Opioid Receptors Modulate Diverse Types of Calcium Channels in the Nucleus Tractus Solitarius of the Rat

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We have investigated the coupling between opioid receptors and different types of Ca^{2+} channels in neurons acutely isolated from the nucleus tractus solitarius (NTS) of the rat. Using fura-2-based imaging we found that Ca^{2+} transients evoked by depolarization with 50 mM KCl were suppressed by the μ -opioid receptor agonist D-Ala², N-MePhe⁴, Gly⁵-ol-enkephalin (DAMGO) and less effectively by the κ -receptor agonist U-69,593. The δ -receptor agonist D-Pen², D-Pen⁵-enkephalin (DPDPE) was ineffective. In whole-cell voltage-clamp recordings from these neurons, depolarizing voltage steps elicited high-threshold Ca^{2+} currents that could be distinguished pharmacologically into different components. Part of the current could be blocked by dihydropyridines, part by ω -conotoxin-GVIA and part by ω -agatoxin-IVA. This suggests that the neurons contained L-, N-, and P/Q-type Ca^{2+} channels. DAMGO and U-69,593 both blocked part of the Ca^{2+} current but DPDPE was ineffective. Perfusion of GTP- γ -S into the cells produced a rapid rundown of the Ca^{2+} current and occluded further effects of the opioid agonists, suggesting the involvement of a G-protein in the coupling mechanism. Inhibition of L-channels did not alter the effect of DAMGO. On the other hand inhibition of N-channels occluded about 80% of the effect of DAMGO. Inhibition of the P/Q-current occluded the remainder of the DAMGO effect. Thus, it appears that activation of opioid receptors can inhibit N- and P/Q-type Ca^{2+} channels but not L-channels in these cells. It is likely that such effects are important in opioid-mediated inhibition of transmitter release in the brain.

[Key words: enkephalin, autonomic nervous system, μ -opioid receptor, ω -conotoxin-GVIA, dihydropyridine, ω -agatoxin-IVA]

Endogenous opioids and opioid drugs produce many effects on the nervous system by interacting with a widely distributed receptor system. Receptors for μ -, δ -, and κ -selective opioids show a unique distribution in the brain (Reisine and Bell, 1993). Activation of these receptors produces a number of effects on neurons at both pre- and postsynaptic sites (North, 1993a,b).

For example, opioids have often been observed to hyperpolarize neurons due to the activation of a K^{+} conductance (North, 1993a). A second widely reported effect of opioids is their ability to inhibit the release of neurotransmitters in both the central and peripheral nervous systems (North, 1993b). Indeed, this latter effect formed the basis of most of the classical bioassays for opioid agonist activity. How are opioids able to do this? Three major possibilities have been suggested. Stimulation of presynaptic opioid receptors may activate a K^{+} conductance, thereby hindering the movement of action potentials into the presynaptic terminal (North, 1993a). Second, opioids may block Ca^{2+} influx directly by inhibiting the Ca^{2+} channels that mediate Ca^{2+} influx into the nerve terminal (Kennedy and Henderson 1991; Seward et al., 1991; North, 1993a,b). Finally, activation of opioid receptors may produce direct effects on the intracellular machinery that regulates transmitter vesicle exocytosis (Capogna et al., 1993; Reikling, 1993).

It has been demonstrated that several different types of Ca^{2+} channels exist in neurons (Snutch and Reiner, 1992; Miller, 1993; Zhang et al., 1993). From the functional point of view Ca^{2+} channels have been classed into "high" and "low" threshold on the basis of the voltage range at which they are activated. Low-threshold Ca^{2+} channels have also become known as T-type and exhibit a unique pharmacological profile (Herrington and Lingle, 1992). On the other hand, high-threshold Ca^{2+} currents can be grouped into various classes according to their sensitivities to dihydropyridines (L-type), ω -conotoxin-GVIA (ω -CgTx-GVIA, N-type), ω -agatoxin-IVA (ω -Aga-IVA, P- or Q-type) or none of these, for example, the R-type currents of cerebellar granule cells. We also know that these activities result from the expression of a family of related Ca^{2+} channel molecules that exist as multisubunit complexes. Neurotransmitter secretion seems to be preferentially coupled to one or more of these channel types under different circumstances (Luebke et al., 1993; Takahashi and Momiyama, 1993; Toth et al., 1993).

