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Spermine-induced negative inotropic effect in isolated rat heart, is mediated through the release of ATP

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Abstract

Putrescine, spermidine and spermine are natural compounds found in up to millimolar concentrations in eukaryotic and prokaryotic cells. At physiologic pH, the polyamines are protonated (+2, +3 and +4 charges), their polycationic properties lead to the assumption that they could affect physiological systems by binding to anionic sites of the cellular membrane and/or by modulating ion channels. At the cardiovascular level, their effects are not completely understood. However, these compounds may be able to exert the induction of synthesis and release of cellular mediators. In an attempt to explore this possibility, we used the isolated and perfused rat heart, Langendorff, model in order to evaluate the inotropic effects of these polyamines, putrescine, spermidine and spermine. Dose–response curves (0.1–0.6 mM) for putrescine, spermidine and spermine were constructed; with the finding that spermine had the largest negative effect. The obtained effects were not blocked by nitric oxide synthesis inhibitors (L-NAME), H_1 and H_2 receptor antagonists (Brompheniramine and Cimetidine) or by Glibenclamide, an antagonist of ATP-sensitive K^+ channels.

We found that spermine-induced and increased ATP concentration in cardiac effluents. Reactive Blue, a P_{2y} purinoreceptor antagonist and Aminophylline, an unspecific adenosine receptor antagonist, blocked the spermine-induced effects. These results showed that ATP, at least in part, is responsible of the spermine cardiovascular effects. Adenosine was shown to also play an important role on those effects.

Keywords: Polyamines; Spermine; Heart; Ventricular contraction force; Inotropism; Pressure

1. Introduction

Putrescine, spermidine and spermine are natural polyamines found in up millimolar concentrations in eukaryotic and prokaryotic cells [1]. Polyamines have been involved in normal and pathologic functions of eukaryotic cell. As well, they are essential for normal cell growth, proliferation and differentiation [2].

At physiologic pH, the polyamines are protonated (+2, +3 and +4 charges, respectively). Their polycationic properties led to the assumption that they could affect physiological systems by binding to the membrane's anionic sites [3] and by regulation ion channels, ej. Intracellular polyamines have recently been shown to cause the inward rectification of several strong inward rectifying potassium channels [4,14,15]. Polyamines have been proposed as natural calcium antagonist. A modulatory effect of calcium channel conductance by polyamines has been described. Accumulating evidence indicates that polyamines modulate cytosolic Ca^{2+} homeostasis in different cell types [5,6].

At the cardiovascular level, their exact role remains to be elucidated; however, several polyamine-induced effects have been described. Exogenous polyamines inhibit vascular contraction, while intracellular polyamines increase vascular contraction and it has been proposed that polyamines, especially spermine, are able to interact with membrane Ca^{2+} channels [2,7]. In isolated left ventricular rat cardiac myocytes, exogenously administered polyamines

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influence myocardial contractility and cardiac homeostasis. It has been suggested that the polyamine action may be exerted at the cell membrane level [8]. In isolated heart preparations, polyamines induced a negative inotropic effect [8,9] and it has been proposed that this effect might be due to a competitive inhibition of calcium influx.

Based on those reports, we reasoned that when applied within the coronary arteries, a direct interaction of polyamines with myocardial myocytes is not likely involved in their negative inotropic effects. In order for the polyamine transport system as described to establish contact with myocytes, a transport system into endothelial cells must be present, followed by extrusion into the endothelial ablminal side. A transocytosis process for polyamines has not been described. As well, due to their polycationic characteristics, free diffusion into cells is a limited process for polyamines with the endothelial plasmalemma blocking the access to myocytes. All these facts raise the possibility of existence of a second messenger that, in turn, induces the polyamine-induced negative inotropic effect. In this study, we have explored that possibility in an isolated and perfused rat heart model.

The results obtained provide evidence suggesting that polyamine effects are mediated through the release of ATP.

