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POTENTIATION BY BOVINE SERUM ALBUMIN (BSA) OF ENDOTHELIUM-DEPENDENT VASODILATOR RESPONSE TO ACETYL GLYCERYL ETHER PHOSPHORYLCHOLINE (AGEPC)

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Endothelium-dependent vasodilator responses of isolated rat aortic strips precontracted with norepinephrine to acetyl glyceryl ether phosphorylcholine (AGEPC) were compared in the presence and absence of bovine serum albumin (BSA). In the absence of BSA, AGEPC produced endothelium-dependent relaxation at concentrations higher than 10^{-6} M, which was considered to be non-specific because similar relaxations were produced by other phospholipids (*e.g.*, lysolecithin) at the same concentration range. This non-specific relaxation was suggested to be caused by changes in membrane fluidity of endothelial cells, since high concentrations of AGEPC and other phospholipids were found to produce structural changes in endothelial cells by phase contrast and electron microscopic studies; structural changes were never observed after the application of acetylcholine (ACh). In the presence of BSA (2.5 mg/ml), AGEPC caused endothelium-dependent relaxation at concentrations as low as 10^{-9}M; however, relaxations by ACh and lysolecithin were not augmented by the presence of BSA. CV-3988 (10^{-5}M), a specific antagonist of AGEPC, inhibited the relaxations by AGEPC in the presence of BSA. From these results, it is suggested that, in the presence of BSA, AGEPC may produce endothelium-dependent relaxation in a specific manner, which is different from the non-specific relaxations observed in the absence of BSA.

Keywords —acetyl glyceryl ether phosphoryl choline (AGEPC); platelet activating factor (PAF); endothelium-dependent relaxation; rat aorta; endothelial cell; electron microscopy; bovine serum albumin; CV-3988

INTRODUCTION

Acetyl glyceryl ether phosphorylcholine (AGEPC), a potent platelet activating factor (PAF), was shown to produce strong hypotension in various animal species, *e.g.*, normotensive and spontaneously hypertensive rats, rabbits, guinea pigs, and dogs.[1,2] It was suggested that AGEPC produced hypotension mainly by acting on peripheral arterial blood vessels.[1,2] Moreover, it was demonstrated that the presence of endothelial cells was required for the relaxation produced by AGEPC of precontracted rat thoracic aortae,[1,3–5] similarly to the case of acetylcholine (ACh). However, we have suggested[6] that the hypotension produced by AGEPC may not be solely explained by endothelium-dependent vascular relaxation since high concentrations of AGEPC were needed to produce the vascular relaxation and that the endothelium-dependent vascular relaxation produced by high concentrations of AGEPC may be due to the changes in membrane fluidity. On the other hand, AGEPC and other phospholipids were shown to bind strongly to serum albumin and it was suggested that serum albumin may act as a carrier for AGEPC.[7,8] Therefore, in the present study, we decided to examine and to compare the vasodilator responses to AGEPC in the presence and absence of BSA. In addition, the structural changes in endothelial cells (cultured bovine aortic endothelial cells and non-cultured rat aortic endothelial cells) were examined by phase contrast and electron microscopy, since possible changes in membrane fluidity were expected to produce structural changes in endothelial cells.

MATERIALS AND METHODS

1) Relaxation of Rat Aortic Strips —Male Wistar strain rats (250–400 g) were killed by decapitation and the descending thoracic aortae were removed. After dissecting out fat and con-

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nective tissues, helical strips (approximately 2 mm × 20 mm) were prepared. Throughout the procedure for making the spiral strips, care was taken to avoid rubbing the intimal surfaces. Preparations were mounted in an organ bath containing modified Krebs-Henseleit solution with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, NaH_2PO_4 1.2, and glucose 11.1. The solution was gassed with 95% O_2-5% CO_2 and the resting tension of 1.0 g was applied. Tissues were allowed to equilibrate for 60—90 min prior to the addition of drugs and the developed tension was measured isometrically with a force displacement transducer (Nihon Kohden, WI-621G). Tissues were precontracted with 10^{-7}—$10^{-6.5}$ M norepinephrine and agonists to produce relaxation were applied, *i.e.*, ACh, AGEPC, and other phospholipids.

In some experiments, the intimal surface of the strips was rubbed gently with a disposable cotton applicator to remove the endothelial cell layer. Complete removal by rubbing off the endothelial cell lining was ascertained by electron microscopy and it was also confirmed that this procedure did not reduce the contractile responses of the aortic strips.

2) Culture of Bovine Aortic Endothelial Cells —Isolation of bovine aortic endothelial cells was performed according to a previously reported procedure[9] with slight modification. Briefly, segments of descending bovine aorta, 30—40 cm long, were transported to the laboratory under a sterile condition in Dulbeco's phosphate buffered saline (PBS) containing penicillin (300 U/ml), streptomycin (300 μg/ml) and mycostatin (100 U/ml).

