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δ Opioid receptor mediates phospholipase C activation via G_i in *Xenopus* oocytes

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

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

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Cloned mouse δ-subtype opioid receptor (DOR1) was expressed in *Xenopus* oocytes to study the signal transduction. Opioid δ-agonists evoked a calcium-dependent chloride current in oocytes injected with mRNA derived from DOR1, together with that from the α subunit of G_i1. The δ-agonist-induced current was blocked by naltrindol, a δ-specific antagonist. The δ-agonist evoked no or very weak currents in oocytes with the α subunit of G_q or G_o. These findings indicate the functional coupling between the opioid δ-receptor and phospholipase C through an activation of G_i.

δ-Opioid receptor; Pertussis toxin; GTP-binding protein; Signal transduction; Phospholipase C; *Xenopus* oocyte

1. INTRODUCTION

The *Xenopus* oocyte expression system has been widely used for studying signal transduction mechanisms of cloned receptors. In addition to ionotropic receptors [1,2], metabotropic receptors coupled to calcium-dependent chloride channel activation through GTP-binding protein (G-protein) and phospholipase C [3,4] are also good targets for electrophysiological studies. Recent studies reported that most of receptor (G-protein-linked)-mediated phopholipase C activation is mediated by pertussis toxin-insensitive G-proteins such as G_q [5–8]. However, little is known of contribution of pertussis toxin-sensitive G-proteins, such as G_i or G_o to receptor-mediated phospholipase C activation. We have reported that the kyotorphin (a neuropeptide) receptor coupled to G_i1 mediates phospholipase C activation, from reconstitution experiments using receptor in synaptic membranes and purified G-proteins [9]. On the other hand, we have also demonstrated that the stimulation of opioid κ-receptor coupled to inhibition of intrinsic G_i1 or G_i2 activity [10], mediates inhibition of phospholipase C activity in guinea pig cerebellar membranes [11,12]. Thus, it is likely that G_i is positively coupled to stimulation of phospholipase C activity at least in synaptic membranes.

The opioid δ-receptor is well known to inhibit adenylate cyclase activity in neuroblastoma × glioma hybrid NG108-15 cells via the action of G_i [13]. Most recently, the δ-receptor has been cloned from a cDNA library of NG108-15 cells and found to be functional in inhibiting membrane adenylate cyclase activity in COS cells expressing this receptor [14]. Although there is a preliminary report that the opioid δ-receptor is possibly coupled to intracellular Ca^{2+} mobilization, G-protein involvement in such a phospholipase C activation remains to be clarified [15]. Here we report the opioid δ-receptor-mediated phospholipase C activation through G_i, using the oocyte expression system combined with G-protein 'reconstitution' methods.

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

2. MATERIALS AND METHODS

2.1. *Materials*

Opioid agonists used were [D-Ser^2,D-Leu^5]enkephalin-Thr (DSLET), [D-Pen^{2,5}]enkephalin (DPDPE), [D-Ala^2,MePhe^4,Gly^{5}ol]-enkephalin (DAMGO) from BACHEM (Bubendorf, Switzerland) and U-69593 (Upjohn, Japan). The opioid antagonist, naltrindol (NTI) was a gift from Dr. Nagase (Toray, Japan). Inositol 1,4,5- triphosphate (IP_3) was purchased from Sigma (St. Louis, USA). cDNA clones used were mouse δ opioid receptor, DOR1 [14] from Dr. C. Evans (UCLA, USA), α subunits of rat G_i1 and G_o ($G_i1\alpha$ and $G_o\alpha$, respectively) [16] from Dr. H. Ito (Tokyo Institute of Technology, Yokohama, Japan), rat $G_q\alpha$ [17] from M. Simon (California Institute of Technology, Pasadena, USA).

