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We report that *in vivo* injection of endotoxin (EDTX, 6 mg · kg^{-1}) induces cardiovascular alterations in rats that closely mimic the clinical situation, as assessed by *in vivo* hemodynamic measurements in anesthetized and conscious, chronically instrumented animals. The patch-clamp technique was used to characterize the L-type calcium current (I_{Ca}) and its autonomic regulation in isolated cardiac myocytes. The density of I_{Ca} progressively decreased at 12 and 36 h after EDTX injection. However, the dihydropyridine (±)Bay K 8644 (100 nM) enhanced I_{Ca} to levels similar to those in control and EDTX-treated myocytes. In addition, the net stimulatory effect of a β-adrenergic agonist (isoproterenol) on I_{Ca} was increased 12 h after EDTX injection. This change in the β-adrenergic effect declined 24 h later. The potentiation in the β-adrenergic stimulation of I_{Ca} was mimicked by L858051 (10 μM), a direct activator of adenylyl cyclase, but not by IBMX (200 μM), a phosphodiesterase inhibitor. Besides, the antiadrenergic effect of acetylcholine on I_{Ca} was unchanged 12 h after EDTX injection, but increased 36 h after EDTX injection. These results support the hypothesis that time-dependent changes in the adenylyl cyclase pathway in cardiac myocytes may contribute, via the autonomic regulation of I_{Ca}, to the severity of myocardial dysfunction during sepsis. Abi-Gerges N, Tavernier B, Mebazaa A, Faivre V, Paqueron X, Payen D, Fischmeister R, Méry P-F. Sequential changes in autonomic regulation of cardiac myocytes after ***in vivo*** endotoxin injection in rats.

Septic shock is characterized by a vascular hyporeactivity and a depressed myocardial contraction, both phenomena being largely related to bacterial endotoxin (EDTX) (1). Myocardial dysfunction may result from alterations in calcium homeostasis and/or changes in myofilament properties. The results regarding these cellular functions and their contribution to myocardial depression in sepsis appear contradictory. For instance, the sensitivity of cardiac myofilament to calcium was reported to be unchanged (2) or decreased (3, 4) in cardiac myocytes after EDTX challenge. Furthermore, diastolic and/or systolic intracellular free calcium concentrations were found to be either reduced (2, 5), increased (6), or unchanged (4) on *in vivo* or *in vitro* exposure to EDTX or cytokines. Interestingly, the action potential, which determines the amplitude of calcium influx, is

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shortened in myocytes isolated from endotoxemic animals, as compared with control (5, 7, 8; but *see* Reference 9). Likewise, both the apparent number of L-type calcium channels (10) and the density of the cardiac L-type calcium current (I_{Ca}) (5) are reduced in myocytes isolated from animals treated with EDTX. In addition, I_{Ca} is weakened in cardiac myocytes exposed to cytokines *in vitro* (7, 11).

Transduction pathways are altered in cardiac myocytes during sepsis or after a prolonged incubation with cytokines. These include nitric oxide (NO) and cyclic AMP (cAMP) pathways (7, 11). Sepsis is often accompanied by the induction of a calcium-independent NO synthase (iNOS or NOS2), but the role of NOS2 in the myocardial dysfunction during sepsis remains controversial (7, 11). Concerning the cAMP pathway, a number of studies examined the effects of proinflammatory cytokines and/or EDTX on sympathetic and parasympathetic regulation of cardiac adenylyl cyclase and contraction. In one *in vitro* study, the β-adrenergic stimulation of adenylyl cyclase activity was potentiated by proinflammatory cytokines (12) while in three others it was found to be reduced (7, 13). In addition, the cardiac positive inotropic effects of β-adrenergic agonists was either increased (5, 14), reduced (4, 15), or unchanged (16) during EDTX exposure. The β-adrenergic stimulation of I_{Ca} was increased (5) or unchanged (9) during sepsis. Interestingly, adrenergic receptors undergo a biphasic change after cecal ligation i*n vivo*: an initial increase in the density of receptors, followed by a reduction due to receptor internalization (17). In addition, sepsis potentiates the inhibitory effect

of the parasympathetic agonist acetylcholine on adenylyl cyclase activity and on the subsequent reduction in cardiac contractility (18, 19).

The above-mentioned studies illustrate some of the mechanisms of cardiac dysfunction that may occur during sepsis, but provide little information on the time course of heart dysfunction during sepsis. Indeed, the development of hypotension is rapid, but multiple organ failure develops more slowly and myocardial dysfunction usually appears 12 to 48 h after the onset of infection and/or bacteremia in humans (20). On the contrary, in the majority of animal studies, myocardial dysfunction takes place on a much faster time scale owing to the administration of large doses of bacterial EDTX. Similarly, incubation or acute administration of high doses of EDTX and/or proinflammatory cytokines can induce functional alterations in isolated myocytes, on a shorter time scale than in the clinical situation (4, 11). Some of the discrepancies found in the literature are likely due to large differences in the protocols used to trigger or mimic sepsis under *in vivo* and *in vitro* conditions.

We set up a model of conscious endotoxemic rats that reproduces human hemodynamic alterations. Our aim was to investigate the chronology of the cardiovascular alterations, from the time of intravenous administration of EDTX to the time of the typical cardiovascular dysfunction observed at the peak of sepsis. The chronology of the alterations of isolated cardiac myocytes function was also studied. In line with the aforementioned studies, we have followed up on the amplitude of the L-type calcium current (I_{Ca}), the trigger of cardiac contraction, and its regulation by the sympathetic and parasympathetic systems during the development of sepsis.

Part of this work has been published in an abstract form (21).

METHODS

In Vivo Experiments

Experiments were performed in male rats (280–320 g) that were given a 0.3-ml, 1-min injection of either saline or endotoxin (EDTX, 6 mg · kg^{-1}, *Escherichia coli* O111 B4; Sigma, St. Louis, MO) in the dorsal vein of the penis, under brief anesthesia with diethyl ether.* The severity of such a model of unresuscitated, endotoxemic rats was mild.

In a first set of experiments, hemodynamic parameters were measured in rats anesthetized 12 or 36 h after EDTX injection (12-h EDTX and 36-h EDTX rats, respectively). Anesthesia (sodium pentobarbital, 60–100 mg · kg^{-1}, administered intraperitoneally) allowed animal preparation and measurements of arterial pressure and aortic velocity. In the control group, measurements were also performed at 12 or 36 h after saline injection. However, because there was no difference between the two populations of control rats, their data were pooled. Hemodynamic parameters were obtained (*1*) at baseline, 30 min after the stabilization period (that followed the surgery), and (*2*) after volume loading (saline, 6 ml · kg^{-1}, over 5 min) in the three groups of anesthetized rats (control, 12-h EDTX, and 36-h EDTX), in order to test the ability of the heart to respond to increased preload.

In the second set of experiments, the effects of EDTX or saline injection were studied in conscious chronically instrumented rats to avoid interference between hemodynamic alterations related to treatment and those related to anesthesia. After instrumentation, rats were allowed to recover for 3 d before intravenous injection. Measurements were performed before and 12, 24, and 36 h after injection of saline or EDTX.

