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

\textit{Tetracaine Blocks the UO\textsubscript{2}\textsuperscript{2+}-insensitive Pathway}

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\textbf{Abstract} We examined the effect of the local anesthetic tetracaine on the Ca\textsuperscript{2+}-blockable, poorly selective cation channels in the isolated skin of \textit{Rana temporaria} and the urinary bladder of \textit{Bufo marinus} using noise analysis and microelectrode impalements. Experiments with frog skin demonstrated that mucosal concentrations of the compound up to 100 \textmu M did not affect the Na\textsuperscript{+} current through type S channels (slowly fluctuating, UO\textsubscript{2}\textsuperscript{2+}-blockable channels) and the associated noise. On the other hand, 20 \textmu M mucosal tetracaine already suffices to inhibit \sim 50\% of the current carried by Cs\textsuperscript{+} and Na\textsuperscript{+} through channel type F (fast fluctuating, UO\textsubscript{2}\textsuperscript{2+}-insensitive channel) and \sim 50\% of the associated Lorentzian component. With 100 \textmu M of the inhibitor the current and \sim 70\% of the associated noise were reduced by at least 70–80\%. The time course of the response to serosal tetracaine was markedly slower and the effects on the current and \sim 70\% of the associated noise were smaller. Possible effects on the basolateral K\textsuperscript{+} conductance were excluded on the basis of the lack of response of transepithelial K\textsuperscript{+} movements to 100 \textmu M tetracaine. UO\textsubscript{2}\textsuperscript{2+} and tetracaine together blocked the poorly selective cation pathways almost completely. Moreover, both agents retain their inhibitory effect in the presence of the other. In toad urinary bladder, the Ca\textsuperscript{2+}-blockable channel is also tetracaine blockable. The concentration required for half-maximal inhibition is \sim 100 \textmu M in SO\textsubscript{4}\textsuperscript{2-} and \sim 20 \textmu M in Cl\textsuperscript{-}. The data with tetracaine complement those obtained with UO\textsubscript{2}\textsuperscript{2+} and support the idea that the Ca\textsuperscript{2+}-blockable current proceeds through two distinct classes of cation channels. Using tetracaine and UO\textsubscript{2}\textsuperscript{2+} as channel-specific compounds, we demonstrated with microelectrode measurements that both channel types are located in the granulosum cells.

\textbf{Introduction}

The apical membrane of frog skin and toad urinary bladder contains Ca\textsuperscript{2+}-blockable channels that allow the passage of several alkali cations (Aelvoet, Erlij, and Van
Driessche, 1988; Van Driessche, Simaels, Aelvoet, and Erlij, 1988; Van Driessche, Desmedt, and Simaels, 1991). In the companion paper (Desmedt, Simaels, and Van Driessche, 1993) we argued that in frog skin Ca\(^{2+}\)-blockable short-circuit currents (\(I_{sc}^{Ca}\)) can be separated into two components proceeding through two different channel types. Channel type S has relatively slow open-closed kinetics and is almost completely occluded by 100 \(\mu\)M mucosal UO\(^{2+}\). Our data demonstrated that the UO\(^{2+}\) block results from a binding event that closes the channel irreversibly, and not from a decrease of the channel's open probability. Channel type F has faster open-closed kinetics and is markedly less sensitive to UO\(^{2+}\). Whereas both channels are permeable for Na\(^+\), K\(^+\), and Rb\(^+\), we have no indication that Cs\(^+\) passes through channel type S. On the other hand, channel type F allows the passage of Cs\(^+\) currents of approximately the same magnitude as Na\(^+\). In toad urinary bladder, we found only one Ca\(^{2+}\)-blockable pathway with characteristics resembling channel type F: permeability for Cs\(^+\), fast open-closed kinetics (Van Driessche, Aelvoet, and Erlij, 1987; Aelvoet et al., 1988) and UO\(^{2+}\) insensitivity (Desmedt et al., 1993).

