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SPIN-LABELED OUABAIN AS A PROBE FOR CARDIAC GLYCOSIDE RECEPTOR/Na,K-ATPase

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We have synthesized a nitroxide spin-label derivative of the cardiac glycoside, ouabain. It has been tested on purified Na,K-ATPase from dog kidney as a probe to study the physico-chemical interactions of this class of drugs with receptor(s). Spin-labeled ouabain retained the biochemical properties of native ouabain as judged by dose response curves for inhibition of Na,K-ATPase activity and inhibition of [^{3}H]-ouabain binding to receptor. Electron paramagnetic resonance spectra of the spin-labeled ouabain-Na,K-ATPase complex revealed the presence of two types of bound label with differing degrees of immobilization.

Cardiac glycosides are a class of drugs which are widely used in the treatment of heart failure. The exact mechanism of action of the cardiac glycosides is not completely understood but Na,K-ATPase is widely regarded as the pharmacologic receptor (1,2). While cardiac glycosides are potent and specific inhibitors of Na,K-ATPase activity and inhibition appears to correlate with the ionotropic response, other receptors cannot be excluded (1-4). Owing to the importance of the Na,K-ATPase system for normal cell function, cardiac glycoside toxicity is a serious problem and is reflected in an abnormally low therapeutic index for these drugs (5). Thus an understanding of how cardiac glycosides react with their receptor(s) is important for improving the efficacy of therapy and reducing the toxicity of cardiac glycosides.

Abbreviations used: EPR, electron paramagnetic resonance; Hepes, hydroxyethylpiperazime-N'2-ethanesulfonic acid; TEMPO, 2,2,6,6-tetramethyl-piperidenoxyl; SDS, sodium dodecyl sulfate.

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The usefulness of EPR spectroscopy has been greatly extended through use of spin-labeling techniques in which a molecule with a stable nitroxide radical (spin-label) is chemically coupled to a particular effector or biomolecule which then serves as a probe for several aspects of the physico-chemical environment of the particular biomolecule (6). In the initial phase of this study we have synthesized a spin-labeled derivative of ouabain, in which the spin-label is coupled to the carbohydrate moiety of ouabain, and demonstrated the feasibility of using spin-labeled derivatives of cardiac glycosides as probes to study interactions of this class of drugs with their receptor(s).

MATERIALS AND METHODS

Materials. [^{3}H]-ouabain (19.5 Ci/mmol) was purchased from New England Nuclear. Sodium cyanoborohydride and 4-amino-2,2,6,6-tetramethylpiperidinooxyl (4-amino-TEMPO) were purchased from Aldrich Chemical Company. All other chemicals were of the highest grade commercially available.

Enzyme preparation and assay. Na,K-ATPase was prepared from the microsomal fraction of fresh dog kidney outer medulla as described by Jorgensen (7). Latent Na,K-ATPase was activated by incubation of the microsomal fraction with low concentrations of SDS in the presence of ATP (7,8). The amount of detergent necessary to achieve optimal activation was determined for each preparation. Activated samples were applied to gradients of 15-45% (w/v) sucrose in 15mM Hepes, 1mM EDTA, pH 7.4 and centrifuged for 3h at 100,000 x g at 4°C. Peak fractions of Na,K-ATPase were combined, diluted 4-fold with sucrose-free buffer and concentrated by centrifugation. The pellet was resuspended in sucrose-free buffer to a concentration of about 10mg protein/ml. This purified plasma membrane fraction had little or no ouabain-insensitive activity and the specific activity of Na,K-ATPase activity ranged between 6-10 µmoles ATP hydrolyzed per min per mg protein at 37°C. Na,K-ATPase activity was assayed as described previously (9), 1 unit of activity being defined as the amount of enzyme required to catalyse the hydrolysis of 1µMol of ATP per min under the conditions of the assay. Protein was determined by the method of Schaffner and Weissmann (10).

