Cross-regulation of cortisol secretion by adrenocorticotropin and platelet-activating factor in perfused guinea pig adrenals

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Abstract

We examined the cross-regulation of signaling between ACTH- and platelet-activating factor (PAF)-mediated steroidogenesis in the perfused guinea pig adrenal gland. Our method of *in situ* perfusion using an artificial medium can evaluate whether cortisol secretion in response to ACTH and PAF is interactive. Treating adrenal glands with 100 pg/ml ACTH diminished the subsequent cortisol response to 10 nM PAF. By contrast, PAF resulted in subsequent potentiation of ACTH-induced cortisol secretion. A mixture of 50 μ M 1- α -1-oleoyl-2-acetyl-*sn*-glycerol (OAG), an activator of protein kinase C (PKC), and 3.3 μ M calcium ionophore (A23187), or 10 μ M forskolin (FRK) diminished the cortisol response to PAF, whereas that to

ACTH was unaffected. Each of PAF, ACTH, or FRK eliminated the cortisol response to OAG plus A23187, whereas that to FRK was unaffected. These data show that the protein kinase A (PKA)-dependent processes activated by ACTH or FRK can interfere with PAF-induced signal transduction at receptor and post-receptor levels. In contrast, PKC-dependent processes activated by PAF promoted ACTH-signaling at receptor and post-receptor level. Cross-regulation between processes activated by PAF receptor-PKC and by ACTH receptor-PKA might function in the multifactorial regulation of adrenocortical steroidogenesis.

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Introduction

Platelet-activating factor (PAF) is a potent mediator of immune and inflammatory responses, cardiovascular, reproductive, respiratory, metabolic, and nervous systems (Ishii & Shimizu 2000, Stafforini *et al.* 2003). We found that PAF is a potent activator of glucocorticoid secretion from the adrenal glands of dogs (Aikawa *et al.* 1991) and guinea pigs (Shimada *et al.* 2005) perfused with artificial medium. In perfused guinea pig adrenals, PAF acts mainly through a membrane receptor accompanied by protein kinase C (PKC) activation (Shimada *et al.* 2005). On the other hand, ACTH acts through its receptor, melanocortin-2 receptor (Clark & Cammas 1996), accompanied by protein kinase A (PKA) activation. The secretion of glucocorticoids in response to repeated infusions of adrenocorticotrophin (ACTH) or FRK is almost equal in perfused adrenal glands of dogs (Aikawa *et al.* 1986), mice (Yang *et al.* 1995), and guinea pigs (Shimada *et al.* 2005). By contrast, cortisol secretion in response to a second infusion of PAF was diminished (Shimada *et al.* 2005). Thus, PAF desensitizes steroidogenesis in a homologous manner through its own ligand. The mechanism of such receptor-specific homologous desensitization is agonist-dependent phosphorylation of the receptor by specific G-protein-coupled receptor kinases (GRKs). Since a second exposure to the PKC activator, OAG, together with A23187

elicited a diminished response (Shimada *et al.* 2005), we proposed that not only GRKs but also activated PKC eliminates PAF receptor-mediated cortisol secretion. These processes should be associated with the modulation of the receptor and post-receptor compounds. At the receptor level, receptors would be phosphorylated and substantially uncouple from G-protein. At the post-receptor level, PKC or phospholipase C (PLC) might be directly inactivated.

The present study examines cross-regulation of adrenal corticosteroidogenesis by pathways activated by PAF and ACTH.

Materials and Methods

Animals

Adult male Hartley guinea pigs weighing 490–780 g each, were group housed in cages with suspended steel-mesh floors in a well-ventilated room at 25 °C under a 12 h light:12 h darkness cycle. Sterilized laboratory guinea pig chow and water were made available *ad libitum*. The protocols conformed to the guidelines governing animal experiments issued by the Animal Care and Use Committee of Nagasaki University and the Physiological Society of Japan Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences (revised 2002).

