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Mechanism of suppression of cardiac L-type Ca^{2+} currents by the phospholipase A_2 inhibitor mepacrine

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Abstract

Phospholipase A_2 plays a crucial role in the release of arachidonic acid (AA) from membrane phospholipids and in myocardial injury during ischemia and reperfusion. Mepacrine, a phospholipase A_2 inhibitor, has been shown to protect the heart from ischemic injury. In order to examine the mechanism of this protection, we investigated the effects of mepacrine on the L-type Ca^{2+} current ($I_{Ca,L}$) in rat single ventricular myocytes. Extracellular application of mepacrine significantly inhibited $I_{Ca,L}$ in a tonic- and use-dependent manner. The inhibition was also concentration-dependent with an IC_{50} of 5.2 μM. Neither the activation nor the steady-state inactivation of $I_{Ca,L}$ was altered by mepacrine. The mepacrine-induced inhibition of $I_{Ca,L}$ was reversible after washout of the inhibitor. Addition of 1 μM AA partially reversed the mepacrine-induced inhibition of $I_{Ca,L}$. Intracellular dialysis, with 2 mM cAMP, significantly increased $I_{Ca,L}$, but did not prevent the mepacrine-induced inhibition of $I_{Ca,L}$. In addition, extracellular application of isoproterenol or membrane permeable db-cAMP did not reverse the mepacrine-induced inhibition of $I_{Ca,L}$. Biochemical measurement revealed that incubation of ventricular myocytes with mepacrine significantly reduced intracellular cAMP levels. The mepacrine-induced reduction of cAMP production was abolished by addition of AA. Our results demonstrate that mepacrine strongly inhibits cardiac $I_{Ca,L}$. While mepacrine is a phospholipase A_2 inhibitor and reduces cAMP production, its inhibitory effect on $I_{Ca,L}$ mainly results from a direct block of the channel. Therefore, we speculate that the protective effect of mepacrine during myocardial ischemia and reperfusion mostly relates to its blockade of Ca^{2+} channels.

Keywords: Cardiomyocyte; Ca^{2+} channel; Phospholipase A_2; Arachidonic acid; Mepacrine

1. Introduction

Biological membranes are essential in maintaining cell integrity and function. The phospholipid bilayer serves not only as a physical barrier, but also as an important source for release of various biological compounds. Phospholipase A_2 enzymes are critical for the generation of bioactive lipids, such as arachidonic acid (AA) and its conversion products (Rana and Hokin, 1990; Exton, 1994). Myocardial ischemia causes activation of phospholipase A_2 and changes in phospholipid metabolism. An accumulation of AA and a marked increase in lysophosphatidylcholine have been found in ischemic myocardium (Chien et al., 1984; Karmazyn, 1989; Creer et al., 1990; Van der Vusse et al., 1992). The increase in AA and lysophosphatidylcholine may have important deleterious electrophysiologic effects and play a crucial role in membrane damage during myocardial ischemia (Damron and Bond, 1993; Van Bilsen and Van der Vusse, 1995). Cyclooxygenase, lipoxygenase, and cytochrome P450 are the three major enzyme systems involved in metabolism of AA. Cytochrome P450 represents the third major pathway for the production of AA-oxygenated metabolites, such as the four epoxide regioisomers, 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids. These products have been shown to enhance cardiac L-type Ca^{2+} currents (Xiao et al., 1998) and to exacerbate the response to ischemia and reperfusion of the heart (Moffat et al., 1993).

Voltage-gated L-type Ca^{2+} channels are extremely important for cardiac contraction, because these channels are the major pathways for Ca^{2+} entry during excitation. The L-type Ca^{2+} current ($I_{Ca,L}$) in heart cells triggers intracellular Ca^{2+} release, which initiates and regulates the force of the muscular contraction, i.e., excitation–contrac-

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tion coupling. The process underlying the stimulatory action of isoproterenol on $I_{Ca,L}$ involves the β-adrenoceptor, the stimulatory G protein (G_s), and the cAMP-dependent protein kinase A (Mcdonald et al., 1994). In cardiac myocytes, the cAMP-dependent protein kinase A phosphorylation of L-type Ca^{2+} channels results in an increase in Ca^{2+} influx. In the present experiments, we evaluated the effects of the potent phospholipase A_2 inhibitor mepacrine (quinacrine) on voltage-activated L-type Ca^{2+} channels in rat single ventricular myocytes.

Mepacrine is an antimalarial drug, which also inhibits phospholipase A_2 activities. A number of studies have shown that mepacrine, via the phospholipase A_2 pathway, protects the heart during ischemia/reperfusion and hypoxia/reoxygenation (Chiariello et al., 1987, Chiariello et al., 1990; Bugge et al., 1997). Mepacrine at 1 μM completely abolished reperfusion arrhythmias and improved recovery of function after global ischemia, and at 10 μM, prevented accumulation of fatty acids in isolated rat hearts (Van Bilsen et al., 1990). The protective effects of mepacrine on myocardial ischemia injury have also been found in an in situ perfused pig heart model (Atani et al., 1986). In vivo mepacrine reduced myocardial infarct size after coronary occlusion in dogs (Chiariello et al., 1990) and rats (Chiariello et al., 1987). The protective effect on cardiac ischemic injury has mainly been ascribed to the inhibition of phospholipase A_2 by mepacrine. However, other effects of mepacrine have been reported. These effects are antagonistic property of voltage-gated Ca^{2+} channels (Filippov et al., 1989; Nagano et al., 1996) and inhibition of Na^+/H^+ exchange (Seiler et al., 1985) and of Na^+/Ca^{2+} exchange (de la Pena and Reeves, 1987). All of these effects may result in protecting the heart during ischemia or hypoxia (Kloner and Braunwald, 1987; Nishida et al., 1993). A recent study strongly supports the possibility that mepacrine protects the heart from hypoxic injury by a mechanism other than inhibition of phospholipase A_2-induced membrane damage (Bugge et al., 1997). As mepacrine is widely used in studies of myocardial ischemia and reperfusion, this study examined whether the inhibition of $I_{Ca,L}$ by mepacrine resulted from an inhibition of phospholipase A_2 or from a direct block of Ca^{2+} channels in isolated rat ventricular myocytes. We also discuss possible mechanisms of mepacrine-induced protective effects on myocardial injury during ischemia or hypoxia.

