Endothelium-dependent Relaxation Is Independent of Arachidonic Acid Release

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Abstract

Endothelium-dependent relaxation is mediated by the release from vascular endothelium of an endothelium-drived relaxing factor (EDRF). It is not clear what role arachidonic acid has in this process. Inhibition of phospholipase A_2, and diacylglycerol lipase in cultured bovine aortic endothelial cells caused a marked reduction in agonist-induced arachidonic acid release from membrane phospholipid pools, and complete inhibition of prostacyclin production. EDRF release, assayed by measuring endothelium-dependent cGMP changes in mixed endothelial-smooth muscle cell cultures, was not inhibited under these conditions. In fact, EDRF release in response to two agonists, melittin and ATP, was actually increased in cells treated with phospholipase A_2 inhibitors. In addition, pretreatment of rats with high-dose dexamethasone, an inhibitor of PLA_2, did not attenuate endothelium-dependent relaxation in intact aortic rings removed from the animals, or depressor responses in anesthetized animals induced by endothelium-dependent vasodilators. In summary, inhibition of arachidonic acid release from membrane phospholipid pools does not attenuate endothelium-dependent relaxation in rats, or the release and/or response to EDRF in cultured cells.

Introduction

Endothelium-derived relaxing factor (EDRF)[1] is an unidentified labile vasodilator, or series of vasodilators, released by the endothelium in response to a variety of stimuli (1–3). It activates soluble guanylate cyclase in vascular smooth muscle to produce smooth muscle relaxation and vasodilation (4). Although the mechanism of production and release of EDRF remain obscure, possible early events are calcium activation of phospholipases and, arachidonic acid (AA) release from membrane phospholipid pools (5–9). It is not known if AA is a precursor of EDRF or, alternatively, a signal for factor production and/or release. A cytochrome P-450–dependent monooxygenase has been identified in endothelium (10) that can oxidize AA to its monohydroxy metabolites and, to a series of epoxides and their corresponding diols (11–13). Some of these novel products are arterial vasodilators. Thus, by definition they are endothelium-derived relaxing factors. There is, however, recent evidence to suggest that EDRF may be endogenous, biologically produced nitric oxide (14).

AA released from endothelial cell phospholipids is metabolized by cycloxygenase to prostacyclin (PGI_2), and lesser amounts of prostaglandin $F_{2\alpha}$ and thromboxane A_2 (15, 16). According to the evidence cited above (10–13), an undetermined amount of AA also may be metabolized by a cytochrome P-450-dependent monooxygenase to a vasodilator. In endothelial cells, the major sources of AA are hydrolysis of phosphatidylinositol (PI) by activated phospholipase A_2 (PLA_2) and phospholipase C (PLC), and hydrolysis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) by PLA_2 (17). PLC hydrolysis of PI releases diacylglycerol (DAG), which requires cleavage by DAG lipase to yield free AA. In the endothelium as well as other tissues, calcium may play a major role in the activation of PLA_2.

Glucocorticoids inhibit inflammation by inducing the production of a family of soluble exportable proteins called lipocortins that inhibit PLA_2 (18–21). This inhibition of PLA_2 reduces prostaglandin and leukotriene production. Although lipocortin has not yet been purified from endothelium, the capacity of endothelial cells to respond to glucocorticoid treatment has been demonstrated (22). Crutchley and colleagues observed a maximal 65% inhibition of bradykinin-induced PGI_2 release from bovine pulmonary artery endothelial cells following 24-h incubation with dexamethasone (10^{-5}-10^{-9} M) (22). The corresponding maximal inhibition of PGI_2 release induced by the calcium ionophore A23187, was 35%.

Thus if EDRF production is dependent on AA release, and if AA release is inhibited by steroids, then EDRF release also should be steroid sensitive. To test this hypothesis we first confirmed that cultured bovine aortic endothelial cells (BAECs) produce a PLA_2 inhibitor capable of inhibiting AA release and PGI_2 formation in response to steroids. Although cultured BAECs release EDRF (23–27), its production cannot be measured directly. To assay EDRF release by cultured BAECs, we measured cGMP changes in mixed BAEC-smooth muscle cell (SMC) cultures in response to endothelium-dependent vasodilators (24, 28). We also investigated how steroids, the PLA_2 inhibitor quinacrine, and a DAG lipase inhibitor, RHC 80267, affected this system. We monitored the degree of inhibition of AA mobilization obtained with these various inhibitors by measuring [^{3}H]AA and PGI_2 release from the mixed cultures. The effect of corticosteroids on EDRF release also was assessed in endothelium-intact rings of aorta

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cell; DAG, diacylglycerol; EDRF, endothelium-dependent relaxing factor; PC, phophatidylcholine; PE, phosphatidylethanolamine; PGI_2, prostacyclin; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PLC, phospholipase C; SMC, smooth muscle cell; SNP, sodium nitroprusside.

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removed form rats after 48 h of treatment with steroids. The relaxations induced by endothelium-dependent vasodilators in these rings were compared to similarly induced relaxations in rings from untreated control rats. Finally, we measured the cardiovascular responses of steroid treated and control rats to intravenous injections of the endothelium-dependent vasodilator methacholine.

Methods

BAEC monolayer cultures. Bovine thoracic aortae were obtained from a local slaughterhouse and endothelial cells were isolated for culture as previously described (23, 24). BAECs were plated at a density of 2 $\times$ 10^4 cells/cm^2 in Waymouth's media (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) plus penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C under an atmosphere of 95% air, 5% CO_2, and the media changed every other day until the cells became confluent on or about day 12. Confluent cultures were passaged using trypsin (0.01% VMF trypsin; Worthington Biochemicals, Freehold, NJ) and 0.02% ethylenediamine tetraacetic acid to disperse the cells. Only cells obtained from the second to the twelfth passage were used for either monolayer or mixed culture experiments.

