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## Regulation of Contractility by Adenosine A<sub>1</sub> and A<sub>2A</sub> Receptors in the Murine Heart: Role of Protein Phosphatase 2A: A Dissertation

Eugene I. Tikh

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A Dissertation Presented

By

Eugene I. Tikh

Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Sciences  
Worcester, Massachusetts

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

June 21, 2006

Ph.D./M.D. Program in Biomedical Sciences

**REGULATION OF CONTRACTILITY BY ADENOSINE  
A<sub>1</sub> AND A<sub>2A</sub> RECEPTORS IN THE MURINE HEART:  
ROLE OF PROTEIN PHOSPHATASE 2A**

A Dissertation Presented

By

Eugene I. Tikh

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Ph.D./M.D. Program in Biomedical Sciences  
June 21, 2006

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Tikh, E.I., Fenton, R.A. and Dobson, J.G., Jr. Adenosine A<sub>1</sub> and A<sub>2A</sub> Receptor Regulation of Protein Phosphatase 2A in the Murine Heart. *American Journal of Physiology: Heart and Circulatory Physiology* (submitted). Copyright © 2006 The American Physiological Society.

Note: References following each manuscript are specific to that manuscript. References for the introductory sections and discussion are located in the “Bibliography” section.

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## **Abstract**

Adenosine is a nucleoside that plays an important role in the regulation of contractility in the heart. Adenosine receptors are G-protein coupled and those implicated in regulation of contractility are presumed to act via modulating the activity of adenylyl cyclase and cAMP content of cardiomyocytes. Adenosine A<sub>1</sub> receptors (A<sub>1</sub>R) reduce the contractile response of the myocardium to β-adrenergic stimulation. This is known as antiadrenergic action. The A<sub>2A</sub> adenosine receptor (A<sub>2A</sub>R) has the opposite effect of increasing contractile responsiveness of the myocardium. The A<sub>2A</sub>R also appears to attenuate the effects of A<sub>1</sub>R. The effects of these receptors have been primarily studied in the rat heart and with the utilization of cardiomyocyte preparations. With the increasing use of receptor knockout murine models and murine models of various pathological states, it is of importance to comprehensively study the effects of adenosine receptors on regulation of contractility in the murine heart. The following studies examine the adenosinergic regulation of myocardial contractility in isolated murine hearts. In addition, adenosinergic control of contractility is examined in hearts isolated from A<sub>2A</sub>R knockout animals. Responses to adenosinergic stimulation in murine isolated hearts are found to be comparable to those observed in the rat, with A<sub>1</sub>R exhibiting an antiadrenergic action and A<sub>2A</sub>R conversely enhancing contractility. A significant part of the A<sub>2A</sub>R effect was found to occur via inhibition of the A<sub>1</sub>R antiadrenergic action. A part of the antiadrenergic action of A<sub>1</sub>R has previously been shown to be the result of protein phosphatase 2A activation and localization to membranes. Additional experiments in the present study examine the effect of adenosinergic signaling on PP2A in myocardial extracts from wild type and A<sub>2A</sub>R knockout hearts. A<sub>2A</sub>R activation was found to decrease the activity of PP2A and enhance localization of the active enzyme to the cytosol; away from its presumed sites of action. In the A<sub>2A</sub>R knockout the

response to  $A_1R$  activation was enhanced compared with the wild type and basal PP2A activity was reduced. It is concluded that  $A_{2A}R$  modulation of PP2A activity may account for the attenuation of the  $A_1R$  effect by  $A_{2A}R$  observed in the contractile studies.



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## **Introduction**

Adenosine plays a role in regulating contractility in the heart by activating adenosine specific receptors. The effects of these receptors mediate primarily the myocardial response to  $\beta$ -adrenergic stimulation. Other functions that have been reported include vasodilation, preconditioning and regulation of myocardial remodeling. As such changes in the activation of adenosine receptors by pharmacological or other means may be useful for both the experimental study of heart function and medical purposes. With the increased use of the murine animal model for scientific experimentation and the availability of transgenic animals which lack one or more adenosine receptors it is imperative to study the function and interaction of the adenosine receptors in the murine heart and also delineate the mechanisms by which these receptors act.

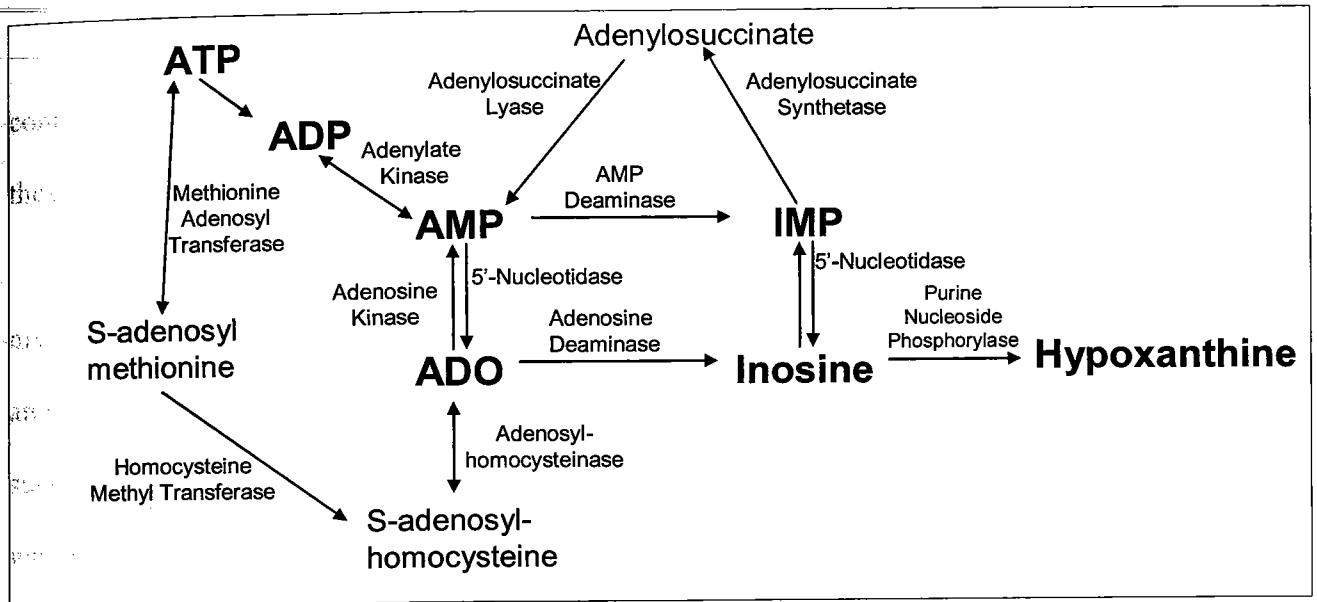
The following dissertation is a study of adenosinergic modulation of contractility in both wild type and adenosine 2A receptor knockout murine hearts. The first work presented focuses on receptor function as seen at the whole organ level using isolated perfused hearts. The second work presented examines one of the recently proposed mechanisms for the manifestation of the adenosinergic effects: the modulation of protein phosphatase 2A activity which has been suggested to be one of the signaling pathways by which adenosine receptors effect alterations in contractile function of the heart. This investigation also compares the observed effects of adenosine receptors between wild type and  $A_{2A}$  receptor knockout models. A brief summary of the known roles of adenosine and its receptors will help to facilitate the integration of the new information obtained into the known framework established through prior study by others.

### Adenosine Metabolism in the Heart

Adenosine is a nucleoside that is a product of AMP metabolism. The metabolism of adenosine in the context of other purines is outlined in Figure A. Adenosine is metabolized to inosine and subsequently hypoxanthine. This pathway is not reversible and leads to eventual transformation of hypoxanthine to xanthine and elimination in the form of uric acid (21). Along this pathway there is an opportunity to “rescue” the adenosine metabolites by conversion of hypoxanthine to inosine monophosphate, which can then be metabolized back to adenosine monophosphate (AMP). This provides a shunt for adenosine that has entered the irreversible pathway to inosine a return to the AMP-adenosine pool. An additional pathway for the metabolism of adenosine is the S-adenosyl-homocystein (49,62). This pathway provides a link between ATP and adenosine without passing through the adenosine diphosphate (ADP) and AMP states. Instead the ATP is reversibly metabolized to S-adenosyl-methionine, which can then be metabolized to S-adenosyl-homocysteine. The S-adenosyl-homocysteine in a reversible reaction then forms adenosine. This pathway is useful for estimation of cellular adenosine content and metabolism rates as S-adenosyl-homocysteine does not participate in other reactions, nor does it get transported out of the cell (63). In addition to being metabolized, adenosine itself can also be transported out of the cell (35,69,87). Once in the interstitial fluid, adenosine can be transported back into the cell or is deaminated to inosine and further metabolized to hypoxanthine. While in the extracellular compartment adenosine is free to interact with and activate adenosine receptors on the cell surface (14,21,82).

Under normoxic conditions the level of extracellular (interstitial) adenosine present in the heart is low. Typical physiological concentrations of adenosine in the heart range 30-120 pmol/ml. However, adenosine levels are rapidly elevated, to as much as ten-fold increase in

response to perturbations of cellular metabolism under conditions such as ischemia or hypoxia (29,32). The stimulus for enhanced adenosine release is a reduction in oxygen levels (3,4,29). Under non-pathological conditions adenosine levels can also be elevated as a result of  $\beta$ -adrenergic stimulation (30).



**Figure A.** Metabolism of adenosine in the context of other purines. ADO-adenosine; ATP-adenosine triphosphate; ADP-adenosine diphosphate; AMP-adenosine monophosphate; IMP-inosine monophosphate

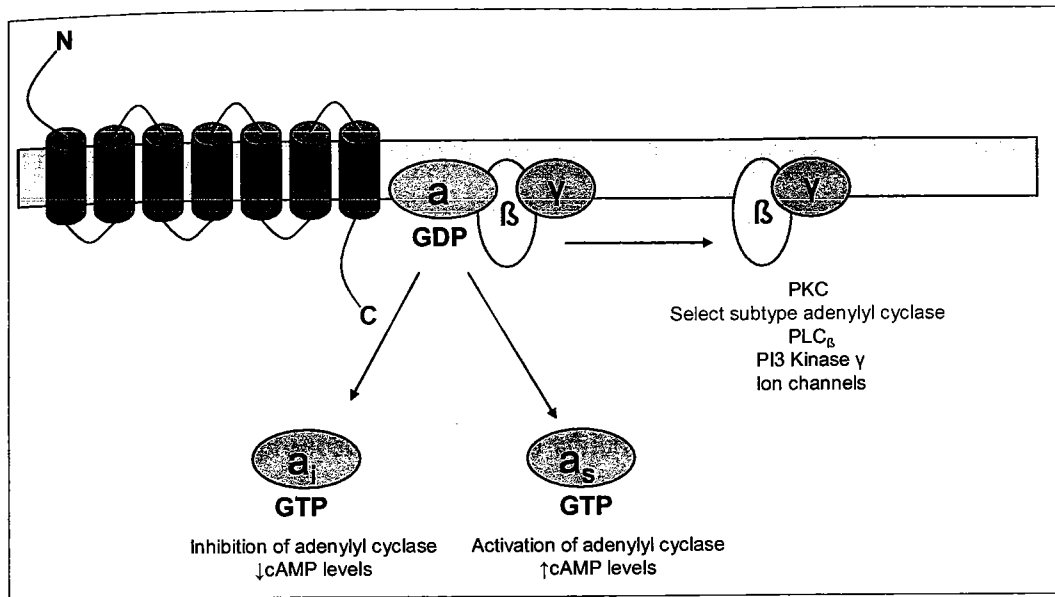
### G-protein coupled signal transduction and modulation of contractility

All adenosine receptors are G-protein coupled (36). As such their effects are believed to be for the main part manifested via modulation of the activity of adenylyl cyclase and subsequent changes in cAMP levels (40,77,85,93). The G-protein coupled receptor (GPCR) is a combination of the receptor associated with the G-protein complex which consists of three subunits termed  $\alpha$ ,  $\beta$  and  $\gamma$  (40). In an inactive state the  $\alpha$  subunit is associated with GDP, which upon receptor activation is replaced by GTP. The  $\alpha$  subunit then disassociates from the  $\beta$  and  $\gamma$  subunits which remain together as a  $\beta\gamma$  complex (81). Both  $\alpha$ -GTP and the  $\beta\gamma$  complex play roles in regulating

the activity of adenylyl cyclase (85) and the  $\beta\gamma$  complex can affect cellular messengers such as p13 kinase and phospholipase C- $\beta$  (PLC $\beta$ ) as well as certain ion channels (10).

Due to the intrinsic GTPase activity of the  $\alpha$  subunit, the GTP bound to it is eventually hydrolyzed and the subunit reassociates with the  $\beta\gamma$  complex resulting in deactivation (40). There are multiple types of subunits and G-proteins are classified on the basis of the specific type of contained subunits. Further description of various G-protein subtypes is beyond the scope of this thesis, but has been previously described (10,25,40).

The G proteins associated with the adenosinergic regulation of contractility in the myocardium are the G<sub>s</sub> and the G<sub>i</sub> proteins, characterized by their contained  $\alpha_s$  and  $\alpha_i$  subunits and named for their stimulatory and inhibitory properties respectively (81). Stimulatory effects such as those resulting from  $\beta$ -adrenergic stimulation are thought to be G<sub>s</sub> mediated (68,95) while inhibitory effects are due to G<sub>i</sub> (46). The alterations in cyclic AMP (cAMP) levels that result from modulation of adenylyl cyclase by the active  $\alpha$ -subunits of G-proteins result in activation or inhibition of protein kinase A (PKA) (26,100) and subsequent changes in phosphorylation level of proteins important to contractile function such as troponin I and phospholamban (22,86). The G-protein regulation of protein kinase C (PKC) is an additional pathway for manifestation of adenosinergic effects as well (44,73). Calcium release is also effected by adenosine (28,31,91) presumably by both downstream effects of the  $\alpha$  as well as  $\beta\gamma$  complex, altering contractility via calcium-dependent mechanisms. A representative diagram of G-protein coupled receptor function and the function some of the key subunits found in the heart are presented in Figure B.



**Figure B.** G-protein coupled receptor function in regulation of contractility in the heart.

#### *Adenosine A<sub>1</sub> receptors and antiadrenergic action*

The A<sub>1</sub> adenosine receptor (A<sub>1</sub>R) is best known for its antiadrenergic action which is its ability to decrease the contractile (27,53) and metabolic (15,16) response of the myocardium to β-adrenergic stimulation (2,6,17,18). This effect has been considered to be manifest primarily through inhibition of adenylyl cyclase activity (41,79,83). PKC activation also appears to be involved in the antiadrenergic effects of A<sub>1</sub>R as evidenced by its enhanced localization to the t-tubular system in response to A<sub>1</sub>R activation in rat ventricular myocytes (73). However, recently studies have shown that in addition to modulating adenylyl cyclase activity levels a significant part of the antiadrenergic effect of A<sub>1</sub>R may be due to its apparent ability to activate protein phosphatase 2A (60), which results in dephosphorylation of proteins important in the regulation of contractility.

The A<sub>1</sub>R are also known to be implicated in the transduction mechanisms leading to other effects. One of these is cardiac preconditioning (1,13,38,50,71), a phenomenon by which



exposure of the heart to a stimulus (such as brief periods of ischemia in ischemic preconditioning or activation of certain adenosine receptors) prior to ischemia results in decreased tissue damage and possibly improved functional recovery (55,58). For over a decade adenosine has been noted to be one of a number of cardioprotective agents released endogenously in response to ischemic/hypoxic episodes (23,24,37,52,67). This effect most recently has been attributed to the action of specific adenosine receptors including the A<sub>1</sub>R (51,55,92). The adenosine activated A<sub>1</sub>R initiates various proposed mechanisms which provide cardioprotection for the ischemic heart.

#### *Adenosine A<sub>2A</sub> receptors*

The A<sub>2A</sub> adenosine receptor (A<sub>2A</sub>R) is known to increase myocardial contractile response to β-adrenergic stimulation (54,74,75) indirectly by the attenuation of the antiadrenergic effects of the A<sub>1</sub>R (78,96). In addition the A<sub>2A</sub>R has been noted to directly increase contractility in the absence of β-adrenergic stimulation (20,74,96). The major pathway for the effects of A<sub>2A</sub>R has been generally thought to be the modulation of adenylyl cyclase activity (68,96) with a subsequent alteration in the cellular cAMP level. A cAMP independent mechanism for the effects of A<sub>2A</sub>R has been proposed as well (56) possibly involving G<sub>s</sub>-mediated effects on calcium ion channels. While with β-adrenergic stimulation the observed enhancement of contractility by A<sub>2A</sub>R activation appears to be closely related to the release of calcium, under non-adrenergic conditions the same effect appears to be calcium-independent (94) suggesting that there may be a difference in the predominant pathways by which the A<sub>2A</sub>R exerts its effects. Another major effect of the A<sub>2A</sub>R not directly related to contractility is the vasodilation of the

coronary vasculature which is also in part due to the A<sub>2B</sub>R but is considered mainly due to the activation of A<sub>2A</sub>R (75,84).

#### *Other adenosine receptors*

The functions of the A<sub>2B</sub> (A<sub>2BR</sub>) and A<sub>3</sub> (A<sub>3R</sub>) adenosine receptors are not nearly as well defined as those of the A<sub>1</sub>R and A<sub>2A</sub>R. It is thought by some that the effect on contractility of A<sub>2B</sub> receptors is minor (54,75). A<sub>2B</sub>R appears to be coupled to the same adenylate cyclase/cAMP pathway as the A<sub>2A</sub>R. However it requires much higher levels of interstitial adenosine to become activated (54). The A<sub>2B</sub>R has also been shown important in the regulation of fibroblast activity in myocardial remodeling which is the reconstruction of myocardial tissue in order to preserve heart function following an infarction (5,8).

While even less is known about the A<sub>3</sub> adenosine receptor, research suggests that it plays a strong role in preconditioning and mediation of ischemic damage in myocardium (12,59,65,89,101). At present there are no published studies focused on the possible effects of the A<sub>3</sub>R on myocardial contractility. In a study of antiadrenergic effects of adenosine in isolated rat hearts administration of A<sub>3</sub>R did not result in alterations in contractile response to stimulation of the A<sub>1</sub>R and A<sub>2A</sub>R (78). This observation suggests that contribution of the A<sub>3</sub>R to regulation of contractility in the heart is either minor so as not to be observable in a setting of β-adrenergic stimulation, or does not play a role in the regulation of myocardial contractility altogether.

Adenosinergic modulation of contractility in the intact heart as published in: "**Contractile effects of adenosine A<sub>1</sub> and A<sub>2A</sub> receptors in isolated murine hearts**" American Journal of Physiology: Heart and Circulatory Physiology 2006 Jan;290(1):H348-56

Over the years, considerable attention has been given to the study of adenosine receptor-mediated regulation of contractility in the heart. Studies published, however, used primarily isolated rat heart or isolated cardiomyocyte models. While several studies have examined the effects of adenosine receptors in murine heart, few have focused on contractility as the primary point of interest, and virtually none have thoroughly explored the interaction of the A<sub>1</sub>R and A<sub>2A</sub>R with regard to contractility. The following manuscript is a study of adenosinergic influence on the contractile function observed in the isolated murine heart under several conditions. The study examines the responses of isolated hearts to adenosinergic stimulation in constant flow and constant pressure preparations using  $\beta$ -adrenergic stimulation, as well as examines the response of the heart to brief periods of decreased perfusion in the presence and absence of A<sub>2A</sub>R activation.

To compare receptor effects between wild type and the available A<sub>2A</sub>R knockout model, it would be necessary to establish a basic scheme of receptor function and validate whether the established information gathered in rat hearts and cardiomyocytes held true in the murine heart. The findings of this study would form the basis for the further study of mechanisms by which the adenosine receptors modulate contractility. A whole organ approach appeared most appropriate for this type of study as the ultimate question of interest is the effect of the adenosine receptors and their interaction with respect to contractility in the physiological setting. As such the isolated heart provides a setting where the interaction of receptor actions can be studied with minimal concern of alteration in functions that may occur as a result of cardiomyocyte isolation.

In order to study the effects of the  $A_1R$  and  $A_{2A}R$  receptors on contractility in the murine heart, and compare their effects in a comprehensive manner, the response to  $\beta$ -adrenergic stimulation was examined under variable adenosinergic stimulation. The use of  $\beta$ -adrenergic stimulation allowed for enhanced manifestation of the effects of these receptors, as the contractile response to adenosinergic stimulation in non-adrenergic conditions is fairly small. In addition because changes in flow and perfusion pressure are known to affect contractility, and  $A_{2A}/A_{2B}R$  modulate vascular resistance, the effects of the  $A_{2A}R$  were examined in the absence of  $\beta$ -adrenergic stimulation by observing responses to decreased flow while stimulating the receptor.

### **Abstract**

The adenosine  $A_1$  receptor ( $A_1R$ ) inhibits  $\beta$ -adrenergic-induced contractile effects (antiadrenergic action), and the adenosine  $A_{2A}$  receptor ( $A_{2A}R$ ) both opposes the  $A_1R$  action and enhances contractility in the heart. This study investigated the  $A_1R$  and  $A_{2A}R$  function in  $\beta$ -adrenergic-stimulated, isolated wild-type and  $A_{2A}R$  knockout murine hearts. Constant flow and pressure perfused preparations were employed, and the maximal rate of left ventricular pressure (LVP) development ( $+dp/dt_{max}$ ) was used as an index of cardiac function.  $A_1R$  activation with 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA) resulted in a 27% reduction in contractile response to the  $\beta$ -adrenergic agonist isoproterenol (ISO). Stimulation of  $A_{2A}R$  with 2-P(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxyamidoadenosine (CGS-21680) attenuated this antiadrenergic effect, resulting in a partial (constant flow preparation) or complete (constant pressure preparation) restoration of the ISO contractile response. These effects of  $A_{2A}R$  were absent in knockout hearts. Up to 63% of the  $A_{2A}R$  influence was estimated to be mediated

through its inhibition of the A<sub>1</sub>R antiadrenergic effect, with the remainder being the direct contractile effect. Further experiments examined the effects of A<sub>2A</sub>R activation and associated vasodilation with low-flow ischemia in the absence of β-adrenergic stimulation. A<sub>2A</sub>R activation reduced by 5% the depression of contractile function caused by the flow reduction and also increased contractile performance over a wide range of perfusion flows. This effect was prevented by the A<sub>2A</sub>R antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385). It is concluded that in the murine heart, A<sub>1</sub>R and A<sub>2A</sub>R modulate the response to β-adrenergic stimulation with A<sub>2A</sub>R, attenuating the effects of A<sub>1</sub>R and also increasing contractility directly. In addition, A<sub>2A</sub>R supports myocardial contractility in a setting of low-flow ischemia.

