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IONIC CURRENTS IN NEURONES CULTURED FROM EMBRYONIC COCKROACH (*PERIPLANETA AMERICANA*) BRAINS

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SUMMARY

Neurones isolated from embryonic cockroach brains were maintained in culture for up to 8 weeks. A single patch electrode was used to record voltage changes in response to injected current, membrane ionic currents under whole-cell voltage-clamp conditions or single-channel currents from isolated membrane patches. The voltage changes in response to injected current that depolarized the cell indicated increases in membrane permeability to calcium and potassium. These observations were confirmed using a voltage clamp. The potassium current observed in the youngest cultures turned on with a delay and was blocked by tetraethylammonium (TEA) and 4-aminopyridine (4-AP). Two kinds of decrease in the outward potassium current were observed. One may be associated with extracellular potassium accumulation, inactivation of the potassium channel or inactivation of a calcium channel. The other appears to be a voltage-dependent inactivation. The magnitude of the calcium permeability appeared to increase as the cultures developed, being most prominent in cultures more than 2 weeks old. Single-channel conductance measured from an analysis of records from six isolated membrane patches ranged from 15 to 110 pS. Except for one channel, the probability of the channels being open did not change appreciably with membrane potential. Our results suggest that much of the increase in potassium permeability may be due an increase in intracellular calcium level.

INTRODUCTION

The insect nervous system affords several advantages for the investigation of membrane excitability, including the large size of the cell bodies, their ease of

Key words: cultured insect neurones, ionic currents, single channels, *Periplaneta americana*.

identification and their ready accessibility to microelectrode recording techniques. It is well known that a few insect neurone somata, such as motor neurones, are capable of generating sodium action potentials (Hagiwara & Watanabe, 1956; Ikeda & Kaplan, 1970) and, under certain conditions, calcium-dependent action potentials (Pitman, 1979). The accessible somata of most insect neurones do not exhibit regenerative responses reminiscent of action potentials, but they do have current–voltage relationships that suggest strong delayed or outward rectification due to activation of voltage-sensitive or calcium-dependent potassium channels (Thomas, 1984). However, little is known about the evolution of excitability or the types and distribution of voltage-sensitive ion channels in the developing insect neurone. One approach to the study of the development of excitability is to investigate embryonic cells during maturation in culture.

Beadle, Hicks & Middleton (1982) and Beadle & Hicks (1985) have described a method for culturing embryonic neurones isolated from the brain of the cockroach, *Periplaneta americana*. Using two high-impedance microelectrodes, one for injecting current and the other for recording voltage changes, they described some of the excitable properties of these cultured neurones during development (Lees, Beadle, Botham & Kelly, 1985). They found that few cells maintained in culture for less than 12 days were capable of generating sodium-like electrical activity. After 12 days, about 50% of the cells investigated generated action potentials believed to be due to an inward sodium current. An increase in membrane conductance during depolarization, thought to be related to an outward potassium current in the form of delayed rectification, was, however, evident in the youngest cells investigated.

Although some information about excitability can be obtained using current-clamp techniques, a thorough analysis of the membrane currents that underlie the voltage changes such as those described by Lees *et al.* (1985) requires the use of the voltage-clamp technique. Since many different kinds of membrane conductance changes can give rise to action potentials and control repolarization, we can understand the excitable properties of the neurone only by isolation of the underlying conductance changes into their individual components. Under culture conditions, the separation of the cells from their neighbours, and from supporting cells, allows an investigation of whole-cell currents as well as single-channel currents with the voltage-clamp technique using the single low-resistance patch electrode (Hamill *et al.* 1981). In this paper we have focused on potassium permeability. However, we have also seen an increase in membrane permeability due to voltage-sensitive calcium channels. Some of these results have been presented in preliminary form (Christensen *et al.* 1985; Pichon *et al.* 1986).

MATERIALS AND METHODS

Culture

The culture method for cockroach embryos used in these studies is a modification of that established by Levi-Montalcini & Chen (1969) and has been described

elsewhere (Beadle *et al.* 1982; Lees *et al.* 1985; Beadle & Lees, 1986). Embryos 20–21 days old were removed under sterile conditions and the brain was dissected free. The cells from the isolated brain were dispersed by a mechanical dissociation procedure that consisted of gentle trituration in a fire-polished Pasteur pipette. Cells were grown either on glass coverslips or directly on plastic culture dishes for 1 week in a '5+4' medium, consisting of five parts of Schneider's *Drosophila* medium (Gibco) and four parts of Eagle's basal medium with Earle's salts and L-glutamine (Gibco). After 7 days, the medium was changed to 'LG' medium, consisting of equal parts of Leibowitz medium (L 15) with L-glutamine and Grace's modified insect medium. The antibiotics penicillin (200 i.u. ml^{-1}) and streptomycin (200 $\mu g\, ml^{-1}$) were used in all media. The cells were maintained at 29°C in a humid atmosphere (Beadle & Hicks, 1985). The development of the cell cultures on glass coverslips was not as prolific as that on plastic culture dishes, probably owing to poor attachment of the cells to the substrate, even though the glass coverslips were coated with polylysine. The age of the cultures used in these studies ranged from 2 days to 6 weeks.

Under the culture conditions described above, large flat cells reminiscent of glial cells found in large numbers in other culture media were absent. We therefore believe that the cultures contained a heterogeneous population consisting mainly of neurones isolated from the brain. At the beginning of the culture period (1–3 days), the cells were small (approximately 10 μm in diameter), round and devoid of processes. After several weeks, approximately 90 % of the cells that appeared to be neurones produced long outgrowths forming a complex network of intertwined neurites, each of which had a diameter of 0·5–2 μm, that continued to grow in length during the period of culture (Beadle & Hicks, 1985). The cell bodies in the older cultures remained about the same size, 10–15 μm in diameter. Fig. 1A,B shows low-power phase-contrast micrographs of cultures from two stages of development. In the early stage (Fig. 1A), before the first change in medium (usually 7 days from the original plating), outgrowth from the soma was sparse and relatively short neurites were only occasionally visible. However, following the first medium change, growth was rapid and extensive proliferation of neurites was established. Individual cells are indicated by arrowheads and clumps of cells growing together are also present.

Experimental procedure

Before an experiment, the culture medium was replaced with a physiological solution containing (in mmol l^{-1}): NaCl, 210; KCl, 3·1; $CaCl_2$, 10; Hepes, 10; pH adjusted to 7·2. Cell culture dishes were placed directly onto the stage of a Nikon inverted-phase-contrast microscope and glass coverslips were placed in a chamber designed for superfusion and attached to the microscope stage. Complete exchange of solutions containing specific ion-channel blockers was estimated to occur within 2–3 min, although effects of blockers could be observed within 30 s. The types and concentrations of the specific blockers utilized are indicated with the appropriate

