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Models of Calcium Regulation in Neurons

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I. Introduction

It is becoming increasingly clear that calcium is a critical second messenger involved in the regulation of synaptic plasticity (see Chapters 4, 10, 14, 15, and 18, this volume). Consequently, in order to understand fully the plastic capabilities of neurons it is necessary to understand in detail the mechanisms by which calcium enters cells, its subsequent subcellular effects, and its subcellular distribution and regulation.

Calcium acts as an intracellular trigger of many important processes in neurons. The two most familiar examples are regulation of electrical activity and activation of synaptic transmitter release. In neurons, the primary source of intracellular calcium is influx through voltage-dependent membrane channels. Calcium is initially highly localized to calcium channel mouths, and must reach nearby or distant sites of action by diffusion in cytoplasm, where it is also rapidly bound to native buffers. To maintain homeostasis, the calcium that has entered a neuron during a depolarization must be removed, first from cytoplasm and eventually from the cell as a whole. This is accomplished by a variety of mechanisms, including uptake into intracellular organelles and extrusion by surface membrane pumps. In order to understand how calcium operates to regulate neuronal function, it has become necessary to construct models of intracellular calcium diffusion, binding, uptake, and extrusion. In this chapter, I shall describe several of these models, including tests of their validity and their application to control of electrical activity and transmitter release.

II. Calcium-Dependent Currents and Electrical Activity

Calcium-dependent potassium currents ($I_{K(Ca)}$) are present in a variety of cell types (Petersen and Maruyama, 1984). In neurons, this current is often activated by action potentials, and contributes to spike repolar-

ization, after-hyperpolarization, and neuronal adaptation (Meech, 1978). Calcium-activated nonspecific cationic current ($I_{NS(Ca)}$) has also been observed in several types of cells (Petersen and Maruyama, 1984). Its function is less clear. Finally, calcium current itself is subject to regulation by intracellular calcium in many neurons, whereby internal calcium accumulation leads to inactivation of the channels admitting calcium (Eckert and Chad, 1984).

A. Three Distinct Currents Regulate Bursting

I have concentrated on molluscan neurons in which all three calcium-dependent currents operate—bursting pacemaker neurons in the dorsal upper left quadrant of the abdominal ganglion of *Aplysia californica*. Figure 1 illustrates these currents after a depolarizing pulse, and the normal pattern of bursting in left upper quadrant neurons of *Aplysia*. Each current plays a role in shaping the endogenous action potential bursts in these cells:

1. An external tetraethylammonium (TEA) sensitive form of $I_{K(Ca)}$ is the dominant current for the first 50–100 msec following spikes in a burst and the end of the burst (Kramer and Zucker, 1985b). This early outward current, along with the voltage-dependent delayed-rectifier potassium current, repolarizes each action potential and generates a 50- to 100-msec hyperpolarizing after-

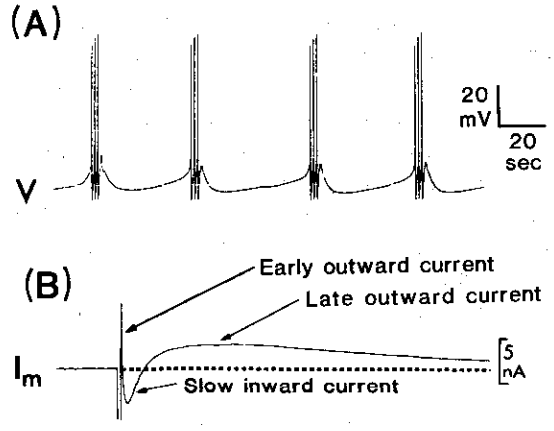


Figure 1.
(A) Endogenously generated action potential bursts in a dorsal upper left quadrant bursting pacemaker neuron in an *Aplysia* abdominal ganglion. (B) Current following a 50msec depolarizing voltage-clamp pulse to 0 mV from a holding potential of −35 mV. [Adapted from Kramer and Zucker (1985a).]

potential. At low temperatures and in some other molluscan bursting neurons (Deitmer and Eckert, 1985; Thompson *et al.*, 1986; Smith and Thompson, 1987), a TEA-insensitive form of $I_{K(Ca)}$ persists for seconds and contributes to the late outward current that hyperpolarizes cells between bursts. At temperatures near the intertidal water temperature in Southern California where our animals are collected, we do not see a prolonged form of $I_{K(Ca)}$.

2. After $I_{K(Ca)}$ decays to low levels, $I_{NS(Ca)}$ is the dominant current for about 1 sec after each spike and the end of the burst (Kramer and Zucker, 1985a). This slow inward current generates a depolarizing afterpotential, which helps trigger subsequent spikes and serves to accelerate firing during the early part of the burst (Thompson and Smith, 1976; Adams, 1985; Smith and Thompson, 1987). In R15, a different bursting pacemaker neuron from the ones we have studied, this current is triggered partially by axonal spikes even in voltage-clamp studies (Adams and Levitan, 1985).
3. Finally, calcium-dependent inactivation of resting calcium current dominates during the late interburst interval (Adams and Levitan, 1985; Kramer and Zucker, 1985b). The calcium-dependent block of this inward current appears as a late outward current after a depolarization in voltage-clamp records. As this inactivation decays, the low-threshold calcium pacemaker current resumes and the neuron is depolarized until the next burst begins.

The roles played by these currents in shaping electrical neuronal activity depend critically on their time courses. Why should three calcium-activated or inactivated currents have such different durations? If each is dependent on submembrane calcium following influx through calcium channels, why do they decay at different rates? To answer these questions, we must know how submembrane calcium varies as a function of time after an influx, and we must know how each current depends on intracellular calcium.

B. Time Course of Submembrane Calcium Activity Following Influx

The measurement of submembrane calcium activity is no trivial matter. Calcium indicators such as aequorin, arsenazo III, quin-2, and fura-2 provide measures of average calcium activity throughout cytoplasm. Two-dimensional imaging of absorbance or fluorescence signals still produces results affected by calcium all along a line through the cell.