To prepare BAECs for monolayer experiments, cells were plated into 4 cm^2 wells on 12 well tissue culture plates (Costar, Cambridge, MA) at a cell density of 2 $\times$ 10^4/cm^2 in 2 ml/well of media. Between days 12 and 16 the medium was changed for 1 ml/well of a serum-free medium (SFM) consisting of equal parts of Nutrient Mixture F12 and Dulbecco's modified Eagle medium (Gibco) supplemented with insulin (5 mg/liter) and transferrin (35 mg/liter) plus penicillin (100 U/ml) and streptomycin (100 μg/ml) (29). Next, 10 μl of a solution of 10^{-4} or 10^{-5} M dexamethasone (Hexadrol; Organon Pharmaceuticals, West Orange, NJ) in PBS was added to 6 of the 12 wells on each plate (experimental wells) to give a final concentration of 10^{-6} or 10^{-7} M dexamethasone per well. The remaining six wells per plate were control wells and received only 10 μl PBS. Since 24 h exposure to steroids is required for maximal inhibition of PLA_2 in endothelium (22), the cultures were reincubated for a further 24 h.

In order to measure AA release, the BAECs were labeled with 1 μ Ci/well of [^{3}H]AA (Amersham Corp., Arlington Heights, IL). After addition of [^{3}H]AA, cultures were incubated for 6 h to allow the label to be taken up and incorporated into the membrane phospholipid pools of the BAECs. At the end of the 6-h labeling period, the labeled media was removed and set aside for counting. Counts on the labeled media removed after 6 h of incubation showed that 80±2.5% of the label had been incorporated into the cells. In addition labeling was unaffected by dexamethasone treatment. The labeled media was replaced with 1 ml/well unlabeled conditioned media obtained from steroid-treated BAECs. The use of conditioned media was necessary to replace the lipocortins that had diffused into labeled media. Great care was taken at all times to avoid disturbing the monolayer by running the conditioned media slowly down the side of the wells. We found that PLA_2 was activated prematurely by touching or disturbing the monolayer. Even applying conditioned media directly onto the monolayer was sufficient to cause premature release of large amounts of the label.

To prepare the cultures for assay, 50 μl/well of PBS containing 3 mg of delipidated bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was added to all wells. The delipidated albumin traps AA and its metabolites when they are released into the medium. Release of [^{3}H]AA from BAECs was stimulated by the addition of 50 μl/well of PBS containing melittin (final concentration of 2 μg/ml; Sigma, St. Louis, MO). Unstimulated control wells received 50 μl PBS alone. Melittin is a bee venom polypeptide that acts directly to activate the phospholipases and is known to induce EDRF release from rabbit aorta and cultured BAECs (25, 30). Time course experiments showed that [^{3}H]AA release from BAECs peaked 10 min after the addition of melittin. 10 min after the addition of melittin the media and cells were separated by pouring off the media. The media was mixed with 10 ml liquid scintillant and counted to assay ^{3}H release. The endothelial cell monolayer was removed by adding 2 ml boiling 0.2 N NaOH to each well, and scraping with a rubber policeman. The cell associated radioactivity was counted, so that the amount of [^{3}H]AA and [^{3}H]AA metabolites released into the media following phospholipase activation could be calculated as a percentage of the total incorporated ^{3}H using the formula: media counts $\times$ 100/cell associated counts + media counts = % release. To measure the effect of steroids on PGI_2 release from BAECs, 12 well plates were set up and grown to confluence as described earlier. After the change to SFM, 4 wells/plate received 10 μl 10^{-5} M dexamethasone (final concentration, 10^{-7} M) while, the remaining 8 wells received 10 μl PBS alone. The plates were reincubated for 24 h. PGI_2 release was stimulated by the addition of melittin (2 μg/ml final concentration) to the four steroid-treated wells, and to four of the untreated wells. The remaining four control wells on each plate were unstimulated controls, receiving PBS alone. PGI_2 release was measured at 90 s and 10 min after melittin stimulation by radioimmunoassay of the stable PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ (New England Nuclear, Boston, MA) in a 200-μl aliquot of media from each well. The endothelial monolayer was removed by adding 2 ml/well of boiling 0.2 N NaOH and scraping with a rubber policeman. Protein was assayed by the Lowry technique (31). PGI_2 levels were expressed as ng/mg protein for each well.

BAEC-SMC mixed cultures. The effect of corticosteroids on EDRF release from endothelium was assessed by measuring cGMP changes in mixed BAEC-SMC cultures. Endothelium-dependent vasodilators have no effect on cGMP levels in pure BAEC or SMC cultures (24, 28). However, when the two cell types are mixed, EDRF released by the endothelium elevates cGMP levels in the SMCs (24, 28). Rat thoracic aortic SMCs were isolated and cultured according to a previously described method (32). The SMCs were cultured for 10 d in 12 well plates (4 cm^2 wells) in 2 ml of medium-199 (Gibco) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). On the tenth day only 1 ml of media was placed in each well. Flasks (25 cm^2) of 10-16-d-old BAECs were harvested, washed, counted, and resuspended at 6 $\times$ 10^5 cells/ml in Waymouth's media plus 10% FCS. 1 ml of the cell suspension was added to each well of the SMC plates. This produced a mixed culture which at the time of plating contained BAECs (1.5 $\times$ 10^5 cells/cm^2), layered on top of a SMC monolayer (1 $\times$ 10^5 SMCs/cm^2). These mixed cultures were incubated for 48 h in the combined media (Waymouth's/M-199) to allow endothelial cells to attach to and spread over the SMC monolayer. After 48 h, the media was changed for 2 ml/well of SFM. A 20-μl aliquot of various doses of dexamethasone or hydrocortisone (Sigma) in PBS, were added to wells to give final concentrations of 10^{-6}-10^{-8} M dexamethasone, and 10^{-4}-10^{-7} M hydrocortisone, respectively. The remaining wells received 20 μl of PBS alone. Cultures were reincubated for a total of 24 h to allow for maximal production of the steroid inducible PLA_2 inhibitor.

