Electrophysiological effects of anthopleurin-Q on rat hepatocytes

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INTRODUCTION

Anthopleurin-Q (AP-Q) is a newly purified polypeptide toxin extracted from marine invertebrates, Anthopleura. Recently, it was reported that AP-Q had a cardiac antihypertrophy activity in rats[1]. It could attenuate the electrophysiological remodeling in rat hypertrophied myocardium. Carbon tetrachloride (CCl4) was chosen as the hepatic toxicant to induce liver injury. After single CCl4 administration, there was a progressive depolarization in rat liver cells between 3 and 27 h. At 48 h, a definite repolarization occurred. At 72 h, the surviving cells had resting potentials not significantly different from control values[2,3]. Hepatocytes isolated from CCl4-induced cirrhotic rats had a lower membrane potential than normal healthy hepatocytes[4]. From the above, damaged hepatocytes have a depolarized membrane potential, but the mechanisms for maintenance of the resting membrane potential in hepatocytes are not well understood. Previous study showed that CCl4 inhibited K+ efflux through K+ channel[5]. We investigated the effects of AP-Q on acute liver injury induced by CCl4, and on membrane currents of isolated rat hepatocytes in order to explore the mechanisms against CCl4-induced liver injury.

MATERIALS AND METHODS

Materials

AP-Q was provided by Qingdao Marine Biology Research Institute. It is a stable and basic polypeptide consisting of 40 amino acid residues, with a molecular weight of 4840 dalton, and a purity >99%. It is easily dissolved in distilled water. CCl4 was from Beijing Chemical Plant.

Animals and treatments

Kunming strain mice of either sex weighing 18-22 g were used. The animals were divided into five groups, 12 animals each group. Acute liver injury was induced by intraperitoneal injection of a 0.1% (v/v) CCl4 solution in olive oil at a dose of 10 mL per kg body weight. The control group was injected intraperitoneally with an equal volume of olive oil. AP-Q (3.5, 7 and 14 µg/kg) dissolved in saline was intraperitoneally injected once every day, for 7 days. CCl4 treatment was given 1 h after the last dose. The same volume of saline was given to control group in the same manner. Animals of each group were killed by cervical dislocation 16 h after CCl4 treatment, blood was collected from the orbital plexus and stored in a non-heparinized tube. Serum was separated from blood and stored at -20 °C until use.

Biochemical determinations

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using a commercial assay kit (Jian Cheng Co., Nanjing). Enzyme activities were expressed in Karman units (U/L).

Isolation of hepatocytes

Hepatocytes were isolated with the modified method reported by Seglen[6,7]. Briefly, adult Wistar rats of either sex (175±25 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The portal vein and inferior vena cava were cannulated and perfused with oxygenated Ca2+-free Hank’s solution 30 mL/min at 37 °C for 4-5 min followed by perfusion with Ca2+-free Hank’s solution containing collagenase (Type I, Sigma) (0.3 g/L) for 10 min. The liver was chopped in 10 mL Ca2+-free Hank’s solution. The cell suspension was filtered through a 200 mesh gauze and then centrifuged three times (50 g, 2 min) to separate liver cells. The cells were plated onto the coverslips and incubated in KB medium for 2 h and preserved in DMEM at 4 °C.

METHODS

A single dose of CCl4 (10 µg/mL, ip) was injected to induce acute liver injury in rats. Serum aminotransferase activities were determined. Whole cell patch-clamp techniques were used to investigate the effects of AP-Q on delayed outward potassium current (I\text{Ko}), inward rectifier potassium current (I\text{K1}) and calcium release-activated calcium current (I\text{CRAC}).

RESULTS: AP-Q (3.5 and 7 µg/kg) pretreatment significantly reduced ALT and AST activities. AP-Q 0.1-100 nM produced an apparent concentration-dependent increase of I\text{Ko} with EC50 value of 5.55±1.8 nM (n=6). AP-Q 30 nM shifted the I-V curve of I\text{Ko} leftward and upward. CCl4 4 mM decreased I\text{Ko} value of 30 nM attenuated the decrease of I\text{Ko} induced by CCl4 close to normal amplitude. AP-Q 0.01-100 nM had no significant effect on either inward or outward components of I\text{K1} at any membrane potential examined. AP-Q 0.1-100 nM had no significant influence on the peak amplitude of I\text{CRAC}, either, and did not affect the shape of its current voltage curve.

CONCLUSION: AP-Q has a protective effect on CCl4-induced liver injury, probably through selectively increasing I\text{Ko} in hepatocytes.

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Voltage-clamp recording

Whole-cell recordings were performed using a PC-II patch clamp amplifier (Huazhong University of Science and Technology). The recording chamber (1.5 mL) was perfused with the corresponding external solution. The pipettes were pulled in two stages from hard glass capillaries using a vertical microelectrode puller (Narishige, Japan). The electrode had a resistance of 2.5 MΩ for whole-cell recording when filled with electrode internal solution. All experiments were conducted at 22±2 °C.

