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# Substrates of Command Ability in a Buccal Neuron of *Pleurobranchaea*

## I. Mechanisms of Action Potential Broadening

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**Summary.** 1. Bilaterally paired ventral white cells (VWCs) in the buccal ganglion of *Pleurobranchaea* are putative command neurons which in part derive their ability to drive the neural network controlling the buccal mass from the progressive broadening of action potentials during repetitive firing (Fig. 1) (Gillette et al. 1978, 1980). We have conducted an investigation of the parameters and mechanisms of spike broadening using conventional intracellular recording methods, ion substitutions, intracellular injections, and pharmacological agents. In the course of these studies we found evidence for a role for $[Ca^{++}]_i$ in modulating spike broadening.

2. During a current-driven train of broadening spikes, the overshoot amplitudes initially increase progressively and then decline. The rate of broadening is slowest during growth of the overshoot and maximal during overshoot decline (Fig. 2), suggesting that changes in overshoot amplitude contribute to spike broadening. However, a continuous decline in spike undershoot amplitudes throughout the train suggests a relation between the factors underlying progressive spike broadening and progressive decline of undershoot (Fig. 3).

3. Progressive spike broadening is dependent on the presence of external $Ca^{++}$ (Fig. 4), indicating that late $Ca^{++}$ current supports the prolonged depolarization of the broadened spike. In contrast, the progressive decay of the amplitudes of the $K^+$-dependent undershoots is not dependent on external $Ca^{++}$ (Fig. 5). Tetraethylammonium ion (TEA) applied extracellularly or injected intracellularly causes extreme spike prolongation (Fig. 6), indicating the presence of the delayed $K^+$ current known to inactivate with depolarization in other molluscan neurons (Aldrich et al. 1979a). Both by analogy with a previous study of spike broadening (Aldrich et al. 1979b) and directly, these data suggest that spike broadening during repetitive firing is largely due to a progressive decrease in a delayed $K^+$ current, which thus permits spike prolongation by inward $Ca^{++}$ current.

4. While the major contribution to spike broadening appears to arise from decrement of the TEA-sensitive $K^+$ current, internal $Ca^{++}$ levels appear to have a significant role in modulating the rate and extent of spike broadening. This role may be effected through a $Ca^{++}$-activated $K^+$ current ($I_{K,Ca}$), and possibly by regulation of the $Ca^{++}$ conductance itself. $I_{K,Ca}$ is demonstrable in the undershoots of single, unbroadened action potentials, whose waveforms and amplitudes are affected by agents and treatments known to suppress $I_{K,Ca}$ (low $Ca^{++}$, $Ba^{++}$, $Co^{++}$, and injections of EGTA) or enhance it (high $Ca^{++}$, high $Ca^{++}$-buffer injection) (Figs. 7, 8, 9 and 10).

5. Intracellular injection of the $Ca^{++}$ chelator, EGTA, or replacement of external $Ca^{++}$ by $Ba^{++}$ enhances progressive spike broadening (Fig. 11). Conversely, intracellular injection of high $Ca^{++}$ (EGTA) buffers suppresses broadening (Figs. 12 and 13). These experiments suggest a possible role for intracellular $Ca^{++}$ regulation in modulating this form of functional neuronal plasticity (Fig. 14).

## Introduction

In the neurophysiological analysis of behavior, one strategy frequently used has been to locate identifiable or addressable populations of neurons which, when active, exert considerable effect in driving or otherwise modulating the behavior in question. In most cases neural networks mediating specific behavior are too large to reasonably permit thorough analysis, so the identification of critical network sites, at which behavioral expression may be controlled, offers the

*Abbreviations: TEA* tetraethylammonium; *VWC* ventral white cell

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opportunity to characterize important controlling factors at the physiological level, i.e., the synaptic context and intrinsic properties of the critical site.

Such critical sites are also operationally useful as central referents from which to explore the properties of the rest of the neural network(s). In molluscs, this approach has been usefully applied in studies of initiation and motor organization of escape swimming at premotor neurons of *Tritonia* (Taghert and Willows 1978; Getting et al. 1980), defensive inking at motorneurons of *Aplysia* (Carew and Kandel 1977), habituation and sensitization of gill withdrawal at a sensorymotor synapse of *Aplysia* (Castelucci et al. 1970; Pinsker et al. 1970; Klein and Kandel 1978) and the neurohumoral control of egg-laying behavior at neurosecretory neurons of *Aplysia* (Kupfermann 1970; Kaczmarek and Strumwasser 1981), to name a few. In the carnivorous marine slug *Pleurobranchaea californica* two populations of neurons have been found which drive different aspects of buccal motor output: the paracerebral neurons of the cerebropleural ganglion (Davis and Gillette 1978; Gillette et al. 1978c) and the ventral white cells (VWCs) of the buccal ganglion (Gillette et al. 1980). The differentiable command roles of the two populations in the behavior of the buccal mass are reflected in their firing characteristics and intrinsic plasticity in function. The present account considers mechanisms that confer distinctive properties of functional plasticity on the VWCs.

Extreme action potential broadening during repetitive firing and the endogenous production of minutes-long episodes of depolarization and rapid discharge set apart the ventral white cells (VWCs) of *Pleurobranchaea* from their neighboring neurons in the neural network of the buccal oscillator (Gillette et al. 1980). These qualities have been previously shown to confer on the VWCs the ability to command vigorous, coordinated, and rhythmic motor output in the isolated nervous system and in the less-dissected CNS-buccal mass preparation as well. Prolonged VWC burst episodes, triggered by spontaneous EPSPs or short pulses of depolarizing current, can be demonstrated in 75% of preparations; in the remainder the capacity to generate such episodes does not normally appear during observation periods of 4–8 h. However, such bursts may be induced, as is shown in the companion paper to this (Gillette et al. 1982). Thus the VWCs show two functional aspects of neuronal plasticity which may be highly relevant to their behavioral roles: pronounced spike broadening and modulation of bursting capacity. In parallel with investigations into the behavioral role of the VWCs and their natural triggering stimuli we have conducted studies on action potential broadening mechanisms using conventional intracellular recording and stimulating methods, prior to voltage clamp analysis. Evidence is presented that spike broadening in the VWCs proceeds largely from a progressive decrease in spike $K^+$ current which permits $Ca^{++}$ currents to prolong the action potential, and that levels of intracellular $Ca^{++}$ may regulate this process.

## Materials and Methods

*Dissection and Preparation.* After dissection from the animal, buccal ganglia were pinned ventral side-up to Sylgard in a water-jacketed preparation dish for intracellular recording from the VWC soma. Temperature was maintained at 12–14 °C. In some experiments the connective tissue was softened to facilitate electrode penetration by a 12 min incubation in 0.2% pronase (Grade B, Calbiochem) in sea water at room temperature. The outer, muscular connective tissue sheath was routinely removed from the ventral side of the ganglion. Occasionally the transparent sheath was also removed. Surgical isolation of the VWC soma was described previously (Gillette et al. 1980) and was performed without pronase treatment.

