Identification of Dynorphins as Endogenous Ligands for an Opioid Receptor-like Orphan Receptor*

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To identify the endogenous ligands for a cloned orphan receptor that shares high degrees of sequence homology with opioid receptors, this orphan receptor was expressed in Xenopus oocytes and in mammalian cell lines CHO-K1 and HEK-293. The coupling of the receptor to a G protein-activated K+ channel was used as a functional assay in oocytes. Endogenous opioid peptide dynorphins were found to activate the K+ channel by stimulating the opioid receptor. This activation was dose-dependent, with EC50 values at 45 and 37 nM for dynorphin A and dynorphin A(1-13), respectively. The dynorphin effect was antagonized by the non-selective opioid antagonist naloxone but at rather high concentrations in the micromolar range. Naloxone also caused a rightward shift of the dose-response curve for dynorphin A, suggesting a competitive antagonism mechanism. In transiently transfected cells, 5 µM dynorphin A(1-13) inhibited the forskolin-stimulated cyclic AMP increase by 51 and 35% in CHO-K1 and HEK-293 cells, respectively. Other classes of endogenous opioids, i.e. enkephalins and endorphins, caused very little activation of this receptor. These results suggest that this orphan receptor is a member of the opioid receptor family and that dynorphins are endogenous ligands for this receptor.

After the cloning of all three major types of opioid receptors, μ, δ, and κ (1), a novel receptor was cloned from several species by using a homology screening technique (2-8). The amino acid sequence of this receptor is similar to those of the opioid receptors. However, whereas the three opioid receptors share about 70% amino acid sequence similarity among themselves, there is a reduced homology level at about 65% between this receptor and any of the opioid receptors (4). This suggests that this novel receptor may be a member of the opioid receptor family, different from the other three receptors, and was thus designated various names including XOR1 (4). In vitro and in vivo assay systems have been used to find its ligands. A synthetic non-selective opioid agonist etorphine was shown to inhibit adenyl cyclase in CHO-K1 cells transfected with this receptor clone, and diprenorphine and naloxone antagonized the inhibitory action of etorphine (2). However, since no endogenous ligands have been found for this novel receptor, it remains an "orphan" receptor.

To identify endogenous ligands for an orphan receptor, one could perform receptor binding with radiolabeled compounds. This approach has been used for the identification of 5HT1A receptors (9). However, this approach is limited in its scope, since many endogenous ligands are not available in radiolabeled form. An alternative approach is to use a functional assay, in which the orphan receptor is expressed in cells and a measurable cell function is used as a readout of receptor activation, such as changes in second messenger levels or membrane currents. In this way compounds can be tested in unlabeled form and, if a proper cellular function is chosen that the orphan receptor does couple to, there is an opportunity to identify the endogenous ligands.

Xenopus oocytes have been used in many functional studies for membrane receptors and ion channels (10, 11). In particular, opioid receptors have been shown to couple to a cloned G protein-activated K+ channel (KGA) in oocytes (12-15). Because of the high degree of homology of this orphan receptor with the opioid receptors, it may also be capable of functionally coupling to KGA in Xenopus oocytes, thus constituting an assay system for identifying endogenous ligands that can activate this receptor. We took such an approach, using XOR1 cloned from rat brain (4) for oocyte expression. Here, we report the results of this study.

EXPERIMENTAL PROCEDURES

Materials—Opioid ligands were from Peninsula Laboratories Inc., Research Biochemicals International, Bachem Inc., and the National Institute of Drug Abuse. CHO-K1 and HEK-293 cell lines were from the American Type Culture Collection. Xenopus laevis was from Xenopus I and African Fish Farm. Culture media were from HyClone Laboratories Inc., and Life Technologies, Inc. In vitro transcripion kit T7 mMessage mMACHINE was from Ambion. The cyclic AMP assay kit was from DuPont NEN. All other chemicals were from Sigma.

