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Inhibition of Ca-Activated K^+ Channels from Renal Microvillus Membrane Vesicles by Amiloride Analogs

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Summary. The effect of the K^+-sparing diuretic amiloride and two of its hydrophobic analogs, methylisobutyl amiloride (MIA) and ethylisopropyl amiloride (EIPA), on Ca-activated K^+ channels from renal microvillus membrane vesicles incorporated into planar lipid bilayers was investigated. Amiloride did not inhibit currents through Ca-activated K^+ channels. MIA and EIPA, however, inhibited channel currents when added to both the internal and external solutions in concentrations between 10 and 250 μM. Furthermore, when dose-response data for channel inhibition were examined using Hill plots, Hill numbers of $\approx$1.5 were found for both blockers from both sides, suggesting that the mechanism of block involves multiple inhibitory binding sites. A simple kinetic scheme is proposed that can account for the results.

Key Words amiloride · methylisobutyl amiloride · ethylisopropyl amiloride · Ca-activated K^+ channels · inhibition

Introduction

High conductance Ca-activated K^+ channels are found in many kinds of cells, including anterior pituitary cells (Wong & Adler, 1986), muscle cells (Moczydlowski & Latorre, 1983; Vergara & Latorre, 1983), macrophages (Gallin, 1984), and a number of epithelial cell types (Guggino et al., 1987*a*; Hunter, Kawahara & Giebisch, 1988). This class of channels is characterized by high conductance (typically >150 pS), selectivity for K^+ over Na^+, and modulation of open probability by both membrane potential and internal Ca-ion concentration (for a review *see* Latorre, 1986).

In general, inhibitors of ionic channels fall into one of two general categories. Inhibitors can be open channel blockers, in which the effect of the inhibitor is to reduce the channel mean open time, or closed channel blockers, in which the effect of the inhibitor is to increase the mean closed time. Furthermore, open channel blockers can be generally placed into three different categories. The first category of open channel blocker is slow blockers, in which addition of inhibitor results in the appearance of blocked durations that are long in comparison to the resolution of the recording system. Examples of slow blockers of Ca-activated K^+ channels include barium (Vergara & Latorre, 1983; Guggino et al., 1987*b*; Zweifach et al., 1991) and charybdotoxin (Miller et al., 1985; Guggino et al., 1987*b*; Zweifach et al., 1991). Slow open-channel blockers can be distinguished from closed-channel blockers because the length of the long closures is independent of inhibitor concentration. The second category of open channel blockers is intermediate or "flickery" blockers. This class of open channel blockers results in closures that are of about the same duration as the minimum resolvable duration of closures. The final class of open channel blockers is fast blockers. The closures induced by fast blockers are so short in relation to the resolution of the recording system that closures cannot be resolved at all. Instead, the action of these compounds results in a concentration-dependent reduction in the amplitude of single-channel currents. Examples of fast open channel blockers of Ca-activated K^+ channels include tetraethylammonium (Wong & Adler, 1986; Guggino et al., 1987*b*; Villarroel et al., 1988) and quinidine (Guggino et al., 1987*b*; Zweifach et al., 1991). The concentration dependence of these inhibitors of Ca-activated K^+ channels can be described by a simple single-site blocking mechanism.

In a previous report, we demonstrated that Ca-activated K^+ channels from rabbit renal microvillus membrane vesicles can be incorporated into planar lipid bilayers and that these channels can be blocked by methylisobutyl amiloride (MIA) added to the external bathing solution (Zweifach et al., 1991). The

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purpose of the present communication is to examine in more detail inhibition of Ca-activated K^+ channels by hydrophobic analogs of amiloride. We report that hydrophobic analogs of the K^+-sparing diuretic amiloride block maxi-K^+ channels from rabbit renal microvillus membrane vesicles incorporated into planar lipid bilayers. MIA and EIPA added to both the internal and external solutions inhibit channel activity. Amiloride itself, when added to the external solution, has no blocking effect on Ca-activated K^+ channels. Hill plots of inhibition by MIA and EIPA indicate that these blockers interact with multiple sites within the channel. Our data support a mechanism of ion channel blockade that involves multiple interactions between inhibitor molecules and open channel states.

