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SELECTIVITY AND PATCH MEASUREMENTS OF A-CURRENT CHANNELS IN *HELIX ASPERSA* NEURONES

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SUMMARY

1. The ionic selectivity of A-current K^+ channels has been measured in single *Helix aspersa* neurones by recording the reversal potential shift in test solutions containing various monovalent cations.

2. The A-current channel is permeable to Tl^+, K^+, Rb^+, NH_4^+ and Cs^+. The channels may also be sparingly permeable to Na^+ and Li^+. Organic cations have an apparent small permeability as judged from their reversal potentials, but this may be an artifact of K^+ accumulation.

3. A large patch electrode (3 μm tip) isolated a region that appeared to contain only A-current channels. This may indicate that A-current channels are found in the membrane as rafts of at least 3 μm in diameter.

4. The single-channel conductance calculated from single-channel current steps was 14 pS.

INTRODUCTION

The A-current is a fast, transient outward K^+ current that is activated at subthreshold voltages by depolarization from a hyperpolarized membrane potential (Connor & Stevens, 1971). This current was first observed in neurones from the mollusc *Onchidium* by Hagiwara, Kusano & Saito (1961) and has now been found in a variety of cells from several phyla. A recent review by Rogawski (1985) indicates the wide distribution of the A-current.

The A-current, like the delayed rectifier and Ca^{2+}-dependent K^+ currents, has a stabilizing effect on the membrane potential. Activation of the A-current in the subthreshold region of the membrane potential lengthens the interspike interval in a number of cells, e.g. molluscan neurones (Connor & Stevens, 1971), crab walking leg axons (Connor, 1975, 1978) and cultured rat hippocampal neurones (Rogawski, 1985, Fig. 5). The amount of A-current available to be activated will determine the delay to the next spike and thus may regulate the spike frequency. Similarly, the A-current may modify a cell's response to synaptic excitation by its short-circuiting effect on depolarizing current in the subthreshold range (Daut, 1973; Byrne, 1980; Getting, 1983; Dekin & Getting, 1984). In some cells, there is evidence that the A-current contributes to the repolarizing phase of the action potential. The

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shaker mutants of *Drosophila*, for example, produce abnormally long action potentials. Voltage clamp experiments on the flight muscles of wild-type and shaker mutant *Drosophila* revealed that the shaker mutations interfered with the A-current (Salkoff & Wyman, 1981). Also, in certain cardiac tissue the fast phase of repolarization of the action potential appears to result from A-current activation (Kenyon & Gibbons, 1979; Josephson, Sanchez-Chapula & Brown, 1984).

Voltage clamp, ion substitution and pharmacological experiments have shown that the A-current is carried by K^+ ions, can be blocked by 4-aminopyridine and tetraethylammonium ions and may be kinetically described by an expression analogous to the Hodgkin–Huxley equation for the Na^+ current (Hagiwara *et al.* 1961; Nakajima, 1966; Connor & Stevens, 1971; Neher, 1971; Neher & Lux, 1972; Thompson, 1977; Adams, Smith & Thompson, 1980).

Little is known about the permeability properties of A-current channels. The ionic selectivity of single-channel currents was reported in one publication but the channels could not be unequivocally identified as A-current channels (Kazachenko & Geletyuk, 1984). Recently, single-channel analysis of the A-current from rat nodose neurones has been published (Cooper & Shrier, 1985). This paper reports the results of experiments to determine these permeability properties in channels from neurones of the mollusc *Helix aspersa*.

