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Aminoglycoside Blockade of Ca^{2+}-Activated K^+ Channel from Rat Brain Synaptosomal Membranes Incorporated into Planar Bilayers

Kazushi Nomura†, Keiji Naruse‡, Kazuyoshi Watanabe†, and Masahiro Sokabe‡
†Department of Pediatrics, ‡Department of Physiology, Nagoya University School of Medicine, Nagoya 466, Japan

Summary. Ca^{2+}-activated K^+ channels from rat brain synaptosomal membranes were incorporated into planar lipid bilayers, and the effects of aminoglycoside antibiotics on the single channel conductance (258 ± 13 pS at 100 mM K^+) were investigated. Aminoglycosides reduced the single channel conductance from the '*cis*' (cytoplasmic) side in a dose- and voltage-dependent manner. Voltage dependence of the blockade indicated an interaction between positively charged amino residues of aminoglycoside antibiotics and a binding site located within the electric field of the ion-conducting pathway. The order of blocking potency was consistent with that of the number of amino residues of aminoglycosides (neomycin (6) > dibekacin (5) > ribostamycin (4) = kanamycin (4)), while the electrical distance ($z\delta$ = 0.46–0.49) of the binding site kept almost constant for each drug. These $z\delta$s were almost the same with those (0.46–0.51) of alkyldiamine blockers with two amino residues (total net charge of +2) and approximately twice of those (0.25–0.26) of alkylmonoamine blockers (total net charge of +1). Assuming that amino residues of aminoglycosides and alkylamines shared the same binding site located at 25% voltage drop from the cytoplasmic surface of the channel, the site would have to be at least large enough to accommodate one diamino sugar residue of the aminoglycoside in order to simultaneously interact with two positively charged amino groups. Dose- and voltage-dependent blockade of the channel by gallamine, an extremely bulky trivalent organic cation, supported the picture that the channel has a wide mouth on the cytoplasmic side and its 'pore' region, where voltage drop occurs, may also be quite wide and nonselective, suddenly tapering to a constriction where most charged cations block the channel by 'occluding' the K^+-conducting pathway.

Key Words synaptosomal membrane · Ca^{2+}-activated K^+ channel · aminoglycoside antibiotics · alkylamine · channel blockade

Introduction

Aminoglycoside antibiotics are known to block several types of calcium channels (Suarez-Kurtz & Reuben, 1987; Wagner et al., 1987; Gustin & Hennessey, 1988) and K^+ channels from skeletal sarcoplasmic reticulum (Sokabe, 1983; Oosawa & Sokabe, 1986). The transduction channels on hair cells in the inner ear are also blocked by aminoglycosides (Hudspeth & Kroese, 1983; Ohmori, 1985), and the blockade has been proposed as one of the most possible mechanisms of aminoglycoside ototoxicity (Sokabe, Hayase & Miyamoto, 1982; Hudspeth & Kroese, 1983; Sokabe, 1984; Tachibana et al., 1984; Ohmori, 1985). Very recently this hypothesis was confirmed in the hair cells of frog inner ear (Kroese, Das & Hudspeth, 1989). Here we show one more channel, Ca^{2+}-activated K^+ channel from brain synaptosomal membranes, blocked by aminoglycoside antibiotics. It is very difficult to study the properties of single ion channels on brain synaptic membranes by conventional electro-physiological techniques including patch-clamp method because of the small size and inaccessibility of the synapses. Incorporation of ion channels of synaptic membrane vesicles into artificial planar bilayers makes it possible to characterize the synaptic ion channels at the single channel level. By utilizing this method, single channel recordings of several kinds of ion channels from synaptosomal membranes have been made: Na^+ channel (Krueger, Worley & French, 1983), Ca^{2+} channels (Nelson, French & Krueger, 1983) and K^+ channels (Krueger et al., 1982; Nelson, Roudna & Bamberg, 1983; Farley & Rudy, 1988; Reinhart, Chung & Levitan, 1989), including Ca^{2+}-activated K^+ channels which appear to play important roles in the regulation of synaptic membrane excitability to modulate transmitter release from presynaptic nerve terminals (Bartschat & Blaustein, 1985*a*,*b*).

Recent studies have revealed that several types of Ca^{2+}-activated K^+ channels of different single conductances are present in rat synaptosomal membranes (Farley & Rudy, 1988; Reinhart et al., 1989). Of these channels, so-called 'maxi-K^+ channels' (or 'big K^+ channels') described in a variety of cells and tissues (Latorre, 1986, 1989) are popular because they have very similar properties in their large unitary conductance (200–250 pS) and characteristic

fast gating kinetics modulated by intracellular Ca^{2+} and transmembrane voltage.

We report here the effects of aminoglycoside antibiotics on the Ca^{2+}-activated maxi-K^+ channel from rat brain synaptosomal membranes incorporated into planar bilayers. Aminoglycoside antibiotics on the cytoplasmc side reduced the single channel currents in a dose- and voltage-dependent manner. Voltage dependence of the blockade suggests an interaction between positively charged amino residues of the aminoglycoside and a negatively charged binding site in the channel pore. We also investigated the effects of various alkylamines with much simpler structure and known number of amino residues on the channel to estimate the number of amino residues interacting with the site at the same time. The channel appeared to have fairly wide vestibule within the intracellular side of the ion-conducting pathway which can accommodate at least one diamino sugar residue of aminoglycoside. Preliminary results were already reported elsewhere in an abstract form (Nomura, Naruse & Sokabe, 1989).

