A subtype of opioid κ-receptor is coupled to inhibition of Gi1-mediated phospholipase C activity in the guinea pig cerebellum

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Abstract PLC activity was stimulated either by 1–100 μM of GTP or by 100–3,000 μM Ca^{2+} in lysed synaptosomal membranes of the guinea pig cerebellum. The κ-opioid receptor agonist selectively inhibited the PLC activity stimulated by 100 μM GTP, but not by 100–3,000 μM Ca^{2+}. Pretreatment of membranes with PTX abolished such a κ-agonist-induced inhibition of PLC activity. The reconstitution of Gi1, but not of Go purified from porcine brains with PTX-treated membranes showed a complete recovery of the κ-agonist-inhibition of PLC activity. These findings suggest that a novel subtype κ-receptor mediates inhibition of PLC through inhibiting the intrinsic activity of PTX-substrate G-proteins.

Key words: Opioid κ-receptor; Gi1-type G-protein; Phospholipase C

1. Introduction

Several different hormones and neurotransmitters influence phosphatidylinositol metabolism, in various tissues [1]. Diacylglycerol and inositol 1,4,5-trisphosphate (InsP3), both derived from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phosphatidylinositol-specific phospholipase C (PLC) serve as important intracellular second messengers for protein kinase C activation [2] and intracellular Ca^{2+} mobilization [1]. Thus, it has been widely accepted that the receptor-linked regulation of PLC activity is one of major transduction pathways which communicate extracellular signals to intracellular second messengers.

There is accumulating evidence that pertussis toxin (PTX)-sensitive GTP-binding proteins (G-proteins) are involved in the regulation of PLC activity in several receptor mechanisms [3], as well as PTX-insensitive ones, such as Gq [1]. In some cells the receptor-mediated stimulation of PLC was abolished by PTX [4–6], while in other cells there were no changes in PLC activations by such treatments [7,8]. We have reported that the receptor-mediated stimulation of PLC in rat brain synaptic membranes was abolished by PTX pretreatment and recovered by reconstitution with purified Gi1, but not with Go [9]. Most recently we reported that δ-opioid receptor evoked a calcium-dependent chloride current through an activation of Gi1 and PLC in *Xenopus* oocytes [10,11].

On the other hand, some investigators reported the contradictory findings that the PLC activity is inhibited by various receptor stimulations. The receptor-mediated inhibition of PLC as a phenomena was first reported by Canonico et al. [12] for the D2 receptor in pituitary and followed up by several groups [13]. Most recently, we also found that the κ-opioid agonist inhibited the GTP-stimulated PLC activity in synaptic membranes of the guinea pig cerebellum [14]. Of interest is the fact that in such preparations, the κ-opioid agonist inhibits G-protein activity [15,16]. As cloned opioid κ-receptor [17,18] shows a stimulatory action on Gi coupled to inhibition of adenylate cyclase [19] and to PLC activation [11], the κ-subtype to show an inhibition of G-protein activity should be of another subtype. Here we report the involvement of PTX-sensitive G-proteins, such as Gi1 in such an inhibitory coupling between κ-opioid receptor and PLC by reconstitution experiments.

2. Materials and methods

2.1. Materials

U-50,488H was a gift from Upjohn, Japan. Pertussis toxin (PTX) was obtained from Funakoshi (Tokyo, Japan), D-myo-inositol 1,4,5-trisphosphate (InsP3) ^3H assay kit and [^{32}P]NAD from Amersham, Japan.

2.2. Membrane preparation

Male guinea pig weighing 250–350 g were decapitated. The cerebellum was dissected from the whole brain, homogenized in 10 vol. of 0.32 M sucrose containing 5 mM HEPES, pH 7.5 (buffer A) and centrifuged at 1,000 × *g* for 10 min. The supernatant (S1) was further centrifuged at 15,000 × *g* for 20 min to produce pellet (P2) and S2. The P2 was resuspended in 5 ml of buffer A, laid on Ficoll gradient (10 ml of 7.5% w/v and 10 ml of 12% w/v) and centrifuged at 68,000 × *g* for 60 min [20]. Synaptosomes were recovered with a Pasteur pipette at the 7.5%/12% Ficoll interface. The synaptosomes were mixed with 9 vols. of 100 mM Na-phosphate, pH 7.5 (buffer B) and centrifuged at 35,000 × *g* for 20 min. The pellet (synaptosomal lysed membranes) was washed once with buffer B and resuspended in the same buffer to make a protein concentration of 1–2 mg/ml. All procedures were done at 4°C.

