Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 254-261

# GREATER IMPORTANCE OF $CA^{2+}$-CALMODULIN IN MAINTENANCE OF ANG II-AND $K^+$-MEDIATED ALDOSTERONE SECRETION: LESSER ROLE OF PROTEIN KINASE C

Arunabha Ganguly[1]*, Shirley Chiou[1], Naomi S. Fineberg[2] and John S. Davis[3]

[1]Department of Medicine, University of South Florida College of Medicine, James A. Haley Veterans Hospital, Tampa, Florida 33612

[2] Indiana University School of Medicine, Indianapolis, Indiana

[3] University of Kansas Medical Center, Wichita, Kansas

Received November 4, 1991

**Summary**: In this study we have investigated various components of the stimulus-secretion coupling process leading to aldosterone secretion from the calf adrenal glomerulosa cells as evoked by angiotensin II (AII) and potassium ($K^+$). The roles of $Ca^{2+}$, calmodulin and protein kinase C in the sustained phase rather than initiation of aldosterone secretion were of special interest. Our investigations revealed that the reduction of extracellular $Ca^{2+}$ by EGTA or interruption of $Ca^{2+}$ influx by nitrendipine at various time points after stimulation with either AII or $K^+$ markedly compromised aldosterone secretion. Calmodulin inhibitors, calmidazolium and W-7 reduced aldosterone secretion profoundly. Agonists of protein kinase C, phorbol ester or diacylglycerol analogues failed to stimulate aldosterone secretion while the protein kinase C inhibitor, H-7, only partially inhibited aldosterone secretion at a concentration which completely inhibited protein kinase C activity. Calmodulin inhibitors produced significantly greater inhibition of aldosterone secretion than inhibitors of protein kinase C. 

Transient elevations in intracellular calcium ($Ca^{2+}$) are believed to play a role in both potassium- and angiotensin-mediated aldosterone secretion (1,2). Some of the concomitant events associated with aldosterone secretion evoked by angiotensin and potassium are shared, but others are disparate. Both induce a rise in cytosolic free $Ca^{2+}$ concentration in adrenal glomerulosa cells but by different mechanisms. Angiotensin (3-6) but not potassium (7) has been shown to cause hydrolysis of phosphoinositides in adrenal cells liberating at least two putative intracellular mediators -- inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol. Adrenal glomerulosa cells contain receptors for $IP_3$ (8) and $IP_3$ has been demonstrated to release $Ca^{2+}$ from intracellular stores (9) and diacylglycerol is known to stimulate protein kinase C (PKC) activity. Rasmussen and co-workers (10) have postulated that angiotensin-mediated aldosterone secretion involves an initial phase related to a rapid rise in intracellular $Ca^{2+}$ followed by a subsequent sustained phase mediated by the interaction of diacylglycerol with PKC. The rise in intracellular $Ca^{2+}$ in adrenal glomerulosa cells induced by angiotensin occurs as a result of mobilization of intracellular $Ca^{2+}$ (10) as well as receptor-mediated $Ca^{2+}$ influx through plasma membrane $Ca^{2+}$ channels (11). Potassium, on the other hand, causes

* To whom correspondence should be addressed.

0006-291X/92 $1.50

depolarization of the adrenal cell and influx of $Ca^{2+}$ again through $Ca^{2+}$ channels (1,2). Angiotensin but not potassium has been reported to cause redistribution of PKC in the adrenal glomerulosa cells (12), a process associated with PKC activation. Both angiotensin and potassium may require calmodulin as well as PKC; yet the relative importance of calmodulin and PKC may be different for the two secretagogues since diacylglycerol, the putative activator of PKC, is generated by hydrolysis of phosphoinositides in response to stimulation by angiotensin but not by potassium. The relative importance of $Ca^{2+}$ for the sustained phase of aldosterone secretion evoked by angiotensin and potassium has not been systematically evaluated.

