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Role of Protein Kinase C in Mediating Alpha-1-Adrenoceptor-Induced Negative Inotropic Response in Rat Ventricles

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Key Words

α_1-Adrenoceptor · Protein kinase C · Negative inotropic response · Ca^{2+} transient · L-type Ca^{2+} current

Abstract

The aim of this study was to determine the effect of protein kinase C (PKC) activation on intracellular Ca^{2+} transient and its relation to α_1-adrenoceptor (α_1-AR)-stimulated negative inotropic response in rat ventricles. The electromechanical responses to phenylephrine (PE) in rat ventricular muscles were concomitantly examined using the conventional microelectrode method. The responses of intracellular Ca^{2+} transient and cell contractions to PE in the absence of certain pharmacological interventions were ascertained in fura-2-loaded myocytes. The influence of PE on L-type Ca^{2+} current ($I_{Ca,L}$) was also examined using a voltage clamp in a whole-cell configuration. PE did not alter the action potential parameters during the negative inotropic phase. The negative inotropic effect (NIE) was inhibited by prazosin, chloroethylclonidine (CEC) and staurosporine, but was insensitive to pertussis toxin. Desensitization of PKC after prolonged pretreatment of rat ventricles with PDBu also abolished the NIE of PE. Caffeine modulated the NIE, but thapsigargin did not. The evoked intracellular Ca^{2+} transient and cell contraction were initially decreased by PE, while $I_{Ca,L}$ was not altered. Prazosin and staurosporine significantly inhibited the responses. Our data indicated that α_1AR-mediated NIE in rat ventricular muscles was due to the decrease of intracellular Ca^{2+} transients by the modulation of PKC on Ca^{2+}-releasing channels signaling through a CEC-sensitive α_1AR subtype.

Introduction

The mechanical effect of α_1-adrenoceptor (α_1-AR) stimulation in rat ventricular muscles has been reported to induce a biphasic response (an initial decrease followed by a sustained increase of contractility) [3, 37], or even a triphasic inotropic response (an initial increase followed by a transient decline in the contractility prior to the development of a sustained positive inotropism) [29]. The mechanism of the sustained positive inotropic action of the α_1-AR agonists on rat ventricles has been widely researched. The results have shown that the mechanism includes the increase of intracellular Ca^{2+} transients [9, 10] and/or the increase of myofibrillar Ca^{2+} sensitivity either by indirectly inducing intracellular alkalinization or by directly influencing the contractile proteins [11, 37]. The increase of Ca^{2+} transients has been proposed to be due to the reduction of transient outward K^+ current [1, 31, 39] and/or the increase of L-type Ca^{2+} current ($I_{Ca,L}$) [22, 23, 40]. The former action indirectly increases Ca^{2+}

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influx through Ca^{2+} channels via the subsequent prolongation of the action potential. However, the mechanism contributing to the initial transient negative inotropic response is still unclear and has not been studied thoroughly.

The diacylglycerol-protein kinase C (PKC) signal transduction pathway mediates the negative inotropic effect (NIE) of α_1-AR stimulation in rat ventricular myocytes [3, 12, 18] or the left atria [30]. The possible mechanisms of the aforementioned NIE by α_1-AR agonists including the following: increased activity of Na pump [30], stimulation of the forward mode of Na/Ca exchange [35], cytoplasmic acidification [12] or mobilization of cytosolic stored Ca^{2+} by inositol-1,4,5-trisphosphate (IP_3) [29] have been proposed. However, the modifiers of the ryanodine receptors can produce transient effects on systolic Ca^{2+} due to the induced changes in sarcoplasmic reticulum (SR) Ca^{2+} stores [7]. In our study, we focussed on the change of cytosolic Ca^{2+} transient in the initial stimulation of α_1-AR, and evaluated the role of PKC in mediating the transient NIE in rat ventricles. Since the action potential duration affects the excitation-contraction coupling in rat ventricular myocytes [2], the electrophysiological property was also examined. In this study, we provide evidence to indicate that α_1-AR-induced NIE is due to the activation of PKC, which then inhibited ryanodine-sensitive Ca^{2+} release channels which led to a decrease of the intracellular Ca^{2+} transients.

Methods

Electromechanical Response

Adult WKY rats of both sexes (250–300 g) were intraperitoneally injected with sodium pentobarbital (25 mg kg^{-1}) plus heparin (16 mg kg^{-1}). After the rats were deeply anesthetized, hearts were quickly removed. Right-ventricular strips and left-ventricular papillary muscles were quickly dissected from the heart and placed in Tyrode solution gassed with 95% O_2 and 5% CO_2 at 37 ± 0.5 °C. The Tyrode solution contained (m*M*): NaCl 137.0, KCl 5.4, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.33, dextrose 11.0 and $CaCl_2$ 2.0. The phenylephrine (PE)-mediated mechanical responses to certain pharmacological interventions were measured by connecting one end of the right-ventricular strips using a silk thread to a force displacement transducer (type BG 25, Gould Inc., Cleveland, Ohio, USA) and the other end fixed to the bottom of the organ bath. The twitch tension was recorded on a Gould 2200s recorder. The resting tension was adjusted to 1 mN and equilibrated for 1 h. The muscles were stimulated by a 2-ms rectangular voltage pulse with twofold threshold at a frequency of 2 Hz via an isolated Grass SD9 stimulator (Grass Instruments Co., Quincy, Mass., USA).

In the study of the influence of pertussis toxin (PTX) on PE-mediated mechanical response, the rats were pretreated with PTX (150 μg kg^{-1}) injected intraperitoneally. Rats in the control group were treated with saline. The drug- and vehicle-pretreated rats were humanely sacrificed 24 h after injections. The influence of PTX on cardiac tissues was proven by the contractile response of atrial strips to acetylcholine. In the left atrial muscles, acetylcholine (3 μ*M*) significantly decreased the basal contractile force from 0.32 ± 0.04 to 0.13 ± 0.03 mN (n = 4, decrease about 60%, $p < 0.01$ by the paired Student t test) in the control group, whereas an insignificant decrease of contraction from 0.34 ± 0.06 to 0.29 ± 0.06 mN (n = 4, decrease about 15%, $p = 0.58$ by the paired Student t test) in the PTX-treated group.

