Effects of 4-Aminopyridine on Potassium Currents in a Molluscan Neuron

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ABSTRACT The effects of 4-aminopyridine (4-AP) on the delayed K^+ current and on the Ca^{2+}-activated K^+ current of the *Aplysia* pacemaker neurons R-15 and L-6 were studied. The delayed outward K^+ current was measured in Ca^{2+}-free artificial seawater (ASW) containing tetrodotoxin (TTX), using brief depolarizing clamp pulses. External (and internal) 4-AP blocks the delayed K^+ current in a dose-dependent manner but does not block the leakage current. Our results show that one 4-AP molecule combines with a single receptor site and that the block is voltage dependent with an apparent dissociation constant ($K_{4\text{-AP}}$) of ~0.8 mM at 0 mV. $K_{4\text{-AP}}$ increases *e*-fold for a 32-mV change in potential, which is consistent with the block occurring ~0.8 of the distance through the membrane electrical field. The 4-AP block appears to depend upon stimulus frequency as well as upon voltage. The greater speed of onset of the block produced by internal 4-AP relative to when it is used externally suggests that 4-AP acts from inside the cell. The Ca^{2+}-activated K^+ current was measured in Ca^{2+}-free ASW containing TTX, using internal Ca^{2+}-ion injection to directly activate the K^+ conductance. Low external 4-AP concentrations (<2 mM) have no effect on the Ca^{2+}-activated K^+ current, but concentrations of 5 mM or greater *increase* the K^+ current. Internal 4-AP has the same effect. The opposing effects of 4-AP on the two components of the K^+ current can be seen in measurements of the total outward K^+ current at different membrane potentials in normal ASW and during the repolarizing phase of the action potential.

The aminopyridines block voltage-dependent potassium currents of nerve axon (Pelhate and Pichon, 1974; Meves and Pichon, 1975 and 1977; Schauf et al., 1976; Ulbricht and Wagner, 1976; Yeh et al., 1976 *a* and 1976 *b*), soma (Thompson, 1977), and axon-terminal (Llinás, et al., 1976) membranes and of muscle membrane (Gillespie and Hutter, 1975; Fink and Wettwer, 1978; Molgo, 1978). Moreover, they block the voltage-dependent K^+ current in lower concentration and in a different manner than the block produced by tetraethylammonium. In molluscan neurons the K^+ current of the soma membrane can be divided into a component activated by changes in membrane voltage and a component activated by the entrance of Ca^{2+} ions into the cell—the Ca^{2+}-activated K^+ current (Meech and Standen, 1975; Heyer

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and Lux, 1976 *b*). The voltage-dependent K^+ current of the molluscan neuron soma membrane has been further divided operationally into a fast outward K^+ current (Connor and Stevens, 1971; Neher, 1971) and the delayed outward K^+ current (or the "delayed outward rectifier"), which was first described in squid axon by Hodgkin and Huxley (1952 *a*). It has been reported that external 4-aminopyridine (4-AP) selectively blocks the fast K^+ current of the molluscan neuron soma (Thompson, 1977). The experiments reported in this paper were carried out to compare the effects of extracellular and intracellular 4-AP on different components of the K^+ current of a molluscan pacemaker neuron. They show that 4-AP also blocks the delayed outward K^+ current but that, at high concentrations, it increases the Ca^{2+}-activated K^+ current. The results suggest that the aminopyridines can be used to provide a pharmacological separation of the voltage- and Ca^{2+}-dependent components of the K^+ current. A preliminary account of some aspects of this work has appeared elsewhere (Hermann and Gorman, 1978).

METHODS

All experiments were performed on the pacemaker neurons R-15 and L-6 (Frazier et al., 1967) in the abdominal ganglion of *Aplysia californica*. The ganglion was removed, the overlying sheath was dissected away to expose the cells, and the ganglion was pinned to the base of a chamber containing artificial seawater (ASW), where it was maintained at a constant temperature (16°C). The ASW contained (mmol/l) 478 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, and 15 Tris HCl, at pH 7.8. In Ca^{2+}-free ASW, Ca^{2+} was replaced by Mg^{2+} on an equimolar basis, so that the total divalent ion concentration remained constant. The Ca^{2+}-free ASW contained $\sim 4 \times 10^{-6}$ M Ca^{2+} (Gorman and Hermann, 1979). Unless otherwise specified, all ASW solutions contained 5×10^{-5} M tetrodotoxin (TTX), which was added directly to the solution just before its use. 4-Aminopyridine (4-AP) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added to the external solutions from freshly made stock solutions. The exchange time for the chamber was determined with ASW solutions containing different K^+ concentrations and a K^+-sensitive microelectode (see Gorman and Hermann [1979]) located near cell R-15 and was 95% complete in 5 s.

An identified neuron was impaled with up to four microelectrodes for the recording of membrane potential and passing current and for the injection of Ca^{2+} ions and 4-AP. Many of the procedural details have been given previously (see Gorman and Hermann [1979] and Hermann and Gorman, [1979 *a*]); in brief, the microelectrodes used for recording and stimulating were filled with 3 M KCl and had resistances of ~5 and 2 MΩ in ASW, respectively. Membrane potential was measured differentially with respect to a low-resistance extracellular electrode that was independent of bath ground. The circuit used for measurements was similar to one described previously (Thomas, 1977). The rest of the recording, voltage-clamp, and current-measuring circuitry was conventional. Signals were displayed on an oscilloscope and on a three-channel rectilinear pen-recorder for analysis.

Electrodes for intracellular injection were pulled with short shafts (<4 mm) and tips with outside diameters of ~1.5 μm. Ca^{2+}-injection electrodes were filled with a solution of 0.1 M $CaCl_2$ and had a resistance of ~3 MΩ when measured in ASW. 4-AP-injection electrodes were filled with a solution of 0.1 M 4-AP, pH 7.0. At pH 7.0, the 4-AP molecule is predominantly in the cationic form ($pK_a = 9.36$ at 16°C; Albert, 1963). 4-AP, therefore, can be ejected by making the microlectrode positive with

respect to ground. Ca^{2+} and 4-AP were electrophoretically injected into cells in the voltage-clamp mode, so that there was no change in the net flow of current across the membrane during injection. The transport number for the Ca^{2+} electrodes has been determined (Gorman and Hermann, 1979) and on average is 0.29. The transport number for 4-AP electrodes is not known. Electrophoretic injections were made with an electrophoresis unit capable of maintaining a constant current of 10^{-6} A through an electrode resistance of up to 10^8 Ω (model 160, W-P Instruments, Inc., Hamden, Conn.). The injection current was measured continuously and was constant for periods of up to at least 90 s for Ca^{2+}-containing electrodes and at least 250 s for 4-AP-containing electrodes. In some instances, 4-AP was pressure injected from similar microelectrodes, with pressures between 15 and 25 lbs/sq in. There were no obvious differences in the results obtained with the two methods for injecting 4-AP.

The resistance in series with the membrane was determined by two methods. Measurements of the series resistance (r_s) were obtained under current-clamp conditions from $\Delta V/\Delta I$, where V is the amplitude of the initial jump in the voltage transient in response to a brief current step, ΔI (see Moore and Cole [1963]), and under voltage-clamp conditions from $\Delta V/I_c$, where ΔV is the amplitude of a command potential and I_c is the peak capacitive current (see Nathan and DeHaan [1979]). There was reasonable agreement between the two methods. Typically, the low-resistance extracellular recording electrode was positioned as close as possible to the cell to minimize the series resistance, and under these conditions r_s was found to be 1–3 kΩ. No compensation was made for this series resistance.

There is evidence that the interior of R-15 is isopotential under voltage-clamp conditions (Adams and Gage, 1979). In most of the experiments, the axon of R-15 was undercut at a distance of ~400–500 μm from the soma in an attempt to minimize the contribution of current from the axon, but it is most unlikely that the axonal stump was space clamped.

