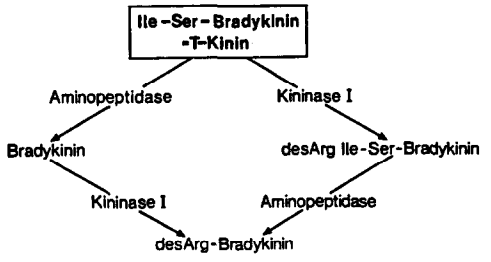

Fig. 3. Proposed pathways for the degradation of Ile-Ser-BK.

Greenbaum [11] to occur after incubation of T-Kinin with aminopeptidase M. As both enzymes (kininase I and aminopeptidase M) occur in human serum it seems likely that they are responsible for the degradation of Ile-Ser-BK. Results of studies in which kininase I was inhibited by HgCl and aminopeptidase M by amastatin and bestatin support this assumption. No change is seen after captopril. This is consistent with the results of Sheik and Kaplan [12] who showed that BK is not a substrate for angiotensin converting enzyme *in vitro*.

For the degradation of Ile-Ser-BK we suggest the model depicted in Fig. 3.

In our experiments we found evidence of Ile-Ser-BK degrading enzymes both in serum and in ascites. In some of the probes we also could detect Ile-Ser-BK in up to micromolar concentrations [6]. Thus, synthesis of Ile-Ser-BK has to exceed degradation considerably. Further studies are needed to clarify how Ile-Ser-BK is generated in human fluids. It is reasonable to assume that Ile-Ser-BK is derived from a third kininogen, different from high- and low-molecular weight kininogens. Whether this third kininogen is a product of the tumor cells or is induced by mediators of the tumor cells is a subject of speculation.

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Effects of calmidazolium and other calmodulin antagonists on adrenal glomerulosa cells

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Angiotensin II (AII*) stimulates aldosterone synthesis by a mechanism that involves alterations in calcium and phosphoinositide metabolism [1–6]. Increases in cytosolic calcium have been observed in adrenal cells exposed to AII, and this calcium may influence steroidogenesis by stimulating calmodulin-dependent enzymes, such as protein kinases [5–8]. Calmodulin is present in the adrenal cortex, and there is evidence to support a role for calmodulin in steroidogenesis [7–13]. A very specific calmodulin antagonist would be useful in defining the calcium–calmodulin-dependent events involved in AII-stimulated steroidogenesis, such as phosphorylation of key proteins. Such a compound would be expected to block a discrete calmodulin-dependent step(s) in AII-stimulated steroidogenesis, but not to have a broad inhibitory effect against adrenal cell function. Several reports have indicated that traditional calmodulin antagonists inhibit steroidogenesis, including AII-stimulated aldosterone synthesis [14–17]. Compound R24571 (calmidazolium) is an

* Abbreviations: AII, angiotensin II; ANP, atrial natriuretic peptide; and W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

antagonist with ostensibly greater specificity than several other compounds in its class [18]. We conducted experiments with several calmodulin reagents and adrenal glomerulosa cells that led us to the unexpected conclusion that calmidazolium has a broader range of effects than some other calmodulin antagonists. However, our results do not contradict the conclusion that calmodulin is critical to AII action on the adrenal.

Materials and Methods

Most reagents were obtained from standard commercial sources or as previously described [14]. Trifluoperazine, calmidazolium, and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) were obtained from the Sigma Chemical Co. (St. Louis, MO). Fluphenazine was obtained from Dr. David L. Nelson, Department of Biochemistry, University of Wisconsin-Madison. Trilostane was a gift from Sterling Winthrop Research Institute (Rensselaer, NY). ^{125}I was purchased from Amersham (Arlington Heights, IL). AII and atrial natriuretic peptide (ANP) [rat (8–33)] were obtained from the Bachem Co. (Torrance, CA) and iodinated as previously described [14, 19].

