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Hypertrophic growth of cultured neonatal rat heart cells mediated by vasopressin V_{1A} receptor

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Abstract

Primary cultures of neonatal cardiac myocytes were used to determine both the identity of second messengers that are involved in vasopressin receptor-mediated effects on cardiac hypertrophy and the type of vasopressin receptor that is involved in vasopressin-induced cell growth. Neonatal rat myocytes were plated at a density of 1×10^6 cells per 60 mm dish and were incubated with serum-free medium for 7 days. Treatment of myocytes with vasopressin significantly increased the RNA-to-DNA ratio, by 18–25%, at culture days 4–6 and the protein-to-DNA ratio by 18–20% at culture days 5–7. Rates of protein synthesis were determined to assess their contribution to protein contents during myocyte growth. Vasopressin significantly accelerated rates of protein synthesis by 25% at culture day 6. Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was transiently increased after vasopressin exposure. After the peak increase in $[Ca^{2+}]_i$ at less than 30 s, there was a sustained increase for at least 5 min. The specific activity of protein kinase C in the particulate fraction was increased rapidly after exposure to vasopressin, and its activity remained higher for 30 min, returning to its control level within 60 min. The activity of protein kinase C in the cytosol was significantly decreased at all times after exposure to vasopressin. After vasopressin treatment, the content of c-*fos* mRNA was increased. The stimulatory effects of vasopressin on these parameters were significantly inhibited by vasopressin V_{1A} receptor antagonist, OPC-21268, but not by vasopressin V_2 receptor antagonist, OPC-31260. These results suggest that vasopressin directly induces myocyte hypertrophic growth via the V_{1A} receptor in neonatal rat heart cells.

Keywords: Vasopressin; Vasopressin V_{1A} receptor; Vasopressin V_2 receptor; Hypertrophy; Myocyte; Cell culture

1. Introduction

Cardiac hypertrophy is a process that occurs in response to various hemodynamic and neurohormoral stimuli (Morgan and Baker, 1991). The neurohormoral factors such as catecholamines, angiotensin II, endothelin 1 and [Arg]vasopressin (vasopressin), which increase in patients with congestive heart failure or hypertension (Cowley et al., 1981; Riegger et al., 1982; Kawano et al., 1997) may play an important role in the development of cardiac hypertrophy through mechanisms related to increased peripheral vascular resistance and increased cardiac afterload. Results of numerous studies suggest that catecholamines, angiotensin II or endothelin 1 directly induces cardiac hypertrophy, independent of increases in peripheral vascular resistance (Simpson, 1983; Aceto and Baker, 1990; Ito et al., 1991). Recently, we have demonstrated that angiotensin II-induced hypertrophic growth is, at least in part, mediated through the type 1 angiotensin II receptor in neonatal rat heart cells (Miyata and Haneda, 1994). However, whether vasopressin directly induces cardiac hypertrophy is not known at this time.

Vasopressin plays an important role in the regulation of body fluid balance through its antidiuretic action. Vasopressin is also known to be a potent vasoconstrictor that regulates the cardiovascular system (Share, 1988; Walker et al., 1988; Schoemaker et al., 1990; Cheng et al., 1993). Two subtypes (V_{1A} and V_2) of peripheral vasopressin receptors have been proven to show both tissue and species variation. The V_{1A} receptor elicits glycogenolysis in liver and vasoconstriction in vascular smooth muscle through an adenosine 3′,5′-monophosphate (cAMP)-independent mechanism coupled to phosphoinositide turnover (Michell et al., 1979; Tolbert et al., 1980; Takeda et al., 1988; Endoh et al., 1992). On the other hand, the V_2 receptor,

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which relates to the antidiuretic response in kidney, acts through a cAMP-dependent mechanism (Bockaert et al., 1973). Recently, V_{1A} and V_2 receptors have been identified in rat myocardium, and it has become clear that the heart is also a direct target for vasopressin. Xu and Gopalakrishnan (1991), Van der Bent et al. (1994) and Reilly et al. (1998) have reported that vasopressin increases intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and protein kinase C activity via the V_{1A} receptor in neonatal rat cardiomyocytes. However, they did not evaluate cell growth subsequent to vasopressin-induced increases in $[Ca^{2+}]_i$ and protein kinase C activity. Furthermore, the role of vasopressin in the development of cardiac hypertrophy still remains to be elucidated.

Neonatal cell culture preparations, composed predominantly of non-dividing cardiac myocytes, are useful models for studies involving myocardial growth induced by hormonal stimulation (Simpson and Savion, 1982; McDermott and Morgan, 1989). Myocardial growth under these conditions occurs primarily through hypertrophy rather than cell division. When neonatal myocytes are maintained in chemically defined media, protein synthesis can be modulated in response to hormone treatment, and rates of protein synthesis and degradation can be evaluated easily and exactly. Therefore, in the present study, primary cultures of neonatal rat cardiac myocytes were used to examine whether vasopressin directly induces cardiac hypertrophy to determine the identity of the second messengers that are involved in the vasopressin receptor-mediated effect on cardiac hypertrophy and the type of vasopressin receptor that is invoved in vasopressin-induced cell growth.

