Biochemical Analysis of the Membrane Topology of the Amiloride-sensitive Na+ Channel*

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A key protein component of the amiloride-sensitive sodium channel has been cloned from rat colon and human lung. It may represent the first member of a new family of ionic channels expressed from nematode to human. The biochemical properties of the rat protein, a 699 amino acids long polypeptide, have been analyzed. Four polyclonal antibodies raised against distinct parts of the channel immunoprecipitated a glycosylated protein of 96 kDa after cRNA expression in oocytes as well as after in vitro translation. When expressed alone into oocytes, the protein was not stable; most of it remains stacked into the endoplasmic reticulum. This results in a very low yield of complete maturation of the protein at the cell surface after expression from the pure cRNA. To determine the membrane topology of the protein, in vitro translation by a rabbit reticulocyte lysate was performed followed by insertion into canine pancreatic microsomes and protease digestion. Analysis revealed a model with only two transmembrane helices and a large extracellular domain of about 500 amino acids. The NH2 and COOH termini are cytoplasmic. Protease digestion results suggest the possible presence of a structural element that could have a function similar to that of the H5 segment in K+ channels. The model indicates that there is no cytoplasmic site for protein kinase A phosphorylation. The well known regulation of the channel activity by hormones that activate this kinase such as vasopressin might thus be situated on another channel component.

Amiloride-sensitive Na+ channels are present in a variety of epithelia (Refs. 1–4 and reviewed in Ref. 5). The main subunit of the Na+ channel protein has recently been cloned from rat colon and called RCNaCh (or arENaCh) (6, 7). The corresponding cRNA was then denatured into 1% SDS, 4% 2-mercaptoethanol at 95 °C and precipitated with protein A-Sepharose CL-4B (Sigma). The beads were extensively washed with the same buffer. The immunoprecipitated material was then denatured into 1% SDS, 4% 2-mercaptoethanol at 95 °C for 5 min and analyzed by SDS-PAGE.

Cell-free Translation—cRNA was added to a rabbit reticulocyte lysate mixture (Promega Corp.), containing (35S)methionine, according to the manufacturer’s protocol, except that the lysate content was lowered to 40% final volume. For microsome insertion, 10 units of dog pancreatic microsomal membranes (Promega Corp.) were added at the start of the translation. For deletion mutants experiments, RNAs transcribed in vitro after linearization of the plasmid with AccI, MscI, and BspEI were used, without introduction of stop codons. This protocol was sufficient to analyze COOH-deleted mutants despite an increase in background.

Protease Digestion—Proteases were added to aliquots of the rabbit reticulocyte lysate translation mixture (final concentration, 100 µg/ml), in the presence or absence of 1% Triton X-100 and incubated for 30 min at 4 °C with trypsin or proteinase K for 1 h at 27 °C with casein or pepstatin A. This protocol was sufficient to analyze COOH-deleted mutants despite an increase in background.

EXPERIMENTAL PROCEDURES

RNA Transcription—cRNA was transcribed in vitro with T7 RNA polymerase as previously described (7). The RCNaCh plasmid was linearized with AccI, MscI, and BspEI to get 3′ truncated cRNAs. The COOH-terminal-deleted proteins were designated as C43S, C521, and C615, respectively, the numbers 438, 521, and 615 corresponding to the last encoded residues (7). The excision of an EcoRI-EcoRI fragment in 5′ of the cDNA and recirculation of the plasmid have permitted the RNA polymerase transcription of a 5′-deleted form of RCNaCh. This last construction, called N108, was translated into protein from Met109 to the end.

Oocytes—Oocytes were prepared and injected according to Ref. 7. For (35S)methionine labeling, oocytes were incubated overnight into 95 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 2 mM MgCl2, 5 mM Heps/NaOH pH 7.4, supplemented with 50 µCi/ml of Trans label kit (ICN Biomedicals).

