Potentiation of the Inotropic Effect of Isoproterenol by Naloxone *in vitro*; Non-opiate Receptor Mechanism

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Directed by Professor Wyun Kon Park

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Abstract

Potentiation of the inotropic effect of isoproterenol by naloxone in vitro; non-opiate receptor mechanism

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The aim of this study was to define the mechanism of non-opiate receptor-mediated action of naloxone, which potentiates the inotropic effect of isoproterenol on the myocardium. Inotropic intervention, measurements of cyclic adenosine monophosphate (cAMP), and electrophysiological methods were used.

Cardiac papillary muscle of the right ventricle was obtained from male guinea pigs (300-400 g) under sevoflurane general anesthesia. Peak force and maximum rate of force development (dF/dt-max) were measured. Cumulative concentration-response curves for isoproterenol as well as for forskolin and 3-isobutylmethylxanthine (IBMX) were obtained by increasing the concentration stepwise. The enhancement of contraction by naloxone in the presence of isoproterenol was further confirmed by sequentially applying increasing concentrations of naloxone in the presence of EC_{50} values of isoproterenol as well as forskolin and IBMX. For cAMP assays, cardiac muscle was homogenized and centrifuged to separate a membranous protein fraction. Naloxone-induced changes in cAMP in the presence of EC_{50} isoproterenol or forskolin were measured with cAMP assay kit. In

electrophysiologic studies, action potential, delayed outward K^+ current (I_k), inward rectifier K^+ current (I_{k1}), and L-type Ca^{2+} current ($I_{Ca,\,L}$) were measured.

Naloxone (30 μM) produced a leftward shift of the isoproterenol concentration-response curve (0.01-2 μM). While naloxone (30 μM) produced a leftward shift of the forskolin concentration-response curve (0.01-2 μM) until 3 μM concentration, markedly enhanced contraction was observed from 10 to 30 μM concentration ranges. Naloxone had no effect on the IBMX concentration-response curve. Concentration-related enhancement of contractile forces following sequential application of naloxone (10, 30, and 300 μM) in the presence of isoproterenol was shown. Naloxone did not alter the cAMP levels induced by application of EC_{50} isoproterenol or forskolin. Naloxone (30 μM), in the presence of isoproterenol, prolonged APD_{50} and APD_{90} while the amplitude and resting membrane potential was unchanged. Naloxone significantly reduced peak outward I_K at +80 mV to 20±3% of isoproterenol-treated group, but did not alter the I_{k1} from -140 mV to 0 mV. At a membrane potential of +10 mV, naloxone reduced the $I_{Ca,\,L}$ by 28±3%. Thus, enhancement of myocardial contractility by naloxone in the presence of isoproterenol is, at least in part, likely due to inhibition of delayed outward K^+ current, resulting in a secondary increase of inward Ca^{2+} current.

Key words: Naloxone, non-opioid receptor-mediated action, myocardial contractility, cAMP, delayed outward K^+ current.

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Ⅰ. INTRODUCTION

Since the discovery of opioid peptides in 1975,[1] the functional role of opiate receptors and their peptide ligands has been extensively researched. In 1978, Holaday and Faden[2] provided the first experimental evidence of opioid peptide involvement in the physiology of septic shock in rat. Subsequent studies in several animal species supported the potential therapeutic efficacy of opioid antagonists, such as naloxone, in different experimental shock states such as endotoxic,[2,3] hypovolemic,[4,5] and spinal shock[6,7]. Since then many papers have been published on the use of naloxone in human shock.[8] Considering that naloxone administration has been documented to be beneficial in some patients, although conflicting clinical results still exist, naloxone still may be a useful adjunct in the treatment of patients with severe septic shock who are unresponsive to other resuscitative measures,[8]

In shock state, it has been postulated that naloxone generates the cardiovascular pressor effect by displacing the endogenous opiate-like peptide ß-endorphin, which is secreted in increased amounts from the adenohypophysis in response to acute stress and binds to the opiate receptors

of the myocardium, resulting in restoration of the normal response to catecholamines released during shock.[9, 10]

In addition to this opioid antagonistic effect of naloxone, an alternative mechanism of action, the non-opiate receptor-mediated effect, which potentiates the adrenergic effect, has been proposed. Lechner[11] in a study using guinea pig (GP) papillary muscles pretreated with isoproterenol, demonstrated that when muscles were exposed to either d-naloxone, the stereoisomer of l-naloxone which does not displace opioids from opiate receptors, or l-naloxone, there was a similar dose-dependent increase in contractility at concentrations of 10 and 30 μM, indicating that the non-opiate receptor-mediated properties predominate at these concentrations. Such a non stereo-specific effect of naloxone in the presence of catecholamines has also been demonstrated in studies using isolated canine renal arteries[12] and *in situ*-isolated heart/lung preparation in dogs.[13] At present, although a non-opiate receptor-mediated effect of naloxone has been proposed, the underlying mechanisms have not yet been defined. The purpose of this study, therefore, was to elucidate the mechanism of naloxone's non-opiate receptor-mediated action on the myocardium, using inotropic intervention, measurements of cyclic adenosine monophosphate (cAMP), and electrophysiological methods.

Ⅱ. MATERIALS AND METHODS

1. Effects on contractility

A. Experiments with modified standard Tyrode solution

According to a procedure approved by the Yonsei University College of Medicine Animal Research Committee, the heart was removed from 350-400 g male GPs following sevoflurane anesthesia (3-4 vol%). The right ventricular papillary muscles were excised, mounted horizontally in a tissue bath, and superfused (8 ml/min) at 37°C with modified standard Tyrode solution (mM: 118 NaCl, 5 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3^-$, 11 glucose, 0.1 EDTA). EDTA was used to chelate any trace contaminant heavy metals. The solution was circulated through the bath from an unsealed reservoirs through which a mixture of 95% O_2 and 5% CO_2 was bubbled (flow rate: 0.5 L/min) to maintain a pH of 7.4±0.05. The tendinous end of the papillary muscle was attached to a Grass FT03 force transducer (Grass Instruments, Quincy, MA, USA) while the other end of the muscle was pinned to the bottom of the tissue bath. The muscle was adjusted to the lowest resting length at which maximum twitch force was obtained and field-stimulated using a Grass S44 stimulator (Grass Instruments, Quincy, MA, USA) at 120% of stimulus threshold at 0.5 Hz for 60 min for stabilization. After a 15-min rest, a rested-state (RS) contraction was elicited followed by stimulation rates of 0.1 Hz, sequentially. At 0.1 Hz stimulation rate, the duration of stimulation was preset and kept constant during the experiment. Effects on contractile function were estimated from the peak force and maximum rate of force development (dF/dt-max).

B. Experimental protocols

Cumulative concentration-response curves for isoproterenol as well as for forskolin (adenylate cyclase activator) and 3-isobutylmethylxanthine (IBMX) [non-selective inhibitor of phosphodiesterase (PDE)] were obtained

by increasing the concentration stepwise. Following baseline measurements after a 10-min rest period, drug concentration was increased after muscle response had been attained with the previous concentration. Exposure to each drug concentration was maintained for 10 min before recording responses. To examine the potentiating effect of naloxone, a second cumulative concentration-response curve was obtained for these three drugs in the presence of naloxone (30 μM). Naloxone was left in contact with the tissue for 10 min before obtaining the second concentration-response curve. Recovery responses were measured following a 20-min wash.

To further confirm the enhancement of contraction by naloxone in the presence of isoproterenol as well as forskolin and IBMX, increasing concentrations of naloxone in the presence of EC_{50} values of these drugs were applied sequentially. Recovery responses were measured following a 20-min wash.

Since both forskolin and IBMX are not water-soluble, we used dimethyl sulfoxide (DMSO) to dissolve these compounds. To exclude the effect of DMSO on contractility, appropriate concentrations of DMSO (1-10 μM), used in the cumulative concentration-response curve for forskolin, were applied. Recovery responses were measured following a 20-min wash.

As the muscles exposed to isoproterenol as well as forskolin and IBMX developed frequent ectopic beats or fatigue at stimulation rates greater than 0.1 Hz, we measured contractile forces only at RS and 0.1 Hz stimulation rate. Each preparation was exposed to only one drug. As the dF/dt-max value was more stable than that of peak force, we used dF/dt-max at 0.1 Hz stimulation rate as the measure of contractility in the results.

After each experiment, the cross-sectional area of each muscle was estimated from the muscle length, weight, and density (1.04 g/ml) assuming a cylindrical form. The mean cross-sectional area was 0.67 ± 0.04 mm^2 (n=73).

