In Vitro Inhibition of Aldosterone-Stimulated Sodium Transport by Steroidal Spirolactones

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SUMMARY

The spirolactones SC 9420 and SC 14266 both are effective inhibitors of the aldosterone-induced increase in *in vitro* sodium transport as measured in the isolated toad bladder. These compounds appear to be specific antagonists of aldosterone as the short-circuit current response to exogenous vasopressin and glucose repletion were not altered by concentrations of spirolactone sufficient to inhibit the electrophysiologic effects of aldosterone. The results of kinetic analysis of aldosterone-spirolactone interaction fulfilled the criteria for competitive inhibition. Assuming that the tissue receptors for aldosterone are homogeneous, the relative affinity for these receptors was estimated by the ratio of the dissociation constants of aldosterone (K_D) and spirolactone (K_i). Using two separate derivations, each from independent data, the resulting ratios for aldosterone: SC 14266 were in reasonable agreement, i.e., 1:235 and 1:336. The average maximal effective aldosterone concentration ($2 \times K_D$) was calculated to be 1×10^{-8} M, while the inhibitor constant (K_i) for the spirolactone SC 14266 was 1×10^{-6} M by direct plot and 2.6×10^{-6} M when derived from a Lineweaver-Burk plot. The K_i for SC 9420 by direct plot was 5.25×10^{-6} M and the $K_D:K_i$ ratio was 1:681.

INTRODUCTION

The contention that spirolactones are competitive inhibitors of aldosterone is primarily based upon evidence obtained from *in vivo* experimentation (1). Under conditions of either enhanced endogenous aldosterone secretion or exogenous administration, spirolactones have been shown to antagonize the hormone's physiologic effect on the kidney (2). In addition, spirolactone antagonism of aldosterone in laboratory animals may be overcome by increasing the concentration of administered aldosterone as judged by changes in urinary electrolyte excretion (3). However, because of the limited information regarding *in vitro* antagonism of the action of aldosterone by spirolactones, I have undertaken a series of experiments using the isolated urinary bladder of the toad. This *in vitro* system was selected since the conditions necessary to demonstrate the mineralcocorticoid action of aldosterone in this membrane are well defined (4, 5).

Objectives of my experiments were (a) to define, if possible, the kinetics of spirolactone inhibition of the aldosterone-induced stimulation of Na^+ transport; (b) to determine whether steroidal spirolactones possess any independent stimulatory action on active sodium transport; and (c) to define the specificity of spirolactone inhibition on the *in vitro* action of aldosterone.

METHODS

Paired urinary hemibladders, removed after rapid double pithing of *Bufo marinus*, were mounted in glass chambers as previously described (5). The temperature of the frog-Ringer solution ($Na^+ = 114$, $K^+ = 3.5$, $Ca^{2+} = 5.4$, $Cl^- = 120$, $HCO_3^- = 2.5$

mEq/liter; osmolality 0.228; pH in air = 8.4) which bathed the hemibladders was maintained at 24.8° ± 0.3° (SD) by circulating constant temperature water through thin-walled glass heating coils immersed in each chamber. The hemibladders were mounted the evening prior to each experiment and left open-circuited overnight, bathed by circulating-aerated frog-Ringer solution containing added glucose (5.5×10^{-3} M), penicillin G (1×10^{-2} μg/ml), and streptomycin (50 μg/ml). The following morning, chamber solutions were replaced with antibiotic-free, glucose-enriched frog-Ringer and the short-circuit current (scc) was measured continuously by the method of Ussing and Zerahn (6). Under the conditions of these experiments it has been shown by ^{22}Na flux measurements that scc measures net active sodium transport (5). After a 30-min stabilization period following the morning solution change, experimental manipulations were commenced.

Various concentrations of *d*-aldosterone (California Biochemical Research Corporation, Los Angeles, California) were added to both the mucosal and serosal surfaces of the hemibladder, ranging from 7×10^{-10} M to 3.5×10^{-6} M with 7×10^{-8} M the most frequent dose administered. 3-(3-Oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl)propionic acid lactone (SC 9420),[1] dissolved in [illegible]% ethanol was added to both the serosal and mucosal surfaces to yield concentrations ranging from 3.5×10^{-7} M to 7×10^{-5} M. To achieve chamber concentrations greater than 7×10^{-5} M, the ethanol content required to maintain SC 9420 solubility was deleterious to the membrane. Potassium 3-(3-oxo-17β-hydroxy-4,6-androstadien-17α-yl) propanoate (SC 14266),[1] a water-soluble spirolactone, was added to both surfaces of the hemibladder in final concentrations ranging from 7×10^{-7} M to 3.5×10^{-3} M. In those experiments in which aqueous vasopressin (Parke, Davis and Co., Detroit, Michigan, Lot No. DG 105-1) was used, sufficient undiluted material was added to the serosal surface to achieve a final chamber concentration of 80 mU/ml and the scc was read at 30-sec intervals until a peak response was evident.

For paired experiments the scc response to aldosterone was expressed as a function of time with the scc ratio (R_t) derived by dividing the scc at any time t (SCC_t) by the scc at the time of aldosterone addition (SCC_0). When spirolactone was added before aldosterone, a minus time notation was used (SCC_{-t}).

Statistical analysis was performed using the student t test for paired observations (7). In addition, for each group of experiments, the scc value in microamperes at the time of aldosterone addition (SCC_0) in the spirolactone-treated hemibladder was compared to its untreated mate, and the absence of a statistical difference confirmed random selection.

RESULTS

1. Effect of spirolactone compounds on basal sodium transport. Table 1 is a tabulation of the short circuit ratio after 6 hr of exposure to the stated concentrations of SC 14266 compared to the paired hemibladders which received only diluent. As can be seen, at a concentration of 3.5×10^{-3} M all hemibladders lost their electrical potential. Therefore, this concentration of SC 14266 is defined as a toxic concentration for this particular biologic system. Since SC 14266 is a potassium salt, the independent effect of this cation was evaluated by determining the scc response to a 3.5 mM increase in potassium content of the bathing media. The increased potassium content, per se, does not account for the toxicity noted at the high concentration of SC 14266 (see Table 1). There was no evidence that any of these concentrations of SC 14266 were capable of stimulating sodium transport when compared to paired control hemibladders.

