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Comparative Effects of a Highly Specific Protein Kinase C Inhibitor, Calphostin C and Calmodulin Inhibitors on Angiotensin-stimulated Aldosterone Secretion

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We have examined the relative roles of the calcium–calmodulin system and protein kinase C in angiotensin-mediated aldosterone secretion. We used a highly specific protein kinase C inhibitor, calphostin C and two well-accepted calmodulin inhibitors, W-7 and calmidazolium. Although both types of inhibitors affected angiotensin-induced aldosterone secretion, as judged by the inhibitory doses of these compounds, angiotensin-evoked aldosterone secretion was more sensitive to calmodulin inhibition than protein kinase C inhibition. Manipulation of intracellular calcium by dantrolene and thapsigargin also modified aldosterone secretion significantly.

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INTRODUCTION

Calcium (Ca^{2+}) is now considered as a second messenger in cellular signaling mechanisms in various cells [1–3]. It has been demonstrated that in the adrenal glomerulosa cells, angiotensin II (Ang II) evokes aldosterone secretion with concomitant increases of Ca^{2+} influx through voltage-dependent Ca^{2+} channels as well as mobilization of intracellular Ca^{2+} presumably from an intracellular organelle by way of the phosphoinositide-derived metabolite, inositol 1,4,5-trisphosphate resulting in an increase in cytosolic Ca^{2+} concentration [4–10]. Ca^{2+} in concert with calmodulin then can activate Ca^{2+}-calmodulin dependent protein kinase(s). However, it has been postulated [4, 11] that in addition to changes in cell Ca^{2+}, activation of protein kinase C (PKC) through another phosphoinositide-derived metabolite, diacylglycerol, also participates in the cellular signaling mechanism responsible for aldosterone secretion from the glomerulosa cells. In this conceptual framework it has been suggested that the initial stimulation of aldosterone secretion is through the increase in cell Ca^{2+} and this is followed by the activation of PKC by diacylglycerol, which sustains the aldosterone-stimulatory effect of Ang II subsequently.

A number of protein kinase C inhibitors have become available recently and this permits investigation of the role of PKC. In this study we have investigated the effect of a highly specific and permeable protein kinase C inhibitor, calphostin C [12, 13], on angiotensin-mediated aldosterone secretion and compared its effect with those of two well-accepted calmodulin inhibitors. We have also examined the effects of two permeable diacylglycerol analogues on aldosterone secretion. Additionally, we investigated the effects of manipulation of intracellular Ca^{2+} stores on aldosterone secretion by agents known to affect such stores.

MATERIALS AND METHODS

Tritiated (1,2,6,7-^{3}H)-aldosterone was obtained from Amersham Co., Arlington Heights, IL, U.S.A. Collagenase was obtained from Worthington Biochemical Co., Freehold, NJ. The diacylglycerol analogues, 1-oleoyl-2-acetylglycerol (OAG) or 1,2-dioctanoylglycerol (DOG) were purchased from Molecular Probes, Eugene, OR. Calphostin C was purchased from Komiya Biomedical Co., Thousands Oaks, CA; calmidazolium and *N*-(6-aminohexyl-5)-chloro-napthalene sulfonamide (W-7) were obtained from Sigma Chemical Co., St Louis, MO and BAY

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K8644 was obtained from Calbiochem Co., La Jolla, CA.

Calf adrenal glomerulosa cells were used for the study. The cells were prepared from microtome-sliced outer layer of the adrenal cortex by collagenase dispersion as described before [14, 15]. This is a widely used method. The viability of the cells was determined by trypan blue exclusion and cells were counted on a hemocytometer. Incubations of the glomerulosa cells were carried out in medium 199 in triplicate in a shaking bath in the presence of 95% O_2 and 5% CO_2 at 37°C for the requisite periods. Each experiment was usually repeated at least once. The results of a representative experiment are shown in the figures. Aldosterone secreted into the medium during the incubation was measured by an assay using a highly specific monoclonal antibody [16].

For experiments involving PKC inhibitor and calmodulin inhibitors, the glomerulosa cells were incubated in the presence or absence of Ang II (10 nM) for 2 h in one set of experiments with either the PKC inhibitor, calphostin C (0.01, 0.05, 0.1, 0.5, 1.0 μM), or calmodulin inhibitor, calmidazolium (1, 10, 30, 50 μM) and in a different set of experiments, the cells were incubated for the same duration in the presence of Ang II (10 nM) with calphostin C in the same doses or calmodulin inhibitor, W-7 (1, 10, 30, 50 μM). Since calphostin C is activated by light [13], the incubation was carried out under a lamp about 9″ above the rack of the tubes in the shaking bath. For experiments involving PKC agonist, the cells were incubated with OAG or DOG alone in doses of 30 or 60 μM or BAY K8644, Ca^{2+} channel agonist alone in a dose of 30 nM or BAY K8644 and OAG or DOG. In some experiments, Ang II (10 nM) was used along with these agents.