Materials and Methods

Synaptosomal Membrane Preparation

Rat brain synaptosomal membranes were prepared by the method of Jones and Matus (1974) with slight modification. Four forebrains from female Wister rats (4-weeks old) were homogenized in 9 volumes of 10% (wt/wt) sucrose by a glass homogenizer. The homogenate was centrifuged at 800 × *g* for 20 min, and the supernatant was removed and centrifuged at 9,000 × *g* for 20 min. The pellet was resuspended in hypotonic buffer (5 mM Tris-HCl, pH 8.1) and incubated at 0°C for 30 min. The lysate (crude mitochondrial fraction) was made up to 34% (wt/wt) sucrose by addition of the appropriate volume of 48% (wt/wt) sucrose, and 10 ml was placed in each of four centrifuge tubes. 28.5% (wt/wt) and 10% (wt/wt) sucrose was placed above the lysate to give a gradient to total volume of 25 ml. These density gradients were centrifuged at 34,000 × *g* for 60 min using a vertical rotor (RPV-30, Hitachi). The middle of the three fractions (pale gray band) were collected and centrifuged with small volume of 0.4 M sucrose, Tris-HEPES, pH 7.4, at 100,000 × *g* for 60 min. The pellet was resuspended in 0.4 M sucrose and stored in small aliquots at −80°C.

Planar Bilayer System

Planar bilayers were formed by applying phospholipid solution (15 mg/ml in *n*-decane) to a hole (300–500 μM in diameter) in a polypropylene partition separating the two aqueous chambers as described elsewhere (Tanifuji, Sokabe & Kasai, 1987). Each chamber was connected to an Ag/AgCl electrode via a glass KCl (3 M) agar bridge, and channel currents were measured under a voltage-clamp condition by means of patch-clamp amplifier (Nihon Koden Model S-3666). All experiments were carried out at room temperature (20–25°C). 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. The buffer solutions usually used were composed of symmetrical 100 mM KCl, 5 mM HEPES, pH 7.2, adjusted with Tris base. In the experiments carried out with low K^+ (<50 mM) solutions, we used buffers with pH adjusted by KOH because a previous report (Vergara, Moczydlowski & Latorre, 1984) indicated that a millimolar amount of Tris had a blocking effect on the Ca^{2+}-activated K^+ channels of muscle T-tubule membranes at very low K^+ concentrations. Ca^{2+} (as chloride salt) was added symmetrically to each chamber. Ca^{2+}-EGTA buffers were used when Ca^{2+} concentration was below 10 μM. Most blocking parameters were obtained from experiments using phosphatidylcholine (PC, commercial grade from soybean) bilayers. In several blocking experiments (with kanamycin and *n*-hexylamine), we also used a mixture of 30% phosphatidylcholine (PC)/70% phosphatidylethanolamine (PE) and found no significant difference in the blocking parameters. Ca^{2+} activation curves (*see* Fig. 2) and conductance-activity relationship (*see* Fig. 9) were obtained from experiments with PC/PE lipid.

Data Analysis

The currents across the bilayer were fed into patch-clamp amplifier, low-pass filtered at 1 kHz by using a two-pole Bessel filter, and recorded on an FM tape recorder. Current recordings obtained at each voltage were digitized at 500 μsec/point for the construction of amplitude histograms by using a home-made software called 'funcf' (by A. Inoue & M. Sokabe). Single-channel current amplitude was measured from peak-to-peak (each peak represents open and closed levels) distance of amplitude histogram or directly from chart records (effective frequency response was 800 Hz). Open probabilities (P_o) were estimated from amplitude histograms as the ratio of open peak area to the total area. Open and closed time distributions were analyzed by the software (PAT) written by Dr. John Dempster (University of Strathclyde, Glasgow, U.K.). The molecular size of the blockers was estimated based on the space-filling model.

Chemicals

Bovine heart phosphatidylethanolamine and phosphatidylcholine were obtained from Avanti Polar Lipids (USA). Soybean asolectin (Type II-S) was purchased from Sigma Chemical (USA). Other chemicals were obtained from the following sources: Sigma Chemical, (USA) (neomycin sulfate, kanamycin sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, gallamine triethiodide); Tokyo Chemical Industry (Japan) (ethylenediamine dihydrochloride, 1,6-diaminohexane dihydrochloride, *n*-butylamine hydrochloride, *n*-hexylamine hydrochloride, tetraethylammonium bromide), Meiji Seika (Japan) (ribostamycin sulfate, dibekacin sulfate). Alkane and salts were of special grade commercially available.

Fig. 1. Single channel currents recorded at a fixed voltage (+35 mV) with symmetrical 100 mM KCl solution. Ca^{2+} concentrations (*cis*) are 1 μM (*A*) and 16.7 μM (*B*), respectively. Addition of 1 mM EGTA (final $[Ca^{2+}] < 3$ nM) eliminates the channel activity (*C*). Records are low-pass filtered at 1 kHz. Zero current levels are indicated at the right of each trace with arrow heads

Results

Characteristics of Ca^{2+}-Activated K^+ Channels from Synaptosomal Membranes

Figure 1 shows typical single channel currents at various Ca^{2+} concentrations under a fixed voltage (35 mV). Increasing Ca^{2+} concentration on the *cis* side increased the time channel stays in the open state (Fig. 1*B*), while chelation of Ca^{2+} with EGTA eliminated channel activities (Fig. 1*C*). Addition of Ca^{2+} or EGTA to the *trans* side had no effect on the conductance and gating of the channel. Most of the time channel incorporation occurred in a 'one-by-one' fashion and Ca^{2+} only on the *cis* side increased the open probability of the channel, suggesting that one synaptosomal vesicle contains one or less channel protein in a fixed orientation; the channels were always incorporated with their Ca^{2+}-activating side ('cytoplasmic' or 'intracellular' side) facing to the '*cis*' aqueous phase. Single channel current-voltage relationship (Fig. 5, dashed line) was linear within the voltage range from −80 to 80 mV in the presence of symmetrical 100 mM K^+ and 100 μM Ca^{2+} to give a constant single channel conductance (258 ± 13 pS, $n = 25$). Channel gating was strictly voltage dependent, i.e., the open probability (P_o) increased with membrane potential (Fig. 2, *e*-fold increase of P_o per 8–11 mV). The theoretical activation curves in Fig. 2 (dotted curves) give zero voltage open probabilities at each Ca^{2+} concentration by which we can get a Hill plot of these values (Fig. 2, inset) with the Hill coefficient (apparent number of Ca^{2+} required for channel activation) of 1.6. Reversal potentials determined from *I-V* relationships in the presence of asymmetric buffer solutions (*cis*, 100 mM KCl; *trans*, 100 mM NaCl) and KCl concentration gradient (*cis* side twofold more concentrated) indicate that the channel is highly selective for K^+ over Na^+ or Cl^- ($P_{Na}/P_K < 0.07$, $P_{Cl}/P_K < 0.03$). All