Synthesis of spin-labeled ouabain. Ouabain (25mM) was incubated with sodium periodate (75mM) in 50% ethanol for 60 min at room temperature and the mixture passed over a 1.5 x 8cm Dowex 1-X8(Cl^-) column. The flow-through fraction was incubated with a 5-fold molar excess of 4-amino-TEMPO and sodium cyanoborohydride in 50mM potassium phosphate, 50% ethanol, pH 7.0 for 24h at room temperature. The reaction mixture was passed over a 1.5 x 8cm

Dowex 1-x8(Cl^-) column and the flow through fraction collected and lyophilized. The spin-labeled ouabain conjugate was further purified by Sephadex LH-20 column (1.5 x 50cm) chromatography with ethanol as the eluting solvent and by silica gel column (2.5 x 25cm) chromatography with chloroform:methanol (4:1) as the eluting solvent. The overall recovery as judged by either cardiac glycoside assay (11) or EPR analysis was about 20%. Product purity was analyzed by TLC on Silica gel 60 with chloroform:methanol (4:1) as the developing solvent. Chromatograms were developed by applying a solution of 2% ceric ammonium sulfate in 2N H_2SO_4 followed by a brief incubation at 70°C (12). A single spot (R_f=0.63) was detected after TLC of the spin-labeled ouabain conjugate. Under identical conditions, periodate-oxidized ouabain and ouabain exhibited R_f values of 0.32 and 0.08, respectively.

Competitive filter binding assay. This procedure was adapted from the procedure described by Yarus and Berg (13). Reaction mixtures contained 3mM ATP, 10^{-8}M [^{3}H]-ouabain (19.5Ci/mMol) and 11µg/ml dog kidney Na,K-ATPase (8.8 units/mg) in 15mM Hepes, 120mM NaCl, 3mM $MgCl_2$ buffer, pH 7.4 and the indicated concentrations of ouabain or spin-labeled ouabain. After 1 h equilibration at room temperature, samples (200µl) were vacuum filtered (nitrocellulose filters, 2.4cm, 0.45µ), washed with 2.5ml of ice-cold 15mM Hepes, 120mM NaCl, 3mM $MgCl_2$, pH 7.4, dried and transfered to scintillation vials for determination of bound radioactivity. Samples without inhibitor had approximately 15,000 cpm bound radioactivity/200µl aliquot. Non-specific binding, determined in the presence of 1mM ouabain, was approximately 0.5% of specific binding.

EPR analysis. EPR spectra were obtained at 19°C, 20mW microwave power and 0.063mT modulation amplitude using a Varian E109 EPR spectrometer operating at 9 GHz. Spin-labeled samples were prepared by incubating Na,K-ATPase (2.9mg/ml, 8.8 units/mg) with 10µM spin-labeled ouabain and 3mM ATP in 15mM Hepes, 120mM NaCl, 3mM $MgCl_2$, pH 7.4, until approximately 80% innibition of initial activity. Samples were centrifuged through a layer of cold 17% (w/v) sucrose in 15mM Hepes, 1mM EDTA, pH 7.4, washed (3x) in cold 15mM Hepes, 1mM EDTA, pH 7.4, to remove free spin-labeled ouabain and resuspended at a concentration of about 40mg protein/ml. Control samples in which the enzyme was pre-treated with excess ouabain (1mM) were prepared under identical conditions.

RESULTS AND DISCUSSION

The scheme for the synthesis of spin-labeled ouabain, analogous to that previously described by Forbush et al. (11) for the synthesis of a photoaffinity derivative of ouabain, is shown in Fig. 1. During the development of this reaction scheme, we synthesized a number of other derivatives, several of which inhibited Na,K-ATPase activity. However, the greatest degree of immobilization of the bound spin label was observed when there

