Adenovirus-Based Phospholamban Antisense Expression as a Novel Approach to Improve Cardiac Contractile Dysfunction

Comparison of a Constitutive Viral Versus an Endothelin-1–Responsive Cardiac Promoter

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***Background*—**A decrease in sarcoplasmic reticulum Ca^{2+} pump (SERCA2) activity is believed to play a role in the impairment of diastolic function of the failing heart. Because the expression ratio of phospholamban (PL) to SERCA2 may be a target to improve contractile dysfunction, a PL antisense RNA strategy was developed under the control of either a constitutive cytomegalovirus (CMV) or an inducible atrial natriuretic factor (ANF) promoter. The latter is upregulated in hypertrophied and failing heart, allowing "induction-by-disease" gene therapy.

***Methods and Results*—**Part of the PL cDNA was cloned in antisense and sense directions into adenovectors under the control of either a CMV (Ad5CMVPLas and Ad5CMVPLs, respectively) or ANF (Ad5ANFPLas and Ad5ANFPLs, respectively) promoter. Infection of cultured rat neonatal cardiomyocytes with Ad5CMVPLas reduced PL mRNA to 30±7% of baseline and PL protein to 24±3% within 48 and 72 hours, respectively. The effects were vector dose dependent. Ad5CMVPLas increased the Ca^{2+} sensitivity of SERCA2 and reduced the time to 50% recovery of the Ca^{2+} transient. A decrease of PL protein was also achieved by infection with Ad5ANFPLas, and the presence of the hypertrophic stimulus, endothelin-1, led to enhanced downregulation of PL. The adenovectors expressing PL sense RNA had no effect on any of the tested parameters.

***Conclusions*—**Vector-mediated PL antisense RNA expression may become a feasible approach to modulate myocyte Ca^{2+} homeostasis in the failing heart. The inducible ANF promoter for the first time offers the perspective for induction-by-disease gene therapy, ie, selective expression of therapeutic genes in hypertrophied and failing cardiomyocytes. **(*Circulation*. 2000;101:2193-2199.)**

Key Words: sarcoplasmic reticulum ■ calcium ■ endothelin ■ atrial natriuretic factor ■ adenovirus ■ gene therapy

The heart adapts to increased workload by responding to mechanical and endocrine, paracrine, and autocrine factors that induce hypertrophy of ventricular myocytes. Although this response is initially compensatory by increasing cardiac output, sustained excessive workload ultimately leads to heart failure.[1] The β-adrenoceptor and sarcoplasmic reticulum (SR) Ca^{2+} pump (SERCA2) genes are downregulated and the atrial natriuretic factor (ANF) gene is strongly upregulated in hypertrophied and failing human myocardium.[2–5] The decrease in SR Ca^{2+} pump activity is believed to be involved in the diastolic dysfunction of the failing heart.[1,4,5] The SR Ca^{2+} pump in the normal heart is inhibited by the regulatory protein phospholamban (PL); this effect is relieved by β-adrenergic stimulation.[6] Studies on (heterozygous) PL-knockout and PL-overexpressing mice have indicated that the expression ratio of PL to SERCA2 is a potential target for improvement of diastolic function of the failing heart.[7] Cardiac overexpression of SERCA2 and PL by efficient adenovirus-mediated gene transfer indeed led to shortening and prolongation of the relaxation phase, respectively.[8,9] These studies used viral promoters, resulting in ubiquitous and constitutive transgene expression. Cardiac troponin T and myosin heavy- and light-chain promoters have also been applied, but only the latter displayed reporter activity exclusively in ventricles.[10,11]

Received October 4, 1999; revision received December 7, 1999; accepted December 22, 1999.

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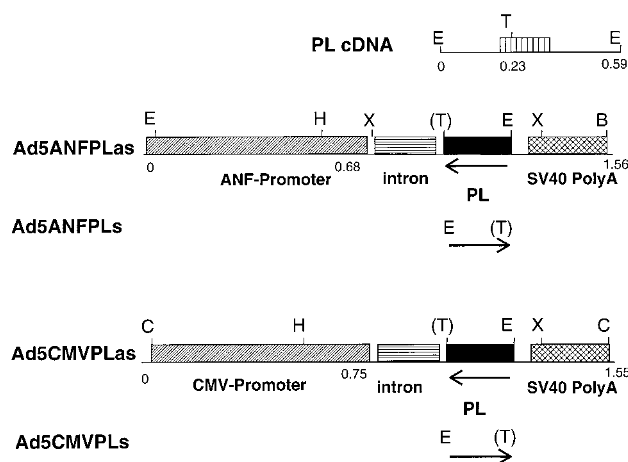

Figure 1. Graphical representation of PL cDNA (top line) and adenovectors. Coding region is indicated as vertically striped box. *Eco*RI-*Taq*I fragment harboring 5′-untranslated and only 47 bp of coding region was used for adenoviral constructs. Names (left) of adenoviral constructs and their compositions (right) are given. B indicates *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; T, *Taq*I; and X, *Xba*I. Brackets indicate sites lost during cloning. Numbers indicate lengths in kb.