Calcium-sensitive microelectrodes respond too slowly to follow changes in submembrane activity following a burst. Thus no method presently exists to measure unequivocally the calcium activity beneath the membrane.

One approach to this problem is to compare signals generated by aequorin and arsenazo III injected into neurons that are depolarized to admit calcium. Depolarizing pulses of equal duration and different amplitude should lead to similar spatial distributions of intracellular calcium with different magnitude, if the processes governing calcium movement in cells (diffusion and pumps) are roughly linear. The absorbance of arsenazo III is linearly related to local calcium concentration, while aequorin photoemission varies as the 2.5 power of local calcium. For any given spatial distribution of calcium in a neuron, the aequorin signal should be proportional to the arsenazo III signal raised to the 2.5 power. This prediction was readily confirmed (Fig. 2A). However, after calcium influx, changes in the spatial distribution of calcium should be reflected by a more rapid drop in the nonlinear indicator aequorin signal than in the linear indicator arsenazo III signal as calcium diffuses away from the membrane (Smith and Zucker, 1980). This prediction was also confirmed (Fig. 2B), and provides direct evidence for diffusional redistribution of calcium in cytoplasm after entry at the surface.

Although confirming the existance of intracellular calcium gradients that change with time, the above results still did not permit the direct estimation of submembrane calcium concentration. For this purpose, we were forced to turn to a model of calcium movements in cytoplasm (Smith and Zucker, 1980). We solved the diffusion equation for cytoplasmic calcium in spherical coordinates, with influx at the surface determined by the magnitude and time course of calcium currents during voltage-clamp pulses. Calcium diffused inward with rapid binding to a fixed cytoplasmic buffer, with a measured binding ratio determined by the ratio of total calcium influx to average calcium concentration change measured with arsenazo III. Calcium was removed in the model either by surface pumps or uptake into organelles at rates measured independently in neuronal tissue (Blaustein *et al.*, 1978; Requena and Mullins, 1979). This model, run with all parameters constrained to narrow ranges determined by independent measurement, successfully predicted the different time courses of aequorin and arsenazo III responses to depolarizing pulses (Fig. 2C). Such simulations, then, are as close as we have come to being able to "measure" submembrane calcium activity after influx through calcium channels.

C. Calcium Removal Mechanisms

The simulations of calcium diffusion away from the membrane showed that the rapid fall of aequorin signals reflected the diffusional dilution of submembrane calcium. The slower decay of arsenazo III absorbance

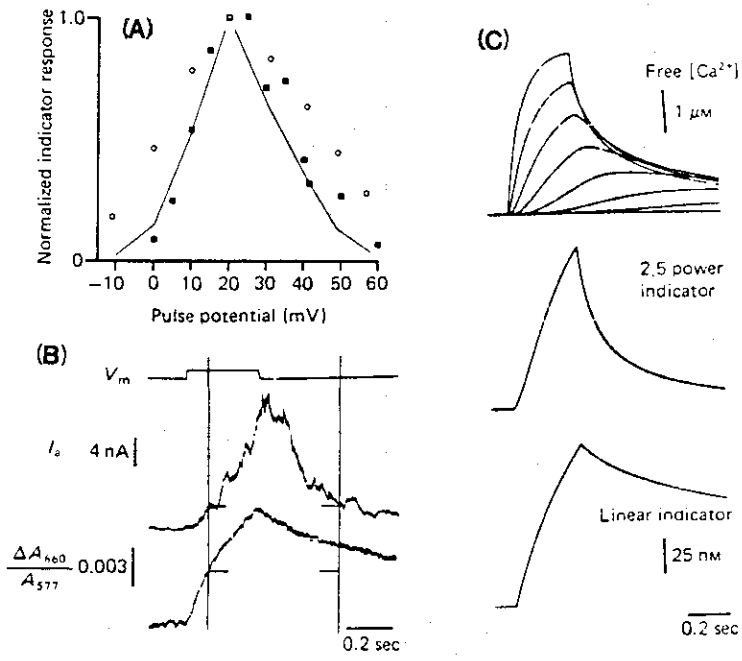


Figure 2.

(A) Normalized arsenazo III absorbance changes (circles) and aequorin photoemissions (squares) as functions of pulse amplitude, for 0.3-sec pulses in a bursting pacemaker neuron. The line plots arsenazo III responses raised to the 2.5 power. (B) Photomultiplier current (I_a) indicating aequorin photoemissions and arsenazo III absorbance changes ($\Delta A_{660}/A_{577}$) from two bursting pacemaker neurons, in response to a +20-mV, 0.3-sec pulse from a holding potential of −45 mV. (C) Theoretical predictions from the Smith and Zucker (1980) model: top trace, predicted calcium concentration changes at depths of 0.2, 0.6, 1.2, 1.8, 2.8, 4.3, 6.5, and 10.8 μm beneath the membrane (from the top), in response to a depolarization to +15 mV for 0.3 sec. The predicted aequorin and arsenazo III signals are shown in the middle and bottom traces respectively. [Adapted from Smith and Zucker (1980).]

reflected true removal of calcium from cytoplasm. What is the mechanism of this removal? A number of possibilities exist: (1) surface extrusion by Na/Ca exchange or an ATP-driven pump (Requena, 1983), (2) uptake into organelles such as endoplasmic reticulum (Blaustein *et al.*, 1978; Henkart *et al.*, 1978), and (3) diffusion past saturated buffers with subsequent additional buffering (Connor and Nikolakopoulou, 1982). Simulations with the diffusion equation indicated that any of these three mechanisms operating with parameters within measured ranges could account for the decay of arsenazo III signals.