Antibodies—The three internal peptides Q44GLGKGDKREEQGLG58, T66IEEELDRITEQTLFDL77C, and G283RGAGAREVAST368 of the RCNaCh sequence are predicted to be highly antigenic (8). They were linked to keyhole limpet hemocyanin and injected to rabbits according to standard protocols (10). An expression protein of 23 kDa corresponding to the COOH-terminal part of the RCNaCh protein (from Ile129 to the COOH terminus) was obtained after induction of BL21(DE3) bacteria transformed with a pET.11c vector (Novagen) containing the 1632–221-base pair fragment of RCNaCh (generated by a digestion by BclI and BamHI). The protein was purified by electrophoresis, checked by NH2-terminal sequencing, and injected into rabbits. Sera were controlled monthly by enzyme-linked immunosorbent assay against pure peptides. Positive ones were those producing immunoprecipitation of the in vitro translated protein. For immunoprecipitation, the labeled material was solubilized in a buffer containing 1% Triton X-100, 0.5% deoxycholate, 140 mM NaCl, 20 mM TrisCl, pH 7.4, 40 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml pepstatin A. After a 1-h preclearing with pansorbin A (Calbiochem), the soluble extract was incubated for 1 h with antibodies diluted 100-fold and then precipitated with protein A-Sepharose CL-4B (Sigma). The beads were extensively washed with the same buffer. The immunoprecipitated material was then denatured into 1% SDS, 4% 2-mercaptoethanol at 95 °C for 5 min and analyzed by SDS-PAGE.

PAP Conditions—The radioactivity from the immunoprecipitation was developed by autoradiography. The density of autoradiographs was read using a densitometer (densitometer, Gilford Instrument Laboratories, model 2500, Bedford, OH) and the data were expressed as a percentage of the total density. The background density was determined and subtracted from each reading. The density values were expressed as means ± S.E. of five experiments. A Student’s t test was used to determine the significance of differences between control and experimental samples. A χ2 test was used to determine the significance of differences between the distribution of density values.

The Abbreviations used are: RCNaCh, rat colon Na+ channel; PAGE, polyacrylamide gel electrophoresis; pAb1, pAb2, pAb3, pAb4, polyclonal antibodies raised against RCNaCh; Eno, endothelial cell. The results are expressed as the mean ± S.E. of at least three independent experiments. The significance of differences between groups was determined by Student’s t test and is indicated by * (p < 0.05) or ** (p < 0.01).
RESULTS AND DISCUSSION

We first intended to establish the topology of the Na+ channel protein in the oocyte membrane, where the channel activity was initially characterized after expression of its cRNA (6, 7). However, expression of RCNaCh in Xenopus oocytes results in a relatively low level of amiloride-sensitive Na+ current (6, 7). The neosynthesized Na+ channel protein was identified after metabolic labeling with [35S]methionine of RCNaCh-injected oocytes; polyclonal antibodies raised against fragments of the protein specifically immunoprecipitated a protein of 96 kDa (Fig. 1). After in vitro translation in the presence of rabbit reticulocyte lysate and canine pancreatic microsomes, the same antibodies precipitated two proteins of 96 and 78 kDa. The 78-kDa protein is the unglycosylated form of the protein (7), because it was also observed when the rabbit reticulocyte lysate was incubated in the absence of canine pancreatic microsomes (see also Fig. 3A). The 96-kDa protein is sensitive to Endo H, an enzyme that cleaves high mannose oligosaccharides from glycoproteins (11). When the immunoprecipitated material from in vitro translation or from oocytes was treated with this enzyme, the 96-kDa protein disappeared and was replaced by the 78-kDa protein (Fig. 1). The sensitivity to Endo H revealed (i) that the protein is N-glycosylated and (ii) that the 96-kDa protein is probably not completely processed, because complete glycosylation usually leads to Endo H-resistant forms of glycoproteins (12). The 96-kDa protein thus does not correspond to the mature form of the channel protein but rather to an intermediate form, most probably stacked into the endoplasmic reticulum, where it will be finally degraded. Analysis of the turnover of the 96-kDa protein supports that view. In oocytes, the half-life of the 96-kDa protein is ~2 h and is not changed by treatments with known blockers of lysosomal degradation (13) such as chloroquine (800 μM), NH4Cl (10 mM), brefeldin A (10 μM), or leupeptin (400 μg/ml) (not shown). The degradation of the 96-kDa protein is thus likely to take place in the endoplasmic reticulum, as previously reported for the asialoglycoprotein receptor (13) and the T cell antigen receptor (14). In these two cases of multi-subunit membrane proteins, several of the subunits have to be co-expressed to stabilize each other. When one subunit is lacking, degradation of the others takes place in the endoplasmic reticulum (13, 14). In the case of the amiloride-sensitive Na+ channel, it is expected that other subunits are necessary for efficient expression at the cell surface. Our current hypothesis is then that a low proportion of the RCNaCh protein reaches full maturation when RCNaCh cRNA is expressed alone, leading to a low level of functional Na+ channels (6, 7).

