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Fast, Local Signal Transduction between the μ Opioid Receptor and Ca^{2+} Channels

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We used patch-clamp methods to describe signal transduction between the μ opioid receptor, the binding site for morphine, and high-threshold Ca^{2+} channels in dorsal root ganglion (DRG) sensory neurons from adult rats. Opioid signaling persists in excised membrane patches, and an activated opioid receptor can only inhibit nearby Ca^{2+} channels; thus, no readily diffusible second-messenger molecule mediates between the μ receptor and Ca^{2+} channels. Inhibition of Ca^{2+} channels begins several hundred msec after application of opioid and it is maximal by 5 sec; this is faster than typical phosphorylation cascades. Blockade of the known serine-threonine kinases and phosphatases does not affect this opioid signaling and, as shown previously by Seward et al. (1991) and Moises et al. (1994a), pertussis toxin eliminates virtually all of the effect. Inhibited channels can open, but their half-activation voltage is unphysiologically positive. The link between the μ receptor and Ca^{2+} channels is clearly unlike the protein kinase C-dependent paths that couple μ receptors to NMDA channels in dorsal horn neurons (Chen and Huang, 1991) and α-adrenergic receptors to Ca^{2+} channels in DRG neurons (Diversé-Pierluissi and Dunlap, 1993). The rapid kinetics and tight localization of the signaling path are properties expected if receptor and channel are linked directly by a G-protein, but these properties do not constitute proof of such a pathway.

[Key words: opioids, opiates, μ (mu) opioid receptor, morphine, Ca^{2+} channels, signal transduction, patch clamp, G-proteins]

Opioid peptides (opioids) diminish pain by decreasing Ca^{2+}-dependent release of neurotransmitters from presynaptic terminals, thereby inhibiting communication between neurons involved in sensory signaling (Jessell and Iverson, 1977; Macdonald and Nelson, 1978; Mudge et al., 1979; Grudt and Williams, 1994). Opioids act through G-protein–coupled receptors to inhibit adenylate cyclase (Sharma et al., 1977), activate K^+ channels (North et al., 1987), or inhibit Ca^{2+} channels (Gross and Macdonald, 1987; Schroeder et al., 1991; Seward et al., 1991); the effects on K^+ and Ca^{2+} channels are responsible for inhibition of evoked neurotransmitter release. The following is known about signal transduction between opioid receptors and Ca^{2+} channels in various cells: the κ and μ opioid receptors inhibit Ca^{2+} channels through a path that does not involve protein kinase A (Gross et al., 1990; Moises et al., 1994a); μ and δ opioid receptors activate a variety of G_i and G_o proteins (Laugwitz et al., 1993), but the link to Ca^{2+} channels is primarily through G_o (Hescheler et al., 1987; Taussig et al., 1992; Moises et al., 1994a).

Here we describe key functional properties of signal transduction between μ receptors and Ca^{2+} channels in DRG neurons to distinguish between two contrasting types of signaling schemes. The schemes are best illustrated by adrenergic and cholinergic modulation of ion channels in the heart. The cyclic AMP (c-AMP)-dependent signaling between the β-adrenergic receptor and cardiac Ca^{2+} channels exemplifies a path in which an activated receptor, by means of a diffusible second messenger, can affect ion channels that are remote from it in the membrane (Hartzell, 1988). In contrast, the cardiac muscarinic acetylcholine receptor activates only K^+ channels that are near it in the membrane and a G-protein may be the only mediator between receptor and channel (Brown, 1993).

In neurons, both local and remote signaling paths have been demonstrated (Kaczmarek and Levitan, 1987; Hille, 1992). Since activation of the μ opioid receptor modulates NMDA channels through protein kinase C in dorsal horn neurons (Chen and Huang, 1991), there is precedence for a phosphorylation-dependent mechanism in μ receptor signal transduction. Moreover, Ca^{2+} channels in DRG neurons can be modulated through protein kinase C and through a different route (Diversé-Pierluissi and Dunlap, 1993). To determine which kind of signaling scheme mediates μ-opioid inhibition of neuronal Ca^{2+} channels, we used patch-clamp techniques and the selective μ receptor agonist DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol) to address four questions: (1) does DAMGO affect channels that are remote from its receptor or just those that are nearby; (2) how fast does DAMGO act; (3) is phosphorylation or dephosphorylation required for the signaling; (4) does inhibition shift the membrane voltage at which Ca^{2+} channels open?

Materials and Methods

Cell culture and preparation. Adult DRG neurons were dissociated and maintained in culture as described previously (Schroeder et al., 1990). All cells were plated on laminin-treated culture dishes and were studied within the first 3 d in culture.

Recording and analysis. Currents were recorded at room temperature and filtered at 2 kHz or 10 kHz (for tail currents) before digital sam-

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pling. Either PCLAMP or AXOBASIC programs (both from Axon Instruments, Foster City, CA) were used for data acquisition and analysis. Least squares curve fitting was done with the NFIT program (University of Texas Medical Branch, Galveston, TX).

Whole-cell patch clamp was performed with the standard broken-patch method (Hamill et al., 1981) or with the perforated patch method (Horn and Marty, 1988; Rae et al., 1991). Pipettes were pulled from borosilicate glass (7052, Garner Glass, Claremont, CA) and fire polished to final resistances of 2–5 MΩ for broken-patch and about 1 MΩ for perforated patch recordings or tail-current recordings. As a control for series resistance artifacts, no recording was accepted unless the Ca^{2+} current tail decayed with a time constant of 2 msec or less. Unless otherwise indicated, the pipette solution for broken-patch recordings was 100 mM CsCl, 1 mM Na_3ATP (equine, Sigma, St. Louis, MO), 0.3 mM GTP (Aldrich, Milwaukee, WI), 10 mM EGTA, 2.5 mM $MgCl_2$, 2 mM $CaCl_2$, 8.8 mM Na-phosphocreatine, 0.08 mM leupeptin, 40 mM HEPES, pH 7.0 (titrated with tetraethylammonium hydroxide). The pipette solution for perforated patch recordings contained amphotericin (final concentration, 0.3 mg/ml) in 55 mM CsCl, 70 mM $CsSO_4$, 7 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, pH 7.3 (titrated with CsOH). Either Na^+-free or Na^+-containing extracellular solution were used on whole-cell recordings. There was less rundown of Ca^{2+} channels in Na^+-containing solution. Unless otherwise indicated, Na^+-free solution contained 140 mM tetraethylammonium chloride, 3 mM $BaCl_2$, 10 mM HEPES, pH 7.3. Na^+-containing solution contained 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.3 (titrated with NaOH). For recordings from either on-cell or outside-out patches, the solution bathing the extracellular face of the membrane was 110 mM $BaCl_2$, 10 mM HEPES, pH 7.3 adjusted with either CsOH or TEA-OH.