Terminology

The outward K^+ current of the molluscan neuron soma membrane can be divided into at least three components, which have been referred to by different names and symbols by various groups. Two of these K^+ currents are activated by changes in voltage across the membrane, whereas the third component is activated by changes in the intracellular Ca^{2+} concentration. The voltage-dependent outward K^+ current that activates quickly and undergoes appreciable inactivation during a voltage step from hyperpolarized potentials is referred to as the *fast outward K^+ current.* It has also been called the transient outward K^+ current and is usually symbolized by I_A. The voltage-dependent outward K^+ current that activates with a delay and is slower to inactivate during a voltage step is referred to as the *delayed outward K^+ current.* It has also been called the voltage-dependent K^+ current, the delayed outward rectifier, and the voltage-activated late K^+ current and has been symbolized by I_K and $I_{K,V}$. The third current depends on a change in the intracellular Ca^{2+} concentration, which can occur after Ca^{2+} influx or after direct internal Ca^{2+} ion injection, and referred to as the *Ca^{2+}-activated K^+ current.* It has also been called the Ca^{2+}-dependent K^+ current, the Ca^{2+}-activated delayed outward K^+ current, and the Ca^{2+}-activated late K^+ current and has been symbolized by $I_{K,Ca}$ and I_C. Finally, the outward current present during positive potential steps under conditions where both the delayed outward K^+ and the Ca^{2+}-activated K^+ currents are recorded simultaneously is referred to as the *total outward current* but has also been referred to as the delayed current and as the delayed K^+ current.

RESULTS

Inhibition of the Delayed Outward K^+ Current by 4-AP

Fig. 1 illustrates the effect of 5 mM external 4-AP on the K^+ outward currents measured with depolarizing clamp pulses of short and long durations and of various amplitudes in Ca^{2+}-free ASW containing 5×10^{-5} M TTX. Ca^{2+}-free ASW was used to abolish the Ca^{2+} current and the Ca^{2+}-activated K^+ current component of the total outward current (Meech and Standen, 1975; Heyer

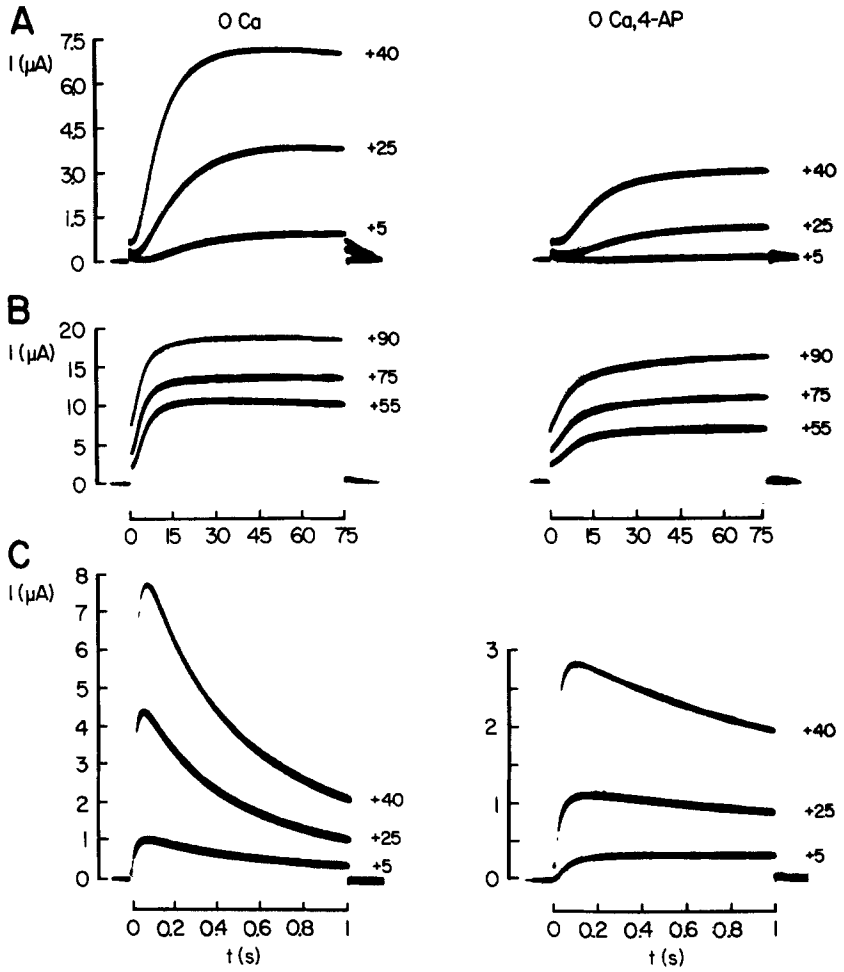

FIGURE 1. Effects of external 4-AP on R-15 membrane outward K^+ currents. The left side shows outward currents measured in Ca^{2+}-free ASW containing 5×10^{-5} M TTX, and the right side shows currents measured after 25 min in 5 mM 4-AP. The holding potential was −45 mV. The membrane potential during the pulse is indicated at the right of each current trace. In *A* and *B* the pulse duration was 75 ms, and in *C* 1 s.

and Lux, 1976 *b*). TTX was used to eliminate the fast early inward current that occurs in Ca^{2+}-free ASW containing Na^+ (Geduldig and Gruener, 1970). Finally, the membrane was held at −45 mV to inactivate the fast outward current (Connor and Stevens, 1971; Neher, 1971). Under these conditions the residual outward current is composed primarily of the delayed outward K^+ current and the leakage current. In the absence of 4-AP, the outward current rose to a peak with a delay and showed a slow decay during maintianed depolarization. The delay can be attributed to the time necessary for the K^+ channel to activate, and the slow decay to the inactivation of the same channel, to K^+ ion accumulation in the extracellular space, or to both processes. External 4-AP has several effects on outward K^+ currents. Fig. 1 shows that the maximum outward current, at any potential, was smaller than the comparable current in the absence of 4-AP but that the reduction of the outward current was less at very positive potentials and at long times. In addition, the rise of the outward current was slowed by 4-AP and its decay was less pronounced. The effects of 4-AP, particularly at high concentrations (~1 mM or greater), were only partially reversible.

Fig 2 *A* shows a plot of the peak K^+ outward current in Ca^{2+}-free ASW after the addition of 5 mM 4-AP. The K^+ outward current was fully suppressed by 4-AP at membrane depolarizations up to about +20 mV, but the block was less effective at more positive potentials. The K^+ outward current, however, includes both the delayed outward K^+ current and leakage current component, which at positive membrane potentials is carried primarily by K^+ ions. The leakage current was estimated from the initial instantaneous jump in current that occurs after the capacitative discharge of the membrane. It was approximately linear between −100 and about +50 mV, but increased nonlinearly at more positive membrane potentials. The leakage current was unaffected (Fig. 2 *B*) or only slightly changed by 4-AP. In six cells, the leakage current was unaffected (two cells), slightly increased (two cells), and slightly decreased (two cells) at positive membrane potentials. Subtraction of the leakage current from the total outward current in Ca^{2+}-free ASW leaves the delayed outward K^+ component, which was inhibited strongly by 5mM 4-AP at all potentials (Fig. 2 *C*).

The fast outward K^+ current or *A* current (Connor and Stevens, 1971; Neher, 1971) is seen in cell R-15 when depolarizing steps to positive potentials are preceded by conditioning hyperpolarizations (Faber and Klee, 1972; Gola, 1976). In normal ASW, the fast outward K^+ current of cell R-15 is fully inactivated at a holding potential of about −50 mV, whereas the delayed outward K^+ current is fully available for activation at this potential (Gola, 1976). Holding potentials between −50 and −45 mV, therefore, should provide a convenient working range for studying the delayed K^+ current in the absence of the fast K^+ current, without the inactivation that occurs at more positive holding potentials. It is important, however, to investigate whether the fast outward current is also fully inactivated at holding potentials of −50 to −45 mV in Ca^{2+}-free ASW. This was investigated in two experiments (performed in collaboration with Dr. D. Tillotson) in Na^+- and Ca^{2+}-free

ASW. The fast outward K^+ current was activated by depolarizing pulses to −30 mV from conditioning steps (300-ms) between −140 and −30 mV. A conditioning hyperpolarization to −100 mV for 300 ms was long enough to remove ~95% of the inactivation of the fast K^+ current present at a holding potential of −45 mV. The normalized peak fast outward K^+ current (the ratio of the peak K^+ current at any conditioning hyperpolarization to the maximum peak K^+ current) was plotted vs. the conditioning hyperpolarizing potential. The results were similar to data obtained by Connor and Stevens (1971) in *Anisodoris* neurons in Na^+- and Ca^{2+}-free ASW. At −45 mV, <1% and at −50

