Multiple Calcium Channels and Neuronal Function

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Recent investigations have demonstrated that neurons have a number of different types of calcium channels, each with their own unique properties and pharmacology. These calcium channels may be important in the control of different aspects of nerve activity. Some of the possibilities can now be discussed.

CALCIUM CLEARLY PLAYS A PIVOTAL ROLE AS AN INTRACELLULAR second messenger. When the concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) rises into the micromolar range, many important cellular events are initiated. Although cells contain a great deal of Ca^{2+}, most of it is not free. Thus, Ca^{2+} can be bound by a variety of proteins and can be sequestered by organelles such as the mitochondria and endoplasmic reticulum (*1*). Furthermore, several pumps and exchange systems remove free Ca^{2+} from the cytoplasm if its concentration begins to rise (*2*). All these factors maintain the resting $[Ca^{2+}]_i$ at very low levels—less than $10^{-7}M$. Because the Ca^{2+} concentration in the extracellular milieu is in the millimolar range, there is a Ca^{2+} concentration gradient of about four orders of magnitude across the cell membrane, much greater than that for Na^+ or K^+.

Transient increases in $[Ca^{2+}]_i$, which act as important intracellular signals, can be initiated in two major ways. (i) Calcium can be released from intracellular storage sites associated with the endoplasmic reticulum (*3, 4*). Several important stimuli (such as hormones and neurotransmitters) act upon cell surface receptors and stimulate the breakdown of the phospholipid phosphatidylinositol bisphosphate (*4*). This generates diacylglycerol and inositol trisphosphate (IP_3). Intracellular receptors for IP_3 exist that, when activated, lead to the release of Ca^{2+} into the cytoplasm (*3*). Although the precise mechanism of this effect is unclear, it is the topic of intensive investigation. (ii) The $[Ca^{2+}]_i$ may also rise due to an increase in the Ca^{2+} permeability of the plasma membrane, which is normally virtually impermeable to Ca^{2+}. This is achieved by the opening of channels through which Ca^{2+} can pass into the cytoplasm down its electrochemical gradient. Some cells have Ca^{2+} channels that can be opened by the action of an agonist on a receptor (*5*). For example, Ca^{2+} can pass through the channels opened by nicotinic cholinergic agonists (*6*) or by the excitatory amino acid *N*-methyl-D-aspartate (NMDA) (*7*) (Fig. 1). However, several cells have channels that open and close (gating) in response to changes in membrane potential. These voltage-sensitive calcium channels (VSCC) (*8*) are found in some types of egg and endocrine cells, the various types of muscle cells, neurons, and a variety of other cell types.

In some cells the functions of VSCC seem rather straightforward. For example, an endocrine cell such as an adrenal medullary cell probably only has one major function—the secretion of hormones. This is reflected by its simple morphology. VSCC in these cells help to provide the Ca^{2+} that triggers the release of catecholamines and peptides (*9*). However, the functions of neurons are altogether more involved, as reflected by their complex morphologies. Not only do they possess the secretory functions of the adrenal cell, but they must also integrate and communicate information. In addition, the functions and properties of different portions of the same neuron differ radically. In some cases, neurons can fire Ca^{2+}-based action potentials (spikes) (*10*). In these cases, Ca^{2+} entering the cell acts as a charge carrier. However, neurons often do not fire Ca^{2+} spikes under normal conditions, although VSCC are clearly present and can be revealed after various pharmacological manipulations. Nevertheless, the influx of Ca^{2+} through these VSCC may play a key role in the regulation of nerve function. For example, the gating of several kinds of ionic channels can be regulated by changes in $[Ca^{2+}]_i$ (*11*). Thus, the influx of Ca^{2+} may lead to important secondary changes in nerve excitability. There are also numerous other targets for the Ca^{2+} that flows into the neuron. The role of Ca^{2+} as a trigger for the release of neurotransmitters is the best known of these (*10*). In addition, Ca^{2+}-dependent enzymes such as Ca^{2+}-calmodulin–dependent protein kinase, protein kinase C, and a variety of other kinases and proteases have been implicated in many neuronal functions (*12*).

In light of the multifaceted role of Ca^{2+} in neurons, it is not surprising that recent research has demonstrated that there are several different types of VSCC. The properties of these VSCC differ considerably in several key respects. Here I shall discuss the properties of these channels and how these properties may allow the channels to regulate the participation of Ca^{2+} in different aspects of nerve function. I have restricted the discussion to results from vertebrate neurons.

The Pharmacology of VSCC

Before discussing neuronal VSCC, I will review some current aspects of the pharmacology of VSCC, as this is essential for an understanding of the field. We know a great deal about the molecular structure of voltage-sensitive Na^+ channels and nicotinic receptors (*13*). One of the keys to this understanding has been the availability of natural toxins that bind to these channels with high affinity and specificity. These toxins can be used as biochemical probes to identify and purify the channel molecules. Until recently, natural toxins that interacted with VSCC had not been unequivocally identified (*14*). However, because of the great importance of VSCC in the control of the cardiovascular system, many synthetic drugs have been developed that interact with VSCC, and these have taken the place of toxins as molecular probes.