2. Materials and methods

2.1. Isolated saline-perfused hearts

Male Wistar rats (250–350 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and heparin sodium (500 U). The animals were artificially ventilated, the chest was opened, and a loose ligature was passed through the ascending aorta. The heart was trimmed of non-cardiac tissue and perfused in a retrograde fashion via a non-recirculating perfusion system at constant flow. 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, experiments were begun and all hearts were perfused at a coronary flow of 10 ± 0.4 mL. The perfusion medium was Krebs–Henseleit solution with the following composition (mM): NaCl 117.8; KCl 6; $CaCl_2$ 1.75; $MgSO_4$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 24.2; glucose 5 and sodium pyruvate 5. The solution was equilibrated with 95% O_2–5% CO_2, pH 7.4 and kept at 37.5°. A pair of stimulating electrodes, made of small stainless steel wire (vascular clamps/Fine Surgical Instruments), were placed 2 mm apart in the apex of the right atrium. Pacing was achieved by applying electrical square pulses of 2 ms duration and at twice the electrical threshold at a rate of 4.5 ± 0.3 Hz.

To measure left intraventricular pressure, a latex fluid filled ballon was placed in the ventricle and connected to a pressure transducer, adapted in turn, to a computerized data acquisition system (Biopac). The pressure developed under control conditions was defined as 100% and all other amplitudes measured under the diverse experimental conditions were expressed as a percent of the control.

2.1.1. Effects of putrescine, spermidine and spermine on isolated rat heart

Effects of each polyamine were assessed in isolated constant flow perfused rat hearts. Dose–response curves (10 nM–1 mM) for putrescine, spermidine and spermine were made separately, one polyamine per heart. Intracoronary continuous infusion (30 s) of each concentration used was followed by a washout period of 15 min.

2.1.2. Inhibition or blockade of possible mediators of polyamine inotropic effects

To study the possible existence of mediators of polyamine-induced inotropic effects, several receptor antagonists and enzymatic inhibitors were tested: Cimetidine [0.1 mM] (H_2 subtype histamine antagonist receptor), Brompheniramine [0.1 mM] (H_1 subtype histamine antagonist receptor) [10,16]; Glibenclamide [1 μM] an antagonist of ATP-sensitive K^+ channels [11]; N^G-nitro-L-arginine methyl ester (L-NAME) [1 μM], an inhibitor of the nitric oxide synthase [17]; Reactive Blue 2 [1 μM] a P_{2y} purinoreceptor antagonist [18]; Aminophylline [10 μM], an adenosine receptors blocker [19]. Each substance tested was continuously perfused during the experiment, initiated 10 min prior polyamine perfusion.

2.1.3. HPLC methods

For the separation and quantification of purines (adenosine, inosine and hypoxanthine) from cardiac effluents, an ODS-C18 column (ultrasphere ODS, Beckman) and an isocratic elution system in a Beckman Gold system were used. The buffer was KH_2PO_4 [100 mM] with 15% (v/v) methanol. Buffer and samples were filtered through Millipores membranes with a pores size of 0.22 μm.

The retention times for hypoxanthine, inosine and adenosine were 4, 6 and 16.3 min, respectively. The concentration of purine was calculated from the area under the curve of HPLC peaks and compared with standard curves developed for each purine. Purines recovered from the heart effluents were concentrated (sep-pak C-18 columns, Fisher) previous to HPLC analysis.

2.1.4. Statistics

Results are expressed as mean ± SEM. 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. Statistical significance was determined using ANOVA and a paired *t*-test with a Bonferroni correction factor for multiple comparisons. A statistically significant difference was defined for values of $P < 0.05$.