The maximum volume of 100% ethanol required to solubilize 7×10^{-5} M SC 9420 in frog-Ringers' resulted in a final chamber concentration of 0.02%, and did not interfere with basal sodium transport rates. SC

[1] Kindly supplied by Dr. L. M. Hoffman, Division of Biological Research, G. D. Searle and Co., Chicago, Illinois.

TABLE 1
Effect of increasing concentrations of SC 14266 on steroid-independent Na^+ transport

SC 14266 concentration (M)	Number of paired experiments	SCC_0 (μamp)	SCC_6/SCC_0
7×10^{-6}	8	C[a] 81 ± 16	0.62 ± 0.05
		E[b] 86 ± 10	0.68 ± 0.07
7×10^{-5}	7	C 58 ± 6	0.48 ± 0.05
		E 64 ± 5	0.60 ± 0.07
3.5×10^{-4}	8	C 39 ± 8	0.55 ± 0.08
		E 36 ± 5	0.61 ± 0.08
3.5×10^{-3}	4	C 43 ± 8	0.53 ± 0.10 (*)
		E 57 ± 19	0
3.5×10^{-3} KCl	4	C 49 ± 8	0.81 ± 0.10
		E 45 ± 5	0.90 ± 0.18

[a] C = Control hemibladders.
[b] E = experimental hemibladder, spirolactone or potassium chloride treated.
(*) $P < 0.05$.

9420 in concentrations up to 3.5×10^{-5} M was without effect on the scc ratio; however, at 7×10^{-5} M a significant depression was recorded (Table 2).

2. Effect of varying the temporal relationship of spirolactone addition to that of aldosterone. 3.5×10^{-5} M SC 9420 or 7×10^{-5} M SC 14266 was added 2.5 hr before to 4 hr after the addition of 7×10^{-8} M aldosterone. The scc ratio for each pair was calculated and statistically analyzed by applying the *t* test to the mean differences 6 hr after aldosterone addition. These data are shown on Table 3 for both spirolactones. As is evident under these experimental conditions unless spirolactone treatment precedes aldosterone addition, clear-cut inhibition of aldosterones' stimulation of scc cannot be demonstrated.

3. Kinetic analysis of the interrelationship between aldosterone and SC 14266. Since this type of analysis requires evaluation of the effect of various concentrations of steroid and inhibitor on sodium transport, the scc ratio is not a satisfactory method of expressing the *in vitro* response since comparison of various paired groups is necessary. Therefore, certain characteristics of the scc response to aldosterone must be identified and the method of expressing these results defined. During the 6–8 hr interval of experimental observation,

TABLE 2
Effect of increasing concentrations of SC 9420 on steroid-independent Na^+ transport

SC 9420 concentration (M)	Number of paired experiments	SCC_0 (μamp)	SCC_6/SCC_0
3.5×10^{-6}	6	C[a] 70 ± 6	0.70 ± 0.08
		E[b] 67 ± 7	0.62 ± 0.06
3.5×10^{-5}	10	C 73 ± 7	0.56 ± 0.05
		E 77 ± 7	0.54 ± 0.04
7×10^{-5}	8	C 85 ± 11	0.62 ± 0.04 (*)
		E 93 ± 13	0.38 ± 0.05
0.2% Ethanol	8	C 88 ± 12	0.63 ± 0.03
		E 88 ± 13	0.58 ± 0.07

[a] C = control hemibladders.
[b] E = experimental hemibladders, spironolactone or ethanol treated.
(*) = $P < 0.05$.

TABLE 3

Effect of varying the time of adding spirolactone on inhibiting the scc response to aldosterone-stimulated Na^+ transport

Time of spirolactone addition (hr)	Number of paired experiments	SCC_0 (μamp)	SCC_6/SCC_0
	7×10^{-5} M SC 14266–7×10^{-8} M aldosterone		
−2.5	9	C[a] 42 ± 8	2.28 ± 0.16 (*)
		E[b] 46 ± 10	1.44 ± 0.15
−1.5	10	C 79 ± 19	2.36 ± 0.15 (*)
		E 83 ± 23	1.54 ± 0.14
−0.25	8	C 41 ± 11	2.80 ± 0.35 (*)
		E 38 ± 6	2.02 ± 0.26
0	10	C 60 ± 5	2.67 ± 0.26
		E 58 ± 5	2.39 ± 0.17
+1.5	8	C 118 ± 24	1.96 ± 0.15
		E 94 ± 21	1.74 ± 0.13
+4	10	C 141 ± 25	1.72 ± 0.21
		E 167 ± 35	1.58 ± 0.23
	3.5×10^{-5} M SC 9420–3.5×10^{-7} M aldosterone		
−1.5	6	C 94 ± 11	1.86 ± 0.09 (*)
		E 88 ± 10	1.13 ± 0.10
0	4	C 100 ± 16	1.90 ± 0.09
		E 105 ± 15	1.97 ± 0.13
+1.5	9	C 88 ± 8	1.49 ± 0.08
		E 85 ± 10	1.48 ± 0.10
+4	6	C 78 ± 10	1.59 ± 0.11
		E 82 ± 13	1.56 ± 0.13

[a] C = aldosterone-treated hemibladders.
[b] E = spirolactone/aldosterone-treated hemibladders.
(*) = $P < 0.05$.

the scc declines at a rate of 5.5%/hr or 33% over a 6-hr period. Therefore, if one is to determine the absolute increase in scc which follows aldosterone, with or without spirolactone, this decline must be accounted for:

$$\Delta SCC_6 = SCC_6 - (0.67)(SCC_0).$$

Secondly, since the current at time zero (SCC_0) cannot be regulated, differences between the SCC_0 for each group must be included in the final expression. This was accomplished by normalizing the derived ΔSCC_6 to a standard SCC_0 value of 100 μamp:

$$\Delta SCC'_6 = \frac{\Delta SCC_6 \times 100}{SCC_0}$$

The rationale for this calculation is shown in Fig. 1, which relates the SCC_6 to the SCC_0. As can be seen, over a SCC_0 range of 0 to 100 μamp there exists a linear relationship between these two current readings. Therefore, the data included in kinetic analysis was limited to SCC_0 of 100 μamp or less.

