Pflügers Arch – Eur J Physiol (2000) 439:559–566
Digital Object Identifier (DOI) 10.1007/s004249900223

ORIGINAL ARTICLE

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Micromolar concentrations of steroids and of aldosterone antagonists inhibit the outwardly rectifying chloride channel with different kinetics

Received: 22 October 1999 / Received after revision and accepted: 15 November 1999 / Published online: 22 December 1999

Abstract We used the patch-clamp technique to analyse the open/close kinetics of single, outwardly rectifying, intermediate-conductance (ORIC) Cl^- channels from cultured epithelial cells under control conditions and in presence of different inhibitors. As observed previously in excised inside/out patches under control conditions, the switching kinetics were characterized by one open-state time constant ($\tau_o \approx 30$ ms) and three closed-state time constants ($\tau_{c1} \approx 0.2$ ms, $\tau_{c2} \approx 2$ ms and $\tau_{c3} \approx 60$ ms). Aldosterone, six further steroids and two aldosterone antagonists inhibited channel open probability (NP_o) concentration dependently with the potency at 10 µmol/l increasing in the sequence: hydrocortisone, corticosterone, β-oestradiol, cortisone, aldosterone, testosterone, progesterone, canrenone, spironolactone. Although all substances decreased τ_o, neither the steroids nor the aldosterone antagonists affected τ_{c1}, τ_{c2} or τ_{c3} or induced additional transitions with additional time constants. Instead, the steroids increased the prevalence of τ_{c2} in the dwell-time histograms and the aldosterone antagonists increased the prevalence of τ_{c3}, both in a concentration-dependent manner. These observations may be explained by a model in which one open state leads to one of three closed states with rate constants α, β and γ, and in which β or γ increase under the influence of steroids or aldosterone antagonists, respectively. Cytosol, which contains a Cl^- channel inhibitor of unknown molecular structure, (Krick et al., Pflügers Arch 418:491, 1991) was also tested, but the results did not conform to the blocker mechanisms described above. This shows that there are even further modes of channel inhibition and argues against the cytosolic Cl^- channel inhibitor being a steroid.

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Key words ORIC Cl^- channel · Open/close kinetics · Three closed time constants · Steroids · Aldosterone antagonists · Cytosolic Cl^- channel inhibitor

Introduction

Although its molecular identity and its physiological role are not yet known, the outwardly rectifying, intermediate-conductance (ORIC) Cl^- channel is a well-characterized ion channel widely distributed in epithelial and non-epithelial cells [2, 6]. Besides the typical current/voltage relation in symmetrical solutions with plasma-like Cl^- concentration, it has further characteristic properties. First, it is virtually only ever observed in excised patches, and it opens either immediately upon excision (if the experiment is performed at 37 °C [13]) or with some delay after application of depolarizing voltages (if the experiment is performed at room temperature [7, 26]). Second, it displays typical switching kinetics with at least one open state and three closed states [20] and third, it is inhibited by a wide variety of substances including various chloride channel blockers [9, 23, 24] and unsaturated fatty acids such as arachidonic acid [1, 10]. Based on its spontaneous activation after excision we have speculated previously that the channel in intact cells probably is blocked by a cytosolic inhibitor that diffuses away after excision. Subsequently, and in collaboration with others [11, 12, 14], we have succeeded in demonstrating the existence of such an inhibitory principle in cytoplasm. It is presumably a low-molecular weight (approximately. 1000 Da), amphiphilic substance but its molecular identity is not yet known.

In the search for possible physiological inhibitor molecules we tested a number of substances, among them steroids that block Cl^- channels in brain [15]. Steroids do indeed block the ORIC Cl^- channel, albeit at higher concentrations than are expected inside cells. Irrespective of their possible physiological role, however, we observed an interesting blocking pattern, insofar as all seven steroids tested shifted the channel into its second closed

state, while the two aldosterone antagonists tested shifted the channel into the third closed state. In addition we observed that the potency of the steroids correlated well with their lipophilicity.

