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Original article

Regulation of voltage-gated cardiac sodium current by epidermal growth factor receptor kinase in guinea pig ventricular myocytes

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Abstract

Voltage-gated cardiac fast sodium channel current (I_{Na}) plays a critical role in the initiation and propagation of the myocardial action potential, and regulation of cardiac I_{Na} by protein tyrosine kinases (PTKs) is not well documented, though it is known that ion channels are among the targets of PTKs. The present study was therefore designed to investigate whether/how cardiac I_{Na} was modulated by PTKs in guinea pig ventricular myocytes using whole-cell patch clamp and immunoprecipitation and Western blotting approaches. It was found that cardiac I_{Na} was enhanced by epidermal growth factor (EGF), and the effect was antagonized by the selective epidermal growth factor receptor (EGFR) kinase inhibitor tyrphostin AG556 while potentiated by orthovanadate (a protein tyrosine phosphatase (PTP) inhibitor). In addition, AG556 inhibited, while orthovanadate increased I_{Na}, and the inhibition of I_{Na} by AG556 was antagonized by orthovanadate. Immunoprecipitation and Western blotting analysis demonstrated that tyrosine phosphorylation level of cardiac sodium channels was enhanced by EGF or orthovanadate, and reduced by AG556. The AG556-induced reduction of phosphorylation level was significantly reversed by orthovanadate. Our results demonstrate the novel information that EGFR kinase enhances, and PTPs reduce native cardiac I_{Na} in guinea pig ventricular myocytes.

Keywords: Protein tyrosine kinases; Epidermal growth factor receptor; Protein tyrosine phosphatases; Voltage-gated sodium current; Ion channels; Guinea pig ventricular myocytes; Heart

1. Introduction

Voltage-gated cardiac fast sodium channel current (I_{Na}) plays a critical role in the initiation and propagation of myocardial action potential, and is a primary target for the development of antiarrhythmic agents. It is generally believed that cardiac I_{Na}, like other types of ion channel currents, is regulated by protein phosphorylation [1,2]. It has been demonstrated that cardiac I_{Na} was modulated by the serine/threonine protein kinases, protein kinase A (PKA) and/or protein kinase C (PKC) [3–8].

Protein tyrosine kinases (PTKs), including receptor PTKs (e.g. EGFR kinase, epidermal growth factor receptor kinase) and nonreceptor PTKs (e.g. Src-related kinases), are associated with long term cellular processes such as cell growth, differentiation, and oncogenesis [1,9]. Recent studies showed that protein phosphorylation of tyrosine residues modulated ion channels [1,10,11], including Ca^{2+}, and several types of K^+ channels [11–14], as well as volume-sensitive Cl^- channels [15] in different types of cells. However, the regulation of cardiac I_{Na} by PTKs is not fully understood. Although several PTK inhibitors were recently shown to inhibit I_{Na} in rabbit ventricular myocytes [16], and a very recent study showed that cloned rat cardiac sodium channel $Na_V1.5$ was modulated by Fyn, a Src-family tyrosine kinase [17], it is unknown whether cardiac I_{Na} would be modulated by EGFR kinase. The present study was therefore designed to use whole-cell patch voltage

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clamp, and immunoprecipitation and Western blotting approaches to investigate whether and how cardiac I_{Na} would be regulated by EGFR kinase in guinea pig ventricular myocytes. We found that activation of EGFR kinase increased cardiac I_{Na} in ventricular myocytes from guinea pig heart.

2. Materials and methods

2.1. Myocyte preparation

Guinea pigs of either gender (250–300 g) were sacrificed by cervical dislocation after anesthetization with pentobarbital (40 mg/kg, i.p.) in accordance with the guideline of Animal Care and Use Committee for Teaching and Research of University of Hong Kong. Ventricular myocytes from guinea pig hearts were enzymatically dissociated by the procedure described previously [18] and the isolated myocytes were kept in a high-K^+ storage medium [18] at room temperature for 1 h before electrophysiological recording.