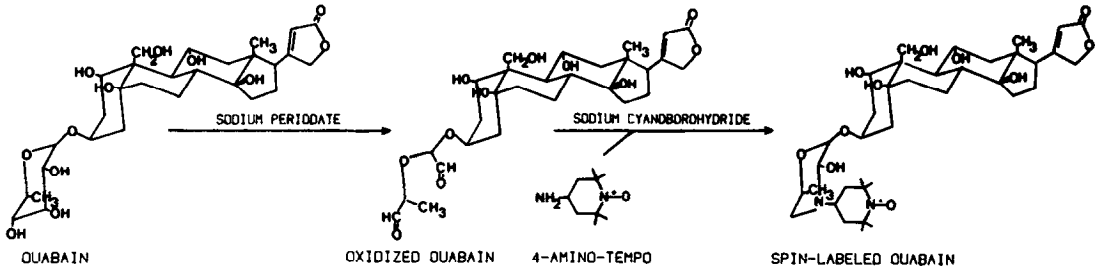

Fig. 1. Scheme for the Synthesis of Spin-Labeled Ouabain. Details are described in MATERIALS AND METHODS.

was no spacer group between the carbohydrate moiety and the TEMPO ring. The high degree of immobilization exhibited by the bound spin-labeled derivative formed from periodate-oxidized ouabain and 4-amino-TEMPO made this an attractive derivative for further studies on factors involved in the interaction of ouabain with its receptor(s).

An important consideration in studies utilizing derivatives of biomolecules as functional probes is retention of biological or pharmacological activity after derivatization. To address this problem we tested spin-labeled ouabain both as an inhibitor of Na,K-ATPase activity and as an inhibitor of [^{3}H]-ouabain binding. As shown in Fig. 2A, spin-labeled ouabain was nearly as effective as ouabain in the inhibition of Na,K-ATPase activity when tested under the same conditions. The concentration of inhibitor which gave 50% inhibition of control activity was slightly lower than 1μM for ouabain and slightly higher than 1μM for spin-labeled ouabain. Spin-labeled ouabain also inhibits the binding of [^{3}H]-ouabain to receptor (Fig. 2B). The I_{50} values for ouabain and spin-labeled ouabain were both in the range of 0.1μM. Preliminary binding experiments established conditions for time required for apparent equilibrium of the binding reaction and linearity with respect to protein concentration. Since inhibition of ATPase activity and competitive binding assays were