2. Materials and methods

2.1. Myocyte culture

Monolayer cultures of 1- to 2-day-old neonatal Sprague–Dawley rat (Japan SLC) myocytes were prepared with modifications as described previously (Haneda and McDermott, 1991). Minced ventricular myocardium was placed in Ca^{2+}- and Mg^{2+}-free Hanks' salt solution buffered with 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4. The cells were dissociated in a water-jacketed Celstir apparatus (Wheaton Scientific) at 37°C with a mixture of partially purified trypsin (2.4 IU/ml, Worthington Biochemical), α-chymotrypsin (2.7 IU/ml), and elastase (0.94 IU/ml, Sigma). After each of five successive 20-min incubations, the dissociated cells were mixed with Eagle's minimal essential medium (MEM) (Gibco) containing 10% newborn-calf serum and were centrifuged and pooled. The dissociated cells were enriched for cardiomyocytes by the technique of differential adhesion for 90 min and plated at a concentration of approximately 1×10^6 cells/60 mm dish. Cultures were incubated in a humidified 5% CO_2–95% air atmosphere at 37°C. After an overnight incubation in MEM containing 10% newborn calf serum and 0.1 mM 5-bromo-2′-deoxyuridine (Sigma), the attached cells were rinsed and maintained in serum-free medium. Briefly, standard MEM was supplemented with MEM amino acids, vitamins, penicillin–streptomycin (Gibco), and 2 mM glutamine. In addition, the medium contained 30 nM $NaSeO_4$, 2.5 μg/ml human insulin, 10 μg/ml human transferrin (Sigma), 0.25 mM ascorbic acid (Sigma) and 0.1 mM 5-bromo-2′-deoxyuridine to minimize the proliferation of non-myogenic cells. The medium was replaced every 2 days with fresh medium over the course of the experiments. More than 90% of the cells were myocytes, as determined by immunofluorescent staining with antimyosin antibodies, and the number of myocytes per culture dish did not change during vasopressin-treatment. Stock solutions of vasopressin (Sigma), a non-peptide selective V_{1A} receptor antagonist (Yamamura et al., 1991), 1-(1-[4(3-acetylaminopropoxy)benzoyl]-4-piperidyl)-3,4-dihydro-2(1*H*)-quinolinone, OPC-21268 (a gift of Otsuka Pharma., Tokushima, Japan) or a non-peptide selective V_2 receptor antagonist (Yamamura et al., 1992), 5-dimethyl amino-1-(4-[2-methylbenzoylamino]benzoyl)-2,3,4,5-tetrahydro-1*H*-benzazepine, OPC-31260 (a gift of Otsuka Pharma.), were prepared in MEM at a concentration of 10^{-3}, 10^{-2} or 10^{-2} M, respectively, stored at −20°C, and diluted in culture medium at the time of use.

2.2. Myocyte protein, DNA and RNA content

Each culture dish was rinsed three times with ice-cold phosphate-buffered saline. The cell layer was scraped from each dish with two 0.5 ml volumes of 1× standard sodium citrate containing 0.25% (wt/vol) sodium dodecyl sulfate and frozen at −80°C. Before assay, the extracts were thawed and vortexed extensively. Protein content was assayed directly in aliquots of each extract by the method of Lowry using crystalline bovine serum albumin as a standard (Lowry et al., 1951). DNA content was measured fluorometrically in aliquots of each extract using calf thymus DNA as a standard (Cesarone et al., 1979). For RNA determination, a 750-μl aliquot from each extract was precipitated with an equal volume of ice-cold 1 N $HClO_4$. The material was centrifuged, and the pellet was washed three times with 0.5 N $HClO_4$. The pellet was hydrolyzed in 0.3 N NaOH for 24 h at 37°C, and the protein was reprecipitated by adding 4 N $HClO_4$ to a final concentration of 1 N. The supernatant was used to measure the absorbance at 260 and 232 nm following centrifugation. The RNA concentration (in μg/ml) was calculated according to the following equation (Munro and Fleck, 1966)

$$\mathrm{RNA} = (A_{260} \times 32.9) - (A_{232} \times 6.11)$$

where A_{260} and A_{232} are the absorbances at 260 and 232 nm, respectively.

2.3. Protein synthesis

The relative amount of protein synthesis was determined by assessing the incorporation of radioactivity into a 10% trichloroacetic acid insoluble fraction after L-[^{14}C]phenylalanine ([^{14}C]Phe: 1 μCi/ml; Amersham) exposure for 2 h. For determination of the specific activity of the labeled protein, the cells were rapidly rinsed three times with ice-cold phosphate-buffered saline, and the protein was precipitated with 1 ml of 10% trichloroacetic acid for 1 h on ice and extracted overnight with 1 N NaOH (Morgan et al.,1971; McDermott and Morgan, 1989). This solution was used for determinations of radioactivity and protein concentrations. Radioactive samples were counted in a liquid scintillation counter (Beckman). Cell protein was determined by the Lowry method (Lowry et al., 1951).

2.4. Intracellular free Ca^{2+}

$[Ca^{2+}]_i$ was measured with the fluorescent dye, fura 2-acetoxymethyl ester (Dojindo), as described (Grynkiewicz et al., 1985). Briefly, laminin (Collaborative Biochemical Products) was diluted in Hanks' buffer to a final concentration of 20 μg/ml. Coverslips were coated with diluted laminin and incubated for 24 h. At the end of the incubation, the remaining solution was removed by aspiration, and coverslips were washed once with incubation medium. Myocytes were diluted to a concentration of approximately 1×10^6 cells/dish, plated onto the laminin-coated cover slips in 60-mm dishes, and cultured as described above. After 24 h, the incubation medium was replaced with serum-free medium, and the cells were cultured for an additional 3 days. To measure $[Ca^{2+}]_i$, the coverslips were transferred to a culture dish and rinsed with HEPES buffer solution containing 140 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, 1 mM Na_2HPO_4, and 30 mM HEPES (pH 7.4) to remove culture medium from the cells. The coverslips were loaded with 5 μM fura 2-acetoxymethyl ester for 30 min and rinsed twice with buffer to remove free fura 2-acetoxymethyl ester from the cells. Measurements of $[Ca^{2+}]_i$ were made at 37°C in a cuvette with 3 ml of HEPES buffer solution. Fluorescence was continuously recorded using an intracellular ion analyzer CAF-110 (Jasco). The ratio of fluorescence during illumination alternatively at 340 and 380 nm was obtained and $[Ca^{2+}]_i$ was calculated as described. Extracellular free Ca was chelated with 10 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA). $[Ca^{2+}]_i$ was calculated with the following formula: $[Ca^{2+}]_i = K_d \times [(R_{340/380} - R_{min})/(R_{max} - R_{340/380})] \times b$. K_d is the dissociation constant of fura 2-acetoxymethyl ester for Ca^{2+} and was assumed to be 224 nM at 37°C. R_{min} is the ratio of corrected 340 and 380 nm signals obtained after EGTA treatment, R_{max} is the ratio obtained after 1 μM ionomycin (Sigma) treatment, and *b* is the ratio of the corrected minimal (EGTA) 340 nm and maximal (ionomycin) 380 nm signals.