After 22 h, 1 μl of either quinacrine, a phospholipase A_2 inhibitor, in DMSO (final concentration 10^{-5} M; Sigma), or 1,6-di-*O*-carbmoyal hexane (RHC 80267), a DAG lipase inhibitor (33), in DMSO (final concentration 4 $\times$ 10^{-5} M; a gift from Rorer Group, Inc., Tuckahoe, NY), or DMSO alone were added to both steroid-treated and untreated wells. To control for possible inhibition of the guanylate cyclase, sodium nitroprusside (SNP), a direct activator of smooth muscle soluble guanylate cyclase was added to certain wells. After 2 h incubation, control unstimulated wells received 20 μl PBS, and positive control wells 20 μl SNP (Sigma; final concentration 10^{-5} M) in PBS. Experimental wells received 20 μl/well of PBS containing either melittin (final concentration 0.5-2 μg/ml), or ATP (Sigma; final concentration 10^{-4} M). After 90 s (24, 34) the medium was removed and 1 ml/well of 0.1 N HCl was added to extract cGMP from the cells. The media from each well was frozen and later assayed for 6-keto-$PGF_{1\alpha}$. Samples were stored at −20°C until cAMP and cGMP were measured, following acetylation, by RIA and results expressed as pmol cGMP or cAMP/mg protein.

To ensure that the observed cGMP changes following melittin and ATP exposure were indeed endothelium dependent, control 10-d-old cultures of rat SMCs alone were treated in exactly the same fashion as the mixed cultures.

Preparation of rat aortic rings. Adult Sprague-Dawley rats (250–500 g) of either sex were injected subcutaneously with 0.4 mg/kg of dexamethasone in 0.1 ml sterile water. This dose corresponded to a final dexamethasone concentration in the animal of $\sim 10^{-6}$ M. Control rats were injected with 0.1 ml of sterile water. 48 h after injection the rats were killed by decapitation and the aortae removed by careful dissection and placed in Krebs-Ringer-bicarbonate buffer (pH 7.4). The composition of the buffer was (millimolar); NaCl, 111; KCl, 5; NaH_2PO_4, 1; $MgCl_2$, 0.5; $NaHCO_3$, 25; $CaCl_2$, 2.5; and dextrose, 11.1. Aortae removed from steroid-treated animals were kept at all times, in buffer containing 164 μM dexamethasone. Periaortic fat was removed and care was taken during dissection to ensure the integrity of the vascular endothelium by avoiding unnecessary stretching or contact of the instruments with the luminal surfaces of the aortae. The aortae were cut into rings (2–3 mm) and suspended under 2 g tension in 10 ml water-jacketed isolated organ baths maintained at 37°C. The rings were equilibrated for 2 h in a solution of Krebs-Ringer-bicarbonate which was gassed continuously with 95% O_2–5% CO_2. The buffer was changed at 30-min intervals during equilibration and the rings stretched to give a final resting tension of 2 g. Isometric force was measured with Grass model FT 03C force transducers (Grass Instruments, Quincy, MA) coupled via a transducer amplifier (model 13-4615-50: Gould Instruments, Cleveland, OH) to Gould 8 channel thermal writing recorder series 8000S.

Relaxation responses were determined in control and experimental rings contracted to a stable plateau tension with 10^{-7} M of the alpha adrenergic agonist phenylephrine (Sigma). This concentration of phenylephrine elicits $\sim$ 40% of the maximum force attainable with this agonist. In all rings the integrity of the endothelium was assessed by observing methacholine (Sigma; 10^{-6} M) induced endothelium-dependent relaxations. Only rings with intact endothelium were used in this study. Rings were then washed three times over 30 min and tension returned to baseline (2 g).

Rings were contracted with 10^{-7} M phenylephrine and relaxations recorded in response to 2 μg/ml melittin and, a range of concentrations of methacholine (10^{-8}-3 $\times$ 10^{-6} M), and A23187 (10^{-8}-10^{-6} M; Sigma). For melittin, the latency, the rate and, the extent of each relaxation was calculated by measuring; the time to onset of contraction, $t_{1/2}$ of relaxation and, the maximum relaxation induced. Maximal relaxations were expressed as a percentage of the developed tension. Only maximal relaxations were recorded for the dose-response curves.

Cardiovascular response to methacholine. Adult male Wistar rats (300 g) were injected with either dexamethasone or sterile water as described above. 72 h after injection, control and experimental rats were anesthetized with 50-60 mg/kg intraperitoneal sodium pentobarbital (Elkins-Sinn, Cherry Hill, NJ). The femoral artery and vein were canulated and the artery connected to a pressure transducer (P23DB; Statham, Oxnard, CA) hooked to a recorder (Brush 220; Gould, Cleveland, OH) for direct arterial pressure recording. Pulse rate was read from a high speed recording. The vein was used for intravenous administration and flushed with 100 μl normal saline (3 vol of cannula dead space) following injection of 100 μl bolus of an aqueous solution of methacholine (10^{-7}-10^{-4} M). Blood pressure and heart rate responses were expressed as percentage fall from the preinjection levels.

Statistical methods. Results were expressed as mean±standard deviation, and were analyzed by "one way" analysis of variance, and Student-Newman-Keuls multiple range tests. Significance was accepted at the 0.05 level of probability.

Results

AA release from BAEC monolayer cultures. 10-min exposure of the [^{3}H]AA labeled BAECs to melittin caused 10±1.1% of the incorporated label to be released into the medium (Fig. 1). The control release after 10 min exposure to PBS was 1.5±0.3%. 24 h pretreatment of BAECs with either 10^{-6} or, 10^{-7} M dexamethasone reduced melittin-stimulated release of label to 6.2±0.7%. With the background (1.5%) subtracted, this amounted to a 45% reduction in AA release. This reduction was significant ($P < 0.01$) and reproducible, provided the labeled media was replaced with conditioned media prior to stimulation.

PGI_2 release from BAEC monolayer cultures. PGI_2 release from BAEC monolayers was assayed at 90 s and 10 min after melittin stimulation by measuring the level of its breakdown product, 6-keto-$PGF_{1\alpha}$, in the media. The results are shown in Fig. 2. 24 h treatment with 10^{-7} M dexamethasone reduced melittin-induced PGI_2 release at 90 s and 10 min by 41% and 43% ($P < 0.01$), respectively. These results are consistent with the 45% reduction in AA release from BAECs shown in Fig. 1 as well as with the anti-inflammatory action of dexamethasone (22).