Drugs and reagents

We purchased 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) from Bachem (Bettendorf, Switzerland). Synthetic adrenocorticotrophic hormone 1–24 (ACTH; Cortrosyn) was obtained from Organon (Boxtel, Holland). Forskolin (FRK) 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.). BSA (fraction V) was obtained from Sigma. Krebs–Ringer bicarbonate glucose (KRBG) contained 121 mM NaCl, 2.37 mM KCl, 1.19 mM MgSO₄·7H₂O, 1.19 mM KH₂PO₄, 2.54 mM CaCl₂·2H₂O, 25 mM NaHCO₃ 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% O₂ and 5% CO₂ as described previously (Shimada *et al.* 2005). Briefly, the 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 iliolumbar, subcostal, internal spermatic, and small vessels on both sides of the lumboabdominal 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 a mini-pump (SJ-1211; Atto, Tokyo, Japan) and connected to a large and smaller flask that contained KRBG plus 0.2% BSA and test compounds dissolved in the same solution respectively. Both flasks were placed in a water bath maintained at 38 °C and bubbled with a gaseous mixture of 95% O₂ and 5% CO₂. 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. Immediately after the aorta was ligated with the suture that had been passed around it below the diaphragm, the bilateral adrenal glands paled during perfusion with the medium. 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 s.c. and we confirmed that the heartbeat had stopped. Another cannula was inserted into the hole in the inferior vena cava and ligated. 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 toward the exterior. 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 procedures. Each of FRK, ACTH, PAF, or OAG plus A23187 was dissolved in KRBG containing 0.2% BSA and perfused into the adrenal glands over a period of 5 min from the smaller flask. Fractions were collected for 5 min before and every 5 min after starting perfusion with ACTH and FRK or for 5 min before and every 2.5 or 5 min after starting PAF and OAG plus A23187.

Perfusates were collected into graduated glass tubes on ice, contaminating blood cells were removed by centrifugation, and the supernatant was extracted with methylene chloride.

The average weight of a pair of perfused adrenal glands was 266.4 ± 4.5 mg (means $\pm$ S.E.M., $n=72$). The respective flow rates (mean $\pm$ S.E.M., $n=6$): during the 5 min before and after perfusion with ACTH and FRK were ACTH (100 pg/ml), 1.84 ± 0.13 and 2.02 ± 0.08 ml/min; and FRK (10 μ M), 1.84 ± 0.07 and 2.02 ± 0.11 ml/min. The respective flow rates (mean $\pm$ S.E.M., $n=6$): during the 5 min before and 2.5 min after perfusion with PAF and OAG plus A23187 were PAF (10 nM), 1.99 ± 0.10 and 2.14 ± 0.17 ml/min; OAG (50 μ M) plus A23187 (3.3 μ M), 1.86 ± 0.12 and 2.07 ± 0.10 ml/min.

Biochemical analysis

Perfusate extracts were purified by thin layer chromatography and then cortisol was quantified by fluorometry (Hirose 1977). We calculated the secretion rate of cortisol (ng/100 mg adrenal weight per min) by multiplying the concentration of the steroid (ng/ml) with the adrenal flow (ml/min) per 100 mg adrenals. We calculated the total secretion of cortisol during the 30- or 15-min after starting perfusion with ACTH, FRK, PAF, or OAG plus A23187 over the basal value (immediately before infusion of compounds).

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 secretion of cortisol were statistically compared by one-way 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 response of cortisol to ACTH and PAF after exposure to PAF and ACTH respectively

To study the effect of ACTH and PAF cross-regulation on the secretory response of cortisol in the perfused guinea pig adrenal, 10 nM PAF was perfused for 45–50 min before 100 pg/ml ACTH (Fig. 1A). We also similarly examined the cortisol response to PAF after ACTH perfusion (Fig. 1B). As the cortisol response to ACTH after saline infusion (vehicle control) was almost equal to the response to first and second infusions of repeated exposure to ACTH, we compared the response to second infusions of ACTH (Fig. 1B) with the first one (Fig. 1A). Exposure to PAF potentially augmented subsequent cortisol release in response to ACTH; the rate of maximal cortisol secretion induced by the second perfusion with ACTH was significantly augmented (Fig. 1A). By contrast, ACTH attenuated subsequent cortisol release in response to PAF; the rate of maximal cortisol secretion induced by the second PAF

infusion was significantly attenuated (Fig. 1B). The total amounts of cortisol secretion in response to the second perfusions with ACTH and PAF were 162.8 and 40.9% when compared with the initial responses (Fig. 1C and Table 1). Thus, PAF augmented ACTH-mediated cortisol secretion, and ACTH diminished PAF-mediated cortisol secretion.