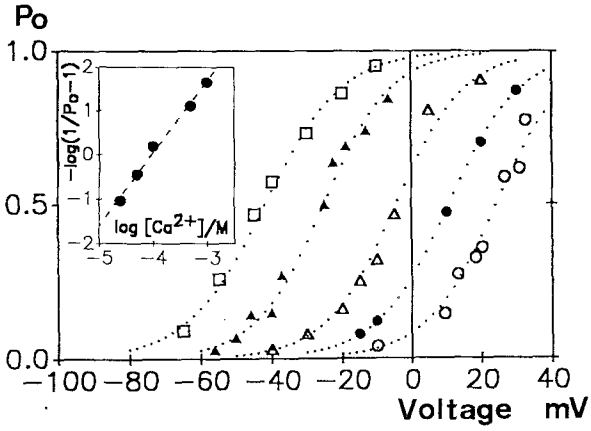

Fig. 2. Channel's open probabilities (P_o) are plotted against the applied voltages at various Ca^{2+} concentrations. Each open probability is obtained from digitized records (500 μsec per point) by the 50% threshold method. The dotted lines are the best fit for data points at each Ca^{2+} concentration to a Bolzmann relation:

$$P_o(V) = \{1 + \exp[-nF(V - Vo)/RT]\}^{-1}$$

where n is an apparent gating charge, Vo is the voltage at which $P_o = 0.5$, V is the applied voltage, and F, R, and T have their usual meanings. Data points were obtained from the same single channel. Ca^{2+} concentration and parameters (n and Vo) for each symbol are as follows, 25 μM Ca^{2+}, $n = 2.5$, $Vo = 24.2$ mV (○); 50 μM Ca^{2+}, $n = 2.4$, $Vo = 10.7$ mV (●); 100 μM Ca^{2+}, $n = 2.5$, $Vo = -4.5$ mV (△); 0.5 mM Ca^{2+}, $n = 2.6$, $Vo = -25.2$ mV (▲); 1 mM Ca^{2+}, $n = 2.3$, $Vo = -41.9$ mV (□). *Inset:* A Hill plot of open probability at zero voltage according to the relation

$$P_o(Ca^{2+}) = [Ca^{2+}]^N/(K + [Ca^{2+}]^N)$$

where K is a constant and N is the Hill coefficient. Open probabilities at zero voltage are 0.083 (25 μM Ca^{2+}), 0.26 (50 μM Ca^{2+}), 0.61 (100 μM Ca^{2+}), 0.93 (0.5 mM Ca^{2+}) and 0.98 (1 mM Ca^{2+}). The least squares regression line (dashed line through symbols) corresponds to $N = 1.63$

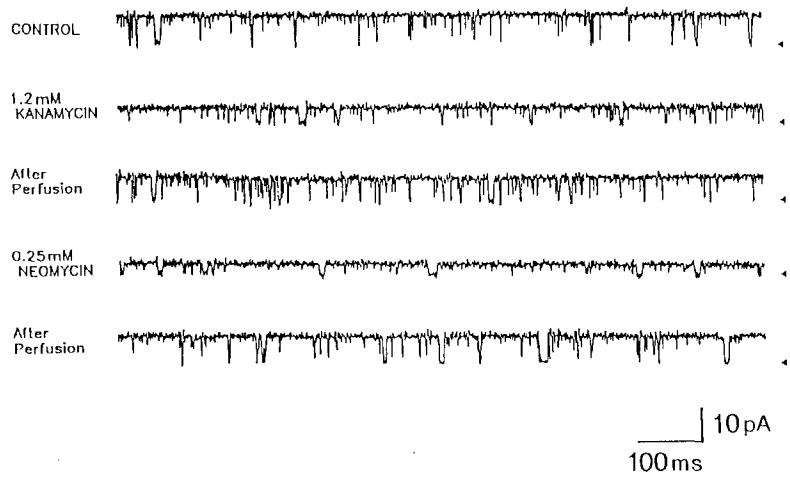

Fig. 3. Reversible blockade of a Ca^{2+}-activated K^+ channel by aminoglycosides. The top trace (*control*) is the record taken with symmetrical solutions containing 100 mM KCl and 100 μM Ca^{2+}. Each aminoglycoside (*kanamycin* and *neomycin*) was added to the *cis* side followed by perfusion with the same solution without drug. Voltage was fixed at +35 mV

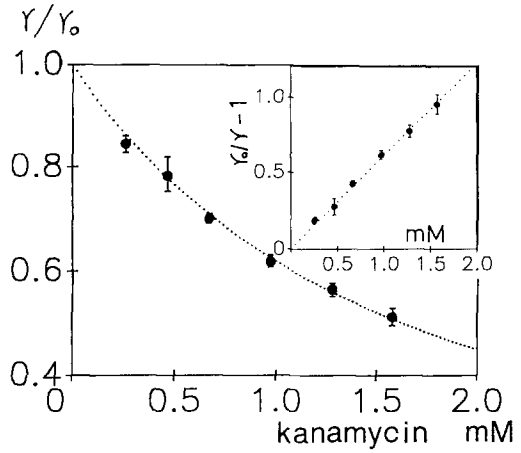

Fig. 4. relative single channel conductance (γ/γ_o) is plotted against the concentration of kanamycin in the *cis* side. (γ_o and γ represent the single channel conductance in the absence and presence of kanamycin, respectively.) Each point represents the mean (±SD) of data from three membranes. Voltage was fixed at +40 mV. *Inset:* Linealized plot of the data. The dotted line is the best fit to the equation:

$$\gamma_o/\gamma - 1 = [KM]/K_d(V)$$

where [KM] is the concentration of kanamicin and $K_d(V)$ is the dissociation constant at that voltage

the characteristics described above (large conductance, Ca^{2+}-sensitivity, voltage dependence and ion selectivity) appear to satisfy the criteria for Ca^{2+}-activated maxi-K^+ channels (Rudy, 1988; Latorre, 1989).

