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THE MECHANISM OF BURSTING IN APLYSIA PACEMAKER NEURONS

ROBERT S. ZUCKER AND RICHARD H. KRAMER

1. SUMMARY

Central neurons in the marine mollusc Aplysia generate endogenous bursts of action potentials as part of their normal pattern of activity. This pattern is thought to arise from an alternation of depolarizing waves due to calcium influx and hyperpolarizing waves due to potassium efflux. We have found that the hyperpolarization that terminates bursts and prevents firing until the next burst is caused not by a potassium current, but rather by a calcium-dependent inactivation of the calcium current underlying the depolarizing phase. In addition, we found that the depolarizing afterpotential at the end of each burst is due to a calcium-activated nonspecific cation current. These results help to resolve several controversies surrounding the mechanism of bursting in these neurons.

2. INTRODUCTION

The endogenous generation of bursts of action potentials due to oscillations in membrane potential (see Fig. 1A) is widespread in neurons, especially neurosecretory cells (1). The physiological mechanism of bursting has been most thoroughly studied in molluscan central pacemaker neurons (2). These cells show an alternation of depolarizing and hyperpolarizing waves, even in the absence of action potentials. Both phases require calcium in the medium. The depolarization is caused by a persistent inward calcium current that is activated at subthreshold voltages. The hyperpolarization is a calcium-dependent process, caused by the accumulation of intracellular calcium and its effect on membrane channels.

The inward current that triggers a burst has been characterized in Aplysia bursting neurons by Eckert & Lux (3) and Gorman et al. (4). They found an inward current carried by calcium ions that inactivates little at potentials near rest. Intracellular calcium accumulation during small depolarizations has also been measured using arsenazo microspectrophotometry (5). A depolarizing afterpotential occurring at the end of each burst is thought to reflect another inward current, carried by sodium and calcium ions, which contributes to the depolarization driving

bursting (6).

The outward current responsible for burst termination has been attributed to calcium-activated potassium channels. Four kinds of evidence support this belief: 1) the post-burst hyperpolarization, as well as a late outward current following depolarization under voltage clamp, are reduced at hyperpolarized membrane potentials. These hyperpolarizing afterpotentials or late outward currents often fail to reverse at large hyperpolarizations (7), but this could be due to the voltage-dependence of calcium-activated potassium current, which is blocked by hyperpolarization (8). 2) Two types of experiments suggest there is an apparent increase in membrane conductance during the hyperpolarizing phase: current pulses generate a smaller voltage change (9,10), and voltage pulses under voltage clamp lead to smaller net currents (11). However, both procedures measure steady-state conductance, in which a decrease in inward current will have exactly the same effect as an increase in outward current. 3) Injecting calcium into bursting cells leads to an unequivocal increase in potassium current, with a reversal potential near the potassium equilibrium potential (12). 4) The interburst hyperpolarization decays as free intracellular calcium is pumped out (13).

We have investigated two aspects of the control of membrane potential in bursting cells: 1) the current responsible for hyperpolarizing the neuron, terminating the burst and holding the cell at negative potentials until the next burst, and 2) the current underlying the depolarizing afterpotential, which appears to contribute to the depolarizing drive in a burst. We have come to conclusions quite different from the consensus summarized above: 1) Bursts are terminated, and interburst hyperpolarization is maintained, primarily by calcium-dependent inactivation of a resting inward calcium current; and 2) the depolarizing afterpotential is caused by a calcium-activated nonspecific inward currrent. Some of our evidence for these conclusions is presented here. More details may be found in Kramer (14) and in manuscripts now in preparation.

3. PROCEDURE

We have studied bursting in the left upper quadrant bursting cells (L2-L6) in Aplysia abdominal ganglia. In these cells, the pattern of membrane potential changes in a burst (Fig. 1B) is mirrored in a similar pattern of membrane current changes following a depolarizing pulse in voltage-clamped cells (Fig. 1C). The post-burst depolarizing and hyperpolarizing afterpotentials are similar in form and duration to slow inward and outward currents following a pulse. Unlike R15, another bursting pacemaker neuron in the abdominal ganglion, the left upper

quadrant bursters usually retain their bursting activity when axotomized near to the soma by the method of Connor (15). They have rather simple large unbranched axons (16); due to the long length constant of Aplysia axons (17), these axotomized neurons are practically isopotential, and are thus especially suited to voltage-clamp studies of membrane currents.

