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# SPHINGOSINE INHIBITS ANGIOTENSIN-STIMULATED ALDOSTERONE SYNTHESIS

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**Summary**—Sphingosine and other protein kinase C inhibitors were tested for their ability to inhibit aldosterone synthesis by bovine adrenal glomerulosa cells. Sphingosine inhibited angiotensin (AII)-stimulated aldosterone synthesis ($IC_{50}$ of 5 $\mu$M). At doses that totally blocked steroidogenesis, sphingosine did not affect protein synthesis or [$^{125}$I]AII binding to cells. Sphingosine also inhibited dibutyryl cyclic AMP (dbcAMP)-stimulated aldosterone synthesis. Sphingosine inhibited pregnenolone synthesis from cholesterol, but not the conversion of progesterone or 20$\alpha$-hydroxycholesterol to aldosterone. These results suggest that sphingosine inhibits steroidogenesis at a locus close to that where stimulation occurs by AII and dbcAMP.

Other protein kinase C inhibitors were tested. Retinal, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), and staurosporine inhibited aldosterone synthesis stimulated by AII and dbcAMP. Retinal and H-7 also inhibited progesterone conversion to aldosterone, and retinal blocked [$^{125}$I]AII binding. Staurosporine was more specific, inhibiting AII-stimulated aldosteronogenesis at concentrations which had little effect on conversion of progesterone to aldosterone. Because they inhibited dbcAMP stimulation, none of the inhibitors was sufficiently specific to use as a probe of the role of protein kinase C. The $IC_{50}$ of sphingosine suggests that this or related products of lipid hydrolysis could act as endogenous regulators of adrenal cell function.

## INTRODUCTION

Sphingolipid breakdown products such as sphingosine and lysosphingolipids have been shown to exert a wide variety of effects in many cell types [1–5]. Cellular activities of sphingosine and lysophospholipids include inhibition of platelet and neutrophil activation, and inhibition of responses to steroid hormones, EGF, and insulin. It is still unclear whether sphingolipid breakdown products play a major role in normal regulation of cell function similar to those played by cyclic nucleotides, calcium, and phosphoinositide metabolites. Sphingosine inhibits protein kinase C, and it has been suggested that protein kinase C may be regulated by both positive (diacylglycerol) and negative (sphingosine) effects of endogenous substances [1, 3].

Angiotensin II (AII) is thought to stimulate aldosterone synthesis by receptor-mediated effects on phosphoinositide and calcium metabolism and subsequent stimulation of protein kinases [6–14]. Little is known about how these changes translate into an increased rate of steroidogenesis.

We tested the effects of sphingosine and various agonists on adrenal glomerulosa cells to see if this lipid derivative is a potential endogenous intracellular inhibitor of aldosterone synthesis. Since sphingosine inhibits protein kinase C, we also performed studies of three other compounds known to inhibit protein kinase C.

If AII stimulation of aldosterone synthesis involves protein kinase C, we reasoned that protein kinase C inhibitors would inhibit AII-stimulated aldosterone synthesis at concentrations similar to those required for kinase inhibition. Processes that do not require direct participation of protein kinase C should not be affected by specific protein kinase C inhibitors. These processes include AII binding to its receptor, conversion of 20$\alpha$-hydroxycholesterol or progesterone to aldosterone, and aldosterone synthesis stimulated by dibutyryl cyclic AMP (dbcAMP). This report presents our results on the effects of sphingosine and other protein

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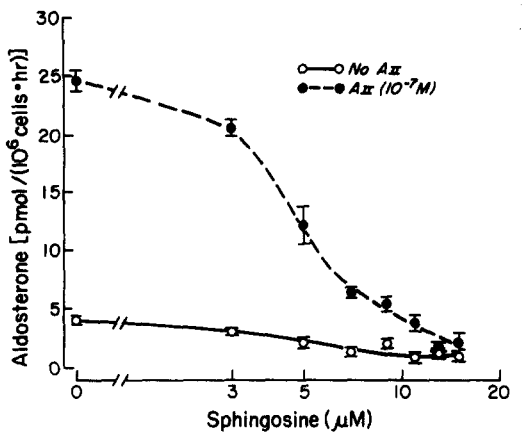


Fig. 1. Effect of sphingosine on aldosterone synthesis. Bovine adrenal glomerulosa cells were incubated as described in the text in the presence or absence of AII, and in the presence of the concentrations of sphingosine indicated in the figure. Aldosterone in the supernatant was measured by radioimmunoassay. Values depicted in this and other figures are the mean ± SEM from five incubation tubes, unless otherwise indicated.

kinase C inhibitors on some of the processes involved in stimulating aldosterone secretion.