The aortic lumen was rinsed thoroughly with PBS and the connective tissues were removed. After making a perpendicular incision, the endothelial lining was scraped gently with a surgical knife. The endothelial cells thus obtained were suspended in Dulbeco's modified Eagle's medium containing 15% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and mycostatin (20 U/ml). Cells were seeded in 60-mm Petri dishes, in which cover glasses (10 × 20 mm) were placed and the dishes were incubated at 37 °C in 95% air-5% CO_2 humidified atmosphere. Subsequently, the cells began to spread, migrate and divide. The medium was changed 3 to 4 d after initial seeding and the cells finally became confluent.

Changes in the shape of the cultured endothelial cells were observed according to the method of Momose, *et al.*[10] The cover glass with attached cultured endothelial cells was placed on a slide glass and the cells were superfused continuously with PBS. Thus, the changes in shape produced by agonists were observed with the aid of phase contrast microscopy (Nikon).

3) Electron Microscopic Study —Immediately after relaxation of the aortic strips, the bathing medium was replaced with aldehyde fixative composed of 2% paraformaldehyde and 2% glutaraldehyde in 0.067 M sodium cacodylate buffer. Aortic strips were kept in the fixative at room temperature for at least 2 h and the fixative was renewed; then, the strips were stored at 4 °C for longer than 24 h. Thus, tissues were subsequently fixed in 2% osmic acid for 2 h at 4 °C and then, block staining was made with 2% uranyl acetate for 1 h at room temperature. After dehydration in graduated alcohols, samples were embedded in Epok 812. Thin sections were stained with uranyl acetate and lead citrate before electron microscopic examinations.

4) Agents —Agents used were acetylcholine chloride (Daiichi), L-norepinephrine bitartrate (Wako), C_{16}-AGEPC (gift of Dr. M. Ohno), C_{18}-AGEPC (Takeda), lyso-AGEPC (gift of Dr. M. Ohno), CV-3988 (Takeda), palmitoyl lyso phosphatidylcholine (Sigma), dilauroyl phosphatidylcholine (gift of Dr. Inoue), dipalmitoyl phosphatidylcholine (gift of Dr. Inoue), phosphatidylethanolamine (gift of Dr. Inoue), bovine serum albumin (Sigma), penicillin G (Meiji Seika), streptomycin sulfate (Meiji Seika), mycostatin (Calbiochem), Dulbeco's modified Eagle's medium (Gibco), glutaraldehyde (Nakarai), osmic acid (Wako), paraformaldehyde (Nakarai), cacodylic acid Na salt (Nakarai), uranyl acetate (Wako), lead citrate EM (Taab) and Epok 812 (Oken).

RESULTS

1) Relaxations in the Absence of BSA

Figure 1 shows dose-response curves for the relaxing effects of C_{16}-AGEPC and other phosphorylcholines and phosphatidylethanolamines on rat aortic strips precontracted with norepinephrine (10^{-7}M) in the absence of BSA. All of these lipids produced concentration-dependent relaxations if the endothelium was intact; how-

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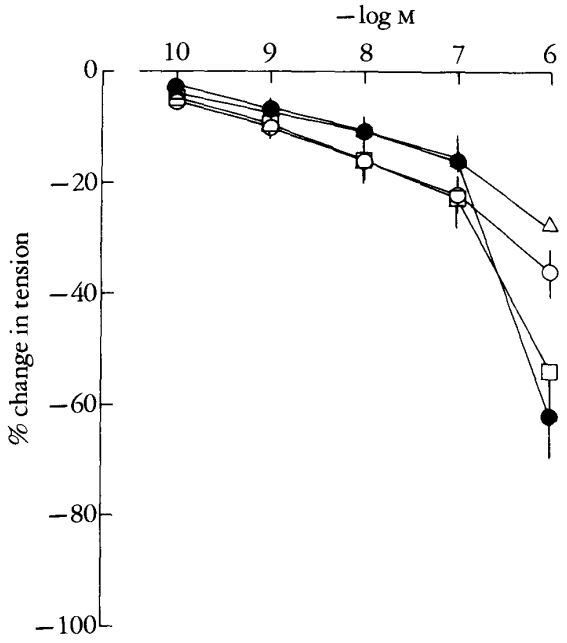

FIG. 1. *Dose-Response Curves for Relaxations of Rat Aortic Strips Complete of Endothelium Produced by AGEPC and Other Phospholipids*

The aortic strips were precontracted with 10^{-7} M norepinephrine (NE).

Ordinate: % relaxation; relaxation to resting tension before NE was expressed as 100%. DLPC, dilauryl phosphatidyl choline; DPPC, dipalmitoyl phosphatidyl choline; PE, phosphatidyl ethanolamine. Numbers of preparations were 3 for AGEPC, 7 for PLPC, 5 for DPPC and 5 for PE.