Effect of Aminoglycosides on the Single Channel Conductance

Figure 3 shows the effect of kanamycin (1.2 mM) and neomycin (0.25 mM) applied to the *cis* side on the single channel currents. The channel currents are apparently reduced, and the effect is completely reversible. No open channel flickering can be observed, suggesting that the blocking reaction is too fast to be resolved by our recording system to give us apparent time-averaged current in the equilibrium between open and blocked states. Generally the open (τ_o) and closed (τ_{c1}, τ_{c2}) time distributions could be fitted with single and double exponential functions, respectively. The time constants, for example, at 30 mV were 15.3 msec for open time and 0.97 and 17.2 msec for closed time. Since kanamycin seemed to have little effect on the channel's mean open time and closed time, analysis was made on the single channel conductance. If the drug interacts with the channel in a one-to-one fashion, relative channel conductance (conductance in the presence of drug divided by conductance in the absence of drug) may be fitted with a single site titration curve according to

$$\gamma/\gamma_o = \{1 + [KM]/K_d(V)\}^{-1} \quad (1)$$

where [KM] is the concentration of kanamycin and $K_d(V)$ is the dissociation constant at a given voltage. As shown in Fig. 4, the data from three membranes are well fitted to an inhibition curve with K_d (40 mV) of 1.63 mM.

The effect of kanamycin was voltage dependent. Current-voltage relationship in the presence of the drug (Fig. 5) indicates that the current is inhibited more strongly as the membrane voltage increases. Since the aminoglycosides contain several positively charged amino residues, the voltage-dependent blockade may be interpreted by Woodhull's channel blocking theory (Woodhull, 1973). If we assume that the binding sites for amino residues locate with the same distance from channel

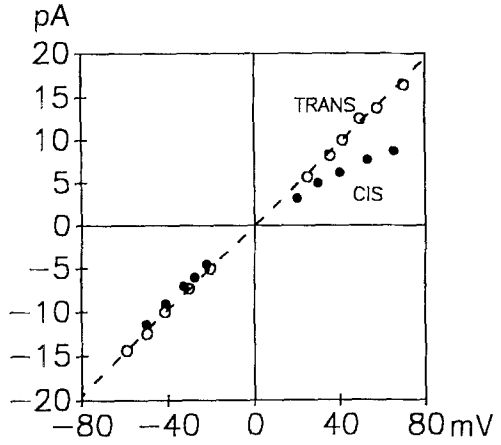

Fig. 5. Single channel current amplitudes are plotted against voltage in the presence of *cis* (filled circles) and *trans* (open circles) 600 μM kanamycin. The dashed line is a linear regression of 12 data points (*not shown*) obtained in the absence of kanamycin (control)

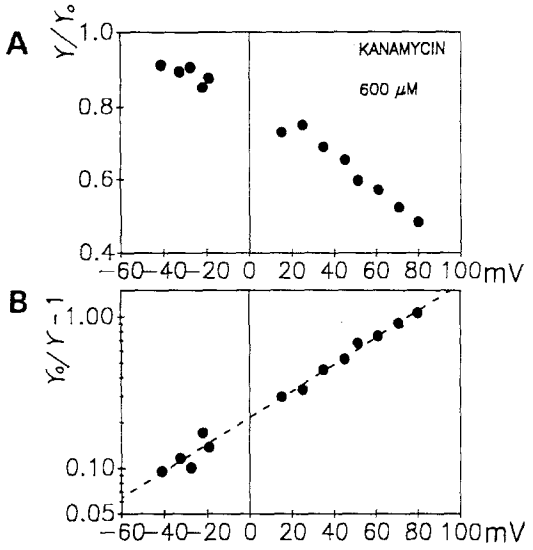

Fig. 6. (*A*) Voltage dependence of kanamycin blockade. Relative single channel conductance (γ/γ_o) in the presence of 600 μM kanamycin (*cis*) is plotted against voltage. (*B*) Linealized plot of the data in *A* according to Eq. (2). The dashed regression line is drawn with the following blocking parameters: $z\delta = 0.51$, $K_d(0) = 2.8$ mM

entrance, the relative conductance (γ/γ_o) could be described as

$$\gamma/\gamma_o = \{1 + [\mathrm{KM}]/K_d(0) \cdot \exp(z\delta FV/RT)\}^{-1} \quad (2)$$

where $z\delta$ is the electrical distance from the *cis* entrance of the channel, $K_d(0)$ is an apparent zero voltage dissociation constant for blockers. In Fig. 6*A*, relative single channel conductances at fixed (600 μM) concentration of kanamycin are plotted against applied voltage, and a linearlized plot by Eq. (2) is shown in Fig. 6*B*. The values of $z\delta$ and $K_d(0)$ for kanamycin are 0.46 ± 0.03 and 2.83 ± 0.32 mM ($n = 6$), respectively. Figure 7 shows the effect of K^+ activity on these blocking parameters. $K_d(0)$ increases linearly with K^+ activity, whereas the values of $z\delta$ remain constant. This indicates a competition between K^+ and kanamycin. If K^+ and kanamycin share the same binding site, $K_d(0)$ is expressed as a linear function of $[K^+]$

$$K_d(0) = K_{d_{KM}}(1 + [K^+]/K_{d_K}) \quad (3)$$

where $K_{d_{KM}}$ and K_{d_K} are dissociation constants for kanamycin and K^+, respectively. From least squares regression line through data points for $K_d(0)$, we can obtain K_{d_K} and $K_{d_{KM}}$ to be 8.0 and 0.22 mM, respectively. The value of K_{d_K} is comparable to K_d (9.9 mM) for K^+ of the 'primary' (high affinity) K^+ binding site of the channel which was obtained from low K^+ activity region of the channel conductance-activity relationship (Fig. 9, *see* discussion). In summary, the channel current is blocked by kanamycin from the cytoplasmic side in a dose- and

