Voltage-gated Calcium Currents in Mouse Spinal Motoneurones: Possible Role in Plateau Potentials

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Doctor of Philosophy

Dedicated to the memories

of

Eugene Carlin

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Abstract

Intracellular studies of the spinal motoneurones in the adult in-vivo cat identified a slowly activating persistent inward current that could be activated by brief depolarizations. The presence of this current provided these cells with two stable membrane potentials – one at the resting potential and the other at a more depolarized potential. Because this second potential is above threshold for sodium spikes, cells will fire repetitively at this second stable membrane potential. If the sodium spikes are blocked, the underlying sustained depolarization can be seen – this stable depolarization resulting from a brief depolarizing stimulus is termed a plateau potential.

The ionic nature of the persistent inward current was difficult to determine in the in-vivo cat. Researchers therefore turned to in-vitro preparations in order to study this current. Due to the difficulties associated with maintaining viable mammalian spinal cord tissue in-vitro, this current was studied in adult turtle spinal motoneurones which are more tolerant of in-vitro conditions. Using this preparation it was determined that the persistent inward current was mediated by a subtype of voltage-gated calcium channels termed L-type channels. Furthermore, it was determined that these channels were located in the dendrites of these cells.

Plateau potentials have since been demonstrated in a number of different cell types in both vertebrate and invertebrate nervous systems. A number of different underlying conductances have been shown to mediate the plateaux in these different cell types. The ionic nature of the current mediating plateau potentials has not been determined in mammalian spinal motoneurones. The purpose of this thesis was to

examine the nature of this current. Specifically, this thesis tested the hypothesis that mammalian spinal motoneurones use L-type voltage-gated calcium channels in the production of plateau potentials.

Using an in-vitro spinal cord slice preparation and whole cell patch clamp recording techniques, voltage-gated calcium currents were characterized in mouse lumbar spinal motoneurones. These experiments demonstrated, for the first time, the presence of a variety of channel subtypes including: T-, N-, P/Q- and R-type channels. Of particular interest was the presence of a dihydropyridine-sensitive L-type current. In a second set of experiments it was demonstrated that a subpopulation of the L-type calcium channels were located in the dendrites of these motoneurones. This result is consistent with the location of L-type channels responsible for plateau potentials in turtle spinal motoneurones. The final set of experiments demonstrated plateau potentials in mouse spinal motoneurones. These plateaux are calcium-mediated and can be elicited by activation of L-type calcium channels.

The demonstration that mammalian spinal motoneurones express L-type channels, a portion of which are located in a subcellular location consistent with their role in the generation of plateau potentials, suggests that mammalian spinal motoneurones use the same ionic mechanisms to generate plateau potentials as do lower species like the turtle. Furthermore, this idea is supported by the ability of L-type calcium channel activators to elicit plateaux in mammalian spinal motoneurones.

General Introduction

Overview

The classic preparation for studying mammalian neurophysiology has been the in-vivo cat spinal cord preparation. This preparation has been used for over 100 years to study the general principles of the nervous system, including chemical transmission, cellular biophysics and signal integration. A great advantage of using this preparation is its fairly well defined input-output, architecture and circuitry. Besides being a good model of the nervous system in general, the spinal cord and its components are an integral part of the CNS. Therefore, by studying the spinal cord we have not only learned a number of general principles about the nervous system but also more specific information about spinal motoneurones.

Early work on spinal motoneurones led to the classic concept of linear integration - in which a cell algebraically adds up the amount of inhibitory and excitatory input and fires an action potential if the amount of excitatory input pushes the membrane potential past threshold. If intracellular current injection is used to mimic synaptic current, this type of response is seen as a linear or bi-linear frequency-current (F-I) relationship (Kernell, 1965). In more recent years it has been shown that under certain conditions spinal motoneurones are capable of non-linear behaviour.

The ability of the mammalian motor system to act non-linearly was described almost 100 years ago. Sherrington (1906) described a prolonged motor nerve discharge following a brief stimulation of afferents in the cat. In 1930 Pollock and Davis described

a similar reflex response in motor nerves of the decerebrate cat in which the motor activity again outlasted the afferent stimulation. This type of prolonged behaviour was later attributed to a reverberating circuit within the spinal cord (Hultborn et al., 1975). It was not until 1986 that Hounsgaard et al used intracellular current injection directly into cat spinal motoneurones to demonstrate that this behaviour was due to an intrinsic property of the motoneurones. With intracellular recording electrodes this intrinsic property can be seen as self-sustained firing after a short depolarizing current pulse. If action potentials are blocked the underlying sustained depolarization becomes evident and is termed a plateau potential.

With the demonstration that spinal motoneurones possessed the ability to produce plateau potentials, the obvious question of mechanism arose. That is, what channels or ionic conductances are responsible for the sustained depolarizing drive? To address this question it is necessary to manipulate the extracellular environment and to use pharmacological agents specific for various membrane channels. Since the extracellular environment cannot be readily controlled in the in-vivo cat preparation, researchers turned to in-vitro spinal cord preparations. The most successful of these at elucidating the mechanisms of plateau potentials in spinal neurons has been the adult turtle spinal cord slice preparation. Using this model Hounsgaard and co-workers have demonstrated that the underlying conductance of the plateau potential is mediated by dihydropyridine-sensitive L-type voltage-gated calcium channels. As the turtle is a reptile, an obvious and important question is, do mammalian spinal motoneurones use the same or a similar mechanism to produce plateau potentials?

Supportive evidence for a similar mechanism underlying plateau potentials in mammalian spinal motoneurones has recently been obtained by Jiang et al (1999a) in the in-vitro mouse whole spinal cord preparation. In this study it was demonstrated that neurochemically evoked lumbar ventral root output became sensitive to both the extracellular calcium concentration and the L-type calcium channel antagonist nifedipine after approximately postnatal day 7. In a second part of this study spinal motoneurones were shown to immunohistochemically stain for both the α_{1C} and the α_{1D} subunits of L-type calcium channels. Consistent with the electrophysiological data, the immunostaining developed during the first 3 postnatal weeks in mouse motoneurones.

These initial results in the mouse spinal cord supported the hypothesis that "mature" mammalian spinal motoneurones produce plateau potentials that are mediated by L-type calcium channels. However, these results could not rule out the possibility that 1) nifedipine was acting at a pre-motoneuronal level and 2) it could not be determined that the immunolabeling represented functional L-type channels. Therefore, experiments were undertaken to determine if, as in the turtle, "mature" mammalian spinal motoneurones use L-type voltage-gated calcium channels in the production of plateau potentials. The hypotheses tested in this thesis are 1) that mammalian spinal motoneurones possess a variety of subtypes of voltage-gated calcium channels including functional dihydropyridine-sensitive L-type channels 2) that a sub-population of L-type channels are located in a subcellular location (dendrites) which is thought to be necessary in the production of plateau potentials and 3) that plateau potentials can be elicited in these cells by means consistent with activation of L-type calcium channels. The rationale

for each of these hypotheses is developed further in the remainder of the INTODUCTION.

Neuronal voltage-gated calcium channels

The function of all calcium channels is to allow calcium entry into the cytoplasm of cells. Normally the concentration of cytosolic free calcium is very low (> 10^{-7} M; Meir et al., 1999), while the concentration in the extracellular fluid is some four orders of magnitude greater. As a result of this sizeable driving force, a selective increase in the permeability of the plasma membrane will allow these charged ions to enter the cell. Also, the normally low concentration of calcium allows this ion to function as an intracellular second messenger. The neuronal voltage-gated calcium channels therefore have the ability to couple changes in the membrane potential to a number of cellular functions. The most well known function of these channels is to trigger calcium-dependent exocytosis of neurotransmitter upon depolarization of nerve terminals. This is but one role these channels play in neuronal functioning. The calcium influx from these channels can also alter the excitability of cells. This can be through either an electrogenic effect such as in the case of plateau potentials (Hounsgaard and Kiehn, 1985), bursting (Williams and Stuart, 1999) or spike initiation (Viana et al., 1993a) or through direct gating effects on other channels (ex. K_{Ca} ; Walton and Fulton, 1986; Viana et al., 1993b). Calcium entry via activation of these channels can also be coupled to activation of various calcium-dependent enzyme systems (ex. calcium-calmodulin-dependent protein kinase ; Aletta et al., 1996) and has been shown to activate gene transcription in some cells (Greenberg et al., 1986).

There are a number of subtypes of neuronal voltage-gated calcium channels. These differ in their activation and inactivation voltage and kinetics, deactivation kinetics, single channel conductance and pharmacological sensitivity. Early work on neuronal voltage-gated calcium channels identified two subtypes: those that were activated at voltages relatively close to the resting membrane potential (ie. at approximately –50 mV) and were thus termed low voltage-activated (LVA) and those that were activated with much larger depolarizations (positive to -10 mV ; Carbone and Swandulla, 1989) and termed high voltage-activated (HVA). The current resulting from the activation of LVA channels was seen to activate and deactivate slowly and inactivate rapidly when compared to the HVA current (Carbone and Swandulla, 1989) which activated more quickly and demonstrated very little if any inactivation. The "transient" LVA channels were therefore termed "T-type" while the "longer lasting" HVA channels were termed "L-type". Single channel analysis of these currents revealed that the LVA channels had a conductance of approximately 8 pS (in 110 mM barium) while the HVA channels had a somewhat larger single channel conductance (approximately 25 pS in 110 mM barium; Nowycky et al., 1985).

With the discovery that the class of compounds called the dihydropyridines (DHP) could specifically antagonize the L-type channels a new DHP-resistant HVA channel was soon shown to co-exist with the other channel subtypes in chick dorsal root ganglion cells (Nowycky et al., 1985). These channels displayed a slope conductance between the T- and L-type channels (13 pS in 110 mM barium) and a time-dependent inactivation. Consequently a strong negative potential was required for complete de-inactivation. Therefore, on the basis of DHP sensitivity and kinetics two distinct HVA

channel subtypes could be distinguished in neurons. Since these latter channels seemed to be specifically expressed in neurons, these DHP-resistant channels were termed "N-type" (Nowycky et al., 1985). It was only two years later when a specific antagonist for these channels was purified from the venom of the hunting snail *Conus geographus*, and the toxin was called ω-conotoxin-GVIA (McCleskey et al., 1987).

Within two years of the N-type channel identification, a third HVA channel subtype was described by Llinas' group in mammalian cerebellar Purkinje cells and in pre-synaptic terminals of the squid giant axon. The non-inactivating current mediated by these channels was resistant to both the DHP's and ω-conotoxin-GVIA but was sensitive to the toxin isolated from venom of the funnel web spider (*Agelopsis aspersa*), FTX (Llinas et al., 1989). More commonly used toxins that specifically inhibit this current component are ω-agatoxin-IVA (Mintz et al., 1992) and ω-agatoxin-IVB (Adams et al., 1993) also known as ω-agatoxin-TK (Teramoto et al., 1995). These latter toxins are also isolated from funnel web spider venom. Having been described first in Purkinje cells, this new channel subtype was termed "P-type".

The fourth channel subtype to be identified was the "Q-type" channel. The current mediated by these channels was first described in cerebellar granule cells as a rapidly inactivating HVA channel (Zhang et al., 1993; Randall and Tsien, 1995) and has been shown to have a much lower sensitivity to block by ω-agatoxin-IVA (Kd ~ 2 nM for P-type vs. > 100nM for Q-type; Bourinet et al., 1999). Since the difference in sensitivity of these two channel subtypes is often difficult to distinguish experimentally, the P- and Q-type channels are often grouped together as ω-agatoxin-sensitive P/Q-type channels.

The last channel subtype to be described in neurons is that termed "R-type". The current mediated by this channel was identified as the current "resistant" to block by DHP, ω-conotoxin-GVIA and ω-agatoxin-IVA and was again first described in cerebellar granule cells (Zhang et al., 1993).

In terms of molecular composition, neuronal calcium channels are constructed with four subunits: α_1, $\alpha_2\delta$ and β. A γ subunit, which is present in muscle tissue, is not normally found in neuronal tissue except in pathological conditions (ex. in the mouse mutant *stargazer*, leading to ataxic and epileptic signs; Jen, 1999) . The $\alpha 1$ subunit forms the calcium selective pore, contains the channel gating mechanism and is the site of action of most pharmacological agents and second messenger regulation (Zhang et al., 1993; Ertel et al., 2000). There are presently 9 identified genes that encode the $\alpha 1$ subunits found in neurones and which form the basis of the various calcium channel subtypes: α_{1A} (P/Q-type), α_{1B} (N-type), α_{1C} and α_{1D} (L-type), α_{1E} (R-type), α_{1G}, α_{1H} and α_{1I} (T-type). An α_{1F} subunit encoding an L-type channel is also expressed in the retina and is mutated in individuals with a type of night blindness (Strom et al., 1998). The α_2 and δ subunits are derived from a single gene, the product of which is cleaved and linked via a disulfide bond in-vivo (De Jongh et al., 1990). There are at least 4 different genes encoding the β subunit (β_1 - β_4; Dunlap et al., 1995) and two encoding the $\alpha_2\delta$ subunit (Gao et al., 2000).

It has been noted that the electrophysiological characteristics of the various voltage-gated calcium channel subtypes can vary from one cell type to another, and a single channel type can display different properties within a given cell (Tottene et al., 2000). For example, N-type channels produce an inactivating current in sensory neurons

(Nowcky et al., 1985) while in pre-synaptic terminals (Stanley and Goping, 1991) and chromaffin cells (Artalejo et al., 1992) the current mediated by N-type channels in non-inactivating. This diversity is thought to be partly the result of alternative gene splicing and therefore the presence of multiple isoforms of the various subunits. Alternative splicing of the α_1 subunit alone has been shown to produce functional channels that differ significantly in terms of inactivation kinetics, toxin sensitivity and second messenger modulation (Bourinet et al.,1999). Combining this with observations of the modulatory effects of the accessory subunits (Bean and McDonough, 1998; Tomlinson et al., 1993; Stea et al., 1993; Williams et al., 1992) leads to a system in which a large diversity of channel functionality can be produced. For example, it has been demonstrated that co-expression of the different isoforms of the β subunit (Zhang et al., 1993; Cahill et al., 2000) and the absence or presence of the two different $\alpha_2(\delta)$ subunits (Stea et al.,1993; Dai et al., 1999; Gao et al., 2000) can modify the electrophysiological properties of the α_1 subunit. These modulatory effects can include changes in whole cell current amplitude, activation and inactivation kinetics, voltage dependency and ability to produce paired pulse facilitation. Moreover, various isoforms of the β subunit have been immuno-precipitated with L-type channels in rabbit and guinea pig brain suggesting that these associations are not an artifact of the expression system and that this heterogeneity exists in-vivo (Pichler et al., 1997).

Voltage-gated calcium channels in motoneurones

Early evidence for the presence of voltage-gated calcium channels in motoneurones was for the most part collected in current clamp mode. In these experiments the effect of current flow through these channels was seen as an afterdepolarization (ADP, also known

as the delayed depolarization DD; Granit et al., 1963), an afterhyperpolarization (AHP; Barrett and Barrett, 1976) or as prolonged action potentials or spikes in the presence of sodium and potassium channel blockers (Barrett and Barrett, 1976; Alvarez-Leefmans and Miledi, 1980; Walton and Fulton, 1986; Ziskind-Conhaim, 1988). Using the in-vitro frog (Barrett and Barrett, 1976) and neonatal rat (Harada and Takahashi, 1983; Walton and Fulton, 1986) spinal cord preparations the ADP was shown to be voltage-dependent, sensitive to the extracellular calcium concentration and could be eliminated by non-specific calcium channel antagonists. Furthermore, the AHP was shown to be dependent on intracellular calcium as it could be eliminated with the intracellular calcium chelator EGTA (Krnjevic et al. 1975). This and other work led to the conclusion that the ADP was mediated directly by voltage-gated calcium channels while the AHP resulted from a potassium conductance activated by a conductance through voltage-gated calcium channels.

Indirect evidence for a voltage-gated "calcium current" was also obtained using two electrode voltage clamp techniques in cat spinal motoneurones (Schwindt and Crill, 1977; 1980a,b). In these experiments the application of slow voltage ramp command produced a region of negative slope in the current-voltage relationship consistent with the activation of a persistent inward current. Because the negative slope conductance increased in amplitude with iontophoretic application of barium and was unaffected by the intracellular fast sodium channel blocker QX-314 (Schwindt and Crill, 1984) the current was attributed to the activation of voltage-gated calcium channels. Although the effects of these drugs are consistent with this current being mediated by voltage-gated calcium channels, our present knowledge of these chemicals make this conclusion less

convincing. Besides having a greater conductance through some voltage-gated calcium channels, barium is known to block a number of potassium channels. The effect of blocking a competing outward current alone could lead to an increase in the negative slope conductance. Furthermore, there is evidence that QX-314 blocks some types of voltage-gated calcium channels (Talbot and Sayer, 1996) and may not readily diffuse throughout the motoneurone (Lee and Heckman, 1999). Therefore, due to the inability to manipulate the extracellular environment and readily apply pharmacological agents to these cells in the in-vivo cat preparation, the ionic nature of this persistent inward current in cat motoneurones is still unconfirmed.

Coinciding with the discovery that multiple subtypes of voltage-gated calcium channels co-existed in neurons (Nowycky et al., 1985), the currents mediated by these channels were subsequently isolated and identified in various types of motoneurones. Originally the various current subtypes were identified by both pharmacological sensitivities and kinetics, later with the refinement of molecular techniques, the channels mediating these currents could be identified through immunohistochemistry (spinal; Westenbroek et al., 1998), RT-PCR (facial; Plant et al., 1998) or in-situ hybridization (spinal; Talley et al., 1999).

Using pharmacological/electrophysiological methods numerous groups have demonstrated the presence of multiple calcium current subtypes in a variety of motoneurones. In spinal motoneurones these include T-, and L- and non-L-types in the chick (McCoob et al., 1989), T-, L- and N-types in cultured embryonic mouse motoneurones (Mynlieff and Beam 1992a,b) and T-, L-, N-, P-, and R-types in embryonic and early postnatal rats (Berger and Takahashi, 1990; Hivert et al., 1995; Gao

and Ziskind-Conhaim, 1998; Scamps et al. 1998). These currents were also isolated and typed in rat brainstem motoneurones including those in the hypoglossal and facial nuclei. In hypoglossal motoneurones all the known subtypes have been identified (Umemyia and Berger 1994; Bayliss et al., 1995). Meanwhile, in facial motoneurones, although mRNA for all the α_1 subunits was identified (including α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, and even α_{1S} - skeletal muscle), an ω-agatoxin-sensitive (P/Q-type) current could not be identified in the somata of these cells (Plant et al., 1998).

The presence of voltage-gated calcium channels in adult rat spinal motoneurones has also been demonstrated immunohistochemically. Using antibodies generated against the various α_1 subunits, Catterall's group has demonstrated both the presence and the subcellular distribution of the various HVA channels including those constructed with the α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} subunits (Westenbroek et al., 1998; see discussion in paper 2). In the mouse, we have recently demonstrated the development and differential subcellular distribution of the both the α_{1C} and the α_{1D} L-type channels (Jiang et al., 1999a). In situ hybridization has revealed the presence of mRNA for both the α_{1G} and α_{1H} subunits and therefore suggests the presence of T-type LVA channels in adult rat spinal motoneurones (Talley et al., 1999).

In phylogenetically lower organisms, motoneurones seem to express a somewhat smaller complement of voltage-gated calcium channels - based on pharmacology alone. In the lamprey, these cells have been found to expressed all of the HVA current subtypes but not the LVA T-type current (Manira and Bussieres, 1997). In crayfish only an L-type (Chrachri, 1995) and P-type (Hong and Lnenicka, 1997) current have been demonstrated. In motoneurones of the lobster stomatogastric ganglion, both L- and P/Q- but not N-type

currents are found (Hurley and Graubard, 1998). In the cockroach both L and non-L-type currents have been identified (David and Pitman, 1995; Mills and Pitman, 1997). In the jellyfish an LVA T-like current and two HVA current components have been isolated (Przysiezniak and Spencer, 1992). The pharmacological identification of channel subtypes in invertebrate preparations may or may not indicate the presence of molecularly identical channels that have been extensively described in vertebrate preparations. In certain instances the IC_{50} values of antagonists are consistent with those in vertebrate preparations while in other instances they are not (for a more in depth discussion on this issue see Hurley and Graubard, 1998).

Plateau potentials

Plateau potentials are defined as prolonged regenerative depolarizations resulting from intrinsic membrane properties (Hartline et al., 1988). The presence of plateau potentials is evidenced by prolonged self-sustained firing of a cell and can be detected with at least 12 different electrophysiological protocols (Hartline and Graubard, 1992), the two most important criteria for establishing the presence of plateau potentials are "the ability to trigger all or none bursts with a brief depolarization and to terminate an ongoing burst in an all or nothing fashion with brief hyperpolarizing pulses" (Hartline et al.,1988). Cells with the capacity to generate plateau potentials feature a region of negative slope in their steady state current-voltage (I-V) relationship - giving the resultant curve a characteristic "N" shape. The points at which the I-V curve crosses the voltage axis with a positive slope represent voltages with zero net current flow and therefore stable membrane potentials. The more hyperpolarized crossing point represents the resting membrane potential while the more depolarized crossing point establishes the potential of

the plateau. If a cell is at rest, a sufficiently large depolarizing current pulse will push the membrane potential into the negative slope region of the current voltage relationship (i.e. the persistent inward current will be activated) and the potential will regeneratively move to the second stable and more depolarized (plateau) potential. The length of time in which the cell remains depolarized at the plateau voltage is partly determined by the inactivation characteristics of the persistent inward current (Lee and Heckman, 1998) and/or the activation of outward currents (Hartline and Graubard, 1992; Lee and Heckman, 1999). Stability of the second zero net current point is lost when the inward current underlying the negative slope can no longer maintain the depolarized potential and the cell repolarizes to the resting potential. The same effect is seen if a sufficiently long and large hyperpolarizing pulse is applied during the plateau phase. The membrane potential is pushed to a point where the persistent inward current is deactivated producing a net outward current flow and the membrane returns to the resting potential. This type of non-linear or bistable behaviour can result in either "on" and "off" firing states or two stable firing states that differ in frequency (bistable firing). The expression of either of these modes depends on the relationship of the spike threshold to the plateau threshold (Lee and Heckman, 1998) such that bistable firing will occur if the plateau threshold is above the firing threshold.

Plateau potentials have been described in a wide variety of both invertebrate and vertebrate cells including motoneurones of the crustacean ventilatory system (DiCaprio, 1997), almost all of the cells of the stomatogastric ganglion (Hartline and Graubard, 1992; Zhang et al., 1995; Elson and Selverston, 1997), and in swimmeret motoneurones of the crab (Chrachri, 1995). In vertebrates plateau potentials have been demonstrated in

spinal neurons of the turtle (Hounsgaard and Kiehn, 1989; Russo and Hounsgaard, 1996), cat (Schwindt and Crill, 1981; Hounsgaard et al., 1984; Paroschy and Shefchyk, 2000), rat (MacLean et al., 1997; Morrisette and Nagy, 1998) and mouse (Jiang et al., 1999b). A few other mammalian cells types in which this membrane property has been observed include: hypoglossal (Mosfeldt-Laursen and Rekling, 1989), ocular (Gueritaud, 1994), and trigeminal (Hsiao et al., 1997), motoneurones, vestibular (Serafin et al., 1991), subthalamic (Beurrier et al., 1999) and ambiguus (Rekling and Feldman, 1997) neurons, cerebellar Purkinje cells (Llinas and Sugimori, 1980a,b) and hippocampal (Hoehn et al., 1993; Fraser and McVicar, 1996) and cortical (Stafstrom et al., 1985) pyramidal cells.

Even though all of the above mentioned cell types are capable of displaying a similar bistable behaviour, the underlying ionic mechanisms vary between cell types. This illustrates the need to study the cell type of interest in order to elucidate the specific mechanisms involved. For this thesis mammalian spinal motoneurones have been studied.

Role of plateau potentials in mammalian motor output

What is the functional significance of plateau potentials? In very general terms Hartline et al (1988; 1992) have outlined the role of this intrinsic mechanism in neuronal networks as follows. In the presence of a mechanism to spontaneously terminate and activate a plateau the cell becomes an endogenous bursting neurone and can play an important role in rhythm generating circuits. Induction of a plateau will increase the gain of an input – amplifying weak inputs into stronger outputs. Because of the trigger-like behaviour, the output then becomes independent of the input. That is, a brief input can produce sustained output. Finally, because in the majority of situations the induction of a

plateau is under the control of neuromodulatory systems, plateau potentials provide a method for regulating the output of a circuit.

If one looks more specifically at the role of plateau potentials in spinal motoneurones, the last cells in the spinal motor circuitry, one can see very similar functions. Although these cells do not spontaneously oscillate as do numerous cells in the crustacean stomatogastric ganglion, the presence of plateau potentials can contribute to the output of the motor circuitry. One of the most obvious roles of plateau potentials in lumbar motoneurones would be in the maintenance of posture (Eken et al., 1989) while in sphincter motoneurones plateau potentials would help maintain continence (Paroschy and Shefchyk, 2000). In this case the motor circuitry would only have to give a brief input to these cells and in response the cells would be able to generate prolonged output (Eken et al., 1989; Kiehn and Eken, 1998). This would be an economical system as the spinal circuitry would not have to provide continuous synaptic excitation in order to produce the sustained muscular contraction required in this system.

Plateau potential mechanisms are also thought to be called upon during phasic output as that seen during locomotion and may serve as part of the recruitment mechanism (reviewed in Hultborn, 1999). Consistent with the induction of a plateau potential in motoneurones during locomotion 1) it can be seen that a high discharge rate is obtained shortly after recruitment when evaluating single motor units in the decerebrate cat during fictive MLR induced (Jordan, 1983) and overground (Gorassini et al., 1999) locomotion 2) the locomotor drive potentials are voltage dependent (Brownstone et al., 1994) and respond to current injection in a similar manner as a plateau potential such that injection of additional current during the depolarized phase does not produce a significant

increase in the firing rate while injection of a small amount of hyperpolarizing current is sufficient to completely eliminate repetitive firing (Brownstone et al., 1992) and 3) similar reductions in the AHPs are seen during the plateau potential (Bennett et al., 1998) and during the depolarized phase of the LDPs (Brownstone et al., 1992). Finally, the induction of plateau potentials in motoneurones may produce a more efficient and stable force output in muscles. This is thought to result from a decrease in the spike variability inherent in the probabilistic nature of synaptic input (Kiehn and Eken, 1998). Furthermore, the initial high firing rates seen during plateau induction are associated with the presence of doublets or triplets in fast motor units (Gorassini et al., 2000). Initial doublet firing has previously been shown to generate greater muscular force than single spikes (Burke et al., 1969; Stein and Parmiggiani, 1979).

Ionic nature of plateau potentials

The ionic conductances responsible for the negative slope conductance (and hence the maintenance of the depolarized phase of the plateau potential) have been determined in a variety of cell types in both vertebrate and invertebrate organisms. In general, neurons seem to use four different conductances to generate plateau potentials. These include a conductance through NMDA receptors, L-type calcium channels, persistent sodium channels and a calcium-activated nonspecific cation conductance (I_{CAN}). Some cell types use more than one of these conductances. For instance the I_{CAN} current has been found coupled to an L-type calcium current and there is some evidence for both a persistent sodium current and a calcium current contributing to the maintenance of the plateau potential. I will briefly review a few representative cell types that use each of these mechanisms and in which the ionic mechanisms have been clearly demonstrated.

Lamprey reticulospinal neurons provide the major descending drive for locomotion. These cells receive sensory information and synapse onto spinal interneurons and motoneurones. Sustained firing in response to a short intense sensory input (ie a physiological input) has been demonstrated in reticulospinal neurons. In these cells both the plateau potential and the resultant calcium transient can be blocked by the specific NMDA (N-methyl-D-aspartate) antagonist AP5 (2-amino-5-phosphonopentanoic acid), demonstrating that they are mediated by a conductance through the NMDA ionophore (Di Prisco et al., 1997).

In turtle spinal motoneurones voltage-dependent plateau potentials have been demonstrated to be dependent on extracellular calcium (Hounsgaard and Kiehn, 1985) and to be sensitive to the L-type calcium channel blocker nifedipine (Hounsgaard and Kiehn, 1989). The sustained depolarization is insensitive to TTX (Hounsgaard and Kiehn, 1985) and has no contribution from I_{CAN} (Perrier and Hounsgaard, 1999). Furthermore, these plateau potentials can be induced in the presence of the L-type calcium channel activator BayK-8644 (Hounsgaard and Kiehn, 1989). From these experiments it has been concluded that plateau potentials in turtle spinal motoneurones are mediated by L-type voltage-gated calcium channels.

