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# Protein Kinase C Involvement in Homologous Desensitization of δ-Opioid Receptor Coupled to $G_{i1}$-Phospholipase C Activation in *Xenopus* Oocytes

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**We have developed the coexpression system of both δ-opioid receptor (DOR1) and $M_2$-muscarinic receptor ($M_2$) which mediate agonist-evoked currents due to common post-receptor mechanisms including $G_{i1}$ and phospholipase C (PLC) activation in *Xenopus* oocytes reconstituted with $G_{i1}\alpha$. The DOR1-currents by 100 nM D-Ser²-leu-enkephalin-Thr⁶ (DSLET) were selectively desensitized by 10 nM phorbol 12-myristate 13-acetate (PMA). The PMA-desensitization of DSLET-currents was abolished in the presence of calphostin C, a protein kinase C inhibitor, or reversed by an intracellular injection of calcineurin, a protein phosphatase 2B. When a higher concentration (3 μM) of DSLET was used, DSLET-currents were rapidly desensitized by repeated challenges of DSLET itself. However, repeated challenges of 10 μM ACh caused no influence on such DSLET- or $M_2$-currents. The desensitization of DSLET-currents was selectively reversed by protein kinase C inhibitors. Similar results were also obtained with various δ-opioid agonists. These resutls suggest that protein kinase C is involved in the homologous desensitization of δ-opioid receptors.**

***[Key words: δ-opioid receptor, $G_{i1}$, phospholipase c, signal transduction, homologous desensitization, protein kinase C,* Xenopus *oocyte]***

δ-Opioid receptor in neuroblastoma × glioma hybrid NG108-15 cells is known to be desensitized by chronic treatments of the agonist through mechanisms via an action of pertussis toxin–sensitive GTP-binding protein (G-protein), $G_i$ (see review, Childers, 1993). Recently, mouse δ-receptor has been cloned from a cDNA library of NG108-15 cells (Evans et al., 1992; Kieffer et al., 1992) and found to be functional in inhibiting membrane adenylate cyclase activity in COS cells expressing this receptor (Evans et al., 1992). As the protein sequence of the receptor was revealed to have several phosphorylation sites for "effector kinases" such as cAMP-dependent kinase (A-kinase) and protein kinase C, in the third intracellular loop and the intracellular region proximal to the seventh transmembrane region (Evans et al., 1992; Kieffer et al., 1992), it is plausible that the phosphorylation of the receptor is one of the desensitization mechanisms, as seen with the case of β-adrenoceptor desensitization (Lefkowitz et al., 1990). However, the phosphorylation by A-kinase is unlikely the mechanism, since the δ-receptor mediates the inhibition of cAMP formation (Evans et al., 1992; Childers et al., 1993). Alternatively, the protein kinase C might be a target for the mechanisms.

Recent studies reported that most of receptor (G-protein-linked)-mediated phospholipase C (PLC) activation is mediated by pertussis toxin (PTX)-insensitive G-proteins such as $G_q$ (Smrcka et al., 1991; Taylor, 1991; Berstein et al., 1992; Berridge, 1993). However, little is known of contribution of PTX-sensitive G-proteins (such as $G_i$ or $G_o$) to receptor-mediated PLC activation. We have reported that the kyotorphin (a neuropeptide) receptor coupled to $G_{i1}$ mediates PLC activation, from reconstitution experiments using receptor in synaptic membranes and purified G-proteins (Ueda et al., 1989). On the other hand, we have also demonstrated that the stimulation of a subtype of κ-opioid receptor which inhibits intrinsic $G_{i1}$ or $G_{i2}$ activity (Ueda et al., 1990), mediates inhibition of PLC activity in guinea pig cerebellar membranes (Misawa et al., 1995). Thus, it is likely that $G_{i1}$ is positively coupled to stimulation of PLC activity at least in synaptic membranes. *Xenopus* oocyte expression system has been widely used for studying signal transduction mechanisms of cloned receptors, measuring calcium-dependent chloride conductance that can readily be used as an indicator of intracellular calcium concentration related to PLC activity (Masu et al., 1991). Using this system, we have reported that the δ-receptor possibly mediates phospholipase C (PLC) activation via $G_{i1}$, but not $G_q$ in *Xenopus* oocytes (Miyamae et al., 1993). Activation of this enzyme leads to an increase in the phosphatidylinositol 4,5-bisphosphate ($PIP_2$) hydrolysis, followed by the generation of two intracellular second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (Berridge, 1993). These second messengers are linked to $Ca^{2+}$ release from intracellular stores (Berridge, 1993) and to protein phosphorylation by activation of protein kinase C (Nishizuka, 1992), respectively. Most recently we reported that the δ-opioid receptor responses were enhanced by the pretreatment with K-252a, a potent inhibitor for protein kinases (Ueda et al., 1994), this suggesting that either δ-opioid receptor or following signaling components are possibly desensitized by protein phosphorylation.

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Here we report the "homologous" desensitization of δ-opioid receptor functions and the involvement of protein kinase C in such mechanisms in *Xenopus* oocytes.

## Materials and Methods

*Materials.* cDNA clones used here were mouse δ-opioid receptor/DOR1 (Evans et al., 1992), porcine muscarinic $M_2$ subtype acetylcholine receptor/HM45 (Fukuda et al., 1987) and G-protein α-subunits, such as rat $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_o\alpha$ (Itoh et al., 1986), mouse $G_q\alpha$ (Strathmann and Simon, 1990) and rat $G_{i1}\alpha/G_{i4}\alpha$ and $G_{i2}\alpha/G_{i1}\alpha$ (Nakamura et al., 1991). Other bioactive reagents were D-Ser$^2$-leu-enkephalin-Thr$^6$ (DSLET), D-Pen$^{2,5}$-enkephalin (DPDPE), selective $\delta_2$- and $\delta_1$-opioid agonist, respectively (Traynor and Elliott, 1993), from BACHEM (Bubendorf, Switzerland), D-Ala$^2$,D-Leu$^5$-enkephalin (DADLE) from Peptide Institute, Inc. (Osaka), inositol 1,4,5-trisphosphate ($InsP_3$), U-73122, U-73343, thapsigargin, and pertussis toxin A-protomer from Funakoshi (Japan), calphostin C and KT5720 from Kyowa Medex Co. Ltd. (Tokyo), protein kinase C inhibitor peptide (19-31), calcineurin/phosphatase 2B from Upstate Biotechnology Inc. (Lake Placid, New York), calmodulin kinase II inhibitor peptide from RBI (Natick, MA) and phorbol 12-myristate 13-acetate (PMA) from Sigma. Dihydroetorphine (DHE) was newly synthesized. Thapsigargin, U-73122 and U-73343 were dissolved in dimethylsulfoxide (DMSO) in a concentration of 1 mM as stock solutions.

Xenopus *oocyte treatments. Xenopus laevis* weighing 60–80 gm was anesthetized in ice-water and a lobe of ovary was removed after a small incision was made in the ventral abdominal surface. Oocytes at stage V and VI (Dumon et al., 1972) were defolliculated with 2 mg/ml of collagenase (Sigma, type I) at room temperature for 3 hr and then incubated in a constant temperature of 19°C in calcium-free Modified Barth's Solution (MBS). Normal 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, as previously reported (Miyamae et al., 1993). 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 hr incubation, oocytes were microinjected at room temperature (25°C) with 50–70 nl of RNAs.

*RNA preparation for oocyte injection.* RNAs of DOR1, $M_2$ receptor and G-protein α-subunits 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. Generated RNAs were then treated with RNase-free DNase and purified according to a protocol of the kit. Finally, purified pellet was dissoved into diethyl pyrocarbonate (DEPC)-treated pure water. Some aliquots were used for determination of RNA concentrations, and others were used for quality-check by 1% agarose gel electrophoresis and ethidium bromide staining. For the injection into oocytes, 10 ng of receptor RNA and 50 ng of G-protein α-subunit RNA were used.

*Preactivation of A-protomer of pertussis toxin.* A-Protomer of pertussis toxin (PTX, 10 μg) was dissolved in 20 μl of 5 mM HEPES (pH 7.5) containing 5 mM DTT and 3 mM ATP, and incubated for 20 min at 37°C. The activated PTX A-protomer was diluted in 100 mM NAD to a concentration of 1 ng/10 nl and used for intracellular injection (20 nl). Vehicle-control without PTX was prepared, as above-mentioned.

*ADP-ribosylation and immunoprecipitation with $G_{i1}\alpha$ antiserum.* Oocytes (10 eggs) which had been injected with $G_{i1}\alpha$ RNA (50 ng) and incubated for 3 d at 19°C were sonicated in 200 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 1000 × *g* for 10 min at 4°C. The supernatant membrane preparations were subjected to PTX-catalyzed ADP-ribosylation by the method of Katada and Ui (1982). 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 (Laemmli, 1970). For immunoprecipitation, commercially available $G_{i1}\alpha$ antiserum (Du Pont-NEN, AS/7), which is specific for $G_i\alpha$, $G_{i1}\alpha$, and $G_{i2}\alpha$, 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 3000 × *g* for 3 min, and the supernatant was mixed with 5 μl of $G_{i1}\alpha$ 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 above-mentioned. 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 above-mentioned. Autoradiography was achieved by exposure of dried polyacrylamide gel to Hyperfilm MP (Amersham) at −80°C with Du Pont Cronex Lightning Plus intensifying screens. SeeBlue (Novex) stained markers were used as molecular standards.

*Electrophysiological recording.* Electrophysiological experiments using *Xenopus* oocyte expression system are essentially as previously reported (Miyamae et al., 1993). After further 2 d 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. Unless otherwise stated, the experiments were carried out at the holding potential of 0 mV. Electrophysiological recordings were made using a conventional two-electrode voltage-clamp technique (CEZ-1200, Nihon Kohden) with both microelectrodes filled with 3 M potassium chloride (resistance 0.5–5 MΩ). Oocytes were placed in 0.1 ml of 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 and with a resting potential of 30–60 mV were used. Current–voltage curve and reversal potential were determined by "Ramp Clamp" method, as reported previously (Harada et al., 1987). Intracellular injection was performed in a volume of 10 nl of distilled water, unless otherwise stated. In standard experiments, bath-applications of test drugs were performed for 1 min every 15 min, unless otherwise stated. In some experiments the chloride ion concentration in the perfusion solution was reduced to 22.5 or 30 mM, compensating the ion balance with sodium isethionate, as reported previously (Harada et al., 1987). In some other experiments we used the perfusion solution substituted of $Na^+$ and $K^+$ with tetramethylammonium.

