The Calcium-activated Potassium Channels of Turtle Hair Cells

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ABSTRACT   A major factor determining the electrical resonant frequency of turtle cochlear hair cells is the time course of the Ca-activated K current (Art, J. J., and R. Fettiplace. 1987. *Journal of Physiology.* 385:207–242). We have examined the notion that this time course is dictated by the K channel kinetics by recording single Ca-activated K channels in inside-out patches from isolated cells. A hair cell's resonant frequency was estimated from its known correlation with the dimensions of the hair bundle. All cells possess BK channels with a similar unit conductance of ~320 pS but with different mean open times of 0.25–12 ms. The time constant of relaxation of the average single-channel current at −50 mV in 4 μM Ca varied between cells from 0.4 to 13 ms and was correlated with the hair bundle height. The magnitude and voltage dependence of the time constant agree with the expected behavior of the macroscopic K(Ca) current, whose speed may thus be limited by the channel kinetics. All BK channels had similar sensitivities to Ca which produced half-maximal activation for a concentration of ~2 μM at +50 mV and 12 μM at −50 mV. We estimate from the voltage dependence of the whole-cell K(Ca) current that the BK channels may be fully activated at −35 mV by a rise in intracellular Ca to 50 μM. BK channels were occasionally observed to switch between slow and fast gating modes which raises the possibility that the range of kinetics of BK channels observed in different hair cells reflects a common channel protein whose kinetics are regulated by an unidentified intracellular factor. Membrane patches also contained 30 pS SK channels which were ~5 times more Ca-sensitive than BK channels at −50 mV. The SK channels may underlie the inhibitory synaptic potential produced in hair cells by efferent stimulation.

INTRODUCTION

Hair cells of the turtle's basilar papilla are tuned to specific frequencies in the acoustic stimulus from ~20 Hz to more than 500 Hz by an electrical resonance (Crawford and Fettiplace, 1981) which, over much of this frequency range, arises from the combined action of a voltage-dependent Ca current and a Ca-activated K current. A major determinant of the resonant frequency is the speed of the Ca-activated K current which relaxes with a time constant varying from ~15 ms at 50

Hz to 0.7 ms at 350 Hz (Art and Fettiplace, 1987). Resonant frequencies <50 Hz are achieved by a slow voltage-dependent K current with an activation time constant up to 150 ms (Art, Fettiplace, and Wu, 1993). For the higher-frequency cells, the time course of the Ca-activated K current could be limited by delays due to the diffusion and buffering of intracellular Ca (Ashmore and Attwell, 1985; Hudspeth and Lewis, 1988b; Oberholtzer, Buettiger, Summers, and Matschinsky, 1988), or by the K(Ca) channel kinetics. We have previously argued, based mainly on measurements of the spectral composition of K(Ca) current noise, that the channel kinetics are indeed the limiting factor (Art and Fettiplace, 1987) and we have now examined this hypothesis by assaying single Ca-activated K channels in inside-out patches from cells tuned to different frequencies.

The turtle's basilar papilla is homologous to the cochleas of higher vertebrates and its hair cells are tonotopically organized with their optimal frequencies increasing with distance from the apical end of the organ (Crawford and Fettiplace, 1980). In addition to the kinetics of the K(Ca) current, other properties of the hair cells, including the size of both the Ca and K(Ca) currents (Art et al., 1993) as well as the dimensions of the hair bundle (Hackney, Fettiplace, and Furness, 1993) display a systematic variation with hair cell resonant frequency and hence cochlear location. A broader question concerns the mechanism by which this panoply of cellular attributes is regulated, and some clue might be derived by elucidating the control of any single property. Both *Drosophila* (Atkinson, Robertson, and Ganetzky, 1991; Adelman, Shen, Kavenaugh, Warren, Wu, Lagrutta, Bond, and North, 1992) and mouse (Butler, Tsunoda, McCobb, Wei, and Salkoff, 1993) Ca-activated K channels have been recently cloned and exhibit the potential for expression of multiple spliced variants which could form the basis of a variation in channel kinetics in turtle hair cells. An alternate possibility is that the hair cell K(Ca) channel kinetics are regulated by some intracellular process such as phosphorylation (Reinhart, Chung, Martin, Brautigan, and Levitan, 1991).

MATERIALS AND METHODS

Preparation and Recording Techniques

Hair cells were isolated from the basilar papilla of the red-eared turtle (*Trachemys scripta elegans*, carapace length 10–12 cm) using a standard procedure (Art and Fettiplace, 1987). Briefly, after decapitation of the turtle, the basilar papilla was dissected out, the tectorial membrane removed and the preparation incubated in a saline containing 0.1 mM Ca supplemented with 0.5 mg/ml papain, 2.5 mM L-cysteine and 0.1 mg/ml serum albumin. Hair cells were collected from identified regions of the papilla and plated onto a glass coverslip which was transferred to the stage of an Zeiss IM-35 inverted microscope fitted with Nomarski optics. Recordings were made only from cells with an intact hair bundle, the dimensions of which were measured before recording with an eyepiece graticule. At the magnification of 1,280 used, the bundle measurements were reproducible to ~0.2 μm. The maximum height of the hair bundle has been found to vary inversely with the cell's resonant frequency (F_o) from 9.5 μm (F_o = 20 Hz) to 5.0 μm (F_o > 350 Hz) (Art and Fettiplace, 1987; Hackney, Fettiplace, and Furness, 1993). The resonant frequency was inferred from the hair bundle height since it was rarely possible to make both whole-cell and detached patch recordings on the same hair cell.

Single-channel currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) from cell-attached or inside-out patches of the basolateral membrane of a hair cell. Borosilicate recording pipettes of resistance 15–25 MΩ and tip diam $\sim$0.5 μm were normally filled with a solution containing (in millimolar): KCl, 135; K$_2$EGTA, 5; K HEPES, 5, pH 7.4. After seal formation the pipette was detached from the cell and the resulting membrane vesicle was broken by contact with a Sylgard ball. This procedure to produce an inside-out patch was performed in an extracellular solution identical to that in the pipette so that the cytoplasmic surface of the membrane was never exposed to high Ca. For an inside-out patch to contain few channels, the patch area needed to be very small, and experiments where a significant membrane omega was drawn into the pipette were rejected. The length of the omega usually ranged between $\sim$1 and 5 μm from the electrode tip. In some experiments, the patch area was determined from the change in capacitance when the electrode was inserted into the Sylgard ball (Sakmann and Neher, 1983), assuming a specific membrane capacitance of 10 fF/μm^2. All membrane currents were stored on a Sony PC-108M instrumentation recorder at a band width of 0–20 kHz. Experiments were performed at 22–24°C.

Solutions

Inside-out patches were exposed to solutions of different composition by means of a six-barreled glass "pan pipe" (Vitro Dynamics, Rockaway, NJ). Each barrel was of square cross-section pulled to an internal diameter of $\sim$50 μm and perfused from a peristaltic pump, a given barrel being selected by means of a remotely controlled miniature solenoid valve (Lee Products, Westbrook, CT). For most of the experiments the ionic composition of the perfusate was (in millimolar): KCl, 130; MgCl$_2$, 0.5; Mg ATP, 1; K HEPES, 5, pH 7.4, with a variable Ca, buffered with 2 mM of the K salt of dibromoBAPTA (Molecular Probes, Inc., Eugene, OR) to give free Ca concentrations of 0.5–50 μM. The proportion of buffer and total Ca to achieve a given free Ca concentration was calculated based on a K'_{Ca} of 2 μM (Bers, Patton, and Nuccitelli, 1993), and all solutions were then calibrated using a MI-100 Ca electrode (Microelectrodes, Inc., Londonderry, NH). DibromoBAPTA was the preferred Ca buffer because its K'_{Ca} lies within the range of Ca concentrations that activate the channel, and also it is relatively insensitive to Mg and pH. In a few early experiments, HEDTA or BAPTA was used as the Ca buffer. The use of BAPTA was discontinued because of the finding that the measured free Ca was often considerably higher than that calculated, probably due to water impurity in the buffer (Harrison and Bers, 1987). For later experiments, the cytoplasmic solutions were supplemented with 0.1 μM okadaic acid (Calbiochem Corp., La Jolla, CA), an inhibitor of PP-1 and PP-2A protein phosphatases. Tyrosine phosphatase catalytic subunit was obtained from Calbiochem and A-kinase catalytic subunit from Promega Corp. (Madison, WI).

To measure simultaneously both single-Ca and K(Ca)-channel currents in cell-attached mode, the recording electrode was filled with a solution similar to that described by Pietrobon, Prod'hom, and Hess (1989) that contained (in millimolar): NaCl, 135; KCl, 2; Na HEDTA, 5; Na TAPS, 5, pH 8.7. To prolong the Ca channel openings, 5 μM ($\pm$) BAY K8644 (Calbiochem Corp.) or FPL-64176 (Zheng, Rampe, and Triggle, 1991), a sample of which was kindly supplied by Fisons (Loughborough, UK), was added to the pipette solution. In some experiments, charybdotoxin (Peninsula Laboratories, Belmont, CA) was applied to the extracellular face of an inside-out patch by pressurized perfusion of the patch pipette (Adams and List Associates, Ltd., Westbury, NY) via a quartz capillary whose tip was sited within 0.6 mm of the end of the pipette.