For experiments involving Ca^{2+} stores, four different sets of incubation conditions were used: (1) and (2) dantrolene (DL), 10 or 30 μM, or thapsigargin (TG), 300 nM or 3 μM, with or without Ang II (10 nM) were added to the cells at the same time and incubation continued for 2 h; (3) Ang II (10 nM) was added initially, then 30 min later DL or TG was added and the incubation continued for a total of 2 h; (4) DL or TG was added to the cells initially, Ang II was added after 30 min and the incubation proceeded for another 2 h after the addition of Ang II.

RESULTS

Effects of protein kinase C and calmodulin inhibitors

The calmodulin inhibitors, W-7 and calmidazolium, on one hand and protein kinase C inhibitor, calphostin C, inhibited aldosterone in different degrees (Figs 1–4). At the highest doses, both types of inhibitors reduced aldosterone secretion by 85–95%.

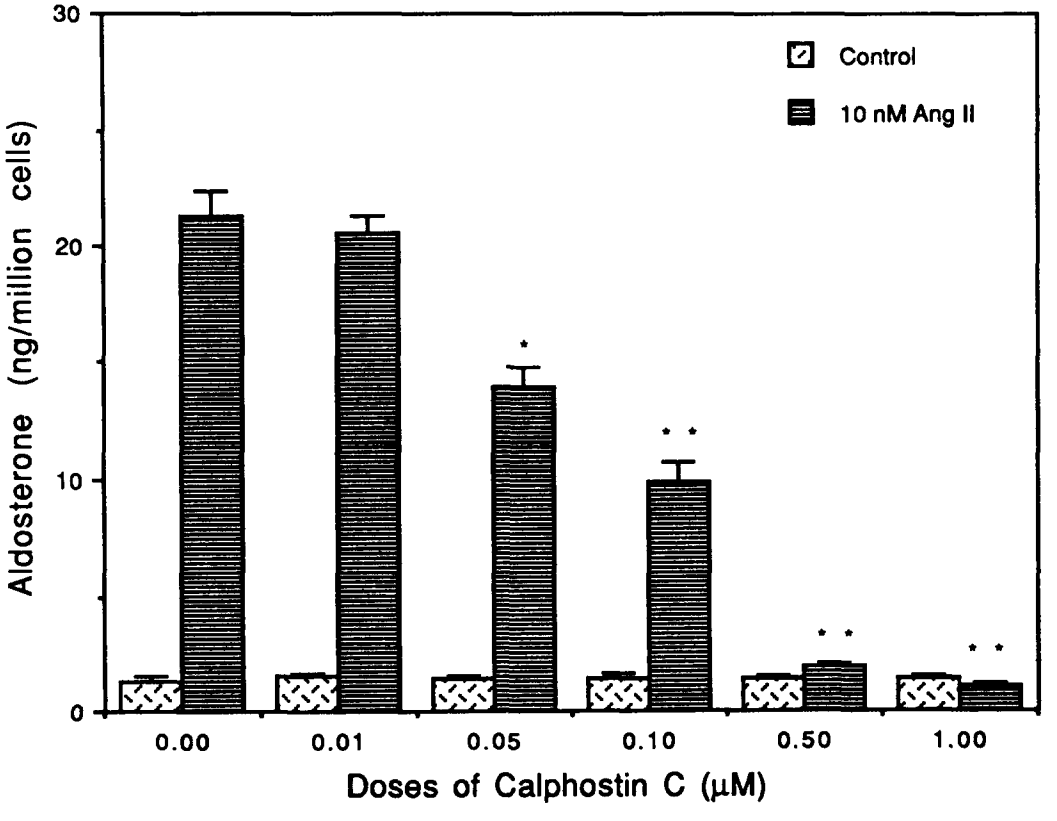

Fig. 1. Effects of calphostin C (in μM) on aldosterone secretion (in ng/million cells) in one set of experiments. Asterisks indicate statistical significance: $*P < 0.05$, $**P < 0.01$ for all figures. Comparisons involve control cells vs cells treated with the respective pharmacological agents ($n = 3$).

