Crosstalk between Na⁺,K⁺-ATPase and a volume-regulated anion channel in membrane microdomains of human cancer cells

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ABSTRACT

Low concentrations of cardiac glycosides including ouabain, digoxin, and digitoxin block cancer cell growth without affecting Na⁺,K⁺-ATPase activity, but the mechanism underlying this anti-cancer effect is not fully understood. Volume-regulated anion channel (VRAC) plays an important role in cell death signaling pathway in addition to its fundamental role in the cell volume maintenance. Here, we report cardiac glycosides-induced signaling pathway mediated by the crosstalk between Na⁺,K⁺-ATPase and VRAC in human cancer cells. Submicromolar concentrations of ouabain enhanced VRAC currents concomitantly with a deceleration of cancer cell proliferation. The effects of ouabain were abrogated by a specific inhibitor of VRAC (DCPIB) and knockdown of an essential component of VRAC (LRRC8A), and they were also attenuated by the disruption of membrane microdomains or the inhibition of NADPH oxidase. Digoxin and digitoxin also showed anti-proliferative effects in cancer cells at their therapeutic concentration ranges, and these effects were blocked by DCPIB. In membrane microdomains of cancer cells, LRRC8A was found to be co-immunoprecipitated with Na⁺,K⁺-ATPase α1-isoform. These ouabain-induced effects were not observed in non-cancer cells. Therefore, cardiac glycosides were considered to interact with Na⁺,K⁺-ATPase to stimulate the production of reactive oxygen species, and they also apparently activated VRAC within membrane microdomains, thus producing anti-proliferative effects.

1. Introduction

Previous epidemiological studies demonstrated that treatment with cardiac glycosides, such as digitoxin and digoxin, prevents cancer recurrence and improves survival in cancer patients [1]. In fact, cardiac glycosides attenuate the development of carcinomas in breast cancer, leukemia, lymphoma and genitourinary tract cancer [2–6]. Thus, Na⁺,K⁺-ATPase, the target of cardiac glycosides, is considered as a potent molecule with clinical benefit in cancer treatment [7]. Na⁺,K⁺-ATPase is composed of a catalytic α-subunit and a glycosylated β-subunit; four α-isoforms and three β-isoforms have been identified in the mammals [8], and the ATPase maintains the membrane potential by establishing Na⁺ and K⁺ gradients across the plasma membrane. A cardiac glycoside ouabain blocks the ion-pumping activity of Na⁺,K⁺-ATPase at micromolar concentrations, whereas submicromolar concentrations of ouabain inhibit cancer cell proliferation without changing the catalytic (pumping) activity [2,6]. It has been reported that submicromolar ouabain affects several key enzymes in the cell [3–5]; however, the mechanisms of ouabain-mediated anti-cancer effects remain unclear.

The volume-regulated anion channel (VRAC), also known as the volume-sensitive outwardly rectifying (VSOR) anion channel, is activated in hypotonic conditions and is ubiquitously involved in cell volume regulation [9,10]. In addition to this house-keeping role, VRAC

most likely contributes to the cell death signaling pathway: that is, abnormal cell shrinkage in isotonic conditions is a pivotal event in apoptosis [11,12]. Leucine rich repeat containing 8 family, member A (LRRC8A), also known as SWELL1, has been identified as an essential component of VRAC [13,14]. Based on the implicated role, it may be hypothesized that VRAC is involved in ouabain-induced anti-cancer effects. In the present study, we found that submicromolar ouabain selectively activates VRAC present in the membrane microdomains of human cancer cells but not in non-cancer cells, and that VRAC activation is essential for eliciting cardiac glycosides-induced anti-proliferative effects.

2. Materials and methods

2.1. Chemicals

Ouabain, digoxin, digitoxin, methyl-β-cyclodextrin (MβCD), anti-β-actin antibody (clone AC-74; A5316), [(dihydroindenyl)oxy]acetic acid (DIOA), apocynin and VAS2870 were obtained from Sigma-Aldrich (St. Louis, MO, USA). 4-(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3 -dihydro-1-oxo-1H-inden-5-yl)oxybutanoic acid (DCPIB) was from Tocris Bioscience (Ellisville, MO, USA). The anti-Na$^+$,K$^+$-ATPase α1 isoform (α1NaK) mouse monoclonal antibody (clone 464.6; sc-21712) and protein A/G-agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Furosemide and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(3,4-*d*)pyrimidine (PP2) were from Wako Pure Chemical Industries (Osaka, Japan). Saracatinib was from Cayman (Ann Arbor, MI, USA). Fetal bovine serum (FBS) was from Nichirei Bioscience Inc. (Tokyo, Japan). Horse-radish peroxidase-conjugated anti-mouse IgG was from Millipore (Bedford, MA, USA). Anti-Src antibody was from Cell Signaling Technology (Beverly, MA, USA). Anti-p-Src antibody was from R&D System (Minneapolis, MN, USA). Alexa Fluor 488-conjugated anti-mouse IgG antibody was from Invitrogen (Carlsbad, CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). ^{86}RbCl was from PerkinElmer (Waltham, MA). All other reagents were of molecular biological grade or the highest grade of purity available.

