Intracellular signaling leads to the hypertrophic effect of neuropeptide Y

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Goldberg, Yaron, Gerhild Taimor, Hans Michael Piper, and Klaus-Dieter Schlueter. Intracellular signaling leads to the hypertrophic effect of neuropeptide Y (NPY) were investigated in adult cardiomyocytes. Reduction of transforming growth factor-β (TGF-β), a cytokine released by adult cardiomyocytes in culture (32). Whether TGF-β is also responsible for induction of hypertrophic responsiveness of adult cardiomyocytes to NPY was investigated.

The previous study (20) also indicated that the effect of NPY differs at least in part from that of β-adrenoceptor stimulation; NPY, but not β-adrenoceptor agonist, induces CK. Such a difference suggests differences in intracellular signaling. The present study, therefore, also aimed to identify key steps of intracellular signals that are activated under NPY and cause either hypertrophic growth (protein and RNA synthesis) or lead to induction of CK in adult cardiomyocytes preincubated in serum-containing media.

Only a few elements of the signal transduction of NPY in mammalian cells are known. NPY can activate pertussis toxin (PTx)-sensitive G proteins and phosphatidylinositol (PI) 3-kinase (4) as well as PTx-insensitive G proteins, coupled to phospholipase C and a subsequent activation of protein kinase C (PKC) (37). We investigated whether one or the other of these signal transduction elements is involved in the hypertrophic response to NPY.

In the same cell system, but for hypertrophic stimuli other than NPY, phosphatidylinositol (PI) 3-kinase and its potential downstream target p70^{65k} were previously identified to be crucial steps in signaling toward regulation of protein synthesis (25). PI 3-kinase can be activated in a PKC-dependent or in an adenyly cyclase-dependent manner, e.g., under α-adrenoceptor stimulation or under β-adrenoceptor stimulation, respectively. In both cases stimulation of protein synthesis requires activation of PI 3-kinase. PI 3-kinase represents therefore a point of convergence for various growth signals. Here, it was investigated whether PI 3-kinase and p70^{65k} are also involved in the growth response to NPY.

Induction of the fetal-type isoform of CK, CK-BB, is a common feature of the hypertrophic response of adult cardiomyocytes stimulated with NPY or α-adrenoceptor agonists (20). In the case of α-adrenoceptor stimulation the induction of CK-BB is mediated through activation of PKC and a secondary activation of mitogen-activated protein (MAP) kinase (26). It is independent of PI 3-kinase and p70^{65k}. Here, we investigated whether NPY causes induction of CK-BB through the same pathway as does α-adrenoceptor stimulation.

MATERIALS AND METHODS

Cell culture. Ventricular heart muscle cells were isolated from 200- to 250-g male Wistar rats as previously described (24, 27). Isolated cells were suspended in FCS-free culture medium and plated at a density of 1.4 × 10^6 elongated
cells/35-mm culture dish (Falcon type 3001). The culture dishes had been preincubated overnight with 4% FCS in medium 199. The basic culture medium consisted of medium 199 with Earle’s salts, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. To prevent growth of nonmyocytes, media were also supplemented with 10 µM cytosine β-o-arabino-furanoside.

Four hours after they were plated, cultures were washed twice with culture medium to remove round and nonattached cells and supplied with basic culture medium supplemented with 20% FCS, in which cells were incubated for 6 days at 37°C. Thereafter, the culture dishes were washed twice, and experiments were carried out in basic culture medium without FCS on day 6 (control), with additions of the agonists at indicated concentrations. Ascorbic acid (100 µM) was added to all cultures as an antioxidant at 1% (vol/vol). In some experiments, cardiomyocytes were pretreated with PTx (1 µg/ml) for 6 h before experiments were started. We have previously shown that this pretreatment is an effective procedure to inhibit Gq/G11 proteins in adult cardiomyocytes (13).

