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μ and δ receptors belong to a family of receptors that are coupled to potassium channels

(opioids/neuron/potassium conductance/guanine nucleotide-binding regulatory proteins/narcotic drugs)

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ABSTRACT **The effects of agonists at μ and δ opioid receptors were compared by measuring membrane currents under voltage clamp from neurons of the rat nucleus locus coeruleus and guinea pig submucous plexus. In each tissue, the appropriate selective agonist (Tyr-D-Ala-Gly-MePhe-Gly-ol for μ receptors in locus coeruleus or Tyr-D-Pen-Gly-Phe-D-Pen for δ receptors in submucous plexus) increased the conductance of an inwardly rectifying potassium conductance and strongly hyperpolarized the membrane. The properties of the potassium conductance affected by the two opioids could not be distinguished. Experiments with intracellular application of guanosine 5′-[γ-thio]triphosphate indicated that a guanine nucleotide-binding regulatory protein was involved in the coupling between opioid receptor and potassium channel, but there was no evidence for activation of either cAMP-dependent protein kinase or protein kinase C. It is noted that a number of vertebrate neurotransmitter receptors are coupled to potassium channels. The potassium conductance associated with these channels has properties similar to the conductance activated by μ and δ opioids; this family includes the following receptors: acetylcholine M_2, norepinephrine α_2, dopamine D_2, 5-hydroxytryptamine 5-HT_1, adenosine A_1, γ-aminobutyric acid $GABA_B$, and somatostatin. It is suggested that this conductance is a conserved neuronal effector coupled to one of the receptor types that mediates the effects of each of several major transmitters. The μ and δ opioid receptors appear to be unusual in that both utilize this same effector mechanism.**

μ and δ opioid receptors were distinguished by the ability of the antagonist naloxone to block the actions, or displace the binding, of [Met]enkephalin and [Leu]enkephalin (1). It is now clear that these two receptors have quite different distributions in nervous tissue (2–4), and the effects of the selective agonists Tyr-D-Ala-Gly-MePhe-Gyl-ol (for μ receptors) and Tyr-D-Pen-Gly-Phe-D-Pen (for δ receptors; Pen = penicillamine) in whole animals reflect the discrete neuroanatomy (5, 6). However, experiments at the cellular level have shown that stimulation of μ and δ receptors increases the potassium conductance in certain neurons. The purpose of the present experiments was to compare the properties of the potassium conductance coupled to μ receptors with that coupled to δ receptors; because a guanine nucleotide-binding regulatory protein (G protein) can also be associated with purified μ or δ receptors (7, 8), we also tested the hypothesis that hydrolysis of GTP was a necessary step in the linkage between occupancy of μ and δ receptors and the subsequent increase in potassium conductance.

MATERIALS AND METHODS

Intracellular recordings were made from neurons of the rat locus coeruleus or guinea pig ileum submucous plexus as described (9, 10). Tissues were continuously superfused at 36–37°C with a physiological saline solution [117 mM NaCl, 4.7 mM KCl (submucous plexus) or 2.5 mM KCl (locus coeruleus), 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 24 mM $NaHCO_3$, 11.5 mM glucose]. This solution was gassed with 95% O_2/5% CO_2. Agonists were applied to the neurons by changing the superfusing solution to one containing the agonist (usually 2–3 min was sufficient for effects to reach steady state). Membrane currents were measured with a single electrode sample-and-hold amplifier (Axoclamp 2, Axon Instruments, Burlingame, CA), which switched between voltage measuring (70%) and current passing (30%) at 1–3 kHz. The potential at the amplifier headstage was continuously monitored on a separate oscilloscope. Steady-state current/voltage (I/V) relations were usually constructed by commanding the membrane potential (x axis) from about −130 mV to −40 mV at a rate of 1–2 mV/s.

