Annu. Rev. Pharmacol. Toxicol. 1992. 32:399–421

THE CENTRAL ROLE OF VOLTAGE-ACTIVATED AND RECEPTOR-OPERATED CALCIUM CHANNELS IN NEURONAL CELLS

Mariella Bertolino

Fidia-Georgetown Institute for the Neurosciences, Georgetown University School of Medicine, Washington DC 20007

Rodolfo R. Llinás

Department of Physiology and Biophysics, New York University Medical School, New York, NY 10016

KEY WORDS: calcium channel, DHP, neurotoxicity, caffeine, ω-conotoxin

INTRODUCTION

It has been known for more than three decades that calcium ions play a fundamental role in the regulation of many cellular processes, including vesicular exocytosis and synaptic transmission (1), muscle contraction (2), and even neurotoxicity and neuronal death. The role of calcium in these macroscopic cellular functions is one of a second messenger, serving to regulate, for example, enzymes, ion channels, the expression of immediate-early genes, and thereby the induction of specific genetic programs that are responsible for such long-term cellular responses as long-term potentiation.

Under normal conditions, the calcium concentration of the extracellular fluid is in the millimolar range, whereas the intracellular free calcium concentration is less than 10^{-7} M (3). Transient increases in the cytosolic calcium concentration can result from the entry of calcium from the external milieu

0362-1642/92/0415-0399$02.00

through receptor-operated channels, voltage-activated channels, or ionic pumps, or from the release of calcium from internal stores (3).

Perhaps the most perplexing observation regarding transmembrane calcium flux is the coexistence of multiple types of calcium channels within the same cell. The different channel types may be distinguished by their particular pharmacological and biophysical properties. Given the fundamental role of calcium channels in most aspects of cellular physiology and biochemistry, it has become important to define the biophysical properties of these channels as well as the mechanisms for their modulation.

This review discusses the regulation of voltage-activated and receptor-operated calcium channels by exogenous and endogenous ligands in neuronal cells of vertebrates.

VOLTAGE-DEPENDENT CALCIUM CHANNELS

Multiple types of voltage-gated calcium channels have been described in most of the excitable cells (4). These channels have been classified according to different criteria, but it is widely accepted that there are at least two classes of voltage-activated calcium channels, as defined by the pattern of channel activation: low voltage-activated (LVA) or low-threshold, and high voltage-activated (HVA) or high-threshold channels. LVA channels are also termed "T" (for transient), and HVA channels have been further divided into three subclasses: "L" (for long-lasting), "N" (for neither T nor L or neuronal) and "P" (for Purkinje cell).

T-Type Channel

CHARACTERISTICS The existence of a low-threshold calcium current was first revealed by Llinás & Yarom in neurons of the guinea pig inferior olivary nucleus (5, 6). Extracellular recordings of field potentials demonstrated a low-threshold calcium conductance localized in the soma and a high-threshold calcium conductance present in the dendrites of these neurons. Subsequently, Carbone et al (7–10) and Nowycky et al (11–13) characterized the T-type calcium channel in chick and rat dorsal root ganglion neurons by whole-cell and single-channel current analysis.

T-type calcium channels only require a weak depolarization for activation and carry a transient current at negative membrane potentials that inactivates rapidly during a prolonged pulse. Whole-cell recording from chick and rat dorsal root ganglion neurons (9, 10, 12, 13) has shown that the T current is activated at approximately −50 mV and reaches its maximum value between −40 and −10 mV. Channel inactivation is eliminated at very negative potentials: at a holding potential more positive than −60 mV, the channel is completely inactivated, but inactivation is progressively eliminated as the

potential decreases from −60 to −100 mV. The time constant of the decay phase is approximately 10 to 50 ms and the current inactivates rapidly and completely during a maintained depolarization. In the presence of 110 mM barium as the main charge carrier, the T-type channel shows a conductance value in the order of 8 pS.

In rat sensory neurons of the cranial dorsal ganglia (14, 15), the T-type calcium current is not affected by the rise in the intracellular free calcium concentration. During a sustained depolarization, most of the calcium current decreases with time, which is apparently due in part to an inactivation of calcium channels caused by an increase in the cytosolic calcium concentration. At an intracellular free calcium concentration of 10^{-6} M, the T-type calcium current is still present and can be isolated from the other components, which, in contrast, are blocked by the increase in intracellular free calcium concentration.

The structure of the T-type calcium channel has not been determined, mostly because of the lack of selective ligands. Studies on the expression of calcium channels in *Xenopus* oocytes after injection of heart mRNA (16) have shown that the channel that generates the transient calcium current is a molecular entity separate from the dihydropyridine (DHP)-sensitive (or L-type) calcium channel.

FUNCTIONS Because T-type calcium currents are activated at negative membrane potentials close to the resting potential, the T-type channel is responsible for neuronal oscillatory activity, that is, spontaneous membrane potential fluctuations not mediated by synaptic activity. Such oscillatory activity may have a prominent role in various brain functions, such as wakefulness regulation, motor coordination, and neuronal circuit specification during ontogenesis via oscillatory electrical activity (17).

