The Journal of Neuroscience, June 1994, 14(6): 3842–3851

μ-Opioid Receptor–mediated Reduction of Neuronal Calcium Current Occurs via a G_o-Type GTP-binding Protein

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It has recently been shown that the activation of μ-opioid receptors inhibits several components of calcium channel current in rat DRG sensory neurons. μ-Opioid receptors, acting through the pertussis toxin (PTX)–sensitive substrate G_i, also reduce the activity of neuronal adenylate cyclase, but the relationship of this effect to changes in calcium channel activity has yet to be determined. Using whole-cell recordings from acutely isolated rat DRG neurons, we examined the ability of the μ-opioid–selective agonist Tyr-Pro-NMe-Phe-D-Pro-NH_2 (PLO17) to reduce calcium current after treatment with PTX and in the presence of the nonhydrolyzable GTP analog guanosine 5′-[-thio]triphosphate (GTPγS), to assess the role of G-proteins in the coupling of μ-opioid receptors to calcium channels. Inhibition of current by PLO17 was mimicked or rendered irreversible by intracellular administration of GTPγS, an activator of G-proteins, and was blocked by pretreatment of neurons with PTX. In contrast, when the catalytic subunit of cAMP-dependent protein kinase was included in the recording pipette, calcium currents increased in magnitude throughout the recording without attenuation of responses to PLO17. Thus, the μ-opioid–induced inhibition of calcium current occurs through activation of a G_i- or G_o-type G-protein, but independent of changes in adenylate cyclase activity. As a first step in identifying this G-protein, we compared the ability of several antisera directed against specific regions of G_i and $G_{o\alpha}$ subunits to block the inhibition in current by PLO17. Intracellular dialysis with an antiserum specific for G_o (GC/2) attenuated calcium current inhibition by PLO17 in five of six neurons by an average of 75%. In contrast, there was no attenuation in the response to PLO17 when neurons were dialyzed with an anti-$G_{i1\alpha}/G_{i2\alpha}$ antiserum (AS/7) or antibodies specific for α subunits of G_i proteins (G_{i1}/G_{i2} or G_{i3}) in an identical manner. These results suggest that in rat DRG neurons μ-opioid receptors couple to calcium channels via the PTX-sensitive G_o subclass of GTP-binding proteins.

[Key words: μ-opioid receptor, PLO17, calcium current, G_o proteins, G-protein antibodies, cAMP-dependent protein kinase, DRG sensory neurons]

The activation of μ-opioid receptors inhibits neuronal adenylate cyclase activity (Bhoola and Pay, 1986; Schoffelmeer et al., 1987; Beitner et al., 1989) and increases membrane potassium conductance in a variety of central and peripheral nerve cells (Williams and North, 1984; Surprenant and North, 1985; North et al., 1987; Loose and Kelley, 1990; Wimpey and Chavkin, 1991). Both of these effects are blocked by pertussis toxin (PTX), indicating that they are mediated via G_i- or G_o-type guanine nucleotide–binding proteins (G-protein) (Aghajanian and Wang, 1986; Bhoola and Pay, 1986; North et al., 1987; Wimpey and Chavkin, 1991). In recent work, we have demonstrated along with others that voltage-dependent calcium channels can now be included among the effector systems in neurons to which μ-receptors are functionally coupled. To date, the activation of μ-opioid receptors has been found to produce a voltage-dependent inhibition of high-threshold calcium currents in acutely isolated preparations (Moises and Macdonald, 1992; Moises et al., 1994) and short-term cultures of rat DRG sensory neurons (Schroeder et al., 1991; Schroeder and McCleskey, 1993). In addition, μ-receptors have been reported to inhibit high-threshold N-type calcium currents in retinoic acid–differentiated cells of the SH-SY5Y human neuroblastoma cell line (Seward et al., 1991). In the SH-SY5Y neuroblastoma cells, reductions in calcium current by the μ-selective agonist DAMGO (D-Ala^2,$NMePhe^4$,glyol enkephalin) were prevented by pretreatment with PTX and rendered irreversible by intracellular application of the nonhydrolyzable GTP analog GTPγS. While these kinds of data suggest that the response to the μ-opioid agonist resulted from the activation of a G-protein, the specific subtype of G_i or G_o protein that couples μ-opioid receptors to calcium channels in this cell line has yet to be identified. In biochemical experiments, Ueda et al. (1988) have recently shown that purified μ-opioid receptors from rat brain can be functionally reconstituted in phospholipid vesicles with either G_i- or G_o-type G-proteins. Hence, the inhibitory coupling between μ-opioid receptors and calcium channels in mammalian sensory neurons may also require G-proteins, but this has not yet been demonstrated directly.

Furthermore, little is known about the nature of the signal transduction pathway by which μ-receptors modulate the activity of calcium channels in mammalian neurons. Considerable evidence indicates that cAMP-dependent phosphorylation is an important regulatory pathway of neuronal calcium channels and may function to maintain them in an activatable state (see Armstrong and Eckert, 1987). For example, elevated levels of cAMP or increased activity of the cAMP-dependent protein kinase (AK) have been shown to increase calcium channel activity and currents in dentate granule cells (Gray and Johnston, 1987) and dorsal root and nodose ganglion sensory neurons of rats (Fed-

Received June 4, 1993; revised Nov. 22, 1993; accepted Dec. 21, 1993.

We thank Dr. Mike Uhler of the Department of Biochemistry (University of Michigan) for providing us with purified catalytic subunit of cAMP-dependent protein kinase and Ms. Rebecca Piltch for providing expert technical assistance. This work was supported in part by National Institutes of Health Grants DA03365 to H.C.M. and DA04122 to R.L.M.

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ulova et al., 1985; Gross et al., 1990a,b). Thus, μ-opioid receptors might inhibit calcium currents indirectly by decreasing activity of the adenylate cyclase/cAMP cascade. Alternatively, it is possible that μ-opioid effects on calcium channels are dependent on G-proteins but occur independent of changes in adenylate cyclase.

The present study was designed to determine whether μ-opioid receptors inhibit calcium currents in rat sensory neurons by means of a PTX-sensitive G-protein–dependent pathway, and if so, to characterize G-protein subtypes that might be involved in μ-opioid receptor responses. Whole-cell recordings from acutely dissociated DRG neurons were used to examine the effects of the μ-selective agonist PLO17 (Chang et al., 1983) on calcium currents in the presence of GTPγS and after pretreatment of cells with PTX. In some experiments, the catalytic subunit of cAMP-dependent protein kinase (AK-C) was included in the patch pipette to assess the possibility that the reduction in current by μ-opioids might be linked to an inhibition in activity of the adenylate cyclase/cAMP cascade (see, e.g., Gross et al., 1990a). Responses to the μ-agonist were then reexamined during intracellular dialysis with antibodies directed against α subunits of G_i and G_o to identify which G-protein subtypes constitute the normal pathway. Our results show that in rat DRG neurons μ-opioid receptors inhibit calcium current via the PTX-sensitive G-protein, G_o, and that this occurs independent of changes in the activity of adenylate cyclase.

A preliminary report of some of this work has already appeared (Moises et al., 1992).