2. Materials and methods

2.1. Isolation of single myocytes

Single left ventricular myocytes were isolated from the heart of adult male rats (200–300 g body weight, Wistar, Charles River Breeding Labs, MA, USA) with a similar method as previously described (Xiao and McArdle, 1994). Briefly, the heart was rapidly removed from an anesthetized rat (pentobarbital sodium, 30 mg/kg i.p.) and the aorta was cannulated and connected to a modified Langendorff apparatus with a flow rate of 5–10 ml/min. The heart was initially perfused for 4 min with oxygenated 37°C Tyrode solution containing (in mM): NaCl 137, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, Hepes 10, glucose 10, pH 7.4. The heart was then perfused with a Ca^{2+}-free Tyrode solution for 5 min, and recirculated for 25 min with 50 ml of a Ca^{2+}-free Tyrode solution containing 60 mg collagenase Type I, 2 mg protease Type XIV, and 1 mg/ml bovine serum albumin (Sigma, St. Louis, MO, USA). At the end of enzyme perfusion, the heart was sequentially washed with 50 ml 0.2 mM Ca^{2+} Tyrode solution plus 1 mg/ml bovine serum albumin. After these treatments, several pieces were cut off from the left ventricle and placed into a culture petri dish. The tissue was then minced and gently agitated to separate the cells. These ventricular myocytes were kept in 0.5 mM Ca^{2+} Tyrode solution at room temperature for 1–2 h before use.

2.2. Recording of L-type Ca^{2+} currents

During an experiment, a small volume of the myocyte-containing solution was pipetted into a small chamber containing 0.3 ml bath solution. The chamber with a coverglass bottom was mounted on the stage of an inverted microscope (Nikon, Japan). The chamber was continuously superfused with the Tyrode solution at a rate of 2–3 ml/min. Recording pipettes were made from 1.5 mm o.d. glass tubes (World Precision Instruments, Sarasota, FL, USA) by a two-stage pull on a David Kopf (Model 700D, Tujunga, CA, USA) vertical puller. Electrodes with 2–4 MΩ resistance were connected via an Ag–AgCl wire to an Axopatch 1D amplifier (Axon Instruments, CA, USA). After forming a conventional ''gigaseal'', the capacitance of an electrode was compensated. Additional suction was used to form the whole-cell configuration. Whole-cell membrane capacitance was routinely measured by a method described previously (Xiao and McArdle, 1994). In the present study, the average whole-cell membrane capacitance was 174 ± 6 pF ($n = 135$). After establishment of the whole-cell recording configuration, cells were clamped at -70 mV and allowed a period of 8–10 min for dialysis of the electrode solution. Series resistance was electrically compensated by approximately 90% to reduce artifact distortion. Whole-cell calcium currents were recorded by using Axopatch-1D amplifier and pCLAMP software 8.02 (Axon Instruments). External solutions were exchanged with a puffer–pipette system (Xiao et al., 1998). Experiments were carried out at 22–23°C.

2.3. Measurement of intracellular cAMP content

Freshly isolated rat single ventricular myocytes were suspended in 1 mM Ca^{2+} Tyrode solution. Myocytes were

incubated with drugs (or vehicle only) for 30 min at 22–23°C. The reaction was terminated by rupturing the cells by the addition of trichloroacetic acid (0.1 g per 2 ml cell suspension) and homogenization in a POLYTRON for 0.5 min. The homogenate was centrifuged at 2000 *g* for 15 min at 4°C. The supernatants were then extracted three times with water-saturated ethyl ether to remove excess trichloroacetic acid, then heated at 70°C under a continuous stream of nitrogen to remove the ether. The pH was adjusted between 6 and 7 to avoid the nucleotide hydrolysis in acidic media. The final volume was measured and stored at −80°C until the time of assay.

The nucleotide measurement was carried out using a reverse phase isocratic paired ion technique (Wang et al., 1995). The mobile phase contained 8.3 mM KH_2PO_4, 0.2% triethylamine, 6.7% acetonitrile, and 3.7% methanol. The final pH was adjusted to 4.12. The high pressure liquid chromatography (HPLC) system is made up of ''Waters 515 Pump'' and ''Waters 2487 Dual Wavelength Detector''. Flow rate was maintained at 1.0 ml/min and detection at 258 nm using Waters Millennium 3.05 software (Waters, Milford, MA, USA). Protein content of the homogenate for nucleotide measurement was determined by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.4. Chemicals

Mepacrine, AA and cAMP were obtained from Sigma. Stock solutions of mepacrine in deionized water and AA in ethanol were made weekly and stored in −20°C. The final concentrations of the solvents had no effects on cardiac Ca^{2+} current. The following solutions were used during experiments. For the whole-cell recording, the bath solution contained (in mM): *N*-methyl *d*-glucamine 120, CsCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 10, and pH 7.4 with NaOH. The pipette solution: CsCl 100, CsOH 40, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 11, MgATP 5, HEPES 10, and pH 7.3 with CsOH.

2.5. Data analysis

Recordings of Ca^{2+} currents were made from the same myocyte before, during and after drug application. The amplitude of $I_{Ca,L}$ was measured as the maximal peak inward current from each current trace elicited by a test pulse. The conductance of activation and the curve of the steady-state inactivation of $I_{Ca,L}$ were fit to a Boltzmann equation, $y = 1/\{1 + \exp[(V - V_{0.5})/K]\}$. The dose–response curve was fit to a logistical equation, $y = \{(A_1 - A_2)/[1 + (x/x_0)^p]\} + A_2$. The best-fit procedure was performed with a software program (Origin 4.1, Microcal™ Software, Northampton, MA, USA). Data were presented as mean ± standard error of the mean (S.E.M.). Statistical difference was evaluated with the two-tailed paired or unpaired Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of $I_{Ca,L}$ by mepacrine

The acute effect of mepacrine on $I_{Ca,L}$ of rat left ventricular myocytes was determined by monitoring the amplitude of $I_{Ca,L}$ elicited by repeatedly applying single-step pulses to 0 mV from a holding potential of −50 mV. Fig. 1 shows that extracellular application of mepacrine progressively inhibited the amplitude of Ca^{2+} currents. Exposure of a rat ventricular myocyte to 5 μM mepacrine resulted in an approximately 50% decrease in peak $I_{Ca,L}$ in 3 min (Fig. 1b). $I_{Ca,L}$ was further decreased upon an increase in the concentration of mepacrine to 10 μM (Fig. 1c) and completely inhibited by 200 μM mepacrine (Fig. 1e). The maximal inhibition of $I_{Ca,L}$ by 200 μM mepacrine occurred within 30 s (Fig. 1e). $I_{Ca,L}$ was recovered to >60% of the control level after 1-min washing of mepacrine (Fig. 1d and f). This result was further confirmed in another eight ventricular myocytes, which showed a 79 ± 6% recovery of $I_{Ca,L}$ after 5-min washout of mepacrine. Alteration of the holding potential had no significant effect on the mepacrine-induced inhibition of

