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CHARACTERISTICS OF MULTIPLE VOLTAGE-ACTIVATED K^+ CURRENTS IN ACUTELY DISSOCIATED CHICK CILIARY GANGLION NEURONES

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SUMMARY

1. The properties of voltage-activated K^+ currents were examined using whole-cell recording techniques in acutely isolated chick ciliary ganglion neurones.

2. Application of depolarizing voltage pulses from a holding potential of −60 mV evoked sustained outward currents that inactivated with time constants of hundreds of milliseconds (I_{DR}). Bath application of 10 mM tetraethylammonium (TEA) caused a 70–90% reduction of I_{DR}. Application of depolarizing voltage steps from a holding potential of −120 mV revealed a second class of TEA-resistant outward currents. These currents activated quickly but inactivated completely within tens of milliseconds (I_A). I_A activated at more negative command potentials than I_{DR}. However, I_{DR} exhibited a steeper voltage dependence of activation than I_A.

3. The midpoint of the steady-state inactivation curve of I_A was between −95 and −110 mV. By contrast the midpoint of the steady-state inactivation curve of I_{DR} was between −80 and −90 mV. It was not possible to produce a complete inactivation of I_{DR} using prepulses of up to 2 s duration.

4. The time course of I_A inactivation could only be fitted with double-exponential curves with time constants of 5–18 ms and 30–60 ms at membrane potentials positive to −30 mV. The inactivation of I_A was slower at more positive membrane potentials because of a greater contribution of the long time constant. The individual time constants were not markedly voltage dependent.

5. Bath application of 5 mM 4-aminopyridine (4-AP) caused a 70–100% block of I_A whereas 1 mM 4-AP was ineffective. Bath application of 560 nM α-dendrotoxin (DTX) produced a 50–70% reduction of I_A, but application of 280 nM DTX had no effect on I_A.

6. Application of 1 mM 4-AP produced a reversible 55–80% block of I_{DR} measured at the end of a 500 ms depolarizing pulse. The 4-AP-sensitive components of I_{DR} activated rapidly and exhibited a gradual inactivation with continued depolarization. The 4-AP-resistant components of I_{DR} activated much more slowly and showed very little tendency to inactivate. Significant blockade of I_{DR} was produced by 10 μM 4-AP.

7. The decay of I_{DR} tail currents could only be fitted with double exponential curves with time constants of 3–6 and 40–60 ms, respectively. The fast and slow

components of the tail currents behaved independently with respect to the duration of the depolarizing voltage step.

8. Application of 1 mM 4-AP eliminated the fast, but not the slow component of I_{DR} tail currents. These results suggest that chick ciliary ganglion neurones express a fast 4-AP-sensitive delayed rectifier (I_{DRf}) and a slow 4-AP-resistant delayed rectifier (I_{DRs}). Application of 200 mM DTX caused a 40–60% blockade of I_{DR} but did not appear to discriminate between I_{DRf} and I_{DRs}.

9. Acutely dissociated chick ciliary ganglion neurones express at least three different types of voltage-activated K^+ channels. These include a TEA-resistant I_A as well as two different types of TEA-sensitive delayed rectifiers (I_{DRf} and I_{DRs}) that can be distinguished on the basis of their kinetic properties and their differential sensitivity to 4-AP.

INTRODUCTION

Neurones and virtually all other excitable cells express a variety of voltage-activated K^+ currents that can be separated on the basis of differences in pharmacology, voltage dependence and kinetics. One group of voltage-activated K^+ currents are the so-called delayed rectifier K^+ channels (I_{DR}) (Hodgkin & Huxley, 1952). These currents are characterized primarily by their kinetics, as delayed rectifiers activate slowly and if they show any inactivation at all, it occurs over a time scale of hundreds of milliseconds. A second group includes the A-currents (I_A) (Connor & Stevens, 1971; Thompson, 1977). A-currents activate rapidly but usually become inactivated within tens of milliseconds, although recent studies suggest that some I_A channels can adopt several different kinetic modes (Cooper & Shrier, 1989). In most cells, I_A is resistant to blockade by 10–20 mM external tetraethylammonium (TEA) but is readily blocked by millimolar concentrations of external 4-aminopyridine (4-AP) (Thompson, 1977; Segal & Barker, 1984; Belluzzi, Sacchi & Wanke, 1985; Bossu, Dupont & Feltz, 1985; Numann, Wadman & Wong, 1987).

While the delayed rectifiers are ubiquitous currents, they show considerable variability from cell to cell. For example, in the frog node of Ranvier, half of I_{DR} is blocked by 0·4 mM external TEA (Stanfield, 1983), while 8 mM TEA is required to produce a similar blockade in frog skeletal muscle (Stanfield, 1970). Even within a single cell, I_{DR} may not be a single homogeneous current. At least three components of I_{DR} have been identified in a single node of Ranvier, with different kinetics and pharmacology (Dubois, 1981). Analysis of single-channel recordings has also demonstrated several distinct K^+ channels underlying the voltage-activated K^+ conductance in a single cell (Harris, Henderson & Spitzer, 1988; Quandt, 1988).

A-currents also show considerable variability. For example, I_A in rat sympathetic neurones is blocked by 1–2 mM 4-AP (Belluzzi *et al.* 1985), while I_A in bullfrog sympathetic neurones is insensitive to 4-AP (Adams, Brown & Constanti, 1982). In rat hippocampal neurones, I_A is largely blocked by 50–300 nM α-dendrotoxin (DTX) (Halliwell, Othman, Pelchen-Matthews & Dolly, 1986), while an A-current isolated from the rat cerebral cortex known as RCK-4 is completely resistant to DTX (Stühmer *et al.* 1989).

This diversity in the properties of voltage-activated K^+ channels is now thought to be due in part to the existence of multiple potassium channel gene families. Several

different gene families have been discovered in the last few years that encode delayed rectifier channel proteins (Stühmer *et al.* 1989; Wei, Covarrubias, Butler, Baker, Pak & Salkoff, 1990). In *Drosophila*, the *Shaker* gene undergoes alternative splicing (Schwarz, Tempel, Papazian, Jan & Jan, 1988) thereby producing many related channel proteins with slightly different properties. In vertebrates, alternative splicing of K^+ channel genes has also been reported (Luneau *et al.* 1991). In addition, the products of different genes can form heteromultimeric channel complexes (Christie, Adelman, Douglass & North, 1990; Ruppersberg, Schroter, Sakmann, Stocker, Sewing & Pongs, 1990) potentially resulting in additional K^+ channel diversity.

The chick ciliary ganglion is frequently used as a model system for studying neuronal development (Pilar & Tuttle, 1982). Our laboratory is currently investigating the factors regulating the expression of ionic conductances in chick ciliary ganglion neurones during embryonic development (Dourado & Dryer, 1992). A necessary foundation for this work is a thorough understanding of the currents that are expressed in the mature neurone. We have already described the Ca^{2+}-activated K^+ currents expressed by these cells (Dryer, Dourado & Wisgirda, 1991). The purpose of this study was to examine I_{DR} and I_A in acutely isolated chick ciliary ganglion neurones. The results suggest that these cells express two distinct components of I_{DR} that can be separated by their kinetics and differential sensitivity to 4-AP, as well as an I_A that is relatively resistant to blockade by both 4-AP and DTX.

METHODS

Chick ciliary ganglion neurones were isolated as described previously (Dryer, 1991; Dryer *et al.* 1991; Dourado & Dryer, 1992). Briefly, ganglia were removed from chick embryos between embryonic days 11 and 14 (E11–E14). Embryos were anaesthetized on ice prior to being killed by decapitation. Ganglia were incubated with collagenase (1 mg ml^{-1}) for 10–30 min at 37 °C. Ganglia were rinsed and dissociated by trituration in a cell culture medium consisting of Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine 50 units ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 3% chick embryo eye extract. Dissociated neurones were plated onto poly-D-lysine-coated glass coverslips and allowed to settle for 30 min. Neurones were used within 4 h of plating, at which time the neurones were still free of neurites.