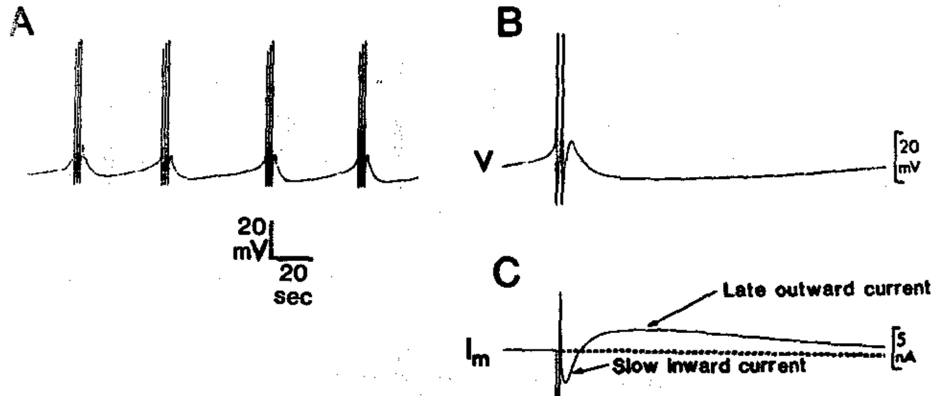

FIGURE 1. A) Endogenous bursting in an upper left qusdrant neuron. B) Membrane potential changes (V) in a single burst. C) Membrane current changes (I_m) following a 50 msec depolarization to 0 mV.

4. RESULTS

4.1 Mechanism of burst termination

4.1.1. Calcium-activated potassium current does not terminate bursts. We first began to doubt that calcium-activated potassium current was responsible for burst termination when we observed that the long-lasting hyperpolarization following a burst fsiled to reverse in cells hyperpolarized by steady current. This occurred even when other potassium-dependent potentials, such as the brief hyperpolarizing afterpotential following eacb action potential, did reverse. We found that the late outward current generated under voltage clamp also fails to reverse, even when the potassium equilibrium potential is elevated by 35 mV in a high-potassium solution (Fig. 2B). This contrasts sharply with the bebavior of calcium-activated potassium current elicited by intracellular calcium injection (Fig. 2A). The latter does reverse at the potassium equilibrium potential, and its reverssl potential shifts almost as much as expected when potassium concentration is raised. Moreover, potassium channels opened by calcium injection are fully blocked by 50 m*M* TEA in

