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Protamine Induces Elevation of Cytosolic Free Ca^{2+} in Cultured Porcine Aortic Endothelial Cells

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Abstract

To test the hypothesis that protamine influences calcium movement in endothelial cells, we measured the concentration of intracellular free calcium ($[Ca^{2+}]_i$) in cultured porcine aortic endothelial (PAE) cells in Krebs solution (2·5 mM Ca^{2+}, pH 7·4) at 37°C, by fura-2 fluorimetry.

The basal $[Ca^{2+}]_i$ of PAE cells was 113 ± 18 nM (n = 6). Protamine increased $[Ca^{2+}]_i$ in a concentration-dependent manner (EC50, the concentration having 50% of the maximum effect, $1{\cdot}4 \pm 0{\cdot}3\ \mu g\ mL^{-1}$, n = 6). The response of PAE cells to $100\ \mu g\ mL^{-1}$ protamine (330 ± 80 nM, n = 6) was blocked by a Ca^{2+} chelator, 5 mM glycoletherdiaminetetraacetic acid (EGTA; 131 ± 16 nM, n = 6), and by a non-selective Ca^{2+} channel blocker, 3 mM Co^{2+} (134 ± 14 nM, n = 6).

These results suggest that Ca^{2+} influx through cell-membrane Ca^{2+} channels is mainly responsible for the protamine-induced Ca^{2+} elevation.

Protamine is commonly applied intravenously to neutralize heparin used as an anti-coagulant for patients during extra-corporeal circulation. However, administration of protamine often induces systemic hypotension, which is hazardous to such patients in an unstable cardiovascular state after cardiopulmonary bypass (Frater et al 1984; Kirklin et al 1986; Katz et al 1987). A recent study using vascular tension bioassay revealed that vascular relaxation induced by protamine is dependent on endothelium and that the inhibition of the nitric oxide (NO) pathway reduced endothelium-derived vascular relaxation induced by protamine, suggesting that protamine stimulates endothelial cells to release NO (Pearson et al 1992). However, the direct action of this compound on vascular endothelial cells has not been discovered. Because the generation of NO from endothelial cells is preceded by an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), which is required for the activation of the constitutive isoform of NO synthase (NOS) (Moncada et al 1991), we examined the protamine-induced change in $[Ca^{2+}]_i$ of endothelial cells.

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Materials and Methods

Materials

DMEM, RPM1 1640, *N*-(2-hydroxyethyl)piperazine- *N′*-(2-ethanesulphonic acid) (HEPES), ampicillin, kanamycin, and trypsin were purchased from Sigma (St Louis MO). Foetal bovine serum was from JRH Bioscience (Lenaxa KS). Protamine was obtained from Shimizu (Shizuoka, Japan). Diiodoacetyl low-density lipoprotein was from Funakoshi (Tokyo, Japan). The acetoxymethyl ester form of fura-2 (fura-2/AM) was from Dojindo Laboratories (Kumamoto, Japan). Verapamil hydrochloride was from Eisai (Tokyo). Disodium ethylenediaminetetraacetate (EDTA) and glycoletherdiaminetetraacetic acid (EGTA) were from Katayama (Osaka, Japan). Other chemicals were of analytical quality.

Cell culture

Isolation and primary culture of porcine aortic endothelial (PAE) cells were performed as described elsewhere (Az-ma et al 1995, 1996). The culture medium used was RD medium (1 : 1 (v/v) RPMI 1640 medium–Dulbecco's modified Eagle's

medium (DMEM)) supplemented with bicarbonate ($2\,mg\,mL^{-1}$), HEPES (15 mM), ampicillin ($90\,\mu g\,mL^{-1}$), kanamycin ($90\,\mu g\,mL^{-1}$) and 10% (v/v) foetal bovine serum equilibrated with 5% CO_2 in air under a humidified atmosphere at 37°C (pH 7·4). PAE cells were subcultured at a 1 : 3 split ratio in collagen-coated $25\,cm^2$ plastic flasks. Endothelial cell identity was confirmed by the uptake of diiodoacetyl-low-density lipoprotein, by means of fluorescence microscopy (> 99% of the cells) (Doan et al 1994). The resulting subconfluent monolayers of PAE cells (passage 2) were harvested with trypsin (0·125% (w/v)) and EDTA (0·02% (w/v)) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS). After washing with the culture medium and centrifugation at 200 *g*, the cells were resuspended at a density of $1-2 \times 10^5$ cells mL^{-1} and seeded on fibronectin-coated glass cover-slips placed in 24-well cluster dishes (0·5 mL/well). Experiments were performed within 24 h of the cells reaching confluent monolayers.

Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ of PAE cells was measured by use of a fluorescent Ca^{2+} indicator dye, fura-2, as previously described, with modification (Az-ma et al 1995). PAE cell monolayers attached to cover-slips were loaded with the acetoxymethyl ester form of fura-2 (3 μM) in the culture medium for 1 h under a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were then washed three times with Krebs solution containing 2·5 mM Ca^{2+}. The fluorescence of fura-2 was measured by fluorescence spectrometry; the spectrometer (CAF-100; Japan Spectroscopy, Tokyo, Japan) was equipped with a thermostatic holder combined with a micro magnetic stirrer (Wickham et al 1988). Cell-attached cover-slips were placed at an angle of 45° to both excitation and emission light paths in a quartz glass cuvette filled with Krebs solution (2 mL, 37°C) stirred at $1000\,rev\,min^{-1}$. The fluorescence intensity ratio with excitation at 340 or 380 nm and emission at 500 nm was continuously recorded on a strip-chart recorder, and was converted to $[Ca^{2+}]_i$ by use of an in-vitro calibration curve obtained from standard Ca^{2+}–EGTA solutions containing 3 μM fura-2-free acid. After 10 min pre-equilibration of PAE cells with Krebs solution to settle the baseline of fluorescence intensities, protamine or other agents, or both, were added cumulatively to the cuvette. All measurements were performed within 1·5 h of addition of fura-2.

Statistical analysis

Data are expressed as means ± s.e.m. Multiple comparison was performed by analysis of variance followed by the *t*-test with Bonferroni's correction ($P < 0·05$).

Results

Application of protamine to PAE cells induced a concentration-dependent increase in $[Ca^{2+}]_i$ (Figure 1). The threshold concentration of protamine which increased $[Ca^{2+}]_i$ was $0·2\,\mu g\,mL^{-1}$. The maximum $[Ca^{2+}]_i$ elevation was obtained by addition of protamine at $\geq 60\,\mu g\,mL^{-1}$. The percent–response curves of $[Ca^{2+}]_i$ against the concentration of protamine were fitted to Hill's equation, and the EC50 for protamine (i.e. the concentration having 50% of the maximum effect) was found to be $1·4 \pm 0·3\,\mu g\,mL^{-1}$ (Figure 2). The basal $[Ca^{2+}]_i$ of PAE cells was 113 ± 18 nM, whereas the maximum $[Ca^{2+}]_i$ induced by 100 $\mu g\,mL^{-1}$ protamine was 330 ± 80 nM (n = 6, Figure 3A). In contrast, preloading of PAE cells with a Ca^{2+} chelator, EGTA (5 mM), for 2 min before addition of $100\,\mu g\,mL^{-1}$ protamine almost completely inhibited $[Ca^{2+}]_i$ elevation (131 ± 16 nM, n = 6; Figure 3B). We also confirmed that protamine did not increase $[Ca^{2+}]_i$ when Ca^{2+}-free Krebs solution was used (n = 3, data not shown). The increase in $[Ca^{2+}]_i$ induced by protamine was also blocked by addition of a non-selective Ca^{2+}-channel blocker (Ikebuchi et al 1991), 3 mM $CoCl_2$ (Co^{2+}; 134 ± 14 nM, n = 6; Figure 3C). It is unlikely that Co^{2+} quenched the Ca^{2+}-associated fluorescence of fura-2 because a transient increase in $[Ca^{2+}]_i$ was observed in the presence of Co^{2+} when PAE cells were stimulated with 100 nM brady-

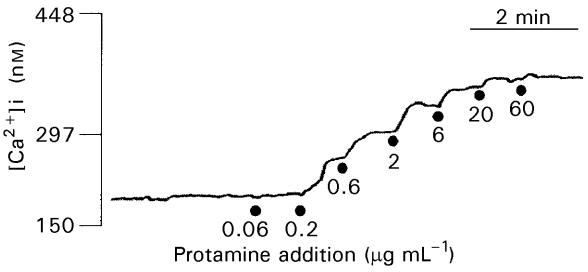

Figure 1. Changes in $[Ca^{2+}]_i$ (nM) in cultured porcine aortic endothelial cells in Krebs solution (2·5 mM Ca^{2+}, pH 7·4) on cumulative addition of protamine at 37°C. $[Ca^{2+}]_i$ was measured by fura-2 fluorimetry. This trace is representative of similar results obtained from three separate cell preparations cultured from different donors. Points at which protamine was added are indicated by '•'.