Whole-cell recordings were made using standard techniques as described previously for K^+ currents (Dryer, 1991; Dryer *et al.* 1991; Dourado & Dryer, 1992, Wisgirda & Dryer, 1993). Pipette salines consisted of (mM): 150 KCl, 2 $MgCl_2$, 10 Hepes/NaOH, 10 EGTA, 5 Mg-ATP and 100 μM leupeptin at a pH of 7·4. Pipette resistances ranged from 4–10 MΩ. It was usually possible to compensate up to 90% of this series resistance without introducing oscillations into the current output of the clamp amplifier (AXOPATCH 1D, Axon Instruments, Foster City, CA, USA). When this was not possible, the cell was abandoned. In these experiments, normal extracellular saline consisted of (mM): 145 NaCl, 5·3 KCl, 6·2 $MgCl_2$, 5-D-glucose and 13 Hepes/NaOH at a pH of 7·4. All salines also contained 500 nM tetrodotoxin to prevent activation of voltage-activated Na^+ currents. External Ca^{2+} was omitted from the external salines in order to prevent activation of Ca^{2+}-activated K^+ currents (Dryer, 1991; Dryer *et al.* 1991; Dourado & Dryer, 1992). All experiments were performed at room temperature (21–23 °C). Drugs (4-AP, TEA, DTX) were dissolved in this saline and were applied by circulation through the entire bath. All drugs and chemicals were obtained from Sigma (St Louis, MO, USA). DTX, however, was obtained from Research Biochemicals Incorporated (Natick, MA, USA; lot number LB-491A) and also from Alomone Labs (Jerusalem, Israel; lot number DE-01).

Voltage commands, data acquisition and data analysis were performed using PCLAMP software version 5·5 (Axon Instruments) and a personal computer as described previously (Dryer *et al.* 1991;

Dourado & Dryer, 1992; Wisgirda & Dryer 1992). Currents were automatically leak-subtracted using on-line P/6 protocols.

RESULTS

Separation of I_{DR} and I_A

Ciliary ganglion neurones express two main classes of voltage-activated K^+ currents that can be separated according to their kinetics and differential sensitivity to external TEA. Fig. 1 shows currents evoked by a depolarizing voltage step to +10 mV from a holding potential of −60 mV (left). This procedure evokes an outward current that inactivates slowly. However, when the same depolarizing step is made from a holding potential of −120 mV (right), a second current is revealed, which inactivates completely and rapidly during the course of the depolarizing voltage step. This rapidly inactivating current appears to be insensitive to bath application of 10 mM TEA, while a large portion of the more sustained current is blocked by 10 mM TEA. Thus, these neurones express sustained TEA-sensitive delayed rectifier currents (I_{DR}) and a rapidly inactivating TEA-resistant A-current (I_A).

These pharmacological and kinetic differences make it possible to examine other characteristics of the two types of currents. Figure 2*A* shows families of currents evoked by application of a series of depolarizing voltage steps (−60 to +30 mV) from a holding potential of −120 mV. In normal saline, the currents remaining at the end of the depolarizing voltage steps (*a*) should consist primarily of I_{DR}, since the majority of I_A will have become inactivated by this time (note time scale). After application of TEA (right), the peak currents (*b*) will consist primarily of I_A, due to the fact that the majority of I_{DR} is blocked by this concentration of TEA, and due to the more rapid activation kinetics of I_A. The normalized conductance–voltage relationship (Fig. 2*B*) is shown for I_{DR} (●) and I_A (▲) with superimposed fitted Boltzmann curves of the form:

$$G/G_{max} = 1/1+\exp(V_h - V/k), \quad (1)$$

where G_{max} is the maximum conductance, V is the command potential, V_h is the voltage at which conductance is one half of the maximum value, and k is a constant related to the steepness of the curve. Conductance was calculated on the basis of a calculated K^+ equilibrium potential of −85 mV. I_{DR} is activated half-maximally at −22 ± 6·2 mV (mean ± S.E.M., $n = 8$ cells). The conductance–voltage relationship for I_{DR} is quite steep ($k = 7$ mV ± 3·2 mV). By contrast, I_A exhibits half-maximal activation at more negative command potentials (−27 ± 4·2 mV) but the conductance–voltage relationship for I_A is not as steep as for I_{DR} ($k = 16$ mV ± 4·3 mV).

The two types of current also exhibit a different voltage dependence of steady-state inactivation, as shown in Fig. 3. In these experiments, currents were evoked by a depolarizing voltage step to +30 mV preceded by a series of 500 ms hyperpolarizing prepotentials (−50 to −140 mV). Again, I_{DR} was measured in normal saline at the end of the depolarizing voltage steps (*a*) while I_A was measured at the peak of the currents recorded in the presence of TEA (*b*). In these salines, I_A exhibits half-maximal inactivation near −100 mV and is essentially completely inactivated at −70 mV. I_{DR} invariably becomes inactivated at more positive membrane potentials, with half-maximal inactivation near −70 mV. But it should be noted that total I_{DR}

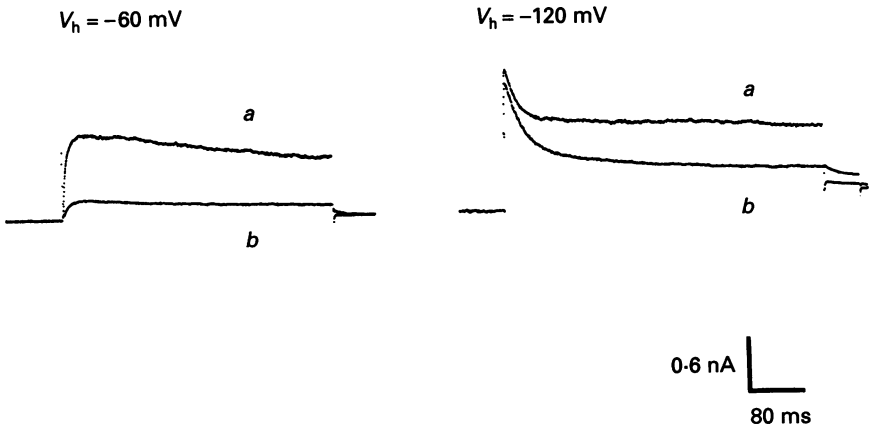

Fig. 1. Voltage-activated K^+ currents in a dissociated chick ciliary ganglion neurone. Left, currents evoked by depolarizing voltage step to +10 mV from a holding potential of −60 mV in the presence of normal saline (*a*) and after application of saline containing 10 mM TEA (*b*). Right, currents evoked in the same cell by a depolarizing step to +10 mV from a holding potential of −120 mV in normal saline (*a*) and after application of 10 mM TEA (*b*). Currents were automatically leak-subtracted.

