J. Enzyme Inhibition, 1987, Vol. 2, pp. 37–45
Photocopying permitted by license only

5β,14β-ANDROSTANE-3β,14-DIOL BINDS TO THE DIGITALIS RECEPTOR SITE ON Na/K-ATPase

WERNER SCHÖNFELD, KARL-HEINZ MENKE, REINHILD SCHÖNFELD and KURT R.H. REPKE*

Central Institute of Molecular Biology, Academy of Sciences of the G.D.R., Berlin, G.D.R.

(Received 6 April 1987)

5β,14β-Androstane-3β,-14-diol, the lead (minimum) structure in digitalis compounds, shows the same characteristics of interaction with Na/K-ATPase as ordinary digitalis compounds judged by the following six criteria: (I) shape of the concentration-inhibition curves, (II) species differences in affinity for the enzyme, (III) apparent competition with K^+, (IV) competition with digitoxigenin for binding to the enzyme, (V) stabilization of phosphoenzyme formed from ATP, and (VI) enhancement of phosphorylation from orthophosphate.

KEY WORDS: 5β,14β-Androstane-3β,14-diol, lead structure, Na^+/K^+-transporting ATPase, digitalis receptor.

INTRODUCTION

Na/K-ATPase (Na^+/K^+-transporting ATPase, EC 3.6.1.37.) has been recognized as the digitalis receptor.[1,2] Digitalis compounds (cardenolides and bufadienolides) bind very specifically to this enzyme. High-affinity binding requires a special enzyme conformation which can be adjusted by suitable ligation of the enzyme.[3] On the other hand, well-defined requirements are made on the compounds to be bound. In a previous paper[4] 5β,14β-androstane-3β,14-diol was proposed to be the lead structure in digitalis compounds. This view was derived from the results of quantitative structure-activity relationships of various steroidal compounds on human heart Na/K-ATPase. The lead structure is devoid of a considerable part of an ordinary digitalis compound -namely the unsaturated lactone ring. This substructure makes an important contribution to the interaction energy of any cardenolide or bufadienolide with the enzyme.[4] Clearly, the lead structure cannot occupy the entire binding area of the receptor site. Hence, the question is quite open as to whether or not the minimum, i.e., lead structure exhibits the same interaction characteristics with the Na/K-ATPase as are known for typical digitalis compounds. The present paper attempts to answer this question.

MATERIALS AND METHODS

[^{3}H]Digitoxigenin was purchased from Isocommerz (Berlin, G.D.R.); [^{32}P]P_i and [γ-^{32}P]ATP were from Central Institute of Nuclear Research (Dresden, G.D.R.). For the source of the steroidal and the other inhibitors used here see references 4 and 6. The auxiliary enzymes and the enzyme substrates were from Boehringer (Mannheim, F.R.G.). All the other chemicals were of analytical grade.

*Correspondence.

TABLE I

Influence of K^+ on the inhibition of Na/K-ATPase from human brain by 5β,14β-androstane derivatives in comparison with digitoxigenin

$[K^+]$ (mM)	% Inhibition of Na/K-ATPase activity by		
	100 μM 5β,14β-Androstane-3β,14-diol	10μM 3β-Rhamnosyloxy-5β,14β-androstan-14-ol	0.1 μM Digitoxigenin
1	60.5	75.9	54.2
2	33.2	63.3	39.7
4	29.2	51.2	37.1
8	30.1	37.0	28.4
16	26.8	27.9	24.9
32	28.8	23.6	23.5

Preparation and determination of Na/K-ATPases

Na/K-ATPase preparations from cardiac muscle and cerebral cortex were obtained from human tissue, taken within 12 hours of post mortem, by the approved procedures cited in reference 5. Na/K-ATPase activities, estimated by the coupled optical test at 37°C as described,[5] amounted to 2.2–11.5 and 46–110 units/mg protein for the cardiac and the cerebral enzyme, respectively. One unit is defined here as the activity splitting one μmole ATP per hour.

