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CALCIUM DEPENDENCE OF VOLTAGE SENSITIVITY IN ADENOSINE 3′,5′-CYCLIC PHOSPHATE-STIMULATED SODIUM CURRENT IN *PLEUROBRANCHAEA*

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SUMMARY

1. Ionophoretic injection of cyclic AMP into a voltage-clamped molluscan neurone caused a transient slow inward current (I_{si}) whose amplitude was enhanced by depolarization. Na^+-replaced salines abolished the current, placing it with cyclic AMP-stimulated Na^+ currents of other gastropod species.

2. I_{si} amplitude was suppressed by extracellular Ca^{2+}. The amplitude increased up to 4-fold at holding potentials of -50 mV in nominally Ca^{2+}-free saline. Ion substitutions showed that Ca^{2+} suppressed I_{si} more effectively than Mg^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Ba^{2+} or Sr^{2+}.

3. Voltage sensitivity of I_{si} was abolished by low-Ca^{2+} salines, by the Ca^{2+} current blocker Co^{2+} and by substitution of Ba^{2+} or Sr^{2+} as Ca^{2+} channel current carriers. In such salines I_{si} showed no appreciable change in amplitude at holding potentials between -70 and -25 mV.

4. Intracellular injection of the Ca^{2+} chelator EGTA both augmented the amplitude of the current and its duration. EGTA injection failed to suppress the Ca^{2+}-dependent voltage sensitivity of I_{si}. Intracellular injection of concentrated 3-*N*-(morpholino) propanesulphonic acid (MOPS) pH buffer to inhibit secondary, Ca^{2+}-dependent intracellular acidification also failed to suppress the voltage sensitivity, as did injections of a mixed EGTA and MOPS solution.

5. While the data indicate a requirement for extracellular Ca^{2+} in conferring voltage sensitivity, they do not support a role for an intracellular action. An extracellular binding site for Ca^{2+} could mediate the voltage sensitivity, either by local depolarization-dependent changes in extracellular Ca^{2+} concentration or through direct voltage-sensitive block of the I_{si} channel.

INTRODUCTION

Interactions among second messenger pathways are increasingly well documented as integral aspects of cell regulation. This extends to single-neurone oscillators where evidence accumulates that second messenger interactions comprise positive and negative feed-back loops that form endogenous oscillatory mechanisms. Notably,

multiple interrelationships have been documented for the pathways of action of cyclic AMP, Ca^{2+} and H^+ (Kramer & Zucker, 1985*a*, *b*; Ewald, Williams & Levitan, 1985; Kaczmarek, Jennings, Strumwasser, Nairn, Walter, Wilson & Greengard, 1980; Green & Gillette, 1983; Gillette, 1987). These interactions determine the expression and character of neuronal bursting activity.

In endogenously oscillatory neurones of the mollusc *Pleurobranchaea* evidence was previously presented for an unknown factor acting in concert with cyclic AMP in stimulating a slow inward current (I_{si}). Specifically, while the cyclic AMP-stimulated I_{si} recorded from the whole neurone showed maximal activation in a region from −40 to −20 mV, single I_{si} channels recorded by patch methods were not sensitive to changing membrane potential (Green & Gillette, 1983). That is, focal depolarization of the channel-containing patch (about 1 μm^2 of membrane) had no effect on the frequency of channel opening. However, depolarization of the whole cell did increase the opening frequency for a period outlasting the depolarization by some seconds. These data were taken to indicate that the I_{si} channels were sensitive to some factor which accumulated as a result of depolarization. This factor would thus impart the observed voltage sensitivity and act synergistically with cyclic AMP in the stimulation of I_{si}. Both the ion dependence of the current and the origin of its voltage sensitivity has remained to be elucidated.

In this report we show that I_{si} is carried exclusively by Na^+. Further, we provide evidence that the factor conferring voltage sensitivity is Ca^{2+}. The data appear to exclude an intracellular site of action for Ca^{2+} in modulating the current.

METHODS

Pleurobranchaea californica were provided by Dr Rimmon C. Fay of Pacific BioMarine, Venice, CA and Mr Michael Morris of Sea-life Supply, Sand City, CA. Buccal ganglia were dissected from animals (20–200 g) and pinned under saline to a layer of Sylgard in the recording chamber. The identifiable, paired ventral white cell somata (Gillette, Gillette & Davis, 1980) were axotomized and isolated with a small clump of adjoining cells. Normal saline composition was (mM): NaCl, 420; $MgSO_4$, 25; $MgCl_2$, 25; KCl, 10; $CaCl_2$, 10, and 3-*N*-(morpholino) propanesulphonic acid (MOPS), 10, adjusted to pH 7·5 at 13 °C. Na^+-replaced saline was made by substituting arginine or tetramethylammonium on an equimolar basis. Replacement of bath Ca^{2+} was done by substituting Mg^{2+}. Nominally Ca^{2+}-free salines contained 6–10 μM-free Ca^{2+}, as measured with Ca^{2+}-selective electrodes. When the effects of other divalent cation substitutes for Ca^{2+} were tested, salines containing 10 mM-Ca^{2+}, Mn^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, Sr^{2+} or Mg^{2+} were used in SO_4^--free, Cl^--substituted salines.