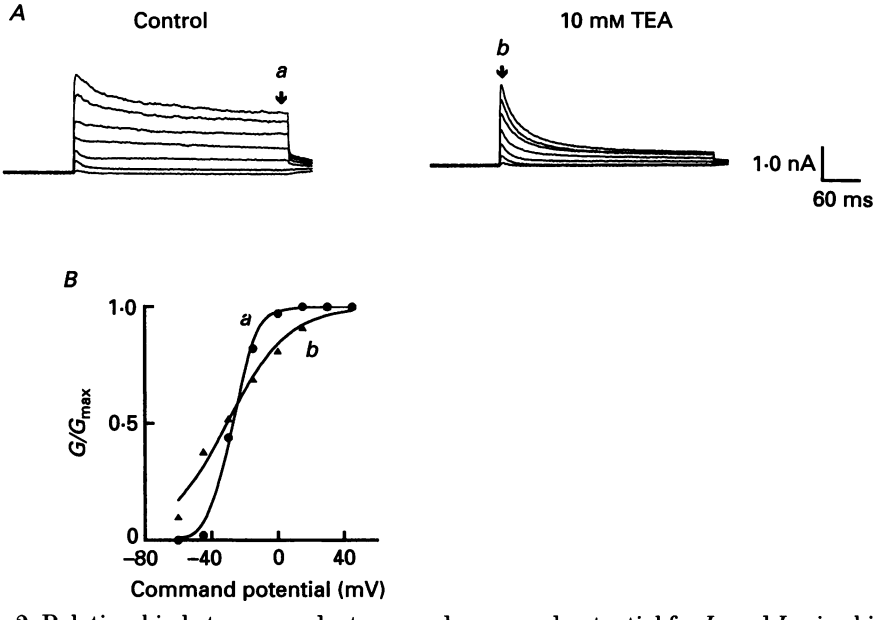

Fig. 2. Relationship between conductance and command potential for I_A and I_{DR} in chick ciliary ganglion neurones. *A*, families of currents evoked by depolarizing voltage steps (−60 to +30 mV) made from a holding potential of −120 mV. Currents were evoked in normal saline (left) and after application of salines containing 10 mM TEA (right). I_{DR} was measured at time *a* (left). I_A was measured at time *b* (right). All currents were automatically leak-subtracted. *B*, plot of normalized conductance *vs.* command potential for I_{DR} (▲) and I_A (●). Lines are fitted according to text eqn (1) with $V_h = -26$ mV, $k = 7{\cdot}1$ mV (I_A), $V_h = -31$ mV and $k = 18{\cdot}5$ mV (I_{DR}). Conductances were calculated from the records shown in *A* assuming an equilibrium potential of −85 mV.

never inactivates completely with these stimulus protocols, although holding the cell at a potential of −40 mV for several minutes does eventually cause the complete inactivation of I_{DR} (Dryer *et al.* 1991). The position of the steady-state inactivation curves for both types of current was not dependent on the voltage step used to activate the currents at the end of the conditioning prepulses (not shown).

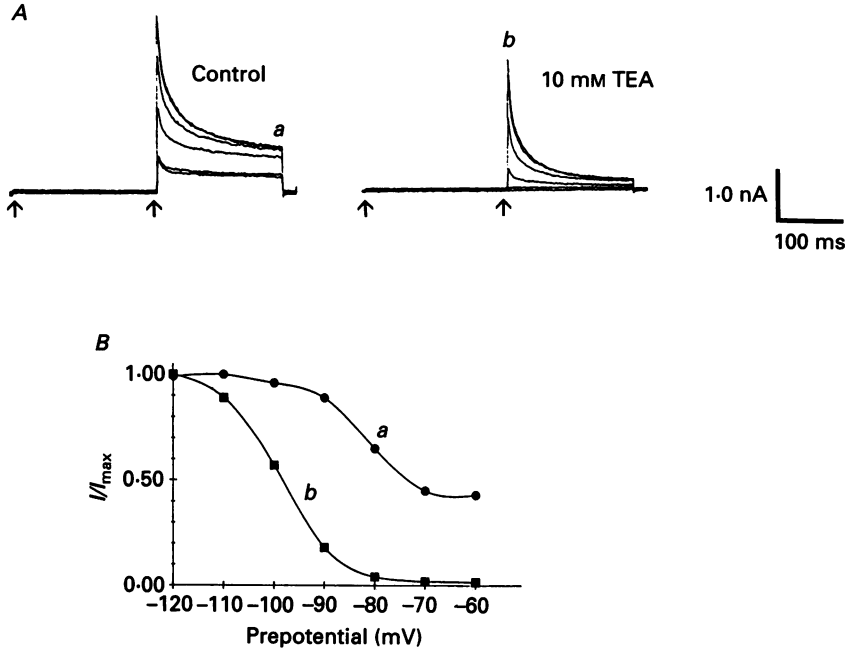

Fig. 3. Steady-state inactivation of I_A and I_{DR} in chick ciliary ganglion neurone. *A*, families of currents were evoked by depolarizing voltage steps to +40 mV from a series of 500 ms hyperpolarizing prepulses (−140 to −50 mV). Arrows on records indicate times when prepulses were applied. Traces shown to the left are for prepulses to −60, −70, −90, −100 and −110 mV in normal saline. I_{DR} was measured at the time marked *a*. Traces to the right are currents evoked from prepulses to −120, −110, −100, −90, −80 and −70 mV in the presence of saline containing 10 mM TEA. I_A was measured at the time marked *b*. All currents were automatically leak-subtracted. *B*, plot of normalized current *vs.* prepulse potential for the same cell shown in *A*. Lines are drawn by cubic-spline interpolation. Steady-state inactivation curve is shown for I_{DR} (●) and I_A (■). Note that I_{DR} does not inactivate completely with these voltage clamp protocols.

Characterization of I_A

Resistance to blockade by TEA is often considered to be a general characteristic of I_A. In addition, many A-currents are highly sensitive to blockade by 4-AP (Thompson, 1977; Segal *et al.* 1984; Belluzzi *et al.* 1985; Bossu *et al.* 1985; Numann *et al.* 1987). I_A in chick ciliary ganglion neurones can also be blocked by 4-AP as shown in Fig. 4. In these experiments, currents were evoked by depolarizing steps from a holding potential of −120 mV in the presence of 10 mM TEA. Application of 1 mM 4-AP for up to 10 min had no effect on the amplitude or time course of these currents in five cells tested (not shown). However, in six cells, application of 5 mM-

4-AP caused a large block of I_A (83±13%). The effect of 5 mM 4-AP could be observed within 1 min of application of the drug and was reversed by several minutes of washing in control saline. Low nanomolar concentrations of DTX can reduce voltage-activated K^+ currents in some preparations (Halliwell *et al.* 1986; Penner,

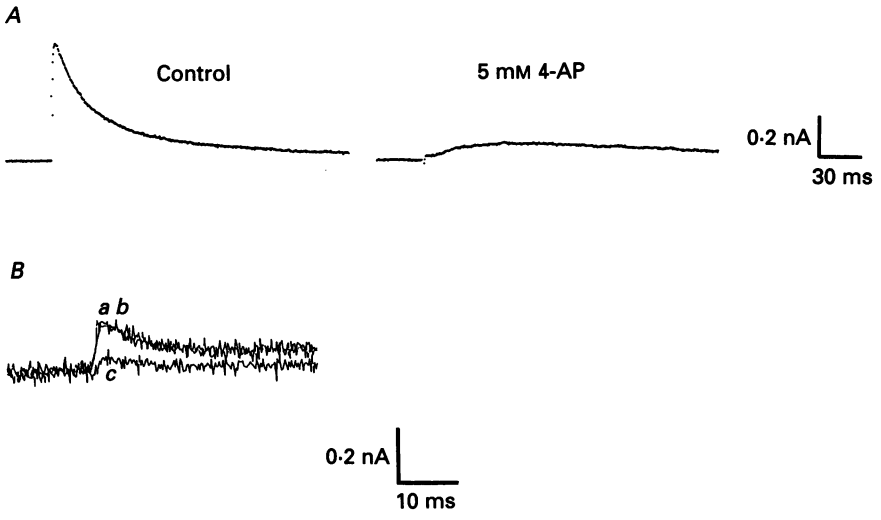

Fig. 4. Pharmacology of I_A in chick ciliary ganglion neurons. *A*, current before (left) and after (right) application of saline containing 5 mM 4-AP. I_A was evoked by depolarizing voltage steps to −20 mV from a holding potential of −120 mV. *B*, current evoked in control saline (*a*), after application of 280 nM DTX (*b*) and after application of 560 mM DTX (*c*). I_A was evoked by depolarizing voltage steps to −30 mV from a holding potential of −120 mV. All currents were automatically leak-subtracted.