Which of these Ca^{2+} channels are regulated by opioid receptors? In the PNS there is evidence that all three types of opioid receptors can influence ω -CgTx-GVIA-sensitive N-type channels and it is this type of Ca^{2+} channel that primarily regulates transmitter release from many peripheral neurons (Lipscombe et al., 1989; Toth et al., 1993). However, nothing is known about the regulation of Ca^{2+} channels by opioid receptors in the CNS where the relationship between Ca^{2+} channel type and transmitter release appears to be more complex (Luebke et al., 1993; Takahashi and Momiyama, 1993). We have therefore examined the situation in the nucleus of the solitary tract (NTS), an area of the brain in which opioids are thought to produce many important effects on the activity of the autonomic nervous system (Van Giersbergen et al., 1992). Our results show that opioid

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receptors can influence the activity of both N- and P/Q-type Ca^{2+} channels, the major types of Ca^{2+} channels thought to be involved in the regulation of transmitter release in the brain.

Materials and Methods

Cell preparation. NTS neurons were isolated using a technique modified from Nakagawa et al. (Nakagawa et al., 1990). Transverse slices (400 μm thick) were prepared from the brainstem of Sprague-Dawley rats (7–21 d old) and placed in a holding chamber filled with 32°C artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 26.2 mM NaHCO_3 , 1 mM NaH_2PO_4 , 3 mM KCl, 1.5 mM MgSO_4 , 1.5 mM CaCl_2 , and 10 mM glucose gassed with 95% O_2 , 5% CO_2 . Slices were transferred to a conical tube containing gently bubbled aCSF at 32°C to which 1.8 U/ml dispase (grade I; 0.75 ml/slice) was added. After 60 min incubation, slices were rinsed with enzyme-free aCSF and transferred for storage to a holding chamber with continuously bubbled aCSF at room temperature. When needed, a slice was removed from the chamber and placed on Sylgard-coated petri dish. Under a dissecting microscope, the NTS region was micropunctured and placed on poly-L-lysine-coated coverslip. The cells were then dissociated by trituration in saline solution containing 146 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, and 10 mM glucose (pH 7.4) using progressively smaller diameter pipettes and allowed to settle on a coverslip for 10 min.

Intracellular Ca^{2+} imaging. The acetoxymethyl-ester form of fura-2 (fura-2/AM; Molecular Probes, Eugene, OR) was used as the fluorescent Ca^{2+} indicator. The cells were loaded with fura-2/AM (5 μM) for 20 min at room temperature 10 min after cells were placed on the coverslips. Cells were rinsed and allowed 20 min to de-esterify the dye. Intracellular free calcium concentration, $[\text{Ca}^{2+}]_i$, in the neurons was calculated by digital video microfluorimetry using an intensified CCD camera (Hamamatsu) coupled to a microscope (40 $\times$ Fluor objective; Nikon Diaphot) and software (Universal Imaging) on a 486 computer. Cells were illuminated using a 150 W xenon arc lamp and excitation wavelengths (340 and 380 nm) were selected by a filter changer (Metaltek). Saline with 50 mM KCl (50K $^{+}$) was made with replacing an equivalent amount of NaCl and applied by a fast U-tube perfusion system controlled by timer (5 sec duration). Data was expressed as mean $\pm$ SEM for the number of cells responding to an opioid agonist (>10%). Cells not responding were not included in this value.

Electrophysiological recordings. Whole-cell voltage-clamp recordings were used (Hamill et al., 1981). Patch electrodes with resistance of 2.5–5 M Ω were filled with the internal solution containing 140 mM CsCl, 1 mM MgCl_2 , 10 mM BAPTA, 3.6 mM Mg-ATP, 14 mM Tris creatine phosphate, 0.1 mM GTP-Tris, and 50 U/ml creatine phosphokinase, pH adjusted to 7.4 with CsOH. The external solution contained 10 mM BaCl $_2$, 150 mM tetraethylammonium (TEA) chloride, 1 mM MgCl_2 , 10 mM HEPES, and 10 mM glucose, pH 7.4 with TEA-OH. Current recordings were obtained using an Axopatch-1D amplifier (Axon Instruments) and filtered at 2 kHz. Ca^{2+} currents were evoked every 15 sec by a 200 msec voltage step from -80 mV to 0 mV. Leak currents were subtracted from the data sweeps by scaling the leak sweep to the data. All data were expressed as the mean $\pm$ SEM.