In this study, we used an antisense RNA strategy directed against de novo synthesis of PL under control of cytomegalovirus (CMV) or ANF promoter. The latter is strongly upregulated in hypertrophied and failing myocardium and therefore is used to evaluate the concept of "induction-by-disease" gene therapy.[3,5] Many studies on signaling involved in hypertrophy and changes in gene expression have used the model of cultured neonatal rat cardiomyocytes stimulated by stretch or agonists such as endothelin-1 (ET-1).[12–14] Therefore, this model was used to test the effectiveness of the newly developed adenovectors on PL expression, SR Ca^{2+} uptake activity, and Ca^{2+} transients.

Methods

Materials

Culture dishes (Costar catalogue No. 3506 and NUNC, catalogue No. 176740) from Becton Dickinson, culture media DMEM and M199 from Gibco-BRL, and fetal bovine and horse sera and all molecular biology enzymes from Boehringer were used. Cloning plasmids were from Promega and Microbix, ET-1 from Peninsula Laboratories, [^{32}P]dCTP and ^{45}Ca from Amersham, and ^{125}I-labeled goat antimouse IgG from ICN.

Isolation of PL cDNA

PL cDNA was obtained by screening of a rat cDNA library (Lambda ZAP, Stratagene) with a probe derived from genomic DNA containing promoter and exon 1 of PL (unpublished). One cDNA (clone 321, 586 bp) appeared to be identical to previously published sequences, with minor differences in the 3′ trailer,[15] and was used for construction of the adenovirus shuttle plasmids.

Development of Recombinant Adenoviral Vectors

Recombinant adenoviral vectors expressing a partial PL antisense mRNA (Ad5CMVPLas) or sense mRNA (Ad5CMVPLs), respectively, under the control of a CMV promoter were developed.[16,17] A 230-bp *Eco*RI-*Taq*I fragment of PL cDNA containing the translation start codon (Figure 1) was inserted into the polylinker of pCMVI in antisense or sense orientation. The resulting CMV promoter expression plasmids were cotransfected with circularized adenoviral genome pJM17 into 293 cells. Viral plaques appeared between 10 and 20 days and were screened for recombinant virus.[16,17] Recombinant vectors expressing a partial PL antisense RNA (Ad5ANFPLas) or sense (Ad5ANFPLs) RNA, respectively, under control of an ANF promoter were developed as follows: The ANF promoter (680-bp *Eco*RI-*Bam*HI), a kind gift from K.R. Chien (University of California, San Diego), was first cloned in pCAT3 (ANF-CAT) to prove its ET-1 inducibility in rat cardiomyocytes transfected by gene gun biolistics by use of a chloramphenicol acetyltransferase (CAT) ELISA assay (Boehringer).[18–20] The partial PL cDNA was inserted into the ANF-CAT plasmid in antisense or sense orientation, thereby eliminating the CAT reporter and introducing a chimeric intron between ANF promoter and PL cDNA. Next, *Eco*RI-*Bam*HI fragments (1521 bp) containing the ANF promoter, followed by the partial PL cDNA and the SV40 poly-A signal, were cloned into the plasmid pΔE1sp1A yielding transfer plasmids with structures similar to those used for Ad5CMVPLs and Ad5CMVPLas, but with ANF instead of CMV promoter. After cotransfection with pJM17 into 293 cells, viral plaques were screened for recombinant virus. Purified positive plaques were grown in large quantities on 293 cell cultures and purified by standard CsCl ultracentrifugation and desalting.[16,17] Absence of replication-competent adenovirus was confirmed by polymerase chain reaction (PCR) for the wild-type adenoviral E1 region and by high-titer infections of nonpermissive EAhy.926 and CHO cells (multiplicity of infection [MOI] 10). Virus titers determined by plaque assay and vector doses are expressed as MOI, defined as plaque-forming units per cell.

