Anesthesiology 2003; 98:223–31

Fentanyl Decreases Ca^{2+} Currents in a Population of Capsaicin-responsive Sensory Neurons

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Background: **Neuraxial opioids produce analgesia in part by decreasing excitatory neurotransmitter release from primary nociceptive neurons, an effect that may be due to inhibition of presynaptic voltage-activated Ca^{2+} channels. The purpose of this study was to determine whether opioids decrease Ca^{2+} currents (I_{Ca}) in primary nociceptive neurons, identified by their response to the algogenic agent capsaicin.**

Methods: **I_{Ca} was recorded from acutely isolated rat dorsal root ganglion neurons using the whole cell patch clamp technique before, during, and after application of the μ-opioid agonist fentanyl (0.01–1 μM). Capsaicin was applied to each cell at the end of the experiment.**

Results: **Fentanyl reduced I_{Ca} in a greater proportion of capsaicin-responsive cells (62 of 106, 58%) than capsaicin-unresponsive cells (2 of 15, 13%; $P < 0.05$). Among capsaicin-responsive cells, the decrease in I_{Ca} was 38 ± 3% (n = 36, 1 μM) in fentanyl-sensitive cells *versus* just 7 ± 1% (n = 15, 1 μM; $P < 0.05$) in fentanyl-insensitive cells. Among capsaicin-responsive cells, I_{Ca} inactivated more rapidly in fentanyl-sensitive cells (τ_h, 52 ± 4 ms, n = 22) than in fentanyl-insensitive cells (93 ± 14 ms, n = 24; $P < 0.05$). This was not due to differences in the types of Ca^{2+} channels expressed as the magnitudes of ω-conotoxin GVIA-sensitive (N-type), nifedipine-sensitive (L-type), and GVIA/nifedipine-resistant (primarily P-/Q-type) components of I_{Ca} were similar.**

Conclusions: **The results show that opioid-sensitive Ca^{2+} channels are expressed by very few capsaicin-unresponsive neurons but by more than half of capsaicin-responsive neurons. The identity of the remaining capsaicin-responsive (and therefore presumed nociceptive) neurons that express opioid-insensitive Ca^{2+} channels is unknown but may represent a potential target of future non–opioid-based therapies for acute pain.**

TRANSMISSION of the sensation of pain from most parts of the body to the central nervous system occurs along the axons of primary sensory neurons whose cell bodies reside in the dorsal root ganglia. These primary nociceptive neurons synapse with secondary neurons in the dorsal horn of the spinal cord. Neuraxial administration of opioids produces analgesia, which may be due, at least in part, to a decrease in neurotransmitter release from primary nociceptive neurons.[1–4]

Opioid-induced decreases in transmitter release may be due to inhibition of voltage-activated Ca^{2+} channels in the presynaptic terminals of primary nociceptive neurons.[5] In *in vitro* cultures of dissociated sensory neurons, however, opioids do not always decrease Ca^{2+} currents (I_{Ca}), and when they do, the effect is quite variable, ranging from 10 to 90%.[6–11] This variability of opioid responsiveness may be a function of the heterogeneity of dorsal root ganglion neurons. Individual sensory neurons may transduce any of a number of different sensations, not only noxious stimuli but also innocuous sensations, such as light touch, warmth (< 43°C), and proprioception. Opioids are generally considered to have little or no effect on such nonnoxious sensations.[12,13]

The purpose of the present study was to determine the opioid sensitivity of Ca^{2+} channels expressed by a subset of sensory neurons identified as being nociceptive based on their response to the algogenic agent capsaicin. Capsaicin elicits burning pain by binding to the VR1 receptor, a nonspecific cation channel expressed almost exclusively by small sensory neurons involved in the transduction of noxious heat.[14–16] The results show that most cells that failed to respond to capsaicin were insensitive to opioids, while over half of the capsaicin-responsive neurons expressed opioid-sensitive Ca^{2+} channels. The significance of these findings is discussed.

Materials and Methods

Isolation of Dorsal Root Ganglion Neurons

Sprague-Dawley weanling rats (40–100 g) of either sex were sacrificed with pentobarbital (0.2 mg/g, intraperitoneal) according to a protocol approved by the Animal Care Use Committee at the University of Wisconsin (Madison, Wisconsin). Dorsal root ganglia were removed from the cervical, thoracic, and lumbar regions of the spinal column using a dissecting microscope and placed in 0.25% trypsin solution (Sigma, St. Louis, MO) containing 2 mg/ml collagenase (Sigma) at 35°C for 45 min. The ganglia were then gently triturated using flame-polished Pasteur pipettes, and the cells were plated on glass coverslips coated with poly-L-lysine (Sigma). Cells were maintained in an incubator at 35°C in a solution of Dulbecco's Modified Eagle Medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma). Experiments were performed between 2 and 36 h after isolation. Responses were not noticeably different at various times after isolation, though after 2 days in culture, neurons developed extensive processes, making it difficult to voltage clamp the cells. Neurons tested ranged in size from approximately 18 to 50 μm in diam-

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Received from the Department of Anesthesiology, University of Wisconsin Medical School, Madison, Wisconsin. Submitted for publication May 10, 2002. Accepted for publication August 23, 2002. Supported by a New Investigator Award from the Foundation for Anesthesia Education and Research, Rochester, Minnesota; the American Society of Regional Anesthesia, Richmond, Virginia; and the Department of Anesthesiology, University of Wisconsin, Madison, Wisconsin. Presented in part at the annual meeting of the American Society of Anesthesiologists, Orlando, Florida, October 21, 1998.

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eter (based on measurement of cell membrane capacitance and assuming spherical cells with specific membrane capacitance of 1 $\mu F/cm^2$).

