Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9704–9708, December 1986
Medical Sciences

Morphological alterations in endothelial cells from human aorta and umbilical vein induced by forskolin and phorbol 12-myristate 13-acetate: A synergistic action of adenylate cyclase and protein kinase C activators

(second messengers/cAMP/diacylglycerol/endothelial cell polymorphism/primary culture)

ALEXANDER S. ANTONOV, MATVEY E. LUKASHEV, YURI A. ROMANOV, VSEVOLOD A. TKACHUK, VADIM S. REPIN, AND VLADIMIR N. SMIRNOV

Institute of Experimental Cardiology, Cardiology Research Center AMS USSR, Moscow 121552, U.S.S.R.

Communicated by I. M. Gelfand, July 21, 1986

ABSTRACT **The morphological effects on human endothelial cells of phorbol 12-myristate 13-acetate (PMA) and of agents that increase intracellular cAMP concentration were studied. The adenylate cyclase activator forskolin (10 μM), the cyclic nucleotide phosphodiesterase inhibitor methylisobutylxanthine (100 μM), dibutyryl-cAMP (10 μM), histamine (10 μM), and PMA (0.1 μM) significantly altered the morphology of human aortic and umbilical vein endothelial cells in primary cultures. These effects reached a maximum 40–80 min after the effector addition and became negligible 30–60 min after its removal. PMA and forskolin were strongly synergistic in altering endothelial cell morphology. All the effects of cAMP-elevating compounds and of PMA were abolished completely by 1 μM colchicine. In explants taken from human adult or child aortas, forskolin and PMA produced alterations in endothelial morphology qualitatively identical to those observed in endothelial cell cultures. Endothelium in these preparations closely resembled that found in zones of expected altered hemodynamic stresses of human aorta. Our data suggest that the morphology of endothelium *in vivo* may be regulated by separate or synergistic action of hormone-dependent adenylate cyclase and of inositol phospholipid turnover systems and might be important for maintenance of endothelial monolayer integrity under normal physiological and pathological conditions.**

Vascular endothelial cells (ECs) comprise a pivotal interface between flowing blood and vessel wall, performing a variety of specific functions to maintain normal blood flow (1). Studies have indicated that some of these functions are closely related to EC morphology. Hemodynamic forces, for example, clearly affect the size, shape, and orientation of endothelium (2–4). Various vasoactive substances have also been shown to cause changes in EC morphology (5–11). On the other hand, the biochemical mechanisms underlying regulation of EC morphology remain poorly investigated.

It is well known that second messengers cAMP, Ca^{2+}, and the products of inositol phospholipid turnover diacylglycerol and triphosphoinositol are intimately involved in regulation of a wide variety of cellular functions. Because EC morphology is thought to play a critical role in vascular function and the morphology of various cells is known to be regulated, at least in part, by second messenger-related systems (12–14), we have studied the morphological effects of activators of the Ca^{2+}- and phospholipid-dependent protein kinase C and of adenylate cyclase on human aortic and umbilical vein ECs in primary cell cultures and in short-term human aortic organ cultures.

MATERIALS AND METHODS

Medium 199 (Earle salts), fetal calf serum, Dulbecco's phosphate-buffered saline (PBS), L-glutamine, penicillin, streptomycin, and sodium pyruvate were from GIBCO. Dispase (neutral protease from *Bacillus polymixa*) was from Boehringer Mannheim. Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate, histamine, 1-methyl-3-isobutylxanthine, N^6,O^2-dibutyryl-cAMP (Bt_2cAMP), colchicine, bovine serum albumin (BSA, essentially fatty acid free) were supplied by Sigma. Forskolin was from Calbiochem. Drugs were dissolved in dimethylsulfoxide or ethanol (Merck) and stored as stock solutions in aliquots at −80°C. Prior to use the aliquots were diluted with medium 199 and 0.5% BSA or with growth medium [medium 199 supplemented with 25 mM Hepes, 2 mM L-glutamine, 1 mM pyruvate, penicillin at 100 units/ml, streptomycin at 100 μg/ml, 10% (vol/vol) fetal calf serum] and sterilized by filtration through 0.22-μm filters (Nalgene).

