Pharmacological Characterization of Protein Phosphatase Activities in Preparations from Failing Human Hearts

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ABSTRACT

β-Adrenoceptor stimulation acts in the heart in part by increasing the phosphorylation state of phospholamban and phospholemman. There is evidence that the β-adrenoceptor-mediated increase in phospholamban phosphorylation is in part due to inhibition of type 1 phosphatases. The aim of the present study was to elucidate which phosphatases dephosphorylate phospholamban and phospholemman in the human heart. In the past, cardiac serine/threonine phosphatases have been studied using phosphorylase a as substrate. Here, type 1 and type 2A phosphatase activities were studied in preparations from failing human hearts using phosphorylated phospholamban and phospholemman as substrates. Phospholamban and phospholemman phosphatase activity was detectable in human cardiac homogenates. Moreover, using a heparin-Sepharose column, the catalytic subunits of type 1 and type 2A phosphatases could be separated from human ventricles. Okadaic acid and cantharidin inhibited phosphatase activities dephosphorylating phospholamban, phospholemman, and phosphorylase a in homogenates in a concentration-dependent manner. However, okadaic acid was more potent. Cantharidin inhibited type 2A and type 1 activities against all substrates studied with IC50 values < 15 nM and > 290 nM, respectively. Okadaic acid inhibited type 1 and type 2A phosphatase activities as effectively but 10–30 times more potently than cantharidin. This work provides evidence that in the human heart, type 1 and 2A phosphatases are involved in the dephosphorylation of phospholamban and phospholemman and could play a role in the effects of β-adrenergic stimulation in the heart.

In the heart, β-adrenergic stimulation increases force of contraction and enhances relaxation. The underlying biochemical mechanism involves the generation of cAMP and the subsequent activation of cAMP-dependent protein kinase. The cAMP-dependent protein kinase phosphorylates regulatory proteins. This triggers the inotropic, clonitropic, and relaxant effects of β-adrenergic stimulation in the mammalian heart (Simmerman and Jones, 1998). Targets for the cAMP-dependent protein kinase have been identified in the sarcolemma and in the sarcoplasmic reticulum of the heart. This approach identified a major phosphoprotein in the sarcoplasmic reticulum called phospholamban and another major substrate of apparent molecular weight of 15,000 in the sarcolemma later called phospholemman (Jones et al., 1979; Palmer et al., 1991). β-Adrenergic stimulation led to an increased phosphorylation state of phospholamban and phospholemman (Lindemann et al., 1983; Presti et al., 1985a). Other work focused on the function of these proteins and their regulation by phosphorylation. Disruption of the phospholamban gene enhanced basal contractility and hastened relaxation (Luo et al., 1994). Moreover, the positive inotropic effect of β-adrenergic stimulation was greatly attenuated (Luo et al., 1994). Unphosphorylated phospholamban inhibits the activity of the SR-Ca2+-ATPase 2a. Thus, less Ca2+ is pumped into the sarcoplasmic reticulum. Phosphorylation relieves this inhibition. The activity of SR-Ca2+-ATPase 2a is enhanced, and more Ca2+ is pumped into the sarcoplasmic reticulum. This is thought to hasten relaxation (reviewed in Simmerman and Jones, 1998).

Recent evidence suggests that phospholemman can act as an ion channel for chloride or taurine (Moorman et al., 1992, 1995). How its function is regulated by phosphorylation remains to be elucidated.

Previous work indicates that serine/threonine phosphatases of type 1, 2A, 2B, and 2C are present in the heart (Cohen, 1989; DePaoli-Roach et al., 1994). More than 90% of phosphatase activity in the heart is contributed by phosphatase 1 and 2A (Cohen 1989, MacDougall et al., 1991). Studies in rabbit hearts indicate that phospholamban from cardiac membranes can be dephosphorylated by phosphatases of type 1 and type 2A, whereas type 2B and 2C are relatively

ABBREVIATION: Tris, tris(hydroxymethyl)aminomethane.
inhibit cardiac type 1 and even more potently type 2A phosphatases in vitro (Li et al., 1993; Neumann et al., 1993, 1995). Moreover, these phosphatase inhibitors increased the phosphorylation state of phospholamban in isolated cardiomyocytes (Neumann et al., 1993, 1994, 1995) and increased membrane currents (Hescheler et al., 1988). Thus, it is likely that phospholamban is dephosphorylated in the intact heart by type 1 and/or type 2A phosphatases.

