Ca^{2+}-blockable, Poorly Selective Cation Channels in the Apical Membrane of Amphibian Epithelia

UO_2^{2+} Reveals Two Channel Types

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ABSTRACT This study deals with the effect of mucosal UO_2^{2+} on the Ca^{2+}-blockable, poorly selective cation channels in the apical membrane of frog skin and toad urinary bladder. Our data show that UO_2^{2+} inhibits the Na^+ currents through the amiloride-insensitive cation pathway and confirm a previously described stimulatory effect on the amiloride-blockable Na^+ transport. Noise analysis of the Ca^{2+}-blockable current demonstrates that the divalent also depresses the low-frequency Lorentzian (f_c = 11.7 Hz) in the power density spectrum (PDS) and reveals the presence of high-frequency relaxation noise (f_c = 58.5 Hz). The action of UO_2^{2+} is not reversed upon washout and is not accompanied by noise, typically induced by reversible blockers. The divalent merely depresses the plateau of the low-frequency Lorentzian, demonstrating a decrease in the number of conductive cation channels. Similarly, with mucosal K^+ and Rb^+, UO_2^{2+} also unmasks the high-frequency Lorentzian by depressing the noise from the *slowly* fluctuating cation channels (type S). In all experiments with mucosal Cs^+, the PDS contains high-frequency relaxation noise (f_c = 75.1 Hz in *Rana temporaria*, and 65.4 Hz in *Rana ridibunda*). An effect of UO_2^{2+} on the Cs^+ currents and Lorentzian plateaus could not be demonstrated, suggesting that this monovalent cation does not pass through type S channels. Experiments with the urinary bladder revealed only a UO_2^{2+}-insensitive pathway permeable for Na^+, K^+, Rb^+, and Cs^+. We submit that in frog skin two cation-selective channels occur, distinguished by their spontaneous gating kinetics, their sensitivity to UO_2^{2+}, and their permeability for Cs^+. In toad urinary bladder, only one kind of cation-selective channel is observed, which resembles the UO_2^{2+}-insensitive channel in frog skin, with *fast* open-closed kinetics (type F).

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INTRODUCTION

Previous studies from our laboratory demonstrated that removal of Ca^{2+} from the mucosal bath reveals the presence of cation-selective channels in the apical membrane of frog skin (Van Driessche and Zeiske, 1985; Van Driessche, Desmedt, and Simaels, 1991) and toad urinary bladder (Aelvoet, Erlij, and Van Driessche, 1988). These channels are amiloride insensitive, but they are blocked by (sub)micromolar concentrations of Ca^{2+}. The concentrations for half-maximal inhibition of the amiloride-insensitive Na^+ current are ~200 and 450 nM in the skin and bladder, respectively (Van Driessche et al., 1991). Another difference between the channels in both tissues concerns their open-closed kinetics. In frog skin the channel flickering gives rise to a Lorentzian component with corner frequencies (f_c) of ~12 Hz, whereas in the toad bladder f_c is 347 Hz (Van Driessche et al., 1991). This variation could result from differences in the channel's environment which modulate its gating and sensitivity to Ca^{2+}. Alternatively, it suggests that more than one channel type exists, a hypothesis dealt with in this article.

In the bladder we extensively studied the permeability for different monovalent cations, whereas in the skin we mainly focused on movements of Na^+ through this pathway. To unequivocally establish whether or not this amiloride-insensitive Na^+ transport was (in part) the consequence of an incomplete amiloride action (Cuthbert and Wong, 1972), we performed a detailed study on the interference of mucosal Ca^{2+} with amiloride block (Desmedt, Simaels, and Van Driessche, 1991). The main conclusions from this study were that (*a*) the inhibitory potency of the diuretic is not affected by external Ca^{2+} per se, and (*b*) differences in amiloride block observed between tight epithelia (frog skin, toad urinary bladder, A6 cells) can be ascribed to the presence or absence of the cation-selective pathway. These results contradict the hypothesis that divalents, and Ca^{2+} in particular, are required to assure full inhibitory potency of amiloride. One of the divalents reported to serve as a substitute for Ca^{2+} in this respect is UO_2^{2+} (Benos, Simon, Mandel, and Cala, 1976). In our opinion, this observation most probably reflects interferences between UO_2^{2+} and the cation-selective pathway. It is the purpose of this report to cover this interaction in detail. The results of this study show that in frog skin, this divalent inhibits mainly the part of the Ca^{2+}-blockable current that is associated with the low-frequency Lorentzian (f_c = 12 Hz) reported previously (Van Driessche et al., 1991). At the same time, UO_2^{2+} reveals the presence of a high-frequency Lorentzian component that was masked by the low-frequency noise. The characteristics of the relaxation noise in the toad urinary bladder resemble those of the high-frequency Lorentzian component in frog skin.

MATERIALS AND METHODS

Tissue Preparation

This study was performed with ventral skins of frog species *Rana temporaria* and *Rana ridibunda*, and the urinary bladders of *Bufo marinus* toads. Frogs were kept at 17°C in plastic containers, whereas toads resided at room temperature on wet peat moss. The animals had free access to tap water. The tissues were dissected from doubly pithed animals and glued with the serosal surface to a Lucite ring with cyanoacryl glue (Hystoacryl; B. Braun Melsungen AG, Melsungen,

Germany) and mounted between two Lucite chamber compartments as described before (De Wolf and Van Driessche, 1986). Both the apical and basolateral compartments had a volume of 1.7 ml, and were continuously perfused at a rate of ~7 ml/min. The tissue area in contact with the bathing media was 0.5 cm^2. The chamber halves were connected to a low-noise voltage clamp through 1 M KCl/agar (3%) bridges, 1 M KCl, and Ag/AgCl wires. The transepithelial potential was clamped to zero, except during the 256-ms voltage steps of 5-mV amplitude, which were applied every 14 s to measure the transepithelial conductance (G_t). Transepithelial currents were continuously recorded on a strip-chart recorder. Short-circuit current (I_{sc}) and G_t were digitized and stored on disk for later analysis. Cation movements from mucosa to serosa are by convention represented as a positive current.

Noise Analysis

The fluctuation in current was recorded and analyzed in the frequency domain. Briefly, the current signal was high-pass filtered with a 24-dB/octave Butterworth type filter with a cut-off frequency of 0.3 Hz. Furthermore, to prevent aliasing the signal was low-pass filtered with a 48-dB/octave Butterworth filter (cut-off frequency = 850 Hz). Power density spectra (PDS) were calculated from Fourier transformed records of 4,096 points collected over a 2-s period yielding a fundamental frequency of 0.5 Hz. Averaged PDSs of 2,048 frequencies were obtained from 50 records. Frequencies >760 Hz were discarded in the further analysis. The number of spectral data points was reduced by calculating the mean of the power densities around frequencies located at equal distances on the logarithmic frequency axis. Two spectra containing 44 and 66 frequency lines were derived with five and eight frequencies per octave, respectively. The 44-line spectrum was used for representation purposes (see figures), whereas the 66-line spectrum was used for numerical analysis. Spectra containing relaxation noise were fitted with the sum of a Lorentzian and an A/f^{α} term according to the following formula:

$$S(f) = \frac{S_o}{1 + (f/f_c)^2} + \frac{A}{f^{\alpha}}$$

The plateau value (S_o) and the corner frequency (f_c) of the Lorentzian function were determined by nonlinear regression analysis.