METHODS

Preparation and experimental chamber

Unidentified cells in the visceral or pleural ganglia of *Helix aspersa* were used in all experiments. Isolated sub-oesophageal ganglia were pinned onto the Sylgard (Dow-Corning) floor of a narrow channel cut through a small perspex wedge. The tough connective tissue sheath and epineurium were removed by tearing with fine forceps to expose the nerve cell bodies. A piece of cover-slip was placed across the front face of the wedge to convert the channel into a microchamber. The usual volume of solution around the ganglion was 75 μl. The wedge was placed in a perspex block such that the exposed cells were viewed horizontally through the cover-slip while micro-electrodes were lowered into the chamber from above (Thomas, 1978). A simple gravity inflow and suction outflow system allowed the exchange of solutions during the experiment. The temperature in the chamber was kept at 20 °C by cooling the solutions *en route* to the chamber.

Isolated ganglia were treated with Trypsin (Sigma type III) 0·2% in normal saline for about 90 min at room temperature prior to attempts to make gigaohm patch seals. The enzyme treatment was performed before desheathing. At the end of the treatment period, the ganglia were continuously superfused in fresh, cold normal saline. On one occasion the membrane potential was monitored during enzyme treatment and was found not to change.

Recording apparatus

Membrane current was measured at the macroscopic level with a home-made two-micro-electrode clamp of conventional design and at the microscopic level with a home-made patch clamp of similar design to that described by Hammil, Marty, Neher, Sakmann & Sigworth, 1981. The current and voltage micro-electrodes were filled with 3 M-KCl and had resistances of about 2 and 10 MΩ, respectively.

Patch electrodes were pulled from filamented thick-walled Pyrex tubing (VWR) and fire polished on a microforge shortly before use. Sylgard was sometimes used to coat the electrode shank to reduce the electrode time constant, although the time constant of thick-walled glass was acceptably short without coating.

Data collection

Two-micro-electrode clamp experiments were controlled by an on-line mini-computer (LM[2]). Membrane current signals were filtered at 2 kHz and sampled at 4 kHz on one channel of a 12-bit analog-to-digital converter. Current records were stored on digital magnetic tape for later analysis.

Patch-clamp current steps were recorded on a Racal FM tape-recorder after the signal was filtered through a four-pole Bessel filter.

Solutions

Standard *Helix* saline contained (mM): NaCl, 80; KCl, 4; $CaCl_2$, 10; $MgCl_2$, 5; glucose, 10; and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 5. The pH was adjusted to 7·8 with Tris (tris(hydrozymethyl)aminomethane).

The experimental solutions for permeation experiments were made by replacing all of the NaCl and KCl by an osmotically equivalent amount of test salt. The following test salts were used: KCl, NaCl, RbCl, NH_4Cl, LiCl, CsCl, methylamine hydrochloride tetramethylammonium (TMA) chloride and $TlNO_3$. Measurements in Tl^+ saline were compared to measurements made in Na^+ and K^+ salines using nitrate salts.

RESULTS

The A-current Helix neurones

Many of the large cells (100–200 μm in diameter) in the visceral and pleural ganglia of *Helix* generate a fast outward current when depolarized under voltage clamp from a negative holding potential. The general characteristics of this current are shown in Fig. 1. The superimposed traces in Fig. 1*A* are macroscopic voltage-clamp currents produced by depolarizing the membrane from a holding potential of −96 mV to voltages ranging from −40 to 8 mV in steps of 8 mV. Depolarizations of the cell body elicit at least four components of current: an inward current, which is usually masked by the simultaneous and larger components of outward current; a rapid transient outward current during the first 10 ms, the A-current; slower components of outward current, which may include delayed rectifier current and Ca^{2+}-activated K^+ current; and finally, brief downward deflections of the trace represent unclamped action potentials in the attached axon.

A characteristic feature of the A-current is its inactivation at relatively negative potentials. So, by selecting an appropriate holding potential most of the A-current can be inactivated while leaving the other components of the membrane current relatively unaffected. When the holding potential (V_h) was changed from −96 to −48 mV and the voltage clamp steps repeated (Fig. 1*B*), the rapid transient outward current disappeared but the other components of the membrane current remained. Digital subtraction of the records taken when $V_h = -48$ mV from those when $V_h = -96$ mV yields a picture of nearly pure A-currents (Fig. 1*C*).