Animal instrumentation. An aortic Doppler probe and carotid arterial catheter were implanted under general anesthesia. After tracheal intubation, rats were mechanically ventilated (tidal volume, 2.5 ml; frequency, 80 cycles · min^{-1}) with a ventilator (Harvard pump 683; Harvard Instruments, Boston, MA) at FI_{O_2} equal to 0.5. After aortic dissection, a 2-mm-diameter silastic cuffed 20-MHz pulsed Doppler was placed around the ascending aorta. For chronically instrumented animals, the electric wires related to the probes were brought subcutaneously to the dorsal face of the neck. A heparin-coated polyethylene (PE 50) fluid-filled catheter was also inserted into the ascending aorta via the right common carotid artery. Its position was checked in postmortem animals. Animal preparation lasted 90–120 min.

Hemodynamic measurement. Aortic velocity was measured by the flow velocity probe connected to a 20-MHz pulsed Doppler flowmeter (engineered by Baylor College of Medicine, Houston, TX). Baseline zero velocity was verified during the diastolic time. This technique allowed continuous measurement of blood flow velocity and its first derivative, the maximal aortic acceleration. Peak values of aortic velocity (V_{max}) and acceleration (G_{max}) were used as indexes of cardiac performance (22, 23).

Arterial pressure was measured using an arterial catheter connected to a pressure transducer (Abbott Laboratories, Chicago, IL). Data were acquired using data acquisition software (AcqKnowledge, version 3.0; Biopac Systems, Goleta, CA) with a sampling rate of 1,000/s and stored in a Macintosh personal computer. Aortic conductance (Gaor) was calculated as the mean aortic blood flow velocity divided by mean arterial pressure (24). Each data point was the average of 10 consecutive beats recorded in stable condition.

Body weight and temperature were recorded in conscious animals before, 12, and 36 h after EDTX administration. Blood gas analysis and ionic composition, including plasma lactate level, showed no difference among the control and the EDTX animals (data not shown).

Electrophysiology

Myocytes from male rats (180–300 g) were dispersed using collagenase A (0.255 mg · ml^{-1}) as described (25). At the end of the perfusion, atria and ventricles were separated, and ventricular myocytes were resuspended, step by step, in a 1 mM Ca^{2+}-containing solution. The cells were kept at 37° C until use, within 1–16 h of isolation. 12-h EDTX and 36-h EDTX myocytes refer to myocytes of rats that received an intravenous injection of EDTX, respectively, 12 and 36 h before cell isolation whereas control myocytes refer to saline-injected or untreated animals.

The whole-cell patch-clamp technique was used to record L-type calcium current (I_{Ca}) in Ca^{2+}-tolerant cells; the routine protocol consisted of a test pulse to 0 mV (400-ms duration) elicited every 8 s from a holding potential of −50 mV. Occasionally (*see* Figure 3), the holding potential was increased to −80 mV and the test pulse was preceded by a short pulse to −50 mV (50-ms duration) in the presence of tetrodotoxin (TTX, 60–90 μM) to inhibit the Na+ current (25). This protocol minimized the antagonistic effect of (±)Bay K 8644 (*see* Reference 26). The time-dependent I_{Ca}, measured as the difference between the peak inward current during the test pulse and the current at the end of the pulse (I_{400}), was attributed to the activity of the L-type calcium channels (25). For the determination of current–voltage relationships for I_{Ca} (*see* Figure 2A) and I_{Ca} inactivation curve (data not shown), a double-pulse voltage-clamp protocol was used (25). Voltage-clamp protocols were generated by a challenger/09-VM programmable function generator (Kinetic Software, Atlanta, GA). The cells were voltage clamped with a patch-clamp amplified (EPC-7; List, Darmstadt, Germany). Currents were sampled at a frequency of 10 kHz with a 12-bit analog-to-digital converter (DT2827; Data Translation, Marlboro, MA) connected to a PC-compatible computer. The experiments were performed at room temperature (24–32° C), and in a given experiment, the temperature did not change by > 2° C.

Solutions. The external Cs^+-containing solution contained 107 mM NaCl, 10 mM HEPES, 20 mM CsCl, 4 mM $NaHCO_3$, 0.8 mM NaH_2PO_4, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM D-glucose, 5 mM sodium pyruvate, 6–9 × 10^{-4} mM TTX (pH 7.4, adjusted with CsOH). External solutions were applied as described (27). The patch pipettes (0.5–1.0 MΩ) were filled with an internal Cs^+-containing solution composed of

* The investigation conforms with the European Community guiding principles in the care and use of animals (86/609/CEE, *CE Off. J.* L358, December 18, 1986) and the French decree 87/748 of October 19, 1987 (*J. Off. République Française*, October 20, 1987, pp. 12245–12248). Authorizations to perform animal experiments according to this decree were obtained from the French Ministère de l'Agriculture et de la Forêt (04226, April 12, 1991).

TABLE 1

HEMODYNAMIC PARAMETERS IN ANESTHETIZED RATS TREATED WITH SALINE OR EDTX*

	Control			EDTX (*12 h*)			EDTX (*36 h*)		
	n	Baseline	After VL	n	Baseline	After VL	n	Baseline	After VL
Body weight, g	43	290 ± 4	—	40	275 ± 3[†]	—	28	259 ± 4[†,‡]	—
Temperature, °C	16	37.2 ± 0.1	—	5	39.5 ± 0.3[†]	—	5	39.5 ± 0.4[†]	—
SAP, mm Hg	5	134 ± 6	141 ± 7[§]	5	91 ± 11[‖]	118 ± 15[§]	5	107 ± 11[‖]	104 ± 11
HR, bpm	5	382 ± 7	368 ± 9	5	368 ± 9	358 ± 17	5	417 ± 35[‖,#]	414 ± 21
Gaor, $cm \cdot s^{-1} \cdot mm\ Hg^{-1}$	5	0.16 ± 0.01	0.17 ± 0.01	5	0.32 ± 0.05[‖]	0.32 ± 0.06	5	0.12 ± 0.01[#]	0.12 ± 0.01

Definition of abbreviations: EDTX = endotoxin; VL = volume loading.

* Body weight and temperature were recorded in awaked rats; systolic arterial pressure (SAP), heart rate (HR), and aortic conductance (Gaor) were recorded in anesthetized animals.

[†] $p < 0.001$ versus control.

[‡] $p < 0.001$ versus 12-h EDTX.

[§] $p < 0.01$ versus baseline for the same condition.

[‖] $p < 0.05$ versus control/baseline.

[#] $p < 0.005$ versus 12-h EDTX/baseline.

119.8 mM CsCl, 5 mM EGTA (acid form), 0.062 mM $CaCl_2$ (pCa 8.5), 4 mM $MgCl_2$, 5 mM disodium phosphocreatine, 3.1 mM Na_2ATP, 0.42 mM Na_2GTP, 10 mM HEPES (pHN 7.3 adjusted with CsOH).

