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Protamine relaxes vascular smooth muscle by directly reducing cytosolic free calcium concentrations in small resistance arteries*

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Abstract: Protamine has been suggested to relax vascular smooth muscle by reducing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, there has been no direct evidence that protamine reduces the $[Ca^{2+}]_i$ of vascular smooth muscle. We therefore studied the effects of protamine on changes in $[Ca^{2+}]_i$ and tension induced by norepinephrine (NE) and high K^+ in endothelium-denuded strips from rabbit small mesenteric artery, using fura-2-fluorometry and isometric tension recording methods. Both NE (1 μM) and high K^+ (40 mM) produced a transient phasic increase, followed by a tonic increase in $[Ca^{2+}]_i$ and tension. Protamine concentration (15–500 $\mu g \cdot ml^{-1}$)-dependently inhibited ($P < 0.05$) the phasic and tonic components of both NE- and high K^+-induced contraction with IC_{50} values of ≈50 $\mu g \cdot ml^{-1}$. Protamine (50 $\mu g \cdot ml^{-1}$) inhibited ($P < 0.05$) the phasic and tonic increases in $[Ca^{2+}]_i$ caused by both NE and high K^+ by ≈40%–60%. We conclude that the direct vasodilator action of protamine is due, at least in part, to reduction of $[Ca^{2+}]_i$ in vascular smooth muscle; this reduction in $[Ca^{2+}]_i$ may be due to inhibition of both Ca^{2+} influx and Ca^{2+} release from intracellular Ca^{2+} stores.

Key words: Protamine, Vascular smooth muscle, Artery, Resistance, Intracellular calcium concentration, Fura-2-fluorometry

Introduction

Protamine is known to cause various adverse cardiovascular responses during reversal of heparin-induced anticoagulation such as systemic hypotension, pulmonary hypertension, and bradycardia [1–3]. The precise mechanisms underlying these cardiovascular effects of protamine have not been fully elucidated, and heterogeneity in their etiology has been suggested [1]. In addition to anaphylactoid reaction to protamine or release of some humoral vasoactive mediators such as prostanoids, direct action of protamine on vascular tissues may also be involved in its adverse cardiovascular effects [1–7].

We have recently reported that protamine has a vasodilator action in isolated human and rabbit small splanchnic resistance arteries [4–7]. In these arteries, protamine inhibited both norepinephrine (NE)- and high K^+-induced contractions either in the presence or absence of endothelium [4–7]. The effects of endothelium-derived relaxing factor (EDRF) pathway inhibitors on vascular responses to protamine have suggested the existence of an EDRF-mediated component in its vasodilating action [4,5]. The lack of effects of protamine on Ca^{2+}-activated contractions in β-escin-membrane-permeabilized muscle suggested that protamine does not affect the Ca^{2+}-calmodulin dependent activation of contractile proteins even if it enters the cell [4]. In addition, heparin (a "polyanionic" substance), applied on washout of protamine, dramatically facilitated "complete" recovery of vascular smooth muscle from protamine (a "polycationic" substance)-induced prolonged inhibition in the presence of either NE or high K^+ [5]. Since heparin is membrane-impermeable, this finding indicates that protamine does not have any significant intracellular

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effect on vascular smooth muscle, also suggesting that protamine does not affect the Ca^{2+} activation of contractile proteins. Therefore, protamine presumably exerts its direct vasodilator action by acting on the cell membrane rather than by directly acting on intracellular contractile proteins [5]. Protamine probably inhibits the high K^+-induced contraction by reducing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle, possibly as a result of inhibition of voltage-gated Ca^{2+} influx [4,5]. Since activation of voltage-gated Ca^{2+} channels is known to be involved in the contractile response to NE [4,8,9], the protamine-induced inhibition of NE contraction is also probably due, at least in part, to a $[Ca^{2+}]_i$-reducing effect as a result of inhibition of voltage-gated Ca^{2+} influx. However, an effect on Ca^{2+} sensitivity of contractile proteins may also be involved in the protamine-induced inhibition of NE contraction, since the NE-induced increase in Ca^{2+} sensitivity could be affected by protamine even if it does not enter the cell [6].