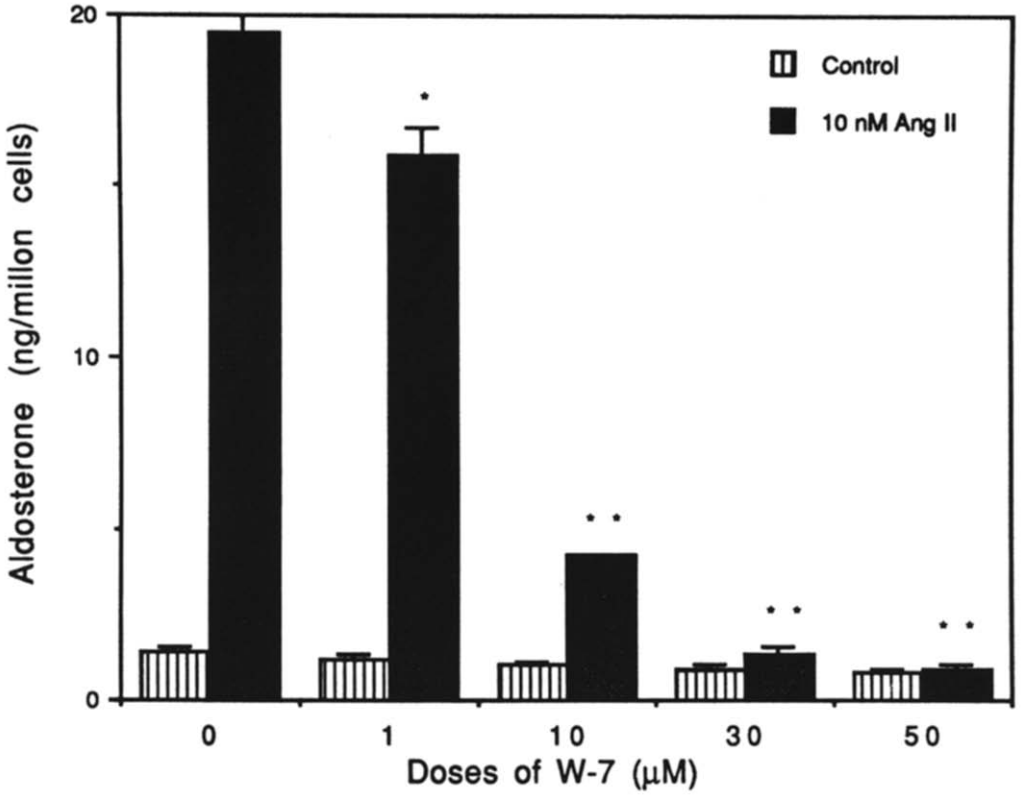

Fig. 2. Comparative effects of W-7 on aldosterone secretion corresponding to the experiment shown in Fig. 1.

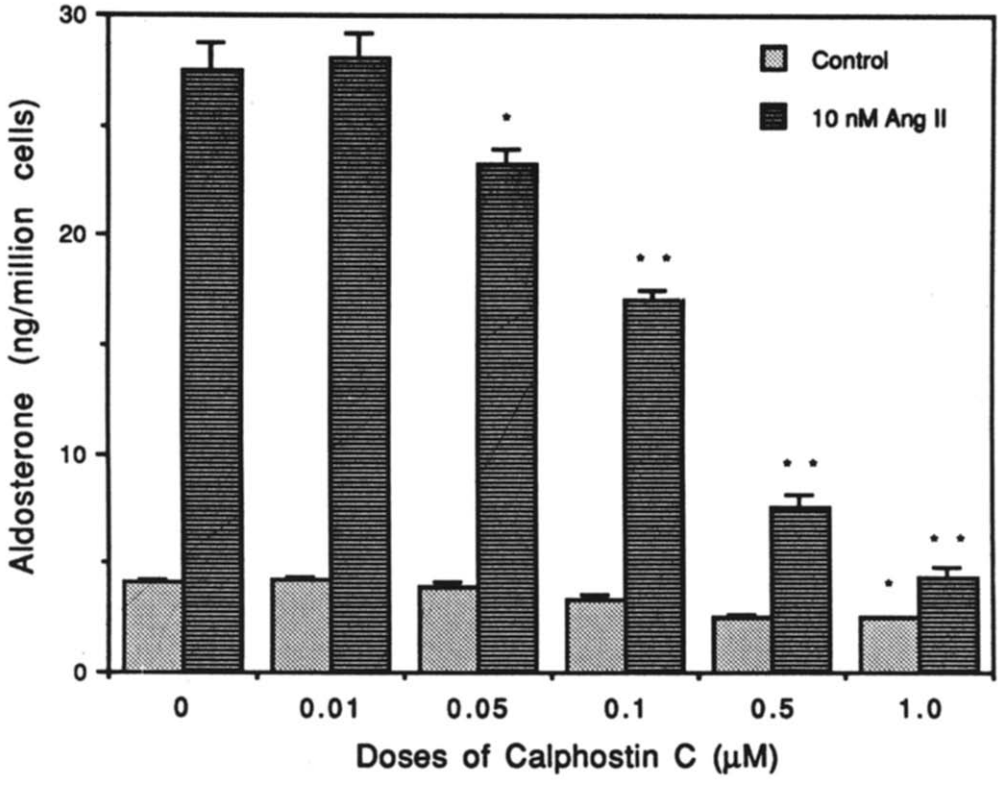

Fig. 3. Effects of calphostin C on aldosterone secretion in another experiment corresponding to the experiment shown in Fig. 4.