The drugs used (and their sources) were as follows: adenosine 5′-[γ-thio]triphosphate (ATP[S]) (Sigma and Boehringer Mannheim), 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304, gift of Pfizer), β-endorphin (Peninsula Laboratories, San Carlos, CA), guanosine 5′-[γ-thio]triphosphate (GTP[S]) (Sigma and Boehringer Mannheim), kelatorphan (gift of B. Roques, Université René Descartes, Paris), [Met]enkephalin (Sigma and Peninsula Laboratories), metorphamide (gift of E. Weber, Institute for Advanced Biomedical Research), and Tyr-D-Ala-Gly-MePhe-Gly-ol and Tyr-D-Pen-Gly-Phe-D-Pen (Peninsula Laboratories).

RESULTS AND DISCUSSION

In neurons of the rat locus coeruleus and the guinea pig submucous plexus, [Met]enkephalin caused a concentration-dependent membrane hyperpolarization or outward current when held close to the resting potential (in both cases about −60 mV). The effective concentrations of [Met]enkephalin in locus coeruleus and in submucous plexus were very similar, after correction had been made for the enzymatic degradation of [Met]enkephalin in the two tissues (11, 12) (Fig. 1). β-endorphin was also approximately equipotent, whereas metorphamide was significantly more effective in the locus coeruleus than the submucous plexus (Fig. 1). The μ agonist Tyr-D-Ala-Gly-MePhe-Gly-ol was fully effective on locus coeruleus cells but inactive at up to 300 times higher concentration in the submucous plexus; conversely, the δ agonist Tyr-D-Pen-Gly-Phe-D-Pen had no effect in the locus coeruleus at concentrations 1000 times greater than those giving half-maximal effects in the submucous plexus (Fig. 1). This finding, along with determinations of antagonist dissociation equilibrium constants (9, 13), allows one to conclude that the rat locus coeruleus neurons express only μ receptors and that

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; Pen, penicillamine.

