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EFFECT OF D-600 ON INHIBITION OF *IN VITRO* RENIN RELEASE IN THE RAT BY HIGH EXTRACELLULAR POTASSIUM AND ANGIOTENSIN II

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SUMMARY

1. Renin was secreted by rat renal cortical slices incubated at 37 °C in a physiological saline solution.

2. Secretion was nearly abolished by incubation in a medium containing 60 mM-K. Secretion could be restored to the control level by the addition of 5×10^{-7} M-D-600 (methoxy verapamil) to 60 mM-K medium.

3. Angiotensin II inhibited renin secretion in a concentration-dependent manner. Concentrations of D-600 ranging from 1 to 3×10^{-6} M (two to sixfold higher than required to block the inhibitory effect of high K) failed to antagonize the inhibitory effect of angiotensin II.

4. Ca is required for the inhibitory effect of angiotensin II, however, as Ca-depletion (incubation of slices in a medium with Na_2EGTA and no added $CaCl_2$) progressively decreased and finally abolished any inhibitory effect.

5. These results confirm and extend previous observations suggesting that Ca plays an inhibitory coupling role in the control of renin secretion from the juxtaglomerular apparatus. Moreover, they suggest that although voltage-sensitive channels exist on juxtaglomerular cells, angiotensin II activates an independent pathway for Ca mobilization.

INTRODUCTION

The granulated juxtaglomerular cells, one of the three components of the renin-secreting juxtaglomerular apparatus, are considered to be the site of renin synthesis, storage, and release (Barajas, 1972). These secretory cells appear to be derived from vascular smooth muscle (Biava & West, 1966). Both their secretory activity and their derivation suggest (Rosenberger & Triggle, 1978) that juxtaglomerular cells possess Ca channels, operated by membrane potential and/or endogenous substances, and that Ca mobilization might play a role in the control of renin secretion. Consistently, both K depolarization (Park & Malvin, 1978) and angiotensin II (Van-Dongen & Peart, 1974) inhibit renin secretion, and Ca chelation abolishes the inhibitory effects. These observations accord with a growing literature suggesting that Ca plays an inhibitory coupling role in the control of renin secretion (*vide infra*).

To date, there are no reports of attempts to block inhibition of renin secretion with 'specific Ca antagonists' (Fleckenstein, 1977) such as verapamil and its methoxy

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derivative, D-600. Such drugs are thought to act by blocking Ca channels regulated by membane potential. Sometimes but not always, such channels may be opened by a natural agonist (Rosenberger & Triggle, 1978). For example, verapamil antagonizes contractions of the uterus elicited by either K-depolarization or angiotensin II (Freer, 1975) suggesting that angiotensin II receptors operate voltage-sensitive Ca channels in this tissue. On the other hand, in vascular smooth muscle, angiotensin II-induced contraction appears to operate independently of voltage-sensitive Ca channels. Verapamil and D-600 antagonize K contracture in vascular smooth muscle (Freer, 1975; Fleckenstein, 1977) but verapamil does not antagonize vascular contractions elicited by angiotensin II (Freer, 1975; Ackerly, Moore & Peach, 1977).

The experiments described below tested the hypothesis that D-600 would block the inhibitory effect of K depolarization, but not the inhibitory effect of angiotensin II, on renin secretion from rat renal cortical slices.

TABLE 1. Lack of effects of angiotensin II and D-600 on renin activity

Sample no.	Renin activity (ng hr^{-1} $ml.^{-1}$)
1 plus H_2O	93·2 ± 0·6 (12)
1 plus angiotensin II	92·3 ± 0·9 (12)
2 plus H_2O	113·4 ± 3·7 (12)
2 plus D-600 in ethanol	117·2 ± 1·7 (4)

Means ± S.E. of means with numbers of observations in parentheses. Samples 1 and 2 were incubation media into which rat renal cortical slices had secreted renin. Water or the same volume of an aqueous solution of angiotensin II was added to sample no. 1 (4×10^{-6} M final). Water or the same volume of a solution of D-600 ethanol was added to sample no. 2 (5×10^{-4} M-D-600; 0·95% ethanol). Then renin activities were determined as described in Methods.