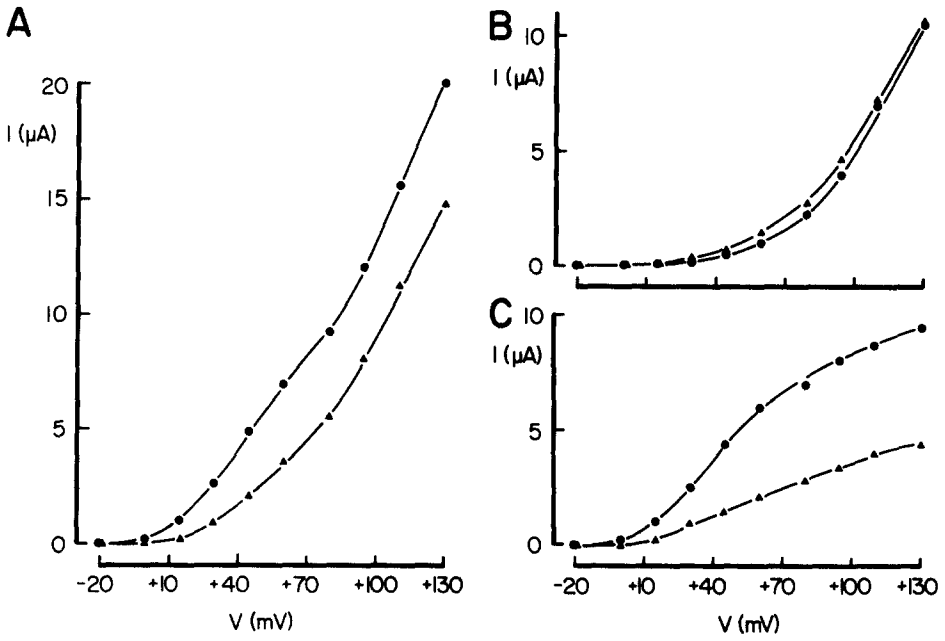

FIGURE 2. Effect of external 4-AP on R-15 membrane outward current in Ca^{2+}-free ASW containing 5×10^{-5} M TTX. (*A*) Current-voltage relation for outward current measured at the end of 75-ms voltage-clamp pulses from −48 mV to the indicated potentials before (closed circles) and after 5 mM 4-AP (closed triangles). (*B*) Current-voltage relation for the leakage current. (*C*) Current-voltage relation for the delayed outward K^+ current for the same neuron. Measurements were made 20 min after the addition of 4-AP.

mV <2% of the fast outward K^+ current was available for activation. With a similar conditioning protocol the cell was also tested with depolarizing pulses to 0, +20, and +70 mV to activate the delayed outward K^+ current. Changes in the rising phase of the current wave form were measurable only for conditioning hyperpolarizations more negative than −55 mV. This is as expected if the fast outward K^+ current is almost fully inactivated at holding potentials of −45 to −50 mV and indicates that the effects shown in Figs. 1 and 2 represent a direct action of 4-AP on the delayed outward K^+ current.

The effects of conditioning hyperpolarization and depolarizing steps between −120 and +30 mV on the delayed outward K^+ current were also studied in Ca^{2+}-free ASW. About 18–22% of the delayed outward K^+ current was inactivated at holding potentials of −50 and −45 mV, respectively. The steady-state inactivation curve (see Hodgkin and Huxley [1952 *b*]) was not altered appreciably by the addition of 5 mM 4-AP to the Ca^{2+}-free ASW.

Voltage Dependence of 4-AP Inhibition

The 4-AP inhibitory effect on the delayed outward K^+ current depends upon voltage as well as upon concentration. The potential dependence of the 4-AP inhibition can be seen in plots of the ratio of the delayed K^+ current in the presence of 4-AP at a given concentration to the K^+ current under control conditions. There was a progressive recovery of the delayed K^+ current during depolarizing steps to positive membrane potentials at each concentration (Fig. 3 *A*). This recovery during depolarization also occurred when 4-AP was injected intracellularly (Fig. 3 *B*). The potential dependence can also be seen in dose-response plots of the current ratio ($I_{K,4\text{-AP}}/I_K$) at different membrane

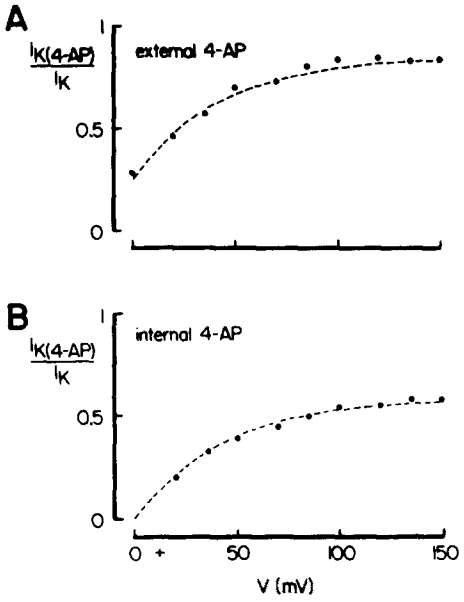

FIGURE 3. Effects of 4-AP on the K^+ current ratio of cell R-15. (*A*) Effects of membrane potential on the ratio of the delayed K^+ current in the presence of 2 mM external 4-AP to control K^+ current, $I_{K,4\text{-AP}}/I_K$ (*B*) Effects of membrane potential on the ratio of the delayed K^+ current in the presence of internal 4-AP to the control K^+ current from a different cell. The 4-AP was injected intracellularly for 1 min at 250 nA. The dashed curves fitted to the experimental points in *B* were calculated from the empirical relation $I_{K,4\text{-AP}}/I_K = 1 - \exp(-V/b)$, where b is a constant. For the cell shown in *A*, b = 40 mV, and for the cell shown in *B*, b = 45 mV.

potentials vs. the logarithm of the external 4-AP concentration (Fig. 4 *A*). If one 4-AP molecule binds with a single receptor site, then the logarithm of the experimental values for the current ratio (expressed as $I_K - I_{K,4\text{-AP}}/I_{K,4\text{-AP}}$) at any potential should fall along a straight line with a slope of 1 when plotted

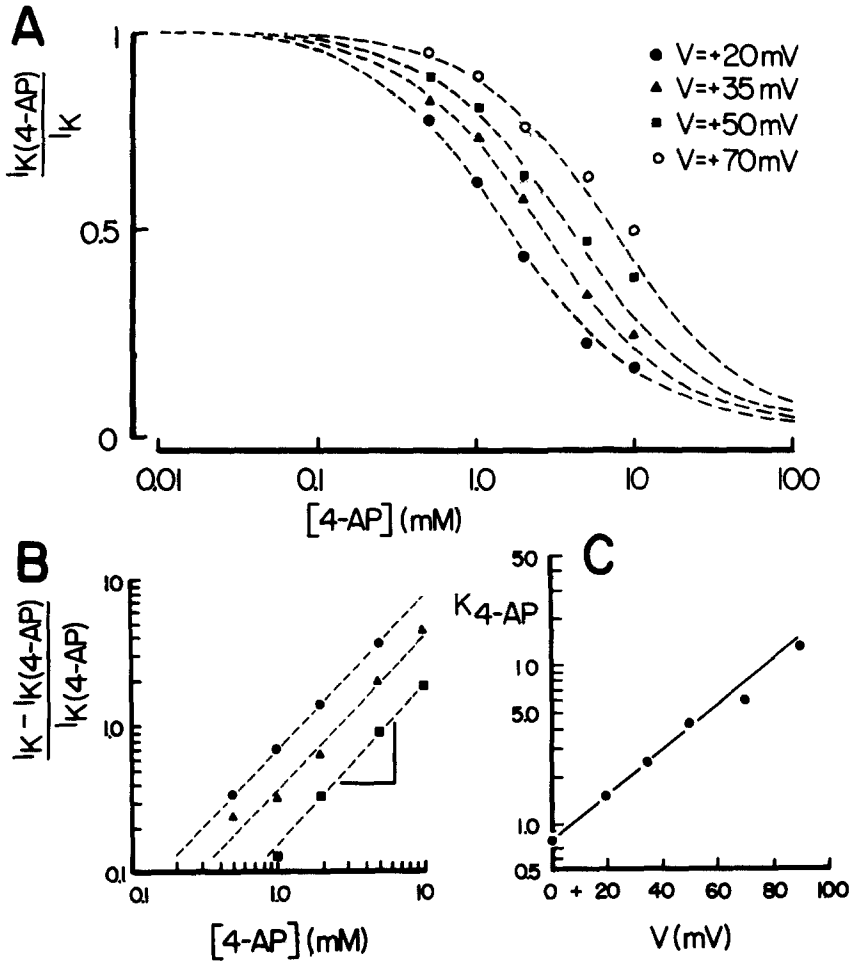

FIGURE 4. Effects of 4-AP concentration and membrane potential on R-15 delayed K^+ current. (*A*) Dose-response plot of the current ratio $I_{K,4\text{-AP}}/I_K$ at four different test pulse potentials vs. the logarithm of the external 4-AP concentration. The theoretical lines drawn through the experimental points were calculated from Eq. 1. (*B*) Double logarithmic plot of the current ratio $(I_K - I_{K,4\text{-AP}})/I_{K,4\text{-AP}}$ for the data in *A* vs. the external 4-AP concentration. The parallel straight lines through the experimental points have a slope of ~1. (C) Semilogarithmic plot of the apparent dissociation constant ($K_{4\text{-AP}}$) vs. membrane potential. The data shown in *A–C* are from the same cell and were obtained in Ca^{2+}-free ASW containing 5×10^{-5} M TTX.

