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OUABAIN RECEPTOR BINDING OF HYDROXYPROGESTERONE DERIVATIVES

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1 A specific and sensitive radioreceptor assay has been devised which is based on high affinity, saturable binding of 9 nM [^{3}H]-ouabain to the total particulate fraction isolated from dog heart. Ouabain and other cardiac glycosides, including the aglycones, were about equipotent in their ability to displace [^{3}H]-ouabain from its receptor, the IC_{50}s ranging from 10 to 30 nM.

2 The only other substances found to compete significantly in the assay were derivatives of hydroxyprogesterone having a 17α-acetate substituent: chlormadinone acetate, megestrol acetate, cyproterone acetate and medroxyprogesterone acetate, with IC_{50}s of 2, 7.4, 9 and 21 μM, respectively. Prednisolone-3,20-bisguanyl-hydrazone, reported to have inotropic activity, gave an IC_{50} of 6.4 μM. Cyproterone-17α-OH was less active (IC_{50} 90 μM) than cyproterone-17α-acetate.

3 A large number of peptide and protein hormones, steroid hormones and their metabolites, amines, and drugs were inactive.

Introduction

A correlation between inhibition of the $(Na^+ + K^+)$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) and positive inotropic responses to cardiac glycosides (CG) has been generally well established (Akera, Larsen & Brody, 1969; Akera, 1970; Besch, Allen, Glick & Schwartz, 1970; Ku, Akera, Pew & Brody, 1974; Schwartz, Lindenmayer & Allen, 1975; Akera, 1977). It appears that this enzyme, closely related to active Na^+ transport, plays a prominent, if not the primary, role in the action of CG to increase contractile force in the mammalian heart (Brody & Akera, 1975). However, a causal relation between enzyme inhibition and inotropism has been disputed (Okita, Richardson & Roth-Schechter, 1973; Murthy, Kidwai & Daniel, 1974; Peters, Raben & Wasserman, 1974).

Ouabain-receptor interactions in $(Na^+ + K^+)$-ATPase preparations from different tissues and species have been extensively studied (Allen & Schwartz, 1969; Schwartz, Allen & Harigaya, 1969; Akera, Ku, Tobin & Brody, 1976) and kinetic constants for the ouabain-receptor subunit determined (Erdmann & Schoner, 1973a, b; Choi & Akera, 1977). Equilibrium binding experiments revealed that CG receptors from different tissues within a given species have the same affinity for ouabain but affinities among species differ (Erdmann & Schoner, 1973a). In the study of ouabain binding to human erythrocyte and cardiac membranes, for example, the dissociation constants of the ouabain-receptor complexes were identical (Erdmann & Hasse, 1975). It was also demonstrated that the specific binding of CG to cell membranes involves a specific binding site or 'receptor' and a cationic site; both are closely attached to or are components of the $(Na^+ + K^+)$-ATPase system (Kyte, 1972; Erdmann & Schoner, 1973a, b; Gardner & Frantz, 1974). It was suggested that the enzyme may exist in two or more conformations in its transport role. In one conformation the enzyme would have a greater affinity for K^+ and in the other for Na^+ (Skou, 1960; 1965).

In vitro studies have indicated that the specific binding of CG to isolated $(Na^+ + K^+)$-ATPase occurs in the presence of several distinct combinations of ligands (Schwartz, Matsui & Laughter, 1968). Although the combinations of Mg^{2+} and Pi promotes binding of CG to the isolated enzyme, this reaction does not appear to occur in beating hearts where ATP, Mg^{2+} and Na^+ are the apparent determinants of digitalis binding *in vivo* (Akera & Brody, 1976).

The recognized potency of the cardiac glycosides, in conjunction with the observation that these agents bind to saturable, high affinity acceptor sites on cell membranes, suggests the possibility that the binding sites may ordinarily accommodate endogenous ligands. The steroid nature of the cardioactive glycosides suggests that a putative ligand might also be a steroid. Thus, in addition to many classes of com-

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pounds, we screened a large number of steroids in a [^{3}H]-ouabain binding assay and identified a few specific compounds with appreciable binding potencies.