## Results

### *DSLET-evoked currents in the oocyte injected with RNAs of DOR1 and various G-protein α-subunits*

Previously we have reported that DSLET, a selective δ-agonist evoked weak outward currents, possibly through PLC in the *Xenopus* oocyte injected with DOR1 RNA (Miyamae et al., 1993; Ueda et al., 1994) and such currents were enhanced by coinjection with RNA of $G_{i1}\alpha$, but not of $G_o\alpha$ or $G_q\alpha$. Figure 1 shows the comparison of evoked currents upon initial challenge of 100 nM DSLET in the oocyte coinjected with various G-protein α-subunit RNAs. Significant increase in the amplitude of evoked currents was observed only with $G_{i1}\alpha$ or $G_{i2}\alpha$ RNA. On the other hand, the coinjection with RNA of pertussis toxin (PTX)-insensitive G-protein α-subunit such as $G_q\alpha$, $G_{i1}\alpha$ ($G_{i2}\alpha$), or $G_{i4}\alpha$ ($G_{i3}\alpha$) significantly decreased the evoked currents, possibly through mechanisms that exogenous G-protein α-subunits could absorb endogenous βγ-subunits and inhibit the DSLET-currents through decreasing the population of heterotrimeric complex of $G_{i1}$ (or $G_{i2}$)α- and βγ-subunits. In the case with oocytes injected with $G_{i3}\alpha$ or $G_o\alpha$ RNA, there was no significant decrease in DSLET-currents. Taken into consideration the report

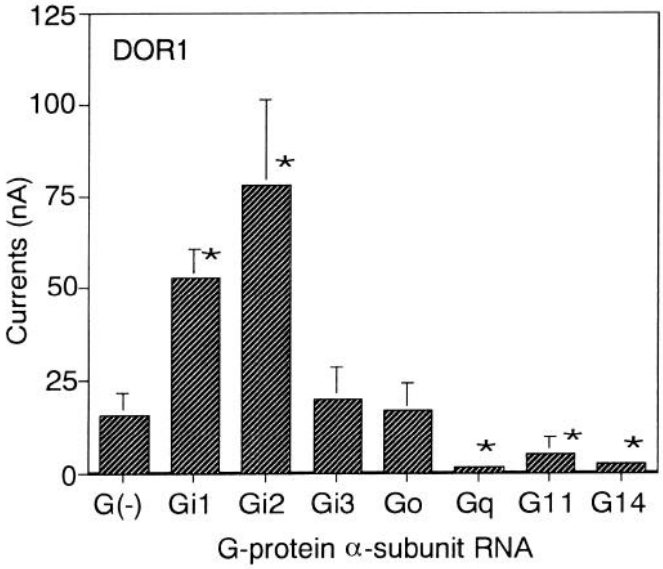


*Figure 1.* Effects of coinjection of various G-protein α-subunit RNAs on DSLET-currents in *Xenopus* oocytes. Results represent the currents (nA) evoked by first challenge of 100 nM DSLET for 1 min in oocytes injected with 10 ng DOR1 RNA and each 50 ng G-protein α-subunit RNA. Other details are described under Materials and Methods. The data are the mean ± SEM from 3–20 separate experiments. *, $p < 0.05$, compared with G(−) (Student's *t* test).

that there are some or significant coupling between δ-receptors and $G_{i3}$ or $G_o$, respectively (Hescheler et al., 1987; Laugwitz et al., 1993), it is suggested that exogenous $G_{i3}$ or $G_o$ to be activated through δ-receptors has a comparable potency in activating PLC with those of endogenous G-proteins to be activated through δ-receptors in *Xenopus* oocytes.

*Constant currents evoked by the lower concentration of δ-agonist in oocytes injected with $G_{i1}\alpha$ RNA*

When 100 nM DSLET was bath-applied to the oocyte not coinjected with any G-protein α-subunit RNA, the responses were gradually attenuated upon repeated challenges of the agonist (Fig. 2*A*). As we detected bigger currents (~1 μA) mediated by muscarinic $M_1$ receptor in oocytes injected with $M_1$ and DOR1 RNAs, coupled to $G_q$-phospholipase C mechanisms (Berridge, 1993), even after such a "tachyphylaxis" of DSLET-currents in the oocyte not coinjected with $G_{i1}\alpha$ RNA (data not shown), it is unlikely that the tachyphylaxis is due to the "run-down" mechanism of ion channels. In the oocyte coinjected with $G_{i1}\alpha$ RNA, on the other hand, the δ-agonist-evoked currents were enhanced, as also seen in Figure 1, and were constant upon at least six repeated challenges (Fig. 2*B*). Table 1 shows the comparison of evoked-currents upon the first and third challenges of DSLET in either oocytes coinjected with or without $G_{i1}\alpha$ RNA, respectively. The ratio of "Third/First" in oocytes injected with $G_{i1}\alpha$ RNA (105.4 ± 20.6%, $n = 5$) was markedly bigger than that without RNA (14.7 ± 12.3%, $n = 4$). Thus, it is evident that DSLET-currents upon the third challenge is due to $G_{i1}\alpha$-mediated mechanisms from this comparison. The minimal amount of exogenous $G_{i1}\alpha$ RNA to show constant δ-agonist-responses was 1 ng. Similar results were obtained with $G_{i2}\alpha$ RNA instead of $G_{i1}\alpha$ RNA, while not with any other G-protein α-subunit RNAs (data not shown). To confirm the expression of exogenous $G_{i1}\alpha$, we performed ADP-ribosylation of membrane G-proteins, followed by immunoprecipitation. Exogenously expressed $G_{i1}\alpha$ protein was detected after immunoprecipitation following ADP-ribosylation by pertussis toxin, only in the oocytes injected with $G_{i1}\alpha$ RNA, but not in those without RNA, as shown in Figure 3. In experiments using oocytes not injected with $G_{i1}\alpha$ RNA, there were significant amounts of ADP-ribosylated materials migrated at 39 kDa, which remain to be identified, but apparently differ from $G_{i1}\alpha$ (41 kDa) or $G_{i2}\alpha$ (40 kDa), since no significant

**Table 1. Comparison of DSLET currents upon the first and third challenge in oocytes injected with DOR1 RNA or with DOR1 and $G_{i1}\alpha$ RNAs**

| | First (nA) | Third (nA) | Third/First (%) |
|---|---|---|---|
| Gα (−) | 22.5 ± 4.3 ($n = 4$) | 3.3 ± 2.4 ($n = 4$) | 14.7 ± 12.3 ($n = 4$) |
| $G_{i1}\alpha$ | 32.4 ± 5.4 ($n = 5$) | 31.6 ± 4.4 ($n = 5$) | 105.4 ± 20.6* ($n = 5$) |

Results represent evoked currents upon the first and third challenge of 100 nM DSLET and their ratios (Third/First) in oocytes injected with DOR1 RNA alone/Gα (−) or with DOR1 and $G_{i1}\alpha$ RNAs/$G_{i1}\alpha$, respectively.
* $p < 0.05$, compared with Gα (−) (ANOVA).

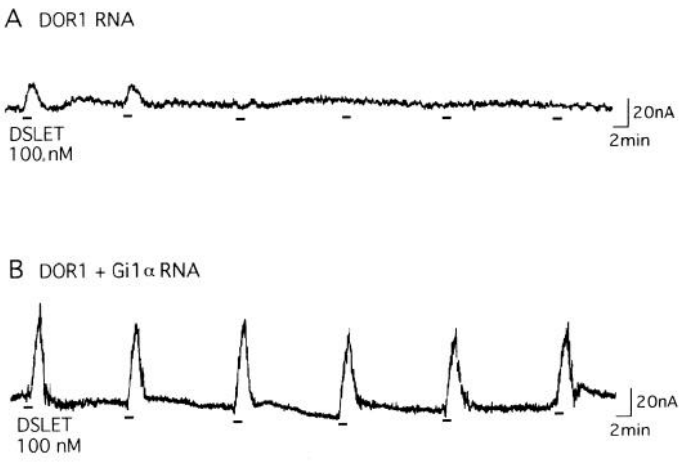


*Figure 2.* Currents evoked upon repeated challenges of DSLET in the oocytes coinjected with $G_{i1}\alpha$ RNA. Results represent evoked currents upon repeated challenges of 100 nM DSLET. DSLET was bath-applied for 1 min periodically every 15 min, as indicated by *underline* in the figure. *A* and *B*, DSLET-evoked currents in the oocyte injected with DOR1 RNA alone (*A*), and with DOR1 and $G_{i1}\alpha$ RNAs (*B*). The *vertical* and *horizontal bar* represent 20 nA and 2 min, respectively.

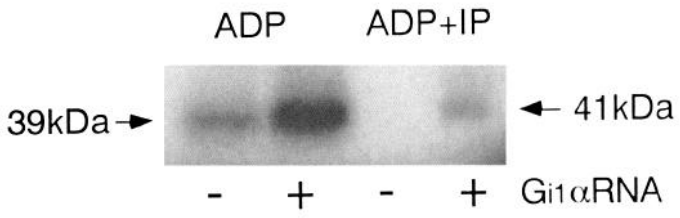


*Figure 3.* ADP-ribosylation and immunoprecipitation with $G_{i1}\alpha$ antiserum. The membrane preparations of oocytes injected with (+) or without $G_{i1}\alpha$ RNA (−) were $^{32}$P-ADP-ribosylated (*left two lanes*) and further immunoprecipitated with $G_{i1}\alpha$ antiserum (*right two lanes*), as described under Materials and Methods. Results represent the autoradiogram of each sample. Substantially same results were reproduced.

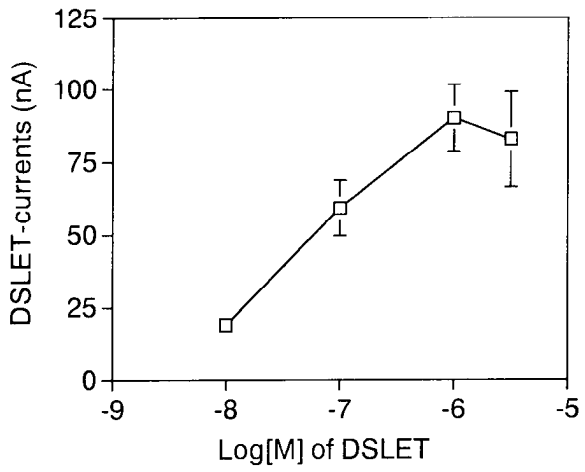


*Figure 4.* Concentration-dependent currents evoked by DSLET in oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs. Results represent the maximal amplitude (nA) of evoked currents. Data are the mean ± SEM of four separate experiments.

activities were observed after immunoprecipitation using antiserum recognizing $G_{i1}\alpha$ or $G_{i2}\alpha$ (Fig. 3).

*Characterization of δ-agonist-evoked currents*

In oocytes coinjected with DOR1 and $G_{i1}\alpha$ RNAs, DSLET evoked currents in a concentration-dependent manner (Fig. 4). The $EC_{50}$ was 32.8 ± 4.2 nM ($n$ = 5). The concentration of DSLET to show maximal responses was 1 μM. Here we characterized the DSLET (300 nM)-evoked currents by pharmacological and electrophysiological approaches. The constant DSLET-currents in oocytes coinjected with $G_{i1}\alpha$ RNA were markedly reduced when 20 nl (2 ng) of preactivated pertussis toxin A-protomer with NAD was intracellularly injected 30 min prior to the following agonist challenges (Fig. 5*A*). Such a marked reduction in DSLET-currents was observed at the time as early as 15 min after the PTX-injection and lasted for more than 1 hr in separate experiments (data not shown). However, the vehicle (NAD)-treatment had no significant effect on such currents upon following challenges of DSLET (Fig. 5*B*).

