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Depression of L-type Ca^{2+} and Transient Outward K^+ Currents in Endotoxin-treated Rat Cardiac Myocytes

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Decreased cardiac contractility occurs in endotoxicosis, but little is known about the ionic mechanism responsible for myocardial dysfunction. In this study, we examined the changes in Ca^{2+} and K^+ currents in cardiac myocytes from endotoxin-treated rat. Ventricular myocytes were isolated from normal and endotoxemic rats (*ex vivo*), that were treated for 10 hours with *Salmonella enteritidis* lipopolysaccharides (LPS; 1.5 mg/kg) intravenously. Normal cardiac myocytes were also incubated for 6 hours with 200 ng/ml LPS (*in vitro*). L-type Ca^{2+} current ($I_{Ca,L}$) and transient outward K^+ current (I_{to}) were measured using whole cell patch clamp techniques. Peak $I_{Ca,L}$ was reduced in endotoxemic myocytes (*ex vivo*; 6.00.4 pA/pF, $P<0.01$) compared to normal myocytes (control; 10.90.6 pA/pF). Exposure to endotoxin *in vitro* also attenuated $I_{Ca,L}$ (8.40.4 pA/pF, $P<0.01$). The amplitude of I_{to} on depolarization to 60 mV was reduced in endotoxin treated myocytes (16.51.5 pA/pF, $P<0.01$, *ex vivo*; 20.00.9 pA/pF, $P<0.01$, *in vitro*) compared to normal myocytes (control; 24.71.0 pA/pF). There was no voltage shift in steady-state inactivation of $I_{Ca,L}$ and I_{to} between groups. These results suggest that endotoxin reduces Ca^{2+} and K^+ currents of rat cardiac myocytes, which may lead to cardiac dysfunction.

Key Words: Endotoxin, Cardiac myocyte, Ca^{2+} current, K^+ current

INTRODUCTION

Septic shock, induced by gram-negative bacterial infection, is associated with complex dysfunction in multiple organ systems, leading to systemic hypotension and circulatory collapse (Abel, 1990; Snell & Parrillo, 1991). These hemodynamic derangements are known to be caused by profound vasodilation and depressed myocardial contractility (Parker & Parrillo, 1983). Endotoxin-induced vasodilation is mediated by nitric oxide, which is released from vascular smooth muscle and endothelium (Kilbourn et al, 1990; Jolou-Schaeffer et al, 1990). However, the mechanism underlying myocardial depression is still obscure. A number of studies have shown that endotoxemia decreases myocardial function both *in vivo* and in isolated cardiac muscle preparation (Parker & Adams, 1985; Sugi et al, 1991). But, it is not clear whether contractile dysfunction is a direct effect of endotoxin-exposure or secondary changes to hemodynamic derangement such as ischemia and acidosis (Parillo et al, 1985; Snell & Parrillo, 1991; Hung & Lew, 1993).

Recently it has been reported that myocytes isolated from endotoxemic animals exhibit reduced cell shortening and decreased action potential duration (Hung & Lew, 1993; Tao & McKenna, 1994; Stein et al, 1996). Some experiments with cat papillary muscle and guinea pig atrial muscle denied the direct effect of endotoxin on contractile performance (Kutner & Cohen, 1966). In contrast, experiments with rat trabeculae muscle and isolated myocytes exhibited significant decreases in peak developed tension and cell shortening after direct exposure to endotoxin *in vitro* (Macnicol et al, 1973; Tao & McKenna, 1994).

In excitation-contraction coupling mechanism of

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cardiac myocytes, Ca^{2+} inward current through voltage-gated Ca^{2+} channels plays an important role. Ca^{2+} influx during the plateau phase of the action potential not only triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) but also replenishes the SR Ca^{2+} stores for subsequent release (Bers, 1991). In this study, we examined changes in L-type Ca^{2+} and transient outward K^+ currents in cardiac myocytes isolated from endotoxemic rats (*ex vivo*) using whole cell patch clamp. We also tested whether *in vitro* exposure to endotoxin directly alters ionic currents of normal cardiac myocytes.