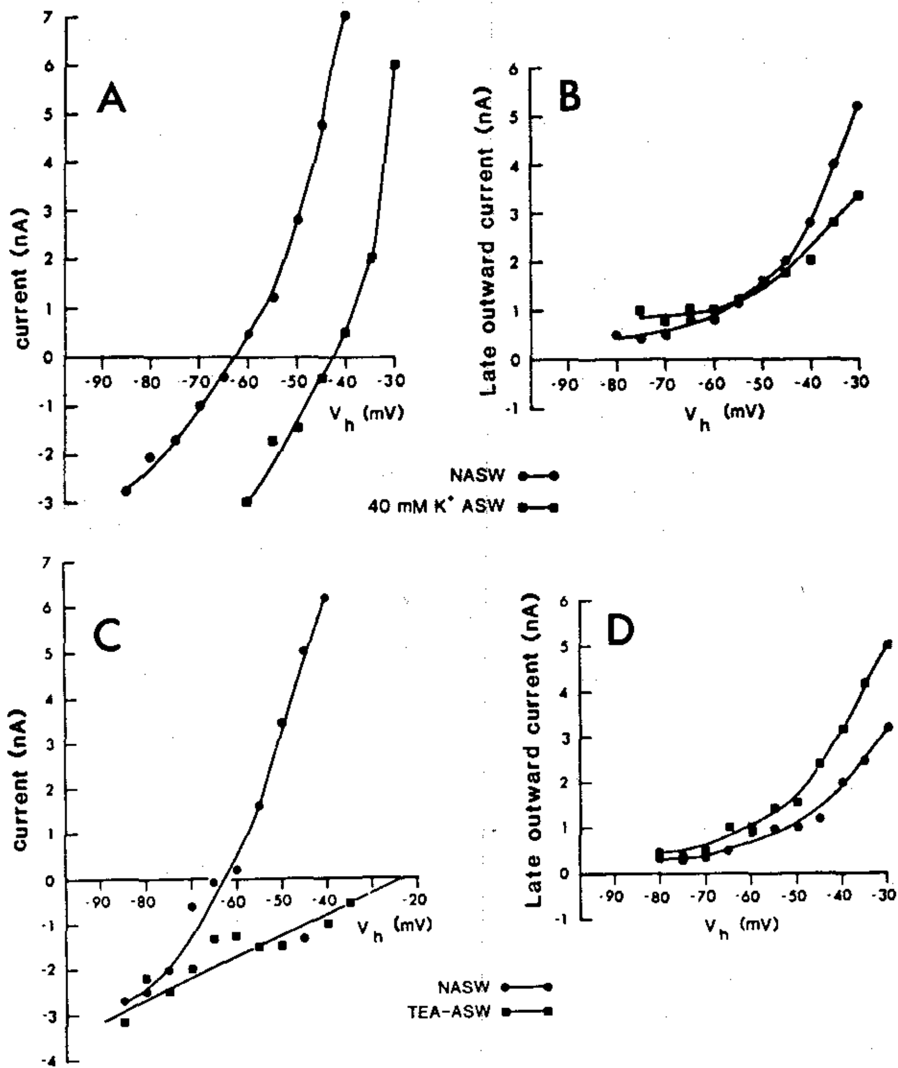

FIGURE 2. A) Currents elicited by calcium injection at different membrane potentials in normal srtificial sea water (NASW) and high potassium sea water. B) Potential-dependence of peak late outward current following a 70 msec pulse to 0 mV. C) Effect of 50 mM TEA on calcium-activated current. D) Effect of TEA on late outward current.

the bath (Fig. 2C), as reported by Hermann & Gorman (18). In contrast,TEA has

little effect on the late outward current following a depolarization (Fig. 2D). Similar results have recently been obtained in R15 by W. B. Adams and I. B. Levitan (personal communication).