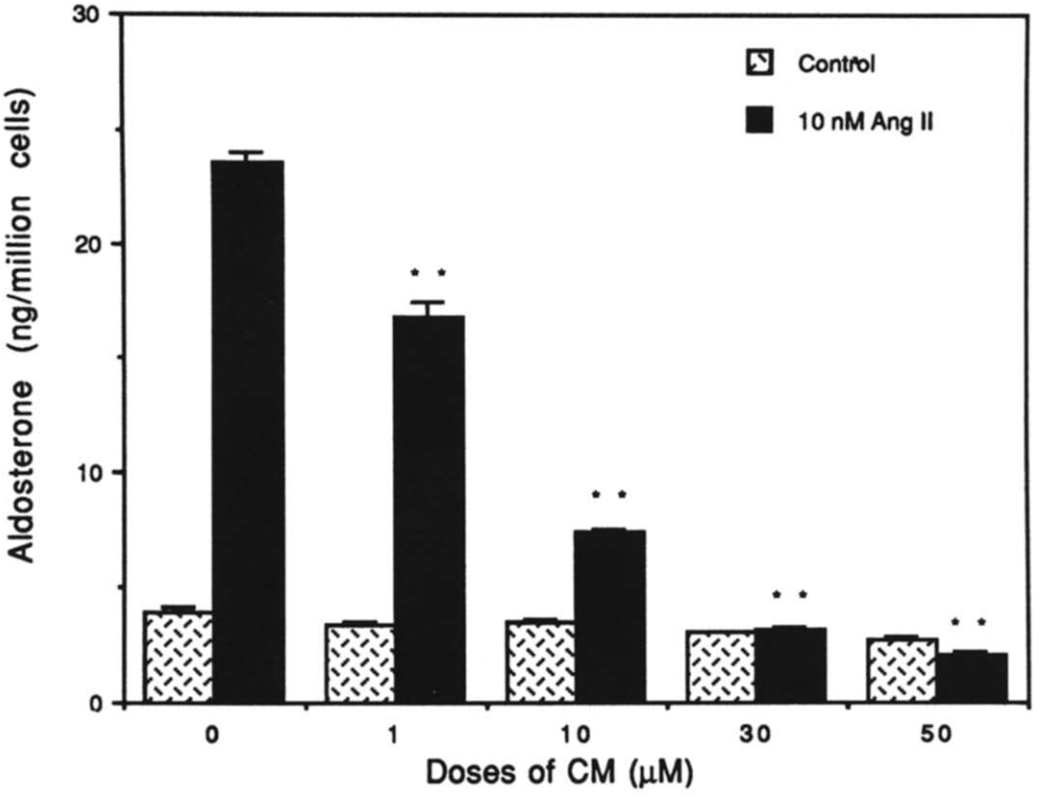

Fig. 4. Comparative effects of calmidazolium on aldosterone secretion corresponding to the experiment shown in Fig. 3.

The ED_{50} for the calmodulin inhibitors for inhibition of aldosterone secretion ranged from 2.4 to 3.1 μM, while the ED_{50} for calphostin C for inhibition of aldosterone secretion was 0.085–0.2 μM. When these ED_{50} values for calmodulin inhibitors were compared with their reported ED_{50} for inhibition of calmodulin or calmodulin-dependent processes (20–30 μM) it was evident that aldosterone secretion was much more sensitive to calmodulin inhibition than inhibition of protein kinase C. The known ED_{50} for inhibition of PKC calphostin C is 0.05 μM while that for inhibition of aldosterone secretion was higher.

Effects of protein kinase C agonists

Protein kinase C agonists, OAG or DOG in doses of 30 or 60 μM with or without BAY K8644 failed to increase aldosterone secretion (Figs 5 and 6). In addition, OAG and DOG tended to inhibit Ang II-induced aldosterone secretion. Similarly, OAG and DOG in doses of 5 or 10 μM produced no appreciable effect on aldosterone secretion (not shown).

