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ACCELERATED COMMUNICATION

Functional Selectivity of Orphanin FQ for Its Receptor Coexpressed with Potassium Channel Subunits in *Xenopus laevis* Oocytes

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SUMMARY

An opioid-like receptor has been cloned by several groups of researchers and recently shown to be activated by an endogenous heptadecapeptide termed orphanin FQ (or nociceptin). We isolated the corresponding mouse cDNA and coexpressed it in *Xenopus laevis* oocytes with the potassium channel subunits Kir3.1 (GIRK1) and Kir3.4 (CIR, rcKATP). Orphanin FQ evoked potassium currents, with 50% of the maximal effect at ~1 nM; [Tyr^1]orphanin FQ was equally effective, and des-Phe-orphanin FQ was without activity. Dynorphin A, dynorphin(1–9), dynorphin(1–13), and α-neoendorphin were >100 times less potent, and other agonists active at μ-, δ-, and κ-opioid receptors had no effect. Naloxone (1 μM) and norbinaltorphimine (1 μM) had no antagonist action. Conversely, oocytes expressing κ receptors responded to dynorphin (half-maximal concentration, 0.3 nM) but not to orphanin FQ. Thus, both κ and orphanin FQ receptors readily couple to potassium channels, but the highly selective activation by dynorphin and orphanin FQ is consistent with distinct functional pathways *in vivo*.

Pharmacological studies have established that there are three main types of opioid receptors in mammals (reviewed in Refs. 1–3). At μ receptors, the enkephalin analog Tyr-D-Ala-Gly-NMePhe-Gly-ol is a selective agonist, and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 is an antagonist. At δ receptors, the enkephalin analogue Tyr-D-Pen-Gly-Phe-D-Pen is a selective agonist, and naltrindole is an antagonist. At κ receptors, U-69593 {D-(5a,7a,8b)-(+)-*N*-methyl-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide} and CI-977 {[(−)-(5b,7b,8a)-3,4-dicholoro-*N*-methyl-*N*[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzo[b]furan-4-acetamide; enadoline} are selective agonists, and binaltorphimine is a selective antagonist. Enkephalin is the probable endogenous ligand at μ and δ receptors, whereas dynorphin is an endogenous ligand selective for κ receptors (1, 4).

Three cDNA clones have been isolated that direct the expression of proteins with all of the pharmacological characteristics of μ, δ, and κ receptors (reviewed in Ref. 3). They are ~60% identical at the amino acid level. Their distribution in the brain and other tissues conforms generally to the distribution of μ, δ, and κ receptors determined with autoradiographic or pharmacological experiments. We (2) and others (5–13) have isolated a fourth cDNA (termed ORN7; GenBank accession No. X91813), which encodes a protein that is clearly homologous to the opioid receptors. The RNA is widely distributed in the central nervous system, including several regions in which opioids are known to have cellular actions. Two groups (14, 15) recently identified an endogenous ligand for this receptor (OFQ or nociceptin: FGGFTGARKSARKLANQ). This basic heptadecapeptide is structurally related to dynorphin (YGGFLRRIRPKLKWDNQ), which raises the question of whether dynorphin or any of its shorter fragments might also activate the OFQ receptor.

A common action of opioids on mammalian neurons is to open membrane potassium channels; they do this by stimulating the production of activated G protein subunits, which then interact directly with inwardly rectifying channels (16). The channel is formed from at least two distinct subunits, termed Kir3.1 [also known as GIRK1 (17) and KGA (18)] and Kir3.4 [also known as CIR (19) or rcKATP (20)]. The heterologous coexpression of opioid receptors and potassium channel subunits thus provides a convenient functional assay for

ABBREVIATIONS: OFQ, orphanin FQ (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GIRK, G protein-activated inwardly rectifying potassium.

ligands at these receptors (17, 21–26). In a recent report (24), it was shown that dynorphin could activate the OFQ receptor in *Xenopus laevis* oocytes coexpressing potassium channels, as measured by the induction of a potassium current, and it was concluded that dynorphins are endogenous ligands for the receptor. We have confirmed the observation that dynorphin can activate the OFQ receptor, but a direct comparison of OFQ with dynorphin and its analogs at both the κand OFQ receptors shows that the ligands are receptor selective over a wide concentration range; this leads us to conclude that dynorphin is unlikely to be a natural ligand at the OFQ receptor.

