EFFECT OF ENDOTHELIN-I ON Na^+/H^+ EXCHANGE IN VASCULAR SMOOTH MUSCLE CELLS

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Endothelial cells of blood vessels, besides having metabolic and antithrombotic properties (for example, prostaglandin formation), also play an important role in the regulation of vascular tone. It has been shown [7] that relaxation of the rabbit aorta in response to acetylcholine is effected by a substance or substances secreted from endothelial cells, such as EDRF. Endothelial cells also are known to form vasoconstrictors [8, 11].

Recently Yanagisawa and co-workers [15] isolated from the supernatant of endothelial cells treated with either an ionophore or thrombin, a peptide which they called endothelin, and which causes considerable and prolonged vasoconstriction both in vitro and in vivo [4, 6, 11, 15]. The endothelin molecule has been shown to consist of 21 amino acid residues and to be formed from its precursor (preproendothelin), which includes residues of 2-3 amino acids, by an unusual proteolytic pathway [11, 15]. However, aside from the presence of specific receptors on the membrane of vascular smooth-muscle cells (VSMC), the molecular mechanisms of its action have not been finally established, and the evidence is contradictory [6, 9, 10, 12, 13].

An important role in the realization of the effects of different vasoconstrictors on VSMC is played by Na^+/H^+-exchange [5, 12]. So far as endothelin is concerned, changes in this exchanger in VSMC have been round in only a single investigation [12], but the mechanisms of involvement of Na^+/H^+-exchange in the response to endothelin were virtually not studied on that occasion.

In the present investigation we compared the effects of endothelin and phorbol ester, a protein kinase C (PKC) activator, on Na^+/H^+-exchange in VSMC. Activity of the exchanger was judged by changes in the intracellular pH (pH_i), with the aid of the specific fluorescent probe BCECF.

EXPERIMENTAL METHOD

Experiments were carried out on aortic SMC of normotensive Wistar-Kyoto rats. Isolation of the cells and their growth in culture were described in detail previously [5, 13]. The culture of VSMC was maintained in modified DMEM medium containing 10% FCS, 1% glutamine, and 1% of antibiotics (penicillin, streptomycin, fungisone), at 37°C in a CO_2 incubator (5% CO_2 + 95% O_2). In the present investigation 3-10 passages of the VSMC were used. The medium was changed 24 h before the investigations for one not containing FCS. On the day of the experiment the VSMC were transferred into Hanks' solution (without magnesium and calcium ions), containing trypsin:EDTA (0.05%:0.02%) for 5-10 min; subsequent addition of FCS halted the effect of trypsin. The VSMC were then sedimented (1000*g*, 5 min) and resuspended in Hanks solution (0.5 mM Ca^{2+} and 1 mM Mg^{2+}), and an aliquot was taken in order to determine cellular autofluorescence and to count the cells. The cell suspension was then incubated with BCECF-AM (1.5 mM) at 37°C for 30 min. After incubation the VSMC were sedimented and the cells

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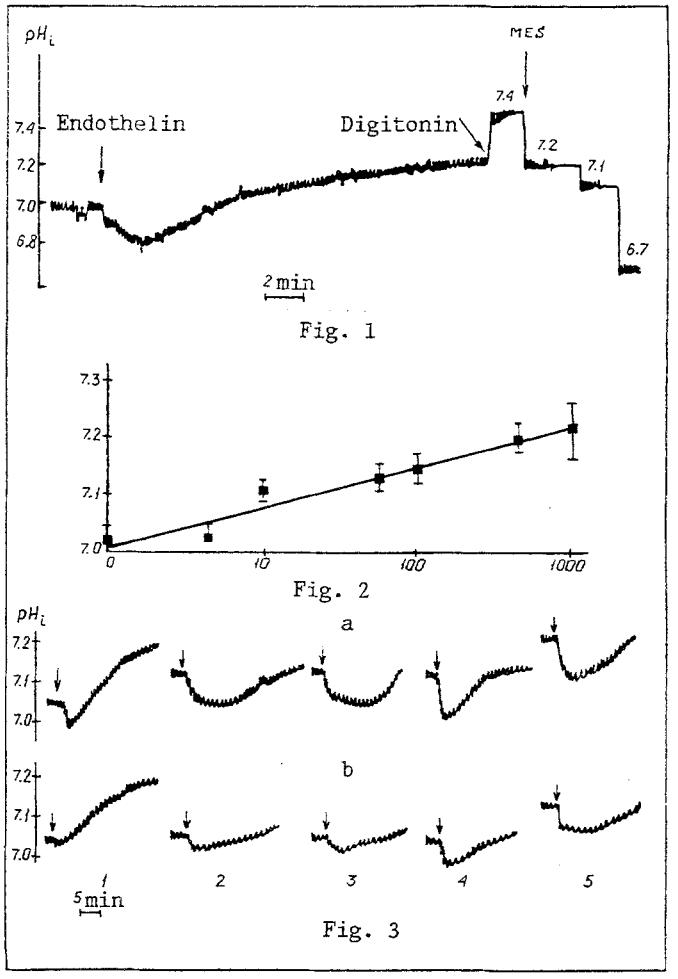

