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Functional Receptor for Platelet-Derived Growth Factor in Rat Embryonic Heart-Derived Myocytes: Role of Sequestered Ca^{2+} Stores in Receptor Signaling and Antagonism by Arginine Vasopressin

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Abstract Platelet-derived growth factor (PDGF) is established to function importantly in the growth, development, and function of most cardiovascular tissues. However, evidence that the factor participates directly in the growth and development of the mammalian myocardium is lacking. H9c2 rat embryonic ventricular myocytes were found to respond to PDGF-BB with a rapid mobilization of cell-associated Ca^{2+} and increased rates of protein synthesis, followed by markedly increased rates of DNA synthesis. PDGF acted as a full mitogen for these myocytes. Evidence is provided that documents the expression of classical PDGF-β, but not PDGF-α, receptors in H9c2 cells. Scatchard analysis revealed the presence of 44,000 β-receptors per myocyte. Cell shortening and clustering of plasmalemmal β-receptors occurred within 30 min of exposure to PDGF-BB. Treatment was also associated with a transient increase in the rate of synthesis of GRP78/BiP, consistent with a transitory release of Ca^{2+} from the sarcoplasmic/endoplasmic reticulum [S(E)R]. Increased rates of protein synthesis at early times of PDGF treatment were additive with those occurring in response to arginine vasopressin, indicating different mechanisms of translational upregulation by these agents. The mitogenic effects of PDGF were delayed by vasopressin, which causes H9c2 myocytes to undergo hypertrophy while promoting the persistent depletion of S(E)R Ca^{2+} stores. In the presence of PDGF, vasopressin did not induce hypertrophy. As compared to untreated myocytes, DNA synthesis in PDGF-treated myocytes was optimized at lower extracellular Ca^{2+} concentrations and was significantly less sensitive to inhibition by ionomycin. H9c2 cells appear to provide a useful embryonic cardiomyocyte model in which to examine both PDGF-activated proliferative and vasopressin-activated hypertrophic events and the importance of transient vs. sustained Ca^{2+} release in these events. J. Cell. Biochem. 84: 736–749, 2002. © 2002 Wiley-Liss, Inc.

Key words: H9c2 cells; platelet-derived growth factor; arginine vasopressin; S(E)R Ca^{2+} stores; rat embryonic cardiomyocytes; hyperplasia/hypertrophy

Platelet-derived growth factor (PDGF) functions importantly in the growth, development, and performance of the cardiovascular system [reviewed in Heldin and Westermark, 1999; Betsholtz et al., 2001]. Vascular endothelial and smooth muscle cells (SMCs), like platelets, produce both the PDGF-A and -B peptide variants of the factor, the homodimers of which act respectively at PDGF-α and PDGF-β receptors in a variety of cell types. Capillary endothelial cells, platelets, and myeloid hematopoietic cells express only one type of PDGF receptor, whereas, vascular SMCs and cardiac fibroblasts express both. PDGF stimulates mitogenesis in vascular SMCs and cardiac fibroblasts. Mitogenic responses are mediated by pathways requiring the autophosphorylation of occupied PDGF receptors and the subsequent binding of critical signaling proteins, such as phosphatidyl inositol 3-kinase (PI 3-kinase), that carry SH_2 domains. Activation of PDGF-β receptors, as for example those in the vasculature, produces a transient increase in $[Ca^{2+}]_i$ attributable to release of S(E)R-associated Ca^{2+} stores and to

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enhanced sarcolemmal Ca^{2+} influx [Lapidot and Phair, 1995; Broad et al., 1999]. Current evidence supports roles for PDGF in angiogenesis, regulation of vascular tone and feedback control of platelet aggregation, and in the progression of atherosclerosis and cardiac fibrosis.

Considerably less information exists regarding the involvement of PDGF in the development and function of the myocardium. PDGF is reported to stimulate the growth of cardiomyocytes isolated from the chicken embryo [Shimizu et al., 1999] and the newt [Soonpaa et al., 1992], but comparable findings with mammalian cardiomyocytes have not been described. Nonetheless, certain findings implicate PDGF in mammalian myocardial development. For example, PDGF-B chain knock out mice were observed to exhibit an increase in the size and trabeculation of the myocardium [Betsholtz and Raines, 1997]. Additionally introduction of anti-PDGF-A neutralizing antibodies into mouse deciduas in utero resulted in development of atrial and ventricular hypertrophy, accompanied by epicardial and endocardial abnormalities, within 48 h [Schattemann et al., 1996].

H9c2 heart-derived myocytes have proven a useful model for studies of the normal embryonic cardiomyocyte. Cloned from embryonic rat ventricular tissue, these cells express sarcolemmal ATPase splice variants characteristic of normal heart [Hammes et al., 1994] and have become an increasingly accepted model for studies of cardiac disease in vitro [He et al., 1999; Su et al., 1999; Aki et al., 2001; Filigheddu et al., 2001; Hong et al., 2001]. H9c2 cells express both cardiac and skeletal isoforms of *l*-type Ca^{2+} channels [Heschler et al., 1991; Sipido and Marban, 1991], typical of the immature myocyte. The cells are also ideal for investigations of the role of intracellular Ca^{2+} signaling in promotion of growth. For example, H9c2 cells respond to arginine vasopressin with a rapid and substantive release of S(E)R Ca^{2+} stores, followed by gradual repletion of these stores over hours-days concurrent with the development of hypertrophy [Reilly et al., 1998; Brostrom et al., 2000, 2001]. H9c2 myocytes possess receptors for insulin-like growth factor-I [Chen et al., 1995; Hong et al., 2001] but not for basic fibroblast growth factor-2 [Sheikh et al., 1997]. We report here that H9c2 cells express classical PDGF-β receptors, PDGF-BB serves as a full mitogen for these cells, and persistent depletion of S(E)R Ca^{2+} stores delays the mitogenic response.

MATERIALS AND METHODS

Materials

H9c2(2-1) cloned rat embryonic ventricular myocytes were obtained from the American Type Culture Collection (passage 12) and utilized through no more than 12 additional passages. Rat recombinant PDGF isoforms, DME/F12 base medium, LY294002, and ionomycin were obtained from Sigma (St. Louis, MO). Polyclonal rabbit antibodies directed against cytoplasmic domains of the human PDGF receptor α and β chains were obtained from Research Diagnostics, Inc. (Flanders, NJ). Cy3-conjugated goat anti-rabbit antibody was from Amersham Pharmaceuticals (Piscataway, NJ). Human recombinant [^{125}I]-PDGF (20 μC/μg) was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). L-[3,4,5-^{3}H(N)]-leucine was from NEN Life Science Products, Inc. (Boston, MA) and [methyl-^{3}H]-thymidine from ICN (Costa Mesa, CA). Arginine vasopressin from Novabiochem (Darmstadt, Germany) was dissolved in water, aliquotted, lyophilized, and stored frozen under desiccation. Freshly reconstituted aliquots were employed for each experiment.

Cell Culture and Incubations

Stock H9c2 myocytes were propagated in DMEM supplemented with 10% fetal bovine serum (FBS) and subcultured weekly. For experiments, cultures in complete medium were allowed to reach confluence (1×10^5 cells/cm^2) and cultured for an additional 24 h without change of medium. Treatments were conducted in DME/F-12 supplemented with normal components except leucine, methionine, and $CaCl_2$, which were adjusted for each experiment and with horse serum as indicated.

