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Research Article

Protein kinase C (PKC) activation in the heart has been linked to a hypertrophic phenotype and to processes that influence contractile function. To establish whether PKC activation is sufficient to induce an abnormal phenotype, PKCbeta was conditionally expressed in cardiomyocytes of transgenic mice. Transgene expression in adults caused mild and progressive ventricular hypertrophy associated with impaired diastolic relaxation, whereas expression in newborns caused sudden death associated with marked abnormalities in the regulation of intracellular calcium. Thus, the PKC signaling pathway in cardiocytes has different effects depending on the timing of expression and, in the adult, is sufficient to induce pathologic hypertrophy.

Expression of Protein Kinase C β in the Heart Causes Hypertrophy in Adult Mice and Sudden Death in Neonates

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Abstract

Protein kinase C (PKC) activation in the heart has been linked to a hypertrophic phenotype and to processes that influence contractile function. To establish whether PKC activation is sufficient to induce an abnormal phenotype, PKCβ was conditionally expressed in cardiomyocytes of transgenic mice. Transgene expression in adults caused mild and progressive ventricular hypertrophy associated with impaired diastolic relaxation, whereas expression in newborns caused sudden death associated with marked abnormalities in the regulation of intracellular calcium. Thus, the PKC signaling pathway in cardiocytes has different effects depending on the timing of expression and, in the adult, is sufficient to induce pathologic hypertrophy. (*J. Clin. Invest.* 1997. 100:2189–2195.) Key words: cardiac hypertrophy • protein kinase C • calcium handling • conditional transgenic

Introduction

Protein kinase C (PKC)[1] comprises a family of serine/threonine kinases that influence a variety of cellular functions. In ventricular cardiocytes, specific isoforms are activated by a number of putative hypertrophic stimuli, including α_1-adrenergic agonists (1, 2), mechanical stretch (3, 4), angiotensin II (5), and endothelin (6, 7). Intracellular PKC phosphorylation targets include proteins in the sarcolemma and sarcoplasmic reticulum that regulate calcium homeostasis as well as sarcomeric proteins that influence the calcium sensitivity of the contractile machinery (8–12). In addition, PKC has been implicated in the modulation of cardiac gene expression and in the induction of cardiac hypertrophy (13–15). While there has been progress towards identifying specific PKC isoform functions in cultured cells, a direct link between enzyme activation and altered myocyte function in the in vivo context has not been established.

To address this, we have developed a binary transgenic mouse model in which a constitutively active PKCβ isoform can be conditionally expressed in the heart. Transient transfection of cultured cardiocytes with this same construct results in transcriptional transactivation of genes, atrial natriuretic factor (ANF), and β myosin heavy chain, which are characteristically activated in pathologic cardiac hypertrophy (16–18). In addition, abnormal expression of PKCβ has been described in the hearts of animals and humans subjected to pathologic loads during postnatal life (19, 20), although this remains controversial. These lines of evidence suggest but do not establish a causal link between kinase activation and cardiac adaptation.

Methods

Animal model. The salient features of the model are shown in Fig. 1 *A*. Two lines of mice were generated using standard techniques in a C57/Bl6 background. Cardiac myocyte specificity in one line is determined by a gene in which ~ 2.9 kb of 5′ flanking sequence from the rat α myosin heavy chain gene drives expression of the tetracycline-controlled transactivator (tTA) (21–23). In the second line, the target gene, under the control of a heptamerized tetracycline operator/CMV minimal promoter, is a bovine PKCβ rendered constitutively active by an internal deletion (aa 6–159) which includes the pseudosubstrate and a portion of the C1 domains of the intact molecule (24). The general strategy and the construct backbones have been described in detail previously (23). The genotype of the offspring was determined either by PCR (primers are shown below) or Southern blotting using standard techniques.

Animals were crossed and subsequently were either maintained on tetracycline (1 mg/ml in drinking water supplemented with 1% sucrose) or were given no drug. To suppress neonatal gene expression, tetracycline was administered to pregnant animals throughout gestation.

