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CHARACTERIZATION OF SINGLE NON-INACTIVATING POTASSIUM CHANNELS IN PRIMARY NEURONAL CULTURES OF *DROSOPHILA*

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Summary

Permeability and gating properties of single, non-inactivating, K^+ channel currents in cultured *Drosophila* neurons were studied using the gigaohm-seal patch-clamp technique. The non-inactivating K^+ currents were activated by depolarizing the membrane to −30 mV or to more positive potentials. The slope conductance of the channel was estimated to be $17{\cdot}6 \pm 3{\cdot}70$ pS when the cytoplasmic side of the inside-out membrane patch was perfused with solutions containing 145 mmol l^{-1} K^+. The single-channel conductance was temperature-sensitive, with a Q_{10} of 1·44 between 10 and 20°C. Single-channel currents could be recorded when the cytoplasmic K^+ was replaced with NH_4^+, Rb^+ or Na^+, but not with Cs^+. The conductance ratio of the channel for these cations was: $K^+(1) > NH_4^+(0{\cdot}53) > Rb^+(0{\cdot}47) > Na^+(0{\cdot}44)$. Tetraethylammonium ($TEA^+$) ions applied at a concentration of 10 mmol l^{-1} to the cytoplasmic side of the membrane increased the frequency of 'blank' traces which contained no channel openings during repetitive depolarization. In addition, single-channel amplitude was reduced by about 20%. The open-time distribution was fitted by a single exponential function, whereas the closed-time distribution required a three-exponential fit. Permeability and gating properties of single, non-inactivating K^+ channel currents in neurons of *eag*, a mutant which has defects in the delayed rectifier K^+ channel, were indistinguishable from those recorded from wild-type neurons.

Introduction

Within the last five years, dramatic advances in the study of membrane channels have been achieved by combining molecular genetics and patch-clamp technology (Sakmann *et al.* 1985; Claudio *et al.* 1987). In *Drosophila*, attempts to identify genes encoding potassium channels by means of transposon tagging and chromosome walking have met with great success (Schwarz *et al.* 1988; Timpe *et al.* 1988). However, there is limited information concerning the physiological and biophysi-

Key words: *Drosophila*, neuron, single channel, K^+ channel, *eag* mutant.

cal properties of neuronal channels in *Drosophila*, as the small diameter of *Drosophila* neurons precludes application of conventional electrophysiological methods (Tanouye *et al.* 1981; Wu *et al.* 1983*b*). The present work provides detailed information about single voltage-gated potassium channels in *Drosophila* neurons by using the gigaohm-seal patch-clamp technique.

Materials and methods

All experiments were carried out with primary cultured neurons of *Drosophila* (Wu *et al.* 1983*b*). Mature third-instar larvae of wild-type (Canton-S) or *eag* (Ganetzky & Wu, 1985) *Drosophila melanogaster* were used. Before dissection, larvae were surface-sterilized by brief immersion in 70 % ethanol and then rinsed three times with sterile distilled water. The brain and ventral ganglion were dissected from larvae in tissue culture medium, and segmental nerves, imaginal discs and other tissues were removed. The culture medium was a revised Schneider medium (Schneider, 1964) from Grand Island Biological Co. (GIBCO; Grand Island, NY), supplemented with heat-inactivated (56°C, 30 min) fetal bovine serum (10 %), penicillin G (50 units ml^{-1}) and streptomycin sulfate (50 $\mu g\,ml^{-1}$) (all obtained from GIBCO). After rinsing, the brains and ganglia were first torn into small fragments in the culture medium with fine dissecting needles and then incubated for 1 h in a saline similar to Rinaldini's (Schneider & Blumenthal, 1978) Ca^{2+}- and Mg^{2+}-free solution (800 mg of NaCl, 20 mg of KCl, 55 mg of $NaH_2PO_4 . H_2O$, 100 mg of $NaHCO_3$, and 100 mg of glucose in 100 ml of distilled water) containing 10 units ml^{-1} of papain (type III, Sigma Chemical Co., St Louis, MO). The fragments were rinsed three times with culture medium supplemented with 10 % fetal bovine serum and then dispersed into single cells in the same medium by repeated flushing through a siliconized glass pipet. To obtain monolayer cultures, 80–200 μl of cell suspension was placed on round glass coverslips (15 mm diameter) in dry Petri dishes. The coverslips were precoated with poly-L-lysine ($M_r \approx 14\,000$; Sigma) to facilitate cell adhesion. Cultures with densities ranging from 0·5 to 5×10^4 cells cm^{-2} were maintained at 25°C in humidified air containing 5 % CO_2. Cells cultured for 1 day to 2 weeks were used for experiments.