To begin to distinguish these possibilities, we have looked at the effects of depolarizations of different amplitude and duration, and com-

pared them to simulations based on different removal processes. For example, the highly saturated buffer model of Connor and Nikolakopoulou (1982) predicts that larger influxes of similar time courses will be removed much more slowly than smaller ones. This prediction was not confirmed (Smith, 1980). If cytoplasmic calcium is taken up into organelles, then the calcium entering during depolarizations of different amplitude or duration will be removed at the same rate. Since removal is slowed by binding to a buffer, saturation of the buffer with large pulses might even cause removal to occur more quickly. We have not observed this (R. S. Zucker, unpublished data). If calcium is removed primarily by surface membrane extrusion, then calcium entering during long pulses, which permit diffusion further from the membrane during influx, will be pumped out more slowly. We have observed such an effect (R. S. Zucker, unpublished data), suggesting an important role for surface pumps, which are present in *Aplysia* neurons (Satin, 1984).

D. Photodynamic Chelators for Control of Cytoplasmic Calcium

We have recently turned to nitr-5, a photolabile calcium chelator (Adams *et al.*, 1988), as a tool for manipulating intracellular calcium and studying calcium regulatory mechanisms. This substance may be loaded with calcium and injected into neurons as a high-affinity buffer. When exposed to a bright flash of ultraviolet light, nitr-5 is photolyzed to a low-affinity form, releasing "caged" calcium into cytoplasm (Tsien and Zucker, 1986).

Figure 3A illustrates the effect of a flash of light on the calcium concentration in a neuron filled with 10 m*M* nitr-5. Because light is absorbed from the front surface of the cell (facing the light) to the back surface, calcium is released nonuniformly, with the greatest increment occurring at the front surface. Subsequently, calcium and the various buffer species diffuse until equilibrium is reached. In order to estimate the time course of surface calcium concentration after a flash, we developed a model (Landò and Zucker, 1989) of nitr-5 photolysis, buffer equilibration, and diffusion that is diagrammed in Fig. 3B. In this model, we represent the cell as a cube, and calculate the light intensity as a function of distance from the front of the cell. Photolysis converts a percentage of nitr-5 proportional to the light intensity at each point. The buffer equations for the high- and low-affinity forms of nitr-5 and the native cell buffer are solved simultaneously at each point, and the resulting free calcium and free and calcium-bound forms of both nitr-5 species and the native cytoplasmic buffer are all allowed to diffuse independently between the front and back of the cell. The free calcium concentration at points on the surface may be related to calcium-dependent membrane currents.

In order to convert submembrane calcium activities to membrane

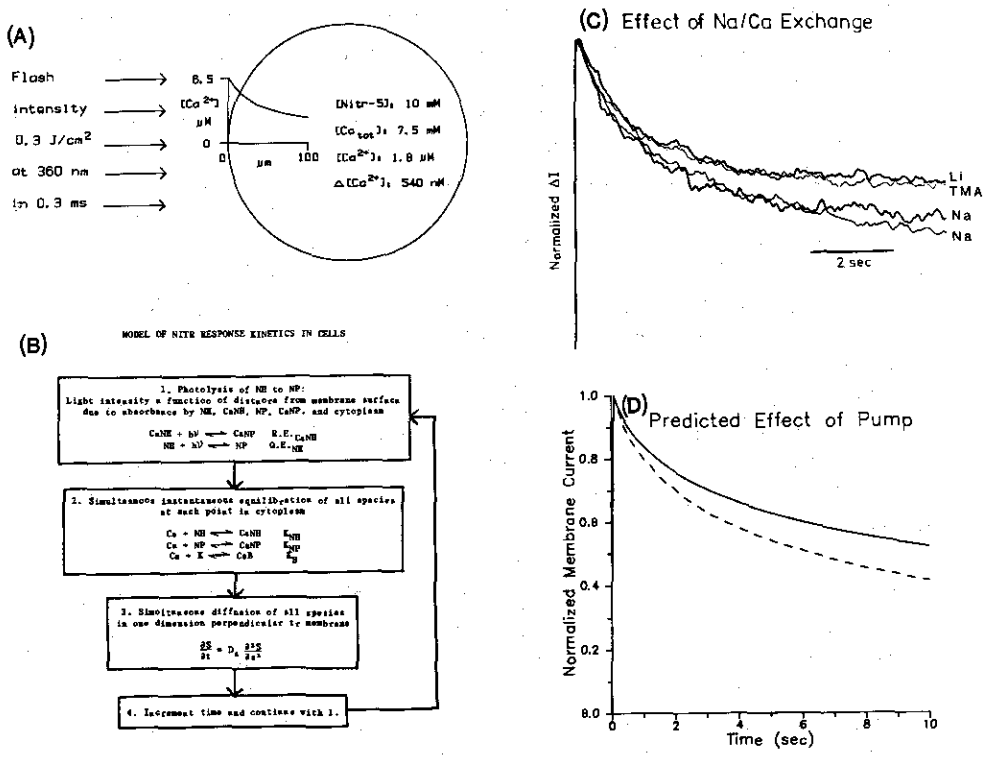


Figure 3.

(A) Effect of light flash on free calcium concentration in a neuron filled with nitr-5. The circle represents the cell, and the curve at its left surface shows the spatial gradient of free calcium at the moment of the flash, whose properties are indicated on the left. The concentrations of nitr-5, total calcium, and free calcium, and the increment in volume-average free calcium caused by the flash, are shown inside the cell. (B) Outline of computational model used to simulate the effect of a flash on calcium concentration in a cell filled with nitr-5. Abbreviations: NH, nitrobenzhydrol (the high-affinity form of nitr-5); NP, nitrosobenzophenone (the low-affinity form of nitr-5); B, native buffer; Q.E.$_s$, quantum efficiency of species s; K_s, dissociation constant of species s; D_s, diffusion constant of species s. (C) The $I_{K(Ca)}$ currents in a cell filled with 5m*M* nitr-5 and 3.75 m*M* calcium caused by 100 J of electrical energy discharged through a flashlamp focussed on the neuron. The traces marked "Na" were recorded in normal-sodium-containing seawater at the beginning and end of the experiment. The traces marked "Li" and "TMA" indicate responses in seawater with sodium replaced by lithium or tetramethylammonium, respectively, to block Na/Ca exchange. (D) Predicted flash responses in a neuron filled with nitr-5, with (dashed line) and without (solid line) a surface calcium extrusion pump (pump rate 0.001 cm/sec). [Adapted from Tsien and Zucker (1986) and Landò and Zucker, 1989.]

