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Anion Channels from Rat Brain Synaptosomal Membranes Incorporated into Planar Bilayers

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Summary. Synaptic membranes from rat brain were incorporated into planar lipid bilayers, and the characteristics of two types of anion-selective channels (type I and type II) were investigated. In asymmetric $BaCl_2$ buffers (*cis*, 100 mM/*trans*, 25 mM), single channel conductances at −40 mV were 70 pS (type I) and 120 pS (type II). Permeability ratios ($P_{Na}:P_{Ba}:P_{Cl}$) calculated from the Goldman-Hodgkin-Katz current equation for type I and type II channels were 0.23 : 0.04 : 1 and 0.05 : 0.03 : 1, respectively. Both channels exhibited characteristic voltage-dependent bursting activities. Open probability for type I channels had a maximum of ~0.7 at about 0 mV and decreased to zero at greater transmembrane potentials of either polarity. Type II channels were relatively voltage independent at negative voltages and were inactivated at highly positive voltages. Type I channels showed spontaneous irreversible inactivation often preceded by sudden transition to subconducting states. DIDS blocked type I channels only from the *cis* side, while it blocked type II channels from either side.

Key Words synaptosomal membrane · anion channels · voltage dependence · DIDS

Introduction

Chloride channels of animal cell membranes are usually classified into three categories (Hille, 1984): (i) background, (ii) transmitter-activated, and (iii) voltage sensitive. Background channels are found in the cell membranes of vertebrate muscle; they show weak voltage dependence and are considered to take part in stabilizing the membrane potential. The typical transmitter-activated Cl channel is anion-permeable GABA (γ-aminobutyric acid)-activated in synaptic membranes of inhibitory neurons (Hamill, Bormann & Sakmann, 1983). Although their physiological roles are unknown, anion channels of the third category, the voltage-sensitive Cl channels, are reported in a variety of tissues including the mammalian central nervous system (Gray, Bevan & Ritchie, 1984; Franciolini & Nonner, 1987; Nowak, Ascher & Berwald-Netter, 1987; Jalonen et al., 1989).

Ion channels on brain synaptic membranes have been investigated by using the planar bilayer method (reconstitution of synaptic membranes into artificial planar bilayers) because of the small size and inaccessibility of synapses. Although there have been detailed studies on synaptic Na channels (Krueger, Worley & French, 1983), Ca channels (Nelson, French & Krueger, 1983), and Ca-activated K channels (Reinhart, Chung & Levitan, 1989; Nomura et al., 1990), only one report (Nelson, 1986) has referred to synaptic Cl channels. In this paper, we describe characteristics of two types of voltage-sensitive anion-selective channels from rat brain synaptic membranes. These channels showed voltage-dependent "inactivation" which resembled that of voltage-sensitive anion channels from various tissues. Preliminary results were reported elsewhere (Nomura & Sokabe, 1990).

Materials and Methods

Biochemicals

Rat brain synaptosomal membranes were prepared from forebrains of female 4-week old Wister rats by sucrose gradient centrifugation as described by Jones and Matus (1974) with minor modifications (Nomura et al., 1990). Briefly, brain homogenate was centrifuged at 800 × *g* for 20 min, and the supernatant was centrifuged at 9000 × *g* for 20 min. The pellet was resuspended in hypotonic buffer (5 mM Tris-HCl, pH 8.1, 0°C) for 30 min. The lysate was made up to 34% (wt/wt) sucrose with 28.5% (wt/wt) and 10% (wt/wt) sucrose placed above to give a gradient. The density gradients were centrifuged at 34,000 × *g* for 60 min using a vertical rotor (RPV-30, Hitachi). The middle of three fractions was collected (synaptosomal fraction) and stored in small aliquots at −80°C. Bovine heart phosphatidylethanolamine and phosphatidylcholine were obtained from Avanti Polar Lipids (Pelham, AL). 4,4′-diisothiocyanostilbene 2,2′-disulfonic acid (DIDS) was

purchased from Pierce (Rockford, IL) and γ-aminobutyric acid (GABA) was from Sigma (St. Louis, MO). Salts were of special grade commercially available.

Planar Bilayer System

Planar bilayers were made as previously described from phospholipid solution (a mixture of 30% phosphatidylcholine/70% phosphatidylethanolamine (wt/wt), 15 mg/ml in *n*-decane) on a hole (300–500 μM in diameter) in a polypropylene partition separating the two aqueous chambers (Tanifuji, Sokabe & Kasai, 1987; Nomura et al., 1990). Channel currents were measured under voltage clamp by a patch-clamp amplifier (Nihon Koden Model S-3666). The side to which vesicles were added was defined as the *cis* side. The opposite side was defined as the *trans* side, and the voltage was referred to the *cis* side with respect to the *trans* side. Usually we used buffer solutions of asymmetric salt concentrations (*cis* side four times the concentration of the *trans* side) because channel incorporation rate was extremely low in symmetrical solutions. The buffers contained 5 mM Tris base adjusted to pH 7.2 by added HCl. All experiments were carried out at room temperature (20 ~ 25°C). Anion channels were always incorporated with a fixed orientation which was confirmed by DIDS sensitivity (type I channels were only sensitive to DIDS added to the *cis* side) and the polarity of voltage dependence (type II channels were inactivated only at positive voltages).

