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CALCIUM-MEDIATED INACTIVATION OF CALCIUM CURRENT IN *PARAMECIUM*

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SUMMARY

1. The Ca current seen in response to depolarization was investigated in *Paramecium caudatum* under voltage clamp. Inactivation of the current was measured with the double pulse method; a fixed test pulse of an amplitude sufficient to evoke maximal inward current was preceded by a conditioning pulse of variable amplitude (0–120 mV).

2. The amplitude of the current recorded during the test pulse was related to the potential of the conditioning pulse. Reduction of test pulse current was taken as an index of Ca current inactivation. The current recorded during a test pulse showed a progressive decrease to a minimum as the potential of the conditioning pulse approached +10 to +30 mV. Further increase in conditioning pulse amplitude was accompanied by a progressive restoration of the test pulse current. Conditioning pulses near the calcium equilibrium potential had only a slight inactivating effect on the test pulse current.

3. Injection of a mixture of Cs and TEA which blocked late outward current had essentially no effect on the inward current or its inactivation.

4. Elevation of external Ca from 0·5 to 5 mM was accompanied by increased inactivation of the test pulse current. The enhanced inactivation of the test pulse current was approximately proportional to the increase in current recorded during the conditioning pulse.

5. Following injection of the Ca chelating agent, EGTA, the inactivation of the test pulse current was diminished; in addition, the transient inward current relaxed slightly more slowly, and the transient was followed by a steady net inward current.

6. The time course of recovery from inactivation in the double pulse experiment approximated a single exponential having a time constant of 80–110 msec. Injection of EGTA shortened the time constant by as much as 50%.

7. It is concluded that interference with the entry of Ca or enhanced removal of intracellular free Ca^{2+} interferes with the process of Ca current inactivation, while enhanced entry of Ca promotes the process of inactivation. While the mechanism of inactivation is unknown, arguments are presented that the accumulation of intracellular Ca influences the Ca channel conductance.

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INTRODUCTION

The voltage-dependent Ca channel has been reported to undergo inactivation in response to maintained depolarization (Standen, 1974; Okamoto, Takahashi & Yoshii, 1976) in a manner qualitatively similar to that originally described for the Na channel in nerve (Hodgkin & Huxley, 1952). Recent studies on the protozoan *Paramecium* have also indicated that inactivation of the Ca channel occurs upon depolarization (Oertel, Schein & Kung, 1977; Brehm & Eckert, 1978). This inactivation was observed as both a relaxation of inward current during a single maintained depolarization and as a depression of peak inward current recorded during the second of paired depolarizations. Unlike the case of the Na channel in nerve however, this inactivation appears to result from the Ca entering upon depolarization rather than from the membrane potential effects alone (Brehm & Eckert, 1978). Thus, we use the term 'inactivation' to refer to the general phenomenon of channel closure and temporary resistance to reopening upon depolarization. The term offers no information as to the mechanism which leads to the conductance decrease.

In the present study we present further evidence for a Ca-mediated inactivation of the Ca channel. Further, we provide evidence that it is accumulation of ionized Ca within the cell and not the flow of Ca current across the plasma membrane which is responsible for the inactivation. Recovery from this inactivation proceeds along an exponential time course and also depends on the level of intracellular free Ca.

METHODS

Cultures of *Paramecium caudatum* were grown on hay infusion medium by methods described elsewhere (Naitoh & Eckert, 1972). Cells were isolated in a solution that consisted of 1 mM-$CaCl_2$ + 4 mM-KCl + 1 mM-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffer adjusted to pH 7·2 with KOH.

Selected cells were voltage clamped by conventional means with intracellular electrodes. The membrane potential was measured between an intracellular glass capillary electrode (10–20 MΩ) and a similar electrode placed in the bath with its tip near the cell surface. The potential difference between the electrodes was controlled through a feed-back amplifier (Datel, AM303) with an open loop gain of 10,000 × and whose output led to a shielded current-passing electrode (10–20 MΩ). The holding potential was always set equal to the resting potential (−22 to −42 mV) by adjusting the holding current to zero level. Command steps from holding level were generally complete within 1·0 msec and the capacitive current transients within 1·5 msec. Membrane current was measured across a 1 MΩ resistor in a virtual ground circuit with a time constant of less than 0·1 msec.