PLA_2 inhibition and melittin-induced PGI_2 and EDRF release. The effect of high-dose dexamethasone (10^{-6} M) on the dose response curves of melittin-induced PGI_2 and EDRF release in BAEC-SMC mixed cultures are shown in Fig. 3. In panel A are the results of the 6-keto-$PGF_{1\alpha}$ assays on the media at 90 s. At all doses (0.5-2 μg/ml) there was significant inhibition of PGI_2 release ($P < 0.01$) by dexamethasone, such that the dose response curve was shifted to the right. Interestingly, basal unstimulated PGI_2 release was also significantly inhibited following 24 h incubation with dexamethasone ($P < 0.01$). The percentage reductions in PGI_2 release from mixed cultures, shown in Fig. 3 *A*, were comparable to the reductions in the BAEC monolayer, shown in Fig. 2. The absolute amounts of PGI_2 released in the two systems were different, because the results were expressed in nanograms per milligram protein.

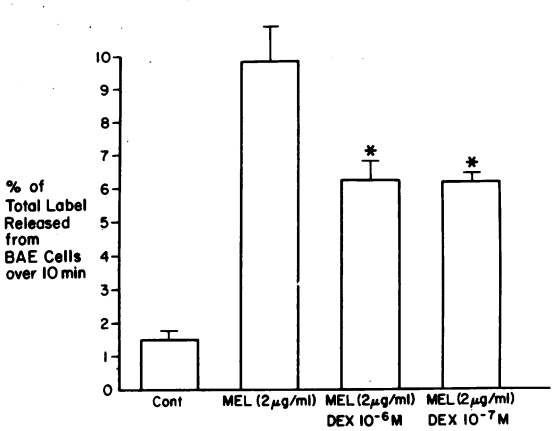

Figure 1. Effect of dexamethasone on AA release from BAECs. BAECs were grown to confluence in 12 well plates then exposed to either dexamethasone in SFM or SFM alone for 24 h and labeled for 6 h with 1 μCi/well [^{3}H]AA. The labeled media was removed, replaced with 1 ml/well conditioned media from mirror-image cultures and phospholipases activated by exposure to melittin for 10 min. The counts released into the media were expressed as a percentage of the total incorporated counts. Shown are the control release (PBS stimulated) and, melittin-stimulated release from dexamethasone-treated and untreated wells. Dexamethasone treatment resulted in a 45% reduction in [^{3}H]AA release (following subtraction of control). This reduction was significant *$P < 0.01$ ($n = 8$).

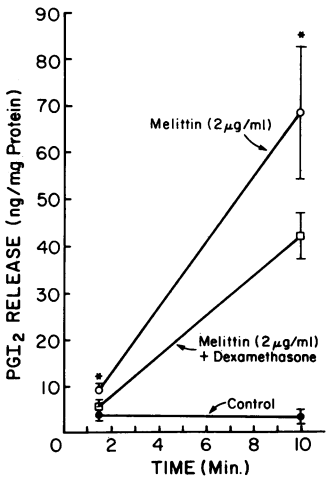

Figure 2. Effect of dexamethasone on PGI_2 release from BAECs. BAECs were grown to confluence in 12 well plates then exposed to either dexamethasone (10^{-7} M) in SFM or SFM alone for 24 h. Cells were stimulated with either PBS (control) or melittin, and 200 = μl aliquots of the media removed at 90 s and 10 min after stimulation. PGI_2 release was measured by determining the level of 6-keto-$PGF_{1\alpha}$ in the media. Protein was determined for each well at the end of the experiment. Shown are the control release, melittin-stimulated release, and the melittin-stimulated release from dexamethasone treated wells at 90 s and 10 min. Dexamethasone treatment lead to a 41 and 43% reduction in melittin stimulated release of PGI_2 from BAECs at the two time points, respectively. Both reductions were significant $^*P < 0.01$ ($n = 8$).

Fig. 3 *B* shows the results of the cGMP extracted from the cells at 90 s. Melittin induced a dose-dependent increase in cGMP over the range 0.5 to 2 μg/ml. In contrast to the PGI_2 results, dexamethasone did not alter melittin-induced increase in cGMP levels. This dose-response curve of cGMP elevations in mixed cultures matched the dose-response curve of melittin-induced endothelium-dependent relaxation seen in intact rings of rat aorta (35, 36).

Basal cGMP in control SMC monolayer cultures, 1.9±0.2 pmol/mg protein, was almost 10-fold less than the levels in the mixed BAEC-SMC cultures. The melittin-induced cGMP elevations seen in the mixed cultures were clearly endothelium dependent, since such changes were not seen in SMC monolayer cultures (not shown).

To determine whether this failure of high-dose dexamethasone to alter melittin-induced EDRF release was a general property of PLA_2 inhibitors, we investigated the effect of quinacrine in this system. Fig. 4 *A* compares the effects of dexamethasone, quinacrine, and a combination of the two on PGI_2 release from BAEC-SMC mixed cultures 90 s after administration of 2 μg/ml melittin. Quinacrine, 10^{-5} M, produced slightly more inhibition of PLA_2 than did 10^{-6} M dexamethasone. While the effect of the combination of the two inhibitors was not additive, it produced a maximum 62% ($P < 0.01$) inhibition of PGI_2 release (Fig. 4). *B* shows the cGMP changes in the cells. Melittin-induced cGMP elevations were not significantly altered by either PLA_2 inhibitor alone. However, a combination of the two potentiated EDRF release by 60% ($P < 0.01$). In addition, SNP-induced (10^{-5} M) cGMP elevations in mixed cultures (not shown) were not affected by dexamethasone or quinacrine.