2.2. *Electrophysiological recordings in* Xenopus *oocytes*

Xenopus laevis were anaesthetised in ice–water and a lobe of ovary was removed after a small incision was made in the ventral abdominal surface. Oocytes (stages 5 and 6, see reference [18]) were defolliculated at room temperature by a 3 h treatment with collagenase (2 mg/ml) in Ca^{2+}-free modified Barth's solution (MBS). MBS contains NaCl 88 (mM), KCl 1, $NaHCO_3$ 2.38, $MgSO_4$ 0.82, $CaCl_2$ 0.41, $Ca(NO_3)_2$ 0.33, Tris-HCl 7.5, pH 7.5. Oocytes were then washed and incubated at a constant temperature of 19°C in MBS containing streptomycin (0.1 mg/ml) and penicillin (100 U/ml). After 24 h incubation, oocytes were microinjected at room temperature (24°C) with 70 nl of mRNAs generated by in vitro transcription primed with cap dinucleotide $m^7G(5')ppp(5')G$ using a Stratagene kit from mouse δ-opioid receptor (DOR1, 10 ng) and from rat $G_i1\alpha$, $G_o\alpha$ or $G_q\alpha$ (each 50 ng). After a further 2 days incubation at 19°C to allow for protein expression,

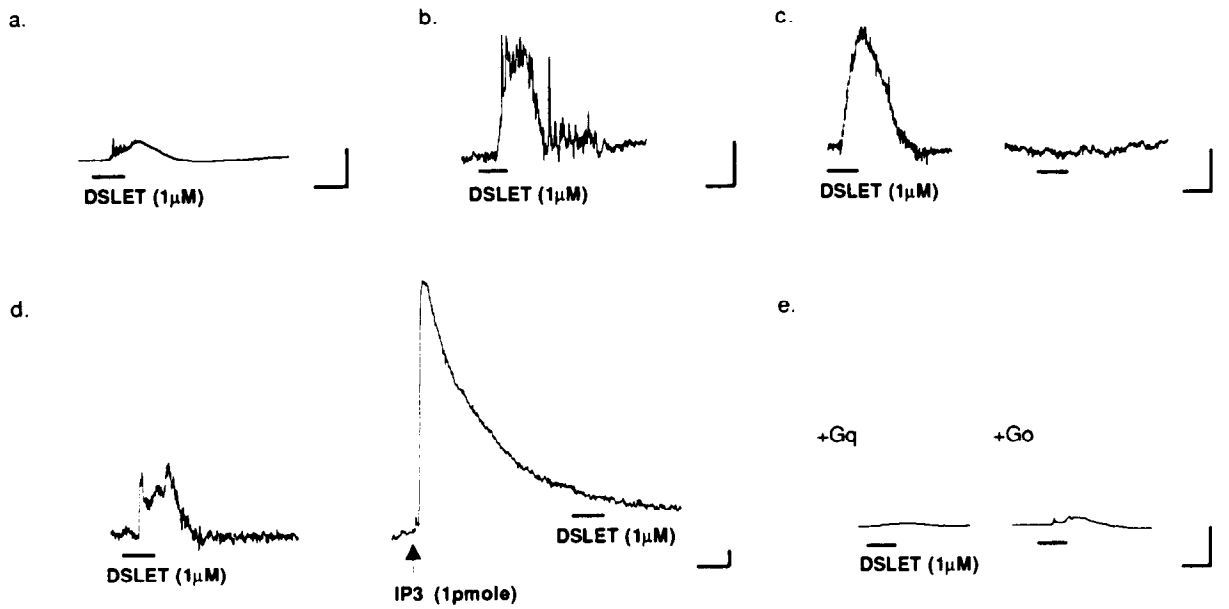

Fig. 1. Typical current responses on the δ opioid agonist, DSLET in DOR1 mRNA-injected oocytes at a holding potential of 0 mV. Panel a and b: DSLET (1 μM)-evoked outward current in oocytes co-injected without and with $G_i1\alpha$ mRNA, respectively. Panel c and d: effects of intracellularly injected EGTA and IP_3 on the DSLET-evoked current in oocytes co-injected with $G_i1\alpha$ mRNA, respectively. Left recordings in panel c and d: control DSLET-responses 15 min before intracellular injection, respectively. Right recordings in panel c and d: DSLET-responses 5 min after injection of 100 pmol EGTA or 1 pmol IP_3, respectively. Panel e: DSLET-responses in oocytes co-injected with $G_q\alpha$ (left panel) or with $G_o\alpha$ (right panel), respectively. The vertical and horizontal bar represent 20 nA and 1 min, respectively.

