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Effects of Protamine on Vascular Smooth Muscle of Rabbit Mesenteric Artery

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Systemic hypotension is commonly observed in association with protamine administration after cardiopulmonary bypass. However, little information is available concerning the action of protamine on vascular smooth muscle. Thus, we investigated the action of protamine on vascular tissues using tension recording and microelectrode methods. Protamine (5–500 μg/ml) inhibited contractions induced by norepinephrine (NE)- or elevated K^+ in a concentration-dependent manner in both endothelium-intact and -denuded strips. Protamine inhibition of NE contractions was less profound after endothelial denudation, whereas protamine inhibition of K^+-induced contractions was less affected by prior denudation. In endothelium-intact strips, the protamine-induced inhibition was significantly reduced by inhibitors of the endothelium-derived relaxing factor pathway, including oxyhemoglobin, methylene blue, or N^G-nitro-L-arginine, whereas the contractile inhibition was enhanced by superoxide dismutase. In endothelium-denuded strips, protamine inhibited Ca^{2+}-induced contraction evoked in Ca^{2+}-free solution containing 100 mM K^+ and inhibited the NE-induced contraction under the following conditions: 1) in Ca^{2+}-free solution; 2) after nifedipine treatment; and 3) after depletion of stored Ca^{2+} by A23187 or ryanodine. In membrane-permeabilized strips, protamine did not modify Ca^{2+}-induced contraction. Protamine (50–500 μg/ml) did not modify the membrane potential of either endothelium-intact or -denuded strips. Furthermore, protamine irreversibly impaired acetylcholine-induced endothelium-dependent relaxant response, implying a toxic effect of protamine on the endothelium. We conclude that protamine exerts its inhibition on vascular smooth muscles in both an endothelium-dependent and -independent manner; *i.e.*, the endothelium-dependent component is mediated probably by endothelium-derived relaxing factor, and direct smooth muscle effects are due to the inhibition of both Ca^{2+}-influx and the NE-induced Ca^{2+} release from intracellular stores. (Key words: Complications, protamine: hypotension. Muscle, smooth: endothelium-derived relaxing factor. Peptides: arginine-rich basic polypeptides.)

PROTAMINE has been widely used to reverse heparin anticoagulation in the practice of cardiovascular surgery.[1–3] During this reversal, protamine causes a number of adverse responses, including systemic hypotension, pulmonary hypertension, bradycardia, bronchoconstriction, thrombocytopenia, and leukopenia.[1,4–6] Many clinical investigations[1,6–8] have demonstrated that these adverse hemodynamic responses following protamine administration after cardiopulmonary bypass vary from mild hypotension (a common response) to life-threatening cardiovascular collapse.

Despite its extensive clinical use, the precise mechanisms of protamine-induced adverse responses have not yet been fully elucidated.[1–5] In the early 1970s, animal investigations implied that protamine-induced systemic hypotension was due mainly to its direct cardiac depressant effect.[1] However, recent investigations have suggested that protamine has no direct cardiac depressant effect and that its effects are more complex.[1,7,9] Whereas the phenomenon of anaphylaxis to protamine or "catastrophic pulmonary vasoconstriction" with protamine are distinct and relatively rare causes of extreme systemic hypotension, modest hypotension is a common side effect of protamine administration.[1,2,6,10]

Recent laboratory studies have reported that arginine-containing basic polypeptides cause endothelium- and cyclic GMP–dependent relaxation of vascular smooth muscles.[11,12] Thus, because protamine is an arginine-rich basic polypeptide, it is possible that protamine causes endothelium-dependent relaxation of vascular smooth muscles. However, some investigators[1,3] have reported that protamine itself could not cause any hemodynamic response *in vivo* in the absence of heparin; therefore, protamine itself may not have any significant action on vascular smooth muscles.

To our knowledge, no systematic study has yet been carried out to examine the action of protamine on vascular smooth muscle tissues.[12,13] Therefore, the present study was designed to investigate the action of protamine on vascular smooth muscles in the absence of both humoral mediators and heparin.

Materials and Methods

Tissue Preparation

With institutional approval, male albino rabbits (2.0–2.5 kg) were given sodium pentobarbital (40 mg/kg intravenously) and exsanguinated. The mesentery in the jejunal region was immediately placed in a dissecting chamber filled with preoxygenated Krebs–bicarbonate

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solution, and the mesenteric artery was rapidly excised. Arterial segments 0.2–0.3 mm in diameter were used for the experiments. Under a binocular microscope, the fat and connective tissue were carefully removed, and circular strips (0.4–0.6 mm long, 0.1–0.12 mm wide, and 0.03–0.05 mm thick) were prepared for tension recording. The length and diameter of each strip prepared for the electrophysiologic experiment were about 10 and 0.2–0.3 mm (without dissection), respectively. In some experiments, the endothelium was removed before the strips were mounted in the chamber: the intimal surface was gently rubbed with the round surface of a small pin (2 mm in diameter) for the tension-recording experiments, and with a cotton ball moistened with Krebs solution (as described by Furchgott and Zawadzki[14]) in the electrophysiologic experiment. Functional removal of endothelium was assessed by disappearance of acetylcholine (ACh)-induced endothelium-dependent relaxation in the tension-recording experiments and by disappearance of ACh-induced endothelium-dependent hyperpolarization[15] in the electrophysiologic experiments. This assessment was performed in all strips.

Recording of Mechanical Activity

Mechanical responses of intact and chemically membrane-permeabilized muscle strips were measured by attaching the strips to a strain gauge (UL-2 type, Shinko Co., Tokyo) in a 0.9-ml chamber. The strip was stretched to about 1.1 times its resting length to maintain resting tension (about 10 μN): under this condition, the contractile response to 128 mM K^+ was maximal. The solution was changed by perfusing rapidly from one end and aspirating simultaneously with a water pump from the other end.[16–18] Most of the tension-recording experiments were performed at 25° C[16–19] because chemically membrane-permeabilized muscle tissues soon began to deteriorate at higher temperatures; furthermore, as shown in figure 1, the effects of protamine at 25° C in intact strips were similar to those at 32 and 37° C. However, some additional experiments were performed at 32 or 37°C (see "Experimental Design"). The effects of protamine on Ca^{2+}-induced contractions in the intact muscle strips were observed in Ca^{2+}-free solution containing 100 mM K^+ buffered with N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) as previously reported.[19] Briefly, the strips were depolarized for 10 min using the HEPES-buffered solution, and various concentrations of Ca^{2+} were cumulatively applied in a step-wise manner.

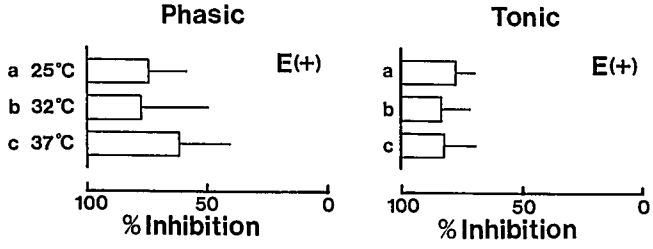

FIG. 1. Effects of temperature (25, 32, 37° C) on protamine (50 μg/ml)-induced inhibition in the presence of 1 μM NE in endothelium-intact (E (+)) strips. Protamine was applied for a sufficient time in order to exert its maximal effect.

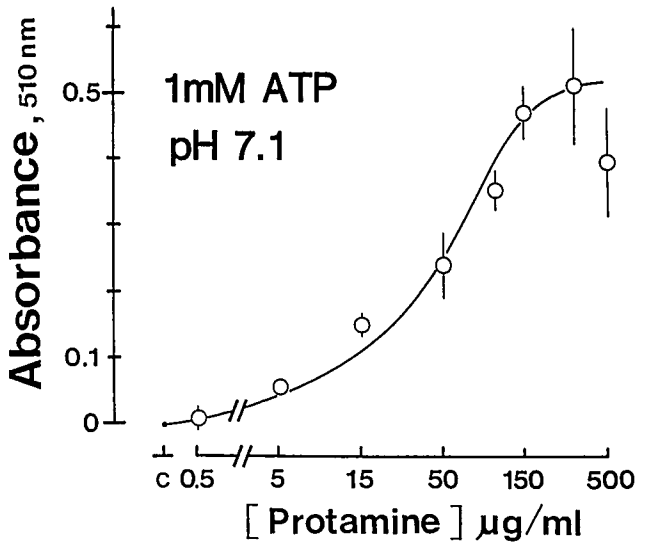

FIG. 2. Effects of protamine (0.5–500 μg/ml) on the turbidity of HEPES-buffered, ATP (1 mM)-containing solution (pH 7.1). Turbidity was measured at 510 nm, volume 3 ml.

Chemically membrane-permeabilized muscle strips were obtained by exposing them to 20 μM β-escin for 30 min in the relaxing solution.[20] Cloudiness developed upon the addition of protamine to the solutions for membrane-permeabilized muscle experiments. Further analysis showed that the cause of this cloudiness was the coexistence of protamine and adenosine triphosphate (ATP) in the solution. We assessed this cloudiness by turbidimetry: as shown in figure 2, protamine (0.5–300 μg/ml) increased the turbidity in HEPES-buffered, ATP (1 mM)-containing solutions in a concentration-dependent manner. (The *p*H was adjusted to 6.8, 7.1, and 7.4 by Tris; data on the experiments at *p*H 6.8 and 7.4 not shown). Protamine is strongly positively charged, whereas ATP is negatively charged. Thus, we speculate that this cloudiness may be due to the formation of complexes between them by ionic force. However, since the dissociation constant for the binding of protamine to ATP is unknown, it is uncertain how much free protamine exists in the relaxing solution. The experiments were performed under the above-mentioned conditions.