Materials and Methods

Membrane vesicles were prepared and bilayers fabricated as described previously (Zweifach et al., 1991). For this study we selected channels that incorporated with their Ca-activation sites (the internal side of the channel) facing the *cis* chamber, or the chamber to which vesicles were added. Therefore, the internal side of these channels always faced *cis* and the external side always faced *trans*. The potential was referenced to the *trans* chamber. Experiments were conducted in symmetrical 200 mM KCL solutions buffered to pH 7.4 with 20 mM MOPS-KOH. Calcium concentrations in the *cis* chamber were 1–5 mM.

Amiloride, methylisobutyl amiloride (MIA) and ethylisopropyl amiloride (EIPA) were prepared as stock solutions in dimethylsulfoxide (DMSO) and added to either the *cis* or *trans* solution chambers to yield the desired final concentration. The final concentration of DMSO was never greater than 2%.

Following incorporation of from one to four Ca-activated K^+ channels, membrane currents were low-pass filtered at either 500 Hz (9/15 bilayers) or 1000 Hz (6/15 bilayers) using an 8-pole Bessel filter (Frequency Devices). The filtered currents were recorded on videotape after being digitized at 44 kHz by a Sony PCM modified to pass DC.

In order to measure inhibition of channel activity, the recorded currents were digitized at either 1 msec/point for records filtered at 500 Hz or 200 μsec/point for records filtered at 1 kHz using a Labmaster Interface (Axon Instruments) and an IBM PC-XT computer. The digitized records (6–10 sec total length) were then analyzed using the SUMS routine of the FETCHAN program (PCLAMP software package, Axon Instruments), which generates an ASCII file containing the sum of the current through the membrane at each of the 512 points in a sampling epoch. The sum file was then itself summed over all 512 points by importing the sum files into LOTUS 123. Dividing the resultant current sum by the total number of sampled points allowed us to measure the time-averaged current through the membrane in the presence and absence of blockers. This procedure is equivalent to integrating the current record. Throughout the rest of this communication integrated currents obtained from this procedure or from filtering the data at 2 Hz (*see below*) are denoted by *I*. Baseline current was measured and subtracted from the time-averaged currents. Some records were filtered at 2 Hz and displayed on a Gould stripchart recorder, a treatment which has been used by others to obtain integrated current records (Latorre, 1986). The two procedures yielded virtually identical results.

Measurements of open and closed time distributions for bilayers containing single channels were obtained using the FETCHAN portion of the PCLAMP program. In order to obtain estimates of open and closed times, we first characterized the frequency response of the recording system. With 1 kHz filtering, we found that we could accurately resolve the duration of openings or closings that were >600 μsec. Therefore, we fit exponential functions to open and closed time distributions for durations >600 μsec. Extrapolation of the fits of closed time distributions to $t = 0$ msec allowed us to obtain an estimate of the number of short closings that were missed due to frequency limitations. This estimate was then used to correct the durations of open times for missed closing events using the relation (Moczydlowski, 1986):

$$t_o = (\#\text{closures}_{obs}/\#\text{closures}_{est}) \cdot t_{o\,obs}. \quad (1)$$

Kinetic parameters for patches with two or three channels were estimated using the program TAC (courtesy of Dr. Frederick J. Sigworth). This program uses half-amplitude threshold-crossing event detection and estimates mean open time for a bilayer with more than one channel as:

$$t_o = \sum \frac{\text{dwell time} \cdot \text{level}}{N_o} \quad (2)$$

where t_o is the mean open time, N_o is the number of openings in the record, and level refers to one channel open, two channels open, etc. In other words, the program considers that an opening starts with a transition to a higher level and terminates when a transition to a lower level is made. For these estimates, it does not matter which of the several channels in the bilayer closes. Using this estimate of mean open time and the measured open probability, we obtained an estimate of mean closed time from the relation:

$$P_o = t_o/(t_o + t_c). \quad (3)$$

When results were fit separately and presented together, values are expressed as mean ± SE.

