FEBS Letters 580 (2006) 2141–2146

A short motif in the C-terminus of mouse bestrophin 4 inhibits its activation as a Cl channel

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Received 16 January 2006; revised 7 March 2006; accepted 8 March 2006

Available online 20 March 2006

Edited by Maurice Montal

Abstract **Bestrophins are a new family of anion channels. Here, we examined the Cl channel activity of mBest4. Surprisingly, wild type mouse bestrophin-4 (mBest4) did not induce functional Cl channels when over-expressed in HEK293 cells. However, deletion of part of the C-terminus (residues 353–669) produced large Cl currents, suggesting the presence of a C-terminal motif that inhibited Cl channel function. Deletion of a short motif (356–364) or substitution of certain residues in this motif with alanines also resulted in expression of robust Cl currents. The channel activity of the mBest4 protein lacking the C-terminus (residues 353–669) was specifically inhibited by co-expression of C-terminal fragments of mBest4 having the inhibitory motif, suggesting that the C-terminal motif blocked mBest4 channel activity probably by interacting with the channel pore.**

Keywords: Ion channel; Anion channel; Chloride channel; Bestrophin; Channel activation; Patch clamp

1. Introduction

Bestrophins are a new family of Cl channels [1,8,11,13] with a different structure than other well-studied Cl channels such as cystic fibrosis transmembrane conductance regulator, voltage-gated Cl channel family channels, and GABA receptors [2]. In humans, there are four bestrophin homologues (hBest1–4) [12,14]. hBest1 was positionally cloned from families with Best vitelliform macular dystrophy, an early-onset form of macular degeneration [5,7]. All four human bestrophins induce Cl currents when over-expressed [13].

Four bestrophin homologues also exist in mice [3]. However, only mBest2 has been reported to induce Cl currents in heterologous expression [8,9]. Wild type mouse bestrophin 4 (mBest4) [1] was first cloned from mouse heart [3]. Its first 363 amino acids are highly homologous to other bestrophins, but it has a unique C-terminus. Here, we identified a short motif located in the mBest4 C-terminus, which blocked mBest4 Cl channel activity.

2. Materials and methods

2.1. Production of mouse bestrophin 4 mutations and heterologous expression

mBest4 (Vmd2l3) cDNA was cloned from mouse heart mRNA by RT-PCR. The sequence agreed with Genbank accession AY450426 and Swissprot accession Q6H1V1. The cDNA was subcloned into pcDNA3.1 (Invitrogen). Site-specific mutations and deletions were made using a PCR-based mutagenesis kit (Quickchange; Stratagene) as described previously [8]. The human ortholog of mBest4 (called hBest3 [14], Genbank accession AAR99656) was provided by Dr. Jeremy Nathans (Johns Hopkins University). cDNAs were cotransfected into HEK293 cells (ATCC) using FuGene-6 transfection reagent (Roche) and pEGFP (Invitrogen) to identify transfected cells. 0.1–0.3 μg cDNA was used to transfect one 35-mm culture dish.

2.2. Electrophysiology

Recordings were performed at 22–24 °C using the whole-cell configuration of the patch clamp [6]. Voltage clamp steps or ramps were used as indicated in the figures with a holding potential of 0 mV. The standard pipette solution (~4.5 μM free Ca) contained (in mM) 146 CsCl, 2 $MgCl_2$, 5 (Ca^{2+})-EGTA, 8 HEPES, 10 sucrose, pH 7.3, adjusted with NMDG. Low intracellular Ca solution (<20 nM free Ca) contained (in mM) 146 CsCl, 2 $MgCl_2$, 5 EGTA, 8 HEPES, 10 sucrose, pH 7.3. The standard extracellular solution contained (in mM) 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose,10 HEPES, pH 7.3 with NaOH. This combination of solutions set E_{rev} for Cl^- currents to zero, while cation currents carried by Na^+ or Cs^+ have very positive or negative E_{rev}, respectively. Anion permeability relative to Cl was determined by measuring the shift in E_{rev} upon changing the bath solution from one containing 150 mM Cl^- to another with 140 mM X^- and 10 mM Cl^-, where X is the substitute anion [10]. The permeability ratio was estimated using the Goldman–Hodgkin–Katz equation:

$$P_x/P_{Cl} = [Cl^-]_i/([X^-]_0 \exp(\Delta E_{rev} F/RT)) - [Cl^-]_0/[X^-]_0,$$

where ΔE_{rev} is the difference between the reversal potential with the test anion X^- and that observed with symmetrical Cl^-, and F, R, and T have their normal thermodynamic meanings.