In Na^+-containing extracellular solution, positive voltage steps initially evoked current through both Na^+ channels (most of which were insensitive to tetrodotoxin and saxitoxin) and Ca^{2+} channels. Ca^{2+} current amplitude was measured after Na^+ channels had inactivated by averaging 5 msec of current beginning 50 msec after the voltage step. Upon repolarization to −70 mV, tail currents decayed smoothly with a time constant of less than 2 msec for all cells used for analysis, indicating that membranes were under good voltage control.

Details for perforated patch recordings largely followed those in Rae et al. (1991). Pipettes were front filled for 300–500 microns by dipping for a few seconds into the pipette salt solution and then were back filled with an amphotericin solution prepared within the previous hour. The amphotericin solution was made as follows: 10 μl of amphotericin stock was added to 20 μl of Pluronic F-127 stock and the mix was vortexed well before adding to 2 ml of filtered salt solution (above) at 37°C. The final mix was vortexed, stored in the dark at room temperature for no more than 1 hr, and was not filtered. Amphotericin stock, made fresh each day, consisted of 6 mg amphotericin B (Sigma) added to 100 μl dimethyl sulfoxide (DMSO); the mix was triturated thoroughly, vortexed, and stored in the dark at room temperature. The Pluronic stock was 2.5 mg Pluronic F-127 (Molecular Probes, Eugene, OR) added to 100 μl DMSO and kept at 37°C. Perforation usually occurred in 5–10 min after seal formation, and series resistance was as low as 3 MΩ. Salt solution containing only Pluronic F-127 alone does not cause membrane perforation (Rae et al., 1991). Sealing of the large pipettes was aided by perfusing elevated divalent ion concentrations (5–10 mM) onto the cell. To assure that no amphotericin flowed from the pipette before sealing, no pressure was applied to the pipette until it was about to touch the cell.

To obtain simultaneous on-cell and whole-cell recordings, we first got a high quality whole-cell recording and then brought the on-cell pipette (about 4 MΩ) into the chamber. Once sealed, the on-cell pipette was grounded since the patch was stimulated through the whole-cell recording pipette.

Solution delivery. Various experiments called for either slow or fast extracellular solution changes or for timed perfusion into the cell. Slow extracellular solution changes were made in about 1 sec by moving a set of six 1 μl pipettes (Drummond "microcaps," VWR Scientific) glued together side by side, supported on a plastic coverslip, and mounted on a rod that was attached to a manipulator. The back ends of the pipettes were connected with polyethylene tubing to stopcocks and different solution reservoirs. The delivery end was positioned within 150 microns of the cell. Solution flowed over the cell at all times.

For fast solution changes, outside-out patches were held in the flow from one of two halves of a theta tube (tip pulled to 650 μm diameter. Sutter Instrument Co., San Rafael, CA) that contained either control or drug solution. Solution changes were made by a rapid step of the theta tube driven by a piezoelectric device (Polytec Optronics, Costa Mesa, CA) that jumped the tube 100 μm in response to a 1000 V step from a power supply triggered by a pulse from the computer. In order to produce the laminar flow required for rapid solution changes, it was necessary to suspend the cell away from the bottom of the chamber. Outside-out patches were used because whole cells were often torn from the recording pipette by the fast solution flow. The two halves of the theta tube were connected to solution reservoirs by polyethylene tubing. Solution changes occurred within 30 msec following a consistent delay that was determined by the distance between the tip of the patch pipette and the plane of the septum of the theta tubing; this distance was 35 μm in all experiments.

During whole-cell patch recordings, compounds were applied to the interior of cells through a second pipette connected to a voltage-measuring amplifier. The pipette was sealed onto the cell before the experiment began and, through a puff of suction, it broke into the cell to deliver the compound at the desired time. The pipette solution contained the test compound dissolved in the broken-patch buffer.

Peptides and drugs. The sources of peptides and drugs were as follows: DAMGO and ω-conotoxin GVIA, Peninsula Laboratories (Belmont, CA); nifedipine, H7, H8, H9, staurosporine, okadaic acid, and 1-nor okadaone, Research Biochemicals, Inc. (Natick, MA); ATPγS, GDPβS, IBMX, dibutyrl cAMP, PKA, and PKAI, Sigma Chemical Co. (St. Louis, MO); pertussis toxin, List Laboratories (Campbell, CA.); oleoyl acetyl glycerol, Avanti Polar Lipids (Alabaster, AL); PKCI, Bachem (Torrance, CA); microcystin LR, GIBCO (Grand Island, NY); calyculin A, Calbiochem (La Jolla, CA).

