J. Membrane Biol. 99, 147–155 (1987)

The Journal of
Membrane Biology

Modification of Ca^{2+}-Activated K^+ Channels in Cultured Medullary Thick Ascending Limb Cells by N-Bromoacetamide

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Summary. Ca^{2+}-activated K^+ channels were studied in cultured medullary thick ascending limb (MTAL) cells using the patch-clamp technique in the inside-out configuration. The Ca^{2+} activation site was modified using N-bromoacetamide (NBA). 1 mM NBA in the bath solution, at 2.5 μM Ca^{2+} reduces the open probability, P_o, of the channel to <0.01, without an effect on single-channel conductance. NBA-modified channels are still Ca^{2+}-sensitive, requiring 25 mM Ca^{2+} to raise P_o to 0.2. Both before and after NBA modification channel openings display at least two distributions, indicative of more than one open state. High Ca^{2+} (1 mM) protects the channels from modification. Also presented is a second class of Ca^{2+}-activated K^+ channels which are normally present in MTAL cells which open infrequently at 10 μM Ca^{2+} (P_o = 0.01) but have a P_o of 0.08 at 1 mM Ca^{2+}. We can conclude (i) that NBA modifies the channel by shifting Ca^{2+}-sensitivity to very high Ca^{2+}, (ii) that NBA acts on a site involved in Ca^{2+} gating, and (iii) that a low affinity channel is present in the apical cell membrane with characteristics similar to those of normal channels modified with NBA.

Key Words loop of Henle · potassium conductance · ion channels

Introduction

Large conductance Ca^{2+}-activated K^+ channels are present in cells from several nephron segments of the kidney, including the apical cell membrane of the *Amphiuma* diluting segment (Kawahara, Hunter & Giebisch, 1987), the thick ascending limb (Guggino et al., 1987*a*) and the cortical collecting duct (Hunter et al., 1984). Although the exact function of these channels in epithelial cells is not fully understood, it is clear that the presence of these K^+ channels gives these cells a pathway for large movements of K^+ from cell to tubule lumen down a favorable electrochemical potential.

For example, it was shown in a primary culture of chick kidney cells that Ca^{2+}-activated K^+ channels recorded in cell-attached patches open very infrequently at the resting membrane potential, but could be stimulated dramatically by application of ADH or forskolin (Guggino et al., 1985). In the stimulated condition, the channels spent a large amount of time in the open state, contributing to K^+ movement across the membrane. Thus, it is possible that Ca^{2+}-activated K^+ channels play a key role in K^+ movements in cells that are stimulated either through receptor activation or possibly by changes in cell volume.

Because of the importance of Ca^{2+} in modulating Ca^{2+}-activated K^+ channel activity and because of the impact that this modulation may have on the K^+ conductance of cells possessing these channels, we addressed our studies to the Ca^{2+} modulation of Ca^{2+}-activated K^+ channels in cultured medullary thick ascending limb (MTAL) cells and to the influence of N-bromoacetamide (NBA) on this modulation. NBA is an agent that modifies proteins at the carboxyl end of cysteine, histidine, phenylalanine, tyrosine, and tryptophan residues. It is known to remove inactivation of Na^+ channels in excitable tissues (Oxford, Wu & Narahashi, 1978; Patlak & Horn, 1982). We chose NBA because it was shown by Pallotta (1985) to remove the Ca^{2+} sensitivity of Ca^{2+}-activated K^+ channels in rat skeletal muscle. We have extended his observations by demonstrating that NBA also modifies Ca^{2+}-activated K^+ channels in MTAL cells. In these cells NBA shifts channel activation to much higher Ca^{2+} concentrations instead of removing completely the Ca^{2+} sensitivity as was postulated for the rat muscle channels (Pallotta, 1985). In addition, we have shown that high Ca^{2+} can protect the channel from NBA modification.

Materials and Methods

Cell Culture

The clone, A_3, of rabbit MTAL cells used in this study were established by Green et al. (1985). The cells were grown in culture according to the techniques described in a previous paper (Guggino et al., 1987*a*). Cultures were used 2 to 7 days after seeding. Cells grown in small groups or near confluency were used for patch-clamp experiments. Excised patch experiments were performed at 22°C.

