5 mRNA); middle, 1 ng (in the case of mGluR5) or 5 ng (in the cases of mGluR1 and M1 receptor) of antisense $G_o\alpha$ DNA was injected together with receptor mRNA; right, the same amount of antisense $G_{i-1}\alpha$ DNA was injected. The bar above each trace indicates the application period of 100 μM Glu or 10 μM ACh. Calibration bars: 500 nA (mGluR1, M1 receptors) or 1000 nA (mGluR5), and 1 min.

Finally we tried to quantify PTX-sensitive G-protein α subunits using [$^{32}$P]NAD. As seen in Fig. 4, PTX-sensitive G-protein ($G_o/G_i$) α subunits in oocytes appeared at the position of about 40 kDa on SDS-PAGE gels. This band disappeared when the labeling reaction was done in the absence of PTX (data not shown). The radioactivity of this band was reduced to approx. 35% of the control when antisense $G_o$ was injected, to approx. 70% when antisense $G_{i-1}$ was injected, and to approx. 25% when both types of antisense DNA were injected (Fig. 4). These results confirmed that the injection of antisense $G_o$ and/or antisense $G_{i-1}$ DNA resulted in the reduction of PTX-sensitive G-protein α subunits in the oocytes. The observation that the reduction by antisense $G_o$ was more pronounced than that by antisense $G_{i-1}$ is consistent with the report that $G_o$ was the most abundant G-protein in this stage of oocytes at mRNA level [21].

These results altogether indicated that the injection of the antisense DNAs suppressed the responses by reducing the corresponding G-proteins but not receptors.

Among the different types of receptors examined, muscarinic receptors [22,23], serotonin receptors [22] and $\alpha_{1B}$-adrenergic receptor [24] were reported to activate PLC via $G_o$, and muscarinic receptor [22] and δ-opioid receptor [25] were via $G_i$ in

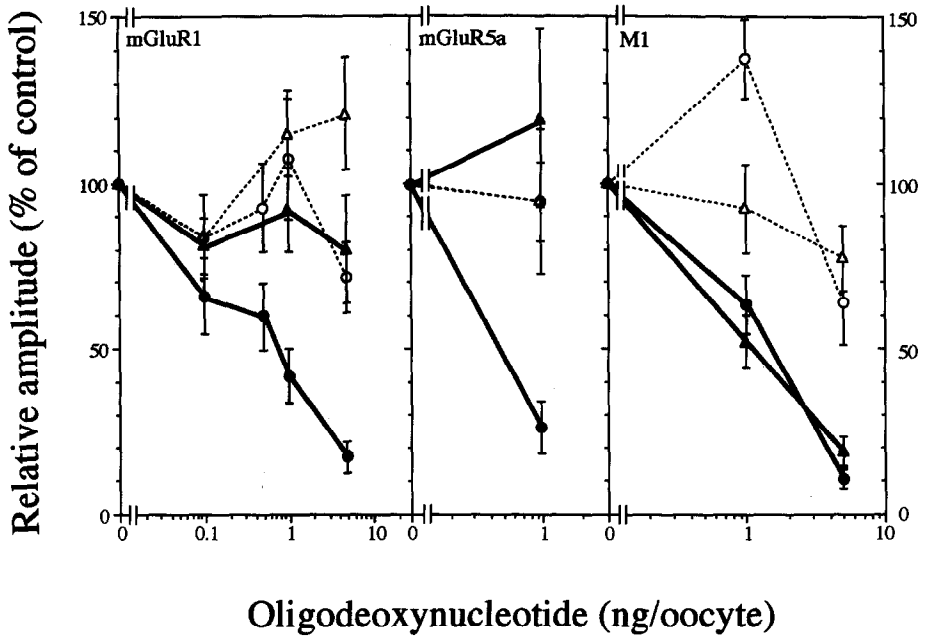


Fig. 2. Dose-dependent effects of antisense DNAs on Glu or ACh responses. A hundred pg (mGluR1 or M1) or 1 ng (mGluR5) of receptor mRNA and the indicated amount of DNA were mixed and injected into an oocyte. The maximum amplitudes of current responses to 100 $\mu$M Glu or 10 $\mu$M ACh were measured at −60 mV and expressed relative to the control, as the means ± S.E.M. ($n$ = 17–35). Open and closed circles indicate sense and antisense $G_o$, respectively. Open and closed triangles indicate sense and antisense $G_{i-1}$, respectively.