2.3. Treatment of synaptic membranes with PTX

Procedures of preactivation of PTX and ADP-ribosylation of synaptic membranes by preactivated PTX were as described [9,21]. After treatment with PTX, the membranes were washed three times, and then suspended in buffer B. Radiolabeling of membrane proteins with [^{32}P]NAD was carried out reported by Katada and Ui [22].

2.4. Reconstitution of Gi1 or Go into PTX-treated membranes

Gi1 or Go was purified (>95%) from cholate extracts of porcine

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Abbreviations: G-protein, heterotrimeric GTP-binding proteins; PLC, phospholipase C; PTX, pertussis toxin; InsP3, inositol 1,4,5-trisphosphate, PIP2, phosphatidylinositol 4,5-bisphosphate.

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brain membranes [23]. The PTX (10 μg/ml)-treated membranes (10–20 μg of protein) was reconstituted with Gi1 or Go (0.25–4 pmol) by incubating at 4°C for 60 min in 20 μl of buffer B.

2.5. Phospholipase C assay

Membrane PLC activity was determined by measuring InsP3 derived from endogenous membrane PIP2, using [^{3}H]InsP3 assay system (Amersham), according to Manufacturer's protocol. In standard experiments, the assay contained synaptosomal lysed membranes (10–20 μg of protein) of guinea pig cerebellum, 1 mM ATP, 100 μM GTP, 10 μM $CaCl_2$, 100 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT in 200 μl of buffer B. The incubation was carried out for 2 min at 37°C, unless otherwise stated. The reaction was terminated with an equal volume of 15% (w/v) of ice-cold trichloroacetic acid. After 30 min standing on ice, the reaction mixture was centrifuged at 35,000 × *g* for 10 min. Supernatants were extracted 3 times with 5 vol. of water-saturated diethyl ether. The aqueous phase was titrated to pH 7.5 with $NaHCO_3$ and then kept at −20°C until the InsP3 assay. The InsP3 was stable for up to 2 weeks at −20°C and all assays were run in duplicate. InsP3 formation was calculated from the difference between InsP3 content at each incubation time and that without incubation (0 time). Proteins were determined by the method of Lowry et al. [24].

3. Results

3.1. Characterization of GTP-stimulation of InsP3 accumulation in guinea pig cerebellar membranes

When synaptosomal membranes were incubated with 100 μM GTP in the presence of 10 μM Ca^{2+}, there was an immediate increase in InsP3 accumulation. As shown in Fig. 1A, the increase in accumulation reached a plateau at 2 min after the initiation of incubation, while there was no increase in the absence of GTP. The InsP3 accumulation measured at 2 min incubation increased as the concentrations of GTP up to 100

