

# *N*-Bromoacetamide Removes a Calcium-dependent Component of Channel Opening from Calcium-activated Potassium Channels in Rat Skeletal Muscle

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**ABSTRACT** Calcium-activated potassium channels from cultured rat skeletal muscle were treated with the protein-modifying reagent *N*-bromoacetamide (NBA) (0.3–1 mM) and studied in excised patches using patch-clamp techniques. After NBA treatment, channels opened only occasionally, and, in contrast to untreated channels, the open probability was no longer sensitive to intracellular surface calcium ions (1 nM to 100  $\mu$ M). Channel activity did, however, exhibit a voltage dependence similar in direction and magnitude to that shown before NBA treatment (increasing *e*-fold with 19 mV depolarization). Distributions of open channel lifetimes revealed that NBA treatment virtually abolished openings of long duration, which suggests that this class of openings requires calcium sensitivity. These effects were not reversed by subsequent washing. Quantitatively similar open probability, voltage dependence, and open-interval distributions were observed in untreated channels in calcium-free medium. These results suggest that NBA removed a calcium-dependent component of channel opening, and that normal channels are able to open in the absence of significant intracellular calcium concentrations.

## INTRODUCTION

An outstanding characteristic of the large-conductance (250–290 pS), calcium-activated potassium channels is their sensitivity to intracellular surface Ca ions and to membrane potential. Raising the concentration of Ca<sup>2+</sup> or depolarizing the membrane increases the amount of time spent by the channel in both long- and short-duration open states (Barrett et al., 1982; Latorre et al., 1982; Wong et al., 1982; Maruyama et al., 1983), although at high concentrations or potentials channel block might occur (Vergara and Latorre, 1983). Since the rates of opening to the long- and short-lived open states depend on the calcium concentration, quantitative descriptions of these effects have been based upon kinetic models in which channel opening depended upon the binding of two or more calcium ions to a succession of closed molecular states of the channel macromolecule (Magleby and Pallotta, 1983). Extrapolation of these results to the low

concentration limit implies that at physiologic intracellular calcium concentrations and resting potentials, the activity of the large-conductance channel would be severely restricted; even in the midst of calcium influx following an action potential, the rapid cellular after-hyperpolarization would rapidly curtail activity.

In this paper it is shown that treatment of calcium-activated potassium channels with the protein-modifying reagent *N*-bromoacetamide (NBA) removes a calcium-dependent component of channel opening; a small, but voltage-sensitive component does, however, remain. Untreated channels bathed in calcium-free solutions show quantitatively similar channel activity, voltage dependence, and open times. Such calcium-independent activity in the normal channel might provide a mechanism for the modulation of channel activity by membrane potential or cytosolic factors in the absence of calcium influx.

#### METHODS

Patch electrodes of N51A borosilicate glass (Drummond Scientific, Broomall, PA) coated with Sylgard (Dow Corning, Midland, MI) were used to record single channel currents (Hamill et al., 1981) from surface membrane patches of rat muscle cells (myotubes) in culture. Myotubes were prepared as described by Barrett et al. (1981), except for the deletion of serum from the culture medium beginning with the third day after plating. All experiments were performed upon "inside-out" excised patches of surface membrane that contained one to four calcium-activated potassium channels. These channels were identified primarily by their characteristic large conductance (typically 250–290 pS) and voltage sensitivity.

Both sides of the excised patch were bathed in solutions containing (mM): 140 KCl, 2 TES [*N*-tris(hydroxymethyl-2-aminoethane sulfonic acid) buffer, 1 EGTA, pH 7.20. The solution bathing the intracellular surface contained in addition either 886 or 929  $\mu$ M total  $\text{CaCl}_2$ , resulting in estimated free calcium ion concentrations of 0.6 or 1  $\mu$ M, respectively (see figure legends). Experiments requiring extremely low  $\text{Ca}^{2+}$  concentrations used an intracellular surface solution similar to that described above except that no  $\text{Ca}^{2+}$  was added. It was estimated that 1.5  $\mu$ M  $\text{Ca}^{2+}$  was introduced as an impurity with the other salts, so it is unlikely that the free calcium ion concentration in this solution exceeded 1 nM. The text reference to this solution is 1 nM  $\text{Ca}^{2+}$ . The solution bathing the extracellular surface of the patch contained 0.01  $\mu$ M free  $\text{Ca}^{2+}$ . The stability constants used in the calculations of free  $\text{Ca}^{2+}$  were from Owen (1976) and Chaberek and Martell (1959). Experiments were performed at room temperature (20–22°C).