Materials and Methods

Ligands were obtained from Bachem (King of Prussia, PA), Peninsula Laboratories (Belmont, CA), Research Biochemicals (Natick, MA), or Sigma Chemical (St. Louis, MO). OFQ and analogs were synthesized using 9-fluorenylmethoxycarbonyl chemistry on a Applied Biosystems peptide synthesizer 420A.

A random- and oligo(dT)-primed cDNA library made from mouse brain in λZAP II cDNA library (Stratagene, La Jolla, CA) was probed at low stringency with a ^{32}P-labeled 280-bp fragment of the mouse μ receptor gene (exon 2, corresponding to transmembrane domains II–IV). After rescreening of the positive clones, DNA inserts were rescued into the pBluescript plasmid with helper phage, restriction mapped, and sequenced. The homology with other sequences in databases was analyzed using the Genetics Computer Group (Madison, WI) program.

OFQ receptor, human κ receptor (27), rat κ receptor (gift of H. Akil, University of Michigan, Ann Arbor, MI) (28), rat Kir3.1 (GIRK, gift of G. Buell, Glaxo Institute, Geneva, Switzerland), and rat Kir3.4 (gift of J. Adelman, Vollum Institute, Portland, OR) (19) cDNAs were linearized and transcribed *in vitro* with T7 polymerase; cRNA was estimated on a formaldehyde gel, aliquoted, and kept frozen at −80° until injection. Female *X. laevis* were anesthetized in 0.4% tricaine and decapitated. Oocytes were defolliculated in a solution containing 87.5 mM NaCl, 2 mM KCl, 5 mM HEPES, pH 7.5, and 2 mg/ml collagenase IA, and injected with 50 nl of the RNA mixture. They were incubated at 18° for 2–7 days in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 5 mM sodium pyruvate, pH 7.5. This solution (flowing at 5 ml/min) was used in electrophysiological recordings, made with two glass microelectrodes containing potassium chloride (3 M). A GeneClamp amplifier and pClamp software (Axon Instruments, Burlingame, CA) were used. In most experiments, oocytes were clamped at a holding potential of −60 mV. Drugs were applied by changing the superfusing solution. The high potassium solution contained 90 mM KCl, 3 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 with NaOH. Currents were generally normalized to that evoked by a fixed concentration of agonist in the same oocyte to account for the variability and time dependence of receptor and channel expression. Averaged values are expressed as mean ± standard error.

Results and Discussion

Isolation of OFQ receptor. One plasmid (ORN7) had a 2-kb insert containing an open reading frame that predicted a protein of 367 amino acids with seven hydrophobic regions; this sequence was 55%, 53%, and 53% identical to the mouse μ (29), δ (30), and κ (31) receptors, respectively, and very similar (93.8–99.5% identical) to the sequence determined for the OFQ receptor from mouse, rat, guinea pig, and humans by others (5–13). A 3.4-kb RNA was detected by Northern blot analysis in mouse brain, cortex, and spinal cord but not in heart, lung, liver, spleen, or kidney.

Coexpression of OFQ receptor with potassium channels. OFQ induced inward currents when applied to oocytes that had been injected with RNAs encoding the OFQ receptor as well as the two potassium channel subunits (Fig. 1); currents were not seen in oocytes injected only with the potassium channel subunits. The current developed within several seconds of the agonist reaching the oocyte, and after a 2-min application, it returned to base-line within 5–10 min. The concentration of OFQ causing the maximal effect was ~30 nM, and the concentration causing half-maximal effect was ~1 nM (Fig. 1B). The OFQ analog in which the amino terminus phenylalanine was replaced by tyrosine ([Tyr^1]OFQ) also evoked currents (at 10 nM, 94 ± 14 nA) that were not different from those caused by OFQ (at 10 nM, 91 ± 22 nA in the same five oocytes). However, the analog lacking the amino-terminal amino acid (des-Phe-OFQ) had no effect in four oocytes that gave robust responses to OFQ.

