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COMPARISON OF OUABAIN RECEPTORS IN SHEEP MYOCARDIUM AND PURKINJE FIBRES

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Abstract—The conducting system of the heart has been reported to be more sensitive to the toxic effects of digitalis than the working myocardium. To investigate the molecular basis of these observations, we have characterized the ouabain receptor in Purkinje fibres and ventricular muscle of the digitalis-sensitive sheep heart using cell membrane preparations, crude homogenates and contracting heart tissues.

[^{3}H]-Ouabain binding has the following characteristics: (1) in sheep left ventricular cell membranes, specific binding was of high affinity (K_D 1.9×10^{-9} M at 37°); was co-incident with an inhibition of ($Na^+ + K^+$)-ATPase activity; and was inhibited by K^+ and unlabelled cardiotonic steroids; (2) in crude homogenates, the maximal binding capacity but not the affinity for ouabain varied in different parts of the sheep heart with Purkinje fibres containing markedly fewer binding sites (0.33×10^{14}/g wet weight; left ventricle, 1.3×10^{14}/g wet weight) and (3) in isolated, contracting Purkinje fibres and right ventricular moderator band strips, concentration-response curves for [^{3}H]-ouabain binding, increase in force of contraction and inhibition of [$^{86}Rb^+$]-uptake were co-incident. In both contracting tissues, a ouabain concentration of 3×10^{-7} M occupied about 50% of the specific binding sites, gave the maximal inotropic effect without toxicity and inhibited [$^{86}Rb^+$]-uptake by about 50%. The maximal binding capacity was lower in contracting Purkinje fibres (2×10^{14} binding sites/g wet weight) than in contracting moderator band strips (3.9×10^{14} binding sites/g wet weight). The maximal inotropic effects were reached slightly faster in Purkinje fibres but toxicity also occurred faster in these fibres.

We conclude that the specific ouabain binding site is the receptor mediating positive inotropy and inhibition of ($Na^+ + K^+$)-ATPase in the sheep heart. Further, this receptor is identical in both the conducting system and working myocardium but the conducting system contains many fewer receptors. This change in receptor number, rather than affinity, may underlie the increased ouabain toxicity observed in Purkinje fibres.

The therapeutic effects of digitalis in congestive heart failure with sinus rhythm are primarily a result of an increased myocardial contractility and cardiac output. Digitalis also decreases the conduction velocity in the Purkinje fibres while increasing the refractory period, excitability and pacemaker automaticity of these conducting fibres. The increased ouabain sensitivity of the Purkinje fibres relative to the working myocardium reported by Vassalle and co-workers [1–3] and Nowak and Haustein [4] may predispose to arrhythmias leading to ventricular tachycardia and fibrillation. In studies by Somberg *et al.* [5], the increased ouabain sensitivity of dog Purkinje fibres was paralleled by an increased sensitivity of [$^{86}Rb^+$]-uptake *in vitro*, a measure of active Na^+/K^+-transport. Administration of quinidine to digoxin-treated dogs further decreased the specific [$^{86}Rb^+$]-uptake in Purkinje fibres but not in ventricular muscle [6].

However, no differences between dog ventricular slices and Purkinje fibres in the [$^{86}Rb^+$]-uptake sensitivity to ouabain could be shown by Rhee [7]. Further, no differences were found in the ouabain sensitivity of the proposed ouabain receptor, ($Na^+ + K^+$)-ATPase, isolated from beef papillary muscles or Purkinje fibres [8]. Previous studies by Kübler and von Smekal [9] had shown that calf Purkinje fibres contained less ($Na^+ + K^+$)-ATPase which was 2–3 times less sensitive to digitalis than ($Na^+ + K^+$)-ATPase isolated from myocardium.

Differences between digitalis effects on the electrical system and the working myocardium may be related to different properties or amounts of the ($Na^+ + K^+$)-ATPase in these tissues. Different receptors have been demonstrated in rat and guinea pig hearts, species of lowered digitalis sensitivity [10, 11].

Sweadner [12] showed that the two different forms of ($Na^+ + K^+$)-ATPase in the brain are located on different cell types. If different ouabain receptors exist in the electrical system and myocardium, then it may be possible by using suitable semisynthetic glycoside derivatives to selectively bind to the receptors on the myocardium rather than those on the Purkinje fibres. Such a selective digitalis derivative may have an increased therapeutic index. The semisynthetic derivative, 16-acetylgitoxin, has been claimed to show an increased therapeutic index resulting from a weaker interaction with the Purkinje fibres [4]. The present studies were undertaken to

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determine whether there are different digitalis receptors (or $(Na^+ + K^+)$-ATPase molecules) in different portions of the heart. We have therefore compared: (1) Ouabain binding in sheep heart left ventricular cell membranes and homogenates from sheep left and right ventricle, moderator band and Purkinje fibres; and the relationship between ouabain binding and inhibition of $(Na^+ + K^+)$-ATPase in these sheep heart cell membranes; (2) Ouabain binding and its effects on force of contraction and $[^{86}Rb^+]$-uptake measured simultaneously and under identical conditions in contracting sheep heart Purkinje fibres and ventricular muscle (moderator band strips).

MATERIALS AND METHODS

Preparation of $(Na^+ + K^+)$-ATPase-containing sheep heart cell membranes

Left ventricular tissue from sheep hearts obtained fresh from the slaughterhouse was divided into portions of about 60 g and frozen at −40°. The partial purification procedure using sodium deoxycholate and sodium iodide has been previously described [10]. The final sediment, homogenized in 1 mM EDTA, pH 7.25 (60 ml), was used for the experiments. The $(Na^+ + K^+)$-ATPase activity was between 0.1 and 0.3 μmol ATP hydrolyzed/min/mg protein at 37°. About 70–90% of the total ATPase activity was inhibited by 1×10^{-3} M ouabain.

Preparation of homogenates of left and right ventricle, moderator band and Purkinje fibres

Tissue (0.3–1.5 g) taken either from left or right ventricle, moderator bands (right ventricular trabecula septomarginalis) or Purkinje fibres, dissected from freshly obtained sheep hearts, was weighed and homogenized in a glass Potter-Elvehjem homogenizer in 12–30 ml 0.25 M sucrose–1 mM EDTA, pH 7.25. The homogenate was centrifuged at 100,000 *g* for 30 min at 0°. The pellet was resuspended in 5–25 ml 1 mM EDTA (pH 7.25). This suspension was then used for $[^3H]$-ouabain binding studies. The $(Na^+ + K^+)$-ATPase activities are given in Table 2.

$[^3H]$-Ouabain binding to sheep heart homogenates or cell membranes

The procedures used for these experiments have been described in detail elsewhere [13]. Bound ouabain was quantitated by a rapid filtration method (Whatman GF/C glass filter membranes) to separate free drug from membrane-bound drug. Non-specific binding, defined as that in the presence of 1×10^{-3} M unlabelled ouabain, was less than 1% of total radioactivity bound to the cell membranes and was subtracted. The homogenates or cell membrane suspensions were incubated in either 3 mM $MgCl_2$ and 3 mM imidazole/PO_4 or 150 mM NaCl, 3 mM ATP and 3 mM $MgCl_2$ together with about $1–2 \times 10^{-9}$ M $[^3H]$-ouabain in 50 mM imidazole/HCl buffer, pH 7.25. Experiments were performed in duplicate assay and at least twice. Incubation times were as follows: 5 hr at 37°, 6 hr at 30°, 12 hr at 20° and 24 hr at 10°, after initial experiments showed that apparent equilibrium was reached within these times.