Petersen, Pierau & Dreyer, 1986; Stansfeld, Marsh, Parcej, Dolly & Brown, 1987). But in five chick ciliary ganglion neurones, DTX produced no detectable blockade of I_A at 280 nM, but caused a 63±14% blockade at 560 nM (Fig. 4*B*).

In Fig. 4, I_A was examined in isolation from I_{DR} by choosing a depolarizing command potential at which I_{DR} is markedly reduced in the presence of 10 mM TEA. However, it is not possible to use this approach to isolate I_A at more positive command potentials due to the incomplete blockade of I_{DR} by 10 mM TEA. Therefore, we have used the following method to isolate I_A artificially for study. The results in Figs 1, 2, and 3 show that external application of 10 mM TEA will produce a large but incomplete block of I_{DR}. For our purposes, we assume that it would be possible to block *all* I_{DR} with some unspecified larger concentration of TEA and that the I_{DR} which remains in 10 mM TEA has properties identical to the I_{DR} that was blocked. In support of this assumption, we have observed that application of 70 mM TEA caused a greater than 95% block of I_{DR} (not shown). Figure 5*A* illustrates the procedure used to isolate I_A. Currents are evoked by a series of depolarizing steps from a holding potential of −120 mV. The currents that remain in the presence of TEA (centre) are digitally subtracted from the currents obtained in control saline (left) to reveal the TEA-sensitive I_{DR} (right). The TEA-resistant currents (Fig. 5*B*, left) consist of the remaining unblocked I_{DR} and *all* I_A. All the

current that remains near the end of the voltage step must be due to I_{DR}, since I_A would have already become fully inactivated (note time scale). Thus we can scale the TEA-sensitive current to match the amplitude of the sustained TEA-resistant current (Fig. 5*B*, centre) and digitally subtract this from the total TEA-resistant

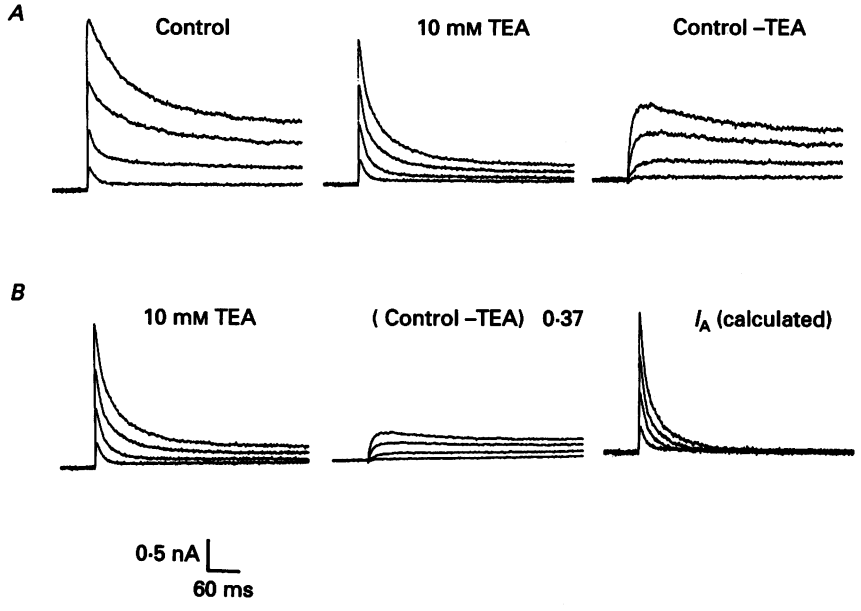

Fig. 5. Isolation of I_A by digital subtraction in a chick ciliary ganglion neurone. *A*, families of currents evoked by a series of depolarizing voltage steps from a holding potential of −120 mV. Recordings were made in normal saline (left) and in saline containing 10 mM TEA (centre). The currents were automatically leak-subtracted. Traces to the right are the TEA-sensitive currents obtained by digital subtraction as indicated. *B*, subtraction procedure for the isolation of I_A. Currents recorded in the presence of TEA (same traces as in the centre panel of *A*) consist of a mixture of I_A and residual I_{DR}. The amplitude of the residual I_{DR} was measured at the end of these traces. TEA-resistant currents were scaled as indicated (centre) and subtracted to remove the residual I_{DR}, resulting in the calculated I_A (right).

current; this subtraction serves to remove the residual unblocked I_{DR}. The result (right) is a TEA-resistant, rapidly inactivating current or I_A.

These calculations allow us to estimate the characteristics of I_A over its entire activation range (Fig. 6). The traces shown in Fig. 6*A* represent I_A calculated in a different cell by the method described above. For a voltage step to −30 mV, the current decay was best fitted to a single exponential curve (Fig. 6*B*). However, for voltage steps to +30, +10, and −10 mV, the time course of the decay of the current was best fitted as the sum of two exponentials, with time constants of 5–9 and 35–50 ms. The correlation coefficient for these fits was greater than 0·998. Neither of the two time constants were markedly voltage dependent, but the relative amplitude of the slow component became *larger* at more depolarized potentials. This caused the overall decay of I_A to actually become *slower* at more positive membrane

