Digoxin Reduces β-Adrenergic Contractile Response in Rabbit Hearts

Ca^{2+}-Dependent Inhibition of Adenylyl Cyclase Activity via Na^+/Ca^{2+} Exchange

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Abstract

Whereas mobilization of intracellular Ca^{2+} stimulates neuronal adenylyl cyclase via Ca^{2+}/calmodulin, mobilized Ca^{2+} directly inhibits adenylyl cyclase in other tissues. To determine the physiologic role of the Ca^{2+}-dependent interaction between Na^+/Ca^{2+} exchange and β-adrenergic signal transduction in the intact heart, digoxin (0.3 mg/kg) was administered intravenously in rabbits. 30 min after the administration, digoxin impaired the peak left ventricular dP/dt response to dobutamine infusions by up to 38% as compared with control rabbits. This impairment was not caused by changes in either β-adrenergic receptor number or in the functional activity of stimulatory guanine nucleotide-binding protein. It was associated with 33–36% reductions in basal and stimulated adenylyl cyclase activities. Animals treated with calcium gluconate (20 mg/kg/min for 30 min) also demonstrated similar reductions in adenylyl cyclase activities. In addition, increasing the free Ca^{2+} concentration progressively inhibited adenylyl cyclase activity in the control, digoxin-treated, and calcium gluconate–treated sarcolemma preparations in vitro. Moreover, digoxin and calcium gluconate pretreatment blunted the increase in cAMP in myocardial tissue after dobutamine infusion in vivo. Thus, digoxin rapidly reduces β-adrenergic contractile response in rabbit hearts. This reduction may reflect an inhibition of adenylyl cyclase by Ca^{2+} mobilized via Na^+/Ca^{2+} exchange. (*J. Clin. Invest.* 1996. 97:6–13.) Key words: cardiac glycosides • cyclic AMP • myocardial contractility • β-adrenergic signal transduction • calcium

Introduction

Cellular responses to hormones and neurotransmitters are regulated by membrane-bound signaling pathways, examples of which include the adenylyl cyclase system, the phospholipase system, and ion channels. Cellular responses are modulated also by interactions between intracellular second messengers activated by these pathways. Previous studies (1–3) have revealed in neuronal cells and tissues that adenylyl cyclase activity is stimulated by physiologic elevations of Ca^{2+} concentrations induced by phosphoinositide breakdown and other Ca^{2+} mobilizing systems. This stimulation has been shown to act indirectly via the Ca^{2+}/calmodulin system (4). In contrast, Brooker et al. (5, 6) and others (7, 8) have demonstrated recently that adenylyl cyclase from nonneuronal sources is inhibited directly by mobilized Ca^{2+}. These differences in the response of adenylyl cyclase to mobilized Ca^{2+} appear to depend upon the identity of the predominant isoform of adenylyl cyclase expressed in each tissue. At present, eight different isoforms of this enzyme have been identified (9) and the isoform that is inhibited by Ca^{2+} mobilization has been cloned from NCB-20 cells (10). Interestingly, this isoform has been shown to be most highly expressed in cardiac tissue (10). However, its physiologic role in the heart has not been determined.

Myocardial cells have a very active sarcolemmal Na^+/Ca^{2+} exchange transporter. Under physiologic conditions, this transporter moves Ca^{2+} out of the cytosol in exchange for Na^+. In contrast, a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) through this transporter (reverse Na^+/Ca^{2+} exchange) occurs during the application of cardiac glycosides and in various pathologic states including ischemia and reperfusion injuries (11–14), myocardial stunning (15), and lusitropic dysfunction in heart failure (16).

The purpose of this study was to determine the potential regulation of β-adrenergic signal transduction by Ca^{2+} mobilized via Na^+/Ca^{2+} exchange in the intact heart. For this purpose, we investigated the effects of the intravenous administration of digoxin on physiologic and biochemical β-adrenergic responsiveness in rabbits.

Methods

Experimental animals. Female Japanese White rabbits weighing between 2.5 and 3.2 kg were anesthetized with sodium pentobarbital (30 mg/kg). Additional sodium pentobarbital (0.5–1.5 mg/kg) was administered as necessary. The trachea was intubated, and the animal was mechanically ventilated with 100% oxygen using a volume-controlled respirator (model SN-485; Shinano Apparatus, Tokyo, Japan). The ventilation rate was 15–20 breaths per minute, and tidal volume was ~ 40 ml. The respiratory rate was adjusted to keep the arterial pH within the physiologic range.

Left ventricular hemodynamics. In 57 animals, a catheter with a Y-type connector was inserted into the left ventricle via the right carotid artery. A 2F micromanometer-tipped catheter (Millar Instruments, Houston, TX) was inserted into a lumen of the connector. The micromanometer pressure tracing was superimposed onto a conventional pressure tracing obtained from the side lumen of the connector with the use of a fluid-filled system attached to a transducer (Satham p-23Db; Gould Inc., Cleveland, OH). The micromanometer catheter was then advanced into the left ventricle. The first derivative of the left ventricular pressure curve (dP/dt) was obtained from a differentiating circuit in the recorder with the high-frequency filter cut-off set at 70 Hz. A precordial electrocardiogram was monitored using bipolar chest leads. Rabbits were allowed at least 10 min after surgical preparation to reach a steady state condition, at which point the left

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ventricular pressure, dP/dt, and electrocardiogram were recorded at a paper speed of 100 mm/s (model R-60; TEAC Co., Musashino, Tokyo, Japan).

After recording left ventricular pressure, 50 rabbits were treated with a 5-min intravenous infusion of saline ($n = 25$) or 0.3 mg/kg of digoxin ($n = 25$). In addition, seven rabbits received an intravenous administration of 20 mg/kg/min of calcium gluconate for 30 min. 30 min after initiating the infusion of each agent, baseline left ventricular hemodynamic measurements were made. A dose of 0.3 mg/kg of digoxin was chosen because it was found to provide stable and maximal left ventricular contractility for at least 1 h without digitalis-induced ventricular arrhythmias (data not shown). An infused dose of 20 mg/kg/min of calcium gluconate was found to have effects on left ventricular peak positive dP/dt similar to those produced by 0.3 mg/kg of digoxin.