Leucine and Thymidine Pulse Incorporations

Incorporations were conducted in multiwell trays (2.4 cm^2/well). Leucine incorporation was measured in 0.5 ml DME/F-12 medium adjusted to contain 10 μM leucine, 115 μM methionine, 0.3 mM $CaCl_2$, and 2 μC [^{3}H]leucine for 30 min. The leucine concentration was then increased to 2 mM and the incubation continued for 2 min. Plates were placed on ice and 1 ml ice-cold 10% trichloroacetic acid (TCA) was added. Following

aspiration of medium wells were washed with 1 ml 5% TCA, incubated on ice for 20 min with 1 ml 5% TCA containing 1 mM unlabeled leucine, and washed again with 5% TCA. Protein was dissolved in 0.5 ml 1% SDS, and radioactivity was determined by liquid scintillation counting. Incubations were performed in triplicate and results are expressed as the average $\pm$ range of values obtained.

Thymidine incorporation was measured after treatments in 1 ml DME/F-12 medium containing 400 μM leucine and 115 μM methionine, and with $CaCl_2$ and horse serum as indicated. Incubations with 4 μC/ml [^{3}H]thymidine were conducted for 45 min. Plates were placed on ice, and medium was aspirated. Wells were washed twice with 0.5 ml ice-cold buffered saline, incubated on ice for 20 min with 1 ml 5% TCA containing 1 mM thymidine, and washed with 1 ml with 5% TCA. Samples were dissolved in 0.5 ml 1% SDS, and radioactivity determined by liquid scintillation counting. Incubations were performed in triplicate and results are expressed as the average $\pm$ range of values obtained. Each experiment has been reproduced at least twice.

Determination of GRP78 Content

Preparations in 12-well (4.8 cm^2/well) plates were subjected to treatments in medium adjusted to contain 400 μM leucine, 115 μM methionine, 0.3 mM $CaCl_2$, and 0.5% horse serum. Lysate preparation, performance of slab gel isoelectric focussing to separate modified from unmodified forms of the chaperone, immunoblotting with polyclonal rabbit anti-GRP78 (StressGen, Victoria, BC), and visualization of complexes have each been described previously [Brostrom et al., 2001].

[^{125}I]PDGF-BB Binding Assay

Myocytes (24-well plates) were washed twice with 0.5 ml Tes-buffered saline. Binding of PDGF-BB was determined in the presence of 1 ml/well of ice-cold binding medium (DME/F12 containing 400 μM leucine, 115 μM methionine, 1 mM $CaCl_2$, 0.5% horse serum, and 0.25% BSA), with varying concentrations of [^{125}I] PDGF-BB, and in the absence or presence of 125 ng/ml unlabeled PDGF-BB. Binding assays were conducted in triplicate. Plates were incubated on ice with gentle shaking for 4 h. Monolayers were then washed three times with 1 ml of ice-cold binding medium, and cell-associated [^{125}I]PDGF-BB was extracted with 0.5 ml solubilization buffer (1% Triton X-100 containing 0.1% BSA and 0.1 N NaOH). Cell-associated radioactivity was measured with a γ-counter. Binding in the presence of unlabeled PDGF-BB was subtracted to calculate specific binding. Scatchard analysis was performed to determine the maximum number of binding sites/cell.

Immunodetection of PDGF Receptors

Myocytes (5×10^6) were lysed with 150 μl 2× Laemmli SDS-sample buffer and lysates (50 μl) were subjected to SDS–PAGE (10% gels). After electrophoresis proteins were electrotransferred onto polyvinylidene difluoride membranes under basic conditions. The immunoblotting for detection of PDGF receptors was performed with rat primary antibodies (1:100) against the α or β receptor chains and chemiluminescence, with goat-anti-rabbit antiserum serving as the secondary antibody. Immune complexes were visualized by using the ECL Western detection kit.

Immunocytochemistry

Cultures grown to confluence on 12 mm poly-L-lysine-coated glass coverslips in 24-well trays were treated for 30 min in the absence or presence of PDGF-BB (0.5 nM). Preparations were then fixed with acetic acid/ethanol (5%/95%) for 5 min at −20°C. Following fixation, coverslips were rinsed in PBS (pH 7.4, 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 4.3 mM $NaHPO_4$) and incubated for 18 h at 4°C with the appropriate primary antibody diluted 1:100 in PBS containing 10% FBS (PBS-serum). After rinsing in PBS, the coverslips were incubated for 30 min at 4°C with Cy3-conjugated goat anti-rabbit secondary antibody diluted 1:100 in PBS-serum. Coverslips were rinsed in PBS, rinsed again with deionized-distilled water, and then mounted in DPX synthetic resin mountant (Fort Washington, PA) on microscope slides. Cultures were examined using a Zeiss Axioplan microscope equipped with an epifluorescence illuminator with an appropriate filter set. Images of the cultures were captured using a Macintosh Quadra 700 with a Scion LG-3 frame grabber board.

Other Procedures

Cell-associated [$^{45}Ca^{2+}$] measurements were performed as described previously [Brostrom et al., 2001]. Incubations were conducted in triplicate, and results are reported as the

average ± range of values obtained. For protein determinations, six replicate samples were treated with 1% SDS for 5 min at 37°C followed by 5 min at 100°C. Aliquots of replicate lysates were then taken for protein measurements with the DC™ protein bioassay (BioRad). Results are expressed as mean ± SE. Viable cell number was determined with a hemocytometer as described [Brostrom et al., 1979]. The acid phosphatase activities of variously treated preparations were measured as described [Connolly et al., 1986] for cells in 24-well plates and for 1 h incubations with substrate. Reactions were performed in triplicate and results, expressed as the mean ± SE, are reported in absorbance units. Pulse-labeling of cellular proteins with [^{35}S]methionine, SDS–PAGE of detergent-solubilized extracts of [^{35}S]methionine-labeled cells, and autoradiography were performed as described [Wong et al., 1993].

RESULTS

Evidence for the Presence of a Functional PDGF-β Receptor in H9c2 Myocytes

Increased rates of mRNA translation are invariably observed during early stages of mitogenic signaling in response to PDGF [Lawrence et al., 1997]. Accordingly, PDGF-BB was found to increase the incorporation of [^{3}H]leucine into proteins of H9c2 myocytes. For incubations conducted in serum-free medium containing 0.3 mM $CaCl_2$, a modest inhibition of incorporation was observed during the first 30 min of PDGF treatment, which was followed by increasing rates of incorporation (Fig. 1A). In incubations conducted at higher (1–5 mM) Ca^{2+} concentrations, only the stimulatory phase was apparent (not shown). At 5–6 h of incubation, 2–2.5-fold stimulations of incorporation were routinely observed for PDGF-treated myocytes as compared with untreated cells. [^{3}H]leucine incorporation was dependent on PDGF concentration, with optimal stimulation obtained at 0.5 nM PDGF-BB (Fig. 1B). Stimulation was additive with that produced by arginine vasopressin (1 μM) (Fig. 1B), a hormone that increases H9c2 cell size while decreasing mitotic indices [Brostrom et al., 2000]. It seemed, therefore, apparent that PDGF-BB and vasopressin upregulated mRNA translation through different mechanisms. To ascertain whether PDGF promoted cell entry into the S phase of the cell cycle, thymidine incorporation