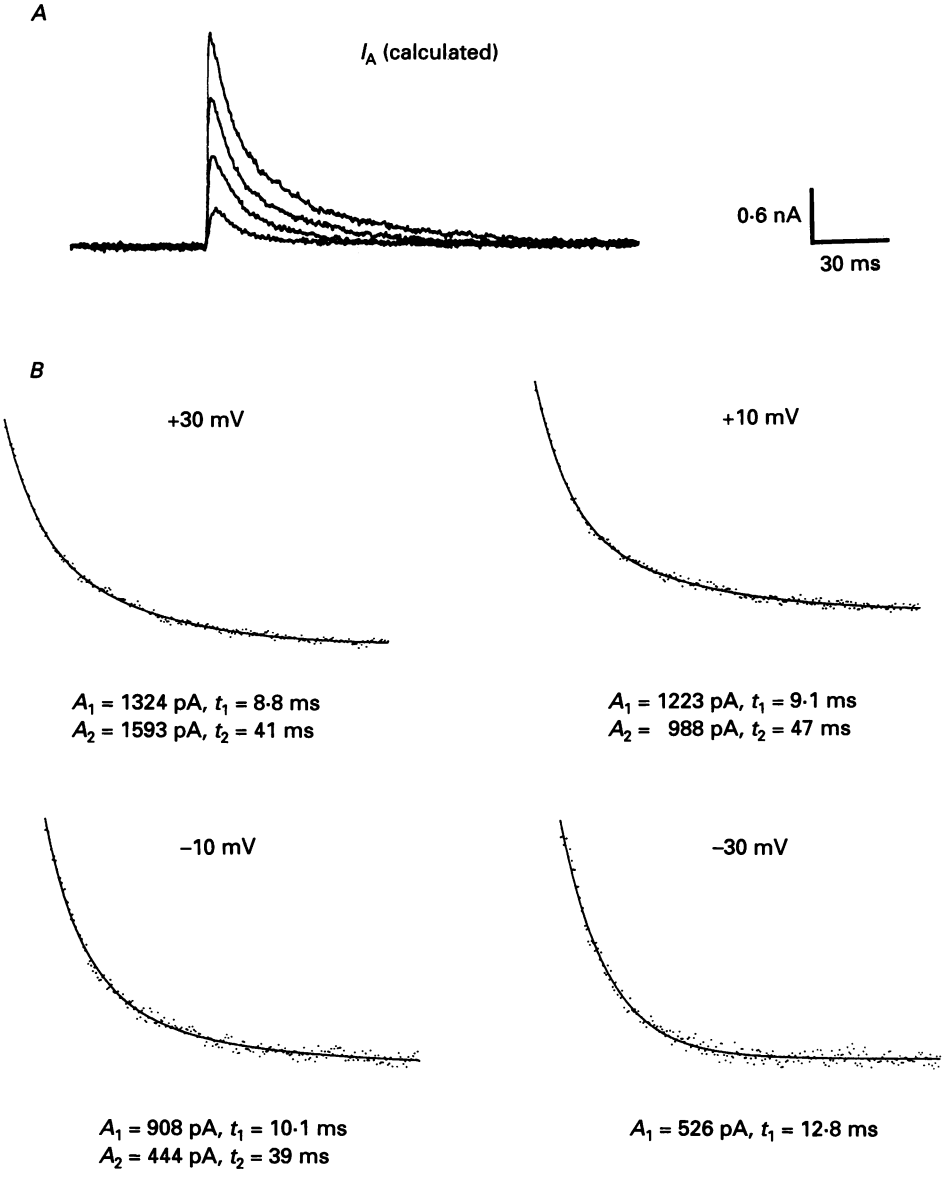

Fig. 6. Voltage dependence of I_A kinetics in a chick ciliary ganglion neurone. *A*, example of I_A isolated as described in Fig. 5 but in a different cell. Currents were evoked by depolarizing voltage steps to −30, −10, +10 and +30 mV from a holding potential of −120 mV. *B*, the time course of the decay of I_A is shown for the same currents at the membrane potentials indicated. Data are shown as points with superimposed exponential curves with amplitudes and time constants as indicated. Double-exponential curves were required to obtain adequate fits to all of the traces except for the current evoked at −30 mV, which could be fitted with a single-exponential curve. Note that the overall process of I_A inactivation was slower at more positive membrane potentials.

potentials. Similar results were obtained in five other cells with time constants ranging from 5–18 and 30–60 ms. In many cells, the slower overall decay time course at more positive membrane potentials could be discerned without resorting to the digital subtraction protocol described above.

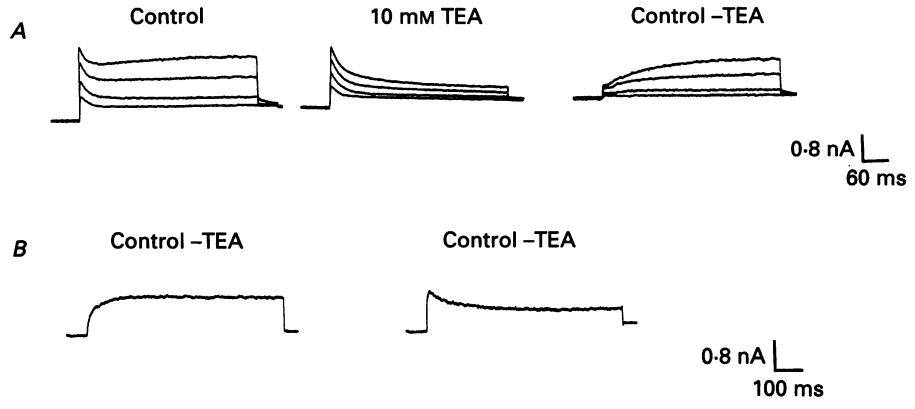

Fig. 7. Different types of I_{DR} in two chick ciliary ganglion neurones. *A*, families of currents evoked from a holding potential of −120 mV in normal saline (left) and after application of 10 mM TEA (centre). TEA-sensitive currents were obtained by digital subtraction as indicated (right). These I_{DR} currents became activated slowly and did not inactivate during the course of 500 ms depolarizing voltage steps. *B*, TEA-sensitive I_{DR} currents obtained by digital subtraction in two different cells. Trace to the left is from the same cell shown in *A*. The trace to the right is from a different cell, with a more typical rapidly activating and slowly inactivating TEA-sensitive I_{DR}. Currents were evoked by a 1 s depolarizing step to +10 mV from a holding potential of −60 mV.

We have noticed that about 10% of chick ciliary ganglion neurones studied express a more rapidly inactivating I_A which is strikingly similar to that of embryonic chick sympathetic neurones (Wisgirda & Dryer, 1993). The inactivation time constants of these currents are 2–6 and 20–30 ms. In these cells, the weight of the fast component is much larger than that of the slow component, leading to a much faster overall rate of inactivation. These results suggest the existence of several types of transient K^+ channels in chick autonomic neurones or that these channels can exhibit several kinetic modes.

Characterization of two types of I_{DR}

In the majority of the chick ciliary ganglion neurones, I_{DR} shows some degree of inactivation over a time scale of hundreds of milliseconds. However, approximately 5% of cells studied exhibited a I_{DR} that showed little or no tendency to inactivate over this time scale. The I_{DR} in these cells also activated much more slowly. An example of this type of behaviour is shown in Fig. 7. The total voltage-activated K^+ current (Fig. 7*A*, left) is biphasic, showing I_A activation and inactivation followed by I_{DR} activation. When the TEA-resistant current (centre) is digitally subtracted from the total current, the TEA-sensitive I_{DR} (right) does not reveal any tendency to inactivate. In Fig. 7*B*, the TEA-sensitive I_{DR} from two different cells is shown on a longer time scale. These recordings were made on the same day and from the same preparation of dissociated cells. The current trace on the left, from the same cell shown in Fig. 7*A*, activates very slowly and shows no sign of inactivating even over

several hundred milliseconds. The current trace on the right, from a more typical cell, activates more quickly, and shows a substantial degree of I_{DR} inactivation during the course of a maintained depolarization. This phenomenon, which was mentioned by Dourado & Dryer (1992), suggests that either a single type of K^+ channel is

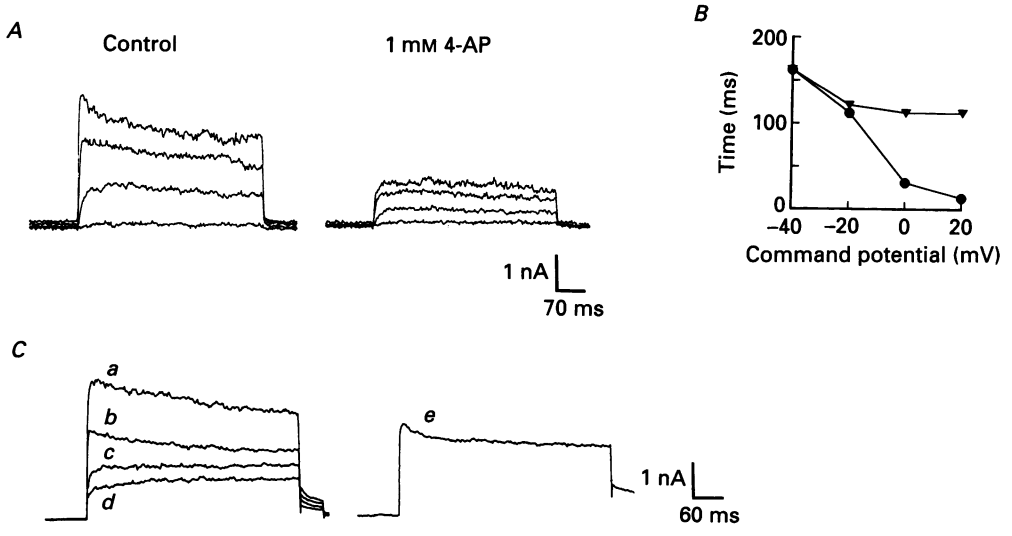

Fig. 8. Effect of 4-AP on I_{DR} in a chick ciliary ganglion neurone. *A*, families of currents evoked by a series of depolarizing steps (−40 to +20 mV) from a holding potential of −60 mV in normal saline (left) and after application of 1 mM 4-AP (right). The currents were automatically leak-subtracted. *B*, plot of the total time required to reach peak current *vs.* the command potential in normal saline (●) and after application of 4-AP (▼). Data were obtained from the records shown in *A*. The currents activate more slowly after application of 4-AP. Note also that the activation kinetics are much less voltage dependent after 4-AP. *C*, currents evoked by a depolarizing step to +20 mV from a holding potential of −60 mV in normal saline (*a*), and 2 min after application of 10 μM 4-AP (*b*), 100 μM 4-AP (*c*) and 1 mM 4-AP (*d*). Partial recovery was observed after 9 min of washing in normal saline. (*e*).

displaying very complex kinetic behaviour, such as that described by Cooper & Shrier (1989), or that more than one channel species is present in these cells. We therefore decided to examine the characteristics of I_{DR} in more detail.

