ELSEVIER

European Journal of Pharmacology 370 (1999) 27–37

ejp

Intravascular adenosine: the endothelial mediators of its negative dromotropic effects

Rafael Rubio [a,*], Guillermo Ceballos [b], Eduardo Balcells [c]

[a] *Department of Physiology, Facultad de Medicina, University Aut. de San Luis Potosi, Av. V. Carranza 2405, 78216, San Luis Potosi, Mexico*
[b] *Postgraduate Department, Escuela Superior de Medicina, I.P.N., Mexico*
[c] *Division of Cardiology, University of Virginia, Charlottesville, VA, USA*

Received 29 June 1998; received in revised form 1 February 1999; accepted 5 February 1999

Abstract

Intravascular adenosine may exert its negative dromotropic effect via activation of luminal coronary endothelial receptors, which suggests the presence of transcellular dromotropic mediators of endothelial origin, perhaps nitric oxide (NO) and prostaglandins. We decided to test this hypothesis in isolated guinea pig hearts retrogradely perfused with Krebs–Henseleit solution. A pair of stimulating electrodes were placed in the right atria and the auricular–ventricular (A-V) delay recorded by means of a recording electrode placed on the left atria and an electrode placed on the tip of the ventricle. Hearts were paced at a rate of 3.8 ± 0.2 Hz and perfused at a coronary flow of 9 ± 0.25 ml/min. To obtain dose–response curves, single doses (as boluses) of different concentrations of adenosine were infused and the maximal increase in A-V delay induced by each dose was determined. Agents that inhibit NO accumulation, such as N^G-nitro-L-arginine methyl ester (L-NAME) and oxyhemoglobin, diminished the effect of adenosine while NO-sparing agents, such as superoxide dismutase and dithiotreitol, enhanced the adenosine effect. Infusion of NO and the NO donor morpholinosydnonimine increased the A-V delay in a dose-dependent manner. In addition, the dose–response curve for adenosine was displaced downward and to the right by indomethacin, indicating also the involvement of prostaglandins. Infusion of L-NAME in addition to indomethacin further diminished the effects of adenosine, indicating that NO and prostaglandins acted simultaneously. To selectively activate intravascular endothelial adenosine receptors, adenosine amino congener (ADAC), an adenosine A_1 receptor agonist, was covalently coupled to 2×10^6 Da dextran. When intracoronarily infused, the dextran–ADAC complex remains in the blood vessel lumen because it is too large to diffuse to the interstitium. On intracoronary administration, the dextran–ADAC complex caused a negative dromotropic effect which was diminished by L-NAME and indomethacin. Our data indicate that the dromotropic effect caused by intracoronarily administered adenosine is the result solely of activation of intravascular endothelial adenosine receptors, possibly type A_1, and that NO and prostaglandins are synergistic endothelial mediators of this effect.

Keywords: Luminal endothelial receptor; Endothelial control; Parenchymal function; Endothelial mediator; Nitric oxide (NO); Prostaglandins, endothelial hyperpolarizing factor; Cardiac function control

1. Introduction

Adenosine has well-defined cardiovascular actions which include coronary vasodilatation (Olsson et al., 1976; Schrader et al., 1977; Rubio et al., 1983; Kroll et al., 1987), a negative chronotropic/dromotropic effect (Clemo, 1986; Belardinelli et al., 1987; Balcells et al., 1992, 1993), and an anti-adrenergic response (Dobson, 1983). While it is now known that most of these actions are mediated via membrane bound receptors (Olsson et al., 1976; Schrader et al., 1977; Jacobson et al., 1985, 1987), the precise mechanisms by which this metabolite exerts its cardiovascular effects have yet to be defined. Difficulties arise secondary to the numerous influences involved in its formation and metabolism. Within the myocardium, adenosine can be produced and metabolized by both the endothelium (Nees et al., 1985a,b; Deussen et al., 1986; Dendorfer et al., 1987) and cardiac myocytes (Deussen et al., 1986; Dendorfer et al., 1987) and therefore is subject to the

* Corresponding author. Tel.: +52-48-26-23-24; Fax: +52-48-17-69-76; E-mail: rrubio@deimos.tc.uaslp.mx

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00079-5

influence of both cell types. In addition, the relative importance of the endothelium becomes evident if one considers the following: adenosine remains confined to the intravascular compartment during its intracoronary infusion, in up to micromolar concentrations, because of the impermeable metabolic barrier imposed by the endothelium (Nees et al., 1985a,b; Tietjan et al., 1990), and yet the vasodilatory and negative dromotropic effects of adenosine are observable at these concentrations (Nees et al., 1985a,b; Clemo, 1986); the pharmacokinetics of macromolecular adenosine analogs are similar to those of their low molecular weight counterparts during intracoronary infusion, suggesting an intravascular site of action (Nees et al., 1985a,b; Balcells et al., 1992, 1993; Smits et al., 1995); and intracoronarily infused adenosine deaminase alters the vasodilator and dromotropic responses attributed to adenosine (Belardinelli et al., 1987; Rubio et al., 1987), but yet does not reach adequate interstitial concentrations to account for its pharmacologic effects (Tietjan et al., 1990). These observations require one to consider whether the adenosine-mediated cardiovascular effects have an endothelial component. Indeed, we have shown (Balcells et al., 1992, 1993) that the selective activation or blockade of intravascular adenosine receptors, via adenosine conjugates or adenosine receptor antagonist bound to latex microspheres of 0.07 μm diameter, caused the same cardiovascular effects as free adenosine or the free adenosine antagonist (Balcells et al., 1992, 1993), and yet these particles are confined to the vascular compartment because of their size. These data suggest that some of the effects of intravascular adenosine could result from the release of transcellular bioactive messengers by the endothelium.

The purpose of this study was to establish whether nitric oxide (NO) and prostaglandins are mediators of the negative dromotropic effects of intracoronarily administered adenosine as a result of selective activation of intravascular endothelial receptors.

Our results for the isolated perfused guinea pig heart show that: (1) Intracoronarily administered adenosine causes a negative dromotropic effect through activation of intravascularly located endothelial receptors, (2) this effect is mediated by the release of at least two mediators: NO and a derivative of the arachidonic acid metabolic pathway.

2. Materials and methods

2.1. Isolated saline-perfused hearts

Male guinea pigs (350–450 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and heparin sodium (500 U). The animals were artificially ventilated, the chests were opened, and a loose ligature was passed through the ascending aorta. The hearts were rapidly removed, immersed in ice-cold physiological saline, retrogradely perfused via a nonrecirculating perfusion system at constant flow, and trimmed of noncardiac tissue. Coronary flow was adjusted with a variable-speed peristaltic pump (Harvard Apparatus, model 1215). An initial perfusion rate of 25 ml/min for 5 min was followed by a 25-min equilibration period at a perfusion rate of 10 ml/min. After this period of equilibration the experiments were started and all hearts were perfused at a coronary flow of 9 ± 0.35 ml/min. The perfusion medium was Krebs–Henseleit solution with the following composition (mM): 117.8 NaCl, 6 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 24.2 $NaHCO_3$, 5 glucose and 5 sodium pyruvate. The solution was equilibrated with 95% O_2–5% CO_2 (37°C at pH = 7.4).