A persistent sodium current has been demonstrated in both invertebrate and mammalian neurons (for review see Crill, 1996). As with the fast sodium current this persistent sodium current is sensitive to TTX. At present it is unclear if this current arises from a distinct set of channels that differ from the fast inactivating sodium channels or if this current arises from a change in gating mode of the same channels. In the mammal, this current has been identified in both neocortical and cerebellar Purkinje cells, and

under appropriate conditions, has been shown to generate prolonged depolarizations in response to short excitatory inputs (Stafstrom et al., 1982; Mittmann et al., 1997; Llinas and Sugimori, 1980a,b). Cells of the stomatogastric ganglion also clearly demonstrate sodium-dependent plateau potentials in response to TEA application. These plateaux are insensitive to low calcium/high magnesium/manganese solutions known to reduce current through voltage-gated calcium channels. The sustained depolarization and spiking can be abolished with perfusion of low sodium or TTX containing solutions (Elson and Selverston, 1997).

The calcium activated nonspecific cation current (I_{CAN}) is known to underlie plateau potentials in a number of cell types including cells of the crab stomatogastric ganglia (Zhang et al., 1995), rat subthalamic neurons (Beurrier et al., 1999) and dorsal horn neurons in the rat (Morisset and Nagy, 1999). In the latter cells the plateau potentials were shown to be calcium-dependent and sensitive to both BAY-K8644 and nifedipine, therefore suggesting a contribution from L-type voltage-gated calcium channels. Furthermore, the plateau potentials were shown to be sodium-dependent and blocked by the intracellular calcium chelator BAPTA (bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid) and by the I_{CAN} antagonist FFA (flufenamate; Morisset and Nagy, 1999). These findings are consistent with the plateau being mediated by both L-type calcium channels and an I_{CAN} current activated by calcium influx through these same L-type channels.

Evidence for calcium-dependent plateau potentials in mammalian spinal motoneurones

As mentioned in the introduction electrophysiological evidence suggesting the presence of plateau potentials in cat spinal motoneurones was first obtained in the early 1900's. The initial reports were of sustained increases in EMG activity in response to short bouts of afferent stimulation (Pollack and Davis, 1930). This same phenomenon was re-examined by the group in Copenhagen (Hultborn et al.,1975; Hounsgaard et al., 1986; Crone et al., 1988) and determined to result from the activation of membrane properties intrinsic to the motoneurone. During this time Schwindt and Crill (1977; 1980ab, 1981) were using voltage clamp techniques to demonstrate a persistent inward current in these cells. The presence of this current gave these cells a characteristic "N" shaped current-voltage relationship. In a portion of these cells the persistent inward conductance was large enough to have the cells demonstrate a net inward current. In these cases there were two "zero current" points with positive slope and therefore the potential for bistability (for a more complete explanation of bistability see section – Plateau Potentials).

Due to the fact the pharmacology of the persistent inward current was consistent with a calcium current and a similar current mediated by calcium was described in bursting pacemaker neurones of the mollusk *Helix* (Eckert and Lux, 1976), Schwindt and Crill (1980b,1981,1984) suggested that the persistent inward current was mediated by calcium. Realizing that in order to study the ionic mechanisms of this current it would be necessary to use an in-vitro preparation such that the extracellular environment could be manipulated, Hounsgaard's group turned to the turtle spinal cord slice preparation. In

spinal motoneurones of the adult turtle, plateau potentials were shown to be calcium-dependent, TTX resistant and sensitive to L-type calcium channel blockers (Hounsgaard and Kiehn, 1989). Since that time calcium-dependent plateau potentials have been identified in a number of mammalian brainstem motoneurones: trigeminal (Hsiao et al.,1998), esophageal (Rekling and Feldman, 1997) and possibly ocular (Gueritaud, 1994) and hypoglossal motoneurones (Mosfeldt-Laursen and Rekling, 1989).

Dendritic location of plateau generating current

Identification of the channel type mediating the persistent depolarizing ionic current responsible for negative slope conductance underlying the plateau potential is but one factor in understanding the cell's ability to produce plateau potentials. A number of modeling studies indicate that a second important aspect of this ability is the subcellular location of these ion channels in relation to the spike generating mechanism (Gutman, 1991; Jaeger et al., 1997; Booth and Rinzel, 1995; Booth et al., 1997; Yuen et al., 1995; Antal et al., 1997; Pinsky and Rinzel, 1994). In these modeling studies it was necessary for the spike generating mechanism to be weakly coupled to the plateau-generating conductance. That is, it was essential for the plateau generating conductance to be in the dendritic compartment(s) at some electrotonic distance from the soma / initial segment in order to simulate realistic firing patterns. Electrophysiological evidence for a dendritic location of channels mediating a persistent inward conductance has also been obtained in a few cell types that display plateau potential behaviour. These include both cerebellar Purkinje cells (Usowicz et al., 1992) and neocortical pyramidal cells (Schwindt and Crill, 1995). In these cell types it has been possible to directly record from dendrites. In turtle

spinal motoneurones evidence for a dendritic location of the channels was obtained using the technique of differential polarization (see below) as opposed to making dendritic recordings (Hounsgaard and Kiehn, 1993). Prior to this thesis, only indirect evidence has been obtained for a dendritic location of the plateau-generating channels in mammalian spinal motoneurones (Schwindt and Crill, 1977, 1980b; Lee and Heckman, 1996; Bennett et al., 1998).

Numerous motoneurone models have simulated the bistable firing patterns seen with the induction of a plateau potential. A common feature in these models is the placement of the persistent inward conductance at some electrotonic distance from the soma (Gutman, 1991; Booth and Rinzel, 1995; Booth et al., 1997). In an early motoneurone model by Gutman (1991; Butrimas and Gutman, 1977), the dendritic tree was modeled as a stick arising from ball-like soma. With a persistent calcium conductance localized to the dendritic compartment, bistability was only achieved when the dendritic compartment was made sufficiently long. Booth and Rinzel (1995) also constructed a two-compartment motoneurone model to address this same question. In this model, a persistent calcium current was localized to the dendritic compartment and the coupling conductance between the two compartments varied to determine its effect on 1) the ability of the model to produce bistable firing and 2) its effect on the frequency-current (F-I) relationship. In cells in which a plateau potential is activated during the ascending limb of a voltage ramp the F-I relationship displays a counter-clockwise hysteresis. That is, for a given amount of injected current the cell fires faster on the descending limb compared to the ascending limb of the ramp.

It was determined that neither the bistable firing nor the counter-clockwise hysteresis could be simulated if the two compartments of the model were tightly coupled - a decrease in the coupling conductance (i.e. an increase in the electrotonic separation) of the two compartments was necessary. This same finding was seen in a second model with additional conductances including an L-like current located in both compartments (Booth et al., 1997). An earlier single compartment motoneurone model presented by Powers (1993) was able to mimic some aspects of bistability once the activation and deactivation time constants of the persistent inward current were increased. This model could simulate hysteretic firing during a current ramp and it was possible to elicit self-sustained firing but it was not possible to obtain bistable firing patterns.

The most convincing evidence that the current identified in the dendrites of these cells is in fact the current mediating the sustained plateau comes from two studies: one using an in-vitro turtle spinal cord slice preparation (Hounsgaard and Kiehn, 1993) and the other using the in-vivo cat preparation (Bennett et al., 1998). In the first study Hounsgaard and Kiehn (1993) created an electric field through a slice of spinal cord while recording intracellularly from the soma of a motoneurone. Because the dendrites of turtle motoneurones projected radially from the soma, there were some dendrites that were orientated towards one pole of the electric field and others that were orientated towards the other. In this manner the dendrites and soma of the cell could be differentially polarized, that is, a set of dendrites could be depolarized or hyperpolarized relative to the soma. Taking advantage of this ability it was demonstrated that a plateau potential could be elicited in the soma not only by transiently depolarizing the soma but

also by hyperpolarizing the soma. This latter result could only be obtained if the plateau generating conductance is located in the relatively depolarized dendrites.

The second study by Bennett et al (1998) used both excitatory and inhibitory synaptic input, which is for the most part dendritically located, to manipulate the *voltage* threshold of plateau potentials elicited by somatic current ramps. In these experiments current ramps were injected into the soma of cells while subthreshold excitatory or inhibitory synaptic input was delivered by stimulating the appropriate afferent system. The addition of subthreshold excitatory synaptic input was seen to reduce the voltage threshold while inhibitory synaptic input was seen to raise the threshold. If the plateau-generating current was localized to the soma, then it would be expected that the addition of synaptic current would reduce the amount of current required from the stimulating electrode to activate the plateau, but the *voltage* of activation would be expected to remain constant. The fact that the somatic *voltage* at which the plateau activated was altered led investigators to suggest that the channels responsible for mediating the plateau were dendritic. The synaptic input was effectively moving these channels either closer or further from their activation voltage independent of the somatic voltage. This would only be possibly if the voltage-gated channels mediating the plateau-generating current were at some electrotonic distance from the soma, that is in the dendrites.

The use of "motor functionally mature" animals

An initial study using an isolated whole spinal cord from the mouse explored the role of L-type calcium channels in mammalian motor output (Jiang et al., 1999a). This study demonstrated that the spinal motor system develops a increasing reliance on L-type

calcium channels in the production of ventral root discharges during the postnatal period. This was demonstrated by a reduction in a neurochemically induced motor output with the application of the L-channel blocker nifedipine. This nifedipine-sensitivity was only evident after approximately postnatal day (P) 7. An examination of the bursts revealed that the nifedipine produced both a reduction in amplitude and a decrease in the duration. This finding is consistent with the block of plateau potentials in the motoneurones but could not rule out a nifedipine action on pre-motor neurons. Immunohistochemical studies using the anti-α_{1C} and anti-α_{1D} antibodies against the pore-forming subunit of both subtypes of L-type channels demonstrated the presence of these subunits in a number of cells including the motoneurones. Consistent with the electrophysiological data, the immunolabeling demonstrated a postnatal development of both the α_{1C} and the α_{1D} L-type channel subunits with each approximating the adult pattern by approximately P18.

The results from these experiments suggested that in the mouse the spinal motor systems use L-type calcium channels in the production of motor output and that these channels develop during the second postnatal week. These results also suggested that the reason a calcium-dependent plateau potential has not been demonstrated using the neonatal (P0 – P9) rat preparation may be due to insufficient development of the L-type channels. Interestingly, mice begin to bear weight and walk with their abdomens suspended at approximately P9, consistent with plateau potentials and/or the plateau mechanism contributing to efficient contraction of both postural and phasically active muscles. If plateau potentials are important in both the maintenance of posture and the generation of locomotor output (Eken et al., 1989; Kiehn and Eken, 1998; Hultborn,

1999), it was reasoned that the mechanisms of plateau potentials would best be studied in animals in which the motor systems have matured to the point that functional output (weight bearing and walking) could be observed. Therefore, only "motor functionally mature" animals were used for the experiments in this thesis.

The use of animals of this age brought a new set of problems. Mammalian motoneurones of any age are very difficult to keep alive in-vitro. "For reasons that are not clear, neurons in the dorsal and intermediate grey matter are more likely to survive than those in the ventral horn. This is true of slices of spinal tissue as well as hemisected cords 'en bloc'" (Somjen and Chez, 1989). One reason for this is they may not be able to recover from the loss of a large percentage of cell volume when the ventral roots are sectioned. When using a slice preparation of sufficient thinness to allow visual identification of cells and rapid drug access, these large cells suffer additional trauma when their dendrites are severed. Furthermore, the older and generally the more caudal the motoneurones, the harder they are to keep alive during the preparation of slices. Possibly some aspect of the rostral-caudal development of the spinal cord is responsible for the increased difficulty of obtaining in-vitro lumbar spinal motoneurones as compared to the greater success that has been obtained with those from the brainstem. However, even postnatal brainstem motoneurones are difficult to keep alive. "In general, it has been difficult to preserve electrophysiologically viable motoneurones in brain slices from adult mammals" (Aghajanian and Rasmussen, 1989). Because of these facts it was necessary to employ various techniques during the harvesting of both the whole spinal cords, and especially the spinal cord slices, to increase the number of viable motoneurones. In general the goal of the preparatory methods was to reduce excessive calcium entry and

cytotoxicity. It has previously been demonstrated that mouse spinal motoneurones have a low calcium buffering capacity compared to other cell types (Palecek et al., 1999). To this end glutamate antagonists were given prior to decapitation and were present in all pre-incubation solutions. Furthermore, the calcium levels were kept low and solutions contained high concentrations of magnesium in order to reduce calcium entry through voltage-dependent calcium channels. More specific details of these techniques can be found in the methods sections of the papers presented in this thesis.

Objectives

To date, the model most successful in elucidating the ionic mechanisms of non-linearity in spinal motoneurones has been the adult turtle in-vitro slice preparation. Using this reptilian preparation Hounsgaard and co-workers have demonstrated that the current underlying the plateau potential is mediated by L-type voltage-gated calcium channels and that these channels are located in the dendrites of these cells. Data obtained from mammalian spinal motoneurones, for the most part in the in-vivo cat, are consistent with these findings but these experiments have been unable to either confirm the identity of the plateau-generating channel type (s) or their subcellular location. Studies using other types of mammalian motoneurones have demonstrated that the ionic mechanisms used to generate plateau potentials are varied and therefore reinforce the idea that it is difficult to generalize between motoneurone types and that ionic mechanisms need to be studied in a cell type specific manner.

The specific question being addressed in this thesis is, do mammalian spinal motoneurones use the same or similar ionic mechanisms to produce plateau potentials as are used in spinal motoneurones of the turtle? More specifically, are L-type calcium

channels a part of the plateau potential mechanism in mammalian spinal motoneurones? Given the postnatal changes in the complement and density of voltage-gated calcium currents outlined in a previous section and the previous almost exclusive use of early postnatal animals by other experimenters, the subtypes of calcium channels present in functionally mature mouse spinal motoneurones were unknown. The hypothesis tested in the first paper was that mouse lumbar spinal motoneurones possessed a variety of subtypes of voltage-gated calcium channels including L-type channels. An absence of this channel subtype would have excluded the possibility that both mammalian and reptilian spinal motoneurones use a common plateau-generating mechanism. These results have been published in the European Journal of Neuroscience (2000) 12 (5):1624-1634.

The second paper tested the hypothesis that a sub-population of functional L-type channels are located in the dendrites of these motoneurones and it would be possible to record this current from the somata of these cells. The finding of L-type channels in this subcellular location would be consistent with the location of these channels in the turtle. Furthermore, numerous modeling studies have shown this location to be necessary in order to replicate firing patterns seen experimentally in spinal motoneurones. This work has been published in the European Journal of Neuroscience (2000) 12 (5):1635-1646.

The third paper is comprised of preliminary unpublished data. Given the presence of L-type calcium channels in mouse spinal motoneurones (paper 1) and the finding that a population of these channels are located in the dendrites of these cells (paper 2), it was hypothesized that under the appropriate conditions these cells would be capable of demonstrating plateau potentials in the spinal cord slice preparation. Furthermore, it was

hypothesized that the plateau potentials in these cells could be elicited by means consistent with activation of L-type calcium channels.

Paper 1

Characterization of calcium currents in functionally mature mouse spinal motoneurones

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Abstract

Motoneurones integrate synaptic input and produce output in the form of trains of action potentials such that appropriate muscle contraction occurs. Motoneuronal calcium currents play an important role in the production of this repetitive firing. Since these currents change in the postnatal period, it is necessary to study them in animals in which the motor system is "functionally mature" – that is, animals that are able to weight bear and walk. In this study, calcium currents were recorded using whole cell patch clamp techniques from large (> 20 μm) ventral horn cells in lumbar spinal cord slices prepared from mature mice. Ninety percent (9/10) of the recorded cells processed for choline acetyl-transferase were found to be cholinergic, confirming their identity as motoneurones. A small number of motoneurones was found to have currents with low voltage-activated (T-type) characteristics. Pharmacological dissection of the high voltage-activated current demonstrated ω-agatoxin-TK- (P/Q-type), ω-conotoxin GVIA- (N-type), dihydropyridine-, and FPL-64176- sensitive (L-type) components. A cadmium-sensitive component of the current that was insensitive to these chemicals (R-type) was also seen in these cells. These results indicate that the calcium current in lumbar spinal motoneurones from functionally mature mice is mediated by a number of different channel sub-types. The characterization of these calcium channels in mature mammalian motoneurones will allow for the future study of their modulation and their roles during behaviors such as locomotion.

Introduction

The importance of neuronal voltage-gated calcium channels in cellular functioning has long been recognized. These channels have been shown to mediate the calcium influx responsible for processes such as firing frequency regulation (Viana et al., 1993a; Wikström & El Manira, 1998), synaptic transmission (Meir et al., 1999), gene expression (Greenberg et al., 1986; Murphy et al., 1991), and initiation of various intracellular signaling pathways (Gosh & Greenberg, 1995). The electrophysiological effects of calcium currents have been studied in various types of motoneurones, where it has been shown that calcium influx through these channels may influence firing rates and patterns through either a direct electrogenic effect or through effects on other neuronal channels. For example, T-type currents in rat hypoglossal motoneurones have been shown to produce rebound depolarisations which can lead to firing in response to inhibitory inputs (Viana et al., 1993b). In cat spinal motoneurones, a current mediated by L-type calcium channels has been proposed to underlie both the negative slope conductance recorded under voltage clamp conditions (Lee & Heckman, 1998; Schwindt & Crill, 1980) and the voltage dependent amplification of synaptic input during locomotion (Brownstone et al., 1994). Current through these same L-type calcium channels has been shown to contribute a persistent inward current in guinea pig trigeminal motoneurones (Hsiao et al., 1998) and underlie plateau potentials in adult turtle spinal motoneurones (Hounsgaard & Kiehn, 1989). Other calcium channels may have different electrophysiological effects. For example, in hypoglossal motoneurones, influx of calcium through both N and P/Q-type calcium channels activates calcium-dependent potassium conductances which regulate firing rate. (Viana et al., 1993a).

Studies of calcium currents in mammalian spinal motoneurones have been limited to embryonic and/or early postnatal animals (Berger & Takahashi, 1990; Hivert et al., 1995; Mynlieff & Beam, 1992a,b; Scamps et al., 1998). It has been demonstrated that the various currents change in the early postnatal period (Mynlieff & Beam, 1992a). Currents in the later postnatal period have not been directly assessed. However, the amplitude of neurochemically-induced ventral root bursting activity in the isolated mouse spinal cord was sensitive to both dihydropyridines and extracellular calcium concentration only after postnatal day 7, indicating a role for L-type calcium channels in the production of this output (Jiang et al., 1999a). Furthermore, it was demonstrated immunohistochemically that both class C and class D L-type channels develop in the first three postnatal weeks (Jiang et al., 1999a), paralleling the development of motor functional maturity – i.e. the stage at which mice can weight bear and walk (postnatal day 9).

Given such post-natal development and the importance of calcium currents in the production of motor output, it has been necessary to develop a preparation where these currents can be recorded from functionally mature motoneurones, and to characterize these currents. Calcium currents recorded from motoneurone somata in spinal cord slices harvested from functionally mature animals are reported in this paper, where T-, N-, P/Q-, L-, and R-type calcium currents are demonstrated. In the companion paper, dendritic L-type calcium currents are reported (Carlin et al., 2000).

Materials and Methods

Slice Preparation

The isolation of the spinal cord is as previously described (Jiang et al., 1999a,b). Briefly, postnatal (P9 – P16) Balb/C mice were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and partially submerged in ice water for approximately 3 – 4 minutes before decapitation and evisceration. The spinal cords were then dissected free by vertebrectomy in a Sylgard-lined petri dish filled with cold (< 4°C) dissecting artificial cerebral spinal fluid (aCSF). The lumbar enlargement was isolated and introduced into a 1% agar solution and quickly cooled. The lumbar enlargement was then blocked and mounted into a Leica (VT 1000E) vibrating microtome filled with cold dissecting aCSF. Slices 150-200 μm thick were prepared and immediately placed into warm (36°C) recovery aCSF for about 45 minutes before being transferred to room temperature aCSF.

Slices were transferred to a 450 μL recording dish mounted in the stage of an upright Olympus BX50 microscope fitted with differential interference contrast optics and epifluorescence. Cells were visualized using an infra-red camera (Hamamatsu C2400-77) and black and white monitor (Dodt & Zieglgansberger, 1990). It was found that there was no need to perfuse the slices during recording but 100% oxygen was continuously blown over the chamber.

All animals were anesthetized, and the experimental procedures were approved by the University of Manitoba Animal Care Committee and conformed to the standards of the Canadian Council of Animal Care. All chemicals were obtained from Sigma (St. Louis, USA) unless otherwise specified.

Patch clamp recordings

Whole cell patch clamp recordings were made using an Axopatch 1D (Axon Instruments) patch clamp amplifier (β = 0.1 headstage) and pClamp7 software (Axon Instruments) running on a Pentium class computer. Data were digitized with a Digidata 1200A A/D converter (Axon Instruments) and stored on the hard drive. Patch pipettes were Sylgaard coated (Dow Corning) and had a resistance of 2.5 – 3.5 MΩ when filled with intracellular solution. Series resistance (typically 10-30 MΩ) was determined in current clamp mode and compensated 70-80%. The series resistance was continually monitored by the software and adjusted as required. Data were low pass filtered at 2 kHz and digitized at 10 kHz. Linear leak correction of current traces was performed by subtraction of an appropriately scaled current determined from the average response of a series of 10 mV hyperpolarising pulses. Reported potentials were corrected for a 3 mV liquid junction potential between the pipette and the recording solution in which the pipette was zeroed before obtaining a gigaohm seal. Whole cell capacitance was determined either by integration of the current transient or directly from the software program. No significant difference was found between the two methods ($p > .05$; paired t - test; $n = 18$). All experiments were performed at room temperature (~22°C). In the current traces shown in the figures, the hyperpolarising transients have been truncated and the depolarizing transients removed for clarity.

Activation curves were constructed with conductance values calculated for each cell using the equation $G = I/(V_T - V_R)$, where V_T is the test voltage, V_R is the reversal potential for that cell, and I is the measured current, and then normalizing the data to G_{max}, the maximum conductance. Inactivation curves were constructed by normalizing the current values to the largest value. Data were fit with Boltzman functions in Microcal

Origin using the equation $G/G_{max} = (A_1 - A_2)/ \{1 + \exp ((V - V_{1/2})/ k\} + A_2$, where A_1 = initial conductance, A2 = final conductance, V = test potential, $V_{1/2}$ = voltage of half (in)activation, and k = slope factor.

Solutions and chemicals

The aCSF solutions were designed to reduce calcium-induced cell damage during processing. The dissecting aCSF was sucrose based (Aghajanian & Rasmussen, 1989) and contained (in mM): NaCl (25), sucrose (188), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $NaHCO_3$ (26), glucose (25). The warm recovery aCSF contained (in mM): NaCl (119), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $CaCl_2$ (1), $NaHCO_3$ (26), glucose (10), kynurenic acid (1.5)(RBI, Natick, MA, USA; Ganong et al., 1983), lactic acid (2) (Schurr et al., 1988) and 3% dextran (Newman et al., 1995). The room temperature aCSF was the same as the recovery aCSF without the kynurenic acid and dextran.

The intracellular and extracellular recording solutions were designed to minimize both sodium and potassium currents. The pipette solution contained (in mM): Cs-methane-sulfonate (100), TEA-Cl (30), $MgCl_2$ (1), EGTA (10), HEPES (10), $CaCl_2$ (0.5), NaCl (5), ATP-Mg (3), GTP (0.3), and leupeptin (0.1) (modified from Umemiya and Berger, 1994). In some experiments 10-20 mM sucrose was added to the intracellular solution to stabilize the series resistance (Jonas et al., 1998). Lucifer yellow (~ 1%; Molecular Probes, Eugene, OR) was used in some experiments as an intracellular marker. The standard extracellular solution contained (in mM): NaCl (105), TEA-Cl (30), KCl (1.9), HEPES (10), $MgCl_2$ (2), glucose (10), 4-AP (4), CsCl (2), $CaCl_2$ (1), 1μM TTX and 0.1% bovine serum albumin (oxygenated with 100% O_2). Changes in extracellular

calcium concentration for certain experiments are noted in the text. (The solutions used for the recording of repetitive firing in current clamp are indicated in the legend of Figure 1.)

Calcium channel toxins ω-agatoxin-TK (Peptides International, Louisville, KY), ω-conotoxin-GVIA (Sigma, St. Louis, USA) and calciseptine (Alomone Labs, Israel) were dissolved in extracellular recording solution and frozen (-80°C long term and –20°C short term) in aliquots that underwent no more than four freeze-thaw cycles. Stock solution of nifedipine (20 mM; RBI), nimodipine (5-20 mM; RBI) and FPL-64176 (5.8 mM; RBI) were dissolved in absolute ethanol. Stock solutions were made fresh daily and protected from light. When added to the recording chamber the vehicle alone had no discernible effect on the calcium currents (n = 3). All antagonists were bath applied with reported concentration values being the final bath concentrations.

Immunohistochemistry

After recording was terminated, slices containing labeled cells were fixed in 4% paraformaldehyde containing 0.1% phosphate buffer over night and then placed in cryoprotectant consisting of 10% sucrose in 50 mM PB. Slices were mounted onto a cryostat chuck and re-sectioned at 7 μm then thaw mounted onto gelatin subbed glass slides. Slides were washed overnight in 0.1M phosphate buffered saline plus 0.3% triton X-100 prior to processing. All dilutions and washes were performed in PBS and incubations included 1% normal horse serum. Slides were incubated in polyclonal rabbit anti-ChAT (Chemicon, Temecula CA, USA) for 2-3 days at 4° C. They were then washed in phosphate buffered saline plus triton X-100, incubated in anti-rabbit Cy3 (Jackson

Labs, West Grove PA, USA) at room temp for 1 ½ hours and washed for 20 min in phosphate buffered saline plus triton X-100 followed by 2 x 20 min washes in 50 mM TRIS-HCL (pH 7.4). Slides were cover slipped with Vectashield (Vector Labs, Burlingame, CA, USA), then studied and digitized using a Nikon Optiphot fluorescent microscope and Neurolucida software (Microbrightfield Inc., Colchester VT, USA).

Results

Identification of motoneurones

The largest cells in the ventral horn were visually targeted as putative motoneurones (Figure 1A; Gao & Ziskind-Conhaim, 1998: Jonas et al, 1998). The somal size criteria of > 20 μm was used in this study as this criteria has been used in the past with postnatal rat lumbar motoneurones (Takahashi, 1990, Thurbon et al, 1998) and is consistent with adult mouse lumbar motoneurone measurements (McHanwell & Biscoe 1981). To further support identification of the targeted cells as motoneurones, a subset of cells were intracellularly labeled with Lucifer Yellow and processed immunohistochemically for the enzyme choline acetyl-tranferase (ChAT). Figure 1B shows the cell illustrated in 1A after recording with Lucifer Yellow in the patch pipette. The slice has been fixed and re-sectioned at 7 μm. Subsequent processing for ChAT revealed that this cell was cholinergic (Figure 1C). A total of 10 cells were examined in this way with 9 (90%) demonstrating positive ChAT immunoreactivity.

An important functional characteristic of motoneurones is their ability to fire action potentials repetitively. This was assessed in the targeted cells where step current injections produced such firing (Figure 1E; 20/20 cells with a stable resting membrane

potential). Note that the firing pattern demonstrates a reduction in spike amplitude with time (sodium channel accommodation), as well as firing rate adaptation; these phenomena are known to occur in adult mammalian spinal motoneurones (Schwindt & Crill, 1984).

Calcium currents

Calcium currents were isolated from motoneurones using a spinal cord slice preparation. Because this preparation permits the cells to retain at least a portion of their dendritic arbour, calcium currents originating in these distal membranes must be taken into consideration. Using a somatically placed electrode, such spatially segregated currents could be identified in a large number of these cells and are considered in the companion paper (Carlin et al.,2000). For the purposes of the present paper, only cells which did not demonstrate spatially segregated calcium currents in the stated recording solutions were studied. The calcium currents originating in the somatic membrane could then be characterized. It is recognized that the absence of visible dendritic current does not guarantee that the distal conductances are not affecting the kinetics of the somatic current (see Discussion).

Motoneuronal calcium currents were isolated by using intracellular and extracellular recording solutions that minimized voltage-gated sodium and potassium currents. In this environment, large inward voltage dependent currents could be recorded in response to step voltage commands. From a holding potential (Vh) of – 60 mV cells within this postnatal period (postnatal day (P)9- P16) typically displayed the persistent graded currents illustrated in figure 2A. The current-voltage (I-V) relationship for this cell

recorded in 1 mM calcium is illustrated in figure 2B. The current was seen to activate at about – 40 mV, peak typically between –10 and 0 mV and reverse at about 40 mV.