Effects of manipulation of Ca^{2+} stores

Both dantrolene (DL) and thapsigargin (TG) affected aldosterone secretion. Basal aldosterone secretion was unaffected by dantrolene, but 30 μM of dantrolene inhibited Ang II-mediated aldosterone secretion (Fig. 7). In contrast, thapsigargin, an inhibitor of Ca^{2+}-ATPase at both doses of 300 nM and 3 μM increased basal aldosterone secretion by 220 and 354%, respectively (Figs 7 and 8), presumably by releasing Ca^{2+} from subcellular organelles and increasing Ca^{2+} influx. Ang II stimulated aldosterone secretion did not differ significantly whether thapsigargin was present or not. However, when one considers the fact that thapsigargin by itself is able to increase aldosterone secretion significantly and if this basal aldosterone production by thapsigargin is corrected, then it appears Ang II-induced aldosterone secretion would actually be lower than that induced by Ang II in the absence of thapsigargin. Thus, it appears that the manipulation of the intracellular calcium pools can affect the magnitude of aldosterone secretion evoked by Ang II.

DISCUSSION

It has been proposed that PKC plays a major role in aldosterone secretion along with the Ca^{2+}–calmodulin system [4, 11]. However, the Ca^{2+}–calmodulin system may be the more influential intracellular messenger system for Ang II-mediated aldosterone secretion than PKC. In studies by one group of investigators in rat glomerulosa cells, in response to Ang II, PKC was transiently translocated from the cytosol to the membrane and aldosterone secretion was concomitantly stimulated, but aldosterone secretion appeared to be unaffected under conditions in which PKC was down-regulated by phorbol ester in the glomerulosa cells

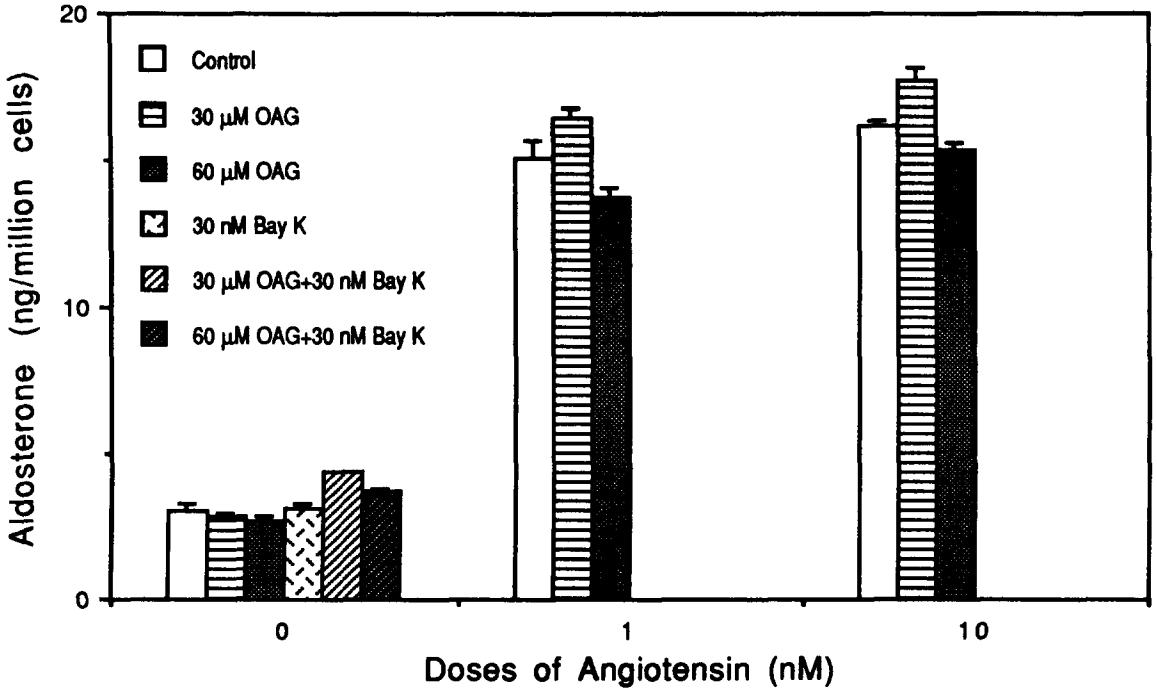

Fig. 5. Effects of OAG and/or BAY K8644 on aldosterone secretion.

[17]. Other investigators (unpublished observations) including ourselves have failed to show an appreciable aldosterone-stimulating effect of PKC agonist, phorbol ester, during the static incubation of acutely dispersed glomerulosa cells. If PKC were involved in aldosterone secretion then the potent PKC agonist should have stimulated aldosterone secretion consistently in acute experiments as seen in some other cells in which a cell signaling role has been assigned to PKC. Thus, the putative role of PKC in cell signaling mechanism for aldosterone secretion has been questioned by some.

