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Effects of AMP579 and adenosine on L-type Ca^{2+} current in isolated rat ventricular myocytes

Xiong WANG[1], Bo-wei WU, Dong-mei WU

Department of Physiology, Shanxi Medical University, Taiyuan 030001, China

Key words

AMP579; adenosine; heart ventricles; cardiac myocytes; L-type calcium channels; patch-clamp techniques

[1] Correspondence to Prof Xiong WANG.
Phn 86-351-469-0162.
E-mail Wxiong@public.ty.sx.cn

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Abstract

Aim: To compare the effects of AMP579 and adenosine on L-type Ca^{2+} current ($I_{Ca\text{-}L}$) in rat ventricular myocytes and explore the mechanism by which AMP579 acts on $I_{Ca\text{-}L}$. **Methods:** $I_{Ca\text{-}L}$ was recorded by patch-clamp technique in whole-cell configuration. **Results:** Adenosine (10 nmol/L to 50 μmol/L) showed no effect on basal $I_{Ca\text{-}L}$, but it inhibited the $I_{Ca\text{-}L}$ induced by isoproterenol 10 nmol/L in a concentration-dependent manner with the IC_{50} of 13.06 μmol/L. Similar to adenosine, AMP579 also showed an inhibitory effect on the $I_{Ca\text{-}L}$ induced by isoproterenol. AMP579 and adenosine (both in 10 μmol/L) suppressed isoproterenol-induced $I_{Ca\text{-}L}$ by 11.1% and 5.2%, respectively. In addition, AMP579 had a direct inhibitory effect on basal $I_{Ca\text{-}L}$ in a concentration-dependent manner with IC_{50} (1.17 μmol/L). PD116948 (30 μmol/L), an adenosine A_1 receptor blocker, showed no action on the inhibitory effect of AMP579 on basal $I_{Ca\text{-}L}$. However, GF109203X (0.4 μmol/L), a special protein kinase C (PKC) blocker, could abolish the inhibitory effect of AMP579 on basal $I_{Ca\text{-}L}$. So the inhibitory effect of AMP579 on basal $I_{Ca\text{-}L}$ was induced through activating PKC, but not linked to adenosine A_1 receptor. **Conclusion:** AMP579 shows a stronger inhibitory effect than adenosine on the $I_{Ca\text{-}L}$ induced by isoproterenol. AMP579 also has a strong inhibitory effect on basal $I_{Ca\text{-}L}$ in rat ventricular myocytes. Activation of PKC is involved in the inhibitory effect of AMP579 on basal $I_{Ca\text{-}L}$ at downstream-mechanism.

Introduction

Recent studies showed that AMP579 was a novel adenosine agonist with high affinity for adenosine A_1 and A_2 receptors[1,2]. Experiments in animal models have demonstrated that AMP579 reduced infarct size by 50% to 98% when administered before a final ischemic event (mediation of ischemic preconditioning) or just before reperfusion (attenuation of reperfusion injury)[3,4]. Further experiments on pigs, dogs, and rabbits suggested that AMP579 was more powerful than adenosine in attenuating polymorphonuclear neutrophil-mediated inflammatory responses, dilating the coronary artery, reducing myocardial contracture and limiting infarct size[5,6]. Although the protective effect of AMP579 required adenosine receptor activation, adenosine could not duplicate the effects.

The difference between pharmacologic effect of AMP579 and adenosine might reflect the differences in ionic mechanisms. It has been established that adenosine could cause an attenuation of basal $I_{Ca\text{-}L}$ only in unstimulated atrial myocytes, but under conditions of isoproterenol stimulation, adenosine could markedly attenuate isoproterenol induced-$I_{Ca\text{-}L}$ in both atrial and ventricular myocytes. However, little is known about the electrophysiological effects of AMP579 so far. This study will examine the effects of AMP579 and adenosine on L-type calcium channel and elucidate the mechanisms underlying the cardioprotective effect of AMP579 and its utility in treatment of myocardial ischemia-reperfusion injury.

Materials and methods

Rat myocardial cell isolation Ventricular myocytes were obtained from Wistar male rats (250–300 g) by enzymatic

isolation procedure. In brief, rats were killed by cervical dislocation and the heart was then immediately removed, cannulated through the aorta and perfused through the coronary artery with Ca^{2+}-free Tyrode's solution for 10 min. The composition of Ca^{2+}-free Tyrode's solution was: NaCl 140.0 mmol/L, KCl 5.4 mmol/L, $MgCl_2$ 1.0 mmol/L, NaH_2PO_4 0.3 mmol/L, glucose 10.0 mmol/L, HEPES 5.0 mmol/L; pH adjusted to 7.4 with NaOH at room temperature. The heart was then perfused with enzymatic solution, which was low Ca^{2+} ($CaCl_2$ 150 μmol/L) Tyrode's solution with collagenase P (0.3g/L) for about 8−10min. The left ventricle was then removed. The cells were isolated by gentle agitation and kept in Krebs buffer (KB) solution, which contained: KOH 85.0 mmol/L, *L*-glutamic acid 50.0 mmol/L, KCl 30.0 mmol/L, taurine 20.0 mmol/L, KH_2PO_4 30.0 mmol/L, $MgCl_2$ 1.0 mmol/L, HEPES 10.0 mmol/L, glucose 10.0 mmol/L and egtazic acid 0.5 mmol/L; pH adjusted to 7.4 by KOH.