Evidence using cell membrane permeant phosphatase inhibitors like okadaic acid, cantharidin, and calyculin A suggests that phosphatases can alter cardiac function by changing the phosphorylation state of cardiac proteins independently of receptor activation. Indeed, phosphatase inhibitors exert positive inotropic effects in nonhuman cardiac preparations and increase the phosphorylation state of phospholamban (Neumann et al., 1993, 1994, 1995). Moreover, we have shown that cantharidin can increase the force of contraction in isolated electrically driven human cardiac preparations (Linck et al., 1996a). This indicates that phosphatase inhibition in the human myocardium can cause a positive inotropic effect and underscores the physiological importance of phosphatases even in the human heart.

However, it is not known whether phosphatases of type 1 or type 2A or both dephosphorylate phospholamban in the human heart. Moreover, it has not yet been reported which phosphatases dephosphorylate phospholemman in any species or tissue. Hence, we studied whether type 1 and type 2A phosphatases are present in the failing human heart and whether they are capable of dephosphorylating key phosphoproteins present in the sarcoplasmic reticulum and in the sarcolemma.

### Experimental Procedures

**Preparation of Human Cardiac Tissue.** Samples were taken from left ventricles of failing human hearts that were explanted in the course of replacement surgery. All patients were male, suffered from idiopathic dilated cardiomyopathy, and their ages ranged from 45 to 57 years. Patients gave informed consent, and the study was approved by the local ethics committee. Medication was comprised of digitalis, diuretics, and angiotensin converting enzyme inhibitors. There is no evidence that these drugs inhibit phosphatase activity as measured in our assay (see below). Moreover, during purification of the catalytic subunits of phosphatases (see below), these drugs are likely lost and should not copurify with the enzyme. Macroscopically visible blood vessels, fatty tissue, and endo- and epicardium were removed from the samples. Samples were then frozen in liquid nitrogen (in most cases within 5 min after explantation). Thereafter, one sample from each heart was homogenized in liquid nitrogen and aliquots (stored at −80°C) were used in additional analysis. The data shown are those from three (Table 1) or from three to four (Table 2) individual hearts. The same hearts were used in Tables 1 and 2. One additional heart was used in Table 2.

**Preparation of Homogenates.** Human left ventricular myocardiun was pulverized in a mortar precooled in liquid nitrogen. The subsequent steps were carried out at 4°C. Five volumes of 50 mM Tris-buffer (pH 7.4) were added to the frozen, pulverized tissue. The powdered tissue was then homogenized three times for 30 s with a Polytron PT-10 (Neumann et al., 1993) at top speed.

**Expression of Recombinant Proteins.** Recombinant canine phospholamban and phospholemman were expressed in insect cells using a baculovirus expression vector system as described (Reddy et al., 1995; Chen et al., 1998). The final concentration of recombinant phospholamban and phospholemman were 1.1 and 0.3 mg/ml, respectively, in a buffer containing 1% (v/v) Triton X-100, 86 mM triethylamine, 80 mM 4-morpholinepropanesulfonic acid, 18 mM glycine, 5 mM dithiothreitol, and 1% (v/v) n-octyl-β-D-glucopyranoside (pH 7.2). Recombinant proteins were diluted 200-fold in 50 mM Tris, 0.1 mM EDTA, and 0.1% (v/v) β-mercaptoethanol for phosphatase studies.

