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κ-Opioid Receptor Activates an Inwardly Rectifying K^+ Channel by a G Protein-Linked Mechanism: Coexpression in *Xenopus* Oocytes

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SUMMARY

cRNAs encoding the κ-opioid receptor and an inwardly rectifying, G protein-coupled, K^+ channel were coinjected into *Xenopus* oocytes. The effects of κ-opioid receptor agonists and antagonists on the membrane currents in these oocytes were studied using the two-electrode voltage-clamp technique. The κ-opioid receptor agonists U69593 and dynorphin A induced a concentration-dependent inward current (EC_{50} of ~0.3 μM and ~30 nM, respectively) after coinjection of both cRNAs, whereas the μ-opioid receptor agonist [D-Ala^2,*N*-MePhe^4,Gly^5-ol]enkephalin (10 μM) and the δ-opioid receptor agonist [D-Pen^{2,5}]enkephalin (1 μM) had no effect. The agonist-induced inward current was reversible upon washing out of the agonists and was inhibited in the presence of the K^+ channel blocker Ba^{2+} (0.1 mM). The specific κ-opioid receptor antagonist norbinaltorphimine (0.1 μM) and the nonspecific opioid receptor antagonist naloxone (1 μM) abolished the agonist-induced currents. Furthermore, the agonist-induced currents exhibited rapid desensitization in the continuous presence of the agonists or after repeated application. Preincubation of the coinjected oocytes with pertussis toxin (400 ng/ml for 3 days or 1.5 μg/ml for 24 hr) abolished most of the agonist-induced activation of the inwardly rectifying K^+ current. We therefore conclude that specific stimulation of the κ-opioid receptor can activate the inwardly rectifying K^+ channel through a pertussis toxin-sensitive G protein.

Opioid receptors are present in various tissues and are especially abundant in neuronal cells (1, 2). Activation of these receptors produces analgesia as well as a variety of endocrine and autonomic responses (1, 3). Extensive studies on opioid pharmacology have suggested that there are at least three types of opioid receptors, designated as μ, δ, and κ (1, 4), and this has been confirmed using molecular biological techniques (2, 5). Stimulation of these receptors has been shown to decrease both Ca^{2+} currents (N and P/Q types) and adenylate cyclase activity throughout the nervous system (1, 3, 6, 7). Several studies have also shown that activation of μ- and δ-opioid receptors can increase one or more K^+ conductances (8–11). This appears to occur by coupling through G proteins to the activation of K^+ channels (7, 9, 10). These effects of opioids on K^+ and Ca^{2+} channels may underlie opioid receptor-mediated hyperpolarization and presynaptic inhibition, respectively, the hallmarks of opioid receptor activation in the nervous system.

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Activation of μ- and δ-opioid receptors has frequently been shown to produce neuronal hyperpolarization, and both of these receptor types can activate GIRK1 when expressed *in vitro* (12, 13). However, such effects have not been widely reported in the case of κ-opioid receptors, even though such receptors are also widely distributed in the nervous system. Recently, Grudt and Williams (14) reported that activation of κ-opioid receptors could indeed increase a K^+ conductance in substantia gelatinosa neurons, although other studies failed to demonstrate effects on K^+ channels in other neuronal cells (15, 16). Direct studies on the possible interaction between κ-opioid receptors and inwardly rectifying K^+ channels, using cDNA clones expressed in reconstituted heterologous systems, have not been reported. Recent molecular cloning of the κ-opioid receptor (17), rat GIRK1 (12, 18, 19), and the mouse brain GIRK family (20) has enabled us to evaluate this possibility. In the present study, we coinjected cRNAs encoding the κ-opioid receptor and rat GIRK1 into *Xenopus* oocytes and characterized receptor stimulation of the K^+ channel using the two-electrode, whole-cell, voltage-clamp technique. The results demonstrate that κ-opioid receptors are also capable of activating this inwardly rectifying K^+ channel.