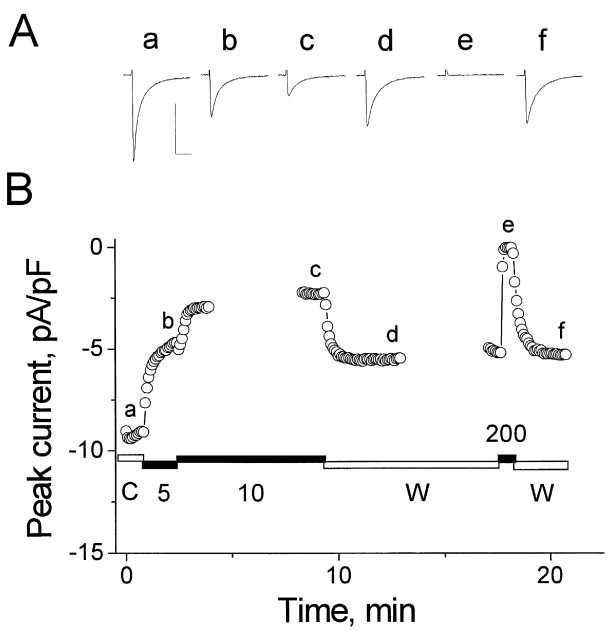

Fig.1. Inhibition of cardiac Ca^{2+} currents by mepacrine. (A) The original current traces of $I_{Ca,L}$ were elicited by 200-ms pulses from a holding potential of −50 to 0 mV every 5 s in the absence (a, control; d and f, washout) and presence of 5 (b), 10 (c), and 200 (e) μM mepacrine. Calibration: 50 ms, 1 nA. (B) The time course of the mepacrine-induced inhibition of peak $I_{Ca,L}$. a, b, c, d, e, and f correspond with the peak values of the current traces in the panel A. The places where the continuous line is interrupted represent the time for measurement of the current–voltage relationship and the steady-state inactivation.

$I_{Ca,L}$. For example, in the presence of 10 μM mepacrine, the inhibition of $I_{Ca,L}$ ($n = 4$) evoked by pulses to 0 mV from a holding potential of −40 or −80 mV was 79 ± 1% and 72 ± 5% ($P > 0.05$), respectively.

The inhibition of $I_{Ca,L}$ by mepacrine is concentration-dependent. Fig. 2 shows the concentration–response relationship for the mepacrine-induced inhibition of $I_{Ca,L}$ activated by single-step pulses from −50 to 0 mV in rat ventricular myocytes. The calculated median inhibitory concentration of mepacrine (IC_{50}) is 5.2 ± 1.1 μM (Fig. 2).

The Ca^{2+} channel blockers verapamil and D-600 are of use-dependent block with little tonic block of cardiac Ca^{2+} channels (Lee and Tsien, 1983; Uehara and Hume, 1985). We examined whether mepacrine-induced inhibition of $I_{Ca,L}$ resulted from tonic- and/or use-dependent block of $I_{Ca,L}$. Fig. 3 shows mepacrine-induced inhibition of $I_{Ca,L}$ without and with depolarizing pulses. Currents were evoked by a voltage command from a holding potential from −70 to 0 mV. After recordings of control currents (Fig. 3, control, $n = 6$), the bath solution containing 10 μM mepacrine was perfused for 3 min with a holding potential of −70 mV. Ca^{2+} currents were then elicited by a train of 30 pulses in the presence of 10 μM mepacrine. Peak $I_{Ca,L}$ elicited by the first pulse (Fig. 3, 1st pulse) of the train was markedly reduced by 52 ± 6% ($P < 0.01$, vs. control). This inhibition is referred to as a tonic block, because this block developed at a holding potential of −70 mV and without the activation of Ca^{2+} channels. During subsequent pulses in the train, an additional use-dependent block developed (Fig. 3, 30th pulse). Peak $I_{Ca,L}$ evoked at 30th pulse was inhibited by 85 ± 6% ($n = 6$, $P < 0.001$, vs. control). The current was significantly smaller than that of the 1st pulse ($P < 0.01$, vs. 1st pulse). Therefore, mepacrine inhibited $I_{Ca,L}$ by acting on closed resting channels, a tonic block, and also, on activated or inactivated channels, a use-dependent block.

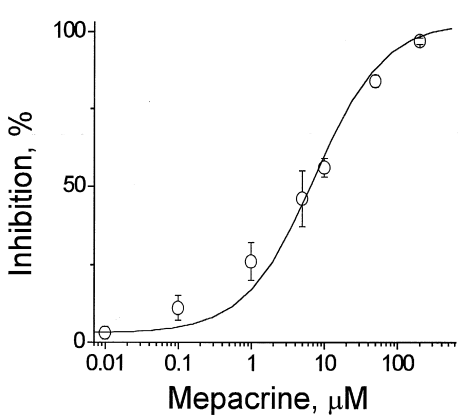

Fig. 2. Concentration-dependent suppression of $I_{Ca,L}$ by mepacrine. Each data point represents mean ± SEM of 6–18 individual myocytes exposed to different concentrations of mepacrine. Currents were evoked by 200-ms pulses from a holding potential from −50 to 0 mV every 10 s.

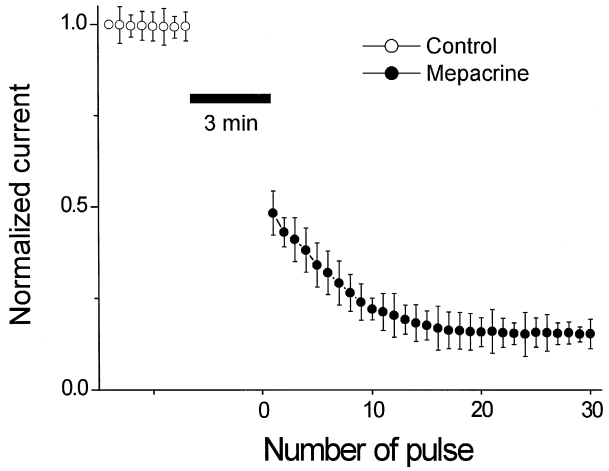

Fig. 3. Mepacrine-induced tonic and use-dependent block of cardiac Ca^{2+} channels. $I_{Ca,L}$ were evoked by 200-ms pulses from a holding potential of −70 to 0 mV every 5 s. Currents were normalized to control. After 2-min recordings of control currents (control), myocytes were perfused with 10 μM mepacrine solution for 3 min without voltage stimulation. Then, a train of 30 pulses were applied to patched myocytes. The amplitude of $I_{Ca,L}$ elicited by 1st to 30th pulses of the train were measured. Tonic block (1st pulse) and use-dependent block (to 30th pulses) of Ca^{2+} channels ($n = 6$) are shown in the presence of 10 μM mepacrine.

