The expression of canine cardiac phospholamban in heterologous systems

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A synthetic phospholamban gene has been cloned and expressed in *Escherichia coli*, producing both native phospholamban and a fusion protein with 81 amino acids of the influenza virus NS1 protein. Both the native phospholamban and fusion proteins produced extensive cell lysis upon induction of gene expression, but only the native protein underwent spontaneous pentamer formation in *E. coli*. Translation *in vitro* of mRNA produced by transcription *in vitro* of phospholamban cDNA was used to demonstrate the spontaneous aggregation of phospholamban to form pentamers in this system also, both in the presence and absence of exogenous microsomes from canine pancreas or heart. Phospholamban produced by translation *in vitro* was apparently susceptible to proteolysis by enzymes present in the particulate fractions in these experiments.

INTRODUCTION

Phospholamban is an integral membrane protein expressed in the sarcoplasmic reticulum (SR) of cardiac (Tada *et al.*, 1974), slow skeletal (Jorgensen & Jones, 1986) and some types of smooth muscle (Raeymaekers & Jones, 1986). When phosphorylated by cyclic AMP-dependent protein kinase, phospholamban stimulates the activity of the Ca^{2+} -stimulated Mg^{2+} -ATPase pump of cardiac SR (Tada *et al.*, 1974). This phenomenon provides one explanation in heart muscle for the effect of agents which elevate cyclic AMP levels, i.e. principally the increased rate of relaxation observed in the heart (Huggins & England, 1985).

Phospholamban has a subunit molecular mass of 6080 Da (Fujii *et al.*, 1987). Primary sequence analysis has shown that each subunit consists of one hydrophilic and one hydrophobic domain (Simmerman *et al.*, 1986; Fujii *et al.*, 1987), the latter being large enough to span the phospholipid bilayer of the SR. On SDS/polyacrylamide gels, phospholamban appears with a range of molecular masses, depending on how the protein is solubilized (Kirchberger & Antonetz, 1982; Wegener & Jones, 1984; Huggins & England, 1987). This behaviour has been interpreted to indicate that the protein has a pentameric structure (Wegener & Jones, 1984), and a model has been generated whereby the monomers are organized into a channel-like pentameric structure in the phospholipid bilayer of the membrane, so that hydrophilic amino acids within the hydrophobic domains protrude into the interior of the putative channel (Simmerman *et al.*, 1986). Ca^{2+} channel activity can be detected in reconstituted preparations of purified phospholamban (Kovacs *et al.*, 1988).

A major problem in analysing the tertiary and quaternary structure of phospholamban, or in performing biophysical studies on the way in which its phosphorylation affects the SR Ca^{2+} pump, has been to isolate SR

in high yields from cardiac muscle (see Fowler *et al.*, 1989) and to purify the protein in a native form. Concerning protein purification, several methods have now been published where phospholamban is denatured in strong detergents or organic solvents (e.g. Le Peuch *et al.*, 1980; Capony *et al.*, 1983; Inui *et al.*, 1985), and the only method which uses mild detergents and reports a homogeneous product (Jones *et al.*, 1985) may purify a partially proteolysed fragment (Simmerman *et al.*, 1986). With these problems in mind, we embarked on studies to attempt to express cardiac phospholamban in heterologous systems with the eventual aim of creating an enriched and abundant source for subsequent purification and reconstitution studies, and to study the behaviour of the protein when expressed in membranes other than cardiac SR. We have succeeded in expressing synthetic DNA encoding canine cardiac phospholamban in *Escherichia coli*, where the expressed phospholamban spontaneously inserts into the bacterial membrane. This event appears to cause cell death. In addition, a cDNA clone containing a region encoding phospholamban and also 3'- and 5'-untranslated regions have been expressed in a reticulocyte lysate expression system *in vitro*, in the presence of pancreatic membranes or cardiac SR. In all systems tested, phospholamban was able to spontaneously aggregate into pentamers, suggesting that this property is intrinsic to the phospholamban molecule and is independent of the phospholipid composition of the membranes into which it spontaneously inserts.