METHODS

Adult male Sprague-Dawley rats (200~300 g) were sedated with inhalation of diethyl ether (Junsei Chemical Co., Tokyo, Japan) and *Salmonella enteritidis* lipopolysaccharides (LPS; 1.5 mg/kg, Difco Laboratories, MI, U.S.A.) or an equivalent volume of sterile saline (control) was injected intravenously via the dorsal vein of the penis. Ten hours after LPS or saline injection, endotoxemic and control rats were anesthetized with sodium thiopental (50 mg/kg, *i.p.*), and heparinized (500 IU/kg, *i.v.*) via the inferior vena cava. After opening the thoracic cavity, hearts were quickly removed and mounted on a modified Langendorff perfusion system for retrograde coronary perfusion. Hearts were perfused for 6 min with nominally Ca^{2+}-free Tyrode solution which contained (in mM) NaCl 140, KCl 5.4, NaH_2PO_4 0.3, $MgCl_2$ 1.0, HEPES 10, glucose 10, titrated to pH 7.4 with NaOH. Hearts were then perfused for 12 min with Ca^{2+}-free Tyrode solution containing 1.2 mg/ml collagenase (CLS-2, Worthington Biochemical Co., Freehold, NJ, U.S.A.) and 0.1 mg/ml protease (Type XIV, Sigma Chemical Co., St. Louis, MO, U.S.A.). Enzymes were washed out by perfusing with Tyrode solution containing 200 mM Ca^{2+} for 6 min. Hearts were then removed from the Langendorff apparatus and the ventricles dissected free. Ventricular tissue was placed in 200 mM Ca^{2+} containing Tyrode solution in a petri dish and gently shaken to disperse cells. For some experiments, dishes of myocytes from control rats were incubated for 6 hours in LPS (200 ng/ml) containing Tyrode solution *(in vitro)*. Only Ca^{2+}-tolerant, rod-shaped cells with clear striations were used for experiments.

The suspension of dissociated cells was transferred to a 0.5 ml chamber placed on the stage of an inverted microscope (IMT-2, Olympus, Tokyo, Japan). After adhering to the bottom of the chamber, the cells were superfused with solution (2~3 ml/min) at room temperature. The Ca^{2+} and K^+ currents were recorded in the whole cell configuration of the patch clamp technique using an Axopatch 1D amplifier (Axon Instruments, Foster city, CA, USA). Patch pipettes were fabricated on a model P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., CA, USA) from glass capillary (G-1.5, Narishige, Tokyo, Japan). Pipettes were fire-polished prior to use and had tip resistances of 1.5~2.0 MΩ when filled with internal solution. The pipette and its holder were connected to the headstage of the amplifier via an Ag/AgCl wire.

The dialyzed patch clamp technique was used to record whole cell currents at room temperature (20~24°C). Voltage-clamp command pulses were generated by an IBM computer using pClamp 6.0 software with a Digidata 1200 analog-to-digital converter (Axon Instruments, Foster City, CA, USA). An 8-pole Bessel filter was used to low-pass filter membrane currents at 5 kHz. To isolate Ca^{2+} currents, the pipette solution contained (in mM) Cs-aspartate 100, CsCl 30, EGTA 10, $MgCl_2$ 1.2, HEPES 10, Adenosine 5'-triphosphate (ATP) 5, Guanosine 5'-triphosphate (GTP) 0.3, creatinine phosphate 10, glucose 10, titrated to pH 7.2 with CsOH. Tyrode solution with 2 mM $CaCl_2$ was used as the external solution for Ca^{2+} current measurements. When recording K^+ currents, the pipette solution contained (in mM) K-gluconate 100, KCl 30, EGTA 10, $MgCl_2$ 1.2, HEPES 10, ATP 5, GTP 0.3, creatinine phosphate 10, glucose 10, adjusted to pH 7.2 with KOH. The external solution for measuring K^+ currents was Tyrode solution with 2 mM $MnCl_2$ instead of $CaCl_2$. Applying a holding potential or prepulse of −40 mV eliminated the fast Na^+ current (Hille, 1992).