2.2. Electrical stimulation and recording procedures

Recording and stimulating electrodes consisted of small stainless steel wire vascular clamps (Fine Surgical Instruments) soldered to a thin flexible wire. The clamps were affixed to the myocardial surface by gently grasping the epicardial tissue layer. In every heart precaution was taken to apply the recording and stimulating electrodes at the same sites in the cardiac chambers. A pair of stimulating electrodes were placed 2 mm apart in the apex of the right atrial appendage. Pacing was achieved by applying electrical square pulses of 2 ms duration and twice the electrical threshold at a rate of 3.8 ± 0.2 Hz. To record the atrial and ventricular electrocardiogram, one electrode was placed in the left atrium and the second electrode was placed on the apex of the left ventricle. These two electrodes were connected to an oscilloscope synchronized with the atrial pacing stimulator. The interval between the application of the stimulus to the atrium and the rising phase of the atrial electrogram had a value of 19.5 ± 0.7 ms and remained constant under control conditions and throughout all of the different pharmacological manipulations used. To measure changes in the delay in the auricular–ventricular (A-V) nodal area, we measured changes in the interval between atrial and ventricular electrograms because of its methodological simplicity and because we (Rubio et al., 1995) and others (Alanis et al., 1958, 1959; Clemo, 1986; Belardinelli et al., 1987) have shown that under a variety of experimental conditions changes A-V delay are due solely to changes in the delay in the A-V nodal area and not to delay within the atria or from the His bundle to the ventricle, because the delay remains constant in these areas. The A-V delay (ms) was continuously monitored and measured as the interval between the application of the stimulus to the atria and the rising phase of the ventricular electrical signal. Under control conditions the A-V delay had a mean value of 84 ± 2 ms.

During adenosine bolus experiments the A-V delay was measured with the aid of a polaroid camera mounted on the oscilloscope display panel. Manual opening of the shutter at the same time as bolus injection captured on film the control A-V delay and its gradual increase for the successive electrocardiographic tracings. For each dose of adenosine, the response amplitude was measured from the film as the maximal increase in A-V delay with respect to control.

2.3. Adenosine dose–response curves

Single doses as boluses of adenosine (range of 0 to 45×10^{-10} mol) were intracoronarily administered and the injection period lasted 0.3 s. Bolus infusions were given by using a programmable infusion pump with a dispensing timer that runs the pump at a pre-established rate of infusion and duration (Harvard App. Model 55-222). Intracoronary administration of an adenosine bolus caused a gradual prolongation of the A-V delay with successive beats, and the longest A-V delay was proportional to the dose. For each dose the amplitude of the response was determined by subtracting the longest A-V delay value minus the A-V delay just prior to adenosine bolus infusion, i.e., the increase in A-V delay. The increase in A-V delay (ordinates) for each adenosine dose (abscissae) was plotted to generate the control dose–response curve. Five groups of six hearts each were used. For the hearts of each of the groups, a control dose–response curve was obtained. In each heart, the control curve was followed by an experimental curve obtained during a sustained intracoronary infusion of either N^G-nitro-L-arginine methyl ester (L-NAME, group 1, 0.5 mM), oxyhemoglobin (group 2, 6 μM), superoxide dismutase (group 3, 100 U/ml), dithiothreitol (group 4, 0.2 mM) or indomethacin (group 5, 10 μM). The perfusion of each one of these agents was started 10 min before the adenosine dose–response curve was recorded.

2.4. Oxyhemoglobin preparation

Oxyhemoglobin was prepared as described by Kelm and Schrader (1990). Briefly, hemoglobin was dissolved in distilled water (pH = 7), equilibrated with 100% oxygen, reduced with a molar excess of sodium dithionite, and purified by gel chromatography (Sephadex G25, Pharmacia).

2.5. NO and morpholinosydnonimine solutions

In order to scavenge higher nitrogen oxides that may contaminate NO, the NO (Aldrich) from its tank was passed into a hermetic glass chamber containing a potassium hydroxide solution (10% w/v) previously equilibrated with 100% nitrogen. The NO from this chamber passed into a second chamber and was bubbled through distilled water previously equilibrated with nitrogen. This solution was defined as 100% saturated. Further dilutions of this solution were made with N_2-equilibrated distilled water. In the case of the NO donor, morpholinosydnonimine (Schlüter et al., 1994), a 0.5-M solution was prepared immediately before use and infused intracoronarily at three different rates to produce final concentrations of 10, 5, and 1 mM. All solutions were collected in air-tight syringes and infused into the coronary perfusion line as close as possible to the coronary entrance.

2.6. Procedure of coupling adenosine amine congener (ADAC) to high molecular weight (2×10^6 Da) dextran

The procedure used was a modification of that published by Haga and Haga (1983, 1985. Briefly, 1 g of dextran (5×10^{-7} mol) was dissolved in 50 ml of distilled water and 1.2 g of $NaIO_4$ (5.55×10^{-3} mol) was dissolved in 20 ml of water. These two solutions were mixed and stirred continuously for 90 min. This reaction caused oxidation of two adjacent carbon atoms with hydroxyl groups per every glucose moiety of the dextran, forming a pair of reactive aldehyde endings. The reaction was terminated by adding 5 ml of glycerol followed by an excess of ice-cold methanol, which was gradually added to precipitate the periodate-oxidized dextran, forming a cloudy suspension which was centrifuged at $3000 \times g$ for 15 min. The pellet of oxidized dextran was dissolved in 50 ml of sodium phosphate buffer at pH = 7, and 1.5 g of 6-aminocaproic acid was added, causing the formation of a Schiff base (between the amino group of aminocaproic acid and the aldehyde). The reaction was allowed to proceed 3 h with continuous stirring. Thereafter, to reduce and stabilize the Schiff base, 1 g of $NaCNBH_3$ was added and the reaction proceeded overnight at room temperature. The final solution was dialyzed against several changes of distilled water, precipitated with methanol, and dried. The product formed, dextran–aminocaproic acid, was dissolved in 50 ml of a mixture of dioxane and 0.1 M sodium phosphate buffer (30/70) and divided into two aliquots. To one of the aliquots (control, solution 1) no other addition was made; to the other aliquot, 250 mg of ADAC (*N*-[4-[2-[[4-[2-[(2-aminoethyl)amino]-2-oxoethyl]phenyl]amino]-2oxoethyl]phenyl]adenosine), an adenosine A_1 receptor agonist, (Jacobson et al., 1985) dissolved in 5 ml dioxane was added under continuous stirring. This solution was further divided into two aliquots and to one of them 1 g of [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] was added, in order to activate the free carboxylate group of aminocaproic acid (DeTar and Silverstein, 1966) to react with the terminal amino group of ADAC, forming a dextran–aminocaproic acid–ADAC (dextran–ADAC) com-

plex. The purpose of the solution that did not receive the carbodiimide was to check for non-covalent binding of ADAC to the dextran–aminocaproic acid complex (control 2). The three different aliquots were left for 3 h at room temperature and were dialyzed against several changes of water/dioxane (70/30). The dextran–aminocaproic complexes of each solution were precipitated with ice-cold acetone and extracted several times with benzene in order to eliminate any adsorbed ADAC.

