Separation of μ -Opioid Receptor Desensitization and Internalization: Endogenous Receptors in Primary Neuronal Cultures

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A close relationship between desensitization and internalization of μ -opioid receptors (MORs) has been proposed based on differential actions of series of agonists. The role that these two processes have in the development of tolerance and dependence to opioids has been a controversial subject that has been studied in a variety of model systems. Here, we examine desensitization and internalization of endogenous MORs simultaneously in primary cultures of locus ceruleus neurons using fluorescently tagged peptide agonists. With the use of two fluorescent opioid peptides, dermorphin-Bodipy Texas Red and dermorphin-Alexa594 (Derm-A594), desensitization was measured electrophysiologically and trafficking was followed by the accumulation of intracellular fluorescent puncta. Blocking endocytosis with concanavalin A eliminated the accumulation of fluorescent puncta but desensitization induced by Derm-A594 was unaffected. Likewise, after treatment with concanavalin A, there was no change in either desensitization or recovery from desensitization induced by [Met]⁵enkephalin. The results demonstrate that desensitization and the recovery from desensitization are not dependent on receptor internalization and suggest that the activity of endogenous MORs in primary neurons can be modulated at the level of the plasma membrane.

Key words: fluorescent-peptide; locus ceruleus; culture; concanavalin A; electrophysiology; two-photon microscopy

Introduction

The activation of μ -opioid receptors (MORs) mediates the analgesic effects of opioids and repeated activation of these receptors results in tolerance so that a higher concentration of opioids are required to achieve the same level of analgesia (Contet et al., 2004). Two early events that follow the activation of MORs are the development of acute desensitization and induction of receptor trafficking (Ferguson, 2001; Connor et al., 2004; Bailey and Connor, 2005). With the use of a series of agonists, it was determined that agonists that cause desensitization also promote receptor trafficking, an observation that has been taken to suggest that the two processes are closely linked (Keith et al., 1996; Alvarez et al., 2002; von Zastrow et al., 2003). Morphine in particular was an agonist that did not cause desensitization or internalization. These results led to the hypothesis that receptor

internalization was a mechanism that prevented adaptive changes that initiate the development of tolerance (Whistler et al., 1999; Finn and Whistler, 2001). Recent work has indicated that, although morphine may not be as efficient as other agonists, it can cause both desensitization (Borgland et al., 2003; Dang and Williams, 2005; Koch et al., 2005) and internalization (Haberstock-Debic et al., 2005). The discrepancy between studies has led to an alternate hypothesis suggesting that internalization plays an important role in the recovery from desensitization (Koch et al., 2005). This alternate hypothesis proposes that internalization is the primary mechanism whereby receptors can be removed from the desensitized state. Because morphine does not cause efficient trafficking it results in much more desensitization than other agonists (Koch et al., 2005). The conflicting theories may well result from marked difference in the experimental models (Connor et al., 2004; Bailey and Connor, 2005). Most studies have used systems in which receptors are overexpressed and that measured both internalization and desensitization in separate experiments with different experimental protocols. Very little work has been done in primary neurons looking at endogenously expressed receptors.

One limitation of using native neurons to study receptor trafficking is difficulty in following the endogenous receptor in real time. Fluorescent opioid ligands have been used successfully to follow receptor internalization in Chinese hamster ovary and human embryonic kidney 293 (HEK293) cells expressing cloned

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MORs (Arttamangkul et al., 2000; Alvarez et al., 2002) as well as rat cortical neurons in cultures (Lee et al., 2002). In this study, fluorescent peptides were used to study MOR activation, desensitization, and internalization in primary neuronal cultures from the mouse locus ceruleus (LC). The LC neurons were identified by the presence of green fluorescent protein (GFP) that was expressed under the tyrosine hydroxylase promoter (TH-EGFP) (Sawamoto et al., 2001; Matsushita et al., 2002). Cultured LC neurons had functional MORs as determined by activation of G-protein-activated inwardly rectified potassium channels (GIRKs), inhibition of voltage-dependent calcium channels, and inhibition of synaptic transmitter release at recurrent synapses (autapses). Both desensitization and internalization were studied on the same cell and the results showed that receptor desensitization and recovery from desensitization were not changed after internalization was blocked with concanavalin A (con A).

