CALCIUM REGULATION OF ION CHANNELS IN NEURONS

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Intracellular calcium modulates the activity of a number of voltage-dependent ion channels in neurons. This modulation is often functionally important, and can influence the responsiveness of cells to natural inputs. In extreme cases, the regulation of ion channels by intracellular calcium can even determine the pattern of electrical activity of neurons. In this review, I will summarize the role of calcium in electrogenesis in bursting molluscan neurons, and in responses to synaptic inputs in rat hippocampal cells and frog sympathetic ganglion neurons.

ENDOGENOUSLY BURSTING NEURONS

There are some molluscan peptidergic neurosecretory neurons which display a remarkable form of endogenous electrical activity. Regularly recurring bursts of spikes separated by periods of silence characterize the firing patterns of these cells. When such cells, for example the left upper quadrant bursters on the dorsal surface of the Aplysia abdominal ganglion, are excised and cultured in isolation, they continue to generate this characteristic pattern of activity in the complete absence of synaptic or hormonal influence.

Each burst is shaped by the interaction of a number of calcium-dependent currents. The burst is triggered by a weak calcium current, activated at resting potentials (-40 to -50 mV), which gradually charges the cell capacitance and depolarizes the cell to threshold (Gorman et al., 1982). Each spike in the burst is followed by a sequence of calcium-dependent afterpotentials which determine the subsequent characteristics of the burst (Adams, 1985). A rapidly decaying (50-100 ms) hyperpolarization is caused by calcium-activated potassium channels (Kramer & Zucker, 1985b). This is followed by a depolarization lasting about a second, which is due to

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calcium-activated nonspecific cation channels (Kramer & Zucker, 1985a; Swandulla & Lux, 1985). The interaction of these two currents determines the frequency of spikes in a burst. Each spike is triggered by the after-depolarization, which reaches a peak only after the more rapidly decaying hyperpolarization has dissipated. Following the depolarizing phase is a very slowly decaying hyperpolarization. This potential is very small after a single spike, but it accumulates with successive spikes in a burst until it is large enough to prevent the depolarizing after-potential following the last spike from reaching threshold. This terminates the burst, and the cell remains silent until this hyperpolarization disappears and the pacemaking calcium current again depolarizes the cell to initiate the next burst.

All three currents, the early outward, inward, and late outward current, are triggered by calcium entry during spikes or depolarizing pulses. They disappear in a calcium-free medium, or when calcium entry is blocked with divalent cations such as manganese and cobalt, or when intracellular calcium accumulation is blunted by EGTA injection. All three display a bell-shaped dependence on pulse potential which resembles the calcium current I-V curve.

THREE CALCIUM-DEPENDENT CURRENTS

The channels underlying the initial hyperpolarization are very sensitive to extracellular TEA, and the current through them reverses at the potassium equilibrium potential, even when the potassium concentration in the medium is raised. This current adds to that carried by the voltage-dependent potassium channels (which are much less TEA-sensitive) and helps to terminate each spike (Kramer & Zucker, 1985b). Simultaneous with this current, and maximal at the end of a spike or depolarizing pulse, is another current which can be seen in isolation when the first current is blocked with TEA. It is carried by sodium, calcium, and potassium ions, reverses at about -20 mV, and lasts about a second (Kramer & Zucker, 1985a; Swandulla & Lux, 1985). It normally appears to start after a delay, but this is due only to its being obscured by the early outward potassium current, which decays somewhat more rapidly. For about 50-100 ms after a spike, the early potassium current dominates, and so a net hyperpolarization is observed. Afterwards, the inward current dominates, so a net depolarization occurs,

which normally triggers the next spike in the burst. Finally, a late hyperpolarization is left after the other two currents have decayed. This current is increased by a depolarizing postpulse and reduced on hyperpolarization. If a postpulse is sufficiently hyperpolarized, it is blocked but not reversed. It is not affected by potassium channel blockers, nor by changes in potassium concentration. Since it shows a voltage dependence (on the voltage after the pulse used to elicit it) similar to the pacemaker calcium current, it appeared that this could be due to a calcium-dependent inactivation of this current (Adams & Levitan, 1985; Kramer & Zucker, 1985b). This was confirmed by showing that the calcium current, which can be activated by small depolarizing pulses or deactivated by small hyperpolarizing pulses, is blocked by pulses in the same way as the late outward current is activated. This block of calcium current is maximal at the peak of the late hyperpolarization, and recovers with the same time course that the late hyperpolarization decays. As calcium accumulates in a burst and reduces the size of the pacemaker current, eventually a point is reaches where it is too small to depolarize the cell (i.e., the net current turns outward as the leak exceeds the pacemaker current). This results in a regenerative hyperpolarization of the cell, as the calcium current is highly voltage-dependent in the region around -40 mV.