## MATERIALS AND METHODS

Most reagents were obtained from standard commercial sources as described [15]. Sphingosine, sphingomyelin, dihydrosphingosine, and retinal were from Sigma Chemical Co. (St Louis, Mo.). Trilostane was a gift from Sterling Winthrop Research Institute, Rensselaer, N.Y., and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was purchased from Seikagaku America, Inc. (St Petersburg, Fla). Aldosterone and pregnenolone antiserum were obtained from Radioassay Systems Laboratories, Inc. (Carson, Calif.). [$^{125}$I]AII was prepared as described [15]. [3,4,5-$^{3}$H]leucine (146.5 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

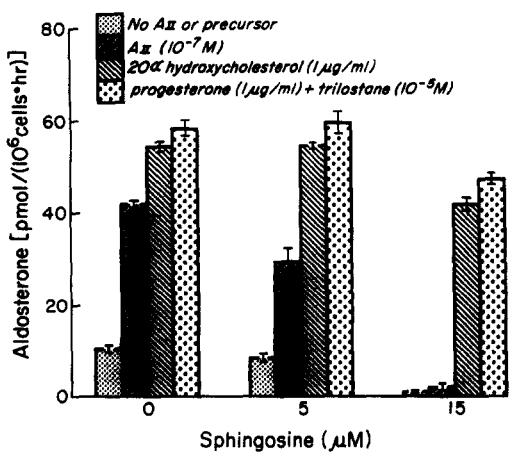


Fig. 2. Effect of sphingosine on aldosterone synthesis stimulated by AII, 20α-hydroxycholesterol, or progesterone. Trilostane ($10^{-5}$ M) was included in the incubations conducted in the presence of progesterone to prevent synthesis of aldosterone from endogenous precursors. Methods are as described in the text.

Bovine adrenal glomerulosa cells were prepared as described previously [15] and suspended in an incubation buffer that contained NaCl (125 mM), HEPES (20 mM), KCl (3.6 mM), glucose (11 mM), $MgSO_4$ (1 mM), $CaCl_2$ (0.5 mM), and bovine serum albumin (0.1%), pH 7.4. Cells (250,000–400,000) were incubated in a final volume of 0.5 ml for 2 h at 37°C with other additions as indicated. Aldosterone was measured by direct radioimmunoassay of the supernatant as described [15]. In some experiments, the early steps of steroid synthesis were assessed by incubating cells with trilostane (10 μM) to prevent conversion of pregnenolone to progesterone; pregnenolone was measured in the supernatant as described [16]. In other experiments, the late steps in steroid synthesis were assessed by incubating cells with progesterone (3.2 μM) and measuring aldosterone production. In these experiments, trilostane (10 μM) was included to prevent the production of progesterone from endogenous precursors. In some experiments, conversion of 20α-hydroxycholesterol to aldosterone was measured by incubating cells with this substrate at 2.5 μM and measuring aldosterone in the supernatant. Binding of [$^{125}$I]AII to adrenal cells and protein synthesis (incorporation of [$^{3}$H]leucine into acid-precipitable material) were measured as previously described [15, 16].

Solutions of H-7 were made in buffer. Retinal and sphingosine were dissolved in ethanol, and staurosporine was dissolved in dimethylsulfoxide. The final concentration of solvent in cell incubations was 0.5% or less, and control tubes were always incubated with the appropriate vehicle. In some incubations, ethanolic solutions of sphingosine were diluted into a 2.5-mM solution of bovine serum albumin, incubated for 1 h at 37°C as described [17], and diluted in incubation buffer for use in a cell incubation.

## RESULTS

Figure 1 shows the effects of sphingosine on aldosterone synthesis. Sphingosine inhibited both basal and AII-stimulated aldosterone synthesis, with half-maximal inhibition observed at approximately 5 μM.

