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Arginine Vasopressin Inhibition of Cytochrome P450c17 and Testosterone Production in Mouse Leydig Cells

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The mechanism of arginine vasopressin (AVP) action in Leydig cells was investigated, and compared to the effects of phorbol-13-myristate-12-acetate (PMA) and interleukin-1β (IL-1β). Previous reports suggested that AVP inhibits Leydig cell testosterone production at the level of 17α-hydroxylase/C17-lyase activity. The present study confirms and extends these observations, and contrasts the effects of AVP to IL-1. In all experiments, macrophage-depleted Leydig cells were isolated from mice and maintained in primary culture for 5 d prior to initiation of treatments. Leydig cells were treated with 8-Br-cAMP plus increasing concentrations of AVP or IL-1β. AVP caused a significant and dose-dependent inhibition of cAMP-stimulated testosterone production and P450c17 mRNA expression. IL-1β completely inhibited cAMP-stimulated testosterone production and P450c17 mRNA expression. PMA is a known activator of protein kinase C (PKC) and has been reported to inhibit Leydig cell steroidogenesis. Leydig cells express type V1 vasopressin receptors, which are coupled to PKC activation. The mechanism of IL-1 action in Leydig cells is not understood, but activation of the PKC pathway has been suggested for IL-1 action in other systems. Therefore, the effects of PMA on cAMP-stimulated steroidogenesis were compared to AVP and IL-1. Similar to the effects of AVP, PMA inhibited cAMP-stimulated testosterone production and P450c17 mRNA expression. To assess the possible involvement of PKC in AVP and IL-1 action in Leydig cells, the PKC inhibitor Calphostin C was tested. cAMP-stimulated testosterone production and P450c17 mRNA expression were significantly inhibited by 10 n*M* AVP ($p < 0.05$), and this inhibition was reversed by treatment with Calphostin C. Analogous experiments were performed to assess the role of PKC in IL-1 action. In contrast to the results for AVP, Calphostin C did not reverse the inhibitory effects of IL-1 on cAMP-stimulated P450c17 mRNA expression. To assess further PKC activation, myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation was analyzed. Only AVP and PMA, but not IL-1β, caused an increase in MARCKS phosphorylation. These results confirm that AVP and PMA activate PKC and indicate that IL-1 likely does not activate PKC in Leydig cells. The implications of AVP-mediated inhibition of steroidogenesis and potential role of MARCKS phosphorylation are discussed.

Key Words: Leydig cells; cytokines; vasopressin; steroidogenesis; protein kinase C; immune-endocrine interactions; neuropeptides.

Introduction

We have demonstrated that injection of mice with the Gram-negative bacterial endotoxin, lipopolysaccharide (LPS) resulted in a biphasic inhibition of steroidogenesis. At 2 h following LPS injection, serum testosterone was decreased by > 80%, and serum testosterone levels were still decreased by 80% after 24 h *(1)*. Preliminary studies indicate that testosterone remains depressed for as long as 9 d after LPS injection *(2)*. At 24 h following LPS, mRNA and protein levels for the steroidogenic enzymes were significantly decreased *(1,3)*. We have shown in vitro that LPS elaborated pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα), cause the transcriptional repression of the steroidogenic enzymes, and suggested that the chronic inhibitory phase following LPS injection is mediated by inhibition of steroidogenic enzyme gene expression.

The systemic response to LPS results in the production of a host of inflammatory mediators and signals that are known effectors of Leydig cell steroidogenesis. LPS induces an acute-phase response, which includes production of cytokines by activated immune cells, stimulation of the hypothalamo–pituitary–adrenal axis (HPA), activation of the sympathetic nervous system, and a rapid elevation of the neurohypophyseal hormone arginine vasopressin (AVP) *(4–9)*. Increased levels of AVP may contribute to

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the pathophysiology of endotoxemia *(6)*. AVP has been shown to inhibit Leydig cell steroidogenesis, but the mechanism of this inhibition has not been determined (for review, *see 10,11*).

Considerable evidence has been presented that demonstrates Leydig cells are a target for AVP (for review, *see 10,11*). Chronic exposure to AVP inhibits testosterone biosynthesis by Leydig cells in vitro. These studies indicated that the site of action of AVP in Leydig cells was at level of 17α-hydroxylase activity *(10)*. Leydig cells are known to possess functional type V_1 vasopressin receptors *(12)*. V_1 vasopressin receptors are coupled to phosphoinositide breakdown, Ca^{2+} mobilization pathways, and activation of protein kinase C (PKC) *(13)*. AVP has been shown to stimulate phosphoinositide turnover in rat Leydig cells *(14,15)*, and mouse tumor Leydig cells *(16)*.

We have shown that both IL-1 and TNFα inhibit cAMP-stimulated testosterone production in Leydig cells primarily by inhibiting the expression of P450c17 *(17–19)*. We *(20)* and others *(21–26)* have shown that the PKC activator phorbol-12-myristate-13-acetate (PMA) inhibits LH/hCG or cAMP-stimulated testosterone production in Leydig cells. Our studies offered us insight into the TNFα mechanism of action and led us to conclude that TNFα action, in part, is mediated via activation of PKC. The similarities between TNFα and PMA included translocation of immunoreactive PKC from cytosol to membrane and reversal of inhibition with the PKC inhibitor Calphostin C *(20)*. In contrast, little information has been gained on the IL-1 mechanism of action in Leydig cells. It has been suggested that IL-1 signals via activation of the PKC pathway in other systems *(27)*. The present study was designed to assess the role of AVP in the regulation of Leydig cell function and to gain further insight into IL-1 signaling mechanisms.

Results

Effect of AVP and IL-1β on cAMP-Stimulated Testosterone Production

To confirm previous reports on the effects of AVP on testosterone production, and to compare the effects of AVP to IL-1, the effects of these agents on cAMP-stimulated testosterone production were analyzed. Macrophage-depleted Leydig cells were maintained in culture for 5 d prior to the initiation of treatments. Treatments consisted of control media, or media that contained 50 μ*M* 8-Br-cAMP (cAMP), cAMP plus increasing concentrations of AVP (1, 10, 100 n*M*), or IL-1β (1 ng/mL). Cells were treated for 24 h, and media were collected for testosterone measurement by RIA, as described under Materials and Methods. As shown in Fig. 1, cAMP caused a 66-fold increase in testosterone production, compared to control (3650 ± 275 ng/10^6 Leydig cells/24 h). AVP caused a significant, and dose-dependent inhibition of cAMP-stimulated testosterone production by 39 ± 17, 80 ± 14, and 84 ± 21%, with 1, 10, and 100 n*M* AVP, respectively. IL-1 ±1 ng/mL) inhibited cAMP-stimulated testosterone production by ~95%. These data confirm that IL-1β and AVP are potent inhibitors of cAMP-stimulated testosterone production, as reported previously *(10,17,18)*.

Effect of AVP and IL-1β on cAMP-Stimulated P450c17 mRNA Levels

Cells from which media were collected for testosterone RIA, shown in Fig. 1, were lysed for RNA extraction and subjected to Northern analysis for P450c17 mRNA quantitation. As shown in Fig. 2A, an equal amount of RNA from each treatment group was analyzed, as confirmed by ethidium bromide fluorescence (upper panel). cAMP caused a marked increase in P450c17 mRNA expression that was inhibited by AVP and IL-1β. Data presented in Fig. 2B were normalized to cAMP-induced P450c17 mRNA levels and demonstrate that AVP caused a significant inhibition by 73, 79, and 82% with 1, 10, and 100 n*M* AVP, respectively. As shown previously *(17,18)*, and confirmed here, IL-1β inhibited cAMP-stimulated P450c17 mRNA expression by 98%.

