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# SEPARATE AND COMBINED EFFECTS OF OUABAIN AND EXTRACELLULAR POTASSIUM ON RENIN SECRETION FROM RAT RENAL CORTICAL SLICES

BY MONIQUE C. CHURCHILL AND PAUL C. CHURCHILL

*From the Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201, U.S.A.*

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SUMMARY

1. Renin secretion of rat renal cortical slices was measured as a function of extracellular K and ouabain concentrations in the incubation medium.

2. A sigmoid relationship was found between renin secretion and log K concentration over the range 1·0–4·0 mM. Secretion was maximal at about 2·25 mM-K and half-maximal at about 1·43 mM-K.

3. In media containing 4·0 mM-K, ouabain at $10^{-8}$, $10^{-7}$, and $10^{-6}$ M did not affect renin secretion. Higher concentrations of ouabain inhibited secretion. A sigmoid relationship was found between % inhibition of secretion and log ouabain concentration ($10^{-6}$–$10^{-3}$ M). Inhibition was half-maximal at $2{\cdot}3 \times 10^{-5}$ M and complete at $10^{-3}$ M-ouabain.

4. Lowering extracellular K concentration from 4·0 to 2·25 mM shifted the dose-effect curve of ouabain to the left. At 2·25 mM-K, inhibition of renin secretion was half-maximal at $10^{-5}$ M-ouabain.

5. The inhibitory effect of $2 \times 10^{-5}$ M-ouabain (twice the dose for 50 % inhibition) in media containing 2·25 mM-K was nearly identical to the combined effect of lowering K to 1·43 mM (the concentration required for 50 % inhibition) and adding $10^{-5}$ M-ouabain. This observation suggests that ouabain and low extracellular K act at a common site, presumably on Na, K-ATPase activity, to inhibit renin secretion.

6. Neither $10^{-3}$ M-ouabain nor K-free medium inhibited renin secretion when the concentration of free Ca in the medium was lowered to $< 10^{-8}$ M. Therefore it is proposed that as a result of Na, K-ATPase inhibition, (*a*) intracellular Na increases, (*b*) intracellular Ca increases via Na-Ca exchange, provided that extracellular Ca exceeds $10^{-8}$ M, and that (*c*) Ca accumulation, in some unknown manner, inhibits renin secretion from rat renal cortical slices.

## INTRODUCTION

There are several reports that ouabain inhibits renin secretion. Infusion of ouabain into the renal artery blocks the stimulation of renin secretion elicited by aortic clamping or ureteral occlusion (Churchill & McDonald, 1974), by stimulation of the renal nerves (Haulica, Petrescu, Branisteanue, Rosca & Balan, 1974), and by administration of furosemide (Blaine & Zimmerman, 1978). Moreover, addition of ouabain to the

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incubation medium almost completely abolishes basal renin secretion from rat and from pig renal cortical slices (Lyons & Churchill, 1974, 1975*a*; Park & Malvin, 1978). Since no biochemical entity other than Na, K-ATPase is directly affected by ouabain (Akera & Brody, 1978; Putney & Askari, 1978), these results suggest that ouabain inhibits secretion by increasing intracellular Na concentration (via inhibition of Na, K-ATPase activity and active transport of Na and K), in accord with a reciprocal relationship between intracellular Na and secretion, as proposed by Vander (1967).

However, ouabain has biphasic effects on renal microsomal Na,K-ATPase activity and active cation transport in some species; high concentrations are inhibitory while low and intermediate concentrations stimulate or have no effect at all, respectively (Palmer & Nechay, 1974). The possibility of a biphasic effect on renin secretion is suggested by the observations that while $10^{-3}$ M-ouabain inhibits secretion from rat kidney slices (Lyons & Churchill, 1974, 1975*a*), $10^{-4}$ M-ouabain transiently stimulates secretion from isolated rat juxtaglomerular cells (Baumbach, Leyssac & Skinner, 1976).