Until now, there has been no direct evidence that protamine reduces the $[Ca^{2+}]_i$ of vascular smooth muscle. In this study, using fura-2-fluorometry and isometric tension recording methods, the effects of protamine on changes in both $[Ca^{2+}]_i$ and tension induced by NE or high K^+ were studied in small splanchnic resistance arteries that have major effects on the regulation of systemic vascular resistance and thus of systemic arterial pressure. The present study represents the first description of the effects of protamine on $[Ca^{2+}]_i$ of vascular smooth muscle.

Materials and methods

Tissue preparation

After institutional approval for this animal study, male albino rabbits (2.0–2.5 kg) were given sodium pentobarbital (40 mg·kg^{-1} i.v.) and exsanguinated. The mesentery in the jejunal region was immediately placed in a dissecting chamber filled with preoxygenated Krebs-bicarbonate solution, and the mesenteric artery was rapidly excised. The distal portion of the third- or fourth-order branches (≈150–250 μm in diameter), which are known to contribute substantially to vascular resistance [10], were used for the present experiments. Under a binocular microscope, the fat and adhering connective tissues were carefully removed, and thin transverse strips (400–600 μm long and 100–120 μm wide) were prepared. In all experiments, the endothelium was removed by gently rubbing the intimal surface with a small pin utilizing its round surface, and the functional removal of endothelium was confirmed by the lack of acetylcholine (10 μM)-induced relaxation, as has previously been reported [4,5,11].

Tension and Ca^{2+} measurement

Mechanical responses were measured by attaching the strip to a strain gauge (UL-2, Shinko, Tokyo, Japan) in a chamber of 0.9 ml capacity as previously described [4–7,11]. Briefly, the strip was horizontally mounted in the chamber on a microscope stage, and then stretched to ≈1.1–1.2 times the resting length to obtain the maximum contractile response to high K^+. The solution was changed by perfusing it rapidly from one end while aspirating it simultaneously from the other end. Since we previously showed that vascular responses to protamine at 25°C are identical to those at 32°C or 37°C [4], all experiments were performed at 25°C to prevent early deterioration of the thin vascular strips. Although the tension-recording experiments were performed separately from the Ca^{2+} measurement experiments, the strips used for tension measurements examining the effects of protamine were also loaded with fura-2 in order to make the experimental conditions identical to those used in the Ca^{2+} measurement experiments. However, the strips used for tension-recording experiments characterizing NE or high K^+-induced contraction were not loaded with fura-2.

In the Ca^{2+} measurement experiments, both ends of the prepared strip were tied with thin silk threads under the microscope. The strip was then firmly fixed onto the quartz glass bottom plate of a chamber (0.6 ml volume) under the same isometric condition as that for the tension measurements (see above) utilizing a Scotch double-sided tape (3M, St. Paul, MN, USA). For loading of fura-2 into vascular smooth muscle cells, the strips were incubated in Krebs solution containing 1 μM acetoxy methyl ester of fura-2 (fura-2AM; 1 mM stock solution in dry dimethyl sulphoxide) for 1 h at room temperature as has been reported [12]. After this period, the solution containing fura-2AM was washed out with Krebs solution for 1 h to ensure sufficient esterification of fura-2AM in the cells. The position of the strip was adjusted to the center of the field with a mask placed in an intermediate image plane to reduce background fluorescence (0.04 mm^2). The fura-2 fluorescence emission at 510 nm using an interference filter (centered at 510 nm and full width at half transmission, 20 nm) was collected through the lens (20 times CF Fluor objective lens, Nikon, Tokyo, Japan) and collected in a photomultiplier tube (R928, side-in type, Hamamatsu Photonics, Hamamatsu, Japan) via a dichroic mirror (DM-400, Nikon) which was substituted for the photochanger in a Nikon Diaphoto-TMD microscope. Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm), were applied by a spectro-

fluorimeter (Spex, Edison, NJ, USA) and the data were analyzed using customized software provided by Spex (DM-3000CM).

The ratio of fura-2 fluorescence intensities excited by 340 nm (F_{340}) to those excited by 380 nm (F_{380}) was calculated after subtracting the background fluorescence. The background fluorescence (including autofluorescence of the strip) as excited by 340 nm and 380 nm u.v. light was measured by application of a solution containing 50 μM ionomycin, 20 mM $MnCl_2$, 110 mM KCl, and 10 mM 3-(N-morpholino) propanesulphonic acid (MOPS) after the experiment. Under these conditions, the background fluorescence (including the autofluorescence of strip) was 10%–15% of the fura-2 signals in smooth muscle strips at either excitation wavelength.