Physiologic measurements. To assay in vivo hemodynamics, animals were anesthetized with isofluorene and intubated. A PE-10 fluid-filled cannula attached to a pressure transducer (Statham Instruments, Hato Rey, PR) was advanced into the right carotid artery to determine baseline arterial pressures. Subsequently the chest was opened and a 2F micromanometer transducer (SPR-407; Millar Instruments, Inc., Houston, TX) was inserted through the apex of the left ventricle for the simultaneous determination of left ventricular pressure. A bolus infusion of isoproterenol (0.1 ml of a 10^{-7} M solution) was then administered via the femoral vein while left ventricular pressure was monitored.

Echocardiograms were obtained on anesthetized mice using a 7.5 MHz transducer (Hewlett Packard, Andover, MA). Left ventricular end systolic and end diastolic dimensions and ejection fractions were calculated from 2D directional M-mode tracings.

Analytic techniques. RNA was purified from the hearts of binary adult animals using Trizol Reagent (GIBCO BRL, Gaithersburg, MD). RT-PCR was performed on RNA according to standard techniques (Gene Amp RNA PCR kit; Perkin Elmer Corp., Norwalk, CT). Primers for the PKCβ transgene (which flank the internal deletion) were: sense, 5′-ATGGCTGACCCGGCCG; antisense, 5′-CTAAATGTTTCGTTCCACTCGGGG, and for the tTA transgene (which recognize the transcriptional initiation site of the α myosin

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1. *Abbreviations used in this paper:* ANF, atrial natriuretic factor; PKC, protein kinase C; SERCA2, slow sarcoplasmic reticular calcium-dependent ATPase; tTA, tetracycline-controlled transactivator.

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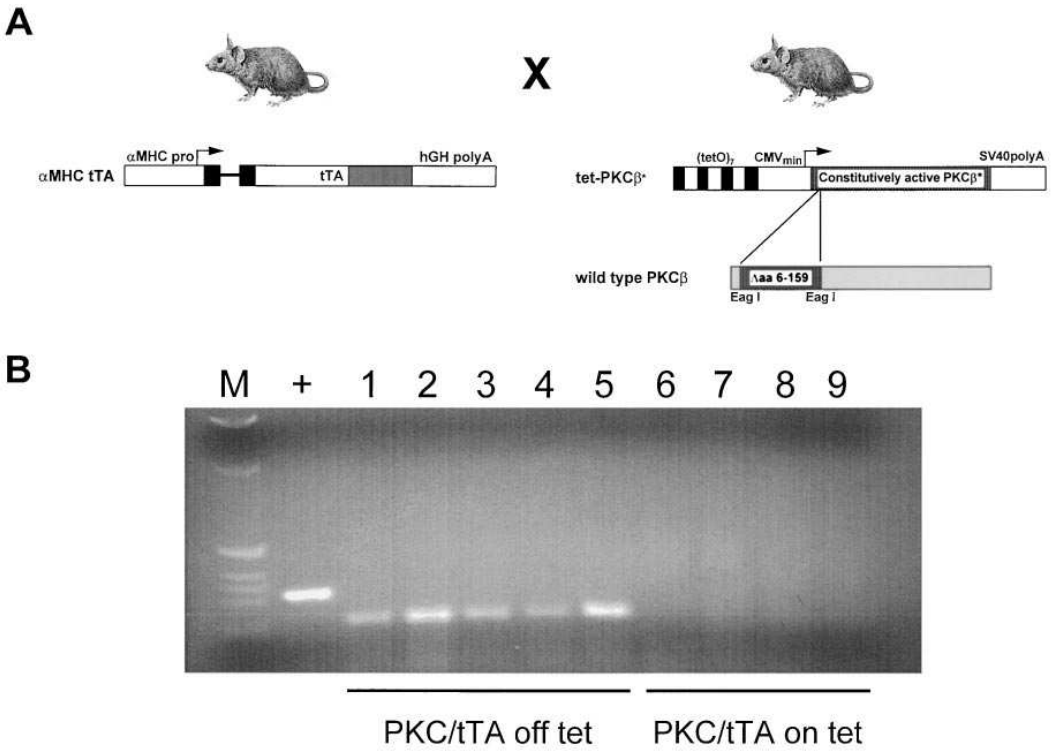