Single-Channel Analysis

Continuous stretches of single-channel data were low-pass filtered at between 5 and 8 kHz (8-pole Bessel) and digitized at 40 kHz. Opening and closing transitions were detected using

half the single-channel amplitude as the threshold criterion. Open and closed time distributions derived from these idealized records were fitted with sums of exponential probability density functions by a maximum likelihood method using a random optimization algorithm (Solis and Wets, 1981). The fit was corrected for the dead time of the recording system (Colquhoun and Sigworth, 1983), estimated as $0.3/f_c$, where f_c is the -3 dB frequency of the filter. Only those events longer than the dead time are shown. Record durations in a given condition were typically long enough to contain between 5,000 and 50,000 events. To display the range of open and closed durations, the distributions are presented as log–log plots (McManus and Magleby, 1991) or using the transformation of Sigworth and Sine (1987). The number of exponentials needed to fit a distribution was determined from the likelihood ratio test, an additional component being added only if the χ^2 test on the likelihood ratios reached a 0.05 level ($P_{\chi^2} < 0.05$ for rejecting the extra component). The stationarity of the data was assessed from the stability of the open probability and mean open time in a given standard condition, usually 4 μM Ca at $\pm$ 50 mV. Transient data were low-pass filtered at 3 kHz and digitized at 12.5 kHz. Leakage and capacitative currents were subtracted using sweeps that contained no openings, and ensemble averages were constructed typically from responses to 300 stimuli presented at 10/s. The number of channels in a patch was estimated from the maximum current for a condition in which the probability of opening was high (Horn, 1991). While patches containing only a single channel could be used for forming open- and closed-time distributions, analysis of the transient response is based on some multichannel patches.

RESULTS

Ca-activated K Channels in Cell-attached Patches

Fig. 1 shows cell-attached patch recording of large-amplitude K channels from two different hair cells, one with a long hair bundle and the other with a short hair bundle. The resonant frequencies of the two hair cells were estimated from previous measurements (Art and Fettiplace, 1987; Hackney et al., 1993) as 50 and 200 Hz, respectively. Both hair cells were immersed in a high K, low Ca solution identical to that in the pipette (see Materials and Methods) so that patches could subsequently be detached from the cell in a quasi-intracellular environment. Under these ionic conditions, the cells probably had zero resting potential which was supported by the observation that the channels had reversal potentials close to zero mV. Fig. 1 shows that at $+50$ mV, the two channels had a similar size of $\sim 12-13$ pA, consistent with their both being BK channels, but that from the short-bundled cell had briefer transitions. As will be shown later, the variations in the kinetics of the BK channels seen in Fig. 1 were retained when the patches were detached and studied in inside-out mode.

To obtain patches with just a few BK channels, it was necessary to use electrodes of internal tip diam 0.5 μm enclosing a small membrane omega. With such electrodes, a variable number of channels/patch was observed. For cells with short hair bundles of length 5.8–6.8 μm, the mean number of channels/patch was 3.0 $\pm$ 2.1 ($N = 43$ patches) and for long hair bundles of length 7.7 to 8.8 μm it was 1.05 $\pm$ 1.26 ($N = 40$). In seven experiments we measured the patch area (see Methods) which ranged from 2 to 13 μm^2, with a mean $\pm$ SD of 9 $\pm$ 4 μm^2. Using this patch area, we estimated that cells with short bundles contained a mean density of 0.33 channels/μm^2 whereas cells with long bundles had a mean of 0.12 channels/μm^2. These

differences are consistent with previous results indicating that cells tuned to high frequencies possess a greater density of K(Ca) channels than cells tuned to low-frequencies (Art et al., 1993).

It was our general impression that the channel density was higher in the basal half of the cell at the nuclear level than in the apical half towards the hair bundle. The measurements on low-frequency cells were mostly from patches in the basal half, but for the high-frequency cells, many of the patches were taken from the apical half in order to obtain some with single channels. This experimental variation would have led to an underestimate in the difference between low- and high-frequency cells. If some BK channels were concentrated into a small number of "hotspots," correspond-
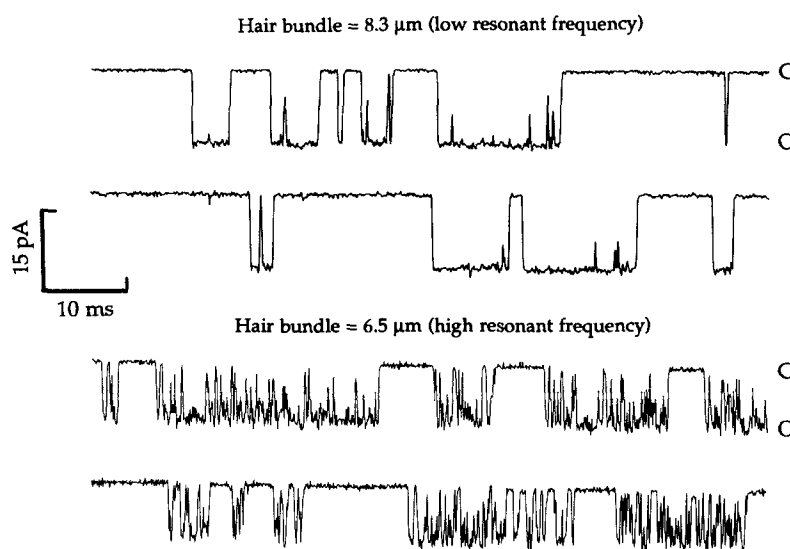

FIGURE 1. Single Ca-activated K channels recorded in cell-attached configuration from different hair cells. Note that the channels were more prolonged from the cell with the long hair bundle (8.3 μm, *top*) than from the cell with the short hair bundle (6.5 μm, *bottom*). Estimated resonant frequencies were 50 Hz (*top*) and 200 Hz (*bottom*). Holding potentials +50 mV, both cells immersed in 135 mM KCl, 5 mM K₂EGTA solution. C and O indicate closed and open levels.

ing to the synaptic release sites (Roberts, Jacobs, and Hudpseth, 1990), it is conceivable that we would rarely have recorded from them. Of the 113 patches studied, one from a cell with a 5.5-μm hair bundle had an unusually high number of BK channels (26); this was not included in the mean-channel densities cited above.

Ca and Ca-activated K Channels Occur in the Same Patch

For the BK channels that we routinely recorded to contribute to the whole-cell current and to experience rapid and substantial changes in intracellular Ca during depolarization, they would need to have neighboring voltage-sensitive Ca channels. We tested for this possibility by searching for both types of channel in the same patch

in cell-attached recordings. To improve the resolution for Ca channels, we measured the Na current through these channels with a pipette solution containing 145 Na, a low divalent cation concentration, and a Ca channel activator, either Bay K8644 or the FPL-64176 (Zheng, Rampe, and Triggle, 1991). In five patches both inward (Na) and outward (K) currents were observed (e.g., Fig. 2), and in another two patches neither type of channel was present. The inward current was activated between −60 and −20 mV, it had an extrapolated reversal potential near 0 mV and it was not observed in more than 20 additional cell-attached experiments in the absence of the Ca-channel activator, all of which endorse its identification as a Ca channel. The slope conductance was 86 pS, and because our previous experiments (Art et al., 1993) have shown that for Ca concentrations <0.1 µM, the current through the Ca channels is increased about sixfold, this yields a channel conductance in 2.8 mM Ca of 14 pS, comparable to previous values reported for the L-type channel (Hess, Lansmen, and Tsien, 1986). The outward current was strongly activated only at membrane potentials positive to −30 mV. By its size and prevalence, it is most likely the Ca-activated K channel which has a lower slope conductance in asymmetric K than the usual 300 pS (Yellen, 1984). It should be noted that at −50 mV, the deduced amplitudes of the Ca and BK single-channel currents are ~0.8 and 2 pA, respectively. The main conclusion from this type of experiment is that the Ca-activated K channels whose properties will be described later have neighboring Ca channels and are likely to contribute functionally to the electrical resonance.

Stationary Behavior of Ca-activated K Channels in Inside-Out Patches

Fig. 3 shows representative segments of records illustrating the effects of Ca concentration on channel activity. At the membrane potential of +50 mV, the channel was fully activated in 5 µM Ca and its amplitude was ~12 pA in the symmetrical 150 mM K solutions. The current-voltage relationship for such channels displayed slight inward rectification, variable from channel to channel (Fig. 6 B), so the slope conductance was characterized at −50 mV, giving 323 pS for this example and a range from 290 to 340 pS in other experiments. The large unitary conductance and the fact that in four experiments the channel was completely blocked by pipette perfusion with 100 nM charybdotoxin establish it as a BK channel (Miller, Moczyd-lowski, Latorre, and Phillips, 1985; Latorre, Oberhauser, Labarca, and Alvarez, 1989; McManus, 1991).

