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DIFFERENT TYPES OF CALCIUM CHANNELS

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SUMMARY

Ca^{2+} channels allow passage of Ca^{2+} ions into the cytoplasm through a selective pore which is opened in response to depolarization of the cell membrane (for reviews see Hagiwara & Byerly, 1981, 1983; Tsien, 1983; Reuter, 1983). The Ca^{2+} flux creates a net inward, depolarizing current and the resulting accumulation of Ca^{2+} in the cytoplasm can act as a chemical trigger for secretion of hormones and neurotransmitters, contraction of muscle and a variety of other Ca^{2+}-sensitive events. Thus, upon sensing membrane potential changes, Ca^{2+} channels simultaneously generate an electrical signal while directly creating an intracellular chemical messenger. This dual ability is unique among the family of ion channels and allows the Ca^{2+} channel to play a variety of roles in excitation–secretion and excitation–contraction coupling.

It has now become clear that versatility of function is reflected by diversity of the types of Ca^{2+} channels on the membrane of individual cells. This article describes the nature of data which have demonstrated multiple channel types, reviews the literature suggesting that many cells have several kinds of Ca^{2+} channels, and discusses newer data regarding a neurotoxin that distinguishes among different Ca^{2+} channels.

MULTIPLE Ca^{2+} CHANNEL TYPES ARE COMMON ON MANY CELLS

Fig. 1 (taken from Nowycky, Fox & Tsien, 1984) illustrates the scheme that has been a useful test for multiple Ca^{2+} channel types on a single cell. The cell is a chick dorsal root ganglion neurone (DRG) and the only current carrier is 10 mmol l^{-1} Ca^{2+} in the extracellular medium. With the cell held at −60 mV by voltage clamp, a pulse to 0 mV elicits a current which does not decline during the several hundred millisecond pulse. When the cell is held at −100 mV, a decaying current is superimposed on the previous current. The hyperpolarization has unmasked a current which was inactivated at a holding potential of −60 mV and which decays within 100 ms during a pulse to 0 mV. The decay appears to be voltage-dependent inactivation because exchanging Ba^{2+} for Ca^{2+} did not alter the decay rate as expected of an inactivation due to accumulation of intracellular Ca^{2+}. The usual screening procedure for multiple channels is, therefore, selective steady-state inactivation.

Key words: calcium channel, patch clamp, multiple types.

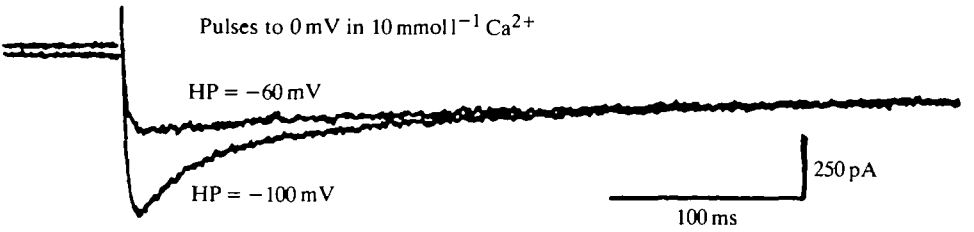

Fig. 1. Calcium currents from cultured chick dorsal root ganglion neurones at two holding potentials (HP), −60 mV and −100 mV. Currents evoked by voltage pulses to 0 mV. Extracellular solution (in mmol l^{-1}): $CaCl_2$, 10; tetraethylammonium(TEA)Cl, 135; Hepes, 10; tetrodotoxin, 200 nmol l^{-1}; pH 7·3. Intracellular solution: CsCl, 120; Cs-EGTA, 10; $MgCl_2$, 2; Hepes, 5; pH 7·3. From Nowycky, Fox & Tsien, 1984.

Like many aspects of the Ca^{2+} channel literature, multiple Ca^{2+} channel types can be traced back to work done by Susumu Hagiwara. The pioneering work, done on starfish eggs and published in 1975, distinguishes two Ca^{2+} current components by steady-state inactivation, activation threshold and selectivity as shown in Fig. 2 (taken from Hagiwara, Ozawa & Sand, 1975). The data connected by solid curves in Fig. 2A plot on the ordinate the peak current in response to voltage pulses to the potentials plotted on the abscissa. Currents were recorded with Ca^{2+}, Sr^{2+} or Ba^{2+} as the charge carrier. In each case, there are two local minima in the current–voltage curve suggesting the presence of two channels which activate at different voltages. Hagiwara called the current peak centred at −30 mV 'Type I' and the current activated at higher potentials 'Type II'. The crossing of curves indicates that the two currents have different selectivity. The type II channel passes greater Ba^{2+} currents than Ca^{2+} currents, as is typical of 'classic' Ca^{2+} channels; the order is reversed for type I channels.