*Bath Solutions.* In all experiments ganglia were initially bathed in an artificial saline (ASW) whose composition was 420 mM NaCl, 25 mM $MgCl_2$, 25 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$ and 5 mM Tris-HCl buffered at pH 7.5. When $Co^{++}$ was used as a $Ca^{++}$ blocker, it was either substituted for $Ca^{++}$ in the saline or a premeasured amount was added to the bath as 1.0 M $CoCl_2$ and gently mixed to a final concentration of 30 mM. Control experiments assessing non-specific effects of increasing divalent ion concentration substituted 1.0 M $MgCl_2$ for $CoCl_2$; no significant effects on the parameters we measured were noted. High $Ca^{++}$ salines were made by equivalent substitution for $Mg^{++}$. Low $Ca^{++}$ saline was made by substituting 10 mM $Mg^{++}$ for $Ca^{++}$. In the absence of chelating agents, such solutions are generally considered to be $10^{-5}$–$10^{-4}$ M in free $Ca^{++}$. For $Ba^{++}$ saline, $CaCl_2$ was replaced by $BaCl_2$ and $MgSO_4$ was replaced by the chloride salt. Prior to addition of $Ba^{++}$ saline to the dish, the bath was flushed with sulfate-free saline to prevent precipitation of $BaSO_4$. Tetraethylammonium (TEA) salines were made by substituting the chloride salt for the equivalent in NaCl.

*Intracellular Injection of EGTA and $Ca^{++}$ Buffers.* Intracellular injections were performed using air pressure from a handheld 50 ml plastic syringe connected to the injection electrode by tightly fitting polyethylene tubing. Recordings from the electrode were taken from a chlorided silver wire entering the tubing near the electrode butt.

EGTA (ethyleneglycol-bis-($\beta$-amino-ethyl ether) N,N'-tetraacetic acid) solutions used in the intracellular injections had the composition of 50 mM EGTA, 180 mM $K^+$ and 10 mM Tris adjusted to pH 7.3 with KOH, or 125 mM EGTA, 250 mM KCl, and 100 mM histidine hydrochloride at pH 7.3. Calcium-EGTA buffers for intracellular injection were prepared as described by Meech (1974) and had a final composition of 125 mM EGTA, 250 mM KCl, and 100 mM histidine hydrochloride with sufficient $CaCl_2$ added to give a calculated final concentration of $5 \times 10^{-7}$ or $2 \times 10^{-6}$ M at the adjusted pH of 7.3.

*Stimulation and Recording.* Intracellular recording methods were conventional, using KCl-filled microelectrodes with resistance of 8–15 megohms. Intracellular current injection was performed either through the recording microelectrode using a balancing bridge circuit or via a second intracellular electrode as noted in the text.

Recordings were photographed from a storage oscilloscope screen, taken directly on a Brush 220 or Brush 2400 chart recorder,

or recorded on tape for later transcription. The response capability of the Brush 220 chart recorder pens (100 Hz for 1 cm and 40 Hz for full scale 4 cm sine waves) caused slight clipping of the amplitude of the fastest action potentials, but comparison with photographic records showed that direct records on the chart recorder adequately reproduced the waveforms of prolonged spikes and their undershoots, the brush 2400 accurately reproduced spike amplitudes.

Since it was found that both spike amplitude and spike duration are affected by slight depolarizations preceding stimulation, conditioning hyperpolarization to $-60$ or $-65$ mV for 60 s preceded stimulation of single or trains of action potentials. The 'resting' potential of the VWC varies from $-50$ to $-60$ mV; the lower values are associated with a high level of background synaptic activity. Intervals of at least 10 min separated the stimulation of prolonged trains of action potentials in which spike broadening and undershoot decrement were studied. In certain experiments the spike frequency of the VWC cell body was entrained by antidromic stimulation of its axon in the stomatograstric nerve using a suction electrode to deliver d.c. shocks of 1 ms in duration.

## Results

### *The Parameters of Action Potential Broadening*

Electrical activity in molluscan neuron somata may be influenced by as many as six distinct ion currents, both inward and outward. These currents vary in their ion dependence ($Na^+$, $Ca^{++}$, $Na^+/Ca^{++}$, $K^+$), kinetics of activation and inactivation, and their contribution to the waveform of the action potential. Variation in the action potential waveform therefore reflects variation in the underlying current species. We attempted to partially characterize the processes involved in spike broadening in the VWC by relating obvious changes occurring in the spike duration, overshoot amplitude, and undershoot amplitude to the effects of ion and pharmacological manipulations.

Progressive broadening of action potentials in nonbursting VWCs is readily observed during prolonged trains of action potentials driven at 2.5 to 5/s by depolarizing current. This range of firing rates and the attendant spike broadening are similar to that occurring in endogenously sustained burst episodes (Gillette et al. 1980). The broadening action potential is characterized by a slowing of the repolarizing phase and by the development of a prominence in the same phase of the waveform (e.g., Fig. 1). During the broadening process consistent changes also occur in the amplitudes of both overshoot and undershoot of the spike. Characteristically, overshoots progressively increase by 5–15 mV during the initial portion of the train and subsequently decline (Fig. 1 shows overshoot decline). In contrast, the undershoots typically decline throughout the train concomitant with spike broadening. Decrement of both undershoot and overshoot during broadening may be noted in Fig. 1. Figure 2 graphically depicts the course of increase and decline of spike overshoots during broadening in a typical test and shows that the maximal rate of broadening is coincident with the decline of overshoot amplitude during the latter part of the train. This observation suggests that the decay of the overshoot may contribute to increase in spike duration, possibly through reduced voltage dependent activation of delayed outward current. However, the fact that appreciable broadening occurs during the initial growth of spike overshoot indicates the presence of one or more additional mechanisms.

In particular, the concurrence of spike broadening and undershoot decrement during the entire train, shown graphically in Fig. 3, suggests that the two phenomena may be causally related. In Fig. 3 the relation of decrement in spike undershoot and the broadening of the spike is plotted for trains driven by four different levels of depolarizing current. At each current level, a parallelism between undershoot decrement and action potential broadening is particularly evident in a region between 30–70 ms spike duration where the relations are notably linear with almost identical slopes, calculated by linear regression (0.17–0.19 mV/ms). Analysis of three other experiments on intact VWCs has shown similar linearity in the same region (slopes estimated at 0.15, 0.17 and 0.23 mV/ms) and of two experiments on the surgically isolated VWC soma (slopes 0.15 and

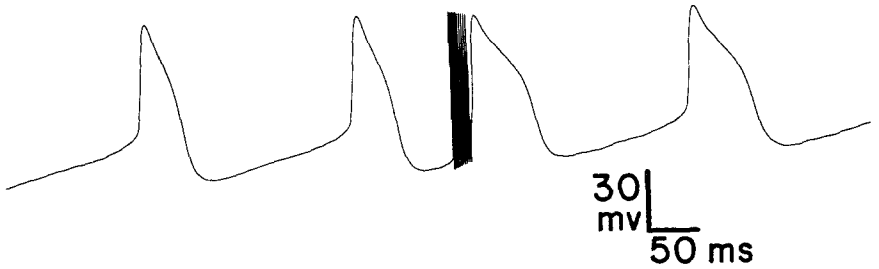


**Fig. 1.** Progressive broadening of the action potential is accompanied by progressive decrease in undershoot amplitude, and, in the later stages of broadening, by a decrease in overshoot amplitude. In this intracellular recording of a current-driven train of spikes in the VWC the chart recorder was temporarily slowed to show progressive decreases in overshoot and undershoot accompanying spike broadening