Primary cultures of human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were obtained as described (15, 16), with minor modification—dispase was used instead of collagenase. Seeding density was 5–8 × 10^4 cells per cm^2. Only confluent (7–9 days after seeding) cultures were used for experiments. The main characteristics of these cultures have been reported (16). For morphometry, cultures were stained with silver nitrate (17) and photographed. Cell shape was analyzed using a MOP-3 digitizer (Reichert, Jung, Vienna) and described in terms of shape index (SI), where SI = $4\pi \times$ area/(perimeter)^2. All the experiments were performed on separate cultures in triplicates. Five randomly selected fields of 200 cells each were analyzed in every culture. Mean SI and percentage of cells with altered morphological features were determined in each experimental group, i.e., for each additive. Morphologically altered cells were defined as those with SI less than the mean SI − 2SDs in control groups. All the experiments were repeated on at least five different isolates. Experiments with short-term human aortic organ cultures were performed on segments excised from human adult (36–65 years, $n = 5$) and child (1–7 years, $n = 5$) thoracic aortas taken at autopsy performed 1–3 hr after sudden death. The vessels were freed of connective tissue, cut along the dorsal side, and washed with PBS. Symmetrical segments (1 × 2 cm) were excised in triplicate from ventral parts of the aortas and incubated

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; HAEC, human aortic endothelial cell; PMA, phorbol 12-myristate 13-acetate; SI, shape index; BSA, bovine serum albumin.

(37°C, 95% air/5% CO_2, 1–3 hr) in medium 199/0.5% BSA with or without additives. After the incubation, endothelium in aortic segments was stained by silver nitrate as described by Zand *et al.* (18), with the exception that physiologic pressure perfusion was omitted.

Primary cultures of intimal smooth muscle cells, hepatocytes, and skin keratocytes were obtained as described (19–22).

RESULTS

Morphological Responses of HUVECs. The adenylate cyclase activator forskolin markedly altered HUVEC morphology as seen by phase-contrast microscopy (Fig. 1 *A* and *B*). A significant proportion of the cells lost their initial "cobblestone" appearance and became elongated and spindle-like (Fig. 1*B*). Visualization of the cell–cell contacts by silver nitrate staining (Fig. 1 *C* and *D*) showed more complex transformations of the cell shape consisted mainly of alterations in cell contours, area, and perimeter (Fig. 1*D*). The overall effect of the morphological responses was a mean SI decrease (Fig. 2). The effects of forskolin were mimicked by agents known to increase intracellular cAMP concentration in ECs and other cells (Table 1). PMA, a potent protein kinase C activator, affected the morphology of HUVECs in a manner similar to that of forskolin but was less potent than either of the cAMP-elevating compounds (Table 1). 4α-Phorbol 12,13-didecanoate, an inactive analogue of PMA, produced no changes in HUVECs at concentrations up to 1 μM.

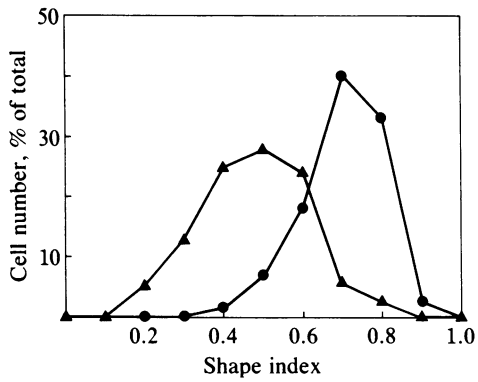

FIG. 2. Change of SI induced by forskolin in HUVEC cultures. Confluent HUVEC monolayers were incubated in medium 199 with 0.5% BSA (●) or with 0.5% BSA and 10 μM forskolin (▲). After the incubation cultures were stained with silver nitrate and SI was determined.