Results

In two experiments we found that amiloride added to the external solution at concentrations as high as 2 mM did not inhibit Ca-activated K^+ channels from renal microvillus membrane vesicles.

Figure 1 shows two different representations of the effect of adding increasing concentrations of MIA to the external solution bathing a bilayer with two Ca-activated K^+ channels. On the left are records filtered at 1 kHz and plotted on a Gould stripchart recorder (effective low-pass corner frequency = 87 Hz). On the right are the same records, filtered at 2 Hz, and displayed on a compressed time scale. The effect of MIA, which can be seen as a concentration-dependent increase in the number of brief closures in the left side of Fig. 1, is visible as

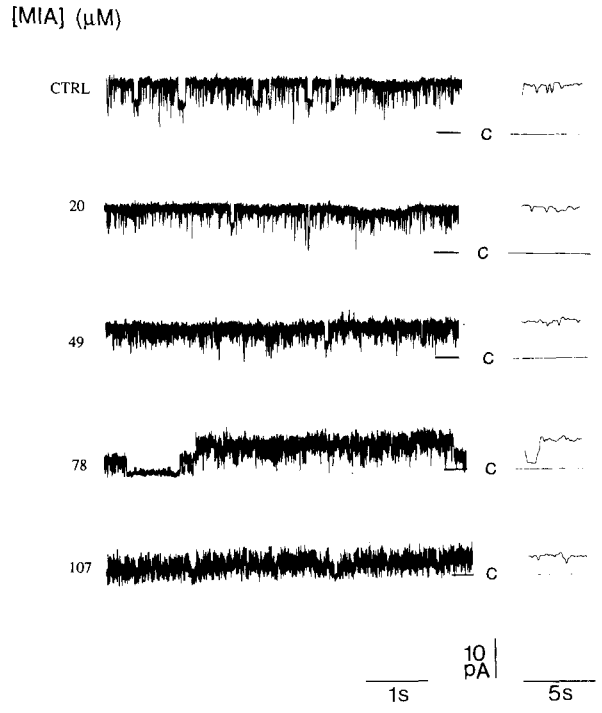


Fig. 1. The left side of the figure shows current records obtained in symmetrical 220 mM K^+ solution in the absence and presence of MIA added to the external solution. The membrane contained two Ca-activated K^+ channels. Openings are upward. The right side of the figure shows sections of the same records filtered at 2 Hz. The closed-channel current level for each trace is denoted by the lowercase letter "*c*" located between the two traces displayed for each condition. The holding potential in this experiment was +20 mV.

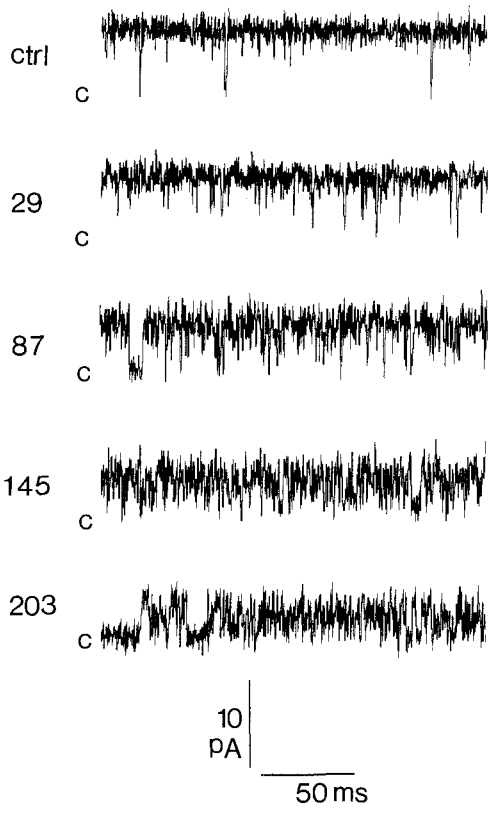


Fig. 2. Current records from a bilayer with a single Ca-activated K^+ channel in the absence and presence of external EIPA. Holding potential was +20 mV. Closed-channel current level is indicated by the lowercase letter "*c*" on the left of each trace. Openings are upward.

