J. Physiol. (1979), **294**, *pp.* 123–134
With 4 text-figures
Printed in Great Britain

POSSIBLE MECHANISM OF THE INHIBITORY EFFECT OF OUABAIN ON RENIN SECRETION FROM RAT RENAL CORTICAL SLICES

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(*Received 9 January 1979*)

SUMMARY

1. The effects of ouabain on renin secretion by rat renal cortical slices were studied.

2. Renin secretion was inhibited by 10^{-3} M-ouabain in the presence of free Ca (10^{-4} to $2{\cdot}6 \times 10^{-3}$ M). Inhibition was blocked at Ca $< 10^{-8}$ M.

3. The effect of free Ca on ouabain-inhibition was shown to be independent of the presence of EGTA, completely reversible, and unrelated to passive leakage of renin from non-viable cells, as assessed by simultaneous release of lactate dehydrogenase activity (LDH).

4. It is proposed that, as a result of inhibition of Na, K-ATPase by ouabain, (*a*) intracellular Na increases in the renin-secreting juxtaglomerular cells, (*b*) intracellular Ca increases, via an Na–Ca exchange mechanism, and (*c*) that Ca accumulation, in some unknown manner, inhibits renin secretion.

INTRODUCTION

It is well known that ouabain, via inhibition of Na, K-ATPase, affects the activities of many specialized cells, including cardiac and smooth muscle, and neuro- and endocrine secretory, cells. In general, the proximal effects are attributable to a rise in intracellular free Ca concentration, which occurs via an Na–Ca exchange mechanism, contingent upon the reduction in Na gradient across the cell membrane (Lee & Klaus, 1971; Blaustein, 1974; Rubin, 1974; Baker, 1976; Akera & Brody, 1978). In particular, ouabain stimulates hormone release from the adrenal medulla (Banks, 1967), the neurohypophysis (Dicker, 1966), the adenohypophysis (Eto, Wood, Hutchins & Fleischer, 1974), and the pancreas (Grodsky), and transmitter release from motor neurones (Birks & Cohen, 1968). These same or other investigators (Kelly, 1968; Hales & Milner, 1968*a*, *b*) have demonstrated the Ca dependency of these stimulatory effects of ouabain.

Within the past few years, several laboratories have reported inhibitory effects of ouabain on renin secretion. When infused directly into the renal artery, ouabain blocked the stimulatory effects of aortic clamping and ureteral occlusion (Churchill & McDonald, 1974), of increased renal nerve activity (Haulica, Petrescu, Branisteanue, Rosca & Balan, 1974), and of furosemide administration (Blaine & Zimmerman,

0022-3751/79/4710-0943 $01.50

1978). More dramatically, ouabain nearly abolished renin secretion from rat (Lyons & Churchill, 1974, 1975) and from pig (Park & Malvin, 1978) renal cortical slices. That Na, K-ATPase is the only known biochemical entity affected directly by ouabain (Lee & Klaus, 1971; Akera & Brody, 1978) strongly suggests that these inhibitory effects of ouabain are contingent upon inhibition of Na, K-ATPase and a reduction in the transmembrane Na gradient (Lyons & Churchill, 1975). Although Na–Ca exchange has not been demonstrated directly in the renin-secreting juxtaglomerular cells, the common derivation of juxtaglomerular and vascular smooth muscle cells (Barajas & Latta, 1967) and the existence of Na–Ca exchange in the latter (Bohr, 1973) suggests that the reduced Na gradient might inhibit renin secretion via Ca accumulation by the juxtaglomerular cells.

The present studies first confirmed that ouabain inhibits the renin secretion of rat renal cortical slices. Then the Ca dependency of this effect was studied by incubating slices, with or without ouabain, in media containing $2{\cdot}6 \times 10^{-3}$ to $< 10^{-8}$ M-free Ca. Extracellular Ca was varied during the course of other incubations, in order to study the reversibility of the effect of Ca. Finally, the accumulation of a cytoplasmic enzyme, lactate dehydrogenase, was used to assess the possibility of non-specific leakage of intracellular contents including renin into the incubation medium.

While these experiments were in progress, Park & Malvin (1978) reported that ouabain had no inhibitory effect on renin secretion of pig renal cortical slices incubated in nominally Ca-free media. The more extensive results presented below, which were obtained from a different species, are in accord with their conclusions.