2.2. Solutions and chemicals

I_{Na} was determined in the solution containing (mM) 5 NaCl, 135 CsCl (or choline Cl), 1 $MgCl_2$, 10 glucose, 10 HEPES, 1.0 $CaCl_2$, 1.0 CoCl (pH adjusted to 7.3 with CsOH). The pipette solution contained (mM) 5 NaCl, 130 CsCl, 1 $MgCl_2$, 10 HEPES, 5 Cs-EGTA, 5 MgATP, 0.1 GTP, with pH adjusted to 7.2 with CsOH. 3-(4-Chlorophenyl)1-(1,1-dimethylethyl)-1h-pyrazolo[3,4-d]pyrimidin-4-amine (PP2, Tocris, Bristol, UK) was prepared as 10 mM stock solution in dimethylsulfoxide (DMSO, Sigma-Aldrich, St Louis, MO). All other reagents were obtained from Sigma-Aldrich. Epidermal growth factor (EGF) was reconstituted using 10 mM acetic acid containing 0.1% BSA to 20 μg/ml stock solution. Tyrphostin AG556 (AG556) and AG1295 were prepared as 100 mM stock solution in DMSO. The stocks were divided into aliquots and stored at −20 °C. Cs-orthovanadate (VO_4^{3-}) stock solution (0.5 mM) was made with distilled water, and pH of VO_4^{3-} working solution was adjusted to 7.3–7.4 with HCl.

2.3. Electrophysiology

I_{Na} was recorded as described previously [18–20] using the whole-cell patch clamp technique with an EPC-9 amplifier and Pulse software (HEKA Electronik, Lambrecht, Germany). Borosilicate glass (1.2-mm OD) pipettes were prepared with a Brown-Flaming puller (model P-97, Sutter Instrument Co, Novato, CA) to produce a tip with resistance of 0.6–0.9 MΩ when filled with the pipette solution. After a giga-ohm seal was obtained, the cell membrane was ruptured by gentle suction to establish the whole-cell configuration. Series resistance (R_s) and capacitance were electronically compensated. Care was taken to ensure that the voltage drop across R_s was <5 mV. Data were collected after 20 min of rupture membrane. When experiments showed any change of R_s or inadequate voltage control, data were discarded. All the experiments were performed under similar conditions (e.g. similar patch duration for each protocol) at room temperature (21–22 °C).

2.4. Immunoprecipitation and Western blot

Ventricular cells were treated with EGF, VO_4^{3-}, AG556, or AG556 plus VO_4^{3-} for 20 min at room temperature, and centrifuged at 4 °C. The cell pellet was then lysed with a lysis buffer containing (mM) 25 Tris, 150 NaCl, 100 NaF, 1.0 EDTA, 1.0 VO_4^{3-}, 1.0 phenylmethylsulfonyl fluoride, and 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 μg/ml leupeptin, and 1 μg/ml aprotenin. Protein quantification of lysates was made using a protein assay reader (Bio-Rad Laboratories, Hercules, CA), and diluted to equal concentrations. Proteins were immunoprecipitated overnight at 4 °C using 2 μg of anti-sodium channel antibody (III–IV loop, Upstate Biotechnology, Lake Placid, NY) and 50 μl of protein A/G beads (Upstate). Immunoprecipitated proteins bound to pelleted protein A/G beads were washed thoroughly with PBS solution, denatured in Laemmli sample buffer, separated using SDS-PAGE, and electroblotted onto nitrocellulose membranes. The immunoblots were probed with anti-phosphotyrosine antibody (1:1000, Upstate) overnight at 4 °C in a blocking solution containing 5% nonfat dry milk in TBS with 0.1% Tween 20, and subsequently treated with goat anti-mouse IgG-HRP antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence (ECL, Amersham Biosciences) and exposed on X-ray film (Fuji Photo Film GmbH, Düsseldorf, Germany). The blots were then stripped and reprobed with the anti-sodium channel antibody to determine total sodium channel protein levels. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK), and analyzed via GeneTools software (Syngene).

2.5. Statistical analysis

Nonlinear curve-fitting was performed using Pulsefit (HEKA) and Sigmaplot (SPSS, Chicago, IL). Paired and/or unpaired Student's two-tailed *t*-test were used as appropriate to evaluate the statistical significance of the differences between two group means, and ANOVA was used for multiple groups. Group data are expressed as means±SE. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Effect of EGF on I_{Na}

Fig. 1A shows the time course of I_{Na} recorded in a representative cell with a 30-ms voltage step from −130 to −35 mV (inset) in the absence and presence of 100 ng/ml EGF. EGF gradually increased I_{Na}, accelerated activation and inactivation, and caused a cross over of current traces (Fig. 1B). The current traces were fitted to mono-exponential function with activation and inactivation time constants (τ_m and τ_h) in the absence and presence of 100 ng/ml EGF (Fig. 1B). The τ_m and