Electrically stimulated Purkinje fibres and moderator band strips from sheep hearts

Fresh sheep hearts, obtained at a local slaughterhouse, were placed in an ice-cold modified Krebs–Henseleit solution of the following composition (in mM): NaCl, 118.0; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 10; continuously gassed with 95% O_2/5% CO_2. Free-running Purkinje fibres, usually from the left ventricle, and moderator band strips cut approximately parallel, were suspended in an organ bath (75 ml) under optimal preload at 35° in modified Krebs–Henseleit solution and continuously gassed with 95% O_2/5% CO_2.

The preparations were stimulated by two platinum electrodes (field stimulation) from either a Grass SD9 or S88 stimulator (frequency, 1 Hz; duration, 5 msec; intensity, 10–20% above threshold). The developed force was measured isometrically with an inductive force displacement transducer (W. Fleck, Mainz, Germany) and recorded on either a Gould or Hellige recorder. The preparations were allowed to equilibrate for at least 60 min before any drug addition. Each preparation was used for only one ouabain concentration.

$[^3H]$-Ouabain binding to and $[^{86}Rb^+]$-uptake into contracting Purkinje fibres and moderator band strips of sheep heart

After equilibration, $[^3H]$-ouabain (about 1.8×10^7 cpm = about 1.2×10^{-8} M) together with differing amounts of unlabelled ouabain was added to the contracting Purkinje fibres or moderator band strips. Force of contraction was measured continuously, and after 120 min (approximate equilibration of positive inotropic effects of $2–5 \times 10^{-8}$ M ouabain), the muscles were rinsed for about 30 sec with distilled water, blotted and weighed. The amount of radioactivity was measured in scintillation counter (Betaszint BF 5000) after dissolving the muscles in Soluene 350 (0.5 ml, Fa. Packard, Zürich, Switzerland) for 2–4 hr at 60° and addition of 10 ml scintillation fluid (Unisolve-1, Koch-Light Laboratories, W. Zinsser, Frankfurt/M., Germany).

All values were measured as counts per minute against an external standard. $[^{86}Rb^+]$-uptake was measured after a stable inotropic effect had been reached with each ouabain concentration. The solution in the organ bath was replaced with gassed, prewarmed Krebs–Henseleit solution containing ouabain and a tracer amount of $^{86}RbCl$ (about 5 μCi = about 1×10^7 cpm). After 15 min incubation, force of contraction was measured and the muscles rinsed, weighed, dissolved and radioactivity counted, as for the $[^3H]$-ouabain binding experiments.

Materials

$[^3H]$-Ouabain, specific activity 14–20 Ci/mmol, was purchased from New England Nuclear, Dreieich, Germany. $^{86}RbCl$, specific activity 0.9–4.6 mCi/mg, was purchased from Amersham Buchler, Braunschweig, Germany. All other chemicals were of analytical grade.

General

Protein was measured by the method of Lowry *et al.* [14], using bovine serum albumin as standard. The $(Na^+ + K^+)$-ATPase activity was determined by the coupled optical assay using the rate of NADH hydrolysis [15]. All values are given as mean ± standard error of the mean. Statistical analyses were performed by the Student's *t*-test. The criterion for statistical significance was a P-value of less than 0.05.

The association rate constants were calculated from the second order rate equation and the dissociation rate constants from the first order equation [16].

RESULTS

[^{3}H]-*Ouabain binding to sheep heart cell membranes*

[^{3}H]-Ouabain binds specifically with high affinity to $(Na^+ + K^+)$-ATPase-enriched heart cell membranes from the digitalis-sensitive species, beef, human and cat [17, 18]. In these species, ouabain binding supported by either (Mg^{2+}, Pi) or (Na^+, ATP, Mg^{2+}) is, under normal conditions, to one site as shown by straight Scatchard plots. The digitalis-sensitive sheep also shows only one binding site under these conditions with an affinity, measured as the dissociation constant (K_D) from Scatchard plots, of 1.9×10^{-9} M for (Mg^{2+}, Pi)-supported ouabain binding at 37° (Table 1). As previously shown with beef and guinea pig cardiac cell membranes, (Na^+, ATP, Mg^{2+})-supported ouabain binding to sheep heart cell membranes is of somewhat lower affinity (K_D 3.3×10^{-9} M at 37°). The affinity (K_D) can be defined as the ratio of the dissociation and association rate constants (Table 1). Both rate constants are decreased at lower temperatures but the decrease in the dissociation rate constant is greater, leading to a higher affinity for ouabain at lower temperatures. Using Arrhenius and van't Hoff plots of these rate constants, ouabain binding to sheep heart cell membranes at 37° can be described by the following parameters: $\Delta G°$, −12.7 kcal mol^{-1}; $\Delta H°$, −15.7 kcal mol^{-1}; $\Delta S°$, −9.7 cal mol^{-1} deg^{-1}; with the transition state values of ΔG^+, 11.0 kcal mol^{-1}; ΔH^+, 18.3 kcal mol^{-1} and ΔS^+, 23.6 cal mol^{-1} deg^{-1} [16, 19].

The higher K_D-values measured by Scatchard analysis of [^{3}H]-ouabain binding at lower temperatures (Table 2) may be due to a lack of true equilibrium conditions.

In digitalis-sensitive species such as human [20], ouabain binding is co-incident with $(Na^+ + K^+)$-ATPase inhibition. Simultaneous measurement of both parameters in sheep heart cell membranes showed this co-incident relationship (Fig. 1); at a concentration of 3×10^{-9} M, about 50% of maximal

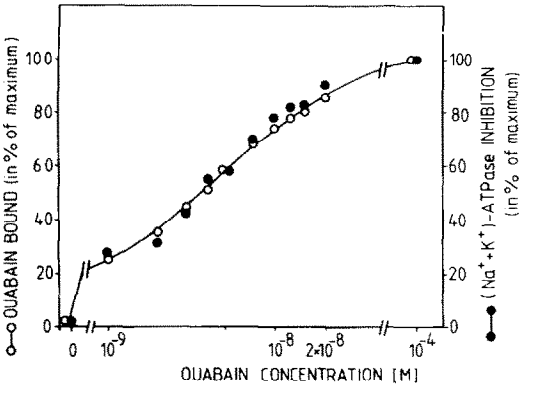

Fig. 1. [^{3}H]-Ouabain binding to sheep heart cell membranes and inhibition of $(Na^+ + K^+)$-ATPase. Heart cell membranes (0.35 mg protein, $(Na^+ + K^+)$-ATPase activity 0.25 μmol ATP hydrolysed/mg protein/min. at 37°) were incubated in 3 mM $MgCl_2$, 3 mM imidazole/PO_4, about 1×10^{-9} M [^{3}H]-ouabain, 50 mM imidazole/HCl (pH 7.25) and increasing amounts of ouabain (1×10^{-9}–1×10^{-3} M) at 37°, total volume 1 ml. After 5 hr, 0.2 ml of the incubation medium was used for determination of $(Na^+ + K^+)$-ATPase activity using a coupled optical assay [15] while the remainder of the incubation medium was filtered through Whatman GF/C filter membranes for determination of [^{3}H]-ouabain binding. $(Na^+ + K^+)$-ATPase activity was determined in the presence of the same ouabain concentration as in the incubation medium. Non-specific[^{3}H]-ouabain binding was defined as that in the presence of 1×10^{-3} M ouabain (less than 1% of the total radioactivity bound) and was subtracted. $(Na^+ + K^+)$-ATPase activity was defined as the ATPase activity which was inhibited by an ouabain concentration of 1×10^{-3} M. Maximal [^{3}H]-ouabain bound determined by a Scatchard analysis [48] and total $(Na^+ + K^+)$-ATPase activity were set as 100%.