METHODS

Experimental procedures for studying renin secretion from rat kidney slices have been described previously (Lyons & Churchill, 1974). Briefly, for each of several experiments, five adult male Sprague–Dawley rats were anaesthetized with ether and nephrectomized. After removing the renal capsule, four thin cortical slices were cut from each kidney, using a razor blade. The forty slices were randomized and kept in incubation medium at room temperature for no longer than 20 min before beginning the incubation. Then, slices were placed in pre-weighed flasks (2 slices/flask) each of which contained 10 ml. medium, which had been equilibrated previously at 37 °C with a 95/5% mixture of O_2 and CO_2. The flasks were stoppered, placed in an oscillating incubator at 37 °C, and gassed continuously during the incubation. Periodically, 200 μl. samples of medium were withdrawn and centrifuged at 4 °C. The supernatants were frozen until renin activity was determined. Following the incubation, the flasks and contents were dried to constant weight. Tissue dry weight was obtained by subtracting the dried weights of the solutes in identical volumes of medium.

The composition of the incubation medium was 125 NaCl, 19 $NaHCO_3$, 4 KCl, 2·6 $CaCl_2$, 1·2 NaH_2PO_4, and 0·8 $MgSO_4$ (mM) and 0·2/100 ml. each of glucose and bovine albumin (United States Biochemical Corp.). Deviations from this composition are noted in the Results section. Angiotensin II (Sigma Chemical Co.) was dissolved in sterile water. D-600 hydrochloride (Knoll Pharmaceutical Co.) was dissolved in ethanol.

Methods for determination of renin activity have been described previously (Churchill, Churchill & McDonald, 1978). Briefly, 50 μl. samples of supernatant were incubated with rat renin substrate at 37 °C for 30 min. It was previously shown that angiotensin I production is linear for up to 2 hr (Churchill, 1979). Radioimmunoassay was used to measure the angiotensin I generated during this incubation. The incubations were performed in duplicate, and angio-

tensin I was assayed in each in duplicate. The renin activity of a sample was expressed in ng of angiotensin II generated per hour of incubation of the sample with renin substrate per ml. sample (ng hr^{-1} $ml.^{-1}$). The total amount of renin secreted at a given time during the incubation of the slices was calculated as the renin activity of the medium (ng hr^{-1} $ml.^{-1}$) multiplied by the volume of the medium (ml.) and divided by the tissue dry weight (mg), yielding the units ng hr^{-1} mg^{-1}. Renin secretion rate of the slices was calculated as the increment in the total amount of renin during a given interval of incubation of the slices, for example, ng hr^{-1} mg^{-1} per 30 min of incubation.

In order to determine if the substances used in these experiments affected the renin activity method *per se*, the following controls were performed. Kidney slices were incubated in the control medium, initially free of angiotensin II, ethanol, and D-600. Angiotensin II dissolved in H_2O or the same volume of H_2O was added to aliquots of one of these renin-containing solutions (no. 1); either D-600 in ethanol or the same volume of H_2O was added to aliquots of another (no. 2). Then the renin activities of these mixtures were determined. The results are presented in Table 1. It can be seen that in comparison with their respective controls, neither angiotensin II nor the D-600-ethanol mixture affected the renin activity measurement.

The unpaired *t* test was used to assess the statistical significance of observed differences.

TABLE 2. Renin secretion of rat renal cortical slices as a function of angiotensin II concentration during three consecutive 30 min periods

	Secretion rate (ng hr^{-1} mg^{-1} per 30 min		
Angiotensin II (M)	0–30	30–60	60–90
0·0	197 ± 20	262 ± 21	265 ± 24
$1·0 \times 10^{-6}$	63 ± 10*	41 ± 10*	91 ± 21*
$2·0 \times 10^{-6}$	61 ± 8*	31 ± 4*	61 ± 11*
$4·0 \times 10^{-6}$	54 ± 3*	28 ± 4*	46 ± 4*

Means ± S.E. of means. $n = 6$ observations for each angiotensin II concentration. Renin secretion rate was estimated as the increment in total renin activity of the incubation medium (ng hr^{-1} $ml.^{-1}$), multiplied by the volume of the incubation medium (ml.), and divided by the dry weight of the kidney slices (mg), during a 30 min period of incubation (ng hr^{-1} mg^{-1} per 30 min). The periods of incubation were 0–30, 30–60, and 60–90 min. * Indicates $P < 0·005$ compared with 0·0 angiotensin II (unpaired *t* test).

