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Differential Presynaptic Effects of Opioid Agonists on Aδ- and C-afferent Glutamatergic Transmission to the Spinal Dorsal Horn

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Background: **Although intrathecal administration of opioids produces antinociceptive effects in the spinal cord, it has not been established whether intrathecal opioid application more effectively terminates C fiber–mediated pain than A fiber–mediated pain. Here, the authors focus on the differences in opioid actions on Aδ- and C-afferent responses.**

Methods: **Using the whole cell patch clamp technique, the authors investigated the presynaptic inhibitory actions of μ-, δ-, and κ-opioid receptor agonists on primary afferent–evoked excitatory postsynaptic currents (EPSCs) in substantia gelatinosa neurons of adult rat spinal cord slices.**

Results: **The μ agonist DAMGO (0.1, 1 μM) reduced the amplitude of glutamatergic monosynaptic Aδ- or C fiber–evoked EPSCs. C fiber–evoked EPSCs were inhibited to a greater extent than Aδ fiber–evoked EPSCs. The δ agonist DPDPE (1, 10 μM) produced modest inhibition of Aδ- or C fiber–evoked EPSCs. In contrast, the κ agonist U69593 (1 μM) did not affect the amplitude of either Aδ or C fiber–evoked EPSCs.**

Conclusions: **These results indicate that opioids suppress excitatory synaptic transmission mainly through activation of μ receptors on primary afferent C fibers. Given that the substantia gelatinosa is the main termination of Aδ and C fibers transmitting nociceptive information, the current findings may partially explain the different potency of opioid agonists.**

EARLY-ONSET first pain, evoked by activation of myelinated Aδ fibers, is sharp and well localized, whereas in contrast, unmyelinated C fiber–mediated secondary pain is dull and much less localized. Postoperative pain is an example of a clinical condition in which distinct sensations can be attributed to either Aδ- or C-fiber activity.[1] Tonic postoperative pain, dominated by C-fiber activity, is generally well controlled by systemic morphine, whereas phasic pain, dominated by Aδ-fiber activity, is less responsive. These clinical findings are supported by previous studies that showed that systemically applied morphine is more potent in attenuating nociception mediated by C fibers than Aδ fibers.[2–4] In addition, electrophysiologic experiments have demonstrated a preferential effect of systemic morphine on C fiber *versus* A fiber (Aβ and Aδ)–evoked dorsal horn neuronal responses.[5–7] However, it has not been established whether intrathecal opioid application more effectively terminates C fiber–mediated pain. Intrathecal administration of opioids produces antinociceptive effects mediated by μ-, δ-, and κ-opioid receptors in the spinal dorsal horn of both laboratory animals and humans.[8–10] Previous physiologic and behavioral studies indirectly suggested that intrathecal administration of opioids suppresses C-fiber responses more effectively than A-fiber responses.[11,12] Whereas electrophysiologic studies have demonstrated that both μ- and δ-opioid receptor agonists inhibit transmission of nociceptive, but not innocuous, information to the rat spinal cord,[13] differences in opioid action on distinct classes of nociceptive afferents (Aδ fiber and C fiber) are not well understood.

The substantia gelatinosa (SG; lamina II) of the spinal cord is thought to modulate nociceptive transmission from peripheral Aδ and C primary afferents.[14–16] Presynaptically, μ- and δ-opioid receptor agonists inhibit synaptic transmission to spinal dorsal horn neurons by blocking voltage-gated calcium channels,[17] thereby reducing transmitter release.[18,19] Postsynaptically, opioids activate μ-, δ-, and κ-opioid receptors, which open G protein–coupled inwardly rectifying potassium channels, thus hyperpolarizing the membrane potential and inhibiting excitatory transmission in spinal cord.[20–22] Such presynaptic and postsynaptic actions of opioids are supported by immunohistochemical and binding studies. These opioid receptors have been localized presynaptically on the central terminals of small-diameter nociceptive primary afferents (Aδ and C fibers), as well as postsynaptically in the superficial layers of the spinal cord, especially lamina II.[23–26] The aim of the current study was to examine the presynaptic effects of μ-, δ-, and κ-opioid receptor agonists on excitatory synaptic transmission, particularly Aδ and C fiber–mediated transmission, to SG neurons in rat spinal cord slices.