Methods

Preparation of total particulate fraction from dog heart

Fresh or frozen heart ventricle from adult male dogs was minced with scissors and 20 g homogenized with a Polytron for 1.5 min in 160 ml of 10% sucrose in 10 mM Tris–HCl buffer, pH 7.4. The homogenate was filtered through a 210 μm nylon mesh screen and centrifuged at 110,000 *g* for 30 min. The pellet was suspended in 160 ml of sucrose-Tris buffer, stirred for 1 h at 4°C and recentrifuged as above. The pellet was resuspended in 320 ml of 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1 mM disodium edetate (EDTA), and 1.25 mM $MgCl_2$.

Assay procedure

Competing test substance or unlabelled ouabain was incubated in a glass culture tube (12 mm × 75 mm, Fisher Scientific Co.) with 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1.25 mM $MgCl_2$, 1.25 mM ATP (freshly added), 9 nM [^{3}H]-ouabain and a total particulate fraction from 12.5 mg dog heart in a total volume of 1 ml. The reaction was started by the addition of the heart preparation and was incubated for 60 min at 37°C. Incubation was stopped by rapid cooling of the assay tubes in an ice bath and centrifuging at 2700 *g* for 20 min. The resulting supernatant containing unbound [^{3}H]-ouabain was discarded by aspiration. The membrane pellet was dissolved in 0.3 ml of 2 N KOH by heating in a 70°C water bath for 10 min, and 0.2 ml transferred to a vial containing 10 ml of scintillation medium (15 g of PPO, 1.5 g of POPOP, 1 litre of methyl-cellosolve in 2 litres of toluene). Radioactivity was counted for 10 min in a Packard Tricarb Liquid Scintillation Spectrometer or Philips Liquid Scintillation Analyser with external standard.

Test compounds were dissolved in the buffer solution and serial dilutions made in the same medium. Compounds insoluble in the buffer were dissolved and diluted in ethanol. The solvent was evaporated under a nitrogen stream and incubation medium was added to the tube and vortexed. Reproducibility of results and the fact that the dose-response curves of soluble and insoluble, actively competing steroids and ouabain are parallel indicated that the insoluble compounds were taken up by the incubation medium.

Drugs and chemicals

[^{3}H]-ouabain was obtained from Amersham, Oakville, Ontario, specific activity (11 Ci/mmol), PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-[2-(5-phenyloxazole)]-benzene were purchased from Syndel Laboratories, Vancouver, B.C. Cardiac glycosides and aglycones were obtained from Sigma Chemical Company, St. Louis, Mo., prednisolone-3,20-bisguanylhydrazone dihydrochloride from Bayer A.G., Wuppertal-Elberfield, Germany, prostaglandins from the Upjohn Company, Kalamazoo, Mich., cyproterone, cyproterone acetate, flutamide and ααα-trifluoro-2-methyl-4-nitro-m-lactotoluidide from Schering A.G., Berlin, through the courtesy of Pentagone Laboratory, Ltd. Montreal, P.Q., chlormadinone acetate from Lilly Res. Labs., Indianapolis, megestrol acetate (Megace-Bristol Labs.) and medroxy-progesterone acetate (Provera-Upjohn) from a local hospital pharmacy, C1-628 from Parke, Davis and Co., Ann Arbor, Mich., SKF 7690 from Smith, Kline and French, Montreal, P.Q. and R02-7239 from Hofmann-La Roche Inc., Vaudreuil, P.Q. Additional steroids were obtained from Steraloids, Inc., Wilton, N.H.; Sigma Chemical Co., St. Louis, Mo.; the Upjohn Company, Kalamazoo, Mich.; and the British MRC Reference Steroid Collection.

Results

Determination of specific binding of [^{3}H]-ouabain

CG specifically bind to membrane-bound receptors located in the sarcolemma, and the binding process has been investigated in detail in human cardiac cell membranes (Erdmann, 1978), cardiac tissue homogenate (Erdmann, 1977) and human red blood cells (Erdmann & Hasse, 1975) as well as different animal tissue preparations rich in ($Na^+ + K^+$)-ATPase.