Electrophysiological measurement Whole-cell patch-clamp was used to record $I_{Ca\text{-}L}$ (L-type Ca^{2+} currents) and membrane capacitance was measured with a P-clamp 5.51 software package (Axon Instruments, USA). Patch electrodes were made from thin-walled glass capillaries (1.5 mm outside diameter) using a two-stage vertical microelectrode puller (model PP-83, Narishige Scientific Instruments, Japan). The electrode resistance ranges 3 MΩ, when filled with pipette solution.

For the measurement of $I_{Ca\text{-}L}$, the extracellular solution contained: NaCl 140.0 mmol/L, $CaCl_2$ 1.8 mmol/L, $MgCl_2$ 1.0 mmol/L, KCl 5.4 mmol/L, glucose 10.0 mmol/L, NaH_2PO_4 0.3 mmol/L, and HEPES 10.0 mmol/L; pH adjusted to 7.4 with NaOH. The pipette solution contained: egtazic acid 10.0 mmol/L, KCl 140.0 mmol/L, Na_2ATP 2.0 mmol/L, HEPES 5.0 mmol/L, 4-AP 5.0 mmol/L, $MgCl_2$ 1.0 mmol/L; pH adjusted to 7.4 with KOH. The calcium current was expressed as membrane current density (pA/pF). The cell capacitance was measured by the method previously described by Coetzee *et al*[9]. $I_{Ca\text{-}L}$ was measured according to the method described by Hartzell *et al*[10]. The AMP579 was a gift from Department of Cardiothoracic Surgery Research Laboratory, Emory University School of Medicine, USA. AMP579 was dissolved in small volumes of Me_2SO, then diluted to the desired final concentration before each experiment.

Statistic analysis Data were expressed as mean±SD. Statistical significance was determined by Student's *t*-test and $P<0.05$ was considered significant.

Results

Detection of L-type calcium channel current The calcium current was activated by depolarizing pulse from a holding potential of -40 mV to +10 mV at 50 mV step-voltage. This inward current could be completely inhibited by 1 μmol/L verapmil, the basic characteristics indicated that the current present in rat ventricular myocytes was L-type Ca^{2+} current .

Effect of AMP579 and adenosine on L-type calcium current In the presence of adenosine at 10 nmol/L, 1, 10, and 50 μmol/L, $I_{Ca\text{-}L}$ varied from 4.9±0.9 to 4.8±0.9, 4.9±0.9, 4.9±0.9, 4.7±0.9 pA/pF, respectively (n=5, $P>0.05$). Adenosine had no effect on basal $I_{Ca\text{-}L}$. However, when $I_{Ca\text{-}L}$ was augmented to 2.7±0.6 pA/pF by 10 nmol/L isoproterenol, adenosine at 10 nmol/L, 1, 10, and 50 μmol/L significantly reduced it to 2.4±0.6, 2.1±0.6, 2.0±0.5, and 1.9±0.5 pA/pF, respectively (n=4, $P<0.05$). Adenosine showed an inhibitory effect on isoproterenol-induced $I_{Ca\text{-}L}$ in a concentration-dependent manner with the IC_{50} of 13.06 μmol/L (Figure 1, 2).

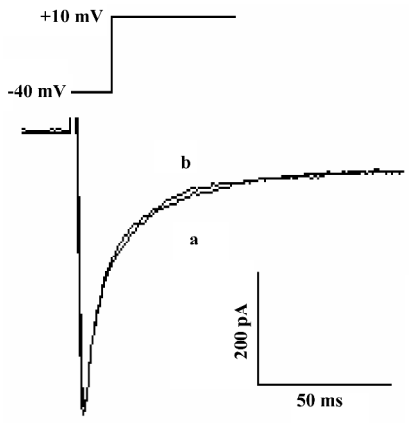

Figure 1. Effect of adenosine on $I_{Ca\text{-}L}$ in isolated rat ventricular myocytes. (a) Control; (b) 50 μmol/L adenosine.