Patch-Clamp Techniques

Pipettes made from flint glass were produced as described in detail by Hamill et al. (1981). Pipettes were pulled in two steps with a Kopf (Tujunga, CA) vertical pipette puller, fire polished on a microforge assembled from a Leitz microscope (Bunton Instruments, MD), and coated with Sylgard (Dow Corning, MI). Pipettes routinely had an open-tip resistance of 5–8 MΩ. Membrane currents were recorded with an extracellular patch clamp (Yale Physiology Department, Mark V, New Haven, CT). Electrical contact between pipette and electrometer was made with a Ag/AgCl wire. A similar wire in the bath was connected to ground. The signal from the patch-clamp amplifier was digitized by a pulse code modulator (Model VP101, JVC, Tokyo, Japan) and recorded on a video tape recorder (Model BR6400U, JVC, Tokyo, Japan). Before being digitized the signal was filtered at 3 kHz with a low pass 8-pole Bessel filter (Frequency Devices, Haverhill, MA).

Seals were made by gently lowering the patch pipette onto the surface of a cell with a Leitz micromanipulator, until a small increase in patch resistance was observed, then gigaseals between 5–10 GΩ were obtained with gentle mouth suction. The patch was excised by quickly pulling the pipette away from the cell. In addition to Ca^{2+} activation and voltage dependence of the channel, inside-out patches were determined by a decrease in amplitude with 20 mM TEA (tetraethylammonium) but not 1 mM TEA. Maxi-Ca^{2+}-activated K^+ channels described in this study showed similar sensitivity to TEA as reported previously (Guggino et al., 1987*b*).

Solutions

In all experiments, the pipette was filled with (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 15 HEPES adjusted to pH 7.4 with NaOH. The bath solution contained (in mM): 135 KCl, 5 NaCl, and 15 mM HEPES adjusted to pH 7.4 with KOH. 10 mM EGTA was added when 1–10 μM free Ca^{2+} was required. The free Ca^{2+} was adjusted with $CaCl_2$ using a Ca^{2+} macroelectrode (F2210Ca, Radiometer, Copenhagen, Denmark). The solutions were perfused in a 1-ml chamber at 17 ml/min, with the patch pipette placed near to solution inflow. The solution near the patch was exchanged in 5 sec, as assessed by a dramatic decrease in channel opening when Ca^{2+} concentration is lowered from 10 to 1 μM. NBA was applied to the intracellular face of excised-inside-out patches for short periods of time (*see* Table 2) and removed. Thus all data analysis was performed in the absence of NBA.

Data Analysis

After the experiment, data digitized at 7.1 kHz were transferred to a computer (Micro-PDP/11, Digital Equipment Corp., Maynard, MA) for analysis. The following two parameters were assessed: (i) Open probability, expressed as the ratio of open time to total time, and (ii) mean open (T_o) times. The latter parameter was determined by plotting frequency histograms of open time versus time. Individual values from the histograms were fitted by the computer using the SAAM program (Berman, Shahn & Weiss, 1962; Berman & Weiss, 1978) running on a VAX 8500 computer (Digital Equipment Corp, Maynard, MA) to the equation

$$Cd = Ae^{-t/T_1} + Be^{-t/T_2} \quad (1)$$

where Cd is the cumulative distribution of open events longer than a given time,[1] t.

Outward current from inside-out, excised patches is designated as upward deflections or positive current. All voltages are applied voltages, designated as the voltage applied at the intracellular face of cell membrane with respect to ground in the pipette. Single-channel conductance was measured from the slope of current *vs.* voltage curves. Typically, between 5 and 7 voltages were used between −40 and +30 mV.

Data are reported as mean ± SE (n = number of observations).

Results

Effect of N-Bromoacetamide

Figure 1 shows a series of tracings from a single channel before and after perfusion of 1 mM NBA on the intracellular face of the membrane. In the left tracings, the channel before NBA modification is distinctly Ca^{2+} sensitive with the opening probability, P_o, increasing from 0.39 at 2.5 μM Ca^{2+} to 0.92 at 10 μM Ca^{2+}. The presence of only one discrete amplitude of channel fluctuations in 10 μM Ca^{2+} (upper left panel of Fig. 1), a concentration which causes normal channels to spend most of the time in the open state, confirms that there is only one channel in the patch. If more than one channel were present, multiple amplitudes would be evident at high Ca^{2+}.

[1] The distributions of open time were fit to two exponentials. However, as shown by others (Magleby & Pallotta, 1983), there is a third component with a very short mean open time. For example, in the NBA-modified channel at 25 mM Ca^{2+} (*see* Table 3), a fit of the opening distributions to three exponentials yields mean open times of 0.49, 5.3, and 27.6 msec, respectively. The latter two are similar to the ones reported in Table 3. We chose not to fit all our data to three exponentials, because in most instances it was not possible for us to resolve accurately the mean open time of events significantly shorter than 0.5 msec.

