Evidence for a metabostatic opioid κ-receptor inhibiting pertussis toxin-sensitive metabotropic glutamate receptor-currents in *Xenopus* oocytes

Hiroshi Ueda*, Takeaki Miyamae, Nobuyuki Fukushima, Shigeru Watanabe, Yoshimi Misu

Department of Pharmacology, Yokohama City University School of Medicine, Yokohama 236, Japan

Received 12 September 1995; revised version received 9 October 1995

Abstract **Glutamate evoked pertussis toxin-sensitive currents in *Xenopus* oocytes expressing metabotropic glutamate receptor subtype 1 (mGluR1) and exogenous Gi1α. The mGluR1-currents were completely blocked by U-73122, a phospholipase C (PLC) inhibitor and by niflumic acid, a chloride channel blocker. In the oocyte further coinjected with poly(A)$^+$ RNA from the guinea pig cerebellum, the mGluR1-currents were inhibited by U-50488H, an opioid κ-agonist, and this inhibition was blocked by norbinaltorphimine, an opioid κ-antagonist. These findings suggest that the mRNA encoding a novel subtype of opioid κ-receptor which inhibits Gi1-PLC-mediated currents is present in guinea pig cerebellar poly(A)$^+$ fractions.**

Key words: Opioid receptor; G-protein; *Xenopus* oocyte

1. Introduction

Until now it has been generally believed that guanine nucleotide-binding protein (G-protein)-coupled receptors, including opioid receptors, always transduce 'stimulatory' signals to G-proteins [1,2]. Recently, δ-, μ- and κ-subtype opioid receptors have been cloned [3–5], and we have demonstrated that these receptors mediate phospholipase C (PLC) activation through stimulation of Gi1 in *Xenopus* oocytes [6–9]. From experiments using reconstitution techniques, however, it was found that in guinea pig cerebellar membranes κ-opioid receptor agonists transduce 'inhibitory' signals to Gi1 (or Gi2), but not Go [10–12], thereby inhibiting PLC [13,14]. It was speculated in such reports that some populations of opioid κ-receptors might be coupled to Gi1 in an inverse (or inhibitory) manner. Here we report the evidence for the presence of mRNA encoding such a novel type of opioid κ-receptor, by use of the *Xenopus* oocyte expression system.

2. Materials and methods

2.1. Xenopus *oocyte experiments*

Details of treatment of *Xenopus laevis* for defolliculation, RNA injection and electrophysiological recording were described previously [6–9]. RNAs except for poly(A)$^+$ RNA to be injected into oocytes were generated by in vitro transcription, primed with cap dinucleotide m^{7}G(5′)ppp(5′)G using Stratagene kit from each cDNA clone. For the injection into oocytes, 100 ng of poly(A)$^+$, 10 ng of metabotropic glutamate receptor type 1 (mGluR1) RNA and 50 ng of Gi1α-subunit RNA [15] were used. After further 3 days incubation at 19°C to allow for protein expression, responses to bath application or intracellular injection of test drugs were detected in injected *Xenopus* oocytes using voltage-clamp recording. The holding potential was −60 mV and the current signals were low-pass-filtered at 5–10 Hz. In standard experiments, glutamate (60 μM or 1 mM) was applied to the bath for 1 min every 15 min unless otherwise stated. Test drugs were applied for 14 min from 7 min prior to the following glutamate challenge, unless otherwise stated.

2.2. *Preactivation of pertussis toxin*

Pertussis toxin (PTX, 50 μg) was dissolved in 20 μl of high K$^+$ buffer (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), containing 40 mM DTT and 3 mM ATP, and incubated for 20 min at 37°C. The activated PTX was diluted to a concentration of 0.25 ng/nl of high K$^+$ buffer, containing 10 mM NAD, and used for intracellular injection (20 nl). Vehicle-control without PTX was prepared, as mentioned above.