In this study we have investigated the role of extracellular $Ca^{2+}$ and $Ca^{2+}$ influx during the sustained phase of aldosterone secretion by manipulating extracellular $Ca^{2+}$ or $Ca^{2+}$ influx at various time points of incubation of adrenal glomerulosa cells after initiation of stimulation with either angiotensin or potassium. Additionally, we have examined the effects of agonists of PKC, phorbol ester and diacylglycerol analogues as well as inhibitors of calmodulin and PKC on aldosterone secretion. The results of these studies implicate $Ca^{2+}$-calmodulin system as the critical mediator of the sustained phase of aldosterone secretion.

## Materials and Methods

Tritiated (1,2,6,7-$^3$H)-aldosterone was obtained from Amersham Co., Arlington Heights, IL. Collagenase was obtained from Cooper Chemicals, Malvern, PA. 1-oleoyl-2-acetylglycerol (OAG) was purchased from Molecular Probes, Eugene, OR and 1,2-dioctanoylglycerol (DOG) was purchased from Life Science Resources, Milwaukee, WI. The protein kinase inhibitor, 1-(5-isoquimoline sulfonyl)-2-methylpiperazine (H-7 in the water-soluble dihydrochloride form) was purchased from Seikagaku America Inc., St. Petersburg, FL. All other chemicals including the calmodulin inhibitors, calmidazolium and N-(6-aminohexyl-5)-chloro-1-napthalene sulfonamide (W-7) were obtained from Sigma Chemicals Co., St. Louis, MO.

Calf adrenals were obtained from a local abattoir. Adrenal glomerulosa cells were prepared by dispersing thin (0.5 mm) slices of the outer adrenal cortex with collagenase (3.0-3.5 mg/ml) and DNAse (50 μg/ml) as described earlier (13). At the end of the dispersion the cells were filtered through nylon mesh and washed twice. The viability of the cells was determined by trypan blue exclusion and the cells counted on a hemocytometer. Cell viability was routinely 90-95%. All incubations were carried out in triplicate in a shaking bath at 37°C in an atmosphere of 95% $O_2$ and 5% $CO_2$. In experiments involving extracellular $Ca^{2+}$ manipulation, in one set of experiments 3.6 mM EGTA was added at 1 or 5 minutes after starting the incubation with AII ($10^{-8}$ M) or $K^+$ (9 mM) to give a ratio of EGTA to $Ca^{2+}$ of 2:1 . In other experiments, the EGTA concentration was kept at 1 mM while $Ca^{2+}$ concentration was varied from 0.25 mM to 2 mM. EGTA was added at 5 or 30 minutes after starting the cell incubation with AII ($10^{-8}$ M) or $K^+$ (9 mM). Cells incubated without EGTA or secretagogues served as unstimulated cells in each experiment. Incubations performed in the presence of AII or $K^+$ without EGTA served as controls for stimulated cells. The cells in each experiment were incubated for a total of 120 minutes. In experiments involving $Ca^{2+}$-channel blockade, nitrendipine was added at 1, 5 or 30 minutes after starting the incubation in the presence or absence of AII or $K^+$. The cells were allowed to incubate up to 2 hours. Incubations without nitrendipine served as controls.

To test the potential involvement of calcium mobilization and PKC activators on aldosterone secretion, adrenal glomerulosa cells were incubated for 2 hours with phorbol ester, 12-O-tetradecanoyl phorbol 13-acetate (TPA) 10 or 100 nM, $Ca^{2+}$-ionophore, A23187 10 or 50 nM, TPA 100 nM + A23187 50 nM, or diacylglycerol analogues, OAG or DOG 10 or 50 μM with or without AII ($10^{-8}$ M). In other experiments adrenal glomerulosa cells were incubated without (basal) or with AII ($10^{-8}$ M) or KCl (9 mM) for 2 hours in the presence or absence (control) of calmodulin inhibitors, calmidazolium or W-7 or PKC inhibitor, H-7 in the water insoluble form or water soluble form, in concentrations of ranging from 100 nM to 100 μM. Aldosterone in all experiments was measured by radioimmunoassay as reported earlier (5). The effect of increasing concentrations of W-7 and H-7 on PKC activity was measured in cytosol prepared from homogenized calf adrenal glomerulosa cells. The preparation of the cytosol and assays of the enzyme activity were as described before (14). Statistical analysis involved analysis of

variance and t tests using the means + sem of each group of data. In some cases 3-way analysis of variance has been used.