Whole Cell Patch Clamp Recording

Patch pipettes were made from borosilicate glass using a two-stage pipette puller (Narishige USA, East Meadow, NY) and were heat-polished on a microforge (ALA Scientific Instruments, Westbury, NY) to a resistance of 1–3 MΩ. Pipettes were coated with Sylgard (World Precision Instruments, Sarasota, FL) to within 100 μm of the tip. Coverslips with cells attached were transferred to the recording chamber and perfused with external solutions. Whole cell currents were recorded in voltage clamp mode using a patch clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) using standard techniques.[17] Data acquisition and analysis were performed using a Digidata 1200 A/D converter and pClamp version 6.0.4 (Axon) running on a microcomputer. Immediately after obtaining a tight seal and rupturing the cell membrane to achieve the whole-cell recording configuration, series resistance (R_s) and membrane capacitance (C_m) were estimated from the settings on the amplifier obtained after eliminating the capacitative current transient recorded during a small voltage step. The values for R_s and C_m obtained in this way closely matched the values calculated by integrating the capacitative transient to measure C_m and fitting the transient to an exponential with a time constant of R_sC_m.

Both I_{Ca} and capsaicin-induced currents were recorded from individual cells at room temperature from a membrane holding potential of −80 mV. I_{Ca} was generally recorded first, but in some experiments, capsaicin was applied first, with no apparent differences in the results. I_{Ca} was elicited by square wave voltage steps of 90 ms duration to potentials ranging from −60 to +30 mV, or by repetitive voltage steps of 90 ms or less in duration to −10 mV. Currents were digitized at 5–10 kHz and filtered at 1–2 kHz. Leak and capacitative currents were subtracted from current records online by adding the currents obtained from four hyperpolarizing pulses each equal to one fourth the magnitude of the depolarizing test pulse (−P/4 protocol). Series resistance compensation was usually used to prevent voltage errors of greater than 5 mV.

Solutions

The pipette solution contained the following: 100–120 mM CsCl, 2.5 mM $MgCl_2$, 10 mM EGTA, 10–40 mM HEPES, 2 mM MgATP, and 0.3 mM LiGTP. The pH was adjusted to 7.3 with tetraethylammonium hydroxide or CsOH, and aliquots were stored at −80°C until use. The external solution used to perfuse the cells between experiments and to record capsaicin-induced currents contained the following: 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH. For recording I_{Ca}, the solution was switched to one containing the following: 135 mM tetraethylammonium chloride, 1–2 mM $CaCl_2$, 0–1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with tetraethylammonium hydroxide or CsOH. In later experiments, sucrose was also added to adjust the osmolality of the external solutions to 310 mOsmol/kg and the osmolality of the pipette solutions to 290 mOsmol/kg. No differences were noted after osmolality correction was instituted.

Capsaicin (N-vanillylnonanamide) stock solution was made in ethanol and refrigerated until use. For 10 μM capsaicin, the concentration of ethanol in the working solution was 0.1% (v/v). Concentrations of ethanol up to 1% (v/v) had either no effect or induced a small outward current. Concentrated stock solutions of fentanyl citrate, ω-conotoxin GVIA, ω-Agatoxin TK, ω-conotoxin MVIIC, and naloxone were made in distilled water and frozen. The nifedipine stock solution was made with DMSO and refrigerated. All reagents were from Sigma.

Agents were applied to cells by several methods. Cells were pretreated with some agents (ω-conotoxin MVIIC, ω-Agatoxin TK) by placing a coverslip of isolated neurons in a small dish filled with a solution containing one of the toxins for at least 20 min before removing the coverslip and placing it in the recording chamber, which did not contain toxin. For rapid applications of capsaicin, fentanyl, and some of the Ca^{2+} channel blockers, cells were gravity perfused with solutions flowing through one of three large capillary tubes glued together in a row. The tubes were rapidly moved with a computer-driven motor (Warner, Hamden, CT), allowing cells to be perfused with different solutions of known concentrations with exchange rates of less than 1 s. Finally, in some experiments, solution changes were accomplished using a gravity-fed system that allowed complete exchange of the solution in the recording chamber in 10–20 s (chamber volume was approximately 700 μl).

Data Analysis

The peak capsaicin-induced current was measured during a 5-s application of capsaicin at a membrane potential of −80 mV. I_{Ca} magnitude was measured 20 ms after the voltage step, when the current was near maximal. Current magnitudes were normalized to cell size by dividing by membrane capacitance. Fentanyl-induced changes in I_{Ca} magnitude were expressed as percent changes from the average of currents recorded before fentanyl application (control) and after washout of fentanyl (recovery). The inactivation time constant of I_{Ca} was estimated by fitting the decaying phase of the current to a single exponential using Clampfit (Axon). Averaged concentration–response relations were fit using

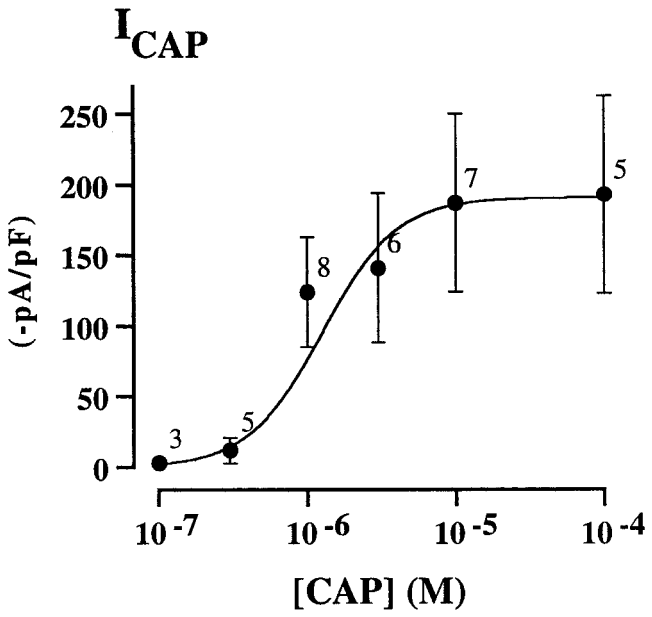

Fig. 1. Dose–response relation for capsaicin (CAP)-induced current density (I_{CAP}) in CAP-responsive sensory neurons. CAP was applied to each cell only once to prevent desensitization. The number of cells studied at each concentration is shown next to each data point. The smooth curve represents the best fit of the data to the Hill equation, with a calculated EC_{50} of 1.3 μM and n_H of 1.7.

nonlinear regression (GraphPad Software, San Diego, CA) to a logistic equation of the form:

$$R = R_{max}/\{1 + 10\text{^}[(\log EC_{50} - C) \times n_H]\},$$

where R is the response at concentration C, R_{max} is the maximum response obtained, EC_{50} is the concentration at which the effect is half-maximal, and n_H is the Hill coefficient.