Oocyte Injection and Electrophysiology—Xenopus oocytes were prepared as described (16). In vitro transcribed RNA was injected into oocytes (1-2 ng/oocyte) by a Drummond automatic micropipette. Oocytes were incubated in 50% L-15 medium supplemented with 0.8 mM glutamine and 10 μg/ml gentamicin at 18°C. Three days after injection, oocytes were voltage-clamped at -80 mV with two glass electrodes (filled with 3 M KC1 and having a resistance of 2-3 MΩ) using an Axodamp-2A (Axon Instruments) under the control of pCLAMP software (Axon Instruments). Oocytes were superfused with either ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2 and 5 mM HEPES, pH 7.5) or a high potassium solution (HK: 96 mM KCl, 2 mM NaCl, 1 mM MgCl2, 1.5 mM CaCl2, and 5 mM HEPES, pH 7.5). The membrane currents were recorded with the aid of the pCLAMP software and on a Gould chart recorder.

Cell Transfection and Cyclic AMP Assay—The CHO-K1 and HEK-293 cells were transiently transfected with either XOR1 cDNA in pcDNA

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† This abbreviations used are: XOR1, rat opioid receptor-like novel receptor; KGA, G protein-activated potassium channel; hK, high potassium solution; dyn, dynorphin; CHO-K1, Chinese hamster ovary cells; HEK, human embryonal kidney cells.
CMV (Invitrogen) or vector only (as control) by the calcium phosphate method (17). Three days after transfection, cells were harvested by trypsin treatment, washed, and resuspended in serum-free medium. Cells were treated with ligands in the presence of 10 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine at 37°C for 20 min. The reaction was terminated by adding 1/3 volume of 0.25 M HCl. The mixture was boiled for 5 min and centrifuged at 14,000 g for 10 min. The supernatant was dried under vacuum and dissolved in assay buffer. The cAMP in cells was determined by the nonacetylated method using the radioimmunoassay kit (DuPont NEN) according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

Orphan Receptor Is Functionally Activated by Some Endogenous Opioid Peptides—Activation of opioid receptors has been shown by their ability to couple to a cloned KGA in Xenopus oocytes (12–15). Due to the sequence similarity of XOR1 to the opioid receptors, it is possible that this orphan receptor may also couple to the KGA channel in oocytes. To test this possibility, XOR1 and KGA were coexpressed in oocytes, and functional coupling of the receptor to the K+ channel was detected by two-electrode voltage clamp. A, membrane current traces recorded at a holding potential of −80 mV. Oocytes were superfused with hK solution in the presence or absence of 300 nM dynA (left trace) or 300 nM dynA-(1–13) (right trace) as indicated. B, bar graph of the membrane currents evoked by different endogenous opioid ligands at a concentration of 1 μM. Data are presented as mean ± S.E., with n of 3–8.

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FIG. 1. Coupling of the opioid receptor-like orphan receptor (XOR1) to a G protein-activated K+ channel (KGA). In vitro transcribed cRNAs of XOR1 and KGA were coinjected into oocytes. Functional coupling of the receptor to the K+ channel was detected by two-electrode voltage clamp. A, membrane current traces recorded at a holding potential of −80 mV. Oocytes were superfused with hK solution in the presence or absence of 300 nM dynA (left trace) or 300 nM dynA-(1–13) (right trace) as indicated. B, bar graph of the membrane currents evoked by different endogenous opioid ligands at a concentration of 1 μM. Data are presented as mean ± S.E., with n of 3–8.

FIG. 2. Dose-response curves of XOR1-KGA coupling activated by dynA and dynA-(1–13). A, an example of membrane current traces showing the calculation method for the ligand-evoked response. Oocytes were superfused with different solutions as indicated. Ligands were dissolved in hK solution and applied as indicated by the bar above the current trace. Spontaneous current (I0) is the current when the K+ concentration is increased by switching the bath solution from ND96 to hK. Receptor-activated current (Ia) is the one when a ligand is applied to activate the receptor. The ratio of Ia/I0 represents the extent of receptor activation by the ligand. B, dose-response curve of dynA-evoked receptor activation. The results are presented as the percentage of the maximum activation. Data are mean ± S.E. (n = 4–5). Each oocyte was used only once to avoid desensitization. The smooth line represents a computer-aided curve fitting to the data using a simple Michaelis-Menten model. The EC50 calculated from the curve was 45 ± 6 nM (mean ± S.E., n = 2). C, dose-response curve of dynA-(1–13)-evoked activation. The EC50 calculated from the curve was 37 ± 9 nM (mean ± S.E., n = 2).

