A Comparison of Calcium-activated Potassium Channel Currents in Cell-attached and Excised Patches

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ABSTRACT   Single channel currents from Ca-activated K channels were recorded from cell-attached patches, which were then excised from 1321N1 human astrocytoma cells. Cells were depolarized with K (110 mM) so that the membrane potential was known in both patch configurations, and the Ca ionophore A23187 or ionomycin (20–100 μM) was used to equilibrate intracellular and extracellular [Ca] (0.3 or 1 μM). Measurements of intracellular [Ca] with the fluorescent Ca indicator quin2 verified that [Ca] equilibration apparently occurred in our experiments. Under these conditions, where both membrane potential and intracellular [Ca] were known, we found that the dependence of the channel percent open time on membrane potential and [Ca] was similar in both the cell-attached and excised patch configuration for several minutes after excision. Current-voltage relations were also similar, and autocorrelation functions constructed from the single channel currents revealed no obvious change in channel gating upon patch excision. These findings suggest that the results of studies that use excised membrane patches can be extrapolated to the K-depolarized cell-attached configuration, and that the relation between [Ca] and channel activity can be used to obtain a quantitative measure of [Ca] near the membrane intracellular surface.

INTRODUCTION

Single channel recordings have revealed that a variety of tissues contain large-conductance (250–300 pS) Ca-activated K channels that are activated by membrane depolarization and micromolar [Ca] at the intracellular membrane surface (Petersen and Maruyama, 1984; Sakmann and Neher, 1984; Latorre et al., 1985; Latorre, 1986). These ubiquitous channels have been well characterized in excised membrane patches and lipid bilayers, two techniques that permit control of the ionic environment on both sides of the channel protein (Hamill et al., 1981; Latorre et al., 1985). Our understanding of the physiological roles of these channels has depended upon the assumption that the channel properties

displayed in the excised patch are similar to those of the channel while attached to the cell. We have tested this assumption in 1321N1 human astrocytoma cells by comparing several properties of Ca-activated K channels in cell-attached patches that were then excised from the cell.

Our experimental approach was to clamp the cell resting potential with high K (110 mM), and then apply the Ca ionophore A23187 or ionomycin to equilibrate intracellular [Ca] with that of the extracellular medium. Under these conditions, where both membrane voltage and intracellular [Ca] were known, we found that the single channel conductance, [Ca] and voltage sensitivities, and gating kinetics were essentially identical in the excised and cell-attached configurations. These results suggest that studies of Ca-activated K channels in excised patches might contribute to our understanding of channel function in the more physiological cell-attached (but K-depolarized) configuration. In addition, if the [Ca] sensitivity of each patch is individually determined (see Methods), then channel activity can provide a novel and quantitative measure of [Ca] near the intracellular surface of the intact cell membrane.

METHODS

Cell Culture

1321N1 astrocytoma cells are subclones of a human astrocyte tumor line (118N1) (Ponten and Macintyre, 1968) first isolated in 1972 (see Clark et al., 1974). Stock cultures were maintained as described in Meeker and Harden (1982) in 25-cm^2 plastic flasks. Before electrophysiologic use, cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and plated onto 35-mm plastic dishes at sufficiently low density that nonconfluent regions could be readily found for 2–6 d after plating.

Single Channel Recording

Patch recording electrodes, fabricated from N51A glass (Drummond Scientific Co., Broomall, PA) and coated near the tips with Sylgard (Dow Corning, Midland, MI), were used to record single channel currents (Hamill et al., 1981). The typical membrane patch from the astrocytoma cells contained 4–20 Ca-activated K channels, identified primarily by their characteristic large conductance (220–250 pS in symmetric 110 mM KCl) and sensitivity to voltage and intracellular [Ca]. Wide variations in [Ca] sensitivities between channels have been described in rat muscle (Methfessel and Boheim, 1982) and lipid bilayers (Moczydlowski et al., 1985). We found the variation in [Ca] sensitivity between patches in astrocytoma cells (at the same voltage) was usually <30% when the percent open times were >50%. When channel activity was very low (<0.5% open time), up to 20-fold differences between patches were occasionally observed, although these large differences might reflect errors in sampling the rare openings.

