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Droogmans, Guy, Jean Prenen, Jan Eggermont, Thomas Voets, and Bernd Nilius. Voltage-dependent block of endothelial volume-regulated anion channels by calix[4]arenes. Am. J. Physiol. 275 (Cell Physiol. 44): C646–C652, 1998.—We have studied the effects of calix[4]arenes on the volume-regulated anion channel (VRAC) currents in cultured calf pulmonary artery endothelial cells. TS- and TS-TM-calix[4]arenes induced a fast inhibition at positive potentials but were ineffective at negative potentials. Maximal block occurred at potentials between 30 and 50 mV. Lowering extracellular pH enhanced the block and shifted the maximum inhibition to more negative potentials. Current inhibition was also accompanied by an increased current noise. From the analysis of the calix[4]arene-induced noise, we obtained a single-channel conductance of 9.3 ± 2.1 pS (n = 9) at −30 mV. The voltage- and time-dependent block were described using a model in which calix[4]arenes bind to a site at an electrical distance of 0.25 inside the channel with an affinity of 220 µM at 0 mV. Binding occludes VRAC at moderately positive potentials, but calix[4]arenes permeate the channel at more positive potentials. In conclusion, our data suggest an open-channel block of VRAC by calix[4]arenes that also depends on the protonation of the binding site within the pore.

endothelium; open-channel properties

VOLUME-REGULATED ANION channels (VRAC) control numerous cell functions, as described in several reviews (4–6, 9, 15). In contrast, with the increasing interest in this channel and its obvious role in diverse biological functions, the elucidation of its gating mechanism, a direct biophysical approach of its open-pore properties, and its molecular identification have so far been largely unsuccessful (5).

In contrast to other ion channels (e.g., the voltage-dependent Na+ channel) in which specific high-affinity ligands have played a pivotal role in the characterization, purification, and cloning of the channel, there are at present no specific high-affinity ligands available for VRAC. Compounds such as tamoxifen, 5-nitro-2-(3-phenylpropylamino)benzoic acid, and dideoxyforskolin inhibit VRAC only in the micromolar concentration range; their mode of action is unclear, and they may also affect other systems (for review, see Ref. 5). A search for VRAC blockers with greater potency and specificity is therefore a necessary step in the development of molecular tools that could be used to probe selective channel features such as open-pore properties.

Recently, a potent block of outwardly rectifying Cl− channels (ORCC) by compounds belonging to the family of calix[4]arenes was described. The calix[4]arene used in this study consists of four para-substituted phenols conjugated by methylenes to form macrocyclic basket (calix)-like molecules (14). Calix[4]arenes are open-channel blockers of ORCC incorporated into planar black lipid membranes (13, 14) and blockers of native ORCC in airway epithelial cells (10). The apparent inhibition constant of this block in the subnanomolar range prompted us to investigate the effects of these calix[4]arenes on the swelling-activated Cl− current (Icl,swell) in endothelial cells.

Our results show that calix[4]arenes induce a voltage-dependent inhibition of Icl,swell, probably by an occlusion of the pore region at moderately positive potentials, which is relieved at more positive potentials at which the negatively charged calix[4]arenes permeate the channel. The pH dependence of the calix[4]arene effect suggests a role for positively charged residues, presumably histidine, in the channel pore.

MATERIALS AND METHODS

The methods used in the present experiments have been described in detail elsewhere (7). Endothelial cells from an established cell line from calf pulmonary artery (cell line CPAE; American Type Culture Collection CCL-209) were grown in DMEM with 2 mM l-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 20% fetal bovine serum at 37°C in a fully humidified atmosphere of 10% CO2 in air. Cells were detached by exposure to 0.05% trypsin in a Ca2+- and Mg2+-free solution and plated on gelatin-coated coverslips for electrophysiological experiments.

