J. Membrane Biol. 148, 79–82 (1995)

The Journal of
Membrane
Biology

Correlation of Open Cell-attached and Excised Patch Clamp Techniques

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Received: 6 February 1995/Revised: 3 July 1995

Abstract. The excised patch clamp configuration provides a unique technique for some types of single channel analyses, but maintenance of stable, long-lasting preparations may be confounded by rundown and/or rapid loss of seal. Studies were performed on the amiloride-sensitive Na^+ channel, located on the apical surface of A_6 cells, to determine whether the nystatin-induced open cell-attached patch could serve as an alternative configuration.

Compared to excised inside-out patches, stable preparations were achieved more readily with the open cell-attached patch (9% *vs.* 56% of attempts). In both preparations, the current voltage (*I-V*) relation was linear, current amplitudes were equal at opposite equivalent clamped voltages, and E_{rev} was zero in symmetrical Na^+ solutions, indicating similar Na^+ activities on the cytosolic and external surfaces of the patch. Moreover, there was no evidence that nystatin altered channel activity in the patch because slope conductance (3–4pS) and E_{rev} (75 mV), when the bath was perfused with a high K:low Na solution (E_{Na} = 80 mV), were nearly equal in both patch configurations.

Our results therefore indicate that the nystatin-induced open cell-attached patch can serve as an alternative approach to the excised inside-out patch when experiments require modulation of univalent ions in the cytosol.

Key words: A_6 cells — Epithelial Na^+ channels — Open cell-attached patches — Nystatin

Introduction

The study of some types of channels using the conventional excised patch technique is complicated by rapid "rundown" of the channel activity and/or loss of the seal (Horn & Marty, 1988). Rundown may be due to loss or alteration of cytosolic components critical to the regulation of ion channel activity (Horn & Marty, 1988: Levitan & Kramer, 1990). An earlier report that open-cell attached patches, obtained on detergent-permeabilized cells, were effective in the analysis of ATP-sensitive K^+ channels (Dunne et al., 1986), prompted us to determine whether this patch configuration, induced by nystatin in the bath solution, could be used to analyze the characteristics of selective Na^+ channels on the apical membrane of cultured A_6 epithelial cells. Because nystatin has been shown to induce small univalent selective pores in plasma membrane (Cass & Dalmark, 1973; Lewis et al., 1977), it seemed likely that an alteration in larger cytosolic components would be minimized, and that open cell-attached patches formed under this condition might serve as an alternative to the excised inside-out patch configuration.

Material and Methods

Cell Culture

Studies were performed on A_6 cells, a cell line derived from the kidney of *Xenopus laevis,* grown on rat tail collagen (Type 1, Collaborative Biomedical Products, Bedford, MA) coated Millipore CM permeable membranes (Millipore Products, Bedford, MA). Standard methods were used for maintaining the A_6 cells in culture (Petzel et al., 1992). Cells were treated continuously with aldosterone (1.5 μM) until experiments were performed 10–16 days after seeding when monolayers achieved maximal resistance.

Solutions

The pipette solution contained (mM): 120 NaMethanesulfonate, 3.5 KCl, 0.5 $CaCl_2$, 1 $MgCl_2$, 10 N-2-hydroxyethylpiperizine-N′-2-ethanesulfonic acid (HEPES). The bath solution contained either (mM): 120 NaMethanesulfonate and 5 KCl or 120 KMethanesulfonate and 5 NaCl;

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both with 10 HEPES. The bath solutions were Ca^{2+}- and Mg^{2+}-free and supplemented with 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) in studies involving excised and open cell-attached patches. To reduce cell swelling in open cell-attached patch experiments, due to the Donnan effect across the permeabilized membrane, sucrose (10 mM) was added to the bath solution. All solutions had a pH of 7.4 and an osmolarity of 210 and 230 mOsmol/kg H_2O.