Materials and Methods

This study was approved by the Animal Research Committee of Niigata University Graduate School of Medical and Dental Sciences in Niigata, Japan. Thick (600-μm) spinal cord slices containing the L4 dorsal root (10–20 mm) were prepared from adult rats (aged 5–7 weeks) as described previously.[27,28] The slice was placed on a nylon mesh in the recording chamber and then perfused at a rate of 10 ml/min with Krebs solution, which was saturated with 95% O_2 and 5% CO_2 maintained at 36° ±

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1°C. The Krebs solution contained 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose.

Substantia gelatinosa was identifiable as a translucent band across the superficial dorsal horn under a dissecting microscope with transmitted illumination. Whole cell patch clamp recordings were made from SG neurons in voltage clamp mode with patch pipette electrodes having a resistance of 10 MΩ. The patch pipette solution contained 110 mM Cs_2SO_4, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 5 mM TEA, 5 mM ATP-Mg salt, and 1 mM guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S). GDP-β-S and K^+ channel blockers (Cs^+ and TEA) were used to inhibit postsynaptic actions of opioid agonists on G proteins and K^+ channels, respectively. Signals were amplified with an Axopatch 200A (Molecular Devices, Union City, CA) and were filtered at 2 kHz and digitized at 5 kHz. Data were collected and analyzed using pClamp8.0 (Molecular Devices). The holding potential used was −70 mV, the reversal potential of glycine and γ-aminobutyric acid receptor–mediated synaptic currents. Excitatory postsynaptic currents (EPSCs) were elicited by dorsal root stimulation using a suction electrode, low-intensity (50–100 μA, 0.05 ms) stimulation for Aδ fibers, and higher-intensity and longer-duration (200–1,000 μA, 0.5 ms) stimulation for C fibers.[29,30] The stimulus intensity necessary to activate Aδ and C fibers and the afferent fiber conduction velocity was determined by extracellular recording of compound action potentials from the dorsal root.[29] Aδ fiber–evoked EPSCs (Aδ-EPSCs) were judged as monosynaptic on the basis of both their short and constant latencies and the absence of failures with repetitive stimulation at 20 Hz. Identification of C fiber–evoked monosynaptic EPSCs (C-EPSCs) was based on an absence of failures with low-frequency (1 Hz) repetitive stimulation.[31,32] Opioids were dissolved in Krebs solution and applied by perfusion without alteration in the perfusion rate or temperature.

Drugs used were [D-Ala^2-N-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO), [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), D-(5α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl] benzeneacetamide (U69593), Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), naltrindole, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and GDP-β-S (Sigma-Aldrich, St. Louis, MO).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical significance was determined as $P < 0.05$ using either the Student paired or unpaired *t* test. In all cases, n refers to the number of neurons studied. The neurons examined were considered to sensitive to the opioids if they exhibited depressions of more than 5%. All statistical calculations were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC).