Whole cell membrane currents were measured using an EPC-9 (Heka-Electronics, Lambrecht/Pfalz, Germany) patch-clamp amplifier and were routinely sampled at 4-ms intervals (2,048 points/record, filtered at 100 Hz). The following standard voltage protocols were used: 1) a “ramp” protocol that was applied every 15 s from a holding potential of 0 mV and consisted of a step to −80 mV for 0.6 s, followed by a step to −150 mV for 0.2 s and a 2.6-s linear voltage ramp to +100 mV; 2) a “step” protocol applied from a holding potential of −50 mV consisting of 2-s steps to voltages ranging from −100 to +100 mV in increments of ±10 mV; the time interval between steps was 6 s. Because the swelling-activated currents are much larger than the background currents, the current traces were not corrected for baseline currents.

For the analysis of current fluctuations, the current at ±30 mV was sampled at 2 kHz and filtered at 1 kHz. Mean current (I) and current variance (σ2) were calculated from consecutive 512-point sweeps. These quantities are related by the expression (11, 12)

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\[ \sigma^2 = i \cdot \frac{I^2}{N} \]  

where \( i \) is the single-channel current amplitude and \( N \) is the total number of channels in the membrane. The single-channel chord conductance can be calculated from \( i \) and the electrochemical gradient, i.e., holding potential minus reversal potential of VRAC.

The standard extracellular solution was a Krebs solution containing (in mM) 150 NaCl, 6 KCl, 1 MgCl\(_2\), 1.5 CaCl\(_2\), 10 glucose, and 10 HEPES, triturated with NaOH to pH 7.4. The inwardly rectifying K\(^+\) current, which is present in CPAE cells, was inhibited by substituting K\(^+\) by Cs\(^+\) in the Krebs solution. The osmolarity, as measured with a vapor pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mosM.

After seal formation, the normal Krebs solution was replaced with another isotonic solution that contained (in mM) 105 NaCl, 6 CsCl, 1 MgCl\(_2\), 1.5 CaCl\(_2\), 10 glucose, 10 HEPES, and 90 mM mannitol, triturated with NaOH to pH 7.4. VRAC was activated by superfusing the cell with the same solution without mannitol, resulting in a 25% hypotonicity.

The pipette solution contained (in mM) 40 CsCl, 100 cesium aspartate, 1 MgCl\(_2\), 5 EGTA, 1.93 CaCl\(_2\), 4 Na\(_2\)ATP, and 10 HEPES, triturated with CsOH to pH 7.2. The calculated free Ca\(^2+\) concentration of the pipette solution was 100 nM.


Diethyl pyrocarbonate (DEPC, Sigma) was used at concentrations between 0.001 and 1 mM.

All experiments were done at room temperature (20–22°C). Data were analyzed in Origin (MicroCal Software). Pooled data are given as means ± SE. Level of significance is 0.05.

**RESULTS**

Voltage-dependent inhibition of VRAC by calix[4]arenes. Figure 1 shows the effect of 100 µM TS-TM-calix[4]arene on the amplitude of the \( I_{Cl,swell} \) in a CPAE cell challenged with a 25% hypotonic solution. Figure 1A shows the time course of the current at different potentials. The current amplitudes at +100, +50, and −100 mV were obtained from repetitively applied voltage ramps. TS-TM-calix[4]arene exerts a fast and reversible inhibition at positive potentials (+50 and +100 mV) but is virtually ineffective at negative potentials (−100 mV). The fraction of the current blocked at +100 mV is obviously smaller than that at +50 mV (Fig. 1A). This voltage dependence of the inhibition is more apparent from a comparison of the instantaneous current-voltage (I-V) curves derived from the voltage ramps during and after application of TS-TM-calix[4]arenes (see Fig. 1, A, filled symbols, and B): current amplitudes at positive potentials are substantially suppressed, whereas those at negative potentials are hardly affected at all. It is also obvious that the current trace in the presence of TS-TM-calix[4]arene is much noisier in the potential range at which the compound exerts its inhibitory action. To quantify the effect of TS-TM-calix[4]arene, we have calculated from these I-V curves the fraction of the current that was inhibited at each potential \( V \) \( \left[ INH(V) \right] \), i.e.