2.2. Cell culture and transfection of siRNA

Human colorectal carcinoma HT-29, human embryonic kidney HEK293 and human fibroblast Hs68 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 10% FBS. Human epidermoid cancer KB cells were maintained in minimum essential medium (MEM) (Sigma-Aldrich) supplemented with 10% FBS. Human hepatocellular carcinoma HepG2 cells were maintained in MEM supplemented with 1% non-essential amino acids and 10% FBS. HT-29 cells (1×10^6 cells) were transfected with 100 pmol of siRNA via Lonza nucleofection using solution R and program W-017. The transfected cells were cultured in growth medium for 48 h. FAM-labeled LRRC8A-siRNA (GGUACAACCACAUCGCCUA) [14] and negative control siRNA were purchased from Nippon Gene. Stealth RNAi siRNAs for α1NaK (HSS181499) and for negative control high GC duplex were obtained from Invitrogen.

2.3. Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature (24–26 °C), as described previously [15]. pClamp software (version 9.2; Axon Instruments) was used for command pulse control, data acquisition, and analysis. Patch electrodes had a resistance of 2–4 MΩ when filled with pipette solution. The access resistance was electrically compensated by 70% to minimize voltage errors. The time course of current activation and recovery was monitored by repetitively applying (every 15 s) alternating step pulses (2 s duration) from a holding potential of 0 to ± 40 mV. To observe the voltage dependence of the current profile, step pulses (2 s duration) were applied from a prepotential of −100 mV to test potentials of −100 to +100 mV in 20 mV increments. Amplitude of the current was measured 1.25 ms after the step pulse onset. The pipette solution contained 110 mM CsCl, 2 mM MgSO$_4$, 1 mM Na$_2$ATP, 1 mM EGTA, 10 mM HEPES, 15 mM Na-HEPES, and 50 mM mannitol (pH 7.3). The control bathing solution (305–320 mosmol/kg H$_2$O) contained 110 mM CsCl, 5 mM MgSO$_4$, 7 mM Tris, 12 mM HEPES, and 75–90 mM mannitol (pH 7.4). The hypotonic solution (270 mosmol/kg H$_2$O) contained 110 mM CsCl, 5 mM MgSO$_4$, 7 mM Tris, 12 mM HEPES, and 40 mM mannitol (pH 7.4).

2.4. Measurement of mitochondrial dehydrogenase activity

Mitochondrial dehydrogenase activity was assessed using an MTT cell proliferation assay kit (Cayman Chemical Company). 2×10^6 cells were seeded in a 6-cm dish. After seeding (24 h later), cells were pretreated with DCPIB for 1 h. Then ouabain and DCPIB were added for 1 h in DMEM or MEM supplemented with 10% FBS. Cells were harvested, counted, and aliquoted in equal numbers (3×10^5 cells), then incubated for 1 h at 37 °C in DMEM or MEM supplemented with 10% FBS. The cells were mixed with MTT reagents. After incubating for 30 min at 37 °C, the cells were centrifuged at $300 \times g$ for 5 min. The supernatants were discarded, and cell pellets were dissolved in crystal dissolving solution. Absorbance was measured at 570 nm using a microplate reader (Sunrise Remote, Tecan).

2.5. Cell proliferation assay

In each well of a 24-well culture plate, 1×10^5 cells were seeded. After seeding (24 h later), cells were pretreated with or without DCPIB for 1 h. Then, the cells were treated with or without cardiac glycoside (ouabain, digoxin, or digitoxin) and/or DCPIB for 1 h, and the cell numbers in each well were counted after the treatment (first counting). When indicated, the cells were treated with H$_2$O$_2$ for 1 h. After washing out of all drugs, the cells were cultured for another 24 h in DMEM or MEM supplemented with 2% FBS in the absence of ouabain, and the cell numbers in each well were counted after the culture (second counting). Cell proliferation was defined as an increased cell numbers between first and second countings.

2.6. Isolation of membrane microdomains

HT-29 cells were lysed with ice-cold MBS buffer (150 mM NaCl, 25 mM Mes-NaOH, pH 6.5) containing 1% CHAPS, 10 μg/ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A for 15 min. The solution was mixed with an equal volume of 66% sucrose in MBS buffer, and the mixture was placed at the bottom of an ultracentrifuge tube. A discontinuous gradient was formed by overlaying this layer with 30% sucrose and the 5% sucrose solutions. The sample was centrifuged at $261,000 \times g$ in a SW41Ti rotor (Beckman) for 18 h at 4 °C. Ten fractions of 1 ml each were collected from the top of the gradient, and proteins were precipitated using acetone before Western blotting was performed. For the depletion of cholesterol, the cells were treated with 10 mM MβCD for 30 min at 37 °C before harvesting.

2.7. Immunoprecipitation

The fractions of membrane microdomains (DRM fractions) and non-membrane microdomains (non-DRM fractions) were lysed with MBS buffer containing 1% Triton X-100 for 30 min on ice. The lysate was pre-cleared with protein A/G-agarose beads for 5 h at 4 °C, and reacted with anti-LRRC8A antibody or rabbit preimmune serum for 15 h at 4 °C. The sample was then incubated with protein A/G-agarose beads for 5 h at 4 °C. The beads were washed and eluted into 250 mM Tris-HCl (pH 6.8) supplemented with 8% SDS, 4% glycerol, and 10% β-