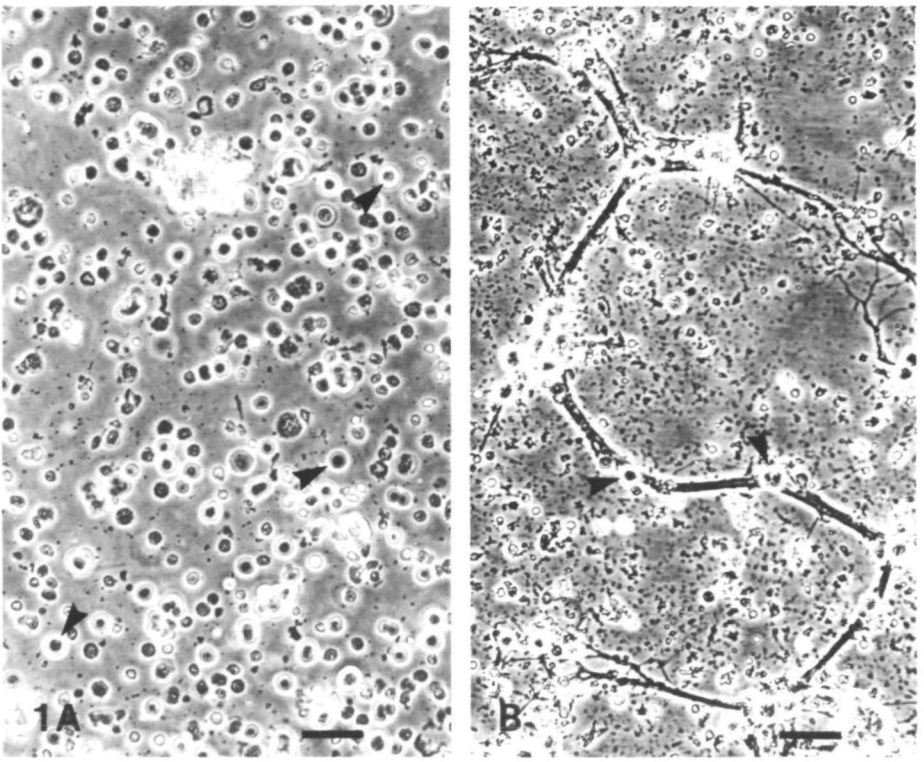

Fig. 1. Low-power phase-contrast photomicrographs showing the cell development at two different stages. (A) One day after plating. Cells are round and devoid of processes. (B) Twenty-nine days after initial plating. The connecting processes between cells or clumps of cells consist of bundles of small-diameter neurites (0·5–2·0 μm). Arrowheads indicate examples of cells. Scale bars, 10 μm.

illustrations. Tetrodotoxin (TTX), at 1–3 μmol l^{-1}, was used in all experiments unless otherwise indicated. All experiments were performed at room temperature.

Recording

Electrodes were fabricated from two types of glass tubing. Hard Pyrex tubing, 1 mm o.d. with a square bore to facilitate filling, was pulled into patch electrodes on a home-made single-stage vertical puller. These electrodes were not fire-polished. Alternatively, electrodes were pulled in two stages from 1·5 mm haematocrit tubing on a modified Kopf (model 150 C) vertical puller. These electrodes were used either polished or unpolished. Electrodes were filled with the external physiological solution used for the cultures, or potassium gluconate or potassium chloride (140 or 240 mmol l^{-1}) solution containing 2 mmol l^{-1} $MgCl_2$, 11 mmol l^{-1} EGTA, 1 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} Hepes, with the pH adjusted to 7·4. The electrode resistances typically measured 2–7 MΩ.

The electrode was advanced towards the cell until gentle contact was made. A small amount of suction applied to the electrode usually resulted in a high-resistance

seal of several GΩ (Hamill *et al.* 1981). If whole-cell currents were to be recorded, further suction or, in some cases, current was passed through the electrode to facilitate rupture of the cell membrane and thus gain access to the interior of the cell.

Whole-cell currents were recorded directly onto digital cassettes using a Hewlett-Packard-based data acquisition system. The command voltage steps were fixed to a pattern of −30 to 100 mV in 10 mV increments and supplied by the Hewlett-Packard-based system. If single-channel activity was to be recorded, the data were fed to a Sony analogue to digital converter and recorded on video tape at full amplifier bandwidth (10 kHz with high-frequency boost on Biologic RK-300 patch amplifier).

Data analysis

Whole-cell currents were analysed as current–voltage graphs following leak subtraction from the response to a 30 mV hyperpolarizing command. The leak current determined from this response was appropriately scaled according to the magnitude of each depolarization. This scaled current was subtracted on a point by point basis by the Hewlett-Packard-based system from the current produced by the depolarization.

The single-channel data were analysed using a computer program called IPROC-2 on an IBM-compatible PC and originally developed by Sachs, Neil & Bakakali (1982). The version used here has been extensively revised by M. Sloderbeck and C. J. Lingle.

RESULTS

Current clamp

The voltage responses during depolarizing and hyperpolarizing current steps demonstrate the rectifying properties of these cultured neurones (Fig. 2). As will be shown below, most of the voltage change during depolarization results from a voltage-dependent increase in membrane conductance to potassium. Hyperpolarization of the cell produced large voltage changes that did not reach steady state during the 25 ms change in membrane potential. The hyperpolarizing voltage changes were plotted to determine the steady-state voltage response, from which the cell input resistance and membrane time constant could be determined. The input resistance estimated from the curves was 850 MΩ and the membrane time constant was 20 ms.

Large depolarizations in the presence of TTX produced a rapid voltage change that peaked and then declined to a steady-state value indicative of an increase in membrane permeability, most probably to potassium, although there may also be some contribution to the damped oscillation voltage change due to an increase in calcium conductance (see below). When the step was turned off, there was an after-hyperpolarization, whose magnitude depended on the size of the depolarization. This hyperpolarization reflected the increase in permeability to potassium that persisted at the end of the current pulse.

In the presence of potassium channel blockers, a regenerative potential change could be seen that resembled calcium action potentials (Pitman, 1979; Lees *et al.*

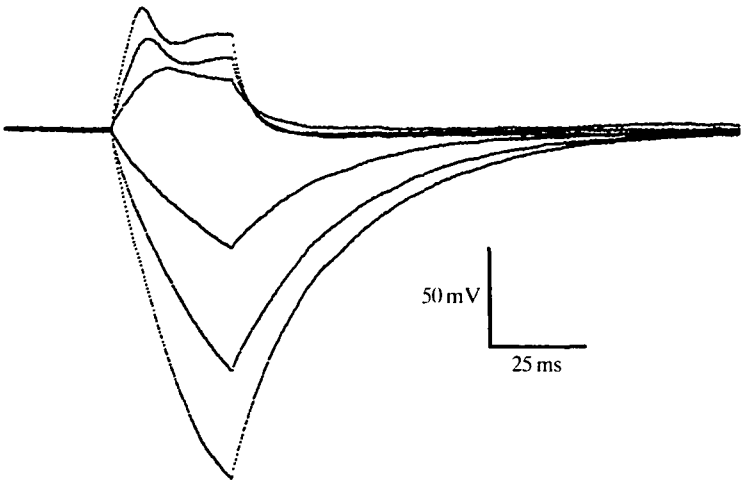

Fig. 2. Voltage response from a cell under current-clamp conditions. The polarizations were to 0·3, 0·2 and 0·1 nA of current. During the depolarizations, the membrane potential reached a peak, then decayed. Resting potential −30 mV, 20-day-old culture.