Table 1. Temperature dependence of kinetic constants of ouabain binding to sheep heart cell membranes

Temperature (°)	10	20	30	37	37
Incubation medium	Mg^{2+}, Pi	Mg^{2+}, Pi	Mg^{2+}, Pi	Mg^{2+}, Pi	Na^+, ATP, Mg^{2+}
Association rate constant, k_{+1} ($M^{-1}sec^{-1}$)	7.2×10^3 (N = 2)	$2.5 \pm 0.3 \times 10^4$ (N = 3)	$5.7 \pm 1.1 \times 10^4$ (N = 5)	$1.2 \pm 0.1 \times 10^5$ (N = 5)	$9.2 \pm 0.9 \times 10^4$ (N = 5)
Dissociation rate constant, k_{-1} (sec^{-1})	1.5×10^{-6} (N = 2)	$7.2 \pm 0.7 \times 10^{-6}$ (N = 4)	$3.5 \pm 0.3 \times 10^{-5}$ (N = 3)	$1.3 \pm 0.1 \times 10^{-4}$ (N = 5)	$1.6 \pm 0.1 \times 10^{-4}$ (N = 5)
$K_D \left(= \frac{k_{-1}}{k_{+1}}\right)$ (M)	2.1×10^{-10}	3.0×10^{-10}	6.2×10^{-10}	1.1×10^{-9}	1.7×10^{-9}
K_D (Scatchard) (M)	1.3×10^{-9} (N = 2)	$3.3 \pm 0.4 \times 10^{-9}$ (N = 3)	$1.2 \pm 0.2 \times 10^{-9}$ (N = 4)	$1.9 \pm 0.2 \times 10^{-9}$ (N = 14)	$3.3 \pm 0.3 \times 10^{-9}$ (N = 6)

Ouabain binding to $(Na^+ + K^+)$-ATPase-containing heart cell membranes, prepared as described in Methods, was measured. $(Na^+ + K^+)$-ATPase activity was between 0.2 and 0.3 μmol ATP hydrolyzed/min/mg protein at 37°. All values, except at 10°, are given as mean ± S.E.M.

Table 2. Kinetic constants and maximal binding capacity in homogenates of sheep heart

	Left ventricle	Right ventricle	Moderator band	Purkinje fibres
K_D ($\times 10^{-9}$ M)	3.6 ± 0.4 (N = 5)	2.4 ± 0.2 (N = 8)	2.1 ± 0.4 (N = 3)	1.9 ± 0.2 (N = 12)
Binding sites ($\times 10^{14}$)/g wet weight	1.3 ± 0.1 (N = 5)	0.86 ± 0.07 (N = 8)	1.1 ± 0.2 (N = 3)	0.33 ± 0.1 (N = 3)
Association rate constant, k_{+1} ($\times 10^4$ M^{-1} sec^{-1})	7.3 ± 0.8 (N = 4)	—	8.2 ± 0.5 (N = 3)	7.9 ± 0.2 (N = 12)
Dissociation rate constant, k_{-1}, ($\times 10^{-4}$ sec^{-1})	1.3 ± 0.3 (N = 4)	—	1.1 ± 0.04 (N = 3)	1.0 ± 0.05 (N = 12)
($Na^+ + K^+$)-ATPase activity (nmol ATP hydrolysed/min/mg protein at 37°)	12 ± 3	10 ± 5	21 ± 10	2.5 ± 0.4

Homogenates were prepared from the different portions of fresh sheep hearts as described in Methods.

ouabain binding was measured, while ($Na^+ + K^+$)-ATPase activity was inhibited by about 50%.

[^{3}H]-Ouabain binding to sheep heart homogenates

The affinities and rate constants for ouabain binding to homogenates of different portions of sheet heart are given in Table 2. The K_D-values are higher than in the cell membrane preparations, by a factor of almost 2 for the left ventricle homogenates. This may be due to small amounts of potassium in the left ventricle homogenates, since the association rate constant, rather than the dissociation rate constant, was decreased. K^+ has been previously shown to preferentially decrease the association rate constant [21]. The number of binding sites/g wet weight in sheep ventricle homogenates (left ventricle, 1.3×10^{14}; right ventricle, 0.86×10^{14}; moderator band, 1.1×10^{14}) is similar to that found in human heart homogenates (left ventricle, 1.5×10^{14}, right ventricle, 0.9×10^{14} [20]).

In contrast, homogenates of sheep Purkinje fibres contain only 0.33×10^{14} ouabain binding sites/g wet weight.

Inhibition of [^{3}H]-ouabain binding by unlabelled cardiotonic steroids and potassium

The potencies of unlabelled digitalis derivatives can be calculated as the K_D-values from the concentration of these derivatives which inhibits [^{3}H]-ouabain binding to heart cell membranes by 50% [18]. The K_D-values of 14 cardiotonic steroids, including ouabain, on sheep and human heart cell membranes are given in Table 3. There is a good correlation between the potencies in these two species ($r > 0.99$), with the sheep heart showing approximately 1.9 times higher affinity.