Microelectrode Measurements

The membrane potential of the smooth muscle cell was measured using a glass capillary microelectrode filled with

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3M KCl (tip resistance, 40–60 MΩ).[17] The tissue segment was pinned onto a rubber plate in a 1.5-ml chamber and superfused with Krebs solution (37° C) at a flow rate of 3 ml/min. The electrode was inserted into the muscle cell from the outer surface of the vessel.

Immunohistochemical Study

In order to verify the morphologic removal of endothelium, an immunohistochemical study was performed for the detection of factor VIII-related antigen and the binding site of *Ulex europaeus* agglutinin-1.

For this study, endothelium-intact (E(+), n = 3) or -denuded (E(−), n = 3) smooth muscle tissues were obtained from the same arterial segments as used for tension recording. The muscle tissues were fixed in 10% neutrally buffered formalin solution, cut into slices, embedded in paraffin, sectioned at 5 μm, and stained immunohistochemically by the avidin–biotin–peroxidase complex (ABC) method of Hsu *et al.*[21]

In the E(+) strips, the endothelial cells were immunoreactive to factor VIII-related antigen and *Ulex europaeus* agglutinin-1, whereas no immunoreactive endothelial cells were found in the E(−) strips (fig. 3; data on immunohistochemical study with factor VIII-related antigen not shown).

Solutions

The Krebs solutions were composed of the following ionic components (millimolar concentration): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_3PO_4^-$ 1.2, Cl^- 134.4, and glucose 11.4. The high-K^+ solution was prepared by replacing NaCl with KCl, isoosmotically. The solution was bubbled with 95% O_2–5% CO_2, and its *p*H was adjusted to 7.3–7.4. In Ca^{2+}-free solutions, $CaCl_2$ was replaced with $MgCl_2$, and 2 mM ethyleneglycol-bis-(β-amino-ethylether)-N,N,N′,N′-tetraacetic acid (EGTA) was added. To prepare the solution containing various concentrations of Ca^{2+}, HEPES buffer was used. The composition of HEPES buffer solution was as follows (millimolar): NaCl 45, K methanesulfonate (Ms) 99, $Mg(Ms)_2$ 1, HEPES 5, glucose 5.6, and various concentrations of $Ca(Ms)_2$. $Ca(Ms)_2$ was simply added to the solution. In Ca^{2+}-free solutions, Ca^{2+} was removed, and 2 mM EGTA was added to the above mixtures. The *p*H of the HEPES buffer solution was kept at 7.4 (adjusted with Tris).

In membrane-permeabilized muscle experiments, the relaxing solution used contained the following components (millimolar concentration): KMs 114, Tris maleate 20, $Mg(Ms)_2$ 5.1, adenosine 5′-triphosphate (ATP) 5.2, and EGTA 4. The *p*H of the solution was adjusted to 7.1 with KOH. Various Ca^{2+} concentrations were prepared by adding the appropriate amount of $Ca(Ms)_2$ to 4 mM EGTA. The binding constants used in the experiment have been previously reported.[22]

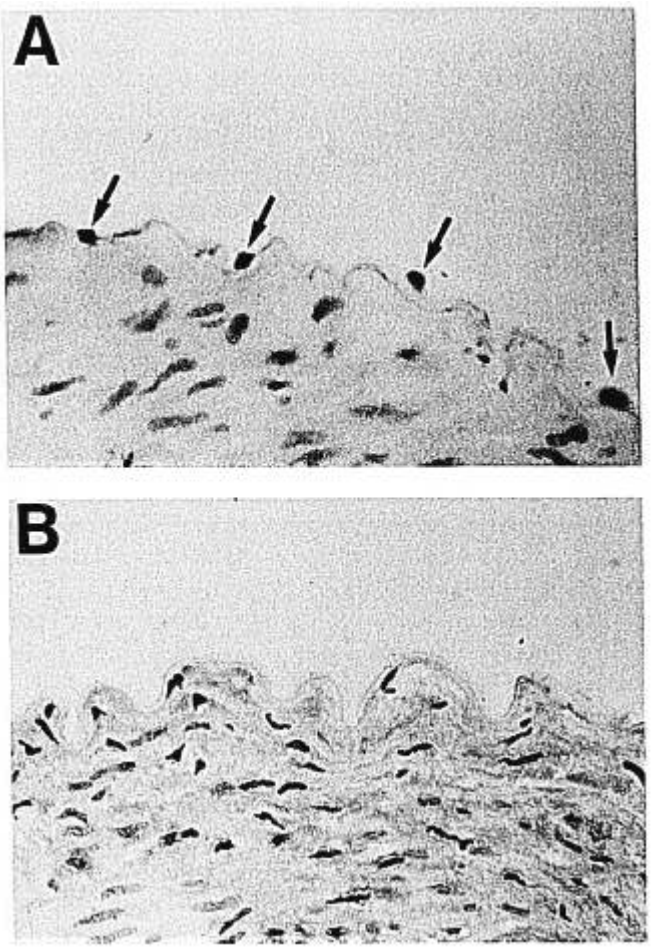

FIG. 3. Immunohistochemical staining (ABC method) of smooth muscle tissues using UEA-1 before (*A*) and after (*B*) the endothial denudation. *A*: Endothelial cells (arrows) were immunoreactive to UEA-1 (original magnification ×600). *B*: No immunoreactive endothelial cells were found (original magnification ×400).

Drugs

Norepinephrine (NE) HCl, indomethacin, poly-L-arginine, superoxide dismutase (bovine liver), methemoglobin (bovine), methylene blue, β-escin, and tetrodotoxin were obtained from Sigma (St. Louis, MO). The other chemicals used were guanethidine HCl (Tokyo Kasei Kogyo, Tokyo), caffeine and propranolol HCl (Wako Pure Chemical Industries, Osaka), EGTA, and HEPES (Dojin Laboratory, Kumamoto, Japan), nifedipine (Bayer Pharmaceutical, Basel, Switzerland), protamine sulfate and cimetidine (Nakalai Tesque, Kyoto, Japan), A23187 (free acid; Calbiochem, San Diego, CA), ryanodine (Agrisystems International, PA), N^G-nitro-L-arginine (L-NNA; Protein Research Foundation, Osaka), anti-factor VIII (rabbit, 1:200; DAKO, San Diego, CA), and biotinylated

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Ulex europaeus agglutinin-1 (1:300, Vector Laboratories, Burlingame, CA). Avidin and biotin reagents were obtained from Vector Laboratories (Vector stain avidin–biotin–peroxidase complex kit PK-4001). Mepyramine maleate was provided by Fujisawa Pharmaceutical (Osaka, Japan).

Protamine was prepared in a normal saline solution just before use. The concentration of protamine was expressed in micrograms per milliliter, because its molecular weight varied from 2 to 12 kd (mean; approximately 4.5 kd).

Nifedipine (0.7 mM) was originally dissolved in 15% ethyl alcohol and 15% polyethylene glycol 400. To avoid any adverse solvent effects, this agent was used in a concentration less than 0.3 μM (such that the concentration of ethyl alcohol was less than 0.01% [2.5 mM]). To prevent inactivation of nifedipine by exposure to daylight, experiments were carried out under either a sodium lamp or conditions of subdued light.[19,23]

Oxyhemoglobin was prepared by reducing commercial bovine hemoglobin containing 75% methemoglobin as described by Martin *et al.*[24]

Experimental Design

Tension-recording Experiments

The preliminary experiments showed that protamine has an inhibitory action on both NE- and high K^+-induced contractions and that its inhibition appeared to develop gradually. Thus, we first studied its time-dependent effect both in E(+) and E(−) strips. Thirty millimolar K^+ and 0.2 μM NE were chosen as the test stimulants, and both contractions consisted of two components—an initial phasic and a subsequent tonic component; the absolute tension levels of both components of each contraction are summarized in table 1. Estimated from the phasic component of NE-induced contraction, 0.2 μM was the EC_{45} and EC_{52} dose in E(+) and E(−) strips, respectively. As shown in fig. 4, each stimulant was applied every 8 min for 2 min, and the experiment was started after the amplitude of each contraction became constant. From this experiment, the application time of protamine was determined. The reversibility and the reproducibility of its inhibition were also studied.

