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CHARACTERIZATION OF THREE TYPES OF POTASSIUM CURRENT IN CULTURED NEURONES OF RAT SUPRAOPTIC NUCLEUS AREA

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SUMMARY

1. Whole-cell, voltage-clamp recordings were obtained from neurones of the supraoptic area of neonatal rats in dissociated cell culture. Recordings were made from neurones having the same morphology as those which were vasopressin or oxytocin immunoreactive.

2. Three types of voltage-activated K^+ current were identified on the basis of their kinetics, voltage sensitivities, Ca^{2+} dependence and pharmacology. The currents corresponded to the delayed rectifier current (I_K), the A-current (I_A), and the Ca^{2+}-dependent current ($I_{K(Ca)}$) described in other neurones.

3. I_K had a threshold of -40 mV, a sigmoidal time course of activation, and was sustained during voltage steps lasting less than 300 ms. The underlying conductance was voltage dependent reaching a maximum at $+30$ mV (mean maximum conductance 4·09 nS). The activation time constant was also voltage dependent declining exponentially from 4·5 ms at -30 mV to 1·8 ms at $+50$ mV.

4. I_A was transient, and was activated from holding potentials negative to -70 mV; the maximum conductance (mean 5·9 nS) underlying the current was obtained at $+10$ mV. The activation and inactivation time constants were voltage dependent: the activation time constant declined exponentially between -40 mV (2·2 ms) and $+40$ mV (0·65 ms).

5. I_K and I_A were attenuated by the K^+ channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP). TEA blocked the conductance underlying I_K but appeared to alter the kinetics of I_A. In contrast, 4-AP blocked the conductance underlying I_A and, to a lesser extent, I_K.

6. I_K and I_A were activated independently of external Ca^{2+} and the voltage activation of Ca^{2+} channels since these currents were recorded in the presence of Co^{2+}, a Ca^{2+} channel blocker.

7. $I_{K(Ca)}$ was recorded only when Ca^{2+} (2 mM) was present in the external medium. From a holding potential of -30 mV, $I_{K(Ca)}$ had a threshold of -20 mV, was maximal at about $+20$ mV and declined at more positive potentials. This current was sustained during voltage steps lasting 100 ms and was abolished by addition of Co^{2+} (2 mM) to the medium.

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8. The possible roles of the three K^+ currents in regulating the characteristic firing behaviour of supraoptic neurones previously recorded *in vivo* and *in vitro* are discussed.

INTRODUCTION

Magnocellular neurones of the supraoptic and paraventricular nuclei synthesize either oxytocin or vasopressin, subsequently releasing these peptide hormones into the blood from their axon terminals in the neurohypophysis. Action potentials generated in the hypothalamic cell bodies are thought to control release from the terminals. A variety of work indicates that the frequency and pattern of firing of action protentials controls the amount and efficiency of terminal release to achieve physiologically active plasma concentrations of the hormones.

There has been a large number of studies to determine how magnocellular neuronal firing patterns are controlled. Extracellular recordings from neurones in the anaesthetized adult rat (see review by Poulain & Wakerley, 1982) and extra- and intracellular recordings from neurones in slices and explants also from adult rats (Mason, 1983; Andrew & Dudek, 1984*a*; Bourque & Renaud, 1985) have been employed. Intracellular recordings have also been obtained from neonatal rat neurones in organotypic explant cultures (Gahwiler & Driefuss, 1979) and from fetal mouse hypothalamic neurones in dissociated culture (Legendre, Cooke & Vincent, 1982). The mechanisms which generate phasic firing in vasopressin neurones and the generation of a high-frequency burst of action potentials by oxytocin cells have been of particular interest. In vasopressin neurones, for example, it has been shown that each burst is associated with a depolarizing plateau potential. This potential is small (< 10 mV) in adult neurones (Mason, 1983; Andrew & Dudek, 1984*a*) but is considerably larger in dissociated, perhaps immature, cultured neurones of fetal mouse (Legendre *et al.* 1982).

To obtain a detailed picture of the mechanisms which regulate firing patterns, it is necessary to have some insight into membrane current flow. However, to date only two voltage-clamp studies on these neurones have been reported (Bourque, 1986, 1988). A major limitation to such work has been the lack of a preparation of cultured magnocellular oxytocin and vasopressin neurones. We have developed methods to culture neurones dissociated from the area of the supraoptic nucleus (SON) of the neonatal rat. We have previously reported that a high proportion of the neurones in these cultures are immunoreactive for oxytocin or vasopressin, and generate characteristic, spontaneous firing patterns (Cobbett & Mason, 1987, 1989). We have also shown that it is possible to voltage clamp these neurones using patch-clamp methodology and to detect voltage-activated inward and outward membrane currents.

We report here the results of a detailed study of the voltage-activated outward K^+ currents found in these cells. We have identified three different K^+ currents, which may be differentiated on the basis of their voltage sensitivity, their calcium dependence and their pharmacology. The reason for our interest in these K^+ currents is that they may contribute to the ionic mechanisms modulating firing patterns generated by these neurones. Aspects of this work have been published in the form of a communication to the Physiological Society (Cobbett, Legendre & Mason, 1987).

METHODS

Dissociated cell cultures of supraoptic neurones from 2- to 4-day-old rats were prepared as described elsewhere (Cobbett & Mason, 1987, 1989). Whole-cell, voltage-clamp recordings using patch-clamp methodology (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were made as previously described (Cobbett, Ingram & Mason, 1987; Cobbett & Mason, 1987). All recordings were made in Na^+-free solutions and the compositions of these solutions are detailed in Table 1. Recordings were made 7–20 days after the neurones were dissociated, at room temperature (18–20 °C). Tetraethylammonium (TEA) and 4-aminopyridine (4-AP; Sigma) were dissolved in the external recording medium and applied by pressure from a micropipette positioned close to the neurone under study. Tetrodotoxin (TTX; Sigma), Ca^{2+} and Co^{2+} were dissolved in external recording medium and added, as required, to the medium bathing the neurone under study.

In the figures all current records are the average of four or five records which have had linear currents subtracted during computer analysis (unless stated otherwise). For clarity, any remaining unsubtracted capacitative transient currents have been removed manually from the records.

TABLE 1. Contents of the external and internal (electrode) solutions. Ionic concentrations (mM) for the contents of the solutions used to suppress Ca^{2+}-dependent K^+ currents (A and B) and for recording total K^+ current i.e. Ca^{2+}-dependent and Ca^{2+}-independent currents (C and D). A and C are internal solutions and B and D are external solutions: all solutions were adjusted to pH 7·3 with KOH giving final internal and external K^+ concentrations of 4·8 and 145 mM respectively

	A	B	C	D
KCl	140	—	140	—
$MgCl_2$	2	2	2	1
EGTA	10	5	10	—
HEPES	10	10	10	10
Choline chloride	—	130	—	130
Glucose	—	10	—	10
$CoCl_2$	—	2	—	—
ATP	—	—	1	—
Cyclic AMP	—	—	2	—
$CaCl_2$	—	—	—	2

RESULTS

Whole-cell, voltage-clamp recordings were made from the largest supraoptic neurones in the cultures. Neurones were identified on the basis of their morphology (phase bright cell body with one to three processes) and immunoreactivity to a specific antiserum against neurofilament protein, a neuronal marker (see Cobbett & Mason, 1989). The somatic diameter was between 11 and 25 μm. The morphology of these neurones corresponded with the majority of cells which were found to be immunoreactive to antiserum against either vasopressin or oxytocin (Cobbett & Mason, 1988). In all cells tested ($n = 52$), no qualitative differences were found in the behaviour of outward currents and the currents were not affected by tetrodotoxin, a specific blocker of voltage-activated Na^+ channels. However, the maximum amplitude for each type of current we shall describe varied up to twofold within the whole population.

