Neuroscience 132 (2005) 239–244

RAPID REPORT

δ-OPIOID RECEPTOR-MEDIATED ACTIONS ON ROSTRAL VENTROMEDIAL MEDULLA NEURONS

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The rostral ventromedial medulla (RVM) forms a component of a descending inhibitory network that modulates nociceptive neurotransmission at the level of the spinal cord dorsal horn (Fields, 2004). The RVM is a crucial site of supraspinal μ-opioid receptor-mediated antinociception. Microinjection of δ-opioid receptor agonists into the RVM also produces antinociception (Satoh et al., 1983; Rossi et al., 1994; Ossipov et al., 1995; Thorat and Hammond, 1997). Previous *in vitro* experiments have characterized the cellular actions of μ- and κ-opioid, but not δ-opioid receptor agonists within the RVM (Pan et al., 1990, 1997; Ackley et al., 2001; Marinelli et al., 2002). We have found that δ-opioid receptor agonists postsynaptically inhibit (1) a subpopulation of μ-opioid responding non-serotonergic RVM neurons which appear to lack lumbar (L3–5) dorsal horn projections and (2) heterogeneous subpopulations of spinally projecting RVM neurons. The overlapping μ- and δ-opioid receptor-mediated actions provide a potential cellular substrate for μ/δ-opioid antinociceptive synergy. However, the findings also suggest complex μ/δ/κ-opioid receptor-mediated interactions in spinally projecting serotonergic and non-serotonergic systems.

EXPERIMENTAL PROCEDURES

All experiments were carried out following the guidelines of the NHMRC 'Code of Practice for the Care and Use of Animals in Research in Australia' and with the approval of the University of Sydney, Animal Care & Ethics Committee. Every effort was made to minimize the number of animals used and their suffering. Experiments were carried out on 10–18 day old male Sprague–Dawley rats (ARC, Perth, Australia), some of which have been described in a previous study on the actions of μ- and κ-opioid receptor agonists (Marinelli et al., 2002). Some animals were anesthetized (1–2% halothane in saturated O_2) and the lumbar spinal cord was exposed between L3 and L5. Two to four injections (10 nl each) of rhodamine-conjugated latex microspheres (Molecular Probes, Eugene, OR, USA) were made bilaterally into the spinal cord through a glass micropipette (tip diameter 20–50 μm) using a calibrated injection system (Drummond Nanoject, Broomall, PA, USA). The incision was closed, a topical antibiotic was applied and the animals were allowed to recover for 2–5 days before *in vitro* experiments were performed.

Brain slice recordings were made from animals as described previously (Marinelli et al., 2002). Briefly, animals were anesthetized with halothane and decapitated. Four to five coronal brain slices (250 μm thick) containing the rostral ventromedial medulla (RVM) were cut in ice-cold artificial cerebrospinal fluid (ACSF). Slices were maintained at 34 °C in a submerged chamber containing ACSF of composition (mM): NaCl 126, KCl 2.5, NaH_2PO4 1.4, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11, $NaHCO_3$ 25, which was equilibrated with a mixture of 5% CO_2 and 95% O_2 (pH 7.3). The brain slices were then transferred to a recording chamber and superfused continuously with ACSF (2 ml min^{-1} at 34 °C). RVM neurons were visualized in the triangular midline region dorsal to the pyramidal tracts using infra-red Nomarski optics on an upright microscope (Olympus BX50). Recordings were made from (1) retrogradely labeled neurons that were identified (under fluorescent epi-illumination) by the presence of fluorescent microspheres in their cell bodies prior to recording, (2) randomly selected neurons from animals that had received spinal cord tracer injections and (3) randomly selected neurons from animals that had not received spinal cord tracer injections. The results of neurons from groups (2) and (3) were similar and their results pooled for analysis. Whole-cell patch clamp recordings of postsynaptic currents (holding potential −60 mV) were performed with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) using patch electrodes (3–5 MΩ) filled with an internal solution of composition K-gluconate 115, KCl 25; NaCl 15, $MgCl_2$ 1, HEPES 10, EGTA 11, MgATP 2, NaGTP 0.25 with 0.01% biocytin (pH 7.3, osmolality 280–285 mosmol l^{-1}). Series resistance was compensated (<15 MΩ) by 80%. Postsynaptic currents were filtered (200 Hz low-pass filter) and sampled (500 Hz) for analysis (Axograph 4; Axon Instruments).