Materials. Dispase (grade I) was purchased from Boehringer Mannheim Co. (Indianapolis, IN). D-Ala 2 , N-MePhe 4 , Gly 5 -ol-enkephalin (DAMGO), D-Pen 3 , D-Pen 5 -enkephalin (DPDPE), and dynorphin A were from Bachem (Torrance, CA). (+)U-69,593 and nor-binaltorphimine (nor-BNI) were from Research Biochemicals International (Natick, MA). D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH $_2$ (CTOP) and ω -conotoxin-GVIA were from Peninsula Laboratories Inc. (Belmont, CA). ω -Agatoxin-IVA was from Peptides International (Japan). Nimodipine was from Miles laboratories (West Haven, CT). Naloxone, Mg-ATP, di(Tris)-phosphocreatine, creatine phosphokinase, GTP-Tris, and GTP- γ -S were from Sigma (St. Louis, MO). Stock solutions of agonists and antagonists were prepared in purified water. Exception to this was U-69,593, which was dissolved in absolute ethanol. All drugs were applied in the perfusate. Stock solutions of toxins were dissolved in purified water and kept at -20°C until used. Final concentrations were made in recording solution containing 0.1 mg/ml ovalbumin (Sigma grade V) as a carrier protein. Toxins were applied by adding 1 or 2 ml of solution at its final concentration directly to the sample chamber (0.5 ml total volume) and stopping flow for 1.5–2.0 min. Data were still collected during this period. Flow was then resumed. Nimodipine was dissolved in DMSO, diluted in recording solution, and applied by means of the perfusion system.

Results

Acute isolation of NTS neurons

NTS neurons were acutely isolated from brainstem slices cut at the level of obex and for several hundred micrometers caudally (see Fig. 1A). The NTS is a heterogeneous cell group that contains a variety of neuronal types with respect to their anatomic (cell size, shape, and packing density), electrophysiological and neurochemical characteristics. It was recently suggested that 10 subdivisions of the NTS could be distinguished in rats (Van Giersbergen et al., 1992). The area of the NTS used in these experiments included mainly the medial portion plus some of the gelatinosa, dorsal, commissural, and intermediate parts. These areas are believed to be involved in the regulation of the cardiovascular and gastrointestinal systems. The majority of the cell somata obtained were round or oval shaped and measured 12–18 μm in diameter (Fig. 1B), thus corresponding in size and shape reported in previous studies (Herbert et al., 1990). Some dissociated cells maintained a few of their original morphological features such as dendritic processes. However, cells without processes were chosen for electrophysiological recordings in order to maintain good space clamp control.

Modulation of evoked calcium signals by opioids

Increases in $[\text{Ca}^{2+}]_i$ evoked by depolarization were first measured using fura-2-based microfluorimetry. Depolarization using a 5 sec exposure to 50K $^{+}$ increased $[\text{Ca}^{2+}]_i$ from a resting level of 41.3 ± 2.1 nM to a peak level of 824.7 ± 54.5 nM ($n = 79$). Figure 2A shows that the μ -selective receptor agonist DAMGO (1 μM) suppressed the 50K $^{+}$ -induced increase in $[\text{Ca}^{2+}]_i$ by $33.5 \pm 3.8\%$ ($n = 29$ cells responding to DAMGO of 79 tested). The κ -selective agonist U-69,593 (1 μM) also produced inhibition but it was less effective ($15.7 \pm 3.4\%$, $n = 3$ cells responding to U-69,593 of 15 tested). However, the selective δ -receptor agonist, DPDPE (1 μM), had no significant effect in 15 tested cells. The DAMGO-mediated inhibition was completely prevented by simultaneous treatment with the nonselective opioid receptor antagonist naloxone (10 μM , $n = 4$) and recovery from this block was observed after about a 5 min washout. The inhibition produced by DAMGO was dose related between 1 nM and 10 μM (Fig. 2B).

Inhibition of high-threshold Ca^{2+} currents by μ -opioids

These results suggested that both μ - and κ -agonists could block Ca^{2+} currents in these neurons. We therefore went on to test this proposal using electrophysiological methods. Whole-cell currents were recorded using Ba $^{2+}$ as the charge carrier, and Cs $^{+}$ and TEA as the predominant internal and external cations, respectively. Figure 3A illustrates the effect of μ -selective opioid agonist DAMGO on the Ba $^{2+}$ current through high-threshold Ca^{2+} channels in one acutely isolated NTS neuron. Application of 1 μM DAMGO caused a 51% inhibition of the peak inward Ba $^{2+}$ current ($I_{\text{Ba}^{2+}}$) that returned to control levels after washout of the agonist (Fig. 3A). In 49 cells of 92 tested, 1 μM DAMGO reduced $I_{\text{Ba}^{2+}}$ by $41.8 \pm 2.2\%$. In a separate set of experiments we observed that $I_{\text{Ba}^{2+}}$ decreased by $49.0 \pm 3.1\%$ upon a second exposure to DAMGO within 2 min of the first exposure ($46.7 \pm 3.5\%$ inhibition produced by the first exposure, $n = 10$). Thus, the inhibition of $I_{\text{Ba}^{2+}}$ did not desensitize. Curiously, in some cells, DAMGO (1 μM) actually produced an increase in the size of $I_{\text{Ba}^{2+}}$ ($17.5 \pm 2.5\%$, $n = 14$ of 92). However, we did not characterize this response further.