The effects of forskolin and PMA were detected at 10 min and maximal by 80 min after the effectors were added (Fig. 3). Thereafter, the cells began to revert to their initial appearance. Forskolin and PMA at concentrations of 1 μM and 10 nM, respectively, were completely ineffective. Surprisingly, when combined at these concentrations, they readily altered HUVEC morphology. This combination produced more rapid and pronounced effects than did either of the effectors at 10-fold higher concentration (Fig. 3, Table 1).

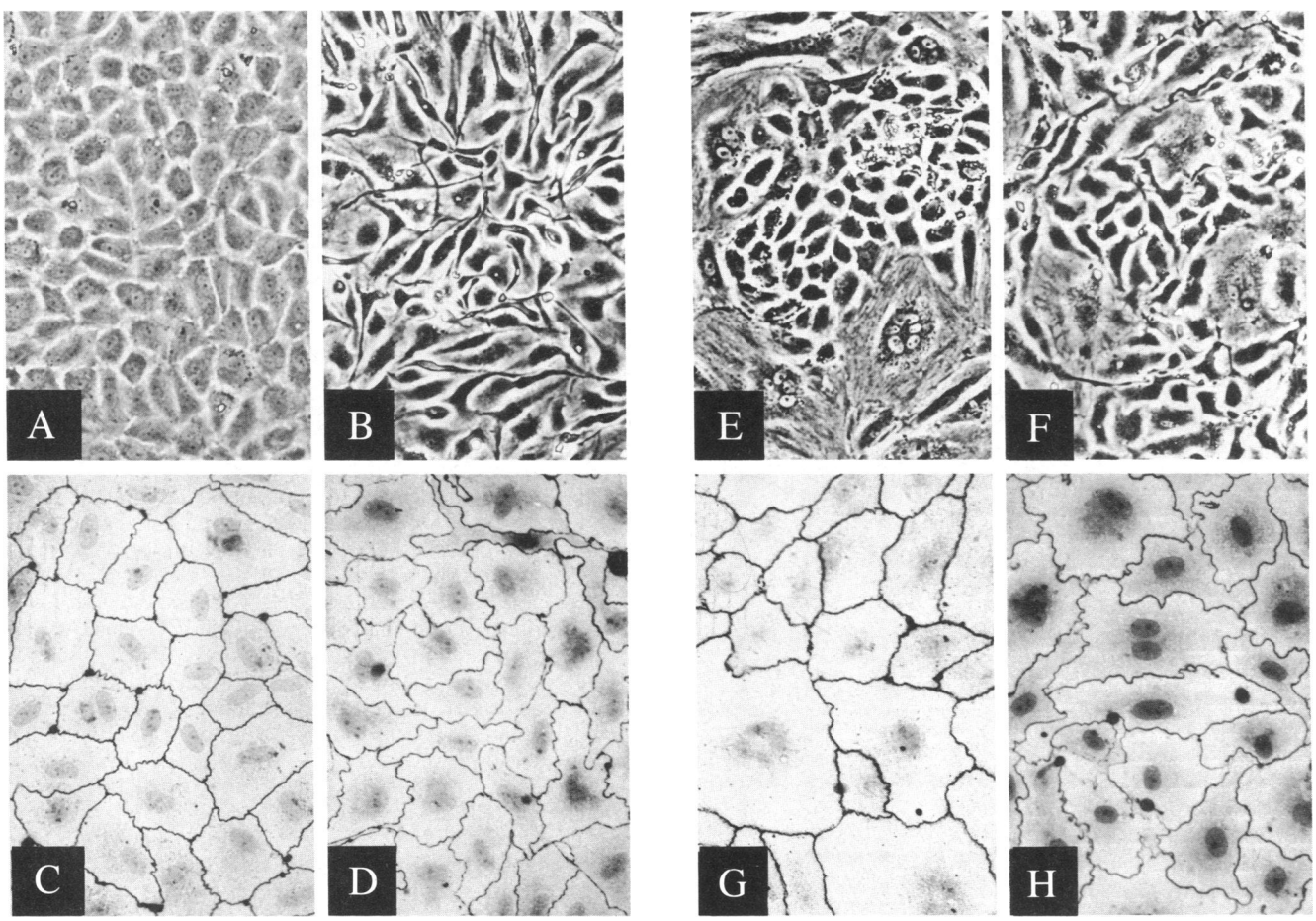

FIG. 1. Effects of forskolin on morphological features of HUVECs (*A–D*) and HAECs (*E–H*) in primary cultures. (*A*, *C*, *E*, and *G*) Control HUVEC and HAEC cultures were incubated for 1 hr in medium 199 with 0.5% BSA. (*B*, *D*, *F*, and *H*) HUVEC and HAEC cultures after incubation with 10 μM forskolin for 1 hr. (*A*, *B*, *E*, and *F*) Phase-contrast micrograph. (×150.) (*C*, *D*, *G*, and *H*) Silver nitrate stained cells. (×260.)

Table 1. Effects of cAMP-elevating compounds and phorbol esters on the percentage of altered cells in primary HUVEC and HAEC cultures

Addition	HUVEC		HAEC	
	SI	% altered cells	SI	% altered cells
None	0.70 ± 0.09	7.6 ± 2.5	0.63 ± 0.13	15.5 ± 9.0
10 μM Fsk	0.48 ± 0.13	68.3 ± 5.7	0.40 ± 0.12	59.6 ± 12
10 μM Fsk/1 μM Col.	0.79 ± 0.08	8.1 ± 1.9	0.65 ± 0.10	14.9 ± 8.3