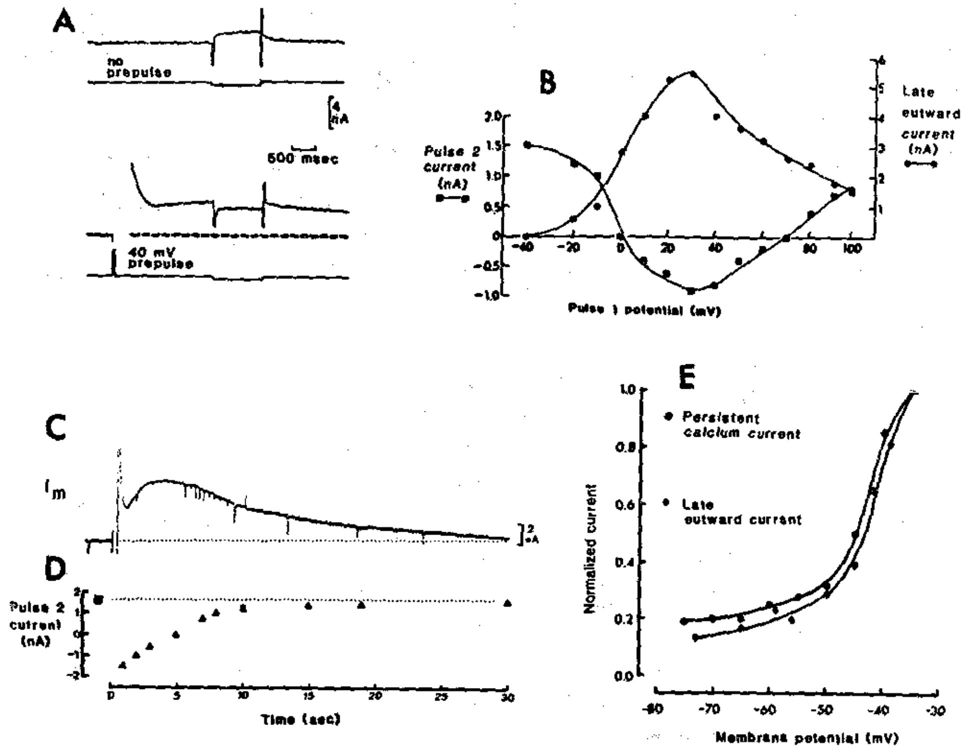

FIGURE 3. A) Effect of a 40 mV prepulse on persistent inward current turned off by a 5 mV test hyperpolarization. B) Dependence of test pulse 2 current and late outward current on size of depolarizing pulse 1. Time course of late outward current (C) and block of persistent inward current turned off by a test hyperpolarization (D) following a 70 msec pulse to +30 mV. E) Voltage-dependence of late outward current and persistent inward current.

4.1.2. <u>Calcium-dependent inactivation of calcium current terminates bursts.</u> Eckert & Lux (3) and Gorman <u>et al.</u> (4) showed that there is a persistent inward current carried by calcium ions that is partially activated at potentials in the pacemaker range (-30 to -50 mV) between the peaks of the depolarizing and hyperpolarizing waves. The steady inward current may be seen in Fig. 3A as a net

outward current in voltage-clamped cells during a small hyperpolarization (the leak current is inward during a hyperpolarization). We now believe that the late outward current underlying a post-burst hyperpolarization is due to the block of this persistent inward calcium current by calcium coming in during the burst.

The magnitude of the late outward current following a depolarizing pulse depends on the size of the pulse. As the pulse amplitude grows, the late outward current grows until the pulse reaches a level of about +25 mV; for larger depolarizations, the late outward current is reduced (Fig. 3B). This bell-shaped dependence of late outward current on pulse amplitude is a characteristic of all processes dependent on calcium influx (19,20). It reflects the rate of calcium entry during a pulse, which grows with depolarization as more calcium channels open, then declines with further depolarization as the calcium equilibrium potential is approached. That the late outward current following a depolarization is due to calcium entry is supported by the fact that it is abolished by calcium antagonists (cobalt and manganese) and by EGTA injection to buffer changes in intracellular calcium.

Fig. 3B shows another interesting result: as the late outward current rises and falls after depolarizations of different sizes, so does the persistent inward current detected by a small hyperpolarizing pulse. Fig. 3C gives another indication that the late outward current is caused by a reduction in persistent inward current. The current elicited by a small hyperpolarization switches from inward (due to leak) back to outward (due to deactivation of persistent inward current) as the test pulse is moved later and later following a depolarization. The time it takes for the persistent inward current to reappear is similar to the time it takes for the slow outward current to disappear. Finally, the slow outward current and persistent inward current display similar voltage-dependence (Fig. 3E). The persistent inward current was measured as in Fig. 3A as the outward current (minus leak current) caused by 5 mV hyperpolarizing pulses from the potential on the absciasa. The peak late outward current was measured at various potentials following a depolarizing pulse.

Taken together, these results strongly suggest that bursts are terminated primarily by the block of resting calcium current by intracellular calcium that has accumulated during the burst.