METHODS

Adult male Sprague–Dawley rats were anesthetized and nephrectomized. The renal capsule was removed and four thin cortical slices were cut by hand from each kidney as previously described, except that the preparation was at room temperature (Lyons & Churchill, 1974). The slices were blotted gently and placed in tared flasks (two slices/flask) each of which contained 10 ml. medium which had been equilibrated at 37 °C with a 95 % O_2 and 5 % CO_2 gas mixture. The flasks were then placed in an oscillating incubator at 37 °C and gassed continuously during the incubation. Every 30 min, 200 μl. samples of medium were obtained, and centrifuged at 4 °C. The supernatants were stored at 4 °C (until determination of lactate dehydrogenase activity) or at -20 °C (until determination of renin activity).

Following most of the incubations, the flasks and contents were dried to constant weight; 4 days at 95 °C were found to be more than adequate. Tissue dry weight was obtained by subtracting the dried weights of the solutes in identical volumes of media. Tissue dry weight averaged $9{\cdot}8 \pm 0{\cdot}3$ mg, $n = 50$ incubations, in this type of experiment. In other experiments, the slices were homogenized in distilled H_2O (Churchill, Bidani, Fleischmann & Becker-McKenna, 1977). The homogenates were centrifuged and samples of supernatant were kept as described above. The remainder of the supernatant, and the pellet, were dried to constant weight. In these experiments, tissue dry weight was $9{\cdot}1 \pm 0{\cdot}5$ mg, $n = 12$ incubations.

The composition of the incubation medium was: 124 NaCl, 19 $NaHCO_3$, 6 KCl, 2·6 $CaCl_2$, 1·2 NaH_2PO_4, and 0·8 $MgSO_4$ (all in mM) and 0·2 g/100 ml. each of glucose and fraction V bovine albumin (United States Biochemical Corp.). In some experiments, Ca- and Na_2-EGTA (ethyleneglycol-bis[beta-amino-ethyl ether]N,N′-tetra-acetic acid; Sigma Chemical Co.) solutions were used, at 2 mM final concentrations, to buffer extracellular Ca (Caldwell, 1970). Ouabain (Strophanthin G; Calbiochem) was used at a concentration of 1 mM, a concentration equal to (Maude, 1969), or less than (Hughes & MacKnight, 1976) that typically employed in studies with the relatively insensitive rat renal cortex (Allison, 1975).

The methods of measuring renin activity differ from those used previously (Lyons & Churchill,

1974, 1975), but have been described and validated in recent publications from this laboratory (Churchill *et al.* 1977; Churchill, Churchill & McDonald, 1978). Additional validation is presented below for the specific use of the methods in measuring renin activity of incubation media. Briefly, 50 μl. samples of medium (undiluted or diluted 2–5 times) and of the supernatants of homogenized renal cortex (diluted 20–50 times) were incubated with rat renin substrate at 37 °C for 30 min. The angiotensin I generated during the incubation was measured by using radioimmunoassay. The incubations with substrate were performed in duplicate and angiotensin I was assayed in each incubation in duplicate. Renin activity was expressed in units of ng hr^{-1} $ml.^{-1}$ (nanogram of angiotensin I generated per hour of incubation with renin substrate per millilitre of sample). The total amount of renin secreted at a particular time during the incubation of the slices was calculated as renin activity of the undiluted medium (ng hr^{-1} $ml.^{-1}$), multiplied by the volume of medium, and divided by the tissue dry weight in mg: ng hr^{-1} mg^{-1}. This is reported as such in some Figures and Tables. In others, the renin secretion rate is reported, that is, the increment in total renin in a 30-min period: ng hr^{-1} mg^{-1}/30-min. Renin activity in tissue was calculated similarly: renin activity of the undiluted homogenate (ng hr^{-1} $ml.^{-1}$) was multiplied by the volume of the homogenate and divided by the tissue dry weight, yielding ng hr^{-1} mg^{-1}.

A commercial kit (Sigma Chemical Co.) was used to measure lactate dehydrogenase activity of the incubation media. For unknown reasons, attempts to measure tissue LDH activity were unsuccessful. Perhaps due to interfering substances, LDH activity in homogenates was unstable, unlike activity in the incubation medium which was stable for several hours at 4 °C. LDH activity was expressed in BB u./ml. According to the brochure of the kit, a BB unit is that amount of LDH required to reduce $4{\cdot}8 \times 10^{-4}$ μM pyruvate per minute at 25 °C. LDH release was calculated like renin release, resulting in the units of BB u. mg^{-1}.