Combined PLA_2 and DAG lipase inhibition. We sought to determine if the residual AA release induced by melittin was due to activation of PLC. If true, then residual AA release should be inhibited by the DAG lipase inhibitor, RHC 80267. Fig. 5 *A* shows the results of the PGI_2 assays on the media. As shown earlier, dexamethasone alone produced 44% ($P < 0.01$)

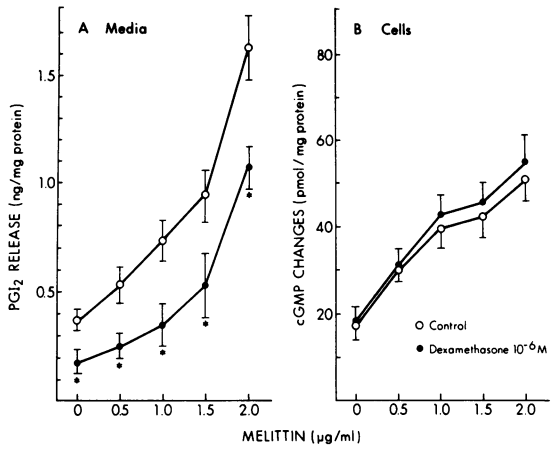

Figure 3. Dose-response curves of melittin-induced PGI_2 release and cGMP changes in mixed BAEC-SMC cultures. BAECs were grown to confluence in 25-cm^2 flasks, harvested with trypsin and plated at 1.5×10^5 cell/cm^2 on top of confluent SMC monolayers (1×10^5 cells/cm^2) in 12 well culture plates. The mixed cultures were grown for 2 d in the presence of serum, then changed to SFM. Dexamethasone (10^{-6} M) was added to half the wells and the cultures reincubated for 24 h. Mixed cultures were stimulated with 20 μl PBS alone, or PBS with increasing doses of melittin (0.5–2 μg/ml). 90 s later, media and cells were separated, cyclic nucleotides extracted from the cells with 0.1 N HCl, and protein determined in each well. PGI_2 release was measured by assaying 6-keto-$PGF_{1\alpha}$ in the media. Shown in A is the amount of PGI_2 released into the media by the dose of melittin indicated. Dexamethasone treatment inhibited PGI_2 release induced by all doses of melittin tested, as well as reducing unstimulated PGI_2 release. All reductions were significant $^*P < 0.01$ ($n = 6$). Shown in B are the melittin-induced endothelium-dependent changes in cGMP in the cells that were not altered by dexamethasone.

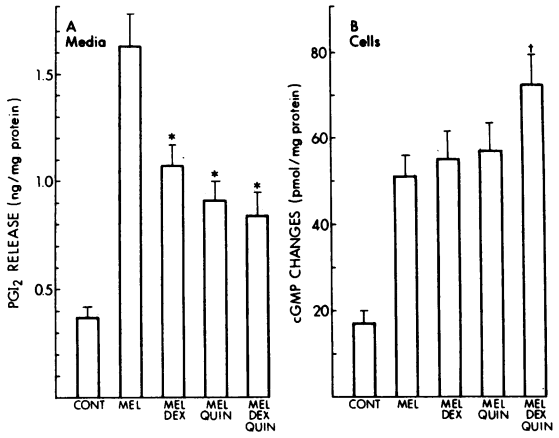

Figure 4. Effect of PLA_2 inhibition on melittin-induced PGI_2 release and cGMP changes in mixed BAEC-SMC cultures. Mixed cultures were prepared and treated with 10^{-6} M dexamethasone (DEX) as described in the previous figure. 2 h before exposure to melittin, 10^{-5} M quinacrine (QUIN) was added to selected wells. 90 s after adding 2 μg/ml melittin (MEL), cells and media were separated and the proteins, cGMP, and 6-keto-$PGF_{1\alpha}$ determined. Shown in A are the amounts of PGI_2 released by PBS alone (CONT), melittin, and by melittin in the presence of dexamethasone, quinacrine, or a combination of the two. All treatments caused a significant reduction in melittin-induced PGI_2 release, $^*P < 0.01$ ($n = 6$). B shows the corresponding cGMP levels in the cells. Combined dexamethasone and quinacrine treatment significantly enhanced cGMP changes, $\dagger P < 0.01$ ($n = 6$) compared to melittin alone, or melittin and individual inhibitors.

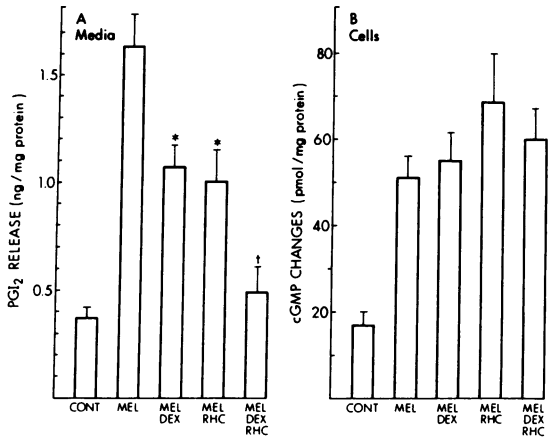

Figure 5. Effect of combined PLA_2 and DAG lipase inhibition on melittin-induced PGI_2 release, and cGMP changes in mixed BAEC-SMC cultures. The cultures were prepared, treated with 10^{-6} M dexamethasone (DEX), and results determined in the same fashion as outlined in Fig. 4, except instead of quinacrine, 4×10^{-5} M RHC 80267 (RHC) was added 2 h before exposure to melittin (MEL). In A are the results from the media; dexamethasone and RHC 80267 alone significantly inhibited melittin-induced PGI_2 release by 44 and 50%, respectively, $*P < 0.01$ ($n = 6$). Combined treatment with RHC 80267 and dexamethasone inhibited PGI_2 release from mixed cultures by 90%. This amount of PGI_2 was significantly different from the amount released by melittin, or by melittin in the presence of either inhibitor alone, $\dagger P < 0.01$. There was no significant difference between control (CONT), PBS alone, and melittin-induced release in the presence of a combination of both inhibitors. In B are the cGMP results from the cells. Melittin-induced cGMP elevations were not affected by any intervention.

