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# Inhibition of $Ca^{2+}$ channel current by $\mu$- and $\kappa$-opioid receptors coexpressed in *Xenopus* oocytes: desensitization dependence on $Ca^{2+}$ channel $\alpha_1$ subunits

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**1** Desensitization of $\mu$- and $\kappa$-opioid receptor-mediated inhibition of voltage-dependent $Ca^{2+}$ channels was studied in a *Xenopus* oocyte translation system.

**2** In the oocytes coexpressing $\kappa$-opioid receptors with N- or Q-type $Ca^{2+}$ channel $\alpha_1$ and $\beta$ subunits, the $\kappa$-agonist, U50488H, inhibited both neuronal $Ca^{2+}$ channel current responses in a pertussis toxin-sensitive manner and the inhibition was reduced by prolonged agonist exposure.

**3** More than 10 min was required to halve the inhibition of Q-type channels by the $\kappa$-agonist. However, the half-life for the inhibition of N-type channels was only $6 \pm 1$ min. In addition, in the oocytes coexpressing $\mu$-opioid receptors with N-type or Q-type channels, the uncoupling rate of the $\mu$-receptor-mediated inhibition of N-channels was also faster than that of Q-type channels.

**4** In the oocytes coexpressing both $\mu$- and $\kappa$-receptors with N-type channels, stimulation of either receptor resulted in a cross-desensitization of the subsequent response to the other agonist. Treatment of oocytes with either H-8 (100 $\mu$M), staurosporine (400 nM), okadaic acid (200 nM), phorbol myristate acetate (5 nM) or forskolin (50 $\mu$M) plus phosphodiesterase inhibitor did not affect either the desensitization or the agonist-evoked inhibition of $Ca^{2+}$ channels.

**5** These results suggest that the rate of rapid desensitization is dependent on the $\alpha_1$ subtype of the neuronal $Ca^{2+}$ channel, and that a common phosphorylation-independent mechanism underlies the heterologous desensitization between opioid receptor subtypes.

**Keywords:** *Xenopus* oocytes; opioid receptors; N-type; Q-type; calcium channels; heterologous desensitization

## Introduction

Desensitization of opioid receptors by continuous stimulation with an agonist is a mechanism underlying the development of opioid tolerance, which is a characteristic problem of opiate drugs in clinical usage. In receptors that couple to G proteins, three basic processes for this desensitization have been postulated, i.e., functional uncoupling by phosphorylation, sequestration of receptors and down-regulation of receptors (Hausdorff *et al*., 1990). Both phosphorylation and sequestration take place rapidly within minutes and are potentially reversible processes. Down-regulation is normally a slower process that takes place over hours, the reversal of which requires synthesis of new receptor molecules. In opioid tolerance, the participation of the latter two processes remains uncertain since no definitive reduction in the number, affinity, or mRNA level of opioid receptors has been observed after chronic morphine treatment *in vivo* (Nishino *et al*., 1990; Abdelhamid & Takemori, 1991; Lutfy & Yoburn, 1991; Brodsky *et al*., 1995). However, chronic opioid treatments cause alterations in the functional coupling to adenylyl cyclase in many brain areas, including the spinal cord (Duman *et al*., 1988; Attali *et al*., 1989). Loss of opioid response is also observed in the G protein-mediated modulation of $K^+$ and $Ca^{2+}$ channels in neurones chronically (Kennedy & Henderson, 1991; Nomura *et al*., 1994) or acutely (Harris & Williams, 1991; Osborne & Williams, 1995) treated with opioids. The acute uncoupling of opioid receptors from the effectors is therefore considered to be the initial stage of the desensitization process which leads to opioid tolerance.

Recently, the mechanism of acute desensitization has been studied in cloned opioid receptors. In cultured cells transfected with the cDNA for $\mu$-receptor, a prolonged exposure to agonist evokes phosphorylation of the receptor proteins (Arden *et al*., 1995; Zhang *et al*., 1996), the time-course of which parallels that of the uncoupling of G protein-coupled inwardly-rectifying $K^+$ channels (GIRKs) expressed in *Xenopus* oocytes (Kovoorm *et al*,. 1995; Zhang *et al*., 1996). The involvement of G protein-coupled receptor kinases (GRKs) in the uncoupling of $\delta$- or $\kappa$-receptors from adenylyl cyclase has been shown (Raynor *et al*., 1994; Pei *et al*., 1995). Several studies indicate the possible participation of $Ca^{2+}$-dependent protein kinases in the reduction of agonist responses after repeated application of agonist (Mestek *et al*., 1995; Ueda *et al*., 1995). However, the mechanism is more complicated in these expression studies since opioid receptors can activate multiple intracellular metabolic pathways (Johnson *et al*., 1994; Kaneko *et al*., 1994b).

With regard to the inhibition of voltage-dependent $Ca^{2+}$ channels by opioids, the mechanism of uncoupling has been studied in cultured or acutely-dissociated neurones in which multiple subtypes of opioid receptors and $Ca^{2+}$ channels are constitutively expressed (Kennedy & Henderson, 1991; Nomura *et al*., 1994; Osborne & Williams, 1995). Using cloned $\kappa$-receptors and P/Q-type $Ca^{2+}$ channels, we have previously shown that these exogenous molecules can couple to endogenous G proteins when expressed in *Xenopus* oocytes (Kaneko *et al*., 1994a). The activation of $\mu$- and $\kappa$-receptors selectively inhibits N- and P/Q-type $Ca^{2+}$ channels in neurones isolated from the nucleus tractus solitarius (Rhim & Miller, 1994) and dorsal root ganglia (Schröder *et al*., 1991), where the opioid-mediated inhibition of transmitter release has physiological consequences. The present work was therefore designed to investigate the specified coupling and uncoupling phenomena between an opioid receptor subtype ($\mu$ or $\kappa$) and a neuronal type (N or P/Q) of $Ca^{2+}$ channel coexpressed in *Xenopus* oocytes. We found that $\mu$- and $\kappa$-receptors were uncoupled

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from $Ca^{2+}$ channel inhibition mainly by a common mechanism which caused cross-desensitization, and that the rate of desensitization was not dependent on opioid receptor subtype but was dependent on the $\alpha_1$ subtype of the neuronal $Ca^{2+}$ channel.

