Effect of elastin peptides on ion fluxes in mononuclear cells, fibroblasts, and smooth muscle cells

(Monocye/ion channels/calcium/sodium/potassium)

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Communicated by Derek Barton, October 20, 1986

ABSTRACT Elastin peptides prepared by alcoholic potassium hydroxide degradation of highly purified fibrous elastin from bovine ligamentum nuchae (κ-elastin) were shown to act on the ion channels of human monocytes, aorta smooth muscle cells, and skin fibroblasts. In small amounts (between 0.1 and 1 μg/ml), elastin peptides strongly increased calcium influx and inhibited calcium efflux by an apparently calmodulin-dependent mechanism. They also were shown to increase sodium influx and to decrease rubidium influx in monocyte preparations obtained from human blood. Only the ouabain-sensitive portion of rubidium influx was inhibited. The action of elastin peptides is strongly concentration-dependent; the maximal activity observed in the above reactions was <1 μg/ml. These results suggest that elastin peptides may play a role in the regulation of the biological activity of mesenchymal cells, in the proximity of which they are released by the action of elastase-type enzymes. Such enzymes were demonstrated in aorta smooth muscle cells (membrane-bound serine protease) and in fibroblasts (metalloprotease). Monocytes and polymorphonuclear leukocytes were also shown to carry elastase-type enzymes. The release of peptides from elastin by elastase-type enzymes and the action of such peptides on the ion fluxes through the cell membrane may well be involved in mechanisms of the modulation of the phenotype of mesenchymal cells during aging as well as in the development of age-dependent pathologies such as artherosclerosis.

The degradation of elastin by elastases was shown to play an important role in several pathological processes such as the development of emphysema (1–3), of atherosclerosis (4–6), and of a variety of skin diseases (7–9). This action was shown to be due to several proteases of the elastase type, that is, proteases that have an affinity for aliphatic amino acid sequences in hydrophobic proteins. Such enzymes have been described on the surface membrane of aorta smooth muscle cells (SMC) (10–12); in fibroblasts obtained from human skin, chicken embryo, pig aorta adventitia, and human vulva (13–15); and also in monocytes and macrophages (16). Platelets (17–19) and leukocytes (20, 21) also have been shown to carry a potent elastase-type protease.

All of these enzymes, although of different nature (metalloprotease or serine protease), are able to attack elastin fibers and release soluble peptides. It also has been shown that soluble elastin peptides, such as κ-elastin prepared by oxalic acid degradation (22) or κ-elastin prepared by KOH hydrolysis in aqueous ethanol (23, 24) or peptides obtained by degradation with elastase-type enzymes, have a variety of interesting biological properties. One of these is the chemo
tactic effect reported by Senior et al. (25). This laboratory has demonstrated their antigenic nature (26, 27). Rabbits immunized with elastin peptides have been shown to develop severe artherosclerosis (28) and also lesions of pulmonary arteries (29). More recently the involvement of elastase type enzymes in the chemotactic movements of mononuclear cells has been shown (30).

Here we report on the effect of elastin peptides on ion fluxes in fibroblasts and SMC as well as in human blood-derived mononuclear cells.

MATERIALS AND METHODS

Patients. Monocytes were obtained from 20 middle-aged healthy men (age 25–52 years) after informed consent. The selection of the subjects was based on the following criteria: good physical and mental health confirmed by clinical, radiological, and biological examinations.

Monocytes were separated by Ficoll/Hypaque gradient centrifugation without any modifications (31, 32).

Cell Cultures. Human fibroblasts were obtained from the skin of a 20-year-old woman (plastic surgery), and SMC were obtained from pig aorta. Cells were cultured in Dulbecco-modified Eagle’s essential medium containing 10% fetal calf serum and 4.5 mg of D-glucose, 100 units of penicillin, and 100 μg of streptomycin per ml at 37°C in 95% air/5% CO2. Medium, serum, penicillin, and streptomycin were purchased from Seromed (Biopro, Strasbourg, France). SMC were used at the seventh passage, and fibroblasts, at the fourth passage. Cells were removed from stock flasks by trypsin-treatment and were seeded onto 30-mm Petri dishes (105 cells per dish). Cells were used for calcium transport experiments at 2 days after subculturing.

