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Influence of extracellular K^+ concentration on the time-course of Na^+/K^+-ATPase inhibition by cardiac glycosides with fast and low binding kinetics [1]

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Abstract

The magnitude of the K^+ antagonism of cardiac glycoside binding to Na^+/K^+-ATPase prepared from porcine heart, was estimated from the enzyme activities determined in the presence of different concentrations of K^+ ($[K^+]$), ouabain, and α-methyl-digitoxigenin-glucoside, the latter showing a 30 fold greater dissociation rate than ouabain. An increase of $[K^+]$ (3–20 mmol/l) prolonged the half-lives of Na^+/K^+-ATPase inhibition and caused a rightward shift of the cardiac glycoside's dose–response curves by the same factor, almost maximal (4 fold) at 14 mmol/l K^+. These data could be verified from the cardiac glycoside-elevated intravesicular Na^+ concentrations of rat brain vesicles. These concentrations declined rapidly in brain vesicles treated with α-methyl-digitoxigenin-glucoside but not with ouabain after K^+ was increased from 3.5 to 14 mM. The results suggest that the magnitude of the K^+ antagonism under physiological conditions is only limited by the lifespan of the cardiac glycoside-binding E2P enzyme conformation reduced by K^+. © 1997 Elsevier Science B.V.

Keywords: ATPase (Na^+, K^+); Cardiac glycoside; α-Methyl-digitoxigenin-glucoside; Ouabain; Therapeutic index

1. Introduction

Up to now cardiac glycosides have been used for the therapy of chronic congestive heart failure in addition to angiotensin-converting-enzyme inhibitors and diuretics (Smith et al., 1993), but the narrow therapeutic range and the risk of intoxication is still a problem with commercially available cardiac glycosides. Toxic doses of the drugs cause an elevation of intracellular levels of Na^+ accompanied by a K^+ efflux (Peters, 1986; Terada et al., 1994) leading to a partial depolarization of cardiac myocytes and an appreciable elevation in diastolic and systolic levels of Ca^{2+}, contributing to arrhythmia. Since binding of cardiac glycosides to their receptor is suppressed by K^+ (Hansen and Skou, 1973; Han et al., 1976; Akera et al., 1985), one can speculate that an increase in extracellular K^+ concentration ($[K^+]_o$) due to toxic cardiac glycoside doses should principally be able to improve the dissociation of the drug from its receptor, thus allowing restoration of cellular ion gradients, followed by rebinding of the drug at normalized $[K^+]_o$. Ideally, such a self-regulating mechanism might prevent or, at least, should diminish intoxication. According to this idea, the cardiac glycoside of choice would be characterized by a high turnover rate of receptor binding. In other words, association and dissociation rates of the drug must be high enough to allow both rapid dissociation that closely follows the increase in $[K^+]_o$ under toxic conditions, and fast association to insure a positive inotropic action at normalized $[K^+]_o$ levels. These criteria might be fulfilled in part by α-methyl-digitoxigenin-glucoside. The α-methyl-digitoxigenin-glucoside's dissociation and association rates are about 30 and 2 times greater than those of ouabain, respectively, as determined by radioligand binding studies (Mohr, 1983; Brown and Erdmann, 1984).

K^+ has been shown to affect the binding of cardiac glycosides in multiple ways including transition of confor-

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[1] Dedicated to Thies Peters (†).

mational states of the Na^+/K^+-ATPase. According to the findings of Albers and Post, Na^+/K^+-ATPase exists in a number of major conformational states (Thomas et al., 1990; Vasilets and Schwarz, 1993) including the cardiac glycoside-binding state E2P whose conversion to nonbinding forms is accelerated by K^+ (Skou and Esmann, 1983; Van der Hijden and De Pont, 1989; Berberian and Beauge, 1991). Concomitantly, the apparent affinity of Na^+/K^+-ATPase for cardiac glycosides is decreased. As shown by [^{3}H]ouabain binding studies with isolated Na^+/K^+-ATPase (Han et al., 1976; Choi and Akera, 1977), K^+ also seems to reduce the association and dissociation rate constants, apparently due to different modes depending on the cardiac glycoside type. Akera et al. (1985) assumed that the magnitude of the K^+ antagonism is proportional to the dissociation rate. This assumption is in line with previous reports that α-methyl-digitoxigenin-glucoside has proved to be less toxic than ouabain or digoxin (Lüllmann et al., 1983). Studies with isolated papillary muscle and atria of guinea pigs showed α-methyl-digitoxigenin-glucoside to have a flatter dose–response curve than ouabain (Tawfik et al., 1985) and a higher positive inotropic maximum than the usual cardiac glycosides before signs of intoxication occur (Lüllmann et al., 1983; Lüllmann et al., 1984). The relatively low toxicity and the flat dose–response curve of α-methyl-digitoxigenin-glucoside, whose slope amounted to 68% of that of ouabain (Tawfik et al., 1985), might be due to its high dissociation rate constant and the K^+ antagonism described above.

Therefore, we investigated the action of ouabain and α-methyl-digitoxigenin-glucoside on the enzyme activities of isolated Na^+/K^+-ATPase at different K^+ concentrations ($[K^+]$) to estimate the magnitude of K^+ antagonism. Additionally, brain vesicles were used as a model to study K^+ antagonism on cellular Na^+/K^+-ATPases which were inhibited by ouabain and α-methyl-digitoxigenin-glucoside as monitored by the intravesicular free Na^+ concentration ($[Na^+]_i$).

