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Topical Review

Molecular and Biophysical View of the Ca Channel: A Hypothesis Regarding Oligomeric Structure, Channel Clustering, and Macroscopic Current

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Introduction

In recent years it has become apparent that not only membrane voltage and external ligands, but also molecular structure, internal messengers, and local ionic conditions can modulate ion channels. Here I focus on the conformation, regulation, and local condition of the L-type Ca channel. I contrast L-type channels with T-type channels and concentrate on data from cardiac cells. For an overview of other types of Ca channels and their expression in different tissues, *see* Nargeot, Dascal and Lester (1992). Finally, I speculate on a connection between the oligomeric structure of L-type channels and the local concentration of Ca ions to explain the diverse kinetics of Ca channels in a variety of cells.

Molecular Structure and Local Conditions Regulate Channel Function

We know that certain intrinsic molecular features of membrane channel proteins can modulate channel kinetics. As examples, consider the cytoplasmic domains that control voltage-gated inactivation, and the multimeric conducting units that influence the activation kinetics of certain K channels (Hoshi, Zagotta & Aldrich, 1990; Zagotta, Hashi & Aldrich, 1990; Choi, Aldrich & Yellen, 1991; Isacoff, Jan & Yan, 1990, 1991; Tytgat & Hess, 1992). The subunits that modify fast Na channels also fall into this category (Kraner, Tanaka & Barchi, 1985; Messner & Catterall, 1985; Krafte et al., 1988). Moreover, we know that local ionic conditions near a channel can alter its kinetics. For example, pH modulates I_{Ca} conductance (Prod'hom, Pietrobon & Hess, 1987, 1989), and Ca modulates I_M activity (I_M is a K current suppressed by muscarinic activation: Marrion et al., 1991). In another case, endothelin-1 stimulated Ca influx exhibits negative feedback through elevated intracellular Ca (Muldoon et al., 1991). Mere proximity may promote interactions between channels, phosphorylation events, and ion pumps, as in the regulation of cAMP by internal Na (Harvey, Jurevicius & Hume, 1991), or the interaction of Na with the Na/Ca exchanger (Leblanc & Hume, 1990; Lederer, Niggli & Hadley, 1990, 1991; Johnson & Lemieux, 1991; Leblanc & Hume, 1991; Hume & Levesque, 1991; Sham, Cleemann & Morad, 1992). Thus, ion currents may help regulate the very gradients that drive them. The two problems I wish to address in this review are how the local concentration of Ca controls the Ca current, and how the molecular structure of the channels helps determine the local concentration.

Action-Potential Patch Clamp Measures Ca Channel Function

Three recent papers exemplify the different strategies now used to study L-type channels: Singer et al. (1991), who co-expressed the pore-forming unit with ancillary subunits to study their function; Hartzell et al. (1991), who combined internal perfusion with whole-cell recording to identify the major regulatory step of cAMP; and finally Yue, Herzig and Marban (1990), who analyzed conditional probabilities of single channels in cell-attached patches to demonstrate Ca-induced inactivation. We may study these same channels not only in these compelling biophysical and biochemical situations, but

Key Words Ca channel subunits · channel clustering · Ca-induced inactivation · whole-cell current · action potential patch clamp · channel regulation · kinetic modes

also during spontaneous action potentials. We do this because in beating heart cells, membrane voltage, membrane-bound regulators, phosphorylating substrates and enzymes, and local ionic conditions vary from moment to moment. Thus it is of interest to know how the channels actually respond in more or less natural circumstances. Before discussing these different approaches in more detail, consider the requirements for studying channels in beating cells.

In our method, we measure action currents and action potentials from the same cell using two patch electrodes, one in the whole-cell mode and the other in the cell-attached mode. To isolate the ionic current, we subtract the capacitive current, $c(dV/dt)$, where c is the patch capacity and $V(t)$ is the action potential (Wellis, DeFelice & Mazzanti, 1990). The cells we use generally come from embryonic chick hearts at different developmental stages (Fuiji, Ayer & DeHaan, 1988). Mazzanti, Galli and Ferroni (1992) have extended the technique to neurons, and the method could apply to any cell—excitable or nonexcitable. The data in Figs. 1 and 3 come from single, 10 to 15 μm diameter, 7-day chick ventricle cells. After dissociation, these cells adhere to the recording dish, round up, and beat spontaneously in physiological solutions. We make the first seal with the whole-cell electrode, which contains an intercellular-like solution. Each cell differs slightly, so we select one with the desired action potential and beat frequency before applying the second electrode. The second patch electrode contains a test solution; only if the test solution equals the bath solution does the channel in the patch experience the same condition as its counterparts in the rest of the cell. By introducing toxins into the cell-attached patch electrode, we can exclude certain channels from measurement. In other situations, we might replace Ca with Ba to enhance Ca channel conductance (Reuter et al., 1982; Cachelin et al., 1983; Cavalie et al., 1983; Tsien et al., 1983), or use high K concentrations to increase K channel conductance (Mazzanti & DeFelice, 1988). In any case, the two electrodes provide a simultaneous record of patch action currents and membrane action potentials in beating cells.