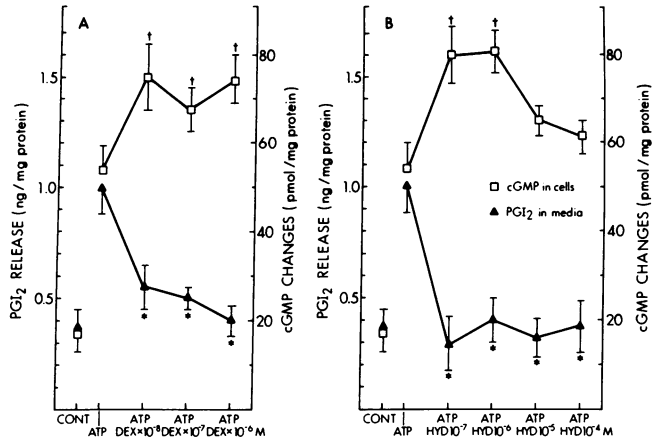

Figure 6. Dose response curves of the effects of dexamethasone and hydrocortisone on ATP-induced PGI_2 release and cGMP changes in mixed BAEC-SMC cultures. The cultures were prepared and results determined as outlined in Fig. 3, except that various doses of dexamethasone (DEX; 10^{-8}-10^{-6} M) or hydrocortisone (HYD; 10^{-7}-10^{-4} M) were added. 24 h later, cells were stimulated for 90 s with 10^{-4} M ATP (ATP), or PBS alone (CONT). Displayed in each panel are the results from both the media and the cells. A shows the response to dexamethasone, and B the response to hydrocortisone. All doses of both steroids completely inhibited ATP-induced PGI_2 release, $*P < 0.01$ ($n = 6$). All doses of dexamethasone and low dose hydrocortisone (10^{-7} M and 10^{-6} M) markedly enhanced ATP-induced cGMP changes in mixed BAEC-SMC cultures by as much as 75%, $\dagger P < 0.05$ ($n = 6$).

inhibition of PGI_2 release. Inhibition of DAG lipase with RHC 80267 produced 50% ($P < 0.01$) inhibition of PGI_2 release from the mixed cultures. A combination of the two inhibitors produced ~ 90% inhibition ($P < 0.01$) of melittin-induced PGI_2 release. This was effectively complete inhibition, because the amount of PGI_2 released at 90 s by 2 μg/ml melittin in the presence of a combination of RHC 80267 and dexamethasone was not significantly different from the unstimulated control (PBS alone). The inhibitors had an additive effect, since the amount of PGI_2 released from the mixed cultures after combined treatment, was significantly less than the amount released in the presence of each inhibitor alone ($P < 0.01$). From Fig. 5 *B* it can be seen that none of these interventions had a significant effect on melittin-induced endothelium-dependent changes in cGMP. In addition, neither RHC 80267, nor a combination of RHC 80267 with dexamethasone altered SNP-induced cGMP changes (data not shown). Thus, marked inhibition of melittin-induced AA release by a combination of inhibitors of PLA_2 and DAG lipase such that PGI_2 production was almost completely inhibited, did not appear to affect EDRF release or VSM response.

PLA_2 inhibition and ATP-induced PGI_2 and EDRF release. From the preceding experiments it can be seen that melittin's action on endothelial cells may involve activation of both PLA_2 and PLC. To investigate the mechanism of action of another endothelium-dependent vasodilator, we studied the effect of PLA_2 inhibitors on ATP-induced release of PGI_2 and EDRF. ATP (10^{-4} M) induced no change in cGMP in either BAEC or SMC monolayer cultures. Fig. 6, *A* and *B* show the effect of increasing doses of dexamethasone and hydrocortisone, respectively, on ATP-stimulated PGI_2 production and cGMP accumulation in mixed cultures. This dose of ATP, 10^{-4} M, was equipotent with 2 μg/ml melittin in terms of EDRF release (54±5 vs. 51±5 pmol cGMP/mg protein), but induced ~ 50% less PGI_2 release ($P < 0.01$; see Fig. 4 for comparison). Dexamethasone and hydrocortisone both caused complete inhibition of PGI_2 production while potentiating EDRF release by as much as 75%. Potentiation of cGMP elevations were not seen with high doses of hydrocortisone (10^{-5} and 10^{-4} M).

Quinacrine (10^{-5} M) and a combination of quinacrine with dexamethasone (10^{-6} M), both agents inhibit PLA_2 but not PLC activity, completely inhibited ATP-induced PGI_2 release from the mixed cultures (not shown). Once again, ATP-induced endothelium-dependent cGMP accumulation was significantly enhanced ($P < 0.01$) by PLA_2 inhibitors (quinacrine alone 80±7, and the combination of quinacrine and dexamethasone 79±6 pmol cGMP/mg protein). SNP-induced cGMP changes were not affected by dexamethasone, hydrocortisone, or quinacrine (not shown).

cAMP changes in mixed BAEC-SMC cultures. The basal cAMP level in mixed cultures (12±4 pmol/mg protein) was not altered by SNP or dexamethasone treatment. Stimulation of the mixed cultures with either ATP or melittin, however, caused minor but significant ($P < 0.05$) increases in cAMP levels to 22±3, and 21±4 pmol/mg protein, respectively. PLA_2 inhibitors have no effect on this response (not shown).

Relaxations in intact rings of rat aorta. The profile of melittin-induced relaxation in rings of endothelium-intact rat aorta is shown in Table I. Dexamethasone treatment (0.4 mg/kg) of rats for 48 h had no significant effect on the latency,

Table I. Effect of Dexamethasone on Melittin-induced Relaxation in Rat Aorta (n = 4)

	Control rings	Steroid-treated rings*
	Melittin	Melittin
	2 μg/ml	*2 μg/ml*
Latency (min).	2±0.5	2±0.5
$t_{1/2}$ (min).	2.5±0.8	2.5±1.2
Maximum relaxation %	47±12	43±11

* Rings were removed from rats 48 h after injection of 0.4 mg/kg dexamethasone s.c.

the rate, or the extent of the relaxations induced by melittin. The maximum relaxations induced by 2 μg/ml melittin were 43±11% and 47±12% for steroid treated and control animals, respectively.

