Original Article

Receptor Occupancy with Digoxin *vs.* Receptor Occupancy with a Putative Endogenous Digitalislike Factor

Thomas A. SCHMIDT*, Henning BUNDGAARD, and Keld KJELDSEN

The possibility that an endogenous ligand for the digitalis receptor might exist has been a source of speculation resulting in efforts over the past decades to identify such a hormone. In the current context it is of interest that prolonged wash of myocardial and skeletal muscular samples from subjects who were not in digoxin treatment generally resulted in small tendencies to increase ^3H -ouabain binding ranging from 2 to 9% and from -2 to 7%, respectively. It may be appreciated that neither wet weight nor water content of left ventricular or skeletal muscular samples have been found to change as a result of the prolonged wash. Although these tendencies most likely are a mere play of chance, it may be argued that the studies did not entirely rule out the possibility of the existence of a quantitatively small amount of endogenous digitalislike factor. However, based on the evaluations of receptor occupancy with digoxin during treatment of 24-34% in the left ventricle and 9-13% in skeletal muscle, it would seem reasonable to expect that a comparatively larger fraction of digitalis receptors should be occupied by a putative endogenous digitalislike factor, if such a factor were to be of any physiological significance.

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Introduction

The digitalis receptor concentration can be determined with high accuracy and precision using vanadate facilitated ^3H -ouabain binding to intact myocardial and skeletal muscular samples (1, 2). The strength of this method is that it can be performed on non purified tissue without enzyme recovery problems; it allows measurements on small samples without intact metabolism, *i.e.* human myocardial and skeletal muscular samples obtained at autopsy as well as *in vivo* using biopsies; and methodological problems can be identified and corrected for (3-5). A problem with studies of ^3H -ouabain binding to tissue samples from patients who have been on digitalis therapy has, however, been that some of the receptors are occupied by

digoxin before ^3H -ouabain binding. Thus, quantification of cardiac glycoside receptor concentration by standard ^3H -ouabain binding in such patients would lead to an underestimation of receptors. However, this problem has been overcome by the development of a method, in which clearance of digoxin from the Na,K-ATPase in myocardial as well as skeletal muscular tissue can be performed by washing samples in buffer containing excess digoxin antibody fragments (F_{ab}) before ^3H -ouabain binding (6). The presence of the cardiac glycoside binding site on Na,K-ATPase has engendered speculation as to whether the sodium pump also serves as a receptor for some native ligand, an endogenous digitalislike factor. The existence of an endogenous digitalislike factor is still open to debate, however if it were present it would like exogenous digoxin have to bind to Na,K-ATPase to exert

From the Departments of Medicine B and *Cardiovascular Surgery RT, The Heart Centre, Rigshospitalet, Denmark.

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Address for Reprints: Thomas A. Schmidt, M.D., D.Sc., Department of Cardiothoracic Surgery RT 2152, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark.

its putative effect and thus be susceptible to an enhanced off-reaction in the presence of digoxin antibody fragments. Thus, the purpose of the present study was to apply a practical method, which would ensure clearance of ligand from the Na,K-ATPase in myocardial and skeletal muscle samples—be it exogenous or endogenous—and thereafter allow subsequent Na,K-ATPase determination by ^3H -ouabain binding. By comparing ^3H -ouabain binding prior to and after wash in digoxin antibody from digoxin and non-digoxin treated patients, respectively, it would thus be feasible to estimate receptor occupancy with digoxin and compare it to receptor occupancy with putative endogenous digitalislike factor.

Methods

The procedure (6) was developed by initially evaluating the net release during wash of specifically bound digitalis glycoside in human left ventricular and muscular samples obtained at autopsy, to which prior complete occupancy of digitalis glycoside receptors by digoxin or ouabain had been obtained *in vitro* by ^3H -digoxin binding, *i.e.* incubating samples in vanadate buffer containing 1×10^{-6} mol/l ^3H -digoxin (10 $\mu\text{Ci/ml}$) and digoxin for 2 h at 37°C . The samples were then transferred to vials containing 3 ml unlabelled buffer, and wash was performed 12 times for 30 min, *i.e.* for a total of 6 h. Half-life time ($T_{0.5}$) for net removal of ^3H -digoxin from its specific receptor in human left ventricular samples as a result of this wash was 32, 7 and 1 h at 0, 30 and 37°C , respectively. Although from these evaluations wash at 37°C might seem attractive for promoting digoxin release from receptors, it was of no avail, because studies of the potential for subsequent receptor quantification revealed that complete ^3H -ouabain binding could not be obtained using the standard assay after wash at this temperature. This was, however possible after wash at 30°C for both left ventricular and muscular tissue. In human skeletal muscular samples obtained post-mortem $T_{0.5}$ for removal of ^3H -digoxin from its receptor as a result of washout in buffer at 30°C was around 5 h.

