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PRELIMINARY COMMUNICATIONS

NITROGLYCERIN STIMULATES THE SARCOLEMMAL Ca^{++}-EXTRUSION ATPase OF CORONARY SMOOTH MUSCLE CELLS

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Nitroglycerin (NTG) has been used successfully in the treatment of angina pectoris for over 100 years. Furthermore, there is now considerable evidence that NTG dilates not only normal but also abnormal coronary arteries, by inducing the relaxation of vascular smooth muscle (for review see ref. 1). Despite of NTG efficacy in the therapy of ischemic heart diseases, the fundamental biochemical mechanisms of NTG action are not well understood. The endothelium-independent vasodilatory effect of NTG has been widely attributed to a "direct" interaction with smooth muscle. However, this knowledge (or lack of knowledge ?) epitomized our paucity of information on the precise mechanism of NTG action (2). The exact subcellular site of NTG action still remains undefined.

A New Look at the Mechanism of NTG Action. The reduction of free intracellular Ca^{++} is the most important element promoting the vascular smooth muscle relaxation. Indeed, very recent studies using phosphorylase activation to monitor changes in intracellular Ca^{++} levels suggested that nitro vasodilators inhibit contraction by reducing free Ca^{++} concentrations in aortic (3) and coronary smooth muscle (P. Galvas, personal communication). Theoretically, NTG could lower free intracellular Ca^{++} by stimulating: a) Ca^{++} uptake by the sarcoplasmic reticulum, b) Ca^{++} binding to the sarcolemma, or c) Ca^{++} extrusion from of the cell. But the mechanisms a and b can be ruled out based on the results reported by Kuriyama and coworkers (4, 5). Therefore, the extrusion of Ca^{++} appears as the best candidate for NTG action at the subcellular level. Because the ATP-fuelled Ca^{++} ejection, operated by a sarcolemmal (SL) Ca^{++}-ATPase pump, is the principal extrusion pathway in vascular smooth muscle (6), it seems attractive to believe that the SL Ca^{++}-ATPase is a molecular target (effector) for NTG action. Some data supporting this hypothesis have been communicated (7).

We present here direct evidence for the existence of a SL Ca^{++}-ATPase in pig coronary artery smooth muscle. This enzyme has the important biochemical properties expected for a typical plasma membrane Ca^{++}-extrusion ATPase, and its specific activity is strongly stimulated by NTG. In short, we are suggesting that the stimulation of SL Ca^{++}-ATPase is causally linked to NTG-induced vasodilatation.

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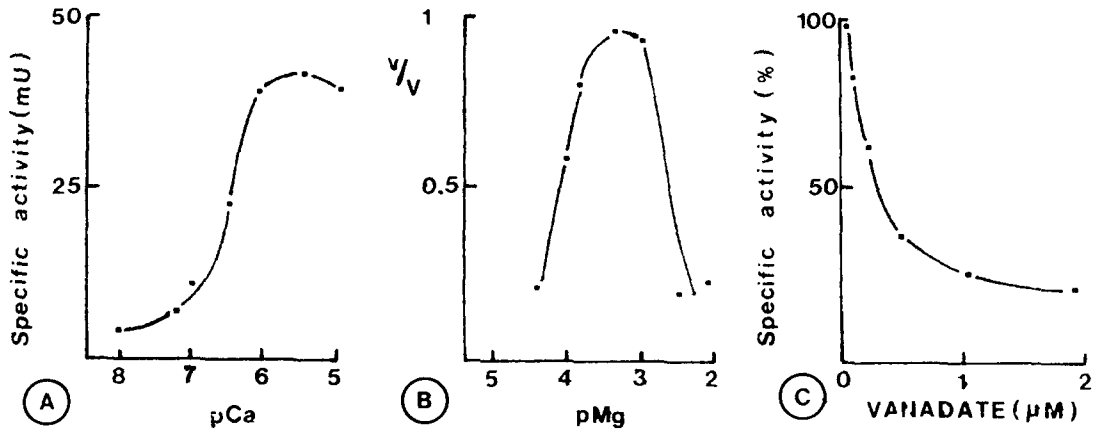