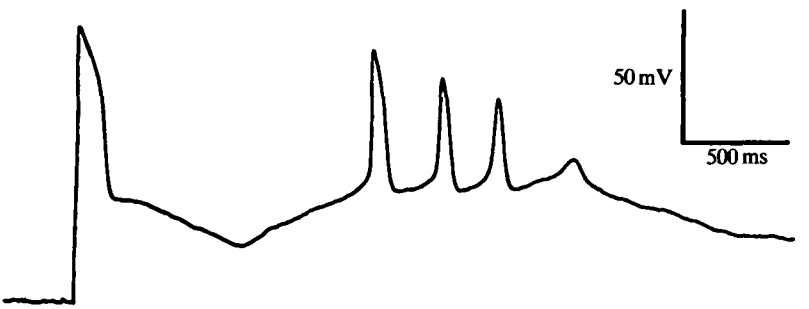

Fig. 3. Action potentials recorded from a 30-day-old culture. The external solution contained 20 mmol l^{-1} $BaCl_2$, 100 mmol l^{-1} TEA and no NaCl. The first action potential was evoked by a 30 ms depolarizing current step. This response is slightly broadened compared to the series of smaller and shorter action potentials that followed. Under these conditions, this cell had a tendency to fire repetitively in the absence of the repolarizing outward potassium current. Resting potential −70 mV.

1985). Fig. 3 shows the action potentials recorded from a 30-day-old culture in an external solution containing 20 mmol l^{-1} $BaCl_2$ (which more readily permeates voltage-gated calcium channels), 100 mmol l^{-1} TEA (tetraethylammonium chloride) to block the potassium current, and no sodium chloride. Under these conditions, the cell has a tendency to fire repetitively.

In this experiment, the first action potential resulting from a small depolarizing current pulse from a membrane potential of −70 mV was broad (160 ms duration)

and was followed by a long after-depolarization, then a series of three shorter action potentials (about 70 ms duration) that occurred spontaneously. The shorter duration of these spontaneous action potentials may reflect some inactivation of the calcium channels. As will be shown later, at least part of these excitability changes could be due to an increase in calcium conductance which appears to be better developed in older cultures.

Voltage clamp

Technical considerations

One of the major problems faced in attempting to voltage-clamp central neurones is neuronal geometry. All recordings were made from nearly spherical cell somata 10–15 μm in diameter, but the number and size of the neurites depended on the age of the culture. Within any one culture dish, it was evident, based on the number and size of the neurites, that there were differences in the stage of morphological development of individual cells. The cable-like structure of the neurites poses a problem for obtaining good space-clamp of the cell. Single electrodes, even those with very low impedances, are incapable of rapidly injecting sufficient current to maintain good voltage control of the cell beyond the very proximal neurites. As the neurones developed in culture the geometry of the neurites became more extensive and complicated (see Fig. 1A,B). Because of the small size of cell somata and small diameter of the neurites [0·5–2·0 μm (Beadle & Hicks, 1985)], we believe that a reasonable space-clamp is, however, maintained in the soma, especially if TTX is used to block the fast sodium currents.

Whole-cell currents

Calcium current

The inward currents recorded under voltage-clamp from a 40-day-old culture are illustrated in Fig. 4A. In this experiment the external solution contained 100 mmol l^{-1} TEA, to block the outward potassium current (see below), and 20 mmol l^{-1} $BaCl_2$, to increase the size of the inward currents. The current activated rapidly and decreased slowly. The decrease was unlikely to be due to slow development of a calcium-dependent potassium current since barium is not known to activate an outward potassium current. The current–voltage relationship for the peak inward current recorded under similar conditions to those shown in Fig. 4A is illustrated in Fig. 4B for three cells. Maximum activation occurred near 0 mV and this did not seem to be affected by age. However, the maximum inward barium current developed did appear to depend on the age of the cell. As illustrated in Fig. 4B, the maximum inward current was between 500 and 550 pA. This was a larger current than that found in younger cultures. For example, the maximum inward current developed in a 14-day-old culture in 10 mmol l^{-1} $BaCl_2$ was only 162 pA (not shown).

In five cells from cultures older than 19 days, in which the outward potassium currents were blocked with 4-aminopyridine (4-AP, 1 mmol l^{-1}) and TEA

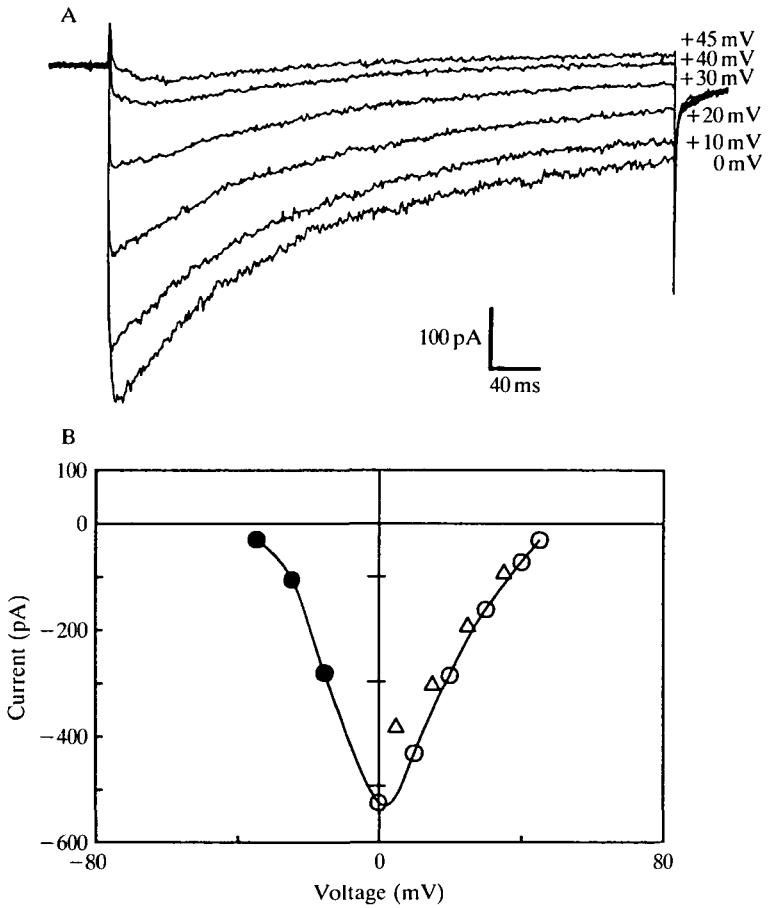

Fig. 4. (A) Inward barium current recorded in a cell from a 40-day-old culture. The inward current had a rapid onset and inactivated slowly, with time constants for the four largest current traces of 115, 116, 98 and 88 ms, indicating that inactivation rate decreased with depolarization. Note the inward tail current when the pulse was turned off. Holding potential −50 mV, external solution (in mmol l^{-1}) $BaCl_2$, 20; TEA, 100; sodium, 0. The membrane potential for each current is indicated next to the trace. (B) I–V relationship showing the peak inward current for three cells under similar conditions to those shown in A. Age of cells ranged from 30 to 40 days. Maximum activation of the inward barium current occurred at 0 mV.

(10 mmol l^{-1}), all exhibited a net inward current in solutions containing elevated barium concentrations. In seven cells from younger cultures (11- and 15-day-old) under the same conditions, only three cells exhibited a small net inward current. Since the soma does not become appreciably larger with age, the increase in inward current from the voltage-sensitive calcium channels may have been due to an increase in channel number or in channel conductance. However, alteration in channel kinetics cannot be discounted.

