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The Na^+/K^+-ATPase and the amyloid-beta peptide $a\beta_{1-40}$ control the cellular distribution, abundance and activity of TRPC6 channels☆

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ABSTRACT

The Na^+/K^+-ATPase interacts with the non-selective cation channels TRPC6 but the functional consequences of this association are unknown. Experiments performed with HEK cells over-expressing TRPC6 channels showed that inhibiting the activity of the Na^+/K^+-ATPase with ouabain reduced the amount of TRPC6 proteins and depressed Ca^{2+} entry through TRPC6. This effect, not mimicked by membrane depolarization with KCl, was abolished by sucrose and bafilomycin-A, and was partially sensitive to the intracellular Ca^{2+} chelator BAPTA/AM. Biotinylation and subcellular fractionation experiments showed that ouabain caused a multifaceted redistribution of TRPC6 to the plasma membrane and to an endo/lysosomal compartment where they were degraded. The amyloid beta peptide $A\beta_{1-40}$, another inhibitor of the Na^+/K^+-ATPase, but not the shorter peptide $A\beta_{1-16}$, reduced TRPC6 protein levels and depressed TRPC6-mediated responses. In cortical neurons from embryonic mice, ouabain, veratridine (an opener of voltage-gated Na^+ channel), and $A\beta_{1-40}$ reduced TRPC6-mediated Ca^{2+} responses whereas $A\beta_{1-16}$ was ineffective. Furthermore, when $A\beta_{1-40}$ was co-added together with zinc acetate it could no longer control TRPC6 activity. Altogether, this work shows the existence of a functional coupling between the Na^+/K^+-ATPase and TRPC6. It also suggests that the abundance, distribution and activity of TRPC6 can be regulated by cardiotonic steroids like ouabain and the naturally occurring peptide $A\beta_{1-40}$ which underlines the pathophysiological significance of these processes.

1. Introduction

One key function of biological membranes is to maintain an uneven distribution of ions such as Na^+, K^+, Ca^{2+}, and Cl^-. Among the various proteins participating in the ionic homeostasis, the Na^+/K^+-ATPase is an essential actor. Its basic function is to maintain the cytosolic concentrations of Na^+ and K^+ by pumping out Na^+ and pumping K^+ into the cytosol [1,2]. Various conditions and actors like Na^+ ions, cytoskeleton proteins, neurotransmitters, peptides, protein kinases, and clinically relevant drugs such as ouabain-like cardiotonic steroids have been shown to influence the activity of the Na^+/K^+-ATPase [1,2]. The pathophysiological relevance of this regulation for neuronal function is illustrated by the fact that amyloid-beta (Aβ) peptides, which are described as playing a central role in Alzheimer's disease, inhibit the activity of this pump [3–5]. This impairs neuronal Na^+ and Ca^{2+} homeostasis and increases the vulnerability of cells to apoptotic insults [6].

The Na^+/K^+-ATPase interacts with several types of channels and transporters of the plasma membrane: AMPA channels (a subtype of ionotropic glutamate receptors), GlyT2 (a glycine transporter), GLAST (a glutamate transporter), Nax (a neuronal Na^+-sensing channel), AQP4 (the water channel aquaporin 4), and TRPC6 (a non-selective cation channel) [7,8]. The functional implications of these protein–protein interactions are just beginning to be understood. For instance, ouabain, a specific inhibitor of the Na^+/K^+-ATPase [1,9], induces the endocytosis and degradation of AMPA receptors and GlyT2 [10,11]. Like these 2 latter proteins, TRPC6 transports Na^+ ions through the plasma membrane [12,13], and associates with the Na^+/K^+-ATPase [7,8]. However, the functional consequences of the interaction between TRPC6 and the Na^+/K^+-ATPase have not been addressed. The present report was undertaken to better understand the role played by the Na^+/K^+-ATPase on TRPC6 activity. This issue is of importance because TRPC6 is found in many tissues and organs, including in the brain where it plays key roles in neuronal survival, dendritic growth and synaptogenesis [14].

☆ Conflict of interest: none.

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2. Materials and methods

2.1. Cell cultures and transfection

HEK-293 and HEK-293 cells stably over-expressing TRPC6 (HEK-TRPC6) were maintained in culture according to experimental conditions described elsewhere [15,16]. HEK cells stably over-expressing TRPM4 channels were cultured as described previously [17]. The culture medium of all cell lines was without penicillin/streptomycin. Primary cultures of cortical neurons were prepared from embryonic (E13) wild type C57BL6 mice according to [18]. The procedures used were approved by the ethical committee of the CEA's Life Sciences Division (CETEA).

2.2. Protein extraction and immunoblotting analysis

Cells were rinsed twice with a phosphate-buffer saline and lysed on ice in 50–100 μl of extraction buffer (10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM KCl, 2,5% Glycerol, 1% triton X-100 and 1:300 protease inhibitor cocktail, Sigma) for 30 min. The lysate was then centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant was collected and frozen at −80 °C for subsequent use. Protein concentration was measured using the Bradford assay. Thirty μg of each extract was boiled with 2× Laemmli buffer, separated by SDS-PAGE and then electrotransferred (Bio-Rad system) onto nitro-cellulose membranes (Bio-Rad). They were blocked with milk 5% in PBS –0.1% Tween overnight. They were then probed with primary antibodies raised against TRPC6 (Alomone labs), α1Na^+/K^+-ATPase subunit, or actin (Sigma). This was followed by incubation with appropriate horse-radish peroxidase-conjugated secondary antibodies (Bethyl). Bands were detected by chemiluminescence (ECL, Pierce) using a Fusion Fx7 apparatus (Vilbert Lourmat). Quantification of protein expression was made with ImageJ software: for a given protein, its expression was normalized to the level of expression of actin. The results presented were the results of at least three measurements from three independent cultures for each condition.