For voltage clamping and intracellular cyclic AMP injection the ventral white cell soma was impaled with a single-barrelled voltage electrode and a double-barrelled current electrode (WPI TST150 thick septum capillaries). One barrel of the current electrode was filled with a solution of 0·2 M-cyclic AMP and 20 mM-Tris buffer, adjusted to pH 7·4 with KOH. Current ranging from 1 to 10 nA was passed between the two barrels to ionophorese cyclic AMP. Using a transport number of 0·1 for 0·2 M-cyclic AMP (Kononenko, Kostyuk & Scherbatko, 1983), the intracellular concentration of cyclic AMP at the end of a 5 s injection may have reached 10–100 μM. Permanent records were made on a Gould 2400 chart recorder. Current records were filtered above 15 Hz to remove noise.

Intracellular pressure injections were performed with a third electrode with a tip broken to 1–2 μm and connected by polyethylene tubing to a manually controlled pressure source. Solutions injected were variously: 200 mM-ethyleneglycol-bis-(β-amino-ethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 250 mM-MOPS buffer at pH 7·4; 1 M-MOPS at pH 7·6; 67 mM-EGTA, 1 M-MOPS at pH 7·4. Measurements were made immediately following an initial injection pulse and subsequently

after repeated pulses. Measurements tended to change very little after one or two pulses; final injected volumes were at least one to several soma volumes, judging from visible swelling of the injected neurone somata.

RESULTS

Na^+ dependence of I_{si}

Ionophoretic injections of cyclic AMP lasting 5–10 s cause a characteristic I_{si} response (Fig. 1) which is relatively constant over a prolonged period. Na^+-free saline in which Na^+ was completely replaced either with arginine or tetramethylammonium reversibly suppressed I_{si} (Fig. 1*A*), an effect not observed after replacement of Ca^{2+}, Mg^{2+} or K^+. Stepwise replacement of Na^+ with tetramethylammonium caused roughly linear and proportional decrease in I_{si} amplitude (not shown).

Figure 1*B* illustrates the characteristic voltage sensitivity of I_{si} between -70 and -20 mV in normal saline, and shows the virtual absence of the current in this voltage range in Na^+-free saline. Partial Na^+ replacement up to 63% did not appreciably affect the voltage sensitivity. Varying external K^+ had no effect on either the amplitude or the voltage sensitivity of the current. Li^+ substituted well for Na^+ as a charge carrier (not shown; Green, 1985). These observations indicate that the cyclic AMP-stimulated I_{si} of *Pleurobranchaea* neurones is carried by Na^+, and they are consistent with the reversal potential of the current estimated under patch and whole-cell voltage clamp (Green & Gillette, 1983).

The voltage sensitivity of the I_{si} is conveniently quantified and compared in different experimental situations as a ratio of the amplitudes of the cyclic AMP-stimulated current recorded at different voltages. For instance, a typical value measured in normal saline for the ratio of I_{si} (-30 mV)/I_{si} (-70 mV) is 1·8. The ratios measured in this way are constant over the low ranges of cyclic AMP injection currents used in these experiments. This procedure is used in analysis of experiments described below.

Ca^{2+} stimulation of I_{si}

The sensitivity of single I_{si} channels to whole-cell, but not focal, depolarization suggested that Ca^{2+} might be the factor that endows I_{si} with sensitivity to voltage (Green & Gillette, 1983). The voltage-activated Ca^{2+} current in the ventral white cell (Gillette *et al.* 1982*a*, *b*) could act agonistically with cyclic AMP in regulation of the ion channel. This hypothesis is supported by the results of bath Ca^{2+} replacement and addition of a Ca^{2+} current blocker.

Replacement of bath Ca^{2+} affected both the amplitude of the current and the voltage sensitivity. Reducing the Ca^{2+} concentration in the bath saline rapidly enhanced the current amplitude, over 4-fold in nominally Ca^{2+}-free saline at holding potentials of -50 mV (Fig. 2*A*; also see Aldenhoff, Hofmeier, Lux & Swandulla, 1983). Varying saline Ca^{2+} concentrations from 10 to 1 mM caused approximately linear decreases in the peak I_{si} (Fig. 2*B*).