## Methods

### *Preparation of RNA-injected oocytes*

Plasmids pSPCBI-2 (Mori *et al*., 1991) and pKCRB3 (Fujita *et al*., 1993) were provided by Dr Y. Mori (National Institute for Physiology, Okazaki, Japan). RNAs for opioid receptors and $Ca^{2+}$ channel subunits were synthesized by *in vitro* transcription by use the recombinant plasmids pSPCBI-2 (rabbit P/Q-type, $\alpha_{1A}$), pKCRB3 (rabbit N-type, $\alpha_{1B}$), pHCaB (human $\beta_2$; Fukuda *et al*., 1996), pKOPR (rat $\kappa$-opioid receptor; Minami *et al*., 1993), and pMOPR (rat $\mu$-opioid receptor; Minami *et al*., 1994). Oocytes of *Xenopus laevis* were defolliculated by collagenase and injected with 50 nl of RNA solution including N- or Q-type $\alpha_1$ subunit (2.5 ng), $\beta$ subunit (2.5 ng), and $\mu$- and/or $\kappa$-opioid receptor (2 ng for $\mu$-receptor, 4 ng for $\kappa$-receptor). The doses of opioid receptor RNA were determined to induce maximum inhibition of $Ca^{2+}$ channel current in the preliminary experiments. The amounts of RNA for $Ca^{2+}$ channel subunits were identical to those of our previous study (Kaneko *et al*., 1994a). The oocytes were maintained at 19–22°C for 2–4 days in sterile modified Birth's solution (MBS composition (mM): NaCl 88, KCl 1, $CaCl_2$ 0.41 $Ca(NO_3)_2$ 0.33, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, sodium pyruvate 2.5 and Tris-HCl 7.5; pH 7.6, supplemented with 10 u $ml^{-1}$ penicillin and 10 $\mu$g $ml^{-1}$ streptomycin).

### *Two-electrode voltage-clamp recording*

An oocyte was placed in a 50 $\mu$l chamber and perfused at a flow rate of 0.9 ml $min^{-1}$ with 50Ba solution (50 mM $Ba(OH)_2$, 50 mM Tris, 5 mM CsOH, adjusted to pH 7.6 with methanesulphonic acid) during recordings. The temperature of the solution was maintained at 20–23°C. The oocytes were voltage clamped at −60 mV by a two-electrode voltage-clamp amplifier (CEZ-1200, Nihon Kohden, Tokyo, Japan), and a MacLab A/D converter was used both for the monitoring of transmembrane current and for command pulse output. Depolarization-induced $Ba^{2+}$ current ($I_{Ba}$) flowing through $Ca^{2+}$ channels was evaluated by a step pulse (500 ms duration) from −100 to +10 mV following a 500 ms-conditioning potential at −100 mV. Capacitive and leak components were subtracted from the current responses using the P/4 procedure.

### *Drugs and chemicals*

U50488H ((3,4-dichloro-*N*-[2-(1-pyrrolidinyl)cyclohexo] benzine acetamide; a gift from Upjohn Company, Kalamazoo, MI) and [D-$Ala^2$, N-$MePhe^4$, $Gly^5$-ol]enkephalin (DAMGO, Research Biochemicals International, Natick, MA, U.S.A.) were dissolved in 50Ba solution and applied by switching the