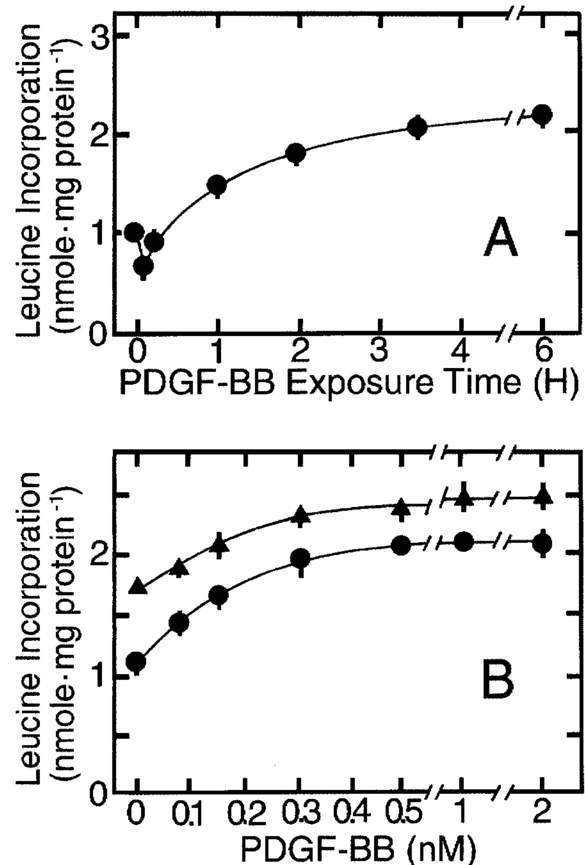


Fig. 1. Incorporation of leucine into proteins of H9c2 myocytes during incubations with PDGF-BB conducted with or without the addition of vasopressin. **A**: Time of treatment with PDGF-BB. Cultures were treated for the indicated times with 0.5 nM PDGF-BB, followed by determination of [^{3}H]leucine pulse-incorporation into proteins. **B**: Effects of PDGF-BB concentration and of incubation in the presence of vasopressin. Cultures were treated with the indicated concentrations of PDGF-BB in the absence (●) or presence (▲) of vasopressin (10 nM). After 6 h of incubation, pulse incorporation of [^{3}H]leucine into proteins was determined. Findings described in A and B have been reproduced on three separate occasions.

was measured after 24 h of incubation (Fig. 2). Increasing concentrations of PDGF-BB provided graded increases in incorporation of thymidine, with an eight-fold stimulation of incorporation observed at 0.4 nM of this isoform. By contrast, PDGF-AA at concentrations up to 5 nM was ineffective. PDGF-AB was modestly stimulatory at low concentrations and at 5 nM provided a stimulation comparable to that observed at 0.2 nM PDGF-BB.

The ability of PDGF-BB, but not PDGF-AA, to stimulate thymidine incorporation in H9c2 cells was consistent with the presence of the β, rather than α, form of the PDGF receptor [Heldin and Westermark, 1999]. To confirm the presence of

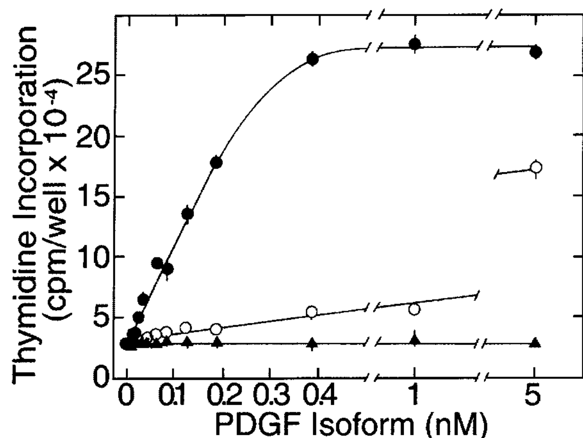


Fig. 2. Incorporation of thymidine into H9c2 myocytes after treatments with PDGF-BB, PDGF-AB, or PDGF-AA at varying concentrations. Preparations in medium containing 0.3 mM $CaCl_2$ and 0.5% horse serum were treated for 24 h with the indicated concentrations of PDGF-BB (●), PDGF-AB (○), of PDGF-AA (▲). Pulse-incorporation of [^{3}H]thymidine was then determined.

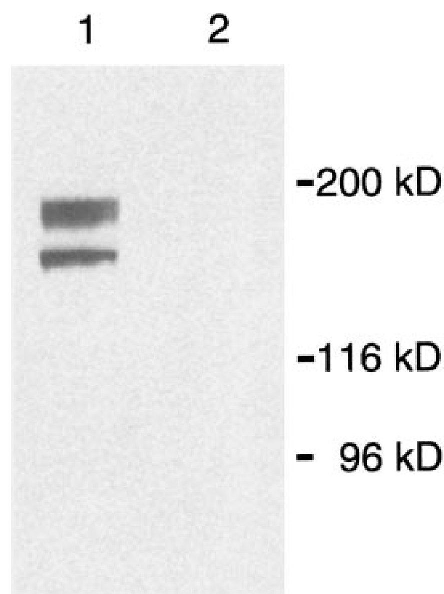
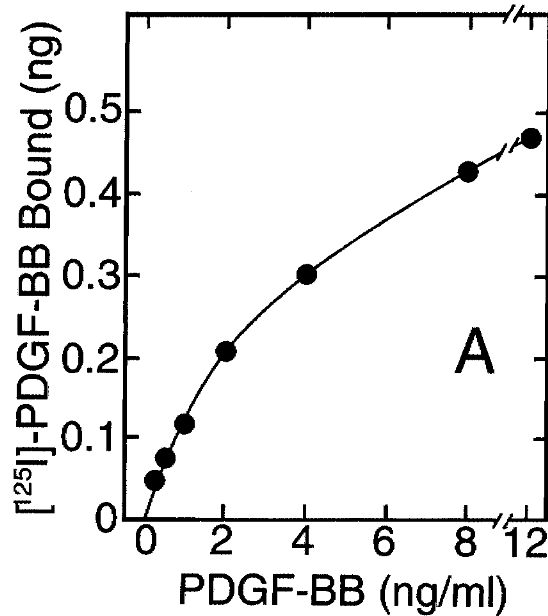


Fig. 3. Forms of the PDGF receptor expressed by H9c2 myocytes. Lysates were subjected to SDS-polyacrylamide gel electrophoresis (10%). Proteins were electrotransferred onto polyvinylidene difluoride membranes, and Western blotting was performed utilizing antibodies directed against the β (**lane 1**) or α (**lane 2**) receptor. The migration positions of molecular weight markers are indicated on the right.

the PDGF-β receptor, immunoblotting with polyclonal antibodies against the α and β forms of the receptor was performed (Fig. 3). As anticipated for the presence of the mature and precursor forms of the β-receptor [Westermark et al., 1989], species of 185 and 160 kDa, respectively, were detected by antibodies against the β-receptor. By contrast, no polypeptides were found to react with antiserum against the α-receptor. Negative findings were obtained even when this antiserum was employed at a five-fold higher concentration than that of antiserum against the β-receptor (not shown). To quantitate the number of PDGF-β receptors expressed by H9c2 cells, myocytes were incubated at 4°C with varying concentrations of [^{125}I]PDGF-BB, and in the absence or presence of an excess of unlabeled PDGF-BB. Binding was found to be saturable and specific (Fig. 4A). Scatchard analysis (Fig. 4B) revealed the presence of 44,000 receptors per cell.