Previously we have reported that the DSLET-currents were abolished by an intracellular injection of 100 pmol of EGTA, a calcium chelating agent, or of 1 pmol of $InsP_3$ in oocytes coinjected with $G_{i1}\alpha$ RNA (Miyamae et al., 1993; Ueda et al., 1994). The involvement of $Ca^{2+}$ mobilization mechanisms in DSLET-currents was confirmed by experiments using thapsigargin, which is a tumor-promoting sesquiterpene lactone and discharges intracellular $Ca^{2+}$ in rat hepatocytes, as it does in many vertebrate cell types. This compound inhibits the endoplasmic reticulum isoform of the $Ca^{2+}$-ATPase ($Ca^{2+}$-pump), while it has little or no effect on the $Ca^{2+}$-ATPase of hepatocyte or erythrocyte plasma membranes or of cardiac or skeletal muscle sarcoplasmic reticulum (Thastrup et al., 1990). As a result, thapsigargin causes a depletion of $Ca^{2+}$ in intracellular $Ca^{2+}$ stores including $InsP_3$-sensitive ones (Lupu-Meiri et al., 1993). When 10 nM thapsigargin was pretreated for 10 min by bath-application from 12 to 2 min before the following DSLET challenge, the evoked-currents were abolished (Fig. 5*C*). Maximal effects of thapsigargin were found at 30 min after the start of application and such reduced DSLET-responses lasted for more than 1 hr. As shown in Figure 6, the ratio of DSLET-currents 30 min after the application of 10 nM thapsigargin to control currents

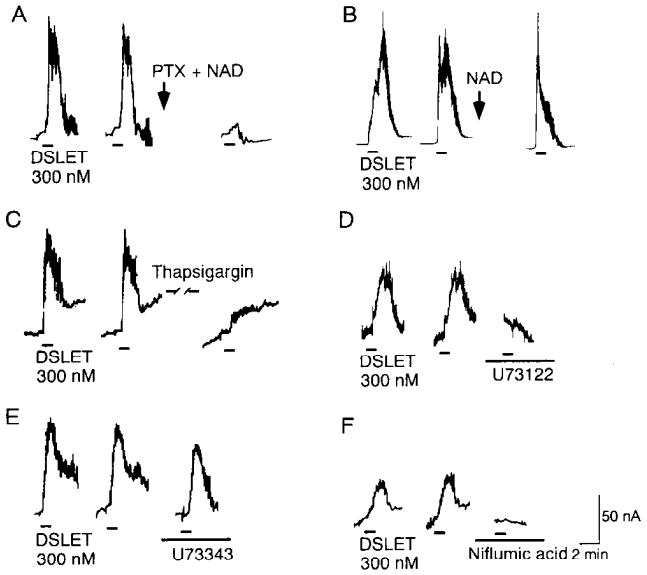


*Figure 5.* Characterization of DSLET-currents by various pharmacological treatments. DSLET at 300 nM was bath-applied for 1 min in oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs, as indicated by *small bar* in the figure in all experiments. *A* and *B*, Effects of intracellular injection of 20 nl of preactivated pertussis toxin A-protomer (0.1 ng/nl) plus 100 mM NAD (*A*) and of NAD alone (*B*) on DSLET-currents. Intracellular injections and following DSLET-application were carried out 5 and 30 min, respectively, after the beginning of preceding DSLET-application. Similar results were reproduced ($n$ = 3). *C*, Effects of bath-application of 10 nM thapsigargin from 12.5 to 2.5 min prior to following DSLET-application. *D* and *E*, DSLET-currents in the presence of 0.5 μM U73122 (*D*) or U73343 (*E*). Bath-application of U-73122 or U-73343 for 5 min was started 2 min before the following DSLET-challenge. *F*, DSLET-currents in the presence of 100 μM niflumic acid (5 min), as described in *D* and *E*. Other details were given in the Figure 2 caption.

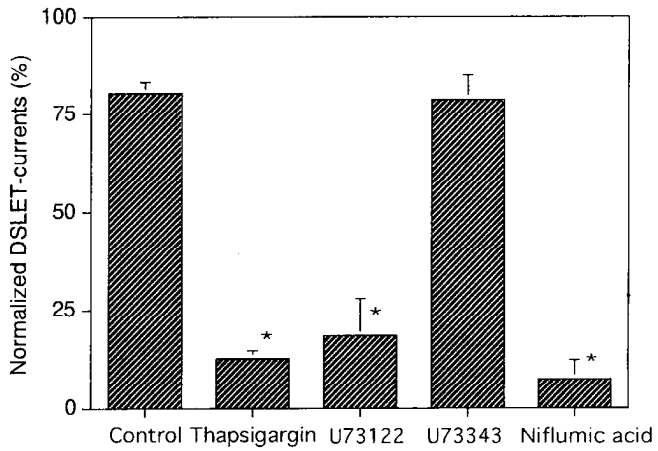


*Figure 6.* Summary of effects of thapsigargin, U73122, U73343 and niflumic acid on DSLET-currents. In this figure, DSLET (100 nM)-challenges were performed four times, and effects of 10 nM thapsigargin, 0.15 μM U73122, 0.15 μM U73343, and 100 μM niflumic acid on DSLET-currents in oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs were compared. Each treatment was started after the second challenge of DSLET, as shown in Figure 5. The currents evoked upon the fourth challenge of DSLET was represented as the percentage of ratio to the control (average of currents upon the first and second challenge of DSLET) in each experiment (normalized DSLET-currents). The control stands for the results without any treatment. Data represents the mean ± SEM from at least four separate experiments. *, $p < 0.05$, compared with control (ANOVA).

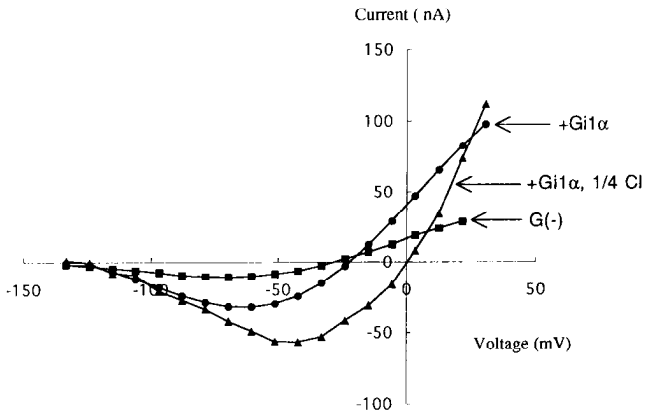


*Figure 7.* Current–voltage relationship and chloride ion-dependency of DSLET-currents. Experiments were performed with individual oocytes injected with DOR1 RNA (*solid square*), with DOR1 and $G_{i1}\alpha$ RNAs under the condition of normal MBS (*solid circle*), or with DOR1 and $G_{i1}\alpha$ RNAs under the condition of one-fourth chloride concentration (22.5 mM) of normal MBS (*solid triangle*), respectively. Results represent the difference between current–voltage (*I–V*) curves of peak currents after bath-application of DSLET (100 nM) and before it. Other details were described under Materials and Methods.

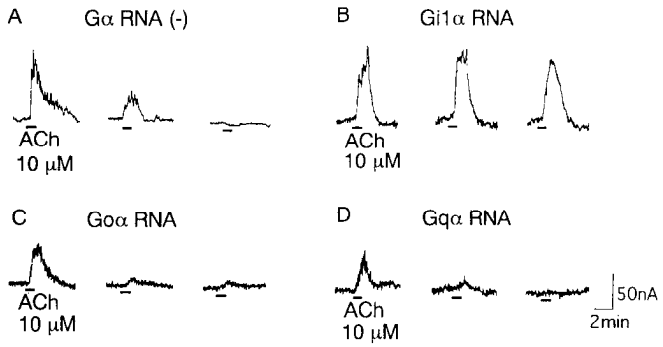


*Figure 8.* Currents evoked upon repeated challenges of ACh in the oocytes coinjected with various G-protein α-subunit RNAs. Results represent evoked currents upon repeated challenges of 10 μM ACh in oocytes injected with muscarinic $M_2$-receptor ($M_2$) RNA alone (*A*), or with $M_2$ + $G_{i1}\alpha$ (*B*), with $M_2$ + $G_o\alpha$ (*C*) or with $M_2$ + $G_q\alpha$ RNAs (*D*). ACh was bath-applied for 1 min periodically every 15 min, as indicated by *small underline* in the figure. Other details were given in the Figure 2 caption.

(average of first and second DSLET-currents before thapsigargin-application) was 13.9 ± 1.9% ($n$ = 4), which was significantly different from the ratio without any treatment (80.4 ± 3.1%, $n$ = 5). In some (not all) preparations, there were transient currents of 50–200 nA during the application of thapsigargin, possibly through inhibition of spontaneous reentry of $Ca^{2+}$ into such stores.

U-73122, an aminosteroid compound, has been found to be a potent inhibitor of aggregation of human platelets induced by a variety of agonists, and this compound was further characterized to be an inhibitor of PLC which is activated through G-proteins (Bleasdale et al., 1990). The use of this compound in blocking receptor-mediated PLC activities has a good advantage, since U-73343, an inactive analog in which pyrrolidinedione was just substituted for pyrroledione is available. As shown in Figure 5*D*, the DSLET-currents were abolished in the presence of U-73122 at 0.5 μM, which was bath-applied from 10 min before the agonist challenge. The inhibitory action of this compound was relatively short-lived and tended to disappear 15–30 min after the cessation of this application. Under the presence of U-73122, the maximal effects of this compound were found 30 min after the application. As shown in Figure 6, the ratio of DSLET-currents in the presence of 0.15 μM U-73122 to the initial one was 18.6 ± 9.6% ($n$ = 4), which was significantly different from the control, as above-mentioned. both DSLET-currents were abolished by U-73122 at 0.15–0.5 μM, a concentration which is much lower than $IC_{50}$ values (5–40 μM) of this compound in blocking aggregation of platelets or $InsP_3$ production or hydrolysis of phosphatidylinositol 4,5-bisphosphate catalyzed by cytosol PLC (Bleasdale et al., 1990; Jin et al., 1994b). To examine the specificity of this inhibitory action, we used U-73343. As shown in Figure 5*E*, the bath-application of 0.5 μM U-73343 had no significant effect on DSLET-currents. In addition, there was no statistical difference between the DSLET-currents in the presence and absence of 0.15 μM U-73343 (Fig. 6).

In order to examine whether other components than chloride currents are included in DSLET-currents, we tested the effect of niflumic acid, a chloride channel blocker (White and Aylwin, 1990). The DSLET-currents were completely blocked in the presence of 100 μM niflumic acid which was bath-applied from 10 min before the agonist-challenge (Fig. 5*F*). The inhibitory effect of this compound was completely reversible after the cessation of bath-application (data not shown). The statistical analysis also demonstrated that 100 μM niflumic acid significantly inhibited the DSLET-currents (Fig. 6).

The current–voltage (*I–V*) relationship of DSLET (100 nM)-currents was studied, using Ramp-Clamp method. As shown in Figure 7, the *I–V* curve in the oocyte not coinjected with $G_{i1}\alpha$ RNA was shallow, but shows characteristics identical to typical calcium-dependent chloride currents found in *Xenopus* oocytes (Dascal et al., 1984) in views of reversal potential (−25 mV) and outward rectification. In the oocyte coinjected with $G_{i1}\alpha$ RNA, the *I–V* curve of evoked currents upon the third challenge with DSLET (which were supposed to be due to $G_{i1}\alpha$-mediated mechanisms, as above-mentioned), was very similar to that without $G_{i1}\alpha$ RNA in views of reversal potential and outward rectification, though the currents at the voltage higher than the reversal potential (−25 mV) were markedly increased. When the chloride ion concentration in the perfusate was reduced to one-fourth (22.5 mM), the *I–V* curve was shifted to the right, while the outward rectification was still observed (Fig. 7). This finding also demonstrates the direct evidence that chloride channel activities are involved in DSLET-currents.