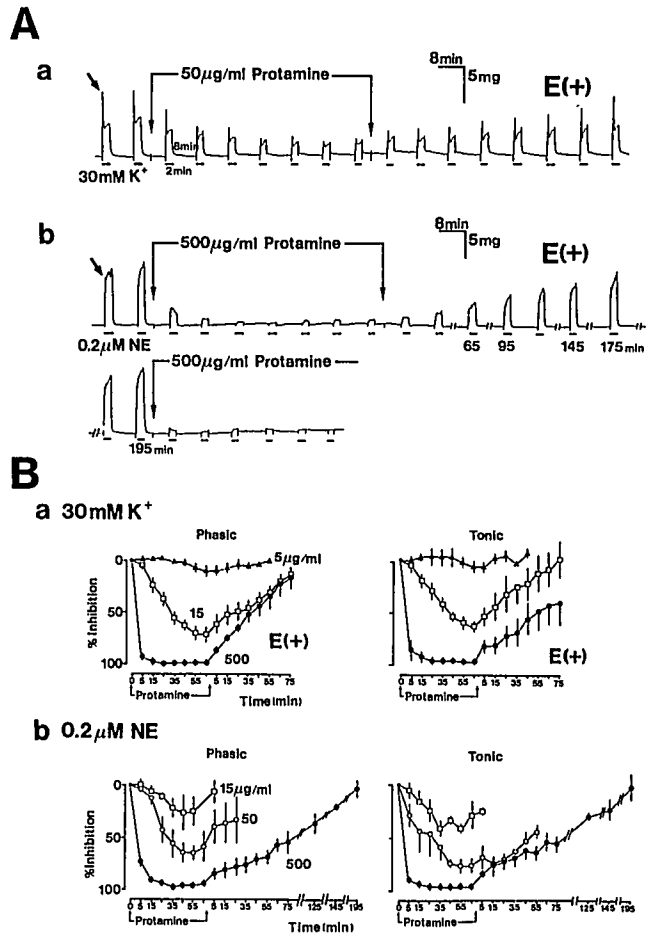

FIG. 4. Time-dependence of protamine-induced inhibition on contractions evoked by 0.2 μM norepinephrine (NE) and 30 mM K^+ in endothelium-intact (E(+)) strips. *A*: Typical examples of the effects of protamine (50 and 500 μg/ml) on 30 mM K^+- (*a*) or 0.2 μM NE- (*b*) induced contraction. Short arrows indicate the phasic component of each contraction. Protamine was applied and removed as indicated by the arrows. *B*: Time-dependent effects of protamine at three different concentrations (high K^+: 5, 15, and 500 μg/ml; NE: 15, 50, and 500 μg/ml) on phasic and tonic components of 30 mM K^+- (*a*) or 0.2 μM NE- (*b*) induced contractions. Vertical bars indicate mean ± SEM; n = 3–10.

TABLE 1. The Absolute Tensions (mg) of the Phasic and Tonic Components of the 30 mM K^+- or 0.2 μM NE-induced Contractions in the Endothelium-intact and -denuded Strips

	30 mM K^+		0.2 μM NE	
	Phasic	Tonic	Phasic	Tonic
E(+)	12.5 ± 3.4 (30)	3.9 ± 2.6 (30)	10.7 ± 3.2 (28)	11.2 ± 4.2 (28)
E(−)	12.9 ± 2.9 (23)	9.3 ± 3.5 (23)	11.2 ± 3.8 (34)	16.3 ± 4.8 (34)

All values are means ± SD (n).
NE = norepinephrine; E(+) = endothelium-intact; E(−) = endothelium-denuded.

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Next, the concentration-dependent effects of protamine on NE- or high K^+-induced contraction were studied in both E(+) and E(−) strips. The concentrations of NE used in this study were, respectively, 0.2 μM (EC_{45}) and 1 μM (EC_{88}) in E(+) strips, and 0.07 μM (EC_{10}, the lowest possible concentration to perform the experiment), 0.1 μM (EC_{21}), and 0.2 μM (EC_{52}) in E(−) strips: each EC value was determined from the phasic contraction. Thirty millimolar K^+ was used as the stimulant in E(+) strips, whereas in E(−) strips, the effects of protamine on various concentrations (20, 30, 39, and 77 mM) of K^+ were observed to examine the possibility that its inhibition is mediated through K^+ channel opening.

The results of the above experiments implied that protamine-induced inhibition consists of both an endothelium-dependent and an endothelium-independent component. Therefore, in order to clarify the characteristics of its endothelium dependence and also to examine the possibility that its inhibition is mediated through β-adrenergic receptors or adrenergic nerve activities, we studied the effects of the following agents on its inhibition in E(+) strips: atropine (1 μM), propranolol (0.3 μM), cimetidine (1 μM), mepyramine (1 μM), guanethidine (3 μM), tetrodotoxin (0.3 μM), indomethacin (3 μM[18]), oxyhemoglobin (10 μM[24]), methylene blue (10 μM[11,12]), L-NNA (100 μM[25]), and superoxide dismutase (100 U/ml[11]).

In order to clarify the endothelium-independent mechanism of the protamine-induced inhibition, the following experiments were performed using the E(−)-strips. First, we studied the effects of protamine on voltage-dependent Ca^{2+}-influx–induced contractions: in Ca^{2+}-free solution containing 100 mM K^+, exogeneously and cumulatively applied Ca^{2+} (0.03–12 mM) generated the contractions in a concentration-dependent manner, and the effects of protamine on these Ca^{2+}-induced contractions were observed. Next, we studied the effects of nifedipine pretreatment (100 nM for 20 min[19,23]) on the protamine-induced inhibition in the presence of NE: under this condition, the NE-induced contraction depends on Ca^{2+} release from the intracellular stores and receptor-operated Ca^{2+} influx. Further, in order to study the effects of protamine on the Ca^{2+} release, we observed its effects on the NE- or caffeine-induced contraction in Ca^{2+}-free, 2 mM EGTA solution: protamine was applied to the strips in the Krebs solution for a length of time sufficient for it to exert its maximal effect before removal of Ca^{2+}, and the NE and caffeine were applied for 2 min, 2 min after removal of Ca^{2+}. Finally, in order to study the effects of protamine on the Ca^{2+}-influx–dependent component of the NE-induced contractions, the intracellular Ca^{2+} stores were depleted by A23187 or ryanodine: 0.1 μM A23187 was pretreated for 30 min,[16] and 30 μM ryanodine was pretreated for 65 min.[26]

Since basic proteins of the protamine and histone types have been reported to enter into living cells regardless of their high molecular weights,[27] it might be possible that protamine gains access to the contractile proteins system. Therefore, to examine the effects of protamine on the contractile proteins system, its effects on Ca^{2+}-induced contractions were observed in chemically membrane-permeabilized muscle strips. After steady contractions induced by 128 mM K^+ were measured, strips were incubated with 20 μM β-escin for 30 min in relaxing solution.[20] After this incubation, the amplitude of 10 μM Ca^{2+}-induced contraction was greater (1.21 ± 0.07 times greater, n = 15) than that induced by 128 mM K^+ in the intact strips, indicating completion of the permeabilization of the muscle tissue.[17] Protamine was applied after the amplitude of Ca^{2+} (10 μM)-induced contraction reached a maximum and steady state.

With the above-mentioned protocol (fig. 4), it is impossible to know the precise onset of protamine-induced inhibition. We, therefore, performed additional experiments to examine its precise onset. The strips were precontracted with 1 μM NE or 40 mM K^+, and after the amplitudes of each contraction reached maximum and a steady state, protamine was applied to the strips for 5 min. This experiment was performed at 37° C in the E(+) strips to better ascertain the clinical relevance of our findings.

Various investigators[12,28–30] have demonstrated that polycationic compounds, including protamine, can cause an endothelial injury. Chang and Voelkel[28] demonstrated that protamine treatment impaired the ACh-induced, endothelium-dependent relaxation in isolated arterial rings. Further, these authors and Wakefield *et al.*[30] showed the histologic evidence of an endothelial cell injury after protamine treatment. However, Rapoport *et al.* reported that protamine had little effect on endothelial integrity and relaxation due to ACh.[12] Therefore, the toxic effect of protamine on endothelium still appears to be controversial. In order to examine the effects of protamine on the functional integrity of endothelium, we observed the effects of protamine on ACh-induced, endothelium-dependent relaxation in the presence of NE (10 μM) and the reversibility of its effects. This experiment was performed at 32° C, because at 37° C the tissue begin to deteriorate a few hours after set-up.

Electrophysiologic Study

In order to examine the possibility that the protamine-induced endothelium-dependent inhibition is mediated through the endothelium-derived hyperpolarizing factor,[15] we studied the effect of protamine on membrane potential in the E(+) strips. Further, in order to examine the possibility that the protamine-induced endothelium-independent inhibition is mediated through K^+ channel

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opening[31] or calcitonin-gene related peptide,[32] we studied the effect of protamine on the membrane potential in the E(−) strips.

Calculation and Statistical Analysis

Results are expressed as means ± standard deviation, except in figure 4 (means ± standard error of the mean).

Protamine-induced inhibitions on the contractions evoked by NE or high K^+ were expressed as percent changes from the amplitudes of each contraction before the application of protamine. The amplitudes of tonic contraction evoked by NE or high K^+ were assessed 2 min after the application of each stimulant. In Ca^{2+}-free (2 mM EGTA) solution, both NE and caffeine evoked only phasic contractions, as previously reported,[17] whereas after A23187 and ryanodine treatment, the NE evoked only tonic contractions, as previously reported.[16] Therefore, in the experiments with Ca^{2+}-free solution, A23187, and ryanodine, the maximal amplitude of each contraction was assessed.

The ACh-induced relaxations in the strips precontracted with NE were assessed 2 min after the application of ACh. The preliminary experiments showed that the ACh-induced relaxation was independent of the amplitude of the NE-induced contraction. Thus, it is inappropriate to assess the ACh-induced relaxation with the percentage change in the amplitude when the amplitude of the NE-induced contraction is different. In this experiment, the application of protamine inhibited the NE-induced contraction, and it was impossible, particularly after the application of a high concentration of protamine, to obtain the same amplitude as the control by increasing the concentration of NE. We therefore assessed the effects of protamine on the ACh-induced relaxation with the amplitude decreased by ACh: the amplitude of 10 μM NE–induced tonic contraction before the application of protamine was normalized as the control (1.0), and the amplitude decreased by ACh was expressed as the relative amplitude.