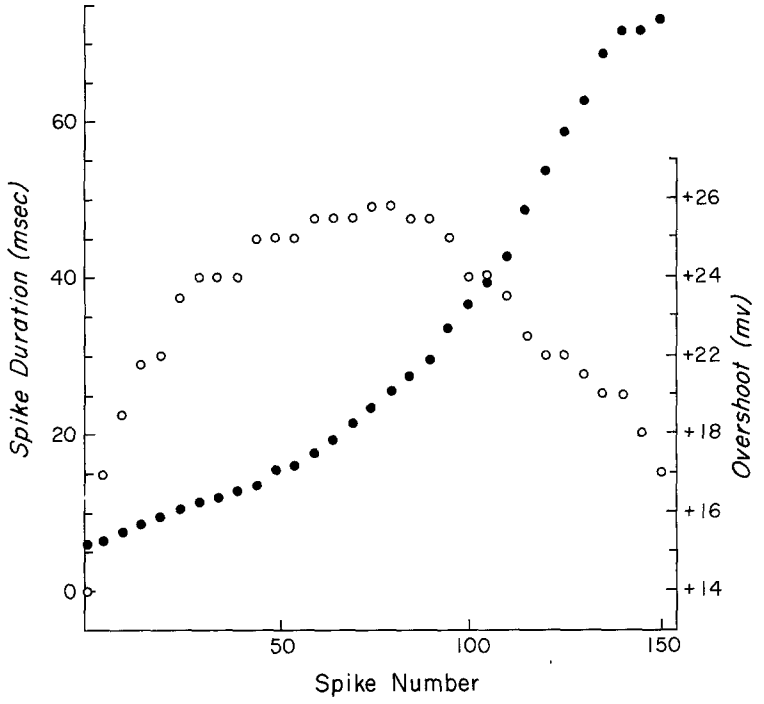


**Fig. 2.** Changes in spike duration and overshoot amplitude during a stimulated train of 150 spikes in the VWC. The rate of progressive spike broadening is least during the progressive increase in overshoot, while the rate of broadening is maximal during decay of the overshoot. This suggests that overshoot amplitude has a negative effect on spike duration, and that decay of the overshoot contributes to spike broadening. *Filled circles:* spike duration; *open circles:* spike overshoot amplitude

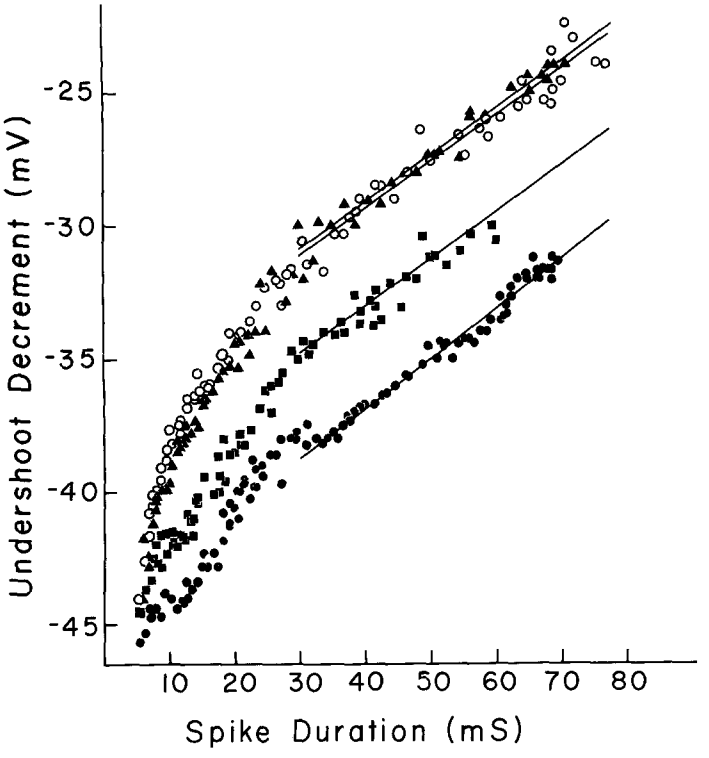


**Fig. 3.** Concurrent decrement of undershoot amplitude and increasing spike duration in the intact neuron. Trains of action potentials were stimulated by steady injection of depolarizing current from a second intracellular electrode at 2 nA (●), 3 nA (■), 4 nA (▲) and 5 nA (○). Slopes were calculated in the linear region by linear regression analysis: 2 nA, 0.188 mV/ms; 3 nA, 0.171 mV/ms; 4 nA, 0.175 mV/ms; 5 nA, 0.171 mV/ms. The vertical axis is the measured maximal potential of the undershoots. Durations of all spikes were measured at the half-amplitude potential of the upstroke of the initial action potential in the 2 nA train. See text for discussion

0.18 mV/ms) where potentially contaminating synaptic inputs are not present. The linearity and constancy of this relation suggest that the same mechanism contributes to both spike broadening and undershoot decrement. Evidence that this is a use-dependent decrease in a $K^+$ current, which permits $Ca^{++}$ current to prolong the spike, is presented in the following paragraphs.

The VWC has the capacity to discharge overshooting action potentials in both $Na^+$-free and $Ca^{++}$-free salines; this capacity is abolished in saline where both ions are replaced by Tris buffer (not shown). This

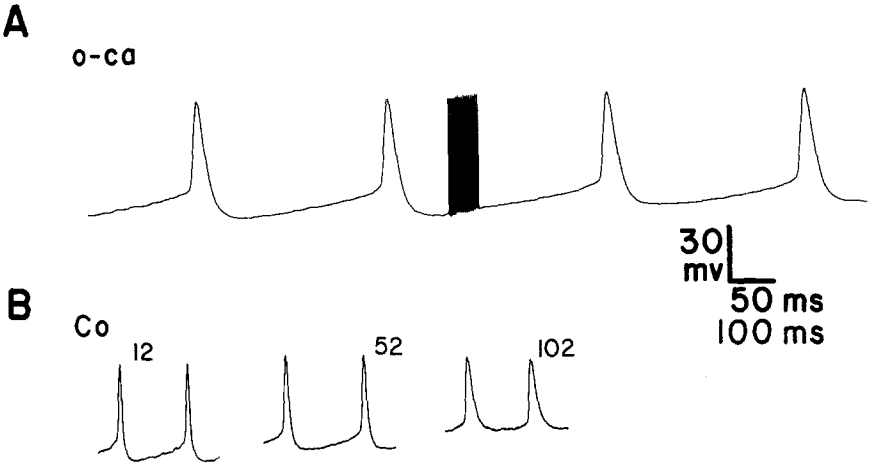


**Fig. 4A, B.** Suppression of spike broadening in low $Ca^{++}$ and $Co^{++}$. Calcium independence of progressive undershoot decrement. **A** In low $Ca^{++}$ (0.1 mM) saline spike broadening is largely suppressed while progressive undershoot decrement is still observed. The record was slowed temporarily to show the progressive decline in undershoot amplitude. **B** Excerpts from a continuous record of a current stimulated train of spikes in the presence of 30 mM $CoCl_2$ showing progressive decline of undershoot amplitude in the absence of appreciable spike broadening. *Superscripts* indicate spike number

indicates that the inward spike currents of the soma are carried by both ion species, as is not uncommon in neurons of many animals. As calcium ion has commonly been found to carry the inward current of prolonged action potentials in many excitable cells, we tested the effects of zero-$Ca^{++}$ salines and $Co^{++}$ (a $Ca^{++}$ current blocker) and found that these treatments largely suppressed progressive spike broadening in the VWC (Fig. 4), indicating that the broadened phase of the spike is mediated by prolonged inward $Ca^{++}$ current. However, during blockade of $Ca^{++}$ current some degree of spike broadening is still observable, although the action potentials lack the prominence in the falling phase attributable to $Ca^{++}$ current. This broadening is also attended by changes in overshoot and undershoot amplitude qualitatively similar to those seen in normal saline. That is, as graphically illustrated in Fig. 5, overshoots rise and subsequently decline, while undershoots decline continuously throughout the stimulated train.

