Ca^{2+}-activated Cl^- Current from Human Bestrophin-4 in Excised Membrane Patches

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Bestrophins are a newly discovered family of Cl^- channels, some members of which are activated by intracellular Ca^{2+}. So far, all studies were carried out with whole-cell recordings from plasmid-transfected cultured cells, so it is unclear whether Ca^{2+} activates bestrophin through a metabolic mechanism or in a more direct way. We report here experiments that addressed this question with excised, inside-out membrane patches. We chose human bestrophin-4 (hBest4) for heterologous expression because it gave particularly large Cl^- currents when expressed, thus allowing detection even in excised membrane patches. hBest4 gave a negligible Cl^- current in a Ca^{2+}-free solution on the cytoplasmic (bath) side, but produced a Cl^- current that was activated by Ca^{2+} in a dose-dependent manner, with a $K_{1/2}$ of 230 nM. Thus, Ca^{2+} appears to activate the bestrophin Cl^- channel without going through a freely diffusible messenger or through protein phosphorylation. Because the activation and deactivation kinetics were very slow, however, we cannot exclude the involvement of a membrane-associated messenger.

The Journal of General Physiology

INTRODUCTION

Bestrophin is the protein product of VMD2, a gene that when defective causes juvenile-onset, autosomal-dominant, vitelliform macular dystrophy (VMD/Best disease) in the retina (Petrukhin et al., 1998; Marquardt et al., 1998). The human genome codes for four members of the bestrophin family, with no obvious homology to any other human protein (Sun et al., 2002; Tsunenari et al., 2003). A number of recent studies using heterologous expressions have shown that bestrophins form Cl^- channels (Sun et al., 2002; Tsunenari et al., 2003; Qu et al., 2003, 2004; Qu and Hartzell, 2004; Fischmeister and Hartzell, 2005). Disease-associated point mutations in bestrophin have also been found to result in severely inhibited Cl^- currents when heterologously expressed (Sun et al., 2002; Qu et al., 2003).

Bestrophin has been localized to the basolateral membrane of the retinal pigment epithelial (RPE) cells (Marmorstein et al., 2000). These cells mediate the transfer of water, ions, and metabolites between the photoreceptors and the heavily vascularized choroid behind the RPE. Because Cl^- flux is generally associated with the transepithelial transport of substances, it is reasonable to think that bestrophin, with its purported location, has an important role in such a function of RPE cells. In the electrooculogram, which is a mass voltage signal rather similar to the common electroretinogram but recorded on a longer time scale, there is a component (the "light peak") that reaches peak in 6–9 min after light onset (Francois et al., 1967; Deutman, 1969; Gallemore et al., 1998b). In patients with Best disease, this light peak is substantially reduced even before the onset of disease symptoms, indicating that the light peak reflects a physiological entity the defect of which is intrinsically associated with (and possibly causes) the disease rather than a consequence of degenerative processes in the course of the disease (Francois et al., 1967; Deutman, 1969; Gallemore et al., 1998b). Moreover, electrophysiological evidence suggests that the light peak reflects an increase in the basolateral Cl^- conductance of RPE cells (Steinberg et al., 1985; Gallemore et al., 1998a). The correlative information from these studies thus corroborates the conclusion from heterologous expression that bestrophin is a Cl^- channel.

A putative agent controlling the Cl^- conductance associated with the electrooculogram light peak is intracellular Ca^{2+} (Steinberg et al., 1985; Gallemore et al., 1998a). Indeed, the Cl^- channel formed by heterologously expressed human bestrophin is opened by a rise in intracellular Ca^{2+} concentration (Sun et al., 2002). *Xenopus* and mouse bestrophin homologues were found to be Ca^{2+} sensitive in a similar way (Qu et al., 2003, 2004). However, all studies so far were performed with the whole-cell, patch-clamp recording method, so the question remains whether the effect of Ca^{2+} on bestrophin goes through an enzymatic reaction such as protein phosphorylation, or is more direct. At the same time, it is difficult to study with the whole-cell recording

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Abbreviations used in this paper: hBest, human bestrophin; mBest, mouse bestrophin; xBest, *Xenopus* bestrophin; RPE, retinal pigment epithelial.

