Expression of the Cystic Fibrosis Phenotype in a Renal Amphibian Epithelial Cell Line*  

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Mutations in a Cl− channel (cystic fibrosis transmembrane conductance regulator or CFTR) are responsible for the cystic fibrosis (CF) phenotype. Increased Na+ transport rates are observed in CF airway epithelium, and recent studies suggest that this is due to an increase in Na+ channel open probability (Po). The Xenopus renal epithelial cell line, A6, expresses both cAMP-activated 8-picosiemen (pS) Cl− channels and amiloride-sensitive 4-pS Na+ channels, and provides a model system for examining the interactions of CFTR and epithelial Na+ channels. A6 cells express CFTR mRNA, as demonstrated by reverse transcriptase-polymerase chain reaction and partial sequence analysis. A phosphorothioate antisense oligonucleotide, complementary to the 5′ end of the open reading frame of Xenopus CFTR, was used to inhibit functional expression of CFTR in A6 cells. Parallel studies utilized the corresponding sense oligonucleotide as a control. CFTR protein expression was markedly reduced in cells incubated with the antisense oligonucleotide. Incubation of A6 cells with the anti-sense oligonucleotide led to inhibition of forskolin-activated amiloride-insensitive short circuit current (Isc). After a 30-min exposure to 10 μM forskolin, 8-pS Cl− channel activity was detected in only 1 of 31 (3%) cell-attached patches on cells treated with antisense oligonucleotide, compared to 5 of 19 (26%) patches from control cells. A shift in the single-channel current-voltage relationship derived from antisense-treated cells was also consistent with a reduction in Cl− reabsorption. Both amiloride-sensitive Isc and Na+ channel P0 were significantly increased in antisense-treated, forskolin-stimulated A6 cells, when compared with forskolin-stimulated controls. These data suggest that the regulation of Na+ channels by CFTR is not limited to respiratory epithelia and to epithelial cells in culture overexpressing CFTR and epithelial Na+ channels.

Cystic fibrosis (CF)1 is the most common lethal genetic disease in the Caucasian population in the United States, affecting approximately 1 in 2500 live births. In 1989 the genetic locus of the disease was determined, and shortly thereafter the gene product was identified (1). This protein, the cystic fibrosis transmembrane conductance regulator (CFTR), is a cAMP-dependent protein kinase-regulated epithelial Cl− channel (2–4). There is now significant evidence that, besides being a Cl− channel, CFTR also alters the activity or properties of other channels. For example, an outwardly rectifying Cl− channel (ORCC) found in many epithelia is not responsive to cAMP-dependent protein kinase in the absence of CFTR (5–7). Recent work suggests that ATP secreted, via CFTR or a pathway that is regulated by CFTR, binds to purinergic receptors and activates ORCC (8). In addition, individuals with CF not only have abnormal Cl− transport but also abnormal Na+ transport (9–13). Examination of airway cells obtained from patients with CF has shown that Na+ transport is significantly increased and Cl− transport is dramatically decreased compared to normal airway cells (13–16). The enhanced rates of Na+ transport across CF airway epithelia are not simply a consequence of altered apical membrane Cl− permeability. Recent work examining CFTR and epithelial Na+ channel (ENaC) co-expression in Madin-Darby canine kidney cells and COS cells supports the role of CFTR as a cAMP-dependent regulator of Na+ channels (17). Mechanism(s) by which CFTR alters Na+ channel function remain unclear. Although one group reported an increase in the number of Na+ channels in CF airway epithelia (18), another reported changes in the open probability of Na+ channels (13). Other studies suggest that cAMP-regulated membrane recycling is altered in CF epithelia, although the changes in exocytosis and endocytosis reported would predict a decrease in the number of Na+ channels in CF airway epithelia (19). Changes in the post-translational modification of Na+ channels or in its intracellular trafficking could also result in altered Na+ channel function, regulation, or cell surface expression.

