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ON THE ACTIVE FORM OF 4-AMINOPYRIDINE: BLOCK OF K^+ CURRENTS IN RABBIT SCHWANN CELLS

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SUMMARY

1. The blocking action of 4-aminopyridine (4-AP) on the outward potassium currents evoked by depolarization of rabbit Schwann cells in short-term primary culture was studied with the whole-cell configuration of the patch-clamp method.

2. We have determined the apparent equilibrium dissociation constant, K, for the action of 4-AP to block potassium currents at a series of different extracellular and intracellular pH values. 4-Aminopyridine is an organic base and exists in both charged and uncharged forms in aqueous solution. Changes in the pH of the extracellular and intracellular solutions therefore also change the extracellular and intracellular proportions of these two forms, and the values of K that were obtained were found to depend in a consistent way on both the extracellular and the intracellular pH.

3. At alkaline extracellular pH, K was decreased. At acidic extracellular pH, K was increased. In contrast, increasing the intracellular pH from 7·2 to 8·1 reduced the apparent potency of extracellularly applied 4-AP (i.e. increased K), and decreasing the intracellular pH (to 6·4) increased this apparent potency (i.e. decreased K).

4. The 4-AP analogues, 2-aminopyridine, 3-aminopyridine and 3,4-diaminopyridine, were also tested. At half-block of the potassium current, the intracellular concentration of the cationic form of the various aminopyridines (applied extracellularly at pH 7·2) varied by a factor of less than five, whereas that of the uncharged form varied by a factor of over 700.

5. The results are inconsistent with the hypothesis that the cationic form of the aminopyridines, acting from the extracellular solution, contributes in any substantial way to potassium channel block. It also seems unlikely that the uncharged form, acting either extracellularly or intracellularly, is solely responsible for the block. However, the results as a whole are consistent with the idea that it is the cation acting from the intracellular side that blocks the 4-AP-sensitive potassium channel and that the affinity with which 4-AP blocks the channel depends on the intracellular pH. The results would be explained if the cation competes with protons for a binding site that has an apparent pK_a of about 7·0.

6. The results, nevertheless, are not inconsistent with the possibility that both the uncharged form and the intracellular charged form of 4-AP are active in blocking the 4-AP-sensitive potassium channel. This can only be so, however, if the uncharged

form is about 150 times more potent than the charged form on a molar basis. In this case, the charged form, although less potent, is so much more abundant at physiological pH that its proportionate contribution to block would be about the same as that of the uncharged form.

INTRODUCTION

4-Aminopyridine (4-AP), which has been widely used to block potassium channels since the original findings of Pelhate & Pichon (1974), is an organic base with a pK_a of 9·2 (Albert, Goldacre & Phillips, 1948). At physiological values of pH, 4-AP is therefore largely in the cationic form, although a small amount (about 1 %) must be present as the uncharged pyridine. It has been argued, on the basis of the finding that the drug blocks more effectively when applied in alkaline solution, that 4-AP blocks the potassium channel from its intracellular side. For example, in experiments on frog skeletal muscle, Gillespie & Hutter (1975) showed that when 1 mM-4-AP was applied at pH 9·8 (where most of the 4-AP exists in the uncharged form and so might be expected to penetrate cells readily) it brought about complete block of the delayed rectifier potassium current, whereas this concentration of 4-AP only reduced the maximum potassium current by 50 % at pH 7·2. A similar pH dependence has also been described by Plant & Standen (1982) for the action of 4-AP on the early outward current of snail neurones. By itself, however, the observation that 4-AP is more effective at alkaline extracellular pH only suggests that it is not predominantly the cation acting from the extracellular solution which produces potassium channel block, and it does not discriminate between several other possible mechanisms by which 4-AP might block potassium channels. For example, the drug might act extracellularly as the uncharged molecule, or it might act from the intracellular surface of the channel as the cation.

In addition, the above-described dependence on extracellular pH is not always found. For example, the blocking action of 4-AP on the potassium current in frog node of Ranvier (Dubois, 1982; Ulbricht, Wagner & Schmidtmayer, 1982) and on the potassium current in squid giant axon (Meves & Pichon, 1977) is reported not to exhibit any marked dependence on extracellular pH. Furthermore, although in many studies the recovery from exposure to 4-AP is complete or nearly so (e.g. Meves & Pichon, 1977), in other experiments with 4-AP and its analogues it has been reported to be incomplete or virtually absent (Ulbricht *et al.* 1982). It seems clear, therefore, that characterization of the blocking action of 4-AP on potassium channels in any particular tissue, such as those present in rabbit cultured Schwann cells (Chiu, Shrager & Ritchie, 1984; Shrager, Chiu & Ritchie, 1985; Howe & Ritchie, 1988), cannot rely on conclusions based on experiments with other preparations.

In the present experiments, we have determined the apparent potency of 4-AP in blocking the potassium currents in rabbit cultured Schwann cells after systematically altering the pH of the extracellular and intracellular solutions, and thereby altering the extracellular and intracellular concentrations of the cationic and the uncharged forms of 4-AP. The results of these experiments permit quantitative considerations of the extent to which the uncharged and cationic forms contribute to the blocking action of 4-AP and some of its analogues.

METHODS

Primary cultures of Schwann cells were prepared from the sciatic nerves of 1- or 2-day-old rabbits as described previously (Chiu *et al.* 1984; Shrager *et al.* 1985; Howe & Ritchie, 1988). Fibroblast proliferation was prevented by the presence of cytosine arabinoside (15 μM) in the culture medium for the first 3 days. Schwann cells, which form the major component of the remaining cells and have a distinctive bipolar morphology, were readily identified from any flat multipolar fibroblasts that remained.

Patch-clamp methods (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were used to record whole-cell currents, and currents in single patches, with a List L/M EPC7 amplifier. Most of the recordings were made after the cells had been in culture for 1–2 weeks. The patch pipettes, which were coated with Sylgard and fire-polished, had an initial resistance of 2–4 MΩ when filled with the solution used for recording. During recording, series resistance compensation was always employed and was always set at 50%. The remaining, uncompensated, series resistance was 3–5 MΩ. Whole-cell currents were low-pass filtered by an eight-pole Bessel-type filter at 2 kHz (−3 dB). The cell was usually held at a membrane potential of −70 mV and then stepped for 20–50 ms to a series of depolarizing test potentials. Currents were sampled at intervals of 50–200 μs and then stored for subsequent analysis in a DEC PDP 11/34 or VAX computer. Leakage currents, taken as linearly scaled responses to 30 mV hyperpolarizing voltage pulses, were subtracted from the digitized records. All test records were the average of five successive sweeps. Outward currents were measured as the average value of the leak-subtracted record during the last 2·0 ms of the test pulse.

The apparent input resistance of the cells was typically 1–2 GΩ, and, because of the small leakage current, most of the total current was active current. Because the block by 4-AP of potassium channels in rabbit Schwann cells is voltage-dependent (Howe & Ritchie, 1988), it was necessary to determine the apparent equilibrium dissociation constant, K, for 4-AP block at a series of membrane potentials; and, for the comparison of K values that were determined under different experimental conditions, it was important to make this comparison at the same membrane potentials. This was complicated by the voltage errors that resulted from current flow across the residual series resistance of the pipette. Furthermore, because most of the total current was active and sensitive to block by 4-AP, the magnitude of these errors was dependent on the 4-AP concentration, and this magnitude decreased as the proportion of the outward current blocked by 4-AP increased. It was therefore important to correct for these voltage errors.

Voltage drops across the remaining series resistance were calculated from the known series resistance at the time the records were obtained (taken as 50% of the reciprocal of the series conductance setting of the patch-clamp amplifier that produced optimal capacitive transient balancing) and the amplitude, at each potential, of the total current (measured at the same time as the corresponding leak-subtracted current). These voltage drops were subtracted automatically from all membrane potential values for the individual I–V curves at the time the data were analysed.

The pipette solution contained (mM): KCl, 140; $MgCl_2$, 2; $CaCl_2$, 1 (buffered by 10 mM-sodium EGTA to give 10^{-8} M-Ca^{2+}); pH buffer, 10. The pH buffer was normally sodium HEPES and the pH of the control solution was 7·2. When necessary, the pH of the pipette solution was brought to pH 8·0 or 8·1 by the addition of KOH. In experiments in which the pH of the solution was 6·4, the buffer was sodium MES (2-(*N*-morpholino)ethanesulphonic acid). In a few experiments, the buffer concentration was increased by isosmotic replacement of KCl with K-HEPES or K-MES. When the pH of the pipette solution was changed to 8·0 and 6·4, the $CaCl_2$ concentration was changed to 8 and 0·03 mM, respectively, to maintain the Ca^{2+} concentration at about 10^{-8} M (Appendix *A*, Blinks, Weir, Hess & Prendergast, 1982). The external Locke solution (normally adjusted to pH 7·2, but altered as noted above to pH 8·0 or 6·4) contained (mM): sodium gluconate, 154; potassium gluconate, 5·6; calcium gluconate, 2·2; sodium HEPES (or sodium MES) pH buffer, 10. Solutions were applied to the individual cells by local superfusion.

