

**ADENOSINE RECEPTOR INTERACTIONS ALTER CARDIAC
CONTRACTILITY IN THE RAT HEART.**

Roselyn B Rose'Meyer

*School of Medical Sciences,
Griffith University, Queensland, Australia, 4222*

Dr Roselyn Rose'Meyer
School of Medical Sciences
Griffith University
Gold Coast Campus
Queensland, Australia, 4222
Tel. 617 55528938
Fax 617 55528908
E-mail address: r.rosemeyer@griffith.edu.au

SUMMARY

The effect of an adenosine (Ado) A₂ receptor agonist, N⁶-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DPMA) on AdoA₁ receptor mediated negative inotropic responses was investigated in rat heart. Hearts from male Wistar rats (250-350g) were perfused with Krebs-Henseleit solution at constant flow in non-recirculating Langendorff mode. Hearts were paced at 5 Hz (5 ms duration, supramaximal voltage) via ventricular electrodes. After 30 mins equilibration (R)-N⁶-phenylisopropyl adenosine (R-PIA) concentration-response curves were acquired in the absence or presence of DPMA.

R-PIA induced concentration-dependent decreases in triple product (HR x left ventricular developed pressure x dP/dt_{max}) in paced hearts. DPMA (1 nM) significantly attenuated R-PIA induced decreases in triple product, with a shift in pEC₅₀ from 8.0 ± 0.51 (n=9) in control hearts to 6.63 ± 1.03 (n=5) in treated tissues (P<0.05). The AdoA_{2A}R antagonist 8-(3-chlorostyryl) caffeine (1 μM) and the adenylyl cyclase inhibitor cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL12330A, 100nM) reversed the effect of DPMA on AdoA₁R mediated negative inotropic actions while the AdoA_{2B}R inhibitor (alloxazine, 3 μM) had no effect on DPMA activity. The results show that stimulation of AdoA₂R attenuates AdoA₁R dependent reductions in inotropic state. The adenosine receptor sub-type involved appears to be the AdoA_{2A}R, and its action involves stimulation of adenylyl cyclase activity.

Key Words: Adenosine, rat heart, cAMP, contractile function

INTRODUCTION

Endogenous adenosine affects many aspects of cardiac function due to activation of the adenosine A₁, A₂ and A₃ receptor (AdoR) subtypes. The adenosine receptor subtypes are found on different regions of the heart. AdoA₁Rs are located predominantly in cardiomyocytes where they exert direct inhibitory effects on chronotropy, dromotropy and inotropy (1, 2). The physiological effects of adenosine mediated by AdoA₁R act to reduce the oxygen demand of the heart, thereby reducing myocardial ischaemia-reperfusion injury (3). The AdoR_{2A}Rs and AdoA_{2B}Rs are mainly located on the endothelium and vascular smooth muscle with a low density of AdoA_{2A}R found on ventricular myocytes (1, 2, 4, 5).

In rat ventricular myocytes, AdoA₁R mediated negative inotropic actions are associated with activation of inward rectifying K⁺ current I_{K(Ado)} and inhibiting isoproterenol stimulated L-type Ca²⁺ current (6). AdoA₂Rs increase levels of intracellular cAMP via a pertussis-toxin insensitive G_s protein (7, 8).

Interestingly, interactions between adenosine receptors in the cardiovascular system have been reported. For example, deletion of AdoA₃Rs augments AdoA_{2A}R induced vasodilation in murine hearts (9). Other studies have demonstrated that AdoA₁R-mediated electrophysiological effects may be modulated by myocardial AdoA₂R (10, 11).

In the chick ventricular heart cells both subtypes of AdoA₂R's have been demonstrated to have greatly different affinities for NECA, coexist and are coupled to the same positive inotropic cardiac responses (12). Furthermore, there is recent evidence of AdoA₂R-mediated modulation of the "indirect" anti-adrenergic effects of AdoA₁Rs (13). In rat

cardiac tissues both AdoA_{2A}R and AdoA_{2B}R are expressed (14, 15). Little work has been published on the role of AdoA_{2B}R in modifying the actions of AdoA₁R in the heart.