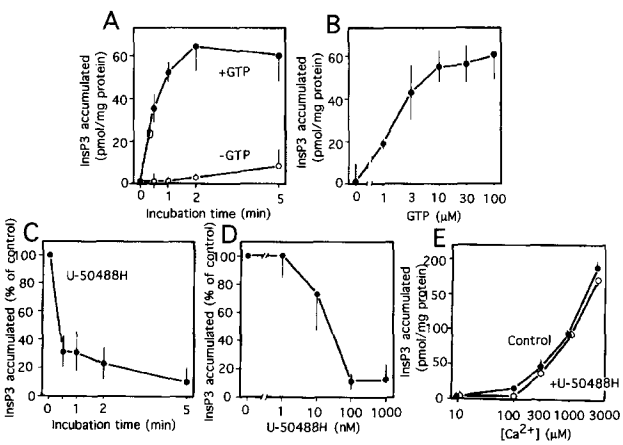

Fig. 1. Effects of the κ-opioid agonist on InsP3 formation stimulated by GTP or Ca^{2+} in guinea pig cerebellar membranes. Measurement of InsP3 accumulation was performed as described in section 2. Amounts of InsP3 accumulated for 2 min in Panel A, B and E are shown as pmol/mg protein, and those in panel C and D were as a% of the control without U-50,488H, respectively. Panel A: Time course of InsP3 accumulation in the presence or absence of 100 μM GTP. Panel B: InsP3 accumulation at various concentrations of GTP. Panel C: Time course of U-50,488H (100 nM)-induced inhibition of InsP3 accumulation in the presence of 100 μM GTP. Results represent the ratio of InsP3 accumulation for various incubation times in the presence of 100 nM U-50488H to that in its absence (control) as a percentage. Panel D: InsP3 accumulation at various concentrations of U-50,488H in the presence of 100 μM GTP. Panel E: InsP3 accumulation at various concentrations of Ca^{2+} in the presence or absence of 100 nM U-50,488H. Experiments were carried out in duplicate. Each point is the mean ± S.E.M. from 3–5 separate experiments.

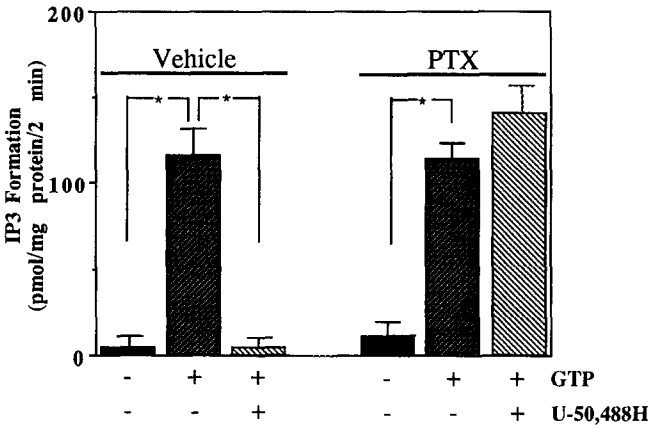

Fig. 2. Abolition of the κ-agonist-inhibition of PLC activity by PTX treatment of guinea pig cerebellar membranes. Measurement of InsP3 accumulation and PTX-treatment of cerebellar membranes were as described in section 2. Membranes treated with vehicle or PTX (as indicated with underline in the figure), were incubated at 37°C for 2 min. The condition in each experiment was depicted in the figure, as the presence (+) and absence (−) of 100 μM GTP and/or 100 nM U-50,488H. Each result represents the mean ± S.E.M. of 3 separate experiments. *The difference is statistically significant ($P<0.05$, Student's *t*-test).

μM (Fig. 1B). We obtained preliminary data that InsP3 binding protein in 'InsP3 assay kit' did not cross-react with 100 μM GTP (data not shown). Such an increase in InsP3 accumulation was also observed with non-hydrolyzable GTP analogs, GppNHp and GTPγS, but not by other nucleotides such as GDP, GMP, CDP and CTP (Table 1). In addition, we have previously reported that the GTP-stimulation of PLC was markedly reduced by the addition of neomycin, an inhibitor of phospholipase C or 0.2 mM EGTA to the assay mixture [14]. The calcium-dependent activation of PLC is consistent with findings of other investigators [7,8]. Thus, the membrane-bound PLC activity seems to be regulated by G-proteins.

3.2. κ-Opioid receptor agonist inhibits the GTP-stimulated PLC activity

In the guinea pig cerebellum, κ-opioid receptors are predominant over μ- and δ-receptors [25]. U-50,488H at 100 nM, a selective κ-opioid receptor agonist [26], showed a complete inhibition of PLC in the presence of 100 μM GTP and within 5 min (Fig. 1C), but not in the absence of GTP (data not shown). The κ-agonist concentration-dependently inhibited GTP (100 μM)-stimulated PLC measured at 2 min in ranges of 1–100 nM (Fig. 1D). The maximal and half maximal inhibitory concentrations of U-50,488H were 100 nM and 65 nM, respectively.