Finally, since these calculations are an expression of the slope of the regression line resulting from such a correlation, a relationship between slope (SCC_6/SCC_0) and the concentration of aldosterone is necessary. Table 4 summarizes the correlation statistics for the several aldosterone concentration. At concentrations below 7×10^{-9}M the difference in intercept precludes the use of slope as an indicator of the *in vitro* response, thus the minimal concentration of aldosterone used in this series of experiments was 7×10^{-9} M.

The data used to obtain the Lineweaver-Burk plot shown in Fig. 2 are summarized on Table 5. From the intercept, the maximum response for $\Delta SCC'_6$ is calculated to 154 μamp, while $K_D = 7.7 \times 10^{-9}$ M and

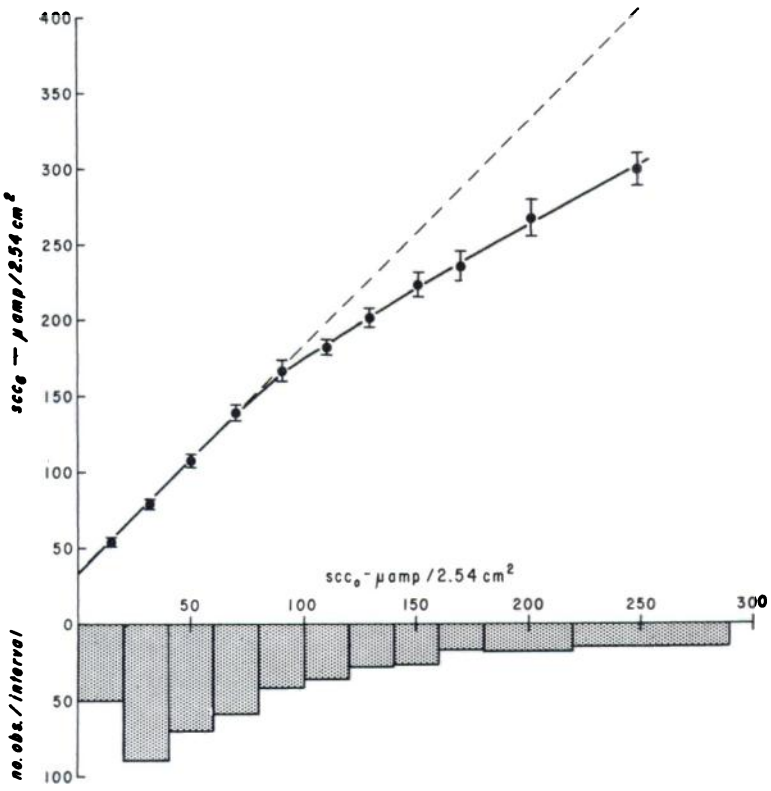

FIG. 1. *Regression line describing the correlation between the short-circuit current 6 hr after aldosterone (SCC_6) and the short-circuit current at the time of addition (SCC_0)*

The mean value with standard deviations are shown for 459 experiments in the upper portion of the figure, and the number of experiments included at each interval are shown at the bottom of the graph. The slope of the first portion of the regression line is 1.48 with an intercept of 33 μamp. The second portion of the regression line ($SCC_0 > 100$ μamp) is 0.83 with an intercept of 93 μamp.

$K_i = 2.6 \times 10^{-6}$ M. These results were obtained by measuring the scc response in paired hemibladders, one member being given 3.5×10^{-5} M SC 14266 and then 30 min later both members of the pair received the indicated concentration of aldosterone (Table 5). However, by fixing the concentration of aldosterone and varying the concentration of SC 14266, K_i can be determined directly from the plotted data by the intercept method of Dixon (8). Experiments using SC 14266 pretreatment were performed at two different concentrations of aldosterone, i.e., 7×10^{-9} M and $7 \times$

TABLE 4

Correlation statistics for various aldosterone concentrations

Aldosterone concentration (M)	None	7×10^{-10}	2×10^{-9}	7×10^{-9}	2×10^{-8}	3.5×10^{-8}	5.5×10^{-8}	7×10^{-8}	3.5×10^{-7}	7×10^{-7}
Number of experiments	71	12	8	50	18	12	10	104	13	68
Slope	0.67	0.78	0.90	1.01	1.42	1.51	1.47	1.52	1.48	1.48
Intercept	−3.0	1.6	−2.0	21.4	28.7	30.8	29.4	33.8	35.4	34.0
Correlation coefficient	0.92	0.84	0.82	0.85	0.89	0.91	0.90	0.91	0.85	0.88

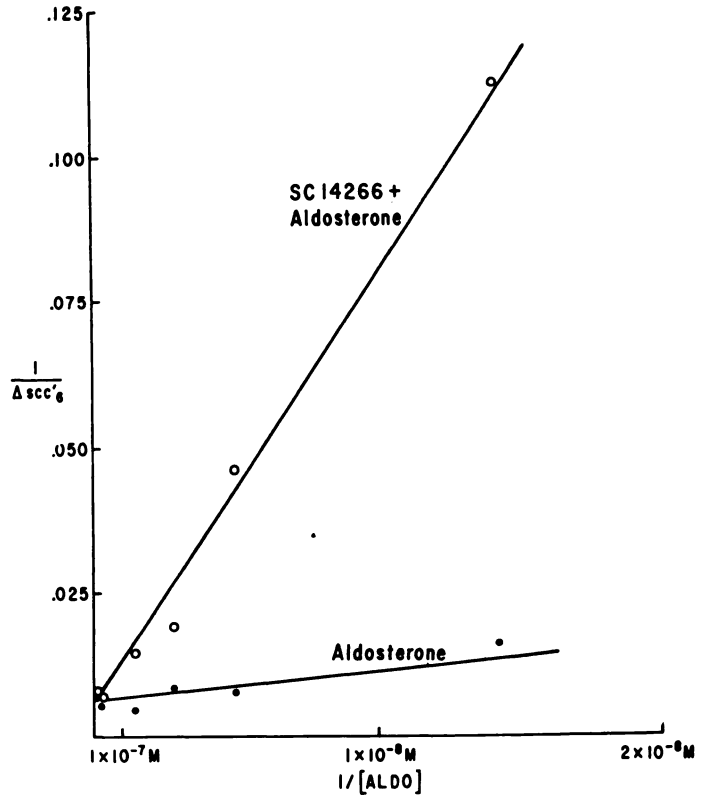

FIG. 2. *Lineweaver-Burk plot of the short-circuit response to various concentrations of aldosterone with and without 3.5 × 10⁻⁵ M SC 14266 pretreatment*