Materials and Methods

Preparation of acutely dissociated neurons. The procedures used for the preparation of acutely dissociated DRG neurons are described in detail in a recent report (Moises et al., 1994). Briefly, DRGs were dissected from the lumbar and thoracic regions of 21–40-d-old rats and treated with collagenase (type II, 3 mg/ml; Sigma Chemical) for 50 min at 37°C, followed by 10 min treatment with trypsin (type I, 1 mg/ml; Sigma Chemical). A 5% solution of bovine serum albumin [20 mg/ml minimal essential medium (MEM); GIBCO Laboratories] was then added to the incubation medium to inhibit the enzymes, and the dissociated ganglia mechanically dispersed by triturating for four or five passages through a fire-polished Pasteur pipette. Cell suspensions (~200 μl/dish) were plated onto collagen-coated culture dishes and incubated at 37°C for 1 hr, after which an additional amount of MEM containing 10% horse serum (Sigma Chemical) and nerve growth factor (50 ng/ml; Boehringer Mannheim) was added to bring the total volume to 2 ml. Neurons were studied between 2 and 10 hr after plating or after 1 d in culture when recordings were made from PTX-treated cells. All recordings were confined to neurons without processes and cells were excluded from the analysis if the recordings showed any sign of inadequate space clamp (e.g., delayed settling of capacitance transient with time constants > 150 μsec and broad tail currents).

Whole-cell, patch-clamp recordings. Voltage-clamp recordings were obtained using the whole-cell variation of the patch-clamp technique (Hamill et al., 1981). Glass recording patch pipettes, prepared from Fisher microhematocrit tubes and having resistances of 0.8–1.5 MΩ, were filled with recording solution of the following composition (in mM): 140 CsCl, 10 HEPES, 10 EGTA, 5 ATP (magnesium salt), and 0.1 GTP (lithium salt) (all reagents from Sigma Chemical). The pH was adjusted with 1 M CsOH to 7.3–7.35 after the addition of ATP and the osmolality (280–300 mOsm) adjusted to 10–15% below that of the bath solution. The neurons were bathed in a solution (pH 7.4, 310–330 mOsm) consisting of (in mM) 67 choline chloride, 100 tetraethylammonium chloride, 5.3 KCl, 5.6 glucose, 5.0 $CaCl_2$, 0.8 $MgCl_2$, and 10 HEPES. Under these conditions sodium and potassium currents were suppressed. In the presence of 200 μM Cd^{2+} to eliminate the calcium currents, no voltage-dependent outward currents were evoked at test potentials as positive as +30 mV.

Recordings were made at room temperature using an Axopatch 1-D patch-clamp amplifier (Axon Instruments, Foster City, CA). Pipette and whole-cell capacitance and series resistance were corrected using compensation circuitry on the patch-clamp amplifier. Initial input resistances were in the range of 500 MΩ to 1.2 GΩ. Series resistance was estimated by cancellation of the capacitance-charging current transient after patch rupture. In most cases, series resistance compensation of 80–90% was obtained without inducing significant noise or oscillation, resulting in final series resistances ranging from 0.1 to 1.2 MΩ. No data were included in the analysis where series resistance resulted in a 5 mV or greater error in voltage commands.

Voltage step commands of 100 msec duration were applied every 30 or 60 sec and the evoked currents filtered with a 12-pole low-pass Bessel filter at 10 kHz (−3 dB). The filtered current records were digitized at 5 kHz, stored and analyzed by a 386-based microcomputer using the program pCLAMP (Axon Instruments). Leak current was estimated as the inverse of the current evoked with 100 msec hyperpolarizing commands of equal magnitude to the depolarizing commands used to evoke the inward currents. This current was digitally subtracted from the relevant inward current to obtain the calcium current. In some cases, Cd^{2+} (200–500 μM) was applied to the neuron at the end of an experiment and always eliminated the inward calcium currents, including any that remained in the presence of the opioid agonist.

Calculations of the magnitude of opioid-induced current inhibition were corrected for the decline in recordable calcium current (rundown) that occurred in some neurons during the course of whole-cell recording. To adjust for rundown, recovery currents were elicited following termination of agonist application until the amplitude of the postdrug currents had plateaued. The response to drug was then expressed as a percentage change in the averaged value of the predrug current and stabilized recovery current amplitudes. This adjustment relies on the fact that the establishment of agonist-induced inhibition in current occurs sufficiently fast (typically within several seconds) that the extent of current rundown during this time contributes marginally, if at all, to the absolute reduction in current measured.

Preparation and delivery of solutions. PLO17 (Peninsula Laboratories, Belmont, CA) was stored frozen (at −20°C) in 10 μl aliquots of lyophilized peptide (dissolved in sterile water) and prepared as a fresh solution immediately before the experiment by dilution with normal extracellular bathing medium. The solution (pH 7.4, 310–330 mOsm) bathing the cells consisted of (in mM) 67 choline chloride, 100 tetraethylammonium chloride, 5.3 KCl, 5.6 glucose, 5.0 $CaCl_2$, 0.8 $MgCl_2$, and 10 HEPES. The μ-opioid agonist was applied either by using pressure ejection (1–5 sec duration) from a blunt-tipped (10–20 μm tip diameter) glass micropipette positioned approximately 50 μm from the neuron or via local superfusion of the cell by means of a U-tube rapid exchange system (see Murase et al., 1989). The effects of GTPγS (lithium salt, Sigma Chemical Co.), AK-C and the different G-protein antibodies (see below) were assessed by introducing them directly into the cell by intracellular dialysis from the recording pipette. For the antibody experiments, the recording pipette solution was modified slightly for its normal composition (in mM: 140 CsCl, 10 HEPES, 10 EGTA, 5 ATP-Mg^{2+}, and 0.1 GTP, pH 7.3 and 280–300 mOsm) by including 0.5% bovine serum albumin and increasing the concentration of GTP (1 mM). Higher concentrations of GTP were used to promote dissociation of holomeric G-proteins in both the resting and ligand-bound state and thus optimize the effectiveness of interaction between antibodies with epitopes on free α subunits (see Asano et al., 1987).

Some neurons were pretreated with PTX for 18–24 hr before recordings were made. PTX was stored as a 50 μg/ml solution in 0.1% bovine serum albumin and was diluted into cultures of freshly dissociated DRG neurons to a final concentration of 150–200 ng/ml. The standard bath solution was used and PTX was included in the pipette solution (100 ng/ml) when recording from pretreated neurons.

Purified AK-C was prepared within 24 hr of the experiment as described elsewhere (Olsen and Uhler, 1989) and stored at 4°C as a 1 mg/ml stock solution. Immediately before the experiment, the AK-C was diluted into the pipette recording solution at the concentrations stated. Experimental solutions of AK-C were stored on ice and retained full activity for several hours (assayed as previously described, Uhler and McKnight, 1987). In some experiments, the peptide inhibitor of AK (protein kinase inhibitor peptide, PKIP; Sigma) was dissolved in recording solution that contained 1 μg/ml (~20 nM) AK-C. To ensure a full inhibitory effect on AK-C activity, an excess of PKIP was added (~50 μM), and the solution used for recording within a few minutes

after briefly heating the mixture at 35°C (see Gross et al., 1990b, for details).

Specific antisera directed against the α subunits of G_o (GC/2) and of G_{i1}/G_{i2} (AS/7) were obtained from Du Pont/New England Nuclear (Boston, MA) and used in initial experiments. These are rabbit antisera that were raised against synthetic decapeptides corresponding to the amino and carboxyl termini of bovine brain $G_{o\alpha}$ (GC/2, Goldsmith et al., 1988; Spiegel, 1991) and transducin-α (AS/7, Goldsmith et al., 1987), respectively. The GC/2 antiserum specifically identifies $G_{o\alpha}$, but does not react with G_i α subunits, whereas AS/7 recognizes both $G_{i\alpha1}$ and $G_{i\alpha2}$ in addition to transducin-α, but does not react with $G_{i\alpha3}$. Experiments were also performed using affinity-purified rabbit antibodies (purchased from Calbiochem, San Diego, CA) that only recognize (carboxyl-terminal epitopes specific to) α subunits of G_{i1}/G_{i2} or $G_{i\alpha3}$, respectively. Nonimmune rabbit serum (Calbiochem), included in the recording pipette at the same dilutions used for antibodies, served as a control.