Dose-response curves for methacholine (10^{-8} to 3×10^{-6} M) induced endothelium-dependent relaxations were constructed for aortic rings removed from dexamethasone treated and control rats. These curves are shown in Fig. 7. Methacholine-induced relaxation of intact rings of rat aorta were not significantly altered by prior treatment of the animals with dexamethasone. The dose response curve (10^{-8} to 10^{-6} M) for A23187, another endothelium-dependent vasodilator, was also not altered by steroid treatment (not shown).

Cardiovascular response to methacholine. The cardiovascular response of steroid treated and control rats to intravenous injections of increasing doses of methacholine is shown in Fig. 8. No appreciable change in heart rate occurred until 100 μl of 10^{-4} M methacholine (final dose 6.5 μg/kg) was injected (top panel). The corresponding blood pressure responses are shown in the bottom panel of Fig. 8. Starting at the lowest dose (10^{-7} M), there was a progressive dose-dependent fall in blood pressure. Steroid treated and control animals displayed the same blood pressure response at all doses of methacholine.

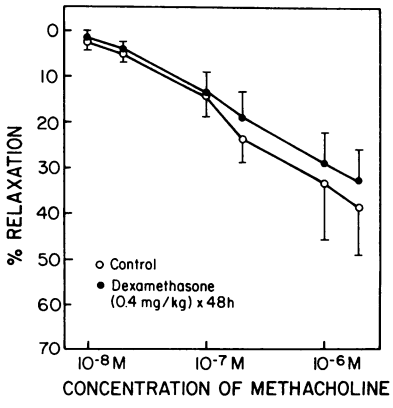

Figure 7. Dose response curve of methacholine-induced relaxation of aortae. Adult Sprague-Dawley rats were injected subcutaneously with either 0.4 mg/kg dexamethasone in 0.1 ml sterile water or sterile water alone (control) and killed 48 h later by decapitation. Aortae were removed, dissected, cut into 2–3 mm rings, suspended under 2 g tension and allowed to equilibrate for 2 h in an isolated-organ bath of oxygenated Krebs buffer maintained at 37°C. The rings removed from dexamethasone treated rats were incubated in buffer containing 164 μM dexamethasone. Relaxation responses to a range of methacholine concentrations were determined in rings from control and dexamethasone treated rats contracted to a stable plateau tension by the addition of phenylephrine (10^{-7} M). Relaxation responses were recorded and expressed as a percentage of the developed tension such that, 100% relaxation would return a ring to its baseline 2 g tension. There was no significant difference in the relaxation responses of rings from control and dexamethasone treated rats (n = 12).

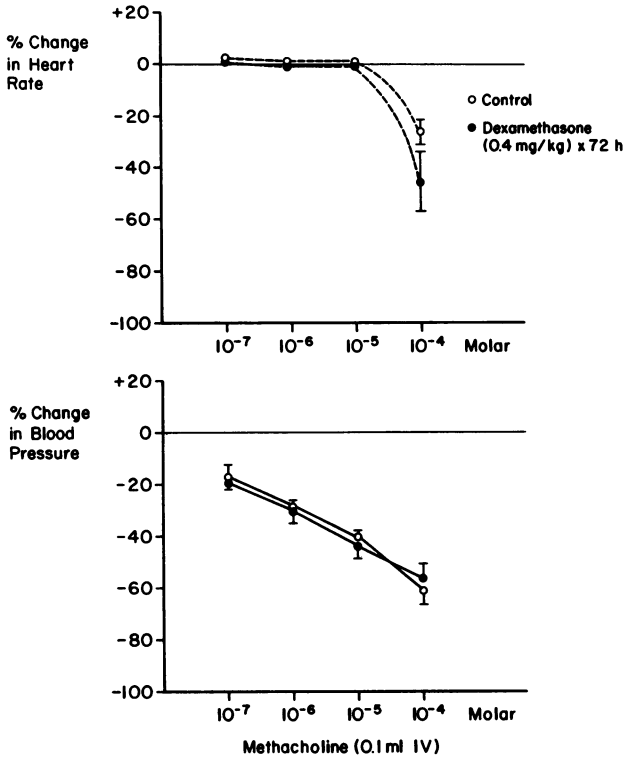

Figure 8. Cardiovascular responses of anesthetized rats to IV methacholine. Adult Wistar rats were injected with either 0.4 mg/kg dexamethasone in 0.1 ml sterile water or, sterile water alone (control). Rats were anesthetized 72 hours later and the femoral artery and vein canulated and used for continuous recording of the heart rate and blood pressure following the injection of 0.1 ml of a solution of methacholine (10^{-7}-10^{-4} M) in normal saline. The heart rate responses are shown in the top panel and the blood pressure changes in the bottom panel. Both results are expressed as percentage change from preinjection level. Dexamethasone caused an exaggeration of the bradycardic response to 10^{-4} M methacholine. Methacholine caused a progressive dose-dependent hypotensive response that was not attenuated by high-dose steroids (n = 5).

Discussion

This study demonstrates that endothelial cells respond to corticosteroids by producing a PLA_2 inhibitor, presumably lipocortin, which is responsible for a reduction of 45% in the amount of AA released from phospholipid pools following exposure to melittin. This inhibition is accompanied by a corresponding fall in the release of a known AA product, PGI_2. Reduced endothelial cell PGI_2 formation is detectable as early as 90 s after stimulation by melittin, by which time EDRF-induced elevation of cGMP in SMCs is maximal (24, 34–36). In mixed cultures melittin causes a dose-dependent, endothelium-dependent increase in smooth muscle cGMP which parallels relaxation responses in endothelium-intact rings of rat aorta (35, 36). Melittin also causes dose-dependent PGI_2 release from endothelial cells in mixed culture. While the dose-response curve for melittin-induced PGI_2 release from mixed cultures is displaced to the right by 10^{-6} M dexamethasone,

EDRF release as measured by cGMP accumulation is unaffected. Quinacrine, another PLA_2 inhibitor, has the same effect as dexamethasone. Marked inhibition of melittin-induced AA release by the use of a combination of PLA_2 and DAG lipase inhibitors, completely blocks PGI_2 production, but does not inhibit EDRF release. The residual melittin-induced AA release that can not be inhibited by PLA_2 inhibitors, appears to be due to activation of PLC and the action of DAG lipase.

