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Regulated Expression of GRP78 During Vasopressin-Induced Hypertrophy of Heart-Derived Myocytes

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Abstract Although the development of cellular hypertrophy is widely believed to involve Ca^{2+} signaling, potential supporting roles for sequestered Ca^{2+} in this process have not been explored. H9c2 cardiomyocytes respond to arginine vasopressin with an initial mobilization of Ca^{2+} stores and reduced rates of mRNA translation followed by repletion of Ca^{2+} stores, up-regulation of translation beyond initial rates, and the development of hypertrophy. Rates of synthesis of the endoplasmic reticulum (ER) chaperones, GRP78 and GRP94, were found to increase preferentially at early times of vasopressin treatment. Total GRP78 content increased 2- to 3-fold within 8 h after which the chaperone was subject to post-translational modification. Preferential synthesis of GRP78 and the increase in chaperone content both occurred at pM vasopressin concentrations and were abolished at supraphysiologic Ca^{2+} concentrations. Co-treatment with phorbol myristate acetate decreased vasopressin-dependent Ca^{2+} mobilization and slowed appearance of new GRP78 molecules in response to the hormone, whereas 24 h pretreatment with phorbol ester prolonged vasopressin-dependent Ca^{2+} mobilization and further increased rates of GRP78 synthesis in response to the hormone. Findings did not support a role for newly synthesized GRP78 in translational up-regulation by vasopressin. However up-regulation, which does not depend on Ca^{2+} sequestration, appeared to expedite chaperone expression. This report provides the first evidence that a Ca^{2+}-mobilizing hormone at physiologic concentrations signals increased expression of GRP78. Translational tolerance to depletion of ER Ca^{2+} stores, typifying a robust ER stress response, did not accompany vasopressin-induced hypertrophy. J. Cell. Biochem. 83: 204–217, 2001.

Key words: H9c2 cells; S(E)R Ca^{2+} stores; mRNA translational regulation; glucose-regulated proteins; ER stress; UPR

The role(s) of Ca^{2+} sequestered by the sarcoplasmic/endoplasmic reticulum [S(E)R] in supporting myocardial cell growth and hypertrophy is poorly defined. A wealth of evidence, however, supports the proposal that Ca^{2+} signaling via increased influx and release from intracellular stores mediates the effects of diverse hypertrophic stimuli. For example, various hypertrophic agonists, myocyte stretch, Ca^{2+} channel agonists, and electrical pacing each increase cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and induce cardiomyocyte hypertrophy [Sadoshima and Izumo, 1997; Sugden, 1999; Molkentin, 2000]. Defects in diastolic Ca^{2+} re-sequestration by the S(E)R and increased basal $[Ca^{2+}]_i$ are both observed in hypertrophic cardiomyopathy leading to heart failure [Dillmann, 1999].

In non-muscle mammalian cells rates of protein synthesis are believed to be coupled to those of protein translocation into the ER for subsequent folding or processing [Laitusis et al., 1999]. Treatments that produce the accumulation of folding or processing intermediates or of misfolded proteins within the ER lumen ("ER stress") result in rapid activation of eIF2 kinase(s) that phosphorylate the α-subunit of eIF2 [Brostrom and Brostrom, 1998; Shi et al., 1998; Harding et al., 2000]. Cycling of eIF2 and mRNA translational initiation are thereby inhibited. Such effects occur, for example, when ER Ca^{2+} stores are released with drugs, such as Ca^{2+} ionophores, to degrees that slow glycoprotein processing and transport competence.

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Persistent depletion of Ca^{2+} stores signals the "unfolded protein response" (UPR) characterized by increased transcription of genes encoding the ER resident chaperones, GRP78 and GRP94 [Little et al., 1994; Chapman et al., 1998; Hampton, 2000]. Transcription of the GRP genes is mediated by the ER-associated protein kinase/endonuclease IRE1, general and specific transcription factors, and the ER stress response element [Yoshida et al., 1998; Foti et al., 1999; Roy and Lee, 1999; Li et al., 2000; Tirasophon et al., 2000; Urano et al., 2000; Yoshida et al., 2000]. GRP78 is an essential component of the translocational machinery and participates in the maturation or degradation of various polypeptides [Gething, 1999]. Continuous GRP78 expression during the UPR is tightly correlated with emergence of translational tolerance to inhibition by drugs that deplete Ca^{2+} stores [Brostrom and Brostrom, 1998; Bertolotti et al., 2000]. Synthesis in excess of needs results in the ADP-ribosylation and inactivation of the chaperone [Ledford and Leno, 1994; Laitusis et al., 1999]. The free, unmodified form of GRP78 has therefore been proposed to serve as a multi-functional modulator of ER-associated processes including the catalysis of protein folding, regulation of mRNA translation, control of *grp78* gene transcription, and targeting of misfolded proteins for degradation [Laitusis et al., 1999].

