Opioid Receptor Modulation of a Metabolically Sensitive Ion Channel in Rat Amygdala Neurons

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We have used single-channel patch-clamp recordings to study opiate receptor effects on freshly dissociated neurons from the rat amygdalohippocampal area (also called the posterior nucleus of the amygdala), an output nucleus of the amygdala implicated in appetitive behaviors. Dissociated cells included a distinct subpopulation that was 30–40 μm in diameter, multipolar or pyramidal in shape, and immunoreactive for neuron-specific enolase, μ opioid receptors, and galanin. In whole-cell perforated-patch recordings, these cells responded to low concentrations of μ opioid agonists with a hyperpolarization. In cell-attached single channel recordings, these cells expressed a large variety of K^+ -permeable ion channels, including 20–100 pS inward rectifiers and 150–200 pS apparent Ca^{2+} -activated K^+ channels, none of which appeared sensitive to the presence of opioid drugs. In contrast, a 130 pS inwardly rectifying channel was selectively activated by μ opioid receptors in this same

subpopulation of cells and was active only in the presence of opioid agonists, and inhibited in the presence of antagonists. Channels identical to the 130 pS channel in conductance and voltage sensitivity were activated in the absence of opioids, when the cells were treated with glucose-free medium or with the metabolic inhibitor rotenone. The sulfonylurea drug tolbutamide inhibited 130 pS channel openings elicited by opioids. Thus, a subpopulation of amygdala projection neurons expresses a metabolically sensitive ion channel that is selectively modulated by opiate receptors. This mechanism may allow opioid neurotransmitters to regulate ingestive behaviors, and thus, opiate drugs to influence reward pathways.

Key words: amygdala; patch-clamp; potassium channel; opiate receptor; opioid receptor; galanin; sulfonylurea; addiction; satiety

Paradoxically, propensity to drug addiction is more prevalent than might be expected for an evolutionarily undesirable behavior. It is widely thought that this observation might be explained by the ability of drugs of abuse to influence reward pathways of behaviors that are needed for survival, such as feeding and reproduction. The amygdala is a limbic brain region implicated in some of the motivational aspects of opiates and other drugs of abuse (Koob et al., 1992; Breiter and Rosen, 1999). A study of the distribution of rat amygdala neurons expressing an inhibitory response to opiates in extracellular recordings *in vivo* found responses to be highly localized to two amygdaloid subnuclei, the central nucleus and the amygdalohippocampal area (AHA) (Freedman and Aghajanian, 1985), which are two major output nuclei of the amygdala (Pitkanen et al., 1997). Because the AHA presents a relatively uniform neuronal population to study, we have now used patch-clamp recordings from dissociated cells to examine the basis of the inhibitory opioid response of AHA neurons.

The AHA has also been called the posterior nucleus of the amygdala (Swanson and Petrovich, 1998) or the posterior portion of the medial nucleus (Konig and Klippel, 1963). It is a densely packed cell layer in the posteromediodorsal amygdala, princi-

pally containing pyramidal or spindle-shaped projection neurons using glutamate as well as various peptides as neurotransmitters (Swanson and Petrovich, 1998). These peptides include galanin (Planas et al., 1994), and galanin release in the amygdala is known to regulate feeding behavior (Krykoulis et al., 1990). Lesions near this area affect feeding and satiety (Rollins and King, 2000), and there is also evidence for an AHA role in sexual behaviors (Demas et al., 1997; Kondo et al., 1997; Petrulis and Johnston, 1999; Heeb and Yahr, 2000).

Several authors have suggested that opiate receptors may be coupled to ATP-sensitive K^+ (K_{ATP}) channels (Ocana et al., 1990; Raffa and Codd, 1994; Shankar and Armstead, 1995; Kang et al., 1998; Lohmann and Welch, 1999; Rodrigues and Duarte, 2000). K_{ATP} channels are regulated by intracellular levels of ATP, and therefore could confer on neurons the ability to detect brain glucose levels (Freedman and Lin, 1996; Seino, 1999). Thus, opioid receptor modulation of K_{ATP} channels could be an attractive explanation of how opiates could affect satiety–reward pathways. However, there is little direct evidence for this idea in the brain; for instance, the ability of a K_{ATP} channel blocker to inhibit an opiate effect does not demonstrate direct coupling of the receptor to the channel. We have recently described an inwardly rectifying 130 pS K^+ -preferring cation channel in dissociated AHA neurons that is activated by μ opioid receptors in a membrane-delimited manner (Chen et al., 2000; Murphy and Freedman 2001). The gating of this channel changes after chronic morphine treatment in a manner that suggests that it may play a role in opiate tolerance (Chen et al., 2000). We now show that this channel is specifically expressed by a subset of cells that appear to be AHA projection neurons and that it is unique among the K^+ -permeable channels of these cells in its μ receptor sensitivity.