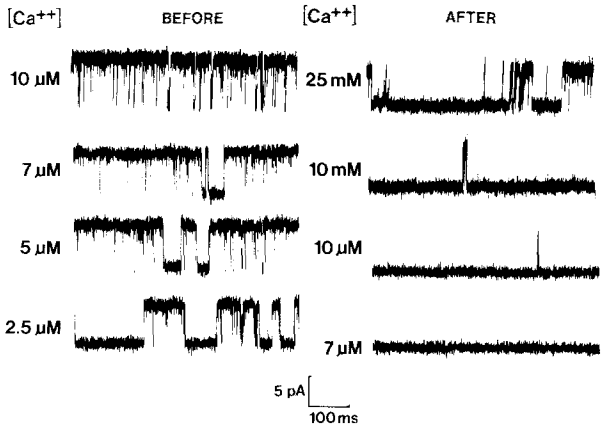

Fig. 1. Ca^{2+} activation of a channel before and after NBA treatment. Recordings are from a single channel at 0 mV before (left panel) and after (right panel) modification with 1 mM NBA at 2.5 μM Ca^{2+}. The open probability before treatment: at 10 μM Ca^{2+}, P_o = 0.92; at 7 μM Ca^{2+}, P_o = 0.85; at 5 μM Ca^{2+}, P_o = 0.81; at 2.5 μM Ca^{2+}, P_o = 0.39; following modification at 25 mM Ca^{2+}, P_o = 0.19; at 10 mM Ca^{2+}, P_o = 0.02; at 10 μM Ca^{2+}, P_o = 0.01; and at 7 μM Ca^{2+}, P_o = 0.00. Evidence that only one channel is present in this patch comes from 10 min of recording time without any evidence of a second level. Control data are from normal activity channels

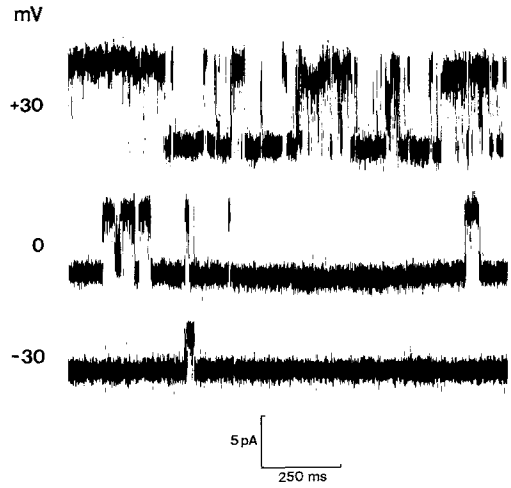

Fig. 2. Voltage activation of a single channel following NBA modification. The open probability at +30 mV, P_o = 0.43; at 0 mV, = 0.14; and at −30 mV, P_o = 0.02. The single-channel conductance estimated from the slope of current *vs.* voltage relationship for this channel in high Ca^{2+} is 81 pS, less than that observed at low Ca^{2+} (149 ± 9 pS, n = 3). We also have observed similar reductions in amplitude in unmodified channels in high Ca^{2+} (single-channel conductance = 79 pS), which may be caused by a fast blocking effect of Ca^{2+} at high concentrations (*see* the discussion for details)

NBA modification of the same channel in the presence of 2.5 μM Ca^{2+} (right panel, Fig. 1) dramatically lowers channel activity such that P_o is 0.000 and 0.009 at a 7 and 10 μM Ca^{2+}, respectively, compared to 0.85 and 0.92 before modification. Importantly, however, the opening probability can be increased (upper two tracings, right panel, Fig. 1) to 0.02 and 0.19 if Ca^{2+} concentrations are increased to 10 and 25 mM Ca^{2+}, respectively, showing that the channels are still Ca^{2+} sensitive following NBA modification. It is important to stress that, although the modified channel is Ca^{2+}-sensitive, P_o of the modified channel at 25 mM is only 21% of that observed at 10 μM Ca^{2+} prior to NBA application. Table 1 summarizes the open probability, P_o, versus Ca^{2+} concentration in four experiments. Although there is considerable variation in the ability to activate channels with Ca^{2+} following NBA modification, P_o always increases with increasing Ca^{2+}. Our results confirm the observations of Pallotta (1985) that NBA modifies Ca^{2+}-activated K^+ channels by dramatically decreasing the opening probability at low Ca^{2+}. However, in contrast to Pallotta, who concluded that NBA removed the Ca^{2+} sensitivity of the channels, we show that NBA modification does not remove Ca^{2+} activation but instead shifts it to much higher Ca^{2+} concentrations.