3. Results

3.1. Negative inotropic effects of polyamines

We performed dose–response curves (10 nM–1 mM) for putrescine, spermidine and spermine (data not shown). However, minimal and maximal effects for each polyamine were obtained in the millimolar range of concentrations and in order to obtain representative dose-responses to polyamines, it was necessary to use concentrations near the millimolar range (0.1–0.6 mM). Figure 1 shows the dose-dependant spermine-induced decrease in ventricular contraction force. In our study, putrescine caused no effect and spermidine-induced only a very small effect. For this reason, the remainders of the experiments were performed exclusively with spermine.

3.2. Analysis of possible mediators of the negative inotropic effect of spermine

Blockage of nitric oxide synthesis with the use of L-NAME [1 μM] induced no effect on spermine-induced negative inotropic effect (data not shown). The same results were obtained in the presence continuous infusion of a mixture of H_1 and H_2 receptor antagonists (Brompheniramine and Cimetidine) [0.1 mM]. The uses of Glibenclamide [1 μM], an antagonist of ATP-sensitive K^+ channels, cause no effect on spermine-induced negative inotropic effect (data not shown).

3.3. Effect of blockage of ATP receptors on spermine-induced effects

The presence of Reactive Blue [1 μM], a P_{2y} receptor antagonist induced a blockage of spermine-induced negative inotropic effects. In the presences of Reactive Blue, the dose–response is displaced downwards and to the right (Fig. 2).

3.4. Effect of adenosine receptors antagonist in spermine-induced negative inotropic effect

Presence of Aminophylline [1 μM], an unspecific adenosine receptor antagonist, induced a partial blockage of spermine effects, with the spermine dose–response curve displaced downward and to the right in its presence (Fig. 2).

3.5. Purines concentration in hearts effluents

Due to rapid ATP catabolism, measurement of its concentration was carried out indirectly. Spermine perfusion induced, in a concentration-dependent manner (Fig. 3), an increase in purines (sum of adenosine, inosine and hypoxanthine) concentration.

4. Discussion

Many cellular functions have been attributed to polyamines; however, their mechanism of action at the

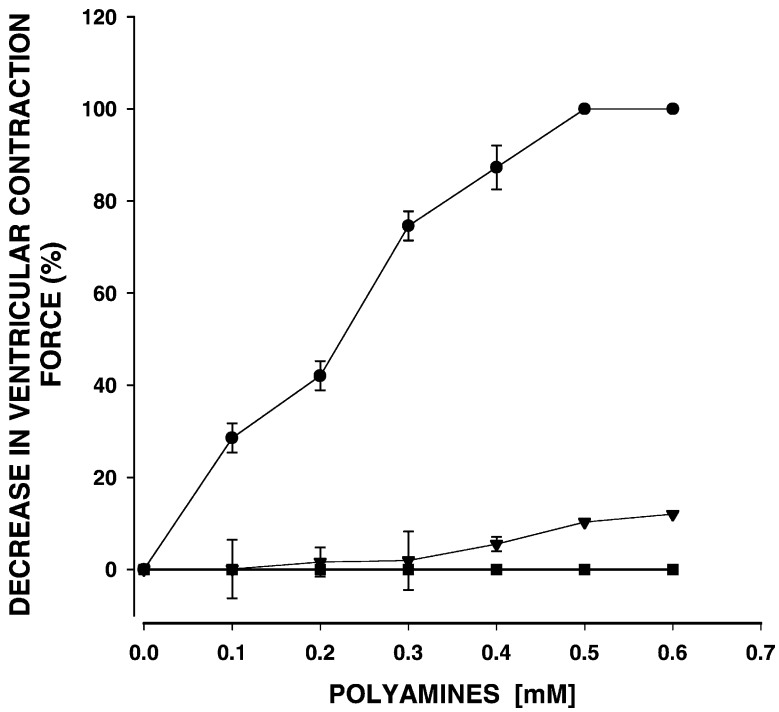

Fig. 1. Effect of putrescine (■), spermidine (▼) and spermine (●) on ventricular contraction force (%). Each point represents at least four assays where each heart was its own control, only one polyamine per heart was used. Data are expressed as means ± SEM. Spermine-induced effects were statistically different from those of the other two polyamines and from its own control ($P \leq 0.01$).

