Platelet-activating factor acts on cortisol secretion by perfused guinea-pig adrenals via calcium-/phospholipid-dependent mechanisms

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Abstract

Bilateral adrenals of the guinea pig were perfused *in situ* with an artificial medium equilibrated with 95% O₂/5% CO₂. Platelet-activating factor (PAF) induced biphasic cortisol responses, which reached a maximum at 10 nM PAF and declined at 100 nM. The effect of the PAF receptor antagonists CV-3988 and CV-6209 on PAF-stimulated cortisol secretion was examined. Prior exposure of adrenal glands to 10 µM CV-3988 or a simultaneous incubation with 10 µM CV-6209 abolished the cortisol response to 10 nM PAF. Lyso-PAF (a PAF precursor and breakdown product) did not affect cortisol secretion. Concentrations of 5–12.5 µM 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a protein kinase C (PKC) inhibitor, abolished subsequent cortisol secretion in response to 10 nM PAF. N-[2-(Methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H-8), a protein

kinase A inhibitor, was less effective. A calcium ionophore (A23187) at 3.3 and 10 µM increased cortisol secretion, but the activator of PKC, 1- α -1-oleoyl-2-acetyl-*sn*-3-glycerol (OAG), at 50 µM had no effect. When infused simultaneously, OAG (50 µM) and A23187 (3.3 µM) stimulated cortisol secretion synergistically. The secretory response of cortisol to repeated infusions of adrenocorticotrophin (100 pg/ml) or forskolin (10 µM) was essentially reproducible. By contrast, cortisol secretion in response to repeated infusions of PAF (10 nM) or OAG plus A23187 was not reproducible and the second response was diminished compared with the first. Our findings suggest that PAF plays a role in the regulation of steroidogenesis via a mechanism mediated by the PAF receptor and PKC.

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Introduction

A potent mediator of inflammatory and allergic processes, platelet-activating factor (PAF) is released by various cells such as macrophages, neutrophils, basophils, mast cells, endothelial cells and platelets (Braquet *et al.* 1987). In addition, PAF is involved in a wide spectrum of biological processes, such as hepatic glycogenolysis (Buxton *et al.* 1984), exocytosis in exocrine glands (Söling *et al.* 1984), ovoidimplantation and labor (Baldi *et al.* 1994). The intracerebroventricular injection of PAF stimulates the release of hypothalamic corticotrophin-releasing factor and increases plasma levels of adrenocorticotrophin (ACTH) and corticosterone in the rat (Rougeot *et al.* 1990). We found that PAF significantly increases glucocorticoid output from the perfused canine adrenal gland (Aikawa *et al.* 1991). Infusing PAF into the perfused adrenal glands of CD1 ICR mice does not induce a significant corticosterone response (Yang *et al.* 1995), whereas N-methylcarbamyl-PAF, a PAF agonist that cannot be metabolized, weakly but significantly stimulates corticosterone release (Han *et al.* 1998). These data indicate a species-dependent difference

in PAF-induced corticosteroidogenesis. Bovine adrenocortical cells can synthesize and release PAF and have high-affinity binding sites for PAF (Pelosin *et al.* 1991).

The binding of PAF to its receptor in many PAF-responsive cells is accompanied by inositol phospholipid hydrolysis by phospholipase C and the rapid accumulation of *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate. These second messengers subsequently promote calcium mobilization and protein kinase C (PKC) activation (Shimizu *et al.* 1992). Thus, PKC might participate in the production of glucocorticoids in fasciculata cells.

The present study examines whether PAF can act on cortisol secretion by the perfused guinea-pig adrenal via the PAF receptor and PKC activation.

Materials and Methods

Animals

Adult male guinea pigs (Hartley), weighing 480–850 g each, were group-housed in cages with suspended steel-mesh floors in a well-ventilated room maintained at 25 °C

under a 12 h light:12 h darkness. Sterilized laboratory guinea-pig chow and water were available *ad libitum*. The protocols conformed to the guidelines governing animal experimentation issued by the Animal Care and Use Committee of our university and by the Japanese Government (Law No. 105, 1 Oct 1973).

