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The dual modulation of GIRK1/GIRK2 channels by opioid receptor ligands

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Abstract

It is well known that activation of the cloned κ-opioid receptor by nanomolar concentrations of U50488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide), a selective κ-opioid receptor agonist, leads to the opening of GIRK1 channels. In this study, we demonstrate that the cloned κ-opioid receptor functionally couples to GIRK1/GIRK2 channels (G-protein-coupled inwardly rectifying K^+ channels), mimicking the probable heteromultimeric state of neuronal GIRK channels. We also show that micromolar concentrations of U50488H reduce GIRK1/GIRK2 current through direct GIRK1/GIRK2 channel block in a voltage-independent manner ($IC_{50} = 70.28 \pm 3.68$ μM). Similarly, it was found that propoxyphene, methadone, and naloxone also can block GIRK1/GIRK2 current. In contrast, elevated concentrations of morphine (up to 1 mM) did not cause channel block. The related inwardly rectifying K^+ channel, IRK1, was not affected by elevated concentrations of these drugs. We conclude that nanomolar concentrations of opioid receptor ligands activate GIRK1/GIRK2 channels through a receptor-mediated pathway, while micromolar concentrations of some opioid receptor ligands inhibit GIRK1/GIRK2 channels by direct channel block.

Keywords: Block; Opioid receptor; GIRK (G-protein activated inwardly rectifying K^+ channel); *Xenopus oocyte*

1. Introduction

Opioid analgesics exert their activity by inhibiting neurotransmission, and this is generally accepted as the mechanism underlying analgesia. The inhibition of neurotransmission is caused by coordinated changes at cellular level, including the closing of voltage-sensitive Ca^{2+} channels (Tallent et al., 1994), activation of G-protein-coupled inwardly rectifying K^+ (GIRK) channels (Ikeda et al., 1995), and alteration of cellular levels of second messengers such as cAMP (Wang and Gintzler, 1994) and inositol 1,4,5-triphosphate (Loh and Smith, 1990). Since activation of the GIRK channel induces membrane hyperpolarization, the GIRK channel is thought to play an important role in decreasing the excitability of neuronal cells and slowing the heartbeat. The cDNAs for the μ-, κ- and δ-opioid receptors (Yasuda et al., 1993; Kaufman et al., 1995) as well as for the GIRK channel subunits (Dascal et al., 1993; Kubo et al., 1993; Lesage et al., 1994) have been cloned. The atrial muscarinic K^+ channel is a heteromultimer of GIRK1 and GIRK4 whilst neuronal G protein-activated K^+ channels function as heteromultimers of GIRK1 and GIRK2 (Kofuji et al., 1995). However, recent evidence suggests that other types of heteromultimers also exist in native cells. For instance, GIRK2 was reported to form functional heteromultimers with GIRK4 (Ferrer et al., 1995) and with GIRK3 (Dissmann et al., 1996). The activation of GIRK channels is mediated by a pertussis toxin-sensitive G protein, via direct membrane-delimited pathway without involving intracellular second messenger systems. The $G_{\beta\gamma}$ subunit plays an important physiological role in the activation by direct binding to multiple regions of GIRK channels (Huang et al., 1997).

Nanomolar concentrations of μ-, κ- and δ-opioids also have stimulatory effects on neurotransmission, including an increased rate of neuronal firing and prolongation of the action potential (Crain and Shen, 1990). In guinea pig hippocampal CA3 neurons, U50488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide) and U69593 ((5α,7α,8β)-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4.5]Dec-8-yl)benzeneacetamide) were found to have both inhibitory and excitatory actions

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in the same concentration range (Alzheimer and ten Bruggencate, 1990). In this study, it was suggested that the non-opioid properties of these κ-opioid receptor agonists result from blockade of Na^+ channels. Kanemasa et al. (1995) previously reported that U50488H inhibits P-type Ca^{2+} channels by two mechanisms, including a high affinity component, which is produced by activation of κ-opioid receptors, whereas a low affinity component is due to its direct action on the P-type Ca^{2+} channel. Because direct actions of opioids have also been reported on K^+ channels (Horrigan and Gilly, 1996), we investigated the effect of different concentrations of selective and non-selective ligands (U50488H, methadone, propoxyphene, morphine, and naloxone) on *Xenopus laevis* oocytes coexpressing GIRK1/GIRK2 (Kir3.1 and 3.2) and cloned κ-opioid receptors by means of the two-microelectrode voltage clamp technique. We demonstrate that U50488H both activates and inhibits GIRK1/GIRK2 channels in a small concentration range (1–25 μM), and that the inhibitory effect is caused by direct channel blockade. Other opioid receptor ligands, including methadone, propoxyphene, and naloxone, but not morphine, display a similar action on GIRK1/GIRK2 channels. The related inward rectifier K^+ channel, Kir2.1 (IRK1), was not affected by elevated concentrations of these drugs.

