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THE EFFECT OF A NEW ANTI-ALDOSTERONE AGENT SC19886 ON ALDOSTERONE-STIMULATED TRANSEPITHELIAL SODIUM TRANSPORT*

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SUMMARY

A new anti-aldosterone compound SC 19886, a 12α, 13β-etiojervane derivative, was evaluated using an isolated toad bladder preparation. Based on analysis of a Lineweaver–Burk plot, SC19886 effect on aldosterone-stimulated short circuit current is compatible with competitive inhibition of the steroid's action on transepithelial sodium transport. At a concentration of $3{\cdot}5 \times 10^{-5}$ M or less, SC 19886 did not distort nonsteroid (basal) sodium transport, nor did it interfere with vasopressin-stimulated short circuit current. By comparing the dissociation constant derived from SC 19886 with values previously obtained for the spirolactone SC 14266 and spironolactone (SC9420), SC 19886 was five times more potent than SC 14266 and 28 times more effective than SC9420 in blocking aldosterone *in vitro*. From the washout characteristics and the results of the late addition of SC 19886 once the aldosterone stimulated transepithelial sodium transport was established, interpretation favored a mechanism of inhibition occurring via nuclear receptor occupancy by SC 19886 with resulting interruption of the aldosterone-induced DNA-dependent RNA synthesis.

INTRODUCTION

SC19886, a 12α, 13α-etiojervane derivative, has recently been reported by Johns and Hofmann to be a potent anti-aldosterone agent [1]. Structurally, SC19886 differs from the aldosterone inhibitors, progesterone and spirolactones, because of a modified steroid nucleus in which the C ring is five membered and the D ring is six membered. As an extension of our previous studies concerning competitive inhibitors of aldosterone stimulated sodium transport [2, 3], we have performed a series of experiments designed to quantitate the inhibitory potency of SC19886 and provide insight as to the cellular mechanism responsible for aldosterone inhibition.

EXPERIMENTAL RESULTS

The isolated toad bladder was employed for these experiments since, from our previous experience [2], it served as an excellent model system to define the mechanism of spirolactone inhibition of aldosterone-induced transepithelial sodium transport. The experimental protocol is essentially identical to that first reported in 1964 [4] and provides consistent, reproducible mineralocorticoid effects on active sodium transport as measured by the short-circuit current (s.c.c.). Figure 1 summarizes the expected s.c.c. response to a maximally effective concentration of aldosterone, i.e. 10^{-7}M, added after overnight incubation of the toad urinary bladder to minimize endogenous mineralocorticoid activity. The s.c.c.

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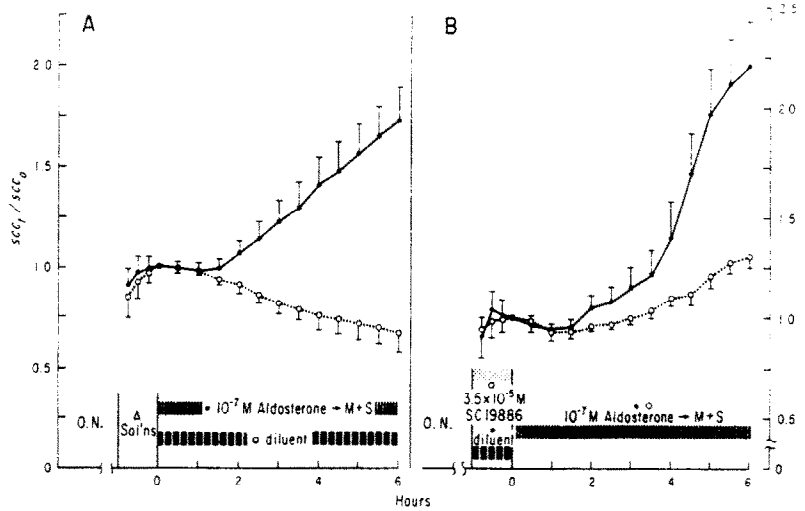

Fig. 1. The short circuit current response to a maximal concentration and its modification by pre-treatment with $3{\cdot}5 \times 10^{-5}$M SC19886. The s.c.c.$_t$/s.c.c.$_0$ ratio, as defined in the text, appears on the ordinate while time related to aldosterone addition, i.e. t_0, is shown on the abscissa. The solid dots represent quarter bladders receiving aldosterone only, while the open circles are either control quarter bladders (A) or $3{\cdot}5 \times 10^{-5}$M SC19886 pre-treatment (B). Each point represents eight paired observations, the vertical bars equal ± 1 standard error of the mean. The experiment sequence is indicated immediately above the abscissa.