Statistical Analysis

Data are expressed as mean ± SEM. The Fisher exact test was used to determine the statistical significance of differences in the proportions of cells among groups. Other values were compared using one-way analysis of variance followed by the Tukey *post hoc* test where appropriate (GraphPad Software). Statistical significance was assumed if *P* was less than 0.05.

Results

Capsaicin induced an inward current in 158 (83%) of 191 sensory neurons tested. Because repeated applications of capsaicin to the same neuron produced currents of progressively smaller magnitude, only one concentration of capsaicin was applied to each cell during the determination of the capsaicin concentration–response curve (fig. 1). The reversal potential of the capsaicin-induced current was approximately 0 mV. At 10 μM, capsaicin produced a near-maximal response. This concentration was used in subsequent experiments to determine the capsaicin response of individual neurons in order to decrease the risk of incorrectly categorizing a capsaicin-responsive cell as being unresponsive. The limits for categorizing cells as being capsaicin responsive or unresponsive were chosen after examining the distribution of capsaicin current responses. Neurons were considered to be responsive to capsaicin if the inward current magnitude was at least 100 pA or 5 pA/pF. Capsaicin-unresponsive cells generated less than 20 pA or 0 pA/pF of inward current. Six cells were excluded from analysis due to intermediate capsaicin responses (inward currents ranging from 30 to 88 pA or 1 to 6 pA/pF).

Table 1. Distribution of Individual Sensory Neurons According to Capsaicin Response and Fentanyl Sensitivity

	Capsaicin +	Capsaicin −	Total
Fentanyl +	62	2	64
Fentanyl −	44	13	57
Total	106	15	121

Individual neurons were classified according to capsaicin response and the sensitivity of Ca^{2+} currents to fentanyl. The number of neurons in each group is shown. The distribution of cells is nonrandom (Fisher exact test, $P < 0.005$).

Fentanyl Sensitivity of I_{Ca} Depends on Capsaicin Response in Individual Cells

Sensitivity to both fentanyl and capsaicin was determined in 121 sensory neurons (table 1). Examples of these responses are illustrated in figure 2. Overall, 106 (88%) of 121 neurons tested responded to capsaicin, while 15 (12%) did not. Of the capsaicin-responsive cells, 62 (58%; fig. 2A) expressed Ca^{2+} channels that were inhibited by fentanyl. In the other 44 capsaicin-responsive cells (42%; fig. 2B), fentanyl had no effect on I_{Ca}. Thirteen (87%) of the 15 capsaicin-unresponsive neurons expressed fentanyl-insensitive Ca^{2+} channels (fig. 2C). In capsaicin-responsive, fentanyl-sensitive cells, the inhibition of I_{Ca} was concentration dependent, with an EC_{50} of 19 nM when fit to the logistic equation with a Hill coefficient of 1 (fig. 3). In fentanyl-insensitive neurons, fentanyl concentrations of up to 1 μM produced less than 10% inhibition of I_{Ca} (figs. 2 and 3). In six fentanyl-sensitive cells, naloxone (10 μM) reduced the fentanyl-induced inhibition of I_{Ca} from 44 ± 10% to 2 ± 1% ($P < 0.01$).

A commonly held theory suggests that the cell bodies of primary nociceptive neurons tend to be relatively small, a reflection of the small axons of unmyelinated C fibers or thinly myelinated Aδ fibers that are thought to transmit noxious sensations. In the present study, assuming that cell capacitance reflects cell size, capsaicin-responsive cells were smaller, on average, than capsaicin-unresponsive cells (25 ± 1 pF, n = 106, *vs.* 38 ± 2 pF, n = 15, respectively; $P < 0.05$). Among the capsaicin-responsive cells, the fentanyl-insensitive cells (21 ± 1 pF, n = 44) were smaller than the fentanyl-sensitive cells

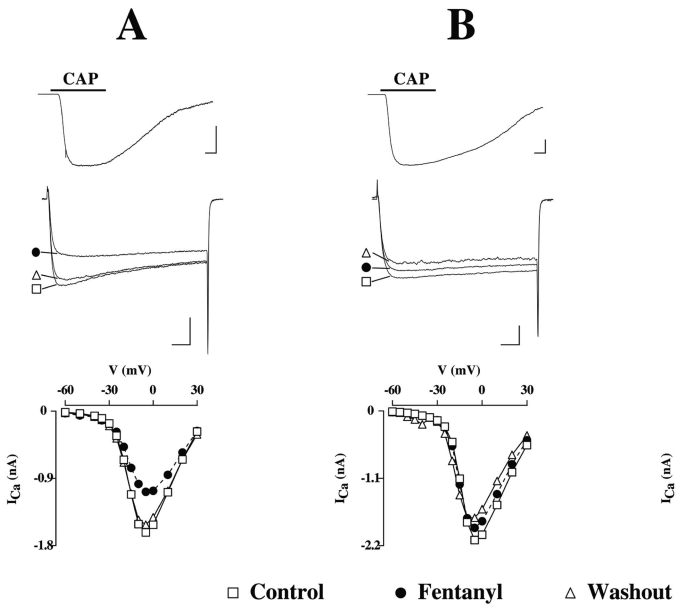

Fig. 2. Responses to capsaicin (CAP) and fentanyl in three different sensory neurons. The cell in *A* was sensitive to both CAP and fentanyl; the cell in *B* was sensitive to CAP but not to fentanyl; the cell in *C* was sensitive to neither CAP nor fentanyl. The top panels show inward currents induced by CAP (10 μM; time of CAP application denoted by bar above each trace). The middle panels show representative high-voltage–activated Ca^{2+} current (I_{Ca}) traces, and the bottom panels show the Ca^{2+} current–voltage (I-V) relations, before (open squares), during (filled circles), and after (open triangles) washout of fentanyl. Fentanyl concentrations were 100 nM in *A* and *B* and 1 μM in *C*. For the CAP-induced current traces, the horizontal scale bars denote 1 s, and the vertical scale bars denote 1 nA. For I_{Ca} traces, the horizontal scale bars denote 10 ms, and the vertical scale bars denote 500 pA.