Fig. 1. Effect of 10^{-7} M endothelin-I on intracellular pH (pH_i) in vascular smooth-muscle cells (VSMC). Ordinate, change of pH_i. Calibration of fluorescence signal of BCECF shown on right.

Fig. 2. Dose-dependent activation of Na^+/H^+-exchange in VSMC by endothelin-I. Abscissa, doses of endothelin-I (in nM); ordinate, changes in pH_i.

Fig. 3. Effect of inhibitors of Na^+/H^+-exchange and of protein kinase C on changes in pH_i in VSMC under the influence of endothelin-I (a) and of phorbol ester (b). Arrow indicates time of injection of substance; 1) effect of agonist in medium containing 130 mM Na^+; 2) in incubation medium with 0 mM Na^+; 3) effect of EIPA (10^{-6} M); 4) effect of substance H-7 (10^{-4} M); 5) effect of staurosporin (10^{-6} M).

resuspended in the same buffer, divided into aliquots ($2 \cdot 10^6$ cells/ml), and left to stand until fluorescence was measured at 20°C in darkness (preliminary experiments showed that under these conditions the cells remain active and no escape of the probe is observed for 3-4 h). Before measurement the samples were heated for 5 min to 37°C and the VSMC were sedimented on a Beckman "Microfuge" (5000*g*, 5 sec) and resuspended in solution of the following composition (mM): NaCl 130, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, HEPES 20, glucose 10, or in the same solution, but in which Na^+ was replaced by an equimolar concentration of choline chloride. Fluorescence of BCECF was measured on a Shimadzu-5000 spectrofluorometer at 37°C. Wavelengths of excitation and emission were 500 and 535

nm respectively, and the width of the monochromator slits was 2.5 nm. The fluorescence signal was calibrated with digitonin (25 μM) and MES (330 mM), and this was followed by recording of the pH [1, 3, 5].

EXPERIMENTAL RESULTS

The basal level of the intracellular pH_i, measured with the aid of the fluorescent probe BCECF, averaged 7.02 ± 0.07 (54 measurements), in agreement with data in the literature [5, 12]. Typical changes in fluorescence of BCECF on the addition of 100 nM endothelin-I are given in Fig. 1. Clearly endothelin-I evoked an initial rapid (30 sec) fall of pH_i, on average by 0.05 unit, followed by alkalification of the cytoplasm. The rise of pH_i, moreover, took place slowly and reached a maximum after 15-20 min. Whereas phase 1 of the action of endothelin-I on pH_i was independent of the dose of agonist (not shown), the degree of alkalification of the cytoplasm was dose-dependent (Fig. 2): IC_{50} was of the order of $3 \cdot 10^{-8}$ M. In other words, endothelin-I caused a biphasic change in pH_i: a small decrease of pH_i, possibly as a result of activation of proton-generating biochemical processes and/or an increase in the intracellular Ca^{2+} concentration, which is characteristic of the action of other vasoconstrictors [5] or of endothelin-I (our own preliminary investigations), and gradual prolonged and sustained rise of pH_i, which, as we showed for the first time, is dependent on the dose of the agonist.

Evidence that the observed changes in pH_i were in fact the result of activation of Na^+/H^+-exchange was given by data obtained when this process was inhibited. The same approaches were used in the present study as in our previous investigations on platelets [1, 3]. In incubation medium not containing Na^+ or after preliminary incubation (5-10 min) of the cells with $5 \cdot 10^{-6}$ M EIPA, a specific inhibitor of Na^+/H^+-exchange [1, 3], endothelin-I (10^{-7} M) did not cause an increase in pH_i – only a decrease took place, and it was stronger and more prolonged than in the control (Fig. 3a). On the other hand, simple alkalification of the cytoplasm with NH_4Cl (data not given) did not lead to any changes characteristic of endothelin-I. It can therefore be postulated that endothelin-I, like any other vasoconstrictor, such as angiotensin II [5], increases the intracellular Na^+ ion concentration as a result of activation of Na^+/H^+-exchange.