Figure 2 presents three recordings from a single NBA-modified channel exposed to 25 mM Ca^{2+} at three different voltages. Importantly, the open probability increases as the applied voltage becomes more positive, indicating that the modified channel is voltage sensitive. Similar observations

Table 1. Open probability of channels treated with NBA

Number	Ca^{2+} concentration	P_o
1	7 μM	0.001
	1 mM	0.004
	10 mM	0.001
	25 mM	0.007
2	5 μM	0
	1 mM	0.06
	10 mM	0.3
3	10 μM	0.001
	5 mM	0.004
	7.5 mM	0.004
	10 mM	0.01
	25 mM	0.2
4	1 mM	0.01
	5 mM	0.4
	10 mM	0.5
	12.5 mM	0.8
	15 mM	0.8

Recording time for the determination of open probability varied between 18 and 88 sec. All results are from single-channel patches.

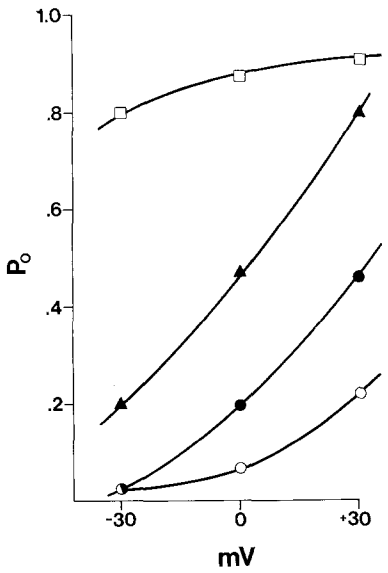

Fig. 3. Open probability *vs.* voltage for normal unmodified and modified channels. Squares represents an unmodified channel in 7 μM Ca^{2+}, triangles, an unmodified channel at 2.5 μM Ca^{2+}, filled circles, a modified channel at 25 mM Ca^{2+}, and open circles, a low activity channel in 1 mM Ca^{2+}

also were made by Pallotta (1985). Plotted in Fig. 3 is the influence of voltage on open probability for the same channel before and after NBA modification. The unmodified channel in 2.5 μM Ca^{2+} shows an open probability that increases at positive voltages, however, at high Ca^{2+} concentrations (10 μM) open probability becomes less sensitive to voltage within the range of voltages described in Fig. 3, a characteristic previously reported for Ca^{2+}-activated K^+ channels in MTAL cells (Guggino et al., 1987*b*). This latter observation is consistent with those of Moczydlowski and Latorre (1983), who showed that Ca^{2+} modulates the voltage activation of the channel. Importantly, P_o of the modified channel also increases with depolarization at 25 mM an effect similar to the untreated channel in 2.5 μM Ca^{2+}. This suggests that the mechanism of voltage activation is unaffected by NBA modification. Our data support the model of Moczydlowski and Latorre (1983) which states that Ca^{2+} binding is the major modulator of channel gating with voltage affecting Ca^{2+} binding.

Finally, similar to the observations of Pallotta (1985), NBA modification does not affect single-channel conductance. The average single-channel conductance of unmodified channels is 141 ± 5 pS (n = 3) and for modified channels is 149 ± 9 pS (n = 3) measured in the presence of 7–10 μM Ca^{2+}. This suggests that NBA affects the gating mechanism without influencing the conduction pathway.

NBA Treatment in High Ca^{2+}

To determine if there is any interaction between the Ca^{2+} binding site and the site of action of NBA, single Ca^{2+}-activated K^+ channels were exposed to NBA in the presence of high Ca^{2+}. An example of such an experiment is given in Fig. 4, which shows tracings from a continuous recording from a single channel. At time 0, the channel is exposed to 10^{-3} M Ca^{2+} for 1 min as a control. Note that in the absence of NBA the channel displays kinetics which are typical of high Ca^{2+}, namely, bursts of flickery openings punctuated by long quiet periods. The channel is then exposed to NBA in 1 mM Ca^{2+} for 5 min. At 1 mM Ca^{2+}, NBA treatment does not have any effect on the open probability in sharp contrast to its effect at 2.5 μM Ca^{2+} (*compare* to Fig. 1). Following 5 min exposure, NBA was removed from the solution for 1 min and reintroduced at 10^{-6} M Ca^{2+}. NBA exposure at low Ca^{2+} dramatically reduces the amount of time that the channel spends in the open state. In fact, in this experiment NBA removes all channel opening events at 1 and 7 μM Ca^{2+}. Similar to the channel shown in Fig. 1, the NBA-modified channel of Fig. 4 could be activated in high Ca^{2+}; however, the open probability in 1 mM Ca^{2+} (0.01) following exposure is much less than before (0.74), more evidence that the channel has been modified by NBA. Thus, it appears that high Ca^{2+} concentration on the intracellular face of the membrane protects the channel from the effects of NBA.

