J. Membrane Biol. 106, 157–172 (1988)

The Journal of
Membrane Biology

Single-Channel Recordings from the Apical Membrane of the Toad Urinary Bladder Epithelial Cell

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Summary. The patch-clamp technique for the recording of single-channel currents was used to investigate the activity of ion channels in the intact epithelium of the toad urinary bladder. High resistance seals were obtained from the apical membrane of tightly stretched tissue. Single-channel recordings revealed the activity of a variety of ion channels that could be classified in 4 groups according to their mean ion conductances, ranging from 5 to 59 pS. In particular, we observed highly selective, amiloride-sensitive Na channels with a mean conductance of 4.8 pS, channels with a similar conductance that were not Na-selective and channels with mean conductance values of 17–58 pS that were mostly seen after stimulation of the tissue with vasopressin or cAMP. When inside-out patches from the apical membrane were exposed to 110 mM fluoride, large conductances (86–490 pS) appeared.

Key Words toad bladder · epithelial ion channels · amiloride · vasopressin · channel phosphorylation

Introduction

The toad urinary bladder has long been studied as a model for tight Na-transporting epithelia (Macknight, Dibona & Leaf, 1980). Most investigators describe this epithelium in terms of two serial membranes (the Koefoed-Johnson-Ussing model). Sodium enters the cell from the urine along its electrochemical potential gradient through Na-selective, amiloride-sensitive ion channels in the apical membrane. An energy-dependent, ouabain-inhibitable Na/K-pump in the basolateral membrane transports cellular Na into the interstitial fluid in exchange for extracellular K (Koefoed-Johnson & Ussing, 1958). In addition, there is a paracellular or shunt conductance with poor ion selectivity in parallel with the transcellular conductance.

A vast amount of information about the toad bladder, acquired by means of a variety of physiological techniques, is consistent with the Koefoed-Johnson-Ussing model. However, a number of findings indicate a greater complexity. For example, Navarte and Finn (1980), from microelectrode studies, have argued for an apical Cl conductance, Palmer (1986) has reported a voltage-dependent apical K conductance and VanDriessche and coworkers, using noise analysis, have described nonselective cation channels and K channels in the apical membrane (Erlij, Schoen & VanDriessche, 1986; VanDriessche, Aelvoet & Erlij, 1987; Aelvoet, Erlij & VanDriessche, 1988).

The patch-clamp technique for the recording of single-channel currents allows the investigation of ion channels in small patches of plasma membrane under controlled conditions (Hamill et al., 1981; Sakmann & Neher, 1983). Current recordings from ion channels in excised patches from the apical membrane permit the investigation of the effects of regulatory agents on both the mucosal and cellular side of the channel. Here we report a method for performing patch-clamp experiments with the toad bladder and initial findings from single-channel recordings from the apical membrane, in which we identified:

–highly selective Na channels with a mean conductance of 4.8 pS that were blocked by amiloride and were sometimes activated by protein kinase A;

–channels with similar conductance and gating kinetics that were neither selective for Na nor amiloride sensitive;

–three groups of channels with mean conductances of 17, 30 and 58 pS that appeared after stimulation of the tissue with vasopressin or cAMP;

–large conductances, in the range of 90 to 490 pS, that were only seen in the inside-out configuration with 110 mM NaF or KF in the bath medium.

Materials and Methods

Animals and Preparation of Tissue

Female Dominican toads (*Bufo marinus*) were kept in a dark room at 16–20°C on sawdust with access to a basin with tapwater and were force fed once a month with minced liver. For experiments, the animals were sacrificed by cutting between two cervi-

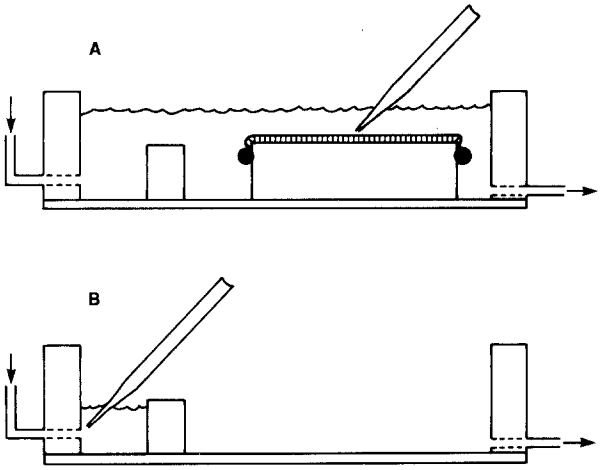

Fig. 1. The experimental chamber. In (*A*) the apical membrane of the stretched tissue, that is mounted on a ring, faces upwards. (*B*) shows the recording configuration for inside-out experiments, with the ring removed from the chamber

cal vertebrae and double pithing. This was done immediately after removing the animals from their containers in order to prevent them from emptying their bladders. During the dissection, care was taken that the bladder remained full, leaving the smooth muscle as relaxed as possible. We found the distended state of the bladder in the living animal to be a prerequisite for sealing pipettes to the apical membrane. Although it was possible to distend bladders both *in situ* and in vitro by filling them with Na-Ringer under hydrostatic pressure at 4°C, we were not able to seal pipettes to such tissues. Thus, empty bladders were of no use for patch-clamp experiments on the apical membrane.

For the preparation of bladder tissue, the peritoneal cavity was opened and the two hemibladders were flushed clear of other tissue with ice-cold Na-Ringer. Both hemibladders were then closed with ligatures that were placed close to their junction. The full hemibladders could then be removed and were transferred into beakers with ice-cold Na-Ringer where they were left for 10 to 20 minutes in order to cool down the urine. After that, one hemibladder was opened, the urine was discarded and the tissue was pinned out on a precooled silicone dish containing ice-cold Na-Ringer, the mucosal side facing upwards. It was important to stretch the tissue evenly in all directions to immobilize it. Four to 8 acetal rings with a diameter of 15 mm were carefully pushed under the tissue and fitting O-rings were then put over tissue and ring from above, so that about 1.8 cm^2 of tightly stretched tissue was mounted on each ring. The rings were cut out of the bladder tissue and transferred into a storage container where they were kept at room temperature (17–23°C) in aerated Na-Ringer until use. They were stored in a vertical position to allow free access of fresh solution to both surfaces of the tissue. The container was designed so that solutions of different compositions could bathe the two surfaces of the tissues when required.

Experimental Chamber and Microscopy

For patch-clamp experiments, we used a perspex chamber with a total volume of 6 ml mounted on a standard microscope slide. The chamber was connected to a perfusion system and contained a partition that made it possible to reduce the volume of the solution to 0.5 ml. For cell-attached recordings, a ring with bladder tissue was placed into the chamber, which was filled with Na-Ringer. Thus, the apical membrane could be reached with the patch pipette (Fig. 1*A*). For inside-out experiments, the patch was first excised, and the chamber was slowly moved until the pipette was in the small compartment. Then the pipette tip was positioned in front of the perfusion inlet and, finally, the chamber was drained leaving the patch in a volume of 0.5 ml and in direct contact with the fresh solution from the perfusion system (Fig. 1*B*). All experiments were conducted at room temperature. The chamber was mounted on the stage of a Zeiss inverted microscope. We used bright-field microscopy at a magnification of 100× to examine the tissue and to position the pipette. This optical arrangement did not enable us to recognize cell borders or the shape of the apical surface. We could, however, identify blood vessels, smooth muscle strands and, most importantly, the network of connective tissue fibers. As explained below, the density of this collagen-network indicates the suitability of a piece of tissue for patch-clamp experiments.

Patch Pipettes and Electrical Recording

Patch pipettes were made from Blu-Tip Micro-Hematocrit capillary tubes (Monoject Scientific, St. Louis, MO) with an inner diameter of 1.1 mm and a wall thickness of 0.2 mm. Before the pipettes were made, the tubes were washed with water, methanol, acetone and again with water and were then kept in chromic acid for about 15 hr. They were then thoroughly rinsed with distilled water and dried at 110°C.

Patch pipettes were pulled in two steps with a horizontal puller that was designed in our laboratory. When filled with Ringer solution, the pipettes had a resistance of 8–10 MΩ, which increased to 10–20 MΩ during fire polishing. Pipettes were coated with Sylgard 184 (Dow Corning, Midland, MI) and were stored in a vacuum desiccator under reduced pressure. Pipettes could be used up to 35 hr later. To record single-channel currents, the solution in the pipette was connected to the headstage of a List EPC-5 patch-clamp amplifier (List-Electronic, Darmstadt, FRG) by an Ag/AgCl-wire. The reference electrode was also an Ag/AgCl-wire that was in contact with the bath solution through a 150 mM KCl-agar-bridge. Signals were low-pass filtered at 3 or 1 kHz and stored on tape either in analog form (Electrodata FM tape-recorder, Mascot, N.S.W., Australia) or in digital form (on videotape after pulse code modulation with a Sony PCM-501 ES modified to pass zero frequency).