In other experiments, addition of Co^{2+} to Ca^{2+}-containing saline further reduced I_{si}, suggesting that the suppressant effects of extracellular Ca^{2+} were a general property of divalent ions. Therefore, in three experiments we tested the effects of

substituting various divalent ions for Ca^{2+}, establishing a sequence of effectiveness of I_{si} suppression in which Ca^{2+} was found to be most effective. The sequence was found to be: $Ca^{2+} > Mn^{2+} > Cd^{2+} > Ba^{2+} > Co^{2+} > Sr^{2+} > Mg^{2+}$. Proportional effectiveness of the divalent ions was approximately 1·0:1·4:1·5:1·8:2·0:2·2:4·7, respectively. These data indicate an appreciable specificity for Ca^{2+} in the suppression of I_{si}.

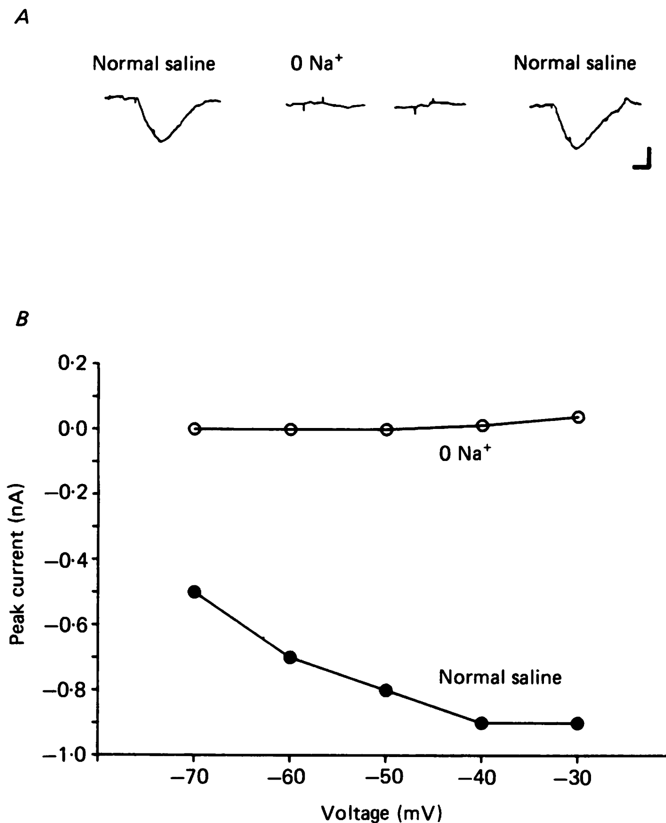


Fig. 1. Na^+ dependence of cyclic AMP-stimulated I_{si}. *A*, the inward current response to injected cyclic AMP in normal saline (left) at −40 mV is eliminated in Na^+-free saline (0 Na^+; middle). The inward current is restored in normal saline (right). Stimulus artifacts mark the 5 s period of cyclic AMP ionophoresis. Calibrations: horizontal, 5 s; vertical, 0·25 nA. *B*, the voltage sensitivity of I_{si} amplitude in normal saline (●) is contrasted with absence of I_{si} in Na^+-free saline over a 40 mV range (○).

Partial or complete replacement of saline Ca^{2+} with Mg^{2+} also caused partial or complete suppression of the voltage sensitivity of I_{si} ($n = 5$). Addition of Co^{2+}, a blocker of Ca^{2+} current, to Ca^{2+}-containing saline also severely reduced the voltage sensitivity ($n = 3$). Since changing the saline Ca^{2+} concentration alters I_{si} amplitude, normalized comparisons were used to measure effects of Ca^{2+} and Co^{2+} on the voltage sensitivity of the current. Figure 3 compares effects of salines containing different Ca^{2+} concentrations and added Co^{2+}. In 10 mM-Ca^{2+} the current response amplitude showed a large increase between −70 and −30 mV. Reduction of bath Ca^{2+} to 2 mM decreased the voltage sensitivity of the current. 10 mM-Co^{2+}, when added to saline

containing 2 mM-Ca^{2+}, suppressed the voltage sensitivity so that the difference in peak amplitude between −70 and −30 mV was virtually eliminated ($n = 3$). Bathing the cell in Ca^{2+}-free (Mg^{2+}-substituted) saline had the same result (not shown).

