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## Functional properties of murine Bestrophin 1 channel

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### Abstract

Bestrophins form  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels when they are expressed heterologously. Here we report the functional characterization of murine bestrophin 1 (mBest1). We isolated mBest1 transcript from mouse heart and analyzed the biophysical properties and expression of this channel protein using a tetracycline inducible system. mBest1 expression is localized at the membrane of transfected HEK cells, in agreement with its role as a channel. Whole-cell patch clamp experiments revealed a calcium sensitive, time independent chloride current. mBest1 current displayed slight voltage dependence, exhibited an anion permeability sequence of  $\text{SCN}^- > \text{I}^- > \text{Cl}^-$  and was sensitive to DIDS- and niflumic acid. Anion replacement studies were also performed on mBest2 and mBest3 and differences were observed in their relative permeability and slope conductance to  $\text{SCN}^-$ . Our study provides the first characterization of the biophysical properties of mBest1 and a framework for the elucidation of the physiological role of bestrophins.

### Keywords

Bestrophin; calcium-activated chloride channel

### Introduction

$\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels ( $\text{Cl}_{\text{Ca}}$ ) are expressed in a variety of tissue types where they have important functions [1]. A complete understanding of these channels is limited by the fact that their molecular identity remains unresolved. Bestrophins are one family of proteins that have been described by a number of independent groups to be  $\text{Cl}^-$  channels which are activated by cytosolic  $\text{Ca}^{2+}$  [2]. In each case  $\text{Ca}^{2+}$ -sensitive  $\text{Cl}^-$  currents ( $\text{I}_{\text{Cl}_{\text{Ca}}}$ ) were recorded using the patch clamp technique [3–10] and their current voltage ( $I$ - $V$ ) relationships displayed either ohmic, inwardly, or outwardly rectifying behavior [2]. Bestrophin  $\text{Cl}^-$  channel function was corroborated with the identification of putative pore domain residues, which when mutated can alter the permeation and selectivity of  $\text{Cl}^-$  [5;6;10–12].

In mouse, three bestrophin homologues (mBest1–3) have been identified in various tissue types using RT-PCR [12;13]. Two independent groups have characterized the biophysical properties of mBest2 in detail [3;5;6;11]. We and others have reported whole-cell patch clamp analysis

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for mBest3 [7;8;12]. To date there are no detailed reports on the electrophysiological properties of mBest1. hBest1, the ortholog of mBest1 sharing 62% amino acid identity, is the prototypic member of the bestrophin family [9;10;14;15]. Mutations in this gene produce Best's vitelliform macular dystrophy, an early onset form of macular degeneration [9]. hBest1 elicits  $I_{ClCa}$  when expressed heterologously [9;10], however there are few comprehensive electrophysiological studies on hBest1 in terms of anion selectivity and pharmacology. Using a gene-silencing approach it was demonstrated that Best1 contributes to  $I_{ClCa}$  in epithelial tissues [15;16]. More recently, mBest1 knockout (*vmd2<sup>-/-</sup>*) mice have been generated [14; 17]. One group suggested that mBest1, together with mBest2, plays a role in  $Ca^{2+}$ -dependent  $Cl^-$  secretion in mouse airways [14]. However, Marmorstein et al. reported that a  $I_{ClCa}$  was still present in retinal pigmented epithelial cells from *vmd2<sup>-/-</sup>* mice [17]. This suggests that either mBest1 plays no role in generating  $I_{ClCa}$  or that other bestrophins could be compensating for the absence of mBest1. Indeed, this group proposed that bestrophin 1 functions as a regulator of L-type  $Ca^{2+}$  channels [17;18]. Since the electrophysiological properties of mBest1 in isolation are unknown, its contribution to  $I_{ClCa}$  could not be investigated further.

In this study, we used a tetracycline regulated expression system in combination with the patch clamp technique to provide a comprehensive report of the biophysical characteristics of recombinant mBest1. We demonstrate that mBest1 is expressed at the membrane of transfected cells, consistent with its role as a transmembrane chloride channel. This study provides evidence that mBest1 encodes a  $Cl_{Ca}$  and it is the first examination of the biophysical and pharmacological properties of this channel. A preliminary report of these results has been presented [19].

## Materials and Methods

### Cloning of mBest1 from heart

Adult BALB/c mice were sedated by exposure to isoflurane prior to cervical dislocation and excision of the heart. This protocol was approved by the University of Nevada Institutional Animal Care and Use Committee. Total RNA was isolated using TRIzol (Invitrogen) and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen). A 1658 bp fragment containing the coding sequence for mBest1 was amplified using *AmpliTaq Gold*<sup>®</sup> (Applied Biosystems) and mBest1 specific primers. For expression studies, the stop codon was removed and mBest1 transcript was ligated into pcDNA4/TO/*c-myc*-HIS vector (Invitrogen) in frame with the C-terminal *c-myc* epitope tag. Recombinant plasmids were sequenced at the Nevada Genomics Center.

