9. Neomycin Blockade of K^+ Channels from Sarcoplasmic Reticulum Membranes Incorporated in Planar Bilayers

By Masahiro SOKABE

Department of Behaviorology, Faculty of Human Sciences,
Osaka University, Suita, Osaka 565

(Communicated by Yasuji KATSUKI, M. J. A., Feb. 12, 1983)

Introduction. Aminoglycoside antibiotics (AGs), like streptomycin (SM) and neomycin (NM), exert acute toxicities such as reductions of cochlear microphonics and neuromuscular blockade; however, the underlying mechanism is still unknown. Recent electrophysiological studies on vertebrate hair cells indicated that SM reversibly inhibited the generation of receptor potentials¹⁾ which originated from a change in the K^+ -permeability of hair cell membranes.²⁾ This finding suggests that AGs block K^+ channels on hair cell membranes. We have proposed such a hypothesis in the previous paper,³⁾ in which it was reported that AGs inhibited the K^+ -conductances of planar bilayers modified with triphosphoinositide that has been considered as a receptor molecule for AGs.⁴⁾ Hitherto, it has not so seriously been considered that AGs may block a channel of biological membranes.

The present study was designed to clarify whether NM behaves as a blocker for K^+ channels of biological membranes by using a fragmented sarcoplasmic reticulum (SR) incorporated in planar phospholipid bilayers. The reasons why we employed this assay system are that the K^+ channel of SR is one of the most elucidated cation channels,^{5),6)} and that single channel experiments which may provide crucial criteria whether the drug acts as a channel blocker can easily be carried out with this system.

NM reversibly inhibited the K^+ -conductances of the planar bilayers containing many K^+ channels from SR membranes. Through single channel experiments, it was clearly shown that the inhibition was due to a blocking action of NM to the ion conducting pathway of the K^+ channel.

Materials and methods. *Materials.* SR vesicles were prepared from rabbit back and leg white muscle by the method of Miller and Rosenberg (1979)⁷⁾ with slight modifications. The vesicles were stored in small aliquots in 0.4 M sucrose-5 mM HEPES-NaOH (pH 7.4) at -75°C . Asolectin (AL) from Sigma Chemical Co. washed

according to Labarca *et al.* (1980)⁵⁾ was used. Phosphatidic acid (PA) was purchased from Serdary Res. Lab. and used without further purification.

Formation of planar bilayers and conductance measurement. Planar bilayers were formed by the painting method of Mueller and Rudin (1969),⁸⁾ i.e., phospholipid solution (30 mM in *n*-decane: 90% AL-10% PA in mole p basis) was applied to a hole (500 μ m diameter) drilled in a polystyrene cup (5 cc) placed in a figure-eight-shaped poly(vinyl chloride) dish. The planar bilayer separates two aqueous solutions composed of 100 mM K⁺ gluconate and 5 mM HEPES adjusted to pH 7.4 with Tris base. Each aqueous chamber was connected to an Ag/AgCl electrode via a glass KCl/agar bridge. The membrane conductance was measured by a current to voltage converting circuit ($\times 10^9$) under a voltage clamp condition. The amplifier output was recorded on a chart recorder and analyzed by hand. Experiments were carried out at room temperature, $25 \pm 2^\circ\text{C}$.

Fusion of SR vesicles. SR vesicles were incorporated into planar bilayers by the Ca⁺⁺-induced fusion process which is dependent upon acidic lipids in planar bilayer and an osmotic gradient across the SR vesicle membrane as described by Miller (1978).⁹⁾ After thinning of the membrane, CaCl₂ (0.5–1 mM final concentration) was added to one side of the chamber, and then SR vesicles (20–60 μ g protein/ml final concentration) were added to the same side (this side was defined as the cis side) with constant stirring. Within a few minutes after an SR addition, the membrane conductance began to increase in discrete steps which reflected fusion of SR vesicles with the planar bilayer. For measurement of steady state conductance, fusion was stopped by merely allowing enough time to pass so that SR vesicles would dissipate their osmotic gradient, or by adding EDTA in excess

Fig. 1. A: Time course of conductance changes induced by fusion of SR vesicles (20 μ g/ml) with a planar bilayer. Each spike-like event might come from fusion of a single SR vesicle. Conductance was measured at -25 mV. Aqueous phase was 5 mM HEPES-Tris (pH 7.2), 0.1 mM EDTA, 1 mM CaCl₂, 100 mM K⁺ gluconate. B: Conductance fluctuations induced by an SR vesicle containing two channels at $+50$ mV (mean single channel conductance was 160 pS). Aqueous phase was the same as in A except that it contained 1.2 mM EDTA.

of Ca^{++} . The chamber to which SR was not added (trans side) was defined as virtual ground.

