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Single-channel currents activated by low intracellular pH in cultured hippocampal neurons of rat

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Patch clamp technique was applied to the plasma membrane of cultured hippocampal neurons of rat. Elementary currents of a cation-selective channel were elicited by low intracellular pH (pH_i 3.5–4.5). Channel activity starts with 1–2 min delay from the application of low pH_i, and persists upon restoration of physiological pH conditions. The channel has a conductance of approx. 110 pS in symmetrical 300 mM NaCl, and is strongly selective for cations over anions. The channel is active over the whole voltage range tested (from +75 mV to −75 mV). Mean open time is function of voltage, increasing with depolarization. Low pH applied extracellularly did not activate the channel.

Introduction

The effects of high proton concentration on ionic channel activity have been extensively studied. Reports broadly fall into three categories.

(1) Inhibition of channel activity. Low (external or internal) pH has been shown to block conduction in the squid giant axon [1], and to decrease conductance or activity of Na^+ channels [2–7], K^+ (delayed rectifier, inward rectifier, Ca^{2+}-activated) channels [4,8–13], Ca^{2+} channels [14–20], Cl^- channels [21–24], and acetylcholine receptor [25]. A few studies have also been done on the effect of intracellular pH on the inactivation of the Na^+ channel. Two apparently conflicting reports are mentioned here. Na^+ current inactivation is removed or greatly reduced by high pH_i in squid giant axon [26], but by low pH_i in frog skeletal muscle [27].

(2) Production of H^+ currents. H^+ currents have been recently described by several authors in different preparations. Mozhayeva and Naumov [28] report a H^+ current at pH lower than 4 that permeates through Na^+ channels. In snail neurons, H^+ currents have been first reported by Thomas and Meech [29], and described in more detail by Byerly et al. [30]. The latter paper shows that H^+ currents go through specific channels which are very selective for H^+.

(3) Transformation of one channel type into another. Lux and co-workers [31,32] found that low external pH (pH_o) activates a transient Na^+ current. Two major observations induced the authors to conclude that the Na^+ current activated by low pH_o flows through a transformed state of a Ca^{2+} channel. First, the voltage-gated Ca^{2+} current disappeared rapidly during the activation of proton-induced Na^+ current. Ca^{2+} current reappeared, however, while Na^+ current inactivated, upon restoration of physiological pH conditions. This 'temporal exclusiveness' of the two currents suggested that both Na^+ and Ca^{2+} currents use the same Ca^{2+} channel in two selectively distinct states. Additional evidence in support of this view was the finding that the proton-activated Na^+ current was blocked by organic (diltiazem, verapamil) and inorganic (Ni and Cd) Ca^{2+} channel blockers at concentrations similar to those required to block the Ca^{2+} currents.

For completeness, one report has also appeared on proton modulation of Cl^- channels reconstituted from *Torpedo* electroplax [33], which shows that lowering pH increases the open probability of the channel, whereas rising it has the opposite effect. A change in pK of certain titratable groups upon opening of the channel is proposed to explain the pH-modulation of the channel gating.

This paper reports on the activation of a cationic channel by low internal pH. The results are discussed in the light of previous reports, to try to determine to which category the effects reported can be ascribed, and

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what possible mechanisms underly the proton-activated cationic channel in cultured hippocampal neurons of rat.

Methods

Experiments were carried out on hippocampal neurons dissected from 18-day-old rat embryos, and plated on collagene/polylysine-coated culture dishes containing basic tissue culture medium (N5) supplemented with a fraction of horse serum [34]. Neurons were kept in culture at 34° C in an atmosphere containing 5% CO_2 for 1–3 weeks before the experiment.

The patch clamp method [35] was used to record single-channel currents in the inside-out configuration, that is, with the cytoplasmic side of the membrane exposed to the perfusing solution. A homemade patch clamp amplifier was used for voltage clamping and current amplification. Single-channel currents were filtered at 0.5 kHz (−3dB), digitized at 400 μs sampling rate, and analyzed with a laboratory computer system (Nova 4, Data General). Single-channel records were taken in either steady-state conditions attained by stepping the membrane voltage to the desired level, and maintaining that value for tens of seconds to minutes, or by stimulating the membrane with a staircase pattern of voltage steps that started with positive steps to activate the channels, and applied over a period of time during which sufficient activity was sustained (for details, cf. Ref. 36).