The Xenopus renal epithelial cell line, A6, expresses a well

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1 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ORCC, outwardly rectifying Cl− channel; ENaC, epithelial Na+ channel; pS, picosiemen(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DIDS, 4,4′-dibromo-5-(3-phenylpropylamino)-4H-1,2,3-triazole; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.
characterized epithelial Na\textsuperscript{+} channel, which is quite similar in structure to epithelial Na\textsuperscript{+} channels present in mammalian lung, kidney, and colon (20–26). A6 cells also express an 8-pS cAMP-regulated Cl\textsuperscript{−} channel, which has functional characteristics of CFTR (23, 27, 28). This cell line provides a potential model system to examine the regulation of ENaCs by CFTR. We provide evidence that CFTR is expressed in A6 epithelia, and that inhibition of its expression leads to the activation of ENaCs, due to increases in Na\textsuperscript{+} channel open probability.

EXPERIMENTAL PROCEDURES

Materials—Ephorpsis reagents were purchased from Bio-Rad. All other reagents were purchased from vendors listed below, or from Sigma. Sense and antisense phosphorothioate oligonucleotides corresponding to the first 25 nucleotides of Xenopus laevis CFTR (29) were synthesized either by the Emory University Microchemical Facility or by the University of Pennsylvania Nucleic Acid Facility. A search of a gene data bank revealed only limited similarity of this 25-mer with Xenopus CFTR (29). A 23-mer peptide (CRKSRPQISALQEE- TEEEVQDTR) corresponding to the carboxyl terminus of passages 90–96, were maintained in plastic tissue culture flasks at X. laevis gene data bank revealed only limited similarity of this 25-mer with X. laevis CFTR (29). A 23-mer peptide (CRKSRPQISALQEE- TEEEVQDTR) corresponding to the carboxyl terminus of Xenopus CFTR but lacking the COOH-terminal leucine was synthesized by Lofstrand Labs (Gaithersburg, MD).

Cell Culture—The methods are similar to those described previously (21–25). Briefly, the A6 subclone 2F3 (a gift from D. B. Rossier and J. P. Kraehenbuhl, University of Lausanne, Lausanne, Switzerland) passages 90–96, were maintained in plastic tissue culture flasks at 28 °C with 5% CO\textsubscript{2} in air in a medium containing Leibovitz's medium L-15 (7 parts)/Com's F-12 medium (3 parts) modified for amphibian cells by adjusting the final HCO\textsubscript{3} concentration to 25 mM and supplemented with 5% fetal calf serum, 0.6% penicillin, and 1.0% streptomycin. The final osmolality was 230 mOsmol/kg H\textsubscript{2}O. For patch clamp experiments, cell monolayers were transferred to a 6-well plate and a final extension at 72°C for 10 min. Productsof the predicted size (9 GAGCTG-3') were excised from A6 cells as described previously (32). A6 cDNA was generated using an oligo(dT) primer, and then amplified by the PCR using an alkaline phosphatase-linked chemiluminescent detection system (Western Light Plus, Tropix, Inc.) according to the manufacturer's instructions, using a 1-h incubation with the monoclonal anti-CFTR antibody (0.2 µg/ml) at room temperature.

Transepithelial Measurements—A6 cell monolayers were transferred to a modified Ussing chamber and bathed in amphibian Ringer's saline containing (in mM): 100 NaCl, 4 KCl, 2.5 NaHCO\textsubscript{3}, 1 K\textsubscript{2}HPO\textsubscript{4}, 1 CaCl\textsubscript{2}, 11 glucose, and buffered with 10 HEPES. Electrical measurements were performed with a modified Ussing chamber and a DVC-1000 voltage clamp (World Precision Instruments) as described previously (31). The short circuit current (Isc) was allowed to stabilize prior to bath additions. The amiloride-sensitive component of the Isc was determined by adding 10 µM amiloride to the luminal bath. Data are reported as mean Isc ± S.E.

Patch Clamp Recording and Analysis—Methods are as described previously (21–25). Patch pipette and extracellular bath solutions consisted of a physiologic amphibian saline containing (in mM): 100 NaCl, 3.4 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 10 HEPES, buffered at pH 7.4 (titrated with 0.1 N NaOH). Experiments were performed at room temperature. Unitary channel currents were measured using a List EPC-7 Patch Clamp (Medical Systems Corp.). The convention for applied voltage to the apical membrane patch (−Vapic) represents the voltage deflection from the patch potential (i.e. the resting membrane potential for cell-attached patches). Inward Na\textsuperscript{+} current (pipette to cell) is represented as downward transitions in single-channel records.

