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Evans Blue is not a suitable inhibitor of the epithelial sodium channel δ-subunit

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ABSTRACT

The Epithelial Sodium Channel (ENaC) is a heterotrimeric ion channel which can be either formed by assembly of its α-, β- and γ-subunits or, alternatively, its δ-, β- and γ-subunits. The physiological function of αβγ-ENaC is well established, but the function of δβγ-ENaC remains elusive. The azo-dye Evans Blue (EvB) has been routinely used to discriminate between the two channel isoforms by decreasing transmembrane currents and amiloride-sensitive current fractions of δβγ-ENaC expressing *Xenopus* oocytes. Even though these results could be reproduced, it was found by precipitation experiments and spectroscopic methods that the cationic amiloride and the anionic EvB directly interact in solution, forming a strong complex. Thereby a large amount of pharmacologically available amiloride is removed from physiological buffer solutions and the effective amiloride concentration is reduced. This interaction did not occur in the presence of albumin. In microelectrode recordings, EvB was able to abrogate the block of δβγ-ENaC by amiloride or its derivative benzamil.

In sum, EvB reduces amiloride-sensitive ion current fractions in electrophysiological experiments. This is not a result of a specific inhibition of δβγ-ENaC but rather represents a pharmacological artefact. EvB should therefore not be used as an inhibitor of δ-ENaC.

1. Introduction

The epithelial sodium channel (ENaC) is a sodium-selective ion channel which is located in the apical membrane of various vertebrate epithelia. ENaCs are the rate-limiting step for apical entry of sodium ions into epithelial cells and thereby play a major role in transepithelial sodium and, consequently, liquid transport. Malfunctions in ENaC activity are associated with severe human diseases such as hypertension, cystic fibrosis and pulmonary edema [1–3].

The "classical" ENaC consists of three homologous subunits (α, β and γ) [4] which likely assemble as a heterotrimer [5] and build a sodium-selective pore in the plasma membrane. In primates, there is a fourth subunit (δ) which can replace the α-subunit and build a functional channel together with the β- and γ-subunits [6]. Remarkably, the substitution of the α-subunit with the δ-subunit changes the biophysical characteristics as well as the molecular regulation of the channel [7]. Compared to α-subunit containing channels, human ENaCs containing the δ-subunit display a higher single channel conductance [8], increased open probability [9], and a decreased sensitivity to the ENaC inhibitor amiloride [6]. In addition, δ-containing ENaCs are more sensitive to changes in the extracellular pH [10,11], have a reduced sensitivity to proteolytic processing [9] and display less negative-feedback regulation by sodium ions (mechanisms referred to as self-inhibition and feedback-inhibition) [11]. Tissue expression of the δ-subunit is different from that of the α-subunit. The δ-subunit is expressed in gonads [6] and neuronal tissues, such as pyramidal neurons in the frontal cortex of the primate brain [8,12]. Especially the neuronal expression of δ-ENaC has recently received much attention. It is speculated that δ-ENaC is involved in the regulation of neuronal excitability [7,13]. Furthermore, elevated expression of δ-ENaC was

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recently suggested to play a role in epilepsy [14].

However, research on δ-ENaC is difficult since rats and mice, the standard models in physiological research, do not express δ-ENaC which complicates its experimental analysis *in vivo* [7]. Therefore, pharmacological discrimination between ENaC isoforms is a major challenge in order to identify functional δ-subunit containing ENaCs in native cells and tissues from primates [15]. The decreased sensitivity of δ-containing ENaCs to amiloride [6] would in principle allow differentiating between α- and δ-containing ENaCs [16], however, the selectivity is not sufficient to inhibit one ENaC isoform without significantly affecting the other.

Yamamura and co-workers were the first to use the azo-dye Evans blue (EvB) to discriminate between α- and δ-containing ENaCs [17]. They showed that EvB inhibited the activity of human δβγ-ENaCs which were heterologously expressed in *Xenopus laevis* oocytes. By contrast, αβγ-ENaCs were activated by EvB. Based on these findings, EvB has been considered and used in various studies as an inhibitor of δ-containing ENaCs [16,18,19].

We now demonstrate that EvB chemically interacts with amiloride and thereby reduces the effective concentration of this standard ENaC inhibitor in experimental solutions. In electrophysiological experiments, such interaction reduces amiloride-sensitive ion current fractions in the presence of EvB. This reduction is not the result of the inhibition of δ-ENaC but rather represents a pharmacological artefact. EvB should not be considered and used as an inhibitor of δ-ENaC.

2. Materials and methods

2.1. Isolation of Xenopus laevis oocytes

Animal experiments were performed in accordance with the German animal welfare law and had been declared to the Animal Welfare Officer of the University of Giessen (Registration No.: M_478). The animal housing facility was licenced by the local authorities. The methods used to euthanise the animals humanely were consistent with the recommendations of the AVMA Guidelines for the Euthanasia of Animals.