Immunocytochemistry was employed to examine the subcellular localization of PDGF receptors (Fig. 5). Because the antibodies used for these studies were directed against carboxy-terminal domains of the β- and α-receptor, fixation of preparations was required. Antiserum against the β-receptor was found to stain the surface and perinuclear regions of H9c2 myocytes, consistent with published work on PDGF receptor immunostaining [Tidball and Spencer, 1993; Hagood et al., 1999]. Myocytes exposed to PDGF-BB for 30 min exhibited a rounder morphology as compared with non-treated myocytes, and immunostaining after incubation with antiserum against the β-receptor revealed significant clustering of receptors in response to the factor. No immunostaining was detected following incubations of untreated or PDGF-treated preparations with antiserum against the PDGF-α receptor.

Activation of PI 3-kinase is one of the best documented and earliest responses to PDGF receptor activation [Heldin and Westermark, 1999]. LY294002, at concentrations reported to inhibit PI 3-kinase selectively [Carpenter and Cantley, 1996], was found to suppress selectively the increases in leucine and thymidine incorporation occurring in response to PDGF. Myocytes were incubated in the absence or presence of 0.5 nM PDGF-BB and with concentrations of LY294002 ranging from 0.1 to 25 μM, followed by measurements of leucine or thymidine pulse-incorporation (Fig. 6A,B). Graded reductions in incorporations were observed for PDGF-treated preparations incubated with increasing drug concentrations, such that incorporation attributable to treatment with the factor was almost completely eliminated at 5 μM LY294002. By contrast, leucine and thymidine



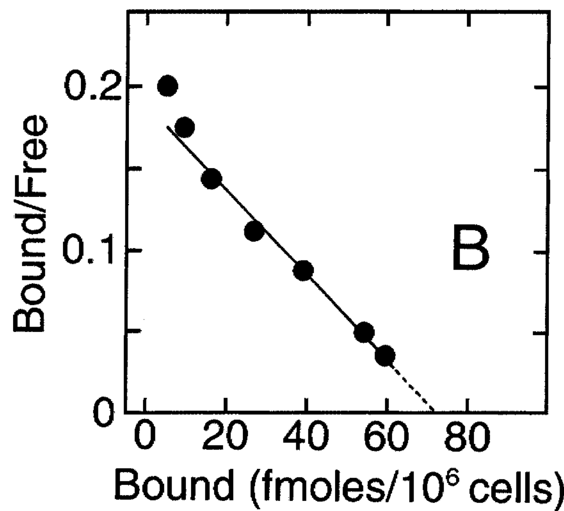


Fig. 4. Binding of [^{125}I]PDGF-BB (**A**) and Scatchard analysis of [^{125}I]PDGF-BB binding (**B**) to H9c2 myocytes. Binding assays were conducted at 4°C as described in Materials and Methods in the absence and presence of unlabeled PDGF-BB to determine specific binding. The number of PDGF receptors/cell (44,000) was unaffected by pretreatment for 30 min or 24 h with vasopressin (not shown).

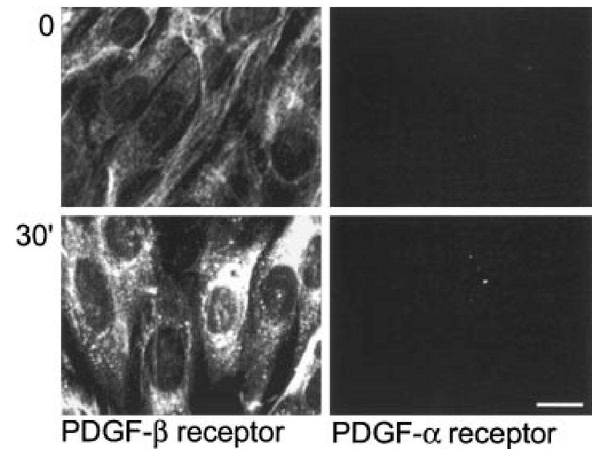


Fig. 5. Localization of the PDGF-β receptor of H9c2 cells and receptor clustering in response to PDGF-BB. Myocytes on coverslips were treated for 0 or 30 min with 0.5 nM PDGF-BB. Fixed preparations were then incubated with antibodies against the PDGF β- or α-receptor as indicated and prepared for examination by fluorescence microscopy. Bar = 10 μm.

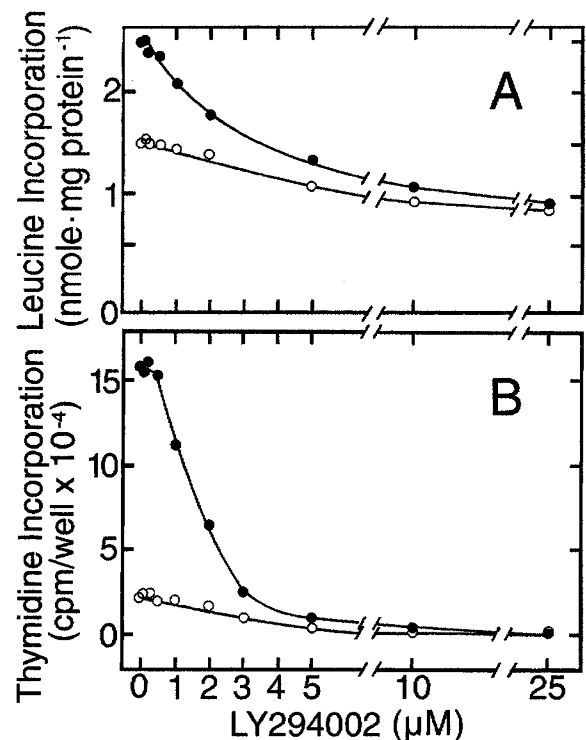


Fig. 6. Selective inhibition by LY294002 of leucine and thymidine pulse-incorporation occurring in response to PDGF-BB. **A**: Leucine incorporation. H9c2 myocytes were pretreated for 5 min with the indicated concentrations of LY294002. Incubations were then conducted for 5 h in the absence (○) or presence (●) of 0.5 nM PDGF-BB prior to determination of [^{3}H]leucine pulse-incorporation into proteins. **B**: Thymidine incorporation. Preparations in medium containing 0.3 mM $CaCl_2$ and 0.5% horse serum were treated with the indicated concentrations of LY294002 in the absence (○) or presence (●) of 0.5 nM PDGF-BB. Pulse-incorporation of [^{3}H]thymidine was determined after 24 h of treatments.

incorporation in non-PDGF-treated H9c2 cells was only modestly reduced by LY294002.