vs. the logarithm of the drug concentration. The experimental data in Fig. 4 *A* are replotted on a double logarithmic scale in Fig. 4 *B* and show that this expectation is reasonably well met over a wide range of membrane potentials.

The reaction of 4-AP with a receptor site, *R*, can be represented by

$$\text{4-AP} + \text{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{4-AP}\cdot\text{R},$$

where k_1 and k_{-1} are the forward and reverse rate constants and $K_{4\text{-AP}} = k_1/k_{-1}$ is the dissociation constant of the reaction. The experimental points in Fig. 4 *A* were fitted with theoretical curves with the equation

$$\frac{I_{K,4\text{-AP}(V)}}{I_{K(V)}} = \frac{K_{4\text{-AP}(V)}}{K_{4\text{-AP}(V)} + [4\text{-AP}]_o}, \tag{1}$$

where $I_{K(V)}$ is the peak delayed K^+ current in Ca^{2+}-free ASW at a given potential V, $I_{K,4\text{-AP}(V)}$ is the peak K^+ current at the same potential in Ca^{2+}-free ASW containing 4-AP, $[4\text{-AP}]_o$ is the external 4-AP concentration, and $K_{4\text{-AP}(V)}$ is the apparent disassociation constant at V. The experimental points in Fig. 4 *A* can be fitted with a series of parallel curves, each with a different dissociation constant. The dissociation constant increases at positive membrane potentials and is plotted in Fig. 4 *C* as log $K_{4\text{-AP}}$ vs. membrane potential. The points fall along a straight line over a wide range of membrane potentials. The voltage dependence of the apparent dissociation constant can be described by

$$K_{4\text{-AP}(V)} = K_{4\text{-AP}(0)} \exp\,(z\mu FV/RT), \tag{2}$$

where $K_{4\text{-AP}(0)}$ is the dissociation constant at zero membrane potential, z is the charge of the 4-AP molecule, which is positive at the pH of the ASW ($z = +1$), μ is a value between 0 and 1 and is the fraction of the potential difference V across the membrane experienced at the blocking site, and $RT/F = 25$ mV. For the cell shown in Fig. 4 C, $K_{4\text{-AP}}$ increased e-fold for a 32-mV change in potential. For the straight line drawn through the points in Fig. 4 *C*, $K_{4\text{-AP}(0)} = 800$ μM and $\mu = 0.78$. Similar values were determined in an experiment on a different cell ($K_{4\text{-AP}(0)} = 850$ μM and $\mu = 0.80$).

During a clamping pulse, a current I will flow across the membrane resistance r_m and across the resistance in series with the membrane r_s (Hodgkin et al., 1952; Taylor et al., 1960); therefore, the voltage clamp pulse V_c will represent not only the voltage drop across the membrane ($V_m = Ir_m$) but also the voltage drop across the series resistance ($V_s = Ir_s$) or $V_c = V_m + V_s$. The error introduced into the current-voltage relation by r_s can be significant when large K^+ currents (>0.5 μA) flow across the membrane. The voltage dependence of the apparent dissociation constant $K_{4\text{-AP}}$ was altered but not abolished when the current ratio ($I_{K,4\text{-AP}}/I_K$) was corrected for the estimated series resistance error, using the relation $V_m = V_c - V_s$. For example, with the data shown in Fig. 4 and an average value of r_s of 1.5 kΩ, $K_{4\text{-AP}}$ increased e-fold for a 29-mV change in potential.

Effects of 4-AP on the Kinectics of the Delayed K^+ Current

The delayed outward K^+ current in Ca^{2+}-free ASW containing TTX rises to a peak ~30–50 ms after the onset of a depolarizing clamp step and thereafter declines slowly during maintained depolarization (see Fig. 1). The addition of 5 mM 4-AP to Ca^{2+}-free ASW slowed the rising phase and the decay of the current wave form at all potentials. These results suggest that 4-AP may affect both the activation and inactivation of the K^+ channel, but there is an alternative explanation. In squid axon (Yeh et al, 1976 *a* and 1976 *b*; Meves and Pichon, 1977) and in frog myelinated axon (Ulbricht and Wagner, 1976), the removal of the 4-AP block is frequency as well as voltage dependent. The frequency dependence has been satisfactorily explained by the hypothesis that 4-AP molecules are displaced from blocking sites during repetitive depolarizing stimuli with a single time constant and rebound afterward, with a longer time constant. In frog myelinated axon, full removal of the 4-AP block takes ~1 s (Ulbright and Wagner, 1976). It is possible that the difference in kinetics in Ca^{2+}-free ASW, with and without 4-AP, reflects the time-course of the removal of the 4-AP block during depolarization. This could account for the decrease in the rising phase of the K^+ current and its slower decay in 4-AP.

Much of our data was obtained at relatively high concentrations of external 4-AP (~5 mM), because the primary purpose of our experiments was to compare its effect on the delayed K^+ current to its effect on the Ca^{2+}-activated K^+ current (see below). High concentrations, however, tend to suppress the frequency-dependent relief of the 4-AP block (Meves and Pichon, 1977). Fig. 5 shows the effect of a train of repetitive (1-Hz) depolarizing pulses to +20 mV from a holding potential of −45 mV on the peak delayed K^+ current in Ca^{2+}-free ASW and in the presence of 5 mM 4-AP. In cell R-15, the delayed K^+ current undergoes a time- and frequency-dependent inactivation during a repetitive train of pulses, and in Ca^{2+}-free ASW the peak outward current was reduced by 45% at the end of 20 s of repetitive stimulation. This is a characteristic feature of outward K^+ currents in many molluscan neurons (Gola, 1974; Lux and Eckert, 1974; Heyer and Lux, 1976 *b*; Aldrich et al., 1979). The addition of 4-AP reduced the peak outward K^+ current measured with the first depolarizing clamp pulse in a repetitive train of pulses to +20 mV by ~70% and also eliminated much of the subsequent frequency-dependent inactivation. This result is as expected if the frequency-dependent inactivation is matched by a frequency-dependent relief of the 4-AP block repetitive stimulation, but it is difficult to compare directly the outward currents measured with identical voltage-clamp pulses under the two conditions because of the marked difference in their amplitudes. A different type of comparison can be made by increasing the size of the depolarizing pulse in 4-AP to +50 mV, so that the outward K^+ current measured at the beginning of the train is similar in magnitude to the current measured in Ca^{2+}-free ASW. Fig. 5 shows that the peak outward K^+ current in 4-AP was reduced by only 15% (to +50 mV) at the end of a 20-s train of repetitive depolarizing clamp pulses. The results are consistent with a frequency-dependent relief of the 4-

AP block but cannot be used to exclude the possibility that 4-AP inhibits directly the frequency-dependent inactivation of the delayed K^+ channel.