Secretory responses of cortisol to PAF and FRK after perfusion with FRK and PAF respectively

To determine whether PKA participates in the cross-regulation of cortisol secretion by ACTH and PAF, FRK, an activator of cAMP–PKA pathways, was perfused before PAF (Fig. 2A). Cortisol secretion induced by 10 nM PAF after perfusion with 10 μ M FRK resulted in potent suppression. The total amount of cortisol secretion in response to the second perfusions with PAF was 15.9% when compared with the initial responses (Fig. 2C). On the contrary, PAF potentially augmented subsequent

Figure 1 Effect of PAF on ACTH-induced cortisol secretion and vice versa. (A) We perfused adrenals with 10 nM PAF for 5 min (bar above curve) then with 100 pg/ml ACTH for 5 min (bar above curve), and monitored cortisol production. (B) Adrenals were perfused with 100 pg/ml ACTH followed by 10 nM PAF and cortisol secretion was monitored. (C) Total amounts of cortisol secretion during first and second perfusions with ACTH and PAF were as follows: 13.6 ± 1.9 and 22.1 ± 2.8 (μ g/100 mg adrenal wt./30 min) for ACTH, and 3.3 ± 0.3 and 1.4 ± 0.4 (μ g/100 mg adrenal wt./15 min) for PAF. All data are shown as means $\pm$ S.E.M. ($n=6$). ** $P<0.01$, * $P<0.05$ versus basal value (immediately before perfusion with compounds); † $P<0.01$, † $P<0.05$ and ## $P<0.01$, # $P<0.05$ first versus second perfusion.

Table 1 Summary of experimental results

	Second application			
	PAF	ACTH	OAG + A23187	Forskolin
First application				
PAF	↓↓	↑↑↑	↓	↑↑
ACTH	↓↓	—	↓	—
OAG + A23187	↓↓↓	↑	↓↓↓	—
Forskolin	↓↓↓	—	↓	—

↓, ↑, or — indicates a decrease, increase, or no change respectively, in the total amounts of cortisol secretion in response to second application of 10 nM PAF, 100 pg/ml ACTH, 50 μM OAG plus 3·3 μM A23187, and 10 μM FRK when compared with those of first applications.

cortisol release in response to FRK (Fig. 2B), and the rate of sub-maximal cortisol secretion induced by the second perfusion with FRK was potentially augmented. PAF caused a consistent, but not statistically significant, augmentation in the total amounts of cortisol secretion in response to the second perfusion with FRK (Fig. 2C). Thus, PAF augmented FRK-mediated cortisol secretion, and PKA activated by FRK participates in heterologous desensitization of PAF-induced cortisol secretion.

Secretory response of cortisol to ACTH after perfusion with OAG plus A23187 and vice versa

To confirm whether PKC participates in the cross-regulation of cortisol secretion by ACTH and PAF, OAG plus A23187, a direct PKC activator, was perfused before ACTH (Fig. 3A). The cortisol response to 100 pg/ml ACTH after 50 μM OAG plus 3·3 μM A23187 was unaffected. By contrast, ACTH potentially attenuated subsequent cortisol release in response to OAG plus A23187 (Fig. 3B). The total amounts of cortisol secretion in response to the second perfusions with ACTH and OAG plus A23187 were 119·7 and 47·0% respectively, when compared with the initial responses (Fig. 3C and Table 1). Thus, ACTH-mediated activation of PKA diminishes the OAG plus A23187-induced cortisol secretion, and PKC activated by OAG plus A23187 did not significantly affect ACTH-mediated one.