Drugs. Collagenase was from Boehringer GmbH (Mannheim, Germany). Tetrodotoxin was from Latoxan (Rosans, France). L855081 (Calbiochem, La Jolla, CA) was dissolved in distilled water at 10 mM and stored at −20° C until single use. All other drugs were from Sigma. Nifedipine and (±)Bay K 8644 were dissolved in ethanol at 1 mM, and stored at −20° C until single use. Drug-containing solutions were prepared at the beginning of each experiment.

Data analysis. During patch-clamp experiments, the maximal amplitude of whole-cell I_{Ca} was measured as previously described (25). Membrane capacitances were calculated as described by Scamps and co-workers (25). On-line analysis of the recordings was made possible by programming a PC-compatible computer in Assembly language (Borland) to determine, for each depolarization, peak and steady state current values, as well as the time to peak and the integral of I_{Ca} (27). Here, the "basal" condition for I_{Ca} refers to the absence of cAMP-elevating agents.

Mean maximal effects (E_{max}) and half-maximal concentrations (EC_{50}) were obtained by fitting sets of individuals values to the Michaelis–Menten equation. Correlation coefficients were found to be > 0.95 for the curves presented in Figures 4 and 6.

Statistical Analysis

Results are expressed as means ± SEM. When appropriate, statistical comparisons of two groups of data were made with a Student *t* test. Comparisons among the three groups (control, 12-h EDTX, and 36-h EDTX) were made using analysis of variance (ANOVA)-factorial or ANOVA-two way for repeated measures.

RESULTS

EDTX-induced Hemodynamic Alterations *In Vivo*

Table 1 shows that *in vivo* injection of EDTX consistently increased body temperature and decreased body weight, as reported in other animal studies (7). The mortality rate was 5% over the 36 h after EDTX administration. Hemodynamic consequences of EDTX injection *in vivo*, studied in anesthetized animals, showed a dominant vascular dysfunction 12 h after EDTX administration and a time-dependent impairment in cardiac function over the 36 h. In the 12-h EDTX group, the large vasodilation (a two-fold increase in Gaor compared with control) induced a decrease in systolic arterial pressure (SAP, −25 ± 3% of control, $p < 0.05$, Table 1) that was partially compensated by volume loading. The indexes of cardiac performance, V_{max} and G_{max}, were slightly diminished after EDTX administration (−10 and −15% of control, respectively) but

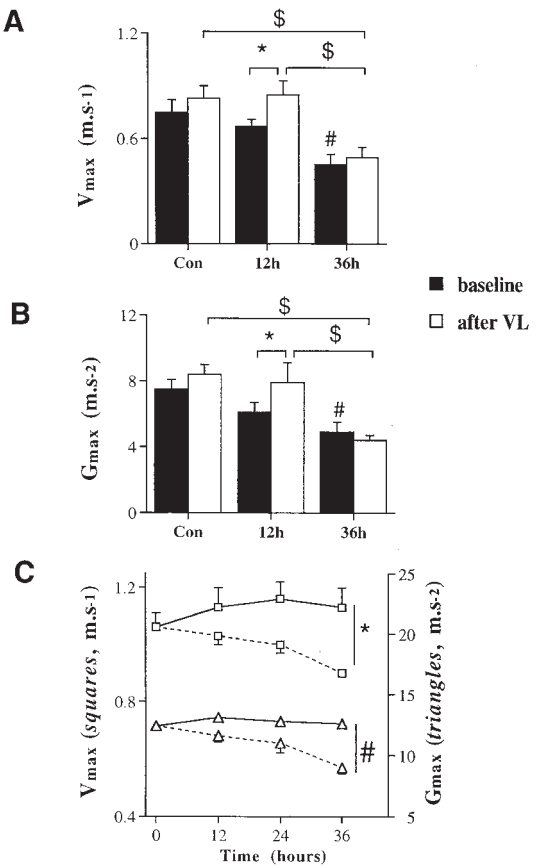

Figure 1. Cardiac function was depressed after endotoxin (EDTX) administration in anesthetized and conscious rats. (*A* and *B*) Cardiac function parameters were measured in rats anesthetized 12 h (n = 5) or 36 h (n = 5) after treatment and compared with rats receiving saline (control, n = 6). V_{max} (*A*) and G_{max} (*B*) were measured before and after volume loading (baseline and after VL, respectively). The *columns* indicate the means and the *bars* indicate the SEM. Statistical significance is indicated as follows: *$p < 0.05$; $^{\$}p < 0.01$; $^{\#}p < 0.01$ versus control and 12 h (Student *t* test). (*C*) Cardiac function parameters V_{max} and G_{max} were measured over a period of 36 h in conscious chronically instrumented rats after saline (*solid lines*, n = 5) or EDTX (*dashed lines*, n = 5) administration. The *symbols* indicate the means and the *bars* the SEM. Differences are indicated as follows: *$p < 0.05$, $^{\#}p < 0.01$ (ANOVA-two way for repeated measures).

were greatly improved by volume loading (Figure 1). Thus the moderate alteration in cardiac performance, likely compensated by the associated decrease in afterload, properly responded to volume loading in 12-h EDTX animals.

Similarly, 36-h EDTX rats had a decreased SAP, as compared with control animals. However, both V_{max} and G_{max} became much more depressed in 36-h EDTX than in control and 12-h EDTX rats. In addition, these indexes of cardiac performance remained unresponsive to volume loading (Figure 1). Accordingly, these data indicate a greater alteration of heart function in 36-h EDTX than in 12-h EDTX rats.

Because anesthesia can distort *in vivo* measurements in an unpredictable manner (28), we repeated these measurements in conscious, chronically instrumented rats (Figure 1C). Although the amplitudes of V_{max} and G_{max} were $\sim$ 25 to 30% higher in awake compared with anesthetized animals, EDTX was found to exert similar hemodynamic effects. V_{max} and G_{max} remained stable in awake control rats whereas EDTX treatment induced a time-dependent decrease in V_{max} and G_{max} (respectively, -30 and -35% versus control at 36 h). Alterations in SAP and aortic conductance after saline or EDTX administration were similar in conscious rats to that observed in anesthetized rats (Table 2). Thus, these data show that myocardial dysfunction was present in all EDTX-treated rats, regardless of anesthesia.

In Vivo Treatment with EDTX Reduces I_{Ca} in Isolated Ventricular Myocytes

By using a routine protocol wherein the cardiac L-type calcium current (I_{Ca}) was elicited at 0 mV, we found that the amplitude of I_{Ca} (814.7 $\pm$ 28.2 pA in control cells, n = 161) tended to be lower in myocytes isolated from 12-h EDTX rats (772.0 $\pm$ 36.3 pA, n = 74), and in myocytes from 36-h EDTX rats (665.9 $\pm$ 25.4 pA, n = 99; $p < 0.01$ versus control and 12-h EDTX rats). Cell-to-cell variability was minimized by normalizing the amplitude of I_{Ca} to cell membrane capacitance (C_m), an estimate of cell membrane area. The density of I_{Ca} (I_{Ca}/C_m) was lower in 12-h EDTX myocytes than in control cells (respectively, 5.04 $\pm$ 0.20 and 5.90 $\pm$ 0.15 pA/pF, n = 70 and 159, $p < 0.001$; Figure 2A). The density of I_{Ca} was even lower in 36-h EDTX myocytes (4.41 $\pm$ 0.14 pA/pF, n = 95, $p < 0.001$ versus control and $p < 0.01$ versus 12-h EDTX). L-type calcium channels being voltage dependent, their behavior was examined at different membrane potentials (Figure 2B). I_{Ca} was reduced in a uniform manner at every potential in 12-h EDTX and 36-h EDTX myocytes, as compared with control cells. Membrane integrity was not affected by EDTX treatment because the "leak current" (i.e., the current measured at the end of the pulse, I_{400}) was similar in the three groups of cells.