The aims of this study were to examine the impact of AdoA₂R activation on AdoA₁R-mediated inotropic responses, to determine the AdoA₂R subtype involved in modifying AdoA₁R induced cardiac actions, and to establish whether cAMP is a second messenger system involved.

METHODS

Animals

Male Wistar rats (250-350 g) were obtained from the central animal house located at the University of Queensland. They were housed in a room maintained at an ambient temperature of 23 ± 2 °C with a twelve hour light-dark cycle. The rats had free access to food and water at all times. The conduct of all experiments conformed to the guidelines for animal experimentation as determined by the National Health and Medical Research Council of Australia and approved by the Griffith University Animal Experimentation Ethics Committee.

Isolated perfused rat hearts

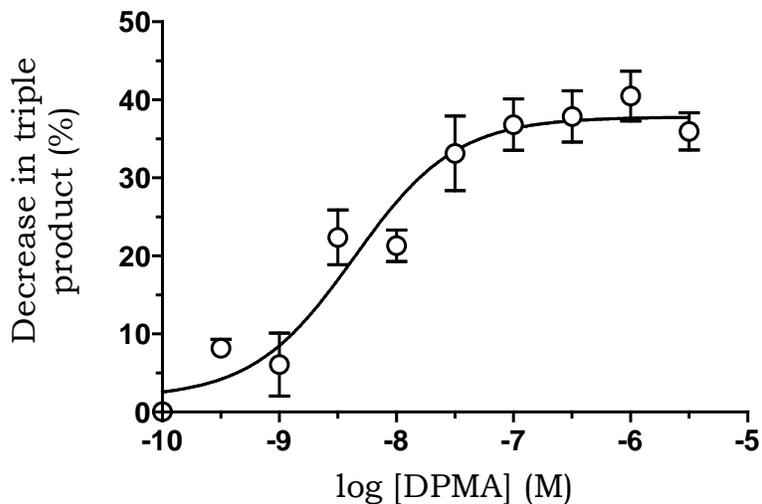
Rats were anaesthetised with pentobarbitone (60 mg/kg, intraperitoneally). Rat hearts were perfused in the non-recirculating Langendorff mode based on a method described by Harrison et al., (16). Briefly, hearts were rapidly excised and immersed into ice-cold Krebs-Henseleit solution containing (in mM): NaCl 118, KCl 4.7, CaCl₂ 1.75, MgSO₄ 1.2, glucose 11, EDTA 0.5 and NaHCO₃ 25, pH 7.4. The aorta was cannulated and heart perfused with Krebs-Henseleit solution gassed with 95% O₂ and 5% CO₂ and kept at 37°C. Ventricular fluid accumulation was prevented by inserting a small piece of polyethylene tubing through the apex of the left ventricle to drain the cavity and ascertain aortic valve patency. A water-filled latex balloon was then introduced into the left ventricle and connected to a pressure transducer (Gould P23-ID, Oxnard Ca). End-diastolic pressure was adjusted to 4-8 mmHg by inflating the balloon. Ventricular pressure and heart rate was monitored continuously on a MacLab data acquisition system

(ADInstruments, Castle Hill, Australia). Coronary perfusion pressure was measured using a pressure transducer connected to a water filled probe inserted into the side arm of the aortic cannula and was recorded using the Maclab data acquisition system. The left ventricular pressure signal was electronically differentiated to measure dP/dt_{max} . Tissues were equilibrated for 30 mins prior to experimentation. For inotropic studies, electrodes were attached to the ventricles and paced at 5Hz (5 ms duration, supramaximal voltage) using a 611 Stimulator (Phipps and Bird Inc, Richmond, VA, USA). To determine myocardial inotropic function a triple product was calculated to incorporate developed pressure and the speed of contraction as follows: HR x Peak systolic ventricular pressure (PSVP) x dP/dt_{max} . As calculation of the triple product results in a very large value ie ($300 \times 100 \times 2000 = 6 \times 10^7$) all decreases in triple product values (TP_R) were shown as a percentage reduction of the paced triple product value prior to the R-PIA concentration-response curve (TP_C) as follows; $(TP_C - TP_R) / TP_R \times 100$.