Fig. 1. Characteristics of Ca^{++}-ATPase from the sarcolemma of pig coronary artery smooth muscle. A: pCa dependence of the specific activity (pCa = -log free (Ca^{++}); mU = miliunits, nmoles P_i/mg protein/min). Five samples were averaged to obtain each point. B: Activation by pMg; v, ATPase activity at given pMg; V, maximal ATPase activity obtained at saturating free-Mg^{++} concentration. Points represent the average of 3-5 samples. C: Vanadate inhibition. The enzyme activity was estimated in the standard assay medium (pCa 6) supplemented with Na_3VO_4. Points represent the average of 3-5 samples. The 100% activity of Ca^{++}-ATPase corresponds to 2.4 μmoles P_i/mg protein/h.

MATERIAL AND METHODS

We took advantage of the recent isolation and characterization of the SL Ca^{++}-extrusion ATPase from human myometrium (8, 9) to identify the corresponding enzyme of pig coronary smooth muscle (tunica media of the left anterior descending artery). The endothelium was removed and then the adventitia was dissected out.

Isolation of SL Sheets. The procedure was essentially as previously described in detail (8) except that the initial homogenization medium was 0.05 M KCl. A progressive increase of 5'-nucleotidase activity, which is a plasma membrane marker, paralleled the separation of sarcolemmae (aprox. 12-fold enrichment over the initial homogenate). It should be noted that the method produces open membrane sheets, which do not permit the study of Ca^{++} transport. In several experiments, the isolated sarcolemmae were solubilized with SDS to unmask the latent activity of Ca^{++}-ATPase (8).

Assay of Ca^{++}-ATPase Activity. The reaction was determined spectrophotometrically by measuring the release of P_i from ATP in an assay medium which simulated the free ionic concentrations of the smooth muscle cytosol (8). A computer program, adopted from Fabiato and Fabiato (10), was used to obtain specific free concentrations in the standard assay medium (mM): Ca^{++}, 0.001; Mg^{++}, 0.5; K^+, 74; Na^+, 7.6; ATP, 0.4; pH 7.4. When the free concentrations of Ca^{++} or Mg^{++} were varied in a given domain (Fig. 1 A or B) the other ionic concentrations were kept constant. Ouabain (0.2 mM) was always added to the assay medium.

RESULTS AND DISCUSSION

Characterization of the SL Ca^{++}-ATPase of Coronary Artery Smooth Muscle. Fig. 1 shows that the enzyme has the characteristic features expected for a plasma-membrane Ca^{++}-extrusion ATPase, according to the criteria formulated repeatedly during the last few years by Schatzmann (11), Penniston (12) and Carafoli et al. (13). The high Ca^{++}-affinity of ATPase activity (Fig. 1 A) is expressed by an apparent K_m(free Ca^{++}) of 0.3 μM. The activation by free Mg^{++} (Fig. 1 B) is

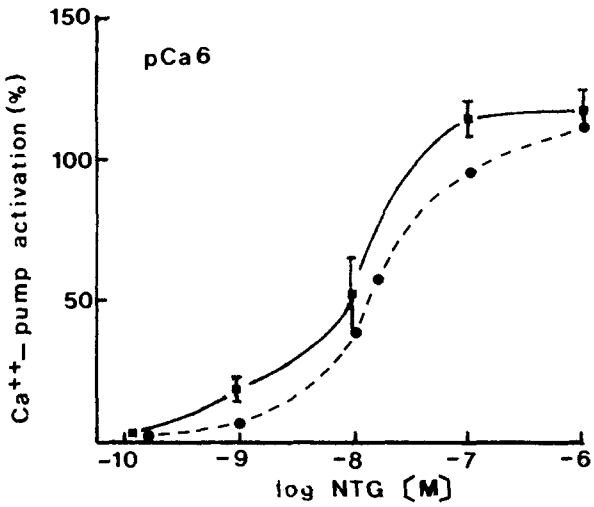

Fig. 2. Dose-response curves (experimental and calculated) of the solubilized sarcolemmal Ca^{++}-pump ATPase activation versus NTG concentration. ▪ mean activating responses to NTG expressed as percentage (± SD) of the basal activity (n = 84, six experiments). ● values for response calculated from Clark's equation:

$$A = A_m \frac{(X)}{(X) + K_x}, \text{ where}$$

A is the activating response, A_m is the maximum activating response, (X) is the concentration of NTG used, and K_x is the apparent dissociation constant of the presumed drug-receptor complex. The ED_{50} (2.5×10^{-8} M) was used as an approximation for K_x.