currents, we must know the relationship between calcium concentration and current level. We determined this by isolating $I_{K(Ca)}$ by recording it at the reversal potential for $I_{NS(Ca)}$ (about -25 mV), and by isolating $I_{NS(Ca)}$ by recording current at the reversal potential for $I_{K(Ca)}$ (about -75 mV). We could also block $I_{K(Ca)}$ with external TEA to measure $I_{NS(Ca)}$. When $I_{K(Ca)}$ was blocked with TEA and $I_{NS(Ca)}$ was eliminated by recording at its reversal potential, light evoked no response at all. When calcium current was isolated in a sodium-free, TEA-substituted medium, and enhanced with barium substituted for calcium, elevation of calcium by release from nitr-5 had little or no effect on subsequent calcium currents. Thus we were unable to elicit the calcium-dependent inactivation of calcium current by elevating calcium with nitr-5, and could not use this method to study the late outward current. This suggests that this effect of calcium on calcium channels occurs only at very high calcium levels, such as are likely to be present at calcium channel mouths (Chad and Eckert, 1984), or in regions of the cell shaded from the light, such as the axon hillock. We were, however, able to selectively activate $I_{K(Ca)}$ or $I_{NS(Ca)}$ using nitr-5.

By using increasing flash intensities, and causing larger and larger increments of calcium activity, we could determine the relationship between calcium concentration and membrane current for these two currents. Both of them depended linearly on the magnitude of a calcium concentration jump, even when the increment in calcium was several times the previous resting calcium level. Neither current showed saturation at calcium concentrations reaching 20 μM. Therefore, both membrane currents should be linearly related to the average submembrane calcium concentration.

E. Evidence for Na/Ca Extrusion

Figure 3C shows flash responses of $I_{K(Ca)}$ in a left upper quadrant bursting pacemaker neuron. The upper two traces show responses in media with lithium or tetramethylammonium in place of sodium to block Na/Ca exchange. These responses are similar to the predicted response time course (upper trace in Fig. 3D) using the model of Fig. 3B. When the cell was bathed in normal-sodium sea water, the nitr-5 response decayed somewhat more rapidly. When we added a surface membrane pump to our model, and adjusted it to remove calcium at a rate of 1 pmol/cm^2 sec per micromolar free calcium (Requena, 1983), we obtained the lower trace of Fig. 3D, which is similar to the experimental records in Fig. 3C. Apparently, Na/Ca exchange affects the decay of nitr-5 signals by removing some of the calcium formed near the surface. This and the preliminary result described in Section II,C suggest that Na/Ca exchange is one important mechanism involved in restoring calcium homeostasis following nervous activity. Whether ATP-dependent extrusion and uptake into or-

ganelles such as endoplasmic reticulum are also important for removing calcium during electrical activity in *Aplysia* neurons remains to be determined.

F. Kinetics of Calcium-Dependent Currents

We still need to explain the origin of the different decay rates of $I_{K(Ca)}$ and $I_{NS(Ca)}$ after a burst or depolarizing pulse. We initially thought these currents were activated by calcium with different stoichiometries, but the results mentioned in Section II,D and other results to be published soon (Landò and Zucker, 1989) indicate that both currents are activated by calcium with a simple first-order stoichiometry. We would then expect them to decay with the same time course as average submembrane calcium. In fact, Fig. 4A shows that the decay of $I_{NS(Ca)}$ after a pulse follows very closely the decay of average submembrane calcium concentration following influx predicted by the Smith and Zucker (1980) model (Section II,B).

Why does $I_{K(Ca)}$ (shown in Fig. 4B) decay faster? The answer is that the conductance underlying $I_{NS(Ca)}$ is not sensitive to voltage (Kramer and Zucker, 1985a; Swandulla and Lux, 1985), while that underlying $I_{K(Ca)}$ is highly voltage-dependent (Gorman and Thomas, 1980). Since calcium activates $I_{K(Ca)}$ linearly (Section II,D), we represented the interaction of calcium with the receptor activating $I_{K(Ca)}$ as a simple first-order binding reaction with voltage-dependent forward and backward rate constants. These rate constants can be determined from measurements of the relaxation time constant of $I_{K(Ca)}$ in response to voltage steps, and steady-state conductance versus voltage measurements. We used nitr-5 photolysis to activate $I_{K(Ca)}$, and measured the responses to a voltage step at potentials near the reversal potential for $I_{NS(Ca)}$. The responses to a voltage step before a light flash were subtracted from those after a flash to obtain relaxations of $I_{K(Ca)}$ to steady-state levels caused by an increment of calcium concentration and change in voltage. These data allowed us to calculate the rate constants for our $I_{K(Ca)}$ reaction scheme (Landò and Zucker, 1989). From this we could predict how $I_{K(Ca)}$ should decay after a depolarizing pulse in a cell containing nitr-5, where submembrane calcium decays according to Fig. 4A. The $I_{K(Ca)}$ experiences both voltage- and calcium-dependent relaxations, and decays somewhat faster than $I_{NS(Ca)}$. Our model fits experimental observations (Fig. 4B). The sum of early outward and slow inward currents gives the characteristic outward–inward tail current sequence normally observed after a voltage-clamp depolarization (Fig. 4C).