Protein synthesis in H9c2 heart-derived myocytes responds biphasically to arginine vasopressin [Reilly et al., 1998; Brostrom et al., 2000]. An initial 50% inhibition attributable to Ca^{2+} release from the S(E)R and the phosphorylation of eIF2α is followed by a recovery that subsequently converts to a 1.4-fold stimulation. The initial suppression of translation is observed at vasopressin concentrations (nM–μM) that cause extensive depletion of Ca^{2+} stores and is diminished at either supraphysiologic extracellular Ca^{2+} concentrations or by phorbol ester, which inhibits Ca^{2+} release. The subsequent stimulation of protein synthesis, in contrast, is unaffected by changes in extracellular Ca^{2+}, depends on gene transcription, is suppressed by a protein kinase C (PKC) pseudosubstrate sequence, and is observed at pM vasopressin concentrations. H9c2 myocytes, like neonatal cardiomyocytes [van der Bent et al., 1994; Aharonovitz et al., 1998; Liu et al., 1999; Xu et al., 1999, 2000] and adult hearts [Fukuzawa et al., 1999; Nakamura et al., 2000], possess all basic features of V1-vasopressin signaling [Tran et al., 1995; Reilly et al., 1998; Chen and Chen, 1999] and undergo hypertrophy in response to the hormone [Brostrom et al., 2000]. Hypertrophy occurs at pM vasopressin concentrations, depends on PKC activity, and precedes full repletion of Ca^{2+} stores.

While Ca^{2+}-mobilizing hormones, such as vasopressin, that produce a persistent depletion of Ca^{2+} stores would be anticipated to signal the UPR, it is unclear that these events are required for the development of vasopressin-induced myocyte hypertrophy. The present study was undertaken to ascertain whether the synthesis of ER chaperone(s) is selectively increased during treatment of H9c2 cells with vasopressin, is controlled by the degree of S(E)R Ca^{2+} depletion, is responsible for translational upregulation during treatment, and renders the cells resistant to translational inhibition by Ca^{2+}-depleting drugs.

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. 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. Polyclonal rabbit anti-GRP78 and monoclonal rat anti-GRP94 were purchased from StressGen (Victoria, B.C.). Monoclonal antibody to eIF2α was the gift of Dr. Lynn O'Brien, University of Rochester. Ampholines were purchased from Gallard (Garden City, NY)-Schlesinger (pH 4-8) and from ESA Inc. (Chelmsford, MA) (pH 3.5–10). SB 203580 and okadaic acid were purchased from Calbiochem (La Jolla, CA). Other agents were obtained from Sigma (St. Louis, MO). Radiochemicals were obtained from NEN Life Science Products, Inc. (Boston, MA).

Cell Culture and Incubations

Stock H9c2 cells were propagated in DMEM supplemented with 10% fetal bovine serum and subcultured weekly. For experiments, cultures were allowed to reach confluence ($2–4 \times 10^4$ cells/cm^2) which was required for

optimal responsiveness to vasopressin. Experimental treatments were conducted in serum-free DME/F-12 base medium (Sigma) supplemented with normal components excepting leucine, methionine, and $CaCl_2$ which were adjusted for each experiment.

Cell-Associated $^{45}Ca^{2+}$ Measurements

Cells in 12-well (4.8 cm^2/well) plates and subjected to various treatments were pre-equilibrated for 2 h in DME/F-12 medium containing 400 μM leucine, 115 μM methionine, 0.2 mM $CaCl_2$, and 10 μC/ml [$^{45}Ca^{2+}$]$CaCl_2$. Medium was aspirated thoroughly at room temperature. Monolayers were then washed twice with 2 ml of pre-filtered ice-cold saline containing 2.5 mM $LaCl_3$, solubilized by incubation for 30 min at 37°C in 500 μl of warm 1% sodium dodecyl sulfate, and analyzed for radioactivity. Incubations were performed in triplicate, and results are reported as the average±range of values obtained. The experiment described in Figure 7 has been reproduced three times.

Amino Acid Pulse Incorporations

Leucine incorporation was measured as described previously [Brostrom et al., 1983] for confluent monolayers in multiwell trays (2.4 cm^2/well) in DME/F-12 medium adjusted to contain 10 μM leucine, 115 μM methionine, 0.3 mM $CaCl_2$, and 4 μC/ml L-[3,4,5-^{3}H(N)]-leucine. Incubations were performed for 30 min in triplicate. The experiment described in Figure 11 has been reproduced four times. For methionine pulse incorporations, monolayers in multiwell trays (4.8 cm^2/well) underwent treatments in DME/F12 medium adjusted to contain 400 μM leucine, 0.3 mM $CaCl_2$ or Ca^{2+} concentrations as specified, and 30 μM methionine. Pulse labeling was conducted for 30 min in the presence of 2 μM methionine and 50 μC/ml of [^{35}S]methionine, and SDS/PAGE and autoradiography were performed as described previously [Wong et al., 1993].