Data Analysis

The channel currents were conditioned by a four-pole Bessel low-pass filter (frequencies given in the legends) and recorded on a PCM digital tape recorder. Amplitude histograms were constructed from digitized (500 μsec/point) 2 min records at each voltage. Steady-state open probabilities for type I channels were estimated from the ratio of open peak area to the total area. Open and closed time distributions of type I channels were analyzed by the PAT (ver.6.2) program written by Dr. John Dempster (University of Strathclyde, Glasgow, U.K.). Since type I channels often showed unstable flickering behavior or spontaneous irreversible inactivation, we only analyzed records that exhibited relatively stable gating behavior.

Results

Incorporation of Two Types of Anion Channels

Two types of anion channels, which we term type I and type II, were observed in asymmetric barium chloride ($BaCl_2$) buffers (Figs. 1 and 2). Incorporation of type I channels almost always occurred in a 'one-by-one' fashion, while the incorporation of 'single' type II channels (Fig. 2) was very rare. Type I channels exhibited fast bursting activity interrupted by silent periods; the latter appeared to become longer as the membrane potential was made negative (Fig. 1*A*). For several seconds after incorporation of type I channels, they often showed unsteady flickering (brief openings) followed by 'stable' open-closed fluctuations. The bursts consisted of openings with brief closures in the order of milliseconds (Fig. 1*B*). Apparent subconducting states were not resolved by our recording system, although we observed spontaneous and irreversible inactivation of channel fluctuations preceded by sudden transitions into smaller subconducting states; 'stable' activities of type I channels usually lasted for several minutes (~10 min) and always resulted in spontaneous irreversible 'inactivation.' An example is shown in Fig. 3. In the top trace, clear subconducting states of multiple levels which are not usually observed appear and channel activity abruptly ceases at the middle of the second trace. It took various times (1 to 15 min) for the channel to be inactivated. Type II channels also showed bursting activities (Fig. 2*A*) consisting of openings with many brief closures or transitions to several subconducting states. The latter were not fully resolved because of the limited bandwidth of our recording system. Initial unsteady flickering behavior and spontaneous inactivation, which were observed in type I channels, were not observed in type II channels.

Conductance and Anion *vs.* Cation Selectivity of Type I Channels

From the single channel current-voltage relationship (Fig. 4*A*) in asymmetrical $BaCl_2$ solutions (*cis*, 100 mM/*trans*, 25 mM) at −40 mV, a single channel conductance of 70 pS was obtained. When an asymmetrical NaCl solutions (*cis*, 200 mM/*trans*, 50 mM) were used, the single channel conductance was 55 pS. The reversal potentials were +26.2 mV and +18.7 mV, from which Ba/Cl permeability ratio (P_{Ba}/P_{Cl}) of 0.04 and Na/Cl permeability ratio (P_{Na}/P_{Cl}) of 0.23 were calculated from the Goldman-Hodgkin-Katz equation. The current-voltage relationship in symmetrical 100 mM $BaCl_2$ (Fig. 4*A*, filled triangles and dashed line) was linear and the estimated conductance from the slope was 93 pS. The conductance-concentration relationship did not show a sign of saturation (Fig. 4*B*) up to 800 mM Cl^-, suggesting that the channel is a single ion channel with a low affinity for Cl^- (Tanifuji et al., 1987) or a multi-ion channel.

Voltage Dependence of Type I Channels

In records such as shown in Fig. 1*A*, long-lasting closed intervals between bursts of type I channels seem to become longer and the open probability (P_o) decreases with increasing potential difference across