Materials and methods

The experiments were performed on subconfluent monolayer cultures of HT29 and T84 cells grown on collagen-coated cover-slips and bathed in NaCl Ringer solution of plasma-like composition as described previously [11, 20]. The bath solution consisted of (in mmol/l): Na^+ 145, K^+ 5, Ca^{2+} 1, Mg^{2+} 1 Cl^- 154 and *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulphonic acid) (HEPES) 5 and was titrated with NaOH to pH 7.4. Conventional patch-clamp techniques were used to measure single-channel currents in excised, inside-out or outside-out patches at room temperature, usually on the 1st or 2nd day after cell seeding. Patch pipettes consisted of borosilicate glass tubing, were filled with 150 mmol/l NaCl buffered with 5 mmol/l HEPES to pH 7.4, and had resistances of 5–15 MΩ. All measurements were performed with a patch-clamp amplifier (EPC9, HEKA Lambrecht, Germany) in conjunction with an Atari Mega ST4 computer. The data were digitized and stored on video tape using a pulse-code modulation device (PCM 501, Sony) operating at 44.1 kHz with 16-bit resolution. They were then replayed as analogue signals, passed through an 8-pole Bessel filter (902 LPF, Frequency Devices, Haverhill, Mass., USA) and stored for further processing. For determination of open probability (P_o) of single channels or NP_o (where N is the number of simultaneously active channels) sampling rate and filter frequency were set at 0.5 and 0.1 kHz respectively. P_o and NP_o were calculated from amplitude histograms collected over at least 30 s (typically ≈1 min) and changes were calculated by referring experimental data to averages from control and recovery periods observed on the same patch. For determination of open/close kinetics, sampling rate and filter frequency were set at 5 and 1 kHz respectively. Open and closed times were analysed with the TAC program (Instrutec, Almond, N.Y., USA) which counts all transitions of the mid level (50% threshold method) between open and closed states and bridges the intervals between data points by spline interpolation. Proper setting of open and closed levels was monitored and, if necessary, adjusted over the entire length of all records (typically 1 min or up to 32768 events, the maximum the program can handle). The steroids were added to the bath perfusate from stock solutions in dimethylsulphoxide (DMSO) to final concentrations of 0.1–100 µmol/l. The maximal DMSO concentration was 0.1%. DMSO concentrations up to 1% did not affect NP_o. Cytosol was collected as described [12]. Data are given as mean values±SD for n measurements. Where n=2, the mean±half the difference is given. The significance of differences was tested with Student's t-test for paired or unpaired samples as appropriate.

Results

Channel inhibition by steroids

Single ORIC Cl^- channels were observed after excision in inside-out membrane patches either spontaneously or after application of a brief hyperpolarizing pulse. As reported previously, the P_o for this channel is rather constant with little indication of run-down, and is independent of membrane voltage, ambient pH or $[Ca^{2+}]$ [20, 25]. Figure 1 shows the effect of 50 µmol/l aldosterone on single-channel currents of an excised inside-out patch of a HT_{29} cell. Essentially identical observations were also made on T84 cells. This experiment was performed

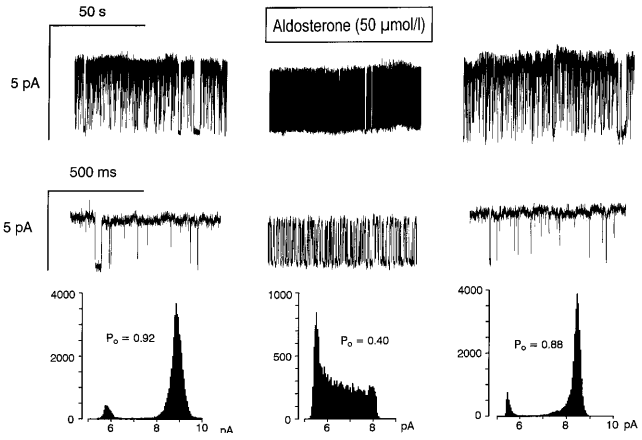


Fig. 1 Effect of aldosterone (50 µmol/l) on an outwardly rectifying, intermediate conductance (ORIC) Cl^- channel in an inside-out membrane patch from an HT_{29} cell. The holding potential was –60 mV. *Upper row:* single-channel currents recorded at a sampling rate of 500 Hz and a filter frequency of 100 Hz. *Upward deflections* of the current trace indicate channel opening. *Middle row:* sections of *upper* trace at a higher time resolution (sampling rate 5 kHz; filter setting 1 kHz). *Lower row:* amplitude histograms used to calculate the open probability (P_o) of the channel