2.3. *Immunochemical detection of exogenously expressed Gi1α and naturally expressed Gqα in oocytes injected with Gi1α RNA*

In order to confirm the expression of exogenous Gi1α, oocytes (40 eggs) which had been injected with Gi1α RNA (50 ng) per oocyte were sonicated in 800 μl of homogenization buffer (20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 250 mM sucrose, 50 μM *p*-(amidinophenyl)methanesulfonyl fluoride and 20 μg/ml leupeptin) and centrifuged 1,000 × *g* for 10 min at 4°C. Aliquots of supernatant membrane preparations (equivalent to 10 eggs) were subjected to PTX-catalyzed ADP-ribosylation by the method of Katada and Ui [16]. Briefly, the membranes (50 μg protein) were incubated in 100 μl of reaction buffer (100 mM Tris-HCl, 10 mM thymidine, 1 mM EDTA, 1 mM DTT, 2.5 μM [^{32}P]NAD (30,000 cpm/pmol) and 1 μg of preactivated PTX) at 30°C for 60 min. The reaction was terminated by the addition of 10 μl of trichloroacetic acid (TCA), and the precipitates were heated at 95°C for 3 min in 25 μl of 10 mM Tris-HCl (pH 7.5) containing 0.2 mM DTT and 2% SDS, added with 10 μl of 10 mM *N*-ethylmaleimide (NEM) and kept at room temperature for 15 min. The sample was mixed with 10 μl of 4× SDS-sample buffer (250 mM Tris-HCl, 400 mM DTT, 8% SDS, 40% glycerol, pH 6.8) and analyzed on 12% polyacrylamide SDS-PAGE [17]. For immunoprecipitation, commercially available Gi1α antiserum (Dupont-NEN, AS/7), which is specific for Gtα, Gi1α and Gi2α, was used. In such experiments ADP-ribosylation reaction (100 μl) was terminated by adding 1 μl of 10% SDS, followed by heating at 95°C for 5 min. The preparation was then mixed with 100 μl of precipitation buffer (100 mM $NaPO_4$, 300 mM NaCl, 4 mM EDTA, 4 mM DTT, 1% SDS, 2% sodium deoxycholate and 2% Triton X-100, pH 7.2), and kept on ice for 15 min. To reduce the background activity, normal goat serum (1 : 100) was added to the preparation and they were further kept on ice for 15 min, followed by incubation with protein G-Sepharose 4FF (40 μl of 50% slurry in 0.4% bovine serum albumin) for 120 min. The mixture was centrifuged at 3,000 × *g* for 3 min, and the supernatant was mixed with 5 μl of Gi1α antiserum. The samples were incubated at 4°C overnight, followed by incubation with protein G-Sepharose 4FF and centrifugation, as above-mentioned. After the Sepharose pellet was washed three times with 50 mM $NaPO_4$, 150 mM NaCl, 1 mM EDTA and 0.5% Triton X-100, pH 7.2, the sample was heated in 2% SDS and 0.2 mM DTT, and treated with NEM, as mentioned above. The suspension was further added with 10 μl of 4× SDS-sample buffer, heated at 95°C for 5 min and added with 10 μg of bovine serum albumin as a carrier. The [^{32}P]ADP-ribosylated materials were then precipitated by TCA, and analyzed by SDS-PAGE, as mentioned above. Autoradiography was achieved by exposure of dried

*Corresponding author. Fax: (81) 45 785 3645.

PII SDI 0014-5793(95)01204-4

polyacrylamide gel to Hyperfilm MP (Amersham) at −80°C with Dupont Cronex Lightning Plus intensifying screens. SeeBlue (Novex) stained markers were used as molecular standards.

For the determination of Gqα, oocyte membrane preparations, as prepared above (equivalent to 30 eggs) were diluted with 3-volumes of TED buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM DTT, pH 7.5) containing aprotinin (25 kallikrein u/ml) and centrifuged at 15,000 rpm × 20 min. The membrane pellets were then resuspended in 100 μl of TED/aprotinin containing 100 mM NaCl and 0.1% sodium cholate, and spun down. The pellets were resuspended in 10 μl of TED/aprotinin, added by 10 μl of 2% sodium cholate without agitation and placed on ice for 1 h. The samples were centrifuged at 15,000 rpm × 30 min and supernatants were carefully removed and applied on 12% acrylamide SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) and probed with anti-Gq antibody. The antibodies bound to the membrane were detected by horseradish peroxidase conjugated goat anti-rabbit IgG (KPL) and the chromogenic substrate 4-chloro-1-naphtol (Sigma).

2.4. Chemicals

U-50488H was a gift from Upjohn Japan (Tokyo), norbinaltorphimine (Nor-BNI) from Dr. H. Nagase (Toray, Japan) and other reagents from Sigma.