All results were expressed as means ± S.E. of means. Both the paired and the unpaired t tests were used to evaluate the statistical significance of any observed differences, as indicated in the text.

RESULTS

Hog renin (Nutritional Biochemicals Corp.) was added to rat renin substrate (Churchill *et al.* 1978) and the reaction was allowed to proceed to completion at 37 °C as judged by identical concentrations of angiotension I at the end of 30 and 60 min. Measured in this way, renin substrate concentration was 1158 ± 25 ng $ml.^{-1}$, $n = 14$ determinations each of which was analysed in quadruplicate. Next, 50 μl. of a renin-containing incubation medium was incubated with 500 μl. of rat renin substrate, and samples were removed every 15 min and assayed for angiotensin I. The results in Table 1 indicated that angiotensin I production was linear and therefore that renin substrate was in excess. All subsequent renin activity measurements were made by incubating 50 μl. sample with 500 μl. substrate for only 30 min. As can be seen in Table 2, the renin method was unaffected by the presence of EGTA, $CaCl_2$, or ouabain at the highest concentrations used in the experiments (A and B in Table 2 were renin-containing media, pooled from several experiments, which initially contained no ouabain and no EGTA (A), or no $CaCl_2$ (B)).

In Fig. 1 is shown, as a function of time, the renin secreted by slices into media containing 2·6 mM-Ca, with and without 1 mM-ouabain. Neither line in this Figure was extrapolated to zero; the slices had been at room temperature and not exposed to ouabain before this time, and it was expected that secretion rate would not stabilize immediately. The increments in renin were almost identical in the control flasks, however: 240 ± 24, 242 ± 29, and 200 ± 35 ng hr^{-1} mg^{-1} during the 0–30, the 30–60, and the 60–90 min periods, respectively. In contrast, only 92 ± 29 ng hr^{-1} mg^{-1} were secreted during the first period of exposure to ouabain, and subsequently, the secre-

tion was completely blocked. The next two 30-min increments were not different from zero.

Experiments were performed in which ouabain was added after the slices had equilibrated for 60 min, in media containing either 2·6 or 0·1 mM-Ca. The latter contained a mixture of Ca- and Na_2-EGTA as described in Methods. As can be seen in

TABLE 1. Generation of angiotensin I from renin substrate

Time (min)	ng angiotensin I/ml.
0	3·9 ± 0·2 (12)
15	49·6 ± 0·5 (9)
30	92·3 ± 1·0 (9)
45	133·7 ± 1·0 (9)
60	170·2 ± 1·3 (9)

Means ± S.E. of means with numbers of observations in parentheses. First column: length of time that 50 µl. sample was incubated with 500 µl. renin substrate at 37 °C. Second column: ng angiotensin I which would have been produced by incubation of 1 ml. sample with proportionately more renin substrate. If the numbers in the second column are divided by 20, the actual amount of the substrate consumed can be calculated; 579 ng-equivalent of substrate were present (see text). Paired increases between 15 and 60 min of incubation were statistically significant ($P < 0·0001$).

TABLE 2. Lack of effects of ouabain, EGTA, and $CaCl_2$ on the renin-activity measurement

Sample	Renin activity (ng hr^{-1} $ml.^{-1}$)
A + H_2O	182 ± 1 (10)
A + 1 mM ouabain	182 ± 1 (10)
A + 2 mM-EGTA	185 ± 3 (10)
B + H_2O	376 ± 5 (10)
B + 2·6 mM-$CaCl_2$	379 ± 4 (10)

Means ± S.E. of means with numbers of observations in parentheses. Rat renal cortical slices were incubated in media without ouabain or EGTA (A) and without $CaCl_2$ (B). Following the incubations, these substances dissolved in H_2O, or the same volume of H_2O, were added and renin activity was determined as described in Methods. Units of renin activity: ng angiotensin I produced during a 1 hr incubation of 1 ml. sample with renin substrate.