Materials and Methods

Cultures. The TH-EGFP mice generated in Dr. Kobayashi's laboratory (Sawamoto et al., 2001) were backcrossed to DBA-2J for at least five generations before these experiments. Only heterozygous animals were used because homozygous mice did not survive for more than a few weeks. Mice (3–5 weeks) were used for these experiments to ensure that the brains were fully developed before the cultures were made. The care and use of animals were conducted in accordance with the National Institutes of Health guidelines and with an approval from the Institutional Animal Care and Use Committee of the Oregon Health and Science University. Animals were anesthetized with halothane and decapitated. The brain was removed and sliced horizontally using a vibratome (Leica, Nussloch, Germany) in ice-cold artificial CSF (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 2.4 CaCl₂, 21.4 NaHCO₃, and 11 glucose. Slices were equilibrated in warm (35°C) oxygenated ACSF solution for 15 min and the area around the LC was microdissected with two 23-gauge needles under a dissecting microscope. The tissues were incubated in dissociation buffer containing papain (20 U/ml; Worthington Biochemical, Lakewood, NJ) for 5–7 min. The dissociation buffer, adjusted to pH 7.4, contained the following (in mM): 81.8 Na₂SO₄, 30 K₂SO₄, 12 MgCl₂, 0.25 CaCl₂, 4 HEPES, 0.2 glucose, and phenol red. Dissociation was done in the growing media containing Neurobasal A, 2% heat-inactivated fetal bovine serum (FBS), 1 mM glutamine, B-27 nutrient mix (all from Invitrogen, San Diego, CA), and 0.5 mM kynurenate (Sigma, St. Louis, MO) by trituration with a series of pasture pipettes having decreasing tip sizes. The cell suspension was plated onto glial feeder previously grown on glass coverslips. A glial feeder layer was prepared from the neonatal cortex and was allowed to grow until a monolayer formed. The media for glial cells contained MEM (Invitrogen), 0.1% mitogenic serum extender (BD Biosciences), 30 mM glucose, and 4% non-heat-inactivated fetal calf serum. After LC neurons were plated onto the feeder layer, media were changed from 2 to 1% FBS of growing media on the next day and then a complete serum-free media (Neurobasal A, 1 mM glutamine, B-27, and 0.5 mM kynurenate) for the following feeding schedule. The cultures were grown in the serum-free media for at least 5 d before being used in the experiments.

Whole-cell recording. Coverslips containing the LC neurons were transferred to a recording chamber and perfused with ACSF solution equilibrated with 95% O₂ and 5% CO₂ to pH 7.4. Noradrenergic neurons were identified as large green fluorescent cells using a Zeiss (Oberkochen, Germany) Axioskop 2 FS fluorescent microscope. Recordings were made with patch pipettes (2 MΩ) containing the following (in mM): 115 potassium gluconate, 20 NaCl, 1.5 MgCl₂, 0.1 EGTA, 5 HEPES, 2 Mg-ATP, 0.5 Na-GTP, and 10 phosphocreatine, pH 7.4 (280 mOsm/kg). To record inward potassium currents, the membrane potential was hyperpolarized from –60 to –100 mV for 30 ms in a high-potassium (10 mM) solution at room temperature (25°C). To record the Ca²⁺/Ba⁺ inward currents, CsCl was substituted for potassium gluconate and the external solution contained BaCl₂ (1 mM) and tetrodotoxin (300 nM; Alomone Laboratories, Jerusalem, Israel). To study the release of transmitter from cultured

LC neurons, autaptic currents were evoked by depolarizing the membrane potential –60 to –20 mV for 2 ms. All drugs and solutions were perfused onto the cultures by gravity flow or a fast-flow valve system (Warner Instrument, Hamden, CT). The peptidase inhibitors bestatin (10 μM) and thiorphan (2 μM) were included in all solutions containing opioid peptides to prevent peptide metabolism (Williams et al., 1987). [Met]⁵enkephalin (ME), thiorphan, and bestatin were obtained from Sigma. 5-Bromo-N-[4,5-dihydro-1H-imidazol-2-yl]-6-quinoxalinamine (UK14304) was obtained from Research Biochemical (Natick, MA). Dermorphin-Bodipy Texas Red (Derm-BTR) and dermorphin-Alexa594 (Derm-A594) were prepared as described in Arttamangkul et al. (2000). Morphine was obtained from the National Institute of Drug Abuse. 3-Dioxobenzo[f]quinoxaline-7-sulfonamide (NBQX) was obtained from Tocris (Ellisville, MO). Whole-cell recordings were made with an Axopatch-1D (Molecular Devices, Union City, CA), filtered at 5 kHz, and digitized with an ITC-16 computer interface (Instrutech, Long Island, NY). Data were sampled at 10 kHz with Axograph 4.5 software. The membrane potential was held at –60 mV. All patch-clamp experiments were done at room temperature. Data are presented as means ± SEM. All statistical comparisons were made using Student's *t* test and the level of significance was considered as *p* < 0.05.