Preparation and Adenoviral Infection of Myocytes

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996). Rat neonatal ventricular myocytes were isolated,[21] preplated, and cultured in 1.8-cm^2 (Western blotting), 10-cm^2 (Ca^{2+} transient measurements), or 20-cm^2 (Northern blotting and Ca^{2+} uptake measurements) dishes (7.5×10^4 cells/cm^2 for the Ad5ANFPLas and Ad5ANFPLs infections and 1.5×10^5 cells/cm^2 for the Ad5CMVPLas and Ad5CMVPLs infections, except for the Ca^{2+} transient measurements: 0.3×10^5 cells/cm^2), up to 24 hours in DMEM/M199 (4:1) supplemented with 5% FCS and 5% horse serum and up to 64 hours with only 5% horse serum. Subsequently, infections were started in 200 μL (1.8-cm^2 dishes) or in 600 μL (20-cm^2 dishes) serum-free medium for 3 hours and continued for 72 hours after addition of 600 or 2600 μL serum-free medium, respectively. When appropriate, after 3 hours, ET-1 (10^{-8} mol/L) was added to induce hypertrophy.[13,18,19,21] Analyses in addition to the plaque assays were carried out to confirm that the relative functional activities of the viruses closely matched: (1) CMV vector doses were tested at the RNA level by hybridizing Northern blots (Figure 2) with SV40 probe specifically recognizing vector-derived RNA, and similar transcriptional activities (relative to GAPDH) were found; and (2) ANF vectors, because of their low expression levels, were tested instead by semiquantitative PCR of infected cells, followed by gel electrophoresis and quantification of the PCR products (not shown).

Northern Blotting

Total cardiomyocyte RNA was isolated by the guanidinium isothiocyanate method, separated on 1% denaturing formaldehyde-agarose gels, and blotted onto Hybond (Amersham). Probes were labeled by random priming with [α-^{32}P]dCTP: PL; the *Taq*I-*Eco*RI fragment of the PL cDNA (excluding the part used for the constructs, Figure 1); GAPDH, developed by RT-PCR on rat heart RNA; and SV40, the SV40 poly A fragment of pCMVI plasmid. Hybridization and washing were carried out as described.[13] Hybridization signals were quantified by Molecular Imager (BioRad) and corrected for GAPDH. Two bands were detected with the PL probe and coquantified.

Western Blotting

Cardiomyocytes were homogenized into 10 mmol/L Tris-HCl, 0.3 mol/L sucrose, 1 mmol/L dithiothreitol, and 3 mmol/L NaN_3 by freeze-thawing. Protein (10 μg) in SDS loading dye (not boiled) was

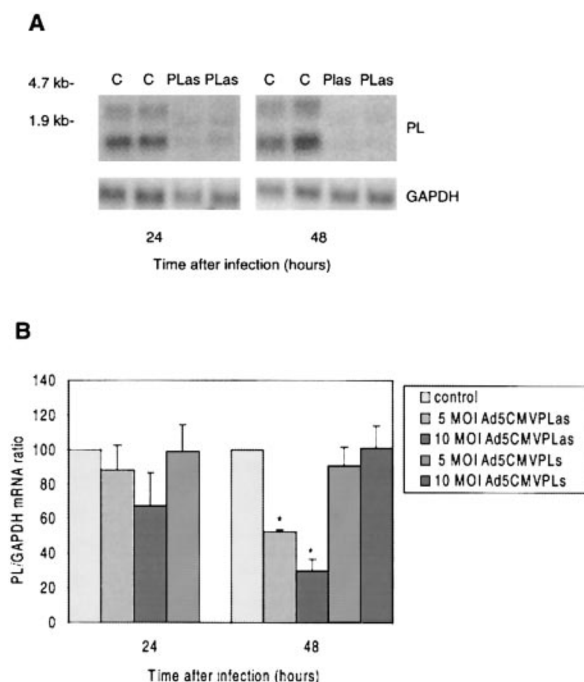

Figure 2. Levels of PL mRNA 24 and 48 hours after infection of cardiomyocytes with Ad5CMVPLas vs Ad5CMVPLs at 2 different doses (MOI 5 and 10). A, Northern blot of a representative experiment (Ad5CMVPLas at MOI 10). C indicates incubation without virus, and duplicate measurements are shown. Markers 18S and 28S are indicated on left. B, Graphical representation of mean PL/GAPDH mRNA ratios expressed as % (±SEM) of control cells obtained from ≥4 independent experiments. *$P<0.05$ vs control cells.

separated with an SDS-PAGE 7.5% to 15% gradient gel and blotted onto PVDF (Amersham). Blots were incubated with monoclonal anti-PL (1:2500) (clone 20/2 [Affinity Bioreagents]) or anti-SERCA2 (1:1000) (clone 2A7-A1, BIOMOL Research Laboratory) as primary antibody followed by ^{125}I-labeled goat antimouse IgG (1:1000) as secondary antibody. After washing, blots were quantified in the molecular imager. The major band (pentameric form of PL, PL_V) was quantified.