## Results

To examine the role for extracellular $Ca^{2+}$ in the sustained phase of aldosterone secretion, adrenal glomerulosa cells were stimulated by AII or $K^+$, and EGTA was then added to the incubation medium at various time points to reduce extracellular $Ca^{2+}$ concentration. In one set of experiments, 3.6 mM EGTA was added to the incubation medium ($Ca^{2+}$ concentration 1.8 mM) either at 1 or 5 minutes after initiation of stimulation of the adrenal cells by AII or $K^+$. Compared with controls (when no EGTA was added), the delayed reduction in extracellular $Ca^{2+}$ with EGTA markedly and comparably reduced AII- and $K^+$-mediated aldosterone secretion (91% and 90% inhibition for AII and 83% and 84% for $K^+$ at 1 or 5 mins respectively). In other experiments, in which the EGTA to $Ca^{2+}$ ratio was varied (from 0.5 to 2.0) EGTA 1mM was added after either 5 or 30 minutes of ongoing incubation of the adrenal cells in the presence or absence of AII or $K^+$. Incubations to which no EGTA was added , served as controls. Again, the degree of inhibition was dependent on the EGTA/$Ca^{2+}$ ratio (or to be specific, the ambient free $Ca^{2+}$ concentration of the medium) and the time point during the incubation when EGTA was added (Fig. 1). When the EGTA/$Ca^{2+}$ ratio was the greatest, the inhibition of the aldosterone secretion was the most profound. Similarly, earlier additions of EGTA caused the greatest inhibition of aldosterone secretion. There was very little difference with regard to the type of secretagogue.

To examine the involvement of $Ca^{2+}$ channels in the sustained phase of aldosterone secretion adrenal glomerulosa cells were stimulated with AII or $K^+$ and nitrendipine was then added at various time points. When a fixed concentration of nitrendipine (10 μM) was added to the incubation media after 1 or 5 minutes following initiation of stimulation with AII or $K^+$, marked inhibition of aldosterone secretion resulted (65% and 69% inhibition for AII and 70% and 76% for $K^+$ at 1 or 5 mins respectively). However, in experiments in which nitrendipine was added in varying concentrations after either 5 or 30 minutes of ongoing incubation, there was greater inhibition of aldosterone secretion when nitrendipine was added at 5 minutes

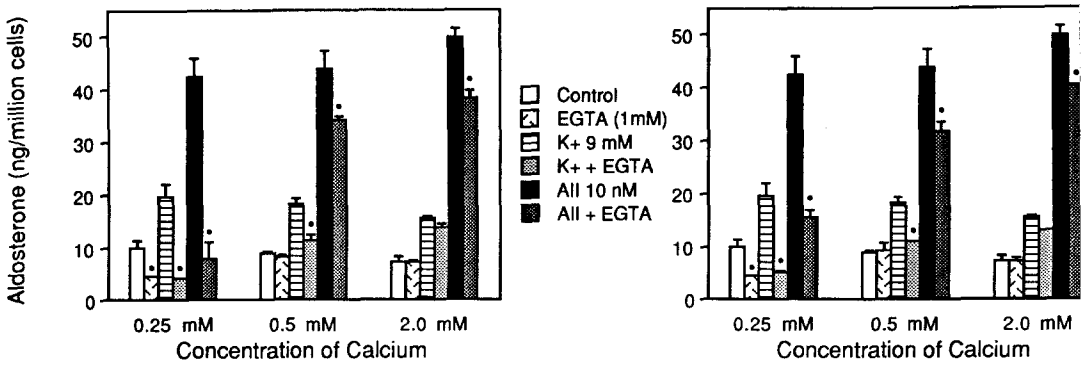


Fig. 1. Effects of delayed alteration of extracellular $Ca^{2+}$ on the AII- or $K^+$-induced aldosterone secretion in the presence of EGTA (1mM) added at 5 (left) or 30 (right) minutes of ongoing incubation. *$p < 0.05$ **$p < 0.01$.

