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ACTIVE SITE-DIRECTED ALKYLATION OF Na^+-K^+-ATPase BY DIGITALIS SULPHONATE DERIVATIVES OF DIFFERENT LIPOPHILICITY

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1 Sulphonate derivatives of k-strophanthidin and digitoxigenin were tested as active site-directed labels of Na^+-K^+-adenosine triphosphatase (Na^+-K^+-ATPase) from guinea-pig heart.

2 Lipophilicity ranged between P = 93 for strophanthidin-3-tosyloxy-acetate (STA) and P = 3028 for digitoxigenin-3-tosyloxy-acetate (DTA).

3 Although the alkylating moiety of STA and DTA was identical, the reversibility of Na^+-K^+-ATPase inhibition varied appreciably (82% and 35% respectively).

4 It is concluded that lipophilicity contributes considerably to the irreversible binding of alkylating cardiotonic steroids to myocardial Na^+-K^+-ATPase.

Introduction

In spite of numerous investigations aimed at elucidating the mode of action of cardiac glycosides, it is still not completely understood. Although it is well established that inhibition of Na^+-K^+-adenosine triphosphatase (Na^+-K^+-ATPase) by digitalis compounds is responsible for their toxic effects, the exact mechanism of the inotropic action remains obscure (Lee & Klaus, 1971).

The Na^+-K^+-ATPase is generally accepted as the most specific binding site ('receptor') for cardiotonic steroids, and interest has recently been focussed on this system. A convenient method for characterization of the cardiac glycoside receptor is its labelling by covalently binding digitalis derivatives, which are supposed to inhibit Na^+-K^+-ATPase irreversibly. There are two standard methods to obtain covalent attachment: (1) Chemical affinity labelling with alkylating compounds containing reactive groups (Hokin, Mokotoff & Kupchan, 1966; Peters, Raben & Wassermann, 1974) or by reductive amination of oxidized ouabain by enzyme amino groups (Hegyvary, 1975); (2) photoaffinity labelling with compounds forming reactive intermediates upon u.v.-irradiation, (Ruoho & Kyte, 1974; Tobin, Akera, Brody & Taneja, 1976; Rogers & Lazdunski, 1979).

Though photoaffinity labelling of the enzyme offers certain advantages over chemical labelling, it has a serious drawback in that the intense u.v.-irradiation necessary for photoactivation unavoidably attacks the Na^+-K^+-ATPase itself, resulting in a considerable loss of enzyme activity (Forbush III & Hoffman, 1979; Rogers & Lazdunski, 1979). In contrast, chemical labelling is more exact and does not affect Na^+-K^+-ATPase activity by unspecific mechanisms.

However, the attempts to label the myocardial glycoside receptor chemically have not been very successful (Fricke & Klaus, 1971; Peters *et al.*, 1974), possibly because of insufficient chemical reactivity or poor penetration of the label to the active centre due to unsuitable physicochemical properties (e.g. lipophilicity). To test this possibility and to overcome these difficulties, two new cardenolides were synthesized: strophanthidin-3-tosyloxy-acetate (STA) and digitoxigenin-3-tosyloxy acetate (DTA), both having a potent alkylating group, but being different in their lipophilicity.

The present paper deals with the inhibitory potency of these two compounds on a microsomal Na^+-K^+-ATPase preparation from guinea-pig hearts and the reversibility of this enzyme inhibition. For comparison, the parent compounds k-strophanthidin (STR) and digitoxigenin (DTG) were included in this study.