Secretory response of cortisol to OAG plus A23187 after prior perfusion with FRK and vice versa

To confirm cross-regulation of post-receptor second messenger-dependent kinases, OAG plus A23187 was perfused before FRK (Fig. 4A). The cortisol response to

Figure 2 Effect of FRK on PAF-induced cortisol secretion and vice versa. (A) Adrenals were perfused with 10 μM FRK for 5 min (bar above curve) followed by 10 nM PAF for 5 min (bar above curve), and cortisol secretion was monitored. (B) Adrenals were perfused with 10 nM PAF followed by 10 μM FRK, and cortisol production was monitored. (C) Total amounts of cortisol secretion during first and second perfusions with PAF and FRK were as follows: 4.1 ± 1.9 and $^{*}0.7 \pm 0.2$ (μg/100 mg adrenal wt./15 min) for PAF, and 3.7 ± 0.7 and 5.2 ± 0.8 (μg/100 mg adrenal wt./30 min) for FRK. All data are shown as means $\pm$ S.E.M. ($n=6$). For significance of differences, see Fig. 1.

Figure 3 Effect of OAG plus A23187 on ACTH-induced cortisol secretion and *vice versa*. (A) Adrenals were perfused with 50 μ M OAG plus 3.3 μ M A23187 for 5 min (bar above curve) followed by 100 pg/ml ACTH for 5 min (bar above curve), and cortisol secretion was monitored. (B) Adrenals were perfused with 100 pg/ml ACTH followed by a mixture of 50 μ M OAG and 3.3 μ M A23187, and cortisol production was monitored. (C) Total amounts of cortisol secretion during first and second perfusions with ACTH and OAG plus A23187 were as follows: 15.0 ± 1.6 and 18.2 ± 1.5 (μ g/100 mg adrenal wt./30 min) for ACTH, and 2.9 ± 0.4 and 1.4 ± 0.3 (μ g/100 mg adrenal wt./15 min) for OAG plus A23187. All data are shown as means $\pm$ S.E.M. ($n=6$). For significance of differences, see Fig. 1.

10 μ M FRK after 50 μ M OAG plus 3.3 μ M A23187 was unaffected. By contrast, the cortisol response to OAG plus A23187 after FRK was diminished (Fig. 4B). The total amounts of cortisol in response to the second perfusions with FRK and OAG plus A23187 were 109.9 and 57.1% when compared with the initial responses (Fig. 4C; Table 1). Thus, PKA activated by FRK diminished OAG plus A23187-induced cortisol secretion, but PKC activated by OAG plus A23187 did not affect the FRK-activated one.

Secretory response of cortisol to ACTH after FRK and *vice versa*

To confirm whether PKA affects ACTH-induced cortisol secretion, FRK was perfused before ACTH (Fig. 5A). The cortisol response to 100 pg/ml ACTH after 10 μ M FRK was unaffected. Similarly, ACTH did not affect subsequent cortisol release in response to FRK (Fig. 5B). The total amounts of cortisol in response to the second perfusions with ACTH and FRK were 79.7 and 104.2% respectively, of the initial responses (Fig. 5C; Table 1). Thus, the ACTH receptor–cAMP–PKA pathway is almost completely free of desensitization.

Secretory response of cortisol to PAF after OAG plus A23187 and *vice versa*

To confirm whether PKC plays an essential role in PAF desensitization, OAG plus A23187 was perfused before PAF (Fig. 6A). The cortisol response to 10 nM PAF after 50 μ M OAG plus 3.3 μ M A23187 was obviously attenuated. Similarly, PAF also attenuated the subsequent cortisol release in response to OAG plus A23187 (Fig. 6B). The total amounts of cortisol released in response to the second perfusions with PAF and OAG plus A23187 were 29.7 and 52.7% respectively of the initial responses (Fig. 6C and Table 1). Activated PKC induces heterologous desensitization of the PAF-activated cortisol secretion, and PAF-mediated activation of PKC down-regulates the OAG plus A23187-activated one.