Patch Clamp Techniques

Patch pipettes were fabricated from borosilicate glass capillary tubing (World Precision Instruments, Sarasota, FL) with a Narishige PP-83 pipette puller (Narishige, Tokyo, Japan). Fire-polished and Sylgard-coated pipettes with a resistance of 12–16 MΩ were employed. Single channel currents were recorded with a List EPC-7 patch clamp amplifier, converted to a video signal with a pulse code modulator (Sony PCM 50IES, AR Vetter, Rebersburg, PA) and were stored on magnetic tape (Beta HGL-750, Sony). The recorded data were played back through an 8-pole Bessel low-pass filter (Frequency Devices, Haverhill, MA) at 100 Hz. Data acquisition and analysis were performed with C-Clamp and C-Crunch programs, respectively (Indec Systems, Capetola, CA) on an IBM AT computer.

Nystatin Permeabilized Cells

After establishing a cell-attached recording, an open cell-attached patch was produced by replacement of the initial bath solution with a solution containing nystatin. An aliquot of nystatin stock solution, 50 mg/ml in dimethyl sulphoxide (DMSO) maintained at −20°C, was added to bathing solution to achieve a concentration of 200 μg/ml and then sonicated. The solution was made fresh before each experiment.

The bath solutions were connected to ground. Currents were defined as negative inward when the direction of flow of positive charges was from pipette into the cell. Recordings were obtained at room temperature (21–23°C). Reagents were purchased from Sigma Chemical (St. Louis, MO) and Aldrich Chemical (Milwaukee, WI) unless specified. Values are given as means ± SEM.

Results

We observed Na^+ channels in the apical membrane of confluent A_6 cells, grown on permeable supports, that exhibited the same features as previously reported (Ohara, Matsunaga & Eaton, 1993). Fig. 1*A* shows a typical current record from a cell-attached patch with long opening and closing times. Downward current deflections were observed in the absence of an applied potential and at negative holding potentials, but not at a positive potential of 120 mV. The conductance was 3.6 ± 0.8 pS (n = 25). Current was inhibited by amiloride (0.5–1 μM) added to the pipette solution (*data not shown*).

Successful excised inside-out recordings were obtained in six of 82 attempts (9%). Fig. 1*B* shows current traces from an excised inside-out configuration with KMethanesulfonate (KMeth) in the bath. This tracing resembles the cell-attached recording (Fig. 1*A*). The *I-V*

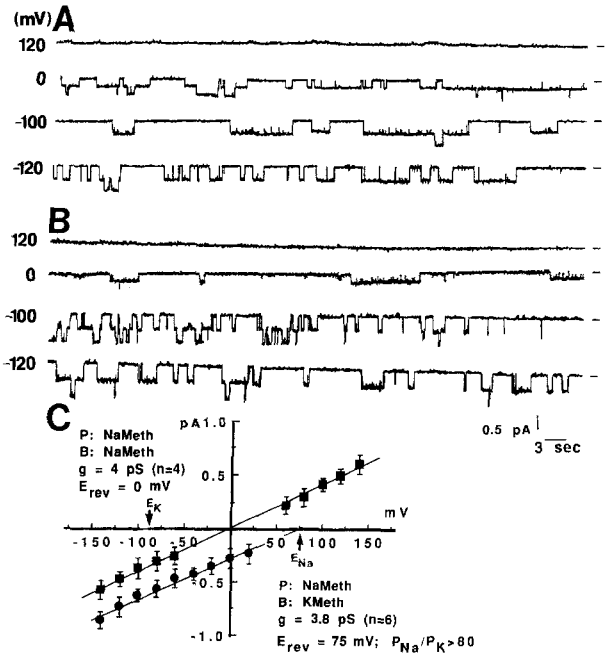

Fig. 1. Single-current traces and current-voltage (*I-V*) relations from cell-attached and excised patches. (*A*) Current traces from cell-attached patch with NaMethanesulfonate (NaMeth) in pipette and bath. Voltages to the left of traces are applied voltages. Bars on right indicate the closed state. Downward current deflections correspond to movement of positive charge from pipette to cell. (*B*) Single-channel current traces from an excised inside-out patch with NaMeth in pipette and KMethanesulfonate (KMeth) in bath. (*C*) The mean current-voltage relations for an excised inside-out patch with symmetrical Na^+ solutions (squares) or asymmetrical Na^+ solutions (circles). Plotted data are means ± SD. The equilibrium potential for K^+ (E_K) and Na^+ (E_{Na}) are indicated by arrows.

curves shown in Fig. 1*C* indicate a linear relation with an E_{rev} close to 0 mV when the excised patch was exposed to symmetrical Na^+ solutions; mean unitary-channel conductance was 4.0 ± 0.1 pS (n = 4). In contrast, when the bath solution contained high K^+ the corresponding E_{rev} was shifted to approximately 75 mV, consistent with an estimated P_{Na}:P_K > 80:1; mean conductance was 3.8 ± 1.1 pS (n = 6).