### Tetracycline regulated expression of mBest1 in mammalian cells

Expression of mBest1 was carried out using a tetracycline regulated expression system (T-Rex<sup>™</sup>, Invitrogen) as described previously for mBest3 [12]. A stable line expressing mBest1-*c-myc* was generated in TRex-293 cells (express tetracycline repressor protein). Transfected and untransfected TRex-293 cells were seeded onto coverslips 24–48 h before recordings or immunocytochemistry. mBest1 channel expression was induced by tetracycline (1 µg/ml) addition to the media. In some instances mBest1, mBest2 or mBest3 were transiently transfected into HEK TsA201 cells for patch clamp analysis. mBest3 in pcDNA4/TO/*c-myc*-HIS vector was generated as described [12]. mBest2 in pCMV-Sport6 (IMAGE clone ID 4989959) was obtained from Invitrogen. Each 35 mm culture dish was transfected with 2 µg plasmid DNA using Polyfect transfection reagent (Qiagen).

### Analysis of mBest1-*c-myc* protein expression

Protein isolated from transfected and untransfected TRex-293 cells was examined by Western blotting in a manner similar to that described previously [12]. mBest1-*c-myc* reactivity was

detected with a *c-myc* mouse monoclonal antibody (1:200 dilution; AbCam) followed by incubation with an alkaline phosphatase conjugated goat anti mouse antibody (1:7500 dilution; Promega). For immunofluorescence labeling, transfected and untransfected TRex-293 cells expressing mBest1-*c-myc* fusion protein were fixed in 4 % paraformaldehyde for 20 min and labeled with *c-myc* monoclonal antibody (1:2000 dilution) followed by secondary labeling with Alexa 488 conjugated chicken anti-mouse antibody (Molecular Probes), as described [12]. Propidium iodide was used as a nuclear stain. Confocal composites were constructed from Z-scans acquired using a Bio-Rad Radiance 2100 Laser scanning confocal microscope with LaserSharp 2000 software (Bio-Rad). Final images were prepared using Adobe Photoshop software.

## Electrophysiological methods and recording solutions

Macroscopic currents were recorded at room temperature in the whole-cell configuration. The bath and pipette solutions were chosen to facilitate the recording of  $\text{Cl}^-$  currents in isolation as described [12]. In all experiments,  $\text{Cl}^-$  currents were studied with  $[\text{Ca}^{2+}]_i$  clamped at known concentrations. Pipette solutions contained 10 mM BAPTA, and free  $[\text{Ca}^{2+}]$  was set to either <1, 250 or 500 nM by the addition of 0.84–8.7 mM  $\text{CaCl}_2$  as determined by the calcium chelator program EQCAL (Biosoft). For recording steady-state *I-V* relationships, cells were held at –50 mV and stepped from –100 to +100 mV in 10 mV increments for 1 s. The ionic nature of the charge carrier was determined from the reversal potential ( $E_{\text{rev}}$ ) values elicited by a ramp protocol. Cells were stepped from –50mV to +60 mV and then stepped to voltages between –100 mV and +100 mV (10 mV increments) followed by a repolarizing step to –60 mV. For external anion replacement experiments, 126 mM  $\text{Cl}^-$  was replaced by an equimolar concentration of thiocyanate ( $\text{SCN}^-$ ), iodide ( $\text{I}^-$ ) or D-gluconate $^-$ . Changes in junction potential were minimized using a 3 M KCl agar-bridge. The relative permeability was determined by measuring the shift in the  $E_{\text{rev}}$  upon changing the bath solution from one containing  $\text{Cl}^-$  to another monovalent anion, where X is the substitute anion. The permeability ratios were estimated using the Goldman-Hodgkin-Katz (GHK) equation as described [12]. All chemicals were obtained from Sigma-Aldrich. DIDS and niflumic acid were prepared in DMSO. Data are reported as the mean of *n* cells  $\pm$  s.e.m.

## Data analysis

$E_{\text{rev}}$  and pharmacological percentage block were determined by curve fitting of individual current traces using *Clampfit* (*PClamp*, version 9.2; Molecular Devices). All data were exported to *Origin 7.5* (OriginLab) or Graphpad *PRISM 3.0* for plotting and curve fitting. *PRISM 3.0* was used to determine statistical significance between groups with one-way ANOVA followed by Dunnett's Multiple Comparison test.  $p < 0.05$  was considered statistically significant.

## Results and Discussion

### mBest1 is a membrane protein and induces $\text{I}_{\text{ClCa}}$ when expressed heterologously

mBest1 cDNA cloned from heart was expressed in a tetracycline inducible expression system to characterize the channel properties. The mBest1 transcript we cloned corresponds to nucleotides 208-1869 of mBest1, Genbank accession # **NM 011913**, and is 100% identical. The mBest1 transcript was inserted into the pcDNA/TO/*c-myc*-His vector, in frame with a C-terminal *c-myc* epitope tag. This vector contains a tetracycline operon (TO) for tetracycline inducible expression. TRex-293 cells which express tetracycline repressor protein were transfected with mBest1-*c-myc* and a stable line was generated. This system enables rapid and efficient tetracycline inducible expression of ion channels in mammalian cell lines [20], as channel expression is under the control of a competition between the constitutively expressed tetracycline repressor protein and exogenously added tetracycline. Figure 1 shows how we