Voltage-dependent Ca and K channels in *Paramecium* were shown to reside in the ciliary membrane (Dunlap, 1977). The soma was found to be isopotential (Eckert & Naitoh, 1970), and calculations based on specific resistance of ciliary membrane suggest that with the membrane at rest the cilia are also isopotential (Dunlap, 1977). At rest the calculated length constant is approximately 14 times the length of a cilium (Dunlap, 1977). The conductance of a single cilium in *P. caudatum* during a 30 mV depolarization (potential at which maximum inward current is observed) is about 17 pS (Eckert & Brehm, 1979), compared to resting ciliary input conductance of 2·5 pS in a cell with a 20 MΩ input resistance. Thus a calculated sevenfold increase in ciliary membrane conductance upon depolarization should reduce the length constant to approximately one half the calculated resting value, leaving the cilium in an isopotential state even at the peak of the inward current. However, with large conditioning steps to potentials near E_{Ca}, a large outward current (60–80 nA) flows during which the cilia may not be adequately space clamped. For this reason membrane currents were only plotted for depolarizations of less than 50 mV.

The standard recording solution contained 4 mM-KCl + 1 mM-$CaCl_2$ + 1 mM-HEPES (pH 7·2). In experiments in which the Ca concentration was altered the ionic strength was held constant by substituting $MgCl_2$ for $CaCl_2$. This procedure essentially eliminated the changes in resting potential that occur with changes in the ionic strength of the recording solution (Satow & Kung, 1979; Eckert & Brehm, 1979).

Solutions for intracellular ionophoresis were introduced into micro-electrodes having tip dimensions similar to the other electrodes. To prevent premature leakage into the cell, the injection electrode was inserted into the cell only after the pre-injection parts of the experiment were completed. These solutions were 50 mM-CsCl + 50 mM-TEA Cl (tetraethylammonium Cl) + 10 mM-Pipes (piperazine-N, N-bis-2 ethanesulphonic acid) buffer (pH 7·0), or 100 mM-K EGTA (ethylene-glycol-bis-aminoethylether-N, N-tetraacetic acid) + 10 mM-Pipes buffer (pH 7·0). Ionophoresis was carried out for periods of 1 min with a 10 nA current while the membrane potential was clamped to holding level. Responses to iontophoretic injection were generally observed within the first 1 min injection. Injections were repeated until no further increase in effect was observed. Experiments were performed at room temperature.

RESULTS

I *Inactivation of Ca current*

A rapidly activating inward current was observed with depolarizations of 5 mV or greater. The maximum transient inward current in a 1 mM-Ca solution was recorded with depolarizations to -10 mV (Fig. 1*A*). In this range of depolarization a nonlinear outward current was also prominent following the inward current. In response to a step depolarization the inward current was fully activated within 3 msec and then relaxed within an additional 5 msec. The evidence that this current is carried by Ca is reviewed elsewhere (Eckert, 1972; Naitoh & Eckert, 1974; Eckert *et al.* 1976; Oertel *et al.* 1977).

The inward current recorded during a test pulse (pulse II) depends on the potential of a prior conditioning pulse (pulse I) (Oertel *et al.* 1977; Brehm & Eckert, 1978). When the potential of pulse I was progressively increased toward $+10$ to $+30$ mV, the inward current recorded during pulse II progressively decreased (Fig. 1*B*).