At each [^{3}H]-ouabain concentration, K^+ reduced the number of counts bound at equilibrium. The IC_{50}-values for K^+-reduction of [^{3}H]-ouabain binding (Table 4) were similar in all sheep heart preparations tested. Scatchard plots show that K^+ reduces the affinity of (Mg^{2+}, Pi)-supported ouabain binding

Table 3. K_D-values from [^{3}H]-ouabain displacement experiments

Compound	Sheep heart cell membranes	Human heart cell membranes
Ouabain	$2.2 \pm 0.3 \times 10^{-9}$	$3.7 \pm 0.3 \times 10^{-9}$
Dihydroouabain	$4.8 \pm 0.6 \times 10^{-8}$	$1.2 \pm 0.3 \times 10^{-7}$
Digitoxin	$1.5 \pm 0.3 \times 10^{-9}$	$2.8 \pm 0.3 \times 10^{-9}$
Dihydrodigitoxin	$2.6 \pm 0.2 \times 10^{-8}$	$2.9 \pm 0.4 \times 10^{-8}$
Digitoxigenin	$1.2 \pm 0.07 \times 10^{-8}$	$1.8 \pm 0.2 \times 10^{-8}$
Anhydrodigitoxigenin	$1.5 \pm 0.1 \times 10^{-6}$	$3.9 \pm 0.5 \times 10^{-6}$
3α-Methyldigitoxigenin glucoside	$6.0 \pm 0.7 \times 10^{-8}$	$7.6 \pm 0.6 \times 10^{-8}$
Neriifolin (digitoxigenin thevetoside)	$6.2 \pm 1.2 \times 10^{-10}$	$1.0 \pm 0.04 \times 10^{-9}$
Digoxin	$2.5 \pm 0.3 \times 10^{-9}$	$6.2 \pm 0.3 \times 10^{-9}$
Dihydrodigoxin	$1.3 \pm 0.1 \times 10^{-7}$	$1.8 \pm 0.3 \times 10^{-7}$
Gitoxin	$1.0 \pm 0.2 \times 10^{-8}$	$1.7 \pm 0.3 \times 10^{-8}$
16-Acetylgitoxin	$1.2 \pm 0.03 \times 10^{-9}$	$2.8 \pm 0.3 \times 10^{-9}$
Pentaacetylgitoxin	$7.7 \pm 0.7 \times 10^{-8}$	$1.4 \pm 0.3 \times 10^{-7}$
Gitaloxin	$1.0 \pm 0.03 \times 10^{-9}$	$1.5 \pm 0.1 \times 10^{-9}$

Values are given as the mean ± S.E.M. for three determinations; for ouabain, N = 12.

Human heart cell membrane values are taken from [18].

K_D-values were calculated according to the methods of [13].

Table 4. K^+-effects on ouabain binding

K^+ concentration (mM)	K_D (M) (B_{max} in picomoles bound/mg protein)			
	Heart cell membranes	Left ventricle homogenate	Moderator band homogenate	Purkinje fibres homogenate
0	3.2×10^{-9}(10.1)	2.5×10^{-9}(1.8)	2.1×10^{-9}(1.1)	1.3×10^{-9}(0.36)
1	6.3×10^{-9}(10.3)	5.4×10^{-9}(1.6)	5.1×10^{-9}(1.0)	4.8×10^{-9}(0.30)
3	1.3×10^{-8}(9.7)	1.6×10^{-8}(1.6)	2.0×10^{-8}(1.0)	1.8×10^{-8}(0.37)
5	1.8×10^{-8}(10.0)	2.9×10^{-8}(1.7)	3.3×10^{-8}(1.0)	3.6×10^{-8}(0.31)
IC_{50}*	1.45	0.95	1.05	1.0

* The IC_{50} is the concentration of K^+ in mM, measured in several separate experiments, which inhibited the binding of [^{3}H]-ouabain (1.1×10^{-9} M) by 50%.

without changing the number of binding sites (Table 4). No significant differences were noted in the response of K^+ between heart cell membranes and homogenates of different heart regions. The reduction in affinity is a result of a markedly reduced association rate constant: in sheep heart cell membranes, the association rate constant was reduced from $1.3 \times 10^5\ M^{-1}\ sec^{-1}$ to $1.9 \times 10^4\ M^{-1}\ sec^{-1}$ in the presence of 3 mM K^+. The dissociation rate constant was not altered.

[^{3}H]-*Ouabain binding and active* Na^+/K^+-*transport in contracting sheep heart muscle*

Contracting sheep heart tissues were used to measure ouabain binding and its effects on force of contraction and [$^{86}Rb^+$]-uptake. These parameters were measured in contracting Purkinje fibres and moderator band strips from sheep hearts. The moderator band was chosen since its muscle fibres are parallel and it is considered to be representative of ventricular muscle [22]. Contracting Purkinje fibres showed a positive staircase when the stimulating frequency was increased from 0.3 to 2 Hz, as shown in other species [23]. In both contracting Purkinje fibres and moderator band strips, ouabain gave positive inotropic effects without evidence of toxicity at concentrations from 2×10^{-8} M to 2×10^{-7} M (Fig. 2). Ouabain concentrations of about 8×10^{-8} M gave half-maximal inotropic effects in both tissues. The maximal positive inotropic effect with 3×10^{-7} M ouabain on Purkinje fibres was not stable, being followed by a gradual decline in developed force without a concomitant increase in the diastolic force. This gradual decline was less marked with moderator band strips. After the maximal positive inotropic effect of 5×10^{-7} M ouabain, Purkinje fibres, in contrast to moderator band strips, showed a variable period of gradual decline before ectopic beats were observed. Higher ouabain concentrations gave similar effects in both tissues: decreases in developed force accompanied by a slow-developing contracture usually with variable periods of ectopic beats. The inotropic effects were very slow in onset, reaching approximate equilibrium after 120 min at the lower concentrations. The time to peak positive inotropic and toxic effects at ouabain concentrations greater than 1×10^{-7} M are given in Fig. 3. At all ouabain

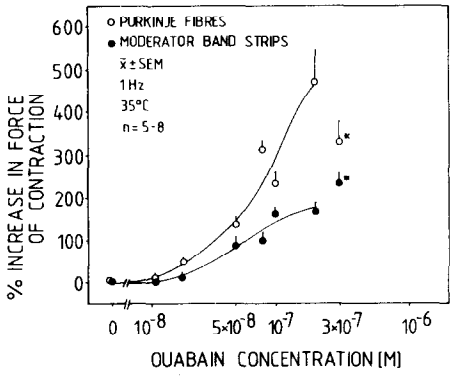

Fig. 2. Concentration–response curves on contracting sheep heart Purkinje fibres and moderator band strips. The inotropic effects of ouabain were measured in isolated Purkinje fibres and moderator band strips from sheep heart at 35° in Tyrode solution containing 2.5 mM Ca^{2+}, stimulated at 1 Hz. Each tissue was used for one ouabain concentration only. All values are given as the mean ± S.E.M. of 5–8 preparations. The initial force of contraction was: Purkinje fibres, 0.45 ± 0.06 mN (N = 50), moderator band strips, 1.9 ± 0.2 mN (N = 50). The Purkinje fibres had an average wet weight of 5.6 ± 0.9 mg; the moderator band strips weighed 8.2 ± 1.3 mg. An asterisk indicates that, at the ouabain concentration of 3×10^{-7} M, initial signs of toxicity were observed, especially with the contracting Purkinje fibres.

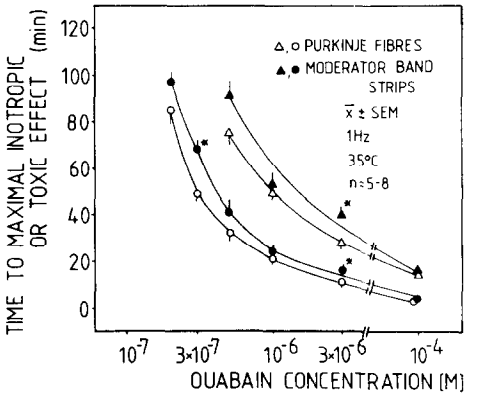

Fig. 3. Time to maximal inotropic effect (○, ●) and contracture (△, ▲) at different ouabain concentrations in contracting sheep Purkinje fibres (open symbols) and moderator band strips (closed symbols). Values are given as the mean ± S.E.M. of 5–8 experiments. An asterisk indicates a significant difference at this concentration ($P < 0.05$, unpaired t-test).