### *$M_2$-muscarinic receptor shares the common signal transduction with δ-receptor in* Xenopus *oocytes*

In the oocyte injected with $M_2$-muscarinic receptor RNA, 10 μM ACh evoked outward currents under the same conditions as the δ-receptor's (Fig. 8*A*). As we defolliculated the oocyte with collagenase treatments to remove endogenous $M_3$-muscarinic receptor (Sugiyama et al., 1985; Kubo et al., 1986), there was no ACh-currents through endogenous muscarinic receptors in non-injected oocytes from each batch.

The average of $M_2$-muscarinic receptor-mediated currents ($M_2$-currents) by 10 μM ACh was 127.3 ± 32.5 nA ($n$ = 8). However, such evoked currents were gradually reduced upon repeated challenges of 10 μM ACh (Fig. 8*A*). There were no currents upon the third challenges in this preparation. As shown in Table 2, the average of ratios of currents by the third ACh-

**Table 2. Comparison of $M_2$ currents upon the first and third challenge in oocytes injected with DOR1 RNA or with DOR1 and $G_{i1}\alpha$ RNAs**

| | First (nA) | Third (nA) | Third/First (%) |
|---|---|---|---|
| Gα (−) | 127.3 ± 32.5 | 20.4 ± 10.3 | 11.7 ± 4.3 |
| | ($n$ = 8) | ($n$ = 8) | ($n$ = 8) |
| $G_{i1}\alpha$ | 104.5 ± 13.3 | 90.3 ± 5.8 | 100.1 ± 10.6* |
| | ($n$ = 13) | ($n$ = 13) | ($n$ = 13) |
| $G_o\alpha$ | 78.7 ± 40.7 | 6.7 ± 6.7 | 4.2 ± 4.2 |
| | ($n$ = 3) | ($n$ = 3) | ($n$ = 3) |
| $G_q\alpha$ | 85.4 ± 34.5 | 17.2 ± 10.8 | 13.9 ± 8.2 |
| | ($n$ = 4) | ($n$ = 4) | ($n$ = 4) |

Results represent evoked currents upon the first and third challenge of 10 μM ACh and their ratios (Third/First) in oocytes injected with $M_2$ RNA alone/Gα (−) or with $M_2$ and $G_{i1}\alpha$ RNAs/$G_{i1}\alpha$, respectively.

* $p < 0.05$, compared with Gα (−) (ANOVA).

challenge to those by the first one (Third/First ratio) was 11.7 ± 4.3% ($n$ = 8) in oocytes not coinjected with any G-protein α-subunit RNA. When $G_{i1}\alpha$ RNA was coinjected into the oocyte, $M_2$-currents were constant upon repeated challenges of ACh (Fig. 8*B*). In most cases constant currents were obtained upon at least six repeated challenges of ACh. Third/First ratio in oocytes coinjected with $G_{i1}\alpha$ RNA was 100.1 ± 10.6% ($n$ = 13), as shown in Table 2. Thus, it is evident that the currents evoked by the third challenges of ACh are due to $G_{i1}$-mediated mechanisms. Under such conditions, ACh evoked currents in a concentration-depedent manner (Fig. 9). The $EC_{50}$ and concentration of ACh to show maximal currents was 1.64 ± 0.34 μM ($n$ = 6) and 10 μM, respectively. In the oocyte coinjected with $G_o\alpha$ or $G_q\alpha$ RNA, on the other hand, such constant currents were not observed (Fig. 8*C,D*). Third/First ratio was 4.2 ± 4.2% ($n$ = 3) or 13.9 ± 8.2% ($n$ = 4), in oocytes coinjected with $G_o\alpha$ or $G_q\alpha$ RNA, respectively (Table 2).

*Characterization of $M_2$-currents*

The $M_2$-currents were abolished by an intracellular injection with 50 pmol of EGTA (Fig. 10*A*). The EGTA-application alone had no effect on the baseline currents. In addition, vehicle (10 nl of pure water) application had also no effect on $M_2$-currents. On the other hand, a direct intracellular injection of 1 pmol of $InsP_3$ evoked bigger currents of 200 nA (Fig. 10*B*). Amplitudes of $InsP_3$-evoked currents were different from experiments to experiments. The average of evoked currents were 512.3 ± 168.1 nA ($n$ = 7). As reported with other metabotropic receptors including δ-opioid receptor (Sugiyama et al., 1985; Miyamae et al., 1993), $M_2$-currents were completely abolished by a prior injection of $InsP_3$ (Fig. 10*B*). The lack of $M_2$-currents after $InsP_3$ challenge was observed in all experiments tested ($n$ = 3).

The bath-application of 10 nM thapsigargin for 10 min abolished the $M_2$-currents (Fig. 10*C*). Such a blockade was statistically significant (Fig. 11). The $IC_{50}$ was 6.3 nM, an equivalent concentration to that in the inhibition of $Ca^{2+}$-dependent ATPase activity (Thastrup et al., 1990). $M_2$-currents were markedly inhibited in the presence of 0.5 μM U-73122, but not by U-73343 (Fig. 10*D,E*). Similarly, $M_2$-currents were abolished in the presence of 100 μM niflumic acid (Fig. 10*F*). All these pharmacological findings were reproduced in separate experiments and statistically significant except for experiments using U-73343 (Fig. 11).

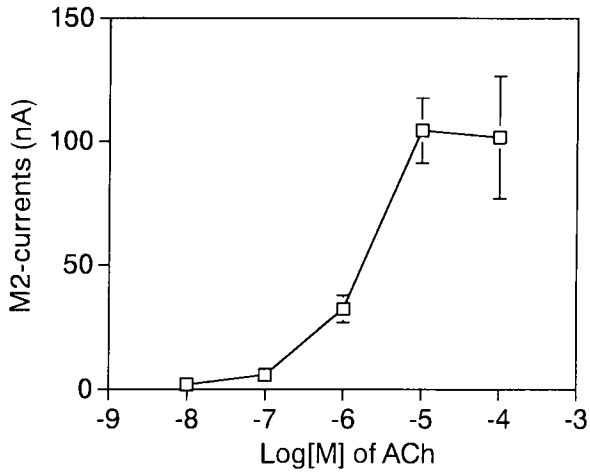


*Figure 9.* Concentration-dependent currents evoked by ACh in oocytes injected with $M_2$ and $G_{i1}\alpha$ RNAs. Results represent the maximal amplitude (nA) of evoked currents. Data are the mean ± SEM of four separate experiments.

The *I–V* curve of $M_2$-currents in the oocyte injected with $M_2$ receptor and $G_{i1}\alpha$ RNAs shows typical features of calcium-dependent chloride currents, in views of reversal potential (−25 mV) and outward rectification (Fig. 12). This view was also supported by the finding that the *I–V* curve was shifted to the right (reversal potential: −8 mV) when the chloride concentration in the perfusate was reduced to one-third (30 mM), suggesting the involvement of chloride channel opening in $M_2$-currents. On the other hand, there was no significant change in the *I–V* curve by substitution of $Na^+$ and $K^+$ with tetramethylammonium in views of reversal potential and outward rectification (Fig. 12). In a very few cases (below 2% probability), however, we detected such nonselective cation currents with a reversal potential of 0–5 mV under normal MBS, as Fukuda et al. (1987) reported. In our experiments, indeed, we adopted the holding potential of 0 mV to minimize such a nonselective cation channel gating in the present experiments.

When we added nitrendipine, a calcium channel blocker at a concentration of 1 or 10 μM, or $Co^{2+}$ at 0.1 mM to perfusion solution, there was no significant change in DSLET- (or $M_2$-) currents nor in effects of various treatments on DSLET- (or $M_2$-) currents throughout experiments presented here (data not shown).

*Phorbol ester selectively causes desensitization of δ-receptor-, but not $M_2$-receptor-currents in* Xenopus *oocytes reconstituted with $G_{i1}\alpha$*

Bath-application of 100 nM DSLET evoked constant currents by repeated challenges in the oocytes coinjected with DOR1 and $G_{i1}\alpha$ RNAs (Fig. 13*A*). When 10 nM PMA, a phorbol ester, was bath-applied from 10 to 5 min before the following DSLET-challenge, the currents were markedly reduced, as time goes (Fig. 13*B*). There were no substantial currents evoked upon the second DSLET-challenges after the PMA-application (Figs. 13*B,C* 14).

When 100 ng of calcineurin, a protein phosphatase 2B (Pallen and Wang, 1984) was injected into oocytes after the complete loss of evoked currents following PMA application, DSLET-currents were recovered (Fig. 13*C*). The recovery of DSLET-currents by this injection was significant when compared to the data without calcineurin-injection (Fig. 14). On the other hand,

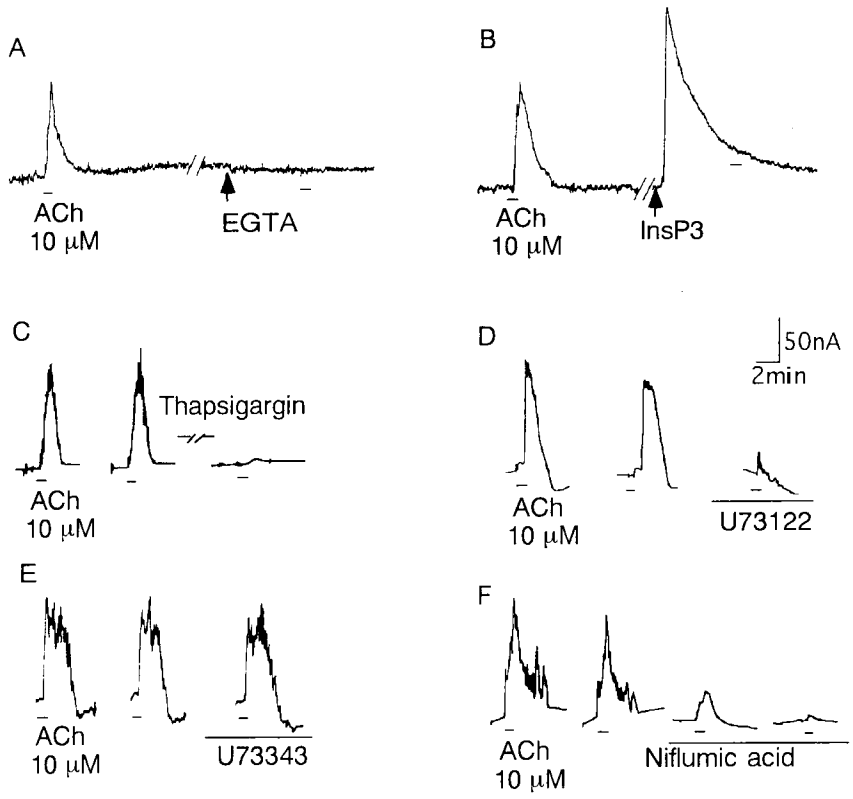


*Figure 10.* Characterization of $M_2$-currents by various pharmacological treatments. ACh at 10 μM was bath-applied for 1 min, as indicated by *small bar* in the figure in all experiments using oocytes injected with $M_2$ and $G_{11}\alpha$ RNAs. *A* and *B*, Effects of intracellular injection of 50 pmol EGTA (*A*) or of 1 pmol $InsP_3$ (*B*) 15 min after the last challenge of ACh. Following challenge of ACh was performed 5 min after such an injection. *C*, Effects of bath-application of 10 nM thapsigargin from 12.5 to 2.5 min prior to following ACh-application. *D* and *E*, ACh-currents in the presence of 0.5 μM U73122 (*D*) or U73343 (*E*). *F*, ACh-currents in the presence of 100 μM niflumic acid. Other details are given in the Figure 5 caption.

the inhibitory actions of PMA were completely blocked in the presence of 100 nM calphostin C (Fig. 15*A*). The $IC_{50}$ of calphostin C in blocking PMA-desensitization was 30 nM. Calphostin C is well known as a most selective, potent and membrane permeable protein kinase C inhibitor, and its 50% inhibitory concentration is 50 nM against protein kinase C, while more than 50 μM against A-kinase (Kobayashi et al., 1989). However, the PMA-induced desensitization was not affected in the presence of 1 μM KT-5720 (Fig. 15*B*), a most selective and membrane permeable A-kinase inhibitor, whose $K_i$ value is 56 nM against protein kinase A, while more than 2 μM against protein kinase C (Kase et al., 1987; Haverstick and Gray, 1992). Thus, it is evident that the PMA-desensitization is through an activation of protein kinase C.