During equilibration, hearts were perfused at a constant pressure of 80 mmHg to allow adequate perfusion of myocardial tissues. For experiments studying the negative inotropic effects of (R)-N⁶-phenylisopropyladenosine (R-PIA, 0.1 nM - 10 μ M) and N⁶-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DPMA, 0.1 nM - 10 μ M), hearts were perfused at a constant flow rate (to give a coronary perfusion pressure of 80 mmHg, approximately 10ml/min/g tissue wet weight). For all experiments only one concentration-response curve was performed per heart. At the completion of concentration-response curve, hearts were removed, blotted and weighed.

The effect of DPMA on coronary vasodilation and contractility.

Initial studies were performed with the AdoA₂R agonist DPMA (0.1 nM - 10 μM). The effect of DPMA on AdoA₁R induced negative inotropic responses are shown in Fig. 1. The pEC₅₀ value for DPMA induced decreases in triple product is 8.36 ± 0.43 (n=6). All further experiments determining the effect of DPMA on AdoA₁R mediated reductions in contractility were performed using the concentration of 1 nM, which induced negligible changes in heart contractility (6 ± 4 %, P>0.05). The concentration of DPMA causing a maximal vasodilator response with less than a 5% variation in heart rate (from baseline) was determined (data not shown). From concentration-response curves it was evident maximal relaxation (and therefore pronounced activation of AdoA₂R) was achieved with 10 nM DPMA, giving a 98 ± 2 % increase in flow with a pEC₅₀ value of 9.64 ± 0.15 (n=6, data not shown). These results are consistent with K_i values for DPMA obtained in male Wistar rats of ~350 nM for AdoA₁R binding versus 2 nM for AdoA₂R binding in mouse brain (17).



Effect of DPMA on R-PIA induced negative inotropic response.

The effect of DPMA on AdoA₁R-mediated inhibition of contractility was examined. The selective AdoA₁R agonist R-PIA (0.1 nM - 10 μM) was infused in the presence or absence of 1 nM DPMA. Additional experiments incorporated selective AdoA₂R antagonists. The AdoA_{2A}R antagonist 8-(3-chlorostyryl) caffeine (CSC, 1 μM) or the AdoA_{2B}R inhibitor alloxazine (3 μM) was combined with DPMA (1 nM) to study R-PIA induced decreases in triple product. Furthermore, the adenylyl cyclase inhibitor cis-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL12330A, 100 nM) was added with DPMA (1 nM) and R-PIA concentration-response curves were performed in paced tissues. To show that the antagonists used do not affect AdoA₁R-mediated inhibition of contractility or paced heart function, concentration-response curves to R-PIA were also completed in the presence of alloxazine, CSC and MDL12330A.

Table 1. Hemodynamic parameters of each treatment group of rat hearts during basal conditions.

Table 1 Haemodynamic parameters for each treatment group under basal conditions

	LVDP (mmHg)	dP/dt _{max} (mmHg.sec ⁻¹)	dP/dt _{min} (mmHg.sec ⁻¹)	CPP (mmHg)	N
Control	93±5	2332±70	-1874±58	88±6	7
DPCPX	101±8	1856±96*	-1830±115	64±10	4
DPMA	74±8	2729±198	-1671±27	52±6*	6
CSC	100±5	2662±123*	-1767±108	72±3	4
Alloxazine	97±8	1604±132*	-1760±187	80±7	4
MDL12330A	102±9	2523±212	-1967±241	69±5	4
DPMA & CSC	90±5	2080±90	-1665±57	64±5*	7
DPMA & Alloxazine	78±3	2039±232	-1587±106	63±3*	7
DPMA & MDL12330A	109±7	2537±158	-1766±227	70±3*	8

All hearts were paced at 5Hz. All values are means \pm S.E.M. LVDP, left ventricular developed pressure; dp/dt_{max} , first derivative of left ventricular pressure; dp/dt_{min} , second derivative of left ventricular pressure CPP, coronary perfusion pressure. *P different from control values ($P<0.05$). The concentrations of the agonist / antagonists are as follows: DPCPX, 50 nM; DPMA, 1 nM; CSC, 1 μ M; Alloxazine, 3 μ M; MDL12330A, 100 nM;

Data analysis.