Drugs and reagents

We purchased 1-*O*-hexadecyl- and octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16} PAF and C_{18} PAF) and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (C_{16} lyso-PAF) from Bachem (Bettendorf, Switzerland). Synthetic adrenocorticotrophic hormone 1–24 (ACTH; Cortrosyn) was obtained from Organon (Boxtel, Holland). The PAF receptor antagonists CV-3988 and CV-6209 were gifts from the Central Research Division of Takeda Chemical Industries (Osaka, Japan). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a PKC inhibitor, and *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H-8), a protein kinase A inhibitor, were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Forskolin was purchased from Wako Pure Chemical Industries (Osaka, Japan) and 1- α -1-oleoyl-2-acetyl-*sn*-3-glycerol (OAG) was obtained from Avanti Polar-Lipids (Alabaster, AL, USA). Krebs–Ringer bicarbonate glucose (KRBG) solution contained 121 mM NaCl, 2.37 mM KCl, 1.19 mM $MgSO_4 \cdot 7H_2O$, 1.19 mM KH_2PO_4 , 2.54 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$ and 10 mM glucose.

Perfusion of adrenal glands

The bilateral adrenal glands of guinea pigs were perfused *in situ* with fresh KRBG containing 0.2% BSA and equilibrated with 95% O_2 /5% CO_2 . Animals were anesthetized with an i.p. injection of pentobarbital sodium (Somnopentyl; 3.8 mg/100 g; Pitman-Moore, Washington Crossing, NJ, USA), and the abdomen was incised through the midline 10 min thereafter. The celiac, superior mesenteric and inferior mesenteric arteries as well as the esophagus, vena portae, bile duct and colon were ligated and resected. The animals were then eviscerated. The renal arteries and veins were ligated at the hilum of the kidney on both sides. We isolated the bilateral adrenal circulation by ligating the ilio-lumbar, subcostal, internal spermatic and small vessels on both sides of the lumbo-abdominal region. The abdominal aorta and the inferior vena cava were separately ligated above the bifurcation and sutures were loosely passed around the aorta and inferior vena cava below the renal vessels. A cannula (i.v. catheter; ATOM, Tokyo, Japan) was inserted into the abdominal aorta. The top of the cannula was placed below the renal arteries and ligated. The cannula was connected to a silicon tube that was covered with a water jacket maintained at 38 °C by circulating water (Thermo

Minder; Taiyo, Saitama, Japan). The tube was attached to the long arm of a silicon T-tube (Phicon no. 5; Fuji Systems, Tokyo, Japan). A mini-pump (SJ-1211; Atto, Tokyo, Japan) was placed between the water jacket and the trifurcation of the T-tube. One short arm of the T-tube was connected to a large flask that contained KRBG plus 0.2% BSA and the other short arm was attached to a smaller flask that contained test compounds dissolved in the same solution. Both flasks were placed in a water bath maintained at 38 °C and bubbled with a gaseous mixture of 95% O_2 /5% CO_2 to maintain the pH at 7.4. One suture was loosely passed around the aorta below the diaphragm and another was passed around the inferior vena cava just above the right adrenal gland. Perfusion medium was introduced into the catheter in the abdominal aorta from the large flask via the mini-pump. The bilateral adrenal glands paled during perfusion with the medium immediately after the aorta was ligated with a suture that had been passed around it below the diaphragm. To stop the outflow from the lumbar arteries to the thoracic region, the muscles around the vertebral column were ligated together with the aorta and vertebral column by a suture at the crura of the diaphragm. Pentobarbital sodium (2 ml, 64.8 mg/ml) was injected subcutaneously and we confirmed that the heartbeat had stopped. Blood was washed out through a small hole cut in the inferior vena cava below the renal veins and perfusion was continued for about 20 min until the effluent perfusate became clear. Another cannula (constructed in-house from a silicon tube and an i.v. catheter) was inserted into the hole in the inferior vena cava and ligated immediately below the renal veins with the suture that had been passed around the inferior vena cava. Finally, the suture that had been passed around the inferior vena cava immediately above the right adrenal gland was ligated to direct the bilateral adrenal venous flow towards the exterior. About 40 min was required from laparotomy to the initial perfusion. The animal was placed in an acrylic chamber maintained at 38 °C. During all experiments, the adrenal glands were perfused for 40 min to allow equilibration before the start of any test procedures. Forskolin, ACTH, PAF, or OAG plus the calcium ionophore A23187 were dissolved in KRBG plus 0.2% BSA and infused into the adrenal glands over a period of 5 min from the smaller flask. Fractions of the perfusate were collected for 5 min before and every 5 min after starting the infusion of ACTH and forskolin or for 5 min before and every 2.5 or 5 min after starting the infusion of PAF and OAG plus A23187. CV-3988 (10 μ M), dissolved in KRBG plus 0.2% BSA, was infused simultaneously with PAF or over a period of 5 min from 20 or 35 min before the PAF infusion. CV-6209 (10 μ M), dissolved in KRBG plus 0.2% BSA, was infused simultaneously with PAF. H-7 (0.25–12.5 μ M) or H-8 (0.5–25 μ M) dissolved in KRBG plus 0.2% BSA was infused over 5 min starting 10 min before the infusion of ACTH or PAF.