ABBREVIATIONS: GIRK, G protein-coupled, inwardly rectifying, K^+ channel; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly^5-ol]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; norBNI, norbinaltorphimine; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

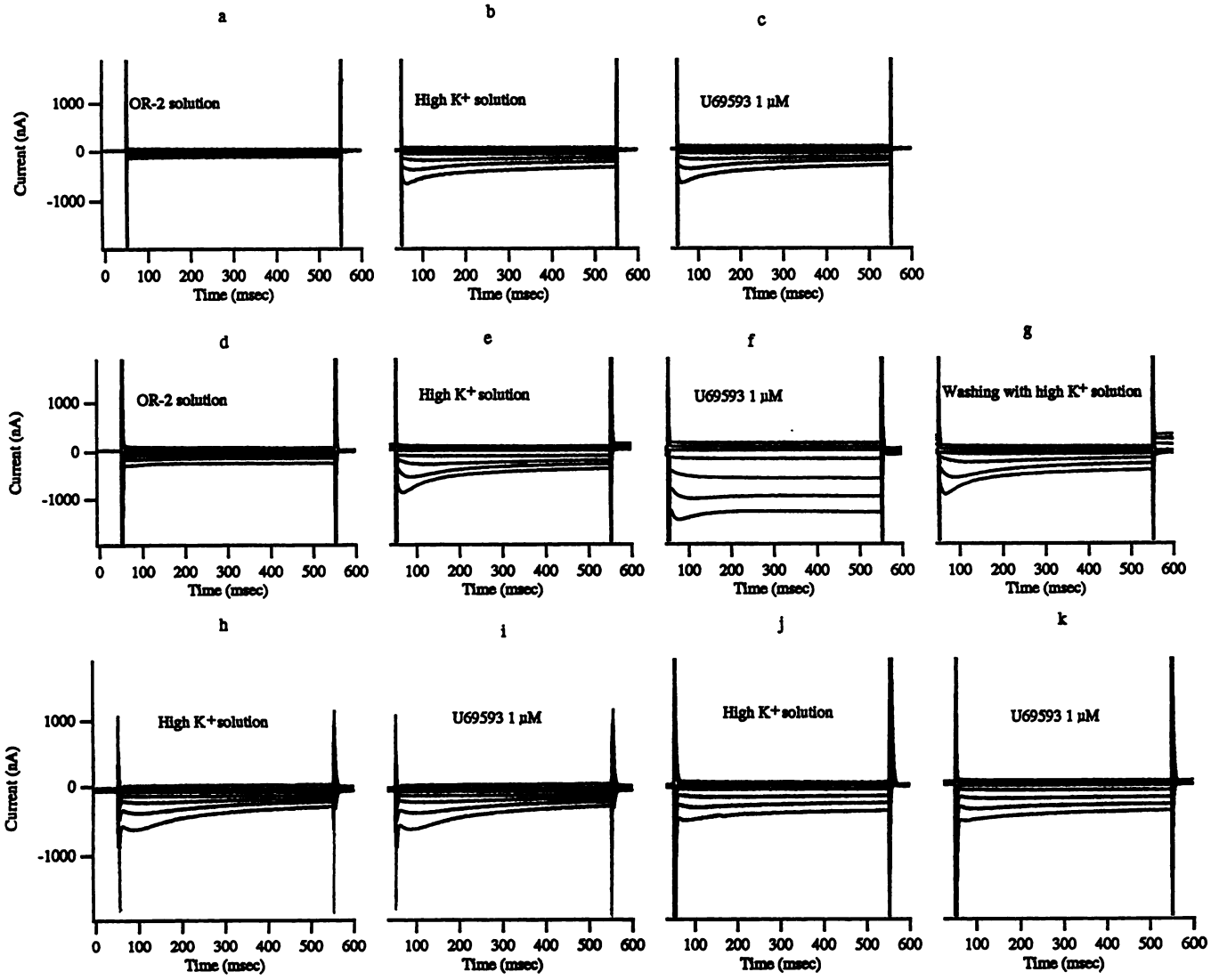

Fig. 1. Inwardly rectifying currents in *Xenopus* oocytes injected with water, coinjected with κ receptor plus GIRK1 cRNAs, or injected with each cRNA alone. a–c, Control oocytes, injected with 50 nl of water; d–g, oocytes coinjected with cRNAs encoding κ-opioid receptor and GIRK1; h and i, oocytes injected with κ-opioid receptor cRNA alone; j and k, oocytes injected with GIRK1 cRNA alone. Currents were recorded on days 6–7 after the injection, using the two-electrode, whole-cell, voltage-clamp technique. Membrane potential was held at 0 mV and stepped to 50, 20, −10, −40, −70, −100, and −130 mV. The recordings were carried out first in OR-2 perfusion solution (2.5 mM K^+) (a and d) and then in high-K^+ solution (50 mM K^+) in the absence (b, e, g, h, and j) or presence (c, f, i, and k) of the κ-opioid receptor agonist U69593 (1 μM), followed by rapid washout of the agonist with high-K^+ solution (g). The results shown represent an example of multiple similar recordings: a–c, n = 8; d–g, n = 24; h and i, n = 6; j and k, n = 18.

Experimental Procedures

Materials. U69593 and norBNI were purchased from Research Biochemicals (Natick, MA), whereas dynorphin A was from Bachem (Torrance, CA). Naloxone, DAMGO, DPDPE, $BaCl_2$, and other chemicals were from Sigma Chemical Co. (St. Louis, MO). The mCAP mRNA capping kit was from Stratagene (La Jolla, CA), the DNA sequencing kit from United States Biochemicals (Cleveland, OH), and T3 RNA polymerase from Ambion (Austin, TX). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The cloning plasmid pGEM-3Zf+ and cDNA miniprep columns were from Promega (Madison, WI), and pBluescript-KSII+ was from Stratagene. DNA and RNA molecular weight markers, cell culture medium, and agarose were from GIBCO-BRL (Grand Island, NY). *Xenopus laevis* were purchased from NASCO (Fort Atkinson, WI).

Subcloning of cDNAs and *in vitro* synthesis of cRNAs. The κ-opioid receptor cDNA, isolated from a mouse brain library, was a gift from Prof. G. I. Bell (University of Chicago) (17). The 1.4-kilobase κ-opioid receptor coding region was excised with *XbaI*/*SalI* and subcloned into pBluescript-KSII+. The sequence was confirmed using the T7 DNA sequencing kit (United States Biochemicals). The κ-opioid receptor cDNA was linearized with *XbaI* and the cRNA was synthesized using T3 RNA polymerase. Similarly, the cDNA encoding rat insulinoma GIRK1 (18) (in the pGEM-3Z vector) was linearized with *NdeI* and the cRNA was synthesized with T3 RNA polymerase.

Expression of cRNAs and two-electrode voltage-clamp recording in *Xenopus* oocytes. *Xenopus* oocyte injection and recording techniques were essentially as described (21). Defolliculated oocytes were incubated in OR-2 solution (2.5 mM KCl, 82.5 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 1 mM Na_2HPO_4, 100 μg/ml gentamicin) at 18° for 1–3 days before injection. Distilled water, κ-opioid receptor cRNA, and/or GIRK1 cRNA (50 nl) were injected into each oocyte (GIRK1 cRNA, 100 ng/μl or 5 ng/cell; κ-opioid receptor cRNA, 1 μg/μl or 50 ng/cell). Oocytes were further incubated

