J. Membrane Biol. 90, 59–65 (1986)

The Journal of
Membrane Biology

Apparent Loss of Calcium-Activated Potassium Current in Internally Perfused Snail Neurons is Due to Accumulation of Free Intracellular Calcium

Edwin S. Levitan and Irwin B. Levitan
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Summary. Internal perfusion of *Helix* neurons with a solution containing potassium aspartate, $MgCl_2$, ATP, and HEPES causes the calcium-activated potassium current ($I_{K(Ca)}$) evoked by depolarizing voltage steps to decrease with time. When internal free Ca^{++} is strongly buffered to 10^{-7} M by including 0.5 mM EGTA and 0.225 mM $CaCl_2$ in the internal solution, $I_{K(Ca)}$ remains constant for up to 3 hours of perfusion. In cells where $I_{K(Ca)}$ is small at the start of perfusion, perfusion with the strongly buffered 10^{-7} M free Ca^{++} solution produces increases in $I_{K(Ca)}$ which ultimately saturate. In cells perfused with solutions buffered to 10^{-6} M free Ca^{++}, $I_{K(Ca)}$ is low and does not change with perfusion. These results lead us to conclude that $I_{K(Ca)}$ is stable in perfused *Helix* neurons and that the apparent loss of $I_{K(Ca)}$ seen initially with perfusion is due to accumulation of cytoplasmic calcium. Since the calcium current (I_{Ca}) provides the Ca^{++} which activates $I_{K(Ca)}$ during a depolarizing pulse, I_{Ca} is also stable in perfused cells when free intracellular Ca^{++} is buffered.

Perfusion with 1 μM calmodulin (CaM) produces no effect on $I_{K(Ca)}$ with either 10^{-7} or 10^{-6} M free internal calcium. Inhibiting endogenous CaM by including 50 μM trifluoperazine (TFP) in both the bath and the internal perfusion solution also produces no effect on $I_{K(Ca)}$ with 10^{-7} M free internal calcium. It is concluded that CaM plays no role in $I_{K(Ca)}$ activation.

Key Words calcium-activated potassium current · calmodulin · internal perfusion · calcium buffering · calcium current · wash out

Introduction

Internal perfusion is a technique with which one can reversibly change the cytoplasmic contents of a cell (Krishtal & Pidoplichko, 1975). It has been used to study ion selectivity of channels (Reuter & Stevens, 1980), the role of nucleotides in single photoreceptor cells (Stern & Lisman, 1982), and modulation of channel activity by cyclic nucleotides (Doroshenko, Kostyuk & Martynyuk, 1982) and phosphorylating enzymes (Depeyer, Cachelin, Levitan, & Reuter, 1982; Doroshenko et al., 1984). A similar technique, whole-cell patch clamping, has allowed internal perfusion of vertebrate cells (Fenwick, Marty & Neher, 1982).

The nature of the site conferring calcium sensitivity to the calcium activated potassium current ($I_{K(Ca)}$) is not known. We wished to use intracellular perfusion to test the hypothesis that cytoplasmic calmodulin, a protein which mediates the calcium sensitivity of a large number of enzymes (Cohen, 1982; Klee, Crouch & Richman, 1980), is required for activation of calcium-activated potassium current. Here we report that $I_{K(Ca)}$ elicited by depolarizing voltage pulses decreases with perfusion in *Helix* neurons. This result is not surprising given that other groups have shown that calcium current (I_{Ca}) is "washed out" with perfusion of molluscan neurons (Byerly & Hagiwara, 1982; Doroshenko et al., 1982). Since active I_{Ca} is required to elicit $I_{K(Ca)}$ with depolarizing voltage pulses, one would expect that $I_{K(Ca)}$ should also appear to decrease with perfusion. However, we show here that with proper calcium buffering of the internal perfusion solution, $I_{K(Ca)}$ remains stable for hours. Loss of $I_{K(Ca)}$ with perfusion is shown not be be due to the loss of cytoplasmic factors but rather is correlated with accumulation of cytoplasmic calcium.

A role for calmodulin (CaM) in activation of $I_{K(Ca)}$ was tested by including 1 μM CaM in the internal perfusion solution with 10^{-7} or 10^{-6} M free calcium. No effect on $I_{K(Ca)}$ was seen in either case. Furthermore, exposure of both the cytoplasmic and extracellular sides of the plasma membrane to trifluoperazine, a calmodulin inhibitor, in the presence of 10^{-7} M free calcium produced no effect on $I_{K(Ca)}$. It is concluded that the activation of $I_{K(Ca)}$ by depolarizing voltage pulses does not require calmodulin.