4.1.3. Role of calcium-activated potassium current. Since potassium currents due to calcium entry or injection have been amply demonstrated in bursting neurones (8,12,19,21), it seemed important to estimate the magnitude and time course of this current during bursting. Fig. 4 shows the membrane current during a simulated burst before and after blocking calcium- activated potassium current with 50 mM

TEA. The difference current is a measure of the calcium-activated potassium current. It rises to a peak during each spike-like pulse, and decays rapidly afterwards. Clearly, calcium- activated potassium current is involved in spike repolarization and the hyperpolarizing afterpotentials following spikes. However, this current has almost completely decayed by the time a burst usually ends with a depolarizing afterpotential (open triangle), and it has entirely dissipated by the usual time of the peak of the interburst hyperpolarization (filled triangle). Thus it plays little if any significant role in burst termination or determination of the interburst interval.

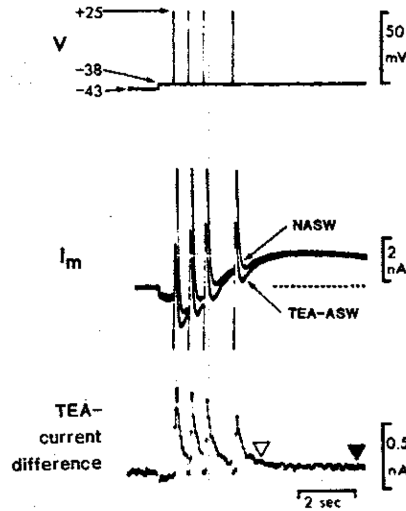

FIGURE 4. Voltage, membrane current in NASW and TEA sea water, and TEA-blocked calcium-activated potassium current during a simulated burst under voltage clamp.

4.2 Basis of depolarizing afterpotential

4.2.1. Calcium-dependence of slow inward current. The depolarizing afterpotential at the end of each burst has been attributed to a slow inward voltage-dependent calcium and sodium current (22). However, our experiments on this current indicate that it is activated by the calcium entering during a depolarization. This current is blocked by EGTA injection or addition of calcium antagonists to the bathing medium (Fig. 5A). Its magnitude shows a bell-shaped dependence on depolarizing pulse amplitude (Fig. 5B), similar to the dependence of the late outward current on pulse potential (Fig. 3B), which is also calcium-

dependent (see above).

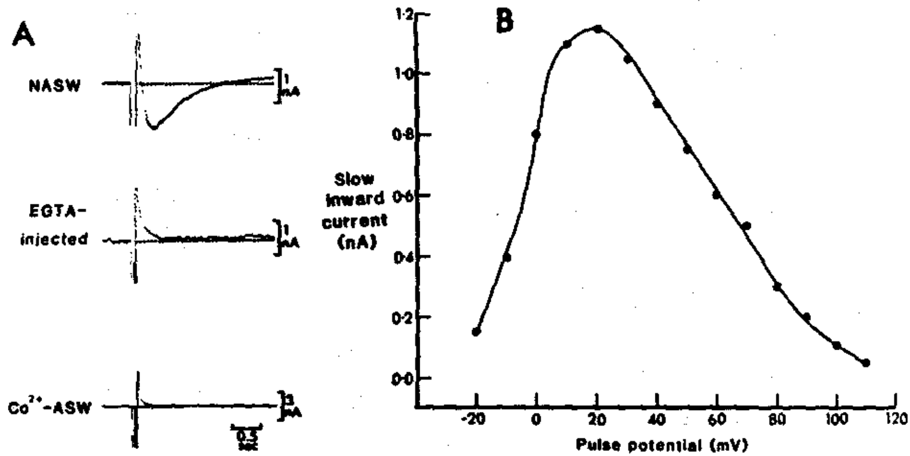

FIGURE 5. A) Slow inward current following a 20 msec pulse to +20 mV in NASW, after EGTA injection, and in sea water with cobalt substituted for calcium. B) Relation between slow inward current and pulse amplitude.

4.2.2. Ions carrying slow inward current. The slow inward current could be a reflection of a modulating effect of intracellular calcium ions on a membrane pump. However, ouabain, which blocks the sodium-potassium pump, and lithium substitution for sodium, which blocks the calcium-sodium pump, have no effect on the slow inward current. The inward current could represent potassium current due to an external accumulation of potassium ions flowing out during a depolarization, shifting the potassium equilibrium potential to more depolarized levels than the resting potential. However, blocking potassium efflux during a pulse with TEA has no effect on the slow inward current.