3. Results

3.1. Partial sensitivity of glutamate-currents to PTX in oocytes injected with mGluR1 receptor RNA

In oocytes injected with mGluR1 RNA, the challenge of 1 mM glutamate evoked sharp inward currents at the holding potential of −60 mV (Fig. 1A), as many other investigators reported [18,19]. Average (±S.E.M.) of glutamate-currents (mGluR1-currents) upon the first challenge was 960 ± 80 nA (n = 8). Repeated challenges of glutamate at 15 min intervals lead to a slow desensitization and showed plateau responses at approximately 60 min after the initial challenge (Fig. 1A). The mGluR1-currents upon the fourth challenge was 360 ± 180 nA (n = 8). However, there was no significant currents evoked by glutamate in oocytes not injected with mGluR1 RNA (data not shown).

When 5 ng of pertussis toxin which had been preactivated was injected together with 10 mM NAD into the oocyte in a volume of 20 nl, mGluR1-currents were partially attenuated (Fig. 1A). However, the vehicle injection with NAD had no significant change in mGluR1-currents, as shown in Fig. 1B. From the replicated experiments it was revealed that the average of mGluR1-currents 20 min after PTX-injection was 49.1 ± 17.9% (n = 3) of the currents just before the injection, which is statistically different (ANOVA) from that of non-treated control (99.8 ± 10.6%, n = 3) or of vehicle control (95.5 ± 13.2%, n = 6), as shown in Fig. 1C.

3.2. Characterization of PTX-sensitive mGluR1-currents in oocytes injected with mGluR1 receptor and Gi1α RNAs

When Gi1α RNA was coinjected together with mGluR1 RNA, the mGluR1-currents upon the first challenge were markedly reduced (290 ± 51 nA, n = 8), compared with the case without Gi1α RNA, as shown in Fig. 2A. However, the attenuation of evoked currents upon repeated challenges was less evident than the latter case. The average of mGluR1-currents upon the fourth challenge was 180 ± 29 nA (n = 8). In oocytes coinjected with Gi1α RNA, the mGluR1-currents were accom-