The characteristic voltage sensitivity of the A-current can be demonstrated in a classical steady-state inactivation experiment. The cell was clamped to a series of conditioning voltages (pre-pulses) from −96 to −48 mV. Following each pre-pulse, the cell was depolarized to a standard test voltage of −32 mV. Superimposed records of the membrane current obtained for each pre-pulse voltage are shown in Fig. 2*A*. When the value of peak membrane current at each pre-pulse level is plotted (Fig. 2*B*) it can be seen that the A-current is already half-inactivated by a pre-pulse to −65 mV. This value agrees well with Neher's study of the fast outward current in *Helix pomatia* neurones (Neher, 1971).

Ionic selectivity measurements

The ionic selectivity of A-current channels was determined by the method of Hille (1973). The reversal potential (E_r) for current passing through the A-current channels was measured first in a K^+ saline and then in a saline in which K^+ was replaced by

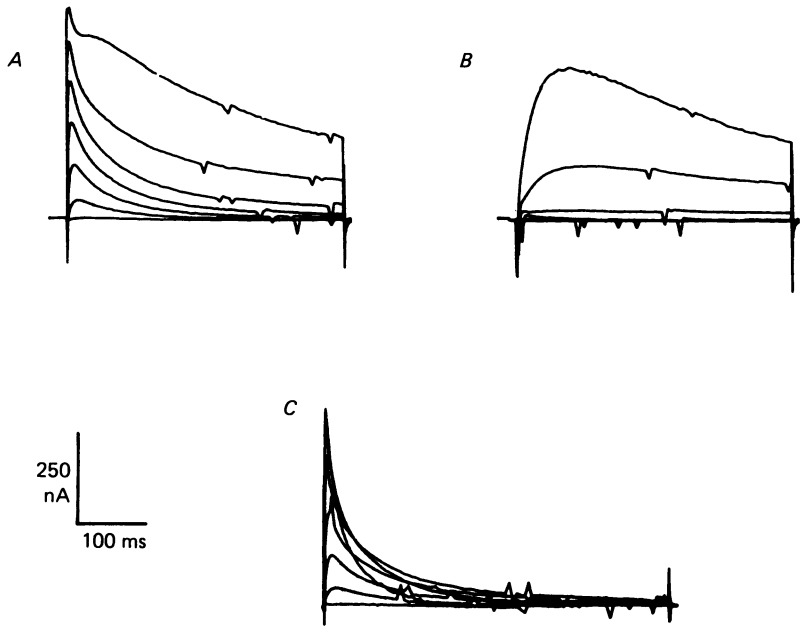

Fig. 1. General features of A-current recorded from a single *Helix* neurone under the control of a two micro-electrode voltage clamp. The cell was clamped in a series of 400 ms depolarizing voltage steps from −40 to 8 mV in steps of 8 mV. The currents in *A* were recorded when the holding potential was −96 mV, while those in *B* were recorded when the holding potential was reduced to −48 mV. The downward deflections on some of the current records are unclamped action potentials produced in the attached axon. *C*, the A-current has been isolated from other components of the outward current by digital subtraction of the current records in *B* from those in *A*.

a test ion. The permeability ratio of the test ion to K^+ was then calculated from the shift in the reversal potential using the relationship:

$$E_{r,x} - E_{r,K} = \frac{RT}{zF} \ln \frac{P_x[x]_o}{P_K[K]_o},$$

where $E_{r,x}$ is the reversal potential when the cell is superfused with the test ion, $E_{r,K}$ is the reversal potential in K^+ saline, $[x]_o$ and $[K]_o$ are the concentrations of the test ion and potassium in the experimental salines, and P_x and P_K are the permeabilities of the membrane to the test ion and to K^+. As a point of reference, P_K is taken as 1.

