μ-Opioid receptor activation inhibits N- and P-type Ca^{2+} channel currents in magnocellular neurones of the rat supraoptic nucleus

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1. The whole-cell voltage-clamp technique was used to examine opioid regulation of Ba^{2+} currents (I_{Ba}) through voltage-sensitive Ca^{2+} channels in isolated magnocellular supraoptic neurones (MNCs). The effects of local application of μ-, δ- or κ-opioid receptor selective agonists were examined on specific components of high voltage-activated (HVA) I_{Ba}, pharmacologically isolated by use of Ca^{2+} channel-subtype selective antagonists.

2. The μ-opioid receptor selective agonist, DAMGO, suppressed HVA I_{Ba} (in 64/71 neurones) in a naloxone-reversible and concentration-dependent manner ($EC_{50} = 170$ nM, $E_{max} = 19{\cdot}5\%$). The DAMGO-induced inhibition was rapid in onset, associated with kinetic slowing and voltage dependent, being reversed by strong depolarizing prepulses. Low-voltage activated (LVA) I_{Ba} was not modulated by DAMGO.

3. Administration of κ- (U69 593) or δ-selective (DPDPE) opioid receptor agonists did not affect I_{Ba}. However, immunostaining of permeabilized MNCs with an antibody specific for κ_1-opioid receptors revealed the presence of this opioid receptor subtype in a large number of isolated somata.

4. μ-Opioid-induced inhibition in I_{Ba} was largely abolished after blockade of N-type and P-type channel currents by ω-conotoxin GVIA (1 μM) and ω-agatoxin IVA (100 nM), respectively. Quantitation of antagonist effects on DAMGO-induced reductions in I_{Ba} revealed that N- and P-type channels contributed roughly equally to the μ-opioid sensitive portion of total I_{Ba}.

5. These results indicate that μ-opioid receptors are negatively coupled to N- and P-type Ca^{2+} channels in the somatodendritic regions of MNCs, possibly via a membrane-delimited G-protein-dependent pathway. They also support a scheme in which opioids may act in part to modulate cellular activity and regulate neurosecretory function by their direct action on the neuroendocrine neurones of the hypothalamic supraoptic neucleus.

Opiates and endogenous opioid peptides (here collectively termed opioids) are known to inhibit the secretion of the neurohormones Arg-vasopressin (AVP) and oxytocin (OT) from the posterior pituitary (see Falke, 1991 for review). Early studies have shown that opioids can modulate this release by acting at the level of the supraoptic nucleus (SON), inhibiting the activity of magnocellular SON neurones (Wakerley *et al.* 1983; Wuarin & Dudek, 1990; Pumford *et al.* 1993; Inenaga *et al.* 1994) either directly or via presynaptic inhibition of their afferent input. Opioids also attenuate release of these neurohormones at the level of the neural lobe through activation of presynaptic opioid receptors located on the nerve endings of the magnocellular SON neurones (Zhao *et al.* 1988; Rusin *et al.* 1997). In addition, recent results obtained from recordings of SON neurones *in vitro* and from microdialysis experiments *in vivo* have strengthened the case for somatodendritic release of AVP and OT from these magnocellular neurones and provided evidence for its inhibitory regulation by opioids (Douglas *et al.* 1995; Ingram *et al.* 1996). Hence, there is ample reason to postulate that opioids act to modulate activity-dependent release of AVP and OT by engaging multiple cellular mechanisms widely distributed within the hypothalamo-neurohypophysial axis.

There is general agreement that opioids can influence neuronal function via three distinct subtypes of opioid receptors, designated as μ-, δ- and κ-opioid receptors. Each of these opioid receptor subtypes shows a unique and preferential pattern of distribution within the central nervous system, with κ-opioid receptors being the

predominant species expressed throughout the rodent hypothalamus and posterior pituitary (Herkenham *et al.* 1986; Mansour *et al.* 1995). Autoradiographic (Sumner *et al.* 1990) and *in situ* hybridization studies (George *et al.* 1994; Minami *et al.* 1994) have further revealed only moderate levels of μ-opioid receptor binding and mRNA within the magnocellular hypothalamic nuclei, while failing to demonstrate the presence of δ-opioid receptors within the hypothalamus or posterior pituitary. Consistent with these anatomical data are the findings from studies *in vivo* that μ-and κ-opioid receptor agonists act at sites in the SON to inhibit the firing rate of identified SON neurones (Ludwig *et al.* 1997). Similarly, only κ-opioid receptor selective agonists and non-selective agonists such as etorphine have been reported to inhibit depolarization-evoked release of AVP and OT from isolated neural lobes and neurosecretosomes prepared from rat neurohypophysis (Dayanithi *et al.* 1992; Kato *et al.* 1992).

Magnocellular SON neurones have recently been shown to express several types of voltage-sensitive calcium channels (VSCCs) that can be distinguished based on their distinct biophysical and pharmacological profiles (Fisher & Bourque, 1995*a*; Foehring & Armstrong, 1996). These include a low voltage-activated (LVA) T-type Ca^{2+} channel and a number of high-voltage activated (HVA) Ca^{2+} channels, including L-, N-, P-, Q- and R-types, similar to those described in many other peripheral and central neurones (see Scott *et al.* 1991 for review). A novel LVA L-type channel has also been described in these neurones (Fisher & Bourque, 1995*a*). Interestingly, Lemos and colleagues (1989, 1994) have shown that the nerve terminals of magnocellular SON neurones express only HVA Ca^{2+} channels. Furthermore, while the latter include a conventional L-type channel, the rapidly inactivating N-type (N_t) (Lemos & Nowycky, 1989; Wang *et al.* 1992) and P-type (Fisher & Bourque, 1995*b*) channel currents expressed in these terminals have biophysical and pharmacological characteristics that differ from those of the corresponding N- and P-type somatic currents. It is tempting to speculate that such differences may reflect distinct Ca^{2+}-dependent functions required for cellular bursting activity and neurosecretion subserved by the somatic and the nerve terminal regions of magnocellular hypothalamo-neurohypophysial neurones, respectively. The differential expression of VSCC subtypes in somatic and terminal regions may also support different capabilities for modulation of these functions by opioid receptors.

It is currently unknown whether modulation of VSCCs represents an important mechanism whereby opioids exert regulatory effects on SON neural excitability and secretory function. Intracellular recordings from magnocellular SON neurones in slices of rat and guinea-pig hypothalamus have revealed both δ- and μ-opioid receptor-mediated reductions in action potential firing (Wakerley *et al.* 1983; Wuarin & Dudek, 1990), although these effects were not accompanied by changes in postsynaptic membrane properties. In contrast, Inenaga (1994) reported that superfusion of rat hypothalamic slices with κ-opioid receptor agonists not only decreased spontaneous firing rates, but also shortened the duration of Ca^{2+} action potentials recorded under conditions where potassium conductances were blocked. There are also initial reports that κ-opioid receptor selective agonists can suppress Ca^{2+} currents in cultured neonatal (Mason *et al.* 1988) and acutely dissociated adult rat SON neurones (Bourque *et al.* 1993). However, mediation of the latter effects by κ-opioid receptors was not established by use of antagonists selective for this opioid receptor subtype. This is of some concern since the κ-opioid agonists used in these studies (U50488H and dynorphin A, respectively), when administered at the concentrations employed, can cross react with μ-, and to a lesser extent δ-opioid binding sites (Goldstein, 1987). The present experiments were designed to determine whether Ca^{2+} channel currents in magnocellular SON neurones are regulated by activation of a particular subtype(s) of opioid receptor and, if so, to identify the specific Ca^{2+} channel subtype(s) that are subject to opioid modulation.

A preliminary report of this has been presented in abstract form (Soldo & Moises, 1996).

METHODS

Neurone preparation

Supraoptic nucleus neurones were acutely isolated from Sprague–Dawley rats (17–25 days postnatal, male and female) using the following dissociation procedure. The animals were anaesthetized with CO_2 and decapitated using a small animal guillotine. The brain was rapidly removed and chilled in ice-cold, artificial cerebral spinal fluid (ACSF) containing (mM): 140 NaCl, 3·5 KCl, 1·2 $MgCl_2$, 0·1 $CaCl_2$, 10 Hepes, 11 glucose (pH 7·2–7·3), and bubbled to saturation with 95% O_2–5% CO_2. The whole brain was coronally blocked at approximately 1 mm anterior and 5 mm posterior to bregma, mounted on the stage of a vibraslicer (World Precision Instruments, Inc., Sarasota, FL, USA) and 400–500 μm thick slices containing the basal hypothalamic nuclei were collected between 0·8 to 1·8 mm posterior to bregma. Slices were allowed to equilibrate for 20 min in oxygenated ACSF. Using a small scalpel blade, tissue containing SON was dissected from regions adjacent to either side of the optic chiasm, transferred to oxygenated Ca^{2+}- and Mg^{2+}-free Hanks' Balanced Salt Solution supplemented with 21 mM $NaHCO_3$ (BSS) and enzymatically treated with trypsin (type I, 1·25 mg ml^{-1}) at 37 °C for 45–60 min. The fragments were then washed repeatedly in BSS containing 8 mM $MgCl_2$, DNAse I (type IV, 4%, w/v), trypsin inhibitor (type II, 0·065%, w/v) and fetal bovine serum (10%, v/v). The tissue was then mechanically dissociated in BSS containing 8 mM $MgCl_2$ and 13% DNAse I using several passages through a fire-polished Pasteur pipette. Dissociated neurones were plated onto poly-L-lysine-coated 35 mm culture dishes that had been modified to include a 10 mm cell well. Neurones were maintained at 37 °C in an atmosphere of 5% CO_2 for approximately 30 min, after which 1 ml growth medium of the following composition was added to each dish: Dulbecco's modified Eagle's medium (with L-glutamine, 40%), Ham F-12 (40%), heat-inactivated fetal calf serum (10%), horse serum (10%), D-glucose (0·35%), penicillin and/or streptomycin (1%) and nerve growth factor (100 ng ml^{-1}). Neurones that had well-rounded cell bodies

(14–38 μm) with no or only a few short extending processes were studied between 17 and 26 h after plating. In several experiments, neurones were examined electrophysiologically within 1–7 h of initial plating and did not differ significantly in their expression of VSCC subtypes or responsiveness to opioids compared with neurones maintained in culture for a longer duration under otherwise identical experimental conditions (16·3 ± 4·6% inhibition of current by a μ-opioid agonist for acute as compared with 16·3 ± 2·5% inhibition for short-term cultured neurones; $n = 7$ for both groups).