In the present study the total particulate fraction from dog heart was employed for the CG radioreceptor assay. It is known that when ($Na^+ + K^+$)-ATPase is incubated with low concentrations (less than 10 μM) of CG, the binding of the latter to the enzyme varies with the concentrations of Mg^{2+}, Na^+, K^+, Pi and ATP present in the medium (Schwartz, *et al.*, 1975). In general, the combinations (Mg^{2+} + ATP) or (Mg^{2+} + Pi) promote binding, and the further additions of Na^+ or K^+ modify the amount of binding. In the presence of either (Mg^{2+} + ATP) or (Mg^{2+} + Pi) the addition of K^+ decreases binding, whereas Na^+ induces further binding in the presence of (Mg^{2+} + ATP), but decreases that promoted by (Mg^{2+} + Pi) (Akera & Brody, 1976). In this assay, we employed the binding condition for ouabain which is thought to occur *in vivo* in the presence of Mg^{2+},

ATP and Na^+ (Akera & Brody, 1976). The medium described earlier by Schwartz *et al.* (1968) was used with some modification in the concentration of components to obtain the maximum binding in our assay system.

The binding of [^{3}H]-ouabain to the receptor was dependent upon the amount of Na^+ present under the experimental conditions employed, and K^+ inhibited ouabain binding in a parallel and dose-related manner (IC_{50} 2 mM) as reported by others.

The results also demonstrated the known characteristics of ouabain binding to heart preparation, i.e. time and temperature-dependence. Binding equilibrium was reached after about 60 min incubation (Figure 1a) and was optimal at 37°C.

The CG-receptor interaction shows a high level (85 to 90%) of specific binding (Figure 1a). The low degree of non-specific binding was determined in the absence of ATP or in the presence of excess (10^{-4} M) non-labelled ouabain. The saturable nature of ouabain binding is shown in Figure 1b.

Activity of cardiac glycosides and aglycones in the radioreceptor assay

A number of compounds, similar in structure and with known inotropic action, were tested in the ouabain radioreceptor assay (Figure 2). The relative potencies in displacing [^{3}H]-ouabain from binding sites range from 10 to 30 nM. The aglycone, digitoxigenin, was the least potent of those cardioactive steroids examined. Rhamnose, the carbohydrate moiety of ouabain, was inactive at 10^{-4} M.

Screening of compounds for competition with [^{3}H]-ouabain in the radioreceptor binding assay

A large number of substances were tested for possible competition with [^{3}H]-ouabain for its binding sites (Table 1). All of the peptides and protein hormones, amines, prostaglandins, and a variety of drugs tested failed to affect ouabain binding.

Over 150 steroid and steroid metabolites of the estrane, androstane, and pregnane series were inactive except for certain hydroxyprogesterone derivatives, particularly those containing a 17α-acetate substituent. All of the other commonly occurring steroids tested, such as oestradiol, testosterone, corticosterone, and aldosterone, were inactive. The actively competing steroids are all semisynthetic compounds and used clinically for progestational activity (Figure 3).

Chlormadinone acetate was the most potent (IC_{50} 2 μM) of the 17α substituted hydroxyprogesterones (Figure 4), but 100 times less potent than ouabain (however, the steroid is only 30 times less potent than ouabain when inorganic phosphate replaces ATP in the incubation medium). The importance of the