adapted this system to investigate mBest1 expression by Western blotting, immunocytochemistry and whole cell patch clamp. For these experiments, mBest1 expression was induced by tetracycline addition to the cell media, and experiments were performed 3–6 hours later. mBest1/*c-myc* protein expression was examined by Western blotting using a *c-myc* antibody (Figure 1A). An immunoreactive signal (~64 kDa) is observed in the TRex-293 cells stably expressing mBest1/*c-myc* and induced with tetracycline. The predicted size of mBest1 protein is 63.8 kDa. For immunocytochemistry, mBest1-*c-myc* expressing cells were probed with a *c-myc* antibody. Figure 1B is a composite confocal image of a single TRex-293 cell expressing mBest1/*c-myc* protein. There is predominant membrane staining consistent with mBest1 being a transmembrane protein. The intracellular staining of mBest1 is likely to be the location of the over-expressed protein on intracellular membranous organelles. Control experiments included both untransfected cells and mBest1 transfected cells without tetracycline addition. In the absence of tetracycline, expression was not induced (data not shown).

To confirm the role of mBest1 as a  $Cl_{Ca}$  we used the whole-cell patch clamp technique. Under conditions designed to minimize the activity of endogenous  $K^+$  currents,  $Cl^-$  currents were studied with free  $[Ca^{2+}]_i$  at fixed concentrations in the pipette solution, a technique used previously [12]. To investigate the requirement of intracellular  $Ca^{2+}$  for the activation of mBest1 currents, cells were treated with tetracycline and dialyzed with pipette solutions containing 10 mM BAPTA and either no added  $Ca^{2+}$  (free  $[Ca^{2+}]_i < 1$  nM), 250 nM  $Ca^{2+}$  or 500 nM  $Ca^{2+}$ . When  $[Ca^{2+}]_i$  was  $< 1$  nM the currents recorded were small (Figure 1C). Increasing  $[Ca^{2+}]_i$  to 250 nM (Figure 1D) or 500 nM (Figure 1E) led to increasingly larger mBest1 currents. Figure 1F shows the mean *I-V* relationships for currents evoked with  $< 1$  nM  $Ca^{2+}$  ( $n = 8$ ), 250 nM  $Ca^{2+}$  ( $n = 7$ ) and 500 nM  $Ca^{2+}$  ( $n = 18$ ) from cells transfected with mBest1 and from untransfected cells with 500 nM  $Ca^{2+}$  ( $n = 8$ ).

The mBest1 currents generated with both 250 and 500 nM  $Ca^{2+}$  displayed an almost linear *I-V* relationship and reversed close to zero as expected for a  $Cl^-$  selective channel (Figure 1F). mBest1  $Cl^-$  currents recorded with 500 nM  $Ca^{2+}$  were largely time- and voltage-independent (Figure 1E), but exhibited small voltage dependence at the extremes of the voltage range (Figure 1F). These biophysical properties are similar to those of mBest2 and mBest3 shown previously in our studies, and in those of other groups [3; 6; 7; 12]. Control experiments were carried out in untransfected cells and in cells transfected with mBest1 but not treated with tetracycline. Neither of these control experiments displayed significant  $I_{ClCa}$  (Figure 1F and 1G respectively). The amplitude of the currents evoked by 500 nM  $Ca^{2+}$  in transfected and tetracycline treated cells was significantly larger at all potentials ( $p < 0.001$ ) than currents evoked by the same pipette solution in these control cells. Figure 1G shows a summary of the mean current densities measured at  $-80$  and  $+80$  mV. This data shows that mBest1 currents elicited with 500 nM  $Ca^{2+}$  display slight outward rectification with current densities at  $-80$  and  $+80$  mV of  $-29.5 \pm 4.6$  pA/pF and  $37.9 \pm 5.9$  pA/pF, respectively ( $n = 18$ ;  $p < 0.001$ ).

There is heterogeneity in channel kinetics among the bestrophin homologues studied to date. Heterologously expressed hBest1, hBest2, dBest1 [9;10], xBest2a and 2b [4], mBest2 [3;6] and mBest3 [7;8;12] display little if any voltage-dependent activation/inactivation. However, expressed ceBest1 [9], hBest3 [10] and hBest4 [10;21] currents display voltage-dependent kinetics. Bestrophin channels are opened by an increase in  $[Ca^{2+}]_i$ . This was demonstrated by the whole-cell patch clamp technique [3–7;9–12;21] and also using excised, inside-out patch recordings [21]. These studies reported a physiological  $K_D$  value for  $Ca^{2+}$  in the nanomolar range, similar to the  $Ca^{2+}$  sensitivity that we observe for mBest1. A recent study has defined specific regions in the C-terminus of human Best1 that play an important role in the  $Ca^{2+}$  regulation of this channel [22]. These regions are highly conserved among the bestrophin family members including mBest1.