For all recordings in the presence of AK-C or G-protein antibodies, the tip of recording pipette was filled to 1–2 mm with standard recording solution, and the pipette was back-filled with the experimental solution. In this way the onset of action of AK-C or antibody loading was delayed, which permitted the measurement of control responses to μ-opioid application within the first few minutes of whole-cell recording. Absence of protein-containing mixtures in the tip of the recording pipette facilitated the formation of gigaohm seals and helped prevent clogging of the pipette tip after patch rupture.

Pilot experiments were performed to determine whether cells could be sufficiently loaded with antibodies by intracellular dialysis via the patch pipette, as previously described (Wiley et al., 1992). Briefly, DRG neurons were plated onto coverslips placed at the bottom of culture dishes and incubated for 1–2 hr before attempting recordings. Some of the cells attached to the coverslips, and in number of these whole-cell recordings were obtained for 5–10 min with patch pipettes containing standard internal solution with or without nonimmune rabbit serum at a 1:10 dilution. The cells were then treated with a fluorescein-conjugated goat anti-rabbit immunoglobulin (Du Pont/New England Nuclear) and prepared for immunohistochemical examination under a fluorescence microscope. Only cells that were dialyzed with pipette solution containing the nonimmune serum showed intense fluorescence under ultraviolet light, indicating the successful loading of the neuron with the rabbit immunoglobulins by this procedure (see Wiley et al., 1992).

Statistical analysis. Statistical comparisons between the inhibitory effect of PLO17 on calcium current in control and PTX-treated neurons were made using Student's two-tailed t test. For experiments involving infusions of G-protein antibodies or AK-C, a paired-sample t test was first used to examine for differences in the mean inhibitions in current produced by application of the μ-opioid agonist when tested within several minutes of patch rupture and again after prolonged dialysis of the neuron with an antiserum or the kinase. Student's two-tailed t test was then used to assess the statistical significance of any differences between the effects on μ-opioid responses produced by intracellular dialysis with a specific G-protein antiserum and by infusion of nonimmune control serum. Values in the text are given as mean ± SEM, unless otherwise indicated.

Results

We recently showed in rat DRG neurons that μ-opioid agonists reduced both the transient, ω-conotoxin GVIA (ωCgTx)–sensitive N-type current as well as more sustained, ωCgTx-resistant components of high-threshold current, but that high-threshold L-type and low-threshold T-type currents were unaffected (Moises et al., 1994). Therefore, we restricted the present experiments to neurons that had little or no observable T-type current, which facilitated the analysis of agonist-induced inhibitory effects following different experimental manipulations that were used to modify activity of G-protein–dependent processes. Unless otherwise indicated, responses to PLO17 were quantified in terms of reductions in peak current amplitude (I_p) and late current amplitude (I_{100}, measured within 2 msec of offset of a 100 msec step used to elicit currents), with the understanding that the opioid-sensitive current may be provided by several types of high-threshold calcium channels that exhibit either transient or more sustained inactivation kinetics.

Experiments with a nonhydrolyzable analog of GTP

In the first series of experiments, we examined the effects of intracellular administration of GTPγS on PLO17-induced reductions in calcium current in untreated DRG neurons. This stable thiol derivative of GTP activates G-proteins directly in the absence of ligand binding (Gilman, 1984), and because it binds to G-proteins irreversibly, has the additional effect of uncoupling them from subsequent activation by neurotransmitter receptors (Breitweiser and Szabo, 1985; Wanke et al., 1987). Thus, we sought to determine whether dialysis of cells with GTPγS, contained in the patch pipette at 100 μM in place of GTP, could potentiate submaximal responses to the μ-opioid agonist and render inhibitory effects of the opioid irreversible. The traces in Figure 1*A* are from a typical experiment (n = 5) and show PLO17-induced reductions in calcium currents elicited by 100 msec commands to +10 mV from V_h = −80 mV at the times indicated during the recording. In this neuron, a 5 sec pressure application of PLO17 (1 μM) reduced peak current (I_p) amplitude only slightly from 4.5 to 3.9 nA and did not affect the rate of current activation when the opioid was initially tested within the first 5 min after patch rupture. Note that there was complete recovery from the opioid response, after which the current continued to increase slowly (Fig. 1*B*), suggesting that an effect of GTPγS had not been established up to this point (see below). In contrast, reapplication of the μ-agonist at 11 min into the recording markedly inhibited I_p amplitude and that of the residual current (I_{100}) measured at the end of the step, and this was associated with a slowing of activation kinetics (also see Gross et al., 1990a; Moises et al., 1994). In addition, the inhibitory effect of PLO17 was rendered irreversible in that washout of the μ-agonist or application of naloxone (1 μM) failed to reverse the opioid-induced reduction in current. Intracellular administration of GTPγS had similar effects on PLO17-induced responses in four additional neurons, although in each of these neurons the effects of the analog showed a much faster onset (5.2 ± 0.4 min). Moreover, in other experiments (n = 3) inclusion of GTPγS (100 μM) in the recording pipette produced a suppression in current much like PLO17 within the first minutes after patch rupture, prior to presentation of the agonist (Fig. 2). In these cells, the reduction by GTPγS was irreversible, however, and application of PLO17 in the presence of the GTP analog was without effect (data not shown).

PTX blocked the response to PLO17

The results obtained with GTPγS indicate that a G-protein was involved in the reduction of calcium current by PLO17. However, GTPγS binds to all subtypes of G-proteins, and thus, little can be inferred about the particular class of G-proteins that couple μ-opioid receptors to calcium channels in DRG neurons. As a first step in identifying the subtype of G-protein involved, we attempted to block the inhibitory effects of PLO17 by pretreating neurons with PTX (150–200 ng/ml) for 18–24 hr at 37°C. The toxin (100 ng/ml) was also included in the internal solution of the patch pipette when recording from PTX-treated neurons. We have previously shown that the procedures used here for treating cells with PTX effectively block the reduction in calcium current by κ-opioids in rat nodose ganglion sensory neurons (Gross et al., 1990a).

