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SHORT COMMUNICATIONS

Effect of cryptosin on Na^+,K^+-ATPase—A ^{31}P NMR spectroscopic study

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Cryptosin is a new cardiac glycoside isolated from the leaves of Indian milkweed, *Cryptolepis buchanani* Roem and Schult [1]. Cryptosin was found to have a novel structure [2, 3], which prompted us to study its effect on mammalian heart.

Cardiac glycosides are believed to interact with membrane bound cardiac Na^+,K^+-ATPase in order to elicit the positive inotropic effect [4, 5]. We studied cryptosin–ATPase interaction by a new approach, using ^{31}P nuclear magnetic resonance (NMR) spectroscopy, and compared the findings with those obtained by the conventional method. A study of cardiac glycoside interaction with Na^+,K^+-ATPase using ^{31}P NMR has not been attempted thus far.

Experimental Procedures

Na^+,K^+-ATPase from guinea pig heart was freshly prepared prior to its use by a method reported previously [6]. The final preparation was a suspension 0.3 M sucrose and 0.05 M Tris buffer (pH 7.4). The preparations had an average Na^+,K^+-ATPase activity of 0.36 μmol/mg protein/min which was consistent with the enzyme used for the chemical assays. The chemical assay was a modification of an earlier method [6]. Briefly, the reaction was carried out in 50 mM Tris–HCl (pH 7.4), in a final volume of 1.5 ml, at 37° for 30 min. The reaction was arrested by trichloroacetic acid (TCA), and the levels of P_i as a complex with molybdate were measured at 660 nm.

Sample preparation. For NMR studies, the enzyme suspension was preincubated for 10 min with cryptosin before the substrate, ATP, was added. After the addition of ATP, the mixture was incubated again for 45 min before recording of NMR spectra.

^{31}P NMR spectroscopy was done at 32.2 MHz using a Varian FT-80A NMR spectrometer; the spectra were recorded in an FT mode. Samples were taken in a 10-mm NMR tube. The reaction mixture (final volume of 2 ml) contained 0.05 M Tris–HCl and 0.3 M sucrose (pH 7.4), NaCl (0.1 M), KCl (0.03 M), $MgCl_2$ (0.01 M), ATP (0.03 M) and cryptosin, if added.

NMR experimental parameters. ^{31}P NMR spectra were recorded using deuterium oxide (D_2O) as the external lock. The number of transients was 2500 with an acquisition time of 1 sec and a pulse width of 10 μsec, for each scan. All chemical shift parameters are with reference to 85% phosphoric acid which was used as the external standard during the recording of the ATP spectra. All spectra were collected under fully relaxed conditions to enable a meaningful comparison of the samples and the control. The external standard was placed in a small probe tube within the sample tube. The resonance intensity of the external standard was calibrated in absolute concentrations of KH_2PO_4 separately, for comparison. All other conditions of the reaction were identical to those used in the chemical assay. The extent of inhibition of the release of phosphate was measured by a comparison of the areas of the peak due to inorganic phosphate in the absence and presence of cryptosin (or ouabain).

Results

The objective of the present studies was to investigate the effect of cryptosin on Na^+,K^+-dependent ATPase activity. We examined by ^{31}P NMR spectroscopy, the hydrolysis of ATP under various conditions (Fig. 1). Panel (a) gives the spectrum of ATP alone under the conditions in which the enzyme was assayed. The spectrum is in agreement with that reported in the literature [7]. Panel (b) shows the spectrum of ATP in the presence of Na^+,K^+-ATPase; panels (c) and (d) demonstrate the effects of 0.1 and 1 mM cryptosin, respectively, on the spectrum of ATP (in the presence of ATPase). A comparison of the spectra in panels (b) to (d) reveals the following salient features:

(i) The spectrum of ATP in the presence of Na^+,K^+-ATPase was characterized by the appearance of a down field peak which could be identified as due to inorganic phosphate from the hydrolysis of ATP [7].

(ii) The peaks due to all three phosphates, α, β and γ, in Fig. 1b were broadened relative to the peaks in the spectrum of ATP alone. At the position corresponding to β and γ phosphates, new peaks appeared which may have been contributed from the product, ADP. The broadening of the peaks can be attributed to either a non-specific viscosity effect of the protein upon the spin–spin relaxation time [8] or association of the substrate, ATP, and the product, ADP, with the enzyme [8].

(iii) The addition of cryptosin in increasing concentrations of 0.1 and 1 mM led to a decrease in the area of the peak due to inorganic phosphate (Fig. 1, c and d).

(iv) The higher concentration of cryptosin (1 mM) also counteracted, to a significant extent, the effect of ATPase on the spectrum of ATP, e.g. the multiple peak spectrum of ATP (Fig. 1b) in the presence of 1 mM cryptosin (Fig. 1d) was, to a major degree, similar to what was observed in the absence of ATPase (Fig. 1a).