Three types of outward K^+ current were discerned in supraoptic nucleus (SON) neurones. In a Na^+- and Ca^{2+}-free external solution, two types of K^+ current were observed depending on the holding potential (V_h): (1) a rapidly activating current which developed during voltage-clamp steps from V_h to various test potentials

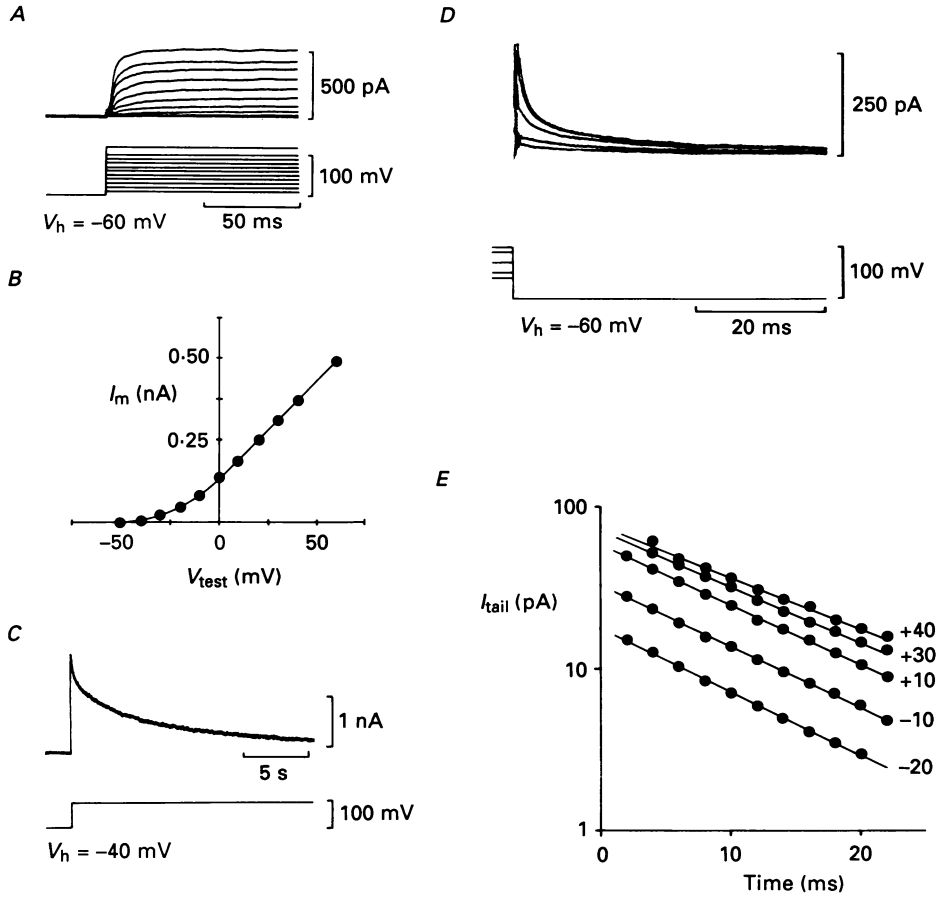

Fig. 1. Whole-cell potassium currents of a cultured supraoptic neurone. *A*, current responses (upper traces) during positive voltage-clamp steps (lower traces) from a holding potential of −60 mV to various test potentials (frequency of stimulation 0·5 Hz). Note that during 100 ms voltage steps, the current did not inactivate. *B*, relationship of the outward membrane current (I_m) and test potential (V_{test}) in *A* measured at the end of the 100 ms voltage step. *C*, inactivation of I_K during prolonged activating voltage steps. *D*, tail currents recorded at −60 mV on termination of 100 ms positive voltage steps to a range of test potentials. *E*, semilogarithmic plots of tail current (I_{tail}) decay with time at several given test potentials showing the independence of current decay and test potential; data plotted from records shown in *C*.

(V_{test}) between −50 and +20 mV (resembling a delayed rectifier current), and (2) a transient outward current which was inactivated at V_h positive to −65 mV (resembling an early transient current). In the presence of external Ca^{2+} (2 mM), a third type of outward current was observed: this was activated from a holding potential similar to that observed for the delayed rectifier current.

These three currents appeared qualitatively similar to the currents previously described in invertebrate cells (Connor & Stevens, 1971; Meech & Standen, 1975; Thompson, 1977). Therefore we have used the conventional terminology I_K, I_A and

$I_{K(Ca)}$ to identify the delayed rectifier current, the early, transient current, and the Ca^{2+}-dependent current, respectively.

Delayed rectifier outward current

In order to isolate I_K from the Ca^{2+}-dependent K^+ current, a Ca^{2+}-free external solution containing 5 mM-EGTA or 1 mM-$CoCl_2$ was used. Furthermore, to inactivate I_A, V_h was set at -60 mV (see below).

Figure 1 illustrates the activation of I_K during a series of voltage steps to several test potentials (V_{test}). I_K had a threshold of -40 mV and a clearly sigmoidal time course (Fig. 1*A* and *B*), as described for the delayed outward current in the squid axon (Hodgkin & Huxley, 1952). During test pulses of 100 ms, I_K developed to a steady-state value and did not inactivate. However, I_K was inactivated with time during a long (> 300 ms) activating voltage step (Fig. 1*C*). In the three cells tested, this time-dependent inactivation of I_K was described by more than one exponential (not shown). On return of the membrane to V_h following a short activating voltage step (100–500 ms), a tail current was recorded (Fig. 1*D*): the amplitude of the current was dependent on V_{test} and its reversal potential was -85 mV (see below). The decay with time of the tail current was described as a single-exponential process for which the time constant was independent of the value of the test potential: the slope of the relationship between I_{tail} and time plotted semilogarithmically was the same for all test potentials (Fig. 1*E*).

The conductance change underlying I_K was calculated by dividing the magnitude of the outward current, observed at the end of a 100 ms activating voltage step at a given test potential (V_{test}), by the driving force $V_{test}-V_K$, where V_K is the reversal potential of the tail current (see below). The conductance increased rapidly from the threshold for I_K and was maximal for V_{test} close to $+30$ mV (Fig. 2*A*). In the fourteen cells tested, the calculated maximum conductance for a test potential of $+30$ mV varied from 2·1 to 7·3 nS (4·9 ± 1·4 nS, mean ± S.E.M.).

Kinetics of I_K

Since the time course of I_K activation appeared sigmoidal, this suggested that I_K in SON neurones followed the Hodgkin and Huxley kinetic model described by the following equation:

$$G_K = n^y G_{Kmax}, \tag{1}$$

where G_K is the macroscopic conductance, G_{Kmax} is the maximum conductance observed and n^y represents the probability, n, for each particle to be in the correct configuration to give rise to an open channel. Therefore I_K develops with time as

$$I_{K(t)} = I_{K(\infty)}\{1-[\exp^{(-t/\tau)}]\}^y, \tag{2}$$

where $I_{K(t)}$ is the current at time t after onset of the voltage step, $I_{K(\infty)}$ the maximum current observed and τ the activation time constant.

With the logarithmic transformation of this equation:

$$\ln\{1-[I_{K(t)}/I_{K(\infty)}]^{1/y}\} = -t/\tau, \tag{3}$$

it is possible to obtain a graphical estimation of y. Figure 2*B* shows plots of this equation taking $y = 2$, 3 or 4. The plot was clearly non-linear for $y = 2$ and 4 and

become more linear with $y = 3$ (for $N = 4$ cells). Thus, taking $y = 3$ we have calculated the activation time constant for different test potentials. The activation time constant was about 4·5 ms at −30 mV and decreased exponentially with voltage from −40 mV to −50 mV (Fig. 2*A*).