Discussion

This study characterizes the functional properties of the short-term cross-regulation of PAF- and ACTH-mediated cortisol secretion using *in situ* perfusion of the guinea pig

Figure 4 Effect of OAG plus A23187 on FRK-induced cortisol secretion and *vice versa*. (A) Adrenals were perfused with a mixture of 50 µM OAG plus 3.3 µM A23187 for 5 min (bar above curve) followed by 10 µM FRK for 5 min (bar above curve), and cortisol secretion was monitored. (B) Adrenals were perfused with 10 µM FRK followed by 50 µM OAG plus 3.3 µM A23187 and cortisol secretion was monitored. (C) Total amounts of cortisol secretion during first and second perfusions with FRK and OAG plus A23187 were as follows: 5.1 ± 0.4 and 5.6 ± 0.4 (µg/100 mg adrenal wt./30 min) for FRK, and 3.6 ± 0.6 and 2.1 ± 0.3 (µg/100 mg adrenal wt./15 min) for OAG plus A23187. All data are shown as means $\pm$ S.E.M. ($n=6$). For significance of differences, see Fig. 1.

adrenal gland. The PAF receptor interacts with multiple G-proteins, $G_{q/11}$ and G_i , leading to the simultaneous stimulation of distinct signaling pathways. While the ACTH receptor interacts preferentially with stimulatory G-protein (G_s) to stimulate adenylyl cyclase (AC), coupling to inhibitory G-protein (G_i) has also been reported (Cote *et al.* 2001, Martini *et al.* 2004). However, the net effect of G_i activation is the stimulation of cAMP production via the $\beta\gamma$ -subunit of G_i and not AC inhibition through the α_i subunit (Cote *et al.* 2001).

Prior perfusion with PAF preferentially enhanced cortisol secretion in response to ACTH (Fig. 1A). Angiotensin II (ANG II) and ACTH are major hormonal regulators of the adrenal zona glomerulosa and zona fasciculata. In adrenal glomerulosa and fasciculata cells, ANG II enhances subsequent ACTH-induced AC activity and the steroidogenic response (Langlois *et al.* 1992, Baukal *et al.* 1994). Since the actions of PAF and ANG II are mediated by PKC activation, we evaluated the effects of an activator of the PKC pathway on the cortisol response to ACTH. OAG plus A23187 did not significantly augment the subsequent cortisol response to ACTH (Fig. 3A). This means that direct PKC activation cannot mimic the potentiating effect of PAF. This finding suggests that $G_{q/11}$ activation by PAF binding to the receptor is required for potent augmentation of the cortisol response to ACTH. Although ANG II potentiates the stimulatory effect

of ACTH in glomerulosa cells, the PKC activator, phorbol 12-myristate 13-acetate (PMA), only partially mimics the effect of ANG II (Langlois *et al.* 1992). Besides, these actions of ANG II and PMA are prevented by pre-incubation with calcineurin (Ca^{2+} /calmodulin-dependent protein phosphatase) inhibitors (Baukal *et al.* 1994). The dual regulation system mediated by Ca^{2+} -calmodulin and PKC might explain why ACTH-induced cortisol secretion can be regulated through phosphorylation and dephosphorylation steps that control the activities of ACTH receptor-coupled G-proteins and/or AC. Figs 2B and 4A show that PAF significantly augmented but OAG plus A23187 did not affect the cortisol response to FRK. Two sites of enhancement of ACTH receptor-mediated signaling should be considered. One is at the receptor and/or G-protein level, namely, a result of greater turnover of G_s or attenuation of α_i subunit of G_i that renders receptors resistant to inactivation and the other is the potentiation of AC activity. The results indicate that PKC-dependent processes activated by PAF promote ACTH signaling at receptor and post-receptor levels.

