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EFFECTS OF ENDOTHELIN ON SODIUM TRANSPORT MECHANISMS: POTENTIAL ROLE IN CELLULAR Ca^{2+} MOBILIZATION

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The effects of endothelin on cellular Ca^{2+} mobilization were examined in cultured rat vascular smooth muscle cells (VSMC). Endothelin (10^{-8}M) induced a rapid transient increase of $[Ca^{2+}]_i$ from 77±3 to 104±5 nM ($p<.05$) in VSMC. Preincubation (60 min) with endothelin ($2x10^{-8}$M) increased basal $[Ca^{2+}]_i$ from 77±3 to 105±8 nM ($p<.05$). Preincubation with endothelin also enhanced vasopressin (10^{-7}M)-stimulated peak levels of $[Ca^{2+}]_i$ (528±20 nM vs 969±21 nM, $p<.01$). Endothelin (10^{-7}M) induced an intracellular alkalinization (7.18±0.03 vs 7.37±0.04, $p<.01$) which was blocked by pretreatment with amiloride. The biphasic effects of endothelin on $[Ca^{2+}]_i$ were similar to those of an endogenous inhibitor of Na-K-ATPase that we examined in a previous study. Therefore, we examined the effects of endothelin on Na-K-ATPase in an enzyme preparation from hog cerebral cortex. At high concentrations, endothelin (10^{-5}M) inhibited Na-K-ATPase in vitro. Thus, endothelin may exert its vasoconstrictor effects at least in part via alterations of cellular Ca^{2+} mobilization in VSMC. While the rapid transient increase of $[Ca^{2+}]_i$ appears to reflect intracellular Ca^{2+} mobilization, the sustained effect on $[Ca^{2+}]_i$ may be related to an increase of intracellular sodium mediated by inhibition of Na-K-ATPase and/or more likely by stimulation of the Na^+/H^+-antiport.

The recently discovered endogenous vasoconstrictor endothelin exerts a potent vasopressor effect in vivo and in vitro in various vascular preparations (1-6). It has been shown that endothelin-induced vasoconstriction is dependent on extracellular calcium and that it is markedly attenuated by calcium channel blockers (1,2,6). Furthermore, it has been found that the peptide sequence of endothelin shows regional homologies to a group of neurotoxins that directly act on voltage-dependent ion channels (1). Thus, it has been suggested that endothelin may affect the intracellular calcium messenger system (1).

Vasopressor-induced contraction of vascular smooth muscle is associated with cellular alkalinization that is mediated by the Na^+/H^+-antiport (7). The

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stimulation of this antiport is thought to play a central role in the sustained phase of smooth muscle contraction.

It has also been proposed that another vasoconstrictor agent, the as yet putative endogenous inhibitor of sodium transport may affect vascular tone by altering cellular calcium mobilization (8,9).

Activation of the Na^+/H^+-antiport and inhibition of Na-K-ATPase may synergistically lead to an increase of intracellular sodium. Accumulation of sodium may then inhibit the Na^+/Ca^{2+}-exchange, a system for cellular Ca^{2+} extrusion (10).

In the present study, we examined in vitro the effects of synthetic endothelin on intracellular calcium and pH in cultured rat vascular smooth muscle cells (VSMC). We also investigated the effects of endothelin on the Na-K-ATPase enzyme.

MATERIALS AND METHODS

Endothelin was obtained from the Peptide Institute, Kyoto, Japan. Fura 2 and a Na-K-ATPase enzyme preparation derived from hog cerebral cortex were purchased from Sigma (St. Louis, MO). 2,7-biscarboxyethyl-5(6)-carboxyfluorescin (BCECF) was from Molecular Probes (Eugene, OR).

Isolation and culture of VSMC

Rat aortic smooth muscle cells were isolated using a modified method (11) originally described by Chamley et al. (12). Briefly, thoracic aortas from male Sprague-Dawley rats were dissected and incubated in Eagle's minimum essential medium (MEM) containing 2 mg/ml collagenase (Cooper Biomedical, Malvern, PA). After dissecting the adventitia, aortas were minced with sterile razor blades and incubated again for 2-3 h in MEM containing collagenase. The resulting single cell suspension was washed and the cells were plated onto 35-mm culture dishes. MEM supplemented with 10 % fetal calf serum and antibiotics was used as culture medium. The cultures reached confluence after 7-10 d and were then subcultured. For experiments, passages 2 to 7 were used.