To confirm that this inward current was indeed mediated by voltage dependent calcium channels, and that the current was not contaminated by other calcium-dependent currents, the current was examined: in different extracellular calcium concentrations; for sensitivity to the non-specific calcium channel blocker cadmium; and by substituting barium for calcium. Figure 2C shows that the addition of 0.5 mM calcium to the extracellular recording solution produced a large increase in the recorded current at potentials above –30 mV. Addition of 200 μM cadmium blocked all inward currents (n = 8). Reducing the extracellular calcium concentration to 0.1 mM also produced a reduction in the current (n = 2; data not shown).

The divalent cation barium is often used when recording currents through calcium channels because of the increased permeability of this ion through HVA channels (Carbone & Lux, 1987) and its inability to activate calcium-dependent processes (Branchaw et al., 1997) and conductances (eg. $I_{K(Ca)}$; Morriset & Nagy, 1999). Figure 2D demonstrates that barium can substitute for calcium as the charge carrier for this inward current. In the cell illustrated here, currents were recorded first in a 3 mM barium solution. The solution was then switched to one containing equimolar calcium, and the same protocol repeated. It can be seen on this plot normalized to peak current amplitude, that both ions produced qualitatively similar current-voltage curves. The peak barium current was approximately double the peak calcium current, not taking run-down into account. Consistent with the effects of barium in other systems, the peak and the reversal potential of the I-V relationship were shifted in the hyperpolarising direction (Bean,

1989; Branchaw et al., 1997; Mills & Pitman, 1997; Mynlieff & Beam 1992b). These reversible effects can be explained by the reduced effectiveness of barium to screen charge on the cell membrane (Byerly & Hagiwara, 1982). Also consistent with currents recorded in other systems, currents recorded in barium consistently displayed less inactivation than those recorded in calcium. In fact, the currents recorded in barium often continued to increase during this relatively short voltage step (Figure 2D, inset).. In other systems this has been generally considered to result from the limited ability of barium, compared to calcium, to produce calcium channel inactivation (Hille, 1992).

The voltage dependency of this current, its sensitivity to extracellular calcium and cadmium and its ability to use both calcium and barium as a charge carrier are all consistent with this current being mediated by voltage dependent calcium channels. In the remaining experiments calcium was used as the charge carrier in low to normal physiologic concentrations (1 – 3 mM).

Properties of the total calcium current

The activation and steady-state inactivation kinetics of the total current are illustrated in figure 2E. Activation of the total current demonstrated a strong voltage dependence between about –40 mV and 0 mV. When fitted with a Boltzman function the resulting values were: $V_{1/2} = -15.8$ mV with $k = 4.7$. Using a one second pre-pulse protocol and a test voltage of 0 mV, the inactivation characteristics of the total current were also examined. As can be seen by the peak current inactivation curve, this current showed complex inactivation kinetics that demonstrated somewhat linear voltage dependence.

This likely results from the contribution of a number of different channel types with different inactivation kinetics (Randall & Tsien, 1995), and is further explored below.

Tonic inhibition of calcium currents has been demonstrated in a number of preparations including chromaffin cells (Hoshi et al., 1984), myocytes (Zygmunt & Maylie, 1990), neuroblastoma cells (Kasai, 1991), sympathetic neurones (Ikeda, 1991; Zhu & Yakel, 1997) and thalamic neurones (Kammermeier & Jones, 1998). This inhibition can be relieved with strong depolarization. Motoneuronal calcium currents in this preparation were also tested for the ability of strong (+100 mV) depolarizing voltage steps to facilitate the total calcium current. In these experiments a standard pre-pulse protocol was employed from a holding potential of –60 mV. Currents were elicited with voltage steps to 0 mV before and 20 ms after a +100 mV depolarization. Figure 2F demonstrates that the pre-pulse failed to produce an enhancement of the current. This same result was obtained in 13/13 cells. As can be seen in figure 2F, the amplitude of the current elicited by the second voltage step was similar to the amplitude of the current at the end of the control voltage step, suggesting that the only difference between the two currents is due to inactivation of the current.

LVA and HVA components of the total current

By varying the holding potential it was demonstrated that some motoneurones in this age range have not only high voltage-activated (HVA) but also a transient low voltage-activated (LVA) calcium current. However, LVA currents were not seen in most motoneurones; alterations in holding potential between –60 and –80 mV changed the amplitude but not the activation voltage (Figure 3A) of the currents. In the remaining

cells, a transient current was seen at low voltages. By using the two step protocol illustrated in figure 3B, this current could be separated from the HVA currents. The LVA current was seen to activate slowly and inactivate rapidly compared to currents activated at more depolarized potentials. This current could be enhanced by a hyperpolarised holding potential (Figure 3C). The presence of this type of low voltage-activated current (operationally defined as a transient current seen at or below –40 mV in 1 or 2 mM extracellular calcium) was seen in 14/87 motoneurones (16 %) with Vh = -60 mV and 4/11 motoneurones (36 %) with a Vh ≤ -70 mV. The LVA current showed sensitivity to low concentrations (100 μM) of nickel (n = 1; data not shown). The voltage of activation, sensitivity to holding potential, slow activation and rapid inactivation kinetics of this current are consistent with the T-type calcium current described in many central neurones (Bean, 1989) including spinal motoneurones (Berger & Takahashi, 1990; McCobb et al., 1989; Mynlieff & Beam, 1992a,b).

The steady-state inactivation of the total calcium currents was explored by separating the total current into transient and sustained components. The sustained component was defined as the current remaining at the end of a 150 ms voltage step while the difference current between the sustained and the peak current amplitude was defined as the transient current (Mynlieff & Beam, 1992b). Using a one second pre-pulse and a test potential of 0 mV, it can be seen that the transient and sustained components inactivated with different kinetics (Figure 3D). While the sustained component of the current demonstrated slight, linear inactivation, the transient component of the current inactivated with steep voltage dependence between –80 and –50 mV. These data could be fit with a Boltzman function (see methods), with $V_{1/2}$ = –66 mV, k = 7, and A_2 = 0.3. The incomplete inactivation of

the transient component would suggest the presence of a voltage-independent inactivation process in these cells (Mynlieff & Beam, 1992b). The half-inactivation voltage of –66 mV is comparable, when measured at 0 mV in low extracellular calcium or barium, to that of N-type channels described in chick DRG cells ($V_{1/2}$ = –64 mV; Kiss & Korn, 1999), R-type channels in both rat cerebellar granule cells ($V_{1/2}$ = –61 mV; Zhang et al., 1993) and amygdalar cells ($V_{1/2}$ = –58 mV; Yu & Shinnick-Gallagher, 1997), and a non-L, non-N, non-P current in hypoglossal motoneurones ($V_{1/2}$ = -62 mV; Umemiya & Berger, 1994). The different steady-state inactivation rates of current components elicited at 0 mV suggest that, as demonstrated in other central neurones as well as in mammalian embryonic motoneurones (Hivert et al., 1995; Mynlieff & Beam, 1992b; Scamps et al., 1998) the current elicited at this potential is mediated by more than one channel sub-type.

Pharmacology of the HVA current

Sensitivity to specific calcium channel blockers is a defining characteristic of the various HVA calcium channel subtypes. N-type calcium channels are selectively blocked by the snail toxin ω-conotoxin-GVIA (Feldman et al., 1987) while P- and Q-type channels are blocked by the spider venom extracts ω-agatoxin-IVA (Mintz et al., 1992) and ω-agatoxin-TK (Teramoto et al., 1995). Sensitivity to dihydropyridines is the hallmark of L-type channels. Since the L-current constitutes only a small percentage of the total current in a number of cell types (Jones & Jacobs, 1990; Mynleiff & Beam, 1992b; Gao & Ziskind-Conhaim,1998), L-channel activators such as Bay K-8644 and FPL-64176 are often used to demonstrate the presence of L-type channels.

To determine whether N, P/Q and L-type channels contribute to the total current in these cells, experiments were undertaken to determine concentrations of the various blockers that would produce a rapid and complete block of the channel subtypes. ω-agatoxin-TK produced an obvious reduction of the total current in 10/11 cells tested. Application of 200 nM agatoxin produced a relatively slow block (Fisher & Bourque, 1995) of a portion of the current (Figure 4A). An additional 200 nM of the toxin produced no further block, indicating that 200 nM was already a saturating concentration (3/3 cells). In this cell the ω-agatoxin blocked both a transient and a sustained component of this current (Figure 4A, inset). To ensure a complete block of P/Q-type channels, and to increase the speed of the block (Randall & Tsien, 1995), a concentration of 400 nM was used in the remaining experiments.

Similar experiments were performed to determine the saturating concentration of ω-conotoxin-GVIA. Application of ω-conotoxin-GVIA was effective in producing an obvious reduction of the total current in 6/7 cells tested. To determine the saturating concentration of this toxin, 3 μM was added repeatedly to the bath. In the cell illustrated in Figure 4B, addition of 3 μM ω-conotoxin-GVIA produced a rapid block of a portion of the total current. Application of additional conotoxin had no additional effect while subsequent application of agatoxin was seen to block a second component of the current. The current blocked by the ω-conotoxin was only slowly inactivating (Figure 4B, inset). In 2/3 cells tested, 3 μM produced a saturating block, but in the remaining cell, a concentration of 6 μM ω-conotoxin-GVIA was necessary for saturation. In the remaining experiments, 6 μM conotoxin was used to ensure a complete block of N-type channels.

The effectiveness of dihydropyridine blockers was also tested in this preparation, usually following co-application of saturating concentration of conotoxin and agatoxin (Figure 4C). Both nifedipine and nimodipine were effective in producing a partial block of the remaining current (nifedipine (20μM) - 6/7 cells; nimodipine (5-20 μM) – 8/9 cells). As illustrated in Figure 4C, application of 20 μM nifedipine reduced the calcium current significantly; an additional 20 μM of this blocker did not produce any further block, indicating that 20 μM nifedipine produced a saturating block of L-type channels (see below). The nifedipine-sensitive component was relatively non-inactivating (Figure 4C, inset). The cadmium-sensitive current remaining after addition of agatoxin, conotoxin and nifedipine likely corresponds to the R-type calcium current described in cerebellar cells (Randall & Tsien, 1995).

The presence of the various calcium channel sub-types was also tested in these cells by sequential applications of saturating concentrations of the various blockers in order to demonstrate non-overlapping components of the HVA current. Figure 4D demonstrates that a portion of the total HVA current is mediated by P/Q-type (agatoxin-sensitive), N-type (conotoxin-sensitive), and L-type (dihydropyridine-sensitive) channels. A resistant (R-type) component can be seen following application of these blockers, which is subsequently blocked by application of cadmium.

L-type channels

The presence of L-type calcium channels is most often identified due to their sensitivity to dihydropyridine blockers. Unfortunately, these compounds have been shown to produce non-specific effects in some preparations (Akaike et al., 1989; Jones &

Jacobs, 1990), most often when used in high concentrations or when used on embryonic cells (Diochot et al., 1995; Mynlienff & Beam 1992b; White et al., 1997). Because of these concerns, the pharmacology of motoneuronal L-type calcium channels was explored further in this preparation using the non-dihydropyridine L-channel activator FPL-64176 (Rampe & Lacerda, 1991), and the L-channel toxin calciseptine (De Weille et al., 1991).

Calciseptine was effective in producing a partial reduction of the current in mouse spinal motoneurones (n = 2 at P4; n = 1 at P10). In figure 5A, after blocking N and P/Q-type components with saturating concentrations of both agatoxin and conotoxin (a – b), addition of 3.3 μM calciseptine produced a small reduction in the calcium current in this cell (b – c). The raw traces reveal that the current component blocked by this toxin is non-inactivating, consistent with the known kinetics of L-channel mediated current. With the addition of the L-channel activator FPL-64176 the remaining current amplitude increased significantly (c – d), indicating that this concentration of calciseptine was not saturating. The saturating concentration of calciseptine in this preparation was not determined but is greater than 5 μM, a finding similar to that found in CA3 pyramidal cells (Avery & Johnston, 1996).

FPL-64176 increases the amplitude of L-channel mediated current by increasing both the probability of opening and the single channel opening time, producing longer channel openings at more hyperpolarised potentials (Kunze & Rampe 1992; Rampe & Lacerda, 1991). The effect of FPL-64176 on the current-voltage relationship of a P10 motoneurone is illustrated in figure 5B. Consistent with its effects in other systems, FPL-64176 produced an increase in the current amplitudes and a hyperpolarising shift in the I-V

relation. Also consistent with known effects of FPL-64176 on L-channel kinetics, the FPL-64176-enhanced currents demonstrated slowed activation (figure 5Binset), and prolonged tail currents (Zheng et al., 1991). These results are all consistent with FPL-64176 enhancing current through L-type calcium channels. Similar effects were seen in 6/7 cells. In agreement with L-type calcium channels being the site of action of FPL-64176, its effects were blocked by prior administration of dihydropyridines (Figure **5C**). A small reduction in the normalized current was seen after the application of the agonist FPL-64176. A similar small reduction in the peak current was demonstrated previously in intracardiac neurones (Jeong & Wurster, 1997) and most likely represents a functional interaction between the dihydropyridine and the FPL 64176 binding sites (Lauven et al., 1999; Usowicz et al., 1995).

Discussion

It has been demonstrated in this paper that motoneurones from functionally mature mouse spinal cords have a number of calcium currents components that can be separated on the basis of current kinetics in the case of the LVA current and sensitivity to pharmacological agents in the case of the HVA currents. Based on these criteria we have identified T, N, P/Q, L, and R-type voltage-activated calcium currents in these cells. Studying these currents in this developmental stage (≥ postnatal day 9) is of particular interest because mice are able to bear weight and walk at this age and therefore can be considered to be "motor functionally mature". L-type calcium channels have previously been shown to develop in the first 3 postnatal weeks using immunohistochemical techniques (Jiang et al., 1999a). Furthermore, a motor rhythm generated by the bath

application of drugs to the in vitro mouse spinal cord has been shown to be nifedipine-sensitive only after postnatal day 7 (Jiang et al., 1999a), indicating that the anatomically-demonstrated development of the L-type channels is of physiological relevance.

Motoneurones in the spinal cord slice

The slice preparation was used for this study because the relative positions of the cells in the spinal cord are maintained as is the natural architecture of at least a portion of the dendritic tree and the surrounding tissue (superstructure). These facts enable visual identification of motoneurones in spinal cord slices by their location and large diameter (Burke et al., 1988; Takahashi, 1990). However, the presence of large interneurones (>20 μm) has recently been demonstrated in the ventral half of the rat lumbar spinal cord (Thurbon et al., 1998). In the present study, using the smaller mouse preparation, putative motoneurones were identified as the largest cells in the ventral horn of the spinal cord (> 20 μm somal diameter). To verify that these were motoneurones, immunohistochemistry for choline acetyl-transferase was carried out, and demonstrated that 9/10 of these cells were positive. The one ChAT negative cell in the subset studied was likely the result of dilatation of this cytosolic protein during patch clamp recording, as it was noted that the longer the cell was patched, the weaker the immunolabeling (note that the patched cell in figure 1B is not the most intensely stained ChAT positive cell). Given these results and the size difference between the mouse and rat, our procedure for visually identifying motoneurones in the ventral horn of the mouse spinal cord would seem to be valid.

Motoneurones are difficult to keep alive in a slice preparation (Aghajanian & Rasmussen, 1989; Somjen & Czeh, 1989). It has been suggested that the truncation of

dendrites in these large cells during the slicing procedure is responsible for their diminished survival rate. Therefore, the task becomes more problematic when using a slice thin enough to permit both rapid drug effects and visual patch clamping of targeted cell populations. Some success has been obtained in very early postnatal rats (P1-P5; Berger & Takahashi, 1990; Takahashi, 1990; Takahashi & Berger, 1990) and more recently in rats up to postnatal day P15 (Thurbon et al., 1998). By taking the precautions outlined to minimize cell death in the dissection, and by the using of a high quality vibrating microtome (Parsley et al., 1998), we are able to consistently obtain whole cell patch clamp data from these mature mouse motoneurones.

The extended structure of cells in slice

The use of a slice preparation, as opposed to the use of dissociated cells, allows the evaluation of channel currents while circumventing the concerns of altering the membrane channels of interest with the use of proteases. This method also mitigates the possible changes in channel expression with exposure to certain culture environments (Jacob et al., 1993; Murrell & Tolkovsky, 1993). It is felt that this method allows a more natural setting for the evaluation of cell specific channel functioning. A consequence of this preparation is that cells are allowed to retain a portion of their dendritic tree. The presence of this extended structure has to be considered under voltage clamp conditions as both distal conductances and the charging of distal membranes can alter the kinetics of currents recorded at the soma (Muller & Lux, 1993). For example, during step depolarisations, the presence of calcium channels in the unclamped dendrites would have the effect of adding extra current to the soma (late-onset currents; Carlin et al., 2000). In

addition, the charging of distal membranes would draw charge away from the recording pipette and produce an apparent inactivation of the evoked current, particularly with large depolarisations. This apparent inactivation may contribute to the voltage independent inactivation recorded under steady-state conditions. Furthermore, the presence of distal calcium conductances may also account for the almost linear steady-state inactivation kinetics of the sustained current component in these cells (Figure 3D). Because of effects such as these, caution must be taken when interpreting current kinetics and quantification data from these cells.

Low voltage-activated channels

A small number of cells in this study displayed transient, low voltage-activated currents consistent with those mediated by T-type calcium channels. In a number of motoneurone preparations the incidence of T-type current and current density mediated by these channels decreases with age (McCobb et al., 1989; Mynleiff & Beam, 1992a; Umemiya & Berger, 1994). The relatively small number of motoneurones with this current is consistent with previous reports (Mynlieff & Beam, 1992a; Scamps et al., 1998). The presence of this current in motoneurones of this postnatal age is also consistent with a recent report of the presence, in adult rat spinal motoneurones, of both α1G and α1H mRNA, two of the α1 subunits encoding T-type channels (Talley et al., 1999). It has been postulated that in hypoglossal motoneurones the postnatal reduction in this current results from a the dendritic localization of the underlying channels coupled to a developmental change in the passive properties of the cells (Viana et al., 1993b). At

present the subcellular localization of these channels in mature mouse motoneurones has not been demonstrated.

High voltage-activated channels

Traditionally, L-, N-, P-, and Q-type channels are classified as high voltage activated channels. They are separated based on their sensitivity to dihydropyridines (L), ω-conotoxin (N), and the ω-agatoxins (P/Q). In this study, mature mouse motoneurones are shown to have each of these components, as well as a toxin resistant (R) component. This finding is consistent with immunohistochemical data where classes A, B, C, and D α1 subunits were detected on spinal motoneurones in the adult mouse (Jiang et al, 1999a; unpublished results), and these plus class E in the adult rat (Westenbroek et al., 1998). The proportion of currents mediated by the various channel subtypes varied greatly from cell to cell. Given the above consideration on the extended structure of these cells, quantification of the various types of currents was not undertaken. However, an attempt was made to gain an understanding of the kinetics of the individual current components by subtracting out the current blocked by the various antagonists (Figure 4A-C insets). In the present study the ω-conotoxin-sensitive current was slowly inactivating while the ω-agatoxin-sensitive current had both rapidly and slowly inactivating components. Although N-type currents are often considered to be rapidly inactivating, slowly-inactivating N-type currents have been reported in a variety of neurones, including lamprey (El Manira & Bussières, 1997) and postnatal rat hypoglossal (Umemiya & Berger, 1994) motoneurones. Although the inactivation kinetics seen in this study may result in part from the extended structure of these cells (see above), they may also result

from the specific subunit composition of these channels. For example, it has been shown that alternative splicing of the α1A subunit gene can produce channels with dramatically different inactivation kinetics (Bourinet et al., 1999; Zhang et al., 1993). Furthermore, there may be cell specific expression (and possibly developmentally controlled expression) of the calcium channel accessory subunits (β or α_2-δ) which modify the channel kinetics. At present, the molecular diversity of calcium channel subunits in mammalian spinal motoneurones has not been fully explored.

One fact that is clear is that in the absence of channel activators, the current recorded at the soma of these cells has a smaller L than non-L component. This is consistent with results obtained in P3 – P6 rat hypoglossal motoneurones (Umemiya & Berger, 1994), E15 – P3 rat spinal motoneurones (Gao & Ziskind-Conhiam, 1998) and embryonic mouse motoneurones (Mynlieff & Beam, 1992b). It is also clear that this current can be greatly enhanced by the addition of channel activators. Whether the relatively small L-type current recorded at the soma results from these channels being in a reluctant state (Bean, 1989; Delgado-Lazama & Hounsgaard, 1999), or from a dendritic localization of these channels cannot be determined in the present study – in both cases, channel activators would have the demonstrated effects. This issue is addressed further in the companion paper (Carlin et al, 2000).

Concluding remarks

Motoneurones from mice of a sufficient developmental stage to weight bear and walk have a complement of voltage-activated calcium channels including T-, N-, P/Q-, L-, and R-types. In other central neurones, these channels have been shown to be important

in mediating firing frequency regulation (Hernandez-Lopez et al., 1997; Viana et al., 1993a; Wikstrom & El Manira, 1998). Motoneurones must fire with sufficient frequency to produce appropriate muscle contraction. During fictive locomotion in the cat, the regulation of this firing rate is modulated, with changes in the post-spike afterhyperpolarisation (Brownstone et al., 1992), voltage threshold (Krawitz et al., 1996), and late adaptation (Krawitz & Brownstone, 1994). Furthermore, there is a voltage-dependent increase in the excitation of motoneurones from spinal rhythmogenic centres (Brownstone et al., 1994). It is plausible that voltage-activated calcium channels and their modulation may play a role in these processes (eg. Jiang et al., 1999a). The characterization of these calcium channels in mature mammalian motoneurones will allow for the future study of their modulation and their roles during behaviors such as locomotion.

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Abbreviations

aCSF – artificial cerebral spinal fluid

ChAT – choline acetyl-transferase

HVA – high voltage-activated

I-V – current-voltage

LVA – low voltage-activated

P – postnatal day

Vh - holding potential

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Figure Legends

Figure 1: The recorded cells have characteristics of spinal motoneurones. **A:** A digital infrared differential interference contrast image of a large ventral horn P14 cell. **B:** Fluorescence image of cell pictured in (A) after whole cell recording with Lucifer Yellow in the patch electrode. Electrode can be seen in the bottom left of the picture. **C:** Lucifer Yellow filled cell pictured in (B) after the slice was fixed and re-sectioned at 7μm. **D:** Immunohistochemical processing reveals that this cell is ChAT positive . **E:** Current injection elicits repetitive firing from a P9 motoneurone. Recording was made using a potassium gluconate based intracellular solution and a sodium chloride based artificial cerebral spinal fluid (aCSF). (Intracellular solution (in mM): K-gluconate (135), Hepes (10), EGTA (10), $CaCl_2$ (1), NaCl (6), Mg Cl_2 (1), ATP (3), GTP (0.3), leupeptin (0.1), glutathione (5), sucrose (20). Extracellular solution (in mM) NaCl (140), KCl (1.9), Hepes (10), $MgCl_2$ (2), glucose (10) $CaCl_2$ (1).)

Figure 2: Total calcium currents in motoneurones. **A:** Inward current elicited from a P14 motoneurone under voltage clamp conditions. The cell was depolarised with 5 mV incremental voltage commands from a holding potential of –60 mV. The current responses to voltage commands up to –5 mV are illustrated. **B:** The peak current-voltage (I-V) relationship from the cell illustrated in (A). **C:** Peak I-V curve from a P9 motoneurone recorded in either 1 (●) or 1.5 mM (o) calcium. Addition of cadmium to the extracellular solution blocks all inward currents (■). Inset shows raw traces recorded at 0 mV in each of the three conditions. **D:** Normalised peak current-voltage relationships in a

P10 motoneurone, recorded in 3 mM barium solution (o) and again after changing to a 3 mM calcium solution (●). Inset shows overlaid currents recorded at 0mV in both solutions. Recordings in barium and calcium were made ~36 minutes apart. **E:** Peak activation and steady-state inactivation curves for the total current. Each point on the activation curve was obtained from averaged data using n = 4 or n = 5 cells. The inactivation curve was constructed using a test potential of 0 mV following 1 second pre-pulse potentials. Each point is from averaged data (n = 4). Error bars are ± standard errors. **F.** Strong depolarizations fail to facilitate the total calcium current. Overlaid current traces from –60 to 0 mV steps (a) before and (b) after a 75 ms depolarization to +100 mV, as illustrated by the waveform in the lower trace.

Figure 3. Some motoneurones have LVA in addition to HVA components of the total calcium current. **A:** I-V curves recorded from a P10 cell (same cell as illustrated in figure 2D) using holding potentials of –60 mV (●) and –80 mV (o) in 3 mM calcium. Current is seen to activate at a similar potential with the different holding potentials suggesting that this cell does not have LVA currents. It can also be seen that at all potentials positive to –30 mV the peak currents are much larger with the more hyperpolarised holding potential, suggesting that some component of the HVA current has been enhanced. Inset shows overlaid traces recorded at –5 mV in both conditions illustrating this enhancement.. **B:** The LVA current is shown in this P10 motoneurone by depolarising from –80 to –50 mV. Subsequent depolarisation to 0 mV reveals a HVA current. Note the difference in the rate of activation and inactivation of the two components. **C:** Current traces elicited in another P10 cell in response to 10 mV x 150 ms voltage steps. The cell was held at either –60 mV

(i) or –80 mV (ii). Current steps from both holding potentials to –40 mV are overlaid in (iii). The overlay illustrates the presence of a low voltage activated transient current that is enhanced by the more hyperpolarised holding potential. **D:** Steady-state inactivation curves constructed using either the sustained (current remaining at the end of a 150 ms test pulse) or transient (difference between peak and sustained currents) components of the current. A one second pre-pulse potential was maintained before the 150 ms test pulse to 0mV. Sustained component of the current is seen to inactivate in a near linear manner (o), while the transient component inactivates with steep voltage-dependence (●). Transient inactivation curve was fit with a Boltzman function (dotted line) with $V_{1/2}$ = – 66 mV, k = 7, and A_2 = 0.3. Data is averaged from 4 cells (error bars = ± SE).

Figure 4: There are P/Q-, N-, L-, and R-type components to the HVA current. **A:** A component of the total calcium current is mediated by agatoxin-TK-sensitive P/Q-type channels. Application of 200 nM of the toxin to this P9 cell produced a partial block of the current. A second application of the toxin produced no further reduction in the current, suggesting that 200 nM is a saturating concentration. Time plot was constructed by plotting the peak current elicited by depolarising the cell to 0 mV from a holding potential of –60 mV. The current amplitude is plotted every 40 seconds. Inset shows difference current obtained by subtracting traces indicated by points a and b. A transient component of the agatoxin-sensitive current was seen in a number of cells. **B:** Time plot demonstrating the effects of ω-conotoxin-GVIA in a P13 cell. A single application of 3 μM conotoxin blocked a portion of the current. A second application had no effect on the current while application of agatoxin was able to block an additional component of the

current. Current in response to voltage steps to 0 mV is plotted every 20 seconds. Conotoxin-sensitive difference current illustrated in inset. **C:** Application of saturating concentrations of agatoxin and conotoxin blocked a large portion of the current in this P15 cell. Application of 20 μM nifedipine blocked a further component, but a second application of nifedipine resulted in no further reduction in the current. Cadmium (200 μM) blocked the remaining current. Nifedipine-sensitive difference current shown in inset. Current amplitude recorded every 20 seconds. **D:** Fast ramp I-V curves (–60 mV to +60 mV; 0.8 mV/ms) illustrating the various current components in a P10 cell by sequential application of channel blockers. Peak current amplitude was monitored and blockers only applied once the blocking effect plateaued from the preceding application. (agatoxin = 400 nM; conotoxin = 6 μM; nimodipine = 20 μM; cadmium = 400μM).

Figure 5: L-type calcium currents in motoneurones. **A:** Time plot (peak current plotted every 20 sec) demonstrating that, following co-application of agatoxin and conotoxin to block the current mediated by N- and P/Q-type channels (a – b), a non-saturating concentration of the L-channel toxin calciseptine (3.3 μM) produced a small reduction in the current amplitude (b – c). The L-channel activator FPL-64176 (11.6 μM) enhanced the remaining current (c – d) in this P10 cell. Raw traces are shown below the time plot. Note the enhanced tail current after FPL-64176 application (*). **B:** The effects of the L-channel activator FPL-64176 on the I-V relationship of a P10 cell. The activator increased peak current amplitude at all potentials and resulted in hyperpolarising shift in the curve. Raw traces recorded at –30 mV are shown in the inset. Note the expected slowed activation of the current and the enhanced tail current. Recordings made in 1 mM

calcium. **C:** The effects of FPL-64176 could be blocked by the dihydropyridine, nifedipine (20 μM). After blocking the N- and P/Q- components of the current, nifedipine reduced the peak current, indicating the presence of L-type channels in this P10 cell. Subsequent addition of FPL-64176 was unable to enhance the current indicating that 1) FPL-64176 and the dihydropyridine are affecting the same population of channels and 2) 20 μM nifedipine is a saturating concentration in this preparation.