Studies on the electrophysiological properties of guinea pig thalamic neurons in a slice preparation (18–20) have shown that voltage-dependent ionic conductances in these cells generate two distinct patterns of activity: repetitive tonic firing and burst firing (or phasic firing). These two activity patterns allow the thalamic neurons to behave as oscillators at two distinct frequencies; and furthermore, changes in membrane potential cause the neuron to switch from one mode of oscillation to the other. Thus, at potentials more positive than −60 mV, the cell exhibits a repetitive firing activity with a frequency of approximately 10 Hz, which is due to the activation of a persistent sodium conductance (19); however, at potentials more negative than −65 mV, a burst of action potentials at a frequency of 5 Hz is triggered, due to the deinactivation of the T-type calcium conductance. Another study conducted on adult neurons isolated from the thalamic dorsal lateral geniculate nucleus (21) confirmed the existence of these two response modes, which

in this instance were evoked by afferent excitation. In agreement with the general view of thalamic function (22), tonic firing activity is most frequently recorded in awake animals and phasic firing corresponds to slow-wave sleep.

Phasic firing is dependent on calcium entry through T-type calcium channels (19–20). In fact, studies on inferior olivary nucleus neurons in brain stem slices (5, 6) demonstrated that intrinsic phasic oscillatory activity can occur as a result of the activation of a low-threshold calcium conductance, which produces an afterdepolarization responsible for the initiation and maintainance of the oscillatory activity.

Studies of the electrophysiological properties of neurons in the pars compacta of the substantia nigra have established the presence of two dendritic calcium conductances, one low-threshold and one high-threshold, among other ion conductances (23). Hyperpolarization of the membrane potential generates a burst of action potentials that are tetrodotoxin insensitive and can be blocked by application of 1 mM cadmium or cobalt. These calcium conductances, probably, mediate the calcium entry that triggers the calcium-dependent release of dopamine from the dendrites of pars compacta neurons.

In rat dorsal root ganglia (24), a subpopulation of neurons exibits burst firing behavior that is generated by an afterdepolarization triggered by an inward current. This inward current is similar in some respect to a low-threshold calcium current, given its negative voltage activation range, its sensitivity to nickel (100 μM), and its resistence to the DHP nifedipine (10 μM).

PHARMACOLOGY The pyrazine diuretic amiloride has multiple blocking effects on transmembrane mechanisms, such as the Na^+/H^+ and Na^+/Ca^{2+} exchange systems (25–27), the sodium channel present in epithelial apical membranes (28), and glutamate-operated cation channels (29). Amiloride also inhibits the T-type calcium channel in mouse neuroblastoma cells and chick dorsal root ganglion neurons (30) in a concentration-dependent manner. Amiloride (250 μM) inhibits the induction of long-term potentiation in the mossy fiber pathway, but not in the commissural-associational pathway, in the CA3 region of the guinea pig hippocampus (31), supporting a role for T-type channels in the induction of long-term potentiation in the mossy fiber pathway. In human neuroblastoma cells (32), T-type channels are weakly inhibited by amiloride (500 μM amiloride only partially blocks LVA current), but the action of the drug is selective for T-type channels.

High molecular weight alcohols (octanol, nonanol, and decanol) exert a selective blocking action on the LVA calcium current in inferior olivary neurons (33) and prevent physiological tremors in normal or harmaline-treated rats (34). In cultured dorsal root ganglion neurons from chick and rat embryos (35), menthol, a cyclic alcohol, affects both LVA and HVA calcium

channels but in a different manner. Menthol (0.1–1 mM) reduces the amplitude of the low-threshold calcium current in a dose-dependent manner without affecting activation kinetics. In contrast, inactivation of the HVA calcium current occurs much more quickly when menthol is applied from the outside of the neurons.

L-Type Channel

CHARACTERISTICS L-type calcium channels (11–13) generate a current that is activated by large depolarizations, particularly from depolarized holding potentials, and inactivates with a slow time course. The biophysical properties of the L-type channel were first described by Nowycky et al (11) for chick dorsal root ganglion neurons. In sensory neurons (12), L-type current shows a small inactivation during a 200 ms depolarizing pulse, the decay constant being higher than 500 ms. The current begins to inactivate at holding potentials more positive than −60 mV and, when elicited from a holding potential of −60 mV, it reaches its maximal amplitude around +10 mV. Single-channel analysis (13) shows that in the presence of 110 mM barium, the unitary slope conductance is 25 pS and that the channel appears to open according to two different gating patterns: mode 1 is characterized by brief openings (mean open time approximately 1 ms) that tend to occur in bursts, and mode 2, which occurs much less frequently, is characterized by much longer openings.

An important characteristic of the L-type calcium channel is that in order to open when the membrane is depolarized, it must be phosphorylated (36–38). In the case of pituitary GH_3 cells (36, 37), the activity of L-type calcium channels in isolated membrane patches is rapidly lost in ionic solutions, and is maintained for long periods only in the presence of phosphorylating agents. The requirement for intracellular components for preservation of calcium channel activity has been demonstrated with a recently developed patch clamp configuration (39). Inclusion of nystatin, a pore-forming antibiotic, in the patch pipette allows the formation of a perforated vesicle that contains a portion of cytoplasm, which usually is lost during the formation of cell-free patches. The activity of L-type calcium channels in the membrane of these small vesicles lasts for at least 15 min without the addition of phosphorylating agents.