Measurement of intracellular free Ca^{2+}

For measurement of $[Ca^{2+}]_i$, VSMC were grown on round glass cover slips (13 mm diam.) as previously described (13). Upon confluence, the cells were washed and loaded with 4μM fura 2 for 30 min in MEM containing no fetal calf serum. The cover slips were then washed three times and rinsed again with buffer containing 2 mM Ca directly before the measurements. For measurements, cover slips were placed into disposable fluorescence cuvettes.

Fluorescence measurements were carried out with a Hitachi F4000 fluorescence spectrophotometer which was set to shuttle between the two excitation

wavelenghts of 340 and 380 nm every 4 sec. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (13).

Measurement of intracellular pH

Intracellular pH was measured using the fluorescent probe BCECF acoording to Rink et al. (14) with minor modifications. VSMC were incubated for 30 min in HEPES buffer containing 1 µM esterified BCECF (BCECF-AM). Cover slips with VSMC were then washed three times and rinsed again in buffer directly before the experiment. Fluorescence was measured in a Hitachi F4000 fluorescence spectrophotometer with excitation wavelength set at 500 nm and emission wavelength at 530 nm. At the end of the experiment cells were lysed with digitonin (50 µM). Calibration of pH was done according to Reid et al. (15).

In vitro effects on Na-K-ATPase

In these experiments, a commercially available enzyme from hog cerebral cortex as well as an ATPase preparation from renal medullary tissue homogenate were employed as previously described (16). Endothelin from the Peptide Institute, Kyoto, Japan and endothelin from Peninsula (St.Helens, UK) were preincubated with the enzyme for 60 min at 4°C; the mixture was then added to the substrate solution in the presence and absence of 1 mM ouabain. The reaction was stopped by adding ice-cold 10 % trichloro-acetic acid (TCA). After centrifugation at 1700 g for 5 min 1.0 ml supernatant was assayed for inorganic phosphate (17).

RESULTS

Intracellular Ca^{2+} Concentrations and pH

Within seconds, endothelin (10^{-8}M) induced a rapid increase of $[Ca^{2+}]_i$ (77±3 nM vs 104±5 nM, $p<.05$) in VSMC. However, compared to the stimulatory effect of arginine vasopressin (AVP, 10^{-7}M) on $[Ca^{2+}]_i$ (77±3 nM vs 528±20, $p<.001$) the increase by endothelin was much smaller (Fig.1). The fast endothelin-induced increase of $[Ca^{2+}]_i$ was only transient; calcium concentrations declined to basal levels within less than 2 min. Preincubation (60 min) with endothelin increased basal $[Ca^{2+}]_i$ from 77±3 to 105±8 nM ($p<.05$) in VSMC (Fig. 2A). This increase of basal $[Ca^{2+}]_i$ persisted for an observation time of 30 min. Preincubation with endothelin also markedly enhanced AVP(10^{-7}M)-stimulated peak levels of $[Ca^{2+}]_i$ (528±20 nM vs 969±21 nM, $p<.01$) (Fig.1). Thus, endothelin and AVP had a potentiating effect on $[Ca^{2+}]_i$.

The Ca^{2+}-mobilizing effects of endothelin resembled those of a previously examined inhibitor of Na-K-ATPase, a "ouabain-like factor" isolated from human urine (11). Thus, we compared the effects of endothelin with those of ouabain, a well known inhibitor of sodium transport. Ouabain did not exhibit a