Solutions

Highly purified water (Milli-Q: Water Purification System; Millipore Corp., Bedford, MA) was used to prepare the solutions. The serosal side of skins and bladders was perfused with Na_2SO_4 Ringer's solution, containing (mM): 115 Na^+, 58.5 SO_4^{2-}, 2.5 K^+, 2.5 HCO_3^-, and 1 Ca^{2+} (pH 8.2). In some experiments Na^+ was partially replaced with K^+ to achieve a final concentration of 37.5 mM K^+ (Fig. 5 and Table III) or 92.5 mM K^+ (Fig. 1 *B* and Table I). Bladders were treated with 0.1 U/ml oxytocin (Sigma Chemical Co., St. Louis, MO) to activate maximally the cation-selective transport (Aelvoet et al., 1988). The mucosal side was bathed with Ca^{2+}-free solutions with the following composition (mM): 115 Na^+, K^+, Cs^+, or Rb^+, 57.5 SO_4^{2-}, 5 HEPES, 0.5 EGTA (ethylene glycol-*O,O'*-bis[2-aminoethyl]-*N,N,N',N'*-tetraacetic acid). pH was adjusted to 7.5 with NaOH, KOH, or Tris. To block the highly specific Na^+ channels, 10 or 60 μM amiloride (Sigma Chemical Co.) was added to the mucosal solutions containing Na^+. The Ca^{2+}-blockable component of I_{sc} (I_{sc}^{Ca}) and the effect of Ca^{2+} on the noise spectra were measured by adding 1.5 mM Ca^{2+} to the EGTA-containing solutions (Fabiato and Fabiato, 1979). UO_2^{2+} was added as nitrate to the mucosal bath in a concentration of nominally 10 or 100 μM. The stoichiometric stability constants for EGTA (Martell and Smith, 1974) indeed indicate that uranyl (UO_2^{2+}) complexes with the chelator, and that, under our experimental conditions, only a small fraction of the divalent (<0.01%) is expected to exist as a free cation.

This fraction is calculated from the published equilibrium constant K_2 between UO_2^{2+} and EGTA.H (Martell and Smith, 1974; Fabiato and Fabiato, 1979). However, one must also take into account the occurrence of uranylhydroxy salts or even anionic uranato complexes, especially at basic pH (Katz and Seaborg, 1957). Consequently, it seems very cumbersome to determine the exact concentrations of free UO_2^{2+} and its complexes. For the purpose of this study, solving these complications was rather irrelevant, and we therefore contented ourselves with using the concentration of UO_2^{2+} in a purely operational sense. Also, no attempt is made to determine which forms of UO_2^{2+} interfere with the cation channels.

RESULTS

Stimulatory Effect of Mucosal UO_2^{2+} on the Amiloride-blockable Na^+ Transport

In the first series of experiments, we tested the influence of 100 μM mucosal UO_2^{2+} on the amiloride-blockable Na^+ channel in the presence and absence of mucosal Ca^{2+} in skins of *R. temporaria*. Experiments in the presence of Ca^{2+} were performed with tissues exposed to Na_2SO_4 solutions on both sides. To investigate the effect of UO_2^{2+} in Ca^{2+}-free conditions we depolarized the basolateral membranes with high K^+-containing solutions (92.5 mM K^+). We used this concentration because it completely abolishes the net current through the Ca^{2+}-blockable, cation-selective pathway in the apical membrane in short-circuited conditions (Desmedt et al., 1991). Consequently, in depolarized skins exposed to Ca^{2+}-free mucosal solutions, changes in I_{sc} elicited by UO_2^{2+} are expected to reflect effects on the amiloride-blockable Na^+ channels. It must be noted that the Ca^{2+} chelator EGTA was always present, in the Ca^{2+}-containing as well as in the Ca^{2+}-free mucosal Ringer's solution. In this way, comparison of the results obtained in different conditions is not biased by any effects due to the chelator itself.

Fig. 1 *A* exemplifies an experiment with a nondepolarized skin. The addition of UO_2^{2+} induced an increase of I_{sc} from 22.0 to 27.5 μA/cm^2. Both the control and the stimulated current were amiloride blockable (10 μM), indicating an effect of UO_2^{2+} on the highly selective Na^+ channels. Fig. 1 *B* represents a similar experiment with a depolarized tissue. Again I_{sc} increased markedly with mucosal UO_2^{2+}, in this case from 23.2 to 34.8 μA/cm^2. As in nondepolarized skins, UO_2^{2+} acted on the Na^+ channel specifically since the current stimulated by UO_2^{2+} was amiloride blockable (10 μM) as well. Mean values in Table I demonstrate that UO_2^{2+} elicits a significant ($P < 0.01$) increase of I_{sc} in the depolarized as well as the nondepolarized skins. From the result obtained with depolarized skins, it is furthermore obvious that the removal of mucosal Ca^{2+} does not affect the inhibitory potency of amiloride. So far, our data indicate that UO_2^{2+} displays a specific stimulatory effect on the amiloride-blockable Na^+ channel.

Inhibitory Effect of Mucosal UO_2^{2+} on Na^+ Currents through the Poorly Selective, Ca^{2+}-blockable Pathway

The influence of mucosal UO_2^{2+} on Na^+ transport through the poorly selective cation pathway was investigated in nondepolarized skins exposed to Ca^{2+}-free mucosal Na_2SO_4 Ringer's solution containing amiloride. To avoid contaminations of our noise spectra with amiloride-induced noise, we increased the concentration of the diuretic

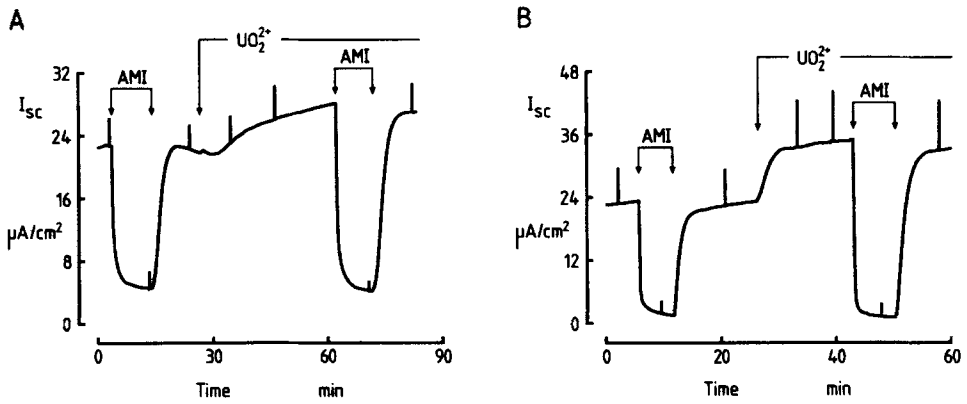

FIGURE 1. Effect of UO_2^{2+} (100 μM) on amiloride-blockable Na^+ currents through frog skin (*R. temporaria*). The amiloride-blockable current was measured by adding 10 μM of the diuretic to the mucosal Na_2SO_4 solution. (*A*) Recording of I_{sc} in nondepolarized skin exposed to mucosal Ca^{2+}-containing Na_2SO_4 solution. (*B*) Ca^{2+} was removed from the mucosal bath and the basolateral membranes were K^+ depolarized (92.5 mM K^+). Here, as well as in the following figures, the vertical current deflections are caused by voltage pulses of 10 mV.

to 60 μM. In the experiment depicted in Fig. 2*A*, the amiloride-insensitive I_{sc} amounted to 9.8 μA/cm². UO_2^{2+} had a different effect on I_{sc} as compared with the response shown in Fig. 1. The amiloride-insensitive current, instead of being stimulated, was reduced to 2.0 μA/cm². The remaining current component was further depressed to 0.9 μA/cm² with Ca^{2+}. The washout of UO_2^{2+} did not restore I_{sc} (unpublished observation). Mean values in Table II demonstrate a significant ($P < 0.01$) inhibition of the amiloride-insensitive I_{sc} by UO_2^{2+}.