A

B

C
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**FIG. 3.** Effect of naloxone on the activation of XOR1 by dynorphins. A, two representative current traces recorded with a holding potential at −80 mV. The dotted line represents a current trace induced by 300 nM dyn A-(1–13) in hK solution (dashed line below the trace). The solid line was recorded with the sequential perfusion of solutions hK, 300 nM dyn A-(1–13) in hK, and 300 nM dyn A-(1–13) plus 1 mM naloxone in hK. B, change of response evoked by 300 nM dyn A-(1–13) in the presence of different concentrations of naloxone. The data are presented as the percentage of the maximum I_{max} (see Fig. 2 legend), shown as mean ± S.E. (n = 4–5). C, naloxone produces a rightward shift of dose-response curve for dyn A. The dose-response curves were generated the same way as in Fig. 2, in the absence (●) or presence (○) of 10 μM naloxone in the dyn A-containing hK solution. The EC_{50} value for dyn A was changed from 45 to 372 nM by naloxone.

Fragments, the potency decreased with the decrease in the peptide length. Also, when the first amino acid tyrosine was missing, such as in dyn A-(2–17) or dyn A-(2–13), there was no receptor activation (data not shown).

**Activation of Receptor by Dynorphins Is Dose-dependent.—**To further study the activation of XOR1 by dynorphins, we chose the most potent peptides dyn A and dyn A-(1–13) to perform dose-response experiments. Due to the variability of individual oocytes in expressing exogenous proteins, we normalized the receptor-mediated response by taking the ratio of the receptor-activated current (I_a) over the spontaneous current in hK solution (I_s), shown in Fig. 2A. As has been observed before (12–15, 19), in cells expressing the KGA channel, there is an increase in membrane K^+ current (the spontaneous current I_s) when the K^+ concentration in the extracellular solution is increased, such as by switching the oocyte bath solution from ND96 to hK. When the KGA channel is coexpressed with a receptor that is capable of coupling to the channel, application of an agonist for the receptor induces a receptor-activated current I_a (Fig. 2A). Since both I_s and I_a are related to the expression level of KGA, their ratio serves as an index that is not heavily influenced by the variability in the expression level in different cells. Using this method, the dose-response relations were determined for dyn A and dyn A-(1–13). As shown in Fig. 2, A and C, both dynorphin peptides induce receptor-mediated activation of the K^+ channel in a dose-dependent manner. A sigmoid curve was fitted to the data for each dynorphin peptide, and an EC_{50} value was calculated to be 45 and 37 nM for dyn A and dyn A-(1–13), respectively (Fig. 2, B and C).

Opioid receptors are capable of regulating membrane conductance in neurons, leading to membrane hyperpolarization and a decrease in the neuronal firing rate or inhibition of neurotransmitter release (20). The KGA has been shown to exist in the brain (12, 19) and was suggested to be the K^+ channel mediating the neuronal effects of neurotransmitters including opioids. The functional coupling of XOR1 to KGA in Xenopus oocytes suggests that this receptor may mediate the activation of the KGA in the central nervous system and function in the neuronal regulation.