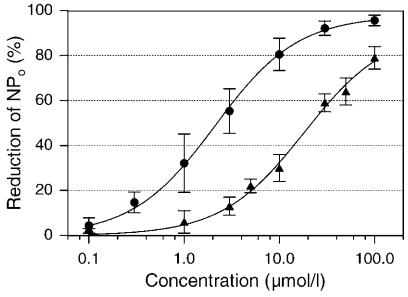


Fig. 2 Concentration dependence of ORIC Cl^- channel inhibition by aldosterone (*triangles*) and spironolactone (*circles*). Reduction of NP_o as a percentage of control (mean of NP_o of pre- and post-experimental control periods) as function of inhibitor concentration on a logarithmic scale. Means±SD, n=2–7 or 5–7 respectively. The data were fitted with single-site inhibition kinetics (*solid lines*)

with virtually identical Cl^- concentrations in pipette and bath at a holding potential of –60 mV. Low- and high-speed recordings are presented as well as current amplitude histograms under control and experimental conditions. As can be seen, aldosterone reversibly induced a typical flicker block with reduction of P_o from almost 0.9 to 0.4. The apparent slight contraction of the current amplitude and the less clear-cut separation of open and closed states in the presence of aldosterone reflect the limited frequency resolution of these measurements. Taking this effect into account there was no significant change in the current/voltage relationship of the channel in presence of the inhibitor. Figure 2 shows (among other data) the concentration dependence of the aldosterone effect. It follows Michaelis-Menten kinetics with a concentration for half-maximal inhibition (IC_{50}) of 18.1 µmol/l.

Table 1 Inhibitory potency of steroids and aldosterone antagonists (all 10 µmol/l). Means±SD for *n* observations. Inhibition is expressed as the percentage reduction in NP_o (N number of active channels, P_o single-channel open probability, $P_{oct/water}$ octanol: water partition coefficient)

[a]Data from [8], [b]data from [19], [c]data from [4], [d]data from [16]

Substance	Reduction of NP_o	*n*	OH groups per molecule	log$P_{oct/water}$
Hydrocortisone	7±3	6	3	1.53–1.68[a]
Corticosterone	18±5	3	2	1.82[b]
β-Oestradiol	24±10	3	2	2.61[a]
Cortisone	29±9	6	2	1.42–1.64[a]
Aldosterone	30±6	7	2	1.08[b]
Testosterone	33±9	4	1	3.32[a]
Progesterone	46±12	6	0	2.48–3.87[a]
Canrenone	70±7	4	0	2.68[c]
Spironolactone	81±7	7	0	2.19[d]

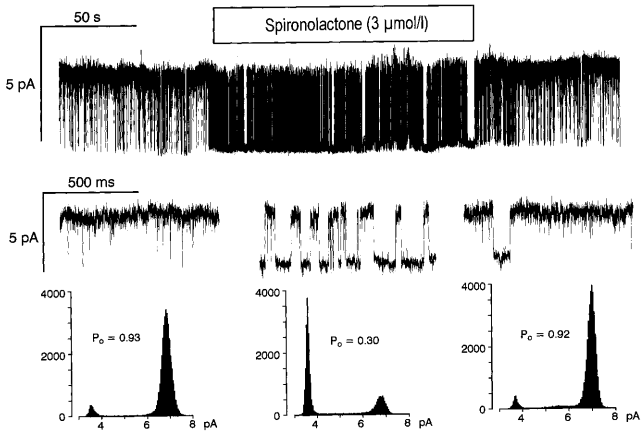


Fig. 3 Effect of spironolactone (3 µmol/l) on ORIC Cl^- channel. Details as in Fig. 1

The observed Cl^- channel inhibition is not restricted to aldosterone, but can also be elicited by other steroids. Six further steroids were tested. At concentrations of 1, 10 or 100 µmol/l they showed similar inhibition kinetics as aldosterone, although the potencies differed. Rather than determining the complete concentration dependence of inhibition for each steroid we focussed on the effects at 10 µmol/l. As shown in Table 1 the order of potency was progesterone>testosterone>aldosterone>cortisone≅ oestradiol>corticosterone>hydrocortisone. Even hydrocortisone, however, which had only a small effect (7% inhibition at 10 µmol/l), reduced NP_o strongly (by 55%) at a 10 times higher concentration.

Figure 3 shows essentially identical experiments as Fig. 1, but here, instead of aldosterone, the aldosterone antagonist spironolactone (3 µmol/l) was used; it was 10 times more potent (IC_{50} 2.1 µmol/l, see Fig. 2). A comparison of Figs. 1 and 3 reveals that spironolactone did not cause a fast flicker block but rather induced longer closed periods without eliciting the typical bursting behaviour often seen in channels with multiple closed states. Very similar observations were also made with the aldosterone antagonist canrenone which reduced NP_o with an IC_{50} of 3.6 µmol/l.