Fig. 2B demonstrates the different inactivation properties of the two currents. The relative current amplitude during a test pulse is plotted against the potential during a conditioning prepulse intended to cause varying levels of inactivation. The triangles and circles are generated with test pulses designed primarily to activate the type I channels; the resulting inactivation curve is monotonic and clearly fitted by the standard Boltzmann distribution (solid curve). The squares are generated with test pulses to +22 mV, which should activate both type I and type II currents. The resulting inactivation curve is unusually broad and appears biphasic in the vicinity of −40 mV. This has been fitted by summation of a scaled curve for the type I channel (solid curve) and a separate Boltzmann curve for the type II channel (dashed curve). This experiment remains the most clearly quantified separation of the inactivation kinetics of two Ca^{2+} channel types.

The properties of different activation thresholds and different inactivation ranges have been applied to a wide range of cells and multiple Ca^{2+} channel types have become the rule rather than the exception (for reviews see Reuter, 1985; Miller, 1985; Tsien *et al.* 1986). The following is an attempt to catalogue the cells in which multiple Ca^{2+} channel types have been observed: starfish eggs (Hagiwara *et al.* 1975), tunicate eggs (Okamota, Takahashi & Yoshii, 1976), *Neanthes* worm eggs

(Fox, 1981; Fox & Krasne, 1984), inferior olivary neurones (Llinas & Yarom, 1981*a*,*b*), neuroblastoma cells (Fishman & Spector, 1981; Tsunoo, Yoshii & Narahashi, 1985; Yoshii, Tsunoo & Narahashi, 1985), rat hippocampal neurones (Halliwell, 1983; Gray & Johnston, 1986), dorsal root ganglion neurones (Nowycky *et al.* 1984, 1985; Carbone & Lux, 1984*a*,*b*; Fedulova, Kostyuk & Veselovsky, 1985), GH_3 and GH_4 cells (Armstrong & Matteson, 1985; Cohen & McCarthy, 1985), sensory cranial neurones (Bossu, Feltz & Thomann, 1985), rat olfactory neurones (Brown *et al.* 1984), protozoan cilia (Deitmer, 1984), dog and frog atrial

