Biochemistry (Moscow), Vol. 68, No. 11, 2003, pp. 1247-1251. Translated from Biokhimiya, Vol. 68, No. 11, 2003, pp. 1550-1555.
Original Russian Text Copyright © 2003 by Matveyeva, Andreyuk, Yermakov.

Transport of Cl^- across the Plasma Membrane during Pollen Grain Germination in Tobacco

N. P. Matveyeva*, D. S. Andreyuk, and I. P. Yermakov

Department of Plant Physiology, Faculty of Biology, Lomonosov Moscow State University, Moscow 119899, Russia;
fax: (7-095) 939-4309; E-mail: plantphys@biophys.msu.ru

Received November 28, 2002
Revision received May 12, 2003

Abstract—The role of Cl^- transport across the plasma membrane was studied in an early step of pollen grain germination in tobacco *Nicotiana tabacum* L. The Cl^- channel blockers, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and niflumic acid, completely suppress the germination with $IC_{50} \sim 8\ \mu M$. At this concentration NPPB reduces the rate of Cl^- efflux out of pollen grain by 1.8-fold in the interval 5-12 min, and niflumic acid reduces the rate 1.2-fold. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid, a known inhibitor of Cl^- channels and antiporters, completely suppresses germination as well ($IC_{50} = 240\ \mu M$), but has no effect on the rate of Cl^- efflux. Inhibitors of chloride co-transporters, such as furosemide, bumetanide, and *bis*(1,3-dibutylbarbituric acid)pentamethine oxonol, suppress the germination by less than 50%. This set of data suggests that NPPB-sensitive anion channels are involved in the activation of pollen grains in the early stage of germination.

Key words: anion channel, anion co-transporter, NPPB, DIDS, furosemide, bumetanide, $DiBAC_4(5)$, pollen germination

Transport proteins such as anion channels, antiporters, and symporters carrying chlorine ions across a membrane play an important role in the biology of plant cells. Anion channels are involved in the transformation of signals induced by hormones, blue light, or interaction with pathogenic or symbiotic organisms [1, 2]. These channels control the level of transmembrane potential and thus the activity of H^+-ATPase in the cell membrane and the transport of ions and metabolites through the membrane [3, 4]. Anion channels are involved in the regulation of cell volume, osmoregulation [5-7], and regulation of growth processes and of the main biosynthetic pathways by blue light [8, 9]. The functions of antiporters and symporters in plant cell are studied relatively poor. Antiporters, along with the channels, are involved in the regulation of intracellular pH [10, 11], and electroneutral cation-chloride symporters control proliferation processes in plant cells [12] as in animal cells [13].

The pollen grain and pollen tube of angiosperm plants represent a relatively well studied model object in the elucidation of the role of plasma membrane transport proteins in the regulation of growth processes. The pulse character of pollen tube growth in tobacco plants is due in part to the periodic activation of chloride ion channels [14, 15]. The blockage of these channels induced an increase in volume of the apical tube and its tearing. The role of proteins transporting anions in activation of growth processes at the initial step of pollen germination is not studied. The activation of tobacco *Nicotiana tabacum* L. pollen grain was shown recently to be accompanied *in vitro* by Cl^- efflux from the grain, and the inhibition of the Cl^- efflux results in germination arrest [16]. The present work was aimed to reveal the transport proteins in plasma membrane providing the Cl^- efflux from pollen grain during its germination. To solve this question, germination was studied under the blockage of various transport proteins (anion channels, antiporters, electroneutral cation-chloride transporters). Several substances were used that are capable of inhibiting the activity of these proteins in animal and plant cells. The degree of inhibition of Cl^- efflux from pollen grains under the action of inhibitors that retard germination was studied.

MATERIALS AND METHODS

The tobacco plants *Nicotiana tabacum* L. Petit Havana SR1 were grown from seeds in a climate chamber

---

Abbreviations: NPPB) 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS) 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; $DiBAC_4(5)$) *bis*(1,3-dibutylbarbituric acid)pentamethine oxonol; DMSO) dimethyl sulfoxide; FDA) fluorescein diacetate.

* To whom correspondence should be addressed.

(25°C, 16-h day) in soil, provided with daily rinsing with mineral salt solution [17]. The pollen was accumulated from completely opened flowers and wetted in a wet chamber during 1 h at 26°C. Thereafter pollen grains were cultivated in liquid nutritive medium [18], which consisted of 0.3 M sucrose, 1.6 mM H_3BO_3, 3 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$, and 1 mM KNO_3 in 25 mM Mes-Tris buffer, pH 5.9. The medium was prepared with bidistilled water using chemically pure reagents.