Several lines of evidence indicate that the depression of the pulse II net inward current results from an actual decrease in the Ca current and not from an increase in outward K current that would partially cancel inward Ca current. First, Fig. 1*C* indicates that the depression of inward current recorded during pulse II was not accompanied by a change in the amplitude of the late outward current. An increase in the late potassium current should be seen during pulse II if an enhanced K conductance were to persist following pulse I. Secondly, the depression of pulse II inward current produced by pulse I was unaffected by ionophoretic injection of Cs and TEA, agents that interfere with K currents (Fig. 1*B*). In seven cells examined the ionophoresis of these two K-conductance blocking agents resulted in strong reduction of the outward current without significantly affecting the relaxation of the early inward current or the depression of the inward current (Fig. 1*A* and *C*).

II *Inactivation is dependent on Ca entry*

Maximum inactivation of the pulse II current was observed following pulse I potentials to $+10$ to $+20$ mV in 1 mM-Ca solution (Figs. 1*B* and 2*B*). Further increases in the potential of pulse I produced progressively less inactivation of the pulse II current (Fig. 2*B*). In a 1 mM-Ca solution, using an 80-msec interval, the

pulse II inward current regained its full strength when pulse I reached approximately +60 mV. However, at shorter intervals (which produce more complete inactivation, e.g., as in Fig. 3*B*) the return of pulse II current to full strength occurred following pulse I potentials much closer to the estimated Ca equilibrium potential (E_{Ca} = +116 mV when 1 mM-Ca is present in the recording solution and the internal Ca concentration is assumed to be 10^{-7} M).