The maximum response derived from the intercept on the Y axis is 154 μamp. The equilibrium constant or half-maximal aldosterone concentration (K_D) is 7.7×10^{-9} M, while the dissociation constant for SC 14266 (K_i) is 2.6×10^{-6} M.

10^{-8} M. The data summarized on Table 6 were used to obtain the plot shown on Fig. 3. The intercept to the left of the Y axis gives $-K_i$ which, by projection on the X axis, indicates a value of 1×10^{-6} M. The K_D derived from the slope of the 7×10^{-9} M aldosterone line is 3×10^{-9} M while the K_D from the 7×10^{-8} M aldosterone line is 5.5×10^{-9} M. The maximum response for $\Delta SCC'_6$, obtained by projection of the intercept on the Y axis, is 145 μamp. Data for the effect of 4 different concentrations of SC 9420 on the scc response induced by 7×10^{-8} M Aldosterone are also shown on Table 6. The reciprocals of $\Delta SCC'_6$ were also plotted according to the method obtained by Dixon (8) and $-K_i$ was determined by projecting a horizontal line at the height of $1/R_{max}$ (taken from the Lineweaver-Burk plot) and then dropping a perpendicular from the point of intersection to the inhibitor line. By this technique K_i for SC 9420 is 5.25×10^{-6} M giving a $K_D:K_i$ ratio of 1:681.

4. Effect of SC 14266 pretreatment on the scc response to vasopressin or glucose repletion. In 30 paired experiments in which diluent or SC 14266 were given 6 hr previously, maximal doses of vasopressin were administered. Figure 4 is a correlation graph of the peak scc response compared to the prevasopressin (baseline) scc value, plotted in a manner identical to that used in a previous publication (9). As can be seen, only one value exceeds the 95% range (stippled area) and it also extends beyond the 99% range (dotted line) for the nontreated control membranes. In addi-

TABLE 5
Data used for Lineweaver-Burk plot

Aldosterone concentration (M)		Number of paired experiments	SCC_0 (μamp)	ΔSCC_6[a] (μamp)	$\Delta SCC'_6$[a] (μamp)	$1/\Delta SCC'_6$	1/[ALDO]
7×10^{-9}	A[b]	12	52.5 ± 13.6	32.3 ± 5.5	61.5	0.0162	1.42×10^8
	SC + A[c]		47.0 ± 9.5	4.2 ± 4.0	8.9	0.1123	
2×10^{-8}	A	8	41.8 ± 6.5	53.6 ± 7.2	128.3	0.0078	0.5×10^8
	SC + A		36.4 ± 7.4	7.9 ± 5.7	21.6	0.0463	
3.5×10^{-8}	A	7	49.0 ± 18.0	58.1 ± 8.6	118.5	0.0084	0.29×10^8
	SC + A		30.6 ± 12.7	15.8 ± 7.2	51.6	0.0193	
7×10^{-8}	A	8	25.7 ± 7.2	49.7 ± 10.0	193.4	0.0051	0.14×10^8
	SC + A		36.0 ± 9.5	25.0 ± 8.8	69.4	0.0144	
3.5×10^{-7}	A	6	34.2 ± 8.2	62.4 ± 11.3	182.5	0.0054	0.028×10^8
	SC + A		42.0 ± 9.9	58.5 ± 9.2	139.3	0.0071	
7×10^{-7}	A	8	53.5 ± 8.6	75.1 ± 8.7	140.4	0.0071	0.014×10^8
	SC + A		62.1 ± 7.4	76.4 ± 6.7	123.0	0.0081	

[a] See text for derivation.
[b] Aldostone
[c] 3.5×10^{-5} M SC 14266 + aldosterone.

tion, in 22 paired experiments in which 7×10^{-5} M SC 14266 was given either 90 min before or simultaneously with 7×10^{-8} M *d*-aldosterone, maximal stimulation by vasopressin was assessed at the conclusion of each experiment. The results are plotted in a similar fashion in Fig. 5. Despite the presence of a sufficient concentration of SC 14266 to interfere significantly with aldosterone enhancement of active sodium transport the anticipated scc response to vasopressin was recorded. Finally, in 8 paired hemibladders in which overnight glucose supplementation was withheld, the

TABLE 6
Data used for Dixon plot

SC14266 Concentration (M)	Number of paired experiments	SCC_0 (μamp)	ΔSCC_6[a] (μamp)	$\Delta SCC'_6$[a] (μamp)	$1/\Delta SCC'_6$
SC 14266			7×10^{-9} M aldosterone		
7×10^{-7}	6	36 ± 10	19 ± 5	53.5	0.0186
1.75×10^{-6}	7	32 ± 5	14 ± 4	42.6	0.0234
3.5×10^{-6}	6	36 ± 12	14 ± 8	37.2	0.0268
7×10^{-6}	7	34 ± 8	6 ± 4	18.5	0.0540
1.75×10^{-5}	8	60 ± 10	−6 ± 4	—	—
SC 14266			7×10^{-8} M aldosterone		
7×10^{-7}	8	60 ± 11	95 ± 12	159.4	0.0062
7×10^{-6}	7	57 ± 10	57 ± 7	99.6	0.0100
1.75×10^{-5}	6	70 ± 14	54 ± 14	76.8	0.0130
3.5×10^{-5}	7	36 ± 7	25 ± 8	69.4	0.0144
SC 9420			7×10^{-8} M aldosterone		
5×10^{-5}	9	53 ± 10	7 ± 5	12.5	0.0800
3.5×10^{-5}	9	63 ± 7	13 ± 4	20.7	0.0484
7×10^{-6}	5	52 ± 14	36 ± 7	68.9	0.0145
3.5×10^{-6}	12	50 ± 4	41 ± 5	80.8	0.0123

[a] See text for derivation.