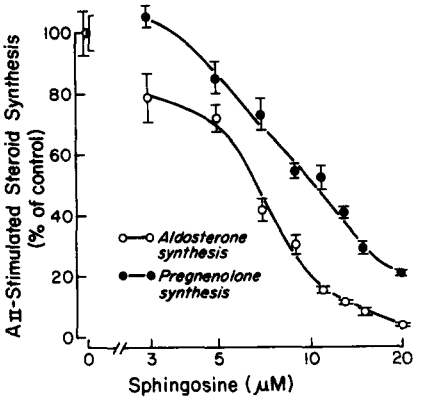


Fig. 3. Effect of sphingosine on aldosterone synthesis and pregnenolone synthesis. Cells were incubated in the presence of AII ($10^{-7}$ M) and with concentrations of sphingosine as indicated. Aldosterone was measured in the supernatant by radioimmunoassay. For assessment of pregnenolone synthesis, trilostane ($10^{-5}$ M) was added to the incubation tube to prevent further metabolism of pregnenolone. Pregnenolone was measured in the supernatant by radioimmunoassay. Control (no sphingosine) aldosterone generation was 3.66 pmol/$10^6$ cells·h, and control pregnenolone generation was 17.7 pmol/$10^6$ cells·h.

In order to determine whether sphingosine inhibition was specific for certain steps in steroidogenesis, we measured sphingosine's effect on aldosterone generation from 20α-hydroxycholesterol and from progesterone. As shown in Fig. 2, sphingosine did not suppress the increment in aldosterone synthesis afforded by either substrate, even at a concentration (15 μM) that completely inhibited AII-stimulated aldosterone synthesis in this experiment. Since sphingosine inhibited aldosterone synthesis from endogenous cholesterol, but not the late steps in aldosteronogenesis, it seemed likely that sphingosine would inhibit pregnenolone synthesis from endogenous cholesterol, which measures the early step in steroidogenesis. As Fig. 3 shows, sphingosine inhibited AII-stimulated pregnenolone synthesis, although this inhibition was slightly less than that of AII-stimulated aldosterone synthesis. Sphingosine also failed to affect binding of [$^{125}$I]AII to glomerulosa cells, even at 30 μM (data not shown).

Compounds structurally related to sphingosine were tested for their effects on adrenal cells. Saturated derivatives of sphingosine inhibited aldosterone synthesis. DL-erythro-dihydrosphingosine and DL-threo-dihydrosphingosine inhibited AII-stimulated aldosterone with a potency and efficacy indistinguishable from that of sphingosine (see Table 1). By contrast, neither bovine brain nor chicken egg yolk sphingomyelin inhibited AII-stimulated aldosterone synthesis, even at a concentration of 100 μM (see Table 1).

Sphingosine has been reported to inhibit $Na^+/K^+$-ATPase [18]. Previous work from our laboratory showed that ouabain inhibits aldosterone synthesis and adrenal protein synthesis, and that this inhibition can be partially overcome by increasing the potassium concentration of the incubation solution [19]. As shown in Fig. 4, basal and AII-stimulated aldosterone production were inhibited by ouabain (0.1 or 1 μM), and this inhibition was partially relieved by increased potassium concentrations. However, the inhibition caused by sphingosine was not relieved by high potassium. Sphingosine did not inhibit protein synthesis, measured as incorporation of [$^3$H]leucine into acid-precipitable material, at concentrations up to 30 μM; it did inhibit however at 100 and 300 μM (data not shown).

Sphingosine and derivatives such as dihydrosphingosine exert nonspecific toxic effects on some cells, including neutrophils [17], when added in a protein-free vehicle. When prepared in a solution with concentrated albumin, however, dihydrosphingosine produces very specific inhibition of agonist-stimulated superoxide generation, with little or no effect on neutrophil viability or other cell functions. Therefore, we prepared spingosine in two ways: (1) in a vehicle yielding a final concentration of 0.5% ethanol;

Table 1. Effects of sphingosine, dihydrosphingosines, and sphingomyelin on aldosterone synthesis

| Compound | Trivial or other name | Potency against AII-stimulated aldosterone synthesis ($IC_{50}$ in μM) |
|---|---|---|
| *trans*-D-erythro-2-amino-4-octadecene-1,3-diol | D-sphingosine | 5 |
| DL-erythro-1,3-dihydroxy-2-aminooctadecane | DL-erythro-dihydrosphingosine; DL-sphinganine | 7 |
| DL-threo-1,3-dihydroxy-2-amino-octadecane | DL-threo-dihydrosphingosine | 5 |
| Sphingomyelin | | >100 |

Cells were incubated as described in the text in the presence of AII ($10^{-7}$ M) and varying concentrations of the compounds listed above. Aldosterone synthesis was determined by radioimmunoassay of the supernatant, and $IC_{50}$s were determined graphically.

