The effect of adrenomedullin on the L-type calcium current in myocytes from septic shock rats: signaling pathway

Xiao-Hui Zhang,1,2 Gui-Rong Li,1 and Jean-Pierre Bourreau1

1Department of Physiology and Institute of Cardiovascular Sciences and Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region of China, 2Department of Pharmacy, Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China

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Zhang X-H, Li G-R, Bourreau J-P. The effect of adrenomedullin on the L-type calcium current in myocytes from septic shock rats: signaling pathway. Am J Physiol Heart Circ Physiol 293: H2888–H2893, 2007—Adrenomedullin (ADM) is upregulated in cardiac tissue under various pathophysiological conditions, particularly in septic shock. The intracellular mechanisms involved in the effect of ADM on adult rat ventricular myocytes are still to be elucidated. Ventricular myocytes were isolated from adult rats 4 h after an intraperitoneal injection of lipopolysaccharide (LPS, 10 mg/kg). Membrane potential and L-type calcium current (I\textsubscript{Ca,L}) were isolated from adult rats 4 h after an intraperitoneal injection of lipopolysaccharide (LPS, 10 mg/kg). Membrane potential and L-type calcium current (I\textsubscript{Ca,L}) were determined using whole cell patch-clamp methods. ADM in LPS group was significantly shorter than control values (time to 50% repolarization: LPS, 169 ± 2 ms; control, 257 ± 2 ms, P < 0.05; time to 90% repolarization: LPS, 220 ± 2 ms; control, 305 ± 2 ms, P < 0.05). I\textsubscript{Ca,L} density was significantly reduced in myocytes from the LPS group (−3.2 ± 0.8 pA/pF) compared with that of control myocytes (−6.7 ± 0.3 pA/pF, P < 0.05). The ADM antagonist ADM-(22-52) reversed the shortened APD and abolished the reduction of I\textsubscript{Ca,L} in shock myocytes. In myocytes from control rats, incubating with ADM for 1 h induced a marked decrease in peak I\textsubscript{Ca,L} density. This effect was reversed by ADM-(22-52). The G\textsubscript{i} protein inhibitor, pertussis toxin (PTX), the protein kinase A (PKA) inhibitor, KT-5720, and the specific cyclooxygenase 2 (COX-2) inhibitor, nimesulide, reversed the LPS-induced reduction in peak I\textsubscript{Ca,L}. The results suggest a COX-2-involved PKA-dependent switch from G\textsubscript{i} coupled to PTX-sensitive G\textsubscript{i} coupling by ADM in adult rat ventricular myocytes. The present study delineates the intracellular pathways involved in ADM-mediated effects on I\textsubscript{Ca,L} in adult rat ventricular myocytes and also suggests a role of ADM in sepsis.

We have previously observed a dual inotropic effect in response to ADM in adult rat ventricular myocytes (25, 26). ADM was found to produce an initial increase in cell shortening and Ca\textsuperscript{2+} transients with a short (<30 min) incubation and a marked decrease in cell shortening and Ca\textsuperscript{2+} transients with a prolonged (>1 h) incubation. In addition to these, ADM was simultaneously demonstrated to decrease intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}) in rabbit ventricular myocytes (14). The inhibitory effects of ADM were also observed in guinea pig ventricular myocytes, and these effects were mediated by a specific ADM receptor (6). Furthermore, ventricular myocytes isolated from guinea pigs (33) or rabbits (12) after in vivo treatment with endotoxin exhibited a reduced cell shortening as well as decreased rates of shortening and relengthening. The decreased cell shortening has been correlated with decreased action potential duration (APD) in endotoxemic rabbit myocytes (12) and reduced free [Ca\textsuperscript{2+}] in endotoxemic guinea pig myocytes (33).

These data suggest that Ca\textsuperscript{2+} influx, specifically Ca\textsuperscript{2+} current through L-type calcium channels of the sarcolemma, may be regulated by ADM during the septic shock. The present study was designed to examine the intracellular pathways involved in the effects of ADM on I\textsubscript{Ca,L} using a rat model of endotoxin challenge.