Figure 1

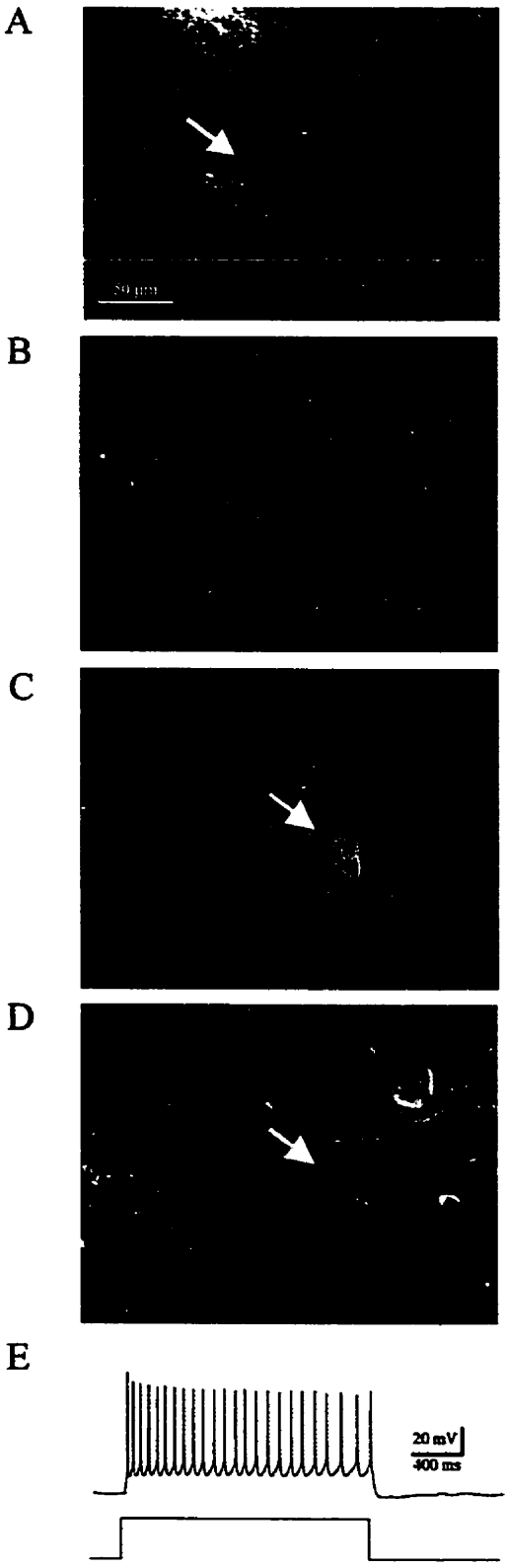
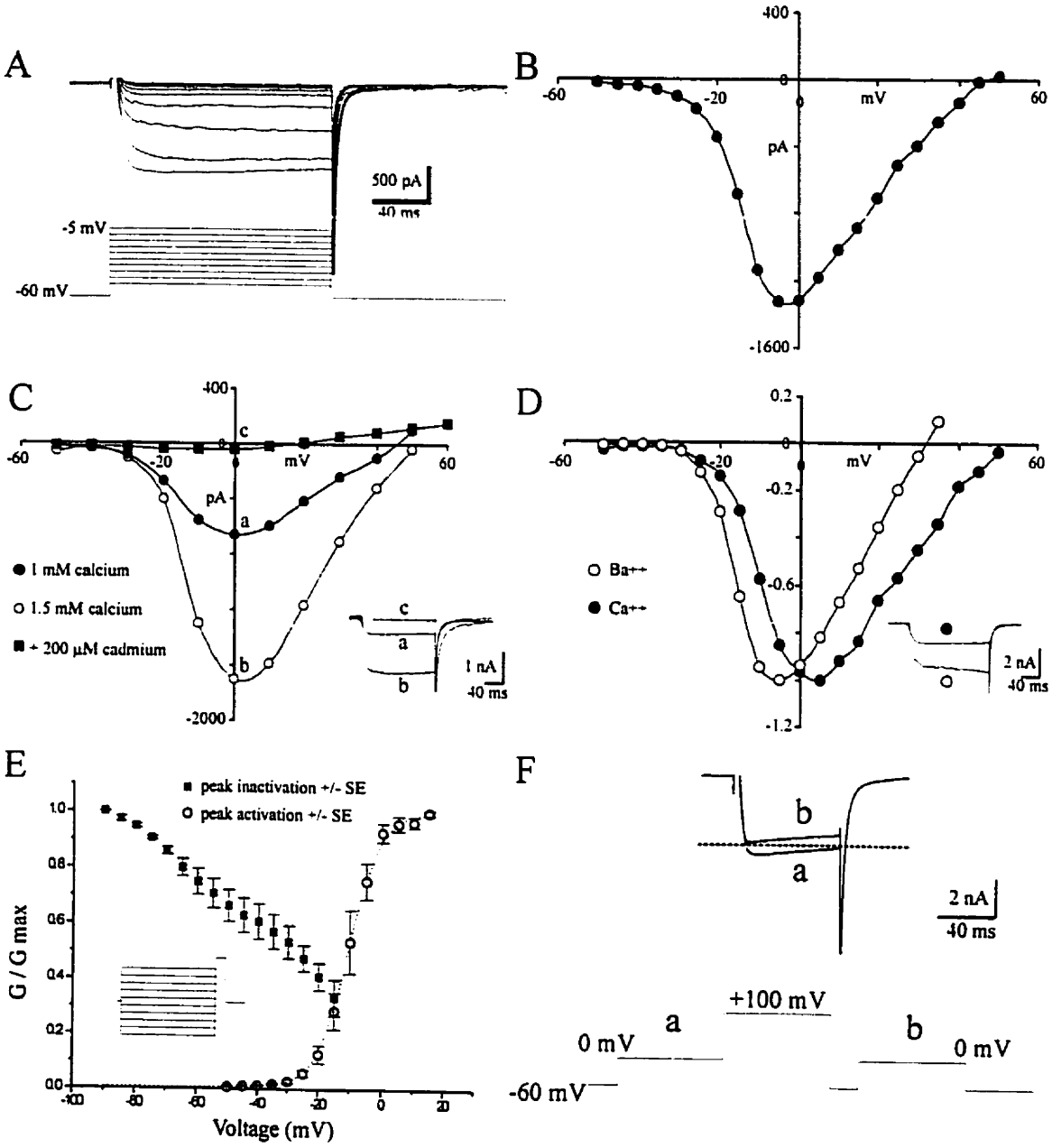


Figure 2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500 pA
40 ms
-5 mV
-60 mV
B
400
-60
-20
mV
60
pA
-1600
C
400
c
-60
-20
mV
60
pA
a
b
-2000
1 mM calcium
1.5 mM calcium
+ 200 µM cadmium
c
a
b
1 nA
40 ms
D
0.2
-60
-20
mV
60
-0.2
-0.6
-1.2
Ba++
Ca++
2 nA
40 ms
E
peak inactivation +/- SE
peak activation +/- SE
G / G max
1.0
0.8
0.6
0.4
0.2
0.0
-100
-80
-60
-40
-20
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20
Voltage (mV)
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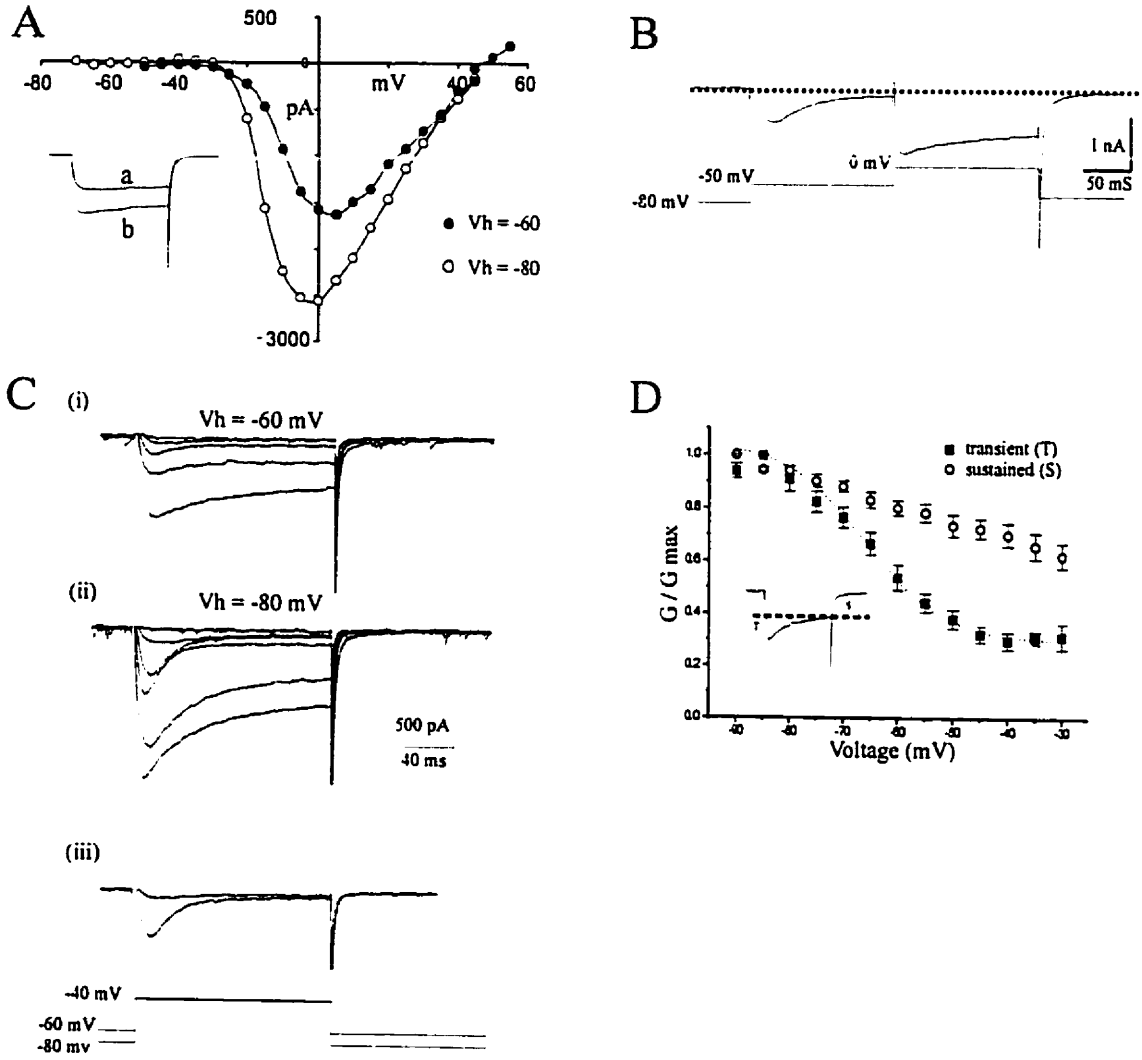
2 nA
40 ms
+100 mV
0 mV
a
b
0 mV
-60 mV

Figure 3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1 nA
50 mS
-50 mV
-80 mV
C
(i)
Vh = -60 mV
(ii)
Vh = -80 mV
500 pA
40 ms
(iii)
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-60 mV
-80 mv
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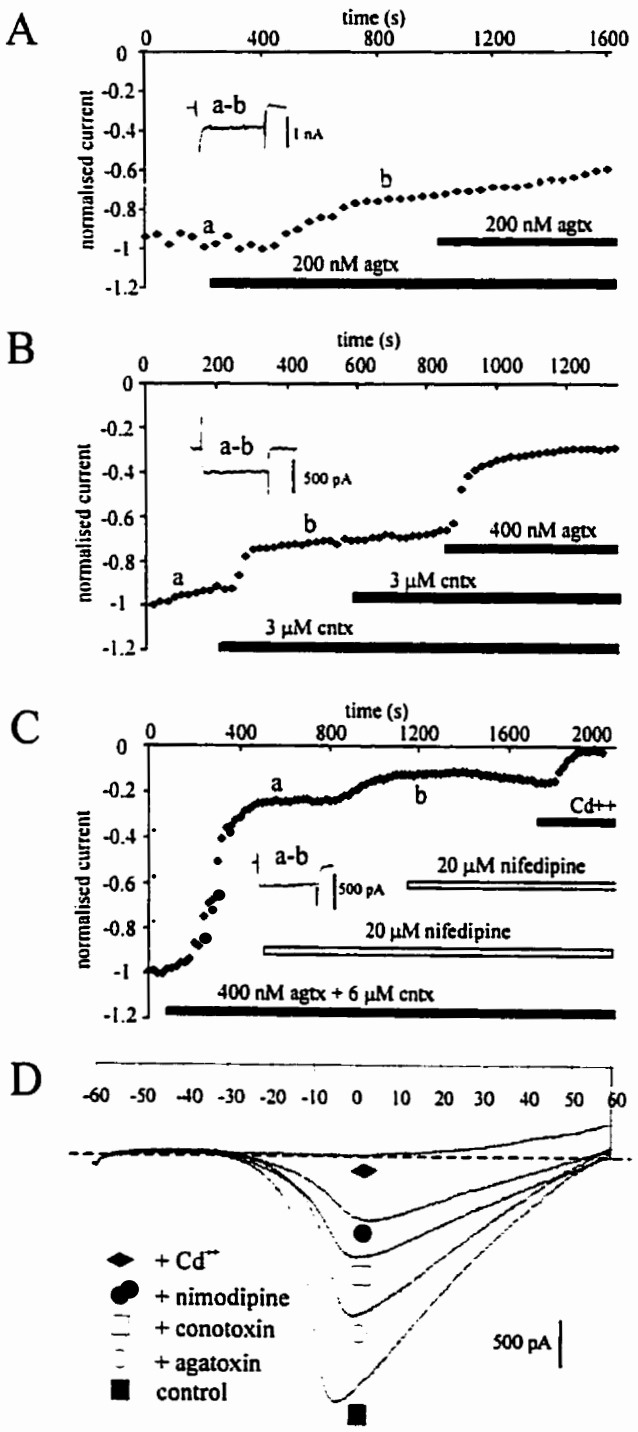
transient (T)
sustained (S)
G / G max
Voltage (mV)

Figure 4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1200
1600
normalised current
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-0.2
-0.4
-0.6
-0.8
-1
-1.2
a-b
1 nA
a
b
200 nM agtx
200 nM agtx
B
time (s)
0
200
400
600
800
1000
1200
normalised current
a-b
500 pA
a
b
400 nM agtx
3 μM cntx
3 μM cntx
C
time (s)
0
400
800
1200
1600
2000
normalised current
a
b
Cd++
a-b
500 pA
20 μM nifedipine
20 μM nifedipine
400 nM agtx + 6 μM cntx
D
-60
-50
-40
-30
-20
-10
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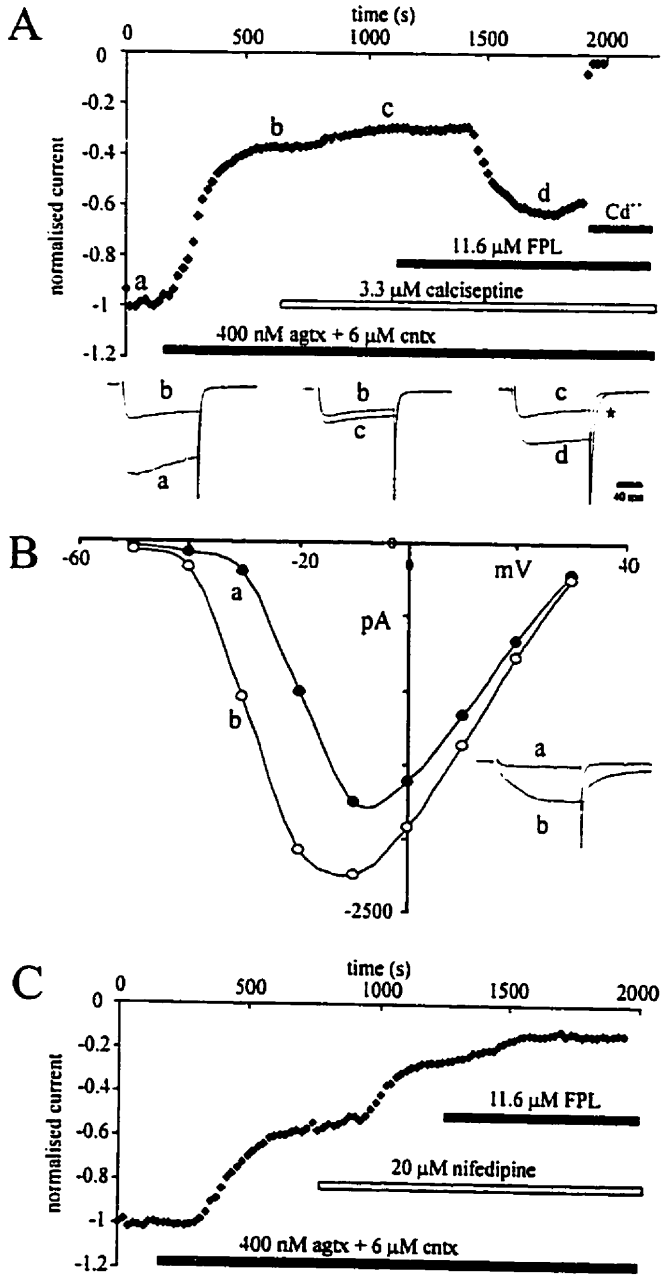
+ Cd++
+ nimodipine
+ conotoxin
+ agatoxin
control
500 pA

Figure 5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-1.2
a
b
c
d
Cd++
11.6 μM FPL
3.3 μM calciseptine
400 nM agtx + 6 μM cntx
B
-60
-20
mV
40
pA
-2500
C
time (s)
normalised current
11.6 μM FPL
20 μM nifedipine
400 nM agtx + 6 μM cntx

Paper 2

Dendritic L-type calcium currents in mouse spinal motoneurones: implications for bistability

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Abstract

The intrinsic properties of mammalian spinal motoneurones provide them with the capability to produce high rates of sustained firing in response to transient inputs (bistability). Even though it has been suggested that a persistent dendritic calcium current is responsible for the depolarizing drive underlying this firing property, such a current has not been demonstrated in these cells. In this study, calcium currents are recorded from functionally mature mouse spinal motoneurones using somatic whole cell patch clamp techniques. Under these conditions a component of the current demonstrated kinetics consistent with a current originating at a site spatially segregated from the soma. In response to step commands this component was seen as a late-onset, low amplitude persistent current while in response to depolarizing-depolarizing ramp commands a low voltage clockwise current hysteresis was recorded. Simulations using a neuromorphic motoneurone model could reproduce these currents only if a non-inactivating calcium conductance was placed in the dendritic compartments. Pharmacological studies demonstrated that both the late-onset and hysteretic currents demonstrated sensitivity to both dihydropyridines and the L-channel activator FPL-64176. Furthermore, the α_{1D} subunits of L-type calcium channels were immunohistochemically demonstrated on motoneuronal dendrites. It is concluded that there are dendritically located L-type channels in mammalian motoneurones capable of mediating a persistent depolarizing drive to the soma and which likely mediate the bistable behaviour of these cells.

Introduction

It has been demonstrated in cat motoneurones that the prolonged discharges seen following brief synaptic inputs (Hultborn et al., 1975) result not from continued activity in pre-motoneuronal circuits, but rather from intrinsic properties of the motoneurones themselves (Crone et al., 1988; Hounsgaard et al., 1984; Lee & Heckman, 1996). This membrane property (plateau potential) provides two stable membrane potentials, one a resting potential and the other a potential which produces sustained firing (ie. bistability). This property is thought to be important for the production of motoneurone output during fictive locomotion (Brownstone et al., 1994), as well as for the maintenance of posture in intact, awake mammals (Eken & Kiehn, 1989), possibly including humans (Kiehn & Eken, 1997). In the cat a voltage-dependent 'persistent' calcium current was surmised to underlie this property (Schwindt & Crill, 1980). Later, non-inactivating L-type calcium channels were demonstrated to mediate this current in turtle motoneurones (Hounsgaard & Kiehn, 1989).

Is an L-type current also responsible for plateau potentials and bistability in mammalian motoneurones? Unfortunately, pharmacological investigations of these currents is difficult in vivo, and calcium-mediated bistability has not been demonstrated in vitro. There are likely for two reasons for the latter. Firstly, most investigators studying mammalian spinal motoneurones in vitro use neonatal animals (eg. MacLean et al., 1997; Takahashi & Berger, 1990; Ziskind-Conhaim, 1988). As both known types of L-type channels (class C and class D) show developmental changes during the post-natal period in mice (Jiang et al., 1999a), younger motoneurones may not have the ability to produce plateau potentials. Secondly, there is evidence from turtle (Hounsgaard & Kiehn, 1993)

and cat motoneurones (Bennett et al., 1998; Lee & Heckman, 1996, 1998b) and from modeling studies (Booth et al., 1997; Gutman, 1991) that these currents originate in the dendrites. Therefore, the use of culture techniques that limit dendritic morphology may preclude their demonstration (eg. Mynlieff & Beam, 1992). To overcome these problems, spinal cord slices from animals which have "functionally mature" motor systems (they are able to weight bear and walk; Jiang et al., 1999b) were used in this study. At this stage, L-type channels contribute to a rhythmic motor output elicited in in-vitro spinal cords (Jiang et al., 1999a).

The hypothesis that mammalian spinal motoneurones have non-inactivating dendritic calcium currents was then examined. As these conductances would be electrotonically distal to the somatic recording site, they would not be adequately voltage clamped. In this situation, the presence of dendritic conductances would be seen as currents with delayed activation in response to constant voltage commands (Müller & Lux, 1993) and as producing a clockwise hysteresis in response to voltage ramp commands (Lee and Heckman, 1998b; Svirskis & Hounsgaard, 1997). Such currents are demonstrated in this study. The pharmacology of this current and the immunohistochemical demonstration of L-type calcium channels on motoneuronal dendrites lead to the suggestion that the presence of dendritic L-type calcium channels is a phylogenetically conserved characteristic of spinal motoneurones. The kinetics and subcellular location of these channels make them ideal candidates to mediate the expression of bistability in mammalian motoneurones.

Methods and Materials

Slice Preparation, Electrophysiology, Solutions and Chemicals

All animals (postnatal (P8 – P15) Balb/C mice) were anaesthetized, and the experimental procedures were approved by the University of Manitoba Animal Care Committee and conformed to the standards of the Canadian Council of Animal Care. All chemicals were obtained from Sigma (St. Louis, USA) unless otherwise specified.
The isolation of the spinal cord, preparation of slices, whole cell patch clamp recordings, and solutions and chemicals were as described in the companion paper (Carlin et al., 2000).

Motoneurones were identified as the largest cells (> 20 μm mean diameter; McHanwell & Biscoe, 1981; Takahashi, 1990) in the ventral horn (Carlin et al., 2000; Gao & Ziskind-Conhaim, 1998; Jonas et al., 1998; but see Thurbon et al., 1998). Voltage step protocols are indicated in the text. The standard voltage ramp protocol consisted of stepping the potential to -120 mV for ~1s from a holding potential of –60 mV. The potential was then ramped to +60 mV and back to -120 mV over a period of 20s. Such slow ramps minimize the effects of transient currents.

Modelling

The three dimensional morphology of a cat lumbar spinal motoneurone (courtesy of R.E. Burke) was incorporated into the NEURON simulation environment (Hines & Carnevale, 1997). A morphometric and electrotonic analysis of this cell has previously been published (FR MG MN 43/5: Cullheim et al., 1987a,b; Fleshman et al., 1988). The passive parameters assigned to the model motoneurone were R_m = 0.25 in the soma and

11 $k\Omega$-cm^2 in the dendrites, C_m = 1 $\mu F/cm^2$ and R_a = 70 Ω-cm (see Fleshman et al., 1988).

Voltage-dependent conductances were modeled using Hodgkin-Huxley formalism. These conductances consisted of two different types of Ca^{2+} channels: a transient high voltage activated N-like conductance and a persistent L-like conductance. The parameters describing the kinetics of these conductances were adapted from Avery & Johnston (1996) and Booth et al. (1997) and are given in Table 1.

The general form of the equations describing these conductances was;

$$I = g_{max} m^x h^y (V_m - E_{rev})$$

$$m' \text{ (or } h') = (m_\infty(V_m) - m)/\tau_m$$

$$m_\infty \text{ (or } h_\infty) = 1/(1 + \exp[(V_m - \theta_m)/\kappa_m]$$

where V_m is the membrane potential, m' is the rate of change of m (i.e. activation or h, inactivation) and m_∞ is the steady state value for activation.

The density of the maximal conductance, g_{max}, for the channels depended on the localization of the channels on the dendrite or soma. The differential equations were solved using the backward Euler method with a time step of 0.025 ms. Voltage clamp simulations were done using similar protocols to the *in vitro* electrophysiology.

Immunohistochemistry

Transcardial perfusion was performed with cold (4°C) pre-fixative solution consisting of 50 mM sodium phosphate buffer, 0.9% saline, 0.1% sodium nitrite and

0.01% heparin. This was followed by perfusion with cold 4% paraformaldehyde, 0.2 M L-lysine, 0.02 M Na-M-periodate solution containing 0.1M phosphate buffer, pH 7.4. The lumbar spinal cords were harvested and post-fixed in this same fixative overnight and then placed in cryoprotectant consisting of 10% sucrose in 50 mM phosphate buffer. The tissue was blocked in OCT embedding compound (Miles, Elkhart, IN) and sectioned transversely on a cryostat to a thickness of 20 μm, then thaw mounted onto gelatin-coated glass slides. Slides were washed overnight in 50 mM Tris-HCl buffered saline prior to processing. All dilutions and washes were performed in Tris-HCl buffered saline and incubations included 1% normal horse serum (Sigma).

Polyclonal rabbit anti-rat α1d subunit antibody (Alamone Labs, Jerusalem) was used to label Class D L-type Ca2+ channels. Sections were incubated in anti-α1d subunit diluted 1:500 for three days at 4°C. Sections were washed for 3 x 20 min. and all slides were incubated for 3 hours with donkey anti-rabbit cy3 (Jackson Labs, West Grove, PA) diluted 1:250. The sections were then washed for 10 minutes in Tris-HCl buffered saline, followed by 2 x 10 minute washes in 50mM Tris-HCl buffer, and coverslipped using Vectashield (Vector Labs, Burlingame, CA). Control procedures were done using sections processed as described above with (a) omission of the primary antibody, and (b) absorption of the peptide. A Molecular Dynamics confocal scanning laser microscope equipped with an argon laser was then used to generate individual optical sections, which were then volume-rendered and presented as look-through projections using Image Space software interpolation on a Silicon Graphics Indigo computer.

Results

Late-onset, persistent currents

Calcium currents were elicited in motoneurones in 150-200 μm mature mouse spinal cord slices using whole cell patch clamp techniques with intra- and extra-cellular solutions designed to eliminate sodium and potassium currents. The high-voltage-activated currents in these cells have components of L-, N-, P/Q- and R-type conductances (Carlin et al., 2000). In a number of cells the presence of a sustained late-onset conductance could be seen (Figure 1A). This conductance could be identified by either an inflection point in the current trace during a single voltage step or as a current with delayed onset (Destexhe et al., 1998; Hirst & McLachlan, 1986; Müller & Lux, 1993; Streit & Lux, 1989). The occurrence of both of these events was increased with the use of higher concentrations of calcium in the recording solution (Figure 2A). Both an inflection point and a current with delayed onset are consistent with a lack of voltage control (space clamp) of an inward conductance that is electrotonically relatively distant. These currents, referred to here as "late currents," were seen in 37/46 motoneurones (80%; 2mM calcium, 500 ms x 2mV increments; Figure 1A), where the delay to activation of this current was variable and depended on the holding potential, the amplitude of the voltage step, and the extracellular calcium concentration (Figure 2A) . They were blocked by extracellular cadmium (not shown). The inability of the somatic repolarisation to rapidly deactivate this conductance also supports a distal origin for this current (large tail currents in Figure 1A).

