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Possible mechanisms of inhibitory action of protamine on contractile activity of rat aorta

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Experiments were performed to determine possible mechanisms of inhibitory action of protamine chloride on noradrenaline (10 μM)-, KCl (40 mM)-, $BaCl_2$ (1 mM)- and $CaCl_2$ (10 mM)-induced contractions in rat aorta. Protamine, La^{3+} and gallopamil (D600), inhibited the K^+-induced contractions more effectively than the noradrenaline-induced responses on the basis of the concentrations giving 40% inhibition. Lanthanum (1–5 mM) reduced tissue Ca content in both normal and Ca^{2+}-depleted Tris-buffered solutions and produced an increase in ^{45}Ca efflux from the aortic strip into the Ca^{2+}-depleted Tris solution. Protamine (1–5 mg ml^{-1}) reduced tissue Ca content in normal Tris solution, but to a lesser extent than La^{3+} in the Ca^{2+}-depleted solution. Furthermore, protamine (3 mg ml^{-1}) produced no increase in ^{45}Ca efflux from aorta. These results suggest that protamine chloride may preferentially inhibit the Ca^{2+} influx stimulated by K^+ depolarization and that its inhibitory action on rat aorta may be due to non-specific displacement of the superficially located bound Ca^{2+} of the cell membrane, which can also be readily removed by treatment with Ca^{2+}-depleted solution.

Protamine is a group of simple, strongly basic, low-molecular weight proteins found in the sperm of certain fish, and its possible clinical use is as an antidote for heparin. Protamine sulphate is known to cause hypotension (Thompson 1900; Jaques 1949; Egerton & Robinson 1961), probably by a direct effect on the vascular tree (Goldman et al 1969; Fadali et al 1974).

Although protamine chloride acts as an inhibitor of the guinea-pig ileal longitudinal smooth muscle contraction induced by various spasmogens (Kubota & Katsuhara 1973), its precise mechanism of action on smooth muscle is obscure. This study was performed to elucidate possible mechanisms of inhibitory action of protamine chloride on the isolated aorta by comparing its inhibitory effects on contractions induced by contractile agents including noradrenaline, KCl, $BaCl_2$ and $CaCl_2$ with those of La^{3+} and gallopamil. The effects of protamine on ^{45}Ca efflux from the tissue and tissue Ca content were also compared with those of La^{3+}.

METHODS

Male Wistar-strain rats (250–450 g) were stunned by a blow to the back of the head and bled via the carotid artery. The thoracic aorta was rapidly dissected, freed of connective tissue and placed in an oxygenated (100%) Tris-buffered solution of the following composition (mM) (Kroeger & Marshall 1974): NaCl 118·5, KCl 4·7, $CaCl_2$ 2·5, $MgCl_2$ 1·2, Tris (hydroxymethyl) aminomethane 23·8, and glucose 5·5. The pH was adjusted to 7·4 with 2N HCl. The Tris buffer was used throughout the experiments to prevent precipitation of lanthanum. The aorta in the solution, bubbled continuously with 100% O_2 at room temperature (24 °C), was helically cut (approximately 35 mm long and 2 mm wide) and suspended in a 10 ml organ bath. The bathing medium was maintained at 32 °C and bubbled with 100% O_2. Isotonic tension was recorded by an isotonic transducer (ME-4012, ME Commercial Co. Ltd, Tokyo). The aortic strip was equilibrated for 60–90 min under a resting tension of 0·3 g.

Agonists used were noradrenaline bitartrate (10 μM), KCl (40 mM), $BaCl_2$ (1 mM) and $CaCl_2$ (10 mM). The response to $CaCl_2$ was obtained in the 100 mM K^+, Ca^{2+}-free solution. The high K^+ solution was of the same composition except that $CaCl_2$ was omitted and NaCl was replaced with equimolar amounts of KCl. These concentrations of the agonists were chosen to produce approximately maximal responses in rat aorta under the conditions of the experiment. Antagonists, protamine chloride, $LaCl_3$ and gallopamil, were allowed to act for 10 min and, subsequently in their presence each agonist was added. A 40%-inhibition concentration for each

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antagonist was graphically determined by applying two doses of the antagonist. All the agonists and antagonists were dissolved in Tris-buffered solution and added directly to the 10 ml organ bath at a volume of less than 0·1 ml.