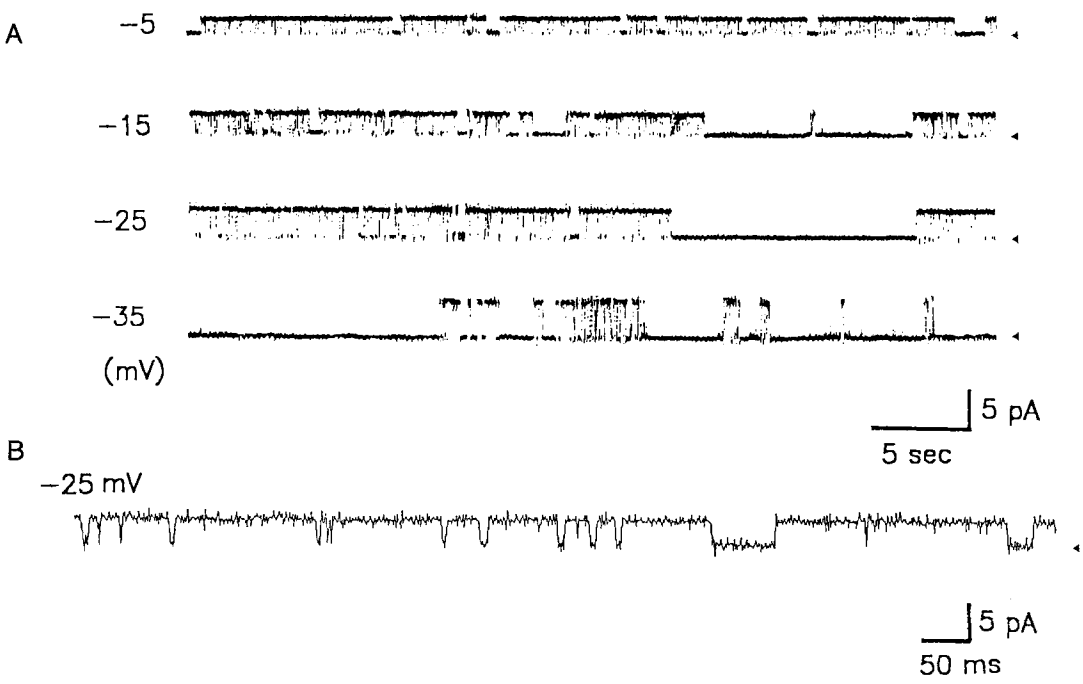


Fig. 1. (*A*) Single channel records of a type I channel at various voltages in asymmetric $BaCl_2$ solutions (*cis*, 100 mM/*trans*, 25 mM). Channel activity occurred in bursts interrupted by protracted closed periods which appeared to become longer as the voltage was made negative. (*B*) Current fluctuations during a burst shown in an expanded time scale. Records are filtered at 500 Hz. Zero current levels are indicated by arrowheads.

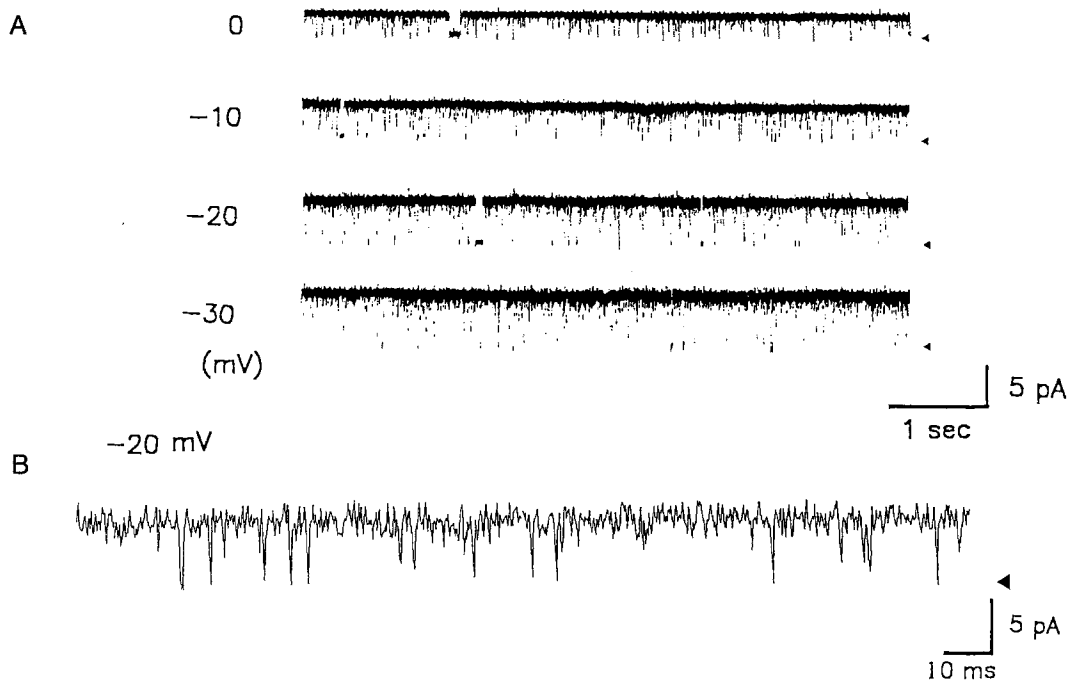


Fig. 2. (*A*) Single channel records of a type II channel in asymmetric $BaCl_2$ solutions (*cis*, 100 mM/*trans*, 25 mM). (*B*) Current fluctuations shown in an expanded time scale show brief (less than 1 msec) shut or partially closed events. Records are filtered at 1 kHz. Zero current levels are indicated by arrowheads.