Calcium currents recorded in PTX-treated cells (n = 10) were

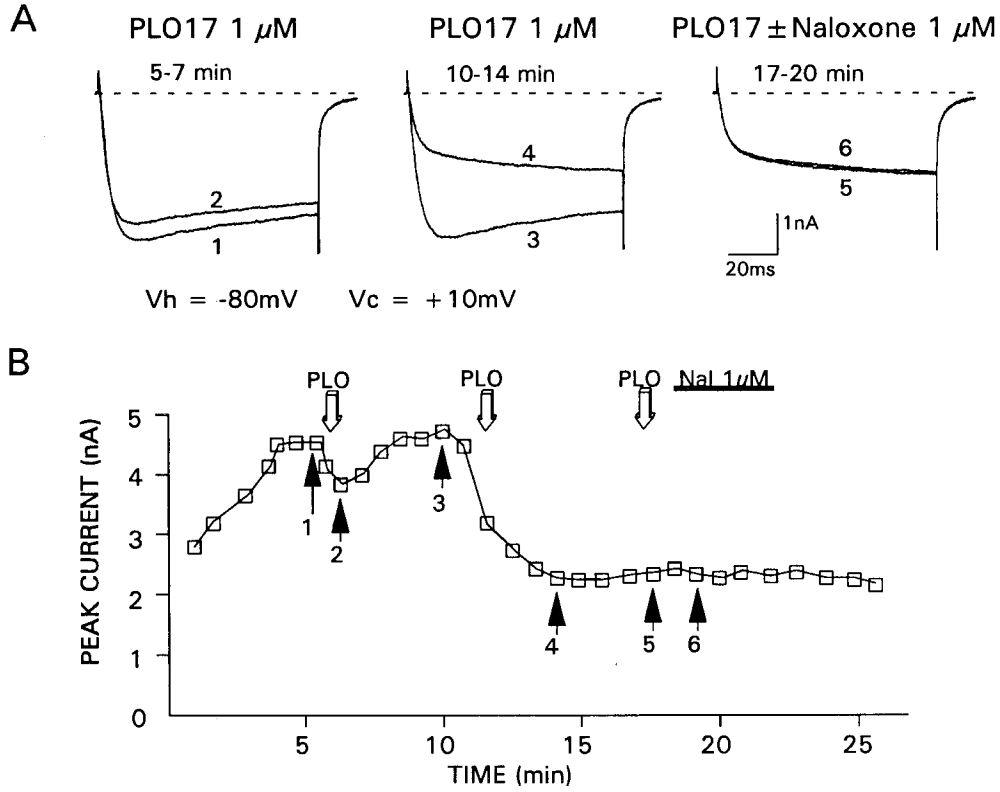

Figure 1. Effects of PLO17 on calcium currents recorded in the presence of GTPγS (100 μM), contained in the recording pipette. *A*, Whole-cell calcium currents evoked by 100 msec voltage steps to +10 mV from V_h = −80 mV at the times indicated after patch rupture. *Traces 2* and *4–6* were recorded within 1 min after a 5 sec puffer application of PLO17 (1 μM) in the absence or presence (*trace 6*) of naloxone (1 μM). Administration of PLO17 within the first 5 min of recording produced only a small reduction in current. Infusion of GTPγS markedly increased the response to a second application of the opioid and rendered its effect irreversible. Note that the reduction in current was now accompanied by a marked slowing in the rate of activation. *B*, Plot of peak current versus time after patch rupture shows the complete time course of the effect of intracellular dialysis with the nonhydrolyzable GTP analog. The *numbers* correspond to the respective currents shown in *A*.

essentially indistinguishable from those in matched control neurons (n = 9) (Fig. 3, compare traces in *A, B*). Thus, for transient and sustained components of high-threshold current evoked by steps to +10 mV from V_h = −80mV, the $I_p$max (3.2 ± 0.4 nA) and the time to I_p (10.8 ± 1.2 msec) in treated neurons were similar to the values for currents in controls ($I_p$max, 2.7 ± 0.5 nA; time to I_p, 11.4 ± 1.2 msec). Similarly, sustained components of high-threshold current that were isolated by evoking currents at +10 mV from reduced V_h = −40 mV showed remarkably little difference between control and treated neurons (Fig. 3). Pretreatment with PTX also appeared to have a negligible effect on the inactivation rates of high-threshold calcium channel currents, given the normal variability in inactivation kinetics found among currents in controls, as well as in treated neurons. On the other hand, the inhibitory effects of PLO17 were markedly attenuated after treatment of DRG neurons with PTX. Whereas calcium currents in 89% of control neurons (eight of nine) were reduced by PLO17 (3 μM), calcium currents were reduced in only 25% of PTX-treated neurons (two of eight), even though the μ-agonist was applied at saturating concentrations (see Moises et al., 1994). Furthermore, for those PTX-treated neurons that did respond, the mean percentage inhibition in calcium current by the opioid (5.9 ± 1.5%) was significantly reduced compared to that in controls (37.3 ± 6.7%, $p < 0.001$). In a follow-up to these experiments, we found that PTX also blocked or greatly attenuated the ability of GTPγS, included in the recording pipette at 100 μM concentration, to mimic the PLO17-induced inhibition of calcium current (n = 2, data not shown). These data enabled us to conclude that the PLO17-induced decrease in calcium current was mediated via activation of a PTX-sensitive G_i- or G_o-type G-protein.

μ-Opioid receptors reduced calcium current independent of changes in adenylate cyclase

As mentioned in the introductory remarks, μ-opioid receptors have been shown to inhibit neuronal adenylate cyclase by a PTX-sensitive mechanism, presumably via the activation of G (Kurose et al., 1983; Birnbaumer et al., 1990). It is known from earlier studies that cAMP-dependent phosphorylation stabilizes calcium currents in whole-cell recordings from rat DRG neurons (Fedulova et al., 1985). In addition, we have recently demonstrated that intracellular dialysis of rat nodose ganglion neurons with the purified catalytic subunit of AK (AK-C) increases N- and L-type calcium currents and prevents the rundown in current normally associated with whole-cell recordings (Gross et al., 1990a,b). Activators of adenylate cyclase (e.g., forskolin and cholera toxin) have also been shown to stimulate calcium influx in rat spinal cord–DRG cocultures and this effect was inhibited by opioids in a PTX-sensitive manner (Attali et al., 1989). Taken together, these results suggest that the reduction in calcium current produced by PLO17 might be secondary to an inhibition in adenylate cyclase and in the activity of the cAMP/AK cascade. To test this hypothesis, we compared the effect of the μ-opioid agonist on calcium currents in neurons during early and later stages of intracellular dialysis with AK-C (1–10 μg/ml) (see Materials and Methods). We reasoned that if a G-protein–mediated reduction in adenylate cyclase activity was the sole mechanism for the PLO17-induced reduction in calcium current, then the effect of the opioid would not be apparent in the presence of exogenous AK-C.

To examine the effects of AK-C on responses to PLO17 we evoked currents with 100 msec steps to +10 mV from V_h =

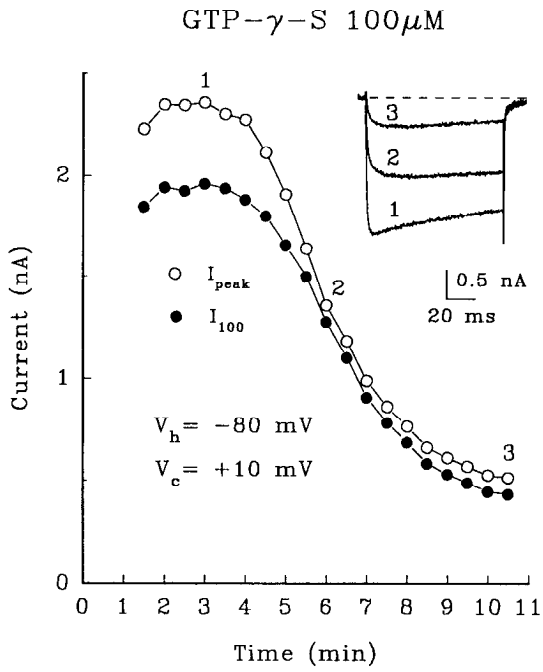

Figure 2. Intracellular dialysis with GTPγS inhibited calcium current and slowed its rate of activation in the absence of an opioid agonist. Amplitudes of peak calcium current (*open circles*) and residual current (*solid circles*), measured just before the end of a 100 msec step to +10 mV from V_h = −80 mV, are plotted versus time after patch rupture. *Numbered traces at right* show currents recorded at times indicated in the graph. Note that the rapidly inactivating component of current was abolished within the first 5 min of recording with pipette solution containing GTPγS 100 μM.