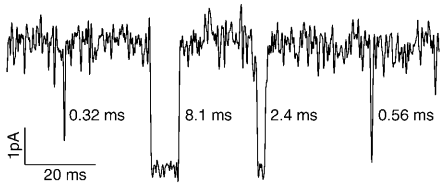


Fig. 4 Typical trace record of control single-channel activity at high time resolution with indication of closed times as analysed with the TAC program (see text for details)

Inhibition kinetics

The mechanism of channel inhibition was investigated by analysing the switching kinetics with dwell-time histograms. As reported previously for control conditions, ORIC Cl^- channels exhibit open/close kinetics which indicate the presence of at least one open and three closed states [20]. Under control conditions the channel opening is characterized by one open time constant $\tau_o \approx 30$ ms, and channel closure by at least three closed time constants: $\tau_{c1} \approx 0.25$ ms, $\tau_{c2} \approx 2$ ms and $\tau_{c3} \approx 60$ ms, where τ_{c1} – because of the limited frequency resolution of the measurements – is only poorly defined although the underlying transition phenomenon is a physical reality (see Fig. 4). When closing, the channel prefers to enter the first closed state (incidence approximately 70–90%) or the second closed state (incidence approximately 10–25%), but is only rarely observed in the third closed state (1% or less).

Figure 5 shows the effect of increasing aldosterone concentrations (10 and 100 µmol/l) on dwell-time histograms analysed according to Sigworth and Sine [22]. Table 2 summarizes the corresponding time constants obtained on three single channels. It can be seen that increasing concentrations of aldosterone decreased τ_o but did not induce transitions into an additional closed state. Rather aldosterone increased the probability of finding the channel in the second closed state while the closing kinetics (τ_{c1}, τ_{c2}, and τ_{c3}) remained virtually unchanged. The same pattern of channel inhibition, which also agrees with our previous observations with glibenclamide [20, 25], was observed with all steroids that could be tested on single channel activity, i.e. – besides aldosterone – progesterone, testosterone, cortisone and hydrocortisone (see also Table 3).

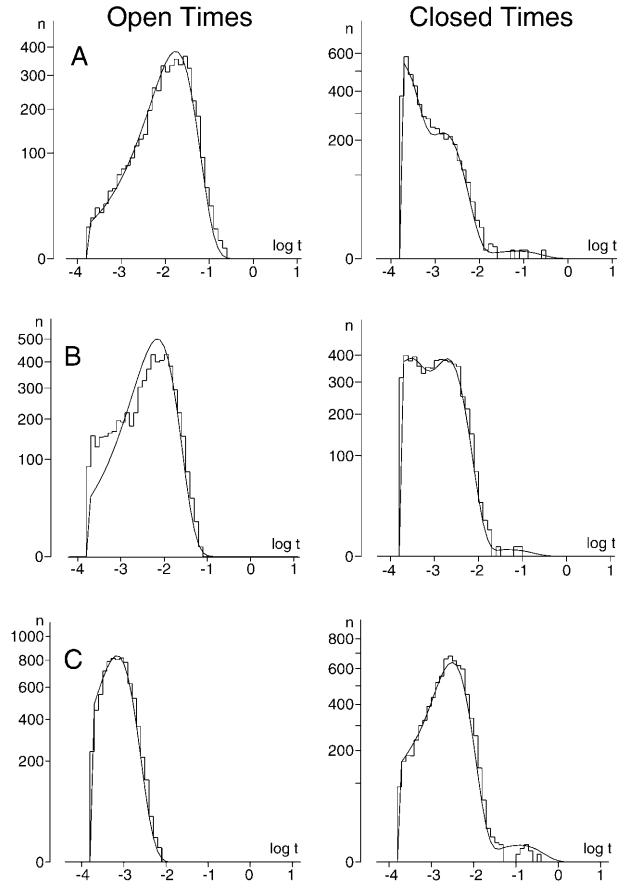


Fig. 5A–C Effect of aldosterone on dwell-time histogram of ORIC Cl^- channel. Open and closed time histograms are presented according to Sigworth and Sine [22] with logarithmic binning of time intervals on the *abscissa* and square-root scaling of the number of events (*n*) on the *ordinate*. **A** Control state, **B** and **C** in the presence of 10 and 100 µmol/l aldosterone respectively

The aldosterone antagonists spironolactone and canrenone, on the other hand, produced very different dwell-time histograms. As shown in Fig. 6 and Table 2, increasing concentrations of spironolactone also progressively decreased τ_o and also left the three closed time constants unchanged. However, it increased progressively the probability of finding the channel in the third closed state. Essentially identical observations were made with canrenone (see also Table 2).