Effect of PMA on cAMP-Stimulated Testosterone Production

PMA is a well-nown activator of PKC that has been reported to inhibit Leydig cell steroidogenesis. Leydig cells express type V_1 vasopressin receptors, which are coupled to phosphoinositide breakdown, Ca^{2+} mobilization pathways, and activation of PKC. Therefore, the effects of PMA on cAMP-stimulated steroidogenesis were compared to AVP and IL-1. Leydig cells, maintained as described above, were treated with 50 μ*M* 8-Br-cAMP (cAMP), or cAMP plus increasing concentrations of PMA. Cells were treated for 24 h, and media were collected for testosterone measurement by RIA, as described under Materials and Methods. The results are shown in Fig. 3. Treatment with 1 and 10 n*M* PMA inhibited cAMP-stimulated testosterone production by 54 and 59%, respectively. Lower concentrations of PMA (10 and 100 p*M*) were not inhibitory. PMA alone had no effect on testosterone production.

Effect of PMA on cAMP-Stimulated P450c17 mRNA Levels

Cells from which media was collected, shown in Fig. 3, were lysed for RNA extraction and subjected to Northern analysis for P450c17 mRNA quantitation. As shown in Fig. 4A, an equal amount of RNA from each treatment group was analyzed, as confirmed by ethidium bromide fluorescence (upper panel). cAMP caused a marked increase in P450c17 mRNA expression that was inhibited by higher concentrations of PMA similar to the effect of PMA on testosterone production. Data presented in Fig. 4B were normalized to cAMP-induced P450c17 mRNA levels and demonstrate that 1 and 10 n*M* PMA completely, and significantly, inhibited

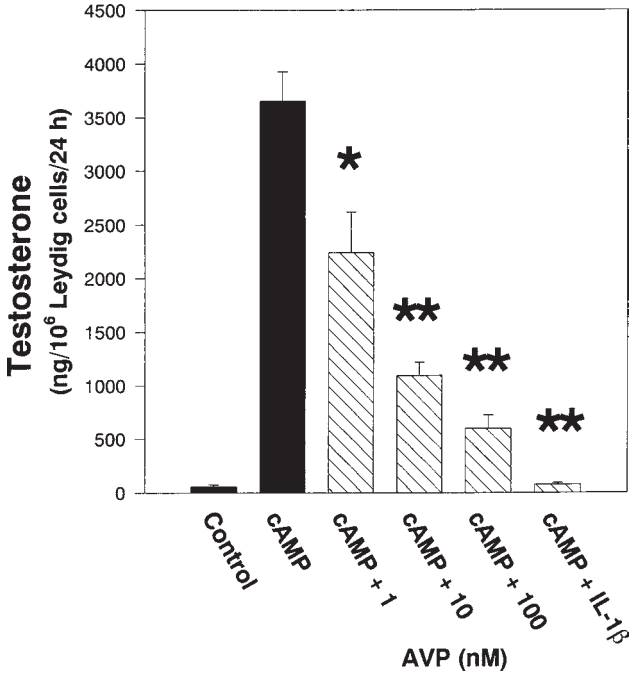

Fig. 1. Effect of AVP and IL-1β on cAMP-stimulated testosterone production. Macrophage-depleted Leydig cells were maintained in culture for 5 d prior to the initiation of treatments. Treatments consisted of control media, or media that contained 50 μ*M* 8-Br-cAMP (cAMP), cAMP plus increasing concentrations of AVP (1, 10, 100 n*M*), or IL-1β (1 ng/mL). Cells were treated for 24 h, and media were collected for testosterone measurement by RIA, as described under Methods. Data represent the mean ± SEM of three separate experiments with replicate samples (* $p < 0.05$; ** $p < 0.01$).

cAMP-stimulated P450c17 mRNA expression, but lower concentrations of PMA did not.

Effect of Calphostin C on AVP-Inhibition of cAMP-Stimulated Testosterone

To further assess the possible involvement of PKC in AVP action in Leydig cells, the highly specific PKC inhibitor Calphostin C was tested. We have shown previously that Calphostin C blocked TNFα and PMA-mediated inhibition of cAMP-stimulated *Cyp17* expression in MA-10 tumor Leydig cells transiently transfected with *Cyp17* promoter constructs, consistent with the hypothesis that both PMA and TNFα act via a PKC pathway to inhibit cAMP-stimulated P450c17 expression *(20)*. *Cyp17* is the gene that encodes P450c17. Cells were maintained as described above, and then treated for 24 h with 50 μ*M* 8-Br-cAMP (10 n*M* AVP ± 1 μ*M* Calphostin C. Media were collected for testosterone determination by RIA as described in Materials and Methods. As shown in Fig. 5, cAMP-stimulated testosterone production was significantly inhibited by 10 n*M* AVP ($p < 0.05$), and this inhibition was reversed by treatment with Calphostin C. AVP alone had no effect on basal testosterone production, and Calphostin C did not affect cAMP-stimulated testosterone.