3.2. Effect of mepacrine on channel conductance and steady-state inactivation

The mepacrine-induced inhibition of $I_{Ca,L}$ may result from alteration of the kinetics of activation and inactivation of cardiac Ca^{2+} channels. Therefore, we examined the effects of mepacrine on the channel gating property. Fig. 4A shows that the original current traces of $I_{Ca,L}$ in the absence and presence of 10 μM mepacrine. The currents were evoked by a group of 200-ms pulses from −60 to 60 mV with 10-mV increments. The results show that mepacrine significantly inhibited the amplitude of peak $I_{Ca,L}$. This inhibition was accompanied by no alteration of the I–V relationship and reversal potential of $I_{Ca,L}$ (Fig. 4B). In addition, mepacrine had no significant effects on the normalized activation curve (Fig. 4C, $n = 9$). The values at the $V_{1/2}$ of the normalized activation conductance curves were −19.5 ± 0.3 mV with a slope factor (k) of 5.0 ± 0.2 mV for control and −20.9 ± 0.2 mV with a k value of 4.6 ± 0.2 mV for 10 μM mepacrine, respectively. This result suggests that mepacrine-induced inhibition of $I_{Ca,L}$ does not alter the activation gating property of cardiac Ca^{2+} channels.

The steady-state inactivation was studied using a double-pulse protocol before and after 10-min application of mepacrine. Myocytes were given a 1000-ms prepulse to potentials from −60 to 20 mV, and then a fixed 200-ms test pulse to 0 mV. The pulse protocol was applied once every 15 s. Fig. 5A shows that mepacrine at 10 μM significantly inhibited the amplitude of $I_{Ca,L}$ elicited by the

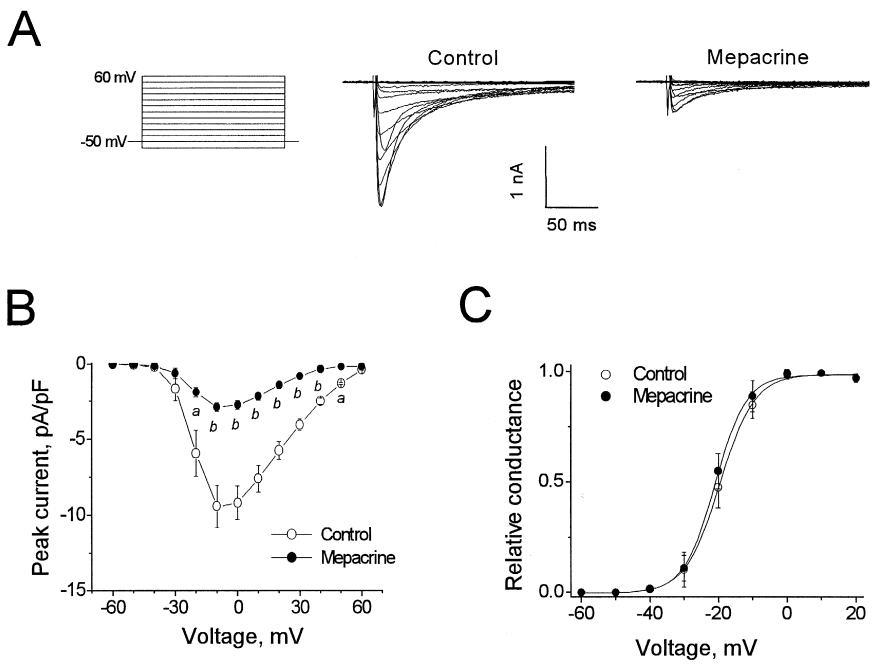

Fig. 4. Effects of mepacrine on the activation of cardiac Ca^{2+} currents. (A) Superimposed current traces in the absence (control) and presence (mepacrine) of 10 μM mepacrine were evoked by 200-ms pulses from −60 to 60 mV with 10 mV increments every 5 s (see inset). The holding membrane potential was set at −50 mV. (B) Current–voltage relationships in the absence (○, control) and presence (●, mepacrine) of 10 μM mepacrine were plotted according to the amplitude of peak $I_{Ca,L}$ elicited by the voltage protocol in the panel (A). (a) $P < 0.05$; (b) $P < 0.01$; vs. control. (C) Relative whole-cell activation conductances ($n = 9$) of $I_{Ca,L}$ in the absence (○) and presence (●) of 10 μM mepacrine. The data fit well with a Boltzmann equation, $y = 1/\{1 + \exp[(V - V_{0.5})/K]\}$.

testing pulses from −60 to 0 mV prepulses. Interestingly, mepacrine also had no significant effect on the steady-state inactivation (Fig. 5B). In the absence of mepacrine, the $V_{1/2}$ of the steady-state inactivation was -27.3 ± 0.1 mV with a k value of 4.1 ± 0.1 mV ($n = 8$). In the presence of 10 μM mepacrine, the $V_{1/2}$ of the steady-state inactivation was -27.6 ± 0.6 mV with a k value of 5.1 ± 0.5 mV. This result suggests that mepacrine does not affect the inactivation gating property of the cardiac Ca^{2+} channel.

3.3. Mepacrine-induced acceleration of channel inactivation

Repeated depolarizing pulses resulted in an additional inhibition of $I_{Ca,L}$ (Fig. 3). This additional inhibition can be due to an acceleration of Ca^{2+} channels into the inactivated state in the presence of mepacrine. To test the effects of mepacrine on the development of inactivation of $I_{Ca,L}$, we used a double-pulse protocol (Fig. 6, inset). Myocytes were held at −70 mV and given a conditioning prepulse to 10 mV with incremental durations from 0 to 50 s. The prepulse was followed by a return to the holding potential for 150 ms and a 200-ms test pulse to 10 mV. Fig. 6 shows that relative peak $I_{Ca,L}$ evoked by test pulses was plotted as a function of the duration of prepulses. The amplitude of $I_{Ca,L}$ was progressively reduced when the duration of conditioning prepulses was gradually prolonged. In the presence of 10 μM mepacrine, the process of inactivation of $I_{Ca,L}$ significantly accelerated (Fig. 6). The data fit well with a double-exponential decay with a fast (τ_f) and a slow (τ_s) time constants. In the absence of mepacrine, τ_f was 0.093 ± 0.008 s with A_f of 0.40 ± 0.02 and τ_s was 5.41 ± 0.94 s with A_s of 0.41 ± 0.02 (open circle, $n = 6$). Mepacrine at 10 μM did not alter the value of τ_f, 0.126 ± 0.036 s with A_f of 0.34 ± 0.05 (solid circle), but τ_s was significantly reduced with a value of 2.05 ± 0.39 s ($A_s = 0.54 \pm 0.05$, $n = 6$, $P < 0.05$, vs. control). The results suggest that mepacrine facilitated the transformation process of Ca^{2+} channels to the inactivated state.