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We furthermore show that the same channel is metabolically sensitive.

MATERIALS AND METHODS

Cell preparation. Amygdala neurons were freshly dissociated by methods similar to those previously described (Greif et al., 1995; Chen et al., 2000). Male rats (Sprague Dawley VAF; Charles River Laboratories, Wilmington, MA), 30–45 d old, were killed by rapid decapitation, and the brains were rapidly and gently removed into an ice-cold solution of (in mM): NaCl, 124; KCl, 4; CaCl₂, 1; MgCl₂, 1; MnCl₂, 0.02; D-glucose, 25; and PIPES-Na, 20, pH 7.0, equilibrated with O₂. Coronal 300 μ m sections of the posteriodorsal amygdala were cut at $\sim$ 0°C on a vibrating tissue slicer. The slices were blocked mediadorsally with a scalpel blade to comprise an $\sim$ 1 $\times$ 3 mm rectangle, including the AHA (Paxinos and Watson, 1986). One edge of the slice was located at the dorsal limit of the AHA, to completely exclude the ventral hippocampus; the cut defining the opposite edge was placed within the medial amygdala so that the slice would include the AHA and part of the regions of the amygdala, principally the cortical nucleus, immediately ventral to the AHA. After slowly warming the slices to 32°C while stirring continuously at 20–25 rpm under 100% O₂, slices as needed for electrophysiology were treated with trypsin (type XI; Sigma, St. Louis, MO; 1000 benzoylarginine ethyl ester U in 0.5 ml) for 5 min, followed by 0.125 mg of soybean trypsin inhibitor for 2 min. Slices were then washed with the above solution in which the Ca²⁺ concentration was reduced to 0.5 mM and triturated with a polished glass pipette. Cells were allowed to settle in a plastic Petri dish for 10 min and were generally used for recording within 1 hr. All recordings reported in this study were made from phase-bright cells, with bleb-free surfaces, and with preservation of some dendritic processes. For immunocytochemistry of dissociated cells, every cell with these morphological criteria was included in counts of cells for the presence or absence of labeling. Cellular diameter was estimated along the major somatic axis, using a calibrated eyepiece reticle.

Immunocytochemistry. Slices or dissociated cells were prepared as above, then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. They were then washed with NH₄Cl, 0.5 mg/ml in PBS, followed by four washes of PBS. Incubations with antibodies were then made in PBS containing also 10% normal goat serum and 1 mg/ml NaN₃ (PBS-NGS). A rabbit polyclonal antibody to neuron-specific enolase was from Polysciences (Warrington, PA). A mouse monoclonal antibody to glial fibrillary acidic protein was from Chemicon (Temecula, CA), as were rabbit polyclonal antibodies to galanin, and to extracellular domains of μ , δ , and κ opioid receptors. The opioid receptor antibodies were used at dilutions that were reported by the supplier to not cross-react with the other opioid receptor subtypes. Opioid receptor antibody incubations were each at 1:500 at room temperature overnight. Incubations with the antibodies to neuron-specific enolase (dilution 1:4000) or glial fibrillary acidic protein (1:400) were 1 hr at 30°C and were preceded by cell permeabilization with 0.01% Triton X-100 in PBS for 1 min. In preliminary experiments, the galanin antibody was not active in fixed tissue, so it was used only in unfixed slices at 1:1000 at 4–8°C overnight. (Unfixed dissociated cells were not stable enough to label.) After primary antibody incubations, the cells or slices were washed four times with PBS-NGS, then incubated with fluorescent secondary antibodies at 1:300 in PBS-NGS for 1 hr at 30°C. For glial fibrillary acidic protein, the secondary antibody was Alexa 488-conjugated goat anti-mouse IgG; for all others, it was Alexa 546-conjugated goat anti-rabbit IgG, both from Molecular Probes (Eugene, OR). After five washes with PBS, fluorescence was viewed with a Nikon Diaphot microscope under mercury lamp illumination and fluorescein (for Alexa 488) or rhodamine (Alexa 546) filter sets. Control determinations of nonspecific fluorescence were made in each experiment by performing sham incubations omitting the primary antibody before treatment with the secondary antibody.