## **Introduction**

Adenosine plays a role in modulating cardiac functions. In the well-oxygenated myocardium, intracellular levels of adenosine are low, but they rapidly increase in response to adrenergic stimulation or pathological conditions, such as ischemia and hypoxia (10, 11). Four adenosine receptor subtypes have been identified and cloned (13). The adenosine A<sub>1</sub> and A<sub>2A</sub> receptors (A<sub>1</sub>R and A<sub>2A</sub>R, respectively) are thought to be particularly important in the regulation of cardiac contractility (4, 7, 9, 19, 26). A<sub>2</sub>R are also known to be responsible for coronary vasodilation, with A<sub>2A</sub>R considered the main contributor to this effect (27, 31). A<sub>2B</sub> receptors are considered more important to fibroblast regulation (3). The role of adenosine A<sub>3</sub> receptors is less certain, although they have been linked to preconditioning (24) and have recently been shown to activate PKB (Akt) in newborn rat cardiomyocytes (14). The A<sub>1</sub>R is known to exhibit an antiadrenergic action reducing the contractile responsiveness of the myocardium to adrenergic stimulation (1, 5, 6, 12). A<sub>2A</sub>R, on the other hand, appears to have a direct inotropic effect on the

myocardium (4, 26, 34) by facilitating a greater response to adrenergic stimulation (19, 33) and an indirect effect by inhibiting the action of the A<sub>1</sub>R (28, 34).

Both A<sub>1</sub>R and A<sub>2A</sub>R are G protein-linked receptors coupled to G<sub>i</sub> and G<sub>s</sub>, respectively (13). The effects of these receptors are thought to be mediated either through the modulation of adenylyl cyclase activity (4, 19) with subsequent activation of PKA (8, 13) or via activation of phosphatidylinositol 3-kinase (PI3K) with the subsequent activation of endothelial nitric oxide synthase (eNOS) (17, 32, 35). A cAMP-independent G<sub>s</sub>-mediated mechanism has been proposed for the stimulatory effects of A<sub>2A</sub>R as well (4, 20). Recently PKC<sub>e</sub> has been shown to be important in the antiadrenergic effect of A<sub>1</sub>R (25). Other recent studies (21, 22) have suggested that a significant component of the antiadrenergic effect of A<sub>1</sub>R is a result of protein phosphatase activation.

The individual effects of A<sub>1</sub>R and A<sub>2A</sub>R on cardiac contractility at the cellular level have been fairly well characterized. However, their individual actions and the interaction between them in the intact heart remain topics of active interest. The main purpose of this study was to examine the interaction of A<sub>1</sub>R and A<sub>2A</sub>R in the perfused murine heart subjected to β-adrenergic stimulation. The study also examined the actions of the A<sub>1</sub>R in the absence of A<sub>2A</sub>R with the use of an A<sub>2A</sub>R knockout (A<sub>2A</sub>RKO) mouse heart and the effect of A<sub>2A</sub>R activation in the absence of β-adrenergic stimulation. In addition, the influence of A<sub>2A</sub>R on ischemic heart function was investigated.

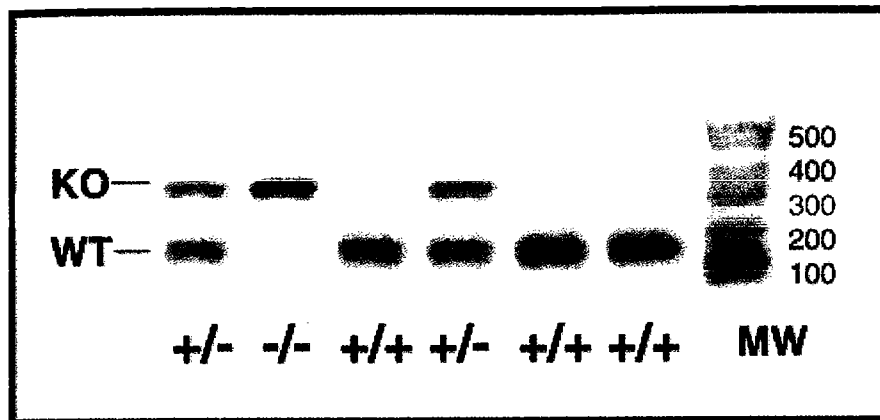
## Materials and Methods

### *Experimental Animals*

Six- to eight-week-old wild-type C57BL/6 male mice (WT) were purchased from Sprague-Dawley. A<sub>2A</sub>RKO mice were obtained from a colony maintained by our laboratory. The animals in this study were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and evaluated and approved according to the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School, Worcester, MA.

### *A<sub>2A</sub>RKO Generation and Verification*

The progenitors for the A<sub>2A</sub>RKO  $-/-$  mice were obtained as a generous gift from Dr. J. F. Chen of Boston University Medical Center (Boston, MA) and were generated as described previously by others (2). The homozygous knockout animals used in the present study were offspring of heterozygous (+/-) breeders. Animals were validated by using DNA isolated from tail tissue with the use of Qiagen DNEasy tissue kit. Isolated DNA was amplified by PCR using Qiagen Taq DNA polymerase and primers: 1) AGC CAG GGG TTA CAT CTG TG, 2) TAC AGA CAG CCT CGA CAT GTG, 3) TCG GCC ATT GAA CAA GAT GG, and 4) GAG CAA GGT GAG ATG AGA GG. Primers 1 and 2 correspond to the WT A<sub>2A</sub>R sequence, whereas primers 3 and 4 correspond to the A<sub>2A</sub>RKO sequence. Products of PCR were resolved by using 1% agarose gel electrophoresis in Tris-boric acid-EDTA buffer and visualized with ethidium bromide staining under UV light (WT, 180 bp; A<sub>2A</sub>RKO, 330 bp). The agarose gels were used to verify the genotype of the mice (Fig. 1).



**Figure 1.** Agarose gel electrophoresis of PCR products used to determine genotype of mice. WT, wild-type (+/+), 180 bp; knockout (KO),  $A_{2A}$ -receptor KO ( $A_{2A}RKO$ ) (-/-), 330 bp; heterozygous breeders (+/-), both 180 and 330 bp; MW, molecular weight marker (100 bp fragment) with 500–100 bands visible top to bottom.

#### *Isolated Heart Preparation*

Mice were euthanized by decapitation and hearts were excised. After the excision, the hearts were rapidly rinsed in saline at room temperature, mounted on the perfusion apparatus, and perfused via the aorta with a physiological saline solution (37°C; PSS) containing (in mM) 118.4 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, and 10 dextrose. The pH of the PSS was maintained at 7.4 by bubbling continuously with a 95% O<sub>2</sub>-5% CO<sub>2</sub> gas mixture. The developed left ventricular pressure (LVP) was monitored by a pressure transducer and a cannula tipped with a water-filled polyethylene balloon that was inserted through the mitral valve after a left atriotomy. Perfusion pressure was monitored by using a transducer connected to a sidearm of the perfusion cannula. The heart was paced with 3 V at 480 times/min via leads on the perfusion cannula and the pulmonary artery. All agents were delivered into the perfusion cannula using infusion pumps (model 22, Harvard Apparatus, Holliston, MA) at the rate required



(1.0% of perfusate flow rate) to achieve the final desired concentration in the perfusion fluid.

The maximal rates of LVP development ( $+dP/dt_{\max}$ ) and relaxation ( $-dP/dt_{\max}$ ) were determined by differentiation of the LVP signal. All data were recorded by using a model RS-3400 Gould polygraph (Chandler, AZ).

### **Protocols**

*General.* For constant flow experiments, the flow rate was adjusted to achieve a LVP of at least 40 mmHg. Flow rates ranged from 2.5 to 2.9 ml/min. For constant pressure experiments, the perfusion pressure was held constant at 60 mmHg and the perfusate flow rate was determined volumetrically. Hearts were allowed to stabilize for at least 15 min before the initiation of experimental protocols. In both constant pressure and constant flow experiments, hearts failing to demonstrate a LVP of at least 40 mmHg on stabilization were excluded from further study. In experiments with  $\beta$ -adrenergic stimulation, isoproterenol (ISO) was infused for 30 s to achieve a final perfusate concentration of  $10^{-8}$  M. Adenosine receptor agonists and antagonists were infused to achieve a final perfusate concentration of  $10^{-7}$  M. Preliminary experiments conducted with the same concentration of ISO at infusion durations of 30 s, 1 min, and 2 min with 15 min washout did not indicate desensitization of  $\beta$ -adrenergic responsiveness with multiple ISO infusions (data not shown).

#### *$\beta$ -Adrenergic-stimulated hearts.*

EFFECT OF  $A_{2A}R$  ACTIVATION ON THE ANTIADRENERGIC EFFECT OF  $A_{1R}$ . After stabilization, hearts were subjected to one 30-s ISO administration, and the peak contractile responses were recorded. After the hearts returned to steady state, infusion of the  $A_{1R}$  agonist 2-

chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA) commenced. After 5 min of CCPA infusion, two additional ISO responses were elicited with a return to steady state between each administration. CCPA infusion was terminated after the second ISO response. CCPA and the A<sub>2A</sub>R agonist 2-(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxyamidoadenosine (CGS-21680) were then administered together for 5 min, whereupon two more ISO responses were elicited while continuing the CCPA and CGS-21680 infusion. This protocol was conducted with both WT and A<sub>2A</sub>RKO hearts. To confirm the response to CCPA as A<sub>1</sub>R specific, in some preparations a combination of CCPA and the A<sub>1</sub>R antagonist 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) was administered.

#### EFFECT OF A<sub>2A</sub>R ACTIVATION IN PRESENCE AND ABSENCE OF A<sub>1</sub>R INHIBITION.

In the first group, after stabilization, hearts were subjected to one 30-s ISO administration, and contractile responses were recorded. On return of contractility to baseline, the A<sub>2A</sub>R agonist CGS-21680 was administered for 5 min, and three 30-s ISO responses were elicited, allowing a return to baseline between stimulations.

In the second group, after stabilization, hearts were subjected to one 30-s ISO administration, and contractile responses were recorded. On return to baseline, the A<sub>1</sub>R antagonist DPCPX was administered for 5 min, and two 30-s ISO responses were elicited, allowing a return to baseline after each stimulation. Subsequently, a combination of DPCPX and A<sub>2A</sub>R agonist CGS-21680 was administered for 5 min, and two 30-s ISO responses were elicited as before.

*Reduced flow experiments.* For flow reduction experiments, the hearts were initially perfused at a rate of 3.0 ml/min. The response to a 1 ml/min flow rate decrease was examined under several experimental conditions. These protocols are depicted in Fig. 7.

#### EFFECT OF A<sub>2A</sub>R ACTIVATION ON CONTRACTILE RESPONSE TO FLOW REDUCTION.

After stabilization, hearts were subjected to three periods of flow reduction, each of 1 min duration separated by periods of normal perfusion (3 ml/min) for ~3 min, allowing a return to baseline contractility (Fig. 7A). At this time, either CGS-21680 or CGS-21680 with the A<sub>2A</sub>R antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385) was administered for 5 min, whereupon the flow-reduction cycle was repeated. Expected changes in perfusion pressures are depicted in Fig. 7, B and C.

#### EFFECT OF CGS-LIKE DECREASE IN PERFUSION PRESSURE ON THE CONTRACTILE RESPONSE TO FLOW REDUCTION.

In the previous experiments, CGS-21680 elicited a fall in perfusion pressure as a result of A<sub>2A</sub>R-induced vasodilation. To investigate the effect of this fall in perfusion pressure independent of CGS-21680 and further differentiate the contractile from the vasodilatory effects of CGS-21680 the experiments were conducted as follows. The response to a 1-ml flow decrease was examined in addition to a manual reduction in flow that achieved a decrease in perfusion pressure comparable to that occurring in response to CGS-21680 administration. After initial stabilization, the heart was subjected to three 1-min periods of flow reduction separated by periods of normal perfusion (3.0 ml/min) for ~3 min, allowing a return of contractile function to

preflow reduction levels. The flow was then reduced to the extent required to produce the same decrease in perfusion pressure as observed with CGS-21680 administration (a decrease of 40–45 mmHg). After stabilization at this new baseline, three more periods of an additional flow decrease of 1 ml/min were administered. Changes in flow with expected changes in perfusion pressure are depicted in Fig. 7, D and E, respectively.

### **Data and Statistical Analysis**

When multiple ISO administrations or flow reductions were used, the responses were averaged. Data are presented as means  $\pm$  SE. Data were analyzed with the use of Prism (GraphPad Software, San Diego, CA) software. Additional statistical analysis was done by using StatMost (Dataxiom, Los Angeles, CA) software. Data were analyzed using one-way ANOVA, Student-Newman-Keuls multiple comparison test, and two-tailed t-test where appropriate. Values were taken to indicate a statistically significant difference at  $P < 0.05$ .

### **Materials**

ISO was dissolved in 0.1% sodium metabisulfite and diluted to the infusion concentration of  $10^{-6}$  M with MilliQ-treated water. CCPA, CGS-21680, ZM-241385, and DPCPX were prepared as 10 mM stock solutions in 100% DMSO and diluted with water to  $10^{-5}$  M that was used for infusion into the perfusion fluid. The resultant perfusion fluid DMSO concentration was not  $>0.05\%$ . Buffer salts were purchased from Fisher Scientific (Fairlawn, NJ). ISO and adenosine receptor agents CCPA, DPCPX, and CGS-21680 were obtained from Sigma-RBI (St. Louis, MO), and the  $A_{2A}R$  antagonist ZM-241385 was purchased from Tocris (Ellisville, MO). Custom primers and DNA reference ladder were acquired from Invitrogen (Carlsbad, CA). Precast 1% agarose

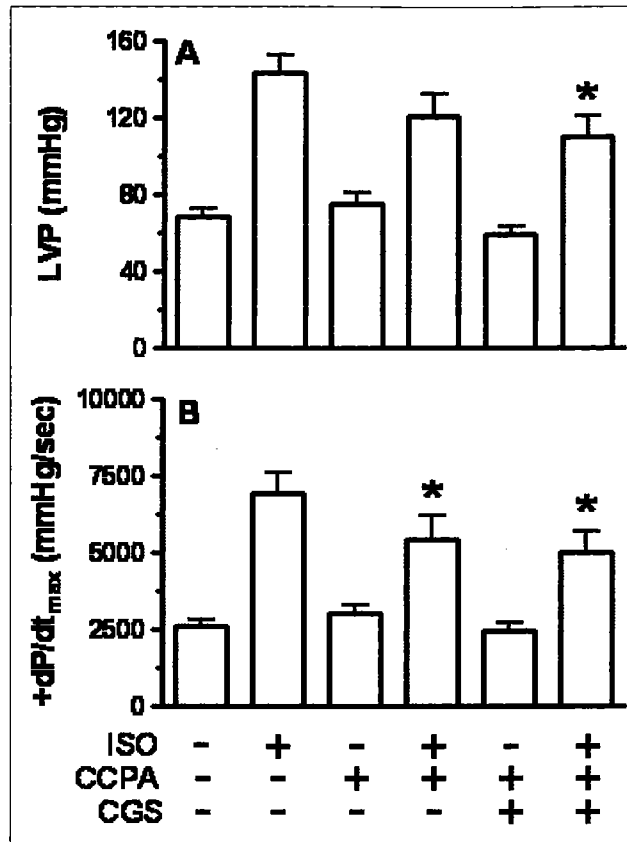
minigels with ethidium bromide were obtained from Bio-Rad (Hercules, CA). Qiagen DNEasy tissue kit and Qiagen Taq DNA polymerase Core kit were purchased from Qiagen (Valencia, CA).

## RESULTS

### *$\beta$ -Adrenergic-Stimulated Hearts*

#### Constant flow perfused wild type (WT) hearts

Effects of  $A_1R$  and  $A_{2A}R$  activation in  $\beta$ -adrenergic-stimulated hearts were investigated. In constant-flow perfused WT hearts, the administration of the  $A_1R$  agonist CCPA reduced the contractile response to ISO stimulation (Fig. 2). Although CCPA reduced the ISO-induced increase in LVP by 15%, this reduction was not statistically significant (Fig. 2A). However, the  $A_1R$  agonist significantly attenuated the ISO-induced increase in  $+dP/dt_{max}$  by 22% (Fig. 2B). The ISO responses in the presence of CGS-21680 (reductions of 23% and 28% for LVP and  $+dP/dt_{max}$ , respectively) remained significantly below those observed in the presence of ISO alone. These values were not significantly different from the CCPA plus ISO responses.

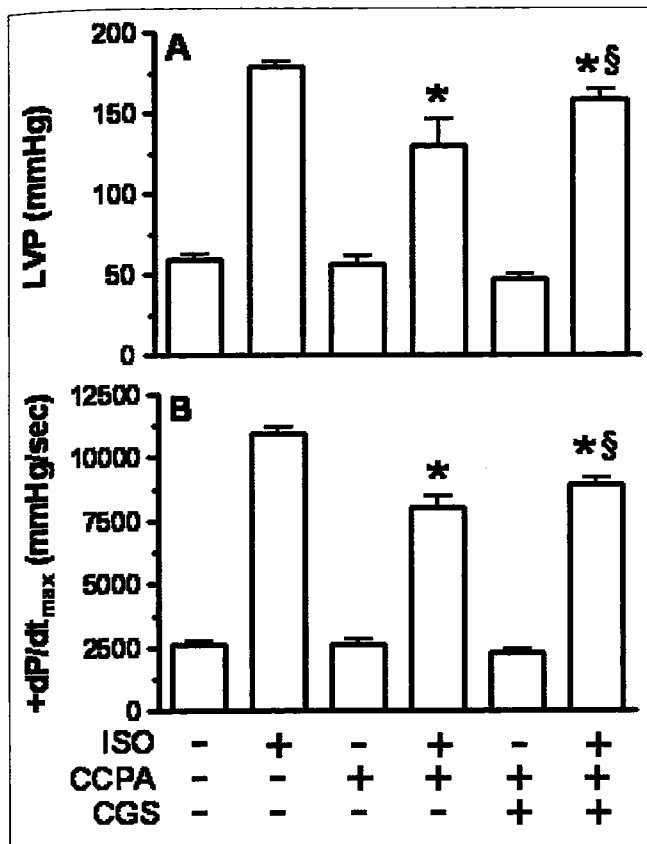


**Figure 2.** Effect of  $A_1R$  and  $A_{2A}R$  activation on left ventricular pressure (LVP; A) and maximum rate of LVP development ( $+dP/dt_{max}$ ; B) responses to  $\beta$ -adrenergic stimulation of the isolated, constant flow-perfused murine heart. ISO, isoproterenol ( $10^{-8}$  M); CCPA, 2-chloro- $N^6$ -cyclopentyladenosine,  $A_1R$  agonist ( $10^{-7}$  M); CGS, 2-P(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxyamidoadenosine ( $10^{-7}$  M; CGS-21680). Data are means  $\pm$  SE for 10 experiments. \*Statistically significant difference from ISO value.

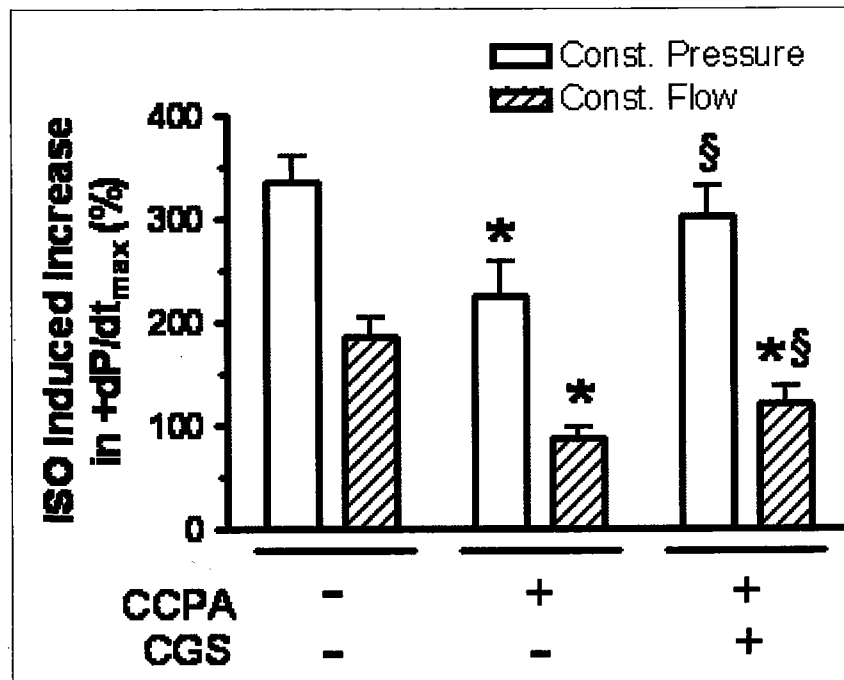
### Constant pressure perfused WT hearts

Experiments were repeated with constant perfusion pressure rather than constant flow (Fig. 3). CGS-21680 administration resulted in an attenuation of the antiadrenergic effect of A<sub>1</sub>R. With CCPA treatment the values for LVP (Fig. 3A) and +dP/dt<sub>max</sub> (Fig. 3B) with ISO stimulation were each reduced by 27%. The antiadrenergic effect of CCPA was fully reversed by an addition of the A<sub>1</sub>R antagonist DPCPX (data not shown). CGS-21680 attenuated the antiadrenergic effect of CCPA. In the presence of CGS-21680, CCPA only reduced the ISO-induced LVP and +dP/dt<sub>max</sub> responses by 12% and 18%, respectively. These values were significantly different from ISO responses observed with CCPA in the absence of CGS-21680. It is concluded that the absence of a CGS-21680 effect in the constant flow preparation (Fig. 2) resulted from the possible influence of a decrease in perfusion pressure resulting from A<sub>2A</sub>R-induced vasodilation. Although CCPA had no effect on perfusion pressure, CGS-21680 administration resulted in a 35% decrease in perfusion pressure (from 100 mmHg) compared with values for the control and CCPA.

The data were further examined as a percentage increase in +dP/dt<sub>max</sub> in response to ISO stimulation (Fig. 4). With data expressed in this manner, attenuation of the antiadrenergic effect of CCPA by CGS-21680 was observed for both constant flow and constant pressure preparations. CCPA reduced the ISO response by 34% and 53% for constant pressure and flow, respectively. However, the ISO responses were decreased only by 10% and 35%, respectively, in the presence of both CGS-21680 and CCPA.