3.4. Effect of mepacrine on recovery from inactivation

The kinetics of recovery of $I_{Ca,L}$ from inactivation in the absence and presence of mepacrine was evaluated by identical two-pulse protocols (Fig. 7, inset). To inactivate cardiac Ca^{2+} channels, we applied a 10 s prepulse to 10 mV from a holding potential of −70 mV. This fixed prepulse was followed by a return to the holding potential with variable durations (Δt) and then by a 200-ms test pulse to 10 mV. In control, the time course of recovery from inactivation of $I_{Ca,L}$ fits well with a two-exponential function and most of the recovery was in the fast component (Fig. 7, open circle). The time constants for recovery from inactivation of $I_{Ca,L}$ were 0.115 ± 0.004 s for τ_f ($A_f = -0.54 \pm 0.01$), and 5.23 ± 0.20 s for τ_s ($A_2 =$

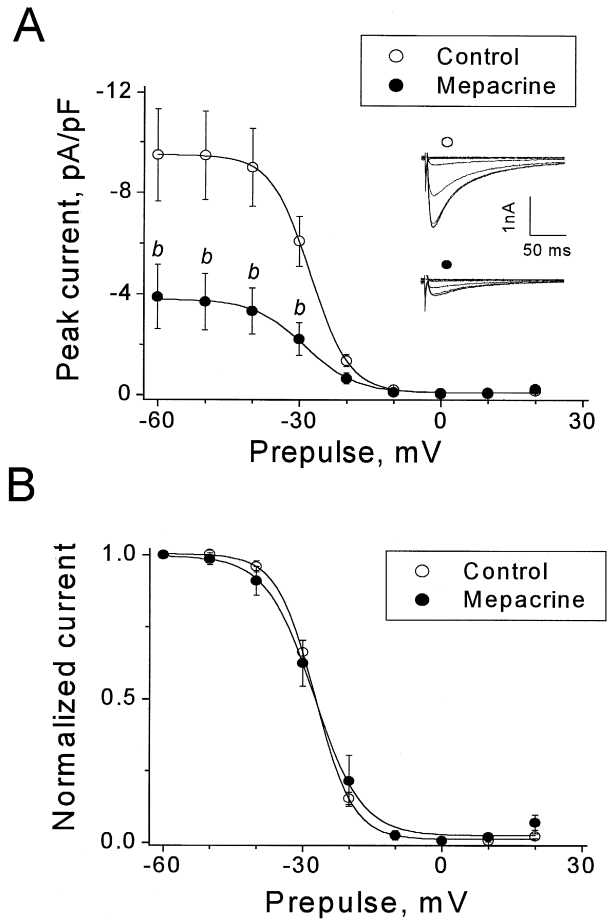

Fig. 5. Effects of mepacrine on the steady-state inactivation of cardiac Ca^{2+} currents. (A) Mepacrine-induced inhibition ($n = 8$) of $I_{Ca,L}$ activated by various conditioning voltages. The inset shows the superimposed original currents recorded from a ventricular myocyte in the absence (○) and presence (●) of 10 μM mepacrine. Currents were elicited with a double-pulse protocol, consisting of a 200-ms testing pulse to 0 mV following a 1000-ms conditioning prepulse varying from −60 to 20 mV in 10 mV increments every 15 s. The membrane potential of myocytes was held at −60 mV. (B) Normalized steady-state inactivation ($n = 8$) of $I_{Ca,L}$ in the absence (○, control) and presence of 10 μM mepacrine (●, mepacrine). The Boltzmann equation was used to fit the data. (b) $P < 0.01$.

-0.46 ± 0.01). In the presence of 10 μM mepacrine, the recovery from inactivation of $I_{Ca,L}$ also fits well with a two-exponential function (solid circle) with the values of 0.296 ± 0.023 s for τ_f ($A_f = -0.53 \pm 0.02$) and 8.76 ± 0.65 s for τ_s ($A_s = -0.48 \pm 0.02$). The differences of τ_f and τ_s between control and mepacrine are significant ($P < 0.05$, $n = 6$). These results indicate that mepacrine prolonged both the fast and slow components of recovery from inactivation of cardiac Ca^{2+} channels.

3.5. Effects of AA on mepacrine-induced inhibition of $I_{Ca,L}$

PKA-mediated Ca^{2+} channel phosphorylation in cardiac myocytes results in an increase in the amplitude of $I_{Ca,L}$. Phospholipase A_2 is responsible for the release of AA. Our recent study showed that the cytochrome P450-mediated metabolite of AA, 11,12-epoxyeicosatrienoic acid, enhanced $I_{Ca,L}$ in rat cardiac myocytes (Xiao et al., 1998). The epoxyeicosatrienoic acid-induced increase in $I_{Ca,L}$ was related to its stimulation of intracellular cAMP production and channel phosphorylation. To determine whether the mepacrine-induced inhibition of $I_{Ca,L}$ involved a reduction of intracellular AA and cAMP levels, which then decreased channel phosphorylation and Ca^{2+} influx, we applied AA or cAMP to cardiac myocytes exposed to mepacrine. In order to suppress most of the $I_{Ca,L}$, we applied a relative higher concentration of mepacrine. Fig. 8 shows that bath perfusion of 50 μM mepacrine solution significantly inhibited the amplitude of peak $I_{Ca,L}$ (Fig. 8, mepacrine). The amplitude of $I_{Ca,L}$ was inhibited from -8.40 pA/pF of control to -1.52 pA/pF for mepacrine (18% of the control). Addition of 1 μM AA to the bath solution gradually increased $I_{Ca,L}$ to -2.90 pA/pF (Fig. 8, AA, 34% of the control). Interestingly, a sharp rebound of $I_{Ca,L}$ (-11.59 pA/pF, 138% of the control) was observed at the early stage of washout of mepacrine and AA (Fig. 8, washout). $I_{Ca,L}$ gradually returned to the control level after continually washing of mepacrine and AA. In eight ventricular myocytes, the averaged increase in $I_{Ca,L}$ after addition of AA was relatively small, but significant ($P < 0.05$), from $19 \pm 7\%$ (mepacrine) to $35 \pm 6\%$ (mepacrine plus AA) of control. The early rebound of $I_{Ca,L}$ after washout of mepacrine and AA was $32 \pm 5\%$ higher than control ($P < 0.05$, $n = 8$). However, in the myocytes