Inhibition of Na/K-ATPase activity

The inhibitory power of the steroids was estimated at 37°C as described.[5] The equilibrium values of inhibition, I_∞, were either determined directly or calculated by nonlinear regression according to the equation:

$$I_t = I_\infty(1 - e^{-kt}) \quad (1)$$

in which t, k, and I_t denote the exposure time, the time constant, and the inhibitory degree at time t, respectively. For studying the influence of K^+ on the inhibition by the steroids the test medium was free of NH_4^+ (cf. reference 5) and contained the $[K^+]$ given in Table I.

TABLE II

Effect of 5β,14β-androstane-3β,14-diol and other inhibitors of Na/K-ATPase on enzyme phosphorylation from $[^{32}P]P_i$. Values are the means ± standard errors of three determinations

Inhibitor (final concentration)	Exchangeable bound ^{32}P (pmole/unit enzyme activity)
Without	0.20 ± 0.15
100 μM Ouabain	0.99 ± 0.08
100 μM Digitoxigenin	1.02 ± 0.15
0.1 μM Digitoxigenin	0.43 ± 0.07
200 μM 5β,14β-Androstane-3β,14-diol	0.46 ± 0.03
20 μM Cibacron Blue F3GA	0.22 ± 0.10
Without	0.15 ± 0.02
100 μM Digitoxigenin	0.99 ± 0.05
0.15 μM Digitoxigenin	0.48 ± 0.03
300 μM 5β,14β-Androstane-3β,14-diol	0.43 ± 0.02
30 μM 3β-Rhamnosyloxy-5β,14β-androstan-14-ol	0.68 ± 0.03
30 μM Cassaine	0.63 ± 0.04
200 μM Cibacron Blue F3GA	0.00 ± 0.02

TABLE III

Effect of 5β,14β-androstane derivatives and cardenolides on the dephosphorylation rate of phosphoenzyme formed from [^{32}P]ATP. Dephosphorylation was measured under turnover conditions (presence of 4 mM $MgCl_2$, 80 mM NaCl, 5 mM KCl, 1 mM ATP, 24 mM imidazole/HCl, pH 7.6 (25°C), and 0.3 mM P_i) at 0°C

Ligation of phosphoenzyme	Rate constant (min^{-1})
Without	18.8 ± 0.4*
5β,14β-Androstane-3β,14-diol	2.8 ± 0.9**
3β-Rhamnosyloxy-5β,14β-androstan-14-ol	1.68 ± 0.15**
Digitoxigenin	0.66 ± 0.02**
Evomonoside	0.60 ± 0.09**

* k_{-1} ± S.D., calculated according to equation (7)
** k_{-2} ± S.D., calculated according to equation (8)

[^{3}H]digitoxigenin binding

The binding of [^{3}H]digitoxigenin (about 17.5 Bq/pmole) to the Na/K-ATPase from human cerebral cortex was followed in the presence of 4 mM $MgCl_2$, 2 mM orthophosphate (P_i), and 40 mM imidazole/HCl buffer, pH 7.4 at 37°C. The reaction was started by the addition of the Na/K-ATPase preparation (51 μg protein) to the medium (final volume: 3 ml). After an exposure time of 20 min the equilibrium was reached at all the [^{3}H]digitoxigenin concentrations used, and the incubation was terminated by removal of the membrane fragments from the medium by vacuum filtration on glass fibre filters (GF/C, Whatman). The radioactivity on the filters was measured directly by liquid scintillation counting (in triplicate). Values for unspecific binding of [^{3}H]digitoxigenin were determined by substituting 20 mM KCl for $MgCl_2$ and P_i.