We compared the amplitudes and current-voltage (I-V) relationships of Ca^{2+} and K^+ current between control and endotoxin-treated *(ex vivo and in vitro)* cardiac myocytes. Current amplitudes were normalized to membrane capacitance and expressed as pA/pF. Membrane capacitance (C_m) was measured by applying a 20 ms, 10 mV hyperpolarizing step from holding potential and calculated according to the following equation: $C_m = I_o \cdot \tau_c / \triangle V_m (1-I_\infty/I_o)$. C_m is the time constant of the capacitative current, I_o is the maximum capacitative current value, V_m is the am-

plitude of a voltage step (10 mV), and I is the amplitude of the steady-state current (Benitah et al, 1993). Series resistance was calculated as $R_s = V_m/I_o$. Average membrane capacitance (C_m) and series resistance (R_s) of ventricular myocytes were 145.1±4.5 pF and 6.2±0.3 MΩ, respectively (n=50). Membrane capacitance of endotoxin-treated myocytes were 150.3 ±41.3 pF (*ex vivo*) and 140.4±26.1 pF (*in vitro*), values that are not statistically different from that of control myocytes.

The steady-state inactivation data were fit by a Boltzmann distribution of the following equation: $I/I_{max} = 1/\{1+\exp[(V-V_{1/2})]/k\}$ where I_{max} is maximal current, $V_{1/2}$ is the membrane potential producing 50% inactivation, and k is the slope factor. All data, including percent inhibition, were expressed as the mean±S.E.M. Differences between groups were compared using the one way ANOVA and $p<0.05$ was considered as significantly different.

RESULTS

L-type Ca^{2+} current of control and endotoxin-treated myocytes

The representative traces shown in Fig. 1A were obtained using depolarizing test pulses between −30 and +50 mV from holding potential of −40 mV. The fast Na^+ current was eliminated by holding at −40 mV, while outward K^+ current was blocked by dialyzing the cell with Cs^+. The current under these conditions was therefore carried mainly by Ca^{2+}. This was confirmed by the observation that voltage-dependent current was completely blocked by 0.1 mM $CdCl_2$ (data not shown). The peak of inward Ca^{2+} currents occured at 0 mV, and the average peak current amplitude was 10.9±0.6 pA/pF (n=14; Fig. 1B).

The magnitude of the Ca^{2+} current diminishes as a function of time during dialyzed patch-clamp recording, a phenomenon termed "run-down". In this study, the rates of run-down were not significantly different between control and endotoxin-treated myocytes, and in most cases, less than 20% reduction was observed throughout a recording period. To properly compare peak $I_{Ca,L}$ in different groups, we measured peak amplitude at the same time period, and all measurements were completed within 15 min after establishment of the whole cell configuration. Peak current density of cardiac myocytes from endotoxemic rats (*ex vivo*) was 6.0±0.4 pA/pF, significantly lower than corresponding values from control myocytes (Fig. 2A, B). Peak current density of endotoxin-incubated myocytes (*in vitro*) was also reduced (8.4± 0.4 pA/pF).

