Rescue of Volume-regulated Anion Current by Bestrophin Mutants with Altered Charge Selectivity

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Mutations in human bestrophin-1 are responsible for several retinopathies, including Best vitelliform macular dystrophy (Marquardt et al., 1998; Petrukhin et al., 1998), adult-onset macular dystrophy (Seddon et al., 2001), autosomal-dominant vitreochoidopathy (Yardley et al., 2004), autosomal-recessive bestrophinopathy (Burgess et al., 2008), and canine multifocal retinopathy (Guziewicz et al., 2007). There is considerable evidence that bestrophins are Cl\(^{-}\)-activated channels (for review see Hartzell et al., 2008). Expression of a variety of different bestrophins in HEK293 cells induces novel Cl\(^{-}\) currents (Sun et al., 2002; Qu et al., 2003, 2004, 2006a, 2006b; Tsunenari et al., 2003, 2006; Qu and Hartzell, 2004; Barro Soria et al., 2006; Chien et al., 2006). Furthermore, mutation of certain amino acids alters the permeability and conductance of the channel (Qu et al., 2004, 2006b; Qu and Hartzell, 2004), and sulfhydryl reagents alter the properties of cysteine-substituted channels in characteristic ways (Tsunenari et al., 2003; Qu and Hartzell, 2004; Qu et al., 2006b). However, the idea that bestrophin-1 is a Cl\(^{-}\) channel has been seriously questioned (Marmorstein et al., 2004a, 2004b, 2006; Rosenthal et al., 2005; Marmorstein and Kinnick, 2007). This challenge is based largely on several observations, the most compelling of which is that Cl\(^{-}\) currents in retinal pigment epithelial cells are not abolished in mouse bestrophin-1 (mBest1) knockout mice (Marmorstein et al., 2006). Furthermore, it is not clear that the Cl\(^{-}\) channel function of bestrophin can explain the human disease phenotypes (Hartzell et al., 2008). These questions, coupled with the observation that human bestrophin-1 (hBest1) can regulate voltage-gated Ca\(^{2+}\) channels (Rosenthal et al., 2005; Yu et al., 2008), has led to the suggestion that hBest1 is not a Cl\(^{-}\) channel but is rather a channel regulator (Marmorstein et al., 2006; Hartzell et al., 2008).

We have previously concluded that Drosophila bestrophin-1 (dBest1) is a Cl\(^{-}\) channel that is dually regulated by Ca\(^{2+}\) and cell volume and mediates regulatory volume decrease (RVD) in Drosophila S2 cells (Chien and Hartzell, 2007). We showed that five different dBest1 RNAi constructs abolished endogenous Cl\(^{-}\) currents activated by intracellular Ca\(^{2+}\) or by cell swelling. The loss of these currents could be rescued by overexpression of wild-type dBest1. However, the rescue experiment alone does not formally prove that dBest1 is the pore-forming...
subunit of the S2 volume-regulated anion channel (VRAC) because overexpression of an essential regulator or accessory subunit of the channel could have the same effect. Here, we address this issue directly by rescuing VRAC in dBest1 RNAi-treated S2 cells with dBest1 mutants that have altered biophysical properties. Residue F81 (F80 in vertebrates; Drosophila has an extra amino acid at position 14) was chosen for this purpose because F81 is invariant among all bestrophins and has been implicated as an important residue in ionic selectivity (Qu et al., 2006b).

Here, we have rescued the VRAC current in S2 cells abolished by dBest1 RNAi by expressing dBest1-F81C. The rescued current is cell volume sensitive but differs from the wild-type current in the shape of the I-V curve, the responsiveness to methanethiosulfonate (MTS) reagents, and anion–cation selectivity. This finding provides strong evidence that dBest1 is indeed the channel pore and is the volume-sensitive Cl− channel in S2 cells. However, VRACs are normal in peritoneal macrophages from mice with both bestrophin-1 and bestrophin-2 disrupted, suggesting that bestrophins are not the classical mammalian VRAC.