a reduction in the mean time-averaged current in the right side of the Fig. 1. A qualitatively similar pattern of blocking is evident in Fig. 2, which shows current records from a bilayer containing a single Ca-activated K^+ channel in the presence and absence of various concentrations of EIPA added to the external solution. Thus, application of methylisobutyl amiloride (MIA) or ethylisopropyl amiloride (EIPA) to the external solutions resulted in a "flickery" block characterized, at low blocker concentrations, by the appearance of brief closures. Increasing the concentration of blocker increased the number of brief closures and appeared to reduce the amplitude of the single-channel currents. Similar patterns of "flickery" block also resulted from addition of MIA or EIPA to the internal solutions. In several experiments in which high concentrations of amiloride analogs were tested, >90% inhibition of channel currents was observed.

We next examined the concentration dependence of inhibition using Hill plots of integrated current records in the absence and presence of different concentrations of inhibitor. Figure 3 shows plots of log $[I_{MIA}/(I_{CTRL} - I_{MIA})]$ *versus* log [MIA] for the experiment of Fig. 1. The best-fit regression line corresponds to an n of 2.1 and a $K_{1/2}$ of 83 μM. Such regression lines were determined for each of three experiments in which at least three concentrations of MIA were tested and indicated a mean Hill number, n, of 1.8 ± 0.2 and a mean $K_{1/2}$ of 52 ± 15 μM. When data from six experiments with external MIA (including the three described above) were pooled and used to determine a single regression line, the Hill number, n, was 1.7 and the $K_{1/2}$ was 60 μM.

Hill numbers for inhibition by EIPA added to the external solution were also >1. Figure 4 shows a plot of log $[I_{EIPA}/(I_{CTRL} - I_{EIPA})]$ *versus* log [EIPA] for the data from Fig. 2. The Hill number in this experiment was 1.4. Regression lines were determined for each of two experiments in which at least three concentrations of EIPA were tested and indicated a mean Hill number, n of 1.5 ± 0.1 and a mean $K_{1/2}$ of 94 ± 12 μM. When the data

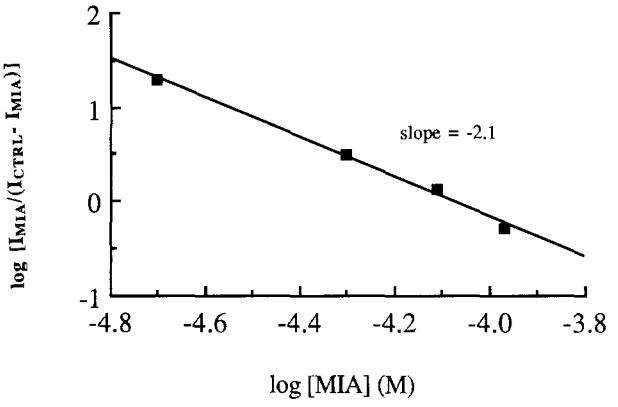


Fig. 3. Hill plot of data from the experiment in Fig. 1. Log($I_{MIA}/I_{CTRL} - I_{MIA}$) is plotted on the ordinate, and the abscissa axis is log[MIA]. The data can be described by a straight line with a slope of 2.1 and a $K_{1/2}$ of 83 μM.

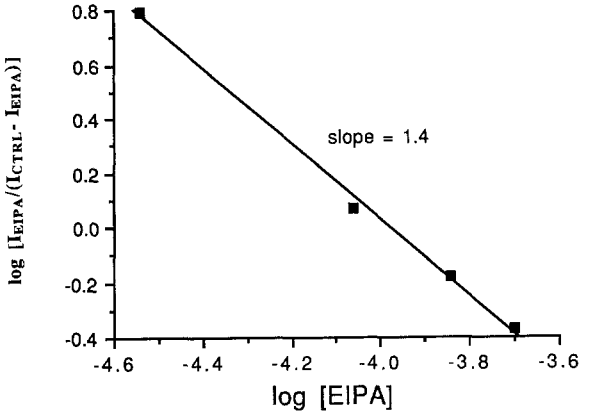


Fig. 4. Hill plot of data from the experiment of Fig. 2. The Hill number, n, for this experiment was 1.4.