Polarity of Signals

In the figures provided, positive current values indicate a cation flux out of, or an anion flux into, the pipette. The potentials are given as the potential of the pipette (V_p) with reference to the ground (bath solution). The total potential over the patched membrane (V_m) is composed of V_p and the cell potential (V_c) and is conventionally given for the cytoplasmic side with reference to the bath solution. Thus, for cell-attached patches $V_m = V_c - V_p$ and for inside-out patches $V_m = -V_p$. Since the toad bladder cells in our experiments are virtually short circuited, a value for V_c of −60 mV can be used (Donaldson, 1986).

Data Processing

The stored signals were played through a low-pass six-pole Bessel filter with variable cut-off frequency and were sampled with an Apple II microcomputer. The mean single-channel current, i,

Table 1. List of solutions used in this study[a]

Name	Na	K	Cl	Ca	Mg	pH	pH buffer	Other
Na-Ringer	115	3	119	1	1	7.4	Phosphate	5 glucose
NaP-sol.	100	20	124	1	—	7.4	5 HEPES/5 Tris	5 bretylium
KB-sol.	20	100	122	—	1	7.2	5 HEPES/5 Tris	1 EGTA
NMDG-sol.	20	—	122	—	1	7.2	5 HEPES/5 Tris	100 NMDG, 1 EGTA
NaFB-sol.	100	4	—	—	—	7.2	10 HEPES/4 KOH	110 fluoride
KFB-sol.	10	104	—	—	—	7.2	10 HEPES/4 KOH	110 fluoride

[a] All concentrations are given in mmol/liter.

was derived from amplitude histograms, which were constructed by digitizing (usually at 2 kHz) selected parts of the recorded signals, and allocating each sampled value to one of 256 bins. The open probability of single channels, P_o, was obtained as $A_o/(A_o + A_c)$ where A_o and A_c are the areas under the open peak and the closed peak, respectively, of the amplitude histogram.

Open and closed times were obtained from recorded signals from single-channel patches. A selected segment was digitized at 0.5–5.5 kHz and up to 32 K samples were stored in computer memory and displayed on a video graphics screen. A window discriminator (Kits et al., 1987) was applied in software: upper and lower threshold cursor lines were overlaid on the display, the placing being adjusted manually to approximately one-third and two-thirds of a single-channel current. The stored trace was then analyzed sequentially to produce a list of times between transitions, *i.e.*, an upward crossing of the upper threshold line preceded by a downward crossing of the lower threshold line, or vice versa. A partial solution to the problem of baseline drift was to allow the threshold lines jointly to be tilted under operator control, thus compensating for drift that was a linear function of time.

A modification of this method allowed recordings of unlimited length to be analyzed on line. Incoming digitized sample values were stored in a circular buffer occupying 26 K of computer memory. The contents of the buffer, together with interactively adjustable upper and lower threshold cursors, were displayed on a video graphics screen. The display was updated every 725 μsec following the acquisition of a sample. The buffer allowed a delay of about 10 sec between the display of a new sample value and its analysis by the window discriminator. Changes in baseline could thus be anticipated by appropriate manual adjustment of the threshold cursors.

Channel kinetics were determined by fitting probability distribution functions with 1 or 2 time constants to the unbinned open and closed times. The maximum-likelihood procedure corrected for sample promotion error and missed events of short duration (McManus, Blatz & Magleby, 1987). If two time constants were necessary for a fit, a mean dwell time (τ_m) was calculated as $(\tau_f A_f + \tau_s A_s)/(A_f + A_s)$ where A_f and A_s are the areas corresponding to fast and slow components of the probability distribution functions.

Materials

The following reagents were obtained from Sigma: lysine-vasopressin; adenosine-3-5-cyclic monophosphoric acid, Na-salt (cAMP); N^6-2-0-dibutyryladenosine 3-5-cyclic monophosphate, Na-salt (dbcAMP); cAMP dependent protein kinase from beef heart, (protein kinase A); adenosine 5-triphosphate (ATP); 3-isobutyol-1-methyl-xanthine (IBMX); ethyleneglycol-bis-(β-amino-ethylether) N,N-tetra-acetic acid (EGTA); N-acetyl-L-cysteine; DL-dithiothreitol (DTT); N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES); N-methyl-D-glucamine (NMDG); crude collagenase and hyaluronidase. Tris(hydroxymethyl)-methylamine (Tris) was purchased from BDH Chemicals. Bretylium was acquired in the form of the drug Bretylol (bretylium tosylate) from American Critical Care. Amiloride was a gift of Merck, Sharp and Dohme (N.Z.) Ltd.

The quaternary ammonium compound bretylium has been shown to increase Na transport in frog skin and toad bladder, probably by releasing the Na self-inhibition (Ilani, Lichtstein & Bacaner, 1982; Ilani, Yachin & Lichtstein, 1984). The presence of bretylium tosylate in the mucosal medium improved the chance of observing Na channels in our patches. Therefore, we routinely included 5 mM bretylium in our pipette solution.

The solutions used in the experiments are shown in Table 1.

Results

Patch Clamping the Apical Membrane

In our attempts to conduct patch clamp experiments on the apical membrane of toad bladder we were faced with several difficulties:

1. The mucosal surface is covered with a thick glycocalyx and a layer of mucus that is constantly secreted by the goblet cells (Bidet et al., 1985). Because several investigators have found that gigaseals form more readily when cell surfaces are cleaned with enzymes (Neher, 1982), we tried to improve the sealing rate in that way. We used the mucolytic agents acetylcysteine (100 mM, pH 8.2, (Sheffner, 1963)) and DTT (5 mM, pH 8.0) as well as hyaluronidase (1.8 mg/ml, pH 7.8) and collagenase (1.0 mg/ml, pH 7.8) at temperatures between 17 and 30°C. None of these agents had any effect on the sealing rate. It seemed necessary only to clean the apical membrane for patch clamping by rinsing the mucosal surface of the stretched tissue thoroughly with the jet from a Pasteur pipette, which was filled with Na-Ringer.

2. The bladder tissue shows periodical contractions of the smooth muscle that can make it difficult to obtain stable cell-attached recordings. We rendered the tissue immobile by keeping it in a tightly

stretched state throughout the course of the experiment.

3. Pipettes with resistances of 3–5 MΩ, which are widely used in patch-clamp studies, did not seal to the apical membrane. Giga-seals did form, however, when pipettes with resistances of 10–20 MΩ were used. Scanning electron microscopy showed that these pipettes had opening diameters of about 0.5 μm, smoothly rounded edges and almost parallel walls at the tip.

4. Not all parts of the bladder surfaces were suitable for patch clamping. The best results were obtained on tissue areas where the density of collagen fibers was low. These areas were probably the most stretched ones and with these tissues the overall sealing rate was 47%. However, the success rate varied from zero to about 90% between bladders from different animals.

5. The main problem with inside-out recordings was the formation, usually immediately after excision, of membrane vesicles at the tip of the pipette. We confirmed the existence of such vesicles by observing distorted single-channel events at high-clamp voltages (80–120 mV). Routinely, we tried to open these vesicles by air exposure or by touching a silicone surface. This, however, in most cases caused the breaking of the giga-seal rather than the opening of the vesicle. The formation of vesicles could, in some cases, be prevented by superfusion of the tissue before the excision of the patch either with a Ca-free Na-Ringer containing 10 mM EGTA or, more reliably, with a Ringer-type solution in which all chloride was replaced by fluoride. Also, in a few experiments, we could reopen vesicles by exposing them transiently to a solution containing 2 μg/ml saponin. But this problem is still largely unsolved and we lost two-thirds of our excised patches through vesicle formation with subsequent breaking of the seal.

The Use of Bretylium

We found it necessary to include 5 mM bretylium in the mucosal (pipette) solution in order to obtain ion channel signals from the apical membrane with any regularity. Without bretylium, patches either contained no conducting channels or showed conductance changes of varying amplitudes that could not easily be interpreted as current signals from gated ion channels (*cf.* Koeppen, Beyenbach & Helman, 1984). In several hundred patches without bretylium we obtained only three single-channel recordings one of which could be analyzed in detail. This patch was obtained from a vasopressin-stimulated tissue and contained a highly selective Na channel with a conductance of 3 pS (with 120 mM Na). The

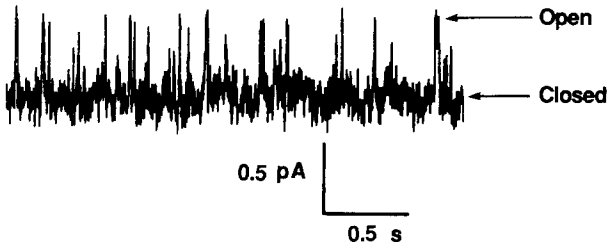

Fig. 2. Single-channel recording from a highly selective apical Na channel in the absence of bretylium tosylate. Inside-out recording with solutions NaP in the pipette (containing 0.5 μM amiloride) and KB in the bath. The pipette potential was +60 mV, filter setting 100 Hz

pipette solution also contained 0.5 μM amiloride. The mean open time of this channel was 3.7 msec (n = 1417), the mean closed time was 195 msec (n = 1417) and the open probability was 0.03. Figure 2 shows a 2-sec trace from this recording. As shown below, Na channels in the presence of 5 mM mucosal bretylium and 0.5 μM amiloride have much longer open and closed times. Another striking difference was that the channel that was observed in the absence of bretylium stayed active in the excised patch for 40 min (until the seal broke) without added protein kinase/cAMP/ATP. In contrast, when bretylium was used, *all* Na channels inactivated shortly after excision and could, in a few cases, be reactivated by protein kinase A (*see below*). Because mucosal bretylium increased our chance of finding Na channels in apical patches from <1 to ≈20%, we conducted all further experiments with 5 mM bretylium in the pipette solution. Therefore, the following results were all obtained in the presence of this drug.