**Figure 3.** Effect of A<sub>1</sub>R and A<sub>2A</sub>R activation on LVP (A) and +dP/dt<sub>max</sub> (B) responses to β-adrenergic stimulation of the isolated, constant pressure-perfused murine heart. Concentrations used are the following: ISO, 10<sup>-8</sup> M; CCPA, 10<sup>-7</sup> M; and CGS, 10<sup>-7</sup> M. Data are means ± SE for 5 experiments. \*Statistically significant difference from ISO value; §statistically significant difference from CCPA+ISO value.

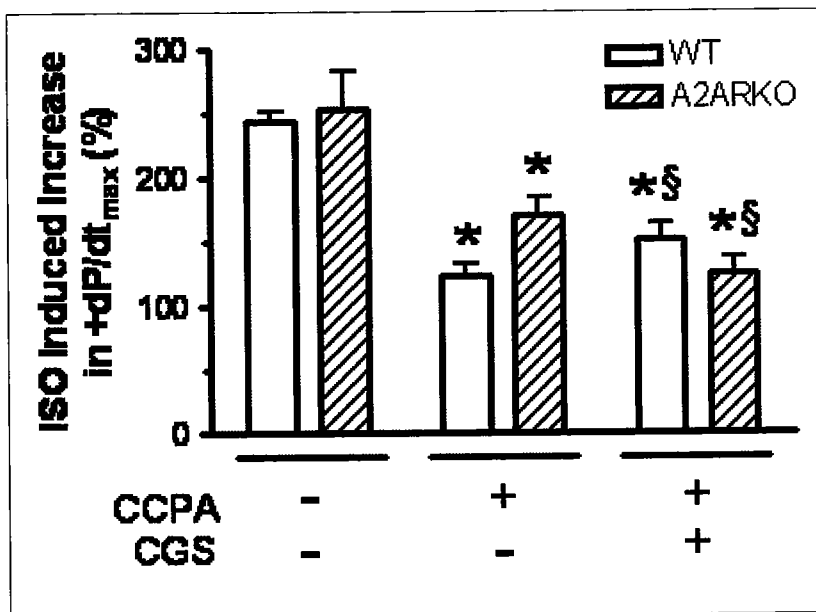


**Figure 4.** Effects of A<sub>1</sub>R and A<sub>2A</sub>R activation on β-adrenergic-induced contractile responses in the murine heart. Concentrations used are the following: ISO, 10<sup>-8</sup> M; CCPA, 10<sup>-7</sup> M; and CGS, 10<sup>-7</sup> M. Data are means ± SE for 10 experiments using constant (Const.) flow (Fig. 2) and 5 experiments using constant pressure (Fig. 3) perfusion. \*Statistically significant difference from respective control (ISO) value; §statistically significant difference from respective CCPA value.



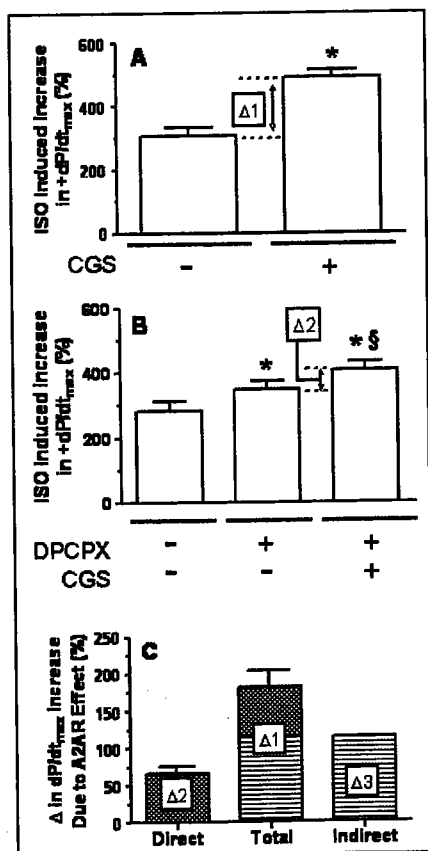
*Effects of A<sub>1</sub>R activation in WT versus A<sub>2A</sub>ARKO hearts.* The response to A<sub>1</sub>R activation, along with β-adrenergic stimulation in the absence and presence of A<sub>2A</sub>R stimulation, was compared between WT and A<sub>2A</sub>ARKO hearts (Fig. 5). In the WT heart, ISO stimulation resulted in a 244% increase in +dP/dt<sub>max</sub>. In the presence of CCPA, ISO only increased +dP/dt<sub>max</sub> by 123%. CGS-21680 attenuated this antiadrenergic action, as evidenced by an ISO+CCPA response of 151%.

In the A<sub>2A</sub>ARKO heart, ISO stimulation resulted in a 253% increase in +dP/dt<sub>max</sub>. In the presence of CCPA, ISO only increased +dP/dt<sub>max</sub> by 169%. CGS-21680 in the presence of CCPA resulted in an ISO response of only 125%. The ISO-induced increase in +dP/dt<sub>max</sub> after administration of both CCPA and CGS-21680 was not significantly different between A<sub>2A</sub>ARKO and WT hearts.



**Figure 5.** Effect of A<sub>1</sub>R and A<sub>2A</sub>R activation on the contractile response to β-adrenergic stimulation of constant flow-perfused WT and A<sub>2A</sub>ARKO murine hearts. Concentrations used are the following: ISO, 10<sup>-8</sup> M; CCPA, 10<sup>-7</sup> M; and CGS, 10<sup>-7</sup> M. Data are means ± SE for 6 experiments. \*Statistically significant difference from ISO value; §statistically significant difference from CCPA+ISO value.

*Direct versus indirect effect of A<sub>2A</sub>R in WT hearts.* To estimate the extent of the A<sub>2A</sub>R effect achieved by a direct increase in contractility, as opposed to its indirect effect through the inhibition of A<sub>1</sub>R, A<sub>2A</sub>R activation in the presence and absence of A<sub>1</sub>R inhibition was examined (Fig. 6). ISO alone resulted in a 308% increase in +dP/dt<sub>max</sub> (Fig. 6A). In the presence of CGS-21680, this response was increased to 490%, reflecting a 182 percentage-point ( $\Delta 1$ ) increase from ISO alone. This response is attributable to both the direct and indirect effects of the A<sub>2A</sub>R. In the presence of an A<sub>1</sub>R blockade by DPCPX, CGS-21680 resulted in an increase of only 67 percentage points ( $\Delta 2$ ) above that of the ISO response (Fig. 6B). It is assumed that the increase in the ISO response with CGS-21680 in the presence of A<sub>1</sub>R blockade by DPCPX is due to the direct effect of A<sub>2A</sub>R on contractility and that the direct and indirect effects of CGS-21680 are independent and additive. The indirect effect of A<sub>2A</sub>R acting on the A<sub>1</sub>R can be estimated by

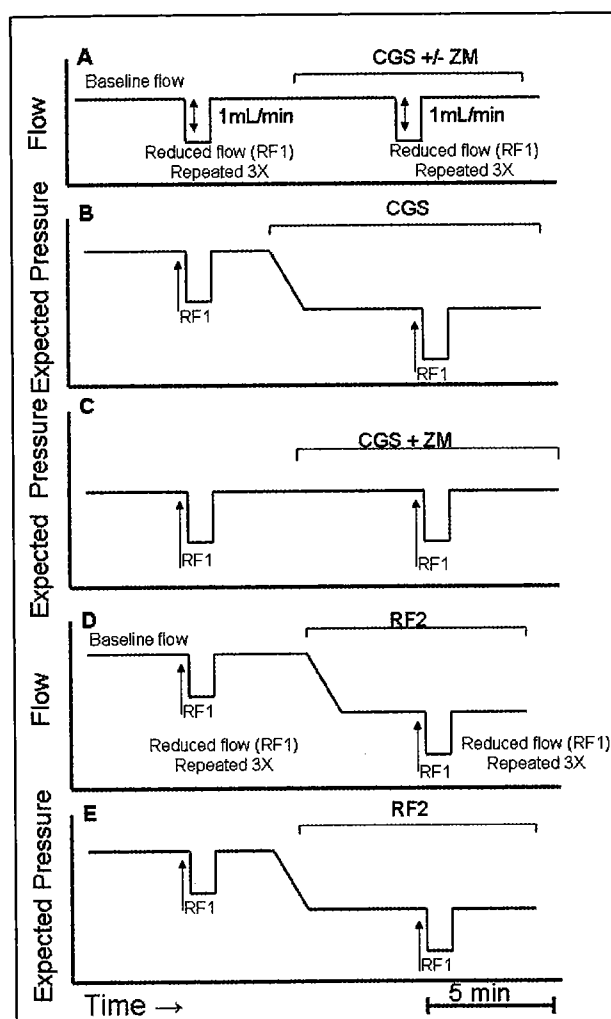


subtracting the direct effect from the value representing both direct and indirect influences ( $\Delta 3$ , Fig. 6C). Determined in this manner, the indirect effect of the A<sub>2A</sub>R through the inhibition of A<sub>1</sub>R is ~63% of the total A<sub>2A</sub>R effect.

**Figure 6.** A: effect of A<sub>2A</sub>R activation on the contractile response to  $\beta$ -adrenergic stimulation of constant flow-perfused murine heart.  $\Delta 1$ , total effect of A<sub>2A</sub>R. B: effect of A<sub>2A</sub>R activation on contractile response to  $\beta$ -adrenergic stimulation in presence of A<sub>1</sub>R blockade.  $\Delta 2$ , direct contractility effect of A<sub>2A</sub>R. C: approximation of A<sub>2A</sub>R effect attributable to its indirect action via A<sub>1</sub>R.  $\Delta 3$ ,  $\Delta 1 - \Delta 2$ , indirect effect of A<sub>2A</sub>R via A<sub>1</sub>R. Concentrations used are the following: ISO,  $10^{-8}$  M; 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX;  $10^{-7}$  M), an A<sub>1</sub>R antagonist; and CGS,  $10^{-7}$  M. Data are means  $\pm$  SE for 6 experiments. \*Statistically significant difference from ISO value; §statistically significant difference from DPCPX value.

## Reduced Flow Experiments

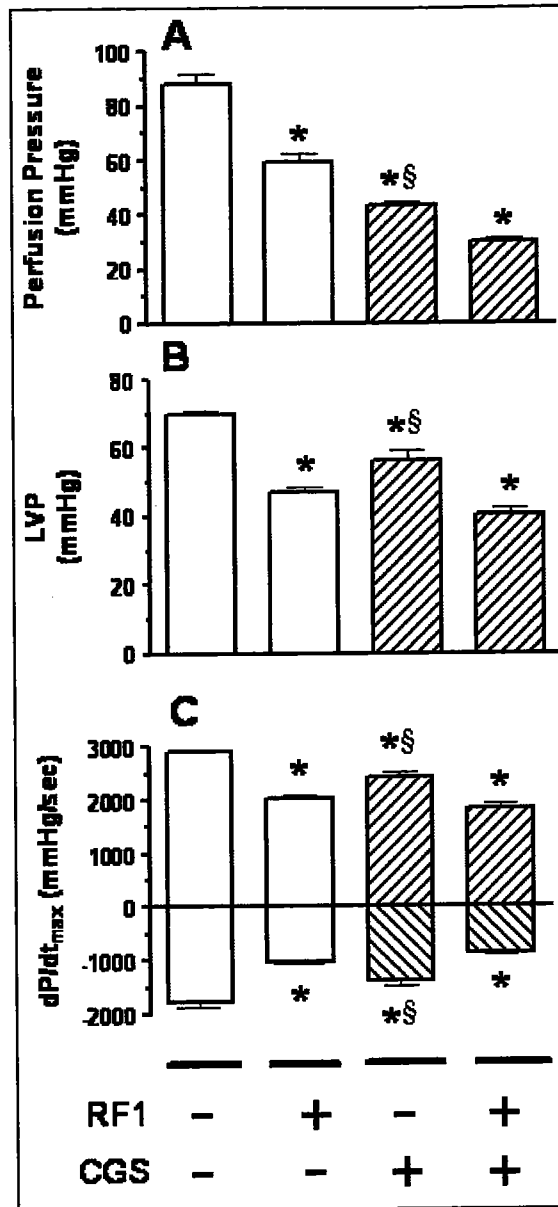
In the absence of  $\beta$ -adrenergic stimulation, the contractile response to  $A_{2A}R$  activation in an intact heart is relatively small (5–10%) compared with that at baseline (26). Furthermore, contractile activity is affected by the fall in perfusion pressure inherent with  $A_{2A}R$ -induced vasodilation in the constant flow preparation. As described in MATERIALS AND METHODS, flow reduction experiments were designed to investigate the effects of  $A_{2A}R$  activation in the absence of  $\beta$ -adrenergic stimulation (Fig. 7, A–C). Additional experiments were designed to examine the effect of the perfusion pressure decrease that occurs with CGS-21680-induced



vasodilation on contractile function (Fig. 7, D and E). A 3.0 ml/min rate of flow was chosen as baseline to permit maximal oxygenation and contractile function without the risk of edema that is caused by higher flow rates.

**Figure 7.** Schematic representation of protocol used to determine effect of  $A_{2A}R$  activation with CGS on contractility change occurring with flow decrease (A) and anticipated perfusion pressure response (B and C). Schematic representation of protocol where perfusion flow was decreased to simulate pressure drop observed with CGS (D) and anticipated perfusion pressure response (E). Baseline flow at stabilization is 3.0 ml/min. RF1, flow decrease of 1 ml/min, 1 min duration; RF2, flow decrease sufficient to lower perfusion pressure by 40–45 mmHg. Concentrations used are the following: CGS,  $10^{-7}$  M; 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM or ZM-241385), and  $A_{2A}R$  antagonist,  $10^{-7}$  M.

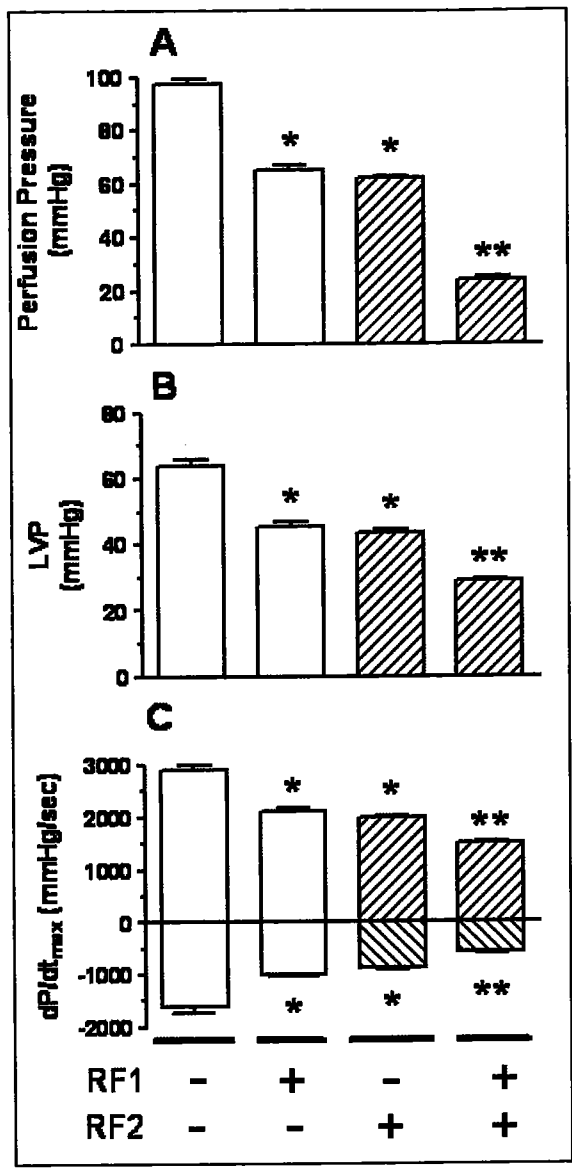
*A<sub>2A</sub>R effect on contractile function with reduced flow.* In the absence of CGS-21680, a flow reduction of 1 ml/min (RF1) resulted in a 32% decrease in perfusion pressure (Fig. 8A), which in turn led to significant 33%, 30%, and 43% decreases in LVP,  $+dP/dt_{max}$ , and  $-dP/dt_{max}$ , respectively, compared with control values (Fig. 8, B and C). In the presence of CGS-21680, a vasodilation elicited a drop in perfusion pressure of 45 mmHg. This represents a 50% decrease from the level observed in the absence of the A<sub>2A</sub>R agonist. This value was significantly lower than that observed after the flow decrease (RF1) in the absence of CGS-21680. In the presence of CGS-21680, the LVP and  $\pm dP/dt_{max}$  were significantly decreased from control values. However, these contractile parameters with CGS-21680 were significantly higher than those seen during RF1 without CGS-21680, despite the lower perfusion pressure. In the presence of CGS-21680, a 1 ml/min flow decrease resulted in a 32% decrease in perfusion pressure compared with the A<sub>2A</sub>R agonist alone (Fig. 8A). This reduction in perfusion pressure was associated with decreases of 27% in LVP (Fig. 8B), 23% in  $+dP/dt_{max}$ , and 39% in  $-dP/dt_{max}$  (Fig. 8C). To verify the CGS-21680 response as A<sub>2A</sub>R specific, experiments were repeated by using the A<sub>2A</sub>R antagonist ZM-241385. The previously observed effects of CGS-21680 were blocked by ZM-241385 (data not shown). These results indicate that the enhanced contractility observed with CGS-21680 is specific to A<sub>2A</sub>R activation and not a result of the decrease in perfusion pressure.



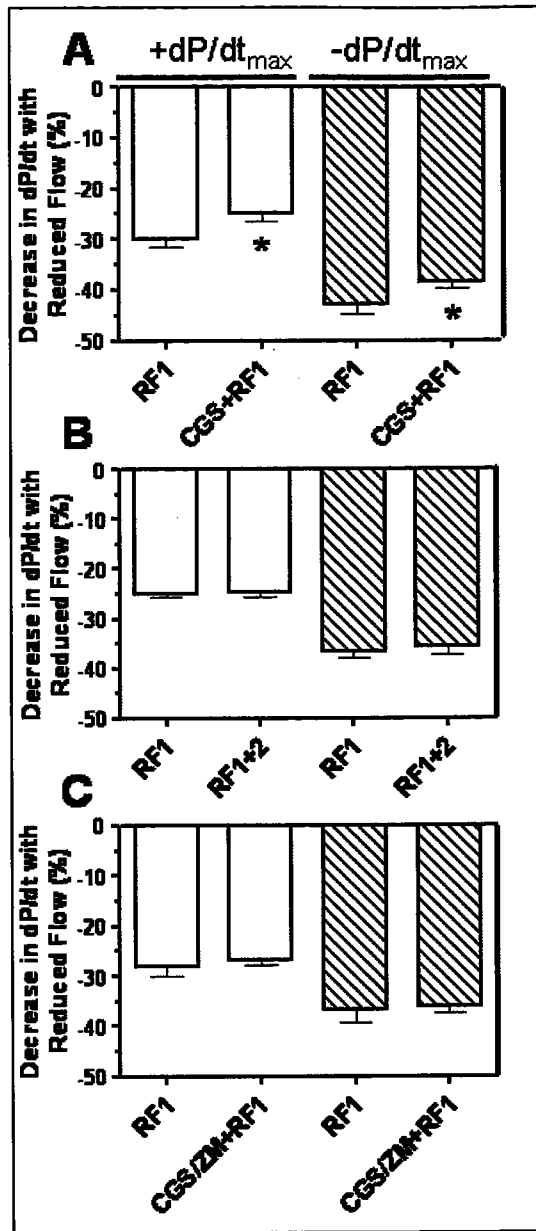
**Figure 8.** Effect of  $A_{2A}R$  activation on effects of a brief flow reduction (RF1) on perfusion pressure (A), LVP (B), and  $\pm dP/dt_{max}$  (C) of the constant flow-perfused murine heart. CGS concentration was  $10^{-7}$  M. Data are means  $\pm$  SE for 6 experiments. \*Statistically significant difference from corresponding control value; §statistically significant difference from RF1 value observed in absence of CGS.

*Effect of A<sub>2A</sub>R-comparable perfusion pressure reduction on contractile function.* Experiments were conducted to ascertain whether the changes in contractile response to flow reduction were due to the effect of CGS-21680 on contractility or as a result of the A<sub>2A</sub>R agonist-induced decrease in perfusion pressure. A protocol was designed with a similar sequence of flow decreases (RF1). However, instead of CGS-21680 administration, the flow was manually reduced (RF2) to the extent required to simulate the decrease in perfusion pressure of 40–45 mmHg that occurs with CGS-21680 (Fig. 8). The changes observed in LVP and  $\pm dP/dt_{\max}$  correlated closely with the decreases in perfusion pressure (Fig. 9). The flow reduction RF2 decreased perfusion pressure, LVP,  $+dP/dt_{\max}$ , and  $-dP/dt_{\max}$  by 36%, 32%, 32%, and 46%, respectively. The decreases in these four parameters showed no significant differences from those observed during RF1.

Further analysis of the data presented in Figs. 8 and 9 reveals that the contractile depression occurring with RF1 is attenuated by CGS-21680. In the absence of CGS-21680, RF1 resulted in decreases of 870 (36%) and 763 mmHg/s (43%) in  $+dP/dt_{\max}$  and  $-dP/dt_{\max}$ , respectively (Fig. 8C). However, after CGS-21680 administration RF1 resulted in decreases of 559 (23%) and 535 mmHg/s (38%) in  $+dP/dt_{\max}$  and  $-dP/dt_{\max}$ , respectively. A significant decrease in response to RF1 in the presence of CGS-21680 may also be observed when examining the percentage decrease in  $\pm dP/dt_{\max}$ , resulting from RF1 (Fig. 10A). The decreases (in %) presented in Fig. 10 are the means of the appropriate differences obtained from Figs. 8 and 9. Attenuation of the contractile response to RF1 observed in the presence of CGS-21680 (Fig. 10A) did not occur in preparations using a manual flow reduction to simulate CGS-21680-induced decrease in perfusion pressure (Fig. 10B). The attenuation of RF1 contractile depression with CGS-21680 was prevented by the A<sub>2A</sub>R antagonist ZM-241385 (Fig. 10C).



**Figure 9.** Effect of a CGS-like perfusion pressure drop (RF2) in conjunction with a brief flow reduction (RF1) on perfusion pressure (A), LVP (B), and  $\pm dP/dt_{max}$  (C) in the constant flow-perfused murine heart. Data are means  $\pm$  SE for 6 experiments. \*Statistically significant difference from control value; \*\*statistically significant difference from RF2 value alone.



**Figure 10.** Percentage decrease of  $\pm dP/dt_{max}$  in response to a brief flow reduction (RF1) in the presence of CGS (A; data from Fig. 8), manual flow decrease sufficient to achieve a perfusion pressure drop similar to CGS (RF2) (B; data from Fig. 9), or a combination of CGS and ZM (C). Concentrations used are the following: CGS,  $10^{-7}$  M; and ZM,  $10^{-7}$  M. RF1+2, combined flow decrease of RF2 and RF1. Data are means  $\pm$  SE for 6 experiments. \*Statistically significant difference from corresponding RF1 value.