Recordings of currents through calcium channels (I_{Ca}) were made from acutely isolated RVM neurons as described previously (Vaughan et al., 2001). Briefly, brainstem slices were prepared as described above, and placed into a solution of composition (mM): Na_2SO_4 82, K_2SO4 30, HEPES 10, $MgCl_2$ 5, glucose 10, containing 20 units ml^{-1} papain, pH 7.3 and incubated for 2–3 min at 35 °C. The slices were then placed in fresh dissociation buffer containing 1 mg ml^{-1} bovine serum albumin (BSA) and 1 mg ml^{-1} trypsin inhibitor. The RVM region was subdissected from each slice and the cells dissociated by gentle trituration, then plated onto plastic culture dishes in dissociation buffer at room temperature (22–24 °C). Whole cell patch clamp recordings of I_{Ca} were made at 22–24 °C. Immediately prior to recording, cells were

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Abbreviations: ACSF, artificial cerebrospinal fluid; BSA, bovine serum albumin; DAMGO, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin; deltorphin-II, [D-Ala^2]-deltorphin II; DPDPE, [D-Pen^2; D-Pen^5]-enkephalin; RVM, rostral ventromedial medulla; TPH, tryptophan hydroxylase; TPH-ir, tryptophan hydroxylase immunoreactivity; 5-HT, 5-hydroxytryptamine.

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doi:10.1016/j.neuroscience.2005.01.015

superfused with a buffer of composition (mM): NaCl 140, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1.5, HEPES 10, glucose 10, pH 7.3 in order to wash off the dissociation buffer. For I_{Ca} recordings, cells were superfused in solution containing (mM): tetraethylammonium chloride 140, $BaCl_2$ 2, $MgCl_2$ 1, CsCl 2.5, HEPES 10, glucose 10, BSA 0.05%, pH 7.3. Recordings were made using patch electrodes (2 MΩ) filled with an intracellular solution of composition (mM): CsCl 110, MgATP 5, Na_2GTP 0.2, EGTA 10, $CaCl_2$ 2, NaCl 5 and HEPES 10, pH 7.3. I_{Ca} were filtered at 2 kHz, sampled at 5–10 kHz for later analysis (PCLAMP; Axon Instruments). Series resistance was compensated (1.5–5 MΩ) by 80%.

All stock solutions for *in vitro* experiments were made in distilled water except for U-69593 (in 0.1% HCl). These solutions were diluted at working concentrations in the extracellular solution immediately before use and applied by superfusion to the recording chamber (brain slice experiments), or via a series of flow pipes positioned above the cells (isolated cell experiments). The selective opioid agonists were applied in a randomized order. δ-Opioid receptor agonists produced similar currents whether they were applied before, or after μ-, or κ-opioid receptor agonists. Neurons were considered to respond to an opioid agonist if it produced an outward current of greater than 5 pA (brain slice experiments), or an inhibition of I_{Ca} greater than 5% (isolated cell experiments) that reversed on washout of the agonist, or addition of an antagonist. [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO), [D-Ala^2]-deltorphin II (deltorphin-II), met-enkephalin and substance P were from Auspep (Parkville, VIC, Australia). Biocytin, [D-Pen^2; D-Pen^5]-enkephalin (DPDPE), 5-hydroxytryptamine (5-HT) and U-69593 were from Sigma (Sydney, NSW, Australia). ICI-174,864 was from Tocris Cookson (Bristol, UK).

Following each recording (<25 min), slices were fixed for 30 min in a phosphate-buffered paraformaldehyde/picric acid solution (75 mM KH_2PO_4; 85 mM Na_2HPO_4; 4% [w/v] paraformaldehyde; 14% [v/v] saturated aqueous picric acid; pH 6.9), then washed six to eight times and stored in a phosphate-buffered sucrose solution (30 mM KH_2PO_4; 70 mM Na_2HPO_4; 10% sucrose [w/v]; 0.01% [w/v] sodium azide; 0.032% [w/v] bacitracin; pH 7.2). Biocytin-filled cells were visualized by incubation with Cy5-labeled streptavidin (Jackson ImmunoResearch, West Grove, PA, USA). Tryptophan hydroxylase (THP) immunoreactivity (TPH-ir) was visualized using a sheep anti-TPH antiserum (Chemicon, Temecula, CA, USA) followed by Cy2-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch). Slices were mounted with coverslips using 85% (w/v) aqueous sucrose. Images of filled cells were collected using a Bio-Rad MRC 1000 or MRC 1024 confocal microscope. Spinal cords from tracer injected animals were fixed, sectioned (100 μm thick) using a freezing microtome and examined using conventional fluorescence microscopy to reconstruct the extent of the injection sites. All numerical data are expressed as means±S.E.M, and statistical comparisons were made using χ^2 tests for differences among proportions.