Incorporation of [14C]phenylalanine and changes in cellular protein and RNA mass. Incorporation of phenylalanine into cells was determined by exposing cultures to [L-14C]phenylalanine (0.1 µCi/ml) for 24 h and determining the incorporation of radioactivity into acid-insoluble cell mass, as described before (22, 28). Nonradioactive phenylalanine (0.3 mM) was added to the medium to minimize variations in the specific activity of the precursor pool responsible for protein synthesis. In incorporation studies, experiments were terminated by removal of the supernatant medium from the cultures and washing three times with ice-cold PBS (composition in mM: 1.5 KH2PO4, 137 NaCl, 2.7 KCl, and 1.0 Na2HPO4, pH 7.4). Subsequently, ice-cold 10% (wt/vol) TCA was added. After storage overnight at 4°C, the acid was removed from the dishes. Radioactivity contained in this acid fraction was taken to represent the intracellular precursor pool. The dishes were then washed twice with ice-cold PBS. The remaining precipitate on the culture dishes was dissolved in 1 N NaOH-0.01% (wt/vol) SDS by an incubation for 2 h at 37°C. In these samples, protein contents (3) and DNA contents (7) were determined, and the radioactivity was counted. RNA was determined from an aliquot of these samples after precipitation with an equal volume of 10% (wt/vol) perchloric acid in the remaining supernatant (18). The RNA content was also expressed relative to the DNA content of the samples.

Analysis of CK activities. Specific activity of the cytosolic CK was determined as described previously (28). Cultures were first washed twice with PBS. After addition of buffer A (composition in mM: 5 magnesium acetate, 0.4 EDTA, 2.5 dithiothreitol, 50 Tris-HCl, and 250 sucrose, pH 6.8) to the dishes, the cells were scraped off, homogenized, and frozen until use at −14°C. For analysis, these samples were thawed, and the resulting suspension was sonicated and centrifuged at 12,000 g for 2 min. The supernatants were used for enzyme analysis. The activity of CK was determined according to Ref. 6, using standard ultraviolet methods.

Distributions of the cytosolic isoenzymes of CK, MM, MB, and BB, were analyzed according to Ref. 14. The supernatants were applied to a 1-ml DEAE-cellulose column that had been equilibrated with buffer B (composition in mM: 20 NaCl, 5 magnesium acetate, 0.4 EDTA, and 100 Tris-HCl, pH 7.9). The CK-MM isoenzyme eluted directly with buffer B, the CK-MB isoenzyme with change of NaCl concentration to 40 mM and pH to 6.4, and CK-BB with change of NaCl concentration to 250 mM and pH to 6.4.

Determination of PI 3-kinase. PI 3-kinase activity was determined in immunoprecipitates as described by Whitman et al. (36). Briefly, cardiomyocytes were washed twice with PBS, and cells were lysed in lysis buffer [composition: 10% (vol/vol) glycerol, 1% (vol/vol) NP-40, and 1 mM phenylmethylsulfonyl fluoride]. After centrifugation (10 min at 10,000 g), the supernatant was used for immunoprecipitation with an antibody against the p85 α-subunit of bovine PI 3-kinase and the immunoprecipitates were sedimented with protein A-Sepharose. The pellets were washed with PBS, twice with buffer A (composition: 0.5 M LiCl, 0.1 M Tris, pH 7.4) and once with buffer B (composition: 10 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and resuspended in 25 µl of buffer B; 1 mg/ml PI (Sigma, Deisenhofen, Germany) was dispersed by sonification in 5 mM HEPES buffer, pH 7.4, and 20 µl of this solution were added to the resuspended immunoprecipitates. After preincubation for 30 min at room temperature, the phosphorylation reaction was started by addition of 20 µCi [γ-32P]ATP in starting buffer containing 50 µM ATP and 5 mM MgCl2. The total volume in the reaction tubes was 50 µl. The reaction mixture was incubated for 20 min at 25°C and terminated by addition of 100 µl of 1 M HCl. Phospholipids were then extracted with 200 µl of CHCl3/MeOH (1:1). The organic phase was spotted onto a silica gel TLC plate pre-treated with 1% (wt/vol) potassium oxalate. Phosphorylated products were separated by TLC in a CHCl3/MeOH/A4 M NH4OH (9:7:2) developing solvent and visualized with a phosphorimag (Molecular Dynamics). To quantify the activity of the immunoprecipitates, TLC plates were scanned densitometrically and the amount of phosphorylated PI was normalized to the spotted radioactivity of the plates, which varied between the reaction tubes (origin).

Determination of PKC. PKC activity was determined in the membrane fraction of cardiomyocytes as described previously (2, 30). Briefly, in experiments in which activation of PKC should be measured, cultures were washed twice with ice-cold PBS at the end of the incubation period after 15 min and the cells were harvested in ice-cold hypotonic lysis buffer and lysed by vortexing the cell suspension vigorously for 2 min. Nuclei and any unlysed cells were sedimented by centrifugation at 1,000 g for 3 min at 4°C. The postnuclear supernatant was centrifuged at 100,000 g for 10 min at 4°C, the resulting supernatant was discarded, and the membrane sediment was suspended in an aliquot (300 µl) of PKC assay solution. The activity of PKC in the membrane suspension was determined by a continuous fluorescence assay as described before (2).