responses to bath application of opioid agonists were detected in injected *Xenopus* oocytes using a voltage-clamp recording. Unless otherwise stated, the holding potential was at 0 mV to get maximal response. Electrophysiological recordings were made using a conventional two-electrode voltage-clamp technique with both microelectrodes filled with 3 M potassium chloride (resistance 0.5–5 MΩ). Oocytes were placed in a 0.1 ml chamber and continuously superfused (flow rate: 3–5 ml/min) with MBS. Electrophysiological recordings were performed at room temperature and only oocytes with an input resistance of 1–5 MΩ were used. The current–voltage relationships were obtained using the 'ramp clamp' technique as described previously [19,20].

3. RESULTS

Bath application of [D-Ser^2,D-Leu^5]enkephalin-Thr/DSLET, a selective opioid δ-agonist (δ2 subtype) at 1 μM to oocytes injected with DOR1 mRNA showed weak outward currents (mean ± S.E.M. = 15.9 ± 5.6 nA) at a holding potential of 0 mV in 5 preparations (Fig. 1a). There was no reproductive response on the second challenge of the δ-agonist even at 10 μM (data not shown).

When oocytes were injected with $G_i1\alpha$ mRNA together with DOR1, the δ-agonist-responses were potentiated 2- to 4-fold (62.5 ± 21.1 nA), as shown in Fig. 1b. The outward current was oscillating and lasted for 2–5 min. The evoked current was blocked by intracellular injection of 100 pmol EGTA, a selective calcium-ion chelating agent (Fig. 1c), or by direct injection of 1 pmol of IP_3 5 min prior to the opioid agonist challenge (Fig.

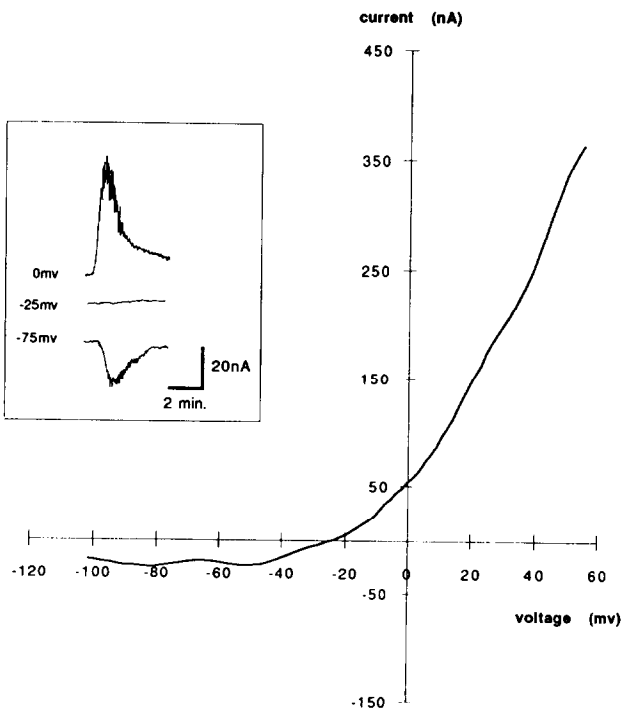

Fig. 2. Current–voltage relationship for the DSLET-evoked current. The data were obtained in oocytes with mRNAs of DOR1 and $G_i1\alpha$, using the voltage ramp (300 mV/s) clamp method [19,20]. The reversal potential (−25 mV) is the voltage where zero current flows through the membrane. The inset shows the traces of DSLET-evoked currents at a holding potential of 0, −25 and −75 mV, respectively.

