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Characterization of fatty acid interaction with ouabain and vanadate binding to $(Na^+ + K^+)$-activated ATPase

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The candidateship of unsaturated fatty acids as endogenous ouabain-like factors was studied. Binding of the artificial ligand vanadate at the intracellular phosphorylation epitope of membrane-bound Na^+/K^+-ATPase was unaffected by linoleic and arachidonic acid. In the $(Mg^{2+} + P_i)$-facilitated system for ouabain binding they were characterized as noncompetitive inhibitors of cardiac glycoside binding, however. The ouabain binding capacity as well as the affinity decreased and the ouabain dissociation rate was accelerated by fatty acids. In the presence of vanadate for facilitation of ouabain binding an increase in ouabain affinity was seen. It is concluded that elementary criteria for the characterization of unsaturated fatty acids as ouabain-like factors are not fulfilled. The ratio between E_2-subconformations of Na^+/K^+-ATPase with different ouabain affinities may be changed by incorporation of fatty acids in the lipid membrane.

Introduction

Cardiac glycosides like ouabain are specific inhibitors of the $(Na^+ + K^+)$-activated ATPase and the ouabain receptor is a well characterized part of the hydrolytic α-peptide of the sodium pump (for Refs. see Refs. 1–3). The receptor, which is located at the extracellular aspect of the cell membrane, has often been anticipated to have a native agonist. A putative ouabain-like agonist carried by the circulation might adjust the pump activity according to specific demands.

Several years of search for the endogenous ouabain-like factor has not succeeded in isolation and characterization of one single compound [4,5]. The main product from such studies has often appeared to be non-esterified fatty acids and lyso-phospholipids [6–8]. Long chain fatty acids and especially the unsaturated ones are known to inhibit the hydrolytic activity of Na^+/K^+-ATPase and to displace [^{3}H]ouabain [9–13]. Fatty acids moreover displace digoxin from anti-digoxin antibody [7]. The Ca^{2+}-ATPase of sarcoplasmic reticulum is not inhibited or may even be activated by fatty acids [14]. The question is whether free fatty acids in every respects fulfil the criteria of a genuine agonist of the digitalis receptor.

Obviously, a compound that inhibits Na^+/K^+-ATPase activity and displaces [^{3}H]ouabain does not necessarily combine with the ouabain receptor since hydrolytic activity and ouabain interaction are dependent on the integrity of the α-peptide and a number of external factors. Ouabain binding under nonhydrolytic conditions is thus facilitated by Mg^{2+} and P_i interaction with the cytosolic aspect of the enzyme. Some generally deleterious effect, e.g. detergent action, by fatty acids may be tested by comparison with their interaction on vanadate binding. Vanadate is a high-affinity agonist of the cytosolic phosphorylation epitope of Na^+/K^+-ATPase. Binding of this transition state analogue of phosphate is also dependent on Mg^{2+} and moreover very efficient in promoting ouabain binding [15].

In the present study the interaction of unsaturated fatty acids with (1) $(Mg^{2+} + P_i)$-facilitated ouabain binding, (2) $(Mg^{2+} + K^+)$-facilitated vanadate binding, and with (3) $(Mg^{2+}$ + vanadate)-facilitated ouabain binding to Na^+/K^+-ATPase is characterized. Fatty acids appear to have opposite effects on ouabain binding in the two systems for facilitation of cardiac glycoside interaction with the enzyme. The observations seem incompatible with a simple competitive role of fatty acids towards the digitalis receptor.

Abbreviations: Na^+/K^+-ATPase, $(Na^+ + K^+)$-activated Mg^{2+}-ATPase (EC 3.6.1.37); pNPPase, *p*-nitrophenylphosphatase.

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Materials and Methods

Na^+/K^+-ATPase was prepared from pig kidney outer medulla according to Jørgensen [16]. The K^+-activated pNPPase activity of the enzyme was 4.73 μmol·(mg protein)$^{-1}$·min^{-1}.

Ouabain was obtained from Sigma Chemical Co. Stock solutions of ouabain were checked spectrophotometrically at 220 nm and assuming a molar absorption coefficient of 16800 [17]. [^{3}H]Ouabain was obtained from New England Nuclear Corp. The isotope was purified by chromatography on Na^+/K^+-ATPase as described elsewhere [18].