● — ●, C_{16} —AGEPC; ○ —○, DLPC; △ —△, DPPC; □ —□, PE.

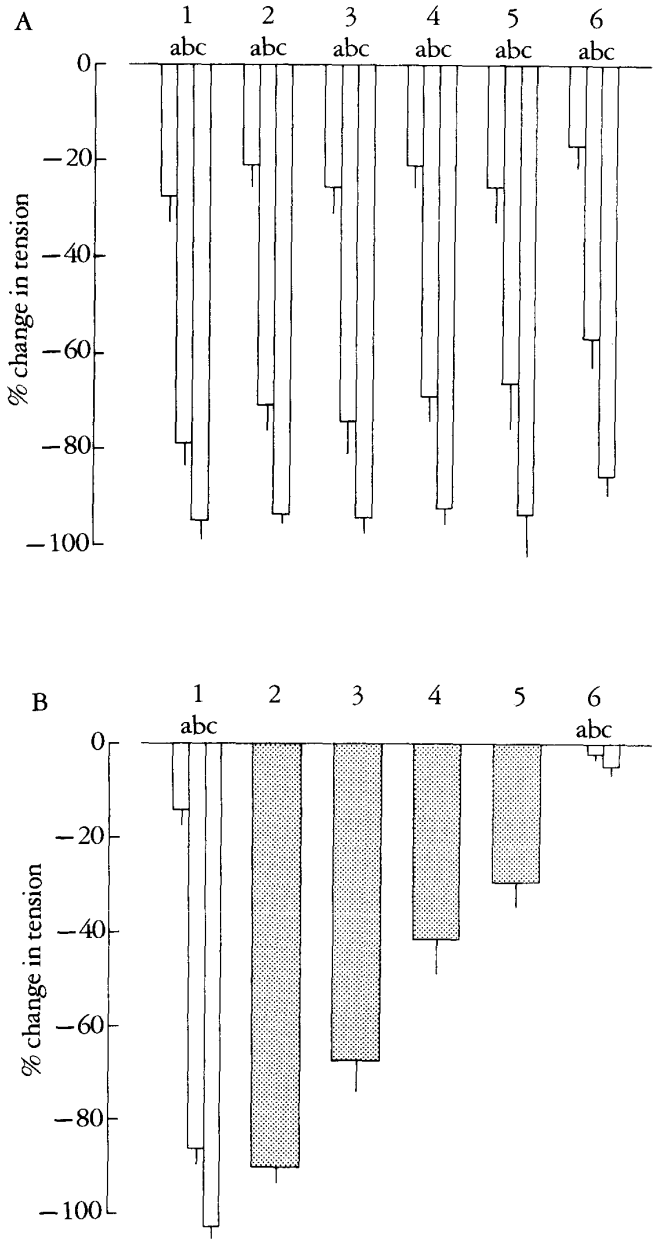

FIG. 2. *Relaxing Responses of Endothelium-Intact Aortic Strips to Repetitive Application of ACh and PLPC (palmitoyl lysophosphatidylcholine)*

The aortic strips were precontracted with 10^{-7} M norepinephrine (NE), and the relaxation to resting tension before NE was expressed as 100% (ordinate). Numbers on abscissa represent the order of repetitive application.

(A) Responses to repetitive application of ACh (total 6 repetitions). Note that no changes in relaxing response were observed.

(B) Diminution of the response to ACh after repeated administration of PLPC. Note that repeated administration of PLPC produced tachyphylactic reduction in the responses not only to PLPC itself but also to ACh. Each column represents the average value and standard error from 5 experiments.

ACh: a, 10^{-8} M; b, 10^{-7} M; c, 10^{-6} M.
PLPC 10^{-4} M.

ever, if the endothelium was removed by gentle rubbing of the intimal surface, none of these lipids produced relaxation (data not shown). As is shown in Fig. 1, it should be noted that these lipids produced endothelium-dependent relaxations only at concentrations higher than 10^{-6} M in the absence of BSA.