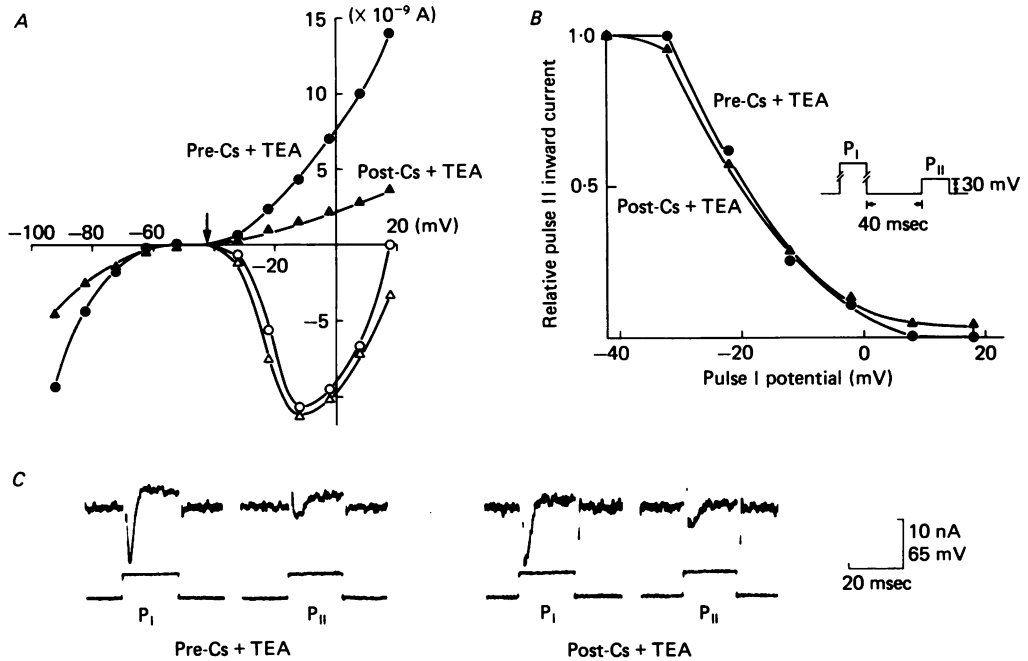

Fig. 1. The effect of ionophoretic injection of Cs^+ and TEA^+ on inactivation of the Ca current. *A*, *I–V* relations of pulse I currents. Peak inward current was measured before (○) and after (△) intracellular ionophoresis (three 1 min injections of 10^{-8} A current) of Cs^+ and TEA to block K current. Late current measured at the end of a 20 msec pulse is shown before (●) and after (▲) injection. Holding potential, indicated by arrow, was set equal to V_{rest} (−42 mV). *B*, inactivation of pulse II inward current as a function of pulse I potential (see inset). A variable amplitude 20 msec depolarization (pulse I) was followed after 40 msec by a 30 mV, 20 msec depolarization (pulse II). Pulse II current amplitude was plotted as a function of pulse I potential and was normalized relative to the pulse II current when pulse I was not applied. Curves indicate relative inactivation in a single cell measured before (●) and after (▲) introduction of Cs and TEA. *C*, representative membrane currents (I_m) with pulses I and II both of the same amplitude before (left) and after (right) injection of Cs and TEA. Bath contained 1 mM-$CaCl_2$ + 4 mM-KCl + 1 mM-HEPES (pH 7·2).

The relationship between pulse I potential and the inward current recorded during pulse II suggested that Ca entry during pulse I may be responsible for the decrease in current during pulse II. Ca entry increases with depolarization up to at least −10 mV, due to voltage-dependent increases in Ca conductance (Fig. 1*A*) (evidence from ciliary activity indicates similar voltage dependence of Ca entry; Machemer & Eckert, 1973). As the potential was further increased toward E_{Ca}, Ca entry during pulse I is presumed to decrease due to a diminished driving force acting on Ca. It

will be noted that the maximum pulse I inward current seems to occur at lower potentials than the maximum depression of pulse II inward current (Figs. 1*A*, *B*, 2*A* and *B*). This apparent discrepancy may result from contamination at high potentials by an outward current. This possibility received support from the low potential at which the early-current plot intercepts the voltage axis. Further support for this explanation comes from a study of *Aplysia* neurones in which a non-linear, voltage-dependent leakage current was revealed in Co-containing solution (Tillotson,

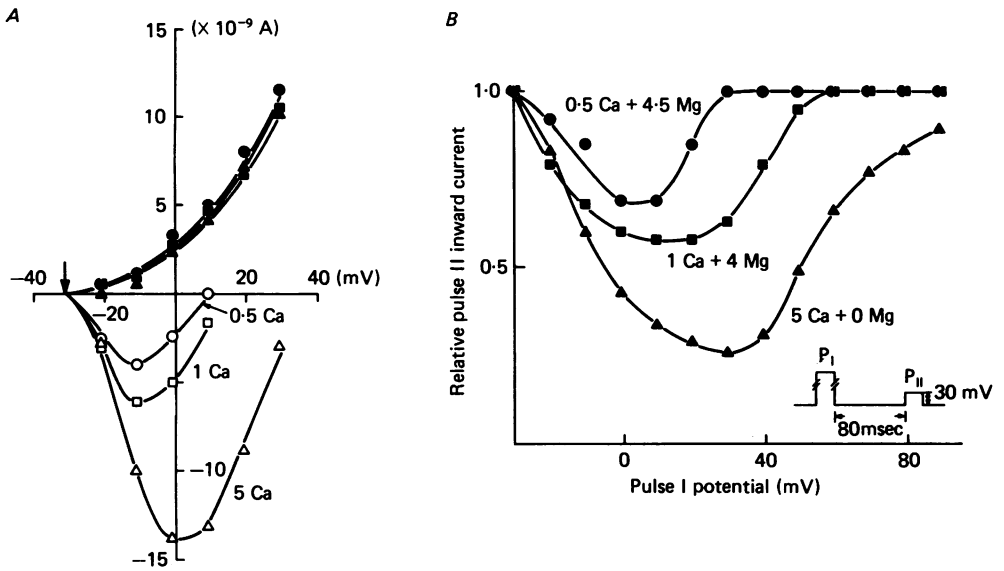

Fig. 2. Ca currents and inactivation at different Ca concentrations. *A*, *I–V* relations for pulse I at three concentrations of extracellular Ca. The peak inward current (open symbols) and outward current at the end of a 20 msec depolarization (filled symbols) are shown for 0·5 mM-Ca + 4·5 mM-Mg (○), 1·0 mM-Ca + 4·0 mM-Mg (□), and 5·0 mM-Ca (△) solutions. All solutions contained 4 mM-KCl + 1 mM-HEPES (pH 7·2). Arrow indicates holding potential. 21 °C. *B*, relative pulse II inward current plotted against pulse I potential at three external Ca concentrations. Same cell as in *A*. Ordinate, pulse II current amplitude relative to pulse II current recorded in the absence of pulse I.