Single-channel Data Analysis—Analysis of data was performed, as described previously (21–25), utilizing locally developed software which closely follows the algorithms presented by Colquhoun and Sigworth (37). The total number of functional channels (N) in the patch were determined by observing the number of transitions in the single-channel recordings. We only used patches in which we had more than a 95% probability of having estimated the number of channels correctly (following the methods of Muramuka and Eaton (see Ref. 38)). As a measure of channel activity, Np (number of channels times the open probability) was calculated as follows.

\[
N_p = \sum_{n=0}^{\infty} nP_n \frac{t_n}{T} \quad \text{(Eq. 1)}
\]

T was the total record time, n was the number of channels open and t\textsubscript{n} was the record time during which n channels are open. All Np values were calculated from 3 min of single-channel recording and are reported as mean Np ± S.D. Significance was p < 0.05. Statistical analysis was performed using SigmaStat (Jandel Scientific, Sun Rafael, CA).
Expression of CFTR in A6 cells was demonstrated by reverse transcriptase-polymerase chain reaction to amplify CFTR message. A predicted product of ~789 base pairs was observed (+ RT lane). This product was not observed when PCR was performed on RNA which was not reverse transcribed (~ RT lane) as a control. This product was subcloned into TA vector (Invitrogen), and partial sequence analysis (201 residues) was obtained. The sequence exhibited ~97% nucleotide identity, and 100% amino acid identity to a previously published X. laevis CFTR sequence (29).

**RESULTS**

A6 Epithelia Express CFTR—A6 epithelia were originally derived from the distal nephron of X. laevis kidney and express apical plasma membrane Cl⁻ channels and Na⁺ channels when grown on permeable supports. Under control conditions (with amphibian saline in the patch pipette and no applied potential), amiloride-sensitive Na⁺ channels, with the same characteristics as mammalian ENaC, are easily identified by their previously described properties (4–5 pS conductance, high Na⁺ selectivity, non-linear current-voltage relationship, long open and closed times) in apical cell-attached patches on confluent, mineralocorticoid-stimulated A6 cells (21–25). Three subunits of the Xenopus epithelial Na⁺ channel, α-, β-, and γ-xENaC, have recently been cloned from A6 epithelia and are similar in structure to ENaCs cloned from mammalian tissues (26). Several Cl⁻ channels with distinct electrophysiologic characteristics have also been observed in the apical membrane of A6 epithelia, although the structural correlates of these Cl⁻ channels have not previously been delineated (23, 28). One of these apical membrane Cl⁻ channels has a number of the same characteristics as mammalian CFTR, including its single-channel conductance (8–10 pS), linear I/V relationship, rapid kinetics at hyperpolarized potentials, response to Cl⁻ channel blockers (e.g. NPPB- and glybenclamide-sensitive, but DIDS-insensitive), and activation by cAMP-producing agonists (23, 27, 28). The Xenopus homologue of CFTR was recently cloned and sequenced (29). We designed oligonucleotide primers based on this sequence and used them to amplify a predicted 762-base pair product from reverse transcribed A6 epithelia mRNA by PCR (Fig. 1). The PCR product was subcloned into TA vector, and partial sequence analysis (201 nucleotide residues) showed ~97% nucleotide identity and 100% amino acid identity with the previously published X. laevis CFTR cDNA and deduced amino acid sequences, confirming that the amplified product was CFTR and providing evidence that CFTR is expressed in A6 epithelia.

The carboxyl-terminal 4-amino acid residue tract of Xenopus CFTR is identical to that of human CFTR (1, 29) and is the primary epitope recognized by a previously characterized monoclonal anti-human CFTR antibody (30). This anti-CFTR antibody was used to further examine CFTR expression in A6 epithelia, by immunolocalization and by immunoprecipitation. CFTR was localized in methanol-fixed A6 epithelia by immunofluorescence microscopy. CFTR was detected on the apical plasma membrane and intracellularly (Fig. 2). CFTR was also immunoprecipitated from A6 epithelia with the anti-CFTR antibody. The specificity of the immunoprecipitate was demonstrated in a parallel experiment in which the immunoprecipitation was performed in the presence of an excess of the 23-mer peptide corresponding to the carboxyl terminus of Xenopus CFTR (but lacking the COOH-terminal leucine). The immunoprecipitate was subjected to SDS-PAGE, transferred to nitrocellulose, and was then probed with the anti-CFTR antibody (Fig. 3). A ~130–170-kDa polypeptide was specifically immunoprecipitated with the anti-CFTR antibody, in reasonable agreement with the size of mammalian CFTR (4, 39).