(28 ± 2 pF, n = 62; $P < 0.05$). As can be seen from the histogram in figure 4, this is partly due to the presence of a number of very large capsaicin-responsive, fentanyl-sensitive neurons. The capsaicin-unresponsive, fentanyl-insensitive cells were the largest on average (39 ± 2 pF, n = 13; $P < 0.05$).

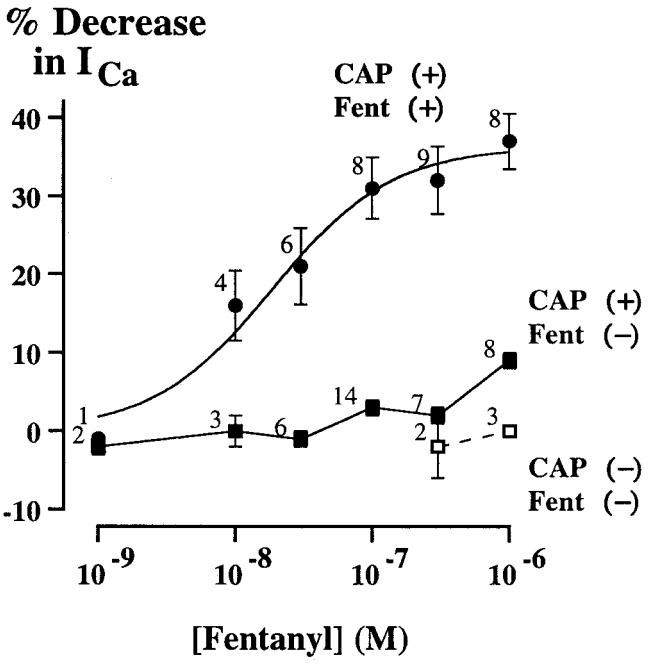

Fig. 3. Dose–response relations for fentanyl-induced inhibition of Ca^{2+} current (I_{Ca}) in the three groups of sensory neurons. CAP (+) = capsaicin responsive; CAP (−) = capsaicin unresponsive; Fent (+) = fentanyl sensitive; Fent (−) = fentanyl insensitive. The number of cells studied at each concentration is shown next to each data point. The smooth line through the filled circles represents the best fit of the data from the CAP (+), Fent (+) cells to the Hill equation.

Differences in Ca^{2+} Currents Recorded from Capsaicin-unresponsive Cells

The average amplitude of capsaicin-induced currents recorded from each group of cells is shown in figure 5A. There was no difference in the current amplitude between the two groups of capsaicin-responsive neurons. The average current in the capsaicin-unresponsive group was an outward current of 1 ± 0 pA/pF.

Capsaicin-unresponsive cells had a significantly greater low-voltage–activated (LVA) Ca^{2+} current density than either of the capsaicin-sensitive groups of cells (figs. 2C and 5B). Not only was the magnitude of LVA current larger, it was expressed in all 5 cells examined, compared to only 12 (55%) of 22 capsaicin-responsive, fentanyl-sensitive cells and 16 (67%) of 24 capsaicin-responsive, fentanyl-insensitive cells. The magnitude of LVA Ca^{2+} current was similar in the two groups of capsaicin-responsive cells. Fentanyl had no effect on LVA Ca^{2+} current.

There were no significant differences in the maximum amplitude of the high-voltage–activated (HVA) Ca^{2+} current density among the three groups (fig. 5C). However, the inactivation rate of the HVA Ca^{2+} current at a test potential of −10 mV was significantly faster in the fentanyl-sensitive group compared to the fentanyl-insensitive group of capsaicin-responsive cells (figs. 2 and 5D). HVA current inactivation was also rapid in the capsaicin-unresponsive neurons (fig. 5D). This may have been due to the presence of residual LVA Ca^{2+} current at this test potential. There was no difference in the rate of HVA Ca^{2+} current activation as assessed by comparing the time from the beginning of the voltage step to the time of peak inward current.

Additional experiments were performed to explore the possibility that the difference in the inactivation rates

