Korean J Physiol Pharmacol
Vol 4: 427–437, December, 2000

N-Type Calcium Channels

Keith S. Elmslie

Department of Physiology, Tulane University Health Science, Center New Orleans, LA, 70112

The early studies of cardiac and smooth muscle cells provided evidence for two different calcium channels, the L-type (also called high-voltage activated [HVA]) and the T-type (low-voltage activated [LVA]). These calcium channels provided calcium for muscle contractions and pace-making activities. As might be expected, the number of different calcium channels increased when researchers studied neurons and the identification of the neuronal calcium channel has proven to be much more difficult than with the muscle calcium channels. There are two reasons for this difficulty; (1) a larger number of different calcium channels in neurons and (2) many of the different calcium channels have similar kinetic properties. This review uses the N-type calcium channel to illustrate the difficulties in identifying and characterizing calcium channels in neurons. It shows that the discovery of toxins that can specifically block single calcium channel types has made it possible to easily and rapidly discern the physiological roles of the different calcium channels in the neuron. Without these toxins it is unlikely that progress would have been as rapid.

Key Words: Ca channel, Modulation, Neuronal

INTRODUCTION

Voltage-dependent calcium channels provide the link between electrical activity and cellular action. Membrane depolarization, as would result from an action potential, activates voltage-dependent calcium channels to generate calcium influx. As the intracellular calcium concentration rises, calcium-sensitive intracellular proteins are activated. These proteins are involved in crucial functions such as muscle contraction, neurotransmitter release and intracellular modulatory pathways. With the diversity of functions controlled by calcium, neurons contain a diverse group of voltage-dependent calcium channels (Table 1). These calcium channels can be differentiated based on kinetics and pharmacology. Based on gene homology they can be grouped into 3 classes, Ca_V1-3. The proteins generated by the transcription and translation of each gene form the pore through which calcium enters the cell. These proteins are called α_1-subunits. Auxiliary subunits are involved in the formation of the functional channels, but these proteins do not form pores. Ca_V1 corresponds to the L-type channels that are sensitive to dihydropyridine (DHP) compounds and there are 4 genes in this group. The expression of some of these channels is tissue specific while others are expressed in many different tissues. For example, $Ca_V1.1$ is expressed only in skeletal muscle, but $Ca_V1.2$ is expressed in both neuronal and nonneuronal cells. Ca_V2 consists of high-voltage activated (HVA) non-L-type calcium channels, including N-type. Channels in this group are almost exclusively found in neurons and are most easily differentiated by their sensitivity to toxins. The Ca_V3 group is comprised of the low-voltage activated (LVA) T-type calcium channels. These channels are noted for activation at voltages near resting membrane potential and for their rapid inactivation. As with the L-channels, T-channels are found in both neuronal and nonneuronal cells.

As a result of the important physiological roles of calcium, the influx of calcium into the cell is tightly regulated. Part of this regulation involves modulation of voltage-dependent calcium channels. It is interesting that the vast majority of modulatory pathways

Corresponding to: Keith S. Elmslie, Department of Physiology SL39, Tulane University Health Science Center, New Orleans, LA 70112. (Tel) 504-584-2445, (Fax) 504-584-2675, (E-mail) kelmslie @tulane.edu

Table 1. Calcium channel classification

Channel class - Pharmacology	Channel class - mRNA cloning	Channel class - Gene homology	Characteristic blocker and/or voltage dependence
L-type (skeletal muscle)	α_{1S}	$Ca_V1.1$	DHP antagonists, HVA
L-type (cardiac muscle)	α_{1C}	$Ca_V1.2$	DHP antagonists, HVA
L-type (neuronal)	α_{1D}	$Ca_V1.3$	DHP antagonists, HVA
L-type (retina)	α_{1F}	$Ca_V1.4$	DHP antagonists, HVA
P/Q-type	α_{1A}	$Ca_V2.1$	ω-AgaIVa, ω-conotoxin MVIIC
N-type	α_{1B}	$Ca_V2.2$	ω-conotoxin GVIA, HVA
R-type	α_{1E}	$Ca_V2.3$	Ni^{2+}, SNX482*, HVA
T-type	α_{1G}	$Ca_V3.1$	Ni^{2+}, LVA
T-type	α_{1H}	$Ca_V3.2$	Ni^{2+}, LVA
T-type	α_{1I}	$Ca_V3.3$	Ni^{2+}, LVA

From Ertel et al (2000); *indicates that R-type current is partially blocked.

inhibit N-channel activity, but L-channel activity is frequently enhanced by modulation. Following recent characterization of single N-channel gating, it appears that one reason for these differences involves gating modes. N-channels predominantly gate in a mode characterized by high open probability (Po) and long open times (Lee & Elmslie, 1999). Modulation places the channels into gating modes with lower Po and shorter open times (Lee & Elmslie, 1999, 2000). L-channels, on the other hand, predominantly gate with a low Po and brief open times (mode 1; Hess et al, 1984) and modulation places these channels in a mode with high Po and longer open times (mode 2; Hess et al, 1984; Yue et al, 1990). As we learn more about the gating of individual calcium channels we will be able to better understand their involvement in regulating calcium-dependent functions within the cell.

EARLY RECORDINGS FROM NEURONAL CALCIUM CHANNELS

In the beginning there was evidence for only a single calcium current in vertebrate neurons. These first voltage clamp recordings were from chick sensory neurons (Dunlap & Fischbach, 1981). Using a holding potential near resting potential (-50 mV), the calcium current in these neurons was found to activate at voltages > -20 mV. This current was slowly inactivating and was inhibited by neurotransmitters (Dunlap & Fischbach, 1981). The results supported only a single type of calcium current.