EXPERIMENTAL

Construction of synthetic phospholamban gene

Six synthetic oligomers were prepared using an Applied Biosystems DNA synthesis machine and reagents according to the manufacturers' instructions. The oligomer sequences correspond to stretches between arrow heads in Fig. 1. Synthetic oligomers were purified using poly-

Abbreviations used: SR, sarcoplasmic reticulum; PAGE, polyacrylamide-gel electrophoresis.

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Fig. 1. Synthetic phospholamban expression plasmids

The DNA sequence of the synthetic phospholamban gene used in this study is shown (b). Arrowheads indicate the limits of individual oligomers which were synthesized, annealed and ligated as described in the Experimental section. Restriction sites were introduced using conservative nucleotide substitutions and are underlined. The initiation ATG of phospholamban is indicated by double underscoring within the *NdeI* site of the synthetic gene. As shown in the upper part of the Figure, insertion of the synthetic gene into *NcoI*-*XhoI* cut pMG1 yielded pSK24, which encodes an NS1-phospholamban fusion protein (see text for details). Digestion of pSK24 with *NdeI* and circularization of the large fragment with DNA ligase yielded pSK32, in which native phospholamban is expressed directly.

acrylamide-gel electrophoresis (PAGE) and electroelution (Gait, 1984). Oligomers were phosphorylated using T4 polynucleotide kinase (New England Biolabs), and 5 µg of each oligomer was mixed and annealed in 100 µl of TM buffer [50 mM-Tris (pH 7.4)/10 mM-MgCl₂]. This yielded 30 µg of the total double-stranded sequence shown in Fig. 1, which was then available for cloning as an *NcoI*-*XhoI* insert in the plasmid pMG1. Restriction digests and ligations were performed using enzymes supplied by BRL using standard conditions (Maniatis *et al.*, 1982). The ligated DNA was transformed into *E. coli* strain AR58 (Gross *et al.*, 1985), and plasmids bearing the required insert were identified by restriction mapping of DNA prepared by the method of Holmes & Quigley (1981).

Cloning of phospholamban cDNA

RNA was prepared from canine heart by extraction of total RNA in guanidinium isothiocyanate, followed by phenol/chloroform extraction, ethanol precipitation of

total nucleic acids, and selective precipitation of RNA by 4 M-LiCl (Maniatis *et al.*, 1982). mRNA was purified by affinity chromatography on oligo(dT)-cellulose and cDNA was synthesized using the Amersham cDNA synthesis kit. The cDNA was cloned in λgt11 using *EcoRI* linkers (New England Biolabs) by the method of Young & Davis (1983). A synthetic DNA probe of 21 nucleotides (sequence: 5'-ACGTGCTTGTTGAGGCATTTC-3') was prepared from the data of Fujii *et al.* (1987) and used to probe approx. 5×10^5 recombinant plaques. Positive plaques appeared at a frequency of approx. 10^{-3} in the canine heart bank, and those containing the longest hybridizing inserts were isolated and retained for further study. The sequence of the cloned phospholamban gene was determined by single-stranded M13 sequencing using an Amersham sequencing kit.

E. coli expression experiments

E. coli strain AR58 contains the *cl857ts* mutation, which renders the pL promoter temperature-sensitive for expression (Gross *et al.*, 1985). For *E. coli* expression experiments, transformants were grown overnight with shaking 32 °C in Luria broth + ampicillin (Maniatis *et al.*, 1982) and inoculated into fresh medium at a 10^{-2} dilution at the start of each experiment. The cells were allowed to grow to an $A_{1\text{ cm}, 600}$ of 0.6 at 32 °C with shaking, and then transferred to a shaking water bath at 42 °C. Aliquots (1 ml) were withdrawn at timed intervals after transfer to 42 °C, pelleted in an Eppendorf centrifuge and either resuspended in Laemmli (1970) loading buffer (10 µl of loading buffer per 0.1 $A_{1\text{ cm}, 600}$ of cells) or suspended in phosphorylation buffer for analysis.