Peptides and drugs were prepared as follows. DAMGO was dissolved in water at 5 mM and 1 nmol aliquots were vacuum dried and stored at −20°C, to be dissolved in extracellular solution on the day of the experiment. Conotoxin was prepared as a 20 μM stock in 1% acetic acid in water, aliquoted, vacuum dried, and stored at −20°C. Nifedipine was prepared as a 5 mM stock in ethanol, stored at −20°C, and diluted into extracellular solution. ATPγS and GDPβS replaced their respective analogs in the pipette solution at 5 mM and 0.3 mM. PTX stock, 100 μg/ml stored at 4°C in sterile filtered water, was diluted to 500 ng/ml into the culture medium to treat cells. H7, H8, and H9 were combined to make a 1 mM stock of all three in external solution and diluted for the experiment. Staurosporine was diluted from a stock of 100 μM in DMSO into external solution. Okadaic acid was prepared as a stock of 500 μM in external solution. IBMX and dibutyryl cAMP were added directly to external solution. PKCI was dissolved in 0.5 M acetic acid to make a stock solution of 1 mM, stored at −80°C in 10 μl aliquots and diluted into internal solution. Internal solutions used for control cells contained 1.5 mM acetic acid to control for any effect it may have had. OAG was obtained as a chloroform solution. The ampule was opened and the chloroform was evaporated under nitrogen. The OAG was redissolved in DMSO as a 60 mM stock solution, stored at −80°C in 10 μl aliquots, and diluted into external solution. PKA and PKAI were obtained at 250 U each, dissolved in water, and divided into 20 μl aliquots to yield 0.164 U/ml and 1.34 U/ml, respectively, and were stored at −80°C. Unless otherwise indicated, all other stock solutions were stored at −20°C.

Results

No slow phase of μ-opioid inhibition of Ca^{2+} channels

Beech et al. (1992) showed that inhibition of Ca^{2+} channels by muscarinic agonists in sympathetic neurons occurs in several phases that correspond to distinct signaling paths. The slowest phase takes about 30 sec to develop, disappears when the cytoplasm is perfused with high concentrations of Ca^{2+}-chelating agents, and uses an unknown, diffusible second messenger (Beech et al., 1991). We looked for the presence of slow, Ca^{2+}-dependent transduction by making whole-cell recordings with the perforated patch method, which does not modify the natural Ca^{2+} buffers of the cell (Horn and Marty, 1988); Figure 1 shows a representative cell. Trace 1 is recorded before and trace 2 is recorded after application of 1 μM DAMGO. The graph plots the amplitude of the Ca^{2+} current against time for many such traces. When DAMGO is applied between two voltage steps that

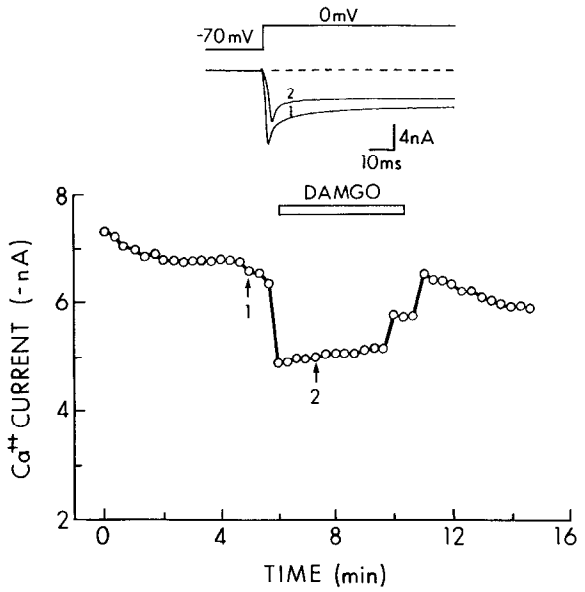

Figure 1. No slow component of Ca^{2+} channel inhibition. Graph of sustained Ca^{2+} current amplitude versus time with 1 μM DAMGO applied when indicated. Traces show currents corresponding to the indicated data. Stimuli initially evoke current through both Ca^{2+} and Na^+ channels and therefore both the initial peak and the sustained components are inhibited. Ca^{2+} current amplitude is measured 50 msec after the depolarizing voltage step, well after both slow and fast Na^+ channels are inactivated (Elliot and Elliot, 1993). Perforated patch method. Na^+-containing extracellular solution. Stimulating pulses: 100 msec duration to 0 mV from −70 mV.

are 20 sec apart, its effect is complete by the second step. Similar results were observed in 15 other perforated patch recordings and more than 200 standard whole-cell recordings. Thus, inhibition by DAMGO is prompt, showing no indication of a slow phase in perforated patch recordings.

A saturating dose (10 μM) of ω-conotoxin, a selective blocker of N-type Ca^{2+} channels (Aosaki and Kasai, 1989), blocks about 50% of high-threshold Ca^{2+} current (52 ± 8%, n = 4) and eliminates much, but not all, of the opioid-sensitive current (Schroeder et al., 1991). In agreement with Moises et al. (1994b), we find that nifedipine, a blocker of L channels, does not affect the opioid-sensitive current (1 μM nifedipine blocks 17 ± 3% of current without affecting the amplitude of inhibition by DAMGO, n = 5), suggesting that L channels are not inhibited by opioids. Similarly, L channels are not affected in the faster modulatory path in sympathetic neurons, whereas they are inhibited in the slow, second-messenger–mediated path (Mathie et al., 1992). Therefore, judging by time course and channel type, μ-opioids act on Ca^{2+} channels in sensory neurons through a mechanism unlike the slow path in sympathetic neurons.

μ-Opioids inhibit only nearby Ca^{2+} channels

As expected from previous results in SH-SY5Y cell lines (Seward et al., 1991), overnight incubation of DRG cultures in 500 ng/ml pertussis toxin virtually eliminated DAMGO inhibition of Ca^{2+} currents: inhibition of Ca^{2+} currents (measured at +10 mV) diminished from 23 ± 2% (n = 40) to 4 ± 1% (n = 30). To ask whether the pertussis toxin-sensitive G-protein might trigger a remotely acting second-messenger cascade, we used two pipettes to simultaneously record activity of all Ca^{2+} channels in the cell and those Ca^{2+} channels within a small patch of membrane that was protected from DAMGO applied to the rest of the cell; modulation of the protected channels by DAMGO would indicate that a readily diffusible second messenger mediates the effect (Brum et al., 1984). We simultaneously used whole-cell and on-cell recordings for two reasons. First, not all DRG neurons are opioid sensitive (Schroeder and McCleskey, 1993), so the whole-cell recording assured that only opioid-sensitive cells were studied. Second, controlling membrane potential with the whole-cell pipette allowed us to bathe the cell and apply the opioid in physiological saline rather than the unphysiological solution (isotonic K^{2+} with EGTA) that is required to obtain stable on-cell Ca^{2+} channel records using a single pipette (Hess et al., 1986). In some experiments, we used the perforated patch method to avoid the loss of small, organic second-messenger molecules from the cytoplasm that can occur with the standard, broken-patch whole-cell method.