Overview of Results

We used the reported method in 1539 trials on the apical membrane. We obtained 723 giga-seals (mean seal resistance: 49.7 GΩ, SD = 37.6 GΩ) from both control tissue and from vasopressin-stimulated tissue. In 242 patches, we could observe signals from ion channels unhindered by vesicle formation. In 181 of these, either the recordings were of too short a duration to characterize the channels or the patches contained more than one channel. The other 61 recordings allowed analysis of single-channel properties. Fifty-three channels from these patches could be classified according to their conductance. Table 2 gives an overview of the results from these patches. In the remaining 8 patches, we observed large conductances in the presence of 110 mM fluoride. In 137 of the 181 patches in which the properties of the channels could not be fully charac-

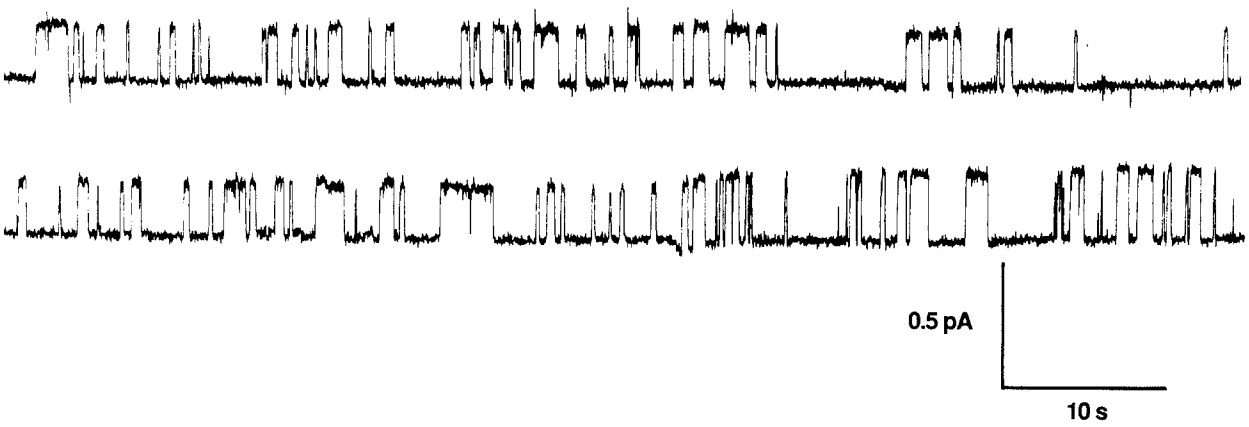

Fig. 3. Cell-attached recording from an apical Na channel. These two consecutive traces were recorded with NaP-solution in the pipette at a pipette potential of 0 mV. The tissue was bathed in Na-Ringer and the filter setting was 10 Hz. Mean single-channel current: 0.2 pA, mean open time: 0.46 sec, mean closed time: 1.16 sec, open probability: 0.29

terized, the conductance and gating properties of the signals were typical of the low conductance channels referred to here and in Table 2 as group 1 channels.

5 pS Highly Selective Na Channels

The predominant ion channels in the apical membrane were small Na-selective channels with long open and closed times (Fig. 3). Most patches contained either no channels or 5–10 or more apparently identical channels. The Na selectivity in such patches was estimated from the reversal potential of the multichannel fluctuations in inside-out recordings with KB-solution (Table 1) in the bath. In 11 recordings, the reversal potential was very close to the calculated Nernst potential for Na (−41 mV), thus indicating a high Na selectivity of the clustered channels. In 8 cases, inside-out patches with single Na channels could be investigated. These channels had a mean conductance of 4.8 pS (SD = 1.2 pS) in symmetrical Na solutions and seemed to be perfectly Na selective. Even with large electrochemical potential differences, no K current could be observed through these channels (Fig. 4*A* and *B*). Both mean open and closed times of the Na channels were significantly reduced by a submaximal concentration (0.1–0.5 μM) of amiloride in the pipette (Fig. 4*C* and *D*).

The dwell-time distributions were analyzed for two long inside-out recordings, one without amiloride and the other with 0.5 μM mucosal amiloride (Fig. 5). The open times in both recordings could be fitted with a single exponential and were 389 msec (n = 252) without amiloride and 65.2 msec (n = 1172) with amiloride. For the fit of the closed-time distributions we needed two exponentials. The two corresponding time constants were 1443 and 2.0 msec (n = 252) without amiloride and 975 and 2.5 msec (n = 1172) in the presence of the drug. The mean closed times, weighted by the areas under their respective exponential curve, were 876 msec without and 869 msec with amiloride.

The open probability of the channels was reduced from 0.25 to 0.07 through the action of 0.5 μM amiloride. Mean open time, mean closed time and open probability were not voltage-dependent in the range of pipette voltages between +30 and +140 mV. The voltage dependence at negative pipette potentials could not be adequately tested under our conditions because of the negligible K conductance (*cf.* Fig. 4*B*, squares).

Na channel activity usually ceased within 0.1–3 min after excision, when channels closed permanently. In 3 out of 9 trials, however, channel activ-

Table 2. Overview of results[a]

Group	Number of channels		Mean conductance ± SD	
	Control	Stimulated	[pS]	Selectivity
1	12	9	5.0 ± 1.6	Na ≫ K, Cl or Na ≈ K
2	1	11	17.2 ± 4.6	Na, K > Cl
3	3	10	30.2 ± 2.2	?
4	0	7	58.7 ± 3.5	(K > Na, Cl)

[a] Channels whose conductances we could measure are classified in 4 groups. The conductance values were derived from linear sections of the *i/V*-curves with maximal slope. The control tissue was kept in Na-Ringer, while the stimulated tissue was treated with vasopressin or cAMP in Na-Ringer. The selectivity of the group 4 channels was inferred from the current rectification in cell-attached patches.