Confocal microscopy. Neurons were submerged in serum-free media plus HEPES buffer (50 mM). Derm-BTR solution containing bestatin (10 μM) and thiorphan (2 μM) was added to the cells with the temperature of the chamber set at 15°C. Images were captured at various times after the temperature was rapidly raised (within 1 min) to 35°C. Images were acquired on a Bio-Rad (Hercules, CA) MRC-1024 confocal microscope equipped with a krypton–argon laser. Cells were visualized under a Plan Apo 60× (1.4 numerical aperture, oil) objective lens. The filters used for scanning green fluorescent protein were 488 (excitation) and 522 nm (emission), and for Derm-BTR they were 568 (excitation) and 585 nm (emission). Acquisition parameters were averaged two frames per image using a direct filter to prevent photobleaching and normal scanning mode. All images were processed using CAS 40 software (Bio-Rad), Image J (National Institutes of Health, Bethesda, MD), and Adobe (San Jose, CA) Photoshop 6.0.

Two-photon microscopy and intracellular recording. Simultaneous recording of membrane potential and imaging was done with an electrophysiological setup equipped with a custom-built two-photon microscope. Intracellular recordings of membrane potential were done with glass electrodes filled with KCl (300 mM) having a resistance of 100–200 MΩ. Experiments with a combination of intracellular recordings and imaging were all done at 35°C. Data collection was done with Power Lab (Chart version 4.1). Drugs were applied by perfusion. Images were acquired using Scan Image software (Pologruto et al., 2003). The Ti:sapphire laser was set at 810 nm. The emission filter sets were 510 nm for EGFP and 600 nm for Derm-A594. Cells were imaged with a z-series taken through the cell at 0.5 μm intervals, averaging two frames per image. Data were processed off-line using Image J and Adobe Photoshop 6.0.

Results

Cultured LC neurons

The LC is a dense and compact group of green fluorescent neurons located on the lateral aspect of the fourth ventricle in slices from the TH-EGFP mice (Fig. 1A,B). The GFP fluorescent cells costained with anti-tyrosine hydroxylase antibodies, thus confirming that they were noradrenergic (Fig. 1C). Cultures were prepared from animals that were 3–5 weeks old and used within 14 d of plating. Generally, there were approximately five GFP-positive neurons per coverslip. Some cells were isolated having no contact with other neurons (Fig. 1D). LC neurons were identified by the presence of GFP and the characteristic morphology when viewed with infrared illumination. Cells were large (>20 μm) with a round cell body and multipolar processes and exhibited dark granulated vesicles. ~90% of cells having these characteristic responded robustly to the α₂-adrenergic agonist UK14304 and of those cells a small percentage (~25%) were equally sensitive to

Figure 1. LC neurons expressing GFP. **A**, The LC is found next to the fourth ventricle ($10\times$). **B**, An enlarged image showing the dense and compact structure of the nucleus ($20\times$). **C**, Colocalization of GFP and anti-tyrosine hydroxylase (mouse monoclonal) labeled with Texas Red-conjugated secondary antibody. **D**, A single neuron in culture for 9 d.

opioid agonists. The reason that many cells responded poorly to opioids is not clear, but was dependent on the culture. Only cells that responded robustly to ME were included in this study.

Opioid actions

Opioids are known to have three different actions on LC neurons, an increase in potassium conductance, an inhibition of voltage-dependent calcium current, and an inhibition of noradrenaline release from terminal regions (Williams et al., 2001). In the present study, each of these actions was studied. As has been observed in slice and acutely dissociated cell preparations, opioid agonists increased GIRK conductance in cultured neurons (Fig. 2A). Experiments were done in a high-potassium (10 mM) solution to increase the amplitude of the GIRK current. Cells were held at -60 mV, stepped to -100 mV, and the inward potassium current measured at -100 mV. ME, morphine, Derm-BTR, and an α_2 -adrenergic agonist UK14304 increased potassium currents. To make comparisons between cells, the currents induced by different opioid agonists were normalized to the current produced by UK14304 ($10 \mu\text{M}$). The amplitude of the ME ($30 \mu\text{M}$) (Fig. 2A) current was similar to that induced by UK14304, having a ratio of 0.91 ± 0.07 ($n = 12$). Morphine ($10 \mu\text{M}$) induced a current that was approximately one-half the size of that induced by UK14304 (ratio = 0.45 ± 0.04 ; $n = 5$). The results suggested that morphine was a weak agonist at MOR in these cultured neurons. The fluorescent peptide agonist Derm-BTR was tested at two different concentrations (0.1 and $1 \mu\text{M}$). Both applications produced potassium currents that were about one-half of current induced by UK14304 (Derm-BTR, $0.1 \mu\text{M} = 0.54 \pm 0.12$, $n = 3$; Derm-BTR, $1 \mu\text{M} = 0.56 \pm 0.08$, $n = 8$) (Fig. 2A). This finding agreed with previous work done in rat brain slices indicating that Derm-BTR was less efficacious than ME (Arttamangkul et al., 2000).