To summarize Section II, we developed a model of calcium diffusing radially inward from the cell membrane, which includes provisions for influx, binding, and extrusion at the surface or uptake into organelles. This model accounts for the time courses of aequorin and arsenazo III

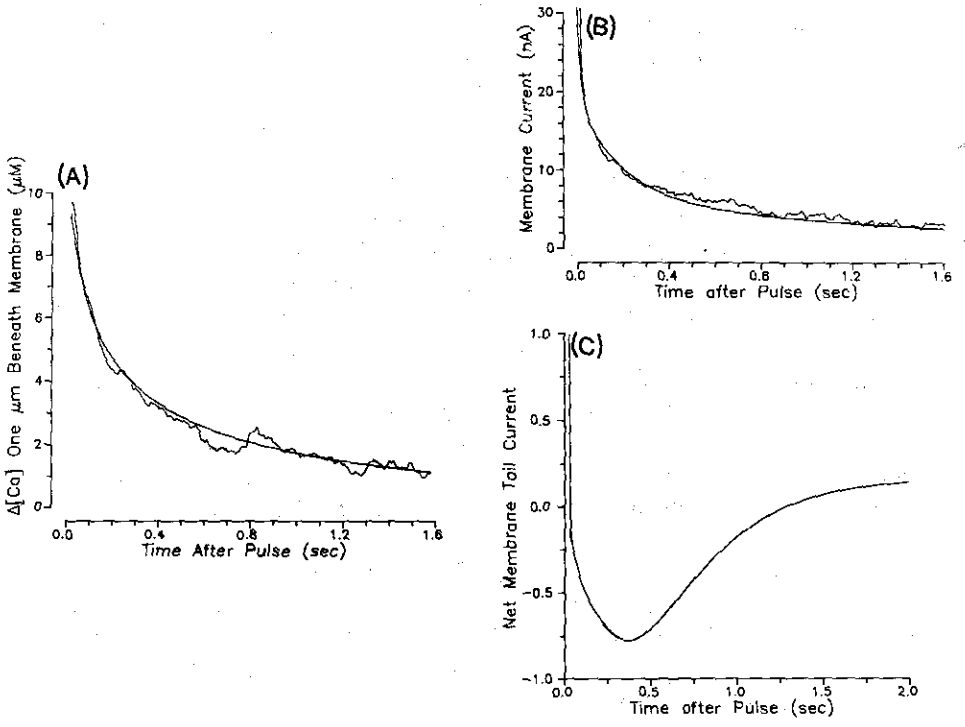


Figure 4.

(A) Predicted decay of submembrane calcium concentration (smooth curve) for a −10-mV, 100-msec pulse, using the Smith and Zucker (1980) model. The noisy curve is normalized $I_{NS(Ca)}$ tail current (inward current plotted upward) following a −10-mV, 100-msec pulse and returning to −77 mV to eliminate potassium currents. The bath also contained 50 m*M* TEA. (B) Predicted decay of $I_{K(Ca)}$ (smooth curve) for a −10-mV, 100-msec pulse, based on the change in submembrane calcium shown in (A) and measurements of the calcium- and voltage dependence of $I_{K(Ca)}$. The noisy curve is normalized $I_{K(Ca)}$ following such a pulse, obtained as the difference current before and after adding 3 m*M* TEA to the bath. (C) Expected net tail current obtained by scaling and mixing predicted currents from (A) and (B). [From Landò and Zucker, 1989].

signals following depolarizing pulses, and the time course of $I_{NS(Ca)}$ after a depolarization. Preliminary results suggest that surface extrusion of calcium entering during electrical activity is more important than internal sequestration into organelles. Nitr-5 experiments indicate that both $I_{K(Ca)}$ and $I_{NS(Ca)}$ depend linearly on submembrane calcium concentration. A model of nitr-5 and calcium diffusion in cells explained the time course of currents evoked by release of "caged" calcium from nitr-5, and also simulated the effect of Na/Ca exchange in speeding the decay of flash responses. Finally, we found that the voltage-dependent relaxation of

$I_{K(Ca)}$ following a depolarization is responsible for its decaying faster than $I_{NS(Ca)}$ and submembrane calcium activity, leading to the characteristic sequence of early outward and slow inward currents following a burst. The reason for the very slow kinetics of late outward current, caused by calcium-dependent inactivation of the calcium pacemaker current, remains to be determined.

III. Presynaptic Calcium and Transmitter Release

My laboratory has also been interested in the effect of presynaptic calcium on transmitter release. One synapse that is amenable to the sorts of biophysical manipulations used in *Aplysia* cell bodies is the giant synapse in the squid stellate ganglion. A great deal is known about this unusually large glutaminergic contact, and it has been a popular preparation in synaptic physiology since its discovery by J. Z. Young (Zucker, 1989).

A. Experiments on the Squid Giant Synapse

My experimental work on this synapse focussed on the role of calcium in synaptic facilitation. During repeated action potentials, successive excitatory postsynaptic potentials (EPSPs) grow as phasic transmitter release by individual action potentials increases (Charlton and Bittner, 1978a). Not due to changes in action potentials, afterpotentials, or calcium currents (Charlton and Bittner, 1978b; Charlton *et al.*, 1982), facilitation was proposed to be a consequence of residual calcium in nerve terminals from prior activity summating with calcium influx during subsequent spikes to evoke more release of transmitter, which is nonlinearly dependent on calcium activity (Katz and Miledi, 1968; Miledi and Thies, 1971). We found that raising presynaptic calcium does facilitate release by spikes, and that residual calcium can be detected in nerve terminals following activity (Charlton *et al.*, 1982).

Just as in *Aplysia* cell bodies, we observed several different calcium-dependent processes and measurements with very different time courses. Presynaptic calcium is supposed to activate phasic transmission directly (Katz, 1969), and this process lasts only 1–2 msec. Yet residual calcium is supposed to cause facilitation, which lasts tens to hundreds of milliseconds. And our measurement of presynaptic calcium activity using arsenazo III microspectrophotometry showed free calcium concentration to remain high for seconds after electrical activity. How can these differences in time course of calcium-dependent events be reconciled?