The biochemical structure of the L-type calcium channel has been widely studied in skeletal muscle (40) because of the high concentration of L-type calcium channels in this tissue (41), and DHPs agents have been used as probes to isolate the protein constituents of the channel. The calcium channel appears to be composed of five different polypeptide subunits, each with different molecular masses (42–45): the $\alpha 1$ subunit (175 kd), which forms the ion channel and contains the DHP and the phenylalkylamine binding site

(46–48); the $\alpha 2$ subunit (143 kd), which is associated with $\alpha 1$ and does not contain any high-affinity binding site (48); and the three low molecular weight subunits, β (54 kd), γ (30 kd) and δ (27 kd) (45, 49, 50; Figure 1). The $\alpha 1$ and β subunits contain phosphorylation sites for cyclic AMP-dependent protein kinase.

Molecular cloning techniques have revealed the complete amino acid sequence of the $\alpha 1$ and $\alpha 2$ (48), β (49), and γ subunits (50). The $\alpha 1$ subunit, like the α subunit of the sodium channel (40), is considered to be the principal structural component of the calcium channel. The sequence of the $\alpha 1$ subunit includes 2005 amino acids and it is 29% homologous to that of the sodium channel α subunit (40). Moreover, as with the sodium channel α subunit, the calcium channel $\alpha 1$ subunit possesses four homologous domains that are predicted to span the cell membrane and to form the channel pore. Each of these four domains is composed of six transmembrane segments (40, 46; Figure 2). Such a structure is believed to be the generic structure of voltage-gated ion channels, a belief supported by the homology that exist between the subunits of the various channels characterized so far. Studies of mutated sodium channels have revealed that one of the transmembrane segments, the S4, serves as the voltage sensor of the channel (51). Furthermore, studies of chimeric potassium channels have suggested that the 21-amino acid segment in the S5-S6 linker is a functional region that forms part of the channel pore (52).

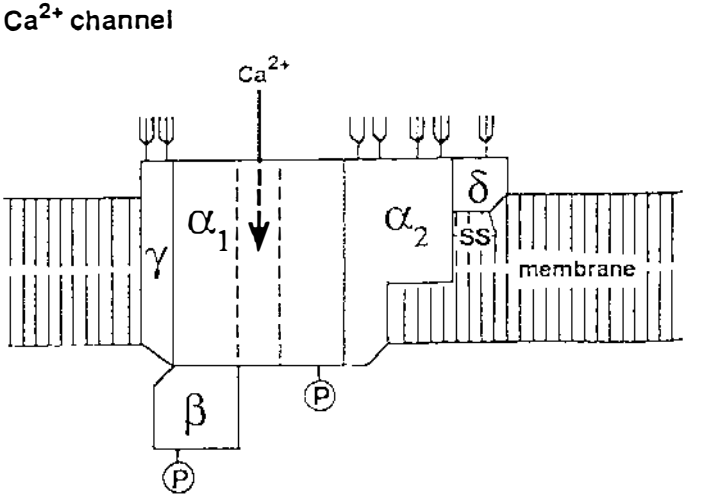

Figure 1 Transmembrane subunit organization of the L-type calcium channel (Reproduced with permission from Ref. 40. Copyright 1988 by the AAAs.)