Figure 1. (*A*) Schematic illustration of the binary transgenic system used. The transactivator mouse expressed the tTA chimeric protein under the control of ~ 2.9 kb of the rat α myosin heavy chain promoter, whereas the target mouse harbored a constitutively active form of the bovine PKCβ gene downstream of the tTA responsive promoter. (*B*) Reverse transcriptase PCR on cardiac tissue from binary transgenic animals maintained on or off tetracycline. Lanes *1–5* represent hearts from individual animals harboring both transgenes but not treated with tetracycline, whereas lanes *6–9* represent hearts from animals with an equivalent genotype but maintained on tetracycline. PKCβ mRNA (282 bp) expression was only seen in untreated animals. A lane showing molecular weight markers (*M*) and a PCR positive control (+) are also shown.

heavy chain gene and the splice acceptor region): sense, 5′-TCAGACCGAGATTTCTCCATCCC; antisense, 5′-CGATCAAAGGACTCTGGTA. Amplification was done for 30 cycles and the annealing temperature was 51°C. Products were electrophoresed on 2% agarose gels and the amplified bands were visualized by ethidium bromide staining.

RNA was run on formaldehyde denaturing gels and blotted using standard techniques. After cross-linking, the filters were hybridized using QuikHyb (Stratagene, La Jolla, CA), and exposed to film. cDNA probes for the human slow sarcoplasmic reticular calcium-dependent ATPase (SERCA2) and mouse atrial natriuretic peptide (ANF) were provided by D. MacLennon (University of Toronto, Toronto, Canada) and J. Seidman (Harvard Medical School, Boston, MA), respectively. An oligonucleotide complementary to sequence within the 3′ untranslated region of the mouse β myosin heavy chain gene was used, 5′-TGTTGCAAAGGCTCCAGGTCTGAGGGCTTC.

Cardiac muscle homogenates were extracted and immunoblotted according to previously published methods (25). The antibody for PKCε was from D. Fabbro (Ciba-Geigy, Basel, Switzerland).

Myofibrillar ATPase assays on murine heart protein were performed as previously described (26).

Histologic assessment was done on 5 μM formalin-fixed paraffin-embedded sections stained with hematoxylin and eosin according to standard techniques. The tissue sections were subjectively evaluated by an experienced pathologist blinded as to animal genotype.

Intracellular calcium transient measurements. To isolate cardiocytes from neonatal hearts, the tissue was initially washed in a dissociation solution containing (mM): NaCl 110, KCl 5.4, $NaHCO_3$ 4, $MgCl_2$ 1.6, NaH_2PO_4 1.8, Hepes 20, glucose 5, L-glutamine 4, taurine 10. The muscle was then minced and bathed in the dissociation solution containing 3 mg/ml trypsin and 5 mg/ml BSA and shaken in a 37°C water bath for 15 min. Five successive digestion–trituration cycles were carried out. The supernatant from the first was discarded and those from the subsequent cycles were pooled and centrifuged. The pelleted cells were resuspended in dissociation solution containing 5 mg/ml BSA. Cells were analyzed within 5 h of preparation. The method for Fura-2 loading and measurement of calcium transients in neonatal cardiocytes has been described previously (27). Cells were isolated from the hearts of six binary PKC/tTA and five single PKC transgenic animals. The investigator performing the analysis was unaware of the genotype of the animals.

Statistical analysis. Unless specifically noted, statistical differences among groups were determined by ANOVA, followed by a Newman-Keul's posthoc analysis. Survival data were analyzed using the χ^2 statistic (28). Significance is reported at the 0.05 level.

Results

PKCβ expression in animals harboring both transgenes should be regulated by the absence or presence of exogenous tetracycline and should be restricted to the cardiomyocytes. Data demonstrating the fidelity of the binary system are illustrated in Fig. 1 *B* and demonstrate regulated expression of the PKCβ gene in hearts from animals harboring both genes. The transgene is expressed only in binary animals maintained in an unsuppressed state, that is, off tetracycline.

To evaluate the effects of PKCβ overexpression, crosses

Table I. Heart and Body Weights in Animals Off Tetracycline for 9 mo (by Gender)

Genotype	*n*	Gender	Heart wt	Body wt	Heart/ Body wt
			mg	*g*	*mg/g*
Binary	5	Male	193±12*	32±1	5.68±0.37*
Single	3	Male	155±5	35±2	4.43±0.41
Binary	3	Female	145±17*	28±2	5.14±0.51‡
Single	6	Female	120±18	26±1	4.51±0.25

Data are mean±SEM. Binary animals harbor both the tTA and the PKCβ transgenes, whereas single animals harbor only the PKCβ transgene. $*P < 0.05$ and $‡P < 0.07$ versus gender-matched single transgenics.