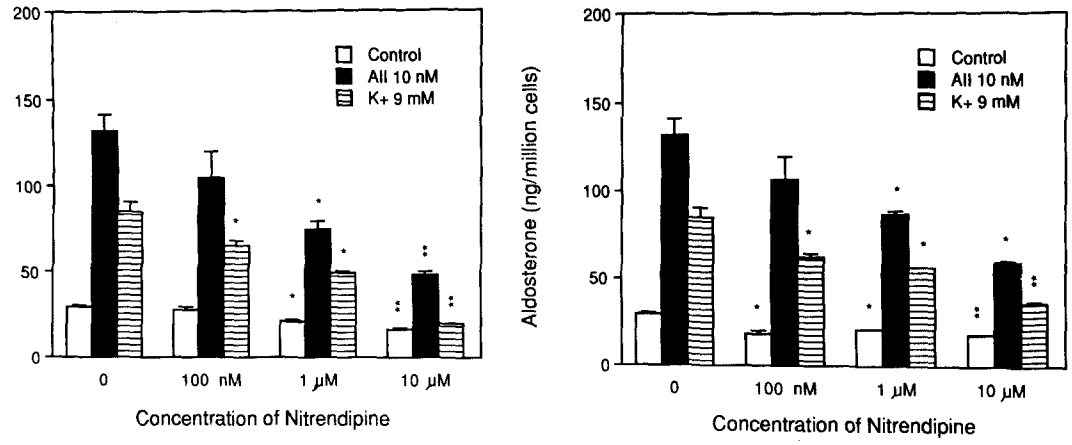


Fig. 2. Dose-responses of nitrendipine (NT) added at 5 (left) or 30 (right) minutes of ongoing incubation on aldosterone secretion over 2 hours in response to AII or $K^+$. *p < 0.05 **p < 0.01.

rather than 30 minutes of incubation (Fig. 2). Nitrendipine caused a concentration-dependent inhibition of aldosterone secretion at both time points.

To examine the potential role of protein kinase C activation, adrenal cells were incubated with phorbol ester, $Ca^{2+}$ ionophore and diacylglycerol analogues on aldosterone secretion. TPA did not stimulate aldosterone secretion (ng/million cells) appreciably (control 66.3±1 vs. TPA 10nM, 69±0.6 or 100nM, 69±1 or A 23187 10nM, 69±1 or 50nM, 70±3). Similarly, diacylglycerol analogues, OAG or DOG in doses of 10 or 50 μM or TPA 100 nM + A23187 50 nM (data not shown) failed to alter aldosterone secretion appreciably. TPA 100nM inhibited AII-stimulated aldosterone secretion significantly (AII 10nM, 131±3.7 vs. AII + TPA 100nM, 110±1).

To further evaluate the role of calcium or PKC in aldosterone secretion, adrenal cells were treated with calmodulin and protein kinase C inhibitors. Calmodulin inhibitors, W-7 (Fig. 3) and calmidazolium (Fig. 4) inhibited aldosterone secretion at doses of 1 μM or higher and at doses of 50 or 100 μM inhibited it by 90-95%. The PKC inhibitor, on the other hand,