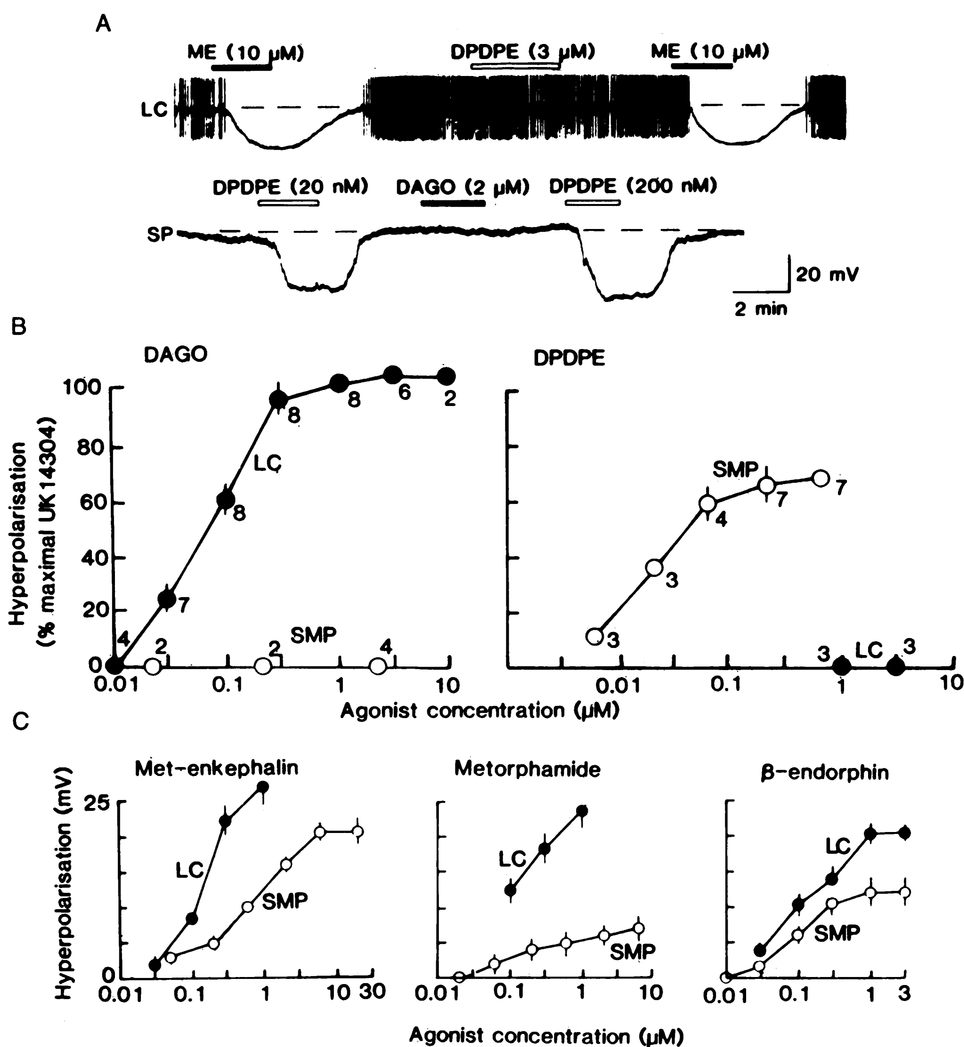

FIG. 1. Selectivity of agonists for μ and δ opioid receptors on individual neurons. (*A*) Intracellular recordings of membrane potential (resting level about −60 mV) of a neuron of rat locus coeruleus (LC) and guinea pig submucous plexus (SP). Locus coeruleus neurons fire action potentials spontaneously (full amplitude not reproduced); [Met]enkephalin (ME) prevented firing and hyperpolarized the membrane, whereas Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE) was ineffective. Submucous plexus cells do not fire spontaneously: Tyr-D-Pen-Gly-Phe-D-Pen caused a large hyperpolarization, whereas Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) was ineffective. (*B*) Results from experiments such as that shown in *A* carried out on several neurons (numbers given beside each point). Tyr-D-Ala-Gly-MePhe-Gly-ol (*Left*) was fully effective on locus coeruleus cells (LC) but ineffective on submucous plexus neurons (SMP). Tyr-D-Pen-Gly-Phe-D-Pen (*Right*) showed the reverse selectivity. Results are expressed as percentage of the hyperpolarization produced in the same neuron by a maximal concentration of the α_2-adrenergic receptor agonist 5-bromo-6-(2-imidazolin-2-ylamino)quinoxaline (UK 14304), which increases the same conductance as the opioid. (*C*) The naturally occurring agonists [Met]enkephalin and β-endorphin showed little selectivity between the two receptor types although metorphamide was more potent on the μ receptors of the locus coeruleus. In each case the points are the mean ± SEM hyperpolarizations observed in four to eight neurons. The applications of [Met]enkephalin and metorphamide were made in the concomitant presence of 20 μM kelatorphan to inhibit degradative enzymes. Kelatorphan had no effect on the response to β-endorphin.

the guinea pig submucous plexus neurons express only δ receptors.

Fig. 2 shows the current induced by a near-maximal concentration of opioid agonist as a function of membrane potential. In both locus coeruleus (μ receptors) and submucous plexus (δ receptors), this current had similar features. The underlying conductance was voltage-sensitive and increased ≈3-fold when the membrane was hyperpolarized beyond the potassium equilibrium potential. Rubidium (1–3 mM) added to the external solution prevented the inward rectification; opioid binding then increased a potassium conductance that was voltage independent between −60 and −130 mV (Fig. 2). Cesium (1 mM) had no effect on the outward current induced by opioids but caused a voltage-dependent block of the inward movement of potassium ions. When the potassium equilibrium potential was changed by increasing the extracellular potassium ion concentration, the inflection on the *I/V* plot shifted according to the Nernst equation; that is, the rectification shown by this conductance was not simply a function of membrane potential but of the difference between the membrane potential and the potassium equilibrium potential. All the locus coeruleus neurons studied (>30 neurons) showed this inward rectification of both the resting membrane current and of the opioid-induced current; in the submucous plexus, 6 of 47 neurons studied did not show inward rectification, and these cells were not hyperpolarized by opioids.