After measuring baseline left ventricular hemodynamics, the hearts of seven rabbits treated with saline, seven with digoxin, and seven with calcium gluconate were quickly excised and perfused with iced saline. The right ventricle was removed, and the left ventricle was frozen in liquid nitrogen and stored at −80°C until preparation of membranes for in vitro biochemical studies.

Left ventricular contractile response to dobutamine and calcium. In 24 rabbits treated with saline ($n = 12$) or digoxin ($n = 12$), the cardiac contractile response to intravenously administered dobutamine or calcium gluconate was examined. Left ventricular hemodynamic measurements were repeated every 3 min during a continuous infusion of graded doses of dobutamine (10, 20, 40, and 50 μg/kg/min) in six saline- and six digoxin-treated rabbits. In six other saline- and six digoxin-treated rabbits, these measurements were performed every 3 min during the infusion of increasing doses of calcium gluconate (10, 20, 40, and 50 mg/kg/min).

Time course of the maximal left ventricular contractile response to dobutamine. In the remaining 12 rabbits treated with saline ($n = 6$) or digoxin ($n = 6$), the time course of the maximal contractile response to the intravenous administration of dobutamine was examined. Left ventricular hemodynamics were measured before and 3 min after a continuous infusion of 50 μg/kg/min of dobutamine, which was started 30 min and 1, 2, 3, 4, 5, and 6 h after treatment with saline or digoxin. Blood was sampled for plasma digoxin measurements before each hemodynamic recording in digoxin-treated animals.

Tissue sampling for measurements of cAMP. In another study, myocardial cAMP concentrations were examined before and 3 min after the continuous administration of 50 μg/kg/min of dobutamine in saline-treated, digoxin-treated, and calcium gluconate–treated animals. In this study, after the induction of anesthesia and intubation (as described above), a left thoracotomy was performed in the fourth intercostal space, and the pericardium was opened. A fluid-filled catheter was placed in the right femoral artery and was connected to a transducer for measurements of arterial pressure. Myocardial tissue samples (10–20 mg) were obtained from an area of the left ventricular wall free of visible vessels using an automatic biopsy needle. Samples were immediately frozen in a clamp precooled in liquid nitrogen, transferred to liquid nitrogen, and stored at −80°C until analyzed for cAMP content. 10 s elapsed between the time of biopsy and transfer to liquid nitrogen. Paired samples were obtained before and after dobutamine infusion in six animals from each of the three groups.

All experiments reported here conformed to the American Physiological Society's guidelines regarding the use and care of laboratory animals and were approved by the institutional animal care committee.

Membrane preparation. Purified cardiac sarcolemmal membranes were prepared from the left ventricle as described previously (17). Briefly, the heart tissue was minced and homogenized in 10:1 (vol/wt) membrane buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM EGTA) using polytron PT10 for 10 s at half-maximal speed at 4°C. This crude homogenate was treated with 750 mM NaCl and centrifuged at 14,000 *g* for 20 min at 4°C. The pellet was then suspended and washed once in buffer containing 10 mM $NaHCO_3$ and 10 mM histidine. The resuspended material was vigorously homogenized in buffer containing 0.25 M sucrose, 10 mM histidine, and 1 mM EGTA (pH 7.5) using polytron PT10 three times for 20 s at half-maximal speed. After centrifugation at 45,000 *g* for 30 min at 4°C, the pellet was resuspended, washed, and centrifuged at 17,000 *g* for 20 min at 4°C. The resulting supernatant was centrifuged at 210,000 *g* for 60 min at 4°C, and the purified sarcolemmal membrane pellet was resuspended in membrane buffer and stored at −80°C.

Radioligand binding assays. Binding of β-adrenergic receptors to [^{125}I]iodocyanopindolol (sp act ≈ 2,200 Ci/mM, New England Nuclear, Boston, MA) was measured as described previously (17) by incubating purified membranes in triplicate with eight concentrations of radioligand (10–640 pM) in a final volume of 250 μl of incubation buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ at 37°C for 1 h. At the end of the incubation, binding was stopped by the addition of 5 ml of incubation buffer and immediate filtration through glass-fiber filters (GF/C; Whatman, Maidstone, United Kingdom). Each filter was washed three times with 5 ml of buffer, and the bound radioactivity was determined using a gamma counter at 75% efficiency. Specific binding was defined as the difference between total binding and binding inhibited by 1 μM (±) propranolol. The maximal binding capacity and dissociation constant were determined by nonlinear curve fitting using the LIGAND binding analysis program (18).

Functional activity of Gs. cyc^- S49 lymphoma cells were propagated in DME containing 10% (vol/vol) heat-inactivated horse serum. Plasma membranes were prepared by using sucrose density-gradient separation to purify the membranes as described by Sternweis et al. (19). The cyc^- membranes were suspended at a concentration of 2 mg/ml in buffer containing 20 mM Hepes (pH 8.0), 2 mM $MgCl_2$, and 1 mM EDTA, frozen, and stored at −80°C.