Materials and Methods

All experiments were performed at room temperature on neurons in the right parietal ganglion of *Helix aspersa*. By analogy with *Helix pomatia* (Lux & Hofmeier, 1982), it is thought that a

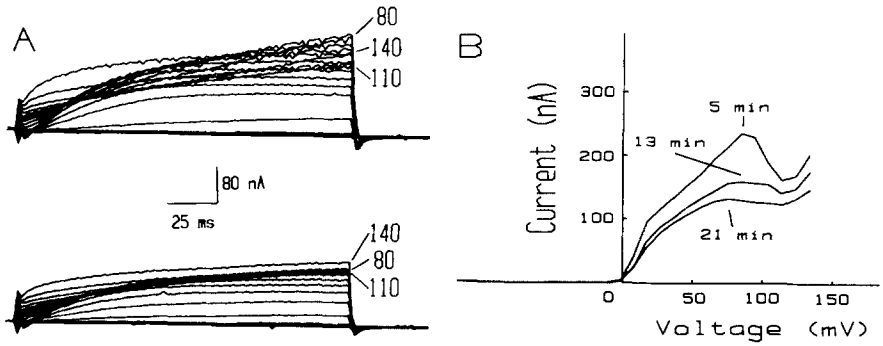

Fig. 1. Internal perfusion without calcium buffering produces apparent loss of $I_{K(Ca)}$. (*A*) Superimposed traces of current responses to depolarizing voltage steps measured after 5 (top) and 21 (bottom) min of internal perfusion. Numbers at right indicate voltages at which several of the current traces were obtained. (*B*) Current *versus* voltage data taken at indicated times of internal perfusion

large proportion of the outward current in these cells is $I_{K(Ca)}$. Cells were made accessible by removing the surrounding connective tissue in the presence of 0.25% trypsin in extracellular saline (80 mM NaCl, 4 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM $NaHCO_3$, 10 mM NaHEPES at pH 7.4). The preparation was then washed extensively to remove the trypsin.

Intracellular perfusion was performed similarly to Lee, Akaike and Brown (1978) and Stern and Lisman (1982). Briefly a fire-polished glass pipette (100 μm o.d., 16 μm i.d.) was brought up to a 50 to 80 μm cell body and with a small amount of suction a 20 to 90 MΩ seal was established. The membrane occluding the pipette tip was permeabilized with a voltage oscillation followed by two pulses of suction. After this molecules in the cell and in the pipette were free to exchange. Suction generated by a gravity trap served both to hold the cell in place and to draw in fresh intracellular buffer (112.5 mM potassium aspartate, 2 mM $MgCl_2$, 0.5 mM Na_2ATP, 5 mM Na_2ATP, 5 mM K^+HEPES at pH 7.1 with amounts of $CaCl_2$ and EGTA as indicated for individual experiments) from a capillary 150 μm from the cell. The volume of solution in the pipette between the cell and the capillary inport could be exchanged quickly at a flow rate of 0.2 μl per minute. Using this configuration intracellular potassium could be replaced with cesium within 7 min as measured by the decrease in outward current in response to depolarizing voltage steps. Cells were used only if peak action potential amplitude was at least 90 mV and if series resistance was less than 600 kΩ.

All current and voltage measurements were made with a single electrode-switching voltage clamp (Almost Perfect Electronics) via a Ag/AgCl wire in a trap connected to the perfusion pipette with tubing filled with 200 mM KCl. The use of a switching voltage clamp eliminates the need for series resistance compensation. Measurements were made relative to a grounded Ag/AgCl wire in the bath.

A holding potential of −40 mV was used when not collecting data. Presentation of pulses and collection and analysis of data were via a PDP 11/23 computer system (Indec Systems). Currents were evoked by a series of 200-msec pulses in 10 mV steps starting from −120 mV and typically reaching +160 mV. Pulses were separated by 5.8-sec intervals at −40 mV. Voltage and current data were collected at a rate of 1 kHz, although only every third point is shown in the figures. The current during the last 5 msec of each pulse was averaged to give "steady state" current. The currents measured between −120 and −90 mV were used to extrapolate leak conductance. Typical leak conductance was 1 to 2% of the peak conductance measured at positive voltages. The current *versus* voltage plots shown are all "steady state" and are leak subtracted. Current *versus* voltage plots were taken at 8 to 12 min intervals usually after an initial 10 min of perfusion. Free calcium concentrations were calculated with a computer program which took into account chelation of Ca^{++} and Mg^{++} by both ATP and EGTA at a specified pH. The program did not take into account either ionic strength or activity coefficients. Thus free calcium concentrations, although consistent from experiment to experiment, are approximate and are only given by order of magnitude. Na_2ATP, trifluoperazine (TFP), potassium aspartate, EGTA, and $CaCl_2$ were obtained from Sigma. Bovine calmodulin was obtained from Biorad.