Electrophysiology. The dissociated cells were superfused at room temperature with (in mM): NaCl, 149; KCl, 3.5; CaCl₂, 2.5; MgCl₂, 1; D-glucose, 10; and HEPES-Na, 10, pH 7.4, oxygenated and adjusted with sucrose to 330–340 mOsmol/kg. Borosilicate glass patch pipettes had tip diameters of 1 μ m. For whole-cell perforated-patch recordings, the pipettes were filled with (in mM): KCl, 140; MgCl₂, 5; HEPES-K, 10, pH 7.2; gramicidin was used as the membrane-permeating agent (Rhee et al., 1994), and drugs were applied by change of solution from a constant-flow macropipette placed near the cell. For cell-attached single-channel recordings, the pipette solution was (in mM): KCl, 140; CaCl₂, 2.5; MgCl₂, 1; and HEPES-K, 10, pH 7.4, and (except for rotenone, which was applied via the external superfusion solution) drugs were added within

Figure 1. Photomicrographs of dissociated amygdala cells. *A* and *B* are phase-contrast photomicrographs. *C* and *D* are fluorescence micrographs showing immunochemical labeling for neuron-specific enolase. *A* and *C* show the larger putative AHA projection neurons. *B* and *D* show examples of smaller cells. Scale bar: *A–D*, 15 μ m.

the patch pipette at a uniform concentration. In a few experiments, glucose was completely omitted from the external superfusion solution, and the osmolality was 325 mOsmol/kg; when glucose and sucrose were both completely omitted, osmolality was 300 mOsmol/kg. One patch was tested per cell. All drugs were obtained from Sigma. Data were collected and analyzed with an Axopatch-1D recording system and pClamp software (Axon Instruments, Foster City, CA), with filtering at 2 kHz lowpass and digital acquisition at 100 μ sec/point. Inward single-channel currents are shown in figures as upward deflections, and membrane potential is expressed relative to cell resting potential. Because cell-attached recordings were performed with approximately symmetrical concentrations (140 mM) of K⁺ across the patch membrane, the reversal potential of a K⁺-permeable channel should occur when the patch is depolarized by an amount equal to the resting membrane potential of the cell. In every cell-attached recording we varied the electrode potential to determine the apparent reversal potential of any channels that were expressed. In recordings from phase-bright cells, the resting membrane potential thus inferred consistently fell between -40 and -70 mV, with most cells at -50 to -55 mV, whereas phase-dark cells were significantly more depolarized, and these results were confirmed by direct measurements of membrane potential in perforated-patch recordings. Except where stated otherwise, all cell-attached recordings were from cells negative to -40 mV, with seal resistance >5 G Ω , and preservation of these parameters and of phase-bright morphology for the duration of the recording of not <10 min. Records in figures are at resting potential unless otherwise stated. All data are expressed as mean $\pm$ SD, and all statistical comparisons were performed by a *t* test, unless otherwise stated. All-points amplitude histograms were used to determine the fractional open probability P_o , as $1/NP_o$, where N was the number of active channels in the patch (determined from the number of peaks) and P_o was the fraction of time that no current was passed. Current amplitudes for determinations of channel conductance were determined from peak-to-peak distances of amplitude histograms.

RESULTS

Cellular identification

The dissociated cell preparation yielded a variety of cellular morphologies when viewed under phase-contrast optics. Some cells had diameters of ~ 30 – 40 μ m and were multipolar or pyramidal in shape (Fig. 1*A*). Others ranged between 5 and 20 μ m and

had a variety of shapes, but were frequently unipolar or bipolar (Fig. 1*B*), and represented the majority of cells in the preparation. Cells of intermediate sizes were not encountered. Cells of both morphologies were immunoreactive for neuron-specific enolase (Fig. 1*C,D*). Among the 30–40 μm cells, 31 of 32 observed (97%) were immunoreactive, as were 365 of 420 of the smaller cells (87%); no nonspecific labeling was observed in either cell group when the primary antibody was omitted from the incubation. In contrast, no labeling for glial fibrillary acidic protein was seen in 12 of the larger cells and 158 of the smaller cells, although the same antibody gives positive labeling of some dissociated cells from the caudate–putamen in our hands (Greif et al., 1995). Thus, a large proportion of the dissociated amygdala cells were neurons.

We used amygdala slices comprising the AHA as well as some regions immediately ventral to it, principally the cortical nucleus of the amygdala, when preparing the dissociated cells, and so it is probable that our preparation included cells from both areas. Others have shown that the principal projection neurons of the AHA are 30–40 μm pyramidal or spindle-shaped cells, whereas neurons from adjacent areas are smaller (Krettek and Price, 1978). We therefore tested whether the larger cells in our preparation might arise from the AHA. When we labeled intact slices of posteriodorsomedial amygdala for μ opioid receptors, there was extensive cellular labeling in the AHA, but sparse labeling in the more ventral areas (Fig. 2*A*). When viewed at higher magnification, cells labeled for μ receptors in the slices had morphologies consistent with AHA projection neurons (Fig. 2*B*) and closely resembled dissociated cells that were similarly labeled (Fig. 2*C*). The neuropeptide galanin is known to be a marker for some projection neurons in the AHA and regions immediately rostral to it (Planas et al., 1994). In intact unfixed slices, galanin immunoreactivity was found in cells within the AHA closely resembling the cells that were labeled for μ receptors (Fig. 2*D*).

