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OUTWARD POTASSIUM CURRENTS OF SUPRAOPTIC MAGNOCELLULAR NEUROSECRETORY CELLS ISOLATED FROM THE ADULT GUINEA PIG

by

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ABSTRACT

OUTWARD POTASSIUM CURRENTS OF SUPRAOPTIC MAGNOCELLULAR NEUROSECRETORY CELLS ISOLATED FROM THE ADULT GUINEA PIG

By

Michael David Hlubek

(1). Whole-cell voltage-clamp recordings revealed several types of outward K^+ current present in somata of magnocellular neurosecretory cells (MNCs) dissociated from the supraoptic nucleus of the adult guinea pig were. These currents were identified on the basis of their voltage dependence, kinetics, pharmacology and Ca^{2+} dependence.

(2) The predominant K^+ current evoked from a holding potential of -40 mV was slowly-activating, long-lasting, tetraethylammonium (TEA)-sensitive and showed little steady-state inactivation. Also, this current was reduced by extracellular Cd^{2+}. These data suggest that in supraoptic MNCs classical Ca^{2+}-insensitive, delayed rectifier channels (K_V) and Ca^{2+}-sensitive, non-inactivating channels ($K_{(Ca)}$) both contribute to the sustained current.

(3) A transient, low-threshold K^+ current which was 4-aminopyridine (4AP)-sensitive and showed significant steady-state inactivation was evoked along with the sustained current from a holding potential of -90 mV. Based on these

characteristics, this current corresponds to the A-current (I_A) described in other neurons.

(4) I_A was activated when Ca^{2+} influx was blocked or when Ca^{2+} was absent from the extracellular medium, suggesting that Ca^{2+} influx is not necessary for activation of the current.

(5) In many recordings, a transient, 4-AP-insensitive outward current was evoked from a holding potential of -40 mV. This high-threshold transient K^+ current was abolished by extracellular Cd^{2+}, Charybdotoxin (ChTX) or tetraethylammonium (TEA) and was absent when extracellular Ca^{2+} was replaced by Sr^{2+}, suggesting that it is a transient Ca^{2+}-dependent K^+ current.

(6) Current-clamp recordings revealed that the temporal activation of several K^+ channel types shapes MNC action potential repolarization. Also, it was shown that frequency-dependent spike broadening in dissociated MNCs results from a reduction in $I_{K(A)}$ during repetitive firing.

(7) We conclude that the presence of multiple types of K^+ current may, in part, underlie the complex firing patterns of oxytocinergic and vasopressinergic MNCs.

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ABBREVIATIONS

ACh	acetylcholine
ω-Aga IVA	ω-agatoxin IVA
AHP	after-hyperpolarization
cAMP	cyclic adenosine monophosphate
4-AP	4-aminopyridine
ATP	adenosine triphosphate
ω-CgTX GVIA	ω-conotoxin GVIA
ChTX	charybdotoxin
CSA	cross-sectional area
DAB	3,3'-diaminobenzidine
DAP	depolarizing after-potential
DHPs	dihydropyridines
DTX	dendrotoxin
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EPSPs	excitatory postsynaptic potentials
GABA	γ-aminobutyric acid
HAP	hyperpolarizing after-potential
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HNS	hypothalamo-neurohypophysial system
5-HT	5-hydroxytryptamine
HVA	high voltage-activated
IbTX	iberatoxin
IPSPs	inhibitory postsynaptic potentials
LVA	low voltage-activated
MNCs	magnocellular neurosecretory cells
NP	neurophysin
OT	oxytocin
OVLT	organum vasculosum lamina terminalis
PIPES	piperazine-N,N'-bis-(2-ethane-sulfonic acid)
PVN	paraventricular nucleus
mRNA	messenger ribonucleic acid
SON	supraoptic nucleus
TEA	tetraethyammonium
TTX	tetrodotoxin
VP	vasopressin

INTRODUCTION

I. Neuronal K^+ Channels and Currents

K^+ channels comprise a family of integral membrane proteins which control membrane excitability in a variety of cell types by selectively catalyzing K^+ current flow. In general, the equilibrium potential for K^+ (E_K = the potential at which there is no net K^+ current flow) is more negative than the resting membrane potential (E_R = the potential at which there is no net current flow) in neurons. K^+ channel currents therefore draw the membrane potential closer to E_K and thus farther from the threshold for action potential initiation. In this way K^+ currents are intrinsically inhibitory in that they oppose excitatory events. The importance of K^+ currents in controlling membrane excitability, along with the tremendous diversity of K^+ channels relative to that of other ion channels, may indicate that the characteristic and diverse electrical behavior exhibited by different neurons is largely defined by their complement of K^+ channels. Accordingly, any attempt to describe the intrinsic mechanisms which underlie the electrical behavior of specific neurons must consider the complement of K^+ channels present in the neuronal membrane.

A. Types of K^+ Channels and Currents

The ionic selectivities of different K^+ channels appear to be identical. They all exhibit an ion permeability sequence of $Tl^+ > K^+ > Rb^+ > NH_4^+$, are relatively impermeable to Na^+ and Ca^{2+}, and are blocked by Cs^+ (Hille, 1992). Nevertheless, K^+ channels may be distinguished based on several different criteria such as voltage dependence of activation and inactivation, kinetics, pharmacology (including Ca^{2+} sensitivity), and molecular structure.

1. Delayed Rectifier K^+ Channels (K_V)

Two main criteria must be met in order for a K^+ channel to be classified as a delayed rectifier (Rudy, 1988). First, the voltage dependence and kinetics of the macroscopic current must be similar to that described by Hodgkin and Huxley (1952) for the "delayed rectifier" current ($I_{K(V)}$) in giant squid axons. The current described by Hodgkin and Huxley exhibited delayed activation and little or no inactivation over the duration of a depolarizing voltage step. Also, the membrane conductance (ease of current flow) changed with voltage, a property called rectification in electric circuit theory. The second criterion which defines K_V channels is one of exclusion. Activation of these channels does not depend on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The non-dependence on $[Ca^{2+}]_i$ distinguishes K_V channels from Ca^{2+}-dependent K^+ channels (Meech and Standen, Hermann and

Hartung, 1983; 1975; Sah, 1996) which produce macroscopic currents that are otherwise indistinguishable from $I_{K(V)}$.

Although all neurons appear to express at least one type of K_V channel, the properties of K_V channels can vary greatly among neurons - an indication that different K^+ channels are probably expressed. Most K_V channels, however, exhibit a "high threshold" for activation, opening at membrane potentials more positive than -40 mV, and are sensitive to block by extracellular tetraethylammonium (TEA) in millimolar concentrations (Thompson, 1977; Numann *et al.*, 1987; Hille, 1992). Functionally, current catalyzed by K_V channels in neurons contributes to action potential repolarization and thus helps determine action potential duration (Hodgkin and Huxley, 1952; Thompson, 1977; Storm, 1987). K_V currents can also contribute to the generation of the after-hyperpolarization (AHP) immediately following individual action potentials (Storm, 1987).

2. A-Type K^+ Channels (K_A)

A rapidly-activating and inactivating current carried through K_A channels was first described in *Anisodoris* snail neurons (Neher, 1971; Connor and Stevens, 1971) and later in many other neurons (Rogawski, 1985). Because K_A channels inactivate rapidly following activation despite a maintained activating stimulus, the current through these channels is transient. The time-dependent inactivation of an A-current ($I_{K(A)}$) typically proceeds with a time constant τ (the time

required for the macroscopic $I_{K(A)}$ to fall to 1/e'th (≈37%) of its peak value) of 10 to 100 ms. K_A channels also exhibit a "low threshold" for activation, opening at potentials subthreshold to that required for action potential initiation, and almost complete steady-state inactivation at membrane potentials more positive than -50 mV. Thus K_A channels can only be activated by depolarizing stimuli after a period in which the membrane is "hyperpolarized" to potentials more negative than -50 mV. The voltage-dependence of K_A channel inactivation is such that in neurons which exhibit an E_R more positive than -50 mV, K_A channels are largely unavailable for activation.

Dendrotoxin (DTX), a peptide toxin present in eastern green mamba snake venom, is a very potent blocker of K_A channels in central neurons (Halliwell *et al.*, 1986; Dreyer, 1990). This compound typically blocks $I_{K(A)}$ with an IC_{50} (the concentration that inhibits the macroscopic $I_{K(A)}$ by 50 percent) of less than 50 nM. The convulsant 4-aminopyridine (4-AP) blocks K_A channels at millimolar concentrations but is less effective at blocking other K^+ channels (Gustafsson *et al*, 1982; Numann *et al.*, 1987). Sensitivity to block by 4-AP is thus commonly used to detect the presence of K_A channels.

Connor and Stevens (1971) were the first to show that $I_{K(A)}$ could regulate the frequency of repetitive action potential firing in neurons. Although K_A channels are largely inactivated (due to steady-state inactivation) near

E_R, the AHP that follows individual action potentials removes a portion of the inactivation. Hence K_A channels are activated as the cell depolarizes and the resultant $I_{K(A)}$ slows the return of the membrane potential toward the threshold for action potential initiation. Over time, however, $I_{K(A)}$ inactivates and the cell is again allowed to depolarize unimpeded. In this way, activation of $I_{K(A)}$ prolongs the interspike interval and slows the firing frequency in a repetitively firing neuron. $I_{K(A)}$ can also contribute to the repolarization of the action potential (Tanouye *et al.*, 1981; Storm, 1987) and to the delay before the generation of an action potential following a depolarizing stimulus (Daut, 1973; Byrne, 1980). These latter functions of $I_{K(A)}$ are observed in neurons which have a sufficient number of K_A channels available for activation near E_R.

3. Ca^{2+}-Dependent K^+ Channels (K_{Ca})

Activation of K_V and K_A channels is voltage-dependent and does not depend on $[Ca^{2+}]_i$. Activation of K_{Ca} channels, on the other hand, is strongly dependent on $[Ca^{2+}]_i$ (Meech and Standen, 1975; Hermann and Hartung, 1983; Sah, 1996). The probability that these channels will activate increases with elevations in $[Ca^{2+}]_i$. Accordingly, the voltage dependence of K_{Ca} channel current ($I_{K(Ca)}$) mirrors that of Ca^{2+} current. This is because changes in Ca^{2+} influx through voltage-dependent Ca^{2+} channels affects $[Ca^{2+}]_i$, and thus the

activation of K_{Ca} channels. Current-voltage (I-V) relationships for K_{Ca} channel currents ($I_{K(Ca)}$) therefore exhibit a characteristic "n-shape" which reflects voltage-dependent changes in Ca^{2+} influx. K_{Ca} channels can also be activated by Ca^{2+} released from intracellular storage sites *via* the action of neurotransmitters coupled to second messenger systems (Nicoll, 1988).

There are at least two types of neuronal K_{Ca} channels (Sah, 1996), the so-called high- or "big"-conductance (BK_{Ca}) channels (Marty, 1981; Blatz and Magleby, 1984; Romey and Lazdunski, 1984) and the low- or "small"-conductance (SK_{Ca}) channels (Romey and Lazdunski, 1984; Blatz and Magleby, 1986). BK_{Ca} channels exhibit single-channel conductances of 100-250 pS and are voltage-dependent even at constant Ca^{2+} concentrations. These channels require 1-10 μM Ca^{2+} for activation at potentials near E_R (-50 to -70 mV). In contrast, SK_{Ca} channels exhibit single-channel conductances of 18-50 pS and little voltage dependence at constant Ca^{2+} concentrations. These channels require 100-400 nM Ca^{2+} for activation, and thus are much more sensitive to Ca^{2+} than BK_{Ca} channels. Both channel types produce macroscopic currents characterized by delayed activation and little time-dependent inactivation, thus resembling $I_{K(V)}$ kinetically.

BK_{CA} and SK_{Ca} channels can also be distinguished based on their pharmacological properties. BK_{Ca} channels can be blocked by nanomolar concentrations (IC_{50}<100 nM) of the

scorpion toxins charybdotoxin (ChTX) and iberatoxin (IbTX), and by low millimolar concentrations of TEA (Miller *et al.*, 1985; Galvez *et al.*, 1990; Sah, 1996). These channels are not sensitive to block by apamin (Romey and Lazdunski, 1984; Pennefather *et al.*, 1985), a peptide toxin isolated from bee venom. SK_{Ca} channels, on the other hand, are not sensitive to block by ChTX, IbTX or TEA, but are blocked by nanomolar concentrations (IC_{50}<50 nM) of apamin (Blatz and Magleby, 1986; Lang and Ritchie, 1990; Park, 1994).

Activation of BK_{Ca} channels produces a current ($I_{BK(Ca)}$) which contributes to action potential repolarization. Block of these channels results in action potentials of increased duration (Romey and Lazdunski, 1984; Pennefather *et al.*, 1985; Storm, 1987). Activation of SK_{Ca} channels produces a current ($I_{SK(Ca)}$) which is thought to underlie the prolonged AHP following individual action potentials, as demonstrated by the action of apamin (Romey and Lazdunski, 1984; Pennefather *et al.*, 1985; Kirkpatrick and Bourque, 1996).

In addition to the above Ca^{2+}-dependent channel currents, there have been reports of a Ca^{2+}-dependent transient K^+ current which contributes to action potential repolarization in some neurons (Ribera and Spitzer, 1987; Neely and Lingle, 1992). This current is distinct from $I_{K(A)}$ in that it is 4-AP-insensitive and can be activated from membrane potentials at which $I_{K(A)}$ is fully inactivated. The pharmacological properties of the current are identical to those of BK_{Ca} channel currents, leading to speculation that

it is carried by a BK_{Ca} channel variant which has as part of its molecular structure a component which confers a mechanism for inactivation.

4. Inward Rectifier K^+ Channels (K_{IR})

Many neurons express K^+ channels which show increased activation during hyperpolarizing rather than depolarizing stimuli (Adams and Halliwell, 1982; Constanti and Galvan, 1983). The I-V relationship for current carried through these channels reveals a decrease in slope conductance with depolarization, a property known as "anomalous" or "inward" rectification. K_{IR} channel currents ($I_{K(IR)}$) are characterized by a lack of time-dependent inactivation and a strongly voltage-dependent block by intracellular Mg^{2+} (Vandenberg, 1987). It is believed that the inward rectification exhibited by these channels results from a voltage-dependent block of K^+ current flow by cytoplasmic Mg^{2+} ions which plug the K_{IR} channels. There are no known pharmacological agents which selectively block K_{IR} channels by acting from the extracellular side of the cell.

K_{IR} channels do pass some outward K^+ current in the range of membrane potentials between E_K and E_R. Under physiological conditions, the membrane potential of a neuron rarely becomes more negative than E_K. It is believed that K_{IR} channels help stabilize E_R near E_K by conducting outward current in the voltage range just positive to E_K. Increased depolarization then increases the block of K_{IR} channels by

Mg^{2+}, thus allowing the membrane potential to change more freely. The biophysical and pharmacological properties of the major voltage- and Ca^{2+}-dependent K^+ channels are summarized in Tables 1 and 2, respectively.

5. Other Types of K^+ Channels

A number of other neuronal K^+ channels have been discovered which cannot be placed in any of the major classes described above. The most notable of these are either receptor-coupled or modulated by intracellular metabolites. Two receptor-coupled K^+ channels have been studied extensively - the muscarinic-inactivated K^+ channel (K_M) and the 5-hydroxytryptamine (5-HT)-inactivated K^+ channel ($K_{5\text{-}HT}$). K_M channels exhibit voltage-dependent, delayed activation at membrane potentials more positive than -65 mV and little inactivation (Brown and Adams, 1980; Brown, 1988). Activation of these channels is inhibited by muscarinic acetylcholine (ACh) receptor agonists. Functionally, these channels contribute a K^+ current ($I_{K(M)}$) that, when decreased by the neurotransmitter ACh, enhances the responsiveness to depolarizing stimuli. $K_{5\text{-}HT}$ channels are weakly voltage-dependent and normally activated at E_R (Klein *et al.*, 1982; Shuster *et al.*, 1985). The neurotransmitter 5-HT inactivates these channels *via* cyclic adenosine monophosphate (cAMP)-dependent phosphorylation, resulting in a decreased K^+ current ($I_{K(5\text{-}HT)}$) and enhanced membrane excitability. In the absence of 5-HT, $K_{5\text{-}HT}$ channel

Table 1

Biophysical and Pharmacological Properties of Voltage-Dependent K^+ Channels

Channel type	K_V	K_A	K_{IR}
Activation threshold (mV)	>-40	<-50	<-50
inactivation kinetics	slow τ = ??	fast τ < 100 ms	slow τ = ??
Single-channel conductance (pS)	5-60	1-20	5-30
Block by TEA	+	-	-
Block by 4-AP	±	+	-
Block by DTX	-	+	-

Data modified from Rudy, 1988; TIPS 1996 Receptor and ion channel nomenclature supplement (seventh ed.).

Table 2

Biophysical and Pharmacological Properties of Ca^{2+}-Dependent K^+ Channels

Channel type	BK_{Ca}	SK_{Ca}
$[Ca^{2+}]_i$ needed for activation	1-10 μM (at -50 to -70 mV)	100-400 nM
Voltage-dependence	+	-
Single-channel conductance (pS)	100-250	18-50
Block by TEA	+	-
Block by ChTX/IbTX	+	-
Block by apamin	-	+

Data modified from Rudy, 1988; Sah, 1996; TIPS 1996 Receptor and ion channel nomenclature supplement (seventh ed.).

currents help stabilize E_R near E_K.

Voltage-insensitive K^+ channels regulated by changes in intracellular adenosine triphosphate (ATP) have also been described in neurons (Ashford *et al.*, 1989; Politi *et al.*, 1989). These inwardly-rectifying, ATP-sensitive K^+ channels (K_{ATP}) tend to be open when the concentration of intracellular ATP is low, but tend to close as the concentration (IC_{50} between 15-100 μM) of this purine nucleotide rises. Although the exact function of K_{ATP} channels in neurons is not known, such channels would contribute a hyperpolarizing current ($I_{K(ATP)}$) as cellular energy stores are depleted.

B. General K^+ Channel Structure

The protein sequences of cloned K^+ channels from invertebrate and vertebrate neurons exhibit a remarkable degree of similarity which has been conserved during evolution (Yokoyama *et al.*, 1989). From analysis of these sequences (see review by Catterall, 1995), it is believed that K^+ channel gene products represent a single subunit that consists of amino- (N) and carboxy-terminal (C) segments which reside in the intracellular compartment, and a core region which contains six membrane-spanning segments (S1-S6) surrounding a pore-forming loop consisting of two short segments (SS1-SS2) that dip into the membrane. Evidence suggests that the SS1-SS2 segment constitutes the entire or a major part of the channel pore. Mutations in

this segment have been shown to modify channel conductance properties (Hartman *et al.*, 1991), ion selectivity and susceptibility to pharmacological block (MacKinnon and Miller, 1989; MacKinnon and Yellon, 1990). Four separate subunits are believed to come together to form a single, functional homomultimeric or heteromultimeric K^+ channel.

The S4 segment in each subunit contains a number of positively-charged amino acid residues that lie within the transmembrane electrical field. It is believed that these residues function as a sensor for voltage-dependent gating of K^+ channels. Mutations which neutralize the amino acid charges shift the voltage dependence of channel gating in a predictable manner (Logethetis *et al.*, 1991; Papazian *et al.*, 1991), indicating that the S4 segment represents a major part of the voltage-sensing apparatus. One theory proposes that the positively-charged amino acid residues move through the membrane in response to changes in transmembrane potential, causing conformational changes of the channel protein which open or close the channel pore (Catterall, 1988).

The N-terminal segment is believed to confer a mechanism for inactivation. Mutations of this segment (Zagotta *et al.*, 1990) or removal of the segment by proteolytic enzymes applied to the intracellular side of the channel (Hoshi *et al.*, 1990) disrupts inactivation. Furthermore, application of synthetic peptides containing amino acid sequences found in this segment to the

intracellular side of the channel can convert a normally non-inactivating channel current to one that inactivates (Zagotta *et al.*, 1990). Observations such as these have led to the belief that a "ball and chain" mechanism leads to inactivation of K^+ channels, as has been proposed for Na^+ channels (Armstrong, 1981). According to this model, an inactivation particle (or "ball"), tethered (by an amino acid "chain") to the channel but free to diffuse in the cytoplasm, moves into the intracellular mouth of the pore and blocks it, resulting in inactivation of the ionic current. Finally, the N- and C-terminal segments each contain many potential sites for phosphorylation by protein kinases (Catterall, 1993), which allow for the regulation of channel behavior under different physiological conditions.

II. Overview of Neuronal Ca^{2+} Channels

Like K^+ channels, voltage-dependent Ca^{2+} channels appear to be ubiquitous components of excitable cells, including neurons (See reviews by Carbone and Swandulla, 1989; Scott *et al.*, 1991; Tsien *et al.*, 1995). Ca^{2+} channels have been studied extensively over the past decade, partly as a result of recognition that they contribute to the regulation of a diverse array of Ca^{2+}-dependent cellular functions. These functions, such as the regulation of membrane excitability, the regulation of enzymes and ion channels (e.g., K_{Ca} channels), and the triggering of exocytosis are carefully regulated, in part by Ca^{2+} channels mediating Ca^{2+} influx.

Voltage-dependent Ca^{2+} channels can be broadly classified on the basis of their voltage range of activation. Those channels which are activated at membrane potentials more negative than -50 mV are often referred to as "low-threshold" or "low voltage-activated" (LVA), while those activated at membrane potentials more positive than -20 mV are often referred to as "high-threshold" or "high voltage-activated" (HVA).

A. Low Voltage-Activated (LVA) Ca^{2+} Channels

To date, only one type (the "T-type") of LVA Ca^{2+} channel has been characterized (Nowycky *et al.*, 1985; Fox *et al.*, 1987a, 1987b). T-type Ca^{2+} channels exhibit a low threshold for activation (<-50 mV) and rapid inactivation kinetics (τ<50 ms). Also, these channels carry tiny and transient (hence the term T-type channel) unitary Ba^{2+} currents (slope conductance $\approx$ 8 pS) and are resistant to organic compounds known to block Ca^{2+} channels. T-type Ca^{2+} channels are more sensitive to block by Ni^{2+} than to Cd^{2+}, whereas the reverse is usually true for HVA Ca^{2+} channels.

B. High Voltage-Activated (HVA) Ca^{2+} Channels

As stated previously, HVA Ca^{2+} channels are activated at membrane potentials more positive than -20 mV. All channels in this class tend to be more sensitive to block by Cd^{2+} than to Ni^{2+}. Subtypes of HVA Ca^{2+} channels can be distinguished based on differences in their inactivation

rates, single-channel conductances, and pharmacology.

"L-type" Ca^{2+} channels (Nowycky *et al.*, 1985; Fox *et al.*, 1987a, 1987b) carry *l*arge unitary Ba^{2+} currents (slope conductance ≈ 25 ps) and produce macroscopic currents which are *l*ong-lasting (hence the term L-type channel). The 1,4-dihydropyridines (DHPs) represent a class of synthetic organic compounds used to detect the presence of L-type Ca^{2+} channels. At low concentrations, DHP antagonists block L-type Ca^{2+} channels but are less effective at blocking other Ca^{2+} channels.

The peptide toxin ω-conotoxin GVIA (ω-CgTX GVIA), isolated from the venom of the cone snail *Conus geographus*, blocks a component of DHP-resistant, HVA Ca^{2+} channel current present in neurons (Aosaki and Kasai, 1987; Plummer *et al.*, 1989; Randall and Tsien, 1995). This ω-CgTX GVIA-sensitive current component is contributed by "N-type" Ca^{2+} channels - so called because they were initially described as being *n*either T nor L and found only in *n*eurons (Nowycky *et al.*, 1985). N-type Ca^{2+} channels exhibit slope conductances of intermediate value (12-18 pS) and inactivation kinetics (τ=50-500 ms) which vary considerably in different neurons (see review by Scott *et al.*, 1991).

First described in cerebellar *Purkinje* neurons (hence the term "P-type" channel), P-type channels are largely resistant to DHPs and ω-CgTX GVIA (Llinás, *et al.*, 1989, 1992a). These channels have conductances between 10 to 20 pS and produce macroscopic currents which exhibit little

inactivation. Low concentrations (IC_{50}≈2 nM) of the peptide toxin ω-agatoxin IVA (ω-Aga IVA), isolated from the venom of the funnel web spider *Agelenopsis aperta*, selectively block P-type channels (Mintz *et al.*, 1992a).

ω-Aga IVA can be used to reveal a second component of HVA current resistant to DHPs and ω-CgTX GVIA (Wheeler *et al.*, 1994; Randall and Tsien, 1995). This component which exhibits slow inactivation (≈35% inactivation over 100 ms) is blocked by high concentrations of ω-Aga IVA (IC_{50}≈100 nM) The channels responsible for the current have been designated "Q-type" to distinguish them from P-type channels. A residual or resistant component of Ca^{2+} current is not sensitive to DHPs, ω-CgTX GVIA, or ω-Aga IVA (Mintz *et al.*, 1992a, 1992b). Consequently, the channels responsible for the current have been designated "R-type". As with the T-type channels, no selective blocker of R-type channels has been identified. Table 3 summarizes the biophysical and pharmacological properties of the types of voltage-dependent Ca^{2+} channels found in neurons.

III. General Hypothalamo-Neurohypophysial System (HNS) Features

A. Historical Overview of HNS Research

Two nonapeptide hormones, oxytocin (OT) and vasopressin (VP), are secreted in the posterior pituitary gland from axon terminals of magnocellular neurosecretory cells (MNCs) which have their cell bodies located in the supraoptic

TABLE 3

Biophysical and Pharmacological Properties of Voltage-Dependent Ca^{2+} Channels

Channel type	T	N	L	P	Q	R
Activation	LVA	HVA	HVA	HVA	HVA	HVA
Inactivation kinetics	fast (τ<50 ms)	moderate (τ<500 ms)	slow (τ=??)	slow (τ=??)	moderate (35% over 0.1 s)	moderate (τ=??)
Single-channel conductance (pS)	≈8	≈25	12-18	???	???	???
Block by DHPs	-	-	+	-	-	-
Block by ω-CgTX GVIA	-	+	-	-	-	-
Block by ω-Aga IVA	-	-	-	+ (IC_{50}≈2 nM)	+ (IC_{50}≈100 nM)	-
Block by Cd^{2+}	-	+	+	+	+	+
Block by Ni^{2+}	+	-	-	-	-	-

Data modified from Scott et al., 1991; Randall and Tsien, 1995; TIPS 1996 Receptor and ion channel nomenclature supplement (seventh ed.).

nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus. These neurohormones play important roles in regulating body-fluid homeostasis and reproductive function.

The first description of the PVN as a distinct hypothalamic nucleus was provided by Malone (1910) who gave the nucleus the name "nucleus paraventricularis". A year later, the SON was described by Cajal (1911) who called it "nucleus tangentalis". The name "nucleus supraopticus" was not applied until twenty years later (Loo, 1931). An anatomical and functional relationship between the cells of the two hypothalamic nuclei and the posterior pituitary gland was not established until many years after the nuclei were described.

Sir Henry Dale (1909) is credited with the earliest description of a posterior pituitary hormone when he reported that extracts from the posterior pituitary gland induced uterine contractions. The discovery that an antidiuretic hormone is also present in the posterior pituitary gland is credited to von den Velden (1913) and Farini (1913). These two clinicians, working simultaneously and independently, found that extracts from the posterior pituitary gland reduced the diuresis in patients with diabetes insipidus. This observation, along with their earlier observations that many patients with diabetes insipidus exhibited signs of pathological pituitary injury, suggested that the posterior gland secreted a hormone which produced an antidiuretic effect.

More than a decade later Verney (1926) provided evidence corroborating the findings of von den Velden and Farini. Verney noted that a kidney included in a Starling "heart-lung" circuit exhibited an exaggerated flow of dilute urine. If extracts from the posterior pituitary gland were injected into the circuit, urine flow decreased and urine concentration increased. Verney concluded that the pituitary gland contributed a factor to the general circulation which was involved in urine regulation at the level of the kidney.

Evidence of an anatomical and functional relationship between the two hypothalamic nuclei and the posterior pituitary gland was first provided by Broers in 1933. He showed that selective destruction of the SON or pituitary stalk produced polyuria. He also noted that either lesion resulted in atrophy of both the SON and PVN. The anatomical studies of the Sharrers in 1939 confirmed the findings of Broers, and led to their concept of neurosecretion (Sharrer and Sharrer, 1940) in which they postulated that hypothalamic neurons can act in an endocrine fashion to induce physiological effects in distal target organs. This concept was later validated by Bargmann and Sharrer (1951) who were able to take advantage of improved staining techniques to establish that posterior pituitary hormones were indeed produced in the hypothalamus. A few years later, the purification, characterization and synthesis of OT (du Vigneaud *et al.*, 1954a) and of VP (du Vigneaud *et*

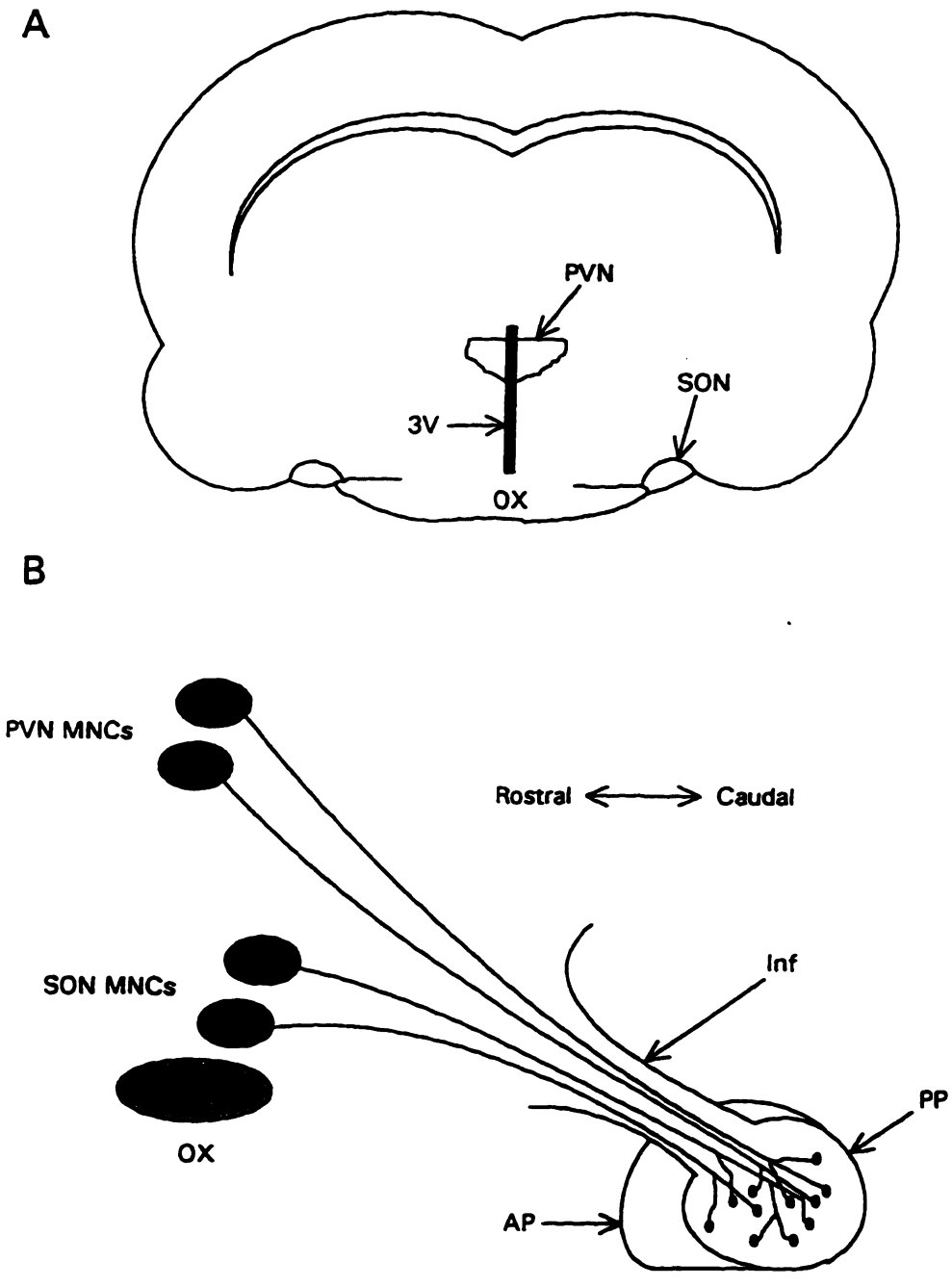
al., 1954b) would define these substances as the principal hormones secreted from the posterior pituitary gland (du Vigneaud, 1956). An enormous growth in HNS research has since followed this epoch of HNS research, contributing to our considerable and growing knowledge of HNS anatomy and physiology.