The amount of mature form was thus too low to permit the analysis of its topology with protocols applicable in situ. Because the protein insertion in the membrane is expected to remain unchanged during processing of RCNaCh, we analyzed the topology of the RCNaCh form detected during in vitro translation experiments. This was done in the presence of rabbit reticulocyte lysate and canine pancreatic microsomes (15). The RCNaCh sequence contains two hydrophobic domains of more than 20 residues (located between residues 110 and 139 and between residues 588 and 613) characterized by a Kyte and Doolittle (16) hydropathy profiles of rat colon and human lung Na+ channels. The window for hydrophobicity analysis is n = 19 residues. B, description of the different constructs used in this study. Boxes indicate the hydrophobic domains, M, the putative first methionine, and V, the potential N-glycosylation sites. "Potential" glycosylation sites at Asn60 and Asn83 are not shown, because they are located before the first hydrophobic domain (intracellular) or too low to permit the analysis of its topology with protocols applicable in situ. Be-
the presence of canine pancreatic microsomes, two major products were observed at 96 and 78 kDa (Fig. 3A, Total). These two bands were immunoprecipitated by the four antibodies (Fig. 3A). When the total translation product was treated with proteinase K, a fragment of 70 kDa was found to be protected from protease degradation (proteinase K, Total, Fig. 3A). pAb1, raised against a peptide located before the first putative transmembrane segment, and pAb3, raised against a peptide located after the second putative transmembrane segment, failed to recognize the 70-kDa peptide. The same observation was made with pAb4, an antibody raised against the COOH-terminal segment of RCNaCh. pAb2 was the only serum that could immunoprecipitate this 70-kDa peptide. The segment between the two transmembrane domains is thus likely to be located inside the microsomes, whereas the NH₂ and COOH termini of the protein probably lie outside the microsomal lumen (Fig. 3B). The absence of reactivity of pAb4 against the 70-kDa peptide was puzzling at first, because this peptide contains a fragment that is located between the two potential transmembrane domains (Fig. 2A). The most probable interpretation is that pAb4 is directed specifically against the COOH-terminal region of the immunogen.

Other bands were observed in the total translation product. Because several of them were recognized by pAb2, pAb3, and pAb4 but not by pAb1, whose epitope is located very near the NH₂ terminus, they probably correspond to translation products beginning at some internal methionine (false starts). For convenience, the following part of the paper deals with the total translation product. Only the highest molecular weight corresponding to the protein translated from the first methionine was considered for structural analyses.

Fig. 4A shows the results of a typical experiment performed on the full-length RCNaCh cRNA after translation and processing of the 96-kDa protein. The 70-kDa fragment generated by proteinase K treatment was deglycosylated with Endo H. The apparent Mᵋ then shifted to 50,000, whereas the calculated molecular mass of the peptide located between residue 110 and residue 613 is 58 kDa (Fig. 4B). The difference between observed and calculated Mᵋ values (8 kDa) could be due to abnormal mobility of the polypeptide in SDS-PAGE. Another possibility is that the peptide obtained after proteolysis is shorter than the 110–613 fragment.

The protection from proteolysis of the 50- and 70-kDa fragments is probably due to microsomal incorporation, because it was not observed when proteinase K treatment was carried out on microsomes solubilized with Triton X-100 or when translation was performed in the absence of microsomes (Fig. 4A).

Carboxypeptidase Y is an exopeptidase starting its catalytic action at the COOH terminus. Its action is known to be stopped at a glycine residue. For RCNaCh, the digestion by carboxypeptidase Y is expected to be stopped by Gly⁶⁰⁸, resulting in an ~2-kDa amputation of the 96-kDa protein. Fig. 4A shows that the mobility of the RCNaCh protein is indeed slightly increased after treatment by carboxypeptidase Y. Because microsomes did not protect the protein against carboxypeptidase Y action, the COOH-terminal part of RCNaCh has to be located outside the microsomal structure, i.e. pointing toward the cell cytoplasm.