Potassium current

As was illustrated in Fig. 2, a hyperpolarizing current pulse results in large voltage changes, indicating that the membranes of these cultured cells have a very low conductance under these conditions. The presence of an inward potassium current (inward rectifier) was investigated under voltage-clamp conditions. Very little, if any, inward rectification was present in these cells at least up to 6 weeks following the initial culture. When hyperpolarizing command steps were applied to the cell only a small inward current could be recorded. The size of this inward current was not changed when barium, known to block inward rectification at low concentrations (Sperelakis, Schneider & Harris, 1967; Standen & Standfield, 1978), was added to the extracellular medium. For this reason, the inward current recorded upon membrane hyperpolarization is considered to consist largely of leakage across the cell membrane, rather than resulting from the activation of voltage-sensitive conductances.

In the standard extracellular solution, the largest membrane current found in these cells was that due to an increase in membrane permeability to potassium. Examples of the outward potassium currents recorded from 15- and 14-day-old cultures under whole-cell voltage-clamp are illustrated in Fig. 5A,B. The shape of the outward current records followed one of two time courses in different cells. The current trace either reached a maximum and was maintained during the command pulse (Fig. 5A), or there was an early peak current with a later decrease (Fig. 5B). We investigated the possibility that, in those cells displaying the decrease illustrated in Fig. 5B, the early peak was due to the activation of a rapid outward (Ia) potassium current (Connor & Stevens, 1971) in addition to the delayed potassium current.

This does not appear to be the case since the time course of the early current does not change at holding potentials more depolarized than −55 mV and the time course is not altered by 4-AP, an antagonist of the rapid outward potassium current.

Tail currents can be seen at the end of the voltage step. The potassium characteristics of these tail currents were investigated by altering the holding potential before the step was applied. As the holding potential was hyperpolarized towards the estimated E_K (see below) the tail currents could be abolished.

To confirm the identity of the outward current as a potassium current, we examined the effect of adding 4-AP and TEA to the extracellular solution. Fig. 5C shows the current traces from the cell illustrated in Fig. 5B in a solution containing 1 mmol l^{-1} 4-AP, 20 mmol l^{-1} TEA and 1 μmol l^{-1} TTX. The outward currents were almost entirely blocked, revealing the small inward current due to the opening of voltage-sensitive calcium channels. The potassium tail currents were also abolished under these conditions. Full recovery of the potassium currents occurred when the extracellular solution was replaced with standard saline (Fig. 5D).

We examined the possibility that potassium accumulation could account for the decrease in membrane current illustrated in Fig. 5B by measuring the potassium equilibrium potential from the potassium tail currents for the four largest voltage steps (+70 to +100 mV) for the two cells illustrated in Fig. 5. If the decrease were

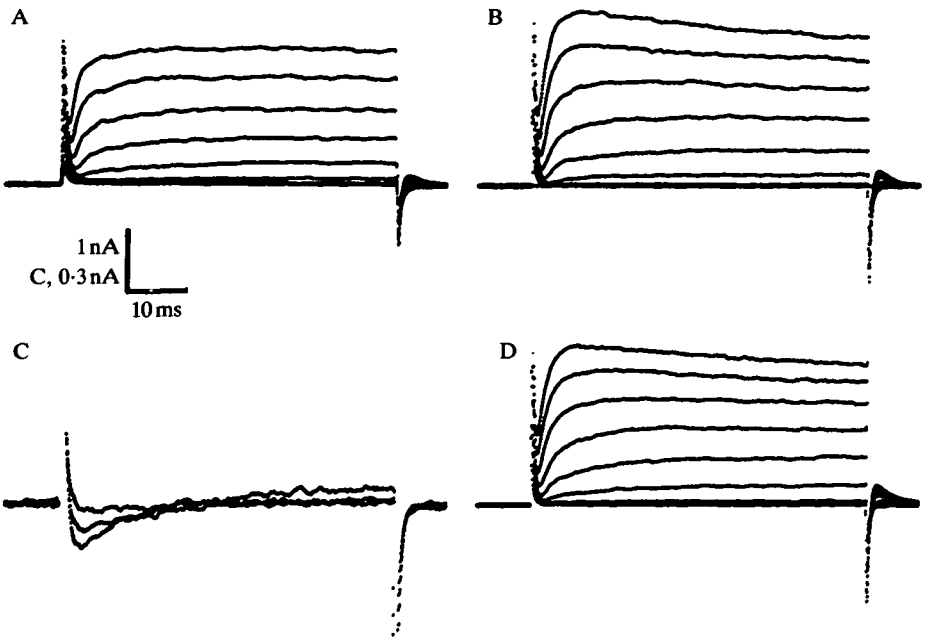

Fig. 5. Whole-cell currents recorded from (A) 15- and (B) 14-day-old cultures showing the time course of the delayed outward potassium currents. (A) Outward potassium currents turned on with a delay typical of the delayed rectification and reached steady state. Following the turn off of the command pulse, potassium tail currents can be seen. (B) Outward currents were similar to A except that following the peak current there was a gradual fall in the current, the magnitude of which increased with depolarization. (C) Inward calcium current recorded from the cell illustrated in B following introduction of 1 μmol l^{-1} TTX, 10 mmol l^{-1} TEA and 1 mmol l^{-1} 4-AP in normal saline. The current traces were recorded 3 min after the start of the superfusion of the solution containing the blockers. (D) Recovery 10 min after the introduction of control solution. Voltage steps for A, B and C are in increments of 10 mV from a holding potential of −50 mV. Maximum depolarization shown was to +30 mV. The two largest steps to +40 and +50 mV are not shown. Voltage steps in C were to −20, −10 and 0 mV.

due to potassium accumulation, then the equilibrium potential for potassium should shift towards more positive potentials, and the tail currents should become relatively smaller with increasing depolarization. Calculation of the potassium equilibrium potential was performed as follows. The time course of the tail current was plotted on a semilogarithmic scale and extrapolated back to the end of the step to determine the size of the tail current at the time that the voltage was returned to the holding potential. All tail currents appeared to decay with a single time constant. The value for E_K for each potential step was calculated from:

$$E_K = (I_1V_2 - I_2V_1)/(I_1 - I_2)\ , \qquad (1)$$

where I_1 is the amplitude of the current just before the end of the step, I_2 is the size of the tail current obtained from the extrapolation, V_1 is the membrane potential during

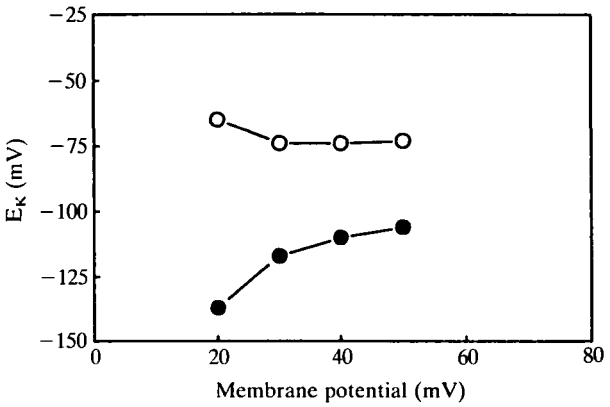

Fig. 6. Plot of membrane potential *versus* calculated E_K as described in the text. Open circles are from data shown in Fig. 5A and closed circles are from data shown in Fig. 5B.