10 μM Bt_2cAMP	0.47 ± 0.10	71.5 ± 4.2	0.41 ± 0.09	57.2 ± 8.4
10 μM Bt_2cAMP/1 μM Col.	0.74 ± 0.10	7.4 ± 3.1	0.64 ± 0.11	15.3 ± 8.4
1 μM iBtMeXan	0.67 ± 0.11	8.1 ± 1.3	ND	ND
100 μM iBtMeXan	0.58 ± 0.12	41.3 ± 5.4	ND	ND
10 μM Histamine	0.58 ± 0.17	36.7 ± 2.3	ND	ND
10 μM Histamine/1 μM iBtMeXan	0.48 ± 0.1	67.5 ± 3.0	ND	ND
10 nM PMA	0.71 ± 0.08	7.8 ± 1.8	0.63 ± 0.11	15.8 ± 8.4
0.1 μM PMA	0.57 ± 0.12	27.2 ± 6.5	0.59 ± 0.12	23.2 ± 8.1
0.1 μM PMA/1 μM Col.	0.81 ± 0.12	6.3 ± 3.0	0.67 ± 0.12	16.1 ± 9.0
1 μM PDD	0.69 ± 0.08	7.9 ± 1.7	0.63 ± 0.10	15.0 ± 1.5
1 μM Fsk	0.69 ± 0.09	8.1 ± 2.1	0.62 ± 0.13	16.4 ± 7.2
1 μM Fsk/10 nM PMA	0.41 ± 0.12	90.7 ± 7.1	0.38 ± 0.12	61.7 ± 10.3
1 μM Fsk/1 μM PDD	0.67 ± 0.08	7.7 ± 1.9	0.64 ± 0.15	16.2 ± 10.2
1 μM Fsk/10 nM PMA/1 μM Col.	0.71 ± 0.12	7.9 ± 2.1	0.62 ± 0.11	15.2 ± 10.1

Cultures were incubated with the additions specified for 1 hr at 37°C in medium 199 containing 0.5% BSA and additives as indicated (Fsk, forskolin; iBtMeXan, 1-methyl 3-isobutyl-xanthine; Col., colchicine; PDD, 4α-phorbol 12,13-didecanoate). After the incubation, cultures were stained with silver nitrate and the percentage of altered cells was determined. ND, not determined. All values are expressed as mean ± SD.

Colchicine (1 μm) completely abolished the effects of cAMP-elevating agents and PMA (Table 1).