Phosphorylation with [^{32}P]P_i

Phosphorylation was performed at 37°C in a medium containing 10 μM [^{32}P]P_i (about 7 Bq/pmole), 3 mM $MgCl_2$, 0.1 mM 1,2-cyclohexanediamine tetraacetic acid, 5 mM imidazole/HCl buffer, pH 7.0 and the additions specified in Table II. The reaction was started by addition of the Na/K-ATPase preparation from human brain (102 μg protein) to the medium (final volume: 0.2 ml) and terminated after 10 min by addition of 3 ml of an ice-cold solution containing 10% trichloroacetic acid (w/v) and 50 mM $Na_4P_2O_7$. The mixture was filtered as described above, but the filters with the membranes were washed twice with 2 ml of 50 mM $Na_4P_2O_7$/HCl, pH 2.0. Unspecific binding of [^{32}P]P_i was obtained by addition of a medium similar to that above but devoid of [^{32}P]P_i and containing 10 mM unlabelled P_i, one minute before stopping the reaction. Blank values were determined also by substituting 20 mM KCl for 3 mM $MgCl_2$ in the medium.

Phosphorylation with [γ-^{32}P]ATP

Phosphorylation was performed at 0°C in a medium containing 10 μM [^{32}P]ATP (about 10 Bq/pmole), 4 mM $MgCl_2$, 80 mM NaCl, 24 mM imidazole/HCl, pH 7.6 (25°C), and the components specified in Table III. The Na/K-ATPase preparation from human brain (102 μg protein) was preincubated for one minute in 0.2 ml of the

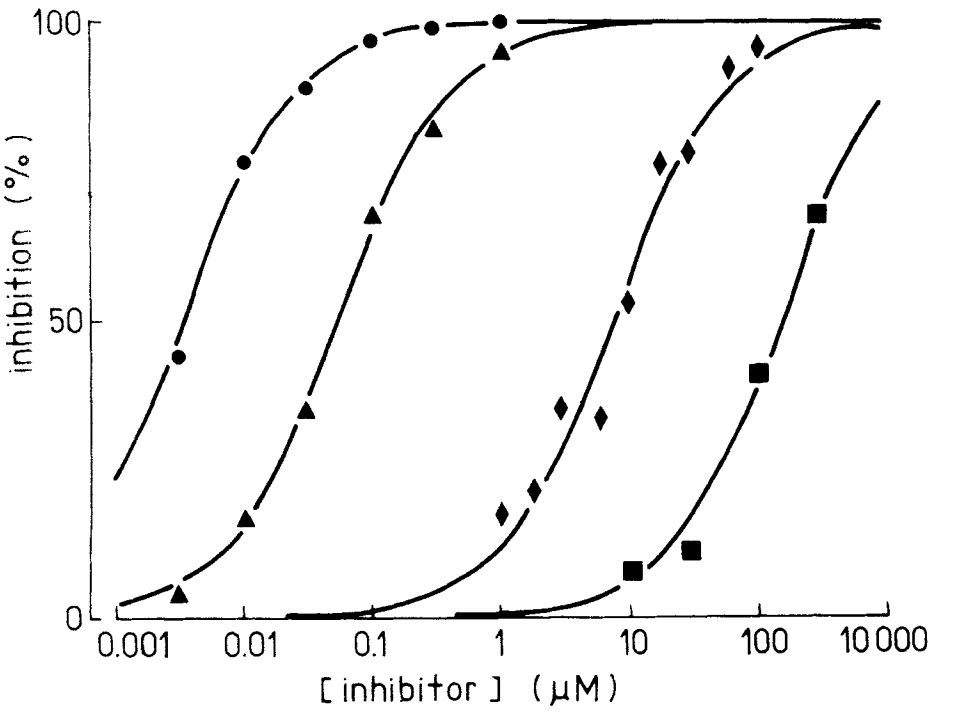

FIGURE 1 Inhibition of Na,K-ATPase from human heart by 5β,14β-androstane derivatives and cardenolides. The data points indicate the equilibrium values of inhibition of Na/K-ATPase activity by 5β,14β-androstane-3β,14-diol (■), 3β-rhamnosyloxy-5β,14β-androstan-14-ol (◆), digitoxigenin (▲), and evomonoside (●). The curves were calculated according to the equation:

$$\text{inhibition} = [\text{inhibitor}]/([\text{inhibitor}] + K'_D) \quad (2)$$

in which K'_D denotes the apparent dissociation constant.

medium. Then the release of ^{32}P from the [^{32}P]phoshoenzyme formed was initiated by addition of 2 μl of a solution containing 100 mM ATP, 500 mM KCl, and 30 mM P_i. The reaction was terminated by denaturation as described above either 10, 20, 30, 40, 50, and 60 seconds after the addition of unlabelled ATP or at zero time (preincubation only). Blank values were obtained by substituting K^+ for Na^+ in the incubation medium.