Further recordings from this same preparation provided evidence for at least two calcium channel types (Carbone & Lux, 1984a, b). The new calcium current was revealed by the use of a more hyperpolarized holding potential (-90 mV vs. -50 mV) and was called LVA because its threshold for activation was near -60 mV. The calcium current characterized by Dunlap & Fischbach (1981) had a threshold for activation of -20 mV was termed HVA. Examination of the voltage-dependence of inactivation showed that the LVA calcium current was completely inactivated by holding potentials of ≥ -60 mV (Carbone & Lux, 1984a). Thus, Dunlap & Fischbach (1981) could have not observed the LVA current from their holding potential of -50 mV. The voltage-dependent inactivation properties of the LVA calcium channels allowed holding potential to be used as a method of isolating LVA and HVA currents.

This holding potential method was later exploited to help provide evidence for a third calcium current in chick sensory neurons (Nowyky et al, 1985; Fox et al, 1987a, b). These researchers found that calcium currents elicited from a holding potential of -100 mV showed both inactivating and sustained components at voltages ≥ 0 mV. The inactivating component of the current was lost if the holding potential

was maintained at −40 mV, leaving only the sustained component. From these observations a hypothesis was developed that chick sensory neurons had three different calcium channels; one that was noninactivating and two that inactivated (Nowyky et al, 1985; Fox et al, 1987a, b). The noninactivating channel was termed L-channel for *long-lasting* and these channels activated at voltages > −10 mV. The two inactivating channels were separated based on the activation voltage (−60 mV vs. −10 mV). The channel that activated at hyperpolarized voltages (−60 mV) was termed T-channel for *transient*. The remaining channel type was termed N-channel for *neither* T nor L (Nowyky et al, 1985; Fox et al, 1987a, b). Single channel recordings in isotonic Ba^{2+} showed three calcium channels that roughly corresponded to the three macroscopic currents. These channels were separated by their single channel conductance of 9 pS, 13 pS and 25 pS for T, N and L, respectively. The identification of these single calcium channels was based on the inactivation properties, just as the whole-cell currents were differentiated. T-channel and N-channel currents inactivated when the patch was held at −40 mV, but L-channels did not. T-channels and N-channels were differentiated by their threshold voltage for activation (−50 mV for T-channels and −20 mV for N-channels) and by their single channel conductance (8 pS for T-channels and 13 pS for N-channels). Finally, L-type channels were shown to be sensitive to dihydropyridines, but N and T channels were insensitive to these compounds (Nowyky et al, 1985; Fox et al, 1987a, b). This pharmacological specificity was exploited in later experiments to demonstrate that inactivation properties could not be used to separate N- and L-channels (Aosaki & Kasai, 1989; Jones & Marks, 1989a; Plummer et al, 1989).

PHARMACOLOGICAL IDENTIFICATION OF THE N-TYPE CALCIUM CHANNEL

The idea that three channel types coexisted in chick sensory neurons was not universally accepted. Swandulla & Armstrong (1988) analyzed tail currents to show that only two channel types (HVA and LVA) were required to explain their results. They also clearly and succinctly described the problems with the use of inactivation to separate N-type and L-type calcium currents. Essentially, these authors argued that the incomplete inactivation of the HVA calcium current could result from fast and slow inactivation processes of a single channel type, as had been demonstrated for sodium channels. In addition, they noted that there was no evidence to equate single N-channel currents with the macroscopic N-current, since the ionic conditions for recording these two currents were radically different (Swandulla & Armstrong, 1988). As we will see this analysis by Swandulla & Armstrong (1988) proved to be basically correct except that the dominant HVA channel type in chick sensory neurons was N-type, and not L-type as they had speculated.

During this same period, researchers at the University of Utah were elucidating the properties of various peptide toxins that had been isolated from the venom of cone snails, *Conus geographus* and *Conus magus* (reviewed by Olivera et al, 1985). One of these toxins called ω-conotoxin GVIA was shown to block synaptic transmission at the frog neuromuscular junction, which was interpreted as a block of the presynaptic calcium channels (Kerr & Yoshikami, 1984). This interpretation was verified in whole-cell voltage clamp recordings from chick sensory neurons that showed that calcium current was blocked, but sodium and potassium currents were relatively unaffected (Feldman et al, 1987; McCleskey et al, 1987). However, not all calcium channels were affected by ω-conotoxin GVIA. McCleskey et al (1987) showed that T-current was not affected by toxin application, but HVA currents were blocked. This block was interpreted as affecting both N-type and L-type current, but this interpretation was based on the use of inactivation to separate N-type from L-type. It was later demonstrated that ω-conotoxin GVIA was specific for the N-type calcium channel (Aosaki & Kasai, 1989; Jones & Marks, 1989a; Plummer et al, 1989). One of the observations used to support this interpretation was that L-type current enhanced by BayK 8644 (a DHP agonist) was insensitive to ω-conotoxin GVIA (Aosaki & Kasai, 1989; Plummer et al, 1989). These papers helped to establish ω-conotoxin GVIA as a specific blocker of N-type calcium channels.

