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Aconitine alters connexin43 phosphorylation status and $[Ca^{2+}]$ oscillation patterns in cultured ventricular myocytes of neonatal rats

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Abstract

Aconitine, a highly poisonous type of alkaloid, has a widespread effect in stimulating the membranes of cardiomyocyte. However, other effects of aconitine on cardiomyocyte are unknown. In this study, we investigated whether aconitine also affects the phosphorylation status of connexin43 (Cx43) and intracellular $[Ca^{2+}]$ oscillation patterns in cultured ventricular myocytes of neonatal rats. As determined by Western blot analysis, a decreased percentage ($47.68 \pm 2.29\%$) of phosphorylated Cx43 (P-Cx43) and a concomitant increased percentage ($52.32 \pm 2.29\%$) of nonphosphorylated Cx43 (NP-Cx43) were found in aconitine-treated cultures, compared to the controls ($82.77 \pm 2.04\%$ for P-Cx43 and $17.23 \pm 2.04\%$ for NP-Cx43). Quantitative immunofluorescent microscopy revealed similar changes in phosphorylation status occurring in Cx43 containing gap junctions in the cultures under the same treatment conditions. Real-time laser scanning microscopy indicated that intracellular $[Ca^{2+}]$ oscillations were relatively stable in control cultures, with occasional calcium sparks; after being treated with aconitine, high frequency $[Ca^{2+}]$ oscillations emerged, whereas typical calcium sparks disappeared. Furthermore, Western blot analysis revealed that, after aconitine treatment, the amount of phosphorylated PKCα decreased significantly. These observations suggest that aconitine not only induces dephosphorylation of Cx43 and PKCα, but also alters intracellular $[Ca^{2+}]$ oscillation patterns in cultured cardiomyocytes.
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1. Introduction

Aconitine is the most toxic alkaloid contained in the tubers of *Aconitium* plants. *Aconitum napellus L.* a member of this genus, is frequently cultivated as an ornamental plant in Europe, due to its attractive dark-blue flowers; while in Asia, aconite tubers and their by-products have been used in traditional herbal medicines or as poison for spears and arrows since ancient times. One gram of the tuber can be fatal and the lethal oral dose of aconitine for humans is estimated to be 1–2 mg (Camps, 1976; Baselt, 2004). Aconitine poisoning is frequently caused by accidental ingestion, mainly in Asian countries; however, it is sometimes used in suicides or homicides worldwide (Elliott, 2002; Van Landeghem et al., 2006). Aconitine suppresses the deactivation of voltage-gated sodium channels, which prevents complete repolarization of the excitable membrane of neural, muscle and cardiac tissues (Ameri, 1998; Wang and Wang, 2003). Therefore, it has been widely used for creating models of cardiac arrhythmia.

Gap junctions, composed of connexin (Cx) protein subunits, form conduits between adjacent cells. They coordinate intercellular communication and allow the transfer of molecules of less than 1000 Da, including ions, amino acids, nucleotides, second messengers, as well as other metabolites, to selectively pass from one cell to another. To date, the connexins comprise a gene family of 20

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members in the mouse and 21 members in the human genome. Cx37, Cx40, Cx43 and Cx45 are the most important in the cardiovascular system (for a detailed overview, see Söhl and Willecke, 2004; Laird, 2005). It is reported that aconitine-induced arrhythmia could be suppressed by the antiarrhythmic peptide (AAP) (Aonuma et al., 1983), a hexapeptide which can improve cellular coupling and increase cardiac gap junction conductance (Dhein, 2002). Cardiac gap junctions are known to be essential for conduction of the electrical impulse among cardiomyocytes, and Cx43 is the primary gap junction protein in the cardiac ventricles. As a phosphorprotein, Cx43 is highly regulated by different protein kinases at different serine or tyrosine residues in its carboxyl terminal domain (Solan and Lampe, 2005; Laird, 2005). There are reports that electrical uncoupling induced by myocardial ischemia is associated with Cx43 dephosphorylation of Ser368 (Beardslee et al., 2000; Axelsen et al., 2006). These pieces of evidence indicate that aconitine-induced electrical uncoupling and arrhythmia among cardiomyocytes could be related, at least in part, to Cx43 dephosphorylation.