2. Materials and methods

2.1. Preparation of Na^+/K^+-ATPase

Partially purified Na^+/K^+-ATPase (EC 3.6.1.37) preparations were obtained with modifications of a previously described method (Matsui and Schwartz, 1966) from left ventricles of pig hearts provided by the local slaughterhouse. Left ventricles were freed from connective tissue and minced with a flesh mill. Minced tissue, 200 g, made up to 1000 ml with homogenization buffer (in mM: 250 sucrose, 1 EDTA, 3 Tris-HCl, pH 7.4, 4°C) in a Waring blendor (New Hartford, CT), was homogenized at 17 000 rpm for 30 s. The homogenate was cleared by a passage through a gauze filter, and the $10\,000 \times g$ (20 min) pellet was resuspended in 800 ml homogenization buffer supplemented with 2.5 mM deoxycholate and 0.0125% (v/v) octanol. After homogenization in the Waring blendor ($3 \times$ 30 s, 13 000 rpm) and centrifugation at $10\,000 \times g$ for 20 min, the supernatant was precipitated at $65\,000 \times g$ for 70 min. The pellet was suspended in 120 ml homogenization buffer supplemented with 1.25 mM deoxycholate, centrifuged at $20\,000 \times g$ for 20 min, and the supernatant was precipitated at $65\,000 \times g$ for 70 min. After resuspending of the pellet in 15 ml sucrose-free homogenization buffer, 30 ml of 6 M NaI, 15 mM EDTA, 7.5 mM $MgCl_2$, 120 mM Tris-HCl, pH 7.4 were added, and the suspension was stirred on ice for 30 min. The suspension was diluted with homogenization buffer without sucrose to a final concentration of 800 mM NaI. Afterwards, the suspension was precipitated at $35\,000 \times g$ for 45 min, and the pellet was washed three times by centrifugation ($35\,000 \times g$, 30 min) and resuspension in sucrose-free homogenization buffer. The final pellet was resuspended in 20 ml of the same buffer, frozen in liquid nitrogen, and stored at −18°C until measurements. The yield of protein amounted to about 16 mg per 200 g tissue.

2.2. Determination of Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was determined photometrically with modifications according to Norby (1971) using an ATP regenerating, enzyme-coupled assay to maintain the ATP concentration constant during measurement. Briefly, Na^+/K^+-ATPase (40 μg protein/ml) was assayed at 37°C in HEPES-buffer containing 1 mM phosphoenolpyruvate, 0.4 mM NADH, 2.5 mM $MgCl_2$, 1 mM EDTA, 3.5 mM ATP, 10 u pyruvate kinase (EC 2.7.1.40), 10 u lactate dehydrogenase (EC 1.1.1.27), 50 mM HEPES, pH 7.2 and various KCl concentrations as indicated in the text. After equilibration for 5 min, the reaction was started by the addition of 100 μl NaCl stock solution to obtain a final volume of 2.3 ml and a concentration of 100 mM NaCl. Afterwards, cardiac glycosides and ouabain (200 μM) were applied sequentially, the latter for the determination of ouabain-sensitive ATP hydrolysis which amounted to 90–95% of the total ATP turnover. Na^+/K^+-ATPase activities were continuously monitored at 340 nm from the oxidation of NADH enzymatically coupled to ATP hydrolysis. Specific enzyme activity was calculated from the rate of decline in NADH extinction using the molar extinction coefficient ($\epsilon_{NADH} = 6.22 \times 10^6$ cm^2/mol).

2.3. Preparation of brain vesicles

The preparation of rat brain vesicles was carried out as described by Gleitz et al. (1996). Briefly, experiments were conducted with adult, male Wistar rats (200–350 g, Charles River, Sulzfeld) killed by cervical dislocation.

Each hemisphere of the brain without cerebellum was homogenized in 15 ml homogenization buffer (320 mM sucrose, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.4) with a Potter (Braun, Melsungen). After centrifugation at $450 \times g$ for 5 min brain vesicles were precipitated at $5000 \times g$ for 5 min. The pellet was resuspended in incubation buffer (in mM: 125 NaCl, 3.5 KCl, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 5 $NaHCO_3$, 10 glucose, 25 HEPES, pH 7.4) to obtain a protein concentration of about 5 mg/ml. The protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

2.4. $[Na^+]_i$ measurement

$[Na^+]_i$ was determined fluorometrically with modifications according to Gleitz et al. (1996) using sodium-binding benzofuran isophthalate (SBFI) as a Na^+-sensitive fluorophore. In brief, the vesicular suspension was incubated at room temperature for 45 min with SBFI-tetra-(acetoxymethyl) ester and Pluronic F127 adjusted to 15 μM and 0.02% (w/v), respectively. Afterwards, vesicles were washed twice with incubation buffer by centrifugation at $5000 \times g$ for 5 min to remove unhydrolysed dye. The final pellet was resuspended in 26 ml incubation buffer, divided into 2 ml portions and centrifuged at $5000 \times g$ for 5 min. Pellets were stored on ice until fluorescence measurements were performed.