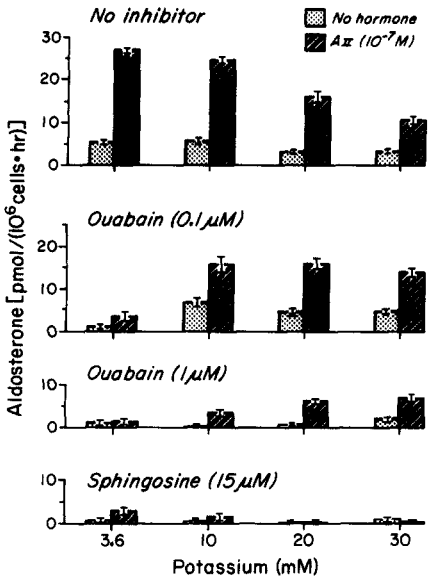


Fig. 4. Effects of ouabain and sphingosine on aldosterone synthesis. Cells were incubated in the presence of ouabain or sphingosine at the concentrations indicated and in solutions containing potassium chloride at the concentrations indicated.

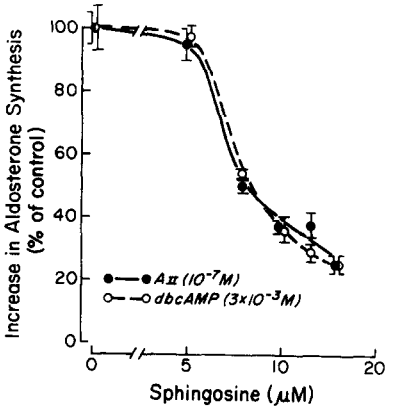


Fig. 6. Effect of sphingosine on aldosterone synthesis stimulated by AII as compared to dbcAMP. Cells were incubated in the presence of sphingosine at the concentrations indicated in the presence or absence of AII or dbcAMP. Aldosterone was measured in the supernatant, and basal aldosterone synthesis was subtracted from values for stimulated cells. Results were expressed as the percentage of increase in aldosterone generation observed in the absence of sphingosine. The control (no sphingosine) AII-stimulated increase in aldosterone synthesis was 10.4 pmol/$10^6$ cells·h. The control dbcAMP-stimulated increase was 15.2 pmol/$10^6$ cells·h.

and (2) in a solution of concentrated albumin as described in the Methods section and in Ref. 17. Figure 5 shows that the two preparations of sphingosine inhibited aldosterone synthesis to a similar extent.

Sphingosine was tested for its ability to inhibit aldosterone synthesis stimulated by dbcAMP. As shown in Fig. 6, sphingosine was equally potent as an inhibitor of AII- and dbcAMP-stimulated aldosterone synthesis.

Other putative protein kinase C inhibitors were tested for their ability to inhibit aldosterone synthesis [20–22]. Figure 7 shows that staurosporine inhibited AII- and dbcAMP-

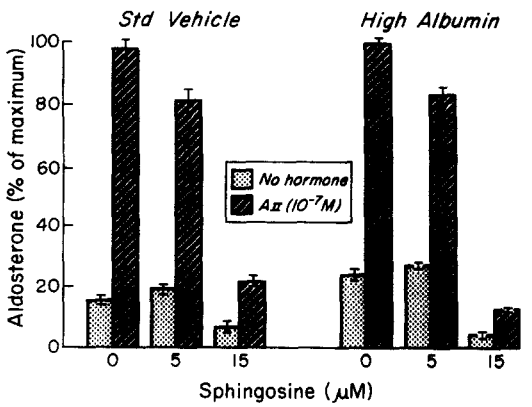


Fig. 5. Effect of two different preparations of sphingosine on aldosterone synthesis. "Std vehicle" refers to sphingosine made in the routine way (dissolved in ethanol and then diluted in incubation solution which contains 0.1% bovine serum albumin). "High albumin" refers to sphingosine which is dissolved in ethanol and then preincubated in a 2.5-mM solution of bovine serum albumin before addition to cells (see text for details). For either preparation of sphingosine, cells were incubated and aldosterone generation was measured as described in the legend to Fig. 1. Maximum aldosterone synthesis was 32.5 pmol/$10^6$ cells·h.