2.7. Effect of dextran–ADAC complex on A-V delay

At zero time, a sustained intracoronary infusion of one of the three dextran–aminocaproic acid complex (controls 1 and 2 and dextran–aminocaproic acid–ADAC), was started, all at a final concentration of 0.2 μM of dextran. The A-V delay was continuously monitored and plotted against time of infusion. These experiments were designed to rule out any effect of the dextran–aminocaproic acid alone (control 1) or non-covalent binding of ADAC to dextran–aminocaproic acid (control 2) and to explore the effects of the dextran–aminocaproic acid–ADAC complex. There were 6 hearts for each of these groups. To rule out the possibility that the dextran–aminocaproic acid–ADAC complex became hydrolyzed to free ADAC during its passage through the heart, 100 ml of venous effluent were collected during a 10-min infusion of dextran–aminocaproic acid–ADAC and was extracted two successive times with 25 ml of benzene. The benzene fractions were combined and evaporated to dryness, and the residue, which could contain free ADAC was resuspended in 100 ml of dioxane/Krebs–Henseleit (1/99). This solution was equilibrated with 95% O2, 5% CO2 and brought to 37°C. The solution was perfused into a second heart preparation and the A-V delay was continuously recorded (control 3, $n = 6$).

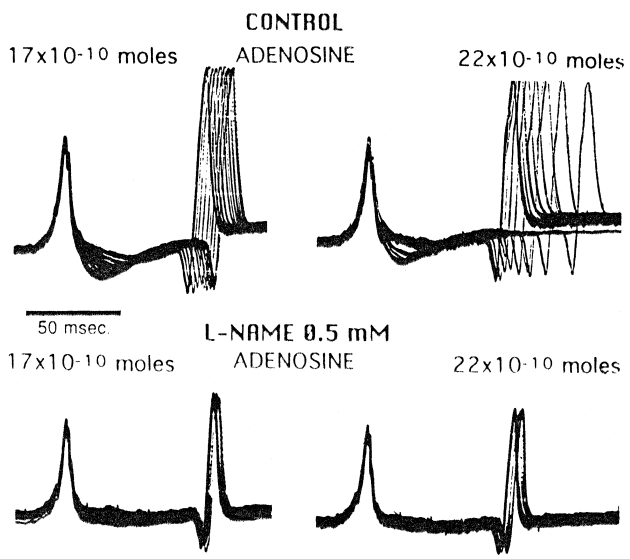

Fig. 1. Negative dromotropic effects of adenosine and diminution of this effect by L-NAME. Electrocardiographic auricular and ventricular traces. The beginning of each trace marks the time the stimulus was applied to the left atria. Effects of doses of 17×10^{-10} and 22×10^{-10} mol of adenosine during control (upper traces) and during sustained infusion of L-NAME (lower traces). Notice that for each dose of adenosine the ventricular delay for the successive beats increased to reach a maximum and that these effects were depressed by L-NAME.

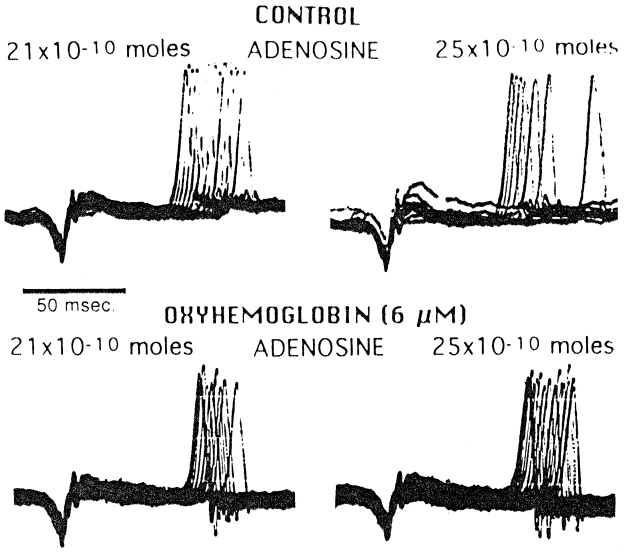

Fig. 2. Negative dromotropic effects of adenosine and diminution of this effect by oxyhemoglobin. Electrocardiographic auricular and ventricular traces. The beginning of each trace marks the time the stimulus was applied to the left atria. Effects of doses of 21×10^{-10} and 25×10^{-10} mol of adenosine during control (upper traces) and during sustained infusion of oxyhemoglobin (lower traces). Notice that upon each dose of adenosine the ventricular delay of successive beats increased to reach a maximum and that these effects were depressed by oxyhemoglobin.

2.8. Statistics

Values are expressed as means ± S.E. In these experiments, each heart and each group served as its own control, and responses under control conditions and during specific manipulations were compared in the same heart. For these reasons, statistical significance was determined using a paired t-test with a Bonferoni correction factor for multiple comparisons. A statistically significant difference was defined for values of $p \leq 0.05$.

3. Results

3.1. Role of NO on adenosine dromotropic effects: effects of agents that inhibit or favor NO accumulation

Application of intracoronary boluses of adenosine under control conditions resulted in a transient dose-dependent