Determination of p42 MAP kinase. The determinations of p42 MAP kinase were done as described in detail (31). Briefly, after stimulation, cells were lysed in lysis buffer [composition in mM: 50 Tris-Cl, pH 6.7, 2% (wt/vol) SDS, 2% (vol/vol) mercaptoethanol, and 1 sodium orthovanadate]. Then, nucleic acids were digested with benzonase (Merck, Darmstadt, Germany). After SDS-PAGE (100 µg protein/well), proteins were transferred onto reinforced nitrocellulose by semidry blotting. The sheets were saturated with 2% (wt/vol) BSA and incubated for 2 h with rabbit polyclonal anti-rat p42 MAP kinase (10 µg/50 ml, Santa Cruz Biotechnology). After sheets were washed, sheep anti-rabbit alkaline phosphatase-labeled IgG (50 µg/50 ml) was added for 2 h. Detection was done by alkaline phosphatase activity recognized by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. For quantification, the blots were densitometrically scanned, and the results were expressed as the ratio of the upper band, with retarded gel mobility of activated and phosphorylated p42 MAP kinase, to the total amount of p42 MAP kinase determined on the Western blots.
Accumulation of cAMP. As an indirect indicator of adenylate cyclase activation, accumulation of cAMP in the cultures over a period of 5 min was investigated in the presence of a phosphodiesterase inhibitor as described previously (30). The cells were incubated with modified Tyrode solution (pH 7.4) containing 125 mM NaCl, 1.2 mM KH2PO4, 2.6 mM MgSO4, 1 mM CaCl2, 10 mM glucose, 10 mM HEPES, and 1 mM IBMX, a phosphodiesterase inhibitor and a nonselective adenosine antagonist. All experiments were performed in the presence of adenosine deaminase (5 U/ml), as adenosine released from the cells may exert an antagonistic effect on adenylate cyclase by interacting with adenosine A1 receptors (11). Experimental incubations were terminated after 5 min by the addition of 1 ml of 1.2 M HClO4 to the contents of the culture dishes. The cells were scraped off and centrifuged for 1 min at 12,000 g. The supernatant was neutralized and quickly frozen in liquid nitrogen. The pellet was redissolved in 0.1 M NaOH, and its protein content was determined by the method of Bradford (3) with BSA as the standard. The frozen supernatants were freeze-dried and redissolved in 100 μl of 0.1 M HEPES (pH 7.4). cAMP content of these samples was determined using a protein binding assay (Amersham-Buchler, Braunschweig, Germany).

TGF-β activity. TGF-β activity in the medium was determined as described (1) by the growth inhibitory effect of TGF-β on the proliferation of microvascular endothelial cells. Isolation and cultivation of microvascular endothelial cells were described earlier (26). Supernatants of the cell culture media were diluted and added to subconfluent monolayers of microvascular endothelial cells plated on 96-well dishes. After 48 h, protein contents of the wells were determined. The growth inhibitory effect was compared with a standard curve using activated TGF-β isolated from porcine platelets. Specificity of the inhibitory effect of medium supernatants to TGF-β was further demonstrated by the use of a neutralizing antibody to TGF-β1, which abolished the growth inhibitory effect.

Statistics. Data are given as means ± SD or SE from n different culture preparations. Statistical comparisons were performed by one-way ANOVA and the use of the Student-Newman-Keuls test for post hoc analysis (8). Differences with P < 0.05 were regarded as statistically significant. All data analyses were computed using SAS software, version 6.11 (SAS Institute, Cary, NC).

Materials. Falcon tissue culture dishes were obtained from Becton-Dickinson (Heidelberg, Germany). Boehringer Mannheim (Mannheim, Germany) was the source for glutamine-Becton-Dickinson (Heidelberg, Germany). Boehringer Mannheim (SAS Institute, Cary, NC).

