Divalent-selective Voltage-independent Calcium Channels in *Lymnaea* Neurons: Permeation Properties and Inhibition by Intracellular Magnesium

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Calcium channels are tightly regulated. Voltage-gated calcium channels open only in response to depolarization, while voltage-independent calcium channels usually open only in response to specific intracellular or extracellular ligands. Voltage-independent calcium channels have been described in several invertebrate neurons. One difficulty in understanding the function of the neuronal channels is that their regulators are unknown. They open rarely in intact cells but are activated by formation of a cell-free patch, suggesting that some intracellular inhibitor usually keeps them closed. This article provides evidence that intracellular Mg^{2+} is one important regulator of the voltage-independent calcium channel (HP channel) in neurons of the pond snail *Lymnaea stagnalis*. Mg^{2+} (1 mM) rapidly and reversibly inhibited activity of this calcium channel when applied to the intracellular side of cell-free membrane patches. The primary effect of the Mg^{2+} was to promote long closings of the channel. The mechanism of the intracellular Mg^{2+} inhibition is distinct from open channel block, a phenomenon seen in a variety of cation channels. Open channel block can also be seen in the HP channels, but only at very positive membrane potentials. Some of the permeability and selectivity characteristics of these channels were also examined. The channels are permeable to Mg^{2+} and Ca^{2+} as well as Ba^{2+} . Outward currents carried by monovalent cations can be observed only at very positive membrane potentials, indicating high selectivity for divalent over monovalent cations. The single channel current–voltage relationship is markedly nonlinear, becoming quite shallow near the reversal potential, and hence is qualitatively similar to that seen in many voltage-activated calcium channels.

Voltage-independent calcium channels are found in a variety of cell types (for review, see Tsien and Tsien, 1990). In general, these channels tend to be tightly regulated, not by the membrane potential, but by various ligands. Examples include the receptor-operated calcium channels described in smooth muscle (Benham and Tsien, 1987) and a fibroblast cell line (Matsunaga et al., 1988), and channels gated by intracellular second messengers

such as the calcium-activated channels in neutrophils (von Tscharner et al., 1986) or the channels gated by inositol 1,4,5-trisphosphate in lymphocytes (Kuno and Gardner, 1987). In the context of this article, “voltage independent” is a relative term, referring to channels either that completely lack voltage dependence, or whose voltage dependence is several orders of magnitude smaller than that seen in L-type voltage-activated calcium channels.

Most studies of voltage-independent calcium channels have used non-neuronal cells, and it is often assumed that, in neurons, calcium enters only through voltage-activated calcium channels. However, voltage-independent calcium channels have been described in several neuronal preparations, including *Aplysia* (Chesnoy-Marchais, 1985), spiny lobster (McClintock and Ache, 1990), and *Lymnaea* (Yazajiian and Byerly, 1989). The latter referred to the channel as the “HP channel,” a term we will also use. A central problem in understanding the function of these channels is that their physiological regulators are unknown. The channels are common (i.e., found in the majority of membrane patches, often in multiple copies) and, at least in mollusks, seem to be rather universally distributed rather than restricted to particular neuronal subtypes. Yet single channel recordings indicate that the channels rarely open during cell-attached recording. In addition, whole-cell and microelectrode recordings do not reveal the high resting conductances and depolarized resting potentials that these channels would be expected to generate when open. Experimentally, HP channel activity can be activated by formation of a cell-free patch, suggesting that some cytoplasmic factor normally keeps the channels closed (Strong et al., 1987; Yazajiian and Byerly, 1989). Coulombe et al. (1989) made similar observations on an analogous channel found in cardiac muscle. In *Lymnaea* and *Aplysia* neurons, a number of potential intracellular inhibitors have been tested but failed to close the HP channel directly in cell-free patches. Substances tested include Mg -ATP, Ca^{2+} (<1 mM), cAMP-dependent protein kinase (Yazajiian and Byerly, 1989), GTP, glucose, and amino acids (Strong et al., 1987). Yazajiian and Byerly (1989) described a number of experimental conditions that can cause the channel to open, including prolonged exposure of cells to high- K^+ divalent-free extracellular solutions, and intracellular perfusion with ATP-deficient solutions or solutions containing high Ca^{2+} concentrations ($pCa = 5.7$). These results suggest that the channel might open under certain “pathological” conditions, but do not resolve the question of when and how the channel normally opens.

This article describes a possible physiological regulator of the *Lymnaea* HP channel, intracellular Mg^{2+} . This possibility was

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suggested by the observation that millimolar intracellular concentrations of Ba^{2+} and Ca^{2+} could close the HP channel (Yaziji and Byerly, 1989). We report that HP channel activity can be inhibited by millimolar concentrations of Mg^{2+} applied to the intracellular face. The primary effect of the Mg^{2+} is to promote long closings of the channel. The inhibition has a weak voltage dependence; the Mg^{2+} is slightly more effective at more negative potentials. Thus, the mechanism of action is distinct from the open channel "flickering" Mg^{2+} block observed in a number of different cation channels. Such open channel block can also be observed in the HP channel, but only at very positive potentials (>100 mV).

A second problem in understanding the physiological function of these channels is to determine which ions they conduct in physiological solutions. In the few instances in which the selectivity of voltage-independent calcium channels was examined, they were found to have relatively poor selectivity for divalent over monovalent cations (von Tscharner et al., 1986; Benham and Tsien, 1987). In general, however, little is known about the selectivity and permeation mechanisms of these channels. This is in contrast to voltage-activated channels, which were discovered much earlier, have been studied intensively (especially in muscle and neuron), and for which detailed models of permeation and selectivity have been developed (e.g., see Almers and McCleskey, 1984; Hess and Tsien, 1984; Hess et al., 1986; Yue and Marban, 1990).