Results

Depolarization elicits two outward noninactivating potassium currents in *Helix* neurons. One of these is due to delayed rectifier channels which open at depolarized potentials. The other current is dependent on both calcium influx and voltage and thus is known as the calcium-activated potassium current ($I_{K(Ca)}$) (Meech & Standen, 1975; Lux & Hofmeier, 1982). This current first appears under voltage clamp at about 0 mV and then rises to a peak value at about 70 mV with 200-msec depolarizing pulses. However, at more depolarizing voltages $I_{K(Ca)}$ decreases. This occurs because, as the calcium equilibrium potential is approached, calcium influx decreases and begins to limit calcium-dependent potassium channel activation (Meech & Standen, 1975). The summation of the delayed rectifier and calcium-activated potassium currents produces a characteristic *N*-shaped current *versus* voltage curve (e.g. Fig. 1*B*, 5 min). The current making up the hump of the *N* shape is $I_{K(Ca)}$ while the monotonically increasing current interpolated from 0 mV to beyond the negative slope region of the curve is due to delayed rectifier and nonlinear leak currents. We have chosen to use the current underlying the *N* shape of the current *versus* voltage curve as a measure of $I_{K(Ca)}$. It should be pointed out that, to our knowledge, there is no blocker of the delayed rectifier current in *Helix* which does not also block $I_{K(Ca)}$ to a significant extent.

The initial characterization of $I_{K(Ca)}$ was done using the two-electrode voltage clamp technique

(Meech & Standen, 1975). We chose to investigate the possible role of calmodulin in $I_{K(Ca)}$ activation by internally perfusing cells. Among the many advantages this technique offers is excellent control of the concentration of agents perfused into cell's cytoplasm. Furthermore, internal perfusion of an agent is reversible. The perfusion electrode also provides an excellent current-passing electrode. Finally, one can study interactions between components of the cytoplasm and the membrane, an option not available with the detached patch recording technique (Hamill et al., 1981).

Internal perfusion of *Helix* neurons with K^+ aspartate, $MgCl_2$, Na_2ATP, and HEPES causes the $I_{K(Ca)}$ component of the current *versus* voltage curve to decrease. Figure 1*A* shows the current records elicited by voltage pulses for one such cell. Early in the experiment, after only 5 min of perfusion, the cell has robust $I_{K(Ca)}$ (Fig. 1*A*, top). The current records later in the experiment are shown in Fig. 1*A*, bottom. There we see that after an additional 16 min of perfusion the currents have become smaller. The outward current flowing at +80 mV has halved. Further depolarization to +120 mV is not accompanied by a large decrease in current as is seen in Fig. 1*A*, top. The current does, however, begin to increase above +120 mV. These points are clearly seen when current is plotted *versus* voltage after different times of perfusion (Fig. 1*B*). Early in the experiment the current *versus* voltage (*I–V*) curve has a distinct *N* shape while after 21 min of perfusion the negative slope region has decreased greatly. This change is indicative of a decrease in the magnitude of $I_{K(Ca)}$ relative to the delayed rectifying potassium current. Note that the current at +140 mV, which is largely delayed rectifying current, also appears to decrease with perfusion in this cell. Some of this decrease may be due to loss of contaminating $I_{K(Ca)}$, and it should also be pointed out that small errors in voltage and fluctuations in leak current would produce dramatic effects on current flow at +140 mV. Furthermore, such a decrease in outward current was not seen consistently at the most depolarized voltages, whereas the current at +70 mV and the *N* shape of the current *versus* voltage curve always decreased with perfusion time. Thus we conclude that only $I_{K(Ca)}$ appears to decrease with perfusion. This loss of $I_{K(Ca)}$ could be due to a decrease in either calcium channel or calcium-activated potassium channel activity.

In fact, a loss of I_{Ca} seems to be more likely. *Helix* calcium-activated potassium channels have been shown to be stable in detached patches (Ewald, Williams & Levitan, 1985). Thus one would not expect internal perfusion to remove some factor required for $I_{K(Ca)}$ activity. On the other hand, molluscan calcium current has been shown to be labile in perfused cells (Byerly & Hagiwara, 1982; Doroshenko et al., 1982). The loss of I_{Ca} has been interpreted to be due to "wash out" of a cytoplasmic factor required for calcium channel activity. An alternative explanation is that the internal perfusion solution supplies some factor which leads to a decrease in $I_{K(Ca)}$ (possibly by decreasing I_{Ca}). One possible factor is calcium. Elevated concentrations of calcium have been shown to cause inactivation of molluscan calcium current (Eckert & Chad, 1984) and block of T-tubule calcium activated potassium channels (Moczydlowski & Latorre, 1983). Either phenomenon would produce a decrease in $I_{K(Ca)}$ elicited by depolarizing voltage pulses.