The functional activity of stimulatory guanine nucleotide-binding protein (Gs)[1] was evaluated by reconstitution experiments as described previously (17). Briefly, 30–50 μg of purified sarcolemma was incubated in 1% cholate for 1 h on ice. After centrifugation at 15,000 *g* for 15 min, the supernatant was heated for 10 min at 30°C to inactivate the solubilized catalytic unit. The inactivated extract was then serially diluted in 0.1% Lubrol, and the Gs fraction was obtained. 10 μl of Gs fraction was mixed with 25 μl (50 μg) of cyc^- membranes. Preactivation of Gs was initiated by the addition of 40 μl of buffer containing 62.5 mM Hepes (pH 8.0), 25 mM $MgCl_2$, 1.25 mM ATP, 0.25 mM GTP, 0.5 mM EDTA, 15 mM phosphocreatine, 180 U/ml creatine phosphokinase, 1.25 mM cAMP, 20 mM NaF, 0.15 mM $AlCl_3$, and 0.625 mM 3-isobutylyl-1-methylxanthine. After incubation for 10 min at 30°C, 25 ml of 5×10^6 cpm [α-^{32}P]ATP was added, and the incubation was continued for 30 min at 30°C. [^{32}P]cAMP accumulation was then measured as described below for the adenylyl cyclase assay. Gs activity was calculated by subtracting the endogenous activity of adenylyl cyclase in the cyc^- membranes from the activity in the reconstituted Gs fraction. The functional activity of Gs was measured as the slope of the curve where adenylyl cyclase activity increases linearly with the amount of added Gs extract. The data reported in this study are from cyc^- membranes harvested at the same time.

Adenylyl cyclase activity. Adenylyl cyclase activity was assayed as described previously (17) in a final volume of 100 μl of buffer containing 12.5 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM DTT, 0.5 mM EGTA, 1 mM cAMP, 1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 10 U/ml creatine phosphokinase, and $2 \sim 3 \times 10^6$ cpm [α-^{32}P]ATP. The reaction was initiated by addition of membranes and was stopped after 15 min of incubation at 37°C by the addition of 150 μl of a stopping solution containing 0.5% SDS, 1.5 mM ATP, 0.5 mM cAMP, and ∼ 10,000 cpm of [^{3}H]cAMP as a recovery marker. [^{32}P]cAMP was separated from ATP by two successive chromatographies on Dowex 50W × 8

1. *Abbreviations used in this paper:* Gs, stimulatory guanine nucleotide-binding protein; K^+-pNPPase, K^+-dependent *p*-nitrophenolphosphatase.

and neutral alumina by the method of Salomon et al. (20). Maximum adenylyl cyclase activity was assessed by measuring cAMP production in the presence of 10 μM (−) isoproterenol, 10 mM NaF, and 100 μM forskolin.

Determination of free Ca^{2+} concentration. In experiments in which Ca^{2+} was used in the assay of adenylyl cyclase activity, the free Ca^{2+} concentration in the assay solutions was determined using EGTA buffers and an iterative computer program which solves equations describing complexes formed in the presence of compounds that affect free divalent cation concentration, i.e., ATP, GTP, EGTA, H^+, Mg^{2+}, Ca^{2+}, and Na^+, using published stability constants (21). The final assay mixture concentrations of $CaCl_2$ (against a background of 0.5 mM EGTA) which gave rise to the free Ca^{2+} concentrations given in parentheses are as follows: 50 (0.045), 100 (0.10), 150 (0.17), 200 (0.27), 250 (0.40), 300 (0.60), 350 (0.93), 400 (1.57), 450 (3.30), and 500 (11.80) μM.

K^+-Dependent p-nitrophenolphosphatase activity. K^+-Dependent *p*-nitrophenolphosphatase activity (K^+-pNPPase) was measured in 50 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM *p*-nitrophenylphosphate, and 20 mM KCl, pH 7.8, at 37°C by the method of Bersohn et al. (22). The K^+-independent phosphatase activity, measured in the above reaction medium, but without KCl, was subtracted from the activity measured in the presence of K^+. The reaction volume was 1 ml and contained ~ 15 μg of protein. The reaction was quenched after 15 min with 2 ml of 1 N NaOH, and an OD of 410 nm was used to measure the amount of *p*-nitrophenol formed.

Protein concentration was measured by the method of Lowry et al. (23) using bovine serum albumin as a standard.

Myocardial tissue cAMP content. The cAMP content was measured in 6% trichloroacetic acid extracts of frozen biopsy specimens using a cAMP assay kit (Yamasa Shouyu Co., Chiba, Japan) based on a sensitive radioimmunoassay (24).

Data analysis. Data in the text, figures, and tables are expressed as mean±SEM. Comparisons in hemodynamic data between the control and digoxin-treated groups were analyzed using an unpaired *t* test. The difference in tissue cAMP content before and after dobutamine infusion was analyzed using a paired *t* test. The statistical significance of difference between the control, digoxin-treated, and calcium gluconate–treated groups and serial hemodynamic changes within each group were determined by one-way ANOVA and multiple comparisons with Tukey's procedure. The differences in the dose–response curves and time courses between control and digoxin groups were analyzed by two-way ANOVA with repeated measures. A P value < 0.05 was considered statistically significant.

Table I. Baseline Left Ventricular Hemodynamics

	Control ($n = 25$)	Digoxin ($n = 25$)	Calcium gluconate ($n = 7$)
HR (beats/min)	275±8	234±6*	252±13
LVSP (mmHg)	97±5	116±4‡	110±6‡
LVEDP (mmHg)	4.1±0.5	5.1±0.8	3.9±0.7
LV peak (+) dP/dt (mmHg/s)	4267±260	5485±296*	5922±388*

Values are mean±SEM. *HR*, heart rate; *LVSP*, left ventricular systolic pressure; *LVEDP*, left ventricular end-diastolic pressure; *LV peak (+) dP/dt*, left ventricular peak positive dP/dt. *$P < 0.01$ and ‡$P < 0.05$ vs. control.

Results

Baseline hemodynamics. Table I compares baseline hemodynamic variables in control, digoxin-treated, and calcium gluconate–treated rabbits. Both digoxin-treated and calcium gluconate–treated groups demonstrated significantly higher left ventricular systolic pressure and peak positive left ventricular dP/dt than did the control group. Heart rate was significantly lower in the digoxin-treated group than in the other two groups. Left ventricular end-diastolic pressure did not differ among the three groups.