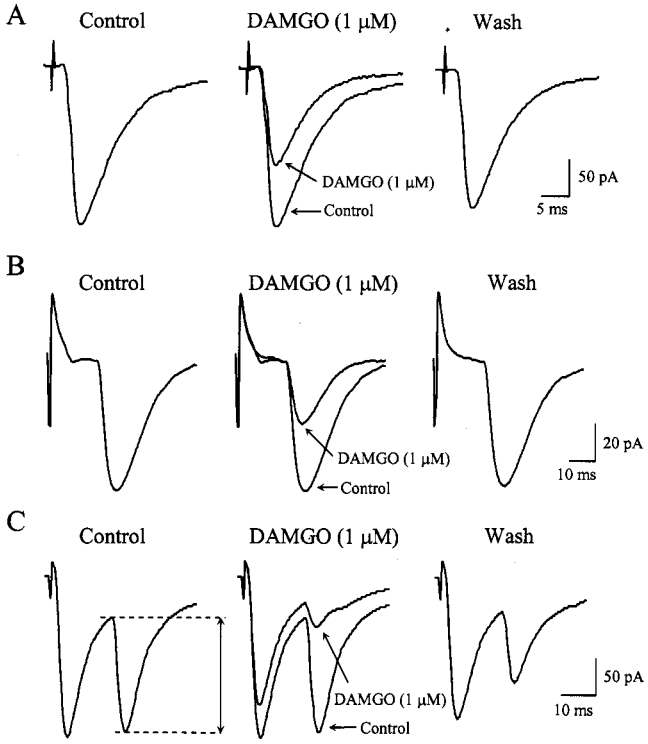

Fig. 1. Effects of DAMGO (1 μM) on monosynaptic excitatory postsynaptic currents (EPSCs) evoked by stimulating Aδ fiber (*A*) and C fiber (*B*) or both Aδ and C fibers (*C*). (*A–C*) Averaged traces of five consecutive evoked EPSCs before (*left*), during (*middle*; where control EPSC on the left is superimposed for comparison), and after (*right*) the action of DAMGO. The EPSCs were evoked at 0.1 Hz. The intensities of the stimuli used in *A–C* were 73, 480, and 380 μA, respectively. An illustration (*C*, *left*) showed how the amplitude of C-EPSC was measured.

Results

Whole cell recordings were obtained from 204 SG neurons. All SG neurons exhibited spontaneous EPSCs at −70 mV as reported previously.[27] In 78 (38%) of 204 neurons examined, stimulating the dorsal root with Aδ-fiber intensity elicited glutamatergic monosynaptic EPSCs (fig. 1A), which displayed no failures and no change in latency when stimulated at 20 Hz. Conduction velocity averaged 6.2 m/s (range, 5.0–9.0 m/s); these values were within the Aδ-fiber range obtained from experiments in dorsal root ganglion neurons.[31,32] Aδ-EPSCs had a mean amplitude of 297 ± 36 pA. Similarly, stimulating the dorsal root at C-fiber intensity induced glutamatergic monosynaptic C-EPSCs (fig. 1B) in 79 (39%) of 204 neurons examined. C-EPSCs had no failures when stimulated at 1 Hz. The average conduction velocity was 0.6 m/s (range, 0.4–0.9 m/s), comparable to values measured in the dorsal root ganglion.[31,32] C-EPSCs had a mean amplitude of 239 ± 34 pA. Six percent (n = 13) of the all neurons tested exhibited both monosynaptic Aδ- and C-EPSCs, as seen in figure 1C. These EPSCs were completely inhibited by CNQX (10 μM, n = 4), indicating the involvement of non–*N*-methyl-D-aspartate glutamate receptors, as reported previously.[27]

Table 1. Proportion of Neurons Exhibiting Inhibition by μ-, δ-, and κ-Receptor Agonists with Respect to the Amplitude of the Aδ- or C Excitatory Postsynaptic Current

Fiber Type	DAMGO (μ Agonist)	DPDPE (δ Agonist)	U69593 (κ Agonist)
Aδ	17/19 (89%)	9/13 (69%)	3/9 (33%)
C	11/12 (92%)	7/20 (35%)	2/10 (20%)

The concentration of DAMGO, DPDPE, and U69593 used was 1 μM. The neurons examined were considered to be sensitive to the opioid agonist if they exhibited depressions of more than 5%.