Solutions and drugs

The ionic concentrations of Krebs solution were as follows (mM): NaCl 111.9, KCl 3.7, $MgCl_2$ 1.2, $CaCl_2$ 2.6, $NaHCO_3$ 25.5, KH_3PO_4 1.2, glucose 11.4. The high-K^+ solution was prepared by replacing NaCl with KCl, isoosmotically. 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. The solution was bubbled with 95% O_2 / 5% CO_2, and its pH was adjusted to 7.3–7.4.

Norepinephrine HCl (NE), acetylcholine (ACh), and tetrodotoxin (TTX) were obtained from Sigma Chemical, St. Louis, Mo., USA. A23187 (free acid) and ryanodine were obtained from Calbiochem (San Diego, CA, USA). Protamine was obtained from Nacalai Tesque, Kyoto, Japan. Protamine was prepared in normal saline solution just before use. The concentration of protamine was expressed as $\mu g \cdot ml^{-1}$, because its molecular weight varied from 2 to 12 kDa. All other reagents were of the highest grade commercially available.

Experimental design

In the tension-recording experiments, both NE and high K^+ produced a transient phasic increase, followed by a tonic increase in tension. To characterize the phasic and tonic components of either NE or high K^+-induced contractions, we first studied the effects of removal of extracellular Ca^{2+} or the effects of functional removal of intracellular Ca^{2+} stores on these contractions. In experiments to examine the effects of removal of extracellular Ca^{2+}, the stimulant was applied ≈30 s after application of Ca^{2+}-free solution containing 2 mM EGTA. Functional removal of the intracellular Ca^{2+} stores was achieved by pretreating the strips with a low concentration of A23187 (0.1 μM, 30 min) or ryanodine (10 μM, 20 min; applied with caffeine) as previously reported [4]. We then studied the effects of protamine (15–500 $\mu g \cdot ml^{-1}$) on NE (1 μM)-and 40 mM K^+-induced contractions in the endothelium-denuded strips, and determined IC_{50} values for protamine-induced inhibition of both types of contractions. In the Ca^{2+} measurement experiments, we examined the effects of protamine on changes in $[Ca^{2+}]_i$ induced by NE (1 μM and 40 mM K^+, employing almost the same concentrations as the above-determined IC_{50} values. Constant vascular responses were observed for at least 12 h and for ≈2 h in the tension and Ca^{2+} measurement experiments, respectively. In both kinds of experiments, after recording the control responses to NE or high K^+, protamine was applied to the strips for a length of time sufficient for it to exert its maximal effects according to our previous findings in this artery [4,5]: 45 min for 15–50 $\mu g \cdot ml^{-1}$ and 15 min for 150–500 $\mu g \cdot ml^{-1}$. All the experiments with high K^+ were performed in the presence of 3 μM guanethidine and 0.3 μM TTX to minimize the influence of peripheral nerve activity.

Calculation and statistical analysis

Although absolute Ca^{2+} concentration could be calculated based on the fura-2 fluorescence ratio and the dissociation constant of fura-2 for Ca^{2+} binding [13], the dissociation constant of fura-2 for Ca^{2+} binding in cytoplasm has been suggested to be rather different (three- to four-fold increase) from that measured in the absence of protein because more than half of the fura-2 molecules in cytoplasm are protein-bound [14]. Therefore, we used the ratio of F_{340} to F_{380} ($R_{340/380}$) as an indicator of $[Ca^{2+}]_i$ [15–20].

Since both NE and high K^+ produced a transient phasic increase, followed by a tonic increase both in tension and $[Ca^{2+}]_i$, the protamine-induced inhibition of tension and $[Ca^{2+}]_i$ are expressed as percentage (%) changes from the corresponding values induced by NE or high K^+ before application of protamine. The tonic component of each contraction was assessed 2 min after application of each stimulant.

The data points in concentration–response relationships for protamine-induced inhibition were fitted according to a four-parameter logistic model described by De Lean et al. [21], and the IC_{50} values (the concentrations that produced 50% of the maximal inhibition) were derived from these fits.