Thus calcium entry during action potentials determines the timing of successive spikes by its effects on calcium-activated potassium and nonspecific cation channels, and terminates bursts by inactivating the calcium current which initiates bursts. The time of the next burst is determined by the recovery of this calcium current from calcium-dependent inactivation. Both the timing of spikes within a burst, and the timing of bursts, are determined by the kinetics of calcium accumulation and dissipation, and the kinetics of the currents which are affected by calcium.

DIFFERENCES IN KINETICS

The question immediately arises: Why do the three calcium-dependent effects (activation of potassium current, activation of nonspecific cation current, and inactivation of calcium current) all decay at such different rates (0.1 sec, 1 sec, and 10 sec respectively)? Shouldn't they all recover at the same rate after a calcium influx during a depolarization, as calcium

diffuses away from the membrane and is pumped out of the cytoplasm? The different recovery kinetics of these calcium-dependent processes are crucial in generating the complex pattern of electrical activity in these cells. Thus, we can only understand this electrogenesis if we can understand the reasons for these differences in kinetics.

Our original idea was that these currents have very different calcium sensitivities and dependencies. Perhaps nonspecific cation currents are activated linearly by calcium, but potassium channels require cooperative calcium action, as in the calcium-activated potassium channels in transverse tubules of muscle. Then the potassium current would decay faster than the nonspecific cation current as submembrane calcium dropped. And perhaps inactivation of calcium channels is very sensitive to small calcium changes and is saturated after a burst; then it might recover very slowly, and only after calcium is nearly fully extruded from cytoplasm.

CAGED CALCIUM AS A PROBE OF CALCIUM-ACTIVATED CURRENTS

To test these possibilites, we needed a method to quantitatively characterize the calcium-dependence of these three currents. For this purpose, we turned to photosensitive calcium chelators, or 'caged calcium' compounds, which can be used to rapidly and quantitatively change submembrane calcium levels and activate or inactivate ion channels (Landò & Zucker, 1989). For most experiments, we used nitr-5, a high-affinity calcium-binding nitrobenzhydrol-substitute member of the BAPTA family which can be photolyzed by ultraviolet light to a low-affinity form (Tsien & Zucker, 1986; Adams et al. 1988). Knowing the quantum efficiency and light intensity of a calibrated light source, it is possible to photolyze a desired fraction of nitr-5, and calculate the free calcium concentration from the mixture of high- and low-affinity chelators present after light exposure. A brief intense flash can be used to rapidly step the calcium concentration from a known level (set initially by the degree of calcium loading of the high-affinity buffer) to another level (determined by the proportion of nitr-5 photolyzed by the light).

The situation in cells is somewhat more complicated, because as the photolyzing light passes through a cell, it is absorbed by the cytoplasm and by the nitr-5 itself, so that it becomes dimmer toward the back surface of the cell away from the light. It is possible, however, to calculate the

calcium concentration changes at the front surface of the cell, or for that matter at any distance from the front surface, and also to calculate the volume average calcium concentration change in calcium to a flash of light. To confirm these calculations, we predicted what sequences of flashes of different light intensity should do to cells filled with nitr-5 of various degrees of calcium loading, and filled with various concentrations of nitr-5/calcium mixtures. We compared our calculations to measurements of calcium concentration changes using the metallochromic indicator arsenazo III, beginning with micro-cuvettes similar in size to the bursting molluscan neurons we were studying. We confirmed quantitatively that brighter flashes release more calcium, that more heavily loaded nitr-5 releases more calcium, and that cells filled with more nitr-5 release *less* calcium. The latter effect is due to the greater absorbance of light by cells filled with more chelator, and so a dimmer average light intensity in the cell or (cuvette). We found that successive flashes cause larger calcium increments, because as the buffer is progressively photolyzed, the remaining buffer is more and more heavily loaded. Once it is fully loaded, successive flashes release smaller amounts of calcium as the amount of remaining nitr-5 drops. Measurements of calcium concentration changes in real neurons also matched our predictions. Now, armed with a method for quantitatively and rapidly changing intracellular calcium, we were ready to study its effects on ion channels.

We first looked at potassium and nonspecific cation currents elicited by flashes of varying intensity. We found that both currents depended linearly on the change in calcium concentration. This was true even when the calcium jump was much larger than the initial level, when nonlinearities would be clearly evident. Neither current showed signs of cooperativity or saturation, with calcium jumps from 0.1 to 20 μM. The dependence on calcium of both currents was linear over a range of voltages for each current (-10 to -30 mV for the potassium current; -50 to -77 mV for the nonspecific cation current). A similar linear dependence of potassium current on calcium in sympathetic neurons (Gurney et al., 1987). Thus, a difference in calcium cooperativity between the channels was not evident.