Methods

Strophanthidin-3-tosyloxy-acetate (STA) and digitoxigenin-3-tosyloxy-acetate (DTA) were prepared from the corresponding 3-bromoacetates of strophanthidin (Hokin *et al.*, 1966) and digitoxigenin (Abramson & Cho, 1971) by reaction with silver *p*-toluenesulphonate in acetonitrile according to the method of Emmons & Ferris (1953). Further details of synthesis

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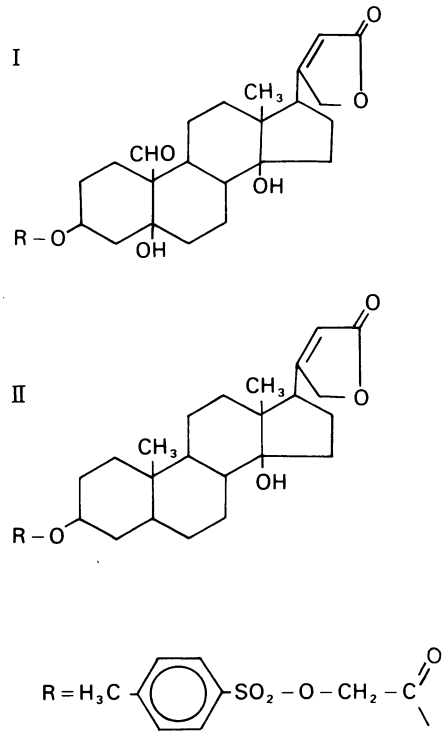

Figure 1 Structures of the alkylating cardiotonic steroids: (I) strophanthidin-3-tosyloxy-acetate (STA) and (II) digitoxigenin-3-tosyloxy-acetate (DTA).

and characterization will be given elsewhere (Rogatti, thesis). The chemical structures are given in Figure 1.

Lipophilicity of the test compounds was estimated by determination of the octanol/water partition coefficients (P). Drug concentrations were determined either in the lipophilic or the hydrophilic phase by means of u.v.-spectrophotometry (Aminco DW2 uv/vis, 220/295 nm).

Na^+-K^+-ATPase was prepared from guinea-pig hearts according to Fricke & Klaus (1974). Enzyme activities were determined at 37°C in the presence of different concentrations of the test substances. Usually 10 µg of protein was preincubated for 10 min in 100 mM imidazole/HCl buffer, pH 7.4, containing Mg^{2+} 5 mM, Na^+ 100 mM, K^+ 5 mM and disodium edetate (EDTA) 1 mM. The reaction was started by the addition of ATP, the final concentration being 2 mM. After 30 min inorganic phosphate was determined by the method of Eibl & Lands (1969). Each assay was performed in duplicate. Na^+-K^+-stimulated ATPase activity was calculated as the difference between total and Mg^{2+}-dependent activity. All results were corrected for blank values without enzyme.

As shown in the flow sheet (Figure 2), the reversibility of drug-induced enzyme inhibition was studied by a modified dilution technique according to Peters *et al.* (1974). The original procedure described by these authors was extended by three control samples (tubes 1, 2, 3 see flow sheet, Figure 2), representing the blank value without enzyme and the ATPase activity in the presence of Mg^{2+} and Na^+ or Mg^{2+}, Na^+ and K^+, respectively. This allowed us to follow the enzyme inhibition by the test compounds in each incubation step. In addition, tubes 4 and 5 contained the test compounds in a concentration that produced an enzyme inhibition of approx. 50 to 70%. Tube 6 differed from tubes 4 and 5 in having a ten times lower drug concentration producing an ATPase inhibition of 20 to 30%. After incubation for 30 min, inorganic phosphate was determined as described above. Simultaneously 0.1 ml of each sample was transferred to a medium of identical electrolyte composition resulting in a ten fold dilution of the enzyme and in addition of the test compound in tube 5 (see flow sheet, Figure 2). After an additional 30 min incubation (in some experiments 60 min of incubation were allowed), a second determination of inorganic phosphate was performed as described above. By comparison of the enzyme inhibition in tube 5 (= ten fold dilution of the drug concentration) to that of tube 4 (higher drug concentration) and that of tube 6 (lower drug concentration) reversibility (stability) of the drug-enzyme-complex was calculated.

Results

Lipophilicity of the drugs studied increased in the following order (P-values are given in parentheses): STR (4.1), STA (93), DTG (216), DTA (3028).

