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CYCLIC GMP-MEDIATED PHOSPHOLAMBAN PHOSPHORYLATION IN INTACT CARDIOMYOCYTES

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SUMMARY: The cGMP-mediated phospholamban phosphorylation was investigated in cardiomyocytes in response to receptor-dependent (atrial natriuretic peptide, ANP) and -independent (sodium nitroprusside; SNP) cGMP generation. ANP (1nM-1μM) induced phospholamban phosphorylation in a concentration-dependent fashion (EC_{50}: 5.0±0.09 nM). Concomitantly, an elevation in cGMP levels was observed. Phospholamban was also dose-dependently phosphorylated in response to SNP, but it required about three orders of magnitude higher concentrations (EC_{50}: 2.9±0.03 μM) than ANP. Treatment of the cells with 8Br-cGMP (10 μM) or with the specific activator of cGMP-protein kinase 8-pCPT-cGMP (1-100 μM) mimicked these effects. The results demonstrate for the first time that a ANP/cGMP signaling pathway exists in neonatal cardiomyocytes which may contribute to modulation of heart contractility.

The biochemical mechanisms how cGMP modulates the cardiac contractility is incompletely understood (1). Recent findings indicate that cGMP may influence the contractility by reducing the cellular Ca^{2+} level caused by a diminished Ca^{2+} influx (2, 3) and by decreased Ca^{2+} sensitivity of myofilaments (4). In addition, it has been shown in vitro that phosphorylation of phospholamban by cGMP-dependent protein kinase increased the affinity of the Ca^{2+} pump for Ca^{2+} in dog ventricular sarcoplasmic reticulum preparations offering the possibility of another potentially cGMP-modulated cellular target (5).

Therefore cGMP may mediate the relaxation of the heart muscle by lowering the free intracellular Ca^{2+} concentration resulting from a cGMP-dependent phospholamban phosphorylation. However,

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ABBREVIATIONS: ANP, atrial natriuretic peptide; 8Br-cGMP, 8-bromo guanosine-3', 5'-cyclic monophosphate; cGMP, guanosine-3', 5'-cyclic monophosphate; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-guanosine-3', 5'-cyclic monophosphate; PLB, phospholamban; ISO, isoproterenol; SNP, sodium nitroprusside.

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there are other reports which did not observe a cGMP-sensitive phosphorylation of this phosphoprotein in vivo (6).

Based on the controverse views of cGMP in mediating phospholamban phosphorylation, we investigated whether different cGMP increasing interventions e.g. 8-bromo-cGMP, 8-pCPT-cGMP, an activator of cGMP-protein kinase, or sodium nitroprusside lead to phosphorylation of phospholamban in intact [^{32}P]-orthophosphate prelabeled neonatal cardiomyocytes. Interest in myocardial cGMP has been reawaked by reports that atrial natriuretic peptide (ANP) has corresponding receptors in the heart. Interaction of ANP with the receptors resulted in an activation of particulate guanylyl cyclase (7) and might be involved in the observed block of Ca^{2+} inward current (3). No reports exist concerning an ANP-linked phosphorylation of phospholamban in intact neonatal heart cells. Consequently we have involved ANP as an extracellular stimulus of a cGMP-pathway in these studies.

MATERIAL AND METHODS

Materials: The sources of substances were as follows: rat ANP (99-126), Peninsula Lab., Heidelberg (FRG); [^{32}P]-orthophosphate (10 mCi/ml) Amersham, Braunschweig (FRG); monoclonal phospholamban antibody, Biomol, Hamburg (FRG); 8-pCPT-cGMP Biolog, Bremen (FRG); 8-bromo-cGMP, Boehringer (FRG), isoproterenol, sodium nitroprusside, (Sigma);XBD-film was from ORWO (FRG); electrophoresis substances were products from BioRad.

Myocytes isolation: The method used to prepare the neonatal rat ventricular myocyte culture have been described in detail elswere (8). Briefly, the ventricles of 2 day old Wistar-rats were disaggregated to single cells by trypsinization and the cells were seeded in 15 mm diameter dishes (350000 cells corresponding to 60-80 μg of protein) and cultivated at 37°C with serum-containing medium for 5 days.