The effects of Ca on the probability of opening at +50 mV is plotted in Fig. 4 A for channels from three cells, two with short hair bundles and one with a long bundle. The smooth curve was calculated from the Hill equation:

$$P_o = P_{max} \, Ca^n / [(K_{0.5})^n + Ca^n] \tag{1}$$

with a half-saturating Ca concentration, $K_{0.5}$, of 2.9 µM and a Hill coefficient, n, of 3.5. In contrast to Eq. 1, a relation without binding cooperativity with the form $P_o = P_{max}[Ca/(K_{0.5} + Ca)]^n$ gave a poor fit to the data and in particular did not predict the sharp corner near saturation. Fits of Eq. 1 to individual data sets for 17 channels gave values for $K_{0.5}$ ranging from 1.1 to 3.9 µM and values for n from 1.9 to 3.7; the maximum probability of opening, P_{max} was between 0.65 and 0.95. There was no evidence that the channel's Ca sensitivity varied systematically with the height of the

A

-110 mV
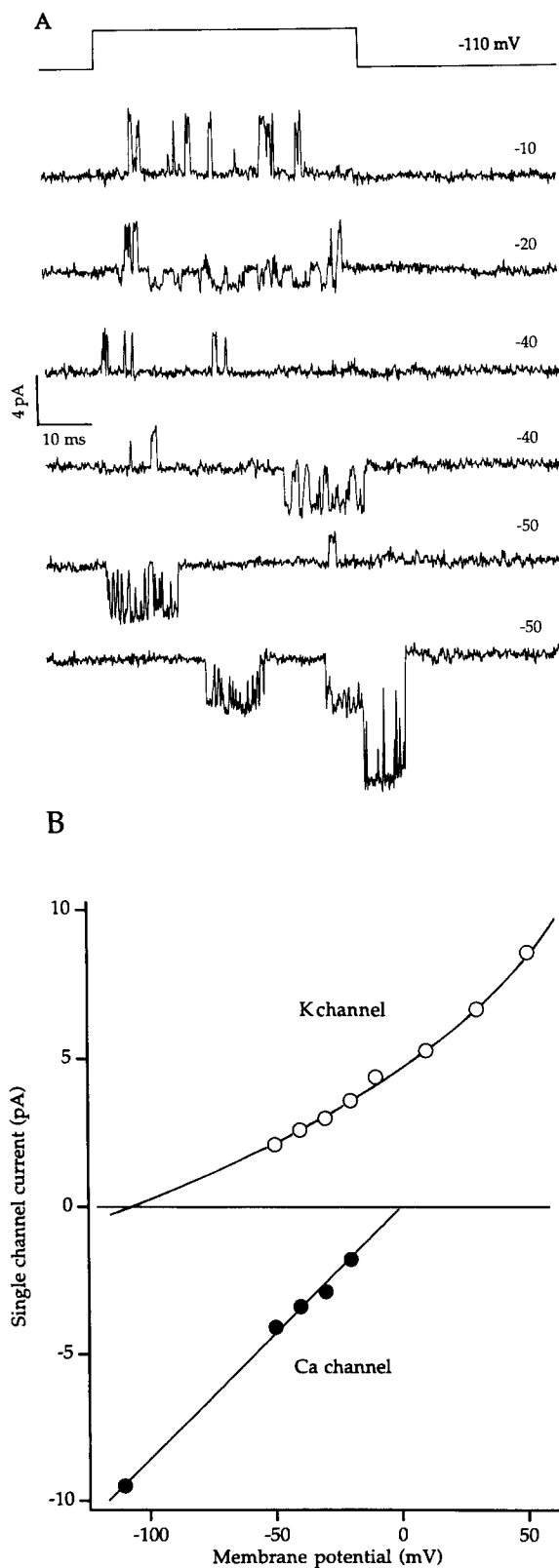
-10

-20

-40

4 pA

-40

10 ms

-50

-50

B

FIGURE 2. (A) Records of both voltage-sensitive Ca channels (negative-going inward currents) and Ca-activated K channels (outward currents) in a single cell-attached patch. The patch was held at −110 mV and stepped to the potential indicated beside each trace. Note the increase in the inward-current channel in the bottom trace on repolarizing to −110 mV. Cell bathed in 135 mM KCl, 5 mM K₂EGTA solution. The pipette solution contain in millimolar: NaCl, 135; KCl, 2; NaHEDTA, 5; NaTAPS, 5, pH 8.7 with 5 μM FPL-64176 to prolong the Ca channel openings. (B) Current-voltage relations for the two types of channel in the experiment shown in A. The smooth curves were drawn by eye, that through the K channel points being extrapolated to an assumed K reversal potential of −107 mV.
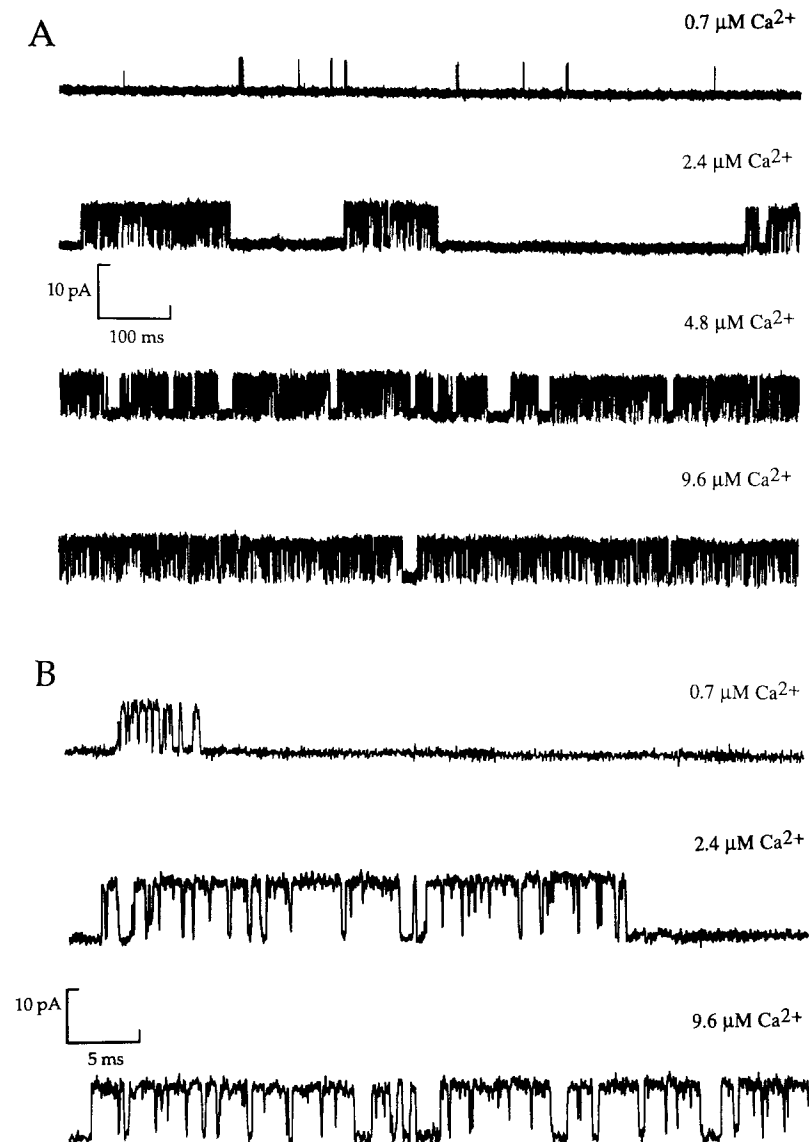
Single channel current (pA)

10

K channel

5

0

Ca channel

-5

-10

-100   -50   0   50

Membrane potential (mV)

FIGURE 3.   Representative stretches of recordings from a single BK Ca-activated K channel in an inside-out patch shown on a slow (A) and fast (B) time scale, in the presence of different cytoplasmic Ca concentrations. Holding potential, +50 mV.

bundle (Fig. 4 B): mean values (±SD) for $K_{0.5}$ were 2.2 ± 0.8 μM (N = 17, +50 mV) and 11.8 ± 5.6 μM (N = 13, −50 mV). The value of $K_{0.5}$ at 0 mV, interpolated from measurements at other membrane potentials (see Fig. 10 A) was 4.9 ± 2.1 μM.

