Temperature-Dependent Immunoreactive Assay to Screen for Digoxin-like Immunoreactive Factor(s)

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Endogenous circulating digoxin-like immunoreactive factors (DLIF) are known to cross-react with antibodies to digoxin and to inhibit Na+/K+-transporting ATPase (Na+/K+ATPase; EC 3.6.1.37). Moreover, increasing the immunoaassay temperature from 4 to 37 °C markedly decreases DLIF from human cord serum. We tested several compounds, including hormonal steroids, bile salts, lipids, and methionine-enkephalin, for their ability to cross-react with two commercially available 125I digoxin RIAs, to inhibit porcine Na+/K+ATPase, and to see whether they present the same incubation temperature dependence as human cord serum. Except for methionine-enkephalin, all compounds were inhibitors of Na+/K+ATPase in the range of 1–10 mmol/L. Progesterone exhibited the highest cross-reactivity in the two RIAs. The apparent digoxin immunoreactivity for the majority of the cross-reacting steroids, bile salts, and linoleic acid was markedly decreased by increasing the incubation temperature from 4 to 37 °C, whereas estradiol, pregnanediol, and nonspecific compounds (e.g., ethanol, human serum albumin) did not appear to be temperature-sensitive. Both lysophosphatidyl lipids gave an increased apparent digoxin concentration with increasing incubation temperature. Our data suggest that numerous weakly cross-reactive compounds can parallel the response of human cord serum. However, the temperature-dependent effect could be an additional criterion for identifying DLIF.

Additional Keyphrases: variation, source of radioimmunoassay, Na+/K+ATPase

The accuracy of digoxin immunoassays is seriously affected by the presence of digoxin-like immunoreactive factors (DLIF) in biological samples (1–4).2 These digoxin-like factors interact specifically with digoxin and digitoxin antibodies, inhibit Na+/K+-transporting ATPase (Na+/K+ATPase; EC 3.6.1.37), displace [3H]ouabain from Na+/K+ATPase, and have anti-natriferic and natriuretic activity (5). Peptides, lipids, lignans, bile salts, and hormonal steroids have been shown to exhibit one or more of the above properties (6). The nature of DLIF is still unknown and many groups agree that DLIF is probably a mixture of compounds. Previously, we demonstrated a specific interaction between DLIF from human cord blood and anti-digoxin antibodies; moreover, the concentrations of DLIF depended on the incubation time and temperature of the RIA (7, 8). Our aim here was to screen a panel of compounds to see whether they exhibited this thermodynamic effect; if so, they could be confirmed as compounds that parallel the response of cord serum, and the temperature-dependent effect could be used as an additional property in defining “true” DLIF. We ran a preliminary cross-reactivity study with these compounds to define the appropriate concentration for assay of the thermodynamic effect. In addition, we investigated the ability of these compounds to inhibit Na+/K+ATPase.

Materials and Methods
Reagents

We used chemicals of reagent grade without purification. Digoxin was a gift from Nativelle Laboratory, Paris, France. Digoxigenin, chlormadinone acetate, cortisol, dehydroepiandrosterone (DHEA), estradiol, pregnanediol, progesterone, aldosterone, taurocholate, glycochenodeoxycholate, taurochenodeoxycholate, L-α-lysophosphatidylcholine, L-α-lysophosphatic acid (oleoyl), linoleic acid, methionine-enkephalin, ouabain, adenosine triphosphate (from porcine brain cortex), human serum albumin (HSA), and adenosine triphosphate (free of metal ions) were purchased from Sigma Chemical Co., St. Louis, MO.

Digoxin Radioimmunoassays

To investigate the cross-reactivity and the thermodynamic effect, we tested two 125I RIA kits: Phadebas digoxin RIA (method A) from Pharmacia Diagnostics AB, Uppsala 1, Sweden, and Gammacoat digoxin RIA (method B) from Clinical Assays, Cambridge, MA 02139. We used kits according to the manufacturers’ instructions and performed all assays in triplicate.