**MATERIALS AND METHODS**

**Rescue in S2 Cells and Heterologous Expression**

Drosophila S2 cells were cultured in Schneider’s Drosophila medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen) at room temperature. The open reading frame of dBest1 was introduced into pAc5.1/V5-HisA Drosophila expression vector (Invitrogen) as described previously (Chien et al., 2006; Chien and Hartzell, 2007). Residue F81 was mutated to cysteine by a PCR-based site-directed mutagenesis method (Quickchange: Stratagene). The F81C mutation was introduced by PCR primers (up: 5'-CATACCCCTGTCCTGCGTGCTTGGTTTC -3'; down: 5'-GAAACCAAGACGGAGACAAGGTTGATG -3'), pAc5.1-dBest1 ORF (open reading frame) was used as the template for high fidelity PCR amplification with Pfu DNA polymerase. The methylated PCR template was digested with the endonuclease Dpn-I, and the nonmethylated PCR product was transformed into XL-1 blue Escherichia coli for amplification. DNA was sequenced to confirm the mutation. For the rescue experiment, 2 · 10^6 S2 cells were treated with 8.5 μg of double-stranded RNA against the 5' untranslated region (UTR) of dBest1 (5U dB1) for 4 d (Chien and Hartzell, 2007). The RNAi-treated cells were then transfected with a mixture of pAc5.1-dBest1 or dBest1-F81C cDNA and pAc5.1-EGFP in a 2:1 ratio using calcium phosphate. Green cells were recorded 2–4 d after transfection. Possible off-target effects of 5U dB1 RNAi have been described in detail previously (Chien et al., 2006; Chien and Hartzell, 2007). The RNAi-treated cells were then transfected with a mix-

**Solutions**

The standard extracellular solution (E300) used for patch clamping S2 cells contained 115 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM KCl, 10 mM HEPES (pH 7.2 with NaOH), and 48 mannitol to achieve 300 mosmol kg\(^{-1}\). The intracellular solution (I300) contained 110 mM CaCl2, 10 mM EGTA, 8 mM MgCl2, 10 mM HEPES, pH 7.2, and 45 mannitol to achieve 300 mosmol kg\(^{-1}\). I300 internal solution was prepared by adding 40 mM mannitol to the I300 solution to achieve 340 mosmol kg\(^{-1}\). Osmotic pressure differences are expressed as ∆ osm mol kg\(^{-1}\) (intracellular osmolality minus extracellular osmolality). Unless indicated otherwise, the osmotically sensitive Cl− current was routinely activated by 340 mosmol kg\(^{-1}\) osmotic pressure (E300/I340). These combinations of solutions set the Erev for Cl− currents to 0 mV, whereas cation currents carried by Cs+ or Na+ would have very negative or positive Erev, respectively. The major cation in the intracellular solution was Cs+, whereas the standard extracellular cation was Na+ unless specified otherwise. Stock solutions of 100 mM 2-trimethylammonioethylmethanethiosulfonate, bromide salt (MTSET+ and sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES−) (Toronto Research Chemicals) were prepared in water and stored at −80°C. Aliquots of the MTS stock solution were thawed and kept on ice for no longer than 10 min. 1 mM of working solution was prepared by diluting the stock with the external recording solution immediately before use. Working solutions of dithiothreitol (DTT; Sigma-Aldrich) were prepared freshly from frozen 1-M stock solutions.

To determine relative cation/Cl− permeabilities, we used a high Ca2+ intracellular solution containing 150 mM CaCl2 (or NaCl), 10 mM HEPES (pH 7.2 with NMDG), and 5 mM Ca-EGTA-NMDG, and extracellular solutions containing different CsCl (or NaCl) (150, 100, 50, 20, or 10 mM), 10 mM HEPES, and 1 mM CaCl2. All solutions were pH 7.2 and adjusted to 304 mosmol kg\(^{-1}\) with mannitol.