As an initial step in understanding the physiological function of these channels, we have begun to characterize the permeability and selectivity of the voltage-independent calcium channel (HP channel) in *Lymnaea* neurons. The main goal of the experiments was to determine what ions should enter through the channel under physiological conditions, as most previous work used Ba^{2+} as the charge carrier. It was found that the channel conducts both Ca^{2+} and Mg^{2+} , is highly selective for divalent over monovalent cations, and hence should conduct primarily Ca^{2+} and Mg^{2+} in physiological solutions. A second aim was to compare the permeation and selectivity properties of this voltage-independent calcium channel with those of the better-studied voltage-activated calcium channels. The *Lymnaea* HP channel was found to share certain qualitative properties with the highly selective voltage-activated calcium channels: monovalent cations can carry outward current through the channel, but only at very positive membrane potentials; selectivity for divalent over monovalent cations is high; and the open channel current-voltage relation is markedly nonlinear, becoming very shallow near the apparent reversal potential. Thus, similar permeation mechanisms may be shared by voltage-activated and voltage-independent calcium channels, and the latter are not necessarily poorly selective.

Some of these results have been presented in preliminary form (Strong, 1990, 1992).

Materials and Methods

Animals and cell culture. *Lymnaea stagnalis* were reared in the laboratory in tanks containing artificial pond water (0.25 gm/liter of Instant Ocean, Aquarium Systems, Mentor, OH), fed lettuce, and maintained on a 12 hr:12 hr light/dark cycle. Adult snails (shell height, >25 mm) were dissected in cold Ringer's solution, and the circumesophageal ganglia of the central nervous system were removed. The ganglia were treated with 0.25% neutral protease (dispase; Boehringer Mannheim, Indianapolis, IN) in supplemented Ringer's solution for 18–20 hr, desheathed, and triturated with polished Pasteur pipettes to obtain isolated cells. In some cases, the dispase treatment was replaced by a much shorter treatment, 0.1% pronase (20 min) followed by 0.25% trypsin

(60 min) (W. L. Byerly, personal communication). Cells were plated onto 35 mm tissue culture plates (Falcon Primaria 3801, Becton Dickinson, Lincoln Park, NJ) in supplemented Ringer's solution, maintained at 22°C, and used from 2 hr to several days after plating. In some cases, the isolated cells were maintained for up to 2 d in 1.5 ml microcentrifuge tubes, in media to which 5 mg/ml BSA had been added in order to prevent the cells from attaching to the plastic. Such "floating" cells were then plated into the usual tissue culture plates 1–5 hr before use, and were attached to the substrate at the time of the recording.

Single channel recording. Standard single channel recording techniques and configurations were used (Hamill et al., 1981). The patch-clamp amplifier was either an Axopatch 1-D (Axon Instruments, Foster City, CA) or a Yale patch clamp (Yale University, Department of Physiology, New Haven, CT). The currents were filtered at 1 kHz, and then digitized, stored, and analyzed with a C-Lab interface system (Indec Systems, Sunnyvale, CA) under the control of dedicated programs written in the laboratory. The open channel current was measured by eye from long, well-resolved openings. In some experiments, this measurement was verified by examining amplitude histograms. Unless otherwise noted, single channel conductances are based on the slope of a line fit to currents measured below -50 mV. The mean channel open time in patches containing multiple channels was estimated as described by Fenwick et al. (1982) and Horn and Lange (1983). Mean closed times were estimated using this estimated mean open time and the measured value of NP_{open} , the number of channels in the patch times the open probability of a single channel. These estimates of closed times, unlike the estimated open times, require an accurate value for the number of channels in the patch. Since the open probability of the channel was relatively high, the number of channels in the patch was readily obtained by finding the maximum number of open channels observed (in 0 Mg) during the experiment, as verified by binomial analysis.

Extracellular solutions. All concentrations are millimolar. The standard *Lymnaea* Ringer's solution contained 50 NaCl, 4 CaCl_2 , 1.5 MgCl_2 , 2 KCl, 5 dextrose, and 10 HEPES (pH 7.8). Penicillin and streptomycin were added to make the supplemented Ringer's used for primary culture. Isotonic Ba^{2+} solution contained 45 BaCl_2 and 10 HEPES (pH 7.8). In some experiments, Mg^{2+} and Ca^{2+} were substituted for Ba^{2+} . Nominally divalent-free Na^+ Ringer's contained 52 NaCl and 10 HEPES (pH 7.8). Divalent ions were added to this solution as needed to make up solutions of varying Ba^{2+} , Mg^{2+} , and Ca^{2+} concentrations. Such solutions are called, for example, "10 Ba^{2+} + Na^+ ." All solutions used had a measured osmolarity of 120 mOsm/liter, except that Na solutions with less than 5 mM divalent added were hypotonic.

Intracellular solutions. The standard K-aspartate solution used on the intracellular side of excised patches contained 5 KCl, 57 K-aspartate, 5 EGTA, and 10 HEPES (pH 7.2). Na^+ , Tris^+ , and Cs^+ were substituted for K^+ as noted.

Activity coefficients referred to in the text were calculated using the "Guggenheim convention" as described in Blinks et al. (1982), giving the following values for $\gamma_{\text{Ba}^{2+}}$: 0.43 (0.5 Ba^{2+} + Na^+ solution), 0.40 (5.0 Ba^{2+} + Na^+), 0.385 (10 Ba^{2+} + Na^+), and 0.31 (isotonic Ba^{2+}). However, all values are reported as concentrations.

All voltages have been corrected to account for the junction potential between the pipette and bath solutions. All such corrections were 10 mV or less.