2. Materials and methods

2.1. Expression in oocytes

Plasmids containing the entire coding sequence for the mouse κ-opioid receptor and for the mouse GIRK1 channel were subcloned into the vector pSP35T and designated as pSPORκ (Ikeda et al., 1995) and pSPOR/GIRK1 (Kobayashi et al., 1995). The mouse GIRK2 channel was subcloned into the vector pBScMXT and designated as pBScMXT/GIRK2 (Kofuji et al., 1995). For in vitro transcription, plasmids were first linearized either with *Eco*RI (for pSPOR/GIRK1), with *Sac*I (for pSPORκ) or with *Sal*I (for pBScMXT/GIRK2). Next, the capped cRNAs were synthesized from the linearized plasmids using the Riboprobe combination system (Promega, USA) with SP6 RNA polymerase (for pSPOR/GIRK1 and pSPORκ) or T3 RNA polymerase (for pBScMXT/GIRK2) in the presence of a cap analogue diguanosine triphosphate (Boehringer, Germany). The subcloning of mouse IRK1 cDNA into pGEM-HE, the synthesis of IRK1 cRNA (Tytgat et al., 1996) and the isolation of *X. laevis* oocytes was as previously described (Liman et al., 1992).

2.2. Electrophysiological recordings and analysis

Oocytes were co-injected with GIRK1 (0.5 ng/50 nl) and GIRK2 cRNA (0.5 ng/50 nl) alone or in combination with pSPORκ cRNA (1 ng/50 nl). Whole-cell currents from oocytes were recorded from 1 to 4 days after injection using the two-microelectrode voltage clamp technique (GeneClamp 500, Axon Instruments, USA). Resistances of voltage and current electrodes were kept as low as possible (0.2–1 MΩ) and were filled with 3 M KCl. Currents were filtered at 200 Hz, using a four-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was actively controlled. Capacitative and leak currents were not subtracted. All experiments were performed at room temperature (19–23°C). The oocytes were superfused with a high K^+ solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, pH 7.5). In this solution, the K^+ equilibrium potential (E_K) is close to 0 mV and enables K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials. Analysis of uninjected cells ($n = 3$), under the same experimental conditions as injected oocytes, revealed an inward rectifier current which mounted maximally 4% as compared to the current measured in injected oocytes in the voltage range of −140 to +40 mV. The pCLAMP program was used for data acquisition, and data files (Axon Instruments) were directly imported, analysed, and visualised with a custom made add-in for Microsoft Excel. Data were fitted to a standard logistic equation to compute the EC_{50}- or IC_{50}-values for analysis of concentration–response relationships. The inhibited or activated current was expressed as the percentage inhibition/control current and activation/control current, respectively. 100% was taken as the control current level. The values are expressed as mean ± S.E.M. and n is the number of oocytes tested. Statistical analysis of differences between groups was carried out with Student's t-test. A probability of 0.05 was taken as the level of statistical significance.

2.3. Compounds

Naloxone, an opioid receptor antagonist and *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide (U50488H) were purchased from Sigma (USA). Morphine hydrochloride was purchased from Bios (Belgium), propoxyphene hydrochloride and methadone hydrochloride were purchased from Federa (Belgium). All compounds were dissolved in high K^+ solution, stored at 5°C until use, and extracellularly applied.