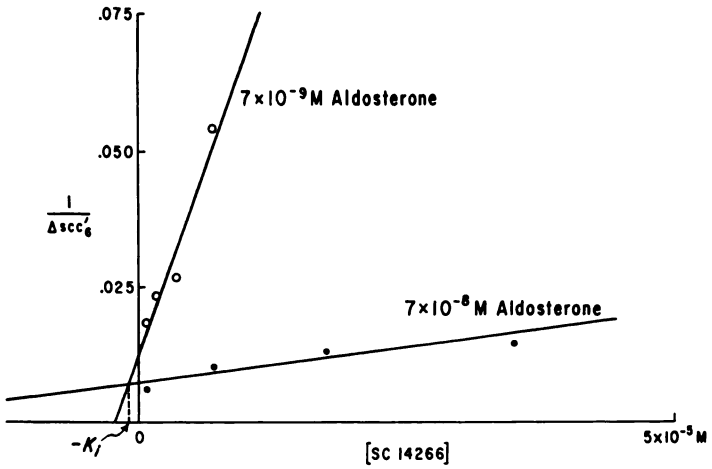

FIG. 3. *Plot of the reciprocal of the short-circuit current to various concentrations of SC 14266*

Two concentrations of aldosterone were used, 7×10^{-9} M and 7×10^{-8} M. The dissociation constant for SC 14266 is obtained by dropping a perpendicular from the intercept to the X axis (8), $K_i = 1 \times 10^{-6}$ M. The equilibrium constant (K_D) derived from the slope of the 7×10^{-9} M line is 5.5×10^{-9} M, while for the 7×10^{-8} M line the K_D is 3×10^{-9} M.

addition of 7×10^{-5} M SC 14266 did not interfere with the expected curvilinear rise in scc ratio (Fig. 6) which normally follows substrate repletion under these conditions (9).

5. Effect of premature removal of SC 14266 on subsequent scc response to aldosterone. In a previous report we demonstrated that a 45-min exposure to aldosterone was sufficient to induce all the electrophysiologic effects of the hormone in presensitized hemibladders (9). As an indirect test of the rate of tissue dissociation of spirolactone, a similar type of experiment was performed. After overnight incubation, one-half of each pair of hemibladders was exposed to 7×10^{-5} M SC 14266 for 90 min and then washed out by three successive 5-min rinses of the reservior chambers with steroid-free frog-Ringer solution. After the washout was completed, 7×10^{-8} M aldosterone was added to all hemibladders and the subsequent scc response was measured. Figure 7 illustrates the results of 8 experiments. From these results it would seem that in order for SC 14266 to manifest its inhibitory effect on aldosterone-stimulated sodium transport, a high concentration of the compound must be maintained in the external solution which bathes the membrane.

6. Effect on the scc response of the addition of SC 14266 following half-maximal concentrations of aldosterone. Figure 8 displays the effect of varying the time of 3.5×10^{-5} M SC 14266 addition relative to 7×10^{-9} M aldosterone. When hemibladders were pretreated with SC 14266 90 min prior to aldosterone, complete inhibition of the steroid-stimulated Na^+ transport occurred (Fig. 8A). Furthermore, the addition of 3.5×10^{-5} M SC 14266 at 3 hr after 7×10^{-9} M aldosterone resulted in a reversal of the increased Na^+ transport after a delay of between 1.5 and 2 hours (Fig. 8B). Finally, the inhibition induced by the late addition of SC 14266 could be reversed by adding a maximally effective concentration of aldosterone, i.e., 7×10^{-7} M (Fig. 8C).

DISCUSSION

In a previous report we concluded that the presence of an acetate or a similar group in the C-21 position was one of the structural requirements for a steroid to stimulate *in vitro* sodium transport (5). Examining the structural formula of SC 9420 and SC 14266 reveals that each com-

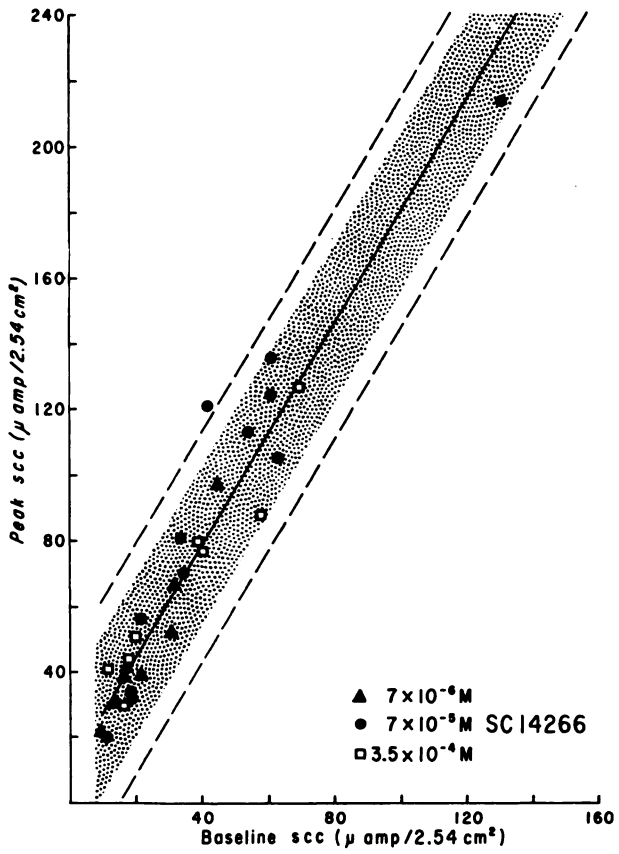

FIG. 4. *Action of vasopressin on Na^+ transport in the presence of SC 14266*

The maximum value of the scc (peak scc) after the addition of vasopressin (0.08 U/ml) to the serosal medium is plotted as a function of the prevasopressin value (baseline scc). The regression line is the mean response of 22 control hemibladders to the same concentration of vasopressin. The shaded area incorporates two standard deviations of the mean of peak scc, while the dotted line represents three standard deviations (99% range). The triangles indicate the response to vasopressin in hemibladders who had received 7×10^{-6} M SC 14266 pretreatment 6 hr before the vasoprssein challenge; the solid circles represent experimental points from hemibladders pretreated with 7×10^{-5} M SC 14266, and the open squares represent experimental points from hemibladders pretreated with 3.5×10^{-4} M SC 14266.