Fig. 2, addition of ouabain to the slices incubating in 2·6 mM-Ca reduced their renin secretion to 60 ± 8 ng hr^{-1} mg^{-1} in the first 30 min of exposure, a value close to that found in the experiments depicted in Fig. 1. The 30–60 and 60–90 min increments in renin, in the absence of ouabain, taken from the experiments of Fig. 1, are shown here for comparison. In a strict sense they cannot be considered 'controls' since no additional medium was added at 60 min. This in no way detracts from the conclusions to be drawn, and moreover, small increases in volume during the incubation did not affect renin secretion in the other experiments, e.g. those performed at 0·1 mM-Ca which are also shown in Fig. 2. In these, ouabain reduced renin secretion to 106 ± 16 ng hr^{-1} mg^{-1} during the first 30 min of exposure. It was concluded that EGTA *per se* does not interfere with the inhibitory response to ouabain.

EGTA was used to buffer Ca to values $< 10^{-8}$ M. In such media, ouabain no longer inhibited renin secretion, as can be seen in Fig. 3. In fact, average renin was

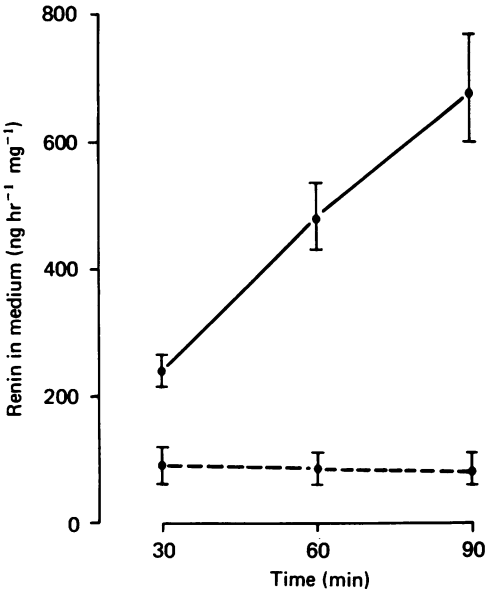

Fig. 1. Renin released into the incubation medium, as a function of time, by rat renal cortical slices. The media contained 2·6 mM-Ca and zero or 1 mM-ouabain. Renin activity of the medium (ng hr^{-1} $ml.^{-1}$) was multiplied by the volume of the medium and divided by the tissue dry weight, giving ng hr^{-1} mg^{-1}. Means ± S.E. of means are shown; n = 6 for each group. ●—●, $2{\cdot}6 \times 10^{-3}$ M-Ca; ● - - - ●, $2{\cdot}6 \times 10^{-3}$ M-Ca ouabain.

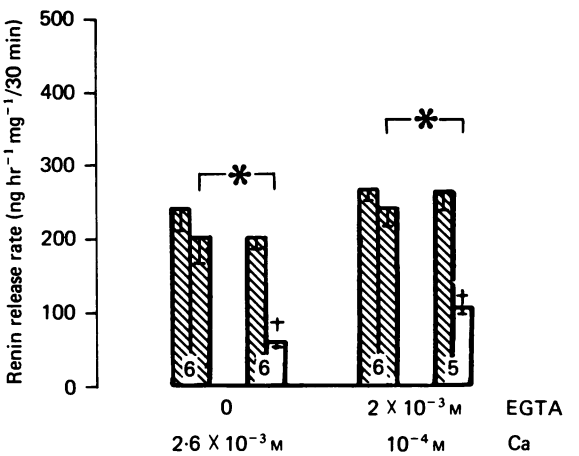

Fig. 2. The effect on renin secretion of adding ouabain during the course of incubation of rat renal cortical slices. The two columns in each pair represent renin release rates during the 30–60 and the 60–90 min periods of incubation. Second and fourth pair from the left: ouabain was added at 60 min. +, the P-values were calculated by comparing rates before (30–60 min period) and after (60–90 min period) the addition of ouabain (paired t test). First and third pair: ouabain was not added. These serve as unpaired 'controls' for the effect of time. *, the P-values were calculated by comparing the rates during the 60–90 min periods with (second and fourth pair) versus without ouabain (first and third pair, respectively). The unpaired t test was used for this comparison. Means ± S.E. of means are shown, with the numbers of experiments within the columns. *, $P < 0{\cdot}01$; +, $P < 0{\cdot}0001$; □, 10^{-3} M-oubain added.

slightly higher in the ouabain-containing media at both 60 and 90 min, but the differences were not statistically significant. It was concluded that the inhibitory effect of ouabain is dependent on a concentration of free Ca greater than 10^{-8} M.