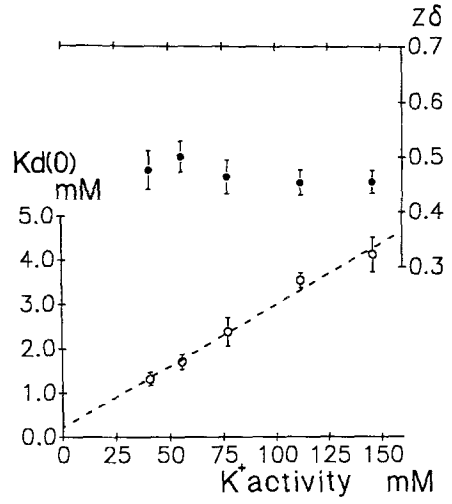

Fig. 7. $K_d(0)$ (open circles) and $z\delta$ (filled circles) measured with solutions of various K^+ activities in the presence of 600 μM kanamycin (*cis*). $K_d(0)$ varies from 1.3 to 4.1 mM as K^+ activity increases from 40.5 to 140 mM. The dissociation constants for K^+ ($K_{d_K} = 8.0$ mM) and that for kanamycin ($K_{d_{KM}} = 0.22$ mM) can be determined from the least squares regression (dashed line) according to a single competitive equation (3) in the text. Each symbol represents the mean (±SD) of data from 3–5 membranes

voltage-dependent manner, and this inhibition is due to the competition for a site locating within the ion-conducting pore where electric potential drop occurs.

We tested the effect of other aminoglycosides

Table 1.

	n	$z\delta$	$K_d(0)$/mM	N
Kanamycin	4	0.46 ± 0.03	2.83 ± 0.32	6
Ribostamycin	4	0.49 ± 0.03	2.39 ± 0.29	6
Dibekacin	5	0.46 ± 0.02	0.600 ± 0.022	4
Neomycin	6	0.48 ± 0.05	0.195 ± 0.028	3

n: number of amino residues; N: number of experiments

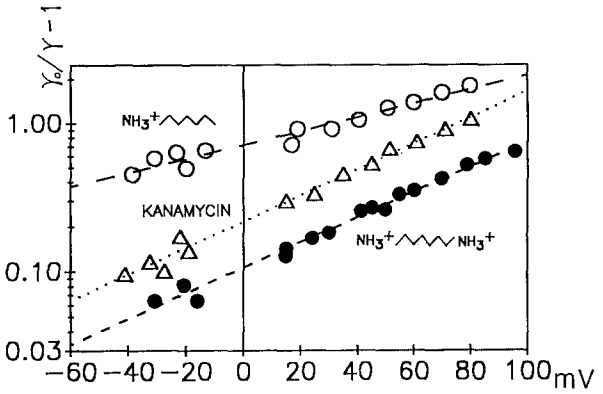

Fig. 8. Voltage dependence of *cis* blockade by *n*-hexylamine (open circles) and 1,6-diaminohexane (filled circles). Each symbol represents a set of data from one membrane. Open triangles are the data for kanamycin in Fig. 6*B*. Regression lines through each symbol correspond to $z\delta = 0.27$ (*n*-hexylamine), $z\delta = 0.49$ (1,6-diaminohexane) and $z\delta = 0.51$ (kanamycin). The concentrations of *n*-hexylamine, 1,6-diaminohexane and kanamycin are 1.0, 2.0, and 0.6 mM, respectively

containing different number of amino residues and found that they also dose- and voltage-dependently blocked the channel. Table 1 shows blocking parameters for each aminoglycoside determined by Woodhull's theory as in the case of kanamycin. The order of blocking potency (neomycin > dibekacin > ribostamycin = kanamycin) parallels that of number of amino residues; neomycin (6 amino residues) is about 15 times more potent than kanamycin (4 amino residues). However, we obtained almost the same $z\delta$ for all aminoglycosides irrespective of the number of their amino residues. This implies, across aminoglycosides tested here, the same number of amino residues interact with the site within the electrical field inside the K^+ conducting pathway (Oosawa & Sokabe, 1986), although we could not determine the value for z and δ separately.

Blockade by Alkylamines

To determine the fraction (δ) of electrical distance involved in the channel blockade by aminoglycosides, we employed *n*-hexylamine, a simpler amino compound with one amino residue, as a model blocker (Sokabe, 1984; Oosawa & Sokabe, 1986). The drug acted as a fast blocker from the *cis* side and the effect was dose and voltage dependent, this is very similar to that of aminoglycosides. In Fig. 8, a linearlized plot of voltage-dependent blockade by *n*-hyexylamine is shown in comparison with that by kanamycin. From this plot the fractional distance (δ) of the amino residue binding site was determined as 0.25, approximately a half value of the electrical distance ($z\delta$) for kanamycin. *n*-butylamine, a shorter alkylmonoamine, also gave a similar value (Table 2). Next we examined the effects of 1,6-diaminohexane, which contains an amino residue at each end of the alkyl chain. This drug also dose- and voltage-dependently blocked the channel, and the resulting value for $z\delta$ was 0.49, almost twice that of *n*-hexylamine. It may be very reasonable to assume that at least one of the amino residue from 1,6-diaminohexane would bind the same binding site for *n*-hexylamine. If so, the electrical distance of the other binding site for the other amino residue of 1,6-diaminohexane becomes about the same as that of the first one. In other words, we can say that the amino residue binding site of synaptosomal Ca^{2+}-activated K^+ channel locates 25% distant of total voltage drop from the *cis* side, and it can bind at least two amino residues at the same time. Blocking parameters for other monovalent and divalent alkylamines with shorter carbon chains are summarized in Table 2. All $z\delta$s for alkyldiamines are about twice those of alkylmonoamines, irrespective of their carbon-chain length. If positively charged amino residues of aminoglycosides share the same binding site with those of alkylamines, only two of them should interact with the site, considering the fact that $z\delta$s for all aminoglycosides are also about twice those of alkylmonoamines (Table 1).

Table 2.

	n	$z\delta$	$K_d(0)$/mM	N
n-butylamine	4	0.26 ± 0.03	14.0 ± 1.5	7
n-hexylamine	6	0.25 ± 0.02	1.65 ± 0.17	7
Ethylenediamine	2	0.49 ± 0.05	21.7 ± 4.4	4
Putrescine	4	0.51 ± 0.03	14.4 ± 0.92	3
Cadaverine	5	0.46 ± 0.04	12.9 ± 1.6	3
1,6-diaminohexane	6	0.46 ± 0.03	17.7 ± 1.6	5

n: number of carbon atoms; N: number of experiments.