The Ca sensitivities and Hill coefficient for the hair cell's BK channel are very similar to those of the well-characterized channels in rat skeletal muscle (Barrett, Magleby, and Pallotta, 1982; McManus and Magleby, 1991). The high Hill coefficient

is indicative of multiple Ca binding sites, a conclusion reinforced by the numbers of exponential components needed to fit the closed- and open-time histograms. The closed time histograms generally required four to six components and the open-time histograms two to three components. Examples of these histograms are illustrated in Fig. 5 for two channels originating from hair cells with long and short hair bundles, which were assumed to be tuned to low and high frequencies, respectively. The open-time histograms tended to be dominated by one of the components which comprised 50–75% of the total area; this major component was ~0.3 ms in the cell with a short bundle and 2.8 ms in the cell with the long hair bundle.
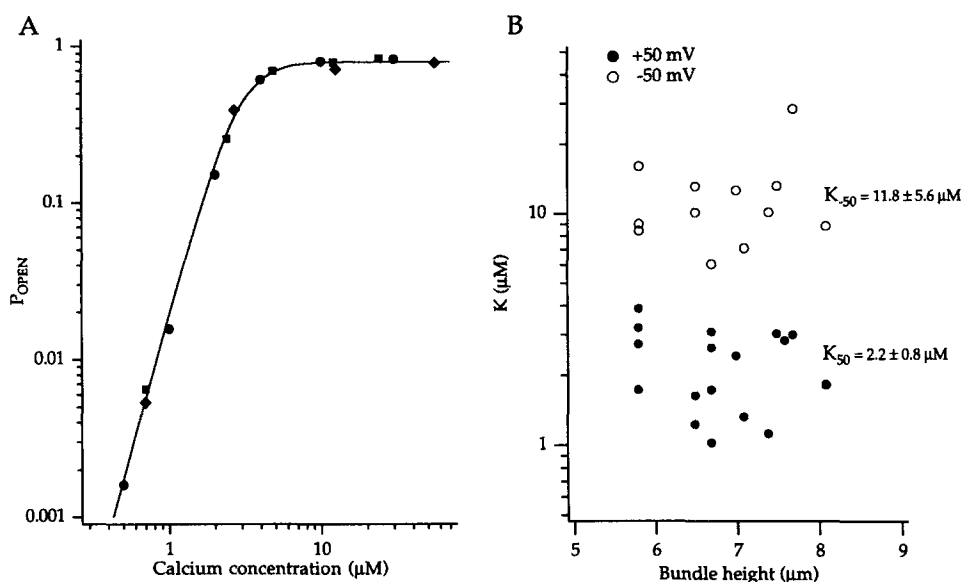

FIGURE 4.   (A) Probability of opening plotted against Ca concentration for three BK channels from cells of different hair bundle length, holding potential +50 mV. Hair bundle lengths: (*squares*) 5.8 μm (same cell as Fig. 3); (*circles*) 6.7 μm; (*diamonds*) 7.7 μm. Smooth curve is the Hill equation (Eq. 1) with P_{max} = 0.81, $K_{0.5}$ = 2.9 μM and n = 3.5. (B) Values of $K_{0.5}$, the half-saturating Ca concentration plotted against hair bundle height of cell of origin; (*filled symbols*) +50 mV; (*open symbols*) −50 mV. The plot includes measurements from both single-channel and multichannel patches.

The major component of the open time at +50 mV is plotted for seven channels against Ca concentration in Fig. 6 A. *For each channel, the open time increased* between two- and fourfold with Ca concentration, but the limiting value varied considerably in different cells from 0.25–12 ms. Such variation was not obviously correlated with the absolute Ca sensitivity of the channel or with the channel conductance at −50 mV. However, channels with longer open times had more linear current-voltage relations (Fig. 6 B). The shorter open times tended to occur in channels originating from cells with shorter hair bundles, though there are probably insufficient results to establish a strict correlation. They do suggest however, that the

kinetic properties of the BK channel are dictated by a separate part of the channel protein from the regions involved in either pore formation or Ca sensing. This observation is reminiscent of the alternatively spliced variants described for the *D-slo* Ca-activated K channel which possess similar unitary conductance and Ca sensitivity but substantially different mean open times (Adelman et al., 1992).
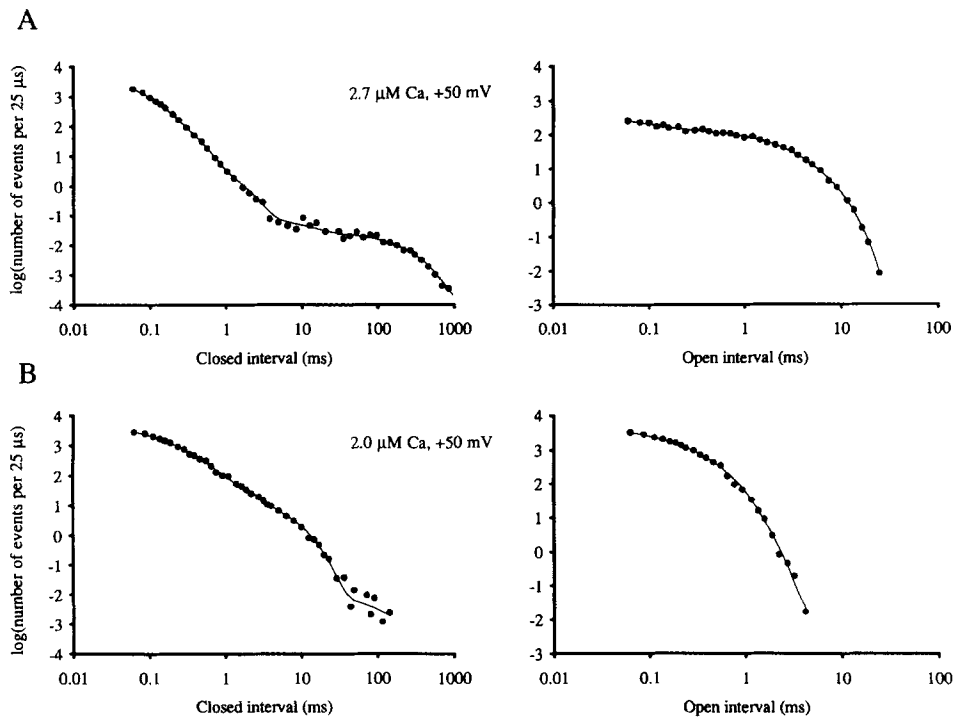

FIGURE 5. Histograms of the closed and open times for BK channels from two cells of different hair bundle lengths at +50 mV. (*A*) Hair bundle, 7.7 μm; Ca, 2.7 μM; probability of opening, 0.39; number of events, 16,686. The smooth lines are the maximum likelihood fits with the sums of six exponential components to the closed intervals and three exponential components to the open intervals. The time constants of the closed and open distribution are (in milliseconds): closed, 0.05, 0.17, 0.77, 8.4, 171, 1067; open .065, 1.3, 2.8, of which 2.8 ms supplied 60% of the area. (*B*) Hair bundle, 6.7 μm; Ca, 2.0 μM; probability of opening, 0.23; number of events, 31,175. The smooth lines are the maximum likelihood fits with the sums of five exponential components to the closed intervals and three exponential components to the open intervals. The time constants of the closed and open distribution are (in milliseconds): (*closed*) .08, .25, 1.0, 4.7, 81; (*open*) .08, .24, .44, of which the 0.24 ms component supplied 71% of the area.

Responses to Voltage Steps

The kinetics of the whole-cell current have previously been inferred (Art and Fettiplace, 1987) from the relaxation at the end of a depolarizing voltage step returning to a holding potential of ~ −50 mV. This potential corresponds to the

average resting potential of turtle hair cells. To compare directly the whole-cell results with single-channel data, ensemble averages of the channel responses to voltage steps were constructed. This allowed the inclusion of some multichannel patches. Examples of the results are illustrated in Fig. 7, *A* and *B*, for patches from short- and long-bundled cells. Each patch was subjected to a 100-mV voltage step from −50 mV in Ca concentrations from 0.5 to 35 or 50 μM which produced a full range of probabilities of opening. The most notable conclusion is that the channel with the longer open time had a much slower relaxation on repolarizing to −50 mV. The principal time constants of the current relaxations for the two cells were ∼1 ms
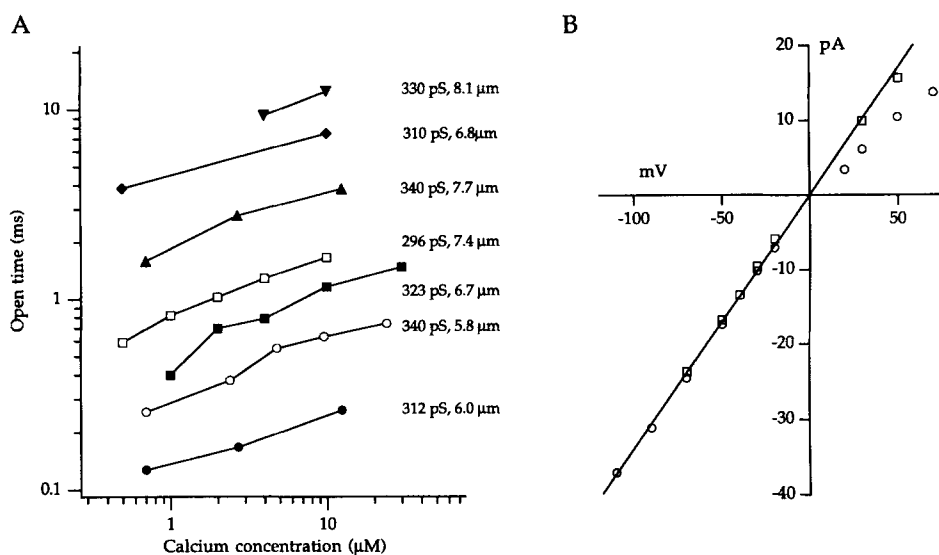

FIGURE 6.   (*A*) The major component of the open time is plotted against Ca concentration for channels from seven different patches, holding potential +50 mV. The single-channel conductance and the hair bundle height from the cell of origin are given alongside each set of points. (*B*) Current-voltage curves for two BK channels from hair cells with bundle heights 6.5 μm (*circles*) and 8.1 μm (*squares*). Same cells as those illustrated in Fig. 7. Each point is the average of the currents at different Ca concentrations. The line through the points corresponds to a unit conductance of 340 pS. Note the inward rectification in one of the channels which represents the most extreme example observed.