RESULTS

Concentration-inhibition curves

The curves with 5β,14β-androstane-3β,14-diol and its 3β-*O*-rhamnoside show the same shape as those with digitoxigenin or its 3β-*O*-rhamnoside (evomonoside) (Figure 1). The above compounds effect half-maximum inhibition of the Na/K-ATPase from human heart at 153, 7.6, 0.054, and 0.0033 μM, respectively. The good fit of the data points to the theoretical curves, calculated according to equations (1) and (2) (see

Figure 1), suggests that with each compound only one class of interaction site on the enzyme exists, i.e., it conforms with the simple model:

$$\text{enzyme} + \text{inhibitor} \rightleftharpoons \text{enzyme} \cdot \text{inhibitor} \quad (3)$$

With 5β,14β-androstane-3β,14-diol at most 68% inhibition was attained due to limited solubility of the compound.

Apparent competition with K^+

As already known for the influence of K^+ on the inhibition of Na/K-ATPase activity by ouabain,[7] the inhibition by 5β,14β-androstane-3β,14-diol, by its 3β-*O*-rhamnoside as well as by digitoxigenin is apparently competitive to K^+ at low concentrations of K^+, whereas at higher concentrations the inhibition becomes more and more non-competitive to K^+ (Table I).

Effect of 5β,14β-androstane-3β,14-diol on binding of [^{3}H]digitoxigenin

The binding of [^{3}H]digitoxigenin to the human brain ATPase was investigated in the presence of Mg^{++} and P_i using five concentrations of digitoxigenin in the range 7–230 nM. The dissociation constant, K_D^*, and the maximum number of binding sites, E_T, amount to 11.9 ± 1.2 nM and 2.13 ± 0.05 pmole/unit enzyme activity, respectively (data not shown). These parameters were calculated according to:

$$[ED^*] = E_T[D^*]/(K_D^* + [D^*]) \quad (4)$$

in which ED* and D* denote the bound and free [^{3}H]digitoxigenin, respectively. [^{3}H]digitoxigenin bound beforehand to the enzyme is displaced by 5β,14β-androstane-3β,14-diol with nearly the same time constant ($k = 0.66\,min^{-1}$, calculated according to equation (5)) as by unlabelled digitoxigenin ($k = 0.50\,min^{-1}$) (Figure 2). If the added unlabelled compound, I, competes with the labelled compound, D*, for common binding sites then the dissociation constant, K_D^I, of the inhibitor, I, can be calculated as follows:

$$K_D^I = K_D^*[I][ED^*]/(E_T[D^*]\text{-}[ED^*][D^*]\text{-}[ED^*]K_D^*) \quad (6)$$

in which ED* denotes the labelled compound bound to the enzyme in equilibrium. Calculated in this manner the K_D values for 5β,14β-androstane-3β,14-diol and digitoxigenin amount to 56 and 0.012 μM, respectively.

Effect on phosphoenzyme formed from [^{32}P]P_i

Phosphorylation of the Na/K-ATPase from [^{32}P]P_i in the presence of Mg^{++} is promoted by digitalis compounds.[8] The gradation of the molar efficacy in enhancing the phosphoenzyme level and in inhibiting Na/K-ATPase activity is roughly the same (cf. Table II and Figure 1). 3β-Rhamnosyloxy-5β,14β-androstan-14-ol is as effective as cassaine, a compound which was described to behave congeneric to cardiotonic steroids.[9] However, another reversible inhibitor of Na/K-ATPase, Cibacron Blue F3GA, has no effect on phosphorylation from P_i at a strong inhibitory concentration (20 μM)[6] or inhibits phosphorylation at a higher concentration (Table II). The dye seems to "chase" [^{3}H]ouabain like unlabelled ouabain but it acts by occupying the catalytic centre of the Na/K-ATPase[6].

