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Voltage-Independent Barium-Permeable Channel Activated in *Lymnaea* Neurons by Internal Perfusion or Patch Excision

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Summary. Isolated nerve cells from *Lymnaea stagnalis* were studied using the internal-perfusion and patch-clamp techniques. Patch excision frequently activated a voltage-independent Ba^{2+}-permeable channel with a slope conductance of 27 pS at negative potentials (50 mM Ba^{2+}). This channel is not seen in patches on healthy cells and, unlike the voltage-dependent Ca channel, is not labile in isolated patches. The activity of the channel in inside-out patches is unaffected by intracellular ATP, Ca^{2+} below 1 mM or the catalytic subunit of cAMP-dependent protein kinase but is reversibly blocked by millimolar intracellular Ca^{2+} or Ba^{2+}. The channel can be activated in on-cell patches by either internal perfusion with high Ca^{2+} or the long-term internal perfusion of low Ca^{2+} solutions not containing ATP. These channels may carry the inward Ca^{2+} current which causes a regenerative increase in intracellular Ca^{+} when snail neurons are perfused with high Ca^{2+} solutions. High internal Ca^{2+}, or long periods of internal perfusion with ATP-free solutions, induces an increase in a resting (−50 mV) whole-cell Ba^{2+} conductance. This conductance can be turned off by returning the intracellular perfusate to a low Ca^{2+} solution containing ATP and Mg^{2+}. The activity of this channel appears to have an opposite dependence on intracellular conditions to that of the voltage-dependent Ca channel.

Key Words barium current · internal perfusion · patch clamp

Introduction

Much of the Ca^{2+} that enters excitable cells does so through voltage-activated Ca channels in the plasma membrane (Hagiwara & Byerly, 1981; Tsien, 1983). This influx of Ca^{2+} causes a localized rise in the intracellular Ca^{2+} concentration. In intact cells, this excess Ca^{2+} is quickly removed by intracellular organelles and buffers and by pumping the Ca^{2+} back out of the cell by Ca^{2+}-ATPases (Beaugé et al., 1981). When cells are internally perfused and voltage clamped (Krishtal & Pidoplichko, 1975), their inherent Ca^{2+} sequestering ability is altered. Byerly and Moody (1984) have shown that the rise of the concentration of intracellular Ca^{2+} in *Lymnaea* neurons becomes regenerative when cells are internally perfused with elevated Ca^{2+} solutions. The excess Ca^{2+} enters the cell through a Ca^{2+}-activated, Cd^{2+}-sensitive conductance which allows Ca^{2+} entry at potentials more negative than those that activate the voltage-dependent Ca channels. The addition of ATP to the intracellular perfusate prevents the regenerative increase in intracellular Ca^{2+}, possibly by increasing the cell's ability to buffer Ca^{2+}.

In this report we have studied a channel that appears to mediate this Ca^{2+} influx using the internal-perfusion voltage-clamp technique (Krishtal & Pidoplichko, 1975; Byerly & Yazejian, 1986) and the patch-clamp technique (Hamill et al., 1981). This channel is normally quiet in healthy, intact cells but becomes active in isolated patches. Its activity is bursty and voltage-independent. This channel is activated in cell-attached patches by internal perfusion of the cell with elevated Ca^{2+} or by prolonged internal perfusion with ATP-free solutions. The control of the channel's activity is most easily studied by measuring the whole-cell Ba current of internally perfused cells clamped to −50 mV. Such studies show that this channel is activated as the voltage-dependent Ca channel is "washed-out" of cells. Conditions that prevent the wash-out of the voltage-dependent Ca current, such as addition of ATP and Mg^{2+} to internal solutions, also prevent the activation of this current.

A preliminary report of this work has been published (Yazejian & Byerly, 1987).

Materials and Methods

Nonidentified neuronal somata from *Lymnaea stagnalis* were used for this study. Essentially all of the procedures for cell isolation and internal perfusion have been described previously

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(Byerly & Yazejian, 1986). Patch electrodes were pulled from thick-walled Kovar glass (Corning 7052) and their shanks were insulated with Sylgard. Electrodes filled with the solutions we used to measure currents had resistances of between 70 and 150 MΩ. Patch currents were recorded with an Axopatch 1A patch-clamp amplifier (Axon Instruments, Burlingame, CA). Currents were filtered at 1 kHz, digitized with a modified digital audio processor (Unitrade) and stored directly onto videotape. Off-line analysis of single-channel currents was made on 100-msec segments of the data triggered by an event-detector and redigitized at 200-μsec intervals. Idealized events were determined and histograms generated using the pCLAMP analysis software (Axon Instruments). In some experiments we combined the whole-cell voltage clamp and patch-clamp techniques. After a stable whole-cell perfusion was set up using the large suction electrode (30–40 μm opening), we then obtained a seal with the patch electrode. With this procedure it was possible to change intracellular solutions by the internal perfusion method while at the same time recording unitary currents from a cell-attached patch.

The standard intracellular solution used in whole-cell studies (referred to as "Cs (or K) aspartate, 100 mM HEPES") contained (in mM) 74 CsOH (or KOH), 28 L-aspartic acid, 100 HEPES and 5 EGTA (pH 7.3). 2 mM ATP and 1 mM $MgCl_2$ (referred to as "Mg ATP") were added where indicated. The solution used to increase the intracellular concentration of Ca^{2+} (referred to as "Cs aspartate, *p*Ca 5.7") contained 5 mM $CaCl_2$, no ATP or $MgCl_2$ and used 10 mM HEDTA as a Ca^{2+} buffer. The Ca^{2+} activity of this solution has been measured to be 5.7 (Byerly & Moody, 1984). The standard extracellular solution (hyperosmotic Tris saline) contained (in mM) 65 Tris Cl, 4 $CaCl_2$, 4 $MgCl_2$ and 60 D-glucose (pH 7.4). The extracellular solution used to bathe the cells when recording with patch electrodes (*Lymnaea* saline) contained (in mM) 50 NaCl, 2.5 KCl, 4 $CaCl_2$, 4 $MgCl_2$ and 10 Tris Cl (pH 7.4). Glucose (60 mM) was added to this solution ("hyperosmotic *Lymnaea* saline") to maintain isosmolarity when studying total inward currents in perfused cells. The solutions used in the patch pipette for recording currents through HP channels contained either 40, 50 or 60 mM $BaCl_2$ plus 10 mM Tris Cl (pH 7.4). Glucose was used to maintain equal osmolarity between these different solutions. Experiments were done with 60 mM Ba^{2+} except where noted. For experiments requiring outside-out patches (Hamill et al., 1981), the solution used to fill the patch electrode ("K aspartate, 5 mM HEPES") was the same as that used in the internal perfusion experiments except for the lowered pH buffer concentration. Catalytic subunit of cAMP-dependent protein kinase (bovine heart) was kindly provided by Irwin Levitan. It was added to the internal solution bathing inside-out patches at a concentration of 0.5 μM.

When testing the selectivity of the conductance activated in the whole cell, we exposed the internally perfused cell to the extracellular test solution (60 mM Ba, 60 mM Ca, etc.) for only 30 sec before switching back to the control solution (hyperosmotic Tris saline). This was done to avoid possible secondary effects a large influx of Ba^{2+} or Ca^{2+} might have on the cell. To quickly and reproducibly change solutions, we held the cell directly in front of the flow tube that was delivering the bath solution and precisely timed the change of solution. The holding current did not reach a new steady value during this short bath exposure; we took the change in current as a measure of the relative conductance for that ion. We estimate that 1–2 min might be required to completely change the solution bathing the cell. The Ba^{2+} conductance was tested with the 60 mM $BaCl_2$ solution used for the patch experiments. 60 mM Ca^{2+} was used in place of the Ba^{2+} to test for the Ca^{2+} selectivity. 45 mM Mg^{2+} plus 15 mM Ba^{2+} was used in testing for the Ba^{2+} *vs.* Mg^{2+} selectivity of the conductance. Hyperosmotic *Lymnaea* saline was used when testing for the Na^+ conductance. Whole-cell holding currents were recorded with 1 Hz low-pass filtering on a Hewlett-Packard 7133A chart recorder. All experiments were done at room temperature (22–25°C).