\[
INH(V) = \frac{I_{cont}(V) - I_{calix}(V)}{I_{cont}(V)} \cdot 100
\]  

where \( I_{cont}(V) \) and \( I_{calix}(V) \) represent corresponding current amplitudes from the instantaneous I-V relationships at the potential \( V \) in the absence and presence of TS-TM-calix[4]arene (Fig. 1C). It is obvious that inhibition increases with depolarization, reaches a maximum at around +30 mV, and decays at more positive potentials. Maximum inhibition was ~60% in this particular cell.
Fig. 2. Time dependence of the TS-calix[4]arene inhibition of I_{Cl,swell}. A: current traces in response to 2-s voltage steps to potentials from −100 to +100 mV (10-mV increment) applied every 6 s from a holding potential of −50 mV. Sampling interval was 2 ms. B: currents recorded in the presence of 30 µM TS-calix[4]arene using the same protocol as in A. Dotted line in A and B, zero-current level. Note the fast current decay when stepped to positive potentials, representing the onset of calix[4]arene block. C: peak currents (I_{peak}) at beginning of voltage step beyond capacitative current transient in the absence (○) and presence (●) of TS-calix[4]arene derived from the current traces shown in A and B. Note that the peak current at any potential is not significantly affected by calix[4]arene. D: steady-state currents (I_{ss}) measured at end of voltage step from current traces in A and B. TS-calix[4]arenes apparently do not affect the current at negative potentials but clearly suppress it at positive potentials.

Figure 2 illustrates the effect of TS-calix[4]arenes on current traces evoked by voltage steps from a holding potential of −50 mV to various potentials between −100 and to +100 mV (step increment = 10 mV). A holding potential of −50 mV was selected because the calix[4]arenes were ineffective at this potential. Figure 2A shows current traces under control conditions, and Fig. 2B shows those in the presence of 30 µM TS-calix[4]arene. The current traces at negative potentials are not significantly affected by TS-calix[4]arene. In contrast, this compound induced a fast decay of the current at positive potentials. The I-V curves of the initial peak current and the steady-state current at the end of the voltage pulse are shown in Fig. 2, C and D. It is apparent that TS-calix[4]arene does not affect the peak I-V curve but clearly suppresses steady-state currents at potentials more positive than +20 mV.

It has been reported that TS-TM-calix[4]arene, a tetramethoxy-TS-calix[4]arene, is a much more potent blocker of ORCC than TS-calix[4]arene (13). From the dose-response relationship of both calix[4]arenes, as shown in Fig. 3, it is, however, obvious that both compounds inhibit VRAC in CPAE cells with the same potency. At +40 mV, the maximal block calculated from the pooled data was 54 ± 5%, and the IC_{50} was 13 ± 3 µM (n between 7 and 16 at each concentration and for each compound). Also, the voltage and time dependencies of the inhibition were similar for both calix[4]arenes (data not shown).

Kinetic analysis of the inhibition by calix[4]arene. The voltage dependence of the block and the increased current noise at positive potentials at which the calix[4]arenes exert their blocking effect (see Fig. 1) suggest that these compounds may act by blocking the open pore. The classical Woodhull analysis of the time and voltage dependence of the block cannot be applied however to our data because the blocking efficacy decreases at strong positive potentials. We have therefore analyzed our data by assuming that the calix[4]arenes not only block the open VRAC channel but also permeate it to some extent at positive potentials, as shown by the kinetic scheme

$$C_0 + VRAC \frac{k_{01}(V)}{k_{02}(V)} C-VRAC \frac{k_{12}(V)}{k_{10}(V)} VRAC + C_i (~0)$$

in which VRAC and C-VRAC represent the fractions of the conducting and blocked channels. C_0 and C_i correspond to the extra- and intracellular calix[4]arene concentrations; the latter is assumed to be zero. Be-
cause the calix[4]arenes have four negative charges
due to the sulfonate groups, the rate constants (k) for
the various transitions are voltage dependent. Using
rate theory, we calculated the fraction of channels
occupied by calix[4]arenes at an extracellular concentra-
tion C and a potential V \( f(C,V) \) by

\[
f(C,V) = \frac{1}{1 + \frac{K_d(0) \cdot \exp(zF \delta V/RT)}{C} + \frac{k_{12}(0)}{k_{10}(0)} \cdot \exp(-zF \delta V/2RT)}
\]

\[ \tag{3} \]

\[
K_d(0) = \frac{k_{30}(0)/k_{02}(0)}{K_d(0)} \]

\[
k_{10}(0) \]

\[
k_{12}(0)/k_{10}(0) \]

\[
\exp(-zF \delta V/2RT)
\]

\[
\frac{1}{1 + \frac{C}{K_d(0)} \cdot \exp(-zF \delta V/RT)} + \frac{k_{12}(0)}{k_{10}(0)} \cdot \exp(-zF \delta V/2RT)
\]

\[ \tag{4} \]