Current amplitude histograms of single-channel recordings were constructed for both stimulation protocols, and used to measure single-channel current and channel open probability. Single-channel current was measured as interpeak distance, and channel open probability calculated from the integral areas of the amplitude histogram peaks.

The solutions used contained either 300 or 1200 mM of bulk salt (NaCl or KCl), 1 mM EGTA, and 2 mM of pH buffer. Solutions where the pH was adjusted to 7.2 were buffered with Mops, and those adjusted to pH 3.5–4.5 were buffered with citric acid. In the text, these solutions are referred to by specifying the concentration and species of the bulk salt, and the pH; for instance, 300 NaCl (pH 4.2). Effective change of test solutions at the cytoplasmic side of the patch in the inside-out configuration was obtained with a perfusion system similar to that described by Franciolini and Nonner [36]. Experiments were carried out at 25–28° C.

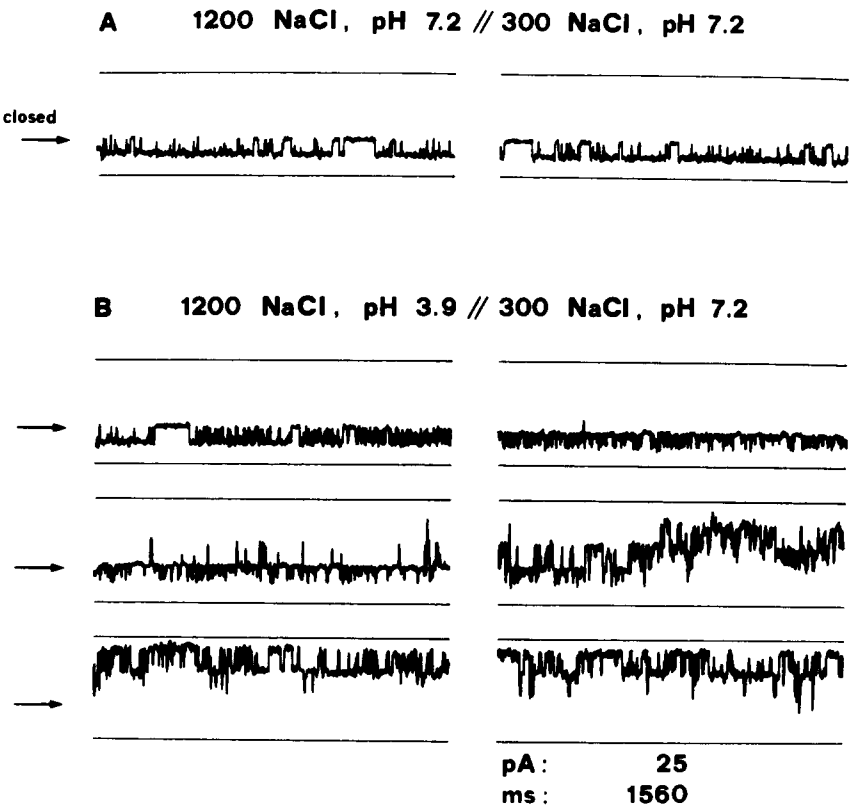

Fig. 1. Activation of the cationic channel by low pH_i. Single-channel recordings from an excised inside-out patch of hippocampal neuron of rat were taken in steady-state conditions at holding potential of 0 mV. The traces are consecutive 1560-ms segments interrupted by 20-ms blank intervals due to data aquisition. Pipette solution was 300 mM NaCl (pH 7.2) and perfusing solution was 1200 mM NaCl (pH 7.2) for the first two control traces (A), and 1200 mM NaCl (pH 3.9) for the following traces (B). The traces shown in (B) were taken 80 s after solution change. Calibration: solid lines enclosing single channel recordings span 25 pA.