As shown in Fig. 1E, the PLC activity was also stimulated by the addition of millimolar ranges of Ca^{2+} in the absence of GTP, as noted by other workers [7,8]. The stimulation was much higher than that observed with GTP in the presence of 10 μM Ca^{2+}. However, the κ-agonist at 100 nM showed no significant change in PLC activity stimulated by Ca^{2+}.

3.3. Abolition of the κ-agonist-induced inhibition of GTP-stimulation of PLC by PTX and its recovery by reconstitution with purified Gi1

To further clarify the involvement of G-protein in the

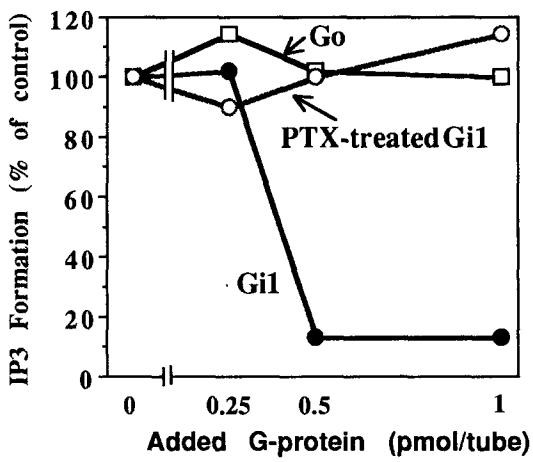

Fig. 3. Recovery of the κ-agonist-inhibition of PLC activity in PTX-treated membranes by reconstitution with purified Gi1, Go or PTX-treated Gi1. Data represnt percentage of control InsP3 accumulation in PTX-treated preparations. Reconstitution of purified Gi1, Go or PTX-treated Gi1 was performed as described in section 2. Symbols represent data in preparations reconstituted with Gi1 (●), Go (□) and ADP-ribosylated Gi1 by PTX (○), respectively. All experiments presented here were carried out in the presence of 100 nM U-50,488H and 100 μM GTP. Each bar (in triplicate) represents the mean from 2 separate experiments. There were no significant changes between both experiments.

κ-agonist-inhibition of GTP-stimulated PLC activity, the cerebellar membranes were pretreated with preactivated PTX (10 μg/ml) at 37°C for 30 min. As shown in Fig. 2, the κ-agonist-induced inhibition of PLC activity was abolished by PTX-treatment, while there was no change in the basal or GTP-stimulated activity of PLC between vehicle- and PTX-treated membranes. These findings are consistent with reported data that the PTX abolished the ability of PTX-substrate G-proteins, such as Gi and Go to functionally couple to receptors [27], but shows no significant effect on the GTPγS binding activities [28].

To reconstitute purified G-proteins into PTX-treated membranes, the addition of detergents to the incubation mixture is required. When various detergents at 0.05% were added to the assay mixture, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid/CHAPS, digitonin and Lubrol-PX reduced PLC activity in the presence of 100 μM GTP to the level below 10% of the control without detergents, while there was no inhibition with sodium cholate. Sodium cholate treatment increased the basal activity from 65 to 110 pmol/mg protein. Thus, we chose sodium cholate for reconstitution of the purified G-proteins with PTX-treated membranes. The control InsP3 formation in the membranes pretreated with 10 μg/mg of PTX in the presence of 100 μM GTP was 136.0 ± 16.9 pmol/mg protein (n = 3), while the reconstitution of purified Gi1, Go or PTX-treated Gi1 (0.25–1 pmol/tube) showed no significant change in InsP3 formation in the presence of GTP. As shown in Fig. 3, the reconstitution of purified Gi1 or Go within 1 pmol of each G-protein/tube into PTX-treated membranes showed no significant change in PLC activity in the presence of GTP, being in consistent with other report [4]. Under such conditions, U-50,488H at 100 nM inhibited the PLC activity in preparations reconstituted with Gi1 at 0.5–1.0 pmol/tube, to a similar extent (by 87% of control), compared to findings in membranes without PTX (Fig. 1A,C,D), but not with Go at 0.25–1.0 pmol/tube. When 1 pmol/tube of purified Gi1 previously ADP-ribosylated by PTX (10 μg/ml, 37°C for 30 min) was reconstituted into PTX-treated membranes, recovery of the κ-agonist-inhibition of PLC was no longer observed.