3. Results

3.1. Activation of GIRK1/GIRK2 via opioid receptors

The functional coupling of G-protein coupled receptors (GPCRs) with GIRK1/GIRK2 channels, mimicking the probable heteromultimeric state of neuronal GIRK channels, is well established and has been demonstrated for dopamine D_2, muscarinic M_2, 5-HT_{1A} receptors (Doupnik

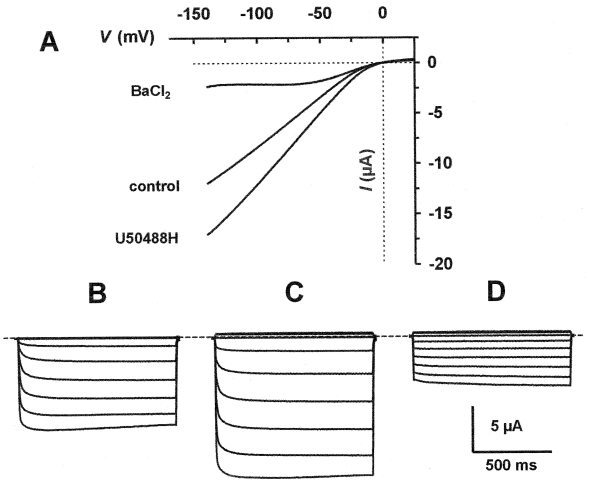

Fig. 1. Enhancement of GIRK current by application of the selective κ-opioid receptor agonist U50488H in *X. laevis* oocytes coexpressing the κ-opioid receptor (KOR), GIRK1, and GIRK2. Whole-cell currents were recorded in a high K^+ solution 3 days post-injection. (A) A 1-s voltage ramp protocol is shown from −140 to +40 mV from a holding potential of −20 mV. (1) Basal inwardly rectifying K^+ current. (2) Activated current after application of 1 μM U50488H. (3) Agonist-gated K^+ conductance after application of 300 μM Ba^{2+}. Representative voltage step experiments are shown in control condition (B) and after addition of 1 μM U50488H (C). Current traces in panel (D) represent the U50488H-induced currents (C minus B). Membrane potential was held at −20 mV, then stepped to 1-s test pulses varying from −140 to +40 mV in 20 mV increments. The most negative test potential (−140 mV) corresponds with the largest inward current. The current remaining after application of 300 μM Ba^{2+} was subtracted from total currents, except for currents shown in panel (A).

et al., 1997; Saitoh et al., 1997). To investigate whether heteromultimeric GIRK1/GIRK2 channels are affected by opioid receptors in a similar way as other neuronal GPCRs, we coexpressed GIRK1, GIRK2 and the cloned κ-opioid receptor in *X. laevis* oocytes. In a first series of experiments, a 1-s voltage ramp protocol was applied from −150 to +60 mV from a holding potential of −20 mV. In the oocytes co-injected with GIRK1, GIRK2, and cloned κ-opioid receptor cRNA, a large basal inwardly rectifying current was observed, which further increased after exposure to U50488H (54.59 ± 5.59%, $n = 12$). This agonist-gated K^+ conductance was blocked by application of 300 μM Ba^{2+} (Fig. 1A). The opioid-induced increase of the GIRK channel conductance requires the coexpression of an opioid receptor and is antagonized by naloxone (data not shown).

Next, we investigated the effect of opioid receptor stimulation on GIRK1/GIRK2 activation kinetic properties. From a holding potential of −20 mV we applied 1-s test pulses ranging from −140 to +40 mV in 20 mV increments. In the oocytes coexpressing GIRK1, GIRK2, and cloned κ-opioid receptor mRNA, a basal inward current was observed which slightly inactivated at −140 mV (Fig. 1B). Application of U50488H increased the inward current (Fig. 1C). The U50488H-induced current showed a slow phase of activation at the most negative test potential (Fig. 1D) and could be blocked by application of 300 μM Ba^{2+}, similarly as in the ramp protocol.

To investigate the relationship between agonist concentration and the activation response, a concentration–response curve was constructed. The agonist concentration required to produce a half-maximal effect (EC_{50}) was 9.81 ± 2.37 nM with a Hill coefficient of 1.00 ± 0.04 ($n = 12$, Fig. 2).

