Anesthesiology 2001; 95:1226–33

Interaction of Protamine with α- and β-Adrenoceptor Stimulations in Rat Myocardium

Jean-Stéphane David, M.D., Benoît Vivien, M.D.,† Yves Lecarpentier, M.D., Ph.D.,‡ Pierre Coriat, M.D.,§ Bruno Riou, M.D., Ph.D.‖*

Background: **Protamine alters the inotropic responses to β-adrenoceptor stimulation, but its mechanism of action is not well-understood. Moreover, its interaction with α-adrenoceptor stimulation and the lusitropic (relaxation) response to β-adrenoceptor stimulation remain unknown.**

Methods: **The effects of protamine (10 or 100 μg/ml) on the responses induced by phenylephrine and isoproterenol were studied in rat left ventricular papillary muscles. Inotropic and lusitropic effects were studied under low and high loads. The authors also studied the interaction of protamine with forskolin (50 μM) and dibutyryl 3',5'-cAMP (0.5 mM). Data are mean percentage of baseline active force ± SD.**

Results: **In control groups, phenylephrine (135 ± 17%, $P < 0.05$) and isoproterenol (185 ± 44%, $P < 0.05$) induced a positive inotropic effect. Isoproterenol induced positive lusitropic effects under low and high loads. Protamine abolished the inotropic responses to α- (102 ± 23%, not significant) and β-adrenoceptor stimulations (99 ± 17%, not significant) but did not modify the lusitropic responses to isoproterenol. Protamine abolished the inotropic responses to forskolin (89 ± 6 *vs.* 154 ± 20%, $P < 0.05$) and markedly decreased that of dibutyryl 3',5'-cAMP (132 ± 31 *vs.* 167 ± 30%, $P < 0.05$) but did not modify their lusitropic responses.**

Conclusions: **Protamine abolished the inotropic responses to α- and β-adrenoceptor stimulations but preserved the lusitropic responses to β-adrenoceptor stimulation. Although protamine may act at several sites on the adrenoceptor stimulation cascade, one of its main sites of action is situated downstream from cAMP-mediated phosphorylation.**

ADMINISTRATION of protamine after cardiac and vascular surgical procedures does not induce marked cardiovascular effects, mainly because protamine binds to heparin, except for rare protamine reactions associated with vasodilatation and decrease in cardiac function.[1–3] Nevertheless, direct negative inotropic and lusitropic effects of protamine have been demonstrated in isolated myocardial preparations and could be related to cytosolic calcium overload.[4–7] A recent study has suggested that the cardiac effects of protamine might be at least partly indirect through the release of mediators, such as tumor necrosis factor.[8] Protamine has also been shown to alter the inotropic response to isoproterenol *in vitro*, particularly in chronic left ventricular dysfunction.[9] However, the precise mechanism by which protamine interferes with β-adrenoceptor stimulation is not completely elucidated, and its interaction with α-adrenoceptor stimulation remains unknown. Moreover, although β-adrenoceptor stimulation induces marked positive lusitropic effects leading to enhancement of cardiac relaxation and diastolic function, the interaction of protamine with these lusitropic effects is not known. Lastly, the interaction of protamine with α-adrenoceptor stimulation has not been previously studied, whereas its transduction pathway markedly differs from that of β-adrenoceptor stimulation.[10]

Therefore, we studied the interaction of protamine with α- and β-adrenoceptor stimulations in isolated rat myocardium. The experimental model used enabled us to investigate the effects on contraction (inotropy) and relaxation (lusitropy) at different loading conditions (isotony *vs.* isometry) and thus the effects on the two main intracellular processes involved in relaxation, *i.e.*, calcium uptake by the sarcoplasmic reticulum (SR) and myofilament calcium sensitivity.[11,12] Lastly, we also studied the mechanisms involved in the interaction of protamine with β-adrenoceptor stimulation.

Materials and Methods

We used adult Wistar rats weighing 250–300 g. Care of the animals conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance with the regulations of the official edict of the French Ministry of Agriculture (Paris, France).

Experimental Protocol

Left ventricular papillary muscles were studied in a Krebs-Henseleit bicarbonate buffer solution (130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.1 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 4.5 mM glucose) maintained at 29°C, as previously reported.[11,12] Preparations were field stimulated at 12 pulses/min by two platinum electrodes with rectangular wave pulses lasting 5 ms just

* Research Fellow, Laboratory of Experimental Anesthesiology, Department of Anesthesiology, Centre Hospitalier Universitaire (CHU) Pitié-Salpêtrière, and Department of Anesthesiology, CHU Edouard Herriot. † Assistant Professor, § Professor of Anesthesiology and Chairman, Department of Anesthesiology, ‖ Director of the Laboratory of Experimental Anesthesiology, Professor of Anesthesiology and Chairman, Department of Emergency Medicine and Surgery, CHU Pitié-Salpêtrière. ‡ Research Director, Institut National de la Santé et de la Recherche Médicale, Palaiseau, and Professor of Physiology, Chairman, CHU de Bicêtre.