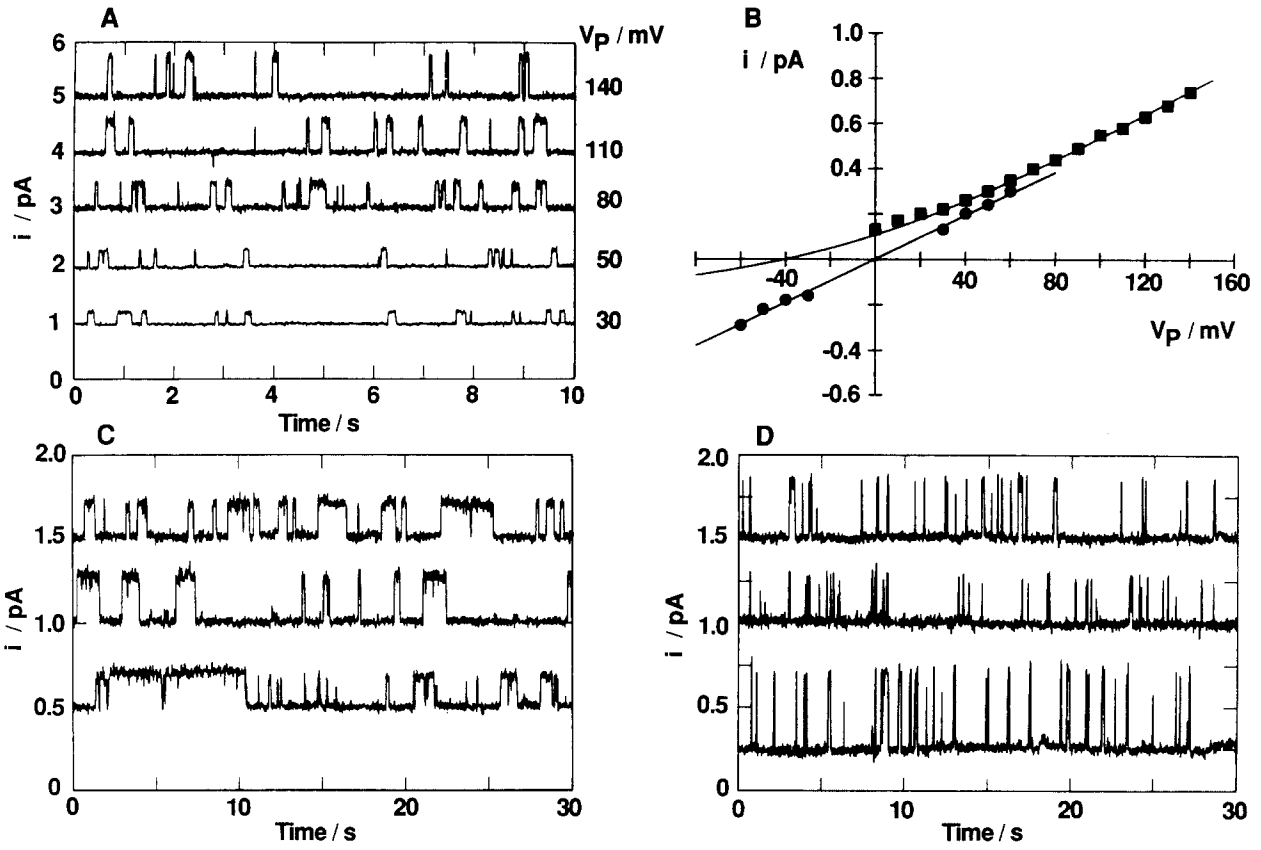

Fig. 4. Inside-out recordings from apical Na channels with solutions NaP in the pipette and KB in the bath. (*A*): Single Na channel at different voltages in the presence of 0.5 μM amiloride in the pipette. Filter setting: 100 Hz for the upper 3 traces and 30 Hz for the lower 2. This recording was obtained after reactivation of the channel with protein kinase A. (*B*): Current/voltage curves of the channel that is shown in *A* with Na-Ringer (circles) and with KB-solution (squares) in the bath. The solid lines are fits by linear regression (circles) and by fitting with the constant field equation (squares) assuming perfect Na selectivity. With KB-solution in the bath, no events could be observed between −10 and −80 mV. (*C*): Recordings from three different single-channel patches without amiloride. The upper trace was recorded at 60 mV, the lower two traces at 40 mV. (*D*): Three other Na channels in the presence of 0.5 μM mucosal amiloride at 60, 40 and 90 mV (from top). The filter setting for *C* and *D* was 30 Hz

ity could be restored and maintained for 25, 50 and 55 min, respectively, until the loss of the giga-seal. This was achieved by the addition of 0.5 μM protein kinase A (specific activity: 2 picomolar units/μg) to the bath solution (KB), which already contained 50 μM cAMP and 500 μM ATP. ATP and cAMP alone had no effect on the activity of the channel. In each of these 3 patches, which were obtained from control tissue, only one channel was active in the cell-attached configuration and for a short while after excision. The reactivation also resulted in single-channel traces. The protein kinase was added after the channels had inactivated despite the presence of both cAMP and ATP on their cellular sides (Fig. 6). The time between the addition of protein kinase and the reactivation of the channels was 1–3 min.

5 pS Channels Without Na Selectivity

Four of the small channels turned out to be nonselective with regard to Na and K. Their kinetic properties resembled that of the Na channel although one of them showed fast closing events during the long open periods (Fig. 7). These channels seemed not to be sensitive to mucosal amiloride up to a concentration of 0.5 μM for they had long open and closed times in the presence of the drug.

Channels in Vasopressin-Stimulated Tissue

When tissue was treated with substances known to stimulate Na transport in toad bladder through a cAMP-dependent pathway (0.1 U/ml vasopressin, 2

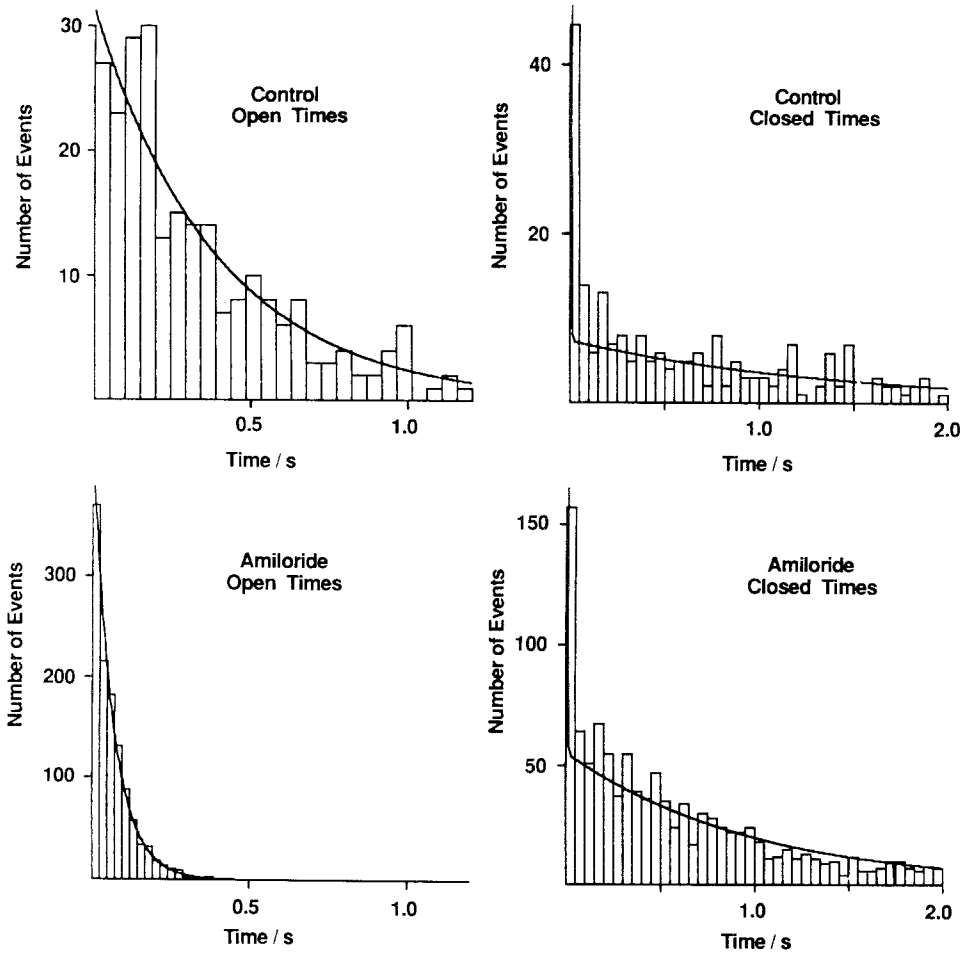

Fig. 5. Dwell-time histograms of two apical Na channels. Inside-out recordings from two channels have been analyzed, one without amiloride (control) and the other one with 0.5 μM mucosal amiloride. The solutions were NaP in the pipette and KB (containing 0.5 μM protein kinase A, 50 μM cAMP and 500 μM ATP) in the bath. Pipette potentials were 60 mV for the control recording and 50–100 mV for the recording with amiloride. The filter setting was 100 Hz, the sample rate was 500 Hz. The curves were fitted to the unbinned data as described in the text and yielded the following time constants. Open times (control): 389 msec; open times (amiloride): 65.2 msec; closed times (control): 1443 msec and 2.0 msec; closed times (amiloride): 975 msec and 2.5 msec. In total, 504 control events and 2344 events in the presence of amiloride were analyzed

mM dbcAMP, 0.1 mM IBMX, applied 3 to 15 min prior to patch clamping), channels with higher conductances appeared relatively frequently in cell-attached patches, in addition to the 5-pS Na channels. A total of 27 stimulated tissues were investigated. In 116 patches of these tissues, we identified 9 group 1 channels (mean conductance 5.4 pS). Two of these were found to be Na selective, two others were nonselective and the selectivity of the remaining five could not be measured. The opening and closing times of the Na channels did not differ significantly from the Na channels that were observed in control tissues. However, the recordings were too short to allow statistical analysis.

In addition to the group-1 channels, larger channels were found in these patches. As shown in Table 2, these larger channels occurred predominantly in patches from stimulated tissue. The group-2 channels (17 pS) closed on excision of the patch but could be reopened in two out of seven cases by addition of 50 μM cAMP and 500 μM ATP to the bath medium (Fig. 8*A*). Both cAMP and ATP, but not protein kinase, were necessary for this reactivation. When we replaced K in an inside-out experiment with NMDG, the shift of the *i/V*-curve (Fig. 8*B*) indicated that this was a cation channel without selectivity with regard to Na and K.