2.3. Preparation of the amyloid-beta peptides $a\beta_{1-40}$ and $a\beta_{1-16}$

$A\beta_{1-16}$ (sequence DAEFRHDSGYEVHHQK) and $A\beta_{1-40}$ peptides (sequence DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) were bought from GeneCust (Dudelange, Luxembourg). They were solubilized in 30 mM NaOH. Peptide concentration was then determined by UV–visible absorption of Tyr10 considered as free tyrosinate ($(\lambda_{293}-\lambda_{360}) = 2130\ M^{-1}cm^{-1}$) [19].

2.4. Subcellular fractionation

First, HEK-293 cells stably expressing TRPC6 were subjected to a slightly modified version of de Duve's subcellular fractionation scheme, as described [20]. The membrane organelle fractions prepared from the post-nuclear supernatant (i.e. M, Heavy mitochondrial; L, Light mitochondrial; and P, microsomal) were then pooled and fractionated in an 18% self-forming Percoll™ (Pharmacia) density gradient, as described [20]. Seven sections were collected from the top of the gradient, and the distribution of a lysosomal marker (β-galactosidase) and a plasma membrane marker (alkaline phosphodiesterase) was determined by enzyme assays [20,21]. The distribution of TRPC6 was assessed by Western blotting.

2.5. Cell surface biotinylation assay

The cell surface level of TRPC6 was measured using sulfo-NHS-SS-biotin [sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate] (Pierce) to label proteins located at the plasma membrane, as described previously [22], except that a concentration of 2 mg/mL of biotin substrate was used and that the cells were lysed in PBS-Triton 1% containing protease inhibitors (Complete Mini Tablets, Roche) [22]. Detection of GAPDH by Western blotting (Sigma antibody) was used to control the impermeability of the cells to the biotin reagent, and to normalize signal intensities.

2.6. Live-cell Ca^{2+} imaging experiments

The fluorescent indicator Fluo-4 was used to monitor cytosolic Ca^{2+} according to [15,23]. Briefly, the culture medium was removed and cells were rinsed twice with a saline containing: (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4 (NaOH). Cells were incubated with the fluorescent probe for 20 min at room temperature before being washed twice with a fluorescent probe-free solution. Fluo-4-loaded cells were kept in the dark at room temperature for 10 min before being placed on the stage of an Axio Observer A1 microscope (Carl Zeiss, France) equipped with a Fluar 40× oil immersion objective lens (1.3 NA) (Carl Zeiss, France). The experimental setup and the recording conditions were as described [15,23].

2.7. Statistics

Throughout the text, data are presented as means ± s.e.m., or ± SD (Figs. 4B and 6E). The software SigmaStat (version 3.5, Systat Software) was used to determine the statistical significance of the difference between groups.

2.8. Materials

Fluo-4/AM as well as tissue culture media were from Molecular Probes (Invitrogen, France). Hyperforin was a kind gift from Dr. Willmar Schwabe GmbH & Co (Karlsruhe, Germany). Unless otherwise indicated, all the other reagents were from Sigma-Aldrich (France).

3. Results

Throughout this study, the activity of the Na^+/K^+-ATPase was inhibited by the glycoside ouabain [1] used at 25 μM, a concentration 2 times smaller than used previously on cultured cortical neurons, cultured brain and spinal cord neurons [10,11], and having no effect on cell viability [11]. To assess the effect of the ouabain-mediated inhibition of the Na^+/K^+-ATPase on TRPC6, HEK cells stably over-expressing TRPC6 channels (HEK-TRPC6) [15,16] were maintained for varying durations at 37 °C in a culture medium supplemented with ouabain before being rinsed twice and collected. Total proteins were extracted and the amount of TRPC6 proteins was analyzed by Western blotting. As illustrated in Fig. 1A, cells exposed to the Na^+/K^+-ATPase inhibitor ouabain showed decreased protein levels of TRPC6. This effect developed in a time-dependent manner (Fig. 1B). For instance, the amount of TRPC6 proteins was reduced by ~30% ($n = 4$, $p < 0.05$) and ~55% ($n = 8$, $p < 0.01$) after a 2 h- and a 4 h-ouabain treatment, respectively.

Next, the question of the mechanisms sustaining the reduction of TRPC6 protein levels was addressed. It is well recognized that the inhibition of the Na^+/K^+-ATPase depolarizes cells. To test the role of the depolarization on the diminution of the abundance of TRPC6 proteins, HEK-TRPC6 cells were maintained in a culture medium in which 20 mM KCl had been added in order to depolarize the membrane. However, after 4 h in a KCl-rich culture medium, the amount of TRPC6 proteins expressed by HEK-TRPC6 cells was unaffected (Fig. 2). This finding is in line with data showing that in cultured cortical neurons 20 mM KCl does not affect the amount of AMPA receptors, which are also associated with the Na^+/K^+-ATPase [11].

Ouabain induces the endocytosis and degradation of GlyT2 in lysosomes [10] whereas the ouabain-dependent degradation of AMPA receptors involves the proteasome [11]. To investigate the intracellular pathway responsible for the ouabain-dependent regulation of TRPC6, experiments were conducted with hypertonic sucrose (which impairs