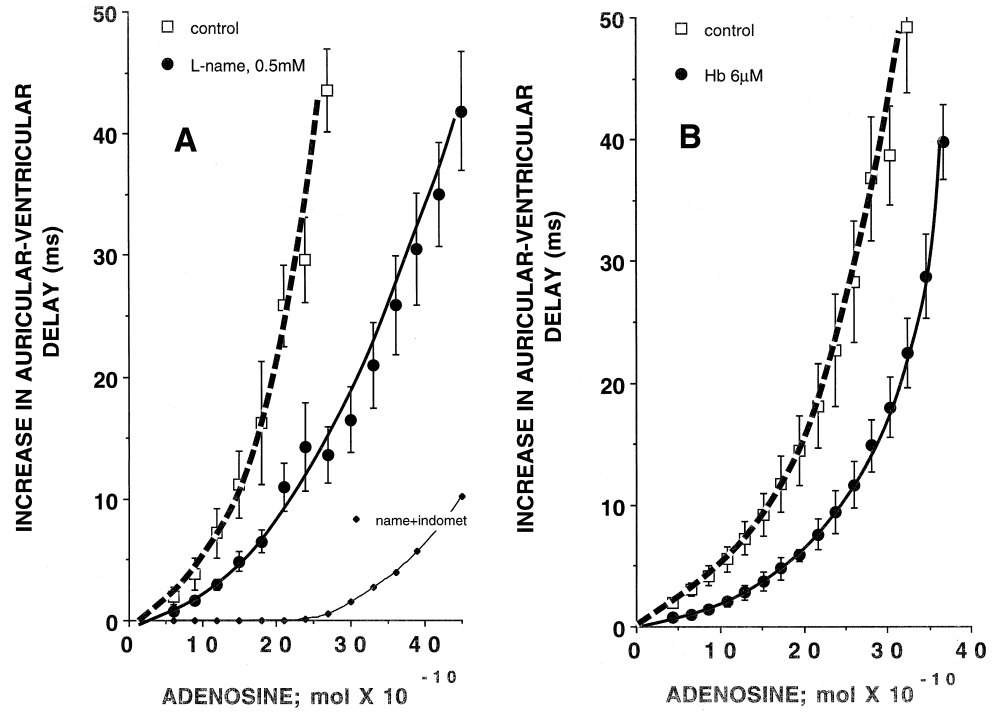

Fig. 3. Diminution of the negative dromotropic effect of adenosine by L-NAME (panel A) and oxyhemoglobin (panel B). Abscissae, doses of adenosine in 10^{-10} mol. Ordinates, maximum increase in A-V delay (ms, mean ± S.E.) caused by each dose of adenosine. Adenosine effects in the absence (open symbols) or in the presence of L-NAME (full symbols, panel A) or oxyhemoglobin (Hb, full symbols, panel B). In A, addition of indomethacin (10 μM) on top of L-NAME (name + indo) further decreased the adenosine effect. In A as in B, the differences between control and experimental curves were statistically different $p < 0.05$.

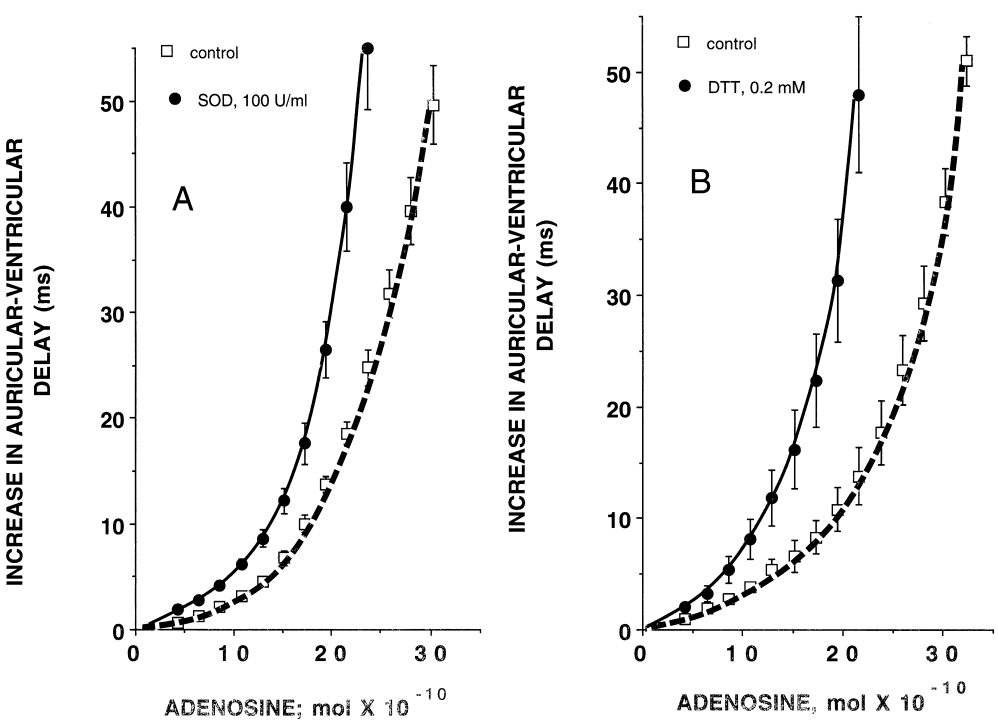

Fig. 4. Potentiation of the negative dromotropic effect of adenosine by superoxide dismutase (panel A) and dithiothreitol (DTT, panel B). Abscissae, doses of adenosine in 10^{-10} mol. Ordinates, maximum increase in A-V delay (ms, mean ± S.E.) caused by each dose of adenosine. Adenosine effects in the absence (open symbols) or in the presence of superoxide dismutse (SOD, full symbols, panel A) or dithiothreitol (DTT, full symbols, panel B). In panel A as in panel B, the differences between control and experimental curves were statistically different $p < 0.05$.

negative dromotropic effect (Figs. 1 and 2). Fig. 1 shows representative electrograms of the effects of 17×10^{-10} and 22×10^{-10} mol doses of adenosine (upper two traces). For each dose, there was a gradual increase in the A-V delay with each successive beat and there was a greater incremental increase in the A-V delay with the larger dose. A similar set of recordings are shown in Fig. 2 for the effects of 21×10^{-10} and 25×10^{-10} mol of adenosine. Control dose–response curves for adenosine are shown in Figs. 3 and 4.

Adenosine administered during the sustained perfusion of agents that inhibit NO accumulation (L-NAME 0.5 mM or oxyhemoglobin 6 μM) had a smaller effect than under control conditions. In Figs. 1 and 2 are shown how the dromotropic effects of adenosine under control conditions (upper traces) were depressed during the sustained infusion of either of these two inhibitors (upper traces). Fig. 3A and B show that the adenosine dose–response curve during sustained infusion of either L-NAME or oxyhemoglobin was displaced downward and to the right as compared with its own control. The inhibitory effect of L-NAME was enhanced when L-NAME was co-perfused with 10 μM indomethacin (Fig. 3A).

Adenosine administered during the sustained perfusion of agents with a NO-sparing effect either superoxide dismutase or dithiothreitol (Gryglewski and Palmer, 1986; Rubanyi and Vanhoutte, 1986) had a greater effect than under control conditions. Fig. 4A and B show that the dose–response curve during sustained perfusion of either 100 U/ml of superoxide dismutase or 0.2 mM dithiothreitol was displaced upward and to the left as compared with its own control.