A dose of ATP, equipotent with melittin in terms of EDRF-induced cGMP accumulation, releases 50% less PGI_2. This is consistent with our finding that melittin-induced PLC activation accounts for about 50% of the AA released in response to this agonist, and suggests that ATP-induced PGI_2 release may be due to PLA_2 activation alone. In keeping with ATP-induced AA release being due solely to PLA_2 activation, it is possible to completely inhibit PGI_2 release with three PLA_2 inhibitors: dexamethasone, hydrocortisone, and quinacrine. Under these conditions, however, EDRF release is significantly augmented by as much as 75%. Furthermore, dexamethasone did not alter endothelium-dependent relaxation in intact rings of rat aorta, nor did it affect methacholine-induced blood pressure changes in the whole animal. This fall in blood pressure is supposedly endothelium-dependent (37). Thus, we conclude that the production and release of EDRF, unlike PGI_2, is not highly correlated with inhibition of phospholipases.

A clear relationship exists between the release of EDRF and the activation of guanylate cyclase in SMCs (3, 4, 38) such that, cGMP changes in intact vascular rings and endothelial cell-SMC mixed cultures are acceptable biochemical assays for EDRF (3, 24, 25, 28, 34). Increased cGMP levels induced by endothelium-dependent vasodilators are associated with relaxation in both a time- and concentration-dependent manner (3, 25, 39). We used cGMP changes in mixed BAEC-SMC cultures to measure release of EDRF induced by endothelium-dependent vasodilators and confirmed the observation that endothelium has a conditioning effect on the basal level of cGMP in SMCs (24, 28).

The increases in cAMP following exposure of mixed BAEC-SMC cultures to melittin and ATP may be, in part, due to adenosine and perhaps PGI_2 release from the BAECs. Adenosine- and PGI_2-induced arterial relaxation is associated with cAMP accumulation in the vessel wall (40, 41). In addition, PGI_2-induced feedback inhibition of prostaglandin production is mediated through elevation of cAMP levels in the endothelium (42).

Our experiments show that melittin activates PLC as well as PLA_2. This was of concern to us since PLC activation represents, through the action of DAG lipase on DAG, an alternative source of AA. To address this concern, we successfully inhibited both PLA_2 and DAG lipase, and inhibited AA release and PGI_2 production by 90%. Despite this, EDRF release was unaffected. Thus, it is possible that melittin-induced EDRF release is mediated through PLC activation and IP_3 release, as suggested by the results of Loeb and colleagues (43).

Our results conflict with the results of previous studies in vessel segments in which certain PLA_2 inhibitors were found to inhibit endothelium-dependent relaxation, as well as the associated rise in smooth muscle cGMP concentration (1, 3, 5, 7, 34, 44–46). Quinacrine and para-bromophenacyl bromide have been shown to have other inhibitory effects on the blood vessel wall (47–51). Part of the inhibitory effect of quinacrine on relaxation responses to methacholine in intact rings appears to be due to inhibition of the muscarinic receptor or receptor coupling, since contractile responses to acetylcholine in denuded rings are also inhibited (47). Quinacrine inhibits other agonist-induced smooth muscle contractions making relaxation responses unreliable (3, 7, 48). In addition, quinacrine does not inhibit A23187-induced endothelium-dependent relaxation (49) and indeed, other recent studies have failed to demonstrate any inhibitory effect of quinacrine on EDRF release (4, 39). We feel, therefore, that measuring cGMP responses in cultured cells is a more accurate way of investigating the action of quinacrine than are recordings of relaxation responses in isolated arterial preparations. Para-bromophenacyl bromide is an alkylating agent that irreversibly inactivates several cellular enzymes including PLA_2 (50). Recently, our laboratory has shown that most of the inhibition of cGMP elevation with this inhibitor is due to its effects on smooth muscle not endothelium (51). Thus the actual mechanism by which para-bromophenacyl bromide inhibits endothelium-dependent relaxation is far from clear. Reviewing these data, we conclude that there is no established correlation between PLA_2 inhibition and inhibition of EDRF release.

Although glucocorticoids are notorious for their multiplicity of actions, our results with quinacrine which we found to be equally effective at inhibiting PGI_2 release and potentiating EDRF release, make it likely that the property they share in common, namely PLA_2 inhibition, is somehow involved in potentiating endothelium-dependent relaxation. We have no good explanation for how this may occur, but we believe it is a real phenomenon since we have observed it in the response to two separate endothelium-dependent agonists using three PLA_2 inhibitors. In addition, our results confirm an earlier observation suggesting that steroids often potentiate ATP-induced EDRF release in aortic rings (47). This potentiation is probably not due to induction of cytochrome P-450-dependent monooxygenases by steroids, since the cytochrome P-450 isozyme identified in endothelium (cytochrome P-450*c*) is not steroid-inducible (10, 52, 53).

Recent reports suggest that EDRF is nitric oxide (14), while other investigators have suggested that EDRF is a metabolite of AA produced by endothelial cells in smaller amounts than prostaglandins (11–13, 54). It is also possible that more than one substance is responsible for endothelium-dependent relaxation (55, 56). Our results suggest, but do not prove, that EDRF produced by cultured cells is some compound other than a metabolite of AA. In particular, the observation that basal unstimulated AA levels within the cell are sufficient for maximal EDRF production is difficult to explain if EDRF is derived from AA. We have proven, however, that even if EDRF is an AA metabolite, its production and/or release from cultured cells is not dependent upon, or regulated by, AA mobilization from membrane phospholipids.

In summary, this study demonstrates that melittin activates both PLA_2 and PLC, while ATP appears to activate PLA_2 alone. Inhibition of endothelial cell phospholipases with a variety of inhibitors, completely inhibits agonist-induced PGI_2 release, but not EDRF release. In fact, both melittin and ATP-induced EDRF release from cultured cells are potentiated by inhibitors of PLA_2. In conclusion, EDRF produced by cultured cells does not appear to be a metabolite of AA, and its production and/or release is independent of, and not regulated by, AA mobilization from membrane phospholipids.

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