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Blepharismins, produced by the protozoan, *Blepharisma japonicum*, form ion-permeable channels in planar lipid bilayer membranes

Yoshinori Muto[a,*], Tatsuomi Matsuoka[b], Akemi Kida[b], Yukio Okano[c], Yutaka Kirino[d]

[a] *Department of Basic Health Science and Fundamental Nursing, Gifu University School of Medicine, 1-1, Yanagido, Gifu 501-1193, Japan*
[b] *Department of Natural Environmental Science, Faculty of Science, Kochi University, Kochi 780-8520, Japan*
[c] *Department of Molecular Pathobiochemistry, Gifu University School of Medicine, Gifu 500-8705, Japan*
[d] *Laboratory of Neurobiophysics, School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan*

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Abstract Blepharismins are polycyclic quinones found in the pigment granules of the ciliated protozoan, *Blepharisma*. Exposure to purified blepharismins results in lethal damage to several other ciliates. We here report that, at cytotoxic concentrations, blepharismins formed cation-selective channels in planar phospholipid bilayer membranes. The channels formed in a diphytanoylphosphatidylcholine bilayer had a K^+/Cl^- permeability ratio of 6.6:1. Single channel recordings revealed the conductance to be quite heterogeneous, ranging from 0.2 to 2.8 nS in solutions containing 0.1 M KCl, possibly reflecting different states of aggregation of blepharismin. Our observations suggest that channel formation is a cytotoxic mechanism of blepharismin's action against predatory protozoa.

Key words: Blepharismin; Ion channel; Planar lipid bilayer; Cytotoxicity; Protozoon

1. Introduction

The free-swimming protozoan, *Blepharisma japonicum*, has numerous pigment granules containing quinone pigments [1], called blepharismins (Fig. 1) [2]; these granules are located just beneath the plasma membrane [3,4]. The pigments are considered to function as photoreceptors modulating the photobehavior of the cell [5,6]. Blepharismins have also been reported to be toxic to certain other kinds of protozoa [2] and to kill a variety of protozoa, including predators which actively feed on *Blepharisma* [7,8]. Furthermore, the study of *Blepharisma–Dileptus* interaction demonstrated that the defensive function of pigment granules in *Blepharisma* against *Dileptus* was based on the discharge of blepharismin in response to the attack by the predator [9,10]. Thus, another function of blepharismins might be defense against predators. The main targets for blepharismin action are mostly unknown. While one mechanism of blepharismin toxicity is thought to be the generation of short-lived oxygen species on exposure to light [11], this may not be the only one, and alternatives have to be considered, especially in order to understand the mechanism of blepharismin toxicity in the dark [8,10].

The complete molecular structure of blepharismin-2 was recently determined [12], quickly followed by those of blepharismin-3 [13] and other types of blepharismins [14]. They possess a naphthodianthrone skeleton with four peri-hydroxyl groups as a common structural component (Fig. 1). This structural information suggests the polarized amphipathic nature of blepharismin molecules, which is indicative of a strong membrane association potential [15,16]. Moreover, hypericin, a quinoid pigment structurally related to blepharismin, has been reported to modify membrane characteristics [17]. It therefore seemed of interest to investigate the interaction of blepharismins with membranes to gain further insights into the mechanism of blepharismin action.

In the present study, using 'solvent-free' planar phospholipid bilayer membranes [18] as a model system, we demonstrate that blepharismins can form cation-selective ion channels in lipid bilayer membranes.

2. Materials and methods

2.1. Extraction and purification of blepharismins

B. japonicum was cultured at 23°C in the dark in an infusion of 0.1% cereal leaves containing *Enterobacter aerogenes* as food. The cells, collected by centrifugation at $150\times g$ for 5 min at room temperature, were suspended and extracted in acetone for 1 min at room temperature. After centrifugation at $800\times g$ for 10 min, the supernatant fluid was concentrated on a rotary evaporator (Rotavapor, Shibata, Tokyo, Japan). Blepharismin-1, blepharismin-2, blepharismin-3, blepharismin-4, and blepharismin-5 were then purified by thin-layer chromatography using silica gel plates (60 F_{254}, Merck, Germany) and a solvent system consisting of ethyl acetate and acetone (4:1, v/v), according to the method of Matsuoka et al. [19]. The purified blepharismins were dissolved in ethanol and stored protected from light at −20°C until use.

