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INDUCTION OF Ca^{2+} SIGNALING AND POSSIBLE EXOCYTOSIS IN ENDOTHELIAL CELLS BY A STABLE LEUKOCYTE-DERIVED FACTOR

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Previous studies have shown that polymorphonuclear leukocytes (PMNs) release a stable factor that inhibits endothelium-dependent relaxation. In the present studies, the effects of the factor on Ca^{2+} signaling and on ultrastructure of endothelial cells were investigated. In the cultured endothelial cells, the PMN-derived factor induced an increase in $[Ca^{2+}]_i$ in a pattern of oscillations. The frequency of the Ca^{2+} oscillations was less than 3 spikes/10 minutes. Removal of extracellular Ca^{2+} by perfusion with Ca^{2+}-free Krebs' solution abolished the spikes. The results of electron microscopy showed that this factor induced an increase in vesicle formation on the luminal surface of the rat aortic endothelium. The increased vesicle formation may represent exocytosis. The structure of the smooth muscle cells was not changed. *In conclusion*, the PMN-derived factor induces a Ca^{2+} influx and possible exocytosis, suggesting that the factor may have other biological functions besides the inhibition of the vascular relaxation.

The interaction between leukocytes and endothelial cells may be important in regulation of vascular function. Activated leukocytes release factors that are capable of causing functional and structural changes in endothelium. Endothelium is a complex organ capable of sensing its environment, transducing signals to the cells within the vasculature or to the surrounding tissue, and releasing local mediators that promote functional or structural responses. We have previously reported that polymorphonuclear leukocytes (PMNs) release a stable factor that produces a potent concentration-dependent inhibition of endothelium-dependent relaxation, but not endothelium-independent relaxation (1). The previous studies suggest that the PMN-derived factor may affect an event after the agonist-receptor interaction and before the

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NO-guanylyl cyclase interaction. It was unknown whether the factor had any effect on a signal-transduction system such as Ca^{2+} signaling. It was also unknown whether the factor caused any change in the endothelial structure.

To further study the biological functions of the PMN-derived factor, the present studies were undertaken to investigate the effect of the factor on Ca^{2+} signaling in a single endothelial cell and its effect on ultrastructure of endothelium.

METHODS

Preparation of PMN-derived factor

PMNs were isolated from human blood obtained from healthy volunteers as previously described (1). The blood was diluted 1:1 with a buffer containing 145 mM NaCl, 5 mM KCl, 10 mM Hepes, 5 mM glucose, and 0.1% bovine serum albumin, at pH 7.4, and 20 ml diluted blood was layered over 15 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden). Centrifugation was carried out at 400 x g for 30 minutes. The upper layer was aspirated, leaving the cell pellet containing red cells and PMNs. Red cells in the cell pellet were then lysed with 150 mM NH_4Cl. The remaining leukocytes were washed twice with the above buffer at 100 x g and resuspended in Krebs' solution at a concentration of 1 - 2 x 10^7cells/ml. Using this procedure, our previous studies showed that the cell preparation consisted of more than 98% PMNs as determined by cytospin and Wright-Giesma staining. The cell viability was more than 98% as tested by Trypan blue exclusion test. PMN-derived supernatants were prepared by incubation of PMNs with Krebs' solution at a concentration of 1 - 2 x 10^7cells/ml for 2 hour at 37°C. At the end of the incubation, it was observed that the polypropylene plastic wall of the tube was fully adhered by PMNs. The cell viability was more than 98% at the end of the incubation. The supernatants were collected by centrifugation at 400 x g for 10 minutes. The cell pellets were discarded. Because the PMN-derived factor is heat-stable (1), the supernatants were heated at 95°C for 30 minutes to denature proteins and unstable molecules before they were used for the experiments. The supernatants were diluted 1 : 10 in Krebs' solution and the final solution was used for perfusion of endothelial cells.