2.5. Protein kinase C activity

The protein kinase C activity of particulate and cytosolic fractions in cultured myocytes was measured with a protein kinase C assay system (Amersham) (Henrich and Simpson, 1988; Allo et al., 1991). Duplicate dishes of myocytes were rinsed three times with ice-cold Hank's balanced salt solution buffer and scraped into 1.0 ml of ice-cold buffer A (20 mM Tris–HCl, 5 mM ethylenediaminetetraacetic acid, 10 mM EGTA, 0.3% β-mercaptoethanol, 10 mM benzamidine, and 50 μg/ml phenylmethylsulfonyl fluoride, pH 7.5). The cells were sonicated twice for 10 s each time and centrifuged at 100,000 × *g* for 60 min. The supernatant was used to measure cytosolic protein kinase C activity. The pellet was resuspended in

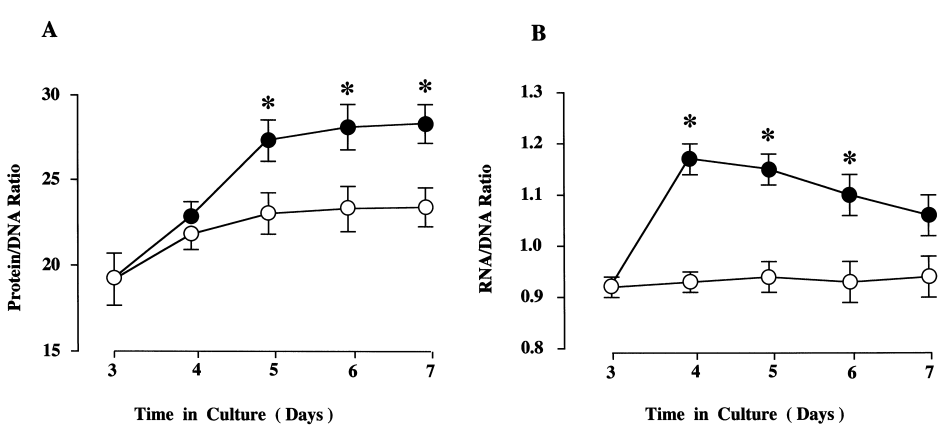

Fig. 1. Effects of AVP on cell growth and RNA content. Cardiomyocytes were plated at 1×10^6 cells/dish and were incubated in serum-free medium, and AVP was added to a final concentration of 10^{-7} M on day 3. The ratios of total protein-to-DNA (A) and RNA-to-DNA (B) were measured at the times shown. Values at each time are means ± S.E.M. from 5 to 10 dishes in four different preparations. (○) Control; (●) AVP-treated myocytes. $^*P < 0.05$, compared with control cells at the same point.

Table 1
Effects of OPC-21268 and OPC-31260 on AVP-induced hypertrophic growth
Values are means ± S.E.M. from 5–10 dishes in four different preparations. Cardiomyocytes were incubated in serum-free medium. OPC-21268 or OPC-31260 was added 30 min before exposure to AVP (10^{-7} M). AVP, OPC-21268 or OPC-31260 was added on day 3 to the final concentrations indicated. The absolute value of DNA and the ratios of total protein-to-DNA and RNA-to-DNA were measured at days 4, 5 and 6.

Agent			DNA, μg/dish			Total protein-to-DNA			RNA-to-DNA		
AVP	OPC-21268	OPC-31260	Day 4	Day 5	Day 6	Day 4	Day 5	Day 6	Day 4	Day 5	Day 6
–	–	–	19.7 ± 0.9	18.9 ± 1.1	19.1 ± 1.3	21.7 ± 1.0	22.9 ± 0.9	23.2 ± 1.2	0.93 ± 0.02	0.94 ± 0.04	0.93 ± 0.04
10^{-7} M	–	–	19.0 ± 1.2	19.1 ± 1.1	19.7 ± 1.0	22.7 ± 0.9	27.1 ± 1.2[a]	27.9 ± 1.3[a]	1.17 ± 0.03[a]	1.15 ± 0.03[a]	1.10 ± 0.04[a]
10^{-7} M	10^{-7} M	–	18.9 ± 1.0	19.3 ± 1.3	19.0 ± 0.9	21.9 ± 1.1	22.3 ± 0.7[b]	23.5 ± 0.9[b]	0.92 ± 0.03[b]	0.91 ± 0.03[b]	0.89 ± 0.04[b]
10^{-7} M	–	10^{-7} M	19.5 ± 0.9	19.1 ± 1.5	18.9 ± 1.0	22.3 ± 0.8	26.9 ± 0.9[a]	27.1 ± 0.7[a]	1.11 ± 0.02[a]	1.10 ± 0.04[a]	1.08 ± 0.03[a]
–	10^{-7} M	–	19.4 ± 1.0	19.0 ± 0.7	19.3 ± 1.3	20.9 ± 1.1	22.1 ± 0.7[b]	22.4 ± 0.8[b]	0.92 ± 0.02[b]	0.91 ± 0.02[b]	0.94 ± 0.04[b]
–	–	10^{-7} M	18.8 ± 0.7	18.9 ± 0.9	19.0 ± 1.4	21.8 ± 0.9	22.3 ± 1.8[b]	23.0 ± 1.0[b]	0.92 ± 0.01[b]	0.93 ± 0.03[b]	0.95 ± 0.02[b]

[a] $P < 0.05$, compared with control cells.
[b] $P < 0.05$, compared with AVP (10^{-7} M)-treated cells.

buffer A, solubilized with 0.1% Triton X-100 for 30 min on ice, and used for measurement of particulate fraction protein kinase C activity.