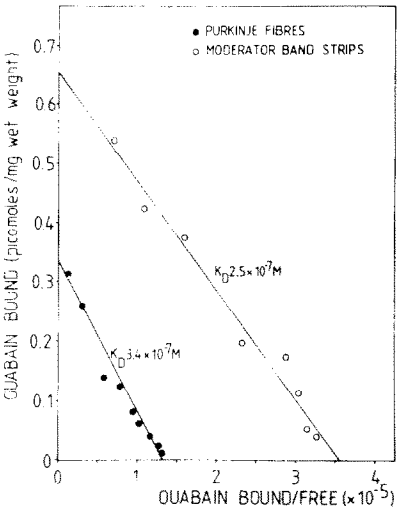

Fig. 4. [^{3}H]-Ouabain binding to contracting Purkinje fibres and moderator band strips from sheep heart. [^{3}H]-Ouabain binding was measured as described in Methods. [^{3}H]-Ouabain (Purkinje fibres, $1.61 \pm 0.17 \times 10^7$ cpm (N = 50), moderator band strips, $1.88 \pm 0.10 \times 10^7$ cpm (N = 50), approximately 1.2×10^{-8} M) was added together with differing concentrations of unlabelled ouabain for 120 min. Each preparation was used for only one ouabain concentration. Each point is the mean of 5–8 determinations. [^{3}H]-Ouabain bound is given as picomoles bound/mg wet weight. Data have been plotted according to Scatchard [48]. Non-specific [^{3}H]-ouabain binding has been defined as that bound in the presence of 1×10^{-4} M unlabelled ouabain (as per cent of maximal radioactivity bound: Purkinje fibres, $9.5 \pm 1\%$ and moderator band strips, $7.7 \pm 0.5\%$). The force of contraction measurements of the contracting tissues in this figure have been given as Fig. 2.

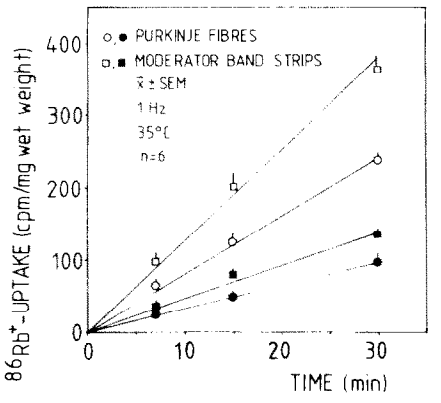

Fig. 5. Time-dependent [^{86}Rb$^+$]-uptake into contracting Purkinje fibres and moderator band strips from sheep heart. [^{86}Rb$^+$]-uptake was measured as described in Methods, using contracting sheep heart Purkinje fibres or moderator band strips, for either 7, 15 or 30 min. Each point is the mean ± S.E.M. of six experiments. The open symbols were obtained in the absence of ouabain, while the closed symbols are experiments carried out after contracture induced by an ouabain concentration of 1×10^{-4} M (= non-specific [^{86}Rb$^+$]-uptake).

concentrations, the time required for the maximal inotropic as well as the toxic effects was less with the Purkinje fibres, although the difference did not reach statistical significance at all concentrations.

[^{3}H]-Ouabain binding was measured after 120 min incubation. The Scatchard plots of [^{3}H]-ouabain binding to contracting Purkinje fibres and moderator band strips are given in Fig. 4. Both tissues show a straight Scatchard plot for one site; for Purkinje fibres, K_D 3.4×10^{-7} M; for moderator band strips, K_D 2.5×10^{-7} M. In contracting sheep heart tissues, similar concentrations gave maximal positive inotropic effects without marked toxicity (Fig. 2). The maximal amount of ouabain bound is about twice as much in contracting moderator band strips (Purkinje fibres, 2.0×10^{14} binding sites/g wet weight; moderator band strips, 3.9×10^{14} binding sites/g wet weight).

The active Na^-/K^+-transport activity in contracting tissues was measured using [^{86}Rb$^+$]-uptake for 15 min after a stable positive inotropic effect had been achieved with each ouabain concentration. [^{86}Rb$^-$]-Uptake in the presence of 1×10^{-4} M ouabain was defined as non-specific uptake unrelated to the (Na^- + K^+)-ATPase. As shown in Fig. 5, [^{86}Rb$^-$]-uptake is linear for at least 30 min with specific uptake being 60–65% of total uptake in both tissues. The specific [^{86}Rb$^+$]-uptake was greater in moderator band strips (4.4 ± 0.6 μmol/g wet weight/15 min, N = 6) than in Purkinje fibres (2.7 ± 0.3 μmol/g wet weight/15 min, N = 6). This increased [^{86}Rb$^+$]-uptake in moderator band strips corresponds to an increased number of specific ouabain binding sites in both contracting moderator band strips and in moderator band homogenates (Table 2). Ouabain inhibited the [^{86}Rb$^+$]-uptake over the same concentration range which gave the positive inotropic effects (Fig. 6). Ouabain

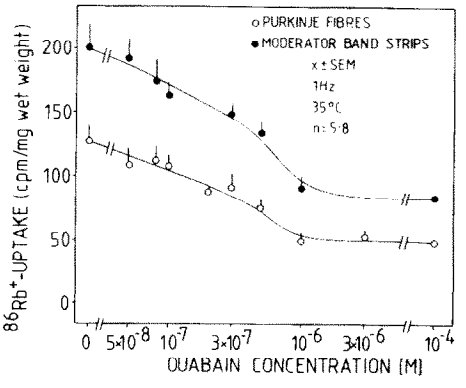

Fig. 6. Concentration–response curves for ouabain inhibition of [^{86}Rb$^+$]-uptake into contracting Purkinje fibres and moderator band strips from sheep heart. [^{86}Rb$^+$]-Uptake was measured as described in Methods, by adding a tracer amount of [^{86}RbCl] (5 μCi; Purkinje fibres, $1.27 \pm 0.06 \times 10^7$ cpm (N = 50); moderator band strips, $1.26 \pm 0.04 \times 10^7$ cpm (N = 50)) for 15 min after the positive inotropic effect of each ouabain concentration was constant. [^{86}Rb$^+$]-Uptake was inhibited by 50% in Purkinje fibres by an ouabain concentration of 2.5×10^{-7} M and in moderator band strips by 3.3×10^{-7} M. Specific [^{86}Rb$^+$]-uptake was 2.7 nmol/mg wet weight/15 min in Purkinje fibres and 4.4 nmol/mg wet weight/15 min in moderator band strips. The force of contraction at different ouabain concentrations of the preparations in this figure is given in Fig. 2.