Cell culture and measurement of cytosolic Ca^{2+}

Endothelial cell culture was carried out as previously described (2, 3). Endothelial cells were isolated from human saphenous vein and umbilical artery and vein by mild collagenase treatment. The cells were seeded into culture flask coated with gelatine and grown to confluence in Medium 199 (Sigma, St. Louis, MO) containing 20% supplemented bovine serum (HyClone, Logan, UT), 50 μg/ml endothelial cell growth supplement and 5 U/ml heparin. The cells were confirmed to be endothelial by their cobblestone morphology, by positive immunofluorescence using an anti von Willebrand factor VIII antibody (Dako, Denmark), and by uptake of acetylated LDL. Such characterised cells were seeded onto glass coverslips and grown to confluence. All the cells used in these studies were in passage 2 - 3. The endothelial cells were loaded with 2 μM Fura-2 in RPMI 1640 medium at 37°C for 30 minutes. Warm Krebs' solution (37°C) was then continuously perfused. After 5 to 10 minutes of equilibration, the PMN-derived supernatants were added to the perfusion medium. The fluorescence was measured from a circular area (15.8 μm in diameter) centred on a single cell (i.e. 100% of total cell area). Fluorescent measurements were made at 340 nm and 380 nm every 5-second with a Zeiss epifluorescent microscope with photometry attachment (Zeiss, Oberkochen, Germany). Cytoplasmic free Ca^{2+} concentration was estimated from the ratio of fluorescence intensities at these two wavelengths.

Morphological study

Rat aortae were isolated as previously described (1), cut into rings and incubated in Krebs' solution aerated with a gas mixture of 95% O_2 + 5% CO_2 at 37 ± 0.1°C for 30

minutes. The supernatants derived from 2 x 10^6cell/ml leukocytes were then added to one group. Another group was without the supernatants as a control. After incubation for 15 minutes, noradrenaline at 3 x 10^{-7}M was added to both groups to mimic the conditions in the organ chambers described previously (1). Incubation was allowed to continue for 60 minutes. The aortic rings were then fixed in 2.5% glutaraldehyde in phosphate buffer at 4°C overnight. The fixed rings were washed with distilled water, dehydrated through graded acetone and embedded in Araldite-Epon resin (CIBA-GEIGY, Lane Cove, NSW, Australia). Thin sections were cut and stained with saturated uranyl nitrate and Reynolds' lead citrate (4) and viewed on a JEOL JEM 1200EX electron microscopy.

Statistical Analysis

Statistical significance was determined by an unpaired Student's *t* test for two-group comparison, with mean value ± SEM. Significance was taken at $p < 0.05$.

RESULTS

Effect of PMN-derived factor on cytosolic Ca^{2+}

To determine whether the PMN-derived factor could induce an intracellular signal in the endothelial cells, the effect of the factor on cytosolic free Ca^{2+} was studied. The results showed that perfusion with the supernatants induced an increase in intracellular

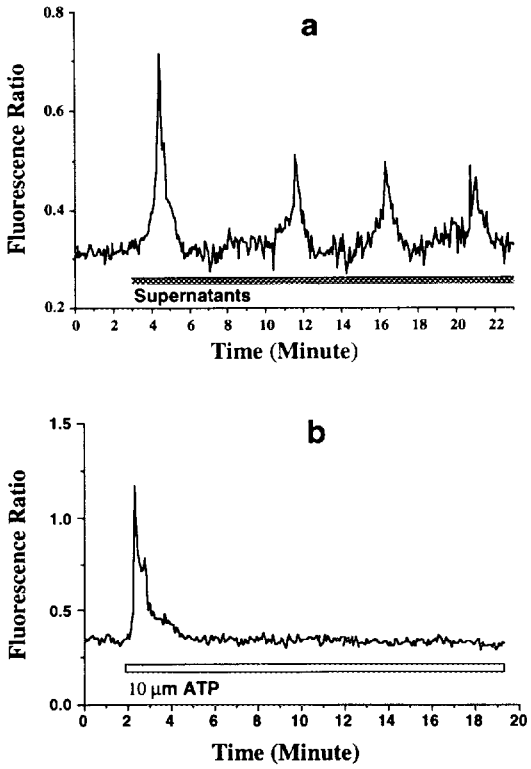

Figure 1. The effect of the PMN-derived factor on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells. (*a*): The supernatants induced an increase in $[Ca^{2+}]_i$ in a pattern of Ca^{2+} oscillations. (*b*): ATP induced an increase in $[Ca^{2+}]_i$ as a single spike. The traces are of direct recording of the fluorescence ratio (340 nm wavelength/380 nm wavelength) from Fura 2-loaded endothelial cells, and are representative of seven similar experiments.