Results

Single-Channel Studies

When gigaohm seals are obtained with patch electrodes containing high concentrations of Ba^{2+} on healthy, intact cells, inward currents through voltage-activated Ca channels are commonly seen. Excision of these patches (to form inside-out patches) into various solutions designed to mimic intracellular conditions causes the eventual loss of the voltage-dependent Ca channel activity. Frequently, the creation of an inside-out patch reveals the existence of another channel, which was not active in the cell-attached patch. Since this channel is the only one active at the holding potential (−50 mV) with high concentrations of $BaCl_2$ in the pipette, we refer to it as the HP channel, for clarity of description. In Fig. 1, a comparison is made between the activity of the two types of Ba^{2+}-current-carrying channels in patches of membrane. To minimize confusion associated with overlapping currents, the records in Fig. 1*A* and *B* are taken from two different patches. However, both types of channels are often seen in the same patch. The pipette contains 50 mM Ba^{2+} for both patches. The currents in Fig. 1*A*, recorded at a potential of +20 mV, are those of the voltage-activated Ca channel. Those in Fig. 1*B*, at −50 mV, are those of the HP channel. In the cell-attached configuration, labeled −1 *min*, the voltage-activated Ca channel is active at +20 mV, while there is no activity of the HP channel (at −50 mV). (All membrane potentials given for the cell-attached configuration of intact cells are estimated, assuming the cell resting potential to be −50 mV.) One minute after creation of the inside-out patch, the HP channel begins to become active and the voltage-activated channel shows little change in activity as compared with the cell-attached configuration. After 3 min, depolarizations to +20 mV activate the Ca channels less frequently, whereas the openings of the HP channel become more common. Finally, after 9 min in the inside-out configuration, the voltage-dependent Ca channel can no longer be activated, while the HP channel remains quite active. Voltage-dependent Ca channel activity was lost upon patch excision in all inside-out patches tested ($n = 22$), even when we fortified the solution bathing the intracellular face of the channels with various combinations of ATP, Mg^{2+} and the catalytic

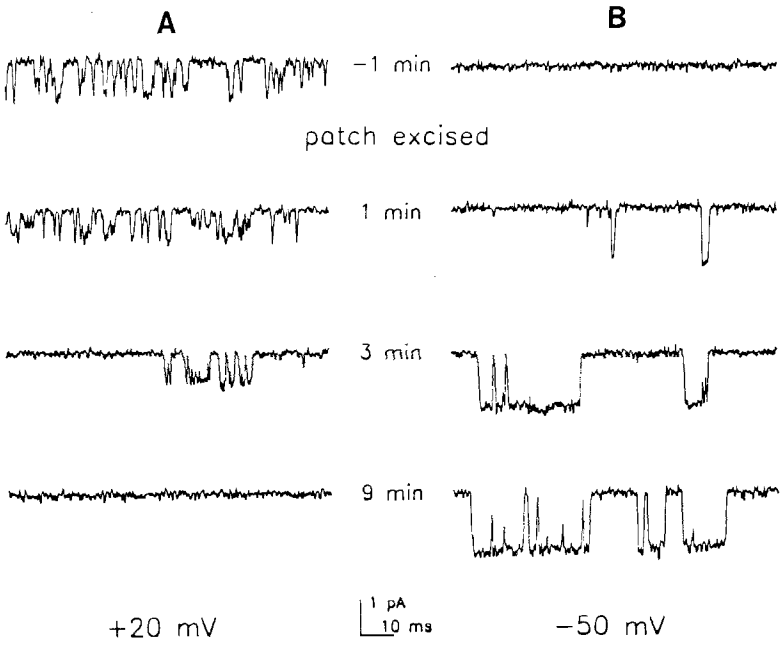

Fig. 1. Patch excision causes the loss of the voltage-dependent Ca channel and the activation of a previously silent channel at the holding potential (HP channel). Patch electrodes contained 50 mM Ba^{2+}. (*A*) Single-channel activity recorded at +20 mV; cell no. 120962. (*B*) Activity at −50 mV in a different patch; cell no. 121061. Patches were excised into K aspartate, 100 mM HEPES. Single-channel activity at −50 mV is not present until after the patch is excised. Several minutes after excision, the activity of the voltage-dependent Ca channel is gone while the HP channel is quite active

subunit of cAMP-dependent protein kinase. HP channels were seen to become active in 31 inside-out patches. We do not think there is a conversion of the voltage-activated channel to the HP channel for two reasons. First, we find many cell-attached patches that have no voltage-activated Ca channels, but which are found to contain one or more HP channels after excision. Secondly, excision of patches that contain the voltage-activated channel do not always reveal HP channel activity.

We find that the activity of the HP channel is usually quite bursty, fluctuating between periods of no activity and periods of high activity. We analyzed single-channel events for 10-sec intervals over a period of 10 min from an inside-out patch containing one of these channels. Figure 2 (upper) shows a plot of the open probability (P_o) for each of these 10-sec intervals. The open probability of the channel varied from 0 to 0.4 during this period. At times the channel was quite active, while at other times it was completely silent. Also plotted in Fig. 2 (lower) is the mean open time for the HP channel during each 10-sec interval. The average open time is about 5 msec and does not seem to be correlated with the probability of opening. Mean open times of about 5 msec were also measured for HP channels from three other patches.

Although the bursty nature of the HP channel makes its activity difficult to quantify, its activity does not seem to have a strong voltage dependence. The HP channel is active over a wide range of potentials; Fig. 3 gives examples of current records taken at potentials between −110 and +70 mV. The

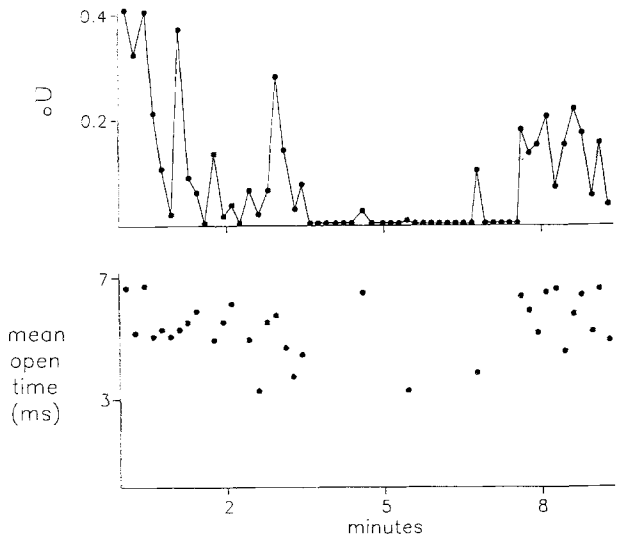

Fig. 2. Bursty nature of HP channel activity. Upper plot: open probability (P_o) of HP channel calculated for consecutive 10-sec periods recorded at −50 mV. Lower plot: mean open times for each period. During some of the periods, the channel did not open. Inside-out patch; electrode contained 50 mM Ba^{2+}; intracellular bathing solution was K aspartate, 100 mM HEPES + Mg ATP; cell no. 121064

current amplitude decreased as the potential became more positive, but we did not establish any clear dependence of open time or open probability on potential. In the inset of Fig. 3, the amplitude of single-channel currents from these records are plotted against voltage. The current exhibits an inward rectification; no outward current is seen at very