Methods

Cross-reactivity analysis. We determined the cross-reactivity of the compounds listed in Table 1. All compounds were dissolved initially either in ethanol or water, at 10 mmol/L. Sequential 10-fold dilutions were made either in 0.01 mol/L sodium phosphate solution (pH 7.6) or in HSA solution (40 g/L), previously dissolved in water and adjusted to pH 7.4 with 0.1 mol/L...
sodium hydroxide. Digoxin and digoxigenin concentrations ranged from 0.1 to 10 nmol/L; concentrations tested were 0.1 μmol/L to 10 mmol/L for all other substances. The cross-reactivity was expressed as the inhibitor concentration that displaced 50% of 125I-labeled digoxin (IC 50).

Effect of changing the incubation temperature. The incubation conditions specified by the manufacturers are 30 min at 21 °C for method A and 60 min at 37 °C for method B; the modified incubation temperatures we used were 30 min at 4 °C for method A and 60 min at 4 °C for method B. For each incubation temperature, a complete calibration curve was prepared and values for apparent digoxin immunoactivity were calculated from the respective calibration curves. Each compound was assayed at a specific concentration (Table 1). We also investigated the effect of changing the incubation temperature with 20 cord sera. Values for apparent digoxin immunoactivity were expressed as digoxin equivalents, in nanomoles per liter.

Effect on Na⁺K⁺ATPase activity. Purified Na⁺K⁺ATPase (from porcine cerebral cortex) was selected as the basic control (9). We added hormonal steroids, bile salts, lipids, and methionine-enkephalin to the mixture (final concentration: per liter, 40 mmol of Tris·HCl, 150 mmol of NaCl, 20 mmol of KCl, and 5 mmol of MgCl₂, with or without 0.75 mmol of ouabain, pH 7.4) in a volume of 1.1 mL. After pre-incubation for 10 min at 37 °C, we added Tris ATP, 4 mmol/L; we stopped the reaction after 15 min by adding 1 mL of ice-cold trichloroacetic acid, 100 g/L. We determined inorganic phosphate in a 0.5-mL aliquot of the supernate according to the method of Fiske and SubbaRow (10). Na⁺K⁺ATPase activity was estimated by subtracting Mg²⁺ ouabain-insensitive ATPase from total ATPase activity. Under such conditions, Na⁺K⁺ATPase activity was linearly related to time of reaction for at least 30 min at 37 °C. We made all determinations in triplicate. Enzyme inhibition was expressed as a percentage of the baseline Na⁺K⁺ATPase values (mean ± 1 SD).

Results

Apparent cross-reactivities of the compounds tested against the anti-digoxin antibodies are shown in Figure 1 for method A, and in Figure 2 for method B. With

![Graph](image-url)
methods A and B, we found that digoxigenin was a more potent inhibitor than native digoxin, with an IC 50 value of 2 and 5 nmol/L, respectively. In contrast, each of the other compounds was a considerably less potent inhibitor, with inhibition occurring only in the 0.1 μmol/L to 10 nmol/L range for method B. Method A was considerably less sensitive to displacement of $^{125}$I-labeled digoxin. Progesterone was most active in the two kits, with 50% displacement at 70 μmol/L for method B and up to 0.1 mmol/L for method A. The other compounds inhibited the 50% binding of $^{125}$I-labeled digoxin at concentrations up to 0.1 mmol/L in method A. Bile salts, linoleic acid, and lysophosphatidyl derivatives exhibited IC 50 values in the range of 1 to 10 mmol/L for method B, whereas methionine-enkephalin did not displace $^{125}$I-labeled digoxin, even at 10 mmol/L. The effect of incubation temperature on the concentration of apparent immunoreactive digoxin is shown in Table 1. When the temperature was increased from 4 to 21°C (method A) or to 37°C (method B), apparent immunoreactive digoxin decreased markedly with the majority of the cross-reacting steroids and bile salts in both RIAs, as did linoleate and methionine-enkephalin in method B. We also performed these assays with 20 cord sera. Digoxigenin was not sensitive to temperature increases, nor were estriol and pregnanediol.