In populations of dissociated cells (Table 1), μ receptor immunoreactivity was expressed by a large percentage of the 30–40 μm cells, as shown in Figure 2*C*, but was much less prevalent in the 5–20 μm cells. Far fewer cells were labeled for δ receptors than for μ receptors, and we obtained only negative results in labeling for κ receptors (Table 1). Taken together, these results indicate that the 30–40 μm dissociated cells are mostly projection neurons from the AHA and tend to express μ receptor immunoreactivity, whereas the 5–20 μm cells represent other types of cells present in our preparation, arising largely from adjacent areas of the amygdala.

Effects of opioid receptor activation

We performed perforated-patch and cell-attached patch recordings from these dissociated cells. In whole-cell current-clamp recordings using perforated-patch, the 30–40 μm cells responded to the opioid peptide met-enkephalin (10–20 μM) with a hyperpolarization (Fig. 3*A*), consistent with the known inhibitory response of AHA cells to this compound *in vivo* (Freedman and Aghajanian, 1985). With a prolonged application, the response was stable for ~ 1 min, after which there was a progressive decline in the amplitude of the response, consistent with some desensitization, but the response to enkephalin was rapidly restored and repeatable after washout (Fig. 3*A*). The maximal amplitude of the hyperpolarization was 3.4 ± 2.1 mV ($n = 36$ trials on 15 cells) and rarely went all the way to the K^+ reversal potential. Previous studies (Chen et al., 2000) showed that this effect was associated with a greater latency to the onset of action potentials, but did not

Figure 2. Fluorescence photomicrographs showing immunocytochemical characterization of amygdala cells. *A*, Low-power view of an intact fixed amygdala slice, labeled for μ opioid receptors, showing extensive labeling in the amygdalohippocampal area (AHA) and sparse labeling in the cortical nucleus of the amygdala (ACo). The left side of the AHA is the dorsal edge of the slice, where the ventral hippocampus was removed. Scale bar, 100 μm . *B*, Higher power view of the slice in *A*. Scale bar, 35 μm . *C*, A dissociated cell, labeled for μ opioid receptors. Scale bar, 15 μm . *D*, An unfixed slice, within the AHA, labeled for galanin. Scale bar, 30 μm .

cause an obvious change in action potential properties. At low concentrations (5–25 nM), the highly potent and selective μ -opioid peptide endomorphin-1 (Zadina et al., 1997) elicited responses similar to those for met-enkephalin (Fig. 3*B*) ($n = 6$ trials on four cells). In contrast, the same compound at concentrations higher than those selective for μ receptors ($>1 \mu\text{M}$) elicited prominent depolarizations (11.5 ± 3.8 mV; $n = 8$ trials on four cells) (Fig. 3*C*).

As previously reported (Chen et al., 2000), a 130 pS channel was observed when met-enkephalin, endomorphin-1, or morphine were applied from within the patch pipette. Figure 4*A* shows examples of channel recordings, with 130 pS channel activity in the presence of agonists, but no activity in the absence of

Table 1. Immunocytochemical labeling of opioid receptor subtypes in dissociated amygdala cells

Receptor subtype	Cells observed	Cells labeled (%)
Cells 30–40 μm		
μ	58	53 (91)
δ	71	5 (7)
κ	26	0 (0)
Control ^a	12	0 (0)
Cells 5–20 μm		
μ	812	31 (4)
δ	672	39 (6)
κ	280	0 (0)
Control ^a	128	0 (0)

^a The primary antibody was omitted in control experiments.

Figure 3. Perforated-patch recordings of whole-cell membrane potential in dissociated 30–40 μm amygdala neurons. The heavy bars indicate the periods of application of the indicated drugs. The dashed lines indicate resting membrane potential, which was -45 to -65 mV in most cells. Scale bar: *A–C*, 5 mV. *A*, Hyperpolarizing response to met-enkephalin, 20 μM , followed by washout and reapplication at 10 μM . *B*, Hyperpolarizing response to a low concentration (25 nM) of endomorphin-1. *C*, Depolarizing response to a high concentration (10 μM) of endomorphin-1.