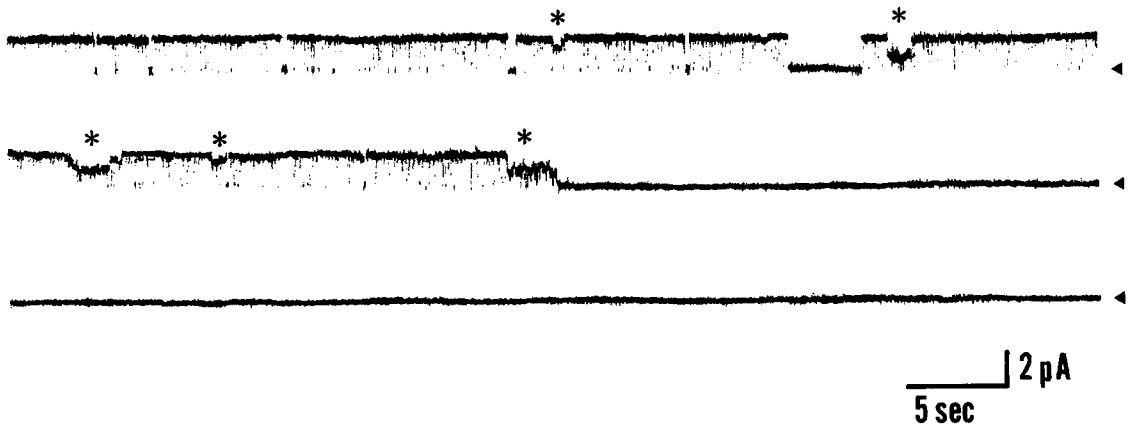
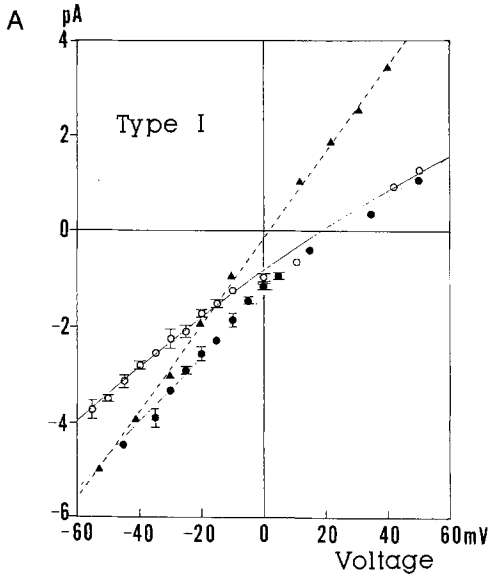
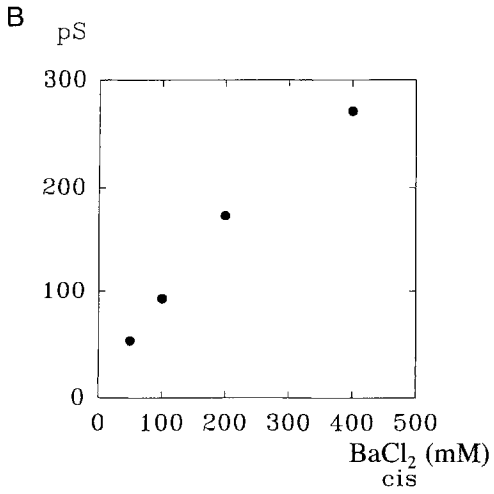


Fig. 3. The process of spontaneous inactivation of a type I channel. The trace shows a serial record (top to bottom) at −20 mV in asymmetrical NaCl solutions (*cis*, 200 mM/*trans*, 50 mM). Emergence of multiple levels of subconducting states (asterisks) is followed by irreversible arrest of channel activity. Zero current levels are indicated by arrowheads.





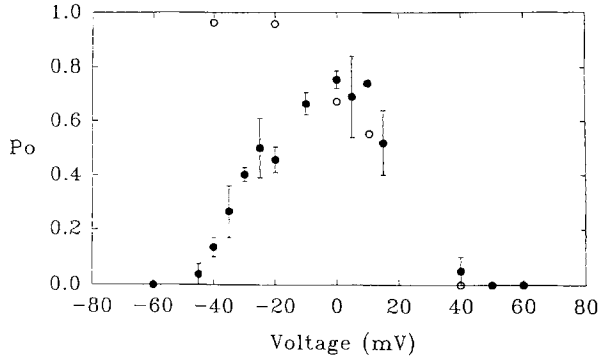


Fig. 5. Filled circles: Voltage dependence of steady-state open probabilities (P_o) for the type I channel in asymmetrical $BaCl_2$ solutions (*cis*, 100 mM/*trans*, 25 mM). P_o was calculated from 2 min single channel records at each voltage. Each symbol with error bar represents the mean ± SE. Open circles: P_o for the type II channel in asymmetrical $BaCl_2$ solutions (*cis*, 100 mM/*trans*, 25 mM). P_o was calculated from 1–2 min single channel records (n = 1). The channel was completely closed (P_o = 0) at levels larger than +40 mV.

Fig. 4. (*A*) Single channel current-voltage relationships of type I channels. In symmetrical 100 mM $BaCl_2$ solutions, the relationship is linear and yielded a single channel conductance of 93 pS (▲). In asymmetrical $BaCl_2$ (●: *cis*, 100 mM/*trans*, 25 mM) and NaCl (○: *cis*, 200 mM/*trans*, 50 mM) solutions, currents reverse at 26.2 and 18.5 mV, respectively. Curves fitted to the data points are drawn according to the Goldman-Hodgkin-Katz current equation using ionic activities. Symbols with error bars represent the mean of 4 ~ 10 membranes ±SE. (*B*) The single channel conductance *vs.* ion concentration relationship of the type I channel. Each conductance (mean of 3 ~ 4 membranes) was obtained from *I-V* relations in asymmetrical $BaCl_2$ solutions (*cis* side was four times the concentration of the *trans* side) at ~40 mV.