RESULTS

The results presented in Table 2 show that slices incubated in a control medium secrete renin at a relatively stable rate during the last 60 min of a 90 min incubation period. Significant inhibition of secretion occurred during all three periods of incubation in media that contained angiotensin II. The lowest secretory rates were found during the 30–60 min period; thereafter, rates increased. Since the average rates during the 30–60 min period of incubation in media containing 1, 2, and 4×10^{-6} M-angiotensin II were not significantly different, it was concluded that concentrations of approximately 1×10^{-6} M were maximally effective.

An angiotensin II dose–response curve was generated by incubating slices in media containing from 3 to 50×10^{-8} M-angiotensin II. The secretory rate during the 30–60 min period of incubation was determined (V) and the effect was calculated as 100% [1–(V – 28)/(262–28)], where 28 and 262 were the mean secretory rates in the presence of 4×10^{-6} M or of zero angiotensin II, respectively (Table 2). The results are shown in Fig. 1.

Two series of experiments were performed to investigate the inhibitory effect of

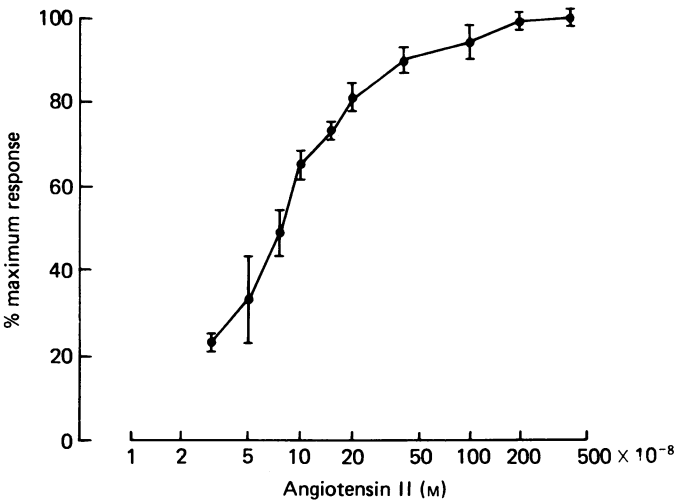

Fig. 1. Effect of angiotensin II on renin secretion from rat renal cortical slices. Angiotensin II concentration is plotted logarithmically against % of maximal response. Maximal response was obtained with 4×10^{-6} M-angiotensin II, at which concentration the rate of secretion was reduced to 11 ± 2 % of the control rate of 262 ± 21 ng hr^{-1} mg^{-1} per 30 min of incubation (see Table 2). Means ± S.E. of means are shown (five to six determinations at each concentration).

TABLE 3. Effect of high K medium on renin secretion from rat renal cortical slices

	Secretion rate (ng hr^{-1} mg^{-1} per 30 min)		
	0–30	30–60	60–90
Low Na, normal Cl medium			
Choline Cl (56 mM) KCl (4 mM)	138 ± 11	142 ± 14	135 ± 15
KCl (60 mM)	55 ± 4*	4 ± 1*	6 ± 2*
Normal Na, high Cl medium			
Choline Cl (56 mM) KCl (4 mM)	165 ± 15	135 ± 15	145 ± 12
KCl (60 mM)	50 ± 4*	8 ± 1*	10 ± 1*

Means ± S.E. of means. $n = 6$ observations for each condition. Secretion rate was estimated as the increment in total renin activity of the incubation medium (ng hr^{-1} $ml.^{-1}$) multiplied by the volume of the incubation medium (ml.) and divided by the dry weight of the kidney slices (mg) during a 30 min period of incubation (ng hr^{-1} mg^{-1} per 30 min). The periods of incubation were 0–30, 30–60, and 60–90 min. In low Na, normal Cl medium, 56 mM of either choline Cl or KCl replaced 56 mM-NaCl (total KCl = 4 or 60 mM). In normal Na, high Cl medium, 56 mM of either choline Cl or KCl was added to medium containing normal concentrations of NaCl (125 mM) and KCl (4 mM). * Indicates that the mean value is significantly lower than that for choline Cl, $P < 0.005$ (unpaired t test).

high extracellular K concentration on renin secretion. In one, 56 mM-NaCl was omitted from the incubation medium and replaced by 56 mM-KCl or choline Cl (KCl = 4 or 60 mM; 'low Na, normal Cl medium'). In the other, 56 mM of either KCl or of choline Cl was added to a medium with normal NaCl (125 mM) and KCl (4 mM) concentrations (KCl = 4 or 60 mM; 'normal Na, high Cl medium'). The rates of renin secretion of slices incubated in the media are presented in Table 3. It is apparent from these data that secretory rate is nearly abolished if the slices are exposed to a high concentration of K, irrespective of changes in osmolality, or in

Na or Cl concentrations. Comparison of the rates found in Tables 2 and 3 reveals that high K media of either type is more efficacious than angiotensin II in inhibiting the secretion of renin.