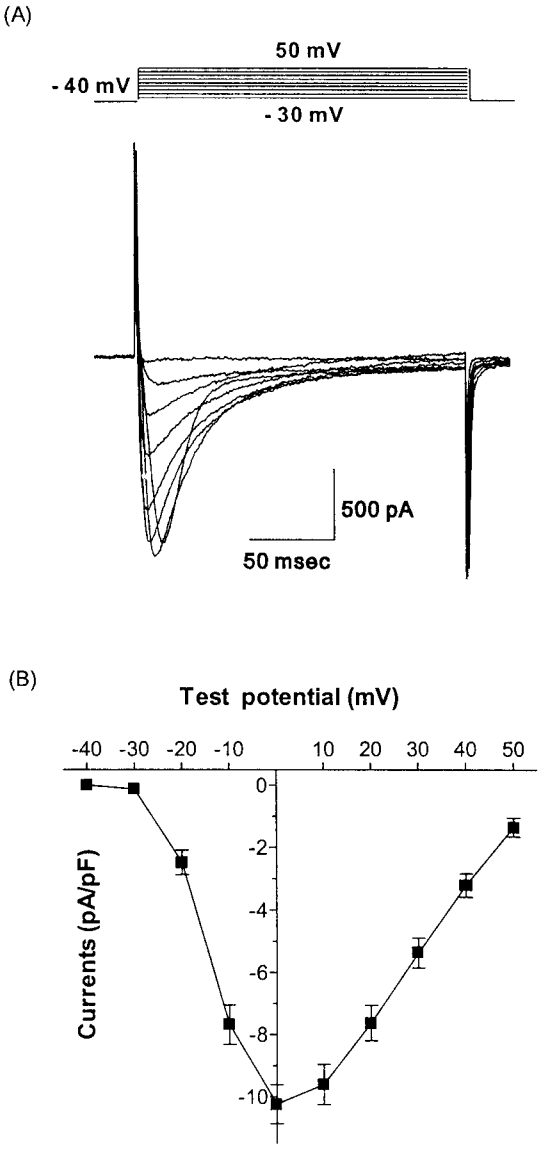

Fig. 1. L-type Ca^{2+} currents in rat ventricular myocytes. Ca^{2+} currents were recorded using the whole cell patch clamp technique. Cells were dialyzed with Cs^+ to block K^+ current and held at −40 mV to inactivate voltage-dependent Na^+ channels. (A) A representative traces of L-type Ca^{2+} current in a single isolated myocyte. (B) Current-voltage relationship of the Ca^{2+} current in myocytes. Group data (n=15) for the peak current at each voltage are shown and currents were normalized to membrane capacitance.

To obtain more information about the possible

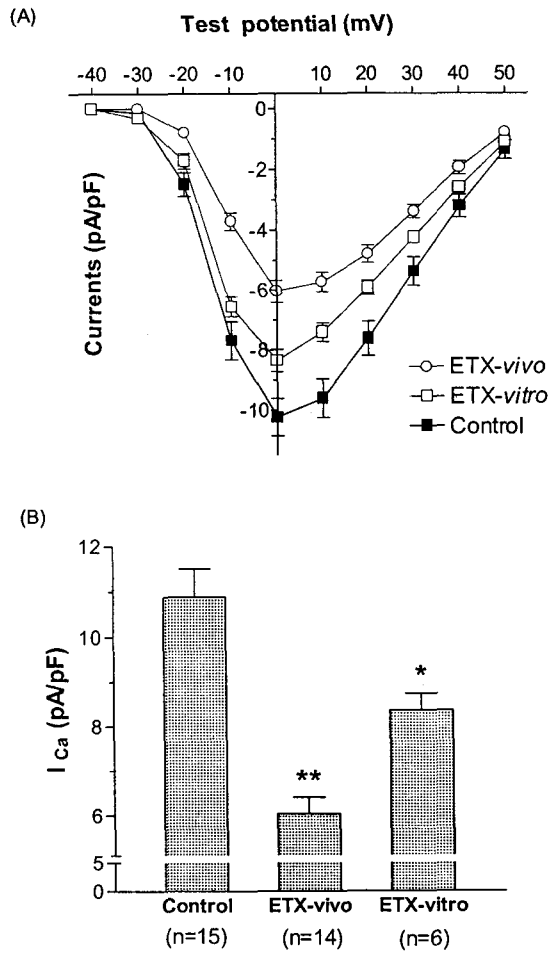

Fig. 2. Effect of endotoxin treatment on the peak amplitude of L-type Ca^{2+} currents. (A) Current-voltage relationships of the Ca^{2+} currents were compared between *ex vivo* or *in vitro* endotoxin-treated myocytes and control myocyte (B) Peak current density (pA/pF) were reduced in endotoxin-treated myocytes. Data points are means ± S.E.M. and n is number of myocytes. Asterisks indicate significant differences from control by one way ANOVA (*: $p<0.05$, **: $p<0.01$).