Unless stated otherwise, all values shown are means \pm S.E.M. All data were analysed using a repeated measures ANOVA followed by the Newman-Keuls post hoc test for individual comparisons when significant effects was detected. Concentration-response curves to the DPMA and R-PIA were analysed by nonlinear regression using the program GraphPad Prism®, EC_{50} and confidence interval (CI) values were derived from these curves. Differences were considered significant at $P<0.05$.

Materials.

R-PIA, DPMA, CSC, alloxazine, DPCPX and MDL-12330A were all purchased from Sigma Chemicals (Castle Hill, Australia). DPMA and CSC was added directly to perfusing fluid from 100 μ M stock (in ~1% DMSO) and diluted to achieve a 10 nM final concentration. DPCPX (0.5 mM) stock was dissolved in DMSO then diluted 1 in 10 000 to give a final concentration of 50 nM. Dilutions of R-PIA were prepared in buffer from a 0.5 mM stock. Alloxazine stock (10 mM) was dissolved in distilled water with 50 μ l NaOH (10 M) and added to the perfusing solution prior to use. MDL 12330A (1 mM) stock was made up in distilled water and stored in frozen aliquots (-80°C) until required.

Results

The effect of drug treatments on basal functional parameters in isolated perfused hearts.

Functional variables under basal conditions are presented in table 1. LVDP and dP/dt_{\min} were comparable in all groups tested. CPP was reduced by 41% in DMPA treated hearts compared to control hearts ($P<0.05$). dP/dt_{\max} was reduced in DPCPX (50 nM) and alloxazine (3 μ M) treated tissues when compared to untreated controls ($P<0.05$).

However, in CSC (1 μ M) treated tissues dP/dt_{\max} values were higher than control data ($P<0.05$). The combination of DMPA and alloxazine or CSC returned dP/dt_{\max} values to control levels.

Effect of adenosine A₂ receptor activation on adenosine A₁ receptor-mediated decreases in heart inotropic responses.

R-PIA induced concentration-dependent reductions in triple product which were inhibited by DPCPX (50 nM, see Table 2). The addition of the AdoA₂R agonist DPMA (1 nM) did not alter contractility in paced hearts, as measured by the triple product ($P<0.05$). DPMA (1 nM) attenuated maximal decreases in cardiac responses to R-PIA from $48.93 \pm 3.00\%$ in control tissues compared to $16.94 \pm 6.20\%$ with DPMA treatment, see table 2 and Fig. 2.

Table 2. The effect of various antagonists on R-PIA induced decreases in triple product.

	pEC ₅₀ ± CI	Maximal response (%)
Control	8.00 ± 0.51	48.93 ± 3.00
DPCPX	7.00 ± 0.94*	29.8 ± 5.67*
DPMA	6.30 ± 1.03*	16.94 ± 6.20*
CSC	7.46 ± 0.51	51.23 ± 6.34
Alloxazine	7.29 ± 0.48	47 ± 1.40
MDL12330A	7.49 ± 0.37	49.26 ± 3.09
DPMA + CSC	7.60 ± 0.30	45.07 ± 5.79
DPMA + Alloxazine	7.76 ± 0.52	19.08 ± 3.19*
DPMA + MDL12330A	7.26 ± 0.32	33.64 ± 4.47

The concentrations of the agonist / antagonists are as follows: DPCPX, 50 nM; DPMA, 1 nM; CSC, 1 μM; Alloxazine, 3 μM; MDL12330A, 100 nM. pEC₅₀ represents -logEC₅₀ values for each R-PIA concentration-response curve with confidence intervals (CI).