(Fig. 7 *A*) and 8 ms (Fig. 7 *B*) in 4 μM Ca. In both cases, the tail current relaxed more rapidly at Ca concentrations <4 μM.

Collected measurements of the relaxation time constant of the average current are plotted against bundle height in Fig. 8 *A*. Each of the measurements was made in 4 μM Ca since this concentration may be comparable to the Ca concentration at the resting potential of intact hair cells (see Discussion). For each patch, the decay in channel activity on repolarization to −50 mV was fitted with one or two exponential components and the major component was plotted. For cleaner records obtained with multichannel patches, two components differing by a factor between 5 and 10
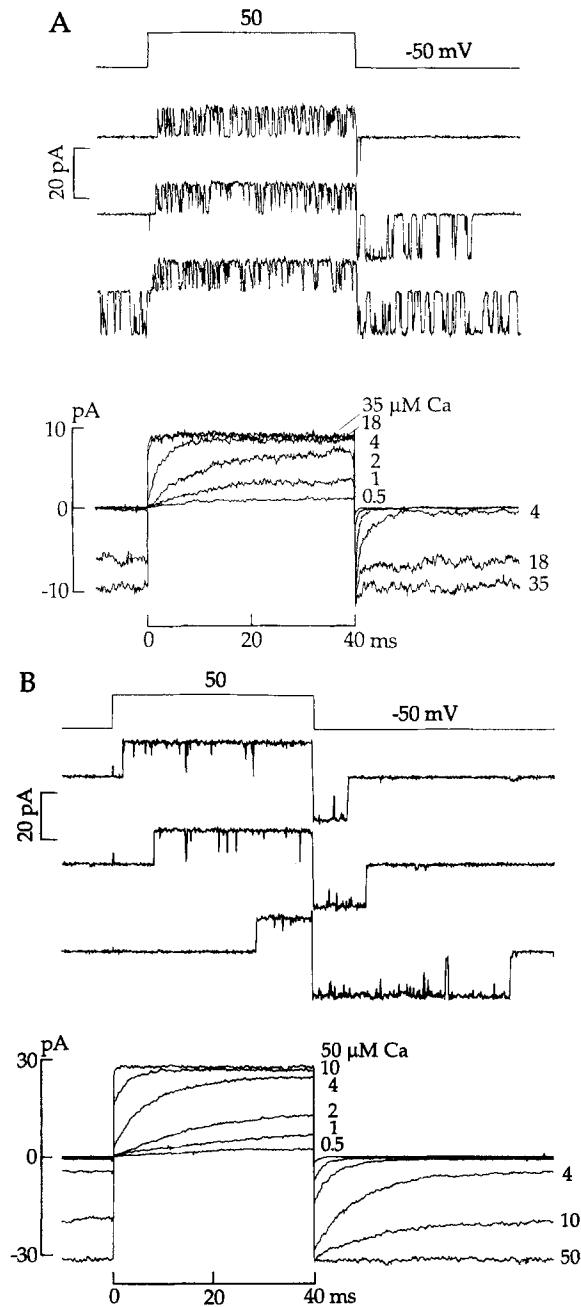
FIGURE 7. Responses to voltage steps of BK channels from two hair cells of bundle heights (*A*) 6.5 μm and (*B*) 8.1 μm. For each part of the figure, the top traces show the voltage step from −50 to +50 mV and three records of the channel behavior in 4 μM Ca. Bottom traces, superimposed ensemble-average currents in Ca concentrations from 0.5 to 35 or 50 μM. The recording in *B* contained two channels.

were usually required, the faster component in most cases being the dominant one. Fig. 8 *A* shows time constants, τ, ranging from 0.4 to 13 ms, which were correlated with bundle height, *h*. The smooth curve describes the parabolic relationship:

$$\tau = 0.5\, h^2/(10.2 - h)^2 \tag{2}$$

An equation of this form is expected if (*a*) the time constant of deactivation of the whole-cell K current varies inversely with the square of the resonant frequency, F_o, i.e., $\tau = k/F_o^2$ (Art and Fettiplace, 1987) where k is a constant and (*b*) the resonant frequency is inversely proportional to bundle height (Hackney, Fettiplace, and Furness, 1993), a relationship $h = a/(b + F_o)$ where a and b are constants, being used to derive Eq. 2. The range of time constants for the single-channel measurements corresponds well to the range of time constants derived for the whole-cell K current, ~15–0.7 ms for resonant frequencies between 50 and 350 Hz. The conclusion from these results is that for hair cells in this frequency range that use BK channels to
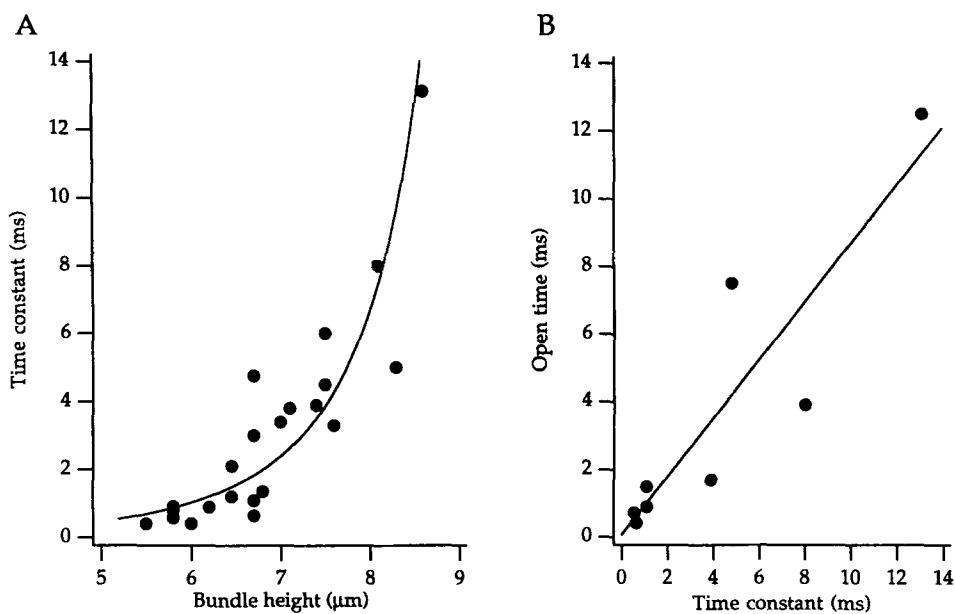

FIGURE 8. (*A*) Collected measurements from 21 patches of the time constant of relaxation of the ensemble-average BK channel current (−50 mV, 4 μM Ca) plotted against the bundle height of the hair cell. Smooth curve fitted using Eq. 2 in text. (*B*) The maximum open times measured at +50 mV in a Ca concentration of 10 μM or more is plotted for eight channels against the time constant of relaxation of the average current at −50 mV in 4 μm Ca. The line is a least-squares fit with regression coefficient 0.89, slope 0.9 and intercept on the *y* axis of 0.067.

generate their resonant properties, the kinetics of the macroscopic current may be varied by regulation of the kinetics of the channels themselves.

The tail current kinetics were measured at a potential of −50 mV, where the BK channels normally contribute to resonance, but most of the single-channel open times were obtained at +50 mV (Fig. 6*A*). While it need not be the case that single-channel kinetic differences seen at +50 mV for high and low-frequency cells extend to −50 mV, in those channels where measurements were available at both membrane potentials, the maximum open times were similar. This suggests that the relaxation of the ensemble average current is determined mainly by the channel open

time. Fig. 8 *B* gives a plot for eight channels of the open time measured at +50 mV against the relaxation time constant of the average current at −50 mV. This shows a reasonable correlation between the two variables, the fitted line having a slope of 0.86 and a regression coefficient of 0.9.

