Systems analysis of digoxin kinetics and inotropic response in the rat heart: effects of calcium and KB-R7943

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Weiss, Michael, Myoungki Baek, and Wonku Kang. Systems analysis of digoxin kinetics and inotropic response in the rat heart: effects of calcium and KB-R7943. *Am J Physiol Heart Circ Physiol* 287: H1857–H1867, 2004. First published May 6, 2004; 10.1152/ajpheart.01121.2003.—To gain more insight into the mechanistic processes controlling the kinetics of inotropic response of digoxin in the perfused whole heart, an integrated kinetic model was developed incorporating digoxin uptake, receptor binding (Na+/K+-ATPase inhibition), and cellular events linking receptor occupation and response. The model was applied to data obtained in the single-pass Langendorff-perfused rat heart for external [Ca^{2+}] of 0.5 and 1.5 mM under control conditions and in the presence of the reverse-mode Na+/Ca^{2+} exchange inhibitor KB-R7943 (0.1 μM) in perfusate. Outflow concentration and left ventricular developed pressure data measured for three consecutive doses (15, 30, and 45 μg) in each heart were analyzed simultaneously. While disposition kinetics of digoxin was determined by interaction with a heterogeneous receptor population consisting of a high-affinity/low-capacity and a low-affinity/high-capacity binding site, response generation was >80% mediated by binding to the high-affinity receptor. Digoxin sensitivity increased at lower external [Ca^{2+}] due to higher stimulus amplification. Coadministration of KB-R7943 significantly reduced the positive inotropic effect of digoxin at higher doses (30 and 45 μg) and led to a saturated and delayed receptor occupancy-response relationship in the cellular effectuation model. The results provide further evidence for the functional heterogeneity of the Na+/K+-ATPase and suggest that in the presence of KB-R7943 a reduction of the Ca^{2+} influx rate via the reverse mode Na+/Ca^{2+} exchanger might become the limiting factor in digoxin response generation.

Na+/K+-ATPase; Na+/Ca^{2+} exchanger; cardiac glycosides; mathematical modeling

IT IS NOW WIDELY ACCEPTED that the cardiac glycosides exert their positive inotropic effect through an inhibition of Na+/K+-ATPase (Na+ pump): the Na+ pump controls the cytoplasmic [Na+], which, in turn, determines [Ca^{2+}] via the Na+/Ca^{2+} exchanger (NCX) (see Refs. 1, 26, and 30 for review). However, the in vivo functional roles of the low (α1)- and then high (α2)-affinity isoforms of the Na+/K+-ATPase in mediating this inotropicism in rodent hearts and the effect of external [Ca^{2+}] ([Ca^{2+}]o) are still a matter of debate (9, 20, 21, 24, 37). The suggestion that in mice the positive inotropic response to ouabain is solely mediated by the α2-isoform (20) has thus been questioned by Schwartz and Petrashevskaya (37), who argued that the relatively high [Ca^{2+}] (2.5 mM) used in this study could have masked the contribution of α1-receptors. Recently, a dominant role of α2-receptors has been also reported by Dostanic et al. (9) in mice. Likewise, although it appears clear that the NCX is essential for the action of cardiac glycosides (32), the quantitative role of NCX in this process is poorly understood in the whole heart. Thus, contrary to expectations, the reverse mode NCX blocker KB-R7943 (KBR) did not influence the inotropic effect of strophanthidin in rat ventricular myocytes (35).

Our study was undertaken to examine the influence of alterations in Ca^{2+} in perfusate as well as the effect of the NCX inhibitor KBR on the processes that underlie inotropic actions of digoxin in the perfused rat heart, namely myocardial uptake, receptor binding (Na+/K+-ATPase inhibition), and postreceptor events. To address this objective, we formulated the following specific questions: 1) what is the functional role of α1- and α2-isoforms; 2) how can the increase in glycoside sensitivity with decreasing [Ca^{2+}]o be explained mechanistically; 3) does NCX inhibition by KBR reduce the inotropic response to digoxin; and 4) how can the KBR effect be modeled at the level of the receptor occupancy-response relationship? By using an extended version of a recently developed modeling approach (21), we have analyzed digoxin outflow concentration and inotropic response data to evaluate in the intact heart the effects of external Ca^{2+} (0.5 and 1.5 mM) and NCX inhibition by KBR (0.1 μM) on the various processes involved in the inotropic effect of digoxin. The model was sufficient to discriminate between the effects of external Ca^{2+} as well as KBR on digoxin receptor binding and postreceptor events, respectively.

METHODS

Materials

[3H]Digoxin (37 Ci/mmol) and digoxin were purchased from Perkin Elmer Life Sciences (Boston, MA) and Sigma-Aldrich (Steinheim, Germany), respectively. KBR was kindly donated by Nippon Organon. Digoxin was dissolved in 70% ethanol and diluted with Krebs-Henseleit buffer. The final concentration of ethanol in vehicle was 0.7%. [3H]Digoxin solution was made by mixing a labeled (1 μCi/ml) compound with an unlabeled (50 μg/ml) one. All other chemicals and solvents were of the highest grade available.