The channels of group 3 (30 pS) were seen in

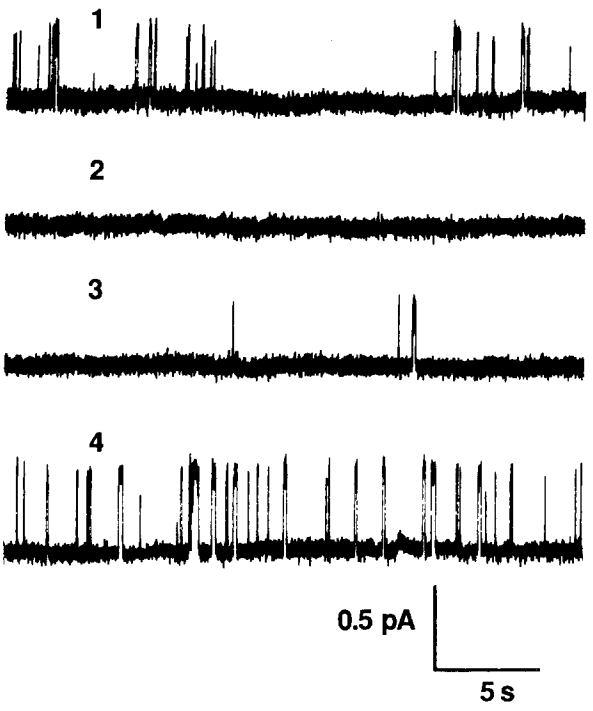

Fig. 6. Activation of an apical sodium channel with protein kinase A. Inside-out recordings with NaP-solution in the pipette and KB-solution in the bath. The pipette solution also contained 0.5 μM amiloride. The pipette potential was +70 mV. Trace *1* was recorded immediately after excision of the patch, trace *2* shows the completely inactive channel 4 min after excision (inactivation occurred 3 min after excision). Trace *3* was recorded 10 min after the bath was perfused with KB-solution, containing 50 μM cAMP and 500 μM ATP (18 min after excision). Trace *4* shows the restored channel activity 2 min after the addition of protein kinase A (final concentration: 0.5 μM, specific activity: 2 pMU/μg) to the bath solution (25 min after excision). The channel's activity continued until the breaking of the seal 25 min after activation of the channel. Filter setting: 30 Hz

three cells of the same control tissue and in 10 cells of stimulated tissue from 9 different animals. Group 4 channels (58 pS) were only seen in stimulated tissue. These two channel species always closed immediately after excision and we were not able to reactivate them in inside-out patches by application of protein kinase/cAMP/ATP. The disappearance of signals from these channels was, at least in 4 cases, not due to vesicle formation, for continuous activity of group 1 channels was observed after excision. The channels in groups 3 and 4 had reversal potentials close to 0 mV in the cell-attached configuration. However, while the 30-pS channels had linear *i/V* curves (Fig. 8*C* and *D*), the 58-pS channels showed pronounced rectification and had a voltage-dependent open probability (Fig. 8*E* and *F*). Another striking feature of the 58-pS channels was a subconductance level at 35% of the maximal channel current.

Large Conductances

In a series of experiments we superfused the tissue while the patch was in the cell-attached configuration with Ringer-type solutions in which all chloride was replaced by fluoride (NaFB- and KFB-solutions) in an attempt to prevent vesicle formation (Horn & Patlak, 1980). Patches were excised after the superfusion was completed. In most cases, vesicles did not form under these conditions and current signals could be recorded from inside-out patches. However, the giga-seals were less stable and usually broke within 1–3 min after excision. Several large conductances were observed in the presence of fluoride that were never seen under other condi-

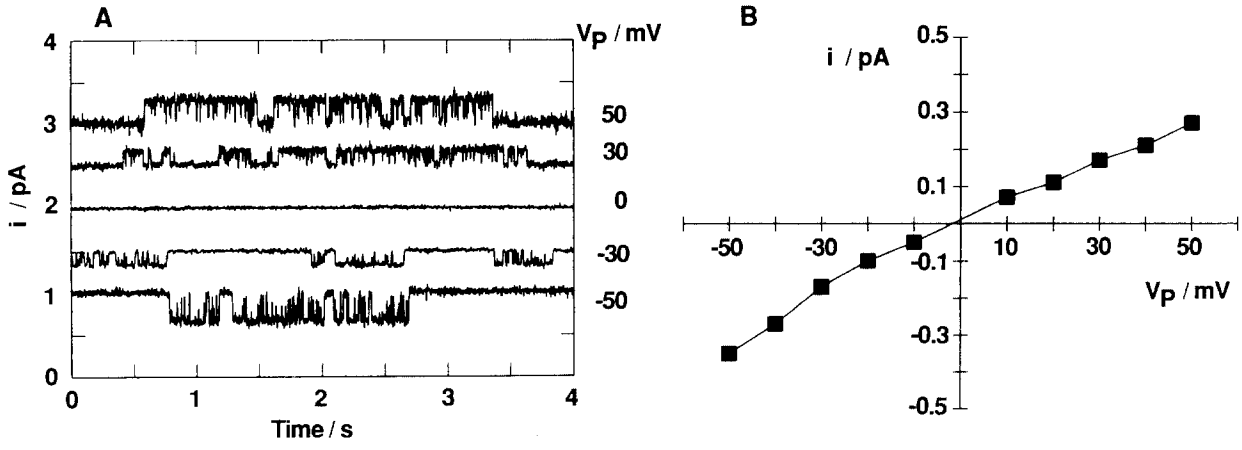

Fig. 7. (*A*): Inside-out recording from an apical group 1 channel with solutions NaP in the pipette and KB in the bath. The filter setting was 100 Hz. The current/voltage-curve (*B*) indicates a conductance of 5.8 pS and no selectivity for Na over K