2.2. Planar bilayer experiments

Planar lipid bilayer membranes were prepared by the folding method described by Montal and Mueller [18]. Briefly, two symmetrical halves of a Teflon chamber with solution volumes of 1.5 ml were divided by a 12.5 μm thick Teflon partition containing a round aperture of about 100–200 μm in diameter; a relatively small aperture (ca. 100 μm) was used to measure single channel fluctuations and a larger aperture to measure macroscopic currents.

Hexadecane in hexane (1%, v/v) was used for aperture pretreatment. The bilayer membranes were formed at room temperature by the union of two monolayers of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) over the aperture facing two aqueous subphases. Unless otherwise specified, the composition of the solution ('standard solution') in both compartments was 100 mM KCl, 10 mM MOPS–Tris (pH 7.2). Membrane formation was monitored by measuring membrane capacitance. Currents, measured using a homemade current/voltage converter, were displayed on an oscilloscope and a chart recorder [20]. The data were stored on a

*Corresponding author. Fax: (81)-58-293 3219.
E-mail address: ymuto@cc.gifu-u.ac.jp (Y. Muto).

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videotape recorder after A/D conversion by a digital audio processor (PCM-501ES, Sony) with a bandwidth extended to zero frequency by various modifications of the input stage [21,22].

A small amount of an ethanolic solution of blepharismin was added to one side of the membrane, defined as the *cis* side, the other being the *trans* side. The potential was applied with agar salt bridges to the *trans* side, while the *cis* side was virtually grounded by the operational amplifier. The voltages of the *trans* side are reported in this study. Currents (cation) flowing from the *trans* to the *cis* compartment were considered positive and plotted in an upwards direction. The *cis* solution was stirred continuously with a magnetic stirrer under applied membrane potential until current fluctuation occurred.

To determine the ionic selectivity of the channel, the reversal potential was measured in asymmetrical solutions of a KCl concentration gradient (200 mM/70 mM KCl, *cis*/*trans*), replacing the 100 mM KCl in the standard solution. The cation/anion permeability ratio was then calculated using the Goldman–Hodgkin–Katz equation [23,24].

3. Results

Fig. 2 shows the effects of addition of blepharismin-2 (final concentration 5 μg/ml) to the *cis* side of a planar lipid bilayer formed by diphytanoylphosphatidylcholine. The trace shows the membrane current as a function of time, with a voltage of 40 mV imposed across the membrane. Initially, there was little current flow, due to the inherent impermeability of lipid membranes to ions, but, after addition of blepharismin-2, the current gradually increased. Following blepharismin addition, channel-like fluctuations of the bilayer conductance were initially seen, which, after several minutes, were replaced by large current fluctuations. Under the conditions defined above, the membranes were significantly destabilized and the current increased continuously until the membrane broke. In the absence of blepharismin, no increase in, or fluctuation of, the membrane current was seen. Since the membrane conductance reflects the permeability to ions in the bathing solution, e.g. K^+ or Cl^-, we concluded that blepharismin-2 increased the ionic permeability of lipid bilayer membranes. For this effect, it was only necessary to add blepharismin-2 to one side of the bilayer. The other four homologous blepharismins (1, 3, 4, and 5) [14] induced similar conductance changes (data not shown). Since blepharismin-2 is the only blepharismin distributed throughout the entire cell body in *Blepharisma* [19], it was used as an example of this class of compounds in the following experiments.

In order to characterize the conductance induced by blepharismin, the *cis* side of the membrane was perfused with

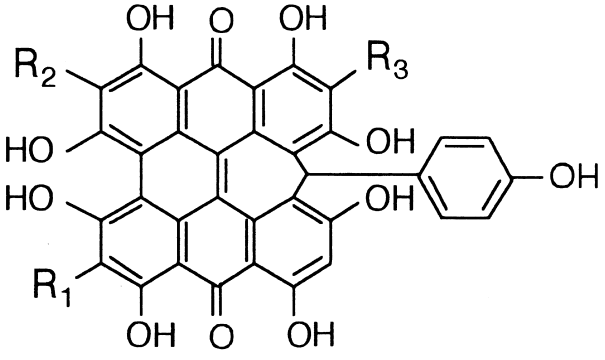

Fig. 1. Structure of blepharismins. Blepharismin-1: $R_1 = R_2 =$ ethyl, $R_3 = H$; blepharismin-2: $R_1 =$ ethyl, $R_2 =$ isopropyl, $R_3 = H$; blepharismin-3: $R_1 = R_2 =$ isopropyl, $R_3 = H$; blepharismin-4: $R_1 =$ ethyl, $R_2 =$ isopropyl, $R_3 =$ methyl or $R_1 =$ isopropyl, $R_2 =$ ethyl, $R_3 =$ methyl; blepharismin-5: $R_1 = R_2 =$ isopropyl, $R_3 =$ methyl.