This paper explores further pharmacological and electrophysiological evidence to corroborate the hypothesis of two channel types. In this respect, it was of special importance to find a compound that selectively blocked channel type F. We tested a number of tertiary amine local anesthetics such as lidocaine, procaine, and tetracaine. It is well documented that these substances and Ca\(^{2+}\) can mimic or antagonize each other in their interactions with the Na\(^+\) conductance in excitable tissue, with biomembrane phospholipids, and with artificial lipid membranes (Frankenhaeuser and Hodgkin, 1957; Aceves and Machne, 1963; Feinstein, 1964; Blaustein and Goldman, 1966; Träuble and Eibl, 1974; Jacobson and Paphahadjopoulos, 1975; Lee, 1976; Cullis and Verkleij, 1979). This initiated the idea that these agents might also be useful tools in the study of the Ca\(^{2+}\)-blockable pathway in frog skin and toad urinary bladder.

**MATERIALS AND METHODS**

**Tissue Preparation and Electrophysiological Methods**

In this study we used ventral skins of *Rana temporaria* and urinary bladders of *Bufo marinus*. The tissues were mounted between two Lucite chamber halves equipped with a continuous perfusion system. Transepithelial short-circuit currents (\(I_{sc}\)), conductances (\(G_{sc}\)), and power density spectra (PDSs) of the fluctuation in current were recorded as described in the preceding paper (Desmedt et al., 1993). In microelectrode experiments PDSs were recorded during microelectrode impalements.

Microelectrode experiments were performed with frog skin to determine the localization of the Ca\(^{2+}\)-blockable pathways. The microelectrode pipettes were pulled from glass fiber-containing capillaries (Hilgenberg GmbH, Malsfeld, Germany) on a Flaming-Brown P-87 puller (Sutter Instrument Co., Novato, CA) and filled with a 3 M KCl solution. Microelectrodes with input resistances (\(R_{in}\)) outside the range of 40–60 MΩ were discarded. Microelectrode resistance was measured during the experiment by placing the microelectrode in a differentiating network with an input capacitor of 10 pF. Triangle-shaped voltage pulses of 20-ms duration and 10-V amplitude were applied at the input and resulted in rectangle voltage pulses whose amplitude was proportional to \(R_{in}\). The tissues were impaled from the mucosal side. The microelectrode was moved by means of a stepping motor micromanipulator. Voltage divider
ratio ($\Delta V_o/\Delta V_l$), $G_t$, and $R_m$ were measured at 7-s time intervals. These measurements were interrupted during periods where we collected the PDSs. $\Delta V_o/\Delta V_l$ and $G_t$ were recorded by clamping the transepithelial potential to 20 mV for 200 ms. $\Delta V_o/\Delta V_l$ is usually assumed to represent the fractional resistance $f_{R_o}$ of the apical membrane. In the presence of phenomena such as time-variant conductance changes (Van Driessche et al., 1991) and voltage-dependent block (Van Driessche and De Wolf, 1991), however, $\Delta V_o/\Delta V_l$ deviates markedly from $f_{R_o}$. Similarly, the ratio of the transepithelial current response to the amplitude of the applied voltage step deviates in these conditions from $G_t$. This nonohmic behavior is generally observed in experiments where Ca$^{2+}$ is removed from the mucosal perfusate. We therefore have deliberately omitted $f_{R_o}$ and $G_t$ data in this report. Moreover, our main conclusions do not depend on these data, and their interpretation will be the subject of a separate study.

The validity of microelectrode impalements has been discussed extensively in the literature (Helman and Fisher, 1977; Nagel, 1978). Briefly, stability of the intracellular potential in steady-state experimental conditions and stability of the $R_m$ are necessary in order to obtain reliable data. Undoubtedly, in studies of Na$^+$ transport, the most important criterion has been that $\Delta V_o/\Delta V_l$ approaches unity in conditions where the apical Na$^+$ conductance was completely blocked. In our experiments we checked this criterion by blocking the highly selective Na$^+$ channels with amiloride and occluding the Ca$^{2+}$-blockable channels with 1 mM Ca$^{2+}$.