3. Results and discussion

3.1. C-terminal deletions allow mBest4 to express functional Cl currents

Because the level of mBest4 transcript was reported to be high in mouse heart [3], we cloned mBest4 cDNA from this tissue to study its physiological function. However, it did not induce significant Cl currents when transiently transfected into HEK293 cells (Fig. 1A). This was surprising because the human

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[1] mBest4 constructs with truncated C-termini were named Δ(residue range), where "residue range" are the positions of the deleted amino acids. C-terminal fragments were named as mBest4(residue range) where "residue range" refers to the amino acid positions that were present in the construct.

Abbreviations: mBest4, mouse bestrophin 4; SCN, thiocyanate; HEK, human embryonic kidney; TMD, transmembrane domain

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.025

ortholog of mBest4 (called hBest3 by Tsunenari et al. [14], Genbank AAR99656) exhibits 85% identity to mBest4 but was reported to induce Cl currents [14]. We found that although hBest3 expression did induce currents, the currents were very small under our standard conditions (Fig. 1C). Transfection with large amounts of hBest3 cDNA and very long (~4 s) and large hyperpolarizations (>−100 mV) were required to see significant currents (Fig. 1D), as was also reported by Tsunenari et al. [14]. When we transfected cells with larger amounts of mBest4 cDNA and gave long and strong hyperpolarizations, we were also able to see similar currents induced by mBest4 (Fig. 1B). We conclude that, although there are quantitative differences between mBest4 and hBest3, neither of these constructs produces significant current with <1 s voltage pulses within a physiological range (Fig. 1A and C).

We hypothesized that the long C-terminus might regulate mBest4 activation. Therefore, we truncated the C-terminus by introducing stop codons at intervals of ~50 amino acids. The residues mutated into stop codons are underlined and indicated with stars as indicated in the alignment. When the protein was truncated at residue 353, the mutant (Δ353–669) expressed significant Cl current (Fig. 1E). The currents were time and voltage independent.

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          10        20        30        40        50        60        70        80        90
  1 MTVTYTARVANARFGGFSQLLLLWRGSIYKLLWRELLCFLGLYMALSAAYRFLLAEEQKRYFEKLVIYCDQYASLIPVSFVLGFYVTLVVHRWWNQYLCM   mBest2
  1 MTVTYSSKVANATFFGFHRLLLKWRGSIYKLLYREFIVFAVLYTAISLVYRLLLTGAQKRYFEKLSIYCDRYAEQIPVTFVLGFYVTLVVNRWWNQFVNL   mBest4
                                       TMD1                                                 TMD2

101 PLPDALMCIVAGTVHGRDDRGRLYRRTLMRYAGLSAVLILRSVSTAVFKRFPTIDHVVEAGFMTREERKKFENLNSSYNKYWVPCVWFSSLAAQARREGR   mBest2
101 PWPDRLMLLISSSVHGSDQHGRLLRRTLMRYVNLTSLLIFRSVSTAVYKRFPTMDHVVEAGFMTADERKLFDHLKSPHLKYWVPFIWFGNLATKARNEGR   mBest4
                                                                                                TMD3

201 IRDNSALKLLLEELNVFRSKCGMLFHYDWISIPLVYTQVVTIAVYSYFLACLIGRQFLDPAQGYKDHTLDLCVPIFTLLQFFFYAGWLKVAEQLINPFGE   mBest2
201 IRDSVDLQSLMTEMNRYRSWCSLLFGYDWVGIPLVYTQVVTLAVYTFFFACLIGRQFLDPTKGYVGHDLDLYVPIFTLLQFFFYAGWLKVAEQLINPFGE   mBest4
                                                                                        TMD4

301 DDDDFETNFLIDRNFQVSMLAVDEMYDDLAMLEKDLYWDAAEARAPYTAATAFLLQQPSFQGSTFDIALAKEDMQFQRLDGVDGPLGEVHGDFLQRLLPA   mBest2
301 DDDDFETNWCIDRNLQVSLLAVDEMHMSLPKMKKDIYWDDSAARPPYTLAAADYCI-PSFLGSTIQMGLSGSNFPAEDWLWNYEKHGNRHSVMRRVKRFL   mBest4
                                                  *353