The slowing repolarization and continuous decline of the spike undershoot in the absence of prolonged $Ca^{++}$ current suggests that a progressive decline in the repolarizing $K^+$ current may account for a large portion of the cause of spike broadening. The initial growth of the spike overshoot could also arise from reduction in outward $K^+$ current. As spike depolarization in these experiments is $Na^+$-dependent, it seems likely that the decline in spike overshoot is a result of accumulating inactivation of $Na^+$ current and can further contribute to spike broadening through less activation of voltage dependent $K^+$ current.

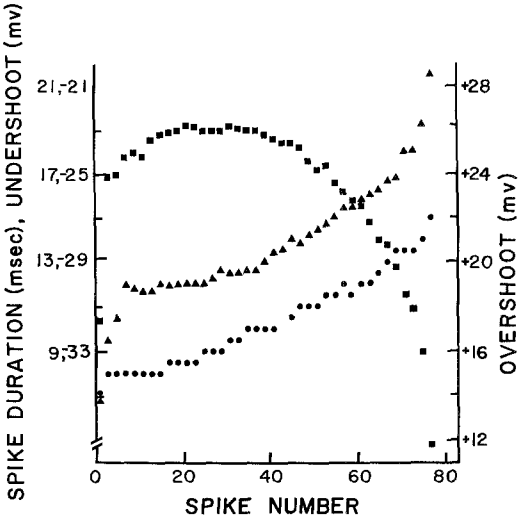


**Fig. 5.** Parallel decrement of undershoot (*triangles*) and increase in spike duration (*circles*), and changes in overshoot amplitude (*squares*) 0-$Ca^{++}$, 10 mM $Co^{++}$ saline

Three outward potassium currents which could contribute to spike broadening have been identified in molluscan neurons: The 'A' current which inactivates at depolarizations greater than −40 mV (Connor and Stevens 1971b), a voltage dependent delayed current which shows slow inactivation (Connor and Stevens 1971a; Thompson 1977), and a current activated by internal $Ca^{++}$ which also shows certain voltage dependence (Meech 1974; Gorman and Thomas 1980). The A current is unlikely to contribute appreciably to spike broadening in the VWC, as this current is inactivated at voltages where progressive spike

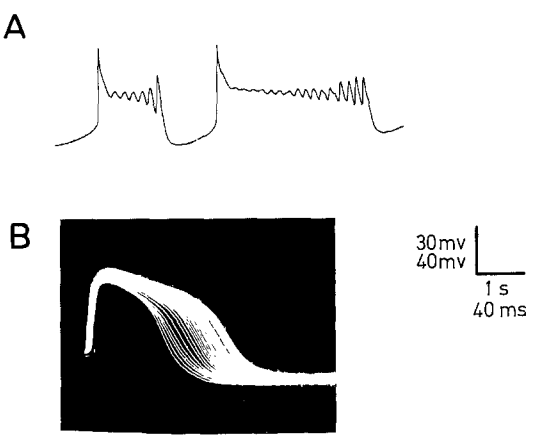


**Fig. 6A, B.** TEA-induced broadening of the VWC spike. **A** 50 mM TEA causes prolonged, waving plateaus. **B** Superimposed oscilloscope sweeps of spikes after raising external $Ca^{++}$ from 10 to 40 mM

broadening and undershoot decrement are conspicuous (Fig. 3). The A current of *Pleurobranchaea* neurons generally resembles that in other molluscs (R.G., unpublished voltage clamp data). The voltage dependent and slowly inactivating $K^+$ current has been shown to underlie spike broadening during repetitive spiking in neurons of dorid nudibranch snails (Aldrich et al. 1979a, b). The inactivation of this current has been shown to be voltage-dependent, to accumulate during repetitive firing, and to specifically correspond with the development of broadened action potentials in neurons with relatively large spike $Ca^{++}$ currents. Aldrich et al. (1979b) have proposed that progressive spike broadening may arise through cumulative inactivation of the delayed $K^+$ current causing prolongation of spike $Ca^{++}$ current. This current is blocked by tetraethylammonium (TEA) ions (Thompson 1977; Connor 1979).

We tested the effects of TEA either injected intracellularly (1 M; $n=2$) or substituted for $Na^+$ in the bath saline (20–100 mM; $n=4$). In each case TEA caused extreme broadening of the action potentials. Figure 6A shows spontaneous action potentials recorded in the presence of 50 mM TEA. The spikes show an extremely prolonged and waving plateau; subsequent addition of $Ca^{++}$ to the saline to raise the $Ca^{++}$ concentration to 40 mM silenced the cell and abolished the waving plateau of stimulated action potentials (Fig. 6B), presumably due in part to an increase in the $Ca^{++}$-activated $K^+$ conductance. The record of Fig. 6B shows TEA-induced broadening of the first and subsequent 29 spikes stimulated at 0.3 Hz in the presence of 50 mM TEA. The action of TEA in exaggerating spike duration is unlikely to be caused by blockade of the Ca-activated $K^+$ current (Hermann and Gorman 1981) since specific blockade of this current by $Ba^{++}$ does not appreciably prolong single, stimulated spikes (see text further, and Fig. 8). Therefore, these data indicate that the VWC possesses a $K^+$ current qualitatively similar to that underlying spike broadening in dorid neurons. It thus seems likely that inactivation of this conductance during repetitive firing has a prominent, but not exclusive, role in spike broadening in the VWC.

Stimulation of the VWC by depolarization at spike rates of 3–5/s for several seconds may trigger either prolonged burst episodes or slowly declining depolarizing afterpotentials, however, these qualities of excitability are somewhat variable both within and among preparations. Prolonged depolarizing potentials in other endogenously bursting molluscan neurons are mediated by slow inward $Ca^{++}$ and/or $Na^+$ currents. In the VWC, the ability to trigger burst episodes or depolarizing afterpotentials is reversibly abolished in spontaneously bursting cells by zero-$Na^+$ saline ($n=2$), 10%-$Na^+$ saline ($n=2$), zero $Ca^{++}$ salines ($n=2$) and $Co^{++}$ salines ($n=6$) (not shown). These results are compatible with the possible presence of slow inward currents. However, the fact that the capacity to sustain burst episodes or lengthy depolarization afterpotentials is labile and the fact, discussed later, that the excitable nature of the cell may be delicately regulated both suggest the possibility of multiple and non-specific effects on the parameters measured in these experiments.

We pursued the possibility that the $Ca^{++}$-activated $K^+$ current $I_{K,Ca}$, present in many neurons (Meech 1978), might influence the course of progressive spike broadening. Action potential duration in neurons of *Helix* and *Aplysia* is sensitive to changes in that current (Meech 1974a, b). The presence of $I_{K,Ca}$ in neurons is often indicated by the presence of a prolonged post-tetanic hyperpolarization, dependent on $Ca^{++}$ influx and sensitive to external $K^+$, following stimulated trains of action potentials, in the VWC the presence of the depolarizing afterpotentials mentioned above precluded this index. However, we found that the $K^+$-sensitive after-hyperpolarizations (undershoots) of single unbroadened action potentials are altered in their amplitude and waveform in a consistent fashion by treatments known to reduce or increase $I_{K,Ca}$.