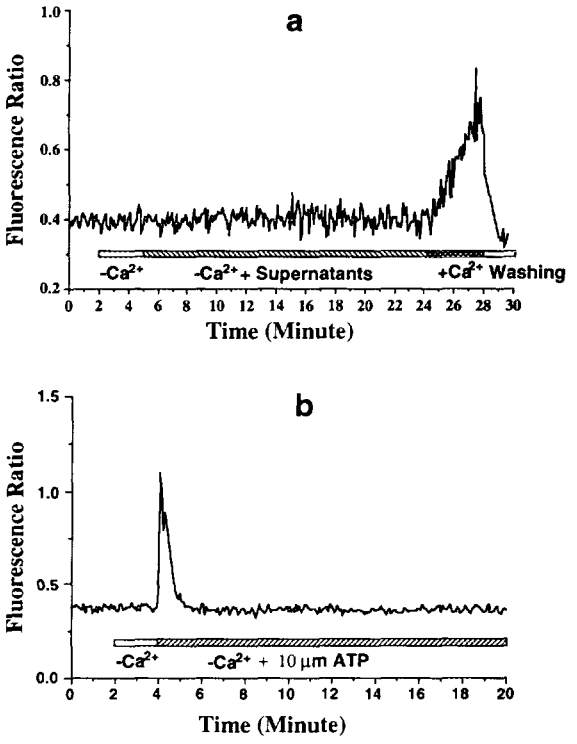

Figure 2. The effect of the PMN-derived factor on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells. (*a*): In the Ca^{2+}-free condition, the supernatants did not induce an increase in $[Ca^{2+}]_i$. When Ca^{2+} was added, a large spike appeared. (*b*): ATP induced an increase in $[Ca^{2+}]_i$ even in the absence of extracellular Ca^{2+}. The traces are of direct recording of the fluorescence ratio (340 nm wavelength/380 nm wavelength) from Fura 2-loaded endothelial cells, and are representative of seven similar experiments.

Ca^{2+} concentration ($[Ca^{2+}]_i$) in a single endothelial cell (Fig. 1 *a*) . The increase in $[Ca^{2+}]_i$ is in a pattern of Ca^{2+} oscillations. The frequency of the oscillations is low, about 2.4 ± 0.14 spikes/10 minutes. In the control (Fig. 1 *b*), however, there was no oscillation when the endothelial cell was perfused with 10 μM ATP. Removal of extracellular Ca^{2+} by perfusion with Ca^{2+}-free Krebs' solution containing 0.5 mM EGTA abolished the spikes although the Ca^{2+}-free supernatants were added (Fig. 2 *a*). When Ca^{2+} was added, a large spike appeared (Fig. 2 *a*). In the control (Fig. 2 *b*), however, ATP induced a large spike even in the absence of extracellular Ca^{2+}.

Morphological study

To determine whether this factor caused any structural change in the endothelial cells, the ultrastructure was examed. It was clearly observed that there was a marked increase in the number of vesicles on the luminal surface of the rat aortic endothelium treated with the PMN-derived factor (Fig. 3 *b* and *d*), as compared with the control (Fig. 3 *a* and *c*). The structure of smooth muscle cells was not changed (data not shown).