In turtle (Svirskis & Hounsgaard, 1998) and cat (Lee & Heckman, 1998a,b) motoneurones that have plateau potentials, as well as in plateau potential modeling studies (Booth et al., 1997), a hysteresis is seen in the current-voltage (I-V) relation in

response to slow depolarizing and hyperpolarising ramp voltage commands. In other words, there is a region on this I-V relation where the inward current during the hyperpolarising ramp is greater than that seen during the depolarizing ramp. Such slow ramp commands were applied to mouse motoneurones in this study, and produced a region of hysteresis in 17/22 (77%) motoneurones. A typical example is shown in figure 1B, where there was a relatively slowly increasing inward current on the depolarizing ramp, followed by an inflection point and a more steeply activating inward current. On the hyperpolarising limb of the ramp, this latter inward current was reduced in amplitude and additional inward current appeared at more hyperpolarised voltages. When the figure was manipulated by overlaying the ascending and descending ramps as illustrated in Figure 1C, this additional current at hyperpolarised voltages led to a clockwise hysteresis, as indicated by the arrows. An inflection point was clearly seen during the hyperpolarising ramp, thus producing a "double hump." Note that this inward calcium current deactivated at a more hyperpolarised point than the potential at which it activated on the ascending limb. This current hysteresis will subsequently be referred to as the hysteretic current. In some cells in which hysteretic currents were not readily evident in 1 mM calcium, they could be demonstrated following the application of additional calcium to the bath (eg. Figure 2B). This is a similar finding to that shown with the late currents (Figure 2A).

It was noted that in all cells with late onset, persistent currents, an hysteretic I-V relation was seen. Experiments were undertaken in 19 cells using both ramp (–120 to +60 to –120 mV over 20 seconds) and voltage step protocols (2 mV x 500 ms steps) in 2 mM calcium to examine the converse: that is, are late currents always seen if there are

hysteretic currents? An hysteretic current was seen in 14/19 (74%) cells; 13 of these had late currents and one did not. In the remaining 5 (26%) cells, neither hysteresis nor late currents were seen (Figure 2C).

The voltage of activation of the channels mediating these currents cannot be determined from the present studies, which rely on the fact that the morphology of the cells precludes space clamp. Nevertheless, it was hypothesized that since the late currents were seen with small depolarizing commands, the voltage at which the negative slope conductance began in the voltage ramps (the "apparent" activation voltage) should be more negative in the cells with hysteresis than in those without. This was found to be the case. Cells with hysteresis had an apparent activation voltage of -47 ± 8.6 mV (mean $\pm$ SD; n=14), which was significantly more hyperpolarized than in the cells without hysteresis (-38 ± 3.8 mV; n=5; t-test, $p<0.05$).

Modelling of the late-onset and hysteretic currents

The hypothesized explanation for the late-onset and hysteretic currents was that they were due to calcium currents originating in the dendrites. To examine this further, a neuromorphic model was constructed using a three-dimensional reconstruction of a cat motoneurone (Cullheim et al., 1987a,b; Fleshman et al., 1988). Although the data are limited regarding the morphological and biophysical properties of mouse motoneurones, it is known that rat motoneurones are biophysically very similar to cat motoneurones (Thurbon et al, 1998). The cat motoneurone data was therefore used as a reasonable first approximation of a mouse motoneurone.

Sodium and potassium conductances were not included in the model. Three calcium conductances were modeled (Figure 3) which differed in activation threshold (high (HVA) vs. low voltage-activated (LVA) L-type) and inactivation kinetics (L-type vs. N-type). The model was used to explore: 1) the effects of spatial segregation of the various calcium conductances on the production of late onset and hysteretic currents; and 2) the effects of voltage threshold and inactivation kinetics on the kinetics of the late and hysteretic currents. Each of the three conductances was uniformly distributed in either the somatic or the dendritic (distal to the third branch point) region of the modeled cell. The densities of the modeled calcium currents were adjusted to give peak current densities of the same magnitude as those seen in the experimental data. The model then simulated the experimental voltage clamp protocols using both step and ramp voltage commands.

During voltage step protocols, a late-onset current was seen only when a non-inactivating current was placed in the dendrites (Figure 3A). This could be modeled with either LVA or HVA properties but a qualitatively better match with experimental results was obtained with the LVA current (see Figure 5Aii). Placement of an N-type conductance in the dendrites produced a slowing of their activation kinetics as expected, but did not lead to a sustained late-onset current. In addition, the deactivation of a distal conductance was slower than the deactivation of the more proximal conductances. This can be seen by the prolonged tail currents produced by both the HVA and to a greater extent by the LVA L-type conductances placed in the dendritic region (Figure 3A, compare to Figure 1A).

With ramp voltage commands, it was necessary to place the voltage-activated channels in the dendrites to obtain clockwise hysteresis (Figure 3B). The difference

between the hysteresis produced by the dendritically located LVA or HVA non-inactivating currents was in the voltage of onset of the negative current slope. With the LVA channels (Figure 3B-ii), the apparent activation voltage was –45 mV while with the HVA channels the activation voltage was –14 mV. On combining an LVA L-type dendritic current with an HVA L-type somatic current, both the voltage steps and ramps produced results strikingly similar to those seen experimentally, including the late-onset current, the prolonged tail current, and the clockwise hysteresis with two current peaks during the hyperpolarising ramp (eg. compare Figures 1C and 3C). This lends support to the hypothesis that these late inward and hysteretic currents result from the activation of a non-inactivating L-type conductance in the dendrites, possibly with a relatively low activation threshold.

Pharmacology of the late onset and hysteretic currents

Cells displaying the late onset currents were exposed to ω-conotoxin-GVIA (3-6 μM) to block N-type (Feldman et al., 1987), ω-agatoxin-TK (400 nM) to block P/Q-type (Teramoto et al., 1995) and nifedipine (20 μM) or nimodipine (10 – 20 μM) to block L-type calcium channels. The L-type calcium channel activator FPL-64176 (5.8 μM) was also used to confirm the contribution of L-type channels (Randall & Tsien, 1995). Cells were either incubated (30 – 70 minutes) in the blockers or had the blockers acutely applied.

To discriminate the effectiveness of each blocker on the late onset currents, acute application experiments were undertaken. The late onset currents were sensitive to both the dihydropyridines nifedipine and nimodipine (7/7 cells; Figure 4A). The current

inflections were completely (n = 2) or partially blocked (n = 1) by application of 20 μM nifedipine. Similarly, application of 10 – 20 μM nimodipine completely blocked (n = 3) or reduced (n = 1) the current inflections.

Since sensitivity to dihydropyridines is the hallmark of L-type calcium channels, these results indicate that at least part of the late onset conductance is mediated through L-type channels. This possibility was further tested with the application of the potent L-type channel activator FPL-64176. Application of FPL-64176 (5.8 μM) to cells with the late onset currents caused an enhancement of these currents (2/2 cells; Figure 4B). In addition to the increased amplitude, this enhancement was characterized by reducing the somatic voltage at which the late current was first seen and by producing large slowly activating currents.

Similar effects of these drugs were seen with the ramp voltage commands. FPL-64176 not only had the expected effect of increasing the amplitude and shifting the peak inward current on the ascending ramp to the right, but it also increased the amplitude of the hysteretic current and delayed its deactivation, as can be seen during the hyperpolarising ramp (2/2 cells; Figure 4C). This current was blocked by nimodipine (20 μM; 2/2 cells; Figure 4C). Given that the apparent activation voltage is more hyperpolarised in cells with the hysteretic current, then if L-type calcium channels are responsible for this current, it is not surprising that the application of nimodipine shifts this apparent activation voltage to the right (Figure 4C).

In contrast to the dihydropyridines, application of ω-conotoxin-GVIA (6 μM) did not eliminate the current inflections (4/4 cells; Figure 5A). Interestingly, following

application of ω-conotoxin, the late onset currents were activated to the same steady-state level and no longer exhibited a relationship to the command voltage level. This is similar to what was seen in the simulation containing only dendritic L-type channels (Figure 3A). This effect was predicted by Müller & Lux (1993) and is thought to result from the regenerative activation of voltage-gated calcium channels in an unclamped region of the cell.

The effects of ω-agatoxin-TK (400 nM) were tested in 6 cells. In four cells the toxin did not block the dendritic currents while the total current amplitude was reduced (Figure 5B). In one cell, the inflection point was abolished while in the last cell the ω-agatoxin rapidly produced a reduction but not block of the amplitude of the late-onset currents.

To rule out the possibility that acute application of the toxins did not allow sufficient time for the toxins to act, cells were incubated in ω-agatoxin TK (400 nM: n = 2) or ω-conotoxin GVIA (3 μM: n = 1; 6 μM: n = 2) for 30 – 70 minutes. Even after prolonged exposure to the toxins, late onset currents were still observed.

In summary, the late-onset currents were not blocked by application of ω-conotoxin-GVIA in 7/7 cells, and following application of ω-agatoxin-TK in 6/7 cells. In contrast, these currents demonstrated sensitivity to dihydropyridines in 7/7 cells and FPL 64176 in 2/2 cells. These pharmacological results support the hypothesis that the late-onset calcium currents are mediated by dihydropyridine-sensitive L-type calcium channels.

The effects of ω-conotoxin-GVIA (6 μM) and ω-agatoxin-TK (400 nM) were also tested on the hysteretic currents. Co-application of these toxins reduced the large inward

current on the ascending limb of the ramp, indicating that there is an N and/or P/Q-type component to this current. However, the hysteretic currents were not affected by these toxins (n=2; Figure 5C). This adds further support to the hypothesis that this current is mediated primarily by L-type channels.

Immunohistochemical localisation of motoneuronal α_{1D} subunits

The presence of L-type calcium channels on motoneurone dendrites was examined with immunofluorescence using a polyclonal antibody to the α_{1D} subunit (n = 4 animals; 3 additional animals were studied using 3,3'-diaminobenzidine tetrahydrochloride immunohistochemistry, data not illustrated). Previous work demonstrated somatic and proximal dendritic labeling with the anti-α_{1C} antibody, and probable dendritic labeling using the anti-α_{1D} antibody (Jiang et al., 1999a). In this study, motoneuronal dendrites were examined for a distance of up to 300 μm from the cell soma. Punctate dendritic labeling of the α_{1D} subunit was seen typically after the second or third dendritic branch point, with less labeling proximal to this (Figure 6). Given the absence of significant dendritic labeling of the α_{1C} subunit (Jiang et al., 1999a; Westenbroek et al., 1998), this leads to the suggestion that the dendritic L-type calcium channels responsible for the late-onset calcium currents are of class D.

Discussion

This study has demonstrated a dihydropyridine-sensitive calcium current (L-type) originating in the dendrites of spinal motoneurones in slices harvested from mice older than postnatal day 7. A similar current can be demonstrated in a motoneurone model by

placing a low-voltage activated, non-inactivating current in the dendrites. Furthermore, the immunohistochemical labeling demonstrates class D L-type calcium channels in motoneurone dendrites.

Evidence for a dendritic calcium current

In the present study, the dendritic origin of the voltage-activated calcium currents is shown indirectly using somatic whole cell recording, and combining this with results using both immunohistochemistry and modeling studies. There is little question that the recorded currents are mediated by calcium. Under the same experimental conditions it has been demonstrated that these currents are voltage dependent, sensitive to the extracellular calcium concentration and cadmium, and are sensitive to the specific channel blockers ω-agatoxin-TK, ω-conotoxin GVIA, dihydropyridines and FPL-64176 (Carlin et al., 2000). The ability of increased extracellular calcium concentrations to make distal currents more visible in the soma would be predicted because the raised calcium not only increases the membrane resistance but also the negative slope conductance (Figures 2B, 2C-2; Müller & Lux, 1993).

In this study it was found that approximately one quarter of cells did not display either late-onset or hysteretic currents even with elevated extracellular calcium. There are a number of possible explanations for this, which can not be distinguished in the current study. For example, it is possible that a subpopulation of motoneurones (eg. subtype) do not possess these dendritic channels, and that these cells would have a different role in motor output than those cells that could express plateau potentials (see below). A second possibility is that the cells in which dendritic currents were not seen had a significant portion of their dendritic trees (and hence α1D channels) removed by slicing.

Although some investigators have recorded calcium currents directly from dendrites (eg. Magee & Johnston 1995; Mouginot et al., 1997), it is unlikely that such an approach would be successful in the distal dendrites of motoneurones where these channels are likely located. On the other hand, recording dendritic calcium currents, or their resultant potential changes, with a somatically placed electrode has also been demonstrated previously. Destexhe et al. (1996) combined electrophysiology with a neuromorphic model to demonstrate LVA channels in the dendrites of thalamic reticular neurones. Hirst & McLachlan (1986) reported a late onset calcium current in voltage clamped sympathetic chain cells. The current was concluded to originate in an electrically distant location due to the change in latency but not amplitude with increasing voltage steps. This is consistent with the experimental data presented here (eg. Figures 5A-ii).

The modeling data from Müller & Lux (1993) suggest that one can deduce the position of a spatially localized inward current from examination of the delay and rate of activation of the somatically-recorded inward current. However, given the limited information regarding the properties of the channels from which this current originates, and the multiple types of calcium currents present, the position of the currents cannot be accurately determined from examination of the trajectory alone. Both the delay and the rate of activation of the late-onset current were variable and depended on the size of the voltage step (eg. Figures 1A, 5A). Near 'threshold' for these currents, the delay to onset of the late current may be as long as several hundred milliseconds (eg. Figure 4A-i). On first examination, such a delay may be difficult to explain based on the relatively electrically compact nature of motoneurones (Thurbon et al., 1998). However, Müller &

Lux (1993) demonstrated delays of up to 2 time constants with conductances placed at 0.5 – 0.8 λ; the majority of cells displaying late-onset currents in this study had time constants < 60 msec. Furthermore, using current clamp protocols in a two compartment model, Booth et al. (1997) demonstrated that several hundred millisecond delays in onset of plateau potentials with current steps near the threshold of activation of the plateaux can be explained using dynamical systems theory. This theory predicts long delays to onset of plateaux near threshold (the onset voltage of the negative slope conductance), and then with increasing current steps, the delay will be shorter. This is similar to the data presented here in voltage clamp (Figures 2A, 4, 5). This would explain why the long delays were more often seen with protocols using 2 mV increments in voltage commands, as the likelihood of a voltage step being close to the threshold (or "saddle-node" in the terminology of dynamical systems theory) would be increased.

Another possible explanation for the delayed currents might be that the somatic channels are in equilibrium between "reluctant" and "willing" states (Bean, 1989). It could be argued that if the channel equilibrium in these cells in this preparation favors the reluctant state, and that if time in a depolarized state is required to shift to the willing state (Delgado-Lezama & Hounsgaard, 1999), then a late-onset current might be seen. This may in fact explain the "wind-up" phenomenon previously reported in turtle motoneurones (Svirskis & Hounsgaard, 1997). Two observations make this possibility less likely. Firstly, it has not been possible to facilitate the total calcium current elicited by voltage steps from –60 to 0 mV following 75 ms pre-pulses to +100 mV in any of the 13 cells tested (Carlin et al., 2000). Secondly, during the hyperpolarising phase of the voltage ramps there are two conspicuous peaks of inward current created by an inflection

point in the current waveform (see Figures 1, 2B, 3C, 4C). It is suggested that this "double hump" represents the separation of a somatic current, which is partly inactivating, and a dendritic current, which is seen as the hysteretic current. The inflection point between these humps indicates that the second (more hyperpolarised) hump must be created either by newly-activating somatic channels or by persistently activated dendritic channels which become a current source for the soma as it is being hyperpolarised. The former possibility would seem unlikely given the absence of evidence that dihydropyridine-sensitive channels can activate (or reactivate) during a slow repolarisation after a prolonged depolarization and then remain open to potentials as low as −100 mV. On the other hand, a more reasonable explanation for this "double hump" would be that the apparent activation is due to the relative depolarization of the non-clamped dendrites, which persists as the soma is hyperpolarised. The more hyperpolarised somatic voltage then acts as a "sink" for the inward current mediated by non-inactivating dendritic channels. This latter hypothesis is supported by the immunostaining of L-channels that are clearly separated from the soma.

Channel type underlying the dendritic currents

The dendritic currents shown here were consistently blocked by dihydropyridines and enhanced by FPL 64176 and are therefore mediated by L-type channels. Previous authors have demonstrated the presence of L-type calcium channels in mammalian spinal motoneurones but have not reported a dendritic calcium current (Gao & Ziskind-Conhaim, 1998; Hivert et al., 1995; Mynlieff & Beam, 1992; Scamps et al., 1998). These cells were either embryonic or neonatal, which may explain why dendritic L-type currents were not reported. This is consistent with the immunohistochemical observation

that both the α_{1C}- and α_{1D}- containing L-type channels develop in the first two postnatal weeks (Jiang et al., 1999a). In the present study, the use of older mice would have facilitated the detection of α_{1D}-mediated dendritic currents.

In addition to L-type calcium channels, it is possible that there are dendritic N- and/or P/Q- type channels as well. It can be seen in figures 5A and 5B that the slope of the late current decreases with the addition of conotoxin and agatoxin. Because the effects of channel rundown are difficult to distinguish in this situation, the possibility that N- and/or P/Q-type channels contribute to this current cannot be ruled out. Furthermore, the ability of agatoxin and conotoxin to block the spike-like current during the slow voltage ramp command (Figure 5C) may indicate the presence of non-L-type channels in the unclamped dendrites. However, the facts that the late and hysteretic currents were not blocked with the application of saturating concentrations of these toxins and that the late currents were still seen in cells incubated in these toxins would indicate that the contribution of current mediated by non-L-type channels to the persistent inward *dendritic* current recorded in the soma (i.e. hysteretic current) was minimal compared to the current mediated by L-type calcium channels.

Voltage of activation of the dendritic current

As the recorded dendritic currents are remote from the patch electrode, the voltage of activation can not be determined in the current study. Nevertheless, it is interesting to note that the somatic voltage of activation of the calcium currents is lower in the cells with dendritic/hysteretic currents than in those without. Furthermore, the modeling data demonstrated a similar lower apparent voltage of activation in the ramp studies in models

with LVA channels (Figure 3B). Interestingly, the voltage of activation of the inward current is –45 mV in the LVA model, and averages –47 mV in the experimental data.

Traditionally, the L-type calcium channels have been considered to belong to the HVA class of channels. However, in hippocampal pyramidal cells, dihydropyridine-sensitive channels have been found to activate at (Davies et al., 1999; Magee et al., 1996) or near (Avery & Johnston, 1996) rest potentials in physiological concentrations of calcium (2 – 2.5 mM). There is also evidence in support of LVA dihydropyridine-sensitive calcium channels in magnocellular neurosecretory cells of the supraoptic nucleus (Fisher & Bourque, 1996), and guinea pig trigeminal motoneurones (Hsiao et al., 1998). Previous studies have looked at either α_{1C} or α_{1D} channels in isolation, inserted into Xenopus embryos. In comparing these studies, it would seem that the threshold of activation of the α_{1D} channels is lower than that of the α_{1C} channels (compare Williams et al., 1992: Figure 6b with Tomlinson et al., 1993: Figures 1b and 3). [Note that subunits other than the α_1 may have some modulatory effect on the voltage of activation (Tomlinson et al., 1993; Klugbauer et al., 1999).] With the immunohistochemical data supporting the dendritic dihydropyridine channels being of class D (whilst class C appear to be more proximal - Westenbroek et al., 1998; Jiang et al., 1999a), it could be speculated that these channels have a lower voltage of activation than the class C channels, and hence account for the findings presented in this study.

Implications for bistability

It has previously been suggested that a persistent dendritic current is responsible for the depolarizing drive underlying self-sustained repetitive firing in motoneurones, and that this current also underlies a second stable membrane potential at a level more

depolarized than rest (hence the term "bistability"). Experimental evidence supporting a dendritic location of this plateau current comes from a number of sources. Schwindt & Crill (1977) first demonstrated a region of negative slope conductance in cat motoneurones, which they attributed to a persistent (non-inactivating) calcium current. In examining these data, Gutman (1991) convincingly argued that this current must be dendritic in origin because the firing continued even when the somatic voltage returned to resting potential. Furthermore, he pointed out that in their voltage-clamp data, the inward current persisted even after returning the soma to resting potential (Gutman, 1991; compare Lee & Heckman, 1996).

Plateau potentials were also demonstrated in cat motoneurones following systemic administration of monoamines, which were thought to unmask the inward current by blocking outward potassium currents (Conway et al., 1988). These potentials were studied further in turtle motoneurones, where they were shown to be sensitive to dihydropyridines (Hounsgaard & Kiehn, 1989). Furthermore, because of the morphology of these cells, application of electrical fields could differentially polarize the soma and dendrites, leading to experiments which demonstrated that the current underlying the plateau potential was dendritic in origin (Hounsgaard & Kiehn, 1993). Interestingly, the activation voltage of plateau potentials in the cat is lower during excitatory synaptic input than with intracellular somatic current injection, which is also consistent with a dendritic location of the plateau mediating channels (Bennett et al., 1998).

Modeling studies simulating bistability also support a dendritic location of the persistent inward current underlying the plateau potential. Booth & Rinzel (1995) and Booth, Rinzel & Kiehn (1997) used a two compartment model based on data from turtle

motoneurones to model bistability. In order to reproduce the bistability seen in experiments, they found that it was essential for the somatic spike-generating mechanism to be electrically distant from (weakly coupled to) conductance underlying the plateau current. This is supported by other models including those of motoneurones (Gutman, 1991) and Purkinje cells (Jaeger et al., 1997).

It was previously reported that a motor rhythm in the whole isolated spinal cord becomes sensitive to dihydropyridines after postnatal day 7 and that L-type calcium channels are best demonstrated in motoneurones after this age (Jiang et al., 1999a). The present study demonstrates dendritic L-type currents in motoneurones after postnatal day 8. It is conceivable that with the development of these currents, motoneurones are able to produce plateau potentials which result in firing rates necessary for functional motor behaviors such as posture and locomotion – activities which mature at postnatal day 9 (Jiang et al, 1999b). In fact, during fictive locomotion in the adult cat, there is a voltage-dependent increase in the excitation of motoneurones by the spinal network for locomotion (Brownstone et al., 1994). Furthermore, motoneurones fire at high rates when first recruited, with little change in these rates with somatic current injection (Brownstone et al., 1992). A possible explanation for these phenomena is that activation of voltage-activated calcium currents led to bistability. The presence of a dendritically-located calcium current such as the one demonstrated here would support this possibility. Preliminary data have demonstrated calcium-mediated plateau potentials in mature mouse motoneurones (Jiang et al., 1999c).

In summary, this study demonstrates a persistent dihydropyridine-sensitive calcium current in mouse motoneurones resulting from activation of dendritically-located

channels. This current may be low voltage-activated, mediated via class D L-type channels, and is likely responsible for the bistable behaviour seen in motoneurones.

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Abbreviations

aCSF – artificial cerebral spinal fluid

HVA- high voltage-activated

I-V – current-voltage

LVA – low voltage-activated

Ri – input resistance

Ra – access resistance

WCC – whole cell capacitance

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Tables

		g_{max} (S/cm^2)	x	y	E_{rev} (mV)	τ_m (ms)	τ_h (ms)	θ_m (mV)	θ_h (mV)	κ_m (mV)	κ_h (mV)
N-like:	soma dendrites	0.05 0.002	2	1	60	4	40	-30	-45	-5	5
L-like HVA	soma dendrites	0.01 0.0003	1	0	60	20		-10		-6	
L-like LVA	soma dendrites	0.01 0.0003	1	0	60	20		-30		-6	

Table 1: Parameters used for modelling calcium conductances in somatic and dendritic compartments. g_{max} , maximum conductance; m , activation state; h , inactivation state; E_{rev} , reversal potential; τ_m , activation time constant; τ_h, inactivation time constant; θ_m, half-activation voltage; θ_h, half-inactivation voltage; κ_m, slope of activation curve; κ_h, slope of inactivation curve.

Figure Legends

Figure 1: Both late-onset and hysteretic calcium currents can be seen in motoneurones. **A:** An example of late-onset, low amplitude persistent currents seen in a post-natal day 12 (P12) motoneurone. The late-onset currents (*) were activated earlier and more rapidly with increasing voltage commands. Voltage steps of 150 ms duration from –60 to –10 mV in 10 mV increments (whole cell capacitance (WCC) 243 pF, input resistance (Ri) 101 MΩ, and access resistance (Ra) 8.1 MΩ). Note the large tail currents (arrow). **B:** Calcium currents elicited from a P10 motoneurone in response to a slow voltage ramp command (from –120 mV to +60 mV to –120 mV over 20 seconds). Ascending limb of the ramp is black and the descending limb is grey. Inflection points indicated by asterisks (*) (WCC 213 pF, Ri 440 MΩ, Ra 10.8 MΩ). **C:** Superimposing the mirror image of the hyperpolarising current response (grey) onto the depolarising current response (black) demonstrates a clockwise calcium current hysteresis (arrows as in **B**). The hysteresis was the result of additional inward current during the hyperpolarising phase of the ramp which was not present during the depolarising phase. The dotted lines mark the onset of the negative slope conductance on the ramp up, and the peak hysteretic current on the ramp down.

Figure 2: Late-onset and hysteretic currents were seen in most but not all motoneurones, and were dependent on extracellular calcium. **A:** Voltage steps of 150 ms duration from –60 to –5 mV in 5 mV increments in a P12 motoneurone (WCC 184 pF, Ri 214 MΩ, Ra 6.9 MΩ). After the addition of extra calcium (Ri increased to 305 MΩ), a late-onset

inward current was seen (arrow) with a progressively shorter delay with increasing voltage commands. **B:** Current responses to voltage ramp commands in a P13 motoneurone before and after the addition of 1 mM calcium (WCC 163 pF, Ri 350 MΩ in 2 mM calcium). The addition of extra calcium enhanced the hysteretic current. The black and grey current traces are as in Figure 1. **C:** In some cells, such as this P10 motoneurone (WCC 236 pF, Ri 447 MΩ, Ra 9.8 mΩ), neither the late-onset (C-1) nor the hysteretic (C-2) currents could be demonstrated, even after increasing the extracellular calcium concentration (Ri increased to 490 MΩ).

Figure 3: The models containing dendritic L-type channels demonstrate currents similar to those seen experimentally. **A:** Neither N-type currents in the soma or dendrites, nor L-type (LVA or HVA) currents in the soma resulted in late onset currents. Such currents were seen only with L-type currents in the dendrites. Note the large, persistent tail currents with LVA L-type currents in the dendrites (middle row, right). **B:** Similarly, hysteretic currents were seen only with L-type currents in the dendrites. Note that the placement of LVA L-type currents in the dendrites results in currents very similar to those seen experimentally, with a low voltage of onset, and delayed deactivation on the downward ramp. **C:** The inclusion of HVA L-type currents in the soma with the LVA L-type currents in the dendrites resulted in hysteretic currents very similar to those seen experimentally, including the "double-hump" on the downward ramp.

Figure 4: The dendritic current is mediated by L-type calcium channels. **A:** The late-onset currents seen in this P9 motoneurone in 2 mM calcium (i) were antagonised after

application of nifedipine (ii). The steps to –16 mV before and 13 minutes after the application of nifedipine were overlaid in iii. **B:** The late-onset currents seen in this P11 motoneurone were still present ~6 minutes after the addition of ω-agatoxin and ω-conotoxin (i) and were enhanced after application of FPL-64176 (ii). The steps to –26 mV are overlaid in iii. In **A** and **B**, voltage steps were 500 ms in duration, from a holding potential of –60 mV to –40 mV then increased in 2 mV steps. Steps from -40 mV to –20 mV are shown in **A** and steps from -32 mV to –16 mV are shown in **B**. **C:** A P13 cell displaying a small hysteretic current in response to a voltage ramp command. The current hysteresis was enhanced with the application of FPL-64176, shown 6 minutes later (ii) and subsequently reduced with nimodipine, shown after an additional 7 minutes (iii). Note that nimodipine application eliminated the inflection points during both the depolarising and hyperpolarising phases of the ramps, and shifted the apparent voltage of activation to the right (dotted line).

Figure 5: The late-onset and hysteretic currents are not blocked by N- and P/Q- type channel blockers. **A:** Addition of a saturating concentration of ω-conotoxin-GVIA reduced the total calcium current in this P15 motoneurone, but the dendritic currents were still evident. Note that the peak current remains constant at increasing voltage steps following toxin application. Holding potential of –50 mV with 150 ms x 5 mV steps from –25 to 0 mV. **B:** In this P12 motoneurone, addition of ω-agatoxin-TK reduced the total current without eliminating the late currents. Holding potential of –60 mV with 150 ms x 5 mV steps to –5 mV. Both **A** and **B** were in 1.5 mM calcium. **C:** Overlaid current traces from a P10 motoneurone before (black) and approximately 3 minutes after (grey)

application of 400 nM ω-agatoxin-TK and 6 μM ω-conotoxin-GVIA. A large reduction in the inward current that was elicited during the ascending phase of the ramp can be seen in the absence of any significant change in the hysteretic current.