Ion-Flux Measurements. For ion-flux measurements, monocyte monolayer cultures were prepared in Nunclon Petri dishes (N-1420) of 30-mm diameter with about 105 monocytes. All incubations and dilutions were made in phosphate-buffered saline (P/NACL; 0.3 mM Na2HPO4/0.4 mM KH2PO4/137 mM NaCl/4.6 mM KCl/1.2 mM MgSO4/1.4 mM CaCl2; pH 7.2) containing 140 mM Na+, 5 mM K+; 1.4 mM Ca2+, and 1.2 mM Mg2+. Before the determinations, cell cultures were incubated for 15–20 min in P/NACL for quiequilibration.

22Na+ influx. Measurements were carried out by the method of Negendank and Shalater (33) with slight modifications (34). Briefly, at zero time, the culture medium was aspirated, and different concentrations of κ-elastin and 1 μCi (1 Ci = 37 GBq) of 22Na+ (Amersham) were added to each dish in 1.0 ml of P/NACL. To prevent Na+ re-uptake from the medium by the cells during the assay, 20 mM dixitone in ethanol was added to the cultures. After 10 min, the monolayers were washed thoroughly with ice-cold P/NACL containing dixitone. After the last washing, the liquid phase was removed by vacuum aspiration. Then 1.0 ml of 0.2% NaDOSO4 was placed on the cells to lyse the monolayers.

Abbreviation: SMC, smooth muscle cells.

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Table 1. Uptake of \(^{45}\text{Ca}^2+\) and \(^{22}\text{Na}^+\) by monocytes obtained from middle-aged subjects in presence of \(\kappa\)-elastin

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>(\text{(^{45}\text{Ca}^2+) uptake}, \text{nmol/10 min per mg of protein})</th>
<th>(\text{(^{22}\text{Na}^+) uptake}, \text{(\mu\text{mol}/10 \text{min per mg of protein})})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(1.86 \pm 0.07)</td>
<td>(41.6 \pm 1.4)</td>
</tr>
<tr>
<td>(\kappa)-Elastin</td>
<td>(124.3 \pm 3.8)</td>
<td>(375.7 \pm 17)</td>
</tr>
<tr>
<td>(0.1 \mu\text{g/ml})</td>
<td>(162.5 \pm 4.5)</td>
<td>(482.4 \pm 19)</td>
</tr>
<tr>
<td>(1.0 \mu\text{g/ml})</td>
<td>(136.8 \pm 4.3)</td>
<td>(420.5 \pm 15)</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of five experiments. Experiments were done in triplicate.

After a 60-min incubation, a 1:5 dilution with distilled water was made, and the radioactivity of 500-\(\mu\text{l}\) aliquots was assayed in a \(\gamma\) NK-350 scintillation counter. The protein content of each monolayer was determined by the method of Lowry et al. (35).

\(\text{\(^{86}\text{Rb}^+\) influx.}\) Measurement was carried out by the method of Horky et al. (36) with slight modifications. In brief, the cell monolayers were preincubated in the presence or absence of 10 \(\mu\text{M}\) ouabain and with or without \(\kappa\)-elastin in different concentrations for 30 min at \(37^\circ\text{C}\) prior to the addition of \(\text{\(^{86}\text{Rb}^+\)}\) (New England Nuclear; specific activity, 2.2–2.4 \(\text{Ci/g}\)). For measurements, we stopped the tracer uptake after 30 min and proceeded as described for \(\text{\(^{22}\text{Na}^+\) influx measurement.}\) The results are presented as \(\text{\(^{86}\text{Rb}^+\) influx in percentage of control level, set at 100.}\)

\(\text{\(^{45}\text{Ca}^2+\) Measurements.}\) \(\text{\(^{45}\text{Ca}^2+\) uptake.}\) This was determined as described by Onozaki et al. (37) with slight modifications (38). To each monolayer, 1.0 ml of \(\text{\(P_i/NaCl\)}\) containing various concentrations of \(\kappa\)-elastin or 1 \(\mu\text{M}\) \(\text{\(^{45}\text{Ca}^2+\)}\) i onophore A23187 and 1.0 \(\mu\text{Ci}\) of \(\text{\(^{45}\text{Ca}^2+\)}\) (Isotope Inst. of the Hungarian Academy of Sciences, 96.37 GBq/g) was added; after 5, 15, 30, or 60 min of incubation, the cells were washed thoroughly with ice-cold buffer containing 4 mM \(\text{LaCl}_3\) and 0.05% bovine serum albumin. Finally, the monolayers were dried by vacuum aspiration and lysed with 1.0 ml of 0.2% \(\text{NaDodSO}_4\). The appropriate dilutions were prepared with distilled water, and radioactivity was determined on 500-\(\mu\text{l}\) aliquots by scintillation counting.