Left ventricular contractile response to dobutamine or calcium gluconate. Table II shows the mean hemodynamic values during dobutamine and calcium gluconate infusions in control and digoxin-treated groups. The percent increase in left ventricular peak positive dP/dt during the two drug infusions is shown in Fig. 1. Dobutamine and calcium gluconate produced incremental increases in left ventricular peak positive dP/dt, accompanied by an increase in left ventricular systolic pres-

Table II. Left Ventricular Hemodynamics during Infusions of Dobutamine and Calcium Gluconate

	Dobutamine (μg/kg/min)					Calcium gluconate (mg/kg/min)				
	Baseline	10	20	40	50	Baseline	10	20	40	50
HR (beats/min)										
Control ($n = 6$)	274±8	279±11	280±14	292±7	296±8*	271±3	271±5	270±8	272±5	275±5
Digoxin ($n = 6$)	220±4‡	225±4§	236±3*‡	252±2§‖	260±3‡‖	228±5§	232±6§	230±6§	235±6§	234±5§
LVSP (mmHg)										
Control ($n = 6$)	95±2	106±3*	113±3‖	114±2‖	119±2‖	93±2	98±3	105±3‖	129±6‖	131±7‖
Digoxin ($n = 6$)	116±5‡	122±3‡	127±3*§	130±2*§	133±2§‖	115±6‡	118±6‡	131±6‡	152±12*‡	162±8‡‖
LVEDP (mmHg)										
Control ($n = 6$)	5.3±1.2	5.7±0.3	5.0±0.6	5.3±0.9	6.0±1.0	5.4±0.7	5.6±0.5	6.0±0.7	7.2±1.3	7.0±1.0
Digoxin ($n = 6$)	6.3±0.9	6.2±0.3	6.3±0.3	7.0±0.6	7.0±0.5	4.8±0.6	5.0±0.8	4.6±0.6	6.6±0.4	6.2±0.6
LV peak (+) dP/dt (mmHg/s)										
Control ($n = 6$)	4235±258	5416±245*	6143±221‖	7064±193‖	7277±230‖	4445±305	5046±323	5703±376*	7729±529‖	8025±676‖
Digoxin ($n = 6$)	5236±110‡	6012±253	6662±253*	7286±331*	7621±258‖	5845±214§	6501±285‡	7868±330*§	10715±1065‡‖	10760±881‡‖

Values are mean±SEM. *HR*, heart rate; *LVSP*, left ventricular systolic pressure; *LVEDP*, left ventricular end-diastolic pressure; *LV peak (+) dP/dt*, left ventricular peak positive dP/dt. ‡$P < 0.05$, §$P < 0.01$ vs. control. *$P < 0.05$, ‖$P < 0.01$ vs. baseline.

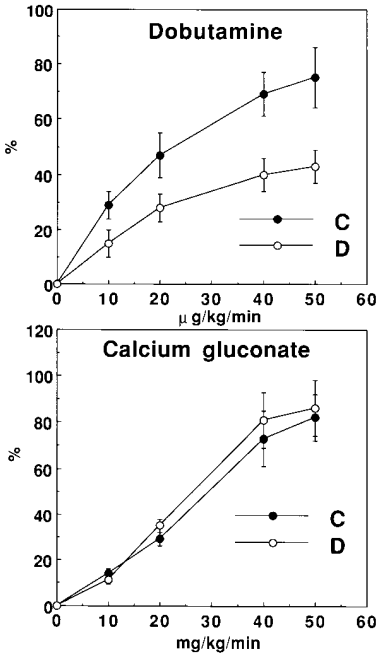

Figure 1. Contractile response to dobutamine and calcium gluconate. Percent increase in left ventricular peak positive dP/dt with 10, 20, 40, and 50 μg/kg/min infusions of dobutamine (*top*) and during 10, 20, 40, and 50 mg/kg/min infusions of calcium gluconate (*bottom*) in control (*C*) (n = 6) and digoxin-treated (*D*) (n = 6) groups. Two-way ANOVA with repeated measures showed a significant F value for the response of both control and digoxin-treated animals to dobutamine infusions (F = 3.35, P = 0.023). The same analysis did not show a significant F value in the response to calcium gluconate infusions (F = 0.21, P = 0.93). Values represent mean±SEM.

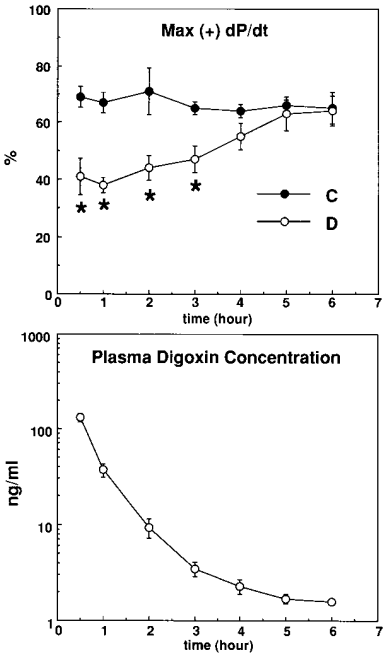

Figure 2. Time courses of the maximal contractile response to dobutamine and of plasma digoxin concentration. Percent increase in left ventricular peak positive dP/dt after intravenous infusions of 50 μg/kg/min of dobutamine 30 min, and 1, 2, 3, 4, 5, and 6 h after treatment with saline (controls, *C*) (n = 6) and digoxin (*D*) (n = 6) (*top*). Two-way ANOVA with repeated measures showed a significant F value for the response of both control and digoxin-treated animals to dobutamine infusions (F = 8.89, P = 0.001). Percent increase in left ventricular peak positive dP/dt was significantly lower in digoxin-treated than in control rabbits for at least 3 h after digoxin treatment. The plasma concentration of digoxin at 30 min after a 0.3 mg/kg intravenous injection was 13±13 ng/ml. It fell rapidly to reach a plateau concentration of 1.6±0.1 ng/ml 6 h after injection (*bottom*). Values represent mean±SEM. * $P < 0.05$ vs. *C*.