During the experiment, cells were bathed in a depolarizing solution of the following composition (mM): 110 KCl, 24 NaCl, 2 TES [N-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid] buffer, 1 EGTA, pH 7.4. The solution contained in addition either 795 or 929 μM total CaCl$_2$, resulting in an estimated free [Ca] of 0.3 or 1 μM, respectively. The solution in the electrode was similar, except that the estimated free [Ca] was 0.01 μM. The stability constants used in the calculations of free [Ca] were from Owen (1976) and Chaberek and Martell (1959). Experiments were performed at room temperature (20–22°C).

A23187 (50 mM) and ionomycin (13.4 mM) (Calbiochem-Behring Corp., La Jolla CA) were kept as stock solutions in dimethylsulfoxide (DMSO) and bath-applied at final concentrations of 20–100 μM in the above solutions (see figure legends). At these dilutions (<0.4%), DMSO alone had no obvious effect on channel activity.

Currents were recorded on FM tape (Racal Recorders, Vienna, VA, or A.R. Vetter, Rebersburg, PA) and later digitized at sampling intervals of 50 μs to 8 ms (see figure legends). Analog filtering (Bessel characteristic, 24 dB/octave) preceded digitizing at one-fifth the sampling frequency (Colquhoun and Sigworth, 1983). Currents for which the autocorrelation function was calculated were filtered with a Butterworth filter (24 dB/octave) at one-fourth to one-half the sampling frequency. Membrane potentials are given as the intracellular potential with respect to bath, and currents are presented such that upward deflections correspond to outward currents.

Percent Open Time

An interval detection routine that sampled at 20 kHz measured open and shut intervals with respect to a 50% amplitude threshold (see Colquhoun and Sigworth, 1983). Depending upon the maximum number of current levels observed, the process was repeated at each current level, since no patches were found that contained only one active channel. The resulting percent open times were then averaged and normalized by the estimated total number of active channels in the patch. Their number was estimated from the maximum number of current levels observed when the channels were most active, i.e., at depolarized voltages in the presence of Ca.

Current Amplitudes

Single channel current amplitudes were estimated from histograms (see Colquhoun and Sigworth, 1983) that were constructed from 4,096–8,192 current samples at each of several voltages (see Fig. 3).

Autocorrelation Function

The autocorrelation and autocovariance functions constructed from macroscopic (Neher and Sakmann, 1976) or microscopic (Liebovitch and Fischbarg, 1985) currents contain information about the average time course of the current fluctuations that arise from channel gating (Colquhoun and Hawkes, 1977; Neher and Stevens, 1977). The covariance function, $C(T)$, can be defined by a shift-and-multiply operation upon the deviations from the mean single channel current:

$$C(T) = \frac{1}{L} \int_0^L [I(t) - \bar{I}][I(t + T) - \bar{I}]dt,$$

where $I(t)$ is the channel current at time t, $I(t + T)$ is the current at a time interval T later, $\bar{I}$ is the mean current, and L is the time interval over which the autocorrelation is calculated. Computation time was greatly reduced in practice by obtaining the covariance from a Fourier transform of the current spectrum (see DeFelice, 1981; Stevens, 1984). The autocorrelation was obtained by normalizing the covariance so that $C(0) = 1$ (Neher and Stevens, 1977). Before these manipulations, the currents were filtered (Butterworth characteristic) at one-fourth to one-half the sampling frequency to minimize aliasing. The autocorrelation functions in Fig. 5 are averages of four to six functions, each calculated from 2,048 sample points (200-μs sampling interval). Segments of current without channel activity were used to calculate a baseline autocorrelation, which was then subtracted from the averaged function.

Quin2 Fluorescence

Cells were suspended in solutions identical to those above, and loaded with the fluorescent Ca-chelator quin2 by incubation with 50 μM quin2-AM for 30 min at 37°C (Tsien et al., 1982). Before the addition of ionomycin (final concentration, 50 μM), cells were twice washed, centrifuged (100 g) to remove extracellular quin2-AM, and then resuspended. Fluorescence changes were monitored with a Perkin-Elmer (Norwalk, CT) MPF-2 fluorescence spectrophotometer. The traces shown in Fig. 2 are digitized chart records from which control (untreated cells) autofluorescence was subtracted.