All results are expressed as the means ± SEM; n denotes the number of animals (= the number of preparations). The statistical assessment of the data was made by an analysis of variance, Scheffé's F-test, and Student's t-test, where appropriate. A level of $P < 0.05$ was considered significant.

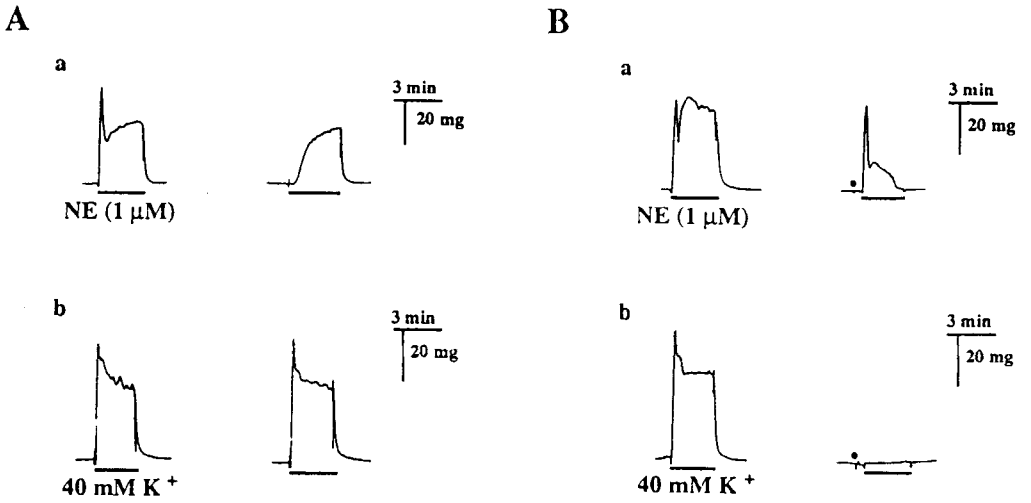

Fig. 1. Effects of functional removal of intracellular Ca^{2+} stores (**A**) and removal of extracellular Ca^{2+} (**B**) on norepinephrine (NE)- and high K^+-induced phasic and tonic contraction in endothelium-denuded strips. **A** Effects of A23187 (0.1 μM, 30 min) on the (**a**) NE- and (**b**) high K^+-induced contraction. Note that A23187 completely eliminated the phasic component of NE-induced contraction, while it had less effect on the tonic component of NE contraction or both components of high K^+ contraction. **B** Effects of removal of extracellular Ca^{2+} on the (**a**) NE- and (**b**) high K^+-induced contraction. Note that this intervention, which completely eliminated the high K^+-induced contraction, strongly inhibited the tonic component of NE-induced contraction, while it had little effect on the phasic component of NE contraction. The *small dots* indicate application of Ca^{2+}-free solution consisting of 2 mM ethyleneglycol-bis-(β-amino-ethylether)-N,N,N′,N′-tetraacetic acid (EGTA)

Results

Both NE and high K^+ produced a phasic increase, followed by a tonic increase in tension in the endothelium-denuded strips (Figs. 1, 2). Both caffeine (20 mM and NE (10 μM) failed to evoke any contractions in Ca^{2+}-free (2 mM EGTA) solution after exposure to A23187 (0.1 μM, 30 min), suggesting that the intracellular Ca^{2+} stores were completely depleted by this treatment. This depletion of intracellular Ca^{2+} stores by A23187 completely eliminated the initial phasic component of NE contraction, but had less effect on the tonic component of NE contraction (n = 4; Figure 1A, Table 1). Identical results were obtained after treatment with ryanodine (10 μM, 20 min) (n = 3, Table 1). In contrast, these interventions to deplete intracellular Ca^{2+} stores had no significant effect on either the phasic or tonic components of high K^+-induced contraction (n = 3–4, Table 1). On the other hand, removal of extracellular Ca^{2+} strongly inhibited the NE-induced tonic contraction, but had

Table 1. Effects of ryanodine, A23187, removal of extracellular Ca^{2+}, and nifedipine on either NE or high K^+-induced contraction