Solutions

Ca²⁺-free Hank’s solution was prepared without Ca²⁺ and Mg²⁺, containing (mM) NaCl 137, KCl 5.4, NaH₂PO₄ 0.5, Na₂HPO₄ 0.58, NaHCO₃ 4.16, and glucose 5.5 (pH 7.3). KB solution contained (mM) glutamic acid 70, taurine 15, KCl 130, KH₂PO₄ 10, HEPES 10, glucose 11, egtazic acid 0.5, pH was adjusted to 7.4 with KOH. The external solution for recording I_{CRAC} contained (mM) NaCl 140, KCl 2.8, CaCl₂ 10, MgCl₂ 0.5, glucose 11, HEPES 10, pH was adjusted to 7.4 with NaOH. The internal solution for recording I_{CRAC} contained (mM) potassium-glutamate 145, NaCl 8, MgCl₂ 1, Mg-ATP 0.5, egtazic acid 10, HEPES 10, pH was adjusted to 7.2 with KOH. The external solution for recording Iₖ contained (mM) NaCl 144, KCl 4, CaCl₂ 1.8, MgCl₂ 0.53, NaH₂PO₄ 0.33, glucose 5.5, HEPES 5, pH was adjusted to 7.4 with NaOH. The internal solution for recording Iₖ contained (mM) KCl 130, KₐATP 5, creatine phosphate 5, HEPES 5, pH was adjusted to 7.2 with KOH. The same external and internal solutions for recording Iₖ contained (mM) KCl 7, MgCl₂ 2, egtazic acid 1, potassium-glutamate 130, HEPES 10, pH was adjusted to 7.4 with KOH.

Statistics

The data were expressed as x±s. Concentration-response relationship was calculated with Hill equation. Statistical significances were analyzed by Student’s t-test. P value <0.05 was considered significant.

RESULTS

Effect of AP-Q on serum ALT and AST activities in CCl₄-treated mice

Serum ALT and AST activities, indexes of liver cell damage of mice receiving a single CCl₄ injection were markedly increased 16 h after the injection. Pretreatment with AP-Q at various concentrations (3.5, 7 μg/kg) significantly reduced ALT and AST levels. By contrast, pretreatment with AP-Q (14 μg/kg) increased ALT and AST levels (Table 1), which were probably due to the intrinsic toxicity of AP-Q.

Table 1: Effects of pretreatment with AP-Q on serum ALT and AST levels in CCl₄-injected mice (n=12)

| Drugs        | ALT(U/L) | AST(U/L) |
|--------------|----------|----------|
| Olive oil    | 26.2±4.6 | 30.5±3.8 |
| CCl₄         | 145.5±25.3 | 110.1±20.6 |
| CCl₄+AP-Q 3.5 μg/kg | 118.7±36.5 | 90.8±29.2 |
| CCl₄+AP-Q 7 μg/kg | 109.8±30.7 | 88.9±30.7 |
| CCl₄+AP-Q 14 μg/kg | 157.3±28.1 | 130.6±15.2 |

*P<0.05 vs control group, P<0.05 vs CCl₄-treated group.

Effect of AP-Q on Iₖ

Iₖ was elicited by depolarizing pulse to +140 mV for 900 ms from a holding potential of -50 mV[9]. The current at the end of the test pulse was measured as the amplitude of Iₖ. At +140 mV, AP-Q 0.1-100 nM produced a concentration-dependent increase of the current, which was partially reversed after washout. EC₅₀ value for AP-Q on Iₖ was 5.55±1.8 nM with the maximal increase of Iₖ up to 35.8±6.6% at AP-Q 30 nM (Figure 1B). The maximal increase in peak Iₖ did not further enhance when the concentration was raised to 100 nM.

Figure 1C shows the effects of AP-Q 30 nM on the steady-state I-V relationship for Iₖ generated by applying depolarizing steps from +30 mV to +140 mV for 900 ms with a 10 mV increment from a holding potential of -50 mV. AP-Q 30 nM shifted the I-V curve of Iₖ leftward and upward.

Cells were exposed to CCl₄ (4 mM) by addition of a concentrated aliquot (dissolved in DMSO) to the culture dish. The final concentration of DMSO was less than 0.1% (v/v). Under this condition, DMSO did not affect membrane current. Exposure to CCl₄ led to a significant decrease in Iₖ. The gradual decrease in Iₖ was detectable 1 min after CCl₄ exposure and the currents remained stable between 5-10 min. CCl₄ 4 mM decreased Iₖ current 28.6±6.5% at 140 mV. After exposure to CCl₄ for 5 min, AP-Q 30 nM attenuated the decrease of Iₖ induced by CCl₄ close to normal amplitude (Figure 1. A, C).

Figure 1. Effects of AP-Q on Iₖ. A: Family of Iₖ recorded with changes in the absence or presence of AP-Q 30 nM (upper) and family of Iₖ recorded with changes after CCl₄ adminstration in the absence or presence of AP-Q 30 nM (bottom). B: Concentration-response curve for the effects of AP-Q on Iₖ, n=6. C: I-V relationship of Iₖ under control (△), AP-Q 30 nM (○), CCl₄ 4 mM (□) and AP-Q 30 nM after CCl₄ 4 mM adminstration (◇). The voltage steps used to elicit Iₖ are shown in the inset of panel (B). n=6.