Each SBFI-loaded vesicular pellet was suspended in 2 ml incubation buffer (37°C) to obtain a protein concentration of 0.5–0.8 mg/ml. $[Na^+]_i$ was determined by the fluorescence ratio method (Minta and Tsien, 1989) carried out at 37°C in a temperature-controlled, stirred, cuvette using a DeltaScan fluorescence spectrophotometer (Photo-Med, Wedel). Calculation of $[Na^+]_i$ and calibration was performed according to Gleitz et al. (1996). Briefly, SBFI fluorescence was monitored at excitation wavelengths of 340 nm and 385 nm with emission at 500 nm. $[Na^+]_i$ was calculated as $[Na^+]_i = (k_D \times S_f/S_b) \times (R - R_{min})/(R_{max} - R)$, where R represents the ratio of fluorescence intensities at 340 nm and 380 nm, R_{min} is the ratio at 0.0 mmol Na^+, R_{max} is the ratio at 125 mM Na^+ and k_D is the Na^+ dissociation constant of SBFI. S_b and S_f are the fluorescence intensities measured at 380 nm excitation in the presence (125 mM Na^+) and absence (0.0 mM Na^+) of sodium.

2.5. Drugs and chemicals

α-Methyl-digitoxigenin-glucoside was generously supplied by Knoll (Ludwigshafen). Bovine serum albumin, ethylendiaminetetraacetic acid (EDTA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), monensin, *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES), choline chloride, NADH (Na salt), phosphoenolpyruvate (Na salt) were purchased from Sigma (Deisenhofen). Lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) were from Boehringer Mannheim (Mannheim). The SBFI-acetoxymethyl ester was supplied by MobiTec (Göttingen). The other chemicals were purchased from Merck (Darmstadt).

2.6. Data analysis and statistics

Results are expressed as mean values ± S.D. Statistical analysis was performed using Student's *t*-test (unpaired, double sided), and differences between means were assumed to be significant if $P < 0.05$ (*).

For the calculation of IC_{50} values, inhibition of ouabain-sensitive Na^+/K^+-ATPase activities by cardiac glycosides was expressed as percent of control, and each dose–response curve was fitted according to the logistic dose–response function: $A = a + b/(1 + (x/c)^d)$, where A is enzyme activity, x represents the cardiac glycoside concentration, and a, b, c and d are fitting constants. The IC_{50} values were calculated from the fitted curves and expressed as means ± S.D.

Half lives ($T_{1/2}$) of Na^+/K^+-ATPase-inhibition due to the action of cardiac glycosides were calculated by a nonlinear fitting of the decline in NADH concentration ([NADH]) according to an exponential–linear function: $[NADH] = a + b \times e^{-x/t} + m \times x$, where x represents time, t is the time constant, and a, b and m are fitting constants. Half life was determined as $T_{1/2} = \ln 0.5/(-1/t)$.

3. Results

3.1. Inhibition of isolated Na^+/K^+-ATPase by ouabain and α-methyl-digitoxigenin-glucoside

The enzyme activities of Na^+/K^+-ATPase were monitored with an ATP-regenerating enzyme assay to insure a constant ATP concentration of 3.5 mM even after incubation times prolonged up to 3 h, because ADP inhibits Na^+/K^+-ATPase with a K_i of 0.1 mM (Apell et al., 1986) and a decline in ATP promotes cardiac glycoside nonbinding conformation states (Lopina et al., 1990; Thomas et al., 1990). The specific enzyme activities were determined photometrically since ATP hydrolysis is coupled to oxidation of NADH as shown in Figs. 1 and 2. Based on the extinction coefficient of NADH, the specific enzyme activity of untreated Na^+/K^+-ATPase amounted to 0.096 nkat/mg protein at 3 mM K^+ and could only be slightly enhanced, to 0.13 nkat/mg protein, at 14 mM K^+.

As illustrated in Fig. 1, Na^+/K^+-ATPase was inhibited by ouabain, and steady states were attained in less than 40 min above 6×10^{-8} M ouabain as monitored by the

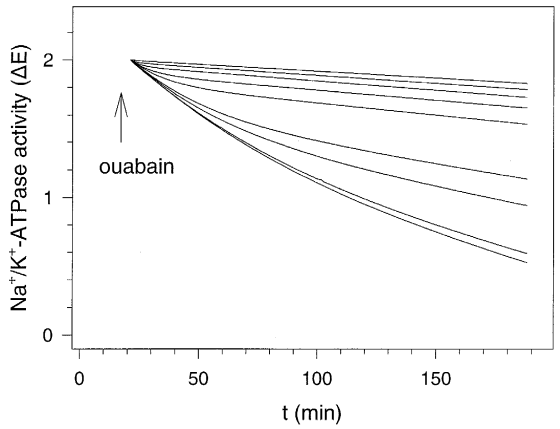

Fig. 1. Time-dependent inhibition of partially purified pig heart Na^+/K^+-ATPase by ouabain. The Inhibition of Na^+/K^+-ATPase at physiological concentrations of K^+ (3 mM), Na^+ (100 mM) and ATP (3.5 mM) was continuously monitored from the decline in NADH which is enzymatically coupled to the hydrolysis of ATP. The figure shows a representative set of traces ($n=1$) obtained with different ouabain concentrations. From top to bottom: 3×10^{-6}, 10^{-6}, 6×10^{-7}, 3×10^{-7}, 10^{-7}, 6×10^{-8}, 3×10^{-8}, 10^{-8} and 10^{-9} M ouabain.