Table II. Heart and Body Weights and Left Ventricular Dimensions in Animals Off Tetracycline for 5 mo

Genotype	Tet	*n*	Body wt	Heart/ Body wt	LVEDD	LVESD	FxShort
			g	*mg/g*	*mm*	*mm*	%
Binary	Off	5	25±4	4.5±0.2*‡	3.6±0.2	1.5±0.3	54±9
Single	Off	5	27±5	3.8±0.2	3.5±0.3	1.6±0.2	55±8
Binary	On	5	31±6	3.8±0.3	3.6±0.4	1.4±0.2	62±4
Single	On	4	33±7*	3.7±0.2	3.6±0.1	1.5±0.2	55±6

Data are mean±SD. Animals were maintained on tetracycline (*Tet*) until 8–10 wk of age at which point half were taken off the antibiotic. Animals were killed and measurements were made 5 mo later. $*P < 0.05$ vs. single/off; $‡P < 0.05$ vs. binary/on. *LVEDD*, Left ventricular diastolic dimension; *LVESD*, left ventricular end systolic dimension; *FxShort*, fractional shortening.

between animals homozygous for the PKCβ transgene and heterozygous for the tTA transgene were made (50% of the offspring would be predicted to harbor both transgenes and 50% to harbor only PKCβ). Animals were maintained on tetracycline throughout gestation and through early postnatal life. At 8–10 wk of age the drug was discontinued in half of the animals. A cohort of animals was killed 4 wk after randomization. A second cohort of animals was maintained for 5 mo and in situ cardiac dimensions and performance were evaluated. In a third group, the antibiotic was discontinued in all animals at 8 wk and the animals were subsequently maintained off tetracycline for 9 mo. None died spontaneously. The animals were killed at the various time points mentioned. Heart and body weight comparisons were made in all groups and the animals at the 5-mo time point underwent a more complete physiologic evaluation.