NBA (Sigma Chemical Co., St. Louis, MO) was applied to the intracellular surface of an excised patch via a perfusion pipette placed into the bath in proximity to the patch electrode. Treatment with NBA was often accompanied by an increase in current noise (cf. Patlak and Horn, 1982), and it was not unusual to lose the patch soon after observing the effects of NBA. All measurements from NBA-treated patches were obtained after washing the patch with NBA-free saline; the effects of NBA were consistent and irreversible, although in some experiments the mean channel activity fluctuated noticeably with time after removal of the NBA. This nonstationarity precluded quantitative discussion of the distributions of shut intervals.

Data recorded with an FM tape recorder (Racal Recorders, Sarasota, FL) were digitized by computer. Open and shut intervals were measured with an interval-detection routine, which sampled at 40 kHz and used a 50% threshold (see Colquhoun and Sigworth, 1983) for determining the durations of the open and shut intervals. Open probability (percent open time) was calculated from these intervals. Open-interval distributions were fit with

sums of exponentials calculated by a maximum likelihood analysis (Colquhoun and Sigworth, 1983).

## RESULTS

### *Treatment with NBA Reduces Channel Activity*

Treatment of membrane patches with 0.3–1 mM NBA had a consistent and irreversible effect on channel activity that appeared suddenly within 2–8 min after perfusion of the intracellular surface of the excised patch with saline containing NBA. Fig. 1 contrasts the channel activity from a patch containing four active calcium-activated potassium channels before (A) and after (B) expo-

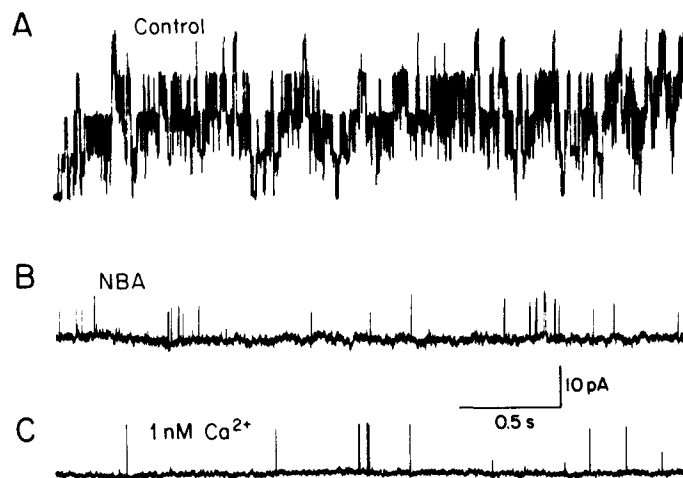


FIGURE 1. NBA reduces channel activity. (A) Control channel activity from an excised inside-out membrane patch in  $0.6 \mu\text{M Ca}^{2+}$  (+40 mV). The four levels of current demonstrate the presence of four active channels. (B) After several minutes of treatment and washout of 1 mM NBA, activity in the same membrane patch was much reduced. Continued washing did not reverse this effect. (C) Channel activity from a normal membrane patch in 1 nM  $\text{Ca}^{2+}$ , which contained three active channels. Note the similarity between the activity in this untreated patch and that seen after NBA treatment (B).

sure to 1 mM NBA. Openings from the modified channels were relatively infrequent and of such brief duration that their amplitudes were limited by the bandwidth of the recording system. Effects similar to this were seen in 21 other excised patches; in six other experiments, channel activity was completely abolished.

### *Single Channel Conductance Is Unaffected by Exposure to NBA*

The characteristic large conductance of the calcium-activated potassium channel can be used to demonstrate that NBA altered the channel kinetics (Fig. 1), rather than blocking the channels entirely to reveal an entirely different channel type.