drug, or when the relatively nonselective opiate antagonist naloxone or the μ -selective antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) (Gulya et al., 1986) were present with the agonists. This pattern of channel activity was maintained in a statistically significant manner when populations of cells were tested (Table 2). In addition, we have previously shown that channel activation occurred directly when agonists were applied by back-filling and that channel open probability increased as a function of agonist concentration (Chen et al., 2000). Thus, it appears clear that channel activation was receptor-mediated. A

Figure 4. Single channel cell-attached patch recordings of the 130 pS channel. *A*, Representative examples of recordings with various drugs in the patch pipette, showing channel activity in the presence of met-enkephalin or endomorphin-1, and the absence of openings in control (no drug) recordings or when antagonists were present. *B*, Voltage insensitivity of channel openings at voltages negative to the reversal potential. Recordings from a single patch, with 10 μM met-enkephalin in the patch pipette, under the voltage protocol shown at the top; pipette voltages are indicated. *C*, Distribution of the number of 130 pS channels per patch. Patches with no channel openings are excluded from the figure.

Table 2. Pharmacology of 130 pS channel expression in 30–40 μ m dissociated amygdala cells

Drugs tested	Patches tested	Patches with channel (%)
Control	73	0 (0)
Agonists		
met-enkephalin (10 μ M)	100	48 (48)
Endomorphin-1 (0.5 μ M)	51	25 (49)
Morphine (1 μ M)	29	9 (32)
Total	180	82 (46)*
Agonists + antagonists		
met-enkephalin (10 μ M) + naloxone (1 μ M)	5	0 (0)
Endomorphin-1 (0.5 μ M) + CTOP ^a (0.1 μ M)	42	3 (7)
Morphine (1 μ M) + naloxone (1 μ M)	20	1 (5)
Total	67	4 (6)**

^a CTOP, D-phe-cys-tyr-D-trp-orn-thr-pen-thr-NH₂.* $p < 0.001$ versus control.** $p < 0.01$ versus total agonists.

comparison of the effects of μ , δ , and κ -selective agonists and antagonists indicated that channel activation was mediated principally by μ receptors (Chen et al., 2000), consistent with the selective expression of μ receptor immunoreactivity by these cells.

The channel was inwardly rectifying and otherwise showed no obvious voltage-dependence. At voltages negative to the reversal potential, the channel carried inward currents, and open probability did not appear to vary with membrane potential (Fig. 4*B*). Outward currents at voltages positive to the reversal potential were not observed, consistent with strong inward rectification. Open probability did not vary over time during a recording, and we saw no evidence of a time-dependent decrease in channel opening paralleling the decrease in the hyperpolarization observed in perforated-patch recordings (Fig. 3*A*).

At appropriate agonist concentrations, 130 pS channel activity was observed in approximately half of recordings from the 30–40 μ m cells (Table 2). Because one does not expect every patch to contain a channel molecule, it is probable that the 130 pS channel was expressed by a large percentage of these cells. Using patch electrodes with tip diameters close to 1 μ m, we consistently encountered one or two active channels per patch (Fig. 4*C*). This result contrasts with observations of an 85 pS dopamine-modulated channel in caudate–putamen neurons, where we typically find two to four channels per patch under equivalent conditions (Greif et al., 1995). Consequently, there may be differences in channel or receptor distributions over the surface of the cell membrane between these two systems. When we instead recorded from the 5–20 μ m diameter cells, we found no evidence for 130 pS channel expression or activation (Table 3). Thus, the opioid-modulated 130 pS channel appears to be expressed selectively by the AHA projection neurons and not by neurons from immediately adjacent areas of the amygdala.

Other channels

With 140 mM KCl as the major component of the patch electrode solution, a large number of distinct single channels could be resolved in cell-attached recordings from these amygdala neurons, of which the 130 pS channel was only one. In recordings

Table 3. Absence of 130 pS channel expression in 5–20 μ m dissociated amygdala cells

Drugs tested	Patches tested	Patches with channel (%)
Control	27	0 (0)
Agonists		
met-enkephalin (10 μ M)	34	0 (0)
Endomorphin-1 (0.5 μ M)	24	0 (0)
Total	58	0 (0)*

* $p < 0.001$ versus “total agonists” in Table 2.