First of all, again just as in *Aplysia* cell bodies, arsenazo III absorbance reports average cytoplasmic calcium activity, not its concentration

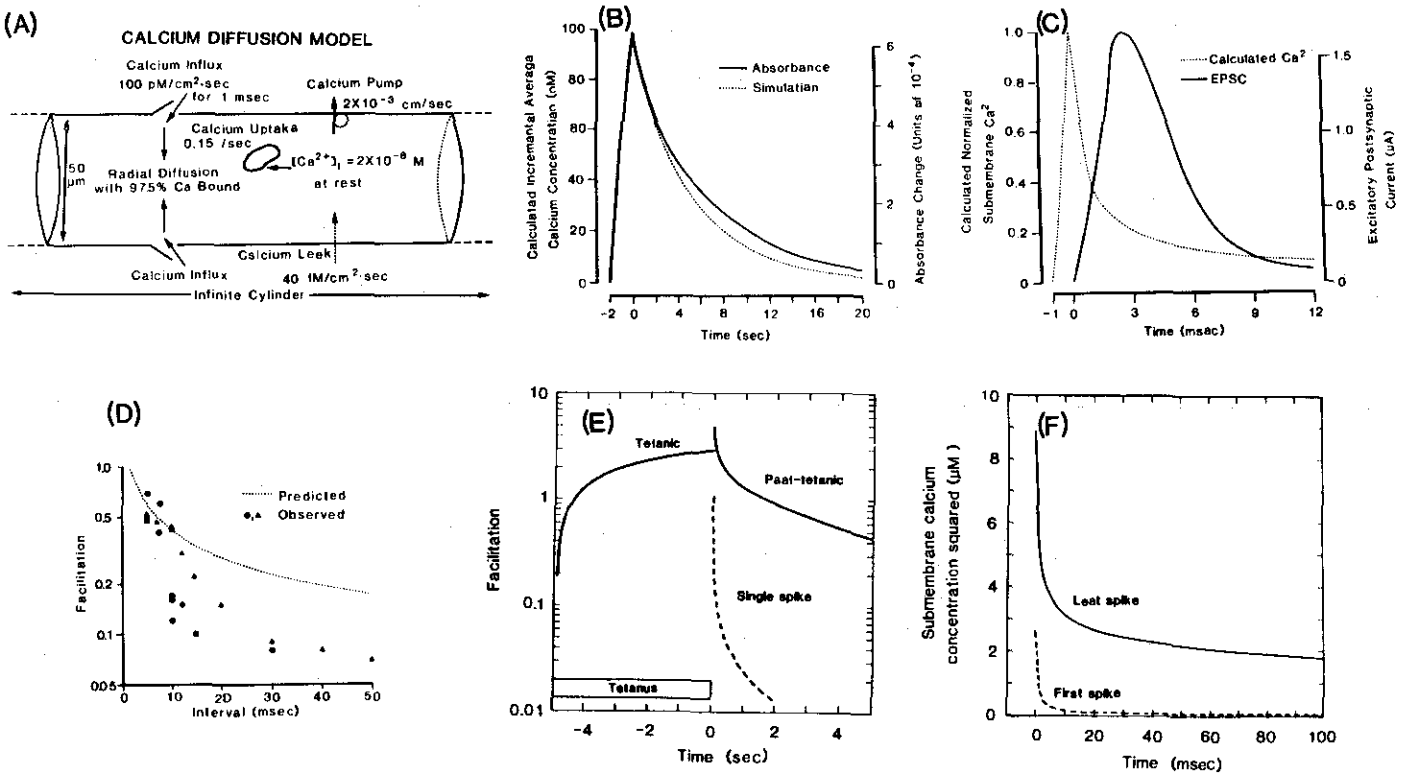
at membrane sites where transmitter is released. The brief synaptic delay (< 0.5 msec) allows calcium to diffuse with binding only about 50 nm from channel mouths before causing transmitter release (Fogelson and Zucker, 1985). Arsenazo III absorbance does not report these local high calcium concentrations responsible for triggering neurosecretion, and this explains the huge difference in time courses of phasic release and presynaptic calcium measurements. As to facilitation, the highly nonlinear relation between transmitter release and calcium would cause a small residual amount of calcium at release sites to have very little effect on transmitter release, which will therefore terminate quickly as residual calcium decays. But its summation with peak calcium in the next spike can greatly facilitate release, as a consequence of the same nonlinearity.

B. One-Dimensional Model of Presynaptic Calcium Diffusion

Although these arguments are qualitatively plausible, it must be shown that the time courses of phasic transmitter release, synaptic facilitation, and presynaptic arsenazo III absorbance changes can be explained quantitatively by what we expect to happen to calcium at presynaptic terminals. As a first approximation, the nerve terminal can be treated as a cylinder with calcium entering uniformly at the surface and diffusing radially inward. We solved the diffusion equation in cylindrical coordinates (Zucker and Stockbridge, 1983), with influx determined by voltage-clamp experiments, and cytoplasmic buffering, uptake into organelles, and extrusion at the surface included with parameters set within the range of reported values (Fig. 5A). Simulations with this model provided a good fit to the arsenazo III absorbance signal (Fig. 5B), and predicted that submembrane calcium would decay rapidly enough to account for phasic transmitter release even with only a square law relating release to presynaptic calcium (Fig. 5C). Facilitation expressed by a second spike

Figure 5.

The one-dimensional model of presynaptic calcium diffusion. (A) Schematic of the model used to predict the effects of spikes on presynaptic calcium concentration. (B) Predicted average calcium concentration change, and observed absorbance change (from Charlton *et al.*, 1982) from a presynaptic terminal injected with arsenazo III, in response to 66 spikes at 33 Hz, ending at time 0. (C) Predicted square of submembrane calcium concentration and observed excitatory postsynaptic current (EPSC). Transmitter release should be somewhat faster than the EPSC, and somewhat slower than the submembrane calcium raised to a power reflecting calcium cooperativity in releasing transmitter. (D) Predicted facilitation of a second response as a function of the interval between it and the first response, and data from two preparations (from Charlton and Bittner, 1978a). (E) Predicted facilitation following a single spike and during and after a 5-sec tetanus at 20 Hz. (F) Predicted square of submembrane calcium for the first and last spikes in the 5-sec train. Transmitter release should be similar to (but somewhat slower than) these curves. [Adapted from Zucker (1987b).]