Portions of I_{DR} are highly sensitive to blockade by 4-AP. In the cell shown in Fig. 8, the time course of activation of I_{DR} is markedly voltage dependent (left). In these experiments, depolarizing voltage commands were made from a holding potential of −60 mV in order to prevent activation of I_A. Note that the inactivation of these currents was faster at more positive command potentials. Within 1–3 min of application of 1 mM 4-AP (right), the current was reduced substantially and its kinetics were changed, so that the *total* time required to reach peak current was greater and much less voltage dependent. This is shown quantitatively in Fig. 8*B*. Moreover, the currents remaining after 4-AP application exhibited much less inactivation during the course of a 500 ms depolarizing voltage step. Similar results were obtained in ten other cells. Therefore, 4-AP appears to be separating two

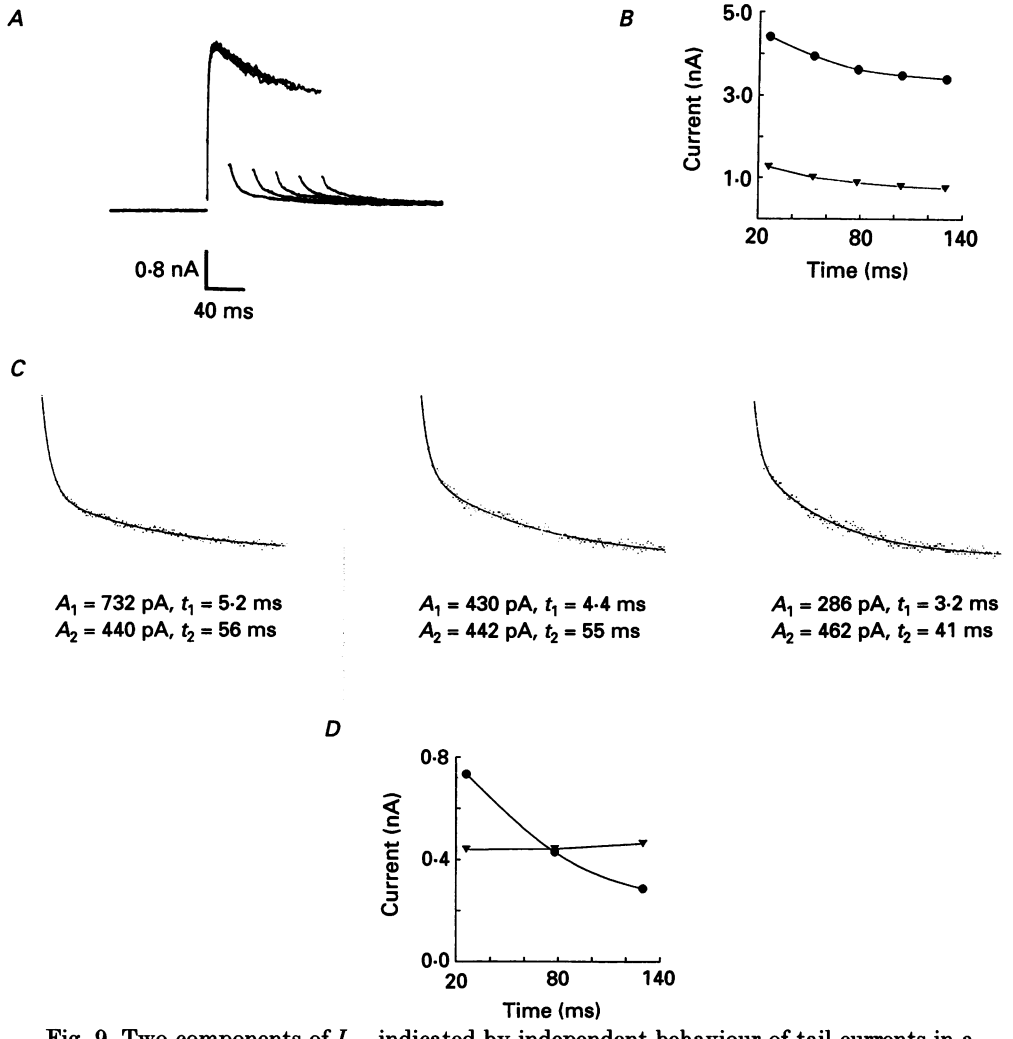

Fig. 9. Two components of I_{DR} indicated by independent behaviour of tail currents in a chick ciliary ganglion neurone. *A*, I_{DR} activated by depolarizing voltage steps to +20 mV of varying duration from a holding potential of −60 mV. Currents were automatically leak-subtracted. *B*, plot of total current remaining at the end of the pulse (●) and peak tail current amplitude (▼) *vs.* the duration of the depolarizing voltage step. *C*, time course of the decay of tail currents evoked by depolarizing steps of varying duration. Data are shown as points with superimposed double-exponential curves with amplitudes and time constants as indicated. Data are shown for depolarizing steps of 20 ms (left), 80 ms (centre) and 140 ms (right). *D*, amplitude of the fast (●) and slow (▼) components of the tail currents *vs.* the duration of the depolarizing pulse. The fast component of tail current becomes smaller as the duration of the depolarizing steps is increased, while the slow component is independent of pulse duration over the time scales tested.

components of I_{DR} that have different kinetics. In four cells, significant blockade of I_{DR} was observed after application of 10 μM 4-AP, with half-maximal blockade usually occurring between 10 and 100 μM 4-AP (Fig. 8*C*, left). The effect of 4-AP was

reversible although this required 10–15 min of washing in normal saline (Fig. 8*C*, right).

The theory that two separate components of I_{DR} are present in ciliary ganglion neurones is supported by experiments on the behaviour of I_{DR} tail currents. Families