Results

Block and inhibition of the HP channel by Mg^{2+}

When isotonic Ba^{2+} is used as the charge carrier, HP channel activity is characterized by large inward openings that are readily observed over a wide range of negative potentials. The single channel conductance extrapolated from potentials below -50 mV is 25–30 pS. Channel activity appears rapidly after a cell-free, inside-out patch is formed by excision into, for example, K-aspartate solutions in which the divalent cations have been strongly buffered. As shown in Figure 1, HP channel activity initiated in this fashion can be reversibly inhibited by addition of 1 mM free Mg^{2+} to the intracellular side of a cell-free patch. A partial effect is seen with lower concentrations of Mg^{2+} . As demonstrated by the sequential records in Figure 1, the primary effect of intracellular Mg^{2+} is to promote long closings of the channel, which are occasionally interrupted by bursts of channel

Figure 1. Effect of intracellular Mg^{2+} on the HP channel. Data are from an inside-out patch; the patch pipette contained isotonic Ba^{2+} solution. HP channel activity in this and subsequent figures was activated by patch excision. Current traces shown in each line are from sequential records of steps to -80 mV. Small numbers to the left indicate the number of open channels. Free Mg^{2+} in the intracellular K-aspartate solution was changed to the indicated values. No openings were seen during 4 min of recording in 1.0 mM Mg^{2+} . In 0.1 mM Mg^{2+} , the overall value of NP_o was reduced to 0.11, and many traces with no or only brief openings were interspersed with occasional records with normal, long openings, as shown. Incomplete reversal of the Mg^{2+} effect in this experiment is probably due to the effective number of channels falling from three to two during the experiment.

activity during which the kinetics and open channel current are similar to that seen in the absence of Mg^{2+} . The onset of the Mg^{2+} effect was too rapid to be resolved with the perfusion system, and in general was rapidly reversible. The lack of complete reversibility seen in Figure 1 is probably due to the loss of one functional channel during the course of the experiment.

In all the data presented in this article, the HP channel was activated by formation of a cell-free patch. As previously reported (Yazajiian and Byerly, 1989), activity of the HP channel was rarely observed during cell-attached recording, but appeared soon after formation of a cell-free patch; in contrast, voltage-activated channel activity was sometimes observed during cell-attached recording but disappeared soon after patch excision. The channel is not stretch sensitive. Overall, 62% of patches showed HP channel activity (see below). HP channel gating was complex; both open and closed time distributions had at least two exponential components. Most data are taken from channels such as those shown in Figure 1, in which the predominant, slowest time constant for both open and closed times was on the order of 5–10 msec. In some patches, channel gating was much more rapid, with the predominant open time constants being on the order of 1 msec, although the open channel current-voltage relation was unchanged. An example is seen in Figure 8, bottom. Occasionally, transitions between these different gating modes could be observed in a single patch. A similar complexity and variability of gating was reported in the analogous channel in *Aplysia* neurons (Chesnoy-Marchais, 1985) as well as in a similar channel in rat cardiac muscle (Coulombe et al., 1989). In the present work, some patches (from which no data are presented) seemed to contain HP channels that were in an even faster gating mode, in which all openings were too rapid to resolve. The channel tended to be in the same gating mode for a given batch of cells.

Yazajiian and Byerly (1989) reported that HP channel was “bursty,” fluctuating on the time scale of minutes between high and very low activity. In our experiments, similar behavior was sometimes observed during the first minutes of an experiment.

However, channel activity then stabilized and burstiness was not observed for the remainder of the experiment (up to 90 min). Data presented above about Mg^{2+} effects were taken after channel activity had stabilized; hence, we cannot determine whether Mg^{2+} also has additional effects on the bursting phenomenon. We often saw an abrupt loss of activity of one of the channels in a multichannel patch, but this was irreversible on the time course of an experiment (30–90 min), and hence distinct from the burstiness reported by Yazajiian and Byerly (1989).

Despite these quantitative kinetic differences between different patches, Mg^{2+} effects qualitatively similar to that described in Figure 1 were seen in all patches studied ($n = 19$). A detailed analysis of the kinetic effect was hindered by the fact that single channel patches were rare (see below). In multichannel patches, the mean open time was estimated as described in Materials and Methods. Figure 2 shows data from seven different patches in which such a quantitative analysis was possible. The mean open time was independent of Mg^{2+} ($p < 0.03$; Fig. 2, bottom), but the mean closed time (Fig. 2, top) increased markedly with Mg^{2+} (note logarithmic scale). For the experiments shown in Figure 2, the average single channel open probability was 0.32 ± 0.03 in 0 mM Mg, 0.18 ± 0.11 in 0.1 mM Mg, and 0.03 ± 0.01 in 1.0 mM Mg. Openings could never be observed in 5.0 mM Mg.

In one experiment in which we were able to study the effects of Mg^{2+} in a single-channel patch, the closed time distribution at -80 mV had two exponential components before and after application of 0.1 mM intracellular Mg^{2+} . The addition of Mg^{2+} increased the slower time constant (from 5.7 to 17.8 msec) as well as increasing the relative contribution of the slow component (from 58% to 71%). The faster time constant was not significantly affected, changing from 0.9 to 1.1 msec.

A variety of cation channels are blocked by intracellular Mg^{2+} (see Discussion). In most of these examples, Mg^{2+} is thought to block the channel by entering the pore. Such open channel (or “flickering”) block is characterized by rapid interruptions of the open channel current or, if these are too rapid to be resolved,

Figure 2. Effect of intracellular Mg^{2+} on estimated mean channel closed time (top) and open time (bottom; note change in scale) measured in seven different inside-out patches. Each symbol represents a different experiment. All but one patch contained more than one channel, so open and closed times were estimated as described in the methods sections. Multiple symbols at one value of $[Mg]$ indicate multiple determinations during an experiment. All data are from measurements at -80 mV, except for two experiments at -55 mV (solid square and solid circle).

by an apparent reduction in the size of the open channel current. In addition, open channel block is more effective at more positive voltages, a voltage dependence that arises because the Mg^{2+} senses some fraction of the transmembrane field when it enters the pore. The data in Figures 1 and 2 suggest that Mg^{2+} inhibits the HP channel by a completely different mechanism, since channel openings are unaffected while long closings are enhanced.