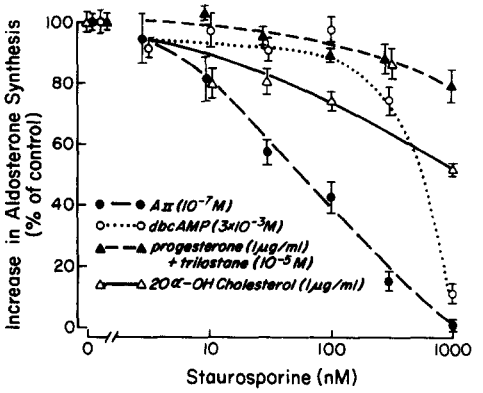


Fig. 7. Effect of staurosporine on aldosterone synthesis. Incubations were carried out with stimuli and staurosporine as indicated. Except for 20α-hydroxycholesterol, all data points represent the mean ± SEM of 15 incubation tubes from three separate experiments. For 20α-hydroxycholesterol, data are means of 10 incubation tubes from two experiments. For each experiment, aldosterone was measured, basal (no stimulus) values were subtracted, and results were calculated as a percentage of the increase in aldosterone generation observed for each stimulus in the absence of staurosporine. The mean control (no staurosporine) values for these experiments were 9.16 pmol/$10^6$ cells·h for the AII-stimulated increase, 19.7 pmol/$10^6$ cells·h for dbcAMP, 35.0 pmol/$10^6$ cells·h for progesterone, and 20.8 pmol/$10^6$ cells·h for 20α-hydroxycholesterol.

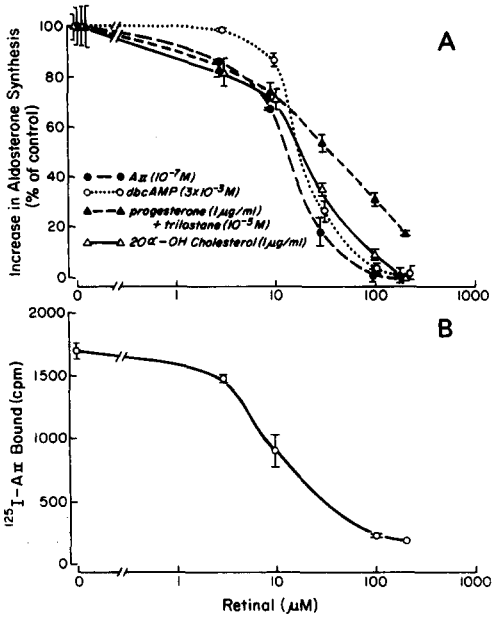


Fig. 8. (A) Effect of retinal on stimulation of aldosterone synthesis caused by AII, dbcAMP, progesterone plus trilostane or 20α-hydroxycholesterol. Cells were incubated, aldosterone was measured, basal (no stimulus) values were subtracted, and the results were expressed as percentage of increase in aldosterone generation observed for each stimulus in the absence of retinal. The control (no retinal) increases in aldosterone synthesis were 21.2 pmol/$10^6$ cells·h for AII, 37.4 pmol/$10^6$ cells·h for dbcAMP, 44.5 pmol/$10^6$ cells·h for progesterone, and 30.6 pmol/$10^6$ cells·h for 20α-hydroxycholesterol. (B) Effect of retinal on binding of [$^{125}$I]AII to adrenal glomerulosa cells. Cells were incubated with the indicated concentrations of retinal and with [$^{125}$I]AII (approx 25 nCi per tube) for 45 min at 37°C. Cells were centrifuged, cell pellets were washed with buffer, and counted in a gamma counter. Nonsaturable binding (in the presence of $10^{-6}$ M unlabeled AII) was systematically subtracted; it amounted to approx 10% of total radioactivity bound.

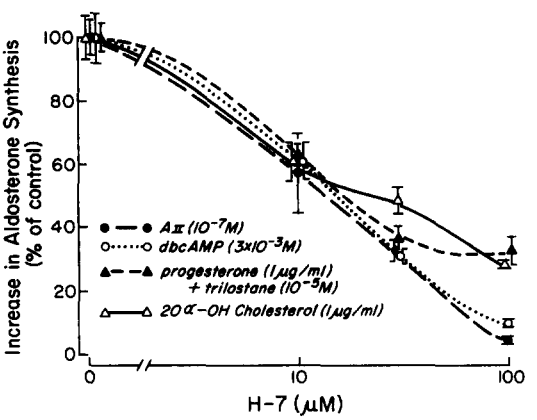


Fig. 9. Effect of H-7 on aldosterone synthesis. Incubations were carried out with stimuli and H-7 as indicated. Results were calculated as described in Fig. 8A. The control (no H-7) increases in aldosterone synthesis were 4.72 pmol/$10^6$ cells·h for AII, 13.6 pmol/$10^6$ cells·h for dbcAMP, 20.0 pmol/$10^6$ cells·h for progesterone, and 16.0 pmol/$10^6$ cells·h for 20α-hydroxycholesterol.