Electrophysiological recording

Voltage-clamp recordings were obtained using the whole-cell variation of the patch clamp technique (Hamill *et al.* 1981). Recording electrodes, prepared from glass microhaematocrit capillary tubes (i.d., 1·1–1·2 mm; o.d., 1·3–1·4 mm) and having resistances of 2–5 MΩ, were filled with an internal recording solution of composition (mM): 102 caesium acetate, 11 CsCl, 1 $MgCl_2$, 5 4-aminopyridine, 40 Hepes, 10 EGTA, 4 ATP (Mg^{2+} salt), 0·1 GTP (Na^{2+} salt), (pH 7·25–7·35, 275–285 mosmol l^{-1}). In several experiments, 0·1 mM GTP-γ-S (tetralithium salt) replaced GTP, as noted in the text. Plated neurones were viewed through an inverted microscope and continuously superfused with an external solution of composition (mM): 140 NaCl, 1·2 $MgCl_2$, 0·1 $CaCl_2$, 10 Hepes, 11 glucose with 2–4 mM Ba^{2+} as charge carrier (pH 7·2–7·3; 300–310 mosmol l^{-1}). The bath solution also contained 1 μM TTX to block inward sodium currents. Inward Ba^{2+} currents are referred to here as Ca^{2+} channel currents or I_{Ba}. In later experiments, 140 mM NaCl was replaced with 100 mM TEACl, 67 mM choline chloride and 3·5 mM KCl. Differences in the biophysical properties of macroscopic Ca^{2+} channel currents or their modulation by opioids could not be distinguished in recordings obtained with NaCl/TTX - compared with TEA-containing external solutions, and therefore the results from these two bath conditions were pooled.

All recordings were made at room temperature using an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA, USA). Pipette and whole-cell capacitances and series resistances were corrected using compensation circuitry on the patch clamp amplifier. Series resistance compensation of 80–90% was typically obtained without inducing oscillations. A series of I_{Ba} were evoked at 30 s intervals by applying depolarizing commands of 100–200 ms duration while holding the cell at −80 mV, a holding potential (V_h) which is sufficiently negative to remove the steady-state voltage-dependent inactivation of all Ca^{2+} channel types. For each neurone, the voltage step that generated the maximum inward I_{Ba} was used throughout. Current records were digitally corrected for leak currents by using an on-line P/4 subtraction protocol. In some experiments, 200 μM Cd^{2+} was applied to the neurone at the end of an experiment to block all VSCCs and verify that no current remained after stepping to test potentials that elicited maximal I_{Ba}. I_{Ba} amplitudes were measured isochronically near the peak of the response (usually 8–12 ms following current activation) for each neurone before, during and after washout of tested drugs. To control for potential confounding effects of rundown in I_{Ba} on quantification of agonist-induced changes in the evoked currents, this time-dependent decline in the recorded whole-cell current was accounted for by using a second-order polynomial equation to fit current amplitudes before drug perfusion and after current recovery following drug washout. Control current amplitudes were interpolated from the fitted equation over the time period of drug perfusion, averaged and compared with the actual mean I_{Ba} amplitudes measured during drug perfusion. Cells showing a rapid rundown of I_{Ba} were not analysed. Otherwise, in the absence of rundown, control current amplitudes were those measured only before drug perfusion. Drug-induced modifications in peak I_{Ba} amplitudes are expressed as:

((control amplitude) − (test amplitude))/(control amplitude)100.

A single component of current decay was estimated in some cases, using a single exponential algorithm of Igor Pro (WaveMetrics, Inc.) of the form:

$$I = K_1 \exp(-x/\tau_1),$$

fitted from the peak current amplitude to 40 ms of current decay, giving an estimate of the amplitude K_1 and time constant τ_1. The duration of the voltage pulses was not sufficient to resolve time constants of current decay beyond 40 ms. All data were analysed using an unpaired Student's t test, unless otherwise indicated, and expressed as means ± S.E.M. in the text.

Drug preparation and delivery

The μ-opioid receptor agonist [D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin (DAMGO, Peninsula Laboratories, Belmont, CA, USA) was prepared as a 10 mM stock solution in sterile water, partitioned into 10 μl aliquots, lyophilized and stored at −20 °C. The Ca^{2+} channel blockers ω-agatoxin IVA (Aga IVA) and ω-conotoxin GVIA (GVIA) were prepared as 100 μM and 167 μM stock solutions, respectively, and stored in the same manner. The κ_1-opioid receptor agonist U69593 (RBI, Natick, MA, USA) was prepared as a 10 mM stock solution in 95% ethanol; the specific μ-opioid receptor antagonist β-funaltrexamine (β-FNA, RBI) was dissolved in methanol to 3 mM, and the L-type Ca^{2+} channel antagonists nicardipine (Nic, Sigma) and nifedipine (Nif, RBI) were dissolved in dimethyl sulphoxide to 10 mM. On the experimental day, stock solutions and freshly weighed amounts of the opioid antagonist naloxone (RBI), the mixed κ_1/κ_2-opioid receptor agonist ethylketocyclazocine (EKC, a generous gift from Dr Jim Woods) and the δ-opioid receptor agonist [D-Pen^{2,5}]-enkephalin (DPDPE, Peninsula Laboratories) were diluted with the standard external solution to the desired concentrations.

Control and drug-containing bath solutions were applied by local superfusion of the individual neurone under study using a modified U-tube delivery system. This consisted of a gravity-fed U-shaped polyethylene tubing, positioned within 100 μm of the neurone. The distal end of the tubing was coupled to a vacuum line and controlled by a digital switching valve. A laminar flow of bathing solution could be maintained across the neurone throughout the recording and this solution could be rapidly exchanged (< 1 s) by operating the switching valve.

Immunocytochemistry

Upon stable adherence to the culture dish, and in some cases following electrophysiological recording, neurones were washed with 0·1 M phosphate-buffered saline (PBS, pH 7·3), exposed to 4% (w/v) paraformaldehyde for 30 min and washed with 50 mM potassium-PBS (KPBS, pH 7·3). Neurones were then stored in KPBS at 4 °C, until being assayed immunocytochemically for the presence of AVP or OT by examining for immunoreactivity to specific antibodies. Immediately prior to immunostaining, neurones were rinsed for 5 min in PBS containing 0·05% BSA and 0·05% azide (rinse buffer), and subsequently permeabilized for 20 min with the addition of 0·005% Triton X-100. Neurones were then incubated in a blocking solution of rinse buffer containing goat serum (1 : 200) for 30 min, followed by rinsing for 5 min.

Arginine vasopressin and oxytocin immunocytochemistry. Identification of AVP-containing neurones was performed by incubating cells overnight at 4 °C in rinse buffer containing a rabbit

polyclonal antibody specific for AVP (1 : 1000; Chemicon, Temecula, CA, USA). In pilot studies, we determined that this primary AVP antibody did not cross-react with the synthetic OT peptide (Sigma) under the conditions of these experiments. Neurones were rinsed for 5 min and incubated in rinse buffer containing a biotinylated, goat anti-rabbit immunoglobulin (Vector Labs, Burlingame, CA, USA) for 30 min at room temperature. After rinsing, neurones were incubated in an Avidin/Biotin complex (diluted 1 : 1000 in blocking solution; Vector Labs) for 60 min at room temperature, washed in PBS, subsequently washed in 0·1 M sodium acetate, then incubated in diaminobenzadine (DAB) and watched carefully for development of brown staining. This reaction was terminated by washing thoroughly in PBS. For OT detection, cells were incubated overnight (4 °C) in the presence of a specific monoclonal antibody directed against OT-neurophysin (PS 36, 1 : 50; kindly provided by Dr H. Gainer), rinsed, and incubated in rinse buffer containing a fluorescein (FITC)-conjugated anti-mouse immunoglobulin (1 : 1000) for 1 h at room temperature. After rinsing three times, AVP- and OT-immunoprocessed cells were visualized using bright-field (AVP) or fluorescence microscopy (OT), photographed and subsequently covered with glycerol gelatin (Sigma) for long-term storage.

κ_1-Opioid receptor immunostaining. Neurones were incubated in the presence of a primary antibody directed against the C-terminal portion of the κ_1-opioid receptor (diluted 1 : 1000 in rinse buffer; generously provided by Dr A. Mansour) overnight at 4 °C. To test for non-specific antibody interactions, this κ_1-opioid receptor antibody was mixed with the purified κ_1-opioid receptor fusion protein (40 μl ml^{-1}) prior to immunostaining. The cells were rinsed for 5 min and incubated in a biotinylated, goat anti-rabbit immunoglobulin (diluted 1 : 1000 in rinse buffer; Vector Labs) for 1 h at room temperature, rinsed and subsequently stained using a DAB procedure (see above).

RESULTS

We began by utilizing specific antibodies to identify AVP- or OT-containing neurones (see Methods) and thereby verify the accuracy of our dissociation procedure for isolating SON neurones from rat hypothalamic slices. Figure 1*A* illustrates specific staining for AVP (Fig. 1*Aa*) or OT (Fig. 1*Ac*) in several large, magnocellular neurones from different platings of acutely isolated neurones. When examined under phase contrast optics (Fig. 1*Ab*), these neurones had large (14–38 μm diameter), phase-bright cell bodies with few or no extending processes. Neurones having these characteristics were designated as magnocellular SON neurones and were of the type targeted for electrophysiological recordings. It was shown previously that > 96 % of magnocellular SON neurones with similar morphological characteristics exhibit positive immunoreactivity for either AVP or OT (Cobbett & Weiss, 1990). Whereas immunocytochemical identification was not performed on all magnocellular SON neurones studied here, it can be safely assumed that the vast majority, if not all electrophysiological recordings were obtained from either AVP- or OT-containing neurosecretory neurones. Therefore, all magnocellular SON neurones will be generally referred to here as magnocellular neurosecretory cells (MNCs), a term commonly used in the literature to describe these neurones.

Characteristics of evoked Ca^{2+} channel currents

Whole cell voltage-clamp recordings were obtained from 85 MNCs. Delivery of 100–200 ms depolarizing commands from a holding potential (V_h) of −80 mV to test potentials in the range −40 to +50 mV evoked inward I_{Ba} that consisted of an initial transient component followed by a longer sustained component (Fig. 1*B*). HVA currents having a threshold of activation ≥ −40 mV were observed in all 85 neurones when depolarizing commands were delivered from a negative V_h between −100 and −80 mV. By contrast, LVA currents with a threshold of activation < −40 mV were observed in a minority of these neurones (12/85). In general, the whole-cell Ca^{2+} channel currents measured in this study corresponded well to those described previously in acutely dissociated MNCs (Fisher & Bourque, 1995*a*; Foehring & Armstrong, 1996) with regard to their kinetics and voltage dependence of activation.

The current–voltage (*I–V*) relationship for I_{Ba} was determined in 54 MNCs by plotting measurements of both the peak current and the amplitude of the steady-state current recorded just prior to termination of the test pulse (~95 ms) against the command potential (Fig. 1*C*). In all these neurones, both the initial transient and sustained current components activated at similar potentials (between −40 mV and −30 mV) and showed concordant changes in amplitude at larger depolarizations. Therefore, the *I–V* relationship was characterized by an absence of a 'shoulder' over the voltage range of −70 to −20 mV. In contrast, the *I–V* relationships for I_{Ba} in many types of neurones have a characteristic 'shoulder' between the test potentials of −70 mV and −20 mV, indicative of activation of a low threshold, rapidly inactivating current contributed by T-type Ca^{2+} channels (Fox *et al.* 1987). Our results indicate that the majority of MNCs in the SON express only HVA channels, although LVA T-type currents were observed in a small subpopulation of neurones (see above). Similar results have been reported for rat MNCs by Foehring & Armstrong (1996).