Inhibition of PDGF-Induced Mitogenesis by Vasopressin

The extent to which PDGF-BB stimulated DNA synthesis was examined under various incubation conditions. For example, treatments were conducted in medium supplemented with

varying concentrations of horse serum and in the absence or presence of vasopressin, followed by measurements of thymidine incorporation. Larger stimulations of incorporation were observed with lower concentrations of serum. As illustrated in Figure 7, thymidine incorporation was increased 15-fold or 50% following incubations with PDGF and 0.25 or 10% horse serum, respectively. Incubations in medium with 0.2% or less of horse serum resulted in some cell death, therefore, treatments were routinely conducted in the presence of 0.5% horse serum. Addition of vasopressin, which stimulates H9c2 cells to undergo hypertrophy, suppressed thymidine incorporation in incubations both without and with the addition of PDGF by 22 and 32%, respectively (Fig. 7). These reductions were largely independent of serum concentration. The effects of vasopressin and PDGF on thymidine incorporation were also measured after various times of incubation (Fig. 8). Incorporation in untreated control preparations did not vary throughout 48 h of incubation. Treatment with vasopressin resulted in modestly decreased incorporation rates (e.g., 15% reduction after 8 h) as compared to controls. PDGF-treated preparations displayed a four-fold increase in incorporation after 16–24 h of incubation. Thereafter, incorporation rates were seen to decline, with a 2.5-fold stimulation observed at 48 h. Incorporation in preparations treated with both PDGF and vasopressin resembled that in vasopressin-treated preparations during the first 8 h, increased by 2.5-fold at 16 h, and remained 2.5-fold elevated through 48 h. Thus, although vasopressin reduced thymidine incorporation in response to PDGF at earlier treatment times, the duration over which PDGF stimulated DNA synthesis was extended, rather than limited, by co-incubation with vasopressin. The PDGF concentration dependence for stimulation of thymidine incorporation was examined after 24 h for incubations conducted with or without vasopressin (Fig. 9). Vasopressin treatment slightly increased the PDGF concentration promoting half-maximal stimulation of incorporation (0.12 nM as compared to 0.09 nM for non-vasopressin-treated samples). By contrast, treatment with the hormone produced significant reductions in thymidine incorporation at all PDGF concentrations examined. Binding of [^{125}I]PDGF-BB to H9c2 myocytes was not altered by vasopressin (not shown).

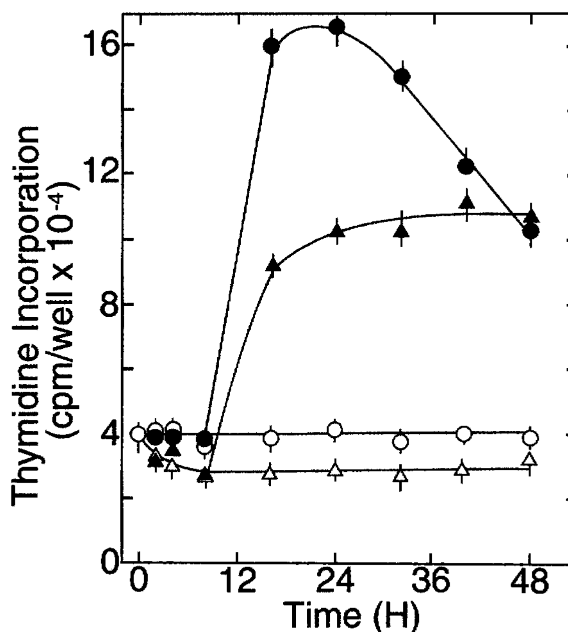


Fig. 8. Thymidine incorporation into H9c2 myocytes after various times of treatment with PDGF-BB, vasopressin, or both agents. Incubations were conducted for the indicated times with no further additions (○), with 0.5 nM PDGF-BB (●), with 10 nM vasopressin (△), or with both PDGF-BB and vasopressin (▲) prior to measurement of [^{3}H]thymidine pulse-incorporation. Medium and agents were replenished at 24 h.

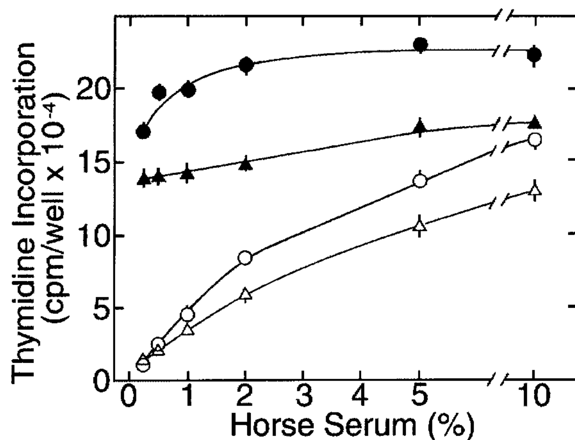


Fig. 7. Thymidine incorporation into H9c2 myocytes following incubations with PDGF-BB, vasopressin, or both agents, and with varying concentrations of horse serum. Incubations were conducted for 24 h in medium containing 0.3 mM $CaCl_2$ and the indicated concentrations of horse serum without further additions (○), with 0.5 nM PDGF-BB (●), with 10 nM vasopressin (△), or with both PDGF-BB and vasopressin (▲). Pulse-incorporation of [^{3}H]thymidine was determined after 24 h of treatments.

It was unclear whether PDGF-BB could serve as a full mitogen for H9c2 myocytes during

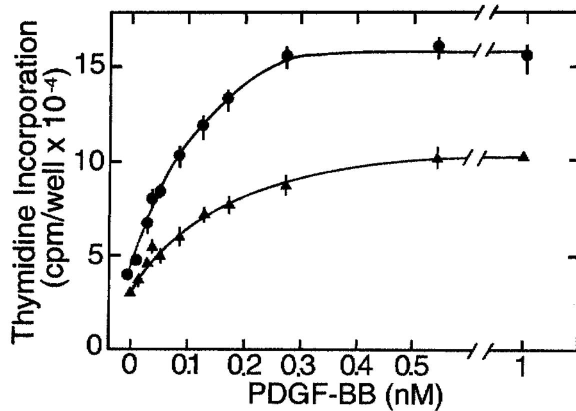


Fig. 9. Thymidine incorporation into H9c2 myocytes following treatments with varying concentrations of PDGF-BB and in the absence or presence of vasopressin. Incubations were conducted for 24 h at the indicated concentrations of PDGF-BB and in the absence (●) or presence (▲) of 10 nM vasopressin prior to measurement of [^{3}H]thymidine pulse-incorporation.

longer treatments. Cells were, therefore, incubated for 1, 2, and 3 days in medium supplemented with 0.5% horse serum, with or without PDGF, and were subsequently analyzed for cell number and protein (Table I). In incubations without PDGF, cell number and protein content rose slightly (35 and 20% increases, respectively, after 3 days). When PDGF was present, larger daily increases in both parameters were observed and cell number doubled within 3 days. Cells exposed to vasopressin for 3 days exhibited the development of hypertrophy in that protein content increased by 75%, while cell number rose by only 26% (i.e., protein content/cell increased by 40%). Combined treatment with PDGF and vasopressin resulted in daily increases in both cell number and protein content. Increases in cell number, however, were less than those observed for PDGF-treated preparations, and increases in protein content were no greater than those observed during treatments with PDGF or vasopressin alone. The mitogenic effects of PDGF were, therefore, slowed by vasopressin, and the hypertrophic action of vasopressin was blocked by PDGF. Acid phosphatase activities of the variously treated preparations were also measured after 3 days as an index of organellar content (Table I). Enzyme activity paralleled protein content in a qualitative fashion: Total activity was higher for each of the treated preparations as compared to non-treated controls, with vasopressin providing an increase in enzyme activity/cell.