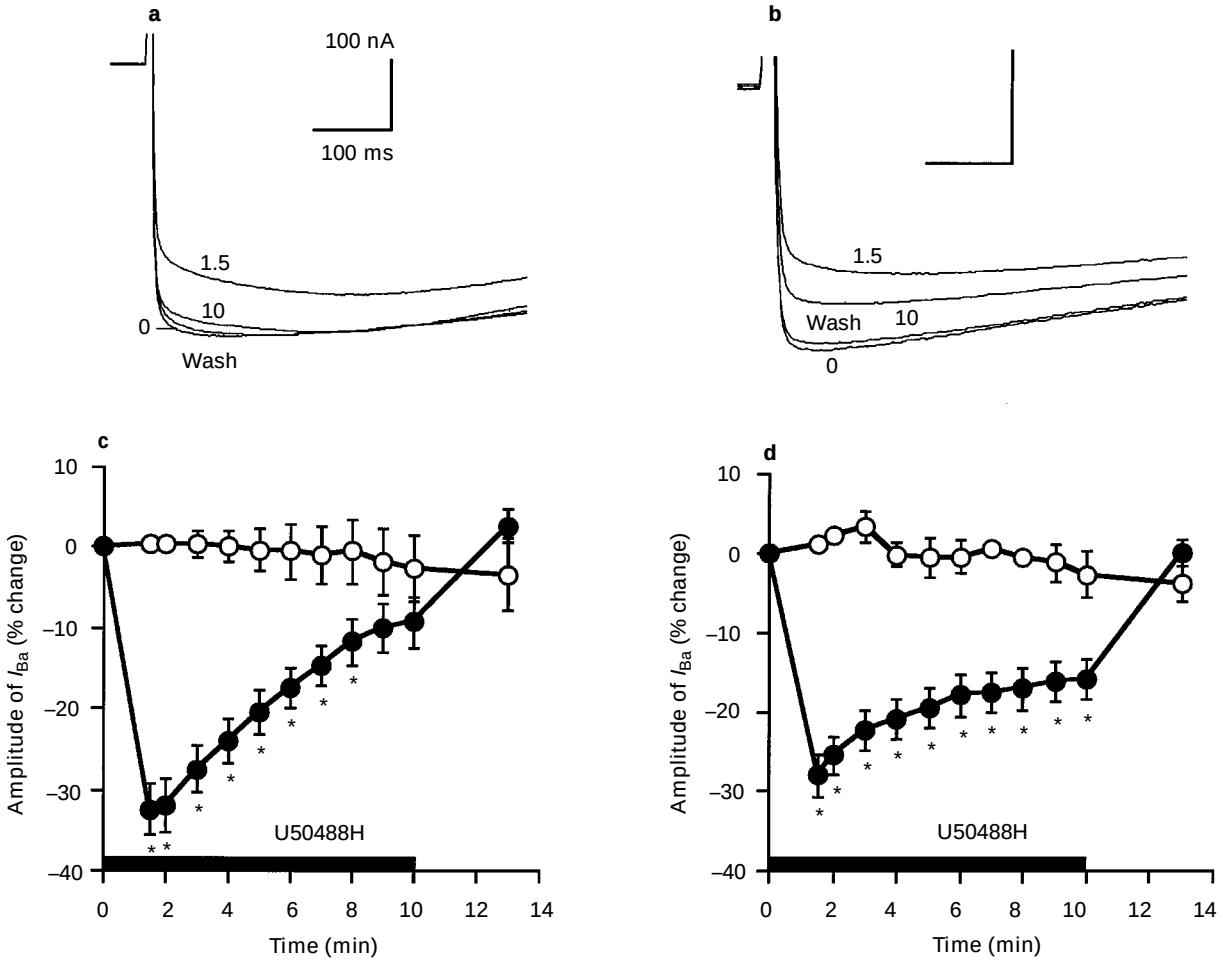


**Figure 1** Desensitization of $\kappa$-opioid receptor-mediated inhibition of voltage-dependent $Ca^{2+}$ channels. $I_{Ba}$ was recorded by a step pulse from −100 to +10 mV in *Xenopus* oocytes coexpressing $\kappa$-receptors with N-type (a, c) or Q-type (b, d) $Ca^{2+}$ channels. Numbers with typical traces are sample time (in min) after the beginning of perfusion with 1 $\mu$M U50488H. The peak amplitudes of $I_{Ba}$ in the presence (●, $n=16$ in c; $n=9$ in d) and absence (○, $n=6$ in c; $n=7$ in d) of U50488H are plotted. *$P<0.05$ vs control $I_{Ba}$ without U50488H at each time.

perfusion line. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), staurosporine, and phorbol 12-myristate-13-acetate (PMA) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N-2-(methylamino)ethyl-5-isoquinolinesulphonamide (H-8) was from Seikagaku Kogyo (Tokyo, Japan). Pertussis toxin (PTX) was from Wakenyaku (Osaka, Japan). Okadaic acid was from Nacalai Tesque (Kyoto, Japan). These drugs were dissolved in MBS for preincubation with oocytes. An anti-$G_o\alpha$ antibody, GC/2 (New England Nuclear, Boston, MA, U.S.A.), directed at the N-terminal region of $G_o\alpha$, was diluted to 1/50 with sterile water and injected in a volume of 50 nl. Tetralithium salt of guanosine-5′-*O*-3-thiotriphosphate (GTP$\gamma$S; Boehringer Mannheim, Tokyo, Japan) was dissolved at 1 mM with water and injected in a 1/100 volume of oocytes.

### *Statistics*

Results are presented as the means ± s.e. Statistical significance was evaluated by use of one-way analysis of variance (ANOVA) with a *post-hoc* Tukey-Kramer test. The level of significance was set at $P<0.05$.

## Results

### *Opioid receptor-mediated inhibition of N-and Q-type $Ca^{2+}$ channels*

Pharmacological phenotypes of $Ca^{2+}$ channels coexpressed with $\kappa$-opioid receptors were confirmed first. The amplitude of $I_{Ba}$ recorded from the oocytes, several days after injection of RNAs for $\kappa$-receptor, $Ca^{2+}$ channel $\alpha_{1A}$ and $\beta$ subunits, was inhibited by 1 $\mu$M $\omega$-conotoxin MVIIC to 10–20% of the control amplitude and inhibited completely by 10 $\mu$M $LaCl_3$, but not by $\omega$-agatoxin IVA or $\omega$-conotoxin GVIA, up to 1 $\mu$M, indicating that the pharmacological phenotype of the $\alpha_{1A}$ channel is Q-type, as previously shown (Sather *et al*., 1993). In contrast, the amplitude of $I_{Ba}$ in the oocytes injected with RNAs for $\kappa$-receptor, $Ca^{2+}$ channel $\alpha_{1B}$ and $\beta$ subunit, was inhibited specifically by 1 $\mu$M $\omega$-conotoxin GVIA and completely by 10 $\mu$M $LaCl_3$, showing the involvement of N-type channels (Fujita *et al*., 1993).

Application of a $\kappa$-agonist, U50488H (1 $\mu$M), inhibited the amplitude of $I_{Ba}$ in the oocytes coexpressing $\kappa$-receptors with N- or Q-type $Ca^{2+}$ channels consisting of $\alpha_1$ and $\beta$ subunits (Figure 1a and b). For both the N- and Q-channels, the U50488H-induced inhibition was accompanied by a delay of channel opening kinetics, and the magnitudes of inhibition were consistently similar to each other. The inhibition of $I_{Ba}$ was diminished both by overnight preinjection of the oocytes with 2 $\mu$g ml$^{-1}$ PTX and by pretreatment (3 h) with $G_o\alpha$-specific antibody, GC/2 (Table 1).