Membrane currents were recorded either from excised inside-out membrane patches or in the whole-cell configuration using the gigaohm-seal patch-clamp technique (Hamill *et al.* 1981). The cells were initially immersed in normal saline containing (in mmol l^{-1}); NaCl, 125; KCl, 5·5; $CaCl_2$, 1·8; $MgCl_2$, 0·8; glucose, 25; sucrose, 20; and Hepes, 10 (pH adjusted to 7·3 with NaOH). For recordings from cell-free patches, the pipet was filled with an 'extracellular solution' containing (in mmol l^{-1}): choline–Cl, 145; $MgCl_2$, 3; ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA), 2; and Hepes, 10 [pH adjusted to 7·3 with tetramethylammonium hydroxide (TMA–OH)]. This Ca^{2+}-, K^+- and Na^+-free extracellular solution was used throughout the study to eliminate Ca^{2+}-activated conductances and Na^+- and K^+-dependent inward currents (Yamamoto & Suzuki, 1987*a*,*b*). The composition

of the internal solution used for the excised patch experiments was: 145 mmol l^{-1} KCl, 1·13 mmol l^{-1} $MgCl_2$, 1·92 mmol l^{-1} EGTA and 10 mmol l^{-1} Hepes. The pH of the internal solution was adjusted to 7·3 with KOH. In experiments to examine ionic selectivity of the channel, KCl was totally replaced with either RbCl, NaCl or NH_4Cl. Some experiments were performed with internal solutions containing either F^- or NO_3^- substituted for Cl^-. In the case of whole-cell recordings, the pipet was filled with an 'internal' solution containing: 145 mmol l^{-1} potassium glutamate, 1·13 mmol l^{-1} $MgCl_2$, 1·92 mmol l^{-1} EGTA and 10 mmol l^{-1} Hepes. All solutions were filtered immediately before use through a membrane filter with 0·45 μm pore diameter (Toyo membrane filters, type TM-2). The temperature of the chamber was controlled by a Peltier device and measured with a thermocouple probe, and was kept within ±0·5 °C of the desired setting. Experiments were performed at 10 °C, unless otherwise indicated.

To record single-channel currents, a Dagan model 8900 patch-clamp/whole-cell-clamp amplifier (Dagan, Minneapolis, MN) with probe type 8930 (feed-back resister: 10 GΩ) was used. All records were filtered at 3 kHz with a six-pole Bessel filter, except when mentioned. The bath potential was measured using a separate reference microelectrode filled with 3 mol l^{-1} KCl. The interior of the pipet was clamped at a command voltage which was generated by a function generator (model 275, Wavetek Inc., San Diego, CA) under microcomputer (model 9816S; Hewlett-Packard Co., Fort Collins, CO) control (Yamamoto, 1986). The current signals were digitized with a digital oscilloscope (2090-3C; Nicolet Scientific Corp., Northvale, NJ) at the sampling frequency of 5 or 20 kHz, and the digitized data were stored on floppy disks. Leakage and capacitative currents were eliminated from the records by subtracting averaged records with no channel openings.

Using half-amplitude threshold analysis (Colquhoun & Sigworth, 1983), we determined the transitions between the open and closed states. Single-channel kinetics were analyzed from records in which overlapping of events due to simultaneous opening of two or more channels did not occur. Probability distribution histograms (Ehrenstein *et al.* 1974) were constructed from open and closed times, and decay rate constants and amplitudes for distributions were calculated by least-squares regression. The fitting was done between the second bin and the bin in which the number of events decayed to 3 % of the total sample number, as the first bin could be underestimated because of the limited frequency response of the recording system (Yamamoto & Yeh, 1984).