In this way we have analyzed the fast Na channel, the early outward K channel, the delayed rectifier K channel, the inward-rectifier K channel, and the L-type Ca channel in neurons and in cardiac cells (Clay & DeFelice, 1983; DeFelice, 1983, 1989; DeFelice & Clay, 1983; DeFelice & Levi, 1984; Fischmeister et al., 1984; DeFelice, Goolsby & Huang, 1985; Levi & DeFelice, 1986; Mazzanti & DeFelice, 1987*a*,*b*, 1988, 1990*a*,*b*; DeFelice, Goolsby & Mazzanti, 1989; Wellis et al., 1990; Liu, DeFelice & Mazzanti, 1992). Figure 1 comes from a recent paper on Ca channel action currents.

In a related technique, one clamps cells with a voltage that follows the shape of the action potential. Starzak and Starzak (1978) used it to study currents in squid axon; Doerr, Deriger and Trautwein (1989, 1990) and Arreola et al. (1991) have applied it to cardiac cells. Doerr et al. and Arreola et al. record current from the entire cell; to measure the Ca curent, they must block all other currents. In the action-potential patch-clamp technique, we block channels only in the patch and let the action potential and other cell events proceed unimpaired. If desired, we can eliminate the perturbing effects of the whole-cell electrode. In this technique, we use only a single cell-attached electrode; we break the patch after recording the currents of interest, switch to the whole-cell recording mode, and record the action potentials before perfusion can occur.

In our previous work on Na and K channels, we made a fundamental assumption that allowed us to correlate time-average kinetics in the patch with ensemble-average kinetics in the cell. Namely, the kinetics of one channel averaged over N beats equal the kinetics of N channels measured over one beat. This assumption turns out to be wrong for Ca channels. Before turning to an explanation of this phenomenon, consider the structure of L-type Ca channels which we believe may bear on this problem.

L-Type Ca Channels Have an Oligomeric Structure

The molecular biology and the biochemistry of Ca channels provide strong evidence that the pore (α1) forms a complex with four other subunits (α2, β, γ, δ). Two main pieces of evidence support the oligomeric model: co-purification and co-immunoprecipitation with antibodies against one of the subunits. The α1 subunit varies in skeletal muscle, cardiac muscle, smooth muscles, and brain tissue (for a recent overview *see* Starr, Prystay & Snutch, 1991). In heart, α1 has a molecular weight of approximately 175 kD. When expressed in naive cells, α1 forms a Ca pore. However, the kinetics of α1 pores differs from that of native Ca channels under comparable conditions (for example, in 40 mM Ba). The α1 subunit has a counterpart in the Na channel (Ellis et al., 1988). The α2 (143 kD) and δ (27 kD) subunits come from the same gene; di-sulfide bonds link these two to form a transmembrane unit written α2/δ. The β subunit (50 kD) extends to the intercellular domain; it contains phosphorylation sites (as does α1)