ratio, shown on the ordinate, is computed for each quarter bladder by dividing the current at any specified time t by the value recorded at the time of aldosterone addition, i.e. t_0. This allows the comparison of the s.c.c. response in paired quarter bladders regardless of the absolute difference in current at the beginning of the experiment. In addition, Fig. 1 depicts the modified s.c.c. pattern which occurs when SC19886* (17β-hydroxy-12α-etiojerv-4-en-3-one) addition precedes aldosterone by 15 min. Although SC19886 did not prolong the 1½ h delay interval which characterizes the s.c.c. pattern in the isolated toad bladder in response to added mineralocorticoids, it significantly impaired the magnitude of the s.c.c. ratio achieved 6 h after steroid addition. As with previous reports [2. 4], the 6 h ratio, i.e. s.c.c.$_6$/s.c.c.$_0$ was used for statistic evaluation of SC19886's inhibition.

To exclude an independent effect of SC19886 to the sodium transporting system of the isolated toad bladder, the experiments summarized on Table 1 were performed. We compared the s.c.c.$_6$/s.c.c.$_0$ in 8 paired quartered bladders one-half receiving $3{\cdot}5 \times 10^{-5}$M SC19886 at t_0 and the other half providing an untreated, control response. No significant stimulation or depression of the s.c.c. was evident during the 6 h of exposure. As an additional observation, the s.c.c.$_6$/s.c.c.$_0$ response to $3{\cdot}5 \times 10^{-6}$M SC19886 addition was evaluated in 6 other paired quarter-bladders with similar results.

To quantitatively define the magnitude of SC19886 inhibition of aldosterone-stimulated transepithelial sodium transport and to evaluate whether or not the

*SC19886 was a gift from Drs. L. Hofmann and W. Johns, G. D. Searle & Co., Chicago, Ill., U.S.A.

Table 1. Effect of SC19886 on the spontaneous short circuit current of isolated toad urinary bladder

Conditions	n	s.c.c.$_0$ (μA)	s.c.c.$_6$/s.c.c.$_0$	P
Control	8	53 ± 10	0·63 ± 0·04	> 0·7
$3·5 \times 10^{-5}$M SC19886		54 ± 11	0·65 ± 0·07	
Control	6	46 ± 7	0·67 ± 0·05	> 0·5
$3·5 \times 10^{-6}$M SC19886		42 ± 9	0·64 ± 0·04	

inhibition was competitive in character, data suitable for analysis using the double reciprocal transformation of Lineweaver-Burk [5] were obtained and plotted as detailed previously [2]. Figure 2 is such a plot in which the reciprocal of the incremental increase in s.c.c. after 6 h (on the ordinate) is expressed as a function of various concentrations of aldosterone with and without pretreatment by $3·5 \times 10^{-5}$M SC19886. The common intercept of the two lines is presumptive evidence of competitive inhibition [6]. From the slope of the line of the uninhibited aldosterone response a half maximally effective concentration (K_D) for aldosterone of $5·6 \times 10^{-9}$M was derived. This value is in good agreement with our previously reported K_D for aldosterone of 7×10^{-9}M [2]. From the slope of the inhibited aldosterone response a dissociation constant (K_i) of $4·6 \times 10^{-7}$M was derived for

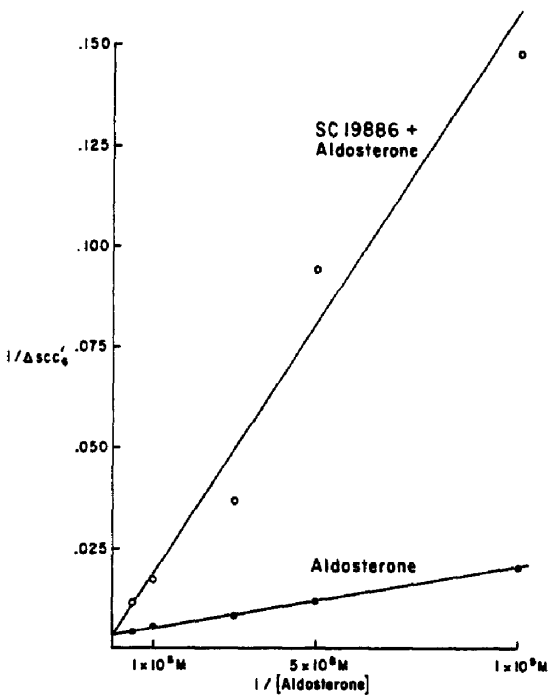

Fig. 2. Lineweaver-Burk plot of the short circuit current response to various concentrations of aldosterone with and without $3·5 \times 10^{-5}$M SC19886 pretreatment. The maximum response derived from the intercept on the Y axis is 313 μA. The equilibrium constant or half-maximal aldosterone concentration (K_D) is $5·6 \times 10^{-9}$M, while the dissociation constant for SC19886 (K_i) is $4·6 \times 10^{-7}$M.