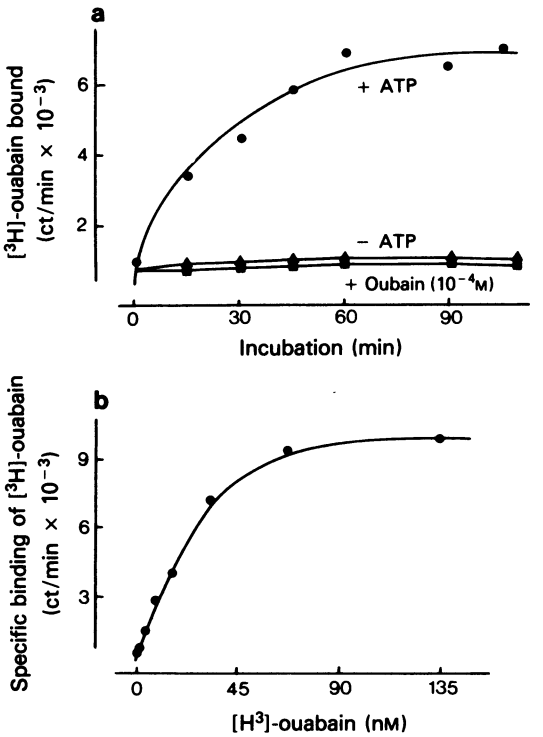

Figure 1(a) Binding of [^{3}H]-ouabain to dog heart preparation. The total particulate fraction from 12.5 mg dog heart was incubated in 50 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1.25 mM $MgCl_2$, and 9 nM [^{3}H]-ouabain (11 nCi/pmol) in a total volume of 1 ml, for the indicated time in the presence of 1.25 mM ATP, (●, total binding) and absence of 1.25 mM ATP (▲, nonspecific binding) or in the presence of 10^{-4} M unlabelled ouabain (■, nonspecific binding). (b) Saturation of specific binding sites by [^{3}H]-ouabain. The indicated concentrations of [^{3}H]-ouabain were incubated for 30 min at 37°C with 50 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1.25 mM $MgCl_2$, and freshly added 1.25 mM ATP (total binding) and either in the presence of 10^{-4} unlabelled ouabain or in the absence of ATP (nonspecific binding). The difference (specific binding) is shown.

17α-acetate substituent is seen in cyproterone acetate (IC_{50} 9 μM) which is more potent than cyproterone (IC_{50} 90 μM) by an order of magnitude. Testing of another available pair of steroids, containing either the 17α-OH or 17α-OAc, showed that 17α-OAc progesterone was more active (IC_{50} 78 μM) than 17α-OH progesterone (IC_{50} > 1000 μM). Estrane, androstane, and adrenocortical steroids containing 17α-OH or 17α-substituents are inactive in the binding assay. Therefore, receptor binding appears to be highly specific for hydroxyprogesterone derivatives.

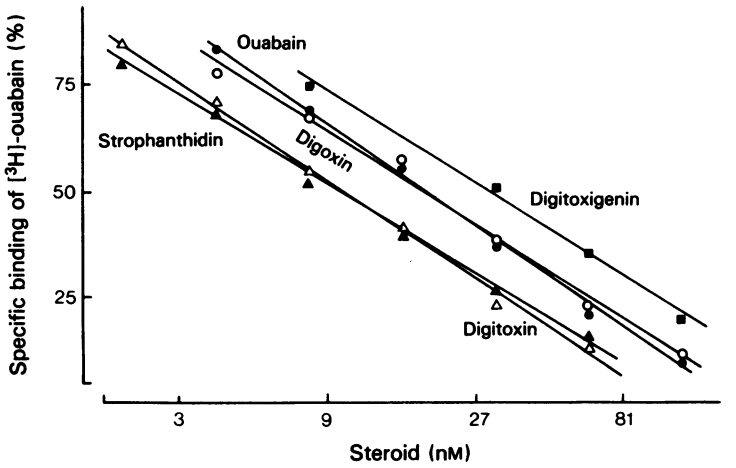

Figure 2 [^{3}H]-ouabain displacement curves for several cardiac glycosides (CG) and aglycones in the radioreceptor assay. Varying amounts of CG were incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1.25 mM $MgCl_2$, 1.25 mM ATP (freshly added), 9 nM [^{3}H]-ouabain and the particulate fraction from 12.5 mg dog heart for 60 min at 37°C in a total volume of 1 ml.