MATERIAL AND METHODS

The animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Male Sprague-Dawley rats, aged 10–15 wk and weighing 250–300 g, were used in this study.

Animal models of septic shock. Endotoxemia was induced by an injection of LPS (10 mg/kg ip), as previously described (25). Observations of conscious rats after LPS treatment were characterized of endotoxemia, i.e., piloerection, apathy, and diarrhea. The rats (10%) that did not exhibit these characteristics were considered LPS-resistant and, consequently, were not used for this study. The sham control rats were injected with saline (1 ml/kg ip).

Cell isolation procedure. Single ventricular myocytes were enzymatically dissociated using a procedure described previously (7). Briefly, male Sprague-Dawley rats (250–300 g) were euthanized by cervical dislocation, and the heart was quickly excised and mounted on a Langendorff apparatus. The heart was retrogradely perfused for 5 min at 37°C with oxygenated Tyrode solution, followed by a nominally Ca\textsuperscript{2+}-free Tyrode solution for 5–10 min and a 20–30-min perfusion with the nominally Ca\textsuperscript{2+}-free solution containing 1.75 mg/ml collagenase (type II, Worthington), 1 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 0.28 mg/ml protease.
(type XXIV, Sigma-Aldrich). Ventricles were then removed from the softened heart and gassed with 5% CO2.

The cells were suspended in a high K+ solution containing (in mM) 10 KCl, 120 K-glutamate, 10 KH2PO4, 1.8 MgSO4, 10 taurine, 10 HEPES, 0.5 EGTA, 20 glucose, and 10 manitol, with pH adjusted to 7.3 with KOH. The isolated myocytes were kept at room temperature in the medium at least 1 h before the experimental study.

Whole cell patch-clamp techniques. A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (1 ml) mounted on the stage of an inverted microscope. Myocytes were allowed to adhere to the bottom of the chamber for 5–10 min and then were perfused at 2 to 3 ml/min with Tyrode solution. Only those myocytes touching the cell. Current and voltage signals were low-pass filtered at 5 kHz and stored in the hard disk of an IBM compatible computer. All experiments were conducted at room temperature (22–24°C). Myocytes were perfused with either Tyrode solution (current clamp) or Na+-free Tyrode solution [N-methyl-d-glucamine (NMDG) replacement, voltage clamp]. Tyrode solution contained (in mM) 136 NaCl, 5.4 KCl, 1.0 MgCl2, 2.0 CaCl2, 0.33 NaH2PO4, 10.0 glucose, and 10 HEPES; pH was adjusted to 7.3 with NaOH. A Na+-free NMDG solution, containing (in mM) 136 NMDG, 5.4 CsCl, 1.0 MgCl2, 2.0 CaCl2, 2.0 NaH2PO4, 10 glucose, and 10 HEPES, was used when ICa,L was recorded; pH was adjusted to 7.3 with NMDG.

The pipette solution for Ca2+ current recording (whole cell voltage-clamp mode) contained (in mM) 20 CsCl, 110 cesium aspartate, 1.0 MgCl2, 10 HEPES, 10 EGTA, 0.1 GTP, and 5 Mg2+ATP; pH was adjusted to 7.2 with CsOH. The pipette solution for action potential recording (current-clamp mode) contained (in mM) 10 NaCl, 120 KCl, 2 MgCl2, 5 Na2ATP, 10 EGTA, 10 HEPES, and 0.1 GTP; pH was adjusted to 7.2 with KOH.

Reagents. Human ADM-(1-52) and human ADM-(22-52) were purchased from Peninsula Laboratories (San Carlos, CA). KT-5720, pertussis toxin (PTX), nimesulide, BSA, and all other chemicals were from Sigma-Aldrich. ADM-(1-52), ADM-(22-52), KT-5720, and PTX were dissolved in buffer. Nimesulide was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO (0.05%) did not affect ICa,L.