from the 30–40 μ m cells, we observed only inward currents with reversal potentials occurring when the patch was depolarized by an amount corresponding to resting membrane potentials observed in perforated-patch recordings, consistent with K⁺ as the charge carrier under these conditions. Examples of some of these channels, recorded in the absence of drugs, are shown in Figure 5. There were inwardly rectifying channels with conductances ranging between 20 and 100 pS (Fig. 5*A*). At voltages negative to the reversal potential, these channels were not depolarization-dependent in their open probabilities. They thus resembled the dopamine receptor-insensitive inward rectifier channels in the caudate–putamen (Greif et al., 1995) except that they included channels of larger conductances. There were also channels of 150–200 pS, whose open probabilities increased conspicuously as the patch membrane was depolarized (Fig. 5*B*). These channels very much resemble large-conductance Ca²⁺-activated K⁺ channels, although we have not characterized their Ca²⁺ sensitivities. There appeared to be a large number of values of conductances of both these groups of channels, reflecting their heterogeneity, and they appeared to be spontaneously active in the absence of applied agonists. Their conductances, determined from current–voltage relationships, did not include values close to 130 pS (Fig. 5*C*). We also observed channels appearing to be Cl[−] channels in a small percentage of recordings from the 5–20 μ m cells, but these were not observed in the 30–40 μ m cells.

In a large number of recordings from the 30–40 μ m cells, these various channels, but not the 130 pS channel, could be observed in the absence of opiate agonists (Fig. 6*A*). When agonists were present within the patch pipette, all channel activities with conductances between 125 and 135 pS behaved as a single population of conductance values, 130 ± 2.8 pS ($n = 35$), normally distributed with a single mode (Kolmogorov–Smirnov normality test). There is consequently no evidence for more than one channel type with a conductance near 130 pS in these cells. In comparisons of recordings in the presence or absence of opioid agonists, the agonist-dependent activation of this 130 pS channel was by far the most conspicuous effect, although smaller effects on some other channels cannot be ruled out (Fig. 6*A,B*). Therefore, the 130 pS channel could be readily distinguished at the single-channel level from the multiplicity of other channels expressed by these cells, by its conductance and lack of depolarization-dependent activation. The effect of μ opioid receptor activation was specific to the 130 pS channel on these cells.

Effects of metabolic depletion and tolbutamide

The 130 pS channel was not observed in the absence of opioids in recordings from phase-bright cells with resting membrane potentials (inferred from reversal potentials) negative to −40 mV (Fig. 6*A*). However, we occasionally recorded from cells that were

Figure 5. Other channels expressed by AHA neurons. *A*, Examples of smaller conductance inwardly rectifying channels, labeled by their conductances. Each record is at resting membrane potential. For these channels, outward currents could not be resolved at voltages positive to the reversal potential. *B*, A large-conductance (186 pS) putative Ca^{2+} -activated K^+ channel, showing its voltage sensitivity. The patch was depolarized (*top*) or hyperpolarized (*bottom*) by 20 mV from resting membrane potential (RMP). *C*, Current–voltage relationships of various channels. Each plot is from a single experiment. The lines were fitted by linear regression of the points negative to the reversal potential (with nonzero inward currents), and the resulting slopes gave the indicated conductances.

Figure 6. Percentages of all patches recorded from 30–40 μm amygdala cells expressing channel openings of various conductances. *A*, No drug present, 73 recordings. *B*, Pooled data from all recordings with met-enkephalin, endomorphin-1, and morphine at all concentrations, 227 recordings. The number of patches expressing channels of a given conductance range are expressed as a percentage of all patches tested.

more depolarized, which we normally would not include in our data out of concern about cellular damage, and we observed spontaneous 130 pS channel activity in some of these cells. Because channel activation was not depolarization-dependent (Fig. 4*B*), this observation suggests that the channel had been activated for some other reason, one possibility being metabolic depletion in a damaged cell. We tested this possibility by performing cell-attached recordings in the absence of opioid drugs, but with removal of glucose from the external medium. We first obtained cell-attached recordings with no drug present and verified the absence of 130 pS openings in the patch. We then changed the external perfusion medium to one from which glucose was excluded. Of eight cells (30–40 μm diameter) for which we made this solution change, five patches (62%) displayed channel openings resembling the 130 pS channel with high open probability (Fig. 7*A*). In two of these cells, we removed sucrose as well as glucose, with no apparent difference in results. Openings occurred within 30–60 sec of the solution change. We were not able to reverse channel activation by restoring glucose because the dissociated cells were generally not stable long enough under these conditions.