Gluconate was substituted for chloride in the extracellular solution in all the experiments in order to eliminate outward chloride currents (Howe & Ritchie, 1988). This caused a junction potential at the tip of the pipette between the external gluconate solution and the pipette solution (which was a chloride solution). This potential was reduced or absent after whole-cell recording was established. This shift in pipette potential is the main cause of the apparent shift (in the hyperpolarizing direction) of the mid-point of the h–infinity curve (Gray & Ritchie, 1986) and of

the similar hyperpolarizing shift in the apparent sodium reversal potential in experiments in which gluconate rather than chloride was the external anion (Howe & Ritchie, 1990). This shift, which is also in part due to the junction potential between the extracellular solution and the 1 M-KCl agar bridge which provided electrical connection to the Ag–AgCl ground pellet, was similar in each of the present experiments because all of the results were obtained in gluconate–Locke solution. The magnitude of the shift was about −10 mV, and a 10 mV correction was applied to all membrane potential values.

The following aminopyridines were tested: 2-aminopyridine, 3-aminopyridine, 3,4-diaminopyridine (all obtained from Sigma), and 4-aminopyridine (obtained both from Sigma and Aldrich). 4-Aminopyridine methiodide, a permanently charged analogue of 4-AP, was made by the method described by Poziomek (1963). All results were obtained at room temperature (22 °C) and at times at which steady-state responses to the drug applications were obtained. Whenever possible, mean values ± the standard errors of the mean (S.E.M.) are given.

RESULTS

Figure 1 illustrates the outward currents evoked at extracellular and intracellular pH 7·2 in response to a series of depolarizing pulses in normal Locke solution (*A*) and the corresponding families of currents recorded after 2 min exposures to each of three concentrations of 4-AP (*B*, 80 μM; *C*, 240 μM; *D*, 2000 μM). The onset of the block by 4-AP was rapid and reached steady state within 45–90 s. On bathing the cell again in solution that was free of 4-AP, the outward current quickly returned to its original value. After correction for series resistance errors (see Methods), the current–voltage (*I–V*) curve in the presence of any given 4-AP concentration was obtained. The values of the fall in current (measured at the end of the test pulse) at any given potential in the presence of a given concentration of 4-AP (control current minus that in 4-AP) were then fitted by an iterative least-squares procedure (Patternsearch; Colquhoun, 1971) to a rectangular hyperbola of the form:

$$I = I_{max}\{[AP]/(K+[AP])\}, \quad (1)$$

where I was the fall in the current in the presence of 4-AP, [AP] was the concentration of 4-AP applied, K was the apparent equilibrium dissociation constant for 4-AP block, and I_{max} was the total 4-AP-sensitive current, that is the control current minus the current remaining at saturating concentrations of 4-AP. This allowed estimations to be made of the apparent equilibrium dissociation constant at a series of test potentials. Three to ten concentrations were tested in each of the sixty-seven cells from which results for 4-AP were obtained. The procedure was essentially identical to that described, and illustrated by Figs 2*b* and 3*a* in Howe & Ritchie (1988), where we showed that most of the outward potassium current in Schwann cells is 4-AP-sensitive (on average, about 80% of the current evoked by a depolarization to a membrane potential of +10 mV).

In previous studies, primarily in the squid giant axon (Yeh, Oxford, Wu & Narahashi, 1976*a*, *b*; Meves & Pichon, 1977), it was assumed that all of the delayed outward current was sensitive to block by the aminopyridines and that the slower activation kinetics of the outward current in the presence of the aminopyridines reflected unblocking of the channel during the test depolarization (as the result of the decreased affinity of the aminopyridines at positive membrane potentials). Our previous analysis (Howe & Ritchie, 1988) as well as the results presented here indicate that in Schwann cells, although most of the outward potassium current is sensitive to block by 4-AP, there is a slowly activating component of outward potassium current that is insensitive to block by the aminopyridine. Although given the voltage dependence of the action of

4-AP on Schwann cell potassium currents (Howe & Ritchie, 1988) some unblocking must occur during the test depolarization, at high concentrations (i.e. many times K) this must be minimal. As a result, we think that the slow activation kinetics of the currents that remain in millimolar concentrations of 4-AP (for example as in Fig. 1*D*) are due primarily to the slow activation kinetics

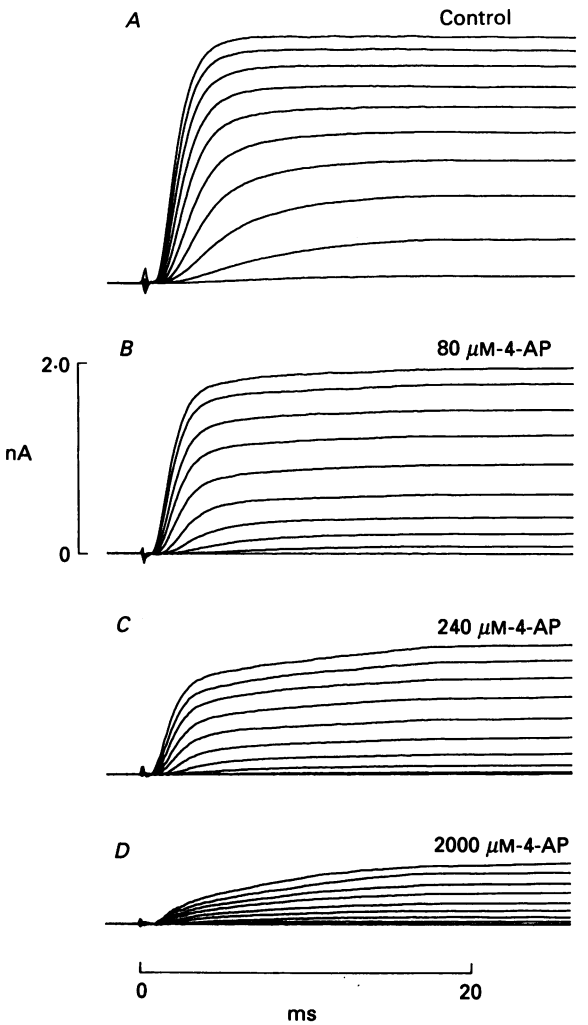

Fig. 1. Outward potassium currents (leak-subtracted) in whole-cell recordings from rabbit cultured Schwann cells in control Locke solution (*A*) and in the presence of various concentrations of 4-AP: *B*, 80 μM; *C*, 240 μM; *D*, 2000 μM. The currents were evoked in response to test depolarizations to a series of nominal membrane potentials between −50 and +40 mV in steps of 10 mV. The extracellular and intracellular solutions were pH 7·2.

of the 4-AP-insensitive potassium current and not to voltage-dependent removal of 4-AP block. This implies that, although the control currents appeared to have reached steady-state values at the end of the test pulse (Fig. 1*A*), in fact the component of the current that is sensitive to 4-AP was declining, and that as a result the measurements were not made under strictly steady-state conditions. Whereas it is true that eqn (1) pertains only under steady-state conditions and only in the case in which the block was caused by 4-AP acting at a single site with a single true equilibrium