Fig. 2 shows that dynorphin also caused an inward current, and this was not seen in oocytes that had been injected only with the Kir3.1/Kir3.4 RNA combination or only with the OFQ receptor RNA. The time course of these currents was similar to that seen with OFQ, and there was little obvious desensitization with repeated applications. However, the effective concentrations were 100–300 times higher than those of OFQ and much higher than those required to elicit currents at the κ receptor (see below). This action of dynorphin was mimicked by α-neoendorphin, carboxyl-terminally truncated analogs (down to dynorphin(1–9)), but not by des-Tyr-dynorphin; the relative actions of these analogs are shown in Fig. 2B. The observation that dynorphin(1–8) lost

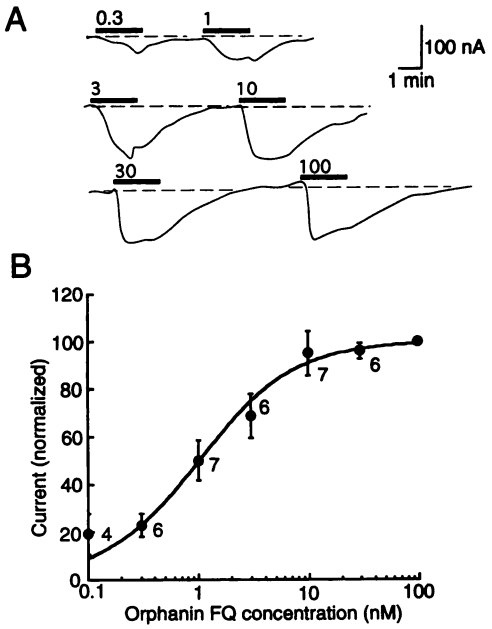

Fig. 1. Potassium currents induced by OFQ in oocytes injected with RNAs encoding OFQ receptor and potassium channel subunits. A, Inward current responses to OFQ applied at the concentration indicated (in nM). *Bars*, durations. The three traces shown are a continuous pen recording (96 mM potassium, −60 mV). B, Results from several experiments such as that shown in A. The amplitude of the inward current is normalized to the current produced by OFQ (100 nM) in the same cells (126 ± 18 nA; seven oocytes). *Points*, mean ± standard error for number of oocytes indicated; curve is best fit to a simple hyperbolic function.

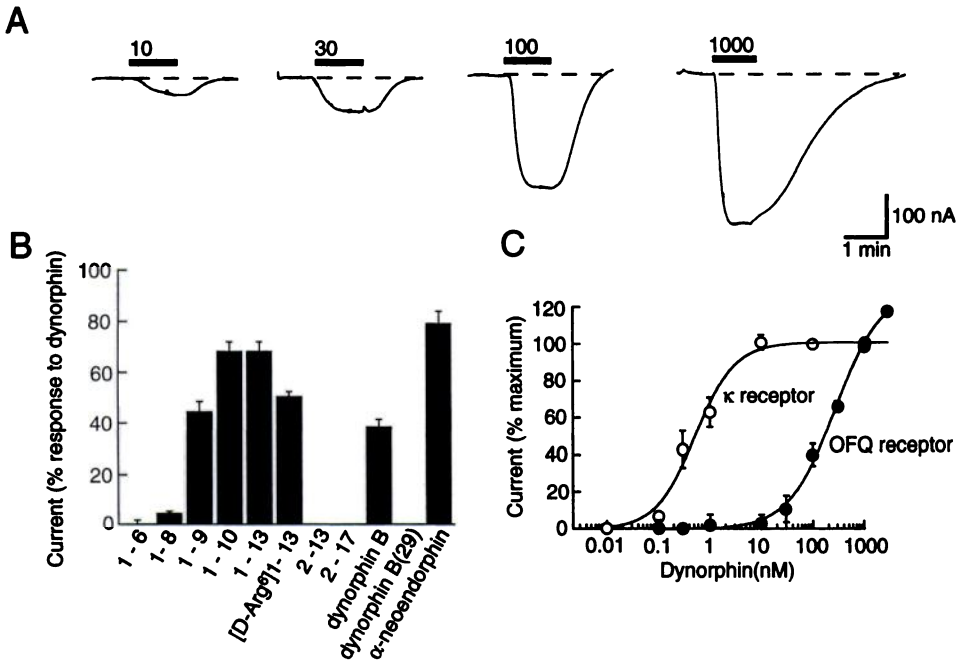

Fig. 2. Potassium current induced by dynorphin and some analogs in oocytes injected with RNAs encoding OFQ receptor and potassium channel subunits. A, Typical inward currents (96 mM potassium, −60 mV). *Bars*, durations. B, Effects of a series of dynorphin analogs on oocytes expressing OFQ receptor. Currents were measured at −60 mV during application of 1 μM peptide and normalized to current evoked in the same oocyte by 1 μM dynorphin; values are mean ± standard error for three to five oocytes for each peptide. C, Results of several experiments such as that shown in A. Currents were measured at −60 mV and normalized to that produced by 1 μM dynorphin (OFQ). Also shown are currents induced by dynorphin (normalized to effect of 100 nM dynorphin) in oocytes injected with κ receptor RNA. Values are mean ± standard error for five oocytes. Curves are best fits to hyperbolas.

almost all activity contrasts with the result at κ receptors similarly expressed (see below).