**Electrophysiology, Cell Volume Determination, and Data Analysis**

S2 cells were allowed to adhere to the bottom of the recording chamber for ~10 min and were then washed and incubated with extracellular solution for ~10 min before whole cell recording. Fire-polished pipettes pulled from borosilicate glass (Sutter Instrument Co.) had resistances of 2–3 MΩ when filled with intracellular solution. For whole cell recording, cells were voltage clamped with a 1-dBest1 or dBest1-F81C cDNA and pEGFP (Invitrogen) were co-

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meanings. All data were analyzed using pClamp 8.2 software and Origin 7.0 and are expressed as mean ± SEM. Two sample t tests (independent tests) were performed with significance levels of 0.05 or 0.01 for statistical analysis.

Phase contrast images of the cells were taken with a Photometrics Cool-Snap camera and analyzed with MetaMorph Imaging software (MDS Analytical Technologies). The volume of the cell was calculated from the measured circumference assuming that the shape of S2 cells is spherical. Some duplicate measurements were performed blind. With an osmotic pressure difference of 40 mosmol kg$^{-1}$, one would expect a 13% increase in cell volume if the cell were a perfect osmometer. However, we observed a larger change in cell volume than expected. This might be explained by the fact that cell swelling is not spherically symmetrical, as assumed. If cell swelling is constrained in the z-plane by mechanical pressure from the patch pipet, the change in cell volume may appear to be larger than it actually is. For this reason, the estimates of cell volume may be subject to some quantitative error. One would also expect that the cell volume would remain unchanged in isosmotic condition. However, we observed an ~13% reduction instead. This could possibly be caused by dilution of cytoplasmic osmolytes into the recording pipet.

Measurement of VRAC in Peritoneal Macrophages

Peritoneal macrophages were isolated by peritoneal lavage. 5 ml of cold RPMI medium containing 10% FBS was injected intraperitoneally into a mouse that had just been killed by an overdose of isoflurane anesthesia. The abdomen was massaged for several minutes, and the fluid was withdrawn and plated onto glass coverslips and cultured at 37°C in a 5% CO$$_2$$, 95% air environment. Round macrophages were patch clamped 2 h to 2 d after isolation at room temperature. The intracellular solution was 95 mM Cs-aspartate, 40 mM CsCl, 1 mM MgCl2, 10 mM HEPES pH 7.4, 4 mM Na/K-ATP, and 5 mM EGTA, and CaCl2 was added to give ~50 mM free Ca$^{2+}$. The hypsomotic extracellular solution was 105 mM NaCl, 6 mM CsCl, 1 mM MgCl2, 1.5 mM CaCl2, 10 mM HEPES, pH 7.4, and 10 mM glucose (234 mosmol kg$^{-1}$). Isosmotic and hypsomotic solutions were made by adding mannitol to the hypsomotic solution to make 266-, 306-, and 326-mosmol kg$^{-1}$ solutions. Recording pipets had 3–6 MΩ of resistances when filled with intracellular 6 mM CsCl, 1 mM MgCl2, 1.5 mM CaCl2, 10 mM HEPES, pH 7.4, and (Merck & Co.) with mBest2 knockout mice (Bakall et al., 2008). Knockout mice were made by breeding mBest1 knockout mice (Chien and Hartzell, 2007). To verify that the dBest1 RNAi was effective in knocking down the current, RNAi-treated cells were patch clamped in each experiment (Fig. 1 B). Then, dBest1 (wild-type, F81C, F81E, or F81L) was expressed by transient transfection. Both the native and rescued VRAC current was <300 pA under isosmotic conditions (Fig. 1 A) but was activated when the extracellular solution was 40 mosmol kg$^{-1}$ hypsomotic relative to the internal solution (I340/E300) (Fig. 1 B). As expected, the dBest1-F81C current had different properties than the native VRAC current. The native VRAC current had an outwardly rectifying, S-shaped I-V curve, whereas the F81C current was inwardly rectifying (Figs. 1 B and 3 D). This inwardly rectifying I-V of F81C was similar to the Ca$^{2+}$-activated dBest1-F81C current expressed heterologously in HEK cells (Chien et al., 2006). Transfection with the F811L mutant produced no current (67.0 ±26.3 pA; n = 5). In contrast, F81C currents increased with time coordinately with cell swelling over several minutes. Immediately after patch break, the F81C currents were 0.4 ± 0.1 nA at +100 mV. The currents then activated slowly to a mean plateau amplitude of 1.0 ± 0.5 nA (n = 7) with an average half-time of ~1.5 min (Fig. 1 C). The activation of the F81C current was coupled to cell swelling. On average, F81C cells swelled 33.6 ± 0.7% (n = 7) when their currents were fully activated with Δ40 mosmol kg$^{-1}$ osmotic pressure (Fig. 1 D). F81C currents were volume sensitive because the current failed to activate under isosmotic conditions (Δ0 mosmol kg$^{-1}$, E300/I300) and remained 0.3 nA (n = 6) throughout ~5 min of recording. Cell swelling was not observed with these cells in isosmotic solutions. Instead, their volume decreased 13.8 ± 2.7% by the end of the recording.