In the oocytes further coinjected with $M_2$ receptor RNA, on the other hand, the PMA (10 nM)-treatment showed no significant effects on the currents evoked by 1 μM ACh, though DSLET-currents were markedly reduced (Fig. 16*A,B*).

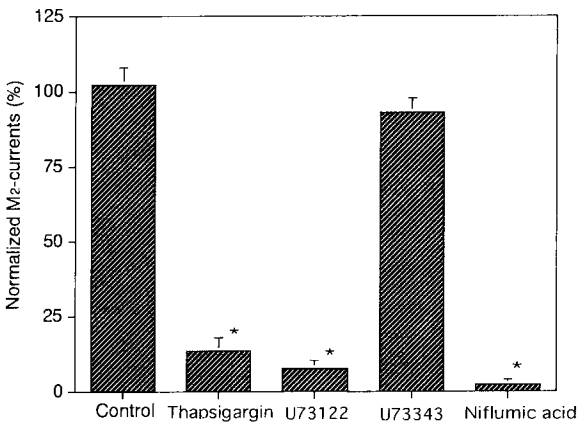


*Figure 11.* Summary of effects of thapsigargin, U73122, U73343 and niflumic acid on ACh-currents. In this figure, ACh (10 μM)-challenges were performed four times, and effects of 10 nM thapsigargin, 0.15 μM U73122, 0.15 μM U73343, and 100 μM niflumic acid on ACh-currents in oocytes injected with $M_2$ + $G_{11}\alpha$ RNAs were compared. Each treatment was started after the second challenge of ACh, as shown in Figure 10. The currents evoked upon the fourth challenge of ACh was represented as the percentage of ratio to the average of currents upon the first and second challenge of ACh in each experiment (normalized $M_2$-currents). The control stands for the results without any treatment. Data represents the mean ± SEM from at least four separate experiments. *, $p < 0.05$, compared with control (ANOVA).

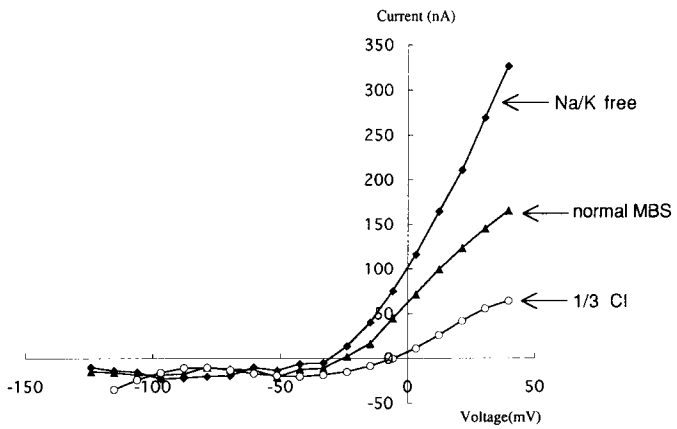


*Figure 12.* Current–voltage relationship of ACh-currents under various conditions. Experiments were performed with individual oocytes injected with $M_2$ and $G_{11}\alpha$ RNAs under the condition of normal MBS (*solid triangle*), of one third chloride concentration (30 mM) of normal MBS (*open circle*) or of $Na^+$ and $K^+$-free MBS (*solid diamond*), respectively. Results represent the difference between current–voltage (*I–V*) curves of peak currents after bath-application of ACh (10 μM) and before it. Other details were described under Materials and Methods.

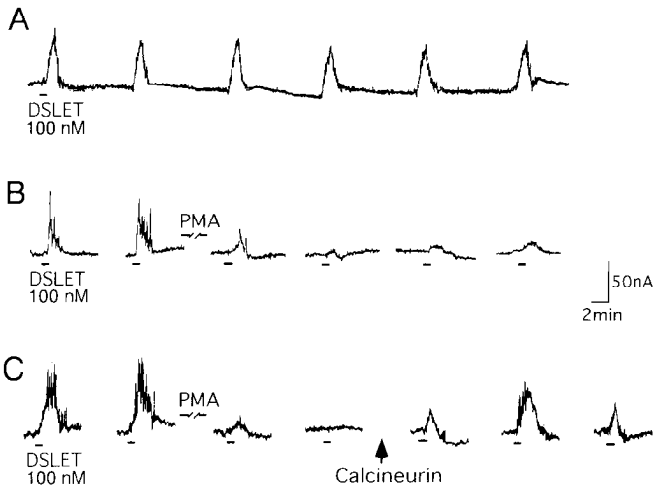


*Figure 13.* Representative results of desensitization of DSLET-currents by phorbol ester and its reversal by calcineurin. DSLET (100 nM)-challenge was carried out by bath-application (1 min) periodically every 15 min. Bath-application of 10 nM PMA for 5 min was started from 10 min prior to the following agonist-challenge. *A*, Constant currents by DSLET in oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs. *B*, Desensitization of DSLET-currents by PMA-pretreatment. *C*. Reversal of PMA-desensitization of DSLET-currents by an intracellular injection of 100 ng calcineurin (50 nl in pure water) 10 min prior to the following DSLET-challenge. The *vertical* and *horizontal bar* represent 50 nA and 2 min, respectively.

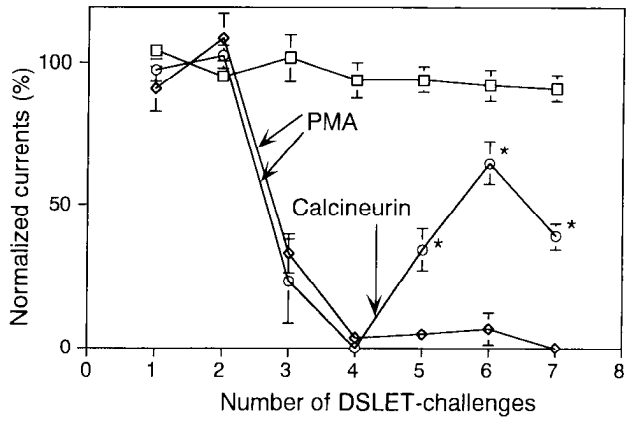


*Figure 14.* Summary of desensitization of DSLET-currents by phorbol ester and its reversal by calcineurin. DSLET (100 nM)-currents evoked upon each challenge (normalized currents) were represented as the percentage of ratio to the average of currents upon the first and second challenge of DSLET in each experiment. Symbols represent control without any treatment (*square*), PMA alone (*diamond*) and PMA + calcineurin (*circle*), respectively. Data represent mean ± SEM from three to six separate experments. Other details are given in the Figure 13 caption. *, $p < 0.05$, compared with PMA alone (ANOVA).

### *Homologous desensitization by repeated challenges of the δ-agonist at higher concentrations*

When a higher concentration of DSLET (3 μM) was used, the evoked currents were rapidly desensitized on repeated challenges in the oocyte coinjected with $G_{i1}\alpha$ RNA (Fig. 17*A*). In this preparation, there were no significant currents evoked upon the third challenges of the agonist. Such a rapid desensitization was reproduced in separate experiments, as shown in Figure 18. When DSLET at various concentrations was repeatedly applied to the oocyte injected with 10 ng of DOR1 RNA and 50 ng of $G_{i1}\alpha$ RNA, constant currents were observed upon repeated challenges of DSLET at 0.1 and 0.3 μM. With 1 μM DSLET, most of preparations showed constant currents, while some preparations did "desensitization," thereby there was a relatively big deviation. However, all preparations tested showed a rapid desensitization with 3 μM DSLET. The minimal concentration showing desensitizing DSLET-currents was 3 μM (Fig. 18).

The desensitized DSLET-currents were recovered when the agonist was applied to the oocyte after 60 min of absence of agonist challenges (Fig. 17*A*). Complete recovery of DSLET-currents was not observed after 30 min or shorter periods of absence of challenges.

Previously, we reported that the constant currents evoked by a lower concentration (1 μM) of DSLET were completely blocked by 100 nM naltrindole (NTI), a specific δ-opioid receptor antagonist (Miyamae et al., 1993). To examine the involvement of specific opioid receptors in such desensitizing DSLET-currents, repeated challenges of DSLET (3 μM) were carried out in the presence of NTI. As shown in Figure 17*B*, there was no or very weak currents evoked upon DSLET-challenges in the presence of 100 nM NTI, while the third challenge of DSLET in the absence of NTI evoked marked currents. Therefore it is evident that evoked currents by 3 μM DSLET and desensitization by repeated challenges of 3 μM DSLET are both mediated through δ-opioid receptors.

In the oocytes injected with $M_2$ receptor RNA together with DOR1 and $G_{i1}\alpha$ RNAs, repeated challenges of ACh (10 μM) did not cause a desensitization of $M_2$-currents (Fig. 19*A*). Furthermore, such treatments also showed no significant change between the initial DSLET-currents and last ones, despite that 10 μM ACh evoked bigger currents (122 nA) than 3 μM DSLET did (58.3 nA). The desensitization of $M_2$-currents was not observed when 1 mM ACh was repeatedly applied (Fig. 19*B*).

On the other hand, repeated challenges of 3 μM DSLET did not affect the $M_2$-currents, though such treatments caused a marked desensitization of DSLET-currents (Fig. 20*A*).

### *Protein kinase C inhibitor selectively recovers the desensitization of δ-agonist responses*

As shown in Figure 20*A*, the DSLET-evoked currents were recovered to the initial level by the addition of calphostin C at 2 μM to the perfusate for 10 min after the ACh challenge, and the rescued δ-agonist responses were constant upon at least three repeated challenges of DSLET. However, there was no change in $M_2$-currents between before and after the calphostin C treatment. As shown in Table 4, the responses to DSLET-challenge 30 min after the bath-application of 2 μM calphostin C were 95.5 ± 16.3% of the initial DSLET-response ($n = 4$). This ratio was significantly different from that without any treatment (6.4 ± 3.6%, $n = 4$). The effects of calphostin C were concentration-dependent in ranges of 100 nM to 2 μM, and there was no significant change between 2 and 5 μM calphostin C. The $EC_{50}$ of calphostin C was 270 nM, a comparable value to that observed in *in vitro* enzymatic assays (Kobayashi et al., 1989), but a little higher than that (30 nM) in blocking PMA-desensitization, as mentioned before. This may come from the difference of periods for calphostin C-application (25 min in Fig. 15 vs 10 min in Fig. 20). Similar recovery of DSLET-currents was also observed when 5 pmol of protein kinase C inhibitor peptide (19-31) (Smith et al., 1990) was intracellularly injected (Fig. 20*B*). The maximal DSLET-currents (84.6 ± 17.2% of the initial DSLET-

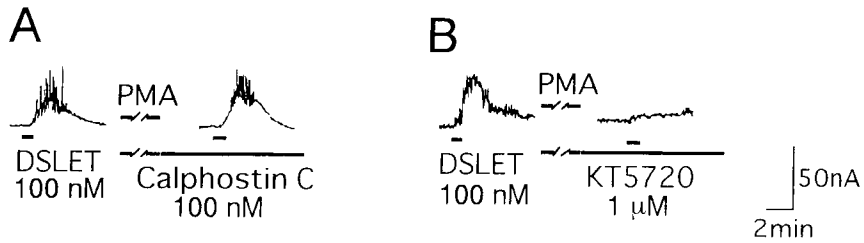


*Figure 15.* Selective blockade of PMA-desensitization of DSLET-currents by calphostin C. Bath-application of PMA (10 nM, 5 min) was carried out, as described in Figure 13. *A*, Blockade of PMA-desensitization of DSLET-currents by a bath-application of 100 nM calphostin C from 25 min prior to the following DSLET-challenge. *B*, No blockade of PMA-desensitization of DSLET-currents by a bath-application of 1 μM KT5720 from 25 min prior to the following DSLET-challenge. Other details are given in the Figure 13 caption.

currents, $n = 3$) were observed 30 min after injection of this inhibitor (Table 4).