CHARACTERISTICS OF THE N-TYPE CALCIUM CURRENT

Through the use of ω-conotoxin GVIA and other blockers, it was found that the whole-cell calcium current in sympathetic (Table 2) and sensory neurons

Table 2. Percentage of total calcium current derived from each component

Component current	Frog sympathetic*	Rat sympathetic† (neonatal)	Rat sympathetic† (adult)
N-type	90%	92%	70%
L-type	5%	6%	5%
R-type§	5%	2%	25%

Whole-cell currents recorded in 2~5 mM Ba^{2+}. *; Elmslie et al, 1992. †; Mintz et al, 1992. †; Zhu & Ikeda, 1993 and Elmslie, unpublished observations. §; 'R'-type in frog sympathetic neurons has been termed E_f-current.

was dominated by N-type calcium current (Aosaki & Kasai, 1989; Jones & Marks, 1989a; Plummer et al, 1989; Regan et al, 1991; Cox & Dunlap, 1992; Elmslie et al, 1992; Mintz et al, 1992). Because of the dominant N-type currents in these cells, experiments could be done to characterize the current without the complication of pharmacological dissection of the calcium current. Thus, sympathetic neurons isolated from frog and rat, and sensory neurons isolated from chick, became preferred preparations for studying N-current. In low concentrations of Ba^{2+} (2~5 mM) N-current activates at voltages > -40 mV and peak current is observed near 0 mV. An equimolar switch to Ca^{2+} causes a 10 mV depolarizing shift in these values, which results from increased screening of the membrane surface charge by Ca^{2+}. Increasing external Ba^{2+} concentration from 2 mM to 100 mM induces a 40 mV depolarizing shift in voltage-dependent channel properties. This is a crucial point since the majority of single calcium channel studies are done using isotonic Ba^{2+} (100~150 mM). Thus, single N-channels recorded in 100 mM Ba^{2+} activate at voltages >0 mV (Elmslie et al, 1994; Elmslie, 1997).

Under control conditions N-current activates monoexponentially following a brief delay (Jones & Marks, 1989a). The activation kinetics have been fit using a m^2 Hodgkin-Huxley model (Sala, 1992), which is a model with two identical and independent closed states on the pathway to open. Deactivation follows single exponential kinetics without a delay as expected for a channel moving directly from the open state to a closed state. Activation kinetics become faster with depolarization from ~0 mV and deactivation kinetics become faster with hyperpolarizations from ~0 mV. Both activation and deactivation kinetics are slowest near 0 mV (the voltage that generates peak current; Jones & Marks, 1989a, b; van Lunteren et al, 1993).

Inactivation of N-current is complex kinetically and mechanistically. As predicted by Swandulla and Armstrong (1988), N-current inactivation has multiple kinetic components. Under control conditions, the fast component has a time constant of ~150 ms at ~0 mV, while a slower component has a time constant of ~1,500 ms (Werz et al, 1993). In addition, inactivation at a holding potential of −40 mV was shown to be slow and incomplete (Jones & Marks, 1989), which demonstrates that holding potential cannot be used to separate N-type and L-type calcium currents.

Inactivation has been shown to have a U-shaped voltage dependence, with N-current showing the largest inactivation at voltages just hyperpolarized to those generating peak current (Jones & Marks, 1989b; Cox & Dunlap, 1994; Patil et al, 1998). A U-shaped voltage-dependence of inactivation has traditionally been associated with Ca^{2+}-dependent inactivation (Chad et al, 1984). In a Ca^{2+}-dependent process inactivation parallels current amplitude so that inactivation is largest at peak current and smallest at the extreme hyperpolarized and depolarized voltages where inward calcium flux is minimal. In addition, treatments that reduced the concentration of free internal Ca^{2+} (e.g. using high concentrations of internal Ca^{2+} buffers like EGTA or BAPTA) were found to decrease Ca^{2+}-dependent inactivation, but not voltage-dependent inactivation (Chad et al, 1984; Gutnick et al, 1989). However, many of tests used to distinguish Ca^{2+}-dependent inactivation from classical voltage-dependent inactivation fail when applied to N-current. For example, N-current inactivation is not greatly altered by switching from Ca^{2+} to Ba^{2+} as the charge carrier (Jones & Marks, 1989b; Cox & Dunlap, 1994; Patil et al, 1998). In addition, changing the concentration of internal Ca^{2+} buffers does not affect N-current inactivation (Jones & Marks, 1989b; Patil et al, 1998). Based on these results purely

voltage-dependent mechanisms have been proposed to explain the U-shaped voltage-dependence of N-current inactivation (Jones & Marks, 1989b; Patil et al, 1998). The recent model from Patil et al (1998) proposed that N-channels inactivate from intermediate closed states on the pathway to open. This model was satisfying since it explained much of the data from frog sympathetic neurons and from N-channels expressed in HEK293 cells (Jones & Marks, 1989b; Patil et al, 1998).

However, results from other systems were more consistent with a true Ca^{2+}-dependent inactivation process. Cox & Dunlap (1994) found that lowering the internal EGTA concentration from 10 mM of 0.1 mM increased the amount of inactivation in chick sensory neurons. In addition, altering external Ca^{2+} concentration was found to alter inactivation with 0.1 mM EGTA internal, but not 10 mM EGTA. Finally, recent experiments examining gating currents of N-channels expressed in tSA-201 cells have demonstrated an apparent Ca^{2+}-dependent inactivation process involved in alteration of gating charge (Shirokov, 1999). However, similar experiments on N-channels expressed in HEK293 cell supported a voltage-dependent process for inactivation (Jones et al, 1999). Thus, the mechanism(s) of inactivation in N-type calcium channels is controversial even after more than 10 years of study.