Received from Laboratoire d' Anesthésiologie, Département d' Anesthésie-Réanimation et Service d' Accueil des Urgences, CHU Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Université Pierre et Marie Curie, Paris, France; Département d' Anesthésie-Réanimation, CHU Edouard Herriot, Hospices Civils de Lyon, Université Claude Bernard, Lyon, France; Institut National de la Santé et de la Recherche Médicale, Laboratoire d' Optique appliqueé-Ecole Nationale Supérieure des Techniques Avancies-Ecole Polytechnique, Palaiseau, France; and Service de Physiologie, Hôpital de Bicêtre, Assistance Publique-Hôpitaux de Paris, Université Paris Sud, le Kremlin-Bicêtre, France. Submitted for publication January 24, 2001. Accepted for publication June 18, 2001. Support was provided solely from institutional and/or departmental sources.

Address reprint requests to Dr. Riou: Département d' Anesthésie-Réanimation, Groupe Hospitalier Pitié-Salpêtrière, 47 Boulevard de l' Hôpital, 75651 Paris cedex 13, France. Address electronic mail to: bruno.riou@psl.ap-hop-paris.fr.

above threshold. The bathing solution was bubbled with 95% oxygen and 5% carbon dioxide, resulting in a pH of 7.4. After a 60-min stabilization period at the initial muscle length at the apex of the length-active isometric tension curve (L_{max}), papillary muscles recovered their optimal mechanical performance. The extracellular calcium concentration ($[Ca^{2+}]_o$) was decreased from 2.5 mM to 0.5 mM because rat myocardial contractility is nearly maximum at 2.5 mM.[11,12]

In control groups, α-adrenoceptor stimulation was induced using cumulative concentrations of phenylephrine (10^{-8} to 10^{-4} M) in the presence of propranolol (10^{-6} M), and β-adrenoceptor stimulation was induced using cumulative concentrations of isoproterenol (10^{-8} to 10^{-4} M) in the presence of phentolamine (10^{-6} M). In the protamine groups (n = 8 in each group), we studied the effects of α- or β-adrenoceptor stimulation in the presence of 10 or 100 μg/ml protamine (protamine sulfate; Sanofi Winthrop, Gentilly, France). In addition, we also studied the effects of the same concentrations of protamine alone (10 and 100 μg/ml) in separate groups of papillary muscles (n = 8 in each group).

To determine the mechanisms of the interaction of protamine with β-adrenoceptor stimulation, we studied the following: (1) the stimulation of adenylate cyclase using forskolin (50 μM; Sigma-Aldrich Chimie, L'Isle d'Abeau Chesnes, France) in the presence (n = 6) or absence (n = 6) of protamine (10 μg/ml); (2) the direct activation of the cAMP-dependent protein kinase system using dibutyryl 3',5'-cAMP (DBcAMP, 0.5 mM; Sigma-Aldrich Chimie) in the presence (n = 8) or in absence (n = 8) of protamine (10 μg/ml); and (3) the response to increased concentrations of $[Ca^{2+}]_o$ in the presence (n = 6) or absence (n = 6) of protamine (10 μg/ml). The quantity of protamine added to the solution did not exceed 1% of the volume of the solution. We have previously shown that this quantity of protamine did not modify the pH and concentrations of the electrolytes (Na^+, K^+, Ca^{2+}) of the Krebs-Henseleit bicarbonate buffer solution.[13] The inotropic and lusitropic responses were recorded 15 min after each dose was added to the bathing solution.

Mechanical Parameters

The electromagnetic lever system has been previously described.[14] Conventional mechanical parameters at L_{max} were calculated from three twitches. The first twitch was isotonic and was loaded with the preload corresponding to L_{max}; maximum shortening ($_{max}Vc$) and lengthening ($_{max}Vr$) velocities were determined from this twitch. The second twitch was abruptly clamped to zero load just after the electrical stimulus, as previously reported[15]; the maximum unloaded shortening velocity (V_{max}) was determined from this twitch. The third twitch was fully isometric at L_{max}; maximum isometric active force normalized per cross-sectional area (AF), and the peak of the positive (+dF/dt) and the negative (−dF/dt) force derivatives at L_{max} normalized per cross-sectional area were determined from this twitch. Because changes in the contraction phase induce coordinated changes in the relaxation phase, $_{max}Vr$ and −dF/dt cannot assess lusitropy; therefore, variations in contraction and relaxation must be considered simultaneously to quantify drug-induced changes in lusitropy.[16] The coefficient R1 (= $_{max}Vc/_{max}Vr$) evaluated the coupling between contraction and relaxation under low load and thus lusitropy under low load.[16] In rat myocardium, R1 tests SR uptake function.[11–13,16] The coefficient R2 (= $+dF \cdot dt^{-1}/-dF^{-1} \cdot dt^{-1}$) evaluated the coupling between contraction and relaxation under high load and thus the lusitropy under high load in a manner that is less dependent on inotropic changes. R2 indirectly reflects myofilament calcium sensitivity.[11–13,17] A decrease in R1 or R2 indicates a positive lusitropic effect.