To promote the net release of specifically bound ^3H -digoxin from its receptor by inhibiting rebinding of released digoxin, excess specific digoxin F_{ab} (5×10^{-7} mol/l) was added to the washing medium. The concentration of digoxin antibody used was calculated to be in excess of the maximum theoretical digoxin binding capacity of the samples. Thus, the effect of adding digoxin antibody on net release of digoxin during wash should be comparable to the effect of adding excess unlabelled digoxin. This was evaluated adding unlabelled digoxin at a concentration of 1×10^{-4} mol/l to the buffer. In both cases $T_{0.5}$ for removal of specifically bound digoxin from left ventricular samples at 30°C was significantly reduced to 3 h. Similarly in both cases $T_{0.5}$ for skeletal muscular samples was significantly reduced to 2 h. That the release of specifically

bound ^3H -digoxin showed no significant difference, whether excess digoxin F_{ab} or excess unlabelled digoxin was added to the buffer indicated that digoxin F_{ab} in the given concentration totally inhibits rebinding of released ^3H -digoxin in left ventricular as well as skeletal muscular samples. On the basis of the determined $T_{0.5}$ values it was calculated that it would take 45 h to reduce specific ^3H -digoxin binding 99% by washing left ventricular samples in buffer at 30°C without digoxin F_{ab} . However, the same reduction might be obtained after only 18 h of wash, if excess digoxin F_{ab} were added to the buffer. For skeletal muscular samples a reduction in specific ^3H -digoxin binding of 99% would require a wash at 30°C for 36 and 12 h in buffer without and with excess digoxin F_{ab} , respectively.

Because samples from digitalised human patients would not exhibit 100% occupancy of receptors with digoxin, the net release of specifically bound digitalis glycoside was also evaluated in samples to which a relatively low occupancy of receptors by ^3H -digoxin had been obtained *in vitro*. Such low occupancy of receptors was obtained by incubating samples in 1×10^{-8} mol/l ^3H -digoxin (0.3 $\mu\text{Ci/ml}$) and digoxin for 25 min at 37°C , which resulted in around 9% of the amount of receptors being occupied by ^3H -digoxin in left ventricle as well as skeletal muscle. Furthermore, because it would be practical to perform the wash of samples overnight, the volume of wash medium was increased from 3 to 12 ml and changes of media omitted. All vials were heat sterilised, and during the wash procedure they were sealed to reduce exposure to the exterior environment and loss of medium due to evaporation. Wash was then performed at 30°C for 8, 16 or 24 h in buffer containing excess digoxin F_{ab} and the remaining specific ^3H -digoxin binding determined. The reduction in specific ^3H -digoxin binding in left ventricular samples was 89, 95 and 95%, respectively; and in skeletal muscular samples it was 94, 97 and 97%, respectively. This was found to be in agreement with the $T_{0.5}$ determinations given above taking the omission of changes of media into consideration. Taking practical laboratory considerations into account, it was found acceptable that 3–5% of a relatively low digitalis glycoside receptor occupancy remained after the wash, and thus subsequently a wash overnight for 16 h at 30°C without changing of media was employed. As noted it had to be ensured that the digitalis receptors were able to rebind digitalis glycoside after a digoxin occupancy had been cleared by wash for 16 h at 30°C in buffer containing digoxin antibody. Thus, experiments were conducted in which specific ^3H -ouabain binding was achieved by applying the standard ^3H -ouabain binding assay and compared to ^3H -ouabain binding obtained after a fraction of the receptors had been occupied by ^3H -digoxin and subsequently removed by the developed wash. In tissues obtained post-mortem this gave values of (means $\pm$ SEM; $n=12$) 370 ± 12 and

378 ± 13 pmol/g wet weight ($p > 0.60$), respectively for left ventricular samples, and for skeletal muscular samples 315 ± 16 and 309 ± 19 pmol/g wet weight ($p > 0.70$), respectively (6). Thus, digitalis receptors were found to maintain capacity for ^3H -digitalis glycoside binding after prior digoxin occupancy had been removed by the present wash. That samples in general were stable to the washing procedure was furthermore evident from later studies on non-digitalised subjects using left ventricular samples obtained post-mortem (7), during heart transplantation (8), and on skeletal muscular samples obtained post-mortem (9) and in life (10). Thus, none of these studies showed any reduction in ^3H -ouabain binding due to the wash.