pound possesses the parent steroid perhydrocyclopentanophenanthrene nucleus which has been modified by substitution of a lactone ring at the C-17 position. In SC 9420, the lactone ring is intact, a fact reflected in the water insolubility of the material, while the lactone ring of SC 14266 has been broken, allowing the formation of a potassium salt and rendering the compound soluble in aqueous media. Both spirolactones can effectively interfere with the *in vitro* mineralocorticord effect of aldosterone, indicating that the substitution at C-17 does not need to be a cyclic radical in order to act as a competitive inhibitor. The results summarized in Table 1 indicate that the spirolactones do not produce any stimulation of *in vitro* sodium transport, a finding similar to that reported by Kagawa using an *in vivo* preparation (10). Despite the absence of mineralocorticoid effect, the similarity in the basic ring structure between aldosterone and spirolactone suggest that this property is important in rendering these compounds effective inhibitors of aldosterone's physiologic effects. This conclusion is strenthened by the observation that progesterone, a steroid

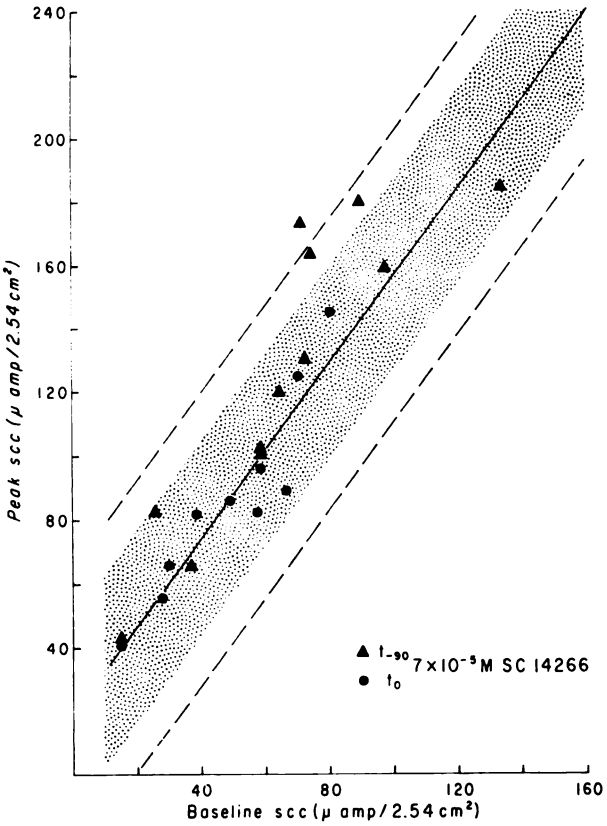

FIG. 5. *Action of vasopressin on Na^+ transport in the presence of SC 14266 and aldosterone*

The regression line is the mean response of 22 control hemibladders which had received 7×10^{-8} M aldosterone 6 hr before the 0.08 U/ml vasopressin was added to the serosal medium. The solid triangles represent the scc response in hemibladders which had received 7×10^{-5} M SC 14266 90 min prior to aldosterone addition, while the solid circles are from hemibladders given 7×10^{-5} M SC 14266 simultaneously with aldosterone addition.

without mineralocorticord effect, but having the basic steroid ring structure, is an effective inhibitor of aldosterone (11). However, the latter observation also suggests that if such a common steroid property as the perhydrocyclopentanophenanthrene nucleus is a significant determinant of competitive inhibitors of natural steroids, then cross-reactions should be possible. Such cross reactivity has been reported by Edgen and Elton, who demonstrated that spirolactones were capable of antagonizing the effect of estrogen on the immature mouse uterus (12).

In addition to structural requirements, the relative tissue affinity of the spirolactone compared to aldosterone is an important factor. Recognizing the limitation of applying kinetic formulas derived from isolated enzyme systems to a complex multicellular system, the information derived from Figs. 2 and 3 does provide insight into the interaction between spirolactone and aldosterone. Evidence as to the existence of a tissue receptor for aldosterone comes from two observations. Using a radioautographic technique we have shown a localization of aldosterone-^{3}H over the nuclei of the epithelial cells of urinary bladder of the toad (9). Fanestil and Edelman extended these observations when they reported perferential uptake of aldosterone-^{3}H by nuclei isolated from the rat kidney (13). If one assumes that the steroid

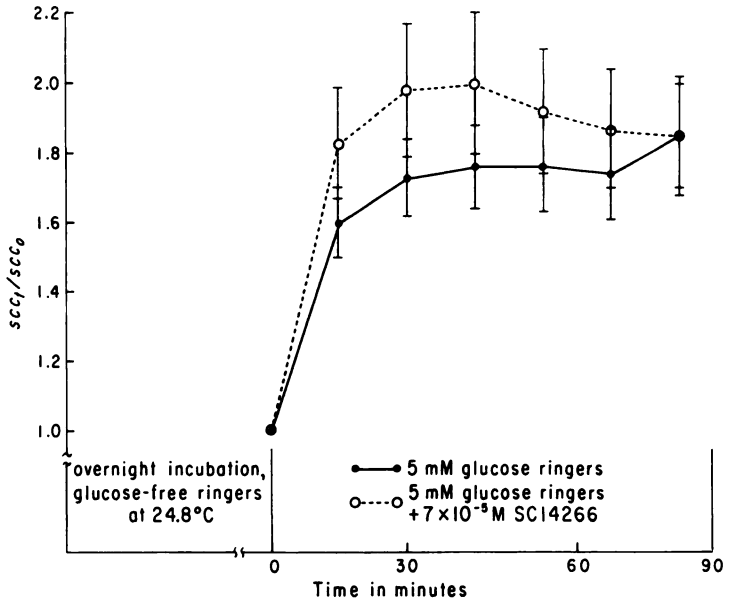

FIG. 6. *Effect of glucose repletion on the scc of hemibladders incubated overnight in glucose-free frog-Ringer solution*