Determinations of GRP78 and GRP94 Contents and of the Extent of Post-Translational Modification of GRP78

Preparations (confluent monolayers) in 12-well (4.8 cm^2/well) plates were subjected to various treatments in medium adjusted to contain 400 μM leucine, 115 μM methionine, and 0.3 mM $CaCl_2$ or Ca^{2+} concentrations as specified. Cells were then lysed with 150 μl urea buffer containing 2.2% ampholines (pH 3.5–10 2D), 4% Nonidet P40, 100 mM dithiothreitol, and 9.9 M urea. Duplicate lysates were pooled and diluted 1:6 with urea buffer, applied to a 6% acrylamide gel (30% acrylamide, 2% bisacrylamide) containing 4% ampholines (1:1 pH 3.5–10:pH 4–8), 9.5 M urea, and Tween 20 (0.5%) and subjected to isoelectric focussing in a Hoeffer SE600 apparatus at 24°C for 16 h set to a limiting voltage of 800 to separate modified and unmodified forms of GRP78. Gels were treated with 1 M Tris, pH 8.8, and blotted onto polyvinylidene difluoride membranes under basic conditions. GRP78 was immunodetected with primary antibody (1:10,000) and chemiluminescence, with goat-anti-rabbit antiserum (1:10,000) serving as the secondary antibody. GRP94 was immunodetected with primary antibody (1:5,000) and chemoluminescence, with goat anti-mouse antiserum (1:10,000) as the secondary antibody. Immune complexes were visualized by using the ECL Western detection kit according to the manufacturer's instructions. Conditions were carefully selected such that linear output with GRP78 or GRP94 content was maintained. Bands of immunoreactive protein were subjected to scanning densitometry as described [Brostrom et al., 1995].

Determination of eIF2α Content

eIF2α was immunodetected with monoclonal antibody to eIF2 and chemiluminescence as described [Reilly et al., 1998] after subjection of lysates to slab gel isoelectric focussing and immunoblotting as specified above for GRP78.

RESULTS

Selective Expression of GRP78 and GRP94 During Treatment with Vasopressin

Pulse labeling of proteins with [^{35}S]methionine, followed by SDS–PAGE and autoradiography, was employed to identify proteins displaying altered rates of synthesis in H9c2 cells during various treatments involving vasopressin (Fig. 1). The effect of vasopressin (1 μM) treatment as a function of time on the total spectrum of polypeptides synthesized by H9c2 cells was initially examined (Fig. 1A). Pulse labeling of proteins of untreated control preparations was sustained through 2 to 16 h of incubation, but decreased modestly at later

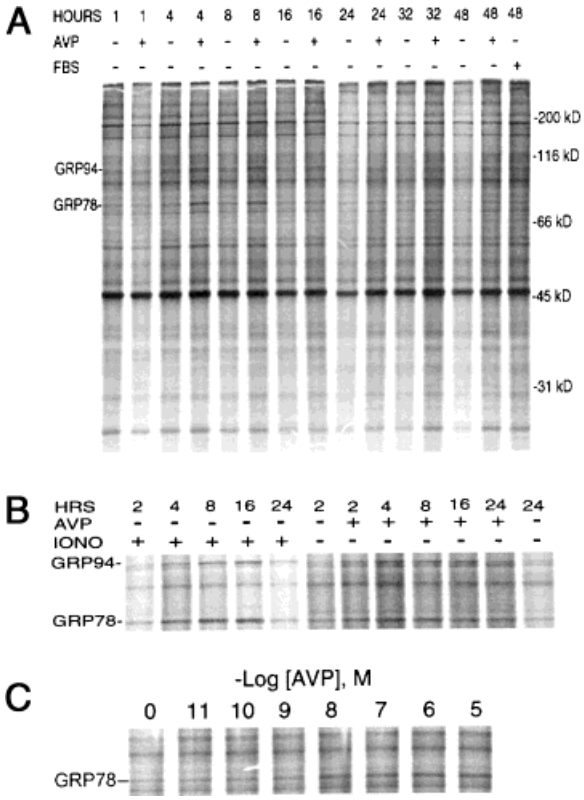

Fig. 1. Preferential synthesis of 78 and 94 kDa polypeptides during treatments with vasopressin or ionomycin. Treatments of H9c2 cells were performed in DME/F12 medium containing 0.3 mM $CaCl_2$. Following treatments, pulse labeling of proteins with [^{35}S]methionine was conducted for 30 min and lysates were analyzed by SDS–PAGE and autoradiography. **A:** Various treatment times with or without vasopressin or serum. Cells were incubated for the times indicated in the absence or presence of vasopressin (AVP, 1 μM) or fetal bovine serum (FBS, 10%). Migration positions of GRP78 and GRP94 and of molecular weight markers are indicated on the left and right ordinates, respectively. **B:** Various treatment times with ionomycin or vasopressin. Cells were treated with ionomycin (IONO, 10 nM) or vasopressin (AVP, 1 μM) for the times indicated. The area corresponding to the migration positions of GRP78 and GRP94 is displayed. **C:** Vasopressin concentration dependence of methionine incorporation into GRP78. Incubations with the indicated concentrations of vasopressin (AVP) were conducted for 4 h. The region corresponding to the migration position of GRP78 is displayed.

