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Properties of single chloride channels in primary neuronal cultures of *Drosophila*

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An anion channel from *Drosophila* neurons had the selectivity sequence: NO_3^- (1.97) > Br^- (1.12) ≈ I^- (1.03) ≈ Cl^- (1) > F^- (0.32) ≫ glutamate(< 0.02) as estimated by the permeability ratio based on the reversal potential measurement. 4-Acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid applied internally at 10 μM blocked the channel. We suggest that the chloride channel identified here may provide a pathway for Cl^- in the resting membrane of *Drosophila* neurons.

Membranes of invertebrate neurons [9,13] and muscles [3] are highly permeable to chloride ions. It has been suggested that Cl^- is free to move across the membrane as the resting membrane potential changes only transiently upon removing external Cl^- [3]. However, the pathways through which Cl penetrates the membrane have not been unequivocally identified in these preparations. In this paper we describe a ligand-independent, weakly voltage-dependent chloride channel which may mediate the resting Cl^- permeability in primary cultured *Drosophila* neurons.

Single-channel currents were recorded from excised inside-out membrane patches using the gigaohm-seal patch-clamp technique. The cells were prepared as described previously [14], and were immersed in normal saline solution containing 125 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 25 mM glucose, 10 mM sucrose, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), and the pH was adjusted to 7.3 with NaOH. The patch pipet was filled with an extracellular solution containing 145 mM choline Cl, 3 mM $MgCl_2$, 2 mM EGTA, and 10 mM 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (Tes), and the pH was adjusted to 7.3 with tetramethylammonium hydroxide. The Ca^{2+}-, K^+- and Na^+-free pipet solution was used throughout the study to eliminate Ca^{2+}-activated conductances and Na^+- and K^+-dependent inward currents [16]. The composition of the internal solution was 145 mM KCl, 1.13 mM $MgCl_2$, 1.92 mM EGTA, and 10 mM Tes. 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 KNO_3, KBr, KI, potassium glutamate, or KF on the equimolar basis. Some experiments were performed with internal solutions containing either Na^+ or Cs^+ as substituted ions for K^+. 4-Acetamide-4′-isothiocyan-stilbene-2,2′-disulfonic acid (SITS) obtained from Nutritional Biochemicals, Ohio, was dissolved in the internal solution at desired concentrations. The temperature of the chamber was controlled by a Peltier device and measured with a thermocouple probe. The temperature was kept at 10 ± 0.5 °C throughout the experiments. To record single-channel currents, the model 8900 Patch Clamp-Whole Cell Clamp amplifier (Dagan, Minneapolis, MN) with a probe type 8930 (feedback resistor: 10GΩ) was used. The low-pass filter (6 pole) was set at 1 kHz. The bath potential was measured using a separate reference microelectrode filled with 3 M KCl. The interior of the pipet was clamped at a command voltage which was generated by the Wavetek model 275 function generator (Wavetek Inc., San Diego, CA) under the control of a microcomputer (model 9816S; Hewlet-Packard Co., Fort Collins, CO) [15]. The current signals were digitized at a sampling frequency of 5 kHz, using a Nicolet digital oscilloscope (2090-3C; Nicolet Scientific Corp., Northvale, NJ). Digitized data were stored on floppy disks. Leakage and capacitive currents were eliminated from the records by subtracting averaged records containing no channel openings.

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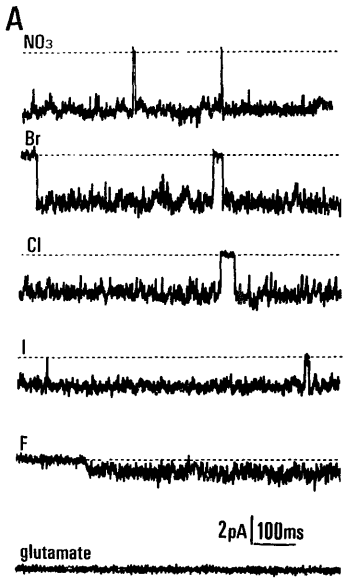

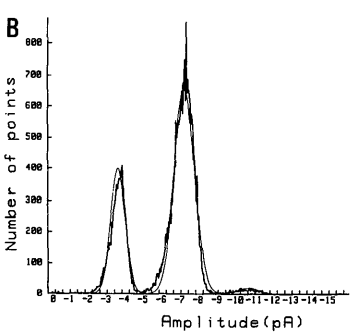

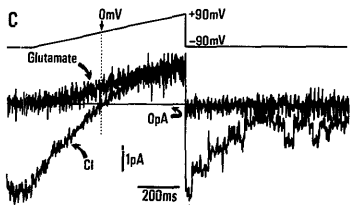

Fig. 1. Effect of Cl^--free internal solutions on the single-channel current. (A) Examples of single-channel currents with internal solutions containing NO_3^-, Br^-, I^-, Cl^-, F^-, or glutamate at 145 mM at a holding potential of −90 mV. Each trace was obtained from different patches. The external solution contained 145 mM chloride. Broken lines represent the zero conductance level. (B) An amplitude histogram (noisy curve) constructed from 32768 sampling points from a 32 s continuous record at −90 mV. The inside-out patch was exposed to the Br^--rich solution internally and the Cl^--rich solution externally. The data points were fitted with a sum of Gaussian distributions. For each peak (corresponding to one current amplitude), the position of the peak and the value of the standard deviation were determined by eye. The peak amplitudes are 3.5, 7.1, and 10.5 pA. (C) Voltage-ramp records from a patch that contained a single chloride channel in the Cl^--containing and Cl^--free (replaced with glutamate) internal solutions. The external solution contained 145 mM Cl^-. Each record represents the average current of six selected traces. The tail current associated with repolarization to −90 mV following a voltage-ramp in the Cl^--containing solution reflects voltage-dependent closures of the channel.