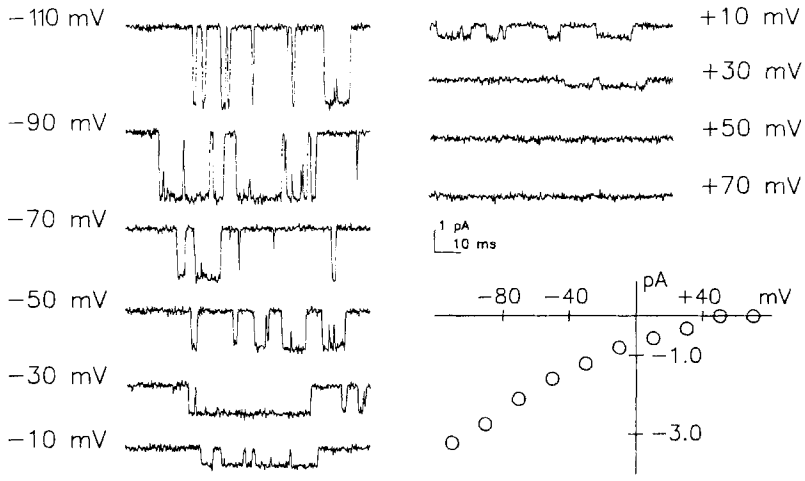

Fig. 3. HP channel activity is not strongly voltage dependent. Recordings of HP channel current at voltages from −110 to +70 mV. Inside-out patch; patch electrode contained 40 mM Ba^{2+}; intracellular bathing solution was K aspartate, 100 mM HEPES. Inset: single-channel current-voltage relationship. Current does not seem to reverse directions above 0 mV. Slope conductance between −110 and −40 mV is 27 pS; cell no. 121064

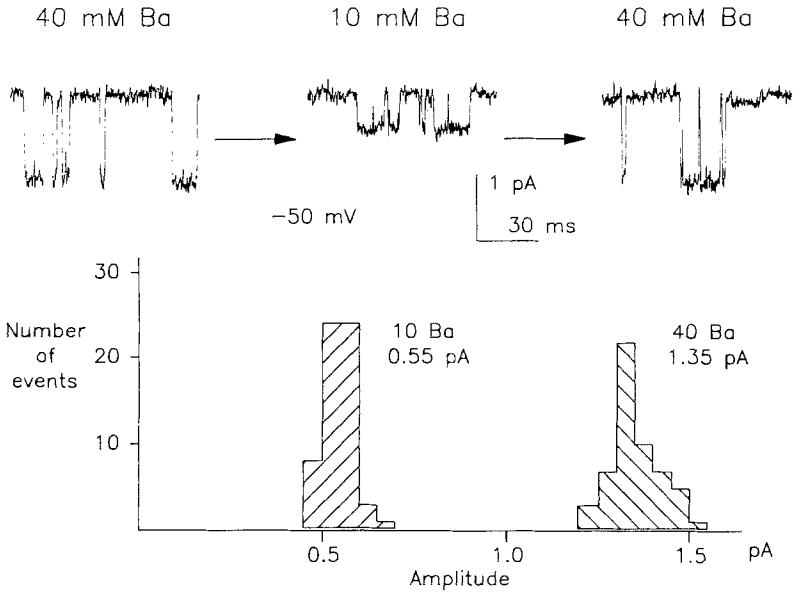

Fig. 4. HP channel passes Ba^{2+} more readily than Mg^{2+}. Top: HP channel activity recorded at −50 mV from an outside-out patch. Patch electrode contained K aspartate, 5 mM HEPES. Bath solution was changed from 40 mM $BaCl_2$ (left) to 10 mM $BaCl_2$ + 30 mM $MgCl_2$ (middle) and then back to 40 mM $BaCl_2$ (right). Histogram shows amplitudes of single-channel currents obtained in the two bathing solutions with mean amplitude levels; cell no. 110467

positive potentials (n = 4). The slope conductance at more negative potentials is 27 pS (range: 25–30 pS, n = 4). There are only two ions that could carry the inward HP channel current seen in the inside-out patches. Either Ba^{2+} moves inward or the current could be carried by the outward movement of aspartate, the major anion bathing the intracellular face of the patch. To test if Ba^{2+} carries the inward HP channel current, we obtained patches of the outside-out configuration so that we could change the concentration of Ba^{2+} at the extracellular face of the channel. We found the HP channel current to be reduced by the partial replacement of Ba^{2+} by Mg^{2+} (Fig. 4). Switching the bathing solution from one containing 40 mM $BaCl_2$ to one where three-fourths of the $BaCl_2$ was substituted with $MgCl_2$ caused the current magnitude to decrease by a factor of 2.5. This effect was reversed by switching back to the 40 Ba^{2+}/0 Mg^{2+} solution. The histograms are of the current amplitudes recorded in the two solutions. This experiment was repeated with similar results in two other patches. This sensitivity of the inward current to the concentration of Ba^{2+} suggests that Ba^{2+} is carrying the inward current. When we changed the intracellular solution from K aspartate to 40 mM $BaCl_2$ (inside-out patch) in an attempt to observe outward current through the channel, we were surprised to discover that the channel's activity was blocked completely. Lowering the intracellular divalent ion concentration to below 1 mM restored inward single channel current activity (Fig. 5). We found the outside-out patch configuration to be very unstable and were unable to test further the selectivity of the HP channel in outside-out

patches. Using the inside-out configuration, however, we tried to see if Na^+ would pass through the HP channel. In eleven patches where NaCl was used in place of $BaCl_2$ in the patch pipette, we were unable to find similar inward single-channel current activity in isolated patches. By contrast, when $BaCl_2$ is used in the pipette, HP channel activity is seen in 57% of inside-out patches (31/54). Thus, either Na^+ cannot carry appreciable current through the HP channel or the channel does not activate upon excision when Na^+ is used in the pipette.

The HP channel does not activate when recording from healthy cells with patch pipettes in the cell-attached configuration, even though later excision of the patches causes activation of the channel (Fig. 1). Thus, it appears that some change in the intracellular environment, such as an increase in the Ca^{2+} concentration or a loss of ATP, causes the turn-on of the HP channel. In an effort to determine what controls the channel's activity, we studied its open probability as a function of the solution bathing the intracellular face of an inside-out patch. We found that varying the concentration of Ca^{2+} from less than 10^{-8} to 10^{-3} M did not appear to affect the frequency of opening or mean open times of the activated channel. Furthermore, addition of ATP and Mg^{2+} (n = 12) or ATP and Mg^{2+} plus the catalytic subunit or cAMP-dependent protein kinase (n = 4) to the intracellular solution did not affect the channel's activity. On the other hand, increasing the concentration of Ca^{2+} or Ba^{2+} above 1 mM reversibly blocked the activity of the channel. This result is shown in Fig. 5. In the top record, the intracellular face of the membrane patch is bathed in a low Ca^{2+}, EGTA-containing solution. Under this condition the channels in the patch are quite active with coincident openings of two channels common. Switching the bathing solution to one containing 3 mM $BaCl_2$ dramatically reduced the frequency of openings without greatly affecting the single channel current amplitude. Return to the low Ca^{2+} solution, in the bottom trace, allows immediate recovery of the previous activity. This experiment was performed 13 times using concentrations of Ca^{2+} or Ba^{2+} from 1 to 50 mM. Concentrations above 5 mM completely blocked HP channels, while those below 1 mM had a minimal effect on their opening frequency.