TABLE I

Compositions of Bath Solutions

	Ca^{2+} solutions						
	NMDG-Cl	Methanesulfonate	$CaCl_2$	EDTA	EGTA	HEDTA	NTA
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Near-zero Ca^{2+}	135			10			
20 nM Ca^{2+}	128.5		3.24	10			
40 nM Ca^{2+}	125.2		4.9	10			
100 nM Ca^{2+}	127.6		3.7		10		
300 nM Ca^{2+}	122.2		6.4		10		
1 μM Ca^{2+}	132.4		1.3			10	
10 μM Ca^{2+}	128.8		3.1			10	
100 μM Ca^{2+}	131.4		1.82				5
Low Cl^-	20	111.4	1.82				5

All solutions contained 20 mM HEPES, and pH was adjusted to 7.2 with NMDG. HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; NTA, nitrilotriacetic acid.

method the kinetics of activation and deactivation of the channel in response to Ca^{2+} changes, and to examine more than one intracellular Ca^{2+} concentration in a single experiment. Accordingly, we have turned to excised, inside-out patch recording. We have chosen human bestrophin-4 (hBest4) for these experiments because, among the gene products of the four human bestrophin genes, hBest4 shows by far the highest whole-cell Cl^- current in transfected cell lines (Tsunenari et al., 2003). A large Cl^- current is important for obtaining any measurable current from excised membrane patches. Our experiments indicate that Ca^{2+} is able to activate hBest4 in a cell-free environment, and provide a clearer dose dependence of the opening of the bestrophin channel on Ca^{2+}.

MATERIALS AND METHODS

Electrophysiology

CHO-K1 cells or HEK293 cells were cotransfected with the hBest4 and EGFP expression plasmids (both in the pRK5 vector) at a 4:1 or 10:1 ratio, by using 3–6 μl FuGENE 6 (Roche Applied Science) at 1–2.5 μg of hBest4 plasmid DNA per 35-mm dish (containing four or five 12-mm circular coverslips plated with cells). The EGFP plasmid (0.1–0.5 μg) alone was also used as a mock-transfection control. Within 3 d after transfection, inside-out patches of plasma membrane were excised from transfected cells identified by EGFP fluorescence and recorded at room temperature (23–25°C). The data were acquired with a DIGIDATA1200 and pClamp6 software (Molecular Devices Corporation). Solutions containing different buffered Ca^{2+} concentrations were applied with a rapid solution changer (RSC-200, Bio-Logic Science Instruments). Liquid junction potentials were <5 mV and have not been compensated. With respect to nomenclature, hBest4 is the human bestrophin family member with the first 12 residues being MTVSYTLKVAEA (NCBI accession no. AAR99657; also see Fig.1 of Tsunenari et al., 2003).

Solutions

Standard bath solution contained (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 Na-HEPES, pH 7.4. The patch-pipette solution contained (in mM) 150 NMDG-Cl, 1 EGTA, and 20 NMDG-HEPES, pH 7.4. The compositions of the various bath solutions are summarized in Table I; in addition, they all contained 20 mM NMDG-HEPES, pH 7.2. The free Ca^{2+} concentration was calculated with the WINMAXC2.5 software (Patton et al., 2004).

RESULTS

When expressed in CHO-K1 cells, hBest4 gave whole-cell currents that often exceeded 20 nA (versus, for example, hBest1, which typically gave currents no more than 1 nA). In the steady presence of 100 nM free Ca^{2+} on the cytoplasmic side, the Cl^- current (measured with $NMDG^+$ as the cation on both sides of the membrane; see Materials and Methods) recorded from a membrane patch with a voltage step resembled in time course the whole-cell current of hBest4 previously reported (cf. Fig.2 of Tsunenari et al., 2003); a −120-mV pulse induced a rapid inward current that decreased by ∼30% over several hundred milliseconds, and a +80-mV pulse induced an outward current that slowly increased over the same duration (Fig. 1 A, bottom, and collected results in Fig. 1 C; 9 cells). These current relaxations probably reflected a mild voltage dependence of the Ca^{2+}-activated Cl^- current. The same patch in the absence of Ca^{2+} showed negligible current (Fig. 1 A, top, and collected results in Fig. 1 C). Experiments on cells mock transfected with the EGFP plasmid alone gave negligible currents in the absence or presence of cytoplasmic Ca^{2+} (Fig. 1, B and C; 7 cells).