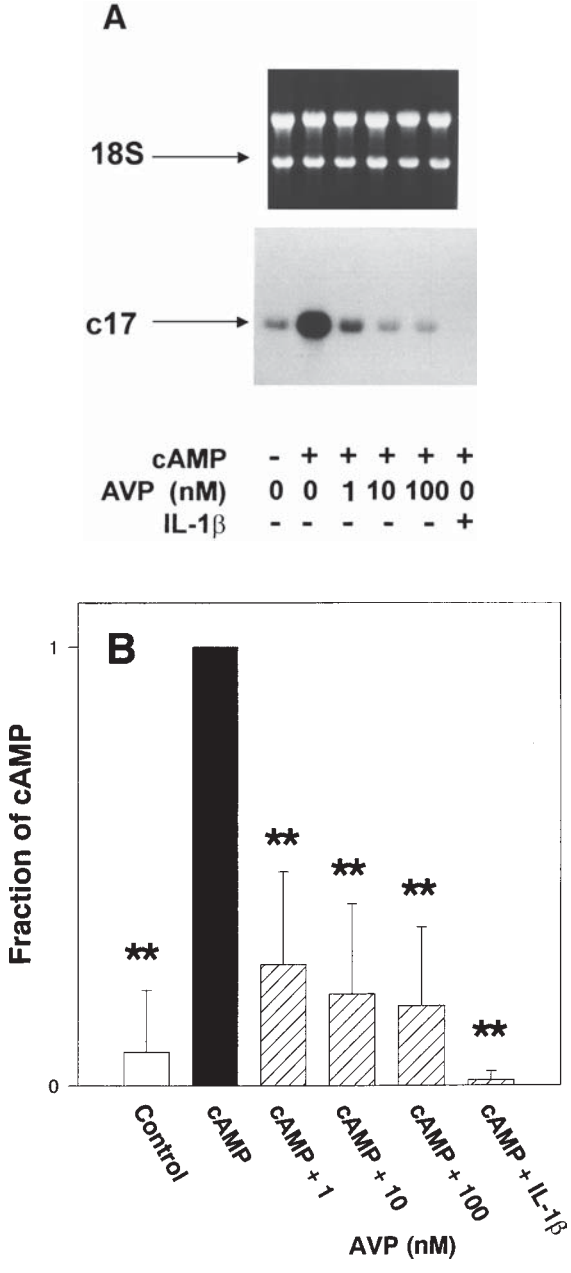

Fig. 2. Effect of AVP and IL-1β on cAMP-stimulated P450c17 mRNA levels. Cells from the treatments described in Fig, 1 were lysed for RNA extraction and subjected to Northern analysis as described under Materials and Methods. **(A)** Each lane contains an equal amount of RNA from each treatment group (10 μg) and appeared equal by ethidium bromide fluorescence (upper panel). Blots were hybridized with ^{32}P-labeled mouse P450c17 cDNA probe, visualized by autoradiography, and quantitated by densitometry. A representative autoradiograph is shown. **(B)** Data are presented as fraction of cAMP-stimulated P450c17 mRNA (SEM of three separate experiments [* $p < 0.05$; ** $p < 0.01$]).

Effect of Calphostin C on AVP-Inhibition of cAMP-Stimulated P450c17

RNA was extracted from dishes from which media were collected for testosterone RIA, shown in Fig. 5. RNA was subjected to Northern analysis for P450c17 mRNA quan-

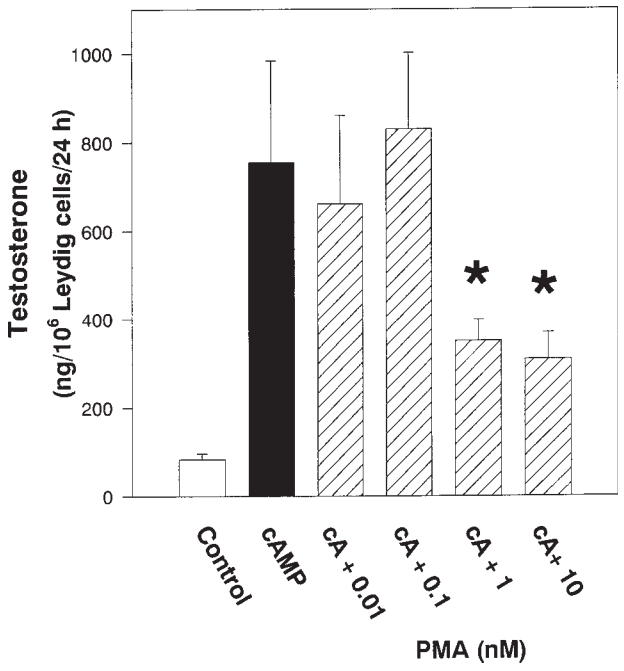

Fig. 3. Effect of PMA on cAMP-stimulated testosterone production. Cells were maintained as described in Fig. 1, and then treated with 50 μ*M* 8-Br-cAMP (cAMP) or cAMP plus increasing concentrations of PMA. Cells were treated for 24 h, and media were collected for testosterone measurement by RIA, as described under Materials and Methods. Data represent the mean ± SEM of three separate experiments with replicate samples (* $p < 0.05$).

titation. A representative autoradiograph is shown in Fig. 6A. Equal loading of RNA samples is shown by ethidium bromide fluorescence (upper panel). Data are presented as integrated optical density of a representative experiment (Fig. 6B). The results are similar to the effects on testosterone production and indicate that Calphostin C can reverse the inhibitory effect of AVP on cAMP-stimulated P450c17 mRNA expression.

Calphostin C Does Not Reverse IL-1 Inhibition of P450c17 Expression

Although much information has been gained on TNFα signaling in Leydig cells, the mechanism of IL-1 signal transduction has remained obscure. To assess the possible role of PKC in IL-1-mediated inhibition of P450c17 expression, experiments utilizing the PKC inhibitor Calphostin C, analogous to those described above, were performed. Cells were maintained as described above, and then treated for 24 h with 50 μ*M* 8-Br-cAMP ± 1 ng/mL IL-1β ± 1 μ*M* Calphostin C. RNA was extracted and subjected to Northern analysis for P450c17 mRNA quantitation. A representative autoradiograph is shown in Fig. 7A. Equal loading of RNA samples is shown by ethidium bromide fluorescence (upper panel). Data are presented as integrated optical density of a representative experiment (Fig. 7B). In contrast to the results shown above for AVP and previously for TNFα , Calphostin C

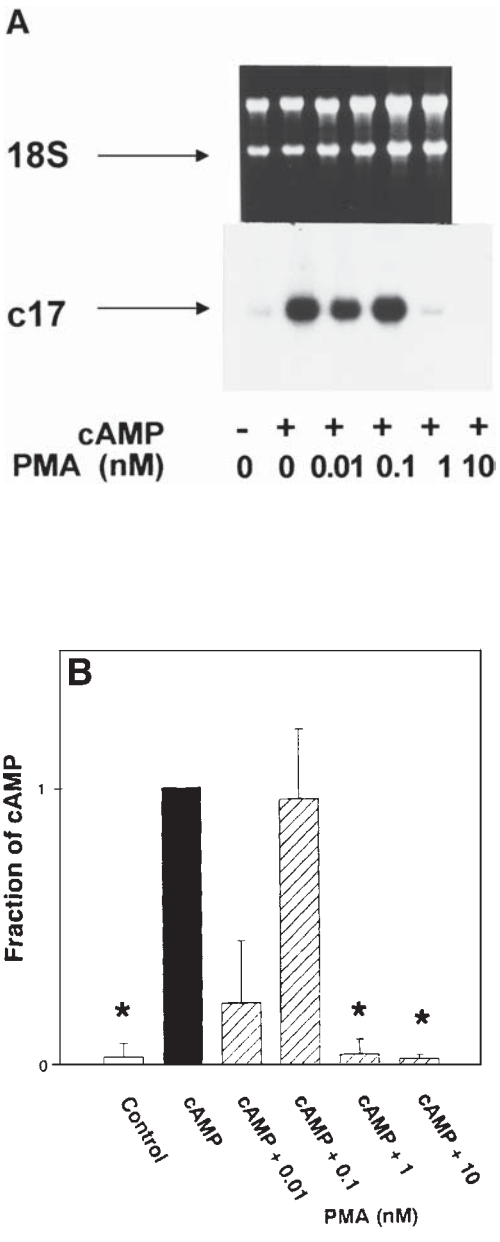

Fig. 4. Effect of PMA on cAMP-stimulated P450c17 mRNA levels. Cells from which media were collected for the data shown in Fig. 3 were lysed for RNA extraction and subjected to Northern analysis as described under Materials and Methods. **(A)** Each lane contains an equal amount of RNA from each treatment group (10 μg) and appeared equal by ethidium bromide fluorescence (upper panel). The blot was hybridized with ^{32}P-labeled mouse P450c17 cDNA probe, visualized by autoradiography, and quantitated by densitometry. A representative autoradiograph is shown. **(B)** Data are presented as fraction of the cAMP-stimulated P450c17 mRNA ± SEM of three separate experiments (* $p < 0.05$).