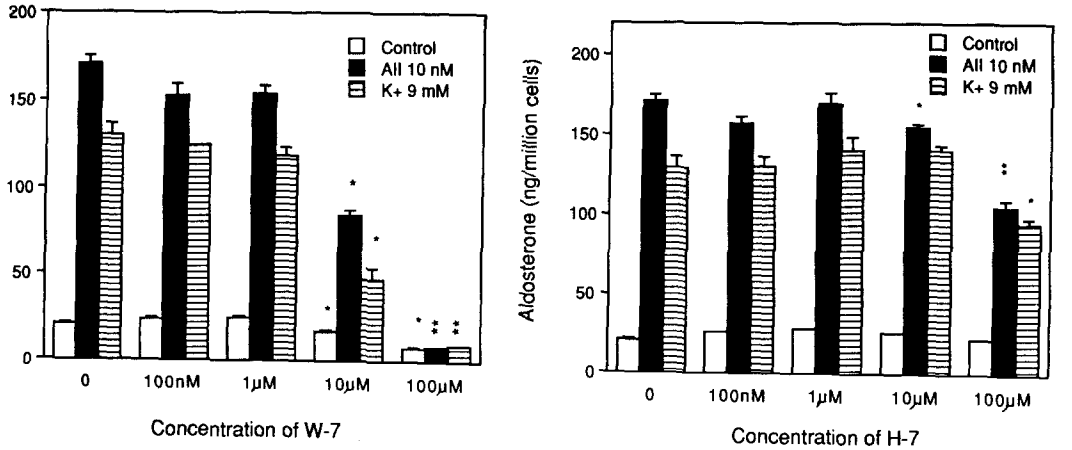


Fig. 3. Effects of increasing concentrations of W-7 and H-7, 1-(5-isoquinoline sulfonyl-2-methyl) piperazine on aldosterone secretion over 2 hours of incubation, in response to AII or $K^+$. *p < 0.05 **p < 0.01.

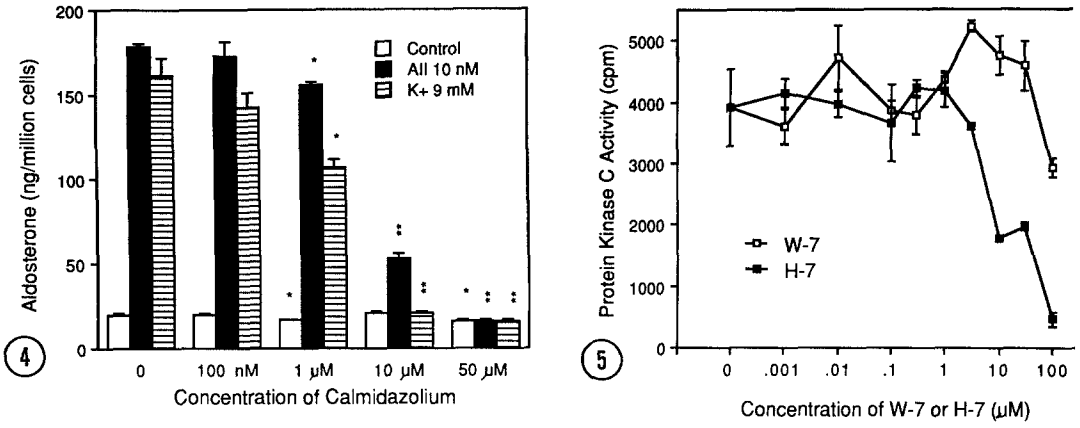


Fig. 4. Effects of increasing concentrations of calmidazolium on aldosterone secretion over 2 hours of incubation, evoked by AII or $K^+$. *p < 0.05 **p < 0.01.

Fig. 5. Effects of varying doses of W-7 and H-7, 1-(5-isoquinoline sulfonyl-2-methyl) piperazine dihydrochloride on protein kinase C activity in cell-free system.

reduced aldosterone secretion to a lesser extent (Fig. 3). W-7 produced significantly ($p<0.001$) greater inhibition of aldosterone secretion than that produced by H-7. Only at doses of 100 μM did H-7 reduce aldosterone secretion below 50%. The water soluble form (dihydrochloride) of H-7 was only slightly more effective than water insoluble form of H-7 solubilized in dimethyl sulfoxide (data not shown). Experiments were performed to examine the effect of W-7 and H-7 on PKC activity under cell-free conditions. H-7 inhibited cytosolic PKC activity in a dose-dependent manner above the dose of 1 μM, while W-7 inhibited PKC activity somewhat at the dose of 100 μM (Fig. 5).