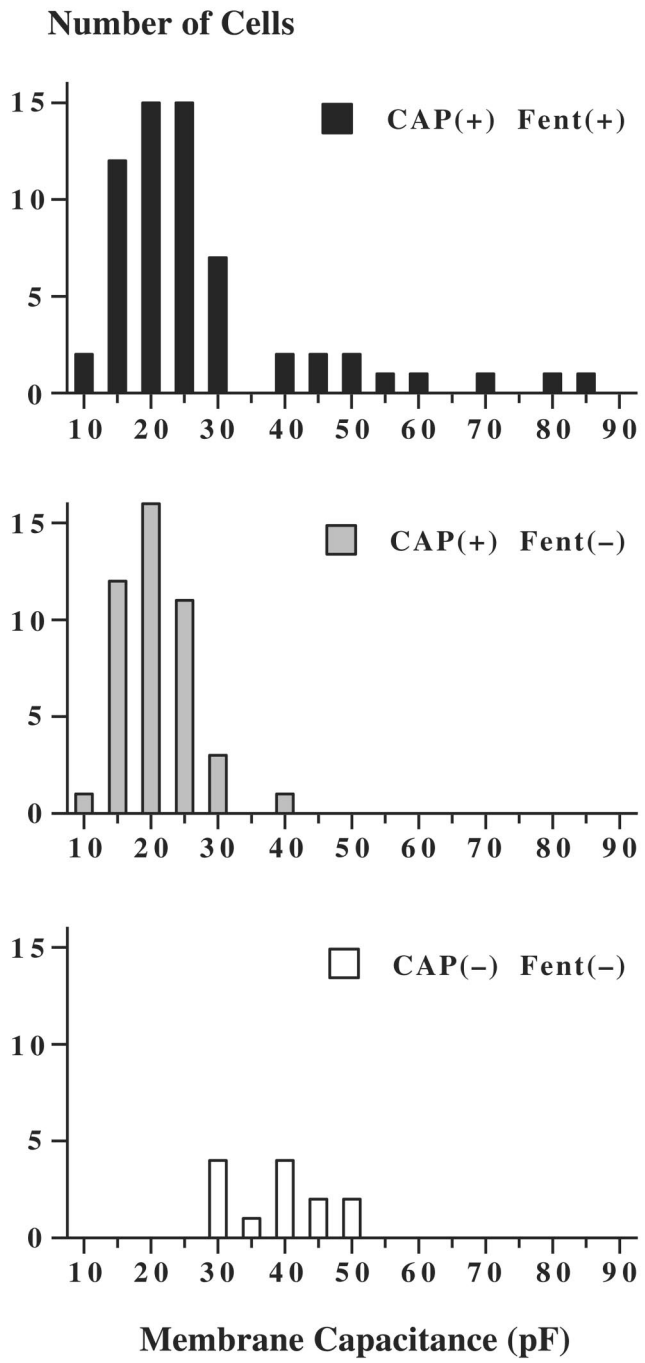

Fig. 4. Histograms show the distribution of membrane capacitance in the three groups of sensory neurons. CAP (+) = capsaicin responsive; CAP (−) = capsaicin unresponsive; Fent (+) = fentanyl sensitive; Fent (−) = fentanyl insensitive. Most of the CAP-sensitive cells were smaller than the CAP-insensitive cells. There was, however, considerable overlap among the groups, with some of the CAP-sensitive, fentanyl-sensitive neurons being much larger than any of the CAP-insensitive neurons.

of I_{Ca} between the two groups of capsaicin-responsive cells was due to a difference in the types of Ca^{2+} channels expressed in these two populations of cells. Figure 6 summarizes the data for all three groups of sensory neurons. ω-Conotoxin GVIA (GVIA; 1 μM) was used to irreversibly block N-type I_{Ca}. Subsequent perfusion with nifedipine (10 μM) blocked L-type current. The current that remained (P-/Q- and/or R-type) was considered GVIA and nifedipine resistant. There were no significant differences in the percentage of L-type, N-type, or GVIA/nifedipine-resistant I_{Ca} between the two groups of capsaicin-responsive cells. On the other hand, the capsaicin-unresponsive neurons expressed significantly less N-type current and more GVIA/nifedipine-resistant current than either of the capsaicin-responsive groups. Capsaicin-unresponsive cells also had significantly less L-type current than the capsaicin-responsive, fentanyl-sensitive cells.

The approximate contributions of P-/Q- and R-type Ca^{2+} channels to the GVIA/nifedipine-resistant current was estimated using another Ca^{2+} channel toxin. ω-Conotoxin MVIIC (MVIIC; 3 μM) blocks P-/Q-type Ca^{2+} channels slowly and irreversibly and blocks N-type channels rapidly and reversibly.[18] Pretreatment of cells with MVIIC, followed by perfusion with MVIIC and nifedipine (10 μM), would thus block all but R-type Ca^{2+} channels. Under these conditions, the average I_{Ca} was -64 ± 13 pA, or a current density of just -3 ± 1 pA/pF (n = 6). This compares to an average I_{Ca} in the absence of any channel blockers of $-3{,}458 \pm 264$ pA, or a current density of -141 ± 9 pA/pF (n = 49) for all groups. Thus, R-type current probably accounts for only about 2% of the whole cell current in these cells. In other words, it is likely that the majority of the GVIA/nifedipine-resistant current is attributable to P-/Q-type Ca^{2+} channels.

Fentanyl Decreases Both N-type and P-/Q-type I_{Ca}

Figure 7 illustrates the effect of fentanyl on I_{Ca} in the presence of various Ca^{2+} channel blockers. In the presence of nifedipine and after MVIIC pretreatment, fentanyl (1 μM) reduced N-type I_{Ca} by 62 ± 9% (n = 3) in sensitive cells (fig. 7A). Subsequent perfusion with MVIIC to block N-type channels eliminated the fentanyl response (fig. 7A), suggesting that R-type channels are not sensitive to fentanyl. In other experiments using pretreatment with ω-Agatoxin TK (500 nM) instead of MVIIC to irreversibly block P- and Q-type Ca^{2+} channels, and in the presence of nifedipine, fentanyl (1 μM) reduced N-type I_{Ca} by a similar amount (49 ± 7%, n = 4). In addition, after application of GVIA and during nifedipine perfusion, fentanyl (1 μM) reduced the P-/Q-type I_{Ca} by 50 ± 8% (n = 4; fig. 7B). Assuming L- and R-type channels are insensitive to fentanyl, a 50% reduction by fentanyl (1 μM) in both N- and P-/Q-type I_{Ca}, which together comprise about 80% of whole cell current in sensitive cells (fig. 6), would produce the 40% reduction in whole cell current seen in earlier experiments (fig. 3).