RESULTS

Ca Ionophores Increase Channel Activity in Cell-attached Patches

1321N1 astrocytoma cells possess large-conductance (220–280 pS) Ca-activated K channels (Pallotta and Oglesby, 1986) that demonstrate [Ca] and voltage sensitivities qualitatively similar to channels in several other preparations (Barrett et al., 1982; Wong et al., 1982; Moczydlowski and Latorre, 1983; Gallin, 1984). Upon the addition of either the Ca ionophore A23187 or ionomycin to the extracellular solution, channel activity in cell-attached patches increased dramatically. Fig. 1 shows a current record from a cell exposed to 50 μM ionomycin and 1 μM free Ca. Channel activity increased to a plateau level (38% open time for each of six channels in the patch) and remained constant after the patch was excised into the solution containing 1 μM Ca. Similar ionophore-induced results were obtained in 25 other patches from cells exposed to ionophores and extracellular [Ca] (0.3–1 μM), 13 of which allowed successful excision of the patch.

As is apparent in Fig. 1, channel activity appeared similar before and after excision from the cell body. This result would imply that channel properties did not change upon excision if the following assumptions were true: (a) intracellular [Ca] was equal to the extracellular [Ca], (b) the membrane potential was the same for the cell-attached and excised patch configurations, and (c) the channel gating kinetics were similar in both patch configurations. As shown below, tests of these assumptions reveal that intrinsic channel properties did not change significantly when the patch was excised.

Quin2 Fluorescence Measurements of Intracellular [Ca]

To test the assumption that Ca ionophores were able to equilibrate the intracellular and extracellular [Ca], cells in suspension were loaded with the Ca fluorophore quin2 (Tsien et al., 1982) and then exposed to ionophore in solutions identical to those used in Fig. 1. Fig. 2A shows the change in fluorescence accompanying 50 μM ionomycin and 1 μM Ca. Within 2 min, the fluorescence reached a plateau and did not change when the cells were lysed with the detergent Triton X-100 in the presence still of 1 μM Ca. Since this [Ca] approaches the saturating region for quin2 (Fig. 2, inset), this result suggests that intracellular [Ca] was at least 1 μM under the conditions of these experiments. Similar results were obtained with 0.3 μM Ca (Fig. 2C) and three additional experiments with 1 μM Ca (including one that used the ionophore A23187).

Cell-attached

Excised
↓

10 s

20 pA
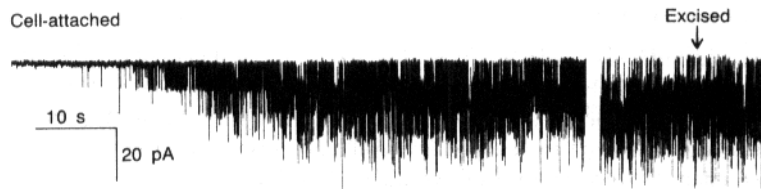
FIGURE 1.   Time course of single channel activity in a cell-attached patch after the addition of 50 μM ionomycin. The extracellular solution (see Methods) contained 1 μM Ca. Note that channel activity did not change when the patch was excised. Ionomycin was bath-applied ~20 s (the dead time of the perfusion system) before the beginning of the above record. The gap in the record corresponds to 1.5 min of data collection at other voltages. Membrane potential, −40 mV; sample interval, 8 ms.
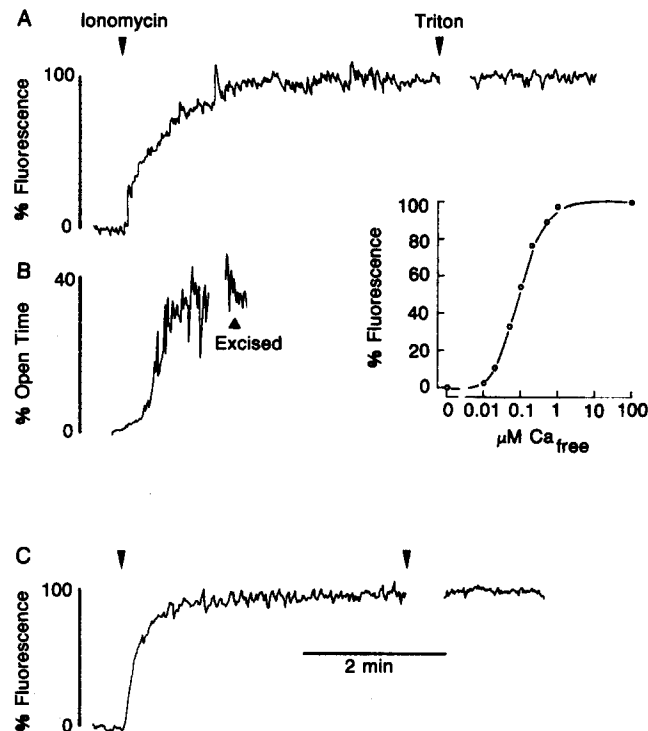
FIGURE 2.   Time course of quin2 fluorescence after the addition of 50 μM iono-mycin. Cell suspensions were loaded with quin2-AM (see Methods) and exposed to 50 μM ionomycin and 1 (A) or 0.3 (C) μM Ca. Cells were lysed with 1% Triton X-100 as indicated; the gaps in the fluorescence records correspond to artifacts that occurred when the cuvettes were opened for detergent injection. (B) Time course of channel percent open time calculated from the data shown in Fig. 1 (assuming six active channels in the patch) and aligned for comparison with the fluorescence records. Inset: calibration curve relating the quin2 fluorescence (percent of maximum) to the free [Ca].