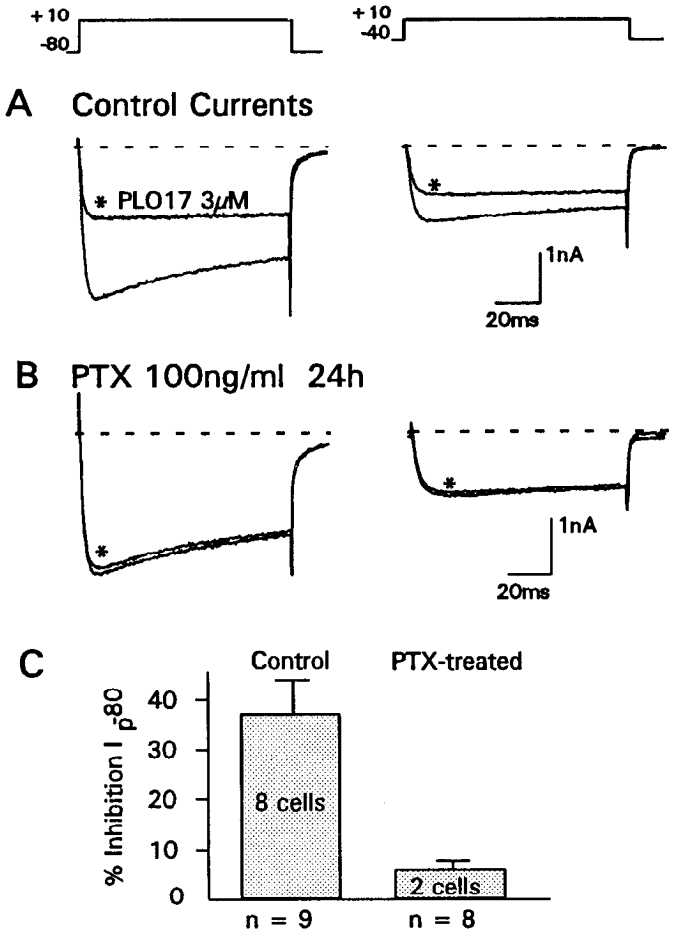

Figure 3. Pretreatment of neurons with PTX blocked the inhibition of calcium current by PLO17. Currents were evoked by stepping to +10 mV alternately from V_h = −80 mV (*traces at left*) and −40 mV (*traces at right*) in the absence and presence of PLO17 (3 μM). At this concentration of the agonist, near-maximal inhibitory effects were produced in control neurons (see Moises et al., 1994). Current traces shown in *A* and *B* were recorded from neurons after 24 hr in culture without or with PTX (100 ng/ml), respectively. Currents recorded in PTX-treated neurons were similar in appearance to those in controls, but were not reduced by PLO17. *C*, The bar graphs compare the percentage inhibition of peak current (V_h = −80 mV) by PLO17 for the stated number of control (eight of nine) and PTX-treated neurons (two of eight) that responded to the opioid. The height of each bar is the mean ± SEM (error bars shown in only one direction).

−80 mV at 30 sec intervals following patch rupture, as illustrated in Figure 4. In the presence of 10 μg/ml AK-C, currents were similar to controls during the first 5–7 min of recording in all eight neurons tested. An initial application of PLO17 (3 μM) during this time period reduced I_p amplitudes an average of 39.5 ± 5.2% (or 1.2 nA, from 3.1 ± 0.3 nA to 1.9 ± 0.2 nA, n = 6). Recovery from the effects of PLO17 typically occurred within 1 min, after which currents recorded in the presence of AK-C increased dramatically in magnitude, whereas those in control neurons characteristically exhibited a steady rate of decline (cf. Fig. 3 from Moises et al., 1994). This effect of AK-C was maximal 12–17 min after patch rupture; thus, I_p values of currents evoked at 15 min were increased 81.5 ± 16.5% (or 3.6 nA, from 3.1 ± 0.3 nA to 6.7 ± 0.8 nA) over maximal I_p values recorded within the first 5 min (n = 6, t = 4.68, df = 5, p < 0.005). When PLO17 was reapplied at this later time, the magnitude of the reduction in current was greater than that initially produced (2.3 nA vs 1.2 nA) (Fig. 4), although the opioid-induced response now averaged only a 34.5 ± 3.8% reduction in I_p (NS compared to value at 5 min). The slight reduction found in the opioid effect is what one would predict if AK-C increased transient high-threshold N-type channel current that was sensitive to regulation by μ-receptors as well as a sustained L-type channel current that was insensitive (see Gross et al., 1990b; Moises et al., 1994). Consistent with this interpretation, the magnitude of the PLO17-sensitive current tended to reach a plateau within the first 5 min of the onset of AK-C's effect, whereas I_p amplitudes continued to grow in an incremental manner in parallel with residual current (Fig. 4*B*). These effects of AK-C on calcium currents appeared to represent a specific action of the cAMP-dependent protein kinase, since they were not obtained with lower concentrations (1–2 μg/ml, four of five neurons) and were reduced or eliminated by incubation of AK-C with PKIP (n = 2; also see Gross et al., 1990b).

Effect of dialysis of G-protein antisera on PLO17 responses

Among the family of G-proteins that serve as substrates for ADP-ribosylation by PTX are three known subtypes of G_i (G_{i1}, G_{i2}, and G_{i3}) and A and B subtypes of G_o, so designated based on sequence differences in their α subunits (see Simon et al., 1991, for review). Rat DRG neurons have been shown to contain at least two PTX substrate G-proteins, identified as G_o and G_{i2}, using immunoblotting procedures with specific antisera raised against peptide sequences unique to different α subunits isolated from bovine brain (Ewald et al., 1989). In a final series of experiments, we tested the effects of PLO17 during dialysis