\(\text{\(^{45}\text{Ca}^2+\) efflux.}\) Measurements were as described by Stabinsky et al. (39). \(\text{\(^{45}\text{CaCl}_2\)}\) (3 \(\mu\text{Ci}\)) was added to the monolayers in 1.0 ml of \(\text{\(P_i/NaCl\)}\) in the presence of 1 \(\mu\text{M}\) A23187 (Calbiochem). After 60 min, the monolayers were washed with ice-cold \(\text{\(P_i/NaCl\)}\) containing 0.1% bovine serum albumin to remove A23187. One milliliter of \(\text{\(P_i/NaCl\)}\) containing or lacking 1 \(\mu\text{M}\) trifluoperazine (Smith Kline & French) and/or \(\kappa\)-elastin was added to the cell monolayers; at minutes 10, 20, 30, and 60 of incubation, the supernatants were removed by vacuum aspiration. Cells were washed vigorously with ice-cold buffer containing 4 mM \(\text{LaCl}_3\) and 0.05% bovine serum albumin. The monolayers were lysed with 1 ml of 0.2% \(\text{NaDodSO}_4\) solution, and after 60 min a 1:5 dilution was carried out with distilled water, and the radioactivity of 500-\(\mu\text{l}\) aliquots was determined.

\(\kappa\)-Elastin. This was prepared from bovine ligamentum nuchae elastin by alkaline hydrolysis in ethanol (24).

**RESULTS**

The \(\text{\(^{45}\text{Ca}^2+\)}\) and \(\text{\(^{22}\text{Na}^+\)}\) uptakes by monocytes obtained from middle-aged healthy subjects after addition of \(\kappa\)-elastin in different concentrations is shown in Table 1. As the \(\text{\(^{45}\text{Ca}^2+\)}\) transport is actively coupled to the \(\text{\(^{22}\text{Na}^+\)}\) transport via the

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**Fig. 1.** \(\text{\(^{86}\text{Rb}^+\)}\) uptake by monocytes in the presence of various concentrations of \(\kappa\)-elastin. \(\otimes\), ouabain-sensitive \((10 \mu\text{M})\) uptake; \(\boxplus\), ouabain-insensitive uptake.

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**Fig. 2.** \(\text{\(^{45}\text{Ca}^2+\)}\) influx in smooth muscle cells in the presence of various concentrations of \(\kappa\)-elastin or ionophore A23187. \(\bullet\), Control; \(\square\), 1 \(\mu\text{M}\) A23187; \(\triangle\), \(\kappa\)-elastin at 1 \(\mu\text{g/ml}\); \(\Delta\), \(\kappa\)-elastin at 10 \(\mu\text{g/ml}\).
Na+/Ca2+ transport mechanism, it was also interesting to study the Na+ uptake by monocytes (Table 1).

Our results demonstrate that κ-elastin in the applied concentrations significantly ($P < 0.001$) enhanced the $^{45}$Ca2+ and $^{22}$Na+ uptakes. The most effective concentration of κ-elastin for the stimulation was 0.5 μg/ml. Thus, it seems that κ-elastin stimulates the $^{45}$Ca2+ and $^{22}$Na+ uptakes in a concentration-dependent manner.

On the other hand, the Na+ transport appears to be coupled to the K+ transport (as shown by the $^{86}$Rb+ uptake) by the Na+,K+-ATPase. κ-Elastin inhibited $^{86}$Rb+ uptake by monocytes through the ouabain-sensitive Na+,K+-ATPase (Fig. 1), demonstrating an important ouabain-like action on monocytes with a maximal effect of κ-elastin at 0.5 and 1 μg/ml.

We also investigated the $^{45}$Ca2+ uptake induced by κ-elastin in SMC (Fig. 2) and fibroblasts (Fig. 3). In both cell types, the ionophore A23187 caused an important $^{45}$Ca2+ influx as compared to controls. κ-Elastin induced in SMC a $^{45}$Ca2+ influx that approached that caused by A23187 in an apparently dose-independent fashion. In fibroblasts, κ-elastin also stimulated the $^{45}$Ca2+ influx. This effect was progressive, attaining the level obtained with A23187.

