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Inhibitory effect of combinations of digoxin and endogenous cardiotonic steroids on Na^+/K^+-ATPase activity in human kidney membrane preparation

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ABSTRACT

Aims: Cardiac glycosides have been extensively used in the treatment of congestive heart failure for more than 200 years. Recently, cardenolides and bufadienolides were isolated from mammalian tissue and are considered as a new class of steroidal hormones. The aim of the present work was to characterize the interaction between the most clinical used cardiac glycoside digoxin and the cardiac glycosides known to exist endogenously, i.e., ouabain, marinobufagin and telocinobufagin, on human kidney Na^+/K^+-ATPase.
Main methods: Inhibition of Na^+/K^+-ATPase activity from crude membrane preparations of human kidney was performed using increasing concentrations of the drugs alone or mixtures of ouabain:digoxin, telocinobufagin:digoxin and marinobufagin:digoxin in a fixed ratio 1:4, 2:3 and 3:2, respectively. The colorimetric method of Fiske and Subbarow was used to measure the inorganic phosphate released.
Key findings: Analyses of inhibition curves showed that the experimental curves for all combinations were superimposed on the theoretical additive curves indicating that an additive effect occurs among distinct cardenolides and bufadienolides combinations on the human $\alpha1\beta1$ Na^+/K^+-ATPase protomer.
Significance: Considering the extensive use of digoxin in the treatment of heart failure and the recent findings that endogenous cardiac glycosides may have altered levels in many diseases, including heart failure, the demonstration of additive effect between cardiac glycosides can help in the understanding of recent clinical observations, including that lower than usual doses of cardiac glycosides are necessary for decreasing mortality in these patients.

Introduction

Cardiac glycosides have been widely used in the treatment of congestive heart failure for more than 200 years due to its positive inotropic effect and benefits on hemodynamics (Hauptman and Kelly, 1999). However, the molecular mechanism of these drugs emerged only in 1963, when Repke and Portius (1963) described the Na^+/K^+-ATPase as their binding target. In heart failure treatment, the most used cardiac glycoside is digoxin, a cardenolide isolated from plants of the genus *Digitalis*. However, a wide diversity of cardenolides has been identified in other plant families (Mijatovic et al., 2007). Furthermore, structurally related steroids, i.e. bufadienolides, have also been identified in amphibians, snakes and fireflies (Eisner et al., 1978; Steyn and Heerden, 1998; Daly et al., 2004; Hutchinson et al., 2007) and differ from plant cardenolides due to the presence of a six-membered, instead of a five-membered lactone ring at the C-17 position of the steroid nucleus (Flier et al., 1980; Mijatovic et al., 2007). In 1991, a cardiac glycoside indistinguishable from ouabain was isolated from human plasma (Hamlyn et al., 1991). Afterwards, other cardenolides and also bufadienolides have been isolated from humans and other mammals (Hamlyn and Manunta, 1992; Lichtstein et al., 1993; Bagrov et al., 1998; Komiyama et al., 2005). They are now considered a new class of mammalian steroidal hormones, but their importance and even their existence have still been a matter of controversy (Bagrov et al., 2009; Nicholls et al., 2009). Nevertheless, the increase of the plasma titer of cardiotonic steroids in several animal models and human disease states (Ferrandi et al., 2005; Schoner and Scheiner-Bobis, 2007) as well as the discovery of novel Na^+/K^+-ATPase signaling functions (Xie and Askari, 2002) give strong support for the (patho)physiological relevance of these cardiac glycosides. Besides, their presence may have implications in current therapies since interactions at the molecular level could happen between endogenous molecules acting in the same receptor/binding site of the drug administered for the treatment of congestive heart failure. It is important to note that patients on digoxin therapy

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frequently display higher levels of endogenous cardiotonic steroids suggesting that endogenous ouabain may contribute to digoxin toxicity (Manunta and Ferrandi, 2006).

Despite the vast literature dedicated to theoretical and experimental aspects of synergism, few works are concerned with drugs that act on the same molecular target. This kind of interaction is not easy to predict since binding to the same receptor might result in simple competition but also in conformational changes that could lead to synergism or antagonism (Bell, 2005).

Recently, we described that ouabain can act synergistically with 8-methoxycoumestrol on the rat kidney enzyme (Pôças et al., 2007). The present study was designed to characterize the interaction between digoxin, the cardiac glycoside extensively used in the therapeutics of congestive heart failure and also considered as an endogenous steroid, and endogenous cardenolide (ouabain) and bufadienolides (telocinobufagin and marinobufagin) in human kidney Na^+/K^+-ATPase activity. Our results show that endogenous cardiac glycosides have an additive effect on human Na^+/K^+-ATPase inhibition by digoxin and the possible consequences are discussed.