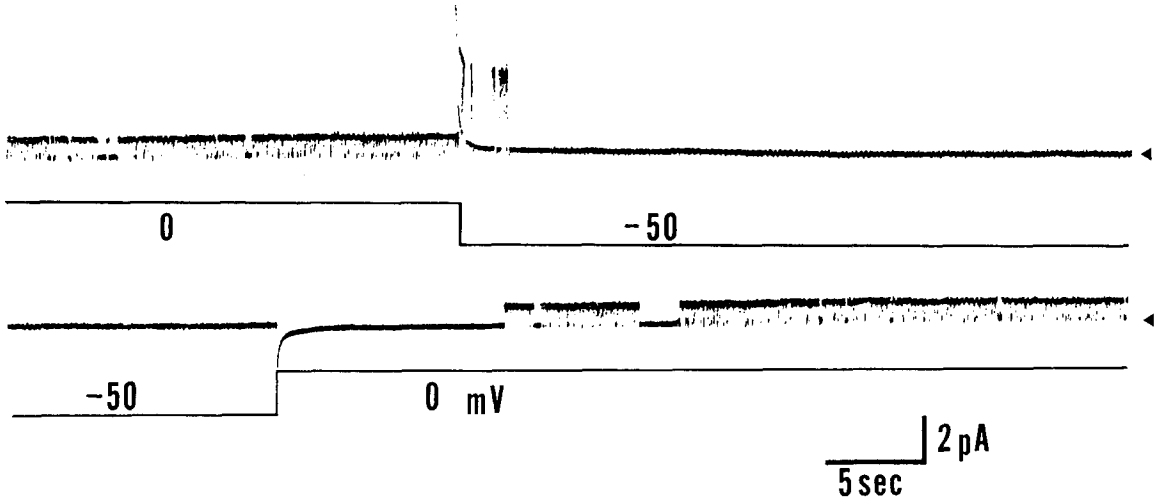


Fig. 6. Reversible voltage-dependent inactivation of a type I channel. The voltage was changed from 0 mV (at which spontaneous bursting activity was seen) to −50 mV (upper trace). Brief openings after the voltage jump were followed by a long silent period (about 4 min). The channel resumed its activity 10 sec after the voltage was restored to 0 mV (lower trace). Arrowheads indicate zero current levels (*cis*, 100 mM/*trans*, 25 mM $BaCl_2$).

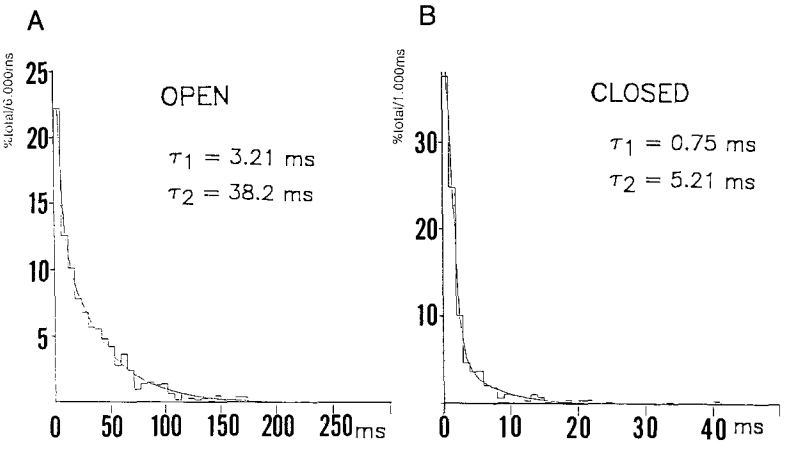


Fig. 7. Open (*A*) and closed (*B*) time histograms obtained from 35-sec records of single type I channel currents at −5 mV. The current was filtered at 500 Hz. Percentage of total events for 6 msec bins (open times) and 1 msec bins (closed times) was calculated. Double exponential functions with two time constants (τ_1 and τ_2) were fitted to the data.

the membrane. Figure 5 shows the steady-state open probability *vs.* voltage relationship for type I channels. P_o takes maximum value of ~0.7 near 0 mV and steeply decreases to zero at larger potentials of either polarity. Current fluctuations at large membrane potentials (> +50 mV or < −50 mV) were observed only when a voltage jump was made from a level at which spontaneous bursting of the channel was seen. Figure 6 shows an example of voltage-dependent inactivation of the type I channel. When the voltage jump was made from 0 mV (at which spontaneous bursting activity usually could be seen) to −50 mV, only brief bursts were observed, which were followed by a long silent period lasting about 4 min. The inactivation represented by long silence was reversible, but the recovery of the channel activity was usually slow (Fig. 6, lower trace). Open and closed time histograms within the burst (*burst* was defined as a group of openings interrupted by closings longer than 100 msec) at −5 mV are shown in Fig. 7, each of which can be fitted with double exponential functions. This indicates that at least two open and two closed states exist within the burst. Two decay time constants (τ_1, τ_2) each for open and closed time histograms are plotted against membrane voltage (0 ~ −45 mV) in Fig. 8. They are essentially voltage independent, suggesting that the decrease of open probability at negative voltages originates from the increase of longer closed state between bursts. We could not analyze channel currents at positive voltages because currents at voltages around the reversal potential (+26.2 mV) were