Opioids have been demonstrated to inhibit calcium (barium) currents in dissociated LC neurons and slices (Ingram et al., 1997;

Figure 2. Effectors sensitive to the activation of MORs. **A**, Activation of GIRK conductance. Whole-cell recordings were made in a high-potassium (10 mM) solution (V-hold -60 mV, step to -100 mV). Inward currents were measured at -100 mV. The superimposed traces show the currents triggered by Derm-BTR (D-BTR; $1 \mu\text{M}$) and UK14304 (UK; $10 \mu\text{M}$). The bar graph summarizes the currents induced by opioid agonists normalized to the current induced by UK14304. Both Derm-BTR ($1 \mu\text{M}$) and morphine ($10 \mu\text{M}$) were partial agonists in this assay. **B**, Inhibition of voltage-dependent calcium (barium) conductance. Whole-cell recordings were made and the inward current induced by a depolarizing step from -60 to -20 mV was measured. Representative traces show the inhibition caused by Derm-BTR ($0.1 \mu\text{M}$). The bar graph summarizes the inhibition of calcium (barium) currents as percentage of control. Derm-BTR caused the same maximal inhibition as ME ($30 \mu\text{M}$) in this assay. **C**, Recurrent synapses (autapses) mediated by glutamate are inhibited by opioids. The superimposed traces show the current induced during depolarization of cells from -60 to -10 mV followed by an EPSC. The EPSCs were inhibition by Derm-BTR ($1 \mu\text{M}$). The bar graph summarizes the inhibition of EPSCs by ME ($10 \mu\text{M}$), Derm-BTR ($1 \mu\text{M}$), UK 14304 ($10 \mu\text{M}$), and NBQX, an AMPA antagonist ($10 \mu\text{M}$). The results indicate that LC cells release glutamate when maintained in an isolated cell culture and opioids inhibit that release. Error bars indicate SE.

Chieng and Bekkers, 1999; Connor et al., 1999; Torrecilla et al., 2002). The membrane potential was depolarized from -60 to -20 mV for 10 ms to activate an inward current carried by calcium and barium. ME ($30 \mu\text{M}$) and Derm-BTR ($0.1 \mu\text{M}$) decreased barium currents by $27.5 \pm 3.6\%$ ($n = 9$) and $26.4 \pm 1.6\%$ ($n = 3$), respectively (Fig. 2B). Unlike activation of GIRKs, Derm-BTR produced the same maximal inhibition as ME. As was suggested in experiments done in acutely dissociated LC neurons, it appears that the coupling of opioid receptors to the inhibition of calcium (barium) currents is more efficient than that required to activate potassium currents (Ingram et al., 1997). The activation of α_2 -adrenergic receptors by UK14304 also reduced the inward calcium (barium) current by $45.8 \pm 2.1\%$ ($n = 3$).

Figure 3. Internalization of Derm-BTR (450 nm). **A**, Binding of Derm-BTR (450 nm) to receptors on the plasma membrane was observed as a fluorescent ring along plasma membrane (15°C). The temperature was then increased to 35°C within 1 min and internalization was monitored for 45 min. **B**, Image of the same cell taken after incubation for 30 min. **C**, After washing the Derm-BTR with a hypertonic acid solution (0.5 M NaCl in acetate buffer, pH 4), extracellular fluorescence outside the cell was removed and fluorescent puncta were observed in the soma. **D, E**, Fluorescent puncta containing Derm-BTR found in a GFP-positive process.

Figure 4. Time course of Derm-BTR internalization and desensitization. **A**, A representative experiment showing the block of Derm-BTR (D-BTR; 100 nm) internalization by naloxone (10 μ M; top row). Naloxone was washed from the bath and Derm-BTR (100 nm) was reapplied to demonstrate the presence of MORs on this cell. Images were taken with a confocal microscope at 2 min intervals. To reduce photobleaching, a single frame was collected at each of 5 z-planes. Only nonspecific staining was found in the presence of naloxone. **B**, Fluorescent intensity in the cytoplasm increased over the first 4–5 min and reached a plateau at 10 min. The fluorescence was normalized to the intensity measured at 10 min. **C**, A representative experiment showing the peak and decline of the potassium current induced by Derm-BTR (1 μ M) during a 10 min application. The experiment was done in a high-potassium solution as in Figure 2A. The inward current was measured during a voltage step from -60 to -100 mV applied once every 20 s. The current is plotted as an outward current to better illustrate the decline in the current. Error bars indicate SE.