4. Discussion

InsP3 accumulation was stimulated by GTP and its unhydrolyzable analogs, but not by other nucleotides, in guinea pig cerebellar membranes. This finding provides the evidence that membrane-bound PLC activity is regulated by G-proteins. The κ-opioid agonist inhibited the GTP-stimulated PLC to the level seen without GTP. The involvement of G-proteins in this κ-agonist-inhibition of PLC is evident from the following observations; (i) the inhibition was specific for PLC activity stimulated by GTP, but not by millimolar Ca^{2+}, (ii) the inhibition was abolished by pretreatment of membranes with PTX, a compound which inactivates substrate G-proteins to uncouple to receptors in membranes by ADP-ribosylation of their α-subunits [29,30], (iii) the selective recovery from the PTX-inhibition of κ-agonist action was observed by reconstitution with purified Gi1.

We have carried out functional reconstitution experiments and found that kyotorphin stimulates GTP-stimulated PLC via an activation of Gi1 in rat brain membranes [9] and that δ-opioid receptor mediates PLC activation through Gi1 in *Xenopus* oocytes [10]. Similar findings were reported by Kikuchi et al. [4] who demonstrated that fMet-Leu-Phe, a chemotactic peptide-stimulation of PLC was abolished by PTX-treatment and was recovered by reconstitution with Gi or Go in human leukemic (HL-60) cell membranes. These findings strongly suggest that PTX-substrate G-proteins are functionally coupled to PLC in a stimulatory fashion. The β-type isozymes of various PLCs are well known to be regulated by G-protein-coupled (metabotropic) receptors. Recent studies have revealed that PLCβ1 is activated by α-subunit of PTX-insensitive G-protein, such as Gq, G11 and G16, PLCβ2 by βγ-subunits which are released from many heterotrimeric G-proteins including PTX-sensitive ones, and PLCβ3 by both Gq/11/16α and βγ-subunits [29]. These findings suggest that βγ-subunits rather than α-subunits of PTX-sensitive G-proteins may play roles in PLC

Table 1
Effects of various nucleotides on InsP3 accumulation in guinea pig cerebellar membranes

Addition	InsP3 accumulated (%) of control
No addition	118.0 ± 10.4
GTP	256.5 ± 49.7*
GppNHp	264.4 ± 30.5*
GTPγS	249.6 ± 10.3*
GMP	105.6 ± 9.7
GDP	104.9 ± 11.8
CDP	91.6 ± 17.7
CTP	93.8 ± 5.6

Guinea pig cerebellar membranes were incubated in the presence of the indicated nucleotides at 100 μM at 37°C for 2 min, as described in section 2. Results represent the percentage of control InsP3 amounts (42.9 ± 5.4 pmol/mg protein, n = 13) without incubation as the S.E.M. of the mean from separate 3–9 experiments. *The difference is statistically significant, compared to no addition (P<0.05, Student's t-test).

activation. However, in our recent findings using electrophysiological detection in *Xenopus* oocyte, the currents evoked through Gi1-coupled δ-opioid receptor were mediated by overexpression of Gi1α [11], but not by βγ-subunits (H. Ueda, T. Miyamae and Y. Misu, unpublished observation). Thus, it may be true that Gi1α per se is also involved in stimulatory regulation of PLC activity. However, there is a problem that no effect of the purified Gi1 was observed on the activation of PLC in the reconstitution experiments. Taken all above-mentioned into consideration, such an unexpected finding may be explained by the view that the activities of related PLC isozymes are fully regulated by G-proteins in vivo, and previously ADP-ribosylated G-proteins which retain their intrinsic activity [28], were just replaced by excess amounts of exogenously added non-ribosylated ones.