[^{48}V]Vanadyl chloride in HCl was a gift from The Cyclotron Department, Niels Bohr Institute, Copenhagen. The solution was neutralized by NaOH, buffered with Tris at pH 8.5, and stirred in the presence of air for conversion of vanadyl to vanadate. A stock solution of 400 mM vanadate was obtained by dissolving $NaVO_3$ (Merck) in 1 M NaOH.

Unless otherwise stated, ouabain binding took place at 37 °C in the presence of 3 mM Mg^{2+}, 3 mM P_i, 40 mM Tris-HCl (pH 7.25) and 15–235 nM [^{3}H]ouabain. After 2 h incubation at equilibrium of binding bound and non-bound [^{3}H]ouabain was separated by filtration as described [18] except that Millipore filters (pore size 0.45 μm) were used. Vanadate binding took place at 25 °C in the presence of 5 mM Mg^{2+}, 10 mM K^+, 40 mM Tris-HCl and 25–500 nM [^{48}V]vanadate. Bound and non-bound [^{48}V]vanadate was determined at equilibrium of binding after 1 h in the same way as ouabain.

Linoleic acid (*cis,cis*-9,12-octadecadienoic acid, 18 : 2($n-6$), sodium salt) and arachidonic acid (eicosatetraenoic acid, 20 : 4($n-6$)) was obtained in sealed ampules from Sigma Chemical Co. The fatty acids were dissolved in methanol, divided among a number of ampules, the methanol evaporated under a stream of nitrogen, and the ampules sealed. After opening of the vials the lipids were dissolved in a small volume of methanol after which a stock solution in 5 mM Tris-buffer (pH 7.25) was prepared. When stored the vials were kept at −20 °C under nitrogen.

Analysis of binding data. The binding isotherms were fitted to the one-component model using a non-linear least-squares fit [19] and a weighting of the minimized Q expression by the square of the bound value [20]. Alternatively, ouabain binding data were fitted to the one-component model by linear regression of the isotherms bound and bound/free ligand.

Theoretical considerations. If ouabain (O) and fatty acid (F) compete for the same Na^+/K^+-ATPase receptor (E), the equilibrium situation is described by:

$$E + O \rightleftharpoons EO \quad \text{i.e.} \quad [EO] = K_{EO}\cdot[O]\cdot[E] \tag{1}$$

$$E + F \rightleftharpoons EF \quad \text{i.e.} \quad [EF] = K_{EF}\cdot[F]\cdot[E] \tag{2}$$

in which K_{EO} and K_{EF} denote association constants for complex formation. The total number of receptors traced by radiolabelled ouabain is defined by:

$$[EO]_{total} = [E] + [EF] + [EO] \tag{3}$$

Substitution of E and EF by derivatives from Eqns. 1 and 2 leads to:

$$[EO] = -\frac{1 + K_{EF}\cdot[F]}{K_{EO}} \cdot \frac{[EO]}{[O]} + [EO]_{total} \tag{4}$$

which is the expression for a Scatchard-type plot. The prefix to [EO]/[O] denotes the slope of the line connecting the ouabain binding isotherms at a certain concentration of fatty acid.

From the apparent dissociation constant obtained at different concentrations of fatty acid a new function may be derived:

$$y = \frac{1 + K_{EF}\cdot[F]}{K_{EO}} \quad \text{or} \quad y = \frac{K_{EF}}{K_{EO}}\cdot[F] + \frac{1}{K_{EO}} \tag{5}$$

A plot of the apparent dissociation constant against the concentration of fatty acid is supposed to be a straight line with the slope K_{EF}/K_{EO} and the ordinate intercept $1/K_{EO}$.