The set of current records shown in Fig. 3 illustrates the experimental approach. The membrane potential was controlled with a two-micro-electrode voltage clamp. The holding potential was −96 mV, at which potential most A-current channels

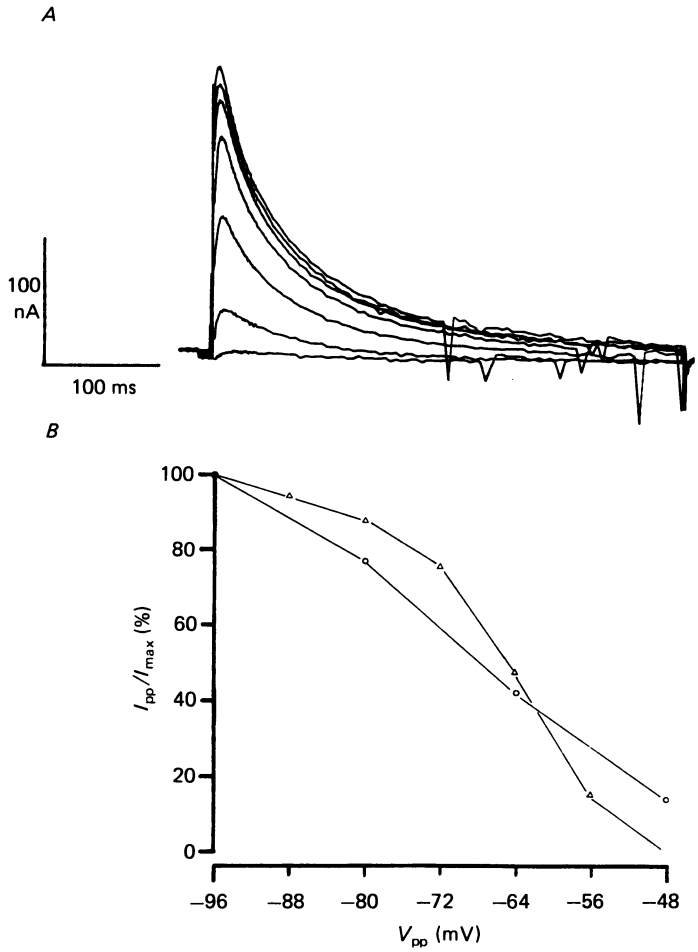

Fig. 2. The steady-state inactivation characteristics of the A-current. *A*, the membrane currents used to produce the macroscopic current inactivation curve in *B*. Each current record is the membrane current obtained in response to a depolarizing voltage clamp step to −32 mV. Preceding this test pulse, the membrane had been depolarized from the holding potential of −96 mV to a pre-pulse conditioning voltage of up to −48 mV for a period of 200 ms. *B*, the current at each pre-pulse voltage (I_{pp}) is plotted as a percentage of the current with no depolarizing pre-pulse step (I_{max}). Triangles, the steady-state inactivation curve for the macroscopic currents shown in *A*. The membrane current was half-inactivated at a voltage of −65 mV. Circles, the steady-state inactivation curve from averaged records of patches containing A-current channels (see Fig. 5*A*). In this case the current was half-inactivated at a membrane potential of −67 mV.

should be available to open. The membrane potential was stepped first to −32 mV for 10 ms to activate a sizeable A-current while minimizing the activation of other outward currents. In the results shown in Fig. 3*A* the A-current during the 10 ms activating pulse is inward because the cell is bathed in isotonic KCl so that E_K is positive to the voltage step. Following the activating voltage step, the membrane potential was depolarized in steps of 8 mV from −96 to 8 mV. Negative to −8 mV

the tail currents are inward and positive to −8 mV these currents are outward, which shows that in this high K^+ saline the A-current reverses near a potential of −8 mV. The second set of currents (Fig. 3*B*) shows the reason for choosing to measure tail current reversal. The reversal potental in NH_4^+ saline is near −40 mV. This is below the potential required to activate a measurable A-current.