Translation of phospholamban mRNA *in vitro*

An *EcoRI* fragment from a λgt11 clone of phospholamban was isolated which contained 237 bp of 5'-untranslated and 257 pb of 3'-untranslated sequence in addition to the phospholamban coding region. This fragment was subcloned in the transcription vector pSPT18 (Pharmacia), and transcribed *in vitro* to give capped mRNA using a Strategene capping kit and T7 RNA polymerase. RNA quality was assessed by agarose-gel electrophoresis and the quantity was determined spectrophotometrically.

Translation experiments *in vitro*

Translation experiments *in vitro* were undertaken using commercially supplied rabbit reticulocyte lysates (Amersham). Translation reactions contained 1.5 µg of mRNA, 10 µl of reticulocyte lysate, 10 µl of microsomes or 10 µl of microsome buffer (184 mM-potassium acetate/1.1 mM-magnesium acetate), 4 µl of [³⁵S]methionine (40 TBq/µmol, Amersham) in a total volume of 30 µl. For polyacrylamide gels, 2.5 µl of reticulocyte lysate products was mixed with 5 µl of sample loading buffer and run in 15% polyacrylamide gels according to the method of Laemmli (1970). Dog pancreas microsomes were obtained from Amersham, and SR vesicles were presented to reticulocyte lysates in microsome buffer at a concentration of approx. 1 mg of protein/ml.

Other methods

Canine cardiac sarcoplasmic reticulum was isolated as described by Chamberlain *et al.* (1983), as modified by Fowler *et al.* (1989). The 'top' fraction of SR, representing those membranes with the highest specific activity

of ATP-dependent Ca^{2+} uptake, were used. The conditions for phosphorylating *E. coli* protein with [γ - ^{32}P]ATP were as described by Huggins *et al.* (1989) except that $2\ \mu\text{M}$ -cyclic AMP and 25 pmol of cyclic AMP-dependent protein kinase/min (assayed using the phosphate acceptor peptide, malantide) were used in these experiments rather than cyclic GMP and its protein kinase. SDS/polyacrylamide gels were run according to Laemmli (1970). Autoradiography was performed using either Kodak X-Mat or Amersham β -Max films.

RESULTS AND DISCUSSION

Two approaches were taken to the heterologous expression of phospholamban in the present work. A synthetic gene was prepared using the sequence described by Fujii *et al.* (1987), which was modified to incorporate convenient restriction sites for DNA sequence manipulation (Fig. 1). The reported cDNA sequence of phospholamban is unusual among integral membrane proteins in lacking an *N*-terminal signal sequence, membrane insertion apparently being mediated solely by the hydrophobic *C*-terminus residues. This unusual characteristic prompted us to isolate canine cardiac phospholamban cDNA. We were able to confirm the original published sequence (Fujii *et al.*, 1987) and the absence of potential signal information in 237 bp of 5'-untranslated cDNA (results not shown). The synthetic gene was used for heterologous expression studies in *E. coli* *in vivo* because of its inbuilt ease of manipulation, while the cDNA was used in translation experiments *in vitro*, because of the presence of appropriate 5'- and 3'-untranslated regions in addition to the phospholamban coding sequence.

Heterologous expression of the synthetic phospholamban gene was arranged by its insertion into the expression vector pMG1 (Fig. 1). The plasmid pMG1 is derived from pMG27 (Gross *et al.*, 1985) by the insertion of a modified DNA sequence encoding the first 101 amino acids of the influenza virus protein NS1 (Young *et al.*, 1983). Expression of the NS1 sequences in pMG1 is driven by the λ pL promoter and is heat-inducible in *E. coli* strain AR58, which contains the temperature-sensitive repressor *cI857ts* (Gross *et al.*, 1985). Gene expression from this vector is thus controlled by a temperature shift from 32 to 42 °C (see the Experimental section).