Results

In an attempt to elucidate the permeation mechanism of the background Cl^- channel in hippocampal neurons of rat, a series of experiments were carried out at low intracellular pH, ranging from 7.0 to 4.0. Low pH_i was found to abolish the activity of the background Cl^- channel (Franciolini and Nonner, unpublished data). Often, however, the activation of a single-channel cationic current with distinctive conductance and kinetics was observed, concurrent with the depression of Cl^- current. This is illustrated in Fig. 1 which shows a typical single-channel recording from an excised inside-out patch of hippocampal neuron and the effect of low pH_i on the patch current.

The traces, from top to bottom, are consecutive segments obtained at a patch membrane potential of 0 mV. Pipette solution was 300 NaCl (pH 7.2). The solution perfusing the cytoplasmic side of the membrane was 1200 NaCl (pH 7.2) for the first two control traces (A), and 1200 NaCl (pH 3.9) for the following traces (B). The control traces show an inward current which, given the zero voltage and the salt gradient conditions, indicates that the underlying channel is anion selective. Single-channel conductance as derived from a current voltage plot (not shown) and channel kinetics indicate that this is the background Cl^- channel frequently seen and already reported in this preparation [36].

When the internal 1200 mM NaCl (pH 7.2) is replaced by the low pH_i solution (1200 mM NaCl (pH 3.9)), an inhibitory effect on the Cl^- current is observed (Fig. 1). This inhibition consists of a depression of single-channel activity. The channel tends to open more rarely, and openings are no longer in bursts but appear as shorter and sparser separate events. Current amplitude also decreases until Cl^- channel activity subsides completely.

Soon after the above changes have been completed, occasional openings appear with current going in the opposite direction, that is, positive with respect to the base line. As patch conditions are unchanged (zero holding voltage, and 1200 mM NaCl (pH 3.9) || 300 mM NaCl (pH 7.2)), the outward current observed must be carried either by Na^+ or H^+ ions. This current was not abolished upon returning to physiological pH conditions, indicating that the activation of the channel by low pH was irreversible.