1d). The current was outwardly rectifying and the reversal potential was approximately −25 mV (Fig. 2), suggesting the involvement of chloride channel opening. All these characteristics are typical to metabotropic receptor-mediated response through phospholipase C, but the current was much weaker, compared to the responses involving G_q (or pertussis toxin-insensitive G-protein)-phospholipase C activation, such as mGR1 metabotropic glutamate receptor responses (approximately 1 μA), which have been preliminarily reported [21] and confirmed by ourselves (unpublished data). However, when mRNA of $G_q\alpha$ or $G_o\alpha$ was injected into oocytes together with DOR1 mRNA, there was no detectable response ($n = 5$, sensitivity limit of approximately 2 nA) or 10.3 ± 3.5 nA ($n = 5$) to the δ-agonist at 1 μM in 5 preparations, respectively (Fig. 1e). There was no significant difference between currents with G_o and without any G-protein mRNA.

As shown in Fig. 3a, the δ-agonist response was completely blocked by naltrindol (NTI), a selective δ-opioid antagonist [22], and then recovered to the initial level by the agonist after the wash of the antagonist. Similarly we tested various opioid agonists, compared to DSLET-response. As shown in Fig. 3b, DPDPE, another specific δ-agonist (δ1-subtype, see reference [22]) evoked an equipotent outward current, while there were no responses with 1 μM DAMGO, a specific μ-opioid agonist [23] or with 1 μM U69593, a specific κ-opioid agonist [24].

4. DISCUSSION

It has been long since the possible involvement of pertussis toxin-sensitive G-proteins in phospholipase C activation was claimed [25]. Actually there are some reports using reconstitution experiments providing evidence that G_i is coupled to phospholipase C activation [9,11,26]. In the last several years, however, most accumulating findings are about the involvement of G_q (or pertussis toxin-insensitive G-protein) in this mechanism [8]. This might originate from the fact that intrinsic activity of G_q in phospholipase C activation is much higher than G_i, as mentioned above. Of interest is the possibility that G_i and G_q mutually interfere with their functional coupling to phospholipase C. A weak δ-agonist response in oocytes without G-protein mRNA injection was abolished by G_q mRNA injection (Fig. 1e). As endogenous G_i-like G-proteins themselves might be partially activated (or G-proteins have their intrinsic activity without receptor stimulation, possibly through an activation by endogenous GTP) in the cell, overexpressed G_q might inhibit the functional coupling of G_i