B. Anatomy of the HNS

The cell bodies of MNCs (15-30 μm in diameter) reside primarily in two pairs of distinct bilateral hypothalamic nuclei - the PVN, which lie adjacent to either side of the dorsal aspect of the third ventricle, and the SON, which cap the lateral aspects of the optic chiasm (Figures 1A and 1B). The PVN and SON receive afferent synaptic inputs from diverse sources within the central nervous system (see review by Hatton, 1990). MNCs of the PVN and SON represent sites of integration for afferent signals concerning the status of electrolyte and water balance, and reproductive function. These cells thus represent a final common pathway for output of information following integration of afferent signals. Unmyelinated axonal fibers from MNCs of the PVN and SON form the paraventriculo-hypophysial and supraoptico-hypophysial tracts, respectively. These fiber tracts run through the internal layer of the median eminence, and subsequently form the infundibulum (pituitary stalk). Axonal projections continue through the infundibulum to the posterior pituitary gland (also referred

Figure 1. Anatomy of the hypothalamo-neurohypophysial system (HNS). A, shown here (in a drawing of a coronal section of brain) are the relationships of the paraventricular nuclei (PVN) and supraoptic nuclei (SON) to the third ventricle (3V) and optic chiasm (OX). B, magnocellular neurosecretory cells (MNCs) of the PVN and SON project axons through the infundibulum (Inf) to the posterior pituitary (PP) which together with the anatomically-distinct anterior pituitary (AP) comprises the mammalian pituitary gland.

Figure 1



to as the neurohypohysis or neural lobe of the pituitary gland) where they terminate near the *basal lamina* - a thin matrix which lines the perivascular space surrounding fenestrated capillaries of the posterior pituitary.

The posterior pituitary consists almost entirely of axon terminals, resident glial cells and a dense network of fenestrated capillaries. Upon entering the posterior pituitary, neurosecretory axons become characterized by numerous focal swellings known as "Herring bodies", which are approximately 20 μm in diameter (Cross *et al.*, 1975). The Herring bodies contain a variety of organelles including lysosomes and mitochondria. The Herring bodies also contain numerous membrane-bound, electron-dense neurosecretory granules with diameters of 150-200 nm (Palay, 1955), each containing packaged neuropeptide. Each neurosecretory axon ultimately gives rise to an estimated 2000 terminals with diameters of 1-12 μm (Nordmann, 1977). This high degree of axonal branching provides an anatomical means for signal amplification at the level of the neurohypophysis. The axon terminals contain numerous neurosecretory granules and other subcellular organelles including lysosomes, mitochondria and small, electron-lucent microvesicles with diameters of 40-60 nm (Smith, 1970).

At least three anatomical characteristics distinguish axon terminals from Herring bodies (Cross *et al.*, 1975). First, Herring bodies are, on the average, larger in diameter than axon terminals. Second, Herring bodies do not

contain the electron-lucent microvesicles which are contained within and perhaps define axon terminals. Third, the axon terminals are found immediately adjacent to the perivascular spaces surrounding fenestrated capillaries of the hypophysial vasculature, whereas Herring bodies are further removed from the capillaries.

Intimate with the axons and terminals in the posterior pituitary are specialized astrocytic glial cells, known as "pituicytes". These cells make up approximately 25-30% of the posterior pituitary volume (Nordmann, 1977), and it has been suggested that they play an active role in modulating neurosecretion. Studies have shown that pituicytes can interact dynamically with other posterior pituitary elements (Tweedle and Hatton, 1980; 1982; 1987). Under basal conditions, these cells interpose their processes between neuronal elements of the posterior pituitary and between the axon terminals and *basal lamina* membrane lining the perivascular space. During conditions of increased hormone demand, the pituicytes can alter their morphology, allowing increased contact between neuronal elements and between axon terminals and *basal lamina*. Observations such as these have led to speculation that pituicytes participate in neurosecretion by modulating inter-neuronal communication and the degree of access that axon terminals have to the hypophysial vasculature. Other ways pituicytes may affect neurohormone release are by modulating the extracellular ionic *milieu* surrounding axons and terminals (Wu and Barish,

1994), and by releasing neuroactive substances which can act on neuronal elements to modulate neurosecretion (Tweedle and Hatton, 1982).

The posterior pituitary is perfused by a dense network of fenestrated capillaries originating from the inferior hypophysial artery. Neurosecretory endings within the posterior pituitary terminate onto the *basal lamina*, separated from the fenestrated capillaries by perivascular space. This anatomical arrangement renders the neuronal elements and glial cells of the posterior pituitary outside the blood-brain barrier. Neurohormones secreted from axon terminals into the extracellular space adjacent to the fenestrated capillaries can freely enter the general circulation and be transported to peripheral target tissues. Also, blood-borne hormones and substances of peripheral origin can freely diffuse across the vasculature and act at receptors located on axon terminals or pituicytes.

C. Physiology of the HNS

As peptide-secreting neurons, MNCs synthesize and package their neurohormones in the cell bodies (somata) within the hypothalamus where the appropriate protein synthetic machinery exists (see review by Richter and Ivell, 1985). Expression of hormone genes in the cell nucleus generates messenger ribonucleic acid (mRNA) transcripts, which are subsequently translated at the rough endoplasmic reticulum in the cell body to produce a large preprohormone.

The signal peptide sequence is removed while the preprohormone is still attached to the ribosome, to yield a prohormone containing the hormone peptide sequence. The prohormone and enzymes necessary for further processing are then packaged into neurosecretory granules within the golgi apparatus, and the granules are shipped *via* fast axonal transport to the posterior pituitary where they await an appropriate signal for release from the axon terminals. Evidence suggests that processing of prohormone into hormone destined for the posterior pituitary occurs within the neurosecretory granule during axonal transport (Gainer *et al.*, 1977a, 1977b). A consequence of hormone processing during transport is that the time required to synthesize hormone and have it available for release in the neural lobe can be quite short. In fact, one study has suggested that this entire sequence of events can occur in less than 2 hours (Jones and Pickering, 1970). Neurosecretory granules which are not released following arrival at the axon terminals, are shipped back to the Herring bodies where they can be stored and later recalled during periods of increased hormone demand (Heap *et al.*, 1975).

In addition to hormone, a hormone-specific peptide known as neurophysin (NP) is also generated by enzymatic cleavage of the VP- or OT-prohormone molecule within the neurosecretory granule during axonal transport (Gainer *et al.*, 1977). The VP-associated neurophysin and OT-associated neurophysin have homologous structures which differ

primarily in their N-terminal portions (Pickering and Jones, 1978). Neurophysins bind with low affinity to their associated hormones and are thus believed to serve as "carrier proteins" during the transport of neurohormones down MNC axons.

Neurosecretion refers to the release of hormone from axon terminals into the general circulation. A variety of physiological stimuli have been shown to evoke VP and/or OT release (see review by Hatton, 1990). VP release occurs in response to changes in plasma osmolarity and/or volume. OT release occurs in response to mechanical distension of the vagina and suckling, and by osmotic stimuli. Release of VP and OT is dependent on the electrical activity of MNCs. Afferent synaptic inputs release chemical neurotransmitters which produce excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) in hypothalamic magnocellular neurons. These inputs are summated at the cell somata, resulting in the generation and propagation of a Na^+- (Dreifuss *et al.*, 1971) and Ca^{2+}-dependent (Bourque and Renaud, 1985) action potential once a threshold depolarization is achieved.

Neurosecretion follows the transmission of an action potential from the cell body in the hypothalamus, down the axon to the nerve terminal where depolarization initiates a series of events resulting in exocytosis of hormone-containing neurosecretory granules. Terminal depolarization results in the opening of voltage-sensitive calcium

channels, allowing extracellular calcium to enter the nerve endings where it triggers exocytosis (Douglas and Poisner, 1964a; Douglas and Poisner, 1964b; Nordmann and Dreifuss, 1972).

The current and longstanding model of Ca^{2+}-dependent exocytosis is derived largely from studies of release of non-peptide neurotransmitters (see review by Südhof and Jahn, 1991). In this model, intraterminal Ca^{2+} interacts with Ca^{2+}-binding proteins which promote fusion of neurotransmitter-containing synaptic vesicles with the presynaptic membrane, resulting in release of transmitter into the synaptic cleft. Empty synaptic vesicles are then recycled by endocytosis and replenished with neurotransmitter at the level of the nerve terminal. Release of non-peptide neurotransmitters is generally localized to presynaptic membrane specializations called active zones. These distinct presynaptic areas are characterized by clusters of synaptic vesicles along the intracellular side of the membrane, an intricate cytoskeletal network which is involved in vesicle trafficking, and a high density of intramembranous particles. The existence of microdomains of high $[Ca^{2+}]_i$ (100 μM or more) in the area of active zones (Llinás, *et al.*, 1992b) has led to speculation that the intramembranous particles are Ca^{2+} channels. Because of this arrangement, release of non-peptides is rapid and tightly-coupled to pathways for Ca^{2+} influx. The identity of the Ca^{2+}-binding

protein(s) involved in triggering exocytosis is not yet known, although synaptotagmin, a protein localized specifically to synaptic vesicle membranes and shown to interact with several different proteins localized to the presynaptic membrane, remains a strong candidate (Matthew *et al., 1981*; Bennett *et al.*, 1992; Brose *et al.*, 1992; *Leveque et al.*, 1992).

Several lines of evidence suggest that peptide release from neurons is mediated by biochemical mechanisms somewhat different from those mediating non-peptide release. First, release of neuropeptides is not confined to a discernible active zone, but can instead occur along all regions of the nerve terminal (Zhu *et al.*, 1986). Second, higher-frequency stimulation is generally required for efficient release of neuropeptides (Dutton and Dyball, 1979; Morris *et al.*, 1995). This is likely because neuropeptide release is not confined to active zones where microdomains of high $[Ca^{2+}]_i$ occur following stimulation. Global elevations in $[Ca^{2+}]_i$ necessary to trigger efficient release of neuropeptides would be more likely to occur during the massive Ca^{2+} entry that occurs only during high-frequency firing of action potentials. Third, vesicles retrieved following exocytosis of neuropeptides must be shipped back to the cell soma for recycling, since the terminal lacks the appropriate protein synthetic machinery necessary to produce new neuropeptide. This may indicate that the proteins responsible for recycling of vesicular membrane in peptidergic neurons may

differ from those in non-peptidergic neurons.

Considerable evidence suggests that exocytotic release of hormone from MNCs is not restricted to axon terminals in the posterior pituitary. Electron microscopy work has demonstrated that MNC dendrites in both the SON and PVN contain numerous neurosecretory granules containing packaged neuropeptide (Pow and Morris, 1989). Moreover, it was estimated that these dendrites contain 70-80% of all the VP and OT in the hypothalamus. These initial observations led to speculation that secretory material can also be released from MNC dendrites within the confines of the hypothalamic nuclei. This possibility was supported by electron microscopy which provided images of neurosecretory granules being exocytosed from MNC dendrites (Pow and Morris, 1989). Although the purpose of dendritic release of secretory material from MNCs is not clearly understood, one possibility is that hormone released within the hypothalamic nuclei by MNC dendrites acts locally as a modulator of MNC electrical activity and thus of hormone release from these cells. Support for this hypothesis has come from a number of observations.

OT (but not VP) administered *via* the third ventricle to anaesthetized rats suckling their litters selectively facilitates burst firing of action potentials in OT neurons and increases the frequency of milk ejections (Freund-Mercier and Richard, 1984). Furthermore, OT release within the SON increases during suckling, as demonstrated *in vivo*

using microdialysis techniques (Moos *et al.*, 1989). These results suggest that a facilitatory influence of dendritically-released OT on OT release from the posterior pituitary is a critical component of the milk ejection reflex.

The exact mechanism by which OT facilitates its own release is not known. To date, the presence of OT receptors in the SON or PVN is not clearly established, although a moderate expression of OT receptor mRNA in both nuclei has been demonstrated (Yoshimura *et al.*, 1993). Nevertheless, there is evidence indicating that OT can act directly on MNCs in an autocrine and/or paracrine manner. Recordings from MNCs in slices of hypothalamic tissue have revealed that application of OT selectively increases the firing rate of putative OT neurons, even when synaptic transmission is blocked (Inenaga and Yamashita, 1986). Also, application of OT to isolated supraoptic MNCs produces a sharp increase in $[Ca^{2+}]_i$ which can be blocked by a specific OT receptor antagonist (Lambert *et al.*, 1994). This is consistent with an action mediated through OT receptors which, in other tissues, are guanine nucleotide-binding protein (G Protein)-coupled receptors which act *via* phosphotidylinositol hydrolysis and mobilization of Ca^{2+} from intracellular stores (Morel *et al.*, 1992). Although no association between OT effects on MNC $[Ca^{2+}]_i$ and on electrical activity has been established, these experimental observations support the possibility that OT selectively facilitates its

own release *via* a direct action on OT neurons.

Like OT, VP released from MNC dendrites in the SON and PVN may act locally on MNCs to modulate their electrical activity and the amount of hormone released from these cells. VP is released in the SON and selectively facilitates its own local release, as demonstrated *in vivo* using microdialysis techniques (Wotjak *et al.*, 1994). Also, application of VP selectively increases the firing rate of putative VP neurons in slices of hypothalamic tissue *via* activation of V_1-type VP receptors located on MNCs (Inenaga and Yamashita, 1986). To date, two subtypes of V_1 receptors (V_{1a} and V_{1b}) have been characterized (Jard *et al.*, 1987). Both are G protein-coupled receptors which act *via* phosphotidylinositol hydrolysis and mobilization of Ca^{2+} from intracellular stores (Morel *et al.*, 1992). *In situ* hybridization studies have revealed that only V_{1a} mRNA transcripts are found in the hypothalamus (Ostrowski *et al.*, 1994). In isolated supraoptic MNCs, VP has been shown to increase $[Ca^{2+}]_i$ and these effects were blocked by a selective V_{1a} receptor antagonist (Dayanithi *et al.*, 1996). These results suggest that supraoptic MNCs express functional V_{1a} receptors which contribute to a selective autoregulation of VP neurons by dendritically-released VP.

Extracellular recordings of electrical activity *in vivo* have provided considerable information regarding MNC firing patterns under physiological conditions (reviewed by Poulain and Wakerly, 1982). Under basal conditions, both VP and OT

rat MNCs are characterized electrophysiologically by slow (<3 Hz), irregular firing patterns. However, in response to physiological stimuli that increase hormone secretion, each type of MNC develops specific firing patterns in which action potentials are fired in bursts. VP neurons exhibit "phasic" patterns of action potential discharge, consisting of bursts of higher-frequency (7-12 Hz) discharge separated by periods of relative inactivity or silence. OT neurons, on the other hand, exhibit firing patterns consisting of bursts of high-frequency (up to 50 Hz) discharge occurring approximately 10 seconds prior to the rise in intramammary pressure which precedes milk ejection in lactating female animals. The OT bursts may be superimposed on a background of "slow irregular" (<3 Hz) or "fast continuous" (3-15 Hz) firing activity. These electrical characteristics are critical to the neurosecretory process. It has been shown that the pattern of electrical activity exhibited by MNCs is an important determinant of intraterminal free calcium concentration (Cazalis *et al.* 1985; Jackson *et al.*, 1991; Stuenkel, 1994) and thus the amount of hormone released from the posterior pituitary (Dutton and Dyball, 1979; Bicknell and Leng, 1981; Bicknell *et al.*, 1982; Cazalis *et al.*, 1985).

D. Mechanisms Underlying the Electrical Behavior of Supraoptic MNCs

The two most important factors controlling MNC electrical behavior and hormone release are extrinsic synaptic inputs and intrinsic membrane properties. Extrinsic control mechanisms are represented by afferent synaptic inputs which regulate the excitability of MNCs. Intrinsic control mechanisms, on the other hand, are represented by the ionic conductances which underlie individual action potentials and other more complex phenomena such as burst firing. My research efforts are directed at the intrinsic electrical properties of supraoptic MNCs. Accordingly, the following review will focus largely on that which is known about these cells. For information regarding extrinsic and intrinsic mechanisms which underlie the electrical behavior of paraventricular MNCs, the reader should consult other sources (see reviews by: Hatton, 1990; Renaud and Bourque, 1991).

1. Preparations Used to Study Mechanisms Which Underlie the Electrical Behavior of Supraoptic MNCs

Several *in vitro* preparations are typically used to study extrinsic and intrinsic mechanisms underlying the electrical behavior of supraoptic MNCs. These preparations include: HNS explants, brain slices, cultured MNCs, and acutely dissociated MNCs. Each preparation offers certain advantages and disadvantages for studying MNC function.

HNS explants are used to study the properties of physically intact supraoptic MNCs (Bourque and Renaud, 1983). Explants are prepared without the use of enzyme treatment and permit the use of high-resolution intracellular recording techniques on MNCs *in situ* with their structure and most of their synaptic contacts preserved. An advantage of the explant preparation is that the preservation of the *in vivo* structure and synaptic contacts permits the study of extrinsic modulation of MNC activity. Also, the neurohypophysis can be retained with the explant, allowing correlative studies on MNC electrical behavior and hormone secretion (e.g., Renaud, 1987). A major problem associated with the use of explants, however, is that the activity of glial cells, presynaptic terminals and other endogenous tissue elements can potentially modulate or mask specific intrinsic neuronal events under study. Explants also do not permit the use of conventional patch-clamp techniques to study specific ionic conductances. This is largely because cells in explants do not exhibit the "clean" membrane surfaces necessary to obtain the kind of high-resistance seal between the patch-clamp pipet and the cell membrane required for patch-clamp recording. Voltage-clamp recordings of cells in explants can be made using intracellular electrodes (Bourque, 1988). However, because MNCs in explants are intact, dendritic processes may contribute to spatial distortions of membrane potential ("space-clamp" errors) when attempting to clamp the membrane

voltage. Thus, although it may represent a more physiologically relevant system, each of the potential problems inherent with explant preparations must be considered when interpreting data obtained from cells in explants.

Intracellular recording techniques can also be applied to MNCs *in situ* in slices of hypothalamic tissue containing SON (Hatton *et al.*, 1978). Additionally, slice preparations offer an advantage over explants in that they permit the use of patch-clamp techniques to record specific ionic conductances from MNCs within the slices (Nagatomo *et al.*, 1995). This is because a relatively simple, mechanical procedure can be applied to "clean" the extracellular membrane surfaces of exposed neurons in thin slices such that a high-resistance seal between a patch electrode and a cell membrane can be easily obtained (Edwards *et al.*, 1989). As with explants, many of the synaptic contacts are preserved within slice preparations, allowing the study of excitatory and inhibitory postsynaptic currents in MNCs. Problems associated with the use of slice preparations are similar to some of those discussed for explants. Because much of the *in vivo* structure is preserved within the slice, the activity of glial cells, presynaptic terminals and other endogenous tissue elements can potentially modulate or mask specific intrinsic neuronal events under study. Also, intact dendritic processes may contribute to space-clamp problems in voltage-clamp experiments.

To circumvent some of the problems associated with explant and slice preparations, some investigators have used MNCs cultured from the area of the SON (Cobbett and Mason, 1987; Cobbett *et al.*, 1989). These neurons have membrane surfaces suitable for patch-clamp recording and are generally devoid of synaptic contacts. However, because the neurons in these cultures are harvested from prenatal or early postnatal animals, the ionic channels in these cells may differ in terms of total complement or behavior from that of MNCs of adult animals.

Recently, methods have been developed to dissociate MNCs of the adult SON using a combination of enzymatic and mechanical procedures (Cobbett and Weiss, 1990; Oliet and Bourque, 1992). These neurons are free of synaptic contacts and glial investments, and exhibit clean membranes that are suitable for patch-clamp recording. Also, only the most proximal dendritic structure is preserved, thus making space-clamp problems less of a concern when conducting voltage-clamp experiments. Available data suggests that the electrical characteristics of these neurons are largely unimpaired by the dissociation procedure. Nevertheless, when interpreting data from acutely dissociated neurons, one must be aware of the possibility that proteolytic digestion by enzymes used in the dissociation procedure can potentially alter the behavior of membrane proteins such as ion channels.

2. Extrinsic Mechanisms Underlying the Electrical Behavior of Supraoptic MNCs

VP and OT release from supraoptic MNCs can be modulated at different levels within the HNS. Within the hypothalamus, afferent synaptic inputs to the SON release chemical neurotransmitters which can act at the dendrites and soma of MNCs to modulate their electrical behavior and thus the amount of hormone released. Neurotransmitters released within the SON may also act at receptors located on terminals of other afferent inputs to the SON and on resident glial cells, thus regulating their participation in the control of hormone release (see review by Hatton, 1990).

Although the primary level of control for hormone release from supraoptic MNCs is within the SON, there is considerable evidence that hormone release can also be modulated by neurotransmitters or circulating hormones at the level of the neurohypophysis. These substances may also act at receptors located on axon terminals or pituicytes within the neurohypophysis to modulate hormone release from MNCs. For a comprehensive review of neurotransmitters and hormones implicated in the modulation of OT and VP release at the level of the neurohypophysis, the reader is referred to Falke (1991). The remainder of this section will address specifically the extrinsic control mechanisms which underlie stimulus-secretion coupling at the level of the SON.

Supraoptic MNCs receive diverse synaptic inputs from select brainstem and forebrain structures. The MNCs thus

represent sites of integration for afferent inputs which help control VP and OT release. Evidence suggests that glutamate and γ-aminobutyric acid (GABA) represent the dominant neurotransmitters released in the SON to regulate MNC activity. The *organum vasculosum lamina terminalis* (OVLT) appears to provide glutamatergic input to the SON (Renaud *et al.*, 1993). Electrophysiology studies have shown that glutamate is an excitatory neurotransmitter mediating fast EPSPs in supraoptic MNCs (Van Den Pol *et al.*, 1990; Oliet and Bourque, 1992). The prominent source of GABAergic input to the SON has not yet been defined. However, electrophysiology studies have shown that GABA mediates fast IPSPs in supraoptic MNCs (Randle *et al.*, 1986; Randle and Renaud, 1987; Oliet and Bourque, 1992).

Many other substances are also thought to provide regulatory influences on the activity of supraoptic MNCs at the level of the SON. Studies have demonstrated that a variety of neurotransmitters are packaged within terminals located presynaptic to MNCs of the SON. The caudal ventrolateral and dorsomedial medulla have been shown to project norepinephrine-containing inputs (from the A1 and A2 cell groups respectively) to the SON (Sawchenko and Swanson, 1981, 1982). Neuropeptide Y appears to be co-localized in norepinephrine-containing inputs from the caudal ventrolateral medulla (Harfstrand *et al.*, 1987). Inputs from dorsomedial medulla neurons also contain the peptide neurotransmitter inhibin (Sawchenko *et al.*, 1988). The SON

receives innervation from 5-HT-containing fibers originating from the B7, B8 and B9 cell groups in the midbrain (Sawchenko *et al.*, 1983) and from angiotensin II-containing fibers originating from the subfornical organ (Jhamandas *et al.*, 1989). Although their exact sources have not yet been defined, other neurotransmitters which may act at the level of the SON to control MNC activity include acetylcholine (Mason *et al.*, 1983), histamine (Panula, 1986), dopamine (Buijs *et al.*, 1984), enkephalin (Martin and Voigt, 1981), cholycystokinin (Vanderhaegen *et al.*, 1981), substance P (Shults *et al.*, 1984) and atrial natriuretic peptide (Standaert *et al.*, 1987). Thus, in addition to glutamate and GABA, a variety of other neurotransmitters are believed to influence the activity of supraoptic MNCs. Available data on these neurotransmitters indicate that they do not mediate fast synaptic potentials as has been described for glutamate and GABA. It therefore seems likely that these substances mediate slow synaptic events and thus function as neuromodulators of MNC activity. For a review of the specific effects of these neurotransmitters on the activity of supraoptic MNCs, the reader is referred to Renaud and Bourque (1991).

3. Intrinsic Mechanisms Underlying the Electrical Behavior of Supraoptic MNCs

Intracellular recordings of membrane potential in *in vitro* preparations (particularly brain slices and HNS

explants) have provided a general picture concerning the electrical properties of supraoptic MNCs. For example, somatically-recorded MNC action potentials are known to consist of TTX-sensitive, Na^+-dependent (Andrew and Dudek, 1984) and Ca^{2+}-dependent components (Bourque and Renaud, 1985). The Ca^{2+} component is represented by a distinct 'shoulder' on the repolarization phase of individual action potentials which contributes to the quite long (up to 5 ms) duration of MNC action potentials. Removal of K^+ from the intracellular and extracellular compartments dramatically prolongs (up to 100 fold) the duration of somatically-generated MNC action potentials (Bourque *et al.*, 1985), suggesting that outward K^+ currents underlie action potential repolarization as in other neurons (see review by Rudy, 1988).

Individual MNC action potentials are immediately followed by a hyperpolarizing after-potential (HAP) that Bourque *et al.* (1985) has suggested may result from the activation of a transient Ca^{2+}-dependent K^+ current (I_{to}). HAPs can be temporally summated following repetitive spikes and may play a role in controlling the firing pattern of MNCs by setting an upper limit on the maximal frequency of firing which can be achieved during burst activity. HAPs may also facilitate the removal of channel current inactivation which occurs during the time course of the action potential.

Following the post spike HAP, a slow depolarizing

after-potential (DAP) is observed (Andrew and Dudek, 1983). Temporal summation of DAPs following repetitive spikes is involved in the generation of a small (<10 mV) sustained depolarization ("plateau potential") which appears to provide the basis for the intrinsic generation of burst activity. Current-clamp studies (Bourque, 1986) suggest that the DAP results from the spike-induced activation of a voltage-gated Ca^{2+} current (I_{dap}).

Andrew and Dudek (1984) have shown that burst firing of spikes in MNCs is followed by a prominent, frequency-dependent AHP, which is believed to result from the activation of a slow Ca^{2+}-dependent K^+ current (I_{ahp}). Current-clamp studies (Bourque and Brown, 1987; Kirkpatrick and Bourque, 1996) have suggested that I_{ahp} is contributed by apamin-sensitive, Ca^{2+}-dependent K^+ channels. Functionally, the AHP acts to regulate intraburst firing frequency and perhaps also firing patterns since it can help negate the late DAP. Burst firing of spikes in MNCs also results in frequency-dependent spike broadening (Andrew and Dudek, 1985; Bourque and Renaud, 1985) which is represented by a progressive increase in spike duration with each successive action potential at the onset of a burst. A similar phenomenon is also observed in MNC axon terminals (Gainer *et al.*, 1986; Jackson *et al.*, 1991) and it may contribute to facilitation of hormone release by enhancing Ca^{2+} accumulation within the terminal (Jackson *et al.*, 1991; Stuenkel, 1994). The exact events which underlie

frequency-dependent spike broadening in MNCs are not clearly understood, although a reduced voltage-activated K^+ current and/or an increased voltage-activated Ca^{2+} current following repetitive stimulation may be involved (O'Regan and Cobbett, 1993).

Clearly, data from intracellular recordings of membrane potential in slice and explant preparations have provided valuable insights into the intrinsic electrophysiology of supraoptic MNCs. A necessary prerequisite to understanding the mechanisms which underlie membrane potential phenomena, however, is a thorough characterization of the specific ionic conductances present in the MNC membrane. These specific ionic events are not easily revealed using intracellular recording techniques. The application of patch-clamp techniques on neurons in slice preparations and on neurons isolated from the SON has facilitated the study of specific ionic conductances which underlie membrane potential phenomena.

Using whole-cell voltage-clamp techniques on MNCs acutely isolated from adult rat SON, Fisher and Bourque (1995a, 1995b) were able to evoke inward macroscopic Ca^{2+} current that exhibited both inactivating and non-inactivating components during prolonged membrane depolarizations from a holding potential (V_H) of -80 mV. Contributions to the inward macroscopic Ca^{2+} current from at least four (possibly five) types of voltage-activated Ca^{2+} channel currents were distinguished based on their

thresholds for activation, rates of inactivation and their sensitivities to a series of calcium channel blockers.

A portion of the inactivating component exhibited a low activation threshold (>-60 mV) and rapid inactivation kinetics (τ = 42 ms at -10 mV). This component was more sensitive to block by the divalent cation Ni^{2+} than to Cd^{2+} and was insensitive to 0.5 μM ω-CgTX GVIA, a peptide toxin reported to produce a preferential block of inactivating N-type Ca^{2+} channels in other neurons (Aosaki and Kasai, 1987; Plummer *et al.*, 1989; Randall and Tsien, 1995). Based on these results, the low-threshold, rapidly-inactivating Ca^{2+} current component in MNC somata was identified as being carried by T-type Ca^{2+} channels which have been described in other neurons (Fox *et al.*, 1987).

A high-threshold, inactivating component of Ca^{2+} channel current was also reported. This component activated at membrane potentials more positive than -30 mV and exhibited slow inactivation kinetics (τ = 1790 ms at -10 mV). Application of 0.5 μM ω-CgTX GVIA blocked a significant portion of this current component. Together, these results were taken to indicate the presence of an N-type Ca^{2+} channel current component.

The non-inactivating component of the macroscopic Ca^{2+} channel current exhibited a low-activation threshold, activating at membrane potentials more positive than -60 mV. A portion of this current component was sensitive to 10 μM nifedipine, a DHP compound which produces a preferential

block of L-type Ca^{2+} channels in other neurons (Fox *et al.*, 1987; Hille, 1992). The activation threshold (>-60 mV) of the nifedipine-sensitive current in MNC somata was low, however, compared to values reported for L-type currents in other cell types (Hille, 1992), suggesting that MNCs express a novel, low-threshold, L-type Ca^{2+} channel. Another portion of the non-inactivating current component was not sensitive to block by nifedipine, but was blocked by ω-Aga IVA, a peptide toxin reported to block non-inactivating P-type Ca^{2+} channels (Mintz *et al.*, 1992) at low concentrations (IC_{50}≈2 nM) and inactivating Q-type Ca^{2+} channels (Wheeler *et al.*, 1994) at higher concentrations (IC_{50}≈100 nM). Since this portion of current was non-inactivating and was blocked by low concentrations of ω-Aga IVA (IC_{50}≈3 nM), it was identified as being carried by P-type Ca^{2+} channels.

Fisher and Bourque (1995a, 1995b) concluded that supraoptic MNC somata of the adult rat express T-, N-, L-, and P-type Ca^{2+} channels. They also noted the presence of a Ca^{2+} current component with an intermediate activation threshold (>-50 mV) and rate of inactivation (τ = 187 ms at -10 mV). This current component was unaffected by the organic Ca^{2+} channel blockers used in their experiments, and thus did not correspond to any identified Ca^{2+} channel type.