The most interesting finding so far is that UO_2^{2+} is able to inhibit Na^+ currents through the poorly selective cation pathway, whereas it stimulates Na^+ movements through the amiloride-blockable channels. The latter process has been reported to be reversible (Zeiske, 1978), whereas our results on the Ca^{2+}-blockable pathway demon-

TABLE I

Effect of UO_2^{2+} on the Amiloride-blockable Na^+ Currents

	Nondepolarized	Depolarized
Control	16.5 ± 1.8	20.3 ± 3.7
Amiloride	1.7 ± 0.8*	0.5 ± 1.7*
UO_2^{2+}	23.2 ± 1.9*	31.7 ± 5.8*
UO_2^{2+} + amiloride	1.9 ± 0.9‡	0.9 ± 1.4‡

I_{SC} (μA/cm²) values recorded while skins (*R. temporaria*) were serosally perfused with either Na_2SO_4 (nondepolarized) or K_2SO_4 (92.5 mM K^+ depolarized). The mucosal solution was Na_2SO_4 with or without Ca^{2+} for nondepolarized or depolarized tissues, respectively. Amiloride concentration, 10 μM; UO_2^{2+}concentration, 100 μM. $n = 5$. All values are given as means ±SEM.

*Values significantly different from control at $P < 0.05$.

‡Values significantly different from the preceding experimental conditions at $P < 0.05$.

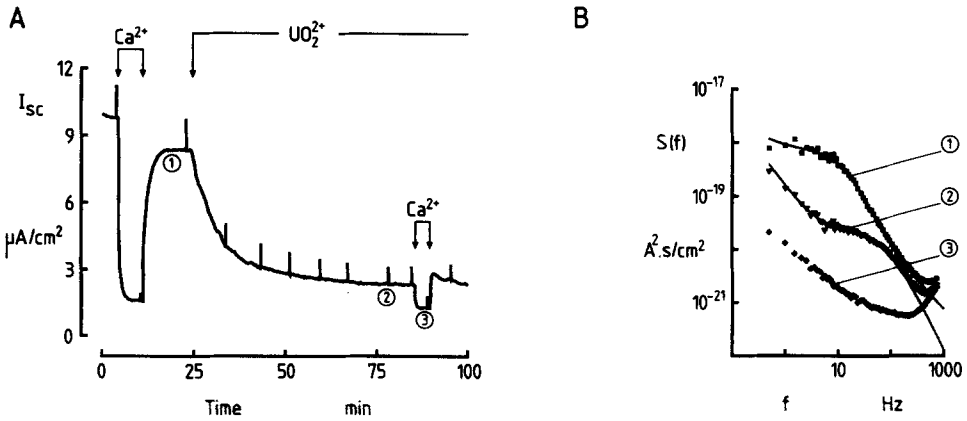

FIGURE 2. Inhibition of I_{sc}^{Ca} and the low-frequency Lorentzian noise by 100 μM mucosal UO_2^{2+}. The skin of *R. temporaria* was bilaterally exposed to Na_2SO_4 solutions. The mucosal bath contained 60 μM amiloride. 1.5 mM Ca^{2+} was added to the EGTA-containing mucosal solution to measure I_{sc}^{Ca}. PDSs in *B* were recorded at the time intervals marked at the current trace in *A*. UO_2^{2+} depresses the low-frequency Lorentzian and unmasks a high-frequency Lorentzian.

strate an irreversible effect. The data in Table II demonstrate that 1 mM Ca^{2+} exerts an additional inhibitory effect in the presence of 100 μM UO_2^{2+}, which might indicate that this dose is too low. Higher amounts of UO_2^{2+} (up to 1 mM) do indeed reduce the Ca^{2+}-blockable I_{sc} further (unpublished observation). However, the results from noise analysis of the UO_2^{2+} block, described below, suggest that the inhibition obtained with higher doses occurs through occlusion of another pathway: up to 100 μM, UO_2^{2+} has a specific inhibitory interaction with only part of the cation-selective channels, whereas concentrations near 1 mM affect all channels, thereby distinguishing between two channel types.

In previous work (Van Driessche et al., 1991), we established that Ca^{2+} removal from the mucosal bath markedly raises noise levels. A Lorentzian component with f_c values in the range of 10–20 Hz was frequently present in the PDS. In this article we will refer to these Lorentzians as low-frequency noise components. The example

TABLE II

Effect of UO_2^{2+} on the Poorly Selective Cation Pathway

Control	15.5 ± 2.9
Amiloride	7.6 ± 1.6*
Amiloride + UO_2^{2+}	3.4 ± 0.8‡
Amiloride + UO_2^{2+} + Ca^{2+}	0.3 ± 0.3‡

I_{sc} (μA/cm²) values were recorded in nondepolarized skins (*R. temporaria*) bathed with Ca^{2+}-free Na_2SO_4 solution on the mucosal side. Amiloride concentration, 60 μM; UO_2^{2+} concentration, 100 μM. n = 6.

All values are given as means ± SEM.

*Values significantly different from control at $P < 0.05$.

‡Values significantly different from the preceding experimental conditions at $P < 0.05$.

demonstrated in Fig. 2 *B* originates from the same experiment as the I_{sc} recording shown in Fig. 2 *A*. The control spectrum contains a Lorentzian component with f_c = 11.4 Hz and S_o = 641 × 10^{-21} A^2·s/cm^2, which is irreversibly depressed by 100 μM UO_2^{2+}. Concomitantly, another relaxation noise component appears at higher frequencies in the PDS. For the experiment shown, the PDS recorded with UO_2^{2+} was fitted with the Lorentzian parameters f_c = 78.4 Hz and S_o = 20.4 × 10^{-21} A^2·s/cm^2. Subsequent administration of Ca^{2+} abolishes this noise component. The appearance of the high-frequency component after UO_2^{2+} is in striking contrast to the complete inhibitory effect of Ca^{2+} (Fig. 2 *B*, and Van Driessche et al., 1991). Means of f_c and S_o together with the corresponding I_{sc}^{Ca} values are listed in Table III (low-frequency component [2.5 K^+]). They convey the idea that UO_2^{2+} inhibits a fluctuating channel

TABLE III

Effect of 10 and 100 μM UO_2^{2+} on the Lorentzian Parameters and the Current of the Ca^{2+}-blockable Channel Types

	n	f_c	$S_o \times 10^{21}$	I_{sc}^{Ca}
		Hz	*A^2·s/cm^2*	*μA/cm^2*
Low-frequency component (2.5 K^+)				
Control	6	11.7 ± 1.0	542.3 ± 139.9	14.2 ± 7.2
10 μM UO_2^{2+}	6	—	—	—
100 μM UO_2^{2+}	6	58.5 ± 4.6*	27.4 ± 6.2*	2.3 ± 0.5*
Low-frequency component (37.5 K^+)				
Control	5	16.0 ± 1.7	121.5 ± 34.9	1.8 ± 0.5
10 μM UO_2^{2+}	4	17.6 ± 1.2	26.5 ± 12.6*	0.4 ± 0.1*
100 μM UO_2^{2+}	5	16.6 ± 2.6	6.0 ± 2.3*	0.2 ± 0.0*
High-frequency component (2.5 K^+)				
Control	10	100.4 ± 6.9	49.0 ± 7.5	10.2 ± 1.6
10 μM UO_2^{2+}	5	98.7 ± 12.6	45.1 ± 8.3	4.3 ± 0.8*
100 μM UO_2^{2+}	10	96.4 ± 7.7	32.5 ± 4.4*	2.5 ± 0.4*

Experiments with skins of *R. temporaria* exposed to Ca^{2+}-free Na_2SO_4 solution mucosally. In the category marked "low-frequency component (37.5 K^+)", 100 μM tetracaine was added to the mucosal bathing solution (see text).