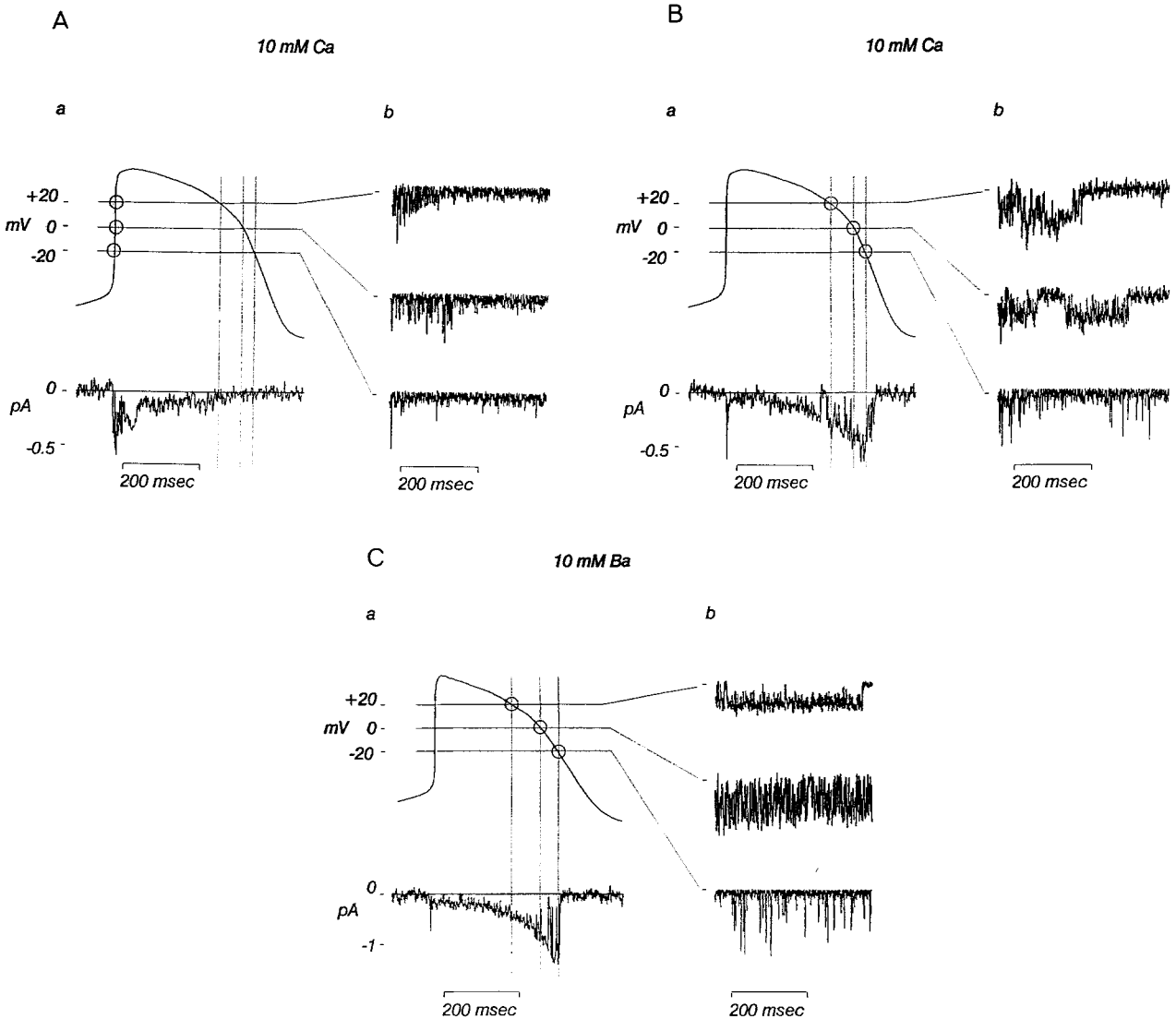

Fig. 1. Spontaneous action-potential patch-clamp technique: L-type Ca channels in seven-day chick embryo ventricle cells (*adapted from* Mazzanti & DeFelice, 1990*a*) (*A*) (*a*) The action potential from a single cardiac cell (top trace), and the simultaneous action current from a patch on the same cell (bottom trace). The whole-cell electrode contains intracellular-like solution (in mM: 120 K, 0.1 Ca, 2 Mg, 1.1 EGTA, 10 HEPES); the cell-attached electrode contains 108 Na, 1.3 K, 10 Ca, 0.5 Mg, 1 Ba, 5 TEA, 10 4AP, 0.1 TTX, 10 HEPES, 5 dextrose. The bath solution is 130 Na, 1.3 K, 1.5 Ca, 0.5 Mg, 10 HEPES, 5 dextrose. (*b*) Currents from the same patch that was subsequently voltage-clamped with steps from a holding potential of −80 mV to test potentials of −20, 0, 20 mV. These test potentials are indicated by the horizontal lines to the corresponding voltages (open circles) in the action potential. Compare action currents in *a* with the step protocol currents in *b*. The step currents correlate with the upstroke currents, not the repolarization currents (vertical lines). (*B*) (*a*) Same conditions as in *A*, except that we have selected a rare event, a long-lasting action current that appears approximately once every 100 beats. This long opening, interrupted by relatively brief closings, we call a mode-2 opening. (*b*) Selected currents from the same patch that was subsequently voltage-clamped with steps from a holding potential of −80 mV to test potentials of −20, 0, 20 mV. Compare action currents in *a* with the step protocol currents in *b*. These step currents correlate with the repolarization currents (vertical lines). (*C*) (*a*) Same conditions as in *A*, except that we have used 10 mM Ba in the pipette in place of 10 mM Ca and 1 Ba, and 110 Na in place of 108 Na. Whereas mode-2 openings in 10 mM Ca are rare, the long-lasting opening shown here occurs with virtually every beat in 10 mM Ba solutions. (*b*) Currents from the same patch voltage-clamped with steps from a holding potential of −80 mV to test potentials of −20, 0, 20 mV. Compare action currents in *a* with the step currents in *b* at the same voltages (open circles). The step currents correlate with the repolarization currents (horizontal lines).