The perfusate was collected in graduated glass tubes on ice, and then contaminating blood cells were removed by centrifugation and the supernatant was extracted with methylene chloride.

We used hexadecyl-PAF (C_{16} PAF) unless otherwise noted. The average weight of a pair of perfused adrenal glands was 290 ± 5 mg (mean $\pm$ S.E.M., $n=204$). The respective flow rates during the 5 min before and 5 min after the administration of ACTH and forskolin were as follows (means $\pm$ S.E.M., $n=6$): ACTH (100 pg/ml), 1.70 ± 0.06 and 1.66 ± 0.14 ml/min; forskolin (10 μ M), 1.78 ± 0.07 and 2.33 ± 0.14 ml/min. The respective flow rates during the 5 min before and 2.5 min after the administration of PAF or OAG plus A23187 were as follows (means $\pm$ S.E.M., $n=6$): PAF (10 nM), 1.94 ± 0.08 and 2.24 ± 0.34 ml/min; OAG (50 μ M) plus A23187 (3.3 μ M), 1.73 ± 0.09 and 2.03 ± 0.07 ml/min.

Biochemical analysis

Cortisol and corticosterone in perfusate extracts were quantified by thin-layer chromatography followed by fluorometry (Hirose 1977). We calculated the secretion rates of cortisol and corticosterone (ng/100 mg adrenal weight per min) by multiplying the concentration of the steroid (ng/ml) by the adrenal flow (ml/min) per 100 mg adrenals. The total secretion of cortisol during the 30- or 15-min period after the onset of infusion of ACTH, forskolin, PAF, or OAG plus A23187 over the basal value (immediately before infusion of compounds) was calculated. We measured catecholamine levels in the perfusate by coulometric electrochemical determination, with a minor modification as reported by Matsumoto *et al.* (1994).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Data were analyzed using STATISTICA software (Stat Soft, Tulsa, OK, USA). Mean secretory rates during each period and the dose-dependent secretion of cortisol were statistically compared by one- or two-way ANOVA, followed by the Newman-Keuls *post hoc* test for multiple comparisons. Student's *t*-test (two-tailed) for paired data compared sets of two values. Statistical significance was established at $P<0.05$.

Results

Secretory responses to ACTH

Figure 1 shows the secretory responses of cortisol to increasing amounts of ACTH (the inset shows the total amount of secreted cortisol). Rates of cortisol secretion increased dose-dependently in response to ACTH infu-

sions of 1, 10 and 100 pg/ml. The cortisol response to ACTH significantly increased at 10 pg/ml and peaked at 100 pg/ml. The peak of the cortisol response to 1000 pg/ml ACTH reached a plateau at 5–10 min that persisted thereafter.

Secretory responses to PAF

Figure 2 shows the secretory responses of cortisol to increasing amounts of PAF (the inset shows the total amount of secreted cortisol). Rates of cortisol secretion in response to 0.01, 0.1, 1 and 10 nM PAF increased dose-dependently. Cortisol secretion peaked 2.5–5 min after the infusion of PAF and then decreased to the basal level within 15–20 min. In response to 10 nM PAF, the duration of cortisol secretion was shorter and the peak was higher and earlier than those induced by 100 pg/ml ACTH. The response to PAF increased significantly at 0.1 nM and maximally at 10 nM. The response to PAF at 100 nM declined as compared with that at 10 nM.