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Several classes of compounds have been shown to interact with VSCC (*15*). The most important of these from the theoretical point of view have been the dihydropyridines (DHP's), typified by the drugs nitrendipine and nifedipine (Fig. 2). Many DHP's potently inhibit VSCC. Some of these VSCC "antagonists" will relax smooth muscle preparations at concentrations below nanomolar. Small structural changes in the antagonist DHP molecule yield novel compounds that keep VSCC open for increased periods of time, allowing a greater than normal flux of Ca^{2+} into the cell (*16*). These drugs, typified by the DHP called BAY k 8644, are known as VSCC "agonists" (Fig. 2). It is interesting that with DHP's such as BAY k 8644 or 202-791, one optical enantiomer acts as a VSCC antagonist and the other as a VSCC agonist—an unusual situation (*17*). Drugs from other chemical classes such as the phenylalkylamines, verapamil, and D-600, as well as others such as diltiazem and bepridil, also act as VSCC antagonists (Fig. 2). However, in general their affinities and specificities are not as great as those of the DHP's (*15*). It is interesting that all these various classes of drugs bind to separate but interacting sites on VSCC, just as toxins interact with different sites on nicotinic receptors or Na^+ channels (*18*). Radiolabeled drugs can be used to biochemically identify and purify VSCC. Indeed, the DHP "receptor" has been purified from skeletal muscle (*19*). It appears to be a multisubunit protein with an approximate molecular mass of 210,000 daltons. Reconstitution of this protein into lipid vesicles is associated with the reconstitution of VSCC activity (*20*). The protein is also a substrate for adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (*21*). In fact, a kinase-mediated modification of VSCC is thought to be the basis for the positive inotropic effects of β-adrenergic agonists in the heart (*22*). Reconstitution of DHP receptor–containing membranes from skeletal muscle and heart into lipid bilayers has revealed subtle differences between the gating properties of the channels from these two sources (*23*). There are also subtle differences in the binding properties of [^{3}H]DHP's in the two tissues (*18*). Thus, within the general class of DHP receptors some small tissue-specific differences may occur.

The interaction between DHP's and VSCC is also voltage-dependent. DHP antagonists block VSCC more potently in depolarized than in polarized tissues (*24*). This seems to be because DHP's bind preferentially to VSCC in an inactivated state favored by depolarization. Thus, DHP's are weak blockers of VSCC in the functioning heart where cells are often highly polarized. However, when binding studies are carried out in membranes isolated from cardiac muscle (no membrane potential), higher affinity interactions are observed. This shift in drug potency with the membrane potential is clear in voltage-clamp studies (*24*) or in biochemical studies with vesicular preparations, which allow experimental manipulations of the membrane potential (*25*).

Dihydropyridine Receptors in the Brain

Theoretically, radiolabeled DHP's seem to be ideal tools for the biochemical investigation of VSCC. When these drugs first became available, several laboratories began to use them for this purpose and studied high affinity [^{3}H]DHP binding sites in various tissues (*18*). Such sites were easily demonstrated on various types of muscle cells, on endocrine cells, and in the nervous system, and they had the properties expected for genuine VSCC. In brain a heterogeneous distribution of [^{3}H]DHP receptors was readily demonstrated with binding assays or autoradiographic techniques (*18, 26*). However, a curious anomaly quickly became evident. VSCC in neurons did not seem to be sensitive to DHP's or indeed to other organic VSCC blockers (*27*).

VSCC in neurons can be analyzed in several different ways (*10*). Electrophysiological methods such as voltage clamping are the most precise, but analysis of the Ca^{2+} spikes observed in many neurons after appropriate manipulations can also be very revealing. Unfortunately, in all but a few instances (invertebrate neurons of colossal size), such recording techniques only give access to the cell soma; in neurons, events in the nerve terminal are of particular interest. However, there are methods for obtaining information about VSCC in terminals. For example, Ca^{2+} fluxes into synaptosomal preparations can be monitored with radioisotopes or optical tech-