Table 3 Effect of different steroids (10 or 100 µmol/l) on τ_o, τ_{c2} and A_2, the relative preference of switching into the second closed state. τ_{c1} and τ_{c3} were not affected, nor did the ratio A_1/A_3 change. Mean of *n* observations

Blocker	Concentration (µmol/l)	*n*	τ_o (ms)	τ_{c2} (ms)	A_2 (%)
Control	–	3	32	1.2	16
Aldosterone	10	1	7.7	2.2	57
	100	2	0.8	2.5	94
Control	–	2	15.3	1.6	12
Cortisone	10	2	5.5	1.6	44
Control	–	1	45	1.6	8.9
Hydrocortisone	100	1	1.5	1.2	90
Control	–	3	13	2.3	12
Progesterone	10	3	4.9	6.7	47
Control	–	1	13	1.3	6.5
Testosterone	10	1	3.8	3	29

Channel inhibition by cytosol

We have shown previously that cytosol isolated from cell cultures, pig kidney cortex or human placenta [11, 12] inhibits the ORIC Cl^- channel either totally or partially by inducing some kind of flicker block, and that by diluting the cytosol we obtain Michaelis-Menten-type inhibition kinetics similar to those observed with aldosterone or spironolactone above. From these and other observations we have concluded that cytosol contains one (or more) natural, low-molecular weight Cl^- channel inhibitors of amphiphilic structure. To characterize further the inhibitor we tested the effect of cytosol on Cl^- channel switching kinetics. As seen clearly in Fig. 7 and Table 2, cytosol inhibited the Cl^- channel with a blocking mechanism different to that of the steroids or the aldosterone antagonists. It also reduced the open time constant, but did not affect the probability of finding the channel in

Table 2 Effect of different inhibitors on single-channel kinetics. Mean±SD for *n* single-channel experiments. *Time* is the total time of channel activity analysed at each concentration. τ_o is the open time constant, τ_{c1}–τ_{c3} are the closed time constants and A_1–A_3 are the relative preferences of dwelling in the closed states C_1–C_3. Observations with 0.1 µmol/l spironolactone are not included because the data were not different from control

Substance	Concentration (µmol/l)	*n*	Time (s)	τ_o (ms)	τ_{c1} (ms)	A_1 (%)	τ_{c2} (ms)	A_2 (%)	τ_{c3} (ms)	A_3 (%)
Control	–	3	175	32±12	0.15±0.02	83±12	1.2±0.5	16±13	46±27	1±0.5
Aldosterone	10	1	74	7	0.23	43	2.2	57	50	0.1
Aldosterone	100	2	93	0.84±0.07	0.26±0.03	5.9±0.2	2.2±1.3	94	85±62	0.2
Control	–	2	1404	40±20	0.13±0.02	91±2.2	1.1±0.05	7.5±1.2	55±1	1.9±1.0
Spironolactone	1	2	209	28±22	0.13±0.04	82±12	1.0±0.1	9.5±3.5	61±9.2	8.6±7.7
Spironolactone	10	2	200	8.0±2.0	0.13±0.05	50±28	1.1±0.9	8.0±2.8	60±6.7	42±25
Control	–	1	135	29	0.15	88	1.7	9.3	56	2.8
Canrenone	10	1	89	5.7	0.11	67	1.3	5	36	28
Control	–	5	261	29±12	0.22±0.06	78±10	1.8±0.4	19±9	84±54	2.7±1.7
Cytosol	–	6	271	6.7±3	0.37±0.1	78±10	4.6±2.0	19±9	77±23	3.5±1.5