One ion substitution that dramatically reduces the slow inward current is sodium replacement (Fig. 6A). Unlike the voltage-dependent sodium channels which generate action potentials, this current is not blocked by tetrodotoxin. Calcium removal also blocks slow inward current, but that is because it is a calcium-activated current. Chloride substitution has no effect, and changing potassium has little effect. The effects of sodium removal and EGTA injection distinguish this current from the persistent inward calcium current that flows at rest (see above). The calcium current is enhanced by EGTA injection, and is not affected by sodium removal (4). Thus it appears that the slow inward current is due mainly to a calcium- activated increase in sodium permeability of the membrane.

4.2.3. Calcium injection elicits an inward current. If calcium influx during depolarization activates an inward current, we should be able to activate these channels by calcium injection. The trouble is that calcium will also open potassium channels in these neurons (12). At hyperpolarized membrane potentials close to the potassium equilibrium potential, calcium injection often leads to a biphasic inward-outward current (23), suggesting the existence of two separate calcium-dependent processes. When the calcium-activated potassium current is blocked with TEA, a calcium-activated inward current is left (Fig. 6B). This response is accompanied by a measurable increase in membrane conductance, and depends linearly on membrane potential, with a reversal potential at about -25 mV (Fig. 6C). Sodium removal always reduces the calcium-activated conductance and shifts the reversal potential to more hyperpolarized levels. Calcium removal has a similar but smaller effect. Removing both sodium and calcium shifts the reversal potential about 20 mV to the left and cuts the conductance to about half, so that what remains appears like a potassium current. Changing chloride concentration or pH have no effect on the calcium-activated inward current. Thus the current responsible for depolarizing afterpotentials resembles that elicited by excitatory neurotransmitters at neuromuscular junctions, namely a relatively nonspecific increase in cation permeability.

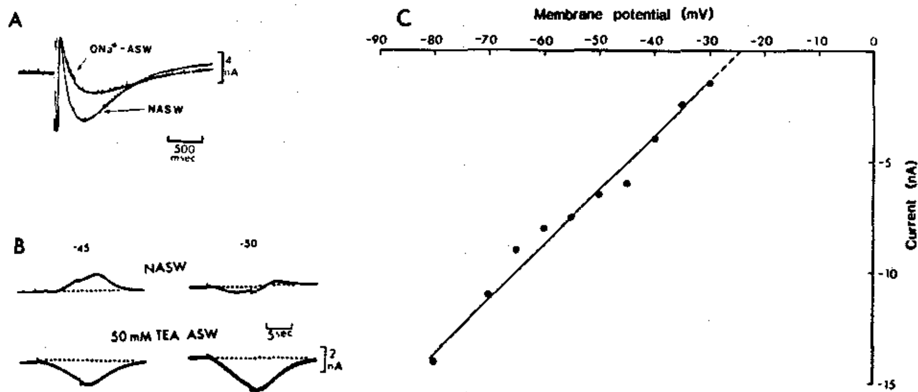

FIGURE 6. A)Effect of tetramethyl ammonium substitution for sodium on slow inward current. B) Calcium-elicited currents in sea water with and without TEA at two membrane potentials. C) Potential-dependence of inward current elicited by calcium injection in TEA sea water.

5. DISCUSSION

5.1. A new model for bursting

We have discovered two new currents in bursting pacemaker neurons -- an outward current due to the calcium-dependent inactivation of resting calcium current, and a nonspecific inward current activated by calcium. Fig. 7 shows how we believe these currents contribute to the pacemaker cycle. A voltage-dependent calcium current (I_{Ca}) causes the depolarizing wave that triggers a burst of spikes. As calcium flows into the cell and accumulates under the membrane ($[Ca]_i$), it activates a nonspecific cation current (called I_B in the figure), which contributes to the depolarizing drive and leads to the depolarizing afterpotential at the end of a burst. Calcium-activated potassium current ($I_{K(Ca)}$) is also activated, which contributes to spike repolarization, and tends to counteract slightly the depolarizing drive. Elevated calcium also blocks the calcium channels which are depolarizing the cell, and eventually this shuts off the depolarizing current, leading to cell hyperpolarization. Since the calcium channels are voltage-dependent, the hyperpolarization is regenerative. Finally, removal of calcium from membrane sites releases the calcium channels from inactivation, and they begin to depolarize the cell again. Bursting therefore arises from the interaction of calcium current, internal calcium accumulation, and three calcium-dependent processes.