**Table 1** Reduction of $\kappa$-receptor-mediated $Ca^{2+}$ channel inhibition after treatment of oocytes with PTX or anti-$G_o\alpha$ antibody (GC/2)

| | *Change in the amplitude of $I_{Ba}$ after U50488H (%)* | |
|---|---|---|
| *Pretreatment* | *κ-N* | *κ-Q* |
| Vehicle-treated | −23.5±1.5 (6) | −26.0±2.2 (7) |
| PTX-treated | −0.3±1.5 (5)* | −0.3±1.7 (5)* |
| Water-injected | −22.6±2.7 (5) | −26.5±4.7 (5) |
| GC/2-injected | −8.9±1.9 (6)* | −6.5±5.0 (5)* |

Oocytes coexpressing $\kappa$-receptors with N- ($\kappa$-N) or Q-channels ($\kappa$-Q) were either subjected to PTX 2 $\mu$g ml$^{-1}$ overnight or injected with GC/2 (1/50 dilution, 50 nl, 3 h before recording). $I_{Ba}$ was recorded by a step pulse from −60 mV to +10 mV, and the effect of U50488H on the amplitude of $I_{Ba}$ was evaluated 90 s after perfusion with 1 $\mu$M U50488H. These treatments did not affect the control amplitude of $I_{Ba}$ by themselves. Numbers of experiments are shown in parentheses. *$P<0.05$ vs vehicle-treated or water-injected.

When $\kappa$-N oocytes ($\kappa$-receptors and N-channels are coexpressed) were subjected to a large depolarization prepulse (to +70 mV, 80 ms duration, 80 ms before test pulse), the amplitude of the following $I_{Ba}$ was potentiated to 120±4.4% of the control value before U50488H and to 134±6.4% in the presence of U50488H ($n=10$, $P<0.05$). Hence, the inhibition of N-channel current by U50488H was significantly decreased to 16.6±1.9% of control value after the prepulse, as compared with the normal test potential (24.8±3.1%). In another experiment, after an intracellular injection of GTP$\gamma$S (10 $\mu$M as a cytosolic concentration) to the $\kappa$-N oocytes, the amplitude of $I_{Ba}$ was gradually decreased to 72.6±7.5%, of the control amplitude, 10 min after injection, and to 59.1±11.4% at 30 min ($n=7$), indicating that there was no recovery or desensitization of GTP$\gamma$S-evoked inhibition in the oocytes up to 30 min

### *Differential time-course of desensitization of $Ca^{2+}$ channel types*

When $\kappa$-N and $\kappa$-Q oocytes were continuously perfused with 1 $\mu$M U50488H for 10 min, the inhibition of $I_{Ba}$ reached a maximum at 90 s and then gradually declined (Figure 1). On washout of U50488H, the amplitude of $I_{Ba}$ was restored to the pretreatment level, or sometimes showed a slight rebound potentiation. Although the degrees of maximum inhibition by U50488H were equivalent, the rate of desensitization in the coupling of $\kappa$-N was faster than that of $\kappa$-Q. During the 10-min perfusion, the percentage of desensitization from the maximal inhibition was 68.7±6.4% ($n=16$) in $\kappa$-N oocytes, whereas that of $\kappa$-Q was 43.8±4.6% ($n=9$, $P<0.05$). Assuming that the rate of desensitization was constant and that the decay could be fitted to a single exponential curve, the estimated time constant for desensitization was 8.1±1.4 min and 18.2±3.4 min in the coupling of $\kappa$-N and $\kappa$-Q, respectively. The calculated half-life of inhibition was 5.6±1.0 min for $\kappa$-N and 12.6±2.4 min for $\kappa$-Q ($P<0.05$). A lower concentration of U50488H (100 nM) caused less inhibition both in the $\kappa$-N (24.3±4.1% at maximum, $n=4$) and $\kappa$-Q (24.5±1.9%, $n=4$) oocytes. Also, the percentage of 10-min desensitization from maximum was significantly different (50.3±2.6% in $\kappa$-N vs 33.3±6.8% in $\kappa$-Q).

A similar comparison was made with oocytes coexpressing $\mu$-opioid receptors with N- or Q-channels (Figure 2). When $\mu$-N and $\mu$-Q oocytes were perfused with 1 $\mu$M DAMGO for 10 min, the degrees of maximum inhibition in the channels were not different. Neither the degree of maximum inhibition nor the rate of desensitization of $\mu$-receptors was different from those of $\kappa$-receptors. However, desensitization of $\mu$-N coupling was significantly faster than that of $\mu$-Q coupling since the percentage of 10 min desensitization was 63.5±4.5% ($n=7$) in $\mu$-N, whereas that of $\mu$-Q was 41.2±6.1% ($n=8$). The estimated time constants for desensitization (and calculated half-lives of inhibition, in parentheses) were 8.7±1.0 min (6.0±0.7 min) and 21.4±4.7 min (14.8±3.3 min) in $\mu$-N and $\mu$-Q, respectively, ($P<0.05$).

### *Recovery from desensitization*

To compare the time-course of recovery from desensitization, U50488H was re-applied after a 3 min washout period following a continuous 10 min exposure to $\kappa$-N or $\kappa$-Q oocytes. When inhibition of N-channels by $\kappa$-receptors was desensitized from 33.3±4.6% (at 1.5 min after the first application) to 11.2±1.7% (at the end of 10 min exposure), the 3 min washout was sufficient to recover the amplitude of $I_{Ba}$ to 105.3±1.6% of the initial amplitude ($n=6$). In this condition, the second application of U50488H for 1.5 min produced 24.9±4.8% inhibition from the recovered amplitude, which was smaller but not significantly different from the first inhibition. In contrast, the inhibition of Q-channels by $\kappa$-receptors was desensitized from a maximum of 29.4±2.4% to 18.1±2.2% during a 10 min exposure. The amplitude of $I_{Ba}$