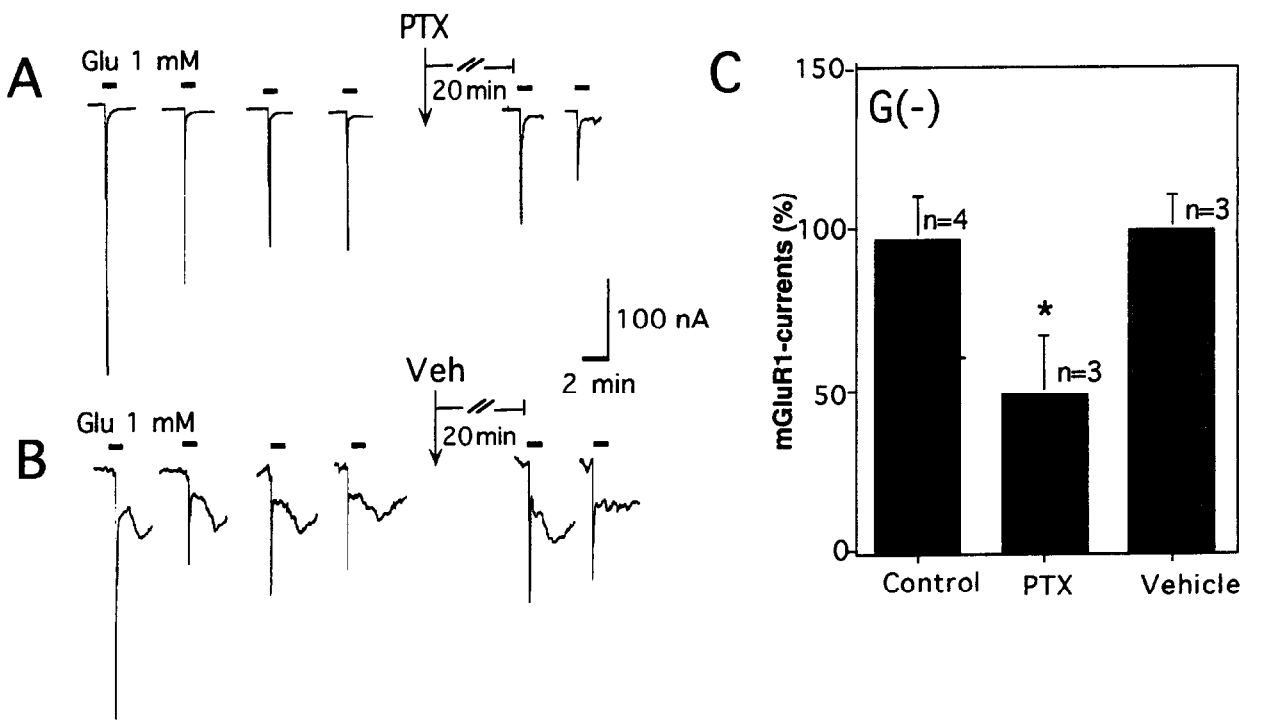

Fig. 1. Partial sensitivity of mGluR1-currents to pertussis toxin (PTX) in oocytes not coinjected with Gi1α RNA. (A) Effects of PTX-injection on mGluR1-currents. Preactivated PTX (5 ng) was injected with 10 mM NAD in a volume of 20 nl of high-K^+ solution into oocytes, 10 min after the fourth challenge of glutamate. The following glutamate-challenge was performed 20 min after the PTX-injection, as indicated in the figure. (B) Effects of vehicle-injection on mGluR1-currents. Vehicle containing 10 mM NAD was injected as described in A. (C) Statistical comparison of effects of PTX-injection on mGluR1-currents with non-injection or with vehicle-injection. Results represent the ratio of mGluR1-currents 30 min after the fourth challenge (Control), 20 min after PTX-injection (PTX) and 20 min after vehicle-injection (Vehicle) to those upon the fourth challenge. *$P < 0.05$, compared with Control or Vehicle (ANOVA).