The proportion of EPSCs inhibited by each opioid was quite different (table 1). Therefore, to compare efficacies of the different opioid receptor agonists, we showed the effects on the amplitudes of Aδ- and C-EPSCs from all recorded neurons. Effects of the μ-receptor agonist, DAMGO (1 μM), on monosynaptic Aδ- and/or C-EPSCs were examined in 28 SG neurons. Superfusing DAMGO reversibly inhibited the peak amplitude of Aδ-EPSCs from 350 ± 39 pA to 242 ± 33 pA in almost all neurons (29 ± 5%, n = 19; $P < 0.01$; figs. 1A and 2A). Similarly, superfusion of DAMGO inhibited the peak amplitude of the C-EPSCs from 164 ± 34 pA to 76 ± 23 pA in almost all neurons (52 ± 9%, n = 12; $P < 0.01$; figs. 1B and 2A). C-EPSCs were more effectively inhibited by DAMGO than Aδ-EPSCs (unpaired *t* test, $P < 0.01$; fig. 2B). Differential effects of DAMGO on Aδ- and C-EPSCs were also observed in neurons with both Aδ- and C-EPSCs (fig. 1C). A dose of 1 μM for DAMGO is well above the EC_{50}[18,33]; we next examined the effects of a lower dose of DAMGO (0.1 μM) on Aδ- or C-EPSCs. DAMGO inhibited Aδ-EPSCs (18 ± 4%, n = 11; $P < 0.01$) and C-EPSCs (37 ± 6%, n = 13; $P < 0.01$), respectively (fig. 2A). Similarly, C-EPSCs were more effectively inhibited by DAMGO (0.1 μM) than Aδ-EPSCs (unpaired *t* test, $P < 0.05$). Inhibitory effects of DAMGO (1 μM) on Aδ- and C-EPSCs were reversed by a μ antagonist, CTAP (1 μM, n = 4; fig. 2C), indicating specific activation of μ receptors by DAMGO. The Aδ- and C-EPSCs were depressed in amplitude by DAMGO with a similar time course, maximal at approximately 2 min after beginning DAMGO superfusion.

We next examined the effects of the δ-receptor agonist, DPDPE (1 μM), on monosynaptic Aδ- and/or C-EPSCs in a total of 30 SG neurons. DPDPE reduced the amplitude of Aδ-EPSC from 314 ± 46 pA to 271 ± 46 pA (12 ± 3%, n = 13; $P < 0.01$; figs. 3A and C), with little affect on the amplitude of C-EPSC (4 ± 3%, n = 20; $P > 0.05$; figs. 3B and C). A dose of 1 μM for DPDPE is not well above the EC_{50} [18]; we next investigated the effects of a higher dose of DPDPE (10 μM) on Aδ- or C-EPSCs. Superfusing DPDPE inhibited Aδ-EPSC (19 ± 5%, n = 14; $P < 0.01$; fig. 4A) and C-EPSC (10 ± 4%, n = 16; $P < 0.05$; fig. 4A). Unlike DAMGO, Aδ- and C-EPSCs were equally inhibited by DPDPE (10 μM) (unpaired *t* test, $P > 0.05$; fig. 4B). The inhibitory effects of DPDPE (10 μM) on Aδ- and C-EPSCs were not seen in the presence of a δ-receptor antagonist, naltrindole (1 μM; 4 ± 4% and 3 ± 4%, respectively, n = 5; data not shown), suggesting specific activation of δ receptors by DPDPE. In contrast to DAMGO and DPDPE, the κ-receptor agonist, U69593 (1 μM), had no effect on the amplitude of Aδ- or C-EPSCs (7 ± 3% and 5 ± 4%, respectively, n = 9 and n = 10; $P > 0.05$; data not shown). Because a dose of 1 μM for U69593 is well above the EC_{50},[21] we did not run the experiments with a higher dose of U69593.

Discussion

The purpose of this study was to compare the presynaptic effects of the μ-, δ-, and κ-opioid receptor agonists on Aδ and C fiber–evoked excitatory transmission to SG neurons of adult rat spinal cord slices. The μ agonist (DAMGO, 0.1, 1 μM) depressed both Aδ- and C-EPSCs; C-EPSCs were more sensitive to DAMGO inhibition than Aδ-EPSCs. In contrast, the δ agonist (DPDPE, 1 μM) produced only moderate inhibition of Aδ-EPSCs and no inhibition of C-EPSCs. At a higher dose of DPDPE (10 μM), Aδ- and C-EPSCs were moderately inhibited equally. However, the κ agonist (U69593, 1 μM) had no effect on either Aδ- or C-EPSCs. The current results that μ and δ but not κ agonist (1 μM) depressed Aδ fiber–mediated excitatory transmission are consistent with our previous study.[19] The current work is the first electrophysiologic report that the presynaptic effect of μ agonist was more potent at C afferents than at Aδ afferents. The results of this study are consistent with our recent study examining the effects of DAMGO on primary afferent–induced extracellular signal–regulated kinase activation in dorsal horn neurons of rat spinal cord slices, a marker for nociceptive-evoked activity. Activation of extracellular signal–regulated kinase induced by C-fiber stimulation[34] was more effectively suppressed by DAMGO than Aδ fiber–induced extracellular signal–regulated kinase activation.[28]