All values are given as means ± SEM.

*Values significantly different from control at $P < 0.05$.

associated with the low-frequency Lorentzian noise (channel type S) reported previously (Van Driessche et al., 1991), and concomitantly reveals the presence of another channel type that generates a high-frequency Lorentzian noise component (channel type F).

Evidence for Two Different Ca^{2+}-blockable Channel Types

As a matter of fact, whereas some tissues display low-frequency Lorentzians, others provide high-frequency Lorentzian components in the absence of mucosal UO_2^{2+}. An example is depicted in Fig. 3. The control spectrum in Fig. 3 *B* consists of substantial $1/f$ noise in the lower frequency part, and a relaxation noise at the higher frequency end with f_c = 135.1 Hz and S_o = 56.6 × 10^{-21} A^2·s/cm^2. The addition of 10 μM

UO_2^{2+} in this case reduces the $1/f$ noise, but leaves the Lorentzian component almost unaltered. Further elevation of the UO_2^{2+} concentration to 100 μM does not change the PDS. Finally, the addition of Ca^{2+} to the mucosal side completely abolishes the relaxation noise, and depresses $1/f$ noise further. Basically, this experiment demonstrates that the influence of UO_2^{2+} on the spontaneous high-frequency Lorentzian is rather limited. I_{sc}^{Ca}, on the other hand, is markedly reduced with 10 μM UO_2^{2+} (Fig. 3 *A*). The elevation of the UO_2^{2+} concentration to 100 μM merely results in a slight additional inhibition of I_{sc} only. Mean values obtained from experiments with this kind of skins (high-frequency component data in Table III) confirm that 10 μM UO_2^{2+} significantly ($P < 0.05$) decreases I_{sc}^{Ca} without altering S_o or f_c ($P > 0.25$). The increase of the dose to 100 μM has a significant ($P < 0.01$), but relatively small (34%), inhibitory effect on the S_o of the high-frequency Lorentzian (compared with the 86% inhibition of the low-frequency Lorentzian in Fig. 5 *B*). The apparent

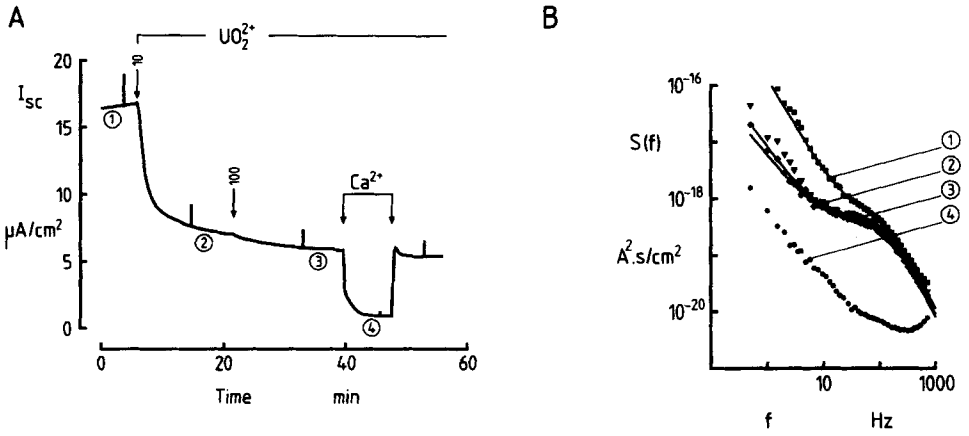

FIGURE 3. Effect of 10 and 100 μM UO_2^{2+} on I_{sc}^{Ca} and the high-frequency Lorentzian noise component. As in Fig. 2, we used the skins of *R. temporaria* perfused with Na_2SO_4 on both sides. Amiloride was present in the mucosal bath. The PDSs in *B* were recorded during the experiment shown in *A* at time intervals marked with numbers at the current trace.

discrepancy between the effect of UO_2^{2+} on I_{sc} and S_o can be understood if one assumes that the $1/f$ noise observed in the control spectrum of Fig. 3 *B* is at least partly related to currents passing through the UO_2^{2+}-blockable channels described before. The observation that the depression of the $1/f$ noise by 10 μM UO_2^{2+} is accompanied by a marked reduction of I_{sc} favors this assumption. Additional support comes from experiments with tissues where $1/f$ noise is not expressed, or only poorly expressed. In these tissues the high-frequency Lorentzians (Fig. 4 *B*), as well as I_{sc}^{Ca} (Fig. 4 *A*), are insensitive to UO_2^{2+}. These findings clearly demonstrate that cation-selective channels with fast open-closed kinetics (type F) are not blocked by 100 μM UO_2^{2+}. The data also show that the lack of UO_2^{2+}-inhibitable $1/f$ noise is correlated with the absence of UO_2^{2+}-blockable currents.

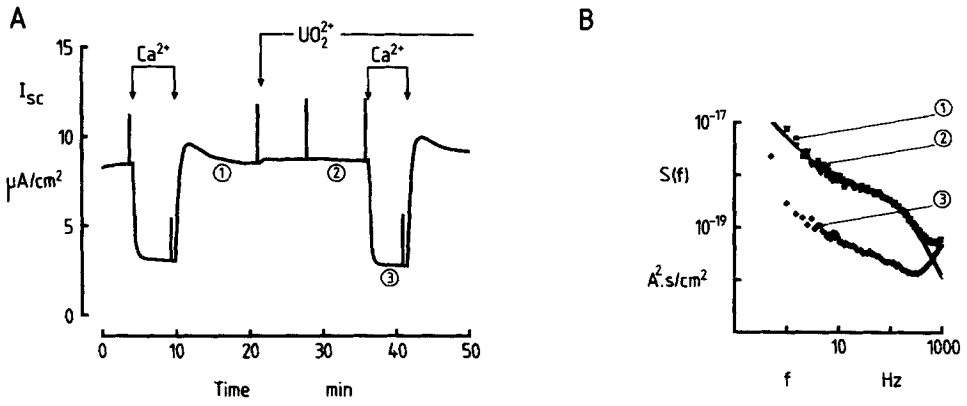

FIGURE 4. Effect of 100 μM UO_2^{2+} on I_{sc}^{Ca} and the high-frequency Lorentzian component. Experiment with the skin of *R. temporaria* perfused with Na_2SO_4 on both sides. Amiloride was given mucosally. In this experiment the low-frequency Lorentzian noise was absent in the PDS and UO_2^{2+} did not influence I_{sc}.