As it was demonstrated that elastin peptides are chemotactic for mononuclear cells (25), it was interesting to study their effect on Ca2+ extrusion in the presence and absence of trifluoperazine, a calmodulin inhibitor. Our results concerning the $^{45}$Ca2+ extrusion from monocytes (Fig. 4) and SMC (Fig. 5) demonstrate that, under κ-elastin stimulation, it is markedly decreased as compared to controls ($P < 0.01$). In the case of monocytes (Fig. 4), trifluoperazine applied concomitantly with κ-elastin did not cause a further inhibition of $^{45}$Ca2+ extrusion as compared with that induced by κ-elastin alone. This seems to be the case for SMC also, but to a lesser extent (Fig. 5). Thus, it seems that the $^{45}$Ca2+ extrusion is inhibited by κ-elastin, possibly through the inhibition of the calmodulin-dependent Ca2+ pump (40).

DISCUSSION

It has been demonstrated in our laboratory (28, 29) that immunization of rabbits with highly purified elastin peptides in complete Freund's adjuvant induced calcified arterial
lesions accompanied by a pronounced fragmentation of the elastic fibers in the aorta and lung vessels. Besides their potential antigenicity, elastin-derived peptides have other biological properties, such as chemotactic activity for monocytes and fibroblasts (25). Monocytes were shown to play a role in the development of arteriosclerosis by penetration in the arterial wall and transformation to foam cells (41). SMC also play an important role in the development of the lesion as a result of their migration towards the intima, proliferation, and synthesis of excess matrix (4–6).

Our results show that elastin peptides stimulated markedly the 45Ca2+ influx and, at the same time, inhibited the 45Ca2+ extrusion by an apparently calmodulin-dependent mechanism. It seems then that κ-elastin acts on ion fluxes in an opposite way than formyl peptides do (38, 42–44). The stimulation of Ca2+ influx may well be an important trigger for the activation of monocytes (e.g., superoxide anion production and intracytoplasmic enzyme release) (39, 45). As the extrusion of Ca2+ is equally important both for cell activation (44) and for regulation of intracellular Ca2+ concentration, its inhibition by elastin peptides may well result in an accumulation of intracellular Ca2+, as suggested by the sustained rise of intracellular free Ca2+ (measured by quin-2) from 115 ± 4 nM Ca2+ per 106 cells in the absence of κ-elastin to 300 ± 12 nM Ca2+ per 106 cells in the presence of κ-elastin at 1 μg/ml (for details, see ref. 40). Our investigations did not permit us to determine whether the increase of intracellular free Ca2+ is due entirely to external Ca2+ influx or if the liberation of Ca2+ from intracellular storage sites is also a participating factor.

It has been demonstrated by Scully et al. (46) that the Ca2+ extrusion pump in monocyte plasma membrane increases 2-fold when the cytoplasmic free Ca2+ increases 2-fold. Thus, the increment in cytoplasmic Ca2+ (either by influx of external Ca2+ or by liberation from the intracellular storage) could account for the increment in Ca2+ extrusion mediated by the Ca2+-transport ATPase. However, this Ca2+ concentration-dependent extrusion mechanism is inhibited by κ-elastin in an apparently calmodulin-dependent fashion. The result would be a net increase of intracellular Ca2+. This contention was verified by the determination of intracellular free Ca2+. As elastin peptides inhibited the Ca2+ extrusion, the increased intracellular free Ca2+ induced an increase in the total Ca2+ content of cells.

Our results concerning the 22Na+ and 86Rb+ fluxes indicate an ouabain-like activity of the elastin peptides. The 22Na+ influx was stimulated, while the 86Rb+ influx was inhibited. These findings further suggest the accumulation of intracellular Ca2+ because of the reduced Ca2+ efflux via the Na+–Ca2+ exchange mechanism. This will decrease because of the rise in intracellular Na+, possibly as a consequence of an inhibited Na+, K+–ATPase (47). The increased intracellular Ca2+ levels in turn could have a direct effect on the inner surface of cell membranes, resulting in an inhibition of Na+, K+–ATPase, as demonstrated on erythrocytes (48). Such an effect would further increase the intracellular Ca2+ level. The above effects of elastin peptides are similar to those of the so-called “natriuretic hormones” (49). On the other hand, the alterations of ion fluxes produced by κ-elastin appear to be similar to those observed in hypertension (50).

This work was supported by Centre National de la Recherche Scientifique (UA 1174) and Conseil Scientifique, Université Paris XII. T.F. is the recipient of fellowships from Conseil Scientifique, Université Paris XII, and Fondation pour la Recherche Médicale Française.


37. Onozaki, K., Takenawa, T., Homma, Y. & Hashimoto, T.