In the phospholamban expression plasmid pSK24, the synthetic phospholamban gene was introduced as a *Nco*I–*Xho*I fragment into the large *Nco*I–*Xho*I fragment of pMG1 (Fig. 1). This plasmid encoded a protein which consisted of the first 81 amino acids of the NS1 protein linked as an *N*-terminal fusion to phospholamban by the dipeptide valylhistidine.

When AR58 cells bearing the pSK24 plasmid were shifted to 42 °C under appropriate conditions, a protein of approx. 16 kDa, corresponding to the expected molecular mass of the NS1-phospholamban fusion protein, was clearly induced (Fig. 2). It should be noted in Fig. 2 that there is an apparent induction of several other proteins in addition to the fusion protein. These bands are all *E. coli* proteins which increase in abundance after temperature shift, regardless of proteins specified by the expression vector. One of these *E. coli* proteins, however, of just over 67 kDa, is more strongly induced in the presence of the fusion construct than in control cells or in cells producing mature phospholamban (results not

Fig. 2. Time course of induction of NS1-phospholamban fusion protein

Cells of strain AR58 were transformed with pSK24 and grown to $A_{1\text{cm},600}$ of 0.6 in Luria broth at 32 °C. The incubation temperature was then increased to 42 °C and samples were withdrawn at intervals for submission to SDS/PAGE (see the Experimental section for details). The Figure shows total protein isolated from transformants at the following times after temperature shift induction: lane a, 0 min; b, 30 min; c, 60 min; d, 120 min; e, 240 min; f, 23 h. The gel was stained using Coomassie Blue. Molecular masses of protein standards are shown.

shown). Nonetheless, the fusion protein is clearly expressed at a high level in *E. coli*.

The plasmid pSK24 was designed in such a way that excision of a small *Nde*I fragment and re-ligation of the large fragment yielded pSK32, in which the expression product is native phospholamban (see legend to Fig. 1). When cells carrying pSK32 were subjected to a temperature shift, no band could be seen in Coomassie Blue-stained total protein extracts which corresponded in molecular mass to phospholamban. Expression of native phospholamban in *E. coli* was therefore monitored by subjecting cell pellets to treatment with 1% Triton X-100/10 mM-Hepes, pH 7.4, and phosphorylating the extract in the presence of cyclic AMP-dependent protein kinase and [γ - ^{32}P]ATP (see the Experimental section).

Fig. 3 shows the results of phosphorylating whole-cell extracts, taken at intervals after induction of the expression of pSK32. At times in excess of 1 h after induction, several phosphorylated proteins were detectable, with a heavily labelled band appearing in the region of the gel which should be occupied by native phospholamban (Fig. 3, tracks a–e). Identification of the lower molecular mass band as phospholamban was confirmed

Fig. 3. Phosphorylation of phospholamban and fusion protein

Cells of strain AR58 were transformed with pSK24 (fusion protein plasmid) or pSK32 (native protein plasmid) and induced to express their encoded protein as described in the legend to Fig. 2. Aliquots (1 ml) were withdrawn at the stated intervals, cells were pelleted in an Eppendorf Microfuge and the pellets were phosphorylated in the presence of cyclic AMP-dependent protein kinase and [γ - 32 P]ATP as described in the text. The products of this reaction were submitted to SDS/PAGE and autoradiographed. Tracks a-e: time course of induction of pSK32 (native protein) after 0 (a), 30 (b), 60 (c), 120 (d) and 240 (e) min. Tracks f-i show phosphorylation after 60 min of induction of pSK32 (f, g) or pSK24 (h, i). Samples were solubilized in SDS at 100 °C (tracks f and h) or at 30 °C (tracks g and i).