Reversal potential of the I_K tail current

The reversal potential of the I_K tail current (I_{tail}) was estimated by a double-voltage-step protocol ($N = 5$ cells). The membrane was first stepped from the holding

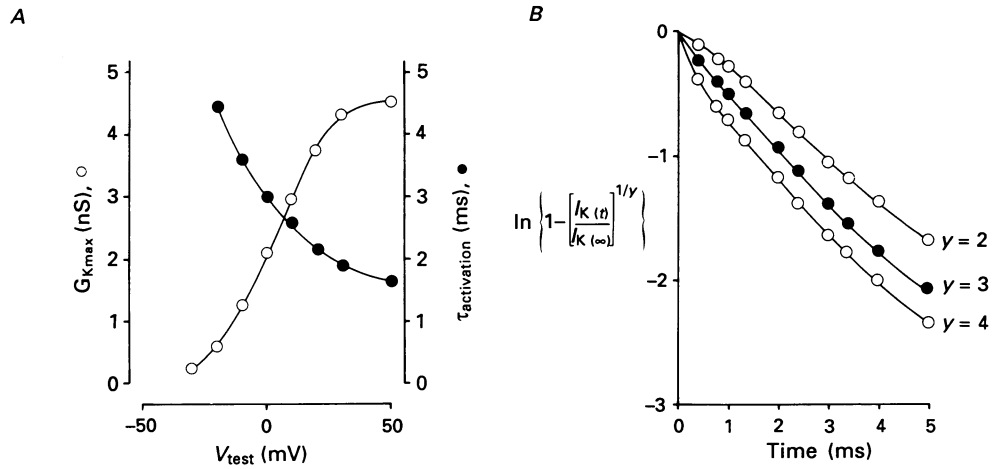

Fig. 2. Kinetics of the delayed outward current. *A*, whole-cell conductance (G_{Kmax}) at the end of the 100 ms voltage step to the test potential calculated from current records in Fig. 1*A* and activation time constants ($\tau_{activation}$; assuming $y = 3$) plotted against test potential (V_{test}). *B*, time course of outward current development evoked by voltage step to 0 mV from a holding potential of −60 mV. This time course was plotted according to eqn (2) and setting $y = 2$, 3 or 4.

potential (−60 mV) to +20 mV to fully activate I_K. I_{tail} was recorded when the potential was then stepped to a series of more negative potentials (V_{tail}; Fig. 3*A*). As shown in Fig. 3*B*, the polarity and amplitude of I_{tail} was dependent on V_{tail}. For inward tail currents, the amplitude was measured at the peak of I_{tail}; for outward tail currents, the amplitude was measured at the same time point as that for the minimum inward tail current. The relationship between tail current amplitude and V_{tail} was linear between −100 and −50 mV, and showed a rectification at more negative potentials. With a 4·8 mM-K^+ external solution, the I_{tail} reversed polarity at −83 mV (Fig. 3*B*) which was close to the equilibrium potential of K^+ (−85 mV).

We also studied the dependence of the reversal potential of the I_K tail current on the external K^+ concentration. An increase of the external K^+ concentration from 4·8 mM to 15 mM and to 25 mM evoked a shift of the reversal potential from −83 mV to −50 mV and to −42 mV respectively (Fig. 3*C*). The slope of the relationship between reversal potential and K^+ concentration was 57·6 mV/decade, close to the estimate of the shift of the K^+ equilibrium potential which is predicted by the Nernst equation for these conditions.

Effect of tetraethylammonium on I_K

Tetraethylammonium (TEA; 10 mM), a potent blocker of I_K channels, was locally applied close to the soma of the cell under study ($n = 4$) using a 50 μm pressure-ejection micropipette. Under these conditions, it was assumed that the concentration of TEA close to the cell membrane (applied by low pressure; > 0·2 lbf/in^2) was close to the concentration of the solution in the micropipette.

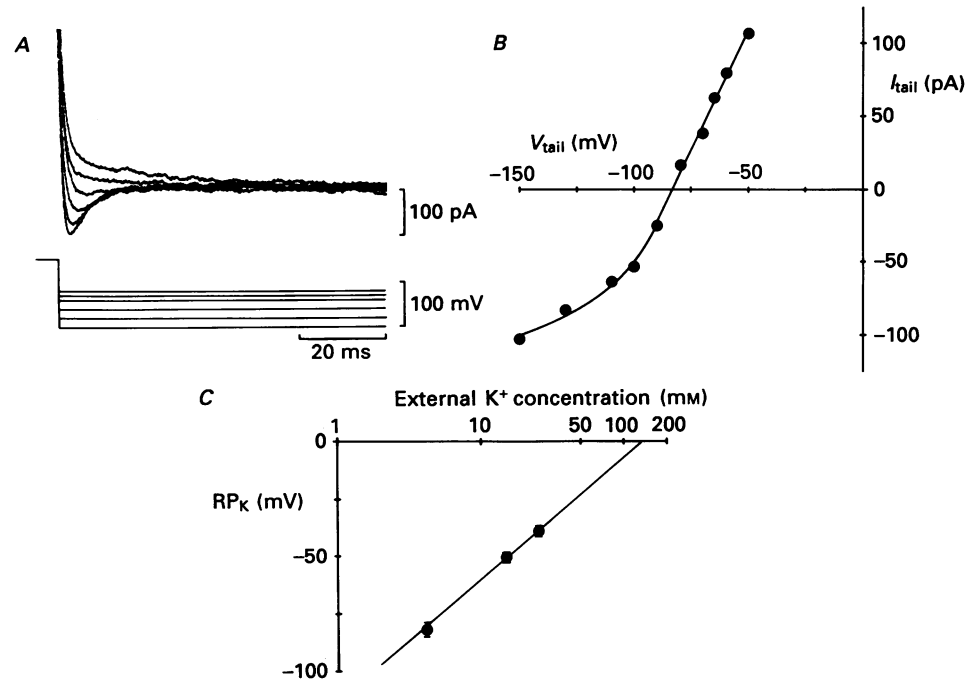

Fig. 3. Reversal potential of I_K tail current. *A*, tail currents (I_{tail}) of I_K recorded upon the offset of an activating voltage step to +20 mV when the membrane was clamped to a potential (V_{tail}) in the range −150 to −50 mV ($V_h = -60$ mV, external K^+ concentration = 4·8 mM). In this figure only records from V_{tail} −150 to −60 mV are shown. *B*, relationship between I_{tail} and V_{tail} for the data shown in *A*. *C*, relationship between the reversal potential of the tail current (RP_K) and K^+ concentration in the external medium. Each point shows averaged data from three cells.

Before TEA application, the *I–V* relationship between I_K and V_{test} was determined (Fig. 4*A*). Then activating voltage steps were applied at 1 s intervals as the TEA-containing micropipette was brought up close to the neurone and a second *I–V* relationship for I_K was determined when the TEA effect was maximal (Fig. 4*A*). I_K decreased within 2 s of onset of TEA application and the effect remained stable until the micropipette was removed: the effect of TEA was reversed by removal of the TEA-containing pipette. As shown in Fig. 4*B*, TEA application did not affect the threshold of I_K but clearly diminished G_{Kmax} which is seen from the different slopes of the *I–V* relationships obtained before and during TEA application. Thus TEA primarily appeared to affect the underlying channel conductance rather than the channel kinetics.

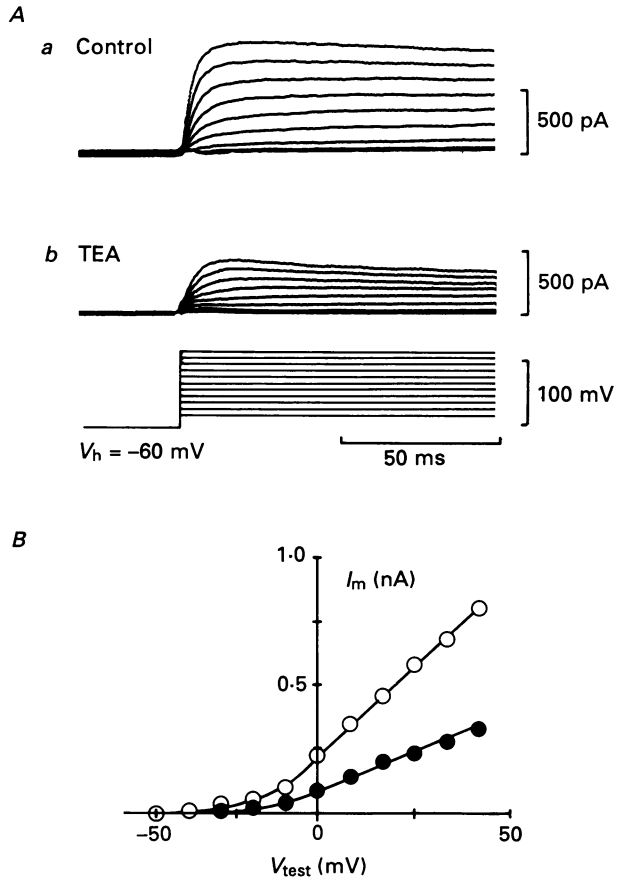

Fig. 4. Effect of TEA application on delayed outward current. *A*, outward currents evoked by voltage steps from V_h −60 mV to test potentials (V_{test}) before and during application of 10 mM-TEA. *B*, current (I_m)–voltage (V_{test}) relationship of delayed outward currents before and during TEA application. ○, control; ●, +TEA.