The following inhibitors of chloride ion transport were used: 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Sigma, USA), niflumic acid, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), furosemide, and bumetanide (ICN, USA), *bis*(1,3-dibutylbarbituric acid)pentamethine oxonol ($DiBAC_4(5)$) (Molecular Probes, The Netherlands). All the inhibitors were diluted in dimethyl sulfoxide (DMSO) (Fluka, Switzerland) to the following concentrations: 40 mM NPPB; 200 mM niflumic acid, DIDS, bumetanide, and furosemide; 20 mM oxonol. Working solutions were prepared from these stock solutions using the medium for germination. The final concentration of DMSO did not exceed 0.5%. Control experiments showed that DMSO does not influence the pollen grain germination at this concentration.

The tubes with pollen grain suspension were incubated in a thermostat at 26°C for 50 min in the experiments on the effect of inhibitors on germination. Then the pollen were harvested by centrifugation for 30 sec at 400*g* and fixed by 4% paraformaldehyde at 6°C in 50 mM phosphate buffer, pH 7.4.

The fixed material was used for the count of the portion of germinated pollen using a light microscope. The pollen grains with pollen tubes no shorter than the diameter of the grain were taken as germinated. No less than 500 pollen grains were counted in each sample. The dependences of germination on the concentration of inhibitor are derived from averaging of 4-7 individual curves.

The total amount of Cl^- emerged from all pollen grains (viable and dead) into the medium during the incubation for 2-12 min with or without inhibitors was measured first in the experiments on the effect of the inhibitors on Cl^- efflux. The number of dead pollen grains was determined simultaneously to introduce a correction in the data obtained for the amount of Cl^- emerged from the cells due to their death.

The suspensions of wet pollen were incubated at 26°C, then the pollen grains were harvested by centrifugation for 30 sec at 400*g*, and the supernatant was used for Cl^- determination. The samples for count of dead pollen grains were prepared by the same way, but the cell suspension was incubated with a dye for 5 min (see below). The samples for determination of Cl^- amount emerged from dead cells were prepared by homogenizing the suspension of pollen grains for 12 min at 31,800 rpm using the DIAX 900 6G homogenizer (Heidolph, Germany). The data of light microscopy confirmed the entire destruction of pollen grains under these conditions. Fragments of decomposed cells were harvested by centrifugation for 10 min at 11,000*g*, and the concentration of Cl^- was determined in the supernatant.

The determination was conducted by potentiometric analysis with a Cl^--selective flow electrode ELIT 261 (Nico, Russia) and pH-meter ELIT 3305 (Jenway, UK). Calibration was performed using KCl solutions in the germination medium. The sample volume was ~120 µl. Each experiment was repeated with 7-11 biological samples, and each of them was analyzed twice.

The portion of dead pollen grains was determined by a conventional method [19] using fluorescein diacetate (FDA) at the final concentration of 20 µM. Following the incubation with the dye, pollen grains were precipitated and photographed in two modes: the first revealing the fluorescence of stained pollen grains and the second revealing (in the same field) all pollen grains, both viable and dead. We used a Leitz-Orthoplan microscope (Leitz, Germany) equipped with the PLOEMOPAK device (10× objective lens) and a Camedia C3030 Z digital camera (Olympus, Japan). Fluorescence was exited by a xenon lamp, the excitation light being passed through a unit I3 input filter (Leitz, Germany). The ratio between viable and dead pollen grains was calculated using the software package Olympus DP-soft (Soft Imaging System GmbH, Germany). About 300 pollen grains were counted in each sample. Six or seven biological repeats were in each variant.

We found from our experiments that 83 ± 3.4 nmol of Cl^- abandon each milligram of dying pollen grains. The portion of dead pollen grains was 5.6 ± 0.1% in control, 6.3 ± 0.1% in the presence of NPPB, 11.8 ± 0.1% in the presence of niflumic acid, and 13.7 ± 0.3% in the presence of DIDS. These data allow the calculation of Cl^- efflux from pollen grains for each sample in nmol per 1 mg wet viable pollen grains.

Data were processed using conventional statistical methods. The significance of differences was evaluated from the Student's *t*-test; mean ± SD values are given.