Similar treatments were carried out with the different truncated forms of RCNaCh. Fig. 5A shows the results obtained with N108. N-glycosylated N108 has a molecular weight of 84,000 (65,000 after Endo H deglycosylation). N108 shares most of the topological properties of RCNaCh. For instance, (i) its COOH-terminal segment is cytoplasmic, as demonstrated by a shift in its mobility after carboxypeptidase Y treatment; (ii) proteinase K digestion generates a 70-kDa peptide (50 kDa after deglycosylation), as previously observed with the full-length RCNaCh protein. Thus the removal of the ~100 first NH₂-terminal residues in N108 does not alter the Mᵋ of the final fragment protected in microsomes from proteinase K digestion.

The difference between the Mᵋ values of N108 before and after treatment with proteinase K represents the Mᵋ of the segment(s) exposed to the protease, i.e. 14,500. Because Met⁶⁰⁸ is located at the very beginning of the first putative transmembrane segment, one should expect that only the 8.9-kDa fragment going from Arg⁶⁰⁸ to the COOH-terminal end would be digested by proteinase K in N108. A 5.6-kDa difference is observed between this calculated mass and the experimental value. On the other hand, the difference between Mᵋ values of RCNaCh and N108 provides the Mᵋ of the deleted NH₂ segment from Met¹ to Arg⁶⁰⁸, i.e. 12,500. In that case, there is a very good agreement with the calculated Mᵋ of 12.4 kDa. The possibility of an abnormal mobility of these proteins in SDS-PAGE is then excluded. The shifts between theoretical masses of the proteinase K peptide and of the COOH-terminal cytoplasmic peptide would then indicate a transmembrane topology different from the one depicted in Fig. 3B. Fig. 5B summarizes the different results obtained with N108.

Three different COOH-terminal-deleted constructs were analyzed and presented in Fig. 6. C615 includes the two hydrophobic segments (Fig. 2) but lacks most of the COOH-terminal part. It has a Mᵋ of 87,000 (67,000 after deglycosylation) (Fig. 6). Proteinase K treatment generates a 69-kDa peptide, which moves at 50 kDa after deglycosylation, and which, therefore, has the same characteristics as the peptide generated by proteinase K from RCNaCh and N108. Thus the removal of the
84 last COOH-terminal residues does not alter the size of the fragment protected in microsomes from proteinase K digestion. Moreover, because C615 is sensitive to carboxypeptidase Y, Arg615 has to be accessible to the exopeptidase and is then cytoplasmic. The difference between the \( M_r \) values of C615 before and after treatment with proteinase K is 18,000. Because Arg615 is located immediately after the second putative transmembrane domain (see the model presented in Fig. 3B), this value is expected to correspond to the size of the NH2-terminal segment (from NH2 terminus to Lys105, \( M_r = 12,500 \)). In that case, as with RCNaCh and N108, there is a difference of 5.5 kDa between the calculated mass of the exposed segment (12.5 kDa) and the experimental \( M_r \) (18,000).

Properties of C438 and C521 are analyzed in Fig. 6. Proteinase K treatment generated a 53-kDa peptide (39 kDa after deglycosylation) from C438 (Fig. 6) and a 58-kDa peptide (46 kDa after deglycosylation) from C521 (Fig. 6). The calculated molecular mass of the peptide (located between residue 110 and residue 148) generated from C438 after proteinase K digestion is 38 kDa, very close to the observed \( M_r \) of 39,000 after deglycosylation. Similarly, the calculated molecular mass for the peptide generated from C521, located between residue 110 and residue 521, is 47 kDa, very close to the observed \( M_r \) value of 46,000. C438 and C521 are not sensitive to carboxypeptidase Y, in good agreement with the model shown in Fig. 3B, which puts their COOH-terminal end within the microsomal lumen. Diff-
ferences between \( M \), values before and after treatment with proteinase K are 15,000 for C438 and 13,000 for C521. These values are very close to the calculated mass of 12.5 kDa for the fragment between Met\(^1\) and Lys\(^{496}\), the exposed NH\(_2\)-terminal segment of RCNaCh.