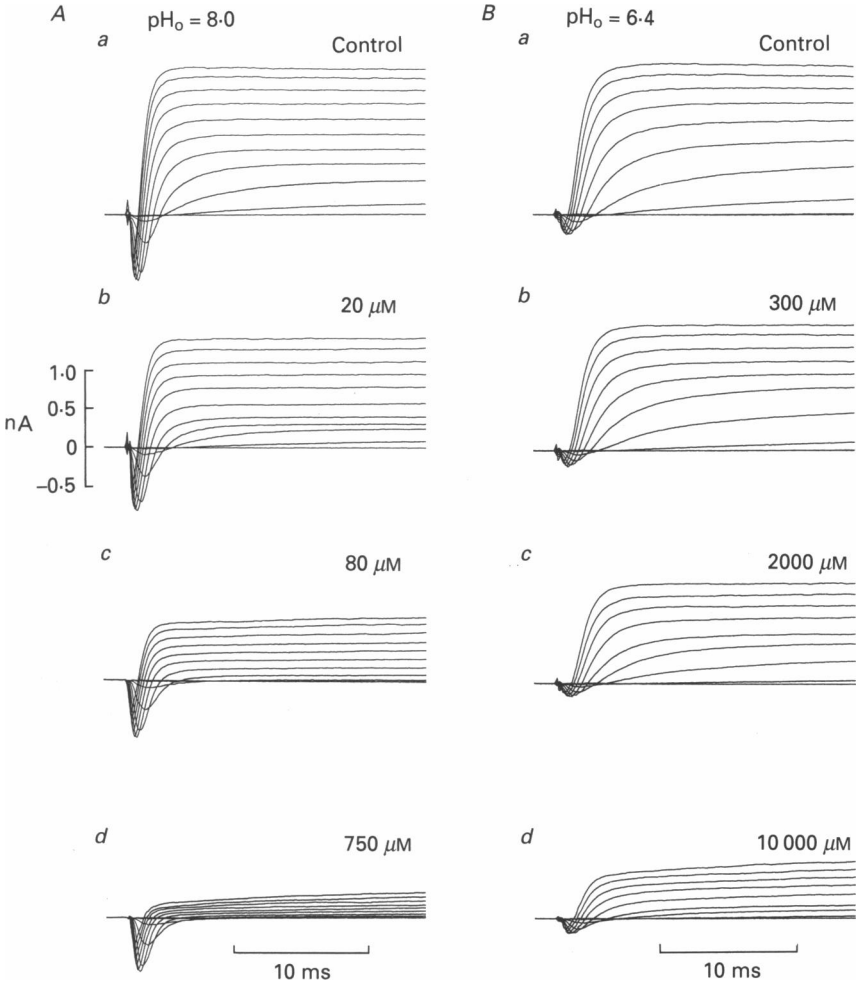

Fig. 2. *A*, ionic currents evoked at external pH 8·0 by depolarizations to nominal membrane potentials of −60 to +40 mV (in steps of 10 mV) in control Locke solution (*a*) and in Locke solution that contained 20, 80 or 750 μM-4-AP (panels *b–d*, respectively). *B*, ionic currents recorded from the same cell that were evoked by the same series of depolarizing test steps after switching to an extracellular solution that was pH 6·4. The control records (panel *a*) were obtained 4 min after washing out the application of 750 μM illustrated in *A*. Panels *b–d* show the currents recorded at pH 6·4 in the presence of 300, 2000 or 10000 μM-4-AP, respectively. Note that the control potassium currents are approximately the same at the two values of external pH but that the inward sodium currents are much smaller at external pH 6·4 than at external pH 8·0. The amplitude of the maximum inward sodium current recorded at external pH 7·2 was intermediate between the values at pH 8·0 and 6·4. It increased from 730 to 840 pA when the external pH was changed to 8·0. The amplitude of the control outward current evoked at external pH 7·2 by a nominal depolarization to +40 mV was 1·89 nA and was similar to the amplitude of the corresponding currents obtained in the absence of 4-AP at pH 8·0 and pH 6·4.

dissociation constant, in the present treatment the equation is simply used empirically to estimate the limiting value to the fall in I, and the concentration at which half-block occurred, as the drug concentration was progressively increased.

In nerve, potassium channel block at low concentrations of 4-AP is removed by successive test depolarizations in a frequency-dependent manner (see, for example, Yeh *et al.* 1976*b*; Meves & Pichon, 1977). However, in Schwann cells this feature is not prominent, and in our experiments successive test pulses were usually repeated at a frequency of 5 Hz.

As we have reported (Howe & Ritchie, 1988), the value of K so determined was found to depend on membrane potential and rose from a value of 114 ± 22 μM at -20 mV to a value of 256 ± 34 μM at $+40$ mV ($n = 12$). These experiments were all done with intracellular and extracellular solutions that were adjusted to pH 7·2, and they served as the control values for the subsequent experiments in which the intracellular and extracellular pH was varied.

The effect of varying extracellular pH

When the above-described procedure was repeated with the cells superfused by alkaline solutions (pH = 8·0), there was a marked increase in the apparent sensitivity of the potassium currents to 4-AP. Conversely, when 4-AP was applied in acidic extracellular solution (pH = 6·4), higher concentrations of 4-AP were required to produce an equivalent degree of block than were required when the extracellular solution was pH 7·2. Figure 2*A* shows families of inward sodium currents and outward potassium currents recorded in extracellular solutions of pH 8·0. Panel *a* shows the currents recorded in the absence of 4-AP and panels *b–d* show the currents recorded in solutions that contained 20, 80 or 750 μM-4-AP. Figure 2*Ba* shows the currents evoked by the same series of depolarizing test steps after switching to an extracellular solution that was pH 6·4 and that contained no 4-AP. The remaining panels in Fig. 2*B* show the currents recorded from the cell at extracellular pH 6·4 in the presence of 300, 2000 or 10000 μM-4-AP (panels *b–d*, respectively). Comparison of these results with those presented in Fig. 1 clearly indicates that the apparent potency of 4-AP to block potassium currents is dependent on the pH of the extracellular solution in which it is applied.

This dependence on extracellular pH was quantified by comparison of the values of K that were determined at the three values of extracellular pH examined. The I–V curves obtained from one cell at pH 8·0 and pH 6·4, both in control solutions and in solutions that contained 4-AP, are shown in Fig. 3*A* (pH = 8·0) and Fig. 3*B* (pH = 6·4). From these curves, the percentage reductions in the outward current in the presence of 4-AP were obtained at a series of membrane potentials, and at each potential these results were used to obtain estimates of K from eqn (1). Panels *C* and *D* show the results obtained at a membrane potential of 0 mV at pH 8·0 and 6·4, respectively. The fits to these data (smooth curves) yielded a K value of 28·3 μM at extracellular pH 8·0 and a K value of 1776 μM at extracellular pH 6·4.

The mean values of K, determined at -20 to $+40$ mV, at extracellular pH values of 6·4 and 8·0, are plotted together with those obtained at extracellular pH 7·2 in Fig. 4 (●, intracellular pH 7·2 in each case). As can be seen, the concentration of 4-AP required to produce 50% block depended on extracellular pH at each membrane potential examined. At extracellular pH 8·0 (curve labelled 8·0/7·2), the value of the apparent equilibrium dissociation constant at -20 mV was $19{\cdot}5 \pm 8{\cdot}6$ μM ($n = 5$) and rose to $36{\cdot}6 \pm 7{\cdot}3$ μM at $+40$ mV, compared with the corresponding values at extracellular pH 7·2 of 114 and 256 μM (see above). When the extracellular pH was