Ca^{2+} Affinity of the SR Ca^{2+} Pump

Oxalate-dependent and thapsigargin (1 μmol/L)–sensitive Ca^{2+} uptake activity at different free Ca^{2+} concentrations was measured in infected and noninfected cardiomyocyte homogenates.[22] Briefly, cardiomyocytes were frozen in liquid N_2 and homogenized in 100 μL 0.3 mol/L sucrose, 20 mmol/L imidazole (pH 7.0), and 10 mmol/L $Na_2S_2O_5$ (to stabilize SR Ca^{2+} pump[23]) with a microdismembrator (Braun). Protein (20 μg) was preincubated at 37°C for 2 minutes in 410 μL 2.5 mmol/L ATP, 50 mmol/L KCl, 10 mmol/L imidazole (pH 7.0), 2.5 mmol/L $MgCl_2$, 5 mmol/L potassium oxalate, and 10 μmol/L ruthenium red. The reactions were started by addition of 40 μL $^{45}Ca^{2+}$ (0.2 Ci/mmol)–EGTA (1 mmol/L) buffers, of which the pCa's were 7, 6.5, 6, and 5. The free Ca^{2+} concentrations were calculated by the computer program WinMAXC version 1.75 (www.stanford.edu/~cpatton) with the following dissociation constants: Ca^{2+}-EGTA, 3.88×10^{-7} mol/L; Ca^{2+}-ATP, 8.16×10^{-5} mol/L; Mg^{2+}-EGTA, 3.10×10^{-2} mol/L; and Mg^{2+}-ATP, 6.86×10^{-5} mol/L. Aliquots (100 μL) were filtered after 1, 2, and 4 minutes through 0.45-μm Millipore filters, and ^{45}Ca was measured by liquid scintillation counting.

Ca^{2+} Transients of Cardiomyocytes

Infected and noninfected beating cardiomyocytes were cultured on 5-cm^2 glass coverslips in 10-cm^2 dishes, washed with HEPES-buffered salt solution (HBSS) containing (in mmol/L) NaCl 125, KCl 5, $MgSO_4$ 1, KH_2PO_4 1, $CaCl_2$ 2.5, $NaHCO_3$ 10, HEPES 20, and probenecid 2.5, pH 7.4, and loaded in HBSS containing 2 μmol/L fura 2-AM (Molecular Probes, Eugene) and 5 mmol/L glucose for 45 minutes at 37°C. Subsequently, the cells were washed, and the coverslip was fitted in a Teflon culture dish,[24,25] incubated in 1 mL 5 mmol/L glucose containing HBSS in a thermostatted chamber, and mounted on the stage of a fluorescence microscope (Leitz Diavert). Fluorescence was recorded at 360- and 380-nm excitation wavelengths with a 490-nm high-pass filter in the emission path with a sensitive video camera (Hamamatsu C2400-08). The mean gray level of video line segments enclosed by the region of interest was sampled at a frequency of 50 Hz by computer. First, the dark current of the camera was recorded and stored, for which fluorescence intensities were corrected. Second, fluorescence at 360 nm was recorded, by which fluorescence at 380 nm was divided and stored as F_{380}/F_{360} ratios. These ratios produce signals nonlinearly related to $[Ca^{2+}]_i$. The time to 50% recovery of the Ca^{2+} transient, but not the time to peak of the Ca^{2+} transient, was dependent on beating frequency. Therefore, myocytes were always stimulated electrically at 1 Hz by 2 platinum electrodes.

Statistical Analysis

One-way ANOVA was performed, followed by multiple comparison by Student-Newman-Keuls test, with the statistical computer program SigmaStat. Significance was set at $P<0.05$ for ≥4 independent experiments.

Results

Cellular PL mRNA and Protein

Cardiomyocytes were infected with 2 different doses of the vectors Ad5CMVPLas and Ad5CMVPLs (MOI 5 and 10) to study their effects on PL expression. A marked and vector-dose–dependent reduction in PL mRNA is apparent after 24 hours of infection with Ad5CMVPLas (88±14% and 68±19% left at MOI 5 and 10, respectively) and becomes more pronounced after 48 hours (53±1% and 30±7% left, respectively) (Figure 2). Subsequently, we examined the effect of the CMV vectors on PL protein. A significant decrease in PL protein content was first visible 48 hours after infection with Ad5CMVPLas (90±11% [P=NS], 63±3%, and 55±6% left at MOI 2.5, 5, and 10, respectively), becoming more pronounced after 72 hours (69±9%, 42±10%, and 24±3% left, respectively) (Figure 3). Like control cells, incubation with Ad5CMVPLs virus produced no effects, indicating specificity of the antisense effect.