Our previous results have indicated that the secretory response of cortisol to repeated infusions of ACTH (Aikawa *et al.* 1986, Yang *et al.* 1995, Shimada *et al.* 2005) or FRK (Shimada *et al.* 2005) is almost equal (Table 1). The results suggest that either desensitizing or potentiating regulation is absent in PKA-induced

Figure 5 Effect of FRK on ACTH-induced cortisol secretion and vice versa. (A) Adrenals were perfused with 10 μ M FRK for 5 min (bar above curve) followed by 100 pg/ml ACTH for 5 min (bar above curve) and cortisol secretion was monitored. (B) Adrenals were perfused with 100 pg/ml ACTH followed by 10 μ M FRK, and cortisol production was monitored. (C) Total amounts of cortisol secretion during first and second perfusions with ACTH and FRK were as follows: 13.8 ± 1.7 and 11.0 ± 1.7 (μ g/100 mg adrenal wt./30 min) for ACTH, and 4.8 ± 0.8 and 5.0 ± 0.9 (μ g/100 mg adrenal wt./30 min) for FRK. All data are shown as means $\pm$ S.E.M. ($n=6$). For significance of differences, see Fig. 1.

steroidogenesis. However, short- and long-term incubations with ACTH have generated contradictory results in terms of regulation of the numbers of ACTH receptors and the production of glucocorticoids. Some investigators have found up-regulation (Penhoat *et al.* 1989, Lebrethon *et al.* 1994), whereas others have found down-regulation or no effect (Sheela Rani *et al.* 1983, Lamberts *et al.* 1987, Kilianova *et al.* 2006). To verify the effect of activated PKA on ACTH-induced cortisol secretion, we perfused adrenal glands with FRK and found that the subsequent cortisol response to ACTH was unaffected (Fig. 5A). Although the ACTH receptor has potential sites for phosphorylation by PKC and PKA in the second and/or third cytoplasmic loops (Clark & Cammas 1996), such sites might not be correlated with susceptibility to desensitization or potentiation.

Within seconds or minutes of PAF challenge, cells can diminish or virtually eliminate agonist-evoked responses. Such desensitization involves phosphorylation of the receptors on one or more intracellular domains and the uncoupling of receptors from signal-transducing G-proteins. Short-term desensitization of receptors can be either homologous or heterologous. The former is restricted to the agonist-occupied form of the receptor, being mediated

through phosphorylation by GRK (Chuang *et al.* 1992). We indicated that repeated PAF infusion desensitizes cortisol secretion in response to PAF in a homologous manner (Shimada *et al.* 2005). Heterologous desensitization occurs independently of receptor binding by a ligand and is mediated through phosphorylation by activated PKC and/or PKA. Agonists that target different receptors or exposure to desensitizing compounds induce this type of desensitization. These phosphorylation phenomena promote the binding of an arrestin to receptors, which prevents subsequent coupling of the receptor to the G-protein. Both homologous and heterologous types of desensitization are adaptive physiological processes that terminate hyper-responsiveness. After 30 min to several hours of exposure to an agonist, short-term desensitization is augmented by internalization of a plasma membrane receptor into intracellular vesicles, and the receptors become down-regulated (Ferguson 2001).

Cross-desensitization among receptors for PAF and peptide chemoattractants (*N*-formylpeptide, a cleavage product of the fifth component of complement (C5a) and interleukin-8) demonstrated that although the response to PAF was cross-desensitized by these peptide ligands, PAF did not cross-desensitize the responses to any of the peptide chemoattractants. The cross-desensitization of PAF receptor

Figure 6 Effect of OAG plus A23187 on PAF-induced cortisol secretion and *vice versa*. (A) Adrenals were perfused with 50 μ M OAG plus 3.3 μ M A23187 for 5 min (bar above curve) followed by 10 nM PAF for 5 min (bar above curve), and cortisol secretion was monitored. (B) Adrenals were perfused with 10 nM PAF followed by 50 μ M OAG and 3.3 μ M A23187, and cortisol production was monitored. (C) Total amounts of cortisol secretion during first and second perfusions with PAF and OAG plus A23187 were as follows: 3.7 ± 0.7 and $^{##}1.1 \pm 0.3$ (μ g/100 mg adrenal wt./15 min) for PAF, and 3.0 ± 0.5 and $^{*}1.6 \pm 0.4$ (μ g/100 mg adrenal wt./15 min) for OAG plus A23187. All data are shown as means $\pm$ S.E.M. ($n=6$). For significance of differences, see Fig. 1.