The above observations indicate that cryptosin inhibited the hydrolysis of ATP by the enzyme and that the inhibitory effect of cryptosin was dependent on its concentration. To obtain a quantitative picture, the percent decrease in the area of the phosphate peak was calculated (from the spectra plotted at an expanded scale) as a function of the concentrations of added cryptosin. The resulting curve is shown in Fig. 2; inhibition of phosphate release plateaued at about 1 mM cryptosin. A similar inhibition curve of ATPase activity (measured by chemical estimation of liberated inorganic phosphate) was reported previously for ouabain [9]; the maximum percentage inhibition was 75% corresponding to a plateau concentration of 1 mM of the exogenous cryptosin. The observation of inhibition of ATP hydrolysis in the presence of cryptosin was also corroborated by the measurement of inorganic phosphate using an available chemical method [10]. Table 1 shows excellent agreement between the values for percent inhibition of the enzyme activity evaluated by the two methods.

The ^{31}P NMR method was extended further to compare the inhibitory effects of cryptosin and ouabain. Figure 3 illustrates the comparative inhibitory effects of the two cardiac glycosides on ATP hydrolysis. From the relative area of the peaks due to inorganic phosphate (in Figs. 3a and 3b), it appears that the effects are comparable. This is also consistent with the values of the inhibition constant (K_i) evaluated from Dixon plot analysis of the ATPase activity measured by the chemical method in the presence of cryptosin or ouabain (Table 2). The results from Table 2 lend further support to the validity of the NMR method

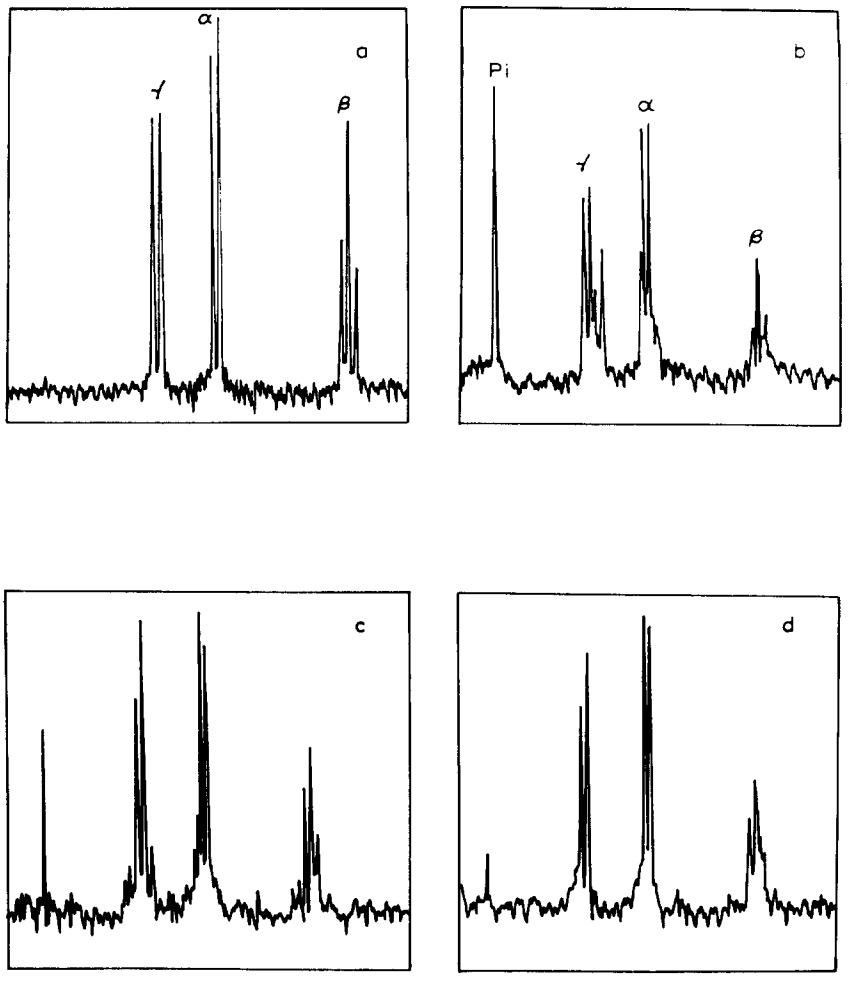

Fig. 1. ^{31}P NMR spectra of ATP under various conditions. (Assay conditions are described in Experimental Procedures). (a) ATP alone; (b) ATP in the presence of ATPase; (c) ATP in the presence of ATPase and cryptosin (0.1 mM); and (d) ATP in the presence of ATPase and cryptosin (1 mM).

Table 1. Inhibition of ATP hydrolysis by Na^+,K^+-ATPase in the presence of cryptosin*

Cryptosin (mM)	% Inhibition*
0.1	19.0 (22.2)†
1.0	78.1 (83.5)†

* A modified chemical assay [6] of Na^+,K^+-ATPase was applied. Inhibition of ATP hydrolysis was calculated from the ratio of initial velocities of the enzyme catalysis (at a substrate concentration of 30 mM) in the presence and absence of cryptosin respectively. The same concentration of substrate was used for the NMR assay.