The time course of the fluorescence change can be compared with the time course of the channel percent open time (Fig. 2 B) obtained from the experiment shown in Fig. 1. The similarities of the time courses, and the results of three experiments where A23187 failed to increase channel activity in the absence of extracellular Ca, support the assumption that the increased channel activity (Fig. 1) reflected changes in intracellular [Ca].

Current-Voltage Relation Compared in Cell-attached and Excised Patches

Since the activity of Ca-activated K channels increases with membrane depolarization, comparisons between the cell-attached and excised patch could not be
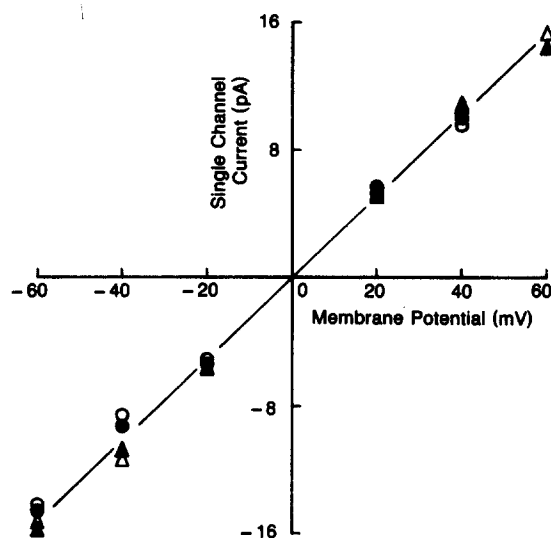

FIGURE 3.   Current-voltage relation from channels in the cell-attached (open symbols) and excised (filled symbols) patch configurations. Cells were depolarized and exposed to 50 μM ionomycin (plus 1 μM Ca) for several minutes before the measurements shown above. Results from two different experiments are shown and the line fitted to all the plotted values indicates a single channel slope conductance of 252 pS.

made unless the membrane potential difference was similar in both configurations. To accomplish this, cells were chemically clamped near 0 mV with a high-K (110 mM) solution. In this solution, currents obtained from cell-attached patches (Fig. 3; open symbols) reversed direction at 0 mV, which suggests that extracellular [K] (110 mM) must be very close to intracellular [K] and that little potential difference remained across the membrane. Similar current-voltage relations were obtained from eight additional experiments. Upon excision, neither the reversal potential nor the slope conductance (252 pS) changed (Fig. 3; filled symbols), which demonstrates that the potential differences across the patch were similar in both configurations.