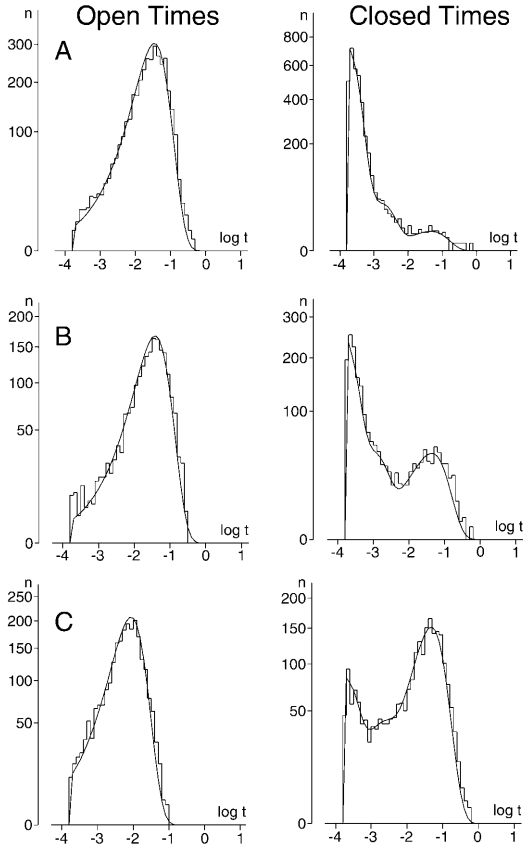


Fig. 6A–C Effect of spironolactone on dwell-time histogram of ORIC Cl^- channel. Details as in Fig. 5 except that concentrations were 1 and 10 µmol/l in panels **B** and **C** respectively. All data were obtained on the same patch

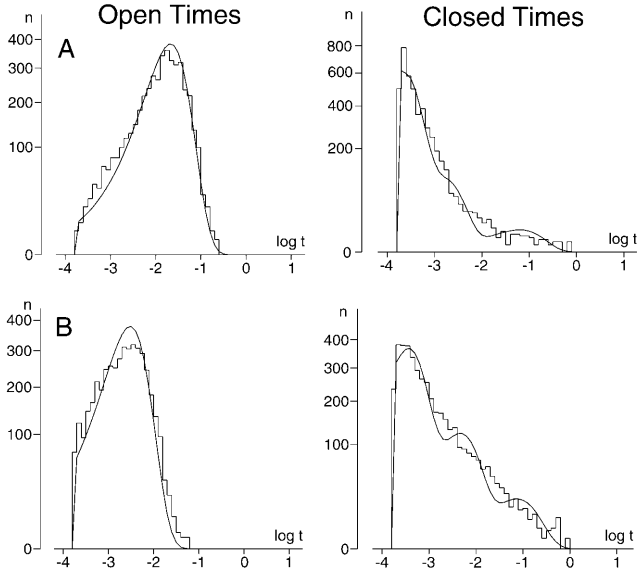


Fig. 7A,B Effect of cytosol on dwell-time histograms of ORIC Cl^- channel. Details as in Figs. 5 and 6. **A** Control, **B** cytosol

one of its natural closed states. Rather, cytosol appeared to induce (at least) one new transition phenomenon with an additional closed time constant located between τ_{c1} and τ_{c2}. Because of the close proximity to τ_{c1}, and because of the limited high-frequency resolution of the measurements, however, the significance of this interpretation is not as clear as in the blocker experiments with steroids and aldosterone antagonists.

Discussion

The present experiments have shown that the ORIC Cl^- channel is blocked by micromolar concentrations of steroids or aldosterone antagonists. This channel has peculiar switching kinetics that are characterized by one open time constant and three closed time constants. Both the steroids and the aldosterone antagonists reduced the open time constant, but did not affect the closed time constants or induce an additional exponential in the closed-state dwell-time histograms, as expected from the classical model of blocker action.

In the classical blocker model [3, 5] the channel is represented by three states: shut (*C*) open (*O*) and blocked (*B*) $C \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} O \underset{k_{+B}\cdot X_B}{\overset{k_{-B}}{\rightleftharpoons}} B$ where α, β‘, k_{-B} and $k_{+B}\cdot X_B$ are transition rates (second^{-1}) and X_B the molar concentration of the blocker. This model predicts firstly, that the open time constant $\tau_o=1/(\alpha+k_{+B}\cdot X_B)$ decreases with increasing blocker concentration; second, that the blocker induces an extra exponential with time constant $\tau_{c2}=1/k_{-B}$ in the closed-channel dwell-time histogram that would not normally be observed and third, that the relative incidence of the blocking events described by this time constant (a_2) should increase with blocker concentration according to the probability density function (pdf_c):

$$pdf_c=a_1\cdot\beta'\cdot\exp(-\beta'\cdot t)+a_2\cdot k_{-B}\cdot\exp(-k_{-B}\cdot t) \quad (1)$$

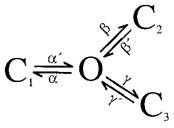
where t is the time and

$$a_1=\alpha/(\alpha+k_{+B}\cdot X_B) \text{ and } a_2=k_+\cdot X_B/(\alpha+k_{+B}\cdot X_B) \quad (2)$$

Although the first prediction would agree with the present observations and, although similar to the third prediction, the relative incidence of staying in one closed state increased with blocker concentration, the second prediction was not fulfilled. It is true, of course, that a fourth exponential would have been difficult to discern against the background of the other three exponentials observed already under control conditions; however the fact that the dwell-time histograms in presence of high concentrations of the steroids or aldosterone antagonists peaked at the same values of τ_{c2} (≈1.5–2.5 ms) or of τ_{c3} (≈60 ms), respectively, that are observed in absence of these blockers speaks strongly against an additional blocked state.