These time constants were estimated from exponential
fits to the current traces recorded in the presence of 100
µM calix[4]arene during voltage steps to various levels
from a holding potential of −50 mV with a duration of
20 ms and sampled at 10 kHz (Fig. 4A). The fraction of
channels blocked at each potential was assessed from
the amplitude of the decaying current component relative
to the total current, which were also obtained from the
exponential fits. Because the onset of block is much
faster than the rate of intrinsic inactivation of I_{Cl,swell} (6,
8), this latter process will affect neither the estimated
fraction of blocked channels nor the time constants for
onset of block.

The results of these analyses are summarized in Fig.
4, B and C. The fit of the fractional current to Eq. 3
yielded the following values for the parameters: \( K_d(0) = 309 \mu M, \delta = 0.24, \) and \( k_{12}(0)/k_{10}(0) = 0.03. \) With the use of
the values for these parameters, we then fitted the
time constants to Eq. 4, which gave a value of 139 s⁻¹
for \( k_{10}(0). \) From the data of six cells, we obtained the
following values for the fitted parameters: \( K_d(0) =
220 ± 50 \mu M, \delta = 0.25 ± 0.05, k_{12}(0)/k_{10}(0) = 0.06 ±
0.01, \) and \( k_{10} = 148 ± 11 s⁻¹. \)

\[ pH \] dependence of the calix[4]arenes block. An alterna-
tive explanation for the relief of inhibition at strong
positive potentials could be that the binding of
calix[4]arenes occurs at a protonated site inside the
channel, which is accessible to extracellular protons
only. Positive potentials will impede this access and
reduce the fraction of protonated binding sites and
These effects would result in a more potent block at low
pH and shift the peak inhibition to more positive
potentials.

Figure 5A shows instantaneous I-V curves obtained
from voltage ramps in the presence and absence of 10
µM TS-calix[4]arene at various extracellular pH val-
ues. The corresponding fractional current inhibition at
each pH is given in Fig. 5B. It is obvious that changes

![Image](http://ajpcell.physiology.org/ by 10.2203.5 on June 17, 2017)
in extracellular pH strongly affect the blocking efficacy of TS-calix[4]arene. Block at positive potentials is reduced by an increase in the extracellular pH and potentiated at acidic pH. Similar effects of extracellular pH were observed in the presence of TS-TM-calix[4]arenes. Changes in extracellular pH also shifted the potential at which maximal inhibition occurs, from $34.4 \pm 1.2$ mV at pH 6.0 ($n = 11$) to $49.5 \pm 1.5$ mV at pH 7.4 ($n = 8$) and $77.5 \pm 1.9$ mV at pH 9.0 ($n = 4$), which is in a direction opposite to that predicted by the above model. Maximum current inhibition, measured at the peak of the inhibition-voltage curve, decreased from $46.1 \pm 3.3$% ($n = 11$) at pH 6.0 to $33.0 \pm 1.5$% ($n = 8$) at pH 7.4 and $2.5 \pm 0.6$% ($n = 6$) at pH 9.0.

The observed, more effective inhibition at acidic pH is compatible with the hypothesis that the inhibitory action of calix[4]arenes requires protonation of an intramembrane binding site, possibly at histidine residues, but the shift of the maximal inhibition to less positive potentials is incompatible. Therefore, it is unlikely that the model of a protonated binding site alone can explain the voltage-dependent inhibition of the calix[4]arenes. To provide additional support for the involvement of histidine residues, we have studied the effects of DEPC, a rather selective histidine reagent, on $I_{\text{Cl,swell}}$. This substance irreversibly inhibited VRAC with an IC$_{50}$ of $0.101 \pm 0.003$ mM ($n = 6$).