Figure 2 shows the relaxations produced by repeated application of ACh and palmitoyl lyso phosphatidylcholine (PLPC), a structural analogue of AGEPC. All the lipids described above showed tachyphylaxis, including AGEPC (data not shown); as representative data, the tachyphylactic decrease in relaxation after repeated application of PLPC is shown in the lower part (B) of Fig. 2. As shown in the upper part (A) of Fig. 2, such tachyphylaxis was never observed in the case of ACh. However, relaxation by ACh almost disappeared after the estab-

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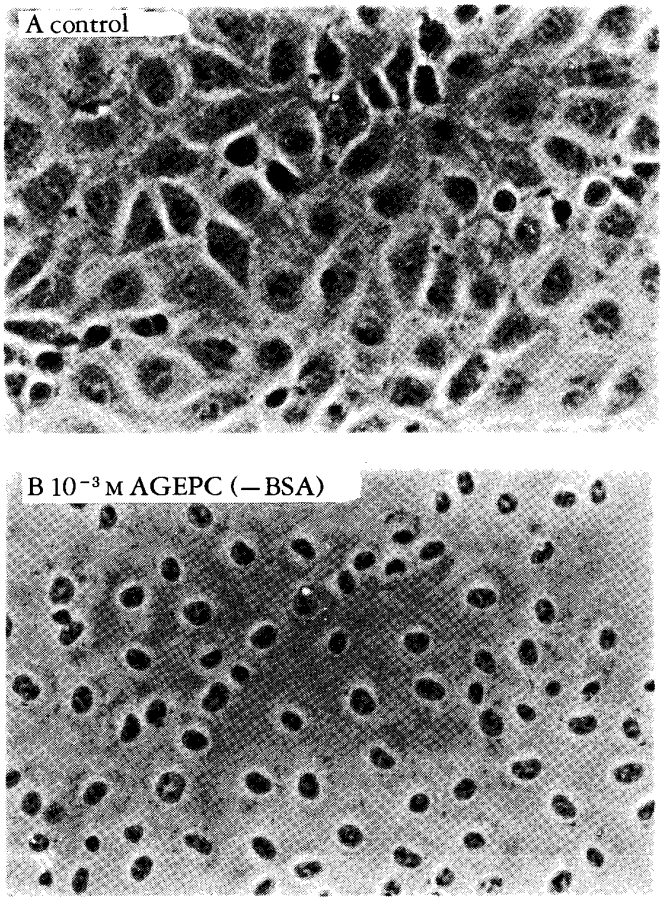

FIG. 3. *Phase Contrast Microscopy of Cultured Endothelial Cells before and after the Application of AGEPC*
(A) control micrograph before the application of AGEPC.
(B) representative micrograph after the application of 10^{-3} M AGEPC.

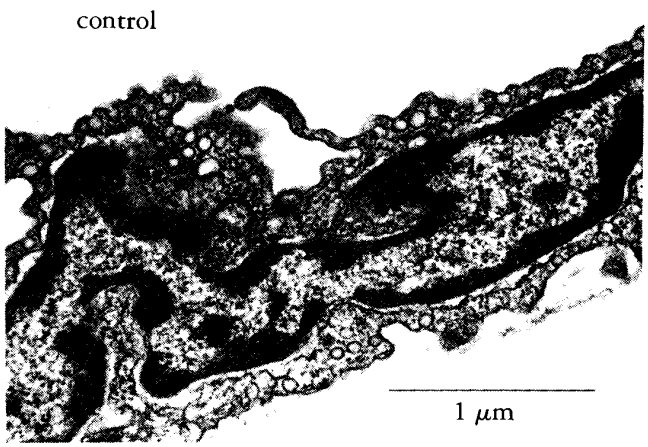

FIG. 4. *Electron Micrograph of Endothelial Cells in the Aortic Strip without Treatment (Control)*

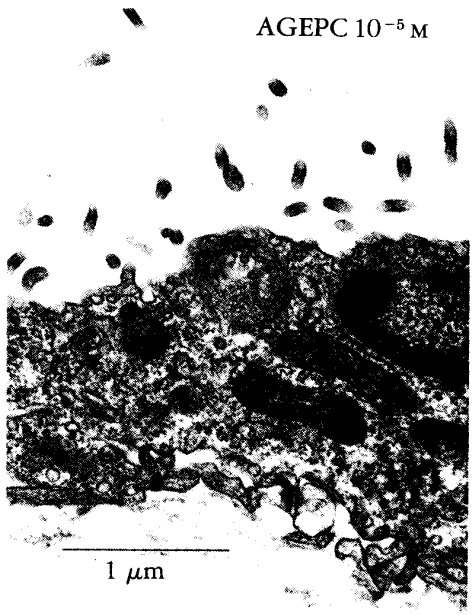

FIG. 5. *Electron Micrograph of Endothelial Cells in the Aortic Strip Having Relaxed in Response to 10^{-5} M AGEPC*
Note the destruction of the endothelial cells.

lishment of tachyphylaxis by repeated application of PLPC, which is shown in the lower part (B) of Fig. 2. In these experiments of repetitive application, a wash-out period of 30 min was allowed between each trial.