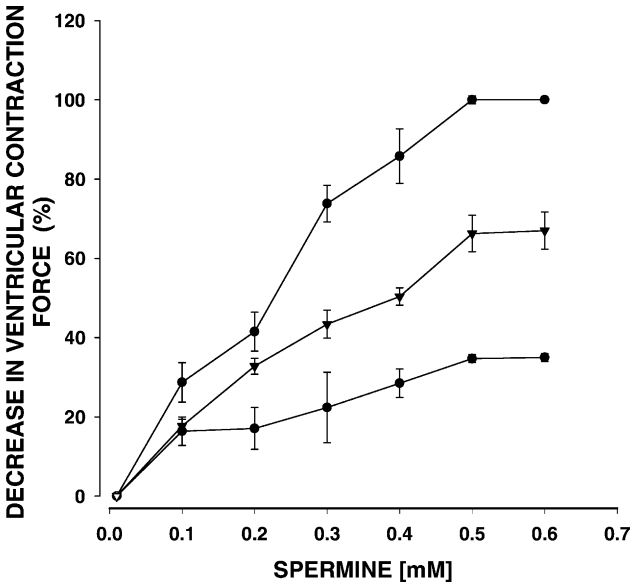

Fig. 2. Dose–response curve to spermine [0.1–0.6 mM] in absence (control, ●) and in presence of: Reactive Blue [1 μM] a P_{2y} receptors antagonist (○) and Aminophylline [1 μM] an unspecific adenosine receptors antagonists (▼). Data are expressed as means ± SEM. Curves in presence of Reactive Blue and Aminophylline were statistically different from control and from each other ($P \leq 0.01$).

molecular level has not been clearly established and is still a matter of controversy.

At physiological pH, polyamines are protonated molecules. They are able to interact with, and bind to, negatively charged cellular macromolecules such as nucleic acids. This interaction and its consequences is a possible mechanism through which polyamine effects may be explained,

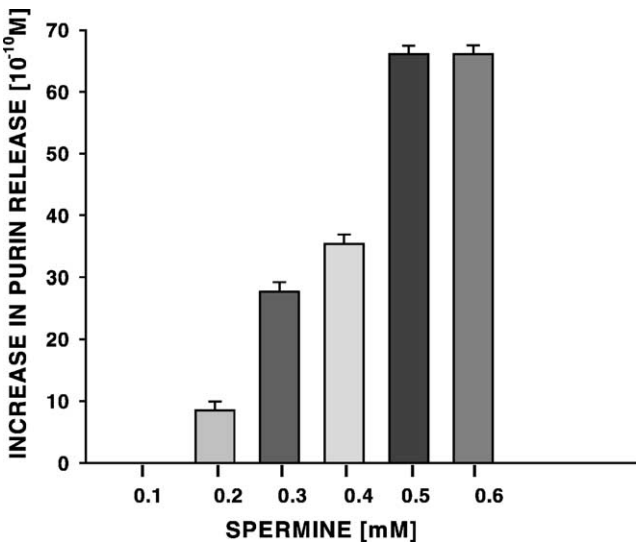

Fig. 3. Spermine-induced increase in ATP concentration, measured here as the sum of adenosine, inosine and hypoxanthine concentrations found in hearts effluents collected during perfusion of spermine [0.1–0.6 mM]. Data are expressed as percentage of increase related to purines concentration in absence of spermine perfusion. Data are statistically different from control ($P \leq 0.01$).

particularly those of intracellular origin, growth and related to development.

Polyamines also interact with negatively charged plasmalemma acidic phospholipids and protein residues affecting membrane properties. As an example, it has been shown that polyamines (in the millimolar range of concentration) are able to protect a microorganism's membrane against osmotic shock [3].