The reduction in I_{Ca} may be explained by an increase in the inactivation rate of the calcium channels. However, an index of the course of I_{Ca} inactivation, the ratio of I_{Ca} time integral over peak I_{Ca} density (29), was unchanged in EDTX myocytes (30.90 $\pm$ 0.65 ms in control, n = 96, 29.91 $\pm$ 0.91 ms in 12-h EDTX, n = 34, 29.76 $\pm$ 0.70 ms in 36-h EDTX, n = 65). Furthermore, the time to peak, the steady state inactivation curves, and the current–frequency relationships of I_{Ca} (in the 0.125- to 2-Hz range) were similar in the three groups of cells (data not shown). Thus, the reduction in I_{Ca} observed in EDTX myocytes was likely due to a reduction in the number of functional calcium channels, with no modifications in the gating properties of individual channels.

Effects of Dihydropyridine Agonists and Antagonists on I_{Ca}

Nifedipine (0.5 μM), a dihydropyridine receptor antagonist that selectively blocks L-type calcium channels (25, 26), inhibited most of the macroscopic I_{Ca} (93.6 $\pm$ 3.2%, n = 7) in control myocytes. The inhibitory effect of nifedipine (0.5 μM) was

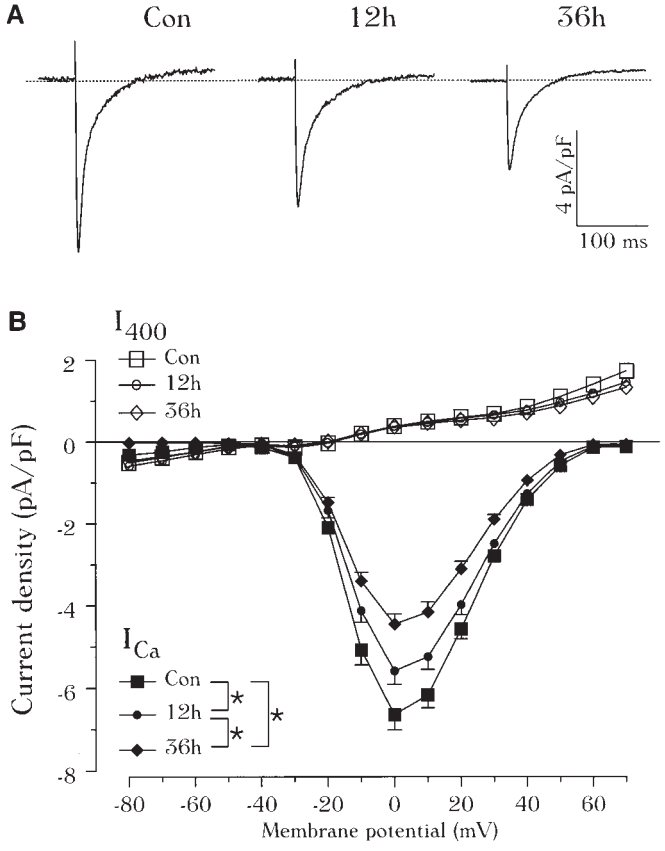

Figure 2. *In vivo* treatment with EDTX reduces I_{Ca} in isolated rat ventricular myocytes. (*A*) Current traces recorded at 0 mV in a control (*left*), 12-hr EDTX (*middle*), and 36-h EDTX myocyte (*right*). The dotted line indicates the zero-current level. (*B*) Current density–voltage relationships of I_{Ca} (*solid symbols*) and I_{400} (*open symbols*) in control (*squares*), 12-h EDTX (*circles*), and 36-h EDTX (*diamonds*) myocytes. *Symbols* represent the means, and *bars* the SEM of 23–33 experiments. Differences in I_{Ca} density among groups, in the -30- to $+50$-mV range, are indicated as follows: *$p < 0.001$ (ANOVA-two way for repeated measures).

TABLE 2

HEMODYNAMIC PARAMETERS IN CONSCIOUS CHRONICALLY INSTRUMENTED RATS BEFORE AND AFTER SALINE OR EDTX ADMINISTRATION*

	Before	12 h	36 h
Control (n = 5)			
SAP, mm Hg	159 $\pm$ 5	154 $\pm$ 4	141 $\pm$ 4
HR, bpm	428 $\pm$ 19	400 $\pm$ 13	418 $\pm$ 23
Gaor, cm $\cdot$ s^{-1} $\cdot$ mm Hg^{-1}	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01	0.10 $\pm$ 0.01
EDTX (n = 5)			
SAP, mm Hg	155 $\pm$ 4	115 $\pm$ 2	117 $\pm$ 7
HR, bpm	420 $\pm$ 12	430 $\pm$ 15	452 $\pm$ 18
Gaor, cm $\cdot$ s^{-1} $\cdot$ mm Hg^{-1}	0.09 $\pm$ 0.01	0.12 $\pm$ 0.01	0.09 $\pm$ 0.01

* EDTX decreased SAP over the 36 h and increased Gaor over the first 12 h ($p < 0.005$ and $p < 0.05$, respectively, both by ANOVA-two way for repeated measures).

similar in 12-h EDTX (92.1 ± 2.0%, n = 8) and 36-h EDTX myocytes (91.1 ± 1.9%, n = 7) as compared with control. Hence, I_{Ca} was carried by the same type of channels in all groups of cells.

(±)Bay K 8644, a dihydropyridine agonist, enhanced I_{Ca} density to the same level in control and EDTX myocytes (Figure 3). The mean stimulatory effect of (±)Bay K 8644 on I_{Ca} was much larger in 12-h EDTX and 36-h EDTX myocytes (350.5 ± 44.44 and 322.4 ± 15.4% of basal, respectively) as compared with control (232.0 ± 9.3% of basal, $p < 0.01$ and $p < 0.001$ versus 12-h EDTX and 36-h EDTX, respectively). Because the macroscopic properties of I_{Ca} were not changed in EDTX myocytes, as compared with control (*see above*), (±)Bay K 8644 appeared to "mobilize" calcium channels made unavailable by the *in vivo* injection of EDTX.