Effect of PAF receptor antagonists, CV-3988 and CV-6209, on PAF-induced cortisol secretion

When concurrently applied with 10 nM PAF, 10 μ M CV-3988 did not inhibit the PAF-induced increase in cortisol secretion (Fig. 3). However, when adrenal glands were exposed to 10 μ M CV-3988 for 15 and 30 min before infusion with PAF, the cortisol response to PAF was inhibited by 43.7 and 41.7% respectively. Thus, the inhibitory effect apparently depended on the duration of the incubation after exposure to CV-3988. By contrast, 10 μ M CV-6209 infused together with PAF significantly inhibited the cortisol response. CV-3988 or CV-6209 did not induce any changes in the cortisol response.

Secretory responses to C_{18} PAF and lyso-PAF

Figure 4 compares the secretory responses of cortisol to C_{18} PAF, C_{16} lyso-PAF and PAF. The rates of cortisol secretion in response to 10 nM C_{18} PAF at 0–2.5, 2.5–5 and 5–10 min were 20.6, 28.4 and 83.7% respectively, as compared with those to 10 nM PAF. The total amount of cortisol secreted in response to C_{18} PAF was 52.5% of that induced by PAF (Fig. 4, inset). Lyso-PAF did not induce any changes in the cortisol response.

Effects of H-7 and H-8 on PAF-induced cortisol secretion

The PKC and protein kinase A inhibitors H-7 and H-8 respectively, did not induce changes in the cortisol response. We infused adrenal glands with 0.25, 0.5, 5 and 12.5 μ M H-7 or with 0.5, 5, 12.5 and 25 μ M H-8 for over 5 min, starting 10 min before the infusion of 10 nM PAF (Table 1). H-7 (5 and 12.5 μ M) significantly and dose-dependently inhibited cortisol secretion. The predicted

Figure 1 Secretory responses of cortisol to ACTH. Vehicle (□) or ACTH at 1 (◆), 10 (●), 100 (▲) or 1000 (■) pg/ml was infused (bar above curves) over 5 min and cortisol secretion was monitored. Inset: total amounts of cortisol secretion for 30 min after ACTH administration. Values are means $\pm$ S.E.M., $n=6$. * $P<0.05$, ** $P<0.01$ versus vehicle control.

value for 50% inhibition (IC_{50}) was about $3.0 \mu M$, and $5 \mu M$ H-7 caused approximately 62% inhibition. H-8 was less inhibitory than H-7 with respect to PAF-induced cortisol secretion.

Synergistic effects of diacylglycerol (OAG) and calcium ionophore A23187 on cortisol secretion

To confirm whether PKC participates in cortisol secretion from perfused guinea-pig adrenals, we examined the effect of a direct PKC activator. Figure 5 shows the cortisol responses to $50 \mu M$ OAG, 3.3 and $10 \mu M$ A23187, and a mixture of $50 \mu M$ OAG and $3.3 \mu M$ A23187. Diacylglycerol did not affect the rate of cortisol secretion. At both tested concentrations, A23187 alone increased cortisol secretion and peaked 5–10 min after the infusion of A23187. However, when $50 \mu M$ OAG and $3.3 \mu M$ A23187 were infused together, the cortisol response increased more than that caused by $3.3 \mu M$ A23187 alone. The rate of secretion peaked 2.5–5 min after infusion of the mixture. The total amount of cortisol secreted after

starting infusion of the mixture ($3.0 \pm 0.2 \mu g/100$ mg adrenals per 15 min) was much greater than the arithmetic sum of the amounts secreted in response to each agent alone ($50 \mu M$ OAG alone, $0.3 \pm 0.1 \mu g/100$ mg adrenals per 15 min; $3.3 \mu M$ A23187 alone, $1.3 \pm 0.3 \mu g/100$ mg adrenals per 15 min; Fig. 5, inset). The concentration of A23187 ($3.3 \mu M$) was critical, since a higher concentration ($10 \mu M$, $1.8 \pm 0.6 \mu g/100$ mg adrenals per 15 min) did not act synergistically with OAG ($50 \mu M$ OAG plus $10 \mu M$ A23187, $2.3 \pm 0.4 \mu g/100$ mg adrenals per 15 min). A23187 might have nonspecifically activated calcium-dependent enzymes by significantly increasing the Ca^{2+} concentration.