Opioid sensitivity of whole-cell Ca^{2+} channel currents

The effects of μ-, δ- or κ-opioid receptor activation on I_{Ba} were examined by stepping to test potentials that yielded maximum current amplitudes at 30 s intervals before, during application and after washout of opioid receptor type-selective agonists.

μ-Opioid sensitivity

Administration of the μ-opioid receptor agonist DAMGO (1 μM) reversibly suppressed the peak amplitude of I_{Ba} in 64/71 neurones examined, as illustrated by the current traces depicted for the neurone in Fig. 2*A*. At this concentration of agonist, the inhibition in current varied greatly among responsive neurones (6–33 %, Fig. 2*B*), with a mean of 17·2 ± 1·1 %. Seven of the neurones did not respond to the

μ-opioid (defined here as < 5% change in control current at this concentration). The DAMGO-induced inhibition in current was rapid in onset and often associated with a slight slowing of current activation (which became pronounced in the presence of GTP-γ-S; see Fig. 4). Testing of the μ-opioid agonist over a 6-log range of concentrations (100 pM to 10 μM) revealed that the suppression in I_{Ba} by DAMGO was concentration dependent with an estimated EC_{50} of 170 nM and mean E_{max} of 19·5% (Fig. 2*C*). It should be noted that the calculated maximal opioid effect might slightly underestimate the actual response due to the correction for the small degree of current rundown that typically occurred over the course of an experiment (see Methods). Administration of DAMGO in very low concentrations (1 nM and below) did not affect I_{Ba}. The opioid receptor antagonist naloxone (1 μM) alone did not modify I_{Ba}, but reversibly blocked the DAMGO-induced inhibition of current ($n = 4$; Fig. 2*A*). After a 5 min washout of the antagonist, re-application of DAMGO again suppressed I_{Ba}. These results indicate that the DAMGO-

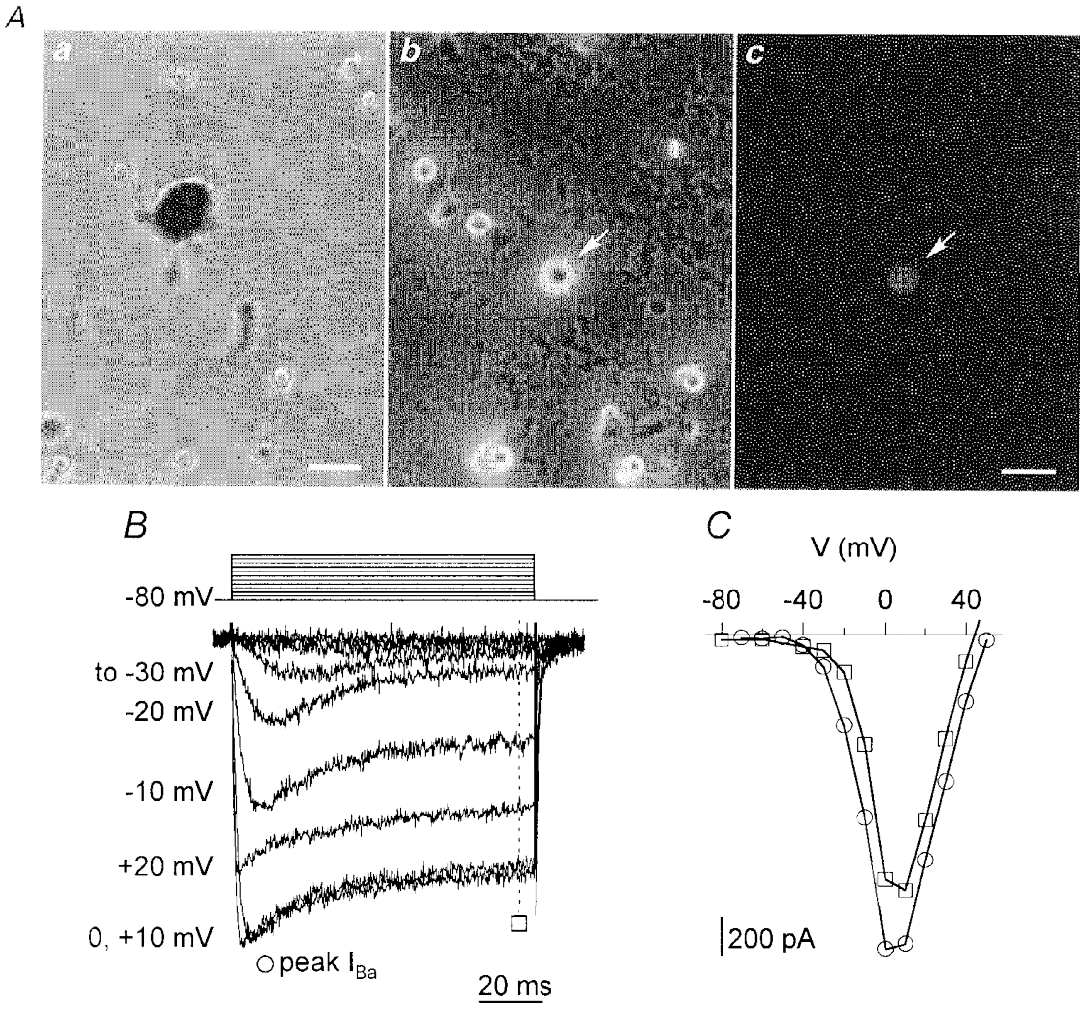

Figure 1. HVA Ca^{2+} channel currents in putative AVP- and OT-containing magnocellular supraoptic neurones

A, examples of neuropeptide-containing magnocellular SON neurones of the type from which recordings were made. *Aa*, diaminobenzadine (DAB)-staining of a magnocellular SON neurone labelled with a specific antibody to AVP. Small cells (< 15 μm) did not label with the AVP antibody. Scale bar 15 μm. *Ab*, phase contrast photomicrograph of an acutely isolated magnocellular neurone (arrow) prepared from SON. Scale bar as *Ac*. *Ac*, immunofluorescence detection for OT-neurophysin in the magnocellular neurone shown in *Ab*. Scale bar 25 μm. *B*, family of inward Ca^{2+} channel currents (lower traces) evoked in a representative MNC by stepping to different test potentials from $V_h = -80$ mV (upper traces). *C*, the *I–V* relationship computed from the current traces shown in *B*. Plotting of the amplitude of peak Ca^{2+} channel current (○) and steady-state current (measured just prior to termination of the test depolarization; □) as a function of test potential revealed a similar range of voltage dependence of activation for each. LVA Ca^{2+} currents were not seen in this MNC, which was typical for all 54 neurones in which *I–V* relationships were constructed.

induced inhibition in I_{Ba} was mediated by the activation of opioid receptors.

The voltage dependency of DAMGO-induced current inhibition was examined by evoking I_{Ba} over a range of test potentials from a holding potential of −100 mV in the absence and presence of the μ-opioid agonist (1 μM; Fig. 3). LVA (T-type) currents were isolated when present, by stepping to test potentials $\leqslant$ −40 mV, whereas HVA current components were maximally activated by commands to test potentials of −10 to +10 mV. Examples of LVA and HVA components of I_{Ba} recorded from the same neurone are shown in Fig. 3*A* and *B*. In this neurone and four additional cells, application of DAMGO inhibited HVA currents, whereas LVA current components were unaffected by the μ-opioid. Examination of the *I–V* relationships further revealed that μ-opioid receptor activation selectively inhibited one or more HVA current components whose activation threshold was $\geqslant$ −30 mV (Fig. 3*C*). Isolation of the DAMGO-sensitive component of I_{Ba} (Fig. 3*D*), computed by subtracting currents evoked in the presence of the opioid from the corresponding pre-drug control current, revealed fast activation characteristics with an initial inactivating component ($\tau = 30{\cdot}6$ ms, $n = 3$) followed by a relatively sustained component (decay time not computed as the pulse durations were insufficient to resolve this portion of the current).

The reduction in peak current amplitude by DAMGO also showed voltage dependence in that the inhibitory effect of the μ-opioid decreased with increasing magnitude of the depolarizing test pulse (Fig. 3*C*). This was further investigated by determining whether large prepulse depolarizations, applied 15 ms before the test pulse (to +10 mV from $V_h = -90$ mV), would reverse the μ-opioid-induced suppression of I_{Ba}. Figure 4 demonstrates that a depolarizing prepulse (to +90 mV for 100 ms) facilitated (or disinhibited) an early, rapidly inactivating component of I_{Ba}. Thus, this component of facilitated current is most likely that which is tonically regulated by a voltage-dependent inhibitory mechanism. In the presence of DAMGO (1 μM), the depolarizing prepulse facilitated a greater proportion of I_{Ba} (Fig. 4*A*). In six similar experiments, the magnitude of the facilitation, measured as the ratio of the integrated facilitated current to that of control, was greater in the presence of DAMGO (ratio: control $1{\cdot}09 \pm 0{\cdot}02$ *vs.* DAMGO $1{\cdot}17 \pm 0{\cdot}02$; $P < 0{\cdot}01$, Student's paired *t* test). These results indicate that the activation of μ-opioid receptors recruits a voltage-dependent process to suppress I_{Ba} in SON

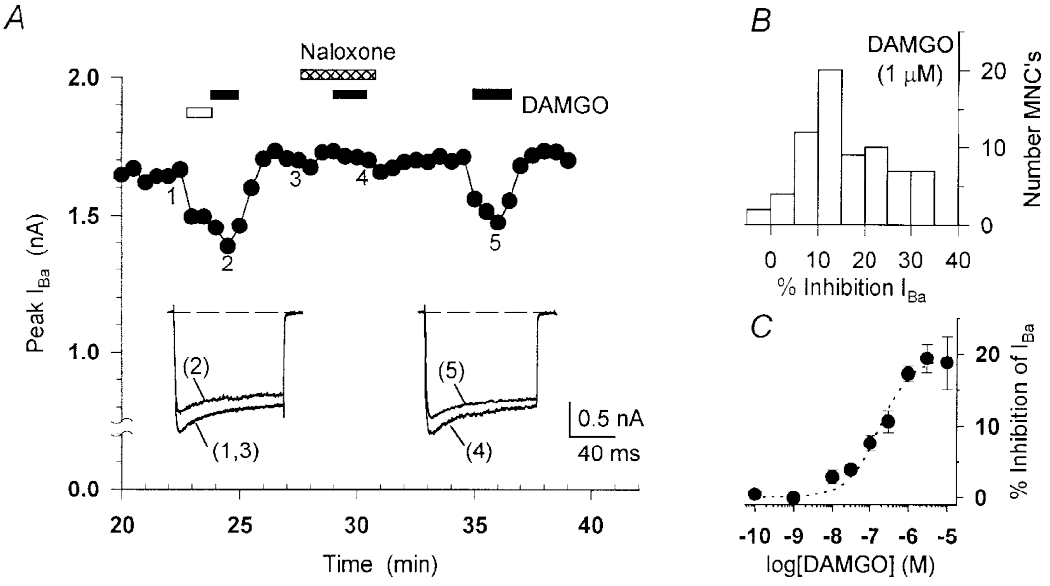

Figure 2. DAMGO reversibly suppressed I_{Ba} in MNCs

A, time course of a representative experiment illustrates the inhibitory effect of DAMGO (3 μM; filled bar) on peak I_{Ba} in one MNC, and blockade of this effect by naloxone (1 μM); insets are means of 2–3 current traces and illustrate the DAMGO effect on Ca^{2+} channel current at the time points and under the experimental conditions indicated. Open bar, 1 μM DAMGO. *B*, cumulative distribution of the DAMGO-induced (1 μM) inhibitory effect on I_{Ba} for all 71 neurones tested. *C*, concentration–response relationship for DAMGO-induced inhibition of I_{Ba} in MNCs. The fit is made using the equation:

$$(E_{max} + [\text{DAMGO}])/(EC_{50} + [\text{DAMGO}]),$$

where E_{max} is the maximum percentage inhibition of current produced by DAMGO, and EC_{50} is the concentration at which DAMGO inhibits 50% of the total inhibitable amount of control current amplitude. Each point represents the mean response recorded for 2–64 neurones.