*P<0.05 vs control, n=4-7 per group.

Effect of inhibitors on adenosine A₁ receptor-mediated decreases in triple product.

Addition of CSC (1 μM) and DPMA (1 nM) reversed effects of DPMA on R-PIA mediated reductions in contractility (see Fig 3A, P<0.05). The attenuation of the R-PIA response by DPMA was not altered by co-treatment with alloxazine (3 μM) (P>0.05, Fig 3A). These data indicate AdoA_{2A}R's are responsible for reducing AdoA₁R mediated decreases in inotropic state in rat heart.

The addition of the adenylyl cyclase inhibitor MDL12330A (100 nM) reversed the effect DPMA on the negative inotropic response to R-PIA (P<0.05, see table 1 and Fig. 3B).

Use of CSC, alloxazine or MDL12330A alone did not alter R-PIA concentration-response curves with pEC_{50} values or maximal decreases in triple product remaining unchanged (see Table 2).

This data implies that AdoA₂Rs reverse AdoA₁R mediated cardiac responses through stimulation of adenylyl cyclase.

Fig. 2

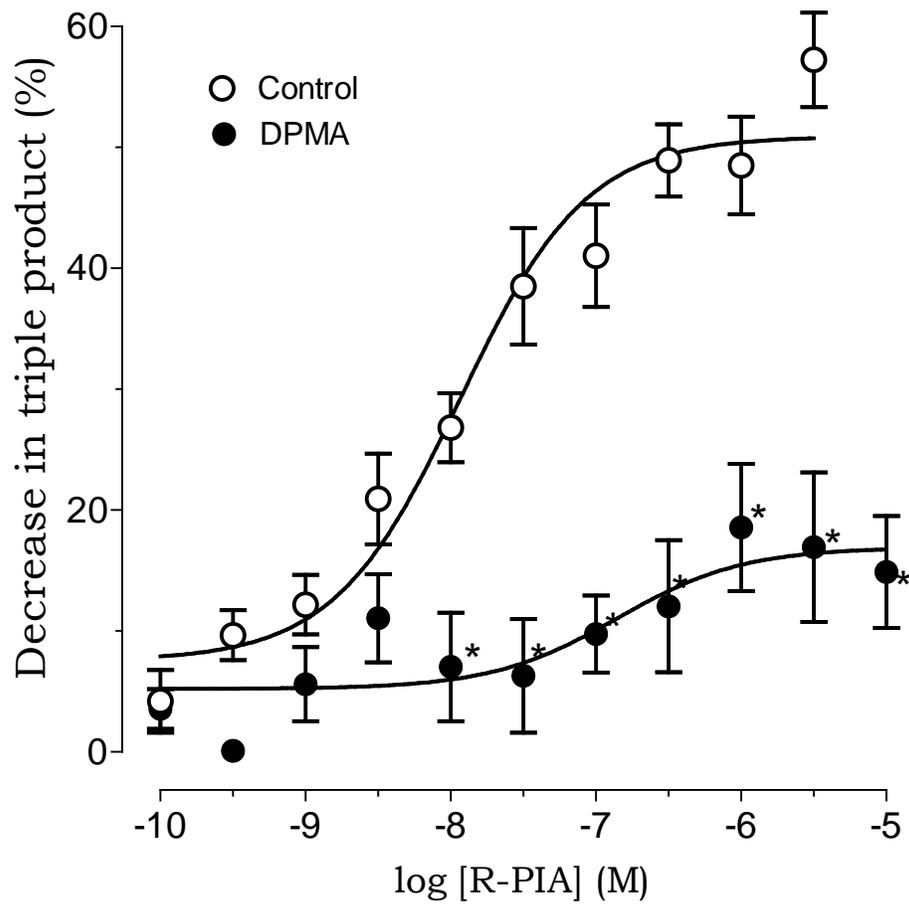
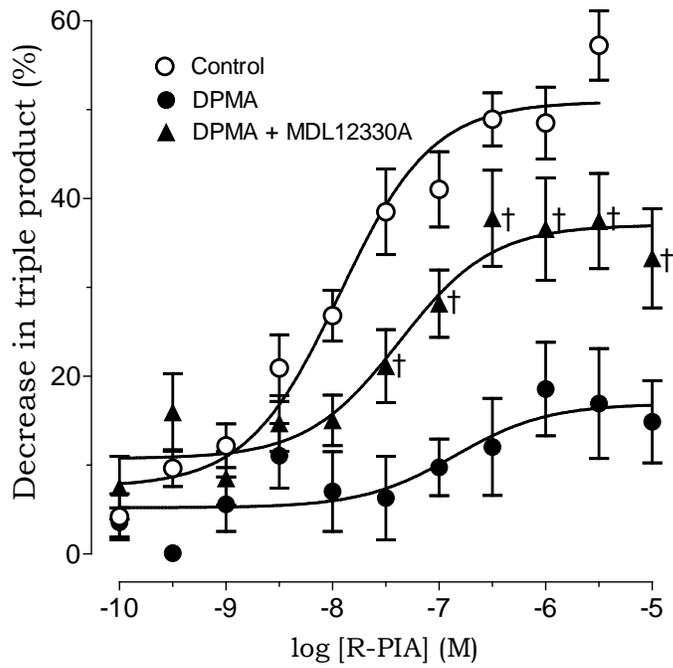
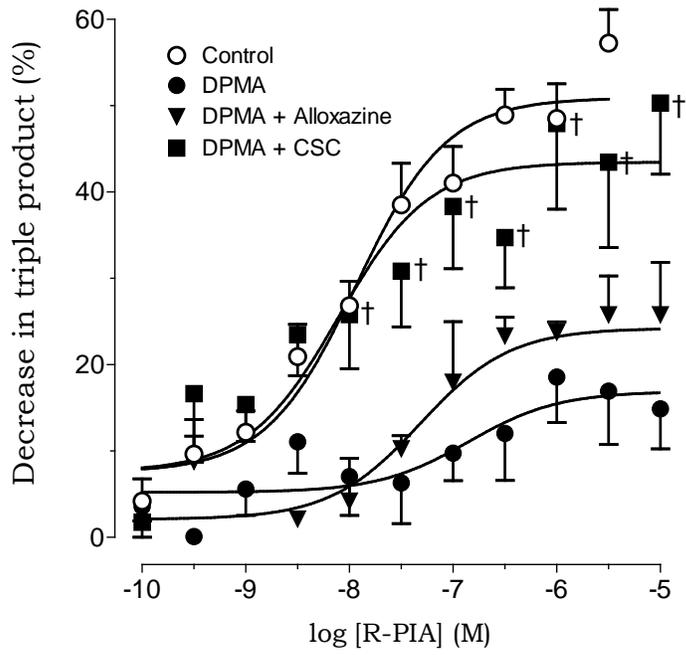