Effect of AP-Q on Iₖ₁

Iₖ₁ was elicited by a number of step pulses (40 ms) from the
holding potential of 0 mV to test potentials from -200 mV to +175 mV with a step of 10 mV[10]. The absolute value at the end of the test pulse was measured as the amplitude of I_{K_1}. AP-Q 0.01-100 nM had no significant effect on either inward or outward components of I_{K_1} at any membrane potential examined.

**Effect of AP-Q on I_{CRAC}**

I_{CRAC} was elicited for 200 ms from the holding potential of 0 mV to various potentials ranging from -100 mV to +80 mV with the step of 20 mV every 5 s[11]. The peak amplitude of I_{CRAC} was -495±127 pA (n=15) and the reversal potential of I_{CRAC} was about 0 mV, which was steady and without run-down in 5 min. AP-Q 0.1-100 nM had no significant influence on the peak amplitude of I_{CRAC} and did not affect the shape of its current-voltage curve.

**DISCUSSION**

The results presented in this study demonstrated that pretreatment with AP-Q 3.5 µg/kg and 7 µg/kg had a protective effect on CCl_4-induced acute liver injury, reflected by changes in serum AST and ALT activities. However, pretreatment with AP-Q 14 µg/kg aggravated the toxicity of CCl_4, probably due to the intrinsic toxicity of AP-Q.

On the basis of its liver protective effect, we investigated the effects of AP-Q on membrane potassium and calcium currents of isolated rat hepatocytes to explore its mechanisms against CCl_4-induced liver injury.

Potassium channels are ubiquitous in eukaryotic cells and play roles in resting membrane potential, frequency of action potential, membrane potential repolarization rates and cell functions. It is noteworthy that small conductance Ca^{2+}-activated K^+ channel played a fundamental role in liver injury[12]. Progesterone induced cholestasis at least in part by inhibition of inwardly rectifying K^+ channel[13,14]. ATP-sensitive K^+ channel regulated proliferation of liver cells[15].

We found CCl_4 decreased I_{K_1} in a time dependent manner. The decrease of I_{K_1} might partly contribute to membrane depolarization. CCl_4-induced hepatocytes injury paralleled with membrane depolarization in damaged hepatocytes[2,4]. Similarly, nicotine blocked multiple types of K^+ currents, elevating the risk for cardiovascular disease and sudden coronary death associated with smoking[16]. Phenoytin (PHT) blocked I_{K_1} resulting in hypoxia-reoxygenation damage[17].

As cells became depolarized, hepatocellular substrates uptake decreased. Conversely, as cells became hyperpolarized, uptake increased[18,19]. Similar to vascular tissues where K^+ channels represent a protective and adaptive mechanism, opening of K^+ channels in liver cells could be beneficial at the early stages of injury since membrane hyperpolarization would stimulate electrogenic uptake of substrates important for regeneration of cellular ATP stores[20]. In addition, K^+ efflux was necessary for recovery from cell swelling[21,22]. Hyperpolarization of the hepatocellular membrane played a role in hepatic cytoprotection[23]. Glycine has been reported to have several beneficial effects, including protection against hepatic toxicity induced by anoxia, oxidative stress, and various toxic agents at cell, organ, and systemic levels. Glycine activated glycine-sensitive chloride channels could lead to hyperpolarization of hepatic parenchymal cell membranes, block the increase in [Ca^{2+}+]_i, due to agonists released during stress to protect against liver injury. Sea anemone toxins were common potassium channel modulators[24-26]. In our study, we investigated the effects of AP-Q on K^+ channels. AP-Q increased I_{K_1} in a concentration-dependent manner, resulting in hyperpolarization of hepatic parenchymal cell membranes, and AP-Q attenuated the decrease of I_{K_1} induced by CCl_4 close to normal amplitude of I_{K_1}, which might be beneficial for CCl_4 induced acute liver injury. AP-Q had no effect on I_{K_1}. It is interesting to note that major sea anemone toxin possessed potassium channel blocking properties except Bainh increased I_{K_1}[26].

Calcium has been demonstrated to play an important role in liver damage[27]. An early disturbance in hepatocellular Ca^{2+} homeostasis might be involved in hepatocellular damages induced by CCl_4[28-30]. Hepatocytes have been found to be short of voltage-dependent Ca^{2+} channels[31] but to possess I_{CRAC}[10,32-34]. In our previous study, we found tetrandrine and palmatine could inhibit I_{CRAC}, protecting hepatocytes from calcium overload[35,36]. AP-Q had no influence on I_{CRAC}, suggesting that AP-Q did not affect the influx of extracellular Ca^{2+}.

In conclusion, AP-Q has a protective effect on CCl_4-induced liver injury, probably by selectively increased I_{K_1}, which in part counteracts the membrane depolarization in CCl_4-induced liver damages.

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