We also recorded in the absence of opioids with the metabolic inhibitor rotenone (5 μM) present continuously in the external superfusion solution. Rotenone is expected to reduce cytoplasmic levels of ATP (Ashcroft et al., 1985; Haworth et al., 1989), but does not otherwise interfere with the ability to record from these neurons. We observed 130 pS channel activity in 10 of 23 record-

Figure 7. Metabolic activation and sulfonylurea inhibition of the 130 pS channel. *A*, Single-channel recordings at resting potential. *Top*, Recording in the absence of opioid drugs, from a patch where we initially verified the absence of channel activity and then changed the external solution to one that was nominally glucose-free. *Middle*, Recording in the absence of opioid drugs, with 5 μ M rotenone applied in the bath solution surrounding the cell. *Bottom*, Recording with met-enkephalin (10 μ M) plus tolbutamide (100 μ M) present within the patch pipette. *B*, Current–voltage relationships for recordings with met-enkephalin (10 μ M in the patch pipette) or the recording conditions in *A*. Membrane potential is expressed relative to RMP. At each point, $n = 5$ –9 patches. The lines were each fitted by linear regression as in Figure 5C, with slopes of 130 ± 1 pS. *C*, Fractional channel open probability P_o as a function of membrane potential; $n = 5$ –9. The rotenone and nominally glucose-free data reached the reversal potential when the patch was depolarized by 40 mV.

ings (43%) from 30–40 μ M cells under these conditions, a frequency similar to that found with opioid agonists. Again, channel openings were observed that closely resembled those obtained with removal of glucose (Fig. 7A).

As expected, rotenone-treated and glucose-depleted cells were slightly depolarized compared with control cells, as revealed by a shift in the reversal potential (Fig. 7B,C). However, the channel conductance of 130 pS was unchanged from that obtained with opioids (Fig. 7B). At voltages negative to the reversal potential, the fractional open probability of the channel was somewhat greater for glucose depletion or rotenone treatment than for opioids, with near-complete channel opening (Fig. 7C). Otherwise, however, the inwardly rectifying behavior of the channel was the same. It thus appears that the same channel could be activated in the absence of opioid agonists, under conditions of reduced concentrations of intracellular ATP.

We also recorded with the sulfonylurea drug tolbutamide (100 μ M) present within the patch pipette along with met-enkephalin. When 130 pS channel openings were present ($n = 9$ cells), openings were much briefer than was observed with met-enkephalin alone (Fig. 7A). Fractional channel open probability was markedly reduced (Fig. 7C), consistent with inhibition of channel gating. However, there was once again no difference in the 130 pS conductance of the channel (Fig. 7B). Thus, the same channel activated by opioid receptors also displayed the metabolic sensitivity and sulfonylurea inhibition associated with K_{ATP} channels.

DISCUSSION

The 130 pS channel

The major findings of our study are that the μ receptor-activated 130 pS channel is expressed selectively by the projection neurons of the AHA, that, of the multiple K^+ -permeable channels expressed by these cells, μ receptor activation is selective for this channel, and that this same channel can be activated in the absence of μ agonists under conditions of metabolic depletion. AHA neurons are known to consistently express inhibitory responses to μ agonists *in vivo* (Freedman and Aghajanian, 1985). We found that low concentrations of μ agonists also elicited hyperpolarizing responses in these cells (Fig. 3A,B). Although high concentrations of endomorphin-1 could also depolarize these cells (Fig. 3C), this effect occurred at concentrations approximately three orders of magnitude higher. The lower endomorphin-1 concentrations, which hyperpolarized the cells and activated the channel, corresponded closely with those selective for μ receptors (Zadina et al., 1997), whereas the depolarizing concentrations were far higher. We therefore believe that the hyperpolarization seen at lower concentrations is more likely to be the receptor response, and the depolarization is likely to be a nonspecific effect of this compound. We previously found that the 130 pS channel can carry both K^+ and Na^+ , but is selective for K^+ over Na^+ (Chen et al., 2000). The fact that the hyperpolarizations observed here did not reach the K^+ reversal potential is consistent with a mixed-ion mechanism. Therefore, the 130 pS channel could contribute significantly to the whole-cell response, although our data certainly do not rule out μ receptor effects on other channels in these cells. The apparent desensitization observed at the whole-cell level (Fig. 3A) was not observed at the single-channel level, and is clearly distinct from the gating change of this channel that may accompany tolerance after chronic morphine treatment (Chen et al., 2000). The absence of desensitization in cell-attached recordings might result from the limited

exposure of the cell surface that occurs when the agonist is only present within the patch pipette, whereas the entire cell membrane is exposed to the agonist in whole-cell recordings.