In order to concomitantly record the effects of PE on the contractile force and transmembrane potential, each rat left papillary muscle was transferred to a tissue bath of 3 ml in volume and superfused at a rate of 15 ml min^{-1} with propranolol (3 μM) containing Tyrode solution (36.5 ± 0.5 °C) gassed with 95% O_2 and 5% CO_2. One end of the muscle was horizontally hooked to the lever arm of a force displacement transducer (HSE-force transducer F30 Type 372) mounted on a micromanipulator, and the other end was pinned to the bottom of the tissue chamber. The developed force was performed under isometric conditions. To obtain the maximum developed tension, an optimal preload was given. Each left-ventricular papillary muscle was stimulated at 1 Hz using rectangular pulses of 1 ms duration with a twofold threshold voltage command. Action potentials were recorded by means of the conventional microelectrode technique using Axoclamp 2B amplifier (Axon Instruments Inc., Foster City, Calif., USA) in the bridge mode. The tip resistances of 3 *M* KCl-filled glass microelectrodes were between 20 and 30 MΩ. The potential was calibrated before each impalement by adjusting the input offset potentiometer. The preparations were allowed to equilibrate for at least 90 min before the microelectrode was impaled. Only the results from experiments in which a single impalement was maintained throughout the experiment are presented herein, and the others are discarded. Both the mechanical and electrical responses were acquired and analyzed using MacLab data acquisition system (AD Instruments Pty. Ltd., Castle Hill, NSW, Australia).

Isolation of Single Ventricular Myocytes

Adult rat ventricular myocytes were isolated according to the experimental procedure described by Mitra and Morad [26] (1985). Briefly, the excised rat hearts were quickly mounted via the aorta on a Langendorff retrograde perfusion apparatus. Hearts were initially perfused with oxygenated Ca^{2+}-free HEPES solution for 5 min in HEPES solution at 37 °C containing (m*M*): NaCl 137.0, KCl 5.4, KH_2PO_4 1.2, $MgSO_4$ 1.22, glucose 22.0 and HEPES 6.0; pH was adjusted to 7.4 using NaOH. The heart was then perfused, followed by the same solution containing 0.3 mg ml^{-1} collagenase (type I, Sigma Chemical Co., St. Louis, Mo, USA) and 0.1 mg ml^{-1} protease (type XIV, Sigma). After 10–20 min of digestion, enzymes were washed out in 0.05 m*M* Ca^{2+} HEPES solution. The ventricles were separated, then cut into small pieces which were resuspended under gentle mechanical agitation and stored in 0.2 m*M* Ca^{2+} HEPES solution at room temperature.

Measurement of Ionic Currents

The whole-cell patch clamp technique was used to record ionic currents in voltage clamp mode with a Dagan 8900 voltage clamp amplifier (Dagan Co., Minneapolis, Minn., USA). A droplet of the cell suspension was placed in a chamber mounted on the stage of an inverted microscope (Nikon, Diaphot, Japan). After settling down, cells were finally exposed to the bath solution containing (m*M*):

NaCl 137.0, KCl 5.4, $MgCl_2$ 1.1, $CaCl_2$ 1.8, HEPES 6.0, glucose 22.0; pH was adjusted to 7.4 using NaOH. All experiments were performed at 30 ± 0.5 °C. For the measurement of I_{Ca}, a pipette was filled with the internal solution containing (m*M*): CsCl 130, Mg-ATP 5, TEA Cl 15, K_2EGTA 15, HEPES 10; pH was adjusted to 7.2 using CsOH. CsCl (5 m*M*) was always added in the bath solution to block the inward rectifier K^+ current (I_{K1}). Heat-polished glass electrodes (tip resistances between 1 and 3 MΩ when filled with pipette internal solution) were used. Junctional potentials were zeroed before the formation of the membrane-pipette seal in bath solution. The series resistance was electronically compensated by about 80% to minimize the duration of the capacitive surge on the current recorded and the voltage drop across the pipette. Currents were elicited and acquired using Digidata 1200 data acquisition system controlled using pClamp software (Axon Instruments). Recordings were low-pass filtered at 10 kHz and stored on the hard disk of an IBM AT-compatible computer.

Measurements of Intracellular Ca^{2+} Transients and Cell Shortening

Ventricular myocytes were loaded with the fluorescent Ca^{2+}-sensitive indicator, fura-2, by incubation in 0.5 m*M* Ca^{2+} HEPES solution containing 3 μ*M* fura-2-acetoxymethylester (fura-2-AM) for 30 min at room temperature. After washing out the excess fura-2-AM, cells were stored in 0.5 m*M* Ca^{2+} HEPES solution. The fura-2-loaded myocytes were transferred to 1.8 m*M* Ca^{2+} containing HEPES buffer for at least 30 min before beginning the experiments. The intracellular Ca^{2+} transients were measured according to the method described by Cleemann and Morad [4, 5]. The fura-2-loaded myocytes were electrically stimulated by a pair of platinum electrodes with a 2-ms and twofold threshold rectangular voltage pulse at 0.5 Hz. The cells were illuminated with ultraviolet light (UV) from a 100-watt mercury arc lamp. The UV beam was split into two wavelengths (335 and 410 nm) for excitation of fura-2 using a vibrating mirror to reflect the UV light to pass through the interference filters (335, 410 and 20 nm bandwidths) at 1,200 Hz. A photomultiplier was used to collect the fluorescence passed through a wide-band interference filter (510 and 70 nm bandwidths). The signal for the photomultiplier was demultiplexed to the two corresponding signals of the respective excitation wavelengths. These signals were acquired using the Digidata 1200 data acquisition system controlled with pClamp software. The level of intracellular calcium was directly expressed as the ratio of the light emitted at 335 nm excitation to that at 410 nm excitation, because the fura-2 ratio was not a linear function of $[Ca^{2+}]_i$ when cells were loaded with the AM ester form of fura-2 [15]. The cell shortening was measured optically by acquiring the image of the contracting myocyte via a CCD camera (MotionScope 8000s, Redlake Imaging Co., USA) mounted on the side port of the microscope. The camera signal was stored on tape for later analysis. The change of cell length between the maximum systolic and diastolic states was defined as cellular contraction. The data were presented as percent change of control after drug treatment.