Figure 1. Location and morphology of acutely isolated nucleus tractus solitarius (NTS) neurons. *A*, NTS neurons were isolated from the shaded areas indicated. *AP*, area postrema; *TS*, solitary tract; *c*, central subnucleus; *10*, dorsal motor vagal nucleus; *12*, hypoglossal nucleus. *B*, Examples of neurons freshly dissociated from an 8-d-old rat. Phase-contrast optics. Scale bars: *A*, 1 mm; *B*, 10 μ m.

Figure 3*B* illustrates $I_{Ba^{2+}}$ plotted during multiple exposures to DAMGO in the presence of nonselective opioid antagonist naloxone. The DAMGO-mediated inhibition of $I_{Ba^{2+}}$ was attenuated by treatment with 1 μ M naloxone ($42.7 \pm 4.5\%$ inhibition observed with 1–3 μ M DAMGO vs $17.0 \pm 5.8\%$ in the presence of naloxone, $n = 5$). The response to the agonist recovered from the block following washout. Figure 3*C* shows the dose/response relationship for DAMGO inhibition of the current. Figure 3*D* illustrates the peak current–voltage (I – V) relation for a neuron in the absence and presence of 1 μ M DAMGO ($n = 5$).

Receptor identification

As also suggested by the fura-2 microfluorimetry experiments, the κ -receptor agonist U-69,593 also inhibited high-threshold Ca^{2+} channel currents in some acutely isolated NTS neurons. Figure 4*A* illustrates $I_{Ba^{2+}}$ inhibition produced by κ -receptor agonists as well as DAMGO. In this particular cell, the application of nonselective κ -agonist, dynorphin A, produced 42% and 56% inhibition of $I_{Ba^{2+}}$ at 30 nM and 100 nM, respectively. In the same cell the selective κ -agonist U-69,593 caused a 62% inhibition of $I_{Ba^{2+}}$ at 1 μ M. More typically, however, the pattern of inhibition produced by μ -, δ -, and κ -selective opioid agonists was as illustrated in Figure 4*B*, in that κ -agonists were less effective than μ -agonists; 1 μ M U-69,593 produced a mean re-

duction in $I_{Ba^{2+}}$ of $21.9 \pm 3.5\%$ in 18 cells from 31 tested. The δ -receptor agonist DPDPE (1 μ M) was not significantly effective in six cells tested.

Because κ -receptor effects were often observed in the same cells as μ -receptor effects, the μ -receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) and κ -receptor antagonist nor-binaltorphimine (*nor*-BNI) were used to determine whether the observed inhibition of $I_{Ba^{2+}}$ was truly the result of activating two separate populations of receptors. Figure 4*C* illustrates some typical effects of these selective antagonists. The mean inhibition of $I_{Ba^{2+}}$ in four neurons exposed to DAMGO was $39.9 \pm 9.8\%$ and this decreased to $11.2 \pm 4.1\%$ following exposure to DAMGO in the presence of 300 nM CTOP. The DAMGO-mediated inhibition recovered fully from this block after around a 7 min washout (Fig. 4*Ci*). However, in the same series of experiments the κ -receptor antagonist *nor*-BNI did not block the DAMGO-mediated inhibition of $I_{Ba^{2+}}$ significantly (Fig. 4*Cii*). The mean inhibition of $I_{Ba^{2+}}$ from four neurons was $45.7 \pm 7.8\%$ following exposure to DAMGO alone, and this decreased to $41.3 \pm 4.9\%$ following exposure to DAMGO in combination with 300 nM *nor*-BNI. On the other hand, exposure to U-69,593 alone caused a $23.3 \pm 1.8\%$ inhibition of $I_{Ba^{2+}}$ and this was inhibited by *nor*-BNI. U-69,593 produced $2.0 \pm 0.7\%$ inhibition following *nor*-BNI ($n = 3$) (Fig. 4*Ciii*).