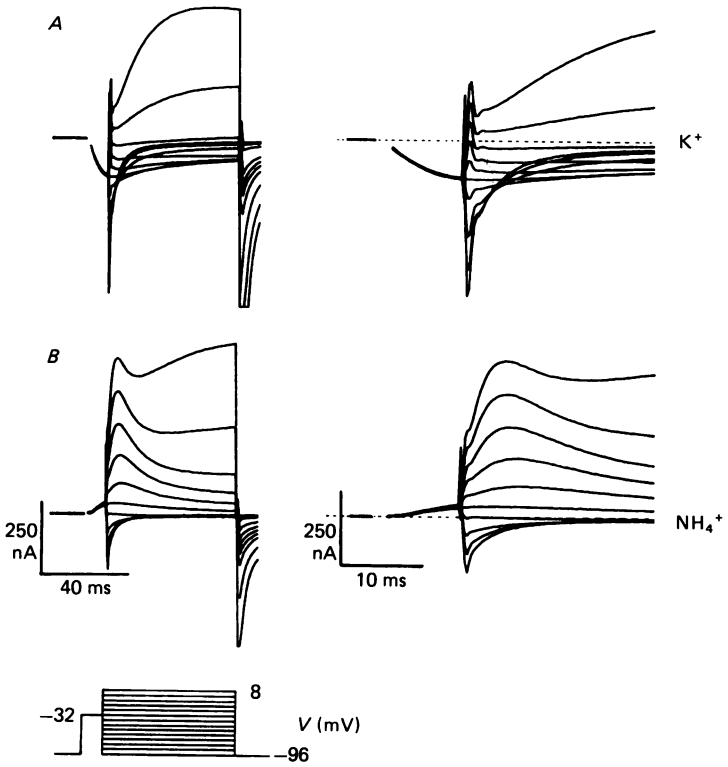

Fig. 3 Reversal potential measurements to determine the selectivity of A-current channels. *A*, the membrane currents flowing across a single snail neurone under voltage clamp in isotonic K^+ saline. The currents during the first 30 ms are shown on an expanded scale with a reference line drawn. The first digital sample point following the step depolarization has been deleted in all four records. Currents below the line are inward, those above the line are outward. *B*, a similar experiment in isotonic NH_4^+ saline. The experimental details are given in the text. The voltage clamp steps used to elicit the currents in *A* and *B* are shown below these records.

Current–voltage curves (Fig. 4) were drawn from the clamp records to determine the reversal potential in each test saline. Ideally, one would measure the direction of the 'instantaneous' current flowing immediately at the beginning of the tail pulse. In practice the size and direction of the tail current were measured soon after the capacity current resulting from the voltage step between activating and test pulses. This was usually 2·5 ms into the test pulse and occasionally 3·75 ms (see the expanded records in Fig. 3). Tail currents were identified as A-current by their sensitivity to depolarizing pre-pulses. Inspection of Fig. 4 shows that the A-current reverses at increasingly more negative values as K^+ saline is replaced successively

