A3 adenosine receptor stimulation modulates sarcoplasmic reticulum Ca$^{2+}$ release in rat heart

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Abstract

Objective: Stimulation of A3 adenosine receptors has been shown to protect cardiac myocytes from ischemic injury, but the mechanism of this action is unknown. We evaluated the effect of adenosine agonists and antagonists on the sarcoplasmic reticulum (SR) Ca$^{2+}$ channels.

Methods: Isolated rat hearts were perfused with control buffer or different adenosine agonists and antagonists. Hearts were then homogenized and used to determine SR Ca$^{2+}$-induced Ca$^{2+}$ release, assayed by quick filtration technique after loading with $^{45}$Ca$^{2+}$, and the binding of $[^3H]$ryanodine, a specific ligand of the SR Ca$^{2+}$ release channel. In parallel experiments, hearts were challenged with 30 min of global ischemia and 120 min of reperfusion, and the extent of tissue necrosis was evaluated by triphenyltetrazolium chloride staining.

Results: Perfusion with the A1$\cdot$.A3 agonist R-PIA and the A3$\cdot$.A1 agonist IB-MECA was associated with reduced $[^3H]$ryanodine binding, due to reduced $B_{\text{max}}$ (by about 20%), whereas $K_d$ and Ca$^{2+}$-dependence of the binding reaction were unaffected. These actions were abolished by the A3 antagonist MRS 1191, while they were not affected by A1 and A2 antagonists. The rate constant of SR Ca$^{2+}$ release decreased by 25–30% in hearts perfused with R-PIA or IB-MECA. Tissue necrosis was significantly reduced in the presence of R-PIA or IB-MECA. Protection was removed by MRS 1191, and it was not affected by A1 and A2 antagonists. Hearts were also protected by administration of dantrolene, a ryanodine receptor antagonist. In the presence of dantrolene, no further protection was provided by IB-MECA.

Conclusion: A3 adenosine receptor stimulation modulates the SR Ca$^{2+}$ channel. This action might account for the protective effect of adenosine.

Keywords: Adenosine; Calcium (cellular); Ion channels; Receptors; SR (function)

1. Introduction

Adenosine is known to exert a cardioprotective effect in myocardial ischemia and reperfusion [1,2], and it has been proposed as mediator of ischemic preconditioning [3,4]. The transduction pathways involved in the response to adenosine have not been definitely established. The protective effect has been initially attributed to stimulation of A1 receptors, since it was reproduced by A1 agonists such as N$^6$-cyclopentyladenosine or N$^6$-R-phenylisopropyladenosine. However, in several experimental models protection was not abolished by selective A1 antagonists. Further studies have shown that many A1 agonists also interact with the A3 receptor [5,6] and that ischemic injury can be reduced by A3 agonists, suggesting a role of A3 receptor stimulation in the protective action of adenosine [7–18].

The effector mechanisms responsible for the beneficial action of adenosine are poorly understood. Hemodynamic changes, metabolic effects, or activation of ATP-dependent K$^+$ channels have been proposed as mediators of A1 responses. In the case of A3 stimulation, the matter is even more obscure. Although A3 receptors are expressed in cardiac myocytes [5,14], their functional role is unknown. On the basis of pharmacological studies, either protein kinase C [11] or $K_A^{\text{ATP}}$ channels [18,19] have been implicated in the response to A3 stimulation.

We have previously reported that sarcoplasmic reticulum (SR) Ca$^{2+}$ release capability is reduced after ischemia–

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reperfusion [20], and this phenomenon is likely to be involved in the pathogenesis of ischemic preconditioning [21]. In the present work we used an isolated rat heart model to characterize the cardioprotective action of adenosine agonists, and to investigate their effects on the SR Ca\(^{2+}\) release channel/ryanodine receptor.