Discussion

Previous studies have shown that, in sensory neurons, the inhibition of I_{Ca} by μ-opioid receptor agonists is

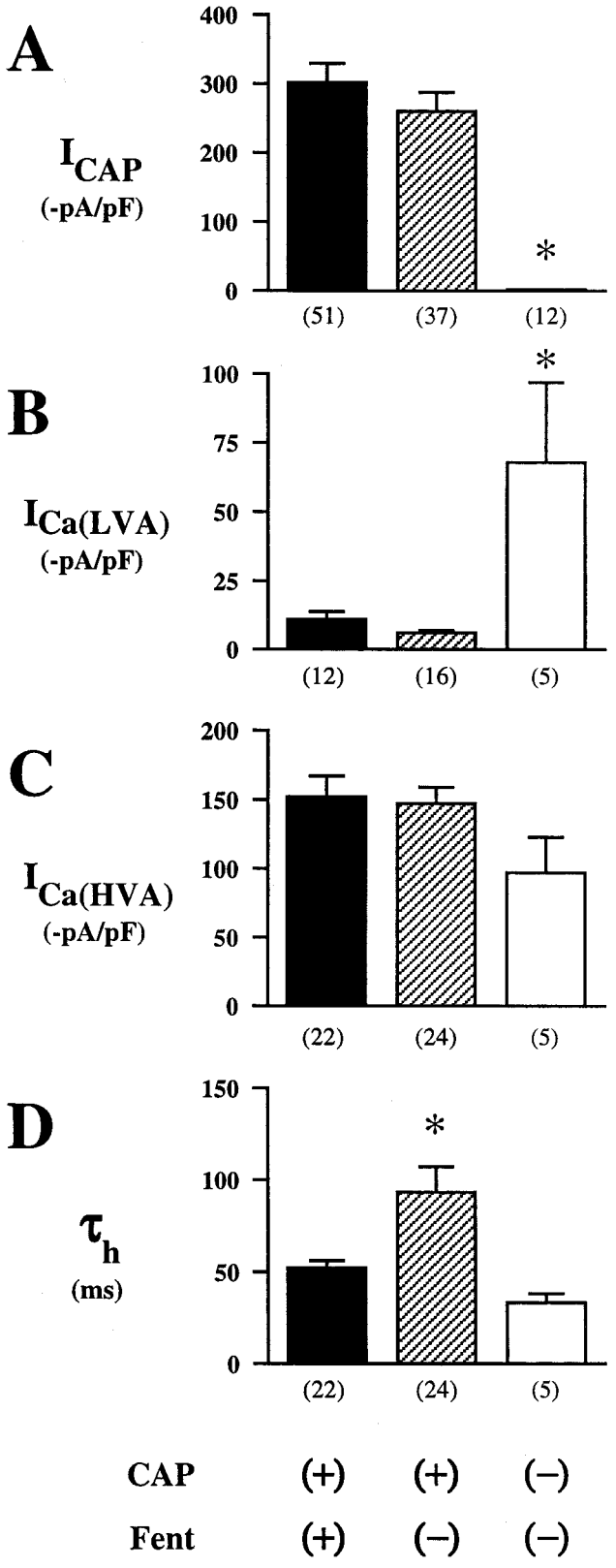

Fig. 5. Differences in the currents expressed among the three groups of sensory neurons. CAP (+) = capsaicin responsive; CAP (−) = capsaicin unresponsive; Fent (+) = fentanyl sensitive; Fent (−) = fentanyl insensitive. The number beneath each bar indicates the number of cells. (*A*) CAP-induced current density (I_{CAP}) recorded during application of 10 μM CAP (*$P < 0.001$ *vs.* both CAP-responsive groups). (*B*) Low-voltage–activated Ca^{2+} current density ($I_{Ca(LVA)}$) recorded at a test potential of about −40 mV (*$P < 0.001$ *vs.* both CAP-responsive groups). (*C*) High-voltage–activated Ca^{2+} current density ($I_{Ca(HVA)}$) recorded at a test potential of about −10 mV. There were no significant differences in the magnitude of HVA I_{Ca} among the three groups. (*D*) The time constant of inactivation of HVA Ca^{2+} current (τ_h) recorded during control conditions (before fentanyl application) at a test potential of about −10 mV (*$P < 0.05$ *vs.* other two groups).

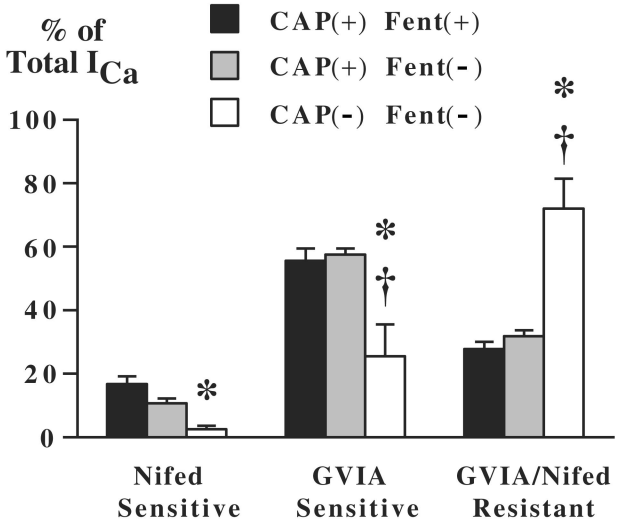

Fig. 6. The percentages of whole cell Ca^{2+} current (I_{Ca}) sensitive to the L-type Ca^{2+} channel blocker nifedipine (Nifed, 10 μM), the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (GVIA, 1 μM), and the percentage that was resistant to a combination of both. CAP (+) = capsaicin responsive; CAP (−) = capsaicin unresponsive; Fent (+) = fentanyl sensitive; Fent (−) = fentanyl insensitive. The CAP-unresponsive, fentanyl-insensitive cells (n = 3) had significantly less L- and N-type I_{Ca} and a greater percentage of I_{Ca} that was resistant to both blockers (*$P < 0.01$ *vs.* CAP-sensitive, fentanyl-sensitive cells, n = 4; †$P < 0.001$ *vs.* CAP-sensitive, fentanyl-insensitive cells, n = 6).

quite variable. In random populations of acutely isolated sensory neurons, between 44 and 90% of cells studied are opioid sensitive (usually defined as ≥ 10% inhibition in I_{Ca}).[6–11] Some of this variability in opioid sensitivity among cells might be explained by differences in the sensory modality transmitted by individual neurons. One might expect nociceptive neurons to be sensitive to the effects of opioids, whereas other neurons that transmit innocuous mechanical and thermal sensations should be relatively insensitive to opioids.[12,13] The challenge, then, is to identify which neurons in an isolated, *in vitro* preparation are nociceptors.