In the cell in Figure 2, application of DAMGO inhibits whole-cell Ca^{2+} current by 18% (upper data) without affecting channels within the patch that is protected from DAMGO by the tight seal of the on-cell pipette (lower data). DAMGO failed to inhibit Ca^{2+} channels within on-cell pipettes on all nine DAMGO-sensitive cells that we studied (three using perforated patch and six using standard whole-cell methods. Whole cell inhibitions ranged from 13% to 87%).

Although we confirmed that each of the nine cells was opioid sensitive, it is theoretically possible that a particular on-cell patch can be enriched in a type of Ca^{2+} channel that is opioid insensitive. We addressed the likelihood of this by applying DAMGO to excised, outside-out patches. Ca^{2+} channels were inhibited by DAMGO in 18 of 29 outside-out patches (see Fig. 3*B* for example), clearly indicating that the machinery for opioid signaling accompanies the patch upon excision (also reported by Shen and Surprenant, 1991). Since patches were excised from cells whose opioid sensitivity was not confirmed, the failure rate (11 out of 29) provides a maximum estimate of the probability that a patch from an opioid-sensitive cell contains only opioid-insensitive Ca^{2+} channels. Therefore, the probability that all nine on-cell patches failed to respond to opioids because they contained opioid-insensitive Ca^{2+} channels is $(11 \text{ out of } 29)^9 = 0.0002$. We conclude that the on-cell patches failed to respond to opioids applied to the rest of the cell because signal transduction is so localized that it cannot reach channels on membrane protected from opioids by the pipette.

μ Opioids inhibit Ca^{2+} channels rapidly

In order to obtain an accurate description of the speed of opioid signaling to Ca^{2+} channels, we excised outside-out patches (Hamill et al., 1981) because they were mechanically stable in the fast flow rates necessary for rapid solution changes. With 110 mM Ba^{2+} in the external solution, current amplitudes through excised patches were macroscopic, but they ran down relatively rapidly compared to whole-cell recordings.

The time course of Ca^{2+} channel inhibition by a saturating dose of DAMGO is described by experiments like that shown in Figure 3*B* (procedure and protocol as in 3*A*). The time indicated above each of the three sets of traces is the interval between the application of 1 μM DAMGO and the second trace, which is marked with an asterisk. The three traces within each set are Ba^{2+} currents that were evoked in succession, 4 sec apart, at the times indicated in the graph, which plots the peak current

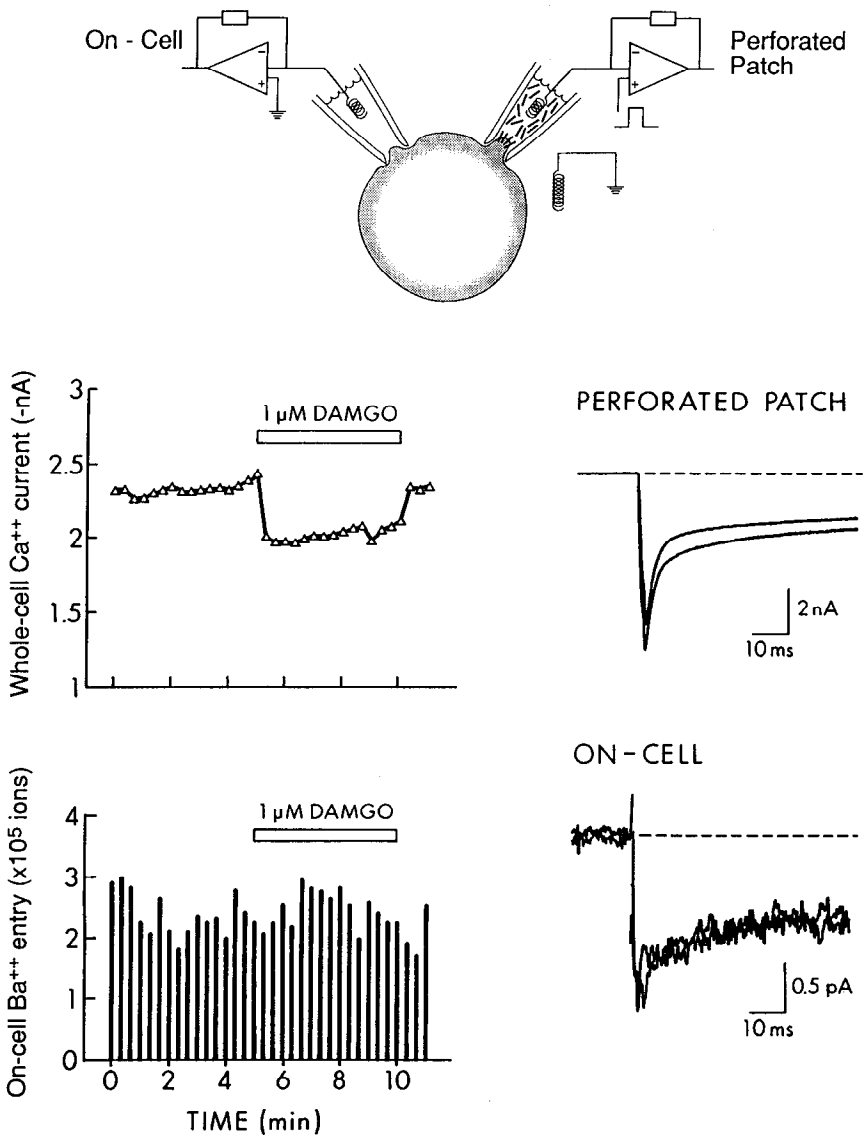

Figure 2. No readily diffusible second messenger mediates Ca^{2+} channel inhibition by μ receptors. Simultaneous perforated patch (*upper data*) and on-cell (*lower data*) recordings from a cell exposed to 1 μM DAMGO at the indicated time. DAMGO inhibits the whole-cell Ca^{2+} current by 18% without affecting channels within the on-cell pipette. *Upper graph:* time course of the whole-cell Ca^{2+} current amplitude; traces show records just before and just after application of DAMGO. *Lower graph,* Time course showing Ba^{2+} entry through the on-cell patch, calculated by integrating each Ba^{2+} current; traces are averages of the five records just before and the five records just after DAMGO application. Stimulating pulses: 60 msec duration to 0 mV from −70 mV applied to the perforated patch electrode. Current carriers are Na^+ and Ca^{2+} (5 mM) in the whole-cell records and Ba^{2+} (110 mM) in the on-cell records. The on-cell and bath electrodes were both grounded.