The components of somatic K^+ current in adult supraoptic MNCs have not been thoroughly characterized. To date, voltage-clamp recordings of voltage-activated outward

K^+ current have been reported in three published studies (Bourque, 1988; O'Regan and Cobbett, 1993; Nagatomo *et al.*, 1995). Using intracellular voltage-clamp techniques on supraoptic MNC somata in explants of adult rat hypothalamus, Bourque (1988) recorded a transient (inactivating) outward K^+ current (I_{TOC}) evoked by depolarizations from a holding potential of -100 mV. This current was significantly reduced by 4-AP (1 mM) or DTX (4 nM), compounds which have both been shown to produce a preferential block of inactivating A-type K^+ channel currents in other neurons (see review by Rudy, 1988). Also, it was reported in the study that I_{TOC} was dependent on the presence of extracellular Ca^{2+}, implying that Ca^{2+} influx into MNCs strongly modulates the gating of TOC channels. This conclusion was in part based on the observation that I_{TOC} was reduced by up to 90% by addition of the Ca^{2+} channel blocker Cd^{2+} (50-400 μM) to the extracellular medium. Also, I_{TOC} was nearly abolished by removal of Ca^{2+} from the extracellular medium. The author concluded that MNCs of the adult rat SON display a transient "A-like" K^+ current which, although similar pharmacologically to A-type K^+ currents reported in other neurons, is novel in that it is dependent on Ca^{2+} influx (see Tables 1 and 2).

O'Regan and Cobbett (1993) reported that whole-cell voltage-clamp recordings from MNCs dissociated from the SON of the adult rat revealed inactivating and non-inactivating components of somatic outward K^+ current. However, although

the non-inactivating component was observed in all dissociated MNCs studied, the inactivating component was observed in only about one-half of the cells. No explanation was offered in the study as to why some MNCs express only non-inactivating K^+ current, nor was there any attempt to characterize the kinetics or pharmacology of the two components of K^+ current.

Whole-cell voltage-clamp recordings from supraoptic MNCs in slices containing hypothalamus have also revealed inactivating and non-inactivating components of somatic outward K^+ current (Nagatomo *et al.*, 1995). The inactivating component of current evoked during membrane depolarizations from a holding potential of -80 mV exhibited a low threshold for activation (-60 mV) and rapid inactivation kinetics which were best fit by a single exponential (τ = 9.5 ms at +40 mV). Also, this current was nearly abolished by 5 mM 4-AP. These results are typical of A-type K^+ channel currents ($I_{K(A)}$) reported in other neurons (see review by Rudy, 1988). The authors also reported that I_A in supraoptic MNCs was reduced by 10 μM angiotensin II. No attempt was made to characterize the non-inactivating component of outward current.

OBJECTIVES

Secretion of OT and VP is directly controlled by the electrical activity of the hormone-containing neurons. Accordingly, one of the fundamental processes underlying neurosecretion is the relationship between electrical activity and hormone release in neurosecretory neurons. My research efforts were directed at the specific ionic events underlying the electrical activity in supraoptic MNCs. Specifically, my aim was to characterize the components of somatic outward K^+ current in MNCs and examine their involvement in the intrinsic regulation of MNC electrical behavior.

The components of somatic K^+ current in adult supraoptic MNCs have not been characterized thoroughly. The preparation I used consisted of MNCs acutely dissociated from the adult guinea pig SON using a combination of enzymatic and mechanical procedures (Cobbett and Weiss, 1990; Oliet and Bourque, 1992). Recordings of somatic K^+ current were made using the tight-seal, whole-cell recording technique described by Hamill *et al.* (1981). In addition, I used a variation of this technique to record membrane potential and examine the involvement of identified K^+ current components in membrane potential phenomena. This

work will add to our understanding of the intrinsic mechanisms which underlie the electrical behavior of supraoptic MNCs.

MATERIALS AND METHODS

I. Preparation of Dissociated Supraoptic MNCs

Dissociated supraoptic MNCs were prepared according to a modification of the method described by Cobbett and Weiss (1990). Adult guinea-pigs (male 250-300 g, obtained from the Michigan Department of Health, Lansing, MI, USA) were decapitated. The brain was rapidly removed and a tissue block containing hypothalamus was prepared. Using a Vibratome, coronal slices 800 μm thick were cut from the tissue block while submersed in cold incubation medium containing (mM): NaCl 120; KCl 5; $CaCl_2$ 1; $MgCl_2$ 1; *d*-glucose 25; and PIPES 20 (pH 7.3). From the slices, SON-containing tissue explants were dissected out and placed in 10 ml oxygenated incubation medium (32°C) supplemented with protease (3 mg/ml, Type I, Sigma) for 60 min. Explants were then rinsed three times with and maintained at room temperature in protease-free incubation medium until required. To dissociate MNCs, an explant was removed from the incubation medium and placed in 1 ml dissociation medium which contained (mM): NaCl 130; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1; *d*-glucose 10; and HEPES 20 (pH 7.3). The tissue was then triturated using a sequence of fire polished pasteur pipettes of decreasing bore size (0.5-0.2 mm inside

diameter). The resulting cell suspension was then plated onto 35 mm polystyrene culture dishes for immediate use in experiments. All experiments were conducted at room temperature (22-25°C).

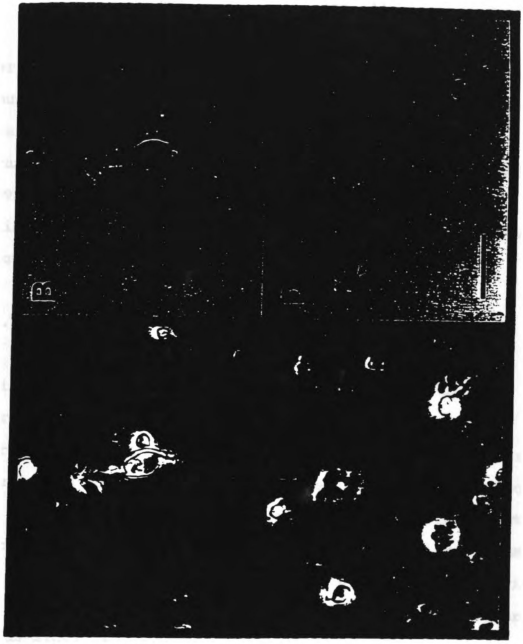
II. Immunocytochemistry and Morphological Examination of Dissociated MNCs

Plated cells were fixed for 30 min with modified Bouin's fixative (4% w/v paraformaldehyde, 0.2% v/v picric acid, 0.1 M phosphate buffer, pH 7.4). The fixed cells were then subjected to three 5-min rinses in phosphate-buffered saline (PBS; pH 7.4). SON neurons were stained at room temperature according to procedures described for the VECTASTAIN® *Elite* ABC Kit system (Vector Laboratories, Burlingame CA). The primary antibody used for the procedure was directed against rat neurophysins (1:10,000; AB948; Chemicon, Temecula, CA). Detection of immunolabelled neurons was accomplished using 3,3'-diaminobenzidine (DAB) provided in a DAB Substrate Kit for Peroxidase (Vector Laboratories, Burlingame CA). Figure 2 shows several dissociated neurons viewed using phase contrast and normal light optics. Note that only the larger neurons were decorated by neurophysin antibody.

To determine if size of isolated guinea pig SON neurons could be used to identify MNCs, the procedure for morphometric analysis of neurons described by Oliet and Bourque (1992) was followed. Briefly, the cross-sectional

Figure 2. Photomicrographs of dissociated supraoptic cells. A, phase contrast photomicrograph of a single cell (*arrow*) obtained after dissociation of the SON of a guinea pig. B, shows the same cell (*arrow*) as in A viewed under transmitted light following immunostaining for NPs. C, phase contrast photomicrograph of smaller cells (*arrows*) obtained in the same preparation used to obtain the cell shown in A and B. D, shows the same cells (*arrows*) as in C viewed under transmitted light following immunostaining for NPs. Note the absence of NP staining in these smaller cells. Bar represents 50 μm.

Figure 2



area (CSA) of isolated neurons was estimated as that of a uniform oval using the following equation:

$$CSA = \pi R_S R_L \qquad \text{eqn (1)}$$

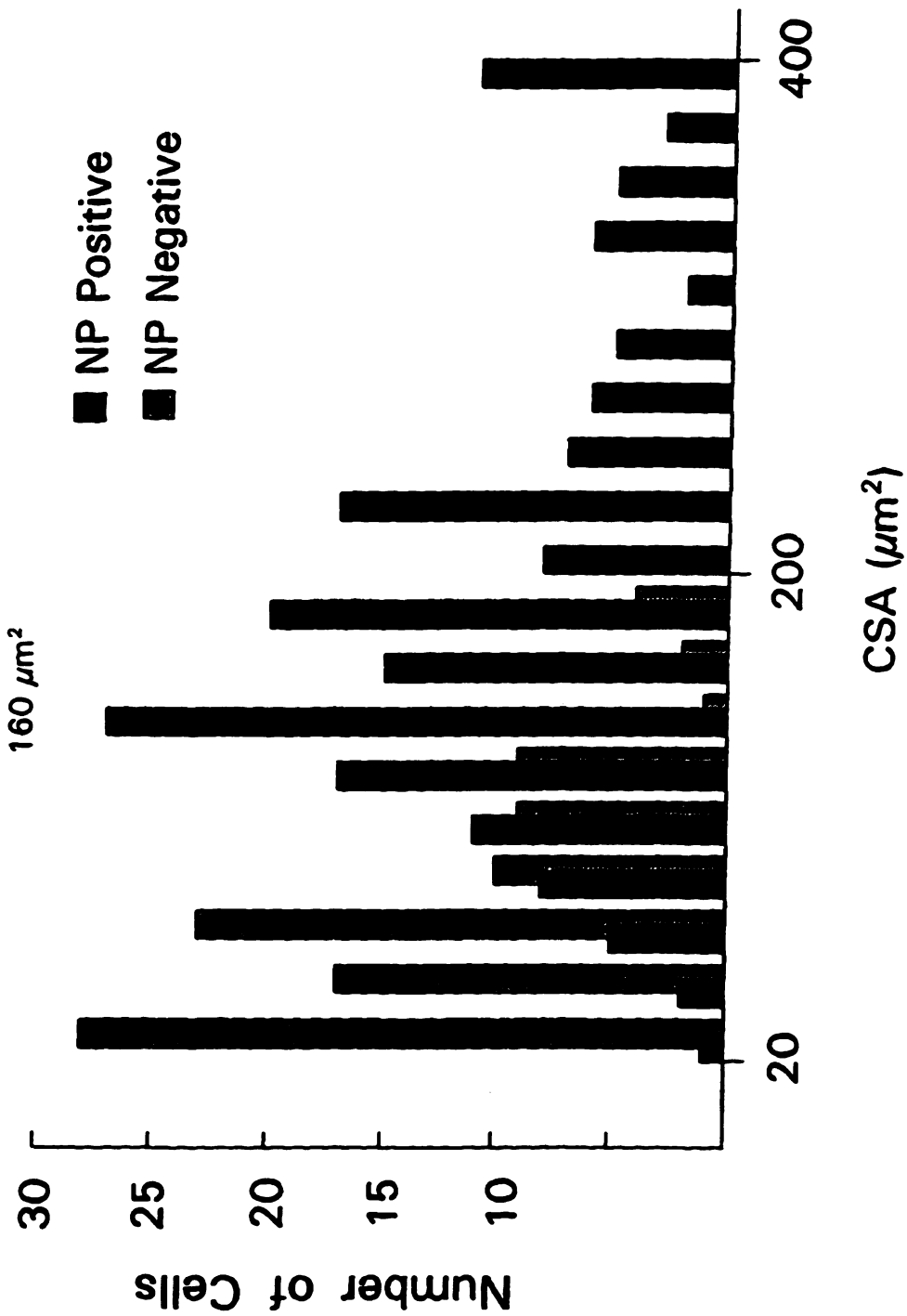
where R_S and R_L are the short and long radius, respectively. Immunostaining and CSA of the neurons were then examined to determine if there was any correlation between the two parameters. Similar to the findings of Oliet and Bourque (1992), 95% (105 of 111 cells) of the neurons which exhibited a CSA $\geq$ 160 μm^2 stained positive for neurophysin (Figure 3).

III. Voltage-Clamp Recording from MNCs

Patch electrodes were double pulled and then fire polished to resistances of 1-5 MΩ. For recording K^+ currents, the dissociation medium supplemented with tetrodotoxin (TTX; 2.5 μM) was used as the extracellular solution in most experiments (see Results and Figure Legends for changes in medium composition in specific experiments). Electrodes were filled with a solution consisting of (mM): KCl 135; $MgCl_2$ 1; *d*-glucose 10; EGTA 0.5; ATP 2; cAMP 0.2; and HEPES 10 (pH 7.3). The electrode solution for recording inward divalent cation current consisted of (mM): CsCl 135; $MgCl_2$ 1; *d*-glucose 10; EGTA 5; ATP 2; cAMP 0.2; and HEPES 10 (pH 7.3). After formation of a tight seal between the electrode and cell membrane, the electrode potential was

Figure 3. Supraoptic MNCs can be identified based on size. This histogram shows the distribution of 279 isolated neurons based on cross-sectional area (CSA) and either positive (dark bars) or negative (light bars) staining by OT and VP neurophysin antibodies. Note that 95% (105 of 111 cells) of the neurons which exhibited a CSA $\geq$ 160 μm^2 stained positive for neurophysin.

Figure 3


NP Positive
NP Negative
160 μm²
Number of Cells
30
25
20
15
10
20
200
400
CSA (μm²)

clamped at -40 mV. The whole-cell configuration was achieved by application of gentle suction to the back of the electrode, and the membrane capacitance and series resistance were then maximally compensated to allow accurate control of membrane voltage. Membrane potential and current signals were amplified (List Electronic EPC-7, Germany) and stored on VHS videotape (recording bandwidth DC to 16 kHz) for off-line analysis. A computer program provided by Dr. John Dempster (University of Strathclyde, Scotland) was used to control voltage protocols and analyze the data.

For analysis, current and voltage signals were filtered at 5 kHz using an 8-pole Bessel filter (Kemo Instruments, Beckenham, U.K.) and then digitized (Cambridge Electronic design 1401, Cambridge, England) at 6 kHz and transferred to the hard disk of a microcomputer. Linear leakage currents and capacitative transients were subtracted from macroscopic current records. The current traces displayed in the figures represent single iterations or averages of two iterations.

IV. Current-Clamp Recording from MNCs

For recording action potentials, the dissociation medium (no TTX present) and electrode solution for recording K^+ current (both described in the previous section) were used as the extracellular and electrode solutions, respectively. After achieving the whole-cell configuration, membrane potential and current signals were amplified using

the current-clamp modes of the amplifier (List Electronic EPC-7, Germany) and stored on VHS videotape (recording bandwidth DC to 16 kHz) for off-line analysis. When necessary, injection of negative current was used to hold the membrane at a slightly hyperpolarized potential (near -60 mV) prior to elicitation of current-evoked action potentials. At this potential the incidence of spontaneous action potential discharge was decreased, permitting current-evoked action potentials to be studied in isolation. Current commands to elicit action potentials were made using a pulse stimulator (W-K Instruments 1831).

For analysis, current and voltage signals were filtered at 5 kHz using an 8-pole Bessel filter (Kemo Instruments, Beckenham, U.K.) and then digitized (Cambridge Electronic design 1401, Cambridge, England) at 6 kHz and transferred to the hard disk of a microcomputer. Action potentials evoked by repetitive stimulation (1-10 Hz) were transferred from VHS videotape to reel-to-reel tape prior to computer analysis. These signals (played at 1/32 recording speed) were filtered at 400 Hz and then digitized at 6 kHz and transferred to the microcomputer hard disk. The membrane potential traces displayed in the figures represent single iterations.

V. Drugs

4-aminopyridine (4-AP; Sigma), tetraethylammonium (TEA; Sigma), charybdotoxin (ChTX; Sigma), $CdCl_2$ and $SrCl_2$ were

dissolved in extracellular recording medium. Drug-containing extracellular medium was applied either by addition to the extracellular medium using a hand-held pipette or by pressure ejection from a micropipette (100 μm inside diameter) positioned immediately adjacent to the neuron from which membrane current or membrane potential was being recorded. All values are given as the mean ± standard error.

RESULTS

I. Outward K^+ Current Components in Dissociated MNCs

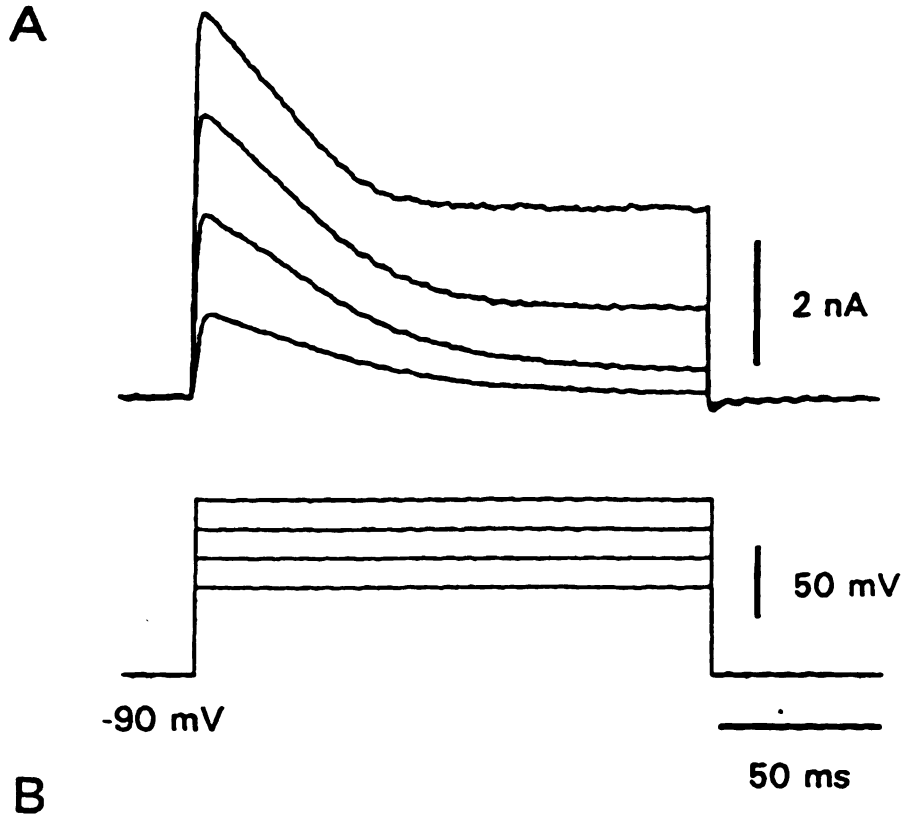
Whole-cell, voltage-clamp recordings were only obtained from cells deemed to be healthy (phase bright) when viewed using phase contrast optics. Recordings were obtained from 101 cells which were considered to be MNCs based on the morphological observation that they all exhibited a CSA $\geq$ 160 μm^2.

A. Profile of Macroscopic Outward Current

Using intracellular and extracellular solutions for recording K^+ current (see Materials and Methods), outward current was elicited from all cells by suprathreshold depolarizing voltage steps from a holding potential of -90 mV (Figure 4A). Voltage steps to test potentials between -60 mV and -30 mV elicited only an early transient current, which I will refer to as the low-threshold transient current. At test potentials more positive than -30 mV, the macroscopic current consisted of an early transient component followed by a sustained component. The current-voltage (I-V) relationship of the early and late current shows that at each test potential the early current amplitude was greater than the late current amplitude

Figure 4. Profile of outward current in supraoptic MNCs evoked by depolarizing voltage steps from a holding potential of -90 mV. A, current (upper records), recorded from a single neuron and evoked by depolarizing voltage steps (lower records) from a holding potential (V_H) of -90 mV, consisted only of a transient component at test potentials between -60 and -30 mV. At test potentials more positive than -30 mV, an additional sustained current component was also activated. B, relationship between current amplitude (I) and test potential (V_T) of the early peak (●) and sustained (■) current components evoked from V_H -90 mV. Note that at each suprathreshold test potential the early current amplitude was greater than the late current amplitude.

Figure 4



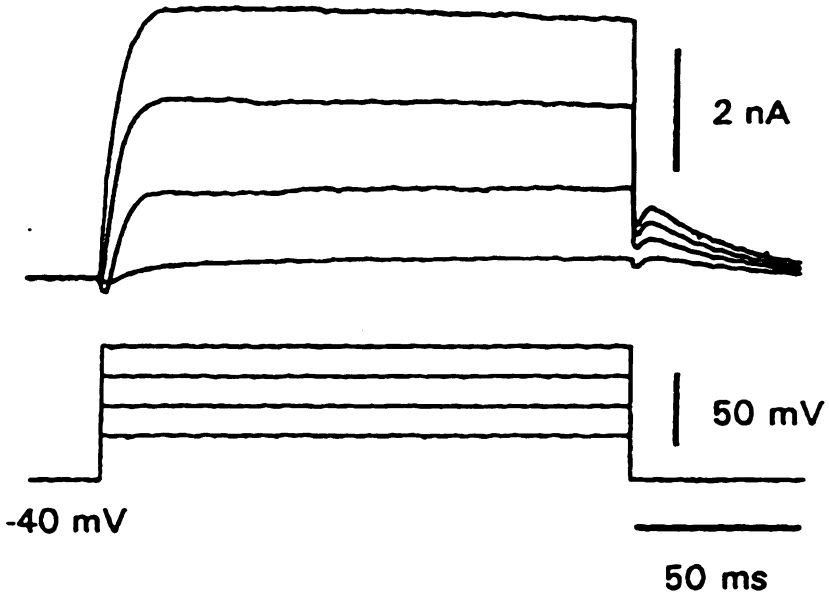
(Figure 4B). These two outward current components were present in all 101 neurons and were likely to be carried by K^+ since no outward current was observed when K^+ was replaced by Cs^+ in the electrode solution (data not shown).

When the holding potential was -40 mV, the sustained current was the predominant current activated (Figure 5A). The I-V relationship of the early and late current amplitudes shows that the outward current evoked from a holding potential of -40 mV decays little over the duration of each voltage step (Figure 5B). In addition to the sustained current, a small, well-defined transient outward current was evoked in many MNCs (67 of 101 cells) by depolarizing voltage steps from a holding potential of -40 mV (Figure 6A): I will refer to this current as the high-threshold transient current. The I-V relationship of the early and late current amplitudes from such recordings reveals two features of the high-threshold transient current. First, at test potentials between +10 and +50 mV, the I-V relationship exhibits an n-shaped hump for the early current, whereas the relationship is nearly linear for the late current over the same voltage range (Figure 6B). These observations suggest that the high-threshold transient component of outward current is Ca^{2+}-dependent. Second, at test potentials between (and including) -30 and 0 mV the sustained component of the current was larger than the transient component, indicating that the activation threshold of the sustained component is more negative than

Figure 5. Profile of outward current in supraoptic MNCs evoked by depolarizing voltage steps from a holding potential of -40 mV. A, in the same neuron as in Figure 4, the sustained current was the predominant current activated by depolarizing voltage steps from V_H -40 mV. B, relationship between current and voltage of the early peak (●) and sustained (■) current components evoked from V_H -40 mV. Note that the current decays little over the duration of each voltage step.

Figure 5

A



B

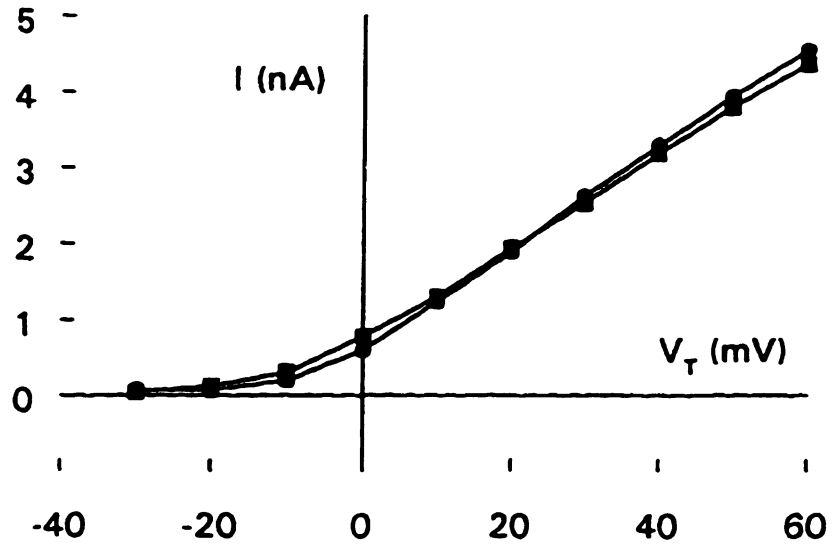
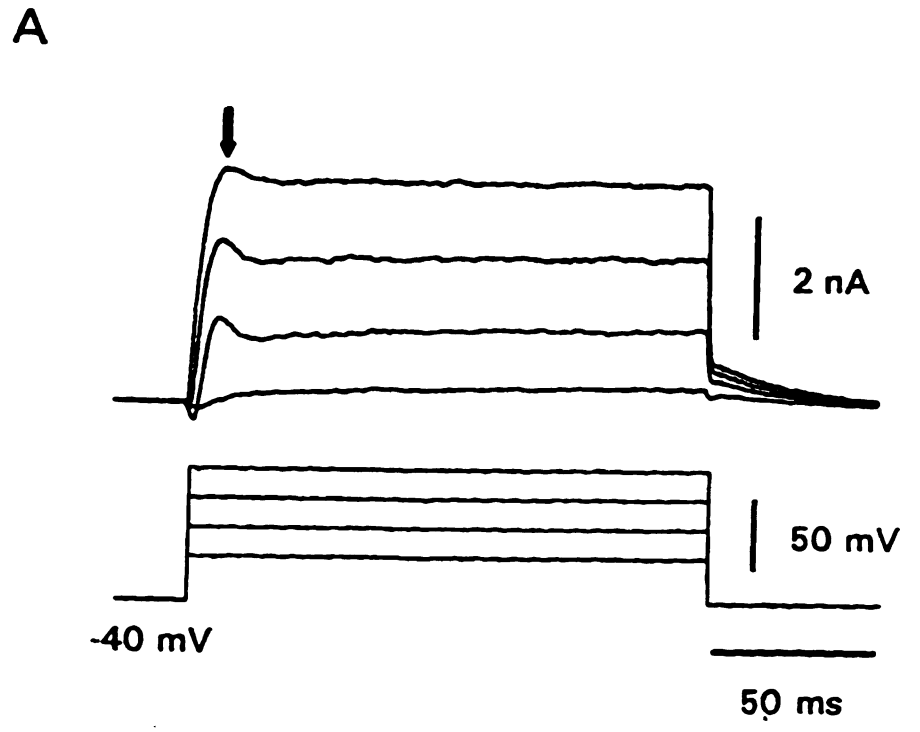


Figure 6. High-threshold transient outward current in supraoptic MNCs evoked by depolarizing voltage steps from a holding potential of -40 mV. A, in addition to the sustained current, a small, well-defined transient outward current (*arrow*) was evoked in many MNCs (67 of 101 cells) by depolarizing voltage steps from V_H -40 mV. B, relationship between current and voltage of the early peak (●) and sustained (■) current components evoked from V_H -40 mV. Note the n-shaped hump at test potentials between +10 mV and +50 mV for the early current and the lack of a similar hump for the late current over the same voltage range.

Figure 6



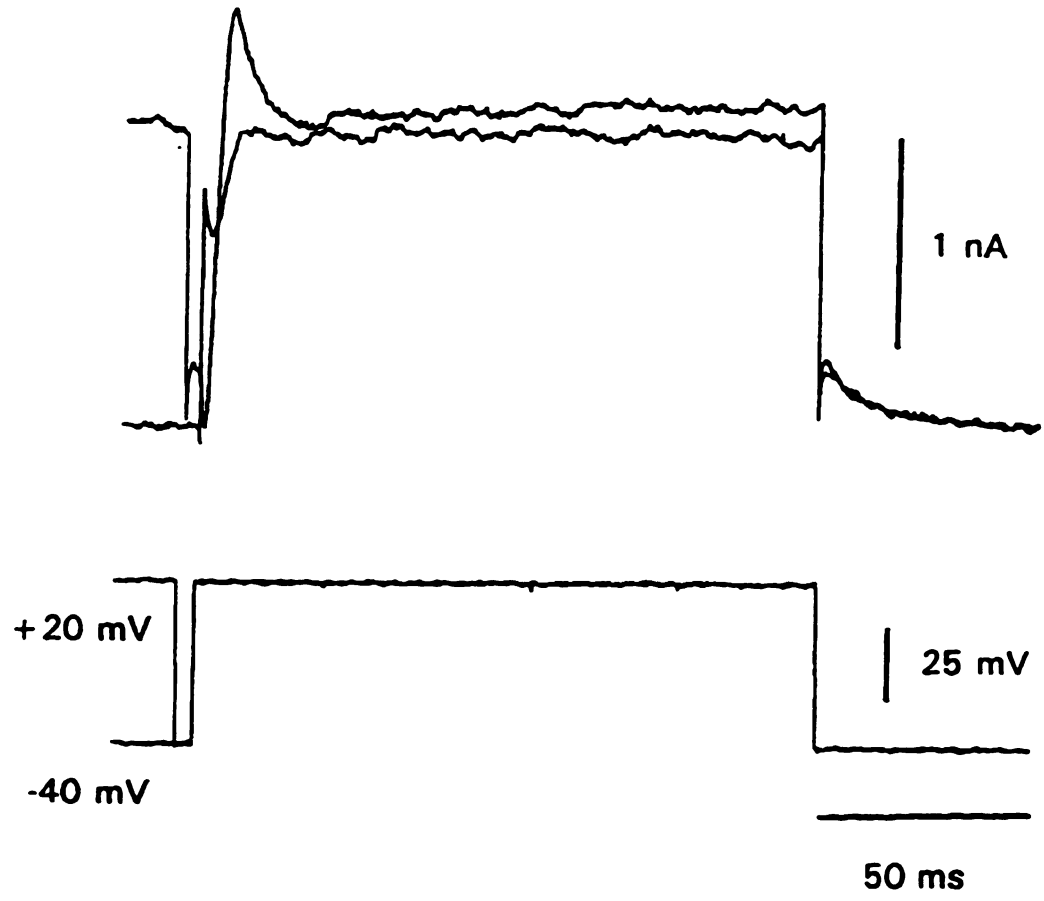
that of the (high-threshold) transient component. These data and evidence that the low- and high-threshold currents appear to have different pharmacological properties as well as different sensitivities to Ca^{2+} and other divalent cations (see **Ca^{2+} sensitivity of outward K^+ current components** below) also indicate that the high-threshold transient current is not simply a reduced low-threshold transient current (reduced due to steady-state inactivation of low-threshold current-conducting channels). Figure 7 shows that inactivation of the high-threshold transient current was produced by a conditioning prepulse to +20 mV. The outward current components were further examined to determine their activation and inactivation kinetics, voltage dependence of activation and steady-state inactivation, pharmacology, and Ca^{2+} sensitivity.

B. Activation and Inactivation Kinetics of the Low-Threshold Transient K^+ Current

Subtracting the current evoked by a depolarizing voltage step from a holding potential of -40 mV from that evoked by a voltage step to the same test potential from a holding potential of -90 mV yields a "subtraction current" (Figure 8A) which is the isolated low-threshold transient current (Connor & Stevens, 1971; Cobbett *et al.*, 1989). Subtraction currents at different test potentials were examined to determine the voltage dependence and the time course of activation and inactivation of the low-threshold

Figure 7. Inactivation of the high-threshold transient current by a depolarizing prepulse. A conditioning prepulse to +20 mV resulted in a large reduction in the high-threshold transient current evoked by a depolarizing voltage step (V_T +20 mV) from V_H -40 mV (lower records illustrate voltage protocol).