Fig. 4 shows the results of the experiments during the course of which concentration of Ca was changed by several orders of magnitude in either direction, either by

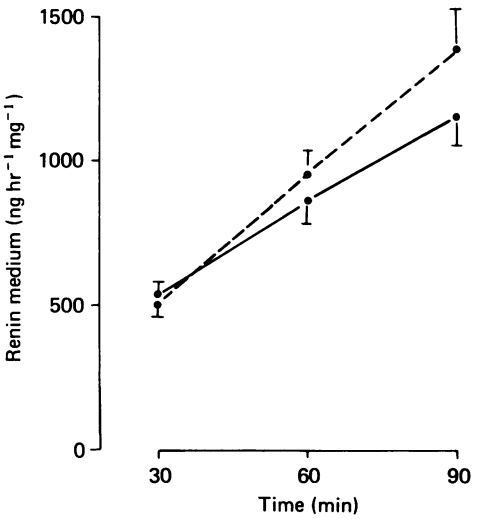

Fig. 3. Lack of effect of ouabain on renin release into media containing less than 10^{-8} M-Ca. Renin release is shown as a function of time, as in Fig. 1. Means ± S.E. of means are shown; $n = 6$ and 5 for 1 mM and zero ouabain, respectively. At no time period were the differences between the two groups statistically significant ($P > 0{\cdot}2$ minimum). The non-ouabain treated slices in this experiment secreted more than those of Fig. 1 at all three time periods: 557 ± 37 *vs.* 240 ± 24 ($P < 0{\cdot}00001$), 860 ± 63 *vs.* 483 ± 52 ($P < 0{\cdot}001$), and 1110 ± 92 *vs.* 683 ± 86 ($P < 0{\cdot}01$) ng hr^{-1} mg^{-1}. ●—●, $< 10^{-8}$ M-Ca; ● - - - ●, $< 10^{-8}$ M-Ca, 10^{-3} M-ouabain.

adding Ca (2·6 mM added) in excess of the EGTA initially present (1 mM), or by adding EGTA (2 mM) in excess of the Ca initially present (1·3 mM). Ouabain was present from the start. It can be seen that an initially high rate of renin release was converted to virtually no release at all by increasing Ca; conversely, in the parallel experiment, there was little or no renin release until Ca was reduced. It was concluded that the Ca-dependency of ouabain's effect is completely reversible. The concentrations of free Ca can be estimated (Caldwell, 1970) using the apparent stability constant of the Ca-EGTA complex: 5×10^6 M^{-1} (pH 7·0, 20 °C, no other divalent cations present). Estimates of the changes during this experiment are $< 10^{-8}$ to about $1{\cdot}6 \times 10^{-3}$ M for the first series, and for the second, about $1{\cdot}3 \times 10^{-3}$ to $< 4 \times 10^{-7}$ M.

LDH release was measured in the same flasks and the results are also shown in Fig. 4, at the bottom. LDH activity increased in both types of experiment. Differences in LDH between the two types were not significant at any time except at 60 min, when the EGTA-containing media had a significantly higher amount. However, the slopes of the two lines, i.e. the rates of release, were not different during any time period. Since the rates of LDH release were not affected in opposite direc-

tions by Ca and EGTA additions but the rates of renin release were, it was concluded that generalized alterations in cell membrane permeability, resulting in non-specific leakage of cell contents including renin, were an unlikely explanation of the results.

The relation between renin and LDH release rates was studied further, in experiments like those of Fig. 1. Following a 2 hr incubation, renin content of the tissue slices was measured. The results are summarized in Table 3. As expected (Fig. 1),