Effect of 4-AP on the Ca^{2+}-activated K^+ Current

Injection of Ca^{2+} ions into molluscan neurons activates an outward current carried by K^+ ions (Meech, 1974) whose amplitude depends on the magnitude of charge passed through the electrode and the holding potential (Gorman and Hermann, 1979). Ca^{2+} was injected electrophoretically (200 nA for 10 s) into the cytoplasm at various membrane potentials between −80 and −30

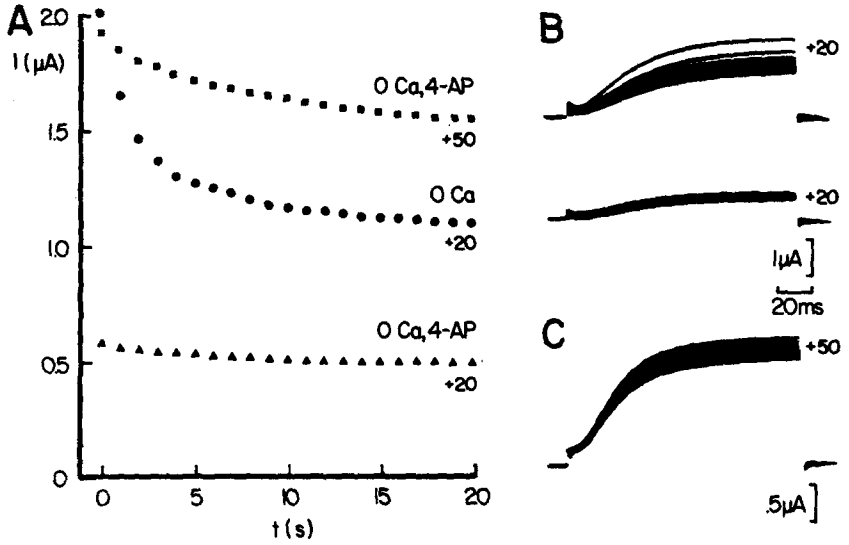

FIGURE 5. Effects of external 4-AP on R-15 delayed outward K^+ current response to repetitive stimulation. (A) Plot of K^+ currents in Ca^{2+}-free ASW containing 5×10^{-5} M TTX before and after 20 min in 5 mM 4-AP measured with repetitive (1-Hz) 75-ms pulses to the indicated membrane potentials. (*B*) Superimposed oscilloscope trace of K^+ currents measured with repetitive (1-Hz) 75-ms pulses to +20 mV before (*top*) and in 5 mM external 4-AP (*bottom*). (*C*) Superimposed K^+ currents measured with repetitive (1-Hz) 75-ms pulses to +50 mV in 4-AP.

mV. The difference between the peak K^+ current and the holding current at each potential in Ca^{2+}-free ASW is plotted in Fig. 6 *A*. At low external 4-AP concentrations (<2 mM) the Ca^{2+}-activated K^+ current was unaffected, but at higher concentrations (~5 mM) it was increased. The increase in the Ca^{2+}-activated K^+ current was proportionately greater when the current was outward but was also apparent when the current was inward. The reversal potential (about −72 mV), however, was unchanged by the addition of 4-AP. The increase might be explained if 4-AP reduced the cell's ability to buffer Ca^{2+} intracellularly. In two experiments (carried out in collaboration with

Dr. D. Tillotson), Ca^{2+} ions were injected into cells containing the Ca^{2+}-sensitive dye arsenazo III, and the differential change in dye absorbance was used to monitor the increase in free intracellular Ca^{2+} during injection. The change in internal Ca^{2+} was not greater in the presence of 5 mM external 4-AP.

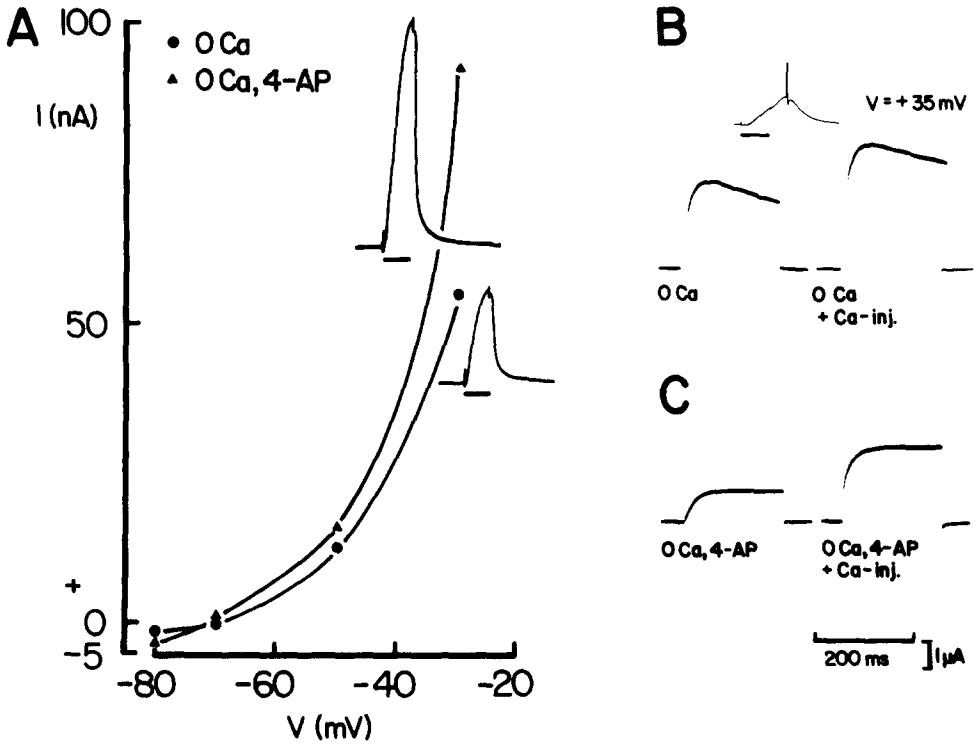

FIGURE 6. Effects of external 4-AP on R-15 Ca^{2+}-activated K^+ currents (*A*) Plot of K^+ current vs. membrane holding potential before and in 5 mM external 4-AP. The current was measured as the difference between the holding current at each potential and the peak current activated by an intracellular injection of Ca^{2+} for 10 s at an intensity of 200 nA. Current responses recorded at −30 mV in Ca^{2+}-free ASW (*bottom*) and in Ca^{2+}-free ASW with 5 mM 4-AP (*top*) are also shown. The injection period is indicated below each record. (*B*) Outward currents measured from a different cell before and at the end of a 10-s, 400-nA intracellular Ca^{2+} injection. (*C*) Outward currents measured from the same cell in 5 mM 4-AP before and at the end of a 10-s, 400-nA Ca^{2+} injection. The pulse amplitude was +35 mV and the holding potential −50 mV in *B* and *C*. All currents were measured in Ca^{2+}-free ASW containing 5×10^{-5} M TTX.

A different type of experiment was used to determine the effect of 4-AP on the Ca^{2+}-activated K^+ current at potentials more positive than −30 mV. It has been shown (Gorman and Thomas, 1980) that the outward currents measured with depolarizing clamp pulses in Ca^{2+}-free ASW are increased

during internal Ca^{2+} injection. Fig. 6 *B* and *C* shows the results from an experiment in which the membrane was briefly depolarized to +35 mV in Ca^{2+}-free ASW at the end of a 10-s intracellular Ca^{2+} injection. The outward current was increased by ~1.4 μA during Ca^{2+} injection (Fig. 6 *B*). The same protocol was repeated in 5 mM external 4-AP, which altered the time-course and reduced the outward current but increased the outward current to ~1.6 μA during Ca^{2+} injection (Fig. 6 *C*).