Figure 6: Class D L-type channels were demonstrated in the dendrites of large ventral horn neurones using an anti-α_{1D} polyclonal antibody. Confocal look-through image from an adult mouse spinal cord slice (20 μm). The labelling was punctate-like, and increased distal to the second or third dendritic branch points (arrows) in this adult mouse spinal cord. An outline of the soma (S) and dendrites is shown on the right. Scale bar = 10 μm.

Figure 1

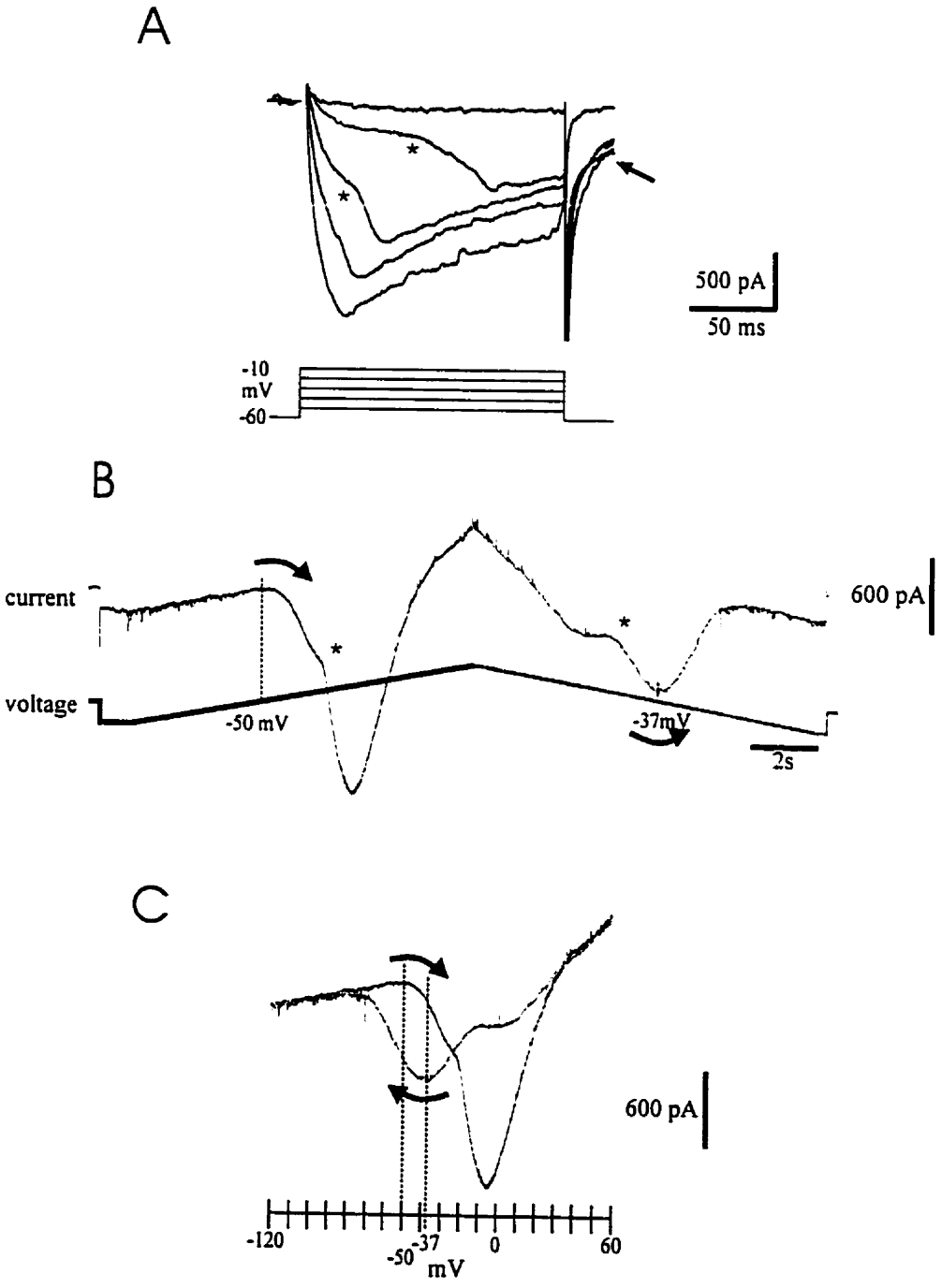

A
500 pA
50 ms
-10
mV
-60
B
current
voltage
-50 mV
-37mV
600 pA
2s
C
600 pA
-120
-50
-37
0
60
mV

Figure 2

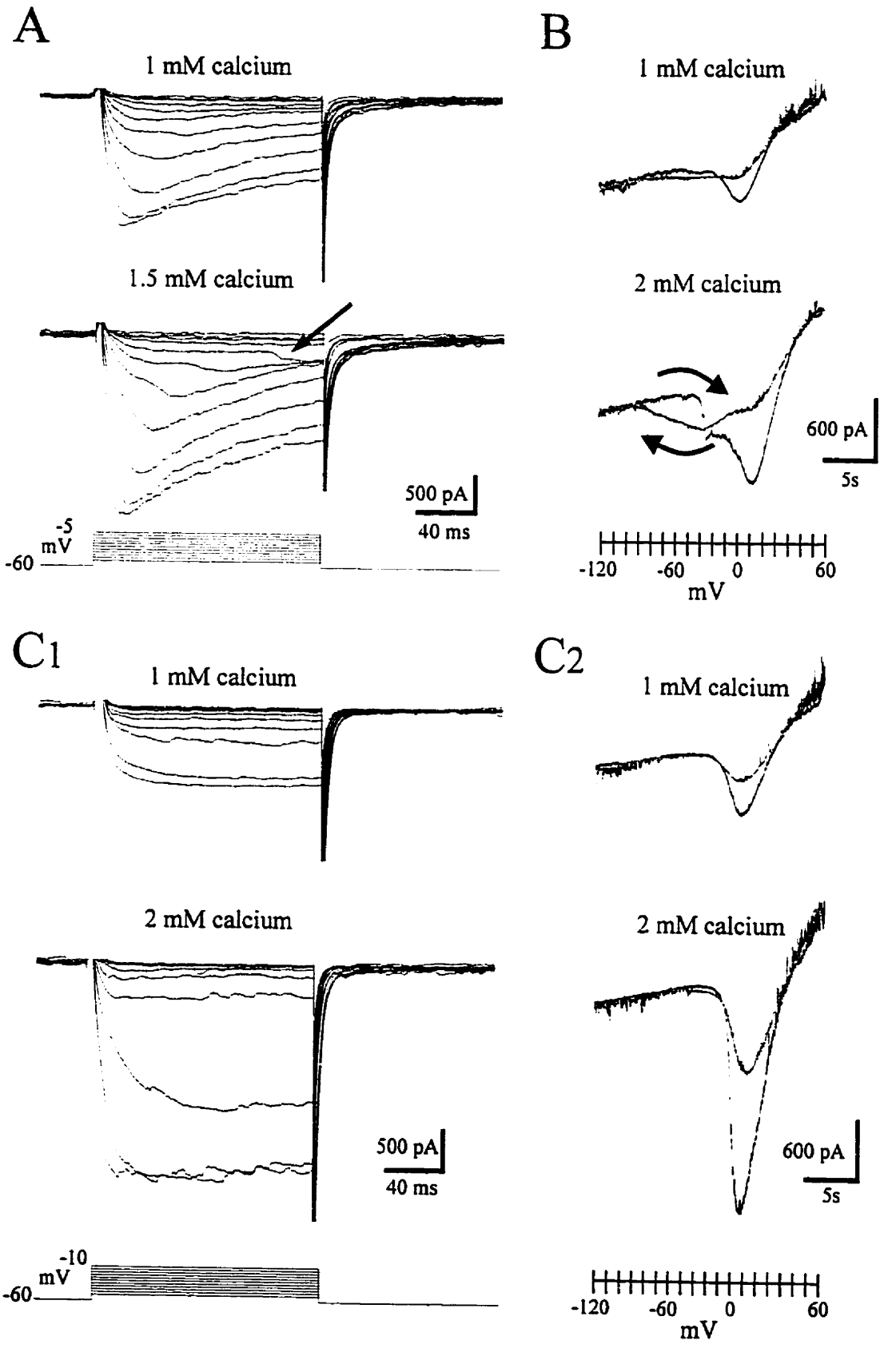

A
1 mM calcium
1.5 mM calcium
500 pA
40 ms
-5
mV
-60
B
1 mM calcium
2 mM calcium
600 pA
5s
-120
-60
0
60
mV
C1
1 mM calcium
2 mM calcium
500 pA
40 ms
-10
mV
-60
C2
1 mM calcium
2 mM calcium
600 pA
5s
-120
-60
0
60
mV

Figure 3

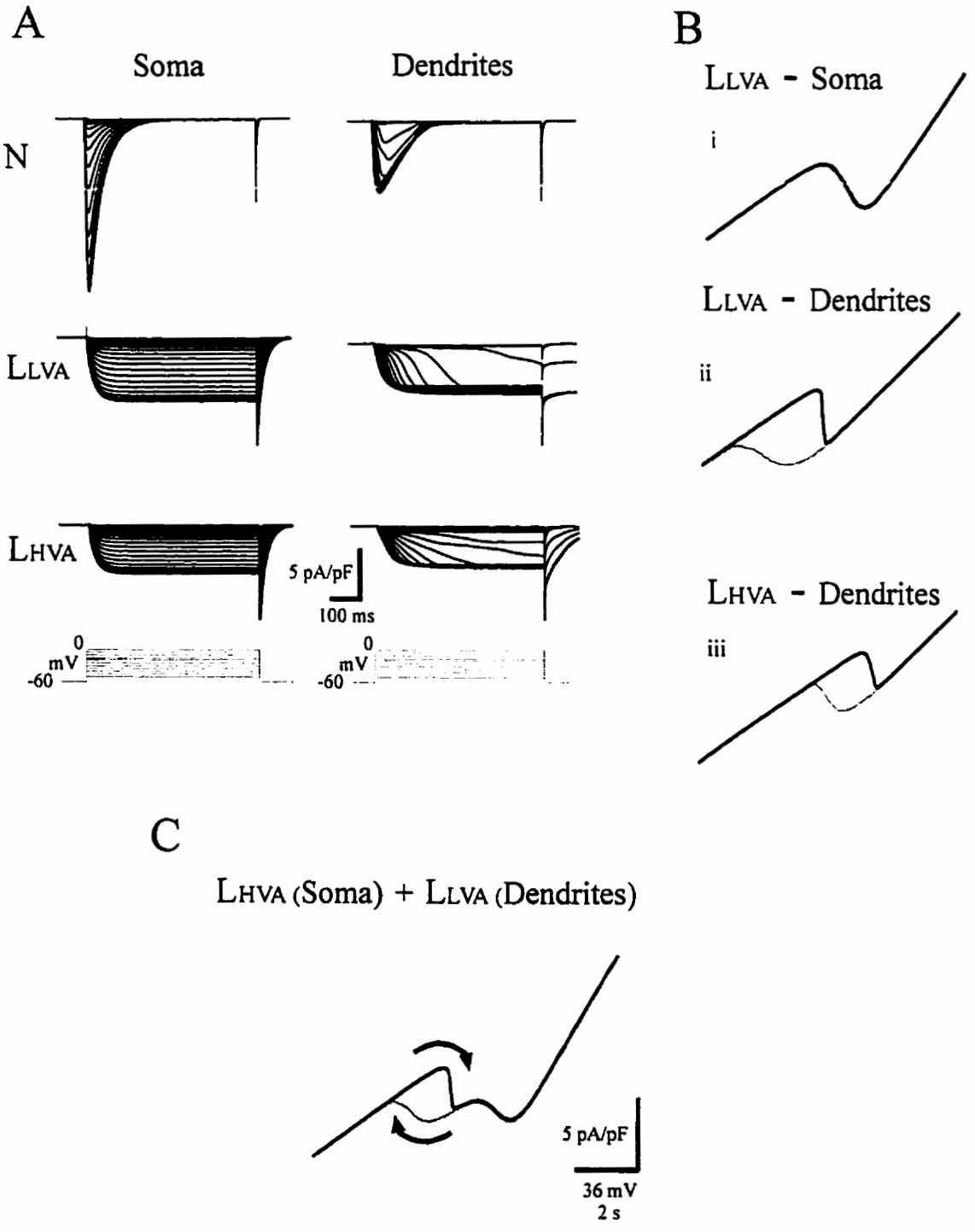

A
Soma
Dendrites
N
LLVA
LHVA
5 pA/pF
100 ms
0
mV
-60
0
mV
-60
B
LLVA - Soma
i
LLVA - Dendrites
ii
LHVA - Dendrites
iii
C
LHVA (Soma) + LLVA (Dendrites)
5 pA/pF
36 mV
2 s

Figure 4

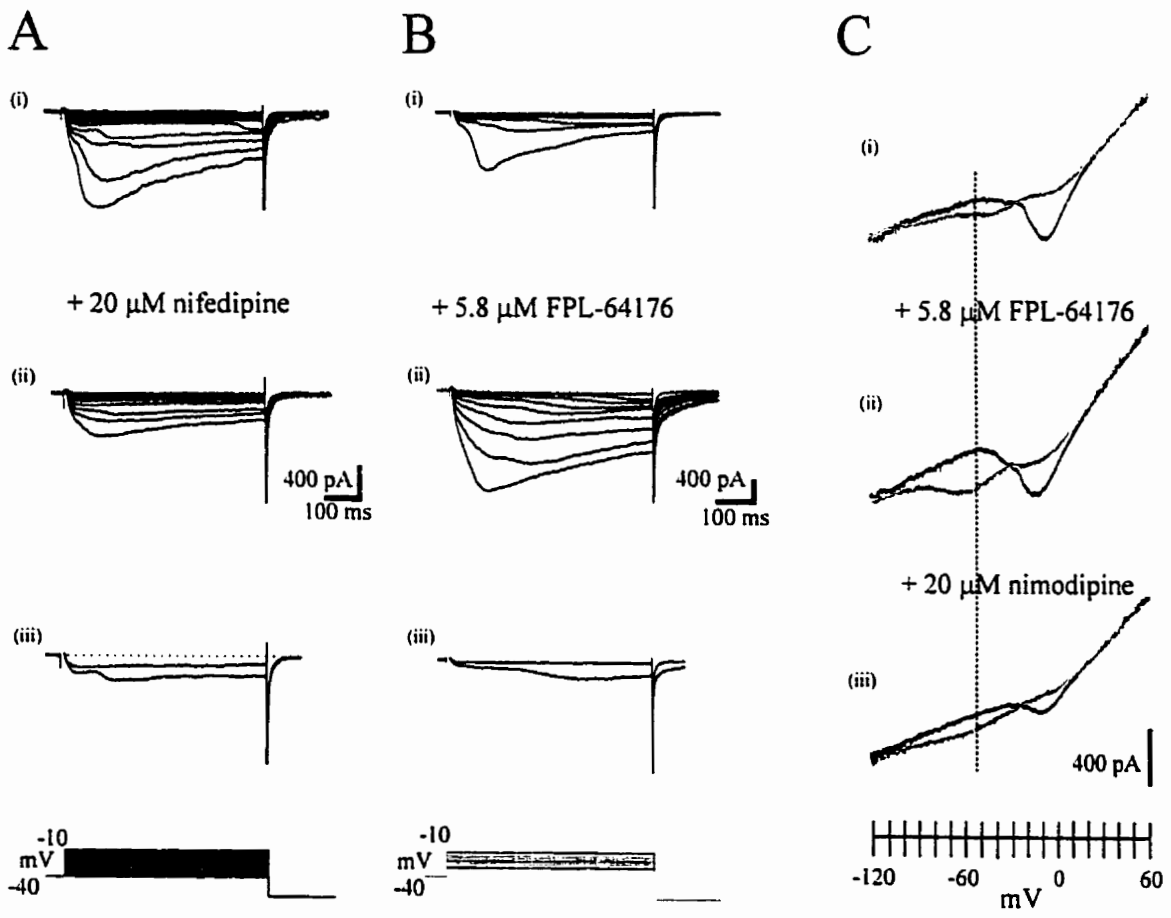

A
B
C
(i)
(i)
(i)
+ 20 μM nifedipine
+ 5.8 μM FPL-64176
+ 5.8 μM FPL-64176
(ii)
(ii)
(ii)
400 pA
100 ms
400 pA
100 ms
+ 20 μM nimodipine
(iii)
(iii)
(iii)
400 pA
-10
mV
-40
-10
mV
-40
-120
-60
0
60
mV

Figure 5

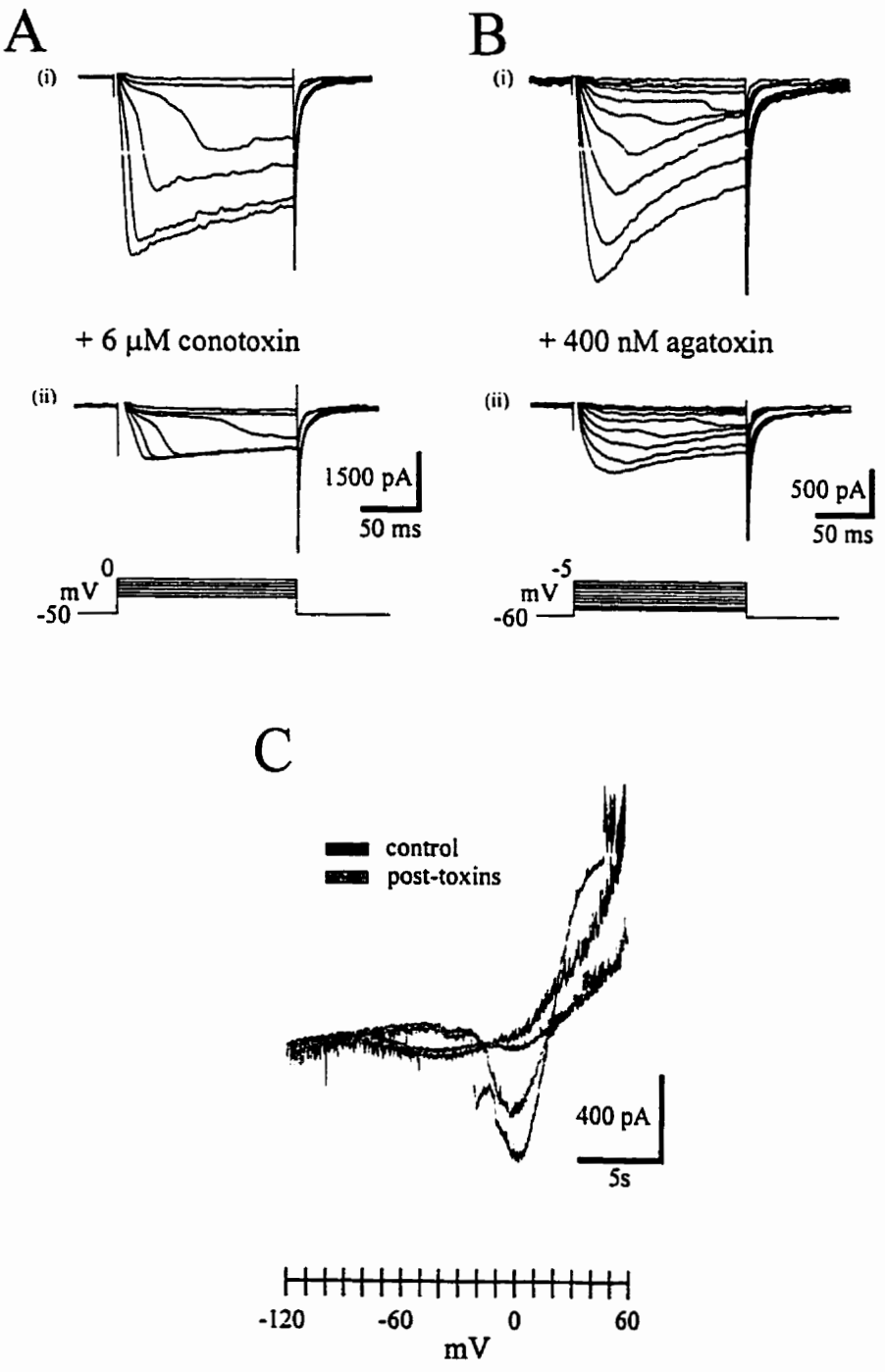
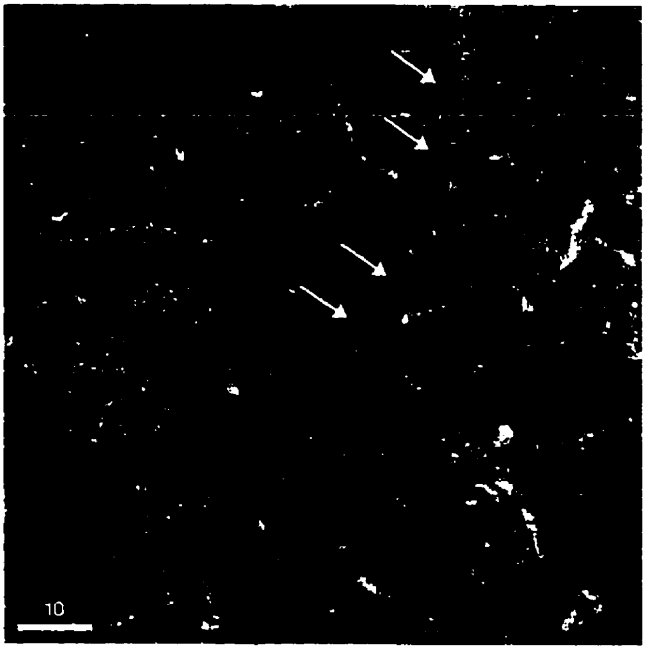

A
(i)
+ 6 μM conotoxin
(ii)
1500 pA
50 ms
0
mV
-50
B
(i)
+ 400 nM agatoxin
(ii)
500 pA
50 ms
-5
mV
-60
C
control
post-toxins
400 pA
5s
-120
-60
0
60
mV

Figure 6



S

Paper 3

Plateau potentials in mouse lumbar spinal motoneurones

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In preparation

Abstract

Spinal motoneurones have complex intrinsic properties that can alter their response to certain synaptic inputs. One such property gives motoneurones the ability to generate sustained output in response to brief inputs. This property results from the activation of a self-sustained depolarization or plateau potential. Plateau properties of mammalian motoneurones were first revealed in the decerebrate cat. However, there are significant difficulties in addressing the cellular mechanisms underlying plateau potentials in the in-vivo cat. In turtle spinal cord slice it has been shown that these potentials are mediated by voltage-activated L-type calcium channels.

In this study plateau potentials are demonstrated in mouse lumbar spinal motoneurones using an in-vitro slice preparation. In the presence of the non-specific potassium channel blocker cesium, or with the application of the L-type calcium channel activator FPL-64176, plateaux can be evoked with short depolarizing current pulses and terminated with hyperpolarizing current pulses. With voltage-gated sodium and potassium channels blocked, calcium-dependent plateaux were able to mimic the behavior of the plateaux recorded in more physiological solutions. These results support the idea that, as in turtle, an L-type calcium current contributes to the plateau potential in mammalian spinal motoneurones.

Introduction

In a number of neuronal cell types the input-output relationship is not linear. That is, in these cells voltage-dependent membrane conductances are activated that amplify either the injected or synaptic current and therefore create a non-linear relationship in terms of amplitude and/or time. One form of non-linearity arises from the presence of a sustained or slowly inactivating inward current that gives the steady-state current-voltage relationship of the cell a region of negative resistance and net inward current. The resulting current-voltage relationship therefore has an "N" shape. In the absence of a repolarizing current this type of relationship gives the cell the capacity for two stable membrane potentials (bistability), a rest potential and a more depolarized potential. Triggering the transition to the depolarized potential with a short current pulse can produce self-sustained firing. If sodium spikes are blocked, the underlying depolarization can be seen as a plateau potential.

Plateau potentials have been demonstrated in a number of different mammalian motoneurone types. These include trigeminal (Hsiao et al., 1998), hypoglossal (Mosfeldt-Laursen and Rekling, 1989), sacral sphincter (Paroschy and Shefchyk, 2000) and ocular (Gueritaud, 1994) motoneurones as well as neurons in the nucleus ambiguus (Rekling and Feldman, 1997). In spinal motoneurones this intrinsic membrane property was first demonstrated in the in-vivo cat (Schwindt and Crill, 1977; Hounsgaard et al., 1984) and later the ionic mechanism of the plateau were defined in the in-vitro turtle preparation (Hounsgaard and Kiehn, 1985). The ionic conductance underlying plateau potentials in the cat is presently unknown but is suspected to be mediated by voltage-gated calcium

channels (Schwindt and Crill, 1981,1984). In the turtle the underlying conductance has been shown to be sensitive to nifedipine and therefore is attributed to L-type calcium channels (Hounsgaard and Kiehn, 1989). As similar plateau potentials have not been demonstrated in mammalian spinal motoneurones in the in-vitro setting, the question remains if mammalian spinal motoneurones use similar ionic conductances to generate plateau potentials.

In both cat and turtle spinal motoneurones, the expression of plateau potentials is a latent property that requires unmasking. In the in-vivo cat plateau potential expression is dependent on the descending monoaminergic systems (Crone et al.,1988; Lee and Heckman, 1999; Paroschy and Shefchyk, 2000) and in the spinal cat on exogenously applied monoaminergic agonists (Hounsgaard et al., 1988; Conway et al., 1988). Similarly in the turtle, plateau potentials can be elicited with application of monoaminergic, muscarinic or metabotropic glutamatergic agonists (Hounsgaard and Kiehn, 1989; Svirskis and Hounsgaard, 1998). In the turtle, plateau expression has also been obtained by manipulating ionic conductances, specifically by reducing potassium currents or increasing L-type calcium currents (Hounsgaard and Mintz, 1988).

In the present study plateau potentials are demonstrated in mouse spinal motoneurones using an in-vitro slice preparation. In these cells, plateau potentials can be unmasked with the application of the non-specific potassium channel blocker cesium or the L-type calcium channel activator FPL-64176. Furthermore, when voltage-gated sodium and potassium channels are blocked, calcium-dependent plateaux can be generated and are able to reproduce the properties of plateau potentials recorded in more

physiological solutions. These results suggest that a conductance through voltage-gated calcium channels may be responsible for mediating plateau potentials in these cells.

Methods

Slice preparation

The preparation of the spinal cord slice is the same as previously described (Carlin et al., 2000a). Briefly, Balb/c mice up to P12 were anesthetized with ketamine and the spinal cord was isolated in a Sylgard-lined Petri dish filled with cold (< 4°C) dissecting artificial cerebral spinal fluid (aCSF). The lumbar enlargement was then blocked and sliced using a Leica (VT 1000E) vibrating microtome filled with cold dissecting aCSF (150-200 um slices). The slices were immediately placed into warm (36 °C) recovery aCSF for 45 min before being transferred to room temperature aCSF.

Recordings were performed in a 450 μL recording dish mounted in the stage of an upright Olympus BX50 microscope fitted with differential interference contrast optics. Cells were visualized using an infra-red camera and black and white monitor. Motoneurones were identified as large ventral horn cells (Carlin et al., 2000a). Whole cell patch-clamp recordings were made using an Axopatch 1D amplifier and pClamp 7/8 software running on a Pentium class computer. All recordings were made in current clamp mode. All experiments were performed at room temperature.

All animals were anesthetized, and the experimental procedures were approved by the University of Manitoba Animal Care Committee and conformed to the standards of the Canadian Council of Animal Care.

Solutions and chemicals

The aCSF solutions were designed to reduce calcium-induced cell damage during processing. The dissecting aCSF was sucrose based and contained (in mM): NaCl (25), sucrose (188), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $NaHCO_3$ (26), glucose (25). The warm recovery aCSF contained (in mM): NaCl (119), KCl (1.9), NaH_2PO_4 (1.2), $MgSO_4$ (10), $CaCl_2$ (1), $NaHCO_3$ (26), glucose (10), kynurenic acid (1.5)(RBI, Natick, MA, USA), lactic acid (2) and 3% dextran. The room temperature aCSF was the same as the recovery aCSF without the kynurenic acid and dextran. The aCSF solutions were bubbled with 95% O_2 / 5% CO_2.

The intracellular solution contained (in mM) - K-gluconate (135), HEPES (10), EGTA (10), $CaCl_2$ (1), NaCl (6), $MgCl_2$ (1), ATP-Mg (3), GTP (0.3), leupeptin (0.1), glutathione (5), sucrose (20). The extracellular solution was composed of (in mM) - NaCl (140), KCl (1.9), HEPES (10), $MgCl_2$ (2), Glucose (10), $CaCl_2$ (1-2). The solutions used for recording isolated calcium currents were as follows: the intracellular solution contained (in mM) Cs-Methane-sulfonate (100), TEA-Cl (30), $MgCl_2$ (1), EGTA (10), HEPES (10), $CaCl_2$ (0.5), NaCl (5), ATP-Mg (3), GTP (0.3), and leupeptin (0.1). In some experiments 10-20 mM sucrose was added to stabilize the series resistance. The extracellular solution contained (in mM) - NaCl (105), TEA-Cl (30), KCl (1.9), HEPES (10), $MgCl_2$ (2), glucose (10), 4-AP (4), CsCl (2), $CaCl_2$ (2), 1μM TTX and 0.1% BSA. All HEPES solutions were oxygenated with 100% O_2.