SINGLE N-CHANNEL STUDIES

In the original description of N-, L-, and T-channels in chick sensory neurons a calcium channel was found that appeared to match the properties of whole-cell N-current (Nowycky et al, 1985; Fox et al, 1987a, b). Like the whole-cell current, this channel activated at voltages > -40 mV, inactivated completely at a holding potential of -40 mV and inactivated rapidly during voltage steps to $+20$ mV (τ = $\sim$50 ms) (Fox et al, 1987b). The unitary current at 0 mV was -0.8 pA and the slope conductance was 13 pS. This channel was identified as the single N-type channel. As discussed by Swandulla & Armstrong (1988), the problem with equating the kinetics of single calcium channels with those of whole-cell calcium current is that the ionic conditions were different. Namely the whole-cell recordings were done in low concentrations (2$\sim$5 mM) of Ca^{2+} or Ba^{2+} whereas single calcium channels were recorded in isotonic Ba^{2+} (90$\sim$150 mM) to maximize the unitary current amplitude. It was later demonstrated that switching the external solution from 2 mM to 90 mM depolarized N-channel activation and inactivation by $\sim$40 mV (Boland et al, 1994; Elmslie et al, 1994; Zhou & Jones, 1995). Thus, N-type calcium current in $\sim$100 mM Ba^{2+} activates at voltages >0 mV (not -40 mV) and peak current is observed near $+40$ mV. In addition, N-current was not strongly inactivated at a -40 mV holding potential in isotonic Ba^{2+}, which is equivalent to -80 mV in 2 mM Ba^{2+} (Elmslie et al, 1994).

An additional problem with the early single channel studies was that the sensitivity of single N-channels to ω-conotoxin GVIA was not tested. One reason was that at the time of some of these studies the correlation of N-current with ω-conotoxin GVIA sensitivity either was not known or was not widely accepted. A second reason was that application of ω-conotoxin GVIA to the isolated N-channel is not as easy as for the hydrophobic DHP modulators of L-channel activity. Bath applied DHPs, like BayK 8644, can modulate L-channels isolated by a recording pipet, since the hydrophobic DHPs can enter and diffuse through the membrane to the channel. This allows channel activity to be assessed before and during application, which greatly facilitated the identification and characterization of L-type channels. Thus, the lack of hydrophobic modulators hampered identification of single N-channels.

Single N-channels in frog sympathetic neurons

Single channel studies on calcium channels of frog sympathetic neurons found a channel with slope conductance of 15$\sim$18 pS and activation at voltages ≥ -40 mV (Lipscombe et al, 1989; Delcour et al, 1993). This channel was identified as N-type based on similarities with the chick calcium channel identified as N-type (Nowycky et al, 1985; Fox et al, 1987a, b), including strong inactivation at a holding potential of -40 mV. Experiments were done to show this channel was DHP insensitive, but the sensitivity of the channel to ω-conotoxin GVIA was not determined (Lipscombe et al, 1989; Delcour et al, 1993; Delcour & Tsien, 1993).

As reviewed above, whole-cell experiments in 100 mM Ba^{2+} demonstrated that N-current activates at voltages >0 mV (Elmslie et al, 1994), which casts doubt on the identification of channel described above

as N-type. In addition, these whole-cell experiments surprisingly demonstrated that a previously unrecognized calcium current was revealed when frog sympathetic neurons were recorded in 100 mM Ba^{2+} (Elmslie et al, 1994). This current shared many properties of the original description of unitary N-current including activation at voltages > -40 mV and nearly complete inactivation at a holding potential of -40 mV (in 100 mM Ba^{2+}). We have called this current E_f-current, because it was hypothesized that the channels were derived from E-class mRNA (Elmslie, 1997). The main conclusion of this work was that the N-channel had been misidentified in previous experiments. Further experiments showed that E_f-channels were the mostly like the channel type described in the original work describing frog calcium channels (see Elmslie, 1997).

The conclusion that N-channels had been misidentified implied that the true N-channel remained to be determined. We used two criteria to positively identify the N-channel in frog sympathetic neurons (Elmslie, 1997). First, we compared the properties of single calcium channels to those of whole-cell N-current under the same conditions (100 mM Ba^{2+}). Second, we tested the ω-conotoxin GVIA sensitivity of the different calcium channels. ω-conotoxin GVIA was applied to isolated calcium channels using 'pipet-diffusion' technique, which allowed us to observe calcium channel before and after ω-conotoxin GVIA (Elmslie, 1997). We found one channel type that was ω-conotoxin GVIA sensitive and that had voltage-dependent properties expected for N-current in 100 mM Ba^{2+}. This channel had a conductance of 20 pS and a unitary current amplitude at 0 mV of ~ 1.4 pA (Elmslie, 1997). This unitary current was $2 \sim 3$ times larger than the channel previously identified as N-type ($0.5 \sim 0.8$ pA; Lipscombe et al, 1989; Delcour et al, 1993; Delcour & Tsien, 1993). The channel activated at voltages >0 mV and it was available to activate from a holding potential of -40 mV in isotonic Ba^{2+} (Elmslie, 1997).

The properties of the frog N-type channel closely match those of mammalian N-channels, which have a single channel conductance of ~ 20 pS, a unitary current at 0 mV near -1.4 pA and an activation voltage of 0 mV (Rittenhouse & Hess, 1994; Carabelli et al, 1996; Delmas et al, 2000). However, Delmas et al. (2000) recently demonstrated an additional ω-conotoxin GVIA sensitive channel in rat sympathetic neurons with a smaller conductance (~ 10 pS) and unitary current at 0 mV (-0.6 pA). They called this the small N-channel and showed that it was preferentially found in dendrites.