Stimulation of I_{Ca} by the β-Adrenergic Agonist Isoproterenol

β-Adrenergic agonists, such as isoproterenol (Iso), produce a well-documented stimulation of I_{Ca} (26). In the presence of Iso (10 nM and 1 μM), I_{Ca} density was somewhat higher in 12-h EDTX myocytes as compared with control and 36-h EDTX myocytes (Figure 4A). Indeed, the net stimulatory effect of Iso (i.e., Iso-stimulated I_{Ca} − basal I_{Ca}) was larger in 12-h EDTX myocytes (+7.9 ± 0.7 pA/pF with 10 nM Iso, and +9.7 ± 1.0 pA/pF with 1 μM Iso) than in control cells (+5.6 ± 0.4 pA/pF with 10 nM Iso, and +7.1 ± 0.6 pA/pF with 1 μM Iso; respectively, $p < 0.01$ and $p < 0.05$ versus 12-h EDTX). However, the net effect of Iso declined in 36-h EDTX myocytes (+5.1 ± 0.3 pA/pF with 10 nM Iso, and +6.36 ± 0.6 pA/pF with 1 μM Iso; respectively, $p < 0.005$ and $p < 0.01$ versus 12-h EDTX). Interestingly, the net stimulatory effect of Iso was as large in control as in 36-h EDTX myocytes, although the basal I_{Ca} was much lower in 36-h EDTX myocytes.

The β-adrenergic regulation of I_{Ca} was studied over a wider range of Iso concentrations. To resolve the changes in the β-adrenergic pathway, the Iso-stimulated I_{Ca} was normalized to the amplitude of the basal I_{Ca}, as summarized in Figure 4B. The stimulatory effect of Iso on I_{Ca} was significantly scaled up in 12-h EDTX myocytes as compared with control, over the whole range of concentrations. The stimulatory effect of Iso was also enhanced in 36-h EDTX myocytes as compared with control, but only in the 0.1–1 μM range. The dose–response curve for Iso appeared shifted to the right in 36-h EDTX myocytes, as compared with 12-h EDTX myocytes. Thus the change in the β-adrenergic stimulation of I_{Ca} appeared more beneficial in 12-h EDTX than in 36-h EDTX myocytes, espe-

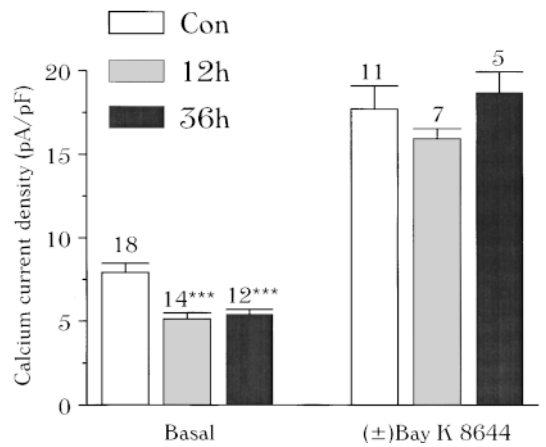

Figure 3. The stimulatory effect of (±)Bay K 8644 on I_{Ca} is enhanced in EDTX myocytes. Summary of I_{Ca} density in the absence (basal) and in the presence of (±)Bay K 8644 (100 nM) in control (Con), 12-h EDTX (12h), and 36-h EDTX (36h) myocytes. *Columns* represent the means, and *bars* the SEM; the number of experiments is indicated above the columns. Significant differences from control are indicated as follows: ***$p < 0.005$ (Student *t* test).

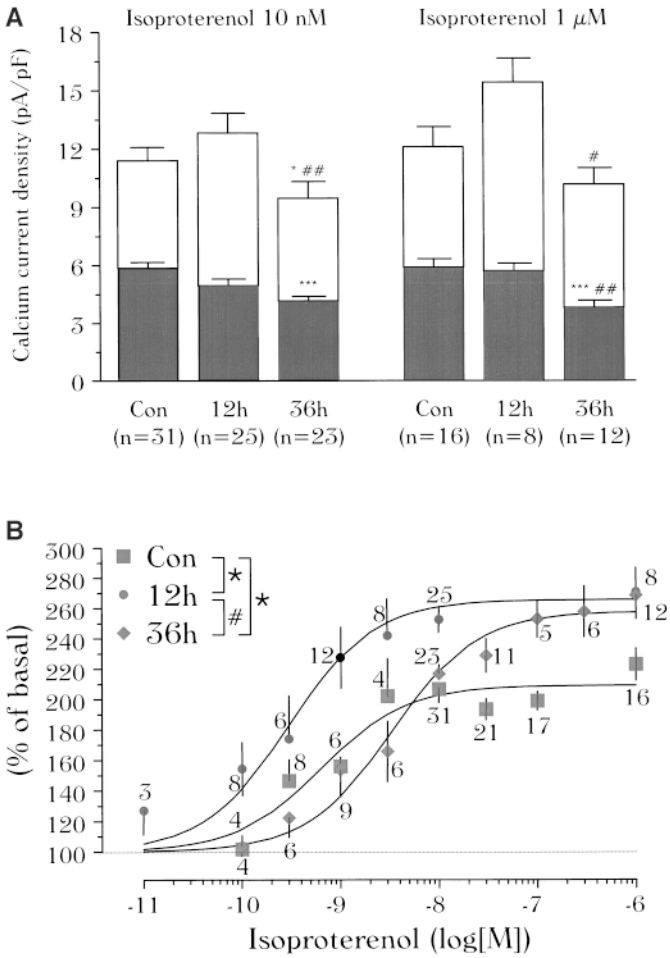

Figure 4. Modifications in the β-adrenergic stimulation of I_{Ca} in EDTX myocytes. (*A*) Summary of I_{Ca} density in the absence (*solid columns*) and in the presence (*open columns*) of either 10 nM or 1 μM isoproterenol in the control (Con), 12-h EDTX (12h), and 36-h EDTX (36h) myocytes. *Columns* indicate the means, and *bars* the SEM of the number of experiments indicated. Significant differences from the control (*) or 12-h (#) group are indicated as follows: $^{*,\#}p < 0.05$; $^{\#\#}p < 0.01$; ***$p < 0.005$ (Student *t* test). (*B*) Dose–response curves for the stimulatory effects of Iso on I_{Ca} in control (*squares*), 12-h EDTX (*circles*), and 36-h EDTX (*diamond*) myocytes. *Symbols* are means, and *vertical lines* the SEM of the number of experiments indicated near the bars. The amplitude of the isoproterenol-stimulated I_{Ca} was normalized to the amplitude of the basal I_{Ca}. The *solid lines* are the fit of the data to the Michaelis–Menten equation. Parameters of the fits (E_{max} and EC_{50}) were 209.1% and 0.6 nM in control myocytes; 265.7% and 0.3 nM in 12-h EDTX myocytes; and 257.9% and 3.3 nM in 36-h EDTX myocytes. Basal I_{Ca} densities in control, 12-h EDTX, and 36-h EDTX myocytes were, respectively, 5.63 ± 0.19 pA/pF (n = 72), 4.94 ± 0.27 pA/pF (n = 39, $p < 0.05$ versus control), and 4.54 ± 0.21 pA/pF (n = 44, $p < 0.0005$ versus control). Significant differences between groups are indicated as follows: *$p < 0.001$; $^{\#}p < 0.05$ (ANOVA-factorial).