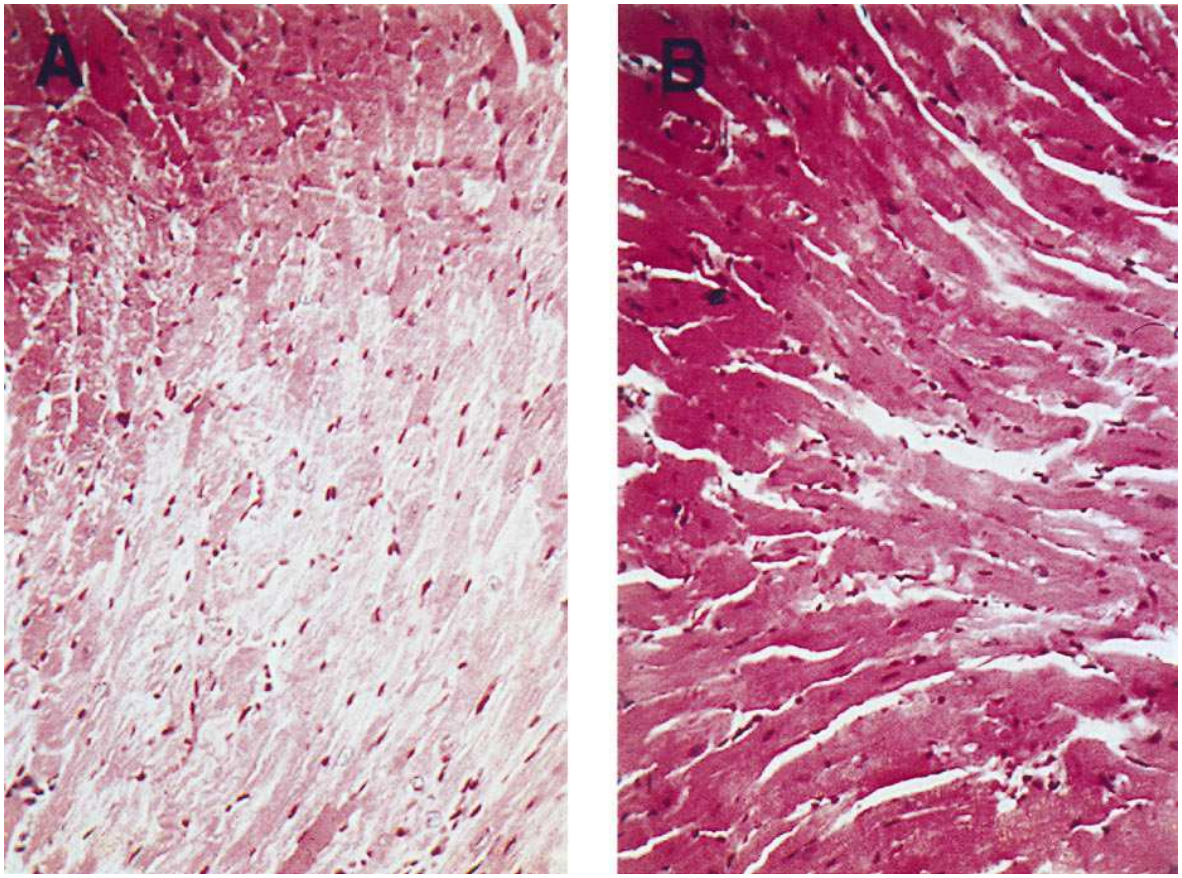

Figure 2. Representative hematoxylin and eosin–stained sections from 7-mo-old adult mouse hearts. *A* is a section from an animal harboring only the PKCβ transgene and *B* is from a binary (PKCβ and tTA) animal. The binary animal evidences mild cellular hypertrophy and no evidence of interstitial fibrosis or cellular necrosis.

Table III. Hemodynamics before and after Isoproterenol Infusion

		HR	BP	LVP	+dP/dt	−dP/dt	LVP	+dP/dt	−dP/dt
Genotype	Tet	Closed chest		Open chest, before isoproterenol			Open chest, after isoproterenol		
Binary	Off	220±69	97±6	78±23	1469±399	1373±479*	92±13*‡	1603±370*	1564±272*‡
Single	Off	240±10	90±10	91±12	1827±247	1844±401	133±40	2103±415	2535±686
Binary	On	200±17	93±12	98±3	1766±68	1837±175	115±23	1846±157	2234±286
Single	On	218±45	99±18	91±15	1698±231	1682±383	138±15	1891±196	2416±511

Data are mean±SD. *$P < 0.05$ vs. single/off; ‡$P < 0.05$ vs. single/on. Animals (and *n*) are exactly the same as those shown in Table II. *Tet*, Tetracycline; *HR*, heart rate; *LVP*, left ventricular pressure.

9 mo after cessation of tetracycline, the binary animals had a significant increase both in heart weight and in heart/body weight ratio (Table I). There was a trend for this increase to be more marked in males (25% increase in heart weight and 28% increase in heart/body weight ratio) than in females (21% increase in heart weight and 14% increase in heart/body weight ratio), although this gender difference did not reach statistical significance. This increase in heart size was gradual: in the group killed 4 wk after randomization, the binary animals off tetracycline had a slight (5%) increase in heart/body weight ratio which was not statistically distinct from the matched tetracycline-treated control groups ($P < 0.08$ versus single PKCβ transgenic animals maintained on tetracycline). 5 mo after randomization, the heart/body weight ratios in the binary animals off tetracycline were increased by ~20% over the single transgenic animals (Table II, $P < 0.05$ vs. other groups). In contrast, there was no increase in this ratio in the binary animals maintained on tetracycline relative to their matched controls. In contrast to the data shown in Table I, half of the binary and single transgenic animals at this time point were maintained on the antibiotic to control for any unanticipated effects of tetracycline or sucrose on cardiac physiology or patterns of gene expression. The increase in body weight seen in these treated animals likely reflects the sucrose added to the drinking water. However, absolute heart weight was significantly increased by 11% in the untreated binary animals relative to the untreated single transgenic group (113 vs. 102 mg, $P < 0.05$). The increase in heart weight was not accompanied by significant changes in internal left ventricular systolic or diastolic dimensions determined by echocardiography, consistent with concentric left ventricular hypertrophy. Ejection fraction did not differ among groups, which is not surprising as this is a relatively insensitive, load-dependent measure of systolic function and the predominant defect in the hearts of the PKCβ expressing mice appears to be diastolic (see below). Histologic evaluation (Fig. 2) confirmed the presence of mild myocyte hypertrophy and did not show any significant intercurrent pathology. Conventional molecular markers of cardiac hypertrophy, an increase in mRNA levels of ANF and β myosin heavy chain and a decrease in SERCA2, were not seen and compensatory changes in the abundance of PKC were not evident. In addition, calcium-dependent myofibrillar ATPase curves did not differ between single and binary off-tetracycline animals.