The extracellular domain of RCNaCh (Fig. 2) contains six consensual putative N-glycosylation sites, corresponding to Asn\(^{311}\), Asn\(^{480}\), Asn\(^{491}\), Asn\(^{494}\), Asn\(^{496}\), and Asn\(^{539}\). The first step in the N-glycosylation of proteins (which occurs in microsomes) results in the branching to asparagine of an oligosaccharide moiety that contains a high number of mannoses. Endo H cleavage occurs between two N-acetylgalosamines leaving one N-acetylgalosamine linked to the polypeptide after removal of the remaining oligosaccharide (11). A mean \( M \), shift of 19,300 is observed for RCNaCh, N108, and C615 after Endo H treatment (Figs. 4–6). On the other hand, a smaller mean \( M \), shift of 13,800 is observed for C438 and C521, which differs from the other constructs by the lack of one putative N-glycosylation site in Asn\(^{539}\). This site is thus clearly glycosylated. Because our attempts to real the number of N-glycosylation sites in RCNaCh are based on SDS-PAGE migrations and because only apparent \( M \), values of the CDs, not the true \( M \), of RCNaCh, it is unfortunately not possible from the available data to provide an exact value. The number of N-glycosylated sites in RCNaCh could vary between four and six.

The secondary structure of the two hydrophobic segments analyzed according to Ref. 17 suggests that they most likely correspond to \( \alpha \)-helices. Accumulations of positive charges were observed immediately before the first transmembrane segment (K\(_\mathrm{a}(\mathrm{HNRMK})_9\)) as well as immediately after the second transmembrane segment (R\(_\mathrm{b}(\mathrm{FRSR})_6\)), as previously reported for many other membrane proteins (18). It is generally believed that segments with an excess of positive charges do not tend to be integrated within the membrane bilayer and that they stabilize adjacent transmembrane segments.

The model, which is presented in Fig. 3B, has cytoplasmic NH\(_2\)- and COOH-terminal segments (both composed of about 100 residues) and a single large extracellular domain (with at least four N-glycosylation sites).

Inward rectifier K\(^+\) channels are other examples of ion channels that have only two transmembrane \( \alpha \)-helical segments (10, 20). An additional H5 segment, also called a P segment, is found in all known K\(^+\) channels (21, 22). This region constitutes a crucial part of the ionic pore. It was therefore of interest to analyze our own data in light of the K\(^+\) channel model. Because all known H5 structures are known to be located in the vicinity of classical transmembrane segments (23), it was logical to think that if such structures exist for the Na\(^+\) channel they would be located either immediately after the first transmembrane domain or immediately before the second transmembrane domain.

The analysis of our \( M \), data has revealed a number of differences between the calculated values for digestion products and the experimental ones that seem to provide indications that a H5-like structure might also exist in the Na\(^+\) channel protein. First, the 80-kDa extracellular segment is smaller than expected for a segment between Thr\(^{162}\) and Leu\(^{213}\). The difference is about 8 kDa. Second, whereas the observed \( M \), values of the C438 and C621 constructs fit well with the two \( \alpha \)-helices model, the \( M \), reduction after proteolysis was about 5 kDa larger than expected for RCNaCh (5.6 kDa), N108 (5.6 kDa), and C615 (5.5 kDa). The general model presented in Fig. 3B, characterized by cytoplasmic NH\(_2\)- and COOH termini and a large extracellular domain, does not have to be deeply changed to include one equivalent of a H5-like segment (see Figs. 4B and 5B). In models presented in Figs. 4B and 5B, a segment crosses the bilayer near a hydrophobic domain. A sensitivity of this small segment to proteinase K digestion would explain the lower experimental \( M \), (as compared with calculated) of the proteinase K-protected fragments, as well as the more extended digestion observed for RCNaCh, N108, and C615. Because \( M \), differences have been observed for digestions of RCNaCh, N108, and C615 but not for C438 and C521, this putative H5-like segment would most likely be located near the second transmembrane segment or formed only when both transmembrane segments are correctly inserted in the bilayer.

Vasopressin is a well known activator of the epithelial Na\(^+\) channel. It acts via phosphorylation by a cAMP-dependent protein kinase. Interestingly, Ser\(^{206}\) and Ser\(^{207}\) from the rat colon sequence are consensuses sites for phosphorylation by cAMP-dependent protein kinase. However the channel topology presented in Figs. 4B and 5B indicates that these two residues have an extracellular localization. Therefore intracellular cAMP-dependent protein kinase probably cannot directly phosphorylate the RCNaCh protein, and phosphorylation induced by vasopressin would have to take place on another protein component, which would either be another subunit of the amiloride-sensitive Na\(^+\) channel or perhaps be a distinct cellular component that would indirectly regulate channel activity.