Slices were incubated in high K medium (normal Na, high Cl) with concentrations of D-600 ranging from 2 to 50×10^{-8} M. Rates of secretion during the 30–60 min

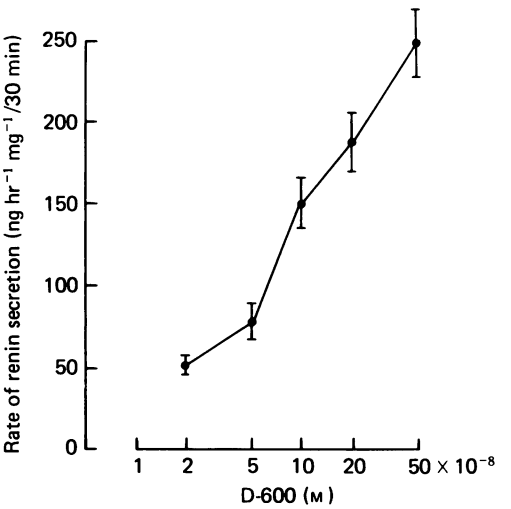

Fig. 2. Effect of D-600 on renin secretion from rat renal cortical slices incubated in high K medium (60 mM). D-600 concentration is plotted logarithmically against the rate of renin secretion. Means ± S.E. of means are shown (five or six determinations at each concentration).

TABLE 4. Lack of antagonism by D-600 of the inhibitory effect of angiotensin II on renin secretion from rat renal cortical slices

		Secretion rate (ng hr^{-1} mg^{-1} per 30 min)		
D-600 (M)	Angiotensin II (M)	0–30	30–60	60–90
1×10^{-6}	—	136 ± 6	256 ± 21	296 ± 32
1×10^{-6}	2×10^{-6}	56 ± 5*	49 ± 4*	95 ± 11*
2×10^{-6}	—	131 ± 13	261 ± 18	250 ± 19
2×10^{-6}	2×10^{-6}	60 ± 5*	48 ± 8*	117 ± 17*
3×10^{-6}	—	136 ± 16	257 ± 26	221 ± 26
3×10^{-6}	2×10^{-6}	65 ± 6*	50 ± 4*	103 ± 13*

Means ± S.E. of means. $n = 6$ observations for each condition. Secretion rate was estimated as the increment in total renin activity of the incubation medium (ng hr^{-1} $ml.^{-1}$), multiplied by the volume of the incubation medium (ml.), and divided by the dry weight of the kidney slices (mg), during a 30 min period of incubation (ng hr^{-1} mg^{-1} per 30 min). The periods of incubation were 0–30, 30–60, and 60–90 min. * Indicates that mean value is significantly lower than respective control value, $P < 0.005$ (unpaired t test).

period of exposure to high K and D-600 were determined, and means ± S.E. of means of the rates are shown in Fig. 2. It is apparent that D-600 can antagonize the inhibitory effect of high K on renin secretion. In the presence of 5×10^{-7} M-D-600, secretion rate was restored to a value not significantly different from the uninhibited rate of secretion (248 ± 21 versus 262 ± 21 ng hr^{-1} mg^{-1} per 30 min).

Slices were incubated in normal K medium, with or without 2×10^{-6} M-angiotensin

II, in the presence of 1 to 3×10^{-6} M-D-600. The results are summarized in Table 4. Secretion rates in the absence of angiotensin II were comparable to secretory rates presented in Table 2, suggesting that D-600 *per se*, at the concentrations used, neither stimulated nor inhibited secretory rate. Whereas 5×10^{-7} M-D-600 almost completely antagonized the inhibitory effect of high K medium on renin secretion, it is apparent from the data in Table 4 that two- to six-fold higher concentrations of D-600 failed to antagonize the inhibitory effect of angiotensin II. Mean rates of secretion in the presence of 2×10^{-6} M-angiotensin II during the 30–60 min period of incubation were similar to the mean rate found previously in the absence of D-600 (Table 2).