The carotid arteries and left ventricles of the same adult mice identified in Table II were cannulated and pressures before and after isoproterenol infusions were determined (Table III and Fig. 3). Closed chest arterial pressures did not differ among groups; however, open chest peak left ventricular pressure and (−) dP/dt_{max} were depressed at baseline and this was accentuated (and dP/dt_{max} was also reduced) in response to an inotropic challenge with isoproterenol.

To assess the effects of transgene expression in early postnatal life, tetracycline was discontinued at midgestation. Survival data from heterozygote tTA/PKC crosses are shown in Fig. 4 (one in four would be predicted to harbor both transgenes). While animals maintained on tetracycline showed no mortality (42/42 live births survived until adulthood), 16/65 (~25%) of the untreated animals died within the first 3 wk of life. Death was sudden and not heralded by evidence of intercurrent disease. The time course is consistent with gene expression driven by the α myosin heavy chain promoter (29, 30). The genotype of the off-tetracycline survivors was (PKCβ/

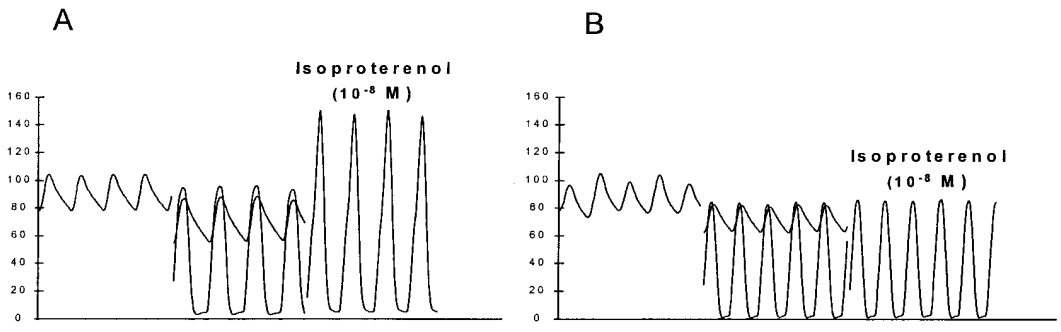

Figure 3. Representative pressure tracings (mmHg) from a binary transgenic either on (*A*) or off (*B*) tetracycline. The first panel in each composite shows closed chest arterial pressure, the second shows simultaneous left ventricular and aortic pressure (open chest), and the third shows left ventricular pressure after bolus infusion of isoproterenol.

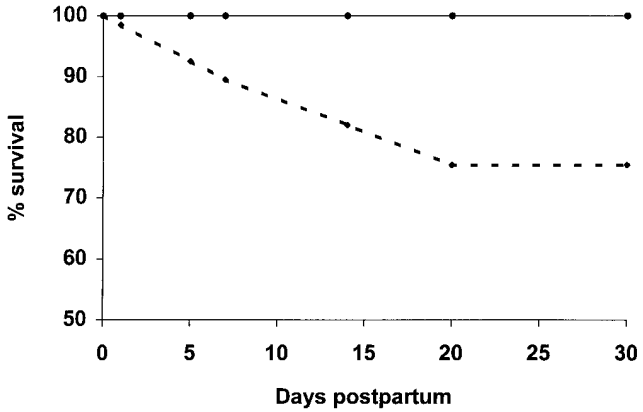

Figure 4. Survival curves of heterozygote crosses indicating that ~25% of live births off tetracycline (*dotted line*) and none of those on tetracycline (*solid line*) died over the first 3 wk of life.

tTA): +/+ = 3; −/+ = 19; +/− = 12; −/− = 15 ($P < 0.02$ by χ^2 analysis). These facts strongly suggest that expression of the constitutively active PKCβ during the early postnatal period is lethal. A second line of PKCβ animals was also crossed with the tTA transactivator mice and showed a similar disproportionate loss of binary animals in early postnatal life (0/12 animals from these crosses surviving to adulthood were binary transgenic animals).