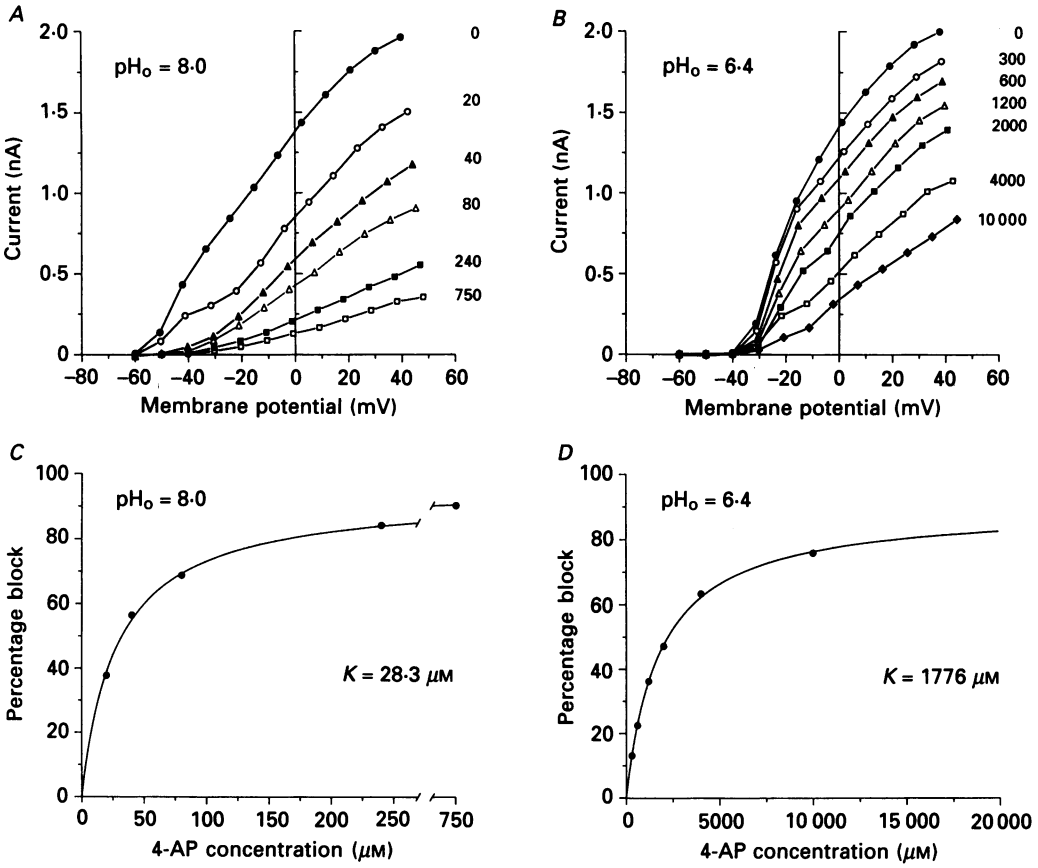

Fig. 3. *A* and *B*, *I–V* curves from the experiment illustrated in Fig. 2. The values of the outward currents measured in each concentration of 4-AP tested are plotted. Panel *A* shows the results obtained at external pH 8·0 and panel *B* shows the results obtained at external pH 6·4. The concentration of 4-AP (in μM) is indicated to the left of each curve. Membrane potential values have been corrected for series resistance errors. The maximum nominal test potential was +50 mV in each case. Note that the apparent activation threshold of the outward currents is different at the two external pH values. *C* and *D*, the data in panels *A* and *B* were expressed, from membrane potentials of −20 to +40 mV (in steps of 10 mV), as fractional reductions of the corresponding control currents, which gave values for the percentage block of the outward current that was produced by a given concentration of 4-AP. Panels *C* and *D* show plots of the values of percentage block so obtained as a function of 4-AP concentration that were determined for a membrane potential value of 0 mV at external pH values of 8·0 (*C*) and 6·4 (*D*). The smooth curves show the least-squares fits to each set of data according to eqn (1). The estimated values of K were 28·3 μM at external pH 8·0 and 1776 μM at external pH 6·4. The fits gave values for I_{max}, the proportion of the total current that was sensitive to 4-AP, of 94% at external pH 8·0 and 90% at external pH 6·4. Note the discontinuity of the *X*-axis in panel *C*.

reduced from 7·2 to 6·4, the value of K was markedly increased; at −20 mV it was 1243±297 μM ($n = 5$) and was 1446±435 μM at +40 mV (Fig. 4, curve labelled 6·4/7·2).

The changes in extracellular pH did not substantially alter the amplitudes of the control potassium currents, whereas the amplitudes of sodium currents recorded

from the cells did change. This is shown clearly in Fig. 2 in which panels *A* and *B* were obtained from the same cell, first at pH 8·0 and then at pH 6·4. The effect of extracellular pH on sodium current amplitudes has been described in detail previously in frog node of Ranvier (Woodhull, 1973). In our experiments the effect of pH on the sodium currents is in part due to changes in surface potential and, at pH 6·4, to a reduction in the peak sodium permeability (P_{Na}, defined as in eqn (3) in Howe & Ritchie, 1990). Altering the extracellular pH from 6·4 to 8·0 had no detectable effect on the corresponding peak potassium permeability, P_K, a result consistent with the work of Hille (1973), who found that potassium conductance was not depressed until the extracellular pH was reduced below 5·0.

Whereas the size of the potassium currents was relatively independent of the pH of the extracellular solution, the voltage at which potassium currents were first detected did vary with extracellular pH. This is apparent from comparison of the *I–V* curves presented in panels *A* and *B* of Fig. 3. An estimate of the apparent activation threshold of potassium currents was obtained by extrapolation, onto the voltage axis, of the line connecting the two points on the control *I–V* curve that represented the smallest two values of the outward current. This procedure showed that, whereas at an extracellular pH of 7·2 activation of the current was first detected at -55 ± 2 mV ($n = 12$), at an extracellular pH of 8·0 the currents were activated at a significantly more negative voltage (-63 ± 1 mV, $n = 5$). Conversely, at an extracellular pH of 6·4, activation required a more positive test depolarization ($-42 \pm 0{\cdot}3$ mV, $n = 5$). The magnitude of these changes was similar to that of the shifts in the half-maximal potential for the P_K–*V* relation.

These changes of apparent membrane potential with changes in extracellular pH are similar to those obtained for the effect of changes in the pH of the solutions bathing frog nodes of Ranvier; and they presumably reflect, at least in part, the neutralization of the negatively charged groups in the surface membrane phospholipid (Hille, 1968). Such buffering by extracellular acidic solutions would reduce the negative surface potential and hence increase the transmembrane potential. This effect can be quite asymmetric. Thus sodium channel activation is shifted by +25 mV by lowering the extracellular pH from 7·0 to 4·5; increasing the pH to 10, however, only changes it by −8 mV (see Hille, 1968, 1984).

The effect of varying intracellular pH

Corresponding experiments to those just described were also carried out in which the extracellular pH was kept constant (at 7·2) while the intracellular pH was increased (to 8·1) or decreased (to 6·4). The intracellular pH was changed by replacement of the normal pipette solution (pH 7·2) by one at pH 8·1 or 6·4. As Marty & Neher (1983) have shown, in whole-cell patch-clamp recording the intracellular concentrations of small inorganic ions equilibrate rapidly with those of the pipette solution, an equilibration that must apply to hydrogen ions. Figure 4 shows the average result of six such experiments in which the intracellular pH was 8·1 and the extracellular pH was 7·2 (curve labelled 7·2/8·1). It is clear that when the intracellular solution was alkaline the apparent equilibrium dissociation constant was greater at all voltages than it was when the intracellular pH was 7·2 (curve labelled 7·2/7·2). It also can be seen that the equilibrium dissociation constant again

depended on voltage, rising from a value of 204 ± 37 μM ($n = 6$) at -20 mV to 510 ± 56 μM at $+40$ mV.

When the intracellular pH was reduced from 7·2 to 6·4 the concentration of extracellularly applied 4-AP required to block outward potassium currents was

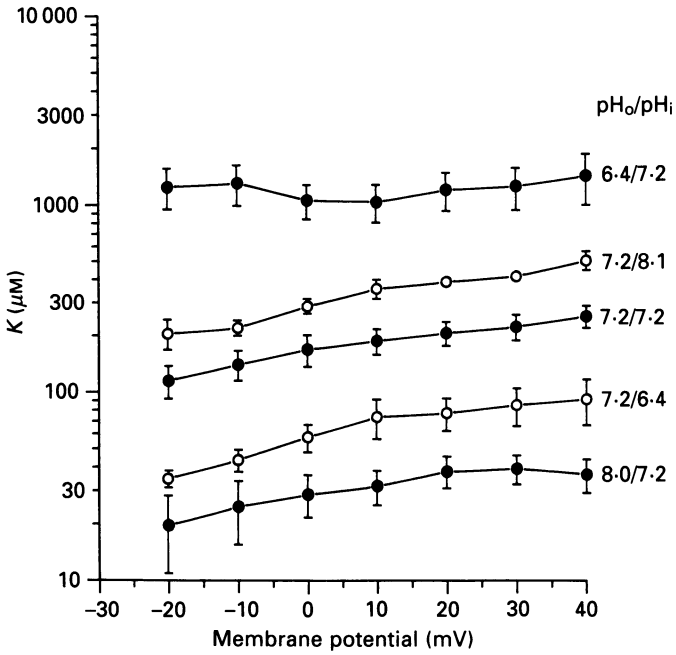

Fig. 4. The mean values of K, the apparent equilibrium dissociation constant, that were obtained with different values of extracellular and intracellular pH at the indicated membrane potentials from the fitting procedure illustrated in Fig. 3. The values of the pH of the extracellular and intracellular solutions (pH_o and pH_i, respectively) are given to the right of the appropriate curve. Each point is the mean of six to twelve values (see text) and the bars give the mean ± 1 S.E.M. The S.E.M. was less than half the symbol size for points without bars. Note the semilogarithmic scale.

much reduced (Fig. 4, curve labelled 7·2/6·4). Thus at -20 mV the value of K was $34{\cdot}6 \pm 3{\cdot}4$ μM ($n = 6$), compared with $168{\cdot}4 \pm 32$ μM ($n = 12$) at pH 7·2; and it rose to $92{\cdot}1 \pm 25{\cdot}1$ μM at $+40$ mV compared with 256 ± 34 μM at pH 7·2.