Voltage and Ca Dependence of Channel Activity

Current records obtained from a cell-attached patch that was later excised are shown in Fig. 4, *A* (+60 mV) and *B* (−60 mV). As in Fig. 1, the records appear qualitatively similar, and this is supported by measurements of the percent open time at several voltages (Fig. 4*C*). Over the voltage range examined in both 1 and 0.3 μM Ca, the sensitivity of the channel percent open time to voltage was
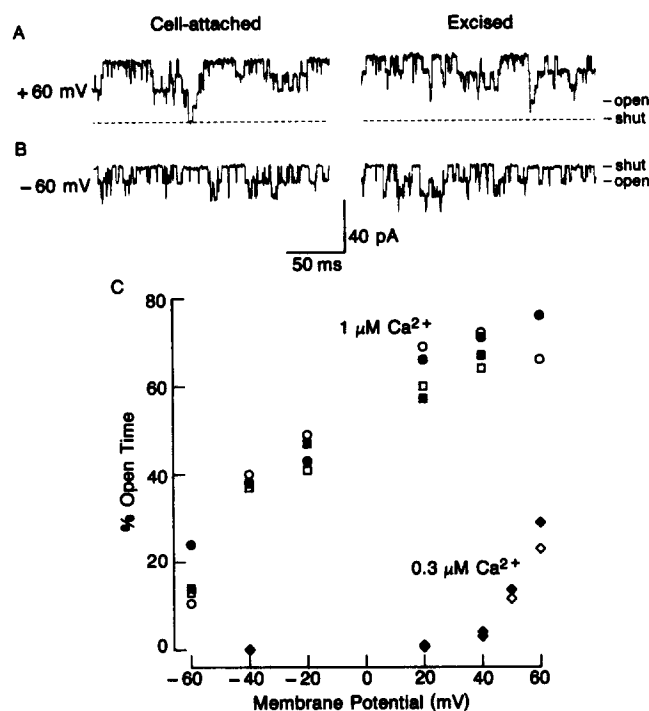

FIGURE 4.   The voltage dependence of the channel percent open time compared in cell-attached and excised patches. Cells were depolarized and treated with 50 μM ionomycin (plus 1 μM Ca) before measurements were taken. Sample currents are shown at membrane potentials +60 mV (*A*) and −60 mV (*B*) in the cell-attached (left panel) and then excised patch configuration (right panel). (*C*) Percent open time as a function of membrane potential in the cell-attached (open symbols) and excised (filled symbols) patch from two experiments with 1 μM extracellular free Ca, and one experiment with 0.3 μM free Ca as indicated. The percent open time has been normalized to the number of active channels in the patch.

similar in cell-attached and excised patches. Similar results were obtained in six additional experiments with 1 μM Ca and either A23187 or ionomycin, and two additional experiments with 0.3 μM Ca and 30 μM ionomycin.

Fluctuation Analysis of Channel Gating

While the channel percent open time did not change with excision (Fig. 4), this result does not preclude significant changes in channel gating. Because the typical

membrane patch contained 4–20 active channels, gating kinetics were character-ized by performing a fluctuation analysis upon the multichannel currents. The autocorrelation function, $C(T)$ (see Methods), reveals any tendencies of the channel current to correlate with itself over time. Since this function is a transform of the common spectral density (see DeFelice, 1981; Stevens, 1984), it is similarly biased toward events of relatively long duration (see Patlak, 1984).

The autocorrelation function constructed from the channel currents is similar in the cell-attached and excised patch configurations. Fig. 5 shows the autocor-relation from a patch clamped at two membrane voltages after the cell was depolarized and treated with 50 μM ionomycin plus 1 μM Ca. Similar results were obtained at several voltages in three additional experiments. In all cases,
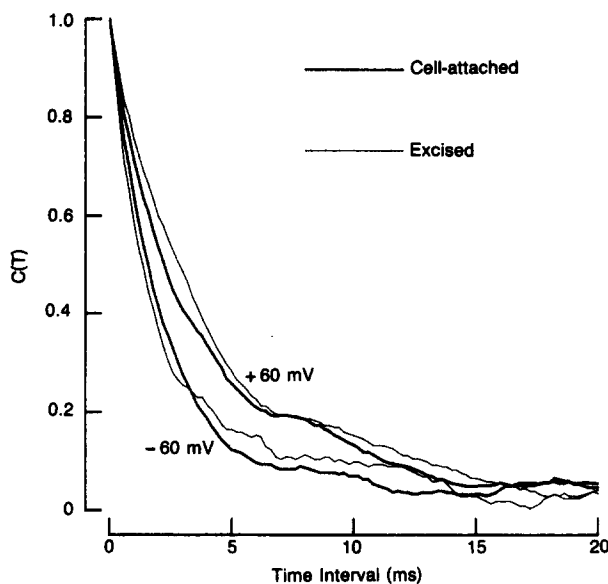