2. Effects on tissue levels of cAMP

To examine whether naloxone-induced potentiation of the inotropic effect of isoproterenol is due to changes in tissue levels of cAMP, we investigated whether naloxone could modify the concentration of cAMP in stimulated GP myocardium.

A. Myocardial membrane preparation and quantitative analysis

Hearts were excised from male GPs (250-300 g) anesthetized with sevoflurane at 3-4 vol%. Blood was evacuated and hearts were kept in ice-cold buffer (0.25 M sucrose, 5 mM Tris/HCl, 1 mM $MgCl_2$; pH 7.4) aerated with 100% O_2. Atrium, aorta, and other extraneous tissues were removed. Two hearts were homogenized with 5 volumes of ice-cold buffer, transferred into a 15 ml conical tube, and centrifuged at 600 g for 10 minutes at 4℃. The supernatant was divided into 6-8 1.5 ml microcentrifuge tubes and centrifuged at 15,000 g for 10 minutes at 4℃. The supernatant was centrifuged again at 100,000 g for 60 minutes at 4℃. The membrane pellet was dissolved in 1 ml of ice-cold incubation buffer (mM: 50 Tris/HCl, 10 $MgCl_2$; pH 7.5) and used for quantitative analysis. Quantitative analysis was performed with Biochronic Acid Assay (BCA) kit (Pierce, Rockford, IL, USA). Protein concentration was measured by the method of Lowry,[14] using a bovine serum albumin (0, 0.25, 0.5, 0.75, 1.0 mg/ml). About 3-4 mg/ml of membranous protein was achieved and kept at -20℃ after freezing with liquid nitrogen.

B. Measurement of cAMP concentration

Each of 30, 100, 300 μM naloxone, 0.05 μM L-isoproterenol or 1.35 μM forskolin and the membranous protein (250 μg) were added to cAMP production buffer (mM: 2.5 Na_2ATP, 5 $MgCl_2$, 1 Tris-EGTA, 20 creatine phosphate, 50 U/ml creatine phosphokinase, 0.8 IBMX, 50 Tris/HCl, pH 7.5) in a total volume of 500 μL. To exclude the effect of

DMSO on cAMP levels, the concentrations of DMSO used for the forskolin and IBMX cumulative concentration-response curves were also tested. After a 10-min incubation at 37℃, the reaction was stopped with 100℃ heat and the sample was centrifuged at 6,000 g for 20 minutes at 4℃. The cAMP concentration in the supernatant was measured using a cAMP assay kit [cAMP Biotrak Enzymeimmunoassay (EIA) System, Amersham, UK].

Working standard was prepared using the standard for acetylation assay, anti-serum, cAMP peroxidase conjugate and wash buffer provided by cAMP assay kit. Samples were diluted 100 times in assay buffer [0.05 M sodium acetate (pH 5.8), 0.02% bovine serum albumin, 0.01% preservative]. Acetylation reagent mixed with 2 volumes of triethylamine and 1 of acetic anhydride was added to all standards (0-128 fmol) and samples. One hundred μL of cAMP antiserum was added to 96 well plates coated with secondary antibody (except blank and non-specific binding wells) and 50 μL of samples or acetylation standard were added to each well. Assay buffer alone (150 μL) was added to non-specific binding wells. Plates were incubated for 60 minutes at 4℃. cAMP peroxidase conjugate (100 μL) were added to all wells except blanks and plates were incubated for 60 minutes at 4℃. Wells were aspirated and washed 4 times with 400 μL wash buffer and TMB substrate (150 μL) was added. After 1-hour shaking at room temperature, the reaction was stopped with 1 M sulfuric acid (100 μL). cAMP concentration was measured at 450 nm with a spectrophotometer (ELISA READER, BERSAmax, Molecular devices, Union city, CA, USA). The experiment was performed in duplicate.

3. Electrophysiologic studies

A. Isolation of ventricular myocytes

Hearts were quickly excised from male GPs (250-300 g) anesthetized with sevoflurane at 3-4 vol% and retrogradely perfused using a Langendorff perfusion system. Hearts were perfused at a rate of 7 ml/min for 5 min at 37ºC with modified Tyrode solution (mM: 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES, 0.18 glucose, pH 7.4). The perfusate was then switched for 5 min to a nominally Ca^{2+}-free Tyrode solution followed by perfusion with the same solution to which collagenase (0.4 mg/ml, Worthington type II, Worthington Biochemical Corporation, Lakewood, NJ, USA) and protease XIV (0.04 mg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) had been added. Following a 10-12-min enzymatic treatment, Kraftbrühe solution (mM: 10 taurine, 10 oxalic acid, 70 glutamic acid, 35 KCl, 10 H_2PO_4, 11 glucose, 0.5 Ethylene glycol-bis (β-aminoethyl ether)-N, N, N, N-tetraacetic acid (EGTA), 10 HEPES, pH 7.4) was perfused for 5 min. Ventricles were then cut off, minced with scissors, and agitated in a small beaker with Kraftbrühe solution. The resulting slurry was filtered through a 200-μm nylon mesh. Isolated ventricular cells were stored in Kraftbrühe solution for 1 hr at room temperature (21-22°C), then kept at 4ºC and used within 8 hours. Only rod-shaped cells with apparent striations that remained quiescent in solution containing 1 mM $CaCl_2$ were used. All the patch clamp experiments were carried out at room temperature.

B. Electrophysiological techniques

Isolated myocytes were allowed to settle to the bottom of a recording chamber mounted on an inverted microscope for approximately 10 minutes where the bathing solutions could be exchanged. The chamber was continuously perfused at a rate of 2 ml/min. Standard whole cell voltage-clamp methods were used.[15] Following the initiation of the whole-cell recording configuration, an interval of 4-6 min was allowed to establish a stable baseline. Voltage clamp measurements were performed using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster city, CA,

USA). Patch electrodes were prepared from a borosilicate glass model KIMAX-51 (American Scientific, Charlotte, NC, USA), which typically have a 2-3 MΩ resistance when filled with internal solution. After fabrication with a two-stage micropipette puller, the pipette tips were heat-polished with a microforge. Data acquisition was performed using a pCLAMP system version 6.0.3 (Axon Instruments, Foster city, CA, USA) coupled with a Pentium-III personal computer.

C. Voltage-clamp protocols

The action potential (AP) was elicited in current-clamp mode by 5-ms, 800 pA current injections at a frequency of 1 Hz. To evaluate the effect of naloxone on delayed outward K^+ current (I_K), the I_K was measured by step depolarizations from -30 to +80mV with a holding potential of -40 mV in 20 mV increments, 5-s intervals, and 4-s pulse duration. The effect on inward rectifier K^+ current (I_{k1}) was verified by measuring the I_{k1} by step depolarizations from -140 mV to 0 mV from a holding potential of -40 mV in 20 mV increments. Pulses were applied for 200 ms at 5-s. Voltage-dependent L-type Ca^{2+} current ($I_{Ca,\ L}$) was evoked by 200 ms step depolarizations from a holding potential of -40 mV to +10 mV in one step at a frequency of 0.1 Hz.

D. Solutions

An external bathing solution (mM: 140 NaCl, 5.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 glucose, adjusted to pH 7.4 with 1 N NaOH) was used before establishing whole-cell recording. For K^+ current measurements, a patch pipette solution (mM: 20 KCl, 110 K-aspartate, 10 EGTA, 10 HEPES, 1 $MgCl_2$, 5 K_2ATP, 1 $CaCl_2$, 10 NaCl, adjusted to pH 7.2 with KOH) was used before establishing the whole-cell recording configuration. After establishing the whole-cell voltage clamp, 0.2 mM $CdCl_2$ was added to the external bathing solution to eliminate any confounding Ca^{2+} current. Inward Ca^{2+} currents were measured using a patch pipette solution (mM: 30 CsCl,

100 aspartic acid, 100 CsOH, 10 BAPTA, 10 HEPES, 10 phosphocreatinine, 1 Na_2GTP, 5 Na_2ATP, 10 glucose, 2 $MgCl_2$, adjusted to pH 7.25 with 1 M CsOH). Upon obtaining whole-cell recording, the external bathing solution was exchanged to a solution (mM: 140 NaCl, 5.4 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.4 with 1 M CsOH) to measure $I_{Ca,\,L}$.

After the baseline measurement with isoproterenol (0.05 μM) in the presence of ascorbic acid (1 mM) to prevent oxidation, myocytes were exposed to naloxone (30 μM) for 2 minutes. Recovery responses were measured after a 2-min wash. A 2-min application of naloxone was sufficient to produce a stable and consistent effect in pilot experiments.