Fig. 2 shows a current-voltage relation derived from two patches that were treated with NBA. Amplitude measurements were taken both before and after treatment with 1 mM NBA. Measurements taken after NBA was washed from the patch for several minutes (solid symbols) were restricted to openings of sufficient duration to be fully resolved. Between  $-30$  and  $+60$  mV, the current-voltage relation is fairly linear. The line drawn in Fig. 2 was derived from a least-squares fit to all the data points and shows that the single channel conductance (267 pS) was unaffected by treatment with NBA.

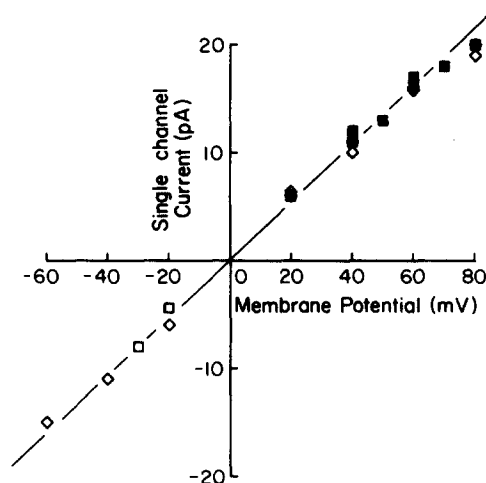


FIGURE 2. Current-voltage relation from channels before (open symbols) and after (solid symbols) treatment with NBA. The straight line drawn through the data suggests an average single channel conductance of 267 pS within the voltage range  $-40$  to  $+60$  mV.

#### *NBA Removes Calcium Sensitivity*

The sparse and brief-duration openings of channels treated with NBA (Fig. 1B) resembled those observed in essentially  $\text{Ca}^{2+}$ -free (1 nM) medium (Fig. 1C). This suggested that channels exposed to NBA were no longer sensitive to intracellular surface calcium ions.

Fig. 3 compares the calcium sensitivity of a channel in an excised patch before (A) and after (B) treatment with NBA. The dependence of channel activity upon intracellular surface calcium ions has been well described (Barrett et al., 1982; Wong et al., 1982; Latorre et al., 1982), and was effectively removed by treatment with NBA (Fig. 3B). Similar results were observed in five other experiments, four of which extended the range of calcium concentrations to 100  $\mu\text{M}$ ; treatment of channels with NBA appeared to completely remove normal calcium sensitivity from the intracellular surface.

#### *Channel Activity Remains Voltage Sensitive After NBA Treatment*

Fig. 4A shows the well-known voltage sensitivity of channel opening (Barrett et al., 1982; Latorre et al., 1982); in three excised patches, channel activity

increased on the average e-fold with every 16 mV of depolarization (open symbols, Fig. 4C). Similar voltage sensitivities in rat skeletal muscle have been previously reported (15 mV, Barrett et al., 1982; 12 mV, Methfessel and Boheim, 1982), as well as the apparent shift in voltage sensitivities between experiments (Methfessel and Boheim, 1982; Moczydlowski et al., 1985).

After treatment with NBA, channel activity is greatly reduced (note the logarithmic scale) but still voltage sensitive (solid symbols, Fig. 4C); activity increased approximately e-fold with 19 mV depolarization, which is similar to that shown by the untreated channels. These results show that despite the loss of calcium sensitivity (Fig. 3), NBA-treated channels retain a component of activity with a voltage sensitivity comparable to that in untreated channels.

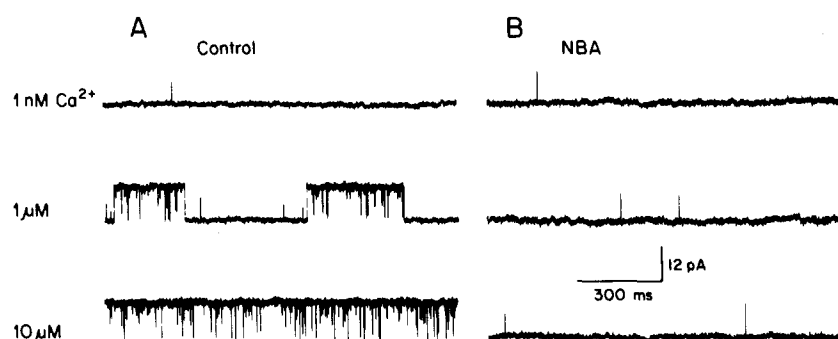


FIGURE 3. Calcium sensitivity of channel opening is removed by treatment with NBA. (A) The activity of an untreated channel (+40 mV) increased with intracellular surface calcium ion over the range 1 nM to 10 μM. (B) After treatment with 1 mM NBA, the same channel showed much-reduced activity that was no longer sensitive to changes in calcium concentration.