1979). Such leakage current measurements could not be made on *Paramecium* due to the inability of Co, Mn, and La to effectively block inward Ca current (Naitoh *et al.* 1972; Friedman & Eckert, 1973).

Inactivation of the inward current was compared in three different Ca concentrations: 0·5 mM (E_{Ca} = +107 mV), 1 mM (E_{Ca} = +116 mV), and 5 mM (E_{Ca} = +136 mV) where Ca was replaced by Mg. The corresponding ratios of inward current maxima were 0·30:0·46:1·0 (Fig. 2*A*). The late outward current was similar for all three extracellular Ca concentrations, further indicating that the outward current is independent of Ca entry (Fig. 2*A*). A plot of inactivation (as measured by the decrease in the inward current during pulse II) at these Ca concentrations (Fig. 2*B*) shows that maximum inactivation increased as the extracellular Ca concentration was elevated. The ratios of maximum inactivation were 0·43:0·56:1·0, for concentrations of 0·5, 1 and 5 mM-Ca respectively.

III *Inactivation retarded by intracellular EGTA*

Membrane currents were recorded in the same cells before and after intracellular ionophoresis of EGTA. Before the injection the rapidly inactivating inward current was followed by a small outward current (Fig. 3*C*). Following ionophoresis of EGTA the early inward current relaxed somewhat more slowly and was followed by a steady inward current that persisted for at least several seconds under maintained de-