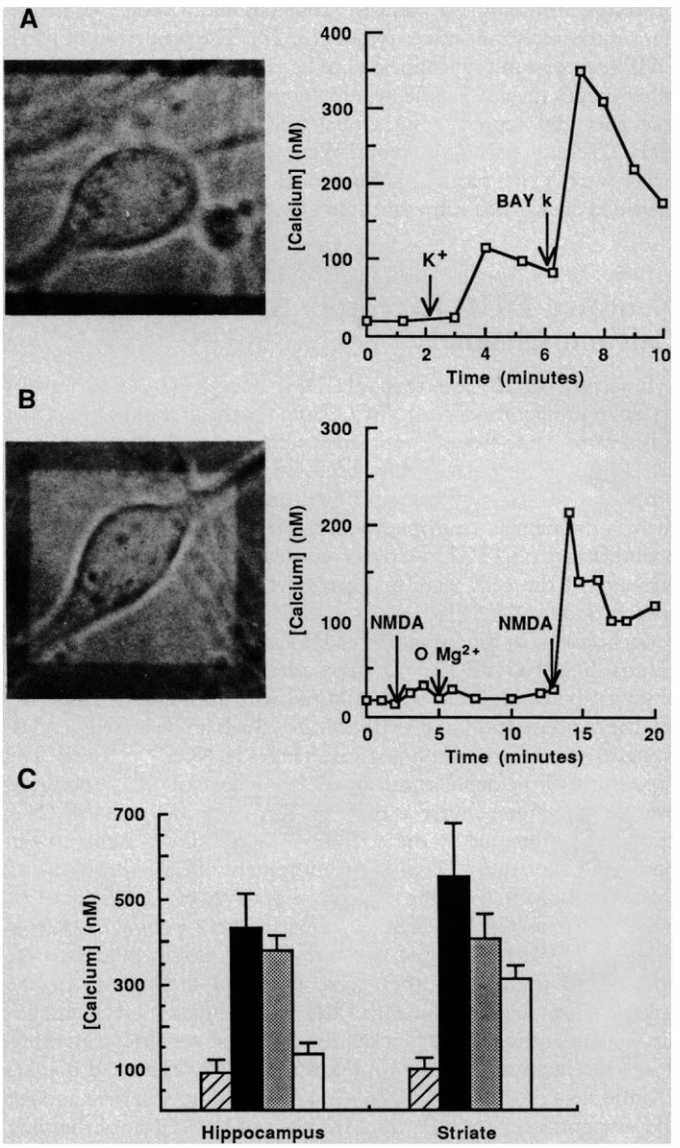
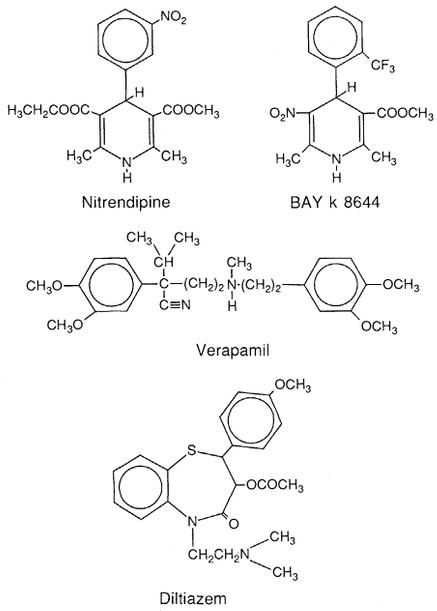


Fig. 1. (**A**) Influx of Ca^{2+} into a single hippocampal neuronal cell body measured with fura-2 microspectrofluorimetry (*67*). The dihydropyridine agonist BAY k 8644 (1 μ*M*) augments the influx of Ca^{2+} into the cell over that produced by K^+ (10 m*M*). (**B**) Effect of the excitatory amino acid NMDA (10^{-5} *M*) on a single striatal neuron. Stimulation of Ca^{2+} uptake occurs in the absence of Mg^{2+}, but not in the presence of physiological concentrations of Mg^{2+} (1.3 m*M*). (**C**) Effect of nitrendipine (10^{-6} *M*) on the uptake of Ca^{2+} into single hippocampal and striatal neurons. Diagonal striped bar, $[Ca^{2+}]_i$ under resting conditions (5 m*M* K^+); black bar, $[Ca^{2+}]_i$ after a depolarizing stimulus (70 m*M* K^+); stippled bar, $[Ca^{2+}]_i$ after a depolarizing stimulus (70 m*M* K^+) in medium in which all Na^+ was replaced by choline; open bar, $[Ca^{2+}]_i$ after a 70 m*M* K^+ stimulus in choline-containing medium in the presence of nitrendipine (10^{-6} *M*). Nitrendipine produced greater inhibition of Ca^{2+} uptake into hippocampal than into striatal neurons.

niques. Furthermore, one of the most important functions of VSCC in neurons is to provide Ca^{2+} for triggering neurotransmitter release. Thus, under appropriate conditions the depolarization-evoked release of a neurotransmitter can be an invaluable de facto assay for the VSCC in nerve terminals.

At the time that [^{3}H]DHP binding assays were first being employed, data indicated that DHP's had no effects upon neurons, no matter what paradigm was utilized (*27*). Some drugs that did have neuronal effects only did so at concentrations where their actions were totally nonspecific (*27*). At elevated concentrations, VSCC blockers such as verapamil also inhibit many other neuronal processes, including Na^+ and K^+ channels and a variety of neurotransmitter receptors and enzymes (*15, 27*). The properties of [^{3}H]-DHP receptors in brain appeared to be virtually identical to those in other tissues such as muscle, where a convincing case could be made that they did represent functional VSCC. What then were the [^{3}H]DHP receptors in the brain? Were they really VSCC and, if so, what were their particular functions? Subsequent research has provided answers to some of these questions.

Fig. 2. Chemical structures of important organic calcium channel modulators.