Materials and methods

Drugs

The bufadienolides telocinobufagin and marinobufagin were isolated from the Brazilian toad *Rhinella schneideri* parotoid glands secretion by chromatographic separation on neutral aluminum oxide column and chemically characterized by spectrometric data, as previously described (Cunha-Filho et al., 2010). Digoxin and ouabain were obtained from Sigma-Aldrich (USA).

Preparation of Na^+/K^+-ATPase from human kidney

Normal human renal tissue specimens (from the unaffected pole) were obtained from patients who underwent unilateral nephrectomy due to well-encapsulated hypernephroma in one kidney pole. All procedures for the use of discarded organ portions were done in accordance to the Institutional Ethical Committee from Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Brazil.

Crude homogenate preparations were obtained as previously described (Quintas et al., 1997; Lopez et al., 2002). Briefly, the tissue was homogenized in a Potter homogenizer with a motor driven Teflon pestle at 4 °C in 2–3 volumes of ice cold 0.25 M buffered sucrose pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) per gram organ. After centrifugation at 100 000 *g* for 1 h, pellets were resuspended with the same buffer but PMSF and were stored in N_2 until use. The protein concentration was measured according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Inhibition of Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase activity was determined by the Fiske and Subbarow method (1925) with slight modifications, as described (Pôças et al., 2007). The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain. The preparation was incubated at 37 °C for 2 h, in a total volume of 0.5 mL. The incubation was performed in the presence of 84 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1.2 mM $ATPNa_2$, 2.5 mM EGTA, 10 mM sodium azide and 20 mM maleic acid buffered to pH 7.4 with Tris in the absence or presence of inhibitor(s). Classical concentration–effect curves were performed with each of the four inhibitors, alone. For the construction of the combination curves, we used increasing concentrations of the mixtures ouabain:digoxin in the fixed ratio 1:4, telocinobufagin:digoxin in the fixed ratio 2:3 or marinobufagin:digoxin in the fixed ratio 3:2. These ratios were calculated according to the method proposed by Tallarida et al. (1997).

Statistical analysis

Inhibition curves

Inhibition curves were fitted using computerized non-linear regression analysis of the data (Prism®, GraphPad Software Inc., version 4.00), assuming a sigmoidal dose–response curve model, where the parameters bottom and top were fixed at 0 and 100% inhibition, respectively, as previously reported for a similar study (Pôças et al., 2007).

Comparison of the composite additive and mixture regression curves

The IC_{50} of the calculated (theoretical) additive curve was obtained by non-linear regression analysis of the theoretical curve constructed considering the theoretical effects calculated for different concentrations of the mixture according to the method of Tallarida et al. (1997), as we reported previously (Pôças et al., 2007).

Isobolographic analysis

For the classical isobolographic analysis, we selected 50% inhibition as the effect level. The experimental combination curves were used to determine the (empirical) pair of drug concentrations eliciting 50% inhibition (E) and their limits of confidence. The theoretical pair of concentrations of the mixture of two drugs at the predetermined fixed ratio that should elicit 50% inhibition if the effect was additive (T), was calculated according to equation 11 described by Tallarida et al. (1997). The concentration of each drug in this mixture (T) was then compared to the experimental combination (E), by a suitable *t* test (Tallarida et al., 1997).

Results

The inhibition curves of Na^+/K^+-ATPase from human kidney (that has only one isozyme, $\alpha1\beta1$) by the combination of different cardenolides and bufadienolides are shown in Fig. 1. As described in Table 1, the IC_{50} values for ouabain and digoxin (Hauck et al., 2009) and marinobufagin (Katz et al., 2010) are in very good accordance to the values described in recent binding studies using expressed human $\alpha1\beta1$ isoform. As far as we know, this is the first description of human Na^+/K^+-ATPase inhibition by telocinobufagin. The mixture (experimental) curves for these combinations were constructed using the proportion 1:4 (ouabain:digoxin) or 2:3 (telocinobufagin:digoxin) and 3:2 (marinobufagin:digoxin). Fig. 1 shows that these curves are superimposed on the additive (theoretical) curves, suggesting that these mixtures of drugs act additively, independently whether cardenolides or bufadienolides were utilized. The same conclusion can be achieved analyzing the isobolograms (Fig. 1, inset) where the experimental pairs of concentrations that achieve 50% inhibition were very close to the additive line.

Discussion

The widespread clinical use of cardiac glycosides, especially digoxin, for the treatment of congestive heart failure and the discovery of several endogenous cardiac glycosides in mammals with still barely known physiological or pathological functions make the investigation of the effect of different combinations of these compounds on their well-known molecular target (i.e., Na^+/K^+-ATPase) an important topic.

In a previous paper we showed that, differently from the non-steroidal Na^+/K^+-ATPase inhibitor 8-methoxycoumestrol, the aglycone ouabagenin acts additively to ouabain on the rat renal Na^+/K^+-ATPase inhibition (Pôças et al., 2007). In the present work we studied the effect of