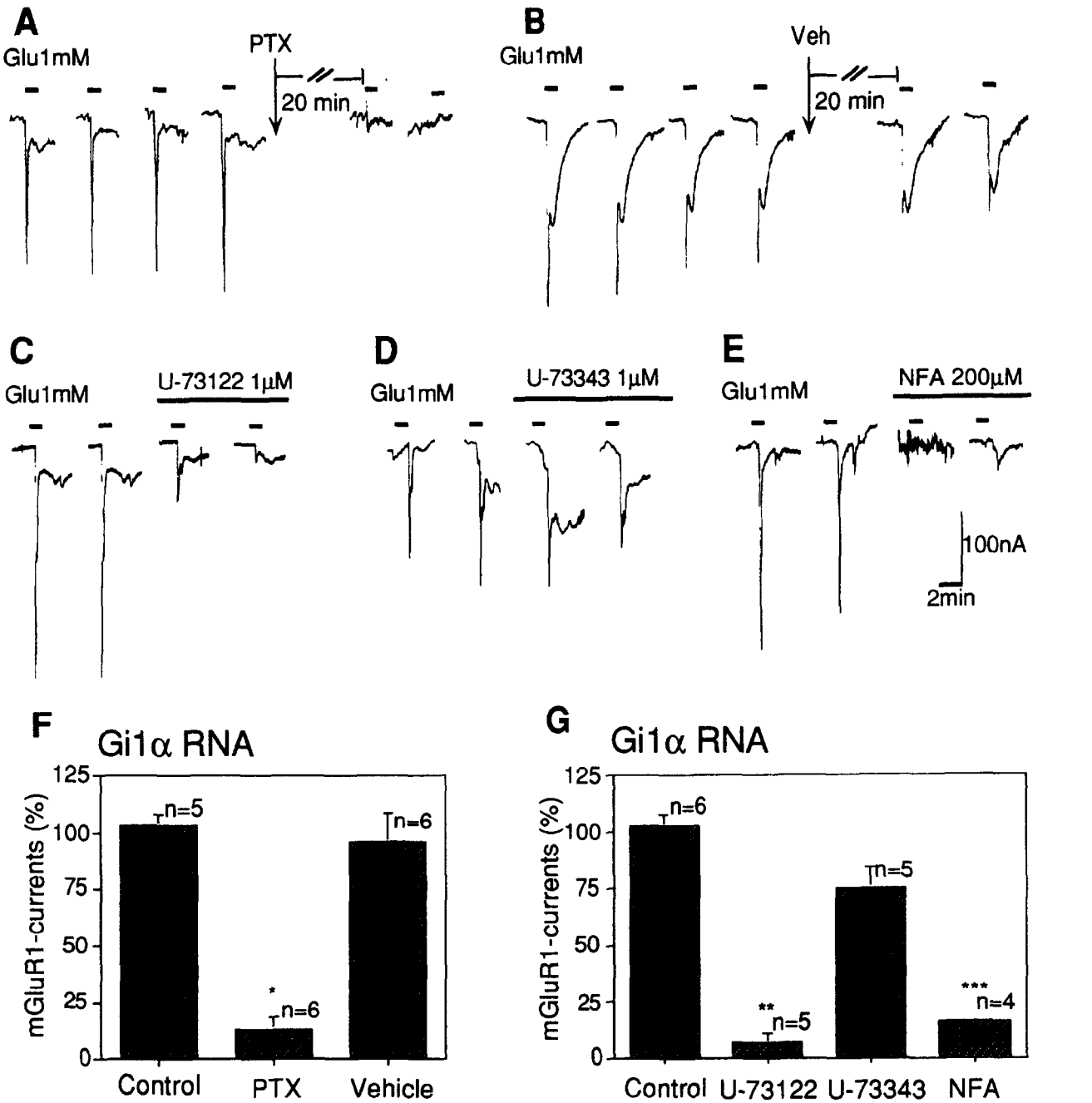

Fig. 2. PTX-sensitive mGluR1-currents through Gi1 and PLC in oocytes injected with Gi1α RNA. (A and B) Effects of PTX- (A) or vehicle-injection (B) on mGluR1-currents in oocytes coinjected with Gi1α RNA. Details are described in the legend of Fig. 1. (C–E) Effects of 1 μM U-73122 (C), 1 μM U-73343 or 200 μM niflumic acid (NFA) on repeated mGluR1-currents. Each drug was bath-applied for 14 min from 7 min prior to the following glutamate-challenge. (F) Statistical comparison of effects of PTX-injection on mGluR1-currents with the non-injection or with the vehicle-injection. Details are described in the legend of Fig. 1. (G) Statistical comparison of effects of various drugs on mGluR1-currents. ** and *** $P < 0.05$, compared to control without any drug (ANOVA).

panied by oscillating currents of relatively slow onset as well as sharp currents of rapid onset. In some cases, no such rapid components were observed. The injection of preactivated PTX (5 ng) together with NAD completely abolished the mGluR1-currents (Fig. 2A). However, the vehicle containing NAD alone showed no significant change (Fig. 2B), as seen in the case with oocytes not injected with Gi1α RNA (Fig. 1B).

To characterize the PTX-sensitive mGluR1-currents, several pharmacological tools were used. The bath-application of U-73122, a specific inhibitor of PLC, completely blocked the mGluR1-currents in Gi1α RNA-injected oocytes (Fig. 2C). However, U-73343, an inactive derivative, showed no significant change (Fig. 2D). Similarly the bath-application of niflumic acid, a chloride channel blocker, abolished the mGluR1-currents (Fig. 2E). Several replicated experiments confirmed such results, as shown in Fig. 2F.

3.3. Expression of exogenous Gilα in oocytes

To detect exogenously expressed Gilα, the oocyte membranes prepared at various time periods after its RNA injection were [^{32}P]ADP-ribosylated by PTX. As shown in Fig. 3A, the radioactivity of [^{32}P]ADP-ribosylated materials in preparations without Gilα RNA was found only at 39 kDa on SDS-PAGE. However, the signal at 41 kDa was growing in preparations injected with Gilα RNA, as the time of incubation. Marked increases were found at 72 and 84 h after the injection of the RNA. To identify the expressed G-protein, [^{32}P]ADP-ribosylated materials at 72 h were incubated with an antibody recognizing Gilα, Gi2α and Gtα, but not Gqα, Goα, Gi3α nor Gsα for immunoprecipitation. As shown in Fig. 3B, there was a significant signal at 41 kDa in preparations injected with Gilα RNA, but not in preparations without it. In Western-blot experiments using an antibody recognizing Gqα and G11α, on the contrary, there was a significant signal at 42 kDa, corresponding to Gqα [20] in preparations not injected with Gilα RNA, while such a signal was markedly reduced in the same preparations at 72 h after Gilα RNA injection, as used in immunoprecipitation experiments.