Changes in the intracellular pH, unlike those in extracellular pH, did not significantly alter the potential at which outward potassium currents were first detected (or the membrane potential at which the P_K–V curve reached half-maximum). At intracellular pH 8·0–8·1, the outward current in the absence of 4-AP was first detected with a test step to -55 ± 2 mV ($n = 6$). The control outward current in the six cells tested at an intracellular pH of 6·4 (extracellular pH 7·2) activated at -53 ± 1 mV (i.e. at a potential not significantly different from the control value of -55 ± 2 mV at pH 7·2).

The intracellular pH was taken to be that of the solution in the patch pipette because there is ample evidence (see Marty & Neher, 1983) that the intracellular solution rapidly equilibrates with the solution in the pipette. Clearly, any calculation

based on that assumption would be vitiated if the intracellular pH was also determined in part by the extracellular pH and so was set at a value intermediate between the pH of the pipette solution and that of the extracellular fluid. In several experiments, therefore, both the extracellular and intracellular pH were shifted

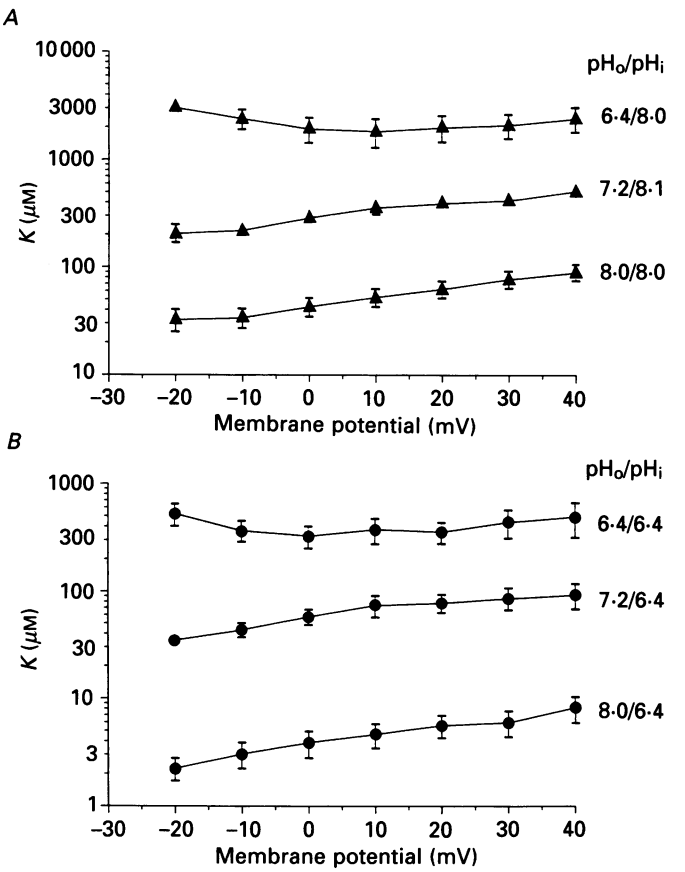

Fig. 5. The effect of varying extracellular pH (pH_o) on the apparent potency of 4-AP when the intracellular pH (pH_i) was alkaline (*A*, pH 8·0–8·1) or acidic (*B*, pH 6·4). The values of pH_o and pH_i are given to the right of each curve. Each point is the mean of four to twelve values (see text) and the bars indicate the mean ± 1 S.E.M. The S.E.M. was less than half the symbol size for points without bars. Note the semilogarithmic scale.

together. As can be seen in Fig. 5*A* (curve labelled 8·0/8·0), half-block of the potassium current (at 0 mV) is attained in alkaline solution (pH = 8·0) with an extracellular concentration of 42·8 ± 8·4 μM (n = 12). In contrast, when both the extracellular and intracellular contents were acidic (pH = 6·4; curve labelled 6·4/6·4, Fig. 5*B*) the corresponding value was 325 ± 76 μM (n = 11). Figure 5 also illustrates the results obtained with the greatest gradient of the pH between the intracellular and extracellular solutions (curve labelled 6·4/8·0, panel *A*; curve labelled 8·0/6·4, panel *B*). When the pH of the extracellular solution was 6·4, and the intracellular solution was at pH 8·0, the cells exhibited the least sensitivity to 4-aminopyridine,

the value of K being 1903 ± 496 μM ($n = 4$). When the extracellular pH was alkaline (8·0) and the intracellular pH acidic (6·4), the greatest apparent sensitivity to 4-AP was obtained, the concentration that produced half-block of the outward potassium current being $3{\cdot}80 \pm 1{\cdot}08$ μM ($n = 6$).

In addition to a possible effect of the extracellular pH on the pH of the intracellular solution, it was also possible that (although the hydrogen ion

TABLE 1. Concentrations of the two forms of 4-aminopyridine present extra- and intracellularly when the potassium current in Schwann cells is half-blocked

	Extracellular		Extracellular 4-AP concentration			Intracellular 4-AP concentration			
	pH_o	pH_i	Total (μM)	[C] (μM)	[U] (μM)	[U] (μM)	[C] (μM)	$K_c/K_u = 142$ $(K_u)_{1\ site}$ (μM)	$K_c/K_u = 157$ $(K_u)_{2\ site}$ (μM)
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
(1)	7·2	7·2	168	166	1·66	1·66	166	2·83	3·27
(2)	8·0	7·2	26	24	1·53	1·53	153	2·60	2·97
(3)	6·4	7·2	1156	1154	1·83	1·83	183	3·12	3·66
(4)	7·2	8·1	287	284	2·84	2·84	36	3·09	3·27
(5)	8·0	8·0	43	40	2·52	2·52	40	2·80	2·97
(6)	6·4	8·0	1944	1941	3·08	3·08	49	3·42	3·69
(7)	7·2	6·4	57	56	0·56	0·56	353	3·04	3·22
(8)	6·4	6·4	363	362	0·57	0·57	362	3·11	3·30
(9)	8·0	6·4	3	3	0·19	0·19	120	1·03	1·01

Column *c* gives the mean value of K determined at the indicated values of extracellular and intracellular pH (columns *a* and *b*, respectively). The number of observations are given in the text and the S.E.M. values are shown in Figs 4 and 5.

concentration of the pipette and intracellular solutions equilibrated rapidly) large intracellular molecules did not exchange rapidly. If this were the case, then the buffering capacity of the intracellular contents may have exceeded that of the pipette solution. Therefore, in some of the experiments, where the extracellular and intracellular solutions were maintained at the same pH, the concentration of buffer was increased 15-fold, in order to ensure further that the intracellular pH was in fact that of the pipette solution. The results from the experiments with the high concentration of buffer were not significantly different from those with the normal concentration, and were included in the calculation of the means.

The intracellular and extracellular concentrations of the charged and the uncharged forms of 4-AP at half-block

The present experiments confirm earlier findings that 4-AP, applied externally, is more potent when the extracellular solution is alkaline (see, for example: Gillespie & Hutter, 1975; Kirpekar, Kirpekar & Prat, 1977; Molgo, Lundh & Thesleff, 1980; Kirpekar, Schiavone & Prat, 1982). They also show that when the extracellular medium is more acidic, externally applied 4-AP is less potent. Table 1 (rows 1–3) shows the extracellular concentrations of 4-AP (column *c*) that were required to produce half-block of the potassium current evoked in response to a depolarization to 0 mV (that is the apparent value of K) when the intracellular pH was kept

constant at 7·2 (column *b*) and the extracellular pH was set at each of the three values tested (column *a*). In Figs 4 and 5, the values for the test potential are nominal values and were not corrected for the shift in transmembrane potential caused by the pH change altering the surface potential generated by the fixed charges in the membrane. The magnitude of this shift was estimated from the change in the apparent activation threshold of the potassium currents, and the values shown in Table 1 are the half-blocking concentrations at zero potential after correction for this shift.

If certain conditions hold, then the relative contribution that the cationic and uncharged forms of 4-AP make, both extracellularly and intracellularly, to the block of potassium currents can be quantitatively assessed from the results reported here. One condition is that equi-effective concentrations imply that the concentrations of the active form of 4-AP are equal. This will be so if changes in the intracellular and extracellular pH do not change the affinity with which the active form of 4-AP binds to its blocking site. An additional condition is that the uncharged form of 4-AP can move freely across the lipid plasmalemma and that, as a result, the extracellular and intracellular concentrations of the uncharged form are the same. If these conditions hold, then (for any given total concentration of 4-AP applied extracellularly) the extracellular and intracellular concentrations of the two forms of 4-AP can be calculated from the known value of its pK_a and from the known values of the extracellular and intracellular pH. If one form of 4-AP is solely responsible for potassium channel block, then the concentration of that active form of 4-AP should be constant when the 4-AP is applied in concentrations that produce equivalent degrees of block. In our experiments these latter concentrations are given by the mean values of K that were determined at the different pH values.