Naloxone Is Low Potency Antagonist for XOR1—Reversibility by the non-selective opioid antagonist naloxone has been considered a major criterion for classification of an "opioid" action (21). To determine the antagonism of naloxone for XOR1, we tested naloxone in oocyte assays. As shown in Fig. 3A, after the receptor-mediated activation of the K^+ current by dyn A-(1–13), inclusion of 1 mM naloxone in the bath solution blocked the current. The blockade of naloxone for XOR1 activation was dose-dependent (Fig. 3B). At concentrations of 10 or 100 μM, which are enough to completely block the other opioid receptor effects, naloxone only partially reversed the activation of XOR1. At a high concentration of 1 mM, naloxone completely blocked the receptor’s activation (Fig. 3, A and B).

Does naloxone antagonize dynorphin effects on this receptor in a competitive manner, as for the other opioid receptors? By...
using naloxone with different concentrations of dyn A to perform dose-response experiments, we found that 10 \( \mu M \) naloxone caused a parallel rightward shift of the dose-response curve for the dyn A-activated response (Fig. 3C). The parallel shift of the dose-response curve suggests that the antagonism by naloxone at the XOR1 is competitive in nature. In this case, the EC_{50} value of dyn A was shifted from 45 nm without naloxone to 372 nm with 10 \( \mu M \) naloxone. These data gave the apparent dissociation constant \( K_a \) of naloxone for the receptor at about 1.37 \( \mu M \) using the Tallarida variation of Schild analysis (22). Compared with the nanomolar affinity values of naloxone for \( \mu \) opioid and \( \kappa \) opioid receptors (23), this value is 2–3 orders of magnitude higher, thus making naloxone a low potency antagonist at this novel receptor.

Activation of XOR1 Can Inhibit Forskolin-stimulated CAMP Increase—A hallmark of the cellular effect for all three major types of opioid receptors, \( \mu \), \( \kappa \), and \( \delta \), is that their activation results in a reduced level of intracellular cAMP (24), an important second messenger in cell functions. This effect is mediated by inhibition of the adenylyl cyclase activity upon opioid receptor activation. Mammalian cells have been used as an efficient expression system for cloned opioid receptors, and it has been shown that all three cloned opioid receptors are negatively coupled to adenylyl cyclase (1). To examine whether this novel receptor is capable of coupling to the cAMP pathway, we transiently transfected CHO-K1 and HEK-293 cells with XOR1 and tested the cAMP level after the treatment by forskolin with or without the endogenous ligand dynorphins. As shown in Fig. 4, 5\( \mu M \) dyn A (1–13) inhibited the forskolin-stimulated cAMP increase by 51\% in CHO-K1 and by 35\% in HEK-293, respectively. These values are significantly different from the forskolin only treated cells (p < 0.01), indicating that XOR1 is capable of negatively coupling to the adenylyl cyclase. The cells transfected with plasmid vector pRC/CMV alone did not show any inhibition to the cAMP increase (Fig. 4). Thus, like the other three major opioid receptors, \( \mu \), \( \delta \), and \( \kappa \), this novel opioid receptor also exerts an inhibitory effect on the cAMP/adenylyl cyclase pathway.

XOR1 Is a Novel Opioid Receptor Distinct from the \( \kappa \) Opioid Receptor—The above results indicate that of the three major classes of endogenous opioid peptides, only dynorphins are capable of activating this orphan receptor, whereas the other two classes, namely endorphins and enkephalins, are not. This raises the question of whether this novel receptor is more closely related to the \( \kappa \) opioid receptor, the receptor type that dynorphins are capable of activating by inhibition of the adenylyl cyclase. Mammalian cells have been used as an efficient expression system for cloned opioid receptors, and it has been shown that all three cloned opioid receptors are negatively coupled to adenylyl cyclase (1). To examine whether this novel receptor is capable of coupling to the cAMP pathway, we transiently transfected CHO-K1 and HEK-293 cells with XOR1 and tested the cAMP level after the treatment by forskolin with or without the endogenous ligand dynorphins. As shown in Fig. 4, 5\( \mu M \) dyn A (1–13) inhibited the forskolin-stimulated cAMP increase by 51\% in CHO-K1 and by 35\% in HEK-293, respectively. These values are significantly different from the forskolin only treated cells (p < 0.01), indicating that XOR1 is capable of negatively coupling to the adenylyl cyclase. The cells transfected with plasmid vector pRC/CMV alone did not show any inhibition to the cAMP increase (Fig. 4). Thus, like the other three major opioid receptors, \( \mu \), \( \delta \), and \( \kappa \), this novel opioid receptor also exerts an inhibitory effect on the cAMP/adenylyl cyclase pathway.