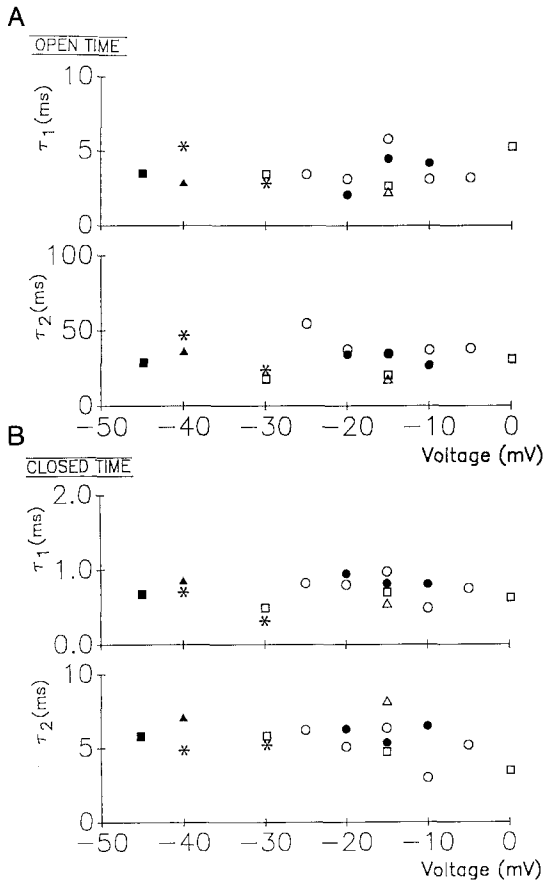


Fig. 8. Open (*A*) and closed (*B*) time constants as a function of voltage (<0 mV). Different symbols represent the data from different membranes.

too small to detect open-close transitions and the channel remained in the closed state at larger positive voltages than +50 mV.

DIDS Blockade of Type I Channels

Figure 9 shows the effect of DIDS, a classical stilbene derivative anion channel blocker, on a type I channel. After the addition of 67 μM DIDS to the *cis* side, the channel open probability gradually decreased time dependently, although the single channel conductance did not change. The effect was irreversible; perfusion with a solution without DIDS could not restore the channel activity. DIDS added to the *trans* side had no effect (~200 μM). The slowly decreasing P_o of type I channel may show transitional process from 'reversible' to 'irreversible' modification of the channel molecule by DIDS. A similar time course of DIDS blocking was reported for colonic secretory Cl channels by Bridges et al. (1989). Calcium (~1 mM) or GABA (~1 mM) added to either side of the membrane did not affect the channel activity.

Characteristics of Type II Channels

Conductance-voltage relationships for type II channels with asymmetric $BaCl_2$ buffer (*cis*, 100 mM/*trans*, 25 mM) yielded a single channel conductance of 120 pS at −40 mV (Fig. 10). The current reversed

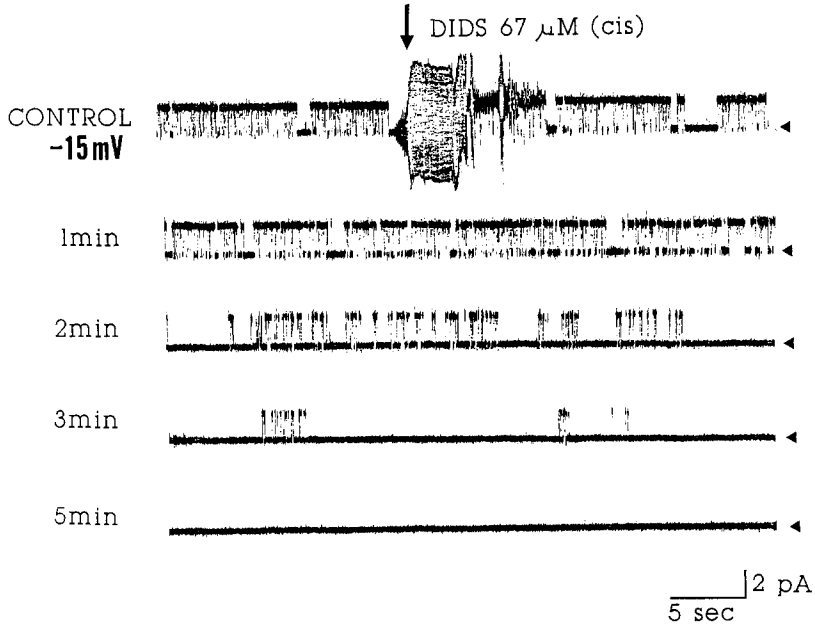


Fig. 9. DIDS (4,4'-diisothiocyano-2,2'-stilbene disulfonate) blockade of a type I channel. Sixty-seven μM DIDS was added to the *cis* side. Channel open probability gradually decreased within 5 min, although the amplitude of channel currents did not change. Arrowheads indicate zero current levels.

at +27.3 mV, which gave a Ba/Cl permeability ratio (P_{Ba}/P_{Cl}) of 0.03. The reversal potential in asymmetric NaCl buffer (*cis*, 200 mM/*trans*, 50 mM) was +28.3 mV, which yielded a Na/Cl permeability ratio (P_{Na}/P_{Cl}) of 0.05. At negative voltages, the channel exhibited fast bursting activity with high open probability ($P_o > 0.95$, Fig. 2). The burst included many brief (<1 msec) shut or partially closed states (not fully resolved because of the limitation of recording equipment). At positive (>0 mV) voltages, long shut periods increased and the channel remained totally closed at highly positive voltages (Fig. 5, open circles). Figure 11 is an example of voltage-dependent slow inactivation of type II channels, where three channels were simultaneously incorporated and the voltage jump from 0 to +50 mV closed all channels. Slow re-activation took place when the voltage was restored to 0 mV. Highly negative voltages did not inactivate the channel. Similar reversible slow inactivation has been reported in anion channels from rat colonic enterocyte (Reinhardt et al., 1987), although the channel was inactivated only at highly 'negative' voltages. Thirty-three μM DIDS from either side of the type II channel caused noisy bursting activity followed by complete blockade within 5 min.