Support for this conclusion was obtained from additional experiments that investigated the voltage dependence of the Mg^{2+} effect. In the experiment shown in Figures 3 and 4, the open probability and open channel size were measured at a holding potential of -80 mV, and during steps to $+160$ mV. A maintained holding potential of $+160$ mV could not be used, as this tended to destroy the patch. Data from the first 6 msec of the depolarizing step to $+160$ were not included; measure-

Figure 3. Voltage dependence of Mg^{2+} effects. Sample traces were taken at the holding potential of -80 mV (bottom; inward currents carried by isotonic Ba^{2+}) and during steps to $+160$ mV (top; outward currents carried by Na^{+}). Lines indicate zero current level. As the intracellular Mg^{2+} concentration was raised from 0 (left) to 0.25 mM (right), the average value of NP, fell from 0.67 to 0.163 at -80 mV, and from 1.65 to 1.34 at $+160$ mV. The first 6 msec after the step to 160 mV were omitted from both the traces and the analysis. Averaged currents indicated that this was sufficient time for the open probability to reach a new steady state value.

ment of averaged currents indicated that this was sufficient time for the open probability to relax to its new value. As shown below, at very positive potentials, outward currents carried by monovalent cations can be observed in the HP channel (see Fig. 5). In the experiment shown in Figures 3 and 4, the outward currents shown at $+160$ mV were carried by Na^{+} .

As shown in Figure 3, 0.25 mM Mg^{2+} reduced the probability of opening at -80 mV by a factor of 4.1, without changing the size of the inward single channel current (Fig. 3, bottom). These results are similar to those shown in Figure 1. The effect of Mg^{2+} on the outward currents at $+160$ mV was more complex. The open channel size was clearly reduced, suggesting that "flickering" open channel block by Mg^{2+} can be seen in the HP channel at very positive potentials. However, at $+160$ mV, Mg^{2+} was actually less effective at inhibiting channel opening—the open probability was reduced by a factor of only 1.2. Thus, two separate effects of Mg^{2+} on the HP channel can be described. Mg^{2+} can reduce the size of the single channel current, in a manner presumably similar to the open channel block described for many types of channels. This effect (which for simplicity will be referred to as "block") is only seen at very positive potentials where current is outward. Second, Mg^{2+} can reduce the open probability of the channel. This effect (which will be referred to as "inhibition") is seen at all voltages and is the only effect seen in the physiological voltage range. As summarized in Figure 4, these two effects of Mg^{2+} are likely to correspond to two different sites of action. Block and inhibition have opposite voltage dependencies. Inhibition is less effective at more positive potentials, and hence cannot be due to entry of Mg^{2+} into the pore since this should be enhanced at more positive potentials. A more plausible explanation for the voltage dependence of Mg^{2+} inhibition is that Mg^{2+} must interact with the closed channel in order to cause inhibition. For example, in the data shown in Figure 4, the probability that the channel is closed is 3.8 times higher, and the Mg^{2+} inhibition is 3.4 times more effective, at -80 mV than at $+160$ mV. Figure 4 also shows the data at $+160$ mV replotted with the Mg^{2+} concentration

"corrected" to the same effective concentration as that seen by the channel at -80 mV (open triangles). The correction is based on the assumption that Mg^{2+} must interact with the closed channel. As shown in the figure, this assumption does a reasonably good job of superimposing the data at $+160$ and -80 mV.

This inability of hyperpolarization to relieve Mg^{2+} inhibition was observed in eight additional experiments. Most of these experiments examined a more narrow voltage range (-100 to $+30$ mV), as patches that could withstand repeated depolarizations to 160 mV were rare.

Outward current can be carried by monovalent cations at very positive potentials

In the above experiments, isotonic Ba^{2+} was used as the extracellular charge carrier. As previously shown (Yaziji and Byerly, 1989), under these ionic conditions, single channel currents were inward at potentials as positive as $+60$ mV. This suggests that the channel has good selectivity for Ba^{2+} over K^+ . In order to characterize further the permeability and selectivity of the HP channel, we looked for outward single channel currents at much more positive membrane potentials. As shown in Figure 5, outward single channel currents could be observed during very strong depolarizations. These outward currents were assumed to pass through the HP channel based on the following observations (see examples of outward single channel currents in Fig. 5). (1) The outward currents had gating characteristics that were qualitatively similar to those observed for inward HP channel currents at negative potentials. (2) In any given patch, the estimated number of HP channels (based on the observed maximum number of overlapping openings) was the same whether based on observations of outward or inward currents (e.g., see Fig. 3). This was particularly obvious in rarely observed single channel patches (see Fig. 8, top, for an example). (3) As shown in Figure 5, if the HP channel was open at the end of a depolarization (where single channel currents were outward) it tended to be open immediately after repolarization to a negative potential (where single channel currents were inward). In the traces shown in Figure 5, outward currents were carried by Na^+ . When K^+ was used as the intracellular cation, additional types of outward openings were sometimes seen. These openings, presumably K^+ channels, had a larger conductance, were not correlated with HP activity observed at negative potentials, and were much shorter (openings were always less than 1 msec). These events were not seen in every patch, and were readily distinguished from HP channel openings. In some patches, measurement of outward currents was precluded by large, time-dependent outward currents that developed at very positive potentials. It was not clear whether these currents were due to other types of small-conductance channels present in high density, or to some physical instability of the membrane patch to large depolarizations.

The size of the outward currents depended on the monovalent cation present on the intracellular side. Figure 6 shows the effect of changing the monovalent cation on the intracellular side, with $10 Ba^{2+} + Na^+$ on the extracellular side. K^+ , the cation that is present physiologically, was less permeant than Na^+ . Outward currents carried by $Tris^+$ could not be measured over the potential range examined (up to 180 mV), though $Tris$ substitution had little effect on the size of the inward currents at negative potentials. Cs^+ permeability was less than K^+ permeability, but measurable (data not shown).