## Discussion

### *A<sub>2A</sub>R and A<sub>1</sub>R Activation in Presence of $\beta$ -Adrenergic Stimulation*

The main finding of this study is that A<sub>2A</sub>R increases the contractile performance of the myocardium through both direct and indirect mechanisms in the  $\beta$ -adrenergic-stimulated mouse heart. The actions of A<sub>2A</sub>R also support myocardial contractility during reduced flow (low-flow ischemia). These results extend the previously reported observations that the A<sub>2A</sub>R can increase contractility in intact hearts and cardiomyocytes that are obtained from rats (4, 19, 26, 34). The A<sub>1</sub>R and A<sub>2A</sub>R interaction has also been reported previously in the rat heart (28) and isolated rat cardiomyocytes (34), although the exact mechanism by which this occurs remains unknown. The antiadrenergic action of A<sub>1</sub>R is thought to be mediated through multiple signaling mechanisms involving a decrease in adenylyl cyclase activity (18), reduction in calcium transients (12, 28), and increased PKC <sub>$\epsilon$</sub>  translocation (25). The inhibition of the A<sub>1</sub>R effect by the A<sub>2A</sub>R may occur by a modulation of any of these processes. Postulated mechanisms for the effects of the A<sub>2A</sub>R have included an activation of adenylyl cyclase (19), calcium-dependent and -independent mechanisms (4, 7, 33), as well as cAMP-independent mechanisms (4, 20). Based on the data presented, the effects of the A<sub>2A</sub>R can be considered as both direct and indirect. Direct effects involve the improvement of contractile performance through positive effects, such as an enhanced activation of adenylyl cyclase, whereas indirect effects involve the inhibition of the antiadrenergic effects of A<sub>1</sub>R. The direct effects of A<sub>2A</sub>R activation in the intact heart can be observed independently of  $\beta$ -adrenergic stimulation as described by Monahan et al. (26). However, the effect of A<sub>2A</sub>R activation becomes more pronounced when examined in the presence of  $\beta$ -adrenergic stimulation.

In both constant flow and constant pressure preparations, A<sub>2A</sub>R stimulation was observed to attenuate the antiadrenergic effects of A<sub>1</sub>R activation. The direct increase in contractile performance observed with the activation of the A<sub>2A</sub>R was consistent with previous reports (26) in which rat hearts were used. Interestingly, in the constant perfusion pressure preparation, A<sub>2A</sub>R activation appeared to have a greater effect than with constant flow. Although CGS-21680 significantly attenuated the effects of CCPA in both constant flow and constant pressure preparations, the +dP/dt<sub>max</sub> returned to control levels in the constant pressure preparation but remained significantly below control level in the constant flow preparation after treatment with CGS-21680 (Fig. 4). This observation is likely due to the increased perfusion flow and vascular filling seen with constant pressure perfusion. Increased regional tissue distension as a result of increased vascular filling may result in ventricular myocytes experiencing enhanced preload conditions (Water hose/Gregg effect; 16, 30).

With respect to the attenuation of the A<sub>1</sub>R effect by A<sub>2A</sub>R, the findings of the present study confirm those reported by Norton et al. (28) in the rat heart. It is possible, however, to further delineate the functional aspect of the A<sub>2A</sub>R effect into direct and indirect components. To estimate the extent to which each of these mechanisms of A<sub>2A</sub>R action occurs in the adrenergic-stimulated mouse heart, the contractile response to CGS-21680 stimulation of the A<sub>2A</sub>R was compared in the presence and absence of A<sub>1</sub>R inhibition. The ISO response in the presence of CGS-21680 is assumed to be affected by both mechanisms in an additive fashion, i.e., A<sub>2A</sub>R is able to both inhibit the manifestation of A<sub>1</sub>R effects and increase contractility directly. The direct action of A<sub>2A</sub>R can be revealed by pretreating the heart with the A<sub>1</sub>R antagonist DPCPX before A<sub>2A</sub>R stimulation. The resulting increase in the contractile response to ISO in this case is due to the direct effect of the A<sub>2A</sub>R on contractility. The observed increase in the ISO response with

CGS-21680 was reduced after pretreatment with DPCPX (Fig. 6). The additional increase in the ISO response was approximately one third of the response seen when CGS-21680 was administered without prior A<sub>1</sub>R inhibition ( $\Delta 1$  vs.  $\Delta 2$ ; Fig. 6). These calculations suggest that a major part of the A<sub>2A</sub>R effect is mediated through the inhibition of the A<sub>1</sub>R (indirect effect) as opposed to its direct effect on contractility. This conclusion is consistent with the observation that A<sub>2A</sub>R activation only has a minor effect on contractility (5–10%) in the absence of adrenergic stimulation. In the presence of adrenergic stimulation where the A<sub>1</sub>R plays a significant role in attenuating the adrenergic response, the A<sub>2A</sub>R exerts a more profound inhibitory effect on the antiadrenergic action of A<sub>1</sub>R. Thus the interaction of the A<sub>2A</sub>R and the A<sub>1</sub>R is of greater importance in the adrenergic-stimulated heart.

#### *A<sub>1</sub>R and A<sub>2A</sub>R Stimulation Response: Comparing Knockout and WT Hearts*

There were two findings of interest when comparing the responses of A<sub>1</sub>R and A<sub>2A</sub>R activation in WT and A<sub>2A</sub>RKO hearts. First, the observed antiadrenergic response to A<sub>1</sub>R activation was greater in the WT than in the A<sub>2A</sub>RKO hearts (Fig. 5). The ISO responses observed were not markedly different between WT and A<sub>2A</sub>RKO hearts, whereas there was approximately a 50 percentage point difference between the average ISO response levels in the presence of CCPA. These findings conflict with previous reports indicating that in a WT rat heart, the inhibition of A<sub>2A</sub>R with ZM-241385 increased the antiadrenergic effects of A<sub>1</sub>R activation (28). The absence of the A<sub>2A</sub>R in the A<sub>2A</sub>RKO heart should result in a situation similar to that where the A<sub>2A</sub>Rs are inhibited pharmacologically. It would be expected that the A<sub>2A</sub>RKO would be more responsive to the antiadrenergic effect of A<sub>1</sub>R. However, the opposite was observed in the present experiments. It is possible that this is due to a modification of A<sub>1</sub>R

signaling in the  $A_{2A}$ RKO. However, the study of vascular smooth muscle responses to adenosine analogs in the  $A_{2A}$ RKO mouse suggested no adaptations (29). The reason for the presently observed enhancement of the  $A_1$ R response in  $A_{2A}$ RKO hearts remains to be explored.

The second notable difference between the WT and  $A_{2A}$ RKO hearts was the response to the CGS-21680 and CCPA combination. In the WT hearts, CGS-21680 produced an increase in contractile response to ISO from that observed in the presence of CCPA, as expected. However, in the  $A_{2A}$ RKO hearts, there was a further decrease in ISO contractile response after treatment with CCPA and CGS-21680 together. A possible explanation is an interaction between CGS-21680 and the  $A_1$ R. A binding of CGS-21680 to tissues in an  $A_1$ R-dependent manner has been reported in the mouse brain (15, 23). The exact nature of this interaction between CGS-21680 and  $A_1$ R remains unknown. The observed decrease in the ISO contractile response seen with CGS-21680 beyond that with CCPA alone may occur in the  $A_{2A}$ RKO hearts, because CCPA together with CGS-21680 activates the  $A_1$ R to a greater extent.

#### *$A_{2A}$ R Supports Myocardial Contractility With Low-Flow Ischemia*

To study the importance of  $A_{2A}$ R activation in providing contractile support in a nonadrenergic-stimulated heart and the role of vasodilation in the contractile effects of  $A_{2A}$ R activation, an experimental protocol was used where the response to brief periods of low-flow ischemia was examined. The effect of  $A_{2A}$ R on contractility in the absence of adrenergic stimulation is small. In the current protocol, where a decrease in flow rather than adrenergic stimulation was applied, it was possible to examine the effect of  $A_{2A}$ R activation on contractility even in the absence of adrenergic stimulation. There were two main findings in this series of experiments. First,  $A_{2A}$ R activation resulted in a higher level of observed contractile performance

at a given perfusion pressure. After the administration of CGS-21680, vasodilation resulted in a perfusion pressure significantly lower than that observed with the 1-ml flow decrease (RF1, Fig. 8A). Despite this result, the observed LVP and  $\pm dP/dt_{\max}$  were both higher than that observed after RF1 in the absence of CGS-21680. This indicates that in the presence of  $A_{2A}R$  stimulation, myocardial contractility is enhanced as evidenced by the increased LVP and  $\pm dP/dt_{\max}$  at a given perfusion pressure. This effect was fully reversible with the  $A_{2A}R$  antagonist ZM-241385 and thus was attributable to  $A_{2A}R$ .

The second observation of interest was that the actual decrease in contractility observed during the administered periods of low-flow ischemia (RF1) was significantly less in the presence of  $A_{2A}R$  stimulation with CGS-21680. This difference was most clearly visible with respect to  $\pm dP/dt_{\max}$  (Fig. 10). To determine whether this observation was due to  $A_{2A}R$  contractile effects, as opposed to the result of the decreased perfusion pressure resulting from CGS-21680-induced vasodilation, another series of experiments used a manual decrease in flow (RF2, Fig. 9) to simulate the decrease in perfusion pressure caused by CGS-21680 administration. This manual perfusion pressure decrease did not attenuate the extent of contractile function reduction seen with flow drop RF1. In fact, the attenuation of response to flow reduction was only seen with CGS-21680 and was prevented by the  $A_{2A}R$  antagonist ZM-24138.

In summary, this study has found that the  $A_{2A}R$  enhances the contractile response to  $\beta$ -adrenergic stimulation in the murine heart directly through an effect on contractility and indirectly by an attenuation of the antiadrenergic actions of the  $A_1R$ . In addition,  $A_{2A}R$  activation supports contractile function during low-flow ischemia, resulting in an increased contractile function at the given reduced perfusion pressure.

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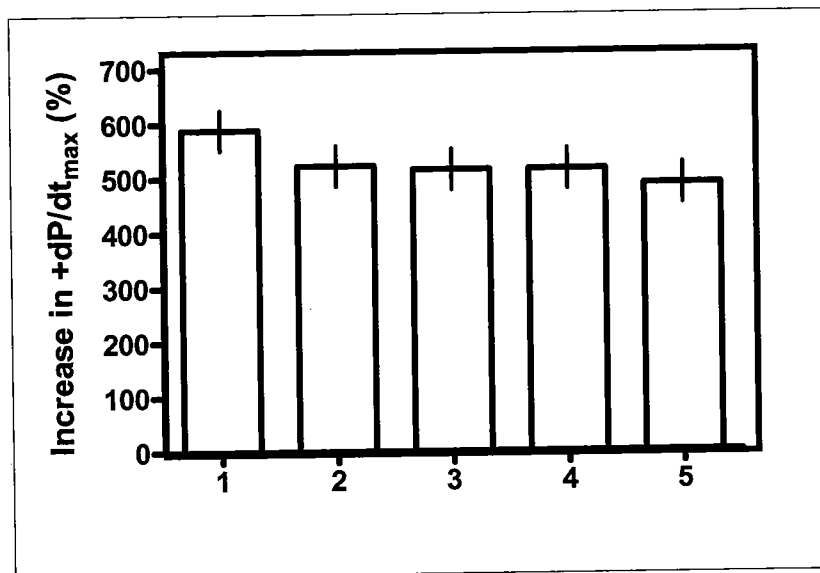
### **Supplemental Material**

Additional preliminary study and supporting experiments for the preceding manuscript are included in this section.

#### *Refractoriness of repeated isoproterenol (ISO) stimulation in isolated hearts*

One of the concerns with the protocols utilized in the present study is the employment of repeated ISO stimulations. This protocol design allowed each heart to be used as its own control. The alterations seen in contractile response to  $\beta$ -adrenergic stimulation following adenosine receptor activation could be then compared immediately in each heart during the experiment to verify the appropriate response to the agent administered. However, with repeated  $\beta$ -adrenergic stimulation desensitization is known to occur (45,90) and the myocardial increase in contractility may be reduced following multiple stimulations. In an experiment designed to examine effects of adenosinergic modulation of contractility, such desensitization effects would result in confounding of the parameter examined. To verify that four ISO administrations in a typical protocol would not result in notable desensitization, experiments were carried out exposing the heart to repeated ISO stimulations on a time scale that was used in the other studies. In addition longer infusions of ISO were also used with the rationale that if desensitization would not be significant following 1 or 2 min ISO infusions, the 30-s ISO infusions would be even less likely to produce desensitization. The results of a representative desensitization study are presented in

supplementary figure S1. Although the response to the second stimulation was observed to be 11% lower than the first, this decrease was not statistically significant. Additional stimulations subsequently resulted in only a further 6% decrease by the 5<sup>th</sup> ISO administration. No significant desensitization occurred following five ISO infusions of 2-min duration. Because only four 30-s infusions per heart were actually used in the studies of contractility outlined in the preceding manuscript, it is concluded that refractoriness to ISO stimulation doesn't influence the interpretation of obtained data.



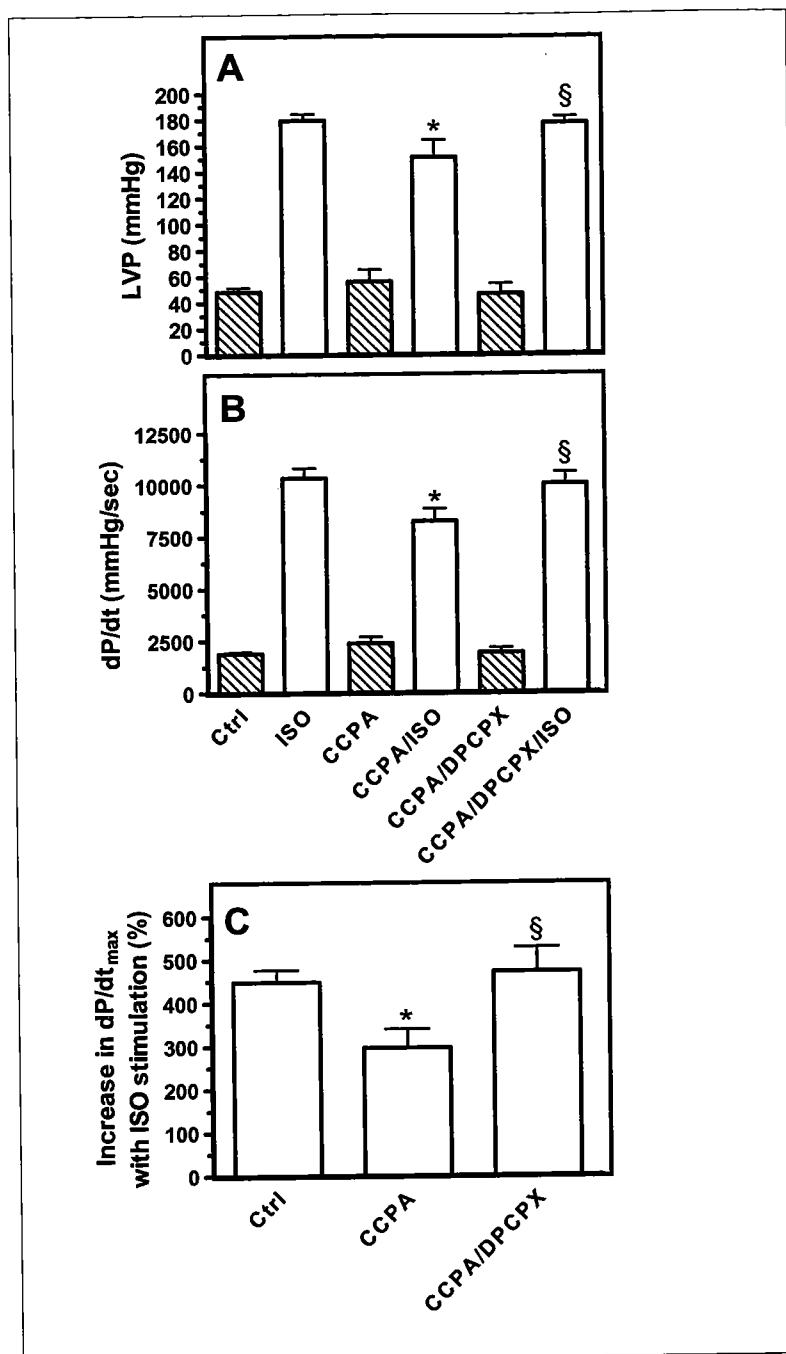
**Figure S1.** Typical contractile response of an isolated constant flow perfused WT murine heart to multiple 2-min stimulations with isoproterenol (ISO,  $10^{-8}$  M). The heart is allowed to stabilize following each ISO administration.

#### *Specificity of A<sub>1</sub>R antiadrenergic response*

In order to verify the reduction in adrenergic-induced contractile responses following administration of CCPA as A<sub>1</sub>R specific, studies were carried out using the A<sub>1</sub>R antagonist DPCPX to block the antiadrenergic action of A<sub>1</sub>R activation (Supplementary Figure S2). DPCPX

administration restored the contractile response to ISO stimulation to levels comparable with those observed in the absence of A<sub>1</sub>R activation. These findings were noted in direct measurements of LVP (Fig. S2 A) and +dP/dt<sub>max</sub> (Fig. S2 B) as well as in the calculated percentage increase of +dP/dt<sub>max</sub> (Fig. S2 C). These results indicate that the decrease in contractile response to ISO stimulation observed following CCPA-induced A<sub>1</sub>R activation is indeed specific to the A<sub>1</sub>R.

As mentioned previously, there are no alterations in contractile function at baseline prior to ISO stimulation as a result of A<sub>1</sub>R stimulation. The observed LVP and +dP/dt<sub>max</sub> levels appear fairly consistent at all points in the experiment. In the isolated heart model, the administration of an A<sub>1</sub>R agonist results in bradycardia as the A<sub>1</sub>R receptor can result in less efficient atrioventricular conduction (42). However, in a heart being electrically paced this effect of A<sub>1</sub>R activation is not visible. With ISO stimulation the antiadrenergic effect of A<sub>1</sub>R activation is clearly evident. These observations suggest that the isolated perfused heart is an appropriate model for study of adenosinergic modulation of contractile response to adrenergic stimulation. In order to study adenosinergic influence and receptor effect interactions on contractility in non-adrenergic conditions it would be advisable to either use an alternate stimulus, or conduct the appropriate studies in isolated cardiomyocytes where the response to adenosinergic stimulation can be measured with greater precision on a smaller scale.



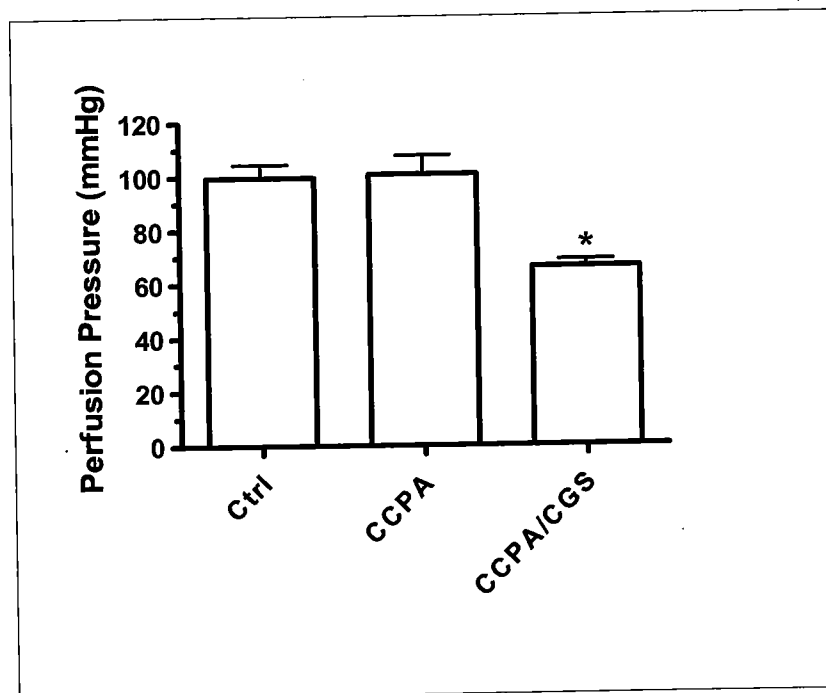
**Figure S2.** Effect of isoproterenol (ISO  $10^{-8}$  M) stimulation on LVP (A),  $+dP/dt_{max}$  (B) and percentage increase in  $+dP/dt_{max}$  (C) in an isolated, constant pressure perfused, murine heart preparation in the absence and presence of CCPA or a CCPA/DPCPX ( $10^{-7}$  M) combination. Data presented are means  $\pm$  SE for 8 experiments. \* denotes statistically significant difference from the corresponding control value; ISO in (A) and (B), Ctrl in (C). § denotes statistically significant difference from the CCPA/ISO value in (A) and (B), and from the CCPA value in (C).

*Effects of A<sub>2A</sub>R stimulation are dependent upon adenosinergic-induced changes in perfusion pressure*

One of the initial observations made during the present study was that under constant flow conditions A<sub>2A</sub>R activation did not appear to attenuate the antiadrenergic effects of A<sub>1</sub>R activation. (See Figure 2 in preceding manuscript.) As described in the preceding manuscript, this finding was subsequently attributed to the decreased perfusion pressure resulting from A<sub>2A</sub>R activation-induced vasodilation. As demonstrated in supplementary Figure S3, the activation of A<sub>1</sub>R with CCPA does not have a notable effect on the perfusion pressure. However, following the administration of both CCPA and CGS-21680 there is a clear reduction in the measured perfusion pressure. Thus A<sub>2A</sub>R stimulation elicits a vasodilation of the coronary vasculature. The observed vasodilatory effect resulted in a 25% decrease in perfusion pressure from the control value.

The decrease in the perfusion pressure following A<sub>2A</sub>R activation results in lower absolute LVP and  $\pm dP/dt_{\max}$  measurements making direct comparison of these values difficult between hearts treated with CGS-21680 and those treated with other adenosinergic agents. Ultimately this issue was resolved via comparison of percentage increases in contractile function (i.e.  $+dP/dt_{\max}$ ) as opposed to direct comparison of measured LVP or  $dP/dt_{\max}$  values. As discussed in the manuscript, this type of analysis results in the same trends visible between the constant flow and constant pressure preparations once the analysis is applied. (See Figures 3 and 4 in preceding manuscript.) An additional conclusion that can be made from these observations is that the decreased perfusion pressure observed in the presence of A<sub>2A</sub>R stimulation-induced vasodilation does not appear to effect contractile function in the same way as a decrease in perfusion pressure resulting from decreased flow. The heart is able to demonstrate a higher level

of contractile response to adrenergic stimulation in the presence of  $A_{2A}R$  stimulation despite the lower observed perfusion pressure, whereas contractile function is not preserved in the absence of  $A_{2A}R$  stimulation. These findings are further supported by the results of the flow decrease experiments.