Typical effects of suppressing $I_{K,Ca}$ by blocking $Ca^{++}$ current with $Co^{++}$ saline ($n=8$) are shown in Fig. 7, and resemble those of zero-$Ca^{++}$ salines ($n=4$). Action potentials stimulated at varying levels of depolarizing current show a characteristic accentuation of the waveform of the undershoot in normal saline (Fig. 7A), whereas the undershoots of action potentials stimulated in $Ca^{++}$ (Fig. 7B) are reduced in amplitude by several millivolts and severely atten-

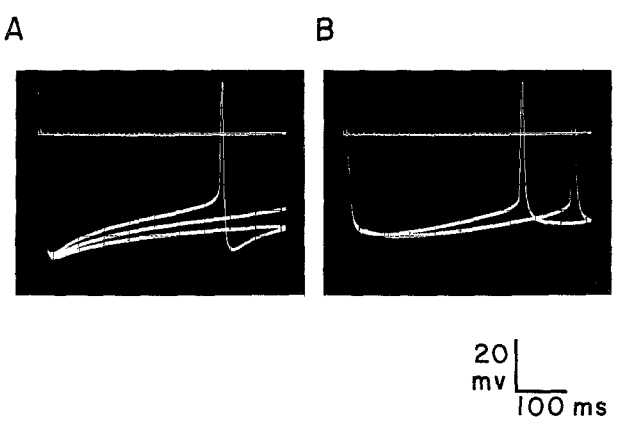


**Fig. 7A, B.** Reduction of the undershoot amplitude and abbreviation of the waveform of the undershoot by blockade of spike $Ca^{++}$ currents with $Co^{++}$. The records are superimposed oscilloscope sweeps triggered on the upstroke of action potentials. The spikes were stimulated by varying levels of injected current after conditioning hyperpolarizations to −65 mV for 1 min. **A** Normal saline. The accentuation of the trough of the undershoot is apparent in spikes stimulated by 1.0, 1.5 and 2 nA of depolarizing current. **B** $Co^{++}$ saline: $Ca^{++}$ is replaced by 10 mM $Co^{++}$. Stimulus currents, 0 and 0.5 nA. The reduction of the undershoot amplitude and abbreviation of the waveform is presumably due to reduction of $I_{K,Ca}$. Reference trace is −4 mV from zero potential; spike heights are clipped

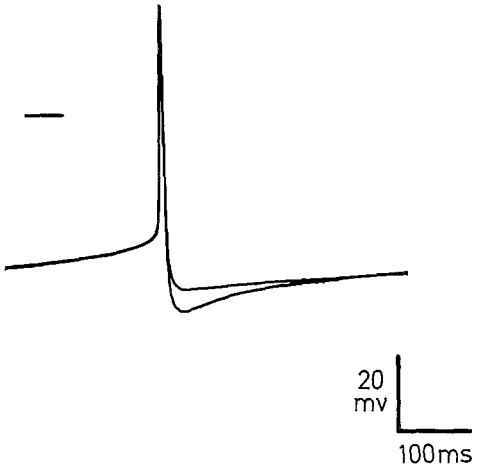


**Fig. 8.** Reduction of the undershoot amplitude and abbreviation of the waveform of the undershoot by replacement of both $Ca^{++}$ with $Ba^{++}$. The records are of two superimposed single action potentials at the beginning of trains driven at nearly identical frequencies. The more accentuated undershoot with greater amplitude is that of the spike recorded in normal saline; that of 6 mV lesser amplitude and abbreviated waveform was recorded in 0 $Ca^{++}$, 10 mM $Ba^{++}$ saline. The $Ba^{++}$ spike is 1 mV higher than that of normal saline. Reference line is zero potential. The abbreviation of the undershoot in $Ba^{++}$ is presumably due to reduction of $I_{K,Ca}$

uated in waveform. Not evident in Fig. 7B is that $Co^{++}$ caused a small decrease in the spike overshoot (4 mV), an effect also true for zero-$Ca^{++}$ salines. This effect might reduce the undershoot through reduced activation of voltage dependent $K^+$ currents.

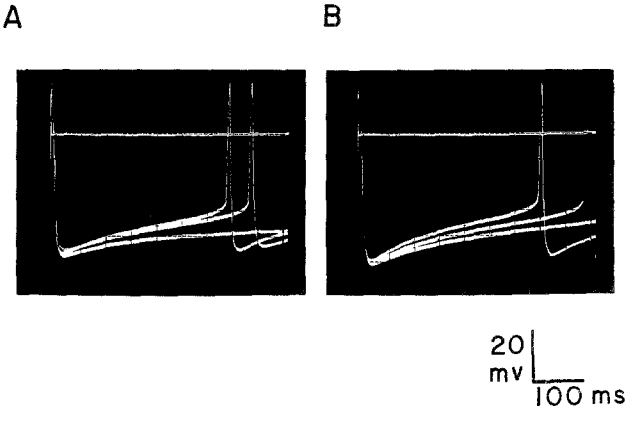


**Fig. 9A, B.** Enhancement of the amplitude and accentuation of the spike undershoot in high $Ca^{++}$. **A** Normal saline. Stimulus currents 0.5, 1.0 and 1.5 nA. **B** $Ca^{++}$ raised to 30 mM ($Mg^{++}$ reduced in equivalent). Stimulus currents 1.0, 1.5 and 2.0 nA. The enhancement of the undershoot in high $Ca^{++}$ is consistent with an increase in $gK_{Ca}$. Spike heights are clipped; reference trace is zero potential

However, replacement of $Ca^{++}$ by $Ba^{++}$ as a carrier of inward current, which does not activate $I_{K,Ca}$ (Hagiwara et al. 1974; Connor 1979), tends to increase the overshoot by 1–2 mV yet has identical effects on the undershoot as $Co^{++}$ and zero-$Ca^{++}$ ($n=3$). The superimposed action potentials of Fig. 8 allow the comparison of undershoots in normal versus $Ba^{++}$ saline.

Conversely, treatments known to augment $I_{K,Ca}$ enhance the amplitude and accentuation of the undershoot. High $Ca^{++}$ salines may enhance $I_{K,Ca}$ both through increasing the $Ca^{++}$ influx of the spike and raising the resting level of free internal $Ca^{++}$ (Standen 1975; Requena et al. 1977). Figure 9 shows the undershoot records obtained in normal saline (Fig. 9A) next to the enhanced undershoots recorded in 30 mM $Ca^{++}$ ($Mg^{++}$-substituted) (Fig. 9B). The superimposed records of the spikes show an overshoot difference of less than 1 mV (greater in high $Ca^{++}$) (Fig. 10). These experiments show that like the post-tetanic hyperpolarizations recorded in other molluscan neurons, the undershoots of single, unbroadened VWC spikes may manifest the presence of $I_{K,Ca}$.

### *Calcium Modulation of Action Potential Broadening*

In testing the effects of altering $I_{K,Ca}$ we found an inverse relationship between progressive spike broadening and $I_{K,Ca}$. That is, decreasing $I_{K,Ca}$ enhanced spike broadening, while increasing $I_{K,Ca}$ suppressed it.