also functionally relevant: the apparent K_m(free Mg^{++}) is about 0.2 mM, and the free cytoplasmic-Mg^{++} in vascular smooth muscle is 0.1-1.0 mM (14). The inhibition by low concentrations of vanadate (Fig. 1 C; $I_{50} < 1$ μM) is another biochemical marker. Last but not least, the enzyme is activated by calmodulin. The specific activity of SL Ca^{++}-ATPase was increased about 4 times, at pCa 6, when highly purified calmodulin (Sigma) was added (1 ng/μg of membrane protein).

Because of the similarity between the properties of the SL Ca^{++}-ATPase from coronary smooth muscle and Ca^{++}-ATPases of erythrocyte membranes, heart sarcolemma (12, 13) or stomach sarcolemma (15), which are well known to function as Ca^{++}-extrusion ATPases (pumps), it is most probable that the coronary enzyme is also a Ca^{++}-extrusion ATPase. Additional support is provided by the work of Furukawa and Nakamura (16) on vascular smooth muscle (bovine aorta). They purified a SL Ca^{++}-ATPase, which has virtually identical properties with "our" SL Ca^{++}-ATPase, and showed that the enzyme reconstituted in liposomes acted as a Ca^{++}-transport ATPase.

NTG Stimulation of Ca^{++}-Extrusion ATPase and Its Significance. Fig. 2 shows that NTG is a potent activator of the detergent-solubilized Ca^{++}-extrusion ATPase isolated from porcine coronary smooth muscle. Moreover, close agreement was found between the experimental dose-response curve and the theoretical curve. NTG also stimulated the activity of the membrane-bound SL Ca^{++}-ATPase: an increase of the specific activity with 181%± 19% (n=12, three experiments) was produced by 1 μM NTG at pCa 6. Apparently, the stimulating effect of NTG does not depend on calmodulin, since the addition of excess calmodulin (100 ng/ml) did not modify significantly the NTG-induced activation. Anyway, the stimulation of coronary SL Ca^{++}-ATPase seems to be a specific response to NTG, because NTG had no effect on the erythrocyte Ca^{++}-extrusion ATPase (7).

The results of this study strongly suggest that the NTG-induced relaxation is causally linked to stimulation of the SL Ca^{++}-extrusion ATPase. Beside the definite indication that nitro compounds produce their vasodilating effect by interference with a yet undefined process within the cell membrane (17), the following arguments can be enumerated in favour of our hypothesis.

1) The ED_{50} that we found in test tube (Fig. 2) is comparable with the concentration of NTG (4.4×10^{-8} M) reported to cause 50% relaxation of coronary strips in organ bath (18).

2) If the activation of the SL Ca^{++}-ATPase is essential for the relaxant effect of NTG, then other nitro vasodilators should be expected to stimulate the SL Ca^{++}-ATPase too. We tested the action of isosorbide-dinitrate and isosorbide-5-mononitrate (L.M.Popescu et al., in preparation) and, indeed, these isosorbide nitrates (10^{-8} M) appeared to stimulate the SL Ca^{++}-ATPase.

3) Recent studies from Kuriyama's laboratory (4, 5) documented the lack of relaxing effect of nitro vasodilators on chemically skinned vascular smooth muscle fibers.

4) Several studies (2, 18-20) demonstrated that cGMP is involved in mediating the coronary smooth muscle relaxation produced by NTG. However, the exact mechanism of cGMP-induced relaxation remained elusive. Very recently, we provided evidence that cGMP activates the SL Ca^{++}-extrusion ATPase of coronary smooth muscle (21). The activation is achieved via the cGMP-dependent protein kinase which phosphorylates the SL Ca^{++}-ATPase.

5) Deeg and Schneider (22) reported that, between the 1st and 5th minute after the intracoronary NTG administration, Ca^{++} concentration increased significantly (about 10%; $p < 0.01$) in the coronary venous blood. Their findings indicate that NTG produces an increase of Ca^{++} efflux from coronary smooth muscle and our data provide a plausible molecular explanation.

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