In addition to its activation through the formation of inside-out patches, the HP channel could also be activated in the cell-attached configuration under certain conditions. These are illustrated in Fig. 6. In Fig. 6*A*, single HP channel activity is seen in a patch on a cell that had been bathed in a high K^+, low Ca^{2+} solution (K aspartate, 5 mM HEPES)

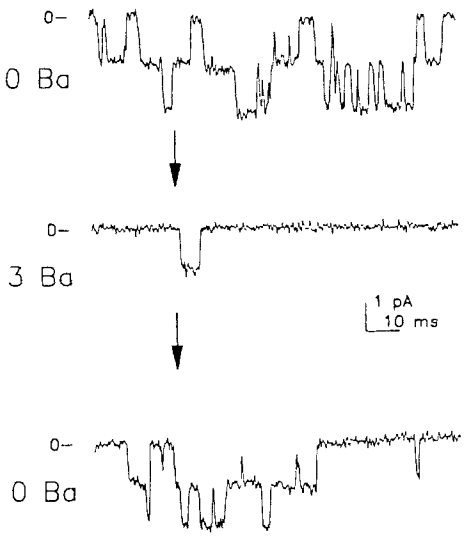

Fig. 5. HP channel activity is blocked by millimolar concentrations of Ba^{2+} on intracellular side. Recordings from an inside-out patch at −50 mV. Patch electrode contains 50 mM Ba^{2+}. Top: intracellular face of patch is bathed with K aspartate, 100 mM HEPES; solution contains 5 mM EGTA. Middle: 1 min after 3 mM $BaCl_2$ (no EGTA) was added to the bathing solution. Bottom: 1 min after return to K aspartate, 100 mM HEPES (0 Ba^{2+}); cell no. 121862

for approximately 15 min prior to patch recording. Apparently, prolonged cell depolarization in low Ca^{2+} solution creates conditions conducive to the turn-on of this channel. The current records in Fig. 6*B* are taken from a patch on a cell that had been perfused with a minimal intracellular solution (no Mg ATP) for about 20 min before patch recording. Here, too, HP channel activity was present. Lastly, as illustrated in Fig. 6*C*, increasing the intracellular Ca^{2+} concentration induces HP channel openings in the cell-attached configuration. In this case, patch recordings were made continuously while the cell was being intracellularly perfused with various solutions. During the first 10 min of perfusion with an ATP-containing solution, no single-channel activity was detected at −50 mV. Then the intracellular perfusate was changed to a high Ca^{2+} solution (pCa 5.7, no ATP). Three minutes later single channel events began to appear. The activity increased dramatically over the course of the next several minutes. After nine minutes of perfusion with the high Ca^{2+} solution, the perfusate was switched back to the low Ca^{2+}, ATP-containing solution. Eleven minutes later, the channel activity was only partly suppressed. Recordings with Ca^{2+}-sensitive electrodes have shown that internal perfusion with low Ca^{2+} solutions brought the intracellular Ca^{2+} concentration to less than 10^{-6} M within 10 min (Byerly & Moody, 1984). It thus appears that the channel is

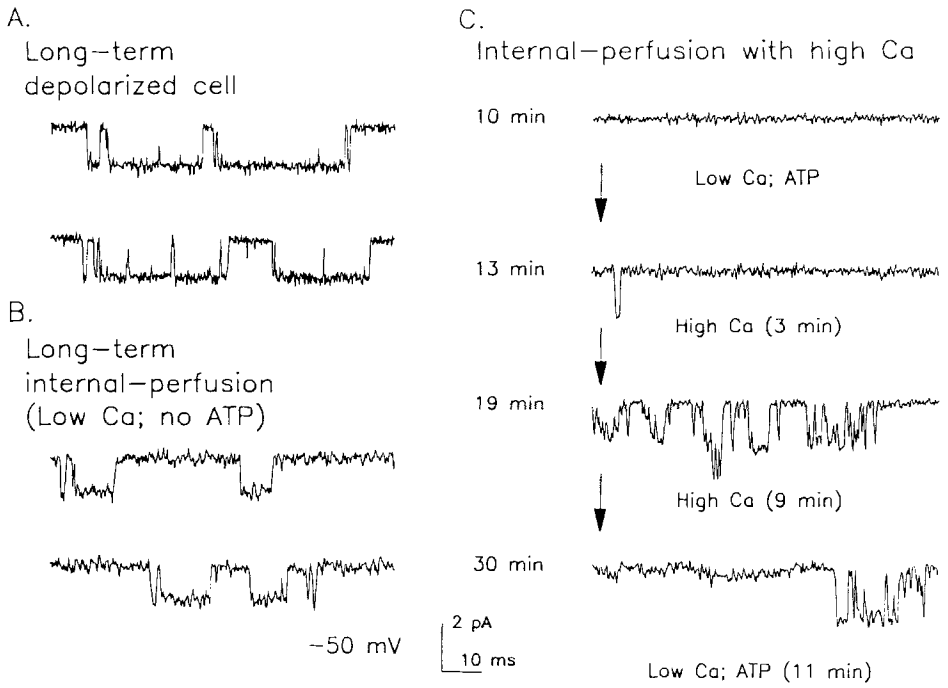

Fig. 6. Activation of HP channel in cell-attached patches. (*A*) Inward single-channel events were recorded at −50 mV after cell was bathed in K aspartate, 5 mM HEPES for 15 min. Patch electrode contained 50 mM Ba^{2+}; cell no. 120864. This result was obtained in three patches. (*B*) Internal perfusion of whole cell with Cs aspartate, 100 mM HEPES for 20 min activates the HP channel recorded by a patch electrode (−50 mV). Patch electrode contained 40 mM Ba^{2+}; cell no. 71062. This result was obtained three times. (*C*) Cell was internally perfused with Cs aspartate, 100 mM HEPES + Mg ATP for 10 min. During this time, no inward single-channel current events were recorded at −50 mV with the patch electrode. The intracellular perfusion solution was then switched to Cs aspartate, *p*Ca 5.7. After 3 min an HP channel became active. After 9 min in the *p*Ca 5.7 solution the HP channel was very active. Eleven minutes after switching back to the low Ca^{2+}, ATP-containing solution, the activity of the HP channel is somewhat decreased but not completely suppressed. Patch electrode contained 60 mM Ba^{2+}; cell no. 52663. This experiment was repeated two more times with similar results

activated by an increase in intracellular Ca^{2+}, but only partially deactivated by return to a low Ca^{2+} internal environment (but Ba^{2+} may remain high under the patch).

Whole-Cell Studies

Since the HP channel was the only channel that we saw open in membrane patches held at −50 mV while exposed to isotonic $BaCl_2$ on the external surface, it occurred to us that the Ba current of the whole-cell membrane when held at −50 mV might be entirely carried by HP channels. If so, control of HP channels could be much more easily studied by measuring the holding Ba conductance in whole-cell experiments, due to the large number of channels involved and the ability to readily change both external and internal solutions. Thus, we monitored the holding Ba conductance of the whole cell while manipulating the intracellular environment in ways that have been shown to affect the HP channel in cell-attached patches (Fig. 6). We then proceeded to study the holding conductance under conditions that had not been studied on single HP channels. In the Discussion we examine the validity of the assumption that the holding current flows through HP channels. To avoid secondary effects a rise in intracellular Ba^{2+} might cause, we only briefly exposed cells to the high Ba^{2+} external solution. We continuously recorded the whole-cell holding current from an internally perfused cell bathed in hyperosmotic Tris saline solution, which contains 4 mM Ca^{2+}. Once every 3–5 min, for precisely 30 sec, we changed the solution flowing into the bath to one containing 60 mM Ba^{2+}. By measuring the relative amplitudes of the inward current transients resulting from those solution changes, we could estimate variations in the Ba^{2+} conductance (at −50 mV) in response to changes in the intracellular perfusate.