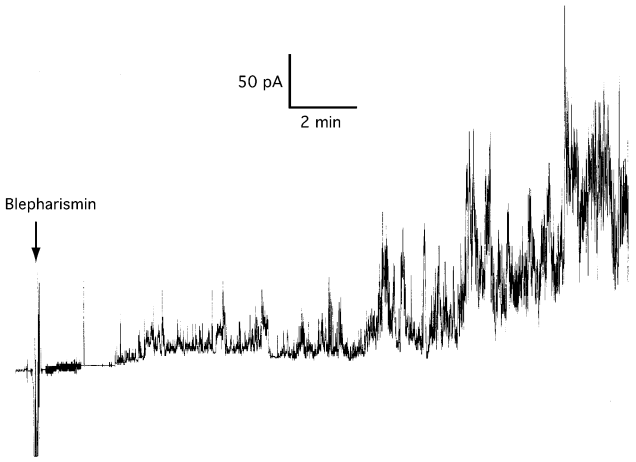

Fig. 2. Induction of an ionic current in a planar lipid bilayer membrane by blepharismin. The membrane current trace is shown as a function of time. The membrane voltage (voltage of the *trans* compartment with respect to the *cis* compartment to which blepharismin was added) was held constant at 40 mV, and the current through an unmodified lipid bilayer membrane was very low. Addition of 5 μg/ml of blepharismin-2 (arrow) to the aqueous phase induced current fluctuations and dramatically increased the membrane current. The lipid bilayer membrane was composed of diphytanoylphosphatidylcholine and the aqueous salt solution in both compartments was 100 mM KCl, 10 mM MOPS–Tris, pH 7.2.

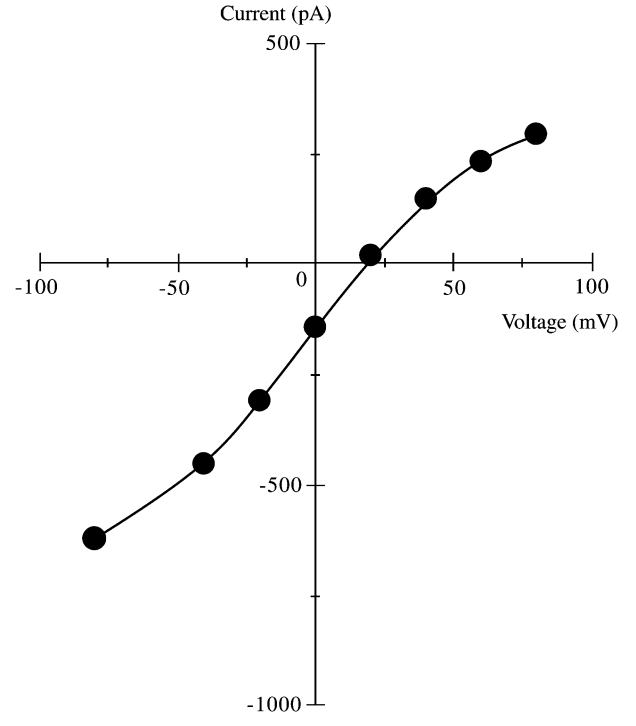

Fig. 3. A macroscopic current–voltage relationship for the blepharismin-induced conductance in an asymmetrical solution of KCl. The solutions were 200 mM KCl, 10 mM MOPS–Tris (pH 7.2) on the *cis* side and 70 mM KCl, 10 mM MOPS–Tris (pH 7.2) on the *trans* side. Before the experiment shown, blepharismin-2 (5 μg/ml final concentration) was added to the *cis* compartment, then the *cis* side of the membrane was perfused with bathing solution. Current amplitudes were measured at the beginning of the voltage changes. The reversal potential was obtained from the intercept on the *x* axis.