Immunohistochemistry and receptor autoradiography have shown that opioid receptors are located on small dorsal root ganglion neurons and in laminae I and II of the dorsal horn,[35–38] suggesting that these receptors are present presynaptically on Aδ- and C-fiber terminals. Moreover, the number of opioid-binding sites in the dorsal horn is reduced 40–70% after dorsal rhizotomy, indicating that opioid receptors are localized to presynaptic terminals.[23,25,39] Reduction of opioid receptors after capsaicin-induced C-fiber degeneration is similar to that observed after dorsal rhizotomy.[40] Furthermore, μ receptors do not colocalize with RT97 (a marker for myelinated afferents, *i.e.*, A fibers), indicating that μ receptors are only localized to neurons that give rise to unmyelinated afferent fibers (*i.e.*, C fibers).[38] However, we demonstrate DAMGO depression of Aδ-fiber EPSCs, indicating that functional μ receptors are expressed on myelinated Aδ afferents. Consistent with the current observation that C-EPSCs are more sensitive than Aδ-

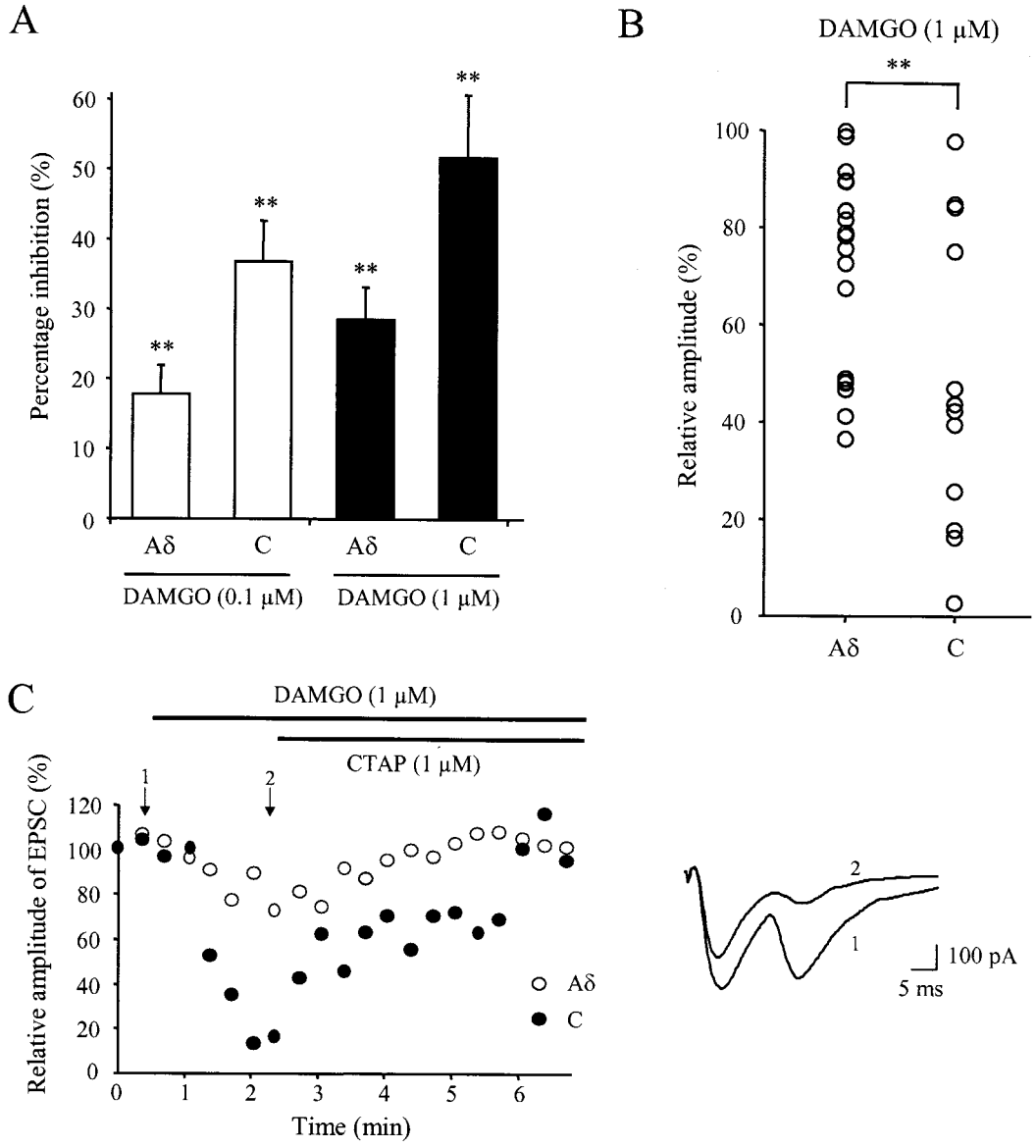

Fig. 2. (*A*) Percentage inhibition of monosynaptic Aδ and C excitatory postsynaptic currents (EPSCs) under the action of DAMGO (0.1, 1 μM). *Vertical bars* show SEM. ** $P < 0.01$, compared with control. (*B*) A distribution of relative amplitude compared with control for Aδ- and C-EPSCs by DAMGO (1 μM). Note that the C-EPSC was more effectively inhibited by DAMGO than the Aδ-EPSC. ** $P < 0.01$, Aδ *versus* C. (*C*) Time course of the peak amplitude of Aδ- (○) and C- (●) EPSCs recorded in the same neuron before and during DAMGO (1 μM). CTAP (1 μM) reversed the inhibition of Aδ- and C-EPSCs during application of DAMGO. Original traces of EPSCs indicated by numbers correspond to the time points on the graph.