Since the cause of death in the binary transgenic animals was presumed to be arrhythmic, myocytes were isolated from 5–8-d-old binary (tTA/PKC) or single (PKC) untreated animals and their ability to regulate intracellular calcium was compared. There were no obvious microscopic differences in the size of the cells isolated from these animals. The cells were loaded with Fura-2 and calcium transients were monitored over a range of stimulation rates (Fig. 5 *B*). Cardiocytes from both groups show the typical rate-dependent increase in the amplitude and decrease in the duration of the calcium tran-

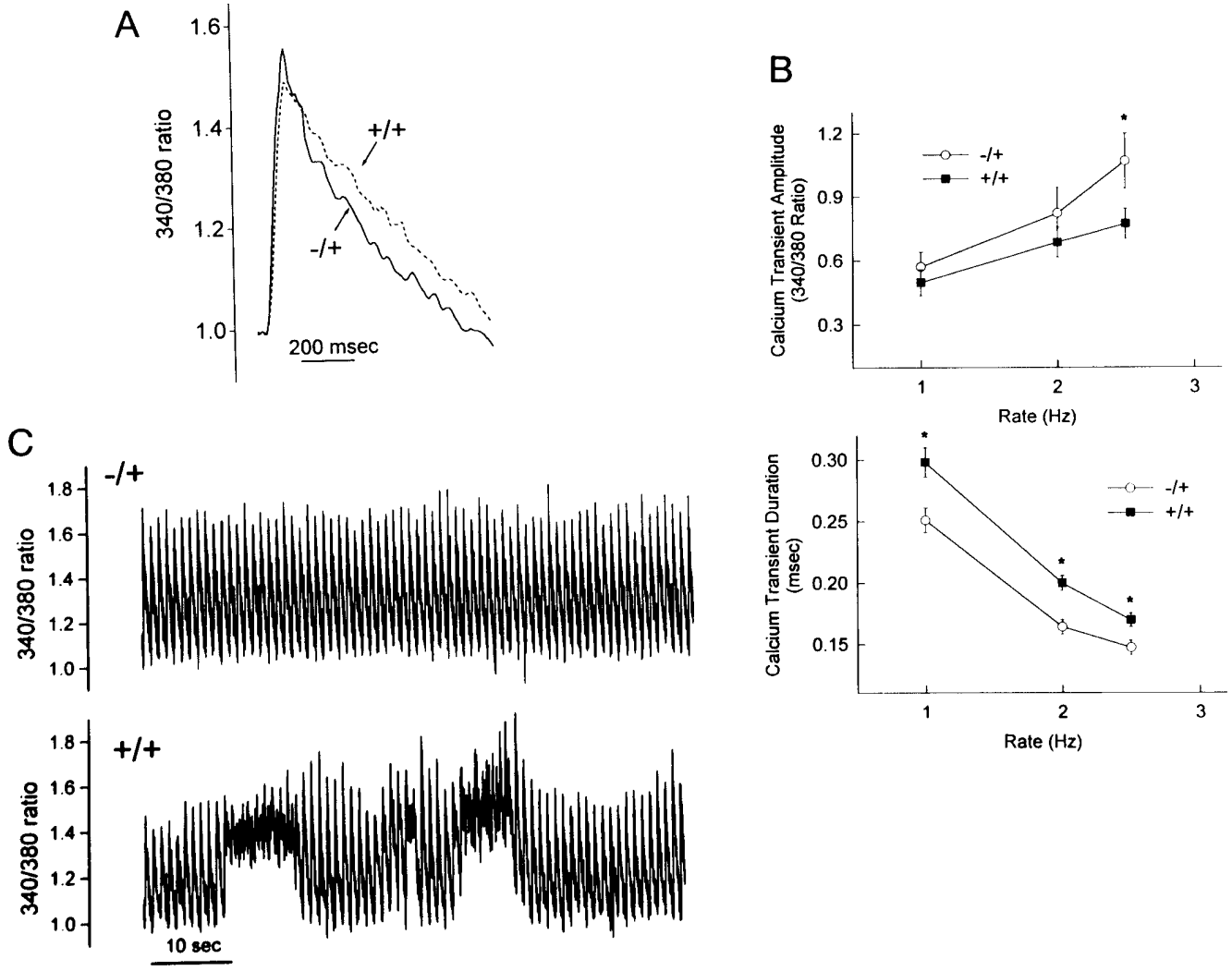

Figure 5. (*A*) Representative calcium transients from a PKCβ expressing and nonexpressing myocyte (tTA/PKCβ: +/+ and −/+). (*B*) At each stimulation rate, a series of six electrically driven calcium transients at steady state was signal averaged for analysis of the amplitude and duration at half-maximal amplitude of the calcium transient. Results of calcium measurements in 47 PKCβ overexpressing and 37 nonexpressing cells are summarized (*$P < 0.05$ vs. matched values). (*C*) A representative record of typical calcium oscillations near the peak systolic value in control (*top*) and PKCβ overexpressing myocytes (*bottom*).