MODAL GATING OF CALCIUM CHANNELS

The first characterization of different gating patterns of a single calcium channel was from the L-type channel (Hess et al, 1984). The different gating 'modes' were observed during repeated steps to the same voltage where the L-channel would switch from a type of gating characterized by low P_o (<0.7) and brief open times (mode 1) to a type of gating characterized by high P_o and long open times (mode 2). A third mode (mode 0) was observed where the channel failed to open during the voltage step, which is called a null sweep. Sweeps exhibiting a particular type of gating were found clustered as if once the channel entered a mode it would tend to remain there. Thus, gating transitions within a mode are faster than transitions between modes. Subsequent recordings of calcium channels in sympathetic neurons have also found multiple types of gating that have been interpreted as modal. The majority of single channel recordings in sympathetic neurons have focused on N-type (Plummer & Hess, 1991; Rittenhouse & Hess, 1994; Lee & Elmslie, 1999) and E_f-type channels (Delcour et al, 1994).

Modal gating in N-type channels

Recording from neonatal rat sympathetic neurons, Rittenhouse & Hess (1994) described 3 modes in actively gating N-channels. In addition, a null gating mode, analogous to the L-channel mode 0, was observed. The 3 modes of active gating were termed SLP, SHP & LLP, where S stood for small unitary current, L stood for large unitary current, LP stood for low P_o and HP for high P_o. Thus, it was concluded that channel conductance as well as P_o changed between gating modes (Rittenhouse & Hess, 1994). However, the precise criteria for differentiating between the different modes were not detailed. The N-channel gating was examined during voltage steps to $+30$ mV, but other voltages have not been extensively studied. In the frog, we observed 3 gating modes that were similar to those described for the L-type channel. The modes were null gating (no

activity), low P_o ($P_o < 0.5$) and high P_o ($P_o \geq 0.5$; Lee & Elmslie, 1999). At +40 mV the high Po mode dominated N-channel activity, followed by null gating and then the low Po mode. Recent recordings of N-channels from IMR32 cells found evidence for only a single gating mode since the histograms of P_o showed only a single peak with a fairly narrow distribution (Carabelli et al, 1996). In addition to activity modes, N-channels in neonatal rats also exhibit modes of inactivation (Plummer & Hess, 1991). This modal inactivation may explain the multiple phases of N-current inactivation that have been observed in whole-cell experiments (Jones & Marks, 1989b; Werz et al, 1993; Cox & Dunlap, 1994).

N-CHANNEL MODULATION

In sympathetic neurons three pathways have been described that mediate neurotransmitter-induced inhibition of N-type calcium current (Hille, 1994). These are the rapid on-set voltage-dependent, rapid on-set voltage-independent and slow on-set voltage-independent pathways. All three pathways have been shown to be involved in calcium current inhibition in rat sympathetic neurons (Hille, 1994), but only the voltage-dependent pathway has been demonstrated in frog sympathetic neurons (Elmslie, 1992; Elmslie et al, 1992).

Voltage-dependent inhibition

In sympathetic neurons the majority of neurotransmitters inhibit N-type calcium current by voltage-dependent mechanism (Hille, 1994). This type of inhibition is characterized by slow activation of the inhibited current and a transient reduction of inhibition following strong depolarization (Elmslie et al, 1990). Several different G proteins have been shown to mediate the neurotransmitter effects (Elmslie, 1992; Zhu & Ikeda, 1994), but the resulting inhibition exhibits identical properties regardless of the G protein involved (Ehrlich & Elmslie, 1995). This striking convergence has been shown to result from the different G protein α-subunits releasing their associated $\beta\gamma$ subunits to inhibit by directly binding to the N-channel (Herlitze et al, 1996; Ikeda, 1996; De Waard et al, 1997; Zamponi et al, 1997). The voltage-dependence of the inhibition appears to result from the state dependent binding of $G\beta\gamma$; where closed N-channels have high affinity for $G\beta\gamma$ but the affinity of $G\beta\gamma$ for the open channel is low (Jones & Elmslie, 1997). Depolarization induces a transient disruption of $G\beta\gamma$-channel binding so that N-channels gate normally once $G\beta\gamma$ has dissociated (Carabelli et al, 1996; Patil et al, 1996; Lee & Elmslie, 2000). However, several observations from whole-cell recordings support the idea that $G\beta\gamma$-channels can open in a new gating mode (the reluctant gating mode, see below). First, N-current deactivation was faster during inhibition than control; implying an open state with different kinetics (Elmslie et al, 1990; Boland & Bean, 1993; Colecraft et al, 2000). Second, activation of inhibited N-current becomes faster than $G\beta\gamma$ dissociation; supporting the idea that $G\beta\gamma$-bound N-channels can open (Jones & Elmslie, 1997; Colecraft et al, 2000). Bean (1989) developed the willing-reluctant model to explain his observations of voltage-dependent inhibition of whole-cell N-current in frog sensory neurons. According to the model N-channels normally gate in the willing mode, but become reluctant during inhibition. The reluctant channels can open, but the threshold for opening is depolarized to that of the willing mode. In addition, when reluctant channels do open they exhibit lower P_o and shorter open times compared to willing openings (Elmslie et al, 1990). The willing-reluctant model was developed to explain results of whole-cell recordings from frog peripheral neurons (Bean, 1989; Elmslie et al, 1990; Boland & Bean, 1993). However, until recently it was uncertain if this model would apply to mammalian N-channels. Whole-cell recordings from NG108 cells failed to show a change in N-current deactivation kinetics between inhibition and control (Kasai, 1992). In addition, tail currents from adult rat sympathetic neurons were not altered during voltage-dependent inhibition (Zhu & Ikeda, 1993). However, these tail currents were induced following 10 ms voltage steps, which tend to drive channels into the willing mode (Elmslie et al, 1990; Boland & Bean, 1993). Therefore, any alteration of deactivation kinetics would be expected to be minor using this protocol. More troubling was that recordings from single mammalian N-channels clearly showed delayed N-channel opening during inhibition, but no new open states were detected (Carabelli et al, 1996; Patil et al, 1996). However, these single channel studies primarily used voltage steps that may have been too hyperpolarized for reluctant channels to open. Whole-cell evidence supporting a reluctant open state for