stimulated aldosterone synthesis, but it was more potent against the former ($IC_{50}$ approximately 50 nM for AII vs >300 nM for dbcAMP). Aldosterone synthesis from exogenous progesterone and from 20α-hydroxycholesterol was relatively resistant to staurosporine.

As shown in Fig. 8A, retinal inhibited aldosterone synthesis stimulated by AII, dbcAMP, 20α-hydroxycholesterol, or progesterone. Figure 8B shows that retinal inhibited binding of [$^{125}$I]AII to adrenal glomerulosa cells at concentrations similar to those that block steroidogenesis.

Compound H-7 was also tested for its ability to inhibit aldosterone synthesis. Figure 9 shows that H-7 inhibited AII- and dbcAMP-stimulated aldosterone synthesis similarly, and was slightly less effective when 20α-hydroxycholesterol or progesterone was added. H-7 did not inhibit binding of [$^{125}$I]AII to adrenal cells or protein synthesis at concentrations up to 200 μM (data not shown).

## DISCUSSION

This study showed that sphingosine inhibits AII-stimulated steroidogenesis in bovine zona glomerulosa (Fig. 1). Sphingosine also inhibited AII-stimulated pregnenolone generation from endogeneous cholesterol. It did not block conversion of progesterone to aldosterone. These results suggest that sphingosine acts specifically on the early steps of aldosteronogenesis. Steroidogenic stimuli such as ACTH and AII increase pregnenolone synthesis by increasing access of endogenous cholesterol to the cytochrome $P450_{scc}$ on the inner mitochondrial membrane [23–26]. 20α-hydroxycholesterol bypasses regulation because it freely penetrates the mitochondrial membrane [23, 27]. Since sphingosine did not affect the utilization of 20α-hydroxycholesterol, we concluded that it does not block the side-chain cleavage enzyme *per se*. Thus, sphingosine appears to inhibit aldosterone synthesis at or near the very site where AII and dbcAMP exert their stimulatory effects, promoting access of endogenous cholesterol to the side-chain cleavage enzyme. Sphingosine did not interfere with AII's interaction with its receptor.

We wondered whether sphingosine's effects might be similar to those of ouabain, since sphingosine inhibits $Na^+/K^+$-ATPase [18]. We previously reported that ouabain inhibition of aldosterone synthesis was partially overcome by

increasing the potassium concentration in the cell incubation solution [19]. Sphingosine inhibition was not relieved at all by increasing the concentration of potassium, despite potassium's ability to partially reverse ouabain's effects in the same experiment. We have not directly measured sphingosine's effects on adrenal $Na^+/K^+$-ATPase, but the results shown here do not suggest an ouabain-like mechanism for sphingosine.

Other researchers have reported on the effects of sphingosine and dihydrosphingosine on neutrophil function [17]. These two compounds inhibited the agonist-stimulated respiratory burst and secretion of specific granules from neutrophils [17]. In these experiments, the reagent in BSA solutions was much more specific than when BSA was absent. When we compared sphingosine's effects in the presence and absence of BSA, it showed similar inhibitory potency in both solutions. This result and the ability of sphingosine to block aldosterone synthesis from endogenous cholesterol but not from 20α-hydroxycholesterol argue against a general cytotoxic effect of sphingosine on adrenal cells.

Sphingosine is an inhibitor of protein kinase C, so the inhibition of AII-stimulated aldosterone synthesis could provide further evidence of a role for protein kinase C in AII's action. However, sphingosine demonstrated equal potency against dbcAMP-stimulated steroidogenesis. This result suggests that sphingosine may inhibit a crucial step in agonist-stimulated steroidogenesis different from the action of protein kinase C.