Perfused Rat Heart

Hearts excised from adult male Wistar rats (250–300 g) were perfused by using the nonrecirculating Langendorff technique as previously described (21). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip). After the onset of general anesthesia, heparin (300 IU) was injected into the tail vein, and a cannula was
bound into the trachea for ventilation. The chest was opened, and an aortic cannula filled with perfusate was rapidly inserted into the aorta to prevent ischemia. The hearts were then subjected to retrograde perfusion with an oxygenated Krebs-Henseleit buffer, pH 7.4, consisting of (in mM) 118 NaCl, 4.7 KCl, 1.66 MgSO4, 24.88 NaHCO3, 1.18 KH2PO4, 5.55 glucose, 2.0 Na-pyruvate, and 0.5 and 1.5 CaCl2, in group 1 and group 2, respectively; it also contained 0.1% bovine serum albumin. The buffer was continuously bubbled with 95% O2:5% CO2 and maintained at 37°C. The pulmonary artery was incised to allow outflow of the perfusate. Coronary perfusion was initiated through a short cannula in the aortic root and maintained at a constant pressure of 60 mmHg. A latex balloon was placed in the left ventricle of the isovolumetrically contracting heart and connected to a pressure transducer line (diastolic pressure was set to 5–6 mmHg). After stabilization, the system was changed to constant flow condition (controlled by a roller pump) maintaining a coronary flow of 9.5 ± 0.4 ml/min. The hearts were beating spontaneously at an average rate of 270 beats/min. Coronary perfusion pressure, the left ventricular (LV) pressure, and heart rate were measured continuously, and a physiological recording system (Hugo Sachs Elektronik; March-Hugstetten, Germany) was used to monitor LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP). LV developed pressure (LVDP) was calculated as LVDP = LVSP – LVEDP. This investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). Prior approval was obtained by the Animal Protection Body of the State of Sachsen-Anhalt, Germany.

Experimental Protocol

The following experiments were performed in two groups of hearts (n = 5 in each) with [Ca2+] in perfusate of 0.5 and 1.5 mM, respectively. After 20-min periods of equilibration, three doses (15, 30, and 45 μg) of [3H]digoxin were administered as 1-min infusions, permitting the sequence of doses with an interval of 15 min. Infusion was performed into the perfusion tube close to the aortic cannula with the use of an infusion device. Outflow samples were collected every 5 s for 2 min and every 30 s for the next 5 min (total collection period, 7 min) and the cardiac response was measured. After an equilibration period of 10 min, these experiments were repeated in the presence of KBR (0.1 μM) in perfusate (starting 15 min after perfusion with KBR-containing buffer). The outflow samples were kept frozen at −20°C until analysis. For determination of [3H]digoxin, the outflow sample (200 μl) was transferred to a vial and 2 ml of cocktail was added. After being vigorously mixed, the radioactivity was measured with a liquid scintillation counter (Perkin Elmer Instruments; Shelton, CT).

Mathematical Model

Myocardial uptake and binding processes. The pharmacokinetic-pharmacodynamic modeling methodology has been described in detail previously (21). Here, this comprehensive approach to analyze myocardial uptake and receptor binding of digoxin has been extended by including a more general model that links Na+/K+-ATPase to inotropic response (Fig. 1). The corresponding differential equations describing changes in the amounts of digoxin in the mixing, capillary, and interstitial compartment as well as in the two compartments representing the two saturable binding sites after infusion of digoxin at the inflow side of the heart (perfused at flow Q in single-pass mode) are presented in the Appendix. As shown in the compartmental model (see Fig. 1), Q and Qout occur in the vascular space (distribution volume; Vvas), transcapsular transport of the unbound drug between vascular and interstitial space is described by rate constants kvi and kvs, respectively, and the apparent permeability surface area or permeation clearance CLvi = kviVvas (net clearance of digoxin from the vasculature) is determined by kvi and Vvas. Assuming passive transport processes, we have kviVvas = kviVapp,vas, where Vapp,vas denotes the apparent volume that governs initial distribution of digoxin in the interstitial space; i.e., exceeding the distribution space Vvas is due to quasi-instantaneous nonspecific tissue binding. Vapp,vas is given by Vapp,vas = Vvas (1 + ksvs), where the equilibrium partition coefficient Keq = koff/kon characterizes nonspecific tissue binding (46).

Kinetics of receptor binding and cellular effectuation. Assuming a reversible interaction between digoxin and two receptor classes R1 and R2 on the Na"" pump ([3H]digoxin binding sites)

\[
d_{1,2} + R \overset{k_{-1}}{\underset{k_1}{\rightleftharpoons}} DR_i, \quad i = 1, 2
\]

the binding probability of digoxin in the interstitial space (unbound amount D_{out,1}) to two saturable binding sites (i = 1, 2) is dependent on the association rate constants k1 and free membrane receptors, which is equal to (R_{tot,1} - DR_i), where R_{tot,1} is the unknown amount of available receptor sites and DR_i denotes the digoxin-receptor complexes (i.e., amount of bound digoxin), i.e., the resulting fractional binding rate K_i = k_i [R_{tot,1} - DR_i] is time dependent (Eqs. A4 and A5). The rate constants for the dissociation of the bound ligand were denoted by k_{-1} (in units of 1/min); k_{off} = k_{-1}/k_1 and K_{off} = 1/K_{eq}, represent the equilibrium dissociation and affinity constants, respectively, of the two receptor systems. Note that due to quasi-instantaneous nonspecific tissue binding the free concentration in the interstitial space is reduced and given by C_{0,i}(t) = D_{1,2}/Vapp,vas instead of D_{1,2}/Vvas (46); because free digoxin concentration governs receptor

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[Fig. 1. Comprehensive kinetic model of cardiac kinetics and inotropic response of digoxin. First-order rate constants of transcapsular transport are denoted by the vascular rate constant (kvi) and interstitial vascular rate constant (kvs). The fractional rate for saturable receptor binding K(t) = k_i [R_{tot,1} - DR_i(t)] is governed by the unbound interstitial digoxin concentration C_{i}(t) = D_{is}(t)/Vapp,vas, where R_{tot,1} is the total interstitial receptor function, DR_i is a function of digoxin receptor complex occupations: D_{is} is interstitial digoxin, and Vapp,vas is the apparent volume that governs the initial distribution of digoxin. The association and dissociation constants are denoted by k_i and k_{-1}, respectively. The chain of postreceptor events determines the effect as a function of DR. The index i = 1, 2 denotes the receptor population α_i.]

[Diagram showing the interplay between the vascular and interstitial compartments, with arrows indicating flow and rate constants.]
binding \((Eqs. \ A2\text{-}A5)\), \(k_i\) is defined in terms of \(C_u\) in units of \(\text{min}^{-1}\text{nmol}^{-1}\text{ml}^{-1}\).