2.6. RNA extraction and Northern blot analysis

Total RNA was extracted from the myocytes at 30 min of exposure to drugs, using the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Total RNA (30 μg) was separated electrophoretically in a 1.2% agarose gel, and the quantity of RNA in each track was verified by ethidium bromide staining before transfer. The RNAs were transferred to nylon membranes (Hybond-N^+, Amersham). v-*fos*, a 1.00-kb *Pst*I/*Pvu*II fragment containing FBJ-murine osteosarcoma virus proviral DNA (Takara, Kyoto, Japan) was used as the probe and was labeled by random priming with [α-^{32}P]cytidine deoxy triphosphate. For standardization, human β-actin, *Hin*fI/*Hin*fI fragment (Wako Seiyaku, Osaka, Japan) was used as β-actin probe. Hybridization to detect c-*fos* mRNA was carried out for 12–24 h at 42°C in a solution containing 5 × saline–sodium phosphate–ethylenediaminetetraacetic acid, 50% formamide, 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate, and 20 μg/ml of sonicated salmon sperm DNA. After washing, the membrane was subjected to autoradiography (X-Omat; Eastman Kodak, Rochester, USA) at −80°C for 2 or 4 days.

2.7. Statistical analysis

All values are expressed as means ± S.E.M. Statistical significance was determined using an unpaired Student's *t*-test or analysis of variance. The results were considered to be significant when $P < 0.05$.

3. Results

3.1. Effects of vasopressin, OPC-21268 and OPC-31260 on cellular hypertrophy

Subconfluent neonatal rat heart cells in culture were used to determine the effects of vasopressin on RNA content and myocyte growth. Vasopressin was added to a final concentration of 10^{-7} M on culture day 3, and protein-to-DNA and RNA-to-DNA ratios were measured at 24-h intervals over the next 4 days in culture. The protein-to-DNA ratio increased over the first day after the addition of vasopressin (Fig. 1A). The protein-to-DNA ratios in vasopressin-treated myocytes were increased significantly, by 18.1%, 20.2% and 20.6%, compared to the controls at days 5, 6 and 7, respectively. The RNA-to-DNA ratios were increased significantly, by 25.8%, compared to the controls 1 day after addition of vasopressin (Fig. 1B).

However, the RNA-to-DNA ratios decreased after 2 days of vasopressin treatment. These findings indicated that vasopressin rapidly increased the capacity for protein synthesis, as measured by the RNA content and that greater capacity was followed by accelerated myocyte growth. vasopressin did not change the absolute values for DNA contents significantly (Table 1).

In the next experiments, the effects of a non-peptide selective V_{1A} receptor antagonist, OPC-21268, and a non-peptide selective V_2 receptor antagonist, OPC-31260, on vasopressin-stimulated increases in protein-to-DNA and RNA-to-DNA ratios were studied to determine which type of vasopressin receptor was involved in vasopressin-mediated hypertrophic growth. OPC-21268 (10^{-7} M) and OPC-31260 (10^{-7} M) were added 30 min before exposure to vasopressin (10^{-7} M). OPC-21268 significantly inhibited the vasopressin-induced increases in protein-to-DNA ratios at culture days 5 and 6, and in RNA-to-DNA ratios at culture days 4, 5 and 6, however, OPC-31260 did not have an inhibitory effect (Table 1). OPC-21268 or OPC-31260 alone did not change the values for DNA contents, protein-to-DNA ratios and RNA-to-DNA ratios (Table 1).

3.2. Effects of vasopressin, OPC-21268 and OPC-31260 on protein synthesis

To determine whether the increase in cellular protein was the result of an increase in the rate of protein synthesis, rates of amino acid incorporation were determined from the [^{14}C]Phe incorporation into total protein (dpm/μg protein). Cultures of neonatal rat myocytes were pulsed for 2 h with [^{14}C]Phe to determine the rates of protein synthesis. Rates of protein synthesis were measured at 2 days after the addition of vasopressin, when rapid increases in protein-to-DNA ratios were observed in this study (Fig. 1A). Dose dependence of the vasopressin response on protein synthesis was determined after exposure of the cells to vasopressin (Fig. 2). The rates of protein synthesis were 49.1 ± 1.1, 49.6 ± 1.4, 57.2 ± 2.2, 61.3 ± 1.1, 62.3 ± 1.0 and 60.1 ± 1.9 dpm/μg protein at concentrations of 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M vasopressin, respectively (Fig. 2). There were significant increases in protein synthesis rates at 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M vasopressin concentrations (18.9%, 24.8%, 26.9% and 22.4% above control, respectively) (Fig. 2). In the next experiments, the effects of OPC-21268 and OPC-31260 to prevent the acceleration of protein synthesis rates by vasopressin were studied. OPC-21268 (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) or OPC-31260 (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) was added first, followed 30 min later by vasopressin (10^{-7} M). The vasopressin-induced increases in rates of protein synthesis were significantly inhibited by OPC-21268 (10^{-8}, 10^{-7} and 10^{-6} M), but not by OPC-31260 (Table 2). OPC-21268 or OPC-31260 did not change the rates of protein synthesis in the absence of vasopressin (Table 2).

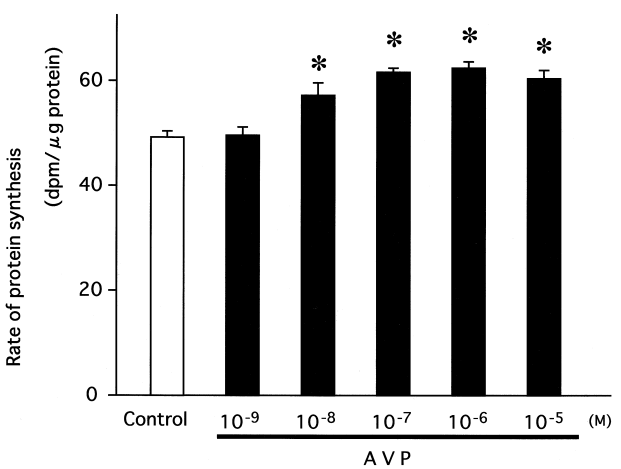

Fig. 2. Effects of AVP on rate of protein synthesis. Cardiomyocytes were plated at 1×10^6 cells/dish and were incubated in serum-free medium, and AVP was added on day 3 to the final concentrations as indicated. Cells were labeled with [^{14}C]phenylalanine (1 μCi/ml) for 2 h. Protein synthesis rates were measured at day 5. Values are means ± S.E.M. from 5 to 10 dishes in five different preparations. * $P < 0.05$, compared with control cells.