We next looked at the kinetics of the responses to light flashes. The two currents rose rapidly to a peak and declined to intermediate levels at identical rates, as predicted by simulations of calcium diffusion from the front of the cell and calculations of the time course of the average submembrane calcium concentration. The simulations also predicted accu-

rately the effect of blocking sodium/calcium exchange (in a sodium-free medium) on the recovery of calcium-dependent current after a flash. These flash-evoked currents contrasted sharply to those following a depolarization, where the potassium current always decayed faster than the nonspecific cation current.

DIFFERENT VOLTAGE-DEPENDENCIES

The key to understanding this dilemma is in the different voltage-dependencies of the two calcium-activated currents. The nonspecific cation current has a linear I-V curve, i.e., the underlying conductance is voltage-independent (Kramer & Zucker, 1985a; Swandulla & Lux, 1985). This current merely senses the average submembrane calcium concentration. Its decay after a depolarization is well described by simulations of radial diffusion of calcium away from the membrane after entering through calcium channels during a pulse. The potassium current, on the contrary, is quite sensitive to voltage, with a much larger conductance as the membrane is depolarized (Gorman & Thomas, 1980). Following a depolarization, this current therefore undergoes a voltage-dependent relaxation, in addition to declining as the average submembrane calcium concentration drops. We modeled the potassium channel as a simple two state channel whose transitions between open and closed state depend on calcium and voltage. The currents elicited by calcium and by voltage steps permitted estimation of the parameters of this model. Simulations of the response of such channels to the calcium concentration changes, averaged over the whole membrane surface of the cell, matched the more rapid decay of potassium current after a depolarization. Thus the differences in voltage sensitivity of these channels is responsible for the sequence of early afterhyperpolarization and depolarization that determines the frequency of spikes in a burst.

CALCIUM-DEPENDENT INACTIVATION

Recently, we have turned our attention to the third effect of calcium, the inactivation of calcium current. Our original idea that this current was very sensitive to small changes in calcium concentration was quickly contradicted by our inability to inactivate calcium currents with calcium

released by photolysis of nitr-5. In order to achieve higher calcium levels, we turned to DM-nitrophen, a caged calcium compound based on EDTA. This substance has more a complicated chemistry than nitr-5 (Delaney & Zucker, 1990). It binds both calcium and magnesium at the same site, and both are released on photolysis. The initial calcium concentration and the final level after photolysis both depend on the relative concentrations of nitrophen and magnesium in the cell, and even on the magnesium bound to ATP. The photolysis products do not bind calcium at all, which then is regulated by the cell's native buffers. Nitrophen is also a slow calcium buffer, so that calcium released by partial photolysis of nitrophen remains free for a few ms before equilibrating with remaining nitrophen, and some remains free even longer, for the tens of ms it takes to displace magnesium from nitrophen. These complications make accurate prediction of the calcium jump on photolysis very difficult, but it may be estimated that a flash will transiently elevate the submembrane calcium to a large fraction of a millimolar, and that the steady-state increase may reach tens of micromolar.

CAGED CHELATORS AS PROBES OF CALCIUM-DEPENDENT CURRENTS

We have found that sudden elevation of calcium during a depolarizing pulse rapidly inactivates the calcium current flowing during the pulse. Similar results have been described recently in snail neurons (Johnson & Byerly, 1990). More to the point is the effect of suddenly reducing the calcium concentration. This has been achieved by use of the substance diazo-4, a diazoacetyl-substituted derivative of BAPTA (Adams et al., 1989). Photolysis of diazo-4 increases its calcium affinity 1,600 times, and a flash delivered during a depolarizing pulse reduces the submembrane calcium at the front surface to less than 1/10 its level prior to the flash. Despite this sudden drop in calcium concentration, there is little or no increase in the calcium current (Fryer & Zucker, 1990). Instead, there is only a reduction in the rate of subsequent inactivation, as is expected from the sudden increase in cytoplasmic buffer capacity. When barium is used as the charge carrier, photolysis of nitrophen has no effect at all on the current, since now there is no calcium accumulation occurring and what little inactivation occurs is apparently due to voltage. Thus we are able to reduce progressive inactivation of calcium current by reducing the accumulation of intracellular calcium, but we are unable to effect a rapid

recovery from inactivation is unaffected by a sudden fall in calcium. It appears that some other calcium-independent process is responsible for recovery from inactivation, and that this process determines the slow recovery of the pacemaker current, and the long interval between bursts. It has been suggested that calcium inactivates calcium channels by a calcium dependent phosphatase, and that recovery from inactivation depends on cyclic-AMP dependent rephosphorylation (Eckert & Chad, 1984), but we have no evidence for or against this hypothesis at this point.

