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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 $\alpha 1\beta 1$ 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

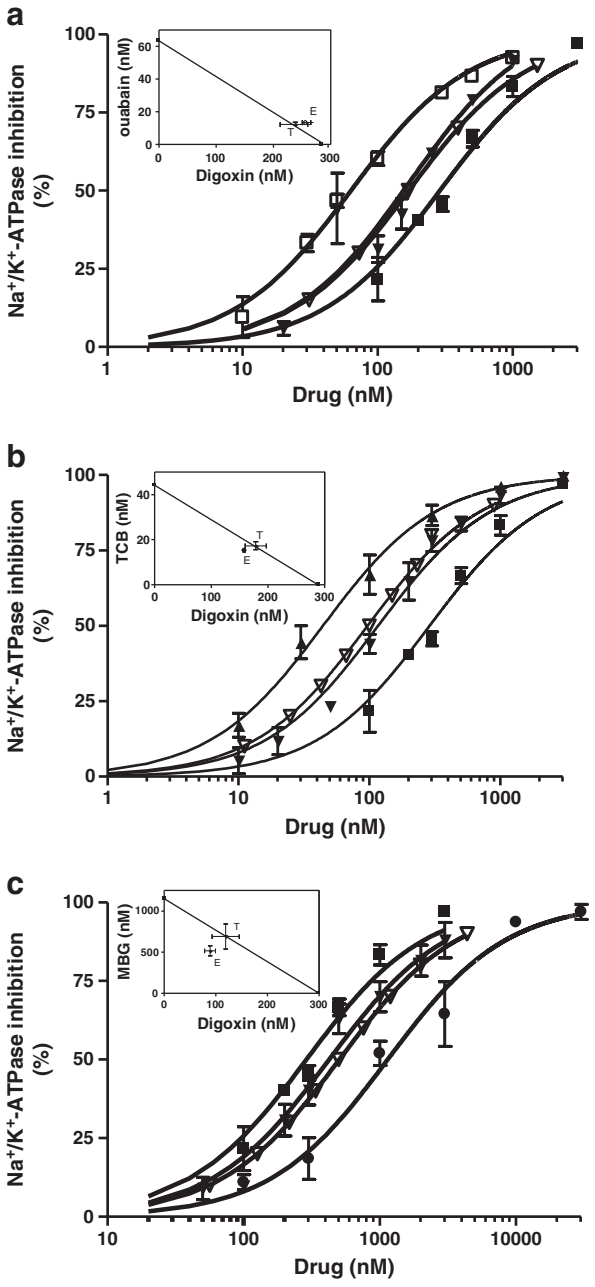

Fig. 1. Inhibition curves of human kidney Na^+,K^+-ATPase by (a) ouabain (□), digoxin (■) and the mixture of both respecting the fixed ratio 1:4 (ouabain:digoxin) (▼). (b) telocinobufagin (TCB, ▲), digoxin (■) and the experimental mixture of both respecting the fixed ratio 2:3 (telocinobufagin:digoxin) (▼). (c) marinobufagin (MBG, ●), digoxin (■) and the mixture of both respecting the fixed ratio 3:2 (marinobufagin: digoxin) (▼). Open triangles (▽) represent the theoretical additive curve for all the combinations analyzed. Curves were fitted by non-linear regression analysis using the model of one binding site. Each point represents the mean ± SEM from at least three experiments performed in triplicate. Inset: Isobolographic analyses of the same mixtures, considering the effect of 50% inhibition. The straight line is the line of additivity, representing all the additive theoretical combinations giving that level of inhibition. Point T represents 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. Point E denotes the concentration pair that gives the same level of effect, experimentally. There was no statistical difference between theoretical and experimental points for all the combinations analyzed ($p > 0.05$).

combinations of digoxin with either ouabain, telocinobufagin or marinobufagin on human renal Na^+/K^+-ATPase for the following reasons: (1) digoxin was chosen since it is the main cardiac glycoside clinically utilized; (2) all of the compounds studied have been identified endogenously in man; and, (3) human kidney Na^+/K^+-ATPase preparation was chosen in order to be closer to clinical conditions and because this organ has only the $\alpha 1\beta 1$ isoform, excluding the possibility of different isoforms with different affinities and interactions that could interfere with simple combination curves or isobolograms. Our findings demonstrate that the inhibitory effect of distinct cardiac glycosides on human Na^+/K^+-ATPase is additive. In a certain way, we expected this behavior since all these compounds are supposed to bind to the same binding site and conformation of the enzyme, but it is well-known that predictions for drug combinations are risky unless they are experimentally proved and the existence of synergism as we reported previously between ouabain and a coumestan could not be ruled out a priori. In addition, our observations have important therapeutic and physiological consequences.