XOR1 Is a Novel Opioid Receptor Distinct from the \( \kappa \) Opioid Receptor—The above results indicate that of the three major classes of endogenous opioid peptides, only dynorphins are capable of activating this orphan receptor, whereas the other two classes, namely endorphins and enkephalins, are not. This raises the question of whether this novel receptor is more closely related to the \( \kappa \) opioid receptor, the receptor type that dynorphins interact with at high affinity (25). To investigate this question, \( \kappa \)-selective agonists were used in the oocyte functional assay. Two \( \kappa \)-selective compounds, U-50,488 and U-69,593, were used, because these compounds have nanomolar affinity at the \( \kappa \) opioid receptor. When applied in the bath solution to stimulate the XOR1, however, neither of the compounds induced any detectable \( K^+ \) current even at micromolar concentration (data not shown). Affinity values of various compounds for the cloned \( \kappa \) opioid receptor have been reported, and Table I shows a comparison of these values between the XOR1 and the \( \kappa \) opioid receptor from rodent species. It can be seen that while dynorphins have subnanomolar affinity values at the \( \kappa \) opioid receptor, its EC_{50} values at this novel receptor are above 30 nm. Also, synthetic \( \kappa \)-selective agonists U-50,488 and U-69,593 do not activate this receptor. In addition, naloxone has a 1.37 \( \mu M \) affinity value at this receptor, whereas it displays nanomolar affinity values at the \( \kappa \) opioid receptor. These data indicate that the XOR1 is distinct from the \( \kappa \) opioid receptor.

It is interesting to note that, while the overall sequence homology between this orphan receptor and each of the cloned \( \mu \), \( \delta \), and \( \kappa \) opioid receptors (23), there is apparent resemblance of the highly negative charges in the second extracellular loop between this receptor and the \( \kappa \) opioid receptor. There are seven negatively charged amino acid residues in this region for both the \( \kappa \) receptor and this receptor, whereas there are only two negatively charged residues in either the \( \mu \) or \( \delta \) receptor (4). In opioid receptors, this loop is the longest among the three extracellular loops with a low level of sequence homology (26), suggesting the possibility that it may be involved in ligand binding specificity for the receptors. Indeed, this region in the \( \kappa \) receptor has been shown to be critical for high affinity binding of dynorphin peptides (27), which are basic peptides with five positively charged amino acid residues for both dyn A and dyn A-(1–13) (18, 28). The highly negative charges in this region of the orphan receptor may contribute to the interaction between dynorphins and the receptor.

What might be the physiological role of this novel receptor? Reports in the literature have provided certain clues. In vivo studies showed that dynorphins caused certain physiological effects that may not be mediated entirely through the \( \kappa \) opioid receptor, such as biphasic anti-nociception effect, motor effects, immunomodulation, inflammation response, and modulation on respiration and temperature control (29–32). Our results suggest the possibility that this novel opioid receptor may play a role in mediating some of the dynorphin effects that are not contributed by the \( \kappa \) opioid receptor.

In conclusion, the data presented in this report indicate that this opioid receptor-like orphan receptor is indeed a novel member of the opioid receptor family, because it can be activated by the endogenous ligand dynorphins. Similar to the other opioid receptors, this receptor also inhibits adenylyl cyclase activity. Unlike the other opioid receptors, however, naloxone does not effectively block this receptor, suggesting that it may mediate some of the "non-opioid" effects of dynorphins. Endorphins or enkephalins are rather ineffective at this receptor, whereas several of the dynorphin peptides can activate it, suggesting that there may be other endogenous ligands for this receptor.

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