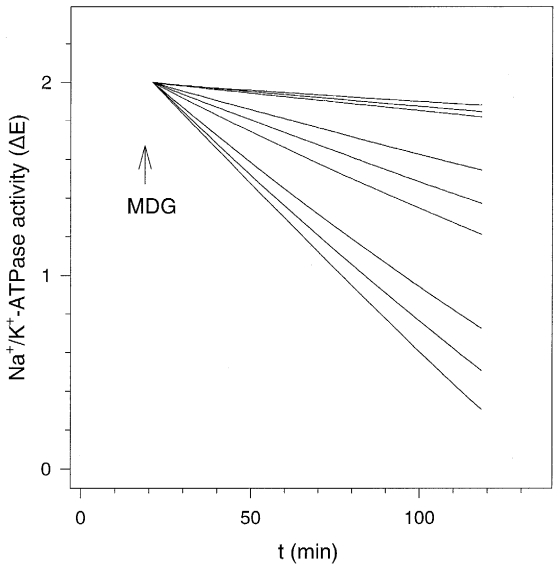

Fig. 2. Time-dependent inhibition of isolated Na^+/K^+-ATPase by α-methyl-digitoxigenin-glucoside. The conditions were the same as described in Fig. 1. The traces ($n=1$) illustrate the rapid attainment of steady state inhibition, within less than 2 min, for all α-methyl-digitoxigenin-glucoside concentrations. From top to bottom: 10^{-4}, 10^{-5}, 6×10^{-6}, 10^{-6}, 6×10^{-7}, 3×10^{-7}, 6×10^{-8}, 3×10^{-8} and 10^{-9} M α-methyl-digitoxigenin-glucoside.

transition from a hyperbolic to a linear decline in NADH extinction. At low concentrations, in the range of 10^{-9}–10^{-8} M ouabain, inhibition equilibrium could not be reached completely even after an incubation time of 3 h (Fig. 1). In contrast to that for ouabain, a steady state of Na^+/K^+-ATPase inhibition was reached with α-methyl-digitoxigenin-glucoside within less than 2 min after its application as shown by the linear decrease in NADH (Fig. 2). This also holds true for low α-methyl-digitoxigenin-glucoside concentrations, at least down to 10^{-9} M α-methyl-digitoxigenin-glucoside.

An increase of the $[K^+]$ in the range of 3–20 mM led to a concentration-dependent prolongation of the time to reach a steady state of Na^+/K^+-ATPase inhibition by ouabain and α-methyl-digitoxigenin-glucoside. For the calculation of $T_{1/2}$ values, depending on both the concentration of ouabain and α-methyl-digitoxigenin-glucoside as well as on $[K^+]$, the enzyme assays were carried out in the presence of different $[K^+]$ (3–20 mM) for each cardiac glycoside concentration, and NADH decline was continuously monitored as depicted in Figs. 1 and 2. The traces for NADH extinction were approximated by a nonlinear fit and $T_{1/2}$ values were calculated from the fitted curves. In general, as shown for ouabain in Fig. 3, the $T_{1/2}$ values increased due to increasing $[K^+]$ at any ouabain concentration, although much higher $T_{1/2}$ values were obtained at low ouabain concentrations. For example, at a ouabain concentration (3×10^{-8} M) closely related to its IC_{50} (2.2×10^{-8} M at 3 mM K^+, Table 1), $T_{1/2}$ increased almost 3 fold, from 50 min at 3 mM K^+ to 135 min at 14 mM K^+ (Fig. 3). Similar results were obtained with α-methyl-digitoxigenin-glucoside, but the $T_{1/2}$ values were

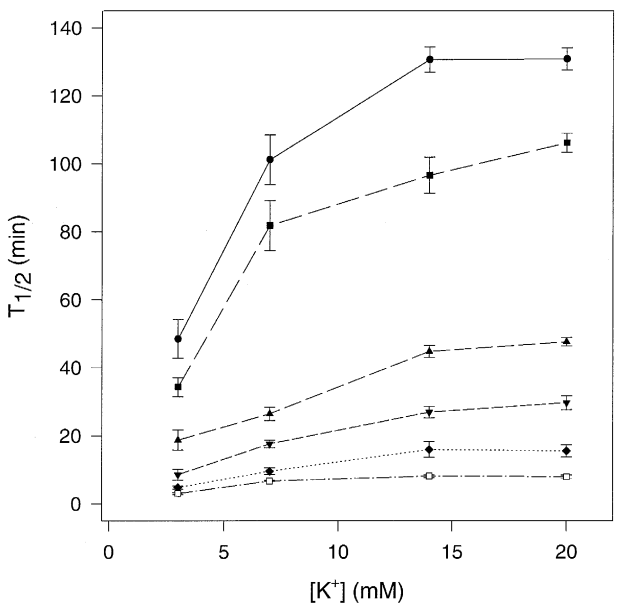

Fig. 3. The influence of $[K^+]$ on the $T_{1/2}$ of ouabain induced Na^+/K^+-ATPase inhibition. Isolated Na^+/K^+-ATPase was incubated in the presence of 3, 7, 14 and 20 mmol/l K^+ and enzyme activities were monitored continuously up to 3 h after the addition of ouabain as illustrated in Fig. 1. Steady state conditions are represented by the transition of the exponential to a linear decline in NADH. For the calculation of $T_{1/2}$, traces of NADH-decline were fitted by an exponential-linear function and $T_{1/2}$ values were calculated as described in the text. Results are shown as means ± S.D. ($n=4$) for 3×10^{-8} (●), 6×10^{-8} (■), 10^{-7} (▲), 3×10^{-7} (▼), 6×10^{-6} (♦) and 10^{-6} (□) M ouabain.