Neuronal DHP Receptors Are Functional Calcium Channels

It is now quite clear that [^{3}H]DHP receptors in the nervous system represent functional VSCC. Some of the first indications that this was so came from experiments with neuronal clonal cell lines (*28*). Struck by the anomaly discussed above, we examined the properties of $^{45}Ca^{2+}$ uptake into neuronal cell lines. In some cells such as the neuron-neuroblastoma hybrid cell line NCB-20 or the neuroblastoma N4TG1, we observed that the uptake of $^{45}Ca^{2+}$ was increased if the cells were depolarized by raising the extracellular concentration of K^+ ($[K^+]_o$) or by a depolarizing alkaloid such as batrachotoxin. In certain other cell lines, such as the neuroblastoma-glioma line NG108-15, no depolarization-induced uptake was observed unless the cells were cultured with stimuli that raised the cellular concentration of cAMP (*28, 29*). Such an increase in cAMP was associated with morphological changes in NG108-15 cells. The appearance of the depolarization-induced uptake of $^{45}Ca^{2+}$ occurred over the same time course as these changes. The increases in $^{45}Ca^{2+}$ uptake were not due to the activation of Na^+-Ca^{2+} exchange (an electrogenic pathway) (*2*) or to the movement of Ca^{2+} through Na^+ channels (*2*). Moreover, the uptake was potently blocked by divalent cations such as Cd^{2+}, which is known to be a potent blocker of VSCC (*8*). We then tested whether DHP's would modulate the uptake and found that they were powerful modulators of the voltage-dependent component of the $^{45}Ca^{2+}$ uptake. DHP antagonists such as nitrendipine blocked the uptake at very low concentrations, and agonists such as BAY k 8644 greatly enhanced uptake. Enantiomers of the DHP 202-791 showed the appropriate agonist and antagonist effects (*17, 28*). In some of the cell lines, binding studies with [^{3}H]DHP's also revealed the presence of DHP receptors that were inducible by cAMP (*29*). The pharmacology of the DHP-sensitive $^{45}Ca^{2+}$ uptake and of the [^{3}H]DHP receptors in brain or muscle correlated closely. Clearly, in these cell lines of neuronal origin, DHP-sensitive VSCC exist. However, would VSCC in authentic neurons also prove to be DHP sensitive?

Recently we have carried out Ca^{2+} flux studies utilizing authentic central neurons in primary culture (*30*) and the Ca^{2+}-sensitive fluorescent dye fura-2 (*31*). The initial observations on the presence of specific VSCC in squid axons were made with the Ca^{2+}-sensitive protein aequorin (*2*). We cultured neurons from different parts of the brain including the striatum, hippocampus, cortex, septum, and cerebellum. Using a microspectrofluorimeter, we could easily measure changes in $[Ca^{2+}]_i$ in the cell bodies of single neurons (Fig. 1). Depolarizing stimuli caused a rapid and long-lasting increase in $[Ca^{2+}]_i$. We then investigated the sensitivity of this Ca^{2+} uptake to the DHP agonist BAY k 8644. Treatment with this agonist unexpectedly enhanced Ca^{2+} uptake in virtually every neuron we studied. In about 100 neurons from various parts of the brain over 90% responded to BAY k 8644. In another type of experiment, we analyzed the effectiveness of the DHP antagonist nitrendipine in blocking the increase in $[Ca^{2+}]_i$ produced by a depolarizing stimulus. Nitrendipine was effective, but to different extents in different brain areas. For example, much more of the increase in $[Ca^{2+}]_i$ was blocked in hippocampal neurons than in striatal neurons. In each type of neuron there was a component of the voltage-dependent Ca^{2+} uptake that was not blocked by the DHP. Thus, DHP-sensitive VSCC exist in many neurons in the central nervous system. Upon depolarization, the Ca^{2+} flux through these channels makes a variable contribution to the total influx of Ca^{2+}. This raises several questions. What is the DHP-insensitive flux? Why do DHP's not influence some neuronal functions such as neurotransmitter release?

Multiple Calcium Channels

One clue to a possible answer to these questions came from experiments with the PC12 rat pheochromocytoma cell line. Normally these cells resemble adrenal medullary cells. Like adrenal medullary cells, they possess DHP-sensitive VSCC. When depolarized, Ca^{2+} can enter the cell through these channels and trigger catecholamine and peptide secretion. Evoked catecholamine release can be potently blocked by nitrendipine and enhanced by BAY k 8644 (*32*). Adrenal medullary cells and sympathetic neurons are closely related. The former can be "transformed" into something resembling the latter by nerve growth factor (*33*). A similar transformation can occur with PC12 cells. After transformation to neuronal growth, the evoked release of catecholamine from PC12 cells became almost completely DHP insensitive, thus mimicking the situation in neurons (*34*). In both growth modes, however, evoked transmitter release was potently blocked by Cd^{2+}. Furthermore, in both growth modes the depolarization-induced influx of $^{45}Ca^{2+}$ into the cells remained very DHP sensitive. Thus in NGF-differentiated PC12 cells, there seemed to be a dissociation in the

Table 1. Properties of multiple calcium channels found in chick dorsal root ganglion neurons. Data summarized from (*36, 40, 43*).

Property	Channel type		
	T	N	L
Activation range (10 m*M* Ca)	Positive to −70 mV	Positive to −10 mV	Positive to −10 mV
Inactivation range	−100 to −60 mV	>−100 to −40 mV	−60 to −10 mV
Approximate single-channel conductance	9 pS	13 pS	25 pS
Cd^{2+} block	Weak; $IC_{50} \simeq 100$ μ*M*	Strong; $IC_{50} \simeq 10$ μ*M*	Strong; $IC_{50} \simeq 10$ μ*M*
ω-CgTx block	Weak	Strong	Strong
DHP modulation	No	No	Yes

properties of evoked transmitter release and $^{45}Ca^{2+}$ influx. The former was DHP insensitive and the latter was DHP sensitive. To explain these observations, we hypothesized that two types of VSCC existed in differentiated PC12 cells—DHP-sensitive channels and DHP-resistant channels. Our $^{45}Ca^{2+}$ flux assay seemed to predominantly measure flux through DHP-sensitive channels. Postulating the existence of DHP-sensitive and -insensitive VSCC explained some of the anomalies in the literature. In particular, we speculated that DHP-insensitive VSCC were linked to the transmitter release process.