Figure 4. Block and inhibition of HP channel as a function of intracellular Mg^{2+} . Data are from the same experiment as shown in Figure 3. In this experiment, trials were taken in the following order of Mg^{2+} values: 0 (1st trial), 1.0, 0 (2nd trial), 0.25, 0.4, 0 mM (3rd trial). Inspection of the data revealed that the effective number of channels suddenly dropped from four to two after the trial in 1.0 Mg^{2+} and then remained stable for the rest of the experiment; hence, NP_0 values taken during the first two trials have been divided by 2. **A**, Block of open channel current at the indicated potentials, defined as the open channel current divided by that observed at the same potential in the absence of Mg^{2+} . For clarity, measurements from only four potentials (indicated on the right) are shown; no effect of Mg^{2+} on inward current amplitude was seen over the entire range of potentials studied, -110 to $+30$ mV. **B**, Inhibition of channel activity, defined as NP_0 divided by the value of NP_0 observed in 0 Mg^{2+} at the same potential. Because so much time was needed to make the NP_0 measurement, values were only obtained at two potentials: -80 mV (the holding potential) and $+160$ mV. NP_0 values for 160 mV were measured during voltage steps (omitting the first 6 msec), since the patches were not stable to maintained depolarizations. The open triangles and dotted line are the data at 160 mV replotted against $[Mg^{2+}]/3.8$, where 3.8 is the factor by which the probability of finding a channel in the closed state is reduced due to the depolarization (in 0 Mg^{2+}). In both **A** and **B**, data from second trial in 0 Mg^{2+} was used as the normalizing value; additional points shown at 0 Mg^{2+} represent values measured during the first and third trials in 0 Mg^{2+} .

Reversal potentials in Figure 6 can be roughly estimated to be between 40 and 50 mV for Na^+ , and between 80 and 100 mV for K^+ , as these are the voltage ranges in which the variance in the single channel current reaches a minimum. These reversal potentials correspond to a selectivity ratio $P_{Ba}:P_{Na}$ ranging from

Figure 5. Examples of outward currents through the HP channel, carried by Na^+ . All measurements are from an inside-out patch, with 10 Ba^{2+} + Na^+ solution on the extracellular side and Na-aspartate on the intracellular side. Ba^{2+} was reduced to 10 mM in order to enhance the size of outward currents (see Fig. 7). Stepping the patch potential to the value indicated on the left revealed outward openings with gating characteristics similar to those seen for the inward HP channel currents. The state of the channel (open or closed) at the end of the step was well correlated with the state observed after repolarization to -40 mV (arrow), where HP currents are inward.

40:1 to 86:1, and a selectivity ratio $P_{\text{Ba}}:P_{\text{K}}$ ranging from 825:1 to 4230:1, respectively, calculated from the constant field equations, assuming that internal and external surface potentials are equal, and using activity coefficients of 1 (see Lee and Tsien, 1984). This range of values is provided for comparison to previous work only; the constant field equations cannot adequately describe the current-voltage relationships presented in this article.

When Na^+ was used as the internal cation, giving a defined value for the Nernst potential for Na^+ , the inward currents were observed at potentials up to 30 mV positive to E_{Na} (Fig. 6B), in spite of the fact that the Na^+ concentration was six times higher than the Ba^{2+} concentration. This provides a direct demonstration of the channel's high selectivity over monovalents, independently of any theory used to calculate actual selectivity ratios.

The single channel current depends on the Ba^{2+} concentration

The selectivity of the channel was further characterized in outside-out patch experiments in which the external Ba^{2+} concentration was lowered by replacement with various monovalent cations.

Figure 7 shows the effect of changing the external Ba^{2+} concentration on the size of the open channel current. As would be expected if Ba^{2+} is the primary permeant ion, at any given potential the size of the inward current becomes smaller as the

Figure 6. Effect of changing intracellular monovalent cation on the single channel current-voltage relation. Records shown are from the same patch as Figure 5. *A*, Full current-voltage relation with Na^+ , K^+ , and Tris^+ as the intracellular cation. No outward currents could be observed with Tris^+ . *B*, Currents in K^+ and Na^+ from *A* are shown on an expanded voltage scale. In the Na-aspartate intracellular solution, E_{Na} has a defined value of -7 mV as indicated by the broken line. Currents in the Tris -aspartate solution (not shown in *B*) essentially overlapped with those seen in the K -aspartate solution. In this and subsequent I - V graphs, the points are connected by an arbitrary polynomial with no theoretical significance.

Ba^{2+} concentration is reduced. Outward currents, carried in this experiment by Na^+ , can be observed at very positive potentials. At any given potential, the outward Na^+ currents are larger when the extracellular Ba^{2+} concentration is smaller, consistent with

Figure 7. Effect of changing extracellular Ba^{2+} concentration on the single channel current-voltage relation. All measurements are from a single outside-out patch. Na-aspartate solution was on the intracellular side. The Ba^{2+} concentration was decreased by substituting Na^+ . The value of E_{Na} in the $10 \text{ Ba}^{2+} + \text{Na}^+$ solution is -7 mV . The limiting slope conductances at negative potentials were 31 pS (45 Ba^{2+}), 25 pS (10 Ba^{2+}), and 23 pS (0.5 Ba^{2+}). Similar results were seen in nine other experiments.

the expected leftward shift of the reversal potential as Ba^{2+} concentration is reduced. However, a precise reversal potential cannot be determined since over a wide range of potentials (30 – 40 mV), no openings can be detected. The current-voltage relation in L-type calcium channels has a qualitatively similar shape (e.g., see Hess et al., 1986). Unfortunately, the ramp method of determining reversal potentials used by those authors was not helpful in the present work, due to the low incidence of single channel patches and the instability of the seal resistance. This precluded a more quantitative description of permeation in the HP channel, which would have permitted a more detailed comparison of permeation models in the HP channel with those developed for voltage-dependent calcium channels.

Although Figure 7 indicates that reducing the Ba^{2+} concentration reduces the size of the inward single channel currents, these currents can be detected in surprisingly low Ba^{2+} concentrations, the limiting slope conductance at negative potentials is very similar over a wide range of Ba^{2+} concentrations (falling by only 25% as $[\text{Ba}^{2+}]$ is reduced 90-fold), and saturation of current size was not observed as the Ba^{2+} concentration was varied over almost 2 orders of magnitude. An Eadie-Hofstee plot of the data in Figure 7 is highly nonlinear (data not shown). This transformation is very sensitive to deviations from the Michaelis-Menton equations, and the observed nonlinearity indicates that a single binding site cannot simply account for the relationship between Ba^{2+} concentration and current size seen