Data analysis. Membrane potential and ICa,L data were collected from two to four myocytes from each animal, and four to five animals were represented in each protocol. Data were expressed as means ± SE, and n refers to the number of myocytes. Nonlinear curve fitting was performed using Pulsefit (HEKA) and Sigmaplot (SPSS). Paired and/or unpaired Student’s t-test was used as appropriate to evaluate the statistical significance of the differences between two group means, and ANOVA was used for multiple groups. P < 0.05 was considered significantly different.

| Table 1. Action potential parameters in rat ventricular myocytes |
|-----------------|---------|-----------------|-----------------|
| Group           | n      | Erest, mV       | Amplitude, mV   | APD50, ms  | APD90, ms  |
| Control         | 18     | −60.5±0.5       | 95.6±0.8        | 257.5±2.2  | 304.9±2.3  |
| LPS             | 14     | −60.4±0.3       | 95.4±0.6        | 168.9±2.2  | 219.9±2.1  |
| ADM-(22-52)     | 13     | −60.5±0.5       | 98.1±0.8        | 233.1±2.2  | 285.5±2.0  |

Values are means ± SE; n = number of myocytes studied. Action potential parameters in ventricular myocytes from control, LPS, and LPS plus adrenomedullin (ADM)-(22-52) groups. Erest, resting membrane potential; APD, action potential duration; APD50 and APD90, time to 50% and 90% repolarization, respectively. *Significantly different from control (P < 0.05).

RESULTS

Effects of LPS treatment and ADM-(22-52) on action potential. LPS treatment induced a significant reduction of APD in rat ventricular myocytes. Characteristics of action potentials for LPS and control groups were summarized in Table 1. No difference was observed in resting membrane potential and action potential amplitude in control and LPS myocytes. However, APD of LPS myocytes was significantly shorter than that of control myocytes. Time to 50% repolarization (APD50) and 90% repolarization (APD90) were decreased by 34% and 28%, respectively, in LPS myocytes (Table 1). Incubating (30 min) the shock cells with 100 nM ADM-(22-52), a specific ADM receptor antagonist, significantly reversed the reduced APD (P < 0.05) (Table 1).

Effect of LPS treatment on ICa,L. It is well known that the APD is determined in part by transmembrane Ca2+ influx through L-type Ca2+ channels. To determine whether the shortened APD observed above in LPS myocytes was related...
nM ADM-(22-52) for 30 min \([-6.4 \pm 0.3 \text{ pA/pF}, n = 8\) from 4 hearts, \(P = \text{not significant (NS) vs. control cells without treatment}\) (Fig. 1).

**Effect of ADM-(1-52) on \(I_{\text{Ca,L}}\) in control myocytes.** To determine whether ADM might mimic the effect induced by LPS, we incubated control cells with 100 nM ADM-(1-52) for 1 h. Interestingly, ADM-(1-52) incubation induced a marked decrease in \(I_{\text{Ca,L}}\). \(I_{\text{Ca,L}}\) was decreased from \(-5.8 \pm 0.3 \text{ pA/pF}\) of control \((n = 8\) from 4 hearts) to \(-2.9 \pm 0.2 \text{ pA/pF}\) in cells with ADM treatment \((n = 10\) from 4 hearts, \(P < 0.05\)). This response was completely abolished by ADM-(22-52), a specific receptor antagonist of ADM (Fig. 2).

**Effect of PTX on \(I_{\text{Ca,L}}\) in shock cells.** To investigate the possible involvement of \(G_i\) in ADM signaling pathway, the shock cells were pretreated with 2 \(\mu\text{g/ml PTX}, an inhibitor of \(G_i\), for 4 h. PTX treatment resulted in a significant reverse in the reduced \(I_{\text{Ca,L}}\) (Fig. 3). \(I_{\text{Ca,L}}\) density was higher in LPS plus PTX myocytes \((-4.7 \pm 0.1 \text{ pA/pF}, n = 11\) from 4 hearts) than that in LPS myocytes \((-3.2 \pm 0.8 \text{ pA/pF}, P < 0.05\), although it was still different from the control value. Pretreatment with PTX had no effect on \(I_{\text{Ca,L}}\) in control cells \((-6.1 \pm 0.3 \text{ pA/pF}, n = 8\) from 4 hearts, \(P = \text{NS vs. control cells without treatment}\).