See text for details of experiments. The scc ratio represents the short-circuit current at time t after glucose addition divided by the short-circuit current at the time of 5.5 mM glucose addition (time zero). The solid circles represent hemibladders receiving glucose only, while the open circles represent paired mates which received 7×10^{-5} M SC 14266 in addition to glucose.

receptors are homogeneous, then the ratio K_D:K_i represents the relative receptor affinities of drug and inhibitor. From the values derived by a Lineweaver-Burk plot (Fig. 2), this ratio for SC 14266 is 1:336, whereas when the K_i of SC 14266 is determined by the intercept method (Fig. 3) the ratio is 1:235. The K_D:K_i ratio for aldosterone:SC 9420 is 1:681. A relationship of similar magnitude is suggested for progesterone-inhibition of aldosterone. Although not as precisely defined as in the current study, we previously found that at equimolar concentrations, progesterone pretreatment failed to inhibit the *in vitro* response to aldosterone (14); however, when the progesterone content was increased a thousandfold, Sharp and Leaf reported definite inhibition of the hormone's effect on scc (15).

That one of the conditions for demonstrating *in vitro* inhibition of maximally effective concentrations of aldosterone (i.e., $>2 \times K_D$) is pretreatment by the inhibitor seems inescapable from the results presented in Table 3. This confirms an earlier report by Sharp and Leaf, who added the spirolactone at least 30 min before aldosterone and were able to demonstrate interference with the biologic activity of the hormone (14). This observation is compatible with exclusion of the spirolactone from a tissue receptor site. The validity of this conclusion was challenged by the experiments plotted on Fig. 8. Using an aldosterone concentration equal to the equilibrium constant derived from the Lineweaver-Burk plot, i.e., $K_D = 7.7 \times 10^{-9}$ M, late addition of 3.5×10^{-5} M SC 14266 produced an inhibition of the aldosterone-stimulated increase in sodium transport (Fig. 8B). Furthermore, this inhibition following late addition of SC 14266 could be counteracted when 7×10^{-7} M aldosterone was subsequently added giving a final K_D:K_i ratio of 1:45 (Fig. 8C).

When dealing with maximally effective concentrations of aldosterone, not only

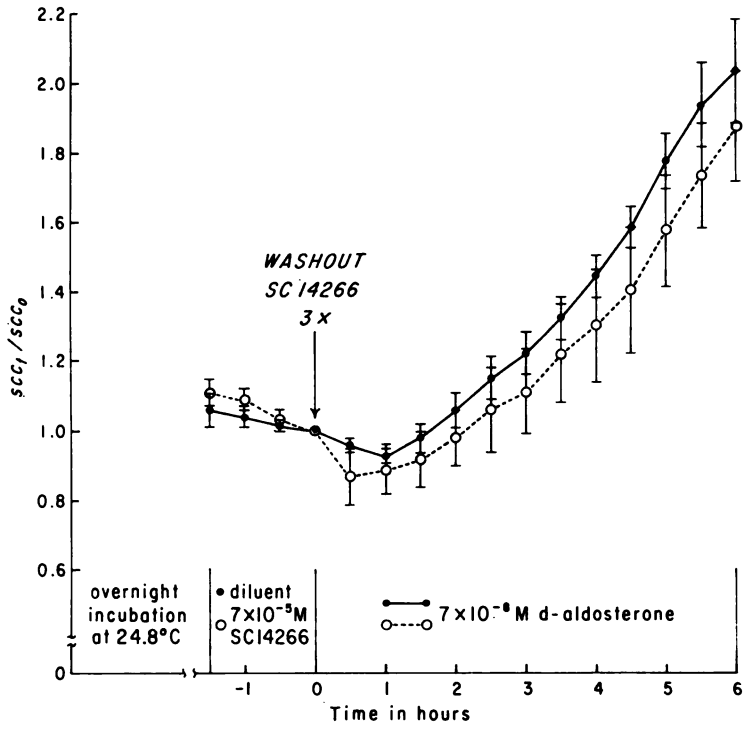

FIG. 7. *Effect of the premature removal of SC 14266 on inhibiting the scc response to aldosterone*

The eight hemibladders represented by the open circles received 7×10^{-5} M SC 14266 90 min before aldosterone was added; the SC 14266 was then washed out by exchanging the chamber fluid three times just before adding 7×10^{-8} M *d*-aldosterone. The solid circles represent the eight mated controls who received aldosterone only.

must membranes be pretreated with spirolactone, but a high extracellular concentration of the inhibitor must be maintained after hormonal exposure if inhibition is to be achieved. Verification of this last statement is shown in Fig. 7, which demonstrates that despite 90 min of pretreatment, when SC 14266 was removed from the external bathing solution at the time of aldosterone addition, the subsequent scc ratios could not be separated from that recorded in the paired hemibladders which did not receive the SC 14266.