Although no calcium was added in these initial experiments, the normal intracellular perfusion buffer was contaminated with about 25 μM Ca^{++}, measured by atomic absorption, arising mostly from the potassium aspartate. The intracellular free calcium concentration in *Helix* neurons, on the other hand, has been found to be about 10^{-7} M (Alvarez-Leefmans, Rink & Tsien, 1981). In order to more closely approximate the cytoplasmic milieu, 50 μM EGTA was added to weakly buffer free calcium to 10^{-7} M. Under these conditions $I_{K(Ca)}$ still decreases with perfusion, albeit at a slower rate. However, increasing the buffering capacity for calcium tenfold, by changing the EGTA concentration to 0.5 mM without increasing free calcium concentration, eliminates the decrease in $I_{K(Ca)}$ associated with perfusion for up to 3 hr. The data shown in Fig. 2*A* were obtained from a cell perfused with this strongly buffered calcium solution. The current *versus* voltage data shows that, although some random shifting occurs, no systematic change in the shape or the magnitude of the $I_{K(Ca)}$ component is seen for 76 min. Thus under these conditions prolonged perfusion produces no change in the outward currents. It should also be noted that examination of the current *versus* time records shows no discernible difference in the kinetics of currents measured in cells perfused with the appropriate buffer for over an hour (data not shown) and currents measured with two-electrode voltage clamp in unperfused cells (Lux & Hofmeier, 1982).

It might be expected that no calcium-activated currents would be evident in the presence of 0.25 mM free EGTA. However, at least 10 mM EGTA is required to completely block $I_{K(Ca)}$ elicited by depolarization (Akaike et al., 1983). Thus the 0.25-mM free EGTA used in the internal solution in the present experiments must saturate near the membrane during depolarizations, but nonetheless is sufficient to maintain a steady-state level of free calcium.

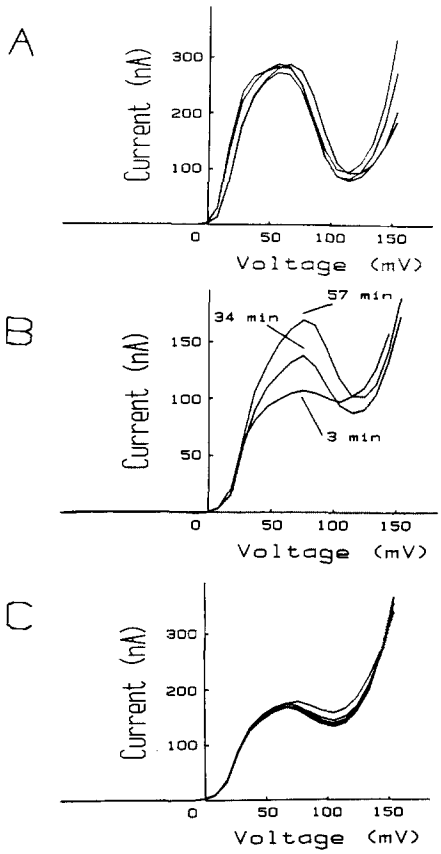

Fig. 2. The effect of buffering free calcium in the internal perfusion solution on maintenance of $I_{K(Ca)}$. (*A*) Inclusion of 0.5 mM EGTA and 0.225 mM $CaCl_2$ in the internal medium to buffer free Ca^{++} concentration to about 10^{-7} M. Current *versus* voltage data obtained after 16, 36, 56, and 76 min of internal perfusion. (*B*) Current *versus* voltage data taken at indicated times of internal perfusion with same buffer as in *A*. (*C*) Current *versus* voltage data after 24, 33, 44, 52 and 61 min of internal perfusion with 10^{-6} M free Ca^{++} (1.0 mM EGTA, 0.95 mM $CaCl_2$). Each set of traces (*A–C*) was taken from a different cell

The stability of $I_{K(Ca)}$ during perfusion with strongly buffered solution is common in cells with initially robust $I_{K(Ca)}$ (e.g., Fig. 2*A*). However, in cells in which $I_{K(Ca)}$ is initially low, intracellular perfusion with solution strongly buffered to 10^{-7} M free Ca^{++} produces *increases* in $I_{K(Ca)}$ without systematically affecting the delayed rectifier current. The data in Fig. 2*B* were taken from a cell perfused with the same buffer as in Fig. 2*A*. The *I–V* plot after 3 min of perfusion is only weakly *N* shaped and thus indicative of a small amount of $I_{K(Ca)}$. After 57 min of perfusion the current at +80 mV increases to 170% of its initial value. Between 80 and 130 mV there is a large decrease in outward current. Further depolarization produces an increase in current similar to that seen at the outset of the experiment (Fig. 2*B*, 3 min). Thus in this cell where $I_{K(Ca)}$ initially appeared to be present in only a small amount, perfusion with a strongly calcium-buffered solution produced a dramatic increase in $I_{K(Ca)}$ without affecting the delayed rectifying potassium current. Typically the increase in $I_{K(Ca)}$ with perfusion, seen in cells with initially low $I_{K(Ca)}$, saturates with time, leaving cells with robust *N* shaped current *versus* voltage curves, and further perfusion produces no change.