Since an important role in the mechanisms of activation of Na^+/H^+-exchange in VSMC is played by PKC [5], we studied the action of its direct stimulator, phorbol ester (PMA), In a concentration of 10^{-7} or $5 \cdot 10^{-8}$ M PMA induced a slow but sustained rise of pH_i (Fig. 3b), analogous to that produced by endothelin-I, which disappeared on inhibition of Na^+/H^+-exchange, but unlike endothelin-I, PMA caused alkalification on the cytoplasm without a preliminary phase of lowering of pH_i. In connection with these results it is interesting to note that contraction of a strip of rat aorta caused by endothelin-I or PMA was just as sustained and as slowly developing [4, 6] as the increase in pH_i.

To answer the question whether PKC is involved in activation of Na^+/H^+-exchange induced by endothelin-I special experiments with PKC blockers were carried out. As will be clear from Fig. 3a, preincubation of VSMC with substance H-7 (10^{-4} M) for 20-30 min inhibited the effect of endothelin-I; the acidification phase, moreover, was more marked: on average it was twice the magnitude of that observed under the influence of the agonist alone. Similar results were obtained by the use of staurosporin (10^{-6} M), another PKC inhibitor. These inhibitors naturally suppressed the effect of PMA (Fig. 3b).

The results are thus evidence that endothelin activates Na^+/H^+-exchange, possibly through activation of PKC, as the results of the present investigation have shown. It can accordingly be postulated that endothelin-I, by interacting with the specific receptor, leads to transmission of the signal through G-proteins to phospholipase C, and forms two secondary messengers: inositol triphosphate, facilitating release of Ca^{2+} from the intracellular depots, and diacylglycerol which activates PKC and, as our results showed, thereby activates Na^+/H^+-exchange. However, this plan of action of endothelin was not confirmed by a number of investigations, because no increase in the turnover of phosphoinositol in VSMC or activation of phospholipase C was found under the influence of endothelin-I [9, 10]. The question of how endothelin-I activates PKC, and through it, Na^+/H^+-exchange, thus remains unanswered.

The results of this investigation are interesting from another standpoint also. On the one hand, as our preliminary investigations and results of other workers [11] showed, endothelin-I stimulates cellular proliferation of VSMC, and on the other hand, Na^+/H^+-exchange is known to have an important role in the regulation of cell proliferation [14]. Hence it can be postulated that endothelin-I, through activation of the exchanger, can participate

in the development of hypertrophy of VSMC, which plays an important role in the pathogenesis of arterial hypertension [2].

REFERENCES

1. M. L. Borin, V. G. Pinelis, S. S. Bykov, et al., Biol. Membr., **5**, 536 (1988).
2. V. G. Pinelis, A. V. Kozlov, T. P. Vaulina, and Kh. M. Markov, Kardiologiya, No. 5, 66 (1983).
3. V. G. Pinelis, M. L. Borin, S. S. Bykov, et al., Byull. Éksp. Biol. Med., No. 9, 304 (1988).
4. M. Auguet, S. Delaflotte, P. E. Chabrier, et al., Biochem. Biophys. Res. Commun., **156**, 156 (1988).
5. B. C. Berk, M. S. Aronow, T. A. Brock, et al., J. Biol. Chem., **262**, 5057 (1987).
6. P. E. Chabrier, P. Roubert, M. O. Longchamp, et al., J. Biol. Chem., **263**, 13199 (1988).
7. R. F. Furchgott and J. V. Zawadzki, Nature, **288**, 373 (1980).
8. K. A. Hickey, G. Rubanyi, R. I. Paul, and R. F. Highsmith, Am. J. Physiol., **248**, 550 (1985).
9. Y. Hirata, R. Yoshimi, S. Takata, et al., Biochem. Biophys. Res. Commun., **154**, 868 (1988)
10. Y. Hirata, J. Cardiovasc. Pharmacol., **13**, Suppl. 5, 157 (1989).
11. T. Masaki, J. Cardiovasc. Pharmacol., **13**, Suppl. 5, 1 (1989).
12. H. Meyer-Lehnert, C. Wauning, H. G. Predel, et al., Biochem. Biophys. Res. Commun., **163**, 458 (1989).
13. P. Roubert, V. Gillard, P. Plas, et al., Am. J. Hypertens, **2**, 62A (1989).
14. Z. Vicentini and M. L. Villereal, Life Sci., **38**, 2269 (1986).
15. M. Yanagisawa, H. Kurihava, and S. Kimura, Nature, **332**, 411 (1988).