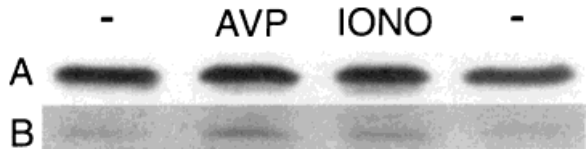

Fig. 2. Increased incorporation of methionine into GRP78 after 2.5 h of exposure to vasopressin or ionomycin. Cells were treated for 2.5 h without additions (–), with ionomycin (IONO, 10 nM), or with vasopressin (AVP, 1 μM) as indicated, followed by pulse labeling of proteins with [^{35}S]methionine for 30 min. Extracts were subjected to isoelectric focussing, followed by western blotting for GRP78 (**A**). The transfer membrane was then subjected to autoradiography (**B**). Regions of the membrane corresponding to the migration position of GRP78 were excised and analyzed for radioactivity by scintillation counting (40 min/sample). Controls: 237 and 226 cpm; vasopressin-treated: 551 cpm; ionomycin-treated, 435 cpm. Comparably treated samples were also analyzed for leucine pulse incorporation into total protein; incorporation was increased 1.2-fold by vasopressin and was reduced 45% by ionomycin.

times. At 1 h of incubation with vasopressin, methionine incorporation into all detectible protein species was found to be reduced. This inhibition was attributable to the rapid and substantial (>40%) release of Ca^{2+} stores by the hormone at concentrations in excess of 1 nM [Reilly et al., 1998; Brostrom et al., 2000]. At 4 h of incubation with vasopressin, a period known to be associated with partial refilling of Ca^{2+} stores [Brostrom et al., 2000], increased pulse labeling was noted for most, if not all, protein populations relative to that of non-treated controls. It was therefore clear that vasopressin promoted higher rates of overall protein synthesis within a relatively short time frame. These increased rates (~40% higher) were preserved through the subsequent 44 h and were comparable to those of preparations maintained for 48 h in medium containing 10% fetal bovine serum. Two polypeptides of 78 and 100 kDa were clearly pulse labeled more intensively than the general population of proteins during vasopressin treatment. The 78 kDa species, which comigrated with authentic GRP78 and interacted with antibody directed against the chaperone (see also Fig. 2), was most intensely labeled at 4–8 h. The 100 kDa species, which comigrated with authentic GRP94 and interacted with antibody directed against that chaperone (see also Fig. 6), was most prominently labeled at 4–16 h.

The extent to which immunoreactive GRP78 was labeled during early treatments with vasopressin or ionomycin, a Ca^{2+} ionophore which reliably activates transcription of the mammalian GRP genes, was examined. Cells were treated 2.5 h without additions, with vasopressin, or with ionophore followed by 30 min of incubation with labeled methionine. Extracts were subjected to isoelectric focussing, immunoblotting for GRP78 was performed, and blots were subjected to autoradiography. A single polypeptide species was found to react

with antibody directed against GRP78, even after prolonged signal development; this species co-migrated with a single radioactively-labeled polypeptide (Fig. 2A and B). Labeling of this polypeptide, but not of others in the region examined, was discernibly increased by vasopressin and by ionomycin at this early incubation time. As assessed by scintillation counting, the amount of radioactivity associated with GRP78 was increased 2.4-fold by vasopressin and 1.9-fold by ionomycin (Fig. 2, legend).

Ionomycin was compared with vasopressin to ascertain the degree to which each agent stimulated synthesis of the GRPs at varying treatment times (Fig. 1B). As compared to untreated controls examined at either 2 or 24 h of incubation, ionomycin-treated cells preferentially synthesized the GRPs at 2, 4, 8, 16, and 24 h, with most prominent synthesis observed at 4–16 h. Overall protein synthesis, however, declined after 24 h with the ionophore and cell viability was significantly reduced (not shown). Vasopressin increased synthesis of both GRPs detectably after 2 h. Relative stimulations of GRP78 synthesis, which were most prominent at 4 h, and of GRP94 synthesis, which were most prominent at 4–16 h, were comparable to those produced by ionomycin at 4–16 h. Viability of vasopressin-treated cells was sustained throughout the 24–48 h period during which protein synthetic rates were stimulated.

The vasopressin concentration dependence capable of stimulating GRP78 synthesis was determined for cells exposed to the hormone for 4 h. Increased synthesis was detectible at concentrations as low as 10 pM (Fig. 1C), with optimal pulse labeling as hormone approached 10 nM. The effect of extracellular Ca^{2+} concentration on chaperone synthesis was also examined (Fig. 3). Incubations were conducted for 4 h with (1 μM) or without hormone in medium containing 0.1, 0.3, 1, 3, or 5 mM $CaCl_2$. Under these conditions vasopressin increased the pulse incorporation of methionine into proteins generally at each of these Ca^{2+} concentrations whereas proteins for controls without hormone were equivalently labeled as a function of increasing Ca^{2+} concentration. Incorporation of the amino acid into GRP78, however, behaved unusually as a function of increasing extracellular Ca^{2+} concentration and hormonal treatment. The chaperone was preferentially labeled in vasopressin-treated preparations incubated from 0.1–1 mM Ca^{2+} whereas selective expression was abolished at either 3 or 5 mM Ca^{2+}. Similar vasopressin and Ca^{2+} concentration dependencies for selective GRP94 expression were observed after 8 h of treatments (not shown).