Figure 7



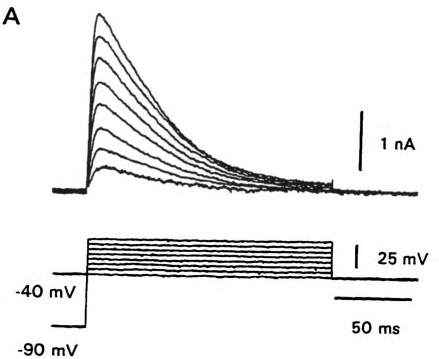
transient current. The subtraction current records reveal that the peak amplitude of the transient current was dependent on the test potential (Figure 8A), increasing as the test potential was made more positive. The time course of the isolated transient current was examined at test potentials in the range of -40 to -5 mV. At these test potentials, the transient current appears to be comprised primarily of the low-threshold transient current (with the high-threshold transient current being largely absent). Rise time (the time taken for the current to rise from 10% to 90% of its peak amplitude) was used as a measure of the time course of activation of the low-threshold transient current. The plot of rise time against test potential shows that as the test potential became more positive, rise time was reduced (Figure 8B). The time-dependent decay of the current was well-fitted by a single exponential function. The plot of the inactivation time constant (τ) against test potential shows that τ was voltage dependent, decreasing as the test potential became more positive than -20 mV (Figure 8B).

Rise time of the sustained current was not examined due to co-activation of the high-threshold transient current over the same range of depolarizing voltage steps. Also, inactivation of the sustained current was virtually undetectable during the current-activating voltage steps (150 ms).

The activation and inactivation properties of the

Figure 8. Separation of low-threshold transient outward current from total outward current. A, computer subtraction of the outward currents evoked by depolarizing voltage steps from V_H -40 mV from those evoked by the same voltage steps from V_H -90 mV gave "subtraction currents" (upper records) which represent the isolated low-threshold transient currents. B, plot of the rise time (■, the time taken for the current to rise from 10% to 90% of its peak amplitude) and inactivation time constant (●, Tau) of the isolated low-threshold transient current at different test potentials (V_T; n=6).

Figure 8


A
1 nA
25 mV
-40 mV
-90 mV
50 ms
B
Rise Time (ms)
Tau (ms)
V_T (mV)
0
1
2
3
4
5
6
7
10
20
30
40
50
-40
-30
-20
-10
0

low-threshold transient current and the sustained current described above are typical of the A-current and delayed rectifier current, respectively, which have been described for many excitable cells. Hence, I will refer to the low-threshold transient current component as $I_{K(A)}$ and the sustained current component as $I_{K(V)}$.

C. Voltage Dependence of Activation and Steady-State Inactivation of $I_{K(A)}$ and $I_{K(V)}$

Figures 9 and 10 show the voltage-dependence of activation and steady-state inactivation of $I_{K(A)}$ and $I_{K(V)}$. For analysis of activation, current (Figure 9A, upper records) was activated from a holding potential of -90 mV by depolarizing voltage steps (to between -80 mV and +60 mV). For $I_{K(A)}$, the peak current at each test potential was converted to peak conductance (g) using the following formula:

$$g = I/(E_K - V) \qquad \text{eqn (2)}$$

where E_K is the equilibrium potential (-85 mV) for K^+ calculated using the Nernst equation. The peak conductance value (g) for each test potential was then normalized to g_{max} (maximal g for that cell) and plotted against test potential to produce an activation curve. The resulting curve is sigmoidal and shows that $I_{K(A)}$ activates at a threshold voltage near -50 mV and is maximal at test potentials more

positive than +40 mV (Figure 9B). The voltage dependence of $I_{K(V)}$ activation was investigated using the same procedure described above for $I_{K(A)}$, except that the current remaining near the end of each voltage step (rather than the peak current) was analyzed. The resulting curve is also sigmoidal and shows that $I_{K(V)}$ activates at a threshold voltage near -30 mV and is maximal at test potentials more positive than +50 mV (Figure 9B).

To examine the steady-state inactivation of $I_{K(A)}$ and $I_{K(V)}$, a 2 sec conditioning prepulse to potentials between -120 mV and 0 mV was applied to allow the inactivation process to reach its steady-state level, and the outward current evoked by a subsequent 100 ms test pulse (V_t = +10 mV) was recorded (Figure 10A). $I_{K(A)}$ became progressively larger when prepulse potentials were more negative than -40 mV, reaching a maximum amplitude when the prepulse potential was more negative than -110 mV. The relationship between amplitude of the transient current to the prepulse potential was sigmoidal (Figure 10B). $I_{K(V)}$ was relatively unaffected by prepulse potentials as positive as 0 mV (Figures 10A and 10B).

Data from these experiments were fitted with a Boltzmann equation:

$$g/g_{max} = 1/\{1 + \exp[-(V-V_{1/2})/\kappa]\} \qquad \text{eqn (3)}$$

for activation and

Figure 9. Voltage dependence of activation of $I_{K(A)}$ and $I_{K(V)}$. A, to examine activation, outward current was recorded during depolarizing voltage steps from V_H -90 mV. Note that following activation, both the early and late current components increase in amplitude as test potentials became more positive. B, relationship between normalized (g/g_{max}) early peak (●, n=7) and late (■, n=7) computer-estimated conductance and test potential (V_T). Activation curves for the data points were fitted by eqn (3).

Figure 9

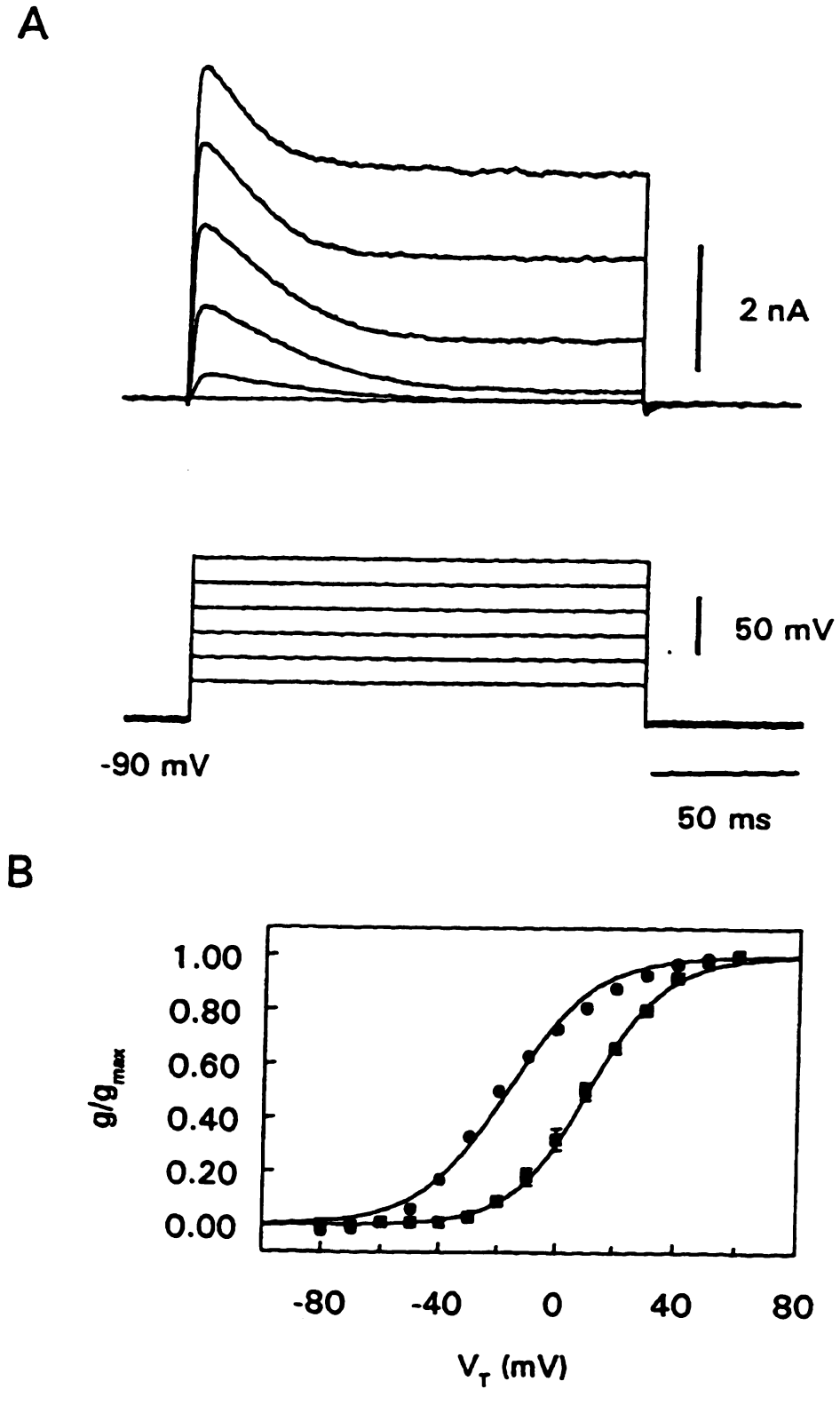
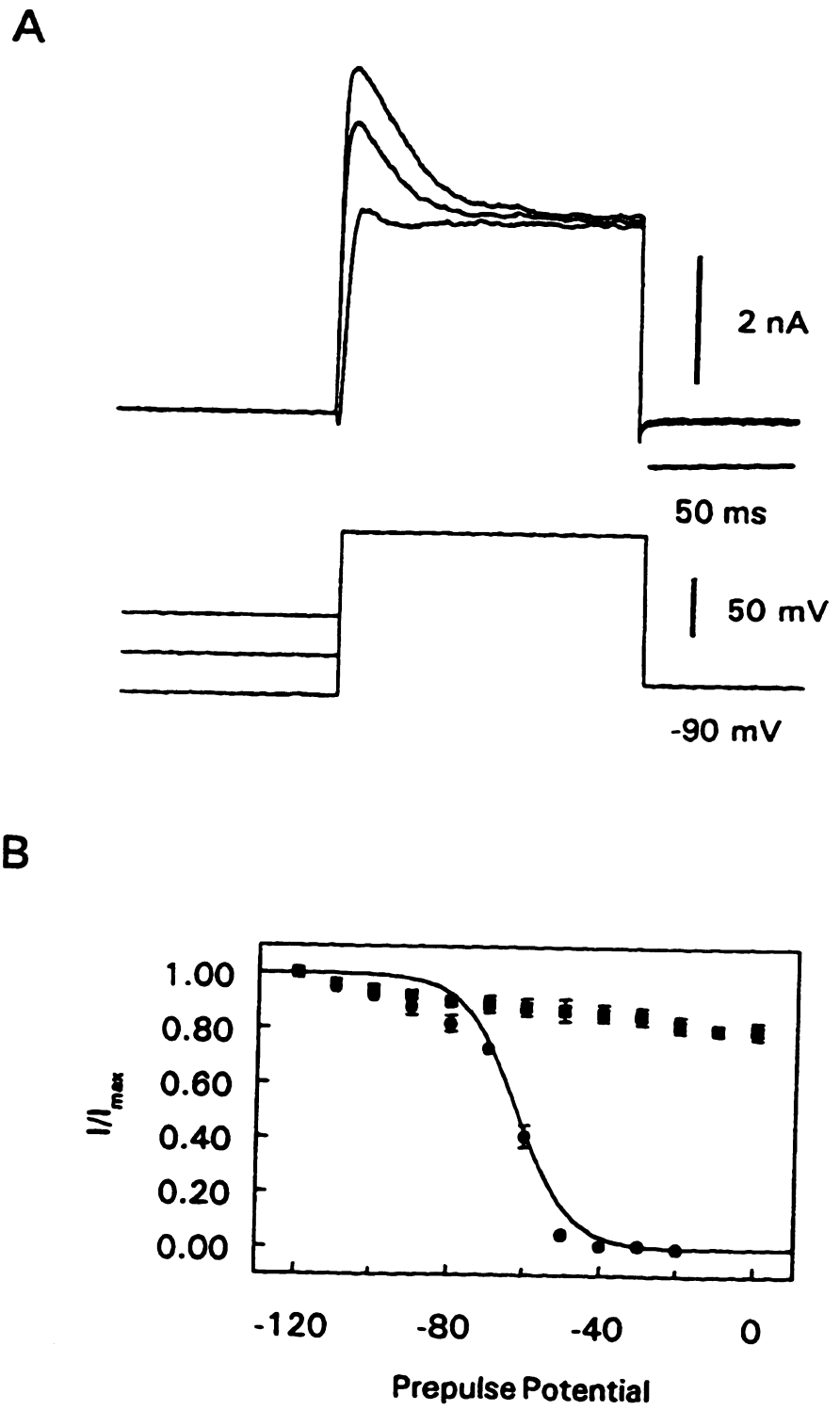

A
2 nA
50 mV
-90 mV
50 ms
B
1.00
0.80
0.60
0.40
0.20
0.00
g/g_max
-80
-40
0
40
80
V_T (mV)

Figure 10. Voltage dependence of steady-state inactivation of $I_{K(A)}$ and $I_{K(V)}$. A, to examine steady-state inactivation, a 1 sec conditioning prepulse to potentials between -120 mV and 0 mV was applied and the outward current evoked by a subsequent depolarizing voltage step (V_T +10 mV) was recorded. Note that $I_{K(A)}$ was larger when prepulse potentials were more negative whereas $I_{K(V)}$ was relatively unaffected by prepulse potential. B, relationship between normalized (I/I_{max}) early peak (●, n=6) and late (■, n=6) current and prepulse potential. The inactivation curve for the $I_{K(A)}$ data points were fitted by eqn (4).

Figure 10


A
2 nA
50 ms
50 mV
-90 mV
B
1.00
0.80
0.60
0.40
0.20
0.00
I/I max
-120
-80
-40
0
Prepulse Potential

$$I/I_{max}=1/\{1 + \exp[(V-V_{1/2})/\kappa]\} \qquad \text{eqn (4)}$$

for steady-state inactivation, where $V_{1/2}$ is the potential at which half of the channels are activated (or inactivated), and κ is the slope factor describing the steepness of the voltage dependence. For $I_{K(A)}$, the data were best fit with Boltzmann functions where the $V_{1/2}$ and κ values were, respectively, -17 mV and 15 for activation (n=7) and -62 mV and 7 for inactivation (n=6). There was some difference between data points and the best-fitting Boltzmann functions for activation and steady-state inactivation in the more negative range of membrane potential which was examined (Figures 10, 12, 14 and 15). Although I was unable to account for these deviations, they were seen in all cells examined. Further, since the same deviations of obtained data from the best-fitting Boltzmann functions were observed for experiments in which the extracellular medium contained either 2 mM Ca^{2+} (Cd^{2+}-free) or 125 μM Cd^{2+} (Ca^{2+}-free), the deviations cannot be due to the contribution of a Ca^{2+}-dependent process. They could, however, be due to a divalent cation-dependent process. The $I_{K(V)}$ activation data (n=7) were best fit with a Boltzmann function where the $V_{1/2}$ and κ values were +11 mV and 13, respectively. No attempt was made to fit the $I_{K(V)}$ inactivation data to a Boltzmann equation due to the non-inactivating nature of this current.

D. Ca^{2+} Sensitivity of Outward K^+ Current Components

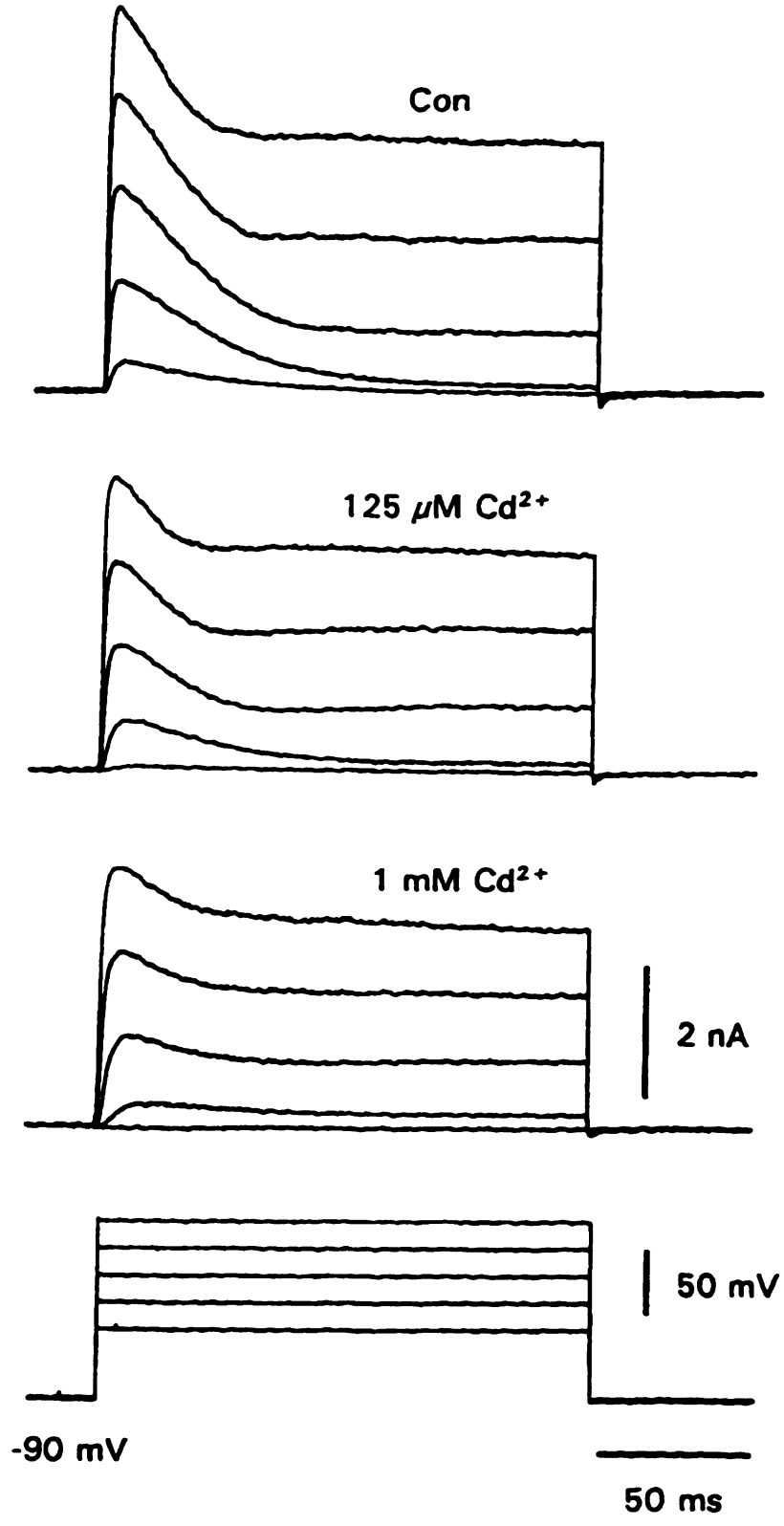
Different studies have suggested that both the low-threshold, A-type transient (Bourque, 1988) and high-threshold sustained (Cobbett *et al.*, 1989) K^+ currents of supraoptic MNCs are modulated by Ca^{2+} influx. I therefore chose to examine the Ca^{2+} sensitivity of outward K^+ current recorded from acutely isolated MNCs of the guinea pig SON by examining the effect of the inorganic Ca^{2+} channel blocker Cd^{2+} on this current.

Recordings of voltage-activated K^+ current evoked by depolarizing voltage steps from a holding potential of -90 mV were made before and after addition of $CdCl_2$ to the extracellular solution. Addition of Cd^{2+} (125 μM or 1 mM) to the Ca^{2+}-containing extracellular solution resulted in an apparent reduction in the amplitude of $I_{K(A)}$ at each potential tested (Figure 11, upper records). For the cell shown in figure 11, at test potentials of -20, 0 and +20 mV the respective peak amplitudes of $I_{K(A)}$ evoked in the presence of 125 μM Cd^{2+} were 46, 63 and 70% of the corresponding peak currents recorded using Cd^{2+}-free extracellular solution.

Several studies have shown that extracellular divalent cations can have a profound effect on the voltage dependence of $I_{K(A)}$ activation and steady-state inactivation (Gilly & Armstrong, 1982; Mayer & Sugiyama, 1988; Carignani *et al.*, 1991; Wisgirda & Dryer, 1993). Accordingly, there was concern that the apparent reduction in the amplitude of $I_{K(A)}$

Figure 11. Effect of extracellular Cd^{2+} on $I_{K(A)}$ and $I_{K(V)}$ amplitudes. Addition of Cd^{2+} (125 μM or 1 mM) to the Ca^{2+}-containing extracellular solution resulted in an apparent reduction in the amplitudes of $I_{K(A)}$ and $I_{K(V)}$ evoked during each depolarizing voltage step from V_H -90 mV.

Figure 11



following addition of Cd^{2+} to the extracellular solution was the result of such an effect, rather than the result of Cd^{2+} blocking Ca^{2+} influx. Addition of Cd^{2+} to the extracellular solution produced positive shifts of the activation and steady-state inactivation curves for $I_{K(A)}$ (Figures 12A and 12B). The lowest concentration of Cd^{2+} (125 μM) shifted the population activation curve by +18 mV (n=5) and the population inactivation curve by +15 mV (n=5) relative to control values. In contrast, this concentration of Cd^{2+} did not affect the voltage dependence of the sustained current (Figure 13).

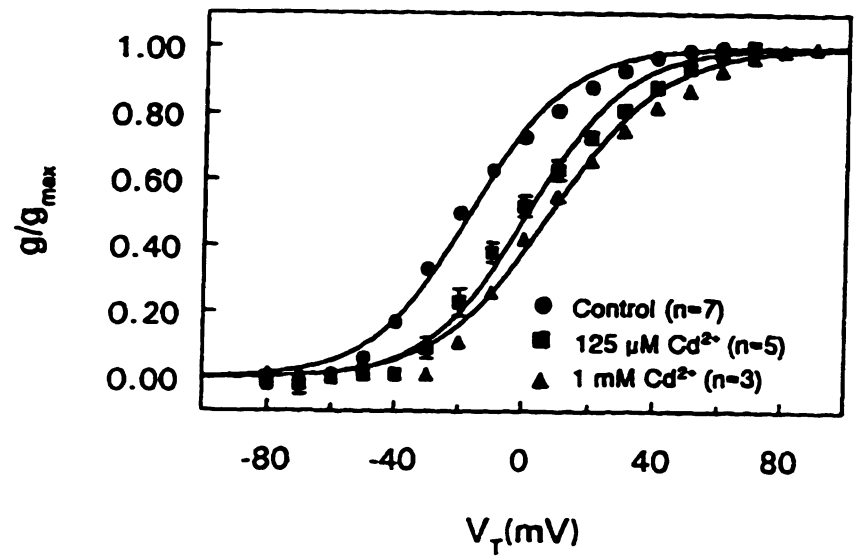
It is conceivable that the shifts in the voltage dependence of $I_{K(A)}$ activation and inactivation induced by Cd^{2+} were not the result of Cd^{2+} directly affecting $I_{K(A)}$, but instead a secondary effect due to a primary effect of Cd^{2+} on an underlying Ca^{2+} current. Accordingly, the effects of extracellular Cd^{2+} on the voltage dependence of activation and inactivation of $I_{K(A)}$ recorded from cells bathed in Ca^{2+}-free extracellular solution were examined.

Removal of Ca^{2+} from the extracellular solution (no Cd^{2+} present) resulted in a -16 mV (n=4) shift of the $I_{K(A)}$ activation curve and -23 mV (n=4) shift of the inactivation curve relative to curves derived from recordings made in the presence of 2 mM extracellular Ca^{2+} (Figures 14A and 14B). Addition of 125 μM Cd^{2+} to the Ca^{2+}-free extracellular solution, resulted in a +22 mV (n=4) positive shift of the activation curve (Figure 15A) and a +28 mV (n=3) positive

Figure 12. Effect of extracellular Cd^{2+} on the voltage dependence of activation and steady-state inactivation of $I_{K(A)}$. A, activation curves for $I_{K(A)}$ data obtained before and after addition of Cd^{2+} (125 μM or 1 mM) to the Ca^{2+}-containing extracellular solution. B, steady-state inactivation curves for $I_{K(A)}$ data obtained before and after addition of Cd^{2+} to the Ca^{2+}-containing extracellular solution.

Figure 12

A


1.00
0.80
0.60
0.40
0.20
0.00
g/g_max
Control (n=7)
125 µM Cd2+ (n=5)
1 mM Cd2+ (n=3)
-80
-40
0
40
80
V_T(mV)

B

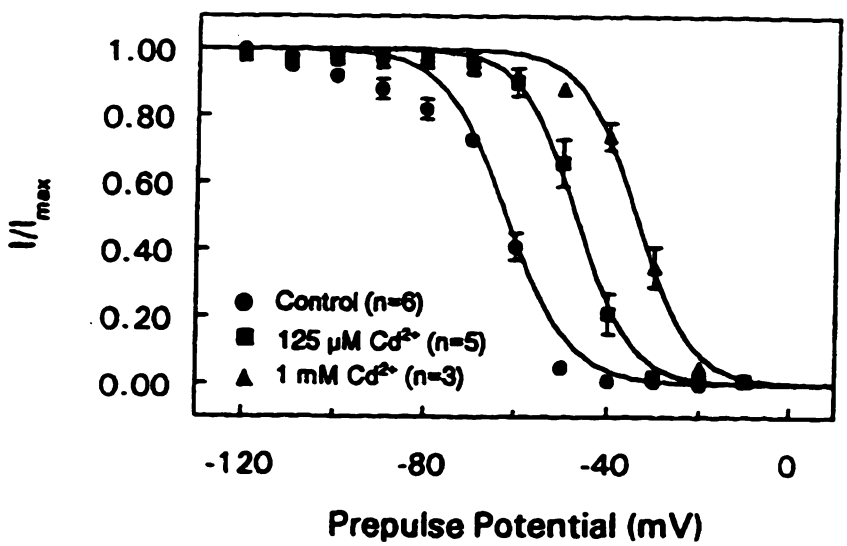

1.00
0.80
0.60
0.40
0.20
0.00
I/I_max
Control (n=6)
125 µM Cd2+ (n=5)
1 mM Cd2+ (n=3)
-120
-80
-40
0
Prepulse Potential (mV)

Figure 13. Effect of extracellular Cd^{2+} on the voltage dependence of $I_{K(V)}$ activation. Activation curves for $I_{K(V)}$ data obtained before and after addition of Cd^{2+} (125 μM) to the Ca^{2+}-containing extracellular solution. Note that extracellular Cd^{2+} produced no change in the voltage dependence of $I_{K(V)}$ activation.

Figure 13

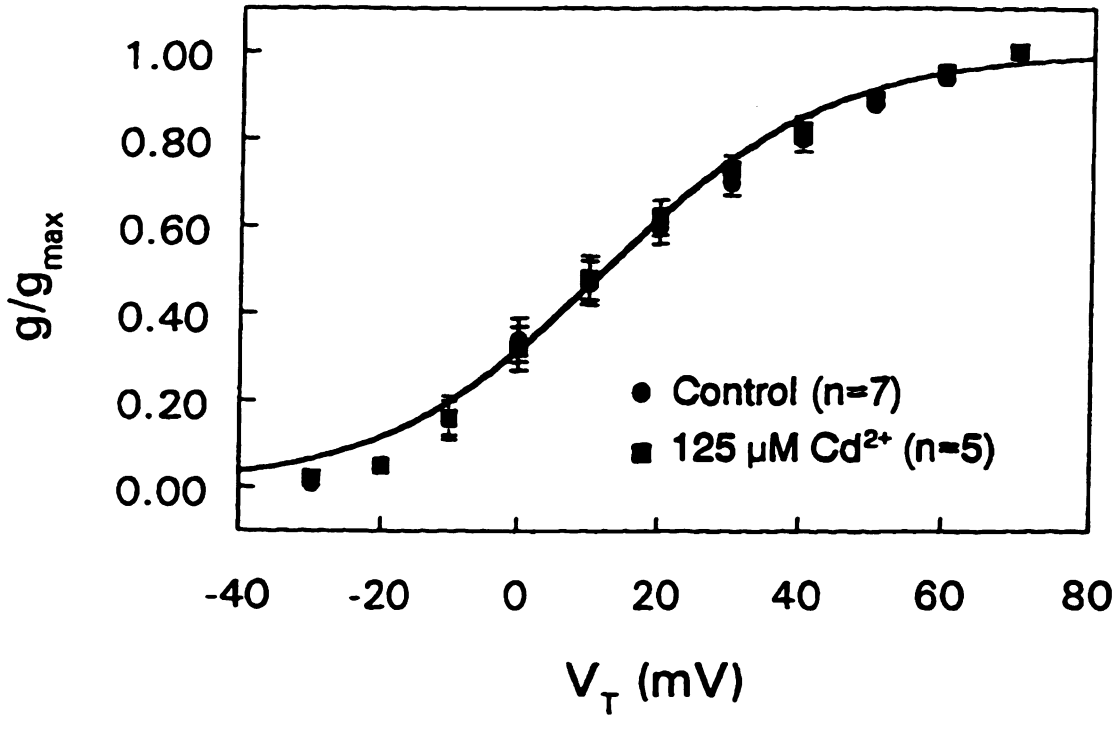
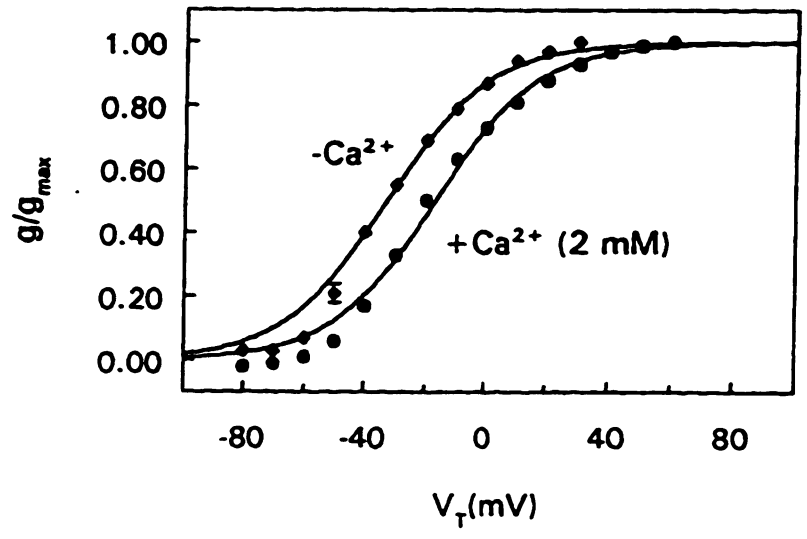

1.00
0.80
0.60
0.40
0.20
0.00
g/g_max
Control (n=7)
125 µM Cd2+ (n=5)
-40
-20
0
20
40
60
80
V_T (mV)

Figure 14. Voltage dependence of activation and steady-state inactivation of $I_{K(A)}$ recorded in Ca^{2+}-free extracellular solution. A, B, removal of Ca^{2+} from the extracellular solution resulted in negative shifts of the activation and steady-state inactivation curves relative to curves fitted to data obtained in the presence of 2 mM extracellular Ca^{2+}.