2. Methods

2.1. Animals and perfusion technique

Male Wistar rats (275–300 g body weight), fed with standard diet, were anesthetized with a mixture of ether and air. After injection of 1000 U sodium heparin in the femoral vein, the heart was quickly excised and perfused according to the working heart technique, as described previously [20]. Experimental procedures were approved by the ethical committee of the University of Pisa. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Adenosine agonists or antagonists were added to the perfusion buffer after 5 min of control perfusion, and hearts were treated with these substances for an additional 15-min period. The following substances were used: N\(^6\)-R-phenylisopropyladenosine (R-PIA), an A1 agonist that is over 100-fold selective for A1 vs. A2 receptors, whose A1/A3 affinity ratio has been reported to be in the range of 50:1 (cloned rabbit receptor) [22] to 1000:1 (cloned sheep cases. A1/A3 affinity ratio has been reported to be in the range of DMSO at 1±10 mmol/l concentration. Final DMSO (without any addition) was always used in the final 120-min of retrograde reperfusion, and it was not recirculated.

2.2. Preparation of cellular fractions

At the end of the perfusions aimed at determining Ca\(^{2+}\)-induced Ca\(^{2+}\) release or \[^{3}H\]ryanodine binding, ventricles were homogenized as described previously [20]. In some experiments, assays were performed in the crude homogenate. In others, the homogenate was used to prepare a microsomal fraction enriched in SR, as described previously [20].

2.3. Assay of \[^{3}H\]ryanodine binding and of SR Ca\(^{2+}\) release

High affinity ryanodine binding was assayed as described previously [20]. Unless otherwise specified, free Ca\(^{2+}\) concentration was 18 \(\mu\text{mol/l}\). The difference between the counts of duplicate samples was <10% in all cases. SR Ca\(^{2+}\)-induced Ca\(^{2+}\) release was determined as described previously [21]. Briefly, vesicles were passively loaded for 120 min at 23°C in a medium containing 10 mmol/l \(^{45}\text{CaCl}_2\) and applied to cellulose nitrate filters. \(^{45}\text{Ca}^{2+}\) release was induced by washing the loaded vesicles, for a preset time, with release buffer, containing 20 mmol/l Hepes-potassium (pH 6.8), 100 mmol/l KCl, 1.01 mmol/l CaCl\(_2\), 1 mmol/l EGTA (free Ca\(^{2+}\) concentration was 15 \(\mu\text{mol/l}\)). A rapid filtration system with time resolution in the order of 10 ms was used (RFS-4, BioLogics, Grenoble, France), and the rate constant of quick Ca\(^{2+}\) release (\(K_r\)) was calculated over the first 100 ms by exponential fitting [21].

2.4. Chemical and radionuclides

R-PIA, CPX, CGS 21680, dantrolene and EGTA were obtained from Sigma Chemicals Co. (St. Louis, MO). IB-MECA and MRS 1191 were purchased from Research Biomedical International (Natick, MA). ZM 241385 was purchased from Tocris Cookson Ltd (Bristol, UK). All adenosine agonists and antagonists were initially dissolved in DMSO at 1–10 mmol/l concentration. Final DMSO concentration was <0.1% in all cases, and an equivalent amount of DMSO was included in the corresponding
control experiments. Ryanodine was purchased from Calbiochem (La Jolla, CA). [3H]Ryanodine and 45CaCl2 were obtained from New England Nuclear–DuPont (Milan, Italy). Free Ca2+ concentration was calculated as described elsewhere, and it was checked by the antipyrilaazo III technique [20].

2.5. Statistical analysis

Results are expressed as mean±S.E.M. Least-squares linear regression analysis was used to calculate binding and release parameters, after appropriate linearizing transformations (respectively, Scatchard transformation and logarithmic transformation). Differences between groups were evaluated as follows. One-way analysis of variance was used as a global test for differences between means. If between-groups variance was significantly (P<0.05) higher than within-groups variance, appropriate post-hoc tests were performed. In experiments aimed at determining differences versus a single control group, Dunnett’s test was applied; in experiments projected for pairwise comparisons of all groups, Student–Neuman–Keul’s test was applied. GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA) was used for data processing and statistical analysis.

3. Results

3.1. Myocardial protection

When control hearts were challenged by 30 min of sustained ischemia and 120 min of reperfusion, the extent of myocardial necrosis, as evaluated by TTC staining, corresponded to 31.6±2.4% of the ventricular mass. We first determined the effect of adenosine agonists on ischemic injury. Both R-PIA, at concentrations ≥15 nmol/l, and IB-MECA, at concentrations >50 nmol/l (Fig. 1), significantly decreased the extent of TTC-negative tissue, whereas CGS 21680 (up to 1 μmol/l) was ineffective (TTC-negative tissue averaged 31.5±3.7% with 1 μmol/l CGS 21680).