Opposite Effects of MTSET$^+$ on dBest1-F81C and Native dBest1

Additional evidence that dBest1 forms the VRAC pore was provided by the finding that MTSET$^+$ had opposite effects on the native VRAC current and the rescued dBest1-F81C current. I-V curves and the time course of current development are shown in Fig. 2. MTSET$^+$ caused a mean ~35% reduction in the amplitude of the native dBest1 VRAC currents (Fig. 3, A and B) over 8–10 min. The effect of MTSET$^+$ on native cells was not reversible by 5 mM DTT. In contrast, MTSET$^+$ caused a dramatic augmentation in the F81C current. On average, the current was transiently increased 15-fold, followed by a gradual decline to a level that was still elevated approximately four- to sevenfold compared with the F81C currents before MTSET$^+$ (Fig. 3, A and B). The stimulation by MTSET$^+$ was at least partly reversible by DTT. In addition to stimulating the current, MTSET$^+$ converted the F81C current from slightly inwardly rectifying to slightly outwardly rectifying and shifted  $$E_{\text{rev}}$$  1.9 ± 0.7 mV in the positive direction (Fig. 3 C). Although this shift was small, it was in the opposite direction to the shift produced by MTSES$^-$ (see below).

MTSES$^-$ Increases the Cation Permeability of dBest1-F81C

The effect of MTSES$^-$ on the I-V relationships of native and F81C-rescued cells is shown in Fig. 4 (A and B).
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Figure 1. Rescue of the volume-regulated Cl⁻ current in S2 cells by expression of dBest1-F81C. Endogenous dBest1 in S2 cells was knocked down by RNAi that targeted the 5' UTR of dBest1 for 4 d. Cells were then transfected with dBest1-F81C and EGFP expression constructs and patch clamped in whole cell configuration with 10-s interval voltage ramps from −100 mV to +100 mV from a holding potential of 0 mV. Currents recorded from native S2 cells, dBest1 RNAi-treated S2 cells, and F81C overexpressed in dBest1 RNAi-treated S2 cells are labeled as native, RNAi, and F81C, respectively. (A) Current–voltage relationship of dBest1 currents in native and F81C-rescued cells recorded with 0 mosmol kg⁻¹ isosmotic solutions. (B) Current–voltage relationship of dBest1 currents in native and F81C-rescued cells stimulated with 40 mosmol kg⁻¹ hyposmotic solutions. (C) Time course of activation of VRAC in S2 cells rescued with F81C. The currents shown were measured at −100 mV (open symbols) and +100 mV (solid symbols) under isosmotic (I300/E300, triangles; n = 6) and hyposmotic (I340/E300, circles; n = 7) conditions. (D) Mean current amplitudes at 100 mV at the onset of whole cell recording (initial current, open bars) and after the currents had reached a peak (final currents, filled bars) and the corresponding cell volume alterations (hatched bars) with hyposmotic (Δ40 mosmol kg⁻¹) and isosmotic solutions. Changes in cell volume are expressed as percent change in cell volume from the initiation of whole cell recording to ∼3–5 min after patch break (isosmotic; n = 6) or when the currents had approached a steady value (hyposmotic; n = 7). Data are represented in mean ± SEM. *, significantly different at P < 0.05 and ** at P < 0.01 level.