3.2. Inhibitory effects of opioid ligands on GIRK channels

When oocytes coexpressing GIRK1, GIRK2, and cloned κ-opioid receptors were voltage-clamped using a 1-s ramp protocol from −150 to +60 mV from a holding potential of −20 mV, an inhibitory effect was observed upon application of 50 μM U50448H (Fig. 3A). To investigate whether this effect was opioid receptor-mediated, oocytes were co-injected with GIRK1 and GIRK2 cRNA, thus, without cloned κ-opioid receptors. It was seen that the basal inwardly rectifying current was again blocked after application of 50 μM U50488H, with the maximal current being 53.68 ± 3.16% of control conditions ($n = 4$). This effect was reversible upon 5 min washout. At the highest concentration tested (1 mM), the maximal current was 14.41 ± 0.54% ($n = 4$). The block of the inward GIRK1/GIRK2 current by U50488H was characterized by an IC_{50}-value and a Hill coefficient of 70.28 ± 3.68 μM and 0.92 ± 0.03 ($n = 4$), respectively (Fig. 2). GIRK chan-

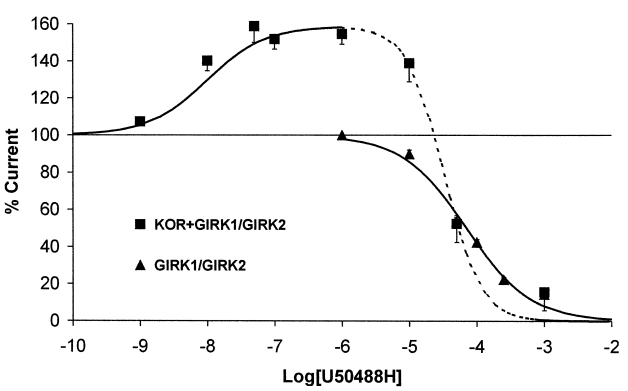

Fig. 2. Concentration–response relationships for the selective κ-opioid receptor agonist U50488H. Application of nanomolar concentrations of U50488H to *X. laevis* oocytes coexpressing the κ-opioid receptor, GIRK1 and GIRK2; application of micromolar concentrations of U50488H to *X. laevis* oocytes coexpressing GIRK1 and GIRK2 mRNA. 100% current refers to control current conditions. The inhibited or activated current was expressed as the percentage inhibition/control current and activation/control current, respectively. The calculated activation curve has been drawn according to the equation $I = I_{max}/[1+(EC_{50}/A)^{n_H}]$ and the inhibition curve according to the equation $I = 100-(100/[1+(IC_{50}/A)^{n_H}])$, where I represents the current response, I_{max} is the maximal current response, A is the concentration of agonist, and n_H the Hill coefficient. The dashed line, which illustrates the simultaneous activation and inhibition, has been drawn by eye.