Opioid receptors of both μ (7) and δ (8) types have been associated with a G protein; activation of either receptor type inhibits adenylate cyclase in cell or membrane preparations by a mechanism that involves hydrolysis of GTP (14–18). We, therefore, recorded from neurons with intracellular electrodes that contained the nonhydrolyzable GTP derivative GTP[S] or, as a control, ATP[S]. Submucous plexus neurons

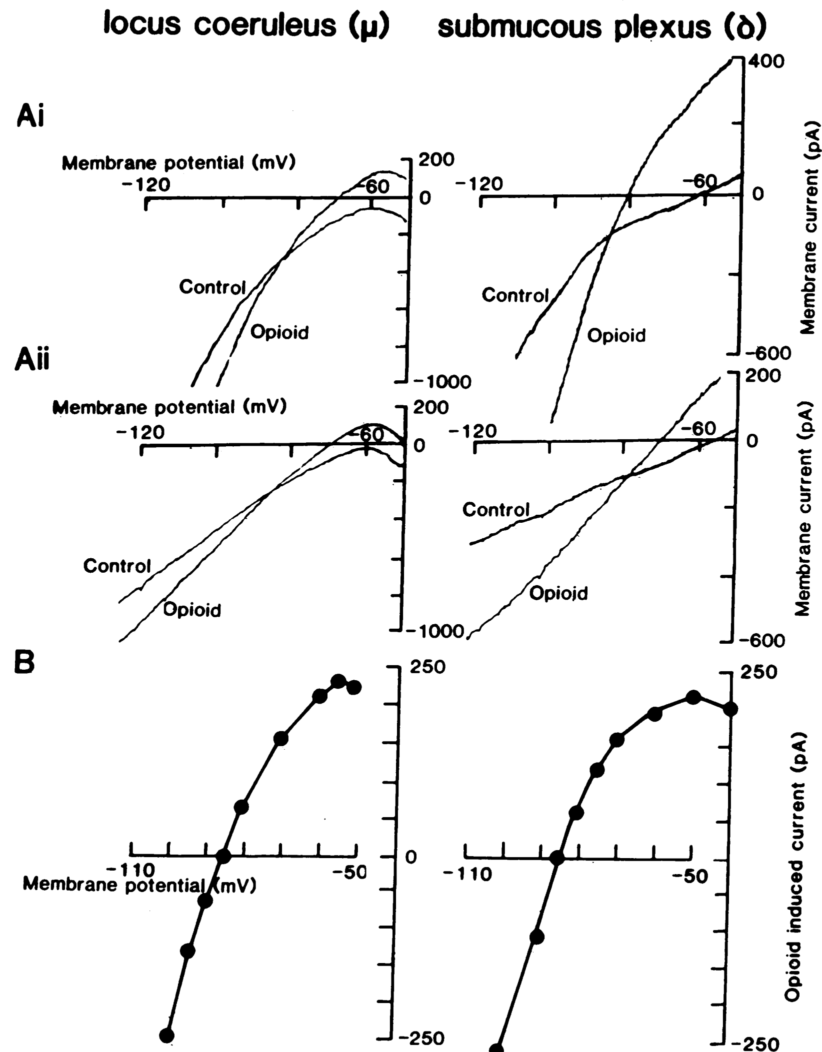

FIG. 2. Opioid-induced currents in neurons of rat locus coeruleus (*Left*) and guinea pig submucous plexus (*Right*). (*Ai*) Typical effects of opioids on μ (*Left*) and δ (*Right*) receptors. In both cases the opioid current reverses at the potassium equilibrium potential (potassium concentration in this experiment on locus coeruleus was 6.5 mM, and for submucous plexus, 4.7 mM); inward rectification is present both before and after the addition of opioid. (*Aii*) After addition of rubidium (2 mM in locus coeruleus and 3 mM in submucous plexus) the rectification was eliminated from the *I/V* plot both before and after addition of opioid. Rubidium often caused a small shift in the reversal potential to a less-negative level, suggesting that it could permeate the channels opened by opioid; this is obvious in the experiment on the submucous neuron. *A i* and *ii* were recorded from the same cells. The agonists were 30 μM [Met]enkephalin in the locus coeruleus and 200 nM Tyr-D-Pen-Gly-Phe-D-Pen in the submucous plexus. (*B*) The opioid-induced current calculated from an experiment such as that shown in *Ai* by subtracting the membrane current in the presence of opioid from that in the absence of opioid ([Met]enkephalin in the locus coeruleus and Tyr-D-Pen-Gly-Phe-D-Pen in the submucous plexus). Examples illustrated were both made in 6.5 mM potassium. Note the close resemblance between the properties of the currents resulting from activation of μ and δ receptors.