RESULTS

The first step of the study was to answer the question what Cl^--transporting proteins are involved in pollen germination. With this aim the effect of transport protein inhibitors on pollen germination was studied *in vitro*. The inhibitors were taken at the concentrations routinely used in studies on animal and plant cells: 1-100 µM NPPB or niflumic acid [3, 8, 9, 20, 21]; 1-800 µM DIDS [11, 22]; 100-1000 µM furosemide or bumetanide [12, 21]; and 0.5-10 µM oxonol $DiBAC_4(5)$ [23, 24].

NPPB, niflumic acid, and DIDS almost completely inhibited the germination (Fig. 1a). The concentration

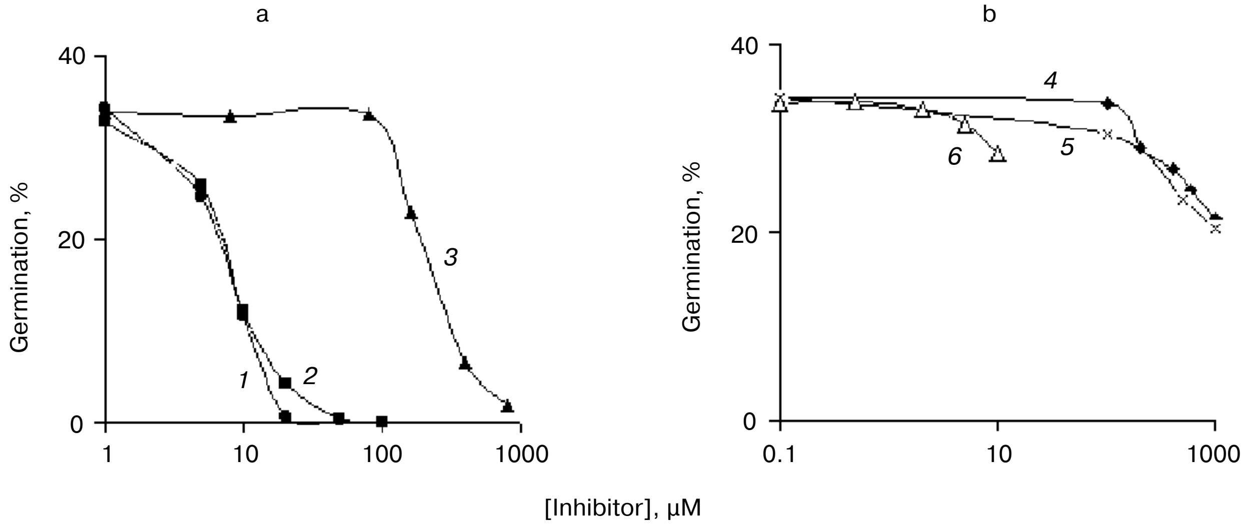

Fig. 1. Inhibitory effect of NPPB (*1*), niflumic acid (*2*), DIDS (*3*) (a), furosemide (*4*), bumetanide (*5*), and DiBAC$_4$(5) (*6*) (b), the inhibitors of Cl⁻ transport across the plasma membrane, on tobacco pollen germination *in vitro*.

curves for NPPB and niflumic acid are virtually identical. Total inhibition was achieved at 20 μM NPPB or 50 μM niflumic acid, and the IC_{50} value was 8 μM for both inhibitors. The effect of DIDS on the germination became visible only at concentrations exceeding 80 μM, and IC_{50} was ~240 μM for this inhibitor.

Furosemide and bumetanide inhibited the pollen grain germination by no more than 40% (Fig. 1b). So, these inhibitors were excluded from consideration along with oxonol, whose inhibitory effect did not exceed 16 ± 0.6% at concentration of 10 μM (Fig. 1b).

Since NPPB, niflumic acid, and DIDS are anion channel inhibitors, one can assume their involvement in pollen germination. Therefore, in the second step we studied the release of Cl⁻ from pollen grains into the incubation medium, as well as the effect of the mentioned inhibitors on the rate of this process.

The data demonstrating the kinetics of Cl⁻ release from pollen grains and the effect of 50 μM NPPB (one of the substances completely inhibiting the pollen germination) on this process are given in the Fig. 2. An intense and NPPB-independent efflux of Cl⁻ occurred predominantly from massive envelopes of pollen grains during the first 2 min of incubation. Within 5-12 min the efflux of Cl⁻ linearly depended on time and significantly decreased in the presence of NPPB. It is the interval that was chosen for the measurements of Cl⁻ efflux rate in further experiments.