Secretory responses to repeated infusion of ACTH, forskolin, PAF, and OAG plus A23187

Figure 6A shows the reproducibility of cortisol and corticosterone secretion in response to repeated infusions of 100 pg/ml ACTH. The values for total cortisol and corticosterone secretion in response to the second infusion

Figure 2 Secretion of cortisol in response to PAF. Vehicle (□) or PAF at 0.01 (◆), 0.1 (●), 1 (▲), 10 (■) or 100 (▼) nM was infused (bar above curves) over 5 min and cortisol secretion was monitored. Inset: total amounts of cortisol secretion for 15 min after PAF administration. Values are means $\pm$ S.E.M., $n=6$. * $P<0.05$, ** $P<0.01$ versus vehicle control.

of ACTH were 99.7 and 74.1% respectively, of the values after the first infusions. We examined cortisol secretion in response to repeated infusions of 10 μ M forskolin (Fig. 6B). The second response to forskolin was 90.1% of the first infusion. The secretory responses of cortisol to ACTH and forskolin were essentially reproducible. We examined whether the adrenal responses of cortisol secretion induced by repeated exposure to 10 nM PAF or 50 μ M OAG plus 3.3 μ M A23187 were reproducible. Figure 7 shows that the second infusion of PAF or OAG plus A23187 only slightly increased cortisol secretion. The total amount of cortisol secreted during the second infusion was 31.7 or 21.7% of that of the first infusion. Thus, the adrenals were desensitized by a second infusion of the two compounds.

Secretory response of epinephrine and norepinephrine to PAF

The administration of 10 nM PAF did not alter catecholamine levels in the perfusate (5–1 min before, and 0–2.5 and 2.5–5 min after PAF administration: adrenaline, 22.6 ± 8.5 , 29.6 ± 12.4 and 19.6 ± 6.7 ng/ml per 100 mg

adrenals per min; noradrenaline, 1.2 ± 0.5 , 1.7 ± 0.8 and 1.0 ± 0.4 ng/ml per 100 mg adrenals per min; means $\pm$ S.E.M., $n=3$).

Discussion

The present study discovered that cortisol output in response to PAF is mediated by a PKC-dependent mechanism and that it can be desensitized.

Surgery involving laparotomy or manipulation of the visceral organs is probably associated with massive secretion of ACTH. However, low basal rates of cortisol secretion that were evident after starting perfusion increased dose-dependently in response to ACTH (Fig. 1). The maximal secretory rate of cortisol in response to 100 or 1000 pg/ml ACTH was greater than the response to approximately 300 pg/ml ACTH in the dog adrenal (323 ± 64 ng/100 mg adrenals per min), perfused with arterial blood from a hypophysectomized/nephrectomized dog (T Aikawa, T Hirose, I Matsumoto & T Suzuki,

Figure 3 Inhibitory effects of CV-3988 and CV-6209 on PAF-induced cortisol secretion. PAF (10 nM) was infused with or without 10 µM CV-3988 or CV-6209 over 5 min (control or pretreatment time 0). CV-3988 was infused over 5 min from 20 or 35 min before PAF infusion (pretreatment time 15 or 30 min). Cortisol secretion in response to PAF with and without (control) inhibitor was compared. Values are means $\pm$ S.E.M., $n=6$. * $P<0.05$ versus control.

unpublished observations). Therefore, our perfusion system using an artificial medium can evaluate both the dynamics of secretion and the acute effects of compounds on the stimulated release of cortisol. The repeated secretory responses of cortisol to 100 pg/ml ACTH at 45 min intervals were very high and reproducible (Fig. 6). This finding indicates that high levels of biosynthesized steroid precursors are located in the adrenals, although the perfusion medium did not contain cholesterol or any other steroid precursors except glucose.