Several groups have used CFTR antisense oligonucleotides to deplete CFTR mRNA and to inhibit cAMP-activated Cl⁻ secretion in both epithelial and non-epithelial cells (40–43). We employed a 25-mer phosphorothioate antisense oligonucleotide, complementary to the 5’ end of the open reading frame of Xenopus CFTR, to inhibit expression of CFTR in A6 cells. This region has been successfully used by other investigators to deplete CFTR mRNA (40). Parallel studies utilized the corresponding sense oligonucleotide as a control. To determine whether treatment of A6 cells with CFTR antisense oligonucleotide led to an inhibition of CFTR expression, CFTR was immunoprecipitated from antisense and sense oligonucleotide-treated A6 cells with the anti-CFTR antibody. CFTR protein expression in the antisense-treated A6 cells was markedly diminished, when compared with control cells treated with the corresponding sense oligonucleotide (Fig. 4).
in CFTR protein expression. 

Inhibition of CFTR Expression Is Associated with Activation of Na⁺ Channels—Under basal conditions (i.e., no forskolin), we did not observe a significant change in amiloride-sensitive $I_{sc}$ in cells treated with the CFTR antisense oligonucleotide when compared with control cells treated with the sense CFTR oligonucleotide (Fig. 5). However, a leftward shift in the single Na⁺ channel current-voltage relationship derived from antisense-treated cells compared to sense-treated cells was consistent with a reduction in apical Cl⁻ reabsorption (Table II). Oligonucleotides did not affect single Na⁺ channel conductance (data not shown). CFTR antisense treatment increased the open probability of amiloride-sensitive 4-pS Na⁺ channels in cell-attached patches by 39%, from 0.41 in cells expressing CFTR to 0.57 in cells not expressing CFTR (Table II and Fig. 6; $p < 0.005$, antisense versus sense oligonucleotide-treated cells).

However, the number of Na⁺ channels observed per individual patch did not significantly differ between the two groups (Table II). Forskolin activates Na⁺ transport in A6 epithelia. We found that forskolin-stimulated, amiloride-sensitive $I_{sc}$ was significantly greater in A6 cells treated with CFTR antisense oligonucleotide (11.8 ± 0.6 μA/cm²) when compared with cells treated with CFTR sense oligonucleotide (9.7 ± 0.7 μA/cm²) ($p < 0.03$, n = 14). We have previously observed that treatment of A6 epithelia with CAMP-stimulating agonists (e.g., arginine vasopressin, arginine vasotocin, or forskolin) led to an increase in the number of Na⁺ channels at the apical cell surface, as demonstrated by immunochemical studies or by direct patch clamp recordings (38, 44). Consistent with our previous studies, forskolin-induced stimulation of Na⁺ transport in A6 epithelia was associated with an increase in the number of Na⁺ channels observed per individual cell-attached patch (Table II and Fig. 6). However, the increase in the number of Na⁺ channels per patch was similar in control cells treated with sense CFTR oligonucleotide (3.1-fold increase) and in cells treated with the antisense CFTR oligonucleotide (2.8-fold increase).

Again, at a single-channel level our observed change in macroscopic amiloride-sensitive $I_{sc}$ was due to a significant 97% increase in Na⁺ channel $P_{o}$ (0.77 ± 0.06) in cells not expressing CFTR, when compared with control cells (0.39 ± 0.06) ($p < 0.0001$; antisense versus sense oligonucleotide-treated cells) (Table II and Fig. 6).

**DISCUSSION**

Epithelial Na⁺ channels mediate the reabsorption of Na⁺ across epithelia lining airway and alveoli, distal nephron, and distal colon (20, 45). Regulated Na⁺ reabsorption has a major role in neonatal lung maturation, extracellular fluid volume homeostasis, and control of blood pressure. Although mechanisms of epithelial Na⁺ reabsorption and its regulatory control have been extensively studied using toad urinary bladder and frog skin, established epithelial cell lines such as A6 serve as convenient models for the study of Na⁺ transport across the mammalian distal nephron and as potentially useful models to study Na⁺ transport across airway and colonic epithelia (20).