Inhibition of transmitter release is a well known action of opioids (Williams et al., 2001). Cultured LC neurons plated at low density often formed recurrent synapses (autapses) (Fig. 2C). When these cells were depolarized from -60 to -20 mV for 2 ms, a sharp-rising inward current was observed. The currents had a

mean amplitude of 2.1 ± 0.6 nA ($n = 13$). The mean duration was ~ 10 ms. This EPSC was blocked by the AMPA receptor antagonist, NBQX (10 μ M; $91 \pm 6.2\%$ inhibition of the control EPSC; $n = 6$). This result suggests that LC neurons, like other monoaminergic neurons (Johnson, 1994; Sulzer et al., 1998), release glutamate when maintained in isolated cell culture. Both opioids and UK14304 decreased the amplitude of the autaptic EPSC. There was no significant difference between the inhibition caused by the opioid or α_2 -adrenoceptor agonists [Derm-BTR, 1 μ M, $53 \pm 15\%$ inhibition ($n = 4$); ME, 10 μ M, $43 \pm 13\%$ inhibition ($n = 4$); UK14304, 10 μ M, $62 \pm 8\%$ inhibition ($n = 8$)].

Agonist-induced internalization

The internalization of Derm-BTR in the LC cultures was examined using a confocal microscope. As was found in experiments done with cell lines expressing Flag-tagged MORs (Arttamangkul et al., 2000; Alvarez et al., 2002), Derm-BTR resulted in the appearance of fluorescent puncta in the LC cultured neurons and was taken as a measure of receptor internalization. This internalization was opioid-receptor dependent because no fluorescent puncta were observed in the presence of naloxone (10 μ M; $n = 4$). Internalization was temperature dependent. When the cell was incubated with Derm-BTR at 15°C, fluorescence was found only at the plasma membrane (Fig. 3A). Cells were incubated in Derm-BTR (450 nm) for a total of 45 min at 35°C. Fluorescent puncta were found in the cytoplasm primarily in the perinuclear region (Fig. 3B). High-resolution images of the internalized Derm-BTR were taken after a hypertonic acid wash. Fluorescent puncta were found in soma, proximal, and distal dendrites (Fig. 3C–E).

To determine the time course of internalization, images were taken at 2 min intervals for 10 min (Fig. 4A). To make comparisons between experiments, intracellular fluorescence was measured at each time point and plotted as a ratio of fluorescence measured at the end of the 10 min incubation. The fluorescence increased quickly (2–4 min) and reached a plateau within 10 min (Fig. 4B) ($n = 6$). The time course of internalization of Derm-BTR in the cultured LC neurons was similar to that measured in HEK cells (Alvarez et al., 2002).

Desensitization and internalization

When recordings were made during the application of Derm-BTR (1 μ M, at 25°C) the potassium current rose to an initial peak and declined in the continued presence of the drug. After 10 min, the current declined to $82 \pm 12\%$ of the initial peak (Fig. 4C) ($n = 4$). Because Derm-BTR is a very hydrophobic molecule, it was not possible to completely wash the high concentration of Derm-BTR, particularly after a prolonged application period. Naloxone (1 μ M) was used to rapidly reverse the current induced by Derm-BTR, to make an accurate measurement of the desensitization (Fig. 4C). Without using a hypertonic acid wash, the continued presence of extracellular fluorescent resulted in images of poor quality. It was therefore not possible to examine both desensitization and internalization in the same experiment.

Two changes in the experimental protocol were necessary to carry out experiments where desensitization and internalization could be examined on the same cell. First, intracellular recordings were used for these experiments because these recordings were stable over 60 min and did not disrupt cell morphology. High-resistance pipettes (100–200 M Ω) were used and membrane potential was measured. All experiments with intracellular recordings in combination with imaging were done at 35°C. As a control, each cell was tested with ME before imaging experiments. The amplitude of the hyperpolarization induced by ME (10 μ M) was 16.1 ± 1.3 mV ($n = 23$), which was similar, but somewhat smaller (5–10 mV) than that measured with intracellular recordings in mouse brain-slice experiments (S. Arttamangkul, unpublished observations). The second change in the experimental protocol required the synthesis of a more hydrophilic fluorescent dermorphin analog, Derm-A594. The advantage of this compound was the increased water solubility, enabling a more rapid (5 min) and complete wash-out, even with the use of high concentrations and prolonged exposures.