sient. However, the increase in amplitude was significantly attenuated in myocytes from the binary animals at the most rapid pacing rate and the duration was prolonged in these myocytes at all pacing rates. The defect in the kinetics of relaxation of the calcium transient led to secondary spikes that delayed the return of intracellular calcium to baseline diastolic levels in 55% of cells from the binary animals (Fig. 5 *C*) and in none of the cells from the single transgenic animals. In 19% of cells, these ionic perturbations lasted for > 10 s.

Discussion

Two major questions are addressed by this study. The first is whether expression of a single PKC isoform in the adult heart is sufficient to effect a cardiac hypertrophic response and the second is whether developmental context influences the physiologic impact of gene expression. The use of the binary transgenic approach for conditional gene expression is ideally suited to answer these questions since genes can be effectively targeted to a specific cell type and expression can be temporally defined. Previous studies using the binary system as well as work from other laboratories using the same promoter indicate that the level of cardiocyte protein expression varies widely throughout the heart and may be at the limits of detection in as many as 40–50% of cells (31–33). In fact, we were unable to consistently detect PKCβ protein expression in binary, unsuppressed animals although we were able to detect mRNA. These facts probably make this general approach impractical for the manipulation of structural genes but it appears to be well suited to modulated expression of regulatory enzymes, such as PKC.

The fact that neonatal and adult cardiocytes manifest very different responses to overexpression of the PKCβ gene is not surprising, although it suggests that extrapolation of data acquired in fetal and neonatal cardiocytes to the adult heart must be done with caution. Both PKC abundance and isoform distribution as well as the downstream components of the signaling pathways activated by PKC are different in neonatal and adult ventricular myocytes and it is also likely that different transcription factors predominate in the different contexts (25, 34–36). In addition, it is quite possible that the activity of the α myosin heavy chain promoter which drives expression of the PKC transgene varies from neonatal to adult life. Beyond this, and likely quite relevant to the phenotypes seen in our study, there are marked developmental differences in the dominant mechanisms that regulate calcium homeostasis. Relaxation of the calcium transient in adult cardiocytes is largely accomplished by reuptake by the sarcoplasmic reticulum, whereas the neonatal cardiocyte, which has a structurally and functionally immature sarcoplasmic reticulum, relies upon the sarcolemmal Na^+/Ca^{2+} exchanger to extrude calcium during the relaxation phase of the cardiac cycle (37, 38).

Our results suggest that sustained activation of PKC in the absence of other intercurrent stimuli is sufficient to induce concentric cardiac hypertrophy associated with a reduced velocity of sarcomeric relaxation. The adaptation occurs, at least at the stage at which mild ventricular hypertrophy is evident, without demonstrable changes in ANF, β myosin heavy chain, or SERCA2 mRNA expression nor with changes in sarcomeric protein isoforms that would alter the calcium sensitivity of the myofibril, demonstrating that these particular alterations (all of which have been described in other models of cardiac hypertrophy) are not necessary for the development of a hypertrophic phenotype. A previous transgenic model, in which p21ras was overexpressed in the postnatal heart, induced a more dramatic hypertrophic phenotype with a similar pattern of diastolic dysfunction which was associated with transcriptional transactivation of the late response genes (39). The identity of the etiologic PKC target(s) is not directly answered, although the striking impairment in relaxation suggests that abnormalities in the regulation of diastolic calcium, a well described function of PKC activation (10, 40), may contribute to the ultimate development of the cardiac adaptation. This speculation is supported by an analysis both of genetic models of cardiac hypertrophy (41, 42), which are characterized by alterations in the sarcomere length/tension relationship which precede overt muscle failure, and acquired models of cardiac hypertrophy (43–46) which manifest altered regulation of diastolic calcium.

Acknowledgments

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