The extracellular concentrations of the charged and uncharged forms of 4-AP (at the total extracellular concentration corresponding to the mean value of K), which were calculated on the basis of pH and the pK_a for 4-AP, are given in columns *d* and *e* in Table 1. Clearly, when the intracellular pH was 7·2, the extracellular concentration of uncharged molecules required to half-block the outward current remained relatively constant (at about 1·7 μM) as the extracellular pH was changed (rows 1–3, Table 1). In contrast, the extracellular concentration of charged molecules present varied by a factor of about 50. This was also the case when the internal pH was kept constant at 6·4 and at 8·0. These findings thus seem to eliminate the possibility that the extracellular cationic form of 4-AP plays any substantial role in the block of the outward current. If it did play a substantial role, then the value of K should have been relatively independent of extracellular pH over the range of pH values tested.

Similar arguments also suggest that neither the uncharged form, nor the charged form of 4-AP acting from the intracellular surface of the channel, are solely responsible for potassium channel blockade. Thus when the extracellular pH was kept constant at 7·2 and the intracellular pH was varied between 8·1 and 6·4 (rows 1, 4 and 7, Table 1) the calculated concentration of uncharged 4-AP, both extracellular and intracellular, varied by a factor of five (columns *e* and *f* in Table 1). The intracellular concentration of the cationic form of 4-AP varied nearly tenfold over the pH range tested (column *g* in Table 1).

Two simple explanations for the variation in both the concentration of the uncharged form and the intracellular concentration of the cationic form are: (1) The uncharged form, or alternatively the intracellular cationic form, might be solely responsible for the observed block, but the affinity that the given form of the molecule has for its binding site depends on intracellular pH. (2) The block of the

TABLE 2. Concentrations of the two forms of 3-aminopyridine present extra- and intracellularly when the potassium current in Schwann cells is half-blocked

				Extracellular 3-AP concentration			Intracellular 3-AP concentration	
	pH_o	pH_i	n	Total (μM) a	[C] (μM) b	[U] (μM) c	[U] (μM) d	[C] (μM) e
(1)	7·2	7·2	5	1291±181	77	1214	1214	77
(2)	8·0	7·2	7	913±161	9	904	904	57
(3)	6·4	7·2	4	3218±464	916	2302	2302	145

potassium currents might be the result of the action of both the uncharged form and the intracellular cationic form. These possibilities are considered in detail in the Discussion where it is shown that if both forms contribute to the block then the uncharged form of 4-AP must have a K of about 3 μM and have about 150 times the apparent affinity that the intracellular cation has. This is so whether the two forms bind to the same site or to different sites (columns h and i of Table 1, respectively). On the basis of the 4-AP results alone it is impossible to decide between these possibilities. We therefore tested other aminopyridines.

Experiments with 3-aminopyridine

3-Aminopyridine (3-AP) is isomeric with 4-AP, but is a much weaker base, having a pK_a value of about 6·0 rather than the 9·2 of 4-AP (see Marshall, 1982). Thus, unlike 4-AP, at all near-physiological values of pH most of the 3-AP molecules will be uncharged. Experiments were therefore carried out to test the blocking action of 3-AP. In these experiments the intracellular pH was kept constant at 7·2.

The potassium channel blocking action of 3-AP was tested at different values of extracellular pH. As Table 2 (column a) shows, at each value of extracellular pH the total concentration of 3-AP required to produce block was considerably greater than the corresponding concentration of 4-AP (cf. rows 1–3, Table 1). Furthermore, the dependence on extracellular pH was a good deal less. Thus, the total extracellular concentration required to half-block the current at pH 6·4 was only 2·9 times higher than the corresponding concentration at pH 8·0, compared with a ratio of about 45 for 4-AP (Table 1, column c). With 3-AP, the extracellular concentration of cation varied by a factor of 100 (column b), whereas the concentration of the uncharged form varied by a factor of only about 2·5 (column c), as the extracellular pH was varied from 6·4 to 8·0. These latter results are similar to the corresponding results obtained with 4-AP, and they provide additional evidence for the conclusion that the cationic form of the aminopyridines does not block the potassium channel from the extracellular solution.

Experiments with 2-aminopyridine and with 3,4-diaminopyridine

Some experiments were also done with 2-aminopyridine (2-AP), which has a pK_a of about 6·7, and with 3,4-diaminopyridine (3,4-DAP), which has a pK_a of 9·1 (Marshall, 1982). The potency of 2-AP was similar to that of 3-AP in that large extracellular concentrations were required to half-block the potassium currents. At a membrane potential of 0 mV, the mean value of K with 2-AP was 1483 ± 318 μM ($n = 6$). 3,4-Diaminopyridine displayed a potency similar to that of 4-AP, the mean value of K at 0 mV being 286 ± 44 μM ($n = 6$).

Recovery after exposure to aminopyridines

Although recovery after exposure to the aminopyridines was not studied systematically, the results from many cells made it clear that complete recovery after exposure to all the aminopyridines could occur, even after the largest concentrations used. With 4-AP about half the current was restored within the first 30 s and it was mostly restored within 2 min. Similarly, relatively fast recovery was obtained after exposure to 2-AP or 3-AP. But with 3,4-DAP, recovery was much slower. Relatively little recovery occurred within the first minute, and a washing period of 10–15 min was required for the potassium current to return to more than about 90% of its control value. The slow recovery with 3,4-DAP was also noted by Kirsch & Narahashi (1978); they found that washing even for 1 h after external application of 3,4-DAP to the squid axon produced only partial recovery.

Experiments with 4-aminopyridine methiodide

As indicated in the Methods section, 4-aminopyridine can readily be converted to the permanently charged methiodide. Experiments were therefore carried out to test whether 4-aminopyridine methiodide (4-APMI) also blocked the potassium currents. 4-APMI, applied in the extracellular solution at a concentration of 10 mM, was tested on nine cells and had no effect on whole-cell potassium currents. (In all of these experiments both the extracellular and the intracellular solutions were pH 7·2.) In five of these cells, 1 mM-4-AP was also tested. At a membrane potential of 0 mV, the mean change in the outward currents (relative to control) that was produced by 4-APMI was $+1{\cdot}6 \pm 4{\cdot}5$% ($n = 9$). In the five cells tested with both 4-APMI and 4-AP, the corresponding mean change was $+0{\cdot}6 \pm 7{\cdot}0$% with 10 mM-4-APMI and $-77{\cdot}3 \pm 4{\cdot}8$% with 1 mM-4-AP. The magnitude of the reduction seen with 4-AP is consistent with the mean K and I_{max} values determined for 4-AP at this membrane potential (168 μM and 88%, respectively).

Several experiments were also done in which 4-APMI was applied to the cytoplasmic surface of inside-out patches. These experiments were complicated by the relatively low potassium current density displayed by rabbit Schwann cells (usually below 1 pA/μm^2) and the consequent small size of the outward ensemble currents obtained (typically only 2–3 pA at test depolarizations to +40 mV). Stable ensemble current records were obtained from seven patches that were tested with 4-APMI (1–10 mM), and there was no indication that 4-APMI had any effect on these currents. By comparison, 4-AP did reduce these currents in a manner similar to that found with whole-cell currents.

Our finding differs from that of Kirsch & Narahashi (1983) who found that internal perfusion of squid giant axons with 4-APMI did block the potassium current. But the potency of the permanently charged methiodide was very weak, being 100 times less than that of 4-AP (which is largely in the cationic form at the pH prevailing in their experiments).

TABLE 3. Voltage dependence of the block of potassium current by extracellular 4-aminopyridine and 3-aminopyridine. The slopes of the semilogarithmic plots in Fig. 4 are given as a function of intracellular (pH_i) and extracellular (pH_o) pH

	Slope (V^{-1}) at pH_i		
pH_o	8·0	7·2	6·4
		4-AP	
8·0	7·96	5·13	7·25*
7·2	5·16	4·57	3·29
6·4	6·06	4·81	3·66
		3-AP	
8·0	—	5·98	—
7·2	—	6·34	—
6·4	—	6·33	—

*Value from same experiments as in row 9, Table 1.

The voltage dependence of aminopyridine action

Figures 4 and 5 show that the apparent voltage dependence of 4-AP action is roughly similar at the different values of intracellular and extracellular pH, especially at positive membrane potentials. The slopes of the semilogarithmic plots in Figs 4 and 5 were determined by least-squares fits to the points in the region of 0 mV, after correction for the shift in the surface potential with changes in extracellular pH (see p. 191). The results of this procedure for the 4-AP experiments, together with the corresponding results from the experiments with 3-AP, are summarized in Table 3.