sure. Heart rate increased during dobutamine infusion but not during calcium gluconate infusion. Interestingly, the positive inotropic response to dobutamine, reflected by the percent increase in left ventricular peak positive dP/dt, was impaired in the digoxin-treated group by up to 38% as compared with the

Table III. Time Course of Left Ventricular Hemodynamic Response to Dobutamine

		Time after the treatment						
		30 min	1 h	2 h	3 h	4 h	5 h	6 h
HR (beats/min)								
Control (n = 6)	Basal	284±8	280±11	270±11	273±8	268±7	274±5	270±8
	Dobutamine	305±9*	310±10*	290±11*	290±9*	287±9*	285±6*	280±8*
Digoxin (n = 6)	Basal	245±11‡	242±9‡	218±14§	216±14§	219±6§	224±11§	226±6§
	Dobutamine	289±13‡‖	268±7‡‖	247±10*§	237±10*§	235±9*§	240±9*§	234±5*§
LVSP (mmHg)								
Control (n = 6)	Basal	102±8	100±6	104±7	109±7	110±6	106±8	104±3
	Dobutamine	116±8*	115±5*	118±9*	118±8*	116±8*	118±2*	115±7*
Digoxin (n = 6)	Basal	124±3‡	117±2‡	115±3‡	109±4	111±2	112±2	111±3
	Dobutamine	143±4§‖	126±4*‡	127±3*‡	126±6*‡	125±4*	125±6*	120±8*
LVEDP (mmHg)								
Control (n = 6)	Basal	3.9±0.5	4.3±0.2	4.2±1.1	4.5±1.0	4.2±0.8	4.0±0.3	3.8±0.5
	Dobutamine	4.4±0.5	4.7±0.4	4.6±0.7	5.0±0.5	4.8±0.3	4.3±0.4	4.2±0.3
Digoxin (n = 6)	Basal	4.2±0.7	4.0±0.7	4.0±0.4	4.1±0.3	4.3±0.4	4.3±0.5	4.2±0.3
	Dobutamine	4.8±0.7	5.2±1.7	4.5±0.6	4.6±0.5	5.1±0.5	4.9±0.4	4.8±0.4
LV peak (+) dP/dt (mmHg/s)								
Control (n = 6)	Basal	4082±274	4035±236	4058±351	3965±297	4299±506	4260±520	3926±510
	Dobutamine	6851±413‖	6733±410‖	6764±524‖	6560±535‖	7084±858‖	7130±722‖	6366±700‖
Digoxin (n = 6)	Basal	5253±429§	5197±248‡	4772±287	4452±210	4228±175	4119±241	3812±130
	Dobutamine	7380±473‖	7287±421‖	6712±312‖	6374±247‖	6638±228‖	7013±374‖	6524±326‖

Values are mean±SEM. *HR*, heart rate; *LVSP*, left ventricular systolic pressure; *LVEDP*, left ventricular end-diastolic pressure; *LV peak (+) dP/dt*, left ventricular peak positive dP/dt. ‡$P < 0.05$, §$P < 0.01$ vs. control; *$P < 0.05$, ‖$P < 0.01$ vs. basal.

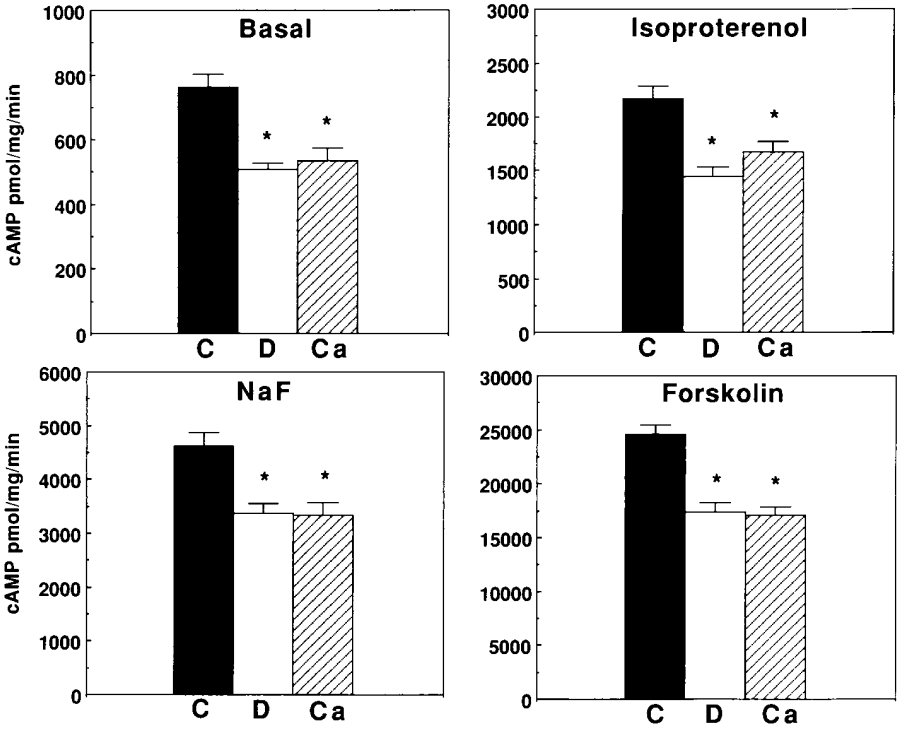

Figure 3. Effects of digoxin and calcium gluconate on adenylyl cyclase activity. Basal adenylyl cyclase activity (*top left*) and the maximal activity stimulated by 10 μM (−)-isoproterenol (*top right*), 10 mM NaF (*bottom left*), and 100 μM forskolin (*bottom right*) in control (*C*) ($n = 7$), digoxin-treated (*D*) ($n = 7$), and calcium gluconate–treated (*Ca*) ($n = 7$) groups. Compared with the control group, all activities were significantly ($P < 0.01$) reduced in the digoxin-treated and calcium gluconate–treated groups. Values represent mean±SEM. * $P < 0.01$ vs. *C*.

control group. In contrast, control and digoxin-treated animals did not differ in their contractile responses during calcium gluconate infusion.