Results

Channel conductance and ionic selectivity

Under our experimental conditions, only one type of channel carrying outward currents was activated by depolarizing voltage steps (Fig. 1A). The outward currents were discernible at −30 mV or at more positive voltages. The channel opened repeatedly throughout the duration of the depolarizing steps (180 ms), producing sporadic bursts of activity separated by long interburst intervals. In the

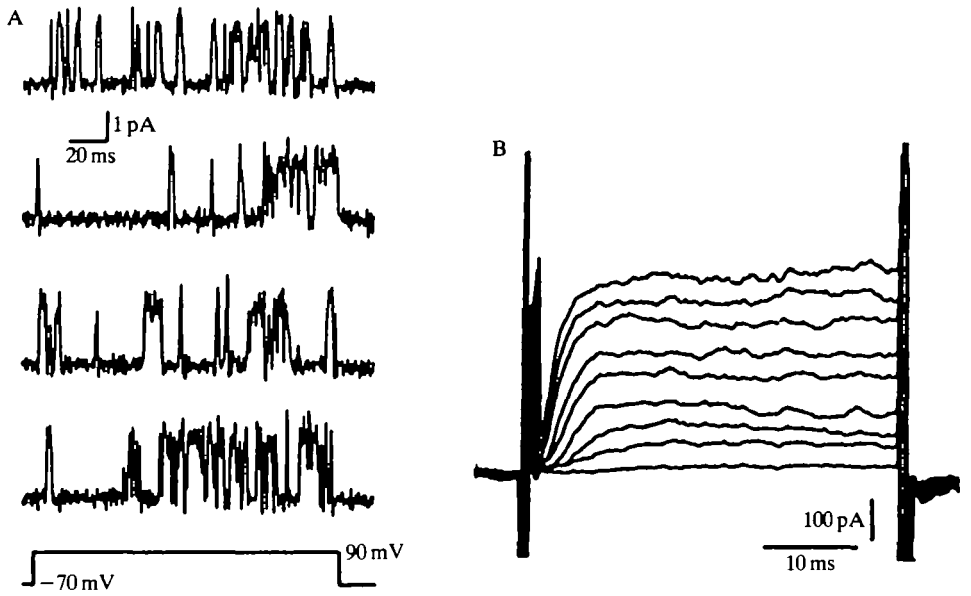

Fig. 1. (A) Examples of single, non-inactivating potassium channel currents recorded from an inside-out membrane patch. The membrane was depolarized to +90 mV from a holding potential of −70 mV for 180 ms at a frequency of 0·4 Hz. Four consecutive traces (top to bottom) are shown. (B) Families of membrane currents recorded by the whole-cell voltage-clamp technique. The membrane was depolarized from a holding potential of −70 mV to −20, −10, 0, +10, +20, +30, +40, +50 and +60 mV.

illustrated example, the mean amplitude of the single-channel current at +90 mV was 2·3pA, as estimated by amplitude histogram analysis.

We are not certain whether the channel has subconductance states, because the rapid kinetics of this channel prevented us from distinguishing possible submaximal levels from very short full openings that were damped by the limited frequency response of the recording system.

A typical example of whole-cell currents from a *Drosophila* neuron is shown in Fig. 1B. The whole-cell current rose just after the onset of the depolarizing voltage step, and stayed at that level during depolarization or even increased slightly towards the end of the step. Thus, the outward current did not show any sign of inactivation, at least over the time scale examined. Ensemble averages of single-channel currents have a similar time course to the whole-cell current (see Fig. 5B).

The amplitude of single-channel currents was temperature-sensitive (Fig. 2). The value of Q_{10} estimated in two different patches was 1·49 and 1·39 between 10 and 20 °C.

The overall kinetics of the channel was speeded by increasing temperature, and many of the openings had a spike-like appearance at temperatures over 25 °C. The amplitude of the single-channel current in this temperature range was estimated from selected open events with prolonged lifetimes.

