

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

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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_i . 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 phospholipase 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_i 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_i 1 or G_i 2 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 \times 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 ex-

pressing 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.

2. MATERIALS AND METHODS

2.1. Materials

Opioid agonists used were [D-Ser²,D-Leu⁵]enkephalin-Thr (DSLET), [D-Pen^{2,5}]enkephalin (DPDPE), [D-Ala²,MePhe⁴,Gly⁵]-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-trisphosphate (IP₃) 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_i 1 and G_o (G_i 1 α and G_o α , respectively) [16] from Dr. H. Ito (Tokyo Institute of Technology, Yokohama, Japan), rat G_q α [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, NaHCO₃ 2.38, MgSO₄ 0.82, CaCl₂ 0.41, Ca(NO₃)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 m⁷G(5')ppp(5')G using a Stratagene kit from mouse δ -opioid receptor (DOR1, 10 ng) and from rat G_i 1 α , G_o α or G_q α (each 50 ng). After a further 2 days incubation at 19°C to allow for protein expression,

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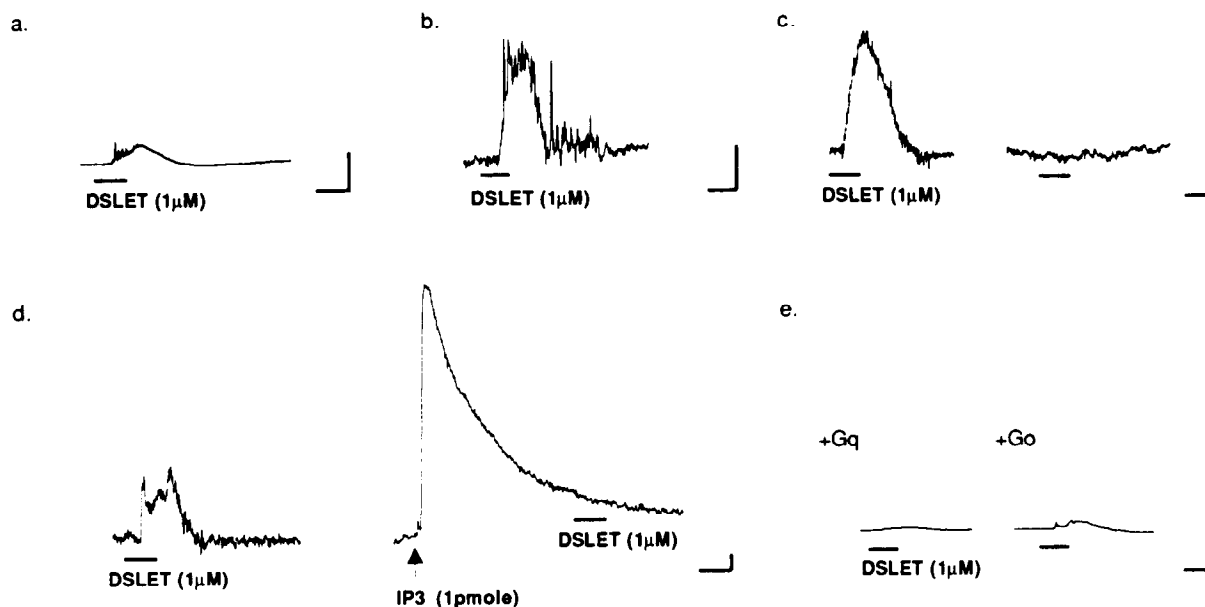


Fig. 1. Typical current responses on the δ opioid agonist, DSLET in DOR1 mRNA-injected oocytes at a holding potential of 0 mV. Panel a and b: DSLET (1 μ M)-evoked outward current in oocytes co-injected without and with $G_{i1\alpha}$ mRNA, respectively. Panel c and d: effects of intracellularly injected 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 $M\Omega$). 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 $M\Omega$ were used. The current-voltage relationships were obtained using the 'ramp clamp' technique as described previously [19,20].

3. RESULTS

Bath application of [D-Ser²,D-Leu⁵]enkephalin-Thr/DSLET, a selective opioid δ -agonist (δ_2 subtype) at 1 μ M to oocytes injected with DOR1 mRNA showed weak outward currents (mean \pm 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 μ M (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.