Table 1
IC_{50} values for human kidney Na^+,K^+-ATPase inhibition by different cardiac glycosides alone and in defined combinations. The proportion of each component in the mixture is described in brackets. See Materials and methods for the determination of the theoretical IC_{50} values for the combinations.

	Experimental IC_{50} (nM)	Theoretical IC_{50} (nM)
Digoxin	289 ± 23	–
Ouabain	63.4 ± 2.9	–
Telocinobufagin	44.2 ± 4.3	–
Marinobufagin	1144 ± 160	–
Ouabain + digoxin (1:4)	172 ± 45	171 ± 2
Telocinobufagin + digoxin (2:3)	117 ± 9	100 ± 0.1
Marinobufagin + digoxin (3:2)	425 ± 37	505 ± 2

Mechanistically, our data are in line with a large body of evidences indicating that cardiotonic steroids, i.e., substances sharing the same molecular backbone structure, interact with Na^+/K^+-ATPase at the same binding site, composed by a shallow groove of the extracellular loops between transmembrane segments 1–2 (major binding site), 3–4, 5–6, and 9–10 of the α subunit, stabilizing the E2-P (phosphorylated) conformation (Pierre and Xie, 2006; Nesher et al., 2007).

In the clinical setting, the Digitalis Investigation Group (DIG) trial, the largest study of the long-term impact of digoxin therapy on heart failure patients, concluded that digoxin reduces the frequency of hospitalizations but had no effect on survival (Digitalis Investigation Group, 1997). Recent post hoc analyses of DIG data, however, account that low (from 0.5 up to 0.8 or 0.9 ng/ml) instead of high (≥ 1.0 ng/ml) serum digoxin levels decrease also mortality rates, revealing a bi-directional effect (Rathore et al., 2003; Gheorghiade, 2006; Ahmed et al., 2006, 2007; Ahmed, 2007). Although neurohormonal modulatory effects (e.g., suppression of sympathetic and renin–angiotensin–aldosterone systems) are sustained as the key factor responsible for this benefit, the additive effect of endogenous cardiac glycosides on digoxin may have a role in counteracting the positive outcome seen at lower serum digoxin concentrations. Noteworthy, endogenous cardiac steroids levels are elevated in heart failure (Jortani et al., 2004; Manunta and Ferrandi, 2006), being suggested as a biomarker for the disease (Jortani et al., 2004). Furthermore, the disease increases the myocardial sensitivity to cardiac glycosides probably by bringing Na^+/K^+-ATPase expression and activity down (Bundgaard and Kjeldsen, 1996; Schwinger et al., 1999) and predisposes hypokalemia, a condition that increases the sensitivity of Na^+/K^+-ATPase to the cardiac glycosides (McDonough et al., 1995).

We are aware that inferences from these findings established in vitro to in vivo conditions should be done with caution. Ionic environment, substrate concentration, associated membrane-bound components and hormonal status along with the action of protein kinases and phosphatases are notorious modulators of the enzyme and may affect cardiac glycosides sensitivity (Therien and Blostein, 2000; Bagrov and Shapiro, 2008). Moreover, our observations were focused on the classical mechanism of cardiac glycosides, i.e., Na^+/K^+-ATPase inhibition. The results of cardiac glycosides mixtures on novel mechanisms, such as signaling transduction through protein–protein

interactions (Xie and Askari, 2002) or scaffolding functions (Aperia, 2007) are unknown. However, as no other cardiac glycosides binding sites have been determined in Na^+/K^+-ATPase and these new mechanisms appear to derive from this interaction, it seems likely that additivity would occur. Finally, there are other Na^+/K^+-ATPase isoforms for the α (α2, α3 and the sperm-specific α4) and for the β (β2 and β3) subunits and several protomer arrangements exist. Unlike what is observed in rodents, though, all the human isoforms have very similar affinities for cardiac glycosides (Crambert et al., 2000; Hauck et al., 2009). Thus, we believe that the additional human isoforms might behave in the same way.

Conclusion

We described the additive effect of cardiac glycosides on the inhibition of human Na^+/K^+-ATPase α1β1 isozyme. These results argue against the possibility of synergistic effect between an endogenous cardiac glycoside and digoxin during the pharmacological treatment of heart failure. The prediction of a therapeutic relevance from an in vitro study may be premature, but this additive effect could be one explanation for the decreased mortality observed only in patients using lower than usual doses of digoxin.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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