Sequestered Ca^{2+} Stores and PDGF Signaling

The Ca^{2+} contents of cells exposed to PDGF-BB or to vasopressin for varying times were determined for the same cell preparation (Fig. 10). As reported previously for H9c2 cells [Reilly et al., 1998; Brostrom et al., 2000, 2001], vasopressin mobilized 55% of cell-associated Ca^{2+} in 30 min. Slow refilling of Ca^{2+} stores was observed, thereafter, such that at 4 h cell-associated Ca^{2+} was 66% of control values. PDGF-BB was also effective in mobilizing cell-associated Ca^{2+}, but mobilizations were briefer. Approximately 45% of stores was mobilized by PDGF within 30 min, but full repletion of stores had occurred by 4 h.

TABLE I. Cell Number, Protein Content, and Acid Phosphatase Activities of H9c2 Preparations During Culture With PDGF, Vasopressin, or Both Agents*

			Protein content		Acid phosphatase activity	
Days	Treatment	Cell number (cells/well × 10^{-5})	μg/well	μg/10^5 cells	Units/well	Units/10^5 cells
0	none	1.97 ± 0.10	132±3	67		
1	none	2.07 ± 0.05	138 ± 3	66		
2	none	2.22 ± 0.02	151 ± 2	68		
3	none	2.68 ± 0.19	167 ± 4	62	0.52 ± 0.03	0.19
1	PDGF	2.48 ± 0.09	175 ± 3	70		
2	PDGF	3.30 ± 0.09	217 ± 3	66		
3	PDGF	4.40 ± 0.18	236 ± 3	54	0.86 ± 0.03	0.19
1	AVP	1.85 ± 0.08	156 ± 3	84		
2	AVP	2.30 ± 0.18	198 ± 3	86		
3	AVP	2.49 ± 0.24	231 ± 2	93	0.65 ± 0.07	0.26
1	PDGF + AVP	2.27 ± 0.10	161 ± 1	71		
2	PDGF + AVP	2.83 ± 0.27	203 ± 5	71		
3	PDGF + AVP	3.58 ± 0.12	249 ± 3	70	0.75 ± 0.02	0.21

*Post-confluent cultures in 24-well plates in DME/F12 medium containing 0.3 mM $CaCl_2$ and 0.5% horse serum were treated for the indicated times with PDGF-BB (0.5 nM), vasopressin (AVP, 10 nM), or both agents as indicated. Medium and agents were replenished daily. Cell number, protein content, and acid phosphatase activities were then determined as described in Materials and Methods.

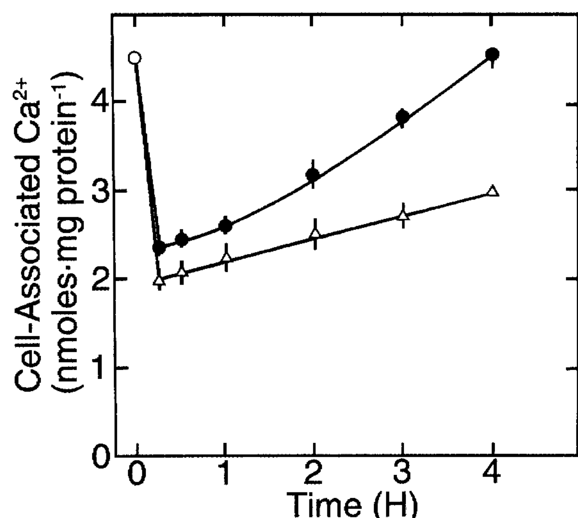


Fig. 10. Cell-associated Ca^{2+} after various times of treatment with PDGF-BB or vasopressin. Preparations were pre-equilibrated for 2 h in serum-free medium containing 0.2 mM $CaCl_2$ and 8 μC/ml $^{45}CaCl_2$. Incubations were conducted for the indicated times in the presence of 0.5 nM PDGF-BB or 10 nM vasopressin, followed by determinations of cell-associated $^{45}Ca^{2+}$. Findings were reproduced on three separate occasions.

The extended depletion of S(E)R Ca^{2+} stores of vasopressin-treated H9c2 myocytes signals the increased expression of the ER chaperone, GRP78/BiP [Brostrom et al., 2001]. Rates of GRP78 synthesis increase preferentially at early times of vasopressin treatment, and total GRP78 content increases 2–3-fold within 8 h, after which the chaperone is subject to post-translational modification. It was of interest to ascertain whether H9c2 cells upregulated GRP78 expression in response to PDGF and whether upregulation was greater during treatments with both PDGF and vasopressin. To this end, myocytes were incubated for 2, 4, or 6 h with no additions, with PDGF-BB, with vasopressin, or with both agents. Pulse-labeling of proteins with [^{35}S]methionine was then performed, followed by SDS–PAGE and autoradiography (Fig. 11A). Increased incorporation of methionine into a 78 kDa species, previously identified as authentic GRP78 [Brostrom et al., 2001], was observed after 2, 4, and 6 h of treatment with vasopressin. Methionine was also preferentially incorporated into GRP78 after 2 h incubation with PDGF, but at longer treatment times GRP78 was not selectively synthesized. Synthesis of GRP78 in preparations treated with both vasopressin and PDGF was no greater than that observed with vasopressin alone. The total GRP78 content of preparations treated for 24 h without agent or with PDGF or vasopressin was also measured. Lysates of treated preparations were subjected to slab gel isoelectric focusing to separate the modified and unmodified forms of the chaperone, followed by Western blotting (Fig. 11B). The GRP78 content of vasopressin-treated myocytes increased two-fold over that of non-treated myocytes, and approximately 40% of the chaperone was found to be modified. By contrast, the chaperone content of PDGF-treated preparations was no greater than that of untreated preparations, and only the unmodified form was discernable.

It was also of interest to ascertain whether extracellular Ca^{2+} affected the stimulation of DNA synthesis by PDGF. Non-treated and PDGF-treated preparations were incubated for 24 h at varying extracellular Ca^{2+} concentrations and in the absence or presence of vasopressin, followed by measurement of thymidine incorporation (Fig. 12A). In non-PDGF-treated preparations (open symbols), thymidine incorporation increased sharply as extracellular Ca^{2+} was raised from 0.1 to 1 mM. Optimal incorporations were observed at 1–2 mM Ca^{2+}. Incorporation in PDGF-treated preparations (filled symbols) also depended on extracellular Ca^{2+}, but optimal incorporation was observed at significantly lower (0.5 mM) Ca^{2+} concentrations. Treatments with vasopressin, either in the absence or presence of PDGF, resulted in

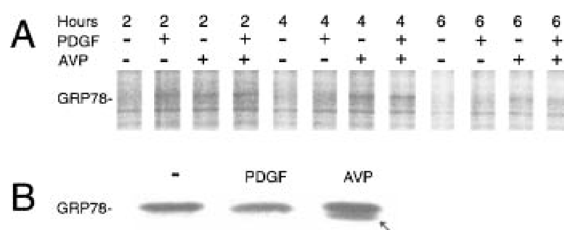


Fig. 11. Methionine incorporation into GRP78 after 2, 4, or 6 h of treatment with PDGF-BB, vasopressin, or both agents (**A**), and total GRP78 content after 24 h incubation with PDGF-BB or vasopressin (**B**). Treatments were conducted in DME/F12 medium containing 0.3 mM $CaCl_2$ and 0.5% horse serum. A: Incorporation of methionine into GRP78. Myocytes were incubated without further addition, with PDGF-BB (0.5 nM), with vasopressin (AVP, 10 nM), or with both agents for 2, 4, or 6 h as indicated. Pulse-labeling of proteins with [^{35}S]methionine was then conducted for 30 min, and lysates were analyzed by SDS–PAGE and autoradiography. The arrow indicates the migration position of authentic GRP78. B: Myocytes were treated for 24 h without addition, with 0.5 nM PDGF-BB, or with 10 nM vasopressin (AVP). Lysates were subjected to slab gel isoelectric focussing followed by Western blotting for GRP78. The arrow indicates the migration position of ADP-ribosylated GRP78.