On the other hand, the bath-application with 2 μM KT5720 showed no effect on the reduced DSLET-responses after repeated challenges of the agonist (Fig. 20*C*). Repeated experiments revealed that both treatments with bath-application of 2 μM KT5720 and with intracellular injection of 1 pmol of calmodulin kinase II (CaMK II) inhibitor (Smith et al., 1992) showed no significant effect on such desensitizing DSLET-currents (Table 4).

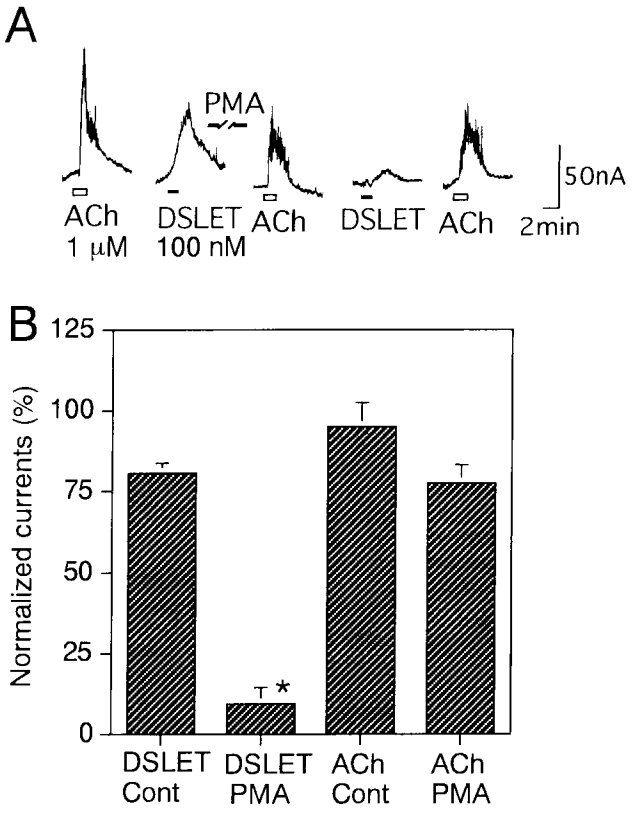


*Figure 16.* Lack of desensitization of $M_2$-currents by PMA-pretreatment. *A*, Bath-applications of ACh (1 μM) or DSLET (100 nM) were carried out by bath-application (1 min) periodically every 15 min in oocytes injected with DOR1, $M_2$, and $G_{i1}\alpha$ RNAs. PMA (10 nM)-pretreatment was carried out as described in the Figure 13 caption. *B*, Experiments were essentially carrried out using oocytes injected with RNAs of $G_{i1}\alpha$ and DOR1 or $M_2$. Other details were as described in *A* except that 300 nM DSLET or 10 μM ACh was used in each preparation. The currents evoked upon the fourth challenge of DSLET or ACh was represented as the percentage ratio to the control (average of currents upon the first and second challenge of each agonist) in each experiment (normalized currents). Data represent mean ± SEM from three to four separate experiments. *, $p < 0.05$, compared to DSLET alone (ANOVA).

In order to further confirm the δ-opioid receptor-involvement in such desensitization mechanisms, 3 μM DPDPE, another selective opioid δ-agonist, was used for δ-receptor stimulation in oocytes coinjected with $G_{i1}\alpha$ RNA. As shown in Figure 21, a marked desensitization of DPDPE-currents was also observed. The time course of desensitization of DPDPE-currents was quite similar to that of 3 μM DSLET (Fig. 18). As is the case with DSLET, there was no significant desensitization with DPDPE at concentrations below 1 μM (data not shown). Figure 21 also shows another example of desensitization with DADLE. In this case, the desensitization became clear when DADLE was used at 10 μM, but not at 3 μM. Therefore the desensitizing concentration of DADLE seems to be higher than those of DSLET and DPDPE in such preparations.

Repeated challenges of dihydroetorphine (DHE) also showed a rapid desensitization in evoking currents (Fig. 22*A*). The desensitization of DHE-currents was so strong that the addition of calphostin C after the complete desensitization had no change in such currents (data not shown). However, such a strong desensitization was partially blocked by pretreatment with calphostin C, as shown in Figure 22*B*. When the ratios of currents upon the second and third challenges of DHE to the first ones were compared, the effects of calphostin C pretreatment were statistically significant (Fig. 22*C*).

In order to study whether agonist-independent phosphorylation through protein kinase C is also involved in δ-receptor desensitization in *Xenopus* oocytes, the effects of calphostin C (2 μM, 10 min) pretreatments on the initial DSLET (100 nM) currents were examined. In experiments using the same batch of oocytes, average of DSLET-currents with or without calphostin C was 55.0 ± 8.5 nA ($n = 6$) or 58.4 ± 8.5 nA ($n = 4$), respectively. There was no significant difference betwen them (Student's $t$ test).

## Discussion

Many neurotransmitters communicate with the cell interior via receptors coupled to phospholipase C (PLC) isozymes. Diacylglycerol (DAG), one of second messengers generated by these enzymes is linked to protein phosphorylation by activation of protein kinase C (Nishizuka, 1992). However, little is known of protein kinase C substrates in metabotropic receptor mechanisms. Considering that protein kinase C is activated locally at the plasma membrane by the action of phospholipids (Nishizuka, 1992), the receptor itself seems to be one of good targets. Despite this idea attracts many investigators' concerns, regarding its acute regulation, many attempts resulted in apparently con-

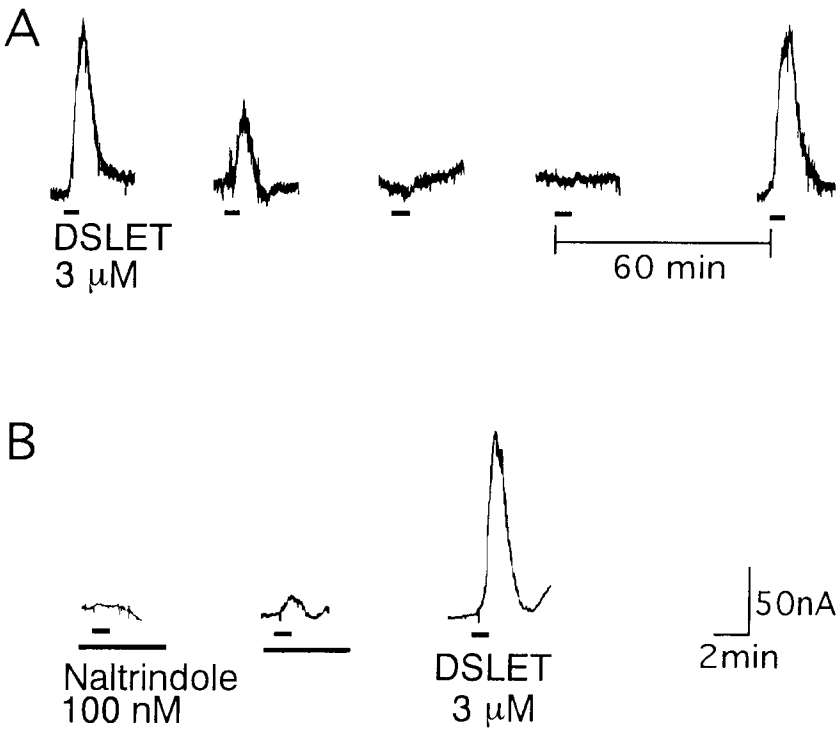


*Figure 17.* Homologous desensitization of DSLET-currents and its blockade by calphostin C. DSLET at 3 μM was bath-applied for 1 min periodically every 15 min, unless otherwise stated, to oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs. *A*, Desensitization of DSLET-currents upon repeated challenges and recovery after the absence of agonist-stimulation for 60 min. *B*, Blockade of DSLET (3 μM)-currents and its desensitization by naltrindole. Naltrindole at 100 nM was bath-applied for 5 min from 2 min before the DSLET-challenge.

tradictory on the susceptibility to desensitization of receptor mechanisms through PLC activation (Eva et al., 1990; Thompson and Fisher, 1990). This may be partly attributed to the facts that the receptor-mediated activation of PLC are also regulated by cross-talk with many other cellular components, such as various kinds of G-protein α- and βγ-subunits (Berridge, 1993; Clapham and Neer, 1993).

In the present study, we developed the "functional reconstitution" experiments in the oocyte system to clarify the coupling between δ-receptors and $G_{i1}$. As shown in Figure 2*A*, DSLET-currents were markedly attenuated by repeated challenges of the agonist at 100 nM. Here we speculated that low density of endogenous $G_{i1}\alpha\beta\gamma$ (or $G_{i2}\alpha\beta\gamma$) heterotrimers is all activated to be dissociated into α- and βγ-subunits, and thereby the "tachyphylaxis" due to the temporary deficiency of such heterotrimers occurs. On this assumption, $G_{i1}\alpha$ RNA was coinjected with DOR1 RNA into oocytes to increase the population of such heterotrimeric forms in the present experiments. In oocytes coinjected with $G_{i1}\alpha$ RNA, the currents were not only enhanced, but also constant in amplitude on at least six times of repeated challenges of the δ-agonist (Fig. 2*B*). When the responses upon the third to sixth challenges of the agonist in oocytes coinjected with or without $G_{i1}\alpha$ RNA were compared, it is evident that such evoked currents are due to $G_{i1}$ (or its α-subunit)-mediated mechanisms. Quite similar conclusion was also obtained in the case with $M_2$-muscarinic receptor which was found to be coupled to $G_{i1}$ in oocytes injected with $G_{i1}\alpha$ RNA (Fig. 8, Table 2). These findings strongly suggest that δ-opioid receptor and $M_2$-muscarinic receptor share the common mechanisms through $G_{i1}\alpha$ in evoking currents in oocytes reconstituted with $G_{i1}\alpha$.

