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Evidence for aconitine-induced inhibition of delayed rectifier K^+ current in Jurkat T-lymphocytes

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ABSTRACT

Aconitine (ACO) is a highly toxic diterpenoid alkaloid and known to exert the immunomodulatory action. However, whether it has any effects on ion currents in immune cells remains unknown. The effects of ACO and other related compounds on ion currents in Jurkat T-lymphocytes were investigated in this study. ACO suppressed the amplitude of delayed-rectifier K^+ current ($I_{K(DR)}$) in a time- and concentration-dependent manner. Margatoxin (100 nM), a specific blocker of $K_V1.3$-encoded current, decreased the $I_{K(DR)}$ amplitude in these cells and the ACO-induced inhibition of $I_{K(DR)}$ was not reversed by 1-ethyl-2-benzimidazolinone (30 μM) or nicotine (10 μM). The IC_{50} value for ACO-mediated inhibition of $I_{K(DR)}$ was 5.6 μM. ACO accelerated the inactivation of $I_{K(DR)}$ with no change in the activation rate of this current. Increasing the ACO concentration not only reduced the $I_{K(DR)}$ amplitude, but also accelerated the inactivation time course of the current. With the aid of minimal binding scheme, the inhibitory action of ACO on $I_{K(DR)}$ was estimated with a dissociation constant of 6.8 μM. ACO also shifted the inactivation curve of $I_{K(DR)}$ to a hyperpolarized potential with no change in the slope factor. Cumulative inactivation for $I_{K(DR)}$ was enhanced in the presence of ACO. In Jurkat cells incubated with amiloride (30 μM), the ACO-induced inhibition of $I_{K(DR)}$ remained unaltered. In RAW 264.7 murine macrophages, ACO did not modify the kinetics of $I_{K(DR)}$, although it suppressed $I_{K(DR)}$ amplitude. Taken together, these effects can significantly contribute to its action on functional activity of immune cells if similar results are found *in vivo*.

1. Introduction

Aconitine (ACO) is an intensely poisonous alkaloid derived from plant species *Aconitium* (Ranunculaceae) (Singhuber et al., 2009; Borcsa et al., 2011; Xu et al., 2011). ACO-containing herbal extracts have been demonstrated to exert the inhibition of proliferation in a variety of neoplastic cells (Chodoeva et al., 2005; Garmanchouk et al., 2005; Dasyukevich and Solyanik, 2007; Yan et al., 2007) and have serious toxicity in rat embryos (Xiao et al., 2007) and in protozoans (Tang et al., 2010). ACO and methyllycaconitine, its structurally related analog, were reported to be antagonists of α_7-nicotinic receptors (Hardick et al., 1995; Mogg et al., 2002; Ivy Carroll et al., 2007; De Rosa et al., 2009). Noroxoaconitine, another structurally related compound, was recently described to be an inhibitor of mitogen-activated protein kinase (Kostenko et al., 2011). In our laboratory, we reported that this compound can block ultrarapid-delayed rectifier K^+ currents in heart-derived H9C2 cardiac myoblasts. However, little information has been reported concerning the effects of ACO on ion currents in immune cells, although it could have immunomodulatory actions (Makino et al., 2009; Singhuber et al., 2009; Zhang et al., 2011).

Voltage-dependent K^+ (K_V) channels are recognized to play a crucial role in the repolarization of membrane potential in nerve and muscle, controlling their excitability. Due to the maintenance of membrane potential, they are involved in the proliferation and activation of immune cells (Chandy et al., 2004; Panyi, 2005; Gilhar et al., 2011). Specifically, $K_V1.3$ performs a key function in the immune system, controlling and leading to proliferation and interleukin-2 synthesis (Chung and Schlichter, 1997; Beeton et al., 2005; Gilhar et al., 2011). Effects memory T-cells, which express high levels of $K_V1.3$ (Helms et al., 1997; Villalonga et al., 2010), are present in autoimmune disorders. $K_V1.3$ channel was reported to show more specificity for autoreactive T cells than any molec-

Abbreviations: ACO, aconitine; 1-EBIO, 1-ethyl-2-benzimidazolinone; FBS, fetal bovine serum; IC_{50}, the concentration required for a 50% inhibition; IK_{Ca} channel, intermediate-conductance Ca^{2+}-activated K^+ channel; $I_{K(DR)}$, delayed-rectifier K^+ current; *I–V*, current–voltage; K_D, dissociation constant; K_V channel, voltage-dependent K^+ channel; MgTx, margatoxin; SEM, standard error of mean; SSR, sum of squared residuals.