For dose–response experiments, we used HEK293 cells for expression because these gave substantially larger Cl^- currents with hBest4 than CHO-K1 cells. In Fig. 2 A, top, which shows one such experiment, there was no detectable current at negative or positive voltages in the absence of Ca^{2+} on the cytoplasmic side (left trace, 10 mM EDTA and no added Ca^{2+}; see also Fig. 2, B and D). Adding 300 nM Ca^{2+} activated a current that gradually increased over ∼1 min (Fig. 2 A, middle trace in top panel, and Fig. 2 B). Increasing the

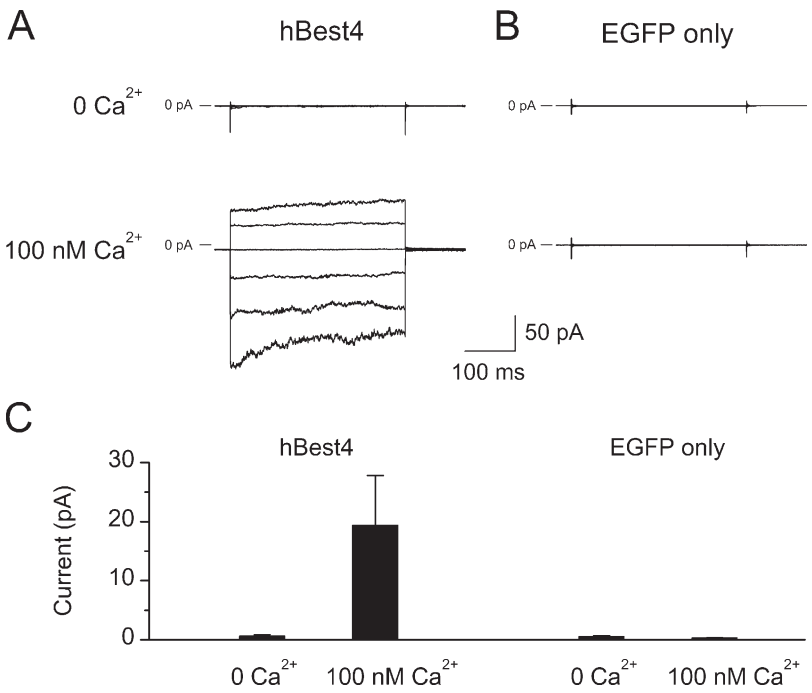

Figure 1. Cl^- current of hBest4 in inside-out membrane patches excised from transfected CHO-K1 cells. (A) Cell cotransfected with hBest4 plasmid and EGFP plasmid. (B) Cell transfected with EGFP plasmid alone (negative control). In each case, the same patch was alternatively exposed to near-zero Ca^{2+} and 100-nM Ca^{2+} in the bath. The current traces were produced by 350-ms voltage steps from a holding potential of 0 mV to voltages between −120 and +80 mV in 40-mV increments. Both of the patch pipette solution and the bath solution contained $NMDG^+$ and Cl^- as major ions. (C) Collected results of experiments shown in A and B, measured at the end of the 350-ms voltage to +80 mV (mean ± SEM). Left, nine patches; right; seven patches.