The pharmacology of myocardial protection was further investigated by using specific antagonists, and through the evaluation of hemodynamic effects, that are shown in Table 1. Preliminary experiments (not shown) revealed that perfusion with adenosine antagonists alone (i.e. 100 nmol/l CPX, 20 nmol/l ZM 241385 or 1 μmol/l MRS 1191) did not affect any hemodynamic variable and did not modify susceptibility to ischemia. The protective effect of R-PIA was preserved in the presence of 100 nmol/l CPX or 20 nmol/l ZM 241385, whereas it was abolished by the A3 antagonist MRS 1191, at 1 μmol/l concentration (Fig. 2, upper panel). Analysis of contractile performance confirmed dissociation between cardioprotection and A1 response. Perfusion with R-PIA determined dose dependent reductions of heart rate, aortic flow and cardiac output, consistent with the well-known effects of A1 receptor stimulation. If R-PIA was used in the presence of 100 nmol/l CPX, negative chronotropic and inotropic actions were abolished, confirming that CPX caused effective A1 blockade. On the other hand, MRS 1191 did not modify the hemodynamic effects of R-PIA, showing no evidence of A1 antagonism.

Perfusion by IB-MECA produced moderate increase in coronary flow, while no A1 effect (particularly, no change in heart rate) was produced. Changes in coronary flow were blocked by ZM 241385 but they were not affected by MRS 1191, suggesting an A2 action. However, the protective effect of IB-MECA was abolished by MRS 1191, while it was not affected by the A2 antagonist ZM 241385 (Fig. 2, lower panel). On the whole, our results suggest

Fig. 1. Protective effect of R-PIA (left) and IB-MECA (right). Hearts were perfused with R-PIA and then subjected to 30 min of global ischemia and 120 min of retrograde reperfusion. The amount of tissue that was not stained by TTC, which is an index of myocardial necrosis, is represented on the vertical axis as percentage of ventricular mass. Data points represent mean±S.E.M. of three to 11 hearts per group. * P<0.05, † P<0.01 vs. control, by ANOVA and Dunnett’s test.
that, in our experimental model, the protective effect of adenosine agonists was mediated by A3 receptor stimulation.

3.2. \[^{1}H\]ryanodine binding

Results of saturation binding experiments performed in crude homogenate are shown in Fig. 3. In the control condition, ryanodine binding was characterized by $K_d=2.5 \pm 0.4 \text{ nmol/l}$ and $B_{\text{max}}=408 \pm 10 \text{ fmol per mg of protein}$. In hearts perfused with 50 nmol/l R-PIA, ryanodine binding was significantly decreased: we observed a 20% reduction in binding site density (339 ± 27 fmol/mg, $P<0.05$), whereas the affinity for ryanodine was not significantly modified ($K_d=1.8 \pm 0.4 \text{ nM}$). The Ca\(^{2+}\)-dependence of ryanodine binding was also unaffected: $EC_{50}$ for Ca\(^{2+}\) averaged 1.4 \text{ \mu M/l}, without significant differences between groups (data not shown). Perfusion with CGS 21680 (1 \text{ \mu mol/l}) did not affect ryanodine binding, whereas IB-MECA (100 nmol/l) determined similar changes as R-PIA ($B_{\text{max}}=342 \pm 22 \text{ fmol/mg, } P<0.05; K_d=2.6 \pm 0.5 \text{ nM}, P=\text{NS}$).

The pharmacological profile of ryanodine receptor modulation, as evaluated by using different adenosine receptor antagonists, was similar to the pharmacological profile of cardioprotection. In particular, the effect of R-PIA was abolished by MRS 1191, while it was not affected by either CPX or ZM 241385 (Fig. 4, upper panel). The effect of IB-MECA was also abolished by MRS 1191 (Fig. 4, lower panel).