**Effect of KT-5720 on \(I_{\text{Ca,L}}\) in shock cells.** To further explore whether PKA is involved in the ADM signaling pathway, we incubated the shock cells with 10 \(\mu\text{M KT-5720}, an inhibitor of PKA, for 30 min. The treatment partially abolished the decrease in \(I_{\text{Ca,L}}\) (Fig. 4). \(I_{\text{Ca,L}}\) density was higher in LPS plus KT-5720 myocytes \((-5.5 \pm 0.2 \text{ pA/pF}, n = 14\) from 4 hearts).
than that in LPS myocytes (-3.2 ± 0.8 pA/pF, P < 0.05), although the current was still smaller than that of control cells. Pretreatment with KT-5720 had no effect on $I_{\text{Ca,L}}$ in control cells (-6.5 ± 0.5 pA/pF, n = 9 from 4 hearts, P = NS vs. control cells).

Effect of COX-2 on $I_{\text{Ca,L}}$ in shock cells. The shock cells were pretreated with the specific cyclooxygenase 2 (COX-2) inhibitor nimesulide (10 μM for 30 min) to study the possible involvement of COX-2 in the ADM signaling pathway. Preincubation of nimesulide partially reversed the decrease of $I_{\text{Ca,L}}$ (Fig. 5). $I_{\text{Ca,L}}$ density was higher in LPS plus nimesulide myocytes (-4.4 ± 0.1 pA/pF, n = 15 from 4 hearts) than that in LPS myocytes (-3.2 ± 0.8 pA/pF, P < 0.05), although it was still smaller than that of control cells. Pretreatment with nimesulide had no effect on $I_{\text{Ca,L}}$ in control cells(-6.2 ± 0.2 pA/pF, n = 8 from 4 hearts, P = NS vs. cells without pretreatment).

DISCUSSION

Endotoxemia consistently produces a deleterious depression in cardiodynamic function. The heart dilates and the ejection fractions of both ventricles decrease with global and regional, as well as systolic and diastolic, dysfunction (18, 19, 23). Our previous work indicated that endotoxin-induced loss of inotropic power can be correlated with decreased amplitude of the Ca$^{2+}$ transient and cell shortening (25). In the present study, we found that ventricular APD shortened and Ca$^{2+}$ currents through L-type calcium channels decreased in myocytes isolated from a rat model of endotoxemia.

It is well-known that both Ca$^{2+}$ and K$^{+}$ currents are the major ionic currents for maintaining cardiac APD (21, 22, 31). Our experiment study did not find changes in the major outward K$^{+}$ currents, transient and steady-state, in LPS myocytes (data not shown). Therefore, the reduced $I_{\text{Ca,L}}$ is most likely responsible for the shortened APD.

The reduction of $I_{\text{Ca,L}}$ could be associated with 1) decreased numbers of L-type Ca$^{2+}$ channels, 2) altered voltage-dependent inactivation, and 3) increased rate of channel decay (2). However, endotoxemia did not affect the voltage-dependent properties of cardiac L-type Ca$^{2+}$ channels in our study. The I-V relationships of $I_{\text{Ca,L}}$ were not altered in LPS myocytes, and no difference in voltage-dependence of steady-state activation and inactivation and recovery of $I_{\text{Ca,L}}$ from inactivation were observed in control and LPS myocytes (data not shown). In addition, Zhong et al. (33) reported that isoproterenol reversed the reduction of $I_{\text{Ca,L}}$ by endotoxin in guinea pig ventricular myocytes and suggested that the number of calcium channels was likely not altered in LPS myocytes (33).