In order to determine whether the inability of weakly buffered solutions to maintain $I_{K(Ca)}$ is due to their inability to hold the free calcium concentration at 10^{-7} M, cells were perfused with solutions buffered to 10^{-6} M free calcium with a high concentration of EGTA. The current *versus* voltage plots for these cells are weakly *N* shaped after only a short time of perfusion, indicating that they possess little $I_{K(Ca)}$ (Fig. 2*C*). Further perfusion with the 10^{-6} M calcium buffer does not increase $I_{K(Ca)}$ in marked contrast to such cells perfused with a solution strongly buffered to 10^{-7} M free calcium (e.g. Fig. 2*B*). These results further indicate that the amplitude of $I_{K(Ca)}$ in response to a depolarizing voltage pulse is regulated by the steady-state level of free cytoplasmic Ca^{++}.

We then investigated whether $I_{K(Ca)}$ is activated in a calmodulin-dependent manner. Calmodulin (CaM) is a soluble 16-kD protein which binds four calcium ions with dissociation constants in the micromolar range (Klee et al., 1980) and thus can act as a calcium buffer. It has also been shown to mediate the calcium sensitivity of a number of enzymes including protein kinases, phosphatases, a phosphodiesterase, and a pump (Cohen, 1982). Perfusion with 1 μM CaM produces no changes in $I_{K(Ca)}$ in the presence of strongly buffered 10^{-7} M free calcium (Fig. 3*A,B*). Since buffering calcium to such submicromolar levels would inhibit steady-state CaM-dependent processes, the experiment was repeated at a calcium concentration at which CaM is active. However, even with 10^{-6} M free internal calcium, inclusion of CaM produces no effect on outward currents (Fig. 3*C,D*). It could be argued that tightly bound endogenous CaM remains present in the cell in sufficient concentration to activate or modulate the $I_{K(Ca)}$ channel. Thus perfusion would not remove CaM and perfusion with CaM would not further activate $I_{K(Ca)}$. To test this possibility cells were exposed to high concentrations of the CaM inhibitor trifluoperazine (TFP). Inclusion of 50 μM TFP in the bath as well as in the perfusion solution for long periods of time produces no effect on $I_{K(Ca)}$. Shown in Fig. 4 are data from a cell perfused with 10^{-7} M free calcium where exposure to TFP for 50 min did not diminish the $I_{K(Ca)}$ component of the current *versus* voltage curve. Both the current

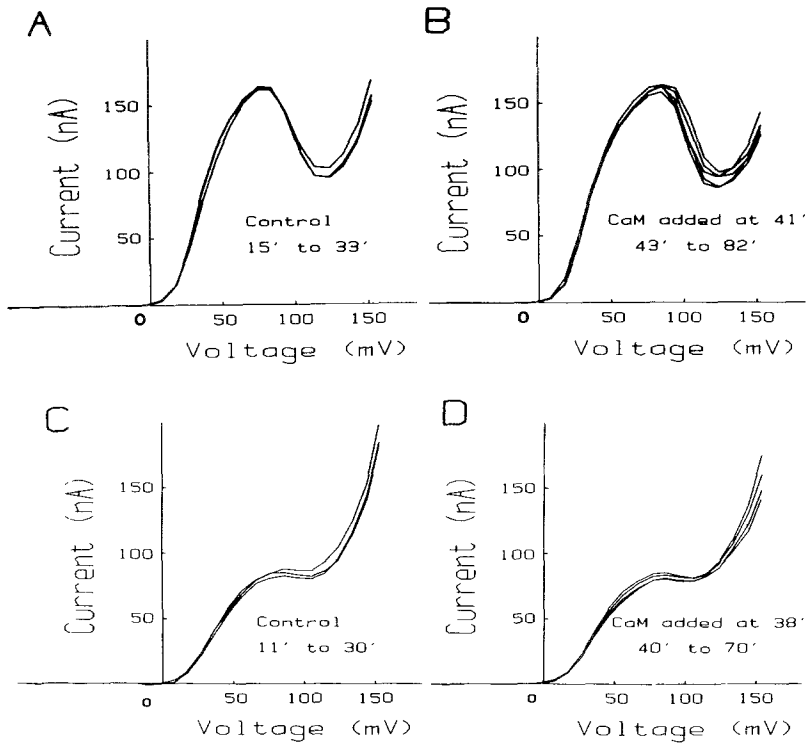

Fig. 3. Addition of 1 μM calmodulin to calcium-buffered internal solutions does not affect $I_{K(Ca)}$. (*A*) Current *versus* voltage data taken after 15, 25 and 33 min of internal perfusion with solution buffered to 10^{-7} M free Ca^{++}. Calmodulin was added to the internal solution after 41 min of perfusion. (*B*) Current *versus* voltage data taken after 43, 51, 59, 69, and 82 min of perfusion (same cell as in *A*). (*C*) Current *versus* voltage data taken after 11, 22 and 30 min of internal perfusion with solution buffered to 10^{-6} M free Ca^{++}. Calmodulin was added to the internal solution after 38 min of perfusion. (*D*) Current *versus* voltage data taken after 40, 51, 61, and 70 min of perfusion (same cell as in *C*)

records and the *I–V* plot appear normal. Thus normal activation of $I_{K(Ca)}$ under voltage clamp does not appear to be a CaM-dependent process in perfused cells. It is also interesting to note that the decreased $I_{K(Ca)}$ seen in cells perfused with 10^{-6} M free calcium is not altered by calmodulin inhibitor (*data not shown*). Thus depression of $I_{K(Ca)}$ by high cytosolic calcium also does not require CaM.