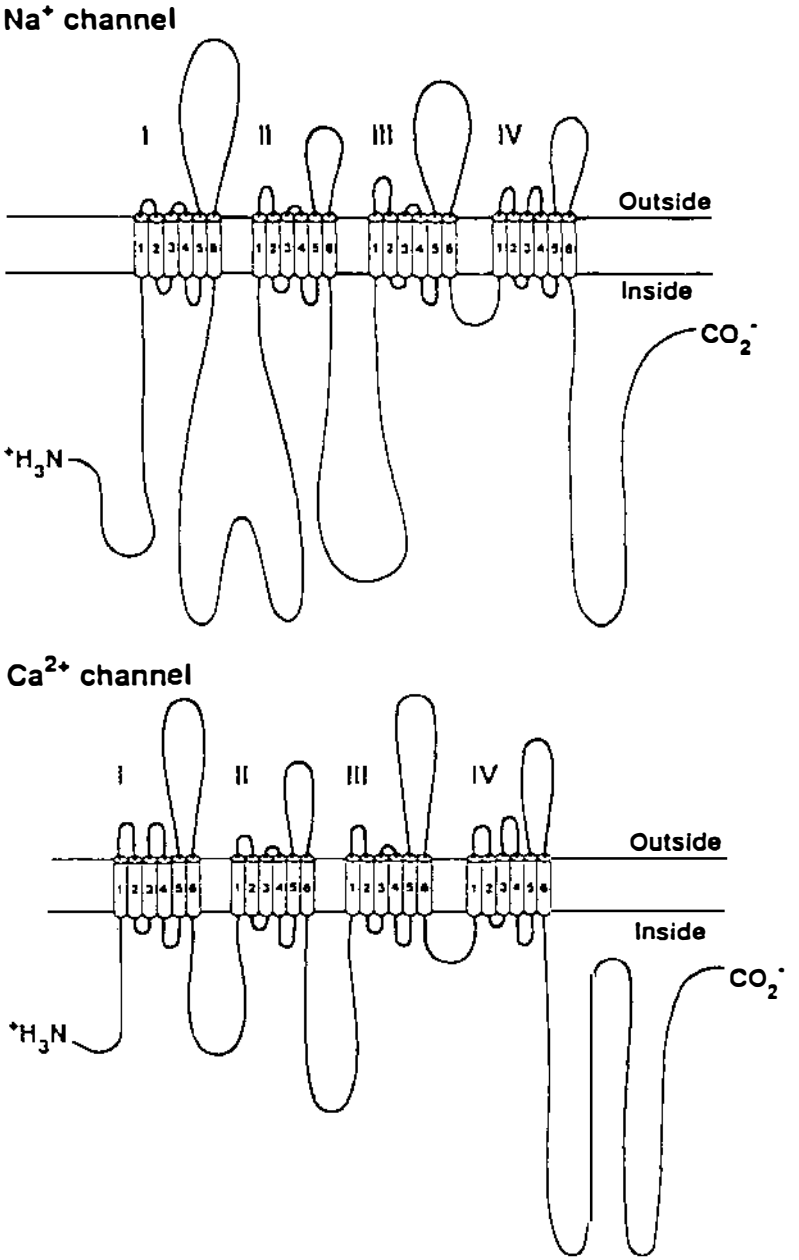

Figure 2 Model of transmembrane arrangement of the L-type calcium channel $\alpha 1$ subunit and sodium channel α subunit (Reproduced with permission from Ref. 40. Copyright 1988 by the AAAs.)

It has recently been shown that expression of various combinations of L-type calcium channel subunits in the LCa.11 cell line (53) give rise to a variety of calcium channels that differ in their calcium permeation properties and in the characteristics of their DHP binding sites. The $\alpha 1$–β combination results in channels with a greater number of DHP binding sites, which probably reflects a contribution of the β subunit to the DHP receptor function. In addition, the β subunit seems to contribute to the activation and inactivation properties of the channel; gating of the channel is accelerated when the β subunit is present.

FUNCTIONS The functions of the L-type calcium channel (54) are related to the generation of action potentials and to signal transduction events at the cell membrane.

The monoclonal antibodies MANC-1 and MANC-2 (55), which recognize subunits of the skeletal muscle L-type calcium channel, have revealed the existence of L-type calcium channels in many regions of the central nervous system (CNS), such as the hippocampus, cerebral cortex, cerebellum, spinal cord, and retina. Microfluorometric imaging studies with brain slices (56–58) have shown that L-type channels are located on the cell bodies and proximal dendrites of neurons, and are clustered at high density at the base of the major dendrites. The wide distribution of the L-type calcium channel, both at the level of the CNS and of a single neuron, reflects the multiple cellular functions of this channel.

PHARMACOLOGY 1,4-DHP derivatives exibit antagonistic or agonistic actions on the L-type calcium channel. These compounds act by binding to a specific recognition site associated with the $\alpha 1$ subunit (46). The DHP agonist Bay K 8644 increases the current generated by L-type channels but not that generated by T- or N-type channels. In chick dorsal root ganglion neurons (13, 59), Bay K 8644 enhances calcium current by changing the gating mode of the channel from mode 1, characterized by brief openings that occur in bursts, to mode 2, having long-lasting openings.

The mechanism of action of DHP calcium antagonists, such as nifedipine and nitrendipine, has been most thoroughly characterized in isolated ventricular myocytes. In this preparation, the action of DHPs is voltage dependent. Moreover, the inhibitory effect of nitrendipine is more potent at depolarized membrane potentials (-10 mV) than at hyperpolarized potentials (-80 mV) (60). Single-channel analysis has shown that DHP calcium antagonists affect channel activity by favoring particular modes of gating rather than by blocking the pore of the channel (61).

It has been shown that DHP binding sites can be up- or down-regulated when they are exposed for a certain period of time to pharmacological agents

(62) and this finding strengthens the idea that DHP binding sites may be regulated by putative endogenous ligands. Furthermore, an endogenous modulator, which interacts with calcium channels by binding to the DHP recognition site, has been isolated from rat brain (63, 64). This modulator inhibits [^{3}H]nitrendipine binding in synaptosomal membranes of rat hippocampus (63), suggesting that it may act as a negative allosteric modulator of the calcium channel. Indeed, biochemical (63) and electrophysiological (65) studies have demonstrated that the nitrendipine-displacing material inhibits calcium influx through voltage-activated channels. Independent of the type of neuron, the endogenous material also affects the neuronal T-type current (65). These experiments strongly suggest that the brain contains an endogenous modulator that can regulate the voltage-activated calcium channels and indicate that some of the voltage-activated calcium channels may be also chemically gated calcium channels.

