Calcium Channels: Critical Targets of Toxicants and Diseases

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The structure and regulation of voltage-dependent calcium channels and their role in inherited and toxicant-induced neurologic disorders was the topic of a conference sponsored and hosted by the National Institute of Environmental Health Sciences, the U.S. Environmental Protection Agency, Sibia Neurosciences, and the Muscular Dystrophy Association on 6–8 December 1999. The conference, "Calcium Channels: Critical Targets of Toxicants and Diseases," brought together experts in a variety of disciplines to discuss our current knowledge of calcium channel structure and function, channel regulation by intracellular signaling molecules, and the effects of mutations, aging, and toxicants on channel function.

Calcium channels play important and diverse roles in neurologic function. It has been known for many years that calcium channels are likely targets of a variety of organic and inorganic toxicants. More recently, calcium channel mutations have been implicated in several inherited neurologic disorders, including migraine headaches, various movement disorders, and some types of malignant hyperthermia. The last decade has seen great strides in determining the subunit structure of calcium channels, how the genetic diversity of subunits generates a variety of functionally distinct channels, and how intracellular signaling pathways regulate channel function. The meeting was convened to guide future research by applying this knowledge of calcium channel biology to further our understanding of how toxicants and mutations alter channel functions.

The presentations discussed four major aspects of calcium channels that are relevant for understanding their roles in genetic and environmental neurologic disorders: the genetics and molecular biology of channel structure; interactions between calcium channels and intracellular signaling pathways; the effects of toxicants on calcium channel function, both through direct interactions with channel subunits and by altering intracellular signaling; and the genetics, molecular biology, and physiology of inherited disorders caused by mutations in the genes encoding calcium channel subunits or from autoimmune disorders caused by the generation of antibodies against subunits of those proteins.

Structure and Function of Calcium Channels

Voltage-dependent calcium channels are composed of four principal subunits: the transmembrane, pore-forming $\alpha 1$ subunits and three accessory subunits that modulate channel function—the glycosylated $\alpha 2\delta$ subunits, the integral membrane γ subunits, and the cytoplasmic β subunits. There are several isoforms of each of these channel subunits, and the composition of the channel complex determines its expression level, localization, kinetics, and pharmacology.

Ten genes on nine different chromosomes encode the $\alpha 1$ subunits. Diversity of $\alpha 1$ subunits gives rise to the familiar classification of calcium channels (i.e., L, N, T, P/Q, R) on the basis of channel kinetics, cation permeability, and pharmacology. Several human disorders have been traced to mutations in the $\alpha 1$ subunit genes, including familial hemiplegic migraine, several types of ataxia, hypokalemic periodic paralysis, and malignant hyperthermia. In most cases, it remains to be established how the mutations produce pathology.

The accessory subunits ($\alpha 2\delta$, β, and γ) regulate the expression, localization, kinetics, and modulation of calcium channels, but relatively little is known of the molecular mechanisms involved. In mice, mutations in the $\beta 4$ (lethargic) and $\gamma 2$ (stargazer) subunits, prominently expressed in cerebellar Purkinje neurons, produce ataxia. Recently, Escayg et al. (*1*) reported that mutations in the $\beta 4$ subunit are associated with some forms of epilepsy and episodic ataxia. It thus appears likely that a variety of human neurologic disorders may be attributable to mutations in the accessory subunits.

Studies of mice with mutant channel subunits are providing clues both to normal channel assembly and to the effects of altered channel function. Of particular importance for the theme of this meeting is that alterations in the expression of one channel subunit are often partially compensated for by changes in expression of other subunits. For example, the absence of the $\beta 4$ subunit in the lethargic mutant mouse causes increased expression of the $\beta 1B$ subunit despite a decrease in the total pool of β subunit protein. The absence of $\alpha 1A$ subunits results in decreased expression of $\beta 4$ subunits and increases in $\beta 1B$ subunits. Nevertheless, animals with either mutation show clear pathologic phenotypes, suggesting that compensatory changes in expression of calcium channel subunits are limited in their ability to restore normal neuronal function. Further, at least some of these compensatory changes may themselves cause cellular dysfunction. This principle, that a primary defect in calcium channel function, either genetic, autoimmune, or toxicant-induced, may produce both "primary" symptoms and a constellation of "secondary" symptoms caused by other, possibly compensatory, changes in neuronal physiology, was repeated several times in various contexts during the presentations.