Discussion

Intracellular perfusion allows exchange of diffusible molecules in the cytoplasm with the contents of the solution in the perfusion pipette (Krishtal & Pidoplichko, 1975; Lee et al., 1978). In contrast to other methods of introducing molecules into cells such as pressure injection and iontophoresis, compounds perfused into cells can later be perfused out. The perfusion pipette also furnishes one with an excellent current-passing electrode for voltage clamping. These advantages have been utilized to study a wide variety of interactions between the contents of the cytoplasm and membrane ion channels ranging from ion selectivity of membrane currents (Reuter & Stevens, 1980) to modulation of ion channels by a protein kinase (Depeyer, et al., 1982; Doroshenko et al., 1984). This last application demonstrates that even large proteins can be perfused into neurons. One would thus expect that any small metabolites and even regulatory enzymes would be "washed out" of a cell during the course of a long perfusion experiment.

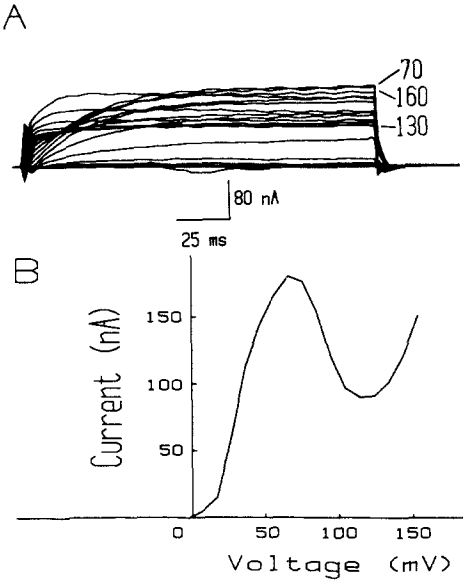

Fig. 4. Inclusion of 50 μM TFP in bathing and internal solutions does not affect $I_{K(Ca)}$. (*A*) Current elicited by voltage pulses after 50 min of exposure to internal and external TFP. (*B*) Current *versus* voltage data after 50 min of exposure to internal and external TFP. Approximate free Ca^{++} concentration was 10^{-7} M. The results are the same as in the absence of TFP (e.g. Fig. 2*A*)

A decrease in calcium current has been reported with internal perfusion in several different cells (Byerly & Hagiwara, 1982; Doroshenko et al., 1982; Fenwick et al., 1982). Such a loss would be reflected in the amplitude of $I_{K(Ca)}$ elicited by depolarization, since $I_{K(Ca)}$ activation is dependent on calcium entry. The fact that *Helix* calcium-activated potassium channels have been found to be stable in detached patches (Ewald et al., 1985) suggests that the loss of $I_{K(Ca)}$ with perfusion (e.g. Fig. 1) may be due to loss of I_{Ca}. However, the decrease of $I_{K(Ca)}$ can be prevented if intracellular free calcium is strongly buffered to 10^{-7} M, and in fact perfusion under these conditions causes $I_{K(Ca)}$ to *increase* in cells where it is initially depressed. Apparent loss of $I_{K(Ca)}$ is seen in cells where free calcium is either unbuffered, buffered only weakly to 10^{-7} M, or buffered to 10^{-6} M. These results lead us to conclude that loss of $I_{K(Ca)}$ with perfusion is not due directly to the loss of a necessary cytoplasmic factor, but rather is due to accumulation of cytoplasmic calcium which in some way causes depression of $I_{K(Ca)}$.

Clearly one could not maintain $I_{K(Ca)}$ activity, elicited by depolarization, for hours in the absence of I_{Ca}. Thus I_{Ca} apparently is not "washing out" under our experimental conditions. This contrasts with previous work on perfused *Helix* neurons. Doroshenko et al. (1982, 1984) have demonstrated that calcium current in *Helix* neurons decreases with perfusion, but that this decrease can be prevented by inclusion of either cAMP or catalytic subunit of cAMP-dependent protein kinase in the perfusion solution. They have therefore concluded that loss of calcium current with perfusion is due to washout of a metabolite required for channel activity, namely cAMP. In contrast to our conditions, these workers did not strongly buffer calcium. Thus it is possible that cAMP acts to modulate the cell's calcium buffering system. If calcium buffering is involved in this phenomenon, perfusion with a buffer similar to ours may prevent "wash out" of calcium current.