**Discussion**

In our present studies, we have demonstrated that the removal of extracellular $Ca^{2+}$ after stimulation of adrenal glomerulosa cell results in decreased aldosterone secretion. Similarly, calcium channel blockade with nitrendipine after stimulation with angiotensin II or potassium was associated with concentration-dependent reductions in aldosterone secretion. These findings underscore the importance of both the continual presence of extracellular $Ca^{2+}$ and the influx of $Ca^{2+}$ for the maintenance of aldosterone secretion after initiation of stimulation with angiotensin or potassium.

Different temporal patterns reported of changes in cytosolic free $Ca^{2+}$ as induced by angiotensin and potassium (15,16), are consistent with the results of our experiments. Removal of extracellular $Ca^{2+}$ or $Ca^{2+}$-channel blockade by nitrendipine has been reported to result in a decrease in the magnitude of the initial rise in cytosolic free $Ca^{2+}$ in response to both potassium and angiotensin (15,16). The secondary sustained rise in cytosolic free $Ca^{2+}$ evoked by angiotensin is attenuated by the absence of extracellular $Ca^{2+}$ (17). These findings strengthen the tenet of our observations that continual influx of $Ca^{2+}$ is a critical concomitant of the sustained aldosterone secretion evoked by both secretagogues. Two different types of voltage-dependent $Ca^{2+}$ channels (L and T channels) have been identified in the bovine adrenal glomerulosa cells by patch clamp techniques and both have been implicated in calcium influx

during aldosterone secretion (18,19). Nifedipine is said to be a specific inhibitor of L channels (20). In the light of our findings, this can be interpreted to mean that nitrendipine inhibits T channels or both L and T channels.

The results of our studies are also supported by other observations (21) showing that the turnover of phosphoinositides, although initially independent of $Ca^{2+}$ influx, require $Ca^{2+}$ influx for its continuation. Taken together with our findings this may mean that continued hydrolysis of phosphoinositides has a bearing on the stimulus-secretion coupling in response to angiotensin II. Continual phospholipid turnover would provide the prolonged increases in diacylglycerol, $IP_3$ and/or inositol 1,3,4,5-tetrakisphosphate ($IP_4$), of which the latter may be critical in promoting $Ca^{2+}$ influx (22). Receptors for $IP_4$ have now been identified in the adrenal glomerulosa cells (23). Diacylglycerol can also be responsible for $Ca^{2+}$-influx (24). Alternatively, elevated $Ca^{2+}$ by itself is able to maintain the increased steroid synthesis and secretion (25) without the need for participation of PKC. Other studies (26) which have shown potentiation of angiotensin-induced aldosterone secretion by $Ca^{2+}$ ionophore, A23187 or $Ca^{2+}$ channel agonist, BAY K8644 seem also to be supportive of such an idea. $Ca^{2+}$ influx may be essential not only to maintain optimal cytosolic $Ca^{2+}$ concentration but to replenish intracellular $Ca^{2+}$ stores (25,27).

In the case of potassium, it was less surprising that removal of extracellular $Ca^{2+}$ and calcium channel blockade promptly compromised its ability to sustain aldosterone secretion since potassium increases cytosolic free $Ca^{2+}$ mainly by influx through voltage-dependent channels and does not cause increased production of diacylglycerol from phosphoinositide hydrolysis.