3.4. Inhibition of PTX-sensitive mGluR1-currents by opioid κ-agonist in oocytes injected with mGluR1 and Gilα and poly(A)$^+$ RNAs from the guinea pig cerebellum

In oocytes injected with mGluR1 and Gilα and poly(A)$^+$ RNAs, glutamate (60 μM or 1 mM) evoked inward currents of approximately 100 nA which are equivalent to the currents in oocytes without poly(A)$^+$ RNA (Fig. 4A and B). In separate experiments such currents were also abolished by PTX-injection. When 0.1 μM U-50488H, an opioid κ-agonist was bath-applied 7 min prior to the following glutamate (60 μM)-challenge to the oocyte, the mGluR1-currents were not affected, as shown in Fig. 4A. However, this opioid κ-agonist at a higher

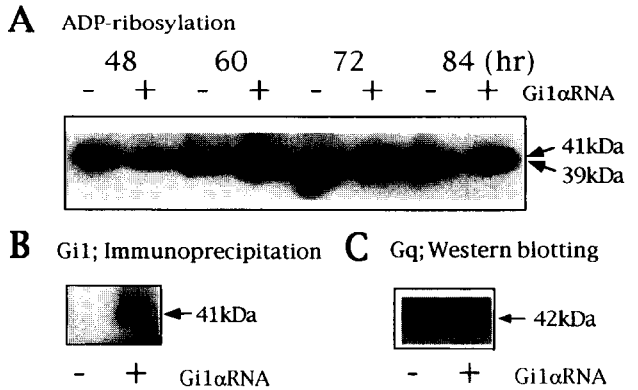

Fig. 3. Gilα and Gqα expression in membranes of oocytes injected with Gilα RNA. (A) Time course of [^{32}P]ADP-ribosylation of membrane G-proteins of oocytes injected with Gilα RNA. Membranes were prepared from non-injected oocytes (−) or from injected ones (+) at 48, 60, 72 or 84 h after the injection. The membranes (50 μg) were incubated with [^{32}P]NAD and preactivated PTX, and subjected to analysis by SDS-PAGE, followed by autoradiography. (B) Immunoprecipitation of [^{32}P]ADP-ribosylated materials using anti-Gilα antiserum. Membranes (50 μg) at 72 h after the Gilα RNA-injection were solubilized with detergent, immunoprecipitated, using anti-Gilα antiserum, SDS-PAGE and autoradiographed. (C) Western-blot of Gqα. Membranes (100 μg), as used in B were solubilized and used for Western-blot experiments combined with visualization with horseradish peroxidase and 4-chloro-1-naphtol.

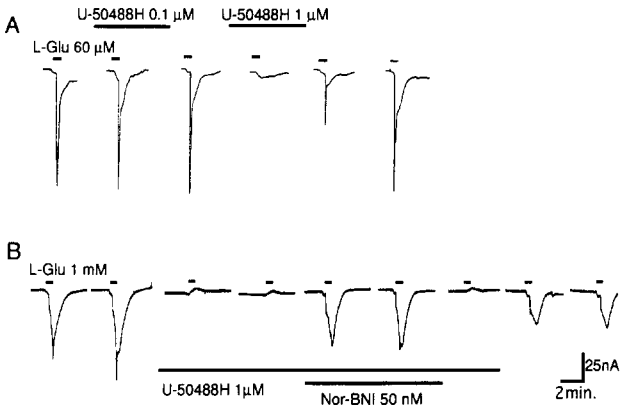

Fig. 4. Opioid κ-agonist-induced inhibition of mGluR1-currents in oocytes injected with mGluR1 RNA, Gilα RNA and poly(A)$^+$ RNA from the guinea pig cerebellum. (A) Representative data of opioid κ-agonist-induced inhibition of mGluR1-currents. Glutamate at 60 μM was used in this experiment. U-50488H (0.1 μM or 1 μM) was bath-applied for 14 min from 7 min prior to the glutamate challenge. (B) Another representative result of U-50488H-induced inhibition of mGluR1 (1 mM)-currents and antagonism by nor-binaltorphimine (Nor-BNI). Test drugs were given as indicated in the figure.

concentration (1 μM) markedly inhibited the mGluR1-currents, and a complete recovery was observed at the second challenge after the washout of U-50488H. In a separate oocyte injected similarly, U-50488H at 1 μM also abolished the mGluR1-currents by 1 mM glutamate, and such inhibitory effects were completely blocked by 50 nM Nor-BNI, a selective opioid κ-antagonist (Fig. 4B). After the cease of Nor-BNI-application, the U-50488H-induced inhibition of mGluR1-currents was observed again. The mGluR1-currents were recovered after the wash of these opioid compounds, though there was a small attenuation. However, there was no inhibition of Gil-mediated mGluR1-currents by U-50488H in oocytes not injected with poly(A)$^+$ RNA (data not shown). Furthermore we have obtained similar results that muscarinic M2-receptor-currents through Gil were also inhibited by this κ-agonist in oocytes injected with poly(A)$^+$ RNA (Ueda et al., unpublished data).