## Anion selectivity of mBest1

The measured reversal potential of the mBest1 currents ( $E_{rev} = -1.3 \pm 0.49$ ,  $n = 8$ ) agrees with the calculated Nernst equilibrium potential for  $Cl^-$  with 126 mM  $Cl^-$  on both sides of the membrane. In our experiments the major extracellular cation was  $Na^+$  and the major intracellular cation was  $Cs^+$ . Therefore, if the mBest1 channel was permeable to these cations, the  $E_{rev}$  would be significantly different from zero. To establish that mBest1 currents are indeed carried by chloride, ion replacement studies were performed. In these experiments, mBest1 currents were recorded with 500 nM  $Ca^{2+}$  in the pipette solution. Figure 2A is a representative trace showing the effect of replacing extracellular  $Cl^-$  with an equimolar concentration of  $SCN^-$  on mBest1 currents. The  $E_{rev}$  shifted to the left suggesting that the mBest1 channel is more permeable to  $SCN^-$  than  $Cl^-$ . This was also the effect of replacing  $Cl^-$  with  $I^-$ . Replacing the extracellular  $Cl^-$  with D-gluconate $^-$  shifted the reversal potential to more positive values, suggesting a substantial  $Cl^-$  permeability. The relative permeability ( $P_x/P_{Cl}$ ) of external ions (X) with respect to  $Cl^-$  was estimated by the shift in the  $E_{rev}$  of the current under bionic conditions and calculated using the GHK equation. Figure 2B shows the relative permeability for  $Cl^-$ ,  $SCN^-$  ( $n = 7$ ),  $I^-$  ( $n = 3$ ) and D-Gluconate $^-$  ( $n = 4$ ). The relative permeability ratio of  $SCN^-:I^-:Cl^-:Gluconate^- = 1.6:1.4:1:0.4$ . The anion selectivity sequence for mBest1,  $SCN^- > I^- > Cl^-$ , is similar to other bestrophins [2] and to native  $Ca^{2+}$ -activated  $Cl^-$  channels [1]. However, the relative permeability ratio of  $SCN^-$  relative to  $Cl^-$  for mBest1 is quite different to what we and others have reported for mBest2 and mBest3 [5–7; 12]. Therefore, we examined and compared the effect of replacing extracellular  $Cl^-$  with  $SCN^-$  on currents recorded from either mBest1 (Figure 3A), mBest2 (Figure 3B) or mBest3 (Figure 3C) expressed transiently in HEK-TsA201 cells in the presence of 500 nM  $Ca^{2+}$ . For each mBest current the  $E_{rev}$  shifted to the left suggesting that in all cases  $SCN^-$  was more permeable than  $Cl^-$ . However, we found the relative permeability ( $P_{SCN}/P_{Cl}$ ) for mBest1 to be significantly less than that for mBest2 and mBest3, in that  $P_{SCN}/P_{Cl}$  of mBest1, 2 and 3 was  $1.6 \pm 0.06$ ,  $3.47 \pm 0.09$  and  $3.51 \pm 0.24$  respectively ( $n = 3-8$ ,  $p < 0.001$ ). This data is summarized in Figure 3D. Figure 3E depicts the average relative slope conductance ratios ( $G_{SCN}/G_{Cl}$ ) obtained from the measured slopes of the  $I-V$  relationships between  $-50$  and  $+50$  mV for each of the murine bestrophins. Substitution of extracellular  $Cl^-$  with  $SCN^-$  produced an increase in conductance for mBest1 ( $1.39 \pm 0.09$ ) and mBest3 ( $1.76 \pm 0.05$ ), whereas it resulted in a decrease in the conductance of mBest2 ( $0.36 \pm 0.05$ ). A decrease in  $G_{SCN}/G_{Cl}$  for mBest2 is in agreement with that previously reported by Qu et al [6].  $G_{SCN}/G_{Cl}$  of mBest1 was significantly greater than that of mBest2 and significantly less than that of mBest3 ( $n = 3-8$ ,  $p < 0.001$ ).

While the  $P_{SCN}/P_{Cl}$  for mBest1 is much lower than the other murine bestrophins, it is very similar to that demonstrated for dBest1 in HEK293 cells [23]. Chien et al attributed the  $P_{SCN}/P_{Cl}$  difference between dBest1 and mBest2 to differences in certain amino acids within the second transmembrane domain, a region shown to be important in anion permeation [5; 11]. Since the  $Cl^-$  channel properties of several bestrophins have not been studied in great detail, it is possible that sequence variability between bestrophins could result in important functional differences. The results of our study indicate that differences exist amongst murine bestrophins in terms of  $P_{SCN}/P_{Cl}$  and  $G_{SCN}/G_{Cl}$ , thus providing each bestrophin with a distinct characteristic which may prove useful during electrophysiological studies in tissues which express multiple bestrophin transcripts. Indeed, in mBest1 knockout mice  $I_{ClCa}$  was not abolished [17], and it was suggested that mBest2 and mBest3 might be compensating for the absence of mBest1, yet this could not be resolved since the electrophysiological properties of mBest1 were unknown.