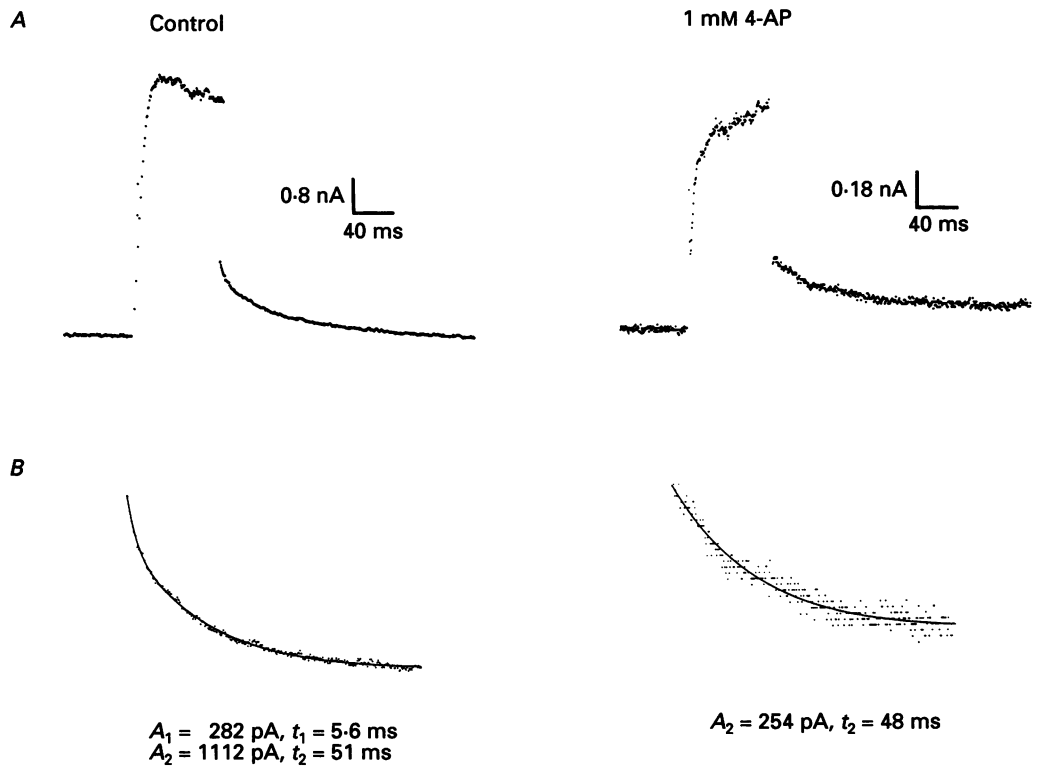

Fig. 10. Effects of 4-AP on I_{DR} tail currents in a chick ciliary ganglion neurone. *A*, currents evoked by a depolarizing voltage step to +20 mV from a holding potential of −60 mV in normal saline (left) and after application of 1 mM 4-AP (right). The currents are shown at a different scale to facilitate comparison of kinetics. The currents were automatically leak-subtracted. Note differences in activation, inactivation and deactivation kinetics after 4-AP. *B*, tail currents of the traces shown in *A* are plotted as points with superimposed exponential curves with amplitudes and time constants as indicated. A double-exponential curve is necessary to fit the tail currents in normal saline, while a single-exponential curve provides an adequate fit after 4-AP.

of tail currents evoked by voltage steps of varying durations to +20 mV are shown in Fig. 9*A*. The envelope of the tail current amplitudes was well correlated with the time course of the total current (Fig. 9*B*). In Fig. 9*C*, tail currents evoked by voltage steps of 20 ms (left), 80 ms (centre) and 140 ms (right) duration are shown. In each case the time course of the tail currents was best fitted as the sum of two exponentials, characterized by a short time constant (3–5 ms) and a longer time constant (40–60 ms). However, the amplitude of the tail current component associated with the short time constant decreased as the duration of the voltage step was increased, while that of the long time constant remained constant (Fig. 9*C* and

D). Similar results were obtained in four other cells. The independent behaviour of the two tail current components is consistent with the existence of two separate channel species underlying the macroscopic current. The fast components of the tail currents appear to be associated with the inactivating components of the total I_{DR}.

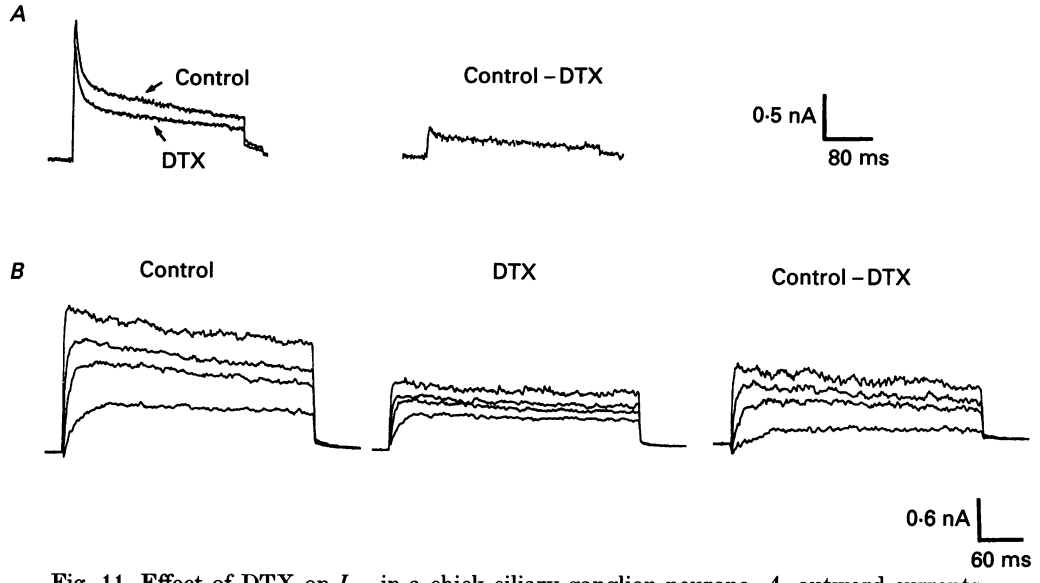

Fig. 11. Effect of DTX on I_{DR} in a chick ciliary ganglion neurone. *A*, outward currents evoked by a depolarizing step to +30 mV from a holding potential of −120 mV in the presence and absence of 260 nM DTX as indicated (left) and the DTX-sensitive currents obtained by digital subtraction. Currents were automatically leak-subtracted. DTX blocks I_{DR} but not I_A. *B*, families of currents evoked by depolarizing voltage steps from a holding potential of −60 mV in normal saline (left) and after application of 200 nM DTX (centre). DTX-sensitive currents were obtained by digital subtraction (right). DTX did not appear to separate kinetically distinct components of I_{DR}.

Since 4-AP appeared to be primarily blocking an inactivating component of I_{DR}, it was of interest to see whether it preferentially blocked the more rapid component of the tail current, as would be predicted if two separate populations of I_{DR} channels were present. Figure 10*A* shows current traces from a single cell before (left) and after (right) application of 1 mM 4-AP. These currents are plotted at a different amplitude scale to facilitate comparison of the time course of the tail currents. Note again that the 4-AP-resistant current (right) activated much more slowly and showed very little tendency to inactivate. The total tail current (Fig. 10*B*, left) requires two exponentials to provide an adequate fit, with the same time constants as seen previously. By contrast, the 4-AP-resistant current (right) can be fitted with a single exponential with a long time constant. These results indicate that 4-AP preferentially blocks the more rapid component of I_{DR}. Similar results were obtained in four other cells. We call the more rapid 4-AP-sensitive component I_{DRf} (for fast delayed rectifier) and the slower, 4-AP-resistant component I_{DRs} (for slow delayed rectifier).

Figure 11*A* shows currents evoked by a depolarizing step to +30 mV from a holding potential of −120 mV before and after application of 200 nM DTX (left).

Note that in these salines, this voltage clamp protocol will cause substantial activation of both I_A and I_{DR}. In five cells, this concentration of DTX caused a $51 \pm 8\%$ blockade of the currents measured at the end of a depolarizing pulse to +30 mV. When the DTX-resistant current is digitally subtracted from the control current, the DTX-sensitive component (right) is revealed to be primarily a sustained current, I_{DR}. Figure 11*B* shows I_{DR} evoked from a holding potential of −60 mV before (left) and after (centre) application of 200 nM DTX. The DTX-resistant currents (centre) and the DTX-sensitive currents (right) have very similar kinetics, and this toxin does not appear to discriminate between I_{DRf} and I_{DRs}. The effects of DTX did not recover after washing for up to 20 min in normal saline.

DISCUSSION

This study has demonstrated that chick ciliary ganglion neurones isolated acutely between E12 and E14 express at least three different types of voltage-activated K^+ channels. These include two TEA-sensitive delayed rectifier currents (I_{DRf} and I_{DRs}) and a transient TEA-resistant A-current (I_A). The two delayed rectifier currents can be separated on the basis of differential sensitivity to blockade by 4-AP, as well as by differences in the kinetics of activation, inactivation and deactivation. Most cells studied between E12 – E14 express all three currents, although about 5% of cells studied expressed only the slow components of I_{DR}. A previous study of chick ciliary ganglion neurones indicated that the expression of I_A is developmentally regulated and is dependent on extrinsic environmental factors (Dourado & Dryer, 1992). I_A is either completely undetectable, or present at very low levels, prior to E9. However, both components of I_{DR} are readily detected prior to E6, as can be ascertained by examining the records presented by Dourado & Dryer (1992). In addition, both types of I_{DR} differ from I_A in that they are expressed at normal levels in chick ciliary ganglion neurones that have been maintained in dissociated cell culture for several days in the absence of other cell types (Dourado & Dryer, 1992).