3.3. Ca\(^{2+}\)-induced Ca\(^{2+}\) release

Reduced \[^{1}H\]ryanodine binding is usually associated with reduced Ca\(^{2+}\) release capability of the SR. To confirm the functional implications of binding experiments, we assayed Ca\(^{2+}\)-induced Ca\(^{2+}\) release in hearts which had been perfused with 50 nmol/l R-PIA or 100 nmol/l IB-MECA. After passive loading, the \(^{45}\text{Ca}\) content of control heart homogenate was on the order of 9–10 nmol/mg of protein. Exposure to the release buffer determined the quick release of 3–4 nmol/mg, which was completed over 100–120 ms. Thereafter the rate of Ca\(^{2+}\) release decreased considerably. The quick component of Ca\(^{2+}\) release was abolished if filters were washed by ‘non-release’ buffer, containing 20 mmol/l Hepes-potassium (pH 6.8), 100 mmol/l KCl, 10 mmol/l MgCl\(_2\) and 10 \mu mol/l ruthenium red, confirming that it represented Ca\(^{2+}\) efflux through the SR release channels (data not shown; results were quite similar to those reported in Ref. [21]).

The extent of \(^{45}\text{Ca}\) loading and of the \(^{40}\text{Ca}\) pool subjected to quick release were not significantly different between the three experimental groups. The experiments described in the previous sections were performed in crude homogenate. We have previously argued, in agreement with other investigators, that the use of a crude preparation does not limit the reliability of these assays, while it avoids the risk of selecting subpopulations of SR vesicles, which may not be representative of the

### Table 1: Hemodynamic variables

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<th>HR (%)</th>
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*Values are mean±S.E.M. derived from a total of 110 hearts (minimum: three hearts per group). HR, heart rate; AF, aortic flow; CF, coronary flow; CO, cardiac output; PAP, peak systolic aortic pressure; HR, heart rate. Hemodynamic variables were measured just before ischemia (i.e. after 20 min of perfusion) and are expressed as percentage of the basal values, that were measured after 5 min of perfusion, i.e. before any addition to the perfusion buffer. Actual basal values averaged as follows (without any significant difference between groups): HR 277±4 beats/min; AF 50.8±0.6 ml/min; CF 17.5±0.3 ml/min; CO 68.3±0.9 ml/min; PAP 131±2 mmHg. * $P<0.05$; $P<0.01$ vs. control, by ANOVA and Dunnett’s test.
Fig. 2. Effect of adenosine receptor antagonists on the protective effect of R-PIA (upper panel) and IB-MECA (lower panel). Hearts were perfused with control buffer, 50 nmol/l R-PIA or 100 nmol/l IB-MECA, in the presence or in the absence of either A1 antagonist CPX (100 nmol/l), A2 antagonist ZM 241385 (20 nmol/l) or A3 antagonist ZM 241385 (1 μmol/l), before being subjected to 30 min of global ischemia and 120 min of retrograde reperfusion. The amount of tissue that was not stained by TTC is represented on the vertical axis as percentage of ventricular mass. Preliminary experiments showed that perfusion with CPX, ZM 241385 or MRS 1191 alone had no effect on the extent of tissue injury. Bars represent mean ± S.E.M. of three to 11 hearts per group. * P<0.05 vs. control, by ANOVA and Dunnett’s test.

Fig. 3. Left: saturation binding curves for [H]ryanodine (specific binding) obtained in homogenates derived from hearts perfused under control conditions (●) or in the presence of 50 nmol/l R-PIA (○), 1 μmol/l CGS 21680 (■), or 100 nmol/l IB-MECA (□). Bmax and Kd were calculated in each experiment by linear regression after Scatchard transformation. Bmax values are shown on the right. Bars represent mean ± S.E.M. of three to 12 experiments in each group. * P<0.05 vs. control, by ANOVA and Dunnett’s test. Kd values were not significantly different between groups (they averaged 2.5±0.4, 1.8±0.4, 2.3±1.2, and 2.6±0.5 nmol/l, respectively).