Bovine adrenal glomerulosa cells were prepared as described in a previous publication [14], and resuspended in a buffer containing HEPES (20 mM), NaCl (125 mM), $MgSO_4$ (1 mM), glucose (11 mM), KCl (3.6 mM), $CaCl_2$ (0.5 mM), and crystalline bovine serum albumin (0.1%), pH 7.4. Calmodulin inhibitors were dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO in all incubation tubes was 0.5%. In experiments in which progesterone was used, the steroid was first dissolved in ethanol, and the final concentration of ethanol in all tubes was 0.1%. Cells (300,000–400,000/tube) were incubated in a final volume of 0.5 mL for 2 hr at 37°. Aldosterone in the supernatant was measured by radioimmunoassay as previously described [14]. Binding of [^{125}I]-labeled AII and of [^{125}I]-labeled ANP to cells was measured as previously reported [14, 19].

Results

Figure 1A shows the effects of calmidazolium on aldosterone synthesis. Calmidazolium alone exerted a biphasic effect on basal steroidogenesis, stimulating at low concentrations and inhibiting at higher concentrations. At all concentrations tested, calmidazolium inhibited the ability of AII to increase aldosterone synthesis.

Figure 1B illustrates experiments with three other putative calmodulin inhibitors, trifluoperazine, W-7, and fluphenazine [18, 20–22]. All three inhibited AII-stimulated aldosterone synthesis. Unlike calmidazolium, these agents alone did not stimulate basal steroidogenesis (data not shown), although a biphasic effect on AII stimulation is suggested by the results with W-7.

Figure 2 depicts an experiment in which all four calmodulin antagonists were tested for their ability to inhibit the late pathway in steroidogenesis, the conversion of progesterone to aldosterone. Cells were incubated with progesterone in the presence of trilostane to prevent the conversion of endogenous precursors to progesterone. Each bar in Fig. 2 shows the increase in aldosterone synthesis caused by addition of AII or progesterone in the presence or absence of antagonist, as indicated. All four calmodulin antagonists strongly inhibited AII-stimulated aldosterone synthesis (panel A). At concentrations which severely inhibited AII-stimulated aldosterone synthesis from endogenous cholesterol, fluphenazine, W-7, and trifluoperazine had much less effect on the increase in aldosterone synthesis caused by the addition of progesterone (panel B). Although these three agents did affect conversion of progesterone to aldosterone to some extent, they were less potent in inhibiting the late path in aldosterone synthesis than in inhibiting AII-stimulated aldosterone synthesis from endogenous cholesterol. In fact, a slight,

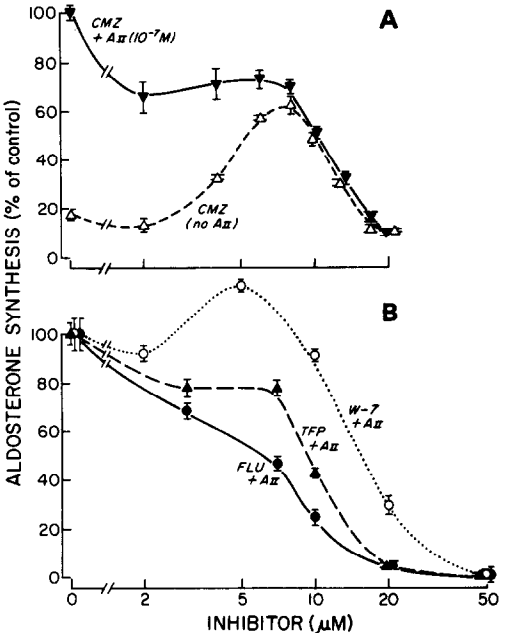

Fig. 1. Effect of calmodulin antagonist on aldosterone synthesis. Each point is the mean ± SEM of five determinations. (A) Effect of calmidazolium. Bovine adrenal glomerulosa cells were incubated as described in the text in the absence or presence of AII (10^{-7} M) for 2 hr, and the aldosterone in the supernatant was measured by radioimmunoassay. Control aldosterone synthesis (with AII, no inhibitor) was 7.2 ng/million cells/hr. (B) Effects of trifluoperazine, W-7, and fluphenazine on AII-stimulated aldosterone synthesis. Each inhibitor was tested in a separate experiment. Glomerulosa cells were incubated for 2 hr in the presence of AII (10^{-7} M) with the indicated concentrations of inhibitor. Aldosterone in the supernatant was measured by radioimmunoassay. Control aldosterone synthesis (with AII, no inhibitor) was 20.0, 13.4, and 13.1 ng/million cells/hr, respectively, for the trifluoperazine, W-7, and fluphenazine experiments.

but not always significant increase in the late pathway usually occurred with low concentrations of fluphenazine, W-7, or trifluoperazine. In contrast, calmidazolium inhibited AII-stimulated aldosterone synthesis and the late path with nearly equal potency (note upper and lower panels).