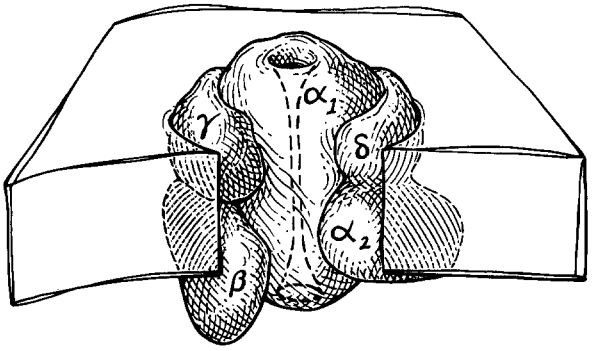

Fig. 2. Oligomeric structure of an L-type Ca channel. L-type Ca channels have five subunits: $\alpha 1$ is the Ca pore, $\alpha 2/\delta$ and γ are membrane proteins, and β is associated with the cytoplasm (*adapted from Catterall*, 1991).

that may regulate the channel. Finally, the γ subunit (30 kD) binds noncovalently with the other subunits.

The complete Ca channel, therefore, consists of four gene products. All subunits indicated in Fig. 2 exist as cDNA clones from skeletal muscle, and various subunits exist as cDNA clones from cardiac muscle, smooth muscle, or brain tissue. At present, no γ subunit appears to exist in cardiac muscle.

Co-Expression of Subunits Helps Define their Function

Two general features result from co-expression of $\alpha 1$ with other subunits: the level of expression increases and the kinetics of the channel becomes more like that of the wild type channel (Tkahashi et al., 1987; Lacerda et al., 1991; Singer et al., 1991; Varadi et al., 1991; Wei et al., 1991; Perez-Reyes et al., 1992). Consider the cardiac β subunit, which Perez-Reyes et al. (1992) cloned and co-expressed in *Xenopus* oocytes with cardiac $\alpha 1$. The voltage-evoked current increased fivefold, and the peak activation curve shifted by -10 mV. Co-expression of $\alpha 1$ and β (Lacerda et al., 1991; Perez-Reyes et al., 1992) speeds the activation kinetics by hundreds of milliseconds when compared to $\alpha 1$ alone. Thus, β not only increases the expression of $\alpha 1$, but it modulates the voltage dependence of $\alpha 1$. However, the effects of this combination can vary, e.g., brain $\alpha 1$ and skeletal muscle β increase expression with no effect on kinetics or voltage dependence (Mori et al., 1991). The co-expression of $\alpha 1$ with γ has the interesting property that γ accelerates the inactivation phase independent of other subunits, and γ shifts the voltage inactivation curve to more negative values (Singer et al., 1991). Contrary to other subunits, γ has little effect on the level of expression of $\alpha 1$. Singer et al. (1991) have tentatively associated γ with Ca-channel inactivation. These data led Catterall (1991) to the conclusion that $\alpha 2/\delta$, β, and γ do not participate directly in the voltage-dependent gating or ionic conductance of Ca channels; rather, they modulate properties that already reside in $\alpha 1$.

Ca Channels Have Different Pharmacology and Regulatory Pathways

L-type Ca channels have three distinguishing features: (1) Dihydropyridines (DHP), like nifedipine or nitrendipine, block L-type currents. Flockerzi et al. (1986) have shown that purified DHP binding sites from skeletal muscle T-tubules represent functional calcium channels. The $\alpha 1$ subunit binds DHPs in muscle (Tanabe et al., 1987; Mikami et al., 1989); furthermore, as Kim et al. (1990) have shown, DHP binds in the absence of the $\alpha 2$ and β subunits. (2) Dihydropyridines can also enhance L-type currents (e.g., BAY K 8644 or (+) PN202-791 prolong single channel openings). (3) Hormones and neurotransmitters regulate L-type channels. T-type Ca channels appear insensitive to DHPs and to β-adrenergic stimulation. Thus, noradrenaline (norepinephrine), or its substitute, isoproterenol, enhances L-type Ca currents but has no effect on T-type Ca currents (Tytgat et al., 1988). Some channels do not easily categorize: a subunit cloned from brain (Mori et al., 1991) acts like $\alpha 1$ in that DHP binds it and $\alpha 2$ and β subunits enhance its expression. The Ca channel formed with the Mori et al. subunit, unlike $\alpha 1$-based channels, does not respond to nifedipine.

Phosphorylation Activates Ca Channels

β-adrenergic stimulation activates cAMP production, and cAMP-dependent phosphorylation events apparently stimulate L-type Ca channels to open (Reuter, 1983; Bean et al., 1984; Trautwein, 1984; Trautwein & Pelzer, 1985; Hartzell, 1988; Lacerda, Rampe & Brown, 1988; Brown & Birnbaumer, 1990; Hartzell et al., 1991). However, no direct evidence exists that cardiac $\alpha 1$ subunits get phosphorylated, or that phosphorylation regulates them (Nastainczyk et al., 1987; Jahn et al., 1988; Nunoki, Flori & Caterall, 1989; *reviewed in* Hartzell & Duchatelle-Gourdon, 1992). Though acetylcholine has no direct action on the Ca current, it decreases the current activated by β-adrenergic pathways (Fischmeister & Hartzell, 1986; Hartzell, 1988). (For the regulatory action of cGMP, *see* Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987; Tohse & Sperelakis, 1991). Mg more effectively blocks cAMP-elevated Ca currents than it blocks basal Ca currents, which suggests that the phosphorylation events may in-

crease the affinity of the channel for Mg (Hartzell & White, 1991).