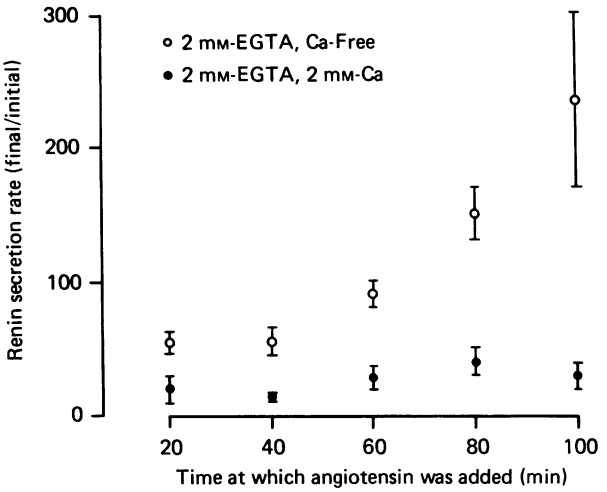

Fig. 3. Effect of Ca depletion on inhibition of renin secretion by angiotensin II. Slices were incubated in media with low or normal Ca, as indicated. Angiotensin II (2×10^{-6} M final) was added at 20, 40, 60, 80 and 100 min. Initial rate of secretion was determined during a 20 min period just before the addition of angiotensin II; final rate was determined during a 20 min period beginning 20 min after angiotensin addition. Means ± S.E. of means of percent final/initial are shown (five or six determinations at each point). Initial rates in normal Ca media were (ng hr^{-1} mg^{-1} per 20 min; means ± S.E. of means): 83 ± 11 (0–20 min), 202 ± 23 (20–40 min), 255 ± 12 (40–60 min), 219 ± 21 (60–80 min), and 167 ± 35 (80–100 min). Initial rates in low Ca media during the same time periods were: 247 ± 26, 233 ± 11, 312 ± 52, 310 ± 38, and 203 ± 13.

To investigate the effect of Ca-depletion, slices were incubated in medium containing 2 mM-Na_2EGTA and no added $CaCl_2$. So that any effect could be attributed specifically to a reduction in Ca concentration and not to EGTA *per se*, other slices were incubated in medium containing 2 mM-CaEGTA and the usual amount of $CaCl_2$. Angiotensin II (2×10^{-6} M final) was added 20, 40, 60, 80 and 100 min after beginning the incubations. Renin secretion rates of each slice were determined twice: during a 20 min period just before the addition of angiotensin II (initial rate) and during a 20 min period beginning 20 min after the addition (final rate). When angiotensin II was added at 40 min for example, the initial rate was the increment in renin found between 20 and 40 min, and the final rate was the increment between 60 and 80 min. Per cent final/initial was calculated for each slice, and the means ± S.E.

of means are presented in Fig. 3. In medium containing 2·65 mM-$CaCl_2$, per cent final/initial was significantly less than 100% ($P < 0{\cdot}001$), irrespective of the time of addition of angiotensin II; the presence of EGTA did not antagonize the inhibitory effect of angiotensin II. In contrast, Ca depletion progressively antagonized the inhibitory effect of angiotensin II. Although angiotensin II still inhibited secretion when added at 20 and 40 min (i.e., percent final/initial was significantly less than 100%, $P < 0{\cdot}01$), the degree of inhibition was clearly less than that observed in medium containing 2·65 mM-$CaCl_2$. Addition of angiotensin II at 60 min failed to inhibit secretion at all; per cent final/initial is not significantly different from 100% ($P > 0{\cdot}05$). Means of percent final /initial exceeded 100% ($P < 0{\cdot}01$) when angiotensin II was added at either 80 or 100 min.

DISCUSSION

In the present study, renin secretion was virtually abolished by incubation of rat kidney slices in 60 mM-K. In previous studies of inhibition of *in vitro* renin secretion by high extracellular K, K was increased by omitting an equivalent amount of Na (Fray, 1978; Park & Malvin, 1978). Reductions in Na *per se* have been shown to inhibit renin secretion from some *in vitro* preparations (Capponi & Vallotton, 1976). However, this cannot be the sole explanation of the inhibitory effect, since in the present study, renin secretion was abolished by 60 mM-K in either low or normal Na media. Although transmembrane potential and renin secretion rate have never been determined simultaneously in the same preparation, the most reasonable explanation of these observations is that high extracellular K depolarizes the renin-secreting juxtaglomerular cell, and that depolarization in some manner inhibits the release of renin, as suggested by Fishman (1976).