Therefore, other mediators would be likely responsible for the reduced $I_{\text{Ca,L}}$ in the septic shock myocytes. In the present study, we found that the specific ADM receptor antagonist ADM-(22-52) could reverse the shortened APD and abolish the decrease of $I_{\text{Ca,L}}$ in septic shock myocytes and ADM-(1-52)-induced $I_{\text{Ca,L}}$ reduction. It has been demonstrated that
ADM receptor is a member of the calcitonin gene-related peptide (CGRP) receptor family. Many biological actions of ADM are mediated by specific ADM receptors or CGRP type 1 (CGRP(1)) receptors (30). However, the ADM effects did not result from the activation of CGRP receptors because CGRP inhibitor CGRP-(8–37) was unable to prevent ADM-(1-52)-induced changes in Ca\(^{2+}\) response and cell contractility, whereas these effects were prevented by the ADM antagonist ADM-(22-52) in our earlier study (13), suggesting that ADM effect is mediated by ADM specific receptor in rat shock myocytes.

We found that the decreased IC\(_{50,L}\) was remarkably reversed by the pretreatment with the PKA inhibitor KT-5720 in rat shock cells, suggesting PKA involvement. In addition, the reduced IC\(_{50,L}\) was partially reversed by the Gi inhibitor PTX in LPS cells, implicating activation of Gi\(_{i}\) pathway. Our previous studies showed that Ca\(^{2+}\) transient reduction induced by ADM-(1-52) was potiated by the adenylyl cyclase (AC) inhibitor SQ-22536 (25), whereas not by the guanylate cyclase inhibitor ODQ (13), suggesting that ADM effects are mediated by the AC/cAMP-dependent pathway but not by cGMP-dependent pathway. However, Du et al. (6) found the ADM-(1-52) effect on IC\(_{50,L}\) was not antagonized by PKA inhibitors in guinea pig ventricular myocytes. The different results may be related to species variation. Furthermore, ADM likely activates one more than one signal pathway. There are several reports suggesting that one receptor is likely coupled to two independent signal mechanisms. The best example would be the prostacyclin receptor (27) and the endothelin receptor (28).

Recently, progressive liberation of prostacyclin and thromboxane has been reported to be induced by LPS, together with myocardial COX-2 mRNA expression in endotoxic rat heart (9). In our present study, we found that the specific COX-2 inhibitor nimesulide significantly reversed the decreased IC\(_{50,L}\) in shock myocytes. Adrenomedullin modulating COX-2 expression was observed in reserpine-injured rat gastric mucosa (3). These observations suggest that ADM effects are likely mediated by activation of COX-2.

We have previously reported that COX-2-induced production of prostacyclin is related to the ADM effects because the prostacyclin synthase inhibitor tranlycypromine abolished the ADM effects in rat shock cardiomyocytes (26). It is well known that prostacyclin exerts its effects by an increase in cAMP levels through an interaction with the inositol phosphate (IP) receptors coupled to the AC system via the Gs protein (1, 10). In the present study, the reduced IC\(_{50,L}\) was significantly reversed by the incubation of LPS cells with the Gi\(_{i}\) inhibitor PTX and the PKA inhibitor KT-5720, which suggests a possible cross talk between COX-2, PKA, and Gi signals.

It is suggested that prostacyclin receptor or ADM receptor is coupled to Gi through initial Gi signaling and cAMP/PKA pathway, leading to an inhibition of AC and cAMP, a switch from G\(_{i}\)-coupled to PTX-sensitive, PKA-dependent Gi coupling in the LPS rat ventricular myocytes (26). Such dual coupling has been reported in \(\beta_1\)-adrenergic receptor (\(\beta_1\)-AR), \(\beta_2\)-AR, and prostacyclin to G\(_{i}\) and G\(_{i}\) pathways (5, 20, 24). Evidence for a B\(_{2}\)-AR/G\(_{i}\) link has been observed in normal rat cardiac myocytes (4), human atria (16) and ventricles (8), and failing rat heart (32). Our results support the notion that the coupling of ADM to G\(_{i}\) switches over to Gi in a regulated manner as ADM receptor to be phosphorylated by PKA before the PTX-sensitive Gi coupling.

In conclusion, a COX-2-involved switch from G\(_{i}\) coupling to PKA-dependent Gi is observed with ADM on IC\(_{50,L}\) in rat ventricular myocytes, which is likely responsible for the cardiac dysfunction during septic shock.

REFERENCES