4. Drugs and chemicals

Isoproterenol, forskolin, IBMX, and naloxone (l-form) were obtained from Sigma-Aldrich Chemicals Company (St. Louis, MO, USA). The cAMP assay system was obtained from Amersham International, UK. Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich Chemicals Company.

Isoproterenol was dissolved in normal Tyrode solution. Forskolin and IBMX were dissolved in DMSO.

5. Statistical analysis

In papillary muscle and isolated myocyte experiments, repeated measures of ANOVA followed by Student-Newman-Keuls test were applied to test for statistically significant differences among the baseline, drug application, and washout period as well as among the stimulation rates. One-way ANOVA followed by Student-Newman-Keuls test was used to compare the cAMP concentrations among each concentration of naloxone. All results were expressed as mean±SEM. A *P* value less than 0.05 was considered significant.

Ⅲ. RESULTS

1. Effects on contractility

A. Effects of isoproterenol and naloxone

Isoproterenol (0.01–2 μM) increased contractile forces in a concentration-dependent manner, producing a maximal effect at 0.5 μM concentration (n=8). In the presence of naloxone (30 μM), isoproterenol increased contractile forces in a concentration-dependent manner, producing a maximal effect at the same concentration (n=6). Naloxone (30 μM) produced a leftward shift of the isoproterenol concentration-response curve and reduced the EC_{50} from 0.05±0.02 μM for isoproterenol alone to 0.02±0.01 μM in the presence of naloxone (Fig. 1A). Despite the visible leftward shift, the EC_{50} values at the same isoproterenol concentration were not statistically different. Baseline values of isoproterenol (0.01 μM) in the absence and presence of naloxone at 0.1 Hz stimulation rate were 4.76±1.36 and 10.94±4.76 $mN/s/mm^2$, respectively.

In preliminary experiments using 10 and 30 μM naloxone to verify its enhancement of contractile forces by naloxone in the presence of isoproterenol (0.03 μM), we observed that 10 and 30 μM naloxone increased contractile force by 28±6% (n=6, $P<0.05$) and 45±8% ($P<0.05$), respectively, when compared to that of isoproterenol alone (Fig. 1B). Complete recovery was shown following a 20-min wash with isoproterenol.

Sequential application of naloxone (30, 100, and 300 μM) in the presence of EC_{50} isoproterenol increased contractile forces by 33±9% ($P<0.05$), 49±13% ($P<0.05$), and 69±13% ($P<0.05$), respectively, when compared to those induced by EC_{50} isoproterenol (n=6). Similar contractile enhancement was shown between 30 and 100 μM naloxone, while 300 μM caused a significant increase in contractility when compared to that of 30 μM (Fig. 1C). Complete recovery was shown following a 20-min wash with

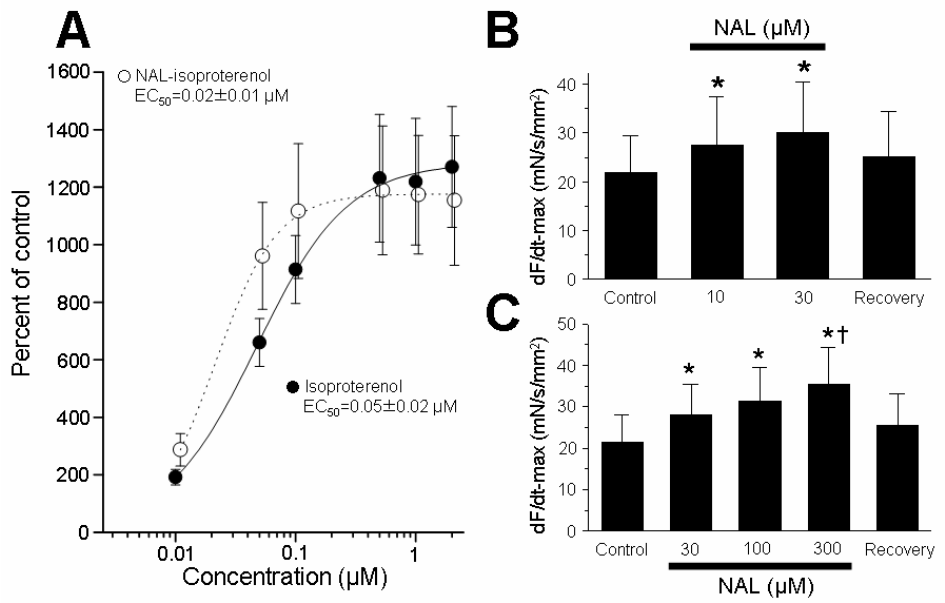

Fig.1. Naloxone potentiation of the positive inotropic effect of isoproterenol in isolated guinea pig ventricular myocardium. A. Isoproterenol cumulative concentration-response curves of isoproterenol in the absence (n=8) and presence of naloxone (NAL) (30 μM) (n=6). Inotropic responses are expressed as a percentage of baseline in the absence of isoproterenol. Each point represents the mean value±SEM. B. Effects of naloxone on the contractile forces at 0.1 Hz stimulation rate in the presence of isoproterenol (0.03 μM) (n=6). dF/dt-max indicates maximum rate of force development per cross sectional area. Error bars indicate SEM. *; $P<0.05$ versus control. C. Effects of higher concentrations of naloxone on the contractile forces at 0.1 Hz stimulation rate in the presence of EC_{50} isoproterenol (0.05 μM) (n=6). dF/dt-max indicates maximum rate of force development per cross sectional area. Error bars indicate SEM. *; $P<0.05$ versus control. †; $P<0.05$ versus 30 μM concentration.

isoproterenol.

B. Effects of forskolin and naloxone

To examine whether naloxone modifies the effect of forskolin, cumulative concentration-response curves were obtained in the absence (n=9) and presence of naloxone (30 μM) (n=8). Forskolin (0.5–30 μM) produced a concentration-dependent increase in contractile forces, producing maximal contractile force at 10 μM. While naloxone (30 μM) produced a leftward shift of the forskolin concentration-response curve between 0.5 and 3 μM, marked enhancement of contraction was observed from 10 to 30 μM. EC_{50} values of forskolin in the absence and presence of naloxone was 1.35±0.17 and 1.63±0.42 μM, respectively (Fig. 2A), which showed no difference (NS). Baseline values of forskolin (0.5 μM) in the absence and presence of naloxone were 7.80±1.54 and 9.94±2.54 mN/s/mm^2, respectively.

Application of 10 and 30 μM naloxone in the presence of EC_{50} forskolin increased contractile forces by 33±10% and 88±26% ($P<0.05$) when compared to that of EC_{50} forskolin alone, respectively (n=6). Complete recovery was shown following a 20-min wash (Fig. 2B).

C. Effects of IBMX and naloxone

To examine whether naloxone modifies the effect of IBMX, concentration-response curves for IBMX were obtained in the absence and presence of naloxone (30 μM). Either IBMX (50-400 μM) alone (n=7) or in the presence of naloxone (30 μM) (n=8) produced a concentration-dependent increase in contractile forces. Application of IBMX in the presence of naloxone did not shift the concentration-response curve obtained in the absence of naloxone (NS). The EC_{50} IBMX (108.84±0 μM) was similar to that in the presence of naloxone (103.30±0 μM) (NS) (Fig. 3A). Baseline values of IBMX (50 μM) in the absence and presence of naloxone were 9.35±3.48 and 3.97±1.68 mN/s/mm^2, respectively.