Superfusion with Derm-A594 (6 μ M) caused a hyperpolarization of 14.8 ± 2.6 mV ($n = 7$), not significantly different from that induced by ME (10 μ M; Student's t test, $p = 0.61$) (Fig. 7C). During a 10 min application of Derm-A594 (6 μ M), the peak hyperpolarization declined by $44 \pm 7\%$ ($n = 5$). This decline was taken as a sign of desensitization. After washing for 5 min, fluorescent puncta were present in the cytoplasm (Fig. 5, top panel). To confirm that the hyperpolarization and internalization were dependent on opioid receptors, experiments were done after treatment with the irreversible opioid antagonist, β -chloral-trexamine (β -CNA; 1 μ M). After β -CNA, Derm-A594 (6 μ M) did not change the resting membrane hyperpolarization and no fluorescent puncta were found in the neurons observed (Fig. 5, bottom panel) ($n = 3$).

With the combination of these measures it was possible to determine the role of receptor internalization on the desensitization process. First, the hyperpolarization induced by ME (300 nM; 12.8 ± 1.1 mV; $n = 14$) was tested before and after a 2 min application of Derm-A594 (6 μ M) (Fig. 6). The hyperpolarization induced by ME (300 nM) tested 5 min after the application of Derm-A594 was $42 \pm 5\%$ ($n = 5$) of control. Thus, as was found with a 2 min application of ME in brain-slice experiments (Dang and Williams, 2004), Derm-A594 caused significant desensitization. After this short application of Derm-A594, no significant fluorescent puncta were detected (Fig. 6B). This may indicate that desensitization was induced in the absence of internalization. It is also possible that the intracellular fluorescence was below the limit of detection. To determine whether desensitization and internalization were separate processes, receptor desensitization was examined under conditions where internalization was blocked.

The application of concanavalin A to block receptor internalization via clathrin-dependent pathways has been shown previously (Xiang et al., 2002; Kim et al., 2004). In the present experiments, all cells were initially tested with ME (10 μ M, 2 min). Concanavalin A (160 or 200 μ g/ml) was superfused for 20 min

Figure 5. Desensitization and internalization in the same neuron. **A**, Intracellular recording and two-photon microscopy were done simultaneously during treatment with Derm-A594 (D-A594). The top left shows an image of a neuron taken with infrared illumination and the same neuron after incubation with Derm-A594 (6 μ M, 10 min, 5 min wash; right). The trace to the right is the recording of the membrane potential of that neuron. ME (10 μ M) and Derm-A594 hyperpolarized this neuron. The hyperpolarization induced by Derm-A594 peaked and declined during a 10 min application. After washing Derm-A594, UK14304 caused a maximal hyperpolarization. **B**, The blockade of the hyperpolarization and internalization by β -CNA (1 μ M).

Figure 6. Desensitization by a short pulse of Derm-A594. **A**, Image of a representative cell where desensitization resulting from a short application of Derm-A594 was measured. **B**, Fluorescence image taken with a two-photon microscope showing the lack of any puncta after a 2 min treatment with Derm-A594 (6 μ M). **C**, Recording of membrane potential from the neuron shown in **A** and **B**. Desensitization was determined by comparing the decrease in the amplitude of the hyperpolarization induced by ME (300 nM) before and after a short pulse (2 min) of Derm-A594 (D-A594; 6 μ M).

followed by treatment with Derm-A594 (6 μ M, in con A) for 10 min. The initial hyperpolarization induced by Derm-A594 was 15.1 ± 1.1 mV ($n = 6$), which declined by $45 \pm 4\%$ over the 10 min application period ($n = 6$) (Fig. 7). After treatment with concanavalin A, neither the peak nor the decline in the hyperpolarization induced by Derm-A594 was significantly different from control (Student's t test, $p = 0.68$ and $p = 0.88$, respectively). The block of internalization was confirmed because no fluorescent puncta were found in the cytoplasm of cells (Fig. 7, supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Thus, concanavalin A blocked internalization but did not change the desensitization induced by Derm-A594, nor was the peak hyperpolarization induced by UK14304 changed (control 19.6 ± 3.6 mV; con A 16.3 ± 1.4 mV; $p = 0.31$) (Fig. 7).

Recovery from desensitization

The recovery from desensitization was determined by comparing the hyperpolarization induced by ME (300 nM, 2 min) before and after application of a saturating concentration of Derm-A594 (6 μ M, 2 min) or ME (10 μ M, 2 and 5 min). The hyperpolarization induced by ME (300 nM) was 42 ± 5 and $61 \pm 6\%$ of control 5 and 20 min after washout of Derm-A594, respectively (6 μ M, 2 min; $n = 5$). After desensitization with ME (10 μ M, 2 min), the hyperpolarization caused by ME (300 nM) was 42 ± 14 and $81 \pm 12\%$