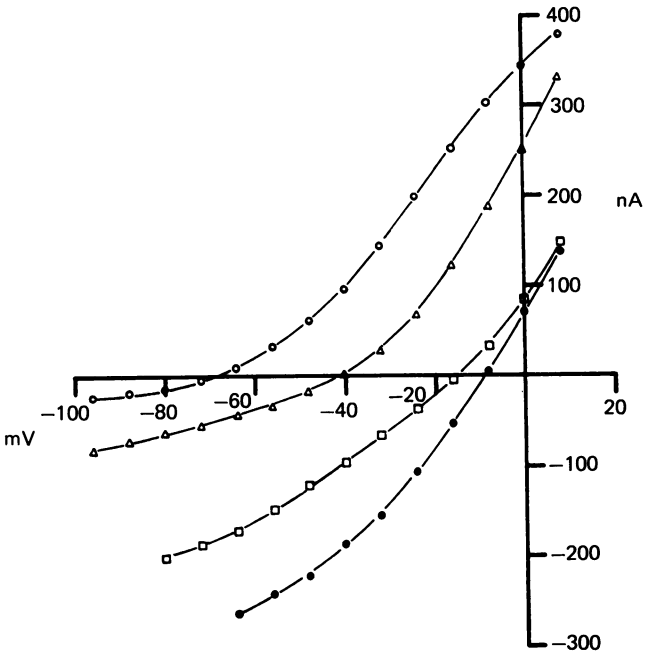

Fig. 4. Current–voltage curves obtained from voltage-clamping a single snail neurone superfused with the indicated experimental salines: filled circles, K^+ saline; squares, Rb^+ saline; triangles, NH_4^+ saline; open circles, Na^+ saline. Each curve crosses the voltage axis at the reversal potential for that test ion. The more negative the reversal potential, the less permeant the ion, so by inspection the selectivity sequence for the A-current channel is $K^+ > Rb^+ > NH_4^+ > Na^+$.

TABLE 1. Reversal potential and permeability ratio data from forty-three experiments

Test ion (x)	Permeability ratio P_x/P_k	$\Delta E_r(E_x - E_k)$ (mV) (mean ± S.E. of mean)
Tl^+	2·04	+18·0 ($n = 1$)
Rb^+	0·73	−8·0 ± 2·3 ($n = 6$)
NH_4^+	0·18	−43·0 ± 3·2 ($n = 5$)
Cs^+	0·14	−49·5 ± 4·8 ($n = 4$)
Na^+ (Cl−)	0·09	−61·5 ± 2·7 ($n = 12$)
Na^+ (NO_3^-)	0·09	−60·5 ± 6·5 ($n = 2$)
Li^+	0·07	−66·5 ± 4·0 ($n = 9$)
Methylamine	0·06	−70·0 ± 2·0 ($n = 2$)
Guanidine	0·05	−75·0 ($n = 1$)
TMA	0·04	−82·0 ($n = 1$)

by Rb^+, NH_4^+ and Na^+ salines. A more negative reversal potential indicates a less permeant ion so the permeability sequence from this experiment was $K^+ > Rb^+ > NH_4^+ > Na^+$. The permeability of the A-current channels to ten cations in all, including K^+, was tested. The reversal potentials and calculated permeability ratios are given in Table 1. According to these results all of the ions tested are permeant. The apparent permeability, however, of ions near the bottom of the list