(A)
CALCIUM DIFFUSION MODEL
Calcium Influx
100 pM/cm²-sec
for 1 msec
Calcium Pump
2X10⁻³ cm/sec
Calcium Uptake
0.15 /sec
[Ca²⁺]i =2X10⁻⁸ M
at rest
50 µm
Radial Diffusion
with 97.5% Ca Bound
Calcium Leek
Calcium Influx
40 1M/cm² sec
Infinite Cylinder
(B)
Absorbance
Simulation
Calculated Incremental Average Calcium Concentration (nM)
Absorbance Change (Units of 10⁻⁴)
Time (sec)
(C)
Calculated Ca²
EPSC
Calculated Normalized Submembrane Ca²
Excitatory Postsynaptic Current (µA)
Time (msec)
(D)
Predicted
Observed
Facilitation
Interval (msec)
(E)
Tetanic
Post-tetanic
Single spike
Tetanus
Facilitation
Time (sec)
(F)
Last spike
First spike
Submembrane calcium concentration squared (µM²)
Time (msec)

was also predicted to be roughly similar to measured values (Fig. 5D). However, when the model was driven by repeated calcium influxes to simulate a tetanus of 100 spikes at 20 Hz (Fogelson and Zucker, 1985), a serious problem developed. Facilitation accumulated during the tetanus and decayed afterward with fast and slow components (Fig. 5E), as observed experimentally at other preparations (Magleby and Zengel, 1982). But the simulations predicted that late spikes in the train would continue to release transmitter at high rates for more than 100 msec (Fig. 5E), clearly contradicting what the synapse actually does.

C. Three-Dimensional Model of Presynaptic Calcium Diffusion

The trouble with the one-dimensional calcium diffusion model is that posttetanic residual calcium is too large a fraction of peak calcium at release sites, and decays too slowly (Fig. 6B). This leads to the critical error of transmitter release lasting a long time for late spikes in a tetanus (Fig. 5F), and to a predicted facilitation after one spike declining too slowly (Fig. 5D). These problems could be reduced somewhat by increasing the cooperativity of calcium action in releasing transmitter, consistent with accumulating experimental evidence (Katz and Miledi, 1970; Lester, 1970; Charlton *et al.*, 1982; Augustine *et al.*, 1985; Augustine and Charlton, 1986). But then our simulations predicted a facilitation that was much larger than observed. Somehow, our simulations were indicating too high a residual calcium compared to the peak at an action potential, or, equivalently, too low a peak calcium at each spike.

This is precisely the error that should occur by representing calcium entry as a spatially uniform flux across the membrane. Calcium actually enters through discrete calcium channels and releases transmitter in their immediate vicinity. Each calcium channel will be surrounded by a cloud of calcium ions, or a "calcium domain," at the end of an action potential and when transmitter release occurs (Fig. 6A). Only after release does each calcium domain collapse by diffusion in three dimensions, with subsequent one-dimensional radial diffusion of residual calcium. Thus a more realistic model would simulate calcium entering at discrete points and diffusing away from each channel in three dimensions into the axoplasm. This would naturally predict larger local calcium concentration peaks that would dissipate more rapidly than in the one-dimensional model (Fig. 6B).

Such a model was constructed by considering calcium diffusion into the presynaptic terminal from arrays of calcium channels (Fogelson and Zucker, 1985). The model included the usual provisions for calcium binding and extrusion. Transmitter release was considered to occur approximately 50 nm from calcium channel mouths, limited on the low side by requiring exocytosis not to obliterate calcium channels, and on

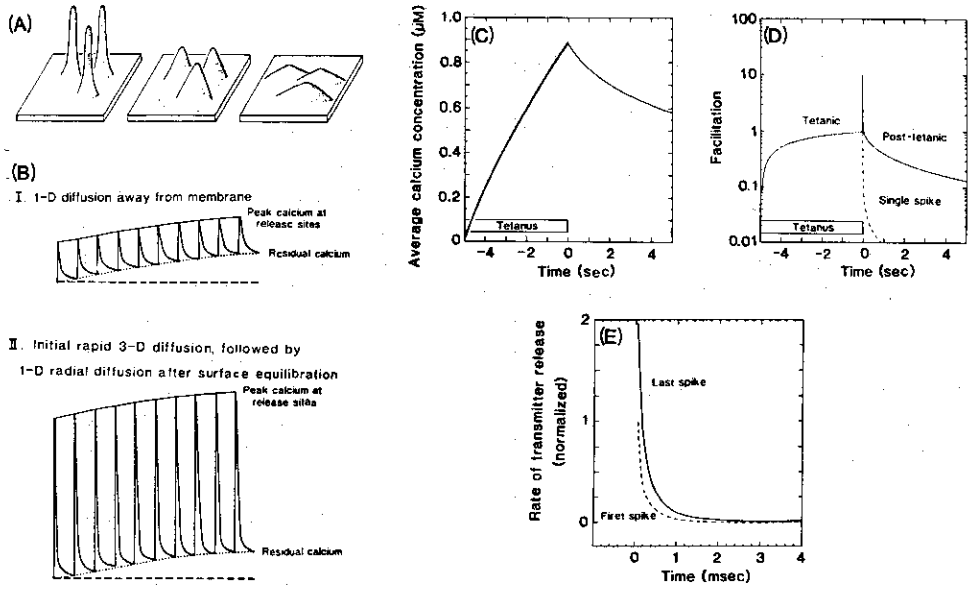


Figure 6.