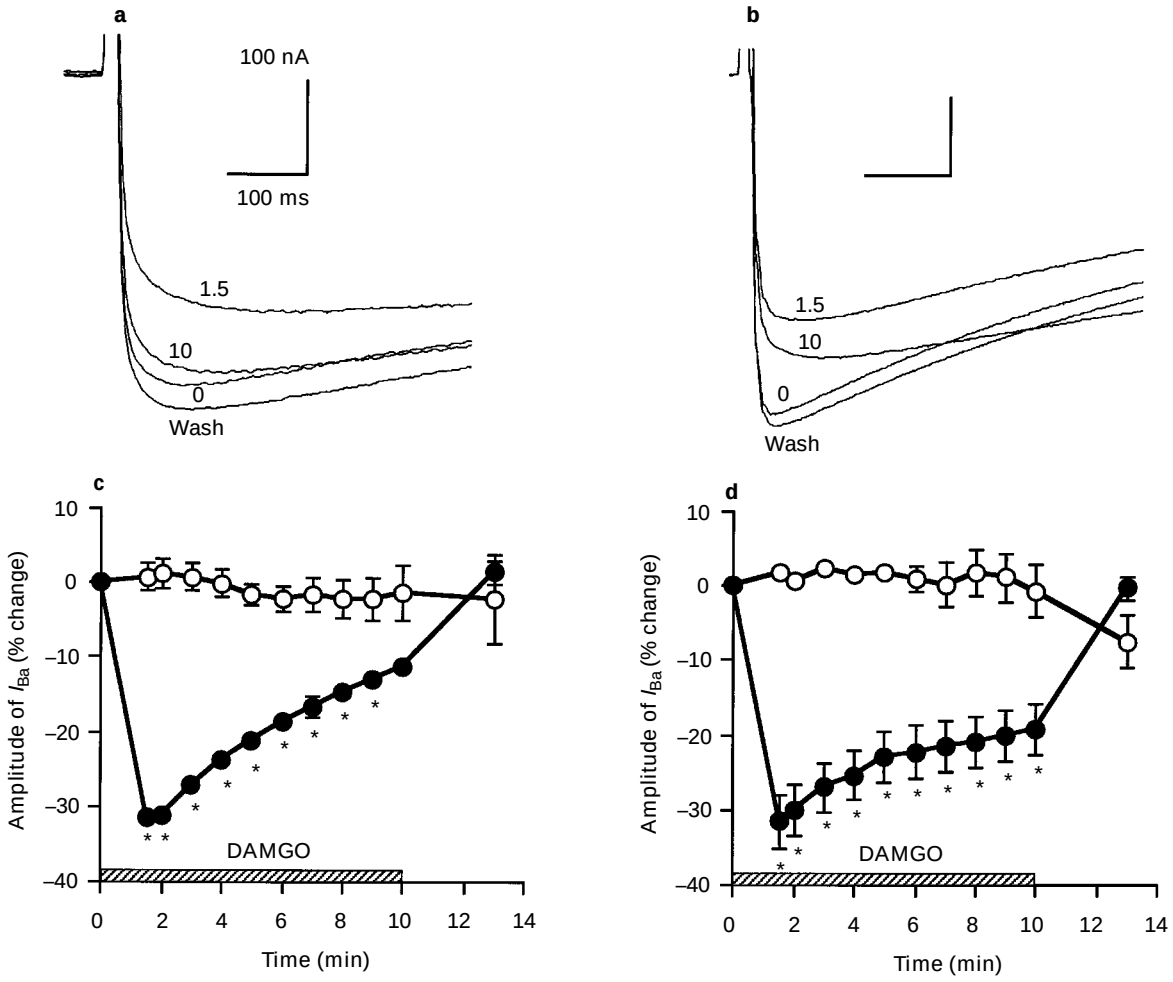


**Figure 2** Desensitization of $\mu$-opioid receptor-mediated inhibition of voltage-dependent $Ca^{2+}$ channels. $I_{Ba}$ was recorded by a step pulse from $-100$ to $+10$ mV in *Xenopus* oocytes coexpressing $\mu$-receptors with N-type (a, c) or Q-type (b, d) $Ca^{2+}$ channels. Numbers with typical traces are sample time (in min) after the beginning of perfusion with 1 $\mu$M DAMGO. The peak amplitudes of $I_{Ba}$ in the presence (●, $n=7$ in c; $n=8$ in d) and absence (○, $n=5$ in c; $n=6$ in d) in DAMGO are plotted. $^*P<0.05$ vs control $I_{Ba}$ without DAMGO at each time.

was restored to $101.4\pm0.8\%$ on washout and reinhibited by $18.2\pm2.4\%$ ($n=6$). The second inhibition in $\kappa$-Q corresponded to 62% of the first one and was lower than in $\kappa$-N (75%); but, the difference was not significant.

Fixation and recovery of desensitization were also examined by pretreating oocytes with an agonist. A time-dependent loss of the $\kappa$-receptor-mediated inhibition was observed when $\kappa$-N and $\kappa$-Q oocytes had been preincubated with U50488H for 0.5–2 h followed by a wash period of 8 min in the recording chamber before the test application of U50488H (Table 2). The pretreatment with U50488H did not affect the basal amplitude of $I_{Ba}$ by itself. The $\kappa$-receptor-mediated inhibition of $I_{Ba}$ was almost completely abolished by the 2 h pretreatment with U50488H in both $\kappa$-N and $\kappa$-Q oocytes, without any apparent difference between the two.

**Table 2** Reduction of $\kappa$-receptor-mediated $Ca^{+2}$ channel inhibition by pretreatment with U50488H

| *Preincubation period* (h) | *Change in the amplitude of* $I_{Ba}$ *after U50488H* (%) $\kappa$-*N* | $\kappa$-*Q* |
|---|---|---|
| 0 | $-19.3\pm2.8$ (7) | $-21.3\pm3.1$ (7) |
| 0.5 | $-10.1\pm0.6$ (4) | $-11.9\pm6.8$ (4) |
| 1 | $-6.0\pm2.4$ (5)* | $-5.1\pm2.2$ (5)* |
| 2 | $-0.2\pm3.1$ (5)* | $-3.4\pm4.8$ (5)* |

Ooocytes coexpressing $\kappa$-receptors with N ($\kappa$-N) or Q-channels ($\kappa$-Q) were preincubated for indicated period with 1 $\mu$M U50488H. The oocytes were placed in the recording chamber and perfused with recording solution for 8 min to remove U50488H completely. $I_{Ba}$ was then recorded and the effect of 1 $\mu$M U50488H was evaluated 90 s after perfusion. Numbers of experiments are shown in parentheses. $^*P<0.05$ vs no preincubation.