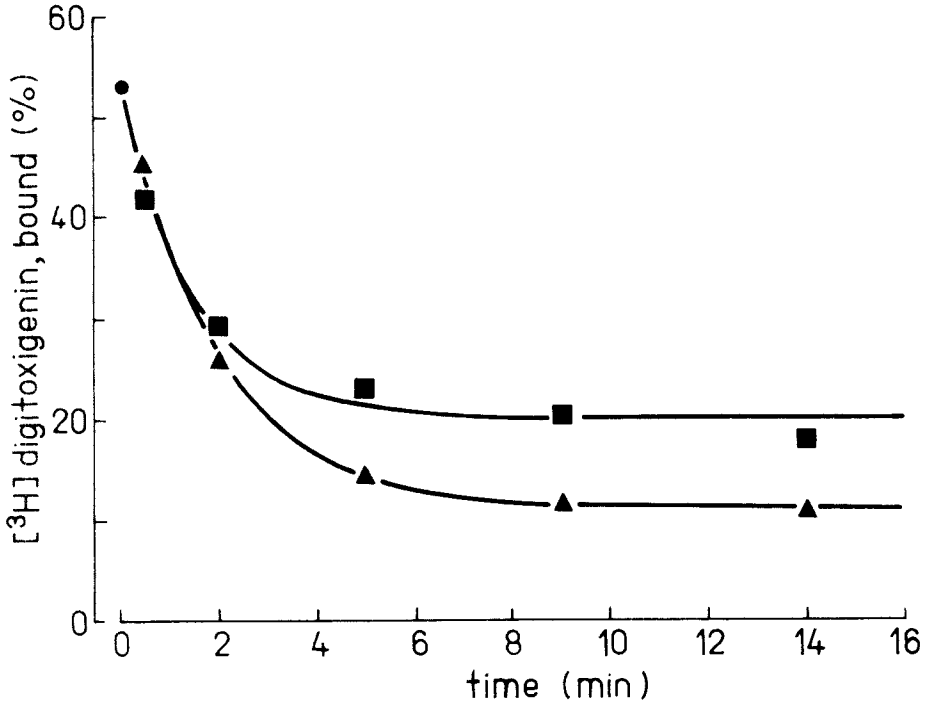

FIGURE 2 Chase of [^{3}H]digitoxigenin by 5β,14β-androstane-3β,14-diol or by digitoxigenin from the enzyme. The Na/K-ATPase preparation was preincubated for 20 minutes in the binding medium containing 13.6 nM [^{3}H]digitoxigenin. At the starting point (●) for the chase of [^{3}H]digitoxigenin by the addition of 200 μM 5β,14β-androstane-3β,14-diol (■) or of 0.1 μM digitoxigenin (▲), the amount of bound [^{3}H]digitoxigenin was 53% of maximum. The curves were calculated according to the equation:

$$B_t = (B_0 - B_\infty)e^{-kt} + B_\infty \quad (5)$$

in which B_0, B_t, and B_∞ denote the amount of [^{3}H]digitoxigenin bound to the enzyme at zero time, at time t, and in equilibrium, respectively; k denotes the time constant. After addition of 5β,14β-androstane-3β,14-diol or digitoxigenin, B_∞ was found to amount to 19.9 and 10.9% of maximum binding, respectively.