All these effects were reversible. Within 30–60 min after the medium supplemented with additive(s) was removed and replaced with standard fresh growth medium, more than 90% of the altered cells returned to their normal morphological configuration. Though changing their shape dramatically, adjacent cells never broke contact and so maintained the monolayer continuity. On the basis of visual inspection of time-lapse photographs, there did not appear to be any significant cell migration or detachment. No signs of injury, overgrowth, sprouting, detachment, rounding, arborization, or cytotoxic effects were detected in HUVEC monolayers cultivated in the medium supplemented with 10 μM forskolin for as long as 2–3 weeks (fresh forskolin was added each time the cultures were fed).

The morphological responses described above could not be reproduced in other cell types. Human intimal smooth muscle cells, when treated by cAMP-elevating compounds, showed strong arborization and in some cases detached from plastic,

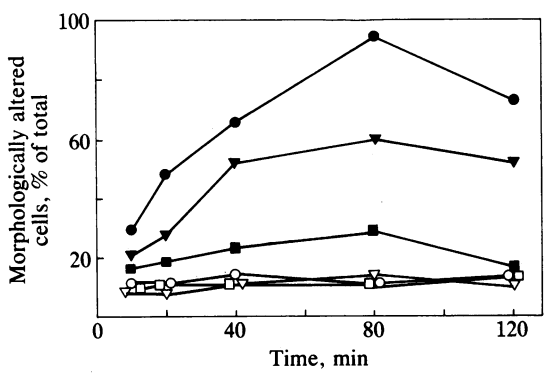

FIG. 3. Synergistic effects of PMA and forskolin on the morphology of HUVECs. Cultures were incubated in medium 199 with 0.5% BSA (○) or in medium 199 and 0.5% BSA supplemented with the following effectors: ▼, 10 μM forskolin; ■, 0.1 μM PMA; ▽, 1 μM forskolin; □, 10 nM PMA; ●, 1 μM forskolin and 10 nM PMA. After the incubation cultures were stained with silver nitrate, and the percentage of altered cells was determined.

while human hepatocytes and keratocytes remained morphologically unchanged.

Morphological Alterations in HAEC Cultures. Forskolin and PMA produced changes in HAEC morphology similar to those produced by these compounds in HUVEC cultures (Fig. 1, *E–H*, and Table 1). HAECs exposed to forskolin significantly changed their appearance (Fig. 1 *E* and *F*), and their contours became strongly tortuous (Fig. 1 *G* and *H*). The onset and reversal kinetics as well as pharmacological specificity of the morphological responses in HAECs were similar to those described for HUVECs. Forskolin and PMA acted synergistically in changing the morphology of HAECs. Taken in subeffective concentrations, they produced a 4-fold increase in the percentage of altered cells upon combined application. Colchicine at 1 μM abolished completely all the effects of forskolin and PMA. The main difference between HUVEC and HAEC morphological responses was heterogeneous response pattern of HAECs. That is, in polymorphous HAEC cultures derived from adult human aortic endothelium, the effects were visually much more prominent in small mononuclear cells of typical endothelial appearance than in large and multinuclear cells (Fig. 1 *E* and *F*). The typical ECs demonstrated morphological alterations virtually identical to those observed in HUVEC cultures. Large and multinuclear cells responded to the stimuli by only minor changes in their shape.

Morphological Responses of HAECs *in Situ*. Forskolin and PMA vividly altered the morphology of ECs in segments excised from human adult and child aortas (Fig. 4). In adult human aortic endothelium *in situ*, as in adult HAEC cultures, small cells demonstrated more pronounced shape changes than large and giant cells did (Fig. 4 *A* and *B*). The morphological alterations resulted in mean SI decrease from 0.63 ± 0.11 in control to 0.48 ± 0.13 in effector-treated segments. The altered cells closely resembled specific local regions of human aortic endothelium (mean SI, 0.46 ± 0.14) where hemodynamic stress may be altered (e.g., bifurcations, ostia, etc.). In aortic segments from children, where endothelium consists of essentially uniform cells, morphological alterations were similar to those described above with the exception that virtually all the cells responded to the applied stimuli.