#### *Short-Duration Openings Predominate After Treatment with NBA*

It would appear from the currents shown in Figs. 1B and 3B that channels treated with NBA open only briefly; this would account for the apparent partial openings seen in the experimental records. The extent to which NBA modified channel open times is shown in Fig. 5, which compares open-interval distributions from an excised patch that contained one channel before (A) and after (B) treatment with NBA. Fig. 5A shows an unconditional open-interval distribution obtained using a half-amplitude threshold (see Methods). The intervals in this distribution were fit with three exponential components using a maximum likelihood analysis of 6,888 total individual open intervals (see Colquhoun and Sigworth, 1983). Although two components are typically fit to this type of distribution (Barrett et al., 1982), with sufficient numbers of intervals, an additional component may be resolved (McManus and Magleby, 1985). In this experiment (0.6 μM Ca<sup>2+</sup>; +50 mV), one relatively long-duration component (time constant, 4.84 ms) made up two-thirds of the openings, while the remaining openings belonged to two briefer components (0.34 and 0.05 ms average duration) (see figure legend).

Fig. 5B shows the open-interval distribution obtained from the same excised patch after treatment (and washout) with NBA. The 998 intervals in this distribution were fit with only two components of 0.38 and 0.05 ms, which were similar to the two brief components observed before NBA exposure (Fig. 5A). Thus, treatment of calcium-activated potassium channels with NBA effectively

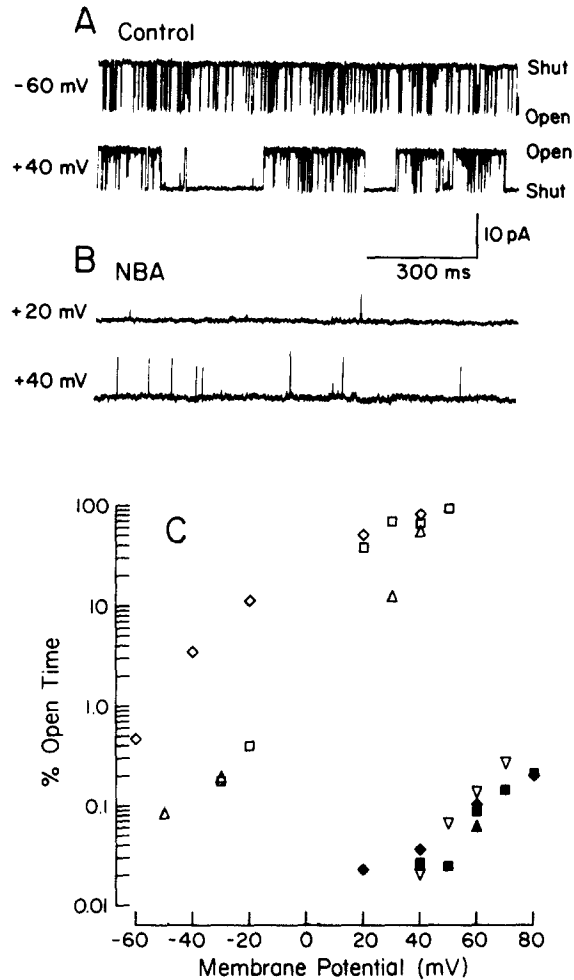


FIGURE 4. Channels exposed to NBA retain a voltage-sensitive open probability. (A) Channel activity from an excised patch ( $0.6 \mu\text{M Ca}^{2+}$ ) at two different voltages. Note the increased open time at +40 mV compared with -60 mV. (B) After treatment with 1 mM NBA (and washout), the channel retained a qualitatively similar voltage sensitivity; openings were more frequent at +40 mV than at +20 mV. (C) Voltage sensitivity of the percent open time in control (open symbols) and NBA-treated (solid symbols) channels. Note the logarithmic scale of the percent open time. The intracellular surface calcium concentration was  $0.6 \mu\text{M}$  in all experiments except one, in which it was 1 nM (inverted triangles).

prevented openings of long duration. This suggests that the components of long and short duration represent distinct conformations of the channel protein, since the short-duration openings were observed in the absence of long-duration openings.