Although the evidence leading to these suggestions arose from pharmacological and neurochemical considerations, the existence of diverse types of VSCC in neurons and other cells such as egg cells has also been postulated on the basis of electrophysiological investigations (reviewed in *8, 10*). For example, Llinas and colleagues had demonstrated that in several types of central nervous system neurons, Ca^{2+}-based action potentials with different properties could be observed. These "high" and "low" threshold spikes seemed to originate in different parts of the neuron. The properties of these spikes led Llinas to postulate the existence of two types of VSCC with different biophysical properties (*10, 35*).

Nowycky *et al.* and several other groups recently advanced our understanding significantly by demonstrating that chick dorsal root ganglion (DRG) neurons in culture possessed three distinct types of VSCC (*10, 36, 37*). The contribution of the three types of VSCC to the overall Ca^{2+} current in these cells is illustrated in Fig. 3. The first type of VSCC, designated T, gives rise to a small transient Ca^{2+} current elicited by small depolarizing steps from negative holding potentials. With stronger depolarizations a second component of inactivating current becomes evident as well as a component of the current that only slightly inactivates over a time course of several hundred milliseconds. Nowycky *et al.* demonstrated that a second type of VSCC (N), of intermediate size, was responsible for the second inactivating phase of the Ca^{2+} current observed at strong depolarizations. The third VSCC (L), which was also activated by strong depolarizations, was responsible for the noninactivating component of the current. The pharmacological properties of these three types of VSCC proved most interesting (Table 1). L channels were modulated by DHP agonists and antagonists but T and N channels were not. L and N channels were potently blocked by Cd^{2+}, whereas T channels were much less sensitive. Finally, in chick DRG cells a 27–amino acid toxin from the venom of the marine snail *Conus geographicus* (ω-CgTx) blocked both N and L channels, but not T channels (*38*).

The L channels identified in these electrophysiological studies are similar to the DHP-sensitive channels mediating Ca^{2+} influx in our fura-2 and $^{45}Ca^{2+}$ flux studies. They are also similar to other DHP-