Table 2
Effects of OPC-21268 and OPC-31260 on AVP-induced increase in rates of protein synthesis
Values are means ± S.E.M. from 5–8 dishes in four different preparations. Cardiomyocytes were incubated in serum-free medium, and AVP (10^{-7} M), OPC-21268 or OPC-31260 was added on day 3 to the final concentrations indicated. OPC-21268 or OPC-31260 was added 30 min before exposure to AVP. Rates of protein synthesis were measured at day 5.

Agents			Rates of protein synthesis dpm/μg protein
AVP	OPC-21268	OPC-31260	
–	–	–	49.4 ± 1.3
10^{-7} M	–	–	62.1 ± 1.1[a]
10^{-7} M	10^{-9} M	–	62.4 ± 2.4[a]
10^{-7} M	10^{-8} M	–	53.1 ± 1.8[b]
10^{-7} M	10^{-7} M	–	49.6 ± 2.2[b]
10^{-7} M	10^{-6} M	–	48.9 ± 1.9[b]
10^{-7} M	–	10^{-9} M	63.6 ± 2.7[a]
10^{-7} M	–	10^{-8} M	61.1 ± 1.9[a]
10^{-7} M	–	10^{-7} M	60.4 ± 2.0[a]
10^{-7} M	–	10^{-6} M	57.9 ± 2.1[a]

[a] $P < 0.05$, compared with control cells.
[b] $P < 0.05$, compared with AVP (10^{-7} M)-treated cells.

3.3. Effects of vasopressin, OPC-21268 and OPC-31260 on $[Ca^{2+}]_i$

The vasopressin-induced Ca^{2+} mobilization in neonatal rat myocytes was examined by measuring the changes in fluorescence of $[Ca^{2+}]_i$ using fura 2-acetoxymethyl ester as an indicator. Vasopressin (10^{-7} M) increased $[Ca^{2+}]_i$ by 80% above basal levels as shown by the representative tracing in Fig. 3A. After the peak increase in $[Ca^{2+}]_i$ at less than 30 s, there was a sustained increase for at least 5

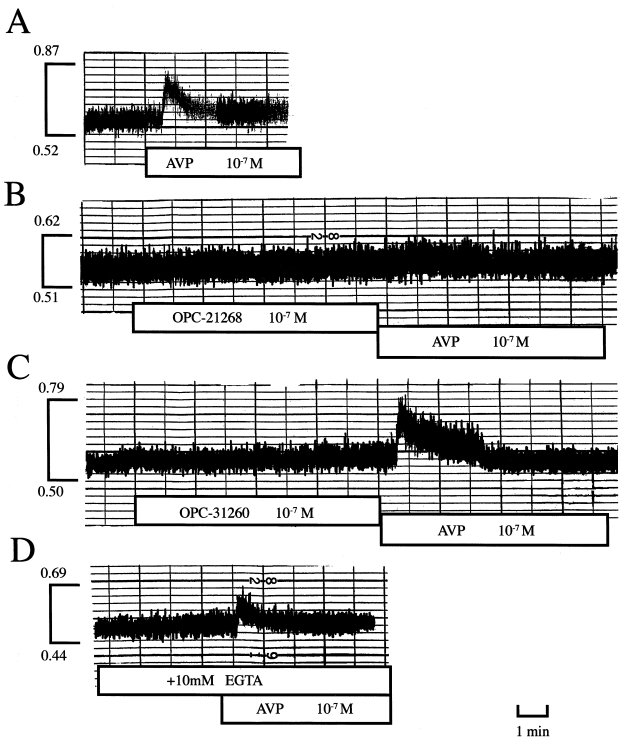

Fig. 3. Effects of AVP (10^{-7} M), OPC-21268 (10^{-7} M) and OPC-31260 (10^{-7} M) on intracellular free Ca^{2+} ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ in separate slides taken from the same preparation of rat cardiomyocytes was measured by loading of fura 2-acetoxymethyl ester. Values are ratios of emitted fluorescence at 340 and 380 nm excitation. (A) AVP increased $[Ca^{2+}]_i$. (B) AVP-stimulated increase in $[Ca^{2+}]_i$ was blocked by pretreatment with OPC-21268. (C) AVP-stimulated increase in $[Ca^{2+}]_i$ was not blocked by pretreatment with OPC-31260. (D) EGTA (10 mM) reduced AVP-stimulated increase in $[Ca^{2+}]_i$. Similar data were obtained in three different experiments.

min. Therefore, the values of $[Ca^{2+}]_i$ were measured 30 s after the addition of vasopressin, the time when the maximal increase in these values was observed. Vasopressin (10^{-7} M) stimulated significantly, by 2.8-fold, compared with the control (Table 3).The vasopressin-stimulated increase in $[Ca^{2+}]_i$ was completely blocked by OPC-21268 (10^{-7} M), when added 7 min before exposure to vasopressin (Fig. 3B and Table 3), but not by OPC-31260 (10^{-7} M) (Fig. 3C and Table 3). The chelation of extracellular Ca^{2+} by addition of 10 mM EGTA significantly reduced the vasopressin-induced transient increases of $[Ca^{2+}]_i$ (Fig. 3D and Table 3).