In one previous report (11), phorbol ester was shown to increase aldosterone secretion in perifused bovine adrenal glomerulosa cells. Diacylglycerol analogue, OAG, has also been reported to stimulate aldosterone secretion (3). In contrast, we were unable to stimulate aldosterone secretion either by phorbol ester itself or in combination with A23187 during static incubation of adrenal cells. Diacylglycerol analogues were similarly ineffective. When added with angiotensin II, phorbol ester inhibited aldosterone secretion slightly at the high dose, as reported also by Kojima et al. (28). While high doses of phorbol ester can produce effects other than stimulation of PKC, failure of low dose of phorbol ester or diacylglycerol analogues to increase aldosterone secretion seems to discount the importance of the PKC. A recent report appears to concur with this assessment (29). Nonetheless, the continued formation of diacylglycerol in the adrenal glomerulosa cells during angiotensin-mediated stimulation of aldosterone secretion (30,31) may serve some as yet undefined function which may even be independent of PKC since the latter is likely to be down-regulated and therefore desensitized by the continual production of diacylglycerol.

The calmodulin inhibitors, calmidazolium and W-7, and PKC inhibitor, H-7, have been used widely by various investigators (32-36). Our studies revealed that for both angiotensin II- and potassium-induced aldosterone secretion, the calmodulin inhibitor, W-7, proved to be more influential as an inhibitor on a molar basis than H-7. In fact, W-7 completely inhibited aldosterone secretion in stimulated cells compared to the partial inhibitory effects of H-7 at comparable concentrations. Furthermore, when the inhibitory effects of W-7 and H-7 on PKC

activity were examined in cytosolic extracts of adrenal glomerulosa cells, we observed that W-7 at concentrations, which completely blocked aldosterone secretion as observed in experiments involving whole cells, minimally inhibited PKC activity. In contrast, H-7 maximally reduced PKC activity at a concentration at which it only partially inhibited aldosterone secretion in experiments involving whole cells. Since H-7 is able to inhibit calmodulin dependent protein kinase at high (Ki= 97μM) concentrations but it inhibits PKC at much lower (Ki= 6μM) concentrations (34), the effect of H-7 at high concentrations on aldosterone secretion could conceivably be attributed to its action on calmodulin-dependent protein kinase rather than PKC inhihibition. Calmidazolium, another inhibitor of calmodulin, also reduced aldosterone secretion in the same manner and to the same extent as W-7. These results further suggest that the effect of W-7 is indeed through its inhibition of calmodulin. Taken together, these findings imply that $Ca^{2+}$ and calmodulin may be more important than PKC in the acute effects of angiotensin and potassium on aldosterone secretion. To put it differently, there is little need to invoke a role of PKC in the intracellular events leading to aldosterone secretion from the glomerulosa cells. The $Ca^{2+}$-calmodulin system by itself may adequately explain the acute cellular basis of aldosterone secretion evoked by angiotensin.

Acknowledgments. These studies were supported by grants from the Medical Research Funds of the Veterans Administration and American Heart Association (Florida affiliate). Authors wish to thank Dr. Alexander Scriabine of Miles Laboratories for providing the nitrendipine and Ms. Linda Sagun for typing the manuscript.