I_1, and V_2 is the holding potential (i.e. the membrane potential during the tail current).

Fig. 6 shows a plot of E_K *versus* membrane potential when there was no fall in the outward current (same cell as illustrated in Fig. 5A, open circles) and when the outward current decreased (same cell as illustrated in Fig. 5B, closed circles). The value for E_K in the absence of current fall is constant at around −75 mV, which compares with that of −74·5 mV obtained for a cockroach axon using the same procedure (Pichon, Poussart & Lees, 1983). However, the calculated value for E_K from the current records that showed a fall is considerably larger and shows a shift towards 0 mV with membrane depolarization, consistent with extracellular potassium accumulation. The membrane conductance at the end of the largest voltage step (+100 mV depolarization) for the data shown in Fig. 5B is 16 nS and E_K was estimated to be −106 mV. If we assume that membrane conductance remains constant during the voltage step, then it is possible to calculate E_K for the peak outward current that occurs near the beginning of the step. This calculation gave a value for E_K of −130 mV at the beginning of the 100 mV depolarization. Therefore, the shift in E_K due to potassium accumulation based on the above assumptions of constant conductance during the largest step corresponds to a change of 24 mV. This shift in E_K is of the same order of magnitude as that estimated for the four largest steps and calculated from the tail currents for this same cell (Fig. 6, filled circles), suggesting that potassium accumulation could be responsible for the observed fall. This does not exclude the possibility that potassium inactivation is present in these neurones. An alternative mechanism that could account for a slow decrease in outward current depends on whether this current is activated by the influx of calcium ions. It appears that the inward calcium current slowly inactivates. In a recent experiment we examined the effect of substituting barium (10 mmol l^{-1}) for calcium in the extracellular solution. Under these conditions, the outward current was unchanged. However, when 500 μmol l^{-1} cadmium was added to the extracellular

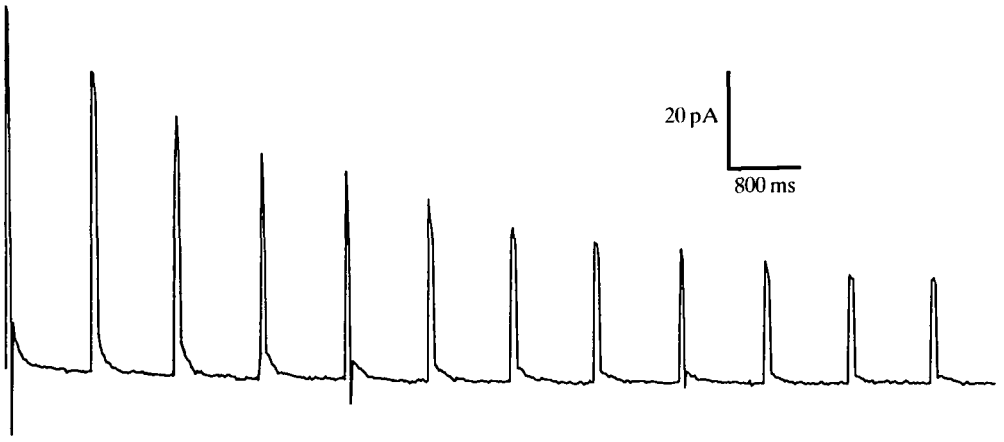

Fig. 7. Voltage-dependent decay of the outward potassium current following a step of −100 mV from a holding potential of −50 mV. The depolarizations are to +50 mV. The cell was from a 17-day-old culture. See text for details.

solution, the outward current was immediately abolished, suggesting that much of this current is activated through the influx of calcium.

A second type of decrease in outward current was seen in some cells. This decrease was observed on repetitive voltage steps to the same potential and appeared to be voltage-dependent. Returning the membrane potential to the holding potential did not relieve the depression, but a hyperpolarizing step removed the block immediately. The current traces illustrated in Fig. 7 are from a cell in which the membrane potential was first stepped to −150 mV then to +50 mV at a repetition rate of $2\,s^{-1}$. The decay in the current magnitude can be described by a single exponential with a time constant of 2·3 s. A hyperpolarizing step to −150 mV removed the depression and the next depolarizing response produced the maximum current (not shown).

Fig. 8 shows the steady-state I–V relationship for four different cells. Outward rectification was turned on near −20 mV, increased with depolarization, and was nearly linear above 0 mV for all cells. The leak response recorded during hyperpolarizing steps is shown for only one cell.

Single channels

Membrane current during the opening of single channels was recorded in cell-attached, cell-free inside-out and cell-free outside-out patches under conditions with high sodium (210 mmol l^{-1} NaCl) or potassium (140 or 240 mmol l^{-1}, KCl or potassium gluconate) levels in the pipette. When recordings were made from cell-attached patches with the external saline (210 mmol l^{-1} NaCl) in the pipette, single-channel currents were outward, and the size of the current increased linearly with depolarization. With high potassium levels in the pipette, single-channel currents were inward until the patch was sufficiently depolarized to exceed the potassium equilibrium potential. The size of the current decreased linearly with patch

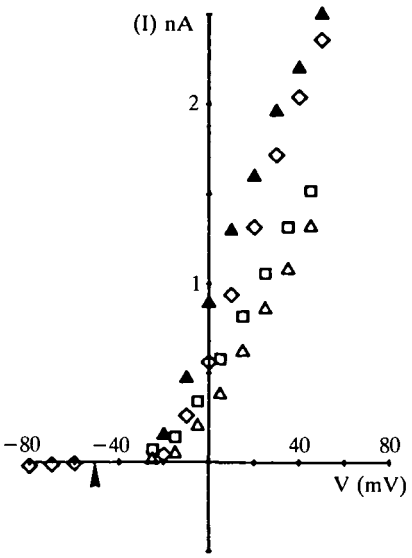

Fig. 8. Isochronal current–voltage relationship obtained from four cells in an external solution containing TTX. Holding potential was −50 mV and is indicated by the arrowhead. Leak was shown for only one cell. The current was measured near the end of the step at about 100 ms. The maximum average whole-cell conductance was determined between −10 and +40 mV from the slopes obtained from a linear regression through these points. This value is 29 ± 6·1 nS (S.D.). For a cell 10 μm in diameter, the average membrane current density for these four cells for 100 mV driving force was 2·9 mA cm^{-2}.

depolarization and reversed at large depolarizations. These results are consistent with the expected direction of membrane current for potassium channels.

The detailed analysis of single-channel activity requires long stable recording periods at several different membrane potentials containing many events. For this reason, data satisfying these criteria were selected for analysis. These experiments include results from three cell-attached and three cell-free patches.

Three main types of single-channel activity were observed. The most common type of channel activity consisted of very rapid openings that we assume were beyond the bandwidth of the recording amplifier, giving the channel opening a triangular appearance. This type of channel activity was seen in almost every record in both cell-attached and cell-free patches. It is possible that this type of rapid opening and closing is part of the normal expression of this channel. This type of channel could not usually be properly analysed because of uncertainties in amplitude. However, as will be described below, in one instance rapid open/close events were included in an analysis in which they appeared to belong to a population of longer-duration events.