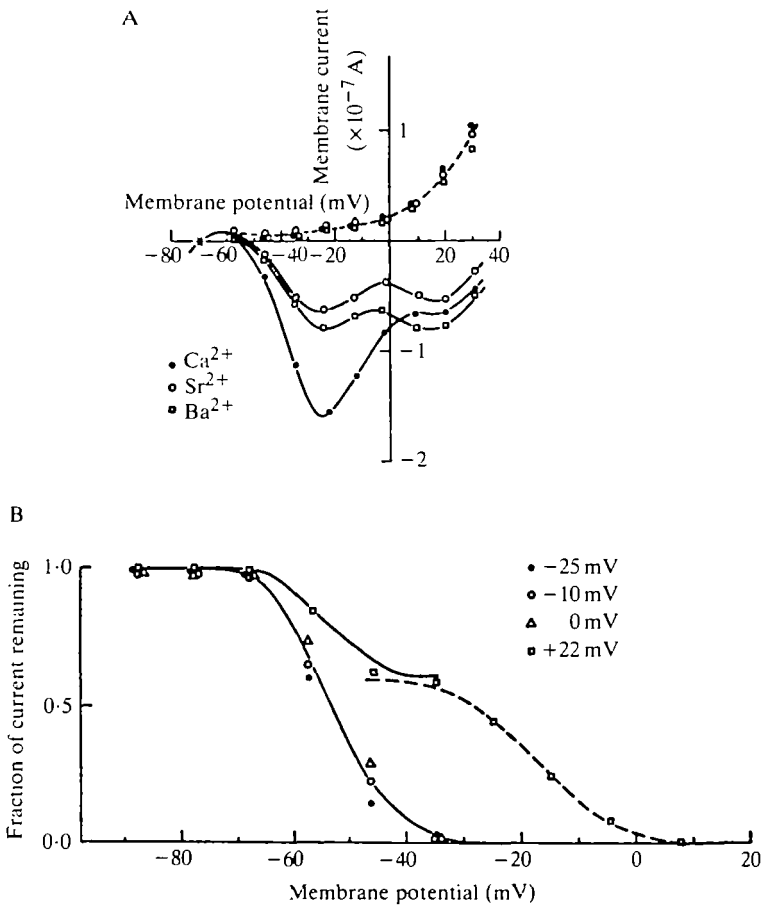

Fig. 2. Data distinguishing two Ca^{2+} channel types in starfish eggs. (A) Activation of type I and type II currents. Data connected by solid curves plot the peak inward current against pulse potential under ionic conditions in which the only inward current carrier was 10 mmol l^{-1} Ca^{2+}, Sr^{2+} or Ba^{2+} as indicated. Note the two minima in the current–voltage curves and that the Ca^{2+} and Ba^{2+} curves cross. (B) Inactivation of type I and type II currents. Plot of the fraction of current remaining (ordinate) after a conditioning voltage pulse to the potential indicated on the abscissa. Circles and triangles plot data in response to test potentials which activate mostly type I current. Squares plot data for test potentials which activate both type I and type II current. Note the biphasic inactivation curve for the more positive pulses. From Hagiwara, Ozawa & Sand, 1975, figs 4, 6.

cells (Bean, 1985), guinea-pig ventricular cells (Nilius, Hess, Lansman & Tsien, 1985; Mitra & Morad, 1985), *Aplysia* bag cells (Chesnoy-Marchais, 1985), rat mesenteric artery smooth muscle (Bean, Sturek, Puga & Hermsmeyer, 1985, 1986), rat venous smooth muscle (Sturek & Hermsmeyer, 1985), rabbit ear artery smooth muscle (Aaronson *et al.* 1986), skeletal muscle myotubes (Beam, Knudson & Powell, 1986; Cognard, Lazdunski & Romey, 1986) and pituitary lactotrophs (DeRiemer & Sakmann, 1986). Cells which appear to have only one type of Ca^{2+} channel are: adrenal chromaffin cells (Fenwick, Marty & Neher, 1982; Hoshi, 1985), hybridoma cells (Fukushima & Hagiwara, 1983) and type II astrocytes (Barres, Chun & Corey, 1985).

THREE Ca^{2+} CHANNEL TYPES ON DRG NEURONES

Whole cell

Fig. 3 shows records from the paper of Nowycky *et al.* (1985) showing whole-cell Ca^{2+} current and associated current–voltage curves from a DRG neurone. As noted before, pulses from a holding potential of -40 mV elicit a non-inactivating current (Fig. 3A, smaller currents) represented by squares on the current–voltage curve (Fig. 3B). This prolonged current has been called 'L' (for Long-lasting, Large Ba^{2+} conductance; Nowycky *et al.* 1985) and 'HVA' (for High Voltage-Activated; Carbone & Lux, 1984*a*,*b*). From a holding potential of -100 mV, pulses to negative potentials elicit a current which inactivates completely and appears to reach its maximum amplitude near -10 mV. This component has been called 'T' (for Transient, Tiny Ba^{2+} conductance; Nowycky *et al.* 1985) and 'LVA' (for Low Voltage-Activated; Carbone & Lux, 1984*a*,*b*). Pulses to positive potentials from the -100 mV holding potential elicit, in addition to the non-inactivating L current, an inactivating current of larger amplitude than expected of the T-type current. The component is like T in that it inactivates and requires a negative holding potential but is like L in its voltage range of activation. Fig. 3C plots the fraction of current which relaxes during the pulse. There are two clear components of the current–voltage curve with peaks at -10 mV and $+20$ mV. The authors proposed that this second peak of inactivating current was due to yet a third Ca^{2+} channel type. The challenge of this hypothesis is to prove the existence of a separate molecular entity despite the fact that it can only be activated under conditions that also activate the other two channels. This component is therefore called 'N' because proof that the current results from a separate channel requires evidence that it is caused by Neither T nor L.

Single channels

The on-cell patch method (Hamill *et al.* 1981) allows the recording of individual ion channels by isolating a sub-microscopic patch of membrane. With this method, Nowycky *et al.* (1985) were able to demonstrate clearly the existence of three distinct Ca^{2+} channel types. Fig. 4 shows results from three different patches that show three