At the end of the study, the muscle cross-sectional area was calculated from the length and weight of papillary muscle, assuming a density of 1. Because there are important differences in baseline values from one muscle to another, inotropic responses were expressed as a percentage of baseline values (*i.e.*, after exposure of protamine) as previously reported.[11,12]

Table 1. Effects of α-(Phenylephrine) and β-(Isoproterenol) Adrenoceptor Stimulations on the Main Mechanical Parameters in Control Groups

Groups	V_{max}	AF	R1	R2
Phenylephrine				
Eff_{max} (% of baseline)	140 ± 10*	144 ± 16*	95 ± 8	98 ± 15
C_{50} (μM)	1.91 ± 1.47	1.38 ± 1.44	—	—
Isoproterenol				
Eff_{max} (% of baseline)	183 ± 24*†	188 ± 45*†	63 ± 7*†	72 ± 10*†
C_{50} (μM)	2.78 ± 1.39	2.54 ± 1.00	0.83 ± 0.35	3.43 ± 2.85

Data are mean ± SD (n = 8 in each group).

* $P < 0.05$ *versus* baseline. † $P < 0.05$ *versus* phenylephrine.

V_{max} = maximum unloaded shortening velocity; AF = isometric active force normalized per cross-sectional area; R1 = ratio of maximum shortening velocity ($_{max}Vc$) to maximum lengthening velocity ($_{max}Vr$); R2 = ratio of the peak of the positive force derivative (+dF/dt) to the peak of the negative force derivative (−dF/dt); Eff_{max} = maximum effect expressed as percent of baseline; C_{50} = concentration that results in 50% of Eff_{max}.

Table 2. Effects of Protamine (10 and 100 μg/ml) on the Main Mechanical Parameters

Concentration of Protamine	V_{max}	AF	R1	R2
10 μg/ml	118 ± 14*	121 ± 13*	128 ± 18*	113 ± 17
100 μg/ml	109 ± 18	117 ± 23	138 ± 18*	114 ± 16

Data are mean percent of baseline ± SD (n = 8 in each group).

* $P < 0.05$ *versus* baseline. There were no significant differences between the two concentrations of protamine.

V_{max} = maximum unloaded shortening velocity; AF = isometric active force normalized per cross-sectional area; R1 = ratio of maximum shortening velocity ($_{max}$Vc) to maximum lengthening velocity ($_{max}$Vr); R2 = ratio of the peak of the positive force derivative (+dF/dt) to the peak of the negative force derivative (−dF/dt).

Statistical Analysis

Data are expressed as mean percentage of baseline ± SD. Concentration–response curves were determined by fitting the data to the Hill pharmacologic model (Origin 5.0; Microcal Software, Northampton, MA) as previously reported.[11] Comparison of two means was performed using the Student *t* test. Comparison of several means was performed using analysis of variance and the Newman–Keuls test. All probability values were two tailed, and a *P* value of less than 0.05 was required to reject the null hypothesis. Statistical analysis was performed with NCSS 6.0 software (Statistical Solutions Ltd., Cork, Ireland).

Results

We studied 104 left ventricular papillary muscles. The mean L_{max} was 6.2 ± 1.4 mm, the mean cross-sectional area was 0.44 ± 0.17 mm^2, the mean ratio of resting force to total force was 0.10 ± 0.03, and the mean R1 was 0.69 ± 0.09, at a $[Ca^{2+}]_o$ of 2.5 mM. A decrease in contractility was observed as $[Ca^{2+}]_o$ was decreased from 2.5 mM to 0.5 mM. The decreases in V_{max} (63 ± 9% of the value at a $[Ca^{2+}]_o$ of 2.5 mM) and AF (52 ± 12% of the value at a $[Ca^{2+}]_o$ of 2.5 mM) were consistent with those previously reported.[11–13] In control groups, α- and β-adrenoceptor stimulations induced a positive inotropic effect under high and low loads, which was more pronounced during β-adrenoceptor stimulation (table 1), which also induced a marked positive lusitropic effect under low and high loads (table 1) as previously described.[11,12] The effects of protamine (10 and 100 μg/ml) were consistent with those observed in our previous study (table 2).[13]