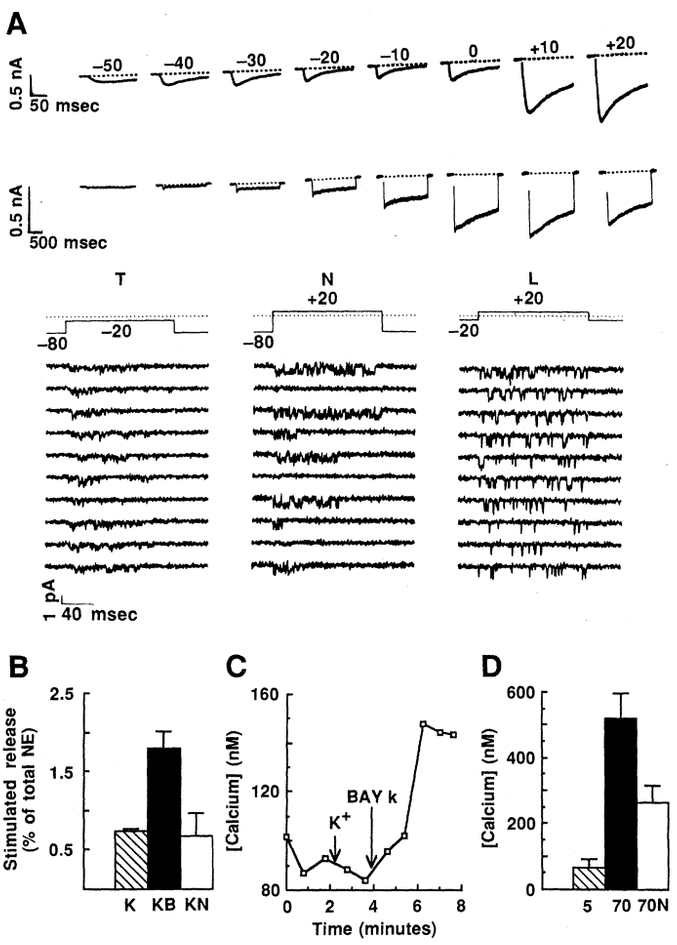


Fig. 3. (**A**) The upper and middle traces illustrate Ca^{2+} currents in whole cell voltage-clamp recordings from a chick DRG neuron and a rat SCG neuron, respectively. In the upper trace, the holding potential was −100 mV and in the middle trace −90 mV. Test potentials are indicated above. External solution contained 135 m*M* tetraethylammonium chloride, 10 m*M* Hepes, 0.2 m*M* tetrodotoxin, and 10 m*M* $CaCl_2$ (upper) or 10 m*M* $BaCl_2$ (middle). Internal solution contained 100 m*M* CsCl, 10 m*M* Cs EGTA, 5 m*M* $MgCl_2$, 40 m*M* Hepes, 2 m*M* adenosine triphosphate, *p*H 7.3. The bottom traces illustrate the three types of unitary Ca^{2+} channel activity in cell-attached patch recordings from a chick DRG neuron with Ba^{2+} as the charge carrier. Patch pipettes contained 110 m*M* $BaCl_2$, 10 m*M* Hepes, and 200 n*M* tetrodotoxin, *p*H 7.4. The cell resting potential was zeroed with an external solution containing 140 m*M* K^+ EGTA, 10 m*M* Hepes, 1 m*M* $MgCl_2$, *p*H 7.4. Data from (*36*) and unpublished observations. (**B**) Release of norepinephrine from cultures of rat SCG neurons. Diagonal striped bar (K), release evoked by elevated K^+ (70 m*M*); black bar (KB), release evoked by elevated K^+ with BAY k 8644 (10^{-6} *M*); open bar (KN), release evoked by elevated K^+ with nitrendipine (10^{-6} *M*). (**C**) Ca^{2+} uptake into a single SCG neuron measured with fura-2 microspectrofluorimetry (*67*). Note the augmenting effect of BAY k 8644. (**D**) Uptake of Ca^{2+} into SCG neurons. Diagonal striped bar (5), $[Ca^{2+}]_i$ in 5 m*M* K^+; black bar (70), $[Ca^{2+}]_i$ in 70 m*M* K^+; open bar (70N) $[Ca^{2+}]_i$ in 70 m*M* K^+ with nitrendipine (10^{-6} *M*).

sensitive channels identified in a variety of other nonneuronal tissues (*39*). Indeed, studies in many of these tissues have identified a second DHP-insensitive Ca^{2+} current that is analogous to the T channel described by Nowycky *et al.* in sensory neurons. However, the discovery of the N-type VSCC was of particular interest. As we shall discuss, it seemed to us that this channel was an ideal candidate for the VSCC that was often linked to neurotransmitter release. Further studies have supported this view.

Calcium Channels and Neurotransmitter Release

To further define the roles of the various VSCC in different neuronal functions, we used sympathetic neurons cultured from the rat superior cervical ganglion (SCG) (*40*). First, we analyzed the types of VSCC in these cells. We found that two of the VSCC in chick DRG cells were also found in rat sympathetic neurons. These were the N and L channels. No T channels were found in these cells (Fig. 3). The properties of the N and L channels in sympathetic neurons were similar to those in DRG cells.

To define which VSCC were involved in neurotransmitter release, we studied evoked [^{3}H]norepinephrine release from these neurons. We observed that release could be potently blocked by Cd^{2+} and partially blocked by ω-CgTx. However, the DHP antagonist nitrendipine produced no inhibition (Fig. 3). Although evoked transmitter release was not blocked by DHP antagonists, it was significantly enhanced by DHP agonists such as BAY k 8644. This extra portion of release could be blocked by nitrendipine. Such a pharmacological pattern had also been observed in a study of serotonin release from brain cortical slices (*41*). Here again, depolarization-evoked release of serotonin could not be blocked by nitrendipine but could be enhanced by BAY k 8644. This may seem curious at first. However, further studies on the sympathetic neurons suggested an explanation.

We next analyzed the depolarization-induced influx of Ca^{2+} into the cell bodies of sympathetic neurons with fura-2 using the same conditions that had been employed in the [^{3}H]norepinephrine release studies (Fig. 3). We observed that the Ca^{2+} influx could be enhanced by BAY k 8644. Significantly, a portion of the Ca^{2+} influx could be blocked by nitrendipine. Thus, just as in the PC12 cells, the pharmacological properties of the Ca^{2+} influx and of the neurotransmitter release differed. Under depolarizing conditions where we saw [^{3}H]norepinephrine release that could not be blocked by nitrendipine, a portion of the Ca^{2+} flowing into the cell soma could be blocked by this DHP. These various results could be explained by a model such as that illustrated in Fig. 4. Vesicle exocytosis occurs by fusion of transmitter-containing vesicles with "active zones" on the plasma membrane. Let us suppose that N channels are localized in clusters near these zones but L channels are not. Upon depolarization, Ca^{2+} will enter the cell via both types of VSCC. However, Ca^{2+} entering through N channels will have a much greater influence on release because of their location. Normally, therefore, nitrendipine would not block release. However, if BAY k 8644 is employed, the flux via L channels will be selectively enhanced. So much Ca^{2+} may now enter through this pathway that there will be a spillover effect and the extra Ca^{2+} will be sensed by the release apparatus. Thus, BAY k 8644 will enhance release, and this portion of the release will be blocked by nitrendipine. In fact, studies with the electron microscope have identified clusters of particles near active zones of transmitter release that may correspond to VSCC (*42*). Second, clusters or "hot spots" of N channels have been found in neurons with patch clamp techniques (*43*). The model in Fig. 4 implies that Ca^{2+} entering through a channel has a very particular sphere of influence. Support for such a view has recently come from several theoretical modeling studies (*44*).