Blockade by TEA and Gallamine

Tetraethylammonium (TEA) and related quaternary ammonium (QA) compounds are known to

block potassium conductances of various biological membranes (Armstrong, 1971). QA compounds of various sizes or lengths are used as probes for the estimation of ion-conducting pore size of several K^+ channels (French & Shoukimas, 1981; Swenson, 1981; Coronado & Miller, 1982; Miller, 1982; Villarroel et al., 1988). Ca^{2+}-activated K^+ channels from various preparations are also blocked by both internally and externally applied TEA (Vergara, Moczydlowski & Latorre, 1984; Latorre, 1989). Most of them are more sensitive to externally applied TEA ($K_d < 1$ mM) (Latorre, 1989), although several Ca^{2+}-activated K^+ channels are reported to have opposite sensitivity (Wong & Adler, 1986; Farley & Rudy, 1988). We examined the effect of TEA and an extremely bulky trivalent organic cation, gallamine (1,2,3 Tris(2-trimethylammonium ethoxy) benzene^{3+}), which contains three ethylammonium groups in its structure. Both compounds voltage-dependently blocked the channel currents as fast blockers from the *cis* side with following blocking parameters: $K_d(0) = 38.6 \pm 7.5$ mM, $z\delta = 0.30 \pm 0.03$ ($n = 4$) for TEA and $K_d(0) = 4.7 \pm 1.1$ mM, $z\delta = 0.66 \pm 0.05$ ($n = 7$) for gallmine. The above result implies that these compounds are able to enter the channel's pore and interact with a site within the electrical field as in the case of aminoglycosides and alkylamines. Assuming that positive charges of both compounds interact with the same site located at a fractional electrical distance of 30% voltage drop from the *cis* side, the site should locate in a fairly wide space to accommodate two or more ethylammonium groups of gallamine at the same time. TEA applied to the *trans* side caused a 'flickering blockade' (K_d at +45 mV = 238 ± 22 μM, $n = 4$) with little or no voltage dependence (*data not shown*), whereas gallamine was not effective at the concentrations we tested (0.1–1.0 mM), suggesting that the size of the *trans* entrance is smaller than that of *cis* (cytoplasmic) entrance. Such an asymmetry in the size of channel entrance was reported in sarcoplasmic reticulum K^+ channel where the cytoplasmic entrance was smaller than the opposite one (Gray et al., 1988).

Discussion

Ca^{2+}-Activated K^+ Channel from Brain Synaptic Membranes

Ca^{2+}-activated K^+ channels with large unitary conductance from rat synaptic membranes have been reported by several authors (Krueger et al., 1982; Farley & Rudy, 1988; Reinhart et al., 1989). Two recent papers have revealed that there are several types of Ca^{2+}-activated K^+ channels with different conductances and Ca^{2+} sensitivities. Farley and Rudy (1988) reported a class of channel (Type I) which had a 200–250 pS single channel conductance (at 200 mM KCl). Reinhart et al. (1989) characterized four types of Ca^{2+}-activated K^+ channels, two of which had similar single channel conductances (242 and 236 pS at 150 mM KCl) but differed in gating kinetics and charybdotoxin sensitivity. These channels showed similar conductances and voltage dependence of open probability (9–11 mV per *e*-fold change) and were activated by '*cis*' Ca^{2+} in micromolar range. Ca^{2+}-activated K^+ channel studied in this paper also has a large unitary conductance (258 ± 13 pS in 100 mM KCl) and voltage dependence of open probability (8–11 mV per *e*-fold change). The fast gating kinetics (open time constant of 15.3 msec at +30 mV) and asymmetric TEA sensitivity (*trans* > *cis*) suggest that the channel is quite similar to the 'fast large conductance channel' reported by Reinhart et al. (1989) except for their Ca^{2+} sensitivity. The Hill coefficient obtained from a Hill plot of P_o *versus* Ca^{2+} concentration is 1.6 for our channel, whereas the 'fast channel' reported by Reinhart appears to be 10-fold or more sensitive to Ca^{2+} than ours and have a Hill coefficient as large as 2.1–2.5. The difference in Ca^{2+} sensitivity may be explained by the fact that they used negatively charged lipid (phosphatidylserine) and internal solution containing 1 mM Mg^{2+}. The presence of negative surface charges on the lipid bilayer can increase apparent Ca^{2+} sensitivity (Moczydlowski et al., 1985) and a milimolar amount of Mg^{2+} in the cytoplasmic side can increase the Hill coefficient (Golowasch, Kirkwood & Miller, 1986; Squire & Petersen, 1987; Oberhauser, Alvarez & Latorre, 1988). The 'Type I channel' (Farley & Rudy, 1988) differs from our channel in its high TEA sensitivity at the cytoplasmic side (K_d = 1–3 mM), although single channel conductance and voltage dependence are similar.

Aminoglycoside Blockade

Ion channel blockade by aminoglycosides are reported in several Ca^{2+} channels, e.g. neomycin blockade of slowly inactivating Ca^{2+} channels in clonal GH3 pituitary cells (Suarez-Kurtz & Reuben, 1987), neomycin blockade of inward Ca^{2+} current of paramecium (Gustin & Hennessey, 1988), aminoglycoside blockade of N-type calcium channel of rat brain synaptosomes (Wagner et al., 1987). Aminoglycosides also block mechano-transduction channels of hair cells (Hudspeth & Kroese, 1983; Ohmori, 1985; Kroese et al., 1989), and the relation to their acute ototoxicity has been discussed. Here we have examined the effect of various aminoglycosides on the Ca^{2+}-activated maxi K^+ channel of syn-