The increase in whole-cell Ca currents following β-adrenergic stimulation causes an increase in the open channel probability, but not in the single channel conductance (Reuter et al., 1982; Cachelin et al., 1983; Cavalie, Pelzer & Trautwein, 1986; Ochi & Kawashima, 1990; Mundina et al., 1991). Yue et al. (1990) interpret isoproterenol enhancement of Ca current as an increase in mode-2 (long-lasting) openings, which Katsushige and Fozzard (1992) have verified. Josephson and Sperelakis (1990) have measured gating currents in isolated cardiac cells (Bean & Rios, 1989; Hadley & Lederer, 1991; Shirokov et al., 1992) and show that Bay K 8644 or isoproterenol (at concentrations sufficient to alter gating kinetics and amplify ionic current) leave the maximum gating charge unaltered. These data suggest that phosphorylation increases the number of active channels and does not otherwise alter the channel. However, experiments on the kinetics of Ca channels in cell-attached *vs.* inside-out patches may modify this explanation. In isolated embryonic chick heart cells, basal Ca channel openings (nonisoproteronol stimulated) have different mean open times in different patches: in a given patch, however, the time constant remains the same after removing the patch from the cell (Mazzanti, DeFelice & Liu, 1991). The inside-out membrane faces a simple, inorganic salt solution free of phosphorylating or dephosphorylating enzymes or substrates. A variable degree of phosphorylation may account for on-cell, patch-to-patch variability, and removing the channel may have no effect on its state of phosphorylation. In other preparations, Ca channels cease to open after removing the patch from the cell. Katsushige and Fozzard (1992) report that MgATP and the catalytic subunit of the cAMP-dependent protein kinase can restore isoproteronol-induced activity.

Ca Channels Have Different Biophysical Properties

Ca channel conductance in normal Ca (1 to 5 mM) is virtually undefined. To study Ca channel conductance, we use high concentrations of Ca, or we replace Ca with other ions that conduct better (Hess & Tsien, 1984; Nilius et al., 1985; Hess, Lansman & Tsien, 1986; Lansman, Hess & Tsien, 1986). For example, in 10 mM Ca and 130 mM Na, the channels have a Ca conductance of 9 pS, whereas in zero Ca and 130 mM Na, the channels have a Na conductance of 50 to 100 pS (embryonic chick heart: Levi & DeFelice, 1986; Mazzanti & DeFelice, 1987*b*, 1990*a*; Kawano & DeHaan, 1989). Na permeation in zero external Ca solutions is probably not a good way to distinguish native differences between channel types. A perhaps more realistc situation uses Ba as the conducting ion. In 1 mM Ba, the conductance of L-type channels is about 7 pS; near 20 mM Ba, the conductance begins to plateau; in 100 mM Ba, it is about 20 pS (Yue & Marban 1990). In 100 mM Ba, L channel conductance is 15 to 20 pS and T channel conductance is 6–8 pS. Neither L-type nor T-type single channels have been observed under physiological conditions, although Klockner and Isenberg (1991) state that physiological temperatures facilitate such measurements. In any case, for concentrations in the range 1 to 5 mM (even if Ba replaces Ca), L- and T-type conductances apparently converge.

L-type channels display current-dependent inactivation, but T-type channels do not. Current-dependent inactivation seemingly occurs even when Ba replaces Ca, as large Ba currents inactivate more rapidly than smaller currents (Richards et al., 1990; Osaka & Joyner, 1991; Mazzanti et al., 1991). Ba-induced inactivation is slower than Ca-induced inactivation; furthermore, inactivation depends on external Ca as well as internal Ca (Kass & Sanguinetti, 1984; Mentrard, Vassort & Fischmeister, 1984). Thus, using Ba or elevated levels of external Ca to increase channel conductance raises formidable problems in interpretation. Nevertheless, we can distinguish L and T currents in whole-cell experiments. Generally, L-type currents inactivate slower, while T-type currents inactivate faster. However, DHP-sensitive currents may have a strong secondary voltage inactivation (in addition to a primary voltage inactivation) caused by the interaction between voltage and current (Mazzanti et al., 1991).

On the basis of single channel properties, voltage and current sensitivity, or voltage-dependent kinetics, cardiac Ca channels do not easily classify into L-type and T-type. Nevertheless, DHP sensitivity and single channel conductances (under extreme conditions) do indicate multiple Ca channel types. *See* Vivaudou et al. (1991) for a discussion of different Ca currents in smooth muscle; *see* Trautwein and Pelzer (1985), Bean (1989), Komori and Bolton, 1991, Tsien et al. (1991), and Nargeot et al. (1992) for a similar discussion in cardiac muscle and nerve tissue.