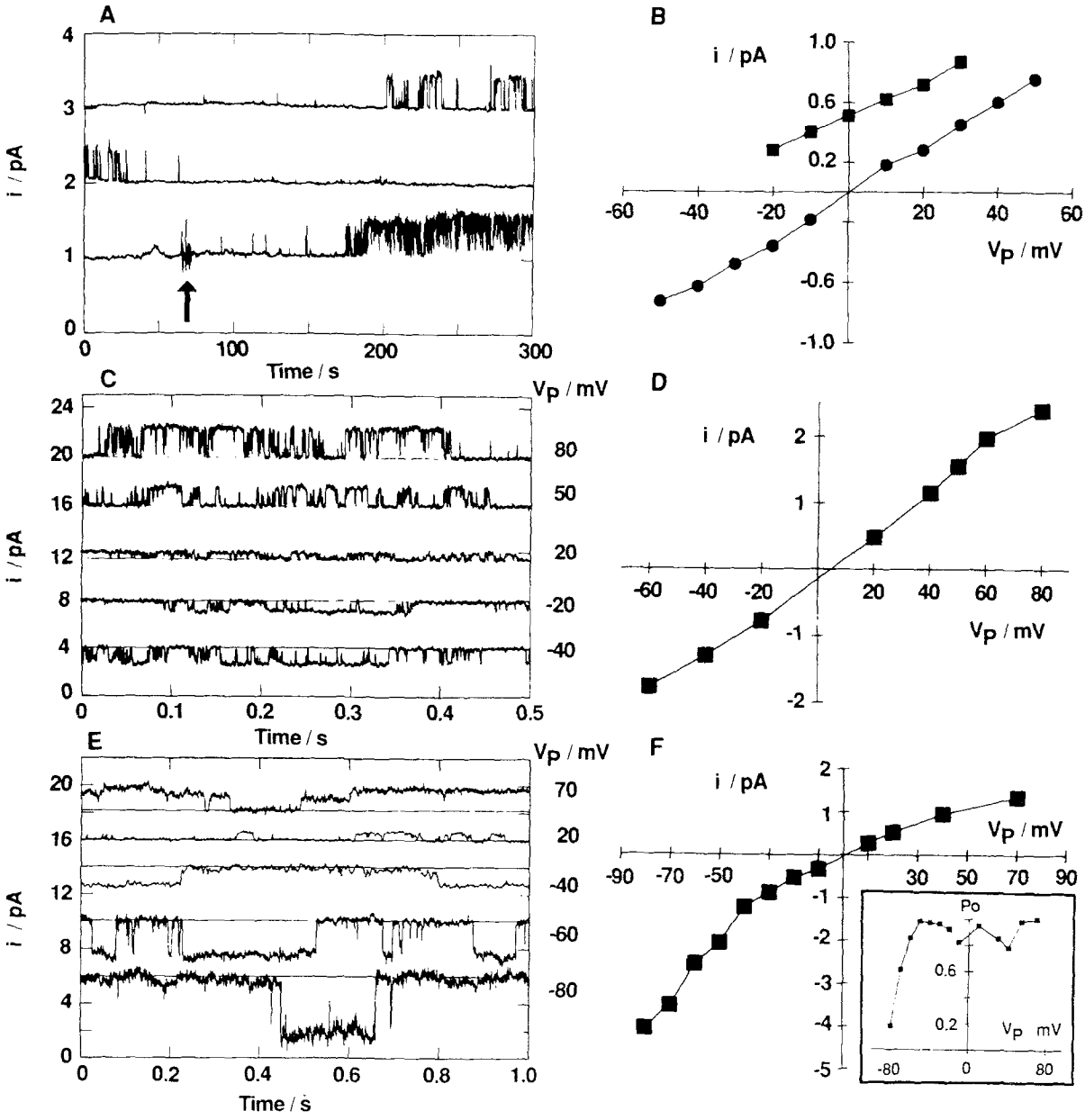

Fig. 8. Ion channels that occur in vasopressin-stimulated tissue. (*A*): Inside-out recording from a 16-pS channel in three consecutive traces with solutions NaP in the pipette and KB in the bath (holding potential: 30 mV, filter setting 30 Hz). The upper and middle traces were recorded with 50 μM cAMP in the bath. At the arrow in the lower trace, 500 μM ATP was added to the solution. After about 100 sec the channel was activated and it stayed active for 18 min. During this time the *i/V*-curves (*B*) were recorded, first with KB in the bath (●), then with NMDG-solution containing 50 μM cAMP and 500 μM ATP (■). In a similar experiment (*not shown*), we perfused the patch first with 500 μM ATP. The channel stayed inactive until 50 μM cAMP was added to the bath solution, which caused a lasting activation. (*C*) and (*D*) show single-channel recordings and the *i/V*-curve of a 31-pS channel in a cell-attached patch. (*E*) and (*F*) are cell-attached recordings from a 58-pS channel, the *i/V*-curve and, as an inset in (*F*), the open probability as a function of the pipette potential. The solid lines in (*C*) and (*E*) indicate the closed state of the channels. The traces in (*C*) and (*E*) were filtered at 300 Hz

tions and that were also never active in cell-attached patches. We could only record from these conductances in a voltage range of −20 to +20 mV because they inactivated as soon as higher potentials were applied.

All of these conductances displayed fluctuations between two major current levels as shown in Fig. 9*A* with a 410-pS conductance. In order to characterize these fluctuations, we recorded amplitude histograms from the current signals at several

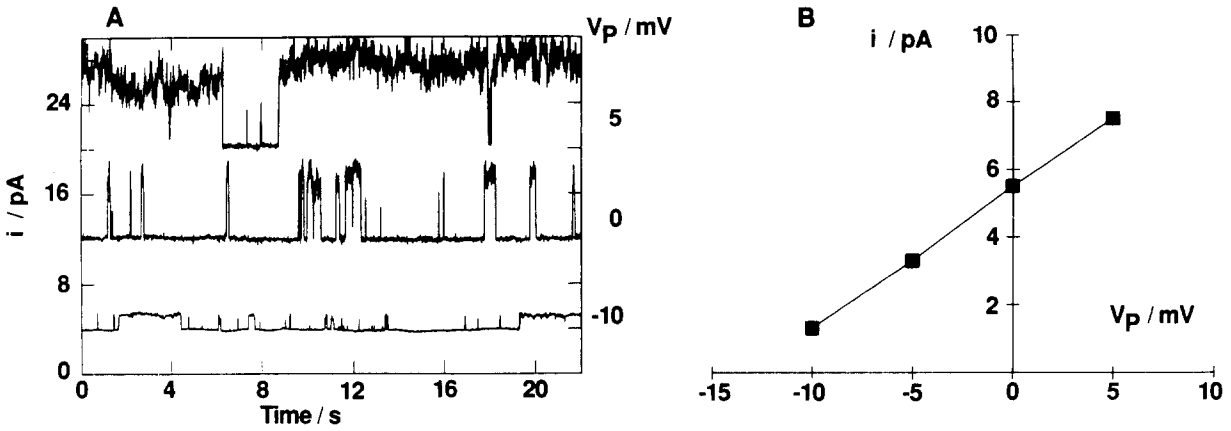

Fig. 9. (*A*): Inside-out recording from a 410-pS conductance in the apical membrane that was observed in the presence of 110 mM fluoride on the cytoplasmic side of the patch. The traces were recorded with solutions NaP in the pipette and KFB in the bath. Filter setting: 300 Hz in the upper two traces, 100 Hz in the lower trace. (*B*) shows the *i/V*-curve of this recording

holding potentials for 10–30 sec. We obtained two-peak histograms representing a conducting and a nonconducting state, as expected from an ion channel with an open-closed gating mechanism. From these histograms we constructed *i/V*-curves and derived mean values of the slope conductances of 86.5 pS (SD = 5.0 pS, $n = 2$); 115.7 pS (SD = 5.1 pS, $n = 3$); and 453.3 pS (SD = 40.4 pS, $n = 3$).

Unfortunately, it was not possible to measure the ion selectivity of these conductances, due to the poor stability of the seals that did not allow changes of the bath solution. The current of all three conductances reversed at small negative potentials (up to −15 mV) regardless of whether NaFB- or KFB-solution was used. This suggests that these large pores do not discriminate between Na and K but have a slight selectivity for F over Cl (which could be explained by the ratio of electric mobilities of F over Cl, which is 1.7).

Discussion

Patch-clamping epithelial tissue seems to be associated with a number of difficulties including poor accessibility of the membranes, covering of mucus, rigidity of luminal membranes and, in some cases, tissue motility. Nevertheless, a number of investigators have worked out procedures that allow single-channel recordings from a variety of intact epithelia and from cultured epithelial cells (for reviews *see* Rae & Levis, 1984; Eaton & Hamilton, 1986). No single-channel data have so far been obtained from toad bladder epithelial cells although this is one of the best characterized of the Na-transporting epithelia. Most of the information about channel gating and selectivity in this tissue comes from fluctuation analysis of spontaneous or blocker-induced noise (e.g., Lindemann & VanDriessche, 1977; and recent reviews by Lindemann, 1984; VanDriessche & Zeiske, 1985; Wills & Zweifach, 1987). The objective of our study was to find a method of preparation that allows the recording of single ion channel currents in this tissue.

We found it possible to apply the patch-clamp technique to tissue that was tightly stretched and thereby flattened and immobilized. It was, however, necessary to use fire-polished patch pipettes with a resistance of 10–20 MΩ which, in their tip section, rather resembled the microelectrodes used for intracellular recording. With these pipettes, we achieved an overall sealing rate of 47%. This success rate was, however, reduced by the formation of vesicles at the pipette tip, which was probably favored by the very geometry we needed for sealing in the first place. The most promising approach to this problem seems to be the superfusion of the tissue in the cell-attached configuration with a Ca-free Ringer-solution that contains 10 mM EGTA. During the subsequent excision of the patch into the Ca-free solution, vesicles formed less frequently.

Since we were not able to see cell borders in the stretched tissue, we cannot say in which kind of cell the channels from which we were recording were located. However, considering that 96% of the mucosal surface consists of apical membrane of the granular cells (Macknight et al., 1980) we can assume that channels that we have observed commonly belong to this type of epithelial cell.

Highly Selective Na Channels

As predicted by noise analysis (Lindemann & VanDriessche, 1977; Li et al., 1982), there are Na channels in the apical membrane with a conductance of

about 5 pS and mean open and closed times of several hundred milliseconds. Mostly, the channels occur clustered in groups of 5–10 or more. Low concentrations of amiloride reduce mean open and closed times by blocking the open channel. The Na selectivity of these channels is very high; the channels seem virtually impermeable to K. This observation is in accordance with the finding of Palmer (1982) that the amiloride-blockable part of the short-circuit current in toad bladder has a selectivity of Na over K of about 1000. This high selectivity, together with the long open and closed times, distinguishes these channels from apical Na channels that have been observed in A6-cell cultures with bilayer reconstitution (Sariban-Sohraby et al., 1984*b*) and with patch clamp (Hamilton & Eaton, 1985). However, the Na channels found by Palmer and Frindt (1986, 1987) in the apical membrane of the rat cortical collecting duct, by Gögelein and Greger (1986) in the apical membrane of the rabbit late proximal tubules, and by Hamilton and Eaton (1986) in A6-cell cultures grown on collagen, were also characterized by long open and closed times and high Na selectivity. Thus it seems that these three properties characterize sodium channels found in native Na-transporting epithelia. It must, however, be emphasized, that the quoted studies were performed without bretylium and that the properties of the Na channels in our study might be influenced by this drug. Hamilton and Eaton (1986) have found highly selective Na channels with a conductance of 2.8 pS, a mean open time of 19.4 msec and a mean closed time of 85.8 msec in the apical membrane of A6 cells. These channels seem to resemble the one that we have observed in the absence of bretylium (Fig. 2). The properties of the apical Na channel have been reviewed by Sariban-Sohraby and Benos (1986); Garty and Benos (1988); and Eaton and Hamilton (1988).

In three experiments, we could restore the activity of Na channels by the addition of protein kinase/cAMP/ATP to the cellular side of the patch. In the absence of protein kinase A, neither cAMP nor ATP activated the channel, suggesting that these compounds do not bind directly to the channel. The longest recording from a Na channel without protein kinase was 3 min, whereas the protein kinase-activated channels were active for 25–55 min when the recordings were ended by loss of the giga-seals. These initial results suggest that a cAMP-dependent protein phosphorylation is involved in the activation of this channel. This is consistent with the model for the action of vasopressin, which proposes that following the binding of the hormone to its basolateral receptor, adenyl cyclase is activated (Hynie & Sharp, 1970) and the rising level of cellular cAMP (Omachi et al., 1974) activates Na channels in the apical membrane through the stimulation of a cytosolic cAMP-dependent protein kinase (Schlondorff & Franki, 1980). However, more often the addition of protein kinase/cAMP/ATP to the bath medium failed to activate Na channels under seemingly identical conditions.