3.2. Infusion of NO or the NO donor morpholinosydnonimine causes a negative dromotropic effect

The effects of continuous infusion of three different NO solutions with a 70%, 80% or 100% saturation at a rate of 0.12 ml/min are shown in Fig. 5A. The effects of continuous infusion of three different solutions of the NO donor morpholinosydnonimine at final concentrations of 10, 5 and 1 mM are shown in Fig. 5B. In the case of NO and morpholinosydnonimine, there was a gradual increase in the A-V delay which was dose dependent. Also dose dependent was the maximum A-V delay. Infusion of the 80% and 100% saturated NO solutions caused a total A-V blockade after 65 and 80 s of infusion, respectively, while the infusion of 70% saturated solution caused a gradual and smaller increase in A-V delay (Fig. 5A). In the case of morpholinosydnonimine, the rate of increase in A-V delay

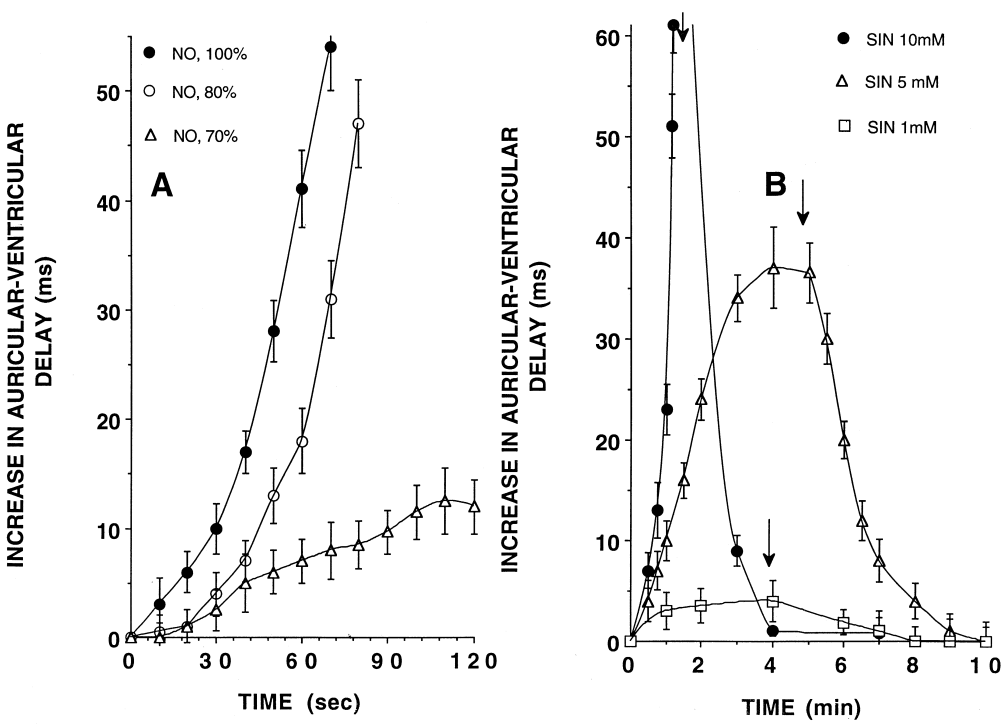

Fig. 5. Time course of the increase in A-V delay (mean ± S.E.) as a consequence of infusion of NO solutions (panel A) and the NO donor morpholinosydnonimine (panel B). At zero time infusion of the agents at various concentrations was initiated. In A, solutions of NO of 100, 80 and 70% saturation were infused at a rate of 0.12 ml/min ($n = 6$). In B, morpholinosydnonimine (SIN-1) was perfused to final concentrations of 10, 5 and 1 mM ($n = 6$). The arrows mark the time SIN-1 infusion was stopped. Notice the reversal of effect.

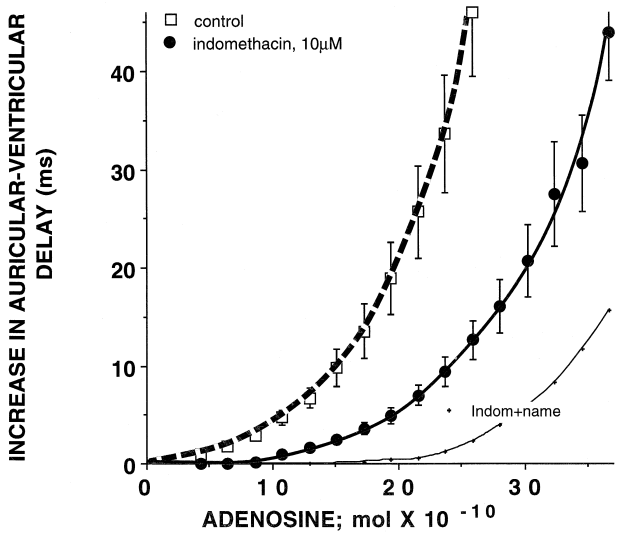

Fig. 6. Diminution of the negative dromotropic effect of adenosine by indomethacin (10 μM). Abscissae, doses of adenosine in 10^{-10} mol. Ordinates, maximum increase in A-V delay (ms, mean ± S.E.) caused by each dose of adenosine. Adenosine effects in the absence (open symbols) or in the presence of indomethacin (full symbols). Addition of L-NAME plus indomethacin (indo + name) further decreased the adenosine effect. The differences between control and experimental curves were statistically different $p < 0.05$.

and its maximum were also concentration dependent, and complete A-V nodal blockade was achieved within 1.5 min of infusion only at the higher concentration of morpholinosydnonimine (10 mM). Upon cessation of infusion of NO or morpholinosydnonimine, the A-V delay returned to control values.

3.3. Effect of indomethacin on adenosine dose–response curves

The presence of indomethacin (10 μM) in the Krebs–Henseleit solution inhibited the effects of adenosine. Indomethacin shifted the curve downward and to the right of the control curve (Fig. 6). This inhibitory effect of indomethacin was enhanced when indomethacin was co-perfused with L-NAME (0.5 mM, Fig. 6).

3.4. The negative dromotropic effect of 2×10^6 Da dextran–6AC–ADAC complex and its blockade by L-NAME and indomethacin

The continuous infusion of 0.2 μM dextran–6AC–ADAC caused a gradual increase in A-V delay that after 2