F81C currents activated by hyposmotic solutions were reduced ∼60% by MTSES⁺, compared with an ∼20% reduction for native dBest1 (Fig. 3, A and B). More importantly, MTSES⁺ consistently produced an E_rev shift of −19.9 ± 1.2 mV (n = 12) in F81C-rescued cells but not in native cells (Fig. 3 C). This negative shift in E_rev could be explained by a changed ionic selectivity of the channel. In these experiments, [Cl⁻] was the same on both sides.

Figure 2. dBest1-F81C-rescued cells respond differently to MTSET⁺ modification than native S2 cells. Whole cell VRAC currents were established in hyposmotic solutions (I340/E300, 40 mosmol kg⁻¹) and were recorded with voltage ramps from −100 to 100 mV. 1 mM MTSET⁺ was applied to the bath solution (E300) after the volume-sensitive current was fully activated. The bath was then replaced with 5 mM DTT to test if the effect of MTSET⁺ was reversible. (A and B) Current–voltage relationships in native (A) and dF81C-rescued (B) S2 cells before and after MTSET⁺ modification. (C and D) Time course data of the effect of MTSET⁺ modification on native (C) and F81C-rescued (D) VRAC currents. These time course data were collected from the same cells shown in A and B.
shifted to $E_{\text{rev}} = -25.1$ mV. When extracellular Na$^+$ was replaced with Cs$^+$, $E_{\text{rev}}$ changed to 1.7 mV. Replacement of extracellular Cs$^+$ with the impermeant NMDG$^+$ produced an $E_{\text{rev}}$ shift to $E_{\text{rev}} = -38$ mV. These observations suggested that the F81C current had become more permeable to Cs$^+$ and, less so, to Na$^+$ after MTSES$^-$ modification.

To quantify relative cation/Cl$^-$ permeability, we performed dilution potential experiments (Franciolini and Nonner, 1987) for native dBest1, F81C, and F81E currents. For these experiments, we chose to activate the current by high intracellular Ca$^{2+}$ because the dBest1 current activated by cell swelling often ran down after the current was fully activated, making several $E_{\text{rev}}$ determinations in the same cell problematic. In contrast, the

Figure 4. MTSES$^-$ increases the cation permeability of F81C currents. Current–voltage relationships from a typical native S2 cell (A) and a S2 cell rescued with F81C (B) before and after MTSES$^-$ modification. Currents were activated with $\Delta 40$ mosmol kg$^{-1}$ hyposmotic solutions. The arrow in B points out the reversal potential of F81C current after MTSES$^-$ treatment. (C) Changes in $E_{\text{rev}}$ of F81C currents under different ionic conditions. The record begins after the dBest1 current had stabilized under hyposmotic solutions (I340/E300, $\Delta 40$ mosmol kg$^{-1}$). MTSES$^-$ was then applied in the bath. Extracellular solution containing symmetrical Cl$^-$ and Na$^+$ as the major cation (E300) was then replaced by solutions with Cs$^+$ or NMDG$^+$ as the major cations as indicated. The osmolality of all the extracellular solutions was 300 mosmol kg$^{-1}$.
Ca\textsuperscript{2+}-activated dBest1 current does not run down significantly for up to 10 min. The nature of this rundown and why it is only seen when dBest1 current is activated by hypotonic cell swelling but not by Ca\textsuperscript{2+} are not clear. Nevertheless, we chose to activate dBest1 current by intracellular application of Ca\textsuperscript{2+} to obtain data to avoid the possible interference of the rundown. Cells were treated with MTSES\textsuperscript{−}, and $E_{\text{rev}}$ was measured with 150 mM CsCl inside and various concentrations of CsCl outside. I-V curves from typical cells recorded with 10, 50, and 150 mM external CsCl are superimposed in Fig. 5 (A–C). For native dBest1 currents (Fig. 5 A), $E_{\text{rev}}$ moved toward positive values as external [CsCl] was decreased, as would be predicted if dBest1 is selectively permeable to Cl\textsuperscript{−}. In addition, the conductance in the outward direction at +100 mV increased significantly with increasing external [CsCl], consistent with Cl\textsuperscript{−} carrying the majority of the outward current. In contrast, the $E_{\text{rev}}$ of F81C shifted toward negative potentials with decreasing external [CsCl] (Fig. 5 B). This negative shift in $E_{\text{rev}}$ showed that F81C had become more selective to Cs\textsuperscript{+} than to Cl\textsuperscript{−} after MTSES\textsuperscript{−} modification. The augmented inward rectification with increasing external [CsCl] was also consistent with a higher Cs\textsuperscript{+} conductance relative to Cl\textsuperscript{−}.