In chick ciliary ganglion neurones, I_A activates at more negative membrane potentials than either type of I_{DR}. Both components of I_{DR} exhibit a steeper voltage dependence of activation than is seen with I_A. We have also observed that the steady-state inactivation curve of I_A is centred around a membrane potential that is considerably negative to that of I_{DR}, a finding similar to that seen in other cell types (Thompson, 1977; Adams *et al.* 1982; Segal & Barker, 1984).

The combined biophysical and pharmacological features of I_A in chick ciliary ganglion neurones are somewhat unusual and allow comparisons to be made with other K^+ currents described previously. For example, the time course of inactivation is complex and the decay phase of the currents at most membrane potentials requires two time constants of 5–18 and 30–60 ms to fit the data adequately. In a previous study of isolated chick lumbar sympathetic neurones (Wisgirda & Dryer, 1993) which express a much larger I_A, we also found that two time constants were required to describe the time course of inactivation. However, in those cells I_A consistently became inactivated approximately twice as fast as that observed in most chick ciliary ganglion neurones. A recent study of clonal pituitary cells also found two time constants in the decay of I_A (Oxford & Wagoner, 1989).

The A-currents of chick ciliary ganglion neurones are blocked by 5 mM 4-AP but are resistant to 1 mM 4-AP. In bullfrog sympathetic neurones, I_A has been reported to be relatively resistant to 4-AP (Adams *et al.* 1982). However, 1 mM 4-AP has been reported to cause substantial blockade of I_A in several other vertebrate preparations at command potentials similar to those used here (Segal & Barker, 1984; Belluzzi *et al.* 1985; Bossu *et al.* 1985; Numann *et al.* 1987). The A-currents of chick ciliary ganglion neurones are also relatively resistant to DTX. Application of 280 nM DTX did not affect the current, while 560 nM DTX produced only a partial blockade. This is similar to the I_A of rat sympathetic neurones (Stansfeld *et al.* 1987) but different from the transient K^+ currents reported in rat hippocampal neurones which are blocked by 50 nM DTX (Halliwell *et al.* 1986).

In recent years, many K^+ channels have been characterized by molecular biological methods and examined in artificial expression systems such as the *Xenopus* oocyte. These studies have led to the identification of several families of K^+ channel genes, most of which consist of more than one gene. Additional K^+ channel diversity in vertebrates has been attributed to the formation of heteromultimeric channels (Christie *et al.* 1990; Ruppersberg *et al.* 1990) as well as by alternative splicing mechanisms (Luneau *et al.* 1991). Although ionic currents expressed in artificial systems such as *Xenopus* oocytes may not completely reflect the native properties of channels found in excitable cells, it is nevertheless interesting to compare the properties of native currents with those of cloned channels. The combined pharmacological and biophysical properties of I_A of chick ciliary ganglion neurones appear to be similar in many ways to the Kv1.4 family of channels cloned recently from the rat cerebral cortex (RCK4 channels; Stühmer *et al.* 1989) and from rat cardiac myocytes (RHK1 channels; Tseng-Crank, Tseng, Schwartz & Tanouye, 1990). For example, all these currents exhibit similar voltage dependence and kinetics of activation and inactivation. Moreover, all are relatively resistant to blockade by DTX and completely resistant to TEA. But it is also possible that the I_A channels of the chick ciliary ganglion are more closely related to channels that have not yet been cloned or that have not been characterized in detail. Unfortunately, at this time, there is no information available on the structure of avian K^+ channels or their genes.

The presence of two components of I_{DR} was suggested by some of the results of a previous study of ionic currents in chick ciliary ganglion neurones (Dourado & Dryer, 1992). In that study, most cells were found to express a TEA-sensitive I_{DR} that became inactivated slowly in the face of a maintained 500 ms depolarization. However, a small number of cells expressed an I_{DR} that had significantly slower activation kinetics and very little tendency to inactivate over a time course of seconds. The observation that only a portion of I_{DR} could be made to inactivate as a result of 500 ms conditioning prepulses also suggested the existence of two distinct types of I_{DR}. This conclusion was supported by the fact that application of comparatively low concentrations of 4-AP was able to separate qualitatively different K^+ currents. The 4-AP-resistant currents (I_{DRs}) activated slowly and showed little or no tendency to inactivate in the face of maintained depolarizing voltage steps. The activation kinetics of these currents resemble those seen in the subset of chick ciliary ganglion neurones that express only non-inactivating I_{DR}. By contrast, the 4-AP-

sensitive currents (I_{DRf}) showed a marked tendency to inactivate and the time required to reach peak current was much more dependent on the command potential. The inactivation time course of I_{DRf} was also voltage dependent, being much faster at more positive membrane potentials. Further support for the presence of two separate components of I_{DR} came from studies of I_{DR} tail currents. For example, the fast component of the tail currents was abolished by 4-AP and was decreased in amplitude during longer depolarizing command pulses where a greater proportion of I_{DRf} was inactivated. The slow components of the tail currents were not dependent on the duration of the depolarizing step and could be detected in the presence of 4-AP.

The two components of I_{DR} of chick ciliary ganglion neurones are much less sensitive to DTX than the delayed rectifier currents described in some other preparations, which can be blocked by as little as 1–10 nM DTX (Penner *et al.* 1986; Stansfeld, Marsh, Halliwell & Brown, 1986). Genes coding for TEA-sensitive delayed rectifiers that are blocked by very low concentrations of DTX have also been identified in the rat brain (Stühmer *et al.* 1989; Swanson *et al.* 1990). However, none of these appear to correspond to the currents described in the present study.

Results similar to those described here for delayed rectifiers have been reported previously in the frog node of Ranvier (Dubois, 1981). These cells express three different voltage-activated K^+ channels, including a 4-AP-sensitive I_{DR} with rapid deactivation kinetics and a non-inactivating 4-AP-resistant I_{DR} with slow deactivation kinetics. Very similar results have also been described for purified lactotrophs of the rat anterior pituitary gland (Herrington, 1992). These cells express two distinct TEA-sensitive delayed rectifiers that can be separated on the basis of differences in activation, inactivation and deactivation kinetics and by their differential sensitivity to 4-AP. The sensitivity of these currents to DTX was not tested. A single-channel study of mouse neuroblastoma cells (Quandt, 1988) has also identified two populations of voltage-activated TEA-sensitive K^+ channels that can be separated on the basis of differences in kinetics and sensitivity to 4-AP. In those cells, as in the present study, the population of K^+ channels that became activated and inactivated more quickly also exhibited a greater sensitivity to blockade by 4-AP. These channels also had a lower unitary conductance.

In summary, we have found that acutely dissociated chick ciliary ganglion neurones express at least three different types of voltage-activated K^+ currents. These include two different types of delayed rectifiers and a transient TEA-resistant A-current. Previous studies have shown that chick ciliary ganglion neurones also express as many as four different types of Ca^{2+}-activated K^+ channels (Dryer *et al.* 1991) as well as non-selective inward rectifier channels (Fletcher & Chiappinelli, 1992). The chick ciliary ganglion contains a relatively homogeneous population of neurones and the number of different K^+ channels expressed by these cells is somewhat surprising. It may be that the electrical properties of these cells are highly 'tuned' to meet a particular physiological function. However, at this time the mechanisms that result in the expression of this particular complement of ionic channels during embryonic development are not well understood.

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