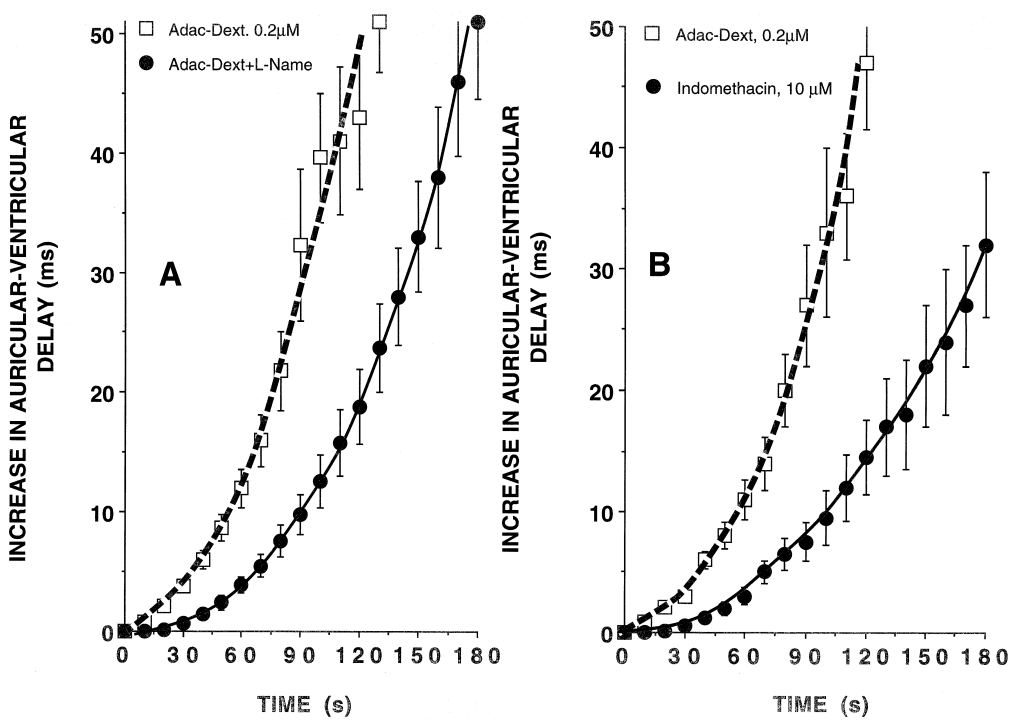

Fig. 7. Time course of the increase in A-V delay (mean ± S.E.) as a consequence of the sustained infusion the complex Adac–Dext (control, open symbols) and the diminution of this effect either by L-NAME (A, full symbols, $n = 6$) or by indomethacin (B, full symbols, $n = 6$). In both cases at zero time, the sustained infusion of the complex Adac–Dext (0.2 μM) was initiated. The differences between control and experimental curves were statistically different $p < 0.05$.

min resulted in complete A-V nodal blockade (Fig. 7A and B). The negative dromotropic effects induced by the sustained infusion of the large size (2×10^3 kDa) dextran–6AC–ADAC polymer were reduced in the presence of L-NAME (0.5 mM, Fig. 7A) and indomethacin (10 μM, Fig. 7B). Infusion of 0.2 μM of the three control solutions was without effect (not shown): dextran–6AC (control solution 1), dextran–6AC pre-incubated with ADAC without the presence of carbodiimide (control solution 2) and control solution 3. These results indicate that dextran–6AC (control 1) was not active and that there was no non-covalent absorption of ADAC to dextran–6AC. In addition, coupled dextran–6AC–ADAC was not hydrolyzed to free ADAC during its passage through the heart because the venous effluents of hearts perfused with dextran–6AC–ADAC had no biological effect.

4. Discussion

Our present data and previous reports (Balcells et al., 1992, 1993) support the hypothesis that the dromotropic effects caused by the intracoronarily administered adenosine and the dextran–ADAC complex are partially mediated by the release of NO and prostaglandins as a result of the activation of endothelial intravascular adenosine receptors.

4.1. Transcellular mediators: NO and prostaglandins

The conclusion that intracoronarily injected adenosine activates endothelial intravascular receptors (Balcells et al., 1992, 1993) suggests the existence of an indirect negative dromotropic effect of adenosine mediated by the release of transcellular mediators of endothelial origin. Nonetheless, adenosine can also act directly on isolated A-V nodal cells (Martynyuk et al., 1995).

4.1.1. Nitric oxide

Recent studies have shown that NO plays an important role in the cholinergic modulation of A-V nodal cells in the adult rabbit. Indeed, immunohistochemical data show that the A-V nodal region has strong endothelial constitutive NO synthase activity associated with endothelial cells and A-V nodal cells (Han et al., 1996). In this study, NO inhibited the transmembrane I_{Ca-L} current of A-V nodal cells, which may result in a prolongation of A-V delay (Han et al., 1996), thus, indicating a possible mechanism by which NO could exert a negative dromotropic effect.

NO accumulation can be reduced either by inhibiting its synthesis with L-arginine analogues such as L-NAME (Rees et al., 1989) or by speeding up its destruction with the NO scavenger oxyhemoglobin (Martin et al., 1985). Conversely, NO accumulation can be increased with NO-sparing agents such as superoxide dismutase, which retards its degradation (Frangos and Juchan, 1991), or dithiothreitol, which increases its half-life by acting as an antioxidant. Our results for these agents show that L-NAME and oxyhemoglobin reduced, while the NO-sparing agents potentiated, the adenosine-induced dromotropic effects. L-NAME also inhibited the negative dromotropic effects of the dextran–ADAC complex. These results indicate that adenosine acting on luminal endothelial receptors stimulates the synthesis and release of NO, which in turn may be responsible for slowing impulse propagation in the A-V node. The negative dromotropic effects of NO were demonstrated during intracoronary infusion of NO or morpholinosydnonimine, a NO donor (Marangos, 1991; Schlüter et al., 1994). The adenosine-activated cell source of NO remains to be established because constitutive NO synthase is found in endothelial cells as well in A-V nodal cells (Han et al., 1996). However, in our study the dextran–ADAC complex could not directly activate the A-V nodal cells because of its intravascular confinement. One would assume that endothelial adenosine receptor activation leads to NO release by the endothelium. However, our study shows that NO may not be the sole mediator of the dromotropic effect of adenosine because L-NAME did not totally block its effects, suggesting the existence of other mediators, perhaps endothelial hyperpolarizing factor. In our experiments the blockade of NO synthesis may have been incomplete, so that higher L-NAME concentrations may be required. Nevertheless, the release of hormone-triggered endothelial mediators other than NO has been suspected because in studies with isolated whole vessel the endothelial-mediated acetylcholine vasodilator response was not fully blocked by L-NAME (Komori et al., 1988; Parkington et al., 1990).

4.1.2. Prostaglandins

Our experiments show that inhibition by indomethacin of cyclooxygenase, a prostaglandin-forming enzyme, depresses the negative dromotropic effect of adenosine. This observation indicates that adenosine upon acting on the endothelium triggers the release of a prostaglandin with a negative dromotropic effect. Thus, the dromotropic action of intravascular adenosine is the result of the sum of the individual negative dromotropic effects of least two mediators: NO and a prostaglandin. This inference is compatible with the fact that indomethacin and L-NAME both individually depress the negative dromotropic effect of adenosine and that their effects are additive.

Interestingly, intracoronary acetylcholine is a negative dromotropic agent which also acts via activation of intravascular muscarinic endothelial receptors (Ceballos and Rubio, 1998). However, in this case indomethacin potentiates the negative dromotropic effects of acetylcholine while L-NAME depresses them. Thus, the dromotropic action of

intravascular acetylcholine is the result of the subtraction of the individual opposite dromotropic effects of at least two mediators: NO, a negative dromotrope, and a prostaglandin-like compound with a positive dromotropic effect. Nevertheless, both acetylcholine and adenosine exert an endothelial-mediated negative dromotropic effect that involves at least NO and prostaglandin synthesis. However, how these two different agonists activate the cyclooxygenase pathway after interacting with their corresponding receptors, resulting in the release of prostaglandin-like compounds with opposite effects, needs further study.