	NE (1 μM)		40 mM K^+	
	Phasic	Tonic	Phasic	Tonic
Ryanodine (10 μM)	8.5±6.7* (3)	86.8±19.2 (3)	105.1±6.6 (3)	95.9±2.7 (3)
A23187 (0.1 μM)	0.0±0.0* (4)	98.4±1.0* (4)	100.3±2.9 (4)	103.6±2.8 (4)
Removal of extracellular Ca^{2+}	93.1±3.4 (4)	13.9±3.5* (4)	0.0±0.0* (4)	0.0±0.0* (4)
Nifedipine (100 nM)	94.8±4.0 (4)	28.2±3.5* (4)	2.2±2.5* (4)	0.0±0.0* (4)

Values, expressed by mean ± SEM (n), are % of control values (values before each intervention).
NE = norepinephrine.
*$p < 0.05$ vs control (100%)

little effect on the NE-induced phasic contraction) ($n = 4$; Fig. 1B, Table 1). Similar results were also obtained after treatment with nifedipine (100 nM) ($n = 4$, Table 1). In addition, these interventions to inhibit Ca^{2+} influxes strongly inhibited both the phasic and tonic components of high K^+-induced contraction ($n = 4$, Table 1).

Protamine concentration (15–500 μg/ml)-dependently inhibited both the phasic and tonic components of either NE or high K^+ contraction in the endothelium-denuded strips (Fig. 2). The IC_{50} values for protamine inhibition of NE-induced phasic and tonic contractions were 52.9 ± 1.46 and 55.8 ± 9.21 μg·ml^{-1}, respectively ($n = 4$), while the IC_{50} values for protamine inhibition of high K^+-induced phasic and tonic contractions were 57.3 ± 1.92 and 42.9 ± 0.21 μg·ml^{-1}, respectively ($n = 3$–4). No significant differences were observed in the protamine-induced inhibition either between NE-induced phasic and tonic components or between high K^+-induced phasic and tonic components.

In the Ca^{2+} measurement experiments, both NE and high K^+ also produced a phasic increase, followed by a tonic increase in $[Ca^{2+}]_i$ in the endothelium-denuded strips (Fig. 3). Both NE- and high K^+-induced changes in F_{340} and F_{380} monitored in all experiments were constantly in opposite directions (not shown). Both phasic and tonic increases in $[Ca^{2+}]_i$ induced by high K^+ corresponded closely to the phasic and tonic components of high K^+-induced contraction, respectively (Fig. 2-a1 *vs* Fig. 3-a1). In contrast, the changes in $[Ca^{2+}]_i$ caused by NE did not appear to correspond exactly to the changes in tension caused by NE; although the phasic increase in $[Ca^{2+}]_i$ appeared to correspond well with the phasic increase in tension, the tonic increase in $[Ca^{2+}]_i$ appeared to be rather small as compared to the tonic increase in tension (Fig. 2-a2 *vs* Fig. 3-a2). This may imply an increase in Ca^{2+} sensitivity during development of the NE-induced tonic contraction. Protamine (50 μg/ml, ≈IC_{50} for protamine inhibition of either NE or high K^+ contraction) inhibited both the phasic and tonic increases in $[Ca^{2+}]_i$ induced by either NE or high K^+ by around 50% (Fig. 3).