The relaxation time constant of the whole-cell K(Ca) current changes e-fold for an ∼25 mV change in the holding potential (Art and Fettiplace, 1987). To confirm the limiting role of the channel properties, we verified that the time constant of the single-channel relaxation possessed a similar voltage dependence. Examples of the single-channel tail currents in 4 μM Ca for repolarization to three different membrane potentials are shown in Fig. 9 *A;* each has been fitted with a single
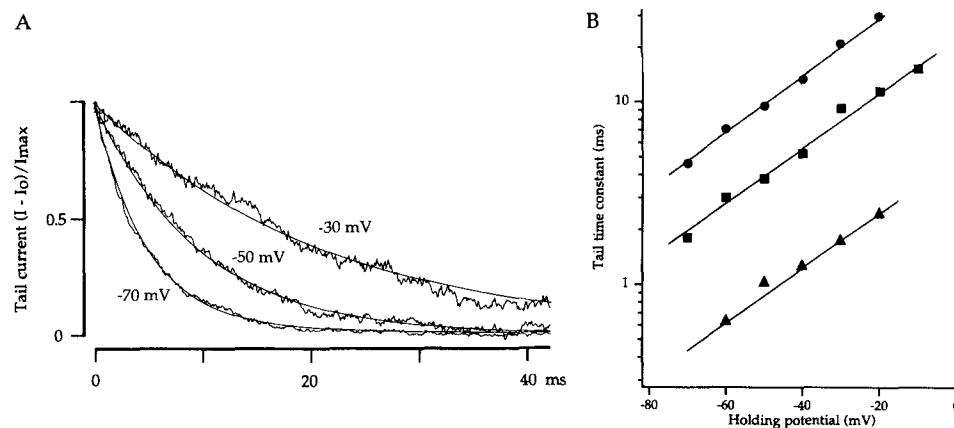

FIGURE 9. Voltage dependence of the relaxation of the ensemble average tail current in 4 μM Ca. (*A*) Averages of the BK single-channel relaxation at −30, −50, and −70 mV after a voltage step to +50 mV. For display, the averages have been scaled to the same maximum value and the steady levels have been subtracted. Smooth curves are single-exponential fits with time constants of 21 ms (−30 mV); 9.5 ms (−50 mV) and 4.6 ms (−70 mV). (*B*) Results of measurements in *A* along with those from two other cells, time constant plotted against holding potential. Lines through points correspond to an e-fold change in time constant in 28, 29 and 29 mV. Bundle heights of hair cells: 8.1 μm (*circles*), 7.4 μm (*squares*), 6.5 μm (*triangles*).

exponential decay. The inferred time constants for this and two other patches are plotted in Fig. 9 *B*. All three sets of data had similar slopes, corresponding to an e-fold increase in time constant for a mean reduction in holding potential of 28.7 ± 0.5 mV.

The Range of Ca Concentrations Occurring Physiologically

The aim of this section is to use the single-channel properties to derive an estimate of the range of Ca concentrations that are needed to activate the K(Ca) channel under physiological conditions. The voltage dependence of the probability of opening, *P*, of a single BK channel obtained from analysis of stationary records is displayed in Fig. 10 *A*. The measurements in different Ca concentrations have been fitted with the

Boltzmann equation:

$$P = P_{max}/[1 + \exp\{(V_o - V)/V_\epsilon\}], \tag{3}$$

where V_o is the voltage for half-maximal activation, P_{max} is the maximum probability of opening (~ 0.77) and V_ϵ is the slope factor, which increased from 10 mV in the lowest Ca concentration to 21 mV in the highest. Mean values for V_ϵ in six patches were 15 ± 4 mV in 4 μM Ca and 18 ± 3 mV in 10 μM Ca, values similar to those reported for BK channels in other preparations (Latorre et al., 1989). The half-saturating Ca concentration depended exponentially on membrane potential, decreasing e-fold for a depolarization of ~ 38 mV (equivalent to a 10-fold change in 86
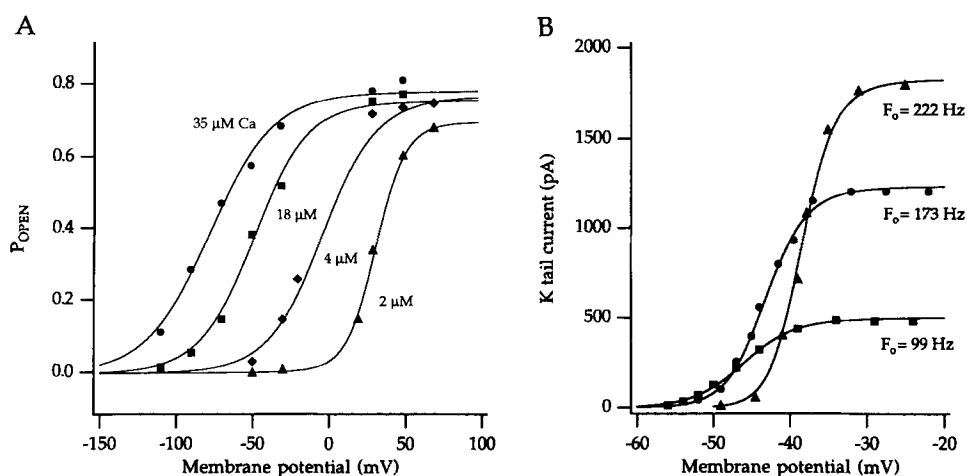

FIGURE 10.   (*A*) Probability of opening of BK channel at different membrane potentials in a range of Ca concentrations. Data obtained from stationary measurements, same cell as Fig. 7 *A*. Smooth curves calculated from Boltzmann equation (Eq. 3) with values of Ca, P_{max}, V_o, V_ϵ: 2 μM, 0.7, 32 mV, 10 mV; 4 μM, 0.77, −3 mV, 18 mV; 18 μM, 0.76, −48 mV, 18 mV; 35 μM, 0.78, −77 mV, 21 mV. (*B*) Plots of the whole-cell K tail current at a holding potential of −50 mV in three hair cells with different resonant frequencies. The smooth curves have been calculated from Eq. 8 of text with the following values of $I_{K(max)}$, *A*, *n*, and resonant frequency: (*triangles*) 1,820 pA, 0.26, 4.2, 222 Hz; (*circles*) 1,230 pA, 0.15, 3.1, 173 Hz; (*squares*) 500 pA, 0.10, 2.3, 99 Hz. The apparent trend in the constant *A* was not obvious in other results.

mV). It is clear from these results that at membrane potentials of −40 to −30 mV, where the whole-cell K(Ca) current normally saturates (Fig. 10 *B*), a maximal probability of opening is achieved for a Ca concentration of 35 μM. Similar estimates for other channels gave a concentration of no more than 50 μM.

An alternative estimate of the Ca concentration can be derived from fitting the voltage dependence of the whole-cell K(Ca) current using the approach described by Martin and Fuchs (1992). Fig. 10 *B* shows activation curves for the whole-cell current, obtained from tail current measurements at −50 mV (see Art and Fettiplace, 1987; Art et al., 1993). These currents are steeply voltage dependent and increase e-fold in 2–3 mV. To describe the activation curves, we shall neglect the intrinsic voltage-

sensitivity of the channel and assume that the voltage dependence arises solely from the voltage dependence of the Ca channel amplified by the cooperative binding of Ca to the BK channel. The activation of the Ca current, I_{Ca}, is described by a Boltzmann equation:

$$I_{Ca} = I_{Ca(max)}/[1 + \exp\{(V_1 - V)/V_2\}] \tag{4}$$

where values for the half-activation, V_1, and slope factor, V_2, of -27 and 7 mV, respectively, have been taken from previous analysis of the hair cell's Ca current (Art and Fettiplace, 1987; Art et al., 1993). Eq. 4 has been corrected for the changes in driving force by assuming an ohmic single-channel conductance and a reversal potential of $+50$ mV:

$$I_{Ca} = \alpha I_{Ca(max)}/[1 + \exp\{(V_1 - V)/V_2\}] \tag{5}$$

where $\alpha = (0.905 - 0.018V)$. Eq. 5 generates the U-shaped steady state current-voltage relationship with a peak at ~ -15 mV as observed experimentally. The average Ca concentration [Ca] at the mouth of the K channel is related to I_{Ca} by

$$[Ca] = \beta\, I_{Ca} f(r, t) \tag{6}$$

where $(1 - \beta)$ is the fraction of Ca instantaneously bound by buffer and $f(r, t)$ reflects attenuation due to diffusion and is a function of distance, r, from the Ca channel and time, t, after it opens; f is assumed to be independent of the Ca concentration, as occurs in simple diffusion models (Smith and Augustine, 1988; Roberts et al., 1990). The Ca concentration can thus be described by

$$[Ca] = \alpha[Ca]_{max}/[1 + \exp\{(V_1 - V)/V_2\}] \tag{7}$$

$[Ca]_{max}$ is the maximum steady state Ca concentration at the K(Ca) channel. Eq. 7 can be substituted into the Hill equation (Eq. 1) and the parameters for the Ca current, V_1 and V_2, inserted giving the voltage dependence of the K(Ca) current, I_K, as

$$I_K = I_{K(max)}/[1 + A^n \alpha^{-n}(1 + \exp\{-(27 + V)/7\})^n] \tag{8}$$

where n is the Hill coefficient and $A = K_{0.5}/[Ca]_{max}$; $K_{0.5}$ is the half-saturating Ca concentration which in the voltage range over which the channel is activated will vary by no more than 50%, and is therefore, assumed to be constant. Eq. 8 gave excellent fits to the whole-cell current (Fig. 10 B) with values of n from 1.8 to 4.2 similar to those obtained from the single-channel results. The mean value of A derived from fitting 10 cells was 0.19 ± 0.06. Assuming that the K(Ca) channels in the whole cell have a Ca sensitivity similar to those assayed in detached patches, their $K_{0.5}$ will be no greater than 11.8 μM (the average value at -50 mV in Fig. 4 B), and therefore $[Ca]_{max}$ is 62 μM. $[Ca]_{max}$ can be substituted in Eq. 7 to give the voltage-dependence of the Ca concentration at the BK channel; the deduced concentration at a membrane potential of -30 mV is 35 μM, which is comparable to the value inferred from the measurements in Fig. 10 A.