The requirement for extracellular Ca^{2+} in the voltage sensitivity of the I_{si} appears absolute. Substitution of saline Ca^{2+} by either Sr^{2+} ($n = 2$) or Ba^{2+} ($n = 2$), ions permeant to all known Ca^{2+} channels, in both cases abolished potentiation of I_{si} by

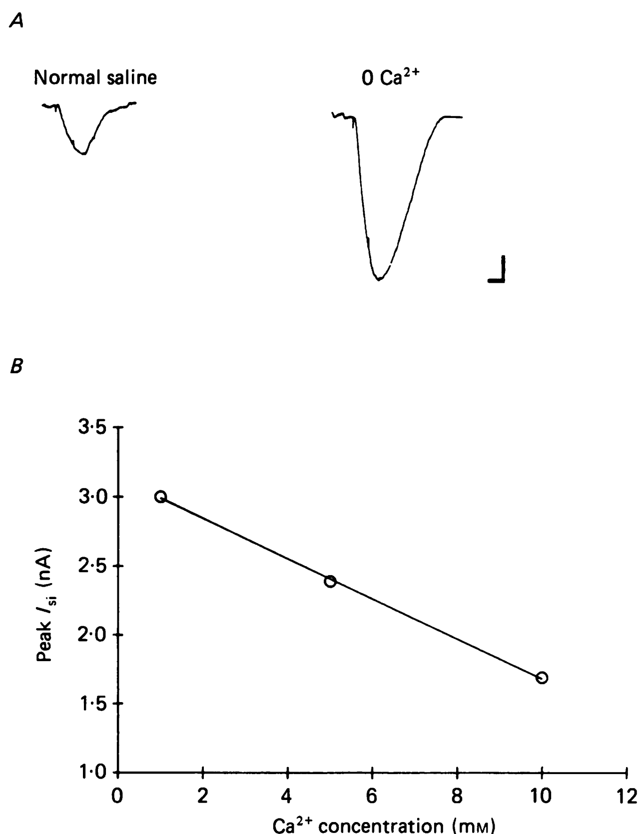


Fig. 2. Reducing extracellular Ca^{2+} enhances I_{si} amplitude. *A*, I_{si} recorded at a holding potential of −50 mV in normal saline and nominally Ca^{2+}-free saline (0 Ca^{2+}). Calibrations: horizontal, 5 s; vertical, 0·5 nA. *B*, graph of I_{si} measured in a sequence of extracellular Ca^{2+} concentrations of 10, 5 and 1 mM.

depolarization (Fig. 4*A* and *B*). The suppression of voltage sensitivity by Sr^{2+} and Ba^{2+} was rapidly reversible upon replacement of the bath solution with Ca^{2+}-containing saline.

These data show that extracellular Ca^{2+} is requisite for the voltage sensitivity of the cyclic AMP-stimulated I_{si} and that conditions blocking Ca^{2+} current are attended by loss of the effects of depolarization. Among several possibilities, these data are compatible with a role for Ca^{2+} entry through voltage-activated Ca^{2+} channels in mediating the voltage sensitivity of the I_{si}.

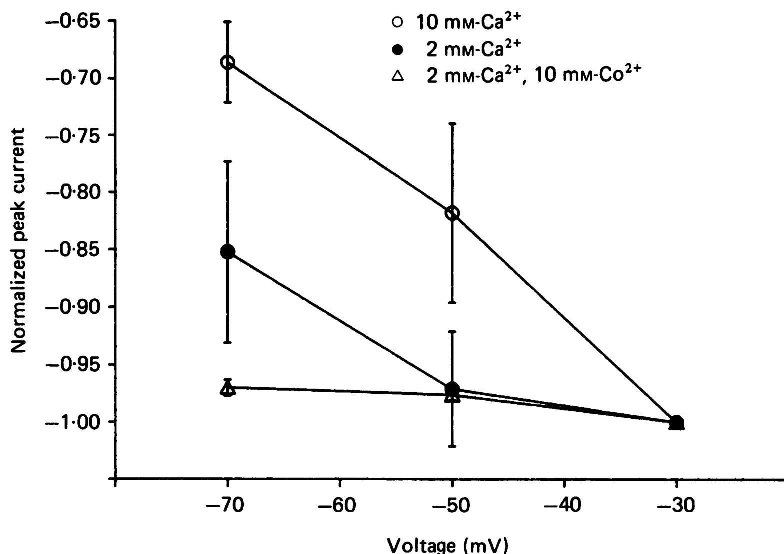


Fig. 3. Suppression of I_{si} voltage sensitivity by reduced extracellular Ca^{2+} levels and by the Ca^{2+} current blocker Co^{2+}. The graph shows the amplitudes of I_{si} at holding potentials of −70, −50 and −30 mV, normalized to the amplitudes recorded at −30 mV. Composite of data from three experiments.