did not reverse the inhibitory effects of IL-1 on cAMP-stimulated P450c17 mRNA expression. At concentrations of Calphostin C >1 μ*M*, cAMP-stimulated testosterone production, and P450c17 mRNA induction were blocked nonspecifically. Calphostin C alone had no effect on basal testosterone production

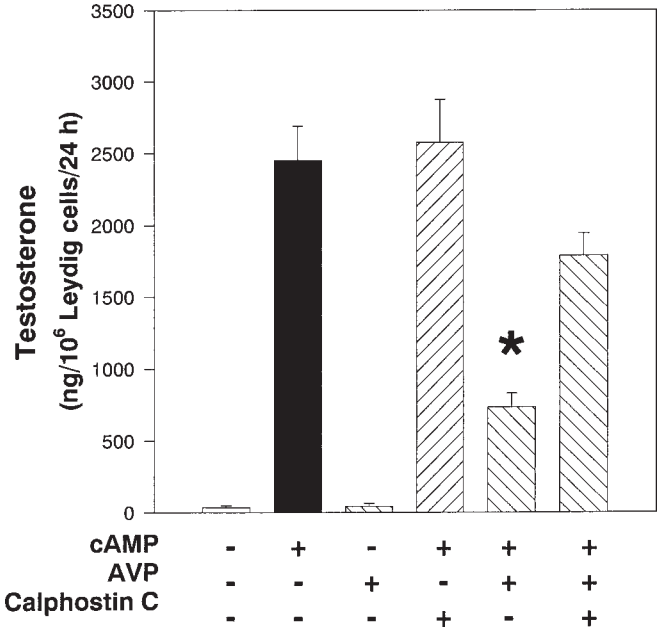

Fig. 5. Effect of Calphostin C on AVP-inhibition of cAMP-stimulated testosterone. Cells were maintained as described in Fig. 1, and then treated for 24 h with 50 μ*M* 8-Br-cAMP, 10 n*M* AVP, cAMP + 1 μ*M* Calphostin C, cAMP + AVP, or cAMP + AVP + Calphostin C. Media were collected for testosterone RIA as described in Methods. Data represent the mean ± SEM of three separate experiments with replicate samples (* $p < 0.05$).

Effect of AVP, PMA, and IL-1 on MARCKS Phosphorylation

The myristoylated alanine-rich C kinase substrate (MARCKS) is a widely distributed PKC substrate that binds both calmodulin and actin *(29,30)*. MARCKS is a highly specific, high-affinity substrate for PKC. It is rapidly and reversibly phosphorylated in intact cells in response to hormonal stimulation, and analysis of MARCKS phosphorylation is a reliable measure of PKC activation by most—but not all—isoforms of PKC in many cell types *(31,32)*. AVP has been shown to stimulate MARCKS phosphorylation in pituitary cells in primary culture, in keeping with the activation of PKC via V_1 vasopressin receptors in that cell type *(33)*. To assess further PKC activation by AVP and PMA in Leydig cells, and to examine the possible involvement of PKC in IL-1 action, MARCKS phosphorylation was analyzed. Leydig cells were maintained in culture as described above. Cells were metabolically labeled with $^{32}P_i$ as described in Materials and Methods, and then treated for 5 min with 1 m*M* 8-Br-cAMP, 100 n*M* AVP, 1 μ*M* 4α-PDD, 10 ng/mL IL-1β, or 1 μ*M* PMA. Cells were quick-frozen on dry ice prior to extracting cellular protein for MARCKS immunoprecipitation. Immunoprecipitates were resolved by SDS-PAGE, and ^{32}P-labeled MARCKS visualized by autoradiography. A representative autoradiograph is shown in Fig. 8A. Similar results were obtained in five independent experiments. In Fig. 8B, autoradiographs were quantitated by densitometry, and data are presented as integrated optical density of a representative

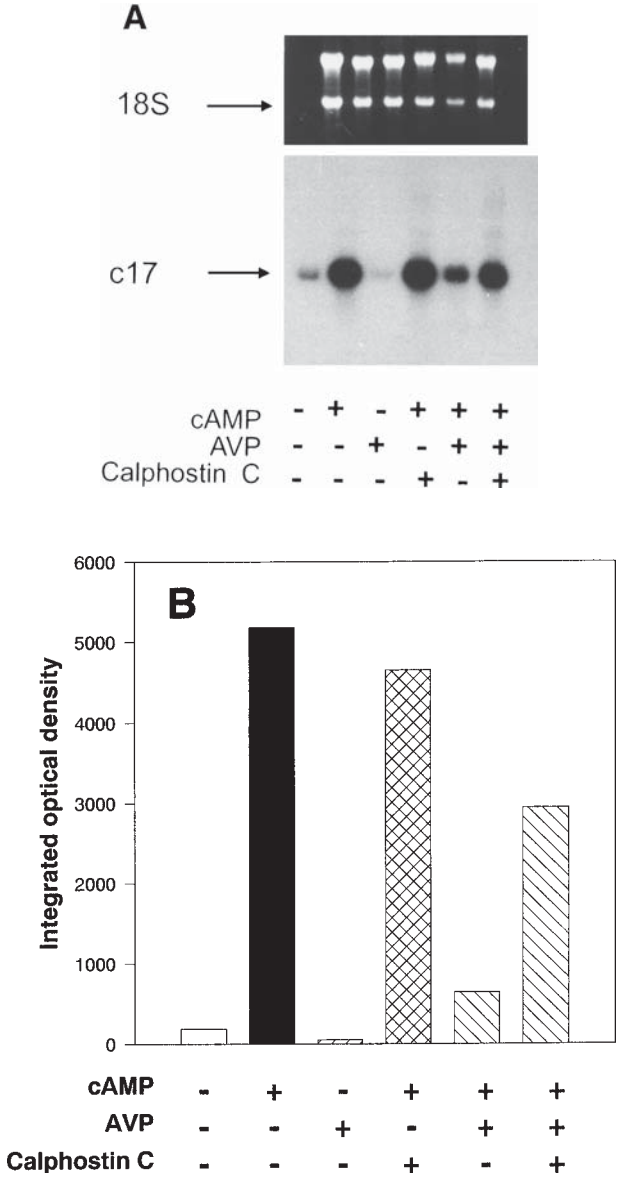

Fig. 6. Effect of Calphostin C on AVP-inhibition of cAMP-stimulated P450c17. **(A)** RNA was extracted from dishes from which media were collected for the testosterone RIA, shown in Fig. 5. RNA was subjected to Northern blot and probed for P450c17, as described in Fig. 2. Equal loading of RNA samples is shown by ethidium bromide fluorescence (upper panel). A representative autoradiograph is shown. **(B)** Data are presented as integrated optical density of a representative experiment.

experiment. Of all of the agents tested, only AVP and PMA caused an increase in MARCKS phosphorylation, consistent with their activation of PKC. Similar results on MARCKS phosphorylation were obtained when 10 n*M* PMA was tested (data not shown). IL-1 did not stimulate MARCKS phosphorylation, confirming the results shown in Fig. 7 above that IL-1 likely does not activate PKC in Leydig cells.