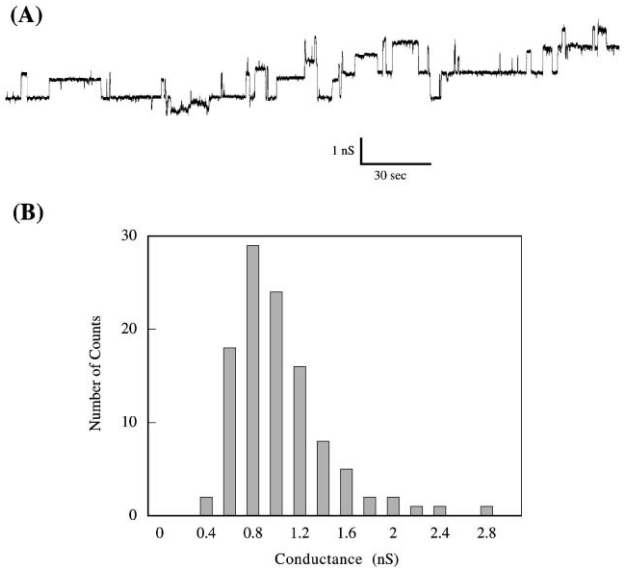

Fig. 4. Single channel currents induced by blepharismin. A: The current due to single channels induced by blepharismin-2 at a final concentration of 0.5 μg/ml is shown. The aqueous salt solutions contained 100 mM KCl, 10 mM MOPS–Tris, pH 7.2, and the membrane was formed from diphytanoylphosphatidylcholine. The membrane voltage was held constant at +40 mV throughout this recording. Note that several different sizes of current jump can be seen, reflecting the heterogeneity of channels induced by blepharismin. B: A histogram of blepharismin single channel conductances. The data are taken from the membrane depicted in A.

bathing solution after addition of blepharismin; this resulted in cessation of the current increase and in a steady-state conductance. All the results described below were obtained in experiments in which a steady-state conductance was observed after perfusion. The ionic selectivity of the bilayers modified with blepharismin-2 was determined by measuring the potential at zero current flow (reversal potential) in the presence of a concentration gradient of KCl across the bilayer (200 mM to 70 mM). As shown in Fig. 3, a reversal potential of +19 mV (with reference to the side with the lower KCl concentration) was observed. The positive sign of the potential indicates preferential cation selectivity. From the Goldman–Hodgkin–Katz equation [23,24], the relative ionic selectivity of the channels for K^+ and Cl^- was estimated to be about 6.6:1. At this concentration difference, an ideally cation-selective channel would yield a potential of approximately +27 mV.

Using a small aperture and a low blepharismin concentration, individual fluctuations, which might constitute the macroscopic membrane current, could be easily resolved. As shown in Fig. 4A, discrete stepwise conductance fluctuations could be routinely observed after addition of a small amount of blepharismin. Since the current fluctuation consisted of unitary digital changes, it might reflect the opening and closing behavior of single channels, suggesting the existence of defined single pores. However, the amplitude of the various steps was highly variable. A histogram of the blepharismin-2 single channel conductances seen under these conditions (Fig. 4B) shows a well-defined peak close to 0.8–1 nS, but the single channel conductance was quite heterogeneous, ranging from 0.2 to 2.8 nS, possibly reflecting different states of aggregation of blepharismin and/or different conformation within the membrane.

We next studied the voltage sensitivity of the blepharismin-induced conductivity using multichannel bilayers, the molecule being applied to the *cis* side. Fig. 5 shows the current response of a blepharismin-treated membrane to a series of various voltage steps. The data show that subjecting the bilayer to positive and negative voltages had no measurable effect on its initial conductivity (instantaneous current), but the current frequently declined with time, accompanying closing events of the single channels, particularly when switching from zero to negative voltage (on the side of the blepharismin-free compartment). It should be noted that a brief reduction in the applied potential (to zero) brings about restoration of the conduction process, i.e. opening of the channels, as is clearly shown in the second or third responses of Fig. 5. The instantaneous current flowing through the membrane re-