We found that the Ba^{2+} conductance of the cell membrane at the holding potential can be reversibly activated by raising the intracellular Ca^{2+} concentration. Figure 7 is a continuous recording of the holding current seen with the cell voltage clamped at −50 mV. The replacement of a Mg ATP-containing, low Ca^{2+} internal solution with a *p*Ca 5.7 (no

Mg ATP) solution causes an increase in the baseline holding current recorded in hyperosmotic Tris saline (4 mM Ca^{2+}) as well as an increase in the amplitude of the current transients recorded during the switch to 60 mM Ba^{2+} (at arrows). The return to the low Ca^{2+} internal solution containing ATP and Mg^{2+} causes a reduction in the Ba^{2+} current transients and a decrease in the holding current recorded in hyperosmotic Tris saline back to near its original level. Since the HP channel is the only channel we find to be active at −50 mV in patches with high Ba^{2+} external solution and since the whole-cell Ba current at −50 mV is activated by elevated intracellular Ca^{2+}, we conclude that the whole-cell Ba current at −50 mV is carried through HP channels. We assume that the increase in the holding current measured in hyperosmotic Tris saline which occurs in response to the elevation of internal Ca^{2+} is carried by Ca^{2+}. We cannot, however, exclude the possibility that at least some of this current is being carried by $Tris^+$.

Long-term perfusion with a minimal intracellular solution (containing no Mg ATP) caused the activation of HP channels in patches (Fig. 6*B*). Such conditions also cause an increase in the holding potential Ba^{2+} conductance (Fig. 8). During the course of perfusion the holding current recorded in hyperosmotic Tris saline becomes increasingly more inward and the magnitude of the Ba current transients increases. The voltage-activated Ca current disappeared during this time, which can be seen as a decrease in the size of the fast inward current transients recorded once per minute during the first half of the experiment (Fig. 8*A*). Figure 8*B* illustrates, for three experiments, the decline in the voltage-activated Ca current magnitude and the increase in the holding current measured during the Ba^{2+} pulses. It is apparent that the holding current increases under the same conditions that the voltage-dependent Ca current washes out, although most of the Ca current is lost before a large increase in the presumed HP channel activity occurs.

Intracellular perfusion with EGTA-containing solutions to which 2 mM ATP and 1 mM $MgCl_2$ (Mg ATP) are added can prevent the progressive loss of the voltage-dependent Ca current in these cells (Byerly & Yazejian, 1986). We have found that the addition of Mg ATP also prevents the dramatic increase in the resting Ba^{2+} conductance seen after prolonged perfusion with an ATP-free internal solution (*compare* Fig. 9 with Fig. 8). Fig. 9 shows a continuous record of the holding current for a cell that is perfused for 90 min with a solution containing Mg ATP. During this time the response to pulses of 60 mM Ba^{2+}, indicated by the arrows, increases very little, and the baseline current is also quite

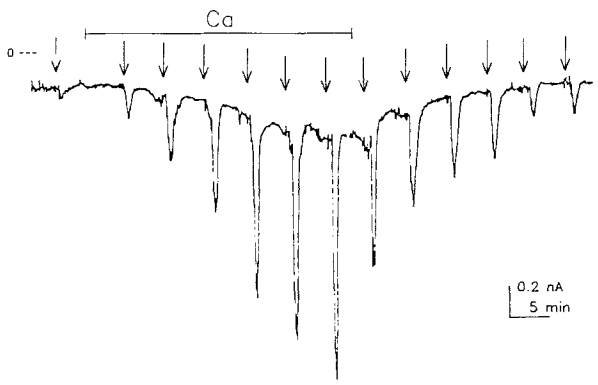

Fig. 7. Ba^{2+} conductance at the holding potential is activated by raising intracellular Ca^{2+}. Continuous record of whole-cell holding current of cell clamped to −50 mV, internally perfused with Cs aspartate, 100 mM HEPES. The current records in this and all following figures are low-pass filtered at 1 Hz. During the time indicated (Ca) the internal perfusate was switched to Cs aspartate, *p*Ca 5.7. Bath solution was hyperosmotic Tris saline except for the times indicated by the arrows when the flow was changed to 60 mM $BaCl_2$ for 30 sec; cell no. 63071. This experiment was repeated in two other cells with similar results

stable. This ability of internal Mg ATP to prevent the activation of HP channel current in the whole cell was seen in all three cells tested. The magnitude of the voltage-activated Ca current was similarly quite stable during the course of the perfusion with Mg ATP (*data not shown*).

To test the selectivity of the whole-cell conductance activated at −50 mV by raising the intracellular Ca^{2+} concentration, we applied 30-sec pulses of various solutions to cells whose resting conductances were previously activated as monitored by inward current responses to Ba^{2+} pulses. We discovered that the current carried by Ca^{2+} is slightly smaller than that carried by Ba^{2+} (Fig. 10*A* and *B*) and that 50 mM Na^+ provides slightly more current than 60 mM Ba^{2+} (Fig. 10*C*). Furthermore, we found that this conductance is selective for Ba^{2+} over Mg^{2+} as was seen for the HP channel in the single-channel studies (Fig. 4). A reduction of the Ba^{2+} concentration from 60 to 15 mM (replacing Ba^{2+} with Mg^{2+}) reduces the size of the current (Fig. 10*D*). We are not certain if these different ions are flowing through the same channels; but in different cells the same relative current ratios are obtained for the different ions, consistent with the involvement of only one channel type. It appears that the membrane conductance studied at the whole-cell level is relatively nonspecific, passing both monovalent and divalent cations. The larger current carried by 50 mM Na^+ as compared with the 60 mM Ba suggests that the active channels are more permeable to Na^+ than Ba^{2+}.

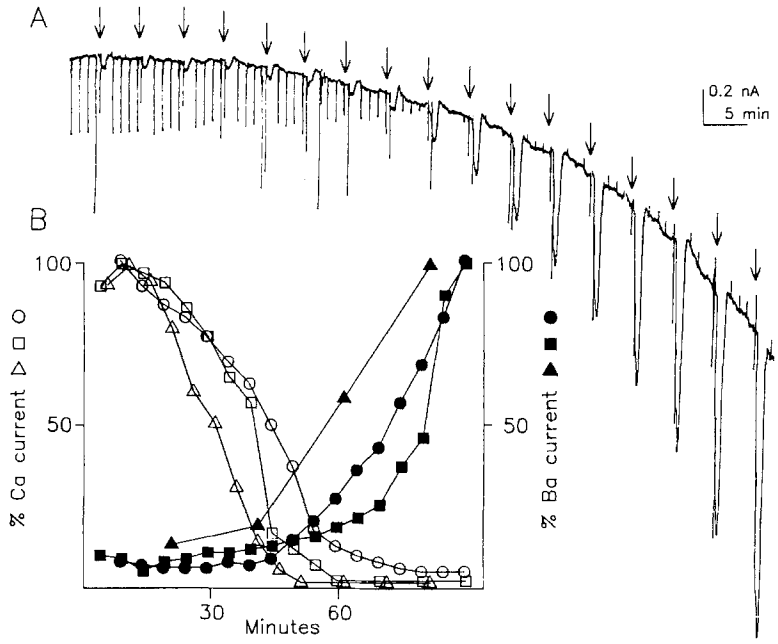

Fig 8. Ba^{2+} conductance at the holding potential is activated with prolonged internal perfusion. (*A*) Continuous whole-cell holding current record. Holding potential was −50 mV. Cell was internally perfused with Cs aspartate, 100 mM HEPES. The total holding current was −0.2 nA at the beginning of the experiment. External solution was hyperosmotic Tris saline except for the times indicated by the arrows when bath was changed to 60 mM $BaCl_2$ for 30 sec; cell no. 61671. (*B*) For three cells, magnitude of peak Ca current at +20 mV (open symbols) and Ba^{2+} conductance at −50 mV (filled symbols) are plotted against perfusion time; circles, cell no. 61671; squares, cell no. 61674; triangles, cell no. 62371