N-Type Channel

CHARACTERISTICS Although many studies have addressed the issue, there is still controversy concerning the distinction between the high-threshold calcium currents mediated by L- and by N-type channels. In dorsal root ganglion neurons (12, 13), the N-type channel is distinguished by having a conductance of 13 pS, a range of inactivation between −120 and −30 mV, and a decay time constant between 50 and 80 ms. Nevertheless, tail current analysis in chick dorsal root ganglion neurons (66) shows the existence of only one HVA calcium channel. The tail current decay is well fitted by the sum of two exponentials, indicating the presence of two components: a slowly deactivating component, which is due to the closing of the LVA calcium channels, and a rapidly deactivating component (well fitted by a single exponential) that is due to the closing of only one type of HVA calcium channel.

The existence of an N-type current has recently been directly demonstrated. In neurons that had been enzymatically dispersed from the dorsal root ganglia of adult rats (67), N-type calcium current was isolated from other calcium currents and recorded simply by adjusting the holding potentials and the test potentials. Single-channel analysis in rat sympathetic neurons (68) has demonstrated the existence of N-type channels with a conductance of 20 pS (similar to that of the L-type channel) and a subconductance state of 13 pS. The N-type calcium channel appears to carry most of the whole-cell calcium current, is insensitive to DHPs, and is blocked by ω-conotoxin (ω-CgTx). Moreover, the N-type calcium current seems to have a slowly inactivating component and a sustained long-lasting component (68), and the same N-type channel seems to be responsible for both of these two components (69). In

fact, a depolarization can elicit either a short burst of openings or long-lasting events in the same channel.

FUNCTIONS Because N-type calcium channels seem to be heterogeneous and because it is evident that they generate a current that inactivates slowly and can be mistaken for an L-type current, the physiological role of N-type channels needs further clarification. Calcium is essential for neurotransmitter release and voltage-activated calcium channels are the preferred pathways by which calcium enters nerve terminals to mediate this process. The N-type channel plays a role in some forms of neurotransmitter release (70). In rat sympathetic neurons (71), norepinephrine release is controlled by a calcium channel that is sensitive to ω-CgTx and is nitrendipine resistant. Direct measurement of calcium currents from the presynaptic terminal of the chicken ciliary ganglion calyx synapse (72) showed that the predominant calcium channel has a pharmacology similar to the N-type channel: it is resistant to DHPs and is sensitive to ω-CgTx.

However, the N-type calcium channel seems to play only a minor role in transmitter release in mammalian CNS neurons, as indicated by the fact that only a slight tremor is observed in rats after injection of ω-CgTx into the cerebrospinal fluid (73).

Because the biophysical properties of N-type calcium channels are similar to those of L-type calcium channels, the identification of a specific channel as N-type or L-type can become challenging, and only pharmacological tools can help to dissect which channel is operative.

PHARMACOLOGY ω-CgTx is one of a group of toxic peptides isolated from a fish-hunting marine snail, *Conus geographus* (74). ω-CgTx irreversibly blocks the stimulus-evoked release of acetylcholine at the frog neuromuscular junction by inhibiting the presynaptic calcium channels. Moreover, studies performed on isolated frog dorsal root ganglion neurons (75) have shown that synthetic ω-CgTx causes a selective depression of calcium current without an appreciable effect on the sodium current. However, identification of the calcium channels affected by ω-CgTx has not been clear cut.

To define the selectivity of the toxin, initial studies were performed on chick dorsal root ganglion neurons (76). These studies showed that ω-CgTx blocks both L- and N-type calcium channels, but it does not affect T-type channels. In a subsequent series of experiments (68, 77), single-channel analysis of chick sensory neurons, rat sympathetic neurons, and rat pheochromocytoma PC12 cells showed that ω-CgTx blocks only the N-type component of the HVA current, leaving the L-type component unaffected. These results suggest that most of the HVA current in these cells is carried by N-type calcium channels. Furthermore, the presence of a single HVA component of

the tail current in dorsal root ganglia (66) can be explained by assuming that most of the current in these neurons is carried by N-type channels, with L-type current being only a minor component.

P Channel

CHARACTERISTICS Direct evidence for the existence of a third type of high-threshold calcium channel in the mammalian CNS was provided by the observation of a calcium conductance that is inhibited by a toxin derived from funnel-web spider poison (FTX), but is not affected by other blockers (78). FTX was used to produce a high-affinity gel and functional channels were isolated from guinea pig cerebellum and squid optic lobe (78). The channels were incorporated into black lipid membranes and their biophysical properties studied with the patch clamp technique. This channel, termed "P" because it was first described in Purkinje cells, has a conductance of approximately 10 to 15 pS in 80 mM barium and 5 to 8 pS in 100 mM calcium. The open probability of the P-type channel is voltage dependent, and the channel is blocked by FTX, cadmium, and cobalt, but not by DHPs or ω-CgTx. Also, as opposed to the N-type channel, the P-type channel has a unique monovalent ion selectivity in the absence of divalent cations. The sequence from the most permeable to the least is: rubidium > sodium > potassium > lithium > cesium (79).