Figure 14

A


1.00
0.80
0.60
0.40
0.20
0.00
g/g_max
-Ca2+
+Ca2+ (2 mM)
-80
-40
0
40
80
V_T(mV)

B

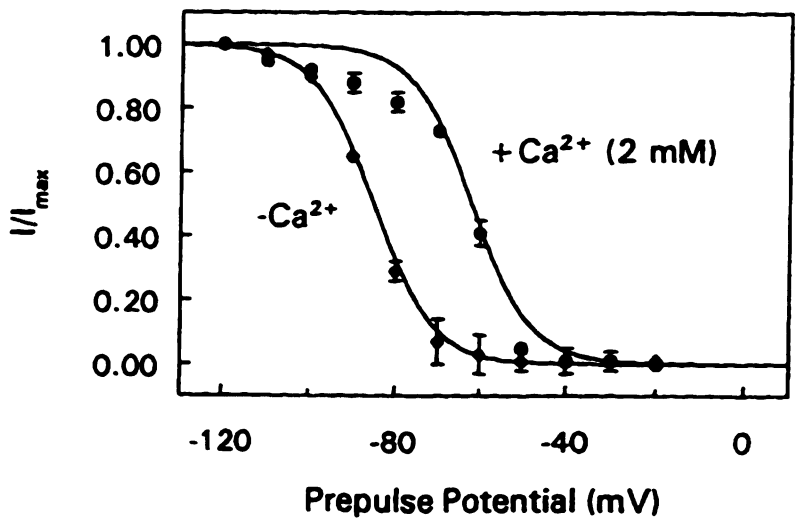
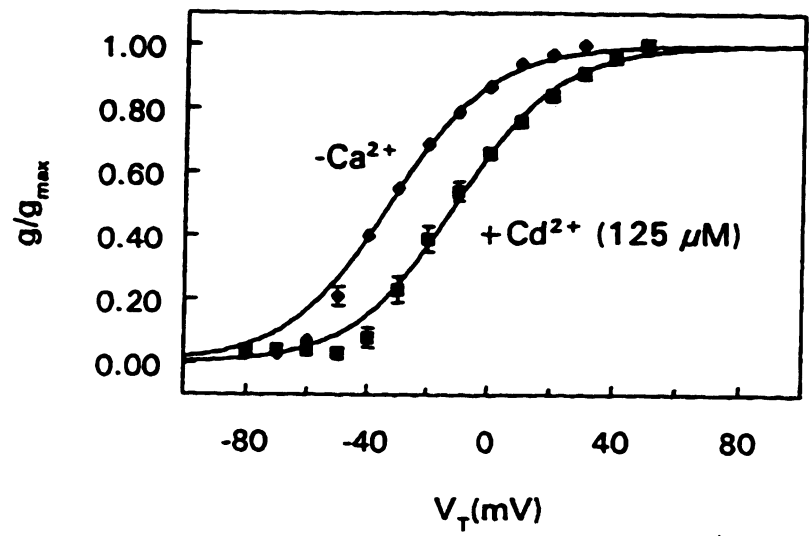

1.00
0.80
0.60
0.40
0.20
0.00
I/I_max
+Ca2+ (2 mM)
-Ca2+
-120
-80
-40
0
Prepulse Potential (mV)

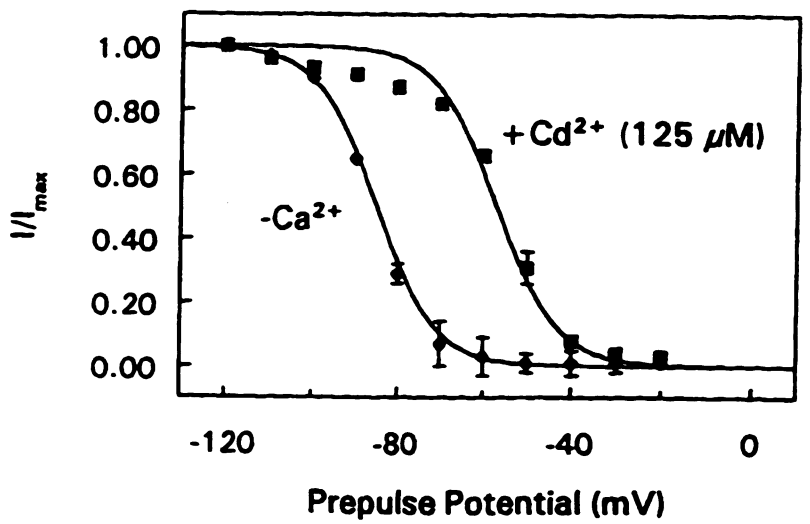
Figure 15. Effect of extracellular Cd^{2+} on the voltage dependence of activation and steady-state inactivation of $I_{K(A)}$ recorded in Ca^{2+}-free extracellular solution. A, B, addition of 125 μM Cd^{2+} to the Ca^{2+}-free extracellular solution resulted in positive shifts of the activation and steady-state inactivation curves relative to curves fitted to data obtained when the extracellular solution contained neither Ca^{2+} nor Cd^{2+}.

Figure 15

A


1.00
0.80
0.60
0.40
0.20
0.00
g/g_max
-Ca2+
+Cd2+ (125 μM)
-80
-40
0
40
80
V_T(mV)

B


1.00
0.80
0.60
0.40
0.20
0.00
I/I_max
+Cd2+ (125 μM)
-Ca2+
-120
-80
-40
0
Prepulse Potential (mV)

shift of the inactivation curve (Figure 15B) relative to values obtained from cells bathed in Ca^{2+}-free, Cd^{2+}-free extracellular solution. In comparison, addition of 125 μM Ca^{2+} to the Ca^{2+}-free extracellular solution did not affect the voltage dependence of $I_{K(A)}$ activation or inactivation (data not shown), suggesting that Ca^{2+} is not as potent as Cd^{2+} in producing changes in the voltage dependence of $I_{K(A)}$.

It also appeared that, in addition to reducing the amplitude and shifting the voltage dependence of $I_{K(A)}$, Cd^{2+} slowed the activation and inactivation of $I_{K(A)}$ (Figure 11); however, this effect was not systematically investigated in the present study.

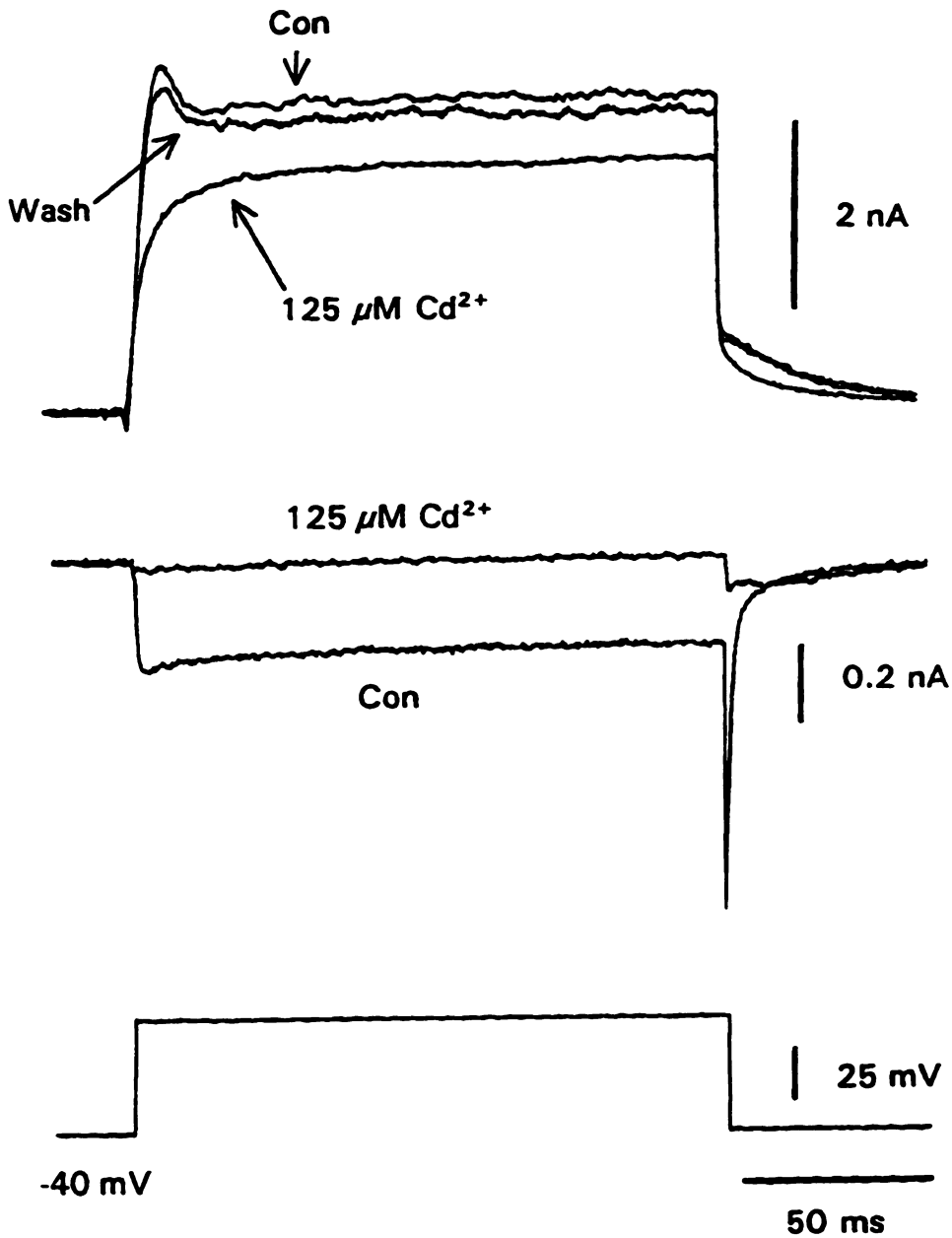
As previously stated, in many recordings from MNCs an early, well-defined transient outward current was evoked by depolarizing voltage steps from a holding potential of -40 mV (see Figure 6). This high-threshold transient current activated at test potentials more positive than -20 mV, and reached a peak within 9 ms and was inactivated within 30 ms during test pulses to +20 mV. To determine the dependence of this current on Ca^{2+} influx, current was recorded from neurons during depolarizing voltage steps from a holding potential of -40 mV to a fixed test potential of +20 mV before and then again after the addition of 125 μM Cd^{2+} to the extracellular solution. Addition of Cd^{2+} to the extracellular solution abolished the early transient K^+ current recorded during the voltage step (Figure 16, upper records; n=6). This effect was not due simply to a shift in

the voltage dependence of current activation and steady-state inactivation, as this current was not activated by depolarizing voltage steps to test potentials as positive as +60 mV. The effect of Cd^{2+} on the high-threshold transient current was reversible in that the current was restored following removal of Cd^{2+} from the extracellular solution. It was also noted that 125 μM Cd^{2+} reduced the amplitude of the sustained current. Under ionic conditions for recording Ca^{2+} current, it was shown that 125 μM Cd^{2+} is sufficient to block all inward Ca^{2+} current recorded during a depolarizing voltage step to +20 mV from a holding potential of -40 mV (Figure 16, middle records). These experiments suggest that the high-threshold transient current is abolished during block of Ca^{2+} influx by Cd^{2+}.

Another approach used to determine the Ca^{2+}-dependence of the high-threshold transient K^+ current observed during depolarizing voltage steps from a holding potential of -40 mV was to make recordings in normal (2 mM) Ca^{2+}-containing extracellular solution and then again after equimolar replacement of Ca^{2+} with Sr^{2+}. I chose to use Sr^{2+}, which (unlike Cd^{2+}) readily permeates voltage-gated Ca^{2+} channels but is less efficacious than Ca^{2+} at activating many Ca^{2+}-dependent processes (Siegelbaum & Tsien, 1980; Barish, 1983), rather than Ba^{2+}, because although Ba^{2+} readily permeates voltage-gated Ca^{2+} channels there are numerous reports that Ba^{2+} produces a pharmacological block of different K^+ current components in a variety of cell types

Figure 16. Effects of extracellular Cd^{2+} on the high-threshold transient outward current. Addition of 125 μM Cd^{2+} to the extracellular solution abolished the early transient current and significantly reduced the sustained current evoked by a depolarizing voltage step (V_T +20 mV) from V_H -40 mV (upper records). This concentration of extracellular Cd^{2+} is sufficient to block all inward current evoked during the voltage step (middle records).

Figure 16



(Armstrong & Taylor, 1980; Latorre & Miller, 1983).

Current was recorded from neurons during depolarizing voltage steps from a holding potential of -40 mV to a test potential of +20 mV before and then again after replacement of extracellular Ca^{2+} with Sr^{2+}. Replacement of extracellular Ca^{2+} with Sr^{2+} nearly abolished the transient K^+ current recorded during the voltage step (Figure 17, upper records; n=6). The effect of Sr^{2+} on the high-threshold transient current was reversible in that the current was restored following replacement of Sr^{2+} in the extracellular solution with Ca^{2+}. Using the same voltage protocol, replacement of extracellular Ca^{2+} with Sr^{2+} produced no change in the current recorded under ionic conditions for recording inward current (Figure 17, middle records). Thus, the high-threshold transient K^+ current evoked during voltage steps from a holding potential of -40 mV is abolished during block of Ca^{2+} influx by Cd^{2+} or when the inward current is carried by Sr^{2+} rather than Ca^{2+}.

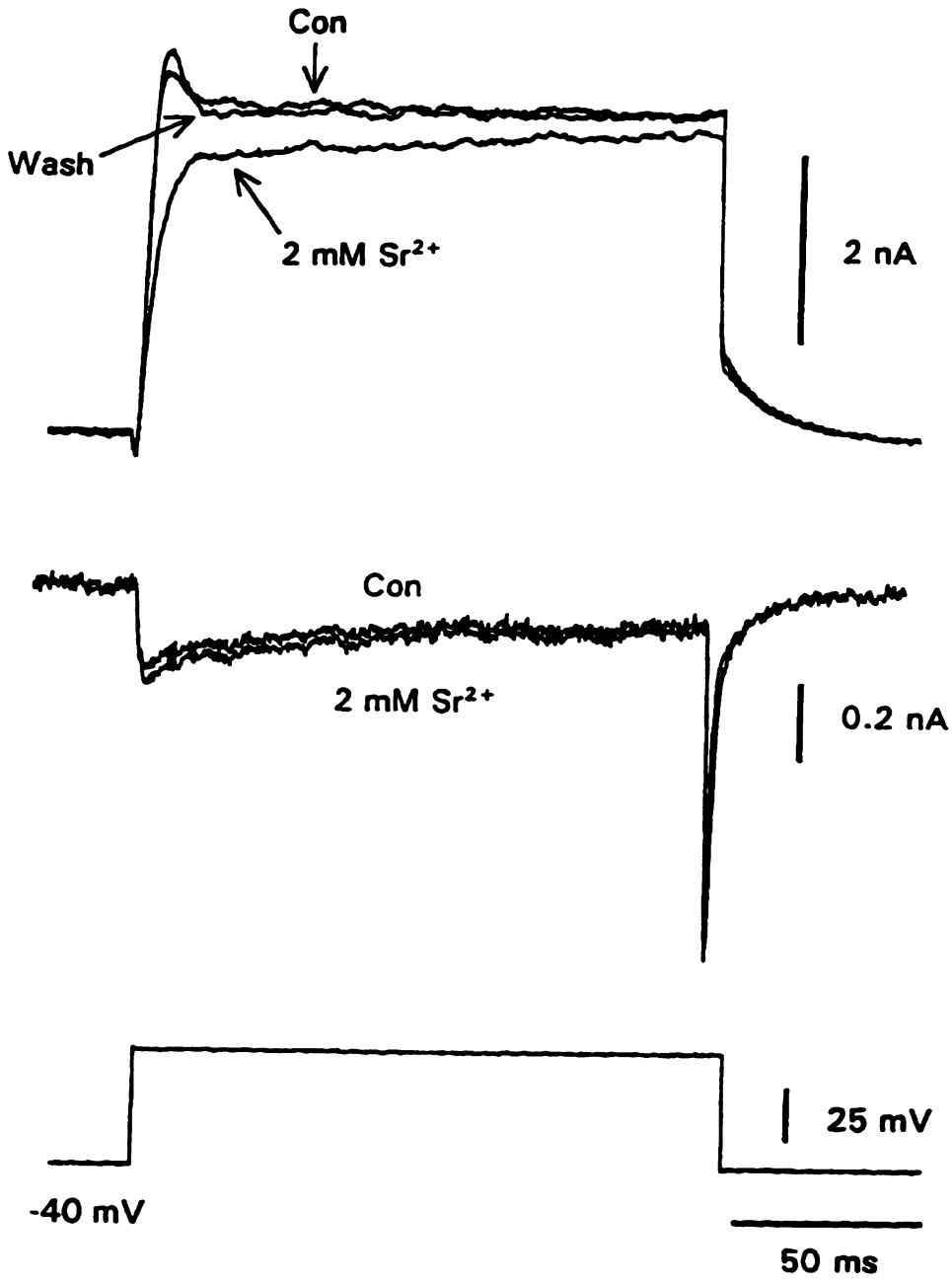
E. Pharmacology of Outward K^+ Current Components

Differential sensitivity to block by external 4-AP and TEA has been used as a criterion to characterize outward potassium current components in several cell types (see review by Rudy, 1988).

Addition of 1 mM 4-AP to the extracellular solution significantly reduced the transient K^+ current evoked from a holding potential of -90 mV (Figure 18A). At a test

Figure 17. Effects of extracellular Sr^{2+} on the high-threshold transient outward current. Equimolar replacement of extracellular Ca^{2+} with Sr^{2+} nearly abolished the early transient current and reduced the sustained current evoked by a depolarizing voltage step (V_T +20 mV) from V_H -40 mV (upper records). This procedure had little effect on the inward current evoked during the voltage step (middle records).

Figure 17



potential of +10 mV, $I_{K(A)}$ in the presence of 1 mM 4-AP was 43.1 ± 6.0% (n=6) of control. As expected, 4-AP was less effective at blocking the sustained current. In the same cells at the test potential of +10 mV, $I_{K(V)}$ in the presence of 1 mM 4-AP was 72.4 ± 6.2% of control.

In contrast, 5 mM extracellular TEA produced a preferential block of the sustained K^+ current (Figure 18B). At a test potential of +10 mV from V_H -90 mV, $I_{K(V)}$ in the presence of 5 mM TEA was 39.0 ± 2.8% (n=5) of control whereas $I_{K(A)}$ in the same cells in the presence of the same concentration of TEA was 90.8 ± 2.3% of control.

The sensitivity to block by external 4-AP and TEA was also examined for the high-threshold, Ca^{2+}-dependent transient current. MNCs which exhibited a prominent high-threshold transient current during a depolarizing voltage step (V_T+10 mV) from a holding potential of -40 mV were selected for these experiments. This current was relatively unaffected by high concentrations (up to 5 mM) of 4-AP (Figure 19A; n=2) but was blocked by 1 mM TEA (Figure 19B; n=4).

For Ca^{2+}-dependent K^+ currents, their tends to be a correlation between sensitivity to block by TEA and by ChTX (Sah, 1996). Accordingly, the sensitivity to block by ChTX of the high-threshold, Ca^{2+}-dependent transient K^+ current recorded from MNCs was examined. High-threshold transient current was evoked by stepping the membrane potential to +10 mV from a holding potential of -40 mV. Addition of 100 nM

Figure 18. Pharmacological properties of $I_{K(A)}$ and $I_{K(V)}$. A, addition of 1 mM 4AP to the extracellular solution preferentially blocked $I_{K(A)}$ evoked by a depolarizing voltage step (V_T +10 mV) from V_H -90 mV. B, addition of 5 mM TEA to the extracellular solution dramatically reduced $I_{K(V)}$ evoked by a depolarizing voltage step (V_T +10 mV) from V_H -90 mV, whereas $I_{K(A)}$ was largely unaffected.

Figure 18

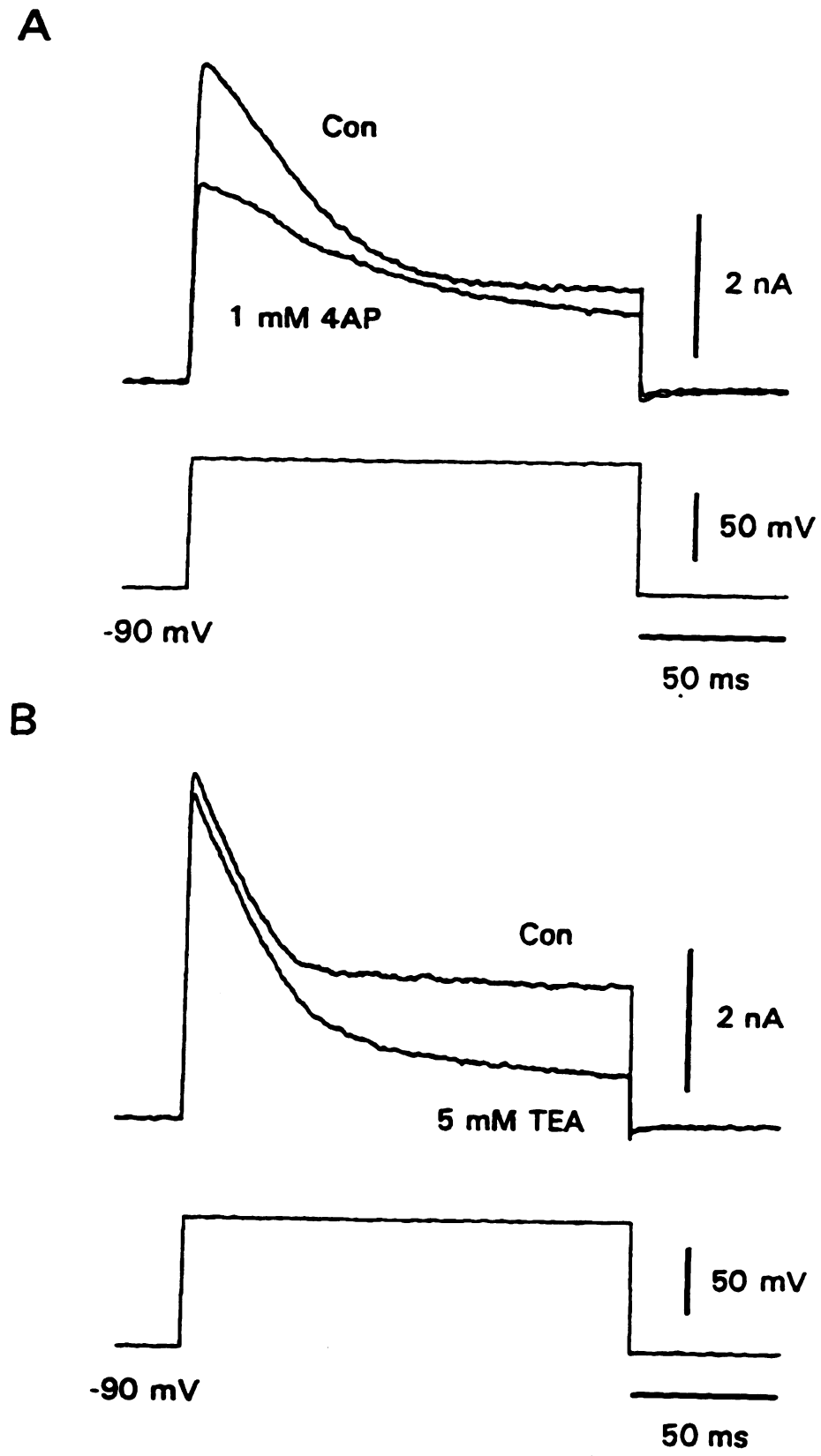
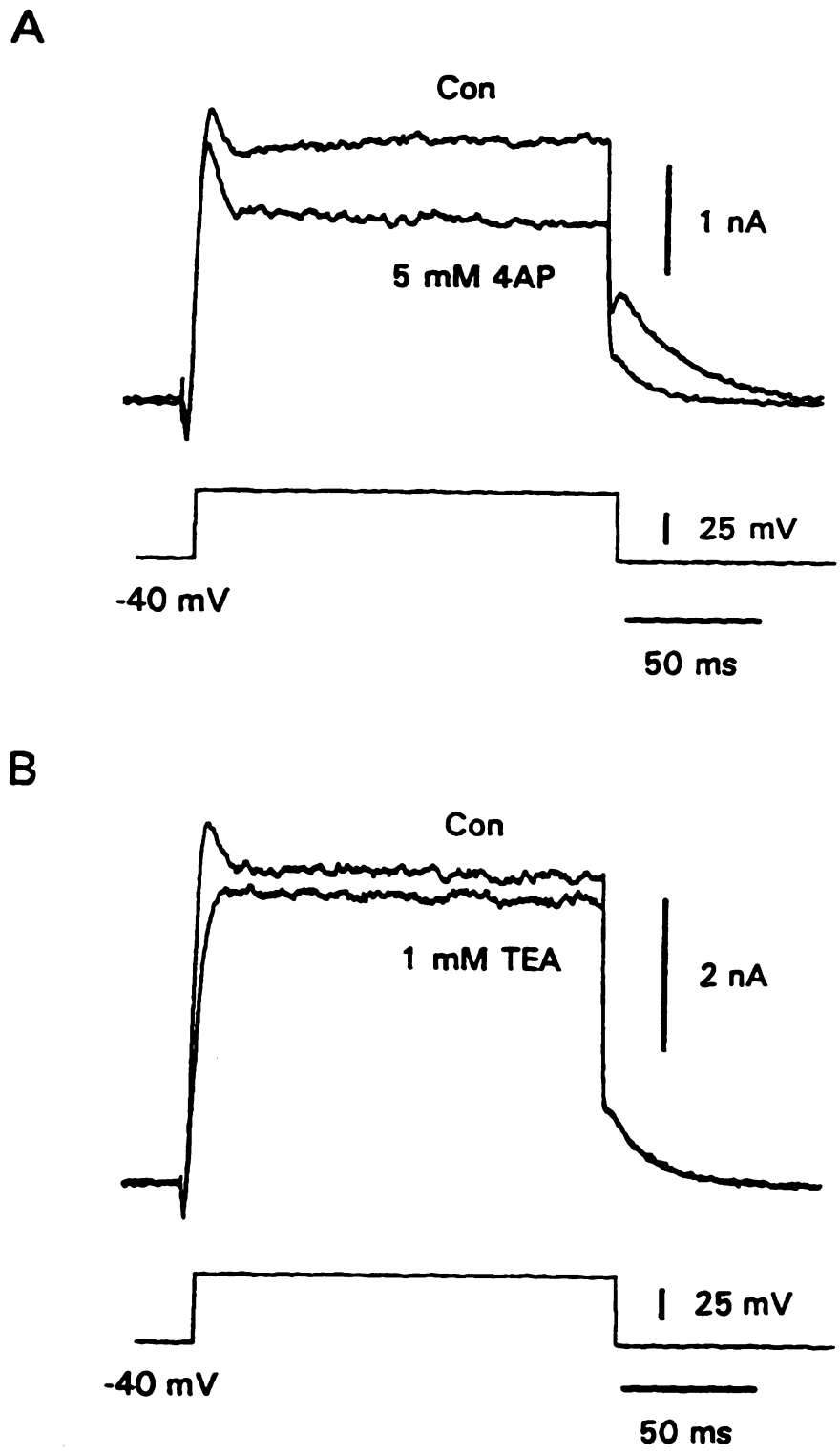

A
Con
1 mM 4AP
2 nA
50 mV
-90 mV
50 ms
B
Con
5 mM TEA
2 nA
50 mV
-90 mV
50 ms

Figure 19. Pharmacological properties of the high-threshold transient outward current. A, application of a high concentration (5 mM) of extracellular 4AP failed to abolish the early transient current evoked by a depolarizing voltage step (V_T +20 mV) from V_H -40 mV. Note that this high concentration of 4AP resulted in a large reduction of the sustained current. B, application of extracellular TEA at a concentration (1 mM) which only slightly reduced the sustained current nearly abolished the early transient current evoked by a depolarizing voltage step (V_T +20 mV) from V_H -40 mV.

Figure 19


A
Con
1 nA
5 mM 4AP
25 mV
-40 mV
50 ms
B
Con
1 mM TEA
2 nA
25 mV
-40 mV
50 ms

ChTX to the extracellular solution abolished the high-threshold transient current (Figure 20B; n=5). To further demonstrate that the high-threshold transient current was not simply a reduced low-threshold transient current, the sensitivity to block by ChTX of the low-threshold transient K^+ current recorded from MNCs was also examined. Current was evoked by stepping the membrane potential to -40 mV from a holding potential of -90 mV. Using this protocol, the low-threshold transient current is the only component of outward current activated (Figure 20A). Addition of 100 nM ChTX to the extracellular solution did not affect the isolated low-threshold transient current (n=5). These results confirm that the low- and high-threshold components of outward current in MNCs are carried by different K^+ channel types.

II. Spike Repolarization and Frequency-Dependent Spike Broadening in Dissociated MNCs

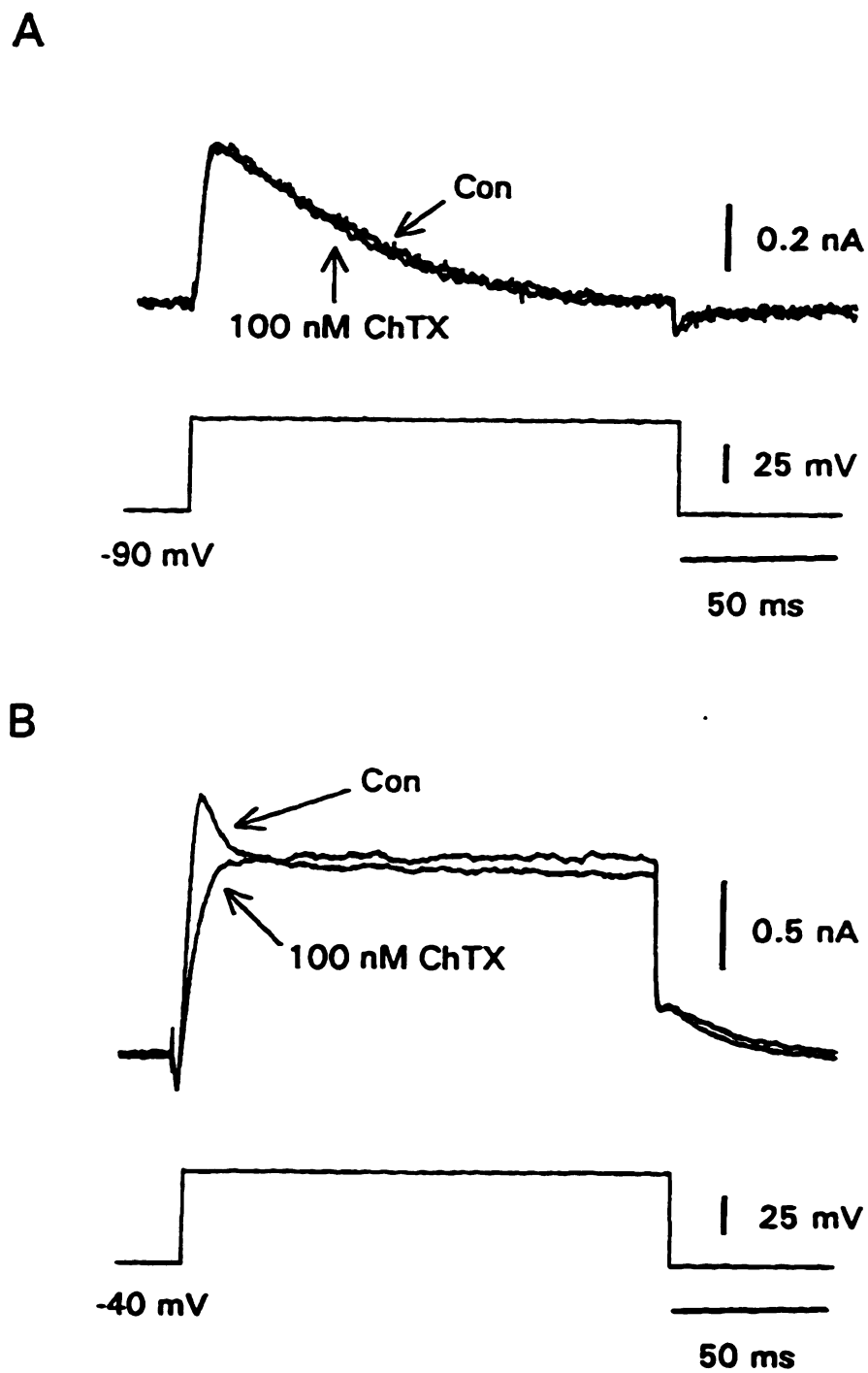
Whole-cell, current-clamp recordings were obtained from 59 cells which were considered to be MNCs based on the morphological observation that they all exhibited a CSA $\geq$ 160 μm^2. Recordings were made using intracellular and extracellular solutions for recording membrane potential (see Materials and Methods).