It is interesting that work in another molluscan system seems to support this explanation. Byerly and Hagiwara (1982) have reported that perfusion produces loss of calcium current in *Lymnaea* neurons. Their buffer contained less than 10^{-8} M free calcium, but unlike ours did not include magnesium or ATP. Byerly and Moody (1984) later reported that inclusion of ATP in the perfusion solution is very important for controlling free calcium in these cells. Byerly and Yazejian (1985) have now found that inclusion of magnesium and ATP along with a strong calcium EGTA buffer is sufficient to prevent loss of calcium current with perfusion, without the addition of cAMP or a protein kinase. Thus this group has found that perfusion conditions, similar to those we have found eliminate loss of $I_{K(Ca)}$ in *Helix* neurons, also prevent loss of I_{Ca} in *Lymnaea* neurons.

There is an apparent paradox in the conclusion that a calcium-dependent ion current is actually depressed by high steady-state concentrations of free intracellular calcium, and thus it is of interest to consider how calcium accumulation might depress $I_{K(Ca)}$. The similarity in sensitivity of calcium current and calcium-activated potassium current to perfusion conditions which allow cytoplasmic calcium to become elevated suggests a mechanism that could account for the apparent "wash out" of these currents with perfusion. It has been shown in a large number of systems that elevated calcium causes inactivation of calcium channels (Eckert & Chad, 1984), and Byerly and Moody (1984) have determined that this occurs in *Lymnaea* neurons when cytoplasmic free calcium reaches 10^{-6} M. Thus under conditions where cytoplasmic calcium is elevated, depolarization results in fewer open calcium channels. Less calcium enters the cell, and in turn fewer calcium activated-potassium channels can be opened. Eckert and Ewald (1982) have shown that this phenomenon occurs in molluscan neurons when cytoplasmic calcium is raised by opening calcium channels with a voltage-clamp prepulse.

Other possible mechanisms also exist. It has been shown that reconstituted calcium-activated potassium channels from skeletal muscle T-tubules are blocked by calcium concentrations in the 50 μM range (Moczydlowski & Latorre, 1983). Although it is unlikely that cytoplasmic calcium ever reaches so high a concentration in our experiments, such a phenomenon might occur at lower concentrations in *Helix* neurons. Single-channel studies may help to distinguish between these two possible mechanisms. Finally, more indirect mechanisms are also possible. For example, it has been shown that phosphorylation increases calcium-activated potassium channel activity (Depeyer et al., 1982; Ewald et al., 1985). Thus elevated cytosolic calcium might decrease $I_{K(Ca)}$ by activating a phosphoprotein phosphatase. Clearly, given the plethora of calcium-dependent enzymes, many alternative mechanisms are possible.

Once conditions were found where $I_{K(Ca)}$ was stable in perfused neurons, we investigated whether calmodulin plays any role in $I_{K(Ca)}$ activation. Calmodulin has been shown to mediate the calcium sensitivity of a number of enzymes including kinases, phosphatases, phosphodiesterase, and the Ca^{++}-ATPase ion pump (Cohen, 1982). It thus seems reasonable that calmodulin might confer calcium sensitivity to the $I_{K(Ca)}$ channel. It was found, however, that calcium-activated potassium current can be maintained without inclusion of calmodulin in the perfusion solution, suggesting that freely dif-

fusible calmodulin is not required for $I_{K(Ca)}$ activity. However, it is conceivable that calmodulin tightly bound to the membrane channel might be necessary for activity. In fact, calmodulin is tightly bound to the enzyme phosphorylase kinase (Cohen, 1982), and such tightly bound calmodulin would not be expected to be washed out with perfusion. In order to address this possibility we simultaneously exposed both the cytoplasmic and the extracellular sides of the plasma membrane to 50 μM TFP, a calmodulin inhibitor. No effect on $I_{K(Ca)}$ activity was observed, although this concentration of TFP completely blocks CaM-dependent enzymes in molluscan ganglia (DeRiemer et al., 1984) as well as in many other systems. Thus calmodulin does not appear to mediate the calcium sensitivity of calcium-activated potassium current in *Helix* neurons. The fact that the calcium sensitivity of $I_{K(Ca)}$ can be maintained in perfused cells and in detached membrane patches (Ewald et al., 1985) suggests that a different factor closely associated with the channel must confer normal calcium sensitivity. This, however, does not rule out the possibility of calmodulin-dependent modulation of $I_{K(Ca)}$ under other physiological conditions such as exposure to neurotransmitters. It is also of interest that inhibition of CaM produces no effect when internal calcium is in the micromolar range. Under these conditions $I_{K(Ca)}$ is depressed, possibly because I_{Ca} is inactivated by elevated cytosolic calcium. This suggests, albeit indirectly, that calcium channel inactivation by cytoplasmic calcium may not require CaM. Finally, it should also be kept in mind that calmodulin may exert effects on calcium-dependent currents indirectly merely by virtue of its calcium-buffering properties. As shown here, such calcium-buffering effects are large when one examines the calcium-activated potassium current.