Previously, we have demonstrated [18] that calmodulin inhibitors produced a greater inhibition of aldosterone secretion than H-7, a PKC inhibitor, and that alteration of extracellular Ca^{2+} or Ca^{2+} influx was more influential than PKC inhibition in affecting even the

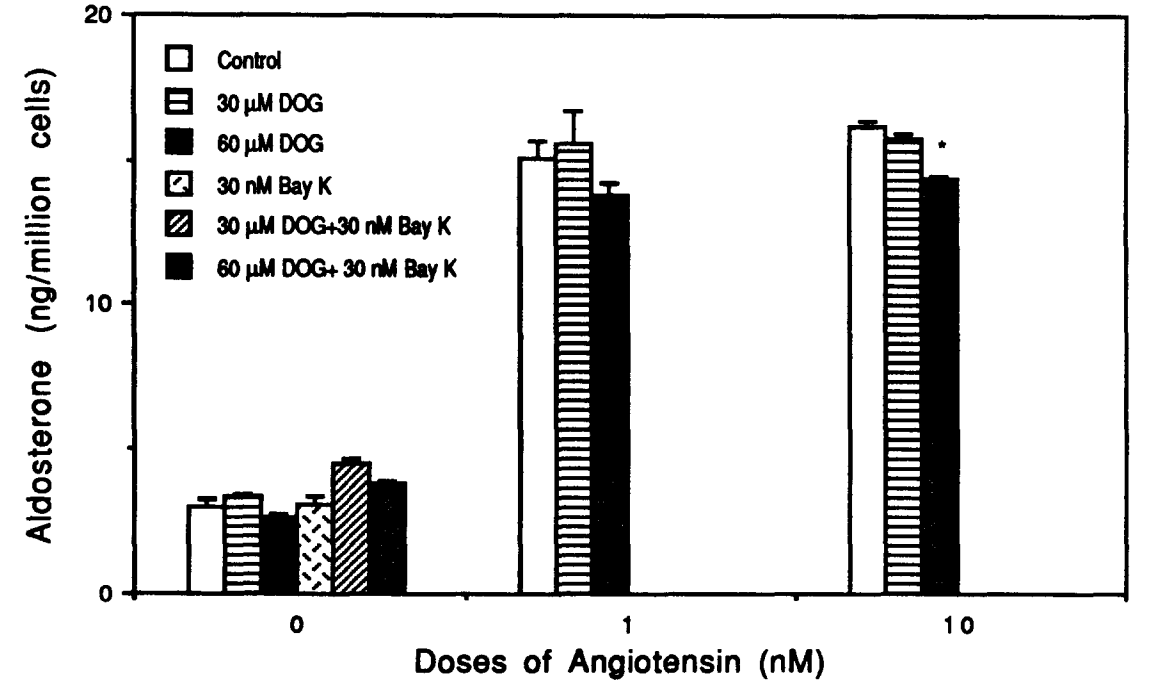

Fig. 6. Effects of DOG and/or BAY K8644 on aldosterone secretion.