To study this current in more detail silent patches

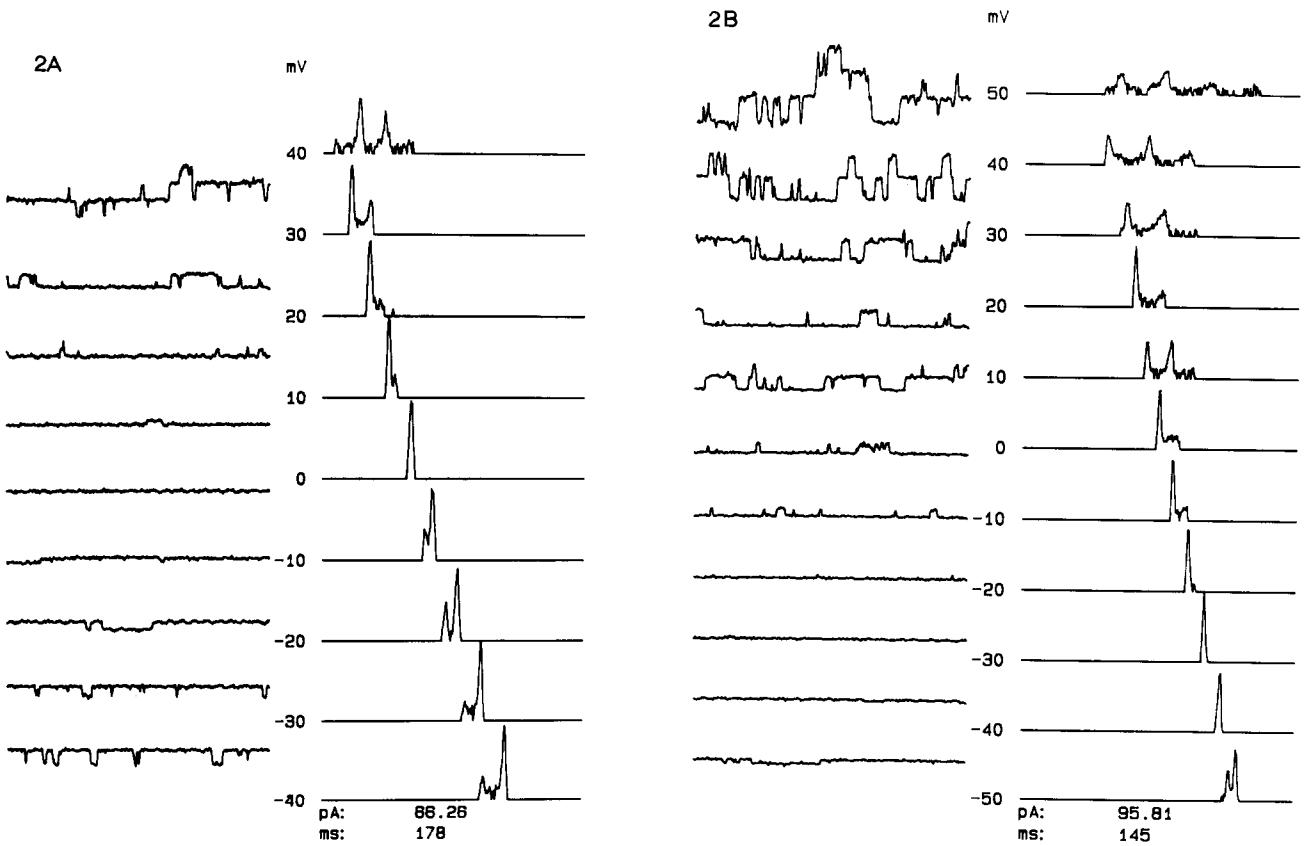

Fig. 2. Single-channel currents of a cationic channel activated by low pH_i and amplitude histograms obtained at different membrane potentials and perfusing conditions. Pipette solution was 300 mM NaCl (pH 7.2) and the solution perfusing the inside-out patch was 300 mM NaCl (pH 4.1) (A), and 1200 mM NaCl (pH 4.1) (B). Current records were obtained by the staircase stimulation protocol (see Methods). The corresponding amplitude histograms shown on the right of each record were constructed by plotting on the ordinate the square root of the number of samples in each bin (bin size 0.08 pA). Calibration is given in the figure.