neurones. However, depolarizing prepulses did not facilitate the entire portion of I_{Ba} suppressed by DAMGO, suggesting that either DAMGO inhibits components of I_{Ba} with different voltage dependencies or the prepulse was not large enough to remove the entire inhibition. We were unable to apply prepulses larger than +90 mV consistently without compromising the integrity of the whole-cell recording configuration. The reversal of responses by depolarizing prepulses is indicative of the involvement of a G-protein-coupled transduction pathway. To confirm the involvement of G-proteins, we examined the inhibitory effects of DAMGO on neurones dialysed intracellularly with GTP-γ-S. Substitution of GTP-γ-S (100 μM) in the patch pipette in place of GTP enhanced the response to DAMGO, as evidenced by a more pronounced slowing of current activation, and rendered the opioid responses irreversible (Fig. 4*B*). Delivery of large depolarizing prepulses transiently reversed the DAMGO-induced current suppression, which redeveloped in the absence of the prepulses.

δ- and κ-opioid sensitivity

The finding that DAMGO inhibited I_{Ba} in MNCs is at odds with the results of an earlier report that κ-opioid, but not δ- or μ-opioid agonists, directly reduced the duration of Ca^{2+} action potentials in these neurones (Inenaga *et al.* 1994). Therefore, to determine whether μ-opioid receptors mediated the DAMGO-induced suppression in I_{Ba}, we compared the abilities of μ-, δ- and κ-opioid selective agonists to inhibit I_{Ba} when applied to the same MNCs. Tests on six neurones revealed that I_{Ba} was unaffected by administration of either the selective δ-receptor agonist DPDPE (1 μM) or the κ_1-opioid selective agonist U69593 (1 μM), whereas DAMGO (1 μM) reduced I_{Ba} in each of these neurones by a mean of 21·4 ± 2·8% (Fig. 5*A*). In a different set of neurones ($n = 5$), administration of EKC (1 μM), which acts at both κ_1- and κ_2-opioid receptors, also failed to affect I_{Ba} in contrast to the 15·0 ± 5·4% mean inhibition induced by DAMGO (1 μM; Fig. 5*B*). The bar graph in Fig. 5*C* provides a comparative summary of μ-, δ- and κ-opioid effects on I_{Ba} for all neurones that were examined for sensitivity to DAMGO and at least one additional agonist. Among the group of 22 neurones examined, DAMGO suppressed I_{Ba} by a mean of 15·7 ± 5·4%, whereas DPDPE ($n = 9$), U69593 ($n = 19$) and EKC ($n = 5$) were without effect. Furthermore, experiments in two additional neurones revealed that the administration of the specific μ-opioid receptor antagonist, β-funaltrexamine (β-FNA, 300 nM) blocked the suppression in I_{Ba} produced by DAMGO. Taken together, these data

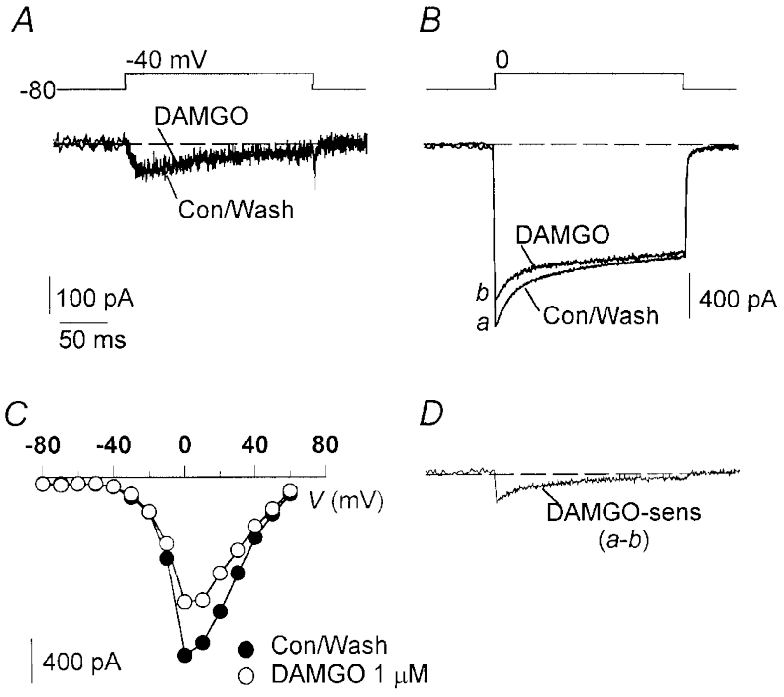

Figure 3. DAMGO suppressed HVA, but not LVA Ca^{2+} channel current in MNCs

A, stepping to −40 mV from $V_h = -80$ mV (200 ms) activated a LVA Ca^{2+} channel current that was unaffected by application of DAMGO (1 μM); whereas the HVA current elicited by stepping to 0 mV was reversibly suppressed by the opioid (*B*). This is representative for 5/5 neurones tested in this manner. Current traces in *A* and *B* and in subsequent figures are means of 2–3 consecutive current records. Con, control current. *C*, the *I–V* relationship for the same neurone demonstrates the range of voltages (−10 to +40 mV) at which Ca^{2+} channel current was suppressed by the μ-opioid. *D*, the μ-opioid-sensitive component of HVA (as shown in *B*) was determined by subtracting the current in the presence of DAMGO (trace *Ba*) from the control current (trace *Bb*). Scale of *D* as *B*.

indicate that the μ-opioid receptors are the only opioid receptor subtype functionally coupled to VSCCs in MNC somata.

Several additional points should be noted about the ability of DAMGO to inhibit I_{Ba} in MNCs. First, more than 90% of all MNCs examined showed a reduction in I_{Ba} in response to this μ-opioid agonist. This observation, coupled with the identification of two populations of MNCs by immunocytochemical staining for AVP and for OT-neurophysin, suggested that μ-opioid receptor activation regulates I_{Ba} in both AVP- and OT-containing neurones. Following immunocytochemical processing, an insufficient number of recorded neurones were recovered to enable us to correlate μ-opioid sensitivity of I_{Ba} with the neurochemical phenotype of each neurone. However, we reasoned that if I_{Ba} in AVP and OT neurones showed a differential sensitivity to modulation by μ-opioid receptor activation, then we would predict a biphasic response profile of DAMGO-induced inhibition among all neurones sampled. Instead, we found that μ-opioid-induced suppression of I_{Ba} showed a unimodal distribution for the entire population of responsive neurones (64/71; see Fig. 2*B*), supporting the likelihood that μ-opioid regulation of I_{Ba} was not differentially expressed between OT- and AVP-containing neurones under the conditions of the present study.

A second issue is that the present results place constraints on the functional significance of the high densities of κ-opioid receptor binding sites that are found in the SON. Our data suggest that a majority of these either have a presynaptic locus or otherwise are likely to be functionally coupled to effectors other than VSCCs in the somata of MNCs. Nevertheless, it is possible that a significant fraction of these κ-opioid receptors is normally found on dendritic processes and/or is somatically expressed, but that these receptors are sheared off or otherwise functionally uncoupled from Ca^{2+} channels during the dissociation procedure. In an effort to distinguish between these possibilities, we examined for the expression of κ-opioid

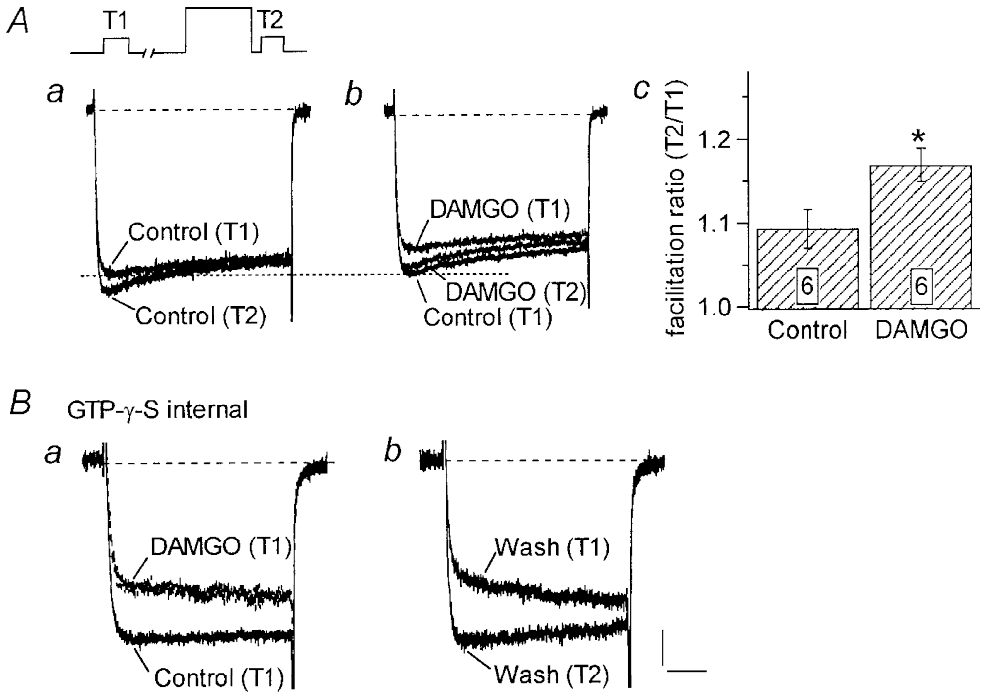

Figure 4. Prepulse depolarizations largely facilitated the portion of I_{Ba} inhibited by μ-opioid receptor activation