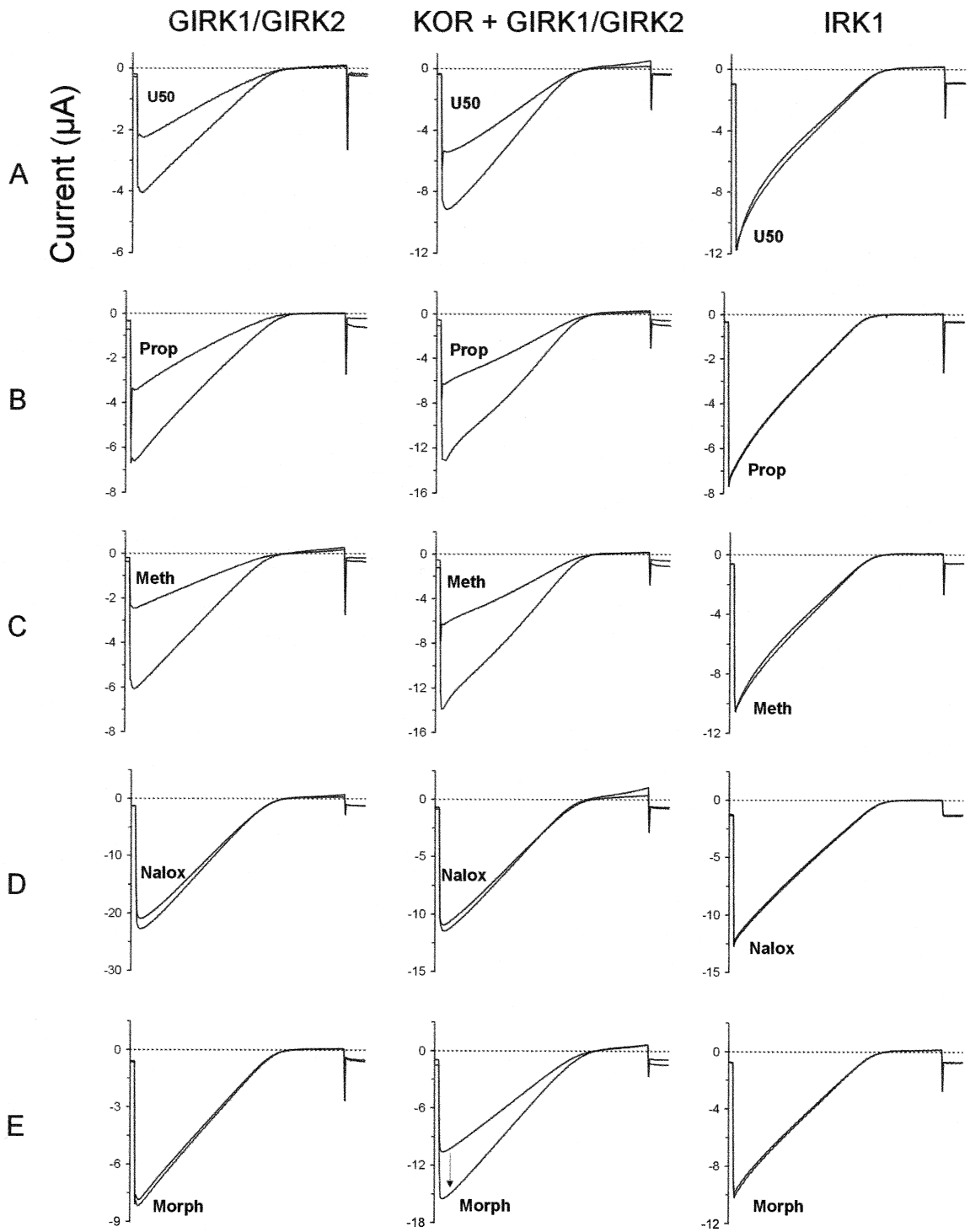

Fig. 3. 1-s voltage ramp protocol from −150 to +60 mV from a holding potential of −20 mV applied to *X. laevis* oocytes coinjected with GIRK1 and GIRK2 (left), or in combination with the cloned κ-opioid receptor (middle), and as compared to injection of IRK1 alone (right). Control responses and blocked GIRK currents are shown after application of 50 μM of U50488H (A), propoxyphene (B), methadone (C), naloxone (D), and morphine (E). The arrow in panel (E) indicates an activated current.

nel block did not show voltage dependency, as was verified by dividing inhibited currents by control currents in the range of −140 to +40 mV ($n = 4$).

In the oocytes co-injected with GIRK1, GIRK2, together with cloned κ-opioid receptor cRNA, the inwardly rectifying current was blocked in a similar way.

In addition to the inhibitory effects of the selective κ-opioid receptor agonist U50488H, we were interested to see whether other non-selective opioid receptor ligands would also block the GIRK current. Micromolar concentrations of propoxyphene and methadone (non-selective μ-opioid receptor agonists), and naloxone (a non-selective opioid receptor antagonist) were applied to oocytes expressing GIRK1 and GIRK2 channels. The effect on GIRK current of 50 μM of each of the respective drugs is shown in Fig. 3. Maximal currents were $49.58 \pm 2.00\%$ ($n = 4$), $49.71 \pm 4.45\%$ ($n = 4$), and $93.61 \pm 2.22\%$ ($n = 4$) of control conditions, for propoxyphene, methadone, and naloxone, respectively. This effect was reversible upon 5 min washout. It is also illustrated in Fig. 3 that GIRK channel block was not influenced by coexpression of the κ-opioid receptor. The GIRK current was blocked in a concentration-dependent and a voltage-independent manner and the calculated IC_{50} values were 53.31 ± 6.01 μM ($n = 4$), 56.12 ± 11.70 μM ($n = 4$), and 513.91 ± 23.61 μM ($n = 4$), respectively, with calculated Hill coefficients of 0.75 ± 0.03, 0.92 ± 0.07, and 1.08 ± 0.08, respectively (Fig. 4). In contrast, elevated concentrations of morphine (a non-selective μ-opioid receptor agonist) up to 1 mM did not show an inhibition. Interestingly, in oocytes coexpressing GIRK1, GIRK2, and cloned κ-opioid receptors, the application of 50 μM of morphine caused an activation of GIRK current, as shown in Fig. 3, panel (E).