Effect on phosphoenzyme formed from [^{32}P]ATP

The interpretation of the influence of digitalis-like compounds on the phosphoenzyme level is more complex in the presence of Na^+, Mg^{++}, and ATP than in the presence of Mg^{++} and P_i. Therefore not the phosphoenzyme level but the kinetics of dephosphorylation of the phosphoenzyme was measured after interrupting rephosphorylation from [^{32}P]ATP by addition of KCl and an excess of unlabelled ATP. If the time course of ^{32}P-release from the phosphoenzyme formed in the absence of a steroidal inhibitor proceeds in a monophasic manner, the reaction will be described by the equation:

$$P_t = (P_0 - P_\infty)\, e^{-k_{-1}t} + P_\infty \quad (7)$$

in which P_0, P_t, and P_∞ denote the amounts of bound ^{32}P at zero time and at the time t after initiation of ^{32}P-release or the amount of unreleasable ^{32}P, respectively. The latter amount of ^{32}P was essentially identical with the ^{32}P-binding in the presence of

K^+ instead of Na^+, i.e., it was unspecifically bound ^{32}P. The rate constant for the dephosphorylation of the phosphoenzyme in the absence of a steroidal inhibitor, k_{-1}, was calculated to be about 19 min^{-1} at 0°C corresponding to a half-life period of 2.2 s (Table III).

In the presence of a steroidal inhibitor, the time course of ^{32}P-release was biphasic due to the co-existence of phosphoenzyme (fast kinetics) and phosphoenzyme-inhibitor complex (slow kinetics). The amounts of these two species were dependent on the concentration of the inhibitors as well as the time of preincubation (not demonstrated). The rate constant for the ^{32}P-release from the phosphoenzyme-inhibitor complex, k_{-2}, was calculated according to:

$$P_t = P_f e^{-k_{-1}t} + (P_0 - P_\infty - P_f)\, e^{-k_{-2}t} + P_\infty \qquad (8)$$

in which P_f and $(P_0 - P_\infty - P_f)$ denote the amount of free phosphoenzyme and phosphoenzyme-inhibitor complex, respectively; for the other terms cf. equation (7). Both digitoxigenin and evomonoside, when bound to the phosphoenzyme, effect an about 30-fold retardation of ^{32}P-release in comparison with the decomposition of the free phosphoenzyme. The stabilizing effect of 5β,14β-androstane-3β,14-diol and of its 3β-*O*-rhamnoside on the phosphoenzyme is striking but seems to be smaller than that of the above cardenolides (Table III). For a possible explanation see DISCUSSION.

DISCUSSION

There are several criteria to characterize the action of a compound as digitalis-like or congeneric to digitalis compounds.[9,10] The two 5β,14β-androstane derivatives investigated here share the following six properties with ordinary digitalis compounds although they are devoid of the lactone ring side chain:

1) The uniform shape of the concentration-inhibition curves. All the typical digitalis compounds inhibit the Na/K-ATPase activity completely. This also applies to 3β-*O*-rhamnosyloxy-5β,14β-androstan-14-ol, i.e. the intrinsic activity of this compound is not different from unity.

II) The ability to reveal the known species differences in the affinity of digitalis compounds to the receptor enzyme[11]. Calculated from the data reported in reference 6, the Na/K-ATPase from human heart is more sensitive to the action of naturally occuring cardenolides and bufadienolides than that from guinea-pig heart by a factor of 8–62. The sensitivity ratio, K'_D (guinea-pig heart enzyme)/K'_D (human heart enzyme), is about 5 and 38 for 5β,14β-androstane-3β,14-diol and its 3β-*O*-rhamnoside, respectively (cf. reference 6 and this paper, section Concentration-inhibition curves).

III) The apparent competition with K^+ and its special dependence on the K^+ concentration. K^+ ions induce an enzyme conformation which either does or does not easily bind digitalis compounds, 5β,14β-androstane-3β,14-diol or its glycoside. Thus, the dependence of the interaction with the enzyme on the enzyme conformation is similar with ordinary digitalis compounds and the lead structure or its glycoside.

IV) The competition with digitoxigenin for common binding sites. Although it is hard to prove a true competition for a common binding site, the results shown in Figure 2 favour this assumption. The attainment of an equilibrium after addition of a

non-saturating amount of 5β,14β-androstane-3β,14-diol is a strong hint at competition with digitoxigenin. Therefore, the application of equation (6) seems to be justified.