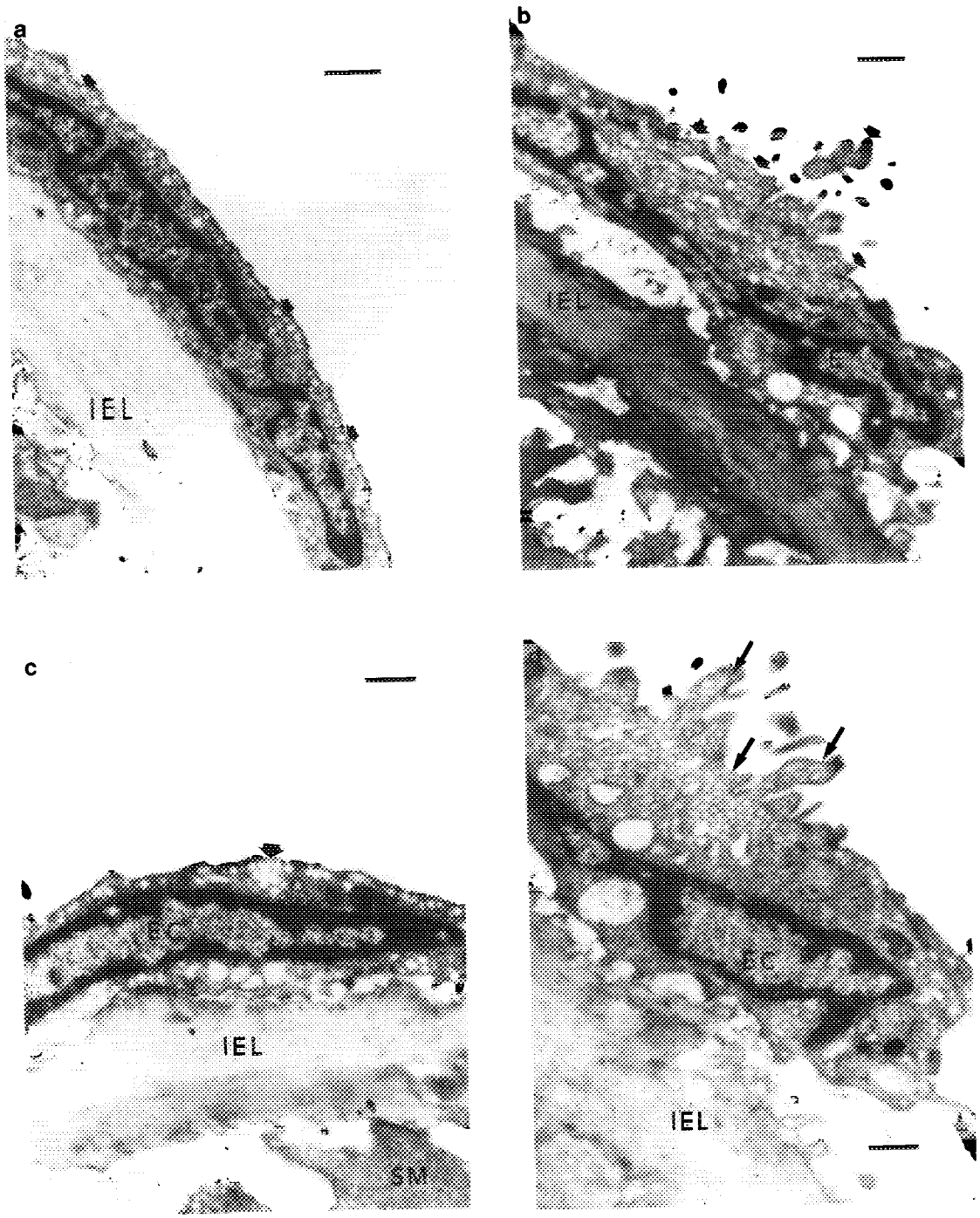

Figure. 3. Electron microscopy photographs showing morphological changes induced by the PMN-derived factor in rat aortic endothelium. There was an increase in vesicle formation on the luminal surface (indicated by the arrows) of the supernatant-treated endothelia (*b, d*). These vesicles may represent exocytosis. Different magnifications of the electron microscopy were used in both the controls (*a, c*) and the treated endothelia (*b, d*). These results are representative of three separate similar experiments. EC: endothelial cell; IEL: internal elastic lamina; SM: smooth muscle. Bar (*a, b*): 1 μm; Bar (*c, d*): 400 nm.

DISCUSSION

These studies have shown that a PMN-derived stable factor induces endothelial Ca^{2+} signaling in a pattern of oscillations and also causes an increase in vesicle formation on the luminal surface of the endothelium.