amplitude versus time throughout the recording. In this cell, Ca^{2+} channel inhibition had not started 250 msec after DAMGO application, inhibition was about 50% complete in 1 sec, and it neared completion in 2 sec.

We varied the interval between opioid application and test pulse in the seven cells studied. Figure 3*C* plots the average normalized inhibition for each of the times for which there were at least three observations. Ca^{2+} current was always unchanged 150 msec after application of the opioid (n = 4), providing a lower limit for the latency of opioid action. Inhibition was greater than 90% complete by 5 sec (n = 7). The data are well fit with an exponential time course starting after a delay of 150 msec and having a time constant of 1.27 sec. Inhibition of Ca^{2+} channels by DAMGO reverses completely but more slowly, taking about 20–30 sec.

Kinase and phosphatase inhibitors have no effect

The preceding data indicate that DAMGO inhibits Ca^{2+} channels through a fast, local signal transduction pathway that does not utilize a readily diffusible second messenger. This does not strictly rule out a role for phosphorylation or dephosphorylation since Ca^{2+} channel phosphorylation can occur very rapidly (Artalejo et al., 1992) and kinases and phosphatases can be tightly localized through specific membrane anchoring proteins (Scott and McCartney, 1994), thereby modulating nearby ion channels (Chung et al., 1991; Rosenmund et al., 1994). Moreover, protein kinase C is reported to mediate μ opioid action in dorsal horn neurons (Chen and Huang, 1991) and protein kinase C can modulate Ca^{2+} channels in chick sensory neurons (Diversé-Pierluissi and Dunlap, 1993).

The ideal way to rule out a role for phosphorylation is to show that signaling remains intact in excised inside-out patches in the absence of ATP. Although possible with K^+ channels (Kurachi et al., 1986), such experiments cannot be done with Ca^{2+} channels, which require ATP to maintain their activity. Therefore, we applied a variety of agents that should modify protein phosphorylation by affecting various serine-threonine protein kinases and phosphatases (Table 1). None of these treatments had