One of the aims of the experiments reported below was to generate ouabain dose–effect curves for renin secretion from rat kidney slices. A second aim was to test the hypothesis that reductions in extracellular concentration of K inhibit renin secretion, presumably by inhibiting Na,K-ATPase activity (Dunham & Glynn, 1961; Skou, 1964, 1975; Whittam & Ager, 1964; Hokin, 1974). Thirdly, since ouabain and extracellular K presumably act at the same site (Na,K-ATPase activity), their interacting effects on renin secretion were investigated. Finally, the Ca-dependencies of the effects of ouabain and K were compared. It is well known that changes in intracellular Ca concentration mediate the effects of ouabain on such cellular activities as contraction and secretion (Rubin, 1970, 1974; Lee & Klaus, 1971; Baker, 1976; Akera & Brody, 1978; Putney & Askari, 1978), and it has been shown previously that Ca is required for an inhibitory effect of ouabain on renin secretion (Park & Malvin, 1978; Churchill, 1979).

## METHODS

All experimental and analytical procedures have been described in detail and validated previously (Churchill, 1979). Briefly, male Sprague-Dawley rats were anaesthetized with ether and nephrectomized. The renal capsule was removed and four thin cortical slices were cut from each kidney with a razor blade. Each time an experiment was performed, the slices from five rats were randomized, blotted gently, and placed in tared 25 ml. flasks (two slices per flask) which contained 10 ml medium previously equilibrated at 37 °C with 95/5 % $O_2/CO_2$. The flasks were stoppered, placed in an oscillating water bath maintained at 37 °C, and continuously flushed with the gas mixture Periodically, 200 $\mu$l. samples of medium were withdrawn, centrifuged at 4 °C, and the supernatants were frozen until determination of renin activity. Following the incubation, the flasks and contents were dried to constant weight. Tissue dry weight was calculated by subtracting the weight of the dried solutes. Tissue dry weight was approximately 10 mg per flask.

The incubation medium consisted of (mM) 124 NaCl, 19 $NaHCO_3$, 4 KCl, 2·6 $CaCl_2$, 1·2 $NaH_2PO_4$, and 0·8 $MgSO_4$, and 0·2/100 ml. each of glucose and fraction V bovine serum albumin (United States Biochemical Corp.). Variations from this composition are indicated in Results. Ouabain (Strophanthin G) was obtained from Calbiochem Corp. Ca- and $Na_2$ -EGTA (ethylene glycol-bis [beta-amino-ethyl ether] N,N′-tetra-acetic acid) were obtained from Sigma ChemicalCo.

Renin activity was measured by incubating 50 $\mu$l. sample with 500 $\mu$l. rat renin substrate at 37 °C for 30 min. Angiotensin I was measured by radioimmunoassay. Units for renin activity are ng $hr^{-1}$ $ml^{-1}$. (ng angiotensin I/hr of incubation of medium with substrate/ml. medium).

Total renin activity secreted by the slices was calculated as renin activity times the volume of the incubation medium divided by the tissue dry weight, yielding the units, ng $hr^{-1}$ $mg^{-1}$. Renin secretion rate of the slices was estimated as the increment in the total renin activity during a given time period of incubation, e.g., ng $hr^{-1}$ $mg^{-1}$/30 min.

The results are presented as means ± S.E. of means. Both paired and unpaired *t* tests, as indicated in Results, were used to assess statistical significance of observed differences or changes.

## RESULTS

During the incubation of rat kidney slices, the total renin activity of the medium progressively increases. Increments in the total renin activity during a given time period were used as estimates of secretion rate. Mean secretion rates during the 0–30, the 30–60, and the 60–90 min periods are given in Table 1. It can be seen that the control rate (uninhibited basal rate) decreases somewhat over time. It can also be seen that although some renin is secreted during the first 30 min of exposure to 1 mM-ouabain, secretion is ultimately completely inhibited. The negative value for secretion rate during the last period was not significantly different from zero and probably represents error in the renin measurement. The increments of $49 \pm 6$, $0 \pm 1$, and $-4 \pm 3$ ng $hr^{-1}$ $mg^{-1}$ per 30 min translate into total renin activities of $49 \pm 6$, $49 \pm 6$, and $45 \pm 5$ ng $hr^{-1}$ $mg^{-1}$ at the end of 30, 60 and 90 min of incubation, respectively. Taken together, the results in Table 1 suggest that the 0–30 min period of incubation should not be taken as base line for a particular condition (e.g., for the effect of 1 mM-ouabain) and also that any comparisons between different conditions should be made during the same time period.