Table 1
IC_{50} values for ouabain- and α-methyl-digitoxigenin-glucoside-induced inhibition of pig heart isolated Na^+/K^+-ATPase at different $[K^+]$

$[K^+]$ (mM)	IC_{50} ouabain (nM)		IC_{50} α-methyl-digitoxigenin-glucoside (nM)	
	2.5 min	50 min	2.5 min	50 min
3	277 ± 46 (100)	22 ± 1 (100) *	269 ± 14 (100)	215 ± 42 (100)
7	559 ± 65 (202)	56 ± 4 (254) *	460 ± 59 (171)	439 ± 66 (204)
14	844 ± 81 (305)	81 ± 1 (368) *	829 ± 49 (344)	797 ± 66 (371)
20	948 ± 86 (342)	86 ± 6 (391) *	926 ± 49 (344)	879 ± 34 (409)

The IC_{50} values were calculated from fitted dose–response curves obtained 2.5 min and 50 min after cardiac glycosides were applied. Data represent means ± S.D. ($n = 4$). Values in percent are shown in brackets. Differences between IC_{50} values determined after 2.5 min and 50 min of incubation with cardiac glycosides were considered significant (Student's *t*-test, double-sided, unpaired) if $P < 0.05$ (*).

of the order of seconds, e.g., at 1 μM α-methyl-digitoxigenin-glucoside ($IC_{50} = 2.2 \times 10^{-7}$ M at 3 mM K^+, Table 1) $T_{1/2}$ amounted to 21.2 ± 4.6 s ($n = 10$) and 146.3 ± 7.1 s ($n = 12$) at $[K^+]$ of 3 and 14 mM K^+, respectively.

As could be expected from the $[K^+]$ dependent-prolongation of the $T_{1/2}$ values, the dose–response curves should have been shifted rightward due to increasing $[K^+]$. In fact, this could be demonstrated with ouabain and α-methyl-digitoxigenin-glucoside as depicted in Fig. 4. The inhibition of Na^+/K^+-ATPase by the cardiac glycosides was determined 2.5 min after their application. As illustrated for ouabain in Fig. 4A, the increase in $[K^+]$ from 3 mM to 14 mM shifted the dose–response curve 3.7 fold to the right as calculated from the IC_{50} values (Table 1). This shift seemed to be maximal at 14 mM K^+ since a higher $[K^+]$ concentration, 20 mM K^+, failed to enhance the IC_{50} significantly (Table 1). Comparable to that for ouabain, the dose–response curves for α-methyl-digitoxigenin-glucoside were also shifted (Fig. 4B) by a factor of 3.7 if $[K^+]$ was raised to 14 mM K^+ (Table 1).

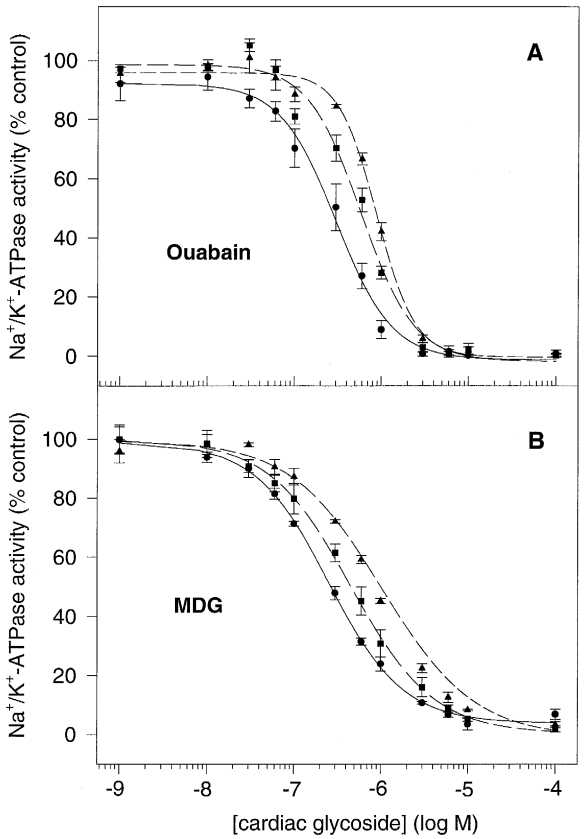

Fig. 4. Dose–response curves for ouabain- and α-methyl-digitoxigenin-glucoside-treated Na^+/K^+-ATPase. Inhibition of Na^+/K^+-ATPase was determined at 3 mM (●), 7 mM (■) and 14 mM (▲) K^+, 2.5 min after the application of ouabain (A) or α-methyl-digitoxigenin-glucoside (B). Enzyme activities were expressed as per cent of control and fitted by the logistic dose–response function. Data are shown as means ± S.D. ($n = 4$).