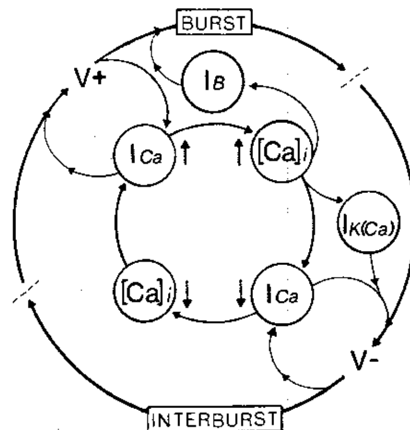

FIGURE 7. Schematic model of the bursting mechanism. Cycles of membrane potential are on the outer circle, and of calcium current and internal calcium levels on the inner circle. Calcium-activated nonspecific and potassium currents are also shown.

How does the cell sort out the three calcium-activated effects? In particular, why does calcium-dependent inactivation of calcium current dominate in the interburst interval, while calcium-activated inward current dominates at the end of the burst and calcium-activated potassium current is prominent only at the end of each spike? We think the three calcium-dependent processes have different calcium sensitivities and stoichiometries. Inactivation of calcium channels is most sensitive. It is present at rest, so that EGTA injection increases calcium currents near rest (4). Thus this effect outlasts the others while submembrane calcium is
dropping after a burst. If calcium-activation of nonspecific cation channels requires only one calcium ion, while calcium-activation of potassium channels requires three calcium ions (24), then the latter current would decay more rapidly as internal calcium declines.

5.2. Some other discrepancies resolved

Our results help to resolve a number of problems in the literature on bursting neurons. For example, estimates of the reversal potential for calcium-activated potassium current have often been more positive than the potassium equilibrium potential (22), while single channel studies of similar channels show a high potassium selectivity (25). Our results indicate that calcium injection activates nonselective as well as potassium channels, so naturally the reversal potential of the mixture is more positive than the potassium equilibrium potential.

Hermann & Gorman (18) reported that low concentrations of external TEA blocked calcium-activated potassium current, but Thompson (26) found that very high concentrations of TEA were needed to block late outward currents attributed to calcium-activated potassium channels. Our results indicate that the late outward current is primarily a calcium-dependent block of calcium current, which is not especially TEA-sensitive.

Eckert & Lux (3) and Gorman et al. (4) report that the inward current leading to depolarization is carried by calcium ions, while others (e.g. 10,27) report that the current is carried by sodium ions. Our discovery of a calcium-activated nonspecific cation channel contributing to the depolarization may be relevant to this controversy. Moreover, we have found that prolonged soaking in sodium-free medium often reduces all calcium-dependent processes. It is possible that block of the sodium-calcium exchange pump leads ultimately to an increase in intracellular free calcium concentration and calcium-dependent inactivation of calcium channels. Then sodium removal will eventually reduce the persistent

inward calcium currenta underlying bursting (see 3), making it appear like a sodium current.

These controversies illustrate the difficulties that have beset the study of bursting mechanisms in the past. We hope that our results will help to clarify these and other problems, and advance our understanding of the mechanisms responsible for generating endogenous repetitive activity in the many neurons that display it.

6. ACKNOWLEDGMENTS

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DISCUSSION OF THE ARTICLE BY R.S. ZUCKER (U.S.)

I. Parnas (Israel): For the late outward current if it is only a decrease in calcium conductance, you should be able to reverse it at the calcium reversal potential. Your lines were parallel. Is it possible that you have an additional slight increase, for e.g., in K conductance and then the current would never reverse?