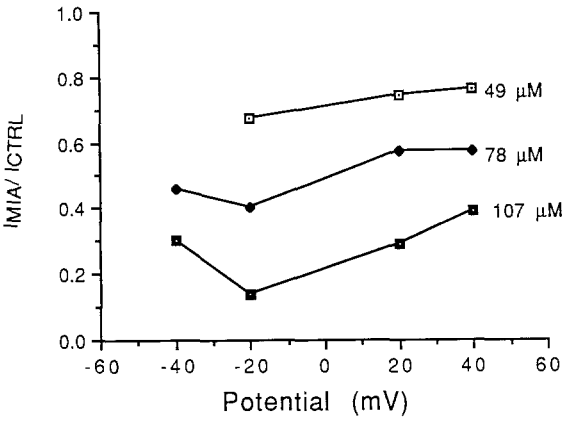


Fig. 5. Inhibition of current as a function of $[MIA]_{out}$, and potential for the experiment shown in the preceding two figures. There is no consistent effect of voltage on the blocking effect of external MIA.

from these two experiments were pooled with the data from a third experiment in which only two concentrations of EIPA were tested, the resulting regression line indicated a Hill number, n, of 1.5 and a $K_{1/2}$ of 100 μM.

Regression lines were determined for each of three experiments in which at least three concentrations of internal MIA were tested and indicated a mean Hill number, n, of 1.9 ± 0.3 and $K_{1/2}$ of 154 ± 31 μM. When data from these experiments were pooled with the data from another experiment in which only two concentrations of MIA were tested, the resulting regression line indicated a Hill number, n, of 1.8 and a $K_{1/2}$ of 128 μM. Regression lines for two experiments in which at least three concentrations of EIPA were added to the internal solution indicated a Hill number, n, of 1.8 ± 0.5 and a $K_{1/2}$ of 128 ± 19 μM. Therefore, in all cases examined, Hill numbers for inhibition by hydrophobic analogs of amiloride were >1 and the $K_{1/2}$ values were in the range 50–150 μM.

Block by MIA and EIPA was not voltage dependent. For example, the results of one experiment in which the block by three concentrations of external MIA was examined as a function of potential over the range of +40 to −40 mV are illustrated in Fig. 5. No voltage dependence of MIA block was evident. In two other experiments, the block by four or six concentrations of external EIPA was found to be independent of applied potential. Similarly, in two experiments the block by two or five concentrations of internal MIA was independent of voltage. In another experiment, block by two concentrations of internal EIPA was unaffected by voltage.

In order to investigate the mechanism of inhibition of Ca-activated K^+ channels by MIA and EIPA we examined the effects of these compounds on channel kinetics. Figure 6 shows the results of such an analysis for the representative experiment of inhibition by external EIPA previously illustrated in Figs. 2 and 4. Figure 6*A* shows the relationship between t_c and [EIPA] for the data from Fig. 2. Note that t_c remained constant at ≈0.3 msec. Figure 6*B* shows the relationship between t_o and [EIPA]. Note that t_o was decreased by EIPA. The number of inhibitor molecules involved in reducing the open time can be estimated using the relation

$$\log(1/t_o - a) = n(\log[\text{EIPA}] + \log k_{on}) \qquad (4)$$

where $1/a$ is the open time in the absence of inhibitor and n is the number of inhibitor molecules. For a simple single-site blocking mechanism, a plot of $\log(1/t_o - a)$ *versus* log[EIPA] should have a slope