EPSCs to a μ agonist, calcium currents in small nociceptive cells of rat tooth pulp were most strongly inhibited by μ opioids.[41] Taken together, this suggests that differences in μ receptor density are likely to underlie the differential sensitivity of C- and Aδ-EPSCs to μ agonists. Alternatively, each of the fiber terminals may express different subtypes of μ receptors. We could not, however, identify different μ receptors on Aδ-fiber and C-fiber terminals with the antagonist CTAP. Differences in DAMGO actions on Aδ-afferent and C-afferent terminals require future examination with a variety of μ-receptor antagonists. Although a cellular mechanism of action was not examined here, it is likely that EPSCs are reduced as a consequence of opioid-induced inhibition of voltage-gated calcium channels, because opioids have been reported to suppress calcium currents in rat dorsal root ganglion neurons.[17,42] However, other possibilities, such as alternate mechanisms of reduced neurotransmitter release, different μ-receptor subtypes, or involvement of potassium channels, cannot be ruled out.

Although the EC_{50} is different for each opioid agonist, rank order potency at the concentration (1 μM) for inhibiting Aδ- and C-EPSCs was $\mu > \delta >> \kappa$.[19] This rank order was the same as that for the proportion of neurons in which each of the opioids inhibited excitatory transmission by more than 5% (table 1). Radioligand binding experiments in the rat spinal cord have demonstrated that the most prevalent type of opioid receptor in laminae I and II