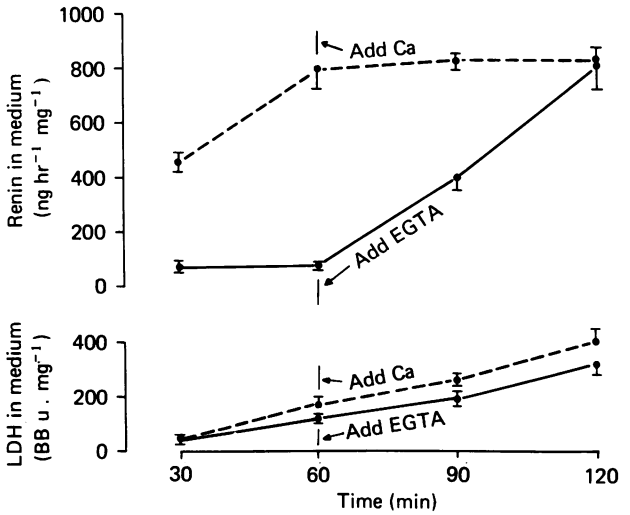

Fig. 4. Reversibility of the Ca-dependent effect of ouabain on renin secretion. Top panel: renin released into the media as a function of time. Media initially contained 1 mM-EGTA and 1 mM-ouabain (dashed line) and 2·6 mM-Ca was added at 60 min, or media initially contained 1·3 mM-Ca and 1 mM-ouabain (continuous line) and 2 mM-EGTA was added at 60 min. Means ± S.E. of means are shown; $n = 5$ in each group. The increments in renin (ng hr^{-1} mg^{-1}) between 30 and 60 min (340 ± 29, dashed line) and between 60–90 and 90–120 min (327 ± 36 and 400 ± 38, continuous line) were statistically significant ($P < 0{\cdot}001$ maximum, paired t test). Other increments were not ($P > 0{\cdot}2$ minimum, paired t test). Bottom panel: lactate dehydrogenase activity of the media as a function of time. All increments were significant for both groups ($P < 0{\cdot}01$, maximum). The differences between the two groups were not significant ($P > 0{\cdot}05$, unpaired t test) except at 60 min ($P < 0{\cdot}05$).

release of renin in the presence of ouabain was low during the first hour and nearly zero during the second. Significantly more renin was secreted by the controls. In this group, secretion during the first hour was about twice the 30-min secretion found previously (Figs. 1 and 2). Secretion was more variable, and the average was higher (but not significantly, $P > 0{\cdot}05$), during the second hour. Tissue renin at the end of the incubation was lower in the control than in the ouabain-treated slices. The total renin presumed to be present at the beginning of the incubation was calculated by adding the measured content and the amounts released by each slice; then renin release, expressed as percent content, was calculated. The means are given in Table 3. That this calculation ignores renin metabolism – synthesis, activation, inactivation – does not detract from the conclusions drawn, since totals calculated in this way were not different from tissue content of non-incubated slices: 3702 ± 579 ng hr^{-1} mg^{-1},

$n = 4$ (two slices/determination). Ouabain-treated slices released less than 4% of their content in a 2 hr period, no matter how this is calculated. Thus, only very small amounts of renin were released by non-specific leak during a relatively long time period. The conclusion that viability of the control slices was similar, despite the higher renin secretion rate, is more tentative; if a renin secretion rate of 677 ± 168 ng hr^{-1} mg^{-1} during a 1 hr period, fifty times that of the ouabain-treated slices,

TABLE 3. Lack of a relationship between renin and lactate dehydrogenase (LDH) release

	Control		Ouabain	
	Renin release (ng hr^{-1} mg^{-1})	LDH release (BB u. mg^{-1})	Renin release (ng hr^{-1} mg^{-1})	LDH release (BB u. mg^{-1})
0–60 min	466 ± 58	264 ± 11	125 ± 32**	227 ± 12*
%	(11 ± 2)	—	(3 ± 1)**	—
60–120 min	677 ± 168	265 ± 8	13 ± 3**	246 ± 15
%	(16 ± 4)	—	$(0{\cdot}3 \pm 0{\cdot}1)$**	—
Content at 120 min	3063 ± 303	—	3676 ± 67*	—
Total content	4206 ± 213	—	3815 ± 48	—

Table entries are the increments in renin and LDH activities in the incubation medium, the tissue renin content following the incubation, and the sum of content and amounts released. Means $\pm$ S.E. of means; $n = 4$ in both groups. After summing content and amounts released for each slice, percent of the sum released in a given time period was calculated; averages are shown in parentheses. * and **, $P < 0{\cdot}05$ and $P < 0{\cdot}01$ compared with control; otherwise the differences between the two groups were not significant ($P > 0{\cdot}05$).

were attributable to differences in viability, then a similar difference between control and ouabain-treated slices should have been found with respect to LDH release, but LDH release was almost identical for the two groups of slices during this same time period.