The data from 11 patches are given in Table 2. Although there is some degree of variation among channels, all were modified by NBA in low Ca^{2+} (assessed by a sudden reduction in open probability). In contrast, when a patch was treated with 1 mM NBA at high Ca^{2+} concentrations, modification was not accomplished for periods as long as 5 min. The average time to modification in 2.5 μM Ca^{2+} was 52 ± 6 sec (n = 7), whereas in 1 mM Ca^{2+} average treatment times without modification were 206 ± 23 sec (n = 6). To make sure that the lack of modification is due to the high Ca^{2+}, two patches containing single channels were exposed sequentially to NBA at high then low Ca^{2+} with modification of both.

It is important to ask whether NBA is capable of working in high Ca^{2+}. Although we have not addressed this question directly, Oxford et al. (1978), have shown that NBA treatment of Na^+ channels in squid axon removes Na^+ channel inactivation when applied to the inside of the axon, but reduces single channel conductance without an effect on inactivation when applied to the outside. The observation that NBA does have an effect when applied to the

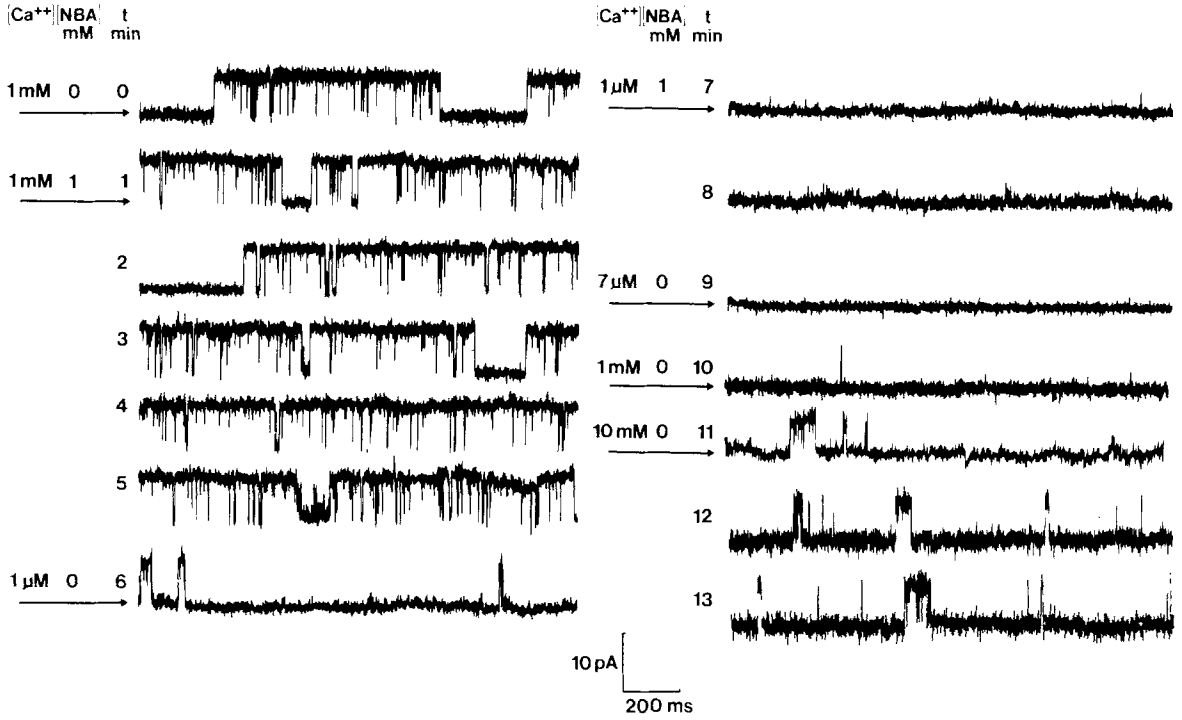

Fig. 4. A continuous recording of a single channel. Each tracing is the last 1.5 sec of the minute specified in the legend. The open probability for each minute is as follows: 0, $P_o = 0.74$; 1, $P_o = 0.80$; 2, $P_o = 0.68$; 3, $P_o = 0.76$; 4, $P_o = 0.90$; 5, $P_o = 0.72$; 6, $P_o = 0.05$; 7, $P_o = 0.00$; 8, $P_o = 0.00$; 9, $P_o = 0.00$; 10, $P_o = 0.01$; 11, $P_o = 0.07$; 12, $P_o = 0.08$; 13, $P_o = 0.07$. Normal activity channels were used in this experiment

seawater face of the membrane (50 mM Ca^{2+}) is evidence that NBA can indeed operate in high Ca^{2+}.