A second class of channel was characterized by relatively short open times that did not change with membrane potential. Three representative records from a cell-attached patch with both these types of records are illustrated in Fig. 9A. The first trace shows a relatively long opening of the second type of channel with one rapid closing. A short-duration opening is seen near the end of this trace and is typical of

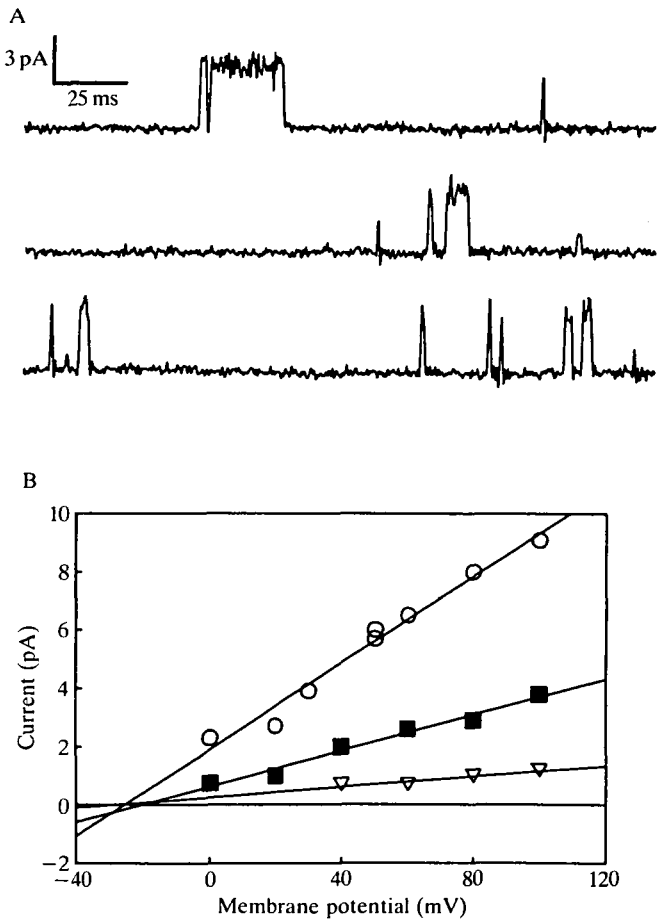

Fig. 9. Single-channel records from cell-attached patch from a 45-day-old culture. Patch potential was held at +100 mV above resting potential. Outward current shown as upwards. See text for details. (B) I–V curves for three cell-attached patches. Filled squares and inverted triangles are from a 45-day-old culture, records shown in A. Open circles are from a 23-day-old culture, records shown in Fig. 10. Lines drawn through points are linear regressions. Zero current intercepts are −30 (▽), −21 (■) and −26 (○) mV.

the rapid open/close events described above. Since the amplitude of this last event was similar to those of the longer openings, they were assumed to occur in the same channel. The second trace shows four openings: the first may be an incomplete opening of the larger channel, the second two events are similar in amplitude to each other and of longer duration, and the fourth is an event from a second, smaller conducting channel in the patch. The third trace illustrates the larger and smaller channel events of varying duration. Observations of superimposed traces on a storage oscilloscope from this experiment suggested that the conformational change into the open and closed states consisted of large, short-duration events, many of which

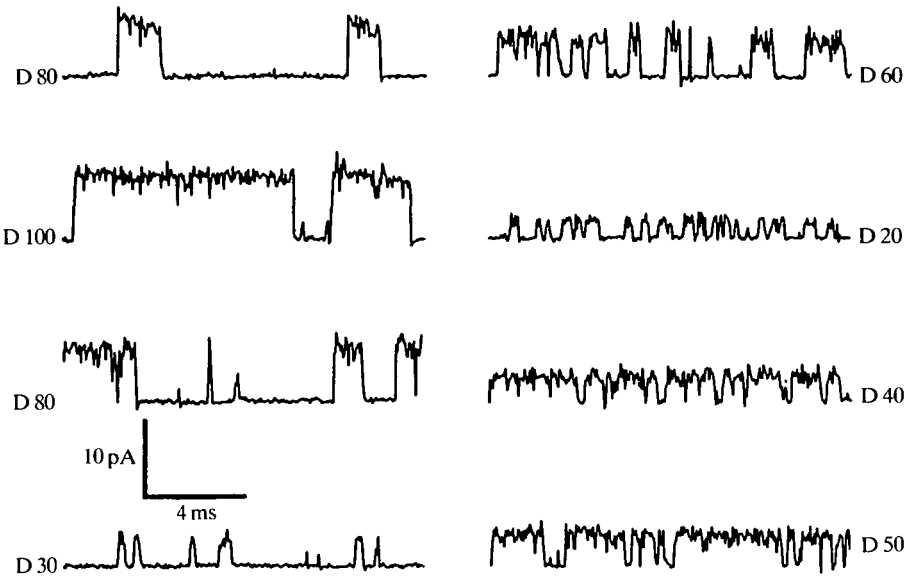

Fig. 10. Single-channel records from a cell-attached patch, 23-day-old culture, data filtered at 5 kHz. The order and magnitude (in mV) of the membrane potential change is as indicated next to each trace reading from top to bottom. D, depolarization. The records at resting membrane potential are not shown. See text for details.

appeared as triangular channel openings. The amplitudes of these openings were similar to those of the longer openings that clearly reached a constant current level and, therefore, the rapid events were considered to belong to this same channel. The small channel events could be identified from the baseline noise at depolarizations greater than 40 mV and had a measured conductance of 15 pS. The larger channel events could be distinguished from baseline noise at all potentials and had a conductance of 30 pS. The mean open times for the small channel were $4{\cdot}7 \pm 0{\cdot}6$ ms (S.D.) and for the large channel $5{\cdot}4 \pm 1{\cdot}1$ ms (S.D.) and were not affected by potential. The current–voltage relationships for these two channels are shown in Fig. 9B, together with that corresponding to another channel from a cell-attached patch whose characteristics will be described below. The channel conductance for this larger channel was 74 pS. The reversal potentials for the single-channel current for these three cell-attached patches were −30, −21 and −26 mV for the smallest to the largest channel, respectively. These values are with respect to the resting potential, which is unknown for these cell-attached patches. However, typical resting potentials for many cells are in the −50 to −60 mV range which would indicate a reversal potential between −70 to −80 mV for these three cell-attached recordings. This is close to the E_K estimated for these cells.

A third type of single-channel activity consisted of channel openings that were larger and occurred in longer bursts of discrete steps. During the open state, the burst was interrupted by gaps that often did not reach the zero current baseline.

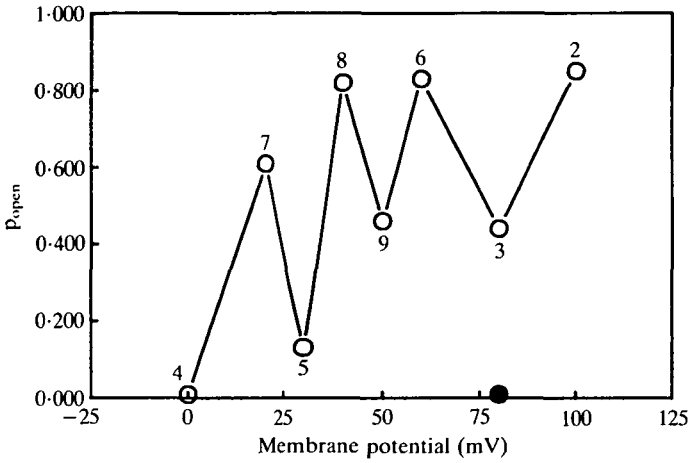

Fig. 11. Open probability for the cell illustrated in Fig. 10. The filled circle represents the measured open probability when the cell was first stepped from resting to +80 mV. The open circles indicate the measured open probabilities, and the numbers associated with each symbol indicate the order of the membrane potential change. See text for details.