The most interesting question was whether these shifts could be preserved even at a steady state of Na^+/K^+ ATPase-inhibition. To answer this question, dose–response curves for ouabain and α-methyl-digitoxigenin-glucoside were calculated in the presence of 3–20 mM K^+ from the degree of ATPase inhibition observed 50 min after the application of the cardiac glycosides. As shown for ouabain in Fig. 5A, the IC_{50} values declined to about 1/10 of their initial values under nonsteady state conditions, but the extent of the IC_{50} shifts was maintained (Table 1). This held true for all $[K^+]$ in the range of 3–20 mM K^+. At 3 mM K^+ the IC_{50} amounted to 22 nM ouabain (Table 1) which was consistent with the IC_{50} values reported by others (Thomas et al., 1990). However, it should be stressed that equilibrium conditions for ouabain binding could not be attained within 50 min of incubation because of the high $T_{1/2}$ values (Fig. 3) and the spontaneous decline of Na^+/K^+-ATPase activity observed during long-term incubation.

In contrast, as could have been expected from the fast equilibration, the IC_{50} values of α-methyl-digitoxigenin-glucoside, determined after 50 min of incubation at 3–20 mmol/l K^+, were almost identical to those seen after 2.5 min of incubation (Fig. 5B).

3.2. The influence of $[K^+]$ on vesicular $[Na^+]_i$ elevated by ouabain and α-methyl-digitoxigenin-glucoside

Brain vesicles were used as a model to study the action of $[K^+]$ on ouabain and α-methyl-digitoxigenin-

glucoside-elevated $[Na^+]_i$. As shown in Fig. 6 (traces A and B), the addition of 10 μM α-methyl-digitoxigenin-glucoside to vesicles, incubated at 3.5 mM K^+ immediately increased $[Na^+]_i$ which tended to level off 200 s after α-methyl-digitoxigenin-glucoside was applied, reflecting the rapid start of action as seen with isolated Na^+/K^+-ATPase (Fig. 2). The α-methyl-digitoxigenin-glucoside-dependent increase in $[Na^+]_i$ was calculated as the difference between basal and α-methyl-digitoxigenin-glucoside-induced $[Na^+]_i$, determined 80 s before and 880 s after the addition of α-methyl-digitoxigenin-glucoside, and amounted to $\Delta[Na^+]_i = 35.5 \pm 15.8$ ($n = 12$). To investigate the action of $[K^+]$ on α-methyl-digitoxigenin-glucoside-elevated $[Na^+]_i$, $[K^+]$ was increased to 14 mM K^+ by the addition of 10.5 mM KCl to brain vesicles. As demonstrated in Fig. 6 (trace B), the application of KCl induced a transient increase in $[Na^+]_i$ which recovered rapidly within 200 s, to 37% of $\Delta[Na^+]_i$, a value closely related to the shift of the dose–response curve by a factor of 3.4 at 14 mM K^+ (Table 1). In contrast to KCl, the reference compound, choline chloride, also provoked a small increase in $[Na^+]_i$, which may have been due to an altered osmolarity, but failed to induce a decline in $[Na^+]_i$ (Fig. 6, trace A).

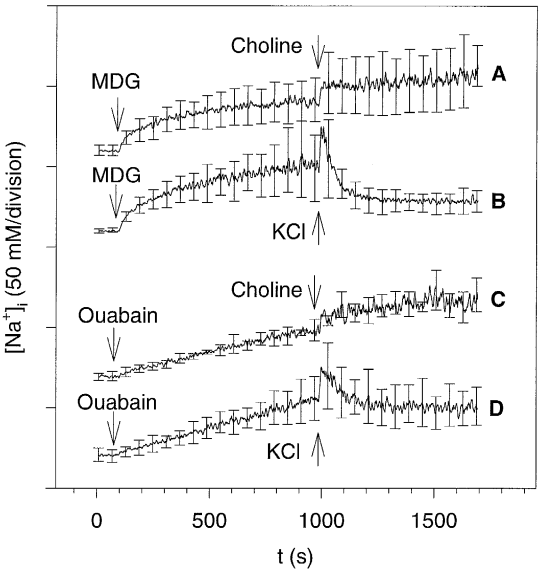

Fig. 6. The effect of K^+ on $[Na^+]_i$ of ouabain and α-methyl-digitoxigenin-glucoside treated brain vesicles. α-methyl-digitoxigenin-glucoside (10 μM) and ouabain (1 μM) were applied to brain vesicles as indicated by arrows. After incubation for 900 s, $[K^+]$ was increased from 3.5 to 14 mM by the addition of KCl as indicated (traces B and D). Control vesicles (traces A and C) were treated with the same concentration of choline chloride (10.5 mM). Traces represents mean ± S.D. ($n = 6$).