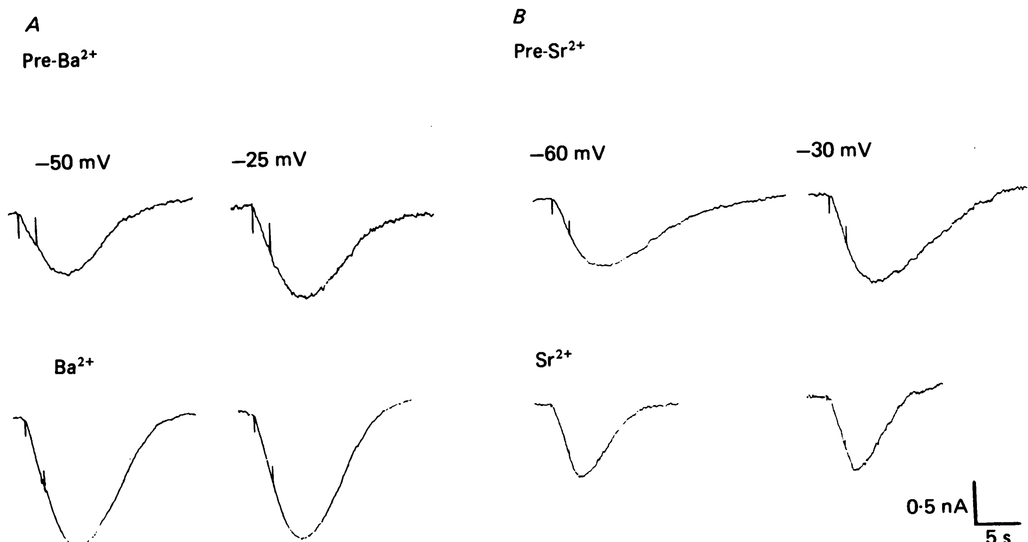


Fig. 4. Substitution for saline Ca^{2+} by Ba^{2+} or Sr^{2+} suppresses the voltage dependence of I_{si}. *A*, control records of I_{si} (upper traces) at holding potentials of −50 and −25 mV compared with records made shortly after saline substitution of Ba^{2+} (lower traces). Cyclic AMP ionophoretic current was reduced in Ba^{2+} to compensate for a large increase in I_{si} amplitude; this did not affect the voltage dependence. *B*, records of a similar experiment where Sr^{2+} substituted for Ca^{2+}. Cyclic AMP ionophoretic current was reduced in Sr^{2+} to compensate for a large increase in I_{si} amplitude.

Effects of intracellular injection of Ca^{2+} chelator and pH buffer

A possible intracellular role for Ca^{2+} in potentiating the cyclic AMP-stimulated I_{si} was tested by intracellular injection of the chelator EGTA. Such experiments also tested the requirement for intracellular Ca^{2+} as a co-factor in the cyclic AMP pathway of action (encompassing phosphorylation and dephosphorylation reactions) in stimulating the Na^+ current. Through chelation of intracellular Ca^{2+}, EGTA would be expected to reduce free levels to less than 10^{-8} M and block depolarization-induced accumulation of the near-micromolar levels sufficient to activate most known Ca^{2+}-activated cell processes.

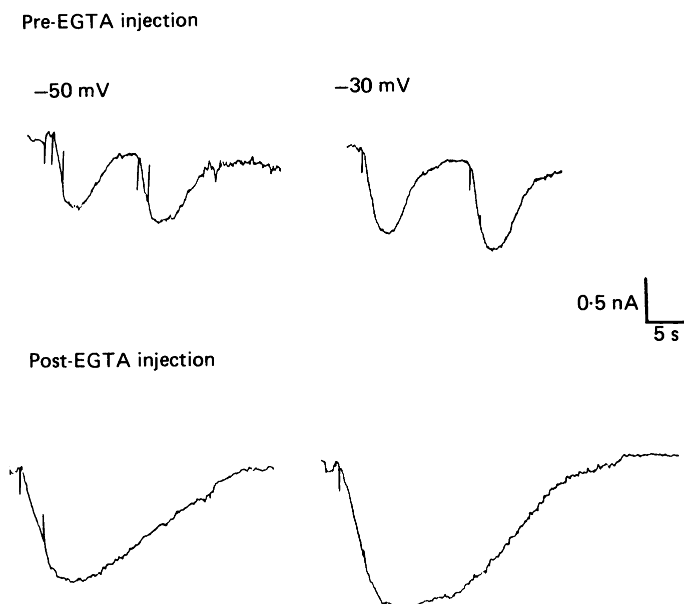


Fig. 5. I_{si} recorded at −50 and −30 mV holding potentials (upper records) is augmented by intracellular pressure injection of 0·2 M-EGTA (lower records). While EGTA injections enhance I_{si} amplitude and duration, the voltage sensitivity of the current remains, reflected in the greater amplitude of the response at −30 mV.