L-Type Ca Channels Have Different Kinetic Modes

Classical models of inactivating currents, like the ones used to describe Na currents, include closed states (C), open states (O), and inactive states (I)

connected by voltage-dependent rate constants. Ca channels have such states, but they also have current-dependent states. The greater the Ca concentration inside the cell, the smaller the Ca current through the membrane. In our model of this phenomenon (Mazzanti et al., 1991), we let Ca ions block the channel, where "blocked" mean "cessation of current," not necessarily conformational change. Thus, the blocked state represents Ca-mediated inactivation. Standen and Stanfield (1982) proposed that the Ca ions that mediate inactivation bind to the channel. In their model, Ca ions enter the cell via I_{Ca} through the cell membrane, and they leave the vicinity of the membrane at a rate proportional to internal concentration; the macroscopic current feeds back on itself uniformly. Our model is stochastic, and it allows for variable channel density. Furthermore, it describes the kinetics actually observed in single channel experiments.

In Ba, long (mode-2) openings last for hundreds of milliseconds, during which only brief closings occur (Hess, Lansman & Tsien, 1984; Kokubun & Reuter, 1984; Nowycky, Fox & Tsien, 1985; Yue et al., 1990; Mazzanti & DeFelice, 1990*a*; Pietrobon & Hess, 1990; Mazzanti et al., 1992). BAY K administration, β-adrenergic stimulation, or large depolarization independently promote the long openings. The action-potential patch clamp tells us that long openings can occur under more normal conditions (Fig. 1). Thus, mode-2 kinetics appears as a regular feature of cardiac excitability. Na channels in cardiac and skeletal muscle have similar kinetic modes (Patlak & Ortiz, 1985, 1986). These low probability events could arise from complex voltage-gated models (Lacerda & Brown, 1989). However, the Ba-long openings occur every beat (Fig. 1), whereas Ca-long openings occur infrequently. An alternative explanation to Lacerda and Brown's, which is implicit in our model, would attribute the long openings to spontaneous lapses in the Ca block of the conduction channel.

L-Type Ca Channels Cooperate via Ca Current

An issue not dealt with here relates to interactions between Ca ions, Ba ions, and binding sites within the Ca channel. Yue and Marban (1990) note a slight tendency for Ca/Ba mixtures to lower open channel probability compared to pure solutions. The mole-fraction paradox remains unresolved; however, it has suggested that Ca binding sites inside the channel mediate Ca inactivation. This story begins with Hagiawara and Hakajima (1966), who initially showed that raising internal Ca from normal levels to 5×10^{-7} M abolishes action potentials in barnacle muscle fibers. Brehm and Eckert (1978) and Brehm, Eckert and Tillotson (1980) tested the idea directly by injecting EGTA into *Paramecium*. EGTA caused the transient inward currents to relax more slowly, probably because increased buffering removes Ca from the vicinity of the membrane (Chad & Eckert, 1984). Ca-induced inactivation also occurs in cardiac cells (Kohlhardt et al., 1975; Hume & Giles, 1983; Josephson, Sanchez-Chapula & Brown, 1984; Mentrard et al., 1984; Lee, Marban & Tsien, 1985; Argibay, Fischmeister & Hartzell, 1988). These results support the idea that Ca-mediated inactivation and voltage-dependent inactivation coexist in heart (Kass & Sanguinetti, 1984; Bean 1985; McDonald et al., 1986; Campbell et al., 1988; Hirano, January & Fozzard, 1989), a notion also supported by gating current measurements (Hadley & Hume, 1987; Hadley & Lederer, 1991).

We have postulated that adjacent Ca channels share Ca. The closer the channels, the more effective the block, and the stronger the inactivation. We tested the hypothesis in 7-day chick ventricle cells by comparing the currents from patches containing different numbers of channels, using 20 mM Ba as the charge carrier (Mazzanti et al., 1991). With five or more channels, Ba currents decline more rapidly than expected by simply scaling the one-channel current. Furthermore, the effect depends on holding potential. At -80 mV, the channels inactivate strongly; at -40 mV, fewer channels open and the channels inactivate weakly. In our model, fewer channels open because of the -40 mV holding potential, and weaker inactivation occurs because there is less Ca current to mediate it.

We have interpreted the evident cooperativity of Ba currents as support for our model of Ca currents (Risso, DeFelice & Goolsby, 1992). Recently, Imredy and Yue (1992) have provided additional evidence for the model in 160 mM Ca. We believe that diverse Ca kinetics exist because Ca channels occur in clusters in which the kinetics do not simply scale by channel number. Under the conditions of Fig. 3, Ba mediates the interaction, but under normal conditions Ca ions would mediate it. Each channel in the cluster shares the total Ca through all pores; effective blocking depends not only on Ca entry but also on its dispersal. Clusters vary in size, and some channels may exist in isolation. This mosaic view of channel density implies that clusters would conduct large transient currents, while individual channels would conduct small long-lasting currents. Thus, we expect L-type Ca currents composed of identical molecular units to have no unique kinetics. Rather, macroscopic I_{Ca} is a mixture of currents at different places in the cell membrane, even though the elementary channel units are exactly the same.