We conclude therefore that, in the present experiments, the blockers force the channel to dwell preferably in the spontaneously occurring closed states 2 or 3 rather than in a new closed state.

As will be justified further below, the most simple model to explain our findings is:



With this model the open time constant in absence of blockers is:

$$\tau_o=1/(\alpha+\beta+\gamma) \quad (3)$$

where α, β, and γ are rate constants of the spontaneous transitions from the open state into each closed state and the distribution of closed times is:

$$\text{pdf}_c=A_1\cdot\alpha'\cdot\exp(-\alpha'\cdot t)+A_2\cdot\beta'\cdot\exp(-\beta'\cdot t) +A_3\cdot\gamma'\cdot\exp(-\gamma'\cdot t) \quad (4)$$

where α‘, β‘, and γ‘ are the rate constants of the reverse (opening) reactions and

$$A_1=\alpha/(\alpha+\beta+\gamma),\ A_2=\beta/(\alpha+\beta+\gamma) \text{ and } A_3=\gamma/(\alpha+\beta+\gamma) \quad (5)$$

codetermine the relative weight of the three exponentials.

In presence of a blocker B_2 which shifts the channel into state C_2 or in presence of a blocker B_3 which shifts it into state C_3 the rate constants β and γ will be $\beta+k_2\cdot B_2$ and $\gamma+k_3\cdot B_3$, respectively, where B_2 and B_3 are the blocker concentrations and $k_2\cdot B_2$ and $k_3\cdot B_3$ the transition rates (second^{-1}) that add to the channels' inherent blocking rate constants. Under these conditions Eq. 3 may be written:

$$\tau_o=1/N \text{ with } N=\alpha+\beta+k_2\cdot B_2+\gamma+k_3\cdot B_3 \quad (6)$$

and Eqs. 5 yield:

$$A_1=\alpha/N,\ A_2=(\beta+k_2\cdot B_2)/N \text{ and } A_3=(\gamma+k_3\cdot B_3)/N \quad (7)$$

In contrast to linear reaction schemes, such as $C_1\leftrightarrow O\leftrightarrow C_2\leftrightarrow C_3$ or similar, our tridirectional model describes the present observations well in as much as it predicts that τ_o should decrease with increasing concentrations of the blocker B_2, as was observed, while the probability of the channel dwelling in state C_2 should increase with B_2, as was also observed, but the relative probability (probability ratio) of finding the channel in the other two states (C_1 and C_3) should not be affected. In view of the relatively large scatter in determining τ_{c3} with the corresponding area under the curve the latter prediction could not be tested rigorously in case of the B_2 blockers. However, the validity of the model is also supported by the experiments with blockers of type B_3, for which corresponding observations hold and in which case the relative probability of the channel staying in state C_1 or C_2 could be tested and was not significantly affected.

The model is further supported by our observations on ORIC Cl^- channels of HT_{29} [20] and M1 [25] cells, in which glibenclamide induced exactly the same blocker effects as the steroids: it decreased τ_o concentration dependently, it did not affect the three closed time constants (τ_{c1}–τ_{c3}) nor did it induce an additional transition phenomenon (with an additional time constant). Moreover, as observed with aldosterone and the other steroids, it concentration dependently increased the probability of finding the channel in closed state C_2.

Whether other blockers might have similar effects is not absolutely clear at present. Hwang et al [10] have analysed the switching kinetics of ORIC Cl^- channels in presence of arachidonic acid which, like other polyunsaturated fatty acids, blocks these channels. In their experiments, however, only one exponential was resolved in both the closed- and open-time histograms. Their data show that τ_o decreased inversely with blocker concentration, while τ_c (approximately 2 ms and thus corresponding to τ_{c2} in the present experiments) was not affected. These findings suggest that arachidonic acid increased the probability of finding the channel in closed state 2 and this implies that arachidonic acid and the other polyunsaturated fatty acids tested (linoleic, oleic and ricinoleic acids) exert the same blocker effect as the steroids and glibenclamide. In addition, Singh et al. have tested three classes of Cl^- channel blockers, anthranilic and indanyl alkanoic acids and disulphonic stilbenes, on colonic epithelial Cl^- channels reconstituted into planar lipid bilayers [23]. These channels exhibit rectifying current/voltage relations and thus may be ORIC Cl^- channels. However, again only one closed-time constant (about 3.6 ms) was identified under control conditions. This may be related to the fact that simple, least-squares fitting to exponential histograms was used rather than the fitting procedure of Sigworth and Sine [22] which should have provided better high- and low-frequency resolution. Because of this limitation it is not possible at present to decide whether the observed flicker block might have corresponded to shifting the channel into the presently observed blocked state C_1.