### *Heterologous desensitization between $\mu$- and $\kappa$-receptors*

To determine whether the desensitization of opioid receptor-mediated oocyte response is homologous or heterologous, both $\kappa$- and $\mu$-receptors were coexpressed with N-channels ($\mu\kappa$-N oocytes). When DAMGO was applied immediately after $\kappa$-receptor-mediated inhibition had been largely desensitized by a 10 min application of U50488H, the additional inhibition by DAMGO was $3.1\pm1.4\%$ ($n=7$, Figure 3a). The $\kappa$-desensitized $\mu$-response in the $\mu\kappa$-N oocytes was significantly smaller than that of the control experiment in which DAMGO was applied to $\mu$-N oocytes following a 10 min perfusion with U50488H ($31.1\pm3.3\%$ inhibition, $n=5$). In addition, U50488H produced $2.8\pm0.7\%$ inhibition following 10 min application of DAMGO to $\mu\kappa$-N oocytes (Figure 3b), which is also significantly smaller than that of $\kappa$-N oocytes ($32.9\pm4.4\%$ inhibition, $n=5$). In $\mu\kappa$-N oocytes, co-application of DAMGO and U50488H produced no additional inhibition ($35.8\pm3.1\%$ in-

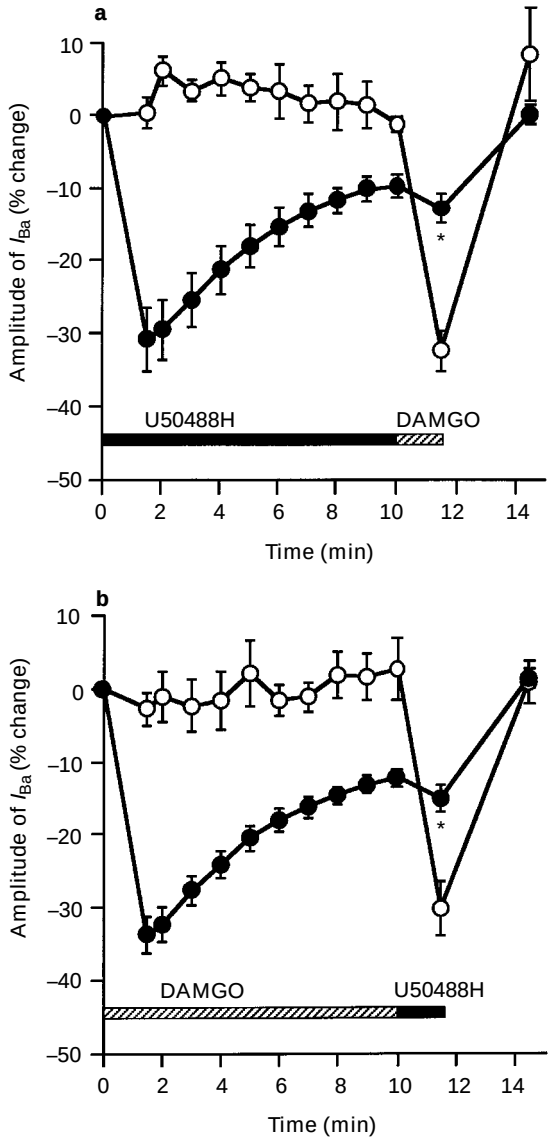


**Figure 3** Cross-desensitization between $\mu$- and $\kappa$-receptor-mediated inhibition of $Ca^{2+}$ channels. (a) $I_{Ba}$ was recorded from oocytes coexpressing N-channels with $\mu$- and $\kappa$-receptors (●, $n=7$) or with $\mu$-receptors (○, $n=5$) in the presence of 1 $\mu$M U50488H (from 0 to 10 min) followed by 1 $\mu$M DAMGO (from 10 to 11.5 min). (b) $I_{Ba}$ was recorded from oocytes coexpressing N-channels with $\mu$- and $\kappa$-receptors (●, $n=7$) or with $\kappa$-receptors (○, $n=5$) in the presence of 1 $\mu$M DAMGO (0–10 min) followed by 1 $\mu$M U50488H (10–11.5 min). $^*P<0.05$ vs $\mu$-N or $k$-N at 11.5 min.

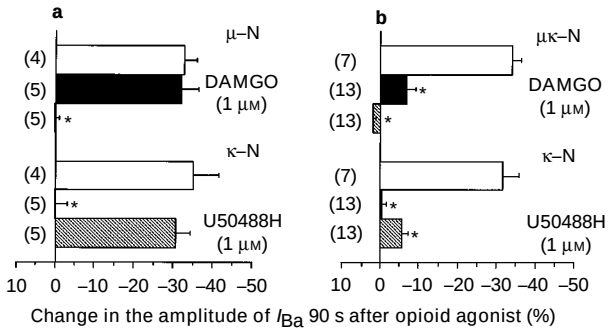


**Figure 4** Cross-desensitization between $\mu$- and $\kappa$-receptor-mediated inhibition after opioid pretreatment. (a) Oocytes expressing N-channels with $\mu$ ($\mu$-N) or $\kappa$ ($\kappa$-N) receptors were preincubated for 2 h with vehicle (open columns), 1 $\mu$M U50488H (solid columns) or 1 $\mu$M DAMGO (hatched columns). Subsequently, effect of 1 $\mu$M DAMGO ($\mu$-N) or 1 $\mu$M U50488H ($\kappa$-N) on the amplitude of $I_{Ba}$ was evaluated 90 s after perfusion. (b) The same experiment was done in oocytes expressing N-channels with $\mu$- and $\kappa$-receptors ($\mu\kappa$-N). Numbers of experiments are shown in parentheses. $^*P<0.05$ vs non-treated.