**Figure S3.** Effect of CCPA or CCPA/CGS ( $10^{-7}$  M) combination on the average perfusion pressure in a constant flow perfused, isolated murine heart preparation. Data presented are means  $\pm$  SE for 10 experiments. \* denotes statistically significant difference from the corresponding control (Ctrl) value.

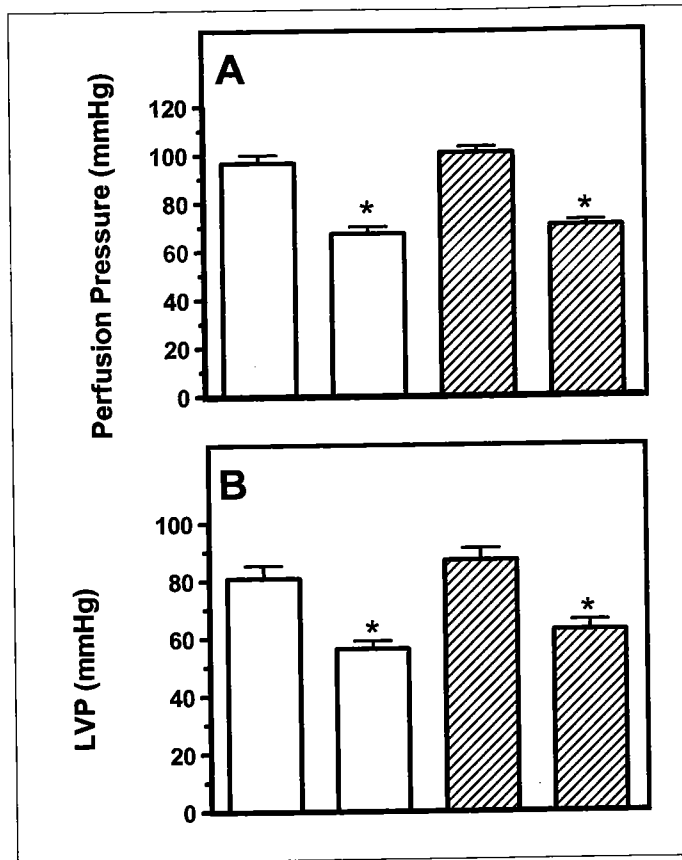
#### *Specificity of $A_{2A}R$ -mediated attenuation of response to flow reduction*

One of the main findings of the reduced flow experiments was that  $A_{2A}R$  activation results in an attenuated contractile depression in response to flow reduction. Hearts pretreated with CGS-21680 displayed a smaller reduction in LVP as well as  $\pm dP/dt_{\max}$  in response to a flow



decrease (See Figures 8 and 10 in preceding manuscript.). In order to confirm these effects as specific to CGS-21680-mediated  $A_{2A}R$  activation, experiments were conducted to block this effect by utilizing a combination of both CGS-21680 and ZM-241385, an  $A_{2A}R$  antagonist. The results are summarized in supplementary Figure S4. Treatment of the hearts with both CGS and ZM resulted in no observable differences between control and treated groups for both perfusion pressure (Fig. S4 A) and LVP (Fig. S4B).

These findings show that the attenuation of contractile response to flow reduction observed with CGS-mediated  $A_{2A}R$  activation is receptor specific. In addition the absence of observed perfusion pressure differences suggests that the addition of ZM is effective in blocking CGS from activating the  $A_{2A}R$ , as one of the first and most easily noted responses observable following  $A_{2A}R$  activation is a profound decrease in perfusion pressure.



RF1	-	+	-	+
CGS/ZM	-	-	+	+

**Figure S4.** Effect of a flow reduction of 1 ml/min (RF1) on perfusion pressure (A), and LVP (B) in an isolated, constant flow perfused, murine heart preparation in the absence and presence of a CGS/ZM ( $10^{-7}$  M) combination. Data presented are means  $\pm$  SE for 6 experiments. \* denotes statistically significant difference from the corresponding control value; Ctrl and CGS/ZM respectively.

## Summary of Key Findings

- 1) A<sub>1</sub>R and A<sub>2A</sub>R play an important role in modulating the response to β-adrenergic stimulation in the murine heart.
- 2) A<sub>1</sub>R exhibits an antiadrenergic effect upon activation, reducing contractile response to β-adrenergic stimulation. Interestingly this effect appears to be less pronounced in the A<sub>2A</sub>R knockout animals.
- 3) A<sub>2A</sub>R attenuates the antiadrenergic action of A<sub>1</sub>R by inhibiting the A<sub>1</sub>R effect and also by directly increasing contractility.
- 4) In the setting of decreased coronary flow A<sub>2A</sub>R activation results in enhanced contractile performance. Contractile activity observed to be higher for a given perfusion pressure in the presence of A<sub>2A</sub>R activation.
- 5) A<sub>2A</sub>R activation decreases the acute response to decreased coronary flow, maintaining a higher level of contractile function. This suggests that the activation of this receptor may be a cardioprotective mechanism in conditions of ischemia or hypoxia.

Adenosinergic influence on protein phosphatases: role of PP2A as submitted for publication in the manuscript entitled: "**Adenosine A<sub>1</sub> and A<sub>2A</sub> Receptor Regulation of Protein Phosphatase 2A in the Murine Heart**" submitted to American Journal of Physiology: Heart and Circulatory Physiology 2006, 22 May. In review.

The best known mechanism for the modulation of cardiac contractility by the A<sub>1</sub>R and A<sub>2A</sub>R is the inhibition or activation of adenylyl cyclase, with subsequent alterations in the cAMP content of the cardiomyocyte (36,96). However, this pathway is not exclusive and a number of other mechanisms have been proposed as well (56,94). If the contractile response to β-adrenergic stimulation and its enhancement by the A<sub>2A</sub>R is mediated in part by the phosphorylation of contractile and regulatory proteins (22) it is conceivable that the antiadrenergic effect. In fact one of the more recent findings is that a significant part of the A<sub>1</sub>R antiadrenergic effect is mediated by the activation of protein phosphatase 2A (PP2A) (60,61).

From previous observations of the effects of the A<sub>2A</sub>R in both literature and the study presented in the preceding manuscript, it was concluded that the A<sub>2A</sub>R is able to attenuate the effects of A<sub>1</sub>R (78). It would be logical to consider whether one of the focal points for A<sub>1</sub>R and A<sub>2A</sub>R interaction is co-regulation of the activity of this protein phosphatase. Thus the goal of the following manuscript was to explore the possible effects of the A<sub>2A</sub>R on PP2A and elucidate the interaction of the A<sub>1</sub>R and A<sub>2A</sub>R at this level. In addition, the A<sub>2A</sub>R knockout model was utilized to study the effects of A<sub>1</sub>R on PP2A in the absence of the influence of the A<sub>2A</sub>R.

Previous research focusing on A<sub>1</sub>R-mediated modulation of PP2A activity utilized the quantification of the carboxymethylation state of the enzyme as an indicator of its activity level. It was presumed that once the enzyme was appropriately modified it would translocate to the site of action and dephosphorylate its target proteins. This observation was supported by measured changes in the phosphorylation state of contractile proteins within the cardiomyocytes (60).

While this approach is quite functional, in the following study the goal was to take this approach

one step further and actually measure the activity of the PP2A directly. In order to achieve this, an assay was utilized where the PP2A in myocardial extracts was allowed to dephosphorylate a specific substrate under conditions optimal for the activity of this phosphatase. This method allows a more direct examination of the activity of PP2A as there is no longer an assumption about its activity state, but rather the activity is explicitly measured.

### **Abstract**

Adenosine plays a role in regulating the contractile function of the heart. This includes a positive inotropic action via the adenosine  $A_{2A}$  receptor ( $A_{2A}R$ ) and an inhibition of  $\beta_1$ -adrenergic receptor-induced inotropy (antiadrenergic action) via the adenosine  $A_1$  receptor ( $A_1R$ ). Phosphatase activity has also been shown to influence contractile function by affecting the level of protein phosphorylation. Protein phosphatase 2A (PP2A) plays a significant role in mediating the  $A_1R$  antiadrenergic effect. The purpose of this study was to investigate the effects of  $A_{2A}R$  and  $A_1R$  on the activities of PP2A in tissues from wild type (WT) and  $A_{2A}R$  knockout ( $A_{2A}RKO$ ) hearts. PP2A activities were examined in myocardial cytoplasm and membrane fractions by measuring phosphate released from a phosphorylated substrate. In WT hearts, treatment with the  $A_1R$  agonist CCPA resulted in an increased PP2A activity and an enhanced localization of active PP2A to the membrane fraction. The  $A_{2A}R$  agonist CGS-21680 decreased PP2A activity and enhanced PP2A localization to the cytosolic fraction. In  $A_{2A}RKO$  hearts the response to  $A_1R$  activation was markedly enhanced whereas the response to  $A_{2A}R$  activation was negligible. In addition control and CGS-21680 treated  $A_{2A}RKO$  hearts demonstrated a significantly reduced PP2A activity level and localization to the membrane fraction. These data

show that A<sub>2A</sub>R and A<sub>1</sub>R regulate PP2A activity thus suggesting an important mechanism for modulating myocardial contractility.

## Introduction

Adenosine (ADO) plays an important role in modulating cardiac function by regulating the contractile responsiveness of the myocardium to adrenergic stimulation (8,40). Of the four subtypes of adenosine receptors identified, the A<sub>1</sub> and A<sub>2A</sub> receptors (A<sub>1</sub>R and A<sub>2A</sub>R, respectively) are thought to be primarily involved in this process (10,11,15,27,33,43). The decrease in contractile response to adrenergic stimulation elicited by A<sub>1</sub>R activation is known as an antiadrenergic action (2,3,9). The A<sub>2A</sub>R have been shown not only to attenuate this antiadrenergic action (37,45), but also to increase contractility directly (11,33,45) and independently of  $\beta$ -adrenergic stimulation (33).

Contractility in the myocardium is closely related to the degree of phosphorylation achieved by various cellular proteins. These include proteins such as troponin I, phospholamban and others that are involved in force development, calcium homeostasis, and mechanisms of receptor signaling (12,14,16,22,41,42,44). The antiadrenergic action of A<sub>1</sub>R has been shown to reduce the level of phosphorylation attained by a number of cellular proteins during adrenergic stimulation (16,19,21,29). This action of adenosine is thought to be mediated, in part, by G<sub>i</sub> proteins with subsequent inactivation of adenylyl cyclase previously activated by  $\beta_1$ -adrenergic receptors ( $\beta_1$ R) (7,28). The resultant reduction in cellular cyclic AMP levels sequentially reduces cellular protein phosphorylation by decreasing the  $\beta_1$ R activated protein kinase A (PKA) activity (6,18). Another protein kinase, PKC<sub>e</sub> has also been reported to be important in the antiadrenergic

action of A<sub>1</sub>R (32). Thus reduced phosphorylation of target cellular proteins is one mechanism by which adenosine exerts an antiadrenergic action.

Enhanced dephosphorylation of proteins previously phosphorylated in response to β<sub>1</sub>R stimulation can be proposed to reduce the adrenergic-induced contractile response. Pharmacological inhibition of protein phosphatases with inhibitors such as cantharidin has been shown to result in an enhanced protein phosphorylation and increased contractile force in myocardial preparations (13,25,34-36). In addition, studies have demonstrated that while overexpression of the catalytic subunit of the serine/threonine protein phosphatase 2A (PP2A) impairs cardiac function (20), elevated expression of protein phosphatase inhibitors PPI-1 and PPI-2 results in cardiac function enhancement (24,38). Recent studies have reported that the A<sub>1</sub>R is able to activate the protein phosphatase 2A (PP2A) and alter its localization within the cell resulting in the decreased phosphorylation of proteins related to contractile function (29,30).

Although reports suggest that A<sub>1</sub>R mediates its effects in part by the activation of PP2A (29,30), it is presently unknown whether A<sub>2A</sub>R modifies A<sub>1</sub>R-induced changes in PP2A activity, directly decreases the PP2A activity or both. An additional rationale for the following study is that A<sub>2A</sub>R has been shown to modulate neutrophil superoxide anion generation through activation of serine/threonine protein phosphatases (39). Such a finding suggests the possibility that A<sub>2A</sub>R may have an effect on PP2A in the myocardium as well.

The purpose of the following study was to examine the effects of A<sub>2A</sub>R and A<sub>1</sub>R stimulation on the PP2A activity in ventricular samples from both wild type (WT) and A<sub>2A</sub>R knockout (A<sub>2A</sub>RKO) mice. The interaction between these two adenosine receptor subtypes in affecting PP2A activity was also examined. In addition the effects of A<sub>2A</sub>R and A<sub>1</sub>R stimulation on the phosphorylation state of Tyr 307, one of the regulatory sites of PP2A (23), were studied.

## Materials and Methods

### Experimental Animals

Six- to eight-week-old wild type C57BL/6 male mice were purchased from Taconic Farms (Hudson, NY). A<sub>2A</sub>ARKO mice were obtained from a colony maintained by our laboratory and were generated and genotyped as previously described (4,43). Briefly, total DNA was isolated from tail clippings, amplified using PCR, resolved using electrophoresis on ethidium bromide containing agarose gels and visualized under UV illumination. The animals in this study were maintained and used in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institute of Health (NIH Publication No. 85-23, Revised 1996) and evaluated and approved according to the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School, Worcester MA.

### Isolated Heart Preparation

Mice were euthanized by decapitation and the hearts were excised. Following excision the hearts were rapidly rinsed in room temperature saline, mounted on the perfusion apparatus and perfused via the aorta with a physiological saline solution (PSS) containing (in mM): 118.4 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, and 10 dextrose. The PSS temperature was held at 37°C and the pH was maintained at 7.4 by bubbling the solution continuously with a 95% O<sub>2</sub>-5% CO<sub>2</sub> gas mixture. The perfusion flow rate was held constant at 2.8 ml/min. The developed left ventricular pressure (LVP) of the hearts was monitored using a cannula tipped with a water-filled polyethylene balloon connected to a pressure transducer and inserted into the left ventricle through the mitral valve following a left atriotomy. Perfusion



pressure was monitored using a transducer attached to a sidearm of the perfusion assembly. Pacing of the heart (3 V at 480 stimulations/min; Grass, Quincy, MA) was accomplished via leads on the perfusion cannula and the pulmonary artery. All agents were infused into the perfusion cannula using an infusion pump (model 22, Harvard Apparatus, Holliston, MA) operating at 1.0% of the perfusate flow rate to achieve the final desired perfusate concentration. Hearts were submerged in 37°C PSS for the duration of the perfusion. Data was recorded using a model RS-3400 Gould polygraph (Chandler, AZ). Hearts failing to develop a LVP of at least 40 mmHg were excluded from further study. The hearts were perfused with PSS containing the agent of interest for a period of 15 min. The heart was rapidly removed from the perfusion apparatus, freeze-clamped and stored in liquid nitrogen prior to being assayed.

#### Effects of A<sub>1</sub>R and A<sub>2A</sub>R on PP2A activity

##### *Isolated heart protocols*

PP2A activities were assayed in ventricular myocardium from WT hearts that were perfused for 15 min with PSS containing the following agents: 10<sup>-6</sup>M A<sub>2A</sub>R agonist 2-*P*(2-carboxyethyl)phenethyl-amino-5'-*N*-ethylcarboxyamidoadenosine (CGS-21680) alone, 10<sup>-6</sup>M A<sub>1</sub>R agonist 2-chloro-*N*<sup>6</sup>-cyclopentyladenosine (CCPA) alone, both CCPA and CGS-21680 together, or the 10<sup>-6</sup>M A<sub>2A</sub>R antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385). Control hearts were perfused with agent-free PSS for the same period of time as the treated hearts prior to freeze-clamping. In addition, PP2A activity was assayed in A<sub>2A</sub>RKO myocardium perfused in the absence (control) or presence of 10<sup>-6</sup>M CGS-21680 or 10<sup>-6</sup>M CCPA.

### *Preparation of Myocardial Fractions*

Ventricular myocardium was homogenized in 2 mL of ice-cold buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 0.1 mM DTT, 0.1 mM benzamidine and 10 µg/ml soybean trypsin inhibitor using a Polytron at a speed setting of 5 (two 10-sec bursts with a 15 sec pause between bursts). A 600 µL aliquot of the homogenate was diluted with an equal volume of the homogenization buffer and centrifuged with a microfuge for 20 min at 12,000 x g. The resulting supernatant is termed the cytosolic fraction containing the PP2A located primarily in the cellular cytoplasm. The pellet was resuspended in 400 µL of homogenization buffer and held on ice for 20 min prior to centrifugation for 20 min at 12,000 x g. This supernatant was termed the particulate fraction containing PP2A solubilized from the cell membranes. All samples were maintained at 4°C during processing.

### *Assay of phosphatase activity*

Supernatants from both cytosolic and particulate fractions were assayed for PP2A activity using the Promega serine/threonine phosphatase assay kit (Promega, Madison, WI). Briefly, 350 µL aliquots of the supernatants were passed through Sephadex G-25 resin spin columns by centrifugation at 600 x g for 4 min to remove endogenous phosphate. Phosphatase activity was determined by the release of free phosphate from a phosphorylated substrate. The phosphate released was detected colorimetrically by the formation of a molybdate:malachite green:phosphate complex. Interference of PP1 and PP2B activity was minimized with the use of a PP2 selective substrate and by inclusion of EGTA, respectively, in the reaction mix. The protein content of the samples was determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL).

### Phosphorylation state of the PP2A Tyr 307 regulatory site

The phosphorylation state of the Tyr 307 site on PP2A was determined with an ELISA assay using a mouse anti-phospho-PP2A (Tyr307) primary antibody and a secondary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Upstate Biotechnology, Lake Placid, NY). Control ventricular samples from WT hearts were compared to those treated with  $10^{-6}$ M CGS-21680 or  $10^{-6}$ M CCPA. Samples were homogenized in a buffer containing 50 mM  $\text{Na}_2\text{CO}_3$  (pH 9.6). A 96-well microtiter plate was layered with ventricular homogenate (90  $\mu\text{g}$  protein/well). Then the plates were incubated at  $4^\circ\text{C}$  overnight. Following this incubation the wells were rinsed twice with a wash buffer containing 150 mM NaCl, 20 mM TRIS (pH 7.4) and 0.05% (by volume) Tween-20. The wells were then blocked for 2 hrs at  $4^\circ\text{C}$  with 5% nonfat dry milk in wash buffer. Blocking solution was removed, the wells were rinsed twice with wash buffer and primary antibody at a dilution of 1:300 in blocking solution was added (100  $\mu\text{L}$  /well). Plates were then incubated for 1 hr at room temperature. Primary antibody was removed and the wells were rinsed twice with wash buffer. Secondary antibody was added at a dilution of 1:500 in blocking solution and incubated 1 hr at room temperature. Secondary antibody was removed and the wells were rinsed four times with wash buffer and once with a Phosphate-Citrate buffer containing 24.3 mM citric acid and 51.4 mM  $\text{Na}_3\text{PO}_4$ . ABTS chromogen substrate solution (100  $\mu\text{L}$ ; Zymed Laboratories, South San Francisco, CA) was added to each well and incubated at room temperature for 55 min. The absorbance of the developed color was read using a plate reader with a 405 nm filter.

### Data and Statistical Analysis

Results are presented as means  $\pm$  SE. Data were plotted using Prism (GraphPad Software, San Diego, CA). Statistical analysis was performed using StatMost (Dataxiom, Los Angeles, CA). Statistical tests applied include ANOVA and Student-Newman-Keuls multiple comparison test. Standard errors for the difference in means as seen in Fig. 3 was calculated as the square root of the sum of variances of the sample means (1). Statistical significance was defined at  $P < 0.05$ .

### Materials

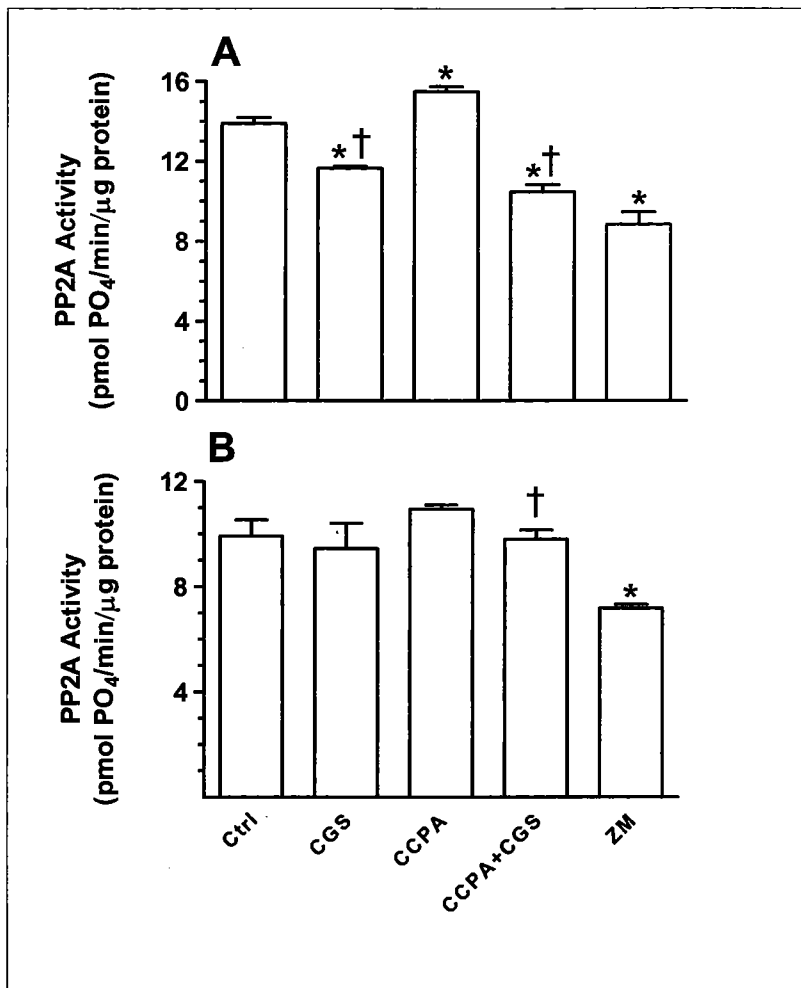
All adenosine receptor agents were prepared as 10 mM stock solutions in 100% DMSO and diluted with milliQ-treated water to  $10^{-4}$  M that was used for injection into the PSS. The resulting concentration of DMSO in the perfusion medium did not exceed 0.01%. Buffer salts and assay multiwell plates were purchased from Fisher Scientific (Fairlawn, NJ). CCPA and CGS-21680 were obtained from Sigma RBI (St. Louis MO) and ZM-241385 was purchased from Tocris (Ellisville, MO).