For determination of non-displaceable calcium by La^{3+} or protamine, a helical strip of aorta was tied at one end with nylon thread, placed into a beaker containing oxygenated (100% O_2) Tris-buffered solution maintained at 32 °C, and allowed to equilibrate for 60 min. The strip was then transferred into the oxygenated Tris solution (10 ml) containing La^{3+} (1 and 5 mM) or protamine chloride (1 and 5 mg ml^{-1}) and incubated at 32 °C for 60 min. During this time, the tissue was washed every 10 min with fresh medium. Similar experiments were carried out in the Ca^{2+}-depleted medium. The control experiments were concurrently carried out in the absence of La^{3+} or protamine. At the end of the 60 min incubation, the muscle was blotted gently with ashless filter paper (Whatman No. 42), dipped rapidly into Ca^{2+}-depleted Tris-buffered solution (50 ml) for 5 s and blotted once again. The tissue was then placed in a quartz test tube and dried at 100 °C for 12 h and weighed. The dried tissue was ashed in a muffle furnace at 550 °C for 16 h, and the ash dissolved in 3 ml of 0·1 M HCl and aliquots were taken for atomic absorption spectrophotometric assay of calcium.

In experiments measuring ^{45}Ca efflux, a helical strip of aorta was attached to a stainless-steel holder with nylon thread under a resting tension of 0·3 g, and equilibrated in oxygenated Tris-buffered solution for 60 min at 32 °C. The tissue was then exposed for 3 h to the ^{45}Ca-containing solution (10 μCi ml^{-1}). After the tissue was washed for several seconds in non-radioactive Ca^{2+}-free Tris solution to remove the radioactive incubation solution adhering to the tissue surface and the organ bath, washout solution was collected after 5, 10, 15 and 20 min, and subsequent successive 10 min intervals over a total washout period of 100 min. Lanthanum (1 mM) or protamine (3 mg ml^{-1}) was applied for 10 min by adding to the washout solution after 50 min. An appropriate control experiment was run simultaneously. The collected samples were freeze-dried before counting. To determine the amount of ^{45}Ca remaining in the tissue at the end of the washout period, the tissue was blotted on filter paper, weighed, placed in 1 ml Protosol (New England Nuclear, Massachusetts) and incubated for 5 h at 50 °C to solubilize the tissue. Washout data for ^{45}Ca were obtained by determining both the ^{45}Ca present in washout samples and the ^{45}Ca remaining in the tissue after the 100 min washout. Curves were then plotted, which express the decline of tissue ^{45}Ca concentration with time.

The data were analysed by Student's *t*-test and statistical significance was set at $P < 0·05$ or better.

The following drugs and chemicals were used; gallopamil (D 600) hydrochloride (Knoll AG), protamine chloride (Sigma Chemical Co. Ltd), (−)-noradrenaline bitartrate (Sigma Chemical Co. Ltd), $^{45}CaCl_2$ (New England Nuclear). All other chemicals were reagent grade.

RESULTS

Table 1 summarizes 40%-inhibition concentrations (IC40) for $LaCl_3$, protamine chloride and D 600 against contractions induced by noradrenaline (10 μM), KCl (40 mM), $BaCl_2$ (1 mM), and $CaCl_2$ (10 mM) in rat thoracic aorta. The IC40 represents the drug concentration required to produce 40% inhibition of the evoked contraction and was used because of the weak inhibitory action of protamine chloride (the values for protamine are expressed as mg ml^{-1}). The inhibitory effects of La^{3+}, protamine,

Table 1. Inhibitory effects of spasmolytics on contractions induced by noradrenaline, KCl, $BaCl_2$ and $CaCl_2$ in rat aorta (IC40 ± 95% confidence limits).