Application of 10 and 30 μM naloxone in the presence of EC_{50} IBMX

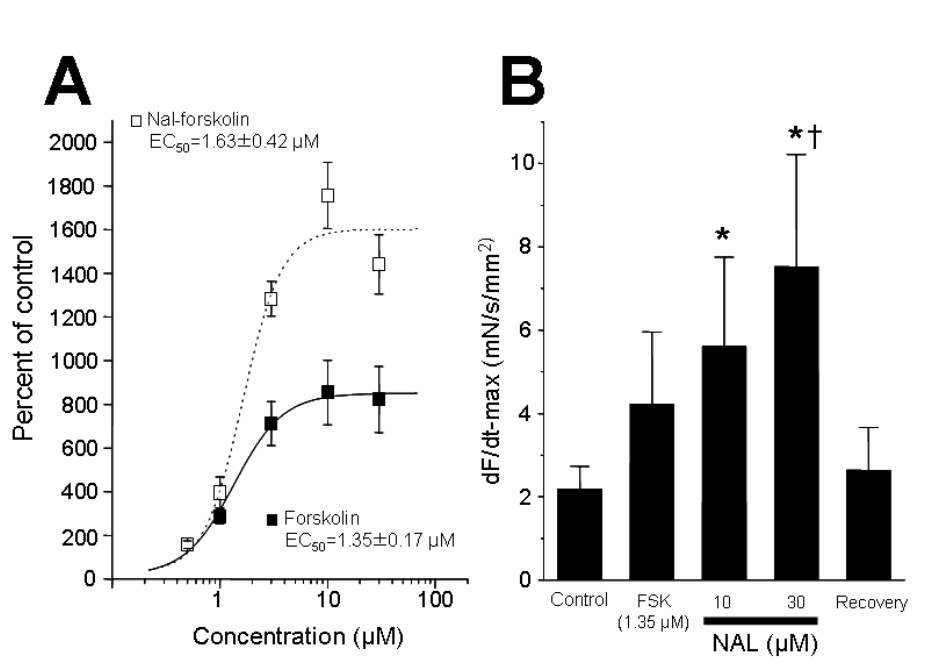

Fig. 2. Naloxone potentiation of the positive inotropic effect of forskolin (FSK) in isolated guinea pig ventricular myocardium. A. Forskolin cumulative concentration-response curves of forskolin in the absence (n=9) and presence of naloxone (NAL) (30 μM) (n=8). Inotropic responses are expressed as a percentage of baseline in the absence of forskolin. Each point represents the mean value±SEM. B. Effects of naloxone on the contractile forces at 0.1 Hz stimulation rate in the presence of EC_{50} FSK (1.35 μM) (n=4). dF/dt-max indicates maximum rate of force development per cross sectional area. Error bars indicate SEM. *; $P<0.05$ versus control. †; $P<0.05$ versus forskolin.

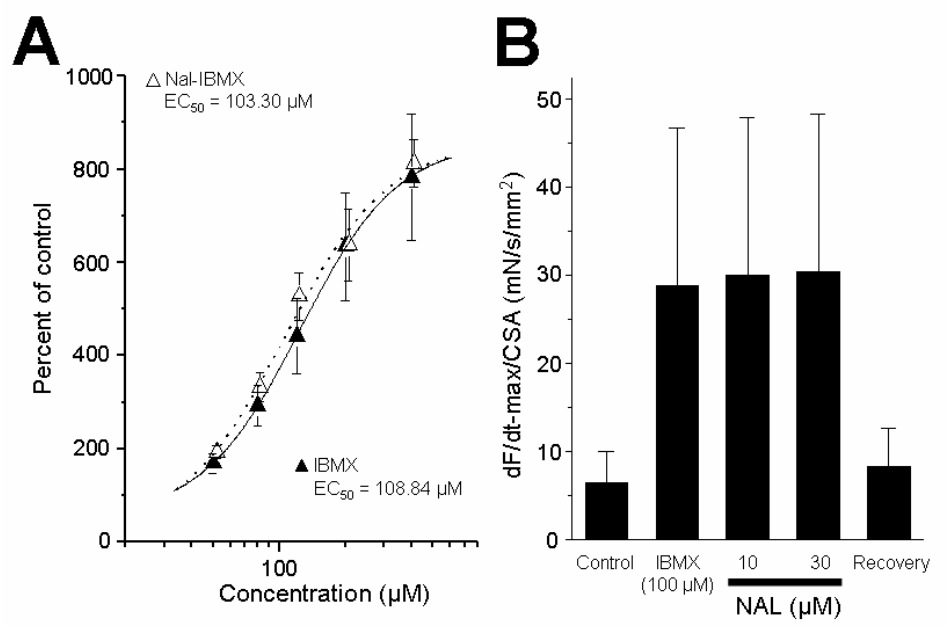

Fig. 3. Naloxone effects on the positive inotropic effect of 3-isobutylmethylxanthine (IBMX) in isolated guinea pig ventricular myocardium. A. IBMX cumulative concentration-response curves in the absence (n=7) and presence of naloxone (NAL) (30 μM) (n=8). Inotropic responses are expressed as a percentage of baseline in the absence of IBMX. Each point represents mean value±SEM. B. Effects of naloxone on the contractile forces at 0.1 Hz stimulation rate in the presence of EC_{50} IBMX (108 μM) (n=4). dF/dt-max indicates maximum rate of force development per cross sectional area. There was no potentiating effect of naloxone in the presence of EC_{50} IBMX. Error bars indicate SEM.

did not alter the contractile forces when compared to that of EC_{50} IBMX alone (N=4, NS). Complete recovery was shown following a 20-min wash (Fig. 3B).

DMSO (1, 3, and 10 μM) did not alter the contractile forces (N=6, NS) (control: 7.55±1.81; 1 μM: 6.80±1.77; 3 μM: 6.78±1.88; 10 μM: 6.30±1.8 $mN/s/mm^2$). In preliminary experiments, we also confirmed that DMSO (30 μM) (n=2) did not alter the contractility (control: 10.97±4.93; 1μM: 10.97±4.93 $mN/s/mm^2$). In the cumulative concentration-response curves, the maximal concentrations of DMSO for forskolin (30 μM) and IBMX (400 μM) in the reservoir solution (200 ml) was 2% and 1.33% by volume, respectively.

II. Effects of isoproterenol, forskolin, and naloxone on the tissue level of cAMP

While the production of cAMP stimulated by the EC_{50} isoproterenol (0.05 μM) increased by approximately 2-fold of the basal level (n=8, $P<0.05$), application of each concentration of naloxone (30, 100, and 300 μM) did not alter the level of cAMP (n=8, NS) (Fig. 3A). Whereas EC_{50} forskolin (1.35 μM) produced a 4-fold increase in basal level of cAMP, each concentration of naloxone (30, 100, and 300 μM) did not alter the level of cAMP (n=6, NS) (Fig. 3B). The vehicle, DMSO (1-10 μM), did not produce any change in tissue level of cAMP (n=8, NS) (control: 452±177; 1μM: 488±186; 3 μM: 489±183; 10 μM: 356±99 fmol/mg protein).

III. Electrophysiologic study

Fig. 5 shows prolongation of AP duration by naloxone (30 μM) in the presence of EC_{50} isoproterenol in a GP ventricular myocyte. Naloxone (30 μM) prolonged the APD_{50} and APD_{90} by 27±8% ($P<0.05$) and 26±8% ($P<0.05$), respectively, following application of the EC_{50} isoproterenol (n=5).

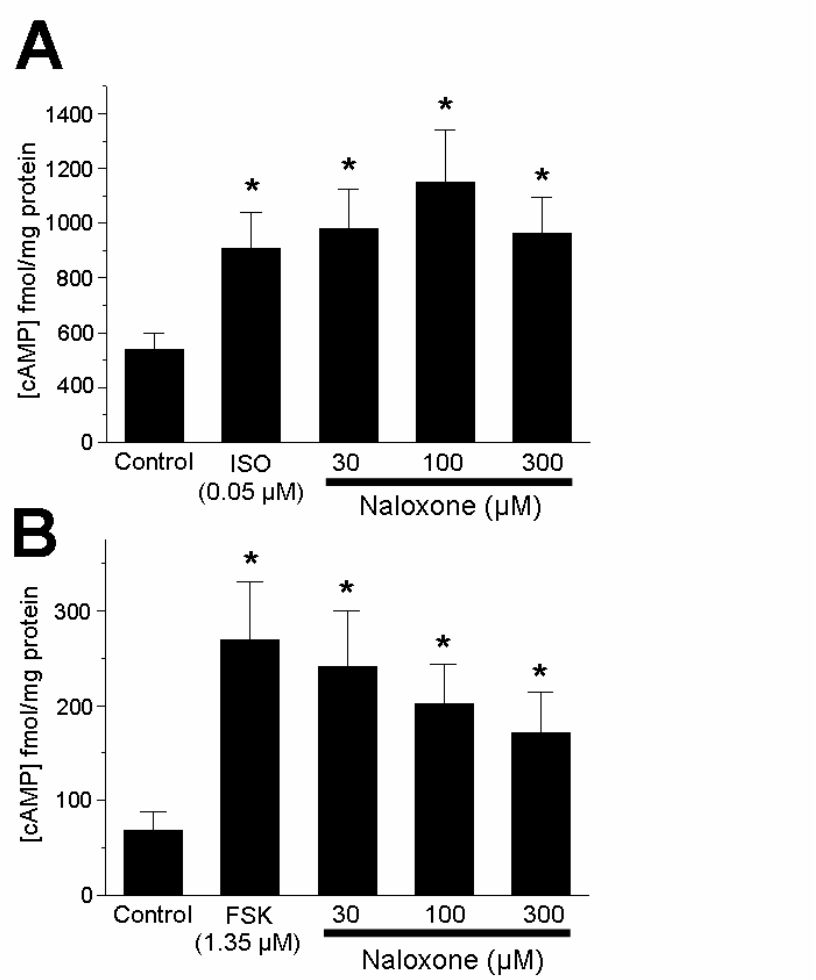

Fig. 4. Effects of isoproterenol (ISO) and forskolin (FSK) on tissue cAMP levels in the absence and presence of naloxone. A. Naloxone cumulative concentration effects in the presence of EC_{50} ISO (0.05 μM) (n=8). B. Effects of cumulative concentration effect in the presence of EC_{50} FSK (1.35 μM) (n=6). Error bars indicate SEM. *; $P<0.05$ versus control.