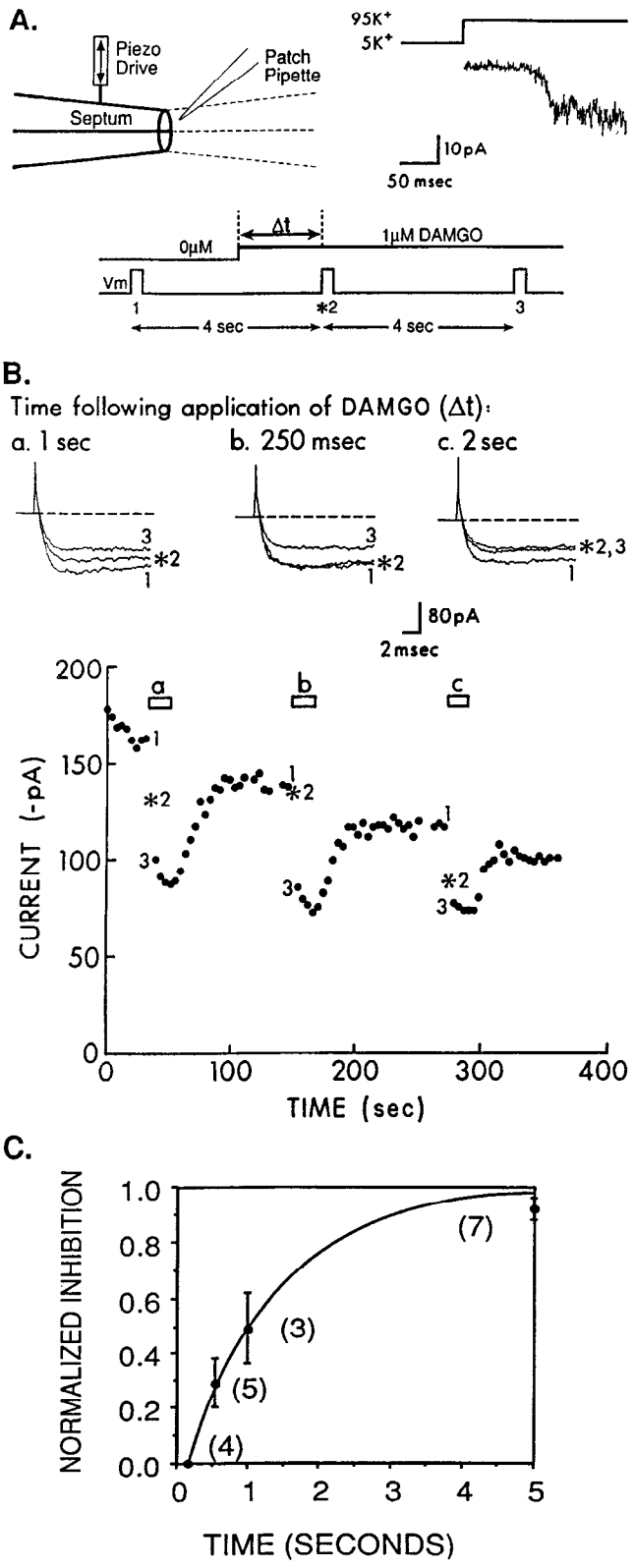

Figure 3. The speed of opioid inhibition of Ca^{2+} channels. *A*, Procedure and protocol for solution exchange. Outside-out patches were excised and positioned within the laminar flow of solutions from a theta tube that could be rapidly repositioned by sending a voltage step to a piezoelectric device on which the tube was mounted (see Materials and Methods). The speed of the system was determined prior to experiments by observing the change in current through resting K^+ channels when K^+ concentration was jumped from 15 to 95 mM with a holding potential of −70 mV (*example on right*). With the recording pipette placed 35 μm from the plane of septum, the solution change was completed in 23 msec (±11 msec, n = 23) following a delay of 120 msec (±16 msec, n = 27); 35 μm was used for all opioid applications. The time (Δt) between opioid application and Ba^{2+} current activation was controlled as shown in the protocol (*bottom panel*). Ba^{2+} currents were activated by voltage steps (15 msec to +30 mV from −70 mV) applied every 4 sec. *B*, A sample experiment. Traces in *a*, *b*, and *c* correspond to the indicated data in the graph of peak Ca^{2+} current versus time. The traces marked with *asterisks* were evoked at the indicated time following application of 1 μM DAMGO to the patch (times corrected for the 120 msec delay in solution change shown in *A*). The other traces were recorded 4 sec before (*1*) and 4 sec after (*3*) the marked trace. In this cell, DAMGO inhibition had not begun at 250 msec, was half complete in 1 sec, and neared completion in 2 sec. Extracellular solution: 110 mM $BaCl_2$, 10 mM HEPES, pH 7.2. Pipette solution: 100 mM TEA-methanesulfonate, 10 mM EGTA, 1 mM Na_2-ATP, 0.3 mM GTP, 2.1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10 mM HEPES, pH 7.0. *C*, Average normalized inhibition (±SEM) of Ba^{2+} current versus time following opioid application. The exponential curve starts after a delay of 150 msec and has a time constant of 1.27 sec. Inhibition was normalized by dividing by the total inhibition measured at least 8 sec following DAMGO application. In rare cases (see application *b* in panel *B*), current continued to slowly decline 8 sec after opioid application, but this was not typical and could not be distinguished from rundown.

a clear effect on inhibition of Ca^{2+} current by DAMGO, suggesting that serine/threonine phosphorylation plays no role in opioid modulation of Ca^{2+} channels.

Inhibitors of protein kinase A (PKA) did not diminish the effect of DAMGO, but enhancing PKA had subtle effects. DAMGO inhibited Ca^{2+} channels to a greater extent when c-AMP was elevated and intracellular perfusion of PKA eliminated the slow rundown of DAMGO inhibition expected during prolonged recordings (Table 1, note b). This agrees with the conclusions of Gross et al. (1990) and Moises et al. (1994a), which showed that κ and μ opioids inhibit Ca^{2+} channels independently of PKA, although the catalytic subunit of PKA upregulates Ca^{2+} channels.

μ Opioids shift the voltage dependence of Ca^{2+} channel activation

In sympathetic neurons, muscarinic receptors inhibit Ca^{2+} channels through several pathways that differ in their biophysical effect on the channels (Beech et al., 1992). A rapid, local inhibition of Ca^{2+} current occurs through a shift in the voltage-dependent gating of the inhibited Ca^{2+} channels so that they open only at unphysiologically positive membrane voltages. In contrast, both a slow pathway and a proposed second fast pathway decrease the maximal activity of Ca^{2+} channels over a wide range of membrane potentials. Since different signaling schemes may cause different biophysical changes in Ca^{2+} channels, we investigated the effect of DAMGO on the voltage dependence of Ca^{2+} channel activation.

Activation of Ca^{2+} channels at various voltages was assessed by measuring the amplitude of tail currents recorded during repolarization to −60 mV following the stimulating pulses (Fig. 4*A*). Arrows point to the records obtained in DAMGO. Inhibition was greater if channels were activated by a pulse to +10 mV than to +100 mV (Fig. 4*A*). The plot of these tail current amplitudes and those for other stimulating voltages (Fig. 4*B*) suggests that more positive voltages are required to open Ca^{2+} channels that are inhibited by DAMGO. The data are fit with a theory, first proposed by Bean (1989), which asserts that inhi-

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Table 1. DAMGO signaling is not suppressed by kinase and phosphatase inhibitors

Compound	Target[a]	DAMGO Control	DAMGO Compound
100 μM (each) H7, H8, H9 (16)	Inhibits PKA, PKG, PKC, CaM kinase II, casein kinase	30 ± 5	30 ± 6
0.164 U/ml PKA[b] (5)		24 ± 7	27 ± 6
0.5 mM dBcAMP + 1 mM IBMX (10)	Activates PKA	22 ± 1	29 ± 1
1 μM staurosporine (10)	Inhibits PKC, PKA, pp60 kinase, EGF receptor kinase	36 ± 8	38 ± 13
1.34 U/ml PKAI[b] (8)	Inhibits PKA	47 ± 6	32 ± 5
3 μM PKCI[b] (12)	Inhibits PKC	34 ± 4	24 ± 4
50 μM OAG[c] (7)	Activates PKC	28 ± 2	26 ± 4
5 μM okadaic acid[d] (13)	Inhibits PP-2A, PP-1	32 ± 4	30 ± 3
20 μM microcystin (8)	Inhibits PP-1, PP2A	24 ± 5	29 ± 8

Data are percentage inhibition (±SEM; number of cells in parentheses) of Ca^{2+} current by 1 μM DAMGO in the absence and presence of the indicated compound. Control and test measurements were always made on the same cell, the contol made first. PKA, PKAI, PKCI, and microcystin were applied by intracellular perfusion; the others were applied to the bath. PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; CaM kinase II, calmodulin-dependent kinase II; IBMX, isobutyl methyl xanthine; EGF, epidermal growth factor; PKAI, protein kinase A inhibitor peptide; PKCI, protein kinase C inhibitor peptide; OAG, oleoylacetylglycerol; PP-1, protein phosphatase 1; PP-2A, protein phosphatase 2A.