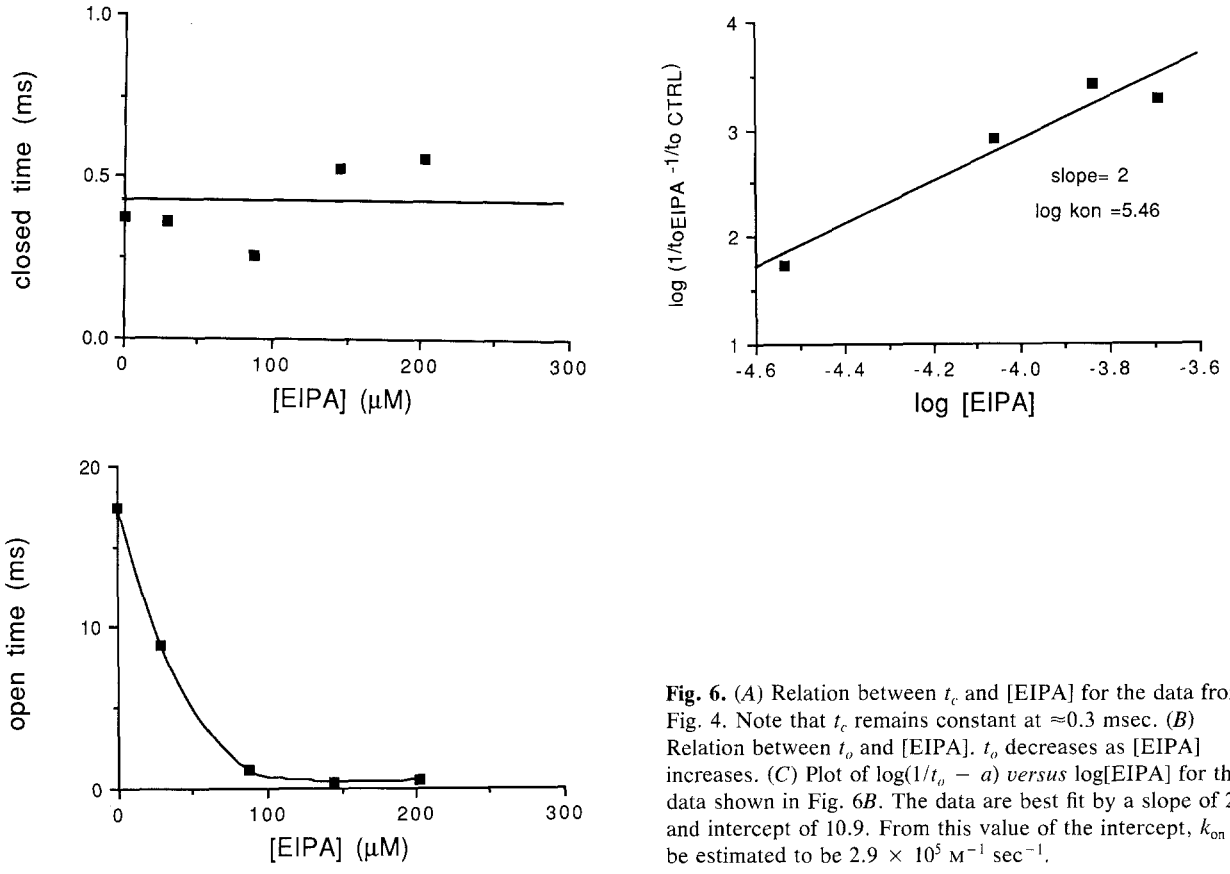


Fig. 6. (*A*) Relation between t_c and [EIPA] for the data from Fig. 4. Note that t_c remains constant at ≈0.3 msec. (*B*) Relation between t_o and [EIPA]. t_o decreases as [EIPA] increases. (*C*) Plot of $\log(1/t_o - a)$ *versus* log[EIPA] for the data shown in Fig. 6*B*. The data are best fit by a slope of 2 and intercept of 10.9. From this value of the intercept, k_{on} can be estimated to be 2.9×10^5 M^{-1} sec^{-1}.

of ≈1 and an intercept of $\log k_{on}$. When the data from the experiment shown in Fig. 2 were plotted in this fashion, however, the slope of the relation was 2 (Fig. 6*C*). Therefore, the relation between $1/t_o$ and [EIPA] can be described by:

$$1/t_o = a + ([\text{EIPA}] * k_{on})^2 \quad (5)$$

where k_{on} is estimated to be 2.9×10^5 M^{-1} sec^{-1}. We performed a similar analysis of the effects of external EIPA on another bilayer that contained three Ca-activated K^+ channels. For this experiment, the slope of the relation between $\log(1/t_o - a)$ and log [EIPA] was 1.6, and k_{on} was estimated to be 3.2×10^5 M^{-1} sec^{-1}.