**Solutions**

SO$_4^{2-}$ Ringer’s solutions with the same composition as in the preceding paper (Desmedt et al., 1993) were used as serosal and mucosal perfusate for all experiments with frog skin. In some experiments with the toad bladder (Table III) we utilized mucosal KCl solution composed of (mM) 120 K$^+$, 115 Cl$^-$, 5 HEPES, and 0.5 EGTA, pH 7.5, and serosal NaCl solution containing (mM) 115 Na$^+$, 2.5 K$^+$, 1 Ca$^{2+}$, 117 Cl$^-$, and 2.5 HCO$_3^-$, pH 8.2. All mucosal solutions contained 0.5 mM EGTA. The 37.5 mM K$^+$ containing serosal solution was prepared by substitution of K$^+$ for Na$^+$ and was utilized to lower the 1/f noise levels. 60 μM amiloride (Sigma Chemical Co., St. Louis, MO) was added to the mucosal bath to block the highly selective Na$^+$ channels. 100 μM UO$^2+$ was added as nitrate to the mucosal side to block channel type S. Ca$^{2+}$-blockable short-circuit currents ($I_{sc}^{Ca}$) were measured by adding 1.5 mM Ca$^{2+}$ to the mucosal solution. Tetracaine was added to the Ringer’s solution from a 100 mM aqueous stock solution.

**RESULTS**

*The Inhibitory Effect of Mucosal and Serosal Tetracaine on Ca$^{2+}$-blockable Cs$^+$ Currents and Noise*

In the first part of this study (Desmedt et al., 1993), we submitted that Ca$^{2+}$-blockable Cs$^+$ currents predominantly, if not solely, pass through type F channels, which open and close randomly and give rise to a high-frequency Lorentzian component in the PDS. To study effects of pharmacological agents that interfere with this channel type we perfused the mucosal side with Ca$^{2+}$-free Cs$^+$ Ringer’s solution. Fig. 1 exemplifies such an experiment with the skin of *R. temporaria* in which we tested the effect of tetracaine. 20 μM of this compound, added to the mucosal bath, rather rapidly reduced $I_{sc}$ from a control value of 1.8 to 1.1 μA/cm$^2$. This decrease represents 46% of the total Ca$^{2+}$-blockable Cs$^+$ current ($I_{sc}^{Ca} = 1.5$ μA/cm$^2$). Concomitantly, the Lorentzian plateau in the PDS (Fig. 1 B) was markedly reduced from 42.3 to 21.9 × $10^{-21}$ A$^2$/s/cm$^2$. The corner frequency $f_c$ increased only slightly in this experiment,
from 74.2 to 77.3 Hz, though the mean value of \( f_c \) was significantly \((P < 0.01)\) augmented, from 74.6 to 87.7 Hz (Table I). Mean values of \( S_o \) and \( I_{sc}^{ca} \) were significantly \((P < 0.05)\) reduced to 41 and 50% of control, respectively (Table I). Increasing the tetracaine concentration to 100 \( \mu \)M evoked a further decrease of \( I_{sc} \) and \( S_o \), and a shift of \( f_c \) to higher values (Table I). For the experiment shown, the remaining \( I_{sc}^{ca} \) was 0.37 \( \mu \)A/cm\(^2\). The corresponding values for \( S_o \) and \( f_c \) were \( 5.0 \times 10^{-21} \) A\(^2\)-s/cm\(^2\) and 95.2 Hz, respectively. With 500 \( \mu \)M, \( I_{sc}^{ca} \) diminished to 0.14 \( \mu \)A/cm\(^2\) and \( S_o \) to \( 1.5 \times 10^{-21} \) A\(^2\)-s/cm\(^2\), and \( f_c \) became 111.1 Hz. However, with this dose the Lorentzians were often too close to background levels to be fitted. Therefore these data are omitted from Table I. From Fig. 1A it is clear that the reversibility of the tetracaine effect is rather poor.

In a separate group of tissues we tested for a possible sidedness of the tetracaine block by applying the compound to the serosal side. Tetracaine inhibited the Ca\(^{2+}\)-blockable \( I_{sc} \) current and Lorentzian noise from this side as well. However, the time course was much slower and the dose dependence shifted to higher concentrations, possibly due to the presence of the corial layer of the tissue. Mean values from five experiments (Table I) show that 20 \( \mu \)M tetracaine decreases \( I_{sc}^{ca} \) and \( S_o \) by only 16 and 27\%, respectively. Increasing the concentration to 100 \( \mu \)M reduces \( I_{sc}^{ca} \) and \( S_o \) further. In experiments where the serosal dose was elevated to 500 \( \mu \)M \((n = 3)\), \( I_{sc}^{ca} \) and \( S_o \) reached final values of 15 and 11\% of control, respectively (not included in Table I). A conspicuous difference with the data obtained with mucosal tetracaine is the relative constancy of \( f_c \) in the experiments with serosal tetracaine. As with mucosal application, the effect of serosal tetracaine was poorly reversible.