Table 3
Effects of AVP, OPC-21268 and OPC-31260 on intracellular free Ca^{2+} in cultured rat myocytes
Values are means ± S.E.M. from 5–8 dishes in four different preparations. Cardiomyocytes were incubated in serum-free medium, and AVP (10^{-7} M), OPC-21268 (10^{-7} M), OPC-31260 (10^{-7} M), or EGTA (10 mM) was added.

Agents				$[Ca^{2+}]$, nM
AVP	OPC-21268	OPC-31260	EGTA	
–	–	–	–	122.9 ± 8.9
10^{-7} M	–	–	–	297.1 ± 9.2[a]
10^{-7} M	10^{-7} M	–	–	120.3 ± 6.7
10^{-7} M	–	10^{-7} M	–	166.9 ± 9.7
10^{-7} M	–	–	10 mM	62.1 ± 5.7[a]

[a] $P < 0.05$, compared with cells in the absence of AVP in each group.

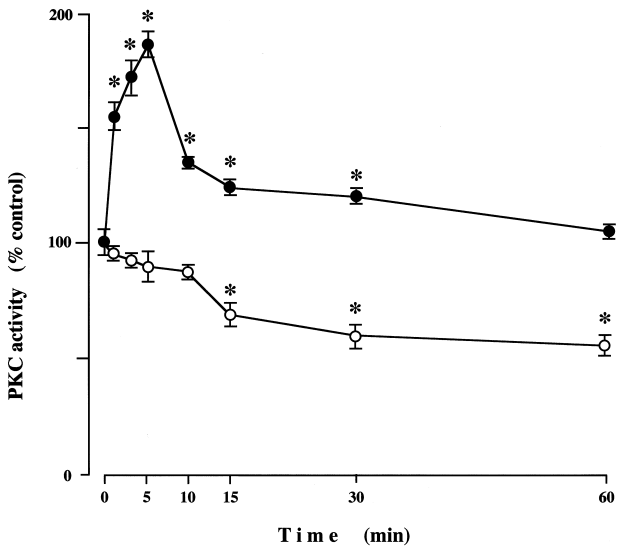

Fig. 4. Effects of AVP (10^{-7} M) on cardiomyocyte protein kinase C activity. Cardiomyocytes were exposed to AVP (10^{-7} M) for the indicated time. Protein kinase C (PKC) activities in both particulate and cytosolic fractions were assayed as described in Section 2. Values at each time are means ± S.E.M. for 24 dishes in four different preparations and are expressed as percentages of control values. (○) Cytosolic fraction; (●) particulate fraction. $^*P < 0.05$, compared with control cells at the same time.

3.4. Effects of vasopressin, OPC-21268 and OPC-31260 on protein kinase C activity

The effects of vasopressin (10^{-7} M) on protein kinase C activities in both particulate and cytosolic fractions were determined. The specific activity of protein kinase C in the particulate fraction from vasopressin-treated myocytes increased rapidly at 1, 3 and 5 min after the addition of vasopressin (+55%, +73% and +86% above control, respectively) (Fig. 4). Its activity remained higher for 30 min after vasopressin exposure and then returned to the control level within 60 min. On the other hand, the specific activity of protein kinase C in the cytosolic fraction of vasopressin-treated myocytes was significantly decreased at 15, 30 and 60 min after the addition of vasopressin (31%, 41% and 45% below control, respectively) (Fig. 4). Its activity in vasopressin-treated myocytes remained lower over the duration of the experiment. In the next experiment, the effects of OPC-21268 and OPC-31260 on vasopressin-induced changes in particulate and cytosolic fractions protein kinase C activitiy were examined. OPC-21268 (10^{-7} M) or OPC-31260 (10^{-7} M) was added first, followed 30 min later by vasopressin (10^{-7} M). OPC-21268 significantly blocked the vasopressin-stimulated in-

creases in particulate fraction protein kinase C activity, but OPC-31260 did not (Fig. 5A). Furthermore, OPC-21268 significantly blocked the vasopressin-stimulated decreases in cytosolic fraction protein kinase C activity, but OPC-31260 did not (Fig. 5B).

3.5. Effects of vasopressin, OPC-21268 and OPC-31260 on c-fos mRNA expression

In a preliminary study, maximal expression of c-*fos* mRNA was reached at 30 min after exposure to vasopressin. The effects of vasopressin, OPC-21268 and OPC-31260 on c-*fos* mRNA expression were evaluated at that time of exposure. As shown in Fig. 6, vasopressin (10^{-7} M) markedly increased c-*fos* mRNA expression. OPC-21268 (10^{-7} M) or OPC-31260 (10^{-7} M) was added to the medium 30 min before exposure to vasopressin. OPC-21268 strongly inhibited the vasopressin-stimulated expression, but OPC-31260 did not (Fig. 6). OPC-21268 or OPC-31260 alone did not affect the expression of c-*fos* mRNA.