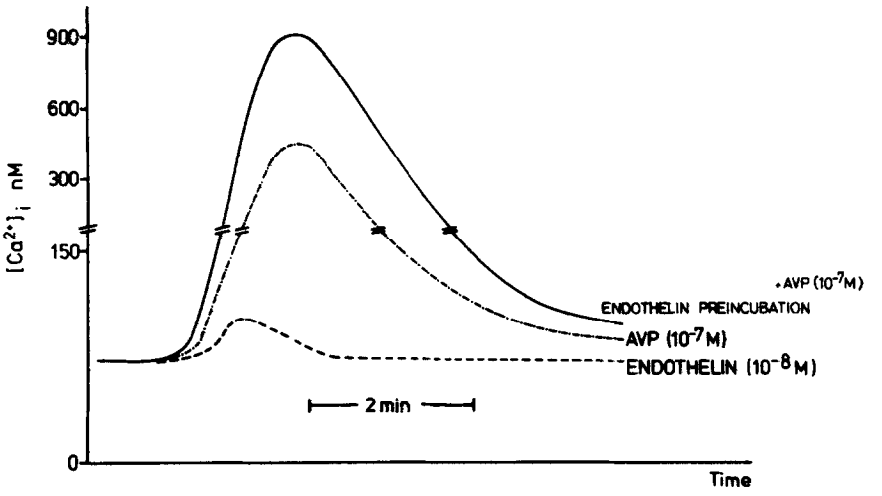

Figure 1. Fura 2 measurements of $[Ca^{2+}]_i$ in vascular smooth muscle cells; each curve has been redrawn and represents a typical time course of $[Ca^{2+}]_i$ with the mean peak value (4-6 experiments per group, mean±S.E.M.).

fast transient effect on $[Ca^{2+}]_i$ (data not shown); however, preincubation with ouabain (10^{-5}M) resulted in a sustained increase of $[Ca^{2+}]_i$ from 77±3 nM to 158±7 nM in VSMC ($p<.01$) (Fig.2A).

Endothelin (10^{-7}M) induced an intracellular alkalinization (7.14±0.03 vs 7.31±0.04 at 20 min, $p<.01$). This effect was blocked by 30 min pretreatment with amiloride (10^{-5}M) (Fig. 2B).

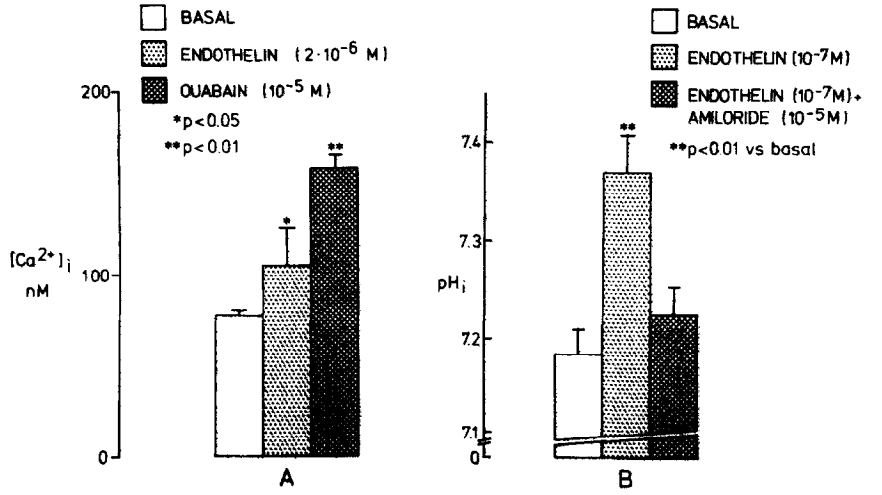

Figure 2. A. Fura 2 measurements of $[Ca^{2+}]_i$ in vascular smooth muscle cells; measurements were taken after 60 min preincubation with buffer, endothelin, and ouabain, respectively (4-6 experiments per grou, mean ± S.E.M.); B. Intracellular pH (pH_i) in VSMC (4 and 5 experiments per group, resp., mean ±S.E.M.).

Na-K-ATPase

Enzyme activity of the hog cerebral Na-K-ATPase preparation was 33.9±0.3 µmol P_i/mg prot x h. Mg-ATPase activity was measured in the presence of 1 mM ouabain and was less than 1.5 % of total ATPase. In a first series of experiments enzyme activity was 31.8±1.9, 27.9±2.4, and 23.6±3.2 µmol P_i/ mg prot x h in the presence of 10^{-9}, 10^{-7}, and 10^{-5} M endothelin from Peptide Institute, respectively; i.e. inhibition of Na-K-ATPase was 6.1%, 17.6%, and 30.5%, respectively. Na-K-ATPase activity in the presence of 1.5 x 10^{-6} ouabain was series of experiments using endothelin purchased from Peninsula incubated with freshly prepared renal medullary tissue homogenate, this degree of enzyme inhibition at 10^{-5}M endothelin as observed in the first series of experiments was not confirmed.