The statistical assessment of the data was made by either Student's *t* test or the Cochran-Cox test, where appropriate. $P < 0.05$ was considered significant.

Results

Time Dependence of Protamine-Induced Inhibition

Protamine inhibited NE- or high K^+-induced contractions both in E(+) and E(−) strips (figs. 4 and 5). Figure 4A shows the typical time course of protamine-induced inhibition in the E(+) strips. As shown in figure 4B, lower concentrations (≤ 50 μg/ml) of protamine gradually reduced the amplitudes of both components of these contractions and required about 55–65 min to exert their maximal inhibitory effects, whereas high concentrations of protamine required only 15–25 min. After rinsing with Krebs solution, the amplitudes of both components of these contractions gradually recovered (fig. 4B), and even at the highest concentration, the protamine-induced inhibitions were almost reversible after 195 min of washing. The time dependence of its inhibition in the E(−) strips was similar to that in the E(+) strips (data not shown). According to above findings, we applied protamine to the intact (not membrane-permeabilized) strips for more than 55 min in the following experiments.

Endothelium Dependence of Protamine-Induced Inhibition

Figure 5 shows the dose-dependent effects of protamine on both components of the NE (0.2 μM)- or high (30 mM)

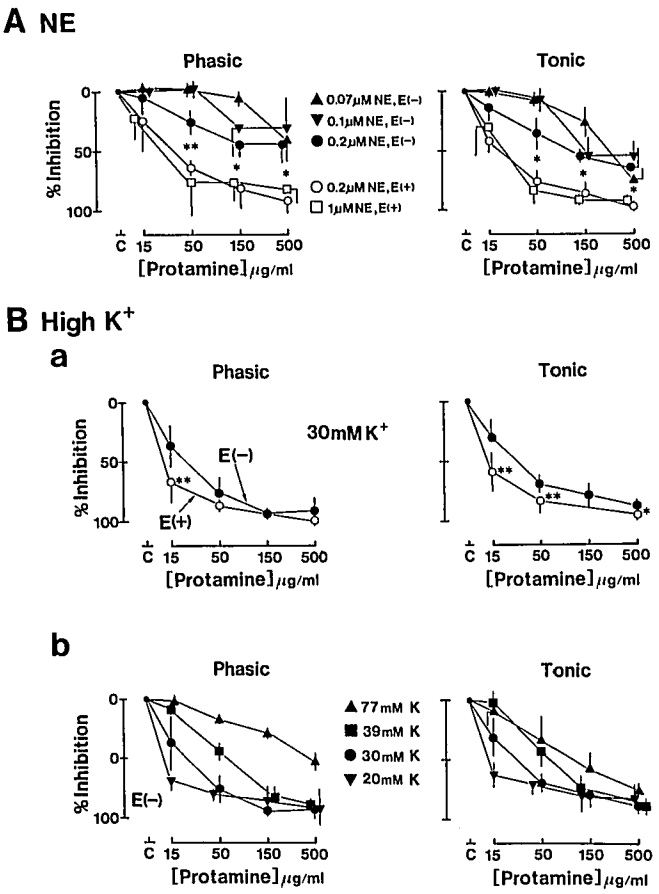

FIG. 5. Dose-dependence of protamine-induced inhibition on the contractions evoked (*A*) by norepinephrine (NE) (0.07–1.0 μM) or (*B*) by high K^+ (20–77 mM) in endothelium-intact (E(+): open symbols) and -denuded (E(−): filled symbols) strips. Protamine was applied 55 min before and during application of each stimulant. Data shown as mean ± SD; n = 3–10. *Significantly different from E(−) strips ($P < 0.05$). **Significantly different from E(−) strips ($P < 0.01$).

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K^+-induced contraction in the E(+) and E(−) strips. The protamine (≥ 50 μg/ml)-induced inhibitions on the NE-induced contraction in the E(+) strips were consistently greater than those in the E(−) strips (fig. 5A). In contrast, the endothelial denudation had less effect on the protamine-induced inhibitions on high K^+-induced contractions (fig. 5B).

Effects of Various Inhibitors and Potentiators on Protamine-Induced Inhibition in Endothelium-Intact Strips

Oxyhemoglobin, methylene blue, and L-NNA slightly increased the resting tension level and significantly ($P < 0.05$) increased the amplitudes of NE (0.2 μM)- and high K^+ (30 mM)-induced contractions, as previously reported.[11,24] At lower concentrations of NE and high K^+, the amplitudes of these contractions did not significantly differ from those of 0.2 μM NE- or 30 mM K^+-induced contraction before exposure to oxyhemoglobin, methylene blue, or L-NNA; therefore, these lower concentrations were used as the stimulants, as follows. The amplitudes of 0.06 μM NE-induced phasic and tonic contractions in oxyhemoglobin-treated strips were 1.11 ± 0.12 (n = 5) and 1.19 ± 0.14 (n = 5) times the control; the amplitudes of 0.1 μM NE-induced phasic and tonic contractions in L-NNA-treated strips were 0.91 ± 0.26 (n = 3) and 1.1 ± 0.22 (n = 3) times the control (fig. 6). In contrast, other various agents did not significantly affect the NE- or high-K^+-induced contractions.

The following various inhibitors had no significant effect on the protamine-induced inhibition in the presence of 0.2 μM NE: atropine (1 μM), propranolol (0.3 μM), cimetidine (1 μM), and mepyramine (1 μM) (fig. 6A). Guanethidine (3 μM) with tetrodotoxin (0.3 μM) had no effect on the protamine-induced inhibition in the presence of 30 mM K^+ (fig. 6B). Indomethacin (3 μM) had no significant effect on the protamine-induced inhibition in the presence of both 0.2 μM NE and 30 mM K^+ (figs. 6A and 6B).

Oxyhemoglobin (10 μM), methylene blue (10 μM), and L-NNA (100 μM) each significantly inhibited the protamine-induced inhibitions in the presence of NE or high K^+ (figs. 6A and 6B). In contrast, superoxide dismutase (100 U/ml) markedly enhanced the protamine-induced inhibition in the presence of 0.2 μM NE (fig. 6A).

Protamine-Induced Inhibition in Endothelium-Denuded Strips

In a concentration-dependent manner, protamine (15–500 μg/ml) inhibited ($P < 0.05$) the Ca^{2+} (0.3–12 mM)-induced contractions evoked in Ca^{2+}-free solution containing 100 mM K^+ and shifted the concentration-response curve for Ca^{2+} to the right. Arunlakshana-Shield plots

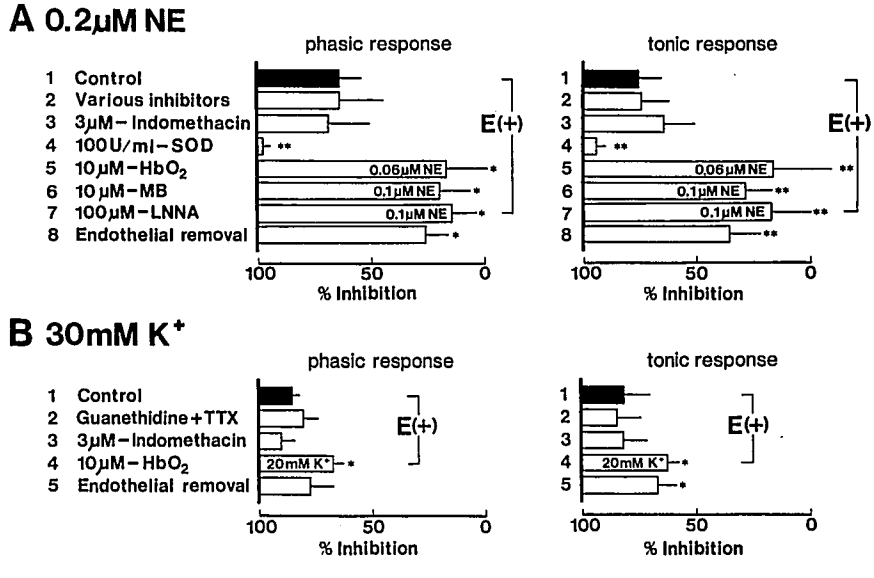

FIG. 6. Effects of various agents on 50 μg/ml protamine-induced inhibition on the contractions evoked by 0.2 μM norepinephrine (NE) (*A*) or 30 mM K^+ (*B*) in endothelium-intact strips and effects of prior endothelium removal on 50 μg/ml protamine-induced inhibition. *A1, B1*: Control inhibitions. *A2*: Effects of various inhibitors. Various inhibitors tested here were 1 μM atropine, 0.3 μM propranolol, 1 μM cimetidine, 3 μM mepyramine, 3 μM guanethidine, and 0.3 μM tetrodotoxin (TTX). These agents were applied together 40 min before and during the experiments. *A3, B3*: Effects of 3 μM indomethacin. Indomethacin was applied 40 min before and during experiments. *A4*: Effects of superoxide dismutase (SOD). SOD was applied 30 min before and during the experiments. *A5, A6, B4*: Effects of oxyhemoglobin (HbO_2) and methylene blue (MB). (HbO_2) and MB were applied 20 min before and during the experiments. In (HbO_2)-treated strips, 0.06 μM NE and 20 mM K^+ were used as stimulants instead of 0.2 μM NE and 30 mM K^+. In MB-treated strips, 0.1 μM NE was applied instead of 0.2 μM-NE. *A7*: Effects of N^G-nitro L-arginine (LNNA). LNNA was applied 20 min before and during the experiments. 0.1 μM NE was used as stimulant instead of 0.2 μM NE. *A8, B5*: Effects of endothelium removal. *B2*: Effects of 3 μM-guanethidine and 0.3 μM TTX. These agents were applied together 40 min before and during experiments. Data shown are mean ± SD; n = 3–10. *A1–6, B1–4*: endothelium-intact strips. *A7, B5*: endothelium-denuded strips. *Significantly different from the control inhibitions ($P < 0.05$). **Significantly different from the control inhibitions ($P < 0.01$).