mammalian N-channels has come from Colecraft et al (2000) using N-channels expressed from human α_{1B} mRNA. These researchers demonstrated that tail currents resulting from large, brief depolarizations were closer to their maximum than currents generated during voltage steps to +10 mV. The relative difference between tail current and step current was interpreted to result from both willing & reluctant channels opening during the large depolarization, but the +10 mV voltage step only activated willing channels (Colecraft et al, 2000). Recently we presented single channel evidence supporting a reluctant gating mode for inhibited N-channels (Lee & Elmslie, 2000). Reluctant openings were only detectable during voltage steps $\geq$ +40 mV, which were depolarized to those used previously. As expected from the willing-reluctant model, reluctant openings had a low Po and brief open times compared to willing mode (Lee & Elmslie, 2000). Thus, the basic predictions of the willing-reluctant model have now been verified.

While the majority of the inhibition can be reversed by strong depolarization, a portion of the inhibition persists in peripheral and central neurons, even at very depolarized voltages (Bean, 1989; Hille, 1994; Jones & Elmslie, 1997). Two hypotheses have developed to explain this voltage-independent part of the inhibition, the multiple pathway hypothesis and the single pathway hypothesis. In the multiple pathway hypothesis, the neurotransmitter simultaneously activates separate voltage-dependent and voltage-independent pathways, which have been shown to exist in rat sympathetic neurons (Hille, 1994). In the single pathway model, it has been suggested that multiple pathways are not needed to explain incomplete recovery of inhibited current by depolarization (Jones & Elmslie, 1997). It is argued that the affinity of $G\beta\gamma$ for the open N-channel is low, but not zero. Thus, a portion of N-channels will remain inhibited even at strongly depolarized voltages. In support of this idea, we have observed N-channels to gate reluctantly following a depolarized voltage step that would maximally recover inhibited current (Lee & Elmslie, 2000). An additional argument supporting the single pathway hypothesis is that pertussis toxin treatments disrupt norepinephrine (NE)-induced voltage-dependent and voltage-independent inhibition in frog sympathetic (Elmslie, 1992) and rat sympathetic neurons (Shapiro et al, 1994a). The simplest explanation is that both components are mediated by the same pathway, namely $G\beta\gamma$ binding directly to N-channels. However, pertussis toxin blocks both Go & Gi and recent evidence supports the idea that each G protein mediates a separate inhibitory pathway. Delmas et al (1999) has shown that the injection of antibodies that block Go disrupts the voltage-dependent portion of NE-induced inhibition, while antibodies directed against Gi disrupts NE-induced voltage-independent inhibition. These results support the multiple pathway hypothesis, since strong depolarization can completely recover inhibited N-current once the voltage-independent component is blocked (Delmas et al, 1999). In addition, the inhibition of N-current in NG108 cells can be completely reversed by strong depolarization (Kasai, 1992), which also supports the multiple pathway model.

Voltage-independent inhibition

In rat sympathetic neurons, most neurotransmitters induce voltage-dependent inhibition, but a few induce a purely voltage-independent inhibition (Hille, 1994). Two types of voltage-independent inhibition can be differentiated by the speed of inhibition and by the involvement of a diffusible second messenger (Hille, 1994). Activation of muscarinic receptors (M1) and Angiotensin II receptors induces an inhibition characterized by slow development (tens of seconds) and by the involvement of an unidentified BAPTA-sensitive diffusible second messenger (Hille, 1994; Shapiro et al, 1994b). The second type of voltage-independent inhibition can be induced by activation of substance P and M1 receptors. This type of inhibition is characterized by rapid development ($\sim$1 sec) and the involvement of a membrane-delimited pathway, two features that also describe voltage-dependent inhibition (Hille, 1994). In addition, recent evidence supports the involvement of $G\beta\gamma$ in fast voltage-independent inhibition (Delmas et al, 1999; Kammermeier et al, 2000). The fast voltage-independent inhibition induced by NE and the muscarinic agonist oxotremorine-M (Oxo-M) is blocked by treatments that reduce the concentration of free $G\beta\gamma$ in the cytoplasm (Delmas et al, 1999; Kammermeier et al, 2000). Delmas et al (1999) proposed that voltage-independent inhibition was mediated by a different $G\beta\gamma$ subunit than voltage-dependent inhibition. They speculated that this different $G\beta\gamma$ subunit may have a stronger affinity for the N-channel (so that it does not dissociate from the open channel) or that it may bind to a different site on the N-channel (to

induce voltage-independent inhibition). However, Kammermeier et al (2000) demonstrated that voltage-independent inhibition required both the G $\beta\gamma$ and G α subunits. These researchers proposed that the G α subunit somehow 'locks' the G $\beta\gamma$ subunit to the N-channel, preventing dissociation during strong depolarization. These experiments demonstrating a common intracellular mediator for both voltage-dependent and voltage-independent inhibition reveal unexpected complexity in the modulation of N-type calcium channels.

CONCLUSIONS

The study of neuronal calcium channels was quite simple in the beginning with only one or two calcium channel types. Upon further study things became more complex and sometimes confusing with the identification of addition channel types. Finally, with the help of toxin blockers, the identification of channel types has become standardized and much of the confusion has gone. The study of identified calcium channels is now being carried out. A few of the interesting areas of study involve modulation, permeation and inactivation of the neuronal calcium channels. The discovery of toxin blockers of HVA calcium channels (N-type and P/Q-type) has also advanced the identification of the calcium channel types involved in neurotransmitter release. These studies continue and it will be very interesting to understand the role of each channel type in the synaptic release of neurotransmitters.