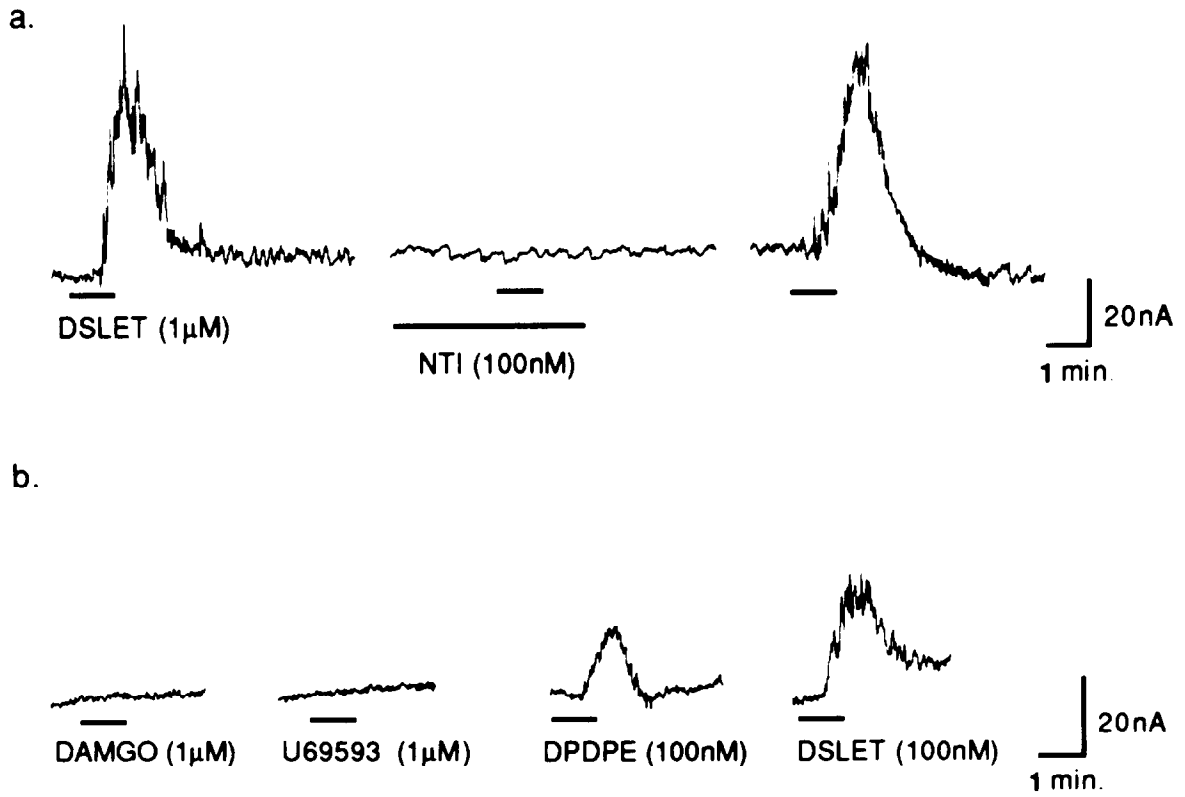

Fig. 3. Effects of various opioid agonists and antagonist in oocytes injected with mRNAs of DOR1 and $G_i1\alpha$ at a holding potential of 0 mV. Agonist applications were performed every 20 min. Panel a: antagonism of DSLET (1 μM)-evoked outward current by 100 nM naltrindol, a δ-opioid antagonist in the same oocyte. Panel b: no detectable effect by 1 μM DAMGO (μ-agonist) or 1 μM U69593 (κ-agonist) and significant outward currents by 100 nM DPDPE (δ1-agonist) or 100 nM DSLET (δ2-agonist).

to phospholipase C, as a competitor for this enzyme. In this respect, it is likely that G_o is not a good competitor for this enzyme, since there was no significant change in δ-agonist response by its mRNA injection (Fig. 1e).

It has been accepted that the opioid δ-receptor mediates the closing of calcium channels in NG108-15 cells through the action of G_o rather than G_i [27]. However, such a weak calcium channel activity is not detected in the present system without major changes of the system, such as mRNA (related calcium channel) injection, ion-balancing in the superfusion solution and addition of some agents to suppress major other ion channel activities. Therefore, from using the present system, it is evident that G_o is not involved in δ-agonist response. In addition, it is unlikely that G_o has much higher intrinsic activity on phospholipase C in the oocyte than G_i. In the previous paper by Moriaty et al. [28], the direct injection of purified G_o potentiated muscarinic acetylcholine response to endogenously expressed muscarinic receptors (it has been characterized to be coupled to G_q or pertussis toxin-insensitive G-proteins) in the oocyte. As G_q had not yet been characterized to be coupled to phospholipase C at that time, the contamination of G_q or related G-proteins in the G_o-preparation might not be excluded, although they proved it to be devoid of G_i. However, it remains to be clarified whether or not, if any, how much G_o is involved in mediating phospholipase C activation.

The major findings in this report are as follows: (1) the opioid δ-receptor activates phospholipase C through the action of G_i1, and it does inhibit adenylate cyclase in the same manner; (2) G_i1 is also involved in phospholipase C activation, but the intrinsic activity to activate this enzyme is less potent, compared to G_q; (3) the contribution of G_o to the activation of phospholipase C remains unclear, but this G-protein is not involved in the opioid δ-receptor-mediated activation of phospholipase C. The direct evidence of δ-receptor coupling to phospholipase C activation through G_i, by G_i-reconstitution experiments in mammalian cell membranes expressing DOR1, are in progress in our laboratory.

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