	40%-Inhibition concn (IC40)		
Spasmogen	$LaCl_3$ (μM)	Protamine (mg ml^{-1})	D 600
Noradrenaline (10 μM)	31·0 (7·8–54·2)	6·5 (4·6–8·4)	2·7 μM (0·8–4·6)
KCl (40 mM)	10·6 (6·0–15·2)	1·5 (0·7–2·3)	12·7 nM (6·8–18·6)
$BaCl_2$ (1 mM)	12·5 (6·6–18·4)	—[a]	24·0 nM (18·8–29·2)
$CaCl_2$ (10 mM)	29·5 (16·7–42·3)	—[a]	40·5 nM (28·3–52·1)

[a] Protamine (3 mg ml^{-1}) marginally inhibited Ba^{2+}-induced contraction and inhibited the Ca^{2+}-induced contraction by 20–30%.

and D 600 on contractions evoked by K^+ (40 mM) in all cases were greater than those on noradrenaline (10 μM)-induced contractions. The IC40 value (2·7 μM) for D 600 against the noradrenaline-induced contraction was 50–150 times larger than those against K^+-, Ba^{2+}- and Ca^{2+}-induced contractions (12·7, 24·0 and 40·5 nM, respectively). La^{3+} and D 600 inhibited the Ba^{2+}-induced contractions more effectively than the Ca^{2+}-induced contractions. The inhibitory action of protamine on the Ba^{2+}- and Ca^{2+}-induced contractions was slight and it was not possible to estimate the IC40 values against them.

Contractile responses after removal of La^{3+} or protamine are illustrated in Fig. 1. A rise in the

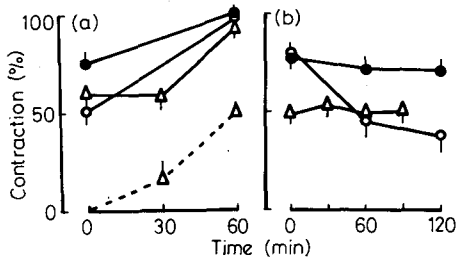

FIG. 1. Comparison of time-response curves for noradrenaline, K^+ and Ca^{2+} after washing out La^{3+} and protamine in rat aorta. Aortic strips were contracted by 10 μM noradrenaline (○), 40 mM K^+ (△) and 10 mM Ca^{2+} (●). Contraction % is expressed as a percentage of the maximum contractile height induced during control contraction by the respective agonist. The contractions at 0 time were determined in the presence of (a) La^{3+} (30 μM for noradrenaline, 10 μM for K^+ and Ca^{2+}) or (b) protamine (0·5 mg ml^{-1} for noradrenaline, 3 mg ml^{-1} for K^+ and Ca^{2+}). The curve (– –△– –) in (a) indicates the rise in the baseline tension with time in the La^{3+}-treated tissue contracted by K^+. Each data point is the mean ± s.e. from five to six experiments. For clarity, the standard error is shown in one direction only.

baseline tension in aorta contracted by noradrenaline or Ca^{2+} after La^{3+}-treatment was negligible, while the tissue contracted by high K^+ exhibited a marked rise in the basal tone after 60 min washout, as shown in Fig. 1A. Therefore, the height of contraction induced by K^+ was measured from the control baseline. The contractile responses of the La^{3+}-treated rat aorta to noradrenaline and Ca^{2+} recovered approximately to the control level after 60 min washout. In contrast, the inhibitory effect of protamine persisted even after it had been removed (Fig. 1B). After washing out protamine, the noradrenaline-induced contractions gradually decreased with washout time.