We measured the effects of the four calmodulin reagents on the binding of [^{125}I]AII to adrenal cells. Only calmidazolium substantially inhibited binding of labeled hormone to cells (Fig. 3), but that effect required higher concentrations than were needed to block aldosterone synthesis. It did not interfere with binding of [^{125}I]ANP to the same cells (data not shown).

Discussion

These results show that calmidazolium and three other calmodulin antagonists inhibit AII-stimulated aldosterone synthesis. Several other reports have described inhibition of steroidogenesis by this class of drugs [14–17]. However, calmidazolium displayed some unexpected effects as well. At low concentrations, the drug stimulated basal aldosterone synthesis. The other calmodulin antagonists did not. Calmodulin-dependent protein phosphatases [23], as well as protein kinases, have been described. If phosphoproteins play a key role in steroidogenesis, it is possible that a calmodulin antagonist would exhibit complex effects, either

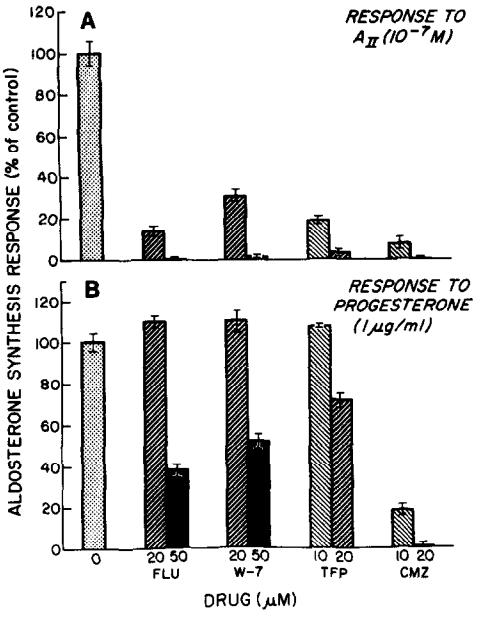

Fig. 2. Effects of calmodulin antagonists on AII-stimulated aldosterone synthesis from endogenous cholesterol (panel A) and on aldosterone synthesis from added progesterone (panel B). Cells were incubated for 2 hr in the presence of AII or in the presence of trilostane (10 μM) plus progesterone (1 μg/mL). Aldosterone was measured by radioimmunoassay of the supernatant. Control aldosterone response was 15.0 ng/million cells/hr in panel A and 20.5 ng/million cells/hour in panel B. Each point is the mean ± SEM of five determinations.

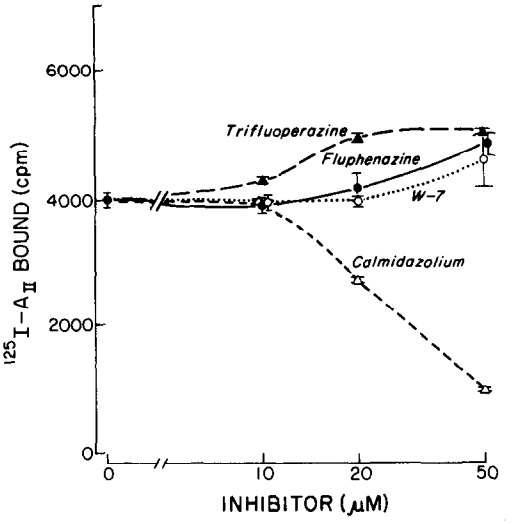

Fig. 3. Effects of calmodulin antagonists on binding of [^{125}I]AII to adrenal glomerulosa cells. Cells were incubated with the indicated concentrations of antagonist and [^{125}I]AII. Binding was measured as described [14]. Each point is the mean ± SEM of three determinations. Another experiment with calmidazolium provided similar results.