A, current traces recorded from one MNC, evoked with a 50 ms test pulse (to +10 mV from $V_h = -90$ mV) before (T1) and 15 ms after (T2) a prepulse depolarization (to +90 mV for 100 ms; see voltage protocol, upper trace), under control conditions (*Aa*) and during the application of DAMGO (1 μM; *Ab*). DAMGO-inhibited I_{Ba} (DAMGO (T1)) was facilitated with a depolarizing prepulse (DAMGO (T2)). *Ac*, summary of six experiments using the same protocol as in *Aa* and *Ab*. The data are expressed as the ratio of integrated currents obtained after (T2) to those obtained before (T1) the depolarizing prepulse. While the integrated currents were facilitated both before and in the presence of DAMGO (control T2/T1 = 1·09 ± 0·02, $P < 0{\cdot}01$; DAMGO T2/T1 = 1·17 ± 0·02, $P < 0{\cdot}001$, *Ac*), facilitation was significantly greater in the presence of DAMGO (* $P < 0{\cdot}01$, Student's paired t test). *B*, in a different MNC under a similar experiment as in *A*, except that GTP-γ-S (100 μM) was included in the internal solution in place of GTP. The DAMGO-induced suppression and kinetic slowing of I_{Ba} was pronounced (DAMGO (T1), *Ba*) and irreversible (Wash (T1), *Bb*), except when the test pulse was preceded by a depolarizing prepulse (Wash (T2)). Scale bars: *A*, 0·2 nA, 20 ms; *B*, 0·1 nA, 10 ms.

receptors in the cell bodies of acutely isolated intact and permeabilized SON neurones by probing with a selective κ_1-opioid receptor antibody (see Methods; Fig. 5*D*). Although the dissociation of SON tissue yielded a heterogeneous population of cells in culture, ranging in size from small cells ($< 10\ \mu$m) to the magnocellular neurones ($> 15\ \mu$m), only MNCs (as previously defined) showed intense immunostaining for κ_1-opioid receptor protein (Fig. 5*Da*). Specificity of the antibody for κ_1-opioid receptors was demonstrated by an absence of positive immunoreactivity in any of the neurones following incubation of the primary antibody with the κ_1-opioid receptor fusion protein (Fig. 5*Db*). In addition, neurones that were not permeabilized prior to exposure to the κ_1-opioid receptor antibody did not demonstrate staining (not shown). These results provide the first immunocytochemical demonstration of κ-opioid receptors in somata of MNCs.

Identification of opioid-sensitive Ca^{2+} channel types

The maximal reductions in I_{Ba} by DAMGO were relatively small (for example, when compared with effects observed in sensory or nucleus tractus solitarius neurones – Rhim & Miller, 1994; Moises *et al.* 1994), rarely exceeding 20% of control current. Importantly, this might occur if an appreciable fraction of opioid-sensitive current was tonically inhibited under basal experimental conditions (see Fig. 4*A*). In fact, we found that a significant portion of total *peak* I_{Ba} was facilitated with depolarizing prepulses ($14{\cdot}1 \pm 2{\cdot}3\%$, $n = 7$, $P < 0{\cdot}001$). Additionally, the small DAMGO effect on I_{Ba} might be explained if μ-opioid receptors modulate a

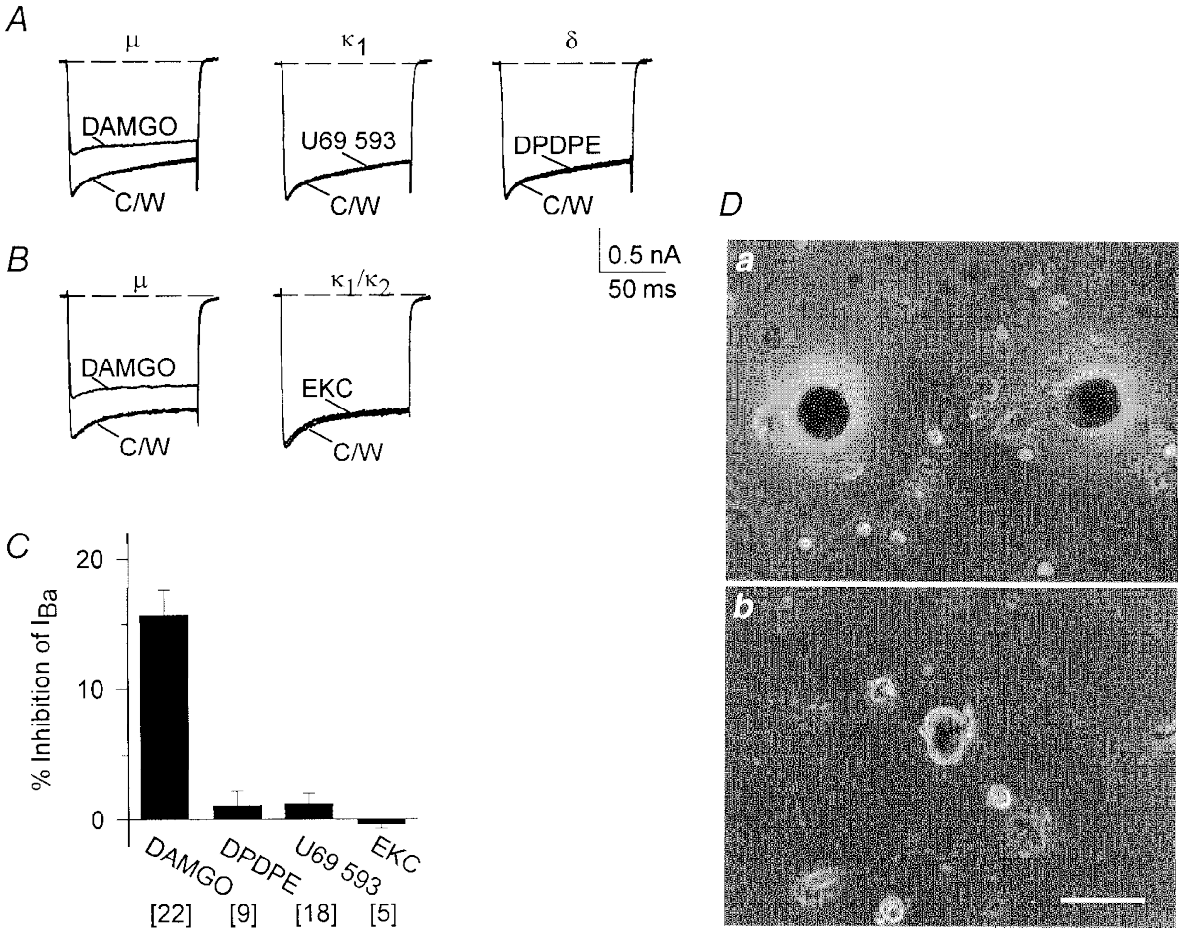

Figure 5. I_{Ba} was suppressed by μ-, but not δ- or κ-opioid-selective agonists

A, traces from one MNC illustrate that DAMGO (1 μM) reversibly inhibited I_{Ba} whereas κ- (U69 593; 1 μM) and δ-selective (DPDPE; 1 μM) agonists were ineffective. *B*, in a different neurone, the mixed κ_1/κ_2-selective agonist ethylketocyclazocine (EKC; 1 μM) did not modify I_{Ba} whereas the current was reduced by μ-opioid receptor activation. Scale bars in *A* are for all traces. *C*, graphical comparison of the effects of μ-, δ- and κ-opioids on a population of MNCs. Numbers of neurones tested are indicated within brackets. *Da*, presence of κ-opioid receptor-like protein in an MNC, demonstrated by DAB staining after incubation of cells with κ_1-opioid receptor primary antibody. *Db*, no κ-opioid receptor-like immunoreactivity was observed when using a κ_1-opioid receptor antibody, pre-incubated with the κ_1-opioid receptor fusion protein (see Methods), indicating specificity of the primary antibody for the κ_1-opioid receptor under the experimental conditions employed. C/W; control and wash currents.

single type or only a small fraction of the different subtypes of HVA Ca^{2+} channels that are expressed in these neurones.

Recent reports have provided pharmacological and biophysical evidence that MNCs express multiple types of HVA Ca^{2+} channels, including classical L-, N-, and P-types (Fisher & Bourque, 1995*a*; Foehring & Armstrong, 1996). Each of these Ca^{2+} channel types can be distinguished by differences in activation kinetics, voltage dependence and sensitivity to Ca^{2+} channel-type selective blockers. To determine the extent to which activation of μ-opioid receptors modulate different Ca^{2+} channel subtypes expressed in MNCs, we compared the ability of DAMGO to inhibit I_{Ba} before and after blockade of specific components of I_{Ba} using various combinations of blockers and organic toxins selective for the different Ca^{2+} channel types.

Dihydropyridine-sensitive Ca^{2+} channel current

Nifedipine (Nif) has been shown to block somatic L-type Ca^{2+} channels in a variety of central and peripheral neurones (Fox *et al.* 1987; Fisher & Bourque, 1995*a*; Foehring & Armstrong, 1996), including those of the SON. Superfusion of neurones ($n = 17$) with Nif at a saturating concentration (10 μM) reversibly blocked peak I_{Ba} by $8{\cdot}6 \pm 1{\cdot}4\%$ (Fig. 6*Aa*). In four of these neurones, the magnitude of current blockade by Nif continued to increase throughout the duration of the test pulse, consistent with the characteristic voltage dependence of dihydropyridine antagonist binding to L-type Ca^{2+} channels (Sanguinetti & Kass, 1984). The onset of current blockade by Nif was rapid with maximal inhibition occurring within 30–60 s. Isolation of the Nif-sensitive current by digital subtraction of current

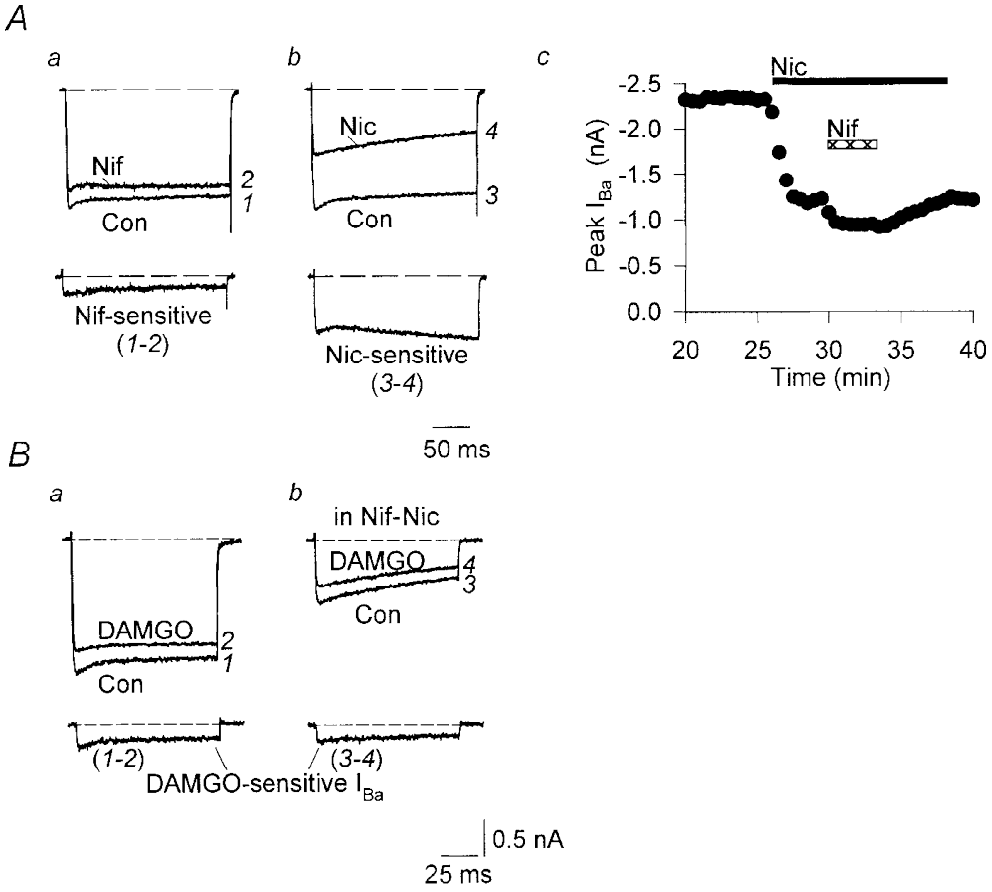

Figure 6. L-type Ca^{2+} channel antagonists blocked a portion of I_{Ba} that was insensitive to DAMGO