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obtained from EC_{50} response were linear (r = 0.99) through the concentration range tested for protamine, but the slope (0.53 ± 0.06) was significantly different from unity ($P < 0.05$). Thus, although this protamine-induced antagonism was surmountable, it was not competitive (fig. 7).

The nifedipine treatment completely inhibited the 30 mM K^+-induced contraction and significantly inhibited the NE-induced tonic, but not phasic, contraction, as previously reported.[19,23] Under this condition, protamine still significantly ($P < 0.05$) inhibited both components of NE (0.2 μM)-induced contraction at 50 μg/ml (phasic: 45 ± 8%, n = 3; tonic: 42 ± 12%, n = 3) and at 500 μg/ml (phasic: 65 ± 9%, n = 3; tonic: 68 ± 8%, n = 3). Similar results were obtained in the presence of 1 μM NE (data not shown).

Protamine significantly ($P < 0.05$) inhibited the NE (0.2 μM)-induced contraction evoked in Ca^{2+}-free solution (50 μg/ml: 45 ± 12%, n = 3; 500 μg/ml: 56 ± 8%, n = 3). On the other hand, protamine had almost no effect on caffeine (2 mM)-induced contractions in Ca^{2+}-free solution (50 μg/ml: −4 ± 7%, n = 3; 500 μg/ml: −3 ± 5%, n = 3).

After the A23187 treatment, neither 10 μM NE nor 10 mM caffeine generated any contractions in Ca^{2+}-free (2 mM EGTA) solution, indicating that intracellular Ca^{2+} stores were completely depleted by this treatment. Under this condition, protamine again markedly inhibited ($P < 0.05$) the NE-induced contractions evoked in Krebs solution (50 μg/ml: 88 ± 6%, n = 3, and 500 μg/ml: 97 ± 2%, n = 3). Similar observations were made after the ryanodine treatment (50 μg/ml: 96 ± 1%, n = 3, and 500 μg/ml: 99 ± 1%, n = 3).

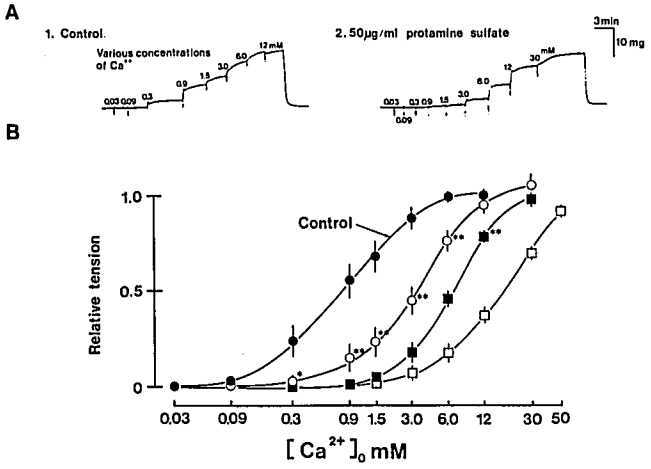

FIG. 7. Effects of protamine on tonic contractions evoked by various concentrations of Ca^{2+} in Ca^{2+}-free solution containing 100 mM K^+ in endothelium-denuded strips. *A1*: Control contractions induced by various concentrations of Ca^{2+} (0.03–12 mM). *A2*: Typical example of protamine (50 μg/ml)-induced inhibitions on the Ca^{2+}-induced contractions. *B*: Inhibitions of protamine at three different concentrations on Ca^{2+}-induced contractions: 15 μg/ml (open circles), 50 μg/ml (filled squares), and 500 μg/ml (open squares). The amplitudes of the tonic contraction evoked by 12 mM Ca^{2+} in the absence of protamine was normalized as 1.0. Protamine was applied 65 min before application of Ca^{2+} (55 min in Krebs solution). Data shown are mean ± SD; n = 3. *Significantly different from the control ($P < 0.05$). **Significantly different from the control ($P < 0.01$).

Effects of Protamine on Calcium-Induced Contractions in Chemically Membrane-Permeabilized Muscle Strips

Protamine (5, 50, and 500 μg/ml) had no significant effect on the Ca^{2+}-induced contraction but markedly inhibited the relaxation of these contractions after removal of Ca^{2+} at higher concentrations (≥ 50 μg/ml): the half relaxation time of 10 μM Ca^{2+}-induced contraction in the control strips was 1.1 ± 0.8 min (n = 5), whereas that in the strips exposed to 500 μg/ml protamine was 28.4 ± 2.1 min (n = 5).

Protamine-Induced Contractions

In addition to its inhibition, higher concentrations (≥ 50 μg/ml) of protamine generated very small but sustained contractions both in the E(+) and E(−) strips. These contractions almost disappeared in Ca^{2+}-free (2 mM EGTA) solution but still developed after the nifedipine or indomethacin pretreatment both in the E(+) and E(−) strips. However, since the amplitudes of the protamine-induced contractions were very small and often fluctuated, it was difficult to estimate their amplitudes precisely and investigate them any further.

The Onset of Protamine-Induced Inhibition at 37° C

At 37° C, protamine (≥ 35 μg/ml)-induced inhibition of the NE or high K^+-induced contraction occurred soon after the application of protamine, but its development was not as fast as the relaxation induced by an agent that acts on the receptor (*e.g.*, ACh) (figs. 8 and 9).

Effects of Protamine on Acetylcholine-Induced Endothelium-Dependent Relaxant Response

Protamine (50–500 μg/ml) inhibited the ACh (0.03–10 μM)-induced relaxation in a concentration-dependent manner in the presence of 10 μM NE (fig. 9). After the washout of protamine, its inhibition gradually recovered. After 195 min of washing, the NE-induced contraction almost recovered from the protamine-induced inhibition, whereas the ACh-induced relaxant response did not completely recover from the inhibition (fig. 9). Although we

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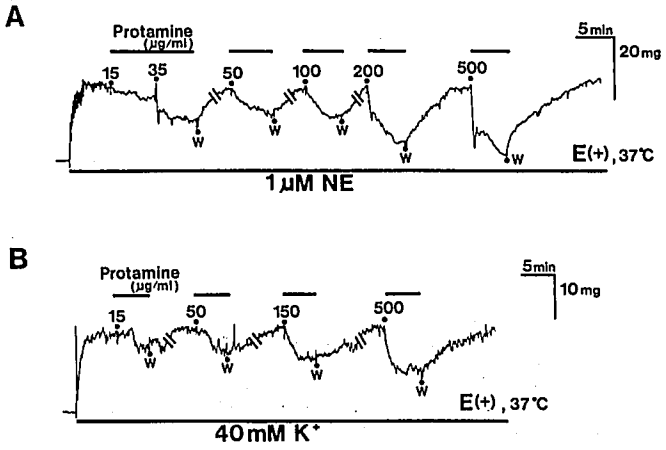

FIG. 8. Protamine-induced inhibitions on norepinephrine (NE) (1 μM)- or 40 mM K^+-induced contractions in endothelium-intact (E(+)) strips at 37° C. Protamine (15–500 μg/ml) was applied for about 5 min after each contraction reached a steady state. Identical results were obtained in the two other strips. W = washout.

further examined its recovery from the inhibition until 255 min after washout, there was no additional sign for the recovery (data not shown).

EFFECTS OF PROTAMINE ON THE MEMBRANE POTENTIAL

Protamine did not significantly affect the resting membrane potential of the smooth muscle cells in either the E(+) or the E(−) strips (table 2).

Discussion

ENDOTHELIUM DEPENDENCE OF PROTAMINE-INDUCED INHIBITION

Protamine inhibited the NE- or high K^+-induced contractions both in E(+) and in E(−) strips in a concentration- and time-dependent manner. The persisting but decreased efficacy after endothelial removal of the protamine-induced inhibition suggests that its inhibition has both an endothelium-dependent and and an endothelium-independent component. In contrast, recent reports[11,12] have demonstrated that endothelial removal abolished all vascular relaxation induced by arginine-rich basic polypeptides. Therefore, the endothelium-dependent component of the protamine-induced inhibition might be due to the common characteristic of an arginine-rich basic polypeptide, whereas its endothelium-independent component might be due to some characteristics of protamine.