The effects of La^{3+} and protamine on tissue Ca content are summarized in Table 2. The La^{3+} concentrations used were higher than IC40 values in order to displace La^{3+}-accessible Ca as much as possible. When rat aortic strips were incubated in Ca^{2+}-depleted medium at 32 °C for 60 min, the amounts of Ca depleted constituted 77–81% of the total tissue Ca. The amounts of the tissue Ca after the treatment with 1 mM and 5 mM La^{3+} in normal medium containing 2·5 mM Ca^{2+} were 5·84 ± 0·32 (n = 6) and 3·35 ± 0·22 (n = 6) μmol Ca g^{-1} dry wt, respectively, indicating a significant and dose-dependent decrease ($P < 0·05$). Furthermore, in the Ca^{2+}-depleted solution the tissue Ca content was significantly further decreased by the treatment with

Table 2. Effects of protamine and La^{3+} on tissue Ca content in rat aorta.

	0 mM	1 mM	5 mM
$LaCl_3$			
Normal soln	11·41±0·45 (12)	5·84±0·32** (6)	3·35±0·22** (6)
Ca^{2+}-depleted	2·63±0·24 (14)	1·09±0·09** (7)	0·98±0·13** (7)
	0 mg ml^{-1}	1 mg ml^{-1}	5 mg ml^{-1}
Protamine			
Normal soln	11·59±0·54 (12)	9·28±0·55* (6)	7·76±0·28** (6)
Ca^{2+}-depleted	2·23±0·07 (12)	1·99±0·75 (5)	1·95±0·21 (6)

Each value (μmol Ca g dry wt^{-1}) represents the mean ± s.e. Numbers in parentheses are the number of independent preparations tested. Significantly different from the corresponding control: *$P < 0·05$, **$P < 0·01$.

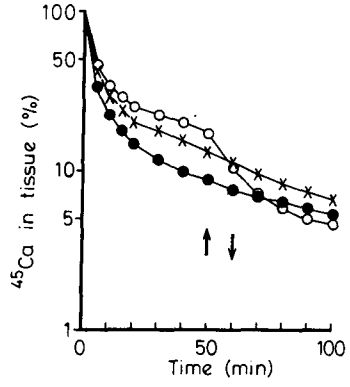

FIG. 2. Effects of protamine and La^{3+} addition on ^{45}Ca efflux from rat aorta. —×—, control; —○—, 1 mM La^{3+}; —●—, 1 mg ml^{-1} protamine. Protamine or La^{3+} was added at an upward pointing arrow and removed at a downward one. Each point is the mean of four determinations.

La^{3+} at concentrations of 1 and 5 mM ($P < 0·01$), although a dose-dependent decrease was not observed. Protamine significantly reduced the tissue Ca content in a similar manner as La^{3+} in normal Tris solution. However, the amounts of Ca removed by protamine were smaller than those by La^{3+} and in the Ca^{2+}-depleted medium no significant effects of protamine on the tissue Ca content were observed.

Fig. 2 shows the effects of La^{3+} and protamine on ^{45}Ca efflux into Ca^{2+}-depleted Tris solution. The results were expressed as the percentage of ^{45}Ca remaining in the tissue against time. Lanthanum or protamine was added at 50 min and left in contact with the tissue for 10 min. After application of La^{3+} (1 mM) a large increase in ^{45}Ca efflux occurred, while protamine (3 mg ml^{-1}) did not influence ^{45}Ca efflux.

DISCUSSION

In the present experiments using rat aorta, protamine chloride as well as lanthanum chloride inhibited high K^+ (40 mM)-induced contractions more effectively than those induced by noradrenaline (10 μM). This suggests that protamine may preferentially inhibit the Ca^{2+} influx stimulated by