TABLE 1. Renin secretion rate of rat kidney slices as a function of time

| | Secretion rate (ng $hr^{-1}$ $mg^{-1}$/30 min) | | |
|---|---|---|---|
| | 0–30 | 30–60 | 60–90 |
| Control ($n$ = 6) | $292 \pm 17$ | $261 \pm 11$ | $210 \pm 16$ |
| 1 mM-ouabain ($n$ = 6) | $49 \pm 6$ | $0 \pm 1$ | $-4 \pm 3$ |

Means ± S.E. of means. Secretion rates were calculated as the increments in total remin activity of the medium from 0–30, 30–60, and 60–90 min of incubation of the kidney slices.

Slices were incubated in media containing 4 mM-extracellular K, $[K]_e$, and from $10^{-8}$ to $10^{-3}$ M-ouabain for 30 min. The increment in total renin from 30–90 min was measured and in order to facilitate comparison with other results, this increment was divided by two to obtain an average 30 min secretion. Control secretion rate in the absence of ouabain (Table 1) is $236 \pm 10$ ng $hr^{-1}$ $mg^{-1}$/30 min, calculated in this manner. Secretion rates were $249 \pm 19$, $229 \pm 36$, and $221 \pm 20$ ng $hr^{-1}$ $mg^{-1}$/30 min in media containing $10^{-6}$, $10^{-7}$, and $10^{-8}$ M-ouabain ($n$ = 6 at each concentration). Since none of these averages differ significantly from the control average ($P > 0.05$, unpaired *t* test), it was concluded that ouabain at less than $10^{-6}$ M has no inhibitory effect on renin secretion.

In media containing 4 mM-$[K]_e$, ouabain over the range $10^{-6}$–$10^{-3}$ M was found to inhibit secretion. Percent inhibition was calculated as $100\,\%\ (1 - V/V_{max})$, where $V$ is the observed secretion rate at a given ouabain concentration and $V_{max}$ is

249 ng $hr^{-1}$ $mg^{-1}$/30 min, the maximum average rate of secretion. Means ± S.E. of means of observed % inhibition are shown in Fig. 1 as a function of the logarithm of ouabain concentration.

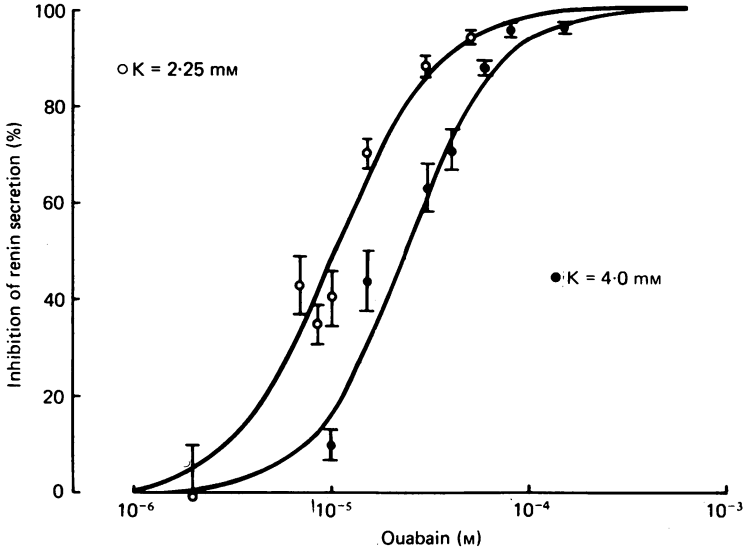


Fig. 1. Effect of ouabain on renin secretion from rat kidney slices. Percent inhibition of secretion is plotted against ouabain concentration (logarithmic scale) at two K concentrations (○, 2·25 mM and ●, 4·0 mM). Means ± S.E. of means, $n$ = 6 observations for each point.