Effect of AMP579 on $I_{Ca\text{-}L}$ Isoproterenol 10 nmol/L augmented $I_{Ca\text{-}L}$ to 3.8±0.7 pA/pF. AMP579 10 μmol/L reduced $I_{Ca\text{-}L}$ to 2.4±0.1 pA/pF ($P<0.05$, n=3, Figure 3), AMP579 also showed an inhibitory effect on isoproterenol-induced $I_{Ca\text{-}L}$. AMP579 and adenosine (both 10 μmol/L) suppressed isoproterenol-induced $I_{Ca\text{-}L}$ by 11.1% and 5.2%, respectively. AMP579 had a stronger inhibitory effect. In contrast to adenosine, AMP579 possessed a direct inhibitory effect on basal $I_{Ca\text{-}L}$ in a concentration-dependent manner with the IC_{50} of 1.17 μmol/L (Table 1, Figure 4).

AMP579 10 μmol/L markedly reduced basal $I_{Ca\text{-}L}$ from 2.5±1.2 to 2.0±1.0 pA/pF (n=5, $P<0.05$). Infusion of PD116948 30 μmol/L, an adenosine A_1 receptor blocker, did not abolish the inhibitory effects of AMP579 on $I_{Ca\text{-}L}$ (1.9±0.6 *vs* 2.0±1.0 pA/pF, $P>0.05$). But under the same conditions AMP579 10

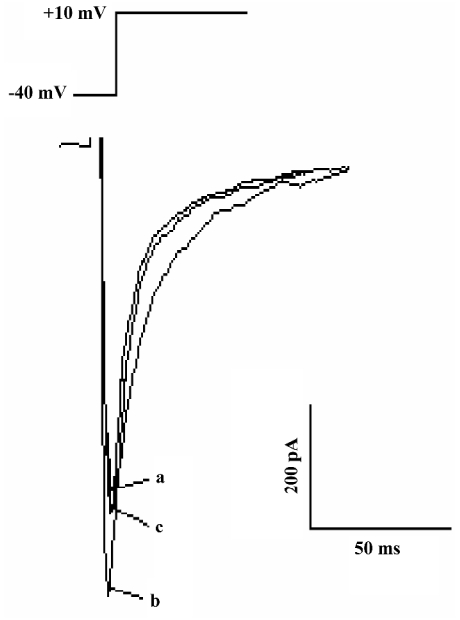

Figure 2. The inhibitory effect of adenosine on $I_{Ca\text{-}L}$ induced by isoproterenol in isolated rat ventricular myocytes. (a) Control; (b) isoproterenol 10 nmol/L; (c) adenosine 50 μmol/L.

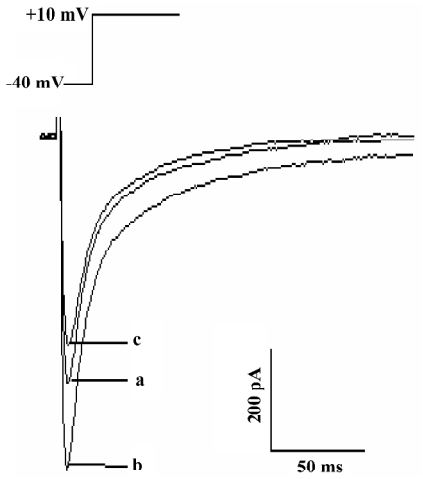

Figure 3. The inhibitory effect of AMP579 on $I_{Ca\text{-}L}$ induced by isoproterenol in isolated ventricular myocytes. (a) Control; (b) isoproterenol 10 nmol/L; (c) AMP579 10 μmol/L.

μmol/L markedly reduced the $I_{Ca\text{-}L}$ from 2.4±0.4 to 1.8±0.4 pA/pF (n=4, P<0.01). Infusion of 0.4 μmol/L GF109203X, a PKC blocker, significantly reversed it to 2.2±0.4 pA/pF (P< 0.05, Figure 5). So GF109203X could abolish the inhibitory effect of AMP579, indicating that the inhibitory effect on basal $I_{Ca\text{-}L}$ by AMP579 was induced through activating PKC but not linked to the adenosine A_1 receptor.

Table 1. Effect of AMP579 on basal $I_{Ca\text{-}L}$ in rat ventricular myocytes. n=5. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* corresponding control group.