Labeling of intact heart cells by [^{32}P] orthophosphate and phosphorylation assay: Before the [^{32}P]-labeling the cell medium was changed to a phosphate-free solution for one hour. The [^{32}P]-labeling procedure (10 μCi [^{32}P]-orthophosphate /350.000 cells) were performed for 2 hours at 37°C. Subsequently the incubation with cGMP-analogues, ANP, SNP, or ISO were performed at 37°C. At the end of the reaction trichloroacetic acid (final concentration 5%) were added and the samples were stored at -20°C until use. Cells were scraped from the dishes and centrifugated at 5000 rpm for 15 min. The pellets were solubilized in 80 μl of electrophoresis "stop mixture", consisting of 2% SDS, 2 mM dithiothreitol, 5% glycerol, 0.1 % bromphenol blue, and were heated for 5 min at 100°C. The protein were determined by (9).

Polyacrylamide gel electrophoresis and autoradiography: Samples were subjected to SDS-polyacrylamide gel electrophoresis according to the method of (10) using a 12% (w/v)-acrylamide gel (65 V overnight at 18°C). Following electrophoresis, the proteins were fixed with 15% trichloroacetic acid, stained with Comassie Blue, dried, and exposed to XBD-ray film and the [^{32}P]-incorporation was analyzed by the use of a densitometric method (PDI-system). Results are expressed in relative units of optical density (ODxMM) measured between unstimulated and stimulated heart cells. Linearity between radioactivity and absorbance was shown in control experiments.

cGMP analysis: Intracellular cGMP concentrations were determined in 65% aethanol- extracted (-40°C) and lyophilized cell samples by radioimmunoassay (11).

Statistics: Data are given as means±SEM. For statistical comparison the Student's t-test for unpaired observations was used. Differences were considered significant at a p-value < 0.05.

RESULTS

The PLB phosphorylation was analyzed in radioactive-prelabeled intact cardiomyocytes after addition of two different receptor specific agonists ISO and ANP. As the autoradiogram demonstrates PLB, identified by Western blot analysis, is not only an ISO- but also an ANP-sensitive phosphoprotein in intact cardiomyocytes (Fig. 1). The time-dependence of the PLB phosphorylation in response to ANP was in the same range as that observed with ISO. A significant 8Br-cGMP-mediated phosphate transfer to PLB could also be detected in cultivated heart cells after 20 min. Fig. 2 (left) demonstrates the dose-dependent effect of ANP on PLB phosphorylation. ANP increased the [^{32}P]-incorporation (optical density; relative units) into PLB from 1.7 ± 0.03 to 8.2 ± 0.6 at 1 nM and 0.1 μM ANP, respectively. In a representative experiment (Fig. 2, right) it was shown that PLB was also phosphorylated by 8-pCPT-cGMP. Taken together, these data demonstrate that ANP induced a rapid and substantial phosphorylation of PLB in intact neonatal cardiomyocytes. This effect was mimicked by both 8Br-cGMP and the activator of endogenous cGMP-dependent protein kinase, 8-pCPT-cGMP. To evaluate whether there is a link between the two regulatory pathways in the heart cells, we measured cGMP levels in intact cardiomyocytes. We found that exposure to ANP for 5 min induced an increase of the