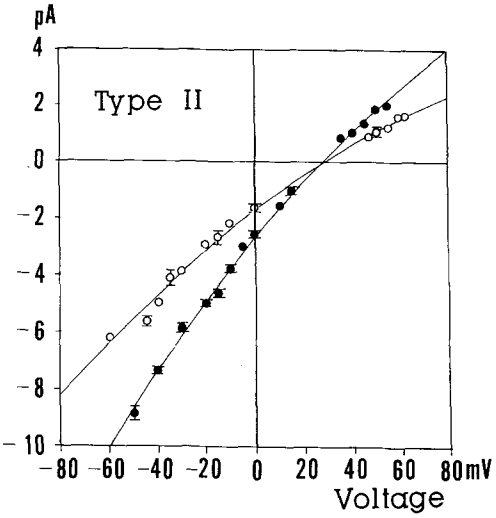


Fig. 10. Single channel current-voltage relationships of the type II channel. In asymmetrical $BaCl_2$ (●: *cis*, 100 mM/*trans*, 25 mM) and NaCl (○: *cis*, 200 mM/*trans*, 50 mM) solutions, currents reverse at +27.3 and +28.3 mV, respectively. Symbols with error bars represent the mean of 3–9 membranes ±SE. Curves are drawn according to the Goldman-Hodgkin-Katz current equation.

Discussion

Anion-Selective Channels from Mammalian Brain

Voltage-dependent anion channels with various single conductances from several mammalian nervous tissues have been reported by using the patch-clamp method: a 450 pS channel from rat cultured Schwann cells (Gray et al., 1984), a 30 pS channel from rat hippocampal neurons (Franciolini & Nonner, 1987), both 5 and 385 pS channels from mouse cultured astrocytes and a 250 pS channel from rat cultured astrocytes (Nowak et al., 1987). Nelson (1986) reported a 400 pS maxi-chloride channel from rat brain synaptosomes incorporated into planar bilayers although its characteristics were not examined in detail. DeBin and Strichartz (1990) have described a 40 pS anion channel from rat brain nerve growth cones which resembled that in rat Schwann cells (Franciolini & Nonner, 1987). These several anion channels are clearly distinguishable from our type I and type II channels by their single conductances at physiological chloride ion concentrations. Single channel current-concentration relationship for type I channels did not saturate at concentrations as high as 400 mM $BaCl_2$ (Fig. 4*B*). Such a nonsaturation relationship was also found in anion channels from hippocampal neurons (Franciolini & Nonner, 1987) and colonic enterocyte plasma membranes (Reinhardt et al., 1987). Usually several type II channels were incorporated at the same time, suggesting that type II channels clustered on synaptic membranes. A chloride channel that showed similar behavior was reported in rat skeletal muscle in a patch-clamp experiment (Blatz & Magleby, 1985). The density of type I channels appeared to be lower than that of type II channels since the incorporation of type I channels always occurred in a 'one by one' fashion. In a few cases, we observed simultaneous incorporation of types I and II channels suggesting that these two channels can co-exist on a membrane vesicle. We observed a few instances of maxi-conductance anion-selective channels (~500 pS) which resembled the maxi-chloride channel reported by Nelson (1986) although the incorporation rate of this type of channel was quite low.

Anion *vs.* Cation Permeability

The selectivity for Cl over Na ($P_{Na}/P_{Cl} = 0.23$) of type I channels was less than that ($P_{Na}/P_{Cl} = 0.05$) of type II channels, while both channels were highly Cl selective over Ba. Many 'maxi'-chloride channels from a variety of tissues have been reported to have significant sodium permeability ($P_{Na}/P_{Cl} > 0.2$) e.g., anion channels from Schwann cells (Gray et al., 1984), macrophages (Schwarze & Kolb, 1984), tra-

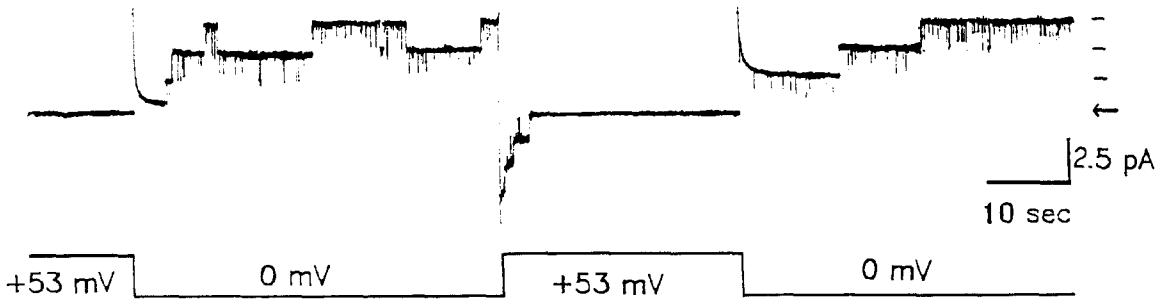


Fig. 11. Reversible inactivation of type II channels (three channels are simultaneously incorporated) at a large positive (+53 mV) voltage. Each channel conductance level (bars) and zero current level (arrow) are indicated at the right of the trace.