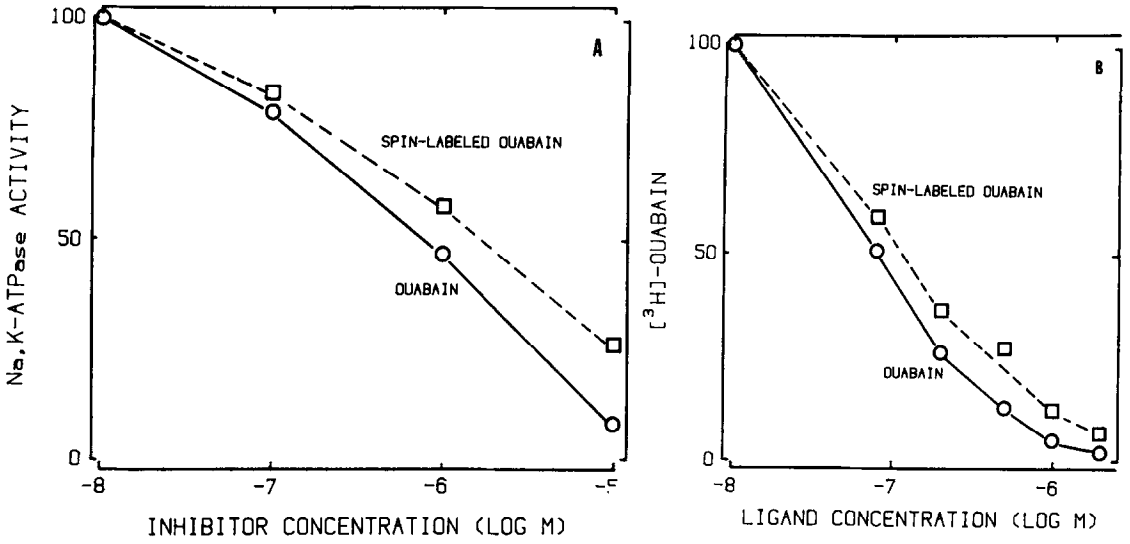

Fig. 2. A: Inhibition of Na,K-ATPase Activity by Ouabain and Spin-Labeled Ouabain. Reactions were started by the addition of enzyme to a mixture containing the indicated concentrations of ouabain or spin-labeled ouabain. 100 represents the relative activity in the absence of ouabain or spin-labeled ouabain. 0 represents the activity in the presence of excess (1mM) ouabain. B: Inhibition of [^{3}H]-Ouabain Binding by Ouabain and Spin-Labeled Ouabain. Reaction mixtures were equilibrated with the indicated concentrations of ouabain or spin-labeled ouabain for 60 min at room temperature. 100 represents the relative amount of bound [^{3}H]-ouabain in the absence of added non-radioactive ouabain or spin-labeled ouabain. 0 represents the amount of bound [^{3}H]-ouabain in the presence of excess (1mM) ouabain.

done under different conditions, the results were not directly comparable. For example the enzyme assays are started by addition of Na,K-ATPase to mixtures containing various concentrations of inhibitor while the binding assays were done after equilibration of Na,K-ATPase with the inhibitor. Since binding of cardiac glycosides to receptor is time dependent (14) lower I_{50} values would be expected under conditions used for the binding assays.

The EPR spectra of free and bound forms of spin-labeled ouabain are shown in Fig. 3. The spectrum of spin-labeled ouabain (B) indicated that motion of the nitroxide function was only slightly restricted in comparison with 4-amino TEMPO (A). In contrast, the spectrum of spin-labeled ouabain-Na,K-ATPase

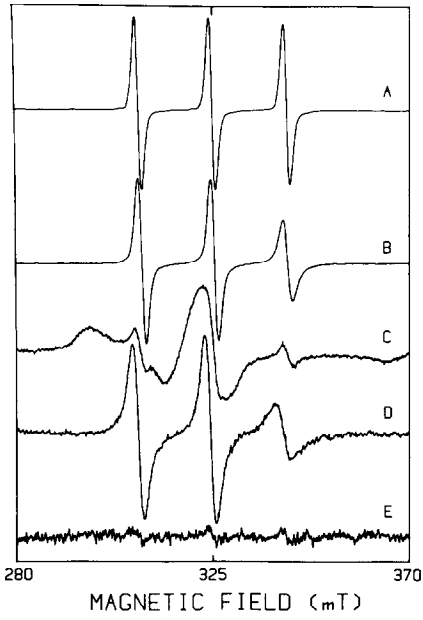

Fig. 3. EPR Spectra of Spin-Labeled Ouabain. A: 4-amino-TEMPO. B: spin-labeled ouabain. C: spin-labeled ouabain-Na,K-ATPase complex. D: same as C but after treatment with 2% SDS. E: same as C but with Na,K-ATPase pre-treated with excess (1mM) ouabain.

complex (C) revealed a dominant strongly immobilized species and a minor, weakly immobilized component. The latter component was unaffected by further washing and was also present in about the same proportions in samples which were inhibited less than 50%. The possible significance of this weakly immobilized component will require further study but could possibly reflect distribution between bound and unbound forms of spin-labeled ouabain at equilibrium or different states or forms (isozymes) of the enzyme (1). The spectrum (D) observed after detergent dissociation of the spin-labeled ouabain-Na,K-ATPase complex was similar to that of free spin-labeled ouabain (B). No EPR signal was observed in samples pre-treated with excess ouabain prior to incubation with spin-labeled ouabain (E) which together with spectrum D indicates the immobilization of the spin-labeled ouabain is due to specific binding with its receptor(s).

These results demonstrate the potential for this and similar spin-labeled cardiac glycosides as probes for studying the interaction of this class of drugs with its receptor(s).

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