All fine chemicals were obtained from either Sigma or Fisher. FPL-64176 (5.8 mM stock) was obtained from RBI.

Results

Large ventral horn cells in lumbar spinal cord slices were studied in current clamp mode using whole cell patch clamp techniques. In these cells intracellular injection of current pulses was able to elicit repetitive firing that ceased with the termination of the pulse (figs. 1A, 2A). In 3 cells an obvious post stimulus depolarization was observed, as illustrated in fig. 1A(i). This transient depolarization lasted between 2 and 4 seconds before the potential returned to baseline values. The amplitude of these depolarizations were such that sodium spikes were not elicited. In one cells the addition of calcium to the bath, although initially blocking spiking, was seen to increase the amplitude of the transient depolarization (fig. 1A).

The effect of cesium (2 mM) was tested on these cells (fig. 1B). The addition of this non-specific potassium channel blocker to the bath was seen to facilitate the expression of both the post stimulus depolarization (n = 3) and in one cell induced a larger amplitude depolarizations that showed no sign of inactivating over a period of seconds. The potential of this plateau-like event was sufficiently depolarized that action potentials were recruited and therefore sustained firing was seen after the termination of the current pulse (fig. 1Bii). The plateau potential could be terminated with a hyperpolarizing current pulse (fig. 1Biii). The development of both the transient depolarizations and the plateau was voltage-dependent as hyperpolarizing the cell with bias current would inhibit their expression while depolarizing bias would facilitate their appearance.

As with the application of cesium, the addition of the L-type calcium channel activator FPL-64176 (11.6 μM) to the bath solution was able change the response of a cell to depolarizing current injection (n = 3). Figure 2A illustrates a cell that

demonstrated repetitive firing only during the period of current injection. With the application of FPL-64176 the same stimulus evoked self-sustained firing (fig. 2B).

With a recording solution designed to block voltage-gated sodium and potassium currents (ie. to isolate voltage-gated calcium currents; Carlin et al., 2000a), prolonged calcium-dependent plateaux could be elicited with depolarizing current pulses (n = 4). The regenerative calcium plateaux were seen to slowly inactivate but could last for tens of seconds. Once activated the plateaux were unresponsive to further depolarizing current injections and small hyperpolarizing current pulses (fig 3A). As with the plateau potentials induced with cesium and FPL-64176, the calcium plateaux could be terminated with a hyperpolarizing current pulse (figs 3B). Furthermore, these calcium plateaux showed a similar voltage-dependency as the non-linear events (post-stimulus depolarizations and plateau potentials) in more physiological solutions. In a number of cells (n = 3) a second plateau level was revealed after either the spontaneous repolarization of the initial plateau (fig 3C) or after a hyperpolarizing current pulse (fig 3D). As with the initial plateau this second plateau could persist for seconds or could be terminated with injection of hyperpolarizing current.

Discussion

The main finding of this study is that motoneurones from the mouse lumbar spinal cord are capable of displaying non-linear membrane properties including both transient and sustained depolarizations in response to short depolarizing current pulses. The ability to trigger and terminate long depolarizations with short current pulses in cells with isolated calcium currents and in cells exposed to either cesium or FPL64176 satisfies the

two main criteria for establishing the presence of plateau potentials (Hartline et., 1988; Hartline and Graubard, 1992). As the expression of these plateau properties required the blockade or enhancement of certain ionic currents these properties can be considered to be latent.

It is likely that the relatively small depolarization seen following a train of action potentials result from the same conductances as the sustained plateau potential. Even though the relationship between these two events was not explored fully in this study similar transient depolarizations have been demonstrated in turtle spinal motoneurones and interneurons. In these cells the transient events developed into sustained depolarizations as either the amplitude of the depolarizing pulse or the amount of injected depolarizing bias current was increased (Hounsgaard and Kiehn, 1989; Hounsgaard and Kjaeruff, 1992).

Plateau potentials have been demonstrated in the lumbar spinal motoneurones in the cat (Hounsgaard et al., 1988; Crone et al., 1988; Lee and Heckman, 1998), rat (MacLean et al., 1997, 1998) and turtle (Hounsgaard et al., 1985; Hounsgaard and Kiehn, 1989). Because pharmacological assessment of currents is difficult in the in-vivo cat the ionic nature of the underlying current has not been confirmed. The voltage-dependence of the current and its response to both barium and tetraethylammonium (TEA) have led to the suggestion that it is mediated by voltage-dependent calcium channels (Schwindt and Crill, 1981, 1984). Using the adult turtle in-vitro preparation plateau potentials have been shown to be mediated by L-type calcium channels (Hounsgaard et al., 1985; Hounsgaard and Kiehn, 1989).

In neonatal rat spinal motoneurones, various forms of non-linearity have been elicited with the glutamate agonist NMDA (MacLean et al., 1998). At present it is not clear if these NMDA-dependent plateau potentials result from similar ionic conductances as those which produce plateau potentials in cat and turtle spinal motoneurones. Due to the fact that NMDA-induced voltage oscillations are dependent on L-type calcium channels in the turtle (Guertin and Hounsgaard, 1998), a potential role of the NMDA ionophore may be to supply an initial depolarizing drive for the activation of L-type channels which in turn could support the plateau-like events.

The present study demonstrates plateau potentials in mouse spinal motoneurones which are analogous to those seen in the in-vivo cat and turtle spinal cord preparations. The precise ionic mechanisms responsible for their production though are not completely resolved. Given that the post stimulus depolarizations and plateau potentials are likely the same phenomenon differing only in magnitude, a number of observations made in this study suggest that voltage-gated calcium channels underlie these non-linear events: 1) the calcium dependence of the post stimulus depolarizations (fig 1A); 2) the voltage dependence of both the post stimulus depolarizations and the plateau potential; and 3) the ability of calcium-dependent plateaux to mimic the properties of the plateau potentials seen in more physiological solutions. Moreover, the ability of an L-type channel agonist to facilitate a plateau response would suggest that L-type channels in particular likely mediate the plateau potentials seen in these cells.

The ability of cesium to promote plateau potentials has been demonstrated in the turtle where it has been suggested that the expression of a plateau depends on a balance between the L-type calcium current and a potassium current (Hounsgaard and Mintz,

1988; Booth et al., 1997). Again, the pharmacological data presented here is consistent with a potassium current counteracting the L-type calcium current in mouse spinal motoneurones. Therefore, either reducing the potassium current or enhancing the L-type calcium current could lead to the expression of a plateau potential.

A dendritic current possibly contributes to the plateau

Evidence from both the cat and the turtle suggest that the channels mediating the current underlying the plateau potential are located in the motoneuronal dendrites (Hounsgaard and Kiehn, 1993; Bennett et al., 1998). L-type calcium channels have been previously demonstrated in the dendrites of mouse spinal motoneurones (Carlin et al., 2000b). Evidence presented in this paper indicates that a portion of the current underlying these plateau may also be dendritic in origin.

Bistability is the result of an "N" shaped I-V curve with the two zero-current crossing points that have positive slopes. In current clamp these crossing points represent stable membrane potentials. When voltage clamping the somata of mouse spinal motoneurones, the presence of relatively electrotonically remote dendritic calcium currents was seen to create a low voltage hysteresis in the current-voltage relationship in a number of cells (Carlin et al., 2000b). This hysteresis occurred during the descending phase of a voltage ramp command and was seen to create a third positive slope zero-current point on the I-V curve. The presence of a third crossing point at a voltage between the stable resting potential and the plateau potential therefore predicted the existence of three stable membrane potentials in these cells - with a stable potential of intermediate voltage being the result of the dendritic currents. As illustrated in figures 3C

and 3D, tri-stability was seen in a number of these cells. This suggests that at least of portion of the initial (larger) calcium plateau was mediated by dendritic currents with the remainder being mediated by somatic conductances. The relative contribution of either somatic or dendritic calcium currents to the plateau potential seen in more physiological solutions is still unclear and will likely depend on interplay of the calcium channels in the two locations with various potassium channels.

Functional significance

Spinal motoneurones innervating the limb and trunk muscles are responsible for the maintenance of posture (Eken et al., 1989) while sacral sphincter motoneurones control the muscles responsible for maintaining continence (Paroschy and Shefchyk, 2000). The ability of these cells to produce self-sustained firing in response to a short stimulus provides an efficient means to produce sustained muscular contraction without continuous synaptic drive. During phasic contractions, as occurs during locomotion, evidence has been shown that the same cellular mechanisms are called upon in order to produce high firing rates upon recruitment and therefore more efficient muscle contraction (Gorassini et al., 1999). A system in which to study non-linear properties in these cells is an initial step in understanding their underlying mechanisms.

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Figure legends

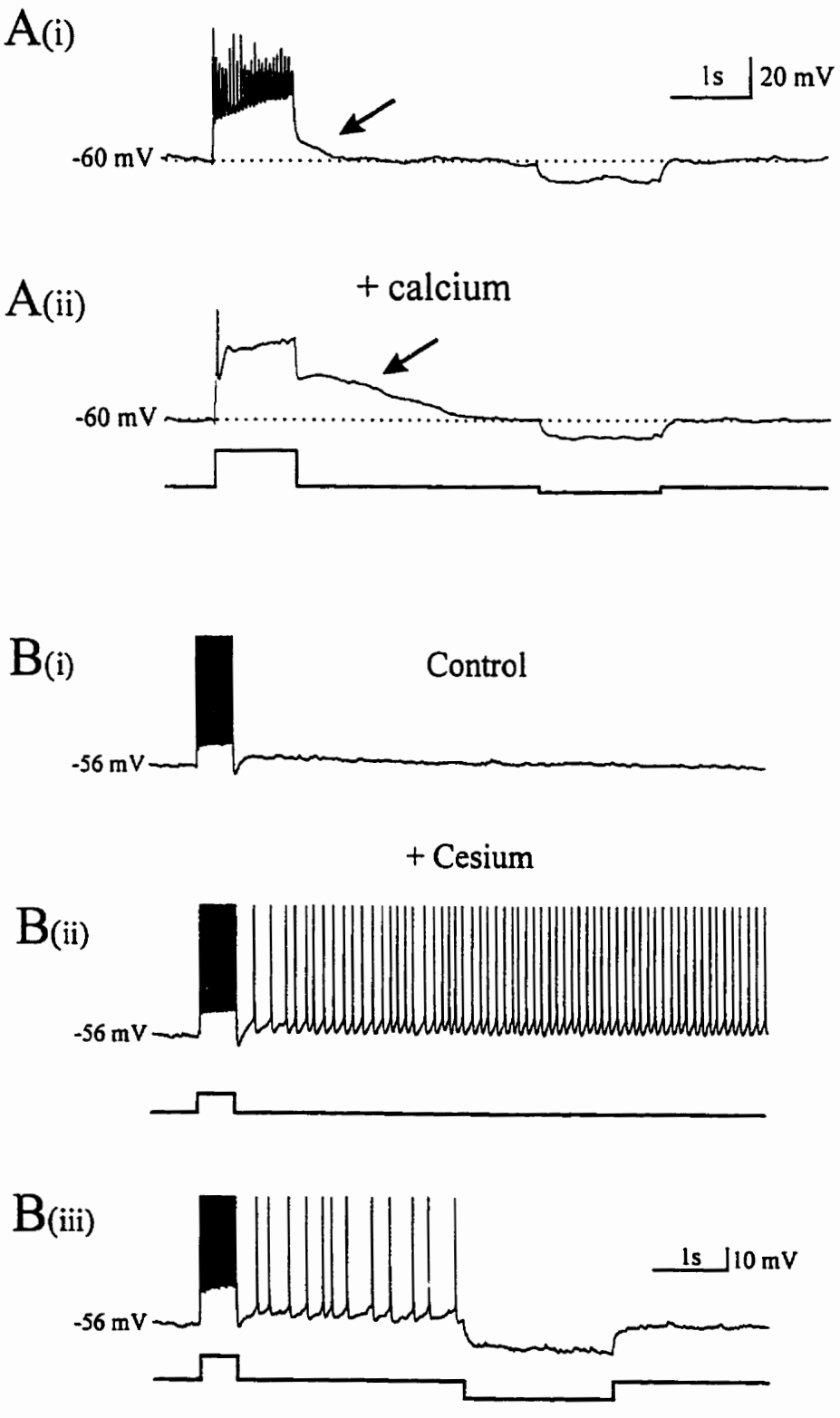
Figure 1: Non-linear membrane properties. **A(i).** A transient depolarization spontaneously occurring at the end of a current pulse (arrow). The amplitude and duration of this transient depolarization was increased with the addition of 1 mM calcium to the bath (**A(ii)**). Records in Ai and ii are both averages of 4 consecutive traces. Depolarizing and hyperpolarizing current pulses are 100 and –20 pA respectively. **B.** Current injection into a P11 cell elicited repetitive firing which ceased with termination of the pulse. **B(i).** After the addition of 2 mM cesium to the bath the same current injection produced repetitive firing that outlasted the stimulus. The repetitive firing could be terminated with a hyperpolarizing current pulse (**B(ii)**). In figure **B** depolarizing and hyperpolarizing current pulses are 600 pA and –100 pA respectively.

Figure 2: Plateau potentials are facilitated with the L-type calcium channel agonist FPL-64176. **A.** A depolarizing current pulse does not produce self-sustained firing in this P9 cell until 11.6 μM FPL-64176 is added to the bath solution (**B**). As with the cesium induced plateau, the underlying depolarization could be terminated with a hyperpolarizing current pulse. Depolarizing and hyperpolarizing current pulses are 400 pA and –50 pA respectively.

Figure 3: Isolated calcium currents can produce plateau potentials with similar properties as those seen in more physiological solutions. **A.** A small depolarizing current pulse elicits a regenerative calcium current and a slowly inactivating plateau potential that lasts for seconds in a P12 cell. Once the plateau is initiated further depolarizing current does

not change the amplitude of the plateau. Similarly, small hyperpolarizing pulses are ineffective in terminating the plateau. **B**. Once the plateau current has inactivated sufficiently the same amplitude hyperpolarizing current pulse can terminate the plateau (same cell as in A). **C**. The initial plateau spontaneously repolarizes to a second plateau level (arrow) in this P10 cell. The second plateau can be terminated with hyperpolarizing current injection. **D**. The presence of a second plateau level (arrow) can be also be revealed with a hyperpolarizing current pulse.

Figure 1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1s
20 mV
-60 mV
A(ii)
+ calcium
-60 mV
B(i)
Control
-56 mV
+ Cesium
B(ii)
-56 mV
B(iii)
1s
10 mV
-56 mV

Figure 2

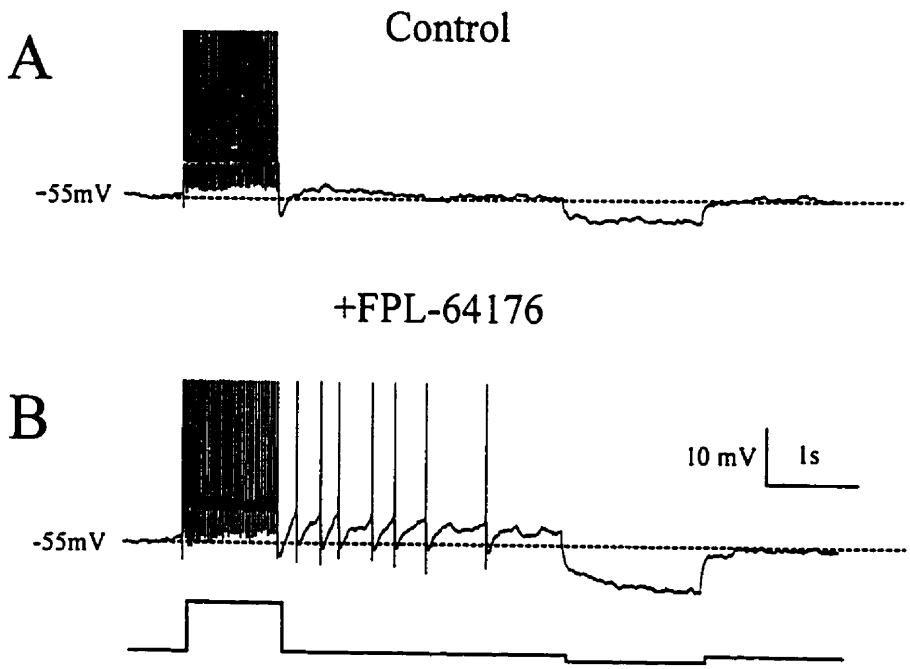
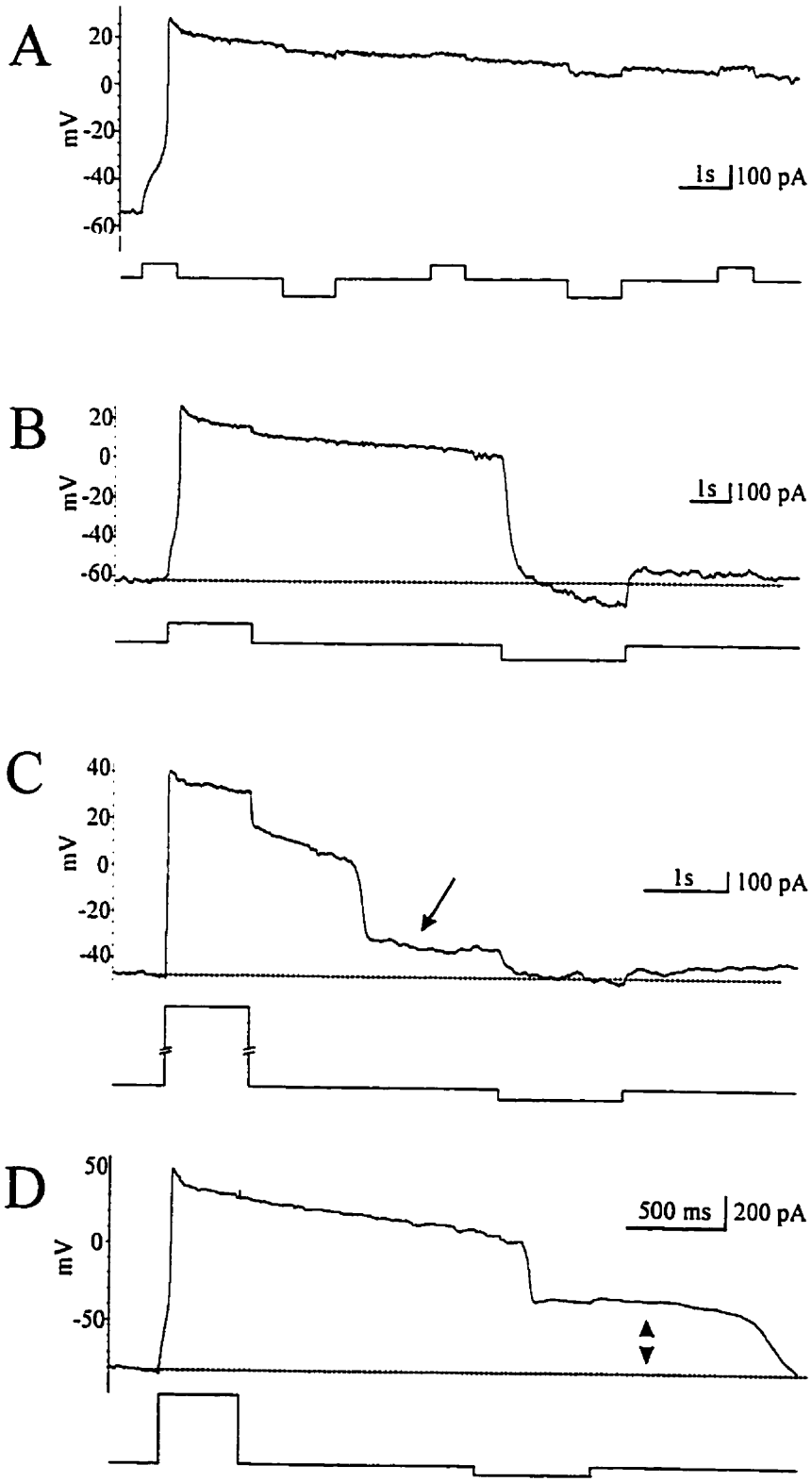


Figure 3


A
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C
40
20
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-20
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1s
100 pA
D
50
0
-50
mV
500 ms
200 pA

General Discussion

Synopsis

Because plateau potentials are thought to be important in both the maintenance of posture and in locomotion, it was reasoned that the ionic mechanisms of plateau potentials in mammalian spinal motoneurones would best be assessed in animals of sufficient age to bear weight and walk. Therefore, in order to directly assess the calcium currents in spinal motoneurones, a "functionally mature" spinal cord slice preparation was developed. With this preparation it was demonstrated that motoneurones older than P9 express a number of different functional calcium channels including L-type (Carlin et al., 2000a). By recording these currents at the soma in a low extracellular calcium concentration (1 mM), it was determined that these cells possess ω-conotoxin-sensitive N-type, ω-agatoxin-sensitive P/Q-type, an antagonist resistant R-type and a dihydropyridine- and FPL-64176-sensitive L-type current. It was noted that a small number of cells also displayed low-voltage activated T-type currents. It was concluded that this was a viable preparation in which to study voltage-gated calcium currents in mammalian spinal motoneurones. More importantly for the evaluation of plateau potential mechanisms, a detectable L-type current was demonstrated in these cells.

Using the same somatic recording procedure and elevated extracellular calcium concentrations (2 mM) it was shown that dendritic calcium currents could be recorded in these cells (Carlin et al., 2000b). These dendritic currents were seen as late-onset low-amplitude currents in response to step depolarizations and as a low voltage clockwise current hysteresis in response to slow voltage ramp commands. Both of these dendritic

current indicators were shown to be sensitive to L-channel pharmacological manipulations: they were blocked by dihydropyridines and enhanced by the L-type channel activator FPL-64176. It was concluded that a subpopulation of non-inactivating L-type channels were present in the dendrites of functionally mature mouse lumbar spinal motoneurones. This finding is consistent with results in turtle spinal motoneurones.

The presence of dendritic non-inactivating L-type currents in these motoneurones would suggest that under the appropriate conditions these cells are able to produce plateau potentials. In modeling studies a dendritic location of the plateau generating current is an essential component of the plateau potential mechanism. Furthermore, this dendritic location is consistent with electrophysiological data collected in both turtle and cat spinal motoneurones, suggesting that this is the same mechanism producing plateau potentials in these cells. In the third paper (Carlin et al., 2000c) non-linear membrane properties were demonstrated in mouse spinal motoneurones under current clamp conditions. In this study these cells were shown to possess the ability to produce post-stimulus depolarizations and plateau potentials in response to various compounds that have been shown to elicit non-linear behaviors in other cells. The ability of these cells to produce plateau potentials with the application of potassium channel blockers and the L-type calcium channel agonist FPL-64176, suggests that the current mediated by L-type calcium channels is capable of producing a plateau potential and that this current may be kept in check by an opposing potassium current in-vivo. The ability of an L-type calcium current to produce a plateau potential is consistent with our demonstration of a dendritic location of L-type channels in mouse spinal motoneurones.

Taken together these studies demonstrate that spinal motoneurones from mice with functionally mature spinal motor systems possess functional L-type calcium channels, a portion of which are located in dendrites of these cells. Given that a dendritic location of the persistent inward current underlying the plateau potential is consistent with electrophysiological and modeling data, the presence of non-inactivating calcium channels in the dendrites of these motoneurones make these calcium channels likely candidates for mediating plateau potentials. Furthermore, the demonstration that a current sensitive to L-channel pharmacological agents is capable of sustaining a plateau potential (paper 3) strongly suggests that the dendritically located L-type calcium channels are responsible for the production of plateau potentials in mammalian motoneurones.

Role of neuromodulators

As stated in the introduction, a plateau potential is produced through a region of negative slope conductance in the steady-state current-voltage relationship of the cell. A plateau potential does not usually occur in the absence of modulation of some sort. Thus, induction of a plateau potential requires unmasking of the negative slope conductance. In theory an increase in the negative slope conductance of a cell can be accomplished by altering the balance between two competing currents, an outward hyperpolarizing potassium current and an inward depolarizing calcium current. In the in-vitro setting this can be evidenced by the induction of plateau potentials with the application of potassium channel blockers like cesium, barium, TEA, apamin or the L-channel activator Bay-K 8644 (Hounsgaard and Kiehn, 1989; Morisset and Nagy, 1999; Carlin et al., 2000c).

Modeling data by Booth et al (1997) support the idea that the plateau is the result of a balance between a calcium and potassium current. These simulations indicate that

modulation (a decrease in the conductance) of K_{Ca} channels by approximately 40% is required for induction of plateaux. This amount can be reduced to approximately 30% if the conductance through L-type calcium channels is increased by 10%. If a concomitant hyperpolarizing shift in the activation curve by as little as 1-2 mV occurred, the reduction of K_{Ca} required would be much less.

A number of neuromodulators have been shown to induce plateau potentials. In the adult turtle spinal cord slice preparation, nifedipine-sensitive plateau potentials can be induced with the application of serotonin (5-HT; Hounsgaard and Kiehn, 1985) muscarine and group I metabotropic glutamate receptor agonist *cis*-ACPD (1-aminocyclopentane-cis-1,3-dicarboxylic acid; Svirskis & Hounsgaard, 1998). Similarly, in guinea pig trigeminal motoneurones 5-HT is capable of inducing plateaux that are partially reduced by dihydropyridines (Hsiao et al., 1998). In addition to 5-HT, L-DOPA and clonidine have been shown to produce plateau potentials in spinal cats (Conway et al., 1988). However, it is unclear if calcium currents were increased or a competing potassium current was blocked. The question that remains unanswered is - which specific channels are being affected by the neuromodulatory substances in the process of generating a plateau?

It is very clear that calcium currents can be modulated by numerous endogenous transmitters and their agonists (for review see. Anwyl, 1991). The vast majority of the modulatory action involves a reduction of current flow via a G-protein mediated pathway. Channel inhibition is seen during serotonergic modulation of N and P/Q-type channels in Xenopus spinal neurones (Sun and Dale, 1998) and caudal raphe neurones (Bayliss et al., 1997). In Rohon-Beard cells of the Xenopus both LVA T-type and N- and

P/Q-type currents are reduced by 5-HT application. In cerebellar granule cells the $GABA_b$ agonist baclofen inhibits not only N- and P/Q-type but also L-type channels (Amico et al., 1995). L-type channels are also inhibited in rat pinealocytes by noaradrenaline and its agonist isoproterenol (Chik et al., 1997). Muscarine and the muscarinic agonist carbachol also reduced the probability of L-channel opening in CA3 hippocampal neurones (Fisher and Johnston, 1990).

Modulation of calcium channels can also lead to an enhancement of the current. For example, isoproterenol enhances the L-current and N-current in CA3 hippocampal neurones (Fisher and Johnston, 1990). Similarly, the α-adrenergic agonist phenylephrine, increases the L-current in rat ventricular cells (Liu et al., 1994ab) while the dopaminergic (D1) receptor agonist enhances the L-current in neostriatal spiny neurones (Hernandez-Lopez et al., 1997). As can be seen from these varying result, the effect of any neuromodulator is likely to be cell type specific.

Serotonin has been shown to directly affect calcium channels in motoneurones. In cervical spinal motoneurones 5-HT leads to increased LVA currents without affecting HVA currents (Berger and Takahashi, 1990), while in hypoglossal motoneurones 5-HT has been shown to decrease currents mediated by N and P/Q-type channels without affecting LVA currents (Bayliss et al., 1995).