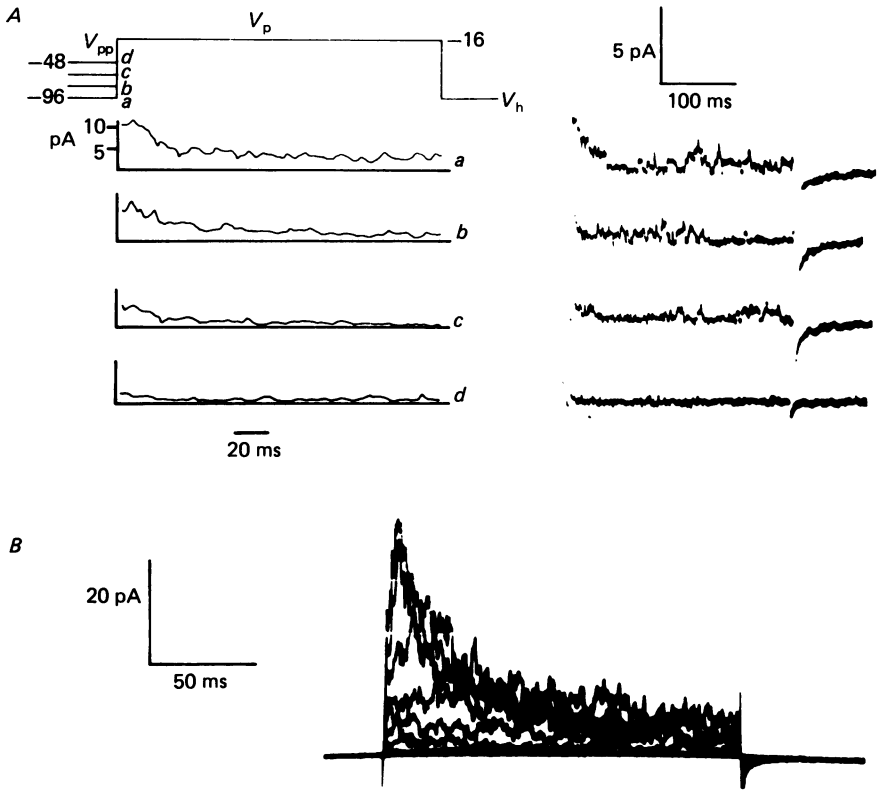

Fig. 5. *A*, a steady-state inactivation experiment to identify A-current channels in a patch of membrane. A standard test pulse to −16 mV was used to open the channels after the membrane was exposed to a 200 ms depolarizing pre-pulse from the holding potential of −96 mV. The records in the right panel of the Figure are examples of the patch current at a pre-pulse of *a*, −96 mV; *b*, −80 mV, *c*, −64 mV; and *d*, −48 mV. The records in the left panel are averages of eight patch recordings at the four pre-pulse levels. Inactivation of the early outward current is quite clear in these records as the pre-pulse is raised from −96 to −48 mV. V_{pp}, pre-pulse voltage; V_p, test voltage. *B*, the membrane current recorded from an on-cell patch using a large (3 μm) electrode. The electrode contained normal *Helix* saline. The currents were elicited by depolarizing the patch in steps of 16 mV from a holding potential of −82 mV.

could be artifactual. The reversal potential measurement in solutions of those ions might be contaminated by the accumulation of K^+ ions in the extracellular space during the 10 ms activating pulse. This possibility is discussed later.

Microscopic A-current measurements

In addition to measuring the selectivity of A-current channels an attempt was made to resolve the A-current at the single-channel level using the patch-clamp technique. This turned out to be very hard on this preparation because few patches contained A-current channels (only five out of thirty-three successful seals) and often patches were too active to make reliable measurements of single-channel characteristics. The results from one successful experiment,, however, are shown in Fig. 5*A*. Because molluscan neurones can possess several components of outward current two

criteria were used to identify patches containing A-current channels. First, channels should open within 10 ms of a depolarizing step. Secondly, the number of channels within this period should be reduced by a depolarizing pre-pulse in the subthreshold range from −96 to −48 mV. The single-channel and averaged records show that both these criteria were met in this patch. The single-channel current in eleven measurements from this patch was 1 pA giving a chord conductance of 14 pS under the experimental conditions of $[K^+]_o = 4$ mM, $[K^+]_i \approx 120$ mM, $E_M = -16$ mV, $T = 20$ °C, where $[K^+]_o$ is the external K^+ concentration, $[K^+]_i$ is the internal K^+ concentration, E_M is the membrane potential and T is the ambient temperature. Since the peak outward current was 9 pA the patch contained at least nine A-current channels.

An attempt was also made to seal quite large (tip *ca.* 3 μm) patch electrodes onto *Helix* neurones to study the distribution of A-current channels. Only two successful seals were obtained, on separate cells known to possess A-current. A family of current records is shown in Fig 5*B*. Because the patch contains a large number of channels, the components of outward current can be recognized from their kinetics. This patch seems to contain mainly A-current channels, as, even at large levels of depolarization, outward current relaxes to the base line. If delayed rectifier channels were present one would expect a maintained outward current at depolarizing steps positive to about −16 mV. It cannot be ruled out, though, that Ca^{2+}-activated channels may be present in the patch but remain unopened because too small an area of membrane is being depolarized to provide the necessary Ca^{2+} entry. The second patch (not shown) also contained A-current channels identified by the early fast outward component of current, but the current did not relax to the base line, suggesting that other outward current channels were present.