Open Channel Kinetics

Ca^{2+}-activated K^+ channels usually display complicated opening and closing kinetics indicative of several different open and closed states (*see* Barrett, Magleby & Pallotta, 1982; Magleby & Pallotta, 1983; Moczydlowski & Latorre, 1983). We have fit the distribution open events to two exponential components, with mean open times given in Tables 3 and 4. Note that both NBA modified (Table 3) and untreated channels (Table 4) display short and long duration open events. Normal channels exposed to 1 mM Ca^{2+} concentrations (Table 4) have a majority of events of longer duration, whereas following NBA modification (Table 3), the mean open time for each class of events shorten considerably. Since, both untreated and modified channels in high Ca^{2+} exhibit short and long duration opening events, the NBA modification does not selectively remove a particular population of events.

It is known that the duration of the individual opening events of Ca^{2+}-activated K^+ channels increase with increasing Ca^{2+} concentration (Moczydlowski & Latorre, 1983). Increasing Ca^{2+} concentration from 1 to 10 mM causes a shift in opening distribution curve, indicating that the duration of

Table 2. Modification of channels with NBA

1 mM NBA–high Ca^{2+} Exposure time (sec)	1 mM NBA–low Ca^{2+} Modification time (sec)
300 −	60 +
175 −	62 +
178 −	50 +
135 −	40 +
223 −	78 +
225 −	35 +
	41 +
206 ± 57	52 ± 23

Exposure time is the period of time that the channel was exposed to NBA. In high Ca^{2+} (1 mM), the minus sign denotes the absence of modification during the exposure time. Channels were considered unmodified if there was no change in the fraction of time that the channels spent open. In low Ca^{2+} (1–2.5 μM), the + sign indicates that channels were modified, as detected by a sharp reduction in fractional open time during the time specified. The first two pairs of data represent experiments in the same channel, for which the channels were first exposed to NBA in high Ca^{2+} without modification and then to NBA in low Ca^{2+}. Only in low Ca^{2+} did NBA modify the channels.

Table 3. Mean open times (msec)

Condition	T_o^1	T_o^2	Fraction T_o^1	Fraction T_o^2
Treated				
1 mM Ca^{2+}	0.8	17.0	0.13	0.92
10 mM Ca^{2+}	10.0	40.0	0.29	0.62
Treated				
10 mM Ca^{2+}	1.1	9.5	0.34	0.51
25 mM Ca^{2+}	4.4	26.1	0.42	0.37

Channel distributions were determined at 1 mM Ca^{2+}, and 0 mV. 803 events were analyzed. The first treated channel distributions were measured at 0 mV, with 234 events analyzed at 1 mM Ca^{2+}, and 522 events analyzed at 10 mM Ca^{2+}. The second treated channel was assessed at +30 mV with 534 events analyzed at 10 mM Ca^{2+} and 675 events analyzed at 25 mM Ca^{2+}. Single-channel experiments from three different patches. Mean open time, T_o, was determined by SAAM curve fitting program (*see* Materials and Methods).

the events has increased. This is reflected in an increase of the mean open time, T_o, of both types of opening events with increasing Ca^{2+} concentrations (Table 3). This demonstrates that not only are two distributions of opening events present following NBA but also that both are Ca^{2+} dependent.

Pallotta (1985) showed Ca^{2+}-activated K^+ channels in muscle modified by NBA when exposed to low Ca^{2+} display a population of short open events whose mean open time was Ca^{2+} independent. Although we cannot eliminate the possibility that Ca^{2+}-independent open events are present in Ca^{2+}-activated K^+ channels in MTAL cells since these events may be shorter than we can resolve,[2] we do show that in high Ca^{2+} the open events are distinctly Ca^{2+}-dependent. Thus, the primary effect of NBA is to reduce but not remove the Ca^{2+}-sensitivity of the channel gating mechanism.

Low Activity Ca^{2+}-Activated K^+ Channels

We observed in 16 of 80 seals, K^+ channels (Fig. 5) with similar single-channel conductance (140 ± 4 pS, $n = 3$ in 7–10 μM Ca^{2+}) as the normal channels but which opened infrequently in the presence of μM concentrations of Ca^{2+}. Table 4 compares the open probability of the normal channel and the lower activity channel. Whereas the normal channel is almost always open in 10 μM or in 1 mM Ca^{2+}, the low activity channel opens much less frequently. Table 4 also gives the mean open times for two open distributions. Similar to the NBA-treated channel,

[2] *See* footnote 1, p. 000.

Table 4. Comparison of normal and low activity channels

	Ca^{2+}	T_o^1	T_o^2	Fraction T_o^1	Fraction T_o^2	P_o
Normal						
	10 μM	6.8	58	0.05	0.95	0.93
	1 mM	6.7	67	0.05	0.89	0.86
Low activity						
	10 μM	0.4	13	0.17	0.31	0.01
	1 mM	1.1	32	0.40	0.57	0.08

Probabilities were analyzed from 57–62 sec of data. The open times T_o^1, T_o^2 for the low activity channel were analyzed from 264 events at 10 μM Ca^{2+} and 404 events at 1 mM Ca^{2+}.