Calcium channel expression also changes with normal aging. For example, in hippocampal neurons, the expression of L-type channels increases with age. This increase in L-type channels is correlated with increased failure of excitatory postsynaptic potentials as well as increased susceptibility to cell death. At the whole-animal level, these changes are correlated with decreased performance on spatial learning tasks. Up-regulation of the expression of the $\alpha 1D$ subunit appears to underlie the increased expression of L-type channels in hippocampal neurons with age; much smaller increases in expression of $\alpha 1C$, and no change in the expression of $\beta 1B$ subunits are observed. This age-related change in channel expression may make the nervous system more susceptible to both pathologic and toxicologic insult.

Interactions between Calcium Channels and Intracellular Signaling

The activity of calcium channels is regulated by a wide variety of intracellular signaling pathways. Probably the three most well understood are binding and activation of

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Received 20 July 2000; accepted 9 August 2000.

calmodulin, phosphorylation by several protein kinases, and binding of G protein βγ subunits. These pathways not only modulate calcium channel activity, all are themselves modulated in various ways by calcium influx through calcium channels, and many interact with one another. Thus, complex feedback mechanisms among calcium channels and intracellular signaling exist and are likely targets for both environmental and genetic disorders.

Calcium channels play key, but far from exclusive, roles in regulating intracellular calcium homeostasis. Both pathologic conditions (stroke, ischemia, AIDS, etc.) and toxicologic insult (e.g., by methylmercury, lead) produce alterations in calcium homeostasis that can lead to neuronal death. Calcium influx through both voltage- and ligand-gated channels (e.g., the *N*-methyl-D-aspartate receptor) contributes to alterations in calcium homeostasis, but other intracellular organelles and calcium ATPases also are part of the machinery responsible for regulation of calcium homeostasis. These intracellular calcium stores and ATPases interact with channel-mediated calcium entry to give rise to locally confined calcium increases, or responses such as calcium spikes or waves. The amplitude, time course, and intracellular distribution of calcium signals are important determinants of the cellular response. All aspects of cellular calcium homeostasis are likely targets for diseases and toxicant-induced disorders.

Calmodulin, a ubiquitous calcium-binding protein, has long been known to mediate many of the intracellular effects of calcium influx through calcium channels, especially L-type channels. For example, calcium entry via the L-type channel appears to be critical for activation of a number of different transcriptional pathways, including those mediated by CREB [cyclic AMP response element binding (protein)], NF-AT (nuclear factor of activated T-cell), and p38 MAP (mitogen-activated protein) kinase. More recently, calmodulin has also been found to act in the feedback regulation of calcium entry through L-type channels. Calmodulin binds to the intracellular carboxy terminal of the α1C subunit. Calcium entering through the channel binds to this calmodulin and reduces further calcium influx. Thus calmodulin can act as a key molecule both in stimulation of gene transcription and other cellular events and in feedback inhibition of calcium channels.

Calcium channels may be phosphorylated by many protein kinases, including calcium/calmodulin-dependent protein kinases and protein kinases A and C. The effects of such phosphorylation depend on the channel subtype under study, the protein kinase, and other signaling pathways that may be active. In recent years considerable progress has been made in understanding the molecular mechanisms of G protein modulation of calcium channels (particularly the N-type, or α1B, channels). The intracellular amino terminal of the α1B subunit has been shown to be essential for G protein effects, but β channel subunits are also required for regulation by G protein βγ subunits. Interactions among all of these intracellular pathways also abound. For example, G protein binding to calcium channels may be modulated by channel phosphorylation.

The Effects of Toxicants on Channel Function

There are two fundamentally different mechanisms whereby neurotoxicants might alter calcium channel function: by direct interaction with channel subunits (e.g., occluding the pore) or by interaction with intracellular signaling pathways that modulate channel activity. Interactions with the channel subunits themselves are well known—for example, heavy metals blocking the pore. Although there is a substantial literature describing effects of neurotoxicants on intracellular signaling, relatively little is known about how such effects might modulate calcium channel activity. The time appears to be ripe for a significant research effort to study the interactions among toxicants, intracellular signaling pathways, and calcium channel activity.

Several papers presented at the meeting discussed the effects of specific toxicants [methylmercury, inorganic lead, polychlorinated biphenyls (PCBs), and ethanol] on calcium channels. Interestingly, all of these toxicants, with the possible exception of PCBs, appear to have effects both directly on channels and indirectly via intracellular signaling. Unfortunately, studies of toxicant effects on calcium channels have used a variety of cell types and experimental approaches, so that unambiguous comparisons among toxicants are often difficult or impossible. Nevertheless, several generalizations can be drawn from the presentations at the meeting.