Time-Course of the 4-AP Block

The aminopyridines are small, lipid-soluble molecules (Christen et al., 1975) and thus might be expected to diffuse rapidly to and into the neuronal membrane when added to the bathing media. The onset of block by 5 mM external 4-AP was studied with 75-ms test pulses to +20 mV applied once per minute. The time-course of the inhibitory action of the drug on the delayed outward K^+ current in Ca^{2+}-free ASW measured at the end of the test pulse was slow (Fig. 7). A steady-state blockade of the current occurred (~77% of the current without 4-AP) after ~15–20 min. In five cells, the average time necessary to produce a 50% block was 1.25 ± 0.17 min (SEM). Reversibility, particularly after prolonged treatment, was poor. The time needed to achieve

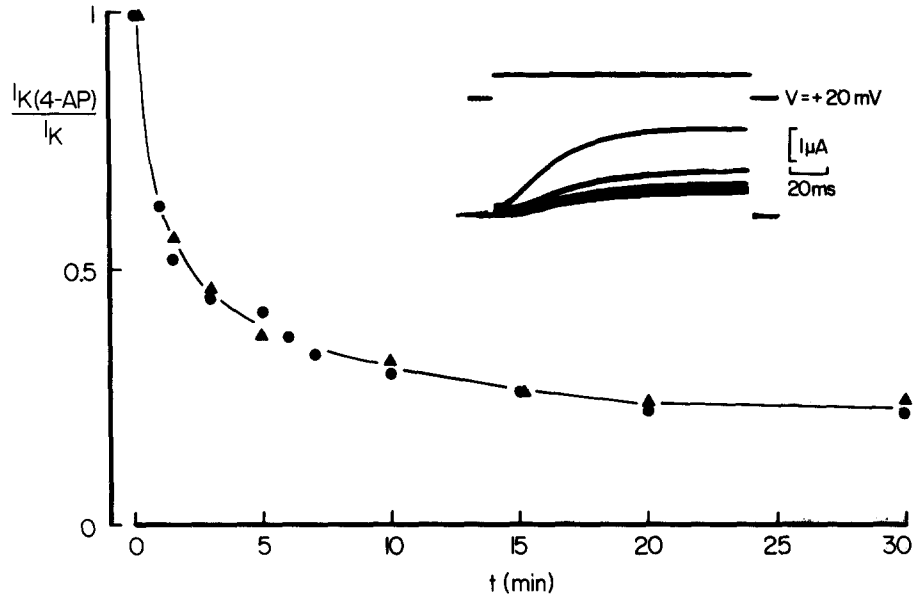

FIGURE 7. Time-course of onset of external 4-AP inhibitory effect on delayed K^+ current. Plot of K^+ current ratio measured from two cells with +20-mV pulses from a holding potential of −45 mV before and at the indicated times after the addition of 5 mM 4-AP to Ca^{2+}-free ASW containing 5×10^{-5} M TTX. The *inset* shows representative oscilloscope current traces from one of these cells recorded at different times in 4-AP. Data from cells R-15 and L-6.

a full block is only slightly longer than the time (~13 min) required for external 4-AP to have the same effect in myelinated axon (Ulbricht and Wagner, 1976).

Effects of Internal 4-AP on the K^+ Current

There is evidence from muscle that intracellular 4-AP blocks the K^+ current (Fink and Wettwer, 1978) and that the effects of extracellular 4-AP occur after passage through the membrane (Gillespie and Hutter, 1975). This might explain the slow time-course of the effects of external 4-AP. The intracellular effect of 4-AP on the delayed outward K^+ current and on the Ca^{2+}-activated K^+ current was investigated. Fig. 8 *A* shows a plot of the delayed K^+ current

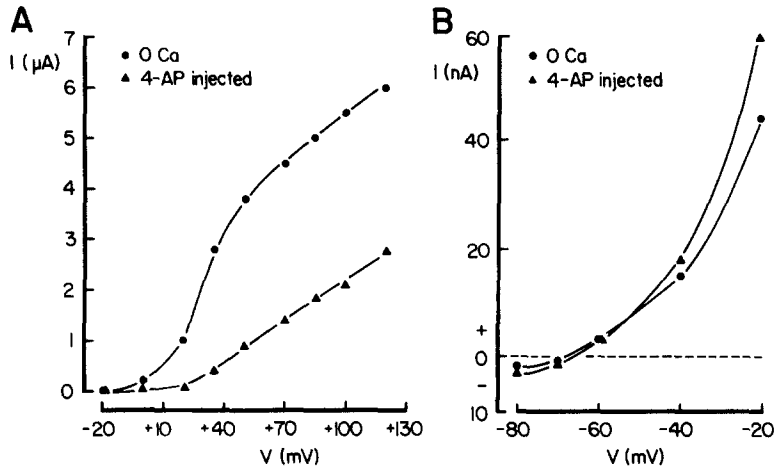

FIGURE 8. Effects of internal 4-AP on R-15 delayed and Ca^{2+}-activated K^+ currents. (*A*). Plot of delayed K^+ current measured at 50 ms before and after intracellular injection of 4-AP vs. membrane potential. The experimental points in 4-AP were measured immediately after a drug injection of 3 min at 250 nA. The leakage current was subtracted from all records (*B*) plot of Ca^{2+}-activated K^+ current vs. membrane holding potential before and after a 3-min, 300-nA intracellular injection of 4-AP. The current was measured as described in Fig. 6*A*. All currents were measured in Ca^{2+}-free ASW containing 5×10^{-5} M TTX.

measured with 200-ms pulses of variable amplitude in Ca^{2+}-free ASW and after electrophoretic injection of 4-AP (250 nA for 3 min). A similar reduction of the K^+ current occurred when 4-AP was pressure injected internally. The K^+ current was decreased at all potentials by internal 4-AP. In most respects the effects of internal and external 4-AP were similar. The magnitude of the block of the current depended on the length of time 4-AP was injected and, thus, presumably on the intracellullar 4-AP concentration. The voltage dependence of the block by internal 4-AP can be seen in the progressive recovery of the delayed K^+ current during depolarizing steps to positive membrane potentials and is shown in Fig. 3 *B*, where the ratio of the current in the

presence of internal 4-AP to the K^+ current under control conditions is plotted vs. membrane potential. The one major difference between external and internal application of the drug is that a steady-state block was established immediately after the electrophoretic or pressure injection of 4-AP. This finding suggests that the receptor site for 4-AP may be more accessible from the inside surface of the membrane. Fig. 8 *B* shows a plot of the K^+ current activated by intracellular Ca^{2+} injection (200 nA for 10 s) at different holding potentials in Ca^{2+}-free ASW and after injection of 4-AP in the same cell. The Ca^{2+}-activated K^+ current was increased by internal 4-AP without any effect on the K^+ current reversal potential. The results are similar to those shown above (Fig. 6 *A*), where a high external concentration of 4-AP was used. The effects of intracellular injection of 4-AP were not reversible, at least not up to 2 h after injection (the longest period studied).

Effects of 4-AP on the Current-Voltage Relation in Normal ASW

The membrane current measured at the end of a (50-ms or greater) depolarizing clamp step to potentials more positive than about −20 mV in *normal* ASW is carried predominantly by K^+ ions (Heyer and Lux, 1976*a*). The current-voltage relation is N-shaped at positive membrane potentials (Fig. 9). The reduction of the outward current between about +80 and +120 mV has been related to the reduction of the Ca^{2+}-activated component of the total outward K^+ current as the membrane potential approaches the Ca^{2+} equilibrium potential (Meech and Standen, 1975; Heyer and Lux, 1976 *a* and 1976 *b*). The net inward flux of Ca^{2+} ions and, therefore, the Ca^{2+}-activated K^+ current is reduced in this region, whereas the delayed K^+ and the leakage currents continue to increase (see Fig. 2). The effects of 4-AP on the current-voltage relation in normal ASW are consistent with its action on the individual components of the K^+ current. Fig. 9 shows that the outward current in external 4-AP (5 mM) was increased between about −20 and +80 mV, where the Ca^{2+}-activated K^+ current predominates. The outward current, however, was reduced at membrane potentials positive to about +80 mV, where the delayed K^+ and leakage currents predominate. The effect of external 4-AP is quite different from the effect of external tetraethylammonium, which blocks both the Ca^{2+}-activated and the delayed K^+ currents and, therefore, reduces the outward current at all potentials (Klee, 1978; Hermann and Gorman, 1979 *b* and 1981). The current measured by depolarizing voltage-clamp pulses to +20 mV contains a small, early inward current component that is increased by the addition of 4-AP (see *inset* records in Fig. 9). It is possible that 4-AP affects the inward current directly, but an equally likely explanation is that, by slowing the onset and reducing the amplitude of the outward K^+ current (see Fig. 1), 4-AP causes an increase in an underlying inward current (Hermann and Gorman, 1979 *b*).