Cyproterone acetate, besides being one of the most potent progestins known, is also a clinically useful antiandrogen. Other antiandrogens tested were inactive; these compounds lack either the steroid nucleus or 17α-acetate substituent (Table 1).

Discussion

The use of radioactively labelled hormones and drugs to study hormone (drug)–receptor interactions at the molecular level in equilibrium binding experiments has been widely applied in the past few years. We have employed a specific and sensitive radioreceptor assay based on the high affinity, saturable binding of [^{3}H]-ouabain to the total particulate fraction isolated from dog heart. It has been shown that cardioactive glycosides significantly displaced [^{3}H]-digoxin binding to ($Na^+ + K^+$)-ATPase from calf heart, while inactive members did not compete (Matsui & Schwartz, 1968). Thus, the active ouabain and scillaren A, with K_i values for ($Na^+ + K^+$)-ATPase inhibition of the same order as digoxin, gave IC_{50} values of about 100 nM. Hexahydroscillaren A, a less efficient inhibitor for the ATPase, showed only minimal displacement of [^{3}H]-digoxin. Non-cardiotonic steroids, such as cholesterol and prednisolone, had no effect on the digoxin binding even at very high concentrations (Matsui & Schwartz, 1968). More recently, the relative affinity of the CG receptor and the inhibitory effect on ($Na^+ + K^+$)-ATPase activity of human cardiac cell membranes was investigated for several CG (Erdmann, 1978). CG in binding to their membrane bound receptors inhibited the ($Na^+ + K^+$)-ATPase in nanomolar concentrations. They displaced receptor-bound, radioactively labelled ouabain or digoxin in a concentration-dependent manner. The relative affinities were as follows: ouabain < digoxin < methyldigoxin < meproscillarin < proscillaridin < digitoxin. This order of potency was the same for the inhibitory effect on the ($Na^+ + K^+$)-ATPase and the displacement of bound [^{3}H]-ouabain.

Binding of CG and aglycones to heart membranes is extremely specific. In the present study all appear to have relatively similar potencies (IC_{50} 10 to 30 nM) and dose-response curves parallel to that of ouabain, indicating a single class of binding site for this family of compounds as suggested by others (Gardner & Kino, 1973; Erdmann & Hasse, 1975).

About 150 steroids, including the major classes of hormones, their metabolites and conjugates, were inactive in this radioreceptor assay. Of the large number of biologically active peptides, proteins, amines, and drugs tested, none was active. Only four steroids of the large number tested were relatively active in the ouabain radioreceptor assay, i.e. 50% displacement of [^{3}H]-ouabain at micromolar concentrations. These compounds are all clinically employed progestational agents, are derivatives of 17α hydroxyprogesterone and share a common structural feature, i.e. 17α-acetate substituent. Chlormadinone acetate was the most potent of the four compounds (IC_{50} 2 μM) although only 1/100th as potent as ouabain. Megestrol acetate, cyproterone acetate, and medroxy progesterone acetate were about 3.5, 4.5, and 10 times,

respectively, less potent than chlormadinone acetate. Unsaturation in ring B of the steroid nucleus appears to be a major determinant for binding of pregnane steroids to the ouabain receptor. Progesterone, hydroxyprogesterone, the 17α-acetate derivative of hydroxyprogesterone, and hydroxyprogesterone 17α-caproate were relatively inactive. Medroxyprogesterone acetate, having a 6α-methyl substituent, is only one-third as effective as megestrol acetate, containing a 6-methyl group but a double bond in B ring as well. Chlormadinone acetate, differing from megesterol acetate in a chlorine rather than a methyl group on position 6, is more than 3 times as potent as the latter steroid. Thus, substitutions, in addition to unsaturation, on ring B contribute to enhanced potency of steroids competing in the ouabain binding assay. 17α-Substituted estrogens and androgens were inactive in the ouabain binding assay. Cyproterone as 17α-OH was one tenth as active as cyproterone 17α-acetate.