Discussion

Previous studies have demonstrated that AVP inhibits Leydig cell steroidogenesis and indicated that the site of

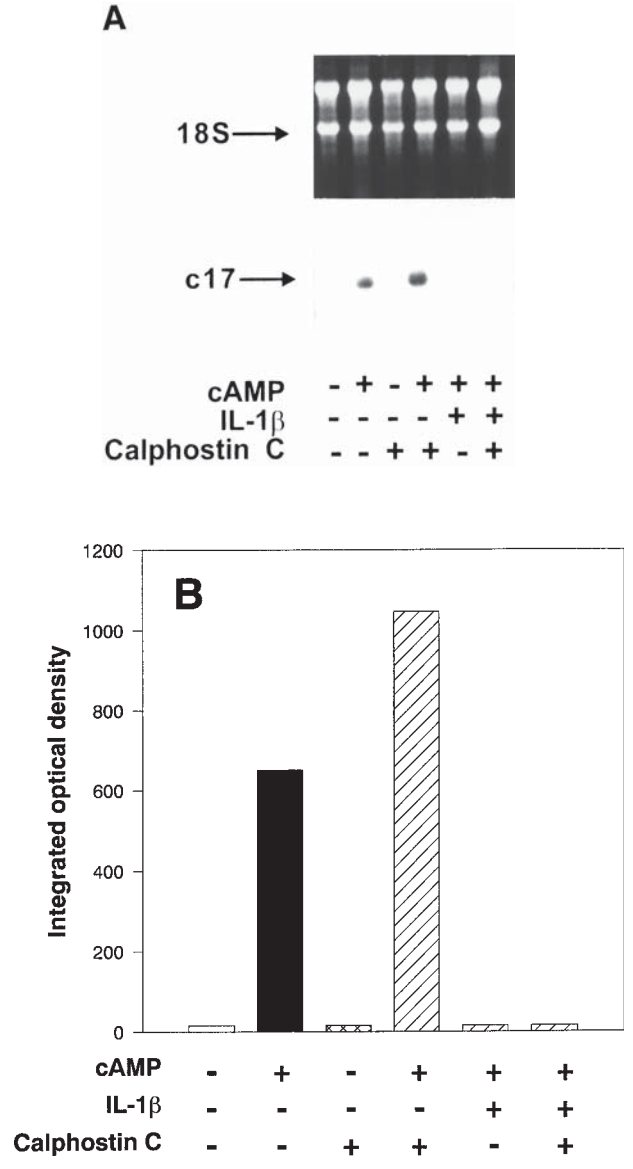

Fig. 7. Calphostin C does not reverse IL-1 inhibition of P450c17 expression. **(A)** Cells were maintained as described in Fig. 1, and then treated for 24 h with 50 μ*M* 8-Br-cAMP, 1 ng/mL IL-1β, cAMP + 1 μ*M* Calphostin C, cAMP + IL-1β, or cAMP + IL-1β + Calphostin C. RNA was extracted and subjected to Northern blot, and probed for P450c17, as described in Fig. 2. Equal loading of RNA samples is shown by ethidium bromide fluorescence (upper panel). A representative autoradiograph is shown. **(B)** Data are presented as integrated optical density of a representative experiment.

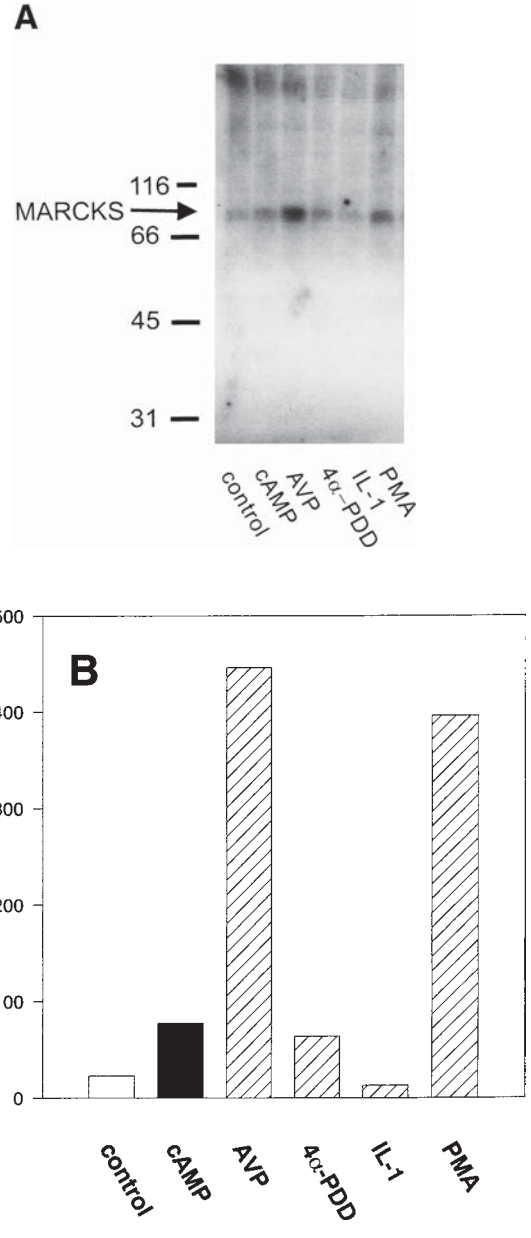

Fig. 8. Effect of AVP on MARCKS phosphorylation. Leydig cells were maintained in culture as described in Fig. 1. Cells were metabolically labeled with $^{32}P_i$ as described in Materials and Methods. Cells were then treated for 5 min with 1 μ*M* 8-Br-cAMP, 100 n*M* AVP, 1 μ*M* 4a-PDD, 10 ng/mL IL-1β, or 1 μ*M* PMA. Media were removed and cells were quick-frozen on dry ice prior to extracting cellular protein for MARCKS immunoprecipitation, as described in Materials and Methods. Immunoprecipitates were resolved by SDS-PAGE and subjected to autoradiography. Data shown are representative of five independent experiments. **(B)** Data are presented as integrated optical density of a representative experiment.

inhibition was at the level of 17α-hydroxylase/C17-20 lyase activity *(10)*. The findings of the present study confirm and extend these earlier observations. AVP caused a dose-dependent inhibition of cAMP-stimulated testosterone production in mouse Leydig cells in primary culture. There was a concomitant decrease in the cAMP-stimulated expression of P450c17 mRNA, consistent with the inhibition of testosterone being mediated primarily at the level of P450c17 expression (Figs. 1 and 2). These results are strikingly similar to our previous studies where we demonstrated that the pro-inflammatory cytokines IL-1 and TNFα both inhibit Leydig cell testosterone production primarily by inhibiting the expression of P450c17 *(17–19)*. Thus, it appears that long-term inhibitory effects of cytokines and vasopressin are mediated by repression of P450c17 expression *(1,3)*.

To gain insight into the signaling mechanisms that mediate cytokine and vasopressin inhibition of P450c17 expression, we examined the effects of the well-known PKC activator, PMA. PMA caused a significant inhibition of cAMP-stimulated testosterone production at the same concentration that it inhibited P450c17 mRNA expression (Figs. 3 and 4). We have shown previously that the PMA inhibition of *Cyp17* transcription in MA-10 tumor Leydig cells was blocked by the PKC inhibitor Calphostin C *(20)*. In addition, PMA stimulated the translocation of immunoreactive PKC from the cytosol to membrane, characteristic of PKC activation. In the present study, we demonstrated that the inhibitory effects of AVP on cAMP-stimulated testosterone production and P450c17 mRNA expression are blocked by Calphostin C, consistent with PKC mediating the inhibitory effects of AVP. Leydig cells express type V_1 vasopressin receptors, which are coupled to phosphoinositide breakdown, Ca^{2+} mobilization pathways, and activation of PKC *(13)*. AVP has been shown to stimulate phosphoinositide turnover in rat Leydig cells *(14,15)* and mouse tumor Leydig cells *(16)*. In addition, AVP stimulation of phospholipase D activity in Leydig cells requires PKC activation *(15,34)*. The present study confirms previous reports on the inhibitory effects of phorbol esters and PKC activation in Leydig cells *(21–26)*.