Blocking action of cytosolic Cl^- channel inhibitor

In contrast to steroids and aldosterone antagonists, the cytosol preparations elicited a completely different blocking pattern: rather than shifting the channel into closed state C_2 or C_3 the cytosol appeared to induce at least one new transition event with a time constant that was slightly larger than τ_{c1}. In view of the limited frequency resolution, however, this time constant could not be separated clearly from τ_{c1}. It is true that our fit to the closed time histograms also indicated an increase in τ_{c2} which, on statistical grounds, was even significant. In view of the rather uncharacteristic shape of the corresponding region in the histogram, however, we are reluctant to accept this as a physically significant effect. We conclude therefore that the blocking mechanism of the cytosolic inhibitor cannot be resolved on the basis of the present experiments. However, the observation that the cytosolic inhibitor blocked differently than all the steroids and aldosterone antagonists tested is of further interest since it allows us to conclude that the inhibitor is most likely neither a steroid nor a polyunsaturated fatty acid.

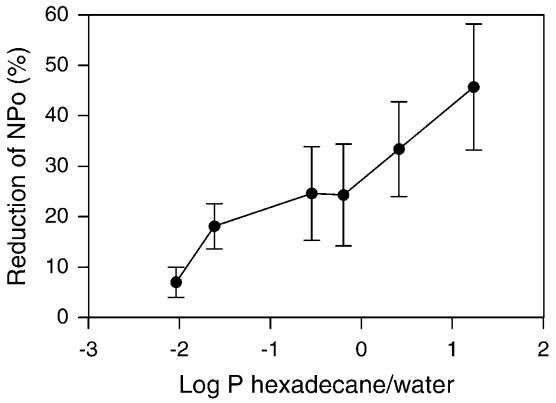


Fig. 8 Inhibitory potency of different steroids on ORIC Cl^- channels (reduction of NP_o in percent) as function of the logarithm of the hexadecane:water partition coefficient [21]. Steroid concentrations were 10 µmol/l throughout, and the data represent (from *left* to *right*) hydrocortisone, corticosterone, cortisone, β-oestradiol, testosterone and progesterone

Mechanism of steroid action

With respect to the mechanism of action of steroids and aldosterone antagonists, two observations are particularly relevant. First, the inhibitory concentrations are high (10–100 µmol/l) and second, the inhibitory potency appears to increase with lipophilicity. The latter follows from a comparison of the percentage reduction of NP_o with the number of OH^- groups per molecule, or with the octanol:water partition coefficient ($P_{oct/w}$, Table 1). This is also supported by our observation with glibenclamide, for which $\log P_{oct/w}$ is 3.08 [18]; it reduced NP_o by 37±7% (n=4) at 10 µmol/l [20]. The characterization of lipophilicity is not straight forward, however, because $\log P_{oct/w}$ values from the literature vary, and because no systematic study of all compounds we used is available. The best correlation is observed if we plot the inhibitory potency against the logarithm of the hexadecane:water partition coefficient (Fig. 8). This plot excludes aldosterone and glibenclamide, for which no data are available.

At the high concentrations required, and in view of the potency increasing with lipophilicity, one might speculate that the steroids flood the lipid phase of the cell membrane and inhibit the channel by altering membrane fluidity [17]. This is unlikely, however, because steroids and aldosterone antagonists exhibited different inhibition patterns. In addition it is unlikely that a change in membrane fluidity following incorporation of lipophilic substances into the membrane would occur instantaneously, and would also reach a new distribution equilibrium as rapidly as channel inhibition occurred (see Fig. 3). Alternatively, we speculate that the channel protein has at least two different lipophilic binding sites: one which binds steroids, glibenclamide or polyunsaturated fatty acids and probably other inhibitors, and another one which binds the aldosterone antagonists and, probably, other inhibitors. Binding then increases the rate of transition into the respective closed state of the channel.

Acknowledgements The authors thank J.P. Koch for help with the data presentation, I. Harward for secretarial assistance and Dr. K-H. Bahringhaus, Hoechst-Marion-Roussel, Frankfurt for information on partition coefficients.

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