UO_2^{2+} Blocks the Slowly Gated Process and Does Not Induce Blocker Noise

In many experimental protocols, the presence of a $1/f$ noise component considerably hampers the study of the Lorentzian noise associated with currents passing through the Ca^{2+}-blockable pathway. For example, the extent to which the low-frequency Lorentzian is depressed with UO_2^{2+} cannot be assessed because quite early in the course of this inhibitory process the low-frequency Lorentzian is masked by $1/f$ noise. Ideally, the investigation of direct effects of UO_2^{2+} on the low-frequency Lorentzian requires low levels of $1/f$ noise. We found that a moderate elevation of serosal K^+

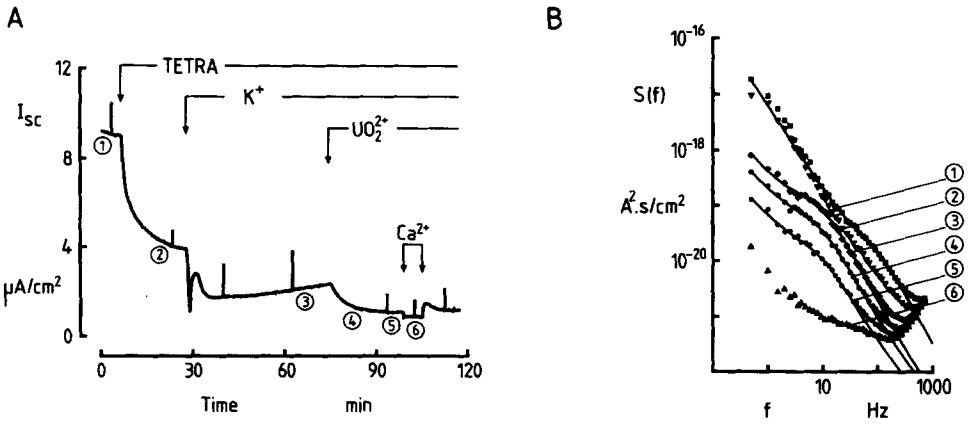

FIGURE 5. Effect of 100 μM UO_2^{2+} on the low-frequency Lorentzian after abolishing the $1/f$ noise by partial K^+ depolarization and blocking the high-frequency Lorentzian by 100 μM tetracaine. Partial K^+ depolarization was achieved by replacing 35 mM serosal Na^+ by K^+. UO_2^{2+} depressed the low-frequency Lorentzian gradually without inducing additional noise. Mucosal Ca^{2+} blocked the Lorentzian component completely.

(37.5 mM) diminishes the $1/f$ amplitudes and unmasks the low-frequency Lorentzian noise (Fig. 5 *B*). A high-frequency Lorentzian with f_c = 93.9 Hz and S_o = 18.9 × 10^{-21} $A^2 \cdot s/cm^2$ is still detectable above the otherwise substantial background noise levels. To investigate the effects on the low-frequency Lorentzian only, we depressed the high-frequency relaxation noise by adding 100 μM tetracaine to the mucosal bath. For the moment, the comparison of spectra 1 and 2 in Fig. 5 *B* should suffice to convince the reader, but in the companion paper (Desmedt, Simaels, and Van Driessche, 1993) we will demonstrate in more detail that this concentration of the local anesthetic indeed selectively abolishes the high-frequency noise component. The subsequent replacement of 35 mM Na^+ in the serosal bath by K^+ depressed the $1/f$ noise and revealed the presence of a low-frequency Lorentzian with f_c = 10.4 Hz and S_o = 127 × 10^{-21} $A^2 \cdot s/cm^2$. The mean values for both S_o and I_{sc}^{Ca} are much lower than in 2.5 serosal K^+ (Table III), which is probably due to a diminished electrical

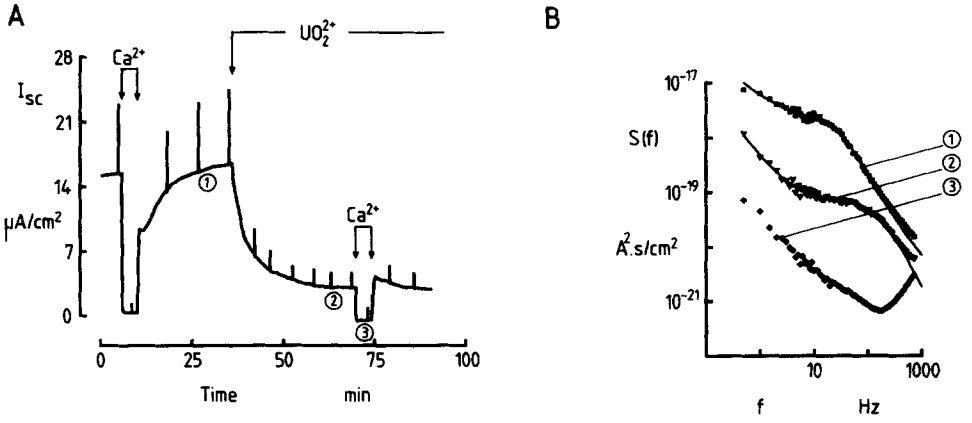

FIGURE 6. Effect of 100 μM UO_2^{2+} on the Ca^{2+}-blockable K^+ current and noise in *R. ridibunda*. Tissues were perfused with Ca^{2+}-free K_2SO_4 and regular Na_2SO_4 solution on the mucosal and serosal sides, respectively. UO_2^{2+} depresses I_{sc}^{Ca} and the low-frequency Lorentzian noise. As in Fig. 2 *B*, the high-frequency relaxation noise is unmasked by UO_2^{2+}.

driving force caused by the K^+ depolarization. The addition of 100 μM UO_2^{2+} further inhibited I_{sc}^{Ca}, and the time-dependent changes in the Lorentzian noise could now be quantified. Clearly, the plateau value of the low-frequency Lorentzian was lowered with UO_2^{2+} to 17.4 × 10^{-21} $A^2 \cdot s/cm^2$ or less, but f_c essentially remained constant. Table III lists the mean values for I_{sc}^{Ca}, f_c, and S_o in control, with 10 and 100 μM UO_2^{2+}. This experiment shows that UO_2^{2+} merely depresses S_o without inducing additional current fluctuation or shifting the f_c. Thereore, the high-frequency component unmasked by UO_2^{2+} (Fig. 2 *B*) cannot be identified with UO_2^{2+}-induced blocker noise. Taken together with the irreversible character of the UO_2^{2+} effect (see above), this result strongly suggests that UO_2^{2+} exerts its blocking action on the low-frequency component by a reduction in the number of conductive units, caused by an irreversible occlusion of the channel pores, and not by changing the open probability of the channels.