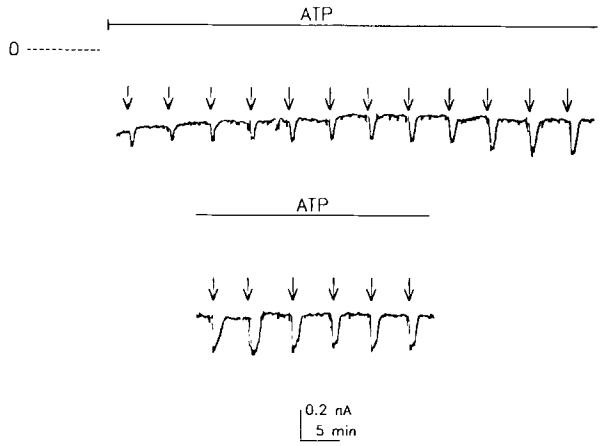

Fig. 9. Intracellular perfusion with Mg ATP prevents increase in Ba^{2+} conductance at the holding potential. Continuous holding current record (−50 mV). Zero current level is indicated by dashed line. Cell was internally perfused with Cs aspartate, 100 mM HEPES + Mg ATP throughout the experiment. Bath solution was normally hyperosmotic Tris saline. Arrows indicate 30 sec change of solution flowing into the bath to 60 mM $BaCl_2$; cell no. 61972

Different permeant ions have distinct secondary effects on the holding potential conductance. The Ba conductance activated by intracellular perfusion with elevated Ca^{2+} cannot be turned off by changing to a low-Ca, Mg ATP containing internal solution if 60 mM Ba^{2+} is maintained in the bath solution throughout the experiment (*data not shown*, $n = 3$). On the other hand, if the bath is changed only briefly from 4 mM Ca^{2+} to 60 mM Ba^{2+} to test for the Ba^{2+} conductance increase during the internal Ca^{2+} load, then the increased conductance can be turned off by the low-Ca^{2+}, Mg ATP solution (Fig. 7). External Ca^{2+} or Ba^{2+} show characteristic current responses when applied for longer periods to cells that have large holding conductances activated. Results from experiments of this type are shown in Fig. 11. A 15-min bath application of 60 mM Ca^{2+} results in a large inward current, which only slowly decays. Returning to 4 mM Ca^{2+} (hyperosmotic Tris saline) reduces the magnitude of the inward current back to the previous level. Switching then to 60 mM Ba^{2+} causes a larger inward current to flow, which shows a much faster rate of decay. When the bath solution is returned to 4 mM Ca^{2+}, the magnitude of the holding current drops below and then increases back to the level seen before the 60 mM Ba^{2+} bath exposure.

Discussion

Byerly and Moody (1984), using Ca^{2+}-sensitive microelectrodes have shown that an elevation of intracellular Ca^{2+} in perfused *Lymnaea* neurons clamped to −50 mV will cause a regenerative increase in the Ca^{2+} concentration, which occurs as Ca^{2+} enters the cell through a Cd^{2+}-sensitive conductance. In this paper we have described a channel that passes Ba^{2+} (and presumably Ca^{2+}), which we think underlies the conductance that produces this

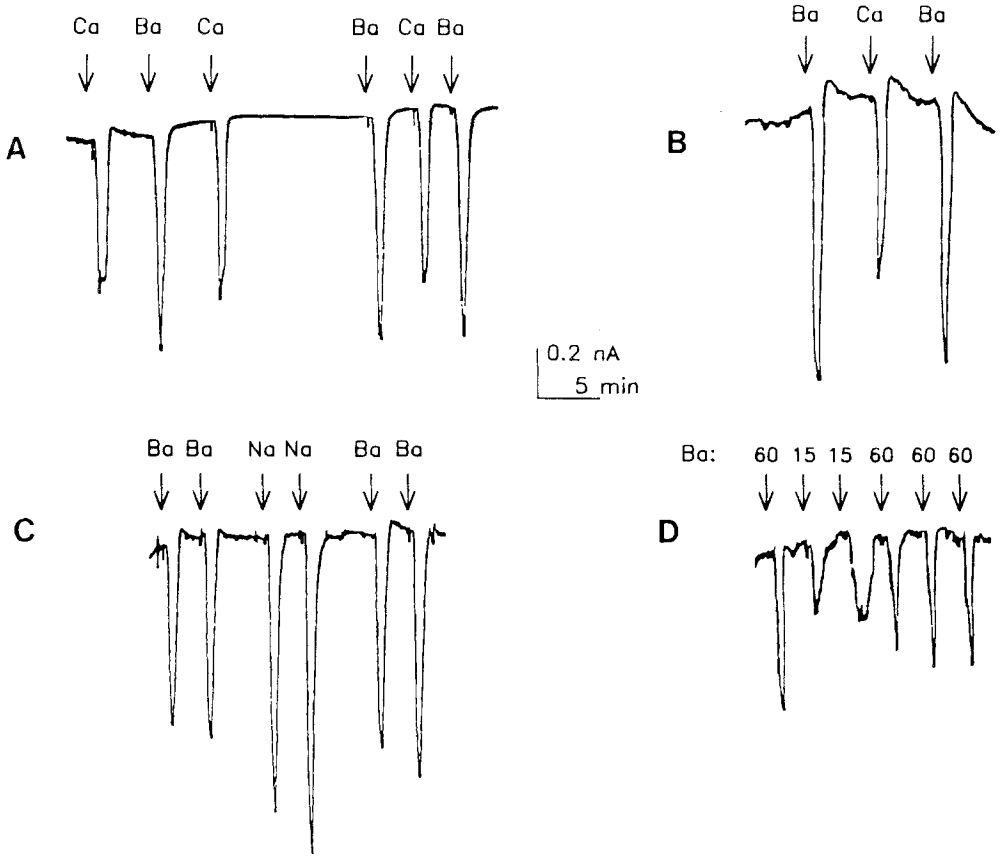

Fig. 10. Selectivity of holding potential conductance. (*A–D*) Holding potential conductance had been activated before these experiments were done by internal perfusion with Cs aspartate, *p*Ca 5.7. When the internal solution was returned to Cs aspartate, 100 mM HEPES + Mg ATP, some of the holding potential conductance remained activated. External solution was hyperosmotic Tris saline except for 30 sec exposures to various solutions (arrows); holding current was typically −0.5 nA for hyperosmotic Tris saline in bath. (*A* and *B*) 30 sec bath exposures to 60 mM $CaCl_2$ or $BaCl_2$ in different cells demonstrates that Ba^{2+} is able to carry more current than Ca^{2+}; cell nos. 61972, 62373. (*C*) Switching bath solution to *Lymnaea* saline (50 mM NaCl) shows that 50 mM Na^+ carries more current than 60 mM Ba^{2+}; cell no. 63072. These results were also obtained in two other cells. (*D*) Conductance passes Ba^{2+} better than Mg^{2+}: smaller current is carried when 15 mM $BaCl_2$ + 45 mM $MgCl_2$ (15) solution is pulsed on the cell for 30 sec than when 60 mM $BaCl_2$ (60) is pulsed onto cell; cell no 63073. These results were also obtained in two additional cells

regenerative increase in internal Ca^{2+}. Since this channel is the only one active at the holding potential (−50 mV) with isotonic $BaCl_2$ in the electrode, we call it the HP channel. In the first half of this paper we characterize this channel at the single-channel level; in the second half we study the whole-cell membrane current while the membrane potential is held at −50 mV, testing the hypothesis that this holding current flows primarily through HP channels.