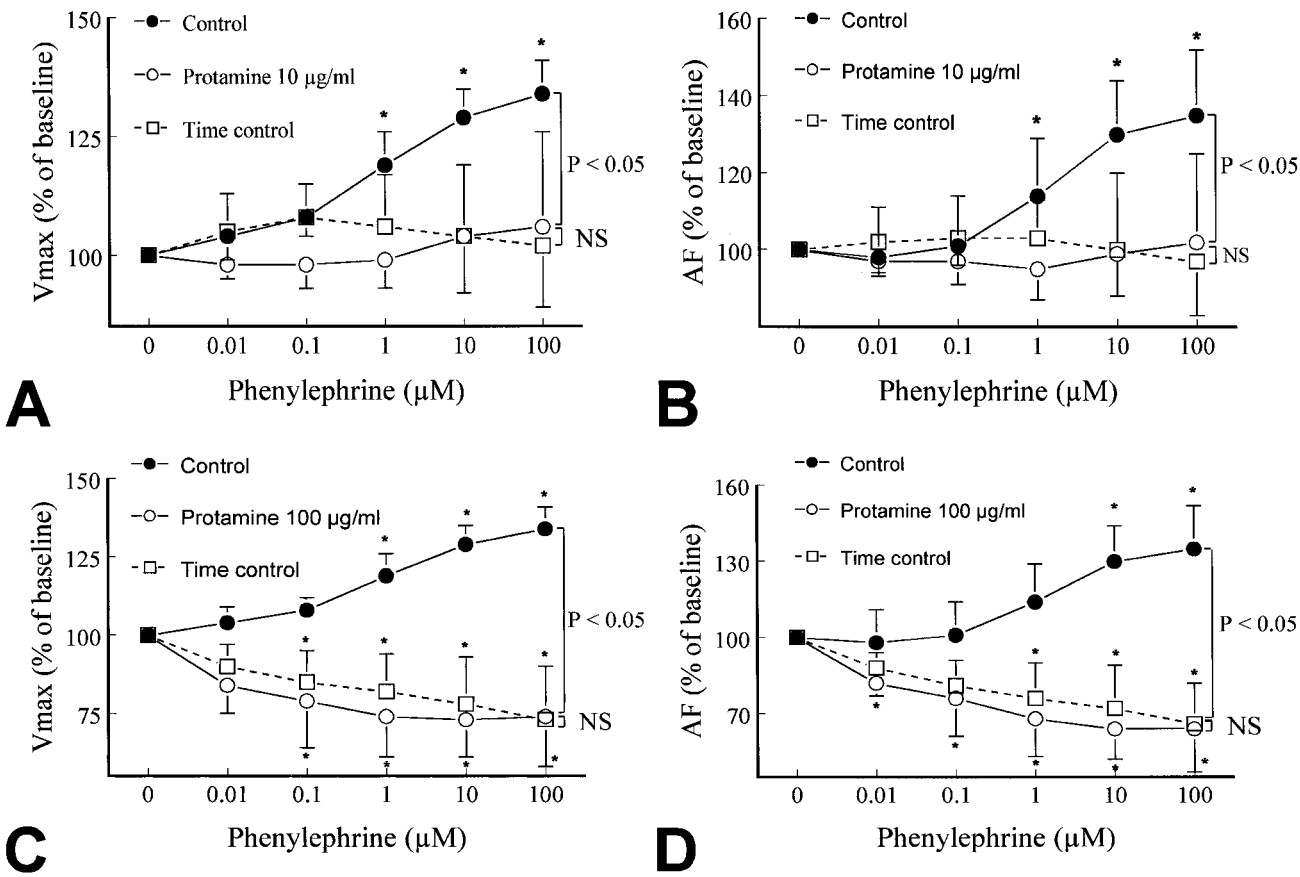

Fig. 1. Effects of protamine (*A* and *B*: 10 μg/ml; *C* and *D*: 100 μg/ml) on the positive inotropic effects of α-adrenoceptor stimulation induced by phenylephrine under low (*A* and *C*) and high (*B* and *D*) loads. V_{max} = maximum unloaded shortening velocity; AF = active isometric force. Data are mean percentage of baseline ± SD; n = 8 in each group. *$P < 0.05$ *versus* baseline. *P* values refer to between-groups differences. NS = not significant.