4. Discussion

Since we firstly reported that opioid κ-agonists inhibit the high-affinity GTPase in guinea pig cerebellar membranes [10], several investigators including ourselves have demonstrated the findings related to such a receptor-mediated inhibition of GTPase activity [21,22]. We have proposed the mechanisms of such inhibitory actions of opioid κ-agonist through a receptor-mediated direct inhibition of GDP-GTP exchange activity (or intrinsic Gil-protein activity) from biochemical reconstitution experiments [11,12].

The major finding in this report is that Gil-mediated mGluR1-currents were markedly attenuated by U-50488H, a specific opioid κ-agonist, and such an inhibition was reversed by Nor-BNI, a specific opioid κ-receptor antagonist. As there was no inhibition of Gil-mediated mGluR1-currents by U-50488H in oocytes not injected with poly(A)$^+$ RNA, it is suggested that a certain opioid receptor of κ-subtype is involved in the inhibition of Gil-mediated currents.

In the present study we used the cross-talk mechanism with metabotropic glutamate receptor, mGluR1 at the level of Gi1 to detect the inhibition of intrinsic G-protein activity. Although mGluR1-currents were reported to be in part sensitive to PTX [18], it has been considered that such currents are mediated through Gqα-PLC mechanisms [19], since PLC is predominantly regulated by Gq [23]. On the other hand, we have reported that Gi1 also plays important roles in such a PLC-activation in biochemical studies in which we adopted the reconstitution experiments using purified Gi1 [14,24]. In our current experiments, we have shown that δ-, μ- and κ-opioid receptors evoked calcium-dependent chloride currents through an activation of PLC in oocytes reconstituted with exogenously expressed Gi1α [6–9]. In such studies, δ-opioid receptor which is known to couple to Gi1, Gi2, Gi3 and Go in some other cells showed enhanced currents in oocytes when injected with RNA of Gi1α or Gi2α, but not of Gi3α nor Goα [9]. These findings suggest that both Gi1 and Gi2 are more potent in an activation of PLC than Gi3 or Go, and that their α-subunits are more potent than βγ-subunits, provided βγ-subunits are common to these heterotrimeric G-proteins. Most recently the former view was confirmed by the report that recombinant Go in an active form failed to stimulate PLCβ, purified from the brain, in reconstituted vesicles [25]. Thus, we attempted to detect PTX-sensitive mGluR1-currents by an overexpression of Gi1α. Indeed, the mGluR1-currents were completely abolished by PTX-injection in oocytes injected with Gi1α RNA, but only partially in those without the RNA. Such a difference in the PTX-sensitivity might be explained by the view that exogenously expressed Gi1α absorbs common βγ-subunits from Gqαβγ heterotrimers, resulting in predominance of Gi1αβγ heterotrimers in injected oocytes. This view was further supported by the present data that Gqα-like immunoreactivities in oocyte membranes were markedly decreased at the time when Gi1α expression maximally increased. This may suggest that Gqα monomer dissociated from βγ-subunits under the condition of Gi1α overexpression is not tightly anchored to the membrane, since isoprenylated γ-subunit is very important for membrane anchoring of heterotrimeric G-proteins [26].

The Gi1-mediated mGluR1-currents were completely blocked by U-73122, an aminosteroid-type PLC inhibitor, but not by U-73343, an inactive derivative which differs by only a single double bond [27]. The bath-application of U-73122 itself showed no significant change in basal currents, suggesting that mGluR1-currents were unlikely abolished by mechanisms secondary to the inhibition of spontaneous PLC activity by U-73122. The mGluR1-currents were also completely blocked by niflumic acid, a chloride channel blocker which is well known to block calcium-dependent chloride channels naturally expressed in *Xenopus* oocytes [28]. These findings are consistent with the data in our previous reports that Gi1- and PLC-mediated currents are through calcium-dependent chloride channels [6–9].