Transient outward current

Figure 5*A* illustrates the different currents activated by a step to 0 mV from V_h −60 mV and V_h −100 mV in an external calcium- and sodium-free solution. Clearly the step from −100 mV activated an early transient current that preceded the delayed rectifier current ($N = 15$ cells). This transient current is similar to the A-current (I_A) described by Connor & Stevens (1971). We have used their protocol to separate I_A from the total outward current as follows. Total outward current was recorded at a series of test potentials from V_h −100 mV, when both I_A and I_K were observed, and V_h −60 mV when only I_K was recorded. The current recorded at a given test potential from V_h −60 mV was subtracted from the current evoked at the same potential after a step from V_h −100 mV: the 'difference' current illustrated in Fig. 5*A* is that portion of the total current evoked from V_h −100 mV which we have considered to be the isolated I_A current. A small transient component is revealed by

TEA at V_h of −60 mV (Fig. 4*A*). However, it should be noted that neither this 'difference' technique nor pharmacological methods to separate I_A and I_K can completely isolate the two currents; indeed, as shown below, agents used to separate the two currents pharmacologically do in fact affect both currents. Thus the 'difference' current isolated by the 'difference' technique cannot be considered completely free of contamination by I_K although in our analysis we have ignored any possible minor contamination.

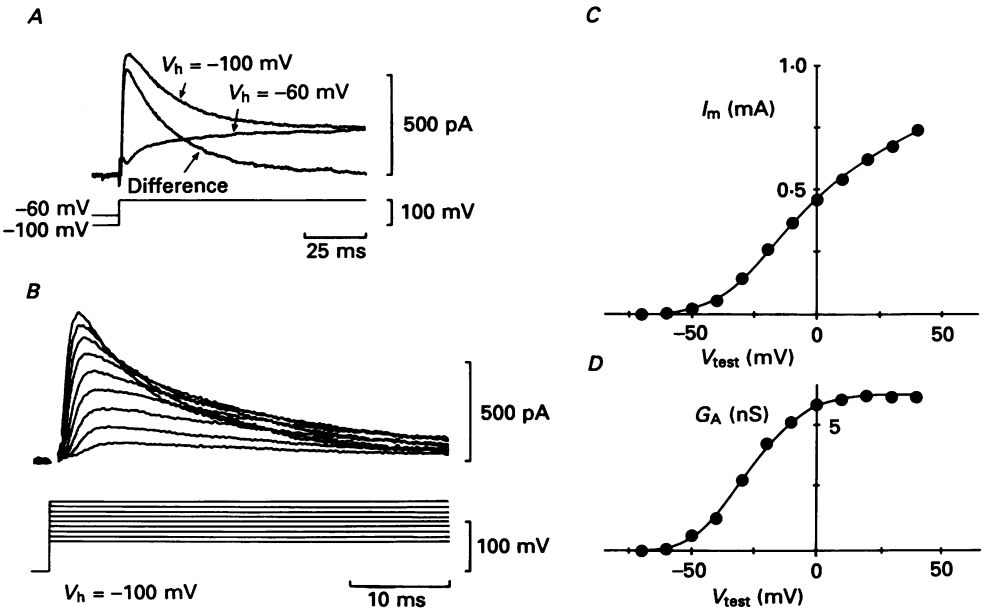

Fig. 5. Separation of I_A from the total K^+ current. *A*, I_A (Difference) was obtained by subtracting the current obtained by a voltage step to 0 mV from a holding potential (V_h) −60 mV from that obtained at the same potential from V_h −100 mV. *B*, I_A evoked by voltage steps to a series of test potentials after subtraction of I_K ($V_h = -100$ mV). *C*, current (I_m)–voltage (V_{test}) relationship of I_A obtained from records in *B*. *D*, conductance (G_A) calculated at the peak of the outward current plotted against test potential (V_{test}); data taken from records in *B*.

The time course and amplitude of I_A can be seen to be dependent on the test potential (Fig. 5*B* and *C*). I_A developed from a threshold close to −60 mV and was characterized by its fast activation and inactivation. The conductance (G_A) underlying I_A was calculated with respect to the equilibrium potential of K^+ as described from G_K. The reversal potential for I_A, estimated by measuring the amplitude of the tail current at a series of values of V_{tail} following a 10 ms, maximally activating voltage step, was close to E_K (−80 mV; $n = 3$; not shown). G_A increased rapidly from −50 mV to +10 mV and remained constant at more positive potentials (Fig. 5*D*). In the eight cells tested the maximum conductance (G_{Amax}) was measured at the peak of the current evoked by a depolarizing step to +10 mV. G_A varied from 3·7 to 7·8 nS amongst the cells tested (5·9 ± 1·6 nS, mean ± S.E.M.; $N = 8$), a similar range to that observed for G_K.

Kinetics of I_A

Since I_A seemed to develop with a sigmoidal rise and inactivated with an exponential fall, it appeared that the kinetics of I_A might be described along the lines proposed by Connor & Stevens (1971). Following the Hodgkin & Huxley (1952) method using an empirical model:

$$G_A = a^n b G_{Amax}, \tag{4}$$

where G_A is the conductance variation, G_{Amax} is the maximum conductance, a^n is the probability a, for each particle n, to be in the correct conformation for channel opening and b represents the inactivation.

Over a range of potentials from -50 to $+20$ mV, I_A decayed as a single-exponential process: the relationship between time and the ratio I_t/I_{max} (where I_t is the current at time t and I_{max} was the maximum current during the response) was linear for the complete decay process when plotted semilogarithmically (Fig. 6*A*; $N = 3$ cells analysed). Since this decay is exponential, the relationship between current and time is

$$\ln I_t/I_{max} = 1 - \exp^{(-t/\tau_B)}, \tag{5}$$

where I_t is the current at time t, t is the time from the peak current (I_{max}) and τ_B is the inactivation time constant. From this equation, τ_B was determined as the time at which I_t/I_{max} was 0·62 and was taken directly from the semilogarithmic plot of I_t/I_{max} against t (Fig. 6*A*).

For more positive potentials, the best fit was obtained with two exponentials: the early and late phases of decay showed a linear relationship between time and I_t/I_{max} plotted semilogarithmically only when plotted separately (Fig. 6*A*). At these more positive potentials, the first process had a time constant smaller than 1·5 ms. The inactivation time constant of I_A in these neurones was partially voltage dependent (Fig. 6*B*); it was independent of membrane potential over a range of -40 to 0 mV but declined exponentially at more positive potentials to reach a value of 13 ms at $+40$ mV (27 ms at -10 mV).