The activation of $I_{K,Ca}$ is primarily dependent on levels of free intracellular $Ca^{++}$ (Eckert and Tillotson 1978; Gorman and Thomas 1980). Intracellular injec-

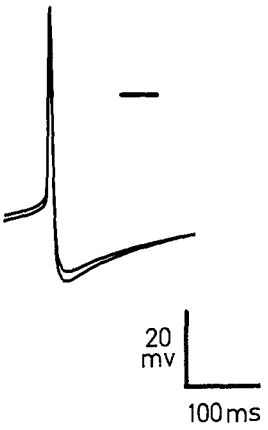


**Fig. 10.** Superimposed action potentials recorded in normal saline and high $Ca^{++}$ saline (30 mM $Ca^{++}$ as in Fig. 12). The undershoot of the spike recorded in high $Ca^{++}$ is 2.8 mV greater in amplitude than that of normal saline and the accentuation of the waveform is enhanced. Reference line is zero potential

tion of the $Ca^{++}$ chelator EGTA ($n=3$) markedly enhanced the rate and extent of spike broadening, as shown in Fig. 11 which compares records from a VWC driven for 40 s by depolarizing current from a second intracellular electrodes before and after intracellular injection of 50 mM EGTA. In all such experiments measuring spike broadening the test stimuli were imposed immediately after a 1 min period of conditioning hyperpolarization to $-60$ mV. Substitution of $Ba^{++}$ for saline $Ca^{++}$ ($n=3$) also strongly enhances spike broadening (not shown). $Ba^{++}$, while able to substitute for $Ca^{++}$ as a carrier of inward current, does not significantly activate $I_{K,Ca}$ (Hagiwara et al. 1974; Connor 1979).

The converse experiment aimed at raising intracellular free $Ca^{++}$, had converse results. Intracellular injections of $Ca^{++}$-EGTA buffer solutions, $5\times10^{-7}$ ($n=3$) or $2\times10^{-6}$ ($n=1$) M in free $Ca^{++}$ caused exaggeration of undershoot amplitude, and suppressed action potential broadening during repetitive activity. These free $Ca^{++}$ concentrations are in the high range of values estimated for gastropod neurons (Gorman and Thomas 1978; Ahmed and Connor 1979). The suppression of spike broadening which results from intracellular injection of $5\times10^{-7}$ M $Ca^{++}$ buffer is illustrated in Fig. 12. The extent of the progressive spike broadening elicited by constant current injection for 2 min (Fig. 12A) is reduced by more than one-third after injection of the high $Ca^{++}$ buffer (Fig. 12B), although the stimulating currents used and the resulting spike frequency of the post-injection trial were actually higher.

The suppression of spike broadening by intracellular $Ca^{++}$ buffer injection is also illustrated by an experiment in which sequential injection pulses were made during a current-stimulated train of broadened

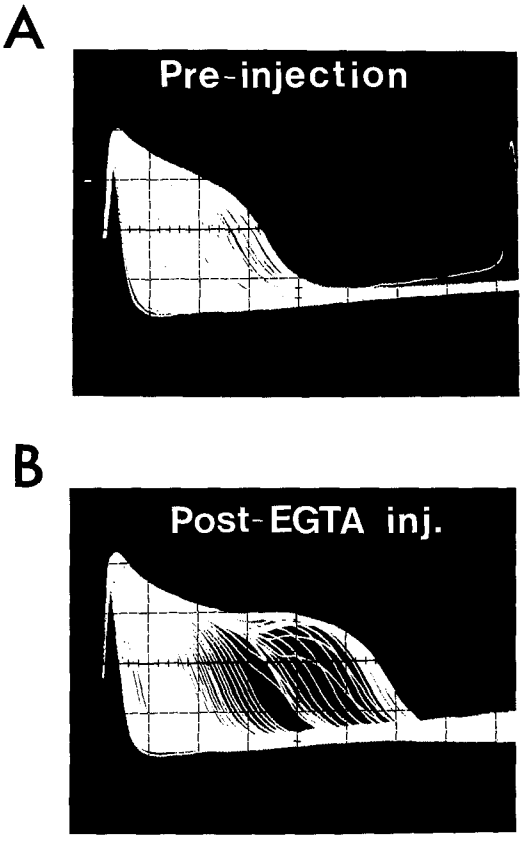


**Fig. 11A, B.** Enhancement of progressive spike broadening after intracellular injection of EGTA. **A** Preinjection. 128 spikes were driven during 40 s by 4.7 nA depolarizing current from a second intracellular electrode. **B** Postinjection of a solution of 50 mM EGTA, 180 mM KCl, and 10 mM Tris buffer, pH 7.3. Stimulation parameters as in **A**, 105 spikes. *Calibration:* grid bars are 20 mV vertical and 20 ms horizontal

spikes. The records of Fig. 13, from top to bottom, are from a continuous train of action potentials in a 90 s interval during which three separate pulses of $5\times10^{-7}$ M $Ca^{++}$ buffer were delivered. The 2nd, 3rd and 4th records down are of spikes recorded within 15 s of each sequential pulse, and they show the cumulative decrease in spike duration and the cumulative enhancement of spike undershoot resulting presumably from the $Ca^{++}$ injections. In this experiment the amplitude of the spike overshoot increased by 3 mV upon the first injection pulse and did not change thereafter. However, the amplitude of the undershoot was successively augmented by 8, 3 and 3 mV with each injection.

Increase of $I_{K,Ca}$ may also be achieved by raising extracellular $Ca^{++}$. This may occur both by a tonic increase in intracellular free $Ca^{++}$ (Requena et al. 1977) and by intensification of spike $Ca^{++}$ current because of the steeper transmembrane gradient of the ion (Standen 1975). In six experiments where the bath saline was raised from 10 mM to 20 ($n=1$), 30 ($n=4$),

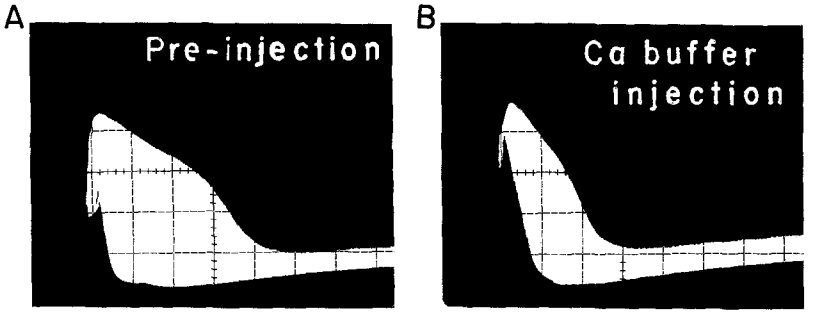


**Fig. 12A, B.** Partial suppression of progressive spike broadening after intracellular injection of a 'high' $Ca^{++}$ buffer.
**A** Preinjection. 394 spikes driven by 6.5 nA depolarizing current during 2 min.
**B** Postinjection of a $Ca^{++}$-EGTA solution $5 \times 10^{-7}$ M in free $Ca^{++}$. 456 spikes driven by 8 nA depolarizing current during 2 min.
*Calibration:* grid bars are 20 mV vertical and 20 mV horizontal


**Fig. 13.** Suppression of broadened action potentials and enhancement of undershoot by intracellular injection of a 'high' $Ca^{++}$ buffer. During a train of previously broadened spikes sequential injections progressively reduced the duration of the spikes while increasing undershoot amplitude. Superimposed are spikes recorded immediately before and after small pressure injections of a CaEGTA solution $5 \times 10^{-7}$ M in free $Ca^{++}$. Reference lines are zero potential

40 ($n=2$) and 50 mM ($n=1$) the notable effect of high external $Ca^{++}$ was that of suppression of progressive spike broadening and undershoot decrement similar to $Ca^{++}$ buffer injection (not shown). While enhancement of $Ca^{++}$ current might have been expected to favor spike broadening in these experiments, the observed suppression is explainable on the basis of a more effective enhancement of $I_{K,Ca}$.