Mean specific activity of the Na^+-K^+-ATPase preparation was 11.4 µmol Pi/mg protein × h and amounted to 40 to 50% of the total activity. The tested drugs caused a dose-dependent inhibition of the Na^+-K^+-ATPase activity; the inhibiting potency of the new cardiotonic steroids STA and DTA was increased two to four fold compared with that of the parent compounds STR and DTG. The respective ID_{50} values are given in Table 1.

Reversibility of the drug-induced Na^+-K^+-ATPase inhibition as determined by the dilution technique is shown in Figure 3. Whereas inhibition of the Na^+-K^+-ATPase by STR and DTG was fully reversible (101.6 ± 2.3%, $n = 6$ and 97.7 ± 6.2%, $n = 6$, respectively) that of the 3-tosyloxy-acetates was only reversible by 81.7 ± 5.1% (STA, $n = 6$) and 34.6 ± 4.2% (DTA, $n = 6$). The differences in reversibility between STR and STA ($P < 0.005$) and DTG and DTA ($P < 0.0005$), respectively were highly significant. There was no change in reversibility when the

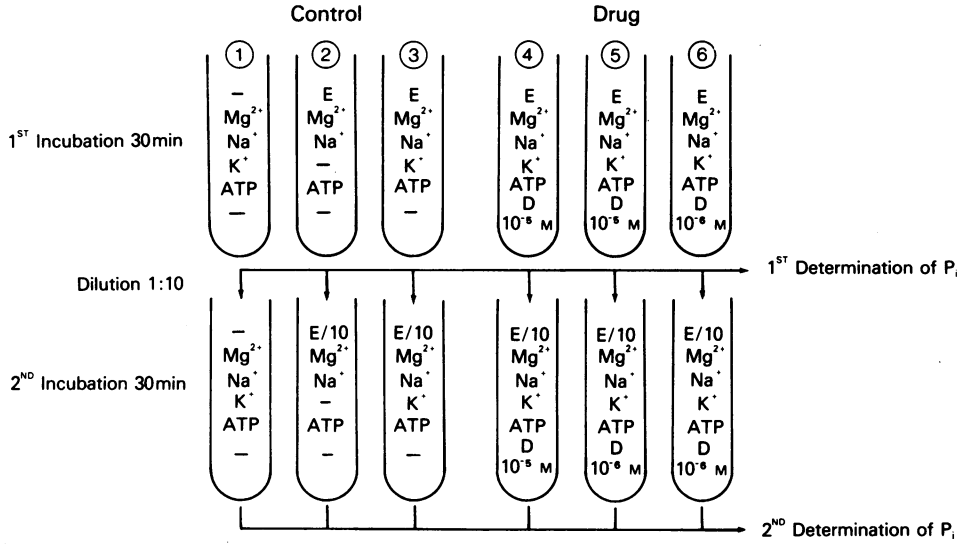

Figure 2 Flow sheet of the experimental procedure for determination of the reversibility of drug-induced Na^+-K^+-ATPase inhibition (further details see methods section). E = enzyme, ATP = adenosine triphosphate, D = test compound studied.

second incubation period (dilution period) was increased to 60 min, indicating the high stability of the drug-enzyme-complex.

Discussion

The present results indicate within the limitations of the method used, that in contrast to the parent compounds a diminished reversibility of the drug-enzyme-complex was obtained with STA and DTA.

Similar results to those observed in this study with the aglycones STR and DTG (i.e. full reversibility of enzyme inhibition) have also been reported recently on the basis of an analogous dilution technique (Yoda & Hokin, 1970; Yoda, 1973; Yoda & Yoda, 1974, Yoda & Yoda, 1977). However, whereas in these studies the aglycone-enzyme-complex was readily reversible, a more stable complex was obtained with cardiac glycosides. This is in accordance with the results of Peters *et al.* (1974), but conflicts with results of the studies of Tobin & Sen (1970), Akera & Brody (1971), and Akera, Ku, Tobin & Brody (1976). These differences may be accounted for by the different sources of the enzyme used (beef brain and heart, dog heart, guinea-pig kidney, rat brain). In our study the cardiac glycoside (ouabain)-induced inhibition of the Na^+-K^+-ATPase proved to be fully reversible (data not shown), unlike that of STA and DTA.