[a] See Hidaka and Kobayashi (1992; H7, H8, H9, and staurosporine), House and Kemp (1987; PKCI), Bialojan and Takai (1988; okadaic acid), Ichinose et al. (1990; microcystin).

[b] These measurements were made following long intracellular perfusions to assure that the protein filled the cell. DAMGO inhibition should diminish over such times (in six untreated cells, inhibition diminished from 38 ± 8% to 28 ± 7% in 13 min). Average times between control and test measurements were 20, 19, and 17 min for PKA, PKAI, and PKCI, respectively. Thus, the decreases in PKAI and PKCI are expected, whereas PKA may have rescued the DAMGO response from the expected rundown.

[c] OAG appeared to cause a slow decline of Ca^{2+} currents but did not affect percent inhibition by DAMGO.

[d] Okadaic acid affects the phosphorylation of cardiac Ca^{2+} channels only at nonsaturating levels of adrenergic receptor stimulation (Hescheler et al., 1988). Therefore, we tested the effect of 5 μM okadaic acid upon signaling by lower concentrations of DAMGO, but found no effect at any DAMGO concentration (10, 50, and 200 nM were tested).

bition involves a decrease in the proportion of Ca^{2+} channels that can open at physiological membrane voltages (from 48% to 22% in this cell; see legend for details).

Although DAMGO causes a clear shift in the activation curve, we found no voltage at which DAMGO was completely ineffective. Figure 4*C* shows the average DAMGO inhibition of Ca^{2+} current tails following depolarizations to +10 mV or +140 mV for seven cells. In each cell, inhibition was less at +140 mV than at +10 mV, but there was still substantial inhibition at the high voltage (average inhibition at +140 mV, 19 ± 3%; average inhibition at +10 mV, 32 ± 5% SEM). Thus, we conclude that DAMGO shifts the voltage dependence of Ca^{2+} channel activation, but this may not explain all inhibition.

Discussion

This work describes three functionally important aspects of opioid signaling to Ca^{2+} channels: (1) transduction is local so that an activated receptor only inhibits nearby Ca^{2+} channels; (2) transduction is fairly rapid, having a latency of several hundred milliseconds and reaching completion in about 5 sec; (3) opioids inhibit Ca^{2+} channels less effectively at very positive voltages.

Localization of opioid action

Two observations indicate that DAMGO acts locally on a cell. (1) DAMGO inhibits Ca^{2+} channels in excised outside-out patches [also seen by Shen and Surprenant (1991) using other opioids]. By itself, this observation does not mean that opioid receptors only act locally since cytoplasmic second-messenger systems can be excised along with outside-out patches (Ertel, 1990). Nevertheless, the experiment shows that all transduction machinery exists in a small part of a cell. (2) Ca^{2+} channels in a small patch of membrane protected from DAMGO by an on-cell pipette are not affected by DAMGO applied to the rest of the cell (Fig. 2). This indicates that no readily diffusible second messenger mediates between the opioid receptor and Ca^{2+} channel, in contrast to β-adrenergic modulation, where cAMP reaches Ca^{2+} channels within an on-cell pipette (Brum et al., 1984). Because of the importance of the experiment, we were careful to apply the opioid in physiological extracellular saline and to confirm that each cell responded to opioids; both of these were made possible by simultaneously recording from whole-cell and on-cell pipettes.

How close must the receptor and Ca^{2+} channel be for modulation to occur? Our experiments allow an upper estimate of this distance. Assuming that Ca^{2+} channels are distributed homogeneously on the membrane, the relative current amplitudes and concentrations of current carriers in Figure 2 indicate that 0.007% of the cell membrane is beneath the on-cell pipette (0.007% = 0.13 × 1 pA/2 nA, where 0.13 is the fraction of current through Ca^{2+} channels expected in 5 mM Ca^{2+} compared to 110 mM Ba^{2+}, Hess et al., 1986). Since the cell was roughly spherical and 33 μm in diameter, the membrane patch was about 0.55 μm in diameter. Thus, signal transduction to Ca^{2+} channels extends less than a fraction of a micron from activated opioid