Cellular SR Ca^{2+} Pump Activity and SERCA2 Protein

We measured oxalate- and ATP-dependent Ca^{2+} uptake in homogenates of control and infected cardiomyocytes at different nonsaturating free $[Ca^{2+}]_i$ (pCa 5 to 7). No significant effect of Ad5CMVPLas (MOI 10) on Ca^{2+} uptake activity was seen 48 hours after infection (Figure 4A), but at 72 hours, the relative Ca^{2+} uptake activity at pCa 6.5 was significantly increased compared with control and Ad5CMVPLs-infected cells (Figure 4B). No effect of Ad5CMVPLas was seen at pCa 6, when Ca^{2+} uptake activity is closer to maximum reached at pCa 5. After 72 hours, Ad5CMVPLas reduced maximal Ca^{2+} pump activity, which cannot be explained by cell loss, because total protein content remained unchanged (legend to Figure 4). A partial reduction of SERCA2 protein after 72 hours of Ad5CMVPLas infection (57.9±6.4% left,

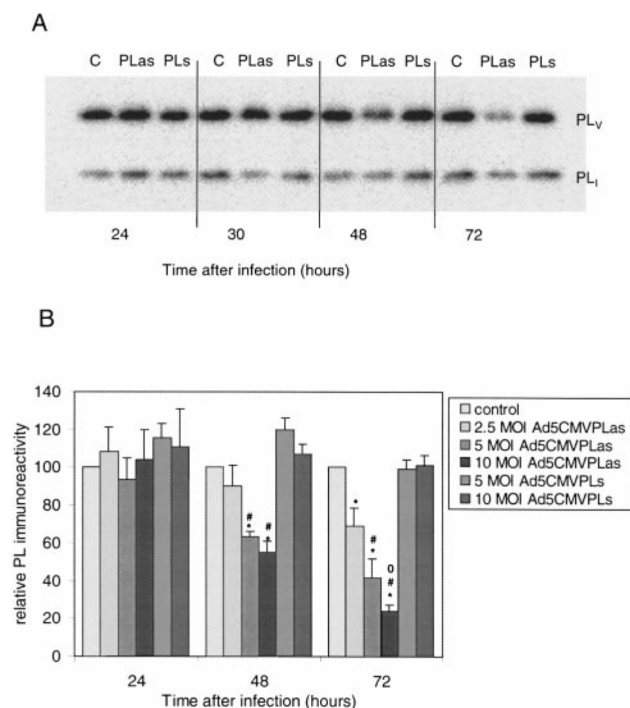

Figure 3. Levels of PL protein 24, 30, 48, and 72 hours after infection of cardiomyocytes with Ad5CMVPLas vs Ad5CMVPLs at 2 different doses. A, Western blot of a representative experiment at MOI 10. C indicates incubation without virus. B, Graphical representation of mean PL protein levels expressed as % (±SEM) of uninfected cells obtained from ≥5 independent experiments. $^*P<0.05$ vs control cells, $\#P<0.05$ vs Ad5CMVPLas (MOI 2.5), $0P<0.05$ vs Ad5CMVPLas (MOI 5).