Relative Cs\textsuperscript{+}/Cl\textsuperscript{−} permeability was calculated by fitting the plots of $E_{\text{rev}}$ versus [CsCl]o to the GHK equation. As shown in Fig. 5 D, the mean $E_{\text{rev}}$ in native S2 cells shifted on average 33.3 ± 3.2 mV with a 15-fold decrease in [CsCl]o (●). The data were well-fitted by the GHK equation, assuming that Cl\textsuperscript{−} was fourfold more permeable than Cs\textsuperscript{+} ($P_{\text{Cs}}/P_{\text{Cl}} = 0.25$). dBest1-F81C, on the other hand, showed a shift in the opposite direction of −22.8 ± 2.3 mV with a 15-fold decrease in [CsCl]o (Fig. 5 D, ▲), which was fitted to $P_{\text{Cs}}/P_{\text{Cl}} = 3.83$. The $P_{\text{Cs}}/P_{\text{Cl}}$ ratio in rescued cells overexpressing wild-type dBest1 was the same as the native S2 current regardless of MTSES\textsuperscript{−} treatment ($P_{\text{Cs}}/P_{\text{Cl}} = 0.24$; not depicted), indicating that the switch in ionic selectivity in F81C currents was not due to an up-regulation of an endogenous cation channel.

The ionic selectivity of dBest1 was explored further by replacing the F81 residue with the negatively charged amino acid, glutamic acid (E). We discovered that the F81E current reversed at −9.4 ± 1.2 mV under conditions of symmetrical Cl\textsuperscript{−} and Cs\textsuperscript{+} inside and Na\textsuperscript{+} outside (Fig. 3 C). This is significantly different from the native current (0.2 ± 0.2 mV). $P_{\text{Cs}}/P_{\text{Cl}}$ for F81E was calculated to be 1.33 from dilution potential experiments (Fig. 5, C and D). Furthermore, the F81E current inwardly rectified (Fig. 3 D). The fact that F81E qualitatively recapitulated most of the properties of MTSES\textsuperscript{−}-modified F81C currents provided additional support that residue F81 is in vicinity to the ion selectivity filter of the dBest1 Cl\textsuperscript{−} channel.

The dilution potential experiment was repeated with NaCl replacing CsCl. The native dBest1 channel was highly selective to Cl\textsuperscript{−} relative to Na\textsuperscript{+} and was fitted with a $P_{\text{Na}}/P_{\text{Cl}}$ ratio of 0.03 (Fig. 5 D). MTSES\textsuperscript{−}-modified F81C, on the other hand, was almost equally permeable to Cl\textsuperscript{−} and Na\textsuperscript{+} ($P_{\text{Na}}/P_{\text{Cl}} = 0.83$) (Fig. 5 D).

![Figure 5](image-url)