Assuming that I_A can be described following the Hodgkin and Huxley method, this transient outward current can be fitted by the following equation (Connor & Stevens, 1971):

$$I_{A(t)} = K[1 - (\exp^{-t/\tau_A})]^N (\exp^{-t/\tau_B}), \tag{6}$$

where I_A is the current in response to a depolarization step, K is an appropriate scaling factor, N is the activation exponent, τ_B is the inactivation time constant and τ_A is the activation time constant, and t is the time after onset of the voltage step. Its logarithmic transformation is thus:

$$\ln\{K - [I_A/(K \exp^{-t/\tau_B})]^{1/N}\} = -t/\tau_A. \tag{7}$$

This permits an estimation of N by plotting the quantity $\ln\{K - [I_A/(K \exp^{-t/\tau_B})]^{1/N}\}$ against t. As shown in Fig. 6*C*, a linear plot was obtained with $N = 4$ for currents evoked by different voltage steps ($N = 3$ cells). In contrast to I_A in molluscan neurones, this analysis showed the activation time constant to be voltage dependent in these neurones: it decreased exponentially from 2·1 to 0·5 ms over the voltage range -40 to $+40$ mV (Fig. 6*D*).

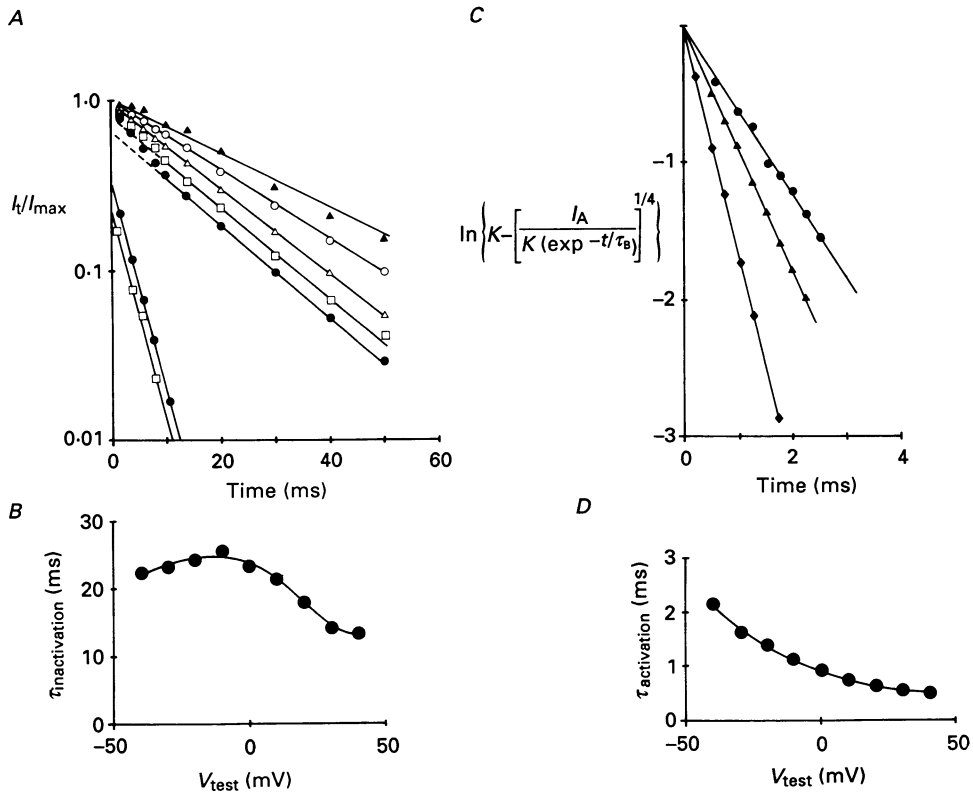

Fig. 6. Voltage dependence of I_A kinetics. *A*, semilogarithmic plots of the decaying phase of outward normalized current (I_t/I_{max}) against time for different test potentials (V_{test}). Note that the decay can be fitted with a single exponential at all potentials except +30 and +40 mV. V_{test}: ▲, −10 mV; ○, +10 mV; △, +20 mV; □, +30 mV; ●, +40 mV. *B*, inactivation time constant ($\tau_{inactivation}$) of I_A plotted against test potential (V_{test}). *C*, rising phase of I_A plotted according to eqn (6) and setting $N = 4$ for different test potentials. V_{test}: ●, −30 mV; ▲, −10 mV; ◆, +20 mV. *D*, activation time constant ($\tau_{activation}$) of I_A calculated according to eqn (7) plotted against test potential (V_{test}).

Voltage-dependent inactivation of I_A

As already noted, activation of I_A requires a holding potential negative to −60 mV. The extent of the steady-state, voltage-dependent inactivation of I_A was studied by recording I_A at two different holding potentials (−40 and −20 mV) following a step (100 ms) to a series of 'pre-pulse potentials' more negative than −50 mV. As shown in Fig. 7*A* and *B*, I_A was activated when the pre-pulse potential was more negative than −70 mV: as the pre-pulse potential was made more negative, the amplitude of I_A increased until a plateau was reached at a pre-pulse potential of −120 mV (Fig. 7*C*). The relationship between current amplitude and the pre-pulse potential is an S-shaped curve: no difference was found in shape between the two steady-state inactivation curves obtained for V_h −20 mV and V_h −40 mV (Fig. 7*C*). The current was half-inactivated when the pre-pulse potential was −80 mV and was completely inactivated at pre-pulse potentials of −65 mV or

more positive. However, for any given pre-pulse potential, the current at V_h −40 mV was smaller than at V_h −20 mV, in line with the voltage dependence of I_A activation ($N = 6$ cells).

Effect of K^+ channel blockers on I_A activation

The effects of 4-aminopyridine (4-AP), a K^+ channel blocker known to decrease I_A in some preparations (Thompson, 1977; Gustafsson, Galvan, Grafe & Wigström, 1982), and TEA on I_A were compared. These two compounds were applied locally with the same protocol as described for the effects of TEA on I_K.

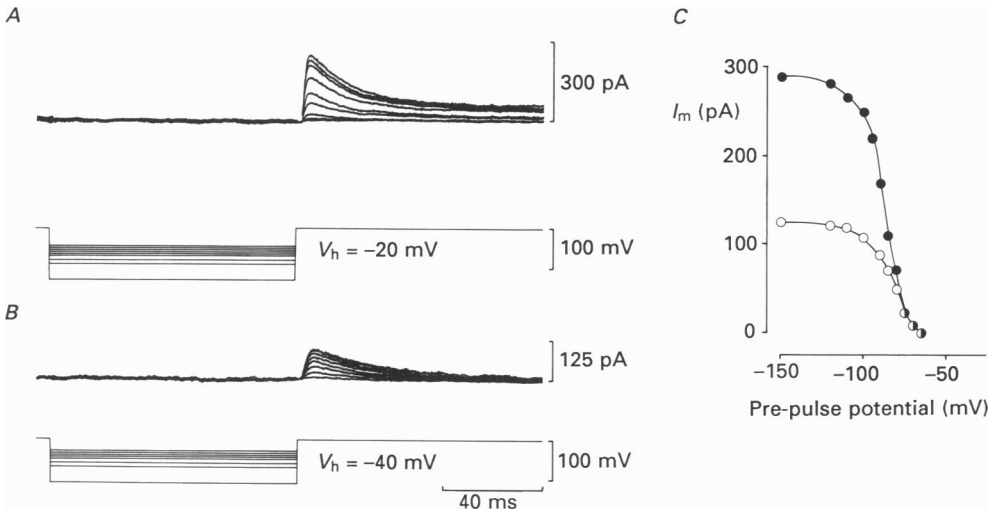

Fig. 7. Steady-state inactivation of the transient current I_A. *A* and *B* show transient current responses (upper traces) on the return of the membrane to the holding potential (V_h; −40 and −20 mV) after a series of negative voltage steps to the pre-pulse potential (lower traces); leakage current was removed by computer subtraction of the response following a 5 mV negative voltage step (which did not reactivate I_A). *C*, plot of peak outward current (I_m) against pre-pulse potential for each holding potential (data obtained from *A* and *B*). V_h = ●, −20 mV; ○, −40 mV.

