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Stimulation by melittin of Na^+-Ca^{2+} exchange current in ventricular myocytes of guinea pigs[1]

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KEY WORDS melitten; patch-clamp techniques; sodium-calcium exchanger; calcium channels; sodium channels; myocardium

ABSTRACT

AIM: To study the mechanism of calcium overload induced by melittin in myocytes. **METHODS**: Whole-cell patch-clamp technique was applied for recording the currents. **RESULTS**: Mel 0.05, 0.1 μmol/L increased the peak amplitude of I_{Na}(nA) from -2.1 ± 0.8 to -3.2 ± 1.0 ($n=7$, $P<0.05$) and -3.7 ± 1.5 ($n=7$, $P<0.05$) respectively at testing potential of -40 mV. Mel 0.05, 0.1, 0.2 μmol/L had no significant effect on I_{Ca}, but enhanced I_{Na-Ca}(pA) from 53 ± 21 to 427 ± 256 ($n=5$, $P<0.05$), 349 ± 147 ($n=5$, $P<0.01$) and 320 ± 97 ($n=5$, $P<0.05$) respectively at a testing potential of +50 mV. **CONCLUSION**: The stimulating effect of Mel on I_{Na-Ca} rather than the effect on I_{Ca} contributes to the calcium overload of myocytes.

INTRODUCTION

Melittin (Mel), a 26 amino acid polypeptide, is the major component of bee venom. Bee venom has long been known to induce cardiac dysfunction[1,2]. It had been proved that Mel was the main constituent to induce cardiotoxicity and the toxic effect was mostly attributable to calcium overload of cardiomyocytes[3].

Generally speaking, three Ca^{2+} transport systems contribute to Ca^{2+} influx in myocytes: *L*-type calcium channels, Ca^{2+} release from the sarcoplasmic reticulum (SR) and Na^+-Ca^{2+} exchange. The Na^+-Ca^{2+} exchanger catalyzes the countertransport of three Na^+ ions for one Ca^{2+} ion across the sarcolemma membrane and plays an important role in the electrical activity of heart. It has two working modes. Normal mode generates an inward current which extrudes Ca^{2+} and reversal mode generates an outward current, which causes Ca^{2+} influx. Its mode of working is sensitive to the intracellular sodium ($[Na]_i$)[4].

It has been reported that Na^+-Ca^{2+} exchanger is the dominant way to bring about Ca^{2+} overload of cultured mouse myocytes exposed to Mel with fluorescent method[3], which was proved by pretreatment with bepridil, an inhibitor of Na^+-Ca^{2+} exchange, abolishing the effect of cardiotoxicity by Mel[3]. Paradoxically, it has been also reported that Mel, as a specific calmodulin antagonist, has no effect on Na^+-Ca^{2+} exchange in guinea pig ventricular myocytes with patch-clamp technique[5]. To elucidate the cardiotoxic mechanisms of Mel, the effect of Mel on I_{Na}, I_{Ca}, and I_{Na-Ca} of ventricular myocytes in guinea pigs was studied.

MATERIALS AND METHODS

Preparation of ventricular myocytes Single ventricular myocytes were isolated from adult guinea pigs, $315 \pm s$ 24 g (supplied by the Experimental Animal Center of Fudan University, Grade Ⅱ), by enzymatic disaggregation. After stunning the guinea pig, the heart was rapidly put into oxygenated calcium-free Tyrode's solution. The aorta was cannulated and the heart was perfused on Langendorff apparatus at 37 ℃. Following perfusion with calcium-free Tyrode's solution for about 5 min, the low calcium (50 μmol/L) Tyrode's solution containing 0.03 % type-II collagenase and 1 % bovine serum albumin (BSA) was used for about 4 min. The ventricles was chopped, minced, and gently agitated to

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obtain myocytes. Cells were filtered through a 200-μm nylon mesh and the calcium concentration was gradually increased.