As summarized in the Table, similar results were obtained in four additional experiments conducted with MIA and EIPA added to the inside or outside solution in which it was possible to low-pass filter the data at 1 kHz. Three of these experiments were bilayers that contained a single Ca-activated K^+ channel. Therefore, for these experiments we were able to fit exponentials to the distributions of open and closed times and correct for the limited resolution of the recording system. The other two experiments were from bilayers with two and three channels, respectively. In all cases, t_c was independent of inhibitor concentration, whereas t_o was reduced with a greater than first-order dependence on inhibitor concentration. These results are consistent with the idea that EIPA and MIA are open channel blockers and that at least two EIPA or MIA molecules are involved in binding to the channel, thereby interrupting channel current.

Discussion

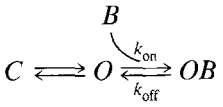
The purpose of the present communication is to expand on our previous observation that MIA inhibits Ca-activated K^+ channels (Zweifach et al. 1991) and to gain insight into the mechanism of inhibition. We found that EIPA also inhibits Ca-activated K^+ channel activity. Inhibition of channel activity results from an increase in the number of brief closures, typical of a "flickery" blocker. Because Hill numbers for the concentration dependence of block were ≈1.5 for MIA and EIPA added to both the internal and external solutions, we suggest that the mechanisms of block are similar for both compounds. The observation that Hill numbers for block by both MIA and EIPA are ≈1.5 provides strong support for the

Table. Summary of kinetic parameters of block

Condition	Slope of log(1/to − a) *vs.* log[Blocker]	k_{on} (M^{-1} sec^{-1})	k_{off} (sec^{-1})	Single channel or multiple channel bilayer
MIA_{out}	2.8	1×10^5	0.7×10^3	Multiple
MIA_{in}	1.75	2.35×10^5	2.5×10^3	2 single
	±0.25	$\pm 0.15 \times 10^5$	$\pm 0.1 \times 10^3$	
$EIPA_{out}$	1.8	3.05×10^5	2.45×10^3	1 single,
	±0.2	$\pm 0.15 \times 10^5$	$\pm 0.05 \times 10^3$	1 multiple
$EIPA_{in}$	1.4	5.9×10^5	2.6×10^3	Single

idea that at least two inhibitor molecules are involved in producing block.

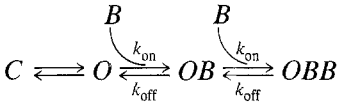
The simplest model of an open channel blocking reaction is



Scheme 1

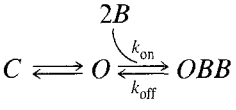
in which transitions between states 1 and 2 represent the kinetic behavior of the channel in the absence of blocker. It has been demonstrated that the behavior of Ca-activated K^+ channels in planar lipid bilayers is consistent with a more complex kinetic scheme involving at least two closed and two open states (Moczydlowski & Latorre, 1983; Zweifach et al. 1991). Under the conditions of these experiments, i.e., 1–5 mM Ca and depolarized membrane potentials (+25 to +50 mV), channel-open probability is high. The kinetic behavior of the channel is well approximated by transitions between states 1 and 2 of scheme 1 under these conditions, as is demonstrated by the single-exponential distributions of both open and closed times obtained under control conditions (*not shown*). According to scheme 1, t_o is expected to decrease as a linear function of the blocker concentration.