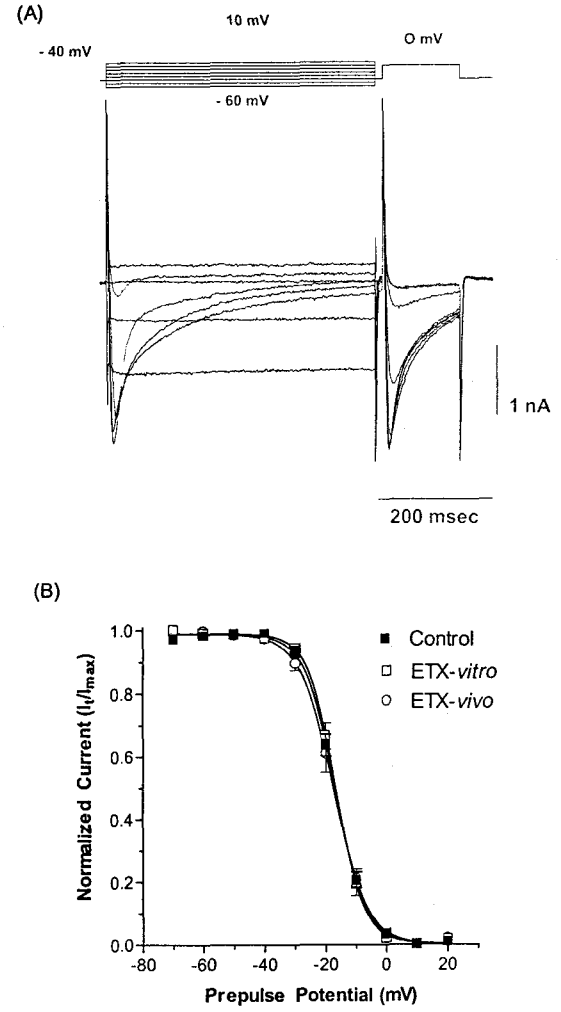

Fig. 3. Effects of endotoxin treatment on the steady-state voltage dependent inactivation of L-type Ca^{2+} currents. (A) The double pulse protocol used and the representative traces obtained. (B) Amplitudes of the currents evoked by test potentials were plotted as a function of the prepulse potential. Currents were normalized to their respective maximal amplitudes. The curves through the data points were drawn according to the Boltzmann equation.

mechanisms of endotoxin-induced current reduction, the effect on steady-state inactivation was examined. The voltage-dependence of steady-state inactivation was determined by using a standard double pulse protocol. This included a 500 msec conditioning step of varying voltage, which was followed by a 200 msec test pulse to 0 mV. Under control conditions the voltage required for half-inactivation ($V_{1/2}$) and slope factor (k) for the Ca^{2+} currents were −16.9 mV and −4.97 (mV per e-fold change). Those values ($V_{1/2}$ and k) of endotoxin-treated myocytes (*ex vivo and in vitro*) were −17.3 mV, −5.55 and −16.67 mV, −4.60, respectively, which were not significantly different from control myocytes (Fig 3A, B).

Transient outward K^+ current of control and endotoxin-treated myocytes

Experiments were performed with equimolar replacement of Ca^{2+} with Mn^{2+} to block Ca^{2+} current and a prepulse to −40 mV to eliminate Na^+ current. Under these conditions, depolarizing pulses evoked voltage-activated outward currents (Fig. 4A), that are

known to reflect a combination of transient outward and delayed rectifier currents (Apkon & Nerbonne, 1991). To isolate I_{to}, we applied 5 mM tetraethylammonium (TEA), which blocked the delayed rectifier current (Fig. 4B, C). The remaining current was assumed to be I_{to}, as it was completely inhibited by 3 mM 4-aminopyridine (data not shown).