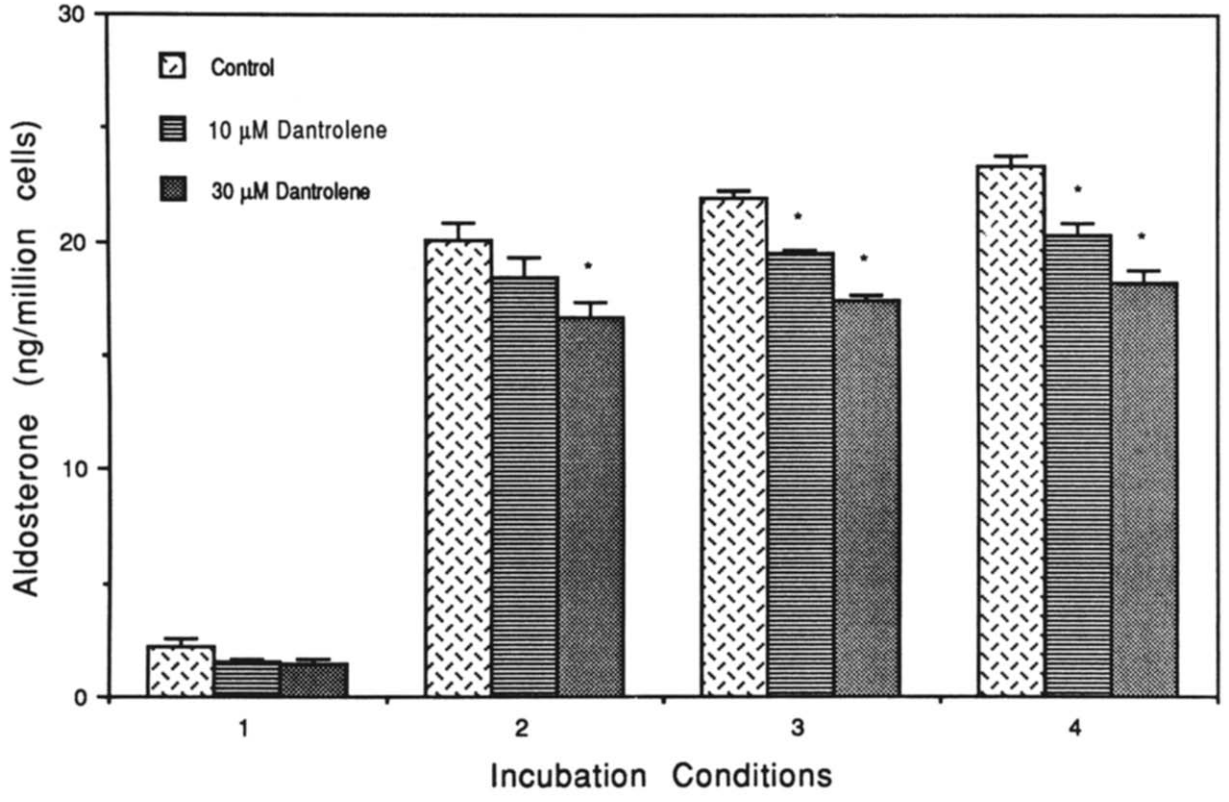

Fig. 7. Effects of dantrolene under different conditions (see text) on aldosterone secretion.

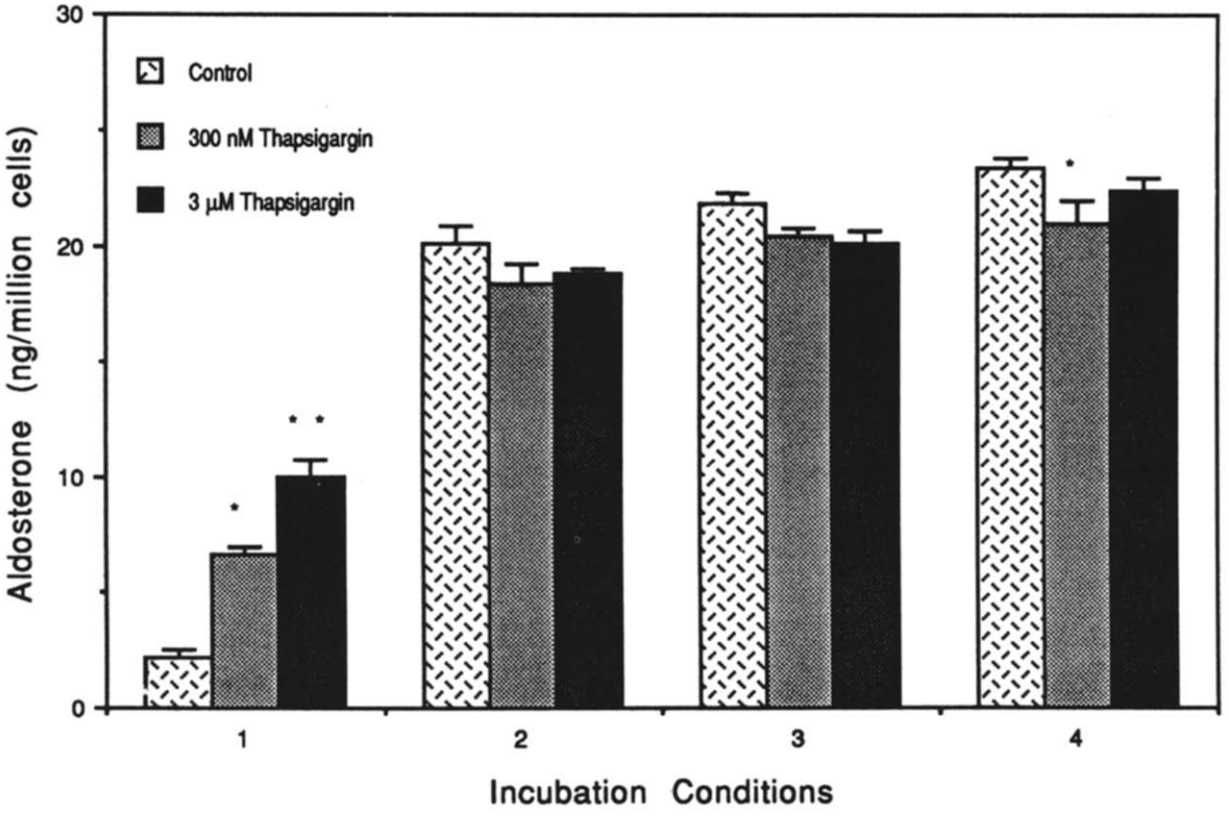

Fig. 8. Effects of thapsigargin under different conditions (see text) on aldosterone secretion.