Drugs

Propranolol (3 μ*M*) was added to block β-AR unless otherwise indicated. In order to irreversibly block α_{1B}-AR, the preparations were incubated in 30 μ*M* chloroethylclonidine (CEC), an alkylating agent, which contained Tyrode solution for 60 min followed by 30 min of washing out. 5-Methyl-urapidil (5-MU), CEC, KN-93, 5-(N-ethyl-N-isopropyl) amiloride (EIPA) wortmannin and thapsigargin was purchased from Research Biochemicals Inc. (Natick, Mass., USA), and fura-2-AM from Molecular Probes, Inc. (Eugene, Oreg.). Other chemicals were purchased from Sigma Chemical Co., (St. Louis, Mo., USA).

Data Analysis and Statistics

Experimental data were presented as the means ± SEM. The unpaired Student t test was used to analyze statistical significance between PE-mediated inotropic responses with or without certain pharmacological interventions. Other data were analyzed using the paired Student t test. p values less than 0.05 were considered significant.

Results

Mechanical and Electrophysiological Response of Rat Ventricular Muscles to α_1-AR Stimulation

Figure 1a shows that, in the presence of 3 μ*M* propranolol, PE elicited a pronounced biphasic mechanical response in rat left papillary muscles. PE produced similar inotropic responses in rat right-ventricular muscles, but monophasic positive inotropism in rat atrial muscles (data not shown). In the initial negative inotropic response to 30 μ*M* PE, the contraction was maximally decreased to 78.9 ± 1.5% of the basal twitch tension (0.25 ± 0.02 mN, n = 25) in the right-ventricular muscles, and to 79.8 ± 4.5% of the basal force (0.34 ± 0.14 mN, n = 10) in the left papillary muscles. There were no significant differences between the extent of decreases in contraction between the two parts of the ventricles. The electrical and mechanical responses of the rat left-ventricular papillary muscles to PE were concomitantly recorded and depicted in figure 1a. After perfusion with PE-containing solution, the resting membrane potential, the maximum upstroke velocity at phase 0, and the action potential duration (APD) at either 50% (APD_{50}) or 90% (APD_{90}) were not significantly altered during the inital transient decline in contracility (table 1).

Influence of α_1-AR Subtype Antagonists and PTX on the NIE of PE

As shown in figure 1b, prazosin 100 n*M* completely inhibited the NIE of PE. In order to further characterize which subtype of α_1-AR mediated the NIE, 5-MU (100 n*M*) and CEC (30 μ*M*) were used. Prazosin, 5-MU or CEC had no significant effects on the basal contraction at their respective concentrations. In CEC-treated right-ventricular strips (as described in Methods), α_1-AR-stimulated NIE was significantly attenuated. However, 5-MU did not affect the NIE of PE.