Results of intracellular EGTA injections ($n = 7$) indicate that appreciable levels of free intracellular Ca^{2+} are not necessary for the stimulation of I_{si} by cyclic AMP. The prominent effect of EGTA injections in these experiments was a large increase in the amplitude and duration of I_{si}, measureable within 10 s of injection (Fig. 5). This increase is attributable to EGTA-induced reduction of cyclic AMP degradation rate (see Discussion) and is evidence of successful injections. However, the voltage dependence of the current remained (Fig. 5) even after multiple injections sufficient to swell the neurone somata visibly by several volumes. In three experiments, peak currents elicited by cyclic AMP injection were compared at holding potentials of −50 and −30 mV. After EGTA injection the averaged ratio of I_{si} recorded at −30 and −50 mV was somewhat reduced, but enhancement of the I_{si} by

depolarization was not abolished (Table 1). The trend in the averaged results reflects an incomplete reduction in the voltage sensitivity in only one of three experiments.

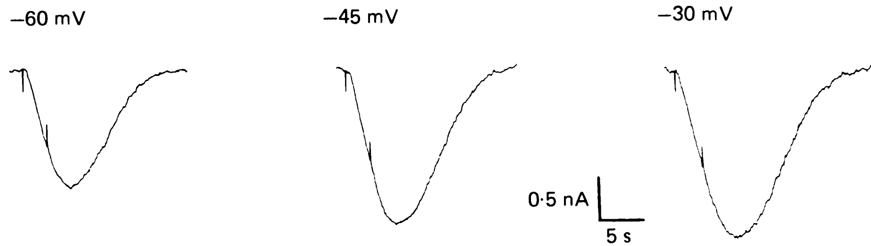
The negative results from intracellular Ca^{2+} chelation suggest that while the event conferring I_{si} voltage sensitivity could be dependent on Ca^{2+} entry, it may proceed in the presence of EGTA. One such process is intracellular acidification, which occurs in many types of cells as a result of Ca^{2+} entry (Ahmed & Connor, 1980; Meech & Thomas, 1980). Intracellular acidification may be potentiated by EGTA as

TABLE 1. Voltage dependence of I_{si} expressed as ratios of the amplitudes of the current recorded at different holding potentials. Averaged values are compared for conditions before and after intracellular injections of Ca^{2+} chelator and/or pH buffer. Numbers in parentheses are 95% confidence intervals

	Pre-injection	Post-injection
EGTA injection ($n = 3$)		
$\frac{I_{si} \text{ at } -30 \text{ mV}}{I_{si} \text{ at } -50 \text{ mV}}$	1·28 (1·23–1·35)	1·18 (1·16–1·20)
MOPS injection ($n = 2$)		
$\frac{I_{si} \text{ at } -30 \text{ mV}}{I_{si} \text{ at } -70 \text{ mV}}$	1·79 (1·76–1·83)	1·64 (1·48–1·82)
$\frac{I_{si} \text{ at } -30 \text{ mV}}{I_{si} \text{ at } -50 \text{ mV}}$	1·26 (1·22–1·30)	1·12 (1·05–1·20)
EGTA and MOPS injection ($n = 3$)		
$\frac{I_{si} \text{ at } -30 \text{ mV}}{I_{si} \text{ at } -60 \text{ mV}}$	1·43 (1·39–1·483)	1·48 (1·39–1·57)
$\frac{I_{si} \text{ at } -30 \text{ mV}}{I_{si} \text{ at } -45 \text{ mV}}$	1·13 (1·08–1·19)	1·24 (1·17–1·31)

a result of dislodgement of protons from the chelator by Ca^{2+} binding; Ahmed & Connor (1980) titrated a release of 2 H^{+} for each Ca^{2+} bound. In present experiments such acidification may not have been blocked by the relatively low ratio of pH buffer to EGTA used. To block intracellular acidification, 1 M-MOPS buffer, pH 7·6, was injected in two experiments. The voltage dependence of I_{si}, measured as the ratios of I_{si} amplitudes at different voltages, was not significantly altered in either experiment (Table 1).

Combined injection of EGTA and a strong MOPS buffer, pH 7·4, in a 1:15 ratio also failed to suppress the voltage dependence of the I_{si} in three experiments (Fig. 6; Table 1). The records of Fig. 6 show that the voltage dependence of the current amplitude, unaffected by EGTA and MOPS injection, was still susceptible to suppression. In spite of repeated injections of the Ca^{2+}chelator and strong pH buffer mixture, I_{si} in the injected neurone was still markedly enhanced by nominally Ca^{2-} free saline and the voltage dependence was abolished (Fig. 7). Neither chelation of intracellular Ca^{2+} nor intracellular 'pH clamp' appeared to suppress the voltage sensitivity.