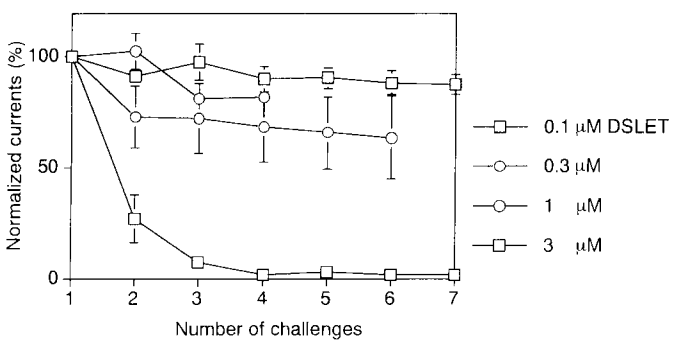


*Figure 18.* Evoked currents upon repeated challenges of various concentrations of DSLET. Results represent normalized currents (%) as the ratio of evoked currents upon each challenge of DSLET (0.1 μM - 3 μM) to those upon the initial challenge in oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs. Data represent the mean ± SEM from at least six separate experiments. Other details are given in the Figure 2 caption.

Next concerns are about downstream mechanisms of both receptor-mediated currents, particularly involvement of PLC activation. Previously we reported a partial characterization of DSLET-currents including sensitivities to prior injection of EGTA or $InsP_3$ into oocytes (Miyamae et al., 1993; Ueda et al., 1994). Here we observed similar sensitivities of $M_2$-currents to intracellular injection of EGTA or $InsP_3$. As the $InsP_3$ receptor on the endoplasmic reticulum is well known to be homologously desensitized (Hajnoczky and Thomas, 1994), such results with $InsP_3$ could be interpreted by the view that both $M_2$- and DSLET-currents are mediated through actions of $InsP_3$ and its receptor, as previously reported in the case with $M_1$ and metabotropic glutamate receptors known to evoke PLC-related currents (Kubo et al., 1986; Sugiyama et al., 1985).

Here we further characterized the PLC-mechanisms and its downstream signalings involved in both DSLET- and $M_2$-currents, using various pharmacological compounds. Thapsigargin, a $Ca^{2+}$ depletor from intracellular $Ca^{2+}$ stores including $InsP_3$-sensitive ones (Thastrup et al., 1990; Lupu-Meiri et al., 1993) completely blocked both currents (Figs. 5*C*, 10*C*). U-73122, a PLC inhibitor, also abolished both currents, while U-73343, an inactive derivative did not (Figs. 5*D,E*, 10*D,E*). Recently, Jin et

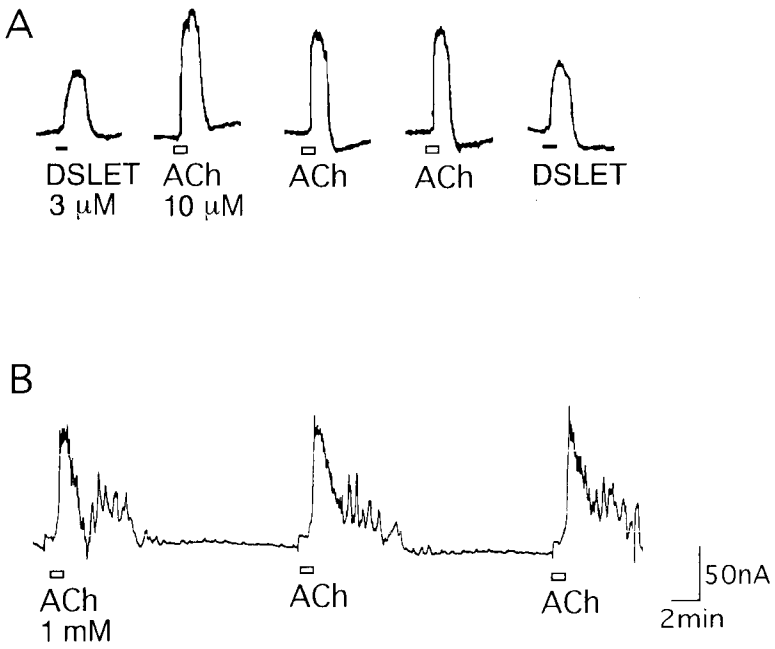


*Figure 19.* Lack of desensitization of DSLET- or $M_2$-currents by repeated challenges of ACh. Details in the present experiments are given in the Figure 2 caption, unless otherwise stated. *A*, $M_2$-currents were not desensitized upon three-repeated challenges of 10 μM ACh in oocytes injected with DOR1, $M_2$, and $G_{i1}\alpha$ RNAs. DSLET-currents were not affected by such repeated ACh-challenges. *B*, $M_2$-currents were not desensitized upon three repeated challenges of higher concentration (1 mM) of ACh in oocytes injected with $M_2$ and $G_{i1}\alpha$ RNAs.

al. (1994a), reported that δ-opioid receptor mediates increases in intracellular calcium mobilization in a pertussis toxin (PTX)-sensitive manner in NG108-15 cells. In such studies, the calcium-mobilization was blocked by thapsigargin or U-73122, being consistent with the present results. The functional coupling between PTX-sensitive G-proteins and PLC found in their report and the present study may be unique, since most of PLC-activating receptors couple to PTX-insensitive G-proteins (Berridge, 1993). However, from reconstitution experiments, there are several reports that $G_{i1}$ is involved in receptor-mediated PLC acti-

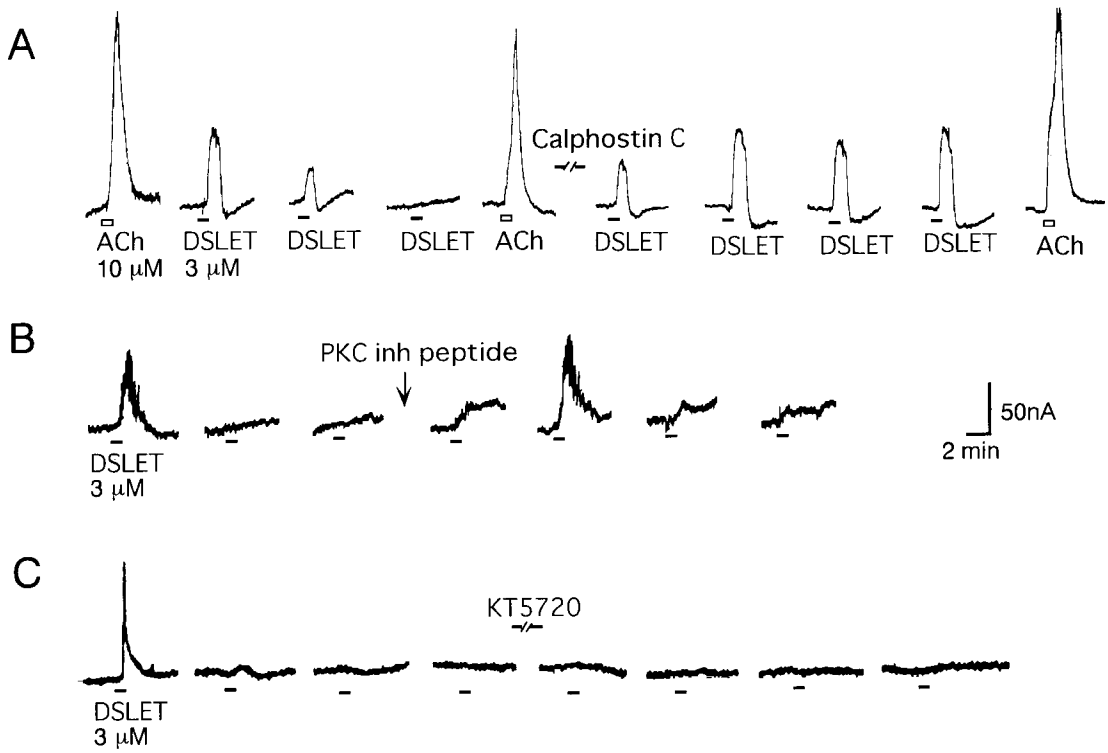


*Figure 20.* Lack of desensitization of $M_2$-currents by repeated challenges of DSLET and recovery of desensitized DSLET-currents by protein kinase C inhibitors. Details in the present experiments are given in the Figure 2 caption, unless otherwise stated. *A*, $M_2$-currents were not affected by three repeated challenges of 3 μM DSLET in oocytes injected with DOR1, $M_2$, and $G_{i1}\alpha$ RNAs. Desensitization of DSLET-currents was reversed by a bath-application of 2 μM calphostin C for 10 min from 15 min before the following DSLET-challenge, while $M_2$-currents were not affected by this inhibitor. *B*, Desensitization of DSLET-currents was reversed by an intracellular injection with 5 pmol of protein kinase C inhibitor peptide (19–31) 5 min before the following DSLET-challenge. *C*, No recovery of desensitized DSLET-currents by a bath-application of 2 μM KT5720 for 10 min, as described in *A*.

**Table 3. Median effective concentration of δ-opioid agonists to evoke currents in oocysts injected with DOR1 and $G_{i1}\alpha$ RNAs**

| | *n* | $EC_{50}$ (nM) |
|---|---|---|
| DSLET | 5 | 32.8 ± 4.2 |
| DPDPE | 3 | 41.3 ± 8.1 |
| DADLE | 3 | 22.3 ± 4.1 |
| DHE | 3 | 4.3 ± 1.5 |

vation in many cases with μ- and κ-opioid receptors and kyotorphin-receptor in the brain (Misawa et al., 1995; Ueda et al., 1988, 1989, 1995) and chemotactic peptide-receptor in human leukemic cells (Kikuchi et al., 1986). From the finding that the coinjection with RNAs of PTX-insensitive G-protein α-subunits, such as $G_q\alpha$ did decrease the DSLET-currents (Fig. 1), it is evident that $G_{i1}$, but not $G_q$ is involved in such evoked currents through PLC-mechanisms in the present $G_{i1}$-reconstituted oocytes. Finally, we provided the evidence that both DSLET- and $M_2$-currents are through $Ca^{2+}$-dependent chloride channels, in pharmacological experiments using niflumic acid, a chloride channel blocker (Figs. 5*F*, 10*F*), and in electrophysiological experiments with currents–voltage relationship analysis (Fig. 7, 12). Thus, it is evident that both δ-opioid receptor and $M_2$-muscarinic receptors share the common post receptor signaling mechanisms including $G_{i1}$, PLC, $InsP_3$-mediated calcium mobilization and calcium-dependent chloride channel gating in such $G_{i1}$-reconstituted oocytes.

Using such reconstitution experiments, we attempted to clarify the mechanisms of homologous desensitization of δ-opioid receptor. One of major findings in the this study is that δ-receptor-responses were desensitized upon repeated challenges of a higher concentration of δ-agonists (Fig. 17*A*), and the mode of desensitization was purely homologous or agonist-dependent. In oocytes coinjected with DOR1, $M_2$ receptor, and $G_{i1}\alpha$ RNA, the currents evoked by a higher concentration (3 μM) of DSLET were not affected by preceding challenges (three times) of 10 μM ACh (Fig. 19*A*). In the present experiments $M_2$-currents by 10 μM ACh were always bigger than DSLET (3 μM)-currents in all preparations tested. Taken into consideration that both currents are mediated by common $G_{i1}$-PLC mechanisms in such oocytes reconstituted with $G_{i1}\alpha$, it is evident that the desensitization of DSLET-currents is not due to the change in $G_{i1}$-PLC or their downstream mechanisms, but to the change in the function of receptor (δ-opioid receptor) itself. In other words, the mode of desensitization in the present experiments is "homologous" or agonist dependent.