According to Goodman & Gilman (1975), if one can assume reversible interaction of drug with receptor, and a resultant effect proportional to receptor occupany, then the equation describing the relationship between drug concentration ($D$) and effect ($E$) is identical in form with the Michaelis-Menten equation in enzyme kinetics: $E = E_{max} D/(K+D)$, where $E_{max}$ is the maximal effect and $K$ is the dissociation constant of drug from its receptor. Accordingly, a double reciprocal plot should be linear with a slope of $K/E_{max}$ and a $Y$-intercept of $1/E_{max}$. The double reciprocal plot of the above data ($1/E$ *vs.* $1/D$, where $E$ = % inhibition and D = ouabain concentration) was concave upward, suggesting by analogy with enzyme kinetics (Segel, 1975), that a relationship of the form $E = E_{max} D^n/(K' + D^n)$ would be a more accurate mathematical description of the experimental results. The linear transform of this equation, $\log [E/(E_{max} - E)] = n \log D - \log K'$, is analogous to the linear transform of the Hill equation (Segel, 1975). In the context of drug-receptor interaction, the Hill equation constants, $n$ and $K'$ can be interpreted as follows: $n$ is the number of drug binding sites per receptor, and $K'$ is a function of both the intrinsic dissociation constant of drug from receptor site and the degree of interaction between sites. Linear regression analysis of a plot of log {% inhibition/(100 % − % inhibition)} *vs.* log ouabain concentration yielded $r^2 = 0{\cdot}92$ ($P < 0{\cdot}0001$), $K' = 380$ μM, and $n = 1{\cdot}9$. The non-integral value for $n$ could suggest two sites per receptor with relatively strong cooperativity, or more than two sites with weaker cooperativity (Segal, 1975). The curve in Fig. 1, for 4 mM $[K]_e$, is a plot of the equation % inhibition = 100 % $D^n/(K' + D^n)$

using the stated values of $K'$ and $n$. It can be seen from the equation that renin secretion is 50 % inhibited when $D$ is $2{\cdot}3 \times 10^{-5}$ M-ouabain (the $ED_{50}$ for ouabain when $[K_e]$ = 4·0 mM).

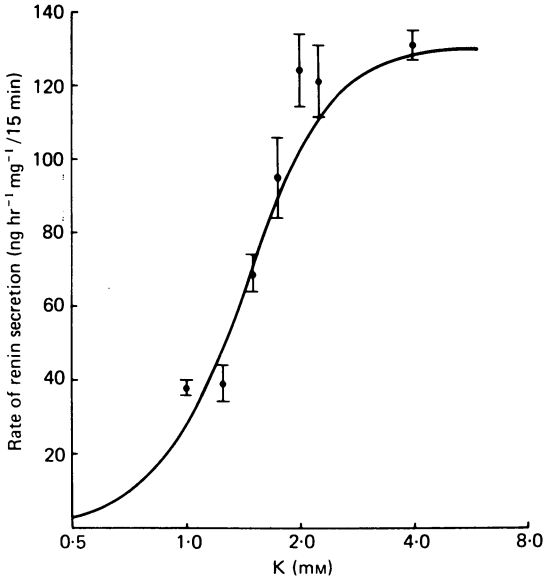


Fig. 2. Effect of extracellular K concentration on renin secretion from rat kidney slices. Rate of renin secretion (the increment in renin activity of the incubation medium over a 15 min period) is plotted against mM-K concentration (logarithmic scale). Means ± S.E. of means; $n$ = 6 observations at each point.

Similar experiments were carried out in media containing 2·25 mM-$[K]_e$, and the means ± S.E. of means of % inhibition are also shown in Fig. 1. The curve is a plot of the above equation, using values of $K'$ and $n$ of 53 $\mu$M and 1·7, respectively. These values were determined by linear regression of a plot of log {% inhibition/(100 % − % inhibition)} *vs.* log ouabain concentration, in which $r^2$ was found to be 0·95 ($P < 0{\cdot}001$). Accordingly, in media containing 2·25 mM-$[K]_e$, 50 % inhibition of renin secretion would occur at $10^{-5}$ M-ouabain. Since at 1, 1·5 and $3{\cdot}0 \times 10^{-5}$ M-ouabain, renin secretion rates were significantly lower in 2·25 than in 4·0 mM-$[K]_e$, $P < 0{\cdot}005$ maximum, unpaired $t$ test, it was concluded that lowering $[K]_e$ shifted the ouabain dose–response curve to the left, increasing the inhibitory effect of ouabain on renin secretion rate.