(3×10^{-7} M) inhibited specific [$^{86}Rb^+$]-uptake by about 50% in both contracting Purkinje fibres and moderator band strips. Blockade of any β-receptors present in Purkinje fibres by L-propranolol (1×10^{-6} M) did not change the effects of a low concentration of ouabain (5×10^{-8} M): force of contraction increased by 150 ± 20% (N = 9), while [$^{86}Rb^+$]-uptake was 2.4 ± 0.2 μmol/g wet weight/15 min. A 30 min incubation with L-propranolol (1×10^{-6} M) alone decreased the developed force of contraction to 44 ± 6% of the initial value.

In both contracting sheep heart Purkinje fibres and moderator band strips, [^{3}H]-ouabain binding is closely correlated with the ouabain-induced positive inotropy and inhibition of [$^{86}Rb^+$]-uptake. Ouabain concentrations which occupy 50% of the binding sites give almost maximal positive inotropic effects without marked toxicity and inhibit active Na^+/K^+-transport by 50%.

DISCUSSION

The increased ouabain sensitivity reported for Purkinje fibres relative to ventricular muscle [1–6] may be related to either quantitative or qualitative differences in the digitalis receptor present in these tissues. We have tested these possibilities by simultaneously measuring ouabain binding and its effects on force of contraction or ($Na^+ + K^+$)-ATPase activity in contracting cardiac preparations or cardiac cell membranes from sheep. In partially purified cardiac cell membranes from left ventricle, [^{3}H]-ouabain bound to one type of binding site with a higher affinity than other digitalis-sensitive species such as human (K_D 3.7×10^{-9} M), beef (K_D 2.7×10^{-9} M) or cat (K_D 4.2×10^{-9} M) [18]; much lower affinities have been reported for the guinea pig heart (K_D 1.1×10^{-7} M) [11] and for the rat heart (K_{D1} 1.05×10^{-7} M; K_{D2} 2.8×10^{-5} M [10]). As in other species, this ouabain binding is supported by (Mg^{2+} + Pi) or ($Na^+ + ATP + Mg^{2+}$); is temperature- and time-dependent; and is fully reversible [10, 11, 13, 17, 18].

As in other digitalis-sensitive species, simultaneous measurement of [^{3}H]-ouabain binding and ($Na^+ + K^+$)-ATPase activity (Fig. 1) showed that the concentration-response curves for ouabain binding and inhibition of ($Na^+ + K^+$)-ATPase activity are co-incident [17, 20]. Because these curves are co-incident, the specific ouabain binding site in cell membranes can be defined as the receptor.

[^{3}H]-Ouabain binds with similar affinity, measured as the dissociation constant (K_D), to homogenates of the left ventricle, right ventricle, moderator band and Purkinje fibres of sheep heart. However, the number of ouabain binding sites/g wet weight is markedly reduced in homogenates of the Purkinje fibres compared with myocardial tissue. This decreased number of binding sites/g wet weight will mean that each Purkinje cell will have fewer binding sites than a ventricular muscle cell since the surface areas are similar (Purkinje cells, 10–30 μm diameter and 20–60 μm long; ventricular cells, 10–15 μm diameter and 30–60 μm long [22, 24]).

Ouabain binding and its effects on force of contraction or [$^{86}Rb^+$]-uptake were measured simultaneously in both contracting, isolated Purkinje fibres and moderator band strips from sheep hearts. In both tissues, [^{3}H]-ouabain bound to one type of binding site with an affinity (K_D) of 2.5–3.5×10^{-7} M. Maximal inotropic effects were seen at concentrations similar to the K_D-value. Half-maximal inotropic effects were measured at 8×10^{-8} M in both tissues. At higher ouabain concentrations, more ouabain was bound specifically but toxicity was observed at concentrations at or above 3×10^{-7} M. The beginning of toxicity manifested as a gradual decline in developed force is difficult to define. Although both maximal inotropy and toxicity occurred earlier in Purkinje fibres than in moderator band strips, these differences were not statistically significant at all concentrations. The clinical relevance of these small differences is questionable.

Specific [$^{86}Rb^+$]-uptake was inhibited over the same concentration range at which inotropic effects could be measured. Since the concentration–response curves for ouabain binding, positive inotropy and inhibition of [$^{86}Rb^+$]-uptake are co-incident (Figs. 2, 4, 6), the specific ouabain binding site in contracting sheep heart Purkinje fibres and moderator band strips can be defined as the inotropic receptor which is part of the ($Na^+ + K^+$)-ATPase, the enzyme responsible for active Na^+/K^+-transport. The affinity of the receptor for ouabain is the same in both contracting Purkinje fibres and moderator band strips; these tissues were taken to be representative of the conducting system and working myocardium, respectively.

However, the number of these receptors/g wet weight is about 50% lower in the Purkinje fibres; these fibres show about 40% lower maximal [$^{86}Rb^+$]-uptake/g wet weight. The reason for the higher number of specific ouabain binding sites in contracting tissue relative to homogenates is unclear but may imply loss of binding sites during membrane disruption.

Ouabain effects on the digitalis receptor in sheep heart Purkinje fibres and moderator band can be compared in Table 5 (for details, see Results).

Variable results have been presented from biochemical and pharmacological studies of ouabain effects on working myocardium and Purkinje fibres. Using driven dog Purkinje fibres and ventricular muscle fibres, Vassalle *et al.* [1] and Nowak and Haustein [4] showed that ouabain-induced toxicity occurred much earlier in the Purkinje fibres. Differences between these two tissues have been shown in the stimulation-induced increase in active ion transport [2], in the Na^+/Ca^{2+}-exchange [3] and in the dependence on extracellular calcium [25] and on intracellular sodium [26]. However, other electrophysiological investigations have not demonstrated substantial differences between Purkinje fibres and ventricular muscle using procedures which change force of contraction by varying the sodium or calcium concentration [27–30]. Qualitatively similar frequency-dependent changes in sodium activity in both tissues have been found in sheep heart [31, 32] and dog heart [33]. The sodium and calcium ion activities in resting sheep ventricular muscles (a^i_{Na} 6.9 mM; a^i_{Ca} 70 nM) were lower than in Purkinje fibres (a^i_{Na} 7.9 mM; a^i_{Ca} 98 nM) [34]; the higher a^i_{Ca} may under-

Table 5.