SC19886. This value was substantially different from the K_i of $2{\cdot}6 \times 10^{-6}$M previously determined for the spirolactone SC14266 (2).

To quantitate the inhibitory potency of SC19886 relative to previously reported aldosterone blocking compounds progesterone and spirolactones[3], a dissociation constant was determined by the direct plot technique of Dixon[7]. This was accomplished by fixing the concentration of aldosterone at either 5×10^{-9}M, i.e. K_D, or 5×10^{-8}M, i.e. $10 \times K_D$, while the concentration of SC19886 added 15 minutes before the aldosterone varied from 0 to 10^{-6}M. The K_i for SC19886 derived by this method was $1{\cdot}9 \times 10^{-7}$M and is incorporated with the previously reported dissociation constants[3] of other aldosterone blocking agents on Table 2. By arbitrarily assigning progesterone, an aldosterone inhibitory potency of 1, SC19886 is 94 times more effective. In addition, SC19886 has approximately 5 times the aldosterone blocking activity of the water-soluble spirolactone SC14266 and nearly 28 times the activity of the more polar spironolactone SC9420.

Table 2. Dissociation constants (K_i) for aldosterone antagonisis derived from Dixon plots

Inhibitor	K_i	Relative potency
SC19886	$1{\cdot}9 \times 10^{-7}$	94
SC14266	1×10^{-6}	18
SC9420	$5{\cdot}3 \times 10^{-6}$	3·4
Progesterone	$1{\cdot}8 \times 10^{-5}$	1

To assess the effect of SC19886 on non-aldosterone-stimulated transepithelial sodium transport a set of 8 paired experiments were performed in which one-half of the matched quarter bladders were exposed to $3{\cdot}5 \times 10^{-5}$M SC19886 and the other half served as controls. Two h after SC19886 addition, a maximally effective concentration of vasopressin, i.e. 80 mU/ml, was added to the serosal surface of all quarter bladders and the s.c.c. recorded at minute intervals until a peak response was registered. A correlation plot of these results was constructed identical to that previously reported using SC14266 (Ref. 2, Fig. 4). The SC19886 pretreatment failed to alter the expected magnitude or pattern of short circuit current in response to the added vasopressin.

Finally, a series of experiments were performed to provide insight as to the intracellular action of SC19886. The first set included varying the sequence of aldosterone/SC19886 addition and are shown in Fig. 3. As predicted by the derived $K_D{:}K_i$ from the Lineweaver-Burk analysis (Fig. 2), 10^{-6}M SC19886, i.e. $5 \times K_i$, completely inhibits a half-maximal concentration of aldosterone (K_D), i.e. 5×10^{-9}M (Fig. 3, panel A). Furthermore, using the same test system but delaying SC19886's addition until after the s.c.c. response to aldosterone is well developed, i.e. $t_{\frac{1}{2}}$ inhibition of the aldosterone-stimulated s.c.c. develops 1½–2 h following SC19886 addition (Fig. 3, panel B). Finally, the reversibility of the SC19886 inhibition by the late addition of 10^{-7}M aldosterone, i.e. $20 \times K_D$, is shown in panel C of Fig. 3. The 1½ h delay between the late addition of SC19886 and subsequent inhibition of the aldosterone-induced increase in s.c.c. (Fig. 3B) is identical to the s.c.c. pattern reported with the late addition of Acti-