Fig. 3



DISCUSSION

In this study, concentration-response relationships for R-PIA mediated negative inotropy were obtained in rat hearts in the presence and absence of selective concentrations of the AdoA₂R agonist, DPMA. DPMA was utilized as it is a non-selective AdoA₂R agonist that has a much greater relative affinity for the AdoA₂R ($K_i = 2$ nM) than for the AdoA₁R ($K_i = 350$ nM, 13). The purpose of this project was to determine the role of both subtypes of Ado₂Rs in modifying AdoA₁R mediated negative inotropic responses in heart. CGS21680 was not employed as it is selective for AdoA_{2A} over AdoA_{2B}R (K_i value of 19 nM *vs.* 10 000 nM at AdoA_{2B}Rs, for review see Muller, 18) and this would have precluded investigations into the potential role of AdoA_{2B}R in modifying cardiac activities of AdoA₁R. There are very few selective AdoA_{2B}R agonists currently available (18). CGS21680 also acts a partial agonist (19) and will alter its binding behaviour in low receptor populations as observed in heart tissue.

Another study has shown that the AdoA₂R modulates the indirect anti-adrenergic effects of AdoA₁R on contractile function (13). In rat ventricular myocytes, AdoA₁R mediated inhibition of cardiac contractility is associated with activation of inward rectifying K⁺ current $I_{K(Ado)}$ and inhibiting isoproterenol stimulated L-type Ca²⁺ current (7). The actions of AdoA₁R are reported to be due to inhibition of adenylyl cyclase (20), or activation of other mediators including nitric oxide and cGMP (21) or phosphoinositide-PKC- ϵ (22, 23).

In our study, DPMA significantly attenuated the negative inotropic effects of R-PIA, demonstrating that AdoA₂R modifies AdoA₁R-mediated inotropic responses in normoxic

heart. Use of selective AdoR antagonists indicate that it is the AdoA_{2A}R subtype that is involved. The AdoA_{2A}R agonist CGS21680 can increase inotropic responses in isolated myocytes an effect that is mediated by cAMP – dependant and independent mechanisms (24), however, another study has found that although rat ventricular myocytes express AdoA_{2A} receptors they do not alter cAMP or contractility (14). AdoA_{2B}Rs mediate an increase in coronary flow and developed pressure in AdoA_{2A}R knockout murine hearts (25). Due to multiple AdoRs in cardiac tissue, the physiological role of myocardial AdoA_{2B}Rs have been difficult to determine and recent investigations have blocked AdoA₁Rs or deleted AdoA_{2A}Rs in order to study cardiac AdoA_{2B}R's (25, 26). Our investigations in isolated electrically paced rat hearts indicate that Ado_{2B}Rs do not modify AdoA₁R mediated decreases in ventricular contractile function.

Also, in the present study, the adenylyl cyclase inhibitor MDL-12330A (27) was employed to determine whether AdoA_{2A}R-mediated attenuation of the negative inotropic effects of AdoA₁R activation was due to an increase in cAMP levels. In support of this possibility, MDL12330A, reversal of the effect of DPMA on AdoA₁R mediated negative inotropic actions. MDL12330A however, did not completely reverse the effects of DPMA, indicating a possible role for other second messengers to be involved.

In conclusion, the results of the present study demonstrate that AdoA₂R significantly attenuates AdoA₁R mediated decreases in inotropic state. The adenosine receptor subtype involved in this response is the AdoA_{2A}R subtype, and its mechanism of action may involve stimulating adenylyl cyclase activity. This study has revealed a more complex interplay between AdoRs in the control of cardiac physiological function than was previously appreciated.

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Figure legends

Fig. 1. Concentration-response curve to DPMA on triple product (heart rate x dP/dt_{\max} x PSVP) in isolated perfused hearts paced at 5Hz (5 ms duration, supramaximal voltage) ($n = 5$). All values represent mean \pm SEM.

Fig. 2. Concentration-response curves to R-PIA on triple product (heart rate x dP/dt_{\max} x PSVP) in isolated perfused hearts paced at 5Hz (5 ms duration, supramaximal voltage) in the absence (\circ , $n = 7$) and the presence of DPMA (10 nM, \bullet , $n = 6$). Values represent mean \pm SEM. * $P < 0.05$ vs. control.

Fig. 3. Panel A; The effects of chlorostyryl caffeine (CSC, $1\mu\text{M}$, \blacksquare , $n = 4$) or alloxazine ($3\mu\text{M}$, \blacktriangledown , $n=4$) and DPMA (1 nM) on concentration-response curves to R-PIA on triple product (heart rate x dP/dt_{\max} x PSVP) in isolated perfused hearts paced at 5Hz (5 ms duration, supramaximal voltage). † $P < 0.05$ vs. DPMA treated data. Panel B; The effects of MDL12330A (100 nM, \blacktriangle , $n = 5$) and DPMA (1 nM) on concentration-response curves to R-PIA on triple product (heart rate x dP/dt_{\max} x PSVP) in isolated perfused hearts paced at 5Hz (5 ms duration, supramaximal voltage). Values represent mean \pm SEM. † $P < 0.05$ vs. DPMA treated data.