† As calculated from the percent decrease in the area of the peak for inorganic phosphate measured from NMR spectra such as shown in panels (b) and (d) of Fig. 1.

Table 2. Dixon plot analysis of Na^+,K^+-ATPase activity in the presence of cryptosin or ouabain

Inhibitor concentration	Substrate concentration	Slope†	Ki* (μM)
Ouabain (1–100 μM)	ATP (10 mM)	0.2597 ± 0.0202	39
	ATP (30 mM)	0.04716 ± 0.0107	
Cryptosin (1–100 μM)	ATP (10 mM)	0.175 ± 0.0115	30
	ATP (30 mM)	0.0376 ± 0.0037	

* Measured from the value of the intercept of the least-square lines on the X-axis.

† Means ± SE, N = 3.

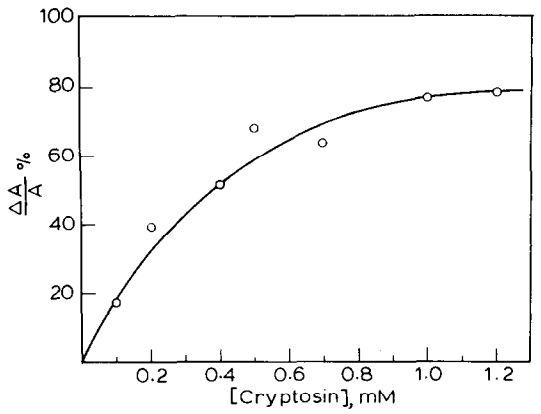

Fig. 2. Effect of cryptosin on ATP hydrolysis. The percent decrease in the area of peak due to inorganic phosphate is plotted as a function of the concentration of cryptosin. "ΔA" denotes the difference in the areas of the peak due to inorganic phosphate in the absence and presence of cryptosin. "A" is the area of the peak due to inorganic phosphate in the absence of cryptosin.

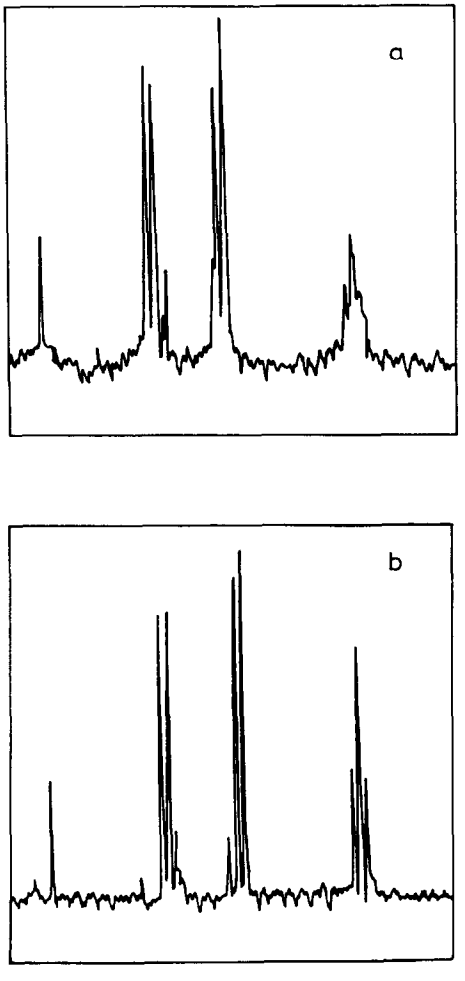

Fig. 3. ^{31}P NMR spectra of ATP (in the presence of ATPase) due to addition of (a) cryptosin (0.7 mM) and (b) ouabain (0.65 mM). The conditions of the experiments are described in Experimental Procedures.

in studying the inhibition and quantitative evaluation of the ATPase inhibitory activity of cryptosin.

Discussion

Studies using two completely different methods, such as the conventional chemical estimation and the ^{31}P NMR spectroscopic method of estimating the inhibition of Na^+,K^+-ATPase by cryptosin gave comparable results and indicate that: (a) cryptosin inhibited the Na^+,K^+-ATPase enzyme, (b) the extent of inhibition was dependent on the concentration of cryptosin, and (c) the inhibitory effect of cryptosin was comparable to that of ouabain, as revealed by the two different assay methods (Table 1).

In accordance with the observation made for other cardiac glycosides, this inhibitory effect of cryptosin on ATPase activity may be one of the factors responsible for its positive inotropic effect [11]. It is important to note that cryptosin and ouabain have comparable ATPase inhibitory effects, thereby indicating that cryptosin would be a very potent cardiac glycoside. However, cryptosin, unlike ouabain, does not produce an arrhythmogenic effect either *in vitro* or *ex vivo* [12]. It also should be mentioned that cryptosin has a higher IC_{50} value (1 μM) than ouabain (0.01 μM), as noted with guinea pig right atrial preparations (data not shown here).

This study suggests that the ^{31}P NMR technique can, in general, be applied to probe the mode of action of the cardiac glycosides. Further, the ^{31}P NMR method can be applied in the quantitative evaluation of chemicals that inhibit ATP hydrolysis *in vitro*.

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