R.S. Zucker (U.S.): Seeing a reversal at the calcium equilibrium potential is almost impossible.

I. Parnas (Israel): But the lines do not approach. When you reduce the late outward current, how do the lines go?

R.S. Zucker (U.S.): I am unclear as to what lines you mean, since I have not shown any voltage-current plots that should cross at the equilibrium potential for the late outward current. I am only able to measure this current at potentials of 100mV below the calcium equilibrium potential, where the calcium conductance is highly voltage-dependent. Therevore, I do not expect it to reverse.

I. Parnas (Israel): Let me ask you a different question. How do you eliminate the possibility that in addition to the decrease in calcium conductance, you have a slight increase in potassium conductance?

R.S. Zucker (U.S.): You may, but it has to be a TEA insensitive one so it would have to be a different channel from the usual calcium-dependent potassium current. It is possible.

B. Minke (Israel): I would like to add that we found a similar phenomenon in insect photoreceptors. When the presentation of intense bright light induces an increase in the intracellular calcium, we also see an afterdepolarization. However, we performed additional measurements. We measured $[Ca^{2+}]$, with a calcium selective microelectrode at the extracellular space, and we saw that this afterdepolarization - which you show as an inward current - is accompanied by large accumulation of calcium in the extracellular space, which is later reversed when you have the hyperpolarization phase. Therefore, we thought that maybe it is involved in sodium-calcium exchange although you eliminated this possibility by using lithium.

R.S. Zucker (U.S.): Yes, we substitute lithium for sodium.

B. Minke (Israel): But you checked only the late phase, the hyperpolarization phase?

R.S. Zucker (U.S.):

No, both phases, and they are not affected.

J. Moore (U.S.): Could you tell me what Gorman and his colleagues missed, was it an interpretation or do you differ in experimental evidence. This was not clear.

R.S. Zucker (U.S.): Many of these experiments have never been done before. There are alot of new experiments being done here. Gorman mainly referred to Junge & Stephens (Junge, D. and Stephens, C.L. (1973). Cyclic variation of potassium conductance in a burst-generating neurone in Aplysia. J. Physiol. 235, 155-181) and he did not himself really study the hyperpolarization. He studied the accumulation of calcium, the removal of calcium, and the fact that when you inject calcium, you do get a potassium current. He quoted Junge & Stephens (1973) as saying that, for example, there is a change in conductance. Junge & Stephens measured the steady-state conductance. They depolarized the cell with current and achieved more or less depolarization. But they did not take into account the fact that there are very sensitive voltage-dependent conductances in these cells which change very rapidly near the resting potential and they did not measure the instantaneous conductance. They measured the steady-state conductance and I have a slide which explains how Jonge & Stephens interpreted their results and how everyone has quoted them. I can repeat their experiments, I can get their results and I can explain them by the voltage-dependent conductances. Junge & Stephens (1973) also claimed that they could reverse the post-hyperpolarization. However, they did not use voltage clamp. When we clamp this current, we get no reversal. When they hyperpolarized a cell after a burst, they thought they saw a reversal of the interburst hyperpolarization. Actually, they saw a relaxation of voltage-dependent calcium conductance. Therefore, I think the evidence is very old, from over one decade ago, and it is quoted by hundreds papers since then while no one has bothered to check it.

D.L. Alkon (U.S.): Just a quick comment. The sensitivity of your conductances to intracellular calcium is quite different from ours and I wonder that maybe the sensitivity of a conductance to inactivation by the elevation of intracellular calcium may have something to do with its state of activation in the first place. In your case, you may have resting calcium conductance in the first place, and perhaps very little resting calcium-dependent K current. In other situations, it may be just the

opposite.

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R.S. Zucker (U.S.): We find that if we look at different cells, the mix of currents is quite different from cell to cell. Other cells, for example, have more calcium-activated K current and less calcium inward current at rest. So there are differences at least in channel density, and possibily also due to differences in the resting calcium concentration.