3.3. Inhibitory effects of opioid ligands on the IRK1 channel

To compare the effect of opioid receptor ligands on GIRK channels with another inwardly rectifying K^+ channel, we applied micromolar concentrations of U50488H, propoxyphene, methadone, naloxone, and morphine on oocytes expressing the IRK1 channel. The effect on the IRK current of 50 μM of each of the respective drugs is shown in Fig. 3. Elevated concentrations up to 1 mM did not cause IRK1 channel block, in contrast to GIRK1/GIRK2 channel block.

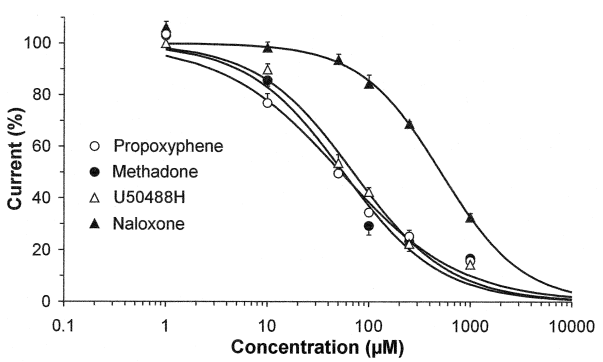

Fig. 4. Concentration–response relationships for the selective κ-opioid receptor agonist U50488H, propoxyphene, methadone and naloxone in *X. laevis* oocytes coinjected with GIRK1 and GIRK2 mRNA. The percentage current is the ratio of the remaining current to the control response after drug application. 100% current refers to control current conditions. Each point represents the mean and S.E.M. of percentages current obtained from four oocytes. Data points are fitted using the same logistic equation as in Fig. 2.

4. Discussion

In the present study, we used *X. laevis* oocytes to coexpress the cloned κ-opioid receptor with heteromultimeric GIRK channels, consisting of GIRK1 and GIRK2 subunits. The functional coupling of GIRK1 with μ-, κ- and δ-opioid receptors was reported by Ikeda et al. (1995) and Henry et al. (1995). Recently, it was found that GIRK channels function as heteromultimers and that neuronal GIRK channels presumably consist of GIRK1 and GIRK2 subunits, since they are expressed in the same areas of the brain (Kofuji et al., 1995). In *Xenopus* oocytes injected with the GIRK1 mRNA alone, the GIRK channel expressed has been shown to form a heteromultimer of GIRK1 and XIR (*X. laevis* inwardly rectifying K^+ channel, GIRK5), which is an endogenous *Xenopus* oocyte polypeptide homologous to rat GIRK4 (Hedin et al., 1996). Our results strongly demonstrate that the κ-opioid receptor functionally couples to GIRK1/GIRK2 channels, mimicking the probable heteromultimeric state of neuronal GIRK channels.

Furthermore, it has been reported that the coexpression of GIRK1 and GIRK2 display new kinetic properties after activation by the muscarinic receptor (Kofuji et al., 1995). It was shown that coexpressed subunits show a slow phase of activation, which cannot be explained as a simple algebraic sum of the phenotypes for GIRK1 and GIRK2 alone. In this study, we show that GIRK activation kinetics are altered in a similar manner by opioid receptor stimulation.

The use of the *Xenopus* expression system also enabled us to study the effects of opioid receptor ligands at nanomolar concentrations on the cloned κ-opioid receptor, functionally coupled to heteromultimeric GIRK channels. Our experiments on *Xenopus* oocytes expressing GIRK1, GIRK2, and cloned κ-opioid receptors show that, the selective κ-opioid receptor agonist U50448H has an EC_{50} of 9.81 nM. When U50488H was applied in micromolar concentrations, we found that GIRK current was blocked up to $85.59 \pm 0.54\%$ ($n = 4$) at the highest concentration tested. This might lead to the wrong conclusion that U50488H, which is a selective κ-opioid receptor agonist at nanomolar concentrations, acts as an inverse agonist when applied at micromolar concentrations. Indeed, when micromolar concentrations of U50488H were applied to *Xenopus* oocytes coexpressing GIRK1 and GIRK2 subunits without cloned κ-opioid receptors, GIRK currents were blocked in a similar way. The calculated IC_{50} value for U50488H was 70.28 ± 3.68 μM. The coexpression of the

κ-opioid receptor with GIRK1/GIRK2 channels produced a simultaneous receptor-mediated GIRK channel activation and direct GIRK channel block in the presence of 1–1000 μM U50488H. From these results, it is likely to assume that GIRK channel activation mainly occurs in the nanomolar range (1–1000 nM), while direct GIRK channel blockade dominates in the micromolar range (25–1000 μM).