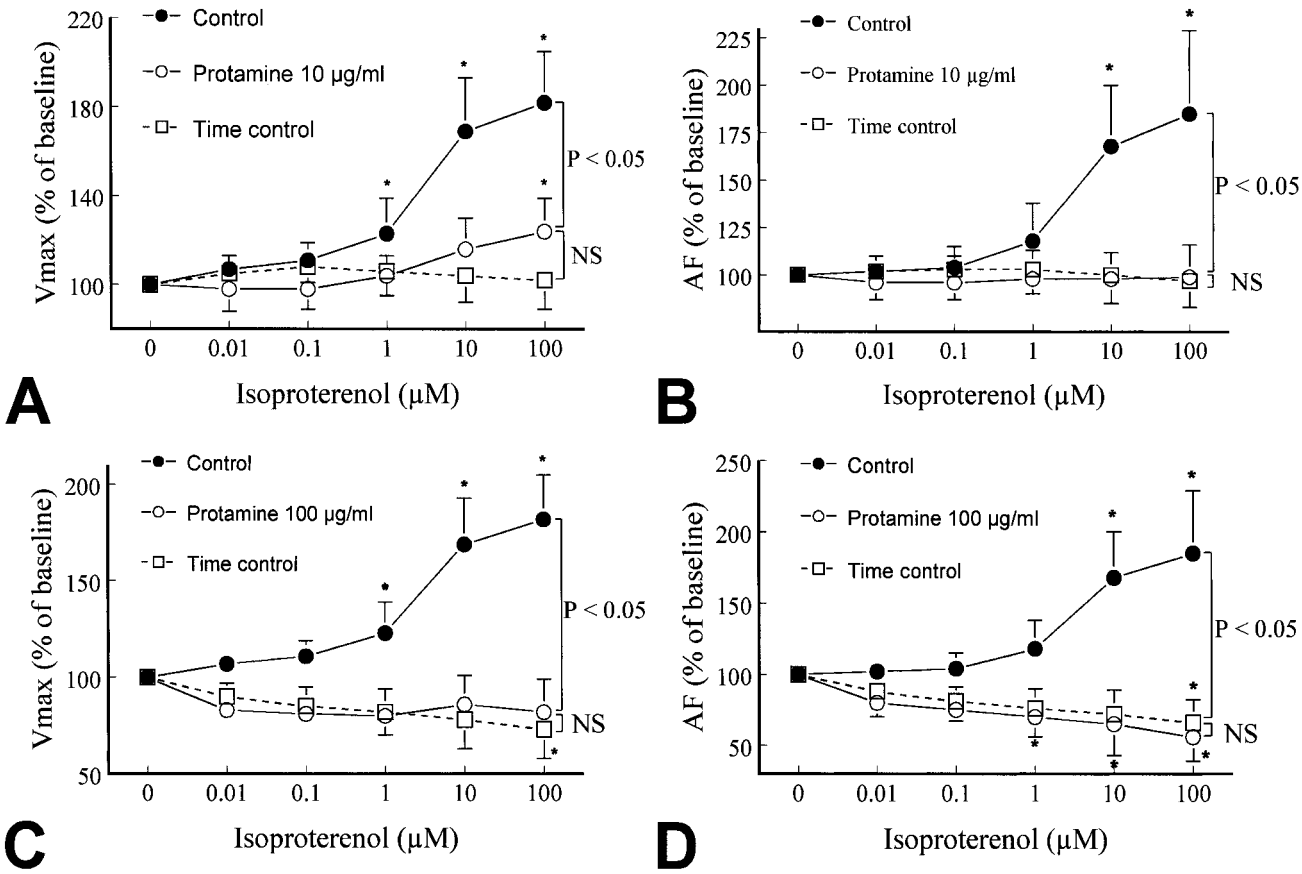

Fig. 2. Effects of protamine (*A* and *B*: 10 μg/ml; *C* and *D*: 100 μg/ml) on the positive inotropic effects of β-adrenoceptor stimulation induced by isoproterenol under low (*A* and *C*) and high (*B* and *D*) loads. V_{max} = maximum unloaded shortening velocity; AF = active isometric force. Data are mean percentage of baseline ± SD; n = 8 in each group. **P* < 0.05 *versus* baseline. *P* values refer to between-groups differences. NS = not significant.

Effects of Protamine on α-Adrenoceptor Stimulation

In the control group, phenylephrine induced a significant positive inotropic effect under low and high loads (table 1). Protamine abolished the inotropic effect of phenylephrine (fig. 1), even at a low concentration (10 μg/ml).

Effects of Protamine on β-Adrenoceptor Stimulation

In the control group, isoproterenol induced positive inotropic and lusitropic effects under low or high loads (table 1). Protamine abolished the positive inotropic effect of isoproterenol (fig. 2), even at a low concentration (10 μg/ml). In contrast, the positive lusitropic effects

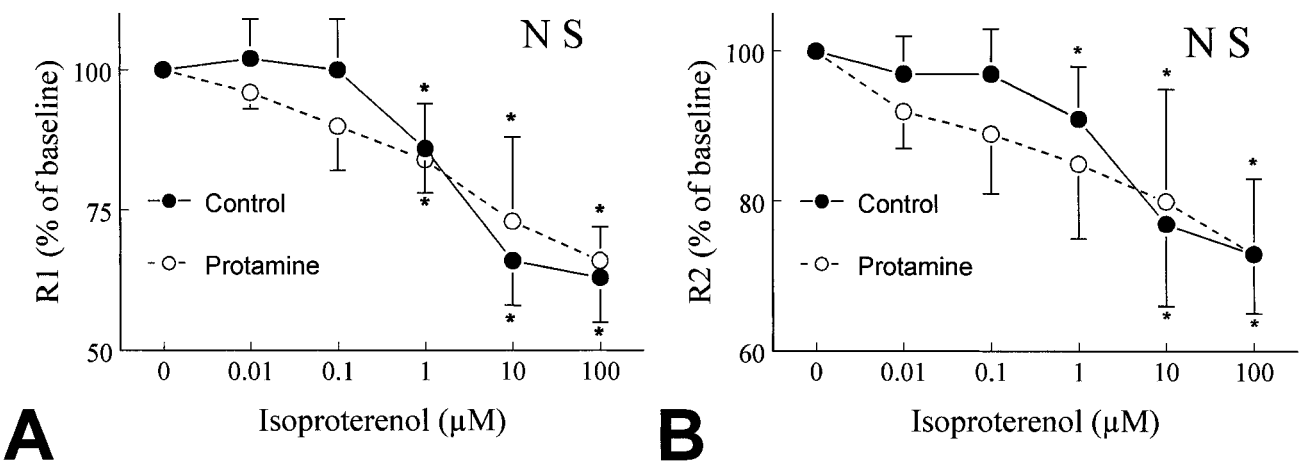

Fig. 3. Effects of protamine (100 μg/ml) on the positive lusitropic effects of β-adrenoceptor stimulation induced by isoproterenol under low (*A*) and high (*B*) loads. R1 = ratio of maximum shortening velocity to maximum lengthening velocity; R2 = ratio of the peak of the positive force derivative to the peak of the negative force derivative. Data are mean percentage of baseline ± SD; n = 8 in each group. **P* < 0.05 *versus* baseline. *P* values refer to between-groups differences. NS = not significant.