Adult female *X. laevis* were purchased from Xenopus Express (Vernassal, France) and housed in 250-L tanks with continuous freshwater supply. Frogs were tranquilised at 4 °C and subsequently killed by decapitation and sounding of the spinal cord. Ovaries were dissected and kept at 16 °C in oocyte culture solution containing 90 mM NaCl, 1 mM KCl, 2 mM $CaCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 0.06 mM penicillin G and 0.02 mM streptomycin sulfate at pH 7.4. Oocytes were isolated by incubating the ovaries for 90 min in 1.5 mg/ml collagenase (NB4, Serva, Heidelberg, Germany) in culture solution at room temperature. Oocytes were washed three times with calcium-free culture solution containing 90 mM NaCl, 1 mM KCl, 5 mM HEPES and 1 mM ethylene glycol tetraacetic acid (EGTA) at pH 7.4 and subsequently incubated for 10 min in this solution. Oocytes were rinsed three times with culture solution and oocytes of stages V/VI (Dumont) were selected for cRNA injection.

2.2. cRNA synthesis and injection

Plasmid constructs for the human δ(1), β and γ subunits were cloned into a pTNT vector (Promega, Mannheim, Germany) as described previously [12,20]. Plasmids were linearized with BamH1 (Promega) and *in vitro* transcribed with the RiboMAX Large Scale RNA Production System (Promega) using T7 RNA polymerase. Obtained cRNAs were combined to a final concentration of 10 ng/μl per ENaC subunit. Thirty-two nanoliters of cRNAs were injected into oocytes which were then cultured for 24–48 h at 16 °C in a low-sodium solution containing 10 mM NaCl, 1 mM KCl, 2 mM $CaCl_2$, 80 mM N-methyl-D-glucamine, 5 mM HEPES, 2.5 mM sodium pyruvate, 0.06 mM penicillin G and 0.02 mM streptomycin sulfate at pH 7.4.

2.3. Two-electrode voltage-clamp (TEVC)

Oocytes were placed in a lucite chamber and perfused with oocyte ringer solution (ORS) containing 90 mM NaCl, 1 mM KCl, 2 mM $CaCl_2$ and 5 mM HEPES at pH 7.4. Chlorinated silver wires served as recording electrodes and were either placed into the lucite chamber for reference, or into borosilicate glass capillaries which were pulled to microelectrodes, filled with 1 M KCl and punctured the oocytes. The electrodes were connected to an oocyte voltage clamp amplifier (OC 725B, Warner Instruments, Hamden, Connecticut, USA) which clamped the oocyte's membrane potential to −60 mV. Transmembrane current signals (I_M) were low-pass filtered at 1000 Hz (Frequency Devices 902, Haverhill, Massachusetts, USA) and were recorded by a strip chart recorder (Kipp&Zonen, Delft, The Netherlands).

2.4. UV/Vis-spectroscopy

Absorption spectra were obtained by UV/Vis-experiments using a Lambda 950 spectrometer (PerkinElmer, Baesweiler, Germany). EvB, amiloride and benzamil solutions (2.5×10^{-5} M in ORS) were placed in plastic cuvettes (d = 1 cm, Vol = 3 ml; Brand, Wertheim, Germany) and absorption spectra were recorded from 800 to 200 nm. For quantification experiments, EvB (10^{-4} M) was dissolved in 1 ml ORS with or without amiloride or benzamil ($5 \times 10^{-5} - 5 \times 10^{-4}$ M), diluted 1:20 in H_2O and filled into plastic cuvettes (d = 1 cm, Vol = 1 mL; LLG, Meckenheim, Germany). The characteristic absorption of EvB at 621 nm was measured with a Vis-spectrophotometer (Kruess Optronic, Hamburg, Germany) immediately following preparation.

2.5. Chemicals

EvB (Sigma, Taufkirchen, Germany) was prepared as a stock solution of 10^{-1} M in Dimethyl sulfoxide (DMSO). Amiloride and benzamil (both from Sigma) were prepared as stock solutions of 10^{-2} M (in H_2O) and 5×10^{-2} M (in DMSO), respectively. All stock solutions were stored at +4 °C. The employed concentrations of DMSO did not affect ENaC activity (data not shown). EvB labelled albumin (EvBA) was prepared by dissolving 0.67 mg/ml EvB in ORS containing 4% bovine serum albumin (BSA, Sigma) [21]. The solution was diluted with ORS to final EvB concentrations of $10^{-5} - 3 \times 10^{-4}$ M.

2.6. Data analysis and statistics

For microelectrode experiments, inward currents are defined as negative current signals and depicted as downward deflections. Data are presented as means ± standard error of the mean. The number of independent experiments is indicated as “n”. Statistical analysis was performed with GraphPad Prism version 5.01 (La Jolla, California, USA). Normal distribution of data was assessed by Kolmogorov–Smirnov normality test. For paired experiments, Student's paired *t*-test or non-parametric Wilcoxon matched-pairs test were used. For multiple comparisons, one-way ANOVA followed by Bonferroni's Multiple Comparison test, or Kruskal–Wallis test followed by Dunn's Multiple Comparison test were employed. P-values ≤ 0.05 were regarded as statistically significant and marked with an asterisk (*).