A simple approach used by many investigators interested in nociceptor function is to focus their studies on small sensory neurons. This is because most nociceptor axons are unmyelinated C fibers or thinly myelinated Aδ fibers, and the cell somata associated with these axons are generally smaller.[19,20] However, many small C- and Aδ-fiber-type sensory neurons do not transmit nociceptive information, and some nociceptors even conduct in

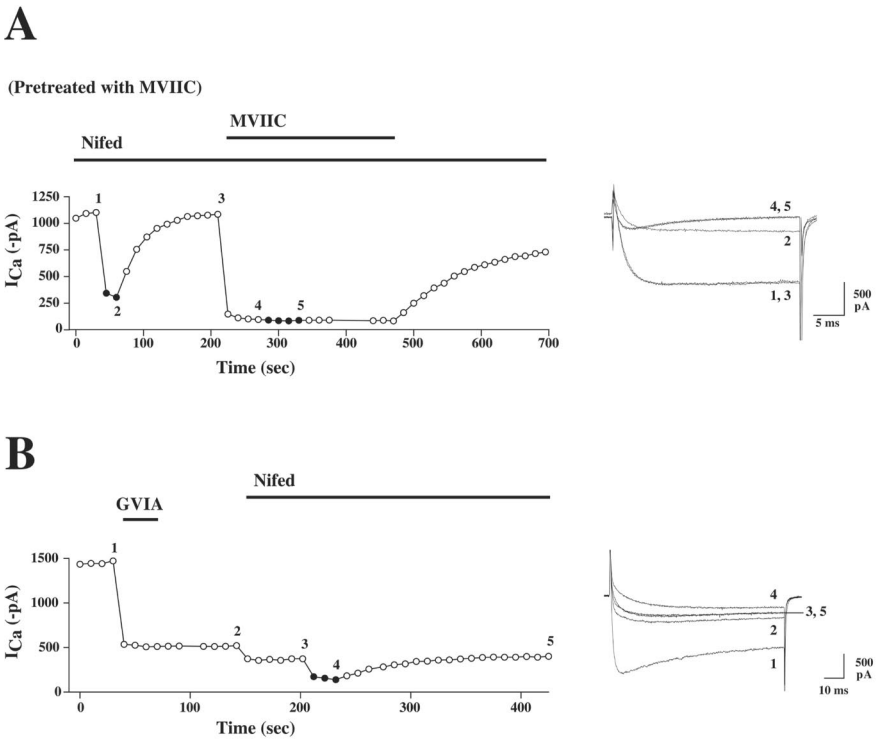

Fig. 7. Fentanyl sensitivity of Ca^{2+} current (I_{Ca}) in two cells treated with Ca^{2+} channel blockers. I_{Ca} was elicited by test pulses to −10 mV every 15 s in *A* and every 10 s in *B*. The numbered points in the graphs on the left correspond to the numbered traces on the right. In *A*, the cell had been pretreated with ω-conotoxin MVIIC (MVIIC, 3 μM). The cell was also continuously perfused with nifedipine (Nifed, 10 μM). Fentanyl (1 μM, filled circles; trace 2) markedly reduced the residual (N- and R-type) current. Subsequent application of MVIIC reversibly inhibited N-type channels, leaving only a small residual R-type current (trace 4). Fentanyl (1 μM, filled circles; trace 5) had no effect on the R-type current. In *B*, the cell was not pretreated with any Ca^{2+} channel blockers (trace 1). Application of ω-conotoxin GVIA (GVIA, 1 μM) irreversibly blocked the N-type current, and Nifed (10 μM) blocked L-type current. Fentanyl (1 μM, filled circles; trace 4) markedly reduced the residual (P-/Q- and R-type) current.

the Aαβ-fiber range.[21,22] Size restriction alone, therefore, does not guarantee a population of nociceptive neurons.

Other characteristics have been used to identify nociceptive neurons in isolated cultures of dorsal root ganglion neurons. These include differences in the shape of the somatic action potential, the presence of neuropeptides such as substance P and calcitonin gene–related peptide, and the presence of tetrodotoxin-resistant sodium channels, as well as other types of ion channels.[23–25] Although these are generally thought of as "nociceptor properties," they do not always cosegregate in individual neurons, and none of these properties have been shown to be specific or sensitive tests for nociceptors.

In the present study, nociceptive neurons were identified by their sensitivity to capsaicin. Capsaicin is a vanilloid compound isolated from hot peppers that produces burning pain when injected intradermally or arterially or applied to mucous membranes. It is an agonist for the vanilloid receptor VR1, a membrane protein involved in the transduction of noxious heat.[14–16] VR1 is expressed primarily by small- to medium-sized neurons of sensory ganglia.[14,15] Single-unit recordings from peripheral nerves innervating the skin demonstrate that capsaicin selectively activates heat-sensitive nociceptors. The majority of heat-sensitive nociceptive neurons are C-fiber polymodal nociceptors, which respond to noxious heat, noxious mechanical stimuli, and noxious chemical stimuli. Some C- and Aδ-fiber-type neurons respond to noxious heat and noxious mechanical stimuli (mechano-heat nociceptors), while purely mechanical nociceptors, which do not respond to noxious heat, are insensitive to capsaicin. Nerve fibers transmitting nonnoxious stimuli, such as light touch and hair sensors, are rarely found to be capsaicin responsive.[26–29] Capsaicin responsiveness, therefore, is a sensitive and specific test for identifying heat-sensitive nociceptors.