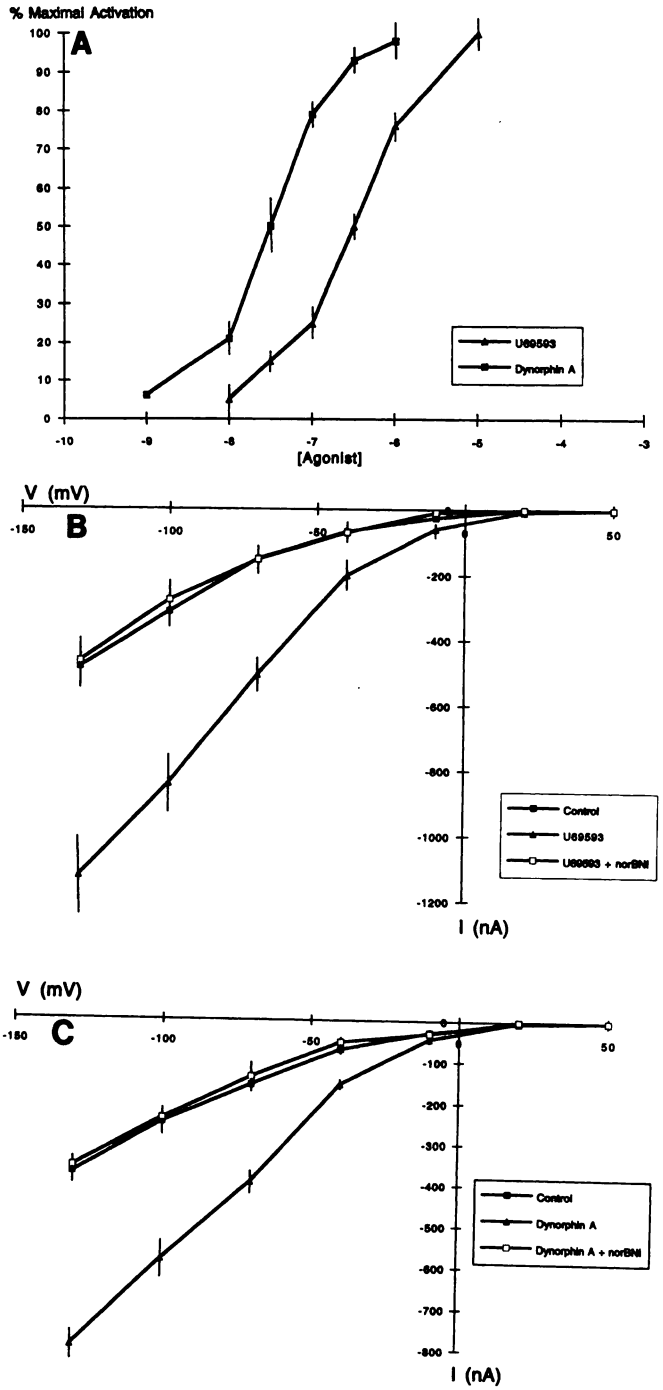

Fig. 2. Effects of the κ-opioid receptor agonists U69593 and dynorphin A and the antagonist norBNI on inwardly rectifying currents in coinjected oocytes. The oocytes were coinjected with κ-opioid receptor and GIRK1 cRNAs and the currents were recorded on days 6–7 after the injection. The membrane potential was held at 0 mV and stepped to 50, 20, −10, −40, −70, −100, and −130 mV. A, Dose-response curves for two κ-opioid receptor agonists. The drugs were added during perfusion with high-K^+ solution, followed by rapid washing out of the agonist (only three or four applications of the agonist were used for each oocyte, to minimize desensitization). The results shown are percentages of the maximal activation of the inwardly rectifying currents by the agonists (mean ± standard error, n = 24 for U69593 and n = 10 for dynorphin A). B and C, Current-voltage curves for U69593 and dynorphin A, respectively. Values are means ± standard errors (*bars*) of five or six individual experiments (five for dynorphin A and six for U69593). No bar is shown if the error is smaller than the symbol.

at 18° for 3–7 days before recording. On the day of recording, oocytes were first perfused and recorded in OR-2 solution, using the two-electrode, whole-cell, voltage-clamp technique, followed by recording in high-K^+ solution (50 mM KCl, 35 mM NaCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6), with or without agonists or antagonists. The recording protocol involved seven pulses (duration, 500 msec; pulse interval, 10 msec, with 50 msec before and after the train; holding potential, 0 mV), stepped to 50, 20, −10, −40, −70, −100, and −130 mV.