The AP amplitude and resting membrane potential remained unaltered. AP duration was partially restored to baseline level following a 2-3 min wash (Table 1).

Peak outward I_K assessed at +80 mV was reduced by 20±3% (n=13, $P<0.05$) by naloxone (30 μM) in the presence of isoproterenol when compared to that of EC_{50} isoproterenol alone (2.0±0.18 nA). Peak outward I_K was not restored to the baseline level (57±9% of the baseline) following washout (Fig. 6).

At membrane potential ranges from -140 mV to 0 mV, naloxone (30 μM) in the presence of EC_{50} isoproterenol did not alter the inward and outward components of I_{KI} (n=5, NS) (Fig. 7A, B).

At a membrane potential of +10 mV, naloxone (30 μM) in the presence of EC_{50} isoproterenol reduced the $I_{Ca,\ L}$ by 28±3% (n=8, $P<0.05$) when compared to that of EC_{50} isoproterenol alone (0.75±0.09 nA) (Fig 8A, B). The effect of naloxone on $I_{Ca,\ L}$ was not reversible following a 2-3 min wash.

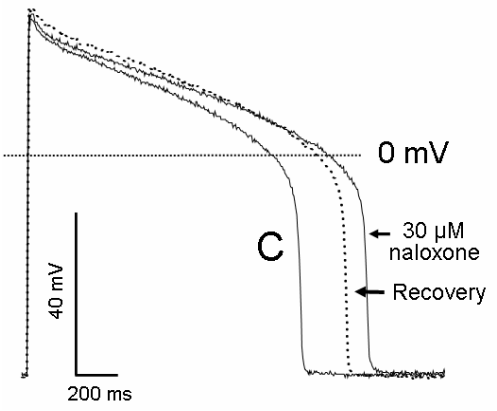

Fig. 5. Effects of naloxone (30 μM) on the action potential in the presence of EC_{50} isoproterenol (0.05 μM) in a guinea pig ventricular myocyte. The dotted line indicates recovery following a 2-3 min wash. C indicates control in the presence of isoproterenol.

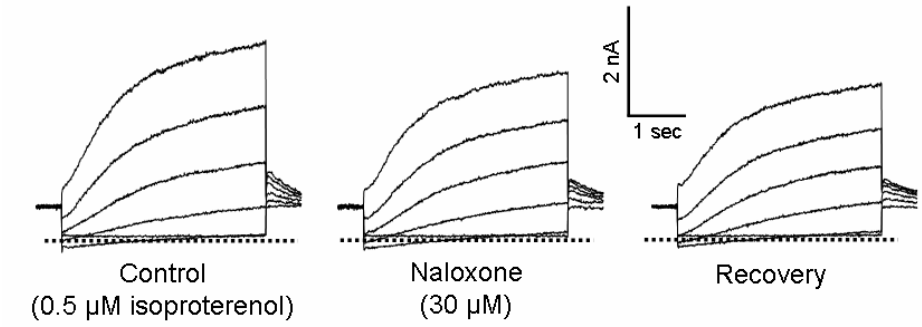

Fig. 6. Effect of naloxone (30 μM) on the delayed outward K^+ current (I_k) in the presence of EC_{50} isoproterenol (0.05 μM) in a guinea pig ventricular myocyte. Control current was obtained in the presence of EC_{50} isoproterenol. I_k was measured by step depolarizations from -30 to +80 mV from a holding potential of -40 mV in 20 mV increments over 5-s intervals. The dotted line indicates zero current level.

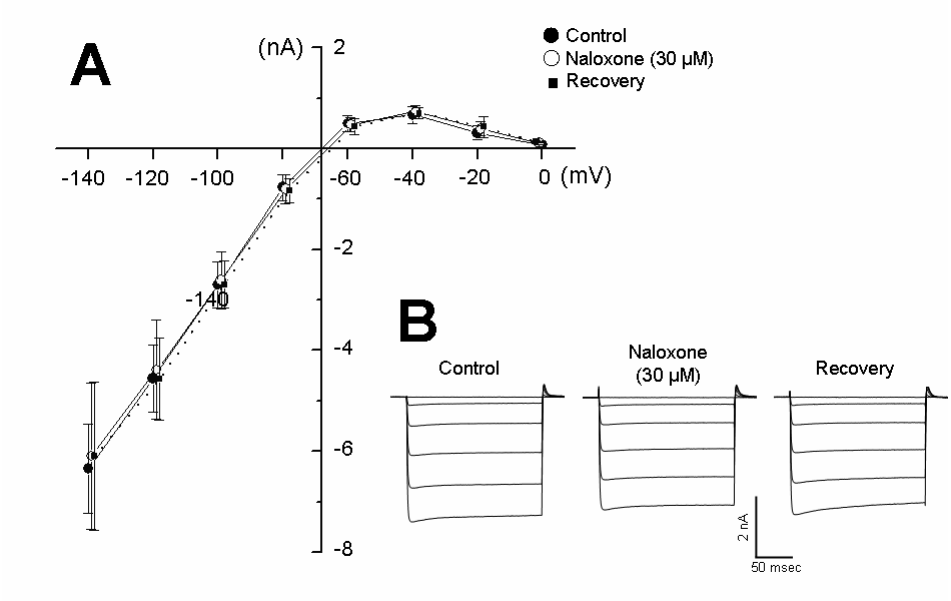

Fig. 7. A. Effects of naloxone (30 μM) on the inward rectifier K^+ current (I_{k1}) in the presence of EC_{50} isoproterenol (0.05 μM) in guinea pig ventricular myocytes. Control current was obtained in the presence of EC_{50} value of isoproterenol. The dotted line indicates recovery following a 2-3 min wash. I_{k1} was measured by step depolarizations from -140 mV to 0 mV from a holding potential of -40 mV in 20 mV increments. The pulse was applied for 200 ms at 5-s intervals. Error bars indicate SEM. B. A representative tracings in a guinea pig ventricular myocyte.

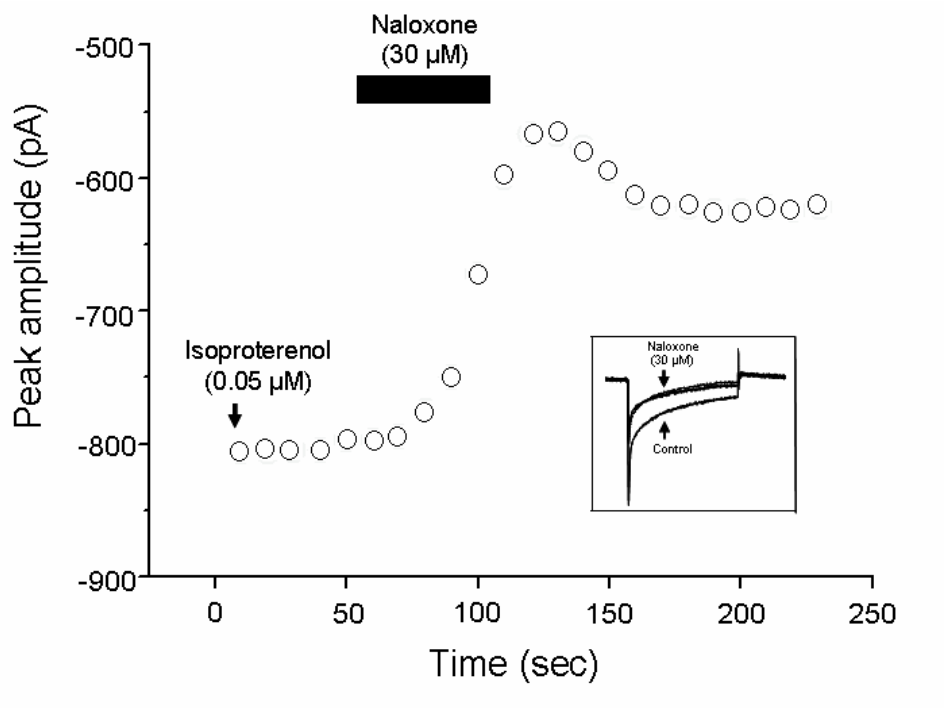

Fig. 8. Effects of naloxone (30 μM) on L-type Ca^{2+} current in the presence of EC_{50} isoproterenol (0.05 μM) in a guinea pig ventricular myocyte. Unfilled circles indicate peak current of an individual record. The horizontal bar indicates the period when naloxone was applied. (Inset) An example of individual currents recorded in the presence of naloxone.