Approximately 80% of all the cells in the present study responded to capsaicin. This is in close agreement with other studies that have reported the capsaicin sensitivity of small sensory neurons from young rats,[30,31] though in older rats, the percentage of capsaicin-sensitive neurons is less, ranging from about 50 to 65%.[24,25,32,33] It also correlates with the finding that approximately 75% of C-fiber single units recorded from a cutaneous nerve in the rat are polymodal nociceptors.[22]

The remaining 20% of the cells in the present study did not respond to capsaicin. Because of their size (calculated diameters of approximately 20–40 μm), these cells may have been mostly C-fiber-type low-threshold mechanoreceptors[19,20] as these are among the most common nonnociceptive unmyelinated fibers found in rat nerves.[22] On the other hand, the two capsaicin-unresponsive cells that were sensitive to fentanyl might have been high-threshold mechanical nociceptors. These neurons may have been underrepresented in this study due to the fact that they often conduct in the Aδ range[21] and therefore may have been larger, on average, than most of the cells studied here.

The capsaicin-unresponsive cells expressed a unique distribution of voltage-activated Ca^{2+} channels. Most of the HVA Ca^{2+} current in these cells was P-/Q-type current, while in both capsaicin-responsive groups, N-type

current was predominant. In addition, all of the capsaicin-insensitive cells expressed a large T-type Ca^{2+} current, an association that has been demonstrated previously.[32–34] While a T-type current was observed in many of the capsaicin-sensitive neurons as well, it was never as large as in the capsaicin-insensitive neurons. Scroggs and Fox[35] also described a population of "medium-sized" (33–37 μm in diameter) sensory neurons with a large T-type Ca^{2+} current component, though capsaicin sensitivity was not tested in that study.

Only about 60% of the capsaicin-responsive neurons were sensitive to fentanyl. The inhibition of I_{Ca} in these cells was concentration dependent and reproducible with little variability from cell to cell. The I_{Ca} in these cells inactivated more rapidly than the I_{Ca} in the fentanyl-insensitive nociceptors. Others have reported that opioids primarily inhibit a transient I_{Ca} component, and this is generally presumed to be due to current flowing through N- and/or P-/Q-type channels.[6,10,36] While the present results confirm these observations, they also show that not all N- and P-/Q-type channels are inhibited by opioids. I_{Ca} was not inhibited by fentanyl when recorded from capsaicin-responsive neurons with slowly inactivating I_{Ca} or when recorded from capsaicin-unresponsive neurons. It is not obvious how the opioid sensitivity of Ca^{2+} channels is related to their inactivation rate. Differences in G-protein expression or the phosphorylation state of the Ca^{2+} channels might be involved as both can markedly alter I_{Ca} kinetics in addition to their ability to affect I_{Ca} magnitude. On the other hand, a recent study in young rats demonstrated that less than half of small sensory neurons in the L4–L5 dorsal root ganglion express μ-opioid receptors.[37] This would explain the fentanyl insensitivity but not the difference in I_{Ca} inactivation rates.

Few other studies have attempted to characterize the opioid sensitivity of putative nociceptive sensory neurons. Schroeder and McCleskey[7] tested the effect of DAMGO, a μ-opioid receptor agonist, on a group of putative nociceptive neurons identified by using antibodies against a specific oligosaccharide present only on cells that project to laminae I and II in the dorsal horn of the spinal cord, the site of most nociceptive input. They found that while the DAMGO response was much less variable in the labeled neurons, the average magnitude of the inhibition of I_{Ca} in the labeled neurons (25%) was actually less than that observed in random samples of neurons (38%). This result may be partly explained by the fact that while primary nociceptive neurons do terminate extensively in laminae I and II, they also project to other areas of the dorsal horn.[21,38] It is also possible that nociceptors projecting to deeper laminae are more likely to be, for example, Aδ mechanical nociceptors, which may be more sensitive to opioids than other types of nociceptors. Another limitation of this labeling technique is that some nonnociceptive neurons also project to laminae I and II, which could explain their finding that not all of the labeled neurons were sensitive to DAMGO.

Using a different approach, Taddese *et al.*[39] studied the opioid responsiveness of neurons from the trigeminal ganglion that were labeled when they took up a fluorescent indicator placed in the tooth pulp. They reasoned that neurons innervating tooth pulp are a pure population of nociceptors based on the assumption that the only sensory modality transmitted from the tooth is pain. They found that DAMGO decreased I_{Ca} by an average of 21% in small labeled neurons compared to 13% in small randomly selected neurons from the trigeminal ganglion. However, their results also show that only 79% of small nociceptors ($\leq$ 30 μm in diameter) were inhibited by DAMGO, and the response rate for nociceptors of all sizes was only 49%. In the present study, 58% of all nociceptors were opioid sensitive using a more diverse population of sensory neurons and, arguably, a simpler method of identifying nociceptors.

Taddese *et al.*[39] found that smaller nociceptors were much more likely to be opioid sensitive than larger nociceptors. They postulated that this may be related to the finding that opioids better alleviate more chronic, aching types of pain that are transmitted by more slowly conducting C-fiber axons, compared to the sharp shooting pain that is likely transmitted by rapidly conducting A-fiber-type axons from larger nociceptive neurons. The present results do not support this theory, however, since the fentanyl-sensitive cells were larger, on average, than the fentanyl-insensitive cells. There were also a few extremely large cells that were opioid sensitive. Nevertheless, it would be beneficial to learn more about the opioid-insensitive nociceptors that have been identified in this and other studies. Such knowledge may make it possible to develop new analgesics that specifically target these nociceptors, which could then be used in combination with neuraxial opioids to provide improved control of acute and chronic pain.

The author thanks Craig Levenick, B.S. (Research Specialist, Department of Anesthesiology, University of Wisconsin, Madison, Wisconsin), for expert technical assistance and Hirochika Komai, Ph.D. (Associate Scientist, Emeritus, Department of Anesthesiology, University of Wisconsin), for numerous discussions and suggestions.

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