To investigate whether GIRK channel block is a general feature of all opioid receptor ligands we applied micromolar concentrations of other non-selective opioids. We found that naloxone, a non-selective opioid antagonist blocks GIRK channels with lower affinity than U50488H (IC_{50} = 513.91 ± 23.61 μM). Propoxyphene and methadone showed a higher affinity block with an IC_{50}-value of 53.31 ± 6.01 μM and 56.12 ± 11.70 μM, respectively. Exceptionally, elevated concentrations of morphine (up to 1 mM) did not cause inhibition but an activation of GIRK currents when cloned κ-opioid receptors were coexpressed. This correlates with previous findings that μ-opioid agonists such as morphine and fentanyl display a very low affinity for δ- and κ-opioid receptors (Ohta et al., 1995).

Our results provide evidence for an as yet undescribed specific interaction of some opioid receptor ligands, when applied at micromolar concentrations, with GIRK1/GIRK2 channels. Indeed, an accumulation of micromolar concentrations of opioids cannot account for a non-specific inihibition of ion channels expressed in oocytes membranes, since (1) not all opioid receptor ligands, used in this study, block GIRK1/GIRK2 channels when applied at micromolar concentrations (e.g., morphine), and (2) none of these opioids showed an effect on the related inwardly rectifying K^+ channel, IRK1. Although IRK1 and GIRK channels have been cloned since 6 years, only very recently, the first high-affinity inhibitor (tertiapin, $K_i = 8.2$ nM) for GIRK1/GIRK4 and ROMK1 (an inwardly rectifying, ATP-sensitive K^+ channel, originally identified in renal outer medulla) channels was reported (Jin and Lu, 1998). Mutagenesis studies showed that tertiapin inhibits the channel from the external side by binding to the ion channel pore region. Interestingly, the related IRK1 channel was found insensitive to tertiapin, suggesting that tertiapin and the drugs used in our study, may block GIRK channels at a common interaction site.

Since the opioid receptor ligands used in this study were shown not to affect IRK1 channels, which are thought to generate most of the neurons' intrinsic inward rectification, the compounds are unlikely to exert substantial influence on the intrinsic properties of neurons. A likely and biologically important corrollary of GIRK channel blockade by opioid receptor ligands is that the potency of other neuromodulators known to recruit GIRK channels will dramatically decline, a possible source of unexpected and paradoxical drug effects.

Although our results may explain some aspects of paradoxical effects caused by opioid analgesics, an important question is whether GIRK channel block by methadone, propoxyphene, and naloxone contributes to toxic effects of these drugs. Propoxyphene is a relatively weak opioid receptor agonist with an analgesic potency 20 to 40 times less than that of (−)methadone (Robbins, 1955) and toxic blood concentrations ranging from 3 to 180 μM have been reported (Cravey et al., 1974). Taken into account that propoxyphene accumulates in several tissues, including brain, reaching the concentration range we have used in this study, GIRK channel block may indeed contribute to toxic and lethal effects of propoxyphene. Whilst propoxyphene may be capable of binding to opioid receptors and blocking GIRK channels at similar concentrations, this is less likely for methadone and naloxone.

In conclusion, we demonstrated that nanomolar concentrations of opioid receptor ligands activate GIRK1/GIRK2 channels through a receptor-mediated pathway, while micromolar concentrations of some opioid receptor ligands inhibit GIRK1/GIRK2 channels by direct channel block. Our results suggest that a concentration-dependent dual modulatory mechanism may help to explain hyperalgesic and paradoxical effects.

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cDNA encoding the mouse IRK1 channel was a gift from L.Y. Jan (Howard Hughes Medical Institute, SF, USA).

Jan Tytgat is a research associate of the F.W.O. Vlaanderen.

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