Whole-cell patch-clamp recording The cells were transferred to a 1 mL pool that was mounted on the stage of inverted microscope (Nikon, Japan). Only cells with rod-shape and clear striations were used in experiments. The pool was continuously perfused with test solution at a rate of 1.5 mL/min. Serial concentrations of Mel solutions were prepared and administered in turn by perfusion for at least 1 min to assure that the drug in the pool reached the anticipated concentrations. Microelectrode were pulled with a microelectrodes puller (Narrishage, Japan), resistance of 3 – 4 MΩ after filled with pipette solution. Ag-AgCl electrode was used as the reference electrode. After gigaseal was formed and membrane ruptured, I_{Na}, I_{Ca}, and I_{Na-Ca} were recorded in the mode of voltage clamping. The Ni^{2+}-sensitive electrogenic I_{Na-Ca} was measured as the difference in current before and after application of 5 mmol/L Ni^{2+}. Axopatch 200A amplifier (Axon Instrument, USA) was employed and pCLAMP 6.0 software (Axon Instrument, USA) was used to produce protocols, acquire, and process data. Series resistance was partially compensated electronically and capacitative transients were removed by applying electronic compensation when recording I_{Na} and I_{Ca}, but because of the slow ramp protocol, no compensation was made for membrane capacitance or series resistance[6].

Chemicals and solution Melittin (Mel), type-Ⅱ collagenase, MgATP, Tris-GTP, CsOH were purchased from Sigma (USA). TEA-Cl, HEPES, and ouabain were Merck products (Germany). 3-(*N*-morpholino) propanesulfonic acid (MOPS), bovine serum albumin (BSA), $CsCl_2$ were purchased from Sino-American Biotechnology company (USA). Tetrodotoxin (TTX) was purchased from Hebei Ocean Product Institute of China. Other reagents of AR grade were products of Shanghai Chemical Reagent Plant.

The calcium-free solution contained: NaCl 100, KCl 10, NaH_2PO_4 1.2, $MgSO_4$ 5.0, glucose 20, taurine 10, MOPS 10 mmol/L; pH was adjusted with KOH to 7.2. Test solution of I_{Na} recording was composed of: choline chloride 100, NaCl 50, KCl 5.4, $MgCl_2$ 2.0, $CaCl_2$ 1.8, HEPES 5, glucose 10 mmol/L; pH was adjusted with NaOH to 7.3. The pipette solution of I_{Na} recording was: $CsCl_2$ 130, NaF 13, $MgCl_2$ 2, egtazic acid 2, HEPES 10 mmol/L; pH was adjusted with CsOH to 7.2. The test solution of I_{Na-Ca} recording was composed of: NaCl 140, $CaCl_2$ 2.0, $MgCl_2$ 2.0, HEPES 5.0, glucose 10 mmol/L; the Na^+-K^+ pump, backgroud currents, K^+ channel and Ca^{2+} channel were blocked with ouabain 20 μmol/L, $BaCl_2$ 1.0 mmol/L, $CsCl_2$ 2.0 mmol/L, and verapamil 5 μmol/L; pH was adjusted with CsOH to 7.2. The pipette solution of I_{Na-Ca} recording was: potassium aspartate 42, $MgCl_2$ 13, egtazic acid 42, $CaCl_2$ 29, K_2ATP 10, Na_2-creatinephosphate 5.0, tetraethylammonium (TEA) 20, HEPES 5.0 mmol/L, pH was adjusted with CsOH to 7.2. Test solution of I_{Ca} recording was composed of: TEACl 140, $MgCl_2$ 2.0, $CaCl_2$ 1.8, HEPES 10, glucose 10, TTX 0.002 mmol/L; pH was adjusted by TEAOH to 7.3. The pipette solution was: $CsCl_2$ 140, egtazic acid 10, HEPES 10, MgATP 3, Tris-GTP 0.4 mmol/L; pH was adjusted by CsOH to 7.2.

Statistics The data were expressed as $\bar{x} \pm s$, n represents the number of cells. The results were analyzed with paired t test.

RESULTS

Effect of Mel on I_{Na} I_{Na} was evoked by a step depolarization from holding potential of – 80 mV to – 40 mV for 30 ms (Fig 1). Mel 0.05, 0.1, 0.2 μmol/L increased I_{Na}(nA) from -2.1 ± 0.8 to -3.2 ± 1.0 ($n = 7$, $P < 0.05$), -3.7 ± 1.5 ($n = 7$, $P < 0.05$) and -3.2 ± 1.3 ($n = 7$, $P > 0.05$) respectively at a testing potential of – 40 mV. The current-voltage relationship was obtained when membrane potential was held at – 80 mV and depolarized to various levels after every 5 mV (Fig 2).