Aa, nifedipine (Nif, 10 μM) inhibited a small portion of Ca^{2+} channel current. The Nif-sensitive current (bottom trace) was computed by subtracting the current trace recorded in the presence of the dihydropyridine (trace 2) from the control trace (trace 1). *Ab*, in the same neurone, nicardipine (Nic, 10 μM) inhibited a much larger portion of I_{Ba}. This is representative of 10/10 experiments. Compare right and left subtraction current traces (lower panel). *Ac*, occlusion experiments performed in a different neurone revealed that Nif reversibly inhibited a component of I_{Ba} in addition to that blocked by a saturating concentration of Nic. *B*, comparison of the effect of DAMGO on I_{Ba} in the absence (*Ba*) and presence of saturating concentrations of Nic and Nif (10 μM; *Bb*) indicated virtually no effect of L channel blockade on the reductions in I_{Ba} by the μ-opioid, which was typical for 4/4 experiments. Inspection of subtraction currents (bottom traces, see Fig. 5) reveals that DAMGO inhibited an equal portion of I_{Ba} in the absence and presence of Nic–Nif.

records obtained before and during application of the antagonist revealed that in most cases it contained both slowly inactivating and sustained components (Fig. 6*Aa*, bottom trace). The Nif-sensitive current represented a much smaller percentage of total I_{Ba} compared with what has been reported previously in MNCs (28%, Foehring & Armstrong, 1996; 23%, Fisher & Bourque, 1995*a*). However, it should be noted that the L-type current in magnocellular nerve terminals is not blocked by Nif but is sensitive to the dihydropyridine nicardipine (Nic; Wang *et al.* 1993). Therefore, additional experiments were performed to determine whether the somatic L-type current might similarly show a preferential sensitivity to blockade by Nic. Application of Nic (10 μM) to MNCs rapidly suppressed I_{Ba}, but its effect was much greater than that of Nif, averaging a 39·7 ± 3·9% reduction in the control peak current ($n = 10$, Fig. 6*Ab*). In addition to blocking an early inactivating component, Nic also exhibited a more pronounced voltage dependence of current blockade, as evidenced by the occurrence of increasing blockade over the duration of the depolarizing pulse in all 10 neurones examined (Fig. 6*Ab*, bottom trace). Furthermore, occlusion experiments performed with maximal concentrations of the two L-type channel blockers revealed that the inhibitory effects produced by Nif and Nic added linearly (46·4 ± 5·4%, $n = 11$, Fig. 6*Ac*). In subsequent experiments, Nic and Nif were co-applied (Nic–Nif) to block L-type channels maximally and to assess more accurately their regulation by activation of opioid receptors.

Blockade of L-type channels by Nic–Nif (10 μM) did not significantly affect μ-opioid-induced inhibition of I_{Ba}. In tests on four neurones, the amount of I_{Ba} suppressed by DAMGO (1 μM) following blockade of L-type current

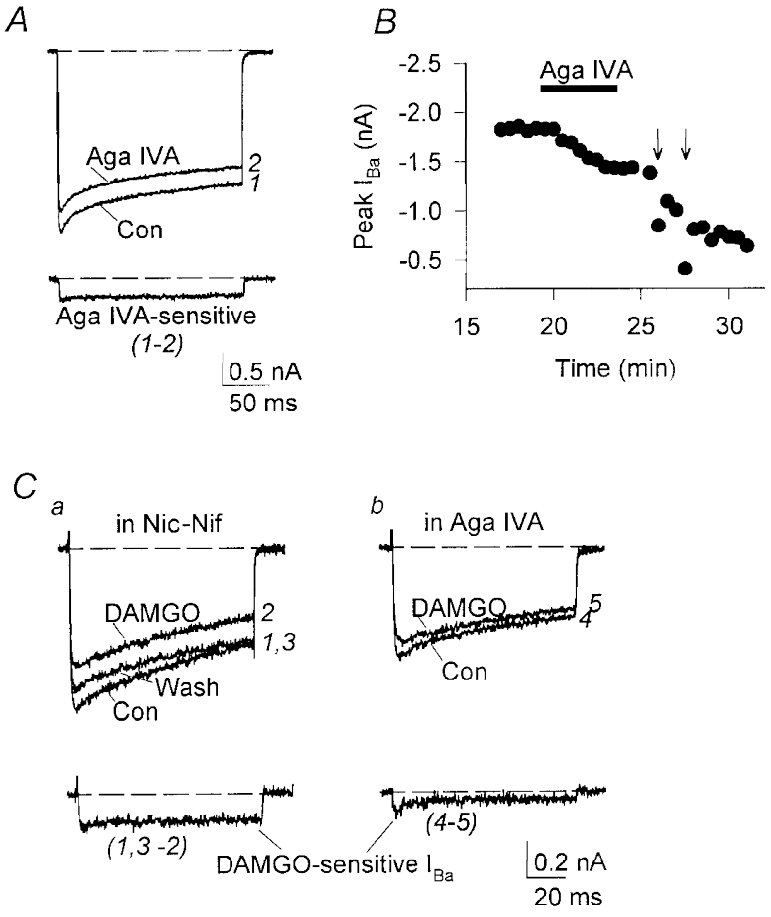

Figure 7. μ-Opioid receptor activation inhibited an Aga IVA-sensitive component of I_{Ba}

A, records from a representative MNC illustrate that Aga IVA (100 nM) inhibited a small, non-inactivating component of I_{Ba}. *B*, plot of peak I_{Ba} amplitude over time course of the experiment in the same neurone showing that delivery of trains of large depolarizing pulses (+120 mV, 2 Hz, 10 s, arrows) after washout of Aga IVA did not relieve the toxin-induced Ca^{2+} channel blockade but did increase current rundown. This was typical of 3/3 experiments. *C*, in a different neurone, DAMGO (1 μM) inhibited approximately 17·8% of I_{Ba} (*Ca*) when tested prior to application of Aga IVA. The portion of DAMGO-induced inhibited current was reduced to 12·3% when re-examined in the presence of Aga IVA (100 nM, *Cb*). A similar effect was seen in four other neurones. The magnitudes of the μ-opioid-sensitive current in the absence and presence of Aga IVA are compared in the subtraction records (bottom traces, see Fig. 5).

(16·7 ± 3·4 %, Fig. 6*Bb*) was not significantly different from the reduction in current produced by the μ-opioid agonist prior to administration of the L-type channel antagonists (18·2 ± 3·1 %, Fig. 6*Ba*). The activation/inactivation kinetics of the DAMGO-sensitive current as determined by digital subtraction (Fig. 6*B*, bottom traces) were also unaffected by blockade of L-type channels.

ω-Agatoxin IVA-sensitive Ca^{2+} channel current

ω-Agatoxin IVA (Aga IVA) at concentrations of 25–200 nM has been reported to block P-type channels in MNCs (Fisher & Bourque, 1995*b*; Foehring & Armstrong, 1996). Administration of Aga IVA (100 nM) suppressed control current amplitude by 19·1 ± 1·2 % and this was attributed to blockade of a non-inactivating current component ($n = 3$, Fig. 7*A*). The inhibitory effect of Aga IVA required several minutes to fully develop (>5 min) and was irreversible upon washout of the toxin, even upon delivery of a train of large depolarizing pulses ($n = 3$). Attempts to reverse the Aga IVA-induced reduction in current were further complicated by the fact that delivery of a 10 s train of depolarizing pulses to +120 mV given at 2 Hz attenuated the amplitude of I_{Ba} (Fig. 7*B*) and accelerated current run-down. To determine whether μ-opioid receptors modulated P-type Ca^{2+} channels, we compared the ability of DAMGO to inhibit I_{Ba} before and after application of Aga IVA (100 nM) in the continuous presence of Nic–Nif. The inhibitory effect of DAMGO was reduced by 48 % after blockade of P-type channels, from an 18·4 ± 4·5 % reduction in control I_{Ba} to a 9·7 ± 2·9 % inhibition in the absence and presence of Aga IVA, respectively ($n = 5$, Fig. 7*Ca* and *Cb*). Inspection of the subtraction currents depicted for the experiment in Fig. 7*C* (bottom traces) demonstrate that a large portion of the μ-opioid-sensitive current was removed after blockade of P-type channels.

ω-Conotoxin-GVIA-sensitive Ca^{2+} channel current

ω-Conotoxin-GVIA (GVIA) has been shown to block N-type Ca^{2+} channels selectively in SON neurones (Fisher & Bourque, 1995*a*). Application of GVIA (1 μM) for 10 s resulted in an irreversible blockade of a slowly inactivating component of I_{Ba} that, on average, accounted for 38·8 ± 3·8 % of the total control current ($n = 11$, Fig. 8*A*). After establishment of N-type channel blockade by GVIA (1 μM), the inhibitory response to DAMGO was significantly reduced (by ~58 %) compared with the effect produced prior to application of the toxin (8·7 ± 2·1 *vs.* 22·5 ± 3·2 %, $n = 5$; Fig. 8*B*). The reduction in total DAMGO-sensitive current after blockade of N-type channels is evident from inspection of the subtraction currents from a typical experiment (Fig. 8*B*; bottom traces).