While it seems likely that the effects in the experiments described above were mediated largely through alterations of $I_{K,Ca}$, there is presently no sure way of excluding possible effects of the experimental manipulations on the activation characteristics of the $Ca^{++}$ current using conventional intracellular recording. Such effects on $Ca^{++}$ current, if significant, might be expected to act agonistically in spike broadening with those effects on $I_{K,Ca}$. For instance, EGTA injection would increase the driving force of $Ca^{++}$ influx while lowering $I_{K,Ca}$; the $Ca^{++}$ buffer injections would have opposite effect. Furthermore, some evidence suggests that inactivation of $Ca^{++}$ current in molluscan neurons could arise from buildup in $[Ca^{++}]_i$ (Kostyuk and Krishtal 1977). Thus, changes in $[Ca^{++}]_i$ may be affecting progressive spike broadening through opposite effects on inward $Ca^{++}$ and outward $K^+$ currents.

## Discussion

### *Mechanisms of Action Potential Broadening*

Progressive action potential broadening in the VWCs appears to proceed largely from mechanisms proposed by Aldrich et al. (1979b) from studies on nudibranch snail neurons. In their model, repetitive firing leads to accumulating inactivation of a delayed, TEA-sensitive $K^+$ conductance and the resulting reduction in outward current permits inward $Ca^{++}$ current to prolong the action potential. Our data are in accord with this model in that progressive spike broadening is substantially $Ca^{++}$ dependent (Fig. 4) and that the continuous decline of $K^+$-sensitive spike undershoots during stimulated trains (Figs. 1 and 3) suggests progressive decay of repolarizing outward currents. Since undershoot decrement proceeds during virtual blockade of $Ca^{++}$ current (Fig. 5), the decrement is likely to reflect in large part a decrease in $K^+$ current. Finally, the pronounced broadening of VWC action potentials induced by TEA indicates the presence of the TEA-blocked and inactivating delayed $K^+$ conductance found in closely related opisthobranch molluscs (Connor and Stevens 1971a; Thompson 1977).

An additional mechanism of spike broadening in the VWC may arise during the progressive decrement in overshoot amplitude seen in the later stages of stimulated trains (Fig. 2). That is, the decrease in the amplitude attained by the overshoot is likely to cause decreasing activation of voltage dependent $K^+$ current and thus may further slow spike repolarization. The decrease in the overshoot amplitude itself probably arises from progressive inactivation of a fast $Na^+$ current and this effect may be augmented by both the prolonging of the depolarization of the spike and by the decreasing amplitude of the undershoot.

An additional contribution to spike broadening may indirectly arise from the activation of slow inward currents during depolarization of the VWC. With the reservation that the VWC's capacity for sustaining burst episodes and depolarizing afterpoten-

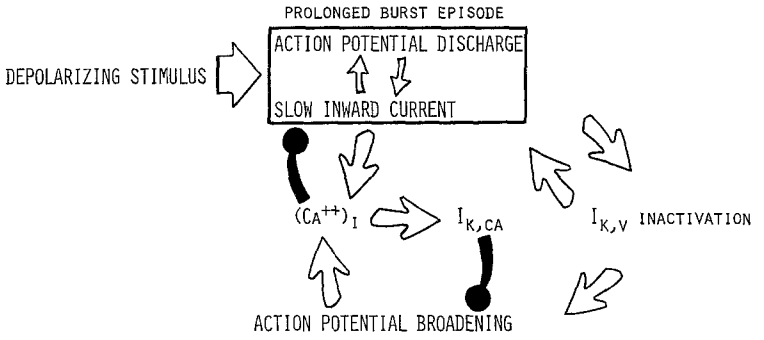


**Fig. 14.** Proposed model for $[Ca^{++}]_i$ influence on VWC activity. See text

tials is labile and possibly subject to non-specific effects of ion substitution, the sensitivity of the bursting capacity of the VWC to $Na^+$ and $Ca^{++}$ substitution suggests that slow inward currents sustain burst episodes, as in other molluscan bursters (Eckert and Lux 1975; Gola 1976; Gorman and Thomas 1978). Activation of these currents during tests of spike broadening driven by the current electrodes can be expected to contribute to net depolarization and thus to the inactivation and activation of the currents involved in spike broadening.

### *$[Ca^{++}]_i$ Modulation of Action Potential Broadening*

It is of interest that the neuronal plasticity involved in spike broadening may be readily modulated through altering internal free $Ca^{++}$. Injection of EGTA enhanced (Fig. 11), while injection of high-$Ca^{++}$ buffers suppressed (Fig. 12), progressive spike broadening. These effects may conceivably arise from either or both of two sources: regulation of the $Ca^{++}$-activated $K^+$ conductance by internal free $Ca^{++}$ (Eckert and Tillotson 1978) or similar regulation of the inactivation of the $Ca^{++}$ current itself (Kostyuk and Krishtal 1977). Both mechanisms can explain the observed effects. Altering the 'set-point' of the $Ca^{++}$-activated $K^+$ current could affect the rate and extent of spike broadening because i) as voltage dependent $K^+$ current inactivates, the $Ca^{++}$-dependent $K^+$ current assumes a relatively greater role in repolarizing the spike, and ii) as the inactivation of voltage-dependent $K^+$ current is both voltage- and time-dependent, the contribution of $I_{K,Ca}$ to spike duration will affect the rate of inactivation. Altering the inactivation characteristics of $Ca^{++}$ current as a function of internal $Ca^{++}$ could affect spike broadening directly through changing inward current intensity and indirectly through the attendant effects on the rate of $K^+$ current inactivation.

The relative contribution of either of these two possible modulatory mechanisms of spike broadening cannot be assessed without the use of a specific blocker of the $Ca^{++}$-activated $K^+$ current or voltage clamp methods. However, the experimental results clearly show that altering internal $Ca^{++}$ markedly affects the functional state of the cell.

A tentative model shown in Fig. 14 summarizes the above considerations in a scheme of the proposed features of the modulation of spontaneous burst episodes and spike broadening in the VWC. Depolarizing synaptic inputs from the feeding oscillator trigger the burst episode through activation of slow inward current and initiation of repetitive firing, both of which are presumed to be mutually reinforcing. The resultant inactivation of $I_{K,V}$ leads to progressive broadening of the action potentials and contributes to the net burst depolarization. Internal $Ca^{++}$ accumulation may directly arise from the inward currents of the broadened spikes and the slow currents supporting the burst. $Ca^{++}$ accumulation may modulate the rate and extent of spike broadening through $I_{K,Ca}$. $Ca^{++}$ accumulation is considered likely to be a major factor in terminating the burst episode via $I_{K,Ca}$, as it does in the shorter bursts of the R15 neuron of *Aplysia* (Gorman and Thomas 1978), and could also reduce the inward current supporting the burst through causing inactivation of $Ca^{++}$ channels. We are currently assessing this model with voltage clamp methods.