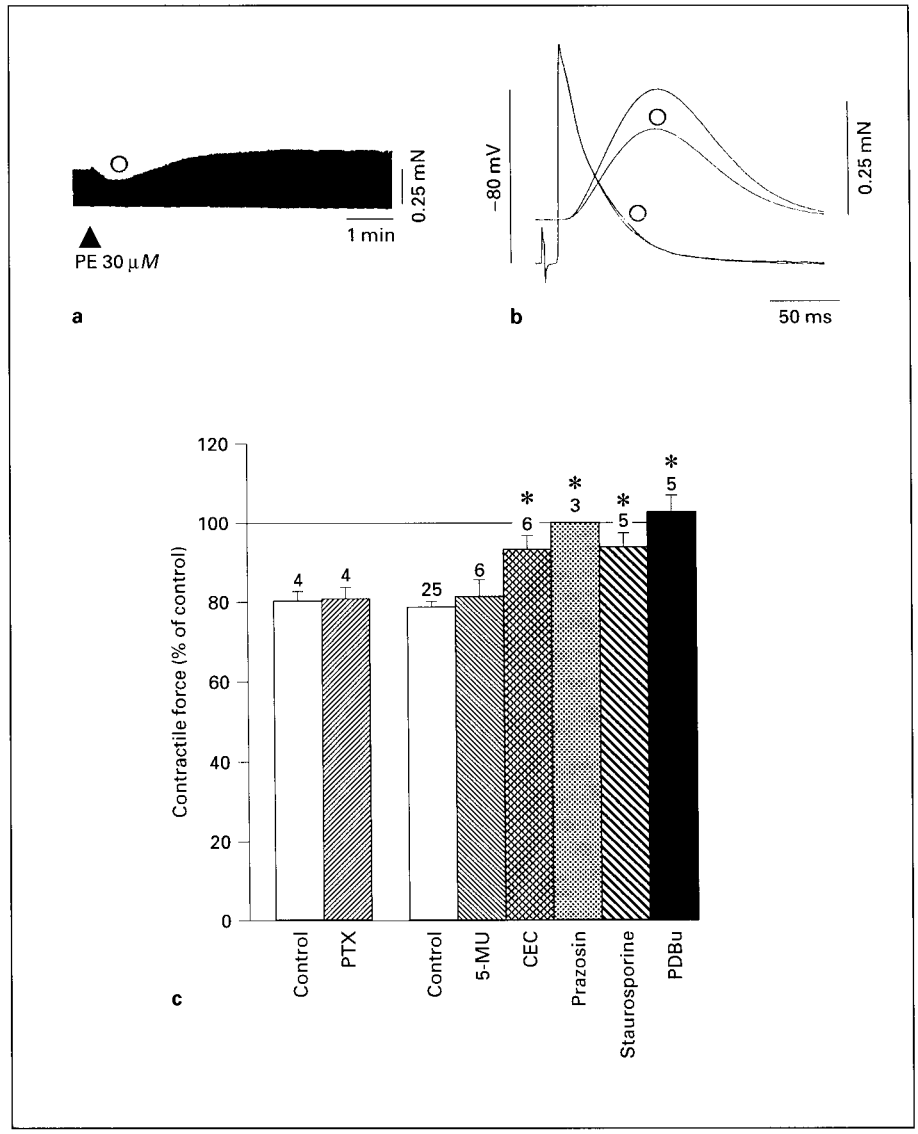

Fig. 1. Electromechanical effects of PE in rat ventricular muscles. The bath solution contained 3 μ*M* propranolol. **a**, **b** The action potential and mechanical responses were concomitantly recorded. The developed force after application of PE was continuously recorded and shown in **a. b** The superimposed recordings show the expanded electrical and mechanical responses of rat left-ventricular papillary muscles in the control group and at the maximum decrease of contraction after 30 μ*M* PE application (○). **c** Influence of PTX, 5-MU (100 n*M*), CEC (30 μ*M*), prazosin (100 n*M*), staurosporine (100 n*M*) and prolonged PDBu (100 n*M*) pretreatment on the negative inotropic response to PE in rat right-ventricular muscles. Ordinate: the maximum negative inotropic response to PE is expressed as the percentage of the contraction before PE addition to rat ventricular strips in the absence or presence of drug pretreatment. The numbers of experiments are indicated above the error bars. Asterisks represent statistical significance from the control group, $p < 0.05$.

Table 1. Influence of PE on action potential parameters during the negative inotropic phase in rat left-ventricular papillary muscles

	RMP	V_{max}	APD_{25}	APD_{50}	APD_{90}
Control	−79.6 ± 1.1	174.4 ± 10.4	9.7 ± 1.0	19.7 ± 0.5	63.3 ± 4.0
NIE of PE	−79.5 ± 1.3	170.6 ± 17.7	10.1 ± 1.0	20.2 ± 1.6	68.8 ± 5.8

The papillary muscles were pretreated with 3 μ*M* propranolol for 10 min before PE application. Action potential parameters were evaluated at the maximum negative inotropic response to 30 μ*M* PE obtained within 2 min after its application. Values are means ± SEM, n = 10.

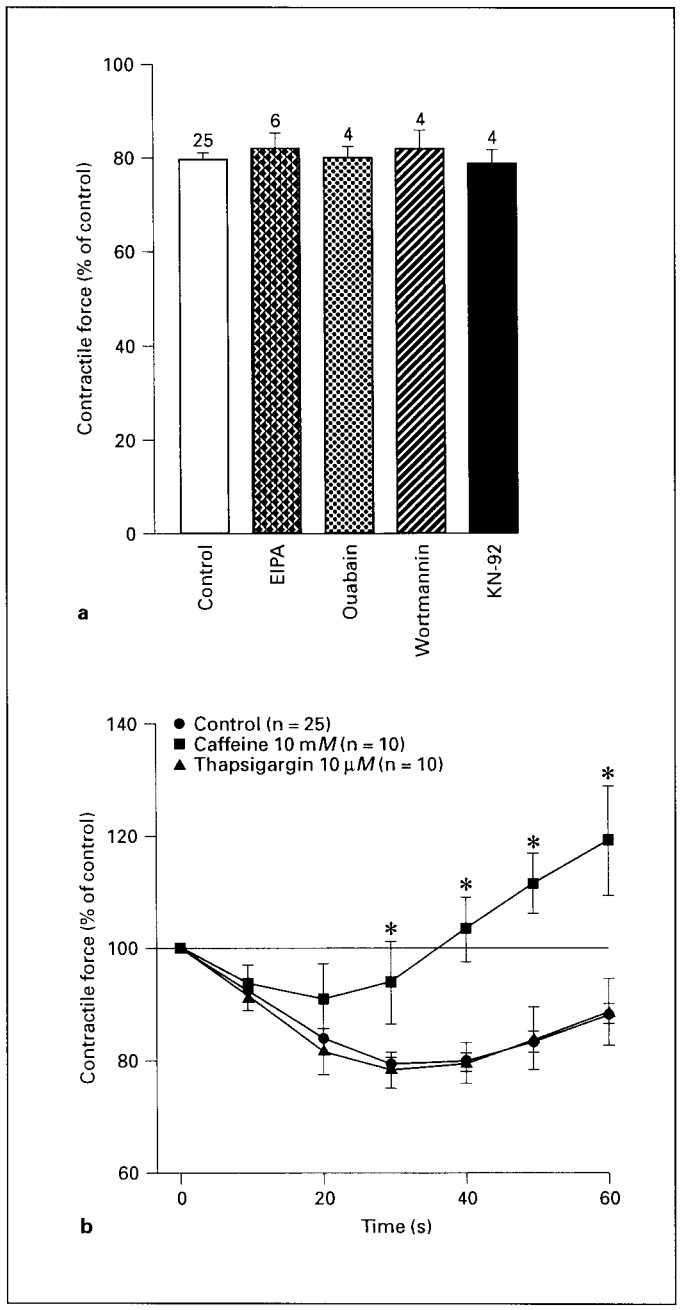

Fig. 2. a Influence of some pharmacological interventions, including EIPA, ouabain, wortmannin, and KN-93, on the decrease of contraction by PE in rat right ventricular muscles. The numbers of experiments are indicated above the error bars. **b** Different effects of intracellular Ca^{2+} store modulators, caffeine and thapsigargin, on the time course of changes in contractile force in response to 30 μ*M* PE in rat right-ventricular muscles. PE-mediated changes in contractile force are plotted as a function of the time after application of PE. Ordinate: the contraction after PE application is expressed as that before PE addition. Abscissa: time in seconds after application of PE. Each point represents means ± SEM of n preparations. Asterisks indicate $p < 0.05$ as significant difference compared with the corresponding control group response to PE.