Table 1. Effects of Naloxone (30 μM) in the Presence of Isoproterenol (0.05 μM) on the Normal Action Potential

	RMP	AMP	APD_{50}	APD_{90}
Control	-66 ± 4	119 ± 3	823 ± 60	847 ± 58
Naloxone	-66 ± 4	118 ± 3	1032 ± 68 *	1005 ± 65*
Recovery	-63 ± 5	118 ± 3	912 ± 46 *	938 ± 45*

Results are presented as mean±SEM. Repeated measures analysis of variance followed by Student-Newman-Keuls test was used to test for differences among groups. RMP = resting membrane potential; AMP = action potential amplitude; APD_{50} and APD_{90} = the duration of the action potential at 50% and 90% of depolarization, respectively. *; $P<0.05$ versus control.

Ⅳ. DISCUSSION

The present results demonstrate that naloxone potentiates the positive inotropic response elicited by isoproterenol in the isolated GP papillary muscles. In the presence of isoproterenol, naloxone modestly, but significantly, reduced the delayed outward K^+ current, resulting in prolongation of AP duration which contributes to potentiation of the positive inotropic response induced by isoproterenol secondary to more enhanced Ca^{2+} entry by naloxone. Naloxone appeared to have no effect on tissue cAMP levels elicited by pretreatment with either isoproterenol or forskolin.

It is postulated that, during shock, ß-endorphins from the anterior pituitary are released into the systemic circulation[16] and bind to opiate receptors in the heart.[17,18] These receptors interact with inhibitory G-protein complexes which subsequently inhibit the adenylate cyclase activity and attenuate the increases in intracellular cAMP levels normally produced by ß-adrenergic receptor stimulation. In this model, when naloxone is administered, the endorphins are displaced from opiate receptors in the myocardium, and, with removal of the opiate effect, the normal response to ß-adrenergic agonists is restored.[9]

Naloxone's action mediated by opiate receptors during shock is supported by evidence that l-naloxone, but not d-naloxone, is effective at increasing myocardial function when delivered directly into the coronary circulation of dogs subjected to hemorrhagic shock.[19] However, these results are in conflict with those of Caffery et al. using isolated canine renal arteries,[12] who showed that potentiation of the adrenergic effect was not stereo-specific. Such a non stereo-specific effect of naloxone has also been demonstrated in dogs using *in situ*-isolated heart/lung preparation.[13] Lechner[11] in a study using GP papillary muscles proposed that naloxone has more than one site of action, suggesting non-opiate receptor-mediated interaction

between naloxone and isoproterenol. He demonstrated that, at low concentration of naloxone (0.1 μM), the beneficial non-opiate receptor-mediated properties of l-naloxone are attenuated by its concurrent action at opiate receptors. However, at higher concentrations (10 or 30 μM), d- and l-naloxone showed similar increase in contractility, indicating that the non-opiate receptor-mediated properties of l-naloxone predominate. Non-opiate receptor-mediated action of l-naloxone in the presence of ß-agonists has also been demonstrated in dogs using renal artery[12] and *in situ* isolated heart-lung preparation.[13]

Lechner,[11] demonstrated that application of 10 and 30 μM l-naloxone to GP ventricular tissues potentiated the positive inotropic effect of isoproterenol by approximately 20% and 32%, respectively. In the preliminary experiment using the same animal preparation, we confirmed that naloxone potentiated contractility in the presence of isoproterenol (Fig 1B). In another experiment using the EC_{50} isoproterenol, we also observed that 30 and 100 μM naloxone increased contractility by 33±9% and 49±13%, respectively (Fig. 1C). Kim et al.[20] in their study assessing the direct myocardial depression by naloxone alone demonstrated that naloxone (50 μM) depressed contractility in GP ventricular tissues by approximately 20%, suggesting little effect at 30 μM. Based on the concentrations used by Lechner[11] and Kim et al.,[20] we decided that 30 μM concentration would be appropriate to use in further experiments because naloxone alone had little effect on contractility

We observed the maximum contraction above 0.5 μM concentration in the isoproterenol cumulative concentration-response curve, which indirectly suggests that production of cAMP induced by isoproterenol at this concentration is maximal. Furthermore, naloxone did not enhance the contractile force above 0.5 μM, suggesting full saturation of the increased cAMP levels. In the forskolin cumulative concentration-response curve,

maximal contractile force was reached above 10 μM concentration, which suggests that maximal cAMP is produced by forskolin alone. Interestingly, however, contraction was markedly enhanced above 10 μM in the naloxone-pretreated group, suggesting that there may be another non-opiate receptor-mediated mechanisms in addition to the inhibitory effect on I_k. Of the possible alternatives, inhibitory effect on Na/K pump as well as on Na^+-Ca^{2+} exchange pump, which contributes to the myocardial contraction, has been investigated. Levin et al.[21] in their study using rat left atria and strips of ventricular muscles suggested inhibition of the sarcolemmal Na/K pump by naloxone, which contributes to the concentration-dependent positive inotropic effect from 10 to 500 μM concentration ranges. Inhibition of Na^+-Ca^{2+} exchange by high concentrations of naloxone (IC_{50}: 4,000 μM) in isolated calf cardiac sarcolemmal vesicles has also been proposed, indicating negligible inhibition of Na^+/Ca^{2+} exchange activity in the concentration range of 30-300 μM which we used in this study.[22] Levin et al.[21] also suggested that naloxone has no effect on Na^+-Ca^{2+} exchange pump function.

ß-agonists bind to ß-adrenergic receptors in the cardiac membrane. This receptor-ligand complex binds to stimulatory guanine nucleotide-binding proteins. These activate adenylate cyclase, which converts ATP to cAMP, the second messenger for inotropic responses to catecholamine. To examine whether the naloxone-induced potentiation of isoproterenol-induced inotropic effects elicited under our experimental conditions is due to the changes in tissue cAMP levels, we investigated whether naloxone could modify the stimulated concentration of cAMP. Our results showed that naloxone does not alter the increased cAMP levels elicited by pretreatment with ß-agonist. We also examined the possibility that naloxone facilitates the effect of isoproterenol on adenylate cyclase by increasing cyclic AMP production. To this purpose, we examined the interaction between forskolin and naloxone. Forskolin is an activator of the catalytic subunit of adenylate cyclase and

increases cyclic AMP production by this mechanism.[21] Our results showed that naloxone has no effect on forskolin-induced cAMP levels. We had initially hypothesized that naloxone would enhance isoproterenol-induced increases in cAMP level, resulting in potentiation of the inotropic effect of isoproterenol. However, unexpectedly, naloxone did not alter the cyclic AMP production induced by EC_{50} isoproterenol or forskolin even though it significantly enhanced contraction, as shown in our contraction experiments. It is well established that catecholamine enhances myocardial contractility by increasing intracellular cAMP levels, and this response is amplified by the presence of PDE inhibitor, IBMX,[23,24] a prototype of non-selective PDE inhibitor.[25] In this study, addition of naloxone did not alter the cumulative concentration-dependent contractility, which indicates that naloxone has no effect on the enzyme cyclic nucleotide PDE.

Since both forskolin and IBMX are not water-soluble, we used DMSO to dissolve these compounds. DMSO has been reported to show some pharmacological activity in several tissues.[26,27] In the innervated GP cardiac atrial tissue, 3% DMSO produced a moderate increase in contractility, which became very marked when the final bath concentration was 6%.[28] In our experiments with GP ventricular tissue, the maximal concentration of DMSO in the reservoir (200 ml Tyrode solution) was about 2%, which showed no alteration of contraction and cAMP levels. Therefore, it is likely that naloxone's potentiating effect on contraction in the presence of forskolin or IBMX could not be attributed to its solvent DMSO.