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**Figure 1.** Inhibition of Ca\(^{2+}\)-blockable \( I_{sc} \) current and noise in frog skin by mucosal tetracaine (TETRA). Mucosal solution: Ca\(^{2+}\)-free \( Cs_2SO_4 \) solution; serosal solution: \( Na_2SO_4 \) as used in all figures presented in this paper. 20, 100, and 500 \( \mu \)M tetracaine gradually depressed \( I_{sc}^{ca} \). PDSs in B were recorded at time intervals marked on the current trace in A. \( S_o \) decreases concomitantly with \( I_{sc}^{ca} \).
Effect of Tetracaine on Apical or Basolateral K⁺ Channels

The previous result raises questions concerning the site and the mechanism of action of tetracaine. In particular, a block of basolateral K⁺ channels, leading to a decrease in the driving force for Cs⁺ uptake, should be considered as an alternative to explain the effects of the local anesthetic, instead of a direct interaction with the poorly selective cation channels. Possible effects of tetracaine on the basolateral membrane conductance should not only influence the cation currents through the Ca²⁺-blockable channels, but also the amiloride-sensitive transepithelial Na⁺ transport and the transepithelial K⁺ currents that pass through the apical Ca²⁺-insensitive K⁺ channels in the skin of R. temporaria. We chose the latter system to test basolateral effects of tetracaine because the whole transcellular K⁺ current has to pass through the native basolateral K⁺ channels. Any effects on the basolateral K⁺ conductance should therefore show up particularly clearly in this system. We deliberately did not use preparations in which the apical membrane was permeabilized with an ionophore because such treatments induce an additional basolateral volume-regulated K⁺ conductance, which is inhibited by various local anesthetics (Germann, Ernst, and Dawson, 1986). A typical experiment is shown in Fig. 2. Tissues were perfused with mucosal K₂SO₄ Ringer's solution containing EGTA and 1 mM unchelated Ca²⁺, which excludes any bias in the results due to the chelator itself and its potential interactions with the anesthetic. For the same reason, we used the same anion (SO₄²⁻) as in the experiments with the Ca²⁺-blockable Cs⁺ currents (Fig. 1). The amount of K⁺ current was determined by replacing mucosal K⁺ by Cs⁺. Control Iₛ amounted to 11.5 μA/cm². Mucosal tetracaine (100 μM) elicited a biphasic effect on Iₛ, initially leading to a small decline to 9.9 μA/cm², followed by a return toward control values. The new steady-state value was 10.8 μA/cm². Subsequent administration of 100 μM tetracaine at the serosal side did not provoke any further changes in

| Table I |
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| **Effect of Mucosal or Serosal Tetracaine on Channel Type F** |
| | n | fi | Sₙ × 10³ | fₑ² |
| Na⁺ (mucosal tetracaine) | | | | |
| Control | 5 | 78.7 ± 8.5 | 25.8 ± 4.1 | 2.7 ± 0.8 |
| 20 μM tetracaine | 5 | 91.5 ± 11.1* | 12.5 ± 2.7* | 1.5 ± 0.9* |
| 100 μM tetracaine | 5 | 101.5 ± 13.0* | 4.7 ± 0.7* | 0.8 ± 0.2* |
| Cs⁺ (mucosal tetracaine) | | | | |
| Control | 8 | 74.6 ± 2.2 | 28.8 ± 10.0 | 1.2 ± 0.3 |
| 20 μM tetracaine | 8 | 87.7 ± 4.2* | 11.7 ± 3.9* | 0.6 ± 0.1* |
| 100 μM tetracaine | 5 | 108.5 ± 9.3* | 2.2 ± 0.7* | 0.2 ± 0.1* |
| Cs⁺ (serosal tetracaine) | | | | |
| Control | 5 | 77.4 ± 5.9 | 51.3 ± 2.4 | 3.2 ± 0.3 |
| 20 μM tetracaine | 5 | 79.1 ± 7.6 | 37.7 ± 3.2* | 2.7 ± 0.4* |
| 100 μM tetracaine | 5 | 84.8 ± 12.4 | 13.8 ± 6.9* | 1.2 ± 0.2* |