Table 1. TGF-β activity and NPY responsiveness under different culture conditions

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>TGF-β Activity, ng/ml</th>
<th>NPY Effect on Protein Synthesis, % above control</th>
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<tr>
<td>20% FCS</td>
<td>1.5 ± 0.2</td>
<td>50.5 ± 4.1*</td>
</tr>
<tr>
<td>20% FCS + aprotinin</td>
<td>0.8 ± 0.2†</td>
<td>210 ± 4.6</td>
</tr>
<tr>
<td>5% FCS</td>
<td>0.4 ± 0.3†</td>
<td>1.2 ± 3.6</td>
</tr>
<tr>
<td>5% FCS + TGF-β</td>
<td>ND</td>
<td>445.5 ± 6.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 culture preparations. Cardiomyocytes were cultured for 6 days under various conditions. These were culture medium with 20% or 5% FCS supplemented with additions of aprotinin (1 ng/ml) or active transforming growth factor-β (TGF-β; 1 ng/ml). TGF-β activity in these culture media was determined after 6 days with a bioassay, as described in MATERIALS AND METHODS. Protein synthesis was stimulated in 6-day-old cultures after removal of serum and other supplements. Protein synthesis stimulated by neuropeptide Y (NPY; 100 nM) was determined as [14C]phenylalanine incorporation and expressed as percentage of a matched control without NPY. ND, not determined. *P < 0.05 vs. control without NPY. †P < 0.05 vs. 20% FCS.

of activated TGF-β isolated from porcine platelets. In the culture media containing 20% FCS (standard conditions), TGF-β activity after 6 days was equivalent to 1.5 ng TGF-β/ml (Table 1). TGF-β activity in the culture medium was reduced either by the addition of the protease inhibitor aprotinin (1 ng/ml), which inhibits proteolytic activation of latent TGF-β, or by lowering the FCS concentration to 5%. In these cultures, the TGF-β activity was equivalent to 0.8 ng/ml or 0.4 ng/ml, respectively (Table 1). It was investigated next to what extent NPY increases protein synthesis in cultures preexposed to the various TGF-β activities. Protein synthesis of cardiomyocytes grown for 6 days in the presence of 20% FCS before stimulation increased by 50.5% with NPY compared with untreated control cultures (Table 1). In the presence of aprotinin during 6 days of precultivation, NPY increased protein synthesis by only 21.0%. When the FCS concentration in the medium was reduced to 5%, NPY was unable to increase protein synthesis. The growth-promoting effect of NPY, however, was restored in these cultures when 1 ng/ml active TGF-β was added. In this case, NPY increased protein synthesis by 44.5% (Table 1). In all subsequent experiments, cardiomyocytes were cultured under standard conditions (20% FCS for 6 days) with high TGF-β activity and used thereafter under serum-free conditions for the subsequent 24 h.

Role of PTx-sensitive G proteins and PKC for stimulation of protein synthesis. Because NPY receptors may either couple to PTx-sensitive proteins (G13/G12) or to Go proteins, leading to subsequent activation of PKC, the influence of PTx or PKC inhibition on hypertrophic growth was studied. It was first analyzed if the PTx-sensitive, antiadrenergic effect of NPY, known for newly isolated cardiomyocytes (13), is preserved in cultures. The inhibitory effect of NPY (100 nM) on isoprenaline (1 μM)-stimulated cAMP accumulation was determined. NPY attenuated the effect of the β-adrenoceptor agonist by 30% (Table 2). The attenuation was abolished in the presence of PTx (Table 2).

After this PTx-sensitive action of NPY in cultured cardiomyocytes was confirmed, the possibility that the
NPy-stimulated increases in protein synthesis and RNA mass were PTx sensitive was analyzed. In the absence of PTx, NPy (100 nM) augmented protein synthesis by 34% and cellular RNA mass by 18% (Fig. 1). These effects of NPy were blunted when the cells were incubated for 6 h before and during [14C]phenylalanine incorporation in the presence of PTx (1 µg/ml). In the same set of experiments, stimulation of α-adrenoceptors by phenylephrine (10 µM) also increased [14C]phenylalanine incorporation (41 ± 12% above control, n = 16 cultures, P < 0.05). This increase was not attenuated by PTx (39 ± 12% above control, n = 16, P < 0.05 vs. control).

We then investigated whether NPy (100 nM) caused an increase in PKC activity and whether the stimulation of protein synthesis by NPy is sensitive for an inhibition of PKC activation. In the particulate fraction, PKC activity increased by 83% within 15 min under NPy (Table 3). The effect of NPy on PKC activity was compared with the effect achieved by addition of phorbol myristate acetate (100 nM), which resulted in a 143% activation of PKC. The presence of the PKC inhibitor bisindolylmaleimide attenuated the activation of PKC by NPy (Table 3). The presence of bisindolylmaleimide did not alter the effects of NPy on protein synthesis and cellular RNA mass (Fig. 1).