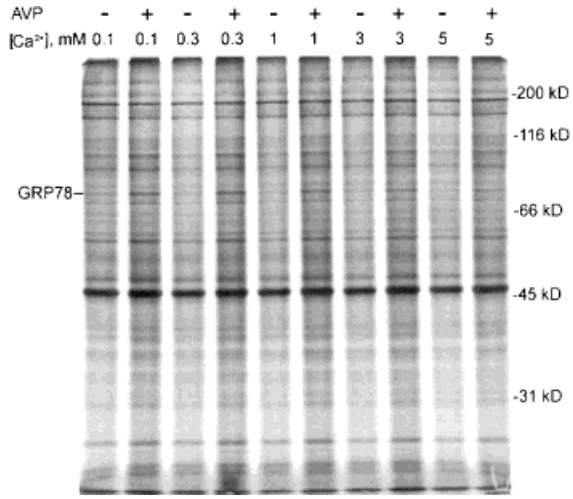

Fig. 3. Extracellular Ca^{2+} concentration dependence of GRP78 synthesis in response to vasopressin. H9c2 cells were incubated for 4 h in medium containing the indicated concentrations of Ca^{2+} and in the absence or presence of vasopressin (AVP, 1 μM). Pulse labeling with [^{35}S]methionine was then performed, and lysates were analyzed by SDS–PAGE and autoradiography. Migration positions of GRP78 and of molecular weight markers are indicated on the left and right ordinates, respectively.

The effects of extended incubations with vasopressin and of varying vasopressin concentrations on the GRP78 content of H9c2 cells were investigated. Extracts of variously treated H9c2 cells were subjected to isoelectric focussing to separate the modified and unmodified forms of the chaperone, followed by Western blotting. Immunoblots from representative experiments are shown in Figure 4A and quantitation by densitometry is provided in Figure 5A. Minimal to modest increases in GRP78 content were observed during 4–24 h of incubation without vasopressin (Figs. 4 and 5A). Approximately one third of total chaperone was converted to the modified form by 24 h. In vasopressin-treated cells, larger increases in chaperone content were observed over 4–24 h, with 2- to 3-fold enhancements apparent at 16–24 h. Approximately one-third of GRP78 was covalently modified at these longer incubation times. For comparison, the eIF2α content of the extracts was also examined by isoelectric focussing and immunoblotting (Fig. 4A). Non-

phosphorylated and phosphorylated forms of eIF2 were detectible before and after vasopressin treatment, but the factor primarily resided in the non-phosphorylated form. Neither total eIF2 content, nor the extent of its phosphorylation, was perceptibly altered by 4–24 h of incubation with hormone. The effect of vasopressin concentration on the GRP78 content of H9c2 cells at 8 h of incubation was also determined (Figs. 4B and 5B). Chaperone content increased approximately 50% at 10 pM hormone and 2- to 2.5-fold at hormone concentrations of 1 nM or greater.

Pulse labeling data for cells treated with vasopressin (Fig. 1) indicated that rates of synthesis of GRP78 were high for the first 8 h of treatment but subsequently declined. It was also clear from Western blotting of GRP78 (Fig. 4A) that GRP78 requirements were reduced during longer term incubations based on the appearance of the modified (inactive) form. It was therefore apparent that cells treated with vasopressin were initially deficient in GRP78 (an adjustment phase) and subsequently in excess during longer term (stable phase) periods of incubation. Since cellular requirements for GRP78 are influenced by the availability of Ca^{2+}, it was of interest to determine how extracellular Ca^{2+} impacted on the induction of GRP78 associated with vasopressin treatment. The effects of extracellular Ca^{2+} concentration on GRP78 content were determined for cells incubated for two time periods (8 and 16 h) either with or without vasopressin, utilizing reference (time 0) values derived from cells in full growth medium (Figs. 4C and 5C). The expression of GRP78 in cells incubated without vasopressin was increased by supraphysiologic Ca^{2+} (5 mM) concentrations during 8 h incubations and by Ca^{2+} concentrations as low as 0.3 mM during 16 h incubations. Cells under these conditions appeared to gradually accumulate GRP78 with the accumulation being accelerated by higher extracellular Ca^{2+} concentrations. Concomitant treatment with vasopressin, however, resulted in GRP78 accumulation varying inversely with extracellular Ca^{2+} concentration. The GRP78 content of cells exposed to hormone for 8 h was sharply boosted by 0.1–1 mM Ca^{2+} but not by either 3 or 5 mM Ca^{2+}. After 16 h exposure to vasopressin, GRP78 contents were increased at each Ca^{2+} concen-