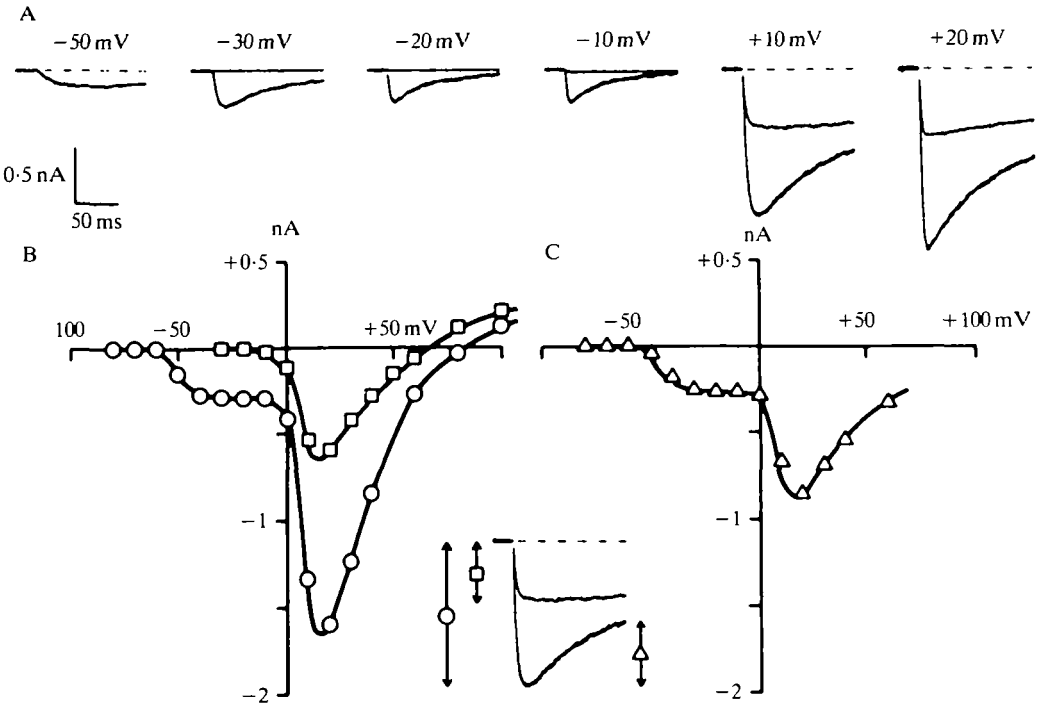

Fig. 3. Ca^{2+} currents from cultured chick DRG neurones at two holding potentials showing three current components. (A) Superimposed current traces from holding potential (HP) = −40 mV (smaller traces) or HP = −100 mV in response to pulses to potentials indicated above. (B) Peak current *vs* pulse potential with HP = −40 mV (squares) or HP = −100 mV (circles). (C) Plot of pulse potential *vs* the subset of current which declines during a pulse from HP = −100 mV. Extracellular solutions as in Fig. 1. Intracellular solution (mmol l^{-1}): CsCl, 100; Cs-EGTA, 10; $MgCl_2$, 5; Hepes, 40; Na_2ATP, 2; cyclic AMP, 0·25; pH 7·3. From Nowycky, Fox & Tsien, 1985, fig. 1.

different kinds of single channel activity. Fig. 4A shows a small-conductance channel activated with a protocol appropriate for T. Activity is bunched towards the beginning of the pulse as expected of an inactivating current. Fig. 4C shows a large-conductance channel activated from a depolarized holding potential that allows only L-type activity. Openings are scattered relatively evenly throughout the pulse as predicted for a non-inactivating channel. Fig. 4B shows a channel which is activated with pulses to positive potentials from a hyperpolarized holding potential as expected for the putative N channel. It has a distinctly larger single-channel amplitude than T yet its activity is towards the beginning of the trace, unlike L. Its single-channel conductance (13 pS) in 110 mmol l^{-1} Ba^{2+} is roughly half that of the L channel (25 pS) and greater than the T channel (8 pS) (making it iNtermediate in Ba^{2+} conductance). Thus, a third channel was demonstrated at the single-channel level with properties that could generate the N-type activity at the whole-cell level. Table 1 summarizes the single-channel conductances and kinetic features of these three channels.

A drug and a neurotoxin that distinguish different channel types

Ligands that distinguish different Ca^{2+} channel types would be used both to dissect the different physiological functions of the channels and to serve as biochemical probes for isolation and purification. Two compounds seem useful in this regard.

Dihydropyridines

Nifedipine and related dihydropyridines are the most potent organic compounds that block Ca^{2+} channels and are used clinically due to the dihydropyridine sensitivity of cardiac and smooth muscle Ca^{2+} channels. The family of dihydropyridines contains both blockers and agonists that enhance Ca^{2+} current. Fig. 5, from Hess, Lansman & Tsien (1984), shows the effect of an agonist, Bay K 8644, on the L-type channel in cardiac cells. The presence of Bay K 8644 causes a dramatic increase in the open time of single Ca^{2+} channels leading to an increase in the overall Ca^{2+} current. This effect occurs only with L-type channels in both DRG neurones (Nowycky *et al.* 1985) and cardiac cells (Nilius *et al.* 1985). Dihydropyridine agonists, therefore, provide a clear assay for the presence of L-type channels.