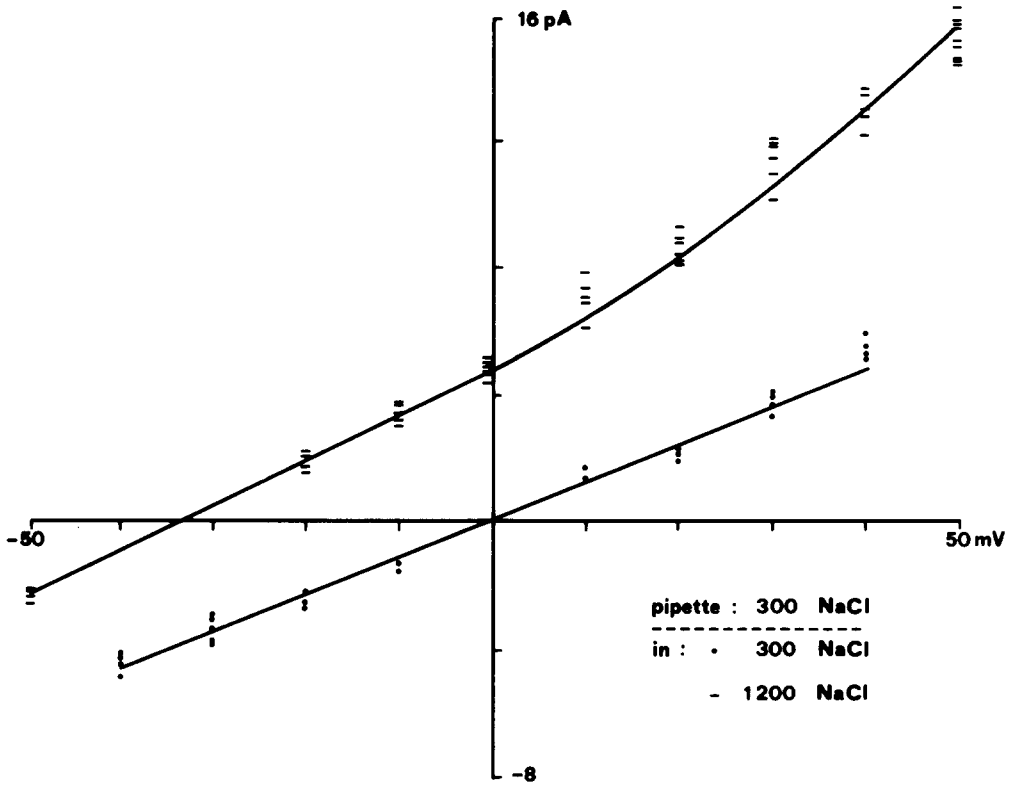

Fig. 3. Current-voltage relations of the proton-activated cationic channel in two different ionic conditions. Data are from the experiment shown in Fig. 2. Pipette solution 300 mM NaCl (pH 7.2). Perfusing solutions 300 mM NaCl (pH 4.1) (dots), and 1200 mM NaCl (pH 4.1) (bars). Single-channel currents were estimated from the amplitude histograms. Each data point represents a separate measurement. The continuous lines were fitted to the data points by eye. In symmetrical 300 mM NaCl (dots), slope conductance is approx. 110 pS. In salt gradient conditions (bars) the interpolated line predicts a reversal potential of −34 mV.

with no observed channel activity were subjected to low pH_i. Fig. 2 illustrates a representative experiment where the excised inside-out patch is perfused with 300 mM NaCl (pH 4.1) (A), and after replacing this solution with 1200 mM NaCl (pH 4.1) (B). Pipette solution in this experiment was 300 mM NaCl (pH 7.2). With symmetrical 300 mM NaCl, but high H^+ gradient, the reversal potential, as shown by single-channel records and amplitude histograms (see also Fig. 3), is near zero, as expected if Na^+ and not H^+ ions were the charge carriers. ymmetrical 150 mM NaCl solutions were occasionally used with the same results. As higher salt concentration produces larger currents, we have predominantly used these solutions). When the same patch is subjected to NaCl gradient conditions (1200 mM NaCl (pH 4.1) || 300 mM NaCl (pH 7.2)) the reversal potential is shifted considerably to negative voltages. As can be seen in Fig. 3 which shows the current-voltage

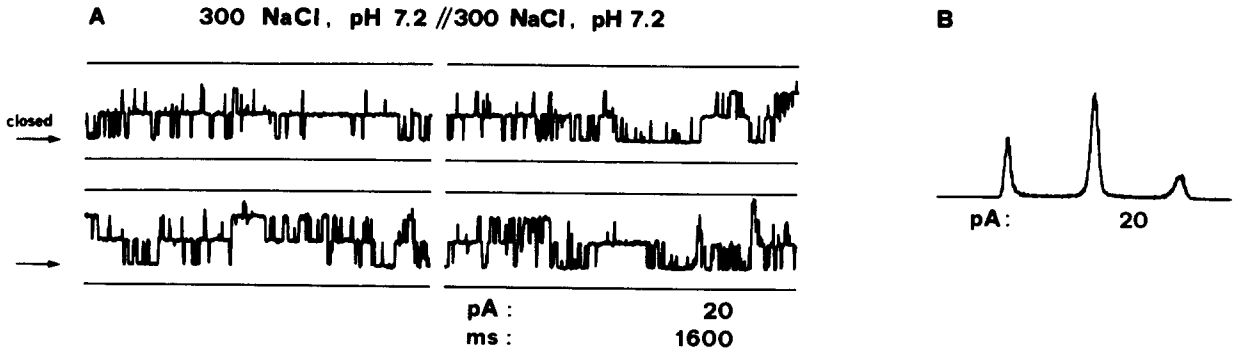

Fig. 4. Steady-state kinetics of the proton-activated cationic channel. (A) Single-channel recording in symmetrycal 300 mM NaCl (pH 7.2). Traces are consecutive 1600-ms segments taken at holding potential of 40 mV. The patch was previously subjected to low pH_i to activate the cationic channel, and then returned to physiological pH. (B) Amplitude histogram from a 8-s segment of continuous recording which includes the two records shown in (A). The histogram abscissa spans 20 pA, and represents also the current calibration (pA between the solid lines enclosing the single-channel records) for (A). The ordinate plots the square root of the number of samples in each 0.04 pA bin.