MECHANISMS OF PROTAMINE-INDUCED ENDOTHELIUM-DEPENDENT INHIBITION

The endothelium-dependent inhibitory action of protamine on vascular smooth muscle is presumably mediated through endothelium-derived relaxing factor (EDRF),[14] which has recently been identified as nitric oxide or its related compounds.[33,34] This view is supported by the following evidence. Oxyhemoglobin, known to bind and inactivate EDRF[24]; methylene blue, an inhibitor of soluble guanylate cyclase activation[24]; and L-NNA, a potent inhibitor of cytosolic nitric oxide synthesis from L-arginine,[25] all significantly decreased the protamine-induced

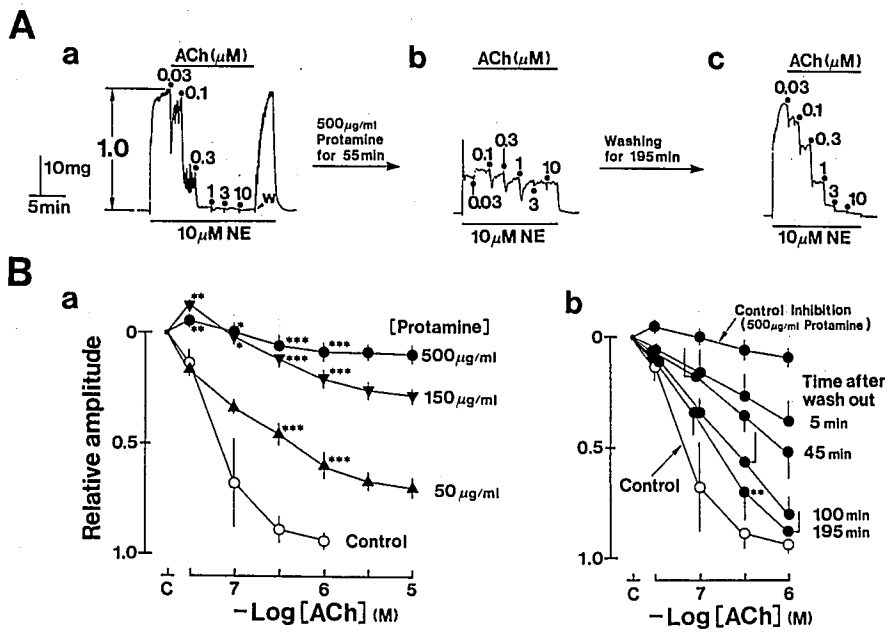

FIG. 9. Effects of protamine (50–500 μg/ml) on acetylcholine (ACh)-induced, endothelium-dependent relaxant response in the presence of 10 μM norepinephrine (NE) in endothelium-intact strips. *Aa*: The ACh (0.03–10 μM)-induced relaxant response before application of protamine (control). *Ab*: The ACh-induced relaxant response after application of protamine (500 μg/ml, 55 min). *Ac*: The ACh-induced relaxant response 195 min after washout of protamine. *Ba*: Concentration-dependent effects of protamine on the ACh-induced relaxant response. The amplitude of tonic component of NE-induced contraction before application of protamine was normalized as 1.0 (see "Calculation and Statistic Analysis" in text). *Bb*: Time course (5–195 min) of the recovery of the ACh-induced relaxant response from the protamine (500 μg/ml)-induced inhibition. W = washout. *Significantly different from the control ($P < 0.05$). **Significantly different from the control ($P < 0.01$). ***Significantly different from the control ($P < 0.001$).

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TABLE 2. Effects of Protamine on the Resting Membrane Potential (mV) in the Endothelium-intact and -denuded Strips

	Control	Protamine (μg/ml)		
		50	150	500
E(+)	−71.0 ± 0.9	−72.7 ± 1.6*	−72.7 ± 2.1*	−69.3 ± 2.1*
E(−)	−70.0 ± 2.6	−71.1 ± 1.2*	−70.5 ± 1.1*	−72.0 ± 1.3*

All values are means ± SD (n=6–8, three preparations).
E(+) = endothelium-intact; E(−) = endothelium-denuded.
* Not significantly different from the control.

inhibition in the E(+) strips, and these effects were similar to those of endothelium removal. The effects of endothelium removal and oxyhemoglobin were less effective on the protamine-induced inhibition in the presence of high K^+ than in the presence of NE. These findings may be consistent with well-known observations that EDRF-induced relaxant responses are greatly reduced in K^+-depolarized vascular preparations.[11] Moreover, superoxide dismutase, known to enhance the action of EDRF by preventing its inactivation,[35] markedly enhanced the protamine-induced inhibition in the E(+) strips. In contrast, indomethacin did not modify the protamine-induced inhibition, suggesting that cyclooxygenase products are not involved. Furthermore, protamine did not affect the membrane potential in the E(+) strip, suggesting that the endothelium-derived hyperpolarizing factor[15] also is not involved.

The precise mechanism by which protamine causes EDRF-mediated inhibition is currently unknown. In the present study, the development of its endothelium-dependent inhibition was much slower than that of relaxation induced by ACh, which interacts with endothelial cell membrane receptor and stimulates the endothelial cell to release EDRF. Therefore, it is unlikely that the protamine-induced endothelium-dependent inhibition is mediated by some selective membrane receptor.

There seem to be three possibilities regarding the mechanisms by which protamine exerts its EDRF-mediated inhibition. First, since nearly 67% of the amino acid composition of protamine is arginine[1] and since arginine has been postulated as the precursor for nitric oxide synthesis,[33] it is tempting to hypothesize that protamine provides the endothelial cell with the arginine required for nitric oxide synthesis. If protamine can enter into living cells, as has been previously reported,[27] it may be possible that it serves as a partial or alternative substrate for an arginine–nitric oxide converting enzyme system, just as Ignarro *et al.*[11] postulated for arginine-rich basic polypeptides. The slow development of its inhibition might reflect the diffusion time for protamine to enter into cells. Conversely, if protamine cannot enter into endothelial cells, some protamine-splitting system should be necessary for protamine to provide the arginine to endothelial cells. However, to our knowledge, it is still unknown whether such a protamine-splitting system exists in the plasma membrane or in the cytoplasm of endothelial cells, although it has been reported[36] that the breakdown of protamine by protaminase in plasma leads to the liberation of arginine. This speculation needs to be investigated further.

Second, Young *et al.* reported that eosinophil "cationic" protein damages the cell membrane and makes it leaky to various substances including divalent ions (Ca^{2+} and Mg^{2+}).[29] Further, several investigators[28–30] showed that various "polycationic" compounds, including protamine, damage the functional and structural integrity of endothelium. In our study, protamine irreversibly impaired the ACh-induced endothelium-dependent relaxant responses, and a high concentration of protamine caused the small contractions previously observed by Chang *et al.*[37] Since only the removal of the extracellular Ca^{2+} could block the protamine-induced contraction, this contraction is mediated probably through Ca^{2+} influxes. Considering the above observations both of the previous and present studies, this Ca^{2+} influx–mediated contraction may reflect the damage of the vascular smooth muscle cell membrane. If this were the case, protamine may evoke the Ca^{2+} influxes also into the endothelial cell by damaging the endothelial cell membrane, and the resultant increase in free cytosolic Ca^{2+} in a endothelial cell may lead to the release of EDRF.

Third, protamine may increase the sensitivity of the vascular smooth muscle to the relaxant effects of basal EDRF.

MECHANISMS OF PROTAMINE-INDUCED ENDOTHELIUM-INDEPENDENT INHIBITION

The results of the membrane-permeabilized muscle experiments and also the lack of the inhibition of protamine on the caffeine-induced contraction in the intact strips suggest that even if protamine can enter into the cell as previously reported,[27] protamine will not affect the Ca^{2+} sensitivity of the contractile-protein system. However, we speculate that protamine may not enter into the smooth muscle cell, for the following reasons. 1) Palade[38] showed in experiments with "isolated" sarcoplasmic reticulum that protamine inhibits the caffeine-induced Ca^{2+}

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release from sarcoplasmic reticulum, suggesting that if protamine can enter into the cell, it will inhibit the caffeine-induced Ca^{2+} release; no such inhibition, however, was observed. 2) If protamine can enter into the cell, it may inhibit the rate of rise of contraction and the rate of its relaxation by binding with adenosine triphosphate, just as observed in the membrane-permeabilized muscle experiments. (Both processes are known to depend greatly on the concentration of adenosine triphosphate.[22]) Such inhibition was not observed in intact strips. 3) It might be difficult for protamine to enter into the cell because of its high molecular weight. Accordingly, we speculate that protamine exerts its endothelium-independent inhibition by acting on smooth muscle cell membrane and by causing a change in intracellular Ca^{2+} mobilization.

In the rabbit mesenteric artery, NE produces a phasic contraction as a result of the Ca^{2+} release from the intracellular store, whereas the tonic contraction probably results from an interplay between increases in Ca^{2+} influx and Ca^{2+} release from the stores.[16,17] Protamine inhibited both components of NE-induced contraction in the E(−) strips, suggesting that protamine inhibits both Ca^{2+} influx and Ca^{2+} release from the intracellular stores. This view is further supported by the following evidence obtained in the E(−) strips: 1) protamine inhibited the NE-induced contraction evoked in Ca^{2+}-free (2 mM EGTA) solution; 2) protamine inhibited the contractions that depend on voltage-dependent Ca^{2+} influx, *i.e.*, high K^+-induced contraction evoked in Krebs solution and the Ca^{2+}-induced contraction evoked in Ca^{2+}-free solution containing high K^+; 3) in nifedipine (a potent voltage-dependent Ca^{2+} channel blocker)-treated strips, protamine still inhibited both components of NE-induced contraction; and 4) after depletion of the intracellularly stored Ca^{2+} by A23187 or ryanodine, protamine markedly inhibited the NE-induced contractions.