All values are given as means ± SEM.
*Values significantly different from control at P < 0.05.
Isc (unpublished observation). On the average (n = 5), 100 μM mucosal tetracaine reduced the K⁺ current from 12.8 ± 2.8 to 12.3 ± 3.0 μA/cm² (not significantly different, P > 0.5). From these experiments, it appears reasonable to exclude basolateral membrane changes as a predominant factor in the block of the Cs⁺ current through the type F channels by tetracaine. Changes in cell potential with tetracaine, as measured with microelectrodes (shown below), will confirm this contention unequivocally.

**Tetracaine Inhibits UO₂²⁺-insensitive Na⁺ Currents**

To substantiate the results with Cs⁺ (Fig. 1) more fully, we tested the effect of tetracaine on the Na⁺ currents passing through channel type F. The mucosal side of the skin was bathed with Ca²⁺-free Na₂SO₄ solution. Channel type S and the highly selective Na⁺ channel were blocked with UO₂²⁺ and amiloride, respectively. An example of such an experiment is displayed in Fig. 3. Control Iₛ was 5.4 μA/cm² and was markedly reduced by 20 μM mucosal tetracaine to 3.2 μA/cm². The accompanying Lorentzian noise was also considerably modified. Sₒ was suppressed from 87.9 to 16.4 × 10⁻²¹ A²s/cm². fₙ increased from 97.5 to 112.5 Hz. Means of control and experimental values of Iₛ, Sₒ, and fₙ were significantly different (P < 0.05; Table I). More pronounced effects were obtained with higher doses of the local anesthetic (100 μM; Table I). These data demonstrate a striking similarity between the effects of tetracaine on the Na⁺ and Cs⁺ currents through type F channels. This result implies that UO₂²⁺ does not prevent the action of tetracaine on the cation-selective channel type F. So far, our data reveal a selective block of channel type F by tetracaine. The local anesthetic does not seem to inhibit the apical and basolateral K⁺ conductance. Therefore, the compound may be useful in the study of poorly selective cation channels.

*Effect of Tetracaine on Type S Channels*

The following experiment aims at the investigation of effects of tetracaine on channel type S. For this purpose we perfused the mucosal surface of the tissues with Ca²⁺-free
mucosal Na₂SO₄ Ringer’s solution containing amiloride. In the experiment illustrated in Fig. 4, control $I_{sc}$ equaled 3.2 µA/cm². The Lorentzian parameters of the associated low-frequency Lorentzian component were $f_c = 15.6$ Hz and $S_o = 279 \times 10^{-21}$ A²·s/cm². When 100 µM tetracaine was applied to the mucosal bath, a rather fast but relatively small inhibitory effect on $I_{sc}$ was observed. $I_{sc}$ stabilized at 2.8 µA/cm² (87% of control) in this case. The low-frequency Lorentzian, on the other hand, was not markedly altered with this dose of the anesthetic compound ($f_c = 14.8$ Hz; $S_o = 263 \times 10^{-21}$ A²·s/cm²). Whereas a small (23%) yet significant ($P < 0.05$) reduction of $I_{sc}$ was observed, mean values of $S_o$ and $f_c$ were not significantly changed ($P > 0.2$; Table II). This apparent discrepancy can be easily understood if it is assumed that type F channels are also present in the tissues we selected, which is a quite reasonable assumption in view of Figs. 2 B and 6 in the companion paper (Desmedt et al., 1993). The high noise power associated with the low-frequency Lorentzian would of course preclude the detection of the open-closed kinetics of these type F channels in the PDS. On the other hand, the block of these channels by tetracaine would become evident in the $I_{sc}$ trace. In other words, $I_{sc}$ changes elicited with tetracaine (mainly) reflect alterations in the open-closed kinetics of type F channels, whereas PDS changes (mainly) reflect influences on the open-closed kinetics of type S channels. Therefore, no correlation is expected to exist between the suppression of $I_{sc}$ and $S_o$. The administration of UO²⁺ suppressed the low-frequency Lorentzian to below background levels and $I_{sc}$ to 0.1 µA/cm² (4% of control). Thus, tetracaine and UO²⁺, when added simultaneously to the mucosal bath, are able to block virtually all of the Ca²⁺-blockable monovalent cation transport through frog skin. Moreover, UO²⁺ retains its inhibitory potency in the presence of tetracaine. Despite this, a large contribution of 1/f noise subsists, which is Ca²⁺ blockable.