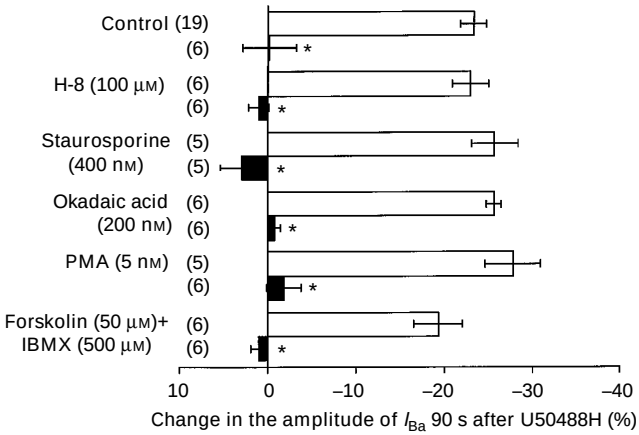


**Figure 5** Effects of various treatments relating protein kinase activities on the desensitization of $\kappa$-receptor-mediated N-type $Ca^{2+}$ channel inhibition. Oocytes coexpressing $\kappa$-receptors with N-channels were subjected to the pretreatments in the presence (solid columns) and absence (open columns) of 1 $\mu$M U50488H for 2 h. After a washout period, inhibition of $I_{Ba}$ by 1 $\mu$M U50488H was evaluated. Numbers of experiments are shown in parentheses. $^*P<0.05$ vs each control (in the absence of U50488H pretreatment).

hibition, $n=10$), as compared with a single application of DAMGO (33.8±2.4%, $n=7$) or U50488H (30.6±4.3%, $n=7$).

Cross-desensitization between $\mu$- and $\kappa$-receptors was further evaluated by a 2 h pretreatment with the agonists. In $\mu$-N and $\kappa$-N oocytes, only pretreatment with their specific agonists, i.e., DAMGO for $\mu$-N and U50448H for $\kappa$-N, caused irreversible desensitization. No effect of pretreatment with U50488H was observed on $\mu$-N or pretreatment with DAMGO on $\kappa$-N (Figure 4a). When $\mu\kappa$-N oocytes were pretreated either with DAMGO or U50488H, the other receptor response was also significantly, but not completely, cross-desensitized (Figure 4b). In these oocytes, any residual agonist that might have been present on the membrane was considered to be negligible after the 8 min washout, since the addition of naloxone (10 $\mu$M) to the 2 h pretreated $\mu\kappa$-N oocytes did not affect the amplitude of $I_{Ba}$ ($n=3$).

### *Involvement of protein kinases in the desensitization*

Since a primary role of second messenger-regulated serine-threonine protein kinases has been postulated for the incidence of heterologous desensitization (Huganir & Greengard, 1990), several agents modulating protein kinase or phosphatase activities were tested (Figure 5). Pretreatment of oocytes with these agents for 2 h did not produce any significant changes in the inhibition of N-channels by U50488H, except that the inhibition of $I_{Ba}$ was relatively reduced in the oocytes treated with forskolin and IBMX since the basal amplitude of $I_{Ba}$ was increased after this treatment. Preincubation of the oocytes with U50488H for 2 h in the presence of these agents did not affect the $\kappa$-agonist-evoked desensitization of $\kappa$-receptor responses. In addition, no change in the rate of acute desensitization during prolonged application of U50488H was observed (data not shown).

## Discussion

Both $\mu$- and $\kappa$-receptors can inhibit the N- and Q-type $Ca^{2+}$ channel current when coexpressed in *Xenopus* oocytes. The $\mu$- and $\kappa$-agonists at the concentrations used (1 $\mu$M) in these experiments are considered to evoke a full inhibition of N- and Q-channels via opioid receptors, since the mixed application of these agonists to $\mu\kappa$-N oocytes induced no further inhibition than a single application. This observation also suggests that N- and Q-channels are inhibited by $\mu$- and $\kappa$-receptors via a common mechanism. With regard to the PTX-sensitive G proteins, $G_o\alpha$ has been shown to mediate the inhibition of N-channels by $\delta$-receptors in cultured NG 108-15 cells (Taussig *et al*., 1992) where different G proteins are involved in the inhibitory effects of other transmitters, such as bradykinin (Wilk-Blaszczak *et al*., 1994) and somatostatin (Taussig *et al*., 1992). The involvement of $G_o\alpha$ in N-channel inhibition by $\mu$-receptors was also shown in rat dorsal root ganglia (Moises *et al*., 1994). In accord with these previous studies, the participation of G proteins, mainly $G_o\alpha$, in the coupling of $\kappa$-receptors with N- and Q-channels was shown by the following findings: (i) $\kappa$-agonist-induced inhibition of N-channels was diminished by a large depolarization prepulse and accompanied by a slowing of channel activation kinetics, both are characteristics of G protein-mediated inhibition of $Ca^{2+}$ channels (Ikeda, 1991); (ii) $\kappa$-agonist-induced inhibition of both N- and Q- channels was equally abolished by pretreatment with either PTX or anti-$G_o\alpha$ antiserum, implying that N- and Q-channels are inhibited by a common population of G proteins in oocytes. Also, we did not find any significant difference between $\mu$- and $\kappa$-receptors, as well as between N- and Q-channels, in the properties of G protein-mediated inhibition of neuronal $Ca^{2+}$ channels by stimulation of these opioid receptors.