### Results

#### *Effects of $A_{2A}R$ and $A_1R$ on PP2A activity in WT hearts*

Administration of the  $A_{2A}R$  agonist CGS-21680 to WT hearts decreased PP2A activity by 16% in the myocardial particulate fraction (Fig 1A). Conversely, the  $A_1R$  agonist CCPA resulted in a significant 12% increase in PP2A activity. Treatment of the hearts with both CCPA and CGS-21680 reduced the PP2A activity level 25% below that of control and 32% below that observed with CCPA alone. This value was not significantly different from the phosphatase

activity observed with CGS-21680 alone. To investigate the effect of pharmacological inhibition of A<sub>2A</sub>R as compared with effects of receptor knockout as shown below, hearts were treated with the A<sub>2A</sub>R antagonist ZM-241385. The antagonist resulted in a 36% decrease in activity as compared to the control value. In the cytosolic fraction of WT hearts (Fig 1B), the effects of adenosinergic stimulation were similar to those of the particulate fraction, though less pronounced. Treatment of the hearts with CGS-21680 and CCPA separately resulted in a 5% decrease and 10% increase in PP2A activity, respectively. However these changes were not statistically significant. Treatment of the hearts with CGS-21680 and CCPA together also did not result in significant changes in activity from that of control. However, this PP2A activity was decreased by 10% from the level seen with CCPA alone. Treatment of the hearts with ZM-41385 resulted in a 28% decrease from control. The observed PP2A activity levels were noted to be higher in the particulate fraction compared with those in the cytosolic fraction for control, CCPA and ZM-241385 groups. These results suggest that the A<sub>2A</sub>R modulates the activity of PP2A both directly and via an interaction with the A<sub>1</sub>R.



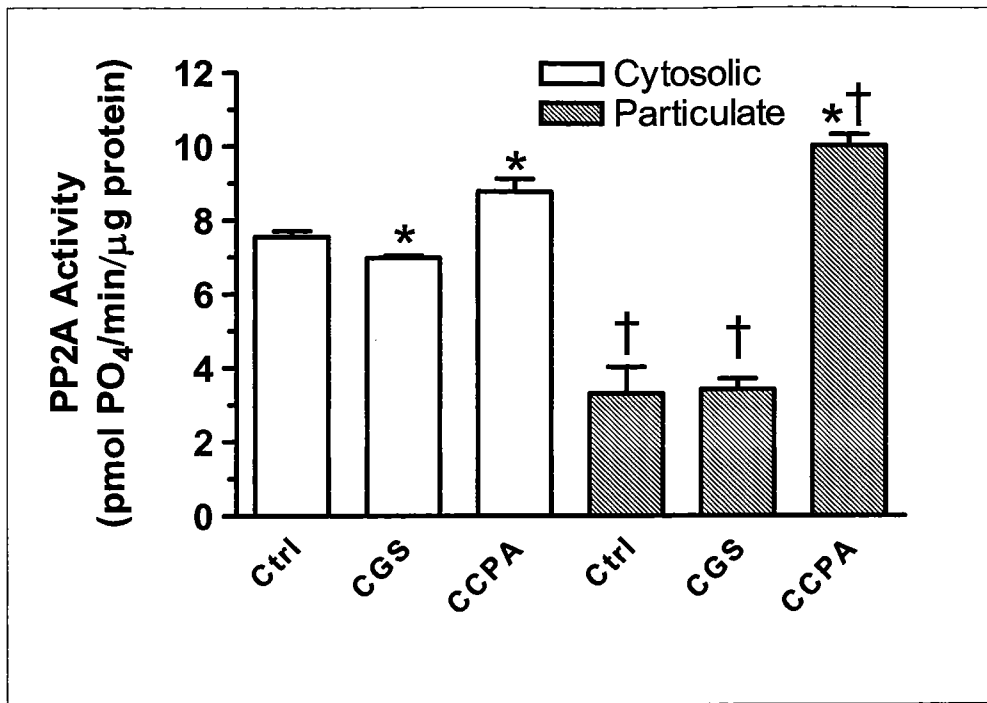
**Figure 1.** Effects of  $A_1R$  and  $A_{2A}R$  stimulation on PP2A activities in particulate (Panel A) and cytosolic (Panel B) fractions of ventricular myocardium from wild type (WT) murine hearts. CGS,  $A_{2A}R$  agonist 2-(2carboxyethyl)phenethyl-amino-5'-*N*-ethylcarboxyamidoadenosine ( $10^{-6}$  M; CGS-21680); CCPA,  $A_1R$  agonist 2-chloro-*N*<sup>6</sup>-cyclopentyladenosine ( $10^{-6}$  M); ZM,  $A_{2A}R$  antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol ( $10^{-6}$  M; ZM-241385). Data are means  $\pm$  SE for 4-9 hearts. \* denotes statistically significant difference from control (Ctrl) value; † denotes statistically significant difference from CCPA value.

### *Effects of A<sub>2A</sub>R and A<sub>1</sub>R on PP2A activity in A<sub>2A</sub>RKO hearts*

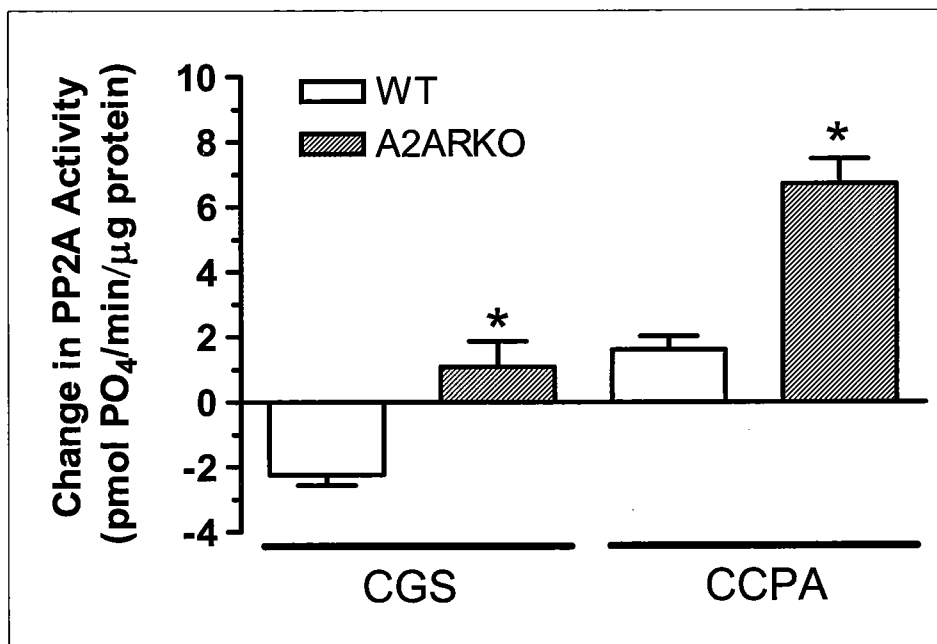
Administration of CGS-21680 to hearts obtained from A<sub>2A</sub>RKO animals (Fig 2) resulted in a 7% decrease in PP2A activity in the cytosolic fraction, whereas in the particulate fraction CGS-21680 had no effect on the phosphatase activity. CCPA administration caused a 16% increase in PP2A activity. In the particulate fraction both control and CGS-21680 treated hearts displayed significantly lower PP2A activity compared with their respective cytosolic counterparts. CCPA treatment of these hearts produced a 205% increase in particulate PP2A activity. This value was 15% higher than that observed in the cytosolic fraction with CCPA.

### *Comparison of WT and A<sub>2A</sub>RKO responses to adenosinergic stimulation*

Major differences in the PP2A response to A<sub>1</sub>R or A<sub>2A</sub>R activation between WT and A<sub>2A</sub>RKO hearts are most readily compared in the particulate fractions as summarized in Figure 3. In WT hearts treatment with CGS-21680 resulted in a decrease in PP2A activity, whereas in the A<sub>2A</sub>RKO CGS-21680 had no significant effect. Both WT and A<sub>2A</sub>RKO hearts demonstrated an increase in PP2A activity in response to CCPA treatment. In the knockout hearts this response was over three-fold (316%) higher than that observed in the WT.



**Figure 2.** Effects of A<sub>1</sub>R and A<sub>2A</sub>R stimulation on PP2A activities in particulate and cytosolic fractions of ventricular myocardium from A<sub>2A</sub>R knockout (A<sub>2A</sub>RKO) murine hearts. CGS (A<sub>2A</sub>R agonist) and CCPA (A<sub>1</sub>R agonist) are used at a concentration of 10<sup>-6</sup> M. Data are means ± SE for 4-8 hearts. \* denotes statistically significant difference from respective control (Ctrl) value; † denotes statistically significant difference from respective cytosolic value.



**Figure 3.** Summary of changes in PP2A activity observed in the particulate fraction of ventricular myocardium from WT and A<sub>2A</sub>RKO murine hearts with A<sub>1</sub>R and A<sub>2A</sub>R activation (derived from data depicted in Figures 1A and 2). CGS, 10<sup>-6</sup> M; CCPA, 10<sup>-6</sup> M. \* denotes statistically significant difference from WT value. See legends of Figures 1 and 2 for additional details.



### *Distribution of PP2A activity between cytosolic and particulate fractions*

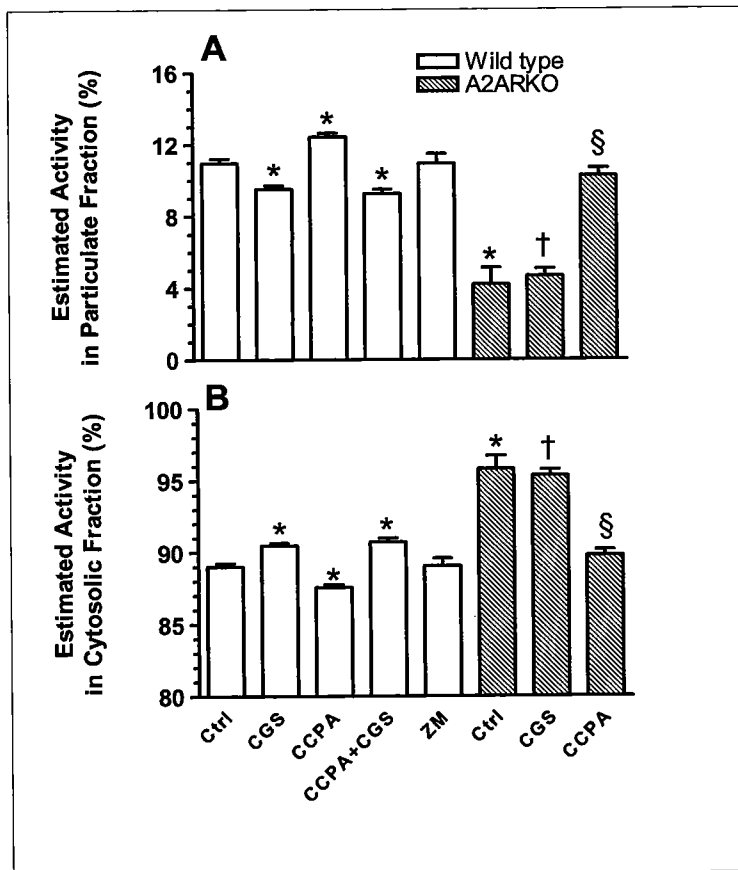
Distribution of the total activity between cytosolic and particulate fractions (Fig 4) was determined by multiplying the protein normalized activity value for each sample by the total volume of the fraction from which the analyzed sample was obtained. The resulting value is an estimate of the total phosphatase activity of the fraction considered. This value was expressed as a percentage of the sum of both cytosolic and particulate fraction activities.

In the WT untreated hearts the particulate fraction (Fig 4A) was determined to contain 10.9% of the total PP2A activity. With hearts treated with CGS-21680 or CCPA the particulate fraction contained 9.5% and 12.4% of total PP2A activity, respectively. The value observed with ZM-241385 was not different from the control value. The particulate fraction of A<sub>2A</sub>RKO hearts showed significantly lower total PP2A activities for both Ctrl and CGS-21680 groups (4.2% and 4.7% respectively) as compared to WT. On the other hand, CCPA-treated knockout hearts, while demonstrating a lower value in the particulate fraction than their WT counterparts, displayed a greater total PP2A activity (10.3%) compared with control.

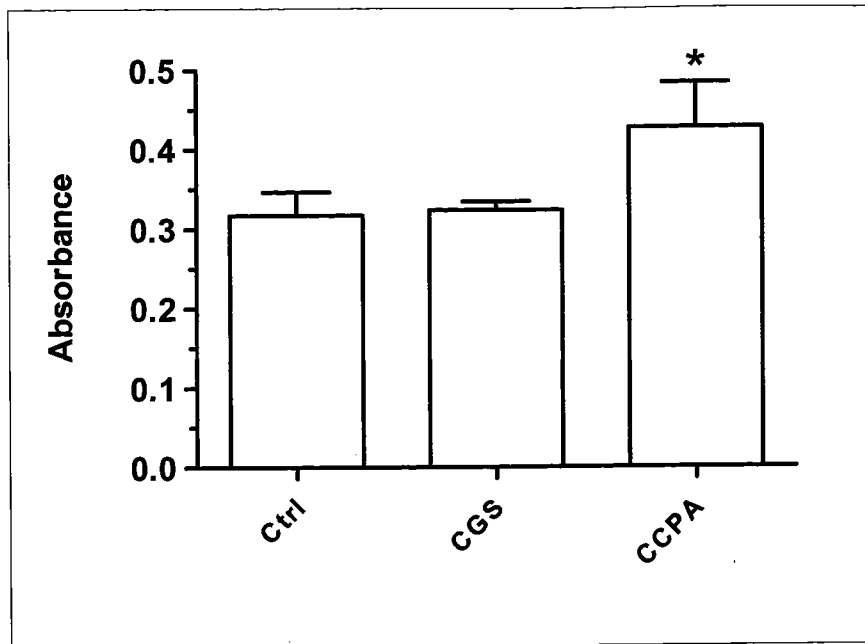
The changes observed in the values for the cytosolic fraction (Fig 4B) follow a converse pattern compared to the particulate fraction (Fig 4A). In WT hearts the cytosolic fraction showed greater total PP2A activities with CGS-21680 alone as well as with CCPA and CGS-2180 administered together. CCPA alone resulted in a reduced total PP2A activity. In the control and CGS-21680 treated A<sub>2A</sub>RKO hearts a higher total PP2A activity value was observed compared with values found in WT hearts, whereas the total PP2A activity with CCPA in A<sub>2A</sub>RKO hearts was lower. These data suggest that translocation of PP2A activity between the cytosolic and particulate fractions is modulated by adenosinergic receptors in the heart.

*Effect of A<sub>1</sub>R and A<sub>2A</sub>R activation on the phosphorylation state of Tyr 307 regulatory site*

An ELISA assay was used to investigate whether the observed decrease in PP2A activity in response to A<sub>2A</sub>R activation results from an increased phosphorylation at the Tyr 307 regulatory site, a known inhibitor of PP2A activity (23) (Fig 5). In WT hearts CGS-21680 did not effect the phosphorylation state of Tyr 307 site. However, treatment with CCPA caused a 35% increase in Tyr 307 site phosphorylation compared with that observed in control hearts. These findings suggest that the Tyr 307 regulatory site is unlikely to be directly involved in the mediation of the presently observed effects of A<sub>2A</sub>R and A<sub>1</sub>R on PP2A activity.



**Figure 4.** Effect of A<sub>1</sub>R and A<sub>2A</sub>R stimulation on the partitioning of PP2A activity between particulate (Panel A) and cytosolic (Panel B) fractions of ventricular myocardium from WT and A<sub>2A</sub>ARKO murine hearts. CGS, CCPA and ZM are each used at a concentration of 10<sup>-6</sup> M; Data are means ± SE for 4-9 hearts. \* denotes statistically significant difference from WT control (Ctrl) value; † denotes statistically significant difference from WT CGS value; § denotes statistically significant difference from WT CCPA value. See legends of Figures 1 and 2 for additional details.



**Figure 5.** Effects of  $A_1R$  and  $A_{2A}R$  activation on the phosphorylation state of the Tyr 307 regulatory site of PP2A in WT murine hearts. ELISA assay was performed on ventricular myocardium homogenates from hearts either untreated (control, Ctrl) or treated with CGS ( $10^{-6}$  M) or CCPA ( $10^{-6}$  M). Data are means  $\pm$  SE for 5 hearts. \* denotes significant difference from Ctrl value. See legends of Figures 1 and 2 for additional details.

## Discussion

### *Adenosinergic modulation of PP2A activity in WT and $A_{2A}RKO$*

The main finding of the present study is that the level of PP2A activity and its localization within the ventricular myocyte is modulated by the  $A_{2A}R$  as well as the  $A_1R$ . This suggests that dephosphorylation of proteins important for contractile activity may play a role in the manifestation of the antiadrenergic action of adenosine.

There is considerable support for a role of protein phosphatases in the regulation of cardiac contractility. Overexpression of the catalytic subunit of PP2A has been shown to impair cardiac function in mice (20), whereas overexpression of PPI-2 has been reported to enhance cardiac contractility (24). The attenuation of protein phosphatase 1 (PP1) activity has been demonstrated to enhance cardiac function in transgenic mice expressing PPI-1 (38).

Protein phosphatases have also been suggested to play a significant role in the adenosinergic regulation of cardiac contractility. Narayan and colleagues have pharmacologically inhibited A<sub>1</sub>R antiadrenergic effects with the protein phosphatase inhibitor cantharidin (34) without significant changes in cAMP levels. Recently, Liu and colleagues have provided evidence in isolated rat cardiomyocytes that the antiadrenergic effect of A<sub>1</sub>R is in part mediated by the activation of PP2A (29). This effect appeared to be transduced through p38 mitogen activated protein kinase (p38 MAPK)(30). In agreement with these observations, acute p38 MAPK activation has been shown to decrease force development in rat ventricular myocytes (5), perhaps as a result of the dephosphorylation of proteins associated with contractile activity. Protein phosphatase inhibition with okadaic acid (OKA) has been demonstrated to foster preconditioning in aged rat hearts by a mechanism that may involve adenosine and is thought to be mediated through changes in cardiac protein phosphorylation (17).

The observations from the present study support the previously reported findings of Liu, et al. suggesting that a significant fraction of the A<sub>1</sub>R antiadrenergic effect may be manifest via protein phosphatase activity (29). In WT hearts, activation of the A<sub>1</sub>R resulted in an increased PP2A activity in both cytosolic (Fig. 1A) and particulate fractions (Fig. 1B). On the other hand, activation of A<sub>2A</sub>R with CGS-21680 resulted in a decrease in PP2A activity in both fractions. Even though the observable trends were the same in both particulate and cytosolic fractions, changes in PP2A activities induced by A<sub>2A</sub>R and A<sub>1</sub>R were more prominent in the particulate fraction. The PP2A enzyme while mainly inactive in the cytosol may become activated upon translocation to its intended destination as is seen with PKC (31). It might be assumed that the higher PP2A activity in the particulate fraction ensures the dephosphorylation of myocardial proteins localized in this fraction.

Another finding of interest in the WT hearts was the observed effect of combined CCPA and CGS-21680 on PP2A activity, where it was observed that PP2A activity was unchanged from CGS-21680 alone. This suggests that CGS-21680 inhibits the A<sub>1</sub>R-induced increase in PP2A activity. The ability of A<sub>2A</sub>R to act both directly and through inhibition of A<sub>1</sub>R effects has been noted previously in contractile studies of both intact hearts and isolated cardiomyocytes (37,43).

In the A<sub>2A</sub>RKO hearts (Fig. 2) A<sub>1</sub>R stimulation with CCPA resulted in a slightly higher PP2A activity increase in the cytosolic fraction (16%) compared with WT (12%). However, in the particulate fraction the increase in PP2A activity with CCPA administration was over two-fold (205%). This observation correlates with previously reported findings that pharmacological inhibition of A<sub>2A</sub>R results in an enhanced response to A<sub>1</sub>R activation (37). Based on the present observations it can be concluded that in the WT heart the A<sub>2A</sub>R would oppose the action of the A<sub>1</sub>R in increasing PP2A activity, whereas in the A<sub>2A</sub>RKO this attenuation is absent resulting in an exaggerated response to A<sub>1</sub>R stimulation. Curiously, CGS-21680 treatment of A<sub>2A</sub>RKO hearts resulted in a small but significant decrease of 7% in PP2A activity, an unexpected result in the absence of A<sub>2A</sub>R protein. However, this finding was not replicable in the particulate fraction.

The presence of A<sub>2A</sub>R may be important in determining partitioning of PP2A activity within the cardiac myocyte. The relative distribution of PP2A activities between the particulate and cytosolic fractions with control and CGS-21680 treatment differs between WT and A<sub>2A</sub>RKO hearts. In the latter hearts, the PP2A activity levels in the particulate fractions from both control and CGS-21680 treated hearts (Fig. 2) were significantly lower than those observed in the cytosolic fraction. In comparison, WT control hearts showed lower PP2A activity in the cytosolic (Fig. 1B) fraction, and with CGS-21680 PP2A activities were similar between cytosolic

and particulate fractions. These observations suggest that the absence of A<sub>2A</sub>R signal may in some way interfere with the PP2A activity as evidenced by the lower activity levels in the particulate fraction of A<sub>2A</sub>RKO hearts. This conclusion is supported by the observation that in both cytosolic and particulate fractions of WT hearts, PP2A activity is significantly reduced by blockade of the A<sub>2A</sub>R with ZM-241385 (Fig. 1). However, this decrease does not appear to be as profound as that observed in the A<sub>2A</sub>RKO. This observation may have resulted from incomplete pharmacological inhibition as compared with receptor absence. The particulate fraction of A<sub>2A</sub>RKO CCPA-treated hearts displayed significantly higher (15%) PP2A activity than the cytosolic fraction, an increase comparable to that seen in the WT. This suggests that despite the decreased PP2A activity observed in the control A<sub>2A</sub>RKO hearts, PP2A is still able to effectively become localized to the particulate fraction and activated following A<sub>1</sub>R stimulation by CCPA.