Ca^{2+} concentration to 100 μM activated more current, again with a slow time course. This slowness was not due to a slow solution exchange, which was near completion within 1 s (Fig. 2 C and legend). The disappearance of the current upon removing Ca^{2+} had a similarly slow time course (Fig. 2 D). In 24 patches, the mean current at +80 mV was 1.2 ± 0.6 (mean ± SD) pA at near-zero Ca^{2+} and 168 ± 136 pA at 100 μM Ca^{2+}. Control experiments with mock-transfected HEK293 cells using the EGFP plasmid alone gave a mean current at +80 mV of 0.6 ± 0.5 pA (6 patches) at near-zero Ca^{2+} and 2.0 ± 1.9 pA (11 patches) at 100 μM Ca^{2+} (see Fig. 2 A, bottom, for example). Thus, any endogenous Ca^{2+}-activated Cl^- current in HEK293 cells should have little effect on the measurements of the bestrophin Cl^- current.

The collective dose dependence of the Cl^- current on Ca^{2+} concentration from multiple experiments is shown in Fig. 3. For each patch, three different Ca^{2+} concentrations were used in the following order: near-zero, an intermediate concentration, and 100 μM. The currents were normalized against that at 100 μM Ca^{2+}, which was saturated. We were unable to test more than one intermediate Ca^{2+} concentration for a given patch because of the slow onset and offset of the current (see Fig. 2, B and D), as well as the general instability of the patches. A high Ca^{2+} concentration such as 100 μM also produced a desensitization-like phenomenon, in that a subsequent exposure to the intermediate concentration of Ca^{2+} elicited a smaller current than before. No apparent difference in the relation between positive and negative voltages was observed. In both cases, the relation can be described by the Hill equation, with a $K_{1/2}$ of 230 nM Ca^{2+} and a Hill coefficient, n, of 0.53. The small Hill coefficient means that the activation of the Cl^- current spans over a 1,000-fold change in free Ca^{2+} concentration; significant activation occurred at 20-nM Ca^{2+}, but the activation still did not reach maximum at 10 μM Ca^{2+}.

As mentioned earlier, the time course of current onset in response to Ca^{2+} application was slow (Fig. 2 B). This time course showed no obvious dependence on voltage or Ca^{2+} concentration (Fig. 4). At +80 mV, for example, the current onset could be described by a single-exponential function with a time constant of 21.5 ± 3.5 s at 20 nM Ca^{2+} (mean ± SD, 3 patches), versus 16.3 ± 8.7 s (4 patches) at 10 μM Ca^{2+}, i.e., a less than twofold change in time course for a 500-fold change in Ca^{2+} concentration. The deactivation of the current when switching the Ca^{2+} concentration from 100 μM to near zero was comparably slow (Fig. 2 D), with a single-exponential time constant of 18.0 ± 7.9 s at +80 mV (n = 17) (and 21.5 ± 7.2 s at −120 mV).

DISCUSSION

Based on the extensive evidence reported so far, it appears that bestrophins are bona fide chloride channels (Sun et al., 2002; Tsunenari et al., 2003; Qu et al., 2003, 2004; Qu and Hartzell, 2004; Fischmeister and Hartzell, 2005). Recently, hBest1 has also been reported to influence the kinetics and voltage dependence of endogenous L-type Ca^{2+} channels when hBest1 was transiently expressed in an RPE cell line (Rosenthal et al., 2006), though what this means remains unclear.