No effect was seen with the following peptides: Tyr-D-Ala-Gly-NMePhe-Gly-ol (10 μM), Tyr-D-Pen-Gly-Phe-D-Pen (10 μM), β-endorphin, morphiceptin, hemorphin-4 (Tyr-Pro-Trp-Thr-OH), Leu-enkephalin, Met-enkephalin, γ-aminobutyric acid, baclofen, histamine, 5-hydroxytryptamine, deamino-[Cys^1,D-Arg^8]vasopressin, arginine vasopressin, formyl-Met-Leu-Phe-OH, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 (a neuropeptide FF analog), Tyr-MIF-I (Tyr-Pro-Leu-Gln-NH_2; [Tyr^0]melanocyte-stimulating hormone release-inhibiting factor), GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2; growth hormone-inducing peptide), neuropeptide Y, oxytocin, neuromedin N, galanin, and somatostatin (all at 1 μM; three to five oocytes for each). No effect was seen with morphine (10 μM, three oocytes) or the κ-selective agonists U-69593 (10 μM; four oocytes) and CI-977 (1 μM; three oocytes). Even high concentrations of naloxone (10 μM) and norbinaltorphimine (1 μM) inhibited only slightly or not at all the responses to dynorphin (100 nM) in oocytes expressing the orphan receptor [26 ± 7% (four oocytes) and 5 ± 2% (three oocytes) inhibition, respectively]. Our findings with dynorphin and naloxone are similar to those reported by Zhang and Yu (24); they estimated a dissociation equilibrium constant for naloxone of ~1 μM, ~100-1000 times higher than that typically seen at μ, δ, and κ receptors.

Coexpression of κ receptor and potassium channels. Oocytes injected with κ receptor RNA did not respond to OFQ, [Tyr^1]OFQ, or des-Phe-OFQ (each at 10 nM; four oocytes); in the same oocytes, α-neoendorphin and dynorphin evoked typical inward currents (10 nM, 165 ± 29 nA). The maximal effect of dynorphin was seen with 10 nM, and the concentration giving half-maximal effect was ~1 nM (Fig. 2B). The nonpeptide κ-selective agonists U-69593 (10 μM; four oocytes) and CI-977 (1 μM; three oocytes) mimicked the action of dynorphin at the κ receptor, and naloxone (1 μM) and norbinaltorphimine (100 nM) completely blocked the effect of dynorphin (10 nM; three oocytes). Norbinaltorphimine often evoked an inward current in these oocytes, suggesting an agonist action under these conditions.

We also examined the actions of several amino-terminally truncated analogs of dynorphin at supramaximal concentrations. Dynorphin(1–8), dynorphin(1–9), dynorphin(1–10), dynorphin(1–13), and [D-Arg^6]dynorphin(1–13) all evoked currents that were 85–95% of that caused by dynorphin; dynorphin(1–6) was only 10% as effective as dynorphin, and dynorphin(2–13) and dynorphin(2–17) were without effect. Strict comparisons are difficult without full dose-response curves, but we noted that the drop in potency between dynorphin(1–8) and dynorphin(1–6) is similar to the situation for native κ receptors in the guinea pig ileum (32). This contrasted with the finding in oocytes expressing the OFQ receptor that dynorphin(1–8) was much less potent than dynorphin(1–9).

Conclusions. OFQ evokes potassium currents when the receptor is coexpressed with potassium channel subunits in *X. laevis* oocytes; the concentration causing half-maximal response is close to 1 nM. Although the amino-terminal phenylalanine residue of OFQ can be substituted with tyrosine (as occurs in dynorphin) without a subsequent loss in activity, dynorphin is effective only at 100–300 times higher concentrations, and carboxyl-terminally shortened versions of dynorphin are similarly ineffective. Naloxone inhibits the actions of agonists at the OFQ receptor only at concentrations that are 100-1000 times higher than those needed at κ receptors (24). Other opioid agonists, including the κ agonists U-69593 and CI-977, and many nonopioid peptide agonists are inactive at the OFQ receptor. Despite the structural similarities of OFQ and dynorphin, the marked functional selectivity reported here strongly suggests that they activate only their corresponding receptors in native cells and thus have separate and quite distinct physiological roles.

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