Fig. 4. Pharmacological characterization of the effect of R-PIA (upper panel) and IB-MECA (lower panel) on [H]ryanodine binding. Hearts were perfused with control buffer, 50 nmol/l R-PIA or 100 nmol/l IB-MECA, in the presence or in the absence of either A1 antagonist CPX (100 nmol/l), A2 antagonist ZM 241385 (20 nmol/l) or A3 antagonist MRS 1191 (1 μmol/l). [H]ryanodine binding was assayed in crude homogenate and Bmax values are represented on the vertical axis. No significant difference in Kd was observed. Preliminary experiments showed that perfusion with CPX, ZM 241385 or MRS 1191 alone had no effect on [H]ryanodine binding. Bars represent mean ± S.E.M. of three to 12 hearts per group. * P<0.05, † P<0.01 vs. control, by ANOVA and Dunnett’s test.
Fig. 5. Ca\(^{2+}\)-induced Ca\(^{2+}\) release determined in crude homogenates derived from hearts perfused under control conditions (●), in the presence of 50 nmol/l R-PIA (○) or in the presence of 100 nmol/l IB-MECA (□). The vertical axis shows the logarithm of the ratio of \(^{45}\text{Ca}\) to \(^{45}\text{Ca}\) at time zero, after subtraction of the amount of \(^{45}\text{Ca}\) not involved in quick Ca\(^{2+}\) release. Since release follows exponential kinetics, slope of regression lines represents rate constant of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Rate constant values were calculated in each experiment and then averaged. Means±S.E.M. values are plotted on the right. The total amount of Ca\(^{2+}\) released over the first 200 ms, which corresponds to the Ca pool subjected to Ca\(^{2+}\)-induced Ca\(^{2+}\) release, averaged 3.8 nmol per mg of protein, without any significant difference between groups (for further details, see Methods and Ref. [21]). Data points represent mean±S.E.M. of four to five experiments in each group.* P<0.05 vs. control, by ANOVA and Dunnett’s test.

whole SR (see Discussion). However, we repeated selected experiments using a microsomal fraction enriched in SR, with similar results. In binding experiments, perfusion with 100 nmol/l IB-MECA produced significant decrease of \(^{3}\text{H}\)ryanodine binding; \(B_{\text{max}}\) averaged 1.29±0.12 pmol/mg of protein in the control group vs. 0.94±0.02 pmol/mg of protein in the IB-MECA group (P<0.05, Student’s t-test, n=3 in each group); \(K_{d}\) averaged 2.3±0.3 vs. 2.1±0.1 nmol/l (P=NS). In release experiments, perfusion with 100 nmol/l IB-MECA significantly reduced the rate constant of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (32.3 vs. 48.9 s\(^{-1}\)).

The microsomal fraction was also used to assess the effects of R-PIA and IB-MECA on \(^{3}\text{H}\)ryanodine binding in vitro. Binding was assayed after including appropriate concentrations (1 nmol/l to 100 \(\mu\text{mol/l}\)) of either R-PIA or IB-MECA in the binding buffer. No significant effect was observed, even at concentrations that exceeded those used in perfusion experiments by several orders of magnitude (data not shown).

3.5. Interaction between IB-MECA and dantrolene

To evaluate the role of ryanodine receptor modulation in the cardioprotective effect of adenosine agonists, we studied their interaction with dantrolene, a ryanodine receptor blocker, whose administration is able to increase resistance to ischemia [26]. Hearts were perfused with 4 \(\mu\text{mol/l}\) dantrolene, 100 nmol/l IB-MECA or 4 \(\mu\text{mol/l}\) dantrolene and 100 nmol/l IB-MECA. After 30 min of ischemia, the extent of TTC-negative tissue averaged 2±2% with dantrolene alone vs. 2±1% with dantrolene and IB-MECA. Since the amount of residual injury might be too low to reveal an additive effect, in other experiments the duration of ischemia was prolonged to 45 min. Results are shown in Fig. 6: under control conditions myocardial necrosis averaged 52.7±10.3% of ventricular mass; both dantrolene and IB-MECA were protective, but their effect was not additive (necrosis averaged 23.5±2.1% with dantrolene and 19.8±1.8% with dantrolene and IB-MECA, P=NS).