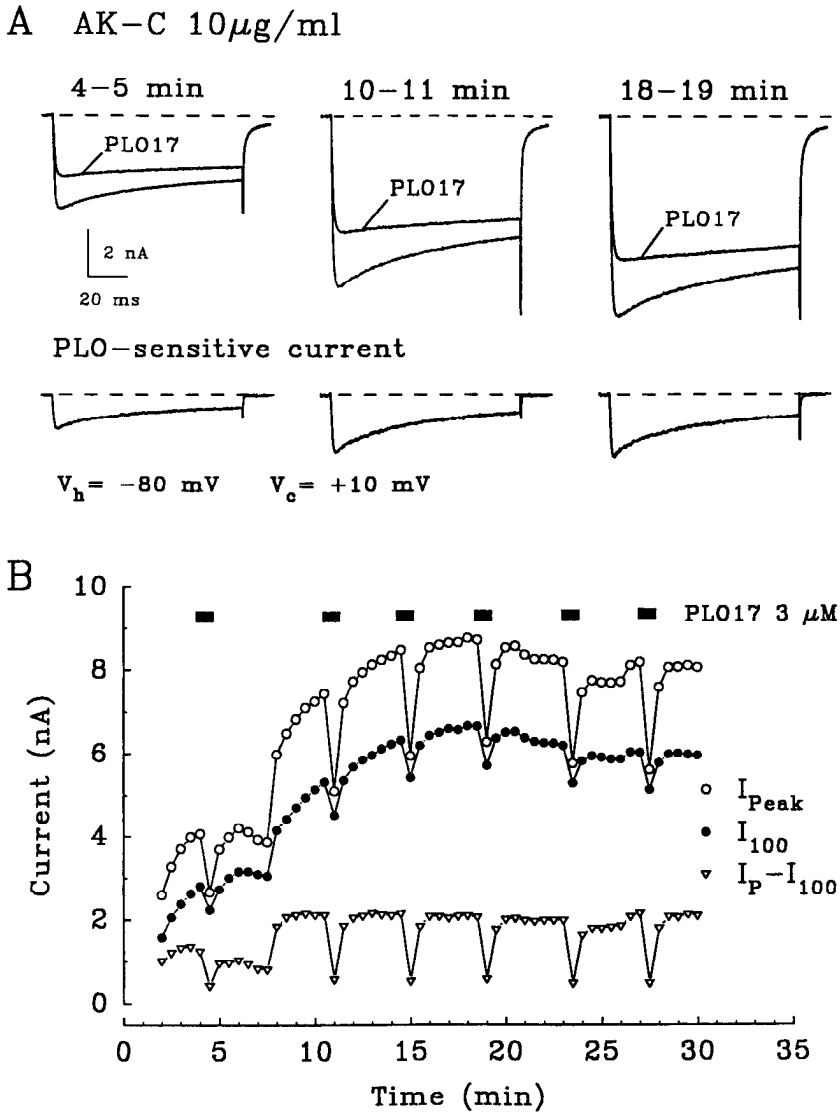

Figure 4. PLO17 reduced calcium current in the presence of AK-C. *A*, Currents were evoked every 30 sec by stepping to +10 mV from V_h = −80 mV in the absence (*lower trace* of each pair) or presence of PLO17. PLO17 (1 μM) was applied to the cell by local perfusion, and the effects of peptide application determined within the first 5 min of recording and then at 4–5 min intervals thereafter during a 30 min infusion of AK-C (10 μg/ml). The tip of the recording pipette was filled with standard solution, and the pipette was then backfilled with standard solution plus AK-C. In this way it was possible to determine the response to PLO17 before and at various times after the AK-C–induced enhancement of calcium current became apparent. Subtraction of currents recorded before and in the presence of the opioid yielded the PLO17-sensitive current (*lower traces in A*). *B*, Amplitudes of peak currents (*open circles*) and residual currents measured at the end of the 100 msec step (*solid circles*) are plotted as a function of time after patch rupture. Subtraction of I_{100} from I_p values yielded an estimate of the transient component of high-threshold current that represented a majority of current that was sensitive to the opioid. *Solid bars* above the graph indicate the period of drug application.

of neurons with G-protein antisera specific for α subunits of G_o, G_{i1}/G_{i2}, or G_{i3} to determine which subtype(s) of PTX-sensitive G-protein(s) is involved in the inhibitory coupling of μ-opioid receptors to calcium channels. To allow for comparisons between effects of the different antisera, responses to the opioid were examined at an early and late stage of recording so that each neuron could serve as its own control. In preliminary experiments we found that administration of a protein load (e.g., bovine serum albumin) to neurons was often sufficient to affect calcium currents, producing stabilization or a slight increase in current amplitudes over the course of a 20 min recording (see Fig. 4*A*). To control for such nonspecific effects that antiserum administration might have on the calcium currents or the responses to the μ-opioid agonist, control neurons were dialyzed with nonimmune rabbit serum at the same dilution (1:20) used for antibody solutions. In these neurons, the amount of calcium current inhibition produced by administration of PLO17 20 min after patch rupture (30.4 ± 5.1%, n = 13) was not significantly different from that produced by opioid application within the first 5 min of recording (32.5 ± 4.6%, $p > 0.5$).

Intracellular administration of the anti-$G_{o\alpha}$ (GC/2) antiserum attenuated the PLO17-induced reduction of calcium current in a concentration-dependent manner (Fig. 5). Infusions of anti-$G_{o\alpha}$ antiserum at a 1:20 dilution over a 20 min period reduced the inhibitory effects of PLO17 in six of seven neurons. In comparison, this antiserum was without effect on control currents or on responses to PLO17 when included in the recording pipette at a 1:100 dilution (n = 3) and attenuated the response to PLO17 in only one of three neurons that were dialyzed with a 1:50 dilution. The pairs of current traces from the neuron shown in Figure 5*B* compare the inhibitions in current produced by application of PLO17 (1 μM) (indicated by asterisks) after 5 and 20 min of recording with a pipette containing the anti-$G_{o\alpha}$ antiserum at a 1:20 dilution. Between 5 and 20 min after patch rupture, the I_p amplitude of control currents increased 20% (from 2.5 nA to 3.0 nA), whereas the PLO17-induced response was reduced to less than 25% of its initial value (from 50.5% to 11.6% inhibition of I_p) during this same period. This attenuation of the opioid effect appeared to be unrelated to changes in control currents or to nonspecific effects associated with the infusion procedure, since administration of nonimmune rabbit serum using the same protocol also increased current amplitudes in some neurons, but did not affect PLO17-induced reductions in calcium current (Figs. 5*A*, 6*A*). Moreover, attenuation of the

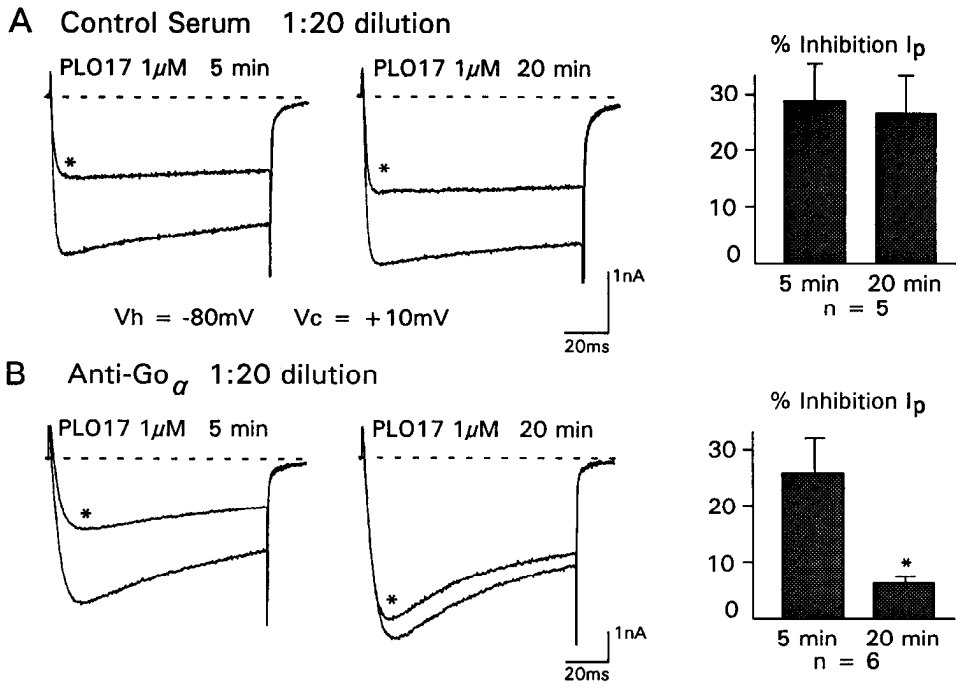

Figure 5. The response to PLO17 was attenuated in neurons dialyzed with anti-$G_{o\alpha}$ antiserum. *A*, Pairs of calcium currents recorded in the absence and presence of PLO17 (1 μM) (indicated by *asterisks*) 5 min and 20 min after initiation of whole-cell recording with a pipette containing nonimmune rabbit serum at a 1:20 dilution. A 20 min infusion of nonimmune serum slightly increased the amplitude of the current, but did not affect the inhibitory response to PLO17. *B*, Calcium currents from another cell recorded in the absence and presence of PLO17 (*asterisks*) 5 and 20 min after dialysis with anti-$G_{o\alpha}$ (GC/2) antiserum at the same dilution. Intracellular loading with the anti-$G_{o\alpha}$ antiserum markedly attenuated the opioid-induced inhibition while also increasing control current amplitude. *Bar graphs at right* compare the mean percentage reduction in peak current produced by PLO17 5 and 20 min after intracellular administration of nonimmune serum (*top*) versus anti-$G_{o\alpha}$ antiserum (*bottom*) for the number of neurons indicated. Currents were evoked by 100 msec steps to +10 mV from V_h = −80 mV. *, $p < 0.001$.