2) Structural Changes of Endothelial Cells

Possible changes in shape or structure produced by high concentrations of phospholipids (AGEPC and PLPC) in the absence of BSA were examined in 2 ways: (1) phase contrast microscopy of cultured bovine aortic endothelial cells and (2) electron microscopy of intact rat endothelial cells. When cultured endothelial cells were exposed to high concentrations of phospholipids, changes in shape were observed within a minute under phase contrast microscopy. Representative photographs showing changes produced by 10^{-3} M AGEPC are shown in Fig. 3. As shown, AGEPC at a high concentration produced marked shrinkage of endothelial cells. The cells with changed shape were stained with trypan blue (data not shown). No changes were observed in the shape of these cultured endothelial cells after the application of ACh, histamine and norepinephrine (concentrations up to 10^{-3} M).

Structural changes were examined further with the aid of an electron microscope. Immedi-

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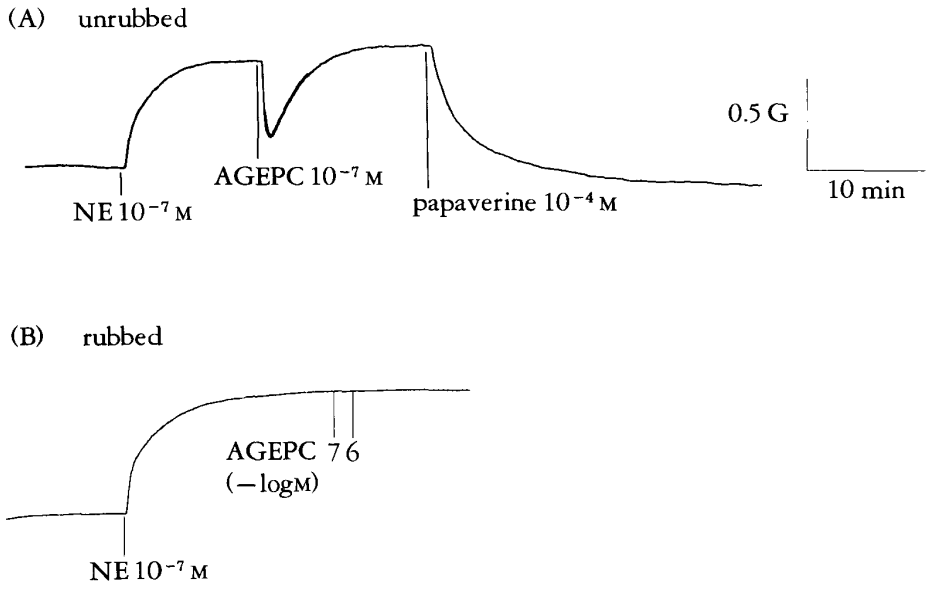

FIG. 6. *Endothelium-Dependent Relaxation of Rat Aortic Strips in Response to Low Concentrations of AGEPC in the Presence of BSA*

The strips were precontracted with 10^{-7} M norepinephrine.

(A) relaxation produced by 10^{-7} M AGEPC of the unrubbed aortic strip which was complete of endothelium.

(B) lack of relaxation in the rubbed preparation which was free of endothelial cells. Both are representative tracings from 4 experiments.

ately after the relaxation of aortic strips was obtained, the tissues were fixed and examined by electron microscopy. As shown in Fig. 5, drastic structural changes were observed to occcur in the endothelial cells of these tissues as compared to those of control endothelial cells (Fig. 4). When the relaxation was elicited with 10^{-5} M ACh, no structural changes were detected (data not shown).

3) Relaxation in the Presence of BSA

In the presence of BSA, AGEPC produced endothelium-dependent relaxation at concentrations lower than 10^{-7} M although the relaxation was transient. In Fig. 6 are shown representative traces showing transient relaxation produced by 10^{-7} M AGEPC in the presence of BSA, which was completely abolished after rubbing the endothelial cells. In contrast, lyso AGEPC or palmitoyl lyso PC did not produced such transient but strong relaxation of endothelium-intact aortic strips even in the presence of BSA, which is shown in Fig. 7. Figure 8 shows the dose-response curves for the endothelium-dependent relaxation produced by AGEPC in the absence and presence of BSA. As shown in this figure, the dose-response curve shifted to the left in the