Rat ventricular myocytes possess a prominent I_{to}, and we tested the effects of endotoxin exposure on this current. The amplitude density of I_{to} on depolarization to 60 mV was reduced in myocytes from endotoxemic rat (*ex vivo;* 16.5±1.5 pA/pF), significantly lower than corresponding values of control myocytes (24.7±1.0 pA/pF). The I_{to} density of endotoxin-incubated myocytes (*in vitro*; 20.0±0.9 pA/pF) was also reduced (Fig. 5A, B). The voltage-dependence of steady-state inactivation was determined and the $V_{1/2}$ and k for I_{to} were −42.26 mV and −6.39. Those values of endotoxin-treated myocytes were not

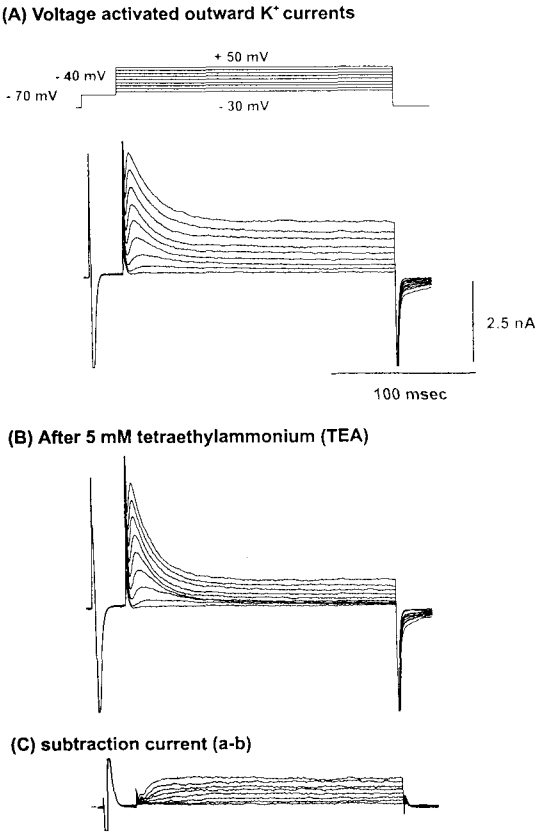

Fig. 4. Voltage activated outward K^+ currents in ventricular myocytes. During depolarization to potentials between −30 and +50 mV from a holding potential of −70 mV, outward currents were elicited in ventricular myocytes. Ca^{2+} current was eliminated by replacing extracellular Ca^{2+} with Mn^{2+}, while a 30 msec prepulse to −40 mV was used to inactivate Na^+ current. (A) Voltage activated outward currents from a representative cell. (B) Tetraethylammonium (5 mM) was applied to the same cell to antagonize delayed rectifier K^+ channels and thus reveal the rapidly inactivating transient outward K^+ current. (C) The TEA-sensitive component, i.e., the delayed rectifier current, was determined by subtracting the current in (B) from that in (A).