The only other steroid substance found to compete in the ouabain binding assay is prednisolone-3,20-bis-guanylhydrazone (PBGH), but it is less than a third as potent as chlormadinone acetate. Prednisolone, on the other hand, is completely inactive. PBGH is known to possess inotropic activity on the isolated heart (Kroneberg, Meyer, Schraufstaetter, Schuetz & Stoepel, 1964; Greeff & Schlieper, 1967; Schuetz, Meyer & Kraetzer, 1969), and, like ouabain, to inhibit $(Na^+ + K^+)$-ATPase and to compete with ouabain

Table 1 Screening of compounds in the ouabain radioreceptor assay

	IC_{50} (nM)	
Ouabain	19	
Strophanthidin	10	
Digoxin	19	
Digitoxin	10	
Digitoxigenin	30	
Rhamnose	No displacement at 10^{-4} M	
*Inactive at 10^{-5} M**		
Morphine	Levorphanol	(−)-Isoproterenol
Naloxone	Levallorphan	(−)-Adrenaline
Methadone	Codeine	Noradrenaline
Nalorphine	Dextrorphan	Dopamine
Prostaglandins E_1, E_2, A_2, $F_{2\alpha}$	Propoxyphene	Diphenylhydantoin
Inactive at 10^{-6} M		
Glucagon	Insulin	
Lys-vasopressin	Arg-vasopressin	
Inactive at 1 μg/ml		
Somatostatin	Thyrotropin releasing hormone	
Prolactin	Thyroid-stimulating hormone	
Growth hormone	Luteinizing hormone	
	Follicle-stimulating hormone	
Steroids, inactive at 10^{-4} M		
Corticosterone	Deoxycorticosterone	
Aldosterone	Prednisolone	
Progesterone	Oestrone	
17α-oestradiol	17β-oestradiol	
Oestriol	Androsterone	
Epiandrosterone	Testosterone	
Cholesterol		

Over 150 other steroids, their sulphates, glucuronides and acetates were inactive at 10^{-4} M.

Ascorbic acid, inactive at 10^{-3} M

Anti-androgens and anti-oestrogens, inactive at 10^{-4} M

Flutamide
Cl-628 (Nitromifene citrate)
SKF 7690
RO 2-7239
ααα-Trifluoro-2-methyl-4-nitro-m-lactotoludide

* Inactive compounds caused less than 10% displacement of [^{3}H]-ouabain at the concentration specified.