A. Spontaneous Action Potentials

Isolated supraoptic MNCs exhibited a mean E_R of approximately -58 $\pm$ 1 mV (n=59). In many of the

Figure 20. Differential sensitivities of the low- and high-threshold transient outward currents to ChTX. A, addition of 100 nM ChTX to the extracellular solution did not affect the isolated low-threshold transient current evoked by a depolarizing voltage step (V_T +40 mV) from V_H -90 mV. B, in the same neuron as in A, 100 nM ChTX abolished the high-threshold transient current evoked by a depolarizing voltage step (V_T +10 mV) from V_H -40 mV.

Figure 20


A
Con
0.2 nA
100 nM ChTX
25 mV
-90 mV
50 ms
B
Con
100 nM ChTX
0.5 nA
25 mV
-40 mV
50 ms

MNCs,spontaneous action potentials were observed immediately after achieving the whole-cell configuration. Figure 21 shows a series of action potentials from a continuously firing cell. The threshold for action potential initiation was between -55 and -45 mV and the mean amplitude (measured from baseline) of spontaneous action potentials was 102 ± 2 mV (n=20). Each action potential was immediately followed by a hyperpolarizing after-potential (HAP) which decayed over 100-200 ms.

B. Current-Evoked Action Potentials

Individual action potentials could be elicited from MNCs (held at a membrane potential near -60 mV) by 3 ms injections of depolarizing current (0.4 nA). Figure 22 shows a single, current-evoked action potential. When evoked at low frequency (<1 Hz), these action potentials exhibited a mean amplitude of approximately 119 ± 2 mV (n=17) and a duration (measured as the time from peak amplitude to one-third peak amplitude during the repolarization phase) of approximately 1.59 ± 0.05 ms (n=17). Figure 23 shows a series of 30 action potentials elicited by repetitive injections of depolarizing current (0.4 nA) applied at 10 Hz. When action potentials were elicited using this stimulation protocol, there was a pronounced broadening of successive action potentials (Figure 24B) which was not observed during lower-frequency (1 Hz) stimulation (Figure 24A). Previous studies have

Figure 21. Spontaneous action potentials in supraoptic MNCs. Sample recording from an MNC which displayed continuous firing activity. Note the presence of the hyperpolarizing after-potential (HAP) at the end of the falling phase of each action potential.

Figure 21

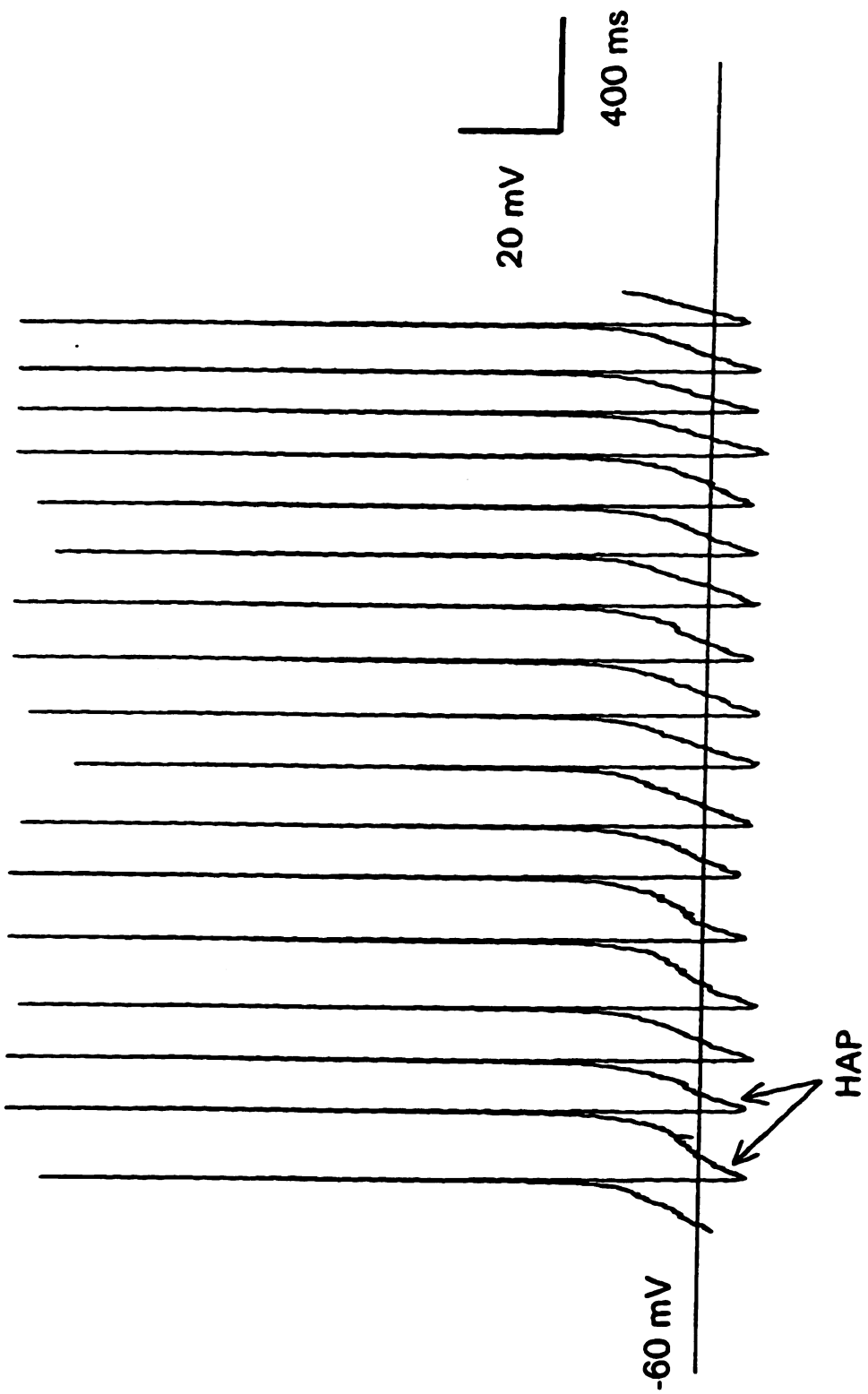


Figure 22. Voltage response of a supraoptic MNC to a single injection of suprathreshold depolarizing current. The spike (upper record) was elicited by injecting a brief (3 ms) pulse of suprathreshold depolarizing current (0.4 nA; lower record). Prior to initiation of the spike, the membrane potential was held at -60 mV by steady injection of current to prevent the occurrence of spontaneous action potentials.

Figure 22

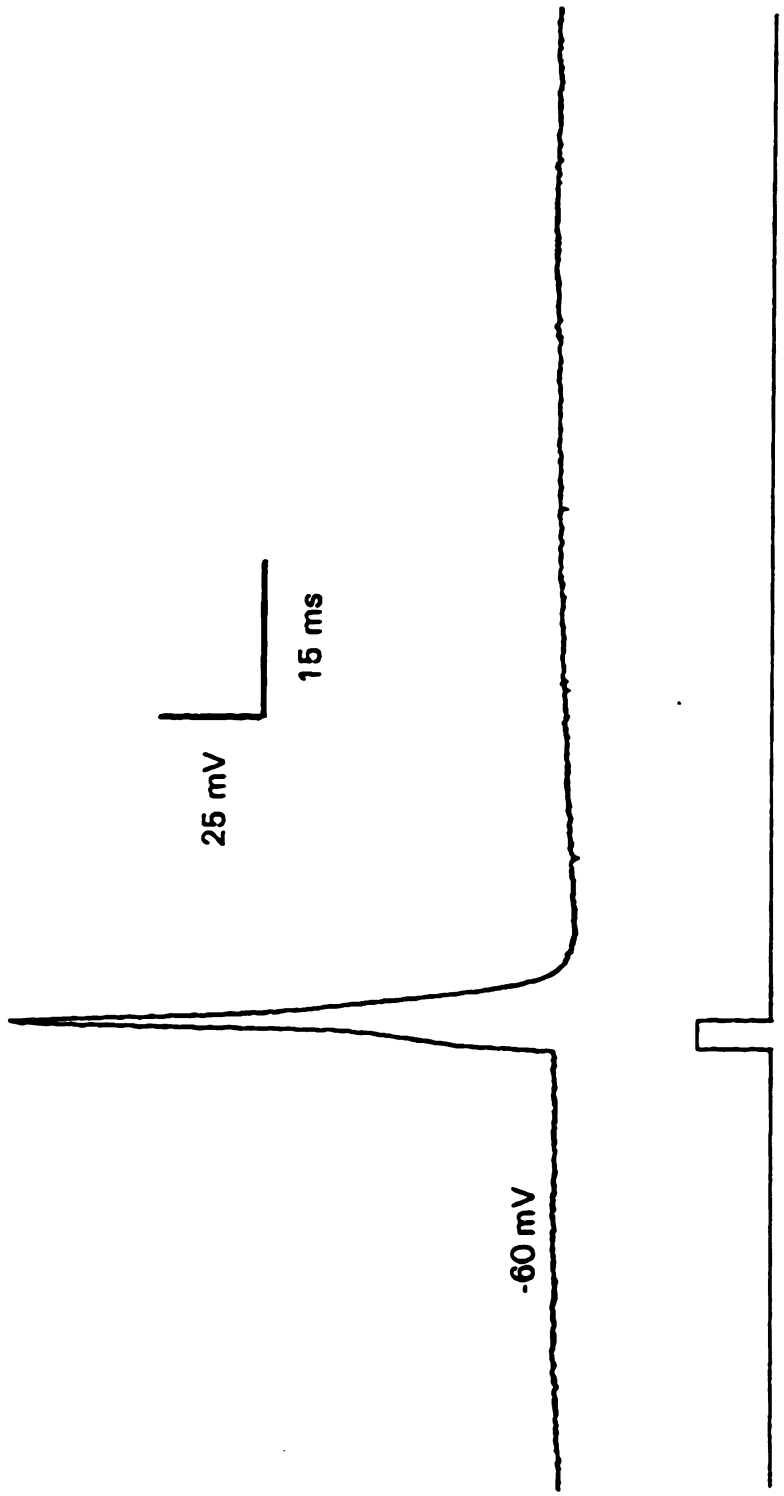


Figure 23. Voltage response of a supraoptic MNC to repetitive injections of suprathreshold depolarizing current. The spikes were elicited by injecting 30 brief (3 ms) pulses of suprathreshold depolarizing current (0.4 nA) at 10 Hz. Note the presence of the hyperpolarizing after-potential (HAP) at the end of the falling phase of each action potential.

Figure 23

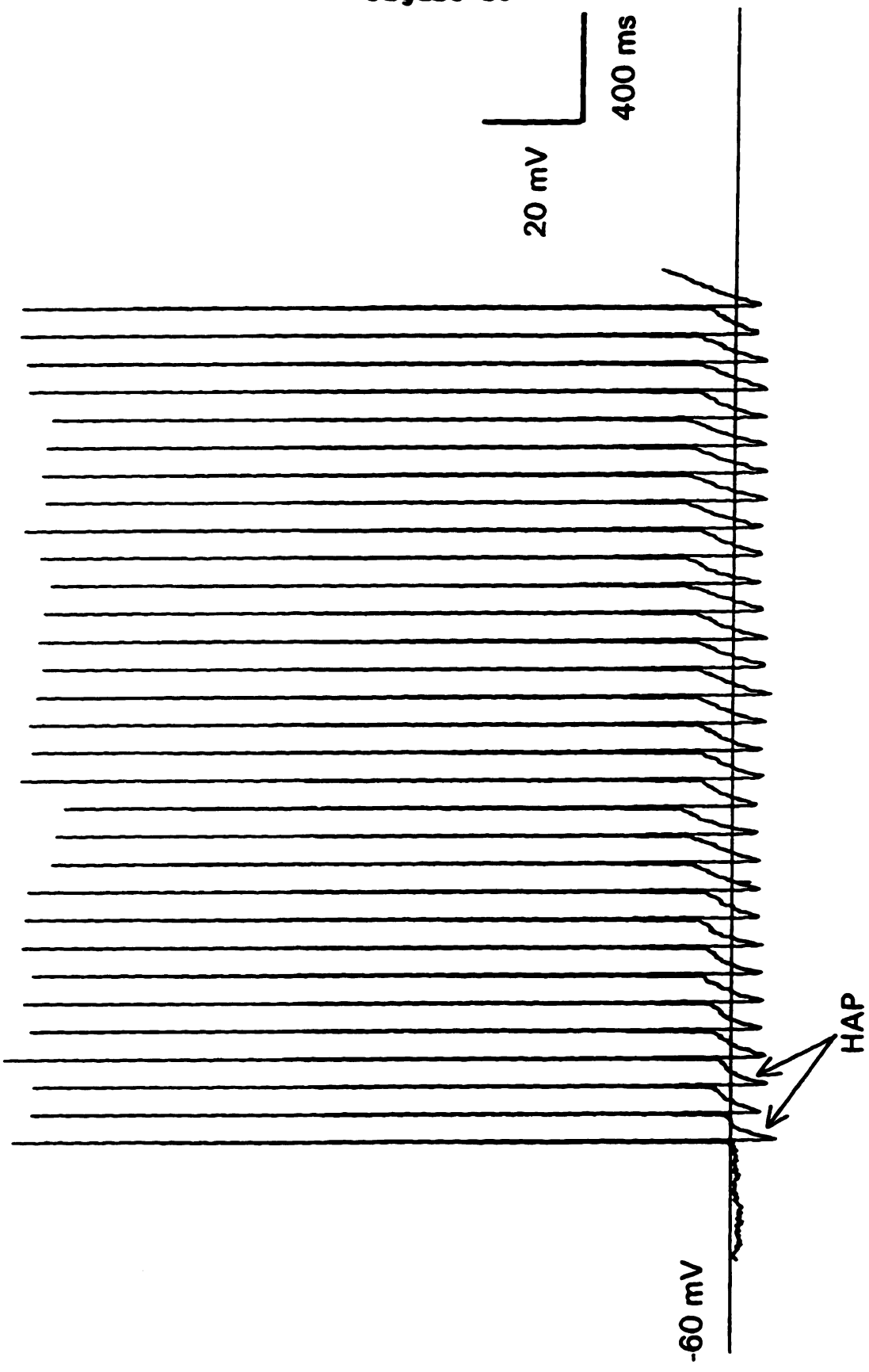
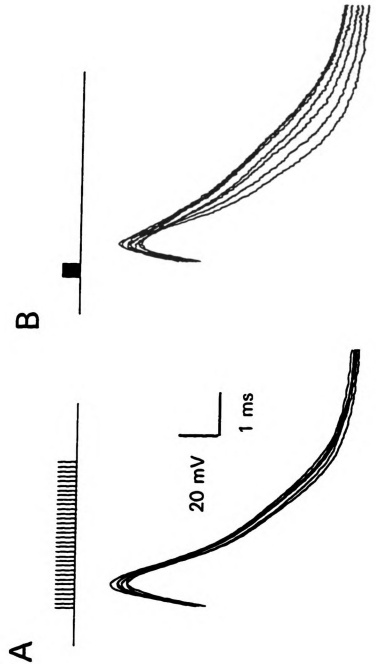


Figure 24. Action potential duration in supraoptic MNCs is frequency-dependent. A, successive action potentials elicited by injecting brief (3 ms) pulses of suprathreshold depolarizing current (0.4 nA) at 1 Hz (upper record) exhibited a similar duration (lower records). B, when the current injections were applied at 10 Hz (upper record), successive action potentials exhibited pronounced broadening after the first spike in the train (lower records).

Figure 24



suggested that frequency-dependent spike broadening in MNCs may result from a frequency-dependent reduction in K^+ current underlying action potential repolarization. Further experiments were conducted to determine the involvement of specific K^+ channel currents in action potential repolarization and frequency-dependent spike broadening.

C. Effects of K^+ Channel Blockers on Action Potential Duration

K^+ currents play a critical role in determining action potential duration because of their involvement in action potential repolarization (see review by Rudy, 1988). To determine which K^+ currents contribute to action potential repolarization in MNCs, the duration of individual action potentials was measured before and after addition of selective K^+ channel blockers to the extracellular medium.

ChTX (100 nM; Figure 25) produced a 29 ± 5% (n=8) increase in action potential duration, suggesting that Ca^{2+}-dependent K^+ channel currents contribute to repolarization in MNCs. TEA (5 mM; Figure 26) and 4-AP (1 mM; Figure 27) produced 334 ± 45% (n=7) and 297 ± 40% (n=7) increases, respectively, in action potential duration, suggesting that both delayed rectifier and A-type channel currents also contribute to repolarization in MNCs. Although both TEA and 4-AP produced pronounced spike broadening, there was clearly a difference in the broadening profiles produced by each compound. 4-AP produced a greater degree of broadening in

Figure 25. Effect of ChTX on the duration of isolated, current-evoked action potentials. Action potential duration was increased by addition of ChTX (100 nM) to the extracellular medium. Note that the broadening effect of ChTX was most pronounced during the latter half of the repolarization phase.

Figure 25

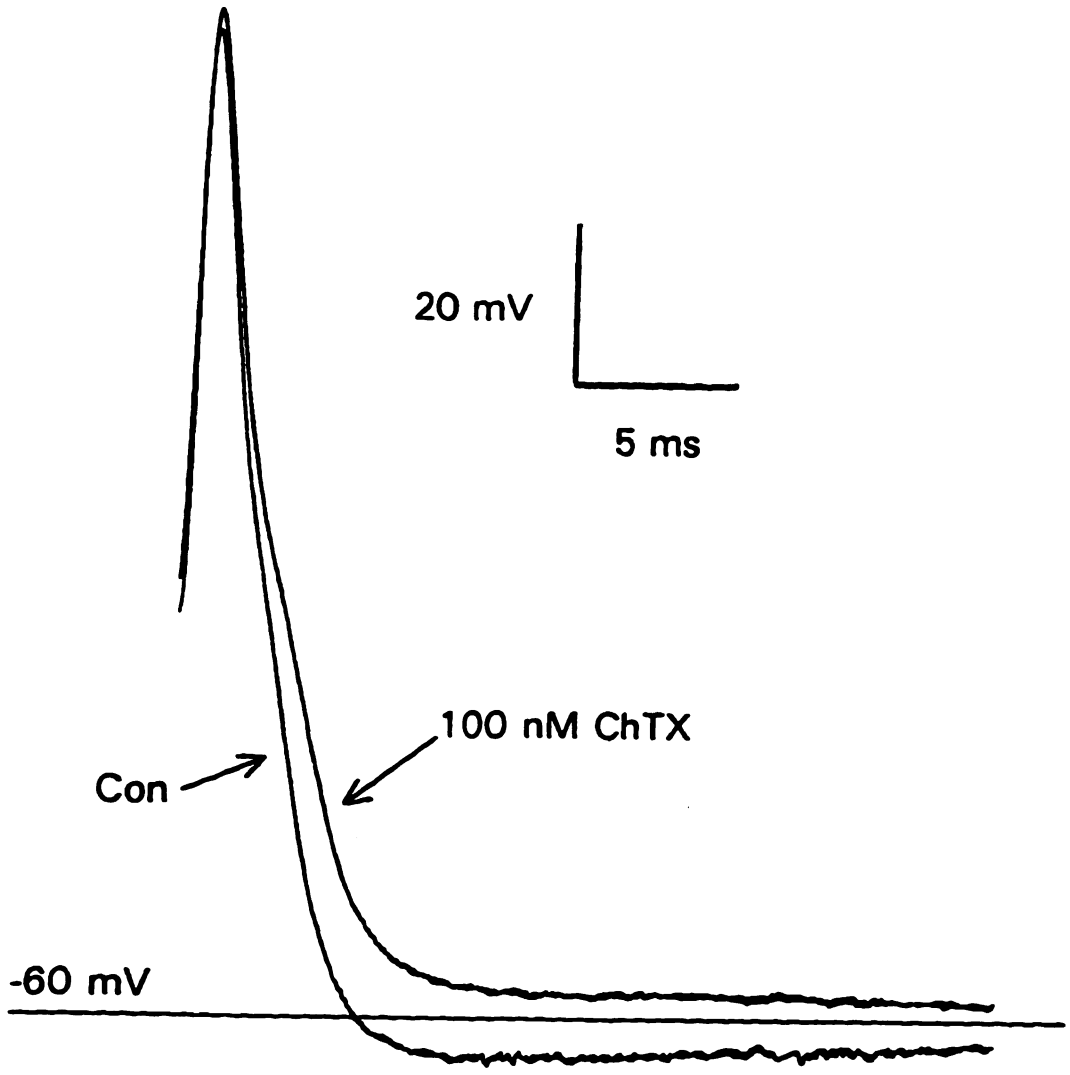


Figure 26. Effect of TEA on the duration of isolated, current-evoked action potentials. Action potential duration was increased by addition of TEA (5 mM) to the extracellular medium. Note that the broadening effect of TEA was evident shortly after the onset of the repolarization phase, but was most pronounced during the latter half of the repolarization phase.

Figure 26

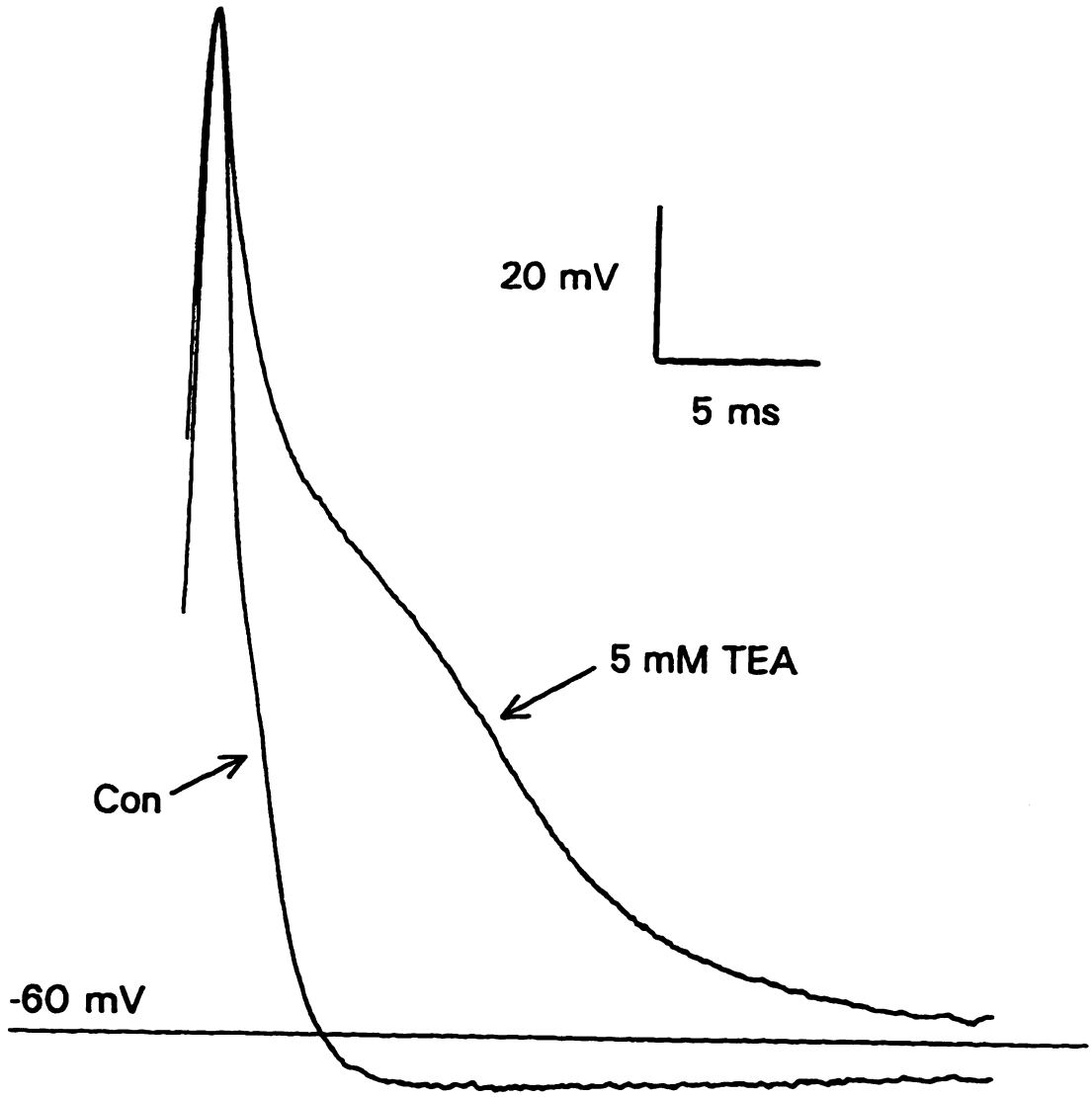
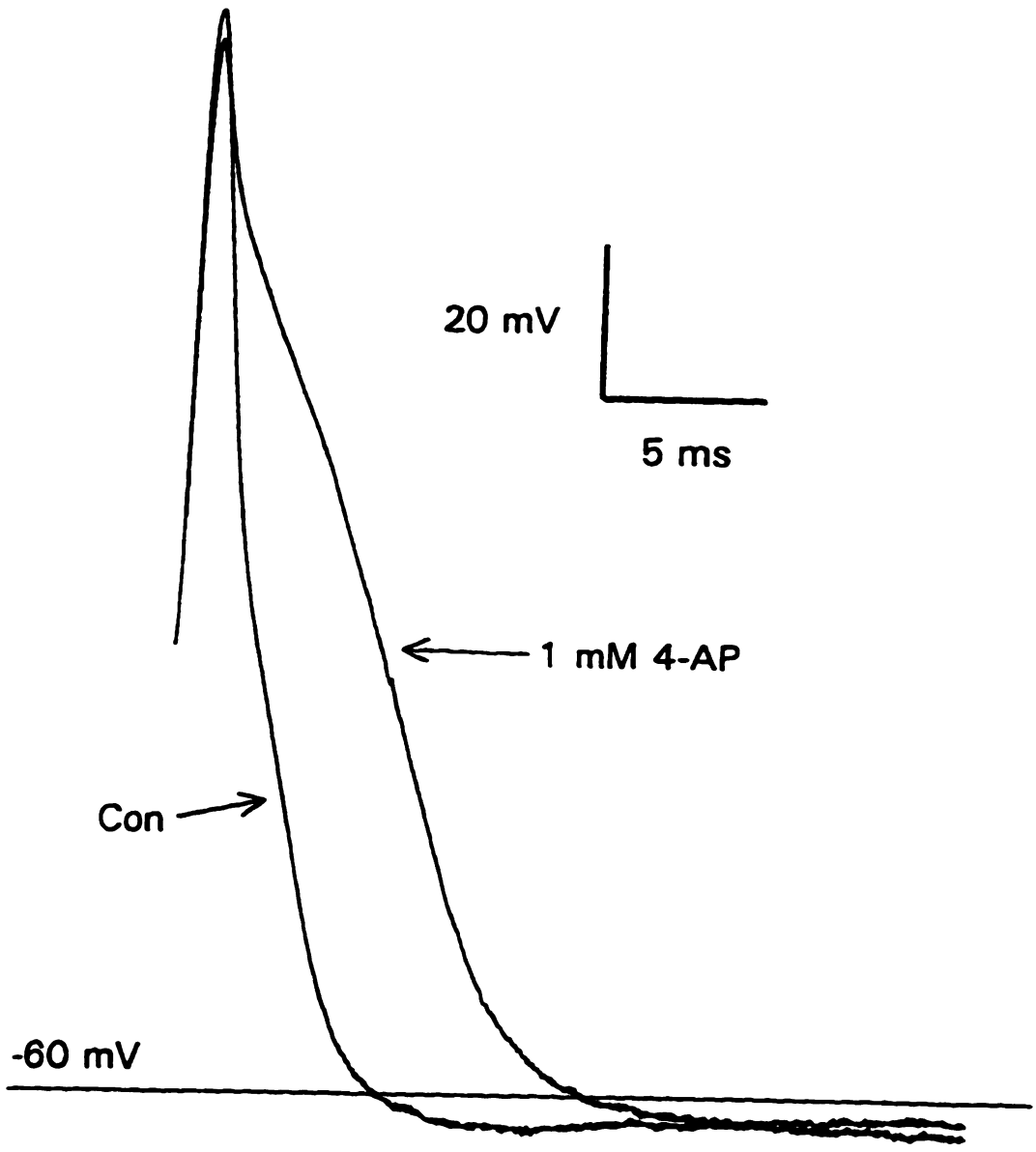


Figure 27. Effect of 4-AP on the duration of isolated, current-evoked action potentials. Action potential duration was increased by addition of 4-AP (1 mM) to the extracellular medium. Note that the broadening effect of 4-AP was pronounced shortly after the onset of the repolarization phase.

Figure 27



the upper part of the spike than did TEA. This difference was likely due to the faster activation of $I_{K(A)}$ and its non-dependence on Ca^{2+} influx during the spike.

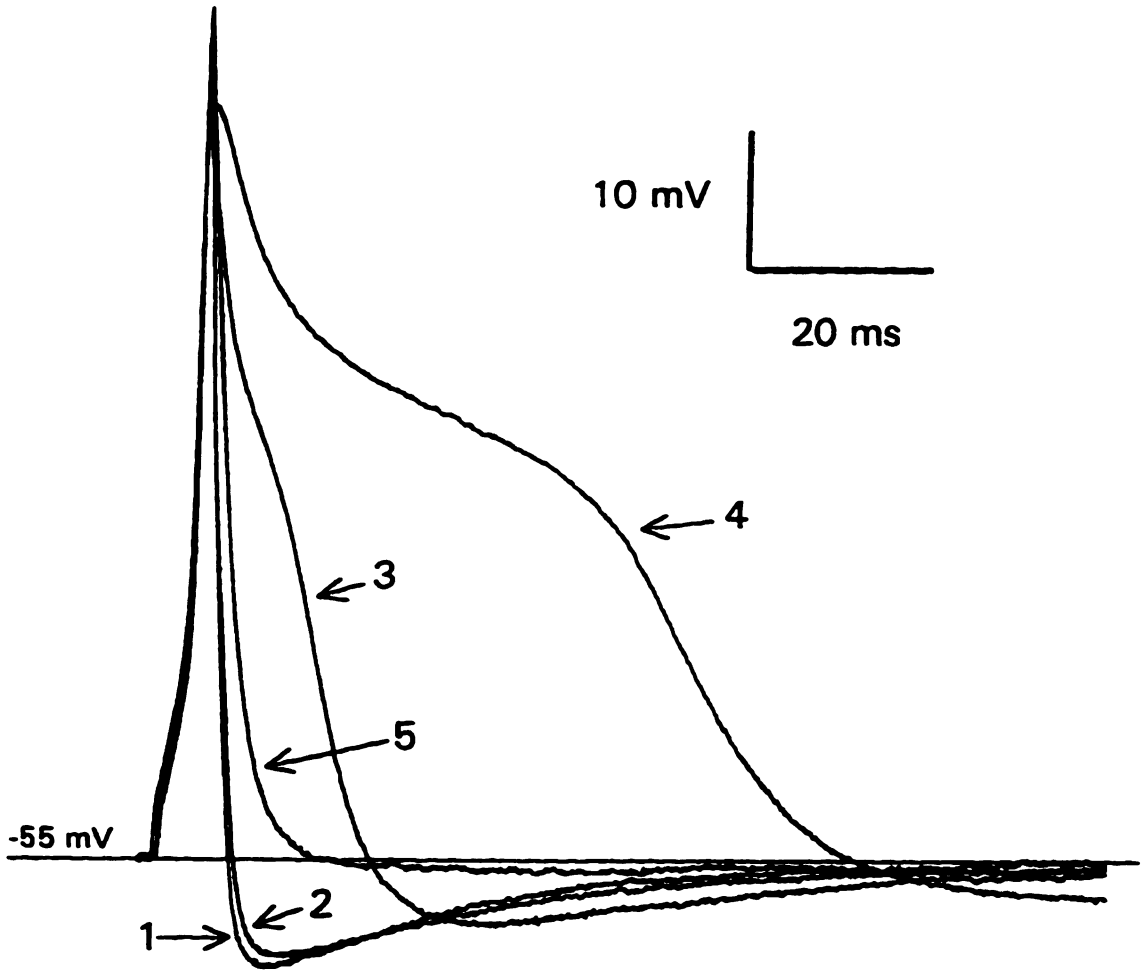
Figure 28 shows the effects of sequential addition of ChTX (100 nM), TEA (5 mM), 4-AP (1mM) and the Ca^{2+} channel blocker Cd^{2+} (125 μM) to the extracellular medium on action potential duration in a single MNC. The sequential addition of ChTX, TEA and 4-AP resulted in a progressive increase in action potential duration. Along with producing an increase in action potential duration, addition of TEA or TEA and 4-AP to the ChTX-containing extracellular medium produced a distinct 'shoulder' on the repolarization phase of the spike. Addition of Cd^{2+} to the extracellular medium to block Ca^{2+} currents abolished the shoulder and prevented most of the action potential broadening produced by the K^+ channel blockers. These results confirm that currents from delayed rectifier, A-type, and Ca^{2+}-dependent K^+ channels all contribute to action potential repolarization in MNCs. They also suggest that Ca^{2+} current is necessary for maximal action potential broadening produced by K^+ channel blockers.