The rate of Cl⁻ efflux was more than three times lower in the presence of 50 μM NPPB and 1.4 times lower in the presence of 50 μM niflumic acid (Table 1). Although DIDS (800 μM) also inhibited germination, no significant difference from the control was observed with DIDS (Table 1), thus this inhibitor was excluded from further consideration.

To compare the effect of inhibitors on the germination and Cl⁻ efflux, the rate of Cl⁻ efflux from pollen grains was studied at various concentrations of NPPB and niflumic acid (Fig. 3). The change in the Cl⁻ efflux rate depending on the inhibitor concentration for each

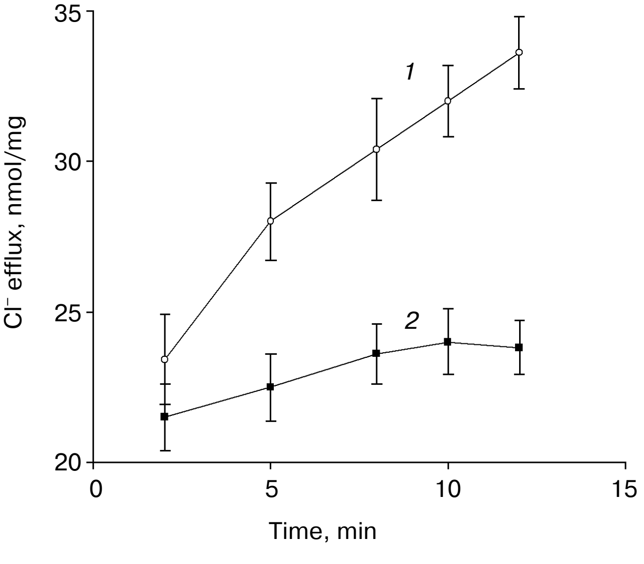

Fig. 2. Efflux of Cl⁻ from tobacco pollen grains (nmol per 1 mg wet viable pollen) at the initial germination stage *in vitro* in the absence (*1*) or presence (*2*) of 50 μM NPPB.

Table 1. Effects of NPPB, niflumic acid, and DIDS on the rate of Cl^- efflux from tobacco pollen grains *in vitro* in the time range of 5-12 min

Inhibitor	Rate of Cl^- efflux, nmol/min per mg pollen
Control (without inhibitors)	0.84 ± 0.03
NPPB (50 μM)	0.23 ± 0.07**
Niflumic acid (50 μM)	0.60 ± 0.09*
DIDS (800 μM)	0.70 ± 0.09

* $p < 0.05$.
** $p < 0.01$.

inhibitor (Fig. 3) correlated with the change in germination (Fig. 1a); in both cases $r = 0.99$. Taken at the concentration of 8 μM corresponding to IC_{50} for the germination (Fig. 1a), NPPB caused 1.8-fold decrease in Cl^- efflux rate in comparison with the control ($p < 0.01$), whereas the niflumic acid caused only 1.2-fold decrease ($p < 0.05$) (Fig. 3). Thus the effective concentrations of NPPB causing half-inhibition of germination and the transport of Cl^- are virtually equal.

In general, the data presented here suggest that Cl^- transport proteins sensitive to NPPB are present in plasma membrane of vegetative pollen grain cells, and the inhibition of the functioning of these proteins completely blocks the germination process.

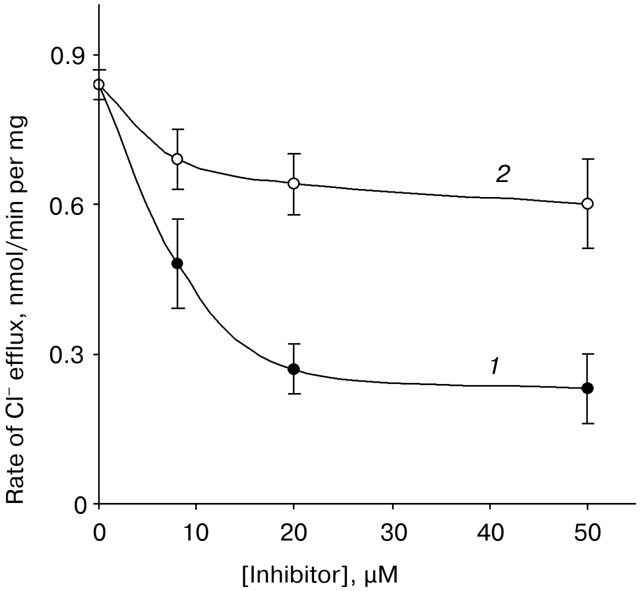

Fig. 3. Effect of NPPB (*1*) and niflumic acid (*2*) on the rate of Cl^- efflux from tobacco pollen grains (nmol/min per mg pollen) during incubation *in vitro* in the range of 5-12 min.