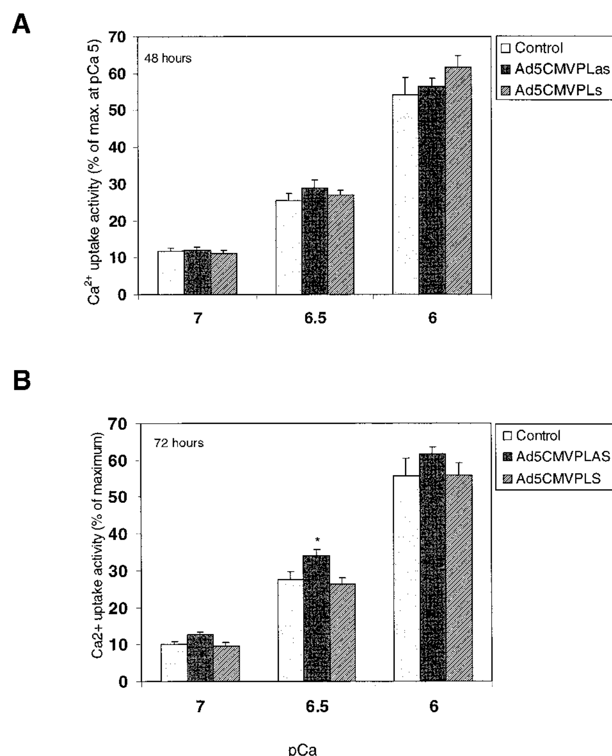

Figure 4. SR Ca^{2+} uptake activity as % of maximal after 48 and 72 hours in noninfected and Ad5CMVPLas- or Ad5CMVPLs-infected (both MOI 10) cardiomyocytes. Maximal activities at pCa 5 (nmol $Ca^{2+}\cdot$ mg protein$^{-1}\cdot$ min^{-1}) 48 hours after infection were control, 12.80±1.90; Ad5CMVPLas, 13.0±0.99; and Ad5CMVPLs, 15.20±2.38; and 72 hours after infection, control, 12.63±1.00; Ad5CMVPLas, 7.52±0.60 ($P<0.05$ vs control and Ad5CMVPLs); and Ad5CMVPLs, 16.70±1.84 (P=NS vs control and $P<0.05$ vs Ad5CMVPLas). Protein contents (mg/mL) of homogenates 48 hours after infection were control, 2.01±0.37; Ad5CMVPLas, 2.39±0.26; and Ad5CMVPLs, 2.44±0.28; and 72 hours after infection, control, 2.52±0.12; Ad5CMVPLas, 2.91±0.15; and Ad5CMVPLs, 2.99±0.10. Values are mean±SEM of samples from 6 (48 hours) or 5 (72 hours) independent experiments. $^*P<0.05$ vs control and Ad5CMVPLs-treated cells.

$P<0.05$ versus uninfected or Ad5CMVPLs-infected cells) was observed, however, which may explain the decrease of maximal Ca^{2+} uptake activity (Figure 5).

Ca^{2+} Transients After Infection With Ad5CMVPLas

To determine whether depletion of PL protein by Ad5CMVPLas also alters the duration of the Ca^{2+} transient of intact cardiomyocytes, the specific fluorescence of fura 2–loaded cells was recorded at fixed beating frequency (1 Hz). Time to 50% recovery of Ca^{2+} transients was already lower after 48 hours in Ad5CMVPLas (MOI 5)–infected cells (245±98 ms; n=42, $P<0.05$ versus uninfected and Ad5CMVPLs-infected cells) compared with uninfected (593±88 ms; n=4) and Ad5CMVPLs-infected cells (564±38 ms; n=7), indicating that by decrease of PL content, the removal rate of Ca^{2+} from myofilaments during relaxation of the cardiomyocytes is increased (see representative average Ca^{2+} transient recordings in Figure 6).

Cellular PL Protein After Infection With Ad5ANFPLas

The ANF promoter fragment used was first tested for ET-1 responsiveness and displayed low activity in nonstimulated gene-gun–transfected cardiomyocytes.[18,19] On ET-1 (10^{-8} mol/L) stimulation, the promoter activity increased 2.5-fold.[18] Figure 6 depicts PL protein expression obtained 48 and 72 hours after infection with Ad5ANFPLas (MOI 10) with or without ET-1 stimulation. Earlier, we showed that in unstimulated cells, ANF mRNA is present in low but significant amounts.[13,19] This explains the decrease in PL protein that was measured in Ad5ANFPLas-infected cells also in the absence of ET-1 (Figure 7, at 72 hours, 86±6% left versus noninfected cells). However, Ad5ANFPLas in the presence of ET-1 resulted in a significant further reduction of PL protein (72±7% left). Ad5ANFPLs had no effect, indicating specificity of the antisense effect.

Discussion

Infection of cardiomyocytes with Ad5CMVPLas resulted in a dramatic decrease in endogenous PL mRNA expression after 48 hours, presumably by increased degradation of the message. The vector generating partial sense mRNA and perhaps truncated PL protein clearly had no effect on endogenous PL mRNA or protein, indicating the specificity of the antisense effect. Downregulation of PL protein