Effects of 4-AP on the Action Potential and on Pacemaker Activity

In cockroach (Pelhate et al., 1972) and squid axon (Llinás et al. 1976; Yeh et al., 1976 *a*) the aminopyridines cause a small depolarization of membrane

potential and spontaneous discharge but prolong only slightly the duration of the action potential (Schauf et al., 1976; Yet et al., 1976 *b*). Their effect on spontaneous pacemaker discharge in cell R-15 was somewhat more complex and depended on the external drug concentration (Fig. 10 *A*). At low external concentrations (>1 mM), 4-AP reduced the number of action potentials per burst. Higher concentrations caused an irregular bursting pattern or a tran-

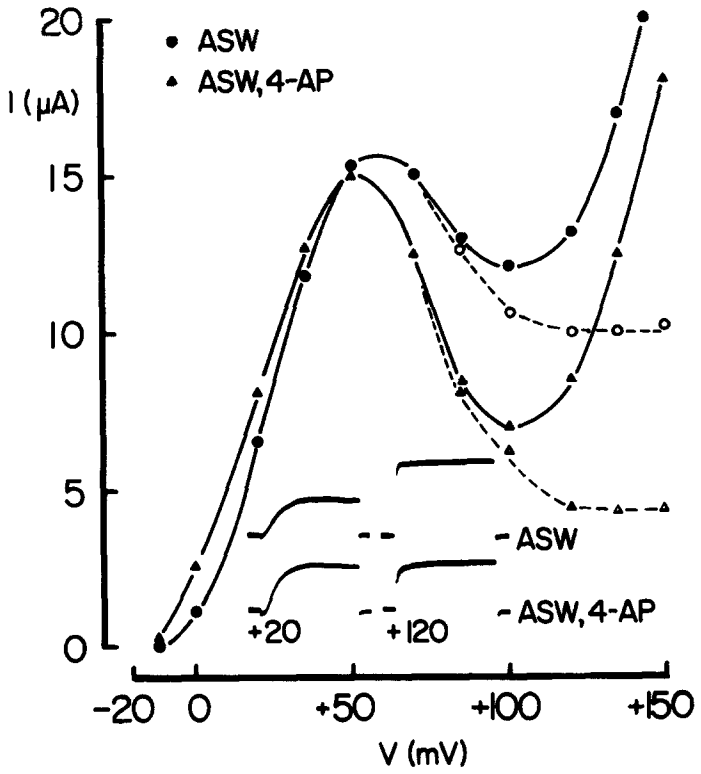

FIGURE 9. Effects of external 4-AP on the R-15 outward current in normal ASW. Plot of the outward current measured at the end of 200-ms pulses before and in 5 mM external 4-AP vs. membrane potential. The holding potential was −50 mV. The open symbols show the current corrected for leakage currents, and the closed symbols show the uncorrected currents. The *inset* shows representative responses to 200-ms clamp pulses to the indicated membrane potential before and in 4-AP.

sition to continuous repetitive discharge. In concentrations of 10 mM or greater, spontaneous discharge of action potentials stopped, and the membrane potential was stable at about −55 mV.

The duration of the R-15 action potential was prolonged by external 4-AP (~2.2-fold) without a significant change in its overshoot, but the afterhyperpolarization was increased (Fig. 10 *B*). These changes are consistent with the

finding that the delayed K^+ current is reduced, whereas the Ca^{2+}-activated K^+ current is increased by 4-AP. For comparison, external 4-AP was tested on a neuron in the same ganglion (cell L-11), in which the contribution of the Ca^{2+}-activated K^+ current to the total outward K^+ current is much smaller than in cell R-15 (Hermann, 1978). External 4-AP at the same concentration also prolonged the duration of the L-11 action potential but reduced the afterhyperpolarization (Fig. 10 *B*).

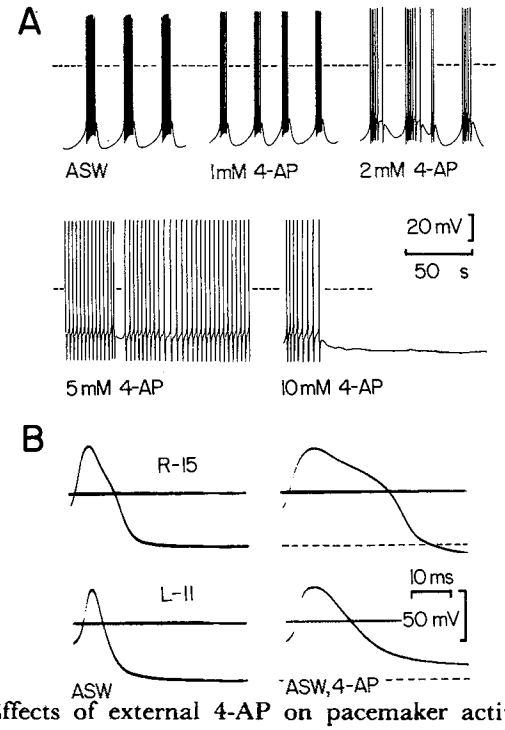

FIGURE 10. Effects of external 4-AP on pacemaker activity and on action potentials. (*A*) Representative records of bursting pacemaker activity from cell R-15 in different external 4-AP concentrations in normal ASW. The broken line indicates 0 mV. (*B*) Superimposed spontaneous action potentials from cells R-15 and L-11 before and in 5 mM external 4-AP in normal ASW. The membrane potential was hyperpolarized to reduce rhythmic discharge in both cases. Each record shows four action potentials. *A* and *B* show data from different cells.

DISCUSSION

Our results show that 4-AP blocks the delayed K^+ current but is either without effect at low external concentrations or, at high concentrations, increases the Ca^{2+}-activated K^+ current of the molluscan neuron soma membrane. They confirm Thompson's (1977) conclusion that the aminopyridines can be

used to pharmacologically separate the voltage-dependent K^+ currents from the Ca^{2+}-activated K^+ current.

The blockade of the delayed K^+ current in axon (Meves and Pichon, 1977; Ulbricht and Wagner, 1976; Yeh et al., 1976*a* and 1976*b*) and muscle (Gillespie, 1977; Molgo, 1978) membrane also depends on voltage, and there is some indication that the block of the fast K^+ current in molluscan neurons is influenced by changes in membrane potential (Thompson, 1977). Our findings suggest that voltage affects a binding site within the membrane. A similar conclusion has been reached for myelinated axon (Ulbricht and Wagner, 1976) and can be inferred from the voltage dependency of the block in squid axon (Meves and Pichon, 1977). Our findings suggest also that the binding site is more accessible from inside the cell, but data are not detailed enough to rule out the possibility that external and internal 4-AP affect different receptor sites (see Kirsch and Narahashi [1978]). Some authors (Gillespie and Hutter, 1975; Thompson, 1977) have suggested that 4-AP has a direct effect on the gating mechanism of K^+ channels. Although this possibility cannot be excluded completely, the time- and frequency-dependent relief of the 4-AP block (Meves and Pichon, 1977; Ulbricht and Wagner, 1976; Yeh et al., 1976 *a* and 1976 *b*) makes it very difficult to draw any firm conclusions about changes in the gating mechanism from the kinetics of membrane currents in the presence of 4-AP.

An alternate explanation is that 4-AP occludes a portion of the delayed K^+ channel, thereby preventing the normal movement of K^+ ions when the channel gate is in the open configuration. The K^+ channel is thought to be a pore with a "selectivity filter" measuring ~3.0–3.3 Å in diameter that allows the passage of small cations (up to 3.0 Å in diameter) but precludes the movement of larger cations (Armstrong, 1975; Hille, 1975). Large, positively charged molecules, such as tetraethylammonium and other quaternary ammonium ions (diameter, ~8.0 Å), are estimated to penetrate ~20% of the way into a wider region of the channel near the inner membrane surface, whereas smaller, positively charged molecules, e.g., methylammonium (diameter, ~3.3 Å), are driven ~70% of the way through the channel when the inside of the cell is made sufficiently positive relative to the outside (Hille, 1975). These estimates suggest that the selectivity filter is closer to the outside than to the inner membrane surface. The 4-AP molecule is a nonplanar, oblong structure composed of a pyridine ring with an attached amine group (NH_2), which, on the basis of the microwave spectra measurements given by Christen et al. (1975), measures ~4.6 × 6.4 Å. At the normal intracellular pH of ~7.2, 4-AP is expected to be almost totally in the cationic form (Albert, 1963). It has been pointed out (Ulbricht and Wagner, 1976) that it is most unlikely for a positively charged molecule the size of 4-AP to be driven through an opening 3.0–3.3 Å in diameter during membrane depolarization. Moreover, the block and its relief during depolarization are independent of the direction and amplitude of the K^+ current through the channel (Ulbricht and Wagner, 1976; Meves and Pichon, 1977), which excludes the possibility that 4-AP is "flushed out" of occupied channels by K^+ ion movement.