We further analyzed AVP and PMA activation of PKC in Leydig cells by measuring MARCKS phosphorylation (Fig. 8). MARCKS is a highly specific, high-affinity substrate for PKC. It is rapidly and reversibly phosphorylated in intact cells in response to hormonal stimulation, and analysis of MARCKS phosphorylation is a reliable measure of PKC activation by most, but not all, isoforms of PKC in many cell types *(31,32)*. The advantages of analyzing MARCKS phosphorylation include that it is an endogenous substrate of PKC whose phosphorylation takes place in intact cells, and its analysis does not require disruption of cells for phosphorylation of artificial substrates in cell-free homogenates. As shown in Fig. 8, only AVP and PMA stimulated MARCKS phosphorylation. cAMP and the nontumor promoting phorbol ester 4α-PDD did not cause an increase in MARCKS. We examined MARCKS phosphorylation after 5 min of exposure to the test compounds. It is likely that AVP-stimulated PKC phosphorylation of MARCKS had achieved peak levels at an earlier time. As reported by Liu et al., AVP-induced increases in MARCKS phosphorylation in pituitary cells peaked within 15 s after cells were treated with AVP . However, in our studies, PMA-stimulated MARCKS phosphorylation continued to increase for as long as 15 min (data not shown). AVP stimulates PKC via a receptor mediated pathway that also activates phosphatases that dephosphorylate PKC substrates *(33)*. PMA directly stimulates PKC by binding to the DAG binding site, and multiple receptor activated signaling pathways are circumvented *(35)*.

To gain insight into IL-1 signal transduction pathways in Leydig cells, we compared the effects of IL-1 to AVP and PMA. As we reported previously, IL-1 inhibits cAMP-stimulated testosterone production and P450c17 expression *(17,18)*. Similar to the effects of TNFα, AVP, and PMA, shown here and previously *(19,20)*, IL-1 inhibits Leydig cell steroidogenesis primarily by inhibiting the expression of P450c17. However, as shown in Fig. 7, in marked contrast to AVP (Fig. 6), PMA, and TNFα *(20)*, Calphostin C does not block the inhibitory effects of IL-1. These results suggest that IL-1 inhibition of P450c17 expression does not depend on PKC activation. Moreover, IL-1 does not stimulate an increase in MARCKS phosphorylation (Fig. 8), consistent with the Calphostin C data, indicating that IL-1 does not act via a PKC mechanism. Most known signal transduction pathways have been suggested as mediating the effects of IL-1 in a wide variety of cells and tissues (for review, *see 27,36,37*). Interestingly, the same pathways have been implicated in TNFα signal transduction *(37–40)*.

Our experiments, summarized in Fig. 8, which examined MARCKS phosphorylation were designed to investigate the possible involvement of PKC activation in AVP and IL-1 signaling. It is possible that phosphorylation of MARCKS may participate in the disruption of steroidogenesis observed in Leydig cells following LPS injection. MARCKS is a calmodulin and actin binding protein. On phosphorylation by PKC, calmodulin is released and associated actin filaments depolymerize *(41)*. The actin cytoskeleton of Leydig cells facilitates intracellular cholesterol movement. Cytochalasins, anti-actin antibodies, and DNase I, compounds that block actin polyermization, inhibit testosterone production by blocking cholesterol transport to the mitochondria *(42)*. It is possible that AVP-stimulated PKC phosphorylation of MARCKS may contribute to the inhibition of testosterone production during the acute phase of LPS-induced experimental endotoxemia.

We have determined that there is a biphasic inhibition of steroidogenesis following injection of LPS. At 2 h following LPS injection, testosterone was decreased by > 80%, and serum testosterone levels were still decreased by 80% after 24 h *(1)*. At 24 h following LPS, mRNA and protein levels for the steroidogenic enzymes are significantly decreased *(1,3,18)*. It is likely that peripheral, as well as testicular, macrophage-elaborated cytokines are produced in response to LPS and that these proinflammatory mediators transcriptionally repress enzyme gene expression in Leydig cells. However, steroidogenic enzyme levels are not decreased acutely and changes in their levels cannot account for the observed inhibition of testosterone production during the initial inhibitory phase. We have shown that steroidogenic acute regulatory (StAR) protein expression is decreased to the same degree as testosterone production at 2 h following LPS *(1)*. We have also shown that testosterone production ex vivo in Leydig cells from mice treated

with LPS can be restored with the permeable hydroxycholesterol intermediate R-22 hydroxycholesterol, consistent with the acute inhibition of steroidogenesis being at the level of cholesterol transport *(2)*. We conclude from these data that inhibition of StAR protein expression contributes to the abrupt cessation of testosterone production. It is entirely possible that other cholesterol transport proteins are also inhibited during the acute phase of the LPS response. Accompanying the release of cytokines, adrenal medulary catecholamines and neurohypophyseal peptides (i.e., AVP) are released rapidly and in large amounts into the peripheral circulation *(4,43)*. AVP serum levels are acutely and markedly elevated following exposure to LPS *(5–9)*. It is possible that AVP, derived from elevated levels in the peripheral circulation or produced locally, may contribute to the acute LPS inhibition of steroidogenesis by disrupting the actin cytoskeleton via PKC phosphorylation of MARCKS.

Immunoreactive AVP is expressed in the testis and has been show to be secreted in vitro by Leydig cells *(44)*. However, AVP expression in Leydig cells is not regulated by factors with known regulatory effects on Leydig cells. For example, neither LH nor PMA had any effect on AVP secretion or mRNA expression in Leydig cells *(44)*. Moreover, physiologic stresses that are well-know effectors of hypothalamic AVP secretion had no influence on testicular AVP. These manipulations included salt loading, dehydration, circadian rhythm perturbation, glucocorticoid, steroid, and gonadotrophin administration *(45)*. There are three mRNA transcripts for vasopressin expressed in the testis. Ivell and coworkers have shown that of these, only one is translated, and it codes for a protein identical to the hypothalamic form of the peptide *(45)*. Sertoli cells are also known to secret immunoreactive AVP, and it has been suggested that AVP is a potent local modulator of Leydig cell function, serving as a signal from the seminiferous tubules to the Leydig cells *(46)*. Although no experimental or pharmacological manipulations were shown to affect testicular AVP expression, vasopressin receptor expression in the testis has been shown to be modulated by opiod pathways *(47)*. In addition, the hypophyseal hormones (LH, GH) and estradiol have been shown to modulate Leydig cell vasopressin receptor expression *(48,49)*. Thus, the regulation of AVP action in the testis appears to be at the level of receptor expression, instead of control of local vasopressin secretion.