Epinephrine directly stimulates cortisol secretion via an α -adrenergic mechanism in perfused adrenal glands of the guinea pig (Mokuda *et al.* 1992). However, 10 nM PAF did not evoke catecholamine release in the adrenal medulla in the present study. Hence, PAF probably stimulates cortisol release via a mechanism that does not include epinephrine.

The secretion of cortisol in response to PAF increased significantly at 0.1 nM, more at 1 nM and maximally at 10 nM and the response to 100 nM PAF was smaller than that to 10 nM PAF (Fig. 2). The maximal cortisol response to 10 nM PAF was higher than that to 100 or 1000 pg/ml ACTH and the response declined sharply within 15 min. The PAF concentrations required to cause 50% maximal platelet aggregation and 20% maximal platelet ATP secre-

tion in guinea pig are 0.41 and 0.56 nM respectively (Coëffier *et al.* 1986). The minimal effective concentration of PAF (0.1 nM) for cortisol secretion was almost identical to that required for platelet aggregation and activation.

PAF acetylhydrolase rapidly metabolizes PAF to an inactive form (Stafforini *et al.* 1987). PAF circulates in the peripheral blood at subnanomolar or nanomolar concentrations in humans, rabbits and rats under physiological conditions (Caramelo *et al.* 1984). Domain II of extracellular albumin is an exclusive acceptor for PAF released by cells (Ammit & O'Neill 1997) and binding to it might increase the half-life of PAF. If the adrenocortical cells are exposed to subnanomolar concentrations of PAF in the perivascular space fluid of the zona fasciculata and zona reticularis under immediate hypersensitivity or severe inflammation, cortisol release will be evoked.

Our previous studies of dogs showed that PAF at 1 nM in the perfused adrenal and 0.1 nM in adrenocortical cells causes significant amounts of cortisol release (Aikawa *et al.* 1991). PAF potently stimulates the adrenal cortex of both guinea pigs and dogs. On the other hand, infusing PAF into the perfused adrenals of mice did not induce a significant corticosterone response (Yang *et al.* 1995). The administration of PAF does not evoke corticosterone production in dispersed rat adrenocortical cells (T Shimada, T Hirose, I Matsumoto & T Aikawa, unpublished observations). These data indicate a species difference in PAF-induced glucocorticoid release. The ACTH-induced secretion of corticosterone in the present study was 1.5% that of cortisol. The adrenal cortex of guinea pigs secretes mainly cortisol. Guinea pigs, dogs and humans are corticosteroid-resistant, and the adrenals of these animals secrete mainly cortisol. On the other hand, the adrenal cortex of corticosteroid-sensitive species such as mice and rats secretes mainly corticosterone, of which the glucocorticoid activity is much weaker than that of cortisol. High doses of glucocorticoids severely suppress the immune response and are lethal for corticosteroid-sensitive animals such as rats and mice.

At subnanomolar or nanomolar concentrations, PAF causes platelet aggregation and cortisol release in guinea pigs and dogs, but not in rats and mice. In guinea pigs and dogs, PAF might play a more important role as a potent mediator of immediate hypersensitivity or inflammation than in rats and mice. Glucocorticoids suppress the production and action of chemical mediators, including PAF, and induce phospholipase A_2 -inhibiting protein (lipocortin I/annexin I) that affects the arachidonic cascade and release of the PAF precursor, lyso-PAF (Flower & Rothwell 1994). Glucocorticoids decrease the activity of PAF acetyltransferase in the rat liver and spleen and increase the plasma PAF acetylhydrolase activity (Ihara *et al.* 1993). Cortisol secretion by PAF that increases under immediate hypersensitivity or more-severe inflammation might represent a life-saving host defense reaction via a negative-feedback mechanism.

Figure 4 Effects of infusion of C₁₈ PAF and lyso-PAF. Vehicle (□), 10 nM C₁₈ PAF (●) or 10 nM C₁₆ lyso-PAF (▲) was infused over 5 min and cortisol secretion was compared with that in response to 10 nM PAF (○). Inset: total amounts of cortisol secretion in response to vehicle, PAF, C₁₈ PAF and C₁₆ lyso-PAF. Values are means $\pm$ S.E.M., $n=6$. †† $P<0.01$ versus PAF; ** $P<0.01$ versus vehicle control.