Fig. 6. Protective effect of IB-MECA and dantrolene. Hearts were perfused with IB-MECA (100 nmol/l), dantrolene (4 \(\mu\text{mol/l}\)) or both dantrolene and IB-MECA, and then subjected to 45 min of global ischemia and 120 min of retrograde reperfusion. The amount of tissue that was not stained by TTC is represented on the vertical axis as percentage of ventricular mass. Hemodynamic effects of dantrolene and IB-MECA were similar, i.e. no significant change was produced except for a slight increase in coronary flow. Data points represent mean±S.E.M. of four hearts per group. * P<0.05, vs. control, by ANOVA and Student–Neumann–Keul’s test. All other pairwise comparisons did not yield statistical significance.
4. Discussion

The protective effect of adenosine agonists in myocardial ischemia or ischemia–reperfusion has been extensively investigated. The rat had been initially considered as an adenosine-insensitive species, since adenosine infusion did not reduce ischemic injury either in vivo or in isolated perfused hearts [27,28]. However, the rate of adenosine catabolism is particularly high in this species, and negative results might be accounted for by extensive breakdown of exogenous adenosine in blood cells, endothelium, or cardiomyocytes. The present findings support this interpretation, since remarkable protection was elicited by non-metabolizable adenosine derivatives, which is consistent with the reported effectiveness of 2-chloro-N^6-cyclopentyladenosine [29].

The signaling pathway involved in the protective effect of adenosine derivatives has not been fully characterized. In particular, the relative role of A1 and A3 receptors is controversial, and species differences might be important [7–18]. Our findings suggest that, in rat heart, cardiac protection is mainly due to A3 receptor stimulation. In fact, the protective action of R-PIA was not modified by CPX, although the latter abolished the functional consequences of A1 stimulation (e.g. heart rate reduction), while it was prevented by A3 antagonist MRS 1191. Involvement of A3 receptors was confirmed by the effectiveness of A3 agonist IB-MECA, whose protective effect was also abolished by MRS 1191. IB-MECA did not produce any negative chronotropic or inotropic action, while it caused slight increase in coronary flow, which was prevented by A2 antagonist ZM 241385. A2-mediated coronary dilation by IB-MECA has also been reported in rat by Lasley et al. [30], and it is probably related to the large A2a receptor reserve for coronary vasodilation [31]. The increase in coronary flow observed in the presence of R-PIA and CPX also might represent an A2 effect, unmasked by inhibition of the A1 response. However, ZM 241385 did not affect the protective effect of IB-MECA or R-PIA, suggesting that A2 stimulation played no role in it.

IB-MECA was effective at concentrations that are higher than its $K_d$, as assessed in vitro, in cells transfected with cloned rabbit A3 receptor (about 2 nmol/l) [22]. However, species differences in the affinity of adenosine receptor agonists have been described, and the pharmacology of rat A3 receptor has not been characterized. In addition, the relationship between intracoronary and interstitial concentration of adenosine is not known, although this is obviously a crucial issue for interpretation of intact heart experiments. Protection by IB-MECA has been observed, at 10–100 nmol/l concentration, in rabbit heart, and in chicken cardiomyocytes [12,13,15], which is consistent with our findings.

The cellular mechanism of the protective effect of adenosine is poorly characterized, and the effectiveness of A3 agonists is puzzling, since the physiological role of cardiac A3 receptors is still unknown. Adenosine has been implicated in the pathogenesis of ischemic preconditioning [3,4], and we have reported that ischemic preconditioning is associated with modifications of the SR Ca^{2+} channel (ryanodine receptor), which might delay the development of cytosolic Ca^{2+} overload [21]. For these reasons, we investigated the effect of adenosine agonists on the ryanodine receptor. Ryanodine binding was reduced, and the pharmacological profile of this action was consistent with an A3 effect. As observed after ischemic preconditioning [21], ryanodine receptor changes consisted in moderate decrease of binding site density, whereas the affinity for ryanodine and the Ca^{2+}-dependence of ryanodine binding were unaffected.

Ryanodine binding is often used as a tool to evaluate the functional state of the SR channel, since increased binding is usually associated with increased open probability of the channel and vice-versa [32,33]. There are, however, some exceptions to this rule [33]. Therefore, we assayed SR Ca^{2+}-induced Ca^{2+} release, confirming that it was decreased by IB-MECA and R-PIA.