In GP ventricular myocytes, we observed that naloxone (30 μM), in the presence of isoproterenol, significantly prolonged AP duration. Naloxone alone has also been reported to prolong the AP duration in GP[20,29] and rabbit[30] cardiac tissues with little effect on the resting membrane potential. In rat ventricular myocytes, naloxone (3-30 μM) prolonged AP duration in a concentration-dependent manner, and a similar effect was shown in GP and

human atrial cells at 10 μM.[31] Increased plateau phase due to prolonged AP duration may permit enhancement of Ca^{2+} entry, resulting in increased contractile force. However, our whole cell voltage clamp experiments revealed a reduction of peak $I_{ce,L}$ by naloxone in the presence of isoproterenol, which highly suggests an effect on the repolarizing K^+ currents.

Brasch[29] in his study using GP atrial and ventricular tissues indirectly suggested inhibition of K^+ outward current as a cause of prolongation of AP duration by naloxone (30-120 μM) alone. Kim et al.[20] also demonstrated AP prolongation by naloxone (100 μM) alone and suggested direct inhibition of K^+ current in GP ventricular myocardium. In other tissues, inhibition of the membrane K^+ conductance in frog node of Ranvier[32] and in squid axons[33] has also been demonstrated. Based on these suggestions and evidences, we speculated that naloxone could affect the repolarizing K^+ currents. In the present study, whole cell voltage clamp experiments revealed that naloxone suppresses the slowly activating current, I_k. Considering the higher voltage used in this study, at which a rapidly activating, inward rectifier current, I_{kr}, is usually deactivated, the effects of naloxone primarily represent depression of I_{ks}. I_k is composed of two different current types: a rapidly activating, inward rectifier current, I_{kr}, and a slowly activating current, I_{ks}.[34] Inhibition of delayed outward K^+ current (28.3±4.5%) by naloxone (10 μM) alone in GP atrial cells at 37°C, similar to the extent of reduction in our results, has also been reported.[31]

The inward rectifier K^+ current is the primary current responsible for maintaining a stable cardiac resting membrane potential near the K^+ equilibrium potential. Inhibition of I_{k1} can result in diastolic depolarization, which in turn can cause increase cardiac excitability[35] and lead to dysrhythmias due to abnormal automaticity.[36] The lack of change in the resting membrane potential following application of naloxone (30 μM) may be attributable to the lack of alteration of I_{k1}. In addition to the inward

current, I_{k1} also contributes to the terminal phase of repolarization via outward current at potentials from -50 or -40 mV to 0 mV.[30,37] Whereas our results demonstrated little alteration of I_{k1} by naloxone from -140 mV to 0 mV, Hung et al.[31] observed increase of inward component of I_{k1} by 10 μM naloxone, approximately 20%, with no alteration of outward component of I_{k1} in rat ventricular myocytes.

Our whole cell voltage clamp experiments revealed a reduction in peak $I_{Ca,\,L}$ despite the prolonged of AP duration. Although inhibition of $I_{Ca,\,L}$ can lead to a reduction of AP duration, the reduction of I_{ks} seems to have a greater effect, resulting in AP lengthening in the present study.

Non-opioid receptor-mediated effect of naloxone alone on the AP as well as contraction has also been proposed in rabbit ventricular tissue and arterially perfused interventricular septa,[38] in GP atrial and ventricular myocardium,[29] and in rat heart.[39]

Naloxone alone (50, 100, and 200 μM) causes a concentration-dependent depression of contractile force in GP ventricular tissues,[20] By contrast, in our results, sequential administration of naloxone (30, 100, and 300 μM) in the presence of isoproterenol showed concentration-related enhancement of contractile forces. Considering that the potential mechanism behind these opposite results could be the same, non-opiate receptor-mediated effect, it is quite perplexing that the effects on contraction are opposite. In GP ventricular tissues, naloxone (100 μM) alone caused prolongation of APD_{50} and APD_{90} by approximately 12%.[20] Based on this, we may assume that the changes in AP duration are minimal or negligible at 30 μM concentration, and produce little effect on repolarizing K^+ current so that the reduction in Ca^{2+} entry by naloxone, approximately 35% by 50 μM concentration,[20] predominates, resulting in depression of contractile force. In the isoproterenol-pretreated group, however, the intracellular Ca^{2+} concentration may be little affected despite direct inhibition of Ca^{2+} entry by

naloxone (30 μM) because intracellular Ca^{2+} is already quite abundant by prior application of isoproterenol. Thus, prolongation of AP duration by naloxone in the presence of isoproterenol would result in more Ca^{2+} entry, followed by enhanced contraction.

In this study, we observed concentration-related enhancement of contraction (30, 100, and 300 μM) by naloxone in the presence of isoproterenol, in which naloxone (300 μM) significantly increased contractility by approximately 70% when compared to that of isoproterenol-treated group. Considering that naloxone in the presence of isoproterenol prolonged AP duration by 26±8%, one may assume that a higher concentration of naloxone (300 μM) may further prolong the AP duration secondary to further inhibition of I_k. This would result in further enhancement of contractility via enhanced Ca^{2+} entry. Under these conditions, as intracellular Ca^{2+} was already much increased by prior administration of isoproterenol, reduction of $I_{Ca,\,L}$ by naloxone (300 μM) may have little effect. In contrast, 200 μM naloxone alone depressed contractility by approximately 60% in the same animal preparation.[20] Considering the AP-prolonging effect of naloxone (100 μM) alone, approximately 12%,[20] it could be assumed that a higher concentration of naloxone (200 μM) may further prolong the AP duration, followed by enhanced contractility secondary to enhanced Ca^{2+} entry. However, naloxone (200 μM) did not increase the contractility but instead caused marked contractile depression. It may be speculated that 200 μM naloxone directly reduces Ca^{2+} entry much more than that of 50 μM, and this may overwhelm the Ca^{2+} entry during the plateau phase induced by naloxone's inhibition of I_k. Further study will be required to validate the effect of high naloxone concentration alone on the $I_{Ca,\,L}$ and repolarizing K^+ current.

Ⅴ. CONCLUSION

In conclusion, the enhancement of myocardial contractility by naloxone in the presence of isoproterenol is, at least in part, likely due to inhibition of delayed outward K^+ current, which results in a secondary increase of inward Ca^{2+} current. Naloxone-induced potentiation of inotropic effects in states of shock may not only be related to reversal of the effects of endorphins but also to its direct inotropic action via a non-opiate receptor-mediated mechanism.

VI. .REFERENCES

1. Hughes K. Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. Brain Res 1975;88:295-308.

2. Holaday JW, Faden AI. Naloxone reversal of endotoxin hypotension suggests role of endorphins in shock. Nature 1978;275:450-1.

3. Faden AI, Holaday JW. Naloxone treatment of endotoxin shock: stereospecificity of physiologic and pharmacologic effects in the rat. J Pharmacol Exp Ther 1980;212:441-7.

4. Faden AI, Holaday JW. Opiate antagonists: a role in the treatment of hypovolemic shock. Science 1979; 205:317-8.

5. Gurll NJ, Vargish T, Reynolds DG, Lechner RB. Opiate receptors and endorphins in the pathophysiology of hemorrhagic shock. Surgery 1981;89:364-9.

6. Holaday JW, Faden AI. Naloxone acts at central opiate receptors to reverse hypotension, hypothermia and hypoventilation in spinal shock. Brain Res 1980;189:295-300.

7. Faden AI, Jacobs TP, Mougey E, Holaday JW. Endorphins in experimental spinal injury: therapeutic effect of naloxone. Ann Neurol 1981;10:326-32.

8. Boeuf B, Gauvin F, Guerguerian AM, Farrell CA, Lacroix J, Jenicek M. Therapy of shock with naloxone: a meta-analysis. Crit Care Med 1998;26:1910-6.

9. Lechner RB, Gurll NJ, Reynolds DG. Naloxone potentiates the cardiovascular effects of catecholamines in canine hemorrhagic shock. Circ Shock 1985; 16:347-61.

10. Allgood SC, Gurll NJ, Reynolds DG. Naloxone requires circulating catecholamines to attenuate the cardiovascular suppression of endotoxic shock. J Surg Res 1988; 44:73-81.