	Purkinje fibres	Moderator band
Homogenates		
K_D	1.9×10^{-9} M	2.1×10^{-9} M
Binding sites/g wet weight	0.33×10^{14}	1.1×10^{14}
Association rate constant	7.9×10^{4} M^{-1}sec^{-1}	8.2×10^{4} M^{-1}sec^{-1}
Dissociation rate constant	1.0×10^{-4} sec^{-1}	1.1×10^{-4} sec^{-1}
Contracting tissue		
EC_{50} (positive inotropy)	8×10^{-8} M	7×10^{-8} M
K_D ([^{3}H]-ouabain binding)	3.4×10^{-7} M	2.5×10^{-7} M
Binding sites/g wet weight	2.0×10^{14}	3.9×10^{14}
IC_{50} ([$^{86}Rb^+$]-uptake)	2.5×10^{-7} M	3.3×10^{-7} M
Specific [$^{86}Rb^+$]-uptake/15 min	2.7 μmol/g wet weight	4.4 μmol/g wet weight
Turnover number	540/min	430/min

line the reported greater sensitivity of the conducting tissue to digitalis. However, Weingart and Hess [35] reported similar calcium concentrations in both tissues from sheep heart. Increases in a^i_{Na} are closely associated with the positive inotropic effects of dihydroouabain [36], ouabain, acetylstrophanthidin and actodigin [37] or strophanthidin [38] in sheep Purkinje fibres or dog Purkinje fibres [39]. Toxic effects usually occurred when the a^i_{Na} was about 30 mM or greater [36]. The threshold ouabain concentration to give an increase in a^i_{Na} in sheep Purkinje fibres was about 1×10^{-7} M [37, 40]; similar concentrations produced non-toxic positive inotropic effects and inhibited active Na^+/K^+-transport in the present study. Further, our findings that the ouabain receptor is identical throughout the sheep heart although the Purkinje fibres contain markedly fewer receptors may explain the higher resting ionic activities in the Purkinje fibres. A reduced number of receptors would also lead to earlier toxicity in these fibres, as shown here and in previous studies by Vassalle *et al.* [1] and Nowak and Haustein [4].

Biochemical studies have not reported consistent differences between the working myocardium and conducting system. Kübler and von Smekal [9], using partially purified beef heart cell membranes, found that Purkinje fibres contained less ($Na^+ + K^+$)-ATPase (about 0.05 U/g) than ventricular muscle (about 0.45 U/g). Further, a very high IC_{50} value using digoxin was reported (Purkinje fibres, 4.1×10^{-6} M; ventricular muscle, 2.4×10^{-6} M). Palfi *et al.* [8] could not show any significant difference in ouabain sensitivity (IC_{50} $4–5 \times 10^{-8}$ M) between beef papillary muscle and Purkinje fibre ($Na^+ + K^+$)-ATPase although the enzyme yield was markedly lower from Purkinje fibres. [$^{86}Rb^+$]-Uptake *in vitro* in dog heart Purkinje fibres and myocardium was measured after infusion *in vivo* of varying digoxin concentrations [5, 6]. Purkinje fibres were more sensitive to ouabain (IC_{50} 4×10^{-7} M; ventricular muscle, 1.4×10^{-6} M) with an increased [$^{86}Rb^+$]-uptake (1.62 compared with 0.49 nmol/mg wet weight/15 min in ventricular muscle) [6]. In chronically digitalized dogs, quinidine increased the serum digoxin concentration which gave a further decrease in [$^{86}Rb^+$]-uptake in Purkinje fibres but not in ventricular tissue [6].

Rhee [7] showed that [$^{86}Rb^+$]-uptake *in vitro* into both dog Purkinje fibres and ventricular muscle was inhibited by 50% by ouabain concentrations of $4–6 \times 10^{-7}$ M, but uptake was 170% greater in the Purkinje fibres. These previous studies have measured [$^{86}Rb^+$]-uptake *in vitro* in tissue slices; a correlation between occupation of the digitalis receptor by ouabain and effects on force of contraction or active Na^+/K^+-transport can only be made using simultaneous measurement of these parameters. We have measured these parameters simultaneously under identical conditions and find no difference in the ouabain sensitivity between contracting Purkinje fibres and moderator band strips. Our studies indicate that the ouabain receptor in contracting Purkinje fibres and moderator band strips is identical. The presence of two different ouabain receptors of widely varying affinities has been shown in the heart [10] and brain [12] of the digitalis-insensitive rat. Further, Sweadner [12] showed that these different receptors were located on different cell types. Different receptors on different cell types would indicate the possibility of producing a semisynthetic cardiac glycoside which preferably binds to the receptor in the myocardium. The semisynthetic derivative, 16-epi-gitoxin, has been proposed to have an increased therapeutic index because of a weaker interaction with the conducting system [4]. No binding studies with 16-epigitoxin have been reported. If this compound binds specifically to the digitalis receptor, then any improvement in therapeutic index probably reflects differences in drug distribution within the heart.

[$^{86}Rb^+$]-Uptake has been widely used for estimating changes in the Na^+/K^+-transport system [10, 11, 41, 42]. In contracting rat [10] and guinea pig heart muscle [11], [$^{86}Rb^+$]-uptake was unaffected by ouabain concentrations causing positive inotropy only but was inhibited by ouabain concentrations which gave toxicity. With the digitalis-sensitive cat heart [43], as in the present study with sheep heart, [$^{86}Rb^+$]-uptake was inhibited over the same concentration range which gave positive inotropic effects. This correlation indicates that steady-state [$^{86}Rb^+$]-uptake measures the Na^+/K^+-pump activity, at least in contracting cat and sheep heart muscle. The arguments of Eisner *et al.* [44] that inhibition of some pump units may not necessarily decrease steady-state [$^{86}Rb^+$]-uptake seem not to

be applicable in the digitalis-sensitive species, cat and sheep.

Propranolol abolishes the stimulatory effect of low ouabain concentrations (about 1×10^{-9} M) on [$^{86}Rb^+$]-uptake *in vivo* in contracting guinea pig left atria [42], although the positive inotropic effects of ouabain concentrations between 3×10^{-8} M and 3×10^{-7} M were not affected by propranolol. The linear relationship between changes in a^i_{Na} and twitch tension in sheep Purkinje fibres was not changed by propranolol [37]. In the present study, low concentrations of ouabain (5×10^{-8} M) did not increase [$^{86}Rb^+$]-uptake. Further, propranolol (1×10^{-6} M) had no effect on the positive inotropy or [$^{86}Rb^+$]-uptake inhibition observed with this ouabain concentration in sheep Purkinje fibres.

The lowest ouabain concentration which gave a significant increase in the force of contraction of sheep Purkinje fibres was 2×10^{-8} M while 50% of the receptors were occupied at a concentration of 3×10^{-7} M in the present study. These concentrations are similar to those giving positive inotropic effects in isolated human ventricular muscle [46] but are 10- to 100-fold higher than the therapeutic free serum concentrations of digoxin, ouabain and digitoxin (about 1.3×10^{-9} M [45]) in man. These serum concentrations are similar to the K_D-values in isolated human and sheep cardiac cell membranes. The [^{3}H]-ouabain binding studies to cell membranes and contracting cardiac muscles were carried out under different experimental conditions. Optimal binding conditions (Mg^{2+}, Pi) were used for the cell membrane studies while [^{3}H]-ouabain bound to the contracting muscles in the presence of Krebs–Henseleit solution containing Na^+, K^+ and Ca^{2+} ions which are known to influence binding. Furthermore, in the contracting muscles, the ($Na^+ + K^+$)-ATPase transports both Na^+- and K^+-ions across an intact cell membrane against their ionic gradients. Another explanation for the discrepancy between inotropic concentrations *in vivo* and in isolated, contracting cardiac muscle might be the existence of two inotropic receptors *in vivo* with the high-affinity receptor being either uncoupled or rapidly inactivated in isolated preparations. Rapid inactivation would lead to a straight Scatchard plot for ouabain binding to isolated cardiac muscles as in Fig. 4 representing the low-affinity receptor. However, we could not show any low-affinity binding site in either sheep heart homogenates or partially purified sheep heart cell membrane preparations under the conditions used. Two digitalis receptors have been shown, however, in mammalian brains [12] and in isolated rat heart ventricular strips [10] or guinea pig left atria [11] although the situation in hearts from digitalis-sensitive species is less clear [47].