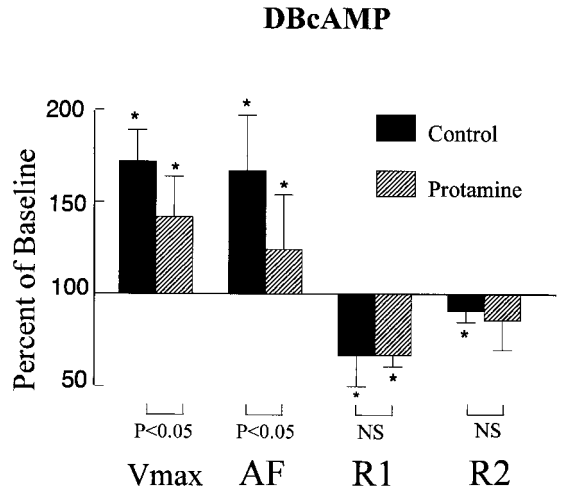

Fig. 4. Effects of protamine (10 μg/ml) on the positive inotropic and lusitropic effects of forskolin (50 μM). V_{max} = maximum unloaded shortening velocity; AF = active isometric force; R1 = ratio of maximum shortening velocity to maximum lengthening velocity; R2 = ratio of the peak of the positive force derivative to the peak of the negative force derivative. Data are mean percentage of baseline ± SD; n = 6 in each group. *$P < 0.05$ *versus* baseline. *P* values refer to between-groups differences. NS = not significant.

of isoproterenol in isotonic (R1) or isometric conditions (R2) were not significantly modified by protamine, even at a high concentration (fig. 3).

Site of the Interaction

Direct activation of adenylate cyclase by forskolin resulted in positive inotropic and lusitropic effects under low and high loads. In the presence of protamine (10 μg/ml), the positive inotropic effects of forskolin were abolished (fig. 4). In contrast, the positive lusitropic effects of forskolin under low (R1) and high (R2) loads were not significantly modified by protamine (fig. 4).

DBcAMP induced positive inotropic and lusitropic effects under low and high loads. Protamine (10 μg/ml) significantly decreased the inotropic effects of DBcAMP, but did not abolish it (fig. 5). In contrast, protamine did not significantly modify the lusitropic effects of DBcAMP under low (R1) and high (R2) loads.

Increasing $[Ca^{2+}]_o$ from 0.5 to 1 mM induced a positive inotropic effect under low (V_{max}: 153 ± 15% of baseline, $P < 0.05$) and high (AF: 183 ± 29% of baseline, $P < 0.05$) loads. Protamine (10 μg/ml) did not significantly modify the positive inotropic effects of $[Ca^{2+}]_o$ (fig. 6).

Discussion

In the current study, we showed that protamine abolished the positive inotropic responses to α- and β-adrenoceptor stimulations, even at a low concentration (10 μg/ml), but did not significantly modify the lusitropic responses to β-adrenoceptor stimulation under low or high loads, even at a high concentration (100 μg/ml).

Protamine has been shown to induce a negative inotropic effect on isolated myocardium.[4–6,9] This effect is thought to be related to an increased permeability of the sarcolemma and an impairment in the Na^+–Ca^{2+} exchange, leading to calcium overload.[6,13] The precise mechanism of this effect is not completely understood but is thought to involve screening of negative sarcolemmal surface charge[18,19] and impairment in SR function.[13]

We observed that protamine abolished the positive inotropic responses to isoproterenol *in vitro* (fig. 2). These results are in accordance with several experimental studies indicating that β-adrenoceptor agonist administration is not efficient in the presence of protamine.[7,20] Binding of β agonists to β adrenoceptors induces the following cascade: stimulation of G_s protein; increase in the activity of adenylate cyclase with subsequent cAMP generation activation of protein kinase A, which phosphorylates several regulatory proteins, including the following: (1) phospholamban, which leads to an increase in the rate of calcium uptake by the SR; (2) troponin I, which results in a decrease in myofilament calcium sensitivity; and (3) calcium channels, which result in an increase in calcium inward.[21–23] The first two of these modifications are responsible for the positive lusitropic effects, whereas the last one is mainly responsible for the positive inotropic effect of β agonists.[17,22,23] We also observed that protamine abolished the positive inotropic effect of forskolin, which directly activates adenylate cyclase, suggesting that the main site of interference is located downstream from the adenylate cyclase in the β-agonist cascade. When cAMP was directly increased by administration of DBcAMP, protamine markedly decreased this positive inotropic effect, suggesting again that one of its main targets was located downstream from cAMP production. The discrepancy between the disappearance of the response to forskolin (fig. 4) and the partial blunt of that of DBcAMP (fig. 5) suggests that protamine might interfere with adenylate cyclase production of cAMP, as previously observed in isolated sarcolemmal preparations.[9] Our study did not enable us to precisely analyze the β-agonist cascade upstream from the adenylate cyclase, *i.e.*, the β adrenoceptor itself and the G-proteins system. Protamine may also interfere at these levels. Indeed, protamine has been shown to decrease β-adrenoceptor binding by 20% without significant changes in affinity.[9]