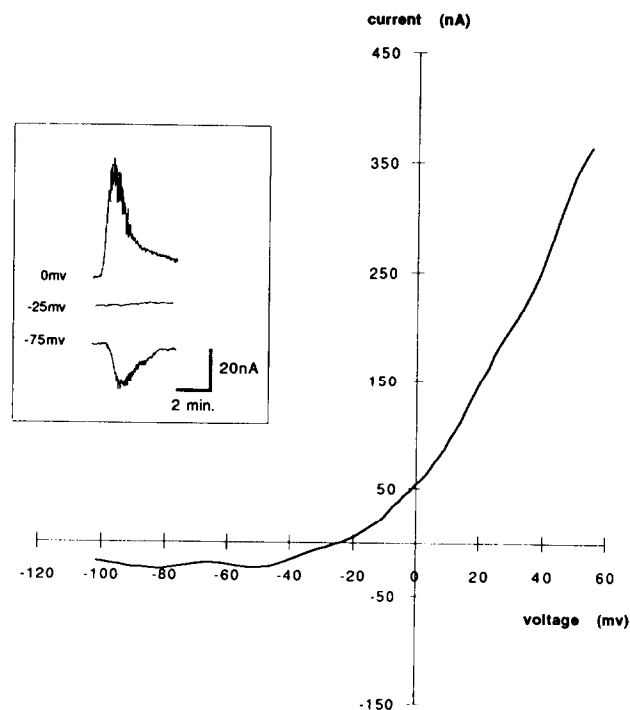


Fig. 2. Current-voltage relationship for the DSLET-evoked current. The data were obtained in oocytes with mRNAs of DOR1 and $G_{i1\alpha}$, using the voltage ramp (300 mV/s) clamp method [19,20]. The reversal potential (–25 mV) is the voltage where zero current flows through the membrane. The inset shows 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 mGRI metabotropic glutamate receptor responses (approximately 1 μ A), 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 μ M 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 ($\delta 1$ -subtype, see reference [22]) evoked an equipotent outward current, while there were no re-

sponses with 1 μ M DAMGO, a specific μ -opioid agonist [23] or with 1 μ M 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 .

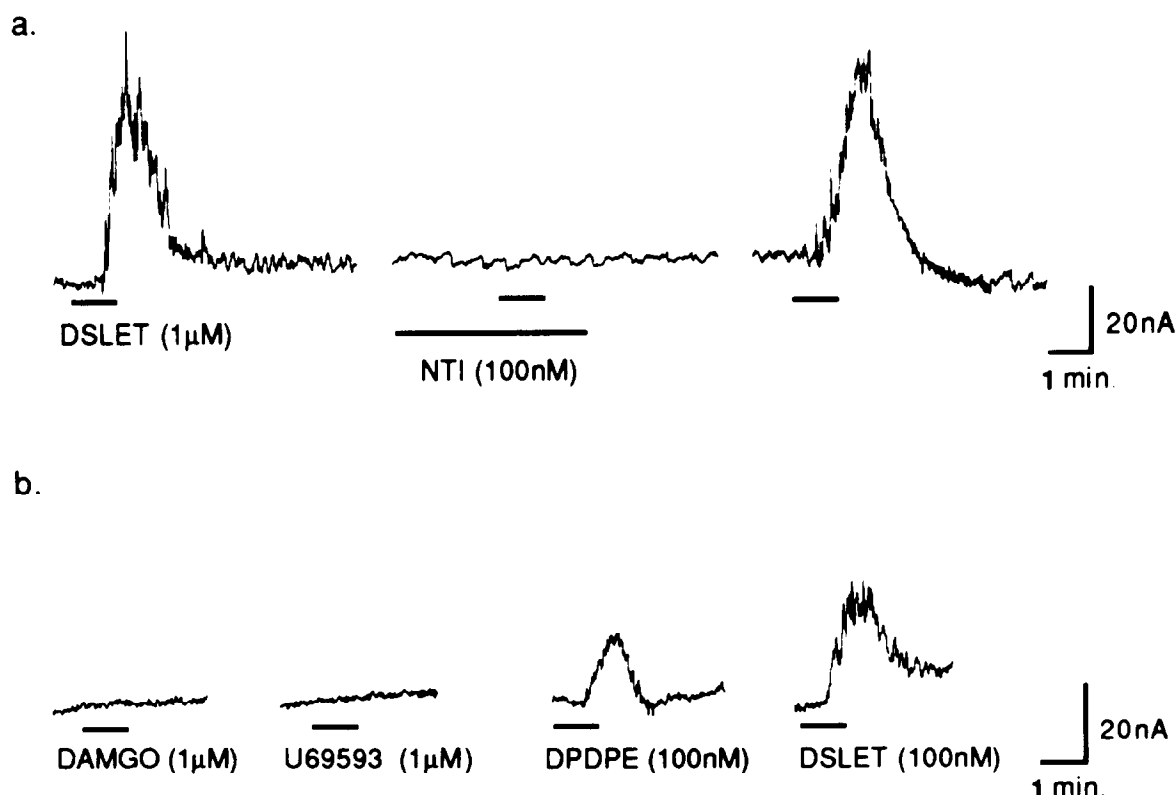


Fig. 3. Effects of various opioid agonists and antagonist in oocytes injected with mRNAs of DOR1 and $G_{i1}\alpha$ at a holding potential of 0 mV. Agonist applications were performed every 20 min. Panel a: antagonism of DSLET (1 μ M)-evoked outward current by 100 nM naltrindol, a δ -opioid antagonist in the same oocyte. Panel b: no detectable effect by 1 μ M DAMGO (μ -agonist) or 1 μ M U69593 (κ -agonist) and significant outward currents by 100 nM DPDPE ($\delta 1$ -agonist) or 100 nM DSLET ($\delta 2$ -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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