Roles of PI 3-kinase and p70s6k for stimulation of protein synthesis. PI 3-kinase and its potential downstream target p70s6k are involved in α-adrenoceptor-mediated increase in protein synthesis. It was investigated whether these two kinases are also involved in the NPy-mediated growth effect in cultured adult cardiomyocytes. Activation of PI 3-kinase by NPy was determined by quantification of the phosphorylation of PI using immunoprecipitates of the p85 subunit of PI 3-kinase. A representative chromatogram demonstrating increased PI 3-kinase activity is shown in Fig. 2. NPy increased PI 3-kinase activity within 15 min by 160 ± 37% (n = 4 cultures, P < 0.01 vs. control). Activation of PI 3-kinase by NPy was abolished in the presence of wortmannin (100 nM), a PI 3-kinase inhibitor, to 31 ± 27% above control (n = 4 cultures, not significant vs. control).

We then analyzed if the inhibitor of PI 3-kinase, wortmannin, influences the NPy-mediated increase in protein synthesis. Wortmannin abolished the effects of NPy on protein synthesis and greatly attenuated its effects on RNA mass (Fig. 3). In the absence of NPy, wortmannin influenced neither protein synthesis (3.0 ± 0.4 vs. 2.9 ± 0.6 dpm × 10^−3 µg DNA, n = 4 cultures, not significant from each other) nor RNA mass of cardiomyocytes (11.7 ± 0.2 vs. 12.0 ± 0.4 mg RNA/mg DNA, n = 4, not significant from each other).

Rapamycin (100 nM), a selective inhibitor of p70s6k, was used to investigate whether this kinase, known as a downstream target of PI 3-kinase, represents another step in intracellular signaling that leads to stimulation of protein synthesis by NPy. In the presence of rapamycin, the stimulation of protein synthesis by NPy was inhibited whether these two kinases are also involved in the NPy-mediated growth effect in cultured adult cardiomyocytes.

Table 2. cAMP accumulation in cardiomyocytes

<table>
<thead>
<tr>
<th>Condition</th>
<th>cAMP, pmol/mg protein</th>
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<tbody>
<tr>
<td>Control</td>
<td>4.9 ± 1.4</td>
</tr>
<tr>
<td>Isoprenaline (100 nM)</td>
<td>37.3 ± 2.7*</td>
</tr>
<tr>
<td>Isoprenaline+NPy (1 µM)</td>
<td>27.5 ± 1.7*</td>
</tr>
<tr>
<td>PTx (1 µg/ml)</td>
<td>21.7 ± 7.4*</td>
</tr>
<tr>
<td>Isoprenaline+PTx (1 µg/ml)</td>
<td>134.1 ± 7.2*</td>
</tr>
<tr>
<td>Isoprenaline+NPy+PTx</td>
<td>138.7 ± 9.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE from 8 culture dishes. Cardiomyocytes were cultured for 6 days in presence of 20% FCS and thereafter incubated for 15 min under serum-free conditions in the presence of the indicated agonists and a phosphodiesterase inhibitor. Accumulation of cAMP was determined and analyzed as mentioned in MATERIALS AND METHODS. For pertussis toxin (PTx) experiments, cardiomyocytes were treated 6 h before the onset of experiments with PTx. *P < 0.05 vs. control. †P < 0.05 vs. isoprenaline.

Table 3. Protein kinase C activity of membrane preparations of cardiomyocytes

<table>
<thead>
<tr>
<th>Condition</th>
<th>PKC Activity, pmol·10 min⁻¹·10 µg protein⁻¹</th>
<th>Activation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120 ± 17</td>
<td></td>
</tr>
<tr>
<td>NPy (1 µM)</td>
<td>219 ± 45*</td>
<td>83 ± 37*</td>
</tr>
<tr>
<td>NPy+BIM (5 µM)</td>
<td>171 ± 41</td>
<td>43 ± 34</td>
</tr>
<tr>
<td>PMA (100 nM)</td>
<td>292 ± 60*</td>
<td>143 ± 50*</td>
</tr>
</tbody>
</table>

Values are means ± SE from 4 different culture preparations. Cardiomyocytes were cultured for 6 days in presence of 20% FCS before the onset of the experiments. Cells were treated for 15 min with indicated agonists. Thereafter, a membrane preparation was prepared as outlined in MATERIALS AND METHODS. Protein kinase C (PKC) activity in this preparation was determined by phosphorylation of fluorescence labeled MARCKS protein and expressed as the amount of phosphorylation within 10 min using 10 µg cell membrane protein. BIM, bisindolylmaleimide. *P < 0.05 vs. control.
reduced to a large extent (Fig. 4). Rapamycin did not reduce the increase in cellular RNA mass induced by NPY (100 nM) (Fig. 4). The effects of rapamycin could not be explained by an effect of rapamycin on its own because in the absence of NPY rapamycin did not influence protein synthesis (3.8 ± 0.2 vs. 3.6 ± 0.3
dpm × 10⁻²/µg DNA) or RNA mass (14.3 ± 0.6 vs. 14.6 ± 0.5 mg RNA/µg DNA, n = 4 cultures, not significant from each other).