## Pharmacology of mBest1 current

We used the  $Cl^-$  channel blockers niflumic acid (NFA) and DIDS to investigate the pharmacological properties of mBest1.  $I_{ClCa}$  was evoked from TRex-293 cells expressing

mBest1 with 500 nM  $[Ca^{2+}]_i$  using a ramp protocol to determine the voltage dependence of the inhibited current. Figure 4A and 4B are representative traces showing the effect of NFA and DIDS on mBest1 currents. Mean data for similar experiments are shown in Figure 4C. 100 $\mu$ M NFA blocked mBest1 currents by  $69.8 \pm 1.6\%$  at  $-80$  mV and  $77.1 \pm 1.7\%$  at  $+80$  mV ( $n = 2$ ). 100 $\mu$ M DIDS blocked mBest1 current by  $81.9 \pm 7.8\%$  at  $-80$  mV and  $84.4 \pm 7.2\%$  at  $+80$  mV ( $n = 4$ ). Inhibition of the mBest1 currents by these drugs appears to be voltage independent.

## Conclusion

This is the first study to characterize the  $Ca^{2+}$ -activated  $Cl^-$  current elicited by the recombinant expression of mBest1. The biophysical properties of this channel indicate that mBest1 functions as a DIDS- and NFA-sensitive  $I_{ClCa}$  when expressed heterologously in HEK 293 cells. mBest1 current is anion selective with a permeability profile of  $SCN^- > I^- > Cl^-$  and is activated by nanomolar concentrations of intracellular  $Ca^{2+}$ . In addition, mBest1 protein is expressed at the membrane of mBest1-transfected HEK cells. This study completes the biophysical characterization of the known murine bestrophin members, mBest1-3. While each murine bestrophin has similar whole cell currents and  $Ca^{2+}$  sensitivity, this study highlights that each isoform has a distinct permeability and conductance to  $SCN^-$ . Our data contributes to the characterization of the biophysical properties of mBest1 and helps further investigations to determine the physiological role of bestrophins.