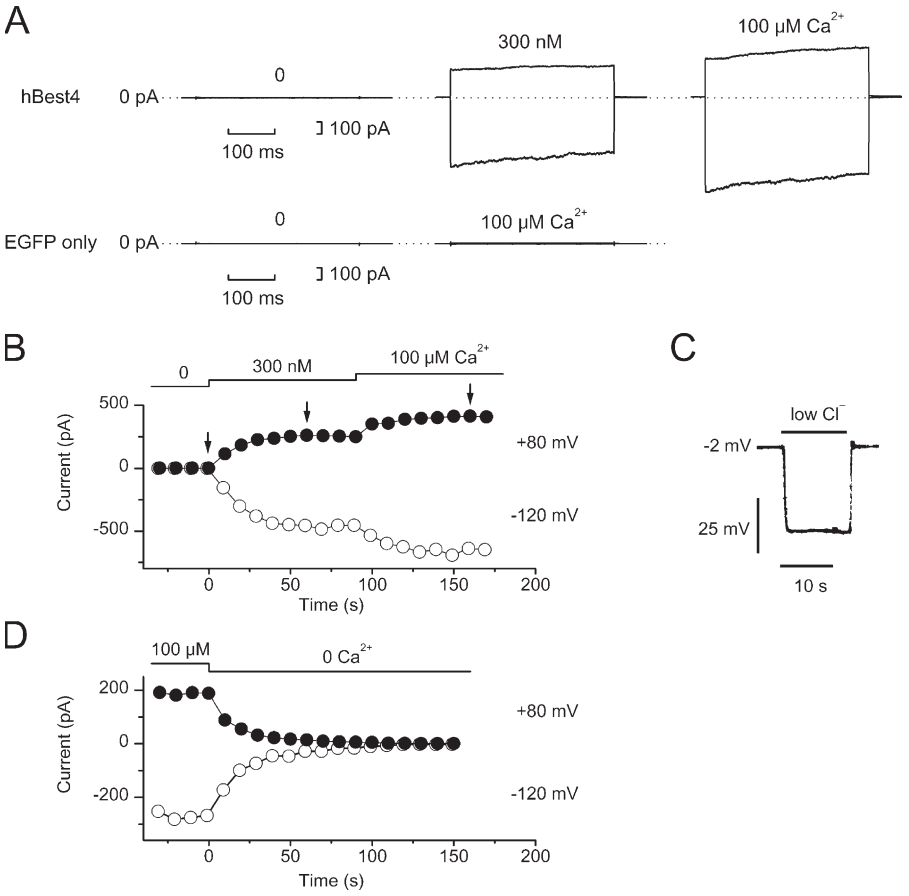

Figure 2. Ca^{2+} dependence of hBest4 Cl^- current. (A) Top, currents recorded from an excised patch of hBest4-transfected HEK293 cell at free Ca^{2+} concentrations of near-zero, 300 nM, and 100 µM. In each panel, the membrane voltage was stepped from 0 to −120 and +80 mV for 350 ms. Bottom, control experiment on an excised patch from a HEK293 cell mock-transfected with EGFP plasmid alone. (B) Complete recordings of the same patch as shown in the top of A to indicate the time course of activation of hBest4 current by Ca^{2+}. Each point was derived from a set of measurements as shown in the top of A, showing the current amplitude at the end of the 350-ms pulse to −120 mV (open symbol) and +80 mV (closed symbol). The arrows indicate the time points at which the measurements in the top of A were obtained. The current activation time course at 300 nM Ca^{2+} in B could be described by a single exponential with time constant of 19 s at −120 mV and 10 s at +80 mV. (C) Solution-exchange time course measured with the same patch shown in A and B. The solution exchange was relatively fast. Membrane voltage was recorded in zero-current clamp mode. A high-Cl^- solution (135 mM Cl^-, E_{Cl} = −2.7 mV) containing 100 µM Ca^{2+} was replaced by a low-Cl^- solution (23.6 mM Cl^-, E_{Cl} = −47 mV) containing the same free [Ca^{2+}]. No compensation for liquid-junction potential has been made in the trace. After compensation, the initial voltage would correspond to −1.5 mV, and reach −45 mV within 1–2 s after the onset of the solution exchange. (D) Same kind of experiment as in B, but from a different patch and showing the decline time course of the current.