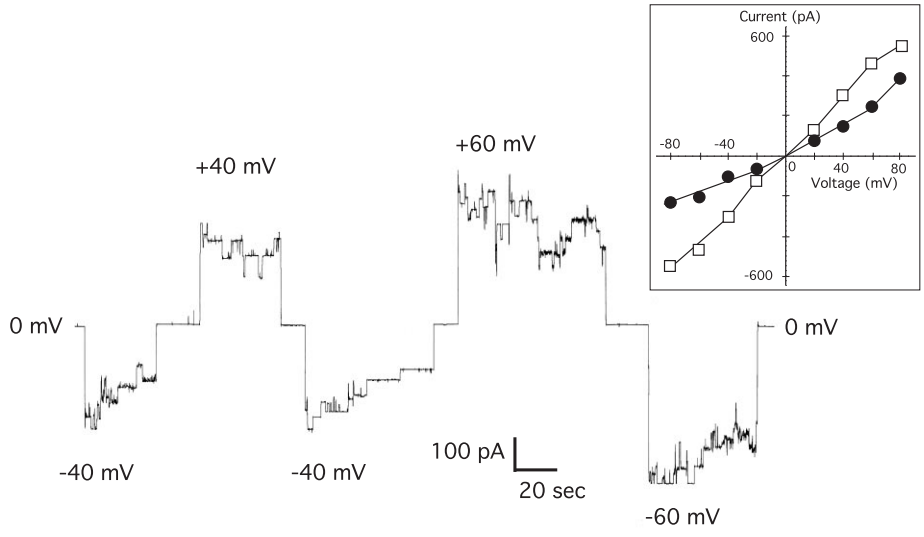

Fig. 5. Multichannel conductance induced by blepharismin at different voltages. The currents in response to a series of voltage steps are shown for a membrane to which blepharismin-2 was added at a final concentration of 2 μg/ml; the other conditions are identical to those in Fig. 4. The inset illustrates the plots for instantaneous (□) and steady-state (●) currents as a function of voltage.

sponded almost linearly to the applied voltage and was symmetrical with respect to the polarity of the electrical field (Fig. 5, inset).

4. Discussion

Planar lipid bilayer membranes are widely used to characterize the interaction of various agents with cellular membranes [25,26]. Many natural products of microbial origin, such as linear and cyclic peptides, polyethers, and polyene macrolides, facilitate ionic transport through lipid bilayer membranes via the formation of pores or carriers, thus enabling passive ion fluxes [27]. In the present study, direct current measurement in planar lipid bilayers treated with blepharismin demonstrated the ability of this molecule to form time-variant cation-selective channels. The discrete conductance fluctuations (Fig. 4A) and the large current fluctuations observed in the multichannel state (Fig. 2) clearly exclude the possibility of a carrier-mediated increase in bilayer conductance. However, the structure of the channels formed by blepharismin is unknown. The molecular structure of blepharismin (Fig. 1) suggests that a single molecule does not form an ion channel in the membrane. In addition, the presence of polar OH groups in the peripheral region should prevent a single blepharismin molecule from being localized in the internal hydrophobic part of a lipid bilayer. Taking into account the fact that blepharismin can form channels of varying conductance levels (Fig. 4B), we hypothesize that differently sized clusters of blepharismin molecules are responsible for the formation of channels of different conductances. Similar single channel heterogeneity has been observed for other small molecule channel formers, such as melittin [28], defensin [29], coumarin antibiotic [30], and beticolin [31].

Several lines of evidence have demonstrated that the induced permeabilization of cell membranes is one of the most common killing mechanisms of cytotoxic molecules. In the case of several peptidic or non-peptidic toxins, the formation of transmembrane pores is considered to be responsible for membrane permeabilization and cell lysis [29,32–34]. The concentration range in which blepharismin induced the conductance increases described here (0.1–10 μg/ml) is the same as that at which it is toxic for various free-swimming protozoa [7,8,10] and mammalian cells [11]. It is thus possible that channel formation, resulting in dissipation of the electrochemical gradient and subsequent cell lysis, might be the main physiological effect of blepharismin on the target cell membrane. In this context, it is interesting to note that Harumoto et al. [10] reported that exposure of the ciliated protozoan, *Dileptus*, to blepharismin induced backward swimming during the early phase of cell killing. Since, as shown in the present paper, blepharismin can form cation-selective channels, the blepharismin-induced backward swimming might be caused by increased cytoplasmic Ca^{2+} levels as a result of Ca^{2+} influx via these channels; however, further work will be required to elucidate the induction mechanism of backward swimming. Although the relative contribution of blepharismin-induced channel formation to cellular toxicity remains to be assessed, this system may play a role in the defensive function of the pigment against predatory ciliates.