**Figure 5.** Quantification of relative cation/chloride permeability. Current-voltage relationships of MTSES\textsuperscript{−}-modified native (A) and F81C (B) currents and unmodified F81E currents (C) in response to different external [CsCl]. Whole cell currents were activated under isosmotic condition (304 mosmol kg\textsuperscript{−1}) with high Ca\textsuperscript{2+} in the pipet and symmetrical CsCl in the bath and pipet (150 mM). The extracellular solution was replaced by solutions containing different [CsCl] as indicated. (D) Changes in $E_{\text{rev}}$ ($\Delta E_{\text{rev}}$) as a function of extracellular salt concentration. $\Delta E_{\text{rev}}$ is $E_{\text{rev}}$ at the indicated salt concentration minus the $E_{\text{rev}}$ with 150 mM extracellular salt. Salt is either CsCl or NaCl as indicated. Each data point represents the mean $E_{\text{rev}}$ ± SEM of two to nine cells. Dashed lines were calculated from the GHK equation ($\Delta E_{\text{rev}} = 25.7 \cdot \ln \left[\left(\left[\text{X}\right]_{\text{in}} + \left[\text{Cl}\right]_{\text{o}} \cdot P_{\text{Cl}} / P_{\text{X}}\right) / \left(\left[\text{Cl}\right]_{\text{in}} + \left[\text{Cl}\right]_{\text{o}} \cdot P_{\text{Cl}} / P_{\text{X}}\right)\right]\right]$, assuming that the channel is exclusively permeable to Cl\textsuperscript{−} ($P_{\text{X}} / P_{\text{Cl}} = 0$) or to the cation X\textsuperscript{+} ($P_{\text{X}} / P_{\text{Cl}} = 0$). Filled symbols: CsCl solutions; ●, MTSES\textsuperscript{−}-treated native dBest1 (n = 2–7); ▲, MTSES\textsuperscript{−}-modified F81C (n = 4–9); ■, F81E (n = 2–5). Open symbols: NaCl solutions; ○, MTSES\textsuperscript{−}-treated native dBest1 (n = 3–5); Δ, F81C (n = 3–8).
DISCUSSION

We have previously shown that the dBest1 current plays an essential role in RVD in S2 cells because RNAi knockdown of dBest1 largely eliminates RVD (Chien and Hartzell, 2007). Our previous data did not clearly establish that dBest1 formed the channel, however, because RNAi knockdown of an essential regulator or β-subunit of the channel would have the same effect as knocking down the channel itself. To establish that dBest1 is the channel itself, it was necessary to rescue the current with a channel that had a biophysical signature that was clearly different than the wild-type channel. Our results here provide solid support that dBest1 is indeed the VRAC in Drosophila S2 cells because we were able to rescue the volume-regulated current by overexpressing a mutant dBest1 with altered biophysical properties. The rescued current is clearly sensitive to cell volume; in hypoxic solution, the current increases with time in parallel with cell swelling. The F81C mutant current has several hallmark features. (1) F81C exhibits altered rectification. The rectification ratio was 0.8 for F81C compared with 1.2 for native currents. (2) MTSES-modification of the F81C channel altered the ionic selectivity of the channel so that the channel became more permeable to cations than to Cl⁻. (3) F81C current amplitude was stimulated by MTSET, whereas the wild-type current was decreased slightly. These three distinguishing features show clearly that the rescued current is mediated by dBest1-F81C. It is difficult to imagine how dBest1 could produce these changes if it were merely a regulatory subunit of the channel. These results with F81C are strengthened by the observation that the F81E mutant also has an increased permeability to cations compared with the wild-type current.

The volume sensitivity of the rescued current (both wild-type and F81C) is slightly different than the native VRAC current. Typically, the native VRAC current immediately after breaking the patch to initiate whole cell recording is 0.1 nA or less at +100 mV, whereas the F81C mutant the current is 0.4 nA. Because the osmotic pressure difference develops only after the patch is broken, the observation that the initial F81C current is larger than native current suggests that the F81C current is partially activated before patch break. Furthermore, the rescued currents seem to activate more quickly than the native current. We believe that these differences are an artifact of overexpression; the channel may be partially uncoupled from its regulatory mechanisms and exhibit a different “set-point.” This is supported by the observation that the apparent uncoupling is related to the level of overexpression. With high levels of overexpression of wild-type dBest1, currents are observed even under isosmotic conditions.