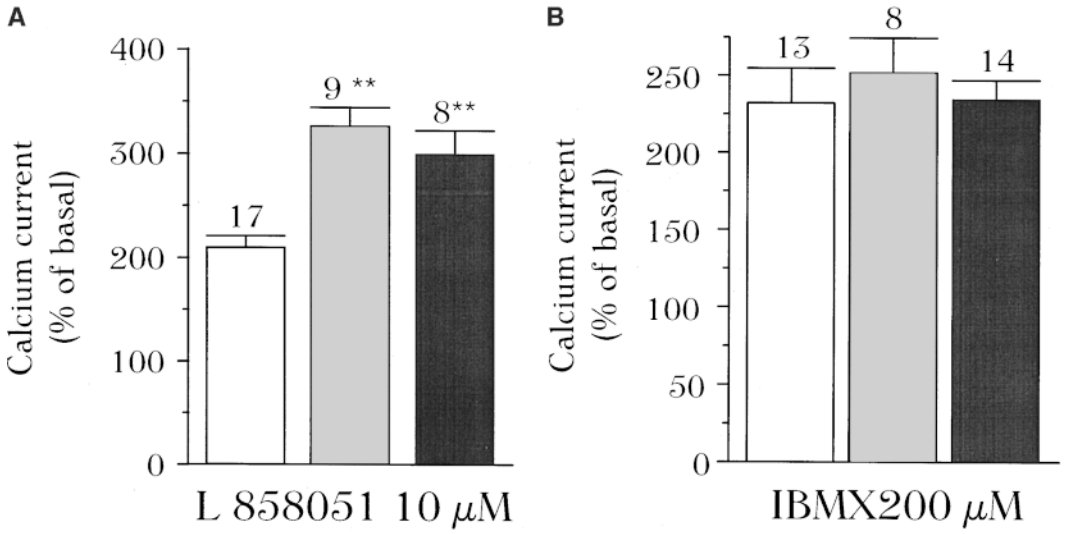

Figure 5. Effects of the cyclic AMP pathway on I_{Ca} in EDTX myocytes. Summary of the effects of the adenylyl cyclase activator L858051 (10 μM; *A*) or the phosphodiesterase inhibitor IBMX (200 μM; *B*) in control (*white columns*), 12-h EDTX (*gray columns*), and 36-h EDTX (*black columns*) myocytes. The amplitude of I_{Ca} in the presence of L858051 (*A*) or IBMX (*B*) was normalized to the amplitude of the basal I_{Ca}. *Columns* represent the means, and *bars* the SEM of the number of experiments indicated near the bars. Significant differences from the control group are indicated as follows: **$p < 0.01$ (Student *t* test).

cially at low concentrations of Iso. The β-adrenergic agonist seemed to "mobilize" voltage-reluctant calcium channels in EDTX myocytes, and this effect can be demonstrated at maximal concentrations of Iso. We next investigated the mechanism accounting for the increase in the maximal effect of Iso in EDTX myocytes.

Effect of the Cyclic AMP Pathway on I_{Ca}

β-Adrenergic receptor density undergoes a biphasic change during sepsis in rat hearts (17). To examine whether changes in β-adrenergic receptors account for the modifications in the effects of Iso on I_{Ca}, we first studied the effect of an adenylyl cyclase activator, the forskolin analog L858051, which bypasses β-adrenergic receptors (30). As illustrated in Figure 5A, 10 μM L858051 induced a twofold increase in basal I_{Ca} in control myocytes, and a threefold increase in myocytes isolated from both 12-h EDTX and 36-h EDTX-treated rats. Thus, the maximal effect of L858051 on I_{Ca} was comparable to the maximal effect of Iso. Hence, a modification in β-adrenergic receptor density does not account for the enhancement of the β-adrenergic stimulation of I_{Ca} in EDTX myocytes.

The regulation of I_{Ca} in EDTX-treated rats appeared to be modified at steps located downstream from cAMP production. One such mechanism could be the cAMP-dependent protein kinase (cA-PK) Because an enhanced activity of cA-PK has been reported in an animal model of sepsis (31). Therefore, we examined the effect of a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, IBMX) that elevates cAMP levels by inhibiting cAMP hydrolysis. As illustrated in Figure 5B, a saturating concentration of IBMX (200 μM) induced a twofold increase in basal I_{Ca} in control myocytes as well as in myocytes derived from EDTX-treated rats, both after 12 and 36 h of *in vivo* treatment. Thus, in the absence of a stimulation of cAMP production, the stimulatory effect of cAMP accumulation on I_{Ca} is not modified in EDTX myocytes, as compared with control.

Muscarinic Regulation of I_{Ca}

Acetylcholine (ACh), the main neurotransmitter of the parasympathetic system, counteracts the effects of β-adrenergic agonists *in vivo*, and antagonizes the β-adrenergic stimulation of I_{Ca} in isolated myocytes (32). This antiadrenergic effect of ACh on I_{Ca} was first studied in the continuing presence of 10 nM Iso (Figure 6A). The density of the Iso-stimulated I_{Ca} was reduced to the same extent by ACh (3 μM) in control and 12-h EDTX myocytes. The density of I_{Ca} was much lower in 36-h EDTX myocytes, in the absence and in the presence of ACh. In these cells, the Iso-stimulated I_{Ca} was reduced almost to the basal level by the muscarinic agonist. The inhibitory effect of ACh was then normalized to the net stimulatory effect of Iso, and further examined at different concentrations of ACh (Figure 6B). The maximal inhibitory effect of ACh was significantly increased in 36-h EDTX myocytes (79.4 ± 13.1% inhibition of the Iso stimulation, $n = 8$), as compared with control and 12-h EDTX myocytes (respectively, 56.0 ± 3.9 and 51.5 ± 4.1% inhibition, $n = 10$ and $n = 6$, both $p < 0.05$ versus 36-h EDTX). Thus, in 36-h EDTX myocytes, the low density of I_{Ca} in the presence of Iso plus ACh was not only due to the low density of the Iso-stimulated I_{Ca}, but also to an enhanced effect of ACh.

The effect of ACh was also studied in the presence of 1 μM Iso, a concentration at which the β-adrenergic stimulation was modified not only in 12-h EDTX myocytes, but also in 36-h EDTX myocytes (*see above*). Under this condition, the antiadrenergic effect of ACh was again similar in control and 12-h EDTX myocytes, and the maximal effect of ACh in 36-h EDTX myocytes was enhanced as compared with control (data not shown). Overall, the *in vivo* treatment with EDTX induces an increase in the inhibitory effect of ACh. This phenomenon was delayed as compared with the changes in the β-adrenergic regulation of I_{Ca}. The next experiments were aimed at illustrating the mechanisms involved in this alteration in the muscarinic regulation.