PTX treatment. PTX, in two experiments, was added to reach two final concentrations; 400 ng/ml PTX was added 3 days before recording and 1.5 μg/ml PTX was added 24 hr before recording. Oocytes were washed at least three times with OR-2 solution immediately before recording.

Results

Oocyte membrane currents were recorded using the two-electrode voltage-clamp technique. We observed an endogenous, oocyte, inwardly rectifying current in high-K^+ solution (50 mM K^+), compared with OR-2 perfusion solution (2.5 mM K^+) (Fig. 1, a and b). The κ-opioid receptor agonist U69593 (up to 10 μM) did not increase these endogenous, inwardly rectifying currents (Fig. 1c). Oocytes coinjected with both κ-opioid receptor and GIRK1 cRNAs exhibited no difference from uninjected oocytes or oocytes injected with water, when exposed to standard OR-2 solution or high-K^+ solution (Fig. 1, d and e). When these oocytes were perfused with U69593 (1 μM), inwardly rectifying currents were increased by 2–3-fold (Fig. 1f), an effect that was reversible when the agonist was washed out (Fig. 1g). Neither the μ-opioid receptor agonist DAMGO (up to 10 μM) nor the δ-opioid receptor agonist DPDPE (up to 1 μM) had any effect in the same cells, which showed a typical response to U69593 (1 μM) (data not shown). In addition, there was no agonist-induced effect on membrane conductance when oocytes were injected with either the κ-opioid receptor or GIRK1 cRNA alone (Fig. 1, h–k).

Two relatively selective κ-opioid receptor agonists, U69593 and dynorphin A, induced inwardly rectifying currents in a dose-dependent manner. The EC_{50} was ~0.3 μM for U69593 and ~30 nM for dynorphin A (Fig. 2A). The κ-opioid receptor-specific antagonist norBNI (0.1 μM) abolished the inward currents induced by the agonist U69593 (Fig. 2B) or by dynorphin A (Fig. 2C). The effect of norBNI was reversible, but only after perfusion with the high-K^+ solution for 10–20 min (data not shown). The nonspecific opioid receptor antagonist naloxone (1 μM) also prevented both agonists from activating the inwardly rectifying currents (data not shown). Both the endogenous, inwardly rectifying currents seen in high-K^+ solutions and the dynorphin A-induced currents were inhibited by Ba^{2+} (0.1 mM), as is typical for inwardly rectifying K^+ channels (Fig. 3).

Maximal current activation after application of agonists occurred within 0.5–1 min and exhibited rapid desensitization after prolonged perfusion with the drug. The agonist-induced maximal current was reduced by 19.2 ± 1.5% (mean ± standard error, n = 6) in the continuous presence of the agonist U69593 (1 μM) for 7 min and by 88.1 ± 5.2% after 22 min of perfusion (mean ± standard error, n = 6) (Fig. 4). Repeated application of an agonist after rapid washout with the high-K^+ solution showed less profound desensitization (10–20% in the fourth application), compared with that ob-