Co-application of Ca^{2+} channel antagonists

Summation of the mean reductions in control I_{Ba} produced by administration of Aga IVA, GVIA or Nic–Nif alone resulted in a cumulative inhibition of ~104 %. By contrast, in five additional experiments, sequential application of Aga IVA, GVIA and Nic–Nif at saturating concentrations resulted in a 79·6 ± 1·6 % reduction in peak current amplitude. Thus, the selectivity of one or more of these channel blockers may not be complete at the concentrations

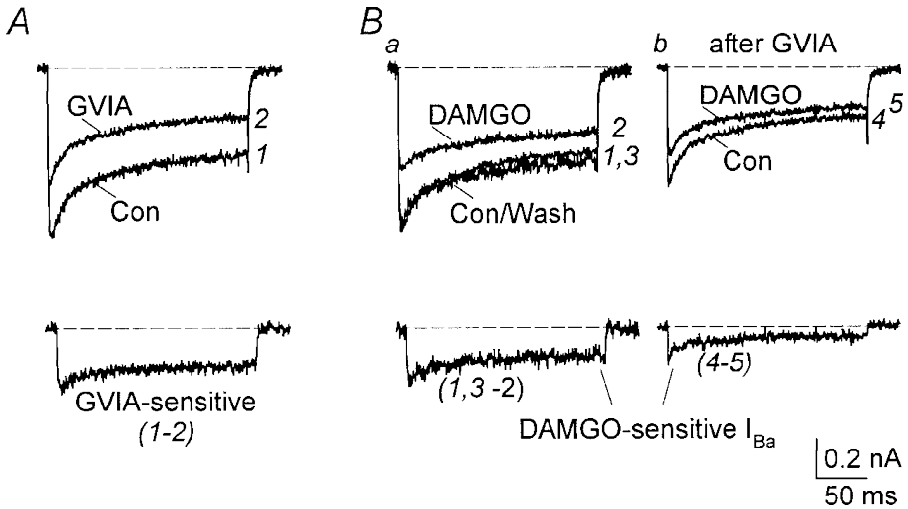

Figure 8. μ-Opioid receptor activation inhibited a portion of GVIA-sensitive N-type Ca^{2+} channel current

A, in this MNC superfusion of the N-type channel blocker GVIA (1 μM, 15 s) irreversibly blocked 36·5 % of the control current, similar to its effect in 10 other neurones. *B*, after irreversible blockade of N-type current by GVIA, the inhibitory effect of DAMGO (1 μM) was reduced by 57 %, from 33·1 % of control current (*Ba*) to 14·2 % suppression (*Bb*). This was similarly observed in four other neurones. The magnitudes of μ-opioid sensitive current before and after application of GVIA are shown in the subtraction traces (bottom traces).

employed here, in which case the proportion of total I_{Ba} contributed by N-, L- and P-type Ca^{2+} channels should be considered as reasonable estimates. The residual current that remained after co-application of the dihydropyridines, GVIA and Aga IVA (approximately 20% of total I_{Ba}; see Fig. 9*C*) could be entirely blocked by application of Cd^{2+} (200 μM) indicating that it was also contributed by VSCCs. Foehring and Armstrong (1996) have described a similar dihydropyridine/toxin-resistant component of current in these neurones that consists of Q-type Ca^{2+} current and a residual, pharmacologically undefined R-type Ca^{2+} channel current.

In the experiment depicted in Fig. 9*Aa* and *B*, we compared the inhibitory effect of DAMGO on I_{Ba} (indicated by the asterisks) before and after the sequential blockade of L-, P- and N-type Ca^{2+} channels by administration of Nic–Nif, Aga IVA and GVIA, respectively. In this neurone, blockade of L-type current with Nic–Nif (10 μM) did not modify the inhibition in I_{Ba} by DAMGO. By contrast, responses to the μ-opioid agonist were attenuated after blockade of P-type channels by Aga IVA (100 nM) and abolished subsequent to the blockade of N-type Ca^{2+} channels by application of GVIA (1 μM). This outcome is demonstrated in a different manner in Fig. 9*Ab* which shows the isolated L-, P- and N-type

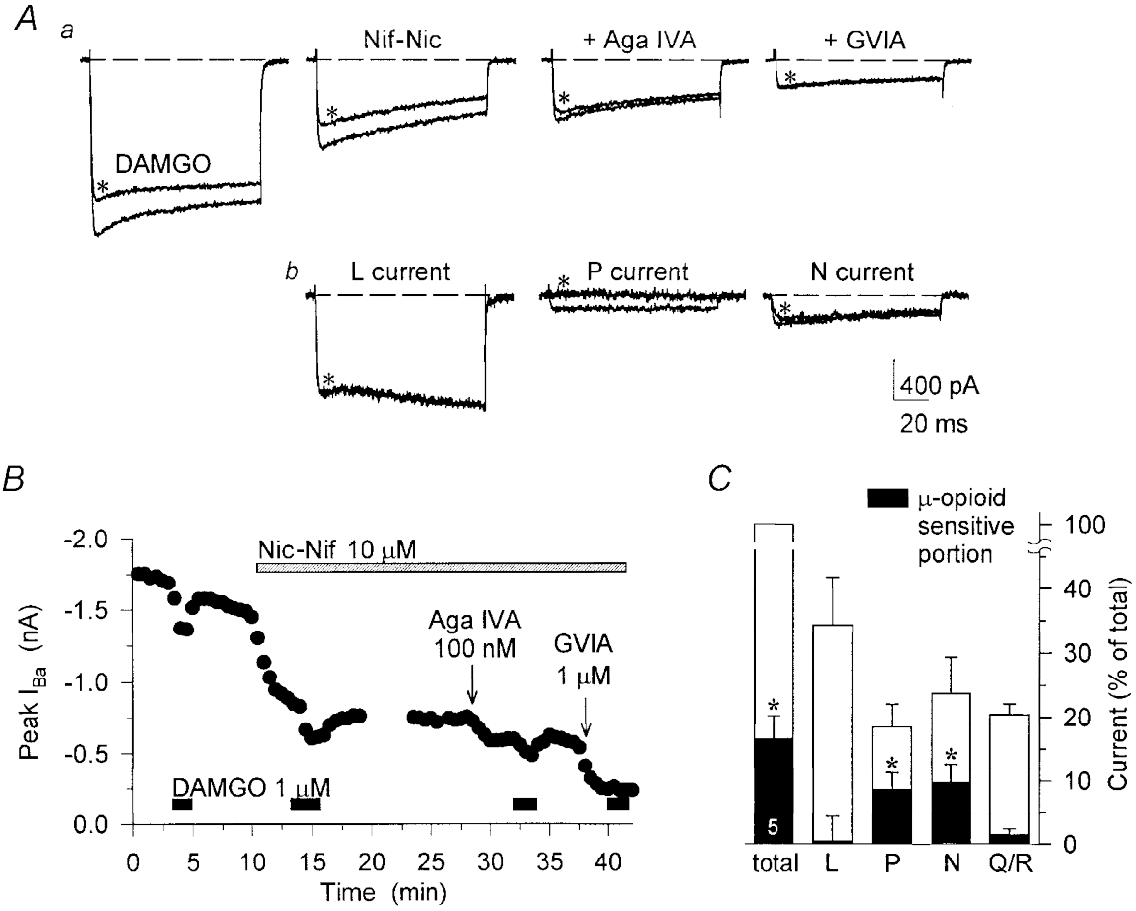

Figure 9. Co-application of N- and P-type channel antagonists eliminated the μ-opioid sensitive portion of I_{Ba} in MNCs

Aa, results from an occlusion experiment performed on a representative MNC reveal a reduction in the degree of DAMGO-induced (1 μM) inhibition of I_{Ba} (*) upon application of the P- and N-type Ca^{2+} channel blockers (100 nM Aga IVA and 1 μM GVIA, respectively) but not with the L-type channel blockers Nic–Nif (10 μM). *Ab*, the portions of total I_{Ba} contributed by L, P and N type Ca^{2+} channels in the same neurone were determined by subtraction of the current traces in *A* and demonstrate that application of the μ-opioid (*) eliminated P current and a portion of N current, but did not modify L current. *B*, time course of this experiment illustrates the reversible, DAMGO-induced reduction in I_{Ba} and the attenuation of the μ-opioid effect after application of Aga IVA and GVIA. The break in the time course corresponds to collection of *I–V* data. *C*, a graphical representation of the different contributions of Ca^{2+} channel subtype to the total I_{Ba} for five MNCs tested (open bars; L = 34·3 ± 7·5%, N = 23·7 ± 5·6%, P = 18·5 ± 3·5%, remainder = 20·4 ± 1·7% of total I_{Ba}), and the portion of each channel subtype current that was inhibited by the μ-opioid (filled bars; see text). * $P < 0{\cdot}05$.

currents, obtained by digital subtraction of corresponding current traces recorded in the absence and presence of the μ-opioid. The analysis clearly shows that DAMGO did not modify dihydropyridine-sensitive (L-type) current, but completely inhibited the Aga IVA-sensitive (P-type) current and reduced a small portion of GVIA-sensitive (N-type) current in this neurone. The bar graph in Fig. 9*C* summarizes the results from five similar experiments and depicts the percentage of control I_{Ba} contributed by each Ca^{2+} channel type (L, P, N and Q/R; open bars) and the corresponding opioid-sensitive portion of each type of Ca^{2+} channel current that contributed to the total DAMGO-induced reduction in I_{Ba} (filled bars). On average, μ-opioid receptor activation inhibited 46·3 $\pm$ 10·7 % of P-type current and 45·7 $\pm$ 11·9 % of N-type current, whereas neither L- nor Q/R-type currents were significantly affected. As the entire inhibitory response to DAMGO was eliminated after Aga IVA and GVIA, it was unlikely that any other Ca^{2+} channel type (i.e. Q/R-type) contributed μ-opioid sensitive current in these neurones. Taken together, these data demonstrate that μ-opioid receptors are negatively coupled to somatic N-and P-type Ca^{2+} channels in magnocellular neurones of the SON.

DISCUSSION

Magnocellular neurones in the SON, here termed as MNCs, project to the neural lobe where they primarily secrete OT into the bloodstream from their nerve endings. Endogenous opioids inhibit this secretion, acting at the level of the SON to reduce the firing rate of MNCs and by directly inhibiting Ca^{2+}-dependent release at their neurosecretory nerve terminals. It has been inferred from early electrophysiological studies that opioids may decrease the excitability of SON neurones by acting presynaptically to suppress excitatory input to the SON (Inenaga *et al.* 1994) and/or by inhibiting somatic Ca^{2+} conductance (Mason *et al.* 1988; Bourque *et al.* 1993; Inenaga *et al.* 1994), leading to suppression in spike discharge. To date, it has not been determined whether any of the ionic conductances that are essential for sustaining and/or driving cellular activity in the SON are specifically modulated by opioid receptor activation. The present study has shown that activation of μ-, but not δ- or κ-opioid receptors reversibly suppressed HVA Ca^{2+} channel currents in acutely isolated MNCs. Further, we have shown that μ-opioid receptors are negatively coupled to N- and P-, but not L- or Q/R-type Ca^{2+} channels in these neurones.