Figure 2. Inhibition of depolarization-induced $[Ca^{2+}]_i$ signals by opioids. *A*, This cell was exposed to $50K^+$ (K) for 5 sec using a fast application system. The increase in $[Ca^{2+}]_i$ was measured using fura-2-based microfluorimetry. DAMGO suppressed the $50K^+$ -induced increase in $[Ca^{2+}]_i$ by 40%. This effect was completely reversed by concurrent treatment with $10 \mu M$ naloxone and recovered again after washout of the antagonist. U-69,593 and DPDPE had no significant effect in this cell. *B*, Dose-response curve for DAMGO inhibition of $50K^+$ -induced increase in $[Ca^{2+}]_i$. Numbers by each point indicate the number of cells treated at each concentration.

Is a G-protein involved in the coupling between receptor and Ca^{2+} channel?

In most cases opioid actions on adenylate cyclase or on ion channels have been reported to be mediated by pertussis toxin-sensitive G-proteins (Taussig et al., 1992). We examined the possible involvement of G-proteins by comparing cells dialyzed with GTP and its nonhydrolyzable analog GTP- γ -S. When cells were dialyzed with $100 \mu M$ GTP, $I_{Ba^{2+}}$ was generally quite stable over time although in some cells a degree of "rundown" was apparent. Furthermore, the inhibition of $I_{Ba^{2+}}$ by $1 \mu M$ DAMGO was readily reversible (Fig. 5*A*). In contrast, if cells were dialyzed with $100 \mu M$ GTP- γ -S, $I_{Ba^{2+}}$ was observed to run down rapidly until it stabilized at a new level. At this point any further inhibition by DAMGO was occluded (Fig. 5*B*; $n = 6$). This result suggests that a G-protein is involved in the coupling between opioid receptor and Ca^{2+} channel.

Types of Ca^{2+} channels inhibited by activation of μ -opioid receptor

We now attempted to find out which types of Ca^{2+} channels were being inhibited in the NTS neurons. Low-threshold transient (T-type) Ca^{2+} currents were not observed in these neurons. Multiple types of high-threshold Ca^{2+} currents were distinguished from each other using pharmacological tools. We found that rat NTS neurons had a Ba^{2+} current component that was inhibited by dihydropyridines such as nimodipine. The extent of block by nimodipine varied from cell to cell (10–42% at $10 \mu M$). The mean reduction of $I_{Ba^{2+}}$ by nimodipine was $24.7 \pm 5.5\%$ in seven neurons. The N-type channel blocker ω -CgTx-GVIA blocked a relatively large component of the current ($66.1 \pm 2.5\%$ inhibition at $1 \mu M$, $n = 7$). In addition, a portion of the current was blocked by ω -Aga-IVA ($31.8 \pm 3.5\%$ at 100 nM , $n = 8$), which suggests that P/Q-type Ca^{2+} channels also existed in the NTS neurons.

To examine which of these different Ca^{2+} channels were inhibited following activation of opioid receptors in the NTS neurons, we tested the actions of DAMGO in combination with these Ca^{2+} channel blockers. The suppression of $I_{Ba^{2+}}$ by DAMGO was measured before and after application of nimodipine; $1 \mu M$ DAMGO suppressed $46.7 \pm 2.0\%$ of $I_{Ba^{2+}}$ before and $46.1 \pm 6.8\%$ after nimodipine block (seven tested cells), suggesting almost none of the current suppressed by DAMGO could be blocked by nimodipine (i.e., was an L-current).