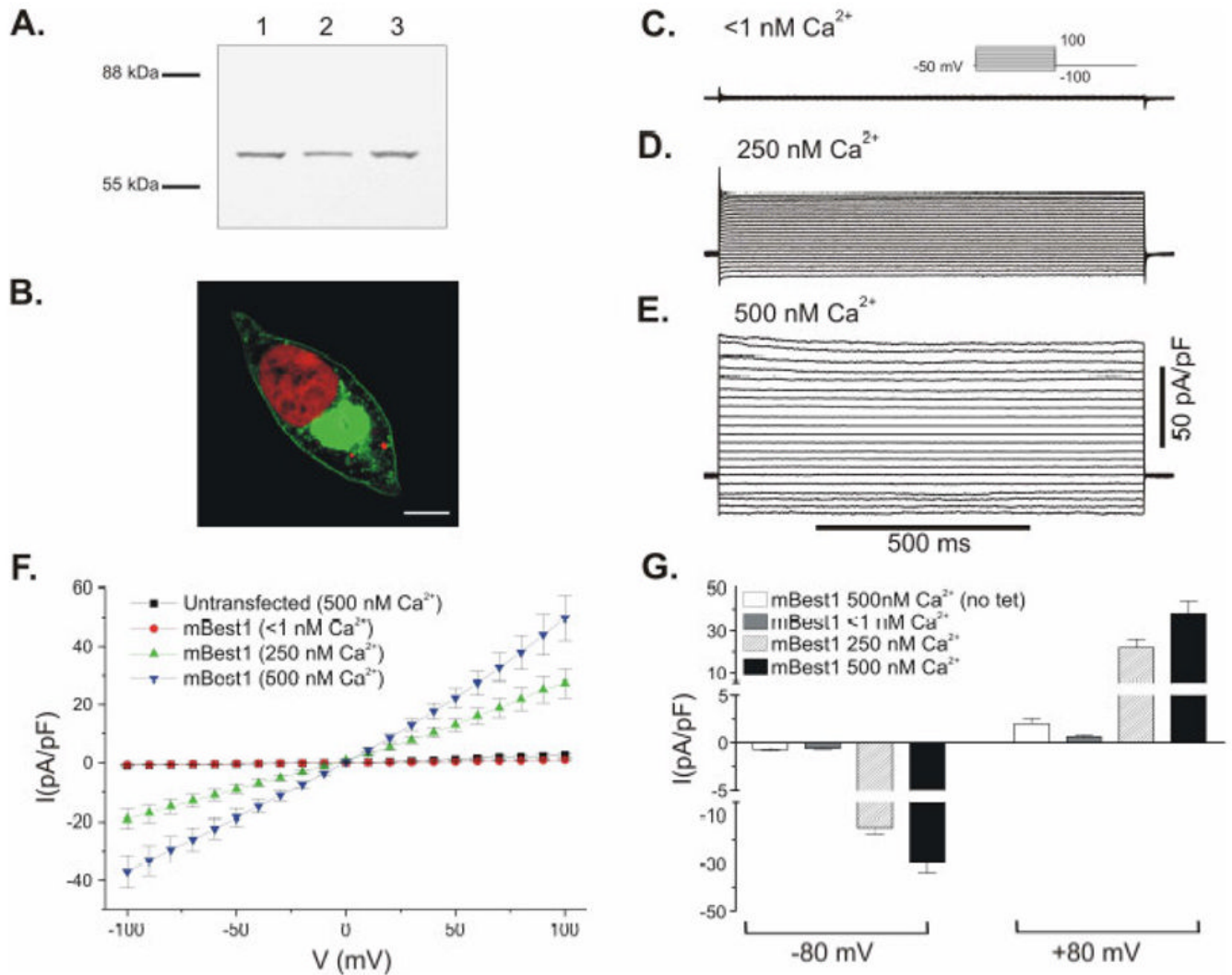
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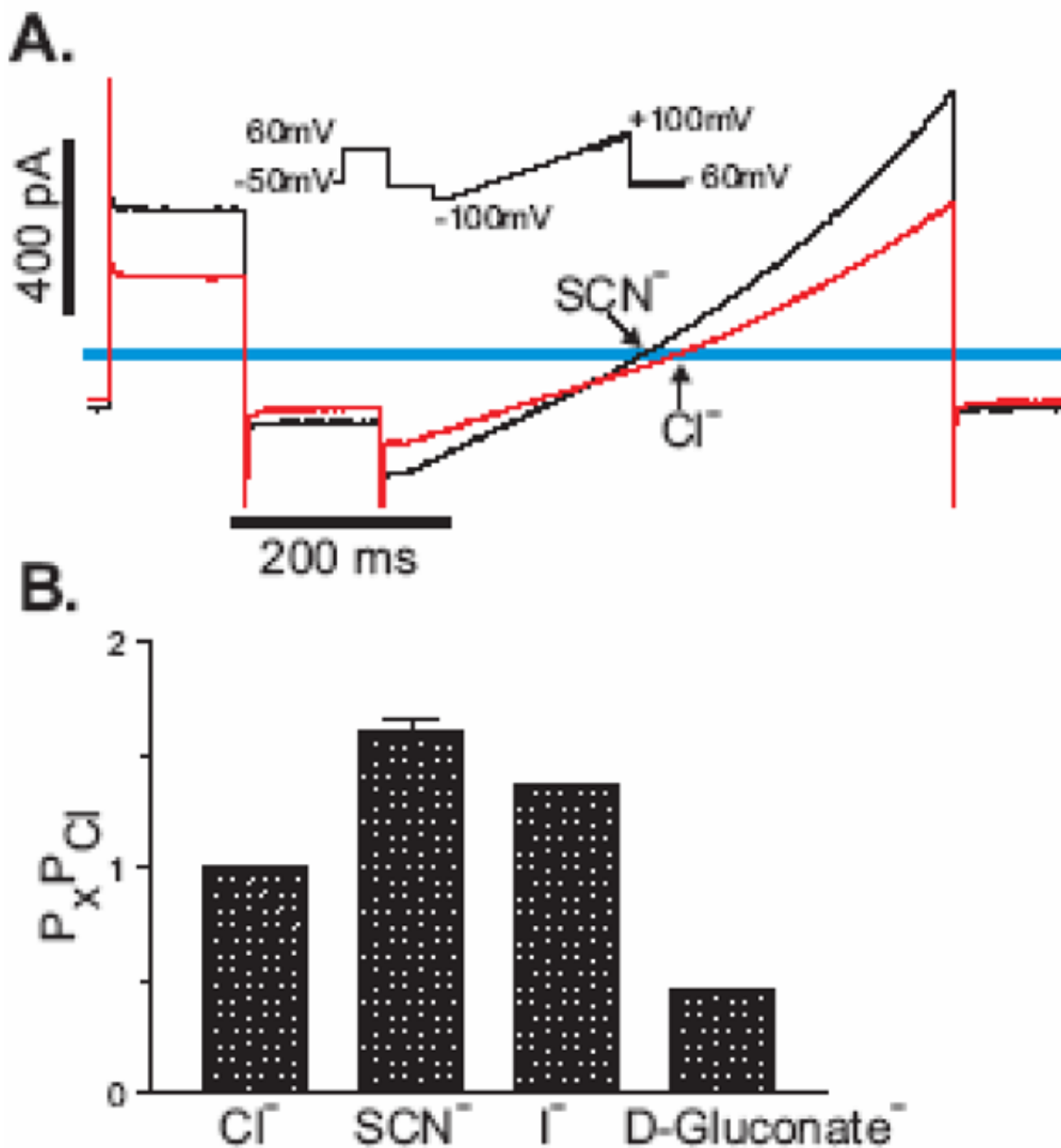
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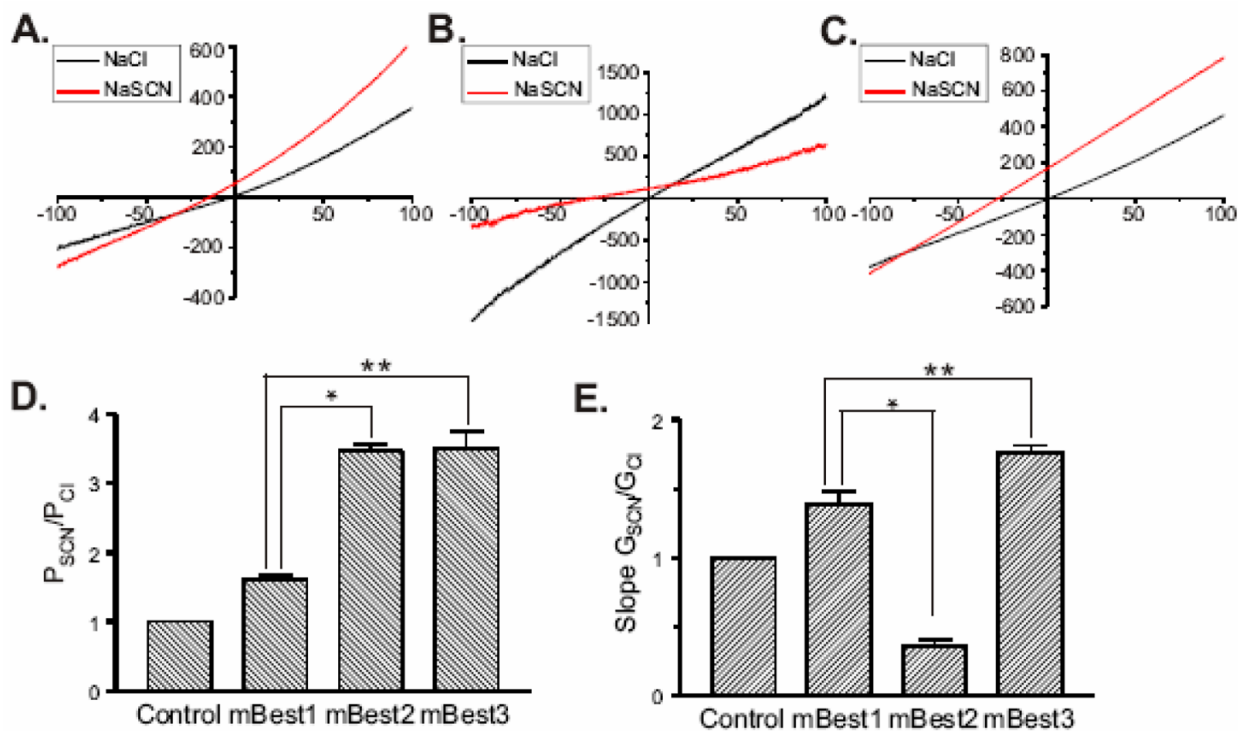
**Fig 1.**