In brief, the changes occurring in PP2A activity with adenosinergic stimulation are most readily compared in the particulate fraction (Fig. 3). Within this fraction stimulation of A<sub>2A</sub>R with CGS-21680 resulted in a decrease of PP2A activity in WT hearts, but not in A<sub>2A</sub>RKO hearts, whereas A<sub>1</sub>R activation had the opposite effect. In the A<sub>2A</sub>RKO hearts the response to A<sub>1</sub>R was exaggerated, displaying a PP2A activity increase approximately threefold higher than that observed in the WT hearts. These findings suggest that both A<sub>1</sub>R and A<sub>2A</sub>R play a role in modulating the activity of PP2A.

#### *Distribution of PP2A between the cytosolic and particulate fractions*

In order to determine whether A<sub>1</sub>R and A<sub>2A</sub>R stimulation affected the distribution of total PP2A activity between the cytosolic and particulate fractions, data was analyzed accounting for the volume of sample in order to estimate the total activity present in each fraction. Significantly

enhanced localization of the total activity to the particulate fraction was observed in the CCPA-treated hearts (Fig. 4A). This finding supports the observations made by Liu, et al. (29) who previously reported translocation of PP2A to the particulate fraction of rat cardiomyocytes following A<sub>1</sub>R activation. Furthermore, A<sub>2A</sub>R activation was observed to have the opposite effect resulting in an enhanced localization of the total activity to the cytosolic fraction. Total PP2A activities in both cytosolic and particulate fractions were similar in the presence of CGS-21680 with or without CCPA. This indicates that the A<sub>2A</sub>R attenuates the actions of the A<sub>1</sub>R with respect to PP2A localization as well as direct modulation of PP2A enzymatic activity as discussed above.

Treatment of WT hearts with ZM-241385 did not result in significant changes in PP2A localization compared with untreated hearts, whereas actual enzyme activity was noted to be decreased following ZM-241385 treatment (Fig. 1). This observation suggests that the decrease in PP2A activity in the absence of A<sub>2A</sub>R protein or the inhibition by an A<sub>2A</sub>R antagonist may be due to a decrease in enzymatic activity rather than altered localization of the enzyme within the ventricular myocyte.

Even though the greatest percentage of total PP2A activity is generally present in the cytosolic fraction, proteins associated with contractile function are localized in the particulate fraction. It is possible to argue that the changes in localization and activity of PP2A observed in the particulate fraction are more reflective of adenosinergic influences on PP2A activity than those changes occurring in the cytosolic fraction.

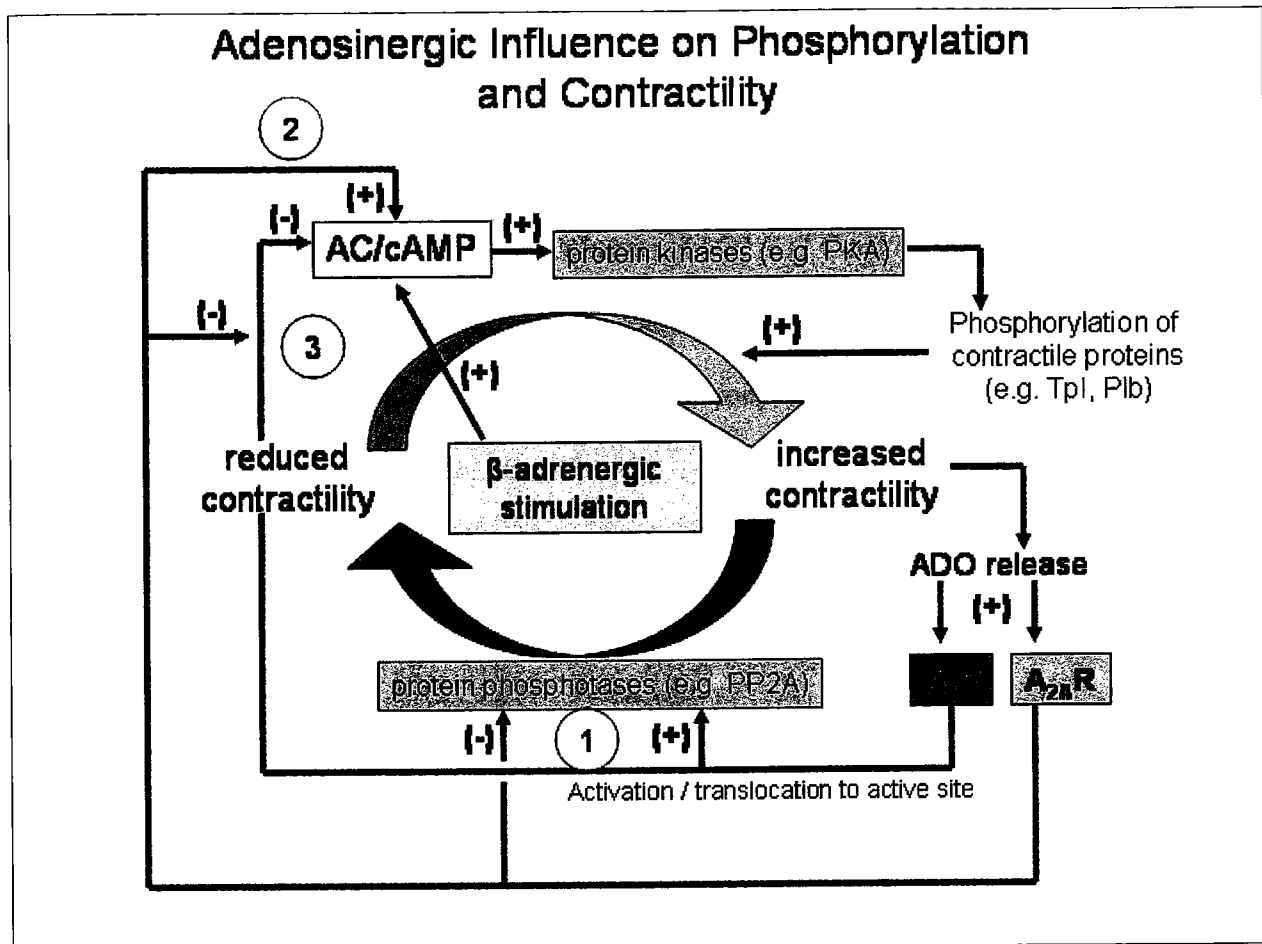
### *Effect of A<sub>1</sub>R and A<sub>2A</sub>R activation on the Tyr 307 regulatory site of PP2A*

The Tyr 307 regulatory site on PP2A is known to decrease the activity level of the enzyme when phosphorylated (23). In order to investigate whether this is a mechanism by which A<sub>1</sub>R or A<sub>2A</sub>R regulate the activity level of the PP2A enzyme, an ELISA assay was employed using an antibody specific for the phosphorylated Tyr 307 of PP2A (Fig. 5). No changes in Tyr 307 phosphorylation were observed with CGS-21680-induced A<sub>2A</sub>R activation. CCPA activation of A<sub>1</sub>R resulted in a significant increase in the phosphorylation of Tyr 307. These results suggest that the Tyr 307 site is unlikely to be involved in the adenosinergic regulation of the PP2A enzyme. If this site is involved in the regulation of PP2A activity by A<sub>1</sub>R and A<sub>2A</sub>R, an increased Tyr 307 phosphorylation following A<sub>2A</sub>R activation and a decreased phosphorylation following A<sub>1</sub>R activation would be expected. As this hypothesis is not supported by the present results, it is concluded that other regulatory sites may be responsible for adenosinergic-mediated changes in PP2A activity. The specific mechanism and sites for this regulation remain to be determined.

In conclusion, this study indicates that A<sub>2A</sub>R as well as A<sub>1</sub>R modulate PP2A activity in the ventricular myocardium. These data indicate another mechanism by which adenosine modulates contractile function in the presence and absence of  $\beta_1$ -adrenergic stimulation. The implications of adenosinergic receptor activation and their effects on protein phosphatases in the regulation of myocardial contractility are summarized in Figure 6 within the context of other adenosinergic mediated events: 1. This mechanism involves activation and translocation of the PP2A enzyme within the ventricular myocyte to its site of action. A<sub>1</sub>R stimulation enhances PP2A function via enzyme activation and/or translocation of enzyme to its active site while A<sub>2A</sub>R stimulation inhibits PP2A by either direct modulation of enzyme activity or translocation, or the inhibition of A<sub>1</sub>R effects on PP2A. 2. A<sub>1</sub>R and A<sub>2A</sub>R directly modulate adenylyl cyclase



activity (26) and subsequently cAMP content of the ventricular myocyte (7). 3.  $A_{2A}R$  activation attenuates the  $A_1R$  antiadrenergic action, indirectly enhancing contractile function (37,43).



**Figure. 6.** Schematic diagram depicting actions of  $A_1R$  and  $A_{2A}R$  in the modulation of cardiac contractility including suggested role of protein phosphatases. See text "Discussion" for details. ADO: adenosine; Tpl: troponin I; Plb: phospholamban.

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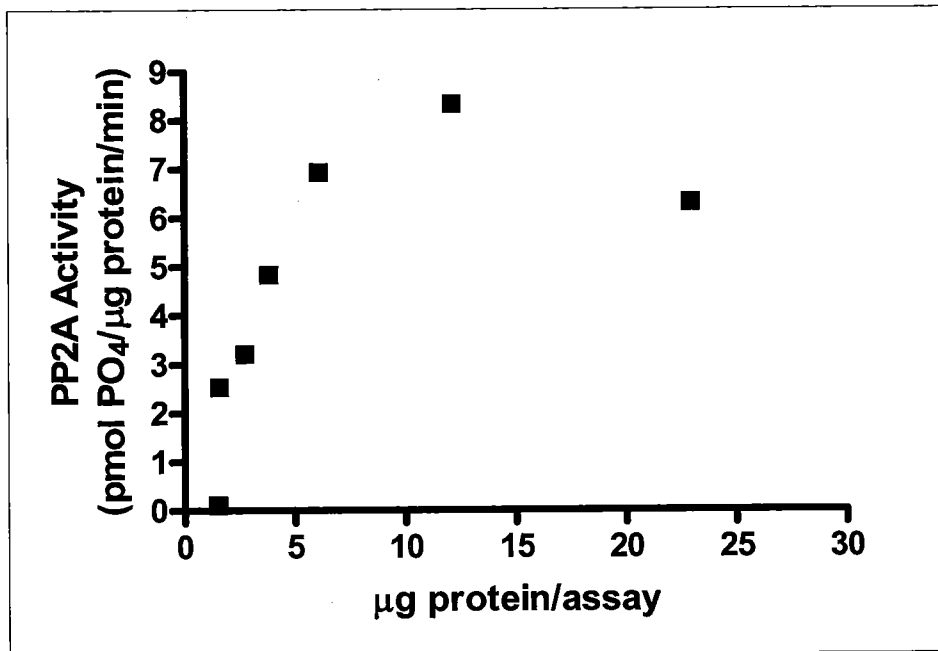
### **Supplemental Material**

Additional preliminary study and supporting experiments for the preceding manuscript are included in this section.

#### *Optimal sample dilution for PP2A assay*

In order to determine the optimal dilution of the myocardial sample in terms of observed PP2A activity, the activity was measured in samples serially diluted by half in the homogenization buffer. The myocardium was initially homogenized in 2 ml of homogenization buffer. The resultant protein concentration in each dilution was determined using a BCA assay and the PP2A activity was plotted as a function of the protein concentration in each respective

dilution. The results are presented in supplementary Figure S5. As can be seen in the figure the optimal sample concentration resulting in highest PP2A activity is between 10-15  $\mu\text{g}$  of protein per assay. To maintain these optimal conditions in further experiments the myocardial homogenate was diluted as appropriate to achieve the desired protein concentration.



**Figure S5.** PP2A activity in a ventricular myocardium sample as a factor of sample dilution. Initially the sample is homogenized in 2 ml of homogenization buffer and then serially diluted by half. The PP2A activity is assayed in each resulting sample concentration and plotted as a function of the protein concentration in that particular dilution.

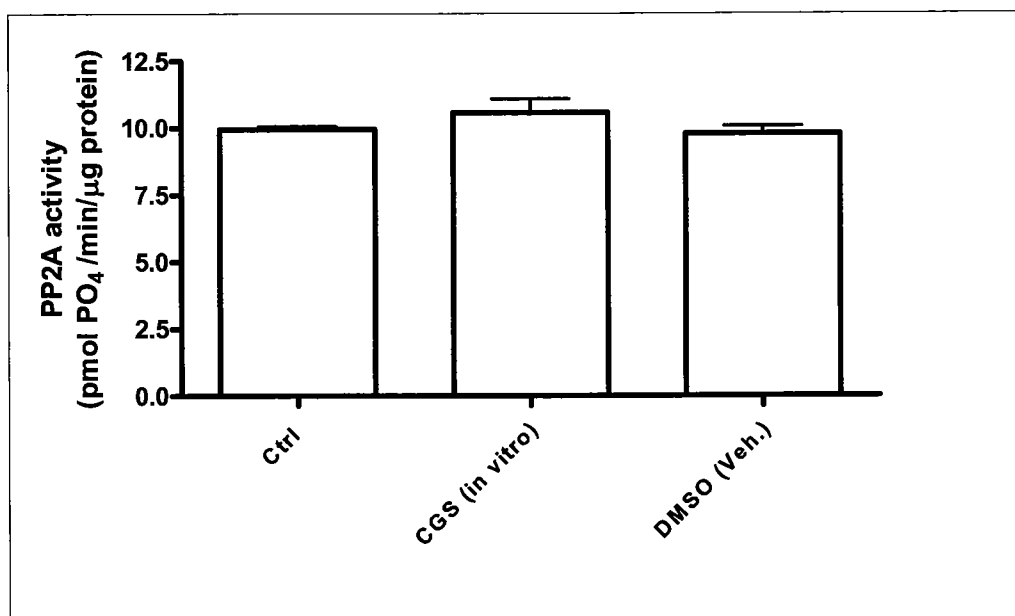
*PP2A activity response to vehicle and in vitro CGS-21680 treatment*

To ascertain that DMSO which was the vehicle used for the administration of adenosine receptor agents did not affect enzymatic activity, the activity of PP2A was assayed in ventricular myocardial extracts from hearts treated with DMSO in concentration and duration similar to that employed in the other experiments. In addition the effect of CGS-21680 treatment of the



homogenate as opposed to perfusion of the heart on PP2A was examined. Representative results from a pair of these experiments are presented in supplementary Figure S6.

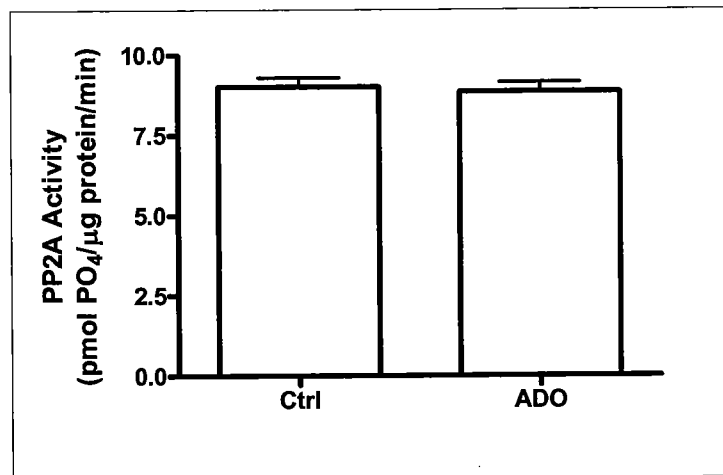
Neither *in vitro* CGS treatment nor DMSO treatment of the perfusing heart resulted in significant changes of PP2A activity observed in the control group. From these observations it can be concluded that DMSO at the concentrations used during administration of adenosinergic agents does not have a significant impact on the activity of PP2A. In addition it appears that the inhibition of PP2A activity by A<sub>2A</sub>R activation requires the intact cell and cannot be reproduced in the disrupted membranes present in the homogenate. This suggests that the transduction pathway for the effects of A<sub>2A</sub>R on PP2A requires a proper spatial arrangement of cellular components as compared to such pathways as β-adrenergic activation of adenylyl cyclase which readily occurs in myocardial membrane preparations (22).



**Figure S6.** Representative results from experiments to examine PP2A activity in ventricular myocardium extracts either untreated (Ctrl) or treated with CGS-21680 (CGS, 10<sup>-6</sup> M) *in vitro* and extracts from hearts treated with the vehicle (DMSO, 0.01%) used for the adenosinergic agents. Results presented are means ± SE for 2 hearts.

### Effect of *in vitro* adenosine treatment on PP2A activity

Myocardial homogenates, such as used in the study of adenosinergic modulation of PP2A activity in the earlier presented study, may contain a significant amount of endogenous adenosine. In most cases this can be remedied by the treatment of the preparation with adenosine deaminase. However in the case of the present study it was desirable to avoid the addition of reagents that were not critical to the assay. The possible direct effects of any endogenous adenosine present in the preparation on PP2A activity were examined by subjecting the ventricular myocardial extract to  $10^{-6}$  M adenosine *in vitro*. Results of this experiment are presented in supplemental Figure S7. The PP2A activity observed following adenosine treatment of the extract was not different from that observed in the control group which was untreated. This observation indicates that adenosine affects the PP2A activity level via acting on the appropriate adenosine receptors and adenosine on its own does not have a direct effect on the PP2A enzyme.



**Figure S7.** PP2A activity in ventricular myocardium extracts either untreated (Ctrl) or treated *in vitro* with adenosine (ADO,  $10^{-6}$  M). Results are means  $\pm$  SE for 5 hearts.

### *Inhibition of PP2A by assay conditions*

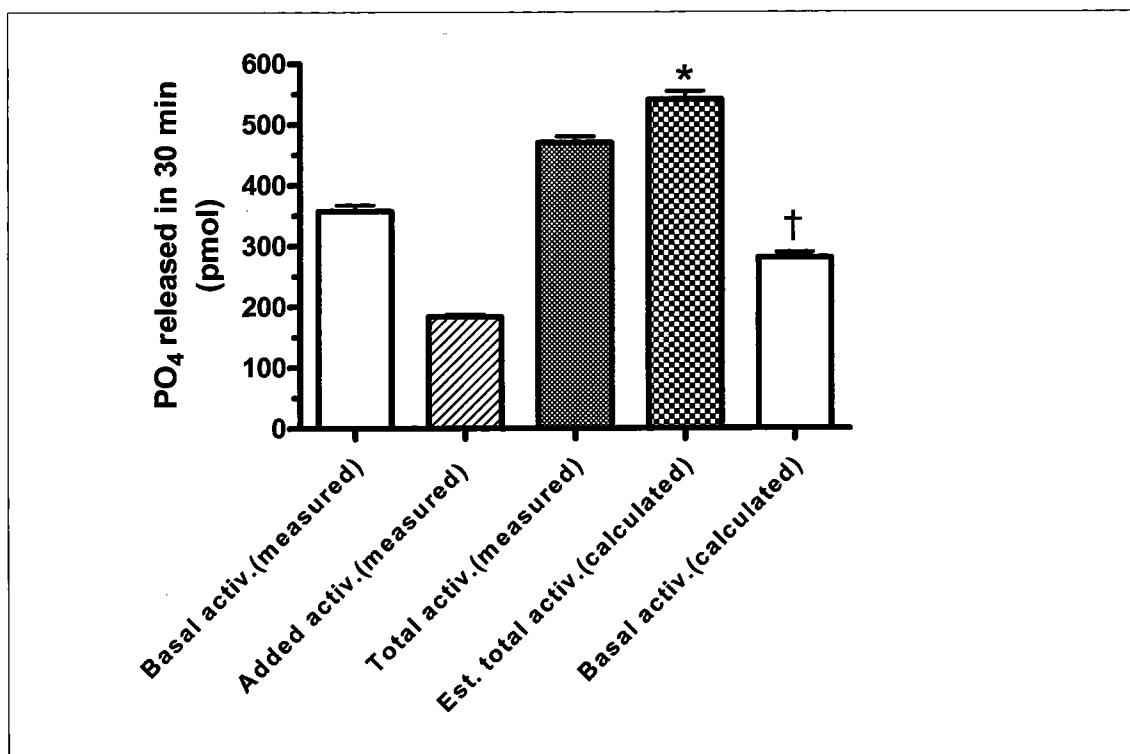
One of the points of interest with regard to the type of phosphatase assay employed is whether the assay conditions themselves cause some degree of inhibition of the phosphatase activity. In order to study whether inhibition of PP2A activity occurs as a result of assay condition, a series of experiments was conducted utilizing added activity from the purified catalytic subunit of PP2A enzyme (Promega, Madison, WI). The results of a representative experiment are presented in supplementary Figure S8.

In the course of the experiment the phosphate released by PP2A activity in a myocardial sample is compared between a “control group” where the sample is the only source of PP2A, and an “added activity group” where a known amount of activity from the purified PP2A catalytic subunit is also present. In theory if no inhibition of PP2A activity occurs, the combined phosphate release should be additive between the added activity and the sample. In fact what is seen is that the observed combined activity is slightly decreased from the estimated level obtained by adding the known sample and added activities. This would suggest a mild inhibition of the phosphatase by the conditions of the assay.

An alternate way of looking at the data is by comparing the estimated sample activity based on the measured total activity and the measured added activity. In this scenario subtracting the known added activity from the total measured activity yield the expected activity in the sample alone. This estimated activity though is lower than the actual sample activity observed. This type of analysis would suggest the contrary: that inhibition of PP2A does not occur.

A direct additive approach appears more appropriate and from that perspective a mild inhibition of PP2A activity appears to occur. Ultimately the level of inhibition observed is small based on the difference in pmols of phosphate released and once the data is corrected for the

protein level as would occur in other experiments, the actual effect on the final PP2A activity level expressed per minute and  $\mu\text{g}$  of protein becomes negligible. Based on these experiments it is concluded that while a minor amount of inhibition does occur under the assay conditions, the inhibition is present across all groups and does not prevent adequate comparison of differently treated samples, nor does it significantly alter the findings of any experiments presented.



**Figure S8.** Representative results from an experiment designed to examine whether the conditions of the PP2A assay result in inhibition of the phosphatase. Additional phosphatase activity is added by supplementing the reaction mixture with purified catalytic subunit of PP2A. Estimated total activity is the sum of measured basal and added activities. Calculated basal activity is obtained by taking the measured Total activity and subtracting the added activity. \* denotes significant difference from the Total activ.(measured) value. † denotes significant difference from Basal activ.(measured) value.

## Summary of Key Findings

- 1) A<sub>2A</sub>R as well as A<sub>1</sub>R is able to affect the activity of PP2A.
  
- 2) A<sub>2A</sub>R stimulation results in decreased PP2A activity as well as enhanced localization of the enzyme to the cytosolic fraction. A<sub>1</sub>R has the opposite effect resulting in increased PP2A activity and enhanced localization to the particulate fraction.
  