FIGURE 5. Autocorrelation functions calculated from single channel currents that were recorded from a patch while cell-attached (thick line) and then after excision (thin line). The currents used in the calculations were recorded at two different voltages (indicated in figure) ~6 min after the cell was depolarized and treated with 50 μM ionomycin plus 1 μM Ca.

$C(T)$ was best described by the sum of two exponential components (not shown). While no attempt was made to relate these components or their voltage depend-ence to a specific molecular mechanism or aspect of channel gating (Colquhoun and Hawkes, 1977), these results do suggest that the time constants of the predominant current fluctuations were similar in the cell-attached and excised patch configurations.

Time Course of Channel Activity after the Patch is Excised

We have shown so far that channel properties are similar in cell-attached and excised patches soon after the patch was excised. Other types of single channel studies, however, might depend upon constant channel properties for much

longer periods of time. Fig. 6 shows the channel percent open time as a function of time after excision in eight experiments. While occasional large transient changes occurred (open diamonds), there was no consistent change in channel activity for several minutes after excision. In one experiment, the percent open time remained constant for 90 min (not shown). Taken together, our results show that channel gating was not obviously altered either immediately upon, or several minutes after, the patch was removed from the intracellular environment of the K-depolarized cell. They also suggest that modulation of the average channel activity by cytosolic factors was not reversible in the excised patch within the time periods we examined.
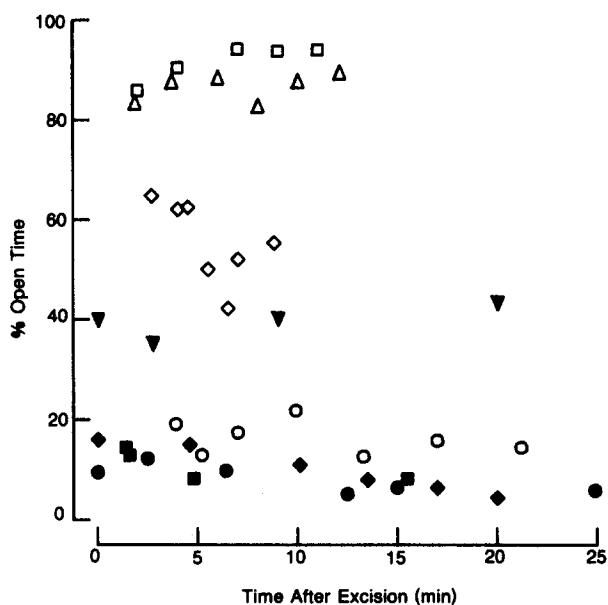

FIGURE 6. Channel percent open time as a function of time after excision. The percent open time was normalized to the number of active channels in the patch. The large differences in percent open time between experiments occurred because different holding potentials were used (range, −70 to +30 mV). The filled symbols correspond to cells that had been exposed to either 50 μM ionomycin or 100 μM A23187.

DISCUSSION

The major finding of this study is that properties of Ca-activated K channels such as sensitivity to [Ca] and voltage in the cell-attached patch were unchanged (Fig. 4) for several minutes after the patch was excised from the cell (Fig. 6). The gating kinetics (Fig. 5) and single channel conductance (Fig. 3) were also similar. This direct comparison thus provides some reassurance that the results from studies that used the inside-out excised patch can be extrapolated to (K-depolarized) cell-attached conditions. Considering the ample precedents for channel modulation by soluble intracellular factors (see review by Kostyuk, 1984), such as ATP (Noma, 1983), cyclic AMP (Siegelbaum et al., 1982; Ewald

et al., 1985; Bartschat et al., 1986), and H (Cook et al., 1984), the results of this study are somewhat surprising, though not unwelcome. While excision from the K-depolarized cell apparently does not affect gating, it is possible that modulation of channel gating by soluble factors occurs in cells with a normal resting potential. Although intracellular [cAMP] and phosphoinositide metabolism in bovine glomerulosa cells were little affected by 8 mM K (Kojima et al., 1985; but see Albano et al., 1974), extrapolation of our results to cells with a normal resting potential might require additional qualification.