The second finding is that protein kinase C is possibly involved in its desensitization mechanisms. Accumulating findings have been reported that the treatment with PMA, an activator of protein kinase C, alters the functions of various kinds of proteins in many types of cells (Nishizuka, 1992). In the present experiment, the responses by DSLET at 100 nM were effectively attenuated by the treatment with 10 nM PMA in a calphostin C-reversible manner (Figs. 13*B*, 15*A*). Such results were consistent with the previous report using NG108-15 hybrid cells (Louie et al., 1990). The PMA-desensitization of DSLET-currents was reversed by a direct injection of calcineurin, a protein phosphatase 2B (Figs. 13*C*, 14), indicating that protein phosphorylation through protein kinases is involved in such a desensitization. On the other hand, $M_2$-currents were not desensitized by 10 nM PMA (Fig. 16*A,B*), this suggesting that putative phosphorylation sites for protein kinase C in $M_2$-receptor unlikely play important roles in the desensitization.

**Table 4. Comparison of various protein kinase inhibitors on the homologous desensitization of δ-opioid receptor-mediated currents**

| Inhibitor | *n* | % of initial current |
|---|---|---|
| None | 4 | 6.4 ± 3.6 |
| Calphostin C | 4 | 95.5 ± 16.3* |
| Protein kinase C inhibitor peptide (19-31) | 3 | 84.6 ± 17.2* |
| KT5720 | 5 | 11.9 ± 8.2 |
| CaMK II inhibitor | 3 | 15.4 ± 12.4 |

Experiments were performed in the oocyte injected with DOR1 and $G_{i1}\alpha$ RNAs. Experiment schedule and other deatils were given in the Figure 12*E* caption. Results were represented as the percentage ratio of current amplitudes obtained at the second challenge of 3 μM DSLET after the application of inhibitor to that obtained at the initial challenge in the beginning of recording. Data were DSLET-evoked currents (nA) after the treatments with protein kinase inhibitors. Both calphostin C and KT5720 were used at 2 μM for 10 min by bath application. Protein kinase C inhibitor peptide (19-31) and calmodulin kinase II (CaMK II) inhibitor peptide were injected intracellularly in amounts of 5 and 1 pmol, respectively. All data given in the figure are mean ± SEM. *n*, Numbers of separate experiments.

* $P < 0.05$, compared with None (ANOVA).

The homologous desensitization of DSLET-currents were reversed by calphostin C-treatment, even after the complete loss of evoked currents (Fig. 20*A*). Similar results were obtained when protein kinase C inhibitor peptide (19-31) (Fig. 20*B*), but not KT-5720 nor CaMK II inhibitor (Fig. 20*C*, Table 4). In addition, the absence of DSLET-challenges for 60 min led to the recovery of the δ-receptor responses from such a homologous desensitization (Fig. 17*A*), possibly through dephosphorylation by endogenous protein phosphatases. All these findings suggest that such a desensitization mechanism is due to a temporary modification (phosphorylation) of δ-receptor by protein kinase C.

It remains to be examined whether receptor kinases are also involved in such mechanisms. However, as $M_2$-mediated cur-

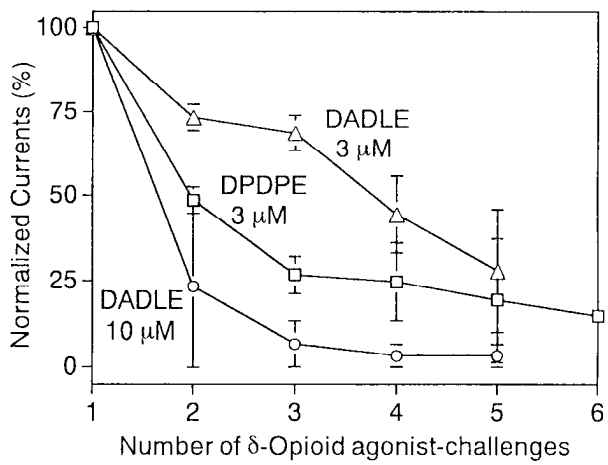


*Figure 21.* Time course of currents evoked by repeated challenges of DPDPE or DADLE. The amplitude of DPDPE (3 μM)- or DADLE (3 and 10 μM)-currents in oocytes injected with DOR1 and $G_{i1}\alpha$ RNAs was represented by the percentage of ratio to that of each initial currents (normalized currents). Data are the mean ± SEM from three to six separate experiments.

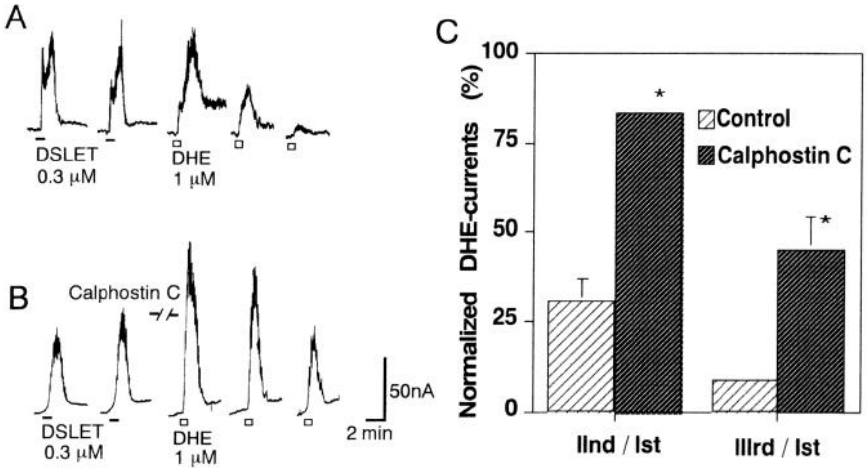


*Figure 22.* Desensitization of DHE-currents upon repeated challenges and its partial protection by caphostin C-treatment. *A*, Desensitization of DHE (1 μM)-evoked currents upon repeated challenges. There was no desensitization with low concentration (0.3 μM) of DSLET in the same preparation. *B*, Inhibitory action of calphostin C pretreatment (2 μM for 10 min) on the desensitization of DHE-currents. Other details were given in the Figure 13 caption. *C*, Summary of inhibitory effect of calphostin C on the desensitization of DHE-currents. Normalized DHE-currents (%) of IInd/Ist or IIIrd/Ist represent the ratio of evoked currents upon the second or third challenge of DHE to those upon the first, respectively. Data are the mean ± SEM from four separate experiments. Average of DHE (1 μM)-evoked currents in the absence or presence of calphostin C was 100.5 ± 25.4 nA or 107.0 ± 27.1 nA, respectively. Twice DSLET (0.3 μM)-challenges precede the DHE-challenges in each experiment. Such DSLET-evoked currents were always constant. The average DSLET-currents were 62.8 ± 10.0 nA ($n$ = 8). *, $p < 0.05$, compared with control (ANOVA).

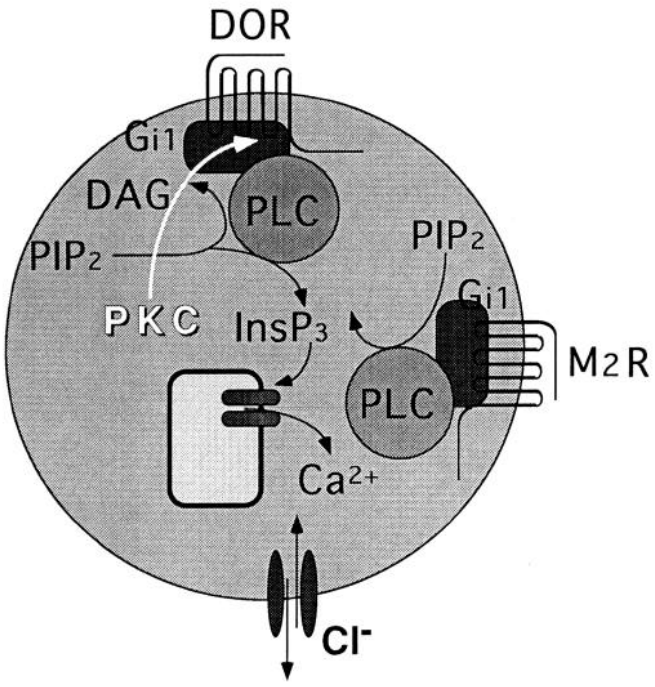


*Figure 23.* Schematic model of homologous desensitization of δ-opioid receptor. Both δ-opioid receptor (*DOR*) and $M_2$ receptor (*$M_2$R*) share the common post receptor signaling mechanisms including $G_{i1}$, phospholipase C (*PLC*), $InsP_3$-mediated $Ca^{2+}$ mobilization and $Ca^{2+}$-dependent chloride channel gating. DOR is phosphorylated and desensitized by protein kinase C (*PKC*) which is activated by PMA or diacylglycerol (*DAG*), a product of PLC reaction. $M_2R$ shows less sensitivity to PKC, since no significant desensitization by PMA was observed in Figure 16, *A* and *B*. Since produced DAG (an activator of PKC) is so hydrophobic that it remains in the membrane, the lateral movement of DAG from $M_2R$ site to DOR site is expected to be slow. In addition, the local concentration of DAG in the membrane may be diluted during movement. Such possibilities may explain why PKC to be activated through $M_2R$-mechanisms did not desensitize DOR, possibly through phosphorylation.

rents through $G_{i1}$ were not affected by repetitive ACh-challenges even at 1 mM (Fig. 19*B*), it is unlikely that such mechanisms through receptor kinases are very important in the present system. This might be explained by the findings that the longer exposure of the receptor to agonists is required for such mechanisms through receptor kinases, such as GRK2 in the cases with β-adrenoceptor and $M_2$-receptor (Lefkowitz et al., 1990, Tsuga et al., 1994). Indeed, we have preliminary findings that $M_2$-currents after longer exposure to 10 μM ACh for more than 30 min were desensitized in oocytes and it was prevented by coexpression with a dominant negative mutant of GRK2 (Ueda et al., unpublished data). On the other hand, the application of calphostin C failed to recover the desensitized DHE-currents, while the pretreatment with this inhibitor significantly, but not completely rescued DHE-currents from rapid desensitization (Fig. 22). DHE is a potent analgesic opiate which has very high affinities for δ, μ, and κ-opioid binding sites (Niwa et al., 1995) and is liable to show a strong tolerance *in vivo* (Tokuyama et al., 1993, 1994) as well as etorphine (see review, Cox, 1993). Taken into consideration the finding that DHE has 100 times higher affinity for δ-opioid binding than DSLET (Niwa et al., 1995), the possibility could not be excluded that receptor kinases are also partly involved in mechanisms of such a desensitization of DHE-currents. Further experiments using dominant negative mutants for δ-opioid receptor-specific receptor kinases should be next subjects to prove this possibility.

We proposed the schematic model for the mechanism of homologous desensitization of δ-opioid receptor in *Xenopus* oocytes (Fig. 23). Although it is unlikely that the calcium-dependent chloride current response, known to be typical in the *Xenopus* oocyte, is a major effector response in the brain, the signal transduction through receptor–$G_{i1}$–PLC and its regulation by protein kinase C may be common in many tissues. Indeed, we have reported the regulation of δ-opioid receptor-responses by protein kinase C in guinea pig striatal membranes, where the δ-receptor-mediated stimulation of GTPase activities were enhanced by treatments with calphostin C (Fukushima et al.,

1994). Therefore, it is true that the desensitization of δ-receptor is also mediated to some extents by protein kinase C in the brain.

The present studies demonstrate that δ-opioid receptor is homologously desensitized through an action of protein kinase C when a higher concentration of agonist was used in *Xenopus* oocytes.

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