DISCUSSION

In the present study we demonstrate that endothelin exhibits a biphasic stimulatory effect on intracellular free calcium concentration, $[Ca^{2+}]_i$, and induces cellular alkalinization in VSMC. Furthermore, at high concentrations endothelin may also inhibit Na-K-ATPase.

Endothelin induced a rapid transient increase of $[Ca^{2+}]_i$ in VSMC. Elevated Ca^{2+} concentrations returned to baseline within less than two minutes. This is in accordance with several studies reporting a rapid transient increase of $[Ca^{2+}]_i$ in VSMC (18,19,20) and in smooth muscle-like mesangial cells (4,21). This effect may be accounted for at least in part by mobilization of Ca^{2+} from intracellular stores via inositol trisphosphate (4,22, 23,24).

In addition to the fast transient increase of $[Ca^{2+}]_i$ we observed a rise of basal $[Ca^{2+}]_i$ in VSMC after 60 min preincubation with endothelin. This observation is again consistent with the findings by others who also observed a sustained effect of endothelin on $[Ca^{2+}]_i$ (18,19). To further elucidate the potential mechanism of action of endothelin in the sustained increase of $[Ca^{2+}]_i$ we examined its effects on the Na^+/H^+-antiport. In the present study, endothelin induced a significant alkalinization in VSMC and this effect was blocked by amiloride, an inhibitor of Na^+/H^+ exchange. A similar effect of endothelin has been reported for mesangial cells (21). Other vasopressors as well have been found to induce an intracellular alkalinization in vascular smooth muscle and this effect is thought to be linked to the sustained contractile response (7).

Recently, we (8) as well as others (9) have shown that endogenous inhibitors of Na-K-ATPase increase intracellular calcium concentrations in vascular smooth muscle. Therefore, we also examined the in vitro-effect of endothelin

on Na-K-ATPase. We found that endothelin at high concentrations may inhibit the Na-K-ATPase enzyme.

Recent evidence obtained in rat renal epithelial cells suggests inhibition of sodium transport by endothelin (25). These authors suggested that the Na-K-ATPase-inhibiting effect may depend on the presence of intact cells (25). Thus, in VSMC the effect of endothelin to inhibit Na-K-ATPase may be more pronounced than under the conditions of the present in vitro-study. However, these as well as our findings will have to be confirmed using different endothelin preparations and the actual inhibition of transmembrane sodium transport still remains to be demonstrated.

The role of extracellular Ca^{2+} in the sustained effect of endothelin cannot be addressed directly by the methods used in the present study, because incubation of cells in zero-Ca^{2+} medium depletes cellular Ca^{2+}. However, there is evidence that transmembrane Ca^{2+} influx also plays a role in the sustained effect of endothelin (18). Increased Ca^{2+} uptake by vasopressor-sensitive intracellular Ca^{2+} stores could explain our finding that the Ca^{2+}-mobilizing effect of AVP was greatly enhanced after preincubation with endothelin, while we did not observe an additive effect of endothelin and AVP on $[Ca^{2+}]_i$ when endothelin was added simultaneously with AVP (data not shown).

In summary, endothelin may exert its vasoconstrictor properties by affecting spontaneous and vasopressor-induced Ca^{2+} kinetics in vascular smooth muscle. The slow and long lasting increase of $[Ca^{2+}]_i$ by endothelin may in part be related to stimulation of Na^+/H^+-antiport and inhibition of Na-K-ATPase. While either effect alone may perhaps not fully account for the tonic elevation of $[Ca^{2+}]_i$, both these effects may synergistically increase intracellular sodium, thus leading to an inhibition of Na-Ca-countertransport, a high-capacity Ca^{2+} transport system that supposedly regulates $[Ca^{2+}]_i$ (26). Further studies will be needed to examine potential effects of endothelin on Ca-Mg-ATPase, the exact role of extracellular Ca^{2+} and the involvement of Ca^{2+} channels in the action of endothelin.