4.2. Activation of intravascular endothelial adenosine A_1 receptors

The vascular endothelium is a necessary and possibly the sole component of the dromotropic effects of intravascular adenosine. This conclusion is based on our data for the intravascularly confined dextran–ADAC complex. In order to ensure intravascular confinement of the adenosine agonist we coupled ADAC to high-molecular-weight dextran, 2×10^6 Da, with an estimated diameter ≥ 0.1 μm. Pore theory modeling studies, using macromolecular dextran or microspheres, in dog myocardium and dog leg have defined an upper limit of the capillary large-pore diameter to be 0.024–0.032 μm for the myocardium (Taylor and Granger, 1984) and 0.04 μm for the leg (Grotte, 1956). Our dextran–ADAC complex, with a mean diameter ≥ 0.1 μm, exceeds the upper limit of the capillary large-pore diameter. Moreover, in our previous studies (Balcells et al., 1992, 1993) ADAC and the adenosine receptor antagonist xanthine amino congener (XAC) were covalently bound to 0.07 μm diameter latex microspheres. After a total of 15.0 min of microsphere infusion electron microscopic studies revealed that the microspheres were uniformly localized around the capillary endothelial surface and were not found within the myocardial interstitium. Therefore, theoretical model studies (Grotte, 1956; Taylor and Granger, 1984) and our experimental evidence support our conclusion that the dextran–ADAC complex and microsphere conjugates, because of their size, are restricted to the intravascular space and thereby could only act by way of the intravascular endothelium.

The control experiments attest to the fact that the dextran–ADAC complex is responsible for the activation of intravascular endothelial adenosine receptors. Venter (1982) has proposed three possible mechanisms by which immobilized drug preparations may yield pharmacological activity: (1) action/effect of non-covalently bound drug (adsorbed drug); (2) action/effect of locally released drug (either from adsorbed or hydrolyzed drug); (3) action/effect of the immobilized drug itself. In addition, one should also consider the possible activity of the matrix used for binding. We found no effect of the binding matrix, the dextran–6AC complex (control 1). In an attempt to rule out the first two mechanisms proposed by Venter, several control studies were performed. For mechanism #1, to test for ADAC adsorption to dextran–AC, we examined the effects of a control dextran–AC preparation (control 2), where the coupling agent was omitted from the reaction mixture (dextran–AC plus ADAC), and found no biological activity. Therefore, these experiments ruled out the possibility of adsorbed ADAC in our preparations. In order to rule out mechanism #2 proposed by Venter we performed a bioassay analysis using both donor and recipient guinea pig hearts. It could be proposed that if the intracoronarily infused dextran–ADAC complex was locally hydrolyzed (in situ), 'free' ADAC would be released into the venous effluent, from which ADAC could be extracted with benzene. The benzene-extracted fraction (control 3) should be biologically active in an assay heart, but this was not the case. The benzene-extracted fraction had no dromotropic effect. These results are similar to those of previous experiments with ADAC and XAC bound to latex microspheres that showed that the venous effluent, once filtered of microspheres and assayed in a recipient heart, had no biological effect (Balcells et al., 1992, 1993). The stability of our dextran–ADAC complex is due to the formation of an amide bond between the carboxylic acid and amine moieties of dextran–aminocaproic acid and ADAC, respectively (DeTar and Silverstein, 1966). This bond is stable during intravascular infusion since there was no in situ hydrolysis of our compound. From the above discussion, it follows then that the biological activity of the dextran–ADAC complex is due to the immobilized ligand which activates intravascular endothelial adenosine A_1 receptors.

In order to define the receptor type, in addition to ADAC, an adenosine A_1 receptor agonist, we covalently coupled the adenosine A_2 receptor agonist CGS-21680 [2-*p*(carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamido adenosine] and the adenosine A_3 receptor agonist APNEA [N^6-2-(4-aminophenyl)ethyladenosine] to dextran–aminocaproic in preliminary experiments. All these three dextran–AC agonists showed vascular, inotropic and dromotropic effects. However, the order of potency of the dromotropic effect of these complexes was dextran–ADAC > dextran–CGS > dextran–APNEA ($A_1 > A_2 > A_3$) In fact, the dextran–APNEA complex was dromotropically inactive in the range of concentrations in which the other two agonists were effective. This order of potency indicates that the intravascular endothelial receptors responsible for the negative dromotropic effects of adenosine are type A_1. The free forms of these three agonist show also had the same order of potency in exerting dromotropic effects: ADAC > CGS > APNEA.

In summary, our results indicate that intravascular adenosine exerts its negative dromotropic actions by way of the vascular endothelium. This effect is mediated by NO, prostaglandins and another unknown mediator, per-

haps EDHF. All these mediators may be of endothelial origin.

Acknowledgements

This work is supported by grants from the Consejo Nacional de Ciencia Y Tecnologia No. 0433P-N and NIH RO1 HL47367-03. The authors are indebted to Dr. Nicholas Handanos for revising and editing this manuscript.

References

Alanis, J., Gonzalez, H., Lopez, E., 1958. The electrical activity of the bundle of His. J. Physiol. Lond. 142, 127–140.

Alanis, J., Lopez, E., Mandoki, J.J., 1959. Propagation of impulses through the atrioventricular node. Am. J. Physiol. 197, 1171–1174.

Balcells, E., Suarez, J., Rubio, R., 1992. Functional role of intravascular coronary endothelial receptors. Eur. J. Pharmacol. 210, 1–9.

Balcells, E., Suarez, J., Rubio, R., 1993. Implications of the coronary vascular endothelium as mediator of the vasodilatory and dromotropic actions of adenosine. J. Mol. Cell. Cardiol. 25, 693–706.

Belardinelli, L., West, G.A., Clemo, S.H.F., 1987. Regulation of atrioventricular node function by adenosine. In: Gerlach, E., Becker, B.F. (Eds.), Topics and Perspectives in Adenosine Research. Springer, Berlin, pp. 344–355.

Ceballos, G., Rubio, R., 1998. Endothelial mediated negative dromotropic effects of intravascular acetylcholine. Eur. J. Pharmacol. 362, 157–166.

Clemo, S.H.F., 1986. Adenosine and AV conduction. Thesis Dissertation, University of Virginia, Charlottesville.

Dendorfer, A., Lauk, S., Nees, S., 1987. New insights into the mechanism of myocardial adenosine formation. In: Gerlach, E., Becker, B.F. (Eds.), Topics and Perspectives in Adenosine Research. Springer, Berlin, pp. 170–184.

DeTar, D.F., Silverstein, R., 1966. Reactions of carbodiimides: II. The reaction of dicyclohexylcarbodiimide with carboxylic acids in the presence of amines and phenols. J. Am. Chem. Soc. 88, 1020–1023.