Does the Holding Current Flow Through HP Channels?

Since the HP channel is the only channel that has been seen to open at −50 mV in the large number of patches studied with isotonic $BaCl_2$ in the electrode, it is reasonable to expect changes in Ba conductance at −50 mV to reflect changes in activation of the HP channels. In fact, we found good agreement between conditions that activated the holding Ba conductance and those that activated HP channels studied in patches. Intracellular perfusion of cells with solutions in which Ca^{2+} was buffered to very low levels and which included ATP and Mg^{2+} (Mg ATP) prevented the activation of HP channels in patches (Fig. 6*C*, top) and prevented the activation of Ba conductance at −50 mV in the whole cell (Fig. 9). Changing the intracellular solution to an elevated Ca^{2+} solution (*p*Ca 5.7, no ATP) caused an activation of HP channels studied in patches (Fig. 6*C*) and an increase in the Ba conductance at the holding potential (Fig. 7). If Mg ATP is not included in the internal solution, prolonged perfusion activates both HP channels in patches (Fig. 6*B*) and the holding Ba conductance (Fig. 8). Also, the rapid

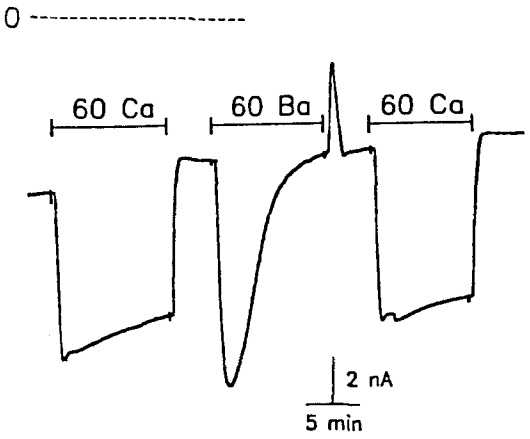

Fig. 11. Holding potential current inactivates more rapidly when Ba^{2+} carries the current than when Ca^{2+} is the current carrier. Conductance was activated by internal perfusion with Cs aspartate, *p*Ca 5.7 before these currents were recorded. Internal solution was Cs aspartate, 100 mM HEPES + Mg ATP during this recording. External solution was 60 mM $CaCl_2$ (60 Ca), 60 mM $BaCl_2$ (60 Ba) or hyperosmotic Tris saline. Large inward current carried by Ca^{2+} inactivates only very slowly, while that carried by Ba^{2+} inactivates quickly. Return to hyperosmotic Tris saline from Ba^{2+}, but not from Ca^{2+}, causes the magnitude of the inward holding current to drop below the level recorded in hyperosmotic Tris saline. Zero current level is indicated by dashed line. Cell no. 61972. This experiment was repeated, with similar results, in a second cell

decline of the holding Ba conductance seen with prolonged exposure to external Ba^{2+} (Fig. 11) is consistent with the block of the HP channel by millimolar concentrations of internal Ba^{2+} (Fig. 5).

On the other hand, when the external solution is not isotonic $BaCl_2$, it is not known if HP channels carry most of the holding current. The membrane may contain other channels which open at −50 mV but don't carry Ba^{2+} or are blocked by it. In particular, the membrane may contain Ca^{2+}-activated cation-selective channels which do not pass divalents (Colquhoun et al., 1981; Yellen, 1982). If so, the holding current measured when the external solution contains Na^+ or other small monovalent cations would not demonstrate only the properties of the HP channels.

What Is the Selectivity of the HP Channel?

From the single-channel studies it is clear that Ba^{2+} passes through the HP channels better than Cl^-, aspartate$^-$, K^+, Cs^+, or Mg^{2+} (Figs. 3 and 4). The whole-cell holding current studies find that Ba^{2+} passes through the activated membrane better than Mg^{2+} (Fig. 10*D*), which supports the conclusion that with divalent cations as the only permeant cations in the external solution, the holding current is primarily carried by HP channels. Since the Ca holding current is slightly smaller than the Ba holding current (Figs. 10*A*, *B*, and 11), it seems reasonable to conclude that Ca^{2+} passes through the HP channels almost as well as Ba^{2+}. However, our present data does not allow a conclusion as to how well Na^+ passes through the HP channel. The large Na conductance of the activated membrane at −50 mV (Fig. 10*C*) does not prove that Na^+ passes through HP channels, since the Na^+ may be passing through different channels that do not carry divalent cations. The absence of HP channel type activity in inside-out patches obtained with pipettes containing primarily NaCl suggests that Na^+ does not pass through the HP channel, but is not conclusive since we do not understand the channel's activation mechanism (*see below*). Additional experiments are required to determine the permeability of the HP channel for Na^+. If outward single-channel currents are seen when the internal cation is Na^+ for an experiment like that of Fig. 3 or if partial replacement of Ba^{2+} by Na^+ in an experiment like that of Fig. 4 does not greatly reduce the single-channel current amplitude, then it could be concluded that the HP channel carries Na current.

What is the Mechanism of Activation of the HP Channel?

It is interesting that the activation of the HP channel appears to have an exactly opposite dependence on intracellular conditions as does the voltage-dependent Ca channel (Byerly & Yazejian, 1986; Yazejian & Byerly[1]). Excision of patches activates the HP channel and causes the loss of voltage-dependent Ca channel activity (Fig. 1). Internal perfusion with a solution that elevates internal Ca^{2+} (and reduces internal ATP) blocks the voltage-dependent Ca current and activates the HP channels; then returning to the low Ca^{2+}, ATP-containing internal solution causes a recovery of the voltage-dependent Ca current and turns off the HP channels (Fig. 7). Prolonged perfusion with internal solutions without ATP causes washout of the voltage-dependent Ca current and activation of the HP channel current (Fig. 8). Since the intracellular control of the voltage-dependent Ca channel is not understood, it is not surprising that control of the HP channel is not understood. In parallel to the present models for control of the voltage-dependent Ca channel, it is reasonable to consider that control of the HP channel may be mediated by direct binding of divalent

[1] Yazejian, B., Byerly, L. ATP allows recovery of calcium current from block by high calcium in internally perfused snail neurons. (*submitted*)

cations to the channel or by phosphorylation of the channel.