Although the paucity of experimental data precludes a proper analysis at this stage, we consider the following working model a possible explanation for this observation. The fact that protein kinase A activated Na channels in only three out of nine single-channel patches indicates that two different kinds of Na channels, which have similar conductance and ion selectivity, are present in the apical membrane. One population is active in the intact cell and closes permanently on excision. Single channels of this species were present in all nine patches that had been treated with protein kinase. In these nine cases, they were observed in the cell-attached configuration and ceased to open shortly after the patches were excised, possibly due to the loss of an unknown component into the bath solution.

In addition to this channel species, the three patches that responded to protein kinase contained a second kind of channel. This second population is inactive (always closed) in the unstimulated cell and also in excised patches. However, in contrast to the first species, these channels are a substrate for cAMP-dependent phosphorylation by protein kinase A and are converted into an active state by phosphorylation. This notion is supported by the finding that the protein kinase-activated channels were the only ones that stayed active for so long as the patch was stable (up to 55 min), while all channels that had been detected in the cell-attached configuration closed permanently within 0.1–3 min after excision.

This model provides a physiological explanation for the mechanism by which vasopressin or aldosterone stimulate Na transport in toad bladder. The first species of Na channels would conduct the baseline Na current in the unstimulated tissue, independent of the cellular cAMP concentration. The other population of channels would be inactive in the unstimulated bladder. However, when vasopressin causes the increase of cellular cAMP, the resulting activation of protein kinase A leads to the opening of these phosphorylation-dependent Na channels, which then give rise to the increase in Na transport.

Several reports from other groups corroborate this model for hormonal stimulation of Na transport. Li et al. (1982) and Palmer et al. (1982) have demonstrated that vasopressin and aldosterone recruit Na channels from a pool of previously inactive apical channels rather than increasing the open

Table 3. Comparison of kinetic parameters derived from single-channel recordings (this study) with results from noise analysis studies on toad bladder (data from Li et al., 1982)[a]

Technique	K_A^{ma} [μM]	K_A^{mi} [μM]	k_{10} [sec^{-1}]	k_{01} [sec^{-1}]	k_{02} [10^7 M^{-1} sec^{-1}]	k_{20} [sec^{-1}]
Patch clamp	—	0.05	0.69	2.57	2.55	1.22
Noise analysis	0.43	0.17	—	—	1.68	2.60

[a] The single-channel data were derived from inside-out recordings with solutions NaP in the pipette and KB in the bath, containing 0.5 μM protein kinase A, 50 μM cAMP and 500 μM ATP (Data from Fig. 5)

probability of Na channels that have already been active prior to the addition of the hormones. Palmer and Edelman (1981) and Park and Fanestil (1980) found that the apical Na channels that mediate the increase of Na transport during stimulation with vasopressin and aldosterone are also present in the apical membranes of unstimulated cells. In contrast, Garty and Edelman (1983), using a similar technique, showed that only aldosterone-activated channels can be found in the apical membrane of unstimulated cells, while the vasopressin-activated channels are inserted during hormonal stimulation. This finding has been supported by the experiments of Kipnowski, Park and Fanestil (1983). Thus, at least aldosterone-activated channels seems to exist in the apical membrane of control tissue in a non-conducting form and are converted into a conducting state through the action of the hormone. The mechanism by which quiescent Na channels are activated is still unknown (Garty, 1986). The release from a Ca-dependent downregulation of Na channels has been suggested (Chase, 1984; Garty & Asher, 1986) as well as methylation of the cytoplasmic side of the channel or the membrane close to the channel (Sariban-Sohraby et al., 1984*a*).

The preliminary findings of our study suggest that this activation could be caused by cAMP-dependent phosphorylation of quiescent apical Na channels. Evidence for the regulation of ion channels by phosphorylation has accrued in recent years through the work of several groups (e.g., Ewald, Williams & Levitan, 1985; Shuster et al., 1985; Armstrong & Eckert, 1987; Avenet, Hofmann & Lindemann, 1988; and *reviews by* Levitan, 1985; Chad, Kalman & Armstrong, 1986).

Comparison with Noise Data

This study was initiated in order to assess the microscopic parameters of the apical Na channels (single-channel conductance, ion selectivity, open- and closed-time distribution and open probability) under various physiological conditions. Most data concerning open-closed kinetics, binding of amiloride, channel density in the apical membrane and ion transport rates in toad bladder have been derived from the comprehensive noise analysis reported by Li et al. (1982) and Palmer et al. (1982). These investigators found that a mean single-channel current of 0.18 pA is conducted by apical Na channels at a mucosal Na concentration of 60 mM. In good agreement, we measured a mean single-channel current of 0.20 pA (at 120 mM Na) from a channel in a cell-attached patch with no applied potential (Fig. 3).

In the noise analysis study, a 3-state model for the competitive blocking of Na channels was used. Current noise (i.e., openings and closings of a large number of Na channels) is generated by mucosal Na and amiloride (Lindemann, 1984). Thus, the channels are either blocked by Na (closed state), or by amiloride (blocked state), or they are open.

$$\text{Closed} \underset{k_{10}}{\overset{[\text{Na}]\ k_{01}}{\rightleftharpoons}} \text{Open} \underset{k_{20}}{\overset{[\text{A}]\ k_{02}}{\rightleftharpoons}} \text{Blocked}$$

where [Na] and [A] are the mucosal concentrations of Na and amiloride, respectively. In our further considerations, we will neglect the binding of Na to the open channel and treat open-to-closed-transitions as a first order reaction. Therefore, the rate constant k_{01} is specifically valid only for our experimental conditions (120 mM Na and 5 mM bretylium in the mucosal solution). Table 3 shows a comparison between the estimates for the rate constants from our study with the values that were found by Li et al. (1982) using noise analysis. The apparent microscopic inhibition constant (K_A^{mi}), which is a measure for the binding kinetics of amiloride to the channel, was derived from the open probabilities without amiloride ($P_o = 0.25$) and with 0.5 μM amiloride ($P_o^A = 0.07$) according to $K_A^{mi} = [A] \cdot P_o \cdot P_o^A / (P_o - P_o^A)$ (Li & Lindemann, 1983). Rate constants were calculated from the mean open and mean closed times that were obtained from the dwell-time distributions (Fig. 5) according to the relations $k_{01} = 1/\tau_o$; $k_{10} = 1/\tau_c$; $k_{02} = (1/\tau_o^A - 1/\tau_o)/[A]$; $k_{20} = k_{02} \cdot K_A^{mi}$. In these equations, τ_o and τ_o^A are the mean open times without and with amiloride and τ_c is the slow time constant of the closed-time distribution function without amiloride. The numerical values for these constants are summarized in Table 3.

Taken together, the comparison of rate constants indicates that the Na channels in the single-channel experiment show a higher affinity for amiloride than expected from noise analysis, an ob-

servation that is clearly expressed by K_A^{mi} which is 3.5 times smaller in the single-channel experiment. A larger on-rate (k_{02}) and a smaller off-rate (k_{20}) give rise to this high affinity. (From the data that were reported by Palmer and Frindt (1986) from rat cortical collecting ($P_o = 0.41$, $P_o^A = 0.14$, [A] = 0.5 μM) we calculate a K_A^{mi} of 0.1 μM which lies between the two estimates for toad bladder in Table 3.) We cannot exclude the possibility that the discrepancy is due to the use of bretylium in our experiments and that the small K_A^{mi} in our experiments might reflect a decrease of the rate of Na binding to the channel. Cuthbert and Shum (1975) found that the macroscopic inhibition constant of amiloride in toad bladder is dependent on the mucosal Na concentration. At 1.1 mM mucosal Na, the inhibition constant was 0.025 μM, while at 111 mM Na, it was 0.34 μM. With low mucosal Na, 0.5 μM amiloride inhibited short-circuit current by approximately 85%, which is in fair agreement with our results (reduction of open probability by 72%). Thus, amiloride seems to compete with Na for a binding site at the channel resulting in a Na-dependence of amiloride inhibition, a view that has been confirmed by noise analysis (Li & Lindemann, 1983). With these findings, it appears possible to explain the high affinity for amiloride in our single-channel experiments. The similarity of our K_A^{mi} and the K_A^{ma} at low mucosal Na in the work of Cuthbert and Shum (1975) suggests that bretylium could occupy a Na-binding site at the channel, thereby attenuating both Na self-inhibition and competition between Na and amiloride. The finding of Ilani et al. (1984), that bretylium prevents the Na-induced closures by competing with mucosal Na for a binding site at the channel, is consistent with this notion.