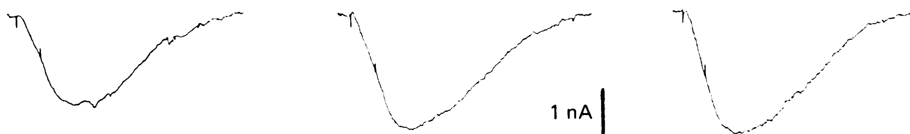


Fig. 6. Intracellular injection of a solution combining EGTA (67 mM) and MOPS (1 M) (pH 7·4) augmented I_{si} at all holding potentials. The combination of both Ca^{2+} chelator and strong pH buffer failed to suppress the Ca^{2+}-dependent voltage sensitivity of the I_{si}. The gain of the responses recorded pre-injection (upper records) is twice that of the post-injection responses (lower records).

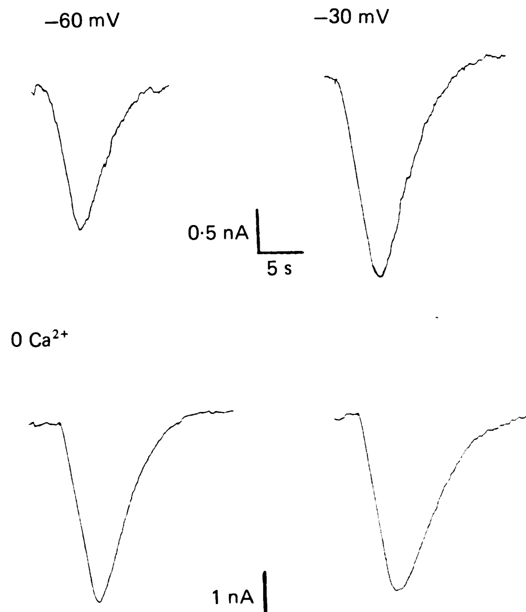


Fig. 7. A neurone injected with combined EGTA and MOPS solution and still showing depolarization enhancement of cyclic AMP-stimulated I_{si} (upper records) was still susceptible to suppression of the voltage sensitivity in Ca^{2+}-free ($0Ca^{2+}$; Mg^{2+}-substituted) saline (lower records). Note that the gain is reduced by one-half in the lower records.

DISCUSSION

Na^+ dependence of the slow inward current

The Na^+ dependence of I_{si} places it with cyclic AMP-stimulated Na^+ current described from neurones of other gastropods (Liberman, Minina & Golubtsov, 1975; Aldenhoff *et al.* 1983; Connor & Hockberger, 1984; Swandulla & Lux, 1984), one that is most pervasive and striking in the molluscan C.N.S. The current is present in many neurones of the feeding motor network in the buccal ganglion of *Pleurobranchaea* (R. Gillette & D. J. Green, unpublished observations). A similar current is activated by neuropeptide hormones in neurones of the buccal ganglion of *Aplysia* (Kirk & Scheller, 1986).

Mechanisms mediating Ca^{2+} dependence of voltage sensitivity

The evidence confirms that cyclic AMP stimulation of I_{si} is potentiated by depolarization (Green & Gillette, 1983), and shows further that potentiation is dependent on extracellular Ca^{2+}. Removal of extracellular Ca^{2+} blocked voltage sensitivity of I_{si} (Fig. 2).

In addition to abolishing the voltage sensitivity of I_{si}, a marked effect of reducing the Ca^{2+} concentration of the extracellular saline was to enhance the current amplitude (Fig. 2); i.e. extracellular Ca^{2+} acted to suppress the current. Ion substitions showed that this was a general property of divalent cations, but that Ca^{2+} was most effective among seven divalent ions tested. These data are compatible with a fairly specific external binding site for Ca^{2+} which may normally influence I_{si}, perhaps directly at the channel or indirectly through somehow modulating the cyclic AMP pathway of phosphorylation and dephosphorylation. In either event, the dependence of the I_{si} amplitude on extracellular Ca^{2+} provides a possible mechanism of the voltage sensitivity of I_{si}, as will be discussed.