**Preparation of Membrane Vesicles.** The membrane vesicles were prepared as described (Ahmad et al., 1989). One g of frozen myocardium was mechanically pulverized in liquid nitrogen. The following steps were carried out at 4°C. The pulverized myocardium was homogenized in 5 ml of buffer containing 1% mercaptoethanol (1% v/v), 4 mM EDTA (at pH 7.4) three times for 30 s with a Polytron PT 10 (Kinematica AG, Luzern, Switzerland) at maximum speed. The homogenate was then sedimented for 20 min at 14,000g in a precooled centrifuge (Centrifuge T-2176; Kontron Instruments, Milan, Italy). The resulting supernatant was sedimented for 30 min at 45,000g. The sediment obtained from this step was resuspended in buffer 1 containing 0.6 M KCl and sedimented again for 30 min at 45,000g. This step was repeated once. The resulting sediment, obtained after the second sedimentation, was resuspended in 220 μl of buffer 2 containing 1% (v/v) β-mercaptoethanol, 50 mM Tris, and 0.1 mM EDTA, homogenized and stored at −80°C.

**Preparation of Radioactively Labeled Substrates.** Phosphorylation with 0.1 mCi [γ-32P]ATP was carried as published (Zimmermann et al., 1996). To eliminate endogenous enzyme activities, the membrane vesicles were heat treated for 30 min at 65°C. Two hundred μl of diluted phospholamban, phospholemman, or membrane vesicles were included in a total volume of 430 μl of the phosphorylation mixture, resulting in final concentrations of 59 U/ml cAMP-dependent protein kinase A, 23 mM Tris, 1 mM MgO4, 0.903 mM d-f-dithiothreitol, and 0.16% (v/v) β-mercaptoethanol. The reaction was started by adding [γ-32P]ATP and was allowed to proceed at 30°C on a thermomixer (Eppendorf). After a 60-min incubation at 30°C, the reaction was stopped on ice. Radioactivity not incorporated into phospholamban or phospholemman was removed by dialyzing the proteins three times for 6 h in 500 ml of a buffer containing 50 mM Tris, 5 mM EDTA, and 0.1% (v/v) Triton X-100. Radioactivity not incorporated into membrane vesicles was removed by sedimenting the proteins for 5 min at 14,000g and resuspending them in buffer 2. The supernatant was discarded, and the pellet was resuspended in buffer 2. This sedimentation and resuspension was repeated until the radioactivity in the supernatant was <1% of the radioactivity in the pellet. The final pellet was resuspended in 220 μl of buffer 2. Preparation of [32P]phosphorylase a followed a published method (Neumann et al., 1991).

**Gel Electrophoresis.** Incubation was stopped by adding stop solution, which consisted of 62.5 mM tris(hydroxymethyl)aminomethane, 10% (v/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 0.6% (w/v) d-f-dithiothreitol, and a trace of bromophenol blue; pH was adjusted to 6.8. Samples were frozen at −20°C. Sodium dodecyl
TABLE 2
IC_{50} Values (in nM) for inhibition of type 2A and type 1 phosphorylase a, phospholamban, and phospholemman phosphatase activities by okadaic acid and cantharidin

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Okadaic acid</th>
<th>Cantharidin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 2A</td>
<td>Type 1</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>11.3 (1.02–122)</td>
<td>14.7 (3.56–55.6)</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>0.45 (0.11–1.81)</td>
<td>37.5 (16.7–84.3)</td>
</tr>
<tr>
<td>Phospholemman</td>
<td>0.29 (0.19–0.44)</td>
<td>35.1 (19.0–67.3)</td>
</tr>
</tbody>
</table>

Protein phosphatases were purified from human left ventricular tissue on a heparin-Sepharose column (as described in Experimental Procedures). Numbers of experiments: phospholamban, n = 4; phospholemman, n = 4; and phosphorylase a, n = 3. Data are means with 95% confidence intervals in nM. Data are from three to four individual hearts.

Materials. Compounds used were [γ-^32P]ATP (Amersham Buchler, Braunschweig, Germany), okadaic acid (Biotechnology, Cologne, Germany), and phospholemman (LC Laboratories, Woburn, MA). All materials for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad (Munich, Germany). All other chemicals were of analytical quality or best commercial grade available.

Statistics. Data shown are means ± S.E.M. or with 95% confidence intervals in parentheses. Statistical significance was estimated with Student’s t test for unpaired observations; P < .05 was considered significant.