Despite this rather compelling evidence that dBest1 is the VRAC in S2 cells, it seems unlikely that bestrophins constitute the VRAC family in mammals because VRAC...
is unaffected in macrophages from mice with both mBest1 and mBest2 knocked out. A less extensive examination of VRAC in microglia also revealed no obvious difference between wild-type and knockout (not depicted). Mice have three functional bestrophin genes, but it seems unlikely that VRAC in microglia and macrophages can be explained by the one bestrophin we have not knocked out. hBest1 and mBest2 have both been shown to exhibit sensitivity to cell volume (Fischmeister and Hartzell, 2005), but if bestrophins are VRACs, one would expect that they would be expressed ubiquitously. But, the data on bestrophin expression suggests that they have rather restricted tissue expression. Publicly available microarray and electronic Northern data (http://www.genecards.org) are limited, and published reports of bestrophin expression by RT-PCR, Northern, and immunoblotting or immunostaining are not always in agreement (Hartzell et al., 2008). At this point in time, the most conservative interpretation is that bestrophins may be one of many different kinds of channels that play a role in cell volume regulation (Nilius et al., 1996; Strange et al., 1996; Lang et al., 1998) or, possibly, that Cl⁻ fluxes associated with cell volume changes are not mediated by specific ion channels, but rather by Cl⁻-interacting proteins that have other primary functions.

The ability to change the anionic selectivity of dBest1 from anionic to cationic by MTSES⁻/H₁₁₀₀₂⁻ modification of a point mutant is surprising and may shed light on the mechanisms involved in ion permeation through bestrophin channels. In S₂ cells, the MTSES⁻/H₁₁₀₀₂⁻-modified F₈₁⁻C current exhibits a P₅₃₉/P₅₃⁺ ratio of 0.83 and a P₅₃⁻/P₅₃⁺ ratio of 2.58. This suggests that the permeability sequence is P₅₃⁻ > P₅₃⁺ > P₅₉⁺. This is supported by the observation that E₉₉₉ shifted to more negative potentials when extracellular Cs⁺ is replaced with the impermeant NMDG⁺ than with Na⁺. Other examples of ion channels where the charge selectivity has been reversed include the nicotinic ACh receptor (Galzi et al., 1992), the GABAₐ receptor (Wang et al., 1999), and the glycine receptor (Keramidas et al., 2000, 2002). Keramidas et al. (2000, 2002)
have converted the glycine receptor from \( \text{Cl}^- \) selective to cation selective by introducing a negative charge in the M2 pore-forming segment by changing A215 to glutamate. The cationic permeability is enhanced by deleting a proline at position 250, which alters the structure and diameter of the pore (Keramidas et al., 2002). The double mutant of the glycine receptor exhibits a permeability sequence of \( P_{\text{Cl}} > P_{\text{Na}} > P_{\text{K}} \) (1.7: 1.0: 0.13). The changes that we observe with the F81C mutant of dBest1 are similar to the single A215G glycine receptor mutant, but less dramatic than those in the glycine receptor double mutant. Perhaps mutation of additional amino acids in dBest1 may produce larger effects. It is significant that changing F81 to glutamate has a smaller effect than modifying the F81C mutant with MTSES\(^{-} \). This may be explained by the fact that MTSES\(^{-} \) is a rather large molecule (1.2 nm × 0.6 nm; Kaplan et al., 2000) compared with the dimensions of glutamate or cysteine (side chain length of <0.5 nm). Thus, the negative charge provided by MTSES\(^{-} \) is likely to be positioned differently than the negative charge provided by glutamate. The reversal in charge selectivity produced by MTSES\(^{-} \), therefore, may involve other regions of dBest1 in addition to transmembrane domain 2, where F81 is located. It is also possible that the incorporation of the MTSES\(^{-} \) moiety disrupts the structure of the pore and changes its diameter or other properties. However, the effect of MTSES\(^{-} \) on ionic selectivity of F81C expressed in HEK cells was less than when F81C was expressed in S2 cells. These data suggest that the bestrophin channel might involve other proteins to form the pore.

The second transmembrane domain of hBest1 is a hotspot for disease-causing mutations (Hartzell et al., 2008). One of the disease-causing mutations is F80L. Although we have not tested this mutation in hBest1, the F81L mutation in dBest1 is nonfunctional. This emphasizes the importance of the second transmembrane domain in bestrophin function.

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