On the other hand, inhibition of $I_{Ba^{2+}}$ by ω -CgTx-GVIA occluded the major portion of the effect of DAMGO. In six neurons, $1 \mu M$ DAMGO suppressed $36.7 \pm 8.5\%$ of $I_{Ba^{2+}}$ before and $10.9 \pm 3.9\%$ after ω -CgTx-GVIA block. These results suggest that most of $I_{Ba^{2+}}$ blocked by DAMGO had the characteristics of an N-current. Schroeder et al. (1991) reported that both ω -CgTx-GVIA-sensitive and ω -CgTx-GVIA-insensitive, high-threshold currents were inhibited by opioids in sensory neurons. We tested whether the actions of DAMGO could also be blocked by P/Q-channel blocker, ω -Aga-IVA. Figure 6*A* illustrates the effects of DAMGO on a neuron sequentially exposed to all three blockers. The suppression of $I_{Ba^{2+}}$ by $1 \mu M$ DAMGO was 45.8% prior to ω -Aga-IVA (100 nM) application, 27.4% after ω -Aga-IVA block, and was virtually absent following ω -CgTx-GVIA treatment, although the cell still responded to nimodipine at this point. Figure 6*B* shows pooled results from a series of similar experiments; $1 \mu M$ DAMGO suppressed $47.3 \pm 2.7\%$ of $I_{Ba^{2+}}$ before and $29.6 \pm 4.7\%$ after ω -Aga-IVA block in six neurons. The DAMGO-mediated $I_{Ba^{2+}}$ inhibition was blocked by cotreatment with ω -CgTx-GVIA and ω -Aga-IVA ($4.0 \pm 1.5\%$ inhibition remaining in seven cells). These results suggest that activation of μ -opioid receptors can inhibit N- and P/Q-type Ca^{2+} channels but not L-channels in the NTS neurons.

Discussion

Voltage-dependent Ca^{2+} channels are the pathway by which Ca^{2+} normally enters the nerve terminal to trigger the release

Figure 3. The inhibitory effects of DAMGO on high-threshold Ca^{2+} channel current in the NTS neurons. **A**, Time course of peak Ba^{2+} current ($I_{Ba^{2+}}$) showing two exposures to DAMGO. Inhibition of $I_{Ba^{2+}}$ was 51% following the first and 44% following the second DAMGO application. *Inset*, Leak-subtracted currents of labeled points from time course of $I_{Ba^{2+}}$. Currents were evoked every 15 sec by a 200 msec voltage step from -80 mV to 0 mV. **B**, Time course of $I_{Ba^{2+}}$ during multiple exposures to DAMGO in the presence of naloxone. In this cell $1 \mu M$ DAMGO inhibited 25% of $I_{Ba^{2+}}$ prior to naloxone application. The effect of DAMGO was blocked by $1 \mu M$ naloxone and recovered from the antagonist block after washout. **C**, Concentration-response relationship for inhibition of $I_{Ba^{2+}}$ by DAMGO. Numbers by each point indicate the number of cells treated at each concentration. **D**, Current-voltage relationship of normalized $I_{Ba^{2+}}$ prior to (○), in the presence (●), and after recovery (△) from DAMGO ($1 \mu M$). Holding potential was -80 mV. Data points represent averages of six experiments for control and drug treatment and five for recovery.

of neurotransmitters. Clearly, regulation of this influx pathway represents a potential mechanism for the modulation of neurotransmission. Indeed there is considerable evidence that this strategy is frequently utilized and that presynaptic receptors can directly couple to Ca^{2+} channels by G-protein-linked mechanisms (Lipscombe et al., 1989; Miller, 1990; Toth et al., 1993). In some instances modulation of presynaptic Ca^{2+} channels may be the sole mechanism employed for the production of presynaptic inhibition. For example, we have recently demonstrated that at peripheral neuroeffector junctions presynaptic inhibition produced by neuropeptide Y is due to the inhibition of Ca^{2+} channels and is not associated with modulation of K^{+} conductances (Toth et al., 1993). Nevertheless it is possible that additional mechanisms may also sometimes be employed (Scanziani et al., 1992; Scholz and Miller, 1992; North, 1993a). The observations presented in this report indicate that opioid receptor-mediated inhibition of Ca^{2+} channels may be a mechanism by which opioids produce presynaptic inhibition in the central as well as the PNS.

Over the last few years it has become clear that central neurons

contain a large number of different voltage-dependent Ca^{2+} channels. At least six genes coding for separate α -subunits, which constitute the pore-forming region of the channel, have been identified (Snutch and Reiner, 1992; Miller, 1993; Zhang et al., 1993). As several ancillary subunits (e.g., α_2/δ , β) that are important for channel function also exist, the total number of possible Ca^{2+} channel complexes is very large indeed (Brust et al., 1993). It has also been shown that these channels differ with respect to their particular neuronal functions; the regulation of transmitter release being a prime example of this. In many peripheral neurons Ca^{2+} channels with the characteristics of N-channels (probably derived from clone α_{1B}) seem to play a major role in the control of transmitter release (Toth et al., 1993; Yawo and Chuhma, 1993). In addition, in the CNS, channels of the P/Q-type (probably derived from α_{1A}) also seem to play an important role in the control of transmitter release (Luebke et al., 1993; Randall et al., 1993; Sather et al., 1993; Takahashi and Momiyama, 1993). If modulation of Ca^{2+} channels plays an important role in the presynaptic inhibitory effects of opioids then it is important to show that these drugs are capable of