The three-dimensional model of presynaptic calcium diffusion. (A) Schematic drawing of the collapse of calcium domains surrounding single calcium channel mouths inside the nerve terminal at the end of the spike and short times later. (B) Comparison of the relative calcium magnitudes of peaks of calcium activity during spikes, and residual calcium activity after spikes, for one- and three-dimensional models. (C) Predicted average free calcium concentration during and after a 5-sec 20 Hz tetanus. (D) Predicted facilitation evoked by one spike or the tetanus. (E) Predicted rate of transmitter release, if this is assumed to follow the fifth power of calcium at release sites 50 nm from calcium channel mouths, for the first and last spike in the train. [Adapted from Zucker (1987b).]

the high side by the time available for calcium diffusion during the synaptic delay. Various arrays of calcium channels were considered, but the one most successful in fitting the data had the channels clustered into active zones, as suggested by ultrastructural studies (Pumplin *et al.*, 1981). Simulations with this model successfully predicted not only the changes in arsenazo absorbance in a tetanus (Fig. 6C), and tetanic and posttetanic facilitation (Fig. 6D), but also the rapid termination of transmitter release after late spikes in a tetanus (Fig. 6E). The latter result in particular required calcium channels to be clustered, leading to two phases of three-dimensional diffusion, away from single channel mouths and then away from each cluster. Spreading channels uniformly on the presynaptic surface led to only one phase of three-dimensional diffusion (away from each calcium channel), and transmitter release still lasted too long after a tetanus.

D. Voltage Dependence of Transmitter Release

Llinás *et al.* (1981) reported a curious characteristic in the relation between transmitter release and presynaptic calcium current at the squid giant synapse. As larger depolarizations are used, calcium influx and transmitter release both increase, and then decline as the calcium equilibrium potential is approached. One would expect equal currents on the rising and falling limbs of the calcium current versus voltage curve to release equal amounts of transmitter. Instead, Llinás *et al.* found that the current caused by the larger depolarization always evoked more transmitter release. Even when calcium tail currents were eliminated by considering only calcium current and release before the end of the pulse, some hysteresis usually persisted in these measurements (Smith *et al.*, 1985; Augustine *et al.*, 1985). To account for this result, Llinás *et al.* (1981) proposed that some of the steps involved in calcium-dependent exocytosis were voltage-dependent.

However, consideration of the effects of voltage on calcium domains leads to a different conclusion (Simon and Llinás, 1985; Zucker and Fogelson, 1986). Large depolarizations open more calcium channels than small pulses, with less calcium entering each channel as the calcium equilibrium potential is approached. This means that synaptic vesicles are exposed to calcium entering several calcium channels. This increases the overlap of adjacent calcium domains: multiple calcium-sensitive sites on vesicles are more likely to be activated, and the peak calcium concentration at release sites will be higher due to domain overlap, despite the fact that less calcium enters each channel (Fig. 7A and B). This leads immediately to a hysteresis in the relationship between transmitter release and calcium current, depending on whether large or small depolarizations are used (Fig. 7C). Calcium diffusion models predict this form of hysteresis without the need to invoke any voltage-dependent steps in neurosecretion.

This result parallels recent work on neuromuscular junctions, in which a number of experiments were interpreted as indicating a voltage dependence of transmitter release (reviewed in Parnas and Parnas, 1986). We have shown that all of these experiments can be explained by a conventional calcium hypothesis of transmitter release, with no need to invoke voltage-dependent steps in the release process (Landò *et al.*, 1986; Zucker and Landò, 1986; Zucker *et al.*, 1986; Zucker, 1987a).

To summarize Section III, we have been able to measure changes in presynaptic calcium activity during synaptic transmission with calcium-sensitive dyes. Theoretical simulations of calcium movements allow us to relate these measurements to calcium-dependent processes such as transmitter release and synaptic facilitation. Improved models of calcium diffusion explain an otherwise surprising relationship between calcium influx and transmitter release at low and high voltages.

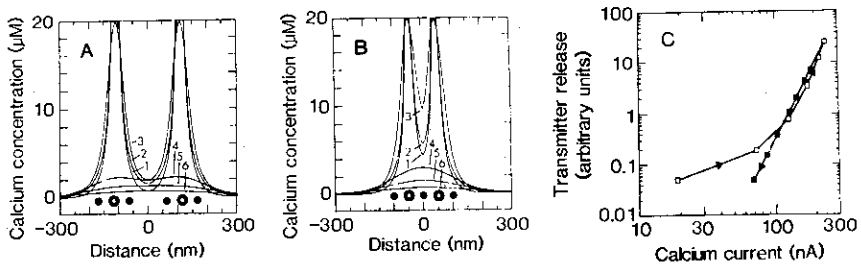


Figure 7.

(A) Predicted spatial profile of free calcium concentration when the average spacing of open calcium channels is 218 nm for a 2.5 msec depolarization to −20 mV. Open circles denote open calcium channels, and closed circles nearby release sites. The single-channel current is 490 fA. Traces 1–6 are for 1.0, 2.0, 2.4, 3.4, 4.4, and 6.5 msec after the beginning of the pulse. Calcium domains show very little overlap. (B) A similar plot for a 2.5-msec depolarization to 0 mV, where open calcium channels are 101 nm apart and the single-channel current is 350 fA. Calcium domains now display significant overlap. (C) Predicted relationship between peak rate of transmitter release and macroscopic presynaptic calcium current at the end of 2.5-msec pulses. Transmitter release is proportional to the number of open calcium channels times calcium concentration 50 nm away raised to the fifth power. Calcium current is proprotional to the number of open calcium channels times the single-channel current. Open squares represent pulse potentials of −25 to O mV (when all channels are open), and filled squares represent pulse potentials from +10 to +60 mV. The arrowheads point in the direction of increasing pulse potential. [Adapted from Zucker and Fogelson (1986).]

IV. Conclusion

Calcium is at the center of a neuron's electrical activity and means of communication. Understanding neural function demands close attention to the movements of calcium within cells. Recent technological innovation permits the measurement and control of intracellular calcium with a precision not even dreamed of 10 years ago. While designing experiments based on new technology, we must constantly revise and refine our formulations of cellular mechanisms under control of calcium. Theoretical modeling and computer simulation have provided as much insight into neural function as have new experimental results. Together the two approaches provide a symbiotic power that dwarfs what can be accomplished with either alone. And fortunately, theoretical and experimental developments seem if anything to be accelerating, promising a good deal more progress and excitement to come in the near future.

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