The blocking effect of 4-AP on the delayed K^+ conductance and the relief of this block during depolarization might be explained if the pyridine ring inserts itself into the lipid phase of the membrane on the *external* side of the selectivity filter such that the amine group partially or fully occludes the channel. This suggestion is consistent with our finding that the block by 4-AP occurs ~0.8 of the way through the membrane electric field, which is 10% further than the beginning of the selectivity filter estimated from the fraction of the field through which methylammonium must be moved (0.7) to block the channel (Hille, 1975). Ulbricht and Wagner (1976) estimated that the 4-AP block was 0.77 of the distance through the electric field across the nodal membrane of myelinated axons, which agrees well with our results. We assume that the pyridine ring of the 4-AP molecule binds to a hydrophobic moiety associated with the blocking site. This would be analogous to the hydrophobic binding of the benzene ring of tetraethylammonium ion derivatives near the internal mouth of the K^+ channel in Armstrong's model (1975) model for the block of K^+ channels by internal quaternary ammonium ions. The delayed K^+ channel is permeable to ammonium ions (NH_4), which are presumed to make hydrogen bonds with oxygen groups lining the channel wall, so that the molecule can move through the selectivity filter (see Hille [1975]). The 4-AP amine group is also capable of making hydrogen bonds that could hold the nitrogen atom in a blocking position on the external side of the filter. The bond strength for a hydrogen bond between hydrogen and oxygen is no more than 2–4 kcal/mol. At 0 mV, the dissociation constant (K_D) for the reaction of 4-AP with the binding site was estimated to be 0.8 mM. Since the free energy $\Delta G^\circ = RT \ln K_D$, ΔG° is ~4 kcal/mol, which suggests that sufficient energy is available to rupture hydrogen bonds. The time constant for the removal of 4-AP block during repetitive depolarization is ~10 ms in squid axon (Yeh et al., 1976 *a*; Meves and Pichon, 1977) and much slower (200 ms) in myelinated axon. The positively charged 4-AP molecule could act like a slow activation gate that swings slowly out of its blocking position during depolarization, or it could be driven off the site into a region of the channel adjacent to the external membrane that can accomodate large molecules, such as tetraethylammonium or other quaternary ammonium ions (Hermann and Gorman, 1981). The model is speculative and is likely to remain so until there is clear evidence that 4-AP does not interfere with channel gating and that the block occurs within the K^+ channel, but the model does take into account the charge of the 4-AP molecule, the relief of the block during membrane depolarization, and the location of the block within the membrane.

The increase in the Ca^{2+}-activated K^+ current in high concentrations was unexpected. We can exclude the possibility that 4-AP decreases the affinity of intracellular sequestering systems for Ca^{2+}, thereby causing a larger increase in *free* intracellular Ca^{2+} per injected load and, thus, an increase in the magnitude of the K^+ current. We can also exclude the possibility that 4-AP produces changes in cell volume causing the internal Ca^{2+}-injection electrode to move closer to the inner membrane surface, thereby increasing the amount of free Ca^{2+} ions available at the membrane to activate the K^+ current (see

Gorman and Hermann [1979]), because a similar increase in the Ca^{2+}-activated K^+ current occurs under conditions where the K^+ current is activated by Ca^{2+} influx rather than by Ca^{2+} injection. It is possible that 4-AP affects directly the activation of the K^+ conductance by Ca^{2+} ions. It has been shown (Gorman and Thomas, 1980) that the Ca^{2+}-activated K^+ conductance increases exponentially with depolarization. This potential dependence has been explained satisfactorily by a model in which a single Ca^{2+} ion binds to a negatively charged site that is about halfway through the electrical field across the membrane and that gates the K^+ channel (Gorman and Thomas, 1980). If 4-AP enters into the K^+ channel then it might alter the position of the binding site in the electrical field or alter its affinity for Ca^{2+} ions, but these and other possibilities need to be examined experimentally.

In a previous study of the effects of 4-AP on the outward K^+ currents in molluscan neurons (Thompson, 1977), the delayed outward K^+ current and the Ca^{2+}-activated K^+ currents were studied together in *normal* ASW rather than separately in Ca^{2+}-free ASW. Most of our findings, therefore, are not directly comparable. The increase of the *total* outward K^+ current in normal ASW containing 4-AP shown in Fig. 9, however, differs from the previous result in *Tritonia* neurons (Thompson, 1977), where a high concentration of 4-AP had no effect on the total outward K^+ current studied under similar conditions. The two results are not necessarily in conflict. Our findings show that high concentrations of 4-AP have opposite effects on the Ca^{2+}-activated and voltage-dependent K^+ currents. In normal ASW, both the Ca^{2+}-activated and the voltage-dependent components of the K^+ current (as well as inward currents) contribute to the total outward current of the molluscan neuron membrane, but the ratio of the Ca^{2+}-activated to the voltage-dependent (delayed outward) K^+ current depends upon cell type as well as upon membrane potential (Meech and Standen, 1975; Heyer and Lux 1976 *a* and 1976 *b*; Hermann, 1978; Aldrich et al., 1979). In cell R-15, a significant fraction of the total outward current is carried by the Ca^{2+}-activated K^+ current; e.g., at +20 mV, ~85% of the current is carried by the Ca^{2+}-activated K^+ current and ~15% by the delayed K^+ current (Hermann, 1978). It is not surprising that there was a net increase in the total outward current of cell R-15, since the increase in the Ca^{2+}-activated K^+ current caused by a high external concentration of 4-AP would be expected to mask any decrease in the delayed K^+ current. The proportion of the total outward current carried by the Ca^{2+}-activated K^+ current appears to be about equal to that carried by the delayed K^+ current in the *Tritonia* neurons studied by Thompson (1977). We do not rule out the possibility that neurons differ in their response to 4-AP, but an alternative explanation for the lack of effect of 4-AP on the total outward current of *Tritonia* neurons is that the decrease in the delayed K^+ current was masked by a proportional increase in the Ca^{2+}-activated K^+ current.

The aminopyridines increase synaptic activity (Schauf et al., 1976; Galindo and Rudomin, 1978; Jacobs and Burley, 1978), enhance muscular contraction (Harvey and Marshall, 1977) and at high concentrations act as a convulsant

in vertebrates and invertebrates (LeMeignan et al., 1969; Pelhate et al., 1972). The effects of 4-AP on the different components of the K^+ current in molluscan neurons may provide an explanation for this apparent diversity of action. In axons or cells in which the Ca^{2+}-activated K^+ current is absent, e.g., squid axon (Begenisich and Lynch, 1974), or weak, e.g., *Aplysia* cell L-11 (see Fig. 10 *B*) and F cells (Klee, 1978), the aminopyridine prolongs slightly the duration of the action potential and abolish the afterhyperpolarization (Yeh et al., 1976 *a*; Llinás et al., 1976; Kirsch and Narahashi, 1978; Klee, 1978) In these cells the resting membrane potential is also depolarized. These effects are consistent with the behavior of the delayed K^+ current at different membrane potentials in the presence of 4-AP. In cells in which the Ca^{2+}-activated K^+ current represents a substantial fraction of the total outward current, the aminopyridines increase slightly (cell R-15) or decrease (Cornwall and Gorman, 1979) action potential duration and increase the afterhypolarization. These effects are consistent with the behavior of the Ca^{2+}-activated current in the presence of 4-AP. Where the action potential depends primarily on an influx of Ca^{2+}, any increase in its duration or amplitude should cause a corresponding increase in the free intracellular Ca^{2+} concentration (Stinnakre and Tauc, 1973; Gorman and Thomas, 1978) and, therefore, an increase in the Ca^{2+}-activated K^+ current (Meech, 1974; Gorman and Hermann, 1979). In a high concentration of 4-AP this increase in the Ca^{2+}-activated K^+ current is further accentuated. This may account for the paradoxical finding in cat cerebellum (Nicholson et al., 1976) that 4-AP increases the duration of slow potentials and the extracellular K^+ concentration. In axon terminals an increase in intracellular Ca^{2+} should also facilitate the release of synaptic transmitter (Katz and Miledi, 1969; Llinás et al., 1976). A similar explanation may apply to the enhancement of muscular contractility. Both nonspecific facilitation of synaptic transmission and increases in extracellular K^+ are likely to cause convulsive activity in the central nervous system.

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