negative or positive, depending on the relative activities of the relevant calmodulin-dependent kinase(s) and phosphatase(s). Another clue related to the ability of calmodulin antagonists to increase basal aldosterone synthesis comes from the work of Ohnishi *et al.* [12]. They showed that calmodulin inhibited the aldosterone-synthesizing activity of purified P-450$_{11\beta}$. Thus, a calmodulin inhibitor might be stimulatory. We do not have any direct evidence to show that either of these mechanisms would account for our observations, or the usefulness of such a system. We also do not know why only calmidazolium stimulated basal aldosterone synthesis.

The rate-limiting steps in steroidogenesis, particularly those stimulated by acute exposure of adrenal cells to AII, involve the transport of cholesterol to the inside of mitochondria and its conversion to pregnenolone. It is this sequence that has been reported to be affected by calmodulin inhibitors such as pimozide [16]. However, we found that calmidazolium also inhibited the late path in steroidogenesis, the conversion of progesterone to aldosterone. At somewhat higher concentrations than are required to inhibit steroidogenesis, calmidazolium also inhibited the binding of [^{125}I]AII to adrenal glomerulosa cells. None of the three other calmodulin antagonists affected the late path or hormone binding at concentrations which inhibited AII-stimulated aldosterone synthesis, although they substantially inhibited progesterone conversion to aldosterone when used at higher concentrations. Wilson *et al.* [16] studied the effects of various calmodulin antagonists, including W-5, W-7, and pimozide, on aldosterone synthesis. Our results with W-7 generally agree with their finding that W-7 inhibits AII-stimulated aldosterone synthesis with greater potency than it inhibits conversion of progesterone to aldosterone. The calmidazolium results could be taken as evidence that calmodulin plays a role in AII binding to its receptors and in the late path as well as the early path. However, this seems unlikely since the other calmodulin inhibitors did not block these steps.

These results suggest that caution be used when drawing conclusions about the role of calcium/calmodulin-dependent processes based on studies of individual calmodulin inhibitors. A truly specific calmodulin antagonist, one which only inhibits calmodulin-dependent processes, might be expected to exhibit a narrower spectrum of action than our results with calmidazolium indicate. Conclusive proof of specific inhibition by a calmodulin antagonist would require detailed studies of particular processes, such as *in vitro* phosphorylation with added calmodulin and antagonists.

Our findings with calmodulin antagonists can be contrasted with the use of protein synthesis inhibitors such as cycloheximide. Cycloheximide blocked AII-stimulated aldosterone synthesis and pregnenolone synthesis, but did not block binding of AII to its receptors, the effects of AII on phosphoinositide turnover or calcium fluxes, progesterone conversion to aldosterone, or conversion of 20α-hydroxycholesterol to aldosterone [24]. Studies with many different protein synthesis inhibitors all pointed to a crucial role for a labile protein in hormone stimulation of the early path of steroidogenesis, and new evidence for such a protein is now accruing [25, 26]. Development of calmodulin antagonists which exhibit a narrower range of action than calmidazolium will be needed to obtain a better definition of calmodulin-dependent processes in steroid-producing and other tissues.

This study of calmodulin antagonists can be summarized as follows: (1) calmidazolium, trifluoperazine, W-7, and fluphenazine inhibited AII-stimulated aldosterone synthesis; (2) calmidazolium inhibited the late pathway in aldosterone synthesis (conversion of progesterone to aldosterone) with a potency similar to that for inhibition of AII-stimulated aldosterone synthesis; (3) the other

agents inhibited AII-stimulated aldosterone synthesis at concentrations that caused little inhibition of the late pathway; (4) of the four agents tested, only calmidazolium stimulated basal aldosterone synthesis; and (5) only calmidazolium inhibited binding of [^{125}]AII to adrenal cells. These results suggest that calmidazolium should be used with caution because it exhibits a broad range of effects, some of which may be unrelated to calmodulin inhibition. However, taken together, the results we obtained with four different antagonists confirm that calmodulin is critically involved in stimulation by AII of the early pathway of aldosterone synthesis.

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