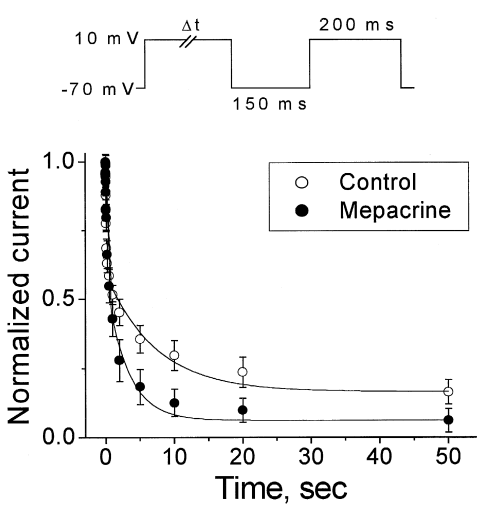

Fig. 6. Mepacrine-induced acceleration of inactivation development of cardiac Ca^{2+} channels. Inactivation development of $I_{Ca,L}$ evoked by the test pulse was plotted as a function of the durations of prepulses in the absence (○, control) and presence (●, mepacrine, $n = 6$) of 10 μM mepacrine. The inset shows the voltage protocol. A prepulse was applied from a holding potential from −70 to 10 mV with incremental durations from 0 to 50 s. The prepulse was followed by a return to the holding potential for 150 ms and then a 200-ms test pulse to 10 mV. The data fit well with a double-exponential decay. The value of τ_s in the presence of mepacrine is much smaller and the difference of τ_s between control and mepacrine is significant ($n = 6$, $P < 0.05$).

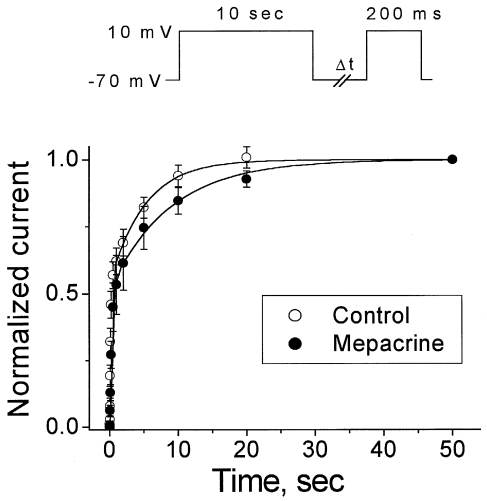

Fig. 7. Kinetics of recovery of $I_{ca,L}$ from inactivation. The inset is the experimental protocol. The pulse protocol was composed of two pulses, one 10-s depolarizing pulse from −70 to 10 mV, followed by a hyperpolarizing pulse to −70 mV with progressively prolonged durations from 0 to 50 s and then a 200-ms test pulse to +10 mV. The membrane holding potential was −70 mV and the rate of pulse was 0.1 Hz. The time course of recovery of peak $I_{Ca,L}$ ($n=6$) from inactivation is shown in the absence (○, control) and presence (●, mepacrine) of 10 μM mepacrine. Currents were normalized to their maximal values of $I_{Ca,L}$ recorded before application of the protocol. Recovery of $I_{Ca,L}$ from inactivation was markedly delayed for both τ_f and τ_s in the presence of 10 μM mepacrine. Data fit well with a two-exponential process.

treated with mepacrine alone, the recovery of $I_{Ca,L}$ after washout was only about 70% (see Fig. 1). Therefore, the rebound of $I_{Ca,L}$ after washout of mepacrine and AA may result from the application of AA. In addition, we found that $I_{Ca,L}$ was increased by $17 \pm 7\%$ ($n=4$, $P=0.08$) after extracellular perfusion of 1 μM AA alone for 5–8 min (data not shown).

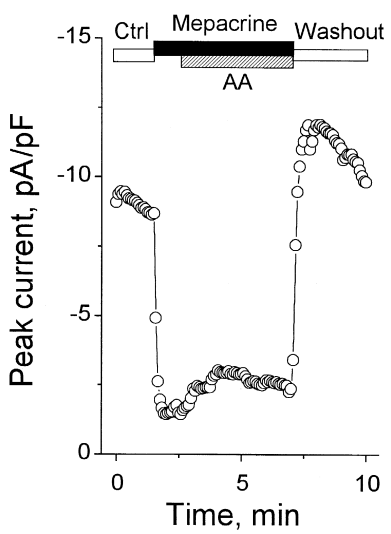

Fig. 8. Effect of AA on the mepacrine-induced inhibition of $I_{Ca,L}$. Currents were evoked by voltage pulses from a holding potential of −50 to 0 mV in the absence (control, Ctrl, and washout) and presence (mepacrine) of 50 μM mepacrine. $I_{Ca,L}$ was markedly inhibited after application of 50 μM mepacrine. Addition of 1 μM AA to the mepacrine perfusion solution partially restored the inhibited $I_{Ca,L}$.

To test the effects of cAMP-dependent phosphorylation of Ca^{2+} channels on the mepacrine-induced inhibition of $I_{Ca,L}$, another series of experiments was performed in cardiac myocytes treated with cAMP or isoproterenol. Fig. 9A shows that $I_{Ca,L}$ was significantly inhibited after application of 10 μM mepacrine, from -9.98 ± 0.88 to -3.39 ± 0.33 pA/pF for mepacrine ($n=4$, $P<0.05$), respectively. Addition of 2 mM membrane permeable dibutyryl cAMP (db-cAMP) did not abolish the mepacrine-induced inhibition of $I_{Ca,L}$ (mepacrine and db-cAMP). The average amplitude of $I_{Ca,L}$ was 4.53 ± 0.53 pA/pF recorded 5 min after application of mepacrine and db-cAMP. We also tested the effects of the β-adrenergic agonist isoproterenol on the mepacrine-induced inhibition of $I_{Ca,L}$. After reaching a steady lower level of $I_{Ca,L}$ in the presence of 10 μM mepacrine, addition of 1 μM isoproterenol did not cause a significant recovery of $I_{Ca,L}$ ($n=3$, data not shown).