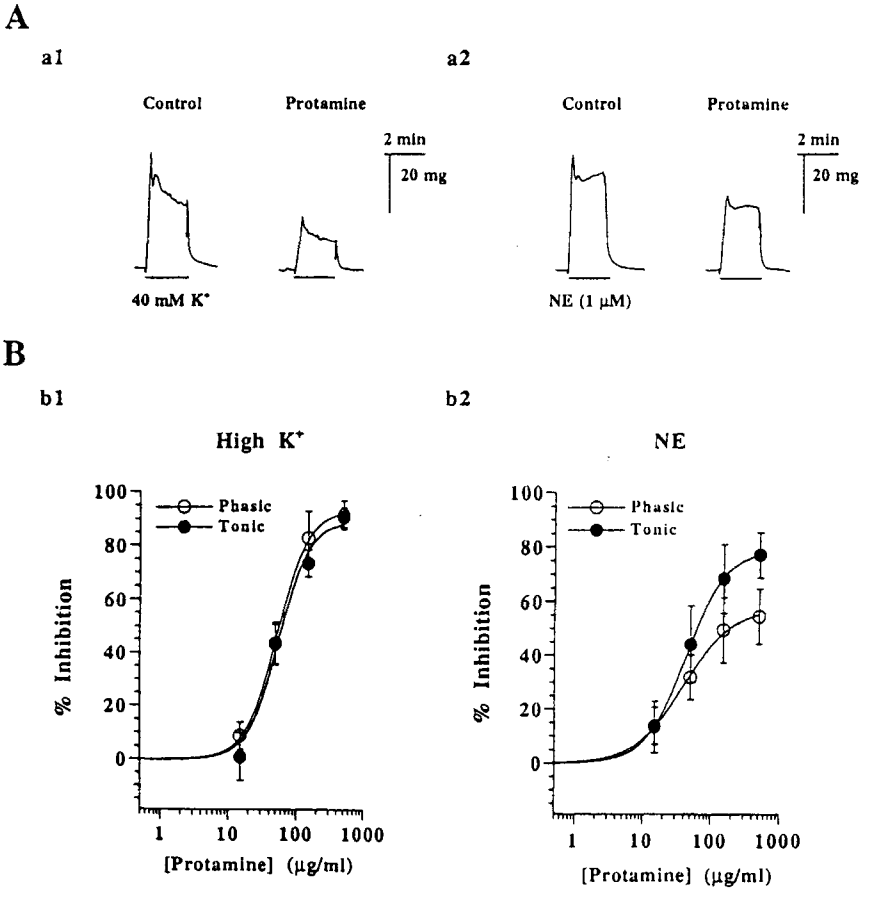

Fig. 2A,B. Effects of protamine (15–500 μg·ml^{-1}) on 40 mM K^+- or NE (1 μM)-induced contractions in endothelium-denuded strips, **A** Representative examples of protamine (50 μg·ml^{-1})-induced inhibition of (**a1**) high K^+- or (**a2**) NE-induced contraction. **B** The concentration–response relationship for protamine-induced inhibition of (**b1**) high K^+- or (**b2**) NE-induced contraction. Protamine (≥50 μg·ml^{-1}) significantly inhibited both high K^+- and NE-induced contractions. All values are displayed as mean ± SEM ($n = 3$–4)

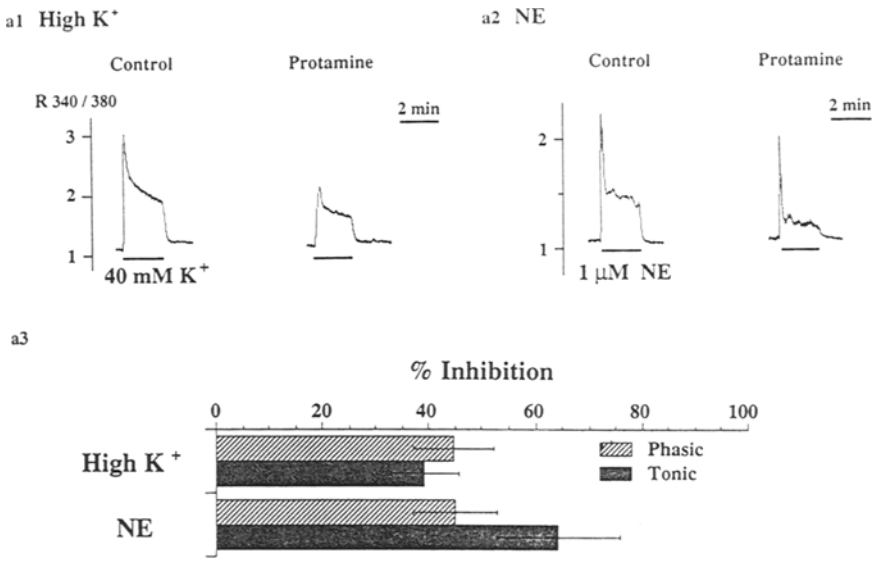

Fig. 3. Effects of protamine ($50\,\mu g \cdot ml^{-1}$) on increases in $[Ca^{2+}]_i$ ($R_{340/380}$) induced by 40 mM K^+ or NE ($1\,\mu M$) in endothelium-denuded strips. **a1**, **a2** Representative examples of protamine-induced inhibition of the increase in $[Ca^{2+}]_i$ ($R_{340/380}$) caused by either 40 mM K^+ or NE. **a3** Protamine-induced inhibition of both phasic and tonic increases in $[Ca^{2+}]_i$ ($R_{340/380}$) caused by 40 mM K^+ or NE. All values are displayed as mean ± SEM (n = 7–8)