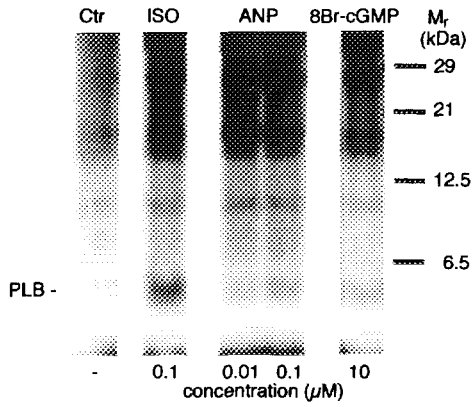

Figure 1. ISO-, ANP- and 8Br-cGMP-mediated phospholamban phosphorylation in intact heart cells (autoradiogram). Neonatal heart cells prelabeled with [^{32}P]orthophosphate were incubated with ISO (0.1 μM), ANP (0.01 and 0.1 μM), or 8Br-cGMP (10 μM) for 5 min (ISO, ANP) and 20 min (8Br-cGMP), respectively. Standard molecular weight markers are shown at the right side. Ctr, control; ISO, isoproterenol; ANP, atrial natriuretic peptide; 8Br-cGMP, 8-bromo guanosine-3', 5'-cyclic monophosphate.

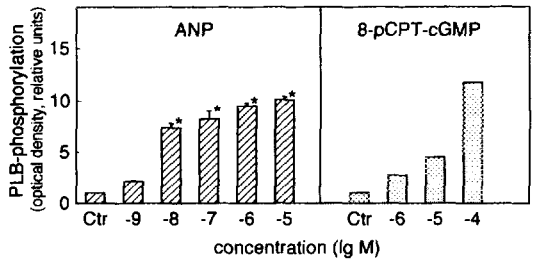

Figure 2. Phospholamban phosphorylation induced by ANP and 8-pCPT-cGMP in neonatal cardiomyocytes. [^{32}P]orthophosphate prelabeled neonatal heart cells were treated with indicated drugs.
Data are means ± SEM from 3-7 separate experiments (*p<0.05 vs control). For the 8-pCPT-cGMP-related PLB phosphorylation a representative experiment is given. Incubation time: ANP 5 min; 8-pCPT-cGMP 20 min. Ctr, control; ANP, atrial natriuretic peptide; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate.

cGMP level (pmoles/mg protein) from a baseline value of 0.82 ± 0.06 to 1.6 ± 0.05 and 3.4 ± 0.1 at 0.01 and 1 μM of ANP, respectively. ISO had no effect on the cGMP level. To evaluate whether the activation of the soluble guanylyl cyclase by SNP leads to a PLB phosphorylation in the neonatal heart cells, the cells were incubated with rising concentrations of this drug. As illustrated by the autoradiogram (Fig. 3) 100 μM SNP caused a significant PLB phosphorylation.

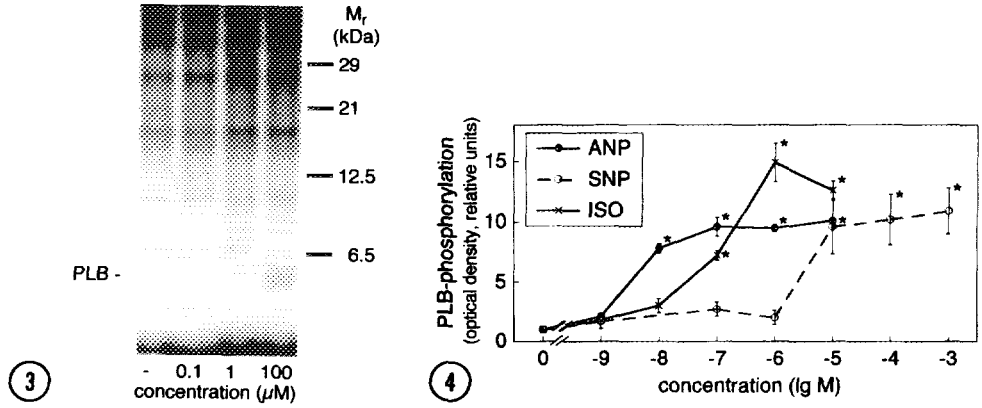

Figure 3. Phosphorylation of phospholamban by sodium nitroprusside in neonatal rat heart cells (autoradiogram). Cells were prepared, phosphorylated and electrophoretically processed as described under Material and Methods. The phosphorylation reaction was started by the addition of 0.1, 1 or 100 μM of SNP for 30 min. Standard molecular weight markers are indicated on the rigth side. SNP, sodium nitroprusside.