K^+ depolarization in rat aorta. It is known that gangliosides bind polycations such as protamine strongly, presumably at the groups of the sialic acid residues, and that the electrical excitability of neurons can be blocked by adding protamine and can then be restored by addition of gangliosides (Lehninger 1968). Furthermore, protamine chloride and La^{3+} inhibit to a greater extent contractile responses of guinea-pig intestinal muscle to agonists such as 5-hydroxytryptamine and acetylcholine, compared with those to K^+ and Ba^{2+} (Kubota & Katsuhara 1973). The observed protamine effects in these experiments were qualitatively similar to those decribed for La^{3+} in the intestinal preparation. La^{3+} is reported to replace Ca^{2+} at superficial binding sites of the cell membrane (Weiss & Goodman 1969) and to block Ca^{2+} influx through the aortic smooth muscle membrane (van Breemen 1969). Putting these observations together, protamine chloride appears to share a common mechanism of inhibitory action with La^{3+} through replacement of Ca^{2+} at the superficial binding sites and prevention of Ca^{2+} influx into the smooth muscle cell. The differential effects of these antagonists on the contractions induced by high K^+ and noradrenaline support the concept that the contractile responses to these stimulants in rat aorta depend on Ca^{2+} from different sources (Hudgins & Weiss 1968; van Breemen 1969; Peiper et al 1971; Massingham 1973; Yamashita et al 1977).

D 600, a potent Ca entry blocker, was much more effective in antagonizing K^+-, Ba^{2+}- and Ca^{2+}-induced than noradrenaline-induced contractions on the basis of IC40 values (Table 1). The selectivity of D 600 for ion-induced effects has also been reported (Massingham 1973). Lanthanum inhibited Ba^{2+}- and Ca^{2+}-evoked contractions, while protamine chloride (3 mg ml^{-1}) marginally inhibited Ba^{2+}-induced contraction and inhibited Ca^{2+}-induced contraction by 20–30% (Table 1). Clement (1981) has suggested that the $BaCl_2$-induced contraction may be due mainly to the movement of membrane-bound Ca^{2+} through a D 600-sensitive Ca^{2+} channels in the guinea-pig ileal longitudinal smooth muscle. More recently, Hansen et al (1984) have indicated that barium enters vascular smooth muscle of the rat via calcium influx channels and produces its contractile effects on the smooth muscle by a direct intracellular interaction with the contractile or regulatory proteins. Our results indicate that protamine may not exert its blocking action through D 600- and La^{3+}-sensitive Ca^{2+} channels. D 600 and La^{3+} inhibited Ba^{2+}-induced contraction more effectively than those induced by Ca^{2+} in the present experiments. Although the reason for this is not clear, these results are indicative of the possibility that Ba^{2+}-induced contraction may be exerted through somewhat different mechanisms from the Ca^{2+}-induced responses in the rat aorta.

The contractile responses of the aorta to noradrenaline and Ca^{2+} after removal of La^{3+} are gradually restored to their control size after washing for 60 min, while the tissue contracted by high K^+ exhibits a marked time-dependent rise in the baseline level during the washout phase. This effect of La^{3+} on K^+-induced contraction remains unclear, but might be due to the different effects of La^{3+} on the redistribution of Ca after contractions induced by these agonists. Meanwhile, the blockade of high K^+-, Ca^{2+}- and noradrenaline-induced contractile responses caused by protamine chloride appeared to be irreversible (Fig. 1B). It is likely therefore that protamine may bind at superficial Ca^{2+} binding sites of the cell membrane in such a manner that protamine is not readily washed out because of its strong positive charge. This may lead to displacement of Ca^{2+} ions by protamine in the superficial membrane and then to non-specific suppression of the membrane Ca^{2+} movements. Our results suggest that the Ca^{2+} fraction displaced by protamine may be required for the development of maximal contractile responses to noradrenaline, K^+ or Ca^{2+}. The contractile response to noradrenaline was inhibited to a greater extent after removal of protamine than in its presence (Fig. 1B), also suggesting that this Ca fraction might be more important for the complete filling of the Ca^{2+} store for noradrenaline-induced contraction compared with those to K^+- and Ca^{2+}. It has been reported that protamine is an effective inhibitor of the various activated forms of adenylate cyclase of liver plasma membrane (Kiss & Zamfirova 1983). The inhibition of adenylate cyclase, if this is the case in rat aorta, it may account for the inhibitory effects of protamine on contraction of smooth muscle, since an increase in cyclic AMP level is reported to lead to relaxation of the vascular smooth muscle (Bär 1974). The protamine-induced hypotension may therefore be produced by its effect on the myocardium and vascular tree, as suggested by Fadali et al (1974), since the inhibitory effect of protamine chloride on isolated rat aorta was not very potent.