The previous experiments established the usefulness of tetracaine in the study of cation-selective transport. The compound displays complementary inhibitory behavior with UO²⁺, apparently without any serious mutual interferences. At concentr-
tions (100 μM) of tetracaine that almost completely occlude channel type F, the UO$_2^{2+}$-blockable $I_{sc}^{Ca}$ and associated Lorentzian noise are little affected.

**Effect of UO$_2^{2+}$ and Tetracaine on the Cell Potential**

The next issue concerns the localization of the UO$_2^{2+}$- and tetracaine-blockable channels. We directly recorded the intracellular potential with microelectrode impalements of the granulosum cells. Effects of UO$_2^{2+}$ and tetracaine on this parameter were used as a criterion to test the cellular localization. Uninterrupted traces of the intracellular potential, $V_o$, are extremely difficult to obtain in the absence of mucosal Ca$^{2+}$. Moreover, in a previous paper (Van Driessche et al., 1991), we commented extensively upon the uncertainties in the interpretation of $fR_0$ and $G_t$ under Ca$^{2+}$-free conditions. We will not repeat this discussion, but will concentrate instead on the traces of $V_o$, since changes in this parameter suffice to decide about the cellular localization.

AVolAVt values were recorded and used as criterion for a valid puncture; i.e., values close to one should be obtained after occlusion of all ion conductances in the apical membrane. Fig. 5 shows the $I_{sc}$ and $V_o$ recordings together with the noise data for an experiment with Cs$^+$ as the main mucosal cation. Removal of mucosal Ca$^{2+}$ resulted in an increase of $I_{sc}$ from 1.0 to 4.1 μA/cm$^2$. At the same time, the intracellular potential was depolarized from $-86$ to $-54$ mV, indicating that the granulosum cells probably mediate at least part of the inward Ca$^{2+}$-blockable Cs$^+$ current. The associated spontaneous Lorentzian noise was fitted with $f_c = 53.9$ Hz and $S_0 = 78.7 \times 10^{-21}$ A$^2$.s/cm$^2$ (Fig. 5 B). Addition of 100 μM UO$_2^{2+}$ to the mucosal bath had no effect on $I_{sc}$, $V_o$, $f_c$, and $S_0$, consistent with the hypothesis that Cs$^+$ passes exclusively through channel type F. On the other hand, 100 μM tetracaine reduced $I_{sc}$ to 1.6 μA/cm$^2$, depressed $S_0$ to $15.1 \times 10^{-21}$ A$^2$.s/cm$^2$, and raised $f_c$ to 118.9 Hz. $V_o$ changed in the hyperpolarizing direction by 19 mV, which is not compatible with
the blockage of a basolateral K+ conductance. 500 µM tetracaine had little further effect besides reducing the Lorentzian noise. In another complete microelectrode recording we also found no effect of UO2²⁺, while tetracaine changed Vo from -63 to -84 mV. These two experiments thus undoubtedly assign the tetracaine-blockable component to the granulosum cell. The lack of a UO2²⁺ effect was demonstrated in four microelectrode experiments.

To localize channel type S, we repeated the same kind of experiment with Na⁺ as the main mucosal cation (Fig. 6). Amiloride was present throughout to block the highly selective Na⁺ pathway. The rise of Isc from 2.5 to 22.0 µA/cm² after Ca²⁺ removal was again accompanied by a marked depolarization of the intracellular potential Vo, from -88 to -46 mV. It is conceivable that this change is due to the opening of tetracaine-blockable channels in the granulosum cells. However, from the spontaneous Lorentzian noise (fc = 6.9 Hz, So = 1,510 × 10⁻²¹ A².s/cm²; Fig. 6 B) it is clear that the UO2²⁺-blockable component is predominantly present in this tissue. We preferred applying UO2²⁺ before tetracaine so as to retain the possibility of detecting the eventual high-frequency component in the noise PDS and relating its subsequent suppression by tetracaine to changes in Isc and Vo. In this skin, a 58%

| Table 11 |
| Effect of Mucosal Tetracaine on Channel Type S |

|     | n | fc × 10¹¹ | So × 10⁻²¹ | Isc/µA/cm² |
|-----|---|-----------|------------|------------|
| Control | 5 | 14.1 ± 1.1 | 406.6 ± 71.1 | 5.5 ± 1.4 |
| 100 µM tetracaine | 5 | 12.8 ± 0.7 | 378.4 ± 80.0 | 4.2 ± 1.0* |