The isolated channel protein has been used to generate polyclonal antibodies, which have revealed the distribution of the P-type channel in the mammalian CNS (80). Purkinje cells (particularly the dendrites and presynaptic terminals), the inferior olivary nucleus, several nuclei in the brain stem, the olfactory bulb, the enthorinal cortex, the hippocampus and the neocortex exibit immunoreactivity with these antibodies. The P-type channel appears to be the most widely distributed calcium channel in the mammalian CNS. P-type channel activity has also been detected in other regions, including the retina (81), the hypophysis (82), and developing granule cells in the cerebellum (83), although P-type channels are not present in granule cells of adult animals (80). The localization of P channels in Purkinje cells has been corroborated by imaging of intracellular calcium (84). Moreover, injection of rat brain mRNA into *Xenopus* oocytes (85) induces a calcium current that has similar pharmacological characteristics to those ascribed to the P-type channel.

Of the calcium channels present in the CNS, the P-type channel is the only one, so far, that has been cloned and sequenced (86, 87). Two different groups have determined the same primary structure of a calcium channel, presumably the P-type channel, that is expressed predominantly in the cerebellum. By injection of mRNA derived from the cloned cDNA into *Xenopus*

oocytes, Mori et al (86) have shown that the encoded protein, terrned "BI", is a functional channel that is permeable to barium, has a conductance of approximately 16 pS, is blocked by crude venom from *Agelenopsis aperta* (FTX), and is insensitive to nickel (100 μM), nifedipine (10 μM), or ω-CgTx (10 μM). Staar et al (87) have terrned the protein encoded by the isolated cDNA the "rbA-I" protein and have shown that it is a calcium channel α1 subunit distinct from the DHP-sensitive calcium channel. The rbA-I protein contains 2212 amino acids, and structural differences from the DHP-sensitive channel may reflect differences in gating properties. Attempts to express rbA-I in *Xenopus* oocytes were not successful, but Northern blot analysis demonstrated that the rbA-I gene is expressed in many areas of the brain and at a high level in the cerebellum.

FUNCTIONS Immunohistochemical studies have shown the P-type channel to be widely expressed in the mammalian CNS, and the channel appears to serve both as a generator of intrinsic activity and as a modulator of neuronal integration and transmitter release. The P-type channel appears to be the channel responsible for high-threshold calcium current that is insensitive to DHP or ω-CgTx (88, 89).

PHARMACOLOGY Spider venoms contain potent neurotoxins (90) that affect neurotransmission in the vertebrate nervous system. One of these toxins, a fraction derived from the venom of the funnel-web spider *Agelenopsis aperta* and called FTX (79, 91), blocks the calcium conductance in cerebellar Purkinje cells and blocks synaptic transmission at the squid giant synapse without affecting the presynaptic action potential (78); this biophysical profile is attributed to the selective block of at least one specific class of calcium channel. Structural analysis of FTX has shown that the toxin does not contain any ring structures and is most probably a polyamine containing an arginine group (79, 91). Synthetic polyamines in which the carbon chain varies in the 4:3, 3:4, and 3:3 manner have been synthesized, with the 3:3 structure being the most potent P-type calcium channel blocker. High concentrations of 3:4 did not produce any calcium channel block, while the potent 3:3 and the 4:3 were able to produce a specific block of calcium channels of the P-type (79).

NEUROTRANSMITTER AND NEUROPEPTIDE MODULATION OF VOLTAGE-ACTIVATED CALCIUM CHANNELS

The negative modulation of HVA calcium channels by neurotransmitters and neuropeptides has been demonstrated in several neuronal types (92). In most instances, the coupling mechanism between the transmitter or peptide recep-

tor and the channel involves the activation of guanine nucleotide-binding proteins (G-proteins). G-proteins may modulate channel activity either by direct interaction with the channel subunits or indirectly by regulating the activity of enzymes that determine the intracellular concentration of second messengers (93–95).

Although the transduction mechanisms by which calcium channel activity is modulated have been well studied, the precise details of how channel function is altered are not known. In bullfrog dorsal root ganglion neurons (96), noradrenaline appears to reduce calcium current by shifting the voltage dependence of channel opening without affecting the number of channels that are operative. These results suggest that the channels can exist in two modes: a "willing" mode, which predominates in the absence of neurotransmitter and in which channels open readily in response to small depolarizations, and a "reluctant" mode, which predominates in the presence of neurotransmitter and in which the channels need larger depolarizations in order to open. The channels can move from one mode to the other and the neurotransmitter enhances the number of channels operating in the reluctant mode. In frog sympathetic neurons, luteinizing hormone-releasing hormone (LHRH) has also been shown to affect the calcium current in such manner (97), and strong depolarization to +70 mV can reverse the inhibitory action of the hormone on the calcium current. It remains to be seen whether such a receptor-mediated modulation of the voltage dependence of channel opening is a mechanism present also in neuronal types different from sensory and sympathetic neurons.