D. Effects of K^+ Channel Blockers on Frequency-Dependent Spike Broadening

As previously stated, studies have suggested that frequency-dependent spike broadening in MNCs may result from a frequency-dependent reduction in K^+ current underlying action potential repolarization. To examine the involvement

Figure 28. Ca^{2+} current is necessary for maximal action potential broadening produced by K^+ channel blockers. Sequential addition of ChTX (100 nM), TEA (5 mM) and 4-AP (1 mM) to the extracellular medium produced a progressive increase in action potential duration. Addition of Cd^{2+} (125 μM) to the extracellular medium to block Ca^{2+} currents prevented most of the action potential broadening produced by the K^+ channel blockers.

Figure 28



(1) Control

(2) ChTX (100 nM)

(3) TEA (5 mM)

(4) TEA (5 mM) + 4-AP (1 mM)

(5) TEA (5 mM) + 4-AP (1 mM) + Cd^{2+} (125 μM)

of specific K^+ channel currents in frequency-dependent spike broadening, the duration of successive action potentials elicited by a 10 Hz train (30 pulses) of suprathreshold current injections (0.4 nA) was measured before and after addition of selective K^+ channel blockers to the extracellular medium.

Addition of ChTX (100 nM) to the extracellular medium did not prevent the occurrence of frequency-dependent spike broadening (Figure 29). In fact, the maximum percent increase in spike duration in the presence of ChTX (91 ± 9%; n=8) was actually greater than that observed under control conditions (54 ± 5%). Figure 30A shows spike duration plotted against spike number before and after addition of ChTX to the extracellular medium. The plotted data for both treatment groups exhibit an asymptotic relationship for spike duration as steady-state duration is approached. Accordingly, when plotted logarithmically (Figure 30B), each data set can be described by a straight line. A comparison of the slopes (κ) of the lines for the ChTX data (κ = 1.19) and for the control data (κ = 0.47) reveals that the rate of spike broadening is greater in the presence of ChTX compared to control.

Extracellular TEA (5 mM) also did not prevent the occurrence of frequency-dependent spike broadening (Figure 31). Like ChTX, the maximum percent increase in spike duration in the presence of TEA (69 ± 9%; n=7) was greater than that observed under control conditions. Another

Figure 29. ChTX does not prevent frequency-dependent spike broadening. Successive action potentials elicited by injecting brief (3 ms) pulses of suprathreshold depolarizing current (0.4 nA) at 10 Hz before (A) and after (B) addition of ChTX (100 nM) to the extracellular medium. Note that frequency-dependent spike broadening occurs even in the presence of ChTX.

Figure 29

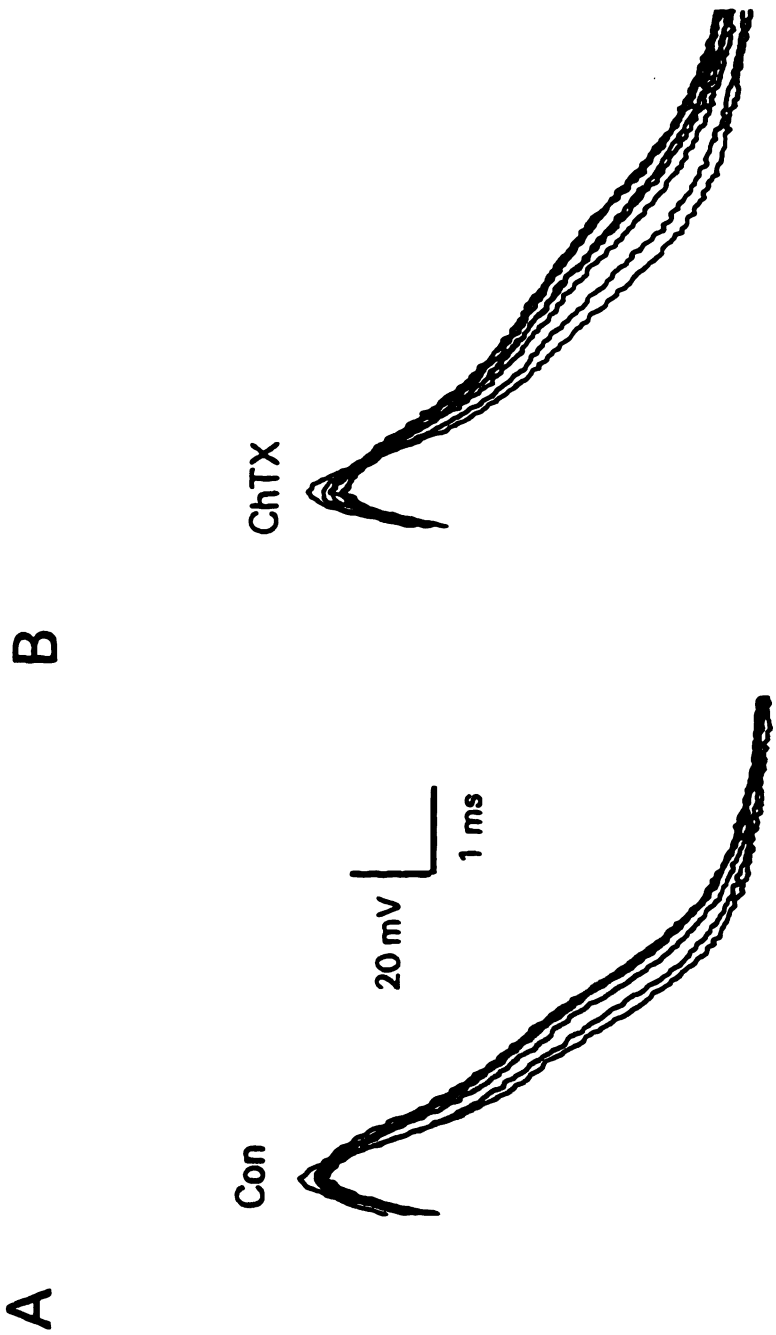
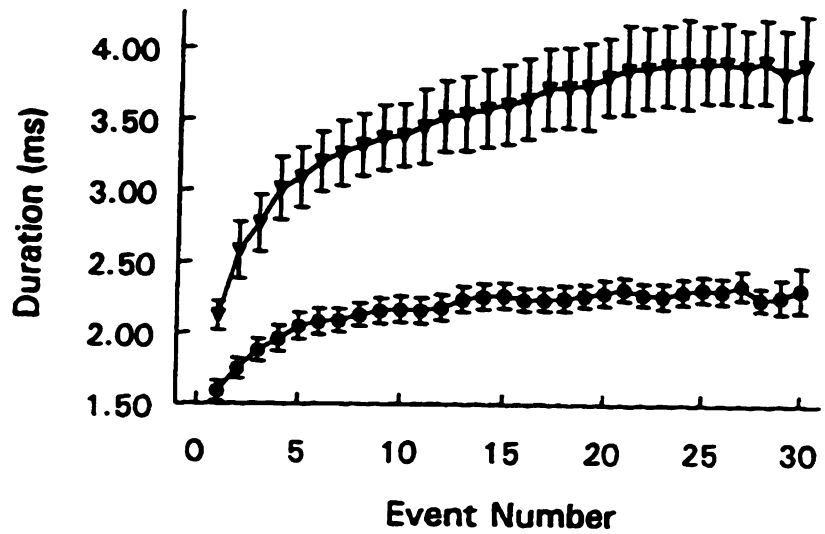


Figure 30. ChTX increases the extent and rate of frequency-dependent spike broadening. A, spike durations plotted against spike number under control conditions (●) and after addition of ChTX (▾; 100 nM) to the extracellular medium. Note that the extent of spike broadening is greater in the presence of ChTX. B, when the x-axis is converted to a log scale, it is evident that the rate of spike broadening is greater in the presence of ChTX.

Figure 30

A


4.00
3.50
3.00
2.50
2.00
1.50
Duration (ms)
0
5
10
15
20
25
30
Event Number

B

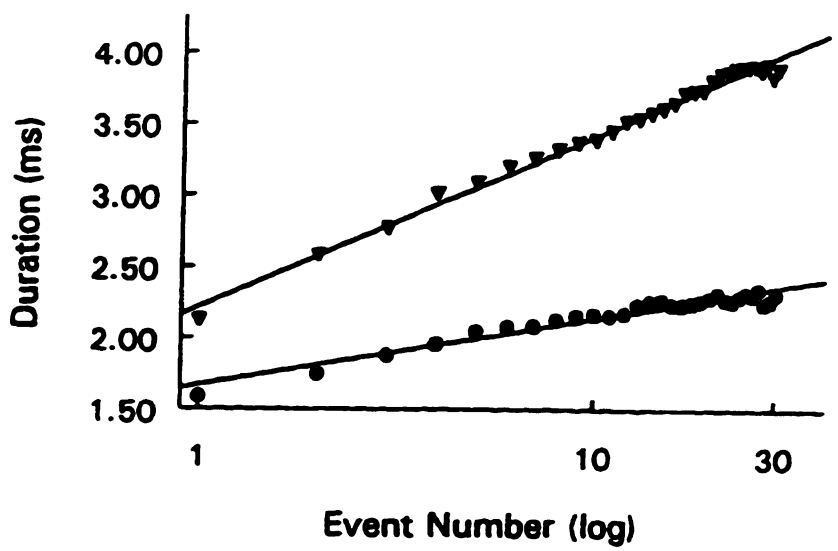
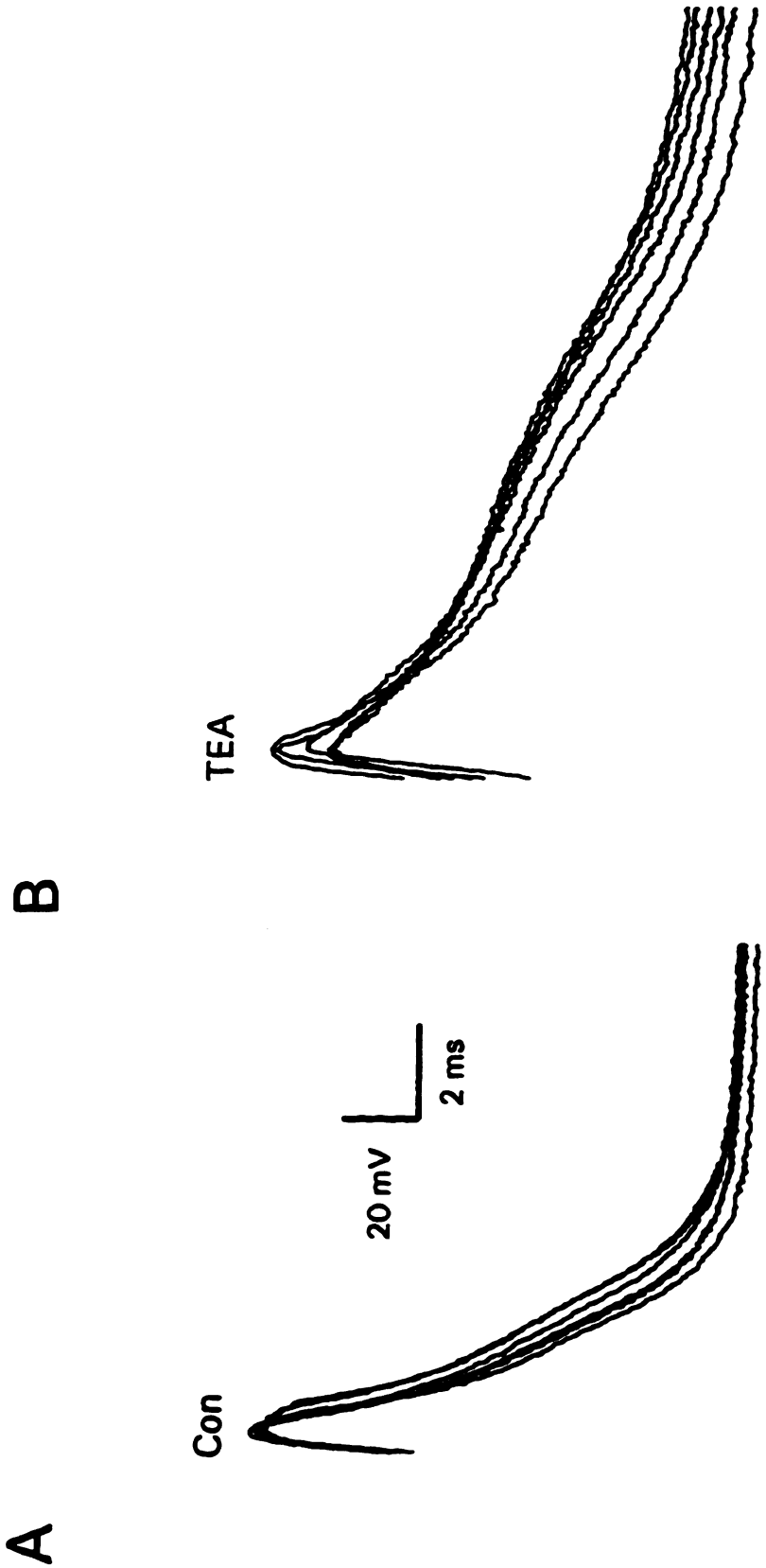

4.00
3.50
3.00
2.50
2.00
1.50
Duration (ms)
1
10
30
Event Number (log)

Figure 31. TEA does not prevent frequency-dependent spike broadening. Successive action potentials elicited by injecting brief (3 ms) pulses of suprathreshold depolarizing current (0.4 nA) at 10 Hz before (A) and after (B) addition of TEA (5 mM) to the extracellular medium. Note that frequency-dependent spike broadening occurs even in the presence of TEA.

Figure 31



notable observation regarding the effects of TEA was that after maximum spike duration was achieved during the train, spike duration began to decrease, returning towards the value observed for the first action potential. Unlike ChTX or TEA, extracellular 4-AP (1 mM) did prevent frequency-dependent spike broadening (Figure 32). In fact, in most cases, the first spike in the 30 pulse train exhibited the longest duration and subsequent spikes decreased slightly in duration. Figure 33A shows spike duration plotted against spike number before and after the addition of TEA or 4-AP to the extracellular medium. Control and ChTX data shown previously in Figure 30 are included in the graph for reference. Figure 33B shows the data from figure 33A plotted logarithmically. The 4-AP data were well fit by a straight line with negative slope value ($\kappa = -0.76$). Because the TEA data appeared to exhibit a polynomial relationship over a 30 pulse train, they could not be described by a straight line. The data points over which progressive broadening occurred, however, were well fit with a straight line with a slope of 5.19, indicating that, as in the presence of ChTX, the rate of spike broadening in the presence of TEA was greater than that observed under control conditions.

Figure 32. 4-AP prevents frequency-dependent spike broadening. Successive action potentials elicited by injecting brief (3 ms) pulses of suprathreshold depolarizing current (0.4 nA) at 10 Hz before (A) and after (B) addition of 4-AP (1 mM) to the extracellular medium. In the presence of 4-AP, the first action potential of the 10 Hz train exhibited the longest duration and subsequent action potentials exhibited a progressive decrease in duration.

Figure 32

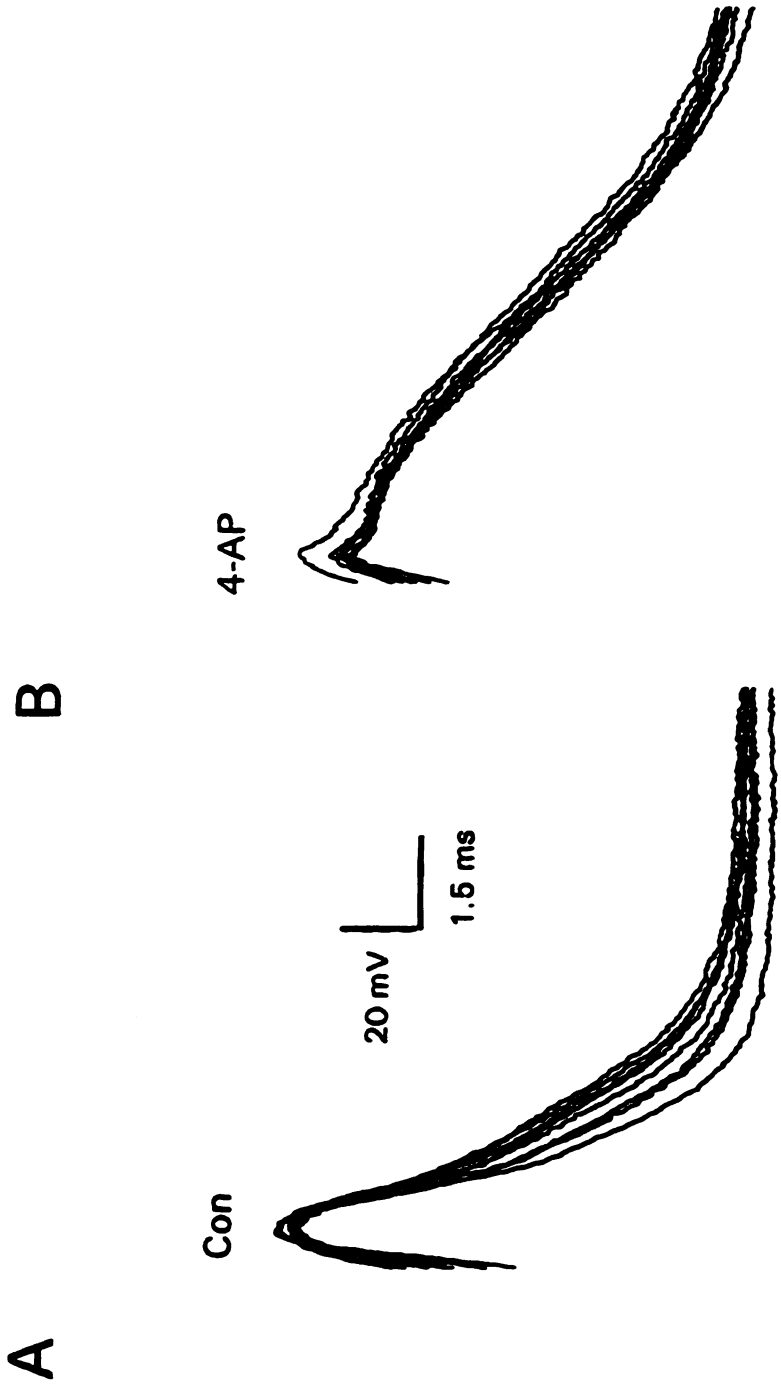
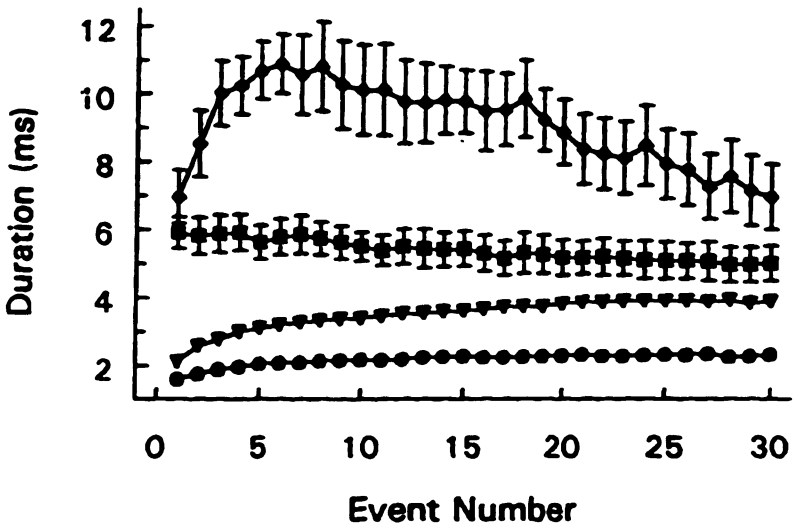


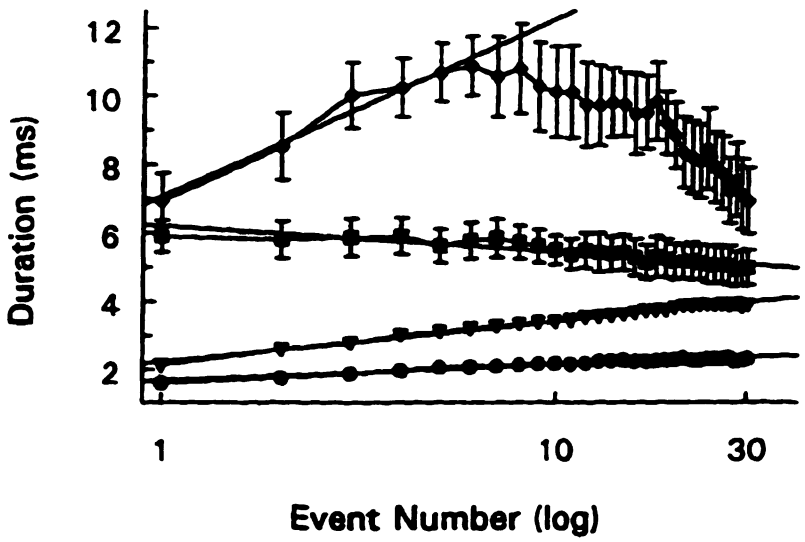
Figure 33. TEA, but not 4-AP, increases the extent and rate of frequency-dependent spike broadening. A, spike durations plotted against spike number after addition of TEA (♦; 5 mM) or 4-AP (■; 1 mM) to the extracellular medium. Control and ChTX data shown previously in figure 29 are included in the graph for reference. Note that the extent of spike broadening is greater in the presence of TEA compared to control and that after maximum spike duration was achieved during a train, spike duration began to decrease. In the presence of 4-AP, the first spike in the 30 pulse train usually exhibited the longest duration and subsequent spikes decreased slightly in duration. B, when the x-axis is converted to a log scale, it is evident that the rate of spike broadening is greatest in the presence of TEA.

Figure 33

A


12
10
8
6
4
2
Duration (ms)
0
5
10
15
20
25
30
Event Number

B


12
10
8
6
4
2
Duration (ms)
1
10
30
Event Number (log)

DISCUSSION

I. Components of Outward K^+ Current in Somata of Supraoptic MNCs

Several components of outward K^+ current recorded from somata of MNCs from adult guinea pig SON were identified on the basis of their voltage dependence, kinetics, pharmacology and Ca^{2+} dependence.

A. Low-Threshold Transient K^+ Current ($I_{K(A)}$)

A low-threshold transient K^+ current was evoked from a holding potential of -90 mV. The current activated at test potentials more positive than -60 mV and was fully inactivated at membrane (holding) potentials more positive than -40 mV. Also, the transient current was preferentially blocked by 4-AP. This transient current therefore resembles the A-current ($I_{K(A)}$) identified in cultured neonatal rat supraoptic neurons (Cobbett *et al.*, 1989), rat supraoptic (Nagatomo *et al.*, 1995) and paraventricular (Li & Ferguson, 1996) magnocellular neurons in hypothalamic slice preparations and other excitable cells (Connor and Stevens, 1971; Neher, 1971; Rogawski, 1985).

It was previously reported (O'Regan & Cobbett, 1993) that $I_{K(A)}$ was evident in only about one half of the

supraoptic neurons acutely dissociated from the adult rat. In contrast, my experiments show that all supraoptic magnocellular neurons acutely dissociated from adult guinea pig exhibit a prominent $I_{K(A)}$. It is tempting to speculate that this discrepancy is the result of species differences between rats and guinea pigs. However, this speculation is not supported by a recent study (Li & Ferguson, 1996) of MNCs of the rat paraventricular nucleus in which it was reported that a prominent $I_{K(A)}$ was always present.

Another possible reason for differences between the data obtained by O'Regan and Cobbett (1993) and the data of the present study is that methods for preparation of the isolated MNCs were different: the compositions of the enzyme-containing media were not the same and SON neurons of rats (but not guinea pigs) were labelled *in vivo* with Evans Blue. It is also notable that, even when present, $I_{K(A)}$ reported by O'Regan and Cobbett (1993) was significantly smaller in amplitude compared to that reported here. Given those conditions, it is possible that an attenuation of $I_{K(A)}$ (by Evans Blue labelling, for example) during preparation of cells may have generated a subpopulation of MNCs in which $I_{K(A)}$ was not detectable. Additionally, recent studies of identified OT and VP supraoptic neurons of the female rat (Stern & Armstrong, 1995; Stern & Armstrong, 1996) propose that OT and VP neurons may differ in terms of the size and/or voltage dependence of $I_{K(A)}$. Given the potential for cell dissociation techniques to influence the amplitude of

recorded currents, it is possible that O'Regan and Cobbett (1993) recorded from MNCs which normally exhibit a greater $I_{K(A)}$. The possibility that subpopulations of MNCs differ in terms of the size and/or voltage dependence of $I_{K(A)}$ was not systematically investigated in the present study.

Interestingly, the A-current identified here was considerably different than the transient outward current (I_{TOC}) identified by Bourque (1988) in whole supraoptic neurons of rat hypothalamic explants in terms of activation threshold, voltage-dependence of steady-state inactivation and sensitivity to divalent cations. He reported a value of -74.5 mV as the activation threshold for I_{TOC} and a value of -82.4 mV as the membrane potential at which half of the channels are inactivated. These values are more negative than the values (-50 mV and -62 mV, respectively) obtained from my experiments. Also, Bourque (1988) reported that I_{TOC} is dependent on the presence of extracellular Ca^{2+}, implying that Ca^{2+} influx into MNCs strongly modulates the gating of TOC channels. This conclusion was, in part, based on the observation that I_{TOC} was reduced by up to 90% by extracellular Cd^{2+} at concentrations (50-400 μM) which apparently did not produce detectable changes in the voltage dependence of current activation or steady-state inactivation. In contrast, my experiments demonstrate that in acutely isolated supraoptic MNCs extracellular Cd^{2+} at concentrations as low as 125 μM not only reduced the amplitude of $I_{K(A)}$, but also produced large positive shifts

in the voltage dependence of both activation and steady-state inactivation of this current. Conversely, removal of Ca^{2+} from the extracellular medium (no Cd^{2+} present) produced large negative shifts in the voltage dependence of both $I_{K(A)}$ activation and steady-state inactivation. Nevertheless, using either Ca^{2+}-free or Cd^{2+}-containing extracellular solution I was still able to evoke a substantial A-current during depolarizing voltage steps. The fact that $I_{K(A)}$ is activated in the absence of extracellular Ca^{2+} suggests that Ca^{2+} influx is not an absolute necessity for activation of the current. The present results were more consistent with those reported by Carignani *et al.* (1991) which suggest that in cultured rat cerebellar granule cells $I_{K(A)}$ behavior is dependent on the inventory of external divalent cations rather than on Ca^{2+} influx and, perhaps $[Ca^{2+}]_i$.

B. Sustained Outward K^+ Current

A slowly-activating, sustained outward K^+ current was co-activated with $I_{K(A)}$ during depolarizing voltage steps to potentials more positive than -30 mV from a holding potential of -90 mV. This current did not inactivate during the test pulse, showed little voltage-dependent steady-state inactivation and was preferentially blocked by TEA. Based on these characteristics, the sustained current probably corresponds in part to the delayed rectifier current ($I_{K(V)}$) originally described in squid giant axons and later in almost all other excitable cells (see review by Rudy, 1988).

The possibility that more than one channel type contributed to the sustained current was supported by experiments which showed that this current could be reduced by 125 μM extracellular Cd^{2+}, a concentration which blocks Ca^{2+} influx but does not affect the voltage dependence of the sustained current. This result was similar to that reported by Cobbett *et al.* (1989) which showed that the sustained outward current recorded from cultured neurons of rat supraoptic nucleus was reduced by addition of the inorganic Ca^{2+} channel blocker Co^{2+} (1-2 mM) to Ca^{2+}-containing extracellular solution. These observations suggest that in supraoptic MNCs a Ca^{2+}-sensitive, non-inactivating K^+ channel ($I_{K(Ca)}$) and a classical Ca^{2+}-insensitive, delayed rectifier channel both contribute to the sustained current. Li and Ferguson (1996) arrived at a similar conclusion regarding the sustained outward K^+ current in rat paraventricular MNCs.

C. High-Threshold (Ca^{2+}-Dependent) Transient Outward K^+ Current

A major finding of my study was a transient outward K^+ current in acutely isolated MNCs which was altogether different from $I_{K(A)}$. This current could be activated during depolarizing voltage steps to membrane potentials more positive than -20 mV from a holding potential (-40 mV) at which $I_{K(A)}$ is fully inactivated. Furthermore, this current was 4-AP-insensitive, TEA- and ChTX-sensitive and appeared

to be absolutely dependent on Ca^{2+} influx. The current was not evident when Ca^{2+} was absent from the extracellular medium or when Ca^{2+} influx was blocked.

There have been reports of high-threshold, Ca^{2+}-dependent transient K^+ currents in other cell types including calf cardiac purkinje fibers (Sigelbaum & Tsien, 1980), rat adrenal chromaffin cells (Neely & Lingle, 1992) and amphibian spinal neurons (Ribera & Spitzer, 1987). However, the Ca^{2+}-dependent transient K^+ current described here (recorded under whole-cell conditions) was different from those previously reported. In calf cardiac purkinje fibers (Siegelbaum & Tsien, 1980) and in rat adrenal chromaffin cells (Neely & Lingle, 1992) the time required to reach peak amplitude of the transient current and the time course of current inactivation appeared to be much longer than the values reported here for MNCs. The time required to reach peak amplitude of the transient current and the time course of current inactivation reported for amphibian spinal neurons (Ribera & Spitzer, 1987) are closer to the values of the high-threshold transient current recorded in this study. However, the transient current in amphibian spinal neurons could be fully activated when extracellular Ca^{2+} was replaced with Sr^{2+}. The Ca^{2+}-dependent transient K^+ current reported here was abolished when extracellular Ca^{2+} was replaced with Sr^{2+}, suggesting an absolute dependence on Ca^{2+} influx.