In fact, it is quite likely that L channels may not be very common in nerve terminals and may be preferentially localized in the cell soma. There are several pieces of evidence for this. For example, it has been shown that, upon careful subcellular fractionation, the concentration of [^{3}H]DHP receptors is much higher in the postsynaptic membrane than on the presynaptic terminal (*45*). In another provocative study, Hanbauer and colleagues examined the effects of lesions on [^{3}H]DHP binding in the striatum (*46*). They found no change in binding when they lesioned the dopaminergic input into the striatum with 6-hydroxy dopamine. However, kainic acid lesions of the striatum, which destroy the cell bodies of intrinsic neurons, completely obliterated [^{3}H]DHP binding. Similar conclusions can be gleaned from lesion studies in the hippocampus (*47*).

Rapid ion flux studies with synaptosomes have also been informative. When the contribution from Na^+-Ca^{2+} exchange is minimized, the properties of the VSCC in synaptosomes are clear. They are potently blocked by Cd^{2+}, are inactivated by prior depolarization, and are insensitive to modulation by DHP's, according to most studies (*48*). These properties are similar to those of N channels. On the other hand, when Ca^{2+} influx into the cell soma is measured, DHP antagonists seem to be effective to a certain extent (*28, 29, 32, 34, 49*). This is true in primary neuronal cultures, brain slices, or in neuronal clonal cell lines (as discussed above). Thus, these data further support the notion that L channels are preferentially localized in the cell soma and that N channels mediate neurotransmitter release from the nerve terminals.

This picture is probably oversimplified in some respects. For example, synaptosomes clearly represent nerve terminals from an enormous variety of neuronal types. Recently, it has been shown that the depolarization-induced influx of $^{45}Ca^{2+}$ into synaptosomes can be blocked by ω-CgTx (*50*). However, different portions of the flux can be blocked over different toxin concentration ranges. Thus, there may be some further heterogeneity among those entities designated as N channels. In fact, some heterogeneity is also present in other "categories" of channels. For example, consider the range of sensitivities of Na^+ channels to tetrodotoxin (*51*).

Furthermore, although the above model fits much of the data in the literature, there are exceptions. The release of substance P from cultured rat sensory neurons from DRG is extremely sensitive to modulation by DHP's under those same conditions in which [^{3}H]norepinephrine release from sympathetic neurons is completely DHP resistant. This is true even after long times in culture (up to 60 days) or if the DRG cells are grown together with their target spinal cord neurons (*52*). However, substance P release from slices of adult rat spinal cord appears to be DHP-insensitive, just as in many of the other cases already referred to. The L channel–dominated release in DRG neurons in culture may have some developmental significance.

A particularly important and exciting aspect of research in this area is the possibility that neuronal VSCC may be modulated by biochemical modifications produced by enzymes such as kinases. There is an obvious precedent for such a mechanism in the β-adrenergic stimulation of the heart, which results from the cAMP-mediated phosphorylation of VSCC in this tissue (*53*). In many vertebrate neurons, drugs and neurotransmitters can reduce the duration of the action potential, an effect that is ultimately reflected in a reduction of neurotransmitter release [(*54*), reviewed in (*8*) and (*10*)]. This may be due to an increased K^+ permeability resulting in more rapid spike repolarization or to the direct blockade of voltage-dependent Ca^{2+} influx. There is great interest in the mechanisms underlying this receptor-mediated inhibition of Ca^{2+} influx. In some cases, a pertussis toxin–sensitive guanine nucleotide binding

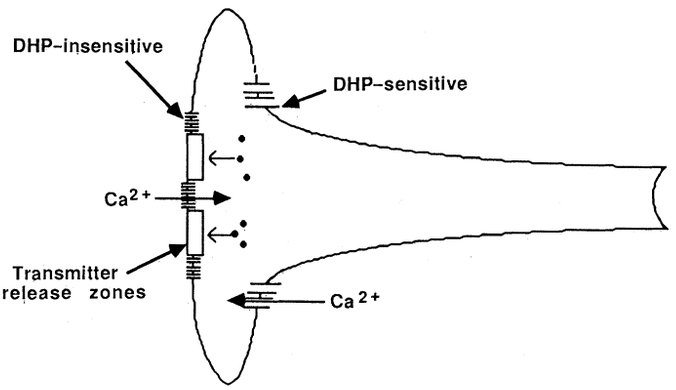


Fig. 4. Hypothetical arrangement of dihydropyridine (DHP)-sensitive (L) and DHP-insensitive (N) type calcium channels in a nerve terminal.

(G) protein seems to be involved in the transduction mechanism (*55*). It is not known whether a diffusible second messenger is also involved. However, phorbol esters can mimic the agonist induced inhibition of Ca^{2+} currents, indicating a possible inhibitory role for protein kinase C in some cases (*56*). We also do not know which types of VSCC are modulated by agonists or phorbol esters or even if it is the same class in all instances. However, if N channels were selectively involved in neurotransmitter release, it would be interesting if they were the specific targets for these inhibitory events. Indeed, some recent studies have suggested that this may be the case (*57*). However, other studies appear to have demonstrated inhibitory effects of phorbol esters on L currents (*58*).