These rapid closures were most prevalent in the one channel in which the open time appeared to be voltage-dependent. This channel showed a dramatic step-like shift in open probability following a step from 80 mV to 100 mV. Fig. 10 shows examples from this patch in the order that the depolarizations were carried out, and Fig. 11 shows the open time probability. The channel was rarely open when it was initially stepped to 80 mV from rest ($p_{open} = 0{\cdot}01$, filled circle). When stepped to 100 mV, the channel was open approximately 80 % of the time. Stepping to a lower depolarization decreased the open probability slightly, but there was a good degree of scatter. However, it is clear that following the step to 100 mV the open probability of the channel increased dramatically over the initial value measured at 80 mV.

The mean open time during a burst in this same experiment showed a tendency to increase with membrane potential (Fig. 12A). In contrast, except for the large decrease in closed time following the initial change in membrane potential to 100 mV that coincided with the increase in open probability, the closed times did not appear to change consistently with membrane potential (Fig. 12B). This suggests that, with larger depolarizations, the number of rapid closures (flickering) that contribute to the closed times decreased, resulting in an increase in open time. The I–V relationship for this patch is shown in Fig. 9B (open circles).

Fig. 13 shows the I–V relationships for three other channels, all from cell-free patches. One was a large conducting (110 pS) and two were smaller conducting channels (30–35 pS). One was from an outside-out patch (large channel) and the membrane potential of the cell was obtained in the whole-cell mode before the excised patch was formed. The I–V is therefore plotted with respect to the measured

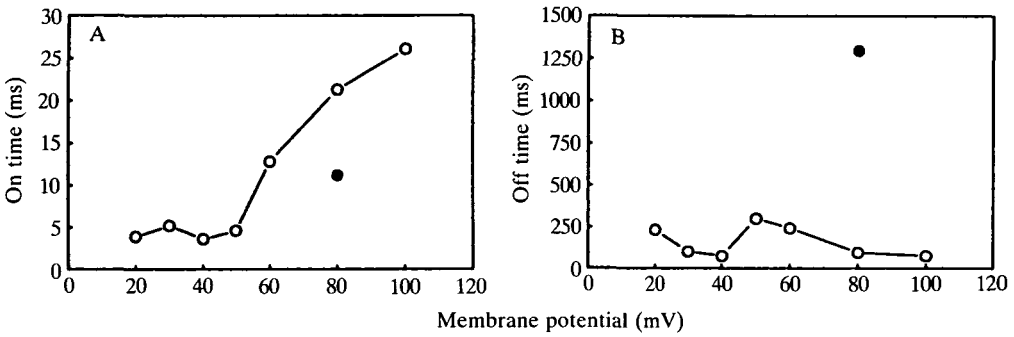

Fig. 12. (A) On time as a function of membrane potential. The filled circle represents the open time following the initial membrane potential change to +80 mV. Open circles indicate the open times at the other membrane potentials. (B) Off time as a function of membrane potential. The closed circle has the same meaning as in A. See text for details.

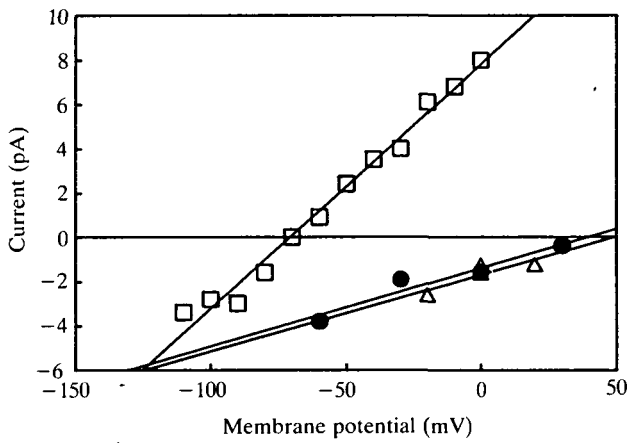

Fig. 13. I–V relationships for cell-free patches. Open squares are from a 45-day-old culture, outside-out patch. The membrane potential of −61 mV was recorded in the whole-cell mode. Filled circles and triangles are for inside-out patches, with 240 mmol l^{-1} K^+ in the electrode. See text for details.

resting potential of −60 mV and the reversal potential was measured as −71 mV. The other two channels were recorded from cell-free inside-out patches with 240 mmol l^{-1} K^+ in the electrode. Their reversal potentials were measured from the linear regressions to be +39 and +49 mV. These reversal potentials were less positive than the +109 mV reversal potential calculated from the known potassium gradient of 240 mmol l^{-1} K^+ on the outside face of the membrane and 3·1 mmol l^{-1} K^+ on the inside face. This suggests that these channels may not be completely selective. Since the gradient for sodium was very large, nominally 0 mmol l^{-1} sodium in the pipette, a small sodium permeability could produce the shift towards zero.

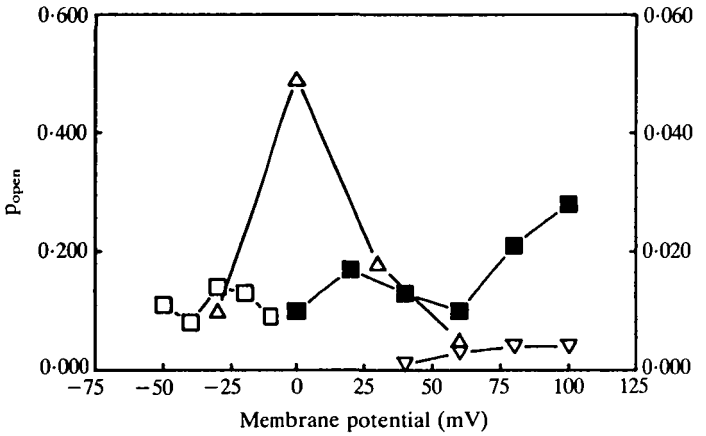

Fig. 14. Open probability as a function of potential. Symbols represent the same cells whose I–V curves are shown in Figs 9B and 13. The scale on the left refers to the open squares and triangles and the scale on the right to the closed squares and inverted triangles. See text for details.

Fig. 14 shows a plot of the open probability as a function of potential for the other recordings analysed and described above (in one patch, the records were not sufficiently long to obtain accurate open–closed time information). There did not appear to be any consistent tendency to increase the open time probability for any of these experiments. The symbols in this figure for each experiment correspond to those shown in the I–V relationships in Figs 9B and 13.