The results presented in Fig. 6 and the Table, however, demonstrate that t_o does not decrease as a linear function of blocker concentration. Examination of the effect of hydrophobic analogs of amiloride on opening and closing rates (Fig. 6 and the Table) indicates that these compounds produce block by interacting with open channel states only and that at least two inhibitor molecules are involved in the blocking interaction. The following kinetic scheme is the most general model that can account for block of Ca-activated K^+ channels by hydrophobic amiloride analogs:



Scheme 2

This model is consistent with the concentration dependence of block by amiloride analogs, because Hill numbers >1 do not necessarily imply cooperative binding of inhibitory ligands (Segel, 1975). This scheme, however, predicts two distributions of closed durations corresponding to mono- and diliganded blocked states (states 3 and 4). We observed only one distribution of blocked times. Because we cannot accurately measure intervals with durations <600 μsec, it is possible that we are missing extremely fast blocking events due to the monoliganded state. A second scheme that can account for our results is



Scheme 3

This scheme is a cooperative binding model. Scheme 2 reduces to scheme 3 if the monoliganded state (state 3 of scheme 2) is extremely short-lived or if the monoliganded state is not a blocked state. The advantages of scheme 3 as a model for block of Ca-activated K^+ channels by MIA and EIPA are that it is simpler than scheme 2, having one less kinetic state, and that it predicts a single distribution of blocked times. Therefore, we conclude that scheme 3 provides an adequate empirical description of block of Ca-activated K^+ channels by hydrophobic amiloride analogs.

MIA and EIPA are significantly more hydrophobic than amiloride itself (Kleyman & Cragoe, 1988).

EIPA, for example, is ≈16 times as lipophilic as amiloride. Since amiloride itself does not block channels, it is possible that the analogs MIA and EIPA bind to hydrophobic sites, perhaps in the wide vestibules that have been postulated to exist in the internal and external sides of the channel (Villarroel et al., 1988; Wong & Adler, 1986). It is also possible that MIA and EIPA partition into the bilayer and then block by interfering with conduction from within the plane of the membrane, perhaps by "poking through" the wall of the pore.

Another reported example of a blocker of Ca-activated K^+ channels that does not follow simple single-site behavior is Ca. Vergara and Latorre (1983) found that Ca added to the intracellular bathing solution introduces long silent periods characteristic of a slow blocker. In addition, millimolar concentrations of Ca added to the intracellular bathing solution cause a reduction in the single-channel current-amplitude characteristic of a fast block. The slow block produced by Ca occurs on a time scale of seconds, while the fast block that reduces the apparent single-channel current-amplitude can be estimated to occur on a time scale of <10 μsec, a difference of at least five orders of magnitude.

A blocking mechanism that involves multiple interactions between an ion channel and an inhibitor has also been reported for excitable Na channels from rat skeletal muscle. Moczydlowski, Uehara and Hall (1986) have described two blocking effects of derivatives of local anesthetics on Na channels incorporated into planar lipid bilayers. The first is also a fast block that reduces current amplitude, and the second effect is a slower block that appears as an increase in mean closed times. The second blocking effect of local anesthetics has been interpreted as a closed-channel block.

Finally, a recent paper by Lane, McBride and Hamill (1991) reports that the mechanosensitive cation channel of *Xenopus* oocytes is blocked by amiloride added to both the external and internal solutions and that Hill numbers for inhibition are ≈2. Amiloride acts as a flickery blocker in concentrations >0.05 mM. Block from the outside is strongly voltage dependent, while inhibition from the inside is independent of applied potential. They demonstrated that their data can be described by a model in which a voltage-dependent conformational change exposes at least two binding sites to external amiloride. Binding of amiloride to these sites is voltage independent.

The mechanism we have suggested on the basis of the results presented in this communication is somewhat similar to the results presented by Lane et al. (1991). The mechanism of block of Ca-activated K^+ channels by hydrophobic analogs of amiloride involves interactions of multiple inhibitor molecules with the open state of the channel on a similar time scale.

A last point, obvious from the results presented in this paper but important nonetheless, is that neither MIA nor EIPA can be considered specific inhibitors of Na^+–H^+ exchange. Inhibition of Ca-activated K^+ channels by MIA or EIPA could result in changes in pH_i in the absence of direct effects on Na^+–H^+ exchange. For example, inhibition of Ca-activated K^+ channels could lead to cellular depolarization, which could cause cellular alkalinization by decreasing the electrochemical driving force for electrogenic bicarbonate exit. Note that this effect on pH_i would be opposite to the effect produced by inhibition of Na^+–H^+ exchange, which leads to cellular acidification.

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