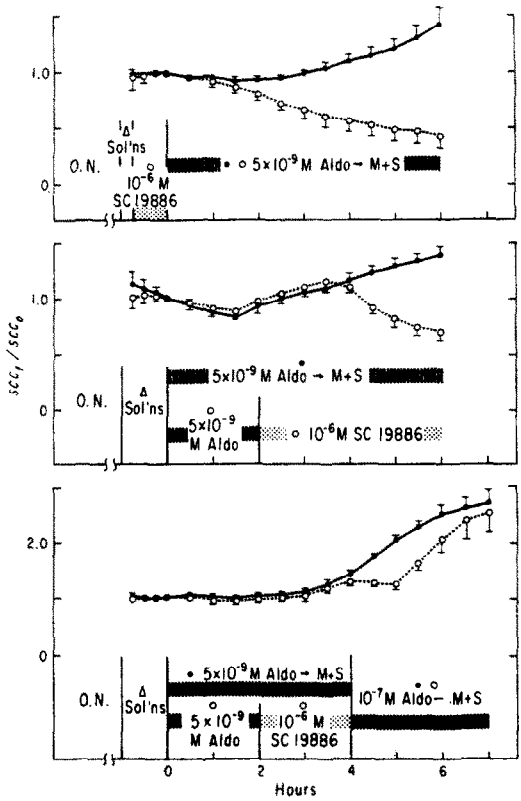

Fig. 3. The short circuit current response in experiments in which the concentration and addition sequence of aldosterone/SC19886 are varied. The format is identical to Fig. 1. The solid dots represent quarter bladders receiving aldosterone only, while the open circles are quarter bladders given SC19886 in addition to aldosterone in the sequence indicated immediately above the abscissa. Format is identical to Fig. 1. The effects of SC19886 pretreatment are shown in graph A, while the delayed addition of SC19886 is shown in B. Reversibility of SC19886 inhibition can be seen in graph C.

nomycin D, an inhibitor of RNA synthesis[3]. To pursue this possibility, the experiments summarized in Fig. 4 were performed. Two sets of experiments were included. In the first set either Actinomycin D (5 μg/ml)[8] or SC19886 (10^{-6}M) was added to paired quarter bladders 15 min prior to the addition of 10^{-7}M aldosterone. One and 1½ h later, chamber solutions were removed, membrane washed twice and s.c.c. monitoring continued for another 4½ h. As can be seen from the lower left hand graph of Fig. 4, both Actinomycin D and SC19886 induced similar changes in the usual aldosterone-induced s.c.c. pattern. The second study involved the use of cycloheximide, an inhibitor of protein synthesis. Both of the paired quarter bladders received 10^{-7}M aldosterone at time zero. Three-quarters of an hour later cycloheximide (0·5 μg/ml)[8] was added to one-half of the quarter bladders and the other half received 10^{-6}M SC19886. Following another ¾ h exposure to either of these compounds, chamber solutions were removed, membranes washed twice and s.c.c. monitored for the ensuing 4½ h. These data are depicted in the right hand graph of Fig. 4 and show that while the 45-min pulse of cycloheximide during the terminal portion of

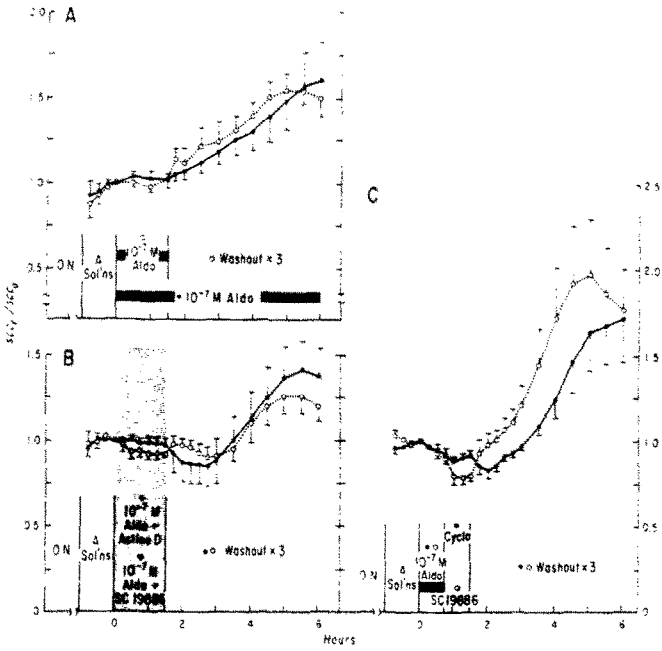

Fig. 4. Comparison of the short circuit current response to SC19886 to that of Actinomycin D or cycloheximide. The format is similar to Fig. 1 with s.c.c.$_t$/s.c.c.$_0$ shown on the ordinate while time relative to aldosterone addition is on the abscissa. The solid dots are the results for the quarter bladders given SC19886 + aldosterone while the open circles are quarter bladders given Actinomycin D or cycloheximide + aldosterone.

aldosterone's latency period delayed the steroid-induced s.c.c. rise by 1 h, no such effect was registered by a short pulse of SC19886.