On the other hand, as one of the most powerful modifiers of the sodium channels, aconitine prolongs the open state of the channels, favoring the entry of a large quantity of Na^+ into cytosol (Peper and Trautwein, 1967; Lu and De Clerck, 1993), which may eventually result in intracellular Ca^{2+} overload. Therefore, real-time measurement of intracellular $[Ca^{2+}]$ changes might be necessary to explain aconitine-induced ionic disequilibrium in cardiomyocytes.

In this work, we investigated the effects of aconitine on Cx43 phosphorylation status and intracellular $[Ca^{2+}]$ oscillation patterns in cultured ventricular myocytes of neonatal rats. Since the protein kinase C-α (PKCα) was reported to have a part in regulating the phosphorylation of Cx43 (Dhein, 2002; Weng et al., 2002), we also detected the phosphorylation status of PKCα simultaneously. We also evaluated the mechanisms underlying these aconitine-induced effects.

2. Materials and methods

2.1. Cell isolation and culture

Primary cultures were prepared from the ventricles of 1-day-old Sprague–Dawley rats, according to the procedures described by Lüss et al. (2002), Zeevi-Levin et al. (2005), with some modifications. Hearts were removed, placed in ice-cold Hanks' balanced salt solution (HBSS, without calcium chloride or magnesium sulfate, Sigma, St. Louis, MO, USA) and washed twice. Ventricles were isolated, minced and then digested in HBSS containing 0.8 mg/ml trypsin (Sigma), 1 mg/ml collagenase type II (Invitrogen-GIBCO, Carlsbad, CA, USA) and 0.5 mg/ml bovine serum albumin (Sigma) by stirring at 37 °C for 10 min; the cell suspension was collected. This process was repeated three times. The first supernatant containing cell debris and blood cells was discarded. Cell suspensions from the three subsequent digestions were centrifuged at 4 °C and 1000 rpm for 10 min, then the resultant pellet was resuspended in 3 ml of Dulbecco's modified Eagle's medium (DMEM, with 4500 mg/L glucose, L-glutamine, and 25 mM HEPES, Sigma), containing 15% fetal bovine serum (FBS, GIBCO), and the procedure was repeated once again. Cells were pooled and resuspended in complete DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (GIBCO) and 15% FBS, then incubated for 1 h at 37 °C, to induce selective attachment of non-myocytes. The unattached cells were plated at a density of 1×10^4 cell/cm^2 on multi-well culture plates (Corning, Acton, MA, USA) pre-coated with collagen type-I (Sigma) and maintained at 37 °C, in 5% CO_2. To inhibit the growth of fibroblast, 0.1 mM 5-BrdU (Sigma–Aldrich) was added to the medium. After 24 h, a confluent monolayer of slowly synchronously beating cardiomyocytes had developed. Cultures were rinsed and replenished with fresh DMEM containing 10% FBS at the moment, then the medium was replenished every 48 h.

Subsequent experiments were performed on day 6, after equilibrating the cells under serum-free conditions for 18 h. During preliminary experiments, we had identified the cultures using anti-cTnI antibodies (Abcam, Cambridge, UK; ab19615) and estimated that most of the cultured cells were cardiomyocytes.

2.2. Preparations of the drugs and experiment conditions

Aconitine was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) and ammonium chloride (NH_4Cl) were purchased from Calbiochem (San Diego, CA, USA) and Sigma, respectively. Antiarrhythmic peptide (AAP) was purchased from United States Biological (Swampscott, MA, USA). All of the drug stock solutions (100×) were prepared according to the manufacturers' instructions and diluted in serum-free DMEM to the desired concentrations immediately before use. The final concentration of DMSO in MG132 solution was less than 0.05%, which had no significant effect on the experiments.

In quantitative Western blot analysis of Cx43 protein isoforms, cultures were treated with aconitine (1 μM, for 1 h), AAP (0.1 μg/ml, for 4 h), NH_4Cl (10 mM, for 4 h) and MG132 (10 μM, for 4 h), respectively; or pretreated with AAP, NH_4Cl and MG132 for 4 h, followed by 1 h of aconitine incubation under the same concentration conditions. NH_4Cl and MG132 were only used in quantitative Western blot analysis of Cx43 protein isoforms.

2.3. Protein extraction and quantitative Western blotting

Cultures were rinsed twice with ice-cold HBSS and harvested by scraping. A membrane protein extracting kit (BioVision, Mountain View, CA, USA) was used according to the recommended protocol. The pellets of cellular

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