The potential for regulation of functional plasticity through direct effects of internal $Ca^{++}$ on membrane conductances is present in a wide variety of excitable cells, particularly those possessing appreciable $I_{K,Ca}$. For instance, the responsiveness to glucose stimulation of bursting activity and insulin release in the $\beta$ cells of the Islets of Langerhans may be modulated by $I_{K,Ca}$ (Atwater et al. 1979). In the subsequent report (Gillette et al. 1982) we discuss the possibility that cyclic AMP may modulate the electrical activity, and thus the command function of the VWCs of *Pleurobranchaea* via internal $Ca^{++}$ regulation.

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## References


Ahmed Z, Connor JA (1979) Measurement of calcium influx under voltage clamp in molluscan neurones using the metallochromic dye Arsenazo III. J Physiol (Lond) 286:61–82

Aldrich RW, Getting PA, Thompson SH (1979a) Inactivation of delayed outward current in molluscan neurone somata. J Physiol (Lond) 291:507–530

Aldrich RW, Getting PA, Thompson SH (1979b) Mechanism of frequency-dependent broadening of molluscan soma spikes. J Physiol (Lond) 291:531–544

Atwater I, Dawson CM, Ribalet B, Rojas E (1979) Potassium permeability activated by intracellular calcium ion concentration in the pancreatic $\beta$-cell. J Physiol (Lond) 288:575–588

Carew TJ, Kandel ER (1977) Inking in *Aplysia californica*. J Neurophysiol 40:692–734

Castelucci V, Pinsker H, Kupfermann I, Kandel ER (1970) Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. Science 167:1745–1748

Connor JA (1979) Calcium current in molluscan neurones: measurement under conditions which maximize its visibility. J Physiol (Lond) 286:41–60

Connor JA, Stevens CF (1971a) Inward and delayed outward membrane currents in isolated neural somata under voltage clamp. J Physiol (Lond) 213:1–19

Connor JA, Stevens CF (1971b) Voltage clamp studies of a transient outward membrane current in gastropod neural somata. J Physiol (Lond) 213:21–30

Davis WJ, Gillette R (1978) Neural correlates of behavioral plasticity in command neurons of *Pleurobranchaea*. Science 199:801–804

Eckert R, Lux WD (1975) A non-inactivating inward current recorded during small depolarizing voltage steps in snail pacemaker neurons. Brain Res 83:486–489

Eckert R, Tillotson D (1978) Potassium activation associated with intraneuronal free calcium. Science 200:437–439

Getting PA, Lennard PR, Hume RI (1980) Central pattern generator mediating swimming in *Tritonia*. Identification and synaptic interactions. J Neurophysiol 44:151–164

Gillette MU, Gillette R, Davis WJ (1978a) Cyclic AMP may modulate prolonged endogenous bursting and spike broadening in the ventral white cell of *Pleurobranchaea*. Soc Neurosci Abstr 4:1209

Gillette R, Kovac MP, Davis WJ (1978b) Command neurons in *Pleurobranchaea* receive synaptic feedback from the motor network they excite. Science 199:798–801

Gillette R, Gillette MU, Davis WJ (1978c) Prolonged endogenous bursting and spike broadening are substrates of command function in the feeding network of *Pleurobranchaea californica*. Soc Neurosci Abstr 4:1210

Gillette R, Gillette MU, Davis WJ (1980) Action potential broadening and endogenously sustained bursting are substrates of command ability in a feeding neuron of *Pleurobranchaea*. J Neurophysiol 43:669–685

Gillette R, Gillette MU, Davis WJ (1982) Substrates of command ability in a buccal neuron of *Pleurobranchaea*: II. Potential role of cyclic AMP. J Comp Physiol 146:461–470

Gola M (1976) Electrical properties of bursting pacemaker neurones. In: Salanki J (ed) Neurobiology of invertebrates. Plenum, New York, pp 381–423

Gorman ALF, Marmor AF (1970) Temperature dependence of the sodium-potassium permeability ratio of a molluscan neurone. J Physiol (Lond) 210:919–931

Gorman ALF, Thomas MV (1978) Changes in the intracellular concentration of free calcium ions in a pace-maker neurone, measured with the metallochromic indicator dye Arsenazo III. J Physiol (Lond) 275:357–376

Gorman ALF, Thomas MV (1980) Potassium conductance and internal calcium accumulation in a molluscan neurone. J Physiol (Lond) 308:287–313

Hagiwara S, Fukuda J, Eaton DC (1974) Membrane currents carried by Ca, Sr, and Ba in barnacle muscle fibers during voltage clamp. J Gen Physiol 63:564–578

Hermann A, Gorman ALF (1981) Effects of Tetraethylammonium on potassium currents in a molluscan neuron. J Gen Physiol 78:87–110

Hodgkin AL, Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol (Lond) 117:500–544

Kaczmarek LK, Strumwasser F (1981) The expression of long lasting after discharge by isolated *Aplysia* bag cell neurons. J Neurosci 1:626–634

Klein M, Kandel ER (1978) Presynaptic modulation of voltage-dependent $Ca^{+2}$ current: Mechanism for behavioral sensitization in *Aplysia californica*. Proc Natl Acad Sci USA 75:3512–3516

Kostyuk PG, Krishtal OA (1977) Effects of calcium and calcium chelating agents on the inward and outward current in the membrane of mollusc neurons. J Physiol (Lond) 270:569–580

Kupferman I (1970) Stimulation of egg-laying by extracts of neuronendocrine cells (bag cells) of abdominal ganglion of *Aplysia*. J Neurophysiol 33:877–881

Meech RW (1974a) Prolonged action potentials in *Aplysia* neurones injected with EGTA. Comp Biochem Physiol [A] 48:397–402

Meech RW (1974b) The sensitivity of *Helix aspersa* to injected calcium ions. J Physiol (Lond) 237:259–277

Meech RW (1978) Calcium-dependent potassium activation in nervous tissues. Annu Rev Biophys Bioeng 7:1–18

Meech RW, Standen NB (1975) Potassium activation in *Helix aspersa* neurons under voltage clamp: a component mediated by calcium influx. J Physiol 249:211–239

Pinsker W, Kupfermann I, Castelucci V, Kandel ER (1970) Habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. Science 167:1740–1742

Requena J, Dipolo R, Brinley FJ Jr, Mullins LJ (1977) The control of ionized calcium in squid axons. J Gen Physiol 70:329–353

Siegler MVS, Mpitsos GP, Davis WJ (1974) Motor organization and generation of rhythmic feeding output in the buccal ganglion of *Pleurobranchaea*. J Neurophysiol 37:1173–1196

Standen NB (1975) Voltage clamp studies of the calcium inward current in an identified snail neurone: comparison with the sodium inward current. J Physiol (Lond) 249:253–268

Stinnakre J, Tauc L (1973) Calcium influx in active *Aplysia* neurons detected by injected aequorin. Nature 242:113–115

Taghert PH, Willows AOD (1978) Control of a fixed action pattern by single, central neurons in the marine mollusk, *Tritonia diomedea*. J Comp Physiol 123:253–259

Thompson SH (1977) Three pharmacologically distinct potassium channels in molluscan neurons. J Physiol (Lond) 265:465–488