Most of our experiments were performed in crude homogenate, because purification procedures may select vesicles that are not representative of the whole SR [20,34]. SR Ca^{2+} release can be reliably measured in crude preparations, since no other structure can support Ca^{2+} release that has $t_{1/2}$ on the order of a few milliseconds and is inhibited by Mg^{2+} and ruthenium red. In any case, we repeated the assay in a microsomal preparation, with similar results.

The functional implications of ryanodine receptor modulation by adenosine are not easy to predict. In our experiments, contractile performance was not affected by IB-MECA, and similar results (i.e. unchanged contractility with reduced ryanodine binding) were obtained after brief ischemia and reperfusion [21]. It seems that, under basal conditions, moderate reduction of SR Ca^{2+} release capability does not affect contractile protein activation. However, under conditions of increased Ca^{2+} cycling, even slight changes in SR channel function might become critical. In particular, inotropic stimulation — produced by either physiological stimuli (e.g. adrenergic effectors) or pharmacological agents (e.g. digoxin) — is usually associated with increased SR Ca^{2+} content and increased SR Ca^{2+} release. Cardiac A3 receptors might modulate the response to inotropic stimuli, which is consistent with the concept that adenosine is a `retaliatory metabolite’, originally introduced with regard to A1 and A2 effects only [35]. Modulation of SR Ca^{2+} release should also determine important consequences during sustained ischemia, since theoretical considerations and experimental findings [36] suggest that ischemia is associated with increased SR Ca^{2+} cycling, and that SR Ca^{2+} release plays a major role in the development of cytosolic Ca^{2+} overload, which is one of the chief determinants of irreversible cellular injury.

It is difficult to prove the existence of a causal relation-
ship between ryanodine receptor modulation and cardiac protection. We have compared the response to IB-MECA and to dantrolene, a ryanodine receptor antagonist which also increases resistance to ischemia [26]. If IB-MECA and dantrolene have different molecular targets, it would be reasonable to expect an additive effect. As a matter of fact, addition of IB-MECA to dantrolene did not produce further reduction in tissue injury. Although this finding is not conclusive, it is consistent with the concept that reduction of SR Ca$^{2+}$ release contributes to the beneficial effect of IB-MECA.

Cardiovascular consequences of A3 adenosine receptor stimulation have often been attributed to mast cell degranulation: either vasodilator [37,38] or vasoconstrictor [39] actions have been reported. It seems unlikely that mast cells are involved in the effects which we have observed. Although coronary flow increased in the presence of 100 nmol/l IB-MECA, this appears to represent an A2 response, since it was inhibited by ZM 241385, and it was not necessary for cardioprotection to occur. In fact, A2 agonist CGS 21680 was not protective, while both R-PIA and IB-MECA were still protective in the presence of A2 antagonist ZM 241385. In addition, there is evidence that mast cell degranulation is either ineffective [40] or detrimental [41] in rat heart models of ischemia-reperfusion.

In other experimental models, the cardioprotective action of A3 agonists was abolished by glibenclamide, an inhibitor of K$_{ATP}$ channels [18,19], suggesting that A3 adenosine receptors may be coupled to K$_{ATP}$ channels. While it is possible that SR Ca$^{2+}$ channels and K$_{ATP}$ channels be different targets of A3 stimulation, it is not clear whether K$_{ATP}$ channel activity or K$^+$ channel modulators may produce some effects on the SR Ca$^{2+}$ channel. Interestingly, there is indirect evidence that K$^+$ channel modulators may affect SR Ca$^{2+}$ release [33].

The transduction pathway that links A3 adenosine receptors to SR Ca$^{2+}$ channels remains to be determined. A3 receptors are coupled to several G proteins, particularly G$_{i}$a-2, G$_{i}$a-3 and G$_{i}$a [42]. A3 stimulation has been reported to activate phospholipases C and D [25]. In some experimental models, the cardioprotective effect of A3 agonists was abolished by chelerythrine, an inhibitor of protein kinase C [11], and the ryanodine receptor is a substrate for several kinases, including protein kinase C, which have complex effects on channel gating [33]. Therefore, transduction mechanisms involving G protein-mediated kinase activation, leading to channel phosphorylation, deserve further investigation.

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