Results

Half-maximum inhibition of the Na^+/K^+-ATPase activity was achieved in the presence of 10–20 μM arachidonic or linoleic acid. If the inhibitory action of the fatty acids were due to their interaction with the digitalis receptor of the enzyme, the affinity of the intracellular receptor for vanadate should not be reduced. Possible cardiac glycoside-like substances could even increase the apparent vanadate affinity [21]. Fig. 1 is a Scatchard-type plot of [^{48}V]vanadate binding iso-

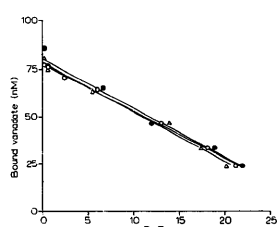

Fig. 1. Scatchard-type plot of equilibrium binding isotherms of bound and bound/free (B/F) [^{48}V]vanadate obtained in the presence of 0 (○), 30 μM (●), and 75 μM (△) linoleic acid. Enzyme concentration 0.027 mg·ml^{-1}. For details, see Materials and Methods.

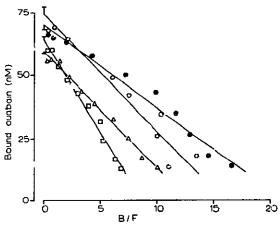

Fig. 2. Scatchard-type plot of equilibrium binding isotherms of bound and bound/free (B/F) [^{3}H]ouabain obtained in the ($Mg^{2+} + P_i$)-facilitated system and in the presence of 0 (●), 40 μM (○), 60 μM (△), and 80 μM (□) arachidonic acid. Enzyme concentration 0.027 mg·ml^{-1}. Bars at the ordinate intercept indicate S.E. For details, see Materials and Methods.

therms obtained in the absence and in the presence of linoleic acid. Binding data fit straight lines and are thus compatible with one homogeneous population of vanadate receptors. It is seen that neither the slope nor the ordinate intercept, i.e., the apparent affinity and the binding capacity, respectively, is changed by linoleic acid.

In contrast to vanadate, the affinity of the extracellular agonist of Na^+/K^+-ATPase, ouabain, is reduced by unsaturated fatty acids in the ($Mg^{2+} + P_i$)-facilitated system for binding. In Fig. 2 this is shown for arachidonic acid. Measurement of ouabain binding in the presence of fatty acids is more capricious than usually seen, but binding isotherms obtained at a fixed concentration of fatty acid still fit the one-component model reasonably well. The ouabain binding capacity is similar to the vanadate capacity in the absence of fatty

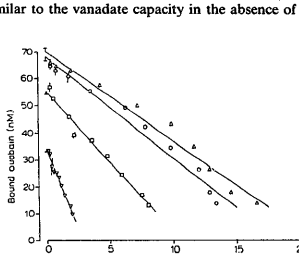

Fig. 3. Scatchard-type plot of equilibrium binding isotherms of bound and bound/free (B/F) [^{3}H]ouabain obtained in the ($Mg^{2+} + P_i$)-facilitated system and in the presence of 0 (△), 15 μM (○), 40 μM (□), and 75 μM (▽) linoleic acid. Enzyme concentration 0.027 mg·ml^{-1}. Bars indicate S.E. of bound ouabain. For details, see Materials and Methods.

TABLE I

Effect of linoleic acid on the apparent ouabain dissociation constant and the ouabain binding capacity of Na^+/K^+-ATPase

At each concentration of linoleic acid two series of [^{3}H]ouabain equilibrium binding data were pooled and analyzed by linear regression of the isotherms bound and bound/free ouabain. The data were obtained at initial ouabain concentrations in the range 15–235 nM.

Linoleic acid (μM)	Apparent enzyme-ouabain dissociation constant ± S.E. (nM)	Ouabain-binding capacity ± S.E. (nmol/mg protein)	Regression coefficient
0	3.25 ± 0.14	2.59 ± 0.05	0.985
10	3.59 ± 0.19	2.60 ± 0.06	0.975
15	3.63 ± 0.18	2.51 ± 0.06	0.980
20	3.59 ± 0.14	2.35 ± 0.04	0.988
30	4.19 ± 0.31	2.03 ± 0.06	0.959
40	5.18 ± 0.22	2.05 ± 0.04	0.986
50	8.09 ± 0.77	1.48 ± 0.05	0.927
75	12.00 ± 0.76	1.27 ± 0.03	0.966

acids, but the ordinate intercept, i.e., the number of receptors, is reduced from 2.59 ± 0.05 in the absence of arachidonic acid to 2.37 ± 0.05 nmol/mg protein at 80 μM arachidonic acid and the apparent dissociation constant increased from 3.25 ± 0.14 to 7.25 ± 0.31 nM (± S.E.).Suggesting that arachidonic acid is a competitive inhibitor of ouabain binding (see Methods: Theoretical considerations) the number of ouabain receptors should of course remain unchanged.