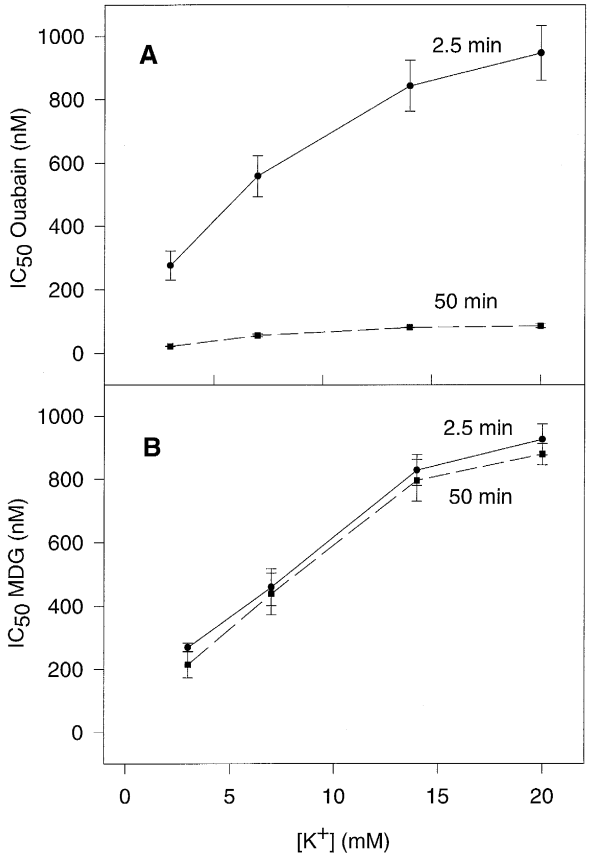

Fig. 5. Time- and $[K^+]$-dependent alterations in IC_{50} values for ouabain- and α-methyl-digitoxigenin-glucoside-treated Na^+/K^+-ATPase. Enzyme activities were calculated 2.5 and 50 min after the application of ouabain (A) and α-methyl-digitoxigenin-glucoside (B) in the presence of 3, 7, 14 and 20 mM K^+. Dose–response curves were calculated for each incubation-time and $[K^+]$ and IC_{50} values were obtained from the fitted curves. Data are shown as means ± S.D. ($n = 4$).

Quite different results were obtained with 1 μM ouabain, a concentration almost equipotent to α-methyl-digitoxigenin-glucoside as estimated by the $\Delta[Na^+]_i = 31.8 \pm 9.7$ ($n = 12$). Consistent with the slow rate of inhibition of isolated Na^+/K^+-ATPase (Fig. 1), ouabain induced a slow, persistent increase in $[Na^+]_i$ which failed to level off even 900 s after its application (Fig. 6, traces C and D). Although an increase of $[K^+]$ to 14 mM K^+ stopped a further increase in $[Na^+]_i$ recovery of $[Na^+]_i$ could not be induced (Fig. 6, trace D).

4. Discussion

As described in more detail in the Introduction, K^+ antagonism of cardiac glycoside binding to Na^+/K^+-ATPase might reduce the toxicity of a cardiac glycoside, provided its dissociation rate constant is high enough to allow a rapid dissociation when $[K^+]_o$ rises due to a toxic cardiac glycoside concentration. This idea is based on previous studies which revealed a $[K^+]$-dependent decline in the steady state levels of cardiac glycoside-binding E2P conformation due to its accelerated turnover (Mardh and Zetterquist, 1974; Hansen, 1984). Since the number of cardiac glycoside-binding receptors remains constant with

an elevated $[K^+]$ (Hansen and Skou, 1973), a $[K^+]$-dependent reduction in the time of availability of the E2P conformation should have at least two consequences for Na^+/K^+-ATPase activity: firstly, the time to reach a steady state of Na^+/K^+-ATPase inhibition by cardiac glycosides should be prolonged by $[K^+]$; secondly, the magnitude of the K^+ antagonism should be limited by the time of availability of E2P, but not by the cardiac glycosides themselves, provided $[K^+]$ failed to affect their association and dissociation rate constants.

In the present study both assumptions could be verified with ouabain and α-methyl-digitoxigenin-glucoside. An increase in $[K^+]$ resulted in a concentration-dependent delay to attain a steady state of Na^+/K^+-ATPase-inhibition which was almost maximal at 14 mM K^+ for both cardiac glycosides, suggesting that the lifespan of E2P was nearly maximally reduced at about 14 mM K^+. This assumption is supported by Skou and Esmann (1983) who demonstrated by stopped-flow fluorescence measurements a $[K^+]$-dependent transition of E2 to E1 conformation with almost maximal rates at 10 mM K^+ in the presence of 30 mM Na^+. The somewhat lower $[K^+]$ the authors found to be maximally effective may have resulted from the relatively low Na^+ concentration they used compared with ours (100 mmol/l Na^+) because Na^+ promotes the formation of the cardiac glycoside-binding E2P (Thomas et al., 1990; Vasilets and Schwarz, 1993).

A reduction in E2P lifespan should also, in turn, suppress the binding of cardiac glycosides to Na^+/K^+-ATPase, which could be verified by radioligand binding studies demonstrating a decline in [^{3}H]ouabain binding to isolated Na^+/K^+-ATPase at elevated $[K^+]$ (Erdmann and Schoner, 1973; Hansen, 1976; Choi and Akera, 1977). However, Han et al. (1976) showed that K^+ was about $8\times$ more effective to reduce phospho-enzyme levels than [^{3}H]ouabain binding. This discrepancy could not be explained, but Akera et al. (1985) suggested that K^+ also reduces the association and dissociation rate constants by apparently different mechanisms, depending on the type of cardiac glycoside. The authors assumed that the extent of the K^+-induced decline in cardiac glycoside-binding is greater when the dissociation rate constant is greater. This assumption could not be verified in the present study. The dissociation rate constant of α-methyl-digitoxigenin-glucoside is about 30 fold greater than that of ouabain (Mohr, 1983; Brown and Erdmann, 1984) but the K^+-dependent shifts of dose–response curves were nearly identical at each $[K^+]$ for both cardiac glycosides, supporting the idea that E2P lifespan is only, or at least mainly, responsible for the shifts. In consequence, the proposed self-regulating mechanism of cardiac glycoside binding mentioned in the Introduction is rather limited since our in vitro studies revealed a maximal $[K^+]$-dependent shift of the dose–response curves by a factor of about 4 at 14 mM K^+. However, such a high $[K^+]$ may have already been attained in the vicinity of the extracellular side of the cell membrane before a massive loss of K^+ could be experimentally detected at toxic cardiac glycoside doses (Peters, 1986), and thus, may reduce toxicity by K^+ antagonism of binding, provided that a cardiac glycoside dissociates rapidly.