Figure 4. Concentration-dependence of ANP-, ISO- and SNP-mediated phospholamban phosphorylation in intact neonatal rat heart cells. Cells were [^{32}P]orthophosphate prelabeled and thereafter stimulated by different concentrations of ANP, ISO or SNP. The incubation times were 5 min for ISO and ANP and 30 min for SNP. Data are means ± SEM from 3-9 separate experiments (*p<0.05 vs control). ANP, atrial natriuretic peptide; ISO, isoproterenol; SNP, sodium nitroprusside.

To compare the ß-adrenergic associated P-incorporation into PLB with that exerted by ANP or SNP in intact heart cells, the myocytes were incubated with ISO (0.001-10 µM), ANP (0.001-10 µM) and SNP (0.1-1000 µM) (Fig. 4). Based on these data showing in Fig. 4 the following EC_{50} values were estimated, ANP 5.0±0.09 nM, ISO 0.1±0.06 µM and SNP 2.9±0.03 µM, respectively.

DISCUSSION

The aim of the present study was to investigate the cGMP/ANP-dependent phosphorylation of PLB in intact neonatal cardiomyocytes in order to identify possible participation of this regulatory protein in the cGMP-related signaling cascade.

Incubation of [^{32}P]-orthophosphate prelabeled neonatal rat heart cells with cGMP-analogues e.g. 8Br-cGMP or 8-pCPT-cGMP and with ANP resulted in a concentration-dependent elevation of the phosphorylation state of PLB. Since ANP increased the cGMP levels, we assume that the observed phosphorylation of PLB occured via a cGMP-mediated pathway. The half-maximal dose of ANP required to induce PLB phosphorylation in rat myocytes was determined at 5.0±0.09 nM being close to the concentration known for the activation of particulate guanylyl cyclase (12) and for cGMP generation in ventricular myocytes (EC_{50}: 0.01 µM) (13, 14).

The activation of membrane-bound guanylyl cyclase by ANP and its induction of PLB phosphorylation differs obviously from the effect of SNP, an activator of the soluble guanylyl cyclase. The observed different potency to phosphorylate PLB could be in part due to differences in the cGMP-compartimentization of ANP-mediated activation of particulate guanylyl cyclase in comparison to that of SNP-activated soluble guanylyl cyclase.

The results that 8Br-cGMP (K_a for cGMP-dependent protein kinase, 0.009 µM) and 8-pCPT-cGMP (corresponding K_a of 0.04 µM, K_i for cGMP-inhibited phosphodiesterase 385 µM) induced PLB phosphorylation may indicate an effect via cGMP-dependent protein kinases at least in neonatal rat heart cells. Findings reported by several groups suggested an involvement of cGMP-dependent protein kinase in the inhibition of cardial Ca^{2+} inward current (2, 15, 16). The level of cGMP-dependent protein kinase in adult rat heart myocytes has been reported to be low (2). The presence of an effective cGMP signal transduction cascade in the neonatal rat heart might enable in part the observed cGMP-regulated PLB phosphorylation (1). From the present studies we can conclude that there is a specific cGMP-mediated PLB phoshorylation in neonatal heart cells. The experiments with 8-pCPT-cGMP support the assumption that a phosphodiesterase pathway is not involved in the induction of PLB phosphorylation.

In summary, several mechanisms may be responsible for the cardiodepressive effect of cGMP/ANP. Beside a cGMP-mediated inhibition of the Ca^{2+} entry into the cells (2, 3, 14), and

the influence of cGMP on Ca^{2+} affinity of myofibrils (4) a stimulation of cGMP on PLB phosphorylation followed by an acceleration of Ca^{2+} sequestration may be involved. These multiple effects seem to indicate that cGMP is a cellular signal to depress the heart contractility by reducing the Ca^{2+} concentration. It should be of interest to investigate in addition to the short-term regulation also the long-term cGMP-modulated heart function with respect to hypertrophic processes in the neonatal and adult myocardium.

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