A moderate reduction of the apparent ouabain affinity is also observed with linoleic acid, which at higher concentrations moreover has a dramatic effect on the binding capacity, Fig. 3 and Table I. The receptor capacity is significantly reduced by 30 μM linoleic acid

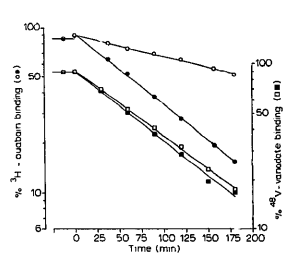

Fig. 4. Effect of arachidonic acid on [^{3}H]ouabain and [^{48}V]vanadate dissociation. Equilibrium binding was established by incubation of 0.027 mg Na^+/K^+-ATPase per ml for 2 h at 37 °C with 50 nM [^{3}H]ouabain (○, ●) or for 1 h at 25 °C with 50 nM [^{48}V]vanadate (□, ■). At the indicated time 0 unlabelled ouabain (○, ●) or vanadate (□, ■) was added to final concn. 10^{-4} M plus (●, ■) or minus (○, □) 80 μM arachidonic acid.

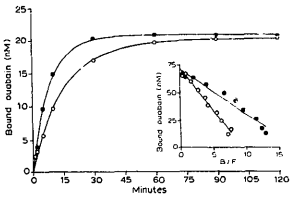

Fig. 5. Time course of ouabain binding facilitated by vanadate in the absence (○) or presence (●) of 50 μM linoleic acid. 0.027 mg Na^+/K^+-ATPase per ml was incubated with 3 mM Mg^{2+}, 40 mM Tris, 100 μM vanadate and 24 nM [^{3}H]ouabain at 37 °C. Inset: Scatchard-type plot of equilibrium binding data obtained under the same conditions with 15–235 nM [^{3}H]ouabain.

whereas 40 μM is needed for a significant reduction in ouabain affinity. Obviously no linear relationship between apparent dissociation constant and fatty acid concentration is seen as expected according to Eqn. 5 from the simple competition model. With reduction of the ouabain binding capacity as well as the ouabain affinity fatty acids would have to be classified as mixed-type inhibitors of ouabain binding.

Non-competitive inhibition of ouabain binding by fatty acids is supported by an experiment in which the release of [^{3}H]ouabain or [^{48}V]vanadate after addition of an excess of the unlabeleld ligand is traced (Fig. 4). Monoexponential dissociations are seen in both cases confirming homogeneity of the enzyme. However, ouabain dissociation is accelerated dramatically by extra addition of arachidonic acid whereas vanadate dissociation is hardly affected. Fatty acids and ouabain thus do not just share a common receptor. The acceleration of dissociation is immediately reversed by addition of an equimolar concentration of albumin (4.5 mg · ml^{-1}) indicating that the fatty acids are easily withdrawn from the membrane-embedded enzyme (not shown).

We have seen that the vanadate affinity was unaffected by fatty acids whereas a decrease in ouabain affinity as facilitated by P_i was noticed. The inhibition of ouabain binding by unsaturated fatty acids could not be generalized, however. When complex formation was facilitated by vanadate instead of P_i it appeared that the apparent ouabain affinity was increased, not decreased by linoleic acid. The equilibrium binding level was increased due to a considerable acceleration of the on-reaction (Fig. 5) in spite of an acceleration of the rate of release of ouabain as well (not shown).

Discussion

For years it has been a mystery why the sodium pump, i.e. the external aspect of the plasma membrane-spanning α-peptide of the Na^+/K^+-ATPase, contains a receptor, which specifically binds digitalis glycosides with very high affinity. The receptor may not justify the existence of a natural agonist, the putative ouabain-like factor, however, since toxic or pharmacological substances by chance could fit a certain protein or lipoprotein structure.