We also examined some other putative protein kinase C inhibitors. Of the inhibitors reported here, only staurosporine showed a greater potency toward AII than toward dbcAMP. Staurosporine's inhibition was also focused in that there was little effect on utilization of progesterone or 20α-hydroxycholesterol. However, the concentration required to inhibit AII's effect by 50% was approximately 50 nM, thus greater than the reported $IC_{50}$ of 3 nM for staurosporine's effect on protein kinase C [20]. Of all the inhibitors tested, retinal exhibited the broadest range of effects. It inhibited both dbcAMP- and AII-stimulated steroidogenesis and the late steps in the pathway. Retinal also inhibited AII binding to adrenal cells. H-7 was somewhat more selective than retinal. Although AII and dbcAMP was similarly affected, H-7 was somewhat less effective at inhibiting conversion of 20α-hydroxycholesterol or progesterone into aldosterone; it did not inhibit AII binding.

We have formulated two general conclusions from the results presented in this report. First, none of the putative protein kinase C inhibitors used was able to provide clear and specific evidence of a role for protein kinase C in the system studied. We had hoped to find an inhibitor that would affect only a key step in steroidogenesis, be effective at a concentration reported to inhibit protein kinase C in other systems, and inhibit AII-stimulated, but not dibutyryl cyclic AMP-stimulated steroidogenesis. None of the inhibitors that we tested fulfilled all of these criteria, but staurosporine came the closest. Solid proof of a role for protein kinase C in steroidogenesis may require use of other inhibitors, or more likely, evidence of an AII-stimulated protein kinase C-phosphorylated protein which can be directly linked to a critical step in steroidogenesis. It should be noted that recent work [28] has cast doubt on the role of protein kinase C in AII-stimulated aldosterone synthesis. That study showed that rat adrenal glomerulosa cells treated with phorbol to deplete protein kinase C retained full AII-stimulated steroidogenesis.

Our second conclusion is that sphingosine inhibits agonist-stimulated aldosterone synthesis at concentrations that inhibit specific cellular functions in many other cell types. This may point at a role for sphingosine or related molecules as endogenous inhibitors of adrenal cell function. Further studies will be needed to determine whether adrenal cells exhibit alterations in levels of sphingosine consistent with a regulatory role for this molecule in aldosterone synthesis.

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## REFERENCES

1. Hannun Y. A., Loomis C. R., Merrill A. H. Jr and Bell R. M.: Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. *J. Biol. Chem.* **261** (1986) 12604–12609.
2. Merrill A. H. Jr, Sereni A. M., Stevens V. L., Hannun Y. A., Bell R. M. and Kinkade J. M. Jr: Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. *J. Biol. Chem.* **261** (1986) 12610–12615.