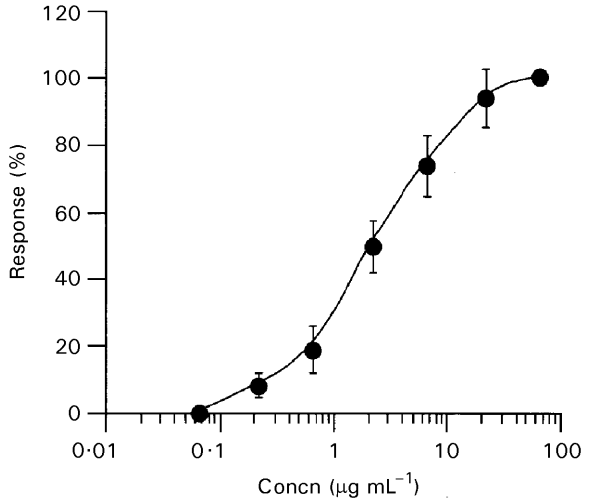

Figure 2. Effect on $[Ca^{2+}]_i$ in cultured porcine aortic endothelial cells of the concentration of protamine in Krebs solution (2·5 mM Ca^{2+}, pH 7·4) at 37°C. $[Ca^{2+}]_i$ was measured by fura-2 fluorimetry. Data are expressed as percent change, assuming basal $[Ca^{2+}]_i$ of 0%, and $[Ca^{2+}]_i$ after addition of 60 μM protamine of 100%. Data are expressed as means ± s.e.m. of results from six separate experiments. The porcine aortic endothelial cells used were obtained from three separate cell preparations cultured from different donors.

kinin, a known agonist of IP_3-dependent Ca^{2+} release from intracellular stores (data not shown, and Az-ma et al 1995). These findings suggested that the protamine-induced elevation of $[Ca^{2+}]_i$ was caused by Ca^{2+} influx through cell-membrane Ca^{2+}

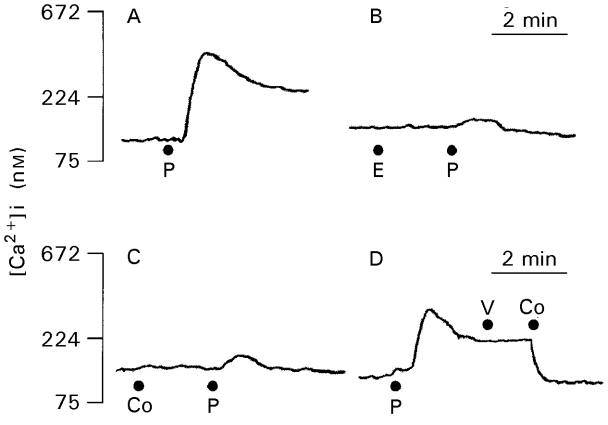

Figure 3. The inhibitory effect of the Ca^{2+} chelator glycol-etherdiaminetetraacetic acid (EGTA) and the non-selective Ca^{2+}-channel blocker, Co^{2+}, on protamine-induced elevation of $[Ca^{2+}]_i$ in cultured porcine aortic endothelial cells. Protamine (100 μg mL^{-1}) was added to the cells in Krebs solution (2·5 mM Ca^{2+}, pH 7·4) at 37°C without inhibitor (A) and after 2-min preloading with 5 mM EGTA (B) and 3 mM Co^{2+} (C). The differential effects of a voltage-gated Ca^{2+} channel blocker, verapamil (10 μM), and Co^{2+} (3 mM), on protamine-induced $[Ca^{2+}]_i$ elevation of porcine aortic endothelial cells is also shown (D). The agents were added as indicated in the figure. $[Ca^{2+}]_i$ was measured by fura-2 fluorimetry. The examples are representative of similar results obtained from at least three separate cell preparations cultured from different donors. '•' indicates times of addition of 100 μg mL^{-1} protamine (P), 5 mM EGTA (E), 3 mM Co^{2+} (Co), and 10 μM verapamil (V).

channels and not by Ca^{2+} release from intracellular stores. After addition of protamine, the $[Ca^{2+}]_i$ of PAE cells continued to rise to a level higher than that observed before protamine administration. Addition of 10 μM verapamil, an L-type voltage-gated Ca^{2+}-channel blocker (Roveri et al 1992), did not influence the sustained increase in $[Ca^{2+}]_i$, whereas Co^{2+} reduced $[Ca^{2+}]_i$ to the level observed before the application of protamine (Figure 3D). Preloading of PAE cells with verapamil for 5 min also failed to inhibit the protamine-induced $[Ca^{2+}]_i$ elevation (n = 3, not shown).