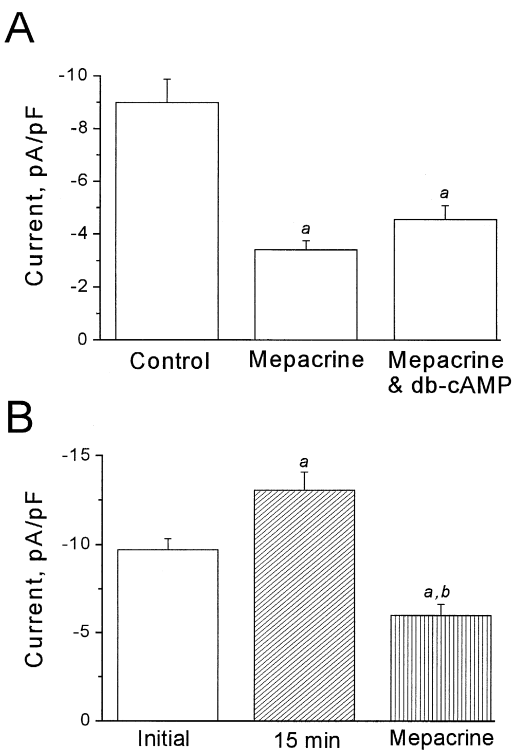

Fig. 9. Effect of cAMP on the mepacrine-induced inhibition of $I_{Ca,L}$. (A) Extracellular application of cAMP did not abolish the mepacrine-induced inhibition of $I_{Ca,L}$ ($n=4$, $P>0.05$). $I_{Ca,L}$ (control) was markedly inhibited after perfusion of 10 μM mepacrine bath solution (mepacrine). Addition of 2 mM db-cAMP (mepacrine and db-cAMP) to the mepacrine perfusion solution partially, but not significantly, recovered $I_{Ca,L}$. (B) Intracellular dialysis with cAMP did not prevent the mepacrine-induced inhibition of $I_{Ca,L}$. Currents immediately recorded after forming the whole-cell configuration were as control (initial). The amplitude of $I_{Ca,L}$ was markedly enhanced after 15 min intracellular dialysis with 2 mM cAMP (15 min) and 10 μM mepacrine significantly inhibited the cAMP-enhanced $I_{Ca,L}$ (mepacrine, $n=4$). Currents were evoked by voltage pulses from a holding potential range from −50 to 0 mV every 10 s. (a) $P<0.05$, vs. control; (b) $P<0.01$, vs. 15 min dialysis.

Table 1
Effects of the phospholipase A_2 inhibitor mepacrine on the nucleotide content (pmol (mg protein)$^{-1}$) in isolated rat ventricular myocytes

Treatment	*n*	cAMP	cGMP	AMP	ADP	ATP
Control	7	60±7	16±8	2124±345	986±152	1118±483
Mepa	9	35±9*	18±7	2257±381	1180±193	833±176
Mepa and AA	6	58±9	21±11	2128±333	936±205	746±193

Values are expressed as mean±S.E.M. *n*, the number of individual measurements carried out in each group. Mepa, mepacrine, 10 μM; mepa and AA, mepacrine (10 μM) plus arachidonic acid (1 μM).
* $P < 0.05$, vs. control.

Pre-phosphorylation of Ca^{2+} channels also did not prevent the mepacrine-induced inhibition of $I_{Ca,L}$. Fig. 9B shows that 15 min after intracellular dialysis with 2 mM cAMP the amplitude of $I_{Ca,L}$ was enhanced by 38 ± 7% ($n = 4$, $P < 0.05$) of the initial current which was immediately recorded after forming the whole-cell configuration. Compared to the value of $I_{Ca,L}$ recorded after 15-min intracellular cAMP dialysis, 10 μM mepacrine inhibited the $I_{Ca,L}$ by 54 ± 6% ($P < 0.01$). Compared to 56 ± 3% inhibition of $I_{Ca,L}$ in the myocytes without intracellular dialysis of cAMP ($n = 18$), there is no significant difference between the two groups. In time-matched control experiments, the amplitude of $I_{Ca,L}$ decreased by 5 ± 5% in 10 min and by 8 ± 10% in 20 min after formation of the whole-cell configuration ($n = 8$, $P > 0.05$, vs. the initial value of $I_{Ca,L}$). Our results suggest that phosphorylation of the channel protein cannot abolish or prevent the inhibition of $I_{Ca,L}$ by mepacrine and that the mepacrine-induced inhibition of $I_{Ca,L}$ may result from a direct block of Ca^{2+} channels.

3.6. Effect of mepacrine on cAMP production

The partial restoration of $I_{Ca,L}$ by addition of AA in the presence of mepacrine probably resulted from an AA-induced stimulation of cAMP production, which then enhanced Ca^{2+} channel phosphorylation. This possibility is further supported by the rebound of $I_{Ca,L}$ after washout of the compounds. To test the hypothesis, we measured intracellular levels of nucleotides in isolated ventricular myocytes incubated with mepacrine. Table 1 summarizes the effects of the phospholipase A_2 inhibitor mepacrine on intracellular nucleotide content in rat ventricular myocytes. Mepacrine at 10 μM ($n = 9$) significantly reduced intracellular cAMP contents ($P < 0.05$). Addition of 1 μM AA abolished the reduction of cAMP contents by mepacrine. In contrast, mepacrine had no significant effect on cGMP, AMP, ADP, and ATP (Table 1).

4. Discussion

In this study, we have shown that the potent phospholipase A_2 inhibitor mepacrine significantly inhibited $I_{Ca,L}$ in isolated rat single ventricular myocytes. The phenomenon of Ca^{2+}-antagonistic properties of mepacrine was found previously in frog atrial trabeculae (Filippov et al., 1989), in guinea pig cardiac and smooth muscle cells (Nagano et al., 1996), and in rat cardiomyocytes (Xiao et al., 1998). It is interesting that Nagano et al. (1996) showed that the potency was about five times higher for mepacrine block of $I_{Ca,L}$ in smooth muscle cells ($IC_{50} = 1.1$ μM) isolated from guinea pig vas deferens and urinary bladder than in cardiac myocytes ($IC_{50} = 5.6$ μM). The IC_{50} of mepacrine block of $I_{Ca,L}$ in guinea pig cardiomyocytes (Nagano et al., 1996) is very close to the value found in this study in rat cardiomyocytes (5.2 μM). In the present experiments, we also found that mepacrine had a tonic- and use-dependent block of cardiac Ca^{2+} channels. In addition, mepacrine accelerated the development of channel inactivation and delayed the recovery from channel inactivation. Mepacrine also showed a greater affinity for inactivated channels, than for resting activatable channels. The acceleration of channel inactivation and delay of recovery from inactivation in the presence of mepacrine support the notion (see Figs. 6 and 7).