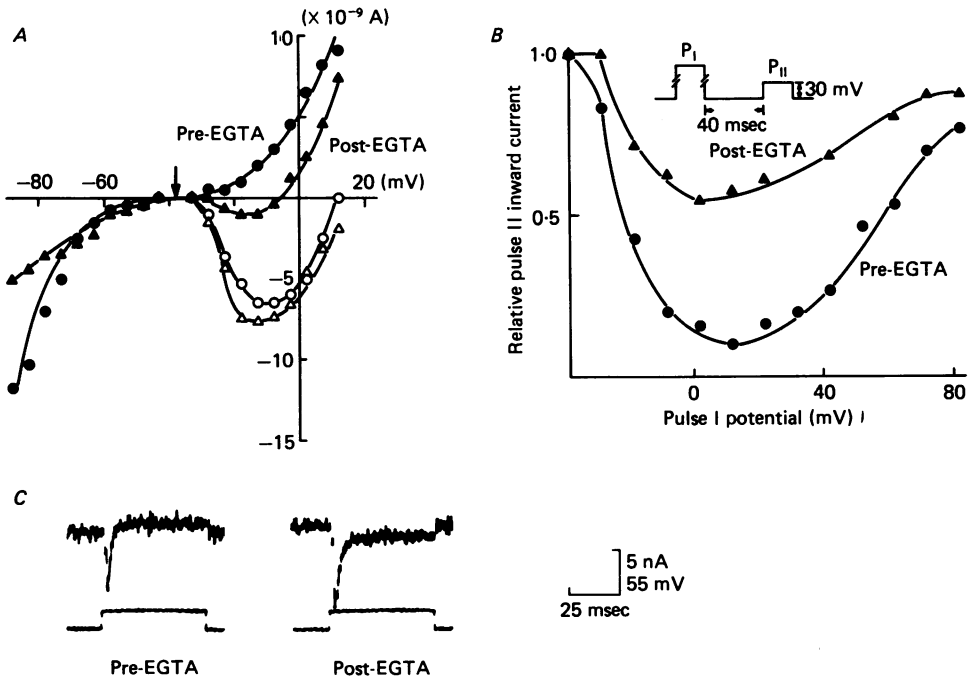

Fig. 3. Effects of injected EGTA. *A*, *I–V* relations before (○) and after (△) EGTA injection, showing early inward current (open symbols) and late current measured at the end of a 50 msec pulse (filled symbols). Holding potential indicated by the arrow. The bath contained 1 mM-$CaCl_2$ + 4 mM-KCl + 1 mM-HEPES (pH 7·2). 21 °C. *B*, inactivation of pulse II Ca current as a pulse I potential before (●) and after (▲) injection of EGTA (see inset). The ordinate gives current relative to pulse II current recorded when pulse I was not applied. *C*, representative membrane currents during single 50 msec, 20 mV depolarization before (left) and after (right) EGTA injection (three 1 min injections of 10^{-8} A current). Same cell used throughout.

polarization. This is seen as a region of negative slope resistance and inward current between −5 and −30 mV in the late *I–V* relations (Fig. 3*A*). The ascending limb of the late outward current was shifted to more positive values. The late inward current grew in size with successive EGTA injections but never exceeded 2 nA.

The *I–V* relations following EGTA injection (Fig. 3*A*) showed no consistent increase or decrease in the peak inward current, nor any change in the rate of inward current development. Thus, the slight increase in peak Ca current in Fig. 3 could not be consistently reproduced. The injected EGTA thus appears to have little or no effect on Ca activation.

Inactivation examined by the paired pulse method in six cells before and after EGTA injection was found to be significantly weaker for all pulse I potentials following injection (Fig. 3*B*).

IV *Recovery from inactivation*

The results described above indicate that Ca inactivation depends in some way on elevation of intracellular free Ca^{2+}. Recovery from inactivation should therefore occur as a direct or indirect consequence of the removal of intracellular free Ca from

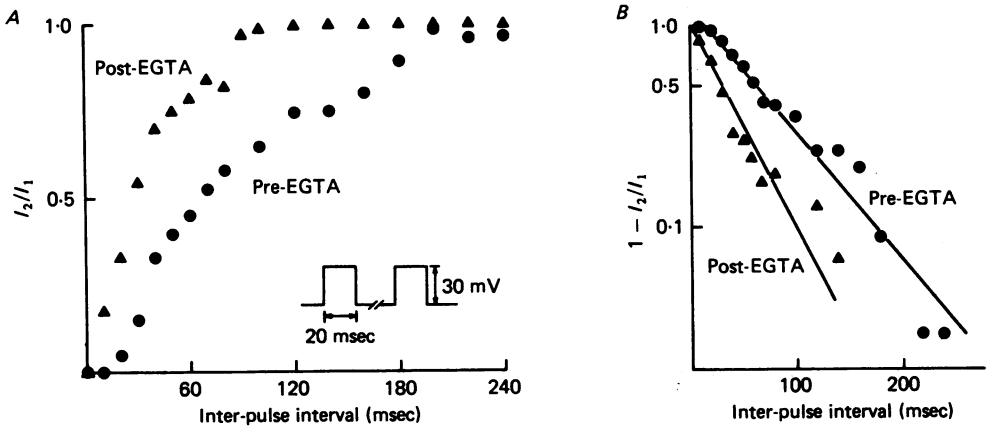

Fig. 4. Time-dependent removal of inactivation. *A*, relative pulse II current plotted against pulse I-pulse II interval (see inset). Pulse amplitudes were both 30 mV and the ratio of pulse II to pulse I current (I_2/I_1) was determined for different pulse intervals before (●) and after (▲) ionophoretic injection of EGTA from a pipette containing 100 mM-EGTA. *B*, same data plotted semilogarithmically against pulse interval before (●) and after (▲) ionophoretic injection of EGTA (three 1 min injections at 10^{-8} A current). Curve fitted by eye. The time constant before injection was 102 msec and after injection was 48 msec. The bath contained 1 mM-$CaCl_2$ + 4 mM-KCl + 1 mM-HEPES (pH 7·2). 21 °C.