The binding of digoxin to receptors (\(Na^+\) pumps) initiates a series of dynamic events that ultimately lead to an increase in the force of contraction. Because a mechanistic model of these postreceptor events as indicated in Fig. 1, showing a higher \(Ca^{2+}\) availability due to activation of \(Ca^{2+}\) influx via NCX after small increase in intracellular [\(Na^+\\)] (\((Na^+)\)), would be necessarily overparameterized, a minimal transduction model, i.e., an ad hoc equation, was selected to mimic the observed behavior (22, 28). Considering initiation of physiological activity on binding to the heterogeneous receptor system, the pharmacological response of the terminology “effect” \(E(t)\) is a function of the number of receptors occupied by drug, i.e., ligand-receptor complexes, \(DR_1(t)\) and \(DR_2(t)\). For the heterogeneous receptor system, the pharmacological response \(E(t)\) induced by the mixture of occupied receptors is a function of the weighted sum of both isoforms

\[
DR(t) = f_1DR_1(t) + (1 - f_1)DR_2(t)
\]

where \(f_1\) is the fraction of \(R_1\)-occupancy contribution. Thus \(DR_1(t)\) is the total functional receptor occupation (stimulus) leading to a response \(E(t) = \Psi[DR(t)]\), where the function \(\Psi\) refers to the cascade of cellular processes, which convert the stimulus into response (22). With the use of a Michaelis-Menten-like hyperbolic function (22) and a delayed response (28)

\[
E(t) = \frac{\phi_{\text{max}}DR(t)}{K_{DR} + DR(t)} * \left(1 - e^{-\tau}\right)
\]

the stimulus-effect relationship is characterized by parameters maximum possible effect (\(\phi_{\text{max}}\)) and \(K_{DR}\) \((DR_2\) producing 50% of \(\phi_{\text{max}}\), and \(\tau\) denotes the convolution operation, which accounts for the transduction delay with time constant \(\tau\) \((Eq. \ A6)\).

The effectuation model greatly simplifies when pump inhibition (rate of receptor occupation) is the rate-limiting step [the effect is in phase with \(DR_2(t)\)] and no saturation occurs, i.e., the effect is proportional to \(DR_1(t)\) and \(Eq. \ 3\) collapses to a linear combination of receptor occupations \(DR_1(t)\) and \(DR_2(t)\)

\[
E(t) = e_T[f_1DR_1(t) + (1 - f_1)DR_2(t)]
\]

where \(e_T\) denotes the total transduction efficiency (or stimulus amplification) with contributions of \(f_1\) and \(f_2 = 1 - f_1\) of \(R_1\) and \(R_2\) receptors, respectively.

Note that in this study, the low-affinity/high-capacity and high-affinity/low-capacity \([\text{H}]\) digoxin binding sites, \(R_1\) and \(R_2\), are assumed to be identical with the \(\alpha_1-\) and \(\alpha_2\)-subunit isoforms of the \(Na^+\text{-K}^+\text{-ATPase, respectively} (26, 29, 42)\.

Data Analysis

To limit the number of parameters to be adjusted, only model structures reasonably consistent with the physiological knowledge of digoxin uptake and action were investigated. However, as already pointed out (21), for our nonlinear model (Fig. 1) we are still faced with the question of its identifiability. Because the information content of the outflow data is inadequate to support such a model with a relative rich parameterization, we take advantage of the fact that a priori information on some of the unknown model parameters is available in the literature. The Bayesian approach to model identification [e.g., maximum a posteriori (MAP) estimation] is a theoretically sound method to incorporate such knowledge in probabilistic terms. The system of differential equations was solved numerically, and MAP Bayesian parameter estimation was performed with the ADAPT II software (6). For all pharmacokinetic and pharmacodynamic fits, an additive plus proportional intraand individual error model was used.

The digoxin outflow concentration data, \(C_{out}(t)\), were first analyzed to obtain estimates of the transport and binding parameters. Those values were then fixed in fitting the change in LVDP over time \([\Delta LVDP(t)]\) data obtained from the same experiments. The latter was used as a measure of inotropic response, i.e., the increase in LVDP, with respect to the baseline (predrug) value \(LVDP_0\).

\[
E(t) = \frac{LVDP(t) - LVDP_0}{LVDP_0}
\]

\(E(t)\) will be used throughout to describe the fractional change in LVDP. As noted above, in Bayesian estimation we made use of a priori knowledge on the ratios of the receptor affinities (\(K_{DR_2}/K_{DR_1}\)) and capacities (\(R_{out,1}/R_{out,2}\)). Because data obtained for digoxin with the use of rat ventricle microsomal preparations (31) alone may not provide reliable information (27), we used more recent binding data (43), and, in addition, receptor affinities estimated in rat cardiac myocytes by measuring the \(Na^+\) pump current (19). Thus we based our analysis on a priori values of the affinity and capacity ratios, \(K_{DR_2}/K_{DR_1} = 45\) and \(R_{out,1}/R_{out,2} = 3\); respectively. We selected a fractional standard deviation of 20% to ensure that the estimates will be both data and a priori knowledge driven. The volume of the vascular compartment was fixed for a literature value (\(V_{\text{vas}} = 0.06\ ml/g\) taken from anatomic data (7). Thus the primary parameters estimated directly were the pharmacokinetic parameters, \(CL_{\text{rev}}, V_{\text{app}}, R_{out,1}, R_{out,2}, k_1, k_{-1}, k_2, k_{-2}\), and the pharmacodynamic parameters \(e_T, f_1, \phi_{\text{max}}, K_{DR}\) under control condition or in the presence of KBR, respectively. One set of parameter starting values was used for all of the data sets. Identifiability was verified by showing convergence to the same solution with alternative sets of parameter starting values. Any model showing a noninvertible Fisher’s information matrix was discarded as nonidentifiable. The assessment of numerical identifiability was guided by the asymptotic fractional standard deviations provided by the fitting procedure, which represent the uncertainty in parameter estimates resulting from the fit and correlation coefficients.

Model selection in fitting the \(C_{out}(t)\) and \(E(t)\) data was based on the generalized information criterion for MAP estimation and the Akaike information criterion, respectively (6).