The effects of protamine on the contractions evoked by various concentrations of K^+ and on the membrane potential in the E(−) strips suggest that the protamine-induced inhibition of the Ca^{2+} influxes is not mediated through K^+ channel opening.[31,32]

It is known that the majority of extracellular Ca^{2+} in smooth muscle exists not as free calcium ion (Ca^{2+}) but rather is bound to the abundant anionic sites on the cell surface: the carboxyl groups of sialic acid in the outer glycoprotein layer of the cell membrane have been proposed as the anionic sites.[39–42] Ishiyama *et al.* showed in experiments with neuraminidase that the interaction between Ca^{2+} and the sialic acid residue may be the first reaction in the process of Ca^{2+} influx.[41] Furthermore, it is known[43] that on the surface membrane of vascular smooth muscles there are two kinds of Ca^{2+} binding sites, which are believed to be related to the Ca^{2+} influx—a low-affinity Ca^{2+} binding site and a high-affinity Ca^{2+} binding site. The voltage-dependent Ca^{2+} influx is believed to depend on the low-affinity bound Ca^{2+}, which is rapidly removed in Ca^{2+}-depleted solution, whereas the receptor-operated Ca^{2+} influx is believed to depend on the high-affinity bound Ca^{2+}, which is not easily removed in Ca^{2+}-depleted solution but is removed by EGTA and La^{3+}. Uruno *et al.* showed[13] in experiments with ^{45}Ca that protamine may replace the superficial low-affinity bound Ca^{2+} but not high-affinity bound Ca^{2+}. Their proposal may thereby explain the protamine-induced inhibition on voltage-dependent Ca^{2+} influx observed in the present study but will not explain its inhibition on the receptor-operated Ca^{2+} influx. The extremely slow recovery from the protamine-induced inhibition after washout may reflect the tight binding of protamine with a superficial Ca^{2+} binding site.

It was recently reported that in smooth muscle cells, ryanodine locks the Ca^{2+}-induced Ca^{2+}-release channel open but has no effect on inositol 1,4,5,-triphosphate (IP_3)-induced Ca^{2+}-release mechanism.[26] In the present study, the ryanodine treatment strongly inhibited the NE-induced contraction in Ca^{2+}-free solution, thereby suggesting that in this tissue, most of the NE-sensitive intracellular Ca^{2+} stores have the Ca^{2+}-induced Ca^{2+}-release channel, *i.e.*, that most of these stores are caffeine-sensitive. Thus, the lack of the inhibition of protamine on the caffeine-induced contraction suggests that the amount of Ca^{2+} of the NE-sensitive intracellular store was not reduced by protamine. Accordingly, the protamine-induced inhibition on the NE (IP_3[44])-induced Ca^{2+} release cannot be explained by a reduced amount of stored Ca^{2+}.

It is now recognized that the inositol-containing phospholipids in the plasmalemma serve as precursors for two important second messengers: IP_3 and 1,2-diacyl-glycerol. In this tissue, the NE-induced Ca^{2+} release from the intracellular store is mediated by IP_3[44]. Some polycationic substances (neomycin, spermine, and poly-L-lysine) have been proposed to bind with phosphatidyl-inositol 4,5-bisphosphate (PIP_2).[45,46] Furthermore, neomycin has been reported to inhibit IP_3-induced Ca^{2+} release by binding with both IP_3 and ATP.[46] Such binding of polycationic substances to anionic PIP_2 or IP_3 is believed to be due to the formation of ionic complexes between them.[45,46] Since protamine is also a polycationic substance, it might be possible that protamine behaves similarly to the above-mentioned polycationic substances: protamine may inhibit the NE-induced Ca^{2+} release by binding with PIP_2 or IP_3. Since, as discussed above, it is unlikely that protamine enters into the cell, protamine may exert its inhibition on the NE-induced Ca^{2+} release by the former mechanism: protamine may inhibit the generation of IP_3 by binding with PIP_2 and by inhibiting phospholipase C-mediated PIP_2 breakdown. Recently it was reported that in some cells, some Ca^{2+} influxes are also mediated by IP_3 and

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IP_4.[47,48] If this tissue also has such second messenger-operated Ca^{2+} channels, to our knowledge never previously reported, it is possible that protamine inhibits such Ca^{2+} influx by binding with PIP_2 and inhibiting the PIP_2 breakdown, in addition to inhibiting the NE-induced Ca^{2+}-release.

Clinical Relevance

As early as 1900, protamine-induced systemic hypotension was first reported in dogs.[49] Later, many investigators compared the cardiovascular effects of protamine in the presence of heparin with those in its absence,[1] and most reported[3,50,51] that protamine (2–3 mg/kg) itself did not cause any hemodynamic effect in the absence of heparin in dogs, pigs, and sheep. However, our results imply the possibility that protamine itself causes hypotension by its direct action on vascular endothelium and smooth muscles, because 1) a plasma concentration of 50 μg/ml was normally achieved after the intravenous administration of 0.75 mg/kg protamine,[4] and 2) the experiment using tritium-labeled protamine showed that it disappeared from blood with a half-life of 2 min after intravenous administration, but that 30% of its radioactivity remained in the blood even 2 h after injection.[52]

Furthermore, it is possible that the inhibitory action of protamine alone on vascular tissues as observed in our study may play some role in its hypotensive effect during heparin reversal. In other words, it is conceivable that protamine can act on the vascular tree not only as a heparin–protamine complex but also as "free" protamine during the heparin reversal, for the following reasons. 1) Since heparin remains complexed only in the presence of an "excess" of protamine, by mass action,[53] even during the "adequate" reversal of heparin some part of protamine may exist in the blood without forming the complex and act as "free" protamine on the vascular tree. 2) Since there are marked (3- or 4-fold) individual variations both in the sensitivity to heparin and in the rate of its clearance,[54] it is possible that the concentration of "free" protamine accidentally increases during heparin reversal. 3) In the case of rapid injection of protamine, it is possible that protamine acts on vascular tissues as "free" protamine before it binds with heparin. 4) The onset of protamine-induced inhibition at 37° C might be consistent with the onset of protamine-induced hypotension *in vivo.*

However, it is still uncertain how much excess free protamine exists in the blood during the heparin reversal, and furthermore, it might be difficult for protamine to act on vascular smooth muscles directly *in vivo* as it does *in vitro,* because the endothelium may serve as the barrier *in vivo:* only the effect of protamine on the endothelium may appear *in vivo.* It may also be possible that protamine prefers to bind with some other anionic substances or with an anioic surface of another kind of cells rather than that of vascular tissue cells.

Nevertheless, considering our data, we may recommend that in order to minimize the inhibitory action of protamine on vascular tissues, heparin anticoagulation should be reversed with the minimum possible amount of protamine and that protamine should be administered slowly as has been generally recommended.[1,55] However, our recommendation regarding the amount of protamine is opposed to the recent recommendation: Shanberge *et al.*[53] suggested that a moderate "excess" (at least 1.5/1 [w/w]) of protamine should be used in the neutralization of heparin in order to prevent the heparin rebound, because excess free protamine is necessary for the stability of the heparin–protamine complexes.

Some investigators[56,57] have suggested that administration of $CaCl_2$ and volume infusion were effective in counteracting protamine-induced hypotensive effects. Such effectiveness of $CaCl_2$ might be explained partly by the present finding that the protamine-induced inhibition on voltage-dependent Ca^{2+} influxes is counteracted by increasing the extracellular Ca^{2+} concentration.

The inhibitory effect of protamine on the ACh-induced endothelium-dependent relaxation in the present study was quite similar to the previous observation in pulmonary artery,[37] and its irreversibility may suggest the toxic effect of protamine on the endothelium, which has been proposed in the previous study.[28,37] This injurious effect of protamine could be attenuated by heparin in the previous studies.[37] Therefore, it might be that protamine should not be administered *via* the central vein or pulmonary artery catheter, because such administration will enable free protamine to act on the endothelium of pulmonary vascular beds in a higher concentration than with peripheral administration.

The above speculations await further investigation, particularly regarding the amount of excess "free" protamine in the blood during heparin reversal and regarding the direct action of heparin–protamine complexes on vascular tissues: the latter is now under investigation. Furthermore, our data suggest the importance of the determination of the amount of protamine required for the precise reversal of heparin.

Conclusion

Protamine itself has an inhibitory action on vascular smooth muscle, and its inhibition may consist of two components—an endothelium-dependent and an endothelium-independent component. Its endothelium-dependent component is probably mediated through EDRF,[14] whereas its endothelium-independent component is attributed to the inhibition of Ca^{2+} influx and the NE-induced Ca^{2+}-release from intracellular stores. These ac-

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tions of protamine on vascular tissues may partly explain its adverse hemodynamic effects during heparin reversal.