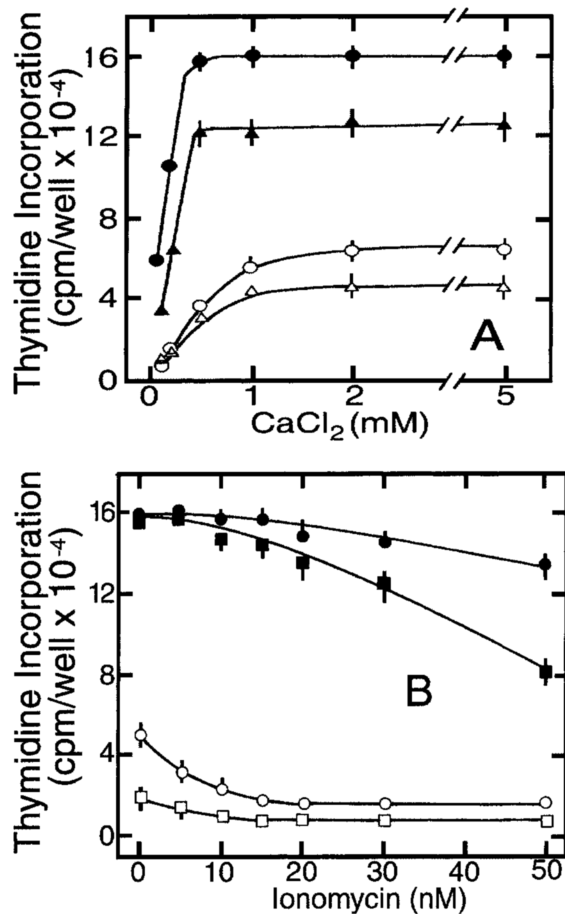


Fig. 12. Ca^{2+} requirements for thymidine incorporation by PDGF-treated as compared to non-treated H9c2 myocytes: effects of vasopressin and ionomycin. **A**: Effects of extracellular Ca^{2+} concentration and vasopressin. Preparations were treated for 24 h at the indicated extracellular Ca^{2+} concentrations with no further addition (○), with 10 nM vasopressin (△), with 0.5 nM PDGF-BB (●), or with both agents (▲). **B**: Effects of ionomycin. Myocytes were treated for 24 h at 0.2 (□, ■) or 2 mM (○, ●) extracellular $CaCl_2$, with the indicated concentrations of ionomycin, and in the absence (□, ○) or presence (■, ●) of PDGF-BB (0.5 nM). Treatments were followed by measurement of [^{3}H]thymidine pulse-incorporation.

reduced thymidine incorporation, but these reductions were largely independent of extracellular Ca^{2+} concentration.

The reduced Ca^{2+} requirement for DNA synthesis in PDGF-treated preparations was consistent with increased availability of intracellular Ca^{2+} for signaling of mitogenesis in these preparations. Ca^{2+} crucial to PDGF signaling could be derived from the extracellular fluid or from sequestered Ca^{2+} stores. To test the latter possibility, PDGF-treated and non-treated preparations were incubated for 24 h with ionomycin at concentrations known to deplete H9c2 Ca^{2+} stores [Reilly et al., 1998], followed by measurement of thymidine incorporation. Incubations with ionomycin were conducted at low (0.2 mM) and high (2 mM) extracellular cation (Fig. 12B). In non-PDGF-treated preparations thymidine incorporation was greater at 2 than at 0.2 mM Ca^{2+}. Increasing ionomycin concentrations promoted increasing degrees of inhibition of incorporation, with maximal inhibition observed at 15 nM ionophore regardless of Ca^{2+} concentration. Treatment with PDGF enhanced thymidine incorporation to equivalent high values (160,000 cpm/well) in preparations incubated at either extracellular Ca^{2+} concentration. Thymidine incorporation in PDGF-treated preparations was remarkably resistant to inhibition by ionomycin at either Ca^{2+} concentration, but greater resistance was observed at 2 mM Ca^{2+}. For example, inclusion of 50 nM ionomycin in incubations with PDGF and 0.2 mM Ca^{2+} resulted in 50% reduction of incorporation, whereas the presence of 50 nM ionophore in incubations with PDGF and 2 mM cation resulted in only 15% reduction in incorporation. The observed differential in sensitivity of thymidine incorporation to inhibition by ionomycin in non-treated vs. PDGF-treated preparations required that the ionophore be present for the first 16 h of incubation with and without the factor (data not shown).

DISCUSSION

This report provides direct evidence for the presence of a functional PDGF receptor in myocardial cells of mammalian origin. H9c2 cloned rat ventricular myocytes were found to respond to PDGF-BB with a rapid mobilization of sequestered Ca^{2+} stores, altered morphology and increased rates of protein synthesis, followed by markedly increased rates of DNA synthesis. Increases in protein and DNA synthesis were exquisitely sensitive to inhibition by LY294002, a selective inhibitor of an enzyme (PI 3-kinase) considered critical to PDGF signaling. More importantly, PDGF-BB was found to operate as a full mitogen for H9c2 myocytes, promoting a doubling of cell number within 3 days of incubation in medium containing non-mitogenic concentrations of horse serum. Other growth factors are not known to be sufficient for H9c2 mitogenesis. IGF-I, which protects H9c2 myocytes against apoptosis during oxidative stress and serum deprivation

[Wang et al., 1998; Hong et al., 2001], provided lesser increases in DNA synthesis as compared to PDGF-BB and did not increase cell number even after several days of incubation in medium supplemented with low serum concentrations [unpublished communications].

This report also documents the presence of a classical PDGF-β receptor in H9c2 cells. In particular, thymidine incorporation was found to be strongly stimulated by PDGF-BB, modestly stimulated by PDGF-AB, and unaltered by PDGF-AA. Two species of molecular mass comparable to those for immature and fully glycosylated forms of PDGF-β receptors were observed upon immunoblotting with antibodies against the PDGF-β receptor. The immunostaining of H9c2 cells with PDGF-β receptor antiserum revealed localization of the receptor to the plasma membrane and clustering of receptors in response to PDGF-BB. The number of PDGF-β receptors expressed by H9c2 cells is closely comparable to that expressed by vascular SMCs [Kasuya et al., 1999]. The ability of PDGF-BB to signal the rapid mobilization of H9c2 Ca^{2+} stores is also fully consistent with signaling promoted by activated β, but not by α, PDGF receptors [Heldin and Westermark, 1999]. No evidence for expression of the PDGF-α receptor was obtained.