Figure 8. Effect of changing the extracellular divalent cation. *A*, Examples of current traces seen in an outside-out patch as the extracellular solution was changed from $10 \text{ Ba}^{2+} + \text{Na}^+$ (left) to $10 \text{ Ca}^{2+} + \text{Na}^+$ (right). Lines indicate the closed state. At any given potential, inward currents were smaller in Ca^{2+} (see example at -80 mV), as were outward currents carried by Na^+ (see example at $+150 \text{ mV}$). *B*, Examples of current traces seen in another outside-out patch as the extracellular solution was changed from $10 \text{ Ba}^{2+} + \text{Na}^+$ (left) to $10 \text{ Mg}^{2+} + \text{Na}^+$ (right). Inward currents were much smaller in Mg^{2+} . This patch provides an example of the channel's faster gating mode.

in Figure 7. The relationship cannot be linearized by using Ba^{2+} activity instead of concentration. One possible explanation for the observed nonlinearity is that the extracellular membrane has a fixed negative surface charge, which leads to a solution-dependent negative surface potential that alters the local Ba^{2+} concentration near the mouth of the channel. Byerly et al. (1985) found evidence for a negative extracellular surface potential in *Lymnaea* neurons. However, it should be noted that similar results in ventricular L-type calcium channels were found to be due to interaction of ions within the pore, rather than to fixed surface charge (Yue and Marban, 1990). Experiments to distinguish these two possibilities were not done in the present work.

Current can also be carried by Ca^{2+} and Mg^{2+}

The preceding experiments were done using Ba^{2+} as the divalent charge carrier because, in many calcium channels, currents carried by Ba^{2+} are much more readily measured than those carried by Ca^{2+} . However, one aim of the present experiments was to determine what ions might be expected to pass through the HP channel in physiological conditions. Permeation by Ca^{2+} and Mg^{2+} is shown in Figures 8 and 9. In the experiment shown in Figure 8, top, the extracellular solution in an outside-out patch was changed from $10 \text{ Ba}^{2+} + \text{Na}^+$ to $10 \text{ Ca}^{2+} + \text{Na}^+$. The resulting inward currents were much smaller, and the current-voltage relation was more linear in the negative potential region (Fig. 9). The single channel conductance fell from 22 pS in 10

Figure 9. Single channel current–voltage relationships in solutions with different divalent cations. Data are from the two experiments shown in Figure 8. The single channel conductance in the negative potential region was 22 pS in the 10 Ba²⁺ solution, 5 pS in 10 Ca²⁺, and 6 pS in 10 Mg²⁺.

Ba²⁺ to 5 pS in 10 Ca²⁺. Because the currents in Ca²⁺ were so much smaller, inward currents above the Na⁺ reversal potential could not be measured. However, in the 10 Ca²⁺ solution, outward currents carried by Na⁺ at very positive potentials were actually reduced at any given potential (Figs. 8, top; 9), indicating that the apparent reversal potential was very positive in Ca²⁺ as well as in Ba²⁺. Similar results were seen in five other outside-out patch experiments, and a similar channel was seen in one inside-out patch experiment using 10 Ca²⁺ + Na⁺ on the extracellular side. Essentially identical results were obtained for Mg²⁺ as for Ca²⁺. Examples of currents carried by Mg²⁺, obtained in a different patch, are shown in Figure 8, bottom. The single channel conductance in 10 Mg²⁺ was 6 pS. In this experiment, the patch broke before outward currents could be measured. Similar results were obtained in three other outside-out patch experiments, and a similar channel was observed in one inside-out experiment using 10 Mg²⁺ + Na⁺ as the extracellular solution. In these four experiments, clearly resolved outward currents at very positive potentials could not be measured due to the difficulties described above. However, in all four experiments, it was observed that outward currents were suppressed at potentials at least as positive as 110 mV, indicating that the reversal potential in 10 Mg²⁺ is very positive, similar to that seen in 10 Ca²⁺. These experiments were greatly hindered by the instability of the outside-out patches.

A second effect of substituting Mg²⁺ or Ca²⁺ for Ba²⁺ was that the open probability of the channel was smaller in Ca²⁺ or Mg²⁺ than in Ba²⁺. This effect was observed in all experiments with outside-out patches, but could not be studied quantitatively, due to the instability of the outside-out patches. The reduction in open probability was obvious at negative potentials but not very noticeable at potentials depolarized enough to drive outward currents.

Localization of the HP channel

In the course of these experiments, information about the localization of the HP channel was also obtained. We found that the channel was found with much higher probability ($p < 0.002$) in patches taken from the neurites than from the soma of cells growing in primary culture. Only 44% of patches ($n = 60$ out of 136) excised from the soma showed HP channel activity, compared with 88% of patches from neurites ($n = 28$ out of 32). The latter patches were taken from all different regions of the neurites and from the growth cone itself, but most were from either the region closest to the soma or the region just before the growth cone at the end of the neurite.

The experiments also indicated that, at least on the soma, the HP channel had a moderate tendency to cluster. Despite the fact that, in one set of experiments, 44% of the patches were “null” (showed no channel activity), single channel patches were rare. In the 56% of patches that did contain channels, the average number of channels was 2.9 ± 0.4 , and only 14% were single channel patches. These measurements are from a subset of the data presented in the preceding paragraph, from 25 soma patches taken from batches of cells in which at least one null patch was obtained. This subset was selected in order to avoid any bias due to the observed variation in channel density between different batches of cells.

This observation suggested the possibility that null patches were found more often in membrane patches from the soma simply because the channel forms clusters there. This possibility was not supported by the data, however, since the increased probability of finding the HP channel in a patch taken from the neurite reflected a genuine increase in channel density. In a subset of data containing patches from neurites that were matched with soma patches taken from the same batch of cells, the average number of channels per patch was 2.9 ± 0.5 in the neurite patches, and 1.0 ± 0.5 in the soma patches ($p < 0.02$; $n = 17$).

Although the HP channel is clearly present in the neurites, we cannot rule out the possibility that the apparent differences in channel density between the soma and the neurites are due to differences in some other membrane property, which result in more membrane being pulled into the patch in the neurite.