Discussion

This study has shown that protamine increases $[Ca^{2+}]_i$ in PAE cells. The increase in $[Ca^{2+}]_i$ induced by protamine was blocked by extracellular Ca^{2+}-free conditions, indicating that Ca^{2+} influx from the extracellular space is responsible for the $[Ca^{2+}]_i$ elevation. The suppression of protamine-induced $[Ca^{2+}]_i$ elevation by a non-selective Ca^{2+} channel blocker, Co^{2+}, further demonstrated that the protamine-induced Ca^{2+} influx passes through cell-membrane Ca^{2+} channels. The type of protamine-sensitive Ca^{2+} channel was unidentified in this study because we could not find a specific blocker of these channels. We observed only that the L-type voltage-gated Ca^{2+} channel is not involved in the protamine-induced Ca^{2+} movement; this is partly confirmed by our previous findings (Az-ma et al 1995) and those of other investigators (for review see Adams et al 1989) that endothelial cells have no voltage-gated Ca^{2+} channel.

The direct action of protamine on the function of endothelial cells is supported by an early observation of Pearson et al (1992) who found that protamine concentration-dependently induces endothelium-derived vascular relaxation in canine coronary, femoral and renal arteries contracted by prostaglandin $F_{2\alpha}$. These investigators also showed that this effect of protamine was suppressed by N^G-monomethyl L-arginine, a competitive inhibitor of NOS, suggesting that endothelial cells have a specific protamine receptor responsible for the protamine-induced release of NO from endothelial cells.

It is well known that the production of NO is triggered by agonist-binding to receptors to open Ca^{2+} channels existing both in the cell membrane and in the intracellular Ca^{2+} stores, because an increase in the $[Ca^{2+}]_i$ of endothelial cells is required for activation of the constitutive isoform of NOS (Moncada et al 1991). Thus, insight into the mode of cytosolic Ca^{2+} movement

induced by protamine is important to understanding the mechanism of action of this compound. It is thus of interest to note that agonist–receptor binding of the common vasoactive compounds (e.g. bradykinin, thrombin, etc.) causes Ca^{2+} release from IP_3-dependent intracellular Ca^{2+} stores. In the current study, the abolition of protamine-induced $[Ca^{2+}]_i$ elevation by Co^{2+} or EGTA suggested that Ca^{2+} release from intracellular Ca^{2+} stores is not involved in the Ca^{2+} movement induced by protamine. However, it is unlikely that intracellular Ca^{2+} stores do not exist in PAE cells because we confirmed that bradykinin (1–100 nM) induced a transient $[Ca^{2+}]_i$ elevation of PAE cells in the presence of EGTA or Co^{2+}. This result puts into question the hypothesis that endothelial cells have a specific protamine receptor.

Other investigators have also suggested that a positive electrical charge on protamine influences endothelial cell function. Akata et al (1993a, b, 1995) reported that the effect of protamine on endothelium-derived vascular relaxation was counteracted by heparin, a negatively charged protein, suggesting that the polycationic property of protamine interferes with the endothelial cell membrane. Evidence is growing that the function of Ca^{2+} channels of several types of cell is regulated by the redox state of thiol residues of channel proteins (Coetzee & Opie 1992; Roveri et al 1992; Boraso & Williams 1994). More recently, we have reported that the redox state of cell-membrane Ca^{2+} channels in endothelial cells is accessible from the extracellular space (Az-ma et al 1999). Because the redox alteration of thiol groups is associated with electron transport to its acceptors, electrically charged compounds such as protamine might interfere with the functional regulation of Ca^{2+} channels. Further investigation is required to resolve the exact mechanisms of action of protamine in influencing Ca^{2+} movement in endothelial cells.

In conclusion, this study has shown the direct action of protamine in increasing $[Ca^{2+}]_i$ of endothelial cells. The increase in $[Ca^{2+}]_i$ was caused mainly by Ca^{2+} influx through cell-membrane Ca^{2+} channels.

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