Fig. 2. Activation of phosphatidylinositol (PI) 3-kinase by NPY. Cardiomyocyte PI 3-kinase was immobilized on protein G-Sepharose beads through PI 3-kinase α-p85 antibodies. Immunoprecipitates were generated from cardiomyocytes under control conditions (C), in the presence of NPY (100 nM), or NPY plus wortmannin (WORT, 100 nM). PI 3-kinase activity in immunoprecipitates was assayed as described in MATERIALS AND METHODS. Lipid products were separated by TLC as described. An autoradiogram of the TLC plate generated on a phosphorimager is shown. Positions of spots corresponding to [3²P]phosphatidylinositol (PIP) and origin (ORI) are indicated.

Fig. 3. Protein synthesis determined as incorporation of [¹⁴C]phenylalanine in cardiomyocytes and RNA mass, determined as RNA-to-DNA ratios of cardiomyocytes during 24-h incubations under control conditions and in the presence of NPY (100 nM) or NPY and wortmannin (WORT, 100 nM). Data are given in percentage of the basal mean values. These correspond for [¹⁴C]phenylalanine to 3.1 ± 0.2 dpm × 10⁻²/µg DNA and for RNA content to 11.7 ± 0.2 µg RNA/µg DNA. Data are means ± SD; n = 16. Differences from control, *P < 0.05; **P < 0.01 from NPY.

Fig. 4. Protein synthesis, determined as incorporation of [¹⁴C]phenylalanine in cardiomyocytes and RNA mass, determined as RNA-to-DNA ratios of cardiomyocytes during 24-h incubations under control conditions and in the presence of NPY (100 nM) or NPY and rapamycin (RAPA, 100 nM). Data are given in percentage of the basal mean values. These correspond for [¹⁴C]phenylalanine to 3.8 ± 0.1
dpm × 10⁻²/µg DNA and for RNA content to 14.1 ± 0.7 µg RNA/µg DNA. Data are means ± SD; n = 16. Differences from control, *P < 0.05; +P < 0.05 from NPY.

Activation of MAP kinase and its influence on CK-BB induction. MAP kinase activation is involved in the a-adrenoceptor-mediated induction of CK-BB. It was therefore investigated whether this kinase is also involved in a NPY-mediated increase in CK-BB activity. MAP kinase activation was investigated by analysis of the reduced gel mobility of the activated compared with nonactivated MAP kinase. Representative Western blots are shown in Fig. 5. MAP kinase activation in the presence of NPY was abolished by bisindolylmaleimide, indicating its dependency on prior activation of PKC (Fig. 5A), and PD-98059, an inhibitor of its upstream activator MAP kinase kinase (Fig. 5B). MAP kinase activation in the presence of NPY was not affected by PTx (Fig. 5A). The effect of NPY on MAP kinase was compared with that of phorbol myristate acetate (100 nM). This caused a larger activation of MAP kinase (Fig. 6).

As reported earlier (17), NPY induces cytosolic CK. Here, we show that this effect is due to a selective induction of the fetal-type isoform CK-BB (Fig. 7). PD-98059 (10 µM), which abolished the activation of p42 MAP kinase by NPY, also abolished induction of total CK and CK-BB by NPY (Fig. 7). PD-98059 did not influence basal CK-BB activity in cardiomyocytes (0.94 ± 0.14 mU/mg protein vs. 1.03 ± 0.23 mU/mg protein).

DISCUSSION

This study characterizes for the first time key steps of signal transduction by which NPY exerts hypertrophic effects on cultured adult ventricular cardiomyocytes. In
the cardiomyocyte model investigated here, hypertrophic responsiveness to NPY is induced by the presence of active TGF-β in culture media. In responsive cells, NPY activates two major signal transduction pathways. The first pathway includes a PTx-sensitive step and leads to stimulation of protein synthesis via an activation of the PI 3-kinase/p70s6k pathway. Part of this pathway (PI 3-kinase) is also involved in the NPY-mediated rise of RNA mass. The second pathway, PTx insensitive, includes activation of PKC and MAP kinase and leads to an induction of the fetal-type isoform of CK, CK-BB. Figure 8 summarizes the signal transduction pathways identified in this study.