Mucosal: Ca²⁺-free Na₂SO₄ solution + amiloride; serosal: Na₂SO₄ Ringer’s solution. All values are given as means ± SEM. *Values significantly different from control at P < 0.05.
Effect of Tetracaine on the Poorly Selective Cation Pathway in Toad Urinary Bladder

Finally, it is worthwhile to examine the effect of tetracaine on the poorly selective cation pathway in toad urinary bladder as well. The results of these experiments should consolidate the suggested parallel between this pathway and the tetracaine-blockable pathway in frog skin. Fig. 7 indeed confirms a tetracaine sensitivity for the cation-selective channel in toad urinary bladder. We used K+ as the main mucosal cation and the cation-selective channels were stimulated with 0.1 U/ml oxytocin (Aelvoet et al., 1988). This yielded an \( I_{sc} \) of 20.6 \( \mu A/cm^2 \), which declined to 15.3 \( \mu A/cm^2 \) with 20 \( \mu M \) tetracaine, to 9.1 \( \mu A/cm^2 \) with 100 \( \mu M \), and to 4.7 \( \mu A/cm^2 \) with 500 \( \mu M \). The plateau value of the associated Lorentzian noise component decreased from 33.9 \( \times 10^{-21} A^2/s/cm^2 \) in control to 28.4, 18.2, and 12.7 \( \times 10^{-21} A^2/s/cm^2 \) with 20, 100, and 500 \( \mu M \) of the local anesthetic, respectively. The reversibility of tetracaine appeared to be markedly better in toad urinary bladder than in frog skin.

![Figure 5](image-url)

**Figure 5.** Localization of type F channels by recording of the \( V_o, I_o, \) and PDSs. Initially the mucosal side was perfused with Ca2+-containing Cs2SO4 solutions. Ca2+ was removed at the time indicated. PDSs in B were recorded at the times indicated in A. The microelectrode impalement was lost during the time marked by the dashed part of the \( V_o \) record.

Mean values (\( n = 6 \)) demonstrate an inhibition of \( I_{sc} \) by 20 and 55% with 20 and 100 \( \mu M \) tetracaine, respectively. The concomitant reduction of \( S_o \) amounted to 12 and 53% (Table III). With Cl− as the main anion in the mucosal and serosal bathing solutions, tetracaine was more potent than in SO42−, leading to inhibition levels quite similar to those encountered in frog skin incubated with SO42− solutions (Table III). Therefore, in spite of the difference in sensitivity for tetracaine between the two tissues, the results add credibility to the identification of the cation-selective channel in toad bladder with the tetracaine-blockable channel in frog skin.

**DISCUSSION**

*Mechanism of the Inhibitory Action of Tetracaine*

The tertiary amine compound tetracaine was found to specifically inhibit Ca2+ and Na+ currents (Figs. 1 and 3, Table I) through type F channels, which display low
sensitivity for UO$_2^+$ (Desmedt et al., 1993). Apparently the local anesthetic was effective from both the mucosal and the serosal sides (Table I). However, we are rather confident that the basolateral membrane is not the prime target of action in view of the fact that transepithelial K$^+$ currents, entering the cell through apical, Ca$^{2+}$-insensitive K$^+$ channels, were hardly affected by tetracaine (Fig. 2). This is an important result, since it is conceivable that tetracaine depresses the basolateral K$^+$ conductance, which would reduce the driving force for Cs$^+$ (Na$^+$) uptake. Studies of the effect of local anesthetics on basolateral membrane K$^+$ conductance of nystatin-treated tissues mainly demonstrated effects on one class of K$^+$ channels which is activated by cell volume expansion, whereas the native K$^+$ pathway is not affected (Germann et al., 1986). An effect on the native basolateral K$^+$ conductance would have rendered a specific interaction hypothesis of tetracaine with the apical cation-selective channels superfluous. As a matter of fact, direct evidence arguing against an effect on basolateral K$^+$ channels comes from the observation that the cell potential hyperpolarizes with tetracaine (Figs. 5A and 6A), in contrast to a depolarization expected from a reduction of the basolateral K$^+$ conductance. The similarity in the time course and the extent of the inhibition of Ca$^{2+}$-blockable Cs$^+$ and Na$^+$ transport (Figs. 1 and 3, Table I) further implies that the Na$^+$-K$^+$ ATPase probably does not play a role in the tetracaine effect either. We submit that the observed inhibition occurs at a site located at the apical membrane. Lowering the mucosal pH from 8.2 to 6.8 potentiates the tetracaine block (unpublished observation). This observation would favor an external site of action and the protonated form of the compound as the active one. The effectiveness from the serosal side, on the other hand, suggests a cytoplasmic target site. Finally, the conspicuous difference in the concentration dependence of $f_c$ on mucosal and serosal tetracaine, even at concentrations that inhibit the same amount of current, might reflect the presence of two sites, one