401 GAGSVGPLGRRLSLLRRKNSCVSEASTAASCGCAGAADGGGVECGCGDPLLDPSLREPELESPACPEPPAPIPGPTPEPFTTVSIPGPRAPAPPWLPSPI   mBest2
401 STHEHPGSPRRRRSFGRQASDSSMFLPPSPARDLLDVPSRNPHRGSPTRKQSRSQEGSPKLHSSMGELSTIRETSRTSTLQSLSPQSSVRSSPTKMPQVP   mBest4
       *405                                        *454

501 GEEEESPA                                                                                                 mBest2
501 EVLITAAEAPAFSADSHQHDSTTSILSLEFTGVQPSGTEQQVEPSGTPPGDPNPQTTSASTERDLFKFEEDLEDDRFPKRWSLPEFLESRHTSLGNLGPD   mBest4
       *504                                        *554

601 PVSPRDALLLPDTETPSETNGIHPGAGSALAPDILYLMESLDKETDILEFNNEHTGESPKGTPQRPRTWF 669                               mBest4
       *604
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However, when mBest4 was truncated at residue 405, 454, 504, 554 or 604, the mutation remained non-functional like wild type (Fig. 1F). These results suggested that there might be a motif between 353 and 405 which somehow blocked mBest4 channel activity or prevented the channel protein from trafficking to the plasma membrane. To test the hypothesis, we deleted amino acids 353–404. As expected, Δ353–404 expressed robust Cl currents (Fig. 2). These results show that the first 352 amino acids of mBest4 are sufficient for channel function and that an inhibitory motif probably exists in the region of 353–405 which inhibits mBest4 activation.

3.2. The inhibitory motif in C-terminus is pinpointed to a short sequence

To locate the critical amino acids in the inhibitory motif, we deleted the first 15 amino acids (353–367) in the region 353–404. The mutant, Δ353–367 expressed large Cl currents (Fig. 2). To determine critical amino acids within 353–367, we substituted alanines for groups of three amino acids. Substitution of $_{356}$IPS$_{358}$, $_{359}$FLG$_{361}$ or $_{362}$STI$_{364}$ with AAA produced significant Cl currents but substitution of $_{353}$DYC$_{355}$ or $_{365}$QMG$_{367}$ with AAA did not (Fig. 2). These results indicate that the residues residing in the $_{356}$IPSFLGSTI$_{364}$ motif are critical for inhibition of mBest4. It is worthy to note that the current amplitudes induced by IPS/AAA, FLG/AAA or STI/AAA mutants were much smaller than those induced by Δ353–404 or Δ353–367, implying that the inhibition of mBest4 may require all residues in the $_{356}$IPSFLGSTI$_{364}$ motif.

The inhibitory short motif in mBest4 is conserved in its human ortholog, hBest3 except at position of 364 where Ile in mBest4 is replaced with Val in hBest3 [14]. We predict that deletion of this motif from hBest3 will increase the amplitude of the currents, but this has not yet been tested. Also, the amino acid sequences flanking the short motif are only ~60% identical between two bestrophins. The small and slow currents produced by hBest3 might be explained by the difference in these flanking regions.

Bestrophins have been reported to interact physically and functionally with protein phosphatase 2A [4], indicating that phosphorylation may play a role in bestrophin regulation. Although there are Ser and Thr residues in the $_{356}$IPSFLGSTI$_{364}$ motif, no phosphorylation consensus sites were found in the motif by Scansite (scansite.mit.edu).

3.3. Co-expression of mBest4 C-terminus reduced Δ353–669-induced Cl currents

To elucidate the mechanism of inhibition of mBest4 by the inhibitory motif, we co-expressed the Δ353–669 mutant with mBest4 C-terminal sequences. If the C-terminal sequences carrying the inhibitory motif can block Δ353–669-induced Cl currents, this would support the hypothesis that the non-functionality of wild type mBest4 may be due to the action of the inhibitory motif on the channel pore. Fig. 3A shows the design of three C-terminal sequences. Δ353–669-induced Cl currents were significantly blocked by the