We are grateful to Drs. Richard Gunning and John Lisman for their critical comments on an earlier version of the manuscript. This work has been presented previously in abstract form (Levitan & Levitan, 1984). This work was supported by grant NS 17910 to IBL from the National Institute of Neurological and Communicative Disorders and Stroke.

References

Akaike, N., Brown, A.M., Dahl, G., Higashi, M., Isenberg, G., Tsuda, Y., Yatan, A. 1983. Voltage-dependent activation of potassium currents in *Helix* neurones by endogenous cellular calcium. *J. Physiol. (London)* **334**:309–324

Alvarez-Leefmans, F.J., Rink, T.J., Tsien, R.Y. 1981. Free calcium ions in neurones of *Helix aspersa* measured with ion selective microelectrodes. *J. Physiol. (London)* **315**:531–548

Byerly, L., Hagiwara, S. 1982. Calcium currents in internally perfused nerve cell bodies of *Lymnaea stagnalis*. *J. Physiol. (London)* **322**:503–528

Byerly, L., Moody, W.J. 1984. Intracellular calcium ions and currents in perfused neurones of the snail *Lymnaea stagnalis*. *J. Physiol. (London)* **352**:637–652

Byerly, L., Yazejian, B. 1985. Intracellular factors for the maintenance of calcium currents in perfused neurones from the snail, *Lymnaea stagnalis*. *J. Physiol. (London) (in press)*

Cohen, P. 1982. The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature (London)* **296**:613–619

Depeyer, J.E., Cachelin, A.B., Levitan, I.B., Reuter, H. 1982. Ca^{2+}-activated K^+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. *Proc. Nat'l Acad. Sci. USA* **79**:4207–4211

DeRiemer, S.A., Kaczmarek, L.K., Lai, Y., McGuinness, T.L., Greengard, P. 1984. Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. *J. Neurosci.* **4**:1618–1625

Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E. 1982. Intracellular metabolism of adenosine 3',5'-cyclic monophosphate and calcium inward current in perfused neurones of *Helix pomatia*. *Neuroscience* **7**:2125–2134

Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E., Kursky, M.D., Vorobetz, Z.D. 1984. Intracellular protein kinase and calcium currents in perfused neurones of the snail *Helix pomatia*. *Neuroscience* **11**:263–267

Eckert, R., Chad, J.E. 1984. Inactivation of calcium channels. *Prog. Biophys. Molec. Biol.* **44**:215–267

Eckert, R., Ewald, D. 1982. Residual calcium ions depress activation of calcium-dependent current. *Science* **216**:730–732

Ewald, D.A., Williams, A., Levitan, I.B. 1985. Protein phosphorylation modulates the activity of single Ca^{++}-dependent K^+ channels. *Nature (London)* **315**:503–506

Fenwick, E.M., Marty, A., Neher, E. 1982. Sodium and calcium channels in bovine chromaffin cells. *J. Physiol. (London)* **331**:599–635

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell free membrane patches. *Pfluegers Arch.* **391**:85–100

Klee, C.B., Crouch, T.H., Richman, P.G. 1980. Calmodulin. *Annu. Rev. Biochem.* **49**:489–516

Krishtal, O.A., Pidoplichko, V.I. 1975. Intracellular perfusion of snail giant neurones. *Neurophysiology (Kiev)* **7**:327–329

Lee, K.S., Akaike, N., Brown, A.M. 1978. Properties of internally perfused, voltage clamped, isolated nerve cell bodies. *J. Gen. Physiol.* **71**:489–507

Levitan, E.S., Levitan, I.B. 1984. Depression of calcium-activated potassium current in internally dialyzed *Helix* neurons is due to accumulation of free intracellular calcium. *Soc. Neurosci. Abstr.* **10**:144

Lux, H.D., Hofmeier, G. 1982. Properties of a calcium- and voltage-activated potassium current in *Helix pomatia* neurons. *Pfluegers Arch.* **394**:61–69

Meech, R.W., Standen, N.B. 1975. Potassium activation in *Helix aspersa* neurons under voltage clamp: A component mediated by calcium influx. *J. Physiol. (London)* **249**:211–239

Moczydlowski, E., Latorre, R. 1983. Gating kinetics of Ca^{++}-activated K^+ channels from rat muscle incorporated into planar lipid bilayers. *J. Gen. Physiol.* **82**:511–542

Reuter, H., Stevens, C.F. 1980. Ion conductance and ion selectivity of potassium channels in snail neurones. *J. Membrane Biol.* **57**:103–118

Stern, J.H., Lisman, J.E. 1982. Internal dialysis of *Limulus* ventral photoreceptors. *Proc. Nat'l. Acad. Sci. USA* **79**:7580–7584

Received 2 August 1985; revised 24 October 1985