A requirement for Ca^{2+} and the suppression of voltage sensitivity of the current by a blocker of Ca^{2+} current (Fig. 3) are consistent with a role for Ca^{2+} entry through conventional voltage-activated Ca^{2+} channels in lending voltage sensitivity to the cyclic AMP-dependent current. However, neither I_{si} itself nor its depolarization-induced potentiation were abolished by injection of the Ca^{2+} chelator EGTA. This also indicates that resting levels of intracellular free Ca^{2+} are not necessary for the operation of the Na^+ channel, or for the kinase and phosphatase enzymes which presumably shape the cyclic AMP dependence of channel operation. More to the point, the data indicate that Ca^{2+} does not act intracellularly to exert potentiating effects, because otherwise the chelator should have prevented significant intracellular Ca^{2+} accumulation.

An alternative to direct intracellular Ca^{2+} regulation of I_{si} is that Ca^{2+}-dependent changes in intracellular pH might underlie the current's voltage dependence. Experiments where strong pH buffer was injected intracellularly were designed specifically to test this possibility. Grounds for suspecting a role for pH were that Ca^{2+} influx during depolarization can decrease intraneuronal pH (Ahmed & Connor, 1980; Meech & Thomas, 1980), and that the I_{si} amplitude is, in fact, enhanced by slight acidification (Green & Gillette, 1985; also see Aldenhoff *et al.* 1983). The effect of intracellular pH is thought to be mediated through a pH-sensitive cyclic AMP

phosphodiesterase (Calhoon & Gillette, 1983; Gillette & Green, 1983*a,b*; Green & Gillette, 1985). However, the possible role of intracellular pH as the intermediary regulator of voltage sensitivity was not borne out, since buffer injection failed to suppress heightened activation of I_{si} at depolarized voltages.

The results of combined EGTA and MOPS injections seem to rule out a remaining possibility for an intracellular mechanism underlying the voltage dependence: that Ca^{2+} or H^{+} can both regulate the current at intracellular sites, and that either could act in the absence of the other. Combined intracellular Ca^{2+} and pH buffering left voltage sensitivity intact. Thus, another action is indicated. Two possibilities are: (1) Ca^{2+} entry through conventional voltage-activated channels could regulate I_{si} through depletion of free Ca^{2+} in the extracellular space, and (2) Ca^{2+} block of the I_{si} channel could be directly sensitive to depolarization.

Enhancement of I_{si} through reduction of extracellular Ca^{2+} (e.g. Fig. 2) potentially confers the observed voltage dependence. Depletion of extracellular Ca^{2+} through neurone depolarization is well documented in vertebrate brain (Nicholson, Phillips, Tobias & Kraig, 1981; Heinemann & Pumain, 1981; Morris, 1981; Prince, Benninger & Kadis, 1981). Failure of Sr^{2+} and Ba^{2+} to confer detectable voltage sensitivity in the absence of Ca^{2+} may reflect greater mobility in the cellular interstices, and hence lesser susceptibility to depletion at the neurone surface. Thus, local extracellular depletion of Ca^{2+} is a potential cause of the voltage sensitivity of I_{si}.

Alternatively, the ion could enter and block the channels in a depolarization-dependent fashion. Notable precedents are the *N*-methyl-D-aspartate-activated ion channels of mammalian neurones blocked by Mg^{2+} in a voltage-dependent fashion (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer & Westbrook, 1985), and a voltage-dependent Ca^{2+} block of Na^{+} channels in neuroblastoma cells (Yamamoto, Yeh & Narahashi, 1984).

The present data also indicate that the mechanisms regulating the voltage sensitivity are yet more complicated and at least dual. In Ca^{2+}-free salines I_{si} shows no voltage sensitivity from -70 to -25 mV, failing to extrapolate towards a likely Na^{+} reversal potential. I_{si} amplitude remains anomalously constant at all holding potentials between -70 and -25 mV, instead of decreasing as the neurone is depolarized towards the expected reversal potential ($+45$ to $+50$ mV; Green & Gillette, 1983). To account for this behaviour another mechanism must be postulated, one independent of both voltage-dependent Ca^{2+} current and changes in intracellular Ca^{2+} and H^{+} concentrations. The nature of such a mechanism is speculative and awaits analysis at the level of the single channel.

Functional significance of cyclic AMP and Ca^{2+} co-regulation of the slow inward current

In bursting neurones such as the ventral white cells of *Pleurobranchaea* (Gillette, Gillette & Davis, 1982*b*; Gillette & Gillette, 1983), Ca^{2+}-dependent voltage sensitivity of I_{si} may act as a positive feed-back mechanism in initiating and sustaining the burst episode. It may also contribute to the triggerability of burst episodes in quiescent cells, where transient depolarization by injected current can trigger a prolonged burst (Gillette *et al.* 1980; Gillette, Gillette & Davis, 1982*a*). Resting

levels of I_{si}, determined by resting levels of cyclic AMP, may be potentiated by Ca^{2+}-dependent voltage sensitivity to support the endogenous burst episode.

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