cheal epithelial cells (Shoemaker et al., 1986), amphibian skeletal muscle (Woll et al., 1987), cardiac cells (Coulombe et al., 1987). Correlation between the ability to discriminate anions from cations and the single channel conductance seems to be poor; the 30 pS channel from hippocampal neurons (Franciolini & Nonner, 1987) showed significant sodium permeability (P_{Na}/P_{Cl} of 0.25) and the 382 pS channel from pulmonary alveolar cells (Schneider et al., 1985) was reported to be highly selective for Cl over Na (P_{Na}/P_{Cl} of 0.015). Our type II channels also showed relatively high selectivity for Cl over Na, although the channel conductance was larger than that of type I channels.

Voltage Dependence

Steady-state open probability as a function of membrane potential for type I channels formed a bell-shaped curve around zero voltage (Fig. 5). At larger potentials of either polarity ($> +50$ mV or < -50 mV), the channel was inactivated. Similar bell-shaped open probability has been reported in anion channels from cultured Schwann cells (Gray et al., 1984) and astrocytes (Nowak et al., 1987). The anion channels not only from nervous tissues but also those from several other tissues open solely in a narrow voltage range around 0 mV (Nelson, Tang & Palmer, 1984; Schwarze & Kolb, 1984; Kolb, Brown & Murer, 1985; Schmid et al., 1988; Soejima & Kokubun, 1988; Hals, Stein & Palade, 1989; Rousseau, 1989; Velasco et al., 1989). Once inactivated, type I channels stayed closed often for several seconds even when the voltage was restored to the voltage at which open probability was high. It is possible that these apparent slow responses to the voltage were not physiological but represented artifactual effects from cell-free environment (excised patch or planar bilayer). Most anion channels which exhibited slow inactivation and/or reactivation processes have been observed only in excised, cell-free patch recordings or bilayer studies. Another problem in interpreting the voltage dependence of anion channels arises from the concentration and/or composition of ions in the buffer solution. Because the channel incorporation rate was extremely low in symmetric solutions, we usually use asymmetric buffers. A recent study (Hals & Palade, 1990) has reported that voltage dependence of the open probability for sarcoplasmic reticulum (sarcoball) anion channels was dependent on the concentration of permeant anions. Although the physiological significance is not clear, type II channels also showed slow inactivation (Fig. 11). In comparison with the type I channel, the type II channel was inactivated only at highly positive voltages. In our study, both type I and type II channels were incorporated with a fixed orientation. We are led to assume that these anion channels were incorporated with their intracellular side facing the *cis* side by the following facts: (i) Type I and type II channels were often simultaneously incorporated with calcium-activated potassium channels in KCl buffers (*data not shown*); (ii) calcium-activated potassium channels in our preparation were almost always incorporated with their intracellular side (calcium-sensitive side) facing the *cis* side (Nomura et al., 1990). Based on the sideness of the channels, we suggest that type I channels open at a narrow range of *trans* membrane voltage near 0 mV under physiological conditions. We also suggest that type II channels have a high open probability at physiological resting potentials and only strong depolarization can inactivate them. Coexistence of anion channels with different voltage sensitivity has been reported for tracheal epithelial cells (Shoemaker et al., 1986), cardiac cells (Coulombe et al., 1987) and astrocytes (Nowak et al., 1987).

Spontaneous Inactivation of Type I Channels

The type I channel often showed unstable flickering for several seconds after incorporation, suddenly switching to stable bursting kinetics. A similar in-

duction of channel conductance has been reported for anion channels in excised membrane patches of amphibian skeletal muscle (Woll et al., 1987). Stable activities of the type I channel usually lasted for several minutes followed by a spontaneous irreversible inactivation. This inactivation was often preceded by transitions to the multiple levels of subconducting states which were not observed during the stable channel activities. Once the channel was inactivated, a voltage step to a value at which open probability was high (e.g.0 mV) did not restore the channel activity. Alteration of gating behavior associated with changes in single channel conductance has been reported for large chloride channels in rat cardiac cells (Coulombe et al., 1987). Instability of gating kinetics for the type I channel implies several possibilities: (i) it is of artifactual, (ii) fragility (or plasticity) of channel protein, (iii) channel modulation by intra-cellular inhibitors or promoters. The first is unlikely because we did not observe spontaneous inactivation of type II channels. In general, anion channels have not been reported in cell-attached patch-clamp recordings, indicating the possible existence of some intracellular inhibitory factors (Welsh & Liedtke, 1986; Frizzell, Rechkemmer & Shoemaker, 1986; Kunzelmann, Pavenstadt & Greger, 1989). Initial instability of type I channel activity may arise from the release of intracellular inhibitors. Spontaneous inactivation (rundown) of reconstituted channels have been reported for an ATP-sensitive potassium channel of skeletal muscle (Parent & Coronado, 1989). The latter channel spontaneously lost its activity within 6 min without GTP-γ-S and Mg^{2+} at the *cis* (intracellular) side suggesting a G protein-dependent regulation. Whether or not similar regulatory factors participate in the inactivation process for the type I channel needs to be elucidated.

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