3. Hannun Y. A. and Bell R. A.: Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243** (1989) 500–507.
4. Zhany H., Buckley N. E., Gibson K. and Spiegel S.: Sphingosine stimulates cellular proliferation via a protein kinase C-independent pathway. *J. Biol. Chem.* **265** (1990) 76–81.
5. Winicov I., Cory R. N. and Gershengorn M. C.: Sphingosine interacts directly with the receptor complex to inhibit thyrotropin-releasing hormone binding. *Endocrinology* **126** (1990) 1668–1672.
6. Spät A.: Stimulus-secretion coupling in angiotensin-stimulated adrenal glomerulosa cells. *J. Steroid Biochem.* **29** (1988) 443–453.
7. Catt K. J., Carson M. C., Hausdorff W. P., Leach-Harper C. M., Baukal A. J., Guillemette G., Balla J. and Aguilera G.: Angiotensin II receptors and mechanisms of action in adrenal glomerulosa cells. *J. Steroid Biochem.* **27** (1987) 915–927.
8. Capponi A. M., Lew P. D., Jornot L. and Vallotton M. B.: Correlation between cytosolic free $Ca^{2+}$ and aldosterone production in bovine adrenal glomerulosa cells. *J. Biol. Chem.* **259** (1984) 8863–8869.
9. Elliott M. E., Siegel F. L., Hadjokas N. E. and Goodfriend T. L.: Angiotensin effects on calcium and steroidogenesis in adrenal glomerulosa cells. *Endocrinology* **116** (1985) 1051–1059.
10. Kramer R. E.: Angiotensin II-stimulated changes in calcium metabolism in cultured glomerulosa cells. *Molec. Cell. Endocr.* **60** (1988) 199–210.
11. Balla T., Hausdorff W. P., Baukal A. J. and Catt K. J.: Inositol polyphosphate production and regulation of cytosolic calcium during the biphasic activation of adrenal glomerulosa cells by angiotensin II. *Archs Biochem. Biophys.* **270** (1989) 398–403.
12. Underwood R. H., Greeley R., Glennon E. T., Menachery A. I., Braley L. M. and Williams G. H.: Mass determination of polyphosphoinositides and inositol trisphosphate in rat adrenal glomerulosa cells with a microspectrophotometric method. *Endocrinology* **123** (1988) 211–219.
13. Barrett P. Q., Kojima I., Kojima K., Zawalich K., Isales C. M. and Rasmussen H.: Temporal patterns of protein phosphorylation after angiotensin II, A23187 and/or 12-O-tetradecanoylphorbol 13-acetate in adrenal glomerulosa cells. *Biochem. J.* **238** (1986) 893–903.
14. Kubo M. and Strott C. A.: Calcium-dependent protein kinase activity and protein phosphorylation in zones of the adrenal cortex. *J. Steroid Biochem.* **29** (1988) 407–413.
15. Elliott M. E., Alexander R. C. and Goodfriend T. L.: Aspects of angiotensin action in the adrenal. Key roles for calcium and phosphatidyl inositol. *Hypertension* **4** (Suppl. II) (1982) 1152–1158.
16. Elliott M. E. and Goodfriend T. L.: Identification of the cycloheximide-sensitive site in angiotensin-stimulated aldosterone synthesis. *Biochem. Pharmac.* **33** (1984) 1519–1524.
17. Lambeth J. D., Burnham D. N. and Tyagi S. R.: Sphinganine effects on chemoattractant-induced diacylglycerol generation, calcium fluxes, superoxide production, and on cell viability in the human neutrophil. *J. Biol. Chem.* **263** (1988) 3818–3822.
18. Oishi K., Zheng B. and Kuo J. F.: Inhibition of Na,K-ATPase and sodium pump by protein kinase C regulators sphingosine, lysophophatidylcholine, and oleic acid. *J. Biol. Chem.* **265** (1990) 70–75.
19. Elliott M. E., Hadjokas N. E. and Goodfriend T. L.: Effects of ouabain and potassium on protein synthesis and angiotensin-stimulated aldosterone synthesis in bovine adrenal glomerulosa cells. *Endocrinology* **118** (1986) 1469–1475.
20. Tamaoki T., Nomoto H., Takahashi I., Kato Y., Morimoto M. and Tomita F.: Staurosporine, a potent inhibitor of phospholipid/$Ca^{2+}$ dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135** (1986) 397–402.
21. Jaffet S. M., Greenfield A. R. L. and Haddox M. K.: Retinal inhibits TPA activated, calcium-dependent, phospholipid-dependent protein kinase ("C" kinase). *Biochem. Biophys. Res. Commun.* **114** (1983) 1194–1199.
22. Hidaka H., Inagaki M., Kawamoto S. and Sasaki Y.: Isoquinoline-sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23** (1984) 5036–5041.
23. Boyd G. S.: Cholesterol regulation and catabolism in the adrenal cortex. In *Hormones and Cell Regulation* (Edited by J. Dumont and J. Nunez). Elsevier/North-Holland Biomedical, New York, Vol. 4 (1980) pp. 197–222.
24. Kramer R., Gallant S. and Brownie A.: Actions of angiotensin II on aldosterone biosynthesis in the rat adrenal cortex: effects on cytochrome P-450 enzymes of the early and late pathway. *J. Biol. Chem.* **255** (1980) 3442–3447.
25. Privalle C., Crivello J. and Jefcoate C.: Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *Proc. Natn Acad. Sci. U.S.A.* **80** (1983) 702–706.
26. Simpson E. R.: Cholesterol side-chain cleavage, cytochrome P-450 and the control of steroidogenesis. *J. Endocr.* **84** (1980) 179–227.
27. Lambeth J. D., Kitchen S. E., Farougin A. A., Tuckey R. and Kamin H.: Cytochrome $P\text{-}450_{scc}$-substrate interactions. Studies of binding and catalytic activities using hydroxycholesterol. *J. Biol. Chem.* **257** (1982) 1876–1884.
28. Nakano S., Carvallo P., Rocco S. and Aguilera G.: Role of protein kinase C on the steroidogenic effect of angiotensin II in the rat adrenal glomerulosa cell. *Endocrinology* **126** (1990) 125–133.