response to PLO17 was observed in neurons (n = 3) that did not exhibit any run-up in current during dialysis with the anti-$G_{o\alpha}$ antiserum. Overall, the inhibitory effect of PLO17 was reduced to 23 ± 8.8% of the initial response level (from 27.0 ± 6.2% to 6.6 ± 1.5% average inhibition of I_p) after 20 min of intracellular administration of the anti-$G_{o\alpha}$ antiserum at a 1:20 dilution (n = 6, t = 5.50, df = 5, $p < 0.001$). In contrast, administration of the anti-G_{i1}/G_{i2} (AS/7) antiserum in an identical manner had no significant effect on the PLO17-induced reduction in calcium current (29.3 ± 6.8% at 5 min compared to 26.3 ± 6.7% at 20 min, n = 5), as illustrated in Figure 6. Similarly, we were unable to demonstrate an attenuation of calcium current inhibition by PLO17 in neurons that were intracellularly dialyzed with affinity-purified antibodies that only recognize α subunits of G_{i1} and G_{i2} (25.6 ± 7.5% at 5 min compared to 23.5 ± 6.7% at 20 min, n = 3) or G_{i3} (23.8 ± 5.5% compared to 24.2 ± 6.2% at 5 and 20 min, respectively, n = 4).

Discussion

The present results indicate that the μ-opioid receptor–mediated reduction of calcium currents in rat DRG sensory neurons occurs through a G-protein–dependent pathway. Thus, the inhibitory effect of PLO17 was mimicked by intracellular administration of the nonhydrolyzable GTP analog GTPγS, and in its presence, the reduction in current by the μ-opioid agonist became irreversible. In addition, pretreatment of neurons with PTX blocked the actions of PLO17, indicating that a G_i- or G_o-type G-protein was required for its effect on neuronal calcium currents.

The finding that the inhibitory coupling between μ-opioid receptors and calcium channels in DRG neurons involves a PTX-sensitive G-protein was not surprising. The ability of PTX to disrupt the coupling of inhibitory receptors to calcium channels has already been well documented for κ- and δ-selective opioids in other types of neurons and neuronal-like cells (nodose neurons, Gross et al., 1990a; submucous plexus neurons, Surprenant et al., 1990; NG108-15, Hescheler et al., 1987). In addition, Seward et al. (1991) showed recently that μ-opioid–induced inhibition of calcium current in SH-SY5Y human neuroblastoma cells occurred through the activation of a PTX-sensitive G-protein. Hescheler et al. (1987) found in reconstitution experiments in NG108-15 cells that intracellular administration of the α subunit of G_o was 10 times more effective than G in restoring the δ-receptor–mediated inhibition in calcium current after treatment with PTX. Using antibodies raised against carboxyl-terminal portions of either $G_{o\alpha}$ or $G_{i1\alpha}/G_{i2\alpha}$, McFadzean et al. (1989) showed further that δ-opioid receptors in these cells were coupled to calcium channels via G_o, but not G_i. It is known from biochemical experiments that purified μ-opioid receptors from rat brain membranes are functionally coupled to both G_i- and G_o-type PTX substrates (Ueda et al., 1988; Wong et al., 1989). However, at least five PTX substrates (G_{oA}, G_{oB}, G_{i1}, G_{i2}, G_{i3}) have now been identified by molecular cloning and sequencing techniques (reviewed by Simon et al., 1991), and thus far, it has been unclear which of these proteins couples μ-opioid receptors to calcium channels.

The results of our experiments in neurons dialyzed with specific G-protein antisera suggest that the reduction in calcium current by PLO17 was mediated by a G_o-type protein. Thus, opioid-induced responses were attenuated in neurons dialyzed with the anti-$G_{o\alpha}$ (GC/2) antiserum, whereas intracellular administration of the anti-G_{i1}/G_{i2} (AS/7) antiserum or antibodies raised against α subunits of G_{i1}/G_{i2} or G_{i3} were without effect. The anti-G_{i1}/G_{i2} antiserum used in our studies has previously been shown to inhibit the reduction in adenylate cyclase activity by δ-opioids in NG108-15 cells (McFadzean et al., 1989). This antiserum, as well as the antibodies that specifically recognize $G_{i\alpha3}$, also attenuated the inhibition in adenylate cyclase pro-

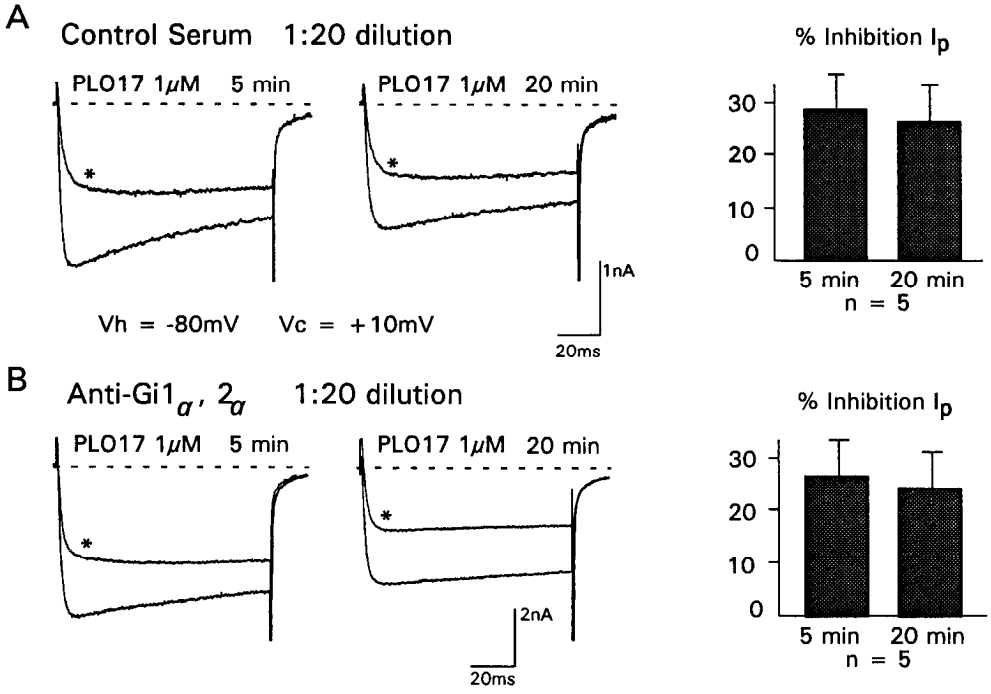

Figure 6. Cells dialyzed with anti-$G_{i1\alpha}$ $G_{i2\alpha}$ antiserum responded normally to PLO17. *A* and *B*, Calcium currents recorded in the absence and presence of PLO17 1 μM (indicated by *asterisks*) at 5 and 20 min after onset of whole-cell recording. In *A* the recording was obtained with a pipette containing nonimmune rabbit serum at a 1:20 dilution. *B*, Recordings obtained in another neuron with a pipette containing anti-G_{i1}/G_{i2} (AS/7) antiserum at the same dilution. Neither of the solutions, when infused for 20 min, attenuated the inhibition of calcium current by PLO17. This same outcome is illustrated in the *bar graphs at right*, which compare the mean PLO17-induced inhibition in peak current obtained 5 min and 20 min after infusion of nonimmune serum (*top*) versus anti-G_{i1}/G_{i2} antiserum (*bottom*) for the number of neurons indicated. Other details are as in Figure 5.

duced by α_2-adrenergic receptor agonists in transfected CHO-K1 cells (Gerhardt and Neubig, 1991). Therefore, it seems unlikely that our inability to demonstrate any effect of either anti-$G_{i\alpha}$ antisera or antibodies on the response to PLO17 was a consequence of the antibodies having little affinity for native G_i proteins. Accordingly, we feel confident in concluding that G_o-type, but not G_i-type, G-proteins play an important role in coupling μ-opioid receptors to calcium channels in rat DRG sensory neurons.