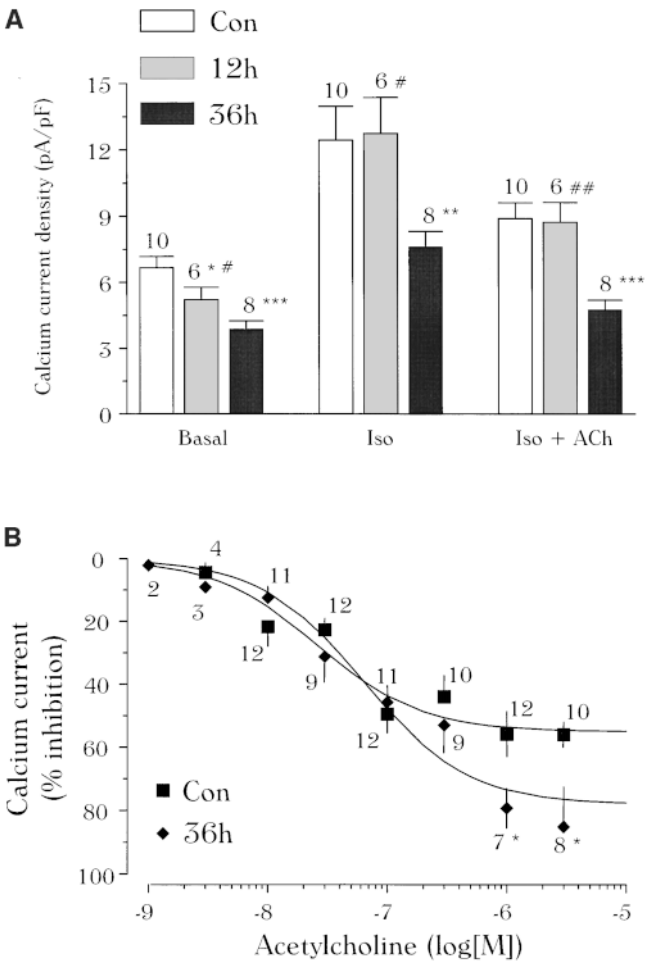

Figure 6. Modifications of the antiadrenergic effect of acetylcholine on I_{Ca} in EDTX myocytes. (*A*) Summary of I_{Ca} density in the absence (Basal) and in the presence of either 10 nM isoproterenol (Iso) or 10 nM Iso plus 3 μM acetylcholine (Iso + ACh) in control (Con), 12-h EDTX (12h), and 36-h EDTX (36h) myocytes. *Columns* represent the means, and *bars* the SEM of the number of experiments indicated. Differences from the control (*) or 12-h (#) group are indicated as follows; $*,^{\#}p < 0.05$; $**,^{\#\#}p < 0.01$; $***p < 0.005$ (Student *t* test). (*B*) Dose–response curve for the inhibitory effect of ACh on Iso-stimulated I_{Ca} in control (*squares*) and 36-h EDTX (*diamonds*) myocytes. *Symbols* indicate means, and *vertical lines* the SEM of the number of experiments indicated near the bars. The reduction in the amplitude of I_{Ca} in the presence of Iso (10 nM) plus ACh was normalized to the net stimulatory effect of Iso. The *solid lines* are the fit of the data to the Michaelis–Menten equation. Parameters of the fits (E_{max} and EC_{50}) were 55.1% and 26.0 nM in control myocytes, and 78.1% and 63.1 nM in 36-h EDTX myocytes. Significant differences are indicated as follows: $*p <$ 0.05 (Student *t* test).

Muscarinic Regulation of the Cyclic AMP-dependent Stimulation of I_{Ca}

The maximal effect of ACh (3 μM) I_{Ca} was examined in the presence of L858051 (Figure 7A). *In vivo* treatment with EDTX potentiated the ACh inhibition of the L858051-stimulated I_{Ca}, an effect that was clearly significant in 36-h EDTX myocytes. These results suggest that β-adrenergic receptors, and/or their coupling efficiency to adenylyl cyclase, are not the primary components responsible for the alteration of the response of I_{Ca} to Iso and ACh in EDTX-treated rats.

The IBMX-stimulated I_{Ca} was also reduced in a reversible manner on application of ACh in control and EDTX myocytes (Figure 7B). Interestingly, the maximal inhibitory effect of ACh (10 μM) on I_{Ca}, observed in the presence of IBMX, was identical in the control and EDTX myocytes. Thus, the muscarinic regulation of I_{Ca} was not modified when cAMP level was elevated by phosphodiesterase inhibition. Altogether, the *in vivo* treatment with EDTX does not affect the cAMP-dependent regulation of I_{Ca} at a step located downstream from cAMP production.

DISCUSSION

We found that a single *in vivo* administration of EDTX induces time-dependent changes in rat cardiovascular function that mimic the clinical situation. At the cellular level, EDTX injection induced a decrease in the basal density of I_{Ca} in isolated rat ventricular myocytes. The autonomic control of I_{Ca} was also modified in a time-dependent manner after EDTX challenge. The β-adrenergic stimulation of I_{Ca} was potentiated at 12 h and to a lesser extent at 36 h after EDTX injection, whereas the antiadrenergic effect of ACh on I_{Ca} was unchanged at 12 h, but strongly increased at the late stage of sepsis. Our findings support the view that sepsis induces a remodeling of the autonomic control of cardiac function.

Septic shock is characterized by hypotension and a low cardiac index in patients before resuscitation. At the early stage of sepsis, fluid resuscitation increases the cardiac index to a normal level or even to a level greater than before sepsis (33). Some patients progress to a more serious cardiac dysfunction and become unresponsive to the usual treatment, including fluid resuscitation and even catecholamine administration (15). In our study, EDTX administration in awake rats induced similar hemodynamic changes and appeared to mimic the cardiovascular dysfunction observed in septic patients.

In vivo injection of EDTX induced a progressive decline in the density of I_{Ca}. Zhong and colleagues (5) also reported a slight slowing of the decay of I_{Ca}, related to a drastic ($\sim$ 50%) reduction in I_{Ca}. The lack of modification in the kinetics of I_{Ca} in the present study is probably related to a smaller reduction ($\sim$ 25%) in I_{Ca} density. The other properties of I_{Ca} were unaffected by EDTX treatment (voltage dependency, steady state inactivation). Furthermore, in myocytes from EDTX-treated rats, I_{Ca} was sensitive to concentrations of nifedipine and (±)Bay K 8644 known to be selective for the L-type calcium channels. These data demonstrated that I_{Ca} was carried by L-type Ca^{2+} channels in EDTX myocytes.

The decline in basal I_{Ca} may participate in the myocardial dysfunction during sepsis. First, EDTX injection was reported to shorten the cardiac action potential (5, 30; but *see* Reference 9). Second, the maximal rate of cell shortening and the rise in intracellular calcium were shown to be considerably reduced in EDTX myocytes (5, 30). Because I_{Ca} is the trigger of cardiac contraction, a reduction in the density of I_{Ca} is likely to exert significant effects on the contraction in EDTX myocytes. Importantly, sarcoplasmic reticulum is unlikely to balance the reduction in I_{Ca} because it was found to be essentially unaffected during sepsis (8). However, the decrease in cardiac myofilament response to calcium (3, 4) might aggravate further the consequences of a reduced calcium influx in cardiac myocytes during sepsis.

The mechanism of I_{Ca} reduction remains unclear. The simplest explanation for this reduction is a diminution in the total number of calcium channels, as already reported in hearts of endotoxemic rabbits (10), with no modification in the voltage-dependent properties of the remaining functional channels.