Effect of UO_2^{2+} on K^+ Currents through the Poorly Selective Ca^{2+}-blockable Channels

To further substantiate the inhibitory action of UO_2^{2+} on the poorly selective cation pathway and the separation of this latter into two components, we tested its effect on other cationic currents passing through the Ca^{2+}-blockable pathways. The experiments with K^+ as the main mucosal cation were performed on skins of *R. ridibunda*. In this frog species the cation-selective pathway is also expressed, whereas K^+ channels as found in the apical membrane of the skin of *R. temporaria* (De Wolf and Van Driessche, 1986) are usually not detectable with noise analysis. Consequently, Ca^{2+}-blockable noise components recorded with K^+ are not expected to be seriously contaminated by the presence of the Ca^{2+}-insensitive K^+ channels. In the experiment shown in Fig. 6, the Ca^{2+}-blockable K^+ current through the cation-selective pathway amounted to 16.9 $\mu A/cm^2$ and yielded a spontaneous Lorentzian noise component

TABLE IV

Lorentzian Parameters and Ca^{2+}-blockable Current with Mucosal K^+ and Cs^+ Solutions

	n	f_c	$S_o \times 10^{21}$	I_{sc}^{Ca}
		Hz	$A^2 \cdot s/cm^2$	$\mu A/cm^2$
K^+ (*R. ridibunda*)				
Control	5	27.6 ± 1.5	1045.7 ± 437.0	9.3 ± 2.4
UO_2^{2+}	5	122.3 ± 14.6*	35.8 ± 9.3*	2.1 ± 0.6*
K^+ (toad urinary bladder)				
Control	4	406.5 ± 8.3	13.0 ± 3.0	9.3 ± 1.8
UO_2^{2+}	4	441.3 ± 17.2	18.6 ± 4.4	7.2 ± 1.4
Cs^+ (*R. ridibunda*)				
Control	5	65.4 ± 4.3	50.5 ± 6.7	3.5 ± 0.6
UO_2^{2+}	5	82.9 ± 8.1	28.3 ± 2.3*	2.1 ± 0.4*
Cs^+ (*R. temporaria*)				
Control	4	75.1 ± 4.3	39.9 ± 6.2	1.7 ± 0.2
UO_2^{2+}	4	86.9 ± 9.3	25.7 ± 3.4*	1.0 ± 0.2*

UO_2^{2+} concentration, 100 μM.

All values are given as means ± SEM.

*Values significantly different from control at $P < 0.05$.

with f_c = 30.5 Hz and S_o = 1,990 × 10^{-21} $A^2 \cdot s/cm^2$ in control conditions. After administration of UO_2^{2+}, I_{sc}^{Ca} reached a steady-state value of 3.4 $\mu A/cm^2$. This experiment also demonstrates that UO_2^{2+} exerts its inhibitory effect most obviously independent of amiloride since the diuretic was absent (see Discussion). Concomitantly, the low-frequency Lorentzian was gradually depressed by UO_2^{2+}, revealing another Lorentzian with higher f_c (f_c = 166.0 Hz; $S_o = 66.5 \times 10^{-21}$ $A^2 \cdot s/cm^2$), which was readily inhibitable with Ca^{2+}. Therefore, also with K^+, the use of UO_2^{2+} seemed to indicate and distinguish between two different gating processes. Means of I_{sc}^{Ca} and the Lorentzian parameters are listed in Table IV. A few additional experiments with Rb^+ as the main mucosal cation yielded similar results (unpublished observation).

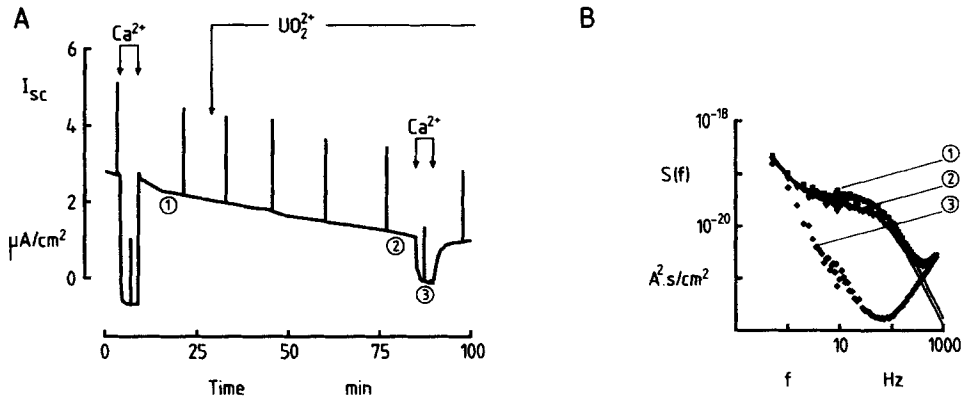

FIGURE 7. Effect of 100 μM UO_2^{2+} on the Ca^{2+}-blockable Cs^+ current and noise in *R. ridibunda*. Mucosal solution: Ca^{2+}-free Cs_2SO_4; serosal solution: Na_2SO_4.

Ca^{2+}-blockable Cs^+ Currents and Noise Are Insensitive to UO_2^{2+}

Finally, we investigated the permeability of the cation-selective pathway for Cs^+ in both *R. ridibunda* and *R. temporaria*. The most remarkable finding was that Cs^+ currents through the cation-selective channels generate relaxation noise components in the high-frequency range of the PDS (e.g., Fig. 4 *B*), whereas the low-frequency Lorentzian was never observed. An example for *R. ridibunda* is shown in Fig. 7 (f_c = 68.4 Hz; $S_o = 36.1 \times 10^{-21}$ A²·s/cm²; I_{sc}^{Ca} = 2.2 μA/cm²). We recorded the effect of UO_2^{2+} for ~60 min. During this time period I_{sc}^{Ca} diminished very slowly to 1.2 μA/cm². This decrease occurred spontaneously, since it was also observed when skins were not exposed to UO_2^{2+} (unpublished observation). Moreover, the I_{sc} trace in Fig. 7 *A* is apparently not altered by the addition of UO_2^{2+}. Means in Table IV were calculated from values recorded at 54 ± 4 min after administration of UO_2^{2+}. The

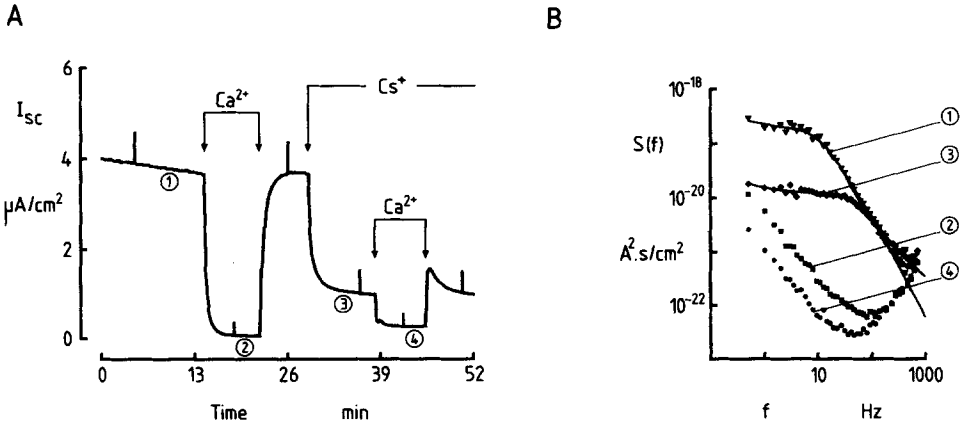

FIGURE 8. Effect of mucosal Na^+ by Cs^+ replacement on the Ca^{2+}-blockable current and noise in *R. temporaria*.

Lorentzian noise also generally underwent gradual changes. In the illustrated experiment f_c varied from 68.4 to 70.3 Hz, while S_o decreased from 36.1 to 23.5 × 10^{-21} $A^2 \cdot s/cm^2$. Spontaneous changes in tissues not subjected to UO_2^{2+} were of comparable magnitude (unpublished observation). Means of the noise parameters and I_{sc}^{Ca} are summarized in Table IV. Although an influence of UO_2^{2+} on the gating kinetics cannot be denied, it is clear that the divalent brings about only a minor modification as compared with the effect on the low-frequency gating process observed with Na^+, K^+, and Rb^+.