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ular target expressed on all T cells (Beeton et al., 2005; Gilhar et al., 2011). Besides that, several important studies have demonstrated that antagonizing the activity of $K_V1.3$ with either highly specific peptides or small-molecule channel blockers can reverse and prevent experimental autoimmune encephalomyelitis in rats (Vennekamp et al., 2004; Beeton et al., 2005; Pegoraro et al., 2009; Gilhar et al., 2011).

The Jurkat T cell line, a CD45-deficient clone derived from the E6-1 clone of Jurkat human T cell leukemic cell line, was demonstrated to express $K_V1.3$-type $I_{K(DR)}$ (Pang et al., 2010; Villalonga et al., 2010). These cells can produce large amounts of interleukin-2 after treatment with phorbol esters. The $K_V1.3$ K^+ channel, that exhibits unique gating properties and voltage dependency, has been described to be an important contributor to the $I_{K(DR)}$ in immune cells (Wu et al., 1998; Pang et al., 2010; Panyi, 2005; Villalonga et al., 2010; Gilhar et al., 2011). Therefore, in this study, we used the Jurkat T leukemia cell line as a model and clearly demonstrated that ACO could suppress $I_{K(DR)}$ in these cells in a concentration- and time-dependent fashion. Our results provide important insights on the still mysterious biological effects of this compound (Singhuber et al., 2009).

2. Materials and methods

2.1. Drugs and solutions

ACO (aconitine or acetylbenzoylaconine, $C_{34}H_{47}NO_{11}$), nicotine and tetrodotoxin were purchased from Sigma Chemicals (St. Louis, MO, USA). The purity of ACO was over 99%. ACO was dissolved in dimethyl sulfoxide and made immediately before the experiments. Amiloride, 1-ethyl-2-benzimidazolinone (1-EBIO), methyllycaconitine were obtained from Tocris Cookson, Ltd. (Bristol, UK), and margatoxin (MgTx) was purchased from Alomone Labs (Jerusalem, Israel). All culture media, FBS, L-glutamine, trypsin/EDTA, penicillin–streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Reagent water that was obtained using a Milli-Q Ultrapure Water Purification System (Millipore, Bedford, MA, USA) was used in all experiments. The composition of bath solution (i.e., normal Tyrode's solution) was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, and HEPES-NaOH buffer 5.5 (pH 7.4). To record K^+ currents and avoid contamination of Cl^- current (Maldonado et al., 1991), the patch pipette was filled with a solution (mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, Na_2ATP 3, Na_2GTP 0.1, EGTA 0.1, and HEPES-KOH buffer 5 (pH 7.2).

2.2. Cell preparations

The Jurkat T cell line, a human T cell lymphoblast-like cell line (clone E6-1), was obtained from the Bioresource Collection and Research Center (BCRC-60255; Hsinchu, Taiwan), and the murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (TIB-71; Manassas, VA, USA). Jurkat T cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (v/v), 100 U/ml penicillin and 10 μg/ml streptomycin. RAW 264.7 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 10 μg/ml streptomycin (Wu et al., 2011). They were maintained at 37 °C in a 95% air and 5% CO_2 humidified atmosphere. The viability of these cells was often assessed by the trypan blue dye-exclusion test. The experiments were made five or six days after cells had been cultured (60–80% confluence).

2.3. Electrophysiological measurements

Before the experiments, either Jurkat T or RAW 264.7 cells were dissociated with 1% trypsin/EDTA solution and an aliquot of cell suspension was thereafter transferred to a recording chamber affixed to the stage of a DM-IL inverted microscope (Leica, Wetzlar, Germany). They were bathed at room temperature (20–25 °C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch electrodes made from Kimax-51 capillaries (Kimble Glass, Vineland, NJ, USA) using a PP-830 puller (Narishige, Tokyo, Japan) had a resistance of 3–5 MΩ when they were filled with different intracellular solutions described above. Patch clamp recordings were performed in the whole-cell configuration using an RK-400 amplifier (Bio-Logic, Claix, France) (Wu et al., 1998; Huang et al., 2011).

2.4. Data analyses

The data were stored online in a TravelMate-6253 computer (Acer, Taipei, Taiwan) at 10 kHz through a Digidata 1322A interface (Molecular Devices, Sunnyvale, CA, USA). The latter device was equipped with an Adaptec SlimSCSI card (Milpitas, CA, USA) via PCMCIA slot and then controlled by pCLAMP9.2 during electrophysiological recordings (Molecular Devices). Currents were low pass-filtered at 3 kHz. The pCLAMP-generated voltage-step protocols were employed to determine *I–V* relationships for ion currents (e.g., $I_{K(DR)}$). Ion currents were analyzed by using Origin 8.0 (OriginLab, Northampton, MA, USA) or custom-made macros built in an Excel 2007 spreadsheet running on Windows 7 (Microsoft, Redmond, WA, USA).