The mechanism of the positive inotropic effect of α_1-adrenoceptor stimulation remains controversial.[10] α_1-Adrenoceptors are coupled with a G_q protein, leading to activation of phospholipase C and then to production of inositol triphosphate and 1,2 diacylglycerol, which increase intracellular calcium concentration and activate protein kinase C, respectively.[10] In the presence of a low concentration of protamine (10 μg/ml), the inotropic responses to phenylephrine were abolished. The precise mechanisms of action of protamine were not studied,

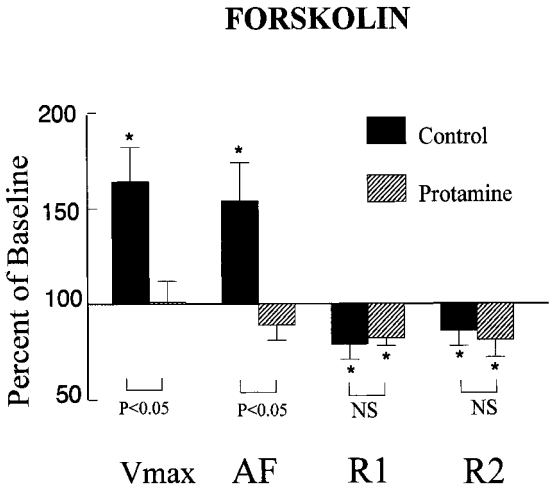

Fig. 5. Effects of protamine (10 μg/ml) on the positive inotropic and lusitropic effects of dibutyryl 3',5'-cAMP (0.5 mM) V_{max} = maximum unloaded shortening velocity; AF = active isometric force; R1 = ratio of maximum shortening velocity to maximum lengthening velocity; R2 = ratio of the peak of the positive force derivative to the peak of the negative force derivative. Data are mean percentage of baseline ± SD; n = 8 in each group. *P < 0.05 *versus* baseline. P values refer to between-groups differences. NS = not significant.

mainly because of the uncertainty regarding those induced by α_1-adrenoceptor stimulation. Nevertheless, the fact that protamine abolished the inotropic responses of many inotropic agents, such as α_1 agonists and ouabain,[9] suggests that protamine interferes with the final commune pathway of action of these different inotropic agents, *i.e.*, the increase in calcium inward. However, we have verified that the response to extracellular calcium was not significantly modified by protamine (fig. 6). This observation implies that the myofilament machinery is still able to respond to calcium in the presence of protamine.

There was a discrepancy between the interaction of protamine with the inotropic and lusitropic effects of β-adrenoceptor stimulation. Even when adenylate cyclase was directly stimulated by forskolin or when DBcAMP was administered to stimulate the cAMP-dependent protein kinase systems directly, we observed that the positive lusitropic effects were not modified by protamine. These lusitropic effects occur at lower concentrations than does the positive inotropic effect[19] and are thought to be an important mechanism of action of β-adrenoceptor stimulation, favoring cardiac relaxation.[24] This has two major implications. First, although protamine is able to block the positive inotropic effect of β-adrenoceptor stimulation, it is not able to block its enhancement of cardiac relaxation. This might have beneficial clinical consequences, especially because protamine *per se* is thought to induce calcium overload, impairment in the calcium uptake by the SR, and thus impairment in cardiac diastolic function.[6,13] This may also be important in diseased or ischemic myocardium in which impairment in cardiac relaxation has been demonstrated.[25] Second, the absence of interaction of protamine with the lusitropic effects of β-adrenoceptor stimulation strongly suggests that the main site of interference of protamine with the inotropic effect of β-adrenoceptor stimulation is mainly situated downstream from the cAMP-mediated phosphorylation.

There are apparent conflicting results in our study because the observed effects on the lusitropic response suggest that production of cAMP was not modified by protamine, whereas those on the inotropic responses to forskolin and DBcAMP (see above) as well as those of Hird *et al.*[9] suggest that cAMP production was decreased by protamine. However, it should be noted that the lusitropic effects of β-adrenoceptor stimulation occur at lower concentrations than do the inotropic effects and plateau at higher concentrations.[17] Consequently, our results are in accordance with the hypothesis that protamine only moderately decreases cAMP production, and we were not able to detect that in our experimental conditions, considering the lusitropic effect of β-adrenoceptor stimulation (fig. 3).