The failure to observe low-frequency Lorentzian noise with Cs^+ strongly suggests that type S channels are not permeable for this cation. This idea is further corroborated by the type of experiment shown in Fig. 8. Initially, the mucosal side was perfused with Ca^{2+}-free Na^+ solution. Under these conditions, I_{sc}^{Ca} amounted to 3.6 μA/cm² (Fig. 8 *A*). The PDS of the fluctuation in I_{sc} contained a low-frequency

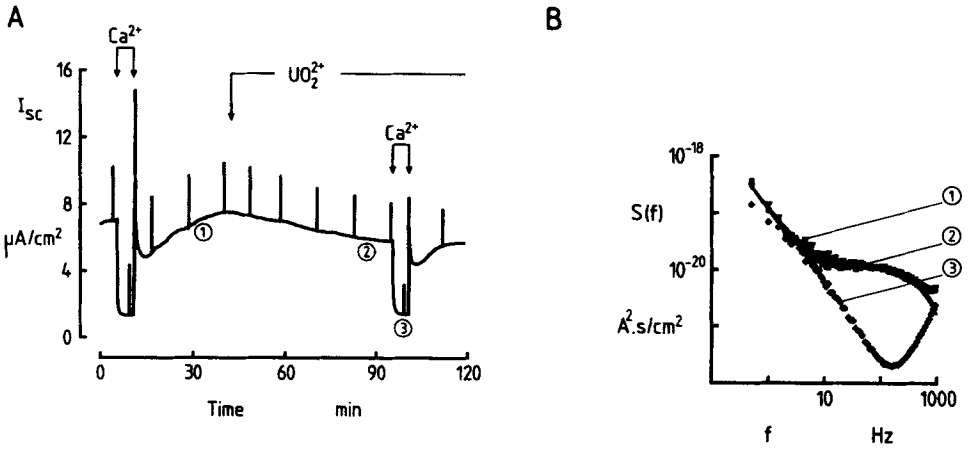

FIGURE 9. Effect of 100 μM UO_2^{2+} on the Ca^{2+}-blockable K^+ current and noise in toad urinary bladder. Mucosal solution: Ca^{2+}-free K_2SO_4; serosal solution: Na_2SO_4 + 0.1 U/ml oxytocin.

Lorentzian with f_c = 12.5 Hz and S_o = 181 × 10^{-21} $A^2 \cdot s/cm^2$ (Fig. 8 *B*). Upon substitution of Cs^+ by Na^+, I_{sc}^{Ca} decreased to 0.8 μA/cm². Concomitantly, the low-frequency Lorentzian was depressed and a high-frequency Lorentzian appeared in the PDS (f_c = 67.1 Hz, S_o = 12.2 × 10^{-21} $A^2 \cdot s/cm^2$). Mean values (n = 5) of I_{sc}^{Ca}, f_c, and S_o in Na^+ solutions were 6.9 ± 1.9 μA/cm², 16.2 ± 1.0 Hz, and 555 ± 163 × 10^{-21} $A^2 \cdot s/cm^2$, respectively. With mucosal Cs^+, I_{sc}^{Ca}, f_c, and S_o amounted to 2.2 ± 0.7 μA/cm², 75.7 ± 4.3 Hz, and 38.3 ± 11.1 × 10^{-21} $A^2 \cdot s/cm^2$, respectively.

Ca^{2+}-blockable Currents and Noise in Toad Urinary Bladder Are UO_2^{2+} Insensitive

The poorly selective cation pathway in toad urinary bladder provides another system to study the effect of UO_2^{2+} (Fig. 9). In this series of experiments bladders were

bathed with Ca^{2+}-free K_2SO_4 Ringer's solution at the mucosal side and with Na_2SO_4 Ringer's solution at the serosal side. Effects of UO_2^{2+} were studied on K^+ currents, because previous studies (Aelvoet et al., 1988) demonstrated that the Lorentzian noise was generally larger with mucosal K^+ than with Na^+ or Cs^+. K^+ currents and noise were stimulated by 0.1 U/ml oxytocin added to the serosal bath. This resulted in control values for I_{sc}, f_c, and S_o of 7.5 $\mu A/cm^2$, 420.6 Hz, and 10.9×10^{-21} $A^2 \cdot s/cm^2$, respectively. About 1 h after the addition of 100 μM UO_2^{2+}, I_{sc} reached a value of 5.9 $\mu A/cm^2$, while f_c equalled 450.9 Hz and S_o became 12.0×10^{-21} $A^2 \cdot s/cm^2$. Mucosal Ca^{2+} reduced I_{sc} to 1.5 $\mu A/cm^2$ and completely abolished the Lorentzian noise. Means of currents and noise data are included in Table IV.

DISCUSSION

The incentive for these experiments with UO_2^{2+} has come from the observation by Benos et al. (1976) that this divalent cation effectively substitutes for mucosal Ca^{2+} in restoring the complete inhibition of Na^+ transport by amiloride. On the other hand, a noise analysis study (Desmedt et al., 1991) on the interference between Ca^{2+} and amiloride revealed that the molecular interaction of the diuretic with the highly specific Na^+ channels is not affected by external Ca^{2+} at all. We ascribed the apparent reduced inhibitory potency, as inferred from I_{sc} measurements, to the presence of conductive cation-selective channels in parallel with the highly specific Na^+ channels. We further reasoned that the result with UO_2^{2+} obtained by Benos and co-workers (1976) therefore probably reflected an interaction of UO_2^{2+} with the Ca^{2+}-blockable pathway.

Inhibitory Effect of UO_2^{2+} on Ca^{2+}-blockable Na^+ Currents

The crucial finding in this study is the inhibition of Na^+ transport by UO_2^{2+} in the absence of Ca^{2+} and in the presence of amiloride (Fig. 2 *A*). On the basis of our previous work, however (Desmedt et al., 1991), the hypothesis of an improved amiloride block by UO_2^{2+} in the absence of Ca^{2+} must be rejected. Moreover, an inhibition of I_{sc} with UO_2^{2+} is also observed in the absence of amiloride in frog skins exposed to Ca^{2+}-free mucosal K_2SO_4 Ringer's solution (Fig. 6 *A*). This demonstrates that it is quite unnecessary to invoke an involvement of amiloride in order to understand the inhibitory effect of UO_2^{2+} on Na^+ transport. The obvious conclusion instead is that the cation-selective channels themselves are blocked by UO_2^{2+}. This is a rather interesting finding in view of the stimulatory action of the divalent on the Na^+ channels (Zeiske, 1978; Benos, Latorre, and Reyes, 1981). It is important, then, to know whether or not the interaction site for UO_2^{2+} is closely associated with the channel proteins, since it is equally conceivable that the divalent exerts its effect on the cation-selective channels indirectly. More specifically, whereas the study of the cation-selective channel entails the use of Ca^{2+}-free mucosal Ringer's solutions containing a Ca^{2+} chelator (EGTA), the Na^+ channel has usually been studied in Ca^{2+}-containing mucosal Na^+ Ringer's solution lacking a Ca^{2+}-chelating agent. Consequently, the observed inhibitory behavior could possibly arise from (*a*) the