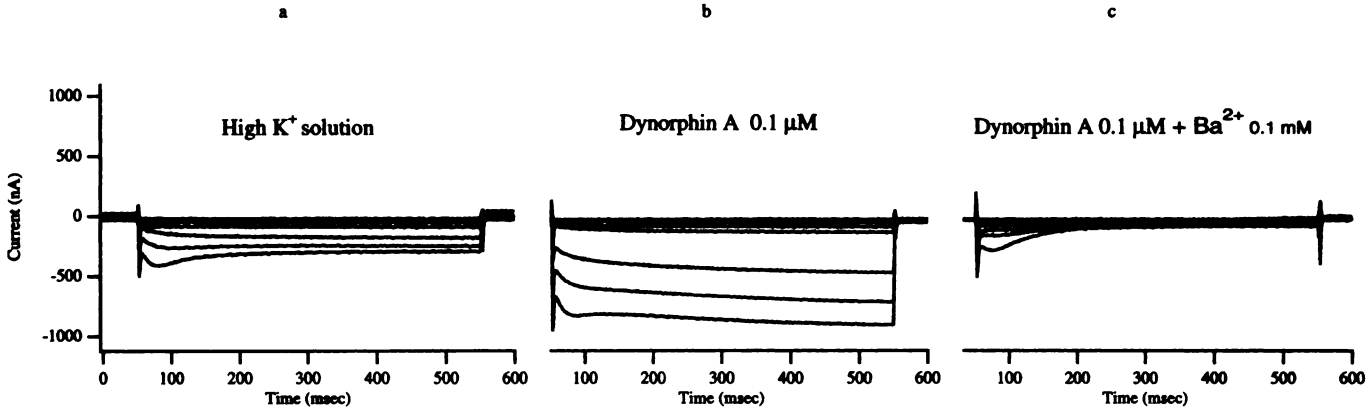

Fig. 3. Effect of Ba^{2+} on the agonist-induced, inwardly rectifying currents in coinjected oocytes. The oocytes were coinjected with both κ-opioid receptor and GIRK1 cRNAs and recorded on days 6–7 after injection. The membrane potential was held at 0 mV and stepped to 50, 20, −10, −40, −70, −100, and −130 mV. The recordings were made during perfusion with high-K^+ solution in the absence or presence of the agonist (0.1 μM) or agonist (0.1 μM) plus Ba^{2+} (0.1 mM). The results represent one of four similar individual oocyte recordings.

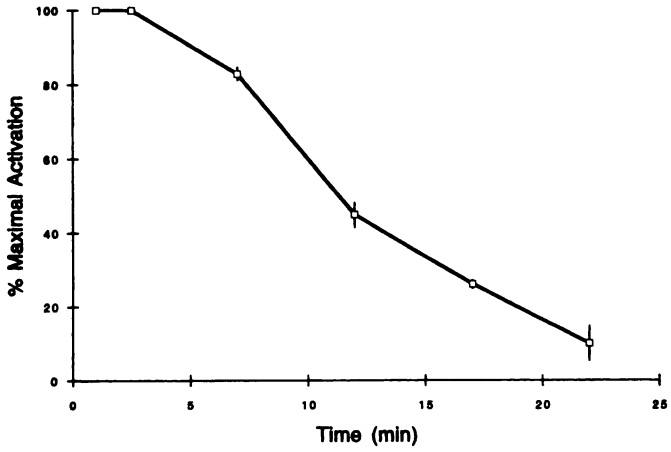

Fig. 4. Desensitization after prolonged perfusion with the agonist in coinjected oocytes. The oocytes were coinjected with κ-opioid receptor and GIRK1 cRNAs and recorded on days 6–7 after the injection. The membrane potential was held at 0 mV and stepped to 50, 20, −10, −40, −70, −100, and −130 mV. The recordings were made after high-K^+ solution perfusion in the continuous presence of the same agonist (1 μM). *Line*, time course of the maximum activation induced by agonist present continuously. The values are means ± standard errors (*bars*) (n = 6); no bar is shown if the error is smaller than the symbol.

served (80–90%) in the continuous presence of the same agonist for 20 min.

Heterotrimeric G proteins that couple opioid receptors to effectors in many systems are sensitive to inhibition by PTX (e.g., Refs. 3 and 7). We therefore examined the endogenous oocyte G proteins that couple the κ-opioid receptor to GIRK1, by preincubating the oocytes with PTX. Oocytes microinjected with either message or coinjected with both κ-opioid receptor and GIRK1 cRNAs were incubated in the absence or presence of PTX (400 ng/ml, for 3 days) and then tested for responses to U69593 (1 μM). The oocytes injected with GIRK1 or κ-opioid receptor cRNA alone, in the PTX-treated or nontreated groups, failed to respond to the agonists (data not shown). In oocytes coinjected with both cRNAs, PTX treatment abolished the agonist-induced activation of GIRK1 currents (Fig. 5). Similar results were obtained after preincubation of the oocytes with PTX at 1.5 μg/ml for 24 hr (data not shown). The results suggest that the κ-opioid receptor-mediated activation of GIRK1 K^+ currents occurred through PTX-sensitive G protein(s).