μ-Opioid receptors are localized to the postsynaptic membrane of magnocellular SON neurones

This study has provided strong evidence that μ-opioid receptors are functionally expressed in the postsynaptic membrane of MNCs. Although binding studies have demonstrated moderate levels of μ-opioid receptor expression in the SON (Sumner *et al.* 1990; Minami *et al.* 1994), it could not be determined whether these receptors were located at pre- or postsynaptic sites. Furthermore, while μ-opioid agonists have been shown previously to suppress the electrical activity of SON neurones directly (Wakerley *et al.* 1983), their site of action remained unclear. The presence of somatic μ-opioid receptors was demonstrated in this study by the ability of the μ-opioid agonist DAMGO to suppress HVA Ca^{2+} channel currents in a concentration-dependent manner and by complete reversibility of this effect by naloxone or the μ-opioid receptor-specific antagonist β-FNA. The inhibitory effect of DAMGO on I_{Ba} in MNCs was also shown to be rapid in onset, often associated with kinetic slowing, voltage dependent and largely reversed with depolarizing prepulses. By virtue of these properties, the modulation of I_{Ba} by activation of μ-opioid receptors conforms to a classical model of channel regulation mediated by a G-protein-coupled, membrane-delimited mechanism, as has been demonstrated for many types of central and peripheral neurones (Beech *et al.* 1992; Dolphin, 1995).

There is conflicting evidence regarding the ability of δ-opioid receptor agonists to regulate the activity of SON neurones. In the present study, administration of the highly selective δ-opioid receptor agonist DPDPE did not affect Ca^{2+} channel currents in MNCs. Moreover, it has been reported that δ-opioid agonists failed to modulate postsynaptic potentials in SON neurones in brain slices *in vitro* (Inenaga *et al.* 1994). In contrast, others have found that δ-opioid agonists act to suppress the firing of these neurones (Wakerley *et al.* 1983). Interpretation of the physiological significance of such δ-opioid receptor-mediated actions must be tempered by an awareness that results of binding studies do not support the presence of δ-opioid receptors in this brain region (Sumner *et al.* 1990; George *et al.* 1994).

We were also unable to demonstrate regulation of somatic Ca^{2+} channel current by κ-opioid agonists. This result is at odds with a previous report that κ-opioids suppress postsynaptic potentials in SON neurones in hypothalamic brain slices and decrease the duration of Ca^{2+} action potentials recorded under conditions of potassium conductance blockade (Inenaga *et al.* 1994). The results of our immunocytochemical experiments with a κ_1-opioid receptor specific antibody revealed the presence of κ-opioid receptor-like protein in dissociated SON somata (Fig. 5*D*), but only after permeabilization of the isolated cells. The failure to detect κ_1-opioid receptor immunoreactivity in non-permeabilized cells is evidence for the specificity of the antibody for the cytoplasmic C-terminal portion of the receptor (see Methods). If a portion of the κ-opioid receptor protein detected by immunostaining represents receptors that are expressed within the somatic membrane of these neurones, then our data would suggest that κ-opioid receptors are not functionally coupled to VSCCs in the cell bodies of SON neurones. Moreover, we have recently demonstrated that κ-opioid receptor agonists selectively modulate HVA Ca^{2+}

currents in isolated neurohypophysial nerve endings and inhibit Ca^{2+}-dependent exocytotic release (Rusin *et al.* 1997). These data, coupled with our failure to demonstrate coupling of the κ-opioid receptors to VSCCs in MNC somata, suggest that at least a portion of somatically localized κ-opioid receptor protein represents receptors that are targeted for transport and expression at the nerve terminal region where they mediate presynaptic actions of opioid receptor activation. Nonetheless, we remain cognizant that restricting electrophysiological recording to isolated SON cell bodies, as done here, would preclude the possibility of measuring responses arising from interactions of opioid agonists with dendritically localized receptors. In summary, our data suggest that previously reported electrophysiological effects by κ-opioid agonists in recordings from SON neurones (e.g. inhibition of Ca^{2+} current) are likely to reflect either the activation of presynaptic or dendritically localized receptors, or the activation of postsynaptic receptors that are coupled with ion channels other than VSCCs or are due to non-specific actions.

Inhibitory coupling of μ-opioid receptors to distinct Ca^{2+} channel subtypes

Five distinct Ca^{2+} channel types (T, L, N, P and Q) have been previously described in MNCs based on their biophysical and pharmacological properties (Fisher & Bourque, 1995*a*; Foehring & Armstrong, 1996). We relied on the use of Ca^{2+} channel blockers specific for L- (dihydropyridines), N- (GVIA) and P-type (Aga IVA) channels to isolate three distinct components of whole-cell I_{Ba}. A detailed kinetic analysis of these currents has already been provided by Fisher & Bourque (1995*a*), and therefore, we focused our attention on the identification of specific Ca^{2+} channel types that serve as targets of μ-opioid action. Nevertheless, the finding here that L-type currents in MNC somata were preferentially sensitive to inhibition by Nic and largely unaffected by Nif is at odds with what has been reported previously, and thus warrants brief discussion.

It is conceivable that the affinity of Nic binding to L-type channels may be more steeply dependent on membrane depolarization than that of Nif (Bean, 1989), in which case the differences observed in inhibitory efficacy between the two dihydropyridines may largely reflect an artifact of experimental conditions. However, it is important to note that the L-type currents recorded in the neurohypophysial nerve endings are inhibited by Nic, but not by Nif (Wang *et al.* 1993). Furthermore, the binding site for dihydropyridine antagonists is localized on the α_1 subunit of both the C and D class of L-type Ca^{2+} channels (Williams *et al.* 1992). Such considerations lead us to suggest the possibility that the L-type Ca^{2+} channels that are expressed in the nerve endings and cell bodies of these MNCs might have different α-subunit composition. Consistent with this are recent findings from immunohistochemical and *in situ* hybridization studies that both the α_{1C} and α_{1D} subunits of L-type channels are localized to the cell bodies and proximal dendrites of central neurones (Hell *et al.* 1993), whereas only the α_{1D} subunit has been detected in the hypothalamus (Ludwig *et al.* 1997). As the D class of L-type channels is known to be the predominant species in neuroendocrine cells, studies are now underway to determine whether the relative contribution of α_{1C} and α_{1D} subunits to somatic and terminal L-type channels is regionally specific in MNCs.

This report reveals a profile for opioid regulation of Ca^{2+} channel currents in somata of magnocellular SON neurones that is very different from that recently reported for Ca^{2+} channels expressed in their neurohypophysial nerve endings. Activation of μ-, but not δ- or κ-opioid receptors was found to selectively inhibit N- and P-type Ca^{2+} channel currents in isolated MNC somata. In contrast, we recently reported that HVA Ca^{2+} currents recorded from isolated neuroendocrine nerve terminals of these neurones are suppressed by activation of κ-, but not δ- or μ-opioid receptors and that these receptors are negatively coupled to L-, N- and P/Q-type HVA Ca^{2+} channels (Rusin *et al.* 1997). Our demonstration of discrete profiles of Ca^{2+} channel-type expression and patterns of regulation by μ- and κ-opioid receptors in these two spatially and functionally distinct domains of MNCs emphasizes the need for caution when attempting to equate information derived from cell bodies to situations that exist at the nerve terminal. The SON of the hypothalamus reveals dense binding of κ-opioid receptor sites, moderate levels of μ-opioid receptor binding and no δ-opioid receptors (Mansour *et al.* 1995). In contrast, the neurohypophysial nerve endings appear to express only κ-opioid receptors (Herkenham *et al.* 1986). Furthermore, while the SON neurones express classical T-, N-, L-, P-, and Q-type Ca^{2+} currents and a pharmacologically unidentified R-type current component, the nerve endings of these cells contain L-, a novel N(t)- and P/Q-type Ca^{2+} currents (see Lemos & Nowycky, 1989; Wang *et al.* 1992; Fisher & Bourque, 1996) as well as a toxin-resistant R-type component (Wang *et al.* 1998). Hence, it seems reasonable to speculate that the different profiles of opioid receptor coupling to particular channel types that occur in the somatic and terminal regions of MNCs might enable more precise regulation of neurosecretion in response to homeostatic requirements and changes in environmental challenges.

Functional significance of μ-opioid regulation of specific Ca^{2+} channel types in MNCs

The finding that opioids act exclusively via μ-opioid receptors to modulate Ca^{2+} channel currents in MNCs adds to a growing body of evidence which highlights the role of these channels as an important effector target of μ-opioid receptor action in both central and peripheral neurones (e.g. Schroeder *et al.* 1991; Rhim & Miller, 1994; Soldo & Moises, 1997). In the hypothalamo-neurohypophysial system, neurohormone release from the nerve endings of MNCs is highly dependent on characteristic bursts of action potentials

generated by these neurones (Dutton & Dyball, 1979; Bicknell & Leng, 1981). This burst firing is, in turn, dependent on entry of extracellular Ca^{2+} through VSCCs (Andrew, 1987; Inenaga *et al.* 1992). Furthermore, the pattern and rate of burst firing that is requisite for neurohormone secretion has been shown to rely on the subsequent activation of Ca^{2+}-dependent potassium conductances (Kirkpatrick & Bourque, 1996). Conceivably, the net effect of suppression of VSCCs by opioids might be to attenuate the Ca^{2+}-activated potassium conductances that provide for auto-inhibitory feedback during continuous or spontaneous burst firing. This effect might in turn be manifested by subtle alterations in intraburst spike patterning, rather than overt changes in the rate of spontaneous firing (Wakerley *et al.* 1983; Wuarin & Dudek, 1990; but see Inenaga *et al.* 1994) which have generally been attributed to opioid activation of an inwardly rectifying potassium conductance and/or to modulation of presynaptic inputs. These considerations, along with our present findings, strongly implicate regulation of VSCC activity as an important cellular mechanism whereby opioids can act to control neuroendocrine cellular activity and neurosecretion. It is also important to consider that changes in this opioidergic tone may influence other Ca^{2+}-dependent processes in the soma such as protein phosphorylation, enzyme activity, and gene expression, thereby providing a potential means for exerting extended modulation of cellular excitability and neurosecretory function.

Lastly, it is tempting to speculate that opioid regulation of voltage-dependent Ca^{2+} influx in MNC somata, while important in providing patterned action potential discharge to the nerve endings, may also be important for regulating somatodendritic release in the hypothalamus. Evidence in support of this has recently been reviewed by Russell *et al.* (1995) who reported differences in opioidergic tone within the hypothalamo-neurohypophysial system of parturient rats. Although κ-opioids act strongly to regulate OT release from the nerve endings of SON and paraventricular nucleus neurones during the early stages of pregnancy, this inhibitory tone is lost during late pregnancy, unleashing parturition. Paradoxically, during these late stages of pregnancy, endogenous opioidergic tone appears to increase at the level of the hypothalamus, as evidenced by the ability of centrally administered naloxone or morphine to regulate OT release from the cell bodies of SON and paraventricular nucleus neurones (Douglas *et al.* 1995; Ingram *et al.* 1996). This opioidergic tone, presumably mediated via μ-opioid receptors, might act as a protective backup system for regulating the OT-generated activity of hypothalamic neuroendocrine neurones and orchestrating the unleashing or prevention of parturition. The results from the present study support and provide a mechanism for this model, demonstrating the inhibition of somatic voltage-dependent Ca^{2+} influx by the activation of μ-opioid receptors on magnocellular neurones of the SON. A further implication of the data presented here is the opportunity for tonic opioidergic inhibition in MNC neurones that may be expressed in both male and female rats, and hence subserve important homeostatic functions in addition to those that are not directly related to parturition.

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