In preliminary experiments, renin secretion was almost abolished by incubation of slices in media with $[K]_e$ of 1 mM or less and secretion was maximal or near maximal at $[K]_e$ = 2 mM. In order to examine more closely the effect of $[K]_e$ on renin secretion, slices were incubated in media containing from 1 to 4 mM-$[K]_e$ and after a 30 min equilibration period, initial secretion rate ($V_i$) was calculated as the increment in renin activity of the medium from 30 to 45 min. Then, so that each slice could serve as its own control, KCl was added to raise $[K]_e$ to 4 mM in all flasks. Following another 30 min equilibration period, the final secretion rate ($V_f$) was calculated as the 75–90 min increment in renin activity. Means of $V_i$ are shown in Fig. 2. The curve in this

Figure is a plot of the equation $V_1 = V_{max} D^n/(K' + D^n)$ where $V_{max}$ is $131 \pm 4$ ng $hr^{-1}$ $mg^{-1}$/15 min, the initial rate when $[K]_e = 4$ mM, $K' = 3{\cdot}74$ mM, $D = [K]_e$ in mM, and $n = 3{\cdot}7$. The values of $K'$ and $n$ were determined by linear regression analysis of a plot of $\log [V_1/(V_{max} - V_1)]$ *vs.* $\log [K]_e$ in mM. The correlation coefficient was 0·95, $P < 0{\cdot}005$. From these data, it was concluded that renin secretion is 50% inhibited at $[K]_e = 1{\cdot}43$ mM ($ED_{50}$ for $[K]_e$).

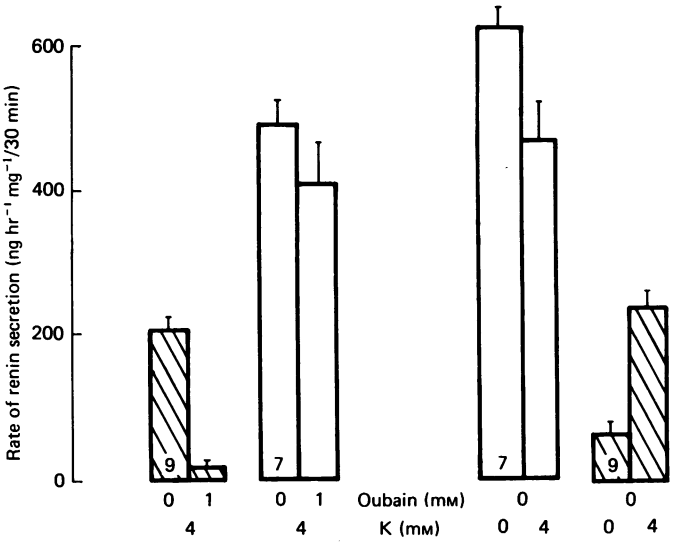


Fig. 3. The Ca dependency of the inhibitory effects of ouabain and of low extracellular K concentration on renin secretion from rat kidney slices. Left to right for the two columns in each pair of columns: renin secretion rates during the 20–40 and the 60–80 min periods of incubation of the slices. Ouabain (first and second pairs of columns) or K (third and fourth pairs) was added to the incubation media at 40 min. All media contained 2 mM-EGTA. Free Ca concentrations of the media were 2·65 mM (first and fourth pairs) and $< 10^{-8}$ M (second and third pairs). Means ± S.E. of means are shown. The number of observations ($n$) is indicated within the columns.

If both ouabain and $[K]_e$ are affecting renin secretion by acting at the same site, presumably on Na,K-ATPase activity, then the combined effect of $10^{-5}$ M-ouabain ($ED_{50}$ for ouabain when $[K]_e = 2{\cdot}25$ mM) and of $[K]_e = 1{\cdot}43$ mM ($ED_{50}$ for $[K]_e$) should approximate the effect of twice the $ED_{50}$ for ouabain when $[K]_e = 2{\cdot}25$ mM. To test this prediction, slices were equilibrated for 30 min in the following media: $[K]_e = 2{\cdot}25$ mM, ouabain $= 2 \times 10^{-5}$ M; $[K]_e = 1{\cdot}43$ mM, ouabain $= 10^{-5}$ M. Secretion rates calculated as the 30–45 min increments in renin activity (multiplied by 2 to conform with the units of ng $hr^{-1}$ $mg^{-1}$/30 min) were found to be $97 \pm 7$ ($n = 10$) and $95 \pm 10$ ($n = 10$) respectively, values which are not significantly different from each other ($P > 0{\cdot}05$).