Epithelial Na⁺ channels are regulated by a number of different hormones or second messengers, such as aldosterone, vasopressin, atrial natriuretic peptide, bradykinin, insulin, endothelin, prostaglandins, α- and β-adrenergic agonists, adenose, cAMP, cGMP, G proteins, and protein kinase C (20, 21, 23, 25, 46–48). In airway epithelia, cAMP-stimulating agonists do
not lead to the activation of Na⁺ channels; rather, transepithelial Na⁺ transport is inhibited. However, airway epithelium expressing a CF phenotype respond to these agonists with an increase in transepithelial Na⁺ transport. This finding has been recapitulated in stable transfectants of Madin-Darby canine kidney cells co-expressing α, β-, and γENaC (rat ENaC) with or without CFTR. In contrast, we and others have shown that A6 epithelium respond to arginine vasopressin, forskolin, or cell-permeable cAMP analogs with an activation of amiloride-sensitive transepithelial Na⁺ transport and amiloride-sensitive Na⁺ channels (20, 31, 38, 44). Our previous studies suggest that this activation of Na⁺ channels is due to an increase in the number of epithelial Na⁺ channels expressed at the apical plasma membrane, and likely represents recruitment of Na⁺ channels from an intracellular pool to the apical membrane, although Benos and co-workers have published data suggesting that protein kinase A directly regulates a Na⁺ channel purified from bovine kidney (49).

We have previously characterized a forskolin-activated, 8-pS, non-rectifying, DIDS-insensitive, and NPPB-sensitive Cl⁻ channel in A6 epithelia (23, 27, 28). These functional characteristics are consistent with that of CFTR. In the present study, we have provided several additional lines of evidence to support CFTR expression in A6 cells, including: 1) reverse transcriptase-PCR amplification, cloning, and sequence analysis of a partial cDNA sequence of A6 cell CFTR (Fig. 1); 2) immunoprecipitation of CFTR from A6 epithelia (Fig. 3); and 3) immunolocalization of CFTR in A6 epithelia (Fig. 2). In addition, treatment of A6 epithelia with a specific CFTR antisense oligonucleotide led to inhibition of CFTR expression, as demonstrated by: 1) a decrease in CFTR protein expression (Fig. 4), 2) a decrease in forskolin-activated, amiloride-insensitive Iₛ (Fig. 5), and 3) a decrease in the frequency of forskolin-activated 8-pS Cl⁻ channels observed in the apical membrane (Table I).

Expression of the CF phenotype in CFTR antisense oligonucleotide-treated A6 epithelia was associated with an activation of Na⁺ channels. Although no difference in amiloride-sensitive Iₛ was observed between control A6 cells and cells expressing the CF phenotype in the absence of forskolin treatment (Fig. 5), a significant 1.4-fold increase in Na⁺ channel open probability was observed in cells expressing the CF phenotype, when compared to controls (Table II, Fig. 6). Following stimulation with forskolin, cells expressing the CF phenotype had a significant 1.3-fold increase in amiloride-sensitive Iₛ and a 2.0-fold increase in Na⁺ channel open probability, when compared to controls (Table II, Fig. 6). Although the magnitude of the increase in Na⁺ channel Pₒ observed in A6 cells expressing the CF phenotype, when compared with controls, is clearly greater than the magnitude of the increase in amiloride-sensitive Iₛ, a measure of transepithelial Na⁺ transport, the direction of change in these parameters associated with the CF phenotype was the same. The observed differences between the fold increase in Na⁺ channel Pₒ and Iₛ in A6 cells expressing the CF phenotype may reflect feedback inhibition of Iₛ associated with activation of Na⁺ transport, or reflect measurement of Na⁺ transport under closed circuit conditions versus open circuit conditions. We have previously shown that forskolin pretreatment increases the number of Na⁺ channels detected in apical membrane cell-attached patches (20, 31, 38, 44). Expression of the CF phenotype in A6 epithelia did not affect this increase in Na⁺ channel density in response to forskolin (Table II, Fig. 6). In addition, the single-channel conductance of amiloride-sensitive Na⁺ channels was not affected in the A6 epithelial CF phenotype (data not shown).