In rat ventricular muscles pretreated with PTX, PE produced marked biphasic inotropic responses. Figure 1b shows that the extent of PE-stimulated NIE was not significantly altered (maximally decreased to 80.7 ± 3.1% of the basal contraction 0.42 ± 0.04 mN, n = 4) as compared with the saline-treated group (maximally decreased to 79.9 ± 2.5% of the basal contraction 0.36 ± 0.04 mN, n = 4).

Dependence of PKC Activation in the NIE of PE

Staurosporine significantly inhibited the NIE of PE in the rat right-ventricular muscles (fig. 1b). The preparations were pretreated with staurosporine 100 n*M* for 60 min. Staurosporine had no significant effects on the basal contractions. In order to desensitize the PKC, the preparations were incubated in a solution containing PDBu (100 n*M*) for 60 min. PDBu (100 n*M*) alone produced a sustained decrease of the basal contractions from 0.17 ± 0.0 mN to 0.10 ± 0.02 mN (n = 5) in rat ventricular muscles. The NIE of PE was absent in preparations pretreated with PDBu.

The Role of Phosphatidylinositol 3-Kinase Ca/Calmodulin Kinase II, Na Pump and Na/H Exchange in the NIE of PE

Figure 2a shows tha α_1-AR stimulated NIE was not inhibited by wortmannin (1 μ*M*), an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), or KN-93 (1 μ*M*), an inhibitor of calcium/calmodulin kinase II. Even when the concentrations of both agents were elevated to 10 μ*M*, the NIE of PE were not affected. The basal contractions were not affected either by wortmannin of KN-93. Since the enhancement of Na/K ATPase [13] and cytosolic acidification [12] have also been proposed as mediators of NIE of PE, the effect of an Na pump inhibitor (ouabain) and Na/H antiport inhibitor (EIPA) on the NIE of PE was examined. As shown in figure 2a, neither ouabain (10 μ*M*) nor EIPA (20 μ*M*) had any effect on the PE-stimulated NIE (The basal contractile force was increased by 10 μ*M* ouabain from 0.26 ± 0.06 mN to 3.88 ± 0.12 mN (n = 4), and 20 μ*M* EIPA decreased the contractile force from 0.18 ± 0.03 to 0.15 ± 0.03 mN, n = 6).

Effects of Caffeine and Thapsigargin on the NIE of PE

Figure 2b shows the influence of the intracellular Ca^{2+} store modulators on the NIE of PE. In the right-ventricular strips of the rats, 10 m*M* caffeine caused a transient increase of contractile force followed by a slight contracture which reached a steady condition after 60 min. The basal contraction decreased from 0.25 ± 0.02 to 0.16 ± 0.05 mN (n = 10). After treating the right-ventricular

strips with caffeine for 60 min, the time course of α_1-AR-mediated transient NIE was altered, and the maximum decrease of contractility was attenuated (fig. 2b). The duration of PE-induced NIE was shortened and the contractile force changed to a positive inotropic response after 40 s. Caffeine, at high concentrations, is also an inhibitor of phosphodiesterase. We further examined the action of the other PDE inhibitors, IBMX (30 μM), on the NIE of PE. IBMX did not significantly affect the NIE of PE (data not shown).

In addition, 10 μM thapsigargin decreased the contraction from 0.27 ± 0.03 to 0.13 ± 0.04 mN (n = 10) and retarded the rate of muscular relaxation after 60 min treatment of right-ventricular strips. The transient NIE of PE in these thapsigargin-treated right-ventricular muscles were the same as in the control (fig. 2b).

Role of the Changes of Intracellular Ca^{2+} Transient in the NIE of PE

The effect of α_1-AR stimulation on intracellular Ca^{2+} transient was examined in isolated ventricular myocytes, and the results are shown in figure 3. We tested the response to PE ventricular myocytes. Only 20 of the 50 examined cells produced biphasic inotropic responses to PE (16 cells produced sustained negatively inotropic responses to PE, and 14 cells exhibited slightly positive inotropic responses to PE). After washing out the PE, the 20 cells were used to examine some pharmacological interventions on PE-elicited effect on the Ca^{2+} transient. As shown in figure 3a, the contraction (cell shortening) of ventricular myocytes decreased by 16.3 ± 7.2% (n = 20) after PE application for 30 s. The intracellular Ca^{2+} transient also decreased in the same period. The integrated area under the Ca^{2+} transient trace was expressed as an index of the intracellular Ca^{2+} releasing level. Figure 3b shows that PE decreased the intracellular Ca^{2+} releasing level to 86.1 ± 2.1% of the control group during the negative inotropic phase. The effect of PE-induced decreases in the intracellular Ca^{2+} releasing levels was attenuated in myocytes pretreated with staurosporine and PDBu, respectively. The myocytes were respectively incubated in staurosporine (100 nM)-containing solution for 20 min and in a solution containing PDBu (100 nM) for 40 min before PE application. A total of 100 nM PDBu reduced the intracellular Ca^{2+} releasing level to 78.0 ± 6.4% (n = 5) of the control In staurosporine-treated myocytes, the inhibitory effect on the intracellular Ca^{2+} releasing level by PDBu was attenuated to be 94.1 ± 5.1% (n = 3) of the control. EIPA did not affect the initial inhibitory effect of PE on the Ca^{2+} transient.