plot constructed with data from this experiment, the reversal potential in NaCl gradient conditions is −34 mV (the average from 11 patches gave a mean ± S.D. of −31 ± 3 mV). This value is very close to the theoretical Nernst potential of −32 mV for a membrane exclusively permeable to Na^+ (activity coefficients of 0.710 and 0.645 have been used, respectively, for 300 and 1200 mM NaCl solutions). This result indicates that the channel is highly selective for Na^+ over H^+. K^+ selectivity was also tested by substituting 300 mM KCl (pH 4.1) for 300 mM NaCl (pH 4.1) on the intracellular side of the patch. In three patches so tested the reversal potential was found to shift 13, 14, and 18 mV respectively, indicating that K^+ is less permeant than Na^+. The mean shift of 15 mV in the reversal potential would be accounted for with the Nernst equation by permeabilities of K^+ relative to Na^+ (P_K/P_{Na}) of 0.69.

Fig. 2 also shows that channel activity is a function of voltage: openings at positive voltages are greater than at negative voltages. Although a quantitative analysis of the voltage dependence of channel activity was not carried out, this apparent voltage sensitivity was seen in virtually all the experiments. In general, at negative voltages the channel opens rarely and many sweeps show no openings. As the patch is depolarized, the channels open more frequently, and double (and more) openings appear, reflecting an increase in open probability.

Fig. 4 illustrates the kinetics of this cationic channel, by showing a segment of continuous recording taken at 40 mV in symmetrical 300 mM NaCl (pH 7.2). The patch was initially subjected to 300 mM NaCl (pH 4.2) solution to activate the cation channel (not shown), then returned to physiological pH. This figure shows that channel activation by low pH_i is not a transient phenomenon, but more likely results from a permanent modification of the channel. An interesting feature of this channel is that for the recording frequency range used, the open-channel noise is not significantly different from the closed channel noise. The amplitude histogram of Fig. 4B made from a 8-s segment of continuous recording which includes the two blocks shown in Fig. 4A illustrates this point more convincingly as well as showing the absence of any subconductance state for this channel. The low open-channel noise, reflecting the little flickering of the channel, indicates the absence of short-lived closed states.

Discussion

This paper reports on a study at the single-channel level of a cationic current elicited by low pH_i applied to membrane patches of rat hippocampal neurons. The channel has a conductance of approx. 110 pS in symmetrical 300 mM NaCl, selects very strongly for Na^+ over Cl^-, and is active within a wide voltage range (from +75 mV to −75 mV). The channel remains active even after physiological pH is restored. The cationic channel is not activated by low pH applied extracellularly.

Several authors have reported H currents in conditions of low intracellular pH values. Mozhayeva and Naumov [28] suggested that in nerve fibers the H^+ current permeates through Na^+ channels (also see, Ref. 30), whereas the presence of a specific H^+ channel has been proposed by Thomas and Meech [29] in the membrane of snail neurons.

Regardless of the type of channel that sustains the current, the cationic current we described in this paper cannot be identified as a H^+ current for the following two reasons. The strongest point is that in conditions of high proton gradient (with expected reversal for H^+ of approx. −180 mV), but otherwise symmetrical salt concentration (300 mM NaCl || 300 mM NaCl), the reversal potential is near zero (Figs. 2A and 3). This result, along with the nearly ideal Nernst reversal potential shift of −31 mV for a salt gradient of 1200 mM NaCl || 300 mM NaCl convincingly indicate that Na^+ ions and not H^+ ions are the current carriers. In addition, the H^+ currents described by other authors showed time-dependent characteristics, inactivating in hundred of ms [30]. The cationic current we describe here is instead active within a wide voltage range, and present in steady-state condition at all voltages tested (from +75 mV to −75 mV), indicating time-independent kinetics.