In a previous study, we showed that the hypertrophic responsiveness to NPY requires a precultivation of cardiomyocytes in the presence of FCS (35). The same requirement has also been described for the induction of hypertrophic responsiveness to β2-adrenoceptor stimulation (22, 32, 38). For the latter case, we showed previously that the induction of hypertrophic responsiveness depends on the activity of TGF-β in the culture medium. TGF-β is released from cultured cardiomyocytes in a latent form and must be activated. One major mechanism to activate latent TGF-β is a proteolytic cleavage caused by the serine protease plasmin (15, 16). As shown before for other cell culture systems, the activation of latent TGF-β can be abolished by addition of the serine protease inhibitor aprotinin (5). Indeed, we found that presence of aprotinin reduces TGF-β activity significantly compared with control cultures. In cultures supplemented with aprotinin, the hypertrophic responsiveness to NPY remained absent. Reduction of serum supplement to the cultures also reduced the appearance of active TGF-β, most likely due to a reduction in the proteolytic activity contained in the serum. Under conditions with low TGF-β activity, obtained by either reduction of the serum supplement or addition of aprotinin, the 6-day cultured cardiomyocytes behave like newly isolated cardiomyocytes in which NPY does not stimulate protein synthesis. The differences in the absolute TGF-β activities seem small.

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**Fig. 5.** A: representative Western blot indicating the activation of mitogen-activated protein (MAP) kinase by NPY. MAP kinase activation was determined by reduced gel mobility of the activated (phosphorylated) MAP kinase compared with nonactivated (nonphosphorylated) MAP kinase. Activated and nonactivated MAP kinases were determined by Western blotting of protein samples from cardiomyocytes using a specific antibody against the p42 MAP kinase. Cardiomyocytes were cultured for 15 min (control, C) alone or cultured in the presence of NPY (100 nM), NPY and PTx (1 µg/ml), NPY and BIM (5 µM), or phorbol myristate acetate (PMA; 100 nM). B: representative Western blot indicating the activation of MAP kinase by NPY. MAP kinase activation was determined by reduced gel mobility of the activated (phosphorylated) MAP kinase compared with nonactivated (nonphosphorylated) MAP kinase. Activated and nonactivated MAP kinases were determined by Western blotting of protein samples from cardiomyocytes using a specific antibody against the p42 MAP kinase. Cardiomyocytes were cultured for 15 min (control, C) or in the presence of NPY (100 nM), NPY and PD-98059 (PD, 10 µM), PMA (100 nM), or PMA (100 nM) and PD.

**Fig. 6.** Activation of MAP kinase under control conditions (C) or after 15 min in the presence of NPY (100 nM), NPY and PTx (1 µg/ml), NPY and BIM (5 µM), or PMA (100 nM). For experiments performed in the presence of PTx, cells were cultured for 6 h before the experiments with PTx. A representative Western blot is given in Fig. 5A. Amount of MAP kinase activation (MAP kinase*) is shown as the ratio of activated to total MAP kinase. Data are means ± SD; n = 5. Differences from control, *P < 0.05; + P < 0.05 from NPY.

**Fig. 7.** Creatine kinase (CK) activity of cardiomyocytes under control conditions (C) or after 24-h incubations in the presence of NPY (100 nM) or in the presence of NPY and the MAP kinase kinase inhibitor PD-98059 (PD, 10 µM). Activities of the specific isoforms (MM, MB, and BB) were determined after ion-exchange chromatography of the initial extract. Data are means ± SD; n = 4. Differences from control, *P < 0.05.
NPY activates either PTx-sensitive G proteins (G\textsubscript{i}/G\textsubscript{o}) or protein kinase C (PKC). Activation of G\textsubscript{i}/G\textsubscript{o} proteins is linked to PI 3-kinase (PI3K) activation and subsequent increase in protein synthesis. Activation of PKC is linked to activation of MAP kinase and leads subsequently to induction of CK-BB.