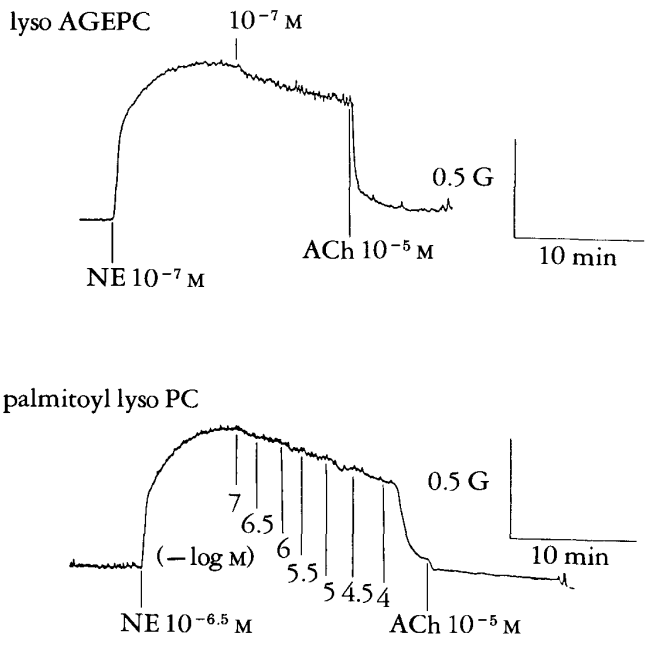

FIG. 7. *Lack of Relaxation Produced by 10^{-7} M Lyso AGEPC (Upper Trace) and Palmitoyl Lyso PC even in the Presence of BSA*

All of the preparations were complete of endothelial cells and precontracted with NE. Both are representative tracings from 5 experiments.

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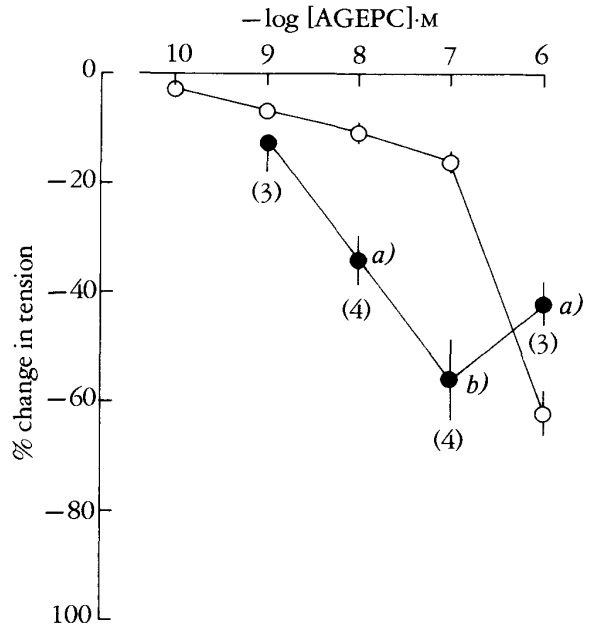

FIG. 8. *Dose-Response Curves for the relaxation of Unrubbed Aortic Strips Complete of Endothelial Cells Produced by AGEPC in the Absence and Presence of BSA*

Note that the dose-response curve shifted to the left by the presence of BSA. Numbers of preparations are shown in parentheses and the statistical significance is indicated by a) $p< 0.05$ and b) $p< 0.01$. ○ —○ *−BSA;* ● — ● *+BSA.*

presence of BSA. In contrast, endothelium-dependent relaxation produced by ACh was not modified by the presence of BSA (Fig. 9A). Relaxation by PLPC was rather suppressed by the presence of BSA (Fig. 9B). Moreover, when the relaxation was obtained by low concentrations of AGEPC in the presence of BSA, no changes were observed in the structure of endothelial cells. Figure 10 shows an electron microscopic photograph of endothelial cells obtained from the aortic strip having relaxed in response to 10^{-7} M AGEPC; as shown, no changes were observed in the endothelial cells.

In Fig. 11 are shown the effects of CV-3988, a specific antagonist to AGEPC, on AGEPC-induced endothelium-dependent relaxation in the presence of BSA. As shown, the relaxation in response to 10^{-7} M AGEPC was inhibited by 10^{-5} M CV-3988, but the relaxation to ACh was not modified by this antagonist.

DISCUSSION

We have previously shown that AGEPC produced endothelium-dependent relaxation of pre-