DISCUSSION

The above results confirm that ouabain inhibits renin secretion from rat renal cortical slices (Lyons & Churchill, 1974, 1975) and demonstrate that this effect is dependent upon extracellular concentration of free Ca. Inhibition was found at or above 10^{-4} M-Ca, no effect was found at $< 10^{-8}$ M-Ca. Inhibition was independent of EGTA *per se* and the inhibitory effect of ouabain, in the presence of Ca, was completely reversible. Since the integrity of the cell membrane depends upon divalent cations (Rubin, 1974; Baker, 1976), it was important to show that the changes in renin secretion were not due to changes in passive leak of cell contents into the incubation medium. The best evidence against this possibility is presented in Fig. 4; a cytoplasmic enzyme did accumulate in the medium, but the rate of accumulation was not affected in opposite directions by manipulations that did affect renin secretion oppositely.

Renin secretion relative to tissue content was surprisingly high, approximately 11–16% per hour in the control slices. This is similar to the range of 8–15% per hour reported by others (DeVito, Gordon, Cabrera & Fasciolo, 1970; Braverman, Freeman & Rostorfer, 1971; Corsini, Crosslan & Bailie, 1974). The decreased renin content after a 2-hr period of secretion (Table 3), whether this conclusion is reached by com-

paring contents of control and ouabain-treated slices or by comparing contents of control and non-incubated slices, suggests that renin synthesis and/or activation do not keep pace with the 'control' *in vitro* release rate. Conceivably, slices could become depleted of renin, and therefore stop secreting, during an experiment, particularly if renin secretion were to be stimulated above the 'control' rate, e.g. by Ca-depletion as indicated in the legend to Fig. 3. It could be argued that renin depletion, rather than inhibited secretion, accounted for the suppressed renin release when Ca was added (experiments of Fig. 4). This is an unlikely possibility, since similarly treated slices continued to secrete at a high rate for an additional 30 min (Fig. 3), and since only 786 ± 66 ng hr^{-1} mg^{-1} of the total content had been secreted by the time Ca was added (Fig. 4) compared to 1110 ± 92 ng hr^{-1} mg^{-1} (Fig. 3). However unlikely, the possibility must be considered, and this emphasizes the importance of demonstrating the reversibility of the effect of Ca, i.e. the parallel experiment depicted in Fig. 4.

The above results are less equivocal than those of Park & Malvin (1978). In their experiments, Ca-depletion with EGTA also stimulated renin release, five- to tenfold above the control rate. Renin release into nominally Ca-free medium (without EGTA) was already depressed below control prior to the addition of ouabain (see legend to their fig. 3, but also Table 1), and ouabain had no further inhibitory effect. On the one hand, the lack of effect could conceivably be due to renin-depletion prior to addition of ouabain. On the other hand, the actual concentration of free Ca in nominally Ca-free medium might be sufficiently low to block an inhibitory effect of ouabain, but also high (or low) enough to have an intrinsic inhibitory effect by itself (see final paragraph).

In dogs, *in vivo* and *in vitro* renin secretion rates appear to be comparable (Park, Malvin, Murray & Cho, 1978). In rats, the 'control' *in vitro* rate is probably at least an order of magnitude higher than *in vivo* (Blendstrup, Leyssac, Poulsen & Skinner, 1975), which raises a question about the physiological relevance of *in vitro* experiments in general: are the results merely 'laboratory curiosities'? The parallel effects of substances on *in vivo* and on *in vitro* renin secretion rates would suggest otherwise. Ouabain inhibits both *in vitro* and *in vivo* rates, as documented above; so do angiotensin II (Vander & Geelhoed, 1965; Veyrat & Rosset, 1972), and increases in extracellular K concentration (Vander, 1970; Park & Malvin, 1978). Catecholamines stimulate rates in both cases (Vander, 1965; Aoi, Wade, Rosner & Weinberger, 1974). In addition, only *in vitro* experiments offer the advantage of controlling some of the multiple factors that stimulate or inhibit renin secretion *in vivo*.

If the results of this study are considered with those of other *in vitro* renin secretion studies, a consistent pattern emerges. It has been amply demonstrated that reductions in extracellular Na inhibit renin release (Braverman *et al.* 1971; Forgacs & Gaal, 1971; Hammersen, Karsunky, Fischinger, Rosenthal & Taugner, 1971; Weinberger & Rosner, 1972; Lyons & Churchill, 1974, 1975). In many of these experiments, both osmolality and Cl concentration were held constant, so that the inhibitory effect can be attributed specifically to a reduction in the transmembrane Na gradient. That ouabain inhibits Na, K-ATPase, resulting in a gradual increase in intracellular Na, and a concomitant reduction in the transmembrane Na gradient, can be considered a well established fact (Akera & Brody, 1978). Like ouabain, low extracellular K concentrations inhibit Na, K-ATPase and both ouabain and low extra-

cellular K concentration inhibit renin secretion from rat renal cortical slices; half-maximal 'activation' of renin secretion occurs between 1 and 2 mM extracellular K concentration (unpublished observations). The common feature of these experiments, in which renin secretion is inhibited, would appear to be a reduced transmembrane Na gradient.