sustained phase of aldosterone secretion, supporting the tenet that aldosterone secretion mediated by Ang II was more dependent on calmodulin and Ca^{2+} than perhaps PKC. However, H-7 is a less specific PKC inhibitor than some others, despite its credentials with regard to cell permeability and potential abilities for even *in vivo* effects [19]. Therefore, in this study we have examined the effect of another PKC inhibitor, calphostin C [12, 13], which appears to be a highly selective and permeable PKC inhibitor, and compared its effect with those of calmodulin inhibitors, calmidazolium and W-7, on Ang II-mediated aldosterone secretion. We observed again that aldosterone secretion was much more sensitive to calmodulin inhibition than to PKC inhibition although calphostin C did also inhibit aldosterone secretion to a significant degree assuming that this inhibition was related solely to PKC inhibition. But the ED_{50} of inhibition of aldosterone secretion for the two groups of inhibitors were strikingly different from their respective inhibitory potencies of PKC and calmodulin, respectively. Since calphostin C is more specific as a PKC inhibitor, we conclude PKC may have some permissive role in aldosterone secretion but calmodulin may exert a more dominant effect. In our studies using OAG or DOG with or without a Ca^{2+} channel agonist we were unable to show any stimulation of aldosterone secretion and at higher concentrations of OAG or DOG there was actually a tendency to inhibition of Ang II-mediated aldosterone secretion. Taking together all of the available data one could argue that perhaps some PKC isoenzyme(s) as they are now known to exist [20], unresponsive to phorbol ester or diacylglycerol analogues, could be involved, although the original concept of a PKC role proposed in aldosterone secretion was based on the effect of phorbol ester [4, 11]. The original study was also conducted using perifused adrenal cells rather than static incubation used by most investigators which could be a confounding variable in that prolonged contact with phorbol ester might desensitize or downregulate PKC activity. In this regard then the question also remains whether diacylglycerol which is continuously produced in the adrenal as a result of persistent hydrolysis of phosphoinositides during Ang II stimulation [21, 22], may also desensitize the PKC in the adrenal cells and render the PKC activity irrelevant. In the presence of low extracellular Ca^{2+} concentration, the initial phase of angiotensin-mediated aldosterone secretion is unimpaired, but both phosphoinositide hydrolysis and aldosterone secretion decline in the face of significant and continued production of diacylglycerol [21, 22]. This seems to suggest that diacyglycerol production alone in the absence of Ca^{2+} influx is incapable of sustaining aldosterone secretion. These and other observations raise more questions about the precise role of PKC. These questions are pertinent and require satisfactory answers. Ang II, however, has been reported to cause redistribution of PKC in adrenal glomerulosa cells [17, 23], an event associated with PKC activation. In rat adrenal glomerulosa cells, in one study [24], Ang II failed to cause redistribution of PKC in Ca^{2+}-free condition but did so in the presence of Ca^{2+}. The increase in cell Ca^{2+} alone clearly is not responsible for the PKC redistribution since potassium stimulation of the cell does not result in the translocation of PKC [23]. It is conceivable then that PKC may serve in some form of supportive function in cell survival and renewal rather than in signaling for steroid secretion and inhibition of PKC activity may modify aldosterone secretion only secondarily. This may also be consistent with a well-recognized role of PKC in cell growth.

Our data on the effect of dantrolene and thapsigargin influencing adrenal cell Ca^{2+} stores differently on aldosterone secretion are of interest. Dantrolene, an inhibitor of the inositol trisphosphate-sensitive pool of Ca^{2+} did inhibit aldosterone secretion at the high dose without affecting basal aldosterone secretion as expected and as shown by others before [4, 9, 25, 26]. But thapsigargin actually increased basal aldosterone secretion as reported earlier also [27]. This occurred predominantly because of increase in cytosolic Ca^{2+} concentration as demonstrated recently [28], resulting presumably from the inhibition of Ca^{2+}-pumps in the membranes of subcellular organelles [29] and/or Ca^{2+} influx [30]. Ang II-induced aldosterone secretion, however, was no greater with thapsigargin than without it at the dose used. Indeed, if one corrects for the basal increase of aldosterone secretion by thapsigargin alone then Ang II-mediated aldosterone secretion was actually blunted and this may be a reflection of depleted inositol trisphosphate-sensitive Ca^{2+} pool. However, at lower doses of Ang II, its effect on aldosterone secretion can be potentiated by thapsigargin [27]. These observations are consistent with the fact that an increase in cytosolic Ca^{2+} in the glomerulosa cell is a stimulus for aldosterone secretion and that depleted Ca^{2+} stores in, presumably, the inositol trisphosphate-sensitive pool or inhibition of mobilization from such a pool results in the attenuation of aldosterone secretion produced by Ang II. Thus, we have been able to extend our studies underscoring again the relative importance of Ca^{2+}-calmodulin branch of the intracellular signaling mechanism for aldosterone secretion.

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