*Xenopus* oocytes. Moreover, the kyotorphin receptor was reported to couple to $G_i$ in rat brain [26], and the fMLP (folmyl Met-Leu-Phe) receptor was reported to couple to both $G_o$ and $G_i$ in HL-60 cell [27] to activate PLC. Our results suggest that mGluR1 and mGluR5 could activate PLC via $G_o$ in *Xenopus* oocyte, and M1 receptor could couple to both $G_o$ and $G_{i-1}$ to activate PLC. Thus $G_o$ and $G_i$ could both participate in the PTX-sensitive PLC signal transduction pathway. Using poly(A)$^+$RNA from rat brains and antisense DNA, Kaneko et al. [22] reported that serotonin receptors were coupled to $G_o$ but not $G_i$ proteins whereas muscarinic ACh receptors preferred $G_i$ rather than $G_o$ proteins in *Xenopus* oocytes. Since they used poly(A)$^+$RNA, however, precise subtypes of receptors and sources of G-proteins (rat or *Xenopus*) were not clear. In the present study, we could define them more precisely using specific cRNAs for mGluR1, mGluR5 or M1 receptors. We also

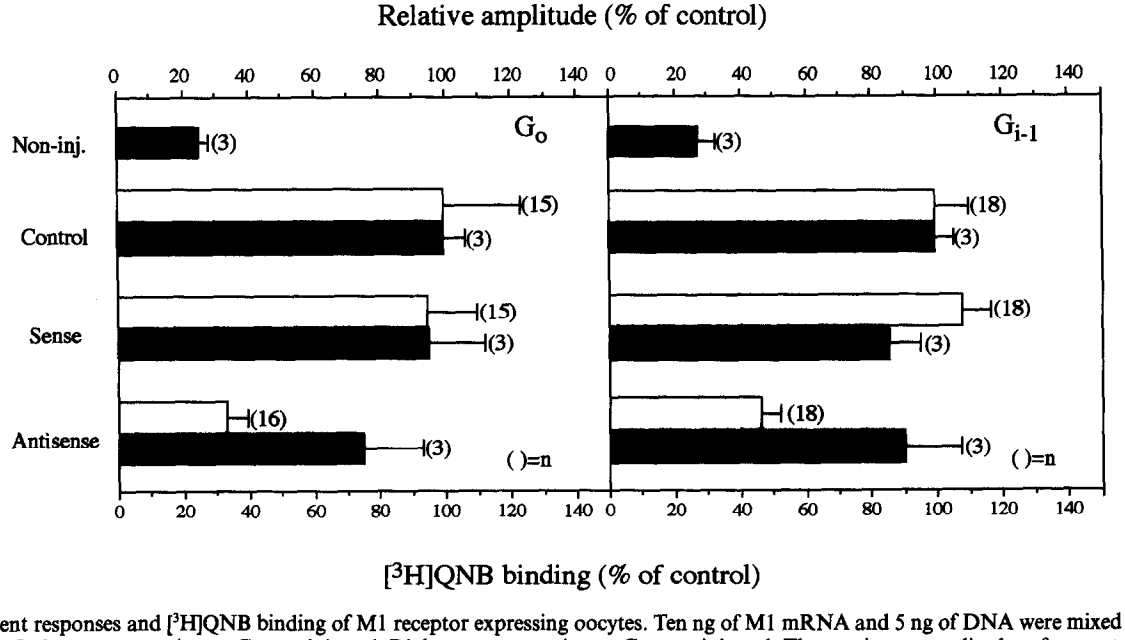


Fig. 3. Current responses and [$^3$H]QNB binding of M1 receptor expressing oocytes. Ten ng of M1 mRNA and 5 ng of DNA were mixed and injected into oocytes. Left: sense or antisense $G_o$ was injected. Right: sense or antisense $G_{i-1}$ was injected. The maximum amplitudes of current responses to 10 $\mu$M ACh were measured at −60 mV and expressed relative to the control, as mean ± S.E.M. (open columns). The specific binding of [$^3$H]QNB was determined as described in section 2, and expressed relative to the control, as mean ± S.E.M. (stippled columns).