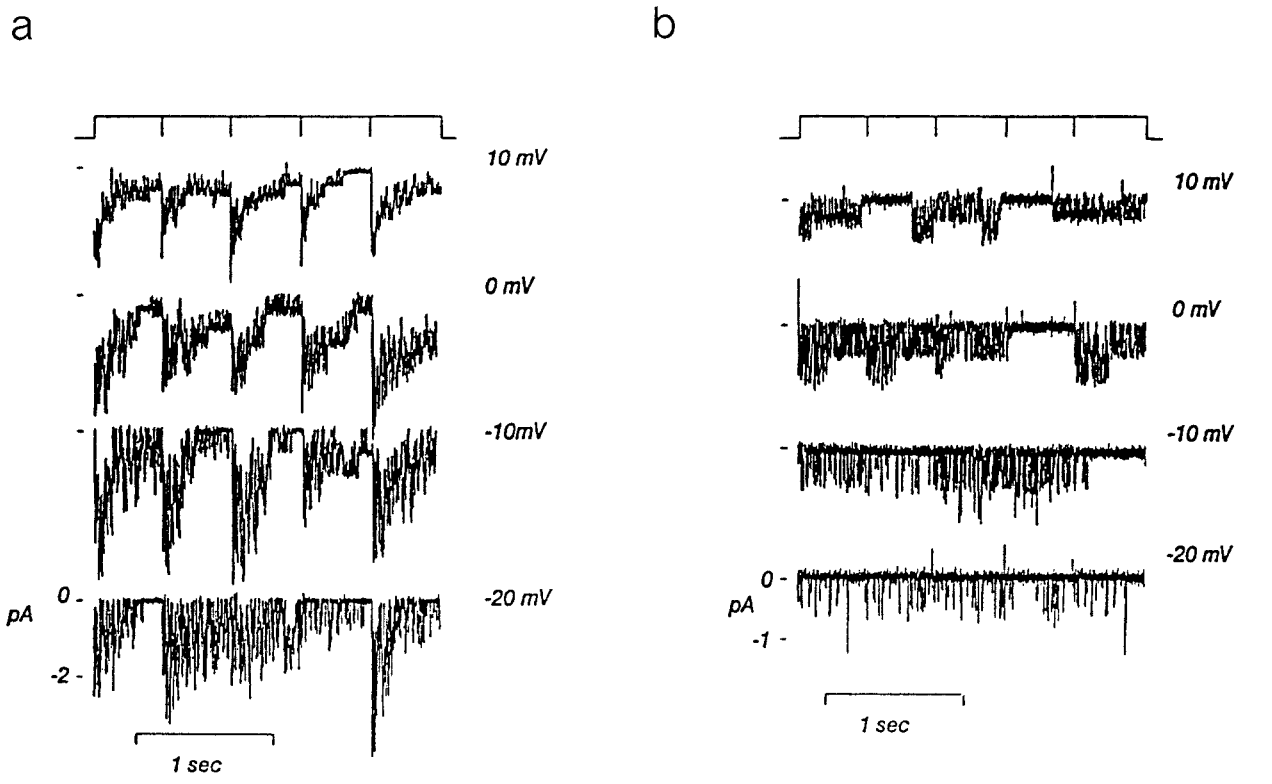

Fig. 3. Cooperativity between L-type Ca channels. Cell-attached patch recordings from L-type Ca channels in seven-day chick embryo ventricle cells (*adapted from* Mazzanti et al., 1991). The area of these patches is 7–10 μm^2. (*a*) This patch has at least five channels (as determined from amplitude histograms). The cell-attached electrode contains (in mM): 90 Na, 1.3 K, 0.5 Mg, 20 Ba, 5 TEA, 10 4AP, 0.1 TTX, 10 HEPES, 5 dextrose. The bath solution is the same as that in Fig. 1. Each trace is a concatenation of five responses to steps from −80 mV to the indicated test potentials. The 1-sec intervals between steps, and the tail currents at the end of each step, are omitted. The inactivation of the channels evident here is eliminated by holding the patch at −40 mV. This effect is reversible. (*b*) Conditions are the same as in *a*, except that the patch contains two channels. Inactivation is reduced, and the holding potential has little effect.

This model provides for a variety of I_{Ca} kinetics. This mechanism would be in addition to the kinetics that may occur as a result of genuinely disparate molecular classes or admixtures of channel subunits. Clustering of apparently similar channels occurs for other channel types. For example, nicotinic ACh receptors cluster in association with a subcortical matrix (Froehner, 1991), and neuronal Ca channel cluster in nerve growth cones, where they stimulate proliferation (Silver, Lamb & Bolsover, 1991).