impaled with GTP[S]-containing electrodes were not noticeably different from control cells; however, brief (typically 2-min) application of an opioid agonist produced hyperpolarizations that reversed only partially or not at all when the agonist application was discontinued. The amplitude and time to peak of the hyperpolarization resulting from the initial application of opioid was not different from that seen in control neurons, but in five of eight cells the control membrane potential was not restored even after 30 min of washing with a solution that did not contain opioid. Second and subsequent applications of opioid to neurons containing GTP[S] produced further smaller irreversible hyperpolarizations until the membrane potential became stable close to the potassium equilibrium potential (about −90 mV) (Fig. 3). In three of the eight cells, the membrane was progressively hyperpolarized even though no opioid agonist was applied. Neurons from which recordings were made with electrodes containing ATP[S] responded normally to [Met]enkephalin.

Locus coeruleus neurons impaled with electrodes containing GTP[S] did not fire spontaneously, and all showed a slowly progressive membrane hyperpolarization whether or not an opioid agonist was applied. (Neurons impaled with electrodes that did not contain GTP[S] fired spontaneously, and the threshold potential from which the action potentials arose remained quite steady during several hours of recording.) [Met]enkephalin hyperpolarized these cells although the amplitude was less than in control cells. Voltage clamp experiments showed that, instead of reversing between −100 and −110 mV (in the control solution containing 2.5 mM potassium), the [Met]enkephalin current became almost voltage independent, and the extrapolated reversal potential became very much more negative. We interpret this finding to indicate that the [Met]enkephalin is still effective at electrotonically distant (dendritic) regions of the cell, which, presumably, were unaffected by the diffusion of GTP[S]. Coupled with the fact that *Bordetella pertussis* toxin prevents