**Figure 6.** Localization of type S channels by recording of the $V_o$, $I_{sc}$, and PDS. Initially the mucosal side was perfused with Ca$^{2+}$-containing Na$_2$SO$_4$ solutions. Ca$^{2+}$ was removed at the time indicated. PDSs in B were recorded at the times indicated in A.
extracellular and the other intracellular (Hille, 1977). Whether the local anesthetic acts directly or indirectly on the site(s) remains to be determined. For the moment we have no clues as to the exact nature of the blocking mechanism. However, the high specificity of tetracaine for type F channels (at least below a concentration of 100 μM) might favor the idea of a direct interaction.

Evidence for Two Different Channel Proteins?

The finding that 100 μM of tetracaine does not influence the UO₂⁺-blockable current component (Fig. 4), whereas it almost completely eliminates the UO₂⁺-insensitive one, adds credibility to the proposal of two physically distinguishable channel types, although the idea of one channel existing in two modes with different tetracaine sensitivity cannot be strictly rejected. Probably the only decisive result in favor of two independent channels (besides single-channel detection with the patch-clamp tech-

| SO₄²⁻ | n | f_c (Hz) | S_x (A²/s/cm²) | I_y (μA/cm²) |
|------|---|---------|---------------|---------|
| Control | 6 | 299.2 ± 12.1 | 13.4 ± 4.5 | 7.5 ± 2.7 |
| 20 μM tetracaine | 5 | 279.1 ± 12.7 | 11.8 ± 4.2 | 6.0 ± 2.4 |
| 100 μM tetracaine | 6 | 325.4 ± 8.9 | 6.3 ± 2.4* | 3.3 ± 1.2* |

| Cl⁻ | n | f_c (Hz) | S_x (A²/s/cm²) | I_y (μA/cm²) |
|------|---|---------|---------------|---------|
| Control | 6 | 404.4 ± 16.9 | 24.2 ± 5.2 | 6.8 ± 1.1 |
| 20 μM tetracaine | 6 | 455.8 ± 31.7 | 11.8 ± 2.7* | 3.4 ± 0.5* |
| 100 μM tetracaine | 4 | 444.3 ± 41.1 | 6.5 ± 1.9* | 1.5 ± 0.3* |

Mucosal solution: Ca²⁺-free K₂SO₄ or KCl Ringer's solution; serosal solution: Na₂SO₄ or NaCl Ringer's solution + 0.1 U/ml oxytocin.

All values are given as means ± SEM.

*Values significantly different from control at P < 0.05.
nique) would have come from the localization in different cell types of the UO$_2^{2+}$-blockable and the tetracaine-blockable component. However, the present microelectrode results favor a localization of both components in the granulosum cells (Figs. 5 and 6), and therefore provide no further argument for two independent channels. In our opinion, the data on toad urinary bladder remain preponderant to settle this question. The sensitivity for tetracaine displayed by the cation-selective pathway in this tissue (Fig. 7) reinforces the contention (Desmedt et al., 1993) that the bladder possesses only one channel type, which closely resembles type F channels in frog skin. By the same token, these data provide the obvious prerequisite for a two-channel concept: namely, that the tetracaine-blockable component can in principle occur as an independent unit.

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