Descriptive data are expressed as means \pm SD. To get a quantitative estimate of the positive inotropic effect that is independent of the model, we calculated the time integral (>7 min) of the developed effect \([\int_0^T E(t)\,dt]\) using a trapezoidal rule. The responses in the two groups were compared using two-way ANOVA with repeated measures performed on the three-dose levels of 0.5 and 1.5 mM \(Ca^{2+}\), followed by a Student-Newman-Keuls post hoc test for multiple comparisons. Differences in parameter estimates between the two \(Ca^{2+}\) groups were assessed by Student’s t-test. For all analyses, a two-tailed \(P\) value of <0.05 was used to indicate statistical significance (SigmaStat, Jandel; San Rafael, CA).

Model Simulations

To visualize the modeling results, \(Eqs. \ A1\text{-}A5\) were used to simulate the time course of \(\alpha_1-\) and \(\alpha_2\)-isoform contributions to inotropic response \(E(t)\); i.e., the contribution of \(\alpha_1 = f_1\cdot DR_1(t)/[\phi_{\text{max}} (DR_1(t) + (1 - f_1) DR_2(t))]\). Phase portraits \([E(t) vs. DR_1(t)]\) were generated using \(Eqs. \ 3\) or \(4\), and the relationship between [\(Na^+\)] and receptor occupancy was calculated as described below \((Eqs. \ 6\) and \(7)\) to explain the relationship between receptor occupation and response. All simulations were carried out with average estimates obtained in each group with the use of MAPLE 8 software (Maplesoft).

RESULTS

Measurements of \(C_{out}\) and Cardiac Performance

Figure 2 shows a typical recording of the LVP response to three consecutive digoxin doses (15, 30, and 45 \(\mu\)g) measured in hearts with [\(Ca^{2+}\)] of 0.5 and 1.5 mM, respectively, in perfusate under control conditions and in the presence of KBR.
Fig. 2. Original recording of the left ventricular pressure after 3 consecutive digoxin doses (15, 30, and 45 μg) under control conditions and in the presence of KB-R7943 (KBR) for experiments with external Ca^{2+} concentrations ([Ca^{2+}]_o) of 0.5 and 1.5 mM. NCX, Na^{+}/Ca^{2+} exchanger.

Fig. 3. Average digoxin outflow concentration (A and B, top) and percent increase in left ventricular developed pressure (LVDP) (A and B, bottom) for 3 digoxin doses of 15 (●), 30 (○), and 45 μg (▲) under control conditions and in the presence of 0.1 μM KBR (open symbols) as observed in experiments with 0.5 and 1.5 mM [Ca^{2+}]_o, respectively.

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The average $C_{\text{out}}(t)$ and inotropic response data ($n = 5$ in each group) are depicted in Fig. 3. The percent increase in LVDP caused by digoxin was significantly enhanced in the 0.5 versus 1.5 mM $[\text{Ca}^{2+}]_o$ group ($P < 0.05$). At digoxin doses of 30 and 45 μg, the presence of KBR (0.1 μM) in perfusate induced a significant decrease in pressure developed (Fig. 3) and its time integral $\int_0^T E(t)dt$ (Fig. 4). An increase in $[\text{Ca}^{2+}]_o$ from 0.5 to 1.5 mM led to an approximately threefold increase ($P < 0.001$) in baseline contractility (LVDP$_0$) and a small decrease ($P < 0.05$) in coronary vascular resistance (CVR). No significant changes in LVEDP were observed, and KBR in perfusate did not affect baseline function of the heart (Table 1). The 1-min infusions of digoxin did not affect CVR and LVEDP.

Estimated Parameters

Representative simultaneous fits of the model to data obtained for three consecutive digoxin doses (15, 30, and 45 μg) and $[\text{Ca}^{2+}]_o$ of 0.5 and 1.5 mM are depicted in Fig. 5. In all control experiments (i.e., without NCX inhibition), the simplest effectuation model (Eq. 4) that assumes additivity of responses mediated by both isoforms (12, 42) was sufficient to describe the data (21). The model was conditionally identifiable, and parameters were estimated with reasonable precision. The averaged model parameters and estimation errors, as a percentage of related parameter estimates, are listed in Table 2. Notably, the increase in digitalis sensitivity with decreasing $[\text{Ca}^{2+}]_o$ is due to a significantly increased efficiency of response generation (the slope of the occupancy-response relationship, $e_T$, increases from 17.7 to 25.6% $\Delta$LVDP/nmol; $P < 0.05$), and this effect is mediated by both isoforms. The fractional contributions $f_1$ and $f_2 (= 1 - f_1)$ of $\alpha_1$- and $\alpha_2$-isoforms, respectively, to stimulus amplification $e_T$ were not significantly different. However, $f_1$ decreased (and $f_2$ increased) significantly by 20% when $[\text{Ca}^{2+}]_o$ was reduced from 1.5 and to 0.5 mM ($P < 0.01$). Furthermore, a decrease in $[\text{Ca}^{2+}]_o$ from 0.5 to 1.5 mM was accompanied by a significant ($P < 0.01$) increase in apparent interstitial distribution volume of digoxin, $V_{\text{app,iso}}$, in the control group, corresponding to an increase in equilibrium partition coefficient $K_{\text{iso}}$ from 3.0 ± 0.6 to 5.2 ± 1.2 [for a volume of the interstitial space of $V_i = 0.15$ ml/g (7)].