Protein kinase C can act as a negative regulator of Na\(^+\) channel activity in A6 cells (24, 25). The model presented in Figs. 4B and 5B indicates that Ser\(^{206}\) is the only site for protein kinase C that is conserved between rat and human (8). Its localization near the cytoplasmic side of the first transmembrane \( \alpha \)-helix suggests a possible involvement in the negative regulation of Na\(^+\) channel activity by protein kinase C.

The primary structure of RCNaCh as well as its membrane topology clearly define a new ionic channel family. Interestingly, RCNaCh is related to the nematode proteins Mec\(_{\alpha}\), Mec\(_{\beta}\) and Deg\(_{\alpha}\), (26, 27). These three proteins called degenerins (26) are present in Caenorhabditis elegans mechanosensory neurons. Point mutations in their sequences induce mechanosensitivity and degenerescence. Their overall homology with RCNaCh is of the order of 15%, but the two hydrophobic segments depicted in Fig. 2 are conserved (26). The highest homology between RCNaCh and degenerins has been found around a cysteine-rich domain located extracellularly between Cys\(^{444}\) and Cys\(^{507}\). Because of their homology with RCNaCh one proposed hypothesis (7, 27) for the physiological role of C. elegans degenerins in touch neurons is that they may correspond to mechanosensitive cationic channels. The different parent degenerins (Mec\(_{\alpha}\), Deg\(_{\alpha}\), and Mec\(_{\beta}\)) all analogous to RCNaCh and with a high homology between them, may be the different subunits forming one single type of ionic channel.

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REFERENCES

1. Hamilton, K. L., and Exton, D. C. (1986) Membr. Biochem. 6, 149–171
2. Palmer, L. G., and Fridlind, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2767–2770
3. Vigne, F., Champion, G., Marsault, R., Barbry, P., Frelin, C., and Lazdunski, M. (1989) Cell. Mol. Biol. 35, 7693–7699
4. Verrier, B., Champion, G., Barbry, P., Gérard, C., Mauclair, J., and Lazdunski, M. (1982) Eur. J. Biochem. 129, 499–505
5. Kaplan, L. G. (1984) Annu. Rev. Physiol. 50, 1–66
6. Canessa, C., Horsberger, J.-D., and Rossier, B. C. (1993) Nature 361, 467–470
7. Lenguan, R., Volley, N., Waldmann, R., and Lazdunski, M. (1993) FEBS J. 318, 95–99
8. Veilley, N., Lenguan, R., Champion, G., Mattei, M.-G., Waldmann, R., Lazdunski, M., and Barbry, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 247–251
9. Parker, J. M. (1985) J. Biol. Chem. 260, 2199–2205
10. Moinier, L., Trimbile, R. B., and Maley, F. (1978) Methods Enzymol. 83, 12985
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12. Roth, J. (1987) Biochim. Biophys. Acta 906, 405–436
13. Amara, J. F., Lederkremer, C., and Lodish, H. F. (1989) J. Cell Biol. 106, 3515–3524
14. Bonifacino, J. S., Cosson, P., and Klausner, R. D. (1990) Cell 63, 503–513
15. Hedgpeth, J., and Lingappa, V. R. (1983) Cell 34, 759–766
16. Kyte, J., and Doolittle, R. F. (1980) J. Mol. Biol. 157, 105–122
17. Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) J. Mol. Biol. 120, 97–120
18. Cramer, W. A., Engelman, D. M., von Heijne, G., and Rees, D. C. (1992) FASEB J. 6, 3397–3402
19. Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M., V., and Hebert, S. (1993) Nature 362, 31–38
20. Kubo, Y., Baldwin, T. J., Jan, Y. N., and Jan, Y. (1993) Nature 362, 127–132
21. Yellen, G., Jarman, M. E., Abramson, T., and MacKinnon, R. (1991) Science 251, 939–942
22. Durell, S. R., and Guy, H. R. (1992) Biophys. J. 62, 238–250
23. Pongs, O. (1992) Physiol. Rev. 72, 569–598
24. Yanase, M., and Handler, J. S. (1986) Am. J. Physiol. 250, C517–C522
25. Ling, B. N., and Eaton, D. C. (1989) Am. J. Physiol. 256, F1094–F1103
26. Chalif, M., Driscoll, M., and Huax, M. (1993) Nature 361, 504
27. Driscoll, M., and Chalif, M. (1991) Nature 349, 588–593