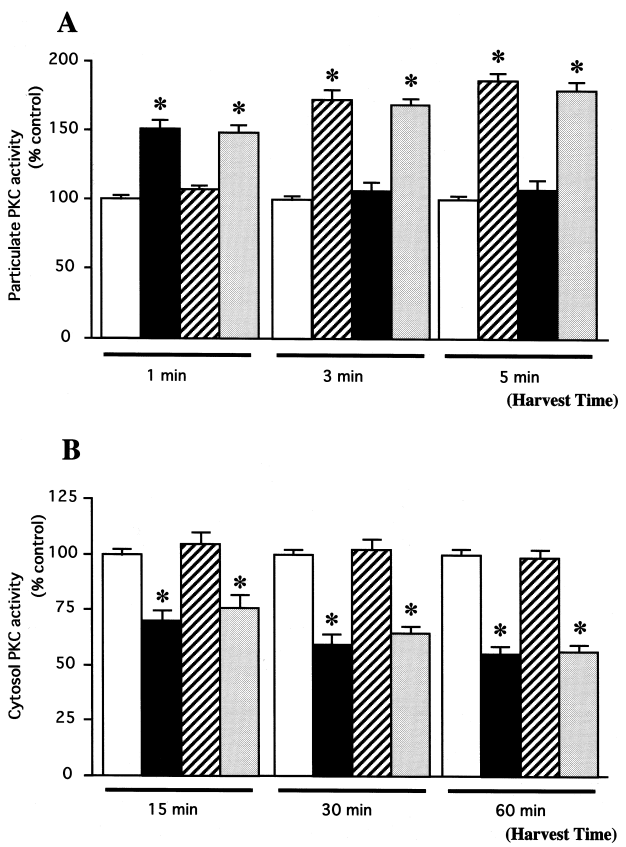

Fig. 5. Effects of OPC-21268 (10^{-7} M) and OPC-31260 (10^{-7} M) on AVP (10^{-7} M)-induced increase in protein kinase C activity. OPC-21268 or OPC-31260 was added 30 min before exposure to AVP. Protein kinase C (PKC) activities in the particulate fraction (A) were assayed after 1, 3 and 5 min of treatment with AVP, and in the cytosolic fraction (B) were assayed after 15, 30 and 60 min of treatment with AVP. Values at each time are means ± S.E.M. for 24 dishes in four different preparations and are expressed as percentage of control values. Open bars, control; solid bars, AVP-treated cells; hatched bars, both AVP- and OPC-21268-treated cells; stippled bar, both AVP- and OPC-31260-treated cells. $^{*}P < 0.05$, compared with control cells at the same point; $^{\#}P < 0.05$, compared with AVP-treated cells at the same point.

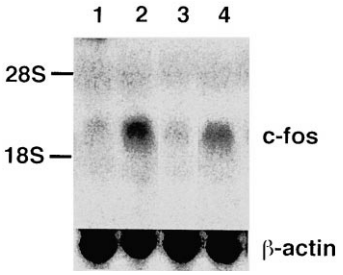

Fig. 6. Effects of AVP (10^{-7} M), OPC-21268 (10^{-7} M) and OPC-31260 (10^{-7} M) on c-*fos* mRNA expression in cardiomyocytes. Expression of c-*fos* mRNA was determined by Northern blot analysis. Total RNA (30 μg) was loaded in each lane. OPC-21268 or OPC-31260 was added 30 min before exposure to AVP. For standardization, human β-actin, *Hin*fI/*Hin*fI fragment was used as β-actin probe. Similar data were obtained in three different experiments. Lane 1: control. Lane 2: exposure to AVP. Lane 3: exposure to AVP with OPC-21268. Lane 4: exposure to AVP with OPC-31260.

4. Discussion

Vasopressin may contibute to cardiovascular regulation by causing vasocontriction and stimulating renal water absorption through V_{1A} and V_2 receptors, respectively. This classification of vasopressin receptor subtypes was originally proposed by Michell et al. (1979), and was based on intracellular mechanisms: cAMP-independent (V_{1A}) and cAMP-dependent (V_2) pathways. Recently, vasopressin has been demonstrated to produce cellular growth as well as contraction of vascular smooth muscle cells and glomerular mesangial cells (Ganz et al., 1988; Takeda et al., 1988; Geisterfer and Owens, 1989; Kribben et al., 1993; Tahara et al., 1997). Vasopressin-induced cell growth in these cells is coupled with the hydrolysis of phosphatidylinositol 4,5-bisphoshate to inositol 1,4,5-trisphoshate and diacylglycerol, with the mobilization of $[Ca^{2+}]$ and the activation of protein kinase C. Xu and Gopalakrishnan (1991) and Van der Bent et al. (1994) reported that vasopressin increases $[Ca^{2+}]_i$ and protein kinase C activity in neonatal rat myocytes. However, the other intracelluar signaling pathway of vasopressin and the effects of vasopressin on protein synthesis and cell growth in cardiac tissue were not clear. The present experiments demonstrated that vasopressin stimulates directly amino acid incorporation and cell growth in neonatal rat heart cells (Table 1, Figs. 1 and 2). The vasopressin-induced growth response was hypertrophic because there was no cellular division, as indicated by stable DNA content (Table 1).

Yamamura et al. (1991) reported on an orally effective, non-peptide V_{1A} receptor antagonist, OPC-21268, that

specifically antagonized responses to vasopressin in vitro and in vivo. Subsequently, via a series of structural conversions of OPC-21268, the selective V_2 receptor antagonist, OPC-31260, was discovered (Yamamura et al., 1991). Furthermore, the presence of two receptor subtypes (V_{1A} and V_2) in rat myocardium has been demonstrated (Xu and Gopalakrishnan, 1991). Therefore, we investigated the effects of OPC-21268 and OPC-31260 on vasopressin-induced stimulation of protein synthesis and cell growth. The present study demonstrated that OPC-21268 inhibited in a dose-dependent manner the increases in rates of protein synthesis and cell growth accelerated by vasopressin, but OPC-31260 did not (Tables 1 and 2). Therefore, these data strongly suggest that vasopressin-induced hypertrophic growth is related to occupancy of the V_{1A} receptor, which couples with phosphatidyl inositol turnover. The next purpose of the present work was to document changes in the components of intracellullar signaling pathways during vasopressin-mediated hypertrophic growth in cultured rat heart cells. The stimulatory effects of vasopressin on protein synthesis and cell growth could occur via several mechanisms, such as increased rates of cell beating, higher concentrations of $[Ca^{2+}]_i$ or inositol 1,4,5-trisphoshate, greater protein kinase C activity, enhanced expression of an immediate early gene, and activation of as yet unknown second or third messengers that could mediate or modulate protein synthesis at a pre- or post-translational level.