Functions of Other Neuronal Calcium Channels

If N channels play an important role in the regulation of neurotransmitter release in many instances, what can be said about the functions of T and L channels? Several authors have speculated on the role of VSCC in the production of rhythmic bursting behavior in neurons. Although it is clear that the modulation of K^+ permeability can play a role in the generation of this phenomenon, other data also implicate VSCC (*10, 35, 59*). Llinas has suggested models of bursting activity in a variety of central neurons (*35*), which involve the participation of a low threshold Ca^{2+} conductance that requires repriming at hyperpolarized membrane potentials for activation. Clearly the T channel has the correct properties for participation in these events. Unfortunately we do not yet know how to specifically inhibit or enhance T channels. The effects of such manipulations on bursting patterns in certain neurons would be most interesting.

The functions of L channels are a subject for even greater speculation at this point. It is reasonable that the cell soma should possess pathways for the injection of large amounts of Ca^{2+} into the cytoplasm as the result of neuronal activity. Ca^{2+} influx does not necessarily make much of a contribution to the action potential in many neurons under normal physiological conditions. However, we know that VSCC are present because Ca^{2+} spikes can usually be revealed after the appropriate ionic and pharmacological manipulations (*10*). Thus, the normal role of this Ca^{2+} influx must be other than the generation of action potentials. As discussed above, it is clear that many ion channels and enzymes such as kinases and proteases depend on Ca^{2+} for their activity (*12*). It will be most instructive to take a careful look at the effects of DHP's on biochemical processes occurring in neurons. For example, it has been suggested that postsynaptic contributions to long-term potentiation (LTP) at central synapses are dependent on the influx of Ca^{2+} into the postsynaptic neuron (*60*). This is thought to reflect the involvement of a Ca^{2+}-dependent protease, calpain, in the postsynaptic cell. Thus, it will be interesting to examine the effects of DHP's in paradigms designed to demonstrate LTP. Would BAY k 8644 enhance this phenomenon?

Another interesting property of L channels is their plasticity. The number of [^{3}H]DHP binding sites in the brain has been shown to change in a number of pathological conditions (for example, aging, cardiomyopathy, and hypertension) (*61*) and after chronic administration of drugs [such as opiates, phencyclidine (PCP), neuroleptics, and alcohol] (*62*). Thus, changes in L channel function may contribute to the changes in nerve excitability associated with such states. Also implicit in these observations is the possibility that DHP's may be of therapeutic value in such situations (*63*). Of course similar changes may also occur in the T and N channels, but at present we have no convenient biochemical probes that can be used as markers for these channels.

Receptor-Operated Channels

In addition to the fact that VSCC in neurons may be altered as the result of the activation of specific receptors, the activation of receptors may also regulate the influx of Ca^{2+} into neurons in other ways as well. Activation of some receptors, such as the nicotinic receptor (*6*), may directly cause the opening of channels that are permeable to Ca^{2+} (as discussed above). Activation of these receptors leads to the opening of a pathway that can accommodate Ca^{2+} as well as many other cations. In contrast, VSCC appear to be rather specific for Ca^{2+}. Receptor-operated channels (ROC's) (*5*) may also exist in smooth muscle where they could participate in agonist-induced muscle contraction.

Another good example of a ROC is the receptor for the excitatory amino acid NMDA. These receptors are found on neurons in many parts of the central nervous system. Activation of NMDA receptors leads to the opening of a channel that allows a large amount of Ca^{2+} to pass into the cell (*7, 64*) (Fig. 1). Other cations also probably pass easily through this channel. Gating of the NMDA channel is actually not a voltage-dependent process, although it behaves as such under normal physiological conditions. However, this is because the channel is blocked in a voltage-dependent fashion by low concentrations of Mg^{2+} (*65*). This block is evident at negative membrane potentials and is relieved as the cell depolarizes. The pharmacology of the NMDA-linked channel is quite different from any of the types of VSCC. In addition to the voltage-dependent block by Mg^{2+}, NMDA channels are also potently blocked by the psychotropic drug PCP (*66*). Calcium entry into neurons through NMDA-linked channels is also important in the generation of LTP (as discussed above) and possibly in the generation of phenomena such as epilepsy (*60*).

Conclusions

Calcium is of such central importance in the integration of the activities of a cell that it is not surprising that multiple methods have evolved for regulating its movement and concentration. In a complex cell such as a neuron, different mechanisms may operate simultaneously in different portions of the cell to regulate separate functions. The multiple types of VSCC's and ROC's offer the cell some flexibility in the way it can modulate the entry of Ca^{2+}. The properties of the different channels allow Ca^{2+} entry to be associated with quite different functions, thus making use of Ca^{2+} in its role as

both a charge carrier and a second messenger. Calcium concentrations may be further localized and compartmentalized as a result of Ca^{2+}-buffering mechanisms within the cell. The physical and anatomical basis for such buffering is not completely understood, but is clearly of great importance. As we have discussed, pharmacological considerations have played a major role in the development of our understanding of these diverse systems. However, we know relatively little about specific ways to modify some of these newly discovered pathways. A search for specific modifiers of the various VSCC would certainly be worthwhile as it will allow us further insights into their particular functions. Moreover, such agents may have unique therapeutic profiles in a number of situations.

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