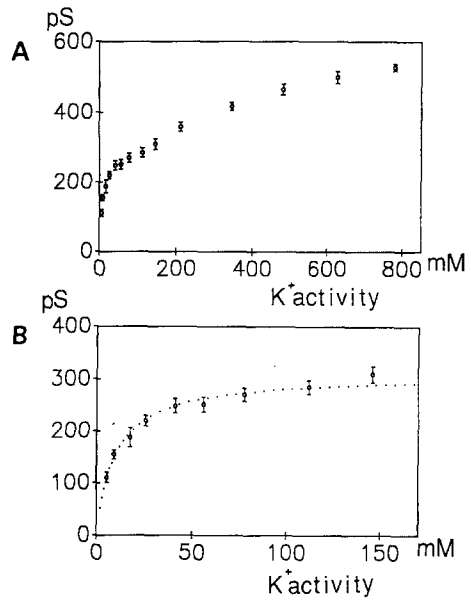

Fig. 9. (*A*) Single channel conductance-activity relationship. Single channel currents were measured with KCl-HEPES buffers adjusted to each K^+ concentration and pH 7.2 with appropriate amount of KOH. Circles with error bars represent the mean ± SD of 4–10 membranes. (*B*) Close-up of low K^+ activity region of *A*. The dotted line is the best fit to the data points below 150 mM K^+ according to a simple saturation function of K^+

$$G/G_{max} = [K^+]/([K^+] + K_d)$$

where G_{max} = 309 pS and K_d = 9.9 mM

aptosomal membranes and found that they acted as 'fast blockers' from the cytoplasmic side. One uninteresting interpretation of the channel blockade is that aminoglycosides just reduced the putative negative surface potential on membranes which may increase the local concentration of K^+ around the channel mouth. However, as mentioned in Materials and Methods, the degree of aminoglycoside blockade was not different in two different kinds of lipid bilayers, one of which carries negative surface charges. In addition, the conductance of chloride channel from synaptic membranes (K. Nomura and M. Sokabe, *unpublished data*) was not affected by aminoglycosides as was reported for sarcoplasmic reticulum chloride channel (Sokabe, 1983). The strongest objection to the contribution of membrane surface potential may be the voltage-dependent nature of the aminoglycoside channel blockade. These three lines of evidence strongly suggest that the idea that aminoglycoside channel blockade originates from specific binding of the drug to the site inside the channel, although there is a possibility of a certain contribution of local surface potential due to the negative charges from the channel protein itself. Voltage dependence of the blockade is well described by Woodhull's ionic blocking theory that positively charged amino residues of aminoglycosides enter the electrical field inside the ion-conducting pathway and compete for a binding site with K^+. A similar voltage-dependent blockade by aminoglycosides is reported in sarcoplasmic reticulum (SR) K^+ channel of skeletal muscle (Sokabe, 1984; Oosawa & Sokabe, 1986). The SR-K^+ channel is known as voltage-dependent slow-gating K^+ channel with large unitary conductance and single K^+-saturable site inside the ion-conducting pathway (Coronado, Rosenberg & Miller, 1980). On the other hand, several recent studies have shown evidence that Ca^{2+}-activated maxi K^+ channels are multi-ion channels with multiple K^+-binding sites (Eisenman, Latorre & Miller, 1986; Cecchi et al., 1987; Neyton & Miller, 1988*a*,*b*). Figure 9*A* shows single channel conductance-activity relationship which cannot be fitted with a single saturation curve, indicating that the Ca^{2+}-activated K^+ channel in the present study is also a multi-ion channel. However, as shown in Fig. 9*B*, we could fit the conductance of low K^+ activity region (10–146 mM) with a simple saturation curve according to

$$G/G_{max} = [K^+]/([K^+] + K_d) \quad (4)$$

where G_{max} is the maximal conductance and K_d is the dissociation constant for a saturable site. We can get a K_d of 9.9 mM, which is comparable to the dissociation constant for K^+ (8.0 mM) obtained from Fig. 7. These results suggest that the channel is occupied by a single K^+ ion at low K^+ concentrations and that aminoglycosides block the channel by competing for the primary K^+ binding site with K^+. However, we should remark here that the apparent single ion channel behavior might result from a combination of complex effects of a multi-ion behavior and surface potential charge with ionic strength, the mechanism of which remains to be solved.

Voltage-Dependent Blockade by Alkylamines

We found several alkylamines with various carbon-chain lengths caused dose- and voltage-dependent 'fast' blockade of the channel from the *cis* side. The voltage dependence of the blockade was well explained by Woodhull's ionic blocking model and $z\delta$s for alkyldiamines were approximately twice those of alkylmonoamines. Assuming that positively charged amino residues interact with a binding site within the ion-conducting pathway, the site is to

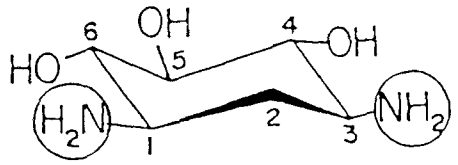

Fig. 10. Chemical structure of 2-deoxystreptamine

locate at 25% voltage drop from the *cis* entrance of the channel, and 'both' charges of alkyldiamines interact with the site simultaneously, irrespective of their chain length.