The amplitude of single-channel current increased on depolarization. The slope

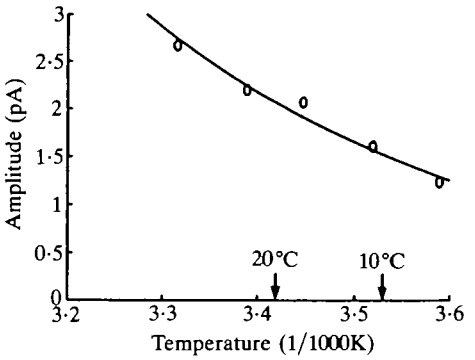

Fig. 2. An Arrhenius plot of the amplitude of single, non-inactivating potassium channel currents in an inside-out membrane patch. The curve is the least-squares fit to data with the equation, $i = A\exp(aE)$, where i is the single-channel amplitude, E is the membrane potential, and A and a are constants. The values for A and a were $25{\cdot}6\times10^3$ and $-2{\cdot}756$, respectively. The Q_{10} obtained in this particular example was 1·49.

conductance calculated between +10 and +90 mV was $17{\cdot}6 \pm 3{\cdot}70$ pS (mean ± S.E., $N = 7$) at 10°C, when K^+ was the major cation in the cytoplasmic side (see Fig. 4). The single-channel current was not seen when K^+ was totally replaced with Cs^+ (not shown). Substitution of Cl^- in internal solutions by F^- or NO_3^- had no appreciable effect on the single-channel current (not shown). These results indicate that the single-channel current under study is carried by K^+. However, smaller outward currents were seen when K^+ was replaced with either Rb^+, NH_4^+ or Na^+ (Fig. 3). The slope conductances were $8{\cdot}2 \pm 1{\cdot}7$ pS with Rb^+ ($N = 3$), $9{\cdot}3 \pm 1{\cdot}1$ pS with NH_4^+ ($N = 3$), and $7{\cdot}7 \pm 1{\cdot}2$ pS with Na^+ ($N = 3$) (Fig. 4).

One question which is of interest in this experiment is whether these permeant cations affect the gating kinetics of the channel. Although channels apparently had longer open times in Na^+, NH_4^+ and Rb^+ solutions (see Fig. 3), the amplitudes of single-channel currents in these solutions were not large enough to permit further investigation of kinetic properties.

Tetraethylammonium (TEA^+) is an effective blocker of potassium channels in other systems (Armstrong, 1975). We examined whether application of TEA^+ to the cytoplasmic side of the membrane had effects on the non-inactivating single K^+ channel currents of *Drosophila*. TEA^+ had two effects on the channel. First, it dramatically increased the frequency of occurrence of blank traces which contained no channel openings when the membrane patch was repeatedly depolarized at 0·4 Hz. In a typical experiment, the frequency of occurrence of blank traces increased from 39 % to 88 % of total trials after the addition of TEA^+ (at 50 mV). A detailed analysis of this action will be presented elsewhere (Yamamoto & Suzuki, 1989). Second, TEA^+ decreased the amplitude of the single-channel current with minimal effect on its kinetics (Fig. 5A). Fig. 5B illustrates the average current of 32 successive single-channel records in the presence and absence of TEA^+, and shows that the combination of these two

actions of TEA^+ produced a marked suppression of macroscopic outward currents. The inhibitory actions were readily detected following washing in of TEA^+, and reversed quickly upon washing out (Fig. 5C).

Channel gating

The probability of a channel being in the open state (P_o) greatly increased as the