Apart from the magnitude of the $[K^+]$ antagonism, the other two requirements for a self-regulating mechanism, namely fast dissociation and association rates, seem to apply to α-methyl-digitoxigenin-glucoside in part. The most prominent characteristic of α-methyl-digitoxigenin-glucoside is its high dissociation rate constant (Mohr, 1983; Brown and Erdmann, 1984) that allows rapid and complete washout from guinea pig isolated papillary muscle within 20 min whereas an equipotent digitoxin concentration of 3×10^{-7} M could not be completely removed even after a washout of 120 min (Lüllmann et al., 1983). In the current experiments, a rapid decline of the α-methyl-digitoxigenin-glucoside-elevated $[Na^+]_i$, within about 200 s, after an increase of the $[K^+]$ from 3.5 to 14 mM K^+ could also be monitored in brain vesicles used as a model of cellular Na^+/K^+-ATPases. The rapid decline in $[Na^+]_i$ after an increase in $[K^+]$ seems to reflect the K^+ antagonism of α-methyl-digitoxigenin-glucoside binding as already seen with isolated Na^+/K^+-ATPase, suggesting that the results obtained with isolated Na^+/K^+-ATPase may also be applicable to cellular Na^+/K^+-ATPases of rat brain vesicles. This assumption is in line with the action of K^+ on the ouabain-induced increase in vesicular $[Na^+]_i$. An increase of $[K^+]$ to 14 mM failed to induce a decline in ouabain-elevated $[Na^+]_i$, presumably due to its low dissociation rate constant (Mohr, 1983; Brown and Erdmann, 1984), but prevented a further increase in $[Na^+]_i$. Further evidence for the comparability of both models is provided by the distribution of different Na^+/K^+-ATPase isoforms. It is well documented that three different cardiac glycoside affinities, presumably due to $\alpha1$, $\alpha2$ and $\alpha3$ Na^+/K^+-ATPase subunits, can be detected both in brain vesicles prepared from adult rats (Sweadner, 1989) and in ventricular tissue of adult pigs (Vasilets and Schwarz, 1993). In both preparations, the $\alpha2$ isoenzyme is the predominant form, amounting to about 70% of total Na^+/K^+-ATPases, followed by the $\alpha1$ isoenzymes (about 20%, Thomas et al., 1990; Sweadner, 1991). However, despite the similar pattern of isoenzymes in both models, some uncertainty remains because, to our knowledge, there is no information about a possible difference in K^+ antagonism for the models used in the current experiments.

Referring to the times observed for ouabain and α-methyl-digitoxigenin-glucoside to attain a steady state of Na^+/K^+-ATPase-inhibition, the equilibrium of α-methyl-digitoxigenin-glucoside action was attained rapidly within seconds, even at low concentrations of 10^{-9} M α-methyl-digitoxigenin-glucoside, whereas steady state conditions for ouabain at concentrations below 3×10^{-7} M were only reached after hours. These results could be verified with α-methyl-digitoxigenin-glucoside- and

ouabain-treated vesicles. In contrast to ouabain, α-methyl-digitoxigenin-glucoside induced a fast increase in $[Na^+]_i$ that levelled off 200 s after its application. The fast start of Na^+/K^+-ATPase inhibition and the rapid attainment of steady state seen with α-methyl-digitoxigenin-glucoside may be attributed to both the 2.4 fold greater association rate constant which determines the velocity of inhibition, and the dissociation rate constant responsible for the rapid attainment of steady state, because the higher the dissociation rate, the faster drug action reaches equilibrium. The rapid inhibition of Na^+/K^+-ATPase by α-methyl-digitoxigenin-glucoside is consistent with the fast start of the α-methyl-digitoxigenin-glucoside-induced maximal increase in force of contraction of guinea pig isolated papillary muscle, which is about 7 times faster than that obtained with ouabain (Lüllmann et al., 1983).

Although inhibition of Na^+/K^+-ATPase by cardiac glycosides is widely accepted as cause of positive inotropism and is formulated as the 'sodium pump lag' hypothesis, the basic mechanism of the inotropic effect is still a matter of debate (Thomas et al., 1990). Lüllmann and Peters (1981) suggested that cardiac glycosides promote the release of plasmalemma-bound Ca^{2+} ions by a disturbance of the Na^+/K^+-ATPase lipid environment due to a cardiac glycoside-ATPase interaction. In this context, the authors assumed an improved therapeutic index for cardiac glycosides with a high turnover rate, such as α-methyl-digitoxigenin-glucoside, since the release of membrane-bound Ca^{2+} was assumed to depend on the process of binding rather than on prolonged binding at the Na^+/K^+-ATPase. However, the relatively low toxicity of cardiac glycosides with high turnover rates (Lüllmann and Peters, 1981; Lüllmann et al., 1983) may also be explained by the K^+ antagonism on the basis of the 'sodium pump lag' hypothesis.

In summary, a maximal K^+-induced shift of a cardiac glycoside's dose–response curve seems to be limited by a factor of 4 under physiological conditions and may depend only on the lifespan of the cardiac glycoside-binding E2P conformation.

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