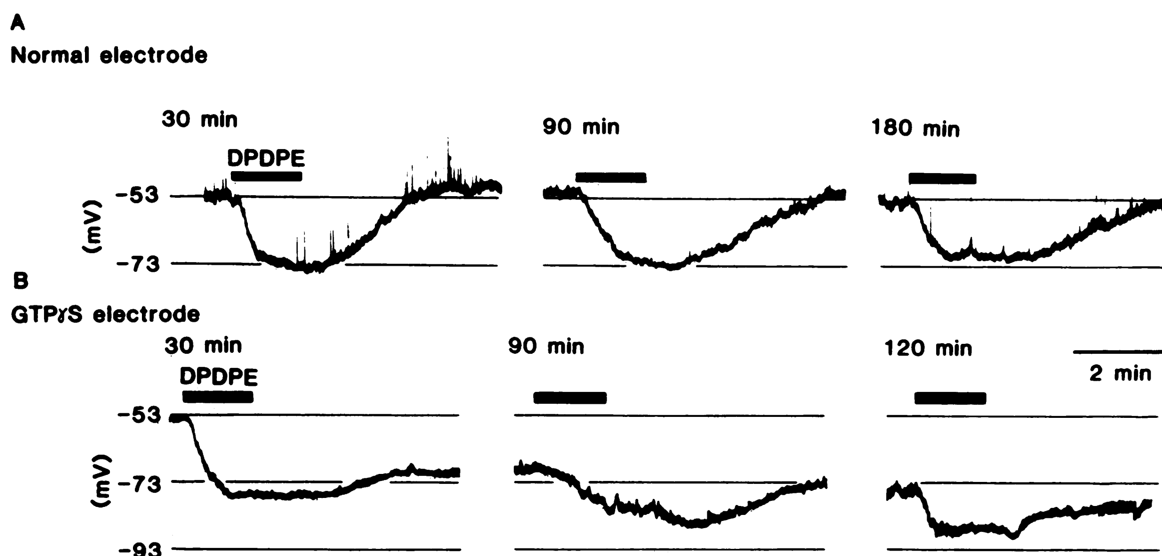

FIG. 3. A G protein is involved in the coupling between opioid receptor and potassium channel. The records are intracellular recordings of membrane potential from two neurons of the submucous plexus. (*A*) The recording from the first neuron was made with an electrode containing 2 M potassium chloride. Repeated applications of 200 nM Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE) caused repeated hyperpolarizations during 3 hr of recording. (*B*) The recording from a different neuron was made with an electrode containing potassium chloride and 20 mM GTP[S] (GTPγS). During the first 30 min of recording, the resting potential and input resistance of this cell were similar to those of the cell in *A*. The first application of 200 nM Tyr-D-Pen-Gly-Phe-D-Pen caused a hyperpolarization that did not recover when superfusion was discontinued; this was never seen in recordings with normal electrodes or electrodes containing 20 mM ATP[S]. The second and third applications of Tyr-D-Pen-Gly-Phe-D-Pen produced further irreversible hyperpolarizations, with the membrane potential approaching the potassium equilibrium potential (between −90 and −95 mV in 4.7 mM potassium). Idazoxan (1 μM) was present to block any effects of spontaneously released norepinephrine that might activate α_2-adrenergic receptors.

the hyperpolarizing action of opioids on rat locus coeruleus neurons if it is injected into the cerebral ventricle 1–2 days earlier (19), these results strongly suggest that agonist binding normally results in GTP hydrolysis and that, when this is prevented, the agonist permanently activates the potassium conductance.

In neither locus coeruleus nor submucous plexus did forskolin (10 nM–10 μM) or N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (up to 1 mM) have any effect on the responses to opioids; in both types of neurons these treatments did have effects of their own, causing a variable but small inward current that was shown in the case of the

Table 1. A family of vertebrate neurotransmitter receptors that is coupled to an increase in membrane potassium conductance

Agonist	Receptor type	Tissues	Ref(s).
Acetylcholine	M_2	Guinea pig cardiac muscle*†‡	20–22
		Rat nucleus parabrachialis*	23
		Rat thalamus reticularis	24
		Frog sympathetic C cells	25
		Mudpuppy cardiac ganglion cells	26
Norepinephrine	α_2	Guinea pig submucous plexus*†‡	27
		Rat locus coeruleus*†	28
		Rat substantia gelatinosa	29
Dopamine	D_2	Rat substantia nigra*	30
5-Hydroxytryptamine	5-HT_1	Rat hippocampus*‡	31
		Rat dorsal raphe*	32, 33
γ-Aminobutyric acid	$GABA_B$	Rat hippocampus*‡	31, 34, 35
Adenosine	A_1	Mouse striatum‡	36
Somatostatin	Unknown	Guinea pig submucous plexus†‡	37
		Guinea pig myenteric plexus	38
Opioid	μ	Rat locus coeruleus*†‡	This study
		Mouse dorsal root ganglion cells*	39
		Rat substantia gelatinosa	40
Opioid	δ	Guinea pig submucous plexus*†‡	This study
		Mouse dorsal root ganglia*	39