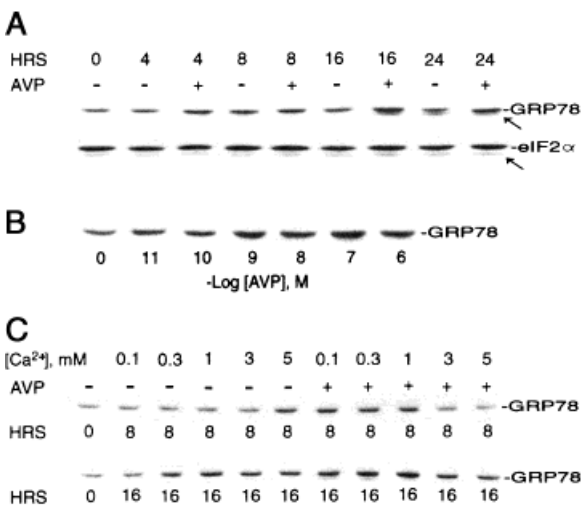

Fig. 4. Contents of the modified and unmodified forms of GRP78 and eIF2α during treatments with vasopressin. **A:** Time of treatment with vasopressin. H9c2 cells were treated for the indicated times with or without vasopressin (AVP, 1 μM). **B:** Vasopressin concentration dependence. Treatments were conducted for 8 h with the indicated concentrations of vasopressin (AVP). **C:** Extracellular Ca^{2+} concentration dependence. Treatments were conducted for 0, 8, or 16 h in medium containing the indicated concentrations of Ca^{2+} and with or without vasopressin (AVP, 1 μM). After treatments, lysates were subjected to slab gel isoelectric focussing followed by western blotting for GRP78 (A–C) or for eIF2α (A). Arrows indicate the migration positions of ADP-ribosylated GRP78 or of phosphorylated eIF2α.

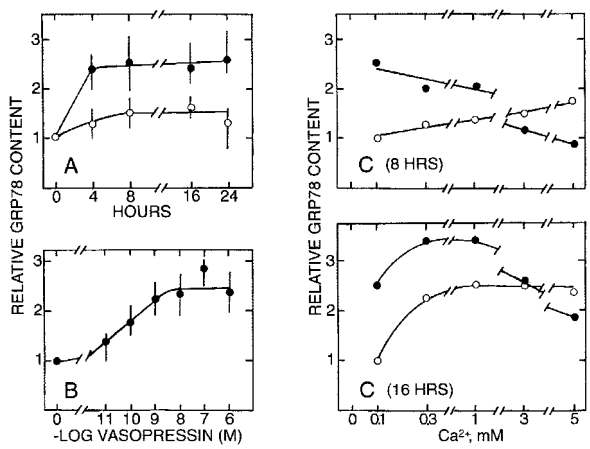

Fig. 5. Quantitation of GRP78 contents of vasopressin-treated cells. Conditions were as described for Figure 4. Immunoreactive bands were subjected to scanning densitometry, and results are expressed as the relative increase in density over the untreated control value at zero time. **A:** Time of treatment without (○) or with (●) vasopressin. The mean±range of values from three separate experiments is displayed. **B:** Vasopressin concentration dependence. The mean±range for three separate experiments is displayed. **C:** Extracellular Ca^{2+} concentration dependence without (○) or with (●) vasopressin. Results from the experiment described in Figure 3C are displayed. Findings have been qualitatively reproduced in two other experiments.

tration tested. However, each increment in chaperone content due to vasopressin treatment was directly comparable to the corresponding increment observed at 8 h. That is, vasopressin-induced increases in GRP78 contents were maximal by 8 h with subsequent changes in values being hormonally independent.

Preparations incubated for 12 h in medium containing 0.1, 1, or 5 mM Ca^{2+} and in the absence or presence of vasopressin were also examined for changes in GRP94 content (Fig. 6). Extracts of treated preparations were subjected to isoelectric focussing, followed by Western blotting and densitometry. A single form of the chaperone was observed under all incubation conditions. GRP94 content of vasopressin-treated preparations was greater than that of untreated controls regardless of extracellular Ca^{2+} concentrations examined. However, larger increases in GRP94 content were observed at 0.1 mM (2.4-fold) as compared to 5 mM (1.3-fold) Ca^{2+}.

Modulation of Vasopressin-Dependent GRP78 Expression by PMA

Acute treatment of H9c2 cells with phorbol 12-myristate 13-acetate (PMA) diminishes Ca^{2+} mobilization and translational inhibition in response to vasopressin, whereas prolonged exposure to phorbol ester to down-regulate PKC in these cells enhances and prolongs the translational inhibition [Reilly et al., 1998; Chen and Chen, 1999]. The relationship between sequestered Ca^{2+} stores and the induction of GRP78 expression by vasopressin was further explored utilizing H9c2 cells subjected to acute and chronic treatments with PMA. The effect of PMA on the Ca^{2+} contents of cells exposed to vasopressin for varying times were initially determined (Fig. 7A). In untreated control preparations, vasopressin mobilized 55% of cell-associated Ca^{2+} in

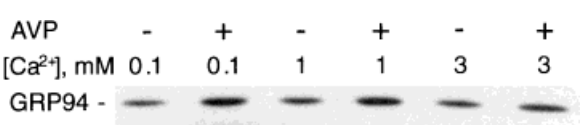

Fig. 6. Contents of GRP94 after incubation with or without vasopressin and at different extracellular Ca^{2+} concentrations. Treatments were conducted for 12 h in the absence or presence of vasopressin (AVP, 1 μM) and at the indicated Ca^{2+} concentrations. Following treatments, lysates were subjected to slab gel isoelectric focussing followed by Western blotting for GRP94.