Though there seems to be some tendency for the voltage dependence to decrease with increasing intracellular acidity, there is no clear relation between voltage dependence and extracellular pH for either 4-AP or 3-AP. The mean slope at an intracellular pH of 7·2 is significantly smaller ($P < 0·01$) with 4-AP ($4·84 \pm 0·16$ V^{-1}) than with 3-AP ($6·22 \pm 0·12$ V^{-1}). These values correspond to an e-fold change in affinity with changes in membrane potential of 90 and 70 mV, respectively.

DISCUSSION

The main outcome of the present experiments is that they allow a quantitative consideration of the molecular basis of action of 4-AP in blocking the potassium current in rabbit cultured Schwann cells. One clear conclusion is that the cationic form of the aminopyridines, acting directly from the extracellular solution, did not make any substantial contribution to the observed reductions in potassium currents. If it had, then the total concentration of extracellularly applied 4-AP required to half-block the potassium current should have been relatively insensitive to changes in extracellular pH, whereas the apparent potency of extracellularly applied 3-AP

should have varied considerably. In fact, just the opposite was the case and, at half-block, the concentration of the cationic form of each of these two aminopyridines varied 50- to 100-fold as the extracellular pH was changed from 6·4 to 8·0. Although it might be argued that the changes in pH changed the apparent affinity of the extracellular cation for its binding site, the size of the variations in the extracellular cation concentration at equivalent degrees of block, together with the relative constancy of the corresponding concentration of the uncharged form, would seem to make this extremely unlikely.

On balance, we believe that the results support the idea that it is the cationic form of the aminopyridines, acting from the intracellular compartment, that brings about the block. But other explanations cannot be fully eliminated. Nevertheless, although the present results do not indicate unambiguously the particular mechanism that is involved, they do define and limit the possibilities. The issue is complex largely because the aminopyridines can exist in two molecular forms (charged and uncharged) and the site may exist in protonated and unprotonated states, so that several possible molecular mechanisms of action exist. For example, either species might act alone, or both species might be active and could act at either the same or at two different sites. Furthermore, if the binding site does contain a titratable group, it may well be that the aminopyridine can bind only when the site is in a particular state – protonated or unprotonated. These possibilities are discussed below.

(1) If both species act at the same site (in a way analogous to the mechanism proposed by Hille (1975) for the block of the sodium channel by both charged and uncharged forms of local anaesthetics), but the affinity of the charged form for the channel was different from that of the uncharged aminopyridine, then pharmacokinetic considerations require that at half-block the concentration of the uncharged and charged form obeys the relation:

$$\{[U]/K_u\}+\{[C]/K_c\} = 1, \tag{2}$$

where K_u and K_c are the respective equilibrium dissociation constants of the uncharged and charged forms, and where [U] and [C] are their respective concentrations. Rearrangement gives:

$$[U] = -\{(K_u/K_c)[C]\}+K_u. \tag{3}$$

Thus, at half-blocking concentrations of 4-AP, a linear relationship between [U] and [C] is expected. This relationship was indeed found experimentally, provided that the intracellular concentration of the cationic form of 4-AP was considered. In Fig. 6, the values of [U] from column *f* of Table 1 are plotted against the corresponding values for the intracellular concentration of the charged form, $[C]_i$, from column *g* of Table 1. As can be seen, the points are fitted reasonably well by a straight line of slope $-0{\cdot}007$ ($K_c/K_u = 142$), apart from the point at one of the extreme cases (extracellular pH 8·0, intracellular pH 6·4) which clearly seems aberrant (for unknown reasons) and which was not included in this or subsequent fits. On the whole, therefore, the results are consistent with the uncharged form having an affinity about 150-fold higher than that of the charged form. The value of K_u estimated from this fit is given from eqn (3) by the intercept on the vertical axis, namely 3·0 μM. Column *h* of Table 1

gives the values of K_u calculated for each individual case from the relation: $K_u = [U] + 0{\cdot}007[C]_i$, and these results illustrate the relatively small scatter of the individual values about the fitted line. (Indeed, even the point at extracellular pH 8·0 and intracellular pH 6·4 is only out by a factor of about 3.) The results thus are

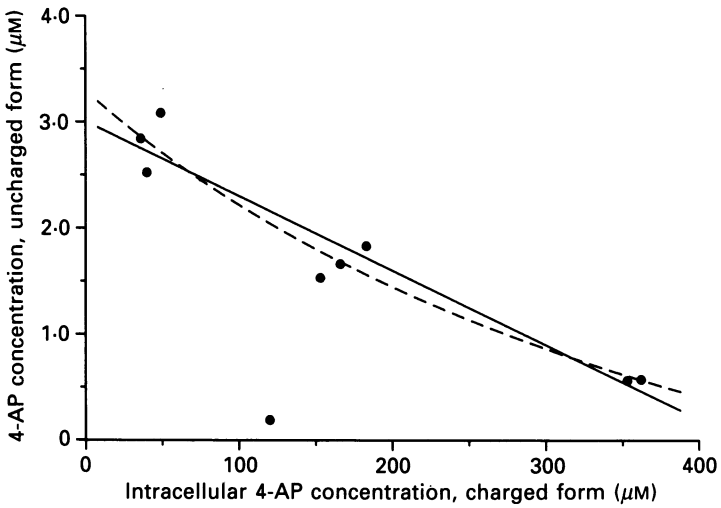

Fig. 6. The relation, at half-block of the current elicited by a depolarization to 0 mV, between the concentration of uncharged molecules ([U]) and the intracellular concentration of charged molecules ($[C]_i$). The straight line is the least-squares fit to eight of the data points (see text) according to eqn (3) and the interrupted curve is the fit to the same points according to eqn (5).

consistent with the hypothesis that the uncharged form of 4-AP is about 150 times more active, on a molar basis, than is the intracellular cation. In contrast to these results, no consistent relationship was found between the concentration of the uncharged form and the extracellular concentration of the charged form. Whether the uncharged form would have to act extracellularly, intramembranally, or from the intracellular side of the channel is unclear from these results, because the concentration (or strictly speaking the thermodynamic activity) of the uncharged form is likely to be the same in all compartments.

(2) A second possibility is that rather than acting at the same site each molecular species acts independently at its own separate site, occupation of either site being sufficient to produce potassium channel block. In this case, at equilibrium it is required that:

$$\{[U]/K_u\} + \{[C]_i/K_c\} + \{[U][C]_i/K_c K_u\} = 1. \quad (4)$$

Rearrangement gives:

$$[U] = K_u \frac{\{1 - ([C]_i/K_c)\}}{\{1 + ([C]_i/K_c)\}}. \quad (5)$$

The least-squares fit to this hyperbola is shown by the interrupted line in Fig. 6. Clearly both models fit the data more or less equally well (again with the omission of the data in row 9, Table 1). The 'best-fit' value of K_u is 3·3 μM and that of K_c

512 μM. These values are similar to the corresponding values of 3·0 and 427 μM obtained for the one-site model, and the value of K_c/K_u is again about 150. Row *i*, Table 1, shows the values for K_u calculated for each individual case on the basis of the 'best-fit' value for the ratio K_c/K_u. As can be seen, there is comparatively little scatter in these individual values. In particular, the values in rows 5 and 8 from the experiments where there was no pH gradient across the cell membrane agree well with the others.

(3) A third possibility is that only a single species of 4-AP is active, but that it can bind to its receptor only when the latter is in a particular state (protonated or unprotonated) determined by pH. For example, the cationic form may bind only to the unprotonated site. In that case, if the 9-fold increase in the intracellular cation concentration necessary for half-block (column *g*, Table 1) at an intracellular pH of 6·4 (compared with the corresponding concentration necessary at pH 8·0) were to be accounted for by protonation of the site, this would require that:

$$[C]_i = K_c + \{(K_c/K_H)[H]\}, \tag{6}$$

where [H] is the hydrogen ion concentration and K_H is the equilibrium dissociation constant for protonation of the site. Given the values of $[C]_i$ at half-block of 358, 167 and 42 μM at intracellular pH values of 6·4, 7·2 and 8·0, respectively (Table 1, but ignoring row 9), a least-squares fits yields a value of 74·8 μM for K_c and gives a pK_a for the binding site of 7·0. A similar calculation based on the assumption that the uncharged molecule binds to the protonated site gives a value for K_u of 0·91 μM and a pK_a for the binding site of 7·7. The existence of a proton-binding site in the delayed rectifier potassium channel with a pK_a of about 7 is supported by data on the effects of pH_i on the potassium conductance in squid giant axon (Wanke, Carbone & Testa, 1979). However, it is noteworthy that Kirsch & Narahashi (1983) found that the blocking potency of 4-APMI applied internally to the squid giant axon did not depend on internal pH. Similar calculations for the two other cases (cation binding to a protonated site, or uncharged aminopyridine binding to an unprotonated site) would require negative calculated values for the equilibrium dissociation constant of the binding site, and so can be neglected.