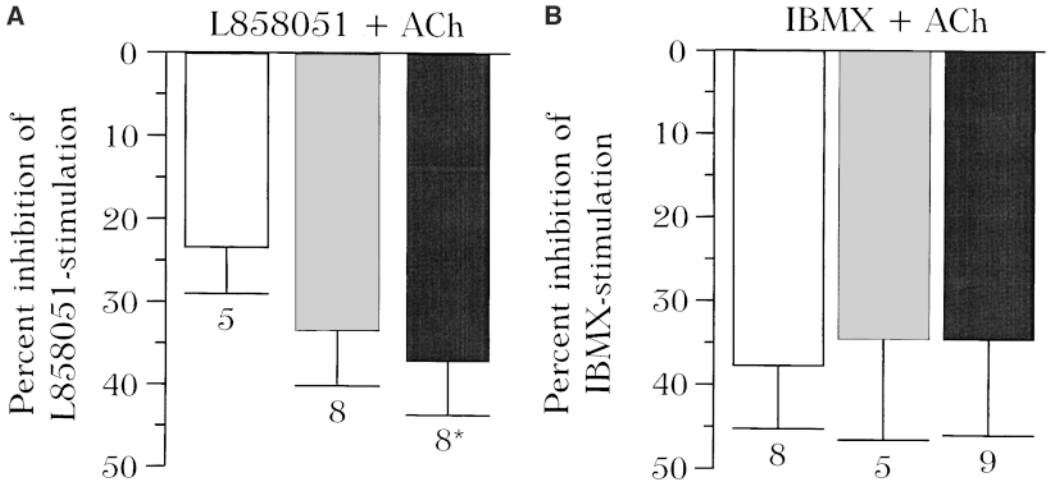

Figure 7. Effect of ACh on the cyclic AMP-dependent stimulation of I_{Ca} in EDTX myocytes. Summary of the inhibitory effects of ACh [3 μM in (*A*), 10 μM in (*B*)] on I_{Ca} in the presence of L858051 (10 μM; *A*) or IBMX (200 μM; *B*) in control (*white columns*), 12-h EDTX (*gray columns*), and 36-h EDTX (*black columns*) myocytes. The reduction in the amplitude of I_{Ca} in the presence of L858051 or IBMX plus ACh was normalized to the amplitude of the stimulation of I_{Ca} with L858051 or IBMX. *Columns* indicate the means, and *bars* the SEM of the number of experiments indicated near the bars. Difference from the control group is indicated as follows: *$p < 0.05$ (Student *t* test).

However, this hypothesis is difficult to reconcile with the results obtained with (±)Bay K 8644 (and with Iso; *see below*). Indeed, the dihydropyridine agonist had a more pronounced stimulatory effect on I_{Ca} density in 12-h EDTX and 36-h EDTX myocytes as compared with control myocytes, thereby canceling the differences in I_{Ca} density between the three groups of cells. Because the voltage-dependent properties of I_{Ca} in the presence of (±)Bay K 8644 were not different in EDTX and control myocytes (data not shown), it looks as if (±)Bay K 8644 increased the number of functional calcium channels, which contradicts its well-established mode of action (26). Further experiments are needed to understand the mechanism of I_{Ca} reduction by EDTX and the "mobilizing" effect of (±)Bay K 8644.

The β-adrenergic stimulation of I_{Ca} increased 12 h after EDTX injection in the rat. A similar finding was obtained in guinea pig hearts as early as 4 h after EDTX injection (5). We show that the dose response to Iso was scaled up at this early stage of sepsis in the rat. These findings might explain the increased positive inotropic effect of Iso observed early after EDTX injection in guinea pig [after 4 h (14)] and rat heart [after 6 h (16)]. In addition, the antiadrenergic effect of ACh on I_{Ca} was unaltered in 12-h EDTX myocytes. Thus, parasympathetic activity may be unable to counteract an increase in the cardiotonic effect of sympathetic activity, at the level of the cardiac myocyte. However, EDTX injection induced further changes because, in 36-h EDTX myocytes, (*1*) the effect of Iso on I_{Ca} was somewhat attenuated, with a reduction in the apparent sensitivity to the β-adrenergic agonist, and, more importantly, (*2*) the antiadrenergic effect of ACh was increased, owing to a larger maximal effect of the muscarinic agonist. These results could provide a cellular mechanism for either (or both) the increased negative inotropic effect of muscarinic agonists observed during sepsis (18, 19), and the impaired β-adrenergic stimulation by dobutamine in late and severe sepsis in humans (15). Overall, contradictory reports on the effects of β-adrenergic agonists during sepsis might be due to the fact that the cardiac preparations used were engaged at different stages of the septic process.

In light of the multiple biochemical changes occurring during sepsis (7), more than a single step in the cAMP cascade might contribute to the remodeling of the autonomic regulation of I_{Ca}. The experiments with Iso and L858051 demonstrated that alterations were taking place at the level of adenylyl cyclase and/or cAMP hydrolysis by phosphodiesterases. Importantly, the regulation of I_{Ca} was identical in control and EDTX myocytes in the presence of IBMX. Thus, a modification at a step beyond cA-PK cannot explain the effects of Iso and ACh on I_{Ca} in EDTX myocytes. Accordingly, the sensitivity of the calcium channels toward cA-PK was not significantly modified by the EDTX challenge. In addition, a change in phosphodiesterase activity alone is also unlikely to account for the effects of EDTX treatment. Indeed, the extent of the β-adrenergic stimulation in EDTX myocytes (∼ 260% of the basal I_{Ca}) cannot be reached in control myocytes, even in the presence of IBMX plus Iso (data not shown). An attractive hypothesis is that EDTX injection induces a hyperdynamic state of adenylyl cyclase. A somewhat similar finding was obtained previously with an *in vitro* application of TNF-α (12), a cytokine known to participate in septic shock (7). In this study, the stimulatory effects of forskolin or the stimulatory G protein (G_s) on cardiac adenylyl cyclase was increased on prolonged exposure to the cytokine (12). After this initial increase in efficacy of Iso observed at 12 h, the potency of the β-adrenergic agonist to stimulate I_{Ca} was markedly reduced 24 h later. A progressive reduction in the number of β-adrenergic receptors could account for this observation. β-Adrenergic receptors are indeed enrolled into an internalization process during the last phase of sepsis (17). This phenomenon may be a consequence of the presence of high levels of circulating catecholamines and/or exaggerated *in situ* sympathetic stimulation (1, 7, 15). Interestingly, both circumstances also increased the expression of inhibitory G proteins (G_i) (7), which might contribute to the increase in the muscarinic inhibition of I_{Ca} observed in 36-h EDTX myocytes.

In conclusion, a decrease in I_{Ca} may be involved in the myocardial dysfunction occurring during septic shock. This alteration in I_{Ca} appeared to be compensated by an early poten-

tiation of the β-adrenergic response. However, this putative compensatory mechanism was not sustained. It was challenged by an increase in the muscarinic response, which seemed to dominate at the late stage of sepsis, when cardiac dysfunction takes place. Overall, our study does not support the view than the myocardial depression is due to a single predominant biochemical change. Instead, sepsis appears to induce a remodeling of multiple cardiac functions, the balance of which might contribute to the severity of the myocardial dysfunction.

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