The voltage-gated L-type Ca^{2+} channel is the major pathway for Ca^{2+} entry, which triggers intracellular Ca^{2+} release. An imbalanced ionic homeostasis in cardiac myocytes may account for tissue injury during ischemia/reperfusion and hypoxia/reoxygenation. Ca^{2+} overload eventually leads to the transition from reversible to irreversible myocardial damage. Cultured neonatal rat heart cells started to accumulate Ca^{2+} within 15 min after metabolic inhibition and ATP levels fell to 10% of control (Barrigon et al., 1990; Post et al., 1993, Post et al., 1998). Activation of Ca^{2+}-dependent proteases and lipases and alteration of physicochemical properties of sarcolemmal phospholipids due to elevation of intracellular Ca^{2+} account for cell damage during myocardial ischemia (Schalkwijk et al., 1995; Silverman and Stern, 1994; Barrigon et al., 1990). Therefore, the inhibition of $I_{Ca,L}$ by mepacrine plays a major role in its protection of the myocardium during ischemia.

Our results show that AA partially reversed the mepacrine-induced inhibition of $I_{Ca,L}$ and caused a rebound of $I_{Ca,L}$ after washout of mepacrine and AA (see Fig. 8). The data suggest that mepacrine reduces phospholipase A_2-mediated AA production and, therefore, decreases AA metabolites, which may increase cAMP production and $I_{Ca,L}$. This hypothesis is consistent with previous data that prostacyclin and 11,12-epoxyeicosatrienoic acid enhanced intracellular cAMP production in human vascular smooth muscle cells (Beasley and Mcgulggin, 1995) and in rat cardiac myocytes (Xiao et al., 1998). In addition, 11,12-epoxyeicosatrienoic acid significantly enhanced intracellular Ca^{2+} signals in isolated guinea pig hearts or single cardiomyocytes (Moffat et al., 1993) and increased $I_{Ca,L}$ in rat cardiomyocytes (Xiao et al., 1998). Increase in intracellular Ca^{2+} level activates

phospholipase A_2, which hydrolyzes the ester bond at the *sn*-2 position of phosphatidylcholine and produces AA and lysophosphatidylcholine (Sharp and White, 1993; Sharp et al., 1991; Van Bilsen and Van der Vusse, 1995). Accumulation of AA and lysophosphatidylcholine is crucial in myocardial damage during cardiac ischemia (Chien et al., 1984; Karmazyn, 1989; Creer et al., 1990; Van der Vusse et al., 1992). Therefore, inhibition of phospholipase A_2 activity is beneficial to ischemic myocardium (Vigo et al., 1979).

The mepacrine-induced inhibition of $I_{Ca,L}$ might result from a direct effect on the Ca^{2+} channel or its regulatory system, including phospholipase A_2. While mepacrine is capable of inhibiting the activity of phospholipase A_2, our data and the findings in guinea pig cardiac and smooth muscle cells (Nagano et al., 1996) strongly support that the inhibition of $I_{Ca,L}$ by mepacrine results from a direct block of the channel, not from modification of channel regulatory proteins or phospholipase A_2 activity. This hypothesis is supported by the time course of the inhibition, which showed that $I_{Ca,L}$ was inhibited to a steady lower level in 30 s after application of mepacrine (see Fig. 1). This result differs from our previous findings, which showed that the inhibition of $I_{Ca,L}$ by the P450 inhibitor clotrimazole requires more than 5 min and involves the cAMP-dependent channel phosphorylation system (Xiao et al., 1998). In this study, cAMP did not prevent or abolish the mepacrine-induced inhibition of $I_{Ca,L}$ (Fig. 9). In addition, since mepacrine acted on channels directly, supplemental AA only restored a small portion of mepacrine-inhibited $I_{Ca,L}$. This small restoration of $I_{Ca,L}$ after addition of AA probably was due to an AA-mediated enhancement of phosphorylation of those unblocked Ca^{2+} channels. Meanwhile, the rebound of $I_{Ca,L}$ after washout of mepacrine and AA might result from unmasking an enhancement of Ca^{2+} channel phosphorylation during AA application. These data suggest that a low concentration of AA enhances $I_{Ca,L}$.

Several studies have indicated that the protective effect of mepacrine on the myocardium during ischemia is related to its inhibition of phospholipase A_2. In isolated rat hearts, mepacrine, with concentrations more than 10 μM, not only protected the heart from injury, but also prevented myocardial accumulation of fatty acids after global ischemia (Van Bilsen et al., 1990). This protective effect has also been found in pigs and dogs (Atani et al., 1986; Chiariello et al., 1990). In sharp contrast, Bugge et al. (1997) reported that the mechanism of mepacrine-induced protection of the heart from hypoxic injury was not by an inhibition of phospholipase A_2. They found that mepacrine at 1 μM did not inhibit phospholipase A_2 activity in an enzyme assay, but protected the heart from hypoxic injury. These opposing experimental results may be due to the different concentrations of mepacrine used in their experiments. However, since mepacrine strongly inhibits $I_{Ca,L}$ and since intracellular Ca^{2+} affects the activity of phospholipase A_2, the mepacrine-induced inhibition of $I_{Ca,L}$ found in the present and previous studies (Filippov et al., 1989; Nagano et al., 1996) may also affect phospholipase A_2 activities through a decrease in intracellular Ca^{2+}. These effects of mepacrine might explain why some studies have ascribed the cardioprotective effects of mepacrine to its inhibition of phospholipase A_2 (Das et al., 1986; Atani et al., 1986; Armstrong and Ganote, 1991). Our present work demonstrates that mepacrine has a tonic- and use-dependent block of cardiac Ca^{2+} channels. This block is mainly caused by a direct effect on the channel protein, not by an inhibition of phospholipase A_2 activity. Therefore, the protective action of mepacrine during myocardial ischemia seems most likely due to its direct blockade of Ca^{2+} channels. This effect helps to reduce intracellular Ca^{2+} accumulation during ischemia, which plays a critical role in heart cell injury. However, mepacrine also inhibits phospholipase A_2 activity, which may result from its direct action on the enzyme or a secondary effect of decreasing intracellular Ca^{2+} because of mepacrine blockade of the channel. The mepacrine-induced inhibition of phospholipase A_2 activity may, thus, also partially contribute to its protection of the heart from ischemic injury.

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