The concentration–response data for inhibition of $I_{K(DR)}$ in Jurkat T-lymphocytes were fitted to the Hill equation. That is,

$$\text{percentage inhibition} = \frac{E_{max} \times [C]^{n_H}}{[C]^{n_H} + IC_{50}^{n_H}},$$

where [C] is the ACO concentration, IC_{50} and n_H are the concentration required for a 50% inhibition and the Hill coefficient, respectively, and E_{max} is the maximal inhibition of $I_{K(DR)}$ caused by ACO.

The inhibitory effect of ACO on $I_{K(DR)}$ in Jurkat T-lymphocytes is explained by a state-dependent blocker that can bind to the open state of the channel according to a minimal kinetic scheme:

$$C \underset{\beta}{\overset{\alpha}{\rightleftarrows}} O \underset{K_{-1}}{\overset{K_{+1}}{\rightleftarrows}} O \cdot B,$$

where α and β are the voltage-dependent rate constants for the opening and closing of K_V channels, k_{+1} and k_{-1} are those for blocking and unblocking by ACO, and [B] is the ACO concentration. C, O and O·B denote the closed, open, and open-blocked states, respectively.

The blocking and unblocking rate constants, k_{+1} and k_{-1}, were determined from the inactivation time constants of $I_{K(DR)}$ in response to the depolarizing pulses. Blocking and unblocking rate constants were estimated using the relation:

$$\frac{1}{\tau_b} = k_{+1} \times [B] + k_{-1}$$

Specifically, k_{+1} and k_{-1}, respectively, result from the slope and the *y*-axis intercept at [B] = 0 of the linear regression interpolating the reciprocal time constant ($1/\tau_b$) versus different ACO concentrations (0.1–10 μM). Based on k_{+1} and k_{-1}, the K_D value (i.e., k_{-1}/k_{+1}) was generated.

The steady-state inactivation curve of $I_{K(DR)}$ in the absence and presence of ACO was plotted against the test potential and fit to the Boltzmann equation:

$$I = \frac{I_{max}}{1 + \exp((V - V_{1/2})/k)},$$

where *V* is the conditioning potential in mV, $V_{1/2}$ is the membrane potential for half-maximal inactivation, and *k* is the slope factor of inactivation curve for $I_{K(DR)}$. The solver subroutine build in Microsoft Excel (Redmond, WA, USA) was used to fit the data by a least-squares minimization procedure (Kemmer and Keller, 2010).

All values are expressed as the mean ± SEM with sample sizes (*n*) indicating the number of cells from which the data were taken, and error bars are plotted as SEM. The paired or unpaired Student's *t*-test and one-way analysis of variance with the least-significant-difference method for multiple comparisons were used to evaluate statistical difference among means. To evaluate the values of sum of squared residuals (SSR), confidence interval was estimated with the use of Fisher's *F* distribution (Kemmer and Keller, 2010). A value of $p < 0.05$ was considered significant. The statistical analyses in this study were performed in SAS 8.2 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effect of ACO on $I_{K(DR)}$ in Jurkat T-lymphocytes

The whole-cell configuration of the patch-clamp technique was initially used to evaluate the effect of ACO on ion currents in Jurkat T-lymphocytes. To record K^+ outward currents and eliminate Ca^{2+}-activated K^+ currents, cells were bathed in Ca^{2+}-free Tyrode's solution. When the cell was held at −50 mV and depolarizing voltage pulses from −50 to +60 mV in 10-mV increments were applied with a duration of 300 ms, a family of outward currents was elicited (Fig. 1). The threshold for elicitation of these outward currents was around −30 mV, and the magnitude became larger with greater depolarizations. This population of outward currents were identified as $I_{K(DR)}$ and to resemble the $K_V1.3$-encoded currents (Matteson and Deutsch, 1984; Wu et al., 1998; Panyi, 2005; Villalonga et al., 2010). Notably, 2 min after exposure to ACO (30 μM), the $I_{K(DR)}$ amplitude was greatly reduced at the potentials ranging from −20 to +60 mV. For example, when depolarizing pulses from −50 to +50 mV were applied, ACO (30 μM) significantly