First, it has long been known that many heavy metals impair current flow through calcium channels, mostly by blocking the pore. However, many metals, in both inorganic and perhaps especially in organic form, have other effects, presumably often mediated through intracellular signaling. Lead is perhaps the best studied of all toxicants with actions on calcium channels. Inhibition of channel current has been demonstrated for acute exposure to low micromolar concentrations of lead in virtually every preparation in which it has been examined, presumably due to blocking the pore. However, lead may also enter the cell through some types of calcium channels (particularly the L-type channel). Intracellular lead may promote channel activity by attenuating the normal channel inactivation by intracellular calcium. This effect occurs at much lower lead concentrations (picomolar to nanomolar) than are required for blocking the pore. Intracellular lead may also activate protein kinase C and various calmodulin-dependent enzymes, such as calmodulin-dependent protein kinase, phosphodiesterase, and calcineurin, at picomolar concentrations. The impacts of such actions on calcium channel activity, or on the pathways that are normally activated by calcium influx through calcium channels, remain largely unknown.

Methylmercury provides another example of complex interactions of toxicants with calcium channels. Unlike most inorganic heavy metals, methylmercury is lipophilic and may permeate cells directly through the phospholipid bilayer or as a cysteine complex and exert intracellular effects in addition to blocking the channel pore. For example, although acute, externally applied methylmercury blocks calcium channels, the block is slowly and incompletely reversible. The lack of reversibility may be the result of prolonged residence in the phospholipid bilayer or the cytoplasm due to the lipophilicity of this organometal, or due to the relatively greater reactivity of methylmercury with sulfhydryl groups on calcium channels. Prolonged exposure (6 days) of PC12 cells to nanomolar concentrations of methylmercury reduces whole cell calcium currents; it is not known whether this decrease in current is caused by direct effects of methylmercury on calcium channels, or to indirect effects, such as altering channel density.

Voltage-gated calcium channels are also prominent targets of ethanol. Acute exposure to ethanol inhibits the function of both L- and N-type calcium channels in several neuronal systems. The mechanisms associated with impaired function of L-type channels involve decreased open channel probability and enhanced inactivation of the channel. With chronic exposure to ethanol, the density of L- and N-type calcium channels increases in PC12 cells and in rodent brain. Up-regulation of L-type channels is thought to contribute to the enhanced neuronal excitability seen during ethanol withdrawal; up-regulation of N-type channels would be expected to enhance transmitter release. Both effects could contribute to the behavioral hyperexcitability seen during ethanol withdrawal. The up-regulation of L- and N-type calcium channels in PC12 cells by ethanol is due in part to effects on protein kinase C (PKC); chronic exposure to ethanol increases PKC-mediated phosphorylation and the abundance of PKC δ and ε isozymes. Interestingly, inhibiting PKC δ prevents up-regulation of L-type channels by ethanol,

while inhibiting PKC ε prevents up-regulation of N-type channels. Thus acute exposure to ethanol inhibits the function of two classes of voltage-gated calcium channels, whereas chronic exposure induces up-regulation of these channels by PKC-dependent mechanisms that involve different PKC isozymes.

The effects of PCBs on the ryanodine receptor provide an illustration of complex effects of toxicants on calcium channels. Ryanodine receptors mediate calcium-induced calcium release from the endoplasmic reticulum. Calcium influx through plasma membrane calcium channels is an important trigger for calcium-induced calcium release; the calcium released from intracellular stores may then cause calcium-induced inactivation of the channels (perhaps via calmodulin; see above). Some non-coplanar PCBs impair the function of the ryanodine receptor and alter intracellular calcium regulation. This would be expected to alter calcium channel function as well, but such effects, if they occur, remain unknown.