by electrophoresis of the 60 min sample both with (track f) and without (track g) boiling the samples, whereupon a molecular mass shift characteristic of phospholamban was observed. Interestingly, the NS1-phospholamban fusion protein is a substrate for cyclic AMP-dependent kinase, but does not show the aggregatory properties of the native molecule (tracks h and i). It is also noteworthy that more than 30 min elapsed after induction before phosphorylated material was visible. The culture $A_{1\text{ cm},600}$ of cells expressing native phospholamban or the phospholamban-NS1 fusion protein dropped significantly in the first 2 h after temperature shift. Viable cell counts in cultures producing native phospholamban dropped to 6% of their pre-shift levels in this period, whereas viable cell counts of cells bearing the parental plasmid (expressing the NS1 protein) increased by 270% under identical conditions. This implies that extensive cell lysis accompanies the expression of phospholamban gene products in *E. coli*, and that it is this lysis, rather than Triton X-100 solubilization, which releases both *E. coli* proteins and phospholamban products to act as substrates for cyclic AMP-dependent protein kinase.

Heterologous expression of phospholamban in *E. coli* therefore demonstrates spontaneous aggregation of phospholamban monomers to the pentamers observed in SR membranes (Wegener & Jones, 1984). The observed cell lysis is suggestive of spontaneous membrane penetration and, ultimately, disruption. This observation may support the notion that phospholamban exists as an ion-channel protein (Simmerman *et al.*, 1986; Kovacs *et al.*, 1988). The spontaneous assembly of pentamers and membrane interactions of phospholamban were further

investigated by translation experiments *in vitro* carried out in the presence of exogenous microsome preparations.

In these experiments, a cDNA encoding phospholamban with 237 bp of 5'-untranslated and 257 bp of 3'-untranslated sequence was inserted into a commercially obtained transcription vector and used to generate large amounts of capped mRNA, which were then subjected to translation *in vitro* (see the Experimental section). Fig. 4 shows the products obtained by translation of phospholamban mRNA *in vitro* under various conditions, after SDS/PAGE. A major protein of approximate molecular mass of 6 kDa is clearly expressed in the absence or presence of pancreatic microsomes or cardiac SR. That this protein is phospholamban is again demonstrated by the appearance of a higher molecular mass band when incubation at 100 °C prior to electrophoresis was omitted (Fig. 4, tracks b, d and f). In these experiments, complete transformation of the monomeric to the pentameric form of phospholamban did not occur. This is probably because only small amounts of protein were present in electrophoresis samples, and phospholamban monomer formation is favoured at high detergent/protein ratios (Le Peuch *et al.*, 1980; J. Huggins, unpublished work). Three additional phenomena are apparent as follows.

(1). SR vesicles dramatically reduced translation efficiencies *in vitro*. This may reflect the presence of proteinases or RNAases contaminating or intrinsic to the SR membrane.

(2). An apparently larger proportion of phospholamban translated *in vitro* is present as pentamers in the dog pancreas microsome preparation (Fig. 4, lane d)

Fig. 4. Translation of the mRNA encoding phospholamban

Approx. 1.5 μ g of capped phospholamban mRNA was added to a rabbit reticulocyte translation system as described in the Experimental section, and the translation products were labelled using [35 S]methionine. A portion (15%) of each reaction was loaded on each track of a 15% SDS/PAGE gel, which was stained, fixed and autoradiographed. On a second gel, translated material *in vitro* (i, j) was compared directly with phosphorylated cardiac SR (g, h) prepared as described by Huggins *et al.* (1989). Lane a, no added microsomes, boiled; b, no added microsomes, unboiled; c, dog pancreas microsomes, boiled; d, dog pancreas microsomes, unboiled; e, dog cardiac SR, boiled; f, dog cardiac SR, unboiled; g, 32 P-labelled cardiac SR vesicles, boiled; h, 32 P-labelled cardiac SR vesicles, unboiled; i, no added microsomes, boiled; j, no added microsomes, unboiled.

than in the microsome-free situation (Fig. 4, lane b), although the total expression of phospholamban was not altered by microsome addition (Fig. 4, lanes a and c). This result is unlikely to be caused by a higher protein/detergent ratio in the presence of microsomes because microsome addition altered the protein concentration only marginally. An interesting possibility is that the phospholipid composition of the pancreatic microsome preparation favoured phospholamban pentamer formation. However, the rabbit reticulocyte lysates used for translation *in vitro* do contain membranes, so that the pentamers seen in lane b (Fig. 4) cannot be said to form in the absence of lipid bilayers.