In this paper, we have demonstrated that hBest4 Cl^- channels on excised membrane patches can be activated by free Ca^{2+} on the cytoplasmic side. These experiments allowed us to obtain a fairly well-defined dose dependence of channel activation on free Ca^{2+} concentration. The $K_{1/2}$ value for the activation was 230 nM Ca^{2+}. Previously, by comparing whole-cell currents elicited with different intrapipette Ca^{2+} concentrations across bestrophin-transfected HEK293 cells, others have estimated the $K_{1/2}$ to be 210 nM for xBest2a and 228 nM for xBest 2b (*Xenopus*), and 230 nM for mBest2 (mouse) (Qu et al., 2003, 2004). These $K_{1/2}$ values are remarkably close to our estimate here, but this agreement should be taken with caution because other bestrophin channels were used in these other studies, and the experimental approach was different. Generally speaking, the dose–response relations derived from whole-cell recordings (Qu et al., 2003, 2004) are somewhat indirect, because they involved comparisons across cells and also assumed perfect Ca^{2+} buffering by the pipette solution dialyzed into the cells. Qu et al. (2003, 2004) used EGTA to buffer Ca^{2+} up to the several-micromolar range, at the upper end of which EGTA might not be very efficient (Patton et al., 2004). One difference between our findings and those of Qu et al. (2003, 2004) is that the Hill coefficient we measured is less than unity, versus 5–7, as can be estimated from the data of Qu et al. (2003, 2004). A Hill coefficient of <1 is rather unusual, typically indicating negative cooperativity. On the other hand, a Hill coefficient of 5–7 seems unusually high as well. Ideally, the excised-patch experiments described here should be repeated with the other bestrophin family members (including those used by Qu et al.), although low levels of functional expression might make this difficult.

We also tested the effect of divalent cations other than Ca^{2+}, and found that Sr^{2+} was about as effective as Ca^{2+}, but Ba^{2+} was only very weakly effective, and Mg^{2+} was practically ineffective (unpublished data). This selectivity conforms with the property of other Ca^{2+}-activated Cl^- channels (e.g., Reisert et al., 2003).

Another unusual feature with hBest4 is that the current activates and deactivates very slowly in response to changes in Ca^{2+} concentration, with a time constant as long as 10–20 s. Previous studies on Ca^{2+}-activated Cl^- currents in excised patches of native membrane of *Xenopus* oocytes and other cell types gave activation

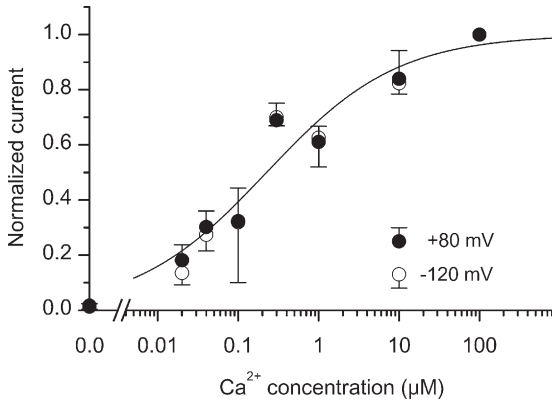

Figure 3. Dependence of hBest4 Ca^{2+}-activated Cl^- current on free Ca^{2+} concentration. Same procedure as in Fig. 2 B, including the order of solution application: near-zero Ca^{2+} followed by an intermediate Ca^{2+} concentration, and then by 100 μM Ca^{2+}. The plotted currents have been normalized with respect to the current at 100 μM Ca^{2+}. Filled circles represent mean values (±SD) at +80 mV, and open circles represent mean values at −120 mV (3–5 patches each). The smooth curve is the Hill equation with $K_{1/2}$ = 230 nM and a Hill coefficient of 0.53.

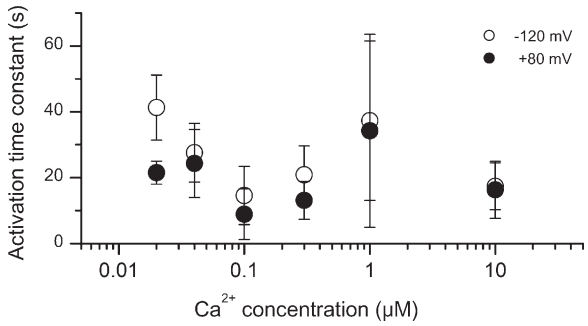

Figure 4. Time constant of current activation at different Ca^{2+} concentrations. Same patches as in Fig. 3. The values are mean ± SD.