Miller (1982) tested quaternary and *bis*-quaternary ammonium ions with various methylene chain lengths on the sarcoplasmic reticulum (SR) K^+ channel and found that the effective valence ($z\delta$) for *bis*-quarternary ammonium ions varied with its chain length; long-chain blockers bound to a blocking site in a 'bent-over conformation' with 'both' charges interacting with the site simultaneously, whereas short-chain blockers bound in an 'extended-chain conformation' with one of their charges leaving the site, resulting in $z\delta$ becoming smaller. Our results for the synaptic Ca^{2+}-activated K^+ channels, however, showed that $z\delta$ for each alkylamine blocker did not change with its alkyl chain length; i.e., 'both' amino residues of all alkyldiamines could bind to one binding site or two binding sites with the same distance. This indicates that these alkylamines are quite flexible to take a 'bent-over conformation' and/or that the size of the conduction pathway of the internal side of the channel is wide enough to accommodate the molecules in that conformation. The latter picture agrees with the fact that the site can simultaneously interact with two amino residues of an aminoglycoside. The aminoglycosides consist of two or more amino sugars in glycosidic linkage to a hexose nucleus. As each sugar has zero to two positively charged amino residues, at least a part of one amino sugar has to enter into the conducting pathway to let two amino residues bind to the binding site. All aminoglycosides used in this study (kanamycin, ribostamycin, dibekacin and neomycin) contain a 2-deoxystreptamine (Fig. 10) moiety as a common structure. If two amino residues of 2-deoxystreptamine moiety of aminoglycosides bind to the site(s), the cross-sectional area around the binding site(s) should have a diameter of at least ~7.5 Å, which is wide enough to accommodate alkyldiamines with four carbon atoms (putrescine, ~7.5 Å) in the 'extended chain conformation'. We also tested one *bis*-quarternary ammonium, hexamethonium, and found that effective valence ($z\delta$) was 0.53 ± 0.02 ($n = 3$), almost the same value of alkyldiamine. This suggests, in synaptic Ca^{2+}-activated K^+ channel, hexamethonium also can form 'bent-over comformation' to allow the two quarternary ammonium residues to bind to the site simultaneously. Villarroel et al. (1988) reported a much smaller value (0.26) of $z\delta$ for hexamethonium blocking in Ca^{2+}-activated K^+ channel from rat skeletal muscle. Although we do not know the reason for the difference, one possible interpretation is that the *cis*-side mouth of the Ca^{2+}-activated K^+ channel from synaptic membranes is wider than that from skeletal muscle. Detailed comparison awaits future study.

Blockade by Bulky Organic Cations and the Structure of the Cytoplasmic Side of the Pore

Ca^{2+}-activated K^+ channels from various cells and tissues are known to be blocked by a millimolar amount of tetraethylammonium (TEA), a large organic cation with a cross-sectional diameter of ~6 Å (Coronado & Miller, 1982). Villarroel et al. (1988) reported that Ca^{2+}-activated K^+ channel from rat muscle T-tubule membranes was voltage-dependently blocked from the cytoplasmic side by quarternary ammonium (QA) ions of various sizes including TEA with almost the same $z\delta$ values (0.27–0.30). They concluded that all QA ions bound to a site located at ~30% voltage drop from the internal surface. The result indicated that a molecule as big as tetrabutylammonium with a diameter of ~10 Å (French & Shoukimas, 1981) could enter the pore to bind to the site. We have found that internally applied TEA was also able to block the synaptic Ca^{2+}-activated K^+ channel with a similar value of $z\delta$ as that of T-tubule channel. Moreover, we have showed gallamine, an extremely bulky trivalent organic cation, blocked the channel. Gallamine consists of three positively charged TEA moieties linked to a benzene nucleus, and its molecular cross-sectional area is as large as ~120 $Å^2$ (Gray et al., 1988). Supposing that gallamine and TEA bind to the same site locating at ~30% voltage drop from the internal side, the cross-sectional area around the site is large enough to accommodate two or more TEA molecules at a time. Figure 11 shows the summary of the values of $z\delta$s for aminoglycosides and other *cis* blockers used in this study. If all these blockers bind to a site located at electrical fractional distance of 25% voltage drop, the right ordinate of the figure ($z\delta/0.25$) will indicate the number of positive charges of each blocker that interacts the site. Then, we can presume a putative structure of the internal side of the ion-conducting pathway of synaptic Ca^{2+}-activated K^+ channel as follows: the channel contains an amino residue

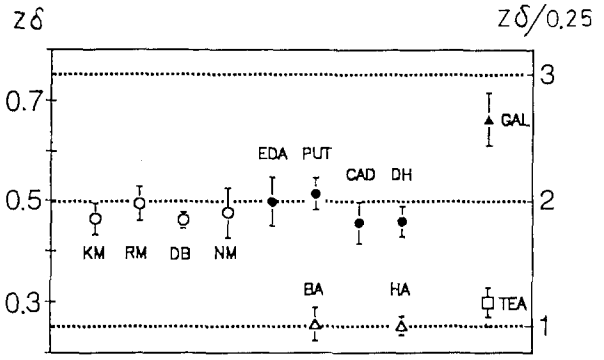

Fig. 11. $z\delta$s for various positively charged *cis* blockers. Each symbol represents the mean (±SD). Number of z is indicated at the right ordinate by assuming δ is 0.25. *Symbols:* open circles = aminoglycosides (*KM* = kanamycin, *RM* = ribostamycin, *NM* = neomycin, *DB* = dibekacin); open triangles = alkylmonoamines (*BA* = butylamine, *HA* = hexylamine); filled circles = alkyldiamines (*EDA* = ethylenediamine, *PUT* = putrescine, *CAD* = cadaverine, *HD* = 1,6-diaminohexane); filled triangle = *GAL* (gallamine); open square = *TEA* (tetraethylammonium)

binding site(s) and TEA binding site(s) which are very closely located at an electrical distance of 25–30% into the voltage drop from the internal side of the channel, and the pathway between the inner mouth of the channel and the site has a fairly wide cross-sectional area large enough to allow at least two ethylammonium groups (each has a diameter of ~6 Å) of gallamine to enter. Aminoglycosides and other cationic blockers block the channel by 'occluding' the ion-conducting pathway at the site from where the narrow K^+-selective region probably begins. Jordan (1984*a*,*b*; 1986) calculated the image potential profile of a simplified cylindrical pore model and showed that a significant voltage drop could occur outside the pore constriction even if the pore's entrance (channel's 'mouth') is quite big. He also showed that the range of voltage drop would be shortened by high ionic strength. We found that the electrical distance of neomycin binding site decreased with increasing ionic strength ($z\delta = 0.36 \pm 0.04$ at 500 mM K^+, $n = 4$), which indicated the shortening of voltage drop range. This finding is well consistent with Jordan's model and very similar to the effect of ionic strength on $z\delta$s for gallamine blockade of SR-K^+ channel, another maxi-K^+ channel, which is also postulated to have a wide channel mouth (Gray et al., 1988). Thus, the big mouth with sudden constriction of the pore may be one of the common properties of the maxi-K^+ channel. Moreover, the size of the mouth on the opposite side of these channels seems much smaller, because gallamine did not block the channel from this side (Gray et al., 1988).

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