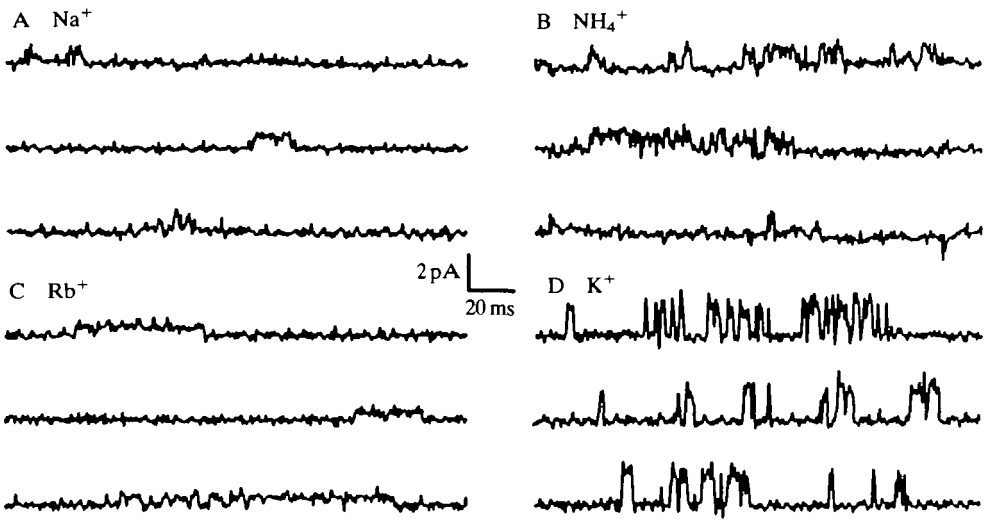

Fig. 3. Examples of single, non-inactivating K^+ channel currents with internal solutions containing Na^+ (A), NH_4^+ (B), Rb^+ (C) or K^+ (D) at 145 mmol l^{-1}. The single-channel currents were elicited by depolarizing the membrane from a holding potential of −70 mV to +110 mV (A–C) or +70 mV (D). Records shown in B–D were from the same inside-out patch, and traces in A were from another patch. Traces were filtered at 1 kHz.

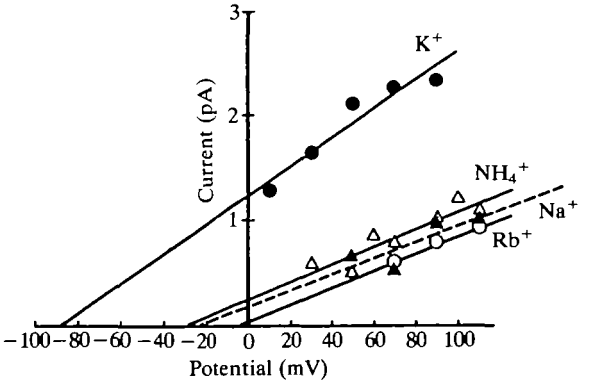

Fig. 4. Single-channel current–voltage (I/V) relationships obtained with internal solutions containing K^+ (●), Rb^+ (○), NH_4^+ (△) or Na^+ (▲) at 145 mmol l^{-1}. I/V relationships were plotted from data collected in the patches from which the traces in Fig. 3 were obtained. The linear regression of data gave slope conductances of 14·0 pS for K^+, 8·3 pS for Rb^+, 8·2 pS for NH_4^+ and 7·0 pS for Na^+ in the illustrated cases.