REFERENCES

Aosaki T, Kasai H. Characterization of two kinds of high-voltage-activated Ca-channel currents in chick sensory neurons. Differential sensitivity to dihydropyridines and ω-conotoxin GVIA. *Pflgers Arch* 414: 150−156, 1989

Bean BP. Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* 340: 153−156, 1989

Boland LM, Bean BP. Modulation of N-type calcium channels in bullfrog sympathetic neurons by luteinizing hormone-releasing hormone: kinetics and voltage dependence. *J Neurosci* 13: 516−533, 1993

Boland LM, Morrill JA, Bean BP. ω-conotoxin block of N-type calcium channels in frog and rat sympathetic neurons. *J Neurosci* 14: 5011−5027, 1994

Carabelli V, Lovallo M, Magnelli V, Zucker H, Carbone E. Voltage-dependent modulation of single N-type Ca^{2+} channel kinetics by receptor agonists in IMR32 cells. *Biophys J* 70: 2144−2154, 1996

Carbone E, Lux HD. A low voltage-activated calcium conductance in embryonic chick sensory neurons. *Biophys J* 46: 413−418, 1984a

Carbone E, Lux HD. A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. *Nature* 310: 501−502, 1984b

Chad J, Eckert E, Ewald D. Kinetics of calcium-dependent inactivation of calcium current in voltage-clamped neurones of *Aplysia californica*. *J Physiol (Lond)* 347: 279−300, 1984

Colecraft HM, Patil PG, Yue DT. Differential occurrence of reluctant openings in G-protein-inhibited N- and P/Q-type calcium channels. *J Gen Physiol* 115: 175−192, 2000

Cox DH, Dunlap K. Pharmacological discrimination of N-type from L-type calcium current and its selective modulation by transmitters. *J Neurosci* 12: 906−914, 1992

Cox DH, Dunlap K. Inactivation of N-type calcium current in chick sensory neurons: calcium and voltage dependence. *J Gen Physiol* 104: 311−336, 1994

Delcour AH, Lipscombe D, Tsien RW. Multiple modes of N-type calcium channel activity distinguished by differences in gating kinetics. *J Neurosci* 13: 181−194, 1993

Delcour AH, Tsien RW. Altered prevalence of gating modes in neurotransmitter inhibition of N-type calcium channels. *Science* 259: 980−984, 1993

Delmas P, Abogadie FC, Buckley NJ, Brown DA. Calcium channel gating and modulation by transmitters depend on cellular compartmentalization. *Nature Neurosci* 3: 670−678, 2000

Delmas P, Abogadie FC, Milligan G, Buckley NJ, Brown DA. $\beta\gamma$ dimers derived from G_o and G_i proteins contribute different components of adrenergic inhibition of Ca^{2+} channels in rat sympathetic neurones. *J Physiol (Lond)* 518: 23−36, 1999

De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, Campbell KP. Direct binding of G-protein $\beta\gamma$ complex to voltage-dependent calcium channels. *Nature* 385: 446−450, 1997

Dunlap K, Fischbach GD. Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. *J Physiol (Lond)* 317: 519−535, 1981

Ehrlich I, Elmslie KS. Neurotransmitters acting via different G proteins inhibit N-type calcium current by an identical mechanism in rat sympathetic neurons. *J*

Neurophysiol 74: 2251−2257, 1995

Elmslie KS. Calcium current modulation in frog sympathetic neurones: multiple neurotransmitters and G proteins. *J Physiol (Lond)* 451: 229−246, 1992

Elmslie KS. Identification of the single channels which underlie the N-type and L-type calcium currents in bullfrog sympathetic neurons. *J Neurosci* 17: 2658−2668, 1997

Elmslie KS, Kammermeier PJ, Jones SW. Reevaluation of Ca^{2+} channel types and their modulation in bullfrog sympathetic neurons. *Neuron* 13: 217−228, 1994

Elmslie KS, Kammermeier PJ, Jones SW. Calcium current modulation in frog sympathetic neurones: L-current is relatively insensitive to neurotransmitters. *J Physiol (Lond)* 456: 107−123, 1992

Elmslie KS, Zhou W, Jones SW. LHRH and GTP-γ-S modify activation of N-type calcium currents in bullfrog sympathetic neurons. *Neuron* 5: 75−80, 1990

Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T, Birnbaumer L, Tsien RW, Catterall WA. Nomenclature of voltage-gated calcium channels. *Neuron* 25: 533−535, 2000

Feldman DH, Olivera BM, Yoshikami D. Omega *Conus geographus* toxin: a peptide that blocks calcium channels. *FEBS Letters* 214: 295−300, 1987

Fox AP, Nowyky MC, Tsien RW. Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J Physiol (Lond)* 394: 149−172, 1987a

Fox AP, Nowyky MC, Tsien RW. Single-channel recordings of three types of calcium channels in chick sensory neurones. *J Physiol (Lond)* 394: 173−200, 1987b

Gutnick MJ, Lux HD, Swandulla D, Zucker H. Voltage-dependent and calcium-dependent inactivation of calcium channel current in identified snail neurones. *J Physiol (Lond)* 412: 197−220, 1989

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA. Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ subunits. *Nature* 380: 258−262, 1996

Hess P, Lansman JB, Tsien RW. Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature* 311: 538−544, 1984