The open cell-attached patch configuration was formed after a successful cell-attached seal was obtained and features typical of the Na^+ channel were identified. The success rate of achieving long-lasting channel recordings after addition of nystatin was 56 percent (25 of 45 attempts). Addition of nystatin resulted in a baseline drift within 30 sec, presumably due to changes in the cell potential, shown by the traces in Fig. 2*A* and *B*. In symmetrical Na^+ solutions (Fig. 2*A*), outward Na^+ current was observed as Na^+ in the bath solution exchanged rapidly with the cell interior. After equilibration was established (1 to 2 min) the tracings show that current jumps were similar for equivalent positive and negative volt-

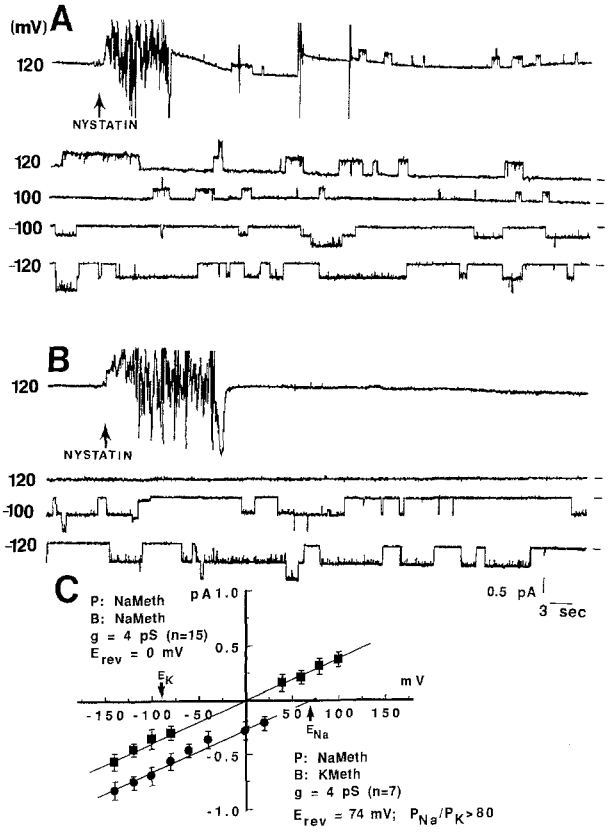

Fig. 2. Single-channel current traces and *I-V* relations from open cell-attached patches. (*A*) Current traces from an open cell-attached patch with NaMeth in pipette and bath. The time of addition of nystatin is indicated by arrows. Note the baseline drift presumably due to change in cell potential. The large spikes are apparently due to artifacts caused by nystatin incorporation into the membrane. (*B*) Current traces obtained from the same open cell-attached patch with NaMeth in pipette and KMeth in bath. (*C*) *I-V* relations from open cell-attached patches exposed to symmetrical Na^+ solutions (squares) or asymmetrical Na^+ solutions (triangles). Plotted data are means ± SD. The solid lines indicate the slope conductance.

ages. Fig. 2*B* illustrates current deflections when KMeth was in the bath. Under this condition, outward deflections were not observed.

The *I-V* relations obtained in open cell-attached patches (Fig. 2*C*) resembled the curves obtained with excised inside-out patches (Fig. 1*C*). When the pipette and bath solutions contained identical concentrations of Na^+ (NaMeth) the *I-V* relation was linear and E_{rev} was close to 0 mV. Substitution of bath solution with KMeth resulted in a shift of E_{rev} to approximately 74 mV, consistent with a P_{Na}:P_K > 80. Conductances and the apparent channel open and close times were similar to these observed in cell-attached and excised patches.