DISCUSSION

In collaboration with Bogoroch and Edelman[9], a hypothesis was formulated that in the isolated toad bladder aldosterone induced DNA directed RNA synthesis which, acting through a protein intermediate, increased the supply of metabolic substrate available for active transepithelial sodium transport. Recently, Edelman and Fanestil[10] have reviewed the biophysical and biochemical data from both rat and isolated toad bladder studies substantiating this concept. When the bioelectric effects of SC19886 are interpreted in the context of this postulated mechanism, it is reasonable to assume that the 1½ h lag between SC19886 addition and inhibition of aldosterone-stimulated s.c.c. involves displacement of the aldosterone from its nuclear receptor and subsequent interruption of the steroid-induced DNA-directed RNA synthesis. Indirect support for a nuclear displacement comes from the recent observation of Fanestil[11] who reported that the simultaneous injection into rats of the aldosterone inhibitor SC14266 and [^{3}H]-aldosterone resulted in a 35% decrease in nuclear accumulation and a 60% decrease in cytosol accumulation of radioactive steroid. In addition, from toad bladder studies, we have recently reported[3] that the rank order of K_D:K_i ratio for a series of variably potent mineralocorticoids and SC14266 was

similar to the hierarchy reported by Herman *et al.*[12] for competitive steroid displacement of [^{3}H]-aldosterone from rat kidney nuclear and cytosol complexes. Finally, Hutchinson and I [13. 14] have reported a significant inhibition of the aldosterone-induced increase in nuclear RNA synthesis of toad bladder epithelial cells when pretreated with the competitive anti-aldosterone blocking agent SC14266. Because of the similarity in the inhibitory pattern of SC19886 and SC14266 on aldosterone-stimulated sodium transport in the isolated toad bladder, it seems likely that they share a common mode of intracellular action.

Finally, examination of the structural formulae of the four competitive aldosterone inhibitors which we have evaluated (Fig. 5) allows speculation as to the

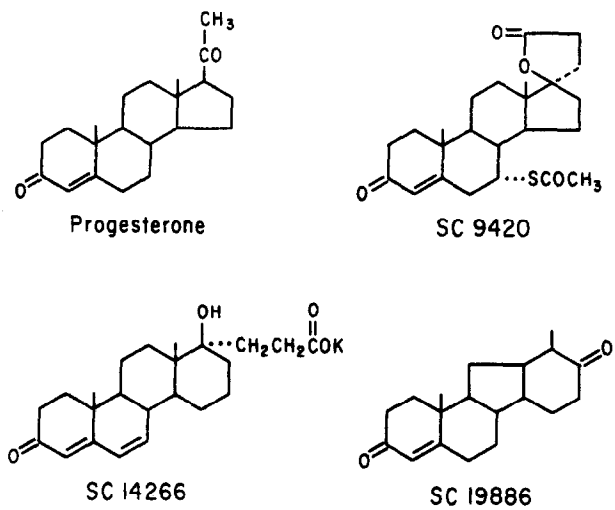

Fig. 5. Comparison of the strucutral formulae of four competitive inhibitors of aldosterone's action on transepithelial sodium transport. SC9420 and SC14266 are spirolactones while SC19886 is a 12α.13β-etiojervane derivative.

binding characteristics of the intracellular mineralocorticoid receptor as measured by physiologic techniques. Since the A-B rings are the only portion of the skeletal stereochemistry which are common to all four compounds, it seems possible that interaction with the tissue receptors occurs at this end of the steroid nucleus. Furthermore, Johns and Hofmann[1] reported that the C-13 methyl of SC19886 must be in the β configuration to have aldosterone-blocking activity since a stable epimer having a 13α-methyl group was without inhibitory activity.

In conclusion, the 12α,13β-etiojervane derivative, SC19886, is a competitive inhibitor of aldosterone's action on transepithelial sodium transport of the isolated toad bladder. SC19886 has no toxic effects on the sodium transport system at concentrations of $3{\cdot}5 \times 10^{-5}$M or less and does not interfere with vasopressin-stimulated short circuit current. Based upon a comparison of dissociation constants (K_i), SC19886 has 5 times the aldosterone blocking activity of the spirolactone SC14266, 28 times the activity of spironolactone (SC9420) and 94 times the inhibitory effect of progesterone. Biophysical data favor SC19886 mechanism of inhibition occurring via nuclear receptor occupancy and interruption of the aldosterone-induced DNA-dependent RNA synthesis.

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DISCUSSION

Edelman: Has this derivative been tried in the response of adrenalectomized rats to aldosterone?

Porter: Yes, Johns and Hoffman of Searle research division have informed me that SC19886 is only effective when given by injection. They postulate that the failure to show a response following oral administration is a result of degradation in the GI tract.

Eigler: Has this compound been tried in frog skin also to see whether it inhibits slough formation?

Porter: To my knowledge investigation of this compound has been limited to the studies I've reported today and those performed by the research division of G. D. Searle. I know of no information regarding its effect on the aldosterone-induced slough in frog skin.