DISCUSSION

Investigation of neuronal excitability in the intact insect nervous system has suggested that the cell membrane includes voltage-gated sodium, calcium and potassium channels (Hoyle & Burrows, 1973; Goodman & Spitzer, 1980; Goodman & Heitler, 1979; Pitman, 1979; Lees *et al.* 1985). In this respect, cultured cockroach neurones do not appear to differ from their intact counterparts. In addition to the delayed potassium current important for repolarization, and demonstrated by Lees *et al.* (1985) in these cultured neurones, action potentials associated with activation of voltage-sensitive calcium channels can be evoked, at least under the special conditions where barium replaces calcium and the outward rectification is blocked. Whether these calcium channels are important for regenerative activity or to serve as a means of calcium entry for activating other channels, for example a calcium-activated potassium channel, remains to be determined.

Inward potassium current

Lees *et al.* (1985) showed a large increase in membrane potential when hyperpolarizing current was injected into intact neurones. Such an increase in membrane potential was not observed in the cultured neurones. The membrane conductance

that would normally reflect this increase in membrane potential would be associated with the turning on of a potassium channel (inward rectifier) and this channel did not appear to be present in the cultured cells. The input impedance measured from hyperpolarizing responses in one cell (illustrated in Fig. 2) under current-clamp conditions was 850 MΩ. When the cells were depolarized the input impedance during maximum activation of the delayed potassium current averaged 33 MΩ, about half that estimated for one cell of similar age investigated by Lees *et al.* (1985). This may reflect the better recording conditions that can be obtained with the patch electrode compared with the high-impedance electrodes.

Delayed outward potassium current

The reduced excitability seen in the cell body of intact insect neurones appears to reside in the large increase in potassium conductance on depolarization of the cell. Potassium channels associated with the delayed outward current are among the earliest to develop in cockroach neurones. This is not surprising since there are several reports using current-clamp methods that have described such properties in insect somata (Hoyle & Burrows, 1973; Goodman & Spitzer, 1980; Goodman & Heitler, 1979).

The outward potassium current seen under whole-cell voltage-clamp conditions either reached a steady state or decreased with time. Although our evidence is consistent with the hypothesis that the decrease is due to potassium accumulation, this is not the only possible mechanism. In the one cell analysed that exhibited this time-dependent decrease, the estimated E_K was near −130 mV, considerably higher than that calculated from our estimate of $[K^+]_i$ or from that calculated for cockroach axons (Pichon *et al.* 1983). In the cell analysed that did not show a decrease, the E_K was close to the value measured from the axon. Although it is not clear how extracellular potassium might be trapped next to the cell surface, since electron microscopic observation of these cultured neurones has shown an absence of supporting glial cells (Beadle *et al.* 1982), cells with an unusually high E_K would be more prone to accumulation. This may be in the form of an unstirred layer, since the large driving force created by the difference between E_K and the command step potential would allow more potassium to be transported across the cell membrane. It is also clearly possible that the decrease represents a true inactivation of the potassium channels. Alternatively, if the outward potassium current were almost entirely dependent on calcium influx, the apparent inactivation could reflect inactivation of the calcium channel. This hypothesis to explain the decrease in outward potassium current seems the most likely, based on our recent observations suggesting that most of the outward current may be dependent on calcium influx.

The outward potassium current was reversibly blocked by the classical potassium channel antagonists TEA and 4-AP. No rapid outward current associated with the potassium A current was seen. In a small number of cells the outward current appeared to fatigue with repetitive depolarization to the same potential. This fatigue could be relieved by a single hyperpolarizing pulse. However, simply returning the membrane potential to the resting level did not relieve fatigue, at least at short times.

A similar fatigue and relief by hyperpolarization has been described in molluscan neurones (Aldrich, Getting & Thompson, 1979; Ruben & Thompson, 1984).

Calcium-activated potassium current

In young cultures, in which the outward current had been blocked, an inward current was revealed that was carried by barium ions. This current is believed to occur during activation of voltage-sensitive calcium channels. The current is small in young cells and becomes larger is older cells and it inactivates at all ages. It is likely that these channels exist within the soma membrane rather than only on the neurite-like processes. In view of the strong outward rectification exhibited by these cells, there does not seem to be sufficient membrane current to generate calcium action potentials, at least in the earlier stages. However, it is equally clear in older cultures that, if the delayed rectification is reduced, trains of calcium-supported action potentials can be generated.

Thomas (1984) has described the presence of a calcium-activated potassium conductance in intact cockroach neurones. However, his evidence suggests that the inward current is very rapid (less than 1 ms) but sufficient to activate the potassium channels. Our evidence suggests that the calcium current should be readily observable in intact cells under appropriate conditions in which the outward current is blocked.

The results from the analysis of our single-channel data suggest that many of the potassium channels are not directly activated by voltage. It seems likely that some, or possibly all, of the outward current is activated by the influx of calcium. It has recently been shown that most of the outward current in GH3 cells, an anterior pituitary cell line, is mediated through calcium influx (Ritchie, 1987). Much of this current is blocked by 30 mmol l^{-1} TEA with an inhibitor concentration that is half maximal at 1 mmol l^{-1}. The similar effects of TEA on the outward current described here would be consistent with a calcium-activated potassium current. In addition, our recent experiments indicating that cadmium can completely block the outward current are consistent with a calcium-activated potassium current. Although it has been reported in several preparations that barium is not able to activate the outward potassium current, a recent report by Ribera & Spitzer (1987) has described an outward potassium current in cultured *Xenopus* neurones in which barium or strontium could substitute for calcium. A similar effect has been reported for snail neurones (Meech, 1974).

Single channels

The major evidence that the single-channel records are from potassium channels is the estimated reversal potential for the current. Although the reversal potential is more positive than the calculated potassium equilibrium potential, we assume that these channels are not entirely potassium-selective or, alternatively, that the estimated $[K^+]_i$ of 210 mmol l^{-1} is incorrect. Since the chloride concentrations on both sides of the membrane were similar in the single-channel recordings, the possibility that these were chloride channels seems remote. Two channels had

measured conductances of 74 and 110 pS, three channels of about 35 pS and one channel of 15 pS. The measured single-channel conductance of potassium channels believed to be associated with delayed rectification is 17 pS in squid (Conti & Neher, 1980) and 16 pS in hippocampal neurones (Rogawski, 1986). Pichon *et al.* (1983) have estimated the voltage-gated single-channel conductance from noise in cockroach axons to be 3·5 pS. If the single-channel conductance in the soma is in this range, it will be difficult to measure with current technology. Gardner (1986) described one of three types of potassium channels in the 100 pS range in chick ciliary ganglion cells. However, they showed no increase in open probability with potential whereas one of our channels did. Rogawski (1986) recorded two channels that conducted in the 30 pS range and had high-frequency bursts, but did not investigate them further. The conductances of the calcium-activated potassium channels described by Ribera & Spitzer (1987) measured 50 pS when activated by calcium and about 80 pS when activated by barium.

The open probability increased as a function of decreasing membrane potential in the single channel recorded from a cell-attached patch. The other channels appeared to be voltage-independent. It is possible that the voltage dependency reflects an influx of calcium ions if this channel is a calcium-activated potassium channel. Since the other patches analysed were cell-free and faced a constant calcium concentration, they might not be expected to show this type of potential dependence. In the case of the cell-attached patch, channel openings were recorded at rest after the patch was initially depolarized. Sufficiently long records were not available at the resting potential to enable us to determine whether the frequency of channel openings decreased with time, which might be expected if this channel were activated by calcium.

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