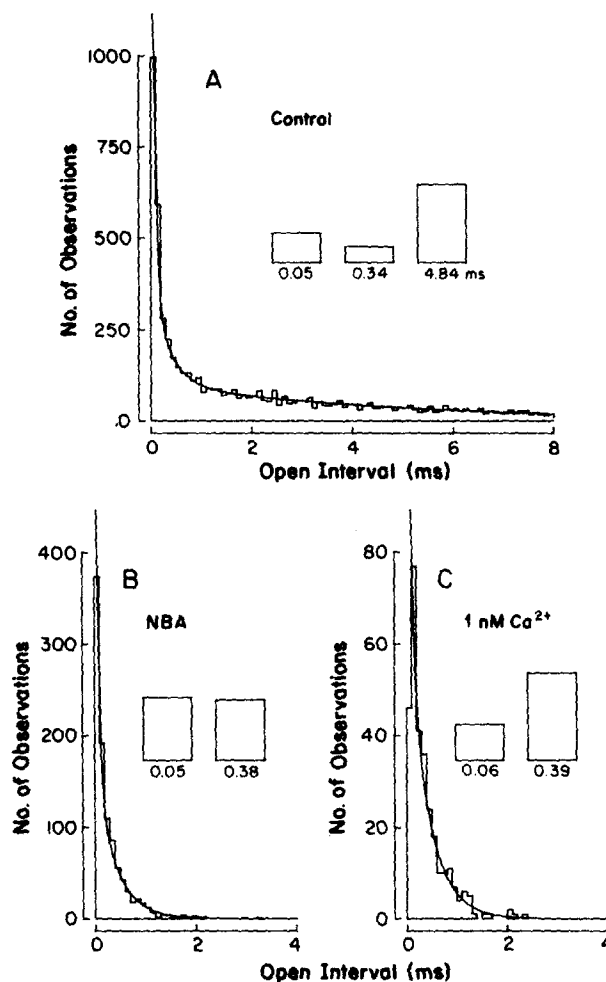


FIGURE 5. Channel openings in Ca<sup>2+</sup>-free medium (C) or after NBA treatment (B) are predominantly of short duration. (A) Open-interval distribution from an excised patch that contained one active channel (+50 mV; 0.6  $\mu$ M Ca<sup>2+</sup>). The 6,888 open intervals were fit with three exponentials with the time constants and relative weights shown in the figure. The long-duration component (time constant, 4.84 ms), which makes up 63% of all the openings in A, could not be resolved after the channel was treated with 1 mM NBA (B). The 998 intervals were well described by two components similar to the two brief components found in the untreated channel (A). (C) Open-interval distribution from another excised patch (+50 mV) that contained one active channel in 1 nM intracellular surface calcium (295 intervals).

*The Kinetic Effects of NBA Are Mimicked in Low Calcium Ion*

The fact that small changes in the intracellular surface calcium ion concentration can greatly affect channel kinetics (Magleby and Pallotta, 1983; Moczydlowski and Latorre, 1983) suggests that the removal of channel calcium sensitivity by NBA might cause profound (but secondary) effects on channel gating. One way to test this hypothesis would be to examine channel kinetics in essentially calcium-free medium.

In the presence of 1 nM intracellular surface  $\text{Ca}^{2+}$ , normal calcium-activated potassium channels open only occasionally (Fig. 1C), which is similar to what is seen in channels treated with NBA. The probability of channel opening, however small, is still voltage sensitive (inverted triangles, Fig. 4C) and is similar to that shown by channels treated with NBA (solid symbols, Fig. 4C). Finally, the open-interval distribution from an untreated channel in low  $\text{Ca}^{2+}$  shows only two brief components (Fig. 5C), with time constants similar to those in channels treated with NBA (Fig. 5B).