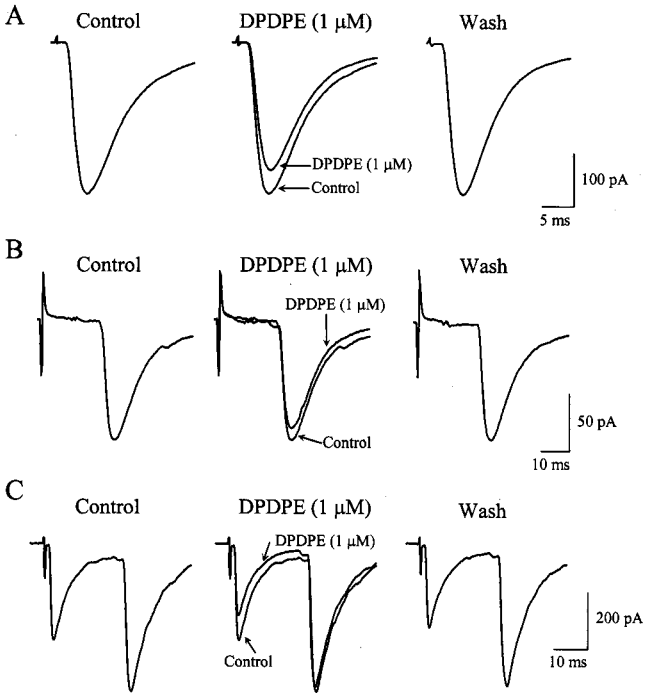

Fig. 3. Effects of DPDPE (1 μM) on excitatory postsynaptic currents (EPSCs) evoked by stimulating Aδ-fiber (*A*) and C fiber (*B*) or both Aδ- and C fiber EPSCs (*C*). (*A–C*) Averaged traces of five consecutive evoked EPSCs before (*left*), during (*middle*; where control EPSC on the left is superimposed for comparison), and after (*right*) the action of DPDPE. The EPSCs were evoked at 0.1 Hz. The intensities of the stimuli used in *A–C* were 56, 380, and 560 μA, respectively.

is the μ receptor (63% or more), with considerably less δ receptor (23% or less) and κ receptor (15% or less),[23,43] precisely reflecting the different potencies.

Previous studies have reported that systemic application of morphine attenuates nociception mediated by C fibers more potently than that mediated by Aδ fibers.[2–4]

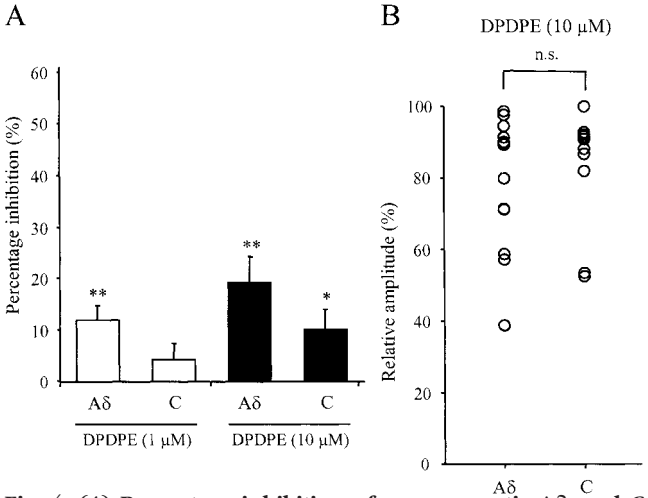

Fig. 4. (*A*) Percentage inhibition of monosynaptic Aδ- and C fiber–evoked excitatory postsynaptic currents (EPSCs) under the action of DPDPE (1, 10 μM). *Vertical bars* show SEM. * $P < 0.05$, ** $P < 0.01$, compared with control. (*B*) A distribution of relative amplitude compared with control for Aδ- and C-EPSCs by DPDPE (10 μM). n.s. = not significant, Aδ *versus* C.

However, systemically applied opioids may produce antinociception *via* both supraspinal and spinal mechanisms. Intrathecal administration of alfentanil, a μ agonist, has a greater inhibitory effect on the C fiber–mediated somatosympathetic reflex than on the Aδ-fiber reflex.[12] Similarly, low doses of intrathecal μ agonist inhibited C fiber–mediated responses more potently than Aδ fiber–mediated responses.[11] Our findings further support the notion of differences in the presynaptic actions of μ opioids on different afferent terminals in the spinal dorsal horn. However, at the spinal level, opioids act on postsynaptic targets[20,22,33] such as excitatory interneurons and projection neurons, as well as on presynaptic inputs from primary afferents. We do not yet understand how inhibitory information from SG neurons, which are known to be excitatory or inhibitory interneurons, is modulated.

In summary, the results presented here provide the antinociceptive presynaptic action of opioid receptor agonists on primary afferent Aδ and C fibers. The differential inhibitory effects of opioids reported here may partially account for differences in clinical analgesic actions of this class of nociceptive agents.

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