**References**

1. Capponi, A.M., Lew, P.D., Jornot, L., and Vallotton, M.B. (1984) J. Biol. Chem. 259, 8863-8869.
2. Braley, L., Menachery, A., Brown, E., and Williams, G. (1984) Biochem. Biophys. Res. Commun. 123, 810-815.
3. Kojima, I., Kojima, K., Kreutter, D., and Rasmussen, H. (1984) J. Biol. Chem. 259, 14448-14457.
4. Farese, R.V., Larson, R.E., and Davis, J.S. (1984) Endocrinology 114, 302-304.
5. Enyedi, P., Buki, B., Mucsi, I., and Spat, A. (1985) Mol. Cell. Endocrinol. 41, 105-112.
6. Guillemette, G., Baukal, A.J., Balla, T., and Catt, K.J. (1987) Biochem. Biophys. Res. Commun. 142, 15-22.
7. Kojima, I., Kojima, K., and Rasmussen, H. (1985) Biochem. J. 228, 69-76.
8. Guillemette, G., Balla, T., Baukal, A.J., Spat, A., and Catt, A.J. (1987) J. Biol. Chem. 262, 1010-1015.
9. Streb, H., Irvine, R.F., Berridge, M.J., and Schultz, J. (1985) Nature 306, 67-69.
10. Kojima I., Lippes, H., Kojima, K., and Rasmussen, H. (1983) Biochem. Biophys. Res. Comm. 116, 555-562.
11. Aguilera, G., and Catt, K.J. (1986) Endocrinology 118, 112-118.
12. Lang, U., and Vallotton, M.B. (1987) J. Biol. Chem. 262, 8047-8050.
13. Ganguly, A., Chiou, S., West, L.A., and Davis, J.S. (1986) J. Hypertension 4 (suppl), S 361-S 363.
14. Davis, J.S., West, L.A., and Farese, R.V. (1986) Endocrinology 118, 2561-2571.
15. Braley, L.M., Menachery, A.I., Brown, E.M., and Williams, G.H. (1986) Endocrinology 119, 1010-1019.
16. Hausdorf, W.P., and Catt, K.J. (1988) Endocrinology 123, 2818-2825.
17. Kramer, R.E. (1988) Mol. Cell. Endocrinol. 60, 199-210.
18. Matsunaga, H., Yamashita, N., Maruyama, Y., Kojima, I. and Kurosawa. (1987) Biochem. Biophys. Res. Commun. 149, 1049-1054.

19. Cohen, C.J., McCarthy, R.T., Barrett, P.Q., and Rasmussen, H. (1988) Proc. Natl. Acad. Sci. USA 85, 2412-2416.
20. Nowycky, M.C., Fox, A.P., and Tsien, R.W. (1985) Nature 316, 440-443.
21. Woodcock, E.A., Smith, A.I., and White, L.B.S. (1988) Endocrinology 122, 1053-1059.
22. Irvine, R.F, and Moor, R.M. (1986) Biochem. J. 240, 917-920.
23. Enyedi, P., and Williams, G.H. (1988) J. Biol. Chem. 263, 7940-7942.
24. Vivandou, M.B., Clapp, L.H., Walsh, J.V., and Singer, J.J. (1988) FASEB J. 9, 2497-2504.
25. Rossier, A.F., Krause, K-H., Lew, P.D., Capponi, P.D., and Vallotton, M.B. (1987) J. Biol. Chem. 262, 4053-4058.
26. Hausdorf, W.P., Aguilera, G., and Catt, K.J. (1986) Endocrinology 118, 869-874.
27. Kojima, I., Shibata, H., and Ogata, E. (1987) J. Biol. Chem. 262, 4559-4563.
28. Kojima, I., Shibata, H., and Ogata, E. (1986) Biochem. J. 237, 253-258.
29. Nakano, S., Carvallo, P., Rocco, G., and Aguilera, G. (1990) Endocrinology 126, 125-133.
30. Hunyady, L., Baukal, A.J., Bor, M., Ely, J.A.,and Catt, K.J. (1990) Endocrinology 126, 1001-1008, 1990.
31. Bollag, W.B., Barrett, P.Q., Isales, C.M., and Rasmussen, H. (1991) Endocrinology 128, 231-241.
32. Raufogalis, B.D., Minoocherhomjee, A.M., and Al-Jabore, A. (1983) Can. J. Biochem. Cell. Biol. 61, 927-933.
33. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23, 5036-5040.
34. Wright, C.D., and Hoffman, M.D. (1986) Biochem. Biophys. Res. Commun. 135, 749-755.
35. El-Saleh, S.C., and Solaro, R.J. (1987) J. Biol. Chem. 262, 17240-17246.
36. Miernyk, J.A., Fang, T.K., and Randall, D.D. (1987) J. Biol. Chem. 262, 15338-15340.