Many smooth muscle and secretory cells are stimulated to contract/secrete upon exposure to depolarising concentrations of K (Rubin, 1974; Fleckenstein, 1977; Rosenberger & Triggle, 1978). It is well known that K-stimulated contractile or secretory activities of these cells are dependent upon influx of extracellular Ca, and it is currently believed that K-depolarization opens voltage-sensitive Ca channels through which Ca influx occurs (Fleckenstein, 1977; Rosenberger & Triggle, 1978). Drugs such as verapamil and D-600, in the 10^{-5}–10^{-7} M range, are thought to act by blocking Ca influx through voltage-sensitive Ca channels. Because of the apparent specificity of such drugs, their blocking of Ca-dependent activities in cells is taken as evidence for the existence of voltage-sensitive Ca channels (Rosenberger & Triggle, 1978).

In the present study, D-600 antagonized the inhibitory effect of K-depolarization on renin secretion. The antagonism was dose-dependent; 7×10^{-8} and 5×10^{-7} M-D-600 were approximately 50 and 100% effective, respectively. Given this sensitivity, and the dose–response relationship, it is unlikely that this antagonism is a non-specific effect. Thus, these observations suggest that depolarization opens voltage-sensitive Ca channels on juxtaglomerular cells and that Ca influx through such channels blocks the secretion of renin.

VanDongen & Peart (1974) reported that the addition of 25 mM-EDTA to the perfusate stimulated renin release from the isolated rat kidney and abolished the

inhibitory effect of angiotensin II. The results of the present study confirm their conclusion that the inhibitory effect of angiotensin II is Ca-dependent. If medium Ca was 2·65 mM, the presence of 2 mM-EGTA had no effect on renin secretion or on its inhibition by angiotensin II. However, when Ca in the medium was buffered to a low value by the same concentration of chelator, free Ca approximately 10^{-8} M (Caldwell, 1970), the inhibitory effects of angiotensin II were decreased and eventually completely abolished. The time course of this effect was not unexpected. Presumably, the amount of Ca associated with the cell membrane and, or, intracellular organelles, and therefore available for influx and/or mobilization, would be reduced only gradually by chelation of Ca in the extracellular spaces (Weiss, 1978). Although chelation blocked the inhibitory effect of angiotensin II, demonstrating Ca-dependency, the specific Ca antagonist D-600 did not, even at concentrations two to sixfold higher than that required to completely antagonize the effect of K depolarization. Thus, inhibition of renin secretion by angiotensin II requires Ca influx and, or, mobilization, but probably not influx through voltage-sensitive Ca channels.

Perhaps most significantly, the present results add to the growing body of evidence that Ca plays an inhibitory, rather than a stimulatory, coupling role in the control of renin secretion. The following contrasts can be cited. (*a*) Ouabain stimulates the secretion of most cells in a Ca-dependent manner (Rubin, 1974). It is well established that ouabain inhibits the secretion of renin (Churchill & McDonald, 1974; Lyons & Churchill, 1974 and 1975; Blaine & Zimmerman, 1978; Park & Malvin, 1978) in a Ca-dependent manner (Park & Malvin, 1978; Churchill, 1979). (*b*) Phenytoin antagonizes the effects of ouabain in many tissues (Schwartz, Lindenmayer & Allen, 1975), perhaps by stimulating Na, K-ATPase activity. Phenytoin inhibits the secretion of insulin and antidiuretic hormone (Goodman & Gilman, 1975) yet stimulates the secretion of renin (Migdal, Slick & McDonald, 1977; Churchill, 1979). (*c*) Decreasing extracellular concentration of Na stimulates secretion from many cells in a Ca-dependent manner, presumably by altering the rate of Na–Ca exchange across the plasma membrane (Rubin, 1974). Decreasing extracellular Na inhibits the secretion of renin *in vitro* (Lyons & Churchill, 1974 and 1975; Capponi & Vallotton, 1976). (*d*) Inorganic ions such as Mg and La have Ca-antagonistic properties on secretory cell activity (Rubin, 1974). Mg (Churchill & Lyons, 1976; Fray, 1976) and La (Baumbach & Leyssac, 1977; Logan, Tenyi, Peart, Breathnach & Martin, 1977) stimulate renin secretion. (*e*) Ca ionophores usually stimulate secretory cell activity (Rosenberger & Triggle, 1978) but they appear to inhibit renin secretion (Baumbach & Leyssac, 1977; Fynn, Onomakpome & Peart, 1977).

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