Additional experiments showed that the HP channel can be found not only in the newly formed neurites seen in primary culture, but also in the adult nerve. Segments of nerve were cut 1–2 mm distal from the soma-containing ganglia, immediately after removal of the ganglia from the animal. This distance was chosen to eliminate the possibility that channels present in the soma *in vivo* could be transported to the axon during dissection and isolation, even by fast axonal transport (e.g., see Schnapp and Reese, 1986). These isolated nerves were then subjected to the same tissue culture procedure that was used for the neurons. The resulting short, asomatic segments of axon attached to the tissue culture dish, survived for up to 2 d, and extended short filaments at both ends. HP channel activity was found in four out of eight membrane patches taken from these acutely isolated segments.

Discussion

Inhibition by intracellular Mg²⁺

One primary finding of this article is that Mg²⁺ can act at the intracellular face to inhibit HP channel opening, at concentrations in the millimolar range, via a mechanism distinct from open channel block. Recent determinations of the intracellular

free Mg^{2+} concentration range from 0.2 to 2 mM. These values are from a variety of tissues including cardiac muscle, skeletal muscle, liver, and red blood cells (see Gupta and Yushok, 1980; Alvarez-Leefmans et al., 1986; London, 1991, and references therein). Thus, it must still be considered an open question whether intracellular Mg^{2+} concentrations are high enough in *Lymnaea* neurons to account for the fact that the HP channel is usually closed in intact cells. The work of Byerly and Yazejian (1986) suggests that an additional regulator must be postulated. In this description of voltage-activated calcium currents in *Lymnaea* neurons, the free Mg^{2+} of the intracellular solutions used for whole-cell perfusion can be calculated to be approximately 0.1 mM, a concentration that according to present results should have been insufficient to inhibit completely the HP channels, yet during these experiments cells perfused for up to 90 min showed no evidence of the large inward currents at negative potentials that open HP channels would be expected to generate. However, another possibility is that intracellular Mg^{2+} was not well controlled in these experiments. In support of this possibility, it is worth noting that intracellular Ca^{2+} was found to be surprisingly difficult to control in whole-cell perfusion experiments unless special precautions were taken (Byerly and Moody, 1984).

Mg^{2+} inhibition may account for the observation that the HP channel opens in cells that have been exposed to high- K^+ , EGTA-containing solutions for many minutes (Yazejian and Byerly, 1989), as this treatment could lower intracellular Mg^{2+} levels. Activation of the channel by intracellular perfusion with ATP-deficient or high- Ca^{2+} (pCa 5.7) solutions cannot be accounted for by Mg^{2+} inhibition in any obvious way; if anything these procedures might be expected to elevate intracellular Mg^{2+} .

Regardless of whether or not intracellular Mg^{2+} is the sole physiological inhibitor of the HP channel, it is clearly important to consider the Mg^{2+} effect in understanding the normal regulation of the HP channel. Most other calcium-permeable channels are found to be strictly regulated, opening only in response to depolarization, or in the presence of specific intracellular or extracellular ligands (for review, see Tsien and Tsien, 1990). This tight regulation presumably reflects the fact that the intracellular concentration of free Ca^{2+} is kept quite low and can be significantly altered by a relatively tiny Ca^{2+} influx. In this vein, it is fortunate for the molluscan neuron that the HP channel is usually closed, since simple estimates of channel density and opening probability indicate that, were all the HP channels to open at once, the resulting Ca^{2+} influx would be much larger than that seen during peak activation of the voltage-activated channels. The HP channel may represent a new class of ligand-gated calcium channel, one in which some as yet unknown ligand acts by disinhibiting Mg^{2+} instead of by simple direct gating.

The Mg^{2+} inhibition of the HP channel described in this article is qualitatively similar to the effects of intracellular Ba^{2+} and Ca^{2+} described by Yazejian and Byerly (1989) on the same channel. Comparison of their data with that presented here suggests that Mg^{2+} is a slightly more effective inhibitor than Ba^{2+} and Ca^{2+} . McClintock and Ache (1990) described two effects of intracellular Mg^{2+} on a similar channel in spiny lobster olfactory receptor neurons. Their fast block and slow block are analogous to the block and inhibition, respectively, described here. Mg^{2+} is a ubiquitous enzyme cofactor, and effects of intracellular Mg^{2+} have been described in many other cation channels. Open channel block, the most common effect seen, occurs in voltage-gated Na^+ channels (Pusch et al., 1989; Albitz et al.,

1990), NMDA receptors (Johnson and Ascher, 1990), and a variety of K^+ channels including the inward rectifier (Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1991), the muscarine-activated K^+ channel (Horie and Irisawa, 1989), and the ATP-sensitive K^+ channel (Findlay, 1987; Horie et al., 1987). High-threshold voltage-activated calcium channels have been shown to be reduced by intracellular Mg^{2+} in heart (Agus et al., 1989; Hartzell and White, 1989) and in *Lymnaea* neurons (Byerly and Yazejian, 1986). These Mg^{2+} effects do not seem to be due to simple open channel block, but have not been studied at the single channel level due to the lability of voltage-activated calcium channels in cell-free patches. Thus, it is not yet clear if Mg^{2+} has a direct inhibitory effect similar to that reported here in HP channels, or whether it acts through some intracellular machinery.

Localization of the HP channel

The finding that the HP channel is present in the processes of neurons growing in primary culture suggests that the channel may play some role in regulating neurite outgrowth. Localized calcium influx has been postulated to regulate neurite outgrowth, although there is considerable disagreement about the nature of this regulation (e.g., see Freeman et al., 1985; Connor, 1986; Cohan et al., 1987; Lockerbie, 1987; Goldberg, 1988; Kater et al., 1988; Silver et al., 1989). Of relevance to this article is the observation that, in these studies, the evidence that calcium enters the neurite through voltage-gated calcium channels is often based on the effects of rather nonspecific blockers such as Co^{2+} . In addition, in most cases the membrane potential of the neurite has not been determined, so it is not clear that the neurite is depolarized enough to open voltage-activated channels. The possibility that HP channels are a pathway for calcium influx into the neurite must be considered.