Rundown in Ca Sensitivity and Open Time

In a number of patches, there were reductions in the Ca sensitivity of the channel and also a shortening of the mean open time throughout the course of a recording. These

changes were not necessarily correlated and one could occur without the other. In view of previous claims that the BK channel may be regulated by phosphorylation (Reinhart, Chung, Martin, Brautigan, and Levitan, 1991; Bielefeldt and Jackson, 1994), most experiments were performed in the presence of 1 mM MgATP and, in later experiments, 0.1 μM okadaic acid, an inhibitor of PP-1 and PP-2A protein phosphatases. The latter maneuver may have slightly augmented the Ca sensitivity, so that the $K_{0.5}$ for Ca activation at +50 mV was 2.6 ± 0.9 μM ($N = 7$) without okadaic acid and 1.9 ± 0.7 μM ($N = 10$) with okadaic acid. In three experiments, 0.3 μM of the catalytic subunit of protein kinase A, added in the presence of okadaic acid, increased the Ca sensitivity by a factor of 1.7 ± 0.4. This enhancement, which took place over a period of ~10 min, is similar to that reported by Reinhart et al. (1991), and may point to a role for A-kinase in modulating the channel. However, such changes in sensitivity were accomplished without a change in channel kinetics and, moreover, a shortening of the mean open time was still observed in the presence of okadaic acid. The channel kinetics were not reproducibly accelerated by prolonged exposure to 300 μM Ca (six experiments) or perfusion with 0.14 μM of the catalytic subunit of tyrosine phosphatase (three experiments). Taken together, these results suggest that the channel kinetics are not regulated by phosphorylation at a site susceptible to the common protein phosphatases.

The speeding up of the channel kinetics was usually, but not always, irreversible. In one such channel, the time constant of relaxation of the tail current at −50 mV was 4.1 ± 0.2 ms during the initial 10 min of a recording and then declined over the next 15 min to 1.3 ms. It might be argued that this stems from a pathological change in the patch: for example oxidation of membrane lipids or loss of cholesterol (Bolotina, Omelyanenko, Heyes, Ryan, and Bregestovski, 1989). However, occasionally, the change was abrupt and reversible, as shown in Fig. 11. In this case the mean open time decreased ~threefold for a period of a few minutes and then reverted to its original condition, and interestingly, the channel in its faster mode had a lower conductance at +50 mV (cf Fig. 6 B). This phenomenon was observed in other patches and is reminiscent of the multiple gating modes reported for the μ1 Na channel (Zhou, Potts, Trimmer, Agnew, and Sigworth, 1991). In those experiments the equilibrium between slow and fast modes of a single Na channel protein expressed in *Xenopus* oocytes was influenced by an intracellular modulator, and also there was a tendency during an extended recording for the channel to migrate into the fast mode. The similar behavior of the BK channel raises the possibility that the range of kinetics of BK channels observed in different hair cells reflects a common channel protein whose kinetics are regulated by an unidentified intracellular factor. It should be noted that, in view of the occasional run down, to characterize a channel's kinetics (Fig. 8), the initial data from a given patch was used, and then only if the channel was stable during the first 10 min of recording (see Materials and Methods).

Other K Channels

In many patches taken from hair cells with a range of bundle sizes, a calcium-dependent leak was evident during the early part of a recording. This was caused by a small channel, the properties of which were characterized in three single-channel recordings (Fig. 12). The channel had a mean conductance at −50 mV of 30 ± 2 pS

and a reversal potential at 0 mV in symmetrical 150 mM K. The reversal potential was shifted positive on substitution of Na for K in the solution bathing the cytoplasmic face of the patch, suggesting that it was a K channel. This K channel was remarkable in being very steeply activated by intracellular Ca. The probability of opening at −50 mV was half-maximal at a Ca concentration of 2.4 ± 0.4 μM, and the Hill coefficient was 4 or more. It was therefore about five times more sensitive to Ca than the BK channel at −50 mV but, in contrast to the BK channel, it had little voltage dependence. The Ca concentration, besides gating the probability of opening, also strongly influenced the open time: for the example illustrated, the mean
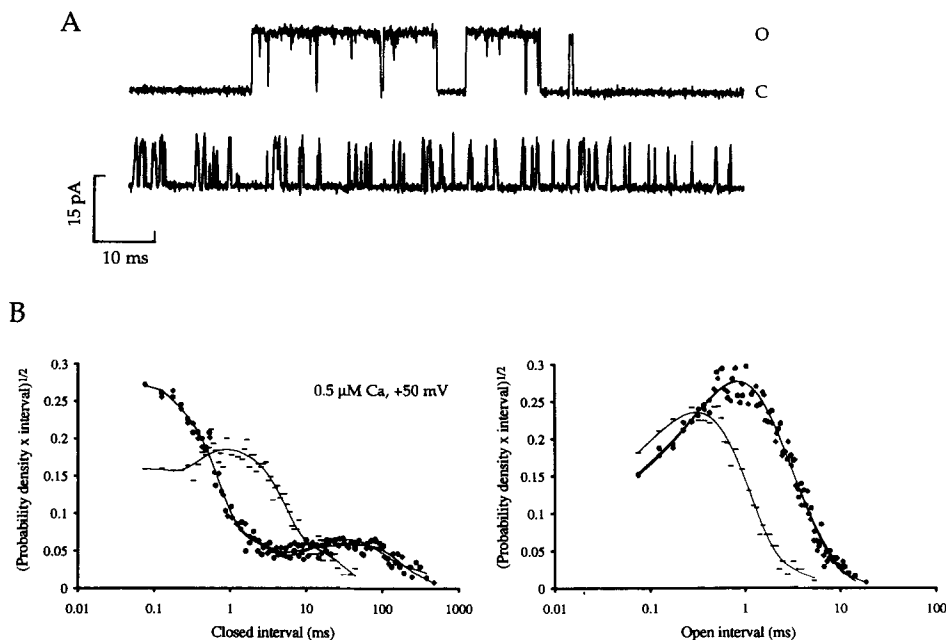

FIGURE 11.   (A) Records showing a spontaneous change in the kinetics of a single BK channel, holding potential +50 mV, 0.5 μM Ca. (B) Closed and open time histograms, displayed using the transformation of Sigworth and Sine (1987). (Horizontal lines) Channel in fast mode. (Circles and diamonds) Channel in slow mode before and after. The smooth curves are fits based on five closed-time and three open-time components. The major component of the open time was reduced from 1.4 to 0.4 ms.

open time increased from 0.6 ms in 0.5 μM Ca to 9 ms in 10 μM Ca. An unusual feature of this SK channel was its complete disappearance after brief (20 s) exposures of the patch to 10–30 μM Ca. It was therefore only observed if the patch was isolated in a low Ca solution (see Materials and Methods) and furthermore, its contaminating effects could be eliminated from a recording by bathing in 30 μM Ca. We had no evidence that brief exposure to high Ca altered the properties of the BK channel.