Neurologic Disorders Mediated by Calcium Channels

A diverse set of clinical syndromes is associated with mutations in the genes encoding the various calcium channel subunits. Some of the disorders affect skeletal muscle or the neuromuscular junction, such as hypokalemic periodic paralysis (HypoKPP) and Lambert-Eaton myasthenic syndrome (LEMS). Other diseases affect the central nervous system, such as familial hemiplegic migraine (FHM), episodic ataxia type 2 (EA-2), and spinocerebellar ataxia type 6 (SCA6). Interestingly, some features seem to be shared by most of the clinical syndromes:

- Most syndromes have episodic features: muscle weakness, ataxia, or migraine attacks
- All syndromes have some features that are chronic and/or progressive, such as muscle damage or cerebellar atrophy
- There seems to be a gradual superimposition of permanent deficits on initially episodic deficits. One exception seems to be SCA6, which lacks episodic features
- All genetic syndromes show reduced penetrance, in that not all carriers of a given mutation are affected clinically, and there is considerable variation in the symptoms. Without doubt, other genetic and/or environmental factors are involved in all of the channelopathies
- Several syndromes are responsive to azetozolamide. Somehow, changing the pH in the extracellular environment and/or blocking the channel pores by the binding of protons seems to affect these channelopathies
- Some general triggers such as stress, fatigue, or certain foods seem to precipitate attacks in different syndromes.

Sometimes the same syndrome is caused by mutations in different genes [e.g., mutations in the ryanodine receptor (RYR1) or the α1S calcium channel cause malignant hyperthermia], and sometimes mutations in the same gene are associated with different clinical syndromes (FHM and EA-2 are both caused by mutations in the α1A calcium channel subunit). This leads to the tantalizing suggestion that trigger or threshold factors might be shared by all or most channelopathies. It will be a major challenge to reveal those common factors. From various presentations during the meeting, it appears that steroid hormones, alcohol, and heavy metals may be candidates worth pursuing.

Several speakers highlighted the difficulties of determining physiologic mechanisms for calcium channelopathies. For example, some types of FHM appear to be caused by mutations in the gene encoding the α1A (P/Q) subunit. Recent electrophysiologic experiments in embryonic kidney cells transfected with FHM genes show that the mutated genes alter channel kinetics. However, some of the mutations decreased channel density and conductance, leading to reduced calcium influx, whereas other mutations caused an increased channel density and increased open probability, leading to increased calcium influx.

A second example of the difficulty of merging clinical and molecular/physiologic studies is illustrated by EA-2. Until recently, EA-2 was thought to be associated with truncating mutations in the α1A gene. However, missense mutations have now been found as well. Further, the severity of the clinical phenotype can dramatically differ between various mutations. For instance, the Gly293Arg mutation is associated with a severe progressive ataxia, whereas other mutations associated with EA-2 are less progressive. Finally, two apparently different syndromes may be caused by the same mutation (i.e., the same mutation of α1A may cause EA-2 and SCA6).

HypoKPP provided a third example of the promise and difficulties of genetic and physiologic analysis of channelopathies. HypoKPP is caused by three different mutations in the calcium channel subunit α1S (skeletal muscle L-type channel). All three known mutations cause substitutions for positively charged arginine residues in the S4 transmembrane domains, which are regions implicated in voltage sensing. These mutations appear to cause reduced current density. It is not understood how reduced calcium currents are related to some of the symptoms of HypoKPP, such as muscle depolarization and reduced serum potassium.

LEMS combines features of both toxicant-induced and inherited channelopathies. It is believed that LEMS begins with an autoimmune response, often evoked by small cell lung cancer. One of the main immunogens is a P/Q-type channel component, probably because P/Q channels are highly expressed on these tumor cells. Circulating autoantibodies recognize P/Q-type channels present at the neuromuscular junction, where they destroy the active zones of nerve terminals, leading to reduced acetylcholine release. A LEMS-like disorder can be elicited in mice by repeated administration of serum, plasma, or immunoglobulins from affected patients. In LEMS immunoglobulin-treated mice, there is a reduction in the amplitude of calcium currents and frequency-dependent facilitation. Mostly P/Q and N-type channels normally carry these calcium currents, but in LEMS immunoglobulin-treated mice an additional L-type current has been identified. Thus, there appears to be a down-regulation of normally expressed channels and a compensatory up-regulation of L-type channels. It is not known whether such changes in channel expression occur in human LEMS patients.

Conclusions and Future Directions

Two dominant themes emerged from the meeting. First, we are in an excellent position to apply our knowledge of the molecular biology and physiology of calcium channels to detailed analyses of genetic defects and toxicant effects. Second, the complex interplay among calcium channels, calcium influx, intracellular calcium homeostasis, and intracellular signaling is likely to be important both in clinical syndromes attributable to calcium channel mutations and in the neurologic effects of a variety of toxicants. Although these two themes are not completely independent, it is useful to discuss them separately. Within each theme, implications for future research in understanding clinical syndromes and neurotoxicant effects will be considered.