30 min. Thereafter, stores gradually refilled such that at 5 h cell-associated Ca^{2+} was 73% of control values. Treatment with vasopressin in combination with PMA resulted in lower initial mobilizations (37% mobilized at 30 min), with rates of Ca^{2+} recovery similar to those of controls. Preparations pretreated for 24 h with PMA responded to vasopressin with 59% mobilization of cell-associated Ca^{2+} in 30 min. Unlike solvent-pretreated controls, however, Ca^{2+} stores of PMA-pretreated preparations were not replenished during the subsequent 4.5 h. Synthesis of GRP78 in comparably treated preparations was examined by pulse labeling of proteins with [^{35}S]methionine, followed by SDS–PAGE and autoradiography (Fig. 7B). The upper panel illustrates the effects of 2, 4, and 6 h incubation without additions, with vasopressin, with PMA, or with vasopressin in combination with PMA. Incorporation into cellular proteins was increased at all treatment times by vasopressin, by PMA, or by the combined treatment. Preferential synthesis of GRP78 was detectible at 2 h and prominent at 4–6 h with vasopressin. In contrast, the chaperone was not selectively synthesized in PMA-treated preparations, and the extent of synthesis observed in vasopressin-treated cells was reduced when PMA was included in incubations with hormone. The lower panel illustrates the effects of 24 h pretreatment with solvent or PMA. In solvent-pretreated controls, methionine incorporation into proteins was reduced by vasopressin at 0.5–1 h but not at 2–6 h wherein increased GRP78 and GRP94 synthesis was apparent. PMA-pretreated cells exhibited larger and more prolonged (0.5–2 h) inhibitions of incorporation and greater GRP78 and GRP94 synthesis at 2–6 h when treated with vasopressin. Cells subjected to acute and chronic PMA treatments were also examined for total GRP78 content before and after exposure to vasopressin (Fig. 7C). GRP78 content was increased at 4 and 8 h of vasopressin treatment, as compared to non-vasopressin-treated controls (lanes 2, 3, 6, and 7). PMA alone did not affect chaperone content at either time (lanes 2, 4, 6, and 8). Co-treatment with PMA abrogated vasopressin-dependent GRP78 expression at 4 h (lanes 2–5) but not at 8 h (lanes 7 and 9) of incubation. Pretreatment with PMA for 24 h did not affect GRP78 content (lanes 1 and 10). The pretreated cells responded to vasopressin with a small increase in GRP78 content at 4 h (lanes 11

and 12) and a prominent increase at 8 h (lanes 13 and 14) of incubation.

Studies With Pharmacologic Probes

GRP78 expression during vasopressin treatment was examined in the presence of agents that suppress the development of myocardial hypertrophy or that alter induction of the chaperone during the UPR. Expression was monitored both by pulse labeling of intact cells after 5 h of treatments (Fig. 8A) and by isoelectric focussing and Western blotting of extracts prepared after 8 h of treatments (Fig. 8B). Neither the Ca^{2+} channel blocker, nifedipine, nor the p38 MAP kinase inhibitor, SB 203580, had any effect on pulse incorporation into proteins, selective synthesis of GRP78 in response to vasopressin, or total GRP78 content during treatments with or without vasopressin. Actinomycin D reduced pulse incorporation into proteins and blocked the up-regulation of protein synthesis and the selective synthesis of GRP78 in response to vasopressin; this inhibitor also lowered the content of GRP78 in cells incubated with or without vasopressin. Okadaic acid (0.2 μM), which enhances GRP78 expression during certain forms of stress [Cao et al., 1995; Hsieh et al., 1996; Chen et al., 1997] did not potentiate induction of GRP78 expression by vasopressin. The phosphatase inhibitor decreased methionine pulse incorporation into H9c2 proteins and reduced the selective labeling and expression of GRP78 in response to the hormone.

Neither PD 098059 (50 μM) to inhibit ERK1/2 nor rapamycin (10 nM) to inhibit activation of p70/85 (S6K) kinase affected pulse incorporation into proteins or synthesis of GRP78 in response to vasopressin (not shown). The phosphoinositide 3-kinase inhibitor wortmannin (100 nM) accelerated the recovery from translational suppression and the up-regulation of translational activity in the presence of hormone. Nonetheless wortmannin treatment did not alter the time course or extent of Ca^{2+} mobilization, selective pulse labeling of GRP78,