Time course of the maximal left ventricular contractile response to dobutamine. Table III represents the left ventricular hemodynamics at baseline and after infusion of 50 μg/kg/min of dobutamine in control (saline) and digoxin-treated animals. Baseline left ventricular peak positive dP/dt remained constant 30 min, and 1, 2, 3, 4, 5, and 6 h after treatment with saline. Compared with controls, baseline left ventricular peak positive dP/dt was significantly higher 30 min and 1 h after treatment with digoxin and gradually returned to control levels 2 h after digoxin treatment. 3 min of 50 μg/kg/min of dobutamine infusion increased left ventricular peak positive dP/dt at each time point in both control and digoxin-treated groups. However, the percent increase in left ventricular peak positive dP/dt after dobutamine infusion was smaller in digoxin-treated than control animals 30 min after treatment (Fig. 2). Moreover, the relative impairment of contractile response to dobutamine infusion in the digoxin-treated group lasted at least 3 h. The plasma concentration of digoxin 30 min after a 0.3 mg/kg intravenous injection was 130±13 ng/ml, after which it fell rapidly to reach a plateau concentration of 1.6±0.1 ng/ml 6 h after injection.

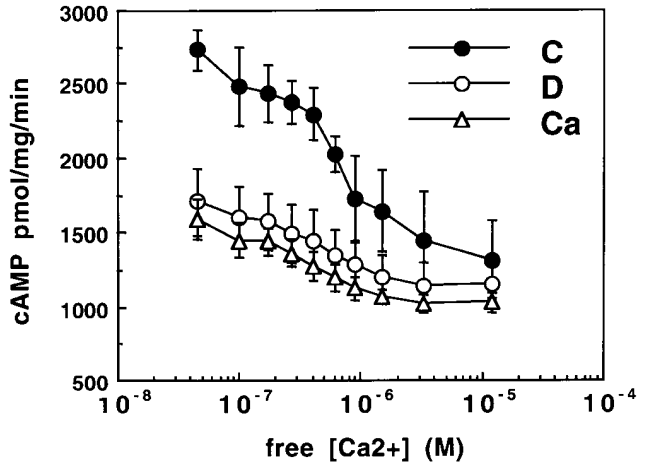

Figure 4. Inhibition of adenylyl cyclase activity by Ca^{2+}. Increasing the free Ca^{2+} concentration from 4.5×10^{-8} M to 1.2×10^{-5} M progressively inhibited adenylyl cyclase activity in sarcolemmal membranes prepared from control (*C*), digoxin-treated (*D*), and calcium gluconate–treated (*Ca*) groups. Adenylyl cyclase activity was measured in the presence of 10 μM of isoproterenol. However, inhibition curves in digoxin-treated and calcium gluconate–treated groups were shallower and were shifted downward as compared with the control group. Two-way ANOVA with repeated measures showed a significant *F* value between control and digoxin-treated groups ($F = 13.08$, $P = 0.0001$) and between control and calcium gluconate–treated groups ($F = 12.99$, $P = 0.0001$). Results are the mean of three separate experiments, each done in triplicate. Values represent mean±SEM.

β-Adrenergic receptor and Gs activity. Despite a reduction in β-adrenergic contractile response, the β-adrenergic receptor number in the sarcolemmal fraction prepared from the digoxin-treated hearts was similar to that in control hearts (341±2 vs. 349±5 fmol/mg protein; NS). The binding affinity did not differ between the two groups (33±3 vs. 37±4 pmol/liter; NS). Similarly, the functional activity of Gs determined by a reconstitution with S49 *cyc*$^-$ lymphoma cells did not differ between the two groups (2.19±0.21 vs. 2.26±0.21 cAMP pmol/mg/min; NS).

Adenylyl cyclase activities. Basal adenylyl cyclase activity and the maximal activity after stimulation with isoproterenol, sodium fluoride, and forskolin were significantly lower in digoxin-treated than in control rabbits (Fig. 3). Calcium gluconate–treated rabbits also demonstrated similar reductions in basal and stimulated activities.

Fig. 4 shows the dose-response curves of adenylyl cyclase

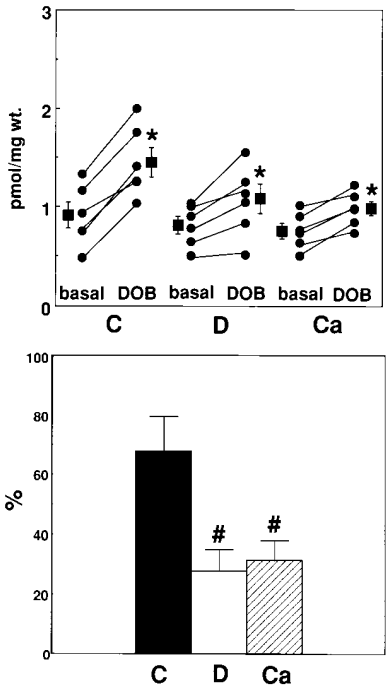

Figure 5. Myocardial tissue cAMP content. Myocardial tissue cAMP content was greater after than before (*basal*) a 50 μg/kg/min infusion of dobutamine (*DOB*) in control (*C*), digoxin-treated (*D*), and calcium gluconate–treated (*Ca*) groups (*top*). Closed square and bar in each group indicates mean±SEM. * $P < 0.01$ vs. basal. However, the percent increase in tissue cAMP content after dobutamine infusion was significantly lower in the digoxin-treated and calcium gluconate–treated groups than that in the control group (*bottom*). # $P <$ 0.01 vs. *C*.

activity to free Ca^{2+} in control, digoxin–treated, and calcium gluconate-treated isoproterenol-stimulated sarcolemma membranes. In all three groups, increasing free Ca^{2+} concentrations from 4.5×10^{-8} M to 1.2×10^{-5} M progressively inhibited adenylyl cyclase activity. However, the dose-inhibition curves in both digoxin-treated and calcium gluconate–treated sarcolemma were shifted downward and were shallower than the control curve. The IC_{50} for Ca^{2+} inhibition of sarcolemmal adenylyl cyclase activity did not differ between control, digoxin-treated, and calcium gluconate–treated groups ($5.03\pm0.29 \times 10^{-7}$ and $4.43\pm0.23 \times 10^{-7}$ vs. $3.97\pm0.42 \times 10^{-7}$ M, respectively).