This report is consistent with our previous and current findings that the opioid κ-agonists inhibits the PLC activity stimulated by GTP through Gi1 reconstituted into PTX-treated membranes and provides the evidence for the presence of opioid κ-receptor inhibiting PLC activity through Gi1. As cloned opioid κ-receptor evokes chloride currents through an activation of Gi1 and PLC in the oocyte [8], the present Gi1-inhibitory, or 'metabostatic' receptor activity is likely through a new receptor different from such a known κ-opioid receptor. However, details must await the expression cloning of this receptor which is now in progress in our laboratory.

References

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[2] Ueda, H., Nozaki, M. and Satoh, M. (1990) Comp. Physiol. Biochem. 98C, 157–169.
[3] Evans, C.J., Keith Jr., D.E., Morrison, H., Magendzo, K. and Edwards, R.H. (1992) Science 258, 1952–1955.
[4] Kieffer, B.L., Beport, K., Gaveriaux-Ruff, C. and Hirth, C.G. (1992) Proc. Natl. Acad. Sci. USA 89, 12048–12052.
[5] Uhl, G.R., Childers, S. and Pasternak, G. (1994) Trends Neurosci. 17, 89–93.
[6] Miyamae, T., Fukushima, N., Misu, Y. and Ueda, H. (1993) FEBS Lett. 333, 311–314.
[7] Ueda, H., Miyamae, T., Fukushima, N. and Misu, Y. (1994) NeuroReport 5, 1985–1988.
[8] Ueda, H., Miyamae, T., Fukushima, N., Takeshima, H., Fukuda, K., Sasaki, Y. and Misu, Y. (1995) Mol. Brain Res. 32, 166–170.
[9] Ueda, H., Miyamae, T., Hayashi, C., Watanabe, S., Fukushima, N., Sasaki, Y., Iwamura, T. and Misu, Y. (1995) J. Neurosci., in press.
[10] Ueda, H., Misawa, H., Fukushima, N. and Takagi, H. (1987) Eur. J. Pharmacol. 138, 129–132.
[11] Ueda, H., Uno, S., Harada, J., Kobayashi, I., Katada, T., Ui, M. and Satoh, M. (1990) FEBS Lett. 266, 178–182.
[12] Ueda, H., Misawa, H., Fukushima, N., Katada, T., Ui, M. and Satoh, M. (1995) J. Neurochem., in press.
[13] Misawa, H., Ueda, H. and Satoh, M. (1990) Neurosci. Lett. 112, 324–327.
[14] Misawa, H., Ueda, H., Katada, T., Ui, M. and Satoh, M. (1995) FEBS Lett. 361, 106–110.
[15] Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Otsuka, E., Kawasaki, H., Suzuki, K. and Kaziro, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 3776–3780.
[16] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. and Nakanishi, S. (1991) Nature 349, 760–765.
[19] Nakamura, K., Nukada, T., Haga, T. and Sugiyama, H. (1994) J. Physiol. 474, 35–41.
[20] Strathmann, M. and Simon, M.I. (1990) Proc. Natl. Acad. Sci. USA 87, 9113–9117.
[21] Negishi, M., Namba, T., Sugimoto, Y., Irie, A., Katada, T., Narumiya, S. and Ichikawa, A. (1993) J. Biol. Chem. 268, 26067–26070.
[22] Ueda, H., Sato, K., Okumura, F., Inoue, A., Nakata, Y., Ozaki, N., Yue, J.-L. and Misu, Y. (1995) Biomedicine and Pharmacotherapy 49, 169–177.
[23] Berridge, M.J. (1993) Nature 361, 315–325.
[24] Ueda, H., Yoshihara, Y., Misawa, H., Fukushima, N., Katada, T., Ui, M., Takagi, H. and Satoh, M. (1989) J. Biol. Chem. 264, 3732–3741.
[25] Nakamura, F., Kato, M., Kameyama, K., Nukada, T., Haga, T., Kato, H., Takenawa, T. and Kikkawa, U. (1995) J. Biol. Chem. 270, 6246–6253.
[26] Clapham, D.E. and Neer, E.J. (1993) Nature 365, 403–406.
[27] Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J. and Bunting, S. (1990) J. Pharmacol. Exp. Ther. 255, 756–768.
[28] White, M.M. and Aylwin, M. (1990) Mol. Pharmacol. 37, 720–724.