The observation that HP channels could be found in acutely isolated nerve segments indicates that the HP channel is likely to be normally present in the adult axon. We cannot as yet exclude the possibility that the channel, though present in the axon, is not inserted into the plasma membrane *in vivo*. In considering possible roles of axonal HP channels, we note that Grinnell and Pawson (1989) provided evidence for a voltage-independent component of calcium entry and neurotransmitter release at the neuromuscular junction in frogs.

Selectivity and permeation

This article also demonstrates that the HP channel in *Lymnaea* neurons has high selectivity for divalent over monovalent cations, and thus would be expected to conduct primarily Mg^{2+} and Ca^{2+} into the cell under physiological conditions.

Qualitatively, the channel was found to share a number of permeation properties with better-characterized voltage-activated calcium channels (e.g., see Kostyuk et al., 1983; Almers and McCleskey, 1984; Hess and Tsien, 1984; Fukushima and Hagiwara, 1985; Hess et al., 1986; Yue and Marban, 1990):

(1) The monovalent selectivity sequence, based on estimated reversal potentials, is $Na^+ > K^+ > Cs^+$. The HP channel differs from most voltage-activated calcium channels in being permeable not only to Ba^{2+} and Ca^{2+} , but to Mg^{2+} as well (however, see Almers and Palade, 1981). The HP-like channel in *Aplysia* is also permeable to Mg^{2+} (Chesnoy-Marchais, 1985). Mg^{2+} permeability has not been examined in most other voltage-independent calcium channels.

(2) The open channel current-voltage relationship is highly

nonlinear, becoming very shallow near the apparent reversal potential.

(3) The single channel current continues to increase as Ba^{2+} concentration is increased over at least 2 orders of magnitude, a relationship that cannot be accounted for by a single binding site.

(4) In voltage-activated calcium channels, high permeability to monovalent ions is unmasked when divalents are removed from the extracellular solution. This critical experiment was not done in the present work because membrane patches proved unstable when the divalents were removed. However, indirect evidence that monovalent permeability in the HP channel is high in the absence of divalents is seen in Figure 6, where the limiting slope conductance at potentials above 120 mV was 108 pS for outward currents carried by Na^+ , and 40 pS for K^+ currents. This suggests that in the absence of divalents (effectively removed in this case by strong depolarization), the limiting conductance for these monovalent cations is actually higher than that for Ba^{2+} . The absolute conductance values for Na^+ and K^+ are somewhat arbitrary, since they are based on linear extrapolation from a rectifying region of the I - V relation.

In voltage-activated channels, the above properties have been accounted for by permeation models in which the channel pore has two or more high-affinity binding sites for divalent cations (Almers and McCleskey, 1984; Hess and Tsien, 1984; Hess et al., 1986; Yue and Marban, 1990). In these models, high selectivity over monovalent cations results from the ability of divalent cations to bind in the pore, effectively blocking monovalent permeation (except at extreme depolarizations). High divalent flux at more negative potentials results from repulsive interactions of multiple divalents within the pore. The results presented in this article suggest that a similar permeation mechanism may be present in voltage-independent channels. A quantitative permeation model, which would allow a more precise comparison of permeation mechanisms, was not possible in the present work due to two limitations of the preparation: the difficulty in measuring reversal potentials precisely; and the instability of patches in divalent-free extracellular media, which precluded experiments testing the ability of low concentrations of divalent cations to block current carried by monovalent cations.

In the HP-like channel in *Aplysia*, Chesnoy-Marchais (1985) observed anomalous mole fraction effects at the single channel level, providing additional evidence that the permeation mechanism in these voltage-independent calcium channels involves interactions between multiple permeant ions.

The observation that the channel's open probability is reduced when Ca^{2+} or Mg^{2+} replaces Ba^{2+} is commonly noted in studies of voltage-activated channels. This dependence of open time on the permeant divalent was well characterized in the HP-like channel in *Aplysia* by Chesnoy-Marchais (1985), who used the term "kinetic selectivity" to describe it. The *Aplysia* channel differs from the *Lymnaea* in certain details, however. The effect of the divalent cation on open channel size is similar ($\text{Ba}^{2+} > \text{Ca}^{2+} = \text{Mg}^{2+}$), but in *Aplysia* long open times are seen in Ca^{2+} as well as Ba^{2+} . In voltage-activated calcium channels, similar behavior is not accounted for by the permeation model discussed above, but has been proposed to result from inactivation of the channel by permeant divalents accumulating at the intracellular face. A similar explanation could be proposed for the *Lymnaea* HP channel, since it is inhibited by intracellular Ba^{2+} , Ca^{2+} (Yazijiian and Byerly, 1989), and Mg^{2+} . However,

one problem with this explanation is that Ba^{2+} and Ca^{2+} have similar potency as intracellular inhibitors and are less potent than Mg^{2+} , while Ba^{2+} produces larger currents and larger open probability than either Mg^{2+} and Ca^{2+} . A second problem is that no obvious kinetic effect was observed when extracellular Ba^{2+} was reduced from 45 to 0.5 mM (data not shown). Another possible explanation for kinetic selectivity is that permeant ions are trapped in the closed channel, so that the free energy of the closed state(s) depends on the charger carrier used.

Although voltage-independent calcium channels have been characterized in a number of different cell types, in most cases little is known about their selectivity, in part because outward currents through these channels are not usually measured (however, see von Tscharner et al., 1986; Benham and Tsien, 1987). It is often assumed that these channels are less selective than their voltage-activated counterparts. The results presented here demonstrate that voltage-independent calcium channels can have high selectivity for divalents. Insofar as other voltage-independent calcium channels may have similar nonlinear current-voltage relations, it would seem unwise to draw strong conclusions about a channel's selectivity based on linear extrapolation of reversal potentials from inward currents measured at negative potentials. Several other voltage-independent calcium channels (e.g., see Chesnoy-Marchais, 1985; Rosenberg et al., 1988; Coulombe et al., 1989; McClintock and Ache, 1990); including some found in internal membranes (e.g., see Ehrlich and Watras, 1988), have nonlinear current-voltage relations for inward currents similar to that seen in the HP channel. Some of these channels may prove to have permeability and selectivity characteristics similar to those of HP calcium channels.

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