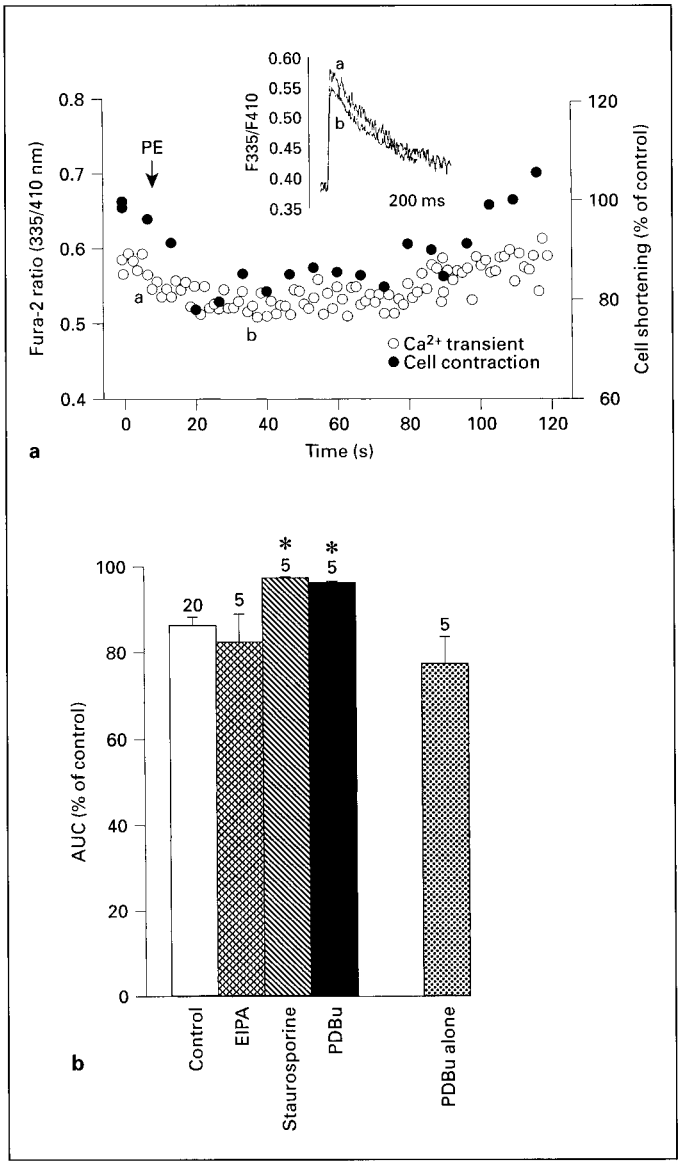

Fig. 3. Effects of PE on Ca^{2+} transients and cell shortening in isolated rat ventricular myocytes during the initial 2 min of PE application in the presence of 3 μM propranolol. **a** The myocytes were electrically stimulated at 0.5 Hz. Abscissa: time in seconds before and after application of PE. Left vertical axis, the peak systolic 335/410 nm ratio of fura-2 fluorescence is expressed as a direct index of the change of Ca^{2+} transients. The superimposed Ca^{2+} transients are recordings on an expanded time scale at the points labeled 'a' (control) and 'b' (PE). Right vertical axis, the stimulation-induced cell shortening after PE application was expressed as a percentage of that before PE addition. **b** The influence of EIPA, staurosporine, and PDBu pretreatment on PE-stimulated decrease of Ca^{2+} transient. Area under the Ca^{2+}-transient trace (AUC) within 750 ms from the rise of intracellular Ca^{2+} level were integrated. The integrated Ca^{2+} transient at 30 s after PE application is expressed as a percentage of that before PE application. The numbers of experiments are indicated above the error bars. Asterisks indicate $p < 0.05$ as significant difference compared with the control group response to PE.

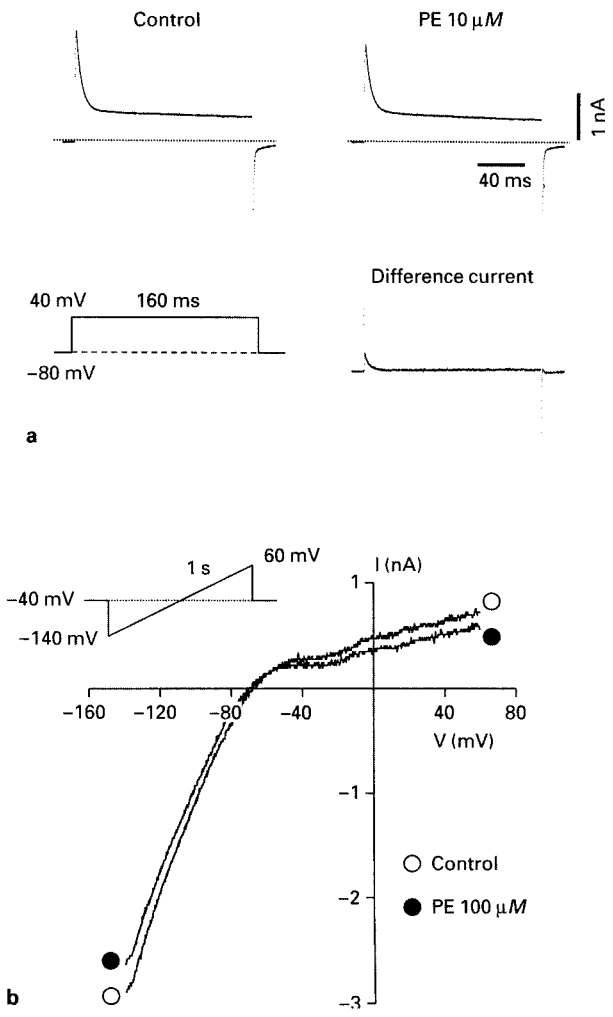

Fig. 4. Effects of PE on the potassium currents in rat ventricular myocytes. **a** I_{to} was elicited during a depolarizing step from holding potential −80 to 40 mV for 160 ms. 1 μ*M* propranolol, 30 μ*M* TTX and 1 m*M* Co^{2+} were present in the bath solution. The current trace in the right panel was recorded at 5 min after exposure to 10 μ*M* PE. **b** The potassium current-voltage relationship curves were elicited by voltage ramps applied from −140 to 60 mV (200 mV/s) before (○) and after (●) 100 μ*M* PE. Membrane potentials of ventricular cells were held at −40 mV.