These results demonstrate that channels treated with NBA behave in a way similar to that of untreated channels bathed in essentially  $\text{Ca}^{2+}$ -free medium, and suggest that the primary action of NBA treatment is to render the channels unresponsive to calcium. The kinetics of channel opening in the  $\text{Ca}^{2+}$ -free solution suggest also that the small, voltage-sensitive component of activity seen after NBA treatment is also exhibited by normal channels.

#### DISCUSSION

The major finding of this study is that calcium-activated potassium channels that had been treated with NBA retained a voltage-sensitive component of opening (Fig. 4), despite the loss of calcium sensitivity (Fig. 3). The fact that these channels behaved in a manner similar to untreated channels that were bathed in calcium-free medium (Figs. 1, 3, 4, and 5) suggests that the channel activity, voltage dependence, and open-interval distribution observed after treatment with NBA were the normal behavior of a channel in the apparent absence of calcium. The calcium-independent component of channel opening might account for the excess activity of this channel at low calcium concentrations ( $0.01 \mu\text{M}$ ), as was previously noted in rat muscle (Barrett et al., 1982). Macroscopic measurements in mammalian smooth muscle, which demonstrated incomplete suppression of the calcium-activated potassium current after intracellular calcium was buffered in the nanomolar range (Mitra and Morad, 1985), might be similarly accounted for. Calcium-activated potassium channels from immature *Xenopus* spinal neurons in culture show burst activity even before they develop calcium sensitivity (Blair and Dionne, 1985), while spontaneous activity of acetylcholine-activated receptors has been shown in the absence of cholinergic agonists (Jackson, 1984).

The results obtained from untreated channels in calcium-free medium also suggest that the extent of the chemical modification by NBA might be confined to that part of the channel macromolecule involved in sensing the presence of calcium. The simplest modification by NBA that would explain the results would be to prevent calcium binding, as this would account for the total loss of calcium



sensitivity. If the channel's affinity for calcium were simply reduced, even by a large extent, some calcium dependence would still have been observed. As evidenced by the similar behavior of normal channels in calcium-free medium, the effects upon the channel open time and open-interval distributions that followed NBA treatment were apparently a consequence of the loss of calcium sensitivity. The verification of such a mechanism would of course require measurements of actual calcium binding.

A comparison of channel voltage sensitivity before and after NBA treatment was restricted to a relatively narrow range of voltages, since membrane patches exposed to NBA did not tolerate voltages more depolarized than +80 mV. A simple depolarizing shift in the voltage dependence would not satisfactorily explain the results, since depolarization results in a larger proportion of long-duration openings (unpublished observations). In contrast, after NBA treatment, the open time arises almost entirely from short-duration openings (Fig. 5). This major difference in kinetics suggests that the voltage sensitivities of percent open time before and after NBA (Fig. 4) reflect different aspects of the channel mechanism and are not strictly comparable.

NBA treatment removed not only the calcium sensitivity of the open probability, but also the long-duration openings. This would be consistent with mechanisms in which these openings arise from the opening of multiliganded channel conformations (Magleby and Pallotta, 1983). Those mechanisms also attributed short-duration openings to a monoliganded (and therefore calcium sensitive) conformation; because of bandwidth limitations, these openings are indistinguishable from the calcium-independent openings observed here. The short-duration openings observed in this study cannot arise from voltage-dependent calcium binding (Wong et al., 1982; Moczydlowski and Latorre, 1983), since such a mechanism would not allow the observed dissociation of calcium from voltage sensitivities. Regardless of the actual mechanism, these results demonstrate that calcium-activated potassium channels can open and be modulated in the absence of significant intracellular calcium ion concentrations. This modulation could occur through changes in membrane potential or cytosolic factors that might shift the voltage dependence toward physiologic ranges.

NBA cleaves peptide bonds on the COOH-terminal side of tryptophan, tyrosine, histidine, and reduced phenylalanine (Means and Feeny, 1971; see Oxford et al., 1978), and, in conjunction with other, more specific protein-modifying reagents, might therefore prove useful in determining which channel protein residues are specifically involved in calcium sensitivity. In addition, with the removal of calcium sensitivity and the long-duration openings that usually dominate channel activity, ion selectivity of the short-duration open state that remains after NBA treatment could be studied, as well as interactions with calcium ions that are not related to channel gating.

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