The voltage-dependent activation of I_A before and during pressure application of 4-AP was studied by applying 100 ms voltage steps to a series of test potentials. Application of 1 mM-4-AP dramatically suppressed the first component of the outward current evoked from V_h −100 mV, corresponding to I_A (Fig. 8*A*; $N = 6$ cells). The threshold for the current was not affected and the *I–V* relation was not shifted, suggesting that I_A blockade by 4-AP was via an effect on single-channel conductance and not on single-channel kinetics. In addition, the shape of the rising phase of the unblocked portion of I_K during 4-AP application led us to suppose that a residual part of I_A was unblocked. Computer subtraction of outward current before and during drug application showed the total current blocked by 4-AP (Fig. 8*A*). The current–voltage (*I–V*) relationship of this fast 4-AP-sensitive current, shown in Fig. 8*B*, was similar to that described for I_A isolated by the 'difference' method (see Fig. 5). However, 4-AP seemed also to partially block I_K by decreasing G_K: that is

the current at the end of a 100 ms activating voltage step was also slightly diminished (Fig. 8*C*).

Application of TEA (10 mM) principally decreased I_K (the current at the end of a 100 ms activating voltage step; Fig. 9*A* and *B*); the change in the slope of the current–voltage relationship for I_K indicates a decreased single-channel conductance

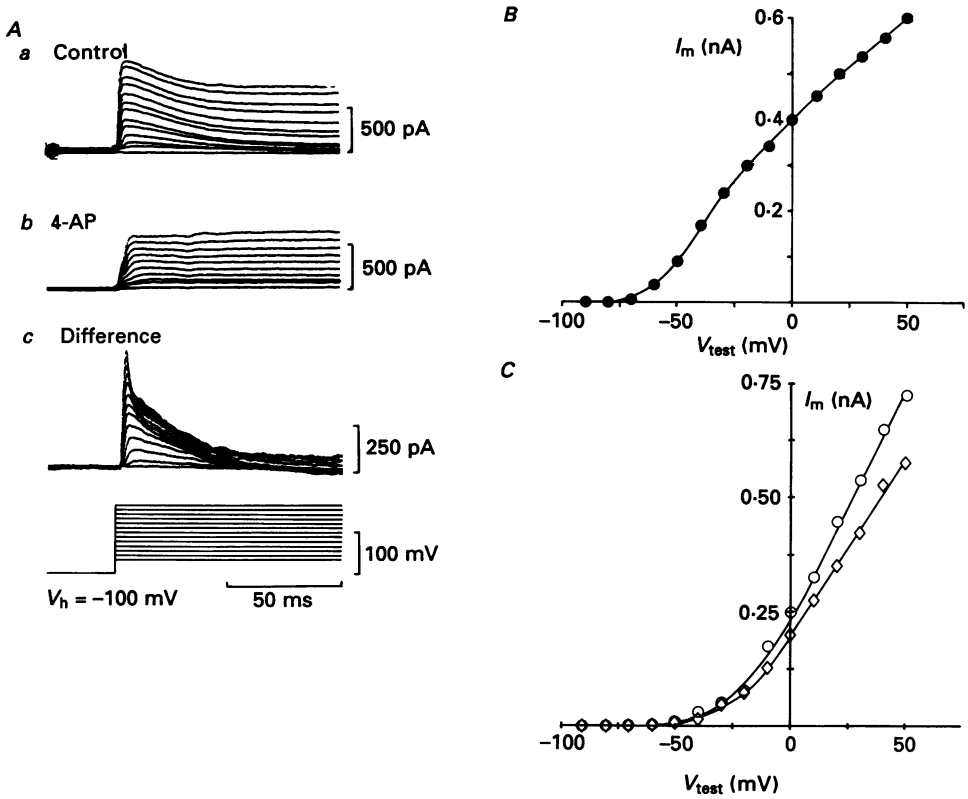

Fig. 8. Effect of 4-AP on steady-state activation of I_A. *A*, outward currents evoked by voltage steps from V_h −100 mV before (*a*) and during (*b*) 4-AP (1 mM) application; *c* shows the current blocked by 4-AP, obtained by computer subtraction of records in *b* from those in *a*. *B*, current (I_m)–voltage (V_{test}) relation of I_A, measured as the peak current in *A c*. *C*, current (I_m)–voltage (V_{test}) relationship of I_K measured at the end of the 100 ms step before and during 4-AP application demonstrating the partial sensitivity of this current to 4-AP. ○, I_K control; ◇, I_K+4-AP.

produced by TEA. At the same concentration, TEA also reduced I_A acting by an apparent decrease in the rate of activation of the current (Fig. 9*C*). The effect on channel kinetics rather than on channel conductance is confirmed by the current–voltage relationship for I_A: the curve is shifted to the right by TEA but has an unchanged slope.

Calcium-dependent K^+ current

A Ca^{2+}-dependent K^+ current was observed when 2 mM-$CaCl_2$ was present in the external medium. The amplitude of the total current activated by 100 ms steps from

a holding potential of −30 mV was greater at each test potential when Ca^{2+} was present. The effect of Ca^{2+} was best observed as a reduction of the current after addition of Co^{2+} (1 mM) which blocks Ca^{2+} channels (Fig. 10*A* and *B*) suggesting that the blocked portion of the total current represented a calcium-dependent K^+ current ($I_{K(Ca)}$). The contribution of this current to the total current was determined by

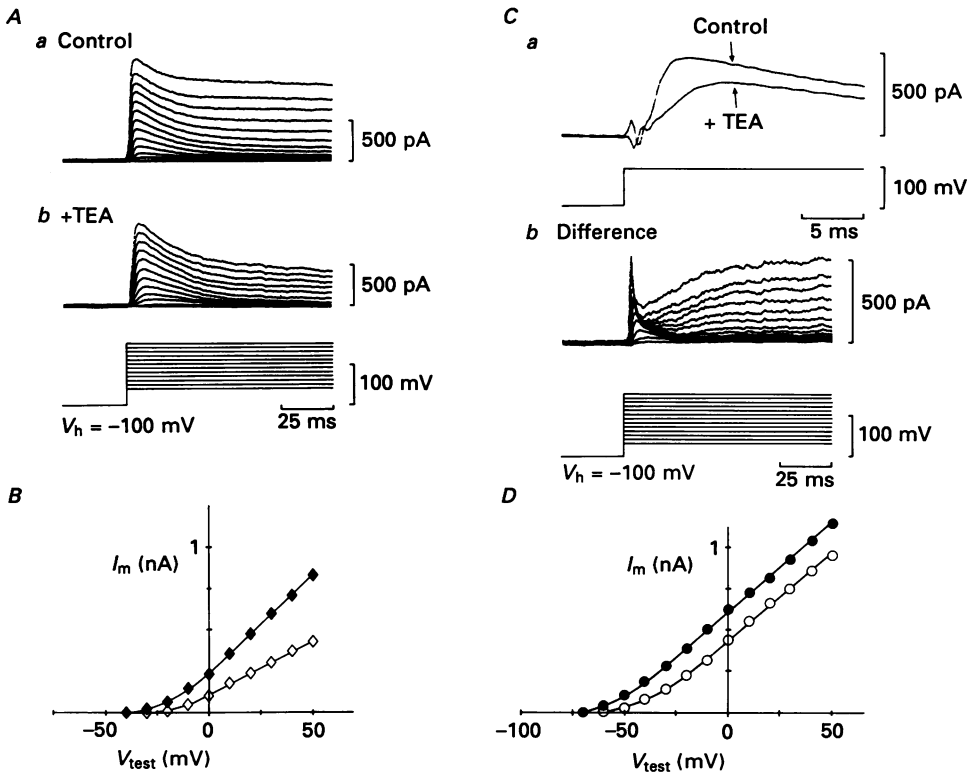

Fig. 9. Effect of TEA application on the steady-state activation of I_A. *A*, total outward current evoked by voltage steps from V_h −100 mV before (*a*) and during (*b*) 10 mM-TEA application. *B*, current (I_m)–voltage (V_{test}) relationship of I_K measured at the end of 100 ms voltage steps before and during TEA application. ◆, I_K; ◇, I_K+TEA. *Ca*, superimposition of total outward current evoked by a voltage step to 0 mV before and during TEA application. *Cb*, separation of the outward current blocked by TEA by computer subtraction. *D*, current–voltage relationship of the peak transient current (I_A) before and during TEA application demonstrating the partial sensitivity of this current to TEA. ●, I_A peak; ○, I_A peak +TEA.

subtracting the current responses obtained in the presence and absence of $CoCl_2$ (Fig. 10*B*). $I_{K(Ca)}$ developed from a threshold of −20 mV, reached a maximum at about +15 mV, and then declined until it was undetectable at −80 mV.