One of the first demonstrations that Ca^{2+} oscillations can occur in non-excitable cells was a study by Cobbold and colleagues using hepatocytes (5). The precise mechanisms are unknown. In the present studies, removing extracellular Ca^{2+} abolished the increase in the cytosolic Ca^{2+} in the supernatant-treated cells but not in the ATP-treated cells. It is well known that ATP-induced increase in $[Ca^{2+}]_i$ is achieved by both release of Ca^{2+} from intracellular stores and cause of Ca^{2+} influx from extracellular media. In the present studies, only ATP induced an increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. This clearly suggests that Ca^{2+} influx plays a major role in the increase in the cytosolic Ca^{2+} induced by the PMN-derived factor. The pathway of this Ca^{2+} influx is unknown. Although Ca^{2+} channels are assumed to exist in non-excitable cells, remarkably little is known about them (6). Recently, it has been reported that depletion of intracellular calcium stores activates a calcium current in mast cells (7). If the store does not communicate directly with a Ca^{2+} influx pathway, then presumably a second messenger is required to activate influx. Tsien and his colleague have recently reported that emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx (8). The small molecule was named CIF (calcium influx factor) (8). Because neither CIF nor the PMN-derived factor has been chemically identified, it is unknown if they are the same factor. Further investigation is needed to determine the chemical nature of this factor.

It has been reported that elevation of intracellular calcium is associated with an induction of exocytosis in excitable cells (9,10) and non-excitable cells (11,12), and with an inhibition of endocytosis (13). In the normal ultrastructure of an endothelial cell, there are a few plasmalemmal vesicles at the periphery of the cell on both the luminal and basal aspects (close to internal elastic lamina, IEL). These vesicles can be seen in the control endothelia in Fig. 3 *a* and *c* in the present studies. In the supernatant-treated aortic endothelia (Fig. 3 *b* and *d*), however, there was an apparent increase in vesicle formation and the increased vesicles were on the luminal surface only. These vesicles appeared to be extruded from cell surface and may represent exocytosis. It has been reported (11) that exocytosis in individual peritoneal mast cell is significantly enhanced as a result of both transient and sustained increases in cytosolic Ca^{2+}. A role of Ca^{2+} oscillations has been proposed in the push-pull model for unidirectional fluid secretion in pancreatic acinar cells (12). The increase in cytosolic Ca^{2+} shown in Fig. 1 *a* might be linked to the phenomenon seen in Fig. 3 *b* and *d*. The endothelial cells appeared to be hyper-functional although what substance was released is unknown. The possible exocytosis might be involved in the mechanisms responsible

for the inhibitory effect of the PMN-derived factor on the endothelium-dependent relaxation observed in the previous studies (1).

In conclusion, the present studies demonstrate that the PMN-derived factor can induce Ca^{2+} signaling and possible exocytosis in endothelial cells. The direct linkage between the Ca^{2+} signaling and the exocytosis awaits further investigation. These studies also indicate that the PMN-derived factor has other biological functions beyond the inhibition of the endothelium-dependent relaxation.

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REFERENCES

1. Liu, J. J., Chen, J. R., Wiley, J., Johnston, C. I., and Buxton, B.F. (1993) Am. J. Physiol. 265, H1454-H1459.
2. Liu, J. J., Casley, D. J., Wojta, J., Dauer, R., Buxton, B. F., and Johnston, C. I. (1993) *J. Hypertension* 11, 977-982.
3. Liu, J. J., Casley, D. J., Wojta, J., Dauer, R., Buxton, B. F., and Johnston, C. I. (1994) *Clin Exper Physiol Pharmacol*, 1994; 21: 49-57.
4. Reynolds, E. S. (1963) *J Cell Biol* 17, 208-212.
5. Woods, N. M., Cuthbertson, K. S. R., and Cobbold, P. H. (1986) *Nature* 319, 600-602.
6. Tsien, R. W., and Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* 6, 715-760.
7. Hoth, M., and Penner, R. (1992) *Nature* 355, 353-356.
8. Randriamampita, C., and Tsien, R. Y. (1993) *Nature* 364, 809-814.
9. Mulkey, R. M. and Zucker, R. S. (1991) *Nature* 350, 153-155.
10. von Gersdorff, H. and Matthews, G. (1994) *Nature* 367, 735-739.
11. Neher, E. (1988) *J Physiol* 395, 193-214.
12. Kasi, H., and Augustine, G. J. (1990) *Nature* 348, 735-738.
13. von Gersdorff, H. and Matthews, G. (1994) *Nature* 370, 652-655.