Calix[4]arene-induced current fluctuations. It was obvious from Fig. 1 that TS-TM-calix[4]arene substantially increases the current noise at the positive potentials at which it effectively blocks VRAC. We have therefore analyzed the current fluctuations induced by


![Fig. 6. Fluctuation analysis of the current noise induced by TS-calix[4]arene. A: time course of the current (bottom) and current variance (top) at +30 mV in a cell stimulated with a HTS at a pH of 6. Application of 30 µM TS-calix[4]arene (arrow) inhibits the current and increases its variance. B: current variance ($\sigma^2$) as a function of the decrease in current amplitude relative to that before TS-calix[4]arene administration, i.e., $\Delta I = I_{\text{calix}} - I_{\text{control}}$. Solid line represents the fit of the data points to Eq. 1. From the parameters of the fit, we obtained a single-channel current amplitude of 0.66 pA, corresponding to a conductance of 10.4 pS (Cl$^-$ equilibrium potential = −32 mV). From the number of channels ($N = 1,109$) and the maximum reduction in macroscopic current, we calculated a 32% decrease in open probability.](http://ajpcell.physiology.org/)
Calix[4]arene block. Currents were activated by challenging the cells with the hypotonic solution, and 30 µM TS-calix[4]arene was added after maximal activation was reached. At this time, all available channels are supposed to be in the open state with an open probability close to 1, resulting in a low current noise level of the macroscopic current (2, 16). The cells were clamped at +30 mV at an external pH of 6 to maximize the calix[4]arene-induced current fluctuations (Fig. 6A). It is obvious that application of TS-calix[4]arene rapidly inhibited the current (bottom trace) and increased the variance of the current (top trace). Figure 6B shows the current variance as a function of Δt = I_{calix} − I_{control}, i.e., the reduction of the macroscopic current relative to its value in the absence of TS-calix[4]arene. In Fig. 6B, the solid line shows the fit of the data points to Eq. 1. The average values from nine cells with a mean membrane capacitance of 38.8 ± 2.9 pF are as follows: I = 0.51 ± 0.07 pA, and N = 1,322 ± 115. The decrease in open probability calculated from the above parameters and the reduction of macroscopic current is 0.46 ± 0.07. The single-channel conductance (γ) calculated from the single-channel current and the driving force (V − Cl− equilibrium potential) is 9.3 ± 2.1 pS. To further validate this noise analysis procedure, we have compared the single-channel current amplitude obtained from this analysis with single-channel measurements. From three patches, excised after full activation of VRAC, we obtained single-channel current amplitudes of 0.37 ± 0.02 and 0.76 ± 0.04 pA at potentials of +20 and +40 mV, respectively.

**DISCUSSION**

VRAC, which regulate several cell functions, are not yet identified at the molecular level. A search for pharmacological tools that bind to VRAC with high affinity may therefore be helpful for the purification and molecular identification of these channels as well as their functional characterization. Calix[4]arenes have been reported to represent a class of high-affinity blockers of the ORCC channel. These compounds, which form basket- or calixalike structures and occupy a space of ~7.5 × 12.5 Å (14), are water soluble and induce a fast block of ORCC within milliseconds.

We could confirm in the present experiments that these substances also induce a fast and reversible voltage-dependent block of VRAC channels in endothelial cells. In contrast with the findings on ORCC channels, we did not observe a significant difference in potency between the TS- and TS-TM-calix[4]arenes. Also, the affinity of these compounds for I_{Cl,swell} consists of the recruitment of channels rather than of an increase of open probability (1–3). Because we first fully activated the current in our experiments and then applied calix[4]arenes to reduce the channel open probability without affecting the number of channels, we could apply the well-established Sigworth analysis to estimate single-channel current amplitude and number of channels from the parabolic relation between current amplitude and current variance (Eq. 1). The similarities of the single-channel current amplitude from this analysis and that from single-channel measurements support the hypothesis of Jackson and Strange (1–3) that cell swelling recruits additional VRAC channels but also lends further support to our contention that calix[4]arenes act by blocking the open-channel pore.

In conclusion, calix[4]arene-related compounds might be a useful tool to probe open-channel properties of VRAC. However, their low-binding affinity for VRAC makes them less suitable for use in biochemical isolation and identification of this hitherto unknown channel.

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