The absence of a symbol indicates that no evidence exists, rather than that a negative result has been obtained. All the receptors listed have been shown to inhibit adenylate cyclase in a variety of tissues.
*Tissues in which the receptor type has been identified most rigorously.
†Tissues in which the potassium conductance exhibits inward rectification at membrane potentials more negative than the potassium equilibrium potential.
‡Tissues in which a GTPase has been shown to be involved.

submucous plexus to result from a reduction in a membrane potassium conductance. Similarly, treatment of the neurons with phorbol 12,13-dibutyrate or phorbol 12-myristate 13-acetate had no effect on the hyperpolarizations or outward currents caused by opioids, whereas treatment clearly had other actions on the neurons (in both tissues an elimination of the calcium component of the action potential and the calcium-dependent afterhyperpolarization that follows it). We conclude from these experiments that cAMP-dependent protein kinase and protein kinase C are not directly involved in the coupling between opioid receptor and potassium channel.

Several neurotransmitters have been shown to increase the potassium conductance of central and peripheral neurons, and although the type of conductance affected has not always been characterized in detail, the available evidence suggests that it often shows inward rectification (Table 1). There is evidence that the coupling to the potassium conductance may also involve a G protein for 5-HT_1 and $GABA_B$ receptors in rat hippocampal cells (31, 34, 35), adenosine and 5-HT_1 receptors in cultured mouse striatal cells (36, 41), and α_2-adrenergic receptors and somatostatin receptors in guinea pig submucous plexus cells (ref. 37, and unpublished observations). Indeed, a single neuron can express several receptors that couple to this conductance [e.g., α_2-adrenergic receptor, δ-opioid, and somatostatin in submucous plexus (9, 37); α_2-adrenergic receptor, μ-opioid, and somatostatin in locus coeruleus (42); 5-HT_1 and $GABA_B$ in hippocampus; D_2 and $GABA_B$ in substantia nigra (30)]. This suggests that the receptor types listed in Table 1 belong to a family for which the common effector is a certain class of potassium channels. There are particularly close similarities between the effects of opioids acting at μ or δ receptors with the actions of acetylcholine at cardiac muscarinic (M_2) receptors (20). Activation of M_2 receptors leads to an increase in an inwardly rectifying potassium conductance, and the coupling between receptor and channel is close, involving GTP hydrolysis by a *pertussis*-sensitive G protein (20–22). All these receptor types (Table 1) are "negatively coupled" to adenylate cyclase, although in the heart cells, as in the present study, changes in levels of cAMP do not seem to be involved in the potassium conductance increase (43). It is possible that the enzyme is inhibited by release of β and γ subunits from the α subunit of the G protein, which is involved in the coupling between receptors and potassium channel (44, 45).

Most mammalian transmitters interact with a number of different receptor types and subtypes. Generally, only one receptor type for each transmitter belongs to the family that couples to this potassium conductance (Table 1); other receptor types for the same transmitter couple to different effector systems. It is intriguing, therefore, that two distinct types of opioid receptor should have an identical effector mechanism at the cellular level, especially since there are no striking differences in the concentration of the natural ligands [Met]enkephalin and β-endorphin that are required to activate them (although metorphamide is clearly more effective at μ than δ receptors).

Our interpretation is that the difference between these opioid receptors is so small as to be of minor physiological consequence, although the discrete tissue localization will allow the difference to be exploited pharmacologically. A similar situation exists for the β_1- and β_2-adrenergic receptors: these are highly homologous, and activation of either stimulates adenylate cyclase in a variety of tissues (46–48). The naturally occurring agonists epinephrine and norepinephrine do not exhibit very great selectivity between β_1 and β_2 receptors, but the distinction nonetheless has had a very important therapeutic impact.

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