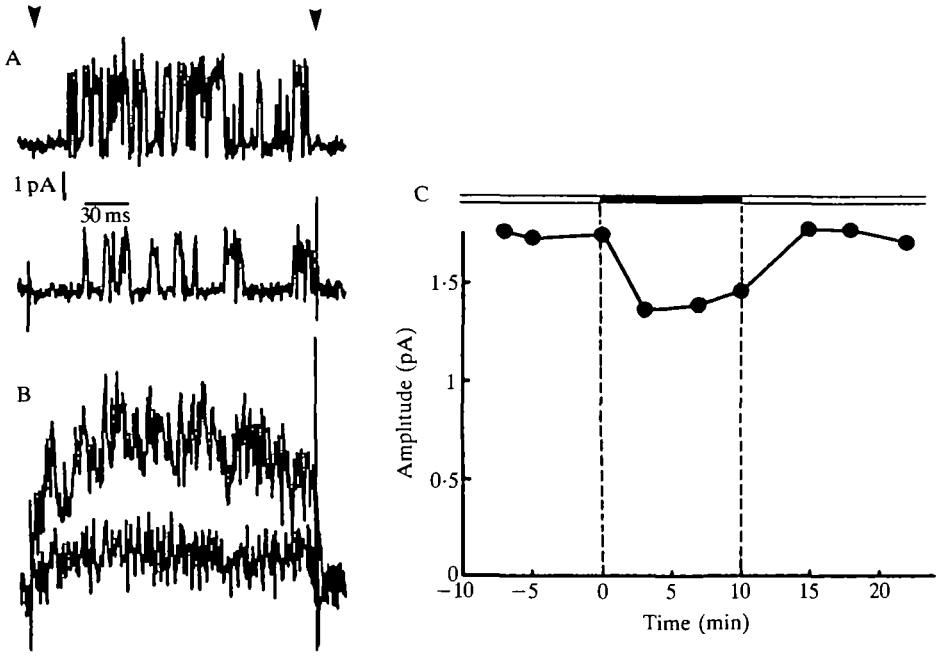

Fig. 5. Effect of TEA^+ on the single, non-inactivating K^+ channel current. (A) Examples of single-channel currents elicited by depolarization of the membrane to +90 mV from a holding potential of −70 mV before (upper trace) and 3 min after (lower trace) addition of 10 mmol l^{-1} TEA^+ to the cytoplasmic side in a single inside-out patch. Traces in B represent the average current of 32 consecutive traces obtained during repetitive depolarizations at 0·4 Hz in the absence (larger current) and presence (smaller current) of TEA^+. Traces with no channel openings were included in the averaging. Depolarizing steps were applied between the two arrowheads. (C) The time course of action of TEA^+ added at 10 mmol l^{-1} to the cytoplasmic side of an inside-out patch. It took approximately 3 min to replace the chamber solution totally in our perfusion system.

membrane was depolarized (Fig. 6). P_o never exceeded 0·25 and did not saturate, even at 90 mV.

To characterize kinetic properties of the non-inactivating K^+ channel, the open- and closed-time histograms were analyzed (Fig. 7). The open-time distribution could be fitted by a single exponential, whereas the closed-time distribution typically required three exponentials to obtain a good fit. The time constant of the open-time distribution as well as the time constants for three components of the closed-time distribution were voltage-dependent (Table 1).

Channel behaviour in a mutant, eag

In *Drosophila*, several single-gene mutants with some defects in K^+ channel functions have been reported (Salkoff & Tanouye, 1986; Wu & Ganetzky, 1986). Mutations at the *Shaker* locus alter or reduce the transient outward current mediated by the A channel in larval and adult muscle (Solc *et al.* 1987). Mutations

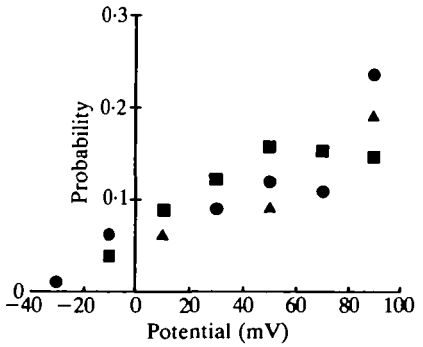

Fig. 6. The probability of a channel being in the open state (P_o) plotted as a function of membrane potential. Different symbols represent data from different patches.

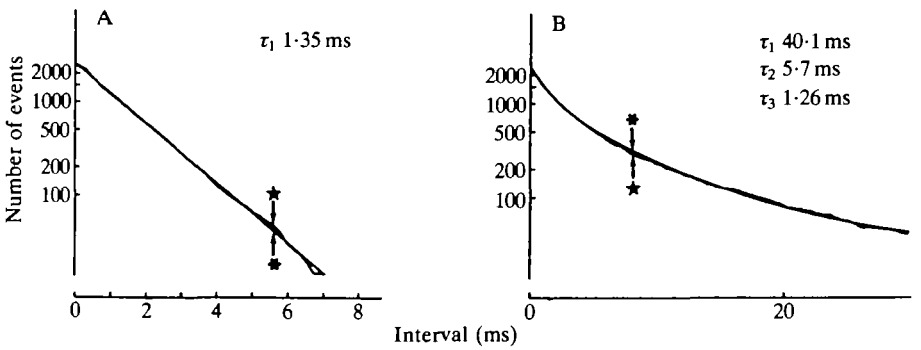

Fig. 7. The open- (A) and closed- (B) time distributions estimated from data obtained with a single inside-out patch. The single-channel currents were elicited by depolarizing the membrane to +70 mV at 20°C. The number of events with a duration longer than that indicated on the abscissa is plotted. Noisy curves (marked with star) represent experimental results, whereas smooth curves (marked with asterisks) are the least-squares exponential fits to the data. The smooth curve in A is drawn using the equation, $N = 2502\exp(-0{\cdot}74t)$ where N is the number of events and t is the duration in ms. The smooth curve in B is drawn using the equation, $N = 82\exp(-0{\cdot}025t) + 1034\exp(-0{\cdot}18t) + 1360\exp(-0{\cdot}8t)$.