Discussion

Opioids give rise to a number of well known effects, including analgesia and sedation. These are presumably the result of opioid receptor-mediated inhibition of neuronal firing and neurotransmitter release in the central and peripheral nervous systems (7, 11). Pharmacological studies have revealed that there are at least three types of opioid receptors, designated as μ, δ, and κ (1, 3, 4). The κ-opioid receptor has been further divided into three types, κ_1, κ_2, and κ_3 (22, 23), whereas the δ- and μ-opioid receptors include subtypes 1 and 2 (24, 25). Three distinct opioid receptor genes, corresponding to the μ, δ, and κ receptors, have been isolated (2, 17, 26). In addition, several other genes have also been cloned and proposed as opioid receptor subtypes (5, 27, 28). However, their functional coupling and pharmacological characteristics remain to be determined.

Activation of μ-, δ-, and κ-opioid receptors inhibits adenylate cyclase activity and Ca^{2+} channels in many parts of the central and peripheral nervous systems (1, 3, 6, 7). Stimulation of μ- or δ-opioid receptors also produces activation of K^+ channels in various neuronal cells, such as those in rat locus ceruleus, guinea pig arcuate hypothalamus, guinea pig submucous plexus, and guinea pig myenteric plexus (7–10), whereas the effect of κ-opioid receptor agonists on K^+ channels has not been widely reported. Indeed, some studies failed to demonstrate κ-opioid receptor-mediated activation of K^+ channels in some neuronal cells (15, 16). It has therefore not been clear whether κ-opioid receptors normally also couple to this response or whether they differ from μ and δ receptors in this respect. For example, Fletcher and Chiappinelli (29) evaluated the effects of the κ-opioid receptor agonist U-50,488 on the membrane properties of presynaptic calyciform nerve terminals of chick ciliary ganglia. Those authors proposed that U-50,488 modulated neurotransmitter release by three mechanisms, including suppression of at least two K^+ conductances. However, activation of the κ-opioid receptor was recently reported to increase a K^+ conductance in the substantia gelatinosa, indicating that it could also activate this signal transduction pathway (14).