The simple transduction model used under control conditions (Eq. 4) failed to describe the effect-time data in the presence of the NCX inhibitor KBR. A reasonable fit was obtained with the nonlinear stimulus-response model (Eq. 3) indicating a decrease in digoxin effect for receptor occupancies $D_{\text{T}}$ above $\approx 0.5 \pm 1 \mu$g (Fig. 6). Because the contribution to total amplification mediated by the $\alpha_1$-receptor (parameter $f_1$) could not be estimated with sufficient reliability in the presence of KBR, this parameter was fixed to the estimate obtained under control conditions. Although this model well captured the major features of the data, it failed to fit the peak of the curve in the case of the 0.5 mM $[\text{Ca}^{2+}]_o$ experiments (Fig. 5). The decrease in $[\text{Ca}^{2+}]_o$ led also under KBR to higher sensitivity to inotropic stimulation here characterized by significant (2.6-fold) increase of $\phi_{\text{max}}$ ($P < 0.001$). In addition, the delay in response generation was significantly increased (Table 2). Although there was some tendency for a higher reduction of stimulus amplification by KBR at 1.5 mM versus 0.5 mM $[\text{Ca}^{2+}]_o$ (Fig. 6 and $\phi_{\text{max,eT}}$ in Table 2), this difference was not statistically significant.

Simulated Response Characteristics

The model analysis indicates that positive inotropic digoxin effect is predominantly mediated by the $\alpha_2$-pumps. This is shown in Fig. 7, where the contributions of both $\alpha$-isoforms to inotropic response for 1.5 mM $[\text{Ca}^{2+}]_o$, were calculated on the basis of the mean parameter estimates. In the transient case of our 1-min infusion as well as in the simulation for a 10-min infusion, the inhibition of the $\alpha_2$-isofrom by digoxin is clearly dominating (>80% of the effect is $\alpha_2$-mediated). An increase in $\alpha_2$-isofrom contribution with decreasing digoxin dose is only suggested by the simulated response to the 10-min infusion where the response at 5 min corresponds to the steady-state situation.

The occupancy-response relationships predicted from the mean parameter estimates under control conditions (Eq. 4) and in the presence of KBR (Eq. 3) visualizes the effect of reverse NCX inhibition on digoxin response generation (Fig. 6). Because of the saturation characteristics (governed by parameters $K_{\text{DB}}$ and $\phi_{\text{max}}$), KBR reduced the efficiency of the cellular transduction process with increasing receptor occupancy. In accordance with the results shown in Fig. 4, this reduction in inotropy disappears (or becomes very small) for $D_{\text{T}} < 0.5 \pm 1 \mu$g. Note that according to Fig. 6, the 15-μg dose led to maximum receptor occupancies, $D_{\text{T}}$, of ~1 and 0.7 μg for 0.5 and 1.5 mM $[\text{Ca}^{2+}]_o$, respectively.
To explain the linear relationship between receptor occupancy and response (Eq. 4) in terms of the underlying effectuation process, we use the equation for the Na\(^+\) pump-mediated Na\(^+\) efflux rate (\(V_{Na}\)) (44)

\[
V_{Na} = \frac{V_{Na,\text{max}}[\text{Na}^+]}{[\text{Na}^+] + K_{Na,\text{tot},1}} + \frac{V_{Na,\text{max}}[\text{Na}^+]}{[\text{Na}^+] + K_{Na,\text{tot},2}}
\]

(6)

where \(K_{Na,\text{tot},1}\) (\(K_{Na,\text{tot},2}\)) denotes the apparent affinities for \([\text{Na}^+]\) of the \(\alpha_1\)- (\(\alpha_2\))-isoform. For a certain pump activity, one can calculate the increase in \([\text{Na}^+]\), with increasing pump inhibition (receptor occupation \(DR\)) by solving Eq. 6 for \([\text{Na}^+]\). Whereas no direct information is available on the relationship between \([\text{Ca}^{2+}]_o\) and Na\(^+\) pump activity, Simor et al. (40) have evaluated the dependence of \([\text{Na}^+]\), from \([\text{Ca}^{2+}]_o\) in isolated perfused rat hearts using nuclear magnetic resonance and could describe it by

\[
[\text{Na}^+] = 11.33 \exp(-[\text{Ca}^{2+}]_o/3.65)
\]

(7)

Thus for a decrease of \([\text{Ca}^{2+}]_o\) from 1.5 to 0.5 mM an increase of \([\text{Na}^+]\), from 7.5 to 9.9 mM is predicted. Substituting these values into Eq. 6 (using \(K_{Na,\text{tot},1} = 12.4\) mM, \(K_{Na,\text{tot},2} = 22\) mM, and \(n = 2.5\) (44)), we finally obtain pump activities of 28 and 48% of the maximum activity for \([\text{Ca}^{2+}]_o\) of 0.5 and 1.5 mM, respectively. As mentioned above, these values were used to simulate the \([\text{Na}^+]\)-\(DR\) relationships shown in Fig. 8. The \([\text{Na}^+]\), increases with increasing pump inhibition (\(\alpha_2\)-receptor occupancy \(DR\)), and the slope of the curve decreases with increasing \([\text{Ca}^{2+}]_o\). For the sake of simplicity, the influence of \(\alpha_1\)-receptor occupation has been neglected in this simulation study.

**DISCUSSION**

Evaluation of the kinetics of digoxin disposition and action in the isolated perfused heart preparation is a useful method to examine the respective roles of myocardial uptake, drug-receptor interaction, and cellular effectuation process in the whole organ with the use of dynamic systems analysis. The results reported suggest the following: 1) the digoxin-induced positive inotropic effect is mainly (>80%) mediated through the \(\alpha_2\)-isoform of the Na\(^+-\)K\(^+-\)ATPase; 2) a decrease in \([\text{Ca}^{2+}]_o\) from 1.5 to 0.5 mM increases the stimulus amplification (slope of the receptor occupancy-response curve) probably due to a steeper relation between \([\text{Na}^+]\), and contractility; and 3) KBR decreases the digoxin action with increasing receptor occupancy to a limiting maximum value indicating an inhibition of Ca\(^{2+}\) influx via NCX.

**Functional Receptor Heterogeneity**

In our previous study, the model of digoxin kinetics and inotropic response was validated using data obtained with 2.5 mM \([\text{Ca}^{2+}]_o\) in perfusate (21). The results obtained here regarding the correlation of physiological response to \(\alpha_1\)- and \(\alpha_2\)-isoform inhibition are in general agreement with these findings if one considers that more refined a priori information on \(K_{A,2}/K_{A,1}\) and \(R_{tot,1}/R_{tot,2}\) ratios were used in the present study. Thus the involvement of the two different
DIOXIN KINETICS AND INOTROPIC RESPONSE

Table 2. Parameters estimated by simultaneous fitting of outflow and inotropic response data after 1-min infusions of 15, 30, and 45 μg digoxin in isolated rat hearts with [Ca2+]o.