chelator itself influencing membrane structure and/or intracellular fluid composition; (*b*) the interaction of the chelator with UO_2^{2+}; or (*c*) the absence of external Ca^{2+} itself changing membrane structure and leading, for instance, to changes in intracellular Ca^{2+}. For this reason, we looked at the effect of UO_2^{2+} on the Na^+ channel with special emphasis on the composition of the bathing solutions. The data (Fig. 1, Table I) show that, as far as the Na^+ channel is concerned, the presence of EGTA per se, its chelating effect on UO_2^{2+}, the absence of Ca^{2+} per se, and, finally, the depolarized state of the tissue do not convert a stimulatory behavior into an inhibitory one. Thus, changes in the experimental conditions alone, without an essential involvement of the channels themselves, probably do not suffice to explain the different response observed for the amiloride-blockable Na^+ pathway and the Ca^{2+}-blockable pathway. It must be mentioned that an inhibitory effect of UO_2^{2+} on active Na^+ transport in frog skin has also been reported (Schwartz and Flamenbaum, 1976; Benos et al., 1981). However, close inspection of the bathing solutions utilized by these groups (Schwartz and Flamenbaum, 1976; Zeiske, 1978; Benos et al., 1981) reveals that stimulatory behavior is observed with the SO_4^{2-} anion, whereas the presence of Cl^- leads to inhibitory behavior of UO_2^{2+}. With respect to the Ca^{2+}-blockable pathway, no such effect was observed. UO_2^{2+} always displays inhibitory behavior on this pathway.

UO_2^{2+} Does Not Induce Blocker Noise of the Type S Channels

More convincing arguments in favor of a direct interaction between UO_2^{2+} and the cation-selective channel proteins come from the noise analysis data. In previous work we showed that the presence of the cation-selective channels was often, though not always, associated with the detection of a spontaneous Ca^{2+}-blockable relaxation noise component with a value for f_c of ~10–20 Hz (Van Driessche et al., 1991), referred to as a low-frequency Lorentzian. In this study we extended this finding and reported that spontaneous Lorentzian components with substantially higher f_c values (60–120 Hz) also occur (Fig. 3 *B* and Table III). At this point it may be useful to digress on the relative occurrence of low- and high-frequency Lorentzians. Experiments for this study have been performed during a 2-yr period, using animals from different batches. We compiled the statistics on such a vast basis because different batches of animals sometimes conspicuously differ from one another with respect to the occurrence of low- or high-frequency Lorentzians. We determined the number of low- and high-frequency Lorentzians in skins subjected to Ca^{2+}-free mucosal Na^+-Ringer solutions containing amiloride. Out of a total of 205 experiments taken into consideration, 96 experiments yielded a PDS without an apparent Lorentzian feature, 58 experiments provided a low-frequency Lorentzian, 49 experiments produced a high-frequency Lorentzian, and 2 experiments allowed fits with a double Lorentzian. However, despite the fact that only very rarely was a double Lorentzian visible, almost every tissue (90%) did express both channel types, as can be inferred from its sensitivity to both UO_2^{2+} and tetracaine. We found that PDSs containing a low-frequency Lorentzian were markedly influenced by UO_2^{2+}. Indeed, 100 μM of this divalent caused the disappearance of the original Lorentzian component together with a substantial reduction of background noise, so as to reveal another Lorentzian component with higher f_c (Figs. 2 *B* and 6 *B*, and Table III). On the other hand, PDSs

displaying a high-frequency Lorentzian feature were not essentially affected, in that the divalent, at a concentration of 100 μM, only moderately suppressed the Lorentzian already present without changing f_c (Figs. 3 *B* and 4 *B*). At this point, the important question that emerged was whether the Lorentzian with higher f_c, unmasked by UO_2^{2+} (Fig. 2 *B*), found its origin in the same gating process as the high-frequency Lorentzian observed in the absence of UO_2^{2+} (Figs. 3 *B* and 4 *B*) or, alternatively, reflected UO_2^{2+}-induced changes in the open-closed kinetics of type S channels. Solving this problem was hampered by the existence of large $1/f$ excess noise in the absence of mucosal Ca^{2+}. More precisely, under our standard experimental conditions it appeared impossible to estimate the extent to which the low-frequency Lorentzian was depressed by UO_2^{2+}, and whether or not the corner frequency shifted during this process. However, we could find a set of appropriate experimental conditions that resolved these difficulties. The result, illustrated in Fig. 5, unequivocally establishes that (*a*) 100 μM, and even 10 μM UO_2^{2+} for that matter, inhibits the low-frequency Lorentzian by 75% at least; (*b*) the open-closed kinetics of the low-frequency Lorentzian component are not modified by UO_2^{2+}; (*c*) UO_2^{2+} does not by itself induce additional fluctuation, because the low-frequency Lorentzian is suppressed without the appearance of a high-frequency Lorentzian. We conclude that the high-frequency Lorentzian unmasked by UO_2^{2+} (Figs. 2 *B* and 6 *B*) must represent an independent gating process besides the low-frequency one, and cannot be attributed to UO_2^{2+}-induced changes in the low-frequency gating process. Also, together with the irreversibility of the depression of the low-frequency Lorentzian by UO_2^{2+}, the previous result strongly suggests that the mode of action of UO_2^{2+} on type S channels consists of an irreversible binding resulting in complete occlusion of the channels. Thus, the decrease of I_{sc}^{Ca} with UO_2^{2+} results from a reduction in the number of conductive units, and not from a shift in the channels' open probability.

Two Channel Hypothesis Versus Two Gating Modes of Only One Channel Protein

So far, we argued that our data can be understood in terms of two gated channels with different kinetics. On the other hand, it is conceivable that the low- and high-frequency Lorentzians reflect different gating modes of one and the same channel type. In any case, a crucial element that has to be accounted for is that fluctuation in Cs^+ current through the cation-selective channels apparently gives rise to high-frequency Lorentzians only (Figs. 7 *B* and 8 *B*). On the other hand, the Ca^{2+}-blockable channels in toad urinary bladder give rise to relaxation noise in the high-frequency part of the PDS that is insensitive to UO_2^{2+} (Fig. 9). Therefore, they seem to belong to the same class as type F channels in frog skin. Finally, it must be repeated that a different UO_2^{2+} sensitivity between the two classes of Lorentzians shows up very clearly. 10 μM UO_2^{2+} affects only the low-frequency Lorentzian, whereas at higher concentrations both Lorentzians are depressed. We want to stress, therefore, that UO_2^{2+} cannot be viewed as an all-or-none agent, but that in a certain range of concentrations it allows us to distinguish between two gating processes. The observation that higher amounts of UO_2^{2+} inhibit both gating processes might be interpreted in favor of one channel type with two gating modes. The data with Cs^+ and with toad urinary bladder, on the other hand, favor the view that two different

channel types are present in the frog skin. A high-frequency component apparently exists in its own right in toad urinary bladder as well as in frog skin (Fig. 4), suggesting that a separate entity is associated with this kind of noise. It is not straightforward to assume that one and the same channel would display two gating modes in frog skin and only one in toad urinary bladder. Also, the result with Cs^+ is more easily understood in terms of two channel types, one permeable (type F), the other impermeable (type S) to Cs^+, than in terms of two modes of operation of the channel, one permitting Cs^+ to pass and the other not. In the following paper (Desmedt et al., 1993), further differences in the pharmacology of both components will be established. Recapitulating, although it cannot strictly be proved, we favor the view of two classes of Ca^{2+}-blockable, cation-selective channels in the frog skin, distinguished by their spontaneous gating kinetics, their sensitivity to UO_2^{2+}, and their permeability for Cs^+.

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