Dryer *et al.* (1989) reported a TTX-sensitive,

Na^+-activated transient K^+ current recorded under whole-cell conditions which closely resembled the Ca^{2+}-dependent transient K^+ current reported here in terms of time course of activation and inactivation. It was later determined, however, that their current was likely due to inadequate voltage control to the extent that unclamped Na^+-dependent action potentials were present during recording (Dryer, 1991). Unclamped Na^+ currents were not a problem during my experiments using isolated MNCs. In my studies, the extracellular solution contained TTX at a concentration (2.5 μM) sufficient to block all Na^+ current. Therefore the Ca^{2+}-dependent transient K^+ current recorded could not have been the result of unclamped Na^+-dependent action potentials.

I also considered was the possibility that the Ca^{2+}-dependent transient K^+ current was the result of an unclamped Ca^{2+}-dependent action potential. However, no indication of poor voltage control was observed during recordings made under conditions for studying Ca^{2+} current. Moreover, the Ca^{2+}-dependent transient K^+ current was not observed when extracellular Ca^{2+} was replaced with Sr^{2+}, although Ca^{2+} and Sr^{2+} currents were of similar magnitude.

II. Comparison of K^+ Current Components in Somata and Axon Terminals of MNCs

Although properties of somatic currents have been characterized in a variety of cell types, the properties of voltage-gated ion currents mediating transmitter or hormone

release from vertebrate axon terminals have been difficult to characterize. This is largely because electrophysiological studies on these channels are limited by the extremely small size of most individual axon terminals. The axon terminals of VP and OT MNCs, however, are sufficiently large to permit the use of conventional patch-clamp techniques to study their ionic channels and currents. Most axon terminals in the rat posterior pituitary have diameters of approximately 2-3 μm, but some have diameters of up to 12 μm (Nordmann *et al.*, 1987). It is these larger terminals which have been used to conduct patch-clamp experiments aimed at providing a better understanding of the ionic events which underlie neurosecretion.

By virtue of their voltage dependence and their ability to be regulated by intracellular second messengers such as Ca^{2+}, K^+ channels have the potential to serve as important transducers for electrical and chemical signals at the level of the axon terminal. Accordingly, the components of outward K^+ current present in MNC axon terminals have been examined in several studies. The laboratories which have studied K^+ current in these terminals, however, have provided conflicting reports regarding the identity of the K^+ currents present.

Whole-cell voltage-clamp experiments on isolated axon terminals ("neurosecretosomes") prepared from rat posterior pituitary have shown that these terminals exhibit both

inactivating (Thorn *et al.*, 1991) and non-inactivating components (Wang *et al.*, 1992) of outward K^+ current. The inactivating component of current evoked during membrane depolarizations from a holding potential of -80 mV exhibited a low activation threshold (>-60 mV) and rapid inactivation kinetics (τ = 21 ms at +30 mV). 4-AP blocked the inactivating current in a concentration-dependent manner ($IC_{50} \approx 3$ mM), while TEA (100 mM) and ChTX (200 nM), organic compounds reported to block various components of non-inactivating K^+ current (Hille, 1992), had no effect on the inactivating current. In contrast to the inactivating K^+ current (I_{TOC}) in rat MNC somata reported by Bourque (1988), the inactivating K^+ current recorded from neurosecretosomes was unaffected by removal of extracellular Ca^{2+} or by addition of the Ca^{2+} channel blocker Cd^{2+} (2 mM) to the Ca^{2+}-containing extracellular medium. These results indicate that MNC axon terminals of the rat express a low-threshold, A-type K^+ channel which is not critically dependent on Ca^{2+} influx.

The non-inactivating component of outward K^+ current in rat neurosecretosmes was studied using both single-channel and whole-cell patch-clamp techniques (Wang *et al.*, 1992). This component exhibited a high activation threshold, activating at membrane potentials more positive than -30 mV. Also, the non-inactivating current was determined to be completely dependent on intracellular Ca^{2+} concentration and could be abolished by extracellular Cd^{2+} (80 μM). 4-AP (7

mM) and dendrotoxin (100 nM) had no effect on this current, nor did apamin (40-80 nM), a peptide toxin reported to block some small-conductance Ca^{2+}-activated channels (Lancaster *et al.*, 1991). The non-inactivating current was reduced by low concentrations of TEA (IC_{50}≈0.5 μM), but was insensitive to ChTX (10-100 nM) which has been reported to selectively block some large-conductance Ca^{2+}-activated channels (Reinhart *et al.*, 1989). Single-channel experiments revealed that MNC axon terminals express a large-conductance (unit conductance = 231 pS in symmetrical 150 mM K^+), Ca^{2+}-activated K^+ channel. Like other reported large-conductance Ca^{2+}-activated K^+ channels (termed maxi-K^+ or BK channels), this channel was blocked by TEA (0.5 mM); but unlike other reported large-conductance Ca^{2+}-activated K^+ channels, this channel was insensitive to block by ChTX (100-360 nM). Together, these results suggest that MNC axon terminals in the rat express a novel large-conductance Ca^{2+}-activated K^+ channel which contributes to the macroscopic non-inactivating K^+ current. No evidence of a non-inactivating, Ca^{2+}-insensitive K_V channel was reported in the study.

Whole-cell experiments on thin slice preparations of rat posterior pituitary confirmed that the macroscopic K^+ channel current recorded during sustained depolarization of the axon terminal membrane consisted of multiple components (Bielfeldt *et al.*, 1992). An inactivating component exhibited a low activation threshold (>-60 mV) and rapid inactivation kinetics (τ = 22 ms at +50 mV). While the

inactivating current recorded from rat neurosecretosomes (Thorn *et al.*, 1991) was not sensitive to block by TEA, the inactivating current recorded from thin slice preparations of rat posterior pituitary was sensitive to block by relatively low concentrations of TEA (IC_{50}≈1 mM). Although it is difficult to account for this discrepancy, one explanation may simply be that K^+ channels behave differently when studied in slices compared to isolated terminals.

The non-inactivating component of K^+ current recorded from thin slice preparations of neurohypophysial tissue exhibited a low activation threshold (>-30 mV) and little inactivation over the duration (500 ms) of a suprathreshold voltage step. This current component was sensitive to block by dendrotoxin (IC_{50}<20 nM) and was less sensitive to block by TEA (IC_{50}<5 mM) than was the inactivating component. The sensitivity to block by dendrotoxin was atypical, however, since this toxin has been shown to produce a preferential block of inactivating A-type channel currents in most other cell types. The non-inactivating current recorded using single- channel techniques was abolished by removal of extracellular Ca^{2+} or by addition of Cd^{2+} (100 μM) to the extracellular medium, suggesting that it was contributed entirely by a non-inactivating, Ca^{2+}-dependent K^+ channel.

Since the functions of K^+ channel types might differ between neuronal compartments, it might be particularly informative to compare the properties of the K^+ currents I

recorded from MNC somata to those recorded from the axon terminals of these neurons. It is difficult to make such a comparison, however, because of the conflicting reports described above regarding the identity of K^+ currents present in axon terminals of the posterior pituitary. Nevertheless, similarities and differences between the complements of K^+ currents in MNC somata and axon terminals are evident. It should be pointed out first, however, that my experiments were performed using guinea pig supraoptic MNCs. Accordingly, the following discussion regarding compartmental localization of K^+ channels and currents in MNCs assumes that the inventory of K^+ channels is the same in guinea pigs and rats.

A low-threshold, inactivating K^+ current is exhibited in MNC somata and in MNC axon terminals. In both neuronal compartments, this current activates between -60 to -40 mV and exhibits rapid inactivation kinetics. However, whereas the current I recorded from MNC somata appears to be insensitive to extracellular TEA (up to 5 mM), the current recorded from axon terminals may be very insensitive (Thorn *et al.*, 1991) or very sensitive (Bielefeldt *et al.*, 1992) to extracellular TEA. The differences in the reported TEA sensitivities of the inactivating current recorded from axon terminals is not due to species differences, since rats were used in both studies. The differences may, however, be due to differences in the preparation used in the studies.

The most striking difference between MNC somata and

axon terminals with respect to their complements of K^+ currents is the apparent lack of a K_V channel current in the latter compartment. Regardless of the preparation used, the non-inactivating current recorded from axon terminals of the posterior pituitary appears to be carried entirely by K_{Ca} channels. In contrast, my experiments suggest that both K_V and K_{Ca} channels contribute to the non-inactivating current recorded from somata of MNCs.

It has been proposed that the inactivating K^+ current is largely responsible for action potential repolarization in MNC axon terminals, whereas the non-inactivating, Ca^{2+}-dependent current acts to uncouple functionally terminal excitability from axonal spikes (Wang *et al.*, 1992; Bielefeldt and Jackson, 1993). Burst firing results in an accumulation of intracellular Ca^{2+} in MNC axon terminals (Jackson *et al.*, 1991). This increase in $[Ca^{2+}]_i$ would tend to activate $I_{K(Ca)}$ maximally in axon terminals and produce a long-lasting hyperpolarization. The hyperpolarization may in turn result in a refractory period during which axon terminals are electrically uncoupled from axonal spikes. Support for this hypothesis was provided by Bielefeldt and Jackson (1993) in a study in which they were able to demonstrate that a K_{Ca} channel current causes frequency-dependent action potential failures in axon terminals of the posterior pituitary. This may explain, at least partly, why the efficacy of hormone secretion from the posterior pituitary declines when the axon terminals are continuously

stimulated at high frequency for >20 s (Bicknell *et al*, 1984; Gainer *et al*., 1986) The functional roles of somatic K^+ current components are discussed below.

III. Involvement of Specific K^+ Channel Currents in Action Potential Repolarization and Frequency-Dependent Spike Broadening

Both OT and VP supraoptic MNCs exhibit complex electrical behaviors characterized by burst firing of action potentials in certain physiological conditions (see review by Poulain & Wakerley, 1982). Voltage-gated Na^+ (Andrew and Dudek, 1984) and Ca^{++} (Bourque and Renaud, 1985) currents both contribute to somatically-generated MNC action potentials. Outward K^+ currents, are believed to play a prominent role in regulating MNC excitability and in modulating firing patterns (see reviews by Renaud & Bourque, 1991; Legendre & Poulain, 1992).

The current-clamp experiments I performed suggest that the temporal activation of several K^+ channel types shapes MNC action potential repolarization as in other central neurons (Storm, 1987; Zhang and McBain, 1995). At least three pharmacologically distinct K^+ channel currents contributed to action potential repolarization in isolated supraoptic MNCs. The 4-AP- ($I_{K(A)}$) and TEA-sensitive ($I_{K(V)}$) currents both contributed substantially to MNC action potential repolarization. However, the two currents contributed differently to the temporal profile of

repolarization. 4-AP produced a pronounced broadening during the early phase of spike repolarization. The broadening produced by TEA, on the other hand, was most pronounced during the latter phase of repolarization. Thus $I_{K(A)}$ appears to contribute to spike repolarization near the onset of the repolarization phase whereas $I_{K(V)}$ contributes primarily during the latter phase of repolarization. These observations are consistent with my voltage-clamp results which revealed a faster activation of $I_{K(A)}$.

ChTX also produced spike broadening, suggesting that a Ca^{2+}-dependent K^+ channel current (perhaps the high-threshold transient current) also contributes to action potential repolarization in supraoptic MNCs. Like TEA, ChTX produced spike broadening primarily during the latter phase of spike repolarization. The degree of broadening produced by ChTX, however, was not as great as that produced by TEA. This is likely due to smaller size of the ChTX-sensitive current. The fact that ChTX produced spike broadening primarily during the latter phase of spike repolarization is not inconsistent with a role for the fast, high-threshold transient current in spike repolarization. The threshold for activation of this current is higher than that of $I_{K(A)}$ and may require the prior activation of a high-threshold Ca^{2+} channel current during a spike. Inward Ca^{2+} currents appear to be expressed during the repolarization phase of the MNC action potential, since block of $I_{K(A)}$ or $I_{K(V)}$ produced a Ca^{2+}-dependent shoulder during this phase.

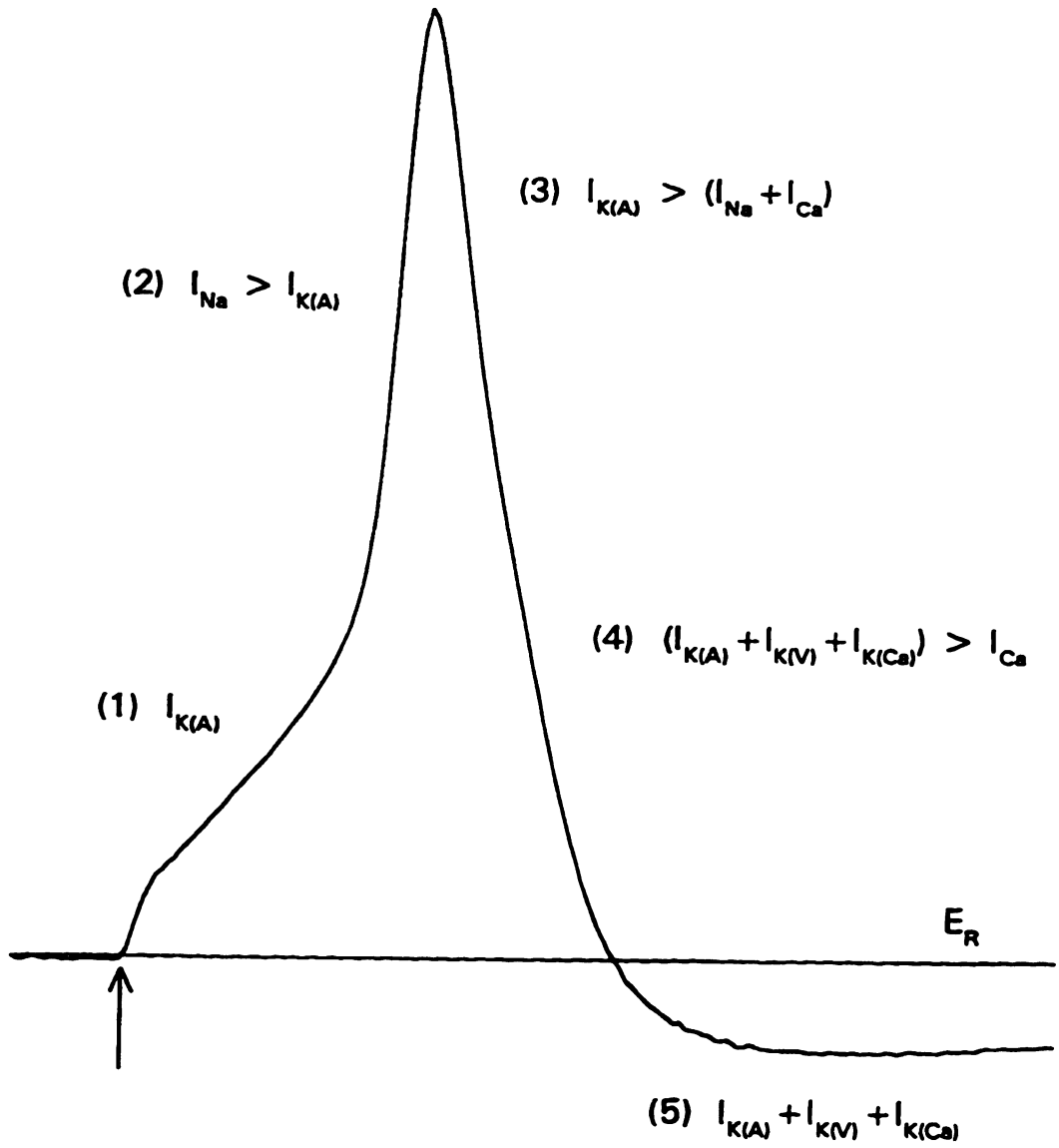
Accordingly, the high-threshold transient current may be expressed predominantly during the latter phase of spike repolarization when $[Ca^{2+}]_i$ is greatest. Figure 34 summarizes the temporal contributions of different ionic currents toward MNC action potential dynamics.

Frequency-dependent spike broadening in repetitively-firing neurons can arise *via* any of several different mechanisms. The mechanism of spike broadening in neurons of the mollusc *Archidoris* involves a frequency-dependent reduction of a K_V channel current which increases the expression of I_{Ca} as a prominent shoulder on the repolarization phase of the action potential (Aldrich *et al.*, 1979). Frequency-dependent spike broadening can occur by one of two mechanisms in neurons of the mollusc *Aplysia californica*. In *Aplysia* bag cell neurons, spike broadening results from a reduction in a slowly-inactivating, TEA-sensitive current carried by K_V channels (Quattrocki *et al.*, 1994). However, in R20 neurons from the abdominal ganglion of *Aplysia*, spike broadening results from a reduction in a rapidly-inactivating, 4-AP-sensitive current carried by K_A channels (Ma and Koester, 1995, 1996). Frequency-dependent spike broadening in both *Aplysia* neuron types was manifested as an increased expression of I_{Ca} as a prominent shoulder on the repolarization phase of the action potential, as in *Archidoris* neurons (Aldrich *et al.*, 1979).

Extracellular recordings *in vivo* (Mason and Leng, 1984) and intracellular recordings *in vitro* (Andrew and Dudek,

Figure 34. Temporal contributions of different ionic currents toward MNC action potential dynamics. (1) Following the onset of a depolarizing stimulus (*arrow*), the membrane potential (E_M) becomes more positive than the resting membrane potential (E_R). The first current activated is the low-threshold $I_{K(A)}$ which contributes a hyperpolarizing influence that slows the depolarization. (2) When the membrane becomes sufficiently depolarized, I_{Na} becomes activated and the E_M rapidly approaches the equilibrium potential for Na^+ (E_{Na}). (3) By the time the action potential reaches its peak, I_{Na} has begun to inactivate, I_{Ca} has begun to activate, and $I_{K(A)}$ has increased. The strong hyperpolarizing influence of $I_{K(A)}$ begins to repolarize the E_M, driving it towards the equilibrium potential for K^+ (E_K). The depolarizing influence of I_{Na} together with an increasing I_{Ca} slows the rate of repolarization. (4) Later in the repolarization phase, $I_{K(V)}$ and $I_{K(Ca)}$ are sufficiently activated such that, along with $I_{K(A)}$, they enhance the repolarizing and drive toward the E_K. (5) Because the E_K is more negative than the E_R, the K^+ currents become active late in the repolarization phase drive the E_M past the E_R, closer to the E_K. The hyperpolarization immediately following MNC spikes is referred to as the hyperpolarizing after-potential (HAP).

Figure 34



1985; Bourque and Renaud, 1985) from supraoptic MNCs have revealed that MNCs exhibit significant action potential broadening following the onset of burst firing. Recordings of action potentials from isolated neural lobes using optical recording techniques and potentiometric dyes indicate that a similar phenomenon occurs at the level of MNC axon terminals (Gainer *et al.*, 1986). Recent studies using voltage-clamp techniques suggest that a reduction in K^+ current during burst firing may contribute to frequency-dependent spike broadening in MNC somata (O'Regan and Cobbett, 1993) and in MNC axon terminals (Jackson *et al.*, 1991). However, at neither level was the identity of the specific component(s) of K^+ current responsible for spike broadening in MNCs determined.

My experiments suggest that frequency-dependent spike broadening in isolated supraoptic MNCs results from a reduction in $I_{K(A)}$ during repetitive firing, based on the observation that 4-AP (but not TEA or ChTX) prevents spike broadening. As $I_{K(A)}$ is reduced during repetitive firing, its contribution to spike repolarization is also reduced and action potentials broaden presumably due to an increased expression of I_{Ca} as a shoulder on the repolarization phase of the action potential. A similar mechanism has been reported for frequency-dependent spike broadening in R20 neurons from the abdominal ganglion of *Aplysia* (Ma and Koester, 1995, 1996).

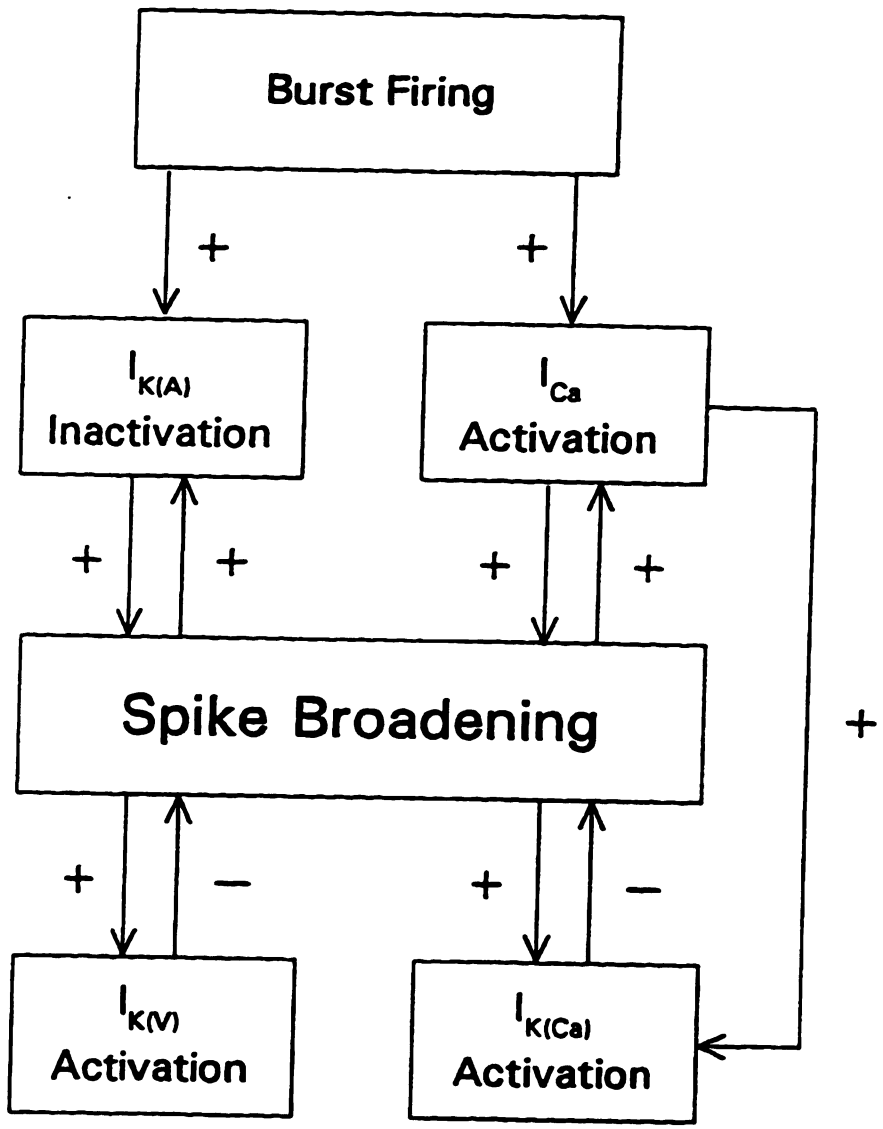
Frequency-dependent changes in TEA- ($I_{K(V)}$) and ChTX-

sensitive K^+ currents ($I_{K(Ca)}$) do not appear to contribute to spike broadening during repetitive firing. Instead, these current components appear to effectively limit the rate and extent of broadening resulting from a frequency-dependent reduction in $I_{K(A)}$. K_V and K_{Ca} channel currents contribute to spike repolarization predominantly during the latter phase of repolarization. Accordingly, these currents would be expected to provide a greater contribution to spike repolarization as spike duration increases following the onset of burst firing in MNCs. The increased contribution of these currents to spike repolarization would effectively act to offset the decreased contribution of $I_{K(A)}$. Figure 35 summarizes the roles of different ionic currents in determining the dynamics of frequency-dependent spike broadening.

The increase in spike duration during repetitive firing may permit Ca^{2+} channels to stay open longer, resulting in an increased accumulation of intracellular Ca^{2+}. Frequency-dependent spike broadening accompanied by an increased accumulation of intracellular Ca^{2+} has been demonstrated in MNC axon terminals (Jackson *et al.*, 1991). Because of the importance of intracellular Ca^{2+} in mediating hormone release, it is possible that the accumulation of intraterminal Ca^{2+} which accompanies spike broadening underlies the facilitation of hormone release during burst firing in MNCs (Dutton and Dyball, 1979; Bicknell and Leng, 1981). If an analogous accumulation of intracellular Ca^{2+}

Figure 35. Roles of different ionic currents in determining the dynamics of frequency-dependent spike broadening. Following the onset of burst firing, $I_{K(A)}$ begins to inactivate (due to steady-state inactivation), decreasing the hyperpolarizing drive responsible for spike (action potential) repolarization, and thus permitting an increased expression of I_{Ca} exhibited as a shoulder on the repolarization phase of the spike. The increased expression of I_{Ca} due to the accumulation of $I_{K(A)}$ inactivation during burst firing results in a progressive increase in the duration of successive spikes - a phenomenon known as frequency-dependent spike broadening. As spikes increase in duration, $I_{K(V)}$ and $I_{K(Ca)}$ are activated more fully. At some point during the burst, the increased repolarizing drive provided by these currents exceeds the depolarizing drive provided by I_{Ca}, thus preventing further spike broadening. Note that in this model the effect of I_{Ca} on spike broadening is two-fold. First, I_{Ca} is responsible for the shoulder which underlies the broadened spike. Second, I_{Ca} is responsible for the increase in $[Ca^{2+}]_i$ which leads to an increased activation of $I_{K(Ca)}$, thus limiting the rate and extent of spike broadening.

Figure 35


Burst Firing
+
+
$I_{K(A)}$ Inactivation
I_{Ca} Activation
+
+
+
+
Spike Broadening
+
+
−
+
−
$I_{K(V)}$ Activation
$I_{K(Ca)}$ Activation

accompanies spike broadening in MNC somata during repetitive firing, it may directly enhance the Ca^{2+}-dependent DAPs in supraoptic MNCs which are recognized to be important in the formation and maintenance of burst firing patterns that promote hormone release from the posterior pituitary (Legendre *et al.*, 1988).

Increased Ca^{2+} influx *via* voltage-dependent Ca^{2+} channels during repetitive firing may also act indirectly to regulate burst firing. Calbindin-D_{28K} (calbindin), a potent cytosolic Ca^{2+} buffer endogenous to supraoptic MNCs, has been shown to play a role in determining intrinsically-generated firing patterns in these cells (Li *et al.*, 1995). The Ca^{2+}-buffering activity of this protein attenuates transient increases in $[Ca^{2+}]_i$ resulting from Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Lledo *et al.*, 1992; Chard *et al.*, 1993). Li *et al.* (1995) showed that in supraoptic MNCs calbindin inhibits the formation of DAPs following individual action potentials and prevents burst firing, presumably by buffering transient increases in $[Ca^{2+}]_i$. Elevations in $[Ca^{2+}]_i$ resulting from increased Ca^{2+} influx during broadened action potentials may help overwhelm the Ca^{2+} buffering capacity of calbindin and thus unmask the DAPs which promote burst firing. Increased Ca^{2+} influx during broadened action potentials may also trigger ryanodine receptor-mediated Ca^{2+} release from internal stores, which enhances DAPs and promotes burst firing in supraoptic MNCs (Li and Hatton, 1997).

Recently, dendrites of supraoptic MNCs have been shown to release hormone by exocytosis into the hypothalamus (Pow and Morris, 1989). It has been hypothesized that hormone released within the SON from MNC dendrites acts locally as a modulator to facilitate MNC electrical activity and thus hormone release from these cells (reviewed in the **INTRODUCTION** section of this dissertation). Perhaps broadened somatic action potentials facilitate hormone release from MNC dendrites by increasing $[Ca^{2+}]_i$, as has been proposed for hormone release from axon terminals. If this were indeed the case, increased hormone release from MNC dendrites resulting from frequency-dependent spike broadening may represent a positive feedback mechanism which contributes to the facilitation of hormone release from MNC axon terminals observed during high-frequency stimulation (Dutton and Dyball, 1979; Bicknell and Leng, 1981; Bicknell *et al.*, 1982; Cazalis *et al.*, 1985).

IV. Other Possible Functions of K^+ Channel Currents

Other potential functions of the outward K^+ currents described in the present study should be considered in light of MNC activity profiles *in vivo* in rats (see review by Poulain & Wakerley, 1982) and in *in vitro* preparations of rat hypothalamus (Mason, 1983; Andrew & Dudek, 1984a,b; Bourque & Renaud, 1985; and others) and guinea pig hypothalamus (Erickson *et al.*, 1993). These studies show that firing patterns exhibited by rat MNCs *in vitro* and

guinea pig MNCs *in vitro* are similar, although there are some minor species differences (such as the range of the duration of bursts of action potentials generated during phasic firing). Much of the following discussion assumes that the electrical behavior of MNCs and the inventory of ion channels in MNCs are essentially the same in both species. Also, it is clear from Figure 2 that primarily current conducted by channels present in the somatic membrane of MNCs are reported in my studies. The following discussion considers only the role of these currents, despite the almost certainty that dendritic channel currents also contribute to the electrical behavior of intact cells.

The A-current may modulate the rate of depolarization between two successive action potentials, as is the case in other excitable cells (Hille, 1992). $I_{K(A)}$ may therefore play an important role in regulating neuronal excitability and determining firing frequency within a burst. The steady-state inactivation characteristics of $I_{K(A)}$ suggest that the efficacy of a depolarizing stimulus (i.e. the ability to evoke an action potential) will be dependent on the membrane potential immediately prior to and at the time of the stimulus. At E_R, $I_{K(A)}$ is largely inactivated, thus requiring a period of hyperpolarization for the removal of inactivation. Removal of steady-state inactivation by a hyperpolarization (such as that provided by an HAP) would allow this transient outward current to counteract a depolarizing stimulus until it is again inactivated.

Conversely, if $I_{K(A)}$ is inactivated or blocked the same stimulus will be unopposed by outward current and thus will generate a greater depolarization (Gustafsson *et al.*, 1982), perhaps sufficient to induce firing.

The transient Ca^{2+}-dependent K^+ current may also play an important role in removing channel inactivation which occurs during MNC action potentials. Each action potential within a burst is immediately followed by a hyperpolarizing afterpotential (HAP) which exhibits an amplitude proportional to the extracellular Ca^{2+} concentration and can be abolished by block of Ca^{2+} influx (Andrew & Dudek, 1984b; Bourque *et al.*, 1985). Bourque *et al.* (1985) have suggested that the HAP may result, from the activation of a transient Ca^{2+}-dependent K^+ current during an action potential. At least part of the transient Ca^{2+}-dependent K^+ current identified here in isolated MNCs is available for activation at relatively depolarized membrane potentials. This current may therefore contribute to the HAP following individual action potentials in supraoptic MNCs. The HAP in turn may facilitate recovery of voltage-dependent ion channels from inactivation.

Current-clamp analysis has demonstrated that the slow afterhyperpolarization (AHP) following bursts of action potentials results from the activation of a sustained Ca^{2+}-dependent K^+ conductance (Kirkpatrick & Bourque, 1996). This conductance has been proposed to modulate firing rate within bursts (Bourque *et al.*, 1985; Kirkpatrick & Bourque,

1996). The gradual accumulation of somatic intracellular Ca^{2+} during a burst of action potentials, as occurs in MNC nerve terminals (Jackson *et al.*, 1991), may contribute to the gradual activation of this K^+ current. Such a gradual activation of the sustained Ca^{2+}-dependent K^+ current could be responsible for the activity dependence of AHP onset during bursts of action potentials. Activation of the sustained Ca^{2+}-dependent K^+ current reported in the present study may be the mechanism underlying the AHP and thus may contribute to the regulation of intraburst firing rate.

In summary, my work identified several components of outward K^+ current recorded from MNCs acutely isolated from the adult guinea pig SON. I propose that the presence of multiple types of K^+ current may underlie the complex firing patterns of OT and VP MNCs, although the exact functional role of each current remains to be clarified.

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