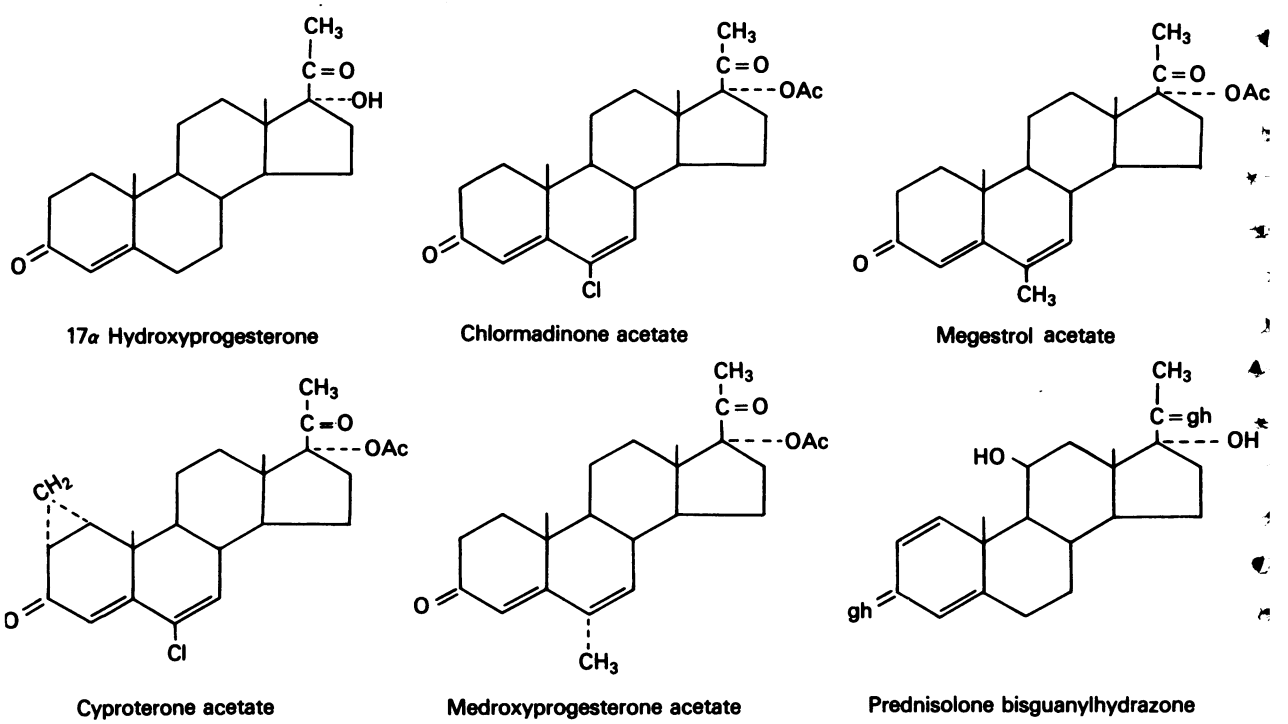

Figure 3 Structures of hydroxyprogesterone and derivatives.

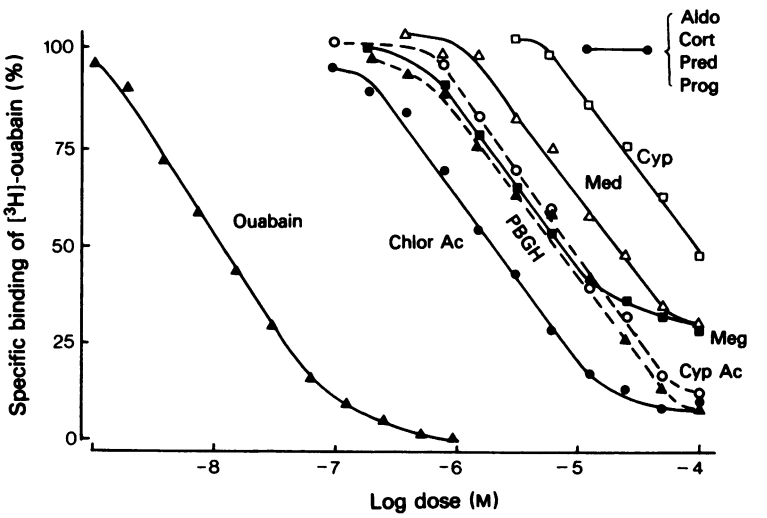

Figure 4 [^{3}H]-ouabain displacement curves for chlormadinone acetate (Chlor Ac), prednisolone-bisguanylhydrazone (PBGH), cyproterone acetate (Cyp Ac), megestrol acetate (Meg), medroxyprogesterone acetate (Med), cyproterone (Cyp), aldosterone (Aldo), corticosterone (Cort), prednisolone (Pred), and progesterone (Prog). Incubation conditions were identical to those in Figure 2.

receptor sites on cell membranes (Dransfeld & Greeff, 1964; Dransfeld, Galetke & Greeff, 1967; Greeff & Schlieper, 1967; Yamamoto, Akera & Brody, 1978a, b). It is unlikely that a steroid conjugate of this type exists in nature. Furthermore, the German workers (Schuetz, *et al.*, 1969; Kroneberg *et al.*, 1964) reported that the guanylhydrazones of several non-steroidal, aromatic compounds also showed inotropic activity.

These observations point to structural requirements for cardiotonic activity which may reside in naturally occurring metabolites of hydroxyprogesterone. Erdmann (1977) has also suggested the possible existence of endogenous substances naturally binding to receptors for cardiac glycosides. Examination of ouabain-like effects of those steroid compounds active in the binding assay is currently being carried out in isolated tissues and in intact animals. The pharmacological effects of these steroids will be presented elsewhere.

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