The observation that the block of the Cl^- channel always preceded the appearance of the cationic channel (cf. Fig. 1) initially led us to consider the possibility of a transformation of the Cl^- channel into a cationic channel by low pH_i. This appeared a possible interpretation as the background Cl^- channel has been shown to have a significant permeability to cations [36], which might be determined by bipolar structures in the channel pore (−OH groups), or by charged amino acids which could be affected by pH. In addition, such a transformation of an ionic channel type into another upon pH manipulation has been reported by other authors. Konnerth et al. [31], for instance, have suggested that in chick dorsal root ganglion cells Ca^{2+} channels assume a distinct conformational state, selective to Na^+, by lowering the internal pH. It is worth noting here that the most convincing evidence provided to support this view was the 'temporal exclusiveness' of the Ca^{2+} and Na^+ currents, a feature we frequently observed in our experiments with the Cl^- and Na^+ currents.

There are, however, at least two reasons why this mechanism is unlikely to be applicable here. The first is that cationic currents can be induced by low pH_i in silent patches. The second is that in experimental protocols similar to that illustrated in Fig. 1, we noticed sometimes, on returning to physiological pH, the reap-

pearance of the Cl^- channel, which would superimpose to the still active cationic channel.

Four observations need to be accounted for in considering a possible mechanism of activation of the cationic current by low pH_i. (1) The high proton concentration (pH_i lower than 4.5) required to activate the cationic current. (2) The inefficacy of low pH when applied extracellularly. (3) The delay in activation of the cationic channel. (4) The persistance of the cationic channel activity upon restoring physiological conditions.

(1) Such a low value of pH could be expected to induce changes in parts of proteins extending from the cytoplasmic side of the membrane. Low pH is known to modify the state of several proteins found in solution [37]. Such an effect is presumably involved in the pH block of the Cl^- channel seen in Fig. 1, and may also be responsible for the activation of the cationic channel.

(2) The sideness of the pH effect also argues for an influence of pH upon the state of the channel through protonation of the exposed cytoplasmic portion of the protein. It seeems less likely that such proton binding sites lie within the permeation path (selectively filter) of the channel, as protons could presumably reach them from either side of the pore. Still it may be possible that protons can access such a pore-oriented activation sites, but are only accessible to the pore from the extracellular side when the channel is in the pore-open conformation.

(3) pH-induced structural changes of proteins are relatively slow processes [37]. The 1–2 min delay between application of the low pH_i solution and activation of the cationic channel argues for a slow pH-induced modification of the channel protein. The finding that pH values higher than 4.5 do not cause activation of the cationic channel indicates that if removal of some steady state inactivation gate were responsible for activation of this channel, then such a gate is relatively resistant to pH-induced changes. It is interesting to notice that in this context the Cl^- channel is more susceptible to modification by pH as it is already blocked at pH values near 5.5 (Franciolini and Nonner, in preparation). This may also argue against the idea that Cl^- channels are being transformed into a cationic channel.

(4) The persistance of the cationic channel upon returning to physiological pH (Fig. 4) shows that the activation of this channel type by low pH_i is irreversible. This is what would be expected if the low pH was indeed removing or altering some steady-state inactivation gate responsible for keeping this channel closed. It is also interesting to notice that the Cl^- channel rarely recovers from the low pH_i exposure, even after a long period of time in the pH 7.2 solution. This may also be due to denaturation of the channel protein by the low pH we used.

Taken together these four points suggest that the activation of the cationic channel is mediated by pH, probably via an unfolding of the channel protein, which releases it from a long-term inactivation state. The effects reported in this paper have been obtained in experimental conditions (excised patches and low pH values) far from those experienced by cells physiologically. These findings cannot therefore be extrapolated to intact neuronal cells. The experiments reported here were directed at biophysical investigation of the ion channel itself, and no physiological significance can be claimed. pH has traditionally been used to probe the functional parameters of ion channels and much useful information has been elucidated from these types of studies [3,6,7,10,18,26,27,31,37]. Through this approach and by other manipulations we hope to further define the relationship of structure to activity in this new channel type.