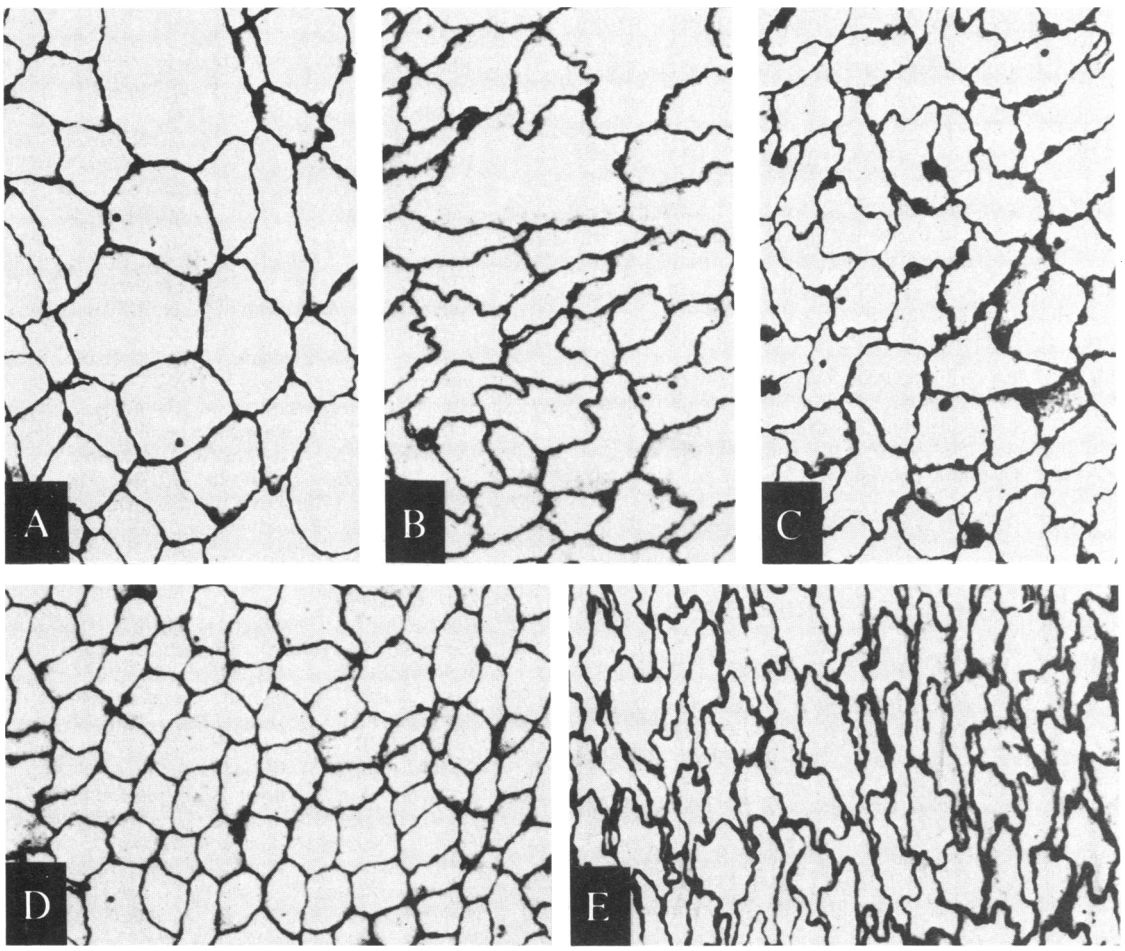

FIG. 4. Effects of forskolin on endothelium from adult human (*A* and *B*) and child (*D* and *E*) aortas in short-term organ culture. (*A* and *D*) Endothelium in explants incubated for 1 hr in medium 199 with 0.5% BSA. (*B* and *E*) Endothelium in explants treated with 10 μM forskolin for 1 hr. (*C*) The altered cells are similar to endothelium in the zone of expected altered hemodynamic stress from the ostia of arteria carotis. (Silver nitrate staining, ×380.)