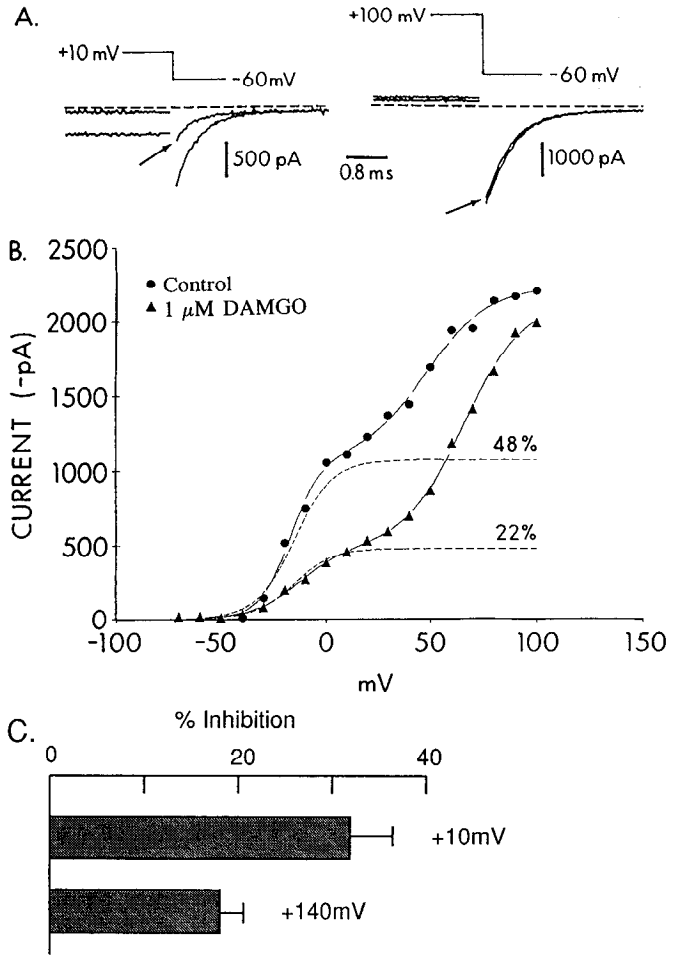

Figure 4. DAMGO shifts the voltage range of Ca^{2+} channel activation. *A*, Traces show tail currents in the presence (*arrow*) and after washout of 1 μM DAMGO recorded at −60 mV following stimulating pulses of 15 msec duration to +10 mV (*left*) and +100 mV (*right*). DAMGO substantially inhibits Ca^{2+} channels that were activated at +10 mV, but has far less effect on channels activated at +100 mV. B, Graph of tail current amplitudes versus the stimulating voltage for the cell in *A*. Tail current amplitudes were measured 450 μsec after the voltage step to −60 mV by averaging the next 150 μsec. Leak and capacity currents were eliminated by subtracting recordings in the presence of 100 μM La^{3+}, a Ca^{2+} channel blocker. Series resistance was 7 MΩ; 50% compensation was applied. C, The average inhibition by 1 μM DAMGO of tail currents following steps to +10 mV or +140 mV (n = 7). Theory in *B*: the tail current amplitude is proportional to the number of Ca^{2+} channels that are open at the end of the stimulating pulse; thus, the graph indicates the relative number of channels that are opened at the various voltages. *Solid curves* are the least squares fits of an equation describing the sum of two Boltzmann distributions:

$$i = M\left[\frac{F_1}{1 + \exp\left(\frac{V_1 - V_m}{s_1}\right)} + \frac{(1 - F_1)}{1 + \exp\left(\frac{V_2 - V_m}{s_2}\right)}\right],$$

where M is the maximum current, F_1 is the fraction of channels that gate in the physiological range, s_1 and s_2 are slope factors for the two Boltzmanns, V_1 and V_2 are the half-activation voltages, and V_m is the membrane voltage. This equation is based on a model proposed by Bean (1989), in which channels exist in two gating modes: in one, channels open at physiological voltages, and in the other, they open only at very positive voltages. The fits suggest that DAMGO decreases the proportion of channels that open in the physiological range from 48% to 22%. The best fit values for the control curve are M = 2229, F_1 = 0.48, s_1 = 7.3, V_1 = −17 mV, s_2 = 14.7, V_2 = 48 mV; and in the presence of DAMGO, M = 2142, F_1 = 0.22, s_1 = 11.0, V_1 = −13 mV, s_2 = 14.3, V_2 = 66 mV.

receptors. The actual distance might be much less. McEnery et al. (1992) have shown that the N-type Ca^{2+} channel coprecipitates with the G_o α subunit of G-proteins, suggesting that the two are linked and that signal transduction may occur without diffusion of intermediates. In any case, experiments like Figure 2 demonstrate that activation of opioid receptors on 99.99% of a cell's membrane fails to create a chemical messenger that diffuses to the remaining 0.01% of membrane that is not exposed to opioid.

Speed of opioid action

The speed at which μ opioids inhibit Ca^{2+} channels is faster than some second-messenger cascades. Opioid inhibition has a latency somewhat greater than 150 msec, is 25% maximal by 500 msec, and is maximal by 5 sec (Fig. 3). This is a far briefer latency and time to completion than the β-adrenergic, c-AMP–dependent phosphorylation of cardiac Ca^{2+} channels, which begins after several seconds and completes after about 30 sec (Frace et al., 1993; but see Yatani and Brown, 1989, for indications of a small, fast component). The time course of the opioid inhibition is only slightly slower than hormonal activation of inward rectifier K^+ channels (Nargeot et al., 1982; Surprenant and North, 1988), a process that may use only a G-protein to couple the receptor and channel (Brown, 1993). Consistent with such a simple signaling scheme, we saw no effect of various serine/threonine kinase and phosphatase inhibitors. Although the analogy is tempting, the rapid time course of opioid action does not prove a direct, G-protein transduction mechanism since more complex signaling cascades, such as phototransduction and muscle contraction, can occur within the latency for initial opioid action.

Voltage dependence of opioid action

The question of the voltage dependence of opioid action is analogous to asking whether inhibition of an enzyme is due to a shift in K_m or a decrease in V_{max}. Our answer is ambiguous. Opioids clearly shift the voltage dependence of Ca^{2+} channel gating to more positive potentials, but we could not find a voltage at which opioids were ineffective. Thus, there may be both a gating shift and a decrease in maximal current. Although voltage-dependent gating shifts are generally cited as a mechanism of Ca^{2+} channel inhibition, failure to fully reverse Ca^{2+} channel suppression at very positive voltages appears common (Boland and Bean, 1993; Zhu and Ikeda, 1994).

Comparison to other neuronal signaling paths

The functional properties—rapid onset, localized effects, gating shifts—typify a fast, G-protein–mediated signaling path to Ca^{2+} channels that has been thoroughly studied in sympathetic neurons (Hille, 1992). The time course is far faster than modulatory pathways to neuronal L-type Ca^{2+} channels that utilize diffusible second messengers (Beech et al., 1992; Mathie et al., 1992). Beech et al. (1992) suggested that there are two rapid routes for Ca^{2+} channel inhibition that differ in their sensitivity to pertussis toxin and in their voltage dependence of inhibition. Because we have never found a voltage that eliminates all opioid inhibition of Ca^{2+} channels, we doubt that all inhibition occurs through shifts in voltage-dependent gating. However, pertussis toxin abolishes almost all of the opioid effect, so it seems safe to conclude that μ opioids use only one of the two rapid paths described by Beech et al. (1992). Without independent evidence, we are reluctant to suggest that the pertussis toxin-sensitive,

voltage-independent fraction of Ca^{2+} channel inhibition represents a new pathway for modulation. The simplest interpretation of our results is that μ opioids inhibit Ca^{2+} channels through a single pathway that is rapid and local and requires neither diffusible second messengers nor phosphorylation.

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