Discussion

The present study confirms that protamine has a direct vasodilator action in small splanchnic resistance arteries and, for the first time, provides direct evidence that protamine reduces $[Ca^{2+}]_i$ of vascular smooth muscle. Protamine at a concentration of $50\,\mu g \cdot ml^{-1}$ ($\approx$ the IC_{50} for protamine-induced inhibition of either NE or high K^+ contraction) significantly inhibited the increases in $[Ca^{2+}]_i$ induced by either NE or high K^+ by ≈40%–60%. This indicates that an effect of reducing $[Ca^{2+}]_i$ is involved in protamine-induced inhibition of both NE and high K^+ contraction. Since the Ca^{2+} measurement experiments were performed separately from the tension-recording experiments, the present study does not directly address whether protamine affects the Ca^{2+} sensitivity of contractile proteins of vascular smooth muscle. However, we have previously shown in the same resistance arteries that protamine affects neither Ca^{2+}-activated contractions in membrane-permeabilized muscle nor caffeine-induced contractions in intact muscle [4]. In addition, we have recently shown that heparin, applied on washout of protamine, dramatically facilitates "complete" recovery of vascular smooth muscle from protamine-induced prolonged inhibitions [5]. Since heparin is believed not to enter the cell because of its high molecular weight and poor lipid solubility [22], the latter finding suggests that protamine does not have any significant intracellular effects [5]. These previous observations [4,5] indicate that protamine does not affect the Ca^{2+}-calmodulin-dependent activation of contractile proteins. Therefore, protamine probably inhibits high K^+-induced contraction largely by reducing $[Ca^{2+}]_i$, possibly by inhibiting voltage-gated Ca^{2+} influx. This idea is consistent with the present data that protamine reduces $[Ca^{2+}]_i$ of vascular smooth muscle in the presence of high K^+ as well as a previous proposal based on experiments with ^{45}Ca [23] that protamine may replace superficial low-affinity bound Ca^{2+} of vascular smooth muscle cells which is known to be essential for voltage-gated Ca^{2+} influx [24]. The present data further indicate that the protamine-induced inhibition of NE contraction is due, at least in part, to a $[Ca^{2+}]_i$ reducing effect. However, there still remains the possibility that an effect on Ca^{2+} sensitivity is also involved in protamine-induced inhibition of NE contraction, because the well-recognized increase in Ca^{2+} sensitivity in contractile responses to NE, which may be mediated through protein kinase C activation [25–29], could be affected by protamine even when it does not enter the cell [5]. However, this study failed to address this issue, since Ca^{2+} and tension were separately, but not simultaneously, measured. Further investigations simultaneously measuring Ca^{2+} and tension would be needed to resolve this question definitively.

The NE-induced phasic contraction was strongly inhibited by the pharmacological interventions to eliminate intracellular Ca^{2+} stores such as A23187 or ryanodine, but little affected by the interventions to block Ca^{2+} influx such as removal of extracellular Ca^{2+} or nifedipine treatment. In contrast, the NE-induced tonic contraction was strongly inhibited by the interventions to block Ca^{2+} influxes, but less affected by the above interventions to deplete intracellular Ca^{2+} stores. Therefore, the NE-induced phasic contraction is probably due mainly to Ca^{2+} release from intracellular Ca^{2+} stores, while the NE-induced tonic

contraction is presumably due to an interplay between Ca^{2+} influxes and Ca^{2+} release from intracellular Ca^{2+} stores as previously proposed [4]. Although the NE-induced phasic increase in $[Ca^{2+}]_i$ appeared to correspond well with the NE-induced phasic increase in tension (Figs. 2, 3), the NE-induced tonic increase in $[Ca^{2+}]_i$ appeared to be rather small as compared to the NE-induced tonic increase in tension (Figs. 2, 3), implying a role for increased Ca^{2+} sensitivity in the generation of NE-induced tonic contractions. This may be consistent with the recent recognition that an increase in Ca^{2+} sensitivity is involved in contractile responses to receptor agonists [25–29]. Protamine inhibited both phasic and tonic increases in $[Ca^{2+}]_i$ induced by NE, suggesting that protamine reduces $[Ca^{2+}]_i$ by inhibiting both Ca^{2+} influxes and Ca^{2+} release from intracellular Ca^{2+} stores.