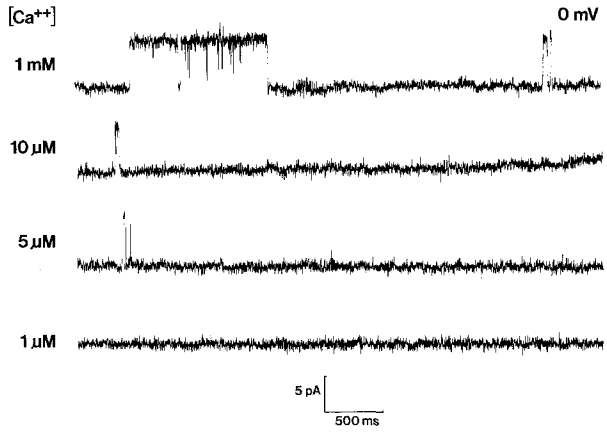

Fig. 5. Ca^{2+} activation of a low activity channel

the mean open times displayed by the low activity channel are shorter than those of the normal channel when compared at the same Ca^{2+} concentrations. The low activity channel is Ca^{2+} dependent since both P_o, T_o^1 and T_o^2, increase with increasing Ca^{2+}. Similar to the normal channel, the open probability of the low activity channel is voltage dependent (*see* Fig. 3). These data demonstrate that MTAL cells possess a population of channels which resemble NBA-treated channels in that the sensitivity to Ca^{2+} is shifted to higher Ca^{2+}.

Discussion

Ca^{2+} Activation of Normal and NBA-Treated Channels

The biophysical characteristics of the gating processes involved in opening and closing Ca^{2+}-activated K^+ channels were examined in detail by Moczydlowski and Latorre (1983). They proposed a

model for channel activation that includes the sequential binding of two Ca^{2+} ions to a binding site on the intracellular face of the channel. The multiple open and closed states were attributed to the binding of either zero, one, or two Ca^{2+} ions to the channel in reversible equilibrium. Similarly, Magleby and Pallotta (1983) also proposed that channel opening depended on the binding of two or more Ca^{2+} ions to the Ca^{2+} binding site of the channel. The important feature of the scheme proposed by Moczydlowski and Latorre (1983) is a voltage-dependent binding of the two Ca^{2+} ions. Moczydlowski and Latorre (1983) viewed the channel as ligand gated, with voltage affecting binding of Ca^{2+} to the channel. The observations critical to the model were obtained from an analysis of medium duration opening and closing events, referred to as the "main gating mode" of the channel. Not considered in the model were the short duration or "flicker events" so typical of this channel or long Ca^{2+} blocking events.

Besides its role in channel activation, Ca^{2+} can also block Ca^{2+}-activated K^+ channels. The relationship is such that low Ca^{2+} concentrations will activate untreated Ca^{2+}-activated K^+ channels (*see* Fig. 1) but high Ca^{2+} will, in addition, tend to block the channels. Ca^{2+} blocks Ca^{2+}-activated K^+ channels in MTAL cells in two modalities, fast and slow block. The fast block, similar to TEA block (Guggino et al., 1987*b*), is evident by such fast closures that the mean closed time is under our resolution limit (0.5 msec). We can detect Ca^{2+} fast block, similar to that observed for Na^+ block (*see* Yellen, 1984), as an apparent decrease in single-channel conductance of an unmodified channel from 150 pS in 10 μM to 88 pS in 25 mM Ca^{2+}. The slow block similar to that reported by Vergara and Latorre, (1983) and Vergara, Moczydlowski and Latorre (1984) appears in MTAL cells as a long residence in a nonconductive state with a mean of 7.2 sec at 1 mM Ca^{2+}, 0 mV. As reported by Vergara and Latorre (1983), the number of block events increases with Ca^{2+}, but the mean block time is not dependent on Ca^{2+} concentration. The combined effect of Ca^{2+} activation and block is that increasing Ca^{2+} concentrations from μM to mM concentrations first increase P_o to a peak value followed by a decline at higher Ca^{2+} concentrations. For example, in unmodified channels at 10 μM Ca^{2+}, P_o = 0.93, whereas at 1 mM, P_o = 0.86 (*see* Table 4).