Influence of PE and Intracellular Released Ca^{2+} on L-Type Ca^{2+} Current

L-type Ca^{2+} current ($I_{Ca,L}$) was elicited as the following description. Cells were held at −80 mV and stimulated at 1 Hz. Following a 60-ms prepulse to −40 mV to inactivate sodium current and $I_{Ca,L}$ was then elicited after the second 120-ms step depolarizing to 0 mV. Ryanodine, which is known to inhibit Ca^{2+} release from Ca^{2+} release channels, was used to check the functional coupling between intracellular Ca^{2+} release channels and L-type Ca^{2+} channels in our experimental condition. Ca^{2+}-induced Ca^{2+} channel inactivation was reduced after the application of ryanodine. The amplitude of $I_{Ca,L}$ was increased and the decay rate of $I_{Ca,L}$ was significantly retarded as shown in table 2. Since the intracellular Ca^{2+} transient was decreased after α_1-AR stimulation, the effect of PE on $I_{Ca,L}$ was examined. To prevent the contamination of PE-mediated β-adrenergic effect of $I_{Ca,L}$, 10 μ*M* propranolol was present in bath solutions. After the rundown of $I_{Ca,L}$ reached a steady state, PE was added. PE (30 μ*M*) produced no significant effects on the density of $I_{Ca,L}$ 1 min after the application even in myocytes pretreated with PDBu. The decay rate of $I_{Ca,L}$ was also not significantly altered by PE (table 2).

Table 2. The current density and the inactivation time constant (τ) of $I_{Ca,L}$ under control and test conditions

	Control	PE 30 μ*M*	Rya 10 μ*M*	PDBu (100 n*M*) pretreated for 1 h	
				control	PE 30 μ*M*
	(n = 20) (after RD)	(n = 20) (30 s)	(n = 5) (3 min)	(n = 6) (after RD)	(n = 6) (30 s)
I, pA/pF	18.2±2.3	17.8±2.6	22.7±1.8	19.6±2.2	19.4±2.6
τ_f, ms	4.4±0.3	4.9±0.4	10.3±1.9*	4.2±0.4	4.3±0.5
τ_s, ms	22.3±0.9	22.9±0.9	31.6±4.9*	23.0±1.2	22.7±0.9

All experiments were performed in the presence of 10 μ*M* propanolol. Values represent means ± SEM; n = number of experiments. Asterisks indicate $p < 0.05$ as significant difference compared with the control group using the unpaired Student t test. Rya = ryanodine; $\tau_{f/s}$ = fast/slow component of $I_{Ca,L}$ inactivation time constant; RD = rundown to a steady state.

Effect of PE on K^+ Currents

Depolarization or hyperpolarization of rat ventricular myocytes from a holding potential of −80 mV resulted in the activation of K^+ currents. In the presence of 30 μ*M* TTX and 1 μ*M* Co^{2+} (to block Na^+ and Ca^{2+} currents), step depolarizations toward potentials more positive than −30 mV produced a rapidly activating and inactivating transient outward current (I_{to}) and a slowly inactivating steady-state K^+ outward current (I_{ss}). When the cell was hyperpolarized to potentials more negative than −80 mV, an inward current through the inward rectifier K^+ channels (I_{K1}) was observed. Typical current traces are shown in figure 4.

In propranolol (1 μ*M*)-treated ventricular cells before exposure to 10 μ*M* PE, the average amplitude of I_{to} and I_{ss} at a depolarization potential of 40 mV was 24.3 ± 2.2 pA/pF and 8.1 ± 0.6 pA/pF (n = 4), respectively. For inward current through K^+ channels, the average amplitude measured at −120 mV before treatment with PE was 18.7 ± 1.2 pA/pF (n = 4). After exposure to 10 μ*M* PE for 1–2 min, the average amplitude of this current was 19.5 ± 1.2 pA/pF (n = 4). When the slope conductance through inward rectifier was measured at potential levels between −80 and −120 mV, an average value in slope conductance was calculated to be 46.7 ± 3.1 and 48.8 ± 3.0 pS/pF before and after exposure to 10 μ*M* PE. These data indicate that the magnitude of potassium currents through I_{to}, I_{ss} and inward rectifier were not significantly affected during the time period during which a significant inhibition of calcium transient and contraction by PE was observed. However, if the currents were measured at 5 min after PE treatment, significant inhibition of potassium currents through I_{to} and inward rectifier was found (fig. 4).

Discussion

In the present study, we found that the α_1-AR-stimulated negative mechanical responses were mainly mediated by PKC activation in rat ventricular muscles. The initial NIE induced by PE was sensitive to the blockade by prazosin and CEC, but insensitive to the blockade by 5-MU. 5-MU and CEC, which respectively have high affinity in binding to α_{1A}- and α_{1B}-AR [13, 14, 16, 28]. Pharmacological classified α_{1A}- and α_{1B}- AR subtypes have been proven to be distributed in rat hearts, and are in the ratio of about 20:80 [24]. Although α_{1D}-AR may play a significant role for vascular contraction, especially in the aorta, it may play a minor role in rat hearts [6]. Our results showed the existence of the different signal transduction pathways after α_1-AR subtype activation were used to mediate the respective inotropic responses. In addition, the number of α_1-AR in myocardium varied from species to species. This may determine the different contractile responsiveness to α_1-ARs stimulation on various mammalian cardiac muscles. In cat papillary muscles, stimulation of α_1-AR induced positive inotropic responses without initial negative inotropic effects [38]. The positive inotropic responses in cat papillary muscles were associated with negative lusitropic effects. In our study of rat papillary muscles, α_1-adrenergic stimulation produced initial NIE followed by late positive inotropic responses. The induction of the initial NIE by PE did not appear to be associated with any significant changes in lusitropic responses.