Lanthanum increased ^{45}Ca efflux from the aorta into Ca^{2+}-depleted medium, while protamine exhibited no effect on ^{45}Ca efflux. Although protamine as well as La^{3+} dose-dependently reduced the aortic

Ca content in normal Tris solution, in the Ca^{2+}-depleted solution, protamine had less influence on the tissue Ca content. The increase in the ^{45}Ca efflux by La^{3+} may be due to a displacement of ^{45}Ca by La^{3+} from cell membrane sites (van Breemen et al 1973; Weiss 1982). Our observations show that La^{3+}-accessible Ca fraction remains unaltered even after treatment of the tissue with Ca^{2+}-depleted solution. It is therefore likely that the inhibitory action of protamine on contraction of rat aorta may be due to non-specific displacement of the superficially located bound Ca of the cell membrane, which is also readily removed by treatment with Ca^{2+}-depleted solution. It is probable that the different spectrum of inhibitory effects of protamine on contractions induced by various stimulants in rat aorta may be related to their dependence upon the Ca fraction sensitive to protamine. These results suggest that the action of protamine may be explicable in terms of a modest removal of membrane-bound Ca^{2+}.

REFERENCES

Bär, H.-P. (1974) in: Greengard, P., Robison, G. A. (eds) Advances in Cyclic Nucleotide Research. Vol. 4, Raven Press, New York, pp. 195–237

Clement, J. G. (1981) Can. J. Physiol. Pharmacol. 59: 541–547

Egerton, W. S., Robinson, C. L. H. (1961) Lancet 2: 635–637

Fadali, M. A., Ledbetter, M., Papacostas, C. H., Duke, L. J., Lemole, G. M. (1974) Ann. Surg. 18: 232–235

Goldman, B. S., Joison, J., Austen, W. G. (1969) Ann. Thorac. Surg. 7: 459–470

Hansen, T. R., Dineen, D. X., Petrak, R. (1984) Am. J. Physiol. 246: C235–C241

Hudgins, P. M., Weiss, G. B. (1968) J. Pharmacol. Exp. Ther. 159: 91–97

Jaques, L. B. (1949) Br. J. Pharmacol. 4: 135–144

Kiss, J., Zamfirova, R. (1983) Experientia 39: 1381–1382

Kroeger, E. A., Marshall, J. M. (1974) Am. J. Physiol. 226: 1298–1303

Kubota, K., Katsuhara, N. (1973) Folia Pharmacol. Japan. 69: 835–842

Lehninger, A. L. (1968) Proc. Nat. Acad. Sci. U.S.A. 60: 1069–1080

Massingham, R. (1973) Eur. J. Pharmacol. 22: 75–82

Peiper, U., Griebel, L., Wende, W. (1971) Pflugers Arch. 330: 74–89

Thompson, W. H. (1900) Hoppe-Seyler's Z. Physiol. Chem. 29: 1–19

Van Breemen, C. (1969) Arch. Int. Physiol. Biochem. 77: 710–716

Van Breemen, C., Farinas, B. R., Casteels, R., Gerba, P., Wuytack, F., Deth, R. (1973) Phil. Trans. R. Soc. Lond. 265: 57–71

Weiss, G. B. (1982) Can. J. Physiol. Pharmacol. 60: 483–488

Weiss, G. B., Goodman, F. R. (1969) J. Pharmacol. Exp. Ther. 169: 46–55

Yamashita, K., Takagi, T., Hotta, K. (1977) Jap. J. Physiol. 27: 551–564