The inhibition of N- and Q-channels by $\mu$- and $\kappa$-receptors was rapidly desensitized by a prolonged application of the opioid agonists. It is known that $\mu$- and $\kappa$-receptors coexist in spinal and central neurones to inhibit both N- and P/Q-channels (Schröder *et al*., 1991; Rhim & Miller, 1994). A desensitization over the order of minutes of neuronal $Ca^{2+}$ channel inhibition by opioids has been shown in neurones (Nomura *et al*., 1994; Rhim & Miller, 1994). However, no difference between receptors was found previously. Therefore, the major finding in this study was the differential time-courses of the development of opioid-evoked desensitization of N- and Q-channel inhibition. In contrast, there was no difference in the time-course of desensitization between $\mu$- and $\kappa$-receptors in conditions where $\mu$- or $\kappa$-agonists produced a similar degree of inhibition of either N- or Q-channels. With both $\mu$- and $\kappa$-receptor-mediated inhibition, N-channel responses were desensitized more rapidly than Q-channel responses. The difference in the time-course between N- and Q-channels was also apparent at a submaximum concentration (100 nM) of the $\kappa$-agonist. The time-course difference was observed only in the developmental phase of desensitization during prolonged agonist exposure, but not during the recovery period on washout after a 10 min to 2 h exposure to the agonists. These results suggest that uncoupling, but not re-coupling, of N-channels from G proteins is more rapid than that of Q-channels.

The time-course of $\mu$- and $\kappa$-receptor uncoupling from $Ca^{2+}$ channels is similar to that of agonist-induced phosphorylation of $\mu$- or $\delta$-receptors exogenously expressed in transfected mammalian cells (Pei *et al*., 1995; Zhang *et al*., 1996). It is suggested, by the use of dominant-negative GRK2, that receptor-specific GRKs play significant roles in the agonist-induced phosphorylation of $\delta$-receptors (Pei *et al*., 1995) and in the short term desensitization of transfected $\kappa$-receptors from adenylyl cyclase inhibition (Raynor *et al*., 1994). These observations indicate a primary role of GRKs in the rapid desensitization of opioid receptors. However, if phosphorylation by GRKs is truly specific to agonist-bound receptors, it will cause an agonist-specific (homologous) desensitization, as has been documented in many G protein-coupled receptors (Huganir & Greengard, 1990). In our experiments with $\mu\kappa$-N oocytes, an agonist-specific component of desensitization could be observed after cross-desensitization during both a 10 min and 2 h exposure to the other agonist. However, it was equivalent to less than 20% of the full desensitization (see Figures 3 and 4). These observations suggest that the ratio of heterologous components is not changed during the 10 min and 2 h-induced desensitization. Although the molecular nature of oocyte-native GRKs is not known, participation of phosphorylation produced via GRKs, is therefore thought to be minor in the uncoupling of $\mu$- and $\kappa$-receptors from $Ca^{2+}$ channels in the oocytes.

Activation of either $\mu$- or $\kappa$-receptors caused a cross-desensitization to each other in the oocytes coexpressing these opioid receptors together, suggesting that a common mechanism underlies the development of desensitization of both receptors. Heterologous desensitization between opioid receptors and other $G_i/F_o$-coupling transmitter receptors, such as $\alpha_2$-adrenoceptors or $\gamma$-aminobutyric acid $(GABA)_B$ receptors has been observed in neurones (Attali *et al*., 1989; Nomura *et al*., 1994). Although second messenger-regulated protein kinases are thought to be involved in the heterologous desensitization (Huganir & Greengard, 1990), no apparent effects of the agents that alter the phosphorylation state of cellular proteins were observed either on the desensitization of $\kappa$-response during a 10 min or 2 h exposure, or on the initial inhibition by the $\kappa$-agonist. These treatments have been shown to affect other signalling pathways in *Xenopus* oocytes (Kato *et al*., 1988; Ito *et al*, 1988; Kaneko *et al*., 1994b; Fukuda *et al*., 1996). Even if the effectiveness of the treatments on the various phosphorylation events is not complete, our results indicate that the heterologous component of $\mu$- and $\kappa$-receptor desensitization is resistant to changes in intracellular second messengers and protein kinases. However, we cannot ignore the possibility that other protein kinases are involved in the desensitization.

Previously, a phosphorylation-independent, rapid desensitization of $\mu$-receptor-mediated N-channel inhibition was shown in rat dorsal root ganglia (Nomura *et al*., 1994). A similar, phosphorylation-independent desensitization of $\mu$-receptors in the activation of GIRK in the oocytes has also been found by Kovoorm *et al*. (1995). The activity of GIRK is known to be regulated by $\beta\gamma$ subunits of G proteins (Reuveny *et al*., 1994). Recently, it was demonstrated that inhibition of $Ca^{2+}$ channel activity is also directly mediated by $G_{\beta\gamma}$ (Ikeda, 1996; Herlitze *et al*., 1996). Although our present results show, as discussed above, that there is no apparent difference in the population of $G_\alpha$ that mediate inhibitory signals from opioid receptors to $Ca^{2+}$ channels, subtypes of $G_{\beta\gamma}$ might differentially couple to N- and Q-channels. However, inhibition of $Ca^{2+}$ channels by each $G_{\beta\gamma}$ may be stable during a prolonged increase in free $G_{\beta\gamma}$ level, since GTP$\gamma$S-induced inhibition of $Ca^{2+}$ channels was not desensitized. A possible mechanism underlying the effector-dependent time-course of desensitization is the difference in the ability of $Ca^{2+}$ channel $\alpha_1$ subunits to 'turn-off' signals from G proteins. A recent study demonstrated a role for the $Ca^{2+}$ channel $\beta$ subunit with L-type $\alpha_1$ subunits in the promotion of GTPase activity during which the $Ca^{2+}$ channel deactivates the inhibitory signal from G proteins (Campbell *et al*., 1995). By supposing that the inhibitory signal of $G_{\beta\gamma}$ to $Ca^{2+}$ channels is turned off by hydrolysis of GTP-bound G$\alpha$ to form GDP which deprives $G_{\beta\gamma}$ of $Ca^{2+}$ channels, it is reasonable to assume that the inhibitory action of non-hydrolysable GTP$\gamma$S was not desensitized. Therefore, the uncoupling rate of $\mu$- and $\kappa$-receptors from the $Ca^{2+}$ channel complex may be determined by the $\alpha_1$ subunit of $Ca^{2+}$ channels. Such an 'effector-dependent' mechanism may be of importance in the heterologous desensitization of receptor-mediated modulation of $Ca^{2+}$ channels.

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