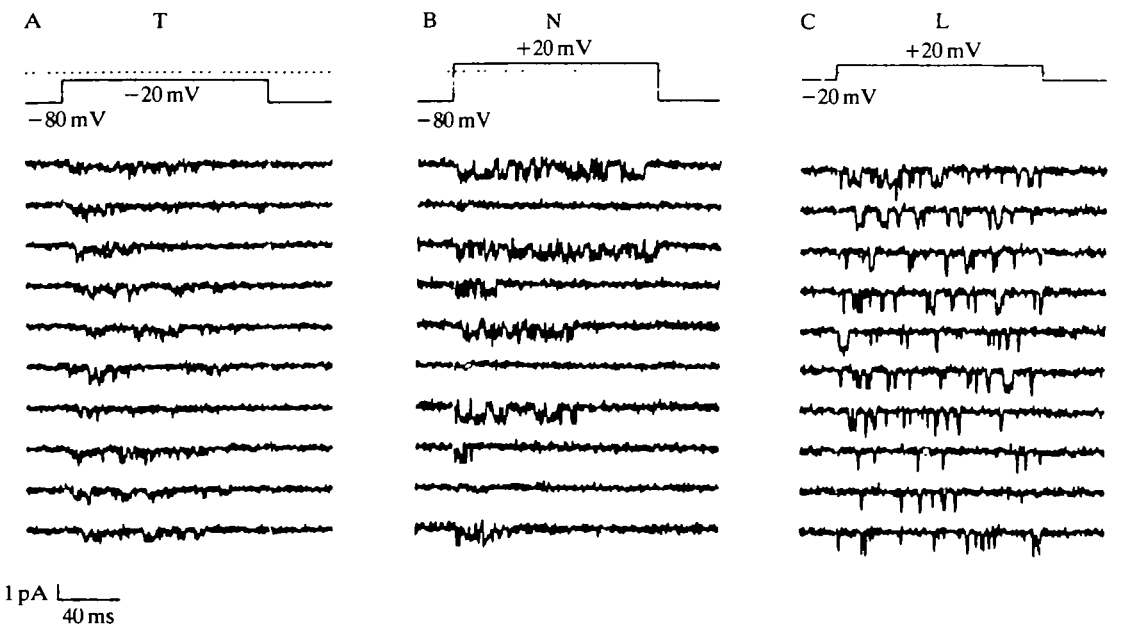

Fig. 4. Three types of single-channel currents from cultured DRG neurones. All recordings with the on-cell patch configuration (Hamill *et al.* 1981) with Ba^{2+} as the charge carrier. (A) T-type channel; (B) N-type channel; (C) L-type channel. Pulse protocols are indicated. Pipette solution (mmol l^{-1}): $BaCl_2$, 110; Hepes, 10; tetrodotoxin, 200 nmol l^{-1}, pH 7·3. Extracellular solution: K-aspartate, 140; K-EGTA, 10; Hepes, 10; $MgCl_2$, 1; pH 7·3. From Nowycky, Fox & Tsien, 1985, fig. 2.

Table 1. *Summary of kinetic properties of T, N and L currents in DRG neurones*

	T	*N*	*L*
Single channel conductance	8–10 pS	13 pS	25 pS
Selectivity	$I_{Ca} > I_{Ba}$		$I_{Ba} > I_{Ca}$
Single channel kinetics (20°C)	inactivating, brief bursts	inactivating, long bursts	non-inactivating
Steady-state inactivation range	−100 mV to −60 mV	−100 mV to −40 mV	−60 mV to −10 mV
Whole cell activation range	−70 mV	−10 mV	−10 mV
Whole cell inactivation rate	20–50 ms	20–50 ms	slow (>700 ms)

While enhancement of single-channel currents by dihydropyridine agonists is clear and dramatic, block by antagonists is not as pronounced. Sensitivity of L-type channels to dihydropyridines varies among cell types and varies with the cell membrane potential (Bean, 1984; Sanguinetti & Kass, 1984). Neurones show relatively less sensitivity to dihydropyridines than does muscle tissue. At the negative holding potentials from which all channels can be activated, L channels in neurones cannot be completely blocked even at the highest dihydropyridine concentrations possible in aqueous media (10^{-5}–10^{-4} mol l^{-1}). The remaining L current is not readily distinguished from N and T currents because dihydropyridines induce a more rapid decay of L current (Lee & Tsien, 1983).