While SK channel were commonly recorded from all classes of hair cell, a third type of K channel was obtained only in patches from cells with hair bundles longer than 7 μm. This channel had a mean conductance at −50 mV of 45 ± 5 pS (N = 5) and a

reversal potential that depended on the internal K concentration. It was activated by depolarizations to membrane potentials between −70 and −30 mV, but was unaffected by the cytoplasmic Ca concentration. A characteristic feature of this purely voltage-dependent K channel was that its openings consisted of prolonged high-frequency bursts, and hence it displayed a slow activation and deactivation in
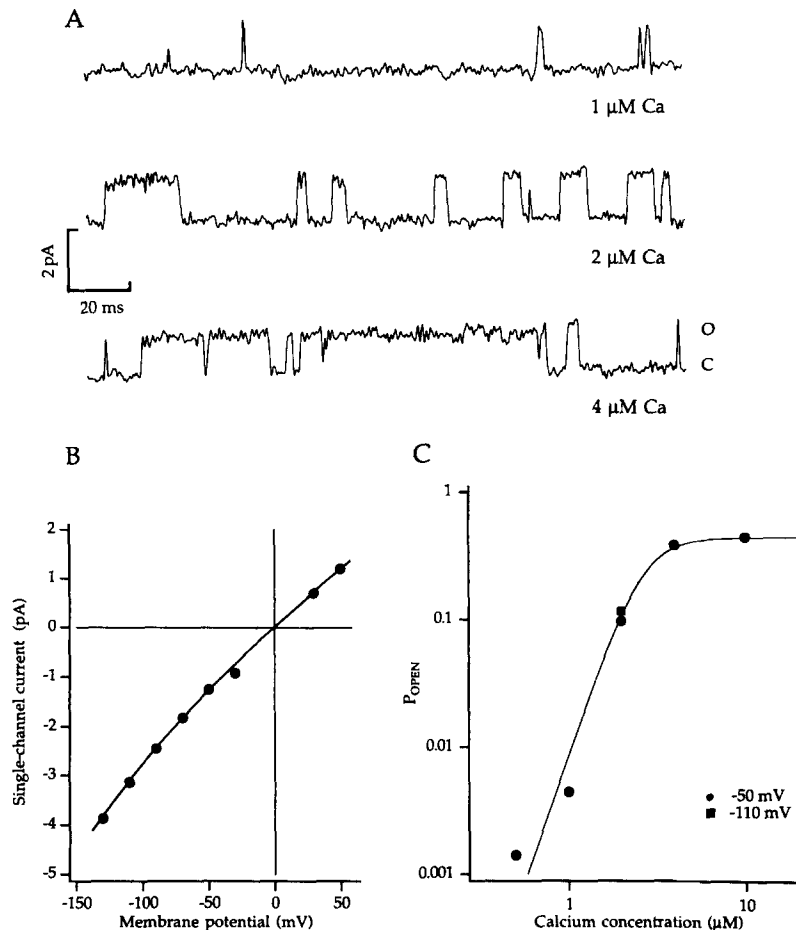

FIGURE 12. Properties of a small (SK) Ca-activated K channel in an inside-out patch. (A) Single-channel records in 1, 2, and 4 μM Ca at a holding potential of −50 mV. (B) Current-voltage relation for this channel, unit conductance of 30 pS at −50 mV. (C) Probability of opening plotted against Ca concentration at −50 mV (*circles*) and −110 mV (*square*). Smooth curve is Hill equation with values for P_{max}, K, and n of 0.43, 2.6 μM, and 4, respectively.

response to voltage steps. The relaxation time constant of the ensemble-average current lay between 18 and 50 ms. Unlike the BK and SK channels, it could be blocked by perfusion of the cytoplasmic surface of the patch with 5 mM 4-aminopyridine. Its restriction to low-frequency cells, its slow kinetics and its pharmacological

profile all suggest that it is the voltage-dependent K channel which tunes hair cells to the very lowest resonant frequencies.

DISCUSSION

Properties and Role of BK Channels in Turtle Hair Cells

The large, 300-pS, Ca-activated K (BK) channels recorded in turtle hair cells have properties remarkably similar to those occurring in rat skeletal muscle (Barrett, Magleby and Pallotta, 1982; Moczydlowski and Latorre, 1983; McManus and Magleby, 1991): they are half-activated by a Ca concentration of ~5 μM at 0 mV, they have a steep voltage sensitivity (e-fold in 15 mV) and the Ca dependence has a Hill coefficient up to 3.7, indicating at least four Ca-binding sites; furthermore the channels are blocked by TEA with a K_i of 0.27 mM (Art J. J, and M. B. Goodman, unpublished results). In spiking cells, the BK channels contribute to repolarization of the action potential (Adams, Constanti, Brown, and Clark, 1982) but in hair cells, which are nonspiking, they form the basis of an electrical resonance which endows the cells with frequency selectivity (Lewis and Hudspeth, 1983; Art and Fettiplace, 1987; Hudspeth and Lewis, 1988*b*; Fuchs, Nagai, and Evans 1988; Fuchs and Evans, 1988). The main conclusion from the present results is that the speed of the macroscopic current, which is the major determinant of the resonant frequency (Art and Fettiplace, 1987), may be largely dictated by the channel kinetics. Both the magnitude (Fig. 8 *A*) and the voltage dependence (Fig. 9) of the time constant of the single-channel relaxation at −50 mV are in quantitative agreement with previous measurements on the macroscopic K(Ca) current.

Since the relaxation time constant for the single channels depends on Ca concentration (Fig. 7), the similar range for the time constants of the single channels in 4 μM Ca and of the whole-cell K(Ca) current may be fortuitous. The previous characterization of the whole-cell K(Ca) conductance (Art and Fettiplace, 1987) indicated that at the resting potential of −50 mV, where the whole-cell kinetics were measured, ~20% of the maximal conductance was activated (mean = 0.18 ± 0.08, N = 10; same cells as used for analysis shown in Fig. 10 *B*). This value may be used to determine the Ca concentration that produces an equivalent probability of opening of single BK channels at −50 mV. From Hill plots at −50 mV for seven channels, this Ca concentration was calculated as 6.1 ± 1.6 μM, a value sufficiently close to 4 μM to permit the comparison between the data in Fig. 8 *A* and earlier measurements of the kinetics of the whole-cell K(Ca) current.

The fast kinetics of the voltage-sensitive Ca current in turtle hair cells (Art and Fettiplace, 1987) can be used to calculate that at the end of a depolarizing voltage-clamp step returning to −50 mV, the Ca current will deactivate with a time constant of ~0.2 ms. Although the time course of the consequent drop in intracellular Ca is unknown, it could in theory diffuse away from a nearby K(Ca) channel with a time constant not much greater than that of the Ca current. Under such conditions, the deactivation of the whole-cell current may be largely determined by the closing rate of the BK channels. This line of argument finds some support from the similarity in the ranges of kinetics for the whole-cell current and single-channel relaxation.

Comparison with Other Hair Cell Preparations

Ca-activated K channels have been recorded in a number of other hair cell preparations. In the frog saccule, BK channels obtained in inside-out patches possessed multiple open and closed times indicative of complex gating, but had a somewhat lower Ca and voltage dependence (Hudspeth and Lewis, 1988a) than those reported here. It was subsequently estimated from whole-cell perfusion with Ca buffers that in order to produce the requisite activation of the whole-cell K(Ca) conductance, the steady state Ca concentration at the K channel needed to be 100 μM at −53 mV and 1 mM at −42 mV (Roberts et al., 1990; Roberts, 1993). The whole-cell K(Ca) conductance in frog hair cells, as in turtle, was maximally activated at ∼ −30 mV. Employing an essentially similar approach, but comparing the activation of the whole-cell K(Ca) conductance with that of single BK channels, we estimate the Ca concentration to be ∼6 μM at −50 mV and 50 μM at −35 mV. The Ca sensitivity of the BK channels in turtle hair cells is thus ∼15- to 20-fold greater than for those in the frog. It might be argued that the Ca sensitivity of channels in our detached patches is much higher than under whole-cell conditions, but this seems unlikely in view of the previous observation that the whole-cell K(Ca) current in turtle cells can still be activated in 10 μM external Ca (Art et al., 1993).

An extensive study of Ca-activated K channels has also been made in goldfish saccular hair cells (Sugihara, 1994). BK channels is these cells possess multiple open and closed times and a range of both unitary conductances (130–320 pS) and Ca sensitivities. The Ca concentration required to produce a half-maximal probability of opening at 0 mV lay between 50 μM and 1 mM. Thus, these BK channels may be more than an order of magnitude less sensitive than those reported here for the turtle. The results on goldfish and frog saccular hair cells taken together suggests a real difference in the BK channels between saccular and cochlear hair cells.

Both BK and SK channels with properties similar to those described for the turtle have also been reported in guinea pig outer hair cells (Ashmore and Meech, 1986). The BK channel of conductance 233 pS was activated by micromolar Ca concentrations at +53 mV, and the SK channel had a smaller unit conductance (44 pS) and a higher Ca-sensitivity than the BK channel at negative membrane potentials. There is no evidence at the present time that the BK channels are employed to electrically tune mammalian hair cells. The SK channel, however, may in all hair cells play a common role of mediating the efferent cholinergic inhibitory post synaptic potentials (Art, Fettiplace and Fuchs, 1984). In cochlear hair cells of guinea pig (Housely and Ashmore, 1991), chick (Shigemoto and Ohmori, 1990; Fuchs and Murrow, 1992) and turtle (Goodman and Art, 1992), acetylcholine is thought to act by causing an elevation in intracellular Ca so activating a K channel; in chick, this K channel is distinct from the BK channel in having a smaller conductance and different ionic selectivity.

The SK channels may also contribute to the whole-cell current in turtle hair cells tuned to lower frequencies since it was found that the current in such cells showed a diphasic dependence on extracellular Ca: most of the current, attributed to the BK channels, disappeared in 1 μM external Ca, but the remainder was only abolished in 0.1 μM Ca (Art et al., 1993). This residual component was originally explained on the

basis of a higher Ca-sensitivity of slow BK channels, but in light of the present results it is more likely due to a separate SK channel.

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