11. Lechner RB. Naloxone potentiates the inotropic effects of isoproterenol in vitro by a nonopiate receptor mechanism. Circ Shock 1992;38:157-64.
12. Caffrey JL, Hathorne LF, Carter GC, Sinclair RJ. Naloxone potentiates contractile responses to epinephrine in isolated canine arteries. Circ Shock 1990;31:317-32.
13. Gu H, Barron BA, Gaugl JF, Caffery JL. (+)Naloxone potentiates the inotropic effect of epinephrine in the isolated dog heart. Circ Shock 1993;40:206-11.
14. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265-75.
15. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 1981;391:85-100.
16. Guillemin R, Vargo T, Rossier J, Minick S, Ling N, Rivier C, Vale W, Bloom F. beta-Endorphin and adrenocorticotropin are secreted concomitantly by the pituitary gland. Science 1977;197:1367-9.
17. Mantelli L, Corti V, Ledda F. On the presence of opioid receptors in guinea-pig ventricular tissue. Gen Pharmacol 1987;18:309-13.
18. Krumins SA, Faden AI, Feuerstein G. Opiate binding in rat hearts: modulation of binding after hemorrhagic shock. Biochem Biophys Res Commun 1985;127:120-8.
19. Lechner RB, Gurll NJ, Reynolds DG. Intracoronary naloxone in hemorrhagic shock: dose-dependent stereospecific effects. Am J Physiol 1985;249:H272-7.
20. Kim KJ, Chung NS, Park WK. Direct myocardial depressant effect of naloxone: mechanical and electrophysiological actions in vitro. Acta Anaesthesiol Scand 2004;48:102-10.
21. Levin G, Gafni M, Roz N, Sarne Y. The involvement of sodium ions in the positive inotropic effect of naloxone. Gen Pharmacol 1993; 24:423-8.

22. Khananshvili D, Sarne Y. The effect of opiate agonists and antagonists on Na^{+}-Ca^{2+} exchange in cardiac sarcolemma vesicles. Life Sci 1992; 51:275-83.

23. Katano Y, Endoh M. Differential effects of Ro 20-1724 and isobutylmethylxanthine on the basal force of contraction and beta-adrenoceptor-mediated response in the rat ventricular myocardium. Biochem Biophys Res Commun 1990;167:123-9.

24. Katano Y, Endoh M. Effects of a cardiotonic quinolinone derivative Y-20487 on the isoproterenol-induced positive inotropic action and cyclic AMP accumulation in rat ventricular myocardium: comparison with rolipram, Ro 20-1724, milrinone and isobutylmethylxanthine. J Cardiovasc Pharmacol 1992;20:715-22.

25. Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 1995;75:725-48.

26. Cherki-Vakil R, Meiri H. Postsynaptic effects of DMSO at the frog neuromuscular junction. Brain Res 1991;566:329-32.

27. Schreiber J, Slapke J. Influence of dimethyl sulfoxide and ethanol on contractions of isolated guinea pig tracheas induced by electrical field stimulation. Pharmazie 1991;46:147-8.

28. Sams WM Jr, Carroll NV, Crantz PL. Effects of dimethysulphoxide on isolated-innervated skeletal, smooth and cardiac muscle. Proc Soc Exp Biol Med 1966;122:103-7.

29. Brasch H. Influence of the optical isomers (+)- and (-)-naloxone on beating frequency, contractile force and action potentials of guinea-pig isolated cardiac preparations. Br J Pharmacol 1986;88:733-40.

30. Oldroyd KG, Hicks MN, Cobbe SM. Influence of hyperkalaemia and ischaemia on non-receptor-medicated cardiac electrophysiological effects of naloxone. Cardiovasc Res 1993;27:296-303.

31. Hung CF, Wu MH, Tsai CH, Chu SH, Chi JF, Su MJ.

Electrophysiological mechanisms for the antiarrhythmic activities of naloxone on cardiac tissues. Life Sci 1998;63:1205-19.

32. Carratu' MR, Mitolo-Chieppa D. Inhibition of ionic currents in frog node of Ranvier treated with naloxone. Br J Pharmacol 1982;77:115-9.

33. Frazier DT, Ohta M, Narahashi T. Nature of the morphine receptor present in the squid axon. Proc Soc Exp Biol Med 1973;142:1209-14.

34. Sanquinetti MC, Jurkiewicz NK. Two components of cardiac delayed rectifier K^+ current. Differential sensitivity to block by class III antiarrhythmic agents. J Gen Physiol 1990;96:195-215.

35. Nichols CG, Makhina EN, Pearson WL, Sha Q, Lopatin AN. Inward rectification and implications for cardiac excitability. Circ Res 1996;78:1-7.

36. The Sicilian Gambit. A new approach to the classification of antiarrhythmic drugs based on their actions on arrhythmogenic mechanisms. Task force of the working group on arrythmias of the European Society of Cardiology. Circulation 1991;84:1831-51.

37. Kass RS, Freeman LC. Potassium channels in the heart: cellular, molecular, and clinical implications. Trends Cardiovasc Med 1993:3:149-59.

38. Koumi S, Wasserstrom JA, Ten Eick RE. Beta-adrenergic and cholinergic modulation of inwardly rectifying K^+ current in guinea-pig ventricular myocytes. J Physiol 1995;486:647-59.

39. Sagy M, Shavit G, Oron Y, Vidne BA, Gitter S, Sarne Y. Nonopiate effect of naloxone on cardiac muscle contractility. J Cardiovasc Pharmacol 1987;9:682-685.

< ABSTRACT(IN KOREAN)>

β-수용체 촉진에 따른 심근 수축력 증가에 대한 naloxone 의 강화 작용: non-opiate 수용체 기전 및 cAMP 의 역활

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장철호

본 연구는 심근의 수축력을 항진시키는 효과를 가진 naloxone의 비아편성수용체 전달반응을 규명하고자 하였다. 심근수축력 측정, cyclic adenosine monophosphate (cAMP)의 측정, 전기생리학적인 방법들이 사용되었다.

수컷 기니픽 (300-400 g)을 sevoflurane으로 전신마취 한 상태에서 우심실 유두근(papillary muscle)을 적출하였다. 최대 장력 및 최대 장력 발생 속도(maximum rate of force development, dF/dt-max)로 심근수축력을 평가하였다. Isoproterenol, forskolin, isobutylmethylxanthine (IBMX)의 용량을 단계적으로 증가시키며 용량-반응 곡선을 얻었다. 각각의 isoproterenol, forskolin, IBMX를 EC_{50} 용량으로 전처치 후 naloxone의 농도를 증가시키면서 수축력이 항진되는 것을 확인하였다. cAMP의 측정을 위해 기니픽의 심근을 분쇄하여 균질화한 뒤 원심분리하여 세포막단백질을 분리하였다. EC_{50}의 isoproterenol 또는 forskolin 과 함께 naloxone을 투여하고 naloxone의 농도에 따른 cAMP 농도를 cAMP assay kit을 이용하여 측정하였다. 전기생리학적인 연구에서는 action potential, delayed outward K^+ current (I_k), inward rectifier K^+ current (I_{k1})

와 L-type Ca^{2+} current ($I_{Ca,L}$)을 측정하였다.

Naloxone (30 μM)은 isoproterenol의 용량-반응 곡선을 좌측으로 이동시켰다. 또한 forskolin의 용량-반응 곡선을 3 μM 까지는 좌측으로 이동시키고 10에서30 μM의 농도에서는 현저하게 증가된 수축력을 보였다. 하지만, IBMX의 용량-반응 곡선에는 영향을 주지 않았다. Isoproterenol의 전처치 후에 10, 30, 300 μM naloxone의 점진적인 투여는 용량에 따른 심근수축력의 증가를 보였다. EC_{50} 용량의 isoproterenol 또는 forskolin과 naloxone을 같이 투여해도 cAMP의 농도에는 영향이 없었다. Isoproterenol 전처치 후 naloxone 30 μM은 APD_{50}과 APD_{90} 을 연장시켰으나 크기나 안정막전위에는 영향이 없었다. Naloxone은 isoproterenol 전처치군에서 peak outward I_k를 +80 mV에서 20±3% 감소시켰으나, -140mV에서 0 mV까지 I_{k1}에는 영향이 없었다. 또한, Naloxone은 막전위 +10 mV에서 $I_{Ca,L}$을 28±3% 감소시켰다. 따라서, isoproterenol 전처치후 naloxone이 심근 수축을 증가시키는 한 원인으로서 I_k의 억제에 의한 활동전위의 연장으로 세포내로의 Ca^{2+} 내향 전류가 증가하여 심근 수축이 증가하는 것으로 생각된다.

핵심되는 말: Naloxone, 비아편성수용체 전달 반응, 심근수축력, cAMP, delayed outward K^+ current.