CALCIUM CHANNELS AND INTRACELLULAR CALCIUM STORES

In response to a depolarizing stimulus, the concentration of cytosolic free calcium undergoes a dramatic increase in neurons, which lasts for a very brief period of time (of the order of milliseconds) and is terminated by the simultaneous action of various mechanisms that involve calcium channels, calcium extrusion systems, and intracellular calcium stores (98, 99).

Little is known about the structure and functions of the intracellular sites where calcium is stored in neurons. Calcium ions seem to be distributed in organelles that are able to sequester calcium and that include mitochondria, the endoplasmic reticulum, and the calciosome (100–103). The role of intracellular calcium stores is not completely clear: although they appear to be mainly involved in the process of calcium uptake after membrane depolarization, there is increasing evidence to suggest their participation in the generation of calcium transients.

Direct evidence for the release of calcium ions from internal stores has been obtained with the use of calcium-sensitive dyes (104, 105). In rat and frog

sympathetic neurons, release of calcium from internal stores can be induced by application of caffeine, a compound that induces calcium release from the sarcoplasmic reticulum of muscle (106). In neurons, caffeine (10 mM) elicits a transient increase in the cytosolic calcium concentration similar to its effects in muscle (104). Anatomical similarities between neuronal endoplasmic reticulum and muscle sarcoplasmic reticulum have been revealed with freeze-fracture techniques and electron microscopy (107). The channel responsible for caffeine-induced calcium release from muscle sarcoplasmic reticulum has been isolated and reconstituted in lipid bilayers (108), and it is likely that a similar channel exists in neuronal endoplasmic reticulum.

Pozzan et al (109) have shown that an internal calcium release mechanism exists and can be activated by the binding of agonists to certain cell surface receptors. The intracellular messenger responsible for mediating this effect has been shown to be inositol 1,4,5-triphosphate ($InsP_3$), which is generated as a result of receptor-dependent activation of phospholipase C (110–112). A putative intracellular organelle termed the "calciosome" was considered to be the calcium store that was responsive to $InsP_3$, but subsequent studies (113) revealed that the neuronal target for $InsP_3$ is the endoplasmic reticulum. In rat cerebellar Purkinje cells, immunocytochemical studies have localized the $InsP_3$ receptor on the membrane of the rough and smooth endoplasmic reticulum, and this receptor has been shown to be associated with $InsP_3$-induced calcium release. Nevertheless, the nature of the organelle responsible for $InsP_3$-mediated calcium release is still controversial (114).

It remains to be investigated whether the caffeine and the $InsP_3$ receptors are coupled to the same calcium channel present on the same organelle or whether there are multiple channels contained in different organelles.

Calcium release from internal stores, particularly from the caffeine-sensitive store, has been considered to be a mechanism that contributes to the generation of spontaneous oscillations of free cytosolic calcium (3, 105). However, it appears that the increase in the cytosolic calcium concentration that occurs after an action potential is due to calcium entry through voltage-gated channels, and the contribution from internal calcium release is minimal (104).

RECEPTOR-OPERATED CALCIUM CHANNELS AND THEIR CONTRIBUTION TO NEURONAL DEATH

The NMDA Receptor-Channel Complex

CHARACTERISTICS Excitatory amino acids such as glutamate and aspartate are among the most important and abundant neurotransmitters at brain synapses (115, 116). They act by binding to two basic types of receptor: (*a*) ionotropic receptors, which are permeable to cations and of which there are several subtypes (117, 118), and (*b*) metabolotropic receptors, which are

functionally linked to stimulation of inositol phospholipid metabolism, possibly by a G-protein coupling mechanism (119–121).

The ionotropic receptor selective for N-methyl-D-aspartate (NMDA) (117, 122) contains multiple binding sites for various ligands at which transmitters, cotransmitters, and pharmacological agents can act either to regulate channel gating or to modulate the channel-gating effects of other ligands. The binding sites associated with the NMDA receptor include: (*a*) the agonist-binding site that is located in the extraneuronal receptor domain and which also binds antagonists that act with apparently competitive kinetics; (*b*) two binding sites located in the extraneuronal receptor domain that bind the endogenous allosteric modulator glycine (123) and the divalent cation zinc (124, 125), respectively; (*c*) two binding sites located in the transmembrane receptor domain that bind magnesium ions (126, 127) and phencyclidine (128), respectively.

The NMDA receptor ion channel has a conductance of approximately 50 pS and a mean open time of 5 ms (117, 122). Studies that have monitored the intracellular free calcium concentration with calcium-sensitive dyes (129) and determination of the reversal potential of the ionic current carried by the NMDA receptor channel (130) have revealed that the channel has a high permeability to calcium ions. The channel is 10 times more permeable to calcium than to sodium, and is approximately 70 times more permeable to calcium than are the other two ionotropic receptors sensitive to excitatory amino acids, which are selective for kainate and quisqualate (131).