ω-CgTX

The only selective blocker known which is both potent and does not alter the kinetics of residual current comes from the venom of a marine snail, *Conus geographus*. This snail feeds on fish by stinging with a venom which causes virtually immediate paralysis and death. B. M. Olivera and his colleagues have characterized the peptides in *Conus* venoms (Olivera *et al.* 1985) and described the ω-VIA *Conus geographus* toxin (ω-CgTX), which has 14 hydroxyl groups among 27 amino acids and a net positive charge of 4 (Olivera *et al.* 1984). Kerr & Yoshikami (1984) showed that ω-CgTX blocks neuromuscular transmission in frogs by decreasing the quantal content of evoked acetylcholine release. They proposed that this was due to decreased Ca^{2+} flux in the presynaptic terminal because Ca^{2+} action potentials in DRG neurones were diminished by the toxin. The inhibition of the action potentials could have been due either to blockade of Ca^{2+} channels or to an enhancement of a hyperpolarizing conductance. Feldman & Yoshikami (1985), using voltage-clamp techniques, proved that the toxin blocked Ca^{2+} channels without an effect on the delayed rectifier potassium current.

Does the toxin distinguish among different channel types? Fig. 6, taken from McCleskey *et al.* (1986), shows recordings of T, N and L currents from DRG neurones recorded before and after a transient application of ω-CgTX. The T current, recorded at higher gain in Fig. 6A, is not blocked whereas the L current in

Fig. 6C and the L and N currents in Fig. 6B are blocked. This selectivity has allowed, for the first time, recording of whole-cell T current on DRG neurones in the absence of N and L currents and provides a clear pharmacological distinction between the two inactivating currents, T and N.

Besides selecting among different channel types on a particular cell, the toxin is able to select among otherwise similar channels in different tissue. Table 2 summarizes results from eight preparations. L currents are blocked in the three neuronal

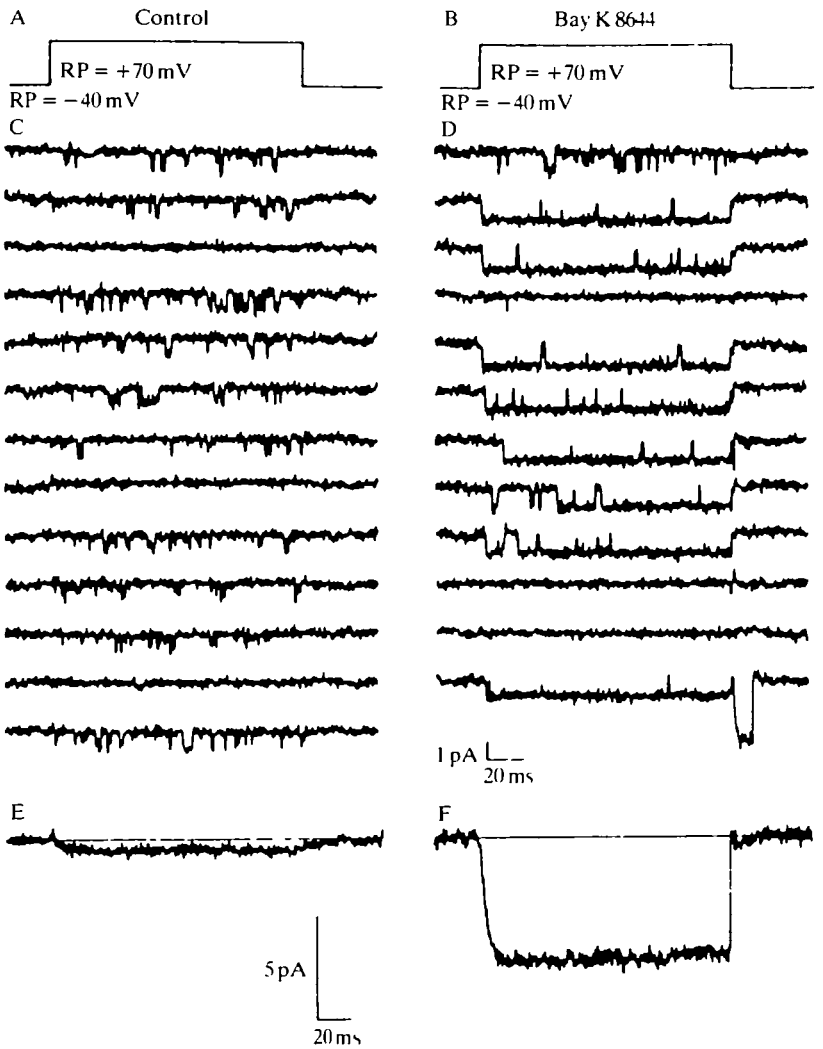

Fig. 5. Bay K 8644 causes prolonged openings of L channels. Cell-attached recordings from guinea-pig ventricle cell before (left) and after (right) adding 5 μmol l^{-1} Bay K 8644 to the bath. Pulse protocol indicated above where RP refers to the cell rest potential. (E),(F) Summations of many sweeps some of which are shown above. Pipette solution as in Fig. 4. Extracellular solution (mmol l^{-1}) NaCl, 135; KCl, 1·4; $CaCl_2$, 1·8; Hepes, 5; pH 7·4. From Hess, Lansman & Tsien, 1984, fig. 2.