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REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., and Masaki, T. (1988) Nature 322, 411-415

2. Kurihara, H., Yanagisawa, M., Yoshizumi, M., Kimura, S., Goto, K., Takaku, F., Masaki, T., and Yazaki, Y. (1988) Int. Soc. Hypertens., Kyoto 0614 (Abstr.)

3. Yanagisawa, M., Kurihara, H., Kimura, S., Goto, K., and Masaki, T. (1988) J. Hypertens. 6(Suppl.4), S188-S191

4. Badr, K.F., Murray, J.J., Breyer, M.D., Takahashi, K., Inagami, T., and Harris, R.C. (1989) J. Clin. Invest. 83, 336-342

5. King, A.J., Anderson, S., and Brenner, B.M. (1988) Kidney Int. 35, 314 (Abstr.)

6. Loutzenhiser, R., Epstein, M., and Hayashi, K. (1988) Kidney Int. 35, 315 (Abstr.)

7. Berk, B.C., Aronow, M.S., Brock T.A., Cragoe E.J.Jr., Gimbrone M.A., Alexander, R.W. (1987) J. Biol. Chem. 262, 5057-5064

8. Wanning, C., Meyer-Lehnert, H., and Kramer, H.J. (1989) Kidney Int. 35, 317 (Abstr.)

9. Goto, A., Yamada, K., Ishii, M., Yoshioka, M., Ishiguro, T., and Sugimoto, T. (1988) Hypertension 11, 645-650

10. Kim, D., Cragoe, E.J.Jr., Smith, T.W. (1987) Circ. Res. 60, 185-193

11. Meyer-Lehnert, H., Caramelo, C., Tsai, P., and Schrier, R.W. (1988) J. Clin. Invest. 82, 1407-1414

12. Chamley, J.H., Campbell, G.R., and Ross, R. (1979) Physiol. Rev. 59, 1-61

13. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450

14. Rink, T.J., Tsien, R.Y., Pozzan, T. (1982) J. Cell Biol. 95, 189-196

15. Reid, J.R., Civitelli, R., Westbrook, S.I., Avioli, L.V., Hruska, K.A. (1987) Kidney Int. 31, 1113-1120

16. Kramer, H.J., Bäcker, A., Weiler, E., and Liddiard, C. (1986) Klin. Wochenschr. 64, 760-766

17. Taussky, H.H., and Shorr, E. (1953) J. Biol. Chem. 202, 675-685

18. Hirata, Y., Yoshimi, H., Watanabe, T.X., Kumagi, S., Nakajima, K., and Sakakibara, S. (1988) Biochem. Biophys. Res. Commun. 154, 868-875

19. Yoshima, H., Harata, Y., Takata, S., Takagi, Y., Fukuda, Y., Kuramochi, M., and Omae, T. (1988) J. Hypertens. 6(Suppl.4), S707

20. Mitsuhashi, T., Morris, R.C., and Ives, H.E. (1989) Kidney Int. 35, 331 (Abstr.)

21. Dunn, M.J., Wann, S., Mene, P., and Nakazato, Y. (1989) Kidney Int. 35, 171 (Abstr.)

22. Miasiro, N., Yamamoto, H., Kanaide, H., and Nakamura, M. (1988) Biochem. Biophys. Res. Commun. 156, 312-317

23. Resink, Z.J., Scott-Burden, T., and Bühler, F.R. (1988) Biochem. Biophys. Res. Commun. 157, 1360-1368

24. Marsden, P.A., Danthuluri, N.R., Brenner, B.M., Ballermann, B.J., Brock, T.A. (1989) Biochem. Biophys. Res. Commun. 158, 86-93

25. Zeidel, M.L., Kone, B., Brady, H., Gullans, S., and Brenner, B. (1989) Kidney Int. 35, 321 (Abstr.)

26. Blaustein, M.P. (1977) Am. J. Physiol. 232, C165-C173