At a holding membrane potential of −90 mV, two distinct types of single inward channels were observed. One channel was identified earlier as a 'fast' chloride channel that had a relatively small unitary conductance of 7 pS and a short open time of 1.2–1.6 ms [17]. The other type of single channel had a much larger amplitude and remained open for tens of seconds. The present paper deals with ionic selectivity of this slow channel.

As illustrated in Fig. 1A, large inward currents were observed with internal solutions containing NO_3^-, I^-, Br^-, or Cl^-. F^- also passed through the channel. In contrast, total replacement of these anions with glutamate completely eliminated the inward current (Fig. 1A). The single-channel current was unaffected by replacing K^+ with either Cs^+ or Na^+ (not shown).

To estimate single-channel conductance, current-voltage (I-V) relationships were constructed for several different patches. Most of the patches studied contained more than one channel, usually three or more. In those cases, the single-channel amplitude was estimated from the amplitude histogram such as shown in Fig. 1B at each membrane potential. When the patch contained only one channel the I-V curve was obtained with a potential ramp. In the illustrated example (Fig. 1C), the

TABLE I

Permeability properties of the single-chloride channel

Anion species	Number of experiments	Reversal potential (mV) (mean ± S.E.)	Permeability ratio P_x/P_{Cl}	Chord conductance (pS) (mean ± S.E.)	Conductance ratio G_x/G_{Cl}
NO_3^-	3	+18.0 ± 2.14	1.97	40.5 ± 1.07	1.15
Br^-	3	+4.4 ± 2.25	1.12	34.8 ± 2.02	0.91
I^-	2	+2.5	1.03	28.0	0.80
Cl^-	5	+1.7 ± 3.29	1	35.2 ± 5.32	1
F^-	3	−27.0 ± 0.21	0.32	18.7 ± 0.54	0.53
Glutamate	3	–	< 0.02	–	0

channel continued to open for the duration of the ramp. The records were leak-subtracted so that the current while the channel was closed is horizontal. The slope of the inclined trace is equal to the single-channel conductance, and the intercept is equal to the reversal potential, E_r. With the Cl^- concentration of 145 mM in both sides of the membrane, the current reversed its polarity from inward to outward at about 0 mV (Fig. 1C). The chord conductance estimated between E_r and −90 mV was 35 pS in this condition. Although the *I-V* curve was practically linear in the potential range between −90 mV and E_r, the current amplitude leveled off at more positive potentials than E_r. This type of rectification of the single-channel *I-V* curve was always seen irrespective of permeant anion species added to the cytoplasmic side. When the internal solution was switched to a Cl^-free glutamate solution (145 mM glutamate) while that of the external solution was fixed (i.e., 145 mM Cl^-), the inward current disappeared, leaving the outward current at depolarized potentials intact.

The results of these experiments are summarized in Table I. From changes in E_r, the ratio of the test cation permeability (P_x) to the chloride permeability (P_{Cl}) was calculated from the constant field equation [6]. The channel had the permeability sequence: $NO_3^- > Br^- \approx I^- \approx Cl^- \gg F^- >$ glutamate. The conductance ratio gave a slightly different selectivity order: $NO_3^- > Br^- = Cl^- > I^- > F^- \gg$ glutamate. The discrepancy between these two parameters would be expected if the channel behaves as a saturable pore. Hille [5] has clearly shown that the permeability ratio depends only upon peak energy differences whereas conductance ratios also depend upon the degree of occupancy of the site within the channel.

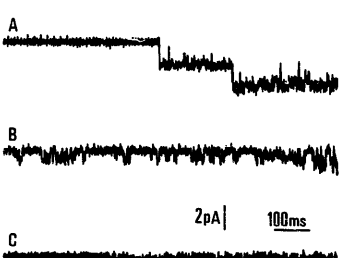

Fig. 2. Single-channel currents recorded before (A), one min (B) and 2 min (C) after perfusing the cytoplasmic surface of a patch with 10 μM SITS. Two independent channels were active.

SITS has been known as an effective blocker of chloride channels in other systems [2,7,8]. We examined the effects of SITS applied to the cytoplasmic side of membrane on the slow chloride channel currents. Following the introduction of 10 μM SITS to the cytoplasmic side of the membrane, repetitive rapid transitions (flickering) between open and blocked states appeared, accompanying a progressive decline in the amplitude of the open channel (Fig. 2). Within a few minutes the current was completely blocked.

The present experiments reveal that the slow chloride channel in *Drosophila* neurons shares some characteristics with the anion transporting mechanisms in other systems. The selectivity sequence of the *Drosophila* slow channel is almost identical to that of anion channels in rabbit epithelium [4] and of the Cl^-/HCO_3^- exchanger in the guinea-pig vas deferens [1]. Another similarity of the *Drosophila* slow channel to many other anion channels resides in its sensitivity to SITS.

The most complete analysis of the permeability sequence for the chloride channels in insect neurons was made on GABA-activated currents by Pinnock et al. [10]. They found that the reversal potential for GABA-activated current was not changed by intracellular injections of F^-, though a positive shift of the reversal potential was seen with injecting I^-, Br^-, and Cl^-. The discrepancy between the F^- permeability in the present experiment and that in their work may reflect the difference between the ligand dependent and independent chloride channels. In fact, a voltage-insensitive, ligand-independent chloride channel in the vascular smooth muscle was highly permeable to F^- (F^-/Cl^-: 0.7) [12]. It should be noted, however, that the data from the cockroach neuron are somewhat qualitative, since the actual intracellular concentration of anions was not determined.

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