DISCUSSION

Each Cl^- transport inhibitor used in our study inhibits specifically a distinct transport mechanism (Table 2). NPPB, niflumic acid, and DIDS are the blockers of anion channels. Niflumic acid and DIDS inhibit functions of anion antiporter. The oxonol dye $DiBAC_4(5)$, being a tool for measurement of membrane potential [27], is the most potent from known inhibitors of Cl^- anion antiporter in animal cells (a protein of band 3) and also is a blocker of Cl^--channels activated by the enlargement of cell volume. Furosemide and bumetanide are the blockers of electroneutral cation-chloride symporters: the first of them preferentially inhibits the K^+-dependent symport of Cl^-, the second one Na^+,K^+-dependent symport of Cl^-.

The data suggest an interrelation between Cl^- transport and pollen germination. The anion channels of plasma membrane, which possess a high affinity to NPPB, are apparently functionally important. In fact, the effect of low NPPB concentrations on these channels completely prevented the germination (Fig. 1a) and inhibited the Cl^- efflux from pollen grains (Figs. 2 and 3, Table 1). Since the concentration of NPPB causing the 50% inhibition of germination (8 μM; Fig. 1a) reduces about twice the rate of Cl^- efflux (Fig. 3), one can conclude that the effect of this inhibitor on Cl^- channels of plasma membrane in vegetative pollen grains is specific.

The ability of niflumic acid and DIDS to completely inhibit the germination (Fig. 1a) along with relatively weak influence on the rate of Cl^- efflux (niflumic acid) (Table 1, Fig. 3) or even in absence of such influence (DIDS) (Table 1) can be explained from the effect of these inhibitors on the intracellular anion-transporting proteins. Niflumic acid-sensitive channels of tonoplast were found in cells of higher plants [2]. In yeasts, DIDS inhibits the transfer of ATP across the endoplasmic reticulum membrane [31]. In trypanosomes, DIDS (50-200 μM) expresses uncoupling activity, eliminating the mitochondrial membrane potential [32].

Activation of electrically neutral cation-chloride symporters appears to be less significant for pollen germination because the inhibition of these transport proteins by furosemide or bumetanide decreased the germination by less than 50% (Fig. 1b). Since this effect of the inhibitors on the germination was only achieved at very high concentration (1 mM), it is likely nonspecific. Effects of antiporters and oxonol-sensitive channels are still less significant (Fig. 1b).

Thus, the anion channels of the plasma membrane of vegetative pollen grain cells contribute appreciably to the transport of Cl^- at the initial germination step. These channels differ from those of the apex of growing tobacco

Table 2. Characteristics of inhibitors of Cl$^-$ transport used in this work and their effect of pollen germination

Inhibitor	Mechanism of transport	Blockage of pollen grain germination	References
NPPB	anion channels	+	[3, 7–9, 15, 21, 22, 25]
Niflumic acid	anion channels, anion antiporter	+	[20, 21, 25, 26]
DIDS	anion channels, anion antiporter	+	[11, 15, 22]
Oxonol DiBAC$_4$(5)	anion antiporter, anion channels activated by the enlargement of cell volume	–	[23, 24, 27, 28]
Furosemide	cation-chloride symporter K$^+$-Cl$^-$	–	[12, 21, 29, 30]
Bumetanide	cation-chloride symporter Na$^+$-K$^+$-2Cl$^-$	–	[12, 29, 30]

pollen tube in sensitivity to Cl$^-$-channel inhibitors [15]. Actually, a complete termination of both tube growth and release of Cl$^-$ from the apex by DIDS was achieved at 80 μM [15], a concentration one order lower than that necessary to arrest the pollen grain germination (800 μM; Fig. 1a); however, both the processes are equally sensitive to NPPB. This fact suggests that distinct Cl$^-$-channels are involved in the activation of pollen grain germination and in the control of pollen tube growth.

This study was supported by the Russian Foundation for Basic Research (grant No. 02-04-49246) and by the Scientific Program "Universities of Russia".