Results

First recombinant phospholamban, recombinant phospholemman, and membrane vesicles from human hearts, which are known to contain phospholamban and phospholemman (Presti et al., 1985a,b), were phosphorylated by cAMP-dependent protein kinase in the presence of radioactive ATP. Samples were separated using gel electrophoresis, and ^32P-labeled proteins were visualized by autoradiography. An autoradiogram (Fig. 1B) shows that phosphoproteins of the expected molecular weights were detectable in membrane vesicles from human hearts. Of note, two bands probably corresponding to phospholamban and phospholemman were phosphorylated in human membrane. The tentative identification of these bands as phospholamban and phospholemman was supported by the fact that boiling of samples before electrophoresis reduced the apparent molecular weight of phospholamban but not of phospholemman, as noted before in rat and canine tissue (Presti et al., 1985a,b). Moreover, we have identified phospholamban before in human hearts using Western blots and specific antibodies (Linck et al., 1996b; Neumann et al., 1997). This finding extends data from canine heart studies that phospholamban and phospholemman are substrates for cAMP-dependent protein kinase in human cardiac membrane vesicles. These data are compatible with the view that phospholemman expression in human heart is substantially lower than phospholamban expression. Recombinant phospholamban also exhibited the expected molecular weight change (reviewed in Simmerman and Jones, 1998) upon boiling (Fig. 1A). Purified phospholemman was also phosphorylated by cAMP-dependent protein kinase. An autoradiogram is shown in Fig. 2. Apparently, recombinant phospholemman is an excellent substrate for phospholamban and phospholemman phosphatase activity, except for a shorter incubation time of 10 min after the addition of phosphorylase a to the assay. Protein was measured as before (Neumann et al., 1991).
substrate for cAMP-dependent protein kinase, as expected
(Presti et al., 1985a,b; Palmer et al., 1991). For comparison,
the classical substrate phosphorylase b was phosphorylated
by phosphorylase kinase to phosphorylase a. After autoradi-
ography, a single band at the expected molecular weight
was detected (Fig. 2). To validate the phosphatase assays,
32P-radiolabeled phospholemman, phospholamban, and
phosphorylase a were dephosphorylated by phosphatases
from diluted homogenates of human left ventricular tissue,
subjected to gel electrophoresis, and autoradiographed (Fig.
2). All three substrates were dephosphorylated by human
cardiac preparations (Fig. 2). In initial experiments, we tried
to dephosphorylate human membrane vesicles by human
cardiac phosphatases. This was very problematic. The radio-
active phosphate incorporation was sufficient for detection on
autoradiograms (Fig. 1) but too low for routine scintillation
counting of radioactivity released by exogenously added hu-
man cardiac phosphatases. Tissue limitation also excluded
human membrane vesicles as routine sources for phospho-
lamban or phospholemman. However, recombinant phospho-
lamban and phospholemman turned out to be excellent sub-
strates and could be used like phosphorylase a for routine
assays. Using these substrates, phosphatase activity in hu-
man homogenates (Table 1) or after column separation of the
catalytic subunits of phosphatases type 1 and 2A (Fig. 3) and
on peak fractions from column separations could be mea-
sured (Table 2).

The dephosphorylation of all substrates was inhibited in a
concentration-dependent manner by okadaic acid and can-
tharidin (Table 1 for phospholamban, phospholemman,
and phosphorylase as substrates). Okadaic acid inhibited phos-
phorylase, phospholamban, and phospholemman phospha-
tase activity in human cardiac homogenates in a concentra-
tion-dependent manner starting at 1 nM.

Likewise, cantharidin inhibited dephosphorylation of phos-
phorylase a, phospholamban, and phospholemman in human
cardiac homogenates in a concentration-dependent manner
but starting at 0.1 μM. The IC50 values for okadaic acid and
cantharidin are provided as Table 1. Okadaic acid and can-
tharidin were equieffective, but okadaic acid was more potent
than cantharidin. The inhibition curves of the phosphatase
activities in homogenates were shallow. This result and our
own previous work on guinea pig cardiac phosphatases (Neu-
mann et al., 1995) indicated the presence of several phospha-
tases that were differently sensitive to these inhibitors.
Hence, the phosphatase activities were further purified, us-
ing an ethanol precipitation step for separation of regulatory
from catalytic subunits and a heparin-Sepharose column to
distinguish type 1 from type 2A phosphatase catalytic sub-
units as described for guinea pig heart (Herzig et al., 1995;
Neumann et al., 1995). The elution profiles for phospholam-
ban, phospholamban, and phosphorylase phosphatase activ-
ities are shown in Fig. 3. The profiles were all very similar
and exhibited two major peaks (peak 1 and peak 2) of phos-
phatase activity.