AMP579 concentration	$I_{Ca\text{-}L}$ value/ pA·pF^{-1}	Change rate/ %
0 (Control)	2.80±0.75	
10 nmol/L	2.66±0.75^b	-5.0
1 μmol/L	2.36±0.71^b	-15.7
10 μmol/L	2.03±0.72^c	-27.5
50 μmol/L	1.78±0.70^c	-36.4

Change rate=(the current value after administration of drug−control value)/control value×100%

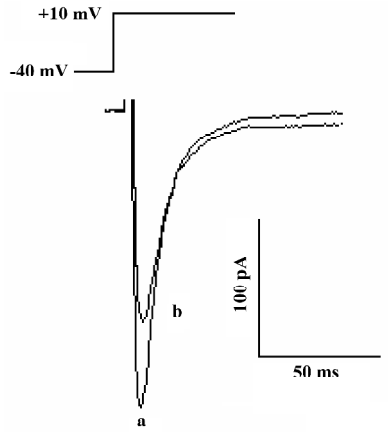

Figure 4. Effect of AMP579 on $I_{Ca\text{-}L}$ in isolated rat ventricular myocytes. (a) Control; (b) AMP579 50 μmol/L.

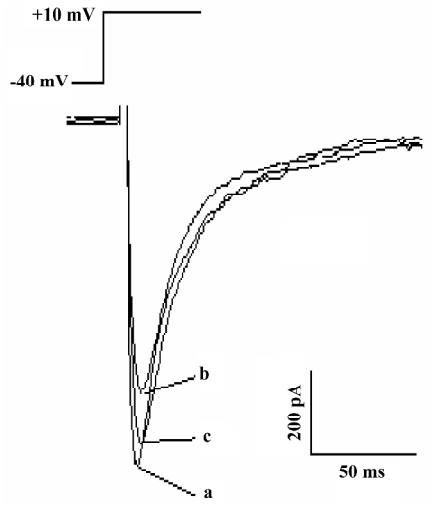

Figure 5. Abolition of inhibitory effects of AMP579 on $I_{Ca\text{-}L}$ by a PKC blocker in isolated rat ventricular myocytes. (a) Control; (b) AMP579 10 μmol/L; (c) GF109203X 0.4 μmol/L.

Discussion

In cardiac tissue, a direct inhibition of basal $I_{Ca\text{-}L}$ by adenosine has only been demonstrated in guinea-pig atrial and ferret ventricular myocytes[11,12]. But in the presence of isoproterenol stimulation, adenosine has prominent inhibitory effects on $I_{Ca\text{-}L}$ in ventricular myocytes[13]. These may reflect differences in receptor-effector coupling mechanisms, the level of basal adenylate cyclase activity, the basal phosphorylated state of Ca^{2+} channels and/or the effect of phosphorylation on the gating of L-type Ca^{2+} channel. Consistent with previous reports, our experiment shows that adenosine has no direct inhibitory effect on basal $I_{Ca\text{-}L}$ in the rat ventricle, but in the condition that isoproterenol was previously administered, adenosine shows an inhibitory effect on the $I_{Ca\text{-}L}$ induced by isoproterenol with an IC_{50} of 13.06 μmol/L, suggesting that adenosine exerts an indirect inhibitory effect on $I_{Ca\text{-}L}$ in the rat ventricle by inhibition of isoproterenol stimulation.

In contrast to adenosine, AMP579 shows a direct inhibitory effects on basal $I_{Ca\text{-}L}$ in the rat ventricle with IC_{50} of 1.17 μmol/L. The blocking of Ca^{2+} influx by L-type Ca^{2+} channel could serve as an efficient method for protecting the ischemic myocyte by minimizing ischemia-induced Ca^{2+} overload and irreversible cell contracture and autodigestion by Ca^{2+}-dependent proteases[14]. Therefore, by reducing both basal $I_{Ca\text{-}L}$ and isoproterenol-induced $I_{Ca\text{-}L,}$ AMP579 will play a more important role in negative chronotropic and negative dromotropic effects. These action mechanism differences between AMP579 and adenosine may account for the contribution of AMP579 in reducing neutrophil-mediated inflammatory reaction, inhibiting cardiac contraction, dilating coronary vessels, attenuating ischemia and reperfusion injury.

Our study does not show that adenosine A_1 receptor is linked to inhibition of AMP579 on basal $I_{Ca\text{-}L}$. At present, available data indicate that three pathways are involved in receptor-linked downstream mechanisms for inhibition of $I_{Ca\text{-}L}$ by adenosine. The first is cAMP-PKA, as PKA increase $I_{Ca\text{-}L}$ by phosphorylation on the gating of the L-type calcium channel, inhibitions of adenylate cyclase and reductions of cAMP and PKA levels by adenosine result in attenuation on $I_{Ca\text{-}L}$[12]. Second is that activation of guanylate cyclase results in increments of intracellular cGMP and PKG concentration, which in turn inhibits phosphorylation on the gating of the L-type calcium channel[15]. The third is modulated by PKC, because there are different PKC subunits which result in different effects[16]. Our experiment finds that special PKC antagonist GF109203X can totally eliminate inhibitory effects of AMP579 on $I_{Ca\text{-}L}$, suggesting that AMP579 exerts a direct inhibitory effects on the L-type calcium channel through the PKC pathway.

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