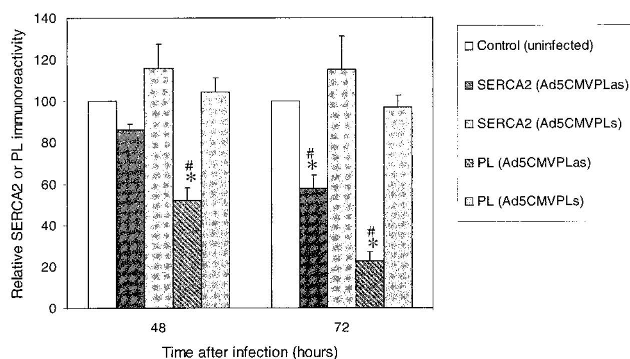

Figure 5. Levels of SERCA2 (and PL) protein 48 and 72 hours after infection of cardiomyocytes with Ad5CMVPLas vs Ad5CMVPLs at MOI 10. Protein levels are expressed as % (±SEM) of uninfected cells obtained from 6 independent experiments. **P*<0.05 vs control cells, #*P*<0.05 vs Ad5CMVPLs infected cells.

lags ≈24 hours behind that of PL mRNA, indicating that no compensatory posttranscriptional mechanisms are present.

Downregulation of SERCA2 in hypertrophied and/or failing myocardium is believed to play a role in the diastolic dysfunction. Several studies reported downregulation of PL in failing heart as well, but generally not as much as SERCA2.[4,5] Because PL in anomalous phosphorylation states may also occur in severe heart failure,[26] antisense suppression of the endogenous PL gene may have an advantage over the enhancement of SERCA2 gene expression. Gene enhancement will not influence preexisting malfunctioning endogenous gene products, whereas antisense-based gene suppression can do so. The reduced expression of PL caused by Ad5CMVPLas results in increased Ca^{2+} affinity of the SR Ca^{2+} pump and in decreased time to 50% recovery of the Ca^{2+} transients of intact cells. This is consistent with a very recent report by He et al,[27] who showed increased shortening as well as relengthening velocity in adult rabbit cardiomyocytes infected with an adenovector expressing a dominant negative mutant PL protein. However, in our present study on monolayer cultures of cardiomyocytes, we were unable to measure relative shortening of the cells during stimulation, because rhythmic displacement of cell boundaries is hard to relate to initial length of the myocytes concerned. Moreover, extrapolation of results on contractile motion of unloaded myocytes to the in vivo situation is doubtful.

Ad5CMVPLas infection (72 hours) of cardiomyocytes not only increased the Ca^{2+} affinity of SR Ca^{2+} pump but also partially reduced its maximal activity. The latter effect is probably due to decreased SERCA2 expression (Figure 5). At present, it is unclear whether this adenovector effect indicates counterregulatory systems in cardiac Ca^{2+} homeostasis.

Conversely, by suppression of endogenous PL protein, the β-adrenergic response becomes blunted. In failing myocardium, however, β-adrenoceptors are reduced and the associated regulatory components functionally altered, causing the attenuated β-adrenergic response.[2] Interestingly, myocytes isolated from hearts of PL-knockout mice retained the relaxant effect evoked by catecholamine.[28] Troponin I phosphorylation may increase the "off rate" for Ca^{2+} exchange, with troponin C compensating for the effect of PL ablation on the β-adrenergic response.

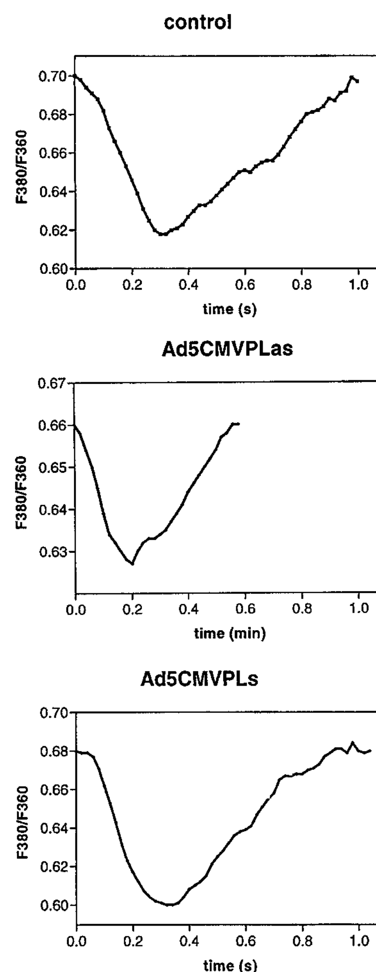

Figure 6. Ca^{2+} transients, represented by time-dependent changes in F_{380}/F_{360} of fura 2–loaded cardiomyocytes stimulated at 1 Hz, of uninfected cardiomyocytes (top) and cardiomyocytes infected with Ad5CMVPLas (middle) and Ad5CMVPLs (bottom) at MOI 5 for 48 hours. To improve signal-to-noise ratio, 5 to 10 subsequent Ca^{2+} transients were averaged per panel. Times to 50% recovery of Ca^{2+} transients are 719 ms (top), 398 ms (middle), and 615 ms (bottom). Note that F_{380}/F_{360} is an inverse measure of intracellular free Ca^{2+} concentration.