<table>
<thead>
<tr>
<th></th>
<th>[Ca2+]o (0.5 mM)</th>
<th>[Ca2+]o (1.5 mM)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>KB-R7943</td>
</tr>
<tr>
<td>Cardiac uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLmax, ml/min</td>
<td>7.959±1.84 (24±9)</td>
<td>9.321±1.61 (27±6)</td>
</tr>
<tr>
<td>Vapp, ml/g</td>
<td>0.930±0.19 (19±10)</td>
<td>0.741±0.19 (21±11)</td>
</tr>
<tr>
<td>Receptor binding</td>
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<tr>
<td>Rtot, nmol/g</td>
<td>30.87±7.46 (41±15)</td>
<td>28.03±8.33 (32±21)</td>
</tr>
<tr>
<td>k1, min -1nmol -1ml</td>
<td>0.021±0.01 (54±33)</td>
<td>0.020±0.01 (49±12)</td>
</tr>
<tr>
<td>k−1, 1/min</td>
<td>2.957±0.98 (83±13)</td>
<td>2.470±1.19 (77±26)</td>
</tr>
<tr>
<td>Rtot, nmol/g</td>
<td>9.857±3.59 (40±14)</td>
<td>9.407±2.57 (31±21)</td>
</tr>
<tr>
<td>k2, min -1nmol -1ml</td>
<td>0.215±0.06 (60±29)</td>
<td>0.310±0.17 (46±25)</td>
</tr>
<tr>
<td>KDR, 1/min</td>
<td>0.674±0.05 (9±2)</td>
<td>0.770±0.10 (9±2)</td>
</tr>
<tr>
<td>Kd, nmol/ml</td>
<td>154.8±61.4</td>
<td>135.2±60.6</td>
</tr>
<tr>
<td>Kd, nmol/ml</td>
<td>3.439±1.36</td>
<td>3.011±1.35</td>
</tr>
<tr>
<td>Kd/Kd2, 1/min</td>
<td>45.04±0.68 (2±0.1)</td>
<td>44.87±0.07 (2±0.1)</td>
</tr>
<tr>
<td>Rtot/Rtot, 1/min</td>
<td>3.244±0.42 (11±0.7)</td>
<td>2.962±0.25 (10±0.4)</td>
</tr>
<tr>
<td>Cellular effecuation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e2, %/nmol</td>
<td>25.59±5.11 (33±27)</td>
<td>17.66±6.9* (59±20)</td>
</tr>
<tr>
<td>f1</td>
<td>0.380±0.04 (29±25)</td>
<td>26.11±5.50 (8±2)</td>
</tr>
<tr>
<td>Kmax, %</td>
<td>0.256±0.09 (18±2)</td>
<td>0.997±0.19</td>
</tr>
<tr>
<td>Kmax, %</td>
<td>0.256±0.09 (18±2)</td>
<td>0.997±0.19</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 hearts per group. CLmax, permeability clearance vascular to interstitial space; Vapp, apparent volume governing interstitial digoxin distribution; Rtot(i), i = 1, 2, amounts of saturable digoxin binding sites (receptors); k1 and k−1, association and dissociation rate constants of receptor i (i = 1, 2); Kd, equilibrium dissociation constants; Kd/Kd2, ratio of receptor affinity constants; Rtot(Rtot, 1)/Rtot, ratio of receptor capacities; e2, slope of the receptor occupancy-response curve; Kmax and KDR, maximal response and receptor occupancy producing 50% of Kmax of the hyperbolic occupancy-response relationship in the presence of KB-R7943; τ, delay time constant. Values in parentheses are the asymptotic coefficients of variation of parameter estimates (mean ± SD) obtained from individual fits. *P < 0.05, †P < 0.01, and ‡P < 0.001 vs. corresponding value in 0.5 mM [Ca2+]o, group. Changes in response to KB-R7943 in each group did not achieve statistical significance.

Na+ pump isofoms, with different kinetic properties, a low-affinity/high-capacity binding site (R1) and a high-affinity/low-capacity binding site (R2) was confirmed (~70% α1 and 30% α2; Table 2). Our observation that the inotropic response to digoxin is >80% mediated by the α2-isoform (Fig. 7) is in accordance with recent results obtained for ouabain in the mouse heart (9) and the general view on the functional role of different isofoms (42). That according to visual examination and a significant change in the Akaike information criterion value, a model without contribution of α1-receptors provided a slightly worse fit to the E(t) data is consistent with the involvement of both isofoms (37). However, a small contribution of α1-isoform was also not excluded by Dostanic et al. (9). Note that the simulation of a steady-state situation (response to a 10-min infusion) gave nearly the same result, except that α2-isoform contribution further increased (to ~90%) for the 15-μg dose (Fig. 7). That similarly the α2-isoform contribution also slightly increased when [Ca2+]o was decreased from 1.5 to 0.5 mM (data not shown) can be explained by the increase in α2-isoform efficacy (f2 = 1 − f1). Thus, although the hypothesis of a functional heterogeneity of the Na+-K+-

Fig. 6. Model simulations of the relationship between total receptor occupancy [DR(t)] and inotropic response (phase-plane plot) under control conditions and after NCX inhibition by KBR (0.1 μM) for the 45- and 15-μg doses of digoxin computed with average parameters estimated for 0.5 and 1.5 mM [Ca2+]o, respectively (Table 2). The dashed lines indicate maximum receptor occupancy for the 15-μg dose and arrows the temporal evolution of response.
ATPase (37) cannot be disproved, the quantitative importance of a $\alpha_1$-isoform contribution appears further open to debate.