DISCUSSION

Ion selectivity measurements

The results presented here may be the first selectivity measurements on A-current channels. The selectivity sequence is in remarkable qualitative agreement with similar measurements obtained from other classes of K^+ channels from a variety of preparations (see Table in Latorre & Miller, 1983). This includes delayed rectifier, Ca^{2+}-activated and inward rectifier K^+ channels. Like the A-current channel, the first two of these exhibit the major permeability sequence $Tl^+ > K^+ > Rb^+ > NH_4^+$ while the inward rectifier has $Tl^+ > K^+ > NH_4^+ > Rb^+$. The A-current channel, however, seems also to be measurably permeable to Cs^+. This observation could be related to the finding of Reuter & Stevens (1980) that the delayed rectifier channels in neurones from *Helix roseneri* were also sparingly permeable to Cs^+ (K^+: Cs^+, 1:0·18). In addition, the measurements of Na^+ and Li^+ permeability ratios discussed above are similar to those of Reuter and Stevens (K^+: Na^+: Li^+, 1 :0·07 :0·09) but rather higher than measurements from other classes of K^+ channels in other animals.

The finding that the organic cations methylamine, guanidine and tetramethylammonium are apparently permeant is surprising. Other studies have suggested that small organic cations are not measurably permeant in delayed rectifier K^+ channels of *Helix* neurones and frog myelinated nerves (Hille, 1973; Reuter & Stevens, 1980).

The most reasonable explanation of the results presented here is that the K^+ ions that leave the cell during the 10 ms activating pulse are trapped in the extracellular space and re-enter the cell during the test pulse. Thus the inward tail current measured in the presence of organic cations may actually be carried by these K^+ ions. If the apparent reversal potential in methylamine is taken as the lower limit (since, of the organic cations it has the least negative reversal potential) then an accumulation of 7 mM-K^+ would provide the measured reversal potential. That guanidine and TMA have more negative apparent reversal potentials than methylamine is to be expected as these ions partially block A-current channels and would reduce the extent of K^+ accumulation.

Microscopic A-current measurements

The results of the small- (1 μm) and large- (3 μm) patch experiments are both consistent with the idea that A-current channels occur as rafts rather than being widely distributed all over the soma. Since one of the large-patch experiments seemed to isolate a region of pure A-current, a minimum diameter of such a raft would be about 3 μm assuming that the membrane under the pipette was not extensively drawn into the tip. There is good evidence for discrete channel localization from a variety of preparations (see review by Almers & Stirling, 1984). Possible mechanisms by which channel localization may occur have been discussed by Fraser & Poo (1982) in relation to the acetylcholine receptor. An attractive idea given by Hille (1984) is that individual channel types are incorporated into the surface membrane as concentrated patches that do not break up. Insufficient data on large patches were obtained in this work to enable any conclusions about the maximum size or number of such rafts to be drawn, but the low success rate of finding A-current channels with small patch electrodes suggests either a relatively low density of small rafts of channels, or perhaps just one or two larger rafts that happened to be located on the unexposed side of a neurone. This seems a particularly interesting question that might best be addressed with the loose-seal patch system of Stühmer, Roberts & Almers (1983) so that regions of a single neurone could be mapped for current type without the problems of damage to the cell when the seal of a large patch electrode is broken.

The single-channel conductance of 14 pS reported here is smaller than the two other published measurements of A-current single-channel conductance. Cooper & Shrier (1985) report an open-channel conductance of 22 pS for A-current channels in rat nodose ganglia cells, while Kazachenko & Geletyuk (1984) report a single-channel conductance of 40 pS for the fast channels in *Lymnaea stagnalis* neurone soma.

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