In cardiomyocytes exposed to active TGF-\(\beta\), NPY increases protein synthesis in a PTx-sensitive way. In newly isolated cardiomyocytes not exposed to TGF-\(\beta\), NPY couples to PTx-sensitive G proteins exemplified by its antiadrenergic effect on adenylate cyclase, which is inhibitable by PTx. This PTx-sensitive effect is still present in cultured cardiomyocytes exposed to TGF-\(\beta\). A concentration of 100 nM NPY, which represents the dose of NPY having maximal hypertrophic effect, had a partially inhibitory effect on adenylate cyclase activation by \(\beta\)-adrenoceptor stimulation. The effect of NPY on protein synthesis is also PTx sensitive but cannot be related to its inhibitory effect on adenylate cyclase. This is because 1) in these cultures activation of adenylate cyclase stimulates protein synthesis and NPY, which inhibits this activation, does not antagonize this effect and 2) NPY stimulates protein synthesis in the absence of a prestimulated adenylate cyclase. The PTx-sensitive G proteins involved in the stimulation of protein synthesis by NPY must therefore be coupled either to signal transduction steps other than adenylate cyclase or to another subset of NPY receptors. This point requires further analysis.

It was also investigated in cultured adult cardiomyocytes which intracellular signals follow the activation of PTx-sensitive G proteins by NPY on the route to a stimulated protein synthesis. We demonstrated, first, that NPY activates PI 3-kinase and, second, that inhibition of PI 3-kinase (by wortmannin) blocks the stimulation of protein synthesis under NPY. These results indicate that activation of PI 3-kinase represents an intermediate step in the intracellular signaling toward stimulation of protein synthesis under NPY. The intracellular targets for PI 3-kinase have not been fully established. Various studies suggest a downstream activation of p70\textsubscript{s6k}, following PI 3-kinase activation. p70\textsubscript{s6k} can be specifically blocked by addition of rapamycin. Rapamycin reduced the stimulatory effect of NPY on protein synthesis. In the same culture model, rapamycin completely abolished the hypertrophic effect to \(\alpha\)-adrenoceptor stimulation (25).

The increase in RNA mass under NPY did not strictly follow the PI 3-kinase and p70\textsubscript{s6k} pathway. This is in contrast to the hypertrophic effects of \(\alpha\)- or \(\beta\)-adrenoceptor stimulation on cultured adult cardiomyocytes (25, 29). In the latter cases, protein and RNA synthesis are both entirely inhibitable by either wortmannin or rapamycin. In the case of NPY, p70\textsubscript{s6k} inhibition by rapamycin attenuates the effect on protein synthesis (translational activity) but not RNA mass (translational capacity). The results indicate that the PI 3-kinase/p70\textsubscript{s6k} pathway toward protein synthesis and control of RNA mass is subject to additional regulatory mechanisms.

NPY activates PKC in cultured cardiomyocytes without involving a PTx-sensitive step. This activation of PKC by NPY is not involved in the growth induction under NPY. This represents a marked difference between the signal transduction of NPY and \(\alpha\)-adrenoceptor stimulation, since in the case of \(\alpha\)-adrenoceptor stimulation the activation of PKC is causally linked to the stimulation of protein synthesis. Activation of PKC in cardiomyocytes by other hypertrophic agonists, e.g., \(\alpha\)-adrenoceptor agonists or parathyroid hormone-related peptide, induce CK-BB in a MAP kinase-dependent way (29, 31). Induction of the fetal BB isoform of CK is another feature of myocardial hypertrophy (33). NPY, like other PKC activators in adult cardiomyocytes, activates MAP kinase and subsequently CK-BB in a MAP kinase-dependent way. From these observations, one may hypothesize that NPY activates only selected isoforms of PKC that are linked to activation of MAP kinase and induction of CK-BB but not some other PKC isoforms that are linked to the PI 3-kinase-dependent pathway toward the regulation of protein synthesis.

In conclusion, this study shows for NPY a dissociation of intracellular signals leading to induction of fetal-type CK-BB and increases in protein synthesis.
and RNA mass. The effects of NPY on protein synthesis and RNA mass require activation of PTx-sensitive G proteins, subsequent PI 3-kinase activation, and, in part, activation of p70^{S6K}. Induction of CK-BB by NPY requires an activation of PKC and, subsequently, of MAP kinase. The importance of our results resides in the observation that NPY, unlike other neurohumoral factors that are coreleased from nerve endings with NPY, increases protein synthesis of cardiomyocytes by a mechanism depending on PTx-sensitive G proteins. During the genesis of heart failure, the expression of NPY, increases protein synthesis of cardiomyocytes by factors that are coreleased from nerve endings with NPY.

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