In the present study, we show that NBA treatment in low Ca^{2+} dramatically reduces the fraction of time that channels spend in the open state by decreasing the mean open times of both populations of long and short opening events. However, the mean open time of both the short and long duration events remain Ca^{2+} sensitive in modified channels. The major difference between modified and unmodified channels is that at the same Ca^{2+} concentration the mean open times of the NBA-modified channels are shorter than those of the normal channels. Likewise, the modified channel retains its normal voltage sensitivity as shown by voltage-dependent increases in P_o (*see* Fig. 3). NBA also does not affect the single-channel conductance. Thus, the modification of the channel produces an effect that is quite specific in reducing only the Ca^{2+} sensitivity.

Site of Action of NBA

An important aspect of these studies is that within the time frame of our experiments NBA modification is protected by exposing the intracellular face of the cell membrane to 1 mM Ca^{2+}, whereas NBA is effective in modifying the channels at 2.5 μM. This lack of modification in high Ca^{2+} raises two hypotheses: (i) NBA modifies amino acids directly involved in Ca^{2+} binding and activation, thus modification cannot take place if the Ca^{2+} binding site is occupied, and (ii) NBA attacks a modifier site on the molecule that can adjust the Ca^{2+} activation of the channel; in high Ca^{2+} when the channel is in an open conformation the modifier site is unavailable for modification. Taken together with the observations that modification is irreversible and that once modified the channel becomes refractory to further modification, it is clear that NBA attacks amino acids directly involved in modulating channel activation by Ca^{2+}.

Low Activity Channels

We have observed that most of the K^+ channels in the apical membrane of cultured MTAL cells are highly Ca^{2+}-activated with a P_o equal to about 0.5 at 5 μM Ca^{2+} in Mg^{2+}-free solutions. However, about 20% of the population appears to require much higher Ca^{2+} concentrations to attain activation. These low activity channels attain a P_o of 0.08 when exposed to 1 mM Ca^{2+} on the intracellular face of the membrane.

Although at the present time we do not know the functional significance of the low activity channels, several possibilities exist. First, it is possible that high and low activity K^+ channels are not related. However, this is unlikely because they both have the same single-channel conductance, display similar multi-state kinetics, are voltage activated, and are inhibited by high doses of TEA on the intracellular side (the latter is the criterion used to ascertain the sidedness of the patch—*see* Materials and

Methods). Thus, it is likely that they are the same molecule in two different functional modes. A second possibility is that the low activity channel of MTAL cells may be present at a stage in the differentiation of these cells. For example, it has been demonstrated that cultured embryonic neurons of the frog, *Xenopus*, have 155 pS K^+ channels which are Ca^{2+}-activated in mature neurons, but appear to lack Ca^{2+}-activation within one day after initiation of the cultures (Blair & Dionne, 1985). It appears that channels attain Ca^{2+} sensitivity as a consequence of cell differentiation in culture. These non-Ca^{2+}-activated channels in *Xenopus* were tested for Ca^{2+}-sensitivity only to 100 μM Ca^{2+}. It is not known whether increasing the Ca^{2+} to millimolar concentrations would reveal a lowered Ca^{2+}-sensitivity similar to the low activity channel in MTAL cells. An example of modulation of K^+ channel activity has also been observed in thrombocytes of the *Newt* (Kawa, 1987). In resting thrombocytes K^+ currents are transient following membrane depolarization suggestive of an inactivation process, whereas during activation of the thrombocytes, the K^+ channels are no longer capable of inactivation. Internal perfusion with NBA also removes inactivation, mimicking a normal cell process. The hypothesis is that one of the normal steps in thrombocyte activation is the modification of a portion of the K^+ channel responsible for inactivation. A similar developmental change may be occurring in MTAL cells in culture. Finally, the low activity channel may represent a nonfunctional state in the life cycle of Ca^{2+}-activated K^+ channels. It is interesting that the low activity channel displays similar Ca^{2+}-sensitivity as the NBA-treated channel. Perhaps there is a site on the channel molecule that can be modified by the cell to decrease the Ca^{2+} sensitivity to a range of Ca^{2+} concentrations well above normal physiological levels similar to the effect of NBA. Such a maneuver would make it extremely unlikely that the modified channel would transport K^+ out of the cell, thereby effectively removing it from the pool of functioning channels. If the cell were able to convert the channel back to a highly Ca^{2+}-sensitive form, or alternately to increase the Ca^{2+} sensitivity of individual channels, it would be able to modulate the number of functioning channels in the membrane without having to change intracellular Ca^{2+} concentrations.

The authors wish to thank Dr. Gary Yellen for his critical reading of the manuscript. We also acknowledge the technical assistance of Patricia Cea and Diane Markakis and the help Dr. Robert Phair provided in introducing us to SAAM programs. This work was supported in part by the National Institutes of Health Grant #DK-32753 and by a Grant-In-Aid from the American Heart Association with funds contributed in part by the American Heart Association, Maryland Affiliate.

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Received 10 June 1987