Fig. 1A shows a typical example of conductance increase induced by SR fusion. When low concentration of SR ($1-5 \mu\text{g/ml}$) was added, sometimes, only a single SR vesicle containing few K^+ channels would fuse, and opening-closing fluctuations of individual channel could successfully be observed (Fig. 1B). When choline Cl^- buffer instead of K^+ gluconate was used, Cl^- -conductance generated by anion channels distinct from K^+ channels⁹⁾ could be measured (data not shown). These results are in harmony with those reported by Miller (1978).⁹⁾

Results and discussion. *Inhibition of macroscopic conductance.* Upon addition of NM to the cis chamber, a steady state K^+ -conductance reduced and reached another steady state level within a minute (see Fig. 2 inset); however, Cl^- -conductances unchanged. Thus, dose *vs* relative-conductance curve could be obtained by successive additions of NM as is shown in Fig. 2. One of the most feasible mechanisms of this conductance inhibition may be surface potential

Fig. 2. Effects of cis-NM on the macroscopic K^+ (●)- and Cl^- (○)-conductances at +25 mV. Aqueous phase was the same as in Fig. 1B except that it contained 100 mM choline Cl^- instead of K^+ gluconate when Cl^- -conductance was measured. Data were taken from at least two membranes. Inset: Time course of inhibitions of a steady state K^+ -conductance by cis-NM (horizontal solid line under the trace indicates zero conductance level).

changes induced by NM. Since the bilayer carries a negative surface charge, a cationic drug, NM, will reduce the magnitude of the surface potential by charge screening or specific binding. This effect will reduce the local concentration of K^+ near the mouth of the channel

and may be able to account for the reduction of the K^+ -conductance. However, this possibility seems to be ruled out because the Cl^- -conductances measured under the same condition were not affected by NM. Therefore, another effect, a blockade of an ion conducting pathway or an inhibition of channel opening reaction, must be considered. These possibilities were tested by single channel experiments described below.

Inhibition of single channel conductance (γ). Single channel conductance (γ) of the K^+ channel was reversibly and equally reduced by NM from either side of the membrane in a dose-dependent manner. In contrast, mean open dwell time of the channel was elongated with NM (Fig. 3). In addition, the degree of the inhibitory effect of NM was absolutely dependent upon applied voltages (data not shown).

Fig. 3. Effects of cis-NM on the single channel conductance (γ) of the K^+ channel at +50 mV. γ_0 indicates the value for γ at 0 mM NM. Aqueous phase was the same as in Fig. 1B. Data were taken from at least three membranes. Inset: Single channel recordings at various NM concentrations; all the recordings were made in different membranes.

These results can consistently be interpreted in terms of open-channel-blocking mechanism where it is assumed that an agent can bind to the blocking site in the open state only.¹⁰⁾ Theoretical analysis based on the voltage dependency of the inhibitory effect suggested that one K^+ channel of SR has two NM-binding sites, which locate near cis and trans side mouth of the channel, respectively (Sokabe, in preparation).

Effect of other aminoglycosides (AGs). Three other AGs, kanamycin (KM), streptomycin (SM), ribostamicin (RM), also showed

similar effect of varying severity: $NM > KM$, $SM > RM$. This order parallels that of ototoxicity¹¹⁾ and of the inhibitory effect to the previously reported lysotriphosphoinositide channel.³⁾ This result raises several interesting problems; first, "Is an acute AGs toxicity caused by a blockade of cation channels of biological membranes?"; second, "Do cation channels of SR and hair cell membranes have similar binding sites for AGs?"; and, "Do the binding sites contain triphosphoinositide?". These problems are now under investigation.

References

- 1) Hudspeth, A. J. (1982): J. Neurosci., **2**, 1.
- 2) Corey, D. P., and Hudspeth, A. J. (1979): Nature, **281**, 675.
- 3) Sokabe, M., Hayase, J., and Miyamoto, K. (1982): Proc. Japan Acad., **58B**, 177.
- 4) Schacht, J. (1976): J. Acoust. Soc. Am., **59**, 940.
- 5) Labarca, P., Coronado, R., and Miller, C. (1980): J. Gen. Physiol., **76**, 397.
- 6) Coronado, R., Rosenberg, R. L., and Miller, C. (1980): *ibid.*, **76**, 425.
- 7) Miller, C., and Rosenberg, R. L. (1979): Biochemistry, **18**, 1138.
- 8) Mueller, P., and Ruden, D. O. (1969): Laboratory Techniques in Membrane Biophysics (eds. Passow, H., and Stampfli, R.). Springer-Verlag, Berlin, 141 pp.
- 9) Miller, C. (1978): J. Membr. Biol., **40**, 1.
- 10) Coronado, R., and Miller, C. (1979): Nature, **280**, 807.
- 11) Lodhi, S. *et al.* (1980): Biochem. Pharmacol., **29**, 597.