For most cell types examined, PDGF-β receptor activation signals a transitory increase in $[Ca^{2+}]_i$ through promotion of Ca^{2+} entry as well as through mobilization of intracellular stores [Broad et al., 1999]. The functional roles of brief elevations of $[Ca^{2+}]_i$ in PDGF signaling are unclear. However, transient increases in $[Ca^{2+}]_i$ occurring in response to various hormonal agents are believed to signal entry into the cell cycle [Whitaker and Larman, 2000]. Findings with PDGF-treated H9c2 cells are consistent with adjustments in intracellular Ca^{2+} homeostasis attributable to the release of S(E)R Ca^{2+} stores over a restricted time period. For example, leucine pulse-incorporation rates, which decrease during significant depletion of S(E)R Ca^{2+} stores [Brostrom and Brostrom, 1998], were reduced at early times of PDGF treatment of preparations in medium supplemented with low, but not with high, extracellular Ca^{2+} concentrations. Additionally, an increase in the rate of synthesis of GRP78/BiP, an S(E)R chaperone expressed at high concentrations during depletion of Ca^{2+} stores, was observed at early times of PDGF treatment. More prolonged chaperone synthesis occurred in response to vasopressin, which produces an extended period of S(E)R Ca^{2+} depletion [Brostrom et al., 2000]. Thymidine incorporation in PDGF-treated and non-treated preparations was reduced by co-incubation with either vasopressin or ionomycin, both of which continuously deplete cell-associated Ca^{2+} stores. These findings are consistent with the hypothesis that DNA synthesis in H9c2 cells is up-regulated by the release of sequestered Ca^{2+} to the cytosol. Overall rates of DNA synthesis in H9c2 cells, however, appeared to reflect more directly the elevation of $[Ca^{2+}]_i$. When compared with untreated preparations, thymidine incorporation in PDGF-treated preparations was optimized at lower extracellular Ca^{2+} concentrations and was considerably less sensitive to inhibition by ionomycin.

Ca^{2+} stores of PDGF-treated preparations were fully repleted at 4 h (Fig. 10), whereas, full repletion of Ca^{2+} stores of vasopressin-treated H9c2 cells requires 48 h of incubation following hormone addition [Brostrom et al., 2000]. The ability of vasopressin to delay, rather than curtail, the period of enhanced DNA synthesis in response to PDGF appears to be exclusively attributable to the less than optimal Ca^{2+} storage at later times of incubation with both agents. Repletion of stores after exposure to both vasopressin and PDGF occurred at 36–48 h [unpublished communications], whereupon rates of DNA synthesis were found to be comparable to those in PDGF-treated preparations (Fig. 8). Additionally, antagonism of PDGF-stimulated DNA synthesis by vasopressin appeared to be greater during incubations at low, as opposed to higher, extracellular Ca^{2+}. For example, vasopressin reduced PDGF-stimulated thymidine incorporation by 40% for incubations conducted at 0.1–0.3 mM Ca^{2+} and by 22% for incubations conducted at 0.5 mM or greater Ca^{2+} concentrations (Fig. 12). Other inhibitory actions of vasopressin, such as impairment of the binding of PDGF to its receptors, were not observed. Ca^{2+}-dependent regulation of PDGF-BB-induced DNA synthesis by a peptide hormone may not be unique to cardiomyocytes. For example angiotensin II, which is known to release Ca^{2+} stores of SMCs through generation of inositol trisphosphate [Rossier and Capponi, 2000], is reported to delay PDGF-BB-stimulated thymidine incorporation in vascular smooth muscle [Dahlfors et al., 1998].

By serving as a paracrine growth factor for many tissues [Heldin and Westermark, 1999; Betsholtz et al., 2001], PDGF is essential to embryonic and postnatal development. Although evidence is more extensive for other tissues, the myocardium is likely to be targeted by PDGF both before and after birth. Growth of the myocardium entails the completion of a finite number of cell divisions during embryonic and fetal stages, followed by emergence of a postmitotic state and hypertrophy of postmitotic myocytes during subsequent stages of cardiac growth. Direct and indirect evidence exists supporting a proliferative role for PDGF in embryonic/fetal myocardial growth. Direct addition of PDGF-BB to embryonic chick cardiomyocyte cultures results in induction of *c-fos*, MAPK activation, and mitogenesis, and a dedifferentiating action of the factor on chick myocytes has also been observed [Shimizu et al., 1999; Velez et al., 2000]. As discussed above, both PDGF-BB knock-out procedures [Betsholtz and Raines, 1997] and introduction of antibodies against the factor during murine fetal growth [Schattemann et al., 1996] result in premature cardiac hypertrophy. PDGF is not thought to be synthesized by normal adult heart cells, but synthesis has been observed in proliferative disorders of the myocardium. Heart cells of patients with hypertrophic cardiomyopathy [Suzuki et al., 1999] or those recovering from myocardial infarction [Wallace et al., 1998] synthesize PDGF-BB, which is proposed to contribute to cardiac remodeling in such patients. The increased synthesis of PDGF-A chains and upregulation of PDGF-α and -β receptors by cardiac myocytes after cardiac transplantation is held responsible for the cardiac tissue remodeling associated with heart allografts [Higgy et al., 1991; Zhao et al., 1994]. Proliferative lesions of chronic Chagasic cardiomyopathy have been attributed to increased synthesis of both PDGF-BB and PDGF-AA by cardiomyocytes of patients with this disorder [Reis et al., 2000]. Similar conclusions were reached regarding the increased PDGF synthesis by cardiac interstitial cells, the activation of post-mitotic cardiomyocytes, and the cardiac remodeling observed in experimental renal failure [Amann et al., 1998]. H9c2 cloned embryonic ventricular myocytes clearly possess the essential features of PDGF-induced mitogenic signaling.

It is of considerable interest to compare and contrast the respective responses of H9c2 cells to PDGF and vasopressin in incubations conducted at low (0.5%) horse serum. At high concentrations, both hormones initially mobilize sequestered Ca^{2+} from the S(E)R and inhibit amino acid incorporation into protein, although vasopressin is somewhat more effective in these actions (Fig. 10) [Reilly et al., 1998; Brostrom et al., 2000]. After approximately 4 h of exposure to PDGF, Ca^{2+} stores recover completely in contrast to the partial recovery observed with treatment by vasopressin. Only slight stimulations of GRP78 synthesis are found with PDGF treatment in comparison to the relatively large increments in chaperone synthesis seen with vasopressin. During this time, the rate of protein synthesis approximately doubles with exposure to either hormone. PDGF stimulates thymidine incorporation and mitogenesis leading to cellular hyperplasia, whereas, vasopressin increases cellular size by roughly 30% while reducing thymidine incorporation, producing hypertrophy and suppression of mitosis. It is clear that the actions of vasopressin in suppressing cell division are readily overwhelmed either by increasing PDGF concentrations or by addition of FBS (10%). Mobilization of S(E)R sequestered Ca^{2+} appears in some manner to relate closely to the actions of both hormones. It is attractive to speculate that the mitogenic actions of PDGF may be triggered by a periodic release and recovery of stored Ca^{2+}, whereas, suppression of mitosis by vasopressin may relate to the chronic depletion of S(E)R Ca^{2+} content. H9c2 cells, therefore, appear to provide a relevant and convenient mammalian cardiomyocyte model in which to explore the biochemical fundamentals of both hormonally activated proliferative (hyperplastic) actions and hormonally driven hypertrophic actions.

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