Deussen, A., Moser, G., Schrader, J., 1986. Contribution of coronary endothelial cells to cardiac adenosine production. Pflug. Arch. 406, 608–614.

Dobson, J.G., 1983. Mechanism of adenosine inhibition of catecholamine-induced responses in heart. Circ. Res. 52, 151–157.

Frangos, J.A., Juchan, M.J., 1991. Fluid flow activates G-proteins that are coupled to Ca-dependent and independent EDRF production in cultured endothelial cells. FASEB J. 5, A1820, (abstract).

Grotte, G., 1956. Passage of dextran molecules across the blood–lymph barrier. Acta Chir. Scand. Suppl. 211, 1–84.

Gryglewski, R.J., Palmer, R.M., 1986. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320, 454–456.

Haga, K., Haga, T., 1983. Affinity chromatography of the muscarinic acetylcholine receptor. J. Biol. Chem. 258 (22), 13575–13579.

Haga, K., Haga, T., 1985. Purification of the muscarinic acetylcholine receptor from porcine brain. J. Biol. Chem. 260 (13), 7927–7935.

Han, X., Kobzik, I.L., Ballingand, J.-L., Kelly, R.A., Smith, T.W., 1996. Nitric oxide synthase (NOS3)-mediated cholinergic modulation of Ca++ current in adult rabbit atrioventricular nodal cells. Circ. Res. 78, 998–1008.

Jacobson, K.A., Kirk, K.L., Padgett, W.L., Daly, J.W., 1985. Functionalized congeners of 1,3-dialkylxanthines: preparation of analogues with high affinity for adenosine receptors. J. Med. Chem. 28, 1334–1340.

Jacobson, K.A., Ukena, D., Padgett, W.S., Kirk, K.L., Daly, J.W., 1987. Molecular probes for extracellular adenosine receptors. Biochem. Pharmacol. 36, 1697–1707.

Kelm, M., Schrader, J., 1990. Control of coronary vascular tone by nitric oxide. Circ. Res. 66, 1561–1575.

Komori, K., Lorenz, R.R., Vanhoutte, P.M., 1988. Nitric oxide, acetylcholine, and electrical and mechanical properties of canine arterial smooth muscle. Am. J. Physiol. 155, H207–H212.

Kroll, K., Schrader, J., Piper, M.H., Henrich, M., 1987. Release of adenosine and cyclic AMP from coronary endothelium in isolated guinea pig hearts: relation to coronary flow. Circ. Res. 60, 659–665.

Marangos, J.P., 1991. Complexes of NO with nucleophiles as agents for the controlled biological release of nitric oxide. J. Med. Chem. 34, 3242–3251.

Martin, W., Villani, M., Jothianandan, D., Furchgott, R.F., 1985. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther. 233, 679–685.

Martynyuk, A.E., Kane, K.A., Cobbe, S.M., Rankin, A.C., 1995. Adenosine increases potassium conductance in isolated rabbit atrioventricular nodal myocytes. Cardiovasc. Res. 30, 668–679.

Nees, S., Herzog, V., Becker, B.F., Bock, M., Des Rosiers, C., Gerlach, E., 1985a. The coronary endothelium a highly active metabolic barrier for adenosine. Basic Res. Cardiol. 80, 515–519.

Nees, S., Herzog, V., Becker, B.F., Bock, M., Des Rosiers, C., Gerlach, E., 1985b. The adenine nucleotide metabolism of the coronary endothelium: implications for the regulation of coronary flow by adenosine. In: Stefanovich, V., Rudolph, K., Schubert, P. (Eds.), Adenosine: Receptors and Modulation of Cell Function. IRl, Oxford, pp. 419–436.

Olsson, R.A., Davis, C.J., Khouri, E.M., Patterson, R.E., 1976. Evidence for an adenosine receptor on the surface of dog coronary myocytes. Circ. Res. 39, 93–98.

Parkington, H.C., Tare, M., Coleman, H.A., 1990. More than one agent is involved in the hyperpolarization induced by acetylcholine in coronary arteries of guinea pigs. Blood Vessels 27, 52–57.

Rees, D.D., Palmer, R.M., Hodson, H.F., Moncada, S., 1989. Specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. Br. J. Pharmacol. 96, 418–424.

Rubanyi, G.M., Vanhoutte, P.M., 1986. Superoxide anions and hyperoxia inactive endothelium-derived relaxing factor. Am. J. Physiol. 250, H822–H827.

Rubio, R., Knabb, M.T., Tsukada, T., Berne, R.M., 1983. Mechanisms of action of adenosine on vascular smooth muscle and cardiac cells. In: Berne, R.M., Rall, T.W., Rubio, R. (Eds.), Regulatory Function of Adenosine. Martinus Nijhoff, Hague, pp. 319–332.

Rubio, R., Knabb, R.M., Ely, S.W., Berne, R.M., 1987. A critique on the use of adenosine deaminase to test the adenosine hypothesis: disregarded implicit assumptions. In: Gerlach, E., Becker, B.F. (Eds.), Topics and Perspectives in Adenosine Research. Springer, Berlin, pp. 443–451.

Rubio, R., Ceballos, G., Suarez, J., 1995. Coronary flow stimulates auricular–ventricular transmission in the isolated perfused guinea pig heart. Am. J. Physiol. 269, H1177–H1185.

Schlüter, K.D., Weber, E., Schraven, E., Piper, H.M., 1994. No donor SIN-1 protects against reoxygenation-induced cardiomyocyte injury by a dual action. Am. J. Physiol. 267, H1462–H1468.

Schrader, J., Nees, S., Gerlach, E., 1977. Evidence for a cell surface adenosine receptor on coronary myocytes and atrial muscle cells. Pflug. Arch. 369, 251–257.

Smits, P., Williams, S., Lipson, D., Banitt, P., Rongen, G., Creager, M.A., 1995. Endothelial release of nitric oxide contributes to the vasodilator effect of adenosine in humans. Circulation 92, 2135–2140.

Taylor, A.E., Granger, D.N., 1984. Exchange of macromolecules across the microcirculation. In: Renkin, E.M., Michel, C.C., Geiger, S.R. (Eds.), Handbook of Physiology, Section 2: The cardiovascular System, Vol. 4: Microcirculation, Part 1. Am. Phys. Soc. Bethesda, pp. 488–499.

Tietjan, C.S., Tribble, J.M., Gidday, J.M., Phillips, C.L., Belardinelli, L., Rubio, R., Berne, R.M., 1990. Interstitial adenosine in guinea pig hearts: an index obtained by epicardial disk. Am. J. Physiol. 259, H1471–H1476.

Venter, J.C., 1982. Immobilized and insolubilized drugs, hormones and neurotransmitters: properties, mechanisms of action and applications. Pharmacol. Rev. 34, 153–187.