Tetracycline inducible expression of mBest1 channel in TRex-293 cells. (A) Western blotting of tetracycline induced mBest1/c-myc fusion protein expression in a TRex-293 stable cell line (Lanes 1–3). Membranes were probed with anti-c-myc. (B) Confocal image of mBest1/c-myc expression in a TRex-293 cell. c-myc (Alexa 488, green) and propidium iodide nuclear stain (red). The confocal image is composite of three Z sections taken from a Z stack (21 x 0.468 $\mu$ m steps) representing a region at the center of the cell as indicated by the presence of the nucleus. Scale bar is 5  $\mu$ m. Panels C–E show families of currents recorded from TRex-293 cells transfected with mBest1 and expression induced with tetracycline. mBest1 currents were evoked with pipette solutions containing (C) <math><1\text{ nM Ca}^{2+}</math>, (D) 250 nM  $\text{Ca}^{2+}$  and (E) 500 nM  $\text{Ca}^{2+}$ . The voltage protocol is shown as inset in C. (F) Mean I-V relationships for mBest1 currents evoked in transfected TRex-293 cells by <math><1\text{ nM Ca}^{2+}</math> (circles, n = 8), 250 nM  $\text{Ca}^{2+}$  (up triangles, n = 7), 500 nM  $\text{Ca}^{2+}$  (down triangles, n = 18) and 500 nM  $\text{Ca}^{2+}$  in untransfected TRex-293 cells (squares, n = 8). (G) Summary of mean current densities recorded at -80 and +80 mV. Error bars represent the s.e.m.