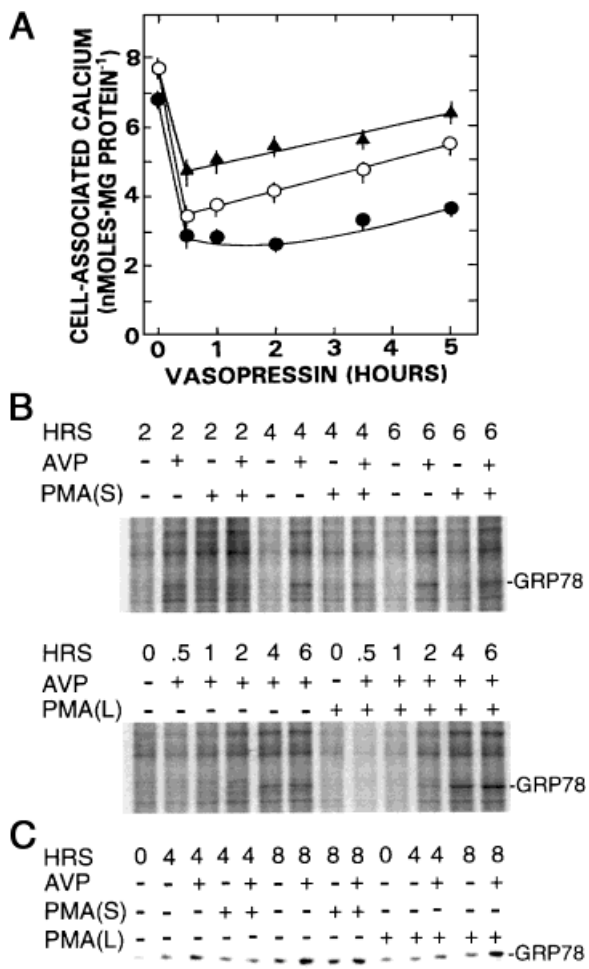

Fig. 7. Alterations in vasopressin-dependent Ca^{2+} mobilization and GRP78 expression by acute and chronic treatments with phorbol myristate acetate. **A:** Effects of acute and chronic phorbol ester treatments on vasopressin-induced mobilization of Ca^{2+} stores. Preparations were pretreated for 24 h with solvent or phorbol myristate acetate (2 μM). Cells were then challenged for the indicated times with vasopressin (1 μM) alone or in combination with phorbol ester (2 μM). Preparations were pre-equilibrated for 2 h with $^{45}CaCl_2$ prior to measurements of cell-associated $^{45}Ca^{2+}$. (○), no phorbol ester during pretreatment or challenge; (▲), phorbol ester present during challenge; (●), 24 h pretreatment with phorbol ester. **B:** Effects of short and long phorbol ester treatments on methionine pulse incorporation into GRP78 during incubations with or without vasopressin. Upper panel, short (S) phorbol ester treatment: incubations were conducted for the indicated times with vasopressin (AVP, 1 μM), phorbol myristate acetate (PMA, 2 μM), or both agents. Lower panel, long (L) phorbol ester treatment: preparations were pretreated for 24 h in growth medium containing solvent or phorbol myristate acetate (PMA, 2 μM). Incubations were then conducted for the indicated times in the absence or presence of vasopressin (AVP, 1 μM). Pulse labeling with [^{35}S]methionine was performed for 30 min, and lysates were analyzed by SDS–PAGE and autoradiography. **C:** Effects of short and long phorbol ester treatments on GRP78 content during incubations with and without vasopressin. Preparations were pretreated for 24 h with solvent or phorbol ester (PMA, 2 μM) or were left untreated. The cells were then challenged for 0, 4, or 8 h with vasopressin (AVP, 1 μM), phorbol ester (PMA, 2 μM), or both agents as indicated. Lysates were subjected to slab gel isoelectric focussing followed by western blotting for GRP78. S, short phorbol ester treatment; L, long phorbol ester treatment. Findings from this experiment were repeated on two other occasions.

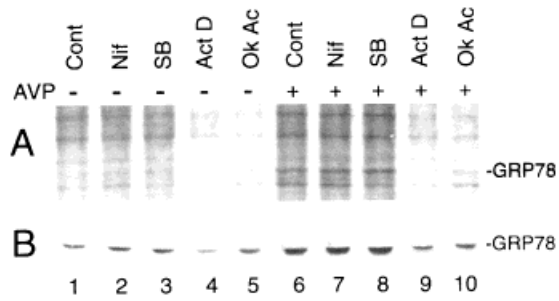

Fig. 8. Vasopressin-dependent GRP78 expression in the presence of nifedipine, SB 203580, actinomycin D, and okadaic acid. **A:** Methionine pulse incorporation into GRP78. Cells were pretreated for 15 min without additions (Cont, 1 and 6) or with 1 μM nifedipine (Nif, 2 and 7), 10 μM SB 203580 (SB, 3 and 8), 1 μg/ml actinomycin D (Act D, 4 and 9), or 0.2 μM okadaic acid (Ok Ac, 5 and 10). Incubations were then continued for 4 h in the absence (1–5) or presence (6–10) of vasopressin (AVP, 1 μM). Pulse labeling with [^{35}S]methionine was then performed, and lysates were analyzed by SDS–PAGE and autoradiography. **B:** GRP78 content. Additional preparations treated as described for 1–10 in A were incubated for 8 h with and without 1 μM vasopressin. Lysates were subjected to slab gel isoelectric focussing, followed by Western blotting for GRP78.