We have considered the hypothesis that the activation of HP channels is through the direct binding of intracellular Ca^{2+} as is the case with the Ca^{2+}-activated K channel (Marty, 1981). Our result of the ability of ATP to turn off the conductance in the whole cell could be explained by a reduction of intracellular Ca^{2+} mediated by a Ca^{2+} pump like that seen in squid axon (Beaugé et al., 1981). The inability of ATP to turn off the increased Ba^{2+} conductance, activated by high internal Ca^{2+}, when the cell is continuously bathed in isotonic Ba^{2+} can be explained by the direct binding hypothesis by assuming that Ba^{2+}, flowing through HP channels, accumulates to micromolar concentrations around the inner face of the channels and that Ba^{2+} also activates the channels. This accumulation of Ba^{2+} may also be occurring with inside-out patches, causing the channels to remain open, once activated. It is possible that the excision of a membrane patch may disrupt intracellular Ca^{2+}-containing organelles, causing a transient rise in the Ca^{2+} concentration near the inner surface of the HP channels, which would initially activate the channels. If the activity of the HP channel is maintained by the internal accumulation of Ba^{2+}, the only way to turn the HP channel off (once activated) would be to take away the high concentration of external Ba^{2+}. This is not possible with inside-out patches, but it is possible in whole-cell experiments. Returning to a low Ca^{2+}, ATP-containing internal solution turns off the holding Ba conductance when the extracellular solution is changed to a Ba^{2+}-free, 4 mM Ca^{2+} solution (Fig. 7), but not if isotonic $BaCl_2$ is always the external solution. Thus, the accumulation of Ba^{2+} on the intracellular side of the HP channels does seem to play a role in maintaining the activity of the channel.

An alternative hypothesis that might explain the control of HP channels is that their activity is suppressed by phosphorylation, as occurs with the serotonin-sensitive K channel (Shuster et al., 1985). The action of ATP in preventing the activation of the HP conductance in response to a rise in the internal Ca^{2+} concentration could be explained by an increase in the level of phosphorylation. Internal perfusion with elevated Ca^{2+} solutions without ATP could act to decrease the level of phosphorylation through activation of Ca^{2+}-activated phosphatases (Klee, Crouch & Krinks, 1979). The inability of Mg ATP to turn off HP channel activity in patches or in whole cells continuously exposed to high external Ba^{2+} could be explained within the phosphorylation hypothesis by a loss of kinase activity. The lack of effect of adding catalytic subunit of cAMP-dependent protein kinase suggests that if phosphorylation does control the channel a different kinase is involved, e.g., protein kinase C (Kaczmarek, 1986).

Although the activation of HP channels is correlated with an increase in the intracellular Ca^{2+} (or Ba^{2+}) concentration, very high levels of divalent cations will block channel activity (Fig. 5). This is also seen in whole cells; the continuous presence of high levels of Ba^{2+} in the bath will cause a decay of the holding potential current. In contrast, when Ca^{2+} is the current carrier, the decay is much less complete (Fig. 11). This result is consistent with the idea that the cell is better able to sequester Ca^{2+} than Ba^{2+} (Connor & Ahmed, 1984) and so when high concentrations of Ca^{2+} are used externally, HP channels are not blocked as much. The fact that HP channels in isolated patches do not inactivate when high levels of Ba^{2+} are used in the pipette (e.g. Fig. 1) may be explained by the faster exchange of solutions bathing the inner face of an excised patch. On the other hand, there may be transient increases in the concentration of Ba^{2+} in the solution bathing the cytoplasmic side of inside-out patches which could account for the bursty nature of the activity of these channels (Fig. 2).

How Does the HP Channel Compare with Other Channels?

Single-channel currents from nonspecific cation channels which are activated by intracellular Ca^{2+} were first recorded in cardiac cells (Colquhoun et al., 1981) and later in neuroblastoma (Yellen, 1982). These currents resemble the HP channels described here, both in their activation by intracellular Ca^{2+} and in the fact that their activation is not strongly voltage dependent. Calcium-activated nonspecific cation channels have since been described in a variety of preparations (Partridge & Swandulla, 1988). Many of these channels differ, however, from HP channels in that they do not seem to be permeable to Ca^{2+}. On the other hand, a Ca^{2+}-permeable conductance in *Helix* neurons was shown to be activated by pressure injection of Ca^{2+} (Swandulla & Lux, 1985), although the Ca^{2+}-permeability of the single channels has not yet been demonstrated (Partridge & Swandulla, 1987). It may be difficult to demonstrate the Ca^{2+}-dependent activation of a channel that passes Ca^{2+}. After a Ca^{2+}-activated channel is opened by raising the intracellular Ca^{2+} concentration, it may not be possible to lower the Ca^{2+} concentration again near the internal side of the channel due to Ca^{2+} entering through the channel and accumulating locally. The extent to which internal Ca^{2+} accumulates depends on both the conductance and the speed of removal of Ca^{2+} from the inner face of the excised patch. Von Tscharner et al. (1986) have demonstrated an ion channel in hu-

man neutrophils that is only activated when the solution bathing the cytoplasmic face of an excised patch contains greater than 10^{-7} M Ca^{2+}. They were able to turn off the conductance by lowering internal Ca^{2+}. These workers report that this channel selects very poorly between Ca^{2+}, Na^+ and K^+ but is impermeable to anions. The rise in intracellular Ca^{2+} which activates the conductance under physiological conditions, seems to be induced by inositol 1,4,5-trisphosphate (IP_3) in these cells. An IP_3-induced rise in intracellular Ca^{2+} has been reported in many systems (Berridge & Irvine, 1984). An ion channel in human T-lymphocytes has also been shown to be activated by IP_3 mobilization (Kuno & Gardner, 1987). This channel is similar to the HP channel described here in that it passes Ca^{2+} quite readily, is not strongly voltage dependent and is blocked by millimolar concentrations of intracellular Ca^{2+}. It is not likely that these are the same as HP channels, however, since their single-channel conductances and open- and closed-time kinetics are quite different. Fink, Connor and Kaczmarek (1987) have demonstrated the existence of a conductance in peptidergic neurons of *Aplysia* that also passes inward current in response to an increase in the intracellular concentration of IP_3.

A divalent-selective channel in *Aplysia* that has several characteristics in common with the HP channels described here has been reported (Chesnoy-Marchais, 1985). The activation of this channel, like HP channels, is not strongly dependent upon voltage, being quite active at negative potentials. The *Aplysia* channel also resembles HP channels in single-channel conductance, open-time kinetics, permeability to divalent cations and its apparent inward rectification. One difference between the *Aplysia* channel and the HP channel is the observation that the former is quickly inactivated when Cs^+ is included in the solution bathing the cytoplasmic side of patches while we routinely record HP channels in Cs^+-containing solutions. Although it is not shown that the *Aplysia* channel is activated by intracellular Ca^{2+}, Chesnoy-Marchais speculates that this channel may underlie the noninactivating inward current described in *Aplysia* bursting pace-maker neurons, which is activated at negative potentials by injection of Ca^{2+} (Kramer & Zucker, 1985). Strong and colleagues have studied a channel from *Aplysia* bag cells which they conclude is the same as that studied by Chesnoy-Marchais (Strong et al., 1987). Their characterization of the channel makes clear its similarity to the HP channel. It is found in virtually every inside-out patch, but is almost never active in cell-attached patches. It does become active in cells following prolonged exposure to low-Ca solution; neither Mg ATP nor Ca^{2+} (up to 10 μM) can inhibit the channel's activity in inside-out patches. Wilson, Jones and Lewis (1986) have reported Ca^{2+}-activated inward currents in *Aplysia* nonbursting neurons that are permeable to both Ca^{2+} and Na^+. Finally, Coyne, Dagan and Levitan (1987) have described Ca^{2+} and Ba^{2+} permeable channels from *Aplysia* reconstituted in lipid bilayers. These channels have several properties in common with HP channels, including the lack of voltage-dependent activation, selectivity for Ba^{2+} over Mg^{2+}, long open-time, slope conductance and episodic gating behavior.

We do not know how closely the HP channel that we see in *Lymnaea* is related to these channels seen in other systems. Furthermore, the function of these divalent-cation permeant channels, which may be activated by intracellular Ca^{2+}, is not clear. Nevertheless, they appear to be common, at least in molluscan neurons, which suggests they may play an important role in neuronal function.

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