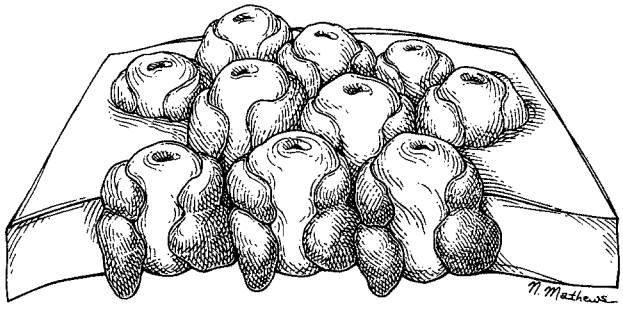

Fig. 4. Clustering of L-type Ca channels: a hypothesis regarding oligomeric structure and macroscopic current. Under this hypothesis, clustering involves the interaction of subunits and provokes cooperativity because adjacent channels share Ca.

Hypothesis

The question remains why some channels cluster and others do not. A clue comes from the strong cooperativity that exists in patches with relatively large areas. The patches in Fig. 3 would have approximately 1 channel per μm^2, if the channels were spaced uniformly. At such low density, we think it is unlikely that the channels would interact via intercellular ions. We therefore believe the channels in such patches are virtually adjacent at the molecular level.

We propose that the subunits help organize the channels into clusters. Under this hypothesis, phosphorylation might regulate not only subunits into

oligomeres, but also oligomeres into larger groups. Such cluster formations would determine the macroscopic current. Most data come from Ba currents or elevated-concentration Ca currents, and we expect significant differences in 1 to 5 mM Ca. Internal Mg accelerates Ba inactivation but slows Ca inactivation (Hartzell & White, 1991); cAMP emulates this Mg effect to some extent, but we have not incorporated these findings into our model.

If Ca-induced inactivation were to occur for a single channel, then open time and the next adjacent closed time ought to correlate reciprocally. Lux and Brown (1984) found no such correlates in 40 mM Ca, and they concluded that individual Ca channels do not inactivate themselves. Yue, Backx and Imredy (1991), using 160 mM Ca, showed that Ca ions do inactivate the very channel they pass through. Both Lux and Brown and Yue et al. selected patches that contained only one or two channels. Our model would predict that the current through one channel would not establish the necessary concentration to block the pore except in very high Ca (Yue et al., 1991; Imredy & Yue, 1992). We believe that Ca channels would normally show very little Ca-mediated inactivation unless they cluster.

In contrast to single channel experiments, whole-cell experiments in normal or slightly elevated Ca generally display Ca-induced inactivation for L-type currents. The amplitude and kinetics of the whole-cell currents vary from cell to cell; however, the larger Ca currents usually inactivate more strongly (*reviewed in* Mazzanti et al., 1991). These whole-cell data fit our model. They also fit other models like the Standen and Stanfield (1982) representation of macroscopic current. However, high density or nonuniform density do not automatically result in Ca-mediated inactivation. Rather, the Ca channels have to be very close, as proposed in Fig. 4, for ions to be shared. The model requires that the conducting ions leave the pore readily (Hess & Tsien, 1984), accumulate near the pore, and exert a negative feedback on Ca flux. The feedback mechanism remains unknown, but it may involve physical restriction and electrostatic repulsion. Such a model would resolve conflicting results from single channel and whole-cell experiments and explain the wide assortment of L-type Ca channel kinetics.

Direct tests will require theoretical calculations (Fischmeister & Horackova, 1983; Risso et al., 1992), experimental measurements of Ca concentrations during transient activity (Callewaert et al., 1991), developmental comparisons in the same tissue (Osaka & Joyner, 1991; Toselli et al., 1991; Tohse, Meszaros & Sperelakis, 1992), and coexpression combined with single channel experiments.

The model may help clarify some unexplained phenomena. For example, voltage dependence and Ca dependence seem to exist in varying proportion in what we presume are intrinsically identical channels. The proportion may depend on experimental conditions, as suggested by Hartzell and White (1991). But it may also depend on extrinsic differences, such as channel clustering and internal buffering, as suggested by our model. Another paradox relates to the persistent observation that the number of DHP binding sites in skeletal muscle and embryonic heart exceeds the number of functional channels (Schwartz, McClesky & Almers, 1985; Curtis & Catterall, 1986; Aiba & Creazzo, 1993), in spite of evidence to the contrary in adult heart (Lew, Hryshko & Bers, 1991). A related observation may be the following: in cell-attached patches with Ca in the pipette, we rarely observe one channel; however, mode-2 openings always appear as one conductance state (Fig. 1). This result is remarkable because the regular openings and the Ca mode-2 opening come from the same patch. Similar remarks apply to L-type channels conducting Na. Thus, Ba or Na may coordinate the cluster to act as a unit, causing a disparity between electrophysiological and pharmacological numbers of channels.

We might propose, therefore, that clustering influences not only the kinetics, but also the pharmacology and the conductance of L-type Ca channels. Such speculations await a more detailed understanding of the molecular structure.

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