- 3) A<sub>2A</sub>R activation appears to block the effects of A<sub>1</sub>R-mediated increase in PP2A activity and localization.
  
- 4) In the A<sub>2A</sub>R knockout animals, PP2A activity increase observed with A<sub>1</sub>R activation is greater suggesting an inhibitory influence of A<sub>2A</sub>R on A<sub>1</sub>R with respect to modulation of phosphatase activity.
  
- 5) The presence of the A<sub>2A</sub>R is required for normal baseline PP2A function as evidenced by decreased PP2A activity and decreased particulate fraction localization in the A<sub>2A</sub>R knockout animals. These observations are supported by pharmacological inhibition of A<sub>2A</sub>R as well.
  
- 6) Despite the alterations in baseline function observed in the A<sub>2A</sub>R knockout, the PP2A enzyme is able to activate and localize appropriately upon A<sub>1</sub>R stimulation.

## **Comprehensive Discussion**

### *Rationale for study of adenosinergic effects in the heart*

The main reason for investigation into the adenosinergic signaling within the heart is that this pathway plays a major role in regulating myocardial contractility and response to adrenergic stimulation (16,18-20). The contractile study presented earlier presents the main findings in the murine heart with respect to adenosinergic effects on the contractility in the intact isolated heart (88).

In addition to regulation of contractility adenosinergic signaling is also involved in both response to acute pathological events (33,92,98,99) as well as chronic conditions (34,39,48,80). The modulation of the effects of adenosine receptor signaling may form the basis of treatment strategies relying on either pharmacological or perhaps genetic manipulation of these effects. An additional benefit to the utilization of adenosinergic signaling as a possible therapeutic pathway is that the adenosinergic effects occur in parallel with the main adrenergic-cholinergic regulation pathway and as such can provide the desired alterations in cardiac function without directly interfering in adrenergic-cholinergic signaling, and with less systemic side effects. This avenue of approach could be useful in conjunction with existing pharmacological treatments for pathological conditions such as heart failure where an increase in contractility is desired, or provide an approach where existing conventional treatments are ineffective as in the case of treatment of resistant hypertension.

The study of adenosinergic regulation of cardiac contractility is particularly convenient in the murine model for several reasons. Current technology allows the monitoring of virtually all the hemodynamic and cardiovascular parameters that can be monitored in a human patient. Combined with reasonable breeding and upkeep costs the murine model is highly valuable and

adaptable model for both in vitro and in vivo experiments. In addition the use of specific receptor knockout genotypes such as the A<sub>2A</sub>R knockout (7), allows for even more intricate investigation into the effects of individual adenosine receptors. The availability of murine models of pathological conditions like heart failure (80) and overload hypertrophy (9,70) makes initial testing of clinical applications feasible in the murine model.

#### Summary of key findings in contractile studies of isolated murine hearts

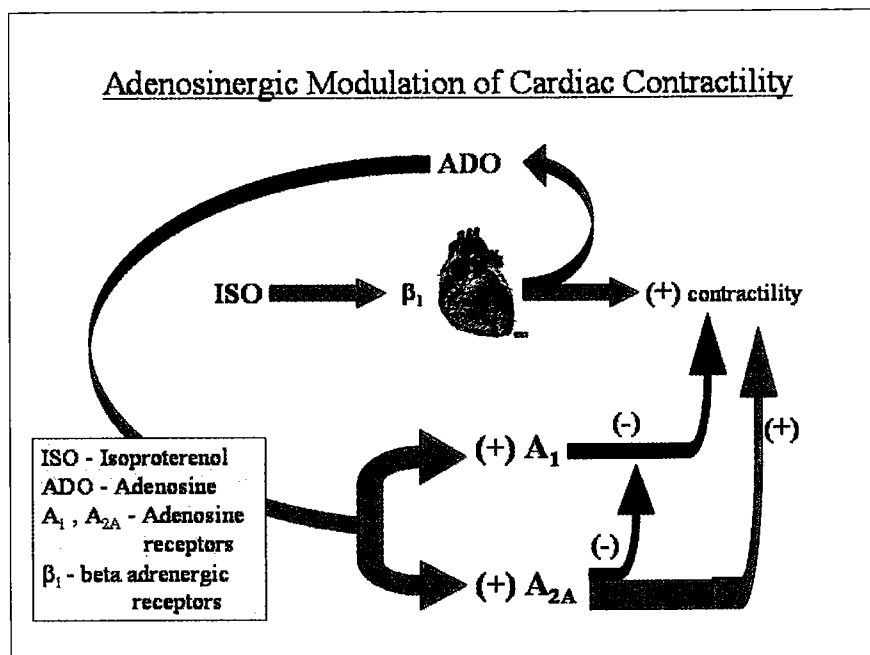
The main finding of the present study is that in the murine heart A<sub>1</sub>R and A<sub>2A</sub>R regulate the contractile response to  $\beta$ -adrenergic stimulation by manifesting opposite effects. As previously observed in the rat model the A<sub>1</sub>R decreases the contractile response to  $\beta$ -adrenergic stimulation (66,78), while A<sub>2A</sub>R attenuates the A<sub>1</sub>R effect and also increases contractility directly (20,96).

From the perspective of an isolated heart it is not possible to directly address what the mechanism behind the A<sub>2A</sub>R-mediated A<sub>1</sub>R inhibition is, however it is possible that the modulation of phosphatase activity as described in the earlier presented study may be a part of that interaction. The adapted scheme of adenosinergic influence on contractility in the heart as outlined in the contractile study is summarized in Figure C.

An additional finding of the study was the effect of A<sub>2A</sub>R activation in the setting of low flow ischemia. A<sub>2A</sub>R activation was noted to significantly maintain contractile function of the heart despite decreased levels of perfusion. The response to a short term acute flow decrease was also found to be reduced following A<sub>2A</sub>R activation. This suggests a cardioprotective effect of A<sub>2A</sub>R that may be related to mechanisms involved in preconditioning, (72,92) or may be the

result of increased phosphorylation of proteins important in contractile function (47,57) or enhanced adenylyl cyclase activation (11,22).

Interestingly the observed response of  $A_{2A}R$  knockout hearts to  $A_1R$  activation was less than that observed in the wild type suggesting that perhaps in the knockout some level of compensation occurs for the absence of  $A_{2A}R$ , as pharmacological inhibition of the  $A_{2A}R$  has been reported to actually increase the response to  $A_1R$  activation (78) and PP2A activity response to  $A_1R$  activation in the knockout is also enhanced. It is possible that this observation is due to an alteration in the response of adenylate cyclase in the  $A_{2A}R$  knockout environment, a concept addressed in further detail in the “future directions” section.



**Figure C.** A schematic of adenosinergic modulation of contractile response to  $\beta$ -adrenergic stimulation in the heart. Adrenergic stimulation results in release of adenosine within the myocardium which in turn activates the  $A_1R$  and  $A_{2A}R$ . The antiadrenergic effect of  $A_1R$  results in decreased contractility, while  $A_{2A}R$  both increases contractility directly as well as modulated the effects of  $A_1R$ .

Summary of key findings in studies of protein phosphatase 2A activity modulation by adenosine

$A_1$  and  $A_{2A}$  receptors

The major finding of this study is that  $A_{2A}R$  as well as  $A_1R$  is able to modulate the activity of PP2A. Like the previously described modulation of PP2A activity by  $A_1R$  (61), the



A<sub>2A</sub>R is able to affect both activity of the enzyme as well as its localization within the cellular fractions. This indicates that both the activity level of the enzyme itself and its location within the cell play a role in regulating the overall activity.

In hearts treated with agonists for both A<sub>1</sub>R and A<sub>2A</sub>R the observed PP2A activity was similar to those treated with A<sub>2A</sub>R agonist alone. The apparent ability of A<sub>2A</sub>R activation to block stimulatory effects of A<sub>1</sub>R on PP2A may be responsible for the observed “indirect effect” described in the contractile studies. If the direct effect of A<sub>2A</sub>R is related to enhanced activation of adenylate cyclase and subsequent increase in cellular cAMP, the decrease in PP2A activity with A<sub>2A</sub>R which is not directly related to cAMP levels may account for the observed increase in contractility with A<sub>2A</sub>R activation even in the absence of large increases in cAMP levels. Further study is needed to determine the relative contribution of each of these effects to the regulation of contractility in the heart. Interestingly, in the A<sub>2A</sub>R knockout the PP2A activity was reduced in the particulate fraction under baseline conditions as well as in the presence of A<sub>2A</sub>R agonist suggesting that the presence of A<sub>2A</sub>R plays a role in the normal activation and translocation of PP2A to its location of activity. Pharmacological deactivation of A<sub>2A</sub>R resulted in similar albeit less pronounced results. The precise function of the A<sub>2A</sub>R to necessitate its presence for normal baseline PP2A activity in the resting state remains to be determined.

#### Wild type and A<sub>2A</sub>R knockout model in study of cardiac contractility

Having established the main effects of adenosine in wild type animals and examined responses in A<sub>2A</sub>R knockout animals as well, the murine heart model can now be used as a platform for a variety of directions for future research. The most interesting prospective avenues of study are discussed in more detail in the “future directions” section but briefly the existing

findings lay the foundations for amongst other things, identification of new adenosinergic agents that are more specific for desired receptors and also synthesis of tissue selective targeting approaches for pharmacological agents; additional study of the impact of protein phosphatases in the regulation of myocardial contractility; the exploration of possible differences in adenylyl cyclase function and response to  $\beta$ -adrenergic stimulation between the wild type and  $A_{2A}R$  knockout animals; long term effect of  $A_{2A}R$  knockout or inhibition on the general functioning of the heart and responses to adrenergic stimuli, and lastly the involvement of adenosine receptors in preconditioning and pathways that are responsible for these effects.

#### Considerations in use of knockout animal models

Certain factors must be considered when utilizing a receptor knockout animal model. Beyond the basic considerations with respect to viability of the animals to a specific age and their ability to reproduce there may be unforeseen effects of receptor deletion that can alter or even interfere with experimental results. However, even the basic considerations of breeding may pose a challenge to successfully obtaining viable knockout animals for studies. For example, the  $A_{2A}R$  knockout animals used in the present study tend to become obese especially at over 6 months of age. While used at a much younger age for the experiments described in the presented studies, even those animals heterozygous for the  $A_{2A}R$  knockout gene would readily become obese when given the increased fat diet typically used for breeders. This would in turn result in decreased litter sizes and at times even failure to produce litters. Use of the standard diet for the breeders alleviated the problem.

In addition to logistical issues, working with a knockout model presents the challenge of interpreting the experimental results with consideration of possible global effect of the absence

of the receptor. The A<sub>2A</sub>R knockout mice are known to exhibit increased heart rate and blood pressure (97) while also manifesting altered characteristics with respect to manifestation of ischemic damage (7) and binding properties of receptor agents (64,88). The enhanced effects of A<sub>1</sub>R activation on PP2A activity as observed earlier in the discussion provide evidence that receptor function and their effects may indeed be different in the absence of other receptors that normally a part of the interaction. Finally, a recent study has shown that adenosine A<sub>1</sub>R deletion may have effects on the expression of other adenosine receptors (76).

Many of these alterations may not significantly change the results of experiments designed for the elucidation of receptor function and transduction pathways for the effects of the receptors, however, these differences between wild type and knockout animals need to be carefully considered if the knockout animals are used to design a treatment or clinical application. While a knockout model is highly useful in exploring the possible interactions between receptor subtypes, or efficiency of pharmacological agents with respect to adenosine receptors, it would be highly advisable to use wild type animals in studies where the goal is to examine the usefulness of receptor action in a more physiologically realistic situation. On the other hand, in considering applications involving long term alteration of receptor physiology such as gene therapy or long term agonist/antagonist treatment, the long term results of receptor deletion in the knockout animal model would be of high value as it would be the best available predictor of the long term consequences of proposed intervention.

### Future Directions

Based on the findings of the preceding studies, there are a number of avenues for continued research to further elucidate the role of adenosine receptors in regulation of cardiac

function particular contractility. In addition, several aspects of the myocardial response to adenosinergic stimulation may be useful clinically and as such may form a basis for treatment of relevant cardiac dysfunction. This section addresses firstly the aspects that merit further investigation from a basic science perspective, with respect to finer mechanisms of adenosine receptor action and co-regulation. Following is a section addressing the possible clinical implications of the findings and possible avenues of investigation to examine the usefulness of the observed responses in context of pathological conditions.

#### Basic Science:

##### *Regulation of PP2A by A<sub>2A</sub>R*

Having demonstrated the ability of the A<sub>2A</sub>R to modulate PP2A activity, the next logical steps would be to determine the mechanism by which the receptor affects the PP2A and what specific changes the phosphatase undergoes in the process of this regulation. As previously discussed it appears that the Tyr 307 site is not directly involved in regulation of PP2A activity by A<sub>2A</sub>R. There are several sites that are also known for altering PP2A activity and may be responsible for mediating the A<sub>2A</sub>R effect. In addition to the Tyr 307 site examined in the present study, the activity of the PP2A can be altered by methylation of the catalytic subunit as well as phosphorylation of other regulatory subunits (43). In order to determine the specific alterations of the PP2A enzyme, myocardial samples of PP2A can be tested with specific antibodies examining the states of the other known regulatory sites. Sites that result in PP2A deactivation and appear to be appropriately altered following A<sub>2A</sub>R activation would become likely candidates for the mediation of A<sub>2A</sub>R effects.

To ensure the specificity of this pathway to the A<sub>2A</sub>R, the experiments can be repeated in an A<sub>1</sub>R knockout animal. Results from this type of investigation may also yield information about the interaction of the A<sub>1</sub>R and A<sub>2A</sub>R at this co-regulatory point. As noted previously the A<sub>1</sub>R effects on PP2A appear to be greater in the A<sub>2A</sub>R knockout animals suggesting an inhibitory effect of A<sub>2A</sub>R on A<sub>1</sub>R. If the A<sub>1</sub>R knockout displays a response to A<sub>2A</sub>R stimulation that is significantly altered from that observed in the wild type, the finding may suggest that A<sub>1</sub>R can modulate the effects of A<sub>2A</sub>R as well.

In addition to the alterations to the PP2A enzyme itself, the pathway mediating the signal leading to that alteration is also of interest. A reasonable starting point would be to examine whether the p38 MAPK pathway previously reported to mediate A<sub>1</sub>R activation of PP2A is also influenced by the A<sub>2A</sub>R. The simplest way to accomplish this would be to examine whether acute A<sub>2A</sub>R activation results in p38 MAPK dephosphorylation. A more circumferential approach that may be used to verify this finding would be to artificially phosphorylate p38 MAPK in a tissue sample or activate it via guanylyl cyclase in an A<sub>2A</sub>R knockout animal. If this results in alterations in PP2A activity similar to those observed on the wild type animal following A<sub>2A</sub>R activation, then that observation would be consistent with the pathway being involved in the regulation of PP2A.

#### *Regulation of PP1 by A<sub>2A</sub>R and A<sub>1</sub>R*

Along with PP2A, PP1 is the other phosphatase mainly responsible for dephosphorylations of cardiac proteins. It would be interesting to ascertain whether PP1 activity is also modulated by adenosinergic pathways. An initial approach would be the repetition of experiments similar to those presented earlier in the study of PP2A. An appropriate substrate and

optimized assay conditions would allow a relatively easy survey of possible A<sub>1</sub>R and A<sub>2A</sub>R effects on the activity of PP1. If indeed such effects are observed, further detailed information may be obtained by more focused studies utilizing A<sub>1</sub>R or A<sub>2A</sub>R knockouts as appropriate. The pathways responsible for the transduction of the signal would also be of interest in the case of positive initial findings.

#### *Adenylyl cyclase activity in A<sub>2A</sub>R knockout*

During the study of adenosinergic modulation of PP2A activity it was noted that in the absence of A<sub>2A</sub>R signal (as seen in the knockout) the response to A<sub>1</sub>R stimulation was enhanced, suggesting that A<sub>2A</sub>R has an inhibitory effect on A<sub>1</sub>R. Enhanced effect of A<sub>1</sub>R activation was also observed following pharmacological inhibition of A<sub>2A</sub>R in previous a previous study of contractility in rat hearts (78). These findings raise the question of whether a similar phenomenon occurs with respect to adenylyl cyclase activation. It would be of interest whether the decrease of adenylyl cyclase activity following A<sub>1</sub>R activation in an A<sub>2A</sub>R knockout animal is greater compared with the wild type. The observed enhanced response to A<sub>1</sub>R activation in the A<sub>2A</sub>R knockout may be due to effects on adenylyl cyclase or another pathway such as activation of PP2A.

In the course of earlier presented contractile studies in isolated mouse hearts, it was noted that the response of the A<sub>2A</sub>R knockouts to A<sub>1</sub>R activation was not as pronounced as that seen in the wild type. The reason for that finding is not entirely clear, as the contrary finding was previously reported, and present studies of adenosinergic modulation PP2A activity also revealed greater effect of A<sub>1</sub>R in the absence of A<sub>2A</sub>R. A possible rationale for the observed decreased responsiveness to A<sub>1</sub>R stimulation in the contractile studies (assuming it is not an isolated

finding) may be a desensitization of adenylyl cyclase to the  $G_i$  mediated inhibitory signal of  $A_1R$  activation. It is possible that in the absence of  $A_{2A}R$  signal, the  $A_1R$  influence alone would result in bradycardia or even heart block if unopposed. This type of detrimental effect could in theory be countered by decreased sensitivity of adenylyl cyclase to  $A_1R$  stimulation. If this is the case it would explain the observations made during the present contractile studies in  $A_{2A}R$  knockouts. The effect of  $A_1R$  activation on adenylyl cyclase activity can be readily assessed by assay of cAMP formed in tissue extracts from wild type or  $A_{2A}R$  knockout myocardium.

#### *Direct interaction between $A_1R$ and $A_{2A}R$*

The present studies have shown that  $A_1R$  and  $A_{2A}R$  have several focal point of co-regulation. Modulation of adenylyl cyclase activity has been thought the "classical" mechanism of the opposing influences of  $A_1R$  and  $A_{2A}R$ , but protein phosphatase has also been shown to play an important role in this process. With the knowledge that there are multiple pathways and levels of interaction between these two adenosine receptors, it is curious whether in addition to downstream regulatory effects, these receptors have a more direct co-regulatory relationship. The question that follows is whether the  $A_1R$  and  $A_{2A}R$  receptors have a direct membrane linkage that alters the binding affinity of either receptor based on the activation state of the other.

An initial method of investigation would be immunoprecipitation studies directed at selectively precipitating the  $A_1R$  or  $A_{2A}R$  in myocardial homogenate preparations. Following immunoprecipitation the resulting product would be probed for the presence of both the receptor targeted by the antibody as well as the other receptor of interest. If  $A_1R$  and  $A_{2A}R$  are found to co-precipitate that may suggest that a membrane linkage may exist between the two. Further analysis of the related proteins may be accomplished via mass spectrometry to identify candidate

proteins. As an advanced step, the importance of these candidate proteins to the co-regulation of the A<sub>1</sub>R and A<sub>2A</sub>R may be verified by creating a genetic knockout of the protein in question, or selectively inhibiting expression of the proteins in question using siRNA techniques if a tissue culture approach is preferred.

An alternate way to begin approaching the issue would be to examine whether the activation state of the A<sub>1</sub>R and A<sub>2A</sub>R, affects the binding affinity for appropriate radiolabeled agonist or antagonist. This type of study may give an initial indication of whether activation of either receptor has a direct effect on its counterpart. The absence of findings in these experiments would suggest that direct interaction between A<sub>1</sub>R and A<sub>2A</sub>R is unlikely and the interaction of their effects occurs downstream of receptor activation. Positive findings however would eventually require an approach such as outlined above in order to elucidate the exact nature of receptor interaction.

#### Clinical Applications:

##### *A<sub>2A</sub>R selective maintenance of contractility in acute heart failure*

As noted in the contractile studies previously described, one of the effects of A<sub>2A</sub>R activation is enhancement of contractility in the presence of reduced blood flow.

It may be possible to make use of this effect to support the contractile function of the heart during an acute event such as myocardial ischemia or hypovolemic shock. In both of these conditions the primary concern is the inadequate blood flow to the myocardium whether local or global.

The use of a reversible A<sub>2A</sub>R agonist may provide support for the myocardium for the period of time necessary to enact treatment of the underlying condition. This type of application



can be tested using a rodent model of local myocardial ischemia or hypovolemic shock. Both of these conditions are readily replicated and the rodent model would allow for easy administration of the receptor agonist as well as monitoring of the response.

#### *A<sub>2A</sub>R-induced vasodilation to treat local ischemia acutely or post-acutely*

One of the most readily notable effects of A<sub>2A</sub>R activation is the coronary vasodilation it causes. This effect may be useful to produce vasodilation during procedures such as angioplasty where the goal is to restore flow to myocardium following restoration of patency in a previously occluded vessel. Local administration of a selective A<sub>2A</sub>R agonist may produce vessel relaxation and enhanced flow to the previously ischemic region. This approach may be combined with techniques such as gradual or “stuttered” reperfusion in order to minimize reperfusion injury to the myocardium. The vasodilatory effect of A<sub>2A</sub>R may also be useful on a chronic level if a low dose agonist is released from the stent placed following angioplasty. The vasodilatory effect may contribute to enhanced flow to regional myocardium and decreased risk of post procedure thrombosis of the stent site. These applications are possible to test using a larger test model (canine or porcine) where the coronary vasculature may be used to simulate conditions and procedures present during catheterization in the human heart.

#### *Protein phosphatase manipulation to treat chronic heart failure*

The observed inhibitory effects of A<sub>2A</sub>R on PP2A suggest that reduced dephosphorylation of proteins important in contractile function contributes to enhanced contractile response observed with A<sub>2A</sub>R activation. These observations combined with the results of previous studies showing enhanced contractility in hearts overexpressing phosphatase

inhibitors suggest that inhibition of PP1 or PP2A may be a functional therapeutic approach to treat chronic heart failure. Initial testing of this methodology may be accomplished by using a murine model of heart failure induced by pressure overload achieved by aortic banding. A phosphatase inhibitor of choice may be administered to one group of animals and the progression of heart failure in that group compared to a control group that is not treated with the inhibitor. Slowed progression of heart failure would suggest that this approach can possibly be used to treat the progression of heart failure occurring from natural causes.

The problem of selective targeting of the phosphatase inhibitor may eventually be solved by utilizing gene therapy as opposed to direct administration of phosphatase antagonists. If it were possible to selectively induce expression of a phosphatase inhibitor in the myocardium, the resultant increase in contractile function could be beneficial to treat end stage chronic heart failure perhaps improving efficacy of a ventricular assist device or providing additional time to acquire a transplant.

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