α_1-Adrenergic activation has been reported to produce negative inotropic responses in rat ventricular myocytes [3, 12, 18] or rat atria 'damaged' by free radicals [30] through the diacylglycerol-PKC signal transduction pathway. The PKC-mediated phosphorylation and subsequent activation of the Na^+/K^+ ATPase may contribute to the NIE of PE. The stimulation of Na^+/K^+ ATPase activity in adult canine Purkinje fibers has been reported to be mediated via a PTX-sensitive pathway [34]. Our results showed that α_1-AR-stimulated NIE was insensitive to PTX but sensitive to staurosporine. Moreover, the NIE of PE in rat ventricular strips was unaffected by ouabain. Therefore, mechanisms other than stimulation of Na^+/K^+ ATPase may by responsible for NIE of PE in rat ventricles. Since the Na^+/K^+ ATPase activity in healthy rat atria was inhibited by α_1-agonist [30], species- and/or tissue-specific differences in α_1-AR-stimulated signal transduction for the inotropic responses may exist.

α_1-AR activation enhances the breakdown of membrane inositol phospholipids resulting in the production of IP_3 or its metabolite inositol-1,3,4,5-tetraphosphate [8]. IP_3 per se has no direct effect on the contraction of the skinned rat ventricular muscles [28]. IP_3 has caused Ca^{2+} mobilization from cardiac SR [17, 25]. In guinea pig ventricular myocytes, low concentrations of IP_3 were found to initiate a transient increase of contraction and high concentrations of IP_3 to induce a sustained negative inotropic response without affecting I_{Ca} in both conditions [32]. In rat ventricular myocytes, Ca^{2+} mobilization by IP_3 was proposed to suppresses $I_{Ca,L}$ and contribute to the NIE of α_1-AR stimulation [29]. While our result shows that $I_{Ca,L}$ was unchanged even though the $[Ca^{2+}]_i$ transient was initially decreased by PE, and the cross-talk between L-type calcium channels and ryanodine-sensitive Ca^{2+} release channels via Ca^{2+}-induced inactivation of L-type calcium channels was checked and proved to exist in our experimental condition.

The inhibition of α_1-AR-induced NIE and the associated decrease of $[Ca^{2+}]_i$ transients by staurosporine or by desensitization of PKC via prolonged pretreatment of the ventricles with PDBu substantiated the importance of PKC in mediating the NIE of PE. The suppression of cell contraction and the decrease of $[Ca^{2+}]_i$ transient through activation of PKC using PDBu also support this notion. The activation of PKC may phosphorylate certain proteins located in the compartment near cytoplasmic membrane, the effect of PKC on the function of Ca^{2+}-releasing channels on the SR is not well illustrated. The whole cell

Ca^{2+} signal detection does have some limitation to observe the detailed Ca^{2+} release signaling. But the functional study in figure 2b found that α_1-AR-mediated NIE was modulated by caffeine, but not by thapsigargin. Caffeine is known to fix the Ca^{2+} release channels in open state. The increase of the opening of Ca^{2+} release channels by caffeine may counteract the initial inhibition of Ca^{2+} release channels by PE. The modulation of the opening of Ca^{2+} release channels by PKC may be the mechanism for the NIE of PE. This notion corresponds with previous observations that the basal PKC activity exerted a tonic inhibition of excitation-contraction coupling in isolated rat ventricular myocytes [27].

Since the elevation of IP_3 may activate PI 3-kinase, the mobilization of Ca^{2+} by IP_3 may activate Ca^{2+}/calmodulin-dependent protein kinase II, which may then modulate the release of Ca^{2+} from ryanodine-sensitive Ca^{2+}-release channels. However, the insignificant effects of PI 3-kinase and Ca^{2+}/calmodulin kinase II inihibitors on the NIE of PE ruled out those possibilities.

Intracellular acidification was also proposed to mediate α_1-AR stimulated NIE in rat ventricular myocytes [12]. Since α_1-agonist stimulated initial decreases of cytoplasmic Ca^{2+} transients were not affected by Na/H exchange inhibitor (EIPA), the modulation of Na/H exchange activity by PE was not involved in the NIE or the decrease of Ca^{2+} transient. In addition to that, cytoplasmic acidification shortened cardiac APD [33]. Moreover, the activation of $I_{K,Ach}$ through the release of arachidonic acid by stimulation of α_1-AR was observed in guinea pig atrial cells [20, 21]. Since $I_{K,Ach}$ channels exist in mammalian ventricular cells [19], it is possible that the shortening of the APD by activation $I_{K,Ach}$ channels occurs in rat ventricular myocytes. In our study, we did not find any significant stimulation of potassium current through inward rectifiers by PE. Nor did we find any effect on other outward potassium currents during the time period in which inhibition of contraction and release of Ca^{2+} transient by PE was observed. These observations were further substantiated by the absence of APD alteration during the induction of NIE by PE. However, we did find significant suppression of I_{to} during the later period of exposure to PE similar to that found by Fedida et al. [10]. Additional mechanisms such as the enhancement of Ca^{2+} efflux through Na^+/Ca^{2+} exchanger [35] or phosphorylation of troponin I and T by PKC to decrease the contraction [36] remain to be clarified.

In conclusion, we found that α_1-AR-mediated NIEs in rat ventricular muscles were due to the decrease of intracellular Ca^{2+} transients via the modulation of PKC on Ca^{2+}-releasing channels signaling through a CEC-sensitive α_1-AR subtype. α_1-AR-mediated decreases of Ca^{2+} transients may provide a protective effect for the heart from the damage of Ca^{2+} overload in the late sustained positive inotropic response by α_1-AR agonists.

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