The present results establish that the alkylating potency of STA and DTA, as reflected in the irreversibility of enzyme inhibition, increases with their lipophilicity. Although the alkylating moiety of these compounds is identical, the reversibility of enzyme inhibition differs appreciably: whereas inhibition by STA is reversible by 82%, inhibition by the more lipophilic DTA shows only 35% reversibility. This is

Table 1 ID_{50} values (concentration for half maximum inhibition of Na^+,K^+-ATPase activity) of the test compounds

	$ID_{50}(\times 10^{-6}$ M)
STR	25.2 ± 4.9
STA	6.3 ± 0.9
DTG	4.9 ± 0.8
DTA	3.0 ± 0.2

Mean values and s.e. mean from 8 experiments for each group are given. STR = k-strophanthidin; STA = strophanthidin-3-tosyloxy-acetate; DTG = digitoxigenin; DTA = digitoxigenin-3-tosyloxy-acetate.

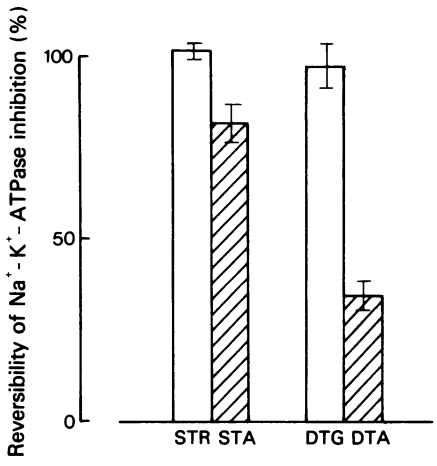

Figure 3 Reversibility of Na^+-K^+-ATPase inhibition in percent. Mean values and standard error of the means from 6 experiments for each drug are shown: STR = k-strophanthidin; STA = strophanthidin-3-tosyloxy-acetate; DTG = digitoxigenin; DTA = digitoxigenin-3-tosyloxy-acetate.

probably the consequence of the easier access of DTA to the receptor site due to its higher lipophilicity.

The irreversible enzyme inhibition of 65% produced with DTA, is the most effective chemical affinity label ever described for myocardial Na^+-K^+-ATPase preparations. This drug may prove to be useful as a tool for studying, in more detail, the digitalis-receptor-interaction and its relevance for the pharmacological action of these compounds.

Very few successful chemical labelling experiments have been reported up till now (Hokin *et al.*, 1966; Ruoho, Hokin, Hemingway & Kupchan, 1968) and these studies were performed on ATPase preparations that did not originate from heart muscle. The properties of ATPase preparations, may differ appreciably depending on their origin (for references see Lee & Klaus, 1971), and the relevance of such results for the interpretation and understanding of the action of cardiac glycosides in the myocardium appears to be doubtful. For example, one label causing almost complete irreversible inhibition of brain Na^+-K^+-ATPase proved to be fully reversible in the myocardial enzyme preparation (Fricke & Klaus, 1971).

An advantage of a chemical affinity label is the possibility of comparing its irreversibility in Na^+-K^+-ATPase inhibition with its biological effects in isolated heart muscle preparations, which seems to be impracticable or at least difficult for photoaffinity labels. It is interesting to note, that in experiments on isolated papillary muscles of guinea-pig, STA and DTA showed complete reversibility of their positive inotropic actions on washout (Fricke, Klaus & Rogatti, 1979). The lack of correlation between reversibility of enzyme inhibition and reversibility of inotropy within these two systems is consistent with the findings of Okita, Richardson & Roth-Schechter (1973) and Peters *et al.* (1974) and further supports the concept of a dissociation of the positive inotropic effect of cardiac glycosides from inhibition of Na^+-K^+-ATPase.

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