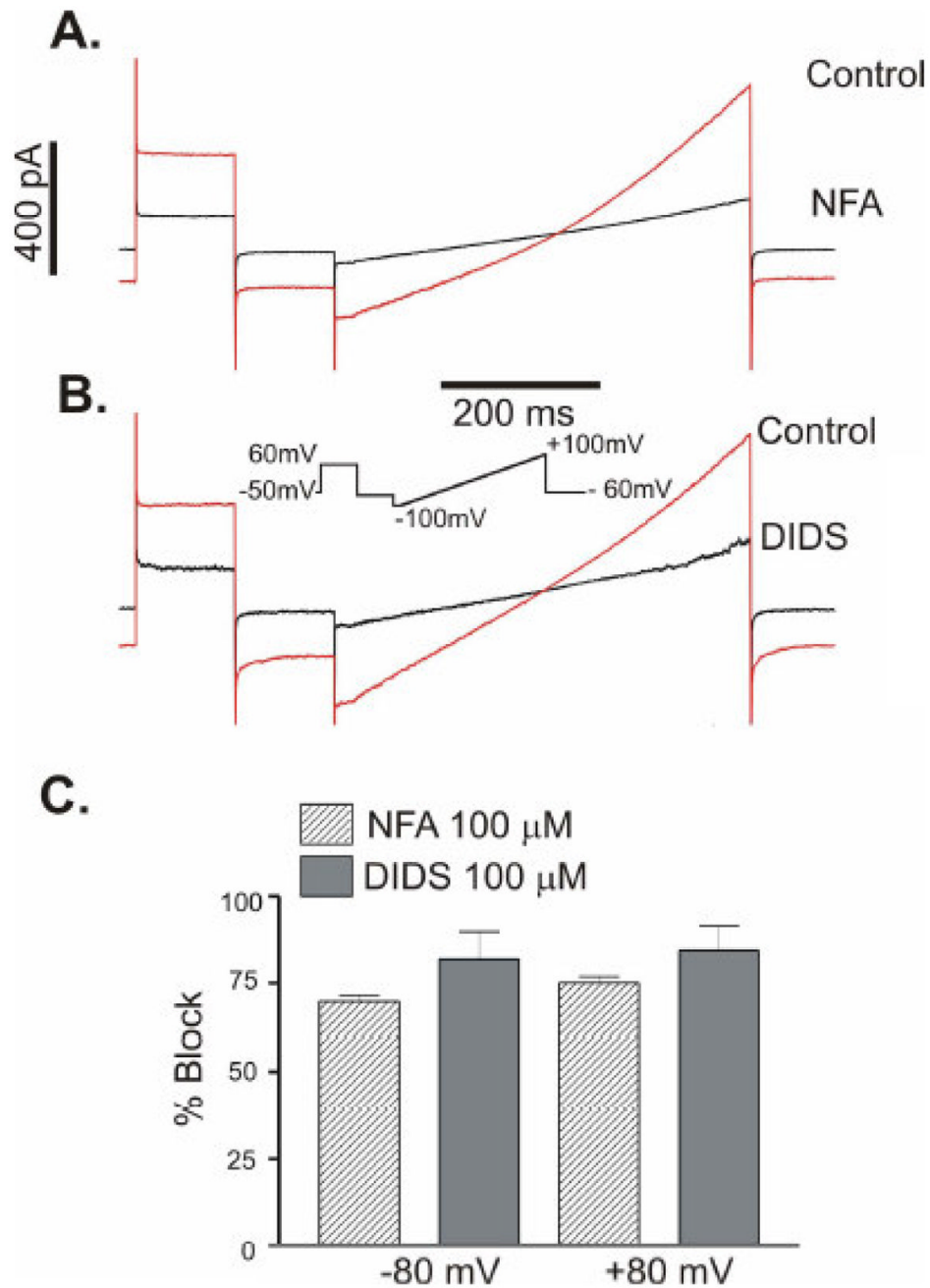


**Fig 2.**

Ionic nature of the mBest1 current. Whole cell mBest1 currents, activated by 500 nM  $[Ca^{2+}]_i$  were measured with bath solutions containing either 126 mM NaCl, NaSCN, NaI, or Na-D-Gluconate. (A) Representative trace of mBest1 currents measured before and after external  $SCN^-$  substitution. The  $I-V$  relationship shows an anion permeability of  $SCN^- > Cl^-$ . The inset shows the ramp protocol used to determine the voltage dependence of the activated current under different external anionic conditions. (B) Relative permeability ratios ( $P_x/P_{Cl}$ ) were calculated using the GHK equation from measured differences in the  $E_{rev}$  between  $Cl^-$  and other anions ( $SCN^-$ ,  $n = 7$ ;  $I^-$ ,  $n = 3$ , D-Gluconate $^-$ ,  $n = 4$ ).

**Fig 3.**

$SCN^-$  selectivity and conductance of the murine bestrophins. Whole cell mBest currents, activated by 500 nM  $[Ca^{2+}]_i$  were measured with bath solutions containing 126mM  $Cl^-$  (black) or bath solutions in which  $Cl^-$  was substituted with 126 mM  $SCN^-$  (red). The effect of external  $SCN^-$  on the  $I-V$  relationship of (A) mBest1, (B) mBest2 and (C) mBest3 is shown. (D)  $P_{SCN}/P_{Cl}$  were calculated using the GHK equation from measured differences in the  $E_{rev}$  between  $Cl^-$  and  $SCN^-$ . (E)  $G_{SCN}/G_{Cl}$  were obtained from the measurement of the slope of the  $I-V$  relationship between  $-50$  and  $+50$  mV from the  $E_{rev}$ . Error bars represent s.e.m.  $P_{SCN}/P_{Cl}$  and  $G_{SCN}/G_{Cl}$  ratios of mBest1 are significantly different than mBest2 (\*) and mBest3 (\*\*) ( $p < 0.001$ ,  $n = 3-8$ ).



**Fig 4.** Niflumic acid and DIDS inhibit mBest1 currents. The effect of (A) NFA and (B) DIDS on currents recorded from TRex-293 cells stably expressing mBest1. Cells were voltage clamped with 500 nM  $[Ca^{2+}]_i$  and the  $I$ - $V$  relationships were obtained with the protocol shown. (C). The mean percentage block of mBest1 current by 100  $\mu$ M NFA ( $n = 2$ ) and 100  $\mu$ M DIDS ( $n = 4$ ) at -80 and +80 mV. Error bars represent the s.e.m.