(3). Translation of phospholamban mRNA *in vitro* is marked by the appearance of multiple bands in the 25.7 kDa region of the gel and by the appearance of [35 S]Met labelled material beneath the phospholamban monomer position. The identity of the band at 6.2 kDa as phospholamban was established by comparing the translation products *in vitro* (Fig. 4, lanes i, j) with 32 P-phosphorylated phospholamban from cardiac SR (Fig. 4, lanes g, h). The simplest explanation of the

Fig. 5. Spontaneous insertion of phospholamban into microsomal membranes

Translation experiments with phospholamban mRNA *in vitro* were carried out in the presence (lanes g-l) or absence (lanes a-f) of dog pancreas microsomes as described in the Experimental section. Aliquots (15 μ l) were withdrawn from the reaction tubes and diluted with 30 μ l of SR buffer (1.17 M-sucrose/10 mM-Hepes, pH 7.2). Diluted samples were centrifuged at 350 000 *g* for 15 min in a Beckman TL100 centrifuge to separate soluble and microsomal material. An SDS/15%-polyacrylamide gel was loaded with either total or fractionated material. Quantities of each sample were chosen to yield material from 2.5 μ l of the original '*in vitro*' translation mix. Lane a, total, 100 $^{\circ}$ C; b, total, 30 $^{\circ}$ C; c, pellet, 100 $^{\circ}$ C; d, pellet, 30 $^{\circ}$ C; e, supernatant, 100 $^{\circ}$ C; f, supernatant, 30 $^{\circ}$ C; g, total, 30 $^{\circ}$ C; h, total, 100 $^{\circ}$ C; i, pellet, 100 $^{\circ}$ C; j, pellet, 30 $^{\circ}$ C; k, supernatant, 100 $^{\circ}$ C; l, supernatant, 30 $^{\circ}$ C.

multiple bands in the translation products is that limited proteolysis occurs in this system, although RNA degradation cannot be ruled out as the source of the apparent artifacts. The light band observed just under 14.3 kDa is residual globin synthesized by the lysate system.

The relationship between pentamer formation and membrane insertion was investigated in the experiment outlined in Fig. 5. In this experiment, 35 S-labelled phospholamban was synthesized *in vitro* in the presence or absence of microsomes. Ultracentrifugation was used to separate particulate from soluble material and the results were again analysed using SDS/PAGE.

It is evident from Fig. 5 that phospholamban spontaneously appears in the particulate fraction in the presence and absence of exogenous pancreatic microsomes. However, the proportion of phospholamban product associated with the particulate fraction is greatly increased by including microsomes during translation (Fig. 5; compare tracks d and f, and tracks j and l). This suggests that the membranes present in the reticulocyte lysate are in some way less effective in inducing the spontaneous insertion of phospholamban into membranes than the pancreas microsomes. This effect could

be due to a saturation phenomenon whereby reticulocyte lysate membranes are unable to 'bind' more than a certain proportion of the expressed phospholamban, or could reflect preferential targeting of the protein to the microsomal membranes.

Also apparent from Fig. 5 is that the proportion of phospholamban present in the pentameric form is greater in the particulate fractions (tracks c, d, i and j) than in the supernatants (tracks e, f, k and l). This indicates the importance of the lipid environment in the assembly or stability of pentamers, a feature which is consistent with the channel model of Simmerman *et al.* (1986).

This paper has described for the first time the heterologous expression of phospholamban in bacterial and mammalian expression systems. It has been shown that the phospholamban spontaneously inserts into membranes and is able to form pentamers there, in a manner which is apparently identical to the phospholamban found in cardiac SR. The ability to selectively express phospholamban will be very useful in studying the function of the protein, especially as the protein inserts itself into exogenously added membranes in a translation system *in vitro*.

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