(4) In addition to a possible effect of pH on the apparent affinity with which 4-AP binds to its blocking site, some effect of pH on the apparent potency of 4-AP might be expected as the result of changes in surface potential. For example, Mozhayeva & Naumov (1972) have shown that changes in the membrane surface potential result in changes in the apparent potency of extracellularly applied tetraethylammonium ions (TEA) in blocking potassium currents in the frog node of Ranvier. However, these changes (which are secondary to changes in the concentration of TEA cations at the membrane surface) are small. The apparent equilibrium dissociation constant for TEA at pH 5·2–5·4 differed from that determined at pH 9·3–9·6 by only a factor of about 2·0 (Mozhayeva & Naumov, 1972), and we have found no substantial difference in the value of *K* determined for the action of TEA to block potassium currents in rabbit Schwann cells over the range of extracellular pH tested in the present experiments (6·4–8·0). Furthermore, although the variation (at half-block and different intracellular pH) in the calculated intracellular concentration of the cationic form of 4-AP is qualitatively what would be expected for pH-dependent

changes in the internal surface potential, the lack of any significant effect in our experiments of intracellular pH on the voltage dependence of potassium current activation, suggests that the internal surface potential was small. For these reasons, it seems unlikely that pH-dependent alterations in surface potential significantly affected the results obtained with 4-AP.

TABLE 4. Concentrations of the two forms of the aminopyridines present extracellularly and intracellularly when the potassium current in Schwann cells is half-blocked

			Extracellular AP concentration			Intracellular AP concentration	
Drug	pK_a	n	Total (μM) a	[C] (μM) b	[U] (μM) c	[U] (μM) d	[C] (μM) e
2-AP	6·71	6	1483±318	356	1127	1127	356±76
3-AP	6·03	5	1291±181	77	1214	1214	77±11
4-AP	9·18	12	168±32	166	1·66	1·66	166±32
3,4-DAP	9·08	6	286±44	282	3·56	3·56	283±43

The extracellular and the intracellular pH was 7·2 in all cases.

Comparison of possible mechanisms of action

Both the one-site model and the two-site model, which postulate that both the uncharged and charged forms of 4-AP are active, can reasonably account for the experimental findings with 4-AP, but only provided the uncharged molecule is about 150 times as active as the charged molecule. However, some doubt that the blocking action is shared by both species of 4-AP is raised by the findings with 2-AP and 3-AP. As Table 4 shows, both 2-AP and 3-AP also block the potassium channel but in much higher externally applied concentrations. For example, at pH 7·2 (internally and externally) about 9 times as much 2-AP and about 7 times as much 3-AP is required to be applied extracellularly, and the intracellular concentration of both uncharged aminopyridines is about 700 times that for 4-AP. It seems more noteworthy that at half-block the intracellular cation concentrations of 2-AP, 3-AP, 4-AP and 3,4-DAP vary by less than a factor of five. In a similar analysis of experiments on squid giant axons, in which the internal pH was varied from 6·3 to 8·6, Kirsch & Narahashi (1983) showed that a cation concentration of 2,3-diaminopyridine ($pK_a = 7·0$) of 12·5 μM produced the same degree of potassium channel block irrespective of how much uncharged molecule was also present.

Although the other possibilities cannot be excluded, the most plausible mechanism of action of the aminopyridines seems to be that the cation interacts with an intracellular receptor to block the potassium channel. If, however, the cation alone is responsible for block it would require that the affinity of the charged form decreases as the intracellular pH is made more acidic (column *g*, Table 1), as indicated in paragraph (3) above.

The lack of any blocking action of the permanently charged aminopyridine 4-APMI when applied to the cytoplasmic membrane surface, although unexpected, does not by itself invalidate the hypothesis that the activity of the aminopyridines resides in the cationic form, for the only evidence available on the ability of the permanently charged analogue to block potassium channels shows that it is

relatively weak, being 100 times less potent than 4-AP itself in the squid giant axon (Kirsch & Narahashi, 1983). Our failure to find an effect of 1–10 μM-4-APMI on Schwann cell potassium currents, where the K for 4-AP is about 200 μM, is therefore not inconsistent with these previous results. In the squid, concentrations of 4-APMI above 10 mM have been reported to produce non-specific and irreversible reductions of both sodium and potassium currents (Kirsch & Narahashi, 1983). Although 4-APMI, like 4-AP, has been found to improve neuromuscular transmission, no clear involvement of potassium channel blockage has been noted. Horn, Lambert & Marshall (1979) did observe an increase in the duration of the frog sartorius action potential with large concentrations of the methiodide. However, it seems clear that the methiodide owes much, if not all, of its effect on muscle to an anticholinesterase action (Horn *et al.* 1979).

If it is indeed the case that the cationic form of the aminpyridines blocks the potassium channel from its intracellular surface (as we believe the rest of the results reported here suggest) then it also seems to be the case that N-methylation, in addition to quarternarizing the ring nitrogen, also greatly reduces the activity of the molecule in blocking potassium channels. This would in turn suggest that the steric constraints on aminopyridine binding (or access) to its blocking site may be quite severe.

The voltage dependence of aminopyridine action

One consistent feature of aminopyridine blockade of potassium channels is that the block depends on membrane potential, the degree of block being reduced by depolarization (Ulbricht & Wagner, 1976; Yeh *et al.* 1976*a*, *b*; Meves & Pichon, 1977; Ulbricht *et al.* 1982). In rabbit Schwann cells, this voltage dependence can be described by an increase in K as the test potential is made more positive (Howe & Ritchie, 1988). Our present experiments show that the apparent affinities with which the other aminopyridines tested (2-AP, 3-AP and 3,4-DAP) block outward potassium currents in these cells show a qualitatively similar dependence on membrane voltage.

As Woodhull (1973) and Strichartz (1973) have pointed out for the blocking action of hydrogen ions and quaternary lidocaine derivatives, respectively, on sodium channels, if blockade of an ion channel requires a cation to bind to a site within the electrical field of the membrane, then it is to be expected that the rate constants of association and dissociation (and consequently K) depend on membrane voltage. Our experiments seem to indicate clearly that the cationic form of 4-AP acts from the intracellular solution and does not have direct access to the potassium channel from the extracellular solution. According to Woodhull (1973), if the cation primarily has access to the blocking site from the cytoplasm, then the apparent affinity of the cation is expected to increase as the membrane potential is depolarized. In contrast, the aminopyridines exhibit the opposite voltage dependence; and therefore this voltage dependence must have other explanations.

The relative potency of 4-aminopyridine and 3,4-diaminopyridine

The potency of externally applied 3,4-DAP (K = 286 μM, Table 4) was not greatly different from that of 4-AP (K = 168 μM). This differs from the conclusion of Kirsch & Narahashi (1978) that 3,4-DAP applied extracellularly is about 50 times more

potent than 4-AP in blocking the potassium channels in the squid giant axon. Their conclusion was based on the observation (Yeh *et al.* 1976*b*) that the extracellular application of 1 mM-4-AP caused a 75% block in the potassium current measured at +100 mV, whereas only 20 μM-3,4-DAP was necessary to produce a similar block of the potassium current evoked by depolarization to +60 mV. However, this comparison may be misleading. Inspection of Table 1 in Yeh *et al.* (1976*b*), which contains the results on which the statement regarding the potency of 4-AP was based, shows that the 75% reduction at 1 mM may well have been near the maximum depression of potassium conductance of which 4-AP was capable, and this result would therefore provide a rather insensitive measure of potency. Indeed, if all the data in Table 1 of Yeh *et al.* (1976*b*) are fitted by a least-squares procedure to rectangular hyperbolae (as in the present experiments and so providing a better basis for comparison), then the value for the equilibrium dissociation constant at 0 mV is 11·0 μM (96·4% of the current being sensitive to 4-AP) whereas at +100 mV the value of K is 15·0 μM (only 77% of the current being sensitive to 4-AP, with the remaining 23% being relatively insensitive to 4-AP). The apparent equilibrium dissociation constant at +60 mV, which presumably lies between these two values, would therefore only differ by a factor of about 2 from the apparent equilibrium dissociation constant of 6 μM found for 3,4-DAP by Kirsch & Narahashi (1978).

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