DISCUSSION

EC morphology is known to be influenced by various factors, including hemodynamic forces (2, 4), blood pressure (3), vasoactive amines (5, 6), arachidonic acid (7), and other substances (8–11); however, the mechanisms mediating hormonal regulation of EC morphology remain unclear.

In this report, PMA and compounds capable of increasing intracellular cAMP concentration have been shown to produce rapid and reversible changes in the morphology of HAECs and HUVECs. The morphological alterations caused by these compounds in cultured ECs were reproduced in endothelium *in situ* but were not seen in other cell types (smooth muscle cells and hepatocytes). The fact that the effects produced by PMA and by cAMP-elevating agents were arrested by the anti-microtubule agent colchicine suggests that they may be due to cytoskeleton rearrangements rather than to shifts in osmotic balance.

These results are in accord with data from other laboratories suggesting that cAMP and protein kinase C may be involved in control of cell morphology in a number of cell types (12–14). cAMP is synthesized from ATP by the membrane enzyme adenylate cyclase and serves as one of the second messengers acting as primary intracellular mediator of various hormones' action. The main function of cAMP within cells is an allosteric activation of cAMP-dependent protein kinases. Protein kinase C plays a crucial role in the transduction of extracellular signals (23, 24). It has been shown to be the receptor for phorbol esters, which activates the enzyme by substituting for the endogenous second messenger diacylglycerol, produced from membrane inositol phospholipids in response to hormonal stimuli (24, 25). Both cAMP-dependent protein kinases and protein kinase C phosphorylate a wide variety of cellular proteins, including cytoskeletal proteins (26, 27). The phosphorylation of cytoskeleton-associated proteins is thought to play a role in cytoskeleton assembly, which is a critical factor defining morphological features of the cell.

An intriguing result of the present study was the finding that PMA strongly potentiated changes of EC morphology produced by the adenylate cyclase activator forskolin. It has been shown that PMA, as well as inositol phosholipid-turnover-activating hormones, potentiate the cAMP accumulation induced by adenylate cyclase activators (6, 28–30). Thus, the synergism of forskolin and PMA may be due either to potentiation by PMA of the forskolin-induced cAMP synthesis or to simultaneous phosphorylation of cytoskeletal proteins by cAMP-dependent protein kinases and protein kinase C. The polymorphous response pattern of adult HAECs may be due either to differences in cAMP metabolism and protein phosphorylation systems or to differences in the cytoskeleton organization of the large, multinuclear ECs.

The physiological relevance of alterations in EC morphology occurring in response to hormones or to direct activators of adenylate cyclase and/or protein kinase C remains a matter of speculation. Vasoactive amines, capable of altering EC morphology (6), can regulate endothelial permeability (5) in animals, and protein kinase C-activating phorbol esters

induce morphological alterations and angiogenesis in bovine microvascular EC cultures (31). We have shown that the altered morphology of human ECs in response to forskolin and PMA is similar to that of cells located in regions of expected altered hemodynamic stresses. We propose that changes in EC morphology in both normal and diseased arteries may be controlled by mechanisms involving interactively functioning second messenger systems. Such mechanisms may be of great importance in the maintenance of endothelial monolayer integrity, both under normal physiological conditions and in pathological states involving endothelial denudation.

We thank N. R. Kabaeva for maintaining the cell cultures and I. I. Tovarova for help in manuscript preparation. We particularly thank Dr. R. G. Gerrity (Cleveland Clinic Foundation, Cleveland, OH), Dr. J. F. Cornhill (Ohio State University, Columbus, OH), and Dr. V. I. Gelfand (Moscow State University, Moscow) for kind and helpful discussions.