the vicinity of the Ca channels. The time course of recovery was determined with paired identical pulses delivered at variable intervals (Fig. 4*A*). The inactivation (i.e., 1-(pulse II current/pulse I current)) was plotted against pulse interval (Fig. 4*B*). Recovery from inactivation occurred as a single exponential with a time constant ranging from 80 to 110 msec (five cells). This is close to the 80–90 msec recovery time reported for *P. tetraurelia* (Oertel *et al.* 1977). EGTA was injected in the same five cells, and in all cases injection of EGTA decreased the time constant for recovery. The decrease in time constant varied between cells, and depended on the amount of EGTA injected. Injection of greater quantitites of EGTA increased the rate of recovery from inactivation, but the effectiveness of the EGTA diminished with time after injection.

DISCUSSION

The inward Ca current activated by depolarization in *Paramecium* undergoes a rapid relaxation (Naitoh & Eckert, 1974; Oertel *et al.* 1977; Brehm & Eckert, 1978; Satow & Kung, 1979). The major fraction of Ca current relaxation has been shown to be a true inactivation of the Ca current, rather than a charge cancellation by superposition of a late outward current upon a non-inactivating Ca current (Oertel *et al.* 1977; Brehm & Eckert, 1978). The evidence presented here together with that reported earlier (Brehm & Eckert, 1978) indicates that the inactivation of the Ca current results from the entry of Ca during depolarization and not as a direct consequence of depolarization. When the pulse II Ca current is plotted against pulse I potential a current minimum is seen for intermediate pulse I potentials, namely for those pulse I potentials expected to produce large Ca entry. As the pulse I potentials approached the estimated Ca equilibrium potential the inward current recorded during pulse II increased again in size to the current strength recorded in the absence of pulse I. The degree of inactivation of the test pulse current therefore appears to parallel the amount of Ca that entered during the prior depolarization (Figs. 2 and 3).

It might be contended that reduction of the inward current results from the augmentation of a facilitating Ca-activated K current during pulse II. Such a phenomenon has been reported to occur in the ampullae of Lorenzini (Clusin, Spray & Bennett, 1975). This effect, if present, would be expected to diminish as pulse I approached E_{Ca}. However, in *Paramecium*, the conditions that enhance inactivation of the inward current do not produce an increase in the late outward current as would be expected if Ca entry during pulse I augments the pulse II potassium current (Fig. 1*B*). The absence of an augmented K current was also reported by Oertel *et al.* (1977).

The Na current in squid axon exhibits less inactivation at large positive conditioning potentials than at intermediate potentials (Chandler & Meves, 1970; Bezanilla & Armstrong, 1977; Meves, 1978). In this case the decreased inactivation at large positive potentials was attributed to direct voltage-dependent effects on the open state of the Na channel (Meves, 1978). A similar voltage dependency of the Ca channel may exist, but appears unlikely for the following reasons. The amount of inactivation recorded with any level of depolarization is related to the extracellular Ca concentration (Fig. 2*B*); moreover, voltage-dependent effects should be expressed equally in the presence of barium (Brehm & Eckert, 1978) or following EGTA injection. In both cases inactivation is strongly decreased.