References

[1] Sevenants, M.R. (1965) J. Protozool. 12, 240–245.
[2] Giese, A.C. (1973) Stanford University Press, Stanford, CA.
[3] Kennedy Jr., J.R. (1965) J. Protozool. 12, 542–561.
[4] Matsuoka, T., Tsuda, T., Ishida, M., Kato, Y., Takayanagi, M., Fujino, T. and Mizuta, S. (1994) Photochem. Photobiol. 60, 598–604.
[5] Scevoli, P., Bisi, F., Colombetti, G., Ghetti, F., Lenci, F. and Passarelli, V. (1987) J. Photochem. Photobiol. B Biol. 1, 75–84.
[6] Matsuoka, T., Matsuoka, S., Yamaoka, Y., Kuriu, T., Watanabe, Y., Takayanagi, M., Kato, Y. and Taneda, K. (1992) J. Protozool. 39, 498–502.
[7] Kato, Y. and Matsuoka, T. (1995) J. Protozool. Res. 5, 136–140.
[8] Terazima, M., Iio, H. and Harumoto, T. (1999) Photochem. Photobiol. 69, 47–54.
[9] Miyake, A., Harumoto, T., Salvi, B. and Rivola, V. (1990) Eur. J. Protistol. 25, 310–315.
[10] Harumoto, T., Miyake, A., Ishikawa, N., Sugibayashi, R., Zenfuku, K. and Iio, H. (1998) Eur. J. Protistol. 34, 458–470.
[11] Kato, Y., Watanabe, Y., Sagara, Y., Murakami, Y., Sugiyama, M. and Matsuoka, T. (1996) J. Photochem. Photobiol. B Biol. 34, 29–33.
[12] Matsuoka, T. (1996) 12th Int. Congress Photobiol., Abstracts, p. 185.
[13] Checcucci, G., Shoemaker, R.S., Bini, E., Cerny, R., Tao, N. and Hyon, J.-S. (1997) J. Am. Chem. Soc. 119, 5762–5763.
[14] Maeda, M., Naoki, H., Matsuoka, T., Kato, Y., Kotsuki, H., Utsumi, K. and Tanaka, T. (1997) Tetrahedron Lett. 38, 7411–7414.
[15] de Kruijff, B. and Demel, R.A. (1974) Biochim. Biophys. Acta 339, 57–70.
[16] Vogel, H. (1981) FEBS Lett. 134, 37–42.
[17] Chaloupka, R., Obsil, T., Plasek, J. and Sureau, F. (1999) Biochim. Biophys. Acta 1418, 39–47.
[18] Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3561–3566.
[19] Matsuoka, T., Sato, M., Maeda, M., Naoki, H., Tanaka, T. and Kotsuki, H. (1997) Photochem. Photobiol. 65, 915–921.
[20] Anzai, K., Hamasuna, M., Kadono, H., Lee, S., Aoyagi, H. and Kirino, Y. (1991) Biochim. Biophys. Acta 1064, 256–266.
[21] Bezanilla, F. (1985) Biophys. J. 47, 437–441.
[22] Fujiwara, T., Anzai, K., Kirino, Y., Nagao, S., Nozawa, Y. and Takahashi, M. (1988) J. Biochem. 104, 344–348.
[23] Goldman, D.E. (1943) J. Gen. Physiol. 27, 37–60.
[24] Hodgkin, A.L. and Katz, B. (1949) J. Physiol. 108, 37–70.
[25] Latorre, R. and Alvarez, O. (1981) Physiol. Rev. 61, 77–150.
[26] Kagan, B.L. and Sokolov, Y. (1994) Methods Enzymol. 235, 691–705.
[27] Hall, J.E. (1981) in: Membrane Transport (Bonting, S.L. and de Pont, J.J.H.H.M., Eds.), pp. 107–121, Elsevier/North-Holland, Amsterdam.
[28] Tosteson, M.T. and Tosteson, D.C. (1981) Biophys. J. 36, 109–116.
[29] Kagan, B.L., Selsted, M.E., Ganz, T. and Lehrer, R.I. (1990) Proc. Natl. Acad. Sci. USA 87, 210–214.
[30] Feigin, A.M., Aronov, E.V., Teeter, J.H. and Brand, J.G. (1995) Biochim. Biophys. Acta 1234, 43–51.
[31] Goudet, C., Benitah, J.-P., Milat, M.-L., Sentenac, H. and Thibaud, J.-B. (1999) Biophys. J. 77, 3052–3059.
[32] Bernheimer, A.W. and Rudy, B. (1986) Biochim. Biophys. Acta 864, 123–141.
[33] Mirzabekov, T.A., Silberstein, A.Y. and Kagan, B.L. (1999) Methods Enzymol. 294, 661–674.
[34] Kourie, J.I. and Shorthouse, A.A. (2000) Am. J. Physiol. Cell Phsyiol. 278, C1063–C1087.