Figure 7. MOR desensitization is not changed after blockade of internalization. **A**, Images of a neuron showing the appearance of fluorescent puncta in a control experiment (top). **B**, Images of cell after treatment with con A. No fluorescent puncta were found. **C**, Recording of membrane potential from a cell treated with con A. All cells were initially tested with ME (10 μ M). Con A (160–200 μ g/ml) was superfused for 20 min. Derm-A594 (D-A594; 6 μ M, in con A) induced membrane hyperpolarization similar in amplitude to that of ME. **D**, Summarized data showing the amplitude of the hyperpolarization induced by Derm-A594 and UK14304 (UK) in the absence and presence of con A. **E**, shows that the decline of the hyperpolarization induced by Derm-A594 was the same in the absence and presence of con A. Error bars indicate SE.

of control after 5 and 20 min, respectively ($n = 3$) (Fig. 8C). The recovery from desensitization was not significantly different after washout of Derm-A594 or ME ($p = 0.14$) and was similar in time course to that found in rat brain-slice experiments (Dang and Williams, 2004).

Recovery from desensitization was next examined 5 and 20 min after the application of ME (10 μ M, 5 min) in the absence and presence of concanavalin A (160 μ g/ml, 20 min preincubation) (Fig. 8A,B). The hyperpolarization induced by ME (300 nM) 5 and 20 min after desensitization with ME (10 μ M, 5 min) was 35 ± 8 and $70 \pm 7\%$ ($n = 4$) of control in the absence of concanavalin A and 40 ± 12 and $65 \pm 10\%$ ($n = 4$) of control in the presence of concanavalin A, respectively. There was no significant difference in the amount of recovery at either time point

($p = 0.71$ after 5 min; $p = 0.66$ after 20 min). The recovery from desensitization was therefore not changed under conditions where internalization was blocked.

Discussion

The results from this study demonstrate functional regulation of MORs in cultured LC neurons from TH-EGFP mice. With the combination of electrophysiological recording and the imaging of fluorescent opioid ligands, both desensitization and internalization were examined on the same cell under identical conditions. The use of primary cultures of neurons and fluorescent agonists made it feasible to study endogenous MORs. The results show that desensitization and the recovery from desensitization were not affected under conditions where internalization was blocked.

Effectors and receptor coupling

The results of the present study indicate that the coupling efficiency between various agonists and effectors can be distinguished. Although Derm-BTR was effective at inducing internalization, and caused a maximal inhibition of voltage-dependent calcium currents and transmitter release, it was not potent at activating GIRKs. By examining the relative action of Derm-BTR and ME on several different effectors on the same cells, it was clear that the activation of GIRKs required the more potent agonists to reach a maximal effect. This difference in agonist/receptor coupling has been observed previously in acutely dissociated LC neurons, where ME was a potent agonist at activating GIRK conductance, but morphine was an antagonist (Ingram et al., 1997). Unlike the activation of GIRK conductance, however, morphine caused an inhibition of calcium currents in acutely dissociated LC neurons (Connor et al., 1999). The coupling efficiency between MORs and the activation of GIRKs suggests that the receptor reserve was limited in this culture assay, thereby decreasing the proportion of neurons that responded to opioids. Similar results were reported in dissociated cell cultures of rat LC cells, where $<50\%$ of cells were hyperpolarized by even high concentrations of [D-Ala², D-Leu⁵]enkephalin (DADLE; 10–30 μ M) (Masuko et al., 1986). In brain-slice experiments on LC neurons, DADLE was a potent agonist that hyperpolarized all neurons through an activation of MORs (Williams and North, 1984). In the present study, each cell was tested with an EC₅₀ concentration of ME (300 nM); only cells that were hyperpolarized by at least 10 mV were selected for further study. The hyperpolarization in these “healthy” cells was similar to that measured in brain-slice recordings, suggesting that the opioid receptor regulation in these neurons was not dramatically changed. Each cell also served as its own control in all

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desensitization and recovery experiments. The decline in the hyperpolarization induced by the test application of ME (300 nM) after treatment with a saturating concentration of agonist was a sensitive measure to detect desensitization. Neither the extent nor the time course of recovery from desensitization was different from what has been measured in rat brain-slice experiments (Dang and Williams, 2004).

Homologous desensitization

Homologous MOR desensitization has been reported in several different preparations (Kovoor et al., 1998; Borgland et al., 2003; Celver et al., 2004). In the present study, the hyperpolarization induced by an α_2 -adrenoceptor agonist was not reduced after MOR desensitization. Similar results have been reported in LC cells recorded in rat brain slices (Bailey et al., 2004; Dang and Williams, 2004) (but see Blanchet and Luscher, 2002). The simplest interpretation of the present results is that a 2–10 min application of an opioid agonist induced homologous desensitization at the receptor. It is possible, however, that desensitization results from a point beyond the receptor but not at the potassium channel. One possibility would be a depletion of G-proteins that are selectively activated by opioid receptors.