Results

By measuring ^3H -ouabain binding to tissue samples from digitalised individuals before (a) and following wash in excess digoxin F_{ab} (b), it was possible to calculate the percentage of receptors specifically occupied, *i.e.* occupancy, by digoxin as a result of in life therapy, *i.e.* $(b - a)/b \times 100$. Specific left ventricular receptor occupancy with digoxin as determined post-mortem, *i.e.* 15 h after death, amounted to 34% ($p < 0.001$) (7), and as determined in life without putative confounding effects of post-mortem receptor degradation it amounted to 24% ($p < 0.05$) (8). The specific left ventricular receptor occupancies with digoxin determined after death and in life are in good accord with the report of a 38% reduction in maximum hyper-polarisation of resting membrane potential in human atrial tissue as a result of digitalisation (11). Specific skeletal muscular receptor occupancy with digoxin as determined post-mortem amounted to 13% ($p < 0.02$) (9). Using vital muscular samples from digitalised patients, muscular receptor occupancy with digoxin as determined in life without putative confounding effects of post-mortem receptor degradation amounted to 9% ($p < 0.05$) (10). Thus occupancy of receptors with digoxin in the heart was 2.6–2.7 fold higher than in skeletal muscle. This seems to be in good agreement with the somewhat higher apparent K_D for ^3H -ouabain binding in human skeletal muscle compared to the myocardium (1, 2). These skeletal muscular digitalis glycoside receptor occupancies seem to accord with the report of 9% lower standard ^3H -ouabain binding to muscular samples from digitalised as compared to non-digitalised patients (12). However, a problem with the evaluation by Dørup and co-workers might be that the occupancy was obtained comparing standard ^3H -ouabain binding in a group of digitalised patients to that of another group of non-digitalised subjects. If the digoxin receptors had been down-regulated due to heart failure in the digitalised subjects, this may have caused an overestimation of digoxin occupancy. Additionally, it would not have been possible to reveal a putative con-

cealed up-regulation of skeletal muscular cardiac glycoside receptor concentration in response to digoxin exposure, which might have resulted in an underestimation of receptor occupancy with digoxin. Studies in man of skeletal muscular content of digoxin as a result of digitalisation have hitherto been carried out by way of radioimmunoassay. These studies reported digoxin concentrations in skeletal muscular samples obtained post-mortem of 20–32 pmol/g wet wt. (13–15). Receptor occupancy by digoxin of 9% in human skeletal muscle corresponds to around 24 pmol/g wet wt. of receptors being occupied by digoxin in response to digitalisation. Compared with the radioimmunoassay studies this would seem to imply that a major part of the digoxin located in skeletal muscle is specifically bound to its receptor.

Discussion

The possibility that an endogenous ligand for the digitalis receptor might exist has been a source of speculation resulting in efforts over the past decade to identify such a hormone (16, 17). It has been reported that endogenous digitalislike factor is in fact ouabain (18, 19). Nevertheless, there are serious points of concern with regard to the putative existence and importance of an endogenous digitalislike factor, not least before ouabain can be elevated from its characterisation as plant toxin to human hormone (20). Thus, it must be established whether the specific enzymes required for the synthesis of the unique steroid structure of the cardiac glycosides are present or inducible in mammalian tissues. Additionally, it would appear to violate most design criteria for hormonal signal transduction, if a ubiquitous important enzyme such as the Na,K-ATPase was to serve as receptor, and not least for its inhibition to be the putative hormone's primary effector mechanism (20). Finally, it has recently been confirmed that ouabain is synthesised in the adrenal glands (21), and that in dogs the secretion rate amounts to 0.68 pmol/min (22). A secretion rate of endogenous ouabain of such a low magnitude has been deemed absurd, because it may be estimated that occupancy of only 1% of the skeletal muscular pool of Na,K-ATPase with endogenous ouabain would consume 26 days of production (23). In the current context it is of interest that prolonged wash of myocardial (6–8) and skeletal muscular (6, 9) samples from subjects who were not in digoxin treatment generally resulted in small tendencies to increase ^3H -ouabain binding ranging from 2 to 9% and from –2 to 7%, respectively. It may be appreciated that neither wet weight nor water content of left ventricular or skeletal muscular samples have been found to change as a result of the prolonged wash (6). Although these tendencies most likely are a mere play of chance, it may be argued that the studies did not entirely rule out the possibility of the existence of a quantitatively small amount of endogenous digitalis-

like factor. However, based on the evaluations of receptor occupancy with digoxin during treatment of 24–34% in the left ventricle and 9–13% in skeletal muscle (7–10), it would seem reasonable to expect that a comparatively larger fraction of digitalis receptors should be occupied by a putative endogenous digitalislike factor, if such a factor were to be of any physiological significance. Recently essential hypertension has been reported to be associated with an increase in plasma concentration of ouabainlike factor (24), however on receptor level no evidence of an endogenous digitalislike factor was observed in spontaneously hypertensive rats when ^3H -ouabain binding to skeletal muscle was compared *in vivo* and *in vitro* as well as after wash of samples (25, 26). Furthermore, in resistance vessels obtained from spontaneously hypertensive rats only a small tendency to a 3% lower concentration of ^3H -ouabain binding sites was observed when compared to age matched controls (25). In comparison K depletion of rats was found to reduce ^3H -ouabain binding site concentration significantly in resistance vessels by 33% and in skeletal muscle by 55%, importantly without the concomitant development of hypertension (25). Taken together ^3H -ouabain binding evaluations have not as yet furnished support for the existence of an endogenous digitalislike factor which could be ascribed any clinical importance.

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