Ca^{2+} has been described as a possible second messenger involved in the process of cell growth (Brostrom et al., 1983). Our data also showed that vasopressin was a full agonist for stimulation of $[Ca^{2+}]_i$ in cultured rat myocytes (Fig. 3A and Table 3). It has been suggested that the vasopressin-mediated increases in $[Ca^{2+}]_i$ occur via Ca^{2+} transport through voltage-activated Ca^{2+} channels (Zhang et al. 1995). In the present study, however, removal of extracellular Ca^{2+} by addition of 10 mM EGTA to the medium reduced the initial vasopressin-stimulated increase in $[Ca^{2+}]_i$ and abolished the sustained elevation of $[Ca^{2+}]_i$ (Fig. 3D and Table 3). These data suggest that the transient increase in $[Ca^{2+}]_i$ may result from both mobilization of intracellular Ca^{2+} stores mediated by inositol 1,4,5-trisphoshate, and Ca^{2+} influx across the membrane via voltage-dependent Ca^{2+} channels. The selective V_{1A} receptor antagonist, OPC-21268, abolished the vasopressin-mediated increases in $[Ca^{2+}]_i$ by interacting with the vasopressin myocardial membrane receptor (Fig. 3B and Table 3), but the selective V_2 receptor antagonist, OPC-31260, did not (Fig. 3C and Table 3). OPC-21268 or OPC-31260 alone did not modify $[Ca^{2+}]_i$ (Fig. 3B,C and Table 3). These data indicate that the vasopressin-mediated increase in $[Ca^{2+}]_i$ is the result of binding to the V_{1A} receptor. There are several reports on the role of $[Ca^{2+}]_i$ in the cardiac hypertrophic response. We have reported that increased Ca^{2+} influx is not involved in stimulation of rates of protein synthesis in perfused rat hearts (Haneda et al., 1989). Baker and Aceto (1990) reported that the antagonist, [Sar^1,Ile^8]angiotensin II, raises $[Ca^{2+}]_i$ but does not stimulate protein synthesis and cell growth in embryonic chick heart cells. However, Sadoshima et al. (1995) reported that $[Ca^{2+}]_i$, rather than protein kinase C, seems to be critical for angiotensin II-induced activation of tyrosine kinase, mitogen-activated protein kinase and 90-kDa S6 kinase in neonatal rat cardiac myocytes. These data suggest that early changes in $[Ca^{2+}]_i$, if then contribute at all, may be permissive but not the sole factor responsible for initiating the protein synthetic response. Whether increased $[Ca^{2+}]_i$ is involved in the vasopressin stimulation of cell growth is still unknown.

Results of numerous studies suggest an important role for protein kinase C as an intracellular mediator of the effects of some hypertrophic growth stimuli (Nishizuka, 1984; Henrich and Simpson, 1988). Protein kinase C is activated by diacylglycerol, formed by the hydrolysis of membrane phosphatidylinositides. The activation of phospholipase C is coupled to agonist–receptor binding and may occur through a guanine–nucleotide binding protein. We have already reported that phorbol ester, which activates protein kinase C, stimulates the hypertrophic growth in cultured heart cells (Haneda and McDermott, 1991). It has been shown that the hypertrophic growth induced by contraction, norepinephrine and phorbol ester results in the translocation of protein kinase C from the cytosolic fraction to the particulate fraction in neonatal rat heart cells in culture (Allo et al., 1991, 1992). We have also reported that angiotensin II rapidly increases protein kinase C activity of the particulate fraction in neonatal rat myocytes (Miyata and Haneda, 1994). Recently, vasopressin has been shown to increase the protein kinase C activity of the particulate fraction from cultured cardiomyocytes (Van der Bent et al., 1994). In the present study of cultured neonatal rat heart cells, vasopressin translocated protein kinase C from the cytosolic to the particulate fraction (Fig. 4). OPC-21268 significantly inhibited the vasopressin-stimulated increase in protein kinase C activity of the particulate fraction (Fig. 5A) and the vasopressin-stimulated decreases in cytosolic fraction protein kinase C activity (Fig. 5B), but OPC-31260 did not (Fig. 5A and B). These data, combined with results previously presented, indicate that the vasopressin-stimulated increase in particulate fraction protein kinase C activity involves the V_{1A} receptor, and that the increased activity of particulate fraction protein kinase C may be involved in stimulating the growth of cardiac myocytes.

The transient induction of growth-related genes, termed early response or immediate-early genes, is one of the earliest detectable effects of growth factor addition to neonatal rat myocytes. The immediate-early genes are characterized by their rapid and transient induction following mitogen stimulation, which does not ordinarily require the synthesis of new or additional proteins. According to these criteria, the protooncogenes, c-*fos* and c-*jun* are central members of an immediate-early gene program.

Recent studies implied an important role for the c-*fos* and c-*jun* protooncogenes in the transcriptional activation of phorbol-inducible genes by the formation of fos/jun heterodimers, which activated transcription after binding to a consensus AP-1 site in the promoter region of these genes (Curran and Franza, 1988). Therefore, increased expression of the c-*fos* gene may play a key role in the control of protein synthesis in cardiac muscle. In this study, we investigated whether vasopressin activated the immediate-early gene program in neonatal rat heart cells. vasopressin increased the expression of c-*fos* mRNA rapidly and transiently, and OPC-21268 inhibited its expression markedly, but OPC-31260 did not inhibit (Fig. 6). These results suggest that vasopressin-mediated expression of c-*fos* mRNA occurs via the V_{1A} receptor in cultured rat myocytes.

In summary, we have demonstrated (1) that vasopressin causes accelerated protein synthesis and cell hypertrophy in neonatal rat myocytes; (2) that vasopressin increases $[Ca^{2+}]_i$ concentrations, particulate fraction protein kinase C activity, and c-*fos* mRNA expression; and (3) that a selective vasopressin V_{1A} receptor antagonist, OPC-21268, inhibits these changes induced by vasopressin, but that a selective vasopressin V_2 receptor antagonist, OPC-31260, did not. These results suggest strongly that vasopressin mediates cardiac hypertrophy through the vasopressin V_{1A} receptor. However, it is not clear which intracellular signaling pathway is the physiological mediator of vasopressin-induced cardiac hypertrophy. Further studies are necessary to determine the mechanisms, including the signal transduction systems, that are responsible for development of vasopressin-induced cardiac hypertrophy.

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