Figure 4. Receptor identification. *A*, Time course of $I_{Ba^{2+}}$ illustrating responses to different opioid receptor agonists. In this cell, 1 μ M DAMGO and 1 μ M U-69,593 (*U-69*) inhibited $I_{Ba^{2+}}$ by 54% and 62%, respectively; 30 nM and 100 nM dynorphin A (*Dyn*) produced 42% and 56% inhibition of $I_{Ba^{2+}}$, respectively. *B*, Responses to μ -, δ -, and κ -selective opioid receptor agonists in another cell. The inhibition of $I_{Ba^{2+}}$ observed was 9% and 30% for U-69 and DAMGO, respectively. DPDPE produced no significant effect. *C*, Selective block of the action of different opioid agonists by corresponding antagonists in different cells. Leak-subtracted $I_{Ba^{2+}}$ elicited by steps to 0 mV from -80 mV. In *i* 1 μ M DAMGO suppressed 31% of $I_{Ba^{2+}}$ before and 10% after addition of 300 nM CTOP. Full recovery observed together with some current rundown. In *ii* DAMGO produced the same degree of inhibition alone (43%) and after addition of 300 nM *nor-BNI* (39%). In *iii* the inhibition of $I_{Ba^{2+}}$ was 27% produced by U-69,593 (*U-69*) alone and this was completely blocked in the presence of *nor-BNI* (300 nM).

Figure 5. A G-protein couples μ -opioid receptors to Ca^{2+} channels. *A*, Time course of $I_{Ba^{2+}}$ illustrating the effect of DAMGO in a cell dialyzed with 100 μ M GTP. *Inset*, $I_{Ba^{2+}}$ at points indicated. *B*, A different cell dialyzed with 100 μ M GTP- γ -S. $I_{Ba^{2+}}$ was measured right after achieving the whole-cell configuration.

modulating the activity of the appropriate Ca^{2+} channels. In peripheral neurons it has been shown that, depending on the preparation used, μ -, κ -, and δ -receptors can all inhibit N-type (and sometimes T-type) Ca^{2+} currents (Gross and MacDonald, 1987; Bean, 1989; Schroeder et al., 1991; Shen and Surprenant, 1991; Taussig et al., 1992). However, no previous data has been available on the CNS. The present results show that the predominant opioid receptor linked to Ca^{2+} channels in the NTS is the μ -receptor. This is in keeping with anatomical data and our previous studies demonstrating the effects of opioid agonists in NTS slices (Rhim et al., 1993; Thompson et al., 1993). As we have now shown activation of μ -receptors in the NTS produces inhibition of both ω -CgTx-GVIA- and ω -Aga-IVA-sensitive components of the Ca^{2+} current. The former component is clearly an N-type current. The identity of the latter is more problematic as it has been recently pointed out that ω -Aga-IVA may be able to block more than one component of the Ca^{2+} current in the brain (Sather et al., 1993; Zhang et al., 1993). At the concentration used in the present studies it seems likely that we are targeting the "P" rather than the "Q" component discussed by Sather and colleagues (1993; see also Mintz and Bean, 1993a). However, as this is not completely clear, we refer to this component as the P/Q-current. It should also be pointed out that it is also not yet clear what the relative roles of P- and Q-currents might be in the control of transmitter release and

Figure 6. Activation of μ -opioid receptor produces inhibition of both ω -Aga-IVA- and ω -CgTx-GVIA-sensitive Ca^{2+} channels. *A*, Time course of $I_{Ba^{2+}}$ illustrating DAMGO effects before and after application of different Ca^{2+} channel blockers. The suppression of $I_{Ba^{2+}}$ by 1 μ M DAMGO was 46% prior to ω -Aga-IVA (100 nM) application, 27% after ω -Aga-IVA block, and subsequently almost disappeared following ω -CgTx-GVIA (1 μ M) treatment; 100 nM ω -Aga-IVA blocked 39% of $I_{Ba^{2+}}$, and 1 μ M ω -CgTx-GVIA blocked 67% of the ω -Aga-IVA-insensitive $I_{Ba^{2+}}$. In addition, 10 μ M nimodipine completely blocked the ω -Aga-IVA- and ω -CgTx-GVIA-insensitive currents. *B*, Pooled results from Ca^{2+} channel blocker experiments illustrating the mean inhibition of $I_{Ba^{2+}}$ by 1 μ M DAMGO following treatment with different Ca^{2+} channel blockers. Numbers by each bar indicate the number of cells treated with each drug.

both types have been implicated (Luebke et al., 1993; Randall et al., 1993; Takahashi and Momiyama, 1993). Thus, it may ultimately prove to be the case that both types of currents are modulated by opioids.