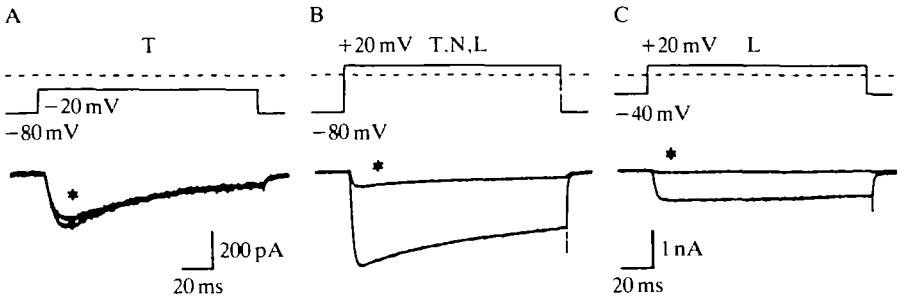

Fig. 6. ω-CgTX blocks particular types of DRG Ca^{2+} channels. Pulse protocols (above) evoke pure T (A), pure L (C), or a mixture of T, N and L currents (B) before and after (star) a transient application of ω-CgTX. Intracellular solution as in Fig. 3 but no cyclic AMP. Extracellular solution as in Fig. 1. From McCleskey, Fox, Feldman, Olivera, Tsien & Yoshikami, 1986.

cells but not blocked in the five muscle cell preparations. This specificity for neural Ca^{2+} channels may serve the purpose of selectively destroying neuromuscular transmission without lowering the toxin concentration through binding to the relatively high concentration of muscle Ca^{2+} channels. The specificity indicates that neuronal and muscle L-type Ca^{2+} channels cannot be entirely identical at the molecular level, though their kinetics and selectivity are similar.

The effect of ω-CgTX on N and L channels in DRG neurones shows little or no reversibility. During recordings lasting up to about 30 min, there has never been any recovery of current after a transient application of toxin. More than 80 % of labelled ω-CgTX remains bound to chick brain synaptosome membranes after 2 h and the apparent dissociation constant with 30-min incubations is about 1 nmol l^{-1} (Cruz & Olivera, 1986). The binding site is distinct from the dihydropyridine and verapamil binding sites (Cruz & Olivera, 1986) and, unlike dihydropyridines, blockage is not voltage-dependent and partial blockage does not alter the kinetics of the remaining current.

Table 2. *List of cell and channel types tested for sensitivity to* ω-CgTX

Preparation	Blocked	Not blocked
Neurone		
chick DRG	N and L	T
rat sympathetic	N and L	
rat hippocampal	'L'	'T'
Muscle		
guinea-pig ventricle	—	L
frog ventricle	—	L
frog atrium	—	L and T
chick myotubes	—	'L'
rabbit ear artery smooth muscle	—	L

Quotation marks indicate tentative assignment of channel type pending further study.

Use of the ω-CgTX as a biochemical ligand for Ca^{2+} channels requires that it binds directly to the channel rather than acting through a second messenger. The toxin is effective at blocking L channels in cell-free, excised patches of membrane when applied to the external surface of the membrane. However, when the toxin is applied to the bulk of the cell surface it fails to block single channels protected from direct contact with the toxin by an on-cell recording pipette. Evidently, binding of ω-CgTX to the external surface of the Ca^{2+} channel is both necessary and sufficient for block.

ω-CgTX has several advantages over dihydropyridines as a biochemical ligand for Ca^{2+} channels. There is no ambiguity that Ca^{2+} channels are its primary target, it binds with little or no reversibility, block is independent of the gating state of the channel, and the toxin is not lipid-soluble. The dihydropyridine binding site of skeletal muscle has been purified (Glossman & Ferry, 1983; Curtis & Catterall, 1984; Borsotto, Barhanin, Norman & Lazdunski, 1984) and Ca^{2+} flux has been reconstituted into vesicles (Curtis & Catterall, 1986). Purification of ω-CgTX binding sites in neurones may provide the opportunity to compare the structures of L and N channels as well as L channels from nerve and muscle.

FUNCTION OF DIFFERENT Ca^{2+} CHANNEL TYPES

L-type

Ca^{2+} channels can make membrane electrical signals, intracellular chemical signals or a combination of both. L-type Ca^{2+} channels in cardiac cells clearly serve both electrical and chemical functions during the heartbeat. The fact that there is only very slow inactivation of L channels allows the maintained Ca^{2+} current that underlies the plateau phase of the cardiac action potential (Reuter, 1967). The Ca^{2+} entering the cell through L channels is necessary both for replenishing the Ca^{2+} stores of the sarcoplasmic reticulum (Chapman & Niedergerke, 1970) and possibly for triggering intracellular Ca^{2+} release.