Receptor Occupancy-Response Relationship

The linear model of the receptor occupancy-response relationship (Eq. 4) used in this study to describe postreceptor events is in principle accordance with the simulated quasi-linear $[\text{Na}^+]_i - DR_2$ relationship (Fig. 8) and the proportionality of digitalis-induced inotropic response and increase in $[\text{Na}^+]_i$ observed in rat (16) and cat (45) ventricular myocytes. This indicates that under control conditions, the process of receptor binding appears to be the rate-limiting step in response generation (i.e., the effect was in phase with receptor occupancy).

The nearly equal fractional contributions $f_1$ and $f_2 (= 1 - f_1)$ of $\alpha_1$- and $\alpha_2$-isoforms, respectively, to stimulus amplification $e_T$ (slope of the relationship between total receptor occupancy $DR_T$ and effect) is consistent with the predicted effect of receptor occupation on the increase in $[\text{Na}^+]_i$ (Eq. 6). It is currently under debate whether the $\text{Na}^+/\text{H}^+$ pump $\alpha_2$-isoforms have specific functional roles (1, 9, 20, 44). Because only for the low $[\text{Ca}^{2+}]_o$ level a slightly higher stimulus amplification was found for the $\text{Na}^+/\text{H}^+$ pump $\alpha_2$-isoform, our findings can hardly be explained by a preferentially $\alpha_2$-mediated response due to a possible colocalization with the NCX in the sarcolemma at sites of restricted diffusion (3, 14, 20). The dominating role of the $\alpha_2$-isoform under our experimental conditions (Fig. 7) was solely due to its higher affinity.

Effect of $[\text{Ca}^{2+}]_o$

In all groups, an increase in $[\text{Ca}^{2+}]_o$ from 0.5 to 1.5 mM resulted in an increase in baseline contractility; the 2.7-fold increase in LVDPo is comparable with the value observed by Gaszner et al. (13) and in accordance with the positive correlation between inotropy and $[\text{Ca}^{2+}]_o$ in rats (11). Note that the $[\text{Ca}^{2+}]_o$ of 1.5 mM corresponds to the physiological level of un-ionized $\text{Ca}^{2+}$ in rats (5).

To our knowledge, there are no reports that give a quantitative mechanistic explanation of the increasing sensitivity to digitalis-induced cardiac inotropy with decreasing $[\text{Ca}^{2+}]_o$ observed in rat (17) and mouse (37) hearts. Our model analysis suggests that a change in $[\text{Ca}^{2+}]_o$ affects solely the cellular effectuation process. The observed 50% increase in the slope of the occupancy-response relationship (i.e., stimulus amplification) after decreasing $[\text{Ca}^{2+}]_o$ from 1.5 to 0.5 mM (Fig. 6) could be attributed to the increase in the slope of the $[\text{Na}^+]_i$-occupancy relationship predicted by Eqs. 6 and 7 (Fig. 8). Because of the uncertainty in the underlying $K_{Na,x}$ values (1) and the assumption of an unchanged $\Delta[\text{Na}^+]_i$-inotropic response relation, this simulation is only a very rough approximation to illustrate the role of $[\text{Ca}^{2+}]_o$. Nevertheless, the results are qualitatively consistent with our experimental data. This finding obtained here by a model analysis of the transient inotropic response to a 1-min infusion of digoxin is in accordance with the biphasic dose-response curves of ouabain mea-

Fig. 7. Model-predicted percent contribution of low-affinity/high-capacity ($\alpha_1$) and high-affinity/low-capacity ($\alpha_2$) receptors to inotropic response after a 1-min infusion (left) and a 10-min infusion (right) of 45 $\mu$g (solid line) and 15 $\mu$g (dotted line) digoxin, computed with average parameters estimated for 1.5 mM $[\text{Ca}^{2+}]_o$ (Table 2).

Fig. 8. Influence of $[\text{Ca}^{2+}]_o$ on the relationship between increase $[\text{Na}^+]_i$, and $\text{Na}^+$ pump inhibition ($\alpha_2$-receptor occupation, $DR_2(t)$), as predicted by Eqs. 6 and 7.
sured for low [Ca\(^{2+}\)]\(_{o}\) in mouse (37) and rat heart (17). A further reduction in stimulation amplification \(e_T\) to a value of \(\sim 10\%\) of the value estimated at 1.5 mM [Ca\(^{2+}\)]\(_{o}\) was found in experiments with 2.5 mM [Ca\(^{2+}\)]\(_{o}\), where the digoxin doses were fivefold higher (21).

Taken together, the increasing sensitivity to inotropic stimulation with decreasing [Ca\(^{2+}\)]\(_{o}\) can be clearly attributed to the cellular effectuation process (parameter \(e_T\)) because no influence of [Ca\(^{2+}\)]\(_{o}\) on receptor binding (\(K_{D,\text{R}}\)) was observed. It should be noted that the terminology of “sensitivity to inotropic stimulation” adopted in this study differs from “cardiac glycoside sensitivity”, which usually refers to receptor affinity alone (e.g., Refs. 1, 26, 30).

In accordance with our previous results (21), the transcapillary exchange clearance of digoxin (\(CL_{vi}, = 6.6 \pm 1.2\) ml\(\cdot\)min\(^{-1}\)\(\cdot\)g\(^{-1}\)) at [Ca\(^{2+}\)]\(_{o}\) = 1.5 mM is consistent with the value of 5.1 \(\pm\) 1.4 ml\(\cdot\)min\(^{-1}\)\(\cdot\)g\(^{-1}\) measured for sucrose in rats (4) indicating barrier-limited uptake by passive transport through interendothelial gaps. Because a reduction in [Ca\(^{2+}\)]\(_{o}\) was suggested to increase endothelial permeability and the vascular surface area available for albumin exchange (8), the 34% increase in the apparent interstitial distribution volume \(V_{\text{app},i}\) of digoxin observed for the decrease of [Ca\(^{2+}\)]\(_{o}\) from 1.5 to 0.5 mM could be attributed to fluid and albumin accumulation in the interstitium. The slight but significant decrease in basal CVR with increasing [Ca\(^{2+}\)]\(_{o}\) (Table 1) may reflect an autoregulatory response to the increased heart work and \(O_2\) consumption.