If we estimate from our values of k_{01} and k_{10} the corner frequency of the power density spectrum in a hypothetical noise analysis experiment in the absence of amiloride ($f_c = (k_{01} + k_{10})/2\pi$), we find a value of 0.52 Hz. This is in good accordance with the results of Lindemann and VanDriessche (1978) who demonstrated that mucosal Na induces a concentration-dependent corner frequency in the range of 0.5 Hz, caused by the blocking of apical Na channels.

5-pS Channels that are not Na Selective

We found channels in the apical membrane that also had conductance values of 5 pS and that, in most cases, showed a gating behavior that was similar to the apical Na channel. They were, however, not Na selective and were not blocked by 0.5 μM amiloride. The similarity of conductance and gating properties suggests that there is some relation between these channels and the Na channels. Lewis (cited by Wills & Zweifach, 1987) has proposed a model for the mammalian urinary bladder in which the apical Na channels undergo a process of hydrolytic degradation that transforms them into unselective "leak" channels that are no longer sensitive to amiloride (*cf.* Fig. 3 of Wills & Zweifach, 1987). It seems possible that the nonselective low-conductance channels in the toad bladder are the products of such a degradation process.

Vasopressin-Induced Channels

It is generally accepted that the stimulation of Na transport by neurohypophysial hormones like vasopressin or oxytocin is mediated by cAMP. Omachi et al. (1974) have shown that a concentration-dependent rise in cellular cAMP follows the addition of vasopressin to the serosal solution. Li et al. (1982) demonstrated that vasopressin increases the number of active Na channels in the apical membrane. However, transport stimulation is not restricted to Na-selective channels. Li et al. (1982) demonstrated that oxytocin increased the amiloride-insensitive apical conductance as well as the sodium conductance. VanDriessche (1987) found that oxytocin induces a Ca conductance in the apical membrane that is activated by nanomolar concentrations of Ag. In the absence of divalent cations from the mucosal solution, this channel permits the passage of monovalent cations, thus forming a transcellular, nonselective cation pathway that is sensitive to micromolar concentrations of Ca and Mg. Erlij et al. (1986) reported a Ba-sensitive K channel in the apical membrane that is also activated by oxytocin. These data show that a complex variety of conductance changes takes place in the apical membrane during stimulation with neurohypophysial hormones.

In our experiments with vasopressin-stimulated tissue, we regularly found three kinds of channels that were rarely seen in unstimulated tissue. A 30-pS and a 58-pS channel could only be studied in the cell-attached configuration because they always inactivated irreversibly on excision. Since we know neither the potential difference across the apical membrane nor the precise cellular ion activities, we do not know the selectivity of these channels. However, assuming standard ion composition of the cytoplasm (141 mM K, 17 mM Na, 47 mM Cl, calculated from Rick et al., 1978), the current rectification in Fig. 7*F* suggests that this channel is K selective. The open probability of this channel was strongly dependent on the applied voltage (V_p), showing a steep decrease at potentials below −50 mV. Assuming a cell potential (V_c) of −60 mV, it appears that this channel responds to the polarity of

the membrane ($V_m = V_p - V_c$). When the cytoplasm is negative with respect to the mucosal solution, the channel is almost constantly open, whereas a reversed polarity (which is the polarity of the open circuited bladder in vivo) keeps the channel closed.

Some preliminary experiments with the 17-pS channel in the inside-out configuration suggest that this is a nonselective cation channel that can be activated by cAMP/ATP in the excised patch. This would require that a cAMP-dependent protein kinase, attached to the membrane in the vicinity of the channel, is excised together with the channel. The observation that both cAMP and ATP are needed for activation rules out the possibility that either one of these compounds might act as an allosteric activator of the channel. Such allosterical activation by cAMP has been described in olfactory epithelia by Nakamura and Gold (1987).

Since all recordings from this channel were obtained in the presence of 1 mM Ca in the mucosal medium, the 17-pS channel seems not to be the Ca channel that was observed by VanDriessche (1987).

Large Conductances

Conductances with 90–450 pS occurred only when inside-out patches were exposed to 110 mM fluoride on their cytoplasmic side. The free concentration of divalent cations must be very low in these solutions ([Ca] $< 10^{-9}$ M, [Mg] $< 10^{-7}$ M) because the solubility of CaF_2 and MgF_2 is 0.2 and 1.2 mM, respectively. This could well be the reason for the low rate of vesicle formation, but it is also a most unusual condition for the cell membrane and its proteins, and it might generate ion conductances that are not present in the intact cell. Another effect of this high fluoride concentration is the inhibition of phosphatase activity (Revel, 1963). Since we have already found evidence for a role of protein phosphorylation in the regulation of channel activity in this membrane, we can also consider the possibility that the large conductances are usually inactivated by phosphatases and that this inhibition is released by fluoride.

The fact that we have never observed these large conductances in the cell-attached configuration does not necessarily mean that they have no function at all in the living cell for there is a considerable amount of literature about large channels in epithelia (Nelson, Tang & Palmer, 1984; Rae & Levis, 1984; Kolb, Brown & Murer, 1985; Kolb, Brown & Murer, 1986; Krouse, Schneider & Gage, 1986; Christensen & Zeuthen, 1987; Gitter et al., 1987; Hunter & Giebisch, 1987).

The absence of any obvious selectivity as well as the huge noise increase in the "open state" of these conductances raises the question whether they may be artifacts of the fluoride treatment, for example fluctuating leaks that are caused by the depletion of divalent cations from the solution bathing the cytoplasmic side of the patch. However, the voltage-dependent inactivation of these conductances suggest that they are membrane integral proteins that can sense the electrical field across the membrane, rather than simple damage to the patched membrane. Conductances of 400 to 1900 pS have been found previously in lens epithelial cells of mice and grass frogs by Rae and Levis (1984) who addressed the possibility that membrane proteins that are nonconducting in the intact cell (e.g., gap junction photochannels) may become conducting as a consequence of perturbations during the patch-clamp experiment, for example the application of a strong electrical field for a long period of time. In our case, such a perturbation could be caused by the removal of Ca and Mg from the cytoplasmic side of the patch.

It will take some time until the channels in the epithelium of the toad bladder are properly characterized and their significance for the regulation of Na transport and cell volume can be assessed. Clearly, in the intact tissue, amiloride-sensitive sodium channels provide the dominant pathway for sodium entry to the cells from the mucosal medium under both control conditions and following hormonal stimulation (Macknight et al., 1980). Indeed, the agreement between measured short-circuit current and net sodium flux (from isotope studies) indicates that nonselective apical membrane cation channels play little part in the movement across this membrane. When amphotericin B is added to the mucosal solution to induce nonselective apical channels, a discrepancy is observed between short-circuit current and net sodium flux (Lichtenstein & Leaf, 1965). Furthermore, though in the patch-clamp studies reported here, treatment of the tissue with vasopressin, cAMP or phosphodiesterase inhibitor (IBMX), was frequently associated with the appearance of larger nonspecific cation channels, these are unlikely to make a major contribution to the increase in transepithelial sodium transport induced by vasopressin, for this hormone does not increase the measured potassium permeability of the tissue (Leaf & Hays, 1962), unlike amphotericin B (Lichtenstein & Leaf, 1965). The contribution that the larger channels make to tissue function is, therefore, unclear. However, patch-clamp studies often reveal an unexpected multitude of channels (e.g., Rae & Levis, 1984; Rae, 1985) and it remains a challenge to equate these observations with macroscopic data.

Conclusion

We have reported a method that allows the recording of single-channel currents from the epithelium of the toad urinary bladder. We applied the patch-clamp technique to the apical membrane after the tissue was tightly stretched. We demonstrated the activity of a surprising variety of ion channels in the apical membrane that occurred under different conditions. Highly selective, amiloride-sensitive Na channels, with a conductance of 5 pS, were present in both untreated and vasopressin-stimulated tissue, mostly in channel clusters. In 3 inside-out patches, single Na channels could be activated with 0.5 μM protein kinase A added to the bath medium containing 50 μM cAMP and 500 μM ATP. Channels without Na selectivity were observed, with a range of conductances from 5 to 58 pS. The 5-pS channels occurred together with Na channels, and 17–58 pS channels were observed during stimulation with vasopressin. Large conductances appeared in inside-out patches in the presence of 110 mM fluoride.

It is a pleasure to thank Dr. Lindsay Gordon for his continuous interest in our work and for many helpful discussions. We are grateful to Prof. Bernd Lindemann for his important contribution to our work during his visit to Dunedin in 1987 and for his critical reading of the manuscript. We thank Dr. Grant Butt for valuable comments on the manuscript and Mr. Noel Still for the preparation of the figures. We also gratefully acknowledge the assistance of Mr. Ron Holdaway and Mr. Tony Bennett. This work was supported by a programme grant from the Medical Reseach Council of New Zealand and the Lottery Board through its Medical Distribution Committee.

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Received 3 June 1988; revised 28 July 1988