The first peak of phosphatase activity, noticed in the
flowthrough, consisted of activities that did not bind to the
column. The second peak was eluted by a NaCl gradient. In
previous studies, type 2A phosphatases did not bind to the
column, whereas type 1 did (Neumann et al., 1995). There-
fore, peak 1 and peak 2 were tentatively identified as type 2A
and type 1 phosphatase activities, respectively. Inhibition
experiments with okadaic acid and cantharidin, as described
below, are consistent with these assumptions.

Fig. 1. A, autoradiogram of a polyacrylamide gel used to identify recom-
binant [32P]phospholamban (PLB). Samples marked (+) were heat treated
for 10 min at 95°C to convert PLB into a low molecular weight
form. On the left, molecular weight standards are indicated. B, autoradi-
ogram of a polyacrylamide gel used to identify [32P]phospholamban
(PLB) and [32P]phospholemman (PLM) in radioactively labeled mem-
brane vesicles from failing human hearts. Samples marked (+) were
heated for 10 min at 95°C before electrophoresis to convert PLB to a low
molecular weight form. Note the time dependence of the phosphorylation
by the cAMP-dependent protein kinase.

Fig. 2. Autoradiograms showing the dephosphorylation of 32P-labeled
phosphorylase a (Phos a), phospholamban (PLB), and phospholemman
(PLM) by phosphatases from human left ventricular tissue. All samples
were heat treated for 10 min at 95°C. On the left and the right, molecular
weight standards are indicated in thousands. Note the different scales of
standards. Samples were preincubated with phosphatases as indicated (+).

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Okadaic acid concentration dependently inhibited type 1 and type 2A phosphatase activities from human heart (Table 2). Similar inhibition curves were obtained for cantharidin (Table 2). Okadaic acid and cantharidin were equeffective, but okadaic acid was more potent than cantharidin. The IC50 values of the inhibition experiments with okadaic acid and cantharidin are summarized in Table 2. Okadaic acid inhibited type 2A phospholamban, phospholemman, phosphorylase phosphatase activities 47, 129, and 133 times more potently than the respective type 1 phosphatase activities. Cantharidin inhibited type 2A phospholamban, phospholemman, and phosphorylase phosphatase activities 20, 25, and 36 times more potently than type 1 phosphatase activities. Comparing the IC50 values of okadaic acid and cantharidin, okadaic acid inhibited type 2A phospholamban, phospholemman, and phosphorylase phosphatase activities 33, 48, and 38 times more potently than cantharidin, whereas type 1 phospholamban, phospholemman, and phosphorylase phosphatase activities were inhibited 14, 9, and 10 times more potently by okadaic acid than by cantharidin. Hence, as reported in guinea pig hearts (Neumann et al., 1995), cantharidin is less selective than okadaic acid for type 2A phosphatase activity. Nevertheless, both inhibitors clearly distinguished between type 1 and type 2A phosphatases.

Discussion

The main finding of the present study is that two important, membrane-localized phosphoproteins in the heart, phospholamban and phospholemman, can be dephosphorylated by the type 1 and type 2A cardiac phosphatases.