REFERENCES

1. Ward, J. M., Pei, Z.-M., and Schroeder, J. I. (1995) *Plant Cell*, **7**, 833-844.
2. Barbier-Brigoo, H., Vinauger, M., Colcombet, J., Ephritikhine, G., Frachisse, J.-M., and Maurel, C. (2000) *Biochim. Biophys. Acta*, **1465**, 199-218.
3. Johannes, E., Crofts, A., and Sanders, D. (1998) *Plant Physiol.*, **118**, 173-181.
4. Sze, H., Li, X., and Palmgren, M. G. (1999) *Plant Cell*, **11**, 677-689.
5. Blatt, M. R. (2000) *Annu. Rev. Cell Dev. Biol.*, **16**, 221-241.
6. Shabala, S. O., Babourina, O., and Newman, I. (2000) *J. Exp. Bot.*, **51**, 1243-1253.
7. Iino, M., Long, C., and Wang, X. (2001) *Plant Cell Physiol.*, **42**, 1219-1227.
8. Cho, M. H., and Spalding, E. P. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 8134-8138.
9. Noh, B., and Spalding, E. P. (1998) *Plant Physiol.*, **116**, 503-509.
10. Kurkdjian, A., and Guern, J. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 271-303.
11. Trofimova, M. S., and Molotkovskii, Yu. G. (1993) *Fiziol. Rast.*, **40**, 91-99.
12. Harling, H., Czaja, I., Schell, J., and Walden, R. (1998) *EMBO J.*, **16**, 5855-5866.
13. Cahalan, M. D., and Lewis, R. S. (1994) in *Chloride Channels* (Guggino, W. B., ed.) Academic Press, San Diego, pp. 103-130.
14. Zonia, L., Cordeiro, S., and Feijo, J. A. (2001) *Sex. Plant Reprod.*, **14**, 111-116.
15. Zonia, L., Cordeiro, S., Tupy, J., and Feijo, J. A. (2002) *Plant Cell*, **14**, 2233-2249.
16. Matveeva, N. P., Andreyuk, D. S., Voitsekh, O. O., and Yermakov, I. P. (2003) *Rus. J. Plant Physiol.*, **50**, 318-323.
17. Nitsch, J. P. (1965) *Bull. Soc. Bot. Fr.*, **112**, 517-522.
18. Benito Moreno, R. M., Macke, F., Alwen, A., and Heberle-Bors, E. (1988) *Planta*, **176**, 145-148.
19. Heslop-Harrison, J., Heslop-Harrison, Y., and Shivanna, K. R. (1984) *Theor. Appl. Gen.*, **67**, 367-375.
20. White, M. M., and Aylwin, M. (1990) *Mol. Pharmacol.*, **37**, 720-724.
21. Nagel, W., Somueski, P., and Katz, U. (2001) *Am. J. Physiol. Cell Physiol.*, **281**, C1223-C1232.
22. Egee, S., Lapaix, F., Cossins, A. R., and Thomas, S. L. (2000) *Bioelectrochemistry*, **52**, 133-149.
23. Knauf, P. A., Law, F. Y., and Hahn, K. (1995) *Am. J. Physiol.*, **269,** C1073-C1077.
24. Alper, S. L., Chernova, M. N., Williams, J., Zasloff, M., Law, F. Y., and Knauf, P. A. (1998) *Biochem. Cell. Biol.*, **76**, 799-806.
25. Marten, I., Zeilinger, C., Redhead, D. W., Awquati, Q., and Hedrich, R. (1992) *EMBO J.*, **11**, 3569-3575.
26. Falke, J. J., and Chan, S. I. (1986) *Biochemistry*, **25**, 7899-7906.
27. Haugland, P. (2001) *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes Inc., Leiden.
28. Arreola, J., Kenneth, R. H., and Knauf, P. A. (1995) *Am. J. Physiol.*, **269**, C1063-C1072.
29. Haas, M. (1994) *Am. J. Physiol.*, **267**, C869-C885.
30. Gillen, C. M., Brill, S., Payne, J. A., and Forbush, B. (1996) *J. Biol. Chem.*, **271**, 16237-16244.
31. Mayinger, P., and Meyer, D. I. (1993) *EMBO J.*, **12**, 659-666.
32. Bernardes, C. F., Meywe-Fernandes, J. R., Saad-Nehme, J., Vannier-Santos, M. A., Peres-Sampaio, C. E., and Vercesi, A. E. (2000) *Int. J. Biochem. Cell Biol.*, **32**, 519-527.