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References

1. Horrow JC: Protamine: A review of its toxicity. Anesth Analg 64: 348–361, 1985
2. Lowenstein E, Zapol WM: Protamine reactions, explosive mediator release, and pulmonary vasoconstriction (editorial views). ANESTHESIOLOGY 73:373–375, 1990
3. Hobbhahn J, Conzen PF, Zenker B, Goetz AE, Peter K, Brendel W: Beneficial effect of cyclooxygenase inhibition on adverse hemodynamic responses after protamine. Anesth Analg 67:253–260, 1988
4. Lindblad B, Wakefield TW, Whitehouse WM, Stanley JC: The effect of protamine sulfate on platelet function. Scand J Thorac Cardiovasc Surg 22:55–59, 1988
5. Wakefield TW, Till GO, Lindblad B, Saenz N, Stanley JC: Complement depletion and persistent hemodynamic-hematologic responses in protamine-heparin reactions. J Surg Res 45:320–326, 1988
6. Morel DR, Zapol WM, Thomas SJ, Kitain EM, Robinson DR, Moss J, Chenoweth DE, Lowenstein E: C5a and thromboxane generation associated with pulmonary vaso- and broncho-constriction during protamine reversal of heparin. ANESTHESIOLOGY 66:597–604, 1987
7. Hines RL, Barash PG: Protamine: Does it alter right ventricular function? Anesth Analg 65:1271–1274, 1986
8. Lowenstein E, Johnston WE, Lappas DG, D'Ambra MN, Schneider RC, Dagget WM, Akins CW, Philbin DM: Catastrophic pulmonary vasoconstriction associated with protamine reversal of heparin. ANESTHESIOLOGY 59:470–473, 1983
9. Taylor RL, Little WC, Freeman GL, Avery DM, Norris SE, Trinkle JK, Grover FL: Comparison of the cardiovascular effects of intravenous and intraaortic protamine in the conscious and anesthetized dog. Ann Thorac Surg 42:22–26, 1986
10. Colman RW: Humoral mediators of catastrophic reactions associated with protamine neutralization (editorial views). ANESTHESIOLOGY 66:595–596, 1987
11. Ignarro LJ, Gold ME, Buga GM, Byrns RE, Wood KS, Chaudhuri G, Frank G: Basic polyamino acids rich in arginine, lysin, ornithine cause both enhancement of and refractoriness to formation of endothelium-derived nitric oxide in pulmonary artery and vein. Circ Res 64:315–329, 1989
12. Rapoport RM, Ashraf M, Murad F: Effects of melittin on endothelium-dependent relaxation and cyclic GMP levels in rat aorta. Circ Res 64:463–473, 1989
13. Uruno T, Matsumoto R, Okushita K, Sunagane N, Kubota K: Possible mechanisms of inhibitory action of protamine on contractile activity of rat aorta. J Pharm Pharmacol 37:476–480, 1985
14. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376, 1980
15. Suzuki H, Chen G: Endothelium-derived hyperpolarizing factor (EDHF): An endogeneous potassium-channel activator. News in Physiological Sciences (Baltimore) 5:212–215, 1990
16. Itoh T, Kanmura Y, Kuriyama H: A23187 increases calcium permeability of store sites than of surface membranes in the rabbit mesenteric artery. J Physiol (Lond) 359:467–484, 1985
17. Itoh T, Kuriyama H, Suzuki H: Differences and similarities in the noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. J Physiol (Lond) 337:609–629, 1983
18. Kanmura Y, Itoh T, Kuriyama H: Mechanisms of vasoconstriction induced by 9,11-epithio-11,12-methano-thromboxane A_2 in the rabbit coronary artery. Circ Res 60:402–409, 1987
19. Itoh T, Satoh S, Ishimatsu T, Fujiwara T, Kanmura Y: Mechanisms of flunarizine-induced vasodilation in the rabbit mesenteric artery. Circ Res 61:446–454, 1987
20. Kobayashi S, Kitazawa T, Somlyo AV, Somlyo AP: Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. J Biol Chem 264:17997–18004, 1989
21. Hsu SM, Raine L, Fanger H: Use of avidin-biodin-peroxidase complex (ABC) in immunoperoxidase technique: A comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 29:577–580, 1981
22. Itoh T, Kanmura Y, Kuriyama H: Inorganic phosphate regulates the contration-relaxation cycle in skinned muscles of the rabbit mesenteric artery. J Physiol (Lond) 376:231–252, 1986
23. Kanmura Y, Itoh T, Suzuki H, Itoh Y, Kuriyama H: Effects of nifedipine on smooth muscle cells of the rabbit mesenteric artery. J Pharmacol Exp Ther 226:238–248, 1983
24. Martin W, Villani GM, Jothianandan D, Furchgott RF: Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J Pharmacol Exp Ther 232:708–716, 1985
25. Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, Herman AG: Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345:346–347, 1990
26. Iino M, Kobayashi T, Endo M: Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. Biochem Biophys Res Commun 152:417–422, 1988
27. De Robertis E, De Robertis EM: The cell membrane and permeability: Intercellular interactions, Cell and Molecular Biology. 7th edition. Philadelphia, WB Saunders Company, 1980, pp 156
28. Chang SW, Voelkel NF: Charge-related lung microvascular injury. Am Rev Respir Dis 139:534–545, 1989
29. Young JD-E, Peterson CGB, Venge P, Cohn ZA: Mechanism of membrane damage mediated by human eosinophil cationic protein. Nature 321:613–615, 1986
30. Wakefield TW, Hinshaw DB, Burger JM, Burkel WE, Stanley JC: Protamine-induced reductions of endothelial cell ATP. Surgery 106:378–385, 1989
31. Standen NS, Quayle JM, Davis W, Brayden JE, Huank Y, Nelson MT: Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. Science 245:177–180, 1989
32. Nelson MT, Huang Y, Brayden JE, Hescheler J, Standen NB: Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. Nature 344:770–773, 1990
33. Palmer RMJ, Ashton DS, Moncada S: Vascular endothelial cells synthesized nitric oxide from L-arginine. Nature 333:664–666, 1988

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34. Myers PR, Minor Jr LM, Guerra Jr R, Bates JN, Harrison DG: Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocyteine than nitric oxide. Nature 345:161–163, 1990
35. Glyglewski RJ, Palmer RMJ, Moncada S: Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 320:454–456, 1986
36. Weiss M, Blass H, Detruit H, Aime L, Baudet B, Fiancette JY, Gaillard MB, Vicaigne MB, Verriest Ch: Determination of "in vitro" degradation of protamine in plasma by three different methods. Thromb Res 41:593–604, 1986
37. Chang SW, Westcott JY, Henson JE, Voelkel NF: Pulmonary vascular injury by polycations in perfused rat lungs. J Appl Physiol 62:1932–1943, 1987
38. Palade P: Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum. J Biol Chem 262:6149–6154, 1987
39. Villamil M, Rettori V, Yeyatil N: Calcium exchange and distribution in the arterial wall. Am J Physiol 224:1314–1319, 1973
40. Loutzenhiser R, Breemen C van: Involvement of extracellularly bound calcium in the activation of arterial smooth muscle. Blood Vessels 20:295–305, 1983
41. Ishiyama Y, Yabu H, Miyazaki E: Changes in contractility and calcium binding of guinea pig *taenia coli* by treatment with enzymes which hydrolyze sialic acid. Jpn J Physiol 25:719–732, 1975
42. Quinton PM, Philpott CW: A role for anionic sites in epithelial architecture. J Cell Biol 56:787–796, 1973
43. Karaki H, Weiss GB: Calcium channels in smooth muscle. Gastroenterology 87:960–970, 1984
44. Hashimoto T, Hirata M, Itoh T, Kanmura Y, Kuriyama H: Inositol 1,4,5-triphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. J Physiol (Lond) 370:605–618, 1986
45. Schacht J: Inhibition by neomycin of polyphosphoinositide turnover in subcellular fractions of guinea-pig cerebral cortex in vitro. J Neurochem 27:1119–1124, 1976
46. Prentki M, Deeney JT, Matschinsky FM, Joseph SK: Neomycin: a specific drug to study the inositol-phospholipid signalling system? FEBS Lett 197:285–288, 1986
47. Morris AP, Gallacher DV, Irvine RF, Peterson OH: Synergism of inositol triphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channel. Nature 330:653–655, 1987
48. Minneman KP: α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. Pharmacol Rev 40:87–119, 1988
49. Thompson WH: Hoppe-Seyl.Z., 29, 1, 1900. In Jaques LB: A study of the toxity of the protamine, salmine. Br J Pharmacol 4:135–144, 1949
50. Fiser WP, Fewell JE, Hill DE, Barnes RW, Read RC: Cardiovascular effects of protamine sulfate are dependent on the presence and type of circulating heparin. J Thorac Cardiovasc Surg 89: 63–70, 1989
51. Morel DR, Mo Costabella PM, Pittet J-F: Adverse cardiopulmonary effects of increased plasma concentrations following the neutralization of heparin with protamine in awake sheep are infusion rate-dependent. ANESTHESIOLOGY 73:415–424, 1990
52. Frimmer M: Über die Verteilung von Tritium-markiertem Protamin im Säugetier-organismus. Med Exp 9:168–172, 1963
53. Shanberge JN, Murato M, Quattrociocchi-Longe T, Neste LV: Heparin-protamine complexes in the production of heparin rebound and other complications of extracorporeal bypass procedures. Am J Clin Pathol 87:210–217, 1987
54. Bull BS, Huse WM, Brauer FS, Korpman RA, Linda L: Heparin therapy during extracorporeal circulation. J Thorac Cardiovasc Surg 69:674–89, 1975
55. O'Reilly RA: Anticoagulant, antithrombotic, and thrombolytic drugs, Goodman and Gilman's The Pharmacological Basis of Therapeutics. 7th edition. Edited by Gilman GA, Goodman LS, Rall TW, Murad F. New York, Macmillan Publishing Company, 1985, p 1344
56. Gourin A, Streisand RL, Greinder JK, Stuckey JH: Protamine sulfate administration and the cardiovascular system. J Thorac Cardiovasc Surg 62:193–204, 1971
57. Fadari MA, Ledbetter M, Papacostas CA, Duke LF, Lemole GM: Mechanism responsible for the cardiovascular depressant effect of protamine sulfate. Ann Surg 180:232–235, 1974

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