CV-3988 and CV-6209 (Terashita *et al.* 1983, 1987) are structural analogs that competitively antagonize PAF at its receptor. The adrenals had to be perfused with CV-3988 before PAF to inhibit PAF-induced cortisol release (Fig. 3). On the other hand, the simultaneous admin-

istration of CV-6209 was an effective inhibitor, because CV-6209 is more lipid-soluble and it binds with higher affinity than CV-3988.

Figure 4 shows that an infusion of lyso-PAF did not induce cortisol secretion and that octadecyl-PAF

Table 1 Inhibitory effects of H-7 and H-8 on the PAF-induced secretion of cortisol. The secretory responses to 10 nM PAF with prior treatment with 0.25, 0.5, 5 and 12.5 μ M H-7 or with 0.5, 5, 12.5 and 25 μ M H-8 were compared to responses without prior treatment with the inhibitor. Numbers in parentheses represent percentage changes compared to the corresponding control (vehicle) value (100%). Values are means $\pm$ S.E.M., $n=6$

	H-7 (μ g/100 mg adrenal wt./15 min)		H-8 (μ g/100 mg adrenal wt./15 min)	
Treatment (μ M)				
Vehicle	4.26 $\pm$ 0.54	(100.0)	4.26 $\pm$ 0.54	(100.0)
0.25	4.00 $\pm$ 0.32	(93.9)	—	—
0.5	2.78 $\pm$ 0.80	(65.3)	4.53 $\pm$ 0.77	(106.3)
5	1.64 $\pm$ 0.41**	(38.5)	3.67 $\pm$ 0.46	(86.2)
12.5	1.65 $\pm$ 0.27**	(38.9)	2.71 $\pm$ 0.29	(63.6)
25	—	—	2.73 $\pm$ 0.55	(64.1)

** $P<0.01$ versus vehicle control.

Figure 5 Synergistic effects of OAG and A23187 on cortisol secretion. Vehicle (□), 50 μM OAG (■), A23187 at 3.3 (▲) or 10 μM (◆), or a mixture of 50 μM OAG and 3.3 μM A23187 (●) was infused (bar above curves) over 5 min and cortisol production was monitored. Inset: total amounts of cortisol secretion in response to vehicle control, 50 μM OAG, 3.3 or 10 μM A23187, and 50 μM OAG plus 3.3 μM A23187. Values are means $\pm$ S.E.M., $n=6$. † $P<0.05$, †† $P<0.01$ versus OAG plus A23187; * $P<0.05$, ** $P<0.01$ versus vehicle control.

stimulated less cortisol release than hexadecyl-PAF. Thus, not only the acetyl moiety at the second position in the triglyceride but also the length of the alkyl side chain at the first position is responsible for binding to the specific PAF receptor on adrenocortical cells.

The interactions between ACTH- and PAF-regulated steroidogenesis are of considerable interest. *N*-Methylcarbamyl-PAF, which cannot be metabolized, induces corticosterone release and a PAF antagonist significantly depresses corticosterone release in response to ACTH, forskolin or dibutyryl cAMP in perfused mouse adrenals (Han *et al.* 1998). PAF and its receptors affect the ACTH-signaling pathway of adrenocortical cells in mice. Our preliminary examinations showed that the cortisol response to PAF after treatment with ACTH seemed much smaller than that after an initial infusion of PAF. By contrast, the cortisol response to ACTH after treatment with PAF seemed to be greater than that after the first infusion. The cortisol response to a mixture of 10 pg/ml ACTH and 1 nM PAF was much lower than the

arithmetic sum of the amounts of each agent secreted separately (10 pg/ml ACTH, 3.0 ± 0.6 μg/100 mg adrenals per 30 min; 1 nM PAF, 2.1 ± 0.4 μg/100 mg adrenals per 30 min; 10 pg/ml ACTH plus 1 nM PAF, 4.0 ± 0.4 μg/100 mg adrenals per 30 min; $n=6$). Furthermore, the adrenal response of cortisol secretion by repeated exposure to the mixture was fairly reproducible (T Shimada, T Hirose, I Matsumoto & T Aikawa, unpublished observations). Consequently, we are examining the intracellular mechanisms of crosstalk between PAF- and ACTH-modulated cortisol secretions in guinea pigs.