NEUROTOXICITY NMDA receptors appear to play a central role in the process of excitotoxicity and neuronal degeneration. A sustained increase in the intracellular free calcium concentration triggers a series of events (including persistent activation of calcium-dependent enzymes, production of toxic metabolities, and disruption of the cytoskeletal network) that cause cytotoxicity and cell death (132). It has been shown that several pathological states, such as anoxia, ischemia, or chronic degenerative diseases (epilepsy, amyotrophic lateral sclerosis) are associated with modified glutamate levels. Extraneuronal concentrations of glutamate in injured brains are dramatically increased because of an abnormal release of the transmitter from damaged nerve terminals and because of reduced glutamate reuptake (133). These increased extraneuronal glutamate concentrations cause paroxysmal stimulation of NMDA receptors, which, in turn, destabilizes neuronal calcium homeostasis (134, 135).

The neurodegenerative processes that result from the application of glutamate to rat cerebellar granule neurons in primary culture can be divided into two phases (136–138): an immediate phase, which occurs within a few minutes of glutamate application, is characterized by cell swelling, is dependent on sodium and chloride influx, and is often reversible; and a delayed

phase, which is dependent on an increase in intracellular free calcium concentration that persists even after cessation of receptor stimulation, and which is almost always followed by neuronal death (with death usually occurring within 24 hr of the initial insult). Measurements of intracellular free calcium with the dye fura-2 (139) have revealed that a neurotoxic dose of glutamate (50 μM) increases the cytosolic calcium concentration of about 10 times. Whereas after a nontoxic dose of glutamate (5 μM), the calcium concentration immediately returns to the basal value when glutamate is withdrawn, after a toxic dose, the calcium concentration remains elevated for longer than 1 hr after glutamate withdrawal (137). The neurotoxic process is prevented by removal of calcium from the extracellular medium during glutamate application or during the first 15 to 30 min immediately after application of glutamate (137). The sustained increase in the cytosolic calcium concentration associated with glutamate neurotoxicity may result from calcium-dependent calcium release from intracellular sites or from decreased calcium extrusion because of damage to transmembrane pumps, such as the calcium-dependent ATPase pump (99). After withdrawal of glutamate from neuronal cultures the sustained calcium increase elicited by glutamate is not blocked by NMDA receptor antagonists or by inhibitors of voltage-activated calcium channels (137).

Because of the high permeability of the NMDA receptor channel to calcium ions, the receptor channel has been considered to be the main pathway of calcium entry in the presence of glutamate. However, although NMDA receptor antagonists applied during the period of persistent glutamate receptor stimulation prevent neuronal degeneration, they do not protect if applied after glutamate withdrawal (134, 137).

The calcium entry that results from the opening of the NMDA channel induces the membrane translocation and activation of protein kinase C (PKC) (140), which, in turn, mediates the phosphorylation of specific membrane proteins. The protracted activation of PKC that results from glutamate receptor abuse is thought to be responsible for the increased cytosolic calcium concentration that persists after glutamate removal. A prolonged activation of PKC has been detected in vivo (137) after brain ischemia and in vitro (141) after glutamate treatment. Prevention of PKC translocation by gangliosides (142) or down-regulation of PKC by phorbol esters (143) protects neurons from glutamate-induced death. Thus, it appears that glutamate neurotoxicity may mainly be mediated by a sustained PKC translocation, which destabilizes cytosolic calcium homeostasis by an unknown mechanism.

A second mechanism for glutamate-induced neurotoxicity, also mediated via an increase in cytosolic calcium, has been described for Purkinje cells (144). In these neurons, glutamate can induce the release of intracellular calcium via the activation of an $InsP_3$-dependent pathway (110–112). With the use of the calcium-sensitive dye fura-2 (84, 145), it is possible to monitor

the intracellular calcium transients that occur during an action potential. Measures of intracellular calcium (following loss of electroresponsiveness after glutamate application) in the absence of extracellular calcium and with 1 mM of EGTA have revealed that the intracellular calcium concentration increases, at first in the soma and then in the dendrites, even in the absence of external calcium. Therefore, Purkinje cell death can be induced by a massive release of calcium from internal stores through the action of $InsP_3$ acting as second messengers of glutamate receptors.

CONCLUDING REMARKS

Voltage-activated and receptor-operated calcium channels are located on the external membrane and on the surface of cytosolic organelles of neurons in most brain areas. Many of these channels appear to have a complex structure comprising the ionophore and multiple ligand binding sites that regulate the activity of the channel. Because calcium ions have a central role in neuronal function, the development of selective drugs that can affect specific calcium channels, while leaving others unaffected and free to perform their physiological role, should be of benefit clinically. Progress in molecular biology has revealed that channels are composed of many different combinations of subunits and that structural subtypes of the same channel may differ in their regulatory properties. Therefore, the various classifications of calcium channels are a simplification and do not reflect the structural heterogeneity of these heterooligomeric integral membrane proteins.

This review has described some of the mechanisms that regulate intracellular calcium transients and how a pathological intracellular calcium overload can trigger metabolic reactions that cause irreversible damage to neurons. Further structural investigations of calcium channels are needed to produce new pharmacological agents that can control and regulate the pathways of calcium homeostasis.

ACKNOWLEDGMENTS

We are grateful to Dr. W. Brocklehurst for helpful comments on the manuscript.

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