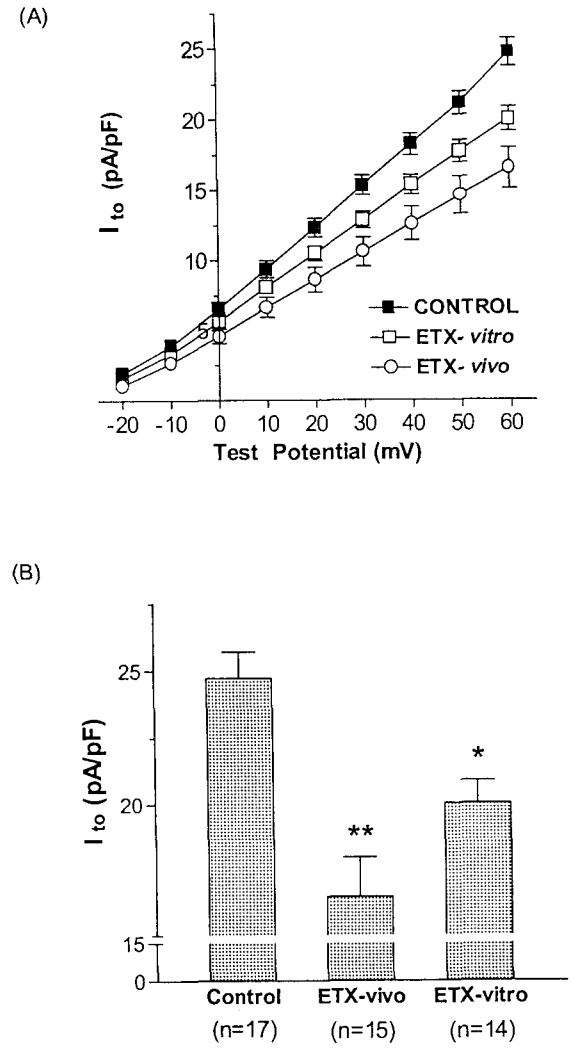

Fig. 5. Effects of endotoxin treatment on transient outward K^+ current. (A) Current-voltage relationships of the transient outward K^+ currents were compared between *ex vivo* or *in vitro* endotoxin-treated myocytes and control myocytes (B) Current density (pA/pF) was reduced in endotoxin-treated myocytes. Asterisks indicate significant differences from control by one way ANOVA (*: $p<0.05$, **: $p<0.01$). Data are means±S.E.M.; n=number of myocytes.

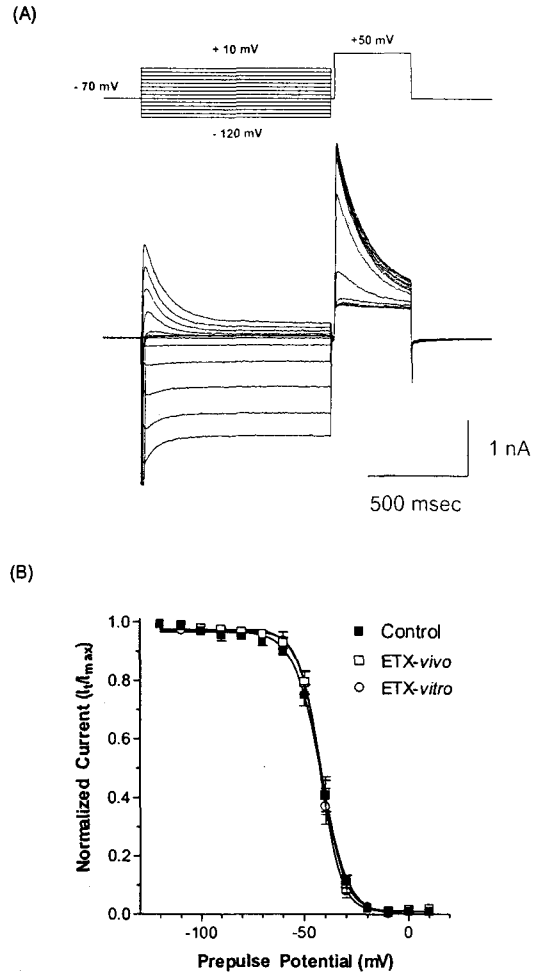

Fig. 6. Effects of endotoxin treatment on the steady-state voltage dependent inactivation of transient outward K^+ current. (A) The representative traces obtained. (B) Amplitudes of the currents evoked by test potentials were plotted as a function of the prepulse potential. Currents were normalized to their respective maximal amplitudes. The curves through the data points were drawn according to the Boltzmann equation.

significantly different from control myocytes (*ex vivo*: −42.04 mV, −5.54; *in vitro*: −42.53 mV, −5.03) (Fig. 6A, B).

DISCUSSION

Septic shock has been increasing in incidence since the 1930s and is presently the most common cause of death in intensive care units (Parker & Parrillo, 1983). Reasons underlying this rising incidence and mortality include increased use of immunosuppressive therapy, increased patient age and the emergence of antibiotic-resistant organism (Snell & Parillo, 1991). During septic shock, the heart exhibits diminished contractility that is characterized by reduced right and left ventricular ejection fractions and increased end-diastolic ventricular volumes (Parker et al, 1990). However, the pathogenetic mechanisms underlying this myocardial dysfunction are complex and unclear.

Two different hypotheses have been offered to account for the myocardial depression in sepsis. The first hypothesis is that coronary hypoperfusion leads to ischemic myocardial dysfunction (Snell & Parillo, 1991). However, it was reported that patients with septic shock had coronary blood flows equal to or greater than those of controls. In addition, there was no difference in myocardial lactate extraction between septic shock patients with and without myocardial depression (Cunnion et al, 1986). This evidence excludes ischemia as the cause of myocardial depression in sepsis. A second postulated mechanism is that endotoxin or other mediators activated by endotoxin could alter cardiac contractility. Several possible mediators of myocardial depression are suggested, such as nitric oxide, interleukin-1, -2 and tumor necrosis factor, but it is still controversial whether they directly affect the contractility (Yokoyama et al, 1993; Schulz et al, 1996).