Based upon criteria derived from isolated enzyme systems (16) the kinetic plots (Figs. 2 and 3) of aldosterone–spirolactone interaction are compatible with competitive inhibition. A decision as to whether or not this competitive inhibition is complete or only partial does not seem justified in view of the multiplicity of uncontrollable factors which operate in a multicellular system such as the one I have used. Regardless of the completeness of the competitive inhibition, it would appear that the intensity of the aldosterone-induced increase in sodium transport can be modified by the availability of intracellular receptors for occupancy. That the condition of overnight incubation, which was a standard procedure in these experiments, may modify the membrane response to higher concentrations of spironolactone is suggested from the observations of Crabbé (4). He reported that when 10^{-4} M SC 9420 was added to freshly mounted hemibladders taken from toads previously exposed to a distilled water environment before sacrifice, there was no evident effect upon the scc (4). We also have administered 7×10^{-5} M

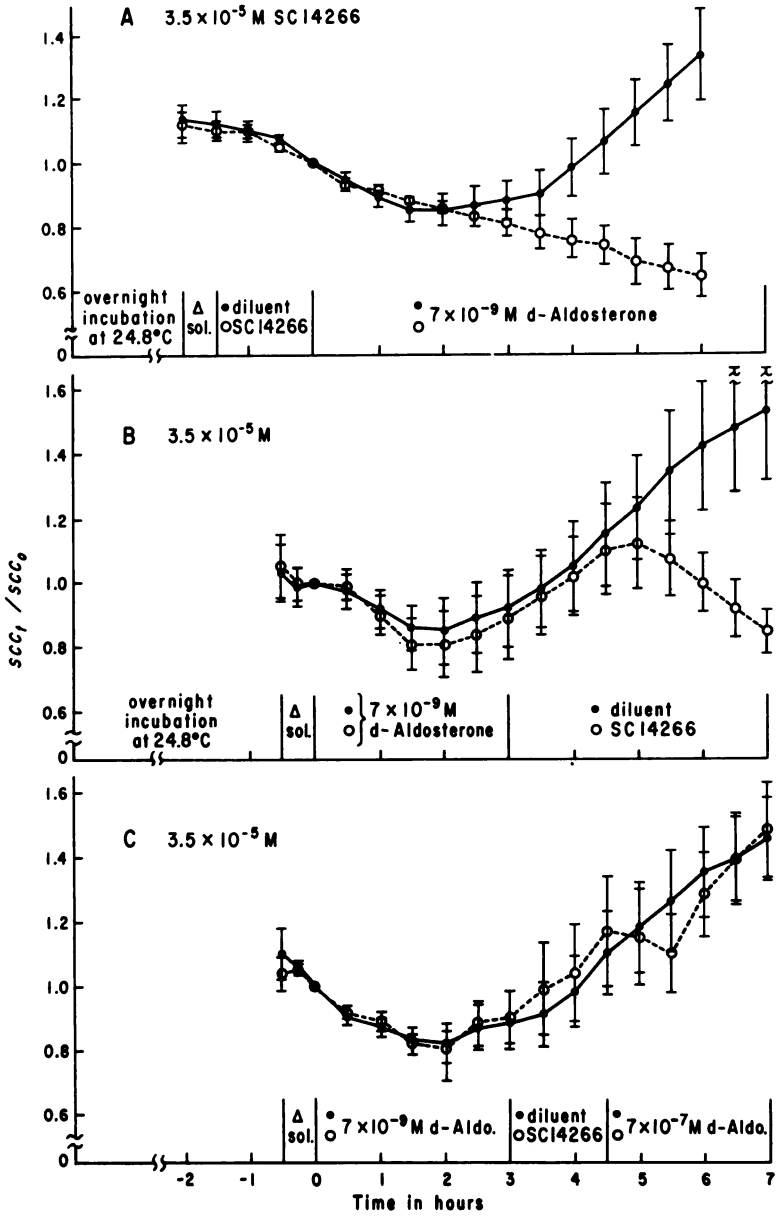

FIG. 8. *The short-circuit response in experiments in which half-maximal concentrations of aldosterone, i.e., 7 × 10⁻⁹ M are completely inhibited by 3.5 × 10⁻⁵ M SC 14266*

The short-circuit current recorded at time t after the addition of aldosterone is denoted by "scc_t"; the short-circuit current at the time aldosterone was added (time zero), by "scc_0". The solid dots represent hemibladders receiving aldosterone only, while the open circles are hemibladders given SC 14266 in addition to aldosterone in the sequence as indicated immediately above the abscissa. Each point includes at least six paired observations, the vertical bars equal ± 1 standard error of the mean. The effects of SC 14266 pretreatment is shown in graph A, while the result of delayed addition of SC 14266 is shown in B. Reversibility of SC 14266 inhibition can be seen in graph C.

SC 9420 to hemibladders which were not incubated overnight and observed that, if given within 3 hr of the time of membrane mounting, the scc was not affected; however, addition beyond 3 hr resulted in a significant decline in scc when compared to untreated control, (G. A. Porter and I. S. Edelman, unpublished observations). That the effect of high concentrations of SC 9420 on toad bladder may be due to inhibition of endogenous aldosterone has been suggested by Crabbé (4). This hypothesis does not explain the observation that the decline in scc which follows the addition of 7×10^{-5} M SC 9420 is completely developed after only 30 min, whereas, if at these high concentrations spironolactone were acting as a competitive inhibitor of aldosterone one would expect a delay of 60–90 min before the inhibition would be manifest. The latter prediction is based upon the inhibitory pattern recorded in Fig. 8B and the pattern which occurs in hemibladders treated with either puromycin or actinomycin; both compounds have been shown to interfere specifically with the mechanism by which aldosterone stimulates active sodium transport (9). This immediate depression of scc resembles the effect of high concentrations of ouabain (2.5×10^{-4} M) (J. Burpee and G. A. Porter, unpublished observations), a cardiac glycoside which is reported to inhibit sodium transport by interfering with membrane ATPase (17). The normal scc response to maximal vasopressin stimulation, despite the presence of concentrations of spirolactone which inhibit the aldosterone response (Figs. 4 and 5), when combined with failure of SC 14266 to inhibit the scc response to substrate repletion (Fig. 6), are taken as evidence that in the presence of inhibitory concentrations of SC 14266 the final common pathway for sodium extrusion by the cell is intact and capable of responding in a normal fashion to these stimuli (9). Therefore, it would seem that the site of spirolactone interference with aldosterone-enhanced Na^+ transport must be intimately associated with the intracellular mechanism (18) by which the hormone exerts its effect.

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