at the *eag* locus produce marked reduction of the steady-state outward current with little change in the transient current (Wu *et al.* 1983*a*). The single non-inactivating potassium channel current could be recorded from neurons dissociated from an *eag* mutant (Fig. 8). A detailed analysis of conductance as well as gating properties failed to detect any significant difference between the wild-type and the *eag* mutant. No evidence was obtained for differences in the density of the channel in neuronal membranes of normal and mutant *Drosophila*.

Discussion

The present experiments reveal that the ion channel mediating the non-

Table 1. *Reciprocal time constants of decay of open- and closed-time distributions*

Membrane potential (mV)	Reciprocal time constants (mean ± S.E.) (ms^{-1})			
	r_o	r_{cf}	r_{ci}	r_{cs}
−10	0·84			
+10	0·72	0·61	0·36	0·02
+30	0·79 ± 0·114	0·65 ± 0·033	0·40 ± 0·138	0·02 ± 0·005
+50	0·71 ± 0·151	0·99 ± 0·240	0·30 ± 0·141	0·03 ± 0·007
+70	0·64 ± 0·088	1·27 ± 0·196	0·17 ± 0·086	0·03 ± 0·004
+90	0·52 ± 0·051	2·03 ± 0·092	0·16 ± 0·059	0·04 ± 0·015

One component for the open time (r_o) and three components (r_{cf}, fast; r_{ci}, intermediate; and r_{cs}, slow) for the closed time are indicated.

The values are the mean and standard error of the mean of estimates from four different patches, except for the values at −10 and +10 mV, where only one patch was used.

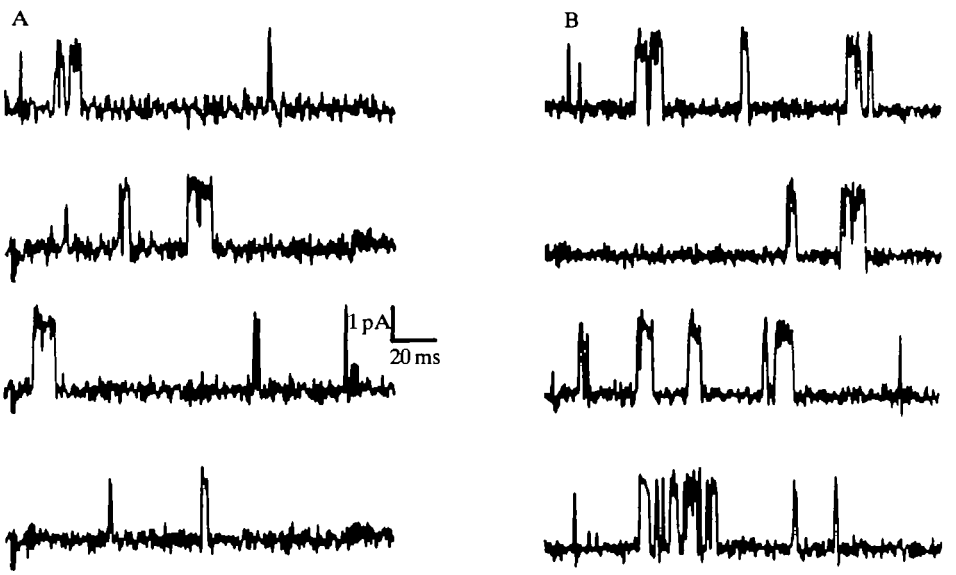

Fig. 8. Examples of single, non-inactivating K^+ channel currents observed in membranes from wild-type (A) or *eag* mutant (B) *Drosophila* neurons. The single-channel currents were elicited by depolarizing the membrane to +70 mV from a holding potential of −70 mV.