It might be argued that the decrease in Ca current that we ascribe to inactivation could occur independent of any change in either channel conductance or numbers of active channels. For example, the accumulation of intracellular free Ca during Ca entry might decrease the driving force on Ca enough to produce the drop in current. However, arguments were presented in an earlier report that such a drop in driving force is unlikely (Brehm & Eckert, 1978). Briefly, calculations of the maximum intraciliary increment in Ca concentration during influx, assuming a uniform distribution of free Ca within the cilium, indicate that Ca accumulation is insufficient to account for the observed relaxation of the Ca current (Brehm &

Eckert, 1978). If it were simply the accumulation of the divalent ion with concomitant drop in driving force that causes reduction of the inward current then the barium current would be expected to undergo inactivation similar to that of the Ca current. The current carried by Ba exhibits far less inactivation than the current carried by Ca (Brehm & Eckert, 1978). Finally, the Ca current is relatively insensitive to elevations in intracellular [Ca] as evident from a consideration of the independence principle (Hagiwara, 1975).

We propose, then, that the Ca-dependent inactivation of the Ca current results from an intracellular action of the Ca ion that leads to an alteration in Ca conductance. The earliest indications that intracellular Ca levels play an important role in determining Ca current independent of driving force were obtained in perfused barnacle muscle fibres (Hagiwara & Nakajima, 1966) and more recently in studies of molluscan neurons (Kostyuk & Krishtal, 1977; Akaike *et al.* 1978) and tunicate eggs (Takahashi & Yoshii, 1978).

The interfering action of injected EGTA on Ca inactivation (Fig. 3) suggests that it is a rise in intracellular free Ca that plays a role in producing the inactivation. However injected EGTA only partially blocked the inactivation. In this regard, it is significant that EGTA injection is seldom adequate to block ciliary reversal during depolarization (Brehm *et al.* 1978). In the present experiments ionophoresis of EGTA never blocked ciliary reversal during depolarization. Thus, in spite of the injection of the Ca-binding agent, the intraciliary Ca concentration still rose to at least 10^{-6} M, the concentration that produces weak ciliary reversal (Naitoh & Kaneko, 1973; Saiki & Hiramoto, 1975), and remained elevated during depolarization. Failure of injected EGTA to buffer the intraciliary Ca concentration may be due to special morphological features of *Paramecium*. The Ca enters through the ciliary membrane (Dunlap, 1977). Calculations of the final intraciliary Ca concentration achieved during peak inward current into the minute volume of the cilium indicate that the final level could reach at least 2×10^{-5} M if uniform distribution within the cilium is assumed (Eckert, 1972; Oertel *et al.* 1977; Brehm & Eckert, 1978). In this case, approximately 10^{-4} M of unbound EGTA would have to be maintained within the cilium in order to buffer the Ca concentration below 10^{-6} M during depolarization (Portzehl, Caldwell & Rüegg, 1964). Because of structural impediments to diffusion such as the basal body present in the base of each cilium, the intraciliary concentration of uncomplexed EGTA may fall significantly out of equilibrium with the concentration present in the bulk of the cell during Ca entry. This would reduce Ca-buffering capacity of the EGTA within the cilium during depolarization.

Partial replacement of extracellular Ca with Ba (Naitoh & Eckert, 1968) or injection of EGTA (Brehm *et al.* 1978; Satow, 1978) both result in prolonged all-or-none action potentials lasting up to 20 sec. These effects were previously attributed to a reduced activation of Ca-dependent K current, although possible effects of injected EGTA on the Ca conductance were discussed (Brehm *et al.* 1978). However, the present study and an earlier one (Brehm & Eckert, 1978) support the findings of Oertel *et al.* (1977) that little or no Ca-dependent K current is measured under voltage clamp within the time scale under study. The actions of Ba (Brehm & Eckert, 1978) and injected EGTA in *Paramecium* appear to be primarily on the Ca-mediated inactivation of the

Ca channel, so that the steady-state inward current carried by Ca^{2+} can exceed the outward K^+ current. In the unclamped cell the amplitudes and time courses of these partial currents undoubtedly determine the duration of the barium and EGTA-induced plateau potentials. In view of the Ca-inactivating action of elevated intracellular Ca demonstrated in *Paramecium* and in molluscan neurones (Tillotson, 1979), special care is indicated to distinguish between this action of Ca and the familiar activation of a Ca-dependent K conductance (reviewed by Meech, 1978).

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