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References

1 Tasaki, I., Singer, I. and Takenaka, T. (1965) J. Gen. Physiol. 48, 1095–1123.
2 Hille, B. (1968) J. Gen. Physiol. 51, 221–236.
3 Woodhull, A.M. (1973) J. Gen. Physiol. 61, 687–708.
4 Schauf, C.L. and Davis, F.A. (1976) J. Gen. Physiol. 67, 185–195.
5 Carbone, E., Testa, P.L. and Wanke, E. (1981) Biophys. J. 35, 393–413.
6 Campbell, D.T. (1982) Nature 298, 165–167.
7 Begenisich, T. and Danko, M. (1983) J. Gen. Physiol. 82, 599–618.
8 Shrager, P. (1974) J. Gen. Physiol. 64, 666–690.
9 Hagiwara, S., Miyazaki, S., Moody, W. and Patlak, J. (1978) J. Physiol. 279, 167–185.
10 Wanke, E., Carbone, E. and Testa, P.L. (1979) Biophys. J. 26, 319–324.
11 Blatz, A.L. (1980) Fed. Proc. 39, 2073.
12 Moody, Jr., W. and Hagiwara, S. (1982) J. Gen. Physiol. 79, 115–130.
13 Cook, D.L., Ikeuchi, M. and Fujimoto, W.Y. (1984) Nature 311, 269–271.
14 Chesnais, J.M., Coraboeuf, E., Sauviat, M.P. and Vassas, J.M. (1975) J. Mol. Cell Cardiol. 6, 627–642.
15 Kohlhardt, M., Happ, K. and Figulla, H.R. (1976) Pfluegers Arch. 366, 31–38.
16 Vogel, S. and Sperelakis, N. (1977) Am. J. Physiol. 233, C99–C103.
17 Yatani, A. and Goto, M. (1983) Jpn. J. Physiol. 33, 403–415.
18 Iijima, T.S., Ciani, S. and Hagiwara, S. (1986) Proc. Natl. Acad. Sci. USA 83, 654–658.
19 Prod'hom, B., Pietrobon, D. and Hess, P. (1987) Nature 329, 243–246.
20 Krafte, D.S. and Kass, R.S. (1988) J. Gen. Physiol. 91, 641–657.
21 Hutter, O.H. and Warner, A.E. (1967) J. Physiol. 189, 403–425.
22 Woodbury, J.W. and Miles, P.R. (1973) J. Gen. Physiol. 62, 324–353.

23 Loo, D.F., McLarnon, J.G. and Vaughan, P. (1981) Can. J. Physiol. Pharmacol. 59, 7–13.
24 Blatz, A.L. and Magleby, K.L. (1985) Biophys. J. 47, 119–123.
25 Landau, E.M., Gavish, B., Nachshen, D.A. and Lotan, I. (1981) J. Gen. Physiol. 77, 647–666.
26 Brodwick, M.S. and Eaton, D.C. (1978) Science 200, 1494–1496.
27 Nonner, W., Spalding, B.C. and Hille, B. (1980) Nature 284, 360–363.
28 Mozhayeva, G.N. and Naumov, A.P. (1983) Pfluegers Arch. 396, 163–173.
29 Thomas, R.C. and Meech, R.W. (1982) Nature 299, 826–828.
30 Byerly, L., Meech, R. and Moody, Jr., W. (1984) J. Physiol. 351, 199–216.
31 Konnerth, A., Lux, H.D. and Morad, M. (1987) J. Physiol. 386, 603–633.
32 Davies, N.W., Lux, H.D. and Morad, M. (1988) J. Physiol. 400, 159–187.
33 Hanke, W. and Miller, C. (1983) J. Gen. Physiol. 82, 25–45.
34 Kaufman, L.M. and Barrett, J.N. (1983) Science 220, 1394–1396.
35 Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pfluegers Arch. 391, 85–100.
36 Franciolini, F. and Nonner, W. (1987) J. Gen. Physiol. 90, 453–478.
37 Privalov, P.L. (1979) Stability of proteins, in Advances of Protein Chemistry, Vol. 33 (Anfinsen, C.B., Edsall, J.T. and Richards, F.M., eds), pp. 167–241, Academic Press, New York.
38 Ehrestein, G. and Fishman, H.M. (1971) Nature New Biol. 233, 16–17.