induced by peptide chemoattractants was a consequence of PAF receptor cross-phosphorylation (Tomhave *et al.* 1994, Richardson *et al.* 1996). We found that ACTH preferentially attenuated the cortisol response to subsequent PAF exposure (Fig. 1B). Prior ACTH exposure interferes with ANG II-induced signaling and aldosterone production in adrenal glomerulosa cells (Yoshida *et al.* 1991). Since the action of ACTH is mediated by PKA activation, heterologous desensitization of PAF and ANG II receptors by PKA might be involved in desensitization of the adrenal fasciculata and glomerulosa cells. Therefore, we evaluated the effects of an activator of the cAMP–PKA pathway and found that prior perfusion with FRK significantly inhibited the cortisol response to PAF (Fig. 2A). Prior incubation with cAMP also interferes with the signaling pathway of ANG II in adrenal glomerulosa cells (Yoshida *et al.* 1991). These results indicate that PKA plays a pivotal role in heterologous desensitization of PAF and ANG II receptors. In contrast to numerous agonist-dependent and -independent PKC phosphorylation sites, the cytoplasmic tail of the human PAF receptor has only one consensus sequence for PKA phosphorylation (Kennelly & Krebs 1991). While differences in phosphorylation sites might be correlated with the difference in susceptibility to desensitization, such sites should play a pivotal role in cross-desensitization. Moreover, ACTH or FRK attenuated the subsequent cortisol response to OAG

plus A23187 (Figs 3B and 4B). This evidence indicates that the PKA-dependent processes activated by ACTH or FRK can interfere with PAF signal transduction at the receptor and post-receptor levels.

We clarified the effect of OAG plus A23187 on the cortisol response to PAF. Prior perfusion with OAG plus A23187 preferentially attenuated the cortisol response to PAF (Fig. 6A), indicating that PKC plays a pivotal role in PAF receptor desensitization. Likewise, PKC activators inhibit PAF binding and signaling (Schwertschlag & Whorton 1988, Yamazaki *et al.* 1989, Ali *et al.* 1994). These results suggest that at least three kinases (most likely PKA, PKC, and GRKs) phosphorylate and desensitize PAF receptors. The other two sites of interference in PAF receptor-mediated signaling are at the PLC and PKC levels. In RBL-2H3 cells that express the PAF receptor, PKC but not PKA phosphorylates PLC β_3 , resulting in the attenuation of responses stimulated by PAF (Ali *et al.* 1997). We found that since the cortisol response to OAG plus A23187 was attenuated by prior exposure to PAF (Fig. 6B) or this mixture (Shimada *et al.* 2005; Table 1), the membrane-associated form of PKC might be readily degraded by proteolysis (Kishimoto *et al.* 1989).

The most fundamental issue is the physiological significance of PAF and ACTH cross-regulation in the adrenal fasciculata. Under physiological conditions, sub-nanomolar or nanomolar concentrations of PAF circulate in the

peripheral blood of humans, rabbits, and rats (Caramelo *et al.* 1984). The blood PAF level increases during endotoxic shock, sepsis, asthma, inflammation, and anaphylaxis (Ishii & Shimizu 2000). Adrenocortical cells can produce and have high-affinity site for PAF (Pelosin *et al.* 1991). Furthermore, i.v. and i.c.v. injections of PAF induce ACTH secretion (Bernardini *et al.* 1989, Rougeot *et al.* 1990). If adrenocortical cells are exposed to sub-nanomolar PAF concentrations and to picomolar ACTH concentrations in fluid of the perivascular space of the zona fasciculata and reticularis under immediate hypersensitivity or severe inflammation, cross-regulated cortisol release will be evoked. Although PAF can induce potent corticosteroidogenesis, the process is rapidly desensitized. In the presence of ACTH, PAF has a permissive action on ACTH-induced glucocorticoid secretion and can maintain high concentrations of this anti-inflammatory stress hormone. These actions of PAF can ameliorate the severity of dysfunction and suppress the immune responses and/or subsequent immune-mediated inflammation.

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