The following conclusions can be taken from the results presented in this study: (1) The specific ouabain binding site is the receptor for positive inotropy and inhibition of ($Na^+ + K^+$)-ATPase in sheep heart cell membranes and contracting muscle preparations.

(2) This receptor in working myocardium and conducting tissue of the sheep heart has the same affinity for ouabain.

(3) The conducting tissue (Purkinje fibres) contains fewer receptors relative to its weight and surface area.

This lowered receptor number could explain the higher sodium and calcium activities reported. These ionic activities are closely related to the inotropic and toxic effects of digitalis. Thus, low serum digitalis concentrations may cause a significant but non-toxic inotropic effect in both tissues. Higher digitalis concentrations may increase the sodium ion activity in the Purkinje fibres above the toxicity threshold before such effects are noted in the working myocardium.

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REFERENCES

1. M. Vassalle, J. Karis and B. F. Hoffman, *Am. J. Physiol.* **203**, 433 (1962).
2. P. I. Polimeni and M. Vassalle, *Am. J. Cardiol.* **27**, 622 (1971).
3. T. Li and M. Vassalle, *Bas. Res. Cardiol.* **78**, 396 (1983).
4. G. Nowak and K.-O. Haustein, *Pharmacology* **14**, 256 (1976).
5. J. C. Somberg, W. H. Barry and T. W. Smith, *J. Clin. Invest.* **67**, 116 (1981).
6. J. C. Somberg, S. M. Knox and D. S. Miura, *Am. J. Cardiol.* **52**, 1123 (1983).
7. H. M. Rhee, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **318**, 344 (1982).
8. F. J. Palfi, H. R. Besch, Jr. and A. M. Watanabe, *J. Molec. Cell. Cardiol.* **10**, 1149 (1978).
9. W. Kübler and P. von Smekal, *Acta Cardiol. Suppl.* **17**, 103 (1975).
10. E. Erdmann, G. Philipp and H. Scholz, *Biochem. Pharmac.* **29**, 3219 (1980).
11. L. Brown, K. Werdan and E. Erdmann, *Biochem. Pharmac.* **32**, 423 (1983).
12. K. J. Sweadner, *J. biol. Chem.* **254**, 6060 (1979).
13. E. Erdmann and W. Schoner, *Naunyn-Schmiedebergs Arch. Pharmac.* **283**, 335 (1974).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. W. Schoner, C. von Ilberg, R. Kramer and W. Seubert, *Eur. J. Biochem.* **1**, 334 (1967).
16. E. Erdmann and W. Schoner, *Biochim. biophys. Acta* **307**, 386 (1973).
17. E. Erdmann, in *Handbook of Experimental Pharmacology* (Ed. K. Greeff), Vol. 56/I, p. 337. Springer, Berlin (1981).
18. L. Brown and E. Erdmann, *Archs Int. Pharmacodyn. Ther.* **271**, 229 (1984).
19. G. A. Weiland and P. B. Molinoff, *Life Sci.* **29**, 313 (1981).
20. E. Erdmann and L. Brown, *Eur. Heart J.* **4**, *Suppl. A*, 61 (1983).
21. E. Erdmann, P. Presek and R. Swozil, *Klin. Wschr.* **54**, 383 (1976).
22. T. N. James, L. Sherf, R. C. Schlant and M. E. Silverman, in *The Heart, Arteries and Veins* (Ed. J. W. Hurst), 5th Edn, p. 22. McGraw-Hill, New York (1982).
23. M. Reiter, in *Handbook of Experimental Pharmacology* (Ed. K. Greeff), Vol. 56/I, p. 187. Springer, Berlin (1981).
24. E. Braunwald, J. Ross, Jr. and E. H. Sonnenblick, in *Harrison's Principles of Internal Medicine* (Eds. K.

J. Isselbacher, R. A. Adams, E. Braunwald, R. G. Petersdorf and J. D. Wilson), 9th Edn, p. 1025. McGraw-Hill, New York (1980).
25. M. Vassalle and M. Bhattacharyya, *Circ. Res.* **47**, 666 (1980).
26. M. Vassalle and C.-I. Lin, *Am. J. Physiol.* **236**, H689 (1979).
27. D. G. Kassebaum, *J. Pharm. Exp. Ther.* **140**, 329 (1963).
28. P. Müller, *Circ. Res.* **17**, 46 (1965).
29. E. Marban and R. W. Tsien, *J. Physiol.* **329**, 589 (1982).
30. G. Hart, D. Noble and Y. Shimoni, *J. Physiol.* **334**, 103 (1983).
31. C. T. January and H. A. Fozzard, *Circ. Res.* **54**, 652 (1984).
32. C. J. Cohen, H. A. Fozzard and S.-S. Sheu, *Circ. Res.* **50**, 651 (1982).
33. J. A. Wasserstrom, D. J. Schwartz and H. A. Fozzard, *Am. J. Physiol.* **243**, H670 (1982).
34. M. G. Lado, S.-S. Sheu and H. A. Fozzard, *Circ. Res.* **54**, 576 (1984).
35. R. Weingart and P. Hess, *Pflüger's Arch.* **402**, 1 (1984).
36. C. O. Lee, D. H. Kang, J. H. Sokol and K. S. Lee, *Biophys. J.* **29**, 315 (1980).
37. J. A. Wasserstrom, D. J. Schwartz and H. A. Fozzard, *Circ. Res.* **52**, 697 (1983).
38. D. A. Eisner, W. J. Lederer and R. D. Vaughan-Jones, *J. Physiol.* **355**, 251 (1984).
39. C. O. Lee and M. Dagostino, *Biophys. J.* **40**, 185 (1982).
40. D. Ellis, *J. Physiol.* **273**, 211 (1977).
41. T. Akera, S. Yamamoto, K. Temma, D.-H. Kim and T. M. Brody, *Biochim. biophys. Acta* **640**, 779 (1981).
42. P. Lechat, C. R. Malloy and T. W. Smith, *Circ. Res.* **52**, 411 (1983).
43. E. Erdmann, G. Philipp and H. Scholz, in *Cell Membrane in Function and Dysfunction of Vascular Tissue* (Eds T. Godfraind and P. Meyer), p. 76. Elsevier/North Holland, Amsterdam (1981).
44. D. A. Eisner, R. D. Vaughan-Jones and W. J. Lederer, *Circ. Res.* **53**, 834 (1983).
45. T. W. Smith and E. Haber, *J. Clin. Invest.* **49**, 2377 (1970).
46. H. Scholz and W. Schmitz, *Basic Res. Cardiol.* **79**, *Suppl.* 134 (1984).
47. E. Erdmann, K. Werdan and L. Brown, *Trends in Pharmac. Sci.* **6**, 293 (1985).
48. G. Scatchard, *Ann. N. Y. Acad. Sci.* **51**, 660 (1949).