Molecular biology and physiology of calcium channels. The variety of physiologically defined calcium channels is now becoming understood at the molecular level. Multiple isoforms of each of the subunits have now been identified, cloned, and sequenced (or will be soon). Studies of calcium channel subunit mutations, both in humans and in animal models, will be important in two different ways. First, and most obviously, understanding the electrophysiologic and cellular consequences of subunit mutations provides at least the potential to design novel, rational treatments that may alleviate the symptoms brought on by the inherited channel defects. Such treatments may be

either at the level of the channel itself or at "downstream" intracellular effects (e.g., on calcium homeostasis or intracellular signaling pathways). The possible necessity of downstream interventions is highlighted by the fact that similar clinical syndromes may be caused by mutations with different effects on channel physiology, and different syndromes may be elicited by the same mutation. Second, electrophysiologic analyses of cells transfected with known subunit combinations, mutated subunits (either naturally occurring or generated by site-directed mutagenesis), and subunits derived from mutant mouse lines will identify key amino acid residues that regulate voltage dependence, channel kinetics, pharmacologic sensitivity, and modulation by intracellular signaling pathways in normal channels. Such analyses, often initially driven by the need to understand the basis of clinical syndromes, have already provided important information about basic channel physiology and offer great promise for the future.

In neurotoxicologic studies, calcium channel diversity should be viewed as an opportunity, not an obstacle. Studies of channel subunit mutations have clearly shown that different effects, both on the cellular and whole-animal levels, occur when different subunits, or different isoforms of the same subunit (especially the α1 subunits) malfunction. It is quite likely that some toxicants preferentially target certain subunits and/or isoforms. Such toxicant selectivity can now be directly analyzed in cells transfected with cloned subunits, rather than by trying to use pharmacologic tools to tease apart effects in cells expressing many channel types.

Several presenters noted that the progression and severity of clinical syndromes vary considerably even within a population bearing the same mutated subunit gene, suggesting that environmental factors may modulate the threshold at which a mutated subunit produces clinical effects. It would be extremely useful, both for clinicians and for neurotoxicologists, to determine if interactions between toxicants and already-defective channels might hasten the onset or increase the severity of symptoms.

Calcium channel regulation, calcium homeostasis, and intracellular signaling. Several speakers pointed out that calcium channels both regulate and are regulated by intracellular calcium homeostasis and intracellular signaling pathways. Further, channel regulation occurs both acutely (e.g., through changes in channel activity by phosphorylation or G protein binding) and chronically (e.g., up- or down-regulation of channel density). Bidirectional regulation may have profound implications for treating genetic calcium channelopathies. First, in many cases the clinical symptoms are caused, not directly by altered channel function per se, but by altered cellular metabolism caused by channel dysfunction. Therefore, understanding the actual cause of the symptomatology and/or designing treatments will require an understanding of the signaling pathways downstream of calcium channel dysfunction. Indeed, it may well be found that some of the symptoms of clinical syndromes not currently thought to be mediated by calcium channels may arise because defective intracellular signaling alters the function of genetically normal channel subunits. Second, cells often up- or down-regulate various channel subunits in response to dysfunction of a given subunit, and the resulting changes in channel types and densities may ameliorate and/or contribute significantly to the clinical symptoms. The mechanisms underlying changes in channel expression, largely unknown at the present time, are likely to be found in the interplay among channel function, intracellular calcium homeostasis, and intracellular signaling pathways.

Neurotoxicants may affect calcium channel function either by directly interacting with the channel subunits or by altering intracellular pathways that regulate channel subunits. A large number of toxicants are known to alter intracellular signaling pathways and/or alter calcium homeostasis, but how these effects impact calcium channel function remains largely unexplored. Such studies are feasible and important. As noted above, in individuals bearing mutated channel subunit genes, toxicants may reduce the threshold at which cellular and organismal dysfunction occurs. These effects may well originate through actions on altered intracellular signaling rather than directly on the channel subunits themselves. It should be noted that aging also changes calcium channel subunit density and susceptibility to insult (e.g., ischemia or excitotoxicity). Although considerable neurotoxicologic research has focused on the developing nervous system, much less attention has been paid to the aging nervous system, which probably also has a unique pattern of susceptibility to toxicants. Interactions between altered intracellular signaling during aging and toxicant exposure, particularly long-term, cumulative exposure, may promote age-related neurologic dysfunction.

References and Notes

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