V) The reduction of the dephosphorylation rate of the phosphoenzyme. The dephosphorylation rate of the phosphoenzyme, when ligated with 5β,14β-androstane-3β,14-diol or with its glycoside, becomes considerably decreased in the turnover system at 0°C. The efficacy of ordinary digitalis compounds to inhibit Na/K-ATPase activity is mostly due to the relatively large half life period of the enzyme-inhibitor complex, which cannot be rephosphorylated by ATP. For instance, the half life period of the complexes of digitoxigenin and evomonoside with the human brain enzyme were determined under turnover conditions at 37°C to amount to 0.9 and 15 min (not shown). Because the ^{32}P-release from the phosphoenzyme ligated with an ordinary digitalis compound is much faster than the dissociation of the inhibitor from the complex, the decomposition of the phosphoenzyme-inhibitor complex is described as follows:

$$I \cdot E\text{–}P^* \rightarrow I \cdot E + P_i^* \rightarrow E + I + P^*_i \qquad (9)$$

The rate constant for ^{32}P-release from the phosphoenzyme-inhibitor complex does not differ for the two cardenolides (Table III), and hence seems to reflect an intrinsic property of the enzyme protein. However, with the two androstane derivatives the rate constant of ^{32}P-release appears to be greater. This might be explained by assuming that the dissociation of the androstane derivatives from the enzyme is much faster than that of the cardenolides, so that the decomposition of their complexes with the phosphoenzyme follows also this pathway:

$$I \cdot E\text{–}P^* \rightleftharpoons E\text{–}P^* + I \rightarrow E + P^*_i + I \qquad (10)$$

VI) The enhancement of the level of phosphoenzyme formed from [^{32}P]P_i. In the presence of Mg^{++} the phosphorylation of the Na/K-ATPase from P_i is known to be much promoted by digitalis compounds.[8] This very characteristic effect is also produced by their lead structure but not by different inhibitor types as shown in the present paper (Table II). The promotion of ^{32}P-incorporation from P_i appears to be the most suitable criterion for classifying a compound as digitalis-like (cf. also reference 12).

Acknowledgements

The authors thank Dr. Bauke (Städtisches Klinikum Berlin-Buch) for providing suitable specimens of cardiac muscle and cerebral cortex from human autopsies.

References

1. Repke, K.R.H. and Portius, H.J. *Experientia,* **19,** 452, (1963).
2. Repke, K.R.H. and Schönfeld, W. *Trends Pharmacol. Sci.,* **5,** 393, (1984).
3. Schwartz, A., Matsui, H. and Laughter, A.H. *Science,* **160,** 323, (1968).
4. Schönfeld, W., Weiland, J., Lindig, C., Masnyk, M., Kabat, M.M., Kurek, A., Wicha, J. and Repke, K.R.H. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **329,** 414, (1985).
5. Schönfeld, W., Schönfeld, R., Menke, K.-H., Weiland, J. and Repke, K.R.H. *Biochem. Pharmacol.,* **35,** 3221, (1986).

6. Schönfeld, W., Menke, K.-H., Schönfeld, R. and Repke, K.R.H. *Biochem. Biophys. Res. Comm.*, **119,** 423 (1984).
7. Akera, T., Larsen, F.S. and Brody, T.M. *J. Pharmacol. Exp. Ther.*, **170,** 17, (1969).
8. Sen, A.K., Tobin, T. and Post, R.L. *J. Biol. Chem.* **244,** 6596, (1969).
9. Tobin, T., Akera, T., Brody, S.L., Ku, D. and Brody, T.M. *Eur. J. Pharmacol.*, **32,** 133, (1975).
10. Wehling, M., Schwartz, A., Whitmer, K., Grupp, G., Grupp, I.L. and Wallick, E.T. *Mol. Pharmacol.*, **20,** 551, (1981).
11. Repke, K.R.H., Est, M., and Portius, H.J. *Biochem. Pharmacol.*, **14,** 1785, (1965).
12. Carilli, C.T., Berne,M., Cantley, L.C. and Haupert, G.T., Jr., *J. Biol. Chem.*, **260,** 1027, (1985).