The selective inhibitors of protein kinases functioned quite differently. H-7 at 5 and 12.5 μM abolished PAF-induced cortisol secretion, whereas inhibition by H-8 was not statistically significant (Table 1). Therefore, the PAF-induced secretion of cortisol seems to involve PKC-activated processes.

Angiotensin II, the action of which involves the phosphoinositide-turnover-mediated activation of PKC,

Figure 6 Reproducibility of cortisol response to repeated infusion of ACTH and forskolin. (A) 100 pg/ml ACTH or (B) 10 μ M forskolin was infused twice (bars above curves) over 5 min and cortisol (●) and corticosterone (○) production was monitored. Total calculated amounts of cortisol secretion during 30 min after the first and second administrations were 9.4 ± 1.5 and 9.4 ± 1.4 μ g/100 mg adrenal weight for ACTH and 4.0 ± 0.6 and 3.6 ± 0.6 μ g/100 mg adrenal weight for forskolin. Values are means $\pm$ S.E.M., $n=6$. * $P<0.05$, ** $P<0.01$ versus basal value (immediately before infusion of stimuli).

stimulates glucocorticoid secretion in bovine (Hadjian *et al.* 1984, Williams *et al.* 1989, Viard *et al.* 1990), human (Lebrethon *et al.* 1994) and guinea-pig (Nishikawa *et al.* 1990) adrenocortical fasciculata cells. In contrast, angiotensin II-induced steroidogenesis is somewhat controversial. In rat (Arola *et al.* 1994), sheep (Viard *et al.* 1990) and human (Ishizuka *et al.* 1988) fasciculata cells that contain active PKC, neither angiotensin II nor PKC activators induce steroidogenesis. The PKC activator OAG (50 μ M) together with A23187 (3.3 μ M) caused cortisol secretion when simultaneously infused in the present study (Fig. 5). The A23187 (3.3 and 10 μ M)-induced release of cortisol might have been mediated by an increase in the cytosolic

Figure 7 Desensitization of cortisol response to repeated infusions of PAF and OAG plus A23187. (A) 10 nM PAF or (B) 50 μ M OAG plus 3.3 μ M A23187 was infused twice (bars above curves) over 5 min and cortisol (●) and corticosterone (○) secretion was monitored. Total calculated amounts of cortisol secretion during 15 min after the first and second responses were 4.7 ± 0.8 and 1.5 ± 0.3 μ g/100 mg adrenal weight for PAF and 3.0 ± 0.2 and 0.7 ± 0.1 μ g/100 mg adrenal weight for OAG plus A23187 respectively ($P<0.01$ for second response versus first response in each case, by paired *t*-test). Values are means $\pm$ S.E.M., $n=6$. * $P<0.05$, ** $P<0.01$ versus basal value (immediately before infusion of stimuli).

concentration of Ca^{2+} ions, with consequent modulation of reactions that are dependent on PKC and/or calmodulin.

Figure 6 shows that the cortisol response to the repeated administration of ACTH or forskolin, an adenylylase activator, was reproducible. These results suggest that protein kinase A-induced steroidogenesis is neither up- nor down-regulated. By contrast, cortisol secretion in response to repeated infusions of PAF or OAG plus A23187 became desensitized, as the second response was much smaller than the first. A G-protein-coupled receptor kinase or activated PKC might exert a negative-feedback effect that results in the direct inactivation of PKC itself or the inhibition of phospholipase C through inactivation of

the receptor by phosphorylation (Izumi & Shimizu 1995, Kishimoto *et al.* 1989). These results support the notion that PAF activates cortisol release via calcium- and phospholipid-dependent PKC activation.

The present study confirmed that PAF at subnanomolar concentrations evokes cortisol output via a mechanism involving PKC in the guinea-pig adrenal gland.

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