Several other neuronal peptides are known to be expressed in the testis and have been shown modulate Leydig cell steroidogenesis in vitro. These include gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), corticotrophin-releasing hormone (CRF), oxytocin (OT), proopiomelanocortin (POMC) and β-endorphin, and proenkephalin B and dynorphin *(11,45,50–52)*. However, the physiological significance of testicular neuronal peptide expression remains obscure. Recently, Turnbull and Rivier provided evidence for the existence of a direct neural-testicular connection *(4)*. They proposed that the inhibitory effects of centrally injected (intracerebroventricular) IL-1 on Leydig cell testosterone production are not mediated by suppression of LH secretion or the result of increases in glucocorticoids. Instead, they suggesedt that loss of testicular responsiveness to LH is owing to direct brain-to-gonad connections that bypass the pituitary *(53)*. In light of the large body of evidence demonstrating the existence of neuronal peptide signaling pathways that affect Leydig cell function, including the present study where we demonstrate that AVP directly inhibits Leydig cell steroidogenesis at the level of P450c17 expression, it is tempting to speculate that these neuronal peptides are the efferent effectors of the direct brain-to-gonad signal produced in response to immune activation.

In summary, the present study demonstrates that AVP inhibits cAMP-stimulated testosterone production and P450c17 expression in Leydig cells via a PKC pathway. Despite the similarity of response to AVP, it is evident that IL-1 does not act via a PKC-dependent pathway to inhibit Leydig cell steroidogenesis.

Materials and Methods

Materials

Multiprimed labeling kit, [^{32}P]orthophosphate, and [α-^{32}P]deoxycytidine triphosphate were purchased from Amersham (Arlington Heights, IL). Testosterone RIA kits were purchased from Diagnostic Products Corp. (Los Angeles, CA). Metrizamide, collagenase, HEPES, BSA (fraction V), bovine insulin, phenylmethylsulfonyl fluoride (PMSF), leupeptin, dithiothreitol (DTT), sodium orthovanadate, EGTA, EDTA, Nonidet P-40 (NP-40) and sodium bicarbonate were purchased from Sigma Chemical Inc. (St. Louis, MO). AVP, Calphostin C, PMA, and 4α-phorbol 12,13-didecanoate (4a-PDD) were purchased from CalBiochem (San Diego, CA). Medium 199, DME/F12, Waymouth's MB752/1, penicillin, and streptomycin were obtained from Gibco-BRL (Gaithersburg, MD). Recombinant mouse IL-1β was purchased from R&D Systems (Minn., MN). Anti-MARCKS antisera was a gift from Perry Blackshear (Duke University Medical Center, Durham, NC), and mouse P450scc complementary DNA (cDNA) was a gift from Keith Parker (Duke University Medical Center, Durham, NC). Mouse P450c17 cDNA was a gift from A. H. Payne (Stanford University, Palo Alto, CA). All other reagents were from sources previously described.

Animals

Adult male outbred pathogen-free CD-1 mice were purchased from Charles River Co. (Wilmington, MA). Mice were housed for at least 1 wk in groups of 5/cage. They were given food and water ad libitum, and maintained on a 14-h light–10-h dark schedule. The animals were procured, maintained, and used in accordance with the Animal Wel-

fare Act and were killed by CO_2 asphyxiation. Experiments were performed when the mice were 60- to 70-d old.

Isolation of Macrophage-Depleted Leydig Cells

Macrophage-depleted Leydig cells were isolated and cultured as described previously *(19)*. Leydig cells were cultured in serum-free DME/F12 culture medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 supplemented with 2.2 g/L sodium bicarbonate, 10 m*M* HEPES, pH 7.4, 500 ng/mL insulin, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 1 mg/mL BSA) and incubated in a humidified atmosphere of 95% air, 5% CO_2 at 32°C. Leydig cell preparations were determined to be about 90% pure by histochemical staining for 3β-HSD as described previously *(18)*. There was <3% contamination by macrophages as determined by DiI-Ac-LDL staining *(54)*.

Treatment of Leydig Cells

Media were changed daily before the initiation of treatments on day 6 of culture. To determine effects of PMA, AVP, and IL-1 on 8-Br-cAMP-stimulated P450c17 mRNA expression and testosterone production, Leydig cells were treated for 24 h with control medium (supplemented DME/F12), 50 μ*M* 8-Br-cAMP, 8-Br-cAMP plus increasing concentrations of PMA (0.01, 0.1, 1.0, and 10.0 n*M*) or AVP (1, 10, and 100 n*M*), or mrIL-1β (1.0 ng/mL). Media were collected for testosterone measurement by RIA, and cells were lysed for RNA extraction. In some experiments, cells were also treated with 1 μ*M* Calphostin C.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method as described previously *(17)*. The RNA was analyzed by Northern blotting as described *(17,19)*. Radioactivity was visualized by autoradiography and quantitated by densitometry.

Testosterone RIA

Culture media were boiled for 5 min and centrifuged at 2000*g* for 20 min at 4°C. The supernatant was stored at –20°C until assayed for testosterone by using Coat-A-Count RIA kits. Results were normalized per 10^6 Leydig cells.

^{32}P-Labeling and MARCKS Immunoprecipitation

Leydig cells were maintained in culture for 5 d prior to the metabolic labeling. Labeling and immunoprecipitation were based on the procedure of Lobaugh and Blackshear *(32)*. Approximately 16 h prior to labeling, media were removed and replaced with Waymouth's MB752/1 medium that contained 1% BSA. All subsequent incubations were conducted in this medium. Cells were incubated for 2 h in media containing 0.1 mCi/mL [^{32}P]orthophosphate. Labeled cells were treated for 5 min with 1 m*M* 8-Br-cAMP, 100 n*M* AVP, 1 μ*M* 4α-PDD, 10 ng/mL IL-1β, or 1 μ*M* PMA before rinsing and homogenizing in 350 μL of extraction buffer (25 m*M* Tris-HCl, pH 7.9, 50 m*M* sodium pyrophosphate, 100 m*M* NaF, 20 NaCl, 10 m*M* EGTA, 5 m*M* EDTA, 0.1% NP-40, 10 μ*M* sodium orthovanadate) containing 1 m*M* PMSF, 1 m*M* DTT, and 50 μg/mL leupeptin. Lysates were cleared by centrifugation (12,000*g* for 20 min). Protein concentration of supernatants was determined by micro-BCA protein assay (Pierce, Rockford, IL). An equal amount of protein from each sample was preincubated with protein A-Sepharose for 1 h at room temperature before pelleting for 5 min at 12,000*g*. Supernatants were incubated with anti-MARCKS antiserum (1:1000 final dilution) overnight at 4°C, before incubation with protein A-Sepharose for 1 h at room temperature. Pellets were collected by brief centrifugation in a table top microcentrifuge, the supernatant removed, and the pellet washed three times in wash buffer (50 m*M* Tris-HCl, pH 8.3, 0.15 *M* NaCl, 1 m*M* EDTA, 0.5 % NP-40). The final pellet was resuspended in SDS-PAGE sample buffer, boiled for 5 min, and subject to electrophoresis in an 8% SDS-polyacrylamide gel according to the method of Laemmli *(55)*. MARCKS phosphorylation was visualized by autoradiography and quantitated by densitometry.

Statistics

Data were presented as means ± SEM of three or more independent experiments. For group comparison, one-way analysis of variance followed by a Student-Newmann-Keuls multiple-range test was performed using the GraphPad InStat, version 2.0, statistical software package (GraphPad Software, San Diego, CA). Differences were considered as significant at $P < 0.05$.