In summary, the A6 epithelial cell line provides a model system for examining potential mechanisms by which epithelial Na⁺ channels are affected in CF. Together with the recent work of Stutts and co-workers (17), our data suggest that the activation of Na⁺ channels associated with the expression of the CF phenotype is not limited to airway epithelial cells. At present, the cellular mechanisms that alter Na⁺ channel open

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**Table I**

| Cells                                      | Pretreatment with 10 μM forskolin (30 min) | Channel activity observed in cell-attached patches | Number of channels per individual patch | Channel open probability |
|--------------------------------------------|-------------------------------------------|---------------------------------------------------|----------------------------------------|--------------------------|
| A6 cells treated with CFTR sense oligonucleotides | No                                        | 1/21 (5%)                                          | 0.67 ± 0.00                            | 0.0 ± 0.00               |
| A6 cells treated with CFTR antisense oligonucleotides | Yes                                       | 5/19 (26%)                                         | 0.87 ± 0.47                            | 0.72 ± 0.07              |

N = number of channels per individual patch; Pₒ = channel open probability.
probability have not been defined. In addition, it is unclear whether epithelial Na⁺ channels and CFTR are associated together in the apical membrane, allowing for direct interactions. However, our preliminary data in A6 epithelia suggest that CFTR and Na⁺ channels may interact with common cytoskeletal proteins. Recent work by Schwiebert and co-workers (8) has suggested that ATP, secreted from airway epithelia by the apical Na⁺ channel activity recorded from amiloride-sensitive, 4-pS Na⁺ channels may occur in A6 epithelia, as shown by glutamate- and pipette solution contained (in mM, pH = 7.4): 100 NaCl, 3.4 KCl, 1.0 CaCl₂, 1.0 MgCl₂, and 10 HEPES. Data were originally recorded at 1 kHz, sampled at 2 ms/point, and software filtered (8). The data mean ± SD.

### Table II

| Cells                          | Pretreatment with 10 μM forskolin (30 min) | Channel activity observed in cell-attached patches | NP₀ᵇ | Number of channels per individual patch | Channel open probability | Reversal potential |
|-------------------------------|-------------------------------------------|--------------------------------------------------|------|----------------------------------------|-------------------------|-------------------|
| A6 cells treated with CFTR sense oligonucleotides | No                                        | 6 /19 (32%)                                      | 0.90 ± 0.41 | 2.2 ± 0.8 | 0.41 ± 0.08 | +65.0 ± 1.8³  |
| A6 cells treated with CFTR antisense oligonucleotides | No                                        | 7 /25 (28%)                                      | 1.60 ± 0.60 | 2.6 ± 0.8 | 0.57 ± 0.07 | +55.4 ± 4.3²  |
|                               | Yes                                       | 8 /22 (36%)                                      | 5.35 ± 1.17 | 7.2 ± 1.8 | 0.77 ± 0.06 | +45.6 ± 4.5²  |

ᵃ NP₀, number of channels per individual patch times channel open probability (mean ± SD).
ᵇ p < 0.005, controlling pre-treatment (sense) vs. control (antisense).
ᶜ p < 0.002, comparing forskolin (sense) vs. forskolin (antisense).
ᵈ p < 0.0001, comparing forskolin (sense) vs. forskolin (antisense).

### Figure 6: CFTR antisense oligonucleotides increase the open probability of amiloride-sensitive, 4-pS Na⁺ channels.

A6 cells were incubated with CFTR sense (left) or with CFTR antisense oligonucleotides (right) prior to patching. Representative single-channel traces show Na⁺ channel activity from 4-pS amiloride-sensitive, 4-pS Na⁺ channels. Excised cell-attached patches were superfused with a solution containing (in mM, pH = 7.4): 100 NaCl, 3.4 KCl, 1.0 CaCl₂, 1.0 MgCl₂, and 10 HEPES. Data were originally recorded at 1 kHz, sampled at 2 ms/point, and software filtered at 100 Hz. Inward current (Na⁺ reabsorption) is represented by downward channel transitions.

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