Increasing the holding potential to −60 mV changed the shape of the *I–V* curve of the current blocked by Co^{2+} ($N = 5$ cells). For any test potential, the total current activated from this V_h was greater than that from −30 mV (Fig. 10*C* and *D*). Subtraction of the current responses obtained in the presence of Co^{2+} from those recorded in the absence of Co^{2+} revealed a small inward current between −50 and

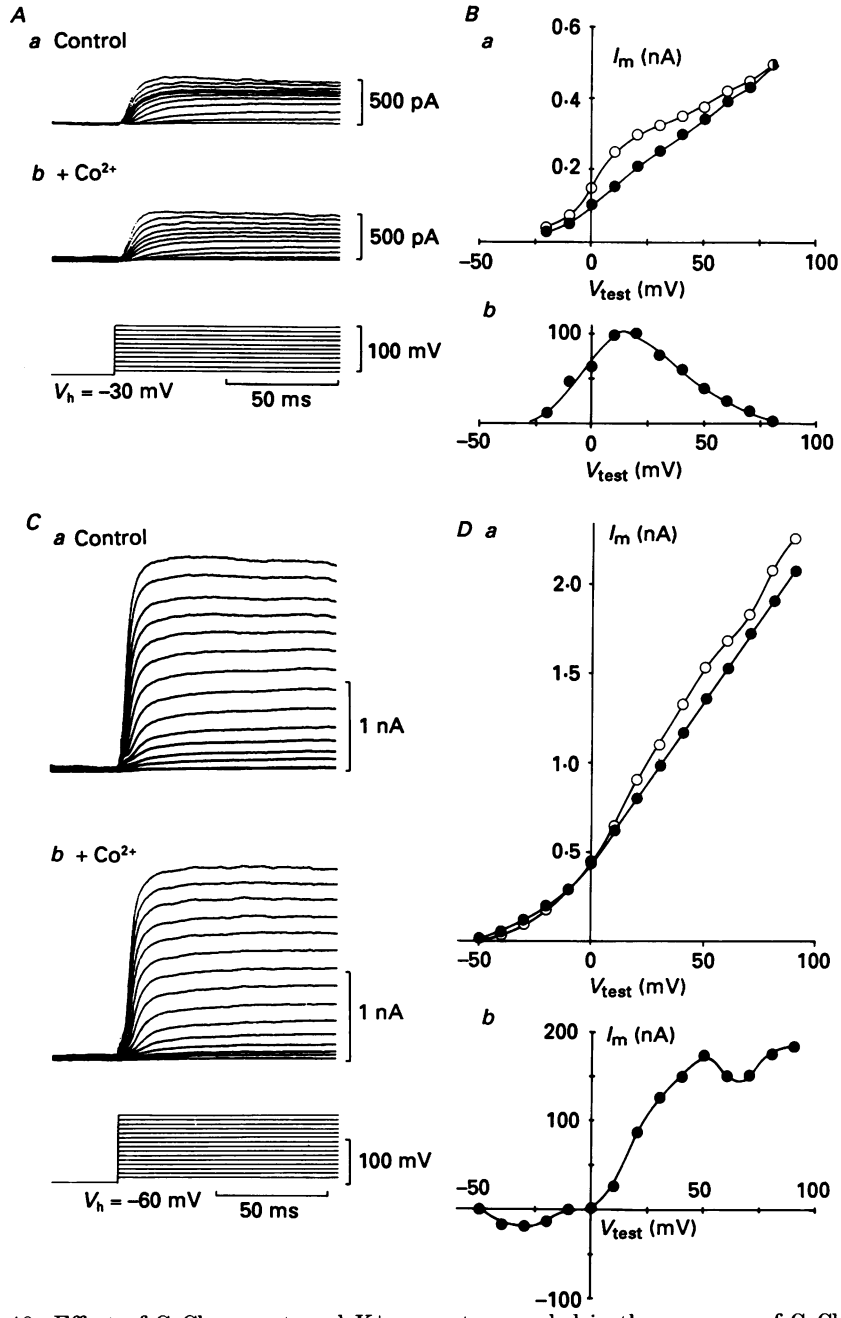

Fig. 10. Effect of $CoCl_2$ on outward K^+ currents recorded in the presence of $CaCl_2$. *A*, outward current evoked by voltage steps to a series of test potentials before (*a*) and during (*b*) 2 mM-$CoCl_2$ application ($V_h = -30$ mV). *Ba*, current (I_m)–voltage (V_{test}) relationship of total K^+ current before and during $CoCl_2$ application. ○, control; ●, $+Co^{2+}$. *Bb*, current–voltage relationship of Ca^{2+}-activated K^+ component of the total current. *C*, outward current evoked by voltage steps to a series of test potentials before (*a*) and during (*b*) 2 mM-$CoCl_2$ application ($V_h = -60$ mV). *Da*, current–voltage relationship of total K^+ current before and during $CoCl_2$ application. ○, control; ●, $+Co^{2+}$. *Db*, current–voltage relationship of Ca^{2+}-activated K^+ component of the total current.

−10 mV, and an outward current at more positive potentials. This outward current reached a maximum value at +50 mV then declined between +50 and +70 mV and increased again at more positive potentials. This complex I–V relation was obtained from five cells tested, and may represent a summation of both inward Ca^{2+} current and outward $I_{K(Ca)}$, suggested by the small inward currents observed between −50 and −10 mV and the outward current at more positive potentials.

DISCUSSION

The present experiments demonstrate that cultured neurones from the SON of 2- to 4-day-old neonatal rats have three types of voltage-activated outward K^+ current: a delayed current, a transient current and a calcium-dependent current.

The delayed K^+ current (I_K) was similar to the delayed rectifying K^+ conductance described in many other cells (for review, see Hille, 1984). It can be described by the equation $G_K = n^y G_{Kmax}$ with $y = 3$. This value is lower than that obtained by Hodgkin & Huxley (1952) but higher than that found, for example, in sympathetic neurones of the bull-frog ($y = 2$; Adams, Brown & Constanti, 1982).

As described in some other cells (Adams *et al.* 1982; Galvan & Sedlmeier, 1984; Kaneko & Tachibani, 1985), I_K decayed during long activating voltage steps. It is unlikely that this decline was due to a shift of E_K caused by a progressive extracellular accumulation of K^+ since such an effect observed in intact systems is the consequence of a small interstitial space (Adams *et al.* 1982; Perez-Armendariz & Atwater, 1985) which is not present in dissociated cell culture. The time-dependent decline of I_K must therefore reflect an inactivation of G_K. Since the time course of I_K inactivation was described by more than one exponential, G_K may reflect the underlying activity of more than one class of ionic channel. Multiple components of G_K, each with different voltage sensitivities, have previously been described for the frog node of Ranvier (Dubois, 1981). The voltage sensitivity of I_K in SON cells suggests that G_K in these neurones is apparently similar to the f2 component of I_K described by Dubois (1981). However, G_K in the node of Ranvier was completely suppressed by 4-AP application, while, in our experiments, 4-AP only partly attenuated I_K. Conversely, TEA blocked only one component of G_K in the node of Ranvier but reduced G_K by 50% (at the same concentration) in the present study. (If more than one type of I_K channel exists in these cultured neurones, then TEA does not appear to preferentially block one type of channel.) The effect of external TEA that we observed is similar to that observed on frog skeletal muscle (Stanfield, 1970) but different to the actions of TEA on I_K in some other cells (Tasaki & Hagiwara, 1957; Hille, 1967; Wong & Binstock, 1980; Rossman & Trube, 1986). These discrepancies are probably due to different external TEA receptors or binding sites on different types of cell.