The question therefore is, do the neuromodulators that produce plateau potentials modulate (specifically facilitate) voltage-gated calcium currents in mammalian spinal motoneurones? The preparation presented in this thesis could be easily used to answer this question. Using this preparation, various neurochemicals could be tested to determine whether or not the voltage-gated calcium channels are being modulated and then could

easily isolate the specific channel subtype(s) affected. This preparation is particularly well suited for this because it uses a mammal, minimizing phylogenetic concerns, and because this preparation uses older animals (i.e. functionally mature mice). The use of older animals is critical for two reasons: 1) developmental changes in both the current density (McCobb et al., 1989) and immunolabeling (Jiang et al., 1999a) of certain calcium channel sub-types have been demonstrated in mammalian spinal motoneurones. Modulation of certain calcium currents may therefore be undetectable in embryonic and early postnatal preparations due to the low channel number. 2) functional maturity at the cellular level may not only include changes in the complement of channel subtypes or their densities but may also include changes in the modulatory system governing the channels. In hypoglossal motoneurones it has been demonstrated that the expression of 5HT receptor subtypes changes during the period when the serotonergic projections reach these cells (Talley et al., 1997). The use of older animals becomes important when one considers that adult-like serotonergic innervation of lumbar motoneurones is established at approximately two (Tanaka et al., 1992) to three (Bregman, 1987) weeks after birth. Therefore, intracellular signaling systems may also be developing between the embryonic stage and the first few postnatal weeks. Assessment of these intracellular signaling pathways in a motor system capable of exhibiting a mature motor behaviour would seem to be critical for extrapolating results to the physiology of adults.

Previous immunohistochemical results demonstrated that the somata of spinal motoneurones express high levels of L-type channels constructed with the α_{1C} subunit while the dendrites of these cells express high levels of L-type channels constructed with the α_{1D} subunit (Jiang et al., 1999b). This subcellular separation of the two functionally

similar L-type channels along with the evidence for a dendritic location of the plateau-generating channels leads to the prediction that the two L-type channels would have different modulation profiles. Given the preferential modulation that certain transmitters have on calcium channel subtypes, it would seem reasonable to suggest that the two different L-type channels may be modulated differently by the same neuromodulator. This system would then allow the two channel types in different parts of the cell to act independently under the same neuromodulatory influence. Support for this suggestion comes from work in the *Xenopus* expression system where the PKA phosphorylation sites on the two α_1 subunits have been determined. The location of these binding sites are such that they would be absent from the α_{1C} subunit because of the known in-vivo post-translational modifications this subunit undergoes but would be present on the α_{1D} subunit (Mitterdorfer et al., 1996). Considering mammalian spinal motoneurones, the prediction would be that the neuromodulators shown to induce plateau potentials would increase the whole cell conductance and/or shift the activation voltage of the α_{1D} L-type channels so as to promote the expression of plateaux while having a limited or a different effect on the α_{1C} L-type channels.

Possible role of non-L channels in dendrites

Experiments described in paper 2 demonstrated that the dendrites of spinal motoneurones express L-type calcium channels that are capable of delivering a sustained current to the cell soma. Some evidence was also presented in that paper suggesting that the dendrites also contain non-L-type calcium channels, in particular N and P/Q- type channels. This was demonstrated by the ability of ω-agatoxin to reduce the late-onset currents in a few cells. Furthermore, during slow ramp voltage commands a few cells

demonstrated a calcium spike on the ascending portion of the ramp which was blocked with the co-application of ω-agatoxin and ω-conotoxin, suggesting the presence of a relatively rapidly inactivating current in the unclamped dendrites. This was concluded because if the conductance was in the clamped soma it would have inactivated during the slow ramp. Immunohistochemical studies in the mouse using anti-α_{1B} antibody provide some evidence for the presence of N-type channels in the dendrites of spinal motoneurones (unpublished observations). However, given the close apposition of synaptic boutons to dendrites and previous demonstration of non-L-type channels in these boutons (Westenbroek et al., 1998), definitive proof is lacking.

The evidence presented in paper 2 for the expression of these non-L-type channels in motoneuronal dendrites has some interesting implications. If in fact N- and P/Q-type channels are in the dendrites, then it would be reasonable to suggest that these channels contribute to the generation of the depolarizing drive underlying the plateau potential. This contribution could be either in the form of actual sustained current drive if the current was a non-inactivating current, or it could be in the form of an initial depolarization to aid in depolarizing the membrane to a potential at which the L-channels were able to activate. As discussed in the introduction, the established molecular diversity of the various calcium channel subunits and the unique modulating effects each has on the kinetic of the resultant current make possible the existence of both non-inactivating and inactivating non-L-type currents. Even though at present very little is known about the specific subunit isoforms expressed in spinal motoneurones, in paper 1 evidence was presented for a somatic ω-agatoxin-sensitive current with both rapidly inactivating and

non-inactivating components, as well as evidence for a non-inactivating N-type current located in the soma of these cells.

If in the steady-state a portion of the non-L-type channels are inactivated, (and therefore require a hyperpolarization to de-inactivate the channels), the role of this current may be to give the initial depolarizing drive to the plateau potential. This scenario would indicate some role for inhibitory input in the generation of plateaux. Evidence for an inactivating "booster" current has been demonstrated in neurons of the subthalamic nucleus (Beurrier et al., 1999). In these cells a T-type current was shown to produce the initial depolarization leading to L-channel activation and a plateau potential. T-type currents will be discussed more in a later section.

If the non-L-type current is non-inactivating it could contribute to the sustained depolarizing drive underlying the plateau potential. Some evidence for a combined plateau generating current has been found in rat dorsal horn neurons (Morisset and Nagy, 1999) and guinea pig trigeminal motoneurones (Hsiao et al., 1998). In these latter cells the sustained current is sensitive to both dihydropyridines and TTX while in the former cells an L-type calcium current and I_{CAN} interact. Evidence from the turtle does not discount the possibility of a combined L-and non-L-type current. The fact that an L-type calcium channel blocker such as nifedipine is capable of blocking the plateau would simply suggest that the contribution by the L-channels is a significant portion of the current drive. A similar argument could be used for the ability of the L-channel activator Bay-K 8644 to induce a plateau. Evidence has not been presented in the turtle that N- or P/Q-type channel blockers are incapable of blocking the plateau.

In considering the possibility of a combined L-and non-L current, it must be considered that the modulatory site for the neuromodulators capable of inducing a plateau could be the non-L-type channels. The possibility also exists that different neuromodulators produce the same result (a plateau) by acting on different calcium channel types. Again, the preparation developed for this thesis could be used to answer these questions.

Low voltage-activated currents

While recording currents from the somata of motoneurones, the presence of low voltage-activated T-type channels was observed in a small percentage (16%; Vh = -60 mV) of functionally mature motoneurones (paper 1). Given that we know the mRNA encoding at least two different α_1 subunits for T-type channels are expressed in adult spinal motoneurones (Talley et al., 1998), one explanation for the low number of cells in which this current is seen is that these channels are located on, or move to, the dendrites during postnatal development. Due to the described postnatal changes in the dendritic morphology of certain motoneurones (hypoglossal - Nunez-Abades et al., 1994; lumbar - Ulfhake et al., 1988), dendritically located channels may be essentially removed from perisomatic regions and may not be detected (or detected infrequently) in these cells using somatic recordings. If this is occurring in spinal motoneurones, then these channels may be in a favorable position to contribute to the initial depolarizing drive as discussed above for an inactivating non-L-type current. Since serotonin has been shown to enhance T-type current in early postnatal spinal motoneurones (Berger and Takahashi, 1990), this co-localization of T- and L-type channels may partly explain the ability of this

neuromodulator to elicit plateau potentials in spinal motoneurones from a number of species.

Placement /pattern of L-channels in the dendrites

When the extracellular calcium concentration was increased from the normal 1 mM to 2 mM a number of cells demonstrated the late-onset and hysteretic currents previously described (see paper 2). A number of cells also demonstrated multiple late-onset currents. That is, with a single voltage step multiple levels of persistent current were activated. We refer to this type of waveform as a "staircase". A total of 11 cells were seen to demonstrate this waveform with the number of "steps" ranging from 2 to 6. One interpretation of this phenomena is that the multiple steps represent different dendrites being activated and sequentially contributing current to the soma. The obvious conclusion from this interpretation is that individual dendrites in a given cell are capable of bistable behaviour. It may therefore be speculated that in order for a given cell to produce a plateau potential a critical number of dendrites need to be "turned on" and contribute current to the soma. A corollary of this would be that cells in which neither late-onset nor hysteretic currents were seen, are cells that are either incapable of producing plateau potentials because they are intrinsically different or during the slicing procedure a critical number of bistable dendrites were removed.

Modeling experiments using the same neuromorphic motoneurone model as used in paper 2 have been initiated to explore further the nature of the staircase waveform. These simulations suggest that the one spatial arrangement of voltage-gated channels capable of demonstrating the staircase waveform is one in which the channels are located at varying distances from the soma on different dendrites. These modeling data excluded

the possibility that the waveform resulted from sequential activation of multiple clusters of channels located on a single dendrite. Experimental data supporting our modeling results has been obtained in neocortical pyramidal cells (Oakley et al., 1999). In this study a calcium-dependent plateau potential was induced in these cells with the iontophoretic application of glutamate to various sites along the apical dendrite. If the plateau was activated at a proximal location, application of glutamate at more distal locations did not increase the amplitude of the plateau. The distal current was effectively shunted by the more proximal conductance. These authors suggested the more distal dendritic arbor was functionally "pruned". On the other hand if a plateau was generated by stimulating a proximal portion of the apical dendrite followed by stimulation of a basal dendrite, the amplitude of the plateau potential was increased. In this case the current produced from the two locations was able to summate. It is hypothesized that a similar shunting of distal currents would occur in our motoneurones if multiple hotspots were located on a single dendrite. This hypothesis seems to be supported by our initial modeling studies.

Consequences for signal integration

If the hypothesis is correct and multiple dendrites are capable of demonstrating bistability, an interesting question that arises is whether all dendrites have this ability or if only a select few are able to demonstrate this behaviour. If all of the dendrites have bistable properties and are "turned on" the cell would no longer be responsive to depolarizing afferent input (Kiehn and Eken, 1998). Essentially this was demonstrated in neocortical pyramidal cells by Oakley et al (1999) and was discussed in a previous section. Briefly, in these cells once the plateau was activated information arriving to the

distal dendrites was effectively shunted. If all the dendrites do not have the potential for bistability then the possibility of differential synaptic integration between dendrites exists. In other words, given the same input to different dendrites and the appropriate neuromodulatory environment, the signal seen at the soma could be quite different depending on the properties of the dendrite. In one instance the dendrite would act linearly and deliver an attenuated post-synaptic potential (PSP) to the soma while in the other case the PSP would be converted to a sustained depolarizing drive to the soma. Evidence suggesting this may occur in the cat has recently been presented in a review by Heckman and Lee (1999). Referring to unpublished data these authors demonstrated the induction of a plateau with group Ia afferent stimulation. During the plateau further group Ia synaptic input was delivered to the cell which produced higher rates of firing during the afferent stimulation. With removal of the stimulus the firing rate returned to the initial plateau level. These authors suggested that the second excitatory input acted on a portion of the dendritic tree that was not in the bistable state and therefore this portion of the tree was still able to deliver current to the soma. This type of behaviour could be explained if some dendrites did not contain plateau-generating channels or if these channels occurred at a lower density and could not sustain a plateau.

There is evidence suggesting that the various dendrites of a given motoneurone have different properties and can function independently. Work with turtle motoneurones has demonstrated that individual dendrites can perform signal integration in an autonomous manner (Skydsgaard and Hounsgaard, 1994). Also the dendrites of single motoneurones have been shown to vary widely in morphology and therefore in passive electrotonic structure (Bras et al., 1987; 1993). Work in neocortical cells has shown that

the type of firing response (train, single spike, plateau, etc.) elicited by dendritic current injection is correlated with the dendritic morphology (Kim and Connors, 1993). This suggests that individual dendrites in a motoneurone may have different functional output based on their morphological differences. These morphological differences may also affect the ability to produce plateau potentials. Simulations using dendrites differing in length and caliber suggest that the ability for a dendrite to demonstrate bistability is governed by its morphology as long and thick dendrites are more stable at the plateau voltage than relatively thinner and shorter dendrites (Gutman, 1991). Therefore, the suggestion that only a fraction of the dendritic tree is capable of bistable behaviour would seem plausible.

Given the above evidence suggesting a difference in the ability of certain dendrites to produce plateau potentials, and the evidence that afferent inputs to motoneuronal dendrites are differentially distributed, a reasonable hypothesis would be that certain inputs are more effective at activating a plateau than others. Burke and Glenn (1996) demonstrated that group Ia afferents projecting onto triceps surae or plantaris motoneurones in the cat were preferentially distributed onto the dendrites oriented in the rostral-caudal axis (63%) while dendrites in the transverse axis contained very few group Ia contacts. A similar differential distribution pattern of vestibulospinal terminals was found on neck motoneurones in the cat (Rose et al., 1995). Furthermore, a functional difference in certain afferent inputs has also been demonstrated in cats treated with nialamide and L-dihydroxyphenylalanine (DOPA cat ; Brownstone et al., 1994). In this study voltage-dependent excitatory post-synaptic potentials (EPSP's) were recorded in motoneurones resulting from stimulation of the ipsilateral group Ib afferents or

contralateral flexor reflex afferents (FRA) but were not detected when stimulating the monosynaptic group Ia afferents. If this scenario holds true and only certain inputs are capable of eliciting a plateau while individual synaptic events onto other dendrites are discriminated by the somatic spike generating mechanism, then the complexity of signal integration possible during bistable behaviour would be greatly increased. The ability to produce bistable responses should therefore not be thought of as simply a digital "on – off" property.

One approach to begin answering the question of whether all dendrites have the potential for bistability would be to perform confocal microscopy on a filled motoneurone that has been labeled with the α_{1D} antibody. Even though this would not definitively demonstrate that the dendrites are bistable an absence of α_{1D} labeling would rule out the possibility of the mechanism discussed in this thesis. The possibility that different dendrites use different mechanisms to produce plateaux still exists as well (ex. non-inactivating P/Q-type calcium channels, persistent sodium channels, NMDA channels). An electrophysiological approach to this question could involve exploring the effects of different afferent input to an ongoing plateau potential. Similar to the experiments of Heckman and Lee (1999), the effects of group Ia input would be assessed when delivered during the plateau but also the effects of other excitatory afferent inputs. If other afferent systems contact a different population of bistable dendrites, then the voltage of the sustained plateau should be increased as the size of the current source is increased (i.e. summation). The demonstration of voltage-dependent EPSP's as a result of contralateral FRA stimulation and the ability of these afferents to elicit plateaux in the

DOPA cat (Corne et al., 1988) make the FRA system a good system to test for this ability.

Evidence for a proximal location of the dendritic plateau generating channels

If the channels responsible for generating the plateau potential are located in the dendrites of motoneurones, one might ask, - where along the dendrite are these channels located? Although there is no definitive evidence indicating any distinct localization of these channels, some information can be derived from plateau behaviour in these and other cell types.

Experiments testing the ability of spatially distant inhibitory inputs to turn off a dendritic calcium conductance were performed in CA3 hippocampal pyramidal cells (Miles et al., 1996). These cells are ideal for such experiments because: 1) it is possible to find inhibitory cells which synapse exclusively onto either the distal dendrites or the proximal dendrites and soma; 2) these cells contain dendritically located voltage-gated calcium channels; and 3) it is possible to record from both the soma and dendrites of these cells. In these experiments it was demonstrated that an inhibitory post-synaptic potential (IPSP) generated in the dendrites was more that three times as efficient in inhibiting a calcium spike as an IPSP generated in the soma. This was true even when the somatic IPSP was adjusted so that its amplitude (measured in the dendrite) was equal to the dendritic IPSP (Miles et al., 1996). It was therefore concluded that effective inhibition was not simply a result of the membrane voltage deflection but rather a crucial aspect was an associated conductance increase in the membrane.

The above data indicating that effective inhibition of a distal conductance requires spatial coupling and the demonstrated effectiveness of inhibitory input to "turn off"

plateau potentials in spinal motoneurones may provide a clue as to the dendritic location of the plateau-generating channels. In the decerebrate cat, plateau potentials can be turned off with various inhibitory inputs including: group Ia inhibitory input (Bennett et al., 1998), Renshaw recurrent inhibitory input (Crone et al., 1988; Baldissera et al., 1991; 1994) and input from group II and III muscle afferents (Hounsgaard et al., 1984, 1988; Crone et al., 1988). Histological data have shown that the majority of inhibitory inputs are located very proximal to the soma of lumbar motoneurones in the cat. Ornung et al (1998) found that 61% of boutons terminated on stem dendrites with 69% of these immunoreactive for GABA and/or glycine. More specifically, Burke et al (1971) concluded that the Ia inhibitory synapses are located very proximal to the soma if not on the soma. Others have suggested a similar location for Renshaw terminals (Fyffe, 1991; Eccles et al., 1954). This proximal location of inhibitory input and its effectiveness in turning off the plateau may suggest that the plateau generating channels are relatively close to the soma as well.

Magee (1998) has suggested that a proximal location of inhibitory inputs may in certain cases be as effective in turning off a distal conductance as more distally located inhibitory inputs. This "amplitude normalization" is thought to occur as a result of the spatial gradient of I_h channels in hippocampal CA1 pyramidal cell dendrites. The seven fold increase in the current density of I_h channels from the soma to the dendrites (a portion of which are open at the resting membrane potential) creates a spatial gradient in the membrane input resistance (Magee, 1998, 1999). If a similar amount of hyperpolarizing current is injected into both the distal and proximal dendrite, initially the IPSP will be larger at the proximal location because of the higher input resistance in this

location compared to the initially smaller IPSP occurring in the high conductance distal membrane. Electrotonic decay of the proximal IPSP as it travels distally over an increasingly leaky membrane will result in an IPSP approximately equal to the initially smaller distally generated IPSP.

Could a similar mechanism occur during a plateau potential? Although the gradient in resting conductance of I_h could be considered qualitatively similar to the difference in conductance between proximal and more distal portions of the dendrite during a plateau potential, there is an important difference. In the case of an activated plateau the more distal dendrite would also be at a more depolarized potential and therefore driving force for the inhibitory IPSP will be greater distally. The result of this greater driving force would be a more effective inhibition in the depolarized portion of the dendrite. This would be equivalent to the results in CA3 pyramidal cells by Miles et al (1996; discussed above) – the inhibitory input is most effective at the site of the depolarization.

The above evidence for a relatively proximal location of the dendritic channels responsible for the plateau potential is consistent with the immunohistochemical data presented in paper 2. As can be seen in the confocal image in figure 6, the intense α1D labeling begins at approximately the second or third branch point in the neurone pictured. In absolute distance this is approximately 50 μm from the soma. Furthermore, modeling studies by Ken Rose, which used morphological data obtained from motoneurones of the cat cervical spinal cord and electrophysiological data reported by Bennett et al (1998), have estimated these channels to be approximately at the second branch point or

approximately 200 μm from the soma of these relatively larger cells (personal communications).

Activation voltage of dendritic L-channels

Evidence in both turtle (Hounsgaard and Khien, 1993) and cat (Lee and Heckman, 1996; Bennett et al., 1998) motoneurones suggest that the persistent current underlying the plateau potential arises, at least partly, from the relatively electrotonically remote dendrites. A number of papers have referred to this persistent current as a low voltage-activated current. One reason for this label is that the potential of the sustained plateau is usually 5 - 20 mV above resting membrane potential (cat - Hounsgaard et al., 1986; turtle - Hounsgaard and Kiehn, 1989), which in absolute terms would place its activation well below the activation voltage of traditional HVA calcium currents (see section – Voltage-gated calcium channels in motoneurones). The persistent inward current has also been demonstrated under voltage clamp during slow voltage ramp commands. In these experiments the onset of the negative slope conductance is seen to activate approximately 3-30 mV above resting membrane potential (Schwindt and Crill, 1977) or at approximately –43 mV to –50 mV (Lee and Heckman, 1998; Paroschy and Shefchyk, 2000) again suggesting a low voltage-activated current. Evidence presented in paper 2 demonstrated that the presence of voltage-gated calcium channels in the dendrites of spinal motoneurones is correlated with a lower voltage of activation of the negative slope conductance as recorded in the soma. That is, the negative slope conductance activated at a significantly lower voltage in cells displaying hysteresis during voltage ramp commands than in cells without hysteresis (-47 mV vs. -38 mV). This would suggest that these dendritic channels provide the low voltage-activated nature of the persistent inward

current. It is still unclear, though, if the dendritic channels mediating plateaux are a unique class of low voltage-activated channels or if it is the dendritic location which confers the appearance of being low voltage-activated. The presence of these channels in the dendrites and the fact that all of the recordings have been made in the somata of these cells makes an accurate determination of the activation voltage of the underlying channels difficult (see discussion in paper 2). The determination of the activation voltage is complicated by a number of factors that may give these dendritic channels the appearance of being low voltage activated while in fact the dendritic channels are activating at traditional high voltage-activated (HVA) potentials.

One factor that arises when evaluating this current in voltage clamp, and that has already been discussed in paper 2, is that these channels are outside of the space clamp and therefore the voltage cannot be controlled in these regions (Lee and Heckman, 1996; Muller and Lux, 1993). Another complicating factor occurring under current clamp arises from the assumption that the entire cell is isopotential. Data obtained with voltage-sensitive dyes has shown that the intramembrane potential may not be uniform within a cell (Bedlack et al, 1994) and therefore the potential seen by dendritic channels may be more positive than that seen by channels in the soma. Moreover, given our present knowledge of the non-uniform distribution of both leak and voltage-gated channels open at resting potentials in certain neurons (Stuart and Spruston, 1998; Magee, 1998; Clements and Redman, 1989), it may not be unreasonable to suggest that the resting membrane potential in the dendrites could be more depolarized than that in the soma (Bedlack et al., 1994; for ex. see Stuart and Spruston, 1998). If the dendritic channels are more depolarized, be it because of lack of space clamp in voltage clamp recordings or

because of a difference in the resting membrane potential in current clamp recordings, then it is possible that when recording this dendritic current or its resultant potential change in the soma the current is only an *apparent* LVA current. Presently information regarding the resting membrane potential of motoneuronal dendrites is lacking.

Similar to the difference in the voltage of activation proposed for the L-type channels in the soma and dendrites of mouse spinal motoneurones, dendritic I_h channels in CA1 pyramidal cells activate at more hyperpolarized potentials than I_h channels located in the soma (Magee et al., 1998). Due to the fact that both the soma and the dendrites can be patch clamped in these cells, it is possible to generate accurate activation curves for channels in both locations. Using these methods Magee found an approximately 10 mV difference in the activation voltage between these two population of channels. Because of the known cAMP modulatory effects on these channels it was speculated that different basal levels of cAMP in the soma and the dendrites likely accounted for the observed differences. A similar basal modulation may account for the low voltage activation of the dendritic L-type current in spinal motoneurones.

A third possibility to explain the low voltage activation of the persistent inward current seen in cat motoneurones is the coupling of a true low voltage-activated current with a high voltage-activated current. The persistent inward current identified in cat motoneurones, although presumed to be a calcium current, has not been identified as a single current. Even in the turtle where this current has been shown to be a calcium current, the current has not been isolated and the kinetics defined. Therefore, a possible explanation for the apparent low voltage activation of this calcium current is through a "coupling" arrangement. This type of arrangement using an LVA calcium current has

been demonstrated in neurons of the subthalamic nucleus (Beurrier et al., 1999) and was discussed in an earlier section. In a similar manner, NMDA-induced oscillations in the turtle can be blocked with application of a variety of L-channel blockers (Guertin and Hounsgaard, 1998). Furthermore, the plateau potential in trigeminal motoneurones can be blocked with nifedipine even though the negative slope conductance in these cells has been shown to be mediated by both a calcium and a sodium current (Hsiao et al., 1998). With this type of channel coupling the initial activation of a low voltage-activated current could lead to the activation of an HVA L-type current, therefore negating the need for an L-type channel with unique activation characteristics.

However, there is evidence for the existence of a true low voltage-activated L-type current in neurons. In magnocellular neurosecretory cells both the soma and the terminals express L-type channels (Fisher and Bourque, 1996). Although channels in both locations had similar inactivation kinetics, somatic channels were seen to activate at –50 mV while those in the terminals did not activate until –30 mV. These authors speculated that this difference might be the result of differential expression of α_{1C} and α_{1D} subunits in the terminals and somata respectively. In dorsal horn neurons of the spinal cord, a dihydropyridine-sensitive current is activated a few millivolts above resting membrane potential (at ~ -55 mV; Morisset and Nagy, 1999). In CA3 pyramidal cells a similar current is seen to activate at approximately –50 mV (Avery and Johnston, 1996) while in CA1 neurons (Magee et al., 1996) and sympathetic neurons (Davies et al., 1999) a dihydropyridine-sensitive calcium current can be detected at the resting membrane potential.

As stated, the immunostaining results presented in paper 2 suggest that L-type channels located in the dendrites are primarily constructed with the α_{1D} subunit while those in the soma are constructed with the α_{1C} subunit. This finding of a differential distribution of L-type calcium channels in mammalian spinal motoneurones would suggest that the resultant channels located in the soma and dendrites might differ in some respect. Early work using the cloned genes for these subunits and expression systems suggested that these two L-type channels differed in their voltage of activation, with the α_{1D} channels activating at a lower potential. When the α_{1C} subunit was expressed in *Xenopus* oocytes along with the $\alpha 2$ and $\beta 1b$ subunits the current activated at –20 mV with a holding potential of –100 mV and had a $V_{1/2}$ of 0.9 mV (Tomlinson et al., 1993). When a similar experiment was performed using the α_{1D} subunit with $\alpha 2b$ and $\beta 2$ accessory subunits, current was seen at –40 mV even from a holding potential of –50 mV. In this case the $V_{1/2}$ was approximately –20 mV (Williams et al., 1992). In both experiments the charge carrier was 40 mM barium. (Note: effects arising from the different subunits used in the two experiments can not be ruled out; see the section "Voltage-gated Calcium Channels" in the Introduction).

Regardless of whether the dendritic current is a traditional HVA L-type current or a unique population of LVA L-type channels, modeling data suggests that the dendritically located channels require a low activation voltage. Various neuronal models have been developed that are capable of simulating plateau potential behaviour (see section - dendritic mechanism of plateau potentials). A number of these have used a low voltage-activated (LVA) persistent current as the current responsible for the depolarizing drive underlying the plateau. In the Booth et al (1997) motoneurone model this

conductance was called an L-type current while in the Jaeger et al (1997) Purkinje cell model this was called a P-type conductance to correspond with the electrophysiologically and pharmacologically defined current. Regardless of its label, it is interesting that two very different cell types use a current with similar LVA kinetics to produce a similar behaviour. The current parameters used were: $V_{1/2}$ = approximately – 40 mV for the Booth et al (1995, 1997) model and a $V_{1/2}$ = approximately – 20 mV with current activation at approximately –40 mV for the Jaeger et al (1997) model. In simulations using our neuromorphic model (paper 2), our experimental data was best mimicked when an LVA L-type current ($V_{1/2}$ = -30 mV) was used compared to when a higher activating current was used ($V_{1/2}$ = -10 mV). The ability of a current with a low voltage of activation to reproduce the plateau potentials demonstrated in spinal motoneurones would suggest that this is an important characteristic of the plateau generating current.

One method to answer these questions and accurately determine both the composition and kinetics of the dendritic calcium current is to patch clamp the dendrites directly. But unlike Purkinje or pyramidal cells which possess thick dendrites that project in a predictable direction from the soma, the dendrites of spinal motoneurones project from the cell body in a seemingly random manner and are relatively thin. Because of these factors, the probability of directly patching a motoneuronal dendrite any distance from the soma in a tissue slice is small compared to the success which has been seen in the aforementioned cell types (but see Luscher et al., 1998). A method that would allow direct dendritic recordings from spinal motoneurones is through the production of "dendrosomes" (Kavalali et al., 1997). These small dendritic segments are generated as a result of the digestion of CNS tissue with proteases. This process is the same as that used

to isolate cell somata to provide a better space clamp during voltage clamp recordings (Kay and Wong, 1986). The dendrosome provides a well clamped system which allows current kinetics to be examined (Kavalali et al., 1997). The use of this method with mammalian spinal motoneurones would allow the voltage threshold and kinetics of the dendritic L-type current to be determined and the presence of T- and other channel types to be assessed.

Concluding remarks

Mammalian spinal motoneurones fire trains of action potentials in order to produce contraction of the muscles they innervate. Effective contraction of these muscles is important during both static motor tasks such as the maintenance of posture and during dynamic events such as during locomotion. Evidence suggests that the nervous system uses the intrinsic mechanism of plateau potential production in order to produce the high firing rates required for these contractions. The purpose of the work presented here was to gain an understanding of the ionic mechanisms underlying plateau potentials in mammalian spinal motoneurones. Data presented in this thesis furthers our understanding of motoneuronal voltage-gated calcium channels and the electrogenic role they play in mature motoneuronal output. Furthermore, this work demonstrates that L-type voltage-gated calcium channels are likely a key component in the production of non-linear behaviour seen in these cells. The fact that this mechanism has been conserved during evolution from the reptiles to the mammals indicates its importance in the functioning of spinal motoneurones. This work has provided insight into spinal motoneurone

functioning at the ionic level and in doing so has deepened our understanding of the complicated tasks that we take for granted such as maintaining posture and walking.

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