Increasing digoxin concentrations from 1.0×10^{-9} M to 1.0×10^{-4} M did not alter in vitro basal adenylyl cyclase activity in sarcolemma prepared from saline-treated animals (814±41, 818±42, 769±58, 774±30, 778±40, and 814±61 cAMP pmol/mg/min for 1.0×10^{-9}, 1.0×10^{-8}, 1.0×10^{-7}, 1.0×10^{-6}, 1.0×10^{-5}, 1.0×10^{-4} M digoxin, respectively).

K^+-pNPPase activity. To rule out the possibility that the decrease in adenylyl cyclase activity in membranes from digoxin-treated and calcium gluconate–treated animals reflected differences in protein yields, another membrane-bound enzyme marker, K^+-pNPPase activity, was assayed. K^+-pNPPase activity did not differ between control, digoxin-treated and calcium gluconate–treated groups (7.6±0.6, 8.3±1.1, and 7.3±1.0 μmol/mg/h, respectively).

Myocardial tissue cAMP content. Fig. 5 demonstrates the myocardial tissue cAMP content before and after 50 μg/kg/min of dobutamine infusion. There was no difference in basal tissue cAMP content among saline-treated control, digoxin-treated, and calcium gluconate–treated animals (0.90±0.13, 0.81±0.09, and 0.76±0.08 pmol/mg wt, respectively). Dobutamine infusion increased tissue cAMP content in all the three groups (1.45±0.15, 1.01±0.15, and 0.97±0.07 pmol/mg wt, respectively). However, the percent increase in cAMP content after dobutamine infusion was less in digoxin-treated and calcium gluconate–treated groups than in controls (28±7, and 32±8 vs. 68±12%, respectively; $P < 0.01$ for both). In this study, baseline hemodynamics measured before the first biopsy did not differ from those measured before the second biopsy in either saline-treated, digoxin-treated, or calcium gluconate–treated animals (peak systolic arterial pressure: 115±5 vs. 113±4 mmHg, 128±6 vs. 123±4 mmHg, and 116±6 vs. 115±4 mmHg, respectively; heart rate: 271±8 vs. 264±8 bpm, 250±8 vs. 246±11 bpm, and 280±3 vs. 281±7 bpm, respectively).

Discussion

This study demonstrates that the intravenous administration of digoxin induces rapid reductions in the myocardial β-adrenergic contractile response and signal transduction in rabbit hearts. To our knowledge, this is the first report describing the physiologic interaction between cAMP-producing (adenylyl cyclase) and Ca^{2+}-mobilizing (Na^+/Ca^{2+} exchange) systems in the intact heart.

Left ventricular contractile response to dobutamine and calcium. We assessed peak positive left ventricular dP/dt to evaluate contractile responses to incremental doses of dobutamine and calcium gluconate in control and digoxin-treated animals. Peak positive left ventricular dP/dt depends not only on contractility but is also influenced by loading conditions and heart rate. In both control and digoxin-treated groups, dobutamine infusion produced equivalent increases in left ventricular systolic pressure but did not change end-diastolic pressure. For reasons we do not understand, dobutamine infusion led to a greater increase in heart rate in digoxin-treated than in control animals. Nonetheless, the percent increase in peak positive left ventricular dP/dt was significantly reduced in digoxin-treated as compared with control rabbits. These observations indicate that the relative reduction in peak positive left ventricular dP/dt in the digoxin-treated group was not caused by changes in preload, afterload, or heart rate, but rather was due to decreased contractility. In this study, infusions of calcium gluconate produced similar incremental increases in peak positive left ventricular dP/dt in both control and digoxin-treated rabbits in association with equivalent changes in heart rate and end-diastolic and peak systolic pressures. These results strongly suggest that the blunted peak positive left ventricular dP/dt response to dobutamine in the digoxin-treated group did not result from an impairment in the ability of contractile proteins to bind Ca^{2+}, but was caused by abnormalities in the membrane-bound β-adrenergic cAMP-producing system. The blunted peak positive left ventricular dP/dt response to dobutamine in the digoxin-treated group persisted for at least 3 h after digoxin treatment.

Effects of digoxin on β-adrenergic signal transduction. Despite the reduced contractile response to β-adrenergic stimulation, in digoxin-treated animals, the β-adrenergic receptor number and binding affinity were the same as in the control group. Although the functional activity of Gs did not differ between the two groups, basal adenylyl cyclase activity and the maximal activity stimulated by isoproterenol, sodium fluoride, and forskolin were all significantly lower in digoxin-treated than in control animals. Similar changes in adenylyl cyclase activities were noted in the calcium gluconate–treated group. These results suggest that digoxin-induced inhibition of adenylyl cyclase ac-

tivity is caused by Ca^{2+} mobilized via Na^+/Ca^{2+} exchange and that this Ca^{2+}-dependent inhibition of adenylyl cyclase is not specific for Ca^{2+} mobilized via Na^+/Ca^{2+} exchange in the rabbit heart. Our data support the concept proposed by Brooker et al. (5) that elevated $[Ca^{2+}]_i$ levels, regardless of whether they are achieved by hormone-stimulated Ca^{2+} release from intracellular stores or by ionophoretic Ca^{2+} influx, adversely affect adenylyl cyclase activity.