The modulation of N-type currents by neurotransmitters in the central as well as the peripheral nervous systems has been quite widely described (Penington et al., 1991; Scholz and Miller, 1991a,b; Swartz and Bean, 1992; Haws et al., 1993; Mintz and Bean, 1993b; Mogul et al., 1993; Sahara and Westbrook, 1993; Swartz et al., 1993). However, the modulation of P/Q-currents has not yet been extensively demonstrated (Mintz and Bean, 1993b; Mogul et al., 1993; Randall et al., 1993). The present results together with those of Mintz and Bean 1993b indicate that modulation of these Ca^{2+} channels will have similar characteristics to the well described modulation of N-channels. Thus, it appears that in both cases the inhibitory pathway utilizes a G-protein link and may involve "direct" interaction of the G-protein and some part of the channel without the

participation of a diffusible second messenger. This results in a change in the kinetics and voltage dependence of the current. Considering the accumulating data suggesting the importance of P/Q-channels in the control of transmitter release in the brain it is likely that receptor modulation of these channels will ultimately be widely observed (Luebke et al., 1993; Randall et al., 1993; Takahashi and Momiyama, 1993).

It should also be pointed out that there are other Ca^{2+} channels in both peripheral and central neurons that are clearly distinct from the N-, P-, and L-types (e.g., the R-currents of cerebellar granule cells; Ellinor et al., 1993; Zhang et al., 1993). These currents can also sometimes be modulated by receptors (Elmslie et al., 1992; Mintz and Bean, 1993b). However, we do not yet know much about them from the molecular point of view. One possibility is that some of them are due to the activity of the recently described α_{1E} clone (Ellinor et al., 1993; Soong et al., 1993). Indeed this Ca^{2+} channel has a structure that is quite closely related to those of α_{1B} and α_{1A} , which are probably responsible for N- and P/Q-channel activity. This may indicate that it is also susceptible to the same type of G-protein coupled modulation by opioid receptors in some cases. It is also interesting to note that both in the present study and in previous studies of peripheral neurons, activation of opioid receptors never produced modulation of L-currents. It may well be that these currents, which are probably due to the expression of the clones α_{1C} and α_{1D} (Williams et al., 1992; Tomlinson et al., 1993) are not susceptible to direct G-protein-linked modulation. This would be interesting as these channels differ from α_{1A} , α_{1B} and α_{1E} structurally, particularly in the presumed intracellular loop that connects domains 2 and 3 (Snutch and Reiner, 1992). This loop thus may represent a potential site for channel/G-protein interaction.

In addition to modulation by μ -receptors we also noted that the Ca^{2+} currents in some cells were also inhibited by κ -receptor agonists. This effect was seen less consistently than the effects of μ -receptor activation. Interestingly, the κ -effects were often observed on the same cells as the μ -effects. Thus some of these neurons may be susceptible to the effects of more than one endogenous opioid. We never observed effects of δ -opioid agonists consistent with our previous observations in the NTS slice (Rhim et al., 1993). The relative paucity of κ - and δ -effects may have more to do with the fact that δ - and κ -receptors are less well represented in this nucleus (Xia and Haddad, 1991) rather than any inability of these receptors to influence Ca^{2+} channels. Thus in peripheral systems both δ - and κ -receptors have been shown to produce robust inhibition of N-currents (Gross and MacDonald, 1987; Bean, 1989; Schroeder et al., 1991; Shen and Surprenant, 1991; Taussig et al., 1992). Nevertheless, results showing coupling of δ -receptors to Ca^{2+} channels in the brain are still lacking.

What do our results tell us about the regulation of the NTS by opioids? Receptor modulation of Ca^{2+} influx into the cell soma may have a variety of effects including the regulation of a variety of Ca^{2+} -dependent ion channels. Effects on gene expression and metabolism are also possible. However, it is also likely that the effects we have demonstrated may also occur on the terminals of the same neurons. Some of these may be interneurons and others may be efferents projecting to various parts of the CNS including parts of the pons, the medulla, the spinal cord, and the hypothalamus (Van Giersbergen et al., 1992). If this is true then this would represent a mechanism by which opioids could regulate transmitter release from these cells.

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