It has been reported that polyamines have similar characteristics to inorganic cations, such as Mg^{2+} and Ca^{2+}. However, some differences have been noted. Polyamines have their positive charges distributed in fixed angles in a carbonated chain, are conformationally flexible and they are not punctual positive charges as are inorganic cations. Polyamines are capable of forming bridges at critical and specific distances (charges in spermidine and spermine are separated by 1.1 and 1.6 nm, respectively). These characteristics confer to polyamines functions and specific interactions which are not shared with inorganic cations [3].

Due to their physicochemical characteristics at physiological pH, it is very improbable that they can diffuse into cells and since their transport is limited to conditions in which their intracellular concentration diminishes, we propose that polyamine-induced effects, when they are intravascularly applied, are the result of their interaction with the closest cell. In the case of isolated organs with endothelial cells, one or more messengers are necessary to induce any effect on parenchymal cells.

In this work we showed that polyamines, spermine particularly, induced a decrease in left ventricular contraction force in an isolated rat heart perfused in a retrograde fashion. These effects were positively related to the number of positive charges in the molecules, being putrescine (+2 charges) without effect, spermidine (+3 charges) with a minimal effect and spermine (+4 charges) with the maximum effect. The spermine-induced decrease in ventricular contraction force was dose-dependent with the necessary concentration to induce the effects found to be in the submillimolar range. These concentrations can be considered as too high and not representative of those found physiologically and perhaps out of site of action. Despite the fact that these substances are essentially distributed in the cytoplasm, cells may also be exposed to extracellular polyamines, since they may be extruded from cells, with the result that the extracellular concentrations of these amines can be highly variable, ranging from nanomolar to micromolar [12].

It has been reported that polyamines induce relaxation effects on precontracted arteries and that $CaCl_2$ counteracts these effects. It has been suggested that polyamine-induced relaxation could be due to inhibition of calcium influx. However, spermine can induce relaxation in the absence of calcium, involving calmodulin or protein kinase C inhibition [2]. In the present work we show that besides calcium modulation, spermine induces ATP release (indirectly measured), which in turn, acting through the stimulus of

P_{2y} receptors subtype, induces its negative inotropic effect. The conversion of ATP into adenosine can also participate in the induction of the obtained effects.

The rapid onset of these effects elicited by exogenously applied spermine and the prompt removal of changes following washout, suggest that its action may be mediated at the level of cell surface binding sites. Such a hypothesis may be based on studies showing that polyamines influence membrane functions, being bound to cell plasma membranes at physiological pH by virtue of their cationic properties [3]. Spermine's site of action (extracellular or intracellular) can not be determined in our studies. Although efficient uptake mechanisms are present in most cell types, studies to date have generally accepted the fact that the transporter is down-regulated in non-dividing cells and that extracellular changes in polyamine concentrations result in little or no change in intracellular levels and submillimolar concentrations of exogenously applied polyamines have been shown to stabilize membranes and it has been proposed that spermine might induce some bridging between integral proteins and membrane lipid binding sites [13] or lipid head groups, leading to a protective effect against lipid peroxidation [3]. Cell targets are also difficult to assess because it has been reported in isolated cardiac myocytes that spermine is able to induce a negative inotropic effect associated with a decrease in the magnitude of the $[Ca^{2+}]_i$ transient [8].

In summary, in order to induce a decrease in ventricular contraction force at least two possible sites of action may be involved: (1) luminal endothelial plasmalemma, where a process increases the activity of these cells and can induce the synthesis and release of messenger(s) which are responsible for effects on the ventricle; (2) myocardial myocytes plasmalemma, in which the effect is probably related to calcium kinetics; or (3) a combination of both. Effectiveness of polyamines seems to be dependent on the size of their positive charges. Electrostatic interactions may be a major parameter in their mechanism of action, which would relate directly to calcium dependent mechanisms. However, it is possible that they can bind specifically to membrane components and by this mechanism, stimulate synthesis and/or release of second messengers such as ATP.

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