The experiments with AK-C confirm earlier findings from this laboratory (Gross et al., 1990a,b) that cAMP-dependent phosphorylation is an important regulatory pathway of calcium channel activity in rat peripheral neurons. In its presence the rundown in current normally associated with whole-cell recording (see Fedulova et al., 1985; Gross et al., 1990a) was reversed, and within 5 min after patch rupture both peak and residual current amplitudes steadily increased throughout the course of a typical 20–30 min recording. The finding that responses to PLO17 were undiminished by intracellular dialysis with AK-C indicates that the acute reduction of calcium current by the μ-opioid agonist occurred independent of an inhibition in the adenylate cyclase/cAMP system. Our inability to attenuate the effects of PLO17 with specific antibodies to G_i proteins, the subclass that inhibits adenylate cyclase (Kurose et al., 1983; Birnbaumer et al., 1989), provides additional support for this conclusion. Nevertheless, other data suggest that cAMP-dependent phosphorylation of calcium channels (Flockerzi et al., 1986; Nunoki et al., 1989) may be an important mechanism to maintain them in an activatable state (Armstrong and Eckert, 1987) that is sensitive to modulation by inhibitory neurotransmitters. For example, we have shown in nodose ganglion neurons that the inhibition in calcium current by the κ-opioid agonist dynorphin A was enhanced in the presence of AK-C (Gross et al., 1990a). In addition, a larger component of PLO17-sensitive current was apparent in DRG neurons after the effects of AK-C had become established. However, in the present study the effect of AK-C was dominant over that of the opioid in that the fractional component of total current inhibited by PLO17 was reduced, relative to control responses determined within the first few minutes of recording. One interpretation of these data is that AK-C may be a potent regulator of multiple types of calcium current in DRG neurons, including that contributed by N- and L-type channels, whereas PLO17 exhibits a different selectivity regarding the particular components of high-threshold current that are affected. Although single-channel recordings will be required to confirm this hypothesis, the results presented here when considered with other findings from our laboratory (see Moises and Macdonald, 1992; Moises et al., 1994) are entirely consistent with the conclusion that in these neurons PLO17 and AK-C modulate ω-conotoxin GVIA–sensitive N-type channels in an opposing manner. The convergence of both regulatory pathways on N-type channels, if occurring within the terminal endings of the neuron, would provide a dual pathway by which μ-opioid receptors could exert their inhibitory control over transmitter release (Schoffelmeer and Mulder, 1983; Schoffelmeer et al., 1987; Hirning et al., 1988).

Our data offer only limited insight regarding the nature of the G-protein–coupled signaling pathway by which μ-opioid receptors affect calcium channel activity. A reasonable speculation derived from the experiments with AK-C is that the inhibitory effects of PLO17 might be mediated through a membrane-delimited pathway, possibly involving a direct action of G-proteins on calcium channels (Yatani et al., 1987; Kasai, 1991; Shen and Surprenant, 1991). However, the present data do not rule out the possibility that other second messenger signaling pathways might be involved. In fact, Hille and coworkers have recently shown that in rat sympathetic neurons multiple pathways, utilizing both membrane-delimited and cytoplasmically diffusible transduction mechanisms, mediate regulation of N-type calcium channels and currents by inhibitory transmitters (Bernheim et al., 1991; Beech et al., 1992; Mathie et al., 1992). Interestingly, in that system the signal in the slow cytoplasmic

pathway did not appear to be carried by any of the four known diffusible messengers (i.e., Ca^{2+}, cAMP, cGMP, or PKC) (Bernheim et al., 1991) and it was insensitive to blockade by PTX (Beech et al., 1992). It will be important in future experiments to determine whether manipulations of these additional signaling pathways (other than adenylate cyclase) modify the inhibition of current by PLO17. In any event, a more conventional G-protein–dependent pathway appears to play the dominant role in coupling μ-opioid receptors to calcium channels in rat sensory neurons, since responses to PLO17 were absent or markedly attenuated in DRG neurons treated with PTX.

Receptors for a variety of neuropeptides and transmitter substances are known to regulate calcium channel currents in DRG sensory neurons by means of PTX-sensitive G-proteins (Holz et al., 1986; Dolphin and Scott, 1987; Ewald et al., 1988; present results). Among those present in rat DRG neurons are distinct receptors for μ- and κ-opioids (Schroeder et al., 1991; Moises et al., 1994), as well as receptors for GABA (acting at $GABA_B$, Dolphin and Scott, 1987), neuropeptide Y, bradykinin, and calcitonin gene–related peptide (Ewald et al., 1988, 1989; Wiley et al., 1992). Given this degree of complexity, it is presumed that each G-protein must interact with a particular receptor and select its appropriate effector to maintain specificity within the signaling network (see, e.g., Hille, 1992; Taussig et al., 1992). Recent evidence suggests that specificity in the linkage of a surface receptor to its effector might be achieved via the intermediation of a unique G-protein, distinguished by a specific heterotrimeric composition of α, β, and γ subunits. Molecular cloning techniques have now identified two splice variants of G_o (G_{oA}, G_{oB}) (Hus et al., 1990; Stratham et al., 1990), and there are known to be four different β polypeptide sequences, in addition to as many as seven distinct isotypes of γ subunits (Simon et al., 1991; Kleuss et al., 1993). In experiments using antisense oligonucleotides to block selectively the expression of specific G-protein subunits, Kleuss et al. (1991) have shown that G_{oA} and G_{oB}, respectively, transduced muscarinic and somatostatin-induced inhibitory effects on calcium currents in GH3 rat pituitary clonal cells. In addition, by constructing an NG108-15 cell line that expressed a mutant α subunit of G_{oA} resistant to PTX, Taussig et al. (1992) demonstrated that G_{oA} transduced the inhibition of ω-conotoxin GVIA–sensitive calcium current mediated by δ-opioid and α_2-adrenergic receptors, but not that by somatostatin. Because the anti-$G_{o\alpha}$ (GC/2) antiserum used here was raised against a peptide corresponding to an amino-terminal sequence of $G_{o\alpha}$, it should recognize both splice variants of G_o, which differ in amino acid sequence only at the carboxyl-terminal half of the protein (Hus et al., 1990; Strathmann et al., 1990). Therefore, we were unable to determine whether or not a distinct isotype of G_o was specifically involved in coupling of μ-opioid receptors to calcium channels in rat sensory neurons. Nonetheless, the present findings are entirely consistent with data obtained in neuronal-like NG108-15 and SH-SY5Y neuroblastoma–glioma cells in showing that μ- and δ-opioid receptors inhibit the activity of N-type calcium channels via a G_o-type G-protein.

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