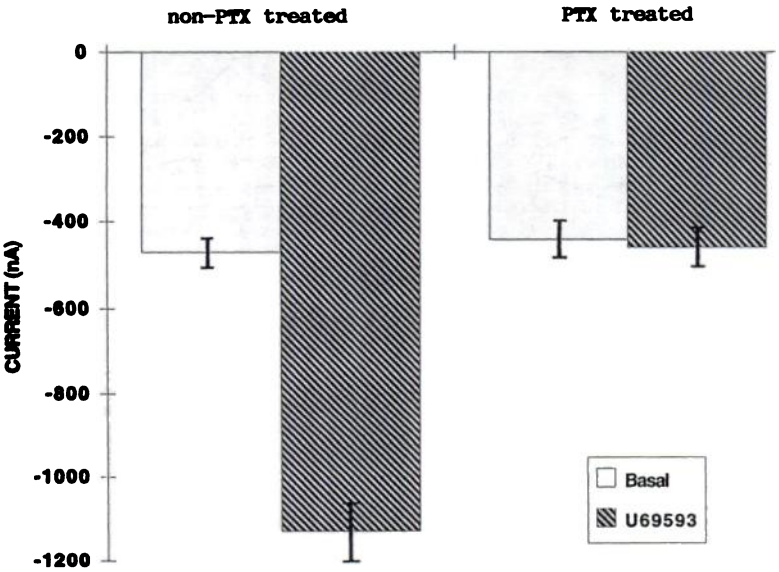

Fig. 5. Effect of PTX preincubation on agonist-induced inwardly rectifying currents in coinjected oocytes. The oocytes were coinjected with κ-opioid receptor and GIRK1 cRNAs and preincubated in the absence (*non-PTX treated*) or presence (*PTX treated*) of PTX (400 ng/ml, for 3 days) before the recordings (made on days 6–7 after the injection). The membrane potential was held at 0 mV and traced to 50, 20, −10, −40, −70, −100, and −130 mV. The currents were recorded after high-K^+ solution perfusion in the absence (*Basal*) or presence of the agonist U69593 (1 μM). The results shown are inward currents recorded at −130-mV membrane potential (means ± standard errors, $n = 10$ for non-PTX-treated or PTX-treated oocytes). The t test was used for comparing agonist-induced responses in the PTX-treated and non-PTX-treated oocytes. The agonist significantly increased the inward currents ($p < 0.01$) in the non-PTX-treated group but not in the PTX-treated group ($p > 0.05$).

The rat insulinoma cell-derived, inwardly rectifying, K^+ channel cDNA used in this study (18) is identical in coding sequence to the GIRK1 or KGA channels cloned from rat heart (12, 30). Stimulation of various G protein-coupled receptors, such as the M_2 muscarinic receptor, μ- and δ-opioid receptors, and serotonin type 1A receptor, can activate GIRK1 channels when coinjected into *Xenopus* oocytes (12, 13, 30).

In the present study, we have coinjected cRNAs encoding GIRK1 and the κ-opioid receptor into *Xenopus* oocytes and, using the two-electrode voltage-clamp technique, recorded the effects of various opioid receptor agonists and antagonists on the inwardly rectifying K^+ currents. The results clearly demonstrate that stimulation of the κ-opioid receptor activates the coexpressed GIRK1.

Many effects of opioids have been shown to be inhibited by PTX treatment, implicating the G proteins G_i and G_o in the coupling mechanism (3, 9, 13, 31, 32). Although some heterogeneity in the specificity of the G proteins involved in opioid receptor coupling has been observed (33), endogenous G proteins in *Xenopus* oocytes that mediate the interactions of several receptors with GIRK1 are at least partially sensitive to PTX (12, 13, 19). Our results are consistent with this finding. We observed that preincubation of oocytes with PTX abolished most of the κ agonist-induced activation of GIRK1.

Chronic opioid treatment has been observed to produce down-regulation of all three opioid receptor types or their receptor-mediated effects (9, 11, 34–37). The desensitization could occur at the level of the receptor, the receptor-G protein interaction, or the effector (13, 35–38). Various kinases may be involved in certain aspects of this desensitization (2, 13). We evaluated the effects of prolonged perfusion with or repeated applications of the κ-opioid receptor agonists on activation of GIRK1. Our data showed rapid desensitization under these conditions. In contrast, a recent report found that κ-opioid receptor-induced increases in K^+ conductance were not decreased after repeated application of the κ-opioid receptor agonists in substantia gelatinosa neurons (14), although such effects have been noted for the hyperpolarizing actions of μ- and δ-opioid receptor agonists (11, 13, 39, 40). This discrepancy may be explained by the involvement of different K^+ channels or different κ-receptor subtypes, or a number of other factors. Nevertheless, our data suggest that desensitization of their actions on K^+ channels may be a common feature of the actions of all opioid receptor agonists.

In summary, we have demonstrated that the κ-opioid receptor can also activate GIRK1 when coexpressed in *Xenopus* oocytes and that the process is sensitive to PTX. Thus, all three opioid receptor subtypes have now been shown to activate this channel, in addition to being able to inhibit adenylate cyclase and Ca^{2+} channels. Specific actions of different opioid receptor types in a given cell would thus depend on the specific cellular coexpression of the relevant effectors or G proteins. The overall paucity of observations describing κ-opioid receptor activation of K^+ conductances could therefore be due to the restricted coexpression of the appropriate signal conduction elements. Furthermore, it is also conceivable that not all subtypes of κ receptors activate the same signal transduction pathways.

Acknowledgments

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