Finally, a series of experiments was performed to compare ouabain and $[K]_e$ with respect to the Ca dependency of their inhibitory effects on renin secretion. Slices were incubated in normal medium to which was added 2 mM-Ca-EGTA. The 20–40 min increment in renin activity was taken as the initial secretion rate; then, at 40 min, ouabain was added (1 mM final concentration) and the 60–80 min increment in renin

activity was taken as the final secretion rate. These two increments, multiplied by 3/2 to conform with the units of ng $hr^{-1}$ $mg^{-1}$/30 min, are shown as the first-from-the-left pair of columns in Fig. 3. The presence of 2 mM-EGTA did not prevent ouabain from completely inhibiting renin secretion ($P < 0{\cdot}0001$, initial *vs.* final secretion rates, paired *t* test). The second pair of columns represents data from an identical experiment except that $CaCl_2$ was omitted from the incubation medium, and $Na_2$- rather than Ca- EGTA was used. Thus, the concentration of free Ca was $< 10^{-8}$ M (Caldwell, 1970). Although average secretion decreased somewhat upon ouabain addition, the decrease was not significant ($P > 0{\cdot}08$, paired *t* test). Therefore, ouabain inhibits renin secretion when Ca = 2·65 mM but not when Ca $< 10^{-8}$ M. Moreover, lowering Ca from 2·65 to $10^{-8}$ M *per se* stimulated secretion, as both columns in the second pair are higher than in the first pair ($P < 0{\cdot}0001$ maximum, unpaired *t* test). Slices were also incubated in K-free medium containing 2·65 mM-$CaCl_2$ and 2 mM-Ca-EGTA, and K was added (4 mM final concentration) at 40 min. The two increments in renin activity, 20–40 min and 60–80 min, multiplied by 3/2 to conform with the units ng $hr^{-1}$ mg $hr^{-1}$/30 min were taken as initial and final secretion rates. These are shown as the fourth pair of columns in Fig. 3. Although secretion was not completely abolished in K-free medium, perhaps because of an inadequate equilibrium period (20 min rather than the usual 30), the rate was clearly less in K-free medium than in the control medium ($P < 0{\cdot}01$, initial rates in column-pairs 1 and 4, unpaired *t* test). Addition of *K* significantly increased renin secretion rate ($P < 0{\cdot}0001$, paired *t* test), and the final rate was comparable to the initial rate in the first column pair ($P < 0{\cdot}05$, unpaired *t* test). The third column pair represents data from an identical experiment except that Ca was $< 10^{-8}$ rather than 2·65 mM. The addition of K did not accelerate renin secretion. In fact, rate was significantly higher before K addition ($P < 0{\cdot}01$, paired *t* test). Therefore, low $[K]_e$ like ouabain, inhibits renin secretion rate when extracellular Ca = 2·65 mM but not when Ca is $< 10^{-8}$ M. Again, lowering Ca from 2·65 mM to $< 10^{-8}$ M *per se* stimulated renin secretion as both columns in the third pair are higher than in the fourth pair ($P < 0{\cdot}001$ maximum, unpaired *t* test).

## DISCUSSION

The above results confirm the observation that ouabain at $10^{-3}$ M inhibits renin secretion from rat renal cortex slices (Lyons & Churchill, 1974, 1975*a*). Moreover, they indicate that, over the range $10^{-8}$–$10^{-3}$ M, ouabain either inhibits or has no effect on renin secretion from this preparation. Although ouabain has been reported to have biphasic effects on Na, K-ATPase activity and on cation transport in some species (Palmer & Nechay, 1964; McClane, 1965), biphasic effects on rat kidney Na,K-ATPase have not been reported, and biphasic effects on renin secretion were not found in the present study. Thus, it is unlikely that the slight stimulatory effect of $10^{-4}$ M-ouabain on renin secretion from isolated rat juxtaglomerular cells (Baumbach *et al.* 1976) or the lack of effect on renin secretion from rat kidney cell suspensions (Lyons & Churchill, 1975*b*) can be explained in this manner. Perhaps some characteristic of the different preparations, such as an intact relationship between juxtaglomerular cells and macula densa cells in the slices but not in the other two preparations, might explain the divergent results.