Whether justified or not a great number of factors has been nominated as ouabain-like since they inhibit the Na^+/K^+-ATPase or even displace ouabain from the enzyme. Due to the complex structure of the membrane embedded Na^+/K^+-ATPase and the great number of ligands or substrates that have to be present for cycling of the system and for digitalis binding [1], their interference with the Na^+/K^+-ATPase could well be at several places or steps during enzyme turnover, however.

In a search of the Na^+/K^+-ATPase inhibitor in plasma, a factor the concentration of which is increased after extracellular volume expansion with saline, isolation procedures led to unsaturated free fatty acids as the main product [7,8]. A similar conclusion was reached by Bidard et al. [6] after extraction from microsomal membranes obtained from the electric organ of *Electrophorus electricus*, which could be a likely source of endogenous ouabain-like factor. The more abundant free fatty acids were arachidonic and linoleic acids which moreover are relatively potent inhibitors of the sodium pump [6,10,13]. Skou in one of the very first publications on Na^+/K^+-ATPase noticed that unsaturated fatty acids inhibited the enzyme whereas stearic acid did not [9]. According to Tamura et al. [7] the unsaturated fatty acids also interfere with anti-digoxin antibody whereas Kelly et al. [8] suggest that the digoxin-like immunoreactivity of extracts from plasma is due to contaminating steroids. An analysis of the question whether free fatty acids could be considered ouabain-like thus seemed reasonable or even urgent although the chemical structure of unsaturated fatty acids is very different from that of digitalis glycosides. The double bond may be the essential common link [4].

The present studies clearly showed that unsaturated fatty acids in no way could be characterized as agonists of the digitalis receptor irrespectively of the fact that binding of another high-affinity ligand, vanadate, was unchanged by fatty acids. This observation may exclude a simple detergent action of fatty acids. Since vanadate binding takes place at the intracellular phosphorylation epitope of Na^+/K^+-ATPase and ouabain binding to an extracellular receptor subsequent to phosphorylation or vanadate binding – in both cases in the presence of Mg^{2+} – the conditions for interaction of the two ligands with the enzyme are intimately related. In the (Mg^{2+} + P_i)-system for facilitation of ouabain binding the fatty acids were non-competitive inhibitors of ouabain binding since also the total number of binding sites decreased

in their presence and since they accelerated ouabain dissociation. Kuske et al. [22] also recently described that unsaturated fatty acid destroy ouabain receptors. Quite unexpected, however, it appeared that in the (Mg^{2+} + vanadate)-facilitated system for ouabain binding an increase in affinity and no reduction in receptor capacity was seen in the presence of unsaturated fatty acids. Inorganic phosphate, vanadate and ouabain probably all interact with the E_2-conformation of Na^+/K^+-ATPase [1,21]. The two opposite effects of fatty acids in the two systems for facilitation of ouabain binding indicate that they change the ratio between E_2-subconformations induced by P_i and vanadate [21]. Swann [13] proposed that fatty acids increase membrane fluidity and reduce the E_2/E_1 ratio in contrast to dimethyl sulfoxide and glycerol. However, the latter reagents increase K^+-activated phosphatase activity and decrease ($Na^+ + K^+$)-activated hydrolysis of ATP, whereas both hydrolytic activities are inhibited by fatty acids. Klausner et al. [23] interpret their binding studies with fatty acids and their fluorescence polarization experiments in the presence of fatty acids as evidence of membrane perturbations when fatty acids intercalate into the membrane lipids. The unsaturated fatty acids preferentially change the packing of lipid molecules of the more fluid-like domains, the saturated fatty acids more so of the gel-like domains of the membrane lipids. The greater potency of unsaturated fatty acids towards Na^+/K^+-ATPase would be reasonable if the enzyme is confined to the more fluid-like domains of the membrane.

Irrespectively of the mechanism free fatty acids inhibit Na^+/K^+-ATPase and interfere with cardiac glycoside binding. The relatively high concentrations needed are probably not reached under physiological conditions, however, due to their association with plasma albumin [8,24,25]. Ischemia associated with increased phospholipase A_2-activity may locally create high concentrations of unsaturated fatty acids which in turn increase tissue damage by inhibition of the sodium pump [24].

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