Using recombinant phospholamban and phospholemman as substrates, we have extended our previous work. Phospholamban phosphorylated in rabbit membranes could be dephosphorylated by phosphatases from rabbit skeletal muscle (MacDougall et al., 1991). Recombinant phospholamban can be dephosphorylated by phosphatases from the guinea pig heart (Zimmermann et al., 1996). However, here we report for the first time that recombinant phospholamban can be dephosphorylated by phosphatases from the human heart. Moreover, dephosphorylation is mediated by both the catalytic subunits of type 1 and type 2A phosphatases. Dephosphorylation of phospholamban by human cardiac homogenates and by purified catalytic subunits of type 1 and type 2A phosphatases from human hearts can be concentration dependently inhibited by cantharidin and okadaic acid, which has not been reported before. For comparison, phosphorylase a dephosphorylation was measured. Cantharidin inhibited dephosphorylation of phosphorylase a in homogenates from human hearts with an IC50 of ~170 nM (this report) and with an IC50 of 540 nM in guinea pig ventricular homogenates (Neumann et al., 1995), which is comparable. In guinea pig preparations, cantharidin inhibited type 1 and type 2A phosphorylase phosphatase activity with IC50 values of 2.7 μM and 130 nM, respectively. In human tissue, type 1 and 2A phosphorylase phosphatase activities were inhibited by cantharidin with IC50 values of 410 and 11 nM, respectively. In guinea pig studies, we reported that okadaic acid inhibited type 1 and type 2A phosphorylase phosphatase activity with IC50 values of 120 and 0.7 nM, respectively (Neumann et al., 1995), whereas in human tissue type 1 and 2A phosphorylase phosphatase activities were inhibited with IC50 values of 40 and 0.3 nM, respectively (this report). In agreement with our previous data (Neumann et al., 1995), we found that cantharidin inhibited human cardiac phosphatases equeffectively but less potently than okadaic acid. It may further be concluded that both cantharidin and okadaic acid inhibited type 2A human cardiac phosphatases about 10 and 100 times, respectively, more potently than type 1 phosphatases. Inhibition of phospholamban phosphatase activities for okadaic acid and cantharidin has not been reported before. It turns out that the IC50 for phospholamban and phosphorylase phosphatase activity are very comparable for human cardiac phosphatases. This validates the work of our group and others that routinely used phosphorylase as a model substrate in the past.

The other new finding of the present work is the characterization of phospholamban dephosphorylation, which has previously been undetectable because of the low expression of phospholamban in tissues. Therefore, no comparable data on human or nonhuman preparations are available in the literature. Phospholamban was dephosphorylated by purified cata-
lytic subunits of type 1 and/or type 2A phosphatases in human cardiac homogenates under our assay conditions. More specifically, phospholamban was dephosphorylated by type 1 and type 2A phosphatases. Both cantharidin and okadaic acid inhibited these dephosphorylations equieffectively, but okadaic acid was more potent than cantharidin. The inhibition of phospholamban and phospholamban dephosphorylation by the phosphatase inhibitors studied (okadaic acid and cantharidin) was very similar. Hence, one would predict that concentrations of okadaic acid or cantharidin that increase the phosphorylation state of phospholamban should in parallel increase phospholamban phosphorylation. However, in our previous studies on isolated cardiomyocytes, we have not unambiguously identified phosphatase inhibitor-induced phospholamban phosphorylation, probably because of its low expression (Neumann et al., 1993).

In fact, the present data support the hypothesis that β-adrenoceptor-stimulated phosphorylation of phospholamban and phospholamban in the heart is mediated at least in part by inhibition of phosphatase type 1, conceivably via altered phospholamban phosphorylation. It was very similar. Hence, one would predict that concentrations of okadaic acid or cantharidin that increase the phosphorylation state of phospholamban should in parallel increase phospholamban phosphorylation. However, in our previous studies on isolated cardiomyocytes, we have not unambiguously identified phosphatase inhibitor-induced phospholamban phosphorylation, probably because of its low expression (Neumann et al., 1993).

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Another interpretation of our data is also warranted. The present functional data confirm and extend previous biochemical analyses showing that mediate the β-adrenergic inotropic effects in the heart.

There is evidence that type 1 mRNA expression (catalytic subunit) and total activity of protein phosphatase is increased in human heart failure (Neumann et al., 1997). We have not yet differentiated which type 1 or type 2A phosphatase activity is elevated in human heart failure.

Preliminary evidence suggests that in some animal models of impaired ventricular function, alterations of phosphatases do occur. Increased phosphatase activity was noted after infarction (Huang et al., 1997) due to chronic β-adrenergic stimulation (which led to hypertrophy; Boknık et al., 1997) and chronic ischemia (Gupta et al., 1997).

In summary, we extend the physiological and functional work of our group by characterizing type 1 and type 2A phosphatase activity in the human heart using substrates that mediate the β-adrenergic inotropic effects in the heart.

References