Effect of Mel on I_{Ca} The membrane potential

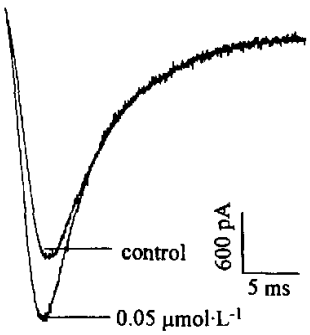

Fig 1. Effect of melittin on I_{Na} current in ventricular myocytes of guinea pigs.

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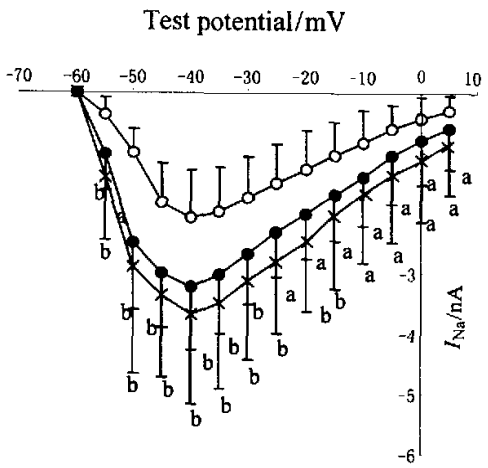

Fig 2. Effect of melittin on current-voltage relationship of I_{Na} (○) control, (●) Mel 0.05 μmol/L, (×) Mel 0.1 μmol/L. $n = 7$ cells from 4 guinea pigs. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* control.

was held at −40 mV and the current-voltage relationship was obtained by depolarizing the membrane potential from −40 to +40 mV in 10 mV increment. The step pulse lasted for 500 ms. Mel 0.05, 0.1, 0.2 μmol/L had no marked effect on the current. Mel 0.05 μmol/L changed the I_{Ca}(pA) from 1094 ± 318 to 1128 ± 500 ($n = 7$, $P > 0.05$) (Fig 3). Though Mel 0.1, 0.2 μmol/L had a tendency to reduce I_{Ca}(pA) from 1094 ± 318 to 770 ± 206 ($n = 7$) and 777 ± 389 ($n = 7$) respectively, the decrease was not significant compared with the "run-down" of control group.

Effect of Mel on I_{Na-Ca} Ramp voltage-clamp

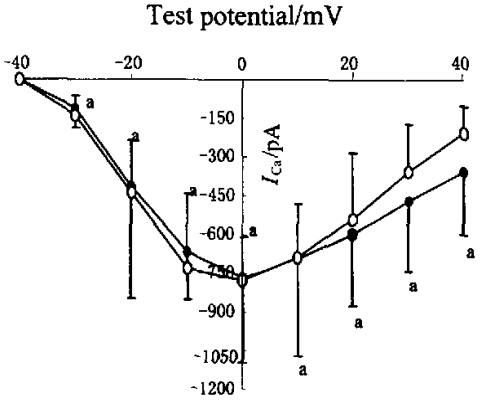

Fig 3. Effect of melittin on current-voltage relationship of I_{Ca}(○) control. (●) Mel 0.05 μmol/L. $n = 7$ cells from 5 guinea pigs. $\bar{x} \pm s$. $^aP > 0.05$ *vs* control.

pulses (−40 to +60 to −120 mV, 250 mV/s) were applied from holding potential of −40 mV. The current was sensitive to Ni^{2+} 5.0 mmol/L. Mel 0.05, 0.1, 0.2 μmol/L resulted in the substantial increase of I_{Na-Ca} (Fig 4). Mel 0.05, 0.1, 0.2 μmol/L enhanced I_{Na-Ca} (pA) from 53 ± 21 to 427 ± 256 ($n = 5$, $P < 0.05$), 349 ± 147 ($n = 5$, $P < 0.01$) and 320 ± 97 ($n = 5$, $P < 0.05$) respectively at testing potential of +50 mV.

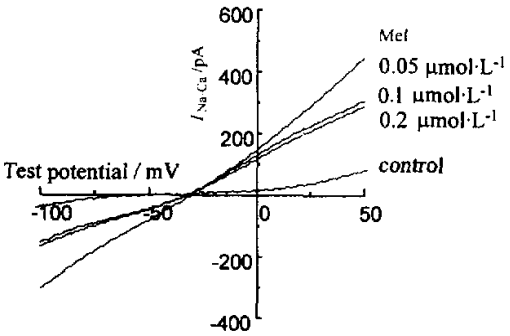

Fig 4. Effect of melittin on current-voltage relationship of I_{Na-Ca}.