However, it is possible that elevation of intracellular Ca^{2+} activates proteases that cleave membrane proteins, thereby nonspecifically lowering sarcolemmal enzyme activities. To test this possibility, we included a protease inhibitor (diisopropyl fluorophosphate) in the myocardial membrane preparation made from control, digoxin-treated, and calcium gluconate–treated animals in three experiments. Although inclusion of the protease inhibitor significantly improved adenylyl cyclase activity, it did so equally in control, digoxin-treated, and calcium gluconate–treated membranes, and adenylyl cyclase activity remained selectively depressed in digoxin-treated and calcium gluconate–treated groups (data not shown). Moreover, in the present study, the activity of K^+-pNPPase, another sarcolemmal membrane marker, did not differ among control, digoxin-treated, and calcium gluconate–treated animals. Thus, it appears that Ca^{2+}-dependent inhibition of enzymatic activity is specific for adenylyl cyclase and is not caused by Ca^{2+}-activated proteolysis of the enzyme.

Ca^{2+}-Dependent inhibition of adenylyl cyclase activity in vitro. In this study, the response of adenylyl cyclase activity to free Ca^{2+} was examined in vitro in sarcolemmal membranes prepared from control, digoxin-treated, and calcium gluconate–treated rabbits. As has been described previously in a study of canine cardiac sarcolemma (25), increasing the free Ca^{2+} concentration progressively inhibited adenylyl cyclase activity. Inhibition curves in digoxin-treated and calcium gluconate–treated groups were shallower and were shifted downward compared with that of the control group. However, the IC_{50} for Ca^{2+} inhibition of adenylyl cyclase activity in sarcolemmal membranes prepared from digoxin-treated or calcium gluconate–treated animals was similar to that in membranes from control animals.

It remains unclear whether the Ca^{2+}-dependent inhibition of adenylyl cyclase activity in membranes prepared from digoxin-treated or calcium gluconate–treated animal hearts is a direct or an indirect effect of Ca^{2+}. It is possible that adenylyl cyclase activity is inhibited by an indirect mechanism such as by phosphorylation via Ca^{2+}-dependent protein kinase C activation (26). To test whether the inhibitory effects of free Ca^{2+} were reversible, we also examined isoproterenol-stimulated adenylyl cyclase activity by washing membranes from control animals with EGTA. Using this approach, we postulated that if the inhibition of adenylyl cyclase activity persists even after chelating the free Ca^{2+}, then the reduced activity observed in the treated animals is probably caused by a direct mechanism. However, as Smith et al. (27) have reported previously using human duodenal specimens, our data also indicated that the Ca^{2+}–EGTA complex was more inhibitory to adenylyl cyclase activity than Ca^{2+} alone (data not shown). Thus, although the inhibitory effects of increasing free Ca^{2+} were not reversible with EGTA treatment, this finding does not necessarily lead to the conclusion that cardiac adenylyl cyclase activity was directly inhibited by Ca^{2+} in digoxin-treated and calcium gluconate–treated animals.

It is unlikely that digoxin affected adenylyl cyclase activity independent of Ca^{2+} since both this study and a previous report (28) have demonstrated that cardiac glycosides did not directly affect adenylyl cyclase activity in vitro.

Response of myocardial cAMP content to dobutamine. To clarify the relationship between the relative impairment of positive inotropic response to dobutamine in vivo and digoxin-induced reduction in adenylyl cyclase activity in vitro, we measured myocardial tissue cAMP content before and after dobutamine infusion in control, digoxin-treated, and calcium gluconate–treated rabbits. Although measurement of tissue cAMP content may be influenced by factors other than receptor agonists, including alterations in phosphodiesterase activity, decreased utilization, and/or washout of cAMP, most of the increases in cAMP in hearts have been shown to result from β-adrenergic stimulation (29). Interestingly, the relative increase in cAMP content induced by the administration of dobutamine was attenuated in digoxin-treated and calcium gluconate–treated animals compared with controls. Accordingly, although the relationship between the rise in $[Ca^{2+}]_i$ induced by digoxin infusion and the inhibition of adenylyl cyclase activity was not assessed in vivo, our findings support the concept that adenylyl cyclase activity is inhibited by Ca^{2+} mobilized via Na^+/Ca^{2+} exchange in intact rabbit hearts.

Clinical implications. Cardiac glycosides, including digoxin, are common therapeutic agents to increase the force of contraction in patients with heart failure. Recent studies have demonstrated that resting concentrations of $[Ca^{2+}]_i$ in myocytes may be as low as 1.0×10^{-7} M and may reach concentrations as high as 0.8×10^{-6} M during contraction (30, 31). This implies that during the normal relaxation–contraction cycle, $[Ca^{2+}]_i$ can reach concentrations sufficient to inhibit adenylyl cyclase, and that these concentrations are even more easily attained during application of digoxin. It is possible in the clinical setting that the positive inotropic effect of cAMP-dependent agents, such as dobutamine, may be attenuated in patients with heart failure who receive cardiac glycosides.

Ca^{2+} overload due to reverse Na^+/Ca^{2+} exchange has been considered to underlie ischemia and reperfusion injuries (11–14), myocardial stunning (15), and lusitropic dysfunction in congestive heart failure (16). The results of our study indicate that Na^+/Ca^{2+} exchange–mediated rise in $[Ca^{2+}]_i$ may relate to these abnormal cellular responses by inhibiting cardiac adenylyl cyclase activity.

In conclusion, intravenous administration of digoxin impaired the peak positive left ventricular dP/dt response to dobutamine in rabbit hearts. This impairment was not caused by changes in the β-adrenergic receptor number or binding affinity, or by changes in the functional activity of Gs. It was associated with an impairment in the cAMP-producing activity of the catalytic unit of adenylyl cyclase. These impairments may reflect an inhibition of adenylyl cyclase activity by a rise in $[Ca^{2+}]_i$, mobilized via Na^+/Ca^{2+} exchange.

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