With respect to the mechanism of action of ouabain, the only biochemical entity known to be directly affected by ouabain is Na,K-ATPase (Akera & Brody, 1978; Putney & Askari, 1978). Accordingly, inhibition of renin secretion can be attributed to inhibition of Na,K-ATPase activity and of active Na,K transport. Na,K-ATPase activity and active Na,K transport can also be inhibited by lowering extracellular concentration of K. (Dunham & Glynn, 1961; Skou, 1964, 1975; Whittam & Ager, 1964; Hokin, 1974; Robinson, 1974). Consistent with expectation then, reductions of extracellular K concentration were found to inhibit renin secretion.

Many of the effects of cardiac glycosides are potentiated by reductions in extracellular K concentration, and in the present study, decreasing the K concentration from 4·0 to 2·25 mM shifted the ouabain dose-effect curve to the left. Moreover, the inhibitory effect of combining the $ED_{50}$s for K and for ouabain was not significantly different from the inhibitory effect of twice the $ED_{50}$ for ouabain alone. This observation suggests that ouabain and low extracellular K concentration were acting at the same site, presumably on Na,K-ATPase activity, to inhibit the secretion of renin.

It has been shown previously that Ca is required for the inhibitory effect of ouabain on renin secretion from pig and from rat kidney slices (Park & Malvin, 1978; Churchill, 1979). The results presented above confirm the observation with respect to the rat, and further demonstrate that the inhibition of renin secretion by low extracellular concentration of K also requires Ca concentrations in excess of $10^{-8}$ M.

It is well known that inhibition of Na,K-ATPase, with either ouabain or low extracellular K, alters the activities of many specialized cells in a Ca-dependent manner (Rubin, 1970, 1975; Baker, 1976; Fleckenstein, 1977; Akera & Brody, 1978). According to current thought, inhibition of Na,K-ATPase activity alters the rate of Na-Ca exchange across the cell membrane, resulting in an increase in intracellular concentration of Ca. Na-Ca exchange can be regarded as either a mechanism for Ca efflux (Na influx down its electrochemical gradient drives Ca efflux against its gradient; Baker, 1976) or as a mechanism for Ca influx (Ca influx down its gradient drives Na against its gradient (Akera & Brody, 1978). In either case, reduced activity of Na, K-ATPase results in a decrease in transmembrane Na gradient, and this favours Ca accumulation; in the first instance by decreased Ca efflux, in the latter by increased Ca influx.

There is evidence for Na-Ca exchange in cardiac and smooth muscle cells, in neurons, and in exocrine and endocrine secretory cells (Blaustein, 1974; Baker, 1976; Akera & Brody, 1978; Rosenberger & Triggle, 1978). The renin-secreting juxtaglomerular apparatus consists, at least in part, of differentiated smooth muscle cells (Barajas, 1971). On this basis, it might be suggested that the inhibitory effect of ouabain and low extracellular K depend upon cellular Ca accumulation via altered Na-Ca exchange. That inhibitory effects failed to occur in media with low Ca concentration ($< 10^{-8}$ M) is consistent with this suggestion. Moreover, several other observations are consistent with an inhibitory coupling role of Ca in the control of renin secretion. Ca is required for inhibition of renin secretion by either angiotensin II (VanDongen & Peart, 1974) or depolarizing concentrations of extracellular K (Park & Malvin, 1978). Ca ionophores, which act as Ca carriers or Ca channel forming species (Rosenberger & Triggle, 1978) inhibit renin secretion (Baumbach & Leyssac, 1977; Fynn, Onamakpome & Peart, 1977). However, the ultimate test of the hypothesis

that Ca plays an inhibitory coupling role will require techniques for measuring intracellular Ca in a pure population of renin-secreting cells. Measurements of tissue Ca are not particularly meaningful, particularly in a kidney slice, as the relevant population of cells comprise somewhat less than 0·005 % of kidney weight (Morris & Johnson, 1976).

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