**Effect of NCX Inhibition by KBR**

The NCX inhibitor KBR (0.1 \(\mu\)M) markedly attenuated the rise in LVPD induced by digoxin doses of 30 and 45 \(\mu\)g (Figs. 3 and 4) but did not influence cardiac performance at basal conditions and the lowest digoxin dose (15 \(\mu\)g). That coadministration of KBR only influences the chain of postreceptor events (it did not affect receptor binding) is in accordance with the well-established central role of NCX in inotropic response generation. In terms of our empirical model, this has two consequences. First, the apparent linear effectuation process (Eq. 4) becomes saturated (Eq. 3), resulting in a decrease in the effect per unit digoxin-receptor occupancy, as shown by the simulated phase portrait of the time course of stimulus-response curves (Fig. 6). The decreasing deviation of the hyperbolic from the linear curves with decreasing total receptor occupancy explains the lack of KBR effect at the lowest dose level of 15 \(\mu\)g (Fig. 4), which corresponds to a receptor occupancy of \(\approx 1\%\). Second, NCX inhibition by KBR leads to a response \(E(t)\) that lags behind the time course of occupancy, \(D_{R}(t)\), as reflected by the counterclockwise hysteresis loop after NCX inhibition (Fig. 6). Thus it is reasonable to assume that the generation of the response becomes the rate-limiting step. Together with the lack of effect of KBR on basal contractility, these findings suggest that the concentration of KBR used in the present experiments in the perfused rat heart (0.1 \(\mu\)M) was able to selectively block the Ca\(^{2+}\) influx mode of the NCX. In this case, the lack of an inhibitory KBR effect on digoxin-induced inotropy for low receptor occupancy (\(< D_{R} \approx 0.5 \, \mu\text{g}\); Fig. 6; or doses \(< 15 \, \mu\text{g}\); Fig. 4) may be explained by the fact that in this situation (small increase in [Na\(^{+}\)]) no net Ca\(^{2+}\) influx via NCX is necessary to increase contractility (36). The inotropic response to increasing digoxin doses would then be effectively limited under KBR to the contribution that is independent of net Ca\(^{2+}\) influx via NCX. The [Ca\(^{2+}\)]\(_{o}\) dependency of \(\phi_{\text{max}}\) can be due to the different slopes of the [Na\(^{+}\)]\(_{i}\)-\(D_{R}\) curves (Fig. 8) (analogous to the [Ca\(^{2+}\)]\(_{o}\) dependency of \(e_T\)). This is consistent with previous results showing that KBR preferentially blocks the Ca\(^{2+}\) influx (reverse) mode of the cardiac NCX rather than the extrusion (forward) mode (2, 10, 25, 39, 45) and that KBR inhibits Ca\(^{2+}\) influx through NCX under Na\(^{+}\)-loaded conditions in rat myocardium (18, 38, 41, 49). Furthermore, it has been shown that Ca\(^{2+}\) influx (via reversal of NCX) is essential for digitalis-induced potentiation of cardiac basal energy expenditure (15). Note also that the selective inhibition of the reverse mode of NCX is especially pronounced at low concentrations of KBR (2, 39, 49) and characterized by an IC\(_{50}\) of 0.15 \(\mu\)M in isolated cardiomyocytes (18). Thus our findings suggest that the differences in the inhibitory effect of KBR among investigators (35) can be explained, in part, by the differences in digitalis dose (apart from the rat strain (49) and the experimental condition (38)).

**Model Validity**

The results of the present and previous experiments (21) suggest that intracellular uptake of digoxin was negligible; i.e., because our model analysis did not account for a contribution of cellular uptake, digoxin was apparently not able to cross the sarcolemma in significant amounts within the 1-min infusion time. Although our result does not exclude that cardiac glycosides may (additionally) act at other intracellular sites of action than the Na\(^{+}\)-pump (33, 34), it was not necessary to postulate such a mode of action to explain the inotropic effect of digoxin in our experiments. This is in accordance with the evidence that the ouabain-induced increase in contractility in mice is solely mediated by the Na\(^{+}\)–K\(^{+}\)-ATPase (9). This theory also holds for a possible activation of signal pathways that may contribute to the effect development at longer timescales (48). Consequently, however, our modeling results obtained in this study for the inotropic response after a 1-min infusion of digoxin may not apply to long-term infusion or a steady-state situation. Note that the validity of a model is always defined in terms of the modeling objectives, which were in our case the explanation of the short-term “quasi-instantaneous” response to a single dose of digoxin.

On the other hand, our kinetic analysis yielded information on digoxin receptor interaction that cannot be obtained by steady-state experiments. The major advantage of this approach is that the study of the interplay between binding kinetics to a heterogeneous receptor population and effectuation processes at the cellular level is possible in the intact heart. Thus the present results underline the heuristic potential of kinetic modeling for providing quantitative insight into the mechanisms underlying system response (23). However, there are several limitations to this approach. Like any model, our model is a great simplification of reality and the results are dependent on model selection and the quality of parameter estimation. Reliability of parameter estimation was improved by using data obtained at three dose levels. Although we have incorporated only those steps in our model known to be essential for modeling of digoxin kinetics and action in the rat.
V_{app,ss} denotes the unbound digoxin concentration in the interstitial space.

While under control conditions $E(t)$ is a linear function of $DR(t)$ and $DR(t)$ (Eq. 4), Eq. 3 holds in the presence of KBR, i.e.,

$$dE(t)/dt = \left[ \frac{\phi_{\text{baso}} DR(t)}{R_{\text{baso}} + DR(t)} - E(t) \right]$$  \hspace{1cm} (A6)

where $DR$ is given by Eq. 2.

Because the short delay $t_0$ and mixing volume $V_0$ needed to fit the initial appearance of outflow concentration (due to the perfusion system and large vessels) had little influence on the estimation of the other parameters, they were set to fixed values, $t_0 = 0.036$ min and $V_0 = 0.30$ ml in the final Bayesian model identification.

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