Hille B. Modulation of ion-channel function by G-protein-coupled receptors. *TINS* 17: 531−536, 1994

Ikeda SR. Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature* 380: 255−258, 1996

Jones LP, DeMaria CD, Yue DT. N-type calcium channel inactivation probed by gating-current analysis. *Biophys J* 76: 2530−2552, 1999

Jones SW, Elmslie KS. Transmitter modulation of neuronal calcium channels. *J Membr Biol* 155: 1−10, 1997

Jones SW, Marks TN. Calcium currents in bullfrog sympathetic neurons. I. Activation kinetics and pharmacology. *J Gen Physiol* 94: 151−167, 1989a

Jones SW, Marks TN. Calcium currents in bullfrog sympathetic neurons. II. Inactivation. *J Gen Physiol* 94: 169−182, 1989b

Kammermeier PJ, Ruiz-Velasco V, Ikeda SR. Voltage-independent calcium current inhibition by muscarinic agonists in rat sympathetic neurons requires both $G\alpha_{q/11}$ and $G\beta\gamma$. *J Neurosci* 20: 5623−5629, 2000

Kasai H. Voltage- and time-dependent inhibition of neuronal calcium channels by a GTP-binding protein in a mammalian cell line. *J Physiol (Lond)* 448: 189−209, 1992

Kerr LM, Yoshikami D. A venom with a novel presynaptic blocking action. *Nature* 308: 282−284, 1984

Lee HK, Elmslie KS. Gating of single N-type calcium channels recorded from bullfrog sympathetic neurons. *J Gen Physiol* 113: 111−124, 1999

Lee HK, Elmslie KS. Reluctant gating of single N-type calcium channels during neurotransmitter-induced inhibition in bullfrog sympathetic neurons. *J Neurosci* 20: 3115−3128, 2000

Lipscombe D, Kongsamut S, Tsien RW. α-Adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating. *Nature* 340: 639−642, 1989

McCleskey EW, Fox AP, Feldman DH, Cruz LJ, Olivera BM, Tsien RW, Yoshikami D. ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc Natl Acad Sci USA* 84: 4327−4331, 1987

Mintz IM, Adams ME, Bean BP. P-type calcium channels in rat central and peripheral neurons. *Neuron* 9: 85−95, 1992

Nowyky MC, Fox AP, Tsien RW. Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440−443, 1985

Olivera BM, Gray WR, Zeikus R, McIntosh JM, Vargas J, Rivier J, de Santos V, Cruz LJ. Peptide neurotoxins from fish-hunting cone snails. *Science* 230: 1338−1343, 1985

Patil PG, Brody DL, Yue DT. Preferential closed-state inactivation of neuronal calcium channels. *Neuron* 20: 1027−1038, 1998

Patil PG, de Leon M, Reed RR, Dubel S, Snutch TP, Yue DT. Elementary events underlying voltage-dependent G-protein inhibition of N-type calcium channels. *Biophys J* 71: 2509−2521, 1996

Plummer MR, Hess P. Reversible uncoupling of inactivation in N-type calcium channels. *Nature* 351: 657−659, 1991

Plummer MR, Logothetis DE, Hess P. Elementary properties and pharmacological sensitivities of calcium

channels in mammalian peripheral neurons. *Neuron* 2: 1453−1463, 1989

Regan LJ, Sah DWY, Bean BP. Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin. *Neuron* 6: 269−280, 1991

Rittenhouse AR, Hess P. Microscopic heterogeneity in unitary N-type calcium currents in rat sympathetic neurons. *J Physiol (Lond)* 474: 87−99, 1994

Sala F. Activation kinetics of calcium currents in bullfrog sympathetic neurones. *J Physiol (Lond)* 437: 221−238, 1991

Shapiro MS, Wollmuth LP, Hille B. Modulation of Ca^{2+} channels by PTX-sensitive G-proteins is blocked by N-ethylmaleimide in rat sympathetic neurons. *J Neurosci* 14: 7109−7116, 1994a

Shapiro MS, Wollmuth LP, Hille B. Angiotensin II inhibits calcium and M current channels in rat sympathetic neurons via G proteins. *Neuron* 12: 1319−1329, 1994b

Shirokov R. Interaction between permeant ions and voltage sensor during inactivation of N-type Ca^{2+} channels. *J Physiol (Lond)* 518: 697−703, 1999

Swandulla D, Armstrong CM. Fast-deactivating calcium channels in chick sensory neurons. *J Gen Physiol* 92: 197−218, 1988

van Lunteren E, Elmslie KS, Jones SW. Effect of temperature on calcium current kinetics in frog sympathetic neurons. *J Physiol (Lond)* 466: 81−93, 1993

Werz MA, Elmslie KS, Jones SW. Phosphorylation enhances inactivation of N-type calcium channels. *Pflgers Archiv* 424: 538−545, 1993

Yue DT, Herzig S, Marban E. β-Adrenergic stimulation of calcium channels occurs by potentiation of high-activity gating modes. *Proc Natl Acad Sci USA* 87: 753−757, 1990

Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP. Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit. *Nature* 385: 442−446, 1997

Zhou W, Jones SW. Surface charge and calcium channel saturation in bullfrog sympathetic neurons. *J Gen Physiol* 105: 441−462, 1995

Zhu Y, Ikeda SR. VIP inhibits N-type Ca^{2+} channels in sympathetic neurons via a pertussis toxin-insensitive but cholera toxin-sensitive pathway. *Neuron* 13: 657−669, 1994

Zhu Y, Ikeda SR. Adenosine modulates voltage-gated Ca^{2+} channels in adult rat sympathetic neurons. *J Neurophysiol* 70: 610−620, 1993