Therefore, with L-α-lysophosphatidylcholine and L-α-lysophosphatic acid (oleoyl), the apparent digoxin concentrations increased when the incubation temperature was increased from 4 to 37°C (method B). Nonspecific modifiers of RIA activity such as ethanol (10 mL/L in solution) or HSA (40 g/L) were not temperature sensitive. The inclusion of a 40 g/L concentration of HSA in some of the compound solutions did not greatly change the equivalent concentrations of digoxin for taurocholic acid, L-α-lysophosphatidylcholine, or linoleic acid. Moreover, the addition of HSA decreased the apparent digoxin for progesterone, L-α-lysophosphatic acid (oleoyl), and methionine-enkephalin. Therefore, the temperature effect reported above without HSA was not qualitatively affected by adding HSA (Table 2).

We screened the same compounds for their ability to inhibit purified Na$^+$K$^+$ATPase (Figure 3). Digoxin inhibited Na$^+$K$^+$ATPase more actively than digoxigenin.

![Graphs showing inhibition of Na$^+$K$^+$ATPase](image)

**Table 2. Effect of Incubation with Human Serum Albumin on Concentration of Apparent Immunoreactive Digoxin**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Apparent digoxin concn, mmol/L</th>
<th>Incubation temp, °C</th>
<th>Without HSA</th>
<th>With HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>0.05</td>
<td>4</td>
<td>7.60</td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>1.60</td>
<td>1.25</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>0.5</td>
<td>4</td>
<td>2.65</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.65</td>
<td>0.70</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>5</td>
<td>4</td>
<td>2.20</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>6.00</td>
<td>5.90</td>
</tr>
<tr>
<td>L-α-Lysophosphatidyl choline</td>
<td>5</td>
<td>4</td>
<td>14.1</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>19.1</td>
<td>14.8</td>
</tr>
<tr>
<td>L-α-Lysophosphatic acid</td>
<td>5</td>
<td>4</td>
<td>2.70</td>
<td>3.00</td>
</tr>
<tr>
<td>(oleoyl)</td>
<td></td>
<td>37</td>
<td>0.85</td>
<td>1.00</td>
</tr>
<tr>
<td>Methionine-enkephalin</td>
<td>5</td>
<td>4</td>
<td>0.75</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>&lt;0.10</td>
<td>0.35</td>
</tr>
</tbody>
</table>
DLIF arise from more than one agent, possibly peptides originating from the hypothalamus and steroids from a peripheral source. The evidence that DLIF are new entities is experimentally blurred because known endogenous compounds are able to exhibit one or more DLIF properties. Thus, unsaturated fatty acids and lysophosphatidyl lipids have been shown to inhibit the Na\(^+\)/K\(^+\) pump, but their high K\(_v\) values and steep dose–response curves suggest that they interact non-competitively \((11, 12)\). Bile acids can also react to antibodies to digoxin, and Pudek et al. \((2)\) found that bile acids can account for 15% of the DLIF in plasma of patients with liver diseases and were significant contributors to DLIF. In fact, bile acids may act like detergents, and not as simple competitive ligands, in their interaction with digoxin antibodies and ATPase.