To determine whether channels were induced in the patch by application of nystatin to the bath solution, cells were exposed to nystatin in 6 experiments when channels were not initially observed in the patch (*data not shown*). Exposure to nystatin did not induce channel-like activity in the patch, consistent with the notion that nystatin did not enter the cell.

Discussion

The general characteristics of Na^+ channels in the apical membrane of A_6 cells, recorded in cell-attached and excised patches, confirm results previously reported when cells were grown on permeable supports (Ohara, Matsunaga & Eaton, 1993). Under this condition, channels exhibit low conductance, long opening and closing times, and a high selectivity for Na^+. In these experiments designed to evaluate the usefulness of open cell-attached patches, we sought to determine: (i) whether the intracellular ion concentration was predictable from the composition of bath solution, (ii) whether the properties of channels in the patch were affected by nystatin in the bath solution, and (iii) whether rapid rundown could be reduced to yield a higher fraction of long-lasting channels compared to excised-patches.

Our results indicate that in nystatin-induced open cell-attached patches the concentration of univalent cations in the cell would be predicted from the bath concentration. With identical Na^+ concentrations in pipette and bath solutions, the I-V plot was linear and the inward and outward current amplitudes were indistinguishable at clamped potentials of opposite polarities, as demonstrated in excised patches. In addition, E_{rev} values in both open cell-attached and excised patches were approximately zero, as expected in the absence of a diffusion potential created by dissimilar Na^+ concentrations across the patch. Although the cell membrane potential was not directly measured in this study, previous reports indicate that the resting membrane potential in nystatin-treated cells is reduced to zero or near zero after nystatin treatment (Lewis et al., 1977; Russell & Eaton, 1993).

The polyene antibiotic, nystatin, has been shown to induce large increases in the permeability of lipid bilayers for univalent ions, including Na^+, K^+, Li^+ and H^+, and for uncharged substances whose Stokes-Einstein radii do not exceed 4*A* (Holz & Finkelstein, 1970). When added to one side of cell membranes the P_{Cl}/P_K is approximately 0.15 and divalent cations are reported to be nonpermeant (Russell, 1993). Nystatin, and amphotericin B which induces similar effects, have been used to control the intracellular concentrations of Na^+ and K^+ in order to study transport processes in cell membranes, as well as the electrical resistance of cell membranes and the paracellular pathway in epithelial cells (Lewis et al., 1977). Prior to the present study, however, nystatin had not been shown to equalize the activities of Na^+ or K^+ across the permeabilized cell membrane, although this effect was

demonstrated in one study on *Necturus* gallbladder epithelial cells by amphotericin B (Graf & Giebisch, 1979). The present study shows that nystatin rapidly equalized the activity of Na^+ and K^+ across the cell membrane, permitting precise alterations in ion activity on the cytosal side of channels isolated by the patch pipette.

These experiments also indicate that the continued presence of nystatin in the bath had no effect on basic properties of the Na^+ channel in the patch because conductance and relative ion selectivity for univalent cations were not different in open cell-attached patches compared to excised inside-out patches. Although a formal analysis of channel kinetics was not performed in these experiments, comparison of tracings of channel activity in cell-attached and open cell-attached configurations suggest that Na^+ channel kinetics were also not affected by nystatin in the bath solution. These results confirm therefore, that nystatin did not enter the cells.

The open cell-attached patch, induced by detergents, was initially described by Dunne and associates in studies designed to analyze ATP-sensitive K^+ channels (Dunne et al., 1986; Harding et al., 1994). In those experiments, the cell membrane was permeabilized by brief exposure to detergents, including digitonin and saponin. In contrast to excised inside-out patches, rundown of channel activity was markedly reduced in open cell-attached patches. Since the physical properties of K channels were not examined in detail, it is not known whether cells permeabilized by detergents are comparable to nystatin-treated cells as experimental models for single-cell analysis.

In sum, this study shows that the nystatin-induced open cell-attached patch configuration was comparable to excised patches in the study of ion selectivity of apical membrane Na^+ channels. In addition, this experimental approach offered a substantial advantage over excised patches in our ability to obtain long-lasting and stable preparations for single channel analysis.

We thank Dr. Olaf S. Andersen for his suggestions in the development of the open cell-attached recording technique. This work was supported by a National Institutes of Health grant (DK-18061)

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