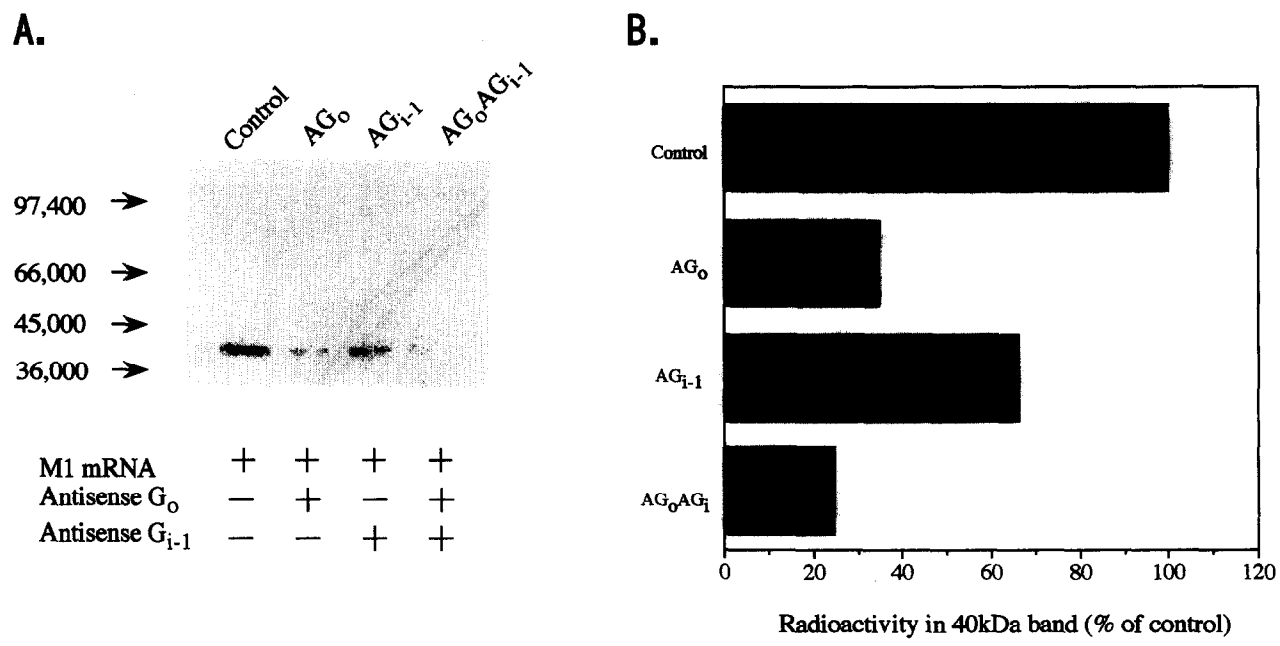


Fig. 4. Depletion of the endogenous PTX-sensitive G-proteins in *Xenopus* oocytes by the injection of antisense DNAs. A: autoradiogram of SDS-PAGE of the oocyte membranes which were [$^{32}$P]ADP-ribosylated in the presence of PTX. M1 receptor mRNA (1 ng) and 10 ng of antisense $G_o\alpha$ and/or $G_{i-1}\alpha$ DNAs were mixed and injected into oocytes. Three days after injection, the membranes were incubated with [$^{32}$P]NAD, as described in section 2. Positions of molecular weight markers are indicated on the left. B: quantitation of radioactivities in 40 kDa bands. The radioactivities of [$^{32}$P]ADP-ribosylated bands (in the autoradiogram in A) were measured by Bio-Image Analyzer, and expressed relative to the control (oocytes in which only M1 receptor mRNAs were injected).

reported here that the oocyte endogenous $G_o$ and $G_i$ could both participate in the PLC signal transduction pathway.

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