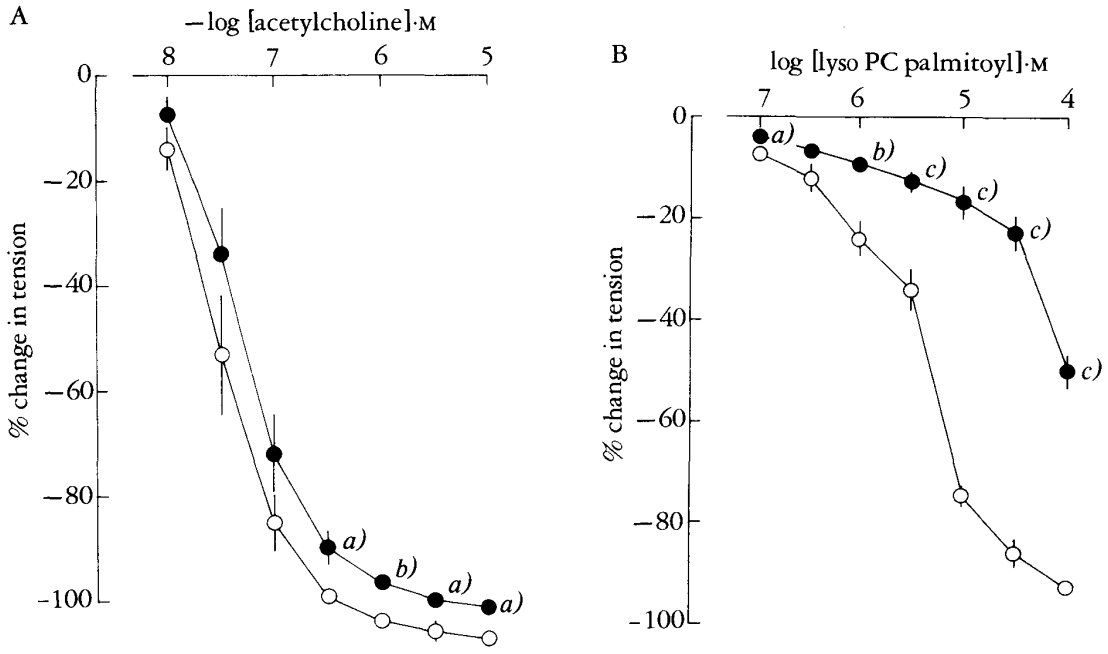

FIG. 9. *Dose-Response Curves for the Endothelium-Dependent Relaxation Produced by ACh and PLPC in the Absence and Presence of BSA*

Preparations were unrubbed (endothelium intact) and precontracted with 10^{-7} M norepinephrine (NE). Ordinate: % relaxation; relaxation to resting tension before NE was expressed as 100%.

(A) Dose-response curves for ACh. Note that the curves were almost identical in the presence and absence of BSA. Number of preparations for each point was 5.

(B) Dose-response curves for PLPC. The relaxation was greatly suppressed by the presence of BSA, which is in contrast to the case of AGEPC (see Fig. 8). Number of preparations for each point was 7 (a) $p< 0.05$, b) $p< 0.01$, c) $p< 0.001$). ○ —○ *−BSA;* ● — ● *+BSA.*

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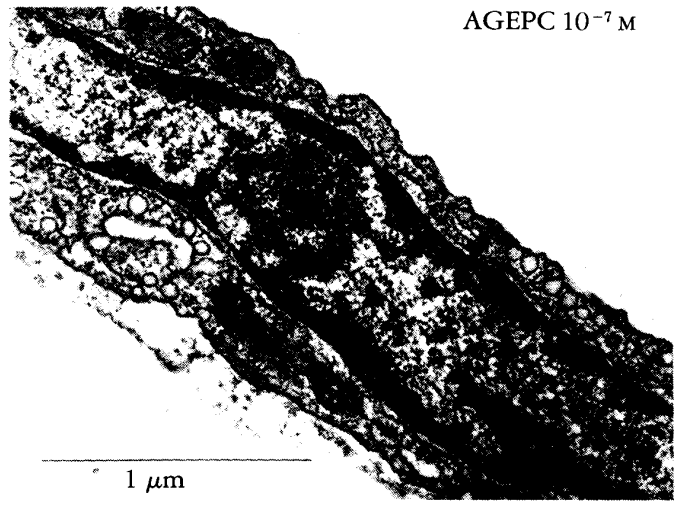

FIG. 10. *Electron Micrograph of the Endothelial Cells of the Aortic Strip Having Relaxed in Response to 10^{-7} M AGEPC in the Presence of BSA*
Note that substantially no structural changes are observed.