RESULTS

When RVM neurons in brain slices were voltage clamped at −60 mV, supra-maximal concentrations of the selective δ-opioid receptor agonists deltorphin-II (300 nM) and DPDPE (300 nM) produced an outward current of 24±3 pA in 38% of neurons tested (Fig. 1a–ci, n=29/77). The deltorphin-II and DPDPE-induced outward currents often displayed rapid desensitization (Fig. 1ai). The δ-opioid receptor antagonist ICI-174,863 (300 nM–1 μM) reversed the outward current produced by deltorphin-II in neurons which did not display significant agonist induced desensitization (Fig. 1bi; n=5). The action potential duration of deltorphin-II responders (width=1.7±0.2 ms, n=20) was less than that of deltorphin-II non-responders (width=2.2±0.2 ms, n=23) when measured in current clamp mode (P<0.05, unpaired t-test).

Fifty-nine of the recordings in brain slices were from randomly selected RVM neurons (e.g. Fig. 1a, b). Deltorphin-II and DPDPE produced an outward current in 36% of these neurons (n=21/59). A supra-maximal concentration of the μ-opioid agonist DAMGO (3 μM) produced an outward current in the majority of both deltorphin-II/DPDPE responding (91%, n=19/21) and non-responding neurons (Fig. 2a; 70%, n=26/37). A supra-maximal concentration of the κ-opioid agonist U-69593 (1–3 μM) produced an outward in smaller percentages of deltorphin-II/DPDPE responders and non-responders (Fig. 2a). Of the 59 randomly selected neurons, 27 were obtained from animals which had received tracer injections into the lumbar spinal cord without first establishing whether they were retrogradely labeled. Ninety-three percent of the recovered randomly selected, biocytin-filled neurons were identified (after electrophysiological recordings) as not containing fluorescent microspheres in their cell bodies (Fig. 1a–bii; n=25/27).

Eighteen of the recordings in brain slices were from neurons which were identified (prior to electrophysiological recording) as being retrogradely labeled from the spinal cord (e.g. Fig. 1c). Deltorphin-II (300 nM) produced an outward current in 44% (n=8/18) of these neurons. DAMGO and U-69593 produced outward currents in 61% (n=11/18) and 56% (n=10/18) of these retrogradely labeled neurons, respectively. Unlike the randomly selected neurons, the deltorphin-II responding and non-responding retrogradely labeled neurons were heterogeneous with respect to their combinations of DAMGO and U-69593 induced responses (Fig. 2b).

We next examined whether the δ-opioid receptor agonist responsive and non-responsive neurons in brain slices were immunoreactive for TPH, a marker of serotonergic neurons (Fig. 1a–cii). Of the 51 recovered randomly selected RVM biocytin-filled neurons, a similar proportion of deltorphin-II responders (11%, n=2/19) and non-responders (9%, n=3/32) were immunoreactive for TPH (Fig. 3; χ^2=0.02, P>0.05). Of the 18 recovered retrogradely labeled RVM biocytin-filled neurons, a similar proportion of deltorphin-II responders (38%, n=3/8) and non-responders (40%, n=4/10) were immunoreactive for TPH (Fig. 3; χ^2=0.01, P>0.05). A number of the randomly selected (n=20/51) and retrogradely labeled (n=8/18) RVM neurons examined for THP-ir responded to more than one of the μ-, δ- and κ-opioid receptor agonists (data not shown).

In recordings from acutely isolated RVM neurons, superfusion of either DPDPE (1 μM, n=7), or deltorphin-II (1–10 μM, n=43) had no effect on I_{Ca} in 96% (n=48/50) of the neurons tested (Fig. 4). In the other two neurons deltorphin-II inhibited I_{Ca} by 6% and 12%. In contrast, I_{Ca} was inhibited by DAMGO, but not by U-69593 in 46% of neurons (n=23/50); by U-69593, but not by DAMGO in 22% of neurons (n=11/50); and by both DAMGO and U-69593 in 10% of neurons (n=4/50). DAMGO and U-69593 had no effect on I_{Ca} in the other 24% of neurons (n=12/50).

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