Our development of inducible ANF promotor vectors for induction-by-disease gene therapy takes advantage of the fact that the cardiac hypertrophic phenotype in vivo and in vitro is characterized by transcriptional upregulation of distinct genes.[3,5,12,13,19,20] Unlike the in vivo situation, a low expression of ANF is present in nonstimulated cardiomyocytes,[12–14,19] which explains the small reduction by treatment with Ad5ANFPLas even in the absence of ET-1. In the presence of ET-1, however, a stronger reduction of PL protein was observed, consistent with the known ET-1 responsiveness of the ANF promoter.[12–14] These results are a first demonstration in cardiomyocytes of the feasibility of using a heart-specific promoter that becomes activated when the cells become hypertrophic. Another advantage is the expected return of this promoter to minimal activity

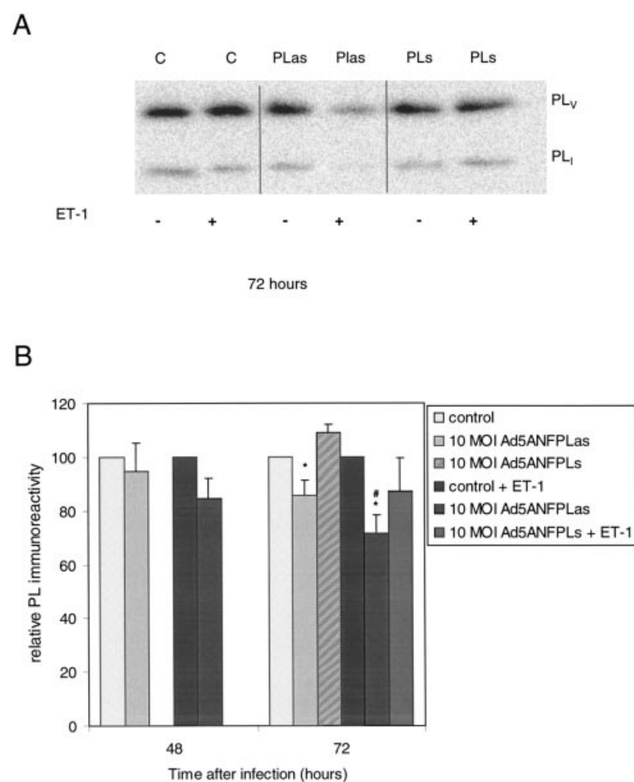

Figure 7. Levels of PL protein 48 and 72 hours after infection of cardiomyocytes with Ad5ANFPLas vs Ad5ANFPLs in absence and presence of ET-1. A, Western blot of representative experiment. Cells were uninfected (C) or infected at MOI 10 for 72 hours in presence (+) or absence (−) of ET-1 (10^{-8} mol/L). B, Graphical representation of mean PL protein levels expressed as % (±SEM) of corresponding control cells (unstimulated noninfected and ET-1–stimulated uninfected, respectively) obtained from 5 independent experiments. *$P<0.05$ vs unstimulated uninfected cells, #$P<0.05$ vs unstimulated cells infected with Ad5ANFPLas.

when the myocytes improve in contractile function because of treatment. Knowlton et al[29] tested the same rat ANF promoter in transgenic mice with hypertrophy induced by pressure overload. In this model, the rat ANF promoter did confer cell-specific expression but not inducibility on pressure overload. Possibly, transgenic mice may compensate for the effects of the transgene during development, or species difference of rat promoter versus transgenic mice may be responsible for the lack of inducibility.[29] Hajjar et al[30] first described a method yielding high adenovector transfer rates in vivo specifically to the rat ventricles. We could confirm those findings using an adenovector expressing green fluorescent protein, thus opening a way to test the PL antisense adenovectors in vivo.[31] In conclusion, the present study suggests that vector-mediated PL antisense RNA expression may become a feasible approach to improve contractile dysfunction of the failing heart. The inducible ANF promoter offers the perspective for induction-by-disease gene therapy by selective expression of therapeutic genes in the hypertrophied and failing myocytes.

Acknowledgments

This work was supported by grant 95.109 from the Netherlands Heart Foundation and by the Deutsche Forschungsgemeinschaft through a Heisenberg fellowship to Dr Poller, grants Po. 378/2-1,2.

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