Desensitization and receptor trafficking

The mechanism underlying desensitization in LC neurons remains unclear but presumably involves receptor phosphorylation by G-protein-receptor kinases and translocation of β -arrestin (Gainetdinov et al., 2004). This sequence could result in a blockade of signaling without internalization, however, the binding of arrestin to the ligand/receptor complex is thought to proceed to endocytosis via clathrin-coated pits (Law and Loh, 1999; Ferguson, 2001; von Zastrow et al., 2003). The separation of internalization and desensitization has been reported for other G-protein-coupled receptors such as β_2 -adrenergic and neurokinin-1 receptors (Pippig et al., 1995; Bennett et al., 2002), but it has also been shown that the desensitization of somatostatin and V1b vasopressin receptors requires internalization (Beaumont et al., 1998; Hassan and Mason, 2005). Although this sequence has been established in many heterologous systems, the role of this process in MOR signaling is the subject of some debate. One theory is that the removal of receptors from the plasma membrane by internalization decreases signaling, thus mediating desensitization (Whistler et al., 1999; Finn and Whistler, 2001). Other work suggests that receptor desensitization is augmented when receptors are not removed from the plasma membrane (Koch et al., 2005). Given the differences in results obtained in model systems and the fact that far less work has been done in primary neurons, it is not a surprise that the basic mechanisms underlying desensitization and receptor trafficking in neurons have not been completely characterized (Connor et al., 2004).

The ability to study receptor signaling on single neurons in the absence of internalization was made possible with the use of Derm-A594. The inhibition of G-protein-coupled receptor internalization with concanavalin A is well established (Xiang et al., 2002; Kim et al., 2004). In the present study, experiments using concanavalin A confirmed the absence of internalized Derm-A594. After blockade of internalization, Derm-A594 still

Figure 8. MOR recovery from desensitization is independent of internalization. **A**, A representative trace showing the recovery from desensitization induced by ME (10 μ M). ME (300 nM) was tested before and 5 and 20 min after application of ME (10 μ M, 5 min). The α_2 -adrenoceptor agonist UK14304 (1 μ M) was applied at the end of the experiment. **B**, The recovery from desensitization induced by ME (10 μ M) was not changed after treatment with concanavalin A (160 μ g/ml, preincubated for 20 min). **C**, Summarized data showing the recovery from desensitization. Left, A short (2 min) application of ME (10 μ M; gray bars) and Derm-A594 (D-A594; 6 μ M; black bars) decreased the hyperpolarization induced by ME (300 nM) to ~40% of control 5 min after washout. After 20 min, significant recovery was observed. Right, ME (10 μ M) was perfused for 5 min and the hyperpolarization induced by ME (300 nM) was tested after 5 and 20 min. The time course or extent of recovery was not changed by concanavalin A (white bars) compared with controls (gray bars). Error bars indicate SE.

caused a hyperpolarization that peaked and declined (desensitized) as in control. Thus, desensitization induced by Derm-A594, a compound that normally is efficient at causing internalization, is completely unaffected after the block of internalization. This experiment also showed that concanavalin A did not interrupt processes such as receptor binding, the coupling to G-proteins, the activation of potassium channels, or the α_2 -adrenoreceptor-dependent increase in potassium conductance. This observation confirms work done in AtT20 cells showing that morphine caused desensitization but was poor at inducing internalization (Borgland et al., 2003) and suggests that the desensitization caused by many, if not all, opioid agonists will proceed as normal under conditions where receptor trafficking is disrupted.

Based on experiments done in HEK293 cells expressing epitope-tagged MORs where the recycling of receptors was blocked with monensin, desensitization induced by DAMGO ([D-Ala², N-Me-Phe⁴, glycyl⁵] enkephalin) was increased, whereas that induced by morphine was not affected (Koch et al., 2005). Treatment of brain slices with monensin also increased the degree of desensitization to both ME and an active metabolite of morphine, morphine-6- β -D-glucuronide (Dang and Williams, 2004). It was concluded that internalization plays a role in recovery from desensitization rather than desensitization itself. The present results show that both the onset and recovery from desensitization occurred in preparations where receptor trafficking was blocked with concanavalin A. This observation suggests that the role of receptor endocytosis and recycling is not the only mechanism responsible for either the initial desensitization or recovery. This does not rule out an action on the prolonged recovery from desensitization seen after chronic morphine treatment (Dang and Williams, 2004). It is equally possible that redundant pathways mediate acute desensitization and blocking one mechanism has little effect on the decrease in downstream signaling.

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