1. Moncada, S. & Vane, J. R. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 66–71.
2. Langille, B. L. & Adamson, S. L. (1981) *Circ. Res.* **48**, 481–485.
3. De Chastonay, C., Gabbiani, G., Elemer, G. & Huttner, I. (1983) *Lab. Invest.* **48**, 45–52.
4. Levesque, M. J., Liepsch, D., Moravec, S. & Nerem, R. M. (1986) *Arteriosclerosis (Dallas)* **6**, 220–229.
5. Shepro, D., Welles, S. L. & Hechtman, H. B. (1984) *Thrombosis Res.* **35**, 421–430.
6. Welles, S. L., Shepro, D. & Heichtman, H. B. (1985) *J. Cell. Physiol.* **123**, 337–342.
7. Sandra, A., Bar, R. S., Dolash, S., Marshall, S. J., Kaduce, T. L. & Spector, A. A. (1985) *Exp. Cell Res.* **158**, 484–492.
8. Galdal, K. S., Evensen, S. A. & Nilsen, E. (1985) *Thrombosis Res.* **37**, 583–593.
9. Galdal, K. S., Evensen, S. A. & Nilsen, E. (1983) *Thromb. Res.* **32**, 57–66.
10. Galdal, K. S., Evensen, S. A. & Brosstad, F. (1982) *Thromb. Res.* **27**, 575–584.
11. Galdal, K. S. & Evensen, S. A. (1981) *Thromb. Res.* **21**, 273–284.
12. Smith, J. B. (1984) *J. Cell. Physiol.* **121**, 375–382.
13. Sulakhe, P. V., Johnson, D. D., Phan, N. T. & Wilcox, R. (1985) *FEBS Lett.* **186**, 281–285.
14. Dedman, J. R., Brinkley, B. R. & Means, A. R. (1979) in *Advances in Cyclic Nucleotide Research*, eds. Greengard, P. & Robinson, G. A. (Raven, New York), Vol. 11, pp. 131–174.
15. Gimbrone, M. A., Cotran, R. S. & Folkman, J. (1974) *J. Cell Biol.* **60**, 673–684.
16. Antonov, A. S., Nikolaeva, M. A., Klueva, T. S., Romanov, Yu. A., Babaev, V. R., Bystrevskaya, V. B., Perov, N. A., Repin, V. S. & Smirnov, V. N. (1986) *Atherosclerosis* **59**, 1–19.
17. Booyse, F. M., Sedlack, B. J. & Rafelson, M. E. (1975) *Thromb. Diath. Haemorrh.* **34**, 825–839.
18. Zand, T., Underwood, J. M., Nunnari, J. J., Majno, G. & Joris, I. (1982) *Virchows Arch. A* **395**, 133–144.
19. Orekhov, A. N., Kosykh, V. A., Repin, V. S. & Smirnov, V. N. (1983) *Lab. Invest.* **48**, 395–398.
20. Babaev, V. R., Antonov, A. S., Romanov, Yu. A., Rukosuev, V. S. & Repin, V. S. (1984) *Bull. Exp. Biol. Med.* **97**, 350–352.
21. Kosykh, V. A., Preobrazhensky, S. N., Ivanov, V. O., Tsibulsky, V. P., Repin, V. S. & Smirnov, V. N. (1985) *FEBS Lett.* **183**, 17–20.
22. Rheinwald, J. G. & Green, H. (1975) *Cell* **6**, 331–344.
23. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851.
24. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698.
25. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360.
26. Vallee, R. B., DiBartolomies, M. J. & Theurkauf, W. E. (1981) *J. Cell. Biol.* **90**, 568–576.
27. Anderson, W. B., Estival, A., Tapiovaara, H. & Gopalakrishna, R. (1985) in *Advances in Cyclic Nucleotide Research and Protein Phosphorylation*, eds. Cooper, D. M. F. & Seamon, K. B. (Raven, New York), Vol. 19, pp. 287–305.
28. Nabika, T., Nara, Y., Yamori, Y., Lovenberg, W. & Endo, J. (1985) *Biochem. Biophys. Res. Commun.* **131**, 30–36.
29. Vanecek, I., Sugden, D., Weller, J. & Klein, D. (1985) *Endocrinology* **116**, 2167–2173.
30. Sibley, D. R., Jeffs, R. A., Daniel, K., Nambi, P. & Lefkowitz, R. J. (1986) *Arch. Biochem. Biophys.* **244**, 373–381.
31. Montesano, R. & Orci, L. (1985) *Cell* **42**, 469–477.