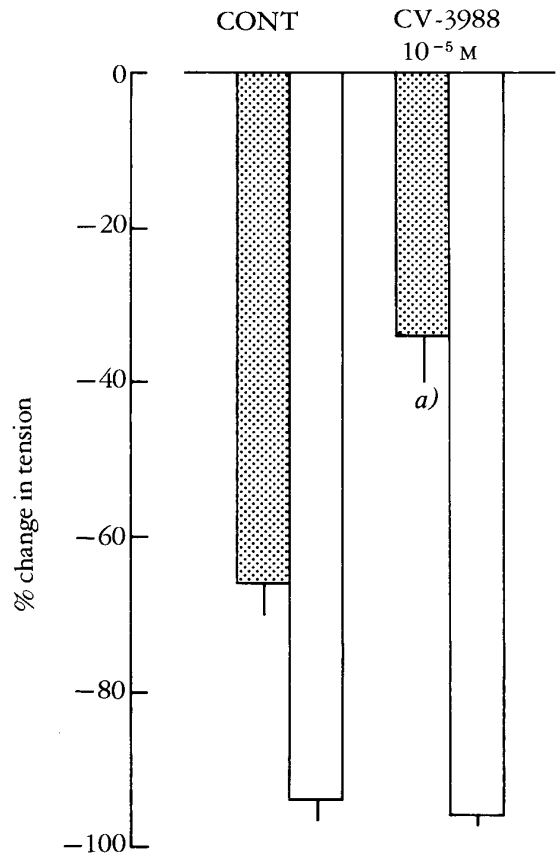

FIG. 11. *Effects of CV-3988, a Specific Inhibitor of AGEPC, on the Endothelium-Dependent Relaxation Produced by 10^{-7} M AGEPC and 10^{-5} M ACh in the Presence of BSA*
The preparations were unrubbed (thus endothelial cells intact) and precontracted with 10^{-7} M norepinephrine (NE). Ordinate: % relaxation; relaxation to resting tension before NE was expressed as 100%. Note that 10^{-5} M CV-3988 inhibited the relaxation produced by AGEPC but did not modify that by ACh. Each column represents the average value and standard error from 5 experiments (a) $p < 0.05$).
▨ *AGEPC: 10^{-7} M;* □ *ACh 10^{-5} M.*

contracted aortic strips similarly to ACh.[3] However, the concentrations of AGEPC necessary to produce endothelium-dependent relaxation were relatively high (10^{-6}—10^{-5} g/ml). Moreover, all of the phospholipids tested also produced endothelium-dependent relaxation at similar concentrations. Therefore, we considered that this relaxation may be produced by some nonspecific mechanisms and speculated that the relaxation might be due to changes in membrane fluidity[6] as was suggested for the relaxation produced by exogenously applied arachidonic acid.[11]

In the present study, it was demonstrated that high concentrations of AGEPC and palmitoyl lyso phosphatidylcholine (PLPC) produced changes in the shape of cultured endothelial cells. Similarly, the endothelial cells obtained from the aortic strips having relaxed in response to high concentrations of AGEPC or PLPC were found to show structural changes by electron microscopic examinations. In contrast, no structural changes were observed after exposure to high concentrations of ACh (*e.g.*, 10^{-5} M). Thus, it is likely that these structural changes may be produced by changes in membrane fluidity.

Another finding obtained in the present study suggesting changes in membrane fluidity or destruction of endothelial cell membrane may be tachyphylaxis observed after repeated exposure to high concentrations of these phospholipids. The response to ACh, which *per se* did not show any tachyphylaxis, also greatly diminished after the establishment of tachyphylaxis by the successive application of these phospholipids. It is possible that the endothelium-derived relaxing factor (EDRF) originally hypothesized by Furchgott[12] could have leaked out completely due to the changes in membrane fluidity or membrane destruction after repeated exposure to lipids and was no longer available.

In the presence of BSA, a much lower concentration of AGEPC produced endothelium-

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dependent relaxation although transient. The relaxation by ACh was not modified and that by PLPC was rather inhibited by the presence of BSA. Moreover, in the presence of BSA, electron microscopic studies showed that AGEPC did not produce substantial changes in membrane structure of endothelial cells at the concentrations enough to produce endothelium-dependent relaxation. Therefore, AGEPC may interact specifically with the endothelial membrane in a bound form with albumin. In contrast, PLPC may not penetrate the endothelial cell membrane in a bound form and thus cannot change membrane fluidity. However, it is not yet clear whether AGEPC, in a bound form with albumin, interacts with a specific receptor or binding site on the endothelial cell membrane. In this connection, it is interesting to note that CV-3988 was found to inhibit the relaxation produced by AGEPC in the presence of BSA. Specific antagonism by CV-3988 to AGEPC has already been reported with respect to AGEPC-induced hypotension[13] and platelet aggregation.[14] In addition, specific receptor sites for AGEPC have been identified by using ^{3}H-AGEPC on rabbit platelets, human platelets, bovine blood polymorphonuclear leukocytes (PMN), guinea pig peritoneal PMN and rabbit ileum.[15]

Since Furchgott and Zawadzki reported the obligatory role of endothelium in vascular relaxation by ACh,[16] many studies have suggested that some chemically undetermined factor(s), EDRF, may be released from endothelial cells in response to various agonists, which act on the adjacent vascular smooth muscle to produce relaxation.[17,18] Therefore, the present results may be explained as follows. In the absence of BSA, high concentrations of AGEPC, like other phospholipids, may elicit changes in membrane fluidity of endothelial cells in a nonspecific manner, which may result in the destruction of the cells and the release of EDRF. Therefore, after repeated exposure to high concentrations of AGEPC or other phospholipids, EDRF may become exhausted and ACh is no longer able to release EDRF. In contrast, in the presence of BSA, low concentrations of AGEPC may act on the endothelial cell membrane in a boud form with BSA and release EDRF somehow more specifically unlike other phospholipids without eliciting changes in membrane structure of the endothelial cells.

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