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References

1. Bosmann, H. B. Hales, K. H., Li, X,. Lui, Z., Stocco, D. M., and Hales, D. B. (1996). *Endocrinology* **137,** 4522–4525.
2. Hales, D. B., Hales, K. H., and Bosmann, H. B. (199). *2nd Internatinal Symposium on Molecular Steroidogenesis*. Monterret, CA.
3. Xiong, Y., and Hales, D. B. (1994). *Endocr. J.* **2,** 223–228.
4. Turnbull, A. V., and River, C. (1996). *Endocrinology* **137,** 455–463.
5. Cronenwett, J. L., Baver-Neff, B. S., Grekin, R. J., and Sheagren, J. N. (1986) *J. Surg. Res.* **41,** 609–619.
6. Egan, J. W., Jugus, M., Kinter, L. B., Lee, K., and Smith, E. F. I. (1989). *Circ. Shock.* **29,** 155–166.
7. Kasting, N. W., Mazurek, M. F., and Martin, J. B., (1985). *Am. J. Physiol.* **248,** E420–424.
8. Kasting, N. W. (1989). *Can. J. Physiol. Pharm.* **66,** 22–26.
9. Schaller, M. D., Waeber, B., Nussberger, J., and Brunner, H. R. (1985). *Am. J. Physiol.* **249,** H1086–1092.
10. Kasson, B. G., Adashi, E. Y., and Hsueh, A. J. W. (1986). *Endocr. Rev.* **7,** 156–168.

11. Saez, J. M. (1994). *Endocr. Rev.* **15,** 574–626.
12. Tahri-Joutei, A. and Pointis, G. (1988). *Life Sciences* **43,** 177–185.
13. Sayeed, M. M. (1996). *New Horm.* **4,** 72–86.
14. Nielson, J. R., Hansen, H. S., and Jensen, B. (1989). *Mol. Cell Endocrinol.* **61,** 181–188.
15. Vinggaard, M. A., and Hansen, H. S. (1991). *Mol. Cell Endocrinol.* **79,** 157–165.
16. Ascoli, M., Pignataro, O. P., and Segaloff, D. L. (1989). *J. Biol. Chem.* **264,** 6674–6681.
17. Hales, D. B. (1992). *Endocrinology* **131,** 2165–2172.
18. Hales, D. B., Xiong, Y., and Tur-Kaspa, I. (1992). *J. Steroid Biochem. Mol. Biol.* **43,** 907–914.
19. Xiong, Y. and Hales, D. B. (1993). *Endocrinology* **132,** 2438–2444.
20. Li, X., Youngblood, G. L., Payne, A. H., and Hales, D. B. (1995). *Endocrinology* **136,** 3519–3526.
21. Dix, C. J., Habberfield, A. D., and Cooke, B. A. (1987). *Biochem. J.* **243,** 373–377.
22. Moger, W. H. (1985). *Life Sci.* **37:** 869–873.
23. Mukhopadhyay, A. K., Bohnet, H. G., and Leindenberger, F. A. (1984). *Biochem. Biophys. Res. Commun.* **119,** 1062–1067.
24. Mukhopadhyay, A. K. and Schumacher, M. (1985). *FEBS Letts.* **187,** 56–60.
25. Papadopoulos, V., Careau, S., and Drosdowsky, M. A. (1985). *FEBS Letts.* **188,** 312–316.
26. Rebois, R. V., and Patel, J. (1985). *J. Biol. Chem.* **260,** 8026–8031.
27. Bankers-Fulbright, J. L., Kalli, K. R., and McKean, D. J. (1996). *Life Sci.* **59**: 61–83.
28. Kikkawa, U. and Nishizuka, Y. (1986). *Annu. Rev. Cell Biol.* **2,** 149–178.
29. Aderem, A. (1995). *Biochem. Soc. Trans.* **23,** 587–591.
30. Aderem, A. (1992). *Cell* **71,** 713–716.
31. Blackshear, P. J. (1993). In: *Handbook of Endocrine Research Techniques.* de Pablo, F., Scanes, C. G., and Weintraub, B. D., (eds.). Academic, San Diego, CA, pp. 339–355.
32. Lobaugh, L. A. and Blackshear, P. J. (1990). *J. Biol. Chem.* **265,** 18,393–18,399.
33. Liu, J. P., Engler, D., Funder, J. W., and Robinson, P. J. (1994) *Mol. Cell Endocrinol.* **105,** 217–226.
34. Vinggaard, A. M. and Hansen, H. S. (1993). *J. Endocrinol.* **136,** 119–126.
35. Nishizuka, Y. (1988). *Nature* **334,** 661–665.
36. Dinarello, C. A. (1996). *Blood* **87,** 2095–2147.
37. Stewart, R. J. and Marsden, P. A. (1995). *Am. J. Kidney Dis.* **25,** 954–966.
38. Riches, D. W., Chan, E. D., and Winston, B. W. (1996). *Immunobiology* **195,** 477–490.
39. Naismith, J. H. and Sprang, S. R. (1996). *J. Inflamm.* **47:** 1–7.
40. Grell, M. (1995). *J. Inflamm.* **47,** 8–17.
41. Aderem, A. (1992). *Trends Biochem. Sci.* **17,** 438–443.
42. Hall, P. F. and Almahbobi, G. (1992). *J. Steroid Biochem. Mol. Biol.* **43,** 769–777.
43. Rivier, C. and Rivest, S. (1991) *Biol. Reprod.* **45,** 523–532.
44. Ivell, R., Hunt, N., Hardy, M., Nicholson, H., and Pickering, B. (1992). *Mol. Cell. Endocrinol.* **89,** 54–61.
45. Foo, N.-C., Carter, D., Murphy, D., and Ivell, R. (1991). *Endocrinology* **128,** 2118–2128.
46. Fillion, C., Tahri-Joutei, A., Hugues, J. N., Allevard, A. M., Taib, N., and Pointis, G. (1994). *Mol. Cell. Endocrinol.* **99,** 25–30.
47. Tahri-Joutei, A., Fillion, C., Bedin, M., Hugues, J.-N., and Pointis, G. (1991). *Mol. Cell Endocrinol.* **79,** R21–24.
48. Tahri-Joutei, A. and Pointis, G. (1989). *FEBS Lett.* **254,** 189–193.
49. Kasson, B. G. and Tuchel, T. M. (1989) *Endocrinology* **124,** 2777–2784.
50. Sharpe, R. M. and Cooper, I. (1987). *J. Endocrinol.* **113,** 89–96.
51. Bardin, C. W., Chen, C.-L. C., Morris, P. L., Gerendai, I., Boitani, C., Liotta, A. S., et al. (1987). *Rec. Prog. Horm. Res.* **43,** 1–28.
52. Fabri, A., Knox, G., Buczko, E., and Dufau, M. L. (1988). *Endocrinology* **122,** 749–755.
53. Turnbull, A. and Rivier, C. (1995). *Neuroimmunomodulation* **2,** 224–235.
54. Pitas, R. E., Innerarity, T., Weinstein, J. N., and Mahley, R. W. (1981). *Arteriosclerosis* **1,** 177–185.
55. Laemmli, U. K. (1970). *Nature* **227,** 680–685.