L-type channels also underlie secretion in some cells. Dihydropyridine-sensitive channels are required for substance P release from DRG neurones (Perney, Hirning & Miller, 1986) and slowly inactivating L-like conductances are responsible for Ca^{2+} influx and transmitter release in adrenal chromaffin cells (Fenwick *et al.* 1982) and the squid giant synapse (Llinas, Steinberg & Walton, 1981; Augustine, Charlton & Smith, 1985).

T-type

A function of T-type Ca^{2+} channels in neurones was proposed before there had been a direct recording of T currents. Llinas & Yarom (1981*a*,*b*), in an elegant study of inferior olivary neurones, demonstrated a rapid Ca^{2+} action potential that could be induced only if the cell was hyperpolarized. This T-like activity would be inactive at all times except during the after-hyperpolarization that follows a burst of action potentials and it was proposed that the conductance helped control the duration of the interburst interval. A full cycle of bursting would utilize two different Ca^{2+}

channels in different ways. The action potential is qualitatively like that in cardiac cells in that it consists of a sodium spike followed by a prolonged calcium plateau caused by a non-inactivating, L-like conductance. A burst of such spikes is terminated when the accumulation of intracellular Ca^{2+} sufficiently activates a Ca^{2+}-dependent potassium conductance. The after-hyperpolarization caused by the increased potassium conductance would unmask the T-type channels which reprime excitability and help trigger the succeeding burst. Thus, T-type channels would play a crucial electrical role in neuronal coding.

N-type

Discussion of the function of N channels is speculative at this time but it is tempting to suggest that N-type channels serve a neurone-specific function since they have only been demonstrated in nerve. Could the N channel be responsible for neurotransmitter release from presynaptic terminals? The pharmacology of evoked release of acetylcholine from the frog neuromuscular junction is similar to N channels in that it is blocked by ω-CgTX (Kerr & Yoshikami, 1984) but not affected by dihydropyridines (Fairhurst, Thayer, Colker & Beatty, 1983). Richard Miller and colleagues have compared the pharmacology of transmitter release in various cultured neurone preparations and found a variety of responses. Potassium-evoked substance P release from DRG neurones is blocked by dihydropyridines but norepinephrine release from sympathetic neurones was not (Perney *et al.* 1986). However, norepinephrine release is blocked by ω-CgTX. We have shown that the L-type channel in sympathetic neurones is dihydropyridine-sensitive and have found a slowly inactivating N-like current that is toxin-sensitive. Thus, toxin-sensitive, N-like channels are a candidate for mediating release in sympathetic neurones whereas the dihydropyridine-sensitive, L-type channels seem to be ruled out. Overall, pharmacological data suggest that the identity of the channel responsible for neurotransmitter release may be L in some tissues and N in others. If so, ω-CgTX, which blocks both L and N channels in neurones but fails to block L channels in muscle, seems ideally designed to inhibit presynaptic release.

CONCLUDING REMARKS

The past 2 years have been a watershed for the description of cells with multiple types of Ca^{2+} channels; a wide variety of neuronal, muscle and endocrine cells have been shown to have at least two kinds of Ca^{2+} channels. Dorsal root ganglion neurones have three types of Ca^{2+} channels, called T, N and L, which are distinguishable by steady-state inactivation, activation threshold, selectivity and pharmacology. A neurotoxin from the *Conus geographus* snail blocks only N- and L-type Ca^{2+} channels in DRG neurones, apparently by binding directly to the external surface of the channel; this promises to be a crucial probe for both the biochemistry and function of different channel types.

The toxin has created new 'sub-classes' of channels by distinguishing between L channels in nerve and muscle. In recording Ca^{2+} currents in different preparations,

one notes that largely similar channels show enough subtle variations that a mere alphabet would be insufficient for cataloguing. On what should we base the nomenclature? Toxicology would be a poor choice. Sodium channels responsible for the rapid action potential in nerve transmission are homogeneous with respect to function, show subtle differences in kinetics and temperature dependencies, and show striking differences in toxicology. Should the sodium channels of tetrodotoxin-producing puffer fish be considered a separate kind of channel because they are tetrodotoxin-insensitive? In the case of sodium channels, function defines type. In the case of Ca^{2+} channels, elucidation of function is lagging behind the description of kinetics and pharmacology. In lieu of clearly defined functions, it seems safest to define Ca^{2+} channels by their most dramatic kinetic features, as these are most likely to reflect function.

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