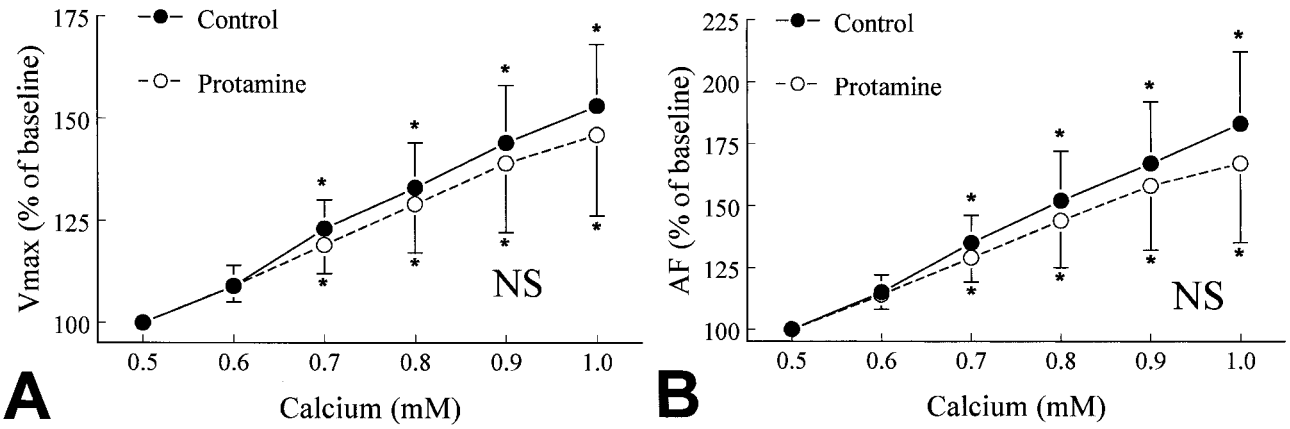

Fig. 6. Effects of protamine (10 μg/ml) on the positive inotropic effects of extracellular calcium under low (*A*) and high (*B*) loads. V_{max} = maximum unloaded shortening velocity; AF = active isometric force. Data are mean percentage of baseline ± SD; n = 6 in each group. *P < 0.05 *versus* baseline. P values refer to between-groups differences. NS = not significant.

Because cAMP-mediated phosphorylation induces an increase in calcium inward through calcium channels,[26] one can hypothesize that protamine either blocks the effects of the cAMP-mediated phosphorylation on calcium channel or blocks the effects of an increase in calcium inward. Indeed, protamine has been shown to modify the function of sarcolemmal channels, such as adenosine triphosphate–sensitive potassium channels through screening of negative sarcolemmal charges[19] and to modify cardiac muscarinic receptors by complex allosteric modulation.[27] Conversely, protamine has also been shown to induce a negative inotropic effect through calcium overload.[6,13] We observed that protamine did not markedly modify the inotropic response to extracellular calcium (fig. 6), suggesting that protamine mainly interferes with the effect of cAMP-mediated phosphorylation on the calcium channel rather than with the calcium channel itself. Further studies are required to elucidate the precise mechanism of action of protamine on the regulation of the calcium channel.

The implications of our findings are potentially of clinical importance because administration of β-adrenergic agents as inotropic support could be less efficient after administration of protamine during cardiovascular surgery. Moreover, in patients with chronic left ventricular dysfunction, protamine may further exacerbate abnormalities in the β-adrenoceptor transduction system.[7,23] Nevertheless, in clinical conditions during cardiovascular surgery, protamine can bind to heparin or albumin; therefore, it remains speculative whether the concentrations of protamine used in the current study exactly reflect protamine concentrations to which cardiac myocytes would be exposed *in vivo*.[13] The following points must also be considered when assessing the clinical relevance of our results. First, this *in vitro* study only dealt with intrinsic myocardial contractility. Observed changes in cardiac function also depend on modifications in venous return, afterload, and compensatory mechanisms. Moreover, there is some evidence that protamine can also have an indirect cardiac effect through the release of mediators, such as tumor necrosis factor.[8] Second, this study was conducted at 29°C and at a low-stimulation frequency. However, papillary muscles must be studied at this temperature because stability of mechanical parameters is not sufficient at 37°C and at a low frequency because high-stimulation frequency induces core hypoxia.[28] Third, it was performed in rat myocardium, which differs from human myocardium. The α-adrenoceptor density and consequently the positive inotropic effect induced by their stimulation are greater in rats than in humans.[29] Nevertheless, the relative importance of α adrenoceptors in cardiac contractility may be increased in the presence of cardiac disease.[30]

In conclusion, in isolated rat myocardium, protamine abolished the positive inotropic effects of α- and β-adrenoceptor stimulations but did not modify the positive lusitropic effects of β-adrenoceptor stimulation. Although protamine acts at several sites on the adrenoceptor stimulation cascade, one of its main sites of action is probably situated downstream from the cAMP-mediated phosphorylation, *i.e.*, the calcium inward.

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