Previous studies that compared ion channel properties in cell-attached and excised patches found differences between the two. Trautmann and Siegelbaum (1983) found a decrease in the mean burst duration of acetylcholine-activated channels from rat myotubes upon formation of an outside-out patch, although channel conductance and gating were unaffected by the formation of an inside-out patch. Trube and Hescheler (1984) described two inwardly rectifying K channels in heart cell membrane whose single channel currents disappeared several minutes after patch isolation. One type of channel (25 pS) spent a larger fraction of time in the open state upon patch isolation, although the authors suggested that this might be a consequence of changes in [ATP] and perhaps [Ca] that occurred at the intracellular membrane surface. Cachelin et al. (1983) found a large (40–50 mV) hyperpolarizing shift in the activation and inactivation kinetics of single Na channels in excised inside-out patches compared with cell-attached patches from cardiac myocytes. Kunze et al. (1985) found that a similar shift occurred in the same preparation when whole-cell Na currents were compared with single channel currents in both cell-attached and excised patches, as did Fernandez et al. (1984) upon comparing single channel currents in excised patches from GH₃ cells with whole-cell currents. Horn and Vandenberg (1986) compared Na channel currents from the same patch in both cell-attached and excised patch configurations. Compared with the cell-attached state, after excision from the GH₃ cell, typical channels were more likely to open during depolarization, with longer open times and more openings per burst. Horn and Vandenberg concluded that the changes in gating occurred as a result of replacing the cytoplasm with internal solution, since the whole-cell currents from dialyzed cells are similar to averaged currents from excised patches. An anomalous observation, however, is the shift in Na channel gating that occurred upon the formation of cell-attached patches in rat myocytes (Kunze et al., 1985). The Ca-activated K channel might therefore be unique because its properties did not appear to change upon excision.

Our results are consistent with the view that at normal, negative cell resting potentials, these channels are relatively insensitive to [Ca] (Fig. 4) compared with otherwise identical channels found in anterior pituitary cells (Wong et al., 1982) and several types of secretory cells (Petersen and Maruyama, 1984). [Ca] and voltage sensitivities similar to ours have been found in rat muscle (Barrett et al., 1982), t-tubule membranes in planar bilayers (Moczydlowski and Latorre, 1983), and human macrophages (Gallin, 1984). Thus, the physiological function(s) these channels fulfill might rely on either extreme depolarization and/or proximity to sites of Ca influx (or release), where the local [Ca] would be sufficiently high for activation to occur.

While the chemical clamp of the membrane potential to near 0 mV with high K was clearly successful (Fig. 3), some uncertainty surrounds intracellular [Ca] after ionophore treatment. For equilibration between intra- and extracellular [Ca] to occur, it was necessary to saturate both the intracellular Ca buffers and any active outward Ca fluxes (Baker et al., 1985; Lew and Garcia-Sancho, 1985). As a result, we used relatively high concentrations of both Ca and ionophores, and tested [Ca] equilibration with fluorescence measurements. These measurements were limited, however, by the saturation of the quin2 fluorescence near the [Ca] of interest (Fig. 2), or by local changes in [Ca] that would not be evident in the fluorescence signal. While it is conceivable that either A23187 or ionomycin released Ca from intracellular organelles, this does not seem likely since ionophores applied in the absence of extracellular Ca (three experiments) or in the presence of 2 mM Mg plus 1 μM Ca (one experiment) failed to induce channel activity. Thus, the available evidence favors the view that [Ca] equilibration occurred under the conditions of our experiments.

An additional consequence of the results of this study is that the activity recorded from cell-attached Ca-activated K channels can provide a quantitative measure of free [Ca] near the intracellular membrane surface if solutions with known [Ca] are used to establish the Ca sensitivity of that particular channel(s) after excision. Since the relation between channel activity and intracellular surface [Ca] is the same in both types of patch configurations, the intracellular [Ca] can be calculated from the activity vs. [Ca] relation, which is easily measured from the excised inside-out patch (cf. Fig. 4).

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