DISCUSSION

Our results showed that Mel increased voltage-gated I_{Na} in ventricular myocytes of guinea pig, which has so far not been reported. The voltage-gated sodium channel can be a source of significant increases in $[Na]_i$. Evidence has accumulated that Mel increases the $[Na]_i$ of cardiomyocytes[3] and active transport of Na^+ in bullfrog cornea[7] and skin of the toad Bufo marinus[8]. But the effect of Mel on increasing I_{Na} may not be the exclusive mechanism for increased $[Na]_i$, the inhibitory effect of Mel on Na^+, K^+-ATPase also contributes to it. A comparative study regarding the effects of Mel, cardiotoxin (a component of snake venom), and ouabain (a typical Na^+-K^+ ATPase inhibitor) on the sodium pump in synaptic membrane isolated from rat cerebral cortex showed that Mel was the most potent inhibitor among the three[9] and the inhibitory effect of Mel has also been observed in other tissues[10]. Both these mechanisms enhance $[Na]_i$ which exerts important influence on I_{Na-Ca}.

I_{Na-Ca} is very sensitive to the intracellular Na^+. A rise in intracellular Na^+ makes the exchanger work in its reversal mode which reflects an increase of Ca^{2+} influx via exchanger and is ultimately responsible for Ca^{2+} release from SR[4]. Furthermore Levi[11] found that the

increased $[Na]_i$ resulted in a progressive outward shift in I_{Na-Ca} during the plateau of action potential, which suggests that the intracellular Ca^{2+} would be increased at the plateau via exchanger. Our results indicate that Mel stimulated I_{Na} and outward I_{Na-Ca}, which is responsible for calcium overload of myocytes.

In addition to the Na^+-Ca^{2+} exchanger, there are two other ways resulting in calcium influx: L-type calcium channels and calcium release from SR. Our results observed that Mel had no significant effect on L-type calcium current. Meanwhile, being a membrane-active peptide, Mel can not affect Ca^{2+} release from SR as the primary effect. Taken together, the calcium overload by Mel is mostly ascribed to its stimulating effect on I_{Na-Ca}.

But it has also been reported by Kimura J[5] that Mel, as a high affinity calmodulin-binding peptide, remained unaffected on Na^+-Ca^{2+} exchanger. The striking difference between Kimura's experiment and ours lies in the mode of Mel administration. In their experiment Mel was applied by internal perfusion to study the interaction of Mel with the calmodulin-binding site of exchanger in plasma of myocytes[12]. However we added Mel in the extracellular solution. Therefore the two experiments are not contradictory and it is suggested that the site of action of Mel on Na^+-Ca^{2+} exchanger was on the external side of the membrane.

The "run-down" phenomenon has long been noticed in recording L-type calcium current. In order to distinguish the decreasing effect caused by drugs from the "run-down" phenomenon, we compared the drug groups with control group respectively[13]. The results showed the three concentrations of Mel all had no significant effect on L-type calcium channel.

Another interesting observation is that the stimulating effect of Mel on Na^+-Ca^{2+} exchanger is not concentration-dependent. As early as in 1970s, Habermann had found that higher doses of Mel were followed by diminished responses[14]. This phenomenon has also been reported in several reports[1,15]. It may be explained by Mel's property of tachyphylaxis.

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蜂毒肽增强豚鼠心室肌细胞 Na^+-Ca^{2+} 交换电流

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关键词 蜂毒肽；膜片箝技术；钠$^+$-钙$^{2+}$ 交换；钙通道；钠通道；心肌

目的：研究蜂毒肽使心肌细胞钙超负荷的机制。

方法：全细胞膜片箝记录。 **结果**：蜂毒肽 0.05，0.1 μmol/L 促进 I_{Na} 峰值（nA）从 -2.1 ± 0.8 分别增加到 -3.2 ± 1.0（$n=7$，$P<0.05$）和 -3.7 ± 1.5（$n=7$，$P<0.05$）。蜂毒肽 0.05，0.1，0.2 μmol/L 对 I_{Ca} 无显著性作用，但在箝制电位为 +50 mV 时，促进 I_{Na-Ca}(pA) 从 53 ± 21 分别增加到 427 ± 256（$n=5$，$P<0.05$），349 ± 147（$n=5$，$P<0.01$）和 320 ± 97（$n=5$，$P<0.05$）。 **结论**：蜂毒肽使心肌细胞内钙增加主要是由于促进 Na^+-Ca^{2+} 交换电流而不是 *L*-型钙电流。

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