In many secretory cells, reductions in the transmembrane Na gradient, produced by lowering extracellular Na or K, or by ouabain, result in cellular accumulation of Ca (mediated by a membrane-bound Na–Ca exchange mechanism), and the increased intracellular concentration of free Ca triggers exocytosis in an as yet unknown manner (Rubin, 1974). The Na–Ca exchange mechanism, first discovered in invertebrate nerve, has been described in a variety of mammalian cell types including cardiac and smooth muscle, both endocrine and exocrine secretory cells, and motor axon terminals (Blaustein, 1974; Baker, 1976; Akera & Brody, 1978; Rosenberger & Triggle, 1978). Na–Ca exchange can be regarded as a mechanism for either Ca efflux or Ca influx, but in either case, cellular accumulation results from a reduced Na gradient. On the one hand, passive Na influx down its electrochemical gradient can drive Ca efflux against its gradient, given the appropriate Na:Ca ratio; reductions in Na gradient then decrease Ca efflux (Baker, 1976). On the other hand, passive Ca influx down its larger electrochemical gradient can drive Na efflux against its gradient, and reductions in the Na gradient facilitate Ca influx (Akera & Brody, 1978).

The existence of Na–Ca exchange in juxtaglomerular cells seems plausible as mentioned in the Introduction, and on this admittedly speculative basis, it could be postulated that Ca accumulation, due to reductions in transmembrane Na gradient, results in inhibition of renin secretion (rather than stimulation, as is observed with other secretory cells). The more general postulate, that renin secretion is inhibited by Ca accumulation by whatever mechanism, has been advanced by others previously, and their results have been consistent: Ca ionophores inhibit renin secretion (Baumbach & Leyssac, 1977; Fynn, Onamakpome & Peart, 1977); angiotensin II and high extracellular K concentrations also inhibit renin secretion in a Ca-dependent manner (VanDongen & Peart, 1974; Park & Malvin, 1978). Taken together, these results argue strongly against Ca-mediated exocytosis as the mechanism controlling renin secretion, and instead argue that Ca influx and accumulation inhibit renin secretion.

Finally, it must be emphasized that there are published results seemingly at variance with an inhibitory role of Ca in renin secretion. Perhaps the most intriguing is the effect on renin secretion of nominally Ca-free medium. Although not consistently observed (Park & Malvin, 1978), renin secretion *in vitro* is often suppressed in 'Ca-free' media (Michelakis, 1971; Churchill, unpublished observations). Interestingly, the diametrically opposite result is often obtained with the insulin-secreting beta cell. Secretion is often enhanced in 'Ca-free' media, and secretion can be inhibited by increasing extracellular Ca (Hales & Milner, 1968*b*). Unless intracellular concentration of Ca actually increases above control level during such experiments, these results seem equally to exclude Ca-mediated exocytosis (insulin secretion) and Ca-mediated inhibition of renin secretion. In fact, increased Ca influx from 'Ca-free' media is not implausible. Cell membrane permeability is increased by reductions in Ca (unless sufficient Mg is present), and extracellular Ca must be reduced to around

10^{-7} M to abolish the gradient for Ca entry (Rubin, 1974). Depending upon impurities in reagents used to make the incubation fluid, the quality of the distilled H_2O, leaching of Ca from glassware, etc., free Ca can be as high as 10^{-5} M in nominally Ca-free fluid (Putney & Askari, 1978). In conclusion, elucidation of the exact role played by Ca in the control of renin secretion awaits the development of techniques for measuring intracellular concentration of ionized Ca in a preparation that consists only of renin-secreting cells. Until this is achieved, evidence of an inhibitory role will necessarily be indirect.

I am very grateful for the expert assistance of my wife, Monique C. Churchill, and Mr James Nacy. This work was supported by the National Science Foundation, grant PCM 77-01134. Salary support from the Skillman Foundation of Detroit and from B.M.A. of Detroit is also gratefully acknowledged.

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