In addition, Vasdev et al. \((13)\) reported that DHEA and DHEA sulfate accounted for 62% to 100% of the total plasma DLIF, and Diamandis et al. \((14)\) demonstrated that part of the digoxin immunoreactivity of cord serum was due to cortisone and progesterone. Recently, by using specific criteria for identifying DLIF activity in human plasma, Lau and Valdes \((15)\) demonstrated that several congeners of fatty acids, phospholipids, hydrocortisone, and DHEA sulfate did not consistently account for the immunoreactive and physical properties of DLIF in human subjects. Moreover, they also reported that by cumulating the highest reported concentrations of these compounds in various patient groups where DLIF is known to be increased, the compounds could contribute only a small fraction at best \((15)\). The "non-specific" interference of known endogenous compounds increases the difficulties of isolating and identifying "true" DLIF, and additional specific criteria for identifying DLIF need to be defined. The aim of our study was to assay various compounds in such a way as possibly to supplement the empirical criteria accounting for DLIF activity, i.e., "apparent" displacement of labeled digoxin from the antibody and inhibition of Na\(^+\)/K\(^+\)ATPase, by demonstrating a sensitivity to incubation temperature in the digoxin RIA. This property could be a specific criterion for DLIF identification.

First, we determined that DLIF concentrations of 20 cord sera were 0.16 ± 0.11 nmol/L with the Phadebas digoxin RIA (method A) and 0.72 ± 0.33 nmol/L with the Gammacoat digoxin RIA (method B) \((16)\). The substantial DLIF concentrations observed with method B correspond to the stronger cross-reactivity of this assay’s components with the individual compounds tested. Progesterone is found in high concentration in cord blood, and its contributions to DLIF concentrations were well correlated with the cross-reactivity results. In contrast, the low DLIF concentration seen with method A is well correlated with the low amount of cross-reactivity observed with this method. Moreover, as reported by Lau and Valdes \((15)\), the addition of HSA to solutions of various compounds tends to diminish their interaction with anti-digoxin antibodies. These cross-reactivity data confirm that many of the substances could affect

did. Methionine-enkephalin did not inhibit the enzyme, but all of the other compounds exhibited inhibition in the range of 1 to 10 mmol/L. The nonendogenous steroid compound, chlormadinone acetate, was the most active.

Discussion

In a recent elaborate review on the source and molecular structure of DLIF, Haddy \((6)\) hypothesized that
the digoxin assays only when present in supraphysiologi-
cal amounts. In addition, the use of a second crite-
ron, the inhibition of Na°K°ATPase, indicates that
similar supraphysiological amounts of compounds are
required to play a role as specific endogenous regulators
of the Na°K° pump.

Second, when we increased the incubation tempera-
ture, the apparent immunoreactive digoxin decreased
for the steroids tested except for estriol and pregnane-
diol. All bile salts, linoleic acid, and methionine-en-
kephalin showed a similar decrease. These data indicate
that the thermodynamic effect described with cord se-
rum (see Table 1) is fully mimicked by a large variety of
compounds. However, nonspecific modifiers of RIA ac-
tivity (e.g., ethanol or HSA) did not exhibit the tem-
perature-dependent effect. Surprisingly, lysophosphatidyl
lipids had an opposite effect, increasing the apparent
digoxin when the temperature increased. For most of
the RIAs, because reaction rates increase with the
temperature, dissociation will be more marked at
higher temperatures, resulting in a lower immuno-
reactivity of the weakly cross-reactive compounds. How-
ever, our data demonstrate that weakly cross-reactive sub-
stances did not systematically behave in the same way;
i.e., apparent digoxin decreased when the temperature of
the digoxin RIA increased. Results for linoleic acid,
methionine-enkephalin, and several steroids and bile
salts parallel those for cord serum. Many of these com-
ounds mentioned above have been identified as po-
tential DLIF contributors in human serum and might
account for the decrease in apparent digoxin that results
from increased RIA temperature (8). In contrast, non-
specific activity modifiers (e.g., ethanol, HSA) and es-
triol, pregnanediol, and lysophosphatidyl lipids did not
demonstrate a temperature-dependent effect.

Finally, these results suggest that the temperature-
effect criterion may be valuable for screening possible
DLIF candidates. Questions of assay specificity and
possible antisera dependence require further study.
However, bearing in mind the low specificity of
immunoreactivity and inhibition of Na°K°ATPase for
identifying DLIF, we suggest that the temperature-
dependent effect could supplement existing criteria for
DLIF identification.

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