The high K^+-induced phasic and tonic contractions were completely or strongly inhibited by the above interventions to inhibit Ca^{2+} influxes, but little affected by the above interventions to deplete intracellular Ca^{2+} stores, suggesting that the high K^+-induced phasic and tonic contractions both appear to be totally dependent on Ca^{2+} influx as previously proposed [4]. Protamine inhibited both phasic and tonic increases in $[Ca^{2+}]_i$ induced by high K^+, suggesting that protamine inhibits the high K^+-induced contraction, probably by inhibiting voltage-gated Ca^{2+} influx. The difference in underlying mechanisms between high K^+-induced phasic and tonic contractions is currently unknown and needs to be further investigated.

The autofluorescence of the strip might be significantly affected by exposure to the drugs (e.g., high K^+) possibly as a result of alteration in redox status [16]. However, background fluorescence intensity (including the autofluorescence of the strips) was only ≈10%–15% of the fura-2 signals in our strips at either excitation wavelength under our experimental conditions as described above and also in a previous study in which Ca^{2+} measurement experiments were performed in the same isolated rabbit mesenteric arteries with the same experimental system as ours [12]. Therefore, we believe that the theoretically possible changes in the autofluorescence of the strip stimulated with the drugs should not make a substantial contribution to our current results. In addition, neither high K^+ nor NE significantly affected the autofluorescence of the same preparations as ours under exactly the same experimental condition as ours (personal communication, Prof. Takeo Itoh, Dept. of Pharmacology, Nagoya City University Medical School, January 5, 1996).

The clinical relevance of our present and previous observations that protamine has a direct vasodilator action in small resistance arteries in the absence of heparin has been fully discussed in our previous studies [4–7]. Briefly, since free protamine is known to exist side by side with a heparin-protamine complex in plasma even during the "adequate" reversal of heparin as judged by clotting time [30,31], the direct vasodilator action of protamine in small "resistance" arteries might contribute to its hypotensive effect in vivo. Although, to our knowledge, no information is available regarding free protamine concentration during heparin reversal, they should be smaller than theoretical concentrations of "total" protamine calculated from plasma volume and the amount of protamine administered (30–200 $\mu g \cdot ml^{-1}$ [32]). Our present and previous studies in both human and rabbit resistance arteries have shown that relatively high concentrations (≥ 15–50 $\mu g \cdot ml^{-1}$) were required for protamine to exert its direct vasodilating action, implying that the direct vasodilator action of protamine may not play a major role in its known hypotensive effect in vivo. However, it is known that there exist marked variations in sensitivity to heparin and the rate of its clearance [33]. In addition, the direct vasodilating action of protamine might have been underestimated because of rather high concentrations of NE or high K^+ used in our experiments which caused maximal contractions; it is unlikely that blood vessels are being maximally contracted in vivo. We therefore speculate that the direct vasodilating action of protamine can play a role in its hypotensive effect, particularly in situations where "free" protamine concentrations may accidentally increase; e.g., in case of administration of an inordinately large amount of protamine, rapid injection of protamine, or administration of protamine to patients with high sensitivity to heparin. This idea would be consistent with the recognition that systemic hypotension is commonly observed with rapid injection of protamine [1]. Again, in this paper, we would recommend that the minimum possible amount of protamine be administered slowly to prevent any unnecessary increase in plasma concentration of free protamine, and we emphasize the importance of determining the required dose of protamine for precise reversal of heparin-induced anticoagulation.

In conclusion, protamine does indeed have a direct vasodilating action in small resistance arteries which may contribute to its hypotensive effect in vivo. The direct vasodilator action of protamine is probably due, at least in part, to its effect of reducing $[Ca^{2+}]_i$ in vascular smooth muscle, possibly due to inhibition of both Ca^{2+} influx and Ca^{2+} release from intracellular stores. The present study represents the first description of the effect of protamine on $[Ca^{2+}]_i$ of vascular smooth muscle.

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