In this context, the inhibition of PTX-substrate G-protein (such as Gi1) activity is presumably involved in the κ-opioid receptor-mediated inhibition of PLC in guinea pig cerebellar membranes. Indeed, this view was supported by our finding that the κ-agonist inhibits G-protein activity, determined by measuring low-K_m GTPase or GDP-GTP exchange activity due to membrane-bound G-proteins in the guinea pig cerebellum [15,16]. Thus, such a novel mechanism through an inhibition of G-protein activity would be involved in the κ-receptor-mediated inhibition of PLC activity. However, as other components in the Gi1-mediated PLC activation may be also involved, further detailed experiments using purified (or recombinant) proteins should be important subjects in future to prove our proposal.

Another important question is why the κ-agonist almost completely inhibited the GTP-stimulated PLC activities in the membranes in a PTX-sensitive manner. The PTX-insensitive Gq/11/16, are now well known to play important roles in the receptor-mediated PLC activation, and parts of GTP-stimulated PLC activities should have been attributed to Gq/11/16-mediated ones which are activated by GTP per se or through unintentionally activated receptors such as ATP or prostaglandin receptors [30,31] in the present preparations. Such a discrepancy, however, may be explained in part by the fact that the intrinsic activity of Gq is very weak, compared with PTX-sensitive G-proteins, such as Gi and Go [1], and thereby the activation of Gq/11/16 by GTP might be limited in such synaptosomal membranes prepared from the guinea pig cerebellum. Alternatively it might be explained by the possibility that βγ subunits released from Gi or abundant Go activated by GTP per se immediately trap the α-subunits released to a small extent from Gq/11/16 by GTP per se or unintentionally activated receptors. Indeed, Go is not only most abundantly found in the brain among many heterotrimeric G-proteins, but also its intrinsic activity (GTPase or GDP-GTP exchange activity) is the highest [32]. Furthermore Goα has no activity to activate PLC in the *Xenopus* oocyte, since evoked currents through δ-opioid receptor which is coupled to Go in neuroblastoma X glioma hubrid NG108–15 cells [33], were not mediated by overexpression of Goα [11]. All these findings suggest that the G-protein activated by GTP per se and responsible for such a GTP-activated PLC activity is PTX-sensitive G-protein, but not Go in *Xenopus* oocytes. Taking into account the previous findings that the κ-opioid agonist inhibited the GDP-GTP exchange activity of Gi1 and Gi2, but not Go which had been reconstituted into the similar membranes prepared from the guinea pig cerebellum [10], it is very likely such Gi1 and Gi2 might be responsible for the κ-receptor-mediated inhibition of PLC activity. We have no data about the contribution of Gi3 in κ-receptor actions. However, as Gi3 known to be coupled to δ-opioid receptor [34] failed to show the functional coupling to PLC in the *Xenopus* oocyte expressing δ-receptor [11], it is unlikely that Gi3 less abundantly found in the brain [23] plays an imporant role in such κ-receptor-mediated actions.

In the present experiments, we observed the κ-agonist-inhibition of PLC activity stimulated by GTP. As GTP exists in a concentration of 0.1–0.3 mM in the cytosol of various cells [35], such a κ-receptor-mediated effect might be related to the inhibition of spontaneous activities of PLC through G-proteins (Gi). Another possible role is in the cross-talk with metabotropic receptors at the level of G-proteins. There are reports that a κ-opioid agonist and an α_2-adrenoceptor agonist showed a mutual antagonism in regulation of intrasynaptosomal free $[Ca^{2+}]i$ [36] or norepinephrine release [37]. As the κ-agonist inhibited $[Ca^{2+}]i$ and α_2-receptor is well known to couple to Gi in a stimulatory fashion [38], it is plausible that a common Gi-PLC system is involved in this antagonism.

In conclusion, the present study provides the evidence that the inhibitory effect of opioid κ-agonist on the PLC in guinea pig cerebellar membranes is attributed to the mechanisms through an inhibitory coupling between the opioid κ-receptor and PTX-sensitive G-proteins such as Gi1. Such a novel mechanism may be involved in regulation of signaling of many other metabotropic receptors through PTX-sensitive G-proteins.

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