inactivating outward current in *Drosophila* neurons shares some characteristics with delayed rectifier potassium channels in other systems. Our estimate of the single-channel conductance, 17·6 pS, with 145 mmol l^{-1} cytoplasmic K^+, is very close to that reported in frog skeletal muscle (Standen *et al.* 1985), human T lymphocytes (Cahalan *et al.* 1985; Bregestovski *et al.* 1986), turtle colon epithelial cells (Richards & Dawson, 1986), bovine adrenal chromaffin cells (Marty &

Neher, 1985) and chick ciliary ganglion neurons (Gardner, 1986). Our experiments indicate that the non-inactivating channel is highly selective for K^+, showing lower permeability to Rb^+, NH_4^+ and Na^+. Cs^+ appears not to permeate the channel. The ratio of slope conductances for these cations relative to K^+ was: $K^+(1) > NH_4^+(0{\cdot}53) > Rb^+(0{\cdot}47) > Na^+(0{\cdot}43) \gg Cs^+(0)$. A permeability ratio based on the reversal potential measurement could not be determined in our experiments since the single-channel current did not reverse its polarity within a manageable potential range (because the practically impermeant choline ion was used as the external cation). The selectivity sequence, based on permeability ratio, for the squid delayed rectifier K^+ channel is: $K^+(1) > Rb^+ > NH_4^+(0{\cdot}3) \gg Cs^+, Na^+$ (Conti *et al.* 1975), and a similar sequence has been reported for delayed rectifier K^+ channels in several other preparations (see Latorre & Miller, 1983). The discrepancy in the selectivity sequence between the present experiments and previous work, particularly the findings for Na^+, may arise from the difference in the parameters (i.e. slope conductance *vs* reversal potential permeability ratio) used to estimate the selectivity sequence (see Eisenman & Horn, 1983). It may be noted that Na^+ can be punched through the delayed K^+ channel of squid axons at very positive potentials, carrying significantly large outward currents (French & Shoukimas, 1985).

Another similarity of the non-inactivating K^+ channel of *Drosophila* to the delayed rectifier resides in its sensitivity to TEA^+. The observation that cytoplasmic TEA^+ reduces the single-channel amplitude is in keeping with previous reports in other preparations (Coronado *et al.* 1984; Standen *et al.* 1985). In addition, we have found an increased frequency of traces with no channel openings during repetitive pulses in the presence of TEA^+ (Yamamoto & Suzuki, 1989).

Voltage-dependent gating of the non-inactivating K^+ channel was clearly demonstrated (Fig. 6; Table 1). The finding that the closed-time distribution is fitted by three exponential functions indicates that the non-inactivating K^+ channel possesses at least three distinct closed conformations.

The permeability and gating properties of the non-inactivating K^+ channel of *Drosophila* neurons, described in this study, are typical of the delayed rectifier class of K^+ channel. We did not find any defect in channel function in neurons of *eag*, which was believed to be a mutant of the delayed rectifier K^+ channel in *Drosophila*. The *eag* mutation causes hyperexcitability of larval presynaptic terminals and reduction of outward currents in the larval muscle fiber (Wu *et al.* 1983*a*), suggesting that the channel associated with the *eag* locus may be present in some axons and terminals of *Drosophila* neurons. If the channel is confined to this region, this could explain why we did not detect it in the somatic membrane. It is also possible that *Drosophila* has several types of delayed rectifier K^+ channel and that our preparation lacks the particular channel type encoded by the *eag* gene because of inadequate culture conditions. Indeed, cell bodies of neurons cultured by our method appear to lack Na^+ channels, which are inducible by adding insulin to the culture media (Solc *et al.* 1986). Alternatively, the *eag* gene might not be a

structural gene encoding the delayed rectifier K^+ channel protein but, rather, might act as a regulator controlling expression of the channel *in vivo*.

Recently, Solc & Aldrich (1988) have reported that larval *Drosophila* neurons possess at least three distinct types of potassium channels. The channel reported here resembles their K_1-channel.

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