The second type of K^+ current (I_A) observed in SON neurones resembles the transient outward current described primarily in molluscan neurones (Connor & Stevens, 1971; Neher, 1971; Thompson, 1977) but also in mammalian neurones (Gustafsson *et al.* 1982; Galvan & Sedlmeier, 1984). The voltage sensitivity of the current is very similar to those observed in the snail (Connor & Stevens, 1971) and in mammalian sympathetic ganglia (Galvan & Sedlmeier, 1984). Some kinetic proper-

ties are also similar. It can be described by the same equation, $G_A = a^N b G_{Amax}$, as in the snail (Connor & Stevens, 1971) or in *Tritonia* (Thompson, 1977) but the activation and inactivation time constants were less than those in invertebrates. The voltage dependence of the inactivation time constant was observed at more positive potentials than in snail and *Tritonia* neurones, and, unlike the situation in the invertebrate neurones, the activation time constant varied exponentially with voltage.

4-AP blocked I_A in SON neurones principally by reducing the conductance (G_A) and it was more potent on these neurones than on snail neurones (Thompson, 1977) where it has various effects on the kinetics of I_A. On the other hand, TEA reduced G_A only slightly but shifted the I–V curve of I_A in the positive direction without modifying its shape, indicating an effect on the kinetics of the current. We have interpreted the data on TEA effects on I_A and I_K by different mechanisms. However, we cannot rule out the possibility that the apparent effect on I_A may in fact be an effect on the kinetics of a contaminating fraction of I_K; only single-channel recordings will resolve this difficulty.

The calcium dependence of I_A has been extensively studied in a variety of preparations. I_A was partially Ca^{2+}-dependent in invertebrate neurones (Thompson, 1977) and in hippocampal neurones (Gustafsson *et al.* 1982) and it was completely suppressed by Cd^{2+} application or by removing external calcium in rat sympathetic neurones (Galvan & Sedlmeier, 1984). More recently Bourque (1988) reported a Ca^{2+}-dependent transient outward K^+ current in adult rat SON neurones. This Ca^{2+}-dependent current was 90% blocked by addition of Cd^{2+}, 50% blocked by 1 mM-4-AP and unaffected by TEA (12 mM). The current designated I_A in the present study was in contrast of similar amplitude in the presence or absence of the Ca^{2+} channel blocker Co^{2+}, almost totally blocked by 1 mM-4-AP, and apparently had slightly altered kinetics in the presence of TEA (10 mM). Clearly the transient current in neonate and adult neurones differs in Ca^{2+} sensitivity. However, where direct comparisons can be made, there appear to be some similarities: inactivation time constant (25–30 ms at −40 mV), latency to peak (about 5 ms at −50 mV), and steady-state inactivation curve 'mid-point' (−80 mV in neonates and −75 mV in adults). Therefore the two currents may in fact be mediated by the same species of channel, but represent different developmental stages of that channel. This difference is detected as different Ca^{2+} and pharmacological sensitivities. Although I_A has been suggested to control a portion of the interspike interval in neurones, whether these apparent differences between the transient K^+ current in neonates and adults affect differentially the generation of action potentials can only be a matter of speculation since recordings of firing activity of neonatal SON neurones *in situ* have not been made.

In SON neurones, the Ca^{2+}-dependent K^+ current ($I_{K(Ca)}$) was recorded in the presence of 2 mM-external Ca^{2+}, and it was suppressed by Co^{2+}. The voltage sensitivity of this current is similar to that described in some other vertebrate cells (Heyer & Lux, 1976; Kaneko & Tachibana, 1985) and the current did not seem to inactivate with time as observed for invertebrates (Aldrich, Getting & Thompson, 1979) and some other vertebrate cells (Frankenhaeuser, 1963; Adams *et al.* 1982; Galvan & Sedlmeier, 1984). The maximum current was recorded at about +15 mV

which is the same potential at which the maximum current passing through voltage-activated Ca^{2+} channels may be recorded (Cobbett & Mason, 1987). The amplitude of $I_{K(Ca)}$ was dependent on the holding potential: these data also reflect the involvement of voltage-operated Ca^{2+} channels since currents through these channels are dependent on the holding potential (P. Cobbett & W. T. Mason, unpublished observations).

The functional significance of the K^+ currents described in the present study must be considered in relation to the inward cationic currents previously noted in these cultured neurones (Cobbett & Mason, 1987), and to the electrophysiological behaviour exhibited by these neurones *in vivo* and also recorded in *in vitro* preparations.

I_K may be activated when the resting or holding potential is -60 mV or more positive and it is only slowly inactivated during prolonged activating voltage steps. These properties therefore suggest that this current is responsible in large part for the repolarization phase of the action potential, as is the case for other neurones (Hille, 1984). The slight voltage- and time-dependent inactivation of the current may, however, underlie part of the prolongation of the action potential during the initial part of a burst of action potentials as recorded from vasopressinergic neurones *in vivo* and *in vitro* (Mason & Leng, 1984; Andrew & Dudek, 1985).

The role of I_A may be complex but it has been suggested that this current controls the rate of firing by modulating the rate of depolarization of the membrane between the after-hyperpolarization of one action potential and the threshold for the next (Connor, 1978; Salkoff & Wyman, 1980; Gustafsson *et al.* 1982; Hille, 1984). A depolarizing stimulus will be reduced in effectiveness when I_A is completely non-inactivated but the same stimulus will depolarize the neurone to a greater extent if I_A is inactivated or blocked (Gustafsson *et al.* 1982) and thus may induce firing. Thus I_A may control burst initiation and firing frequency within bursts of action potentials, maintaining firing at less than about 15 Hz. However, if I_A becomes partially inactivated, which would occur at higher firing frequencies, then the neurone will fire more frequently (a process which will itself further inactivate this current). This may be the underlying mechanism which permits oxytocin neurones to fire at up to 80 Hz during so-called milk-ejection bursts.

The Ca^{2+}-dependent K^+ current is, by definition, dependent on an accumulation of free Ca^{2+} in the cytoplasm and has been suggested to be the underlying mechanism controlling burst duration (Andrew & Dudek, 1984*a*, *b*; Bourque, Randle & Renaud, 1985). Calcium may enter the cell via voltage-activated Ca^{2+} channels (Cobbett & Mason, 1987) in two situations; first, a sustained, if small, influx during the Ca^{2+}-dependent plateau potential which underlies each burst of action potentials in vasopressin neurones; second, influx during each action potential (Bourque & Renaud, 1985) during a burst of action potentials in either oxytocin or vasopressin cells. The accumulated free Ca^{2+} in the cytoplasm will then permit $I_{K(Ca)}$ to be activated and the neurone will hyperpolarize. This burst-induced hyperpolarization has been recorded intracellularly following current-evoked bursts in SON neurones in slices and explants (Andrew & Dudek, 1984*b*; Bourque *et al.* 1985), and the activation of this current may be the underlying mechanism by which bursts may be terminated *in vivo* by antidromic stimulation (Dreifuss, Tribollet, Baertschi &

Lincoln, 1976). $I_{K(Ca)}$ found in vertebrate pacemaker bursting neurones has been suggested to have a similar role in controlling burst durations (Gorman, Hermann & Thomas, 1981).

In conclusion, therefore, we have recorded isolated, voltage-activated K^+ currents from cultured neurones of the supraoptic nucleus of the neonatal rat. Three currents, which may have crucial roles in regulating the firing frequency and firing patterns of these neurones, have been distinguished on the basis of their voltage sensitivity, activation and inactivation characteristics, Ca^{2+} dependence and pharmacology. The role of these three currents in the control of firing activities in the SON neurones is presently under examination.

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