Channels activated by glucose depletion or by rotenone and those activated by μ agonists were remarkably similar. The only difference appears to be a somewhat higher open probability seen with metabolic depletion, which could reflect different mechanisms of channel modulation. Because no other channels of 130 pS conductance were found under control conditions, it is very likely that the same channel was activated in each case. We have not, however, been able to show that internal ATP gates the channel, because this channel proved unstable in inside-out recordings. However, our results are very similar to those for the 85 pS channel modulated by D_2 dopamine receptors in caudate-putamen neurons (Greif et al., 1995). That channel is also activated by rotenone, is sensitive to sulfonylurea drugs in a manner suggestive of a K_{ATP} channel (Lin et al., 1993), and has recently been shown to be directly regulated by ATP (Sun et al., 2000). However, our tolbutamide data reveal one interesting difference between these channels. The 85 pS channel could be largely inhibited by submicromolar concentrations of this drug (Lin et al., 1993), whereas concentrations of $\sim 100 \mu M$ were needed to give the same effect for the 130 pS channel (Fig. 7), more like most K_{ATP} channels (Freedman and Lin, 1996). This finding suggests that different sulfonylurea-binding proteins may be involved in the two systems.

The 130 pS channel has a number of distinctive properties. Based on its conductance, it is clearly not the same as the inwardly rectifying 30–45 pS K^+ channel activated by μ receptors in the locus coeruleus (Miyake et al., 1989; Grigg et al., 1996). However, because 130 pS channel activation is membrane-delimited (Chen et al., 2000), it is likely to be activated either by direct binding of G-protein $\beta\gamma$ subunits (Slesinger et al., 1995) or by their action via a sulfonylurea-binding protein (Wada et al., 2000). Its relative permeability for K^+ and Na^+ is similar to the opioid-sensitive I_h current (Svoboda and Lupica, 1998), but it is not sensitive to cyclic nucleotides (Chen et al., 2000), and it has no voltage sensitivity in its activation. It also somewhat resembles the cation channel modulated by μ receptors in the locus coeruleus (Alreja and Aghajanian, 1993), but is more K^+ -selective and appears likely to contribute to a hyperpolarizing rather than a depolarizing response. Finally, it has some of the properties of a K_{ATP} channel, or at least is metabolically sensitive and inhibited by sulfonylureas.

Functional implications

Our data are limited by the facts that we have used populations of cell-attached recordings to characterize the receptor modulation of channels and that we did not immunocytochemically label the same cells that we recorded electrophysiologically. However, it can be concluded that μ receptors do activate this channel and that it is expressed by many of the AHA projection neurons. What might be its functional significance in the amygdala?

The amygdala contributes to the motivational aspects of opiate drug dependence (Koob et al., 1992), and amygdala lesions suppress some opiate withdrawal signs (Calvino et al., 1979). The amygdala is activated in human addicts during drug craving (Breiter and Rosen, 1999). Within the amygdala, the AHA is one of two regions that express functional inhibitory responses to μ agonists *in vivo* (Freedman and Aghajanian, 1985). There is also evidence for a role of the AHA and regions immediately rostral to it, through which AHA efferent fibers pass (Maragos et al.,

1989), in sexual behaviors. This part of the amygdala is innervated by the accessory olfactory bulb, which in turn receives inputs from the vomeronasal organ and thus might respond to pheromones (Demas et al., 1997; Martinez-Marcos and Halpern, 1999; Petrulis and Johnston, 1999). The AHA projects to areas of the hypothalamus thought to regulate sexual behaviors (Pitkanen et al., 1997; Swanson and Petrovich, 1998). This pathway is activated by sexual anticipation (Kondo et al., 1997), which might be considered to have some resemblance to craving, and lesions of this pathway disrupt sexual behaviors (Heeb and Yahr, 2000). Thus, opioid receptor activation leading to an inhibition of AHA cell firing, partly through the 130 pS channel, might be able to decrease some components of sexual craving and thus contribute in part to opiate drug reward.

There is also evidence for a role of the AHA and adjacent areas in feeding and satiety. At least some of the neurons expressing the 130 pS channel contain galanin as a neurotransmitter (Fig. 2D), and the regulation of galanin release in the amygdala is expected to affect feeding behavior (Krykoulis et al., 1990). Lesions near this area affect feeding and satiety (Rollins and King, 2000). In primates, some amygdala neurons are glucose-sensitive in their firing, and so can sense brain glucose levels (Ragozino and Gold, 1994; Yan and Scott, 1996). Because the 130 pS channel is metabolically sensitive, it can contribute to the ability of AHA neurons to respond to changes in glucose. Animal studies indicate that craving for opiate drugs varies with hunger or satiety (Shalev et al., 2001). Because opioids modulate the 130 pS channel, there thus could be an interaction between opiate craving and food craving. We found that metabolic depletion could activate the 130 pS channel to near-100% opening (Fig. 7C), such that there would be little additional possible activation by opiates. Perhaps in a hungry subject the 130 pS channel would already be activated, and so the neurons would be less responsive to opioid neurotransmission, thus reducing the salience of other types of cravings. Whether this is true will require further studies, but our results clearly show that a specific channel molecule is modulated both by opioids and by metabolism in this limbic brain region.

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