The present study indicated that treatment with endotoxin (*ex vivo* and *in vitro*) attenuated $I_{Ca,L}$ and I_{to} of rat ventricular myocytes. Reduction of $I_{Ca,L}$ and I_{to} could not be explained by difference in cell size, because current amplitudes were normalized to membrane capacitance and expressed as pA/pF. Furthermore, membrane capacitance was not different between endotoxin-treated cells and control cells. Recording conditions for $I_{Ca,L}$ and I_{to} measurement were also managed to prevent contamination by other currents and were confirmed by complete blockade with $CdCl_2$ and 4-aminopyridine, respectively. Futhermore it is unlikely that endotoxemia affects the voltage kinetics of Ca^{2+} and K^+ channels, since endotoxin changed neither the current-voltage relationship nor the voltage-dependence of steady-state inactivation. Even now, it is impossible to predict the mechanisms of current inhibition by treatment with endotoxin.

In previous reports, ventricular myocytes from endotoxemic guinea pigs or rabbits exhibited reduced the amplitudes and rates of shortening and relengthening (Hung & Lew, 1993; Rubin et al, 1994). In addition, they also exhibited shortened action potential

duration in rabbit myocytes (Hung & Lew, 1993). Recently, it was reported that L-type Ca^{2+} current is reduced in cardiac myocytes isolated from endotoxemic guinea-pigs (Zhong et al, 1997). Sarcolemmal Ca^{2+} current could play an important role in determining action potential duration and cardiac contractility, as diminished Ca^{2+} current in their study was correlated with decreased cell shortening and shortened action potential duration. Taken together, the mechanism of myocardial depression induced by endotoxin was related to reduced peak Ca^{2+} current.

Cardiac voltage-activated outward K^+ current consists of I_{to} and delayed rectifier current (I_K), which are sensitive to 4-aminopyridine and tetraethylammonium, respectively (Apkon & Nerbonne, 1991). It has been reported that most voltage-activated outward K^+ current is I_K in frog, guinea pig, and bovine ventricular myocytes (Delpon et al, 1992). However in rat, dog, rabbit, and human myocytes, I_{to} is more prominent than I_K (Josephson et al, 1984; Escande et al, 1987; Delpon et al, 1992), which we demonstrated in rat ventricular myocytes (Fig. 4). In general, inhibition of K^+ current may prolong action potential duration and increase contractility, but it is not the sole factor affecting the duration of an action potential. As discussed above, inhibition of $I_{Ca,L}$ could be a more important factor than that of I_{to} on myocardial contractile function. Another possibility for explaining this discrepancy is the extent of inhibition. Inhibition of $I_{Ca,L}$ by endotoxin was 42.2% (*ex vivo*) and 23.5% (*in vitro*), larger than that of I_{to} (33.2% and 19.0%, respectively). The significance of endotoxin-induced K^+ current inhibition on myocardial function remains unclear.

Recent studies have shown that *in vitro* treatment of cardiac myocyte with endotoxin impaired cell shortening and serum is not required to induce myocyte hypocontractility (Tao & McKenna, 1994). We also found that endotoxin by itself (200 ng/ml for 6 hours) directly reduced the $I_{Ca,L}$ and I_{to} of cardiac myocytes, even though *in vitro* exposure of endotoxin showed less inhibitory effects than *ex vivo treatment.* It is not certain whether nitric oxide or other mediators were involved or not. Several clinical trials employing inhibition of endotoxin by use of antiserum has been associated with improved survival from Gram-negative bacteremia (Ziegler et al, 1982). In summary, treatment with endotoxin reduces the amplitude of Ca^{2+} and K^+ currents of rat cardiac myocytes, events that may lead to cardiac dysfunction. Further research into the pathogenesis of septic shock will lead to reductions in the high mortality of this disease.

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