and deactivation kinetics that are orders of magnitude faster (e.g., Gomez-Hernandez et al., 1997; Reisert et al., 2003). Because the activation time course of hBest4 shows hardly any dependence on Ca^{2+} concentration, and the deactivation kinetics are comparably slow, the underlying rate-limiting step does not appear to be dominated by Ca^{2+} binding/unbinding. One possibility is that the opening/closing transitions of hBest4 after Ca^{2+} binding are very slow. However, the voltage-dependent relaxations of the current were only in the range of hundreds of milliseconds (see Fig. 1; also Fig. 2 in Tsunenari et al., 2003), suggesting that the opening/closing transitions were not rate limiting. The slow kinetics may reflect an effect of Ca^{2+} on the channel that is indirect. We can probably rule out the involvement of a kinase as an intermediate because no ATP was required for the channel activation in our experiments. Nonetheless, the action of Ca^{2+} may still be indirect and act through, for example, a membrane-associated messenger such as lipid or a membrane-associated regulatory protein. Finally, an intermediate scenario is, in principle, also possible; namely, Ca^{2+} binds directly to bestrophin, but this binding indirectly controls the opening/closing of the channel through another entity. A complex activation mechanism may also explain the sublinear dose–response relation. Interestingly, among the various bestrophins that we have studied, heterologously expressed hBest1, hBest2, and dmBest1 (*Drosophila*) showed no obvious voltage-dependent relaxations of the current, whereas hBest3 and ceBest1 (*Caenorhabditis elegans*), like hBest4, both showed very slow (in the range of a second or hundreds of milliseconds) voltage-dependent current relaxations (Sun et al., 2002; Tsunenari et al., 2003). Thus, there is heterogeneity in channel kinetics among the bestrophin homologues. Whether this implies heterogeneity in the gating mechanism for bestrophins, as apparently is the case for the TRP channel superfamily (Clapham, 2003; Nilius et al., 2005), is unclear.

If Ca^{2+} indeed binds directly to bestrophin, where is the binding site? In the case of the large-conductance Ca^{2+}-activated K^+ channel (BK channel), a high-affinity Ca^{2+}-binding site (the Ca^{2+} bowl) has been identified between the S9 and S10 domains, corresponding to the sequence T**E**LVN**D**TNVQFL**DQDDDDD**P**D**TE**LYLTQ (residues 883–910, with the negatively charged glutamates and aspartates in bold face) near the cytoplasmic COOH terminus of mSlo1 (Schreiber and Salkoff, 1997; Schreiber et al., 1999). This sequence has 10 negative charges, 5 of which are consecutive. Interestingly, all members of the human bestrophin family also have five consecutive negative charges, together with three nearby negative charges, in the cytoplasmic COOH terminus (for hBest4: $_{306}$A**E**QIINPFG**EDDDDD**F**E**TNQLI**D**-RNLQV$_{332}$; for hBest1–3: residues 291–317, which are highly homologous to hBest4). We have noticed that *Xenopus* and mouse bestrophins also have a similar sequence: residues 306–332 for *Xenopus* bestrophin 2 (Qu et al., 2003) and residues 291–317 for mouse bestrophin 2 (Krämer et al., 2004; Qu and Hartzell, 2004). Five of the disease-associated human mutations in hBest1 previously studied are in fact situated in this region (Q293K, G299E, E300D, D301E, and T307I; see Sun et al., 2002). All of these mutants produced substantially reduced whole-cell currents in transfected HEK293 cells, suggesting that this region is crucial for channel function. Whether this region indeed has a role in the Ca^{2+} sensitivity of bestrophin Cl^- channels remains to be determined.

We would like to thank members of the Yau laboratory for discussions on the experiments.

This work was supported by NIH grants EY06837 (K.-W. Yau) and EY14367 (J. Nathans) and an award from the Ruth and Milton Steinbach Fund (K.-W. Yau).

Olaf S. Andersen served as editor.

Submitted: 27 February 2006
Accepted: 25 April 2006

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