THE SLOW AFTERHYPERPOLARIZATION IN HIPPOCAMPAL NEURONS

Slow afterhyperpolarizations take many forms in neurons. For example, in bursting neurons of other mollusks, bursts may be terminated by a calcium-activated, voltage-independent, and TEA-insensitive potassium current (Thompson et al., 1986; Müller et al., 1989). We have recently looked at a similar current in rat hippocampal neurons. These cells display early and late post-spike hyperpolarizations, both of which are due to calcium-activated potassium channels (Lancaster & Adams, 1986; Lancaster & Nicoll, 1987). The early component is TEA-sensitive and voltage-dependent, while the late component is neither, but is blocked by noradrenaline or cyclic-AMP derivatives and carbachol. A curious feature of the late current is that it rises slowly to a peak, reached about 0.5 s after the end of a train of spikes. This delay persists when the current is reversed by changing the potassium concentration, so it is not due to the decay of some other early inward current obscuring the beginning of this outward current. The outward current is largely responsible for spike-frequency adaptation to prolonged inputs in these neurons.

We wished to determine the origin of the delay in the current responsible for the late hyperpolarization. One possibility is that it is an artifact of inadequate voltage-clamp control of distal dendrites, although this would not account for the delay of the late afterhyperpolarization in current clamp following spikes, nor could it account for a delay in voltage-clamp as long as 0.75 s in some cells. Another possibility is that calcium activates the current through a cascade of intermediate steps, one of which imposes the delay. In that case, activation of the current by a sudden rise of calcium released by photolysis of intracellular caged calcium would preserve the delay. However, we have found that photolysis of DM-

nitrophen loaded with calcium activates a hyperpolarization without substantial delay. The peak is reached within 50 ms, and this is accounted for entirely by the membrane time constant. In these experiments, the flash-evoked current was sensitive to noradrenaline, so the calcium released from nitrophen apparently activated the channels responsible for the late afterhyperpolarization. We now think that the slow rise of this current after spikes or depolarizing pulses may be due to a geometrical separation between the calcium channels that admit calcium to the cell and the potassium channels that respond to the calcium.

CALCIUM MODULATION OF M-CURRENT

Another current that is modulated by intracellular calcium is the muscarine-blocked voltage- and time-dependent potassium current in sympathetic ganglion neurons, usually called M-current. Reduction of this current underlies the slow muscarinic EPSP and the peptidergic late, slow EPSP in these cells (Adams & Brown, 1982; Jan & Jan, 1982; Kuffler & Sejnowski, 1983). Muscarinic stimulation of these cells results in a rise in intracellular calcium, but blocking this calcium rise does not prevent the electrical response to muscarine (Pfaffinger et al., 1988). Thus the role of calcium, if any, in regulating this current remains unclear.

We used photolysis of calcium-loaded nitr-5 to study the calcium-sensitivity of M-current in bullfrog sympathetic ganglion neurons (Marrion et al., 1991). We confirmed that muscarine blocks M-current and elevates resting calcium concentration by about 50 nM. Removal of muscarine leads to about a 20% over-recovery of the M-current. This over-recovery seems to be due to the calcium rise induced by muscarine, because suppressing the calcium rise with EGTA or BAPTA injection prevents the over-recovery. Elevating intracellular calcium a similar amount by nitr-5 photolysis also enhances M-current by about 20-30%. On the contrary, raising calcium by about 200-300 nM depressed M-current. Raising internal calcium was also seen to depress the response of M-current to muscarine. Thus intracellular calcium has a biphasic dose-dependent effect on resting M-current, as well as an effect on the response of this current to agonist. Physiologically, the rise in calcium accompanying action potentials was found to be sufficient to augment M-current, and this appears to be one mechanism for spike afterhyperpolarization in cells containing M-current (Storm, 1989).

These examples illustrate the roles played by calcium in regulating ion channels. They are not an exhaustive list, by any means. Calcium-activated potassium currents, in particular, have been studied in dozens of neurons, while calcium-activated nonspecific cation currents and calcium-activated chloride currents have also often been described. The ability of calcium to modulate numerous ion channels in distinctly different ways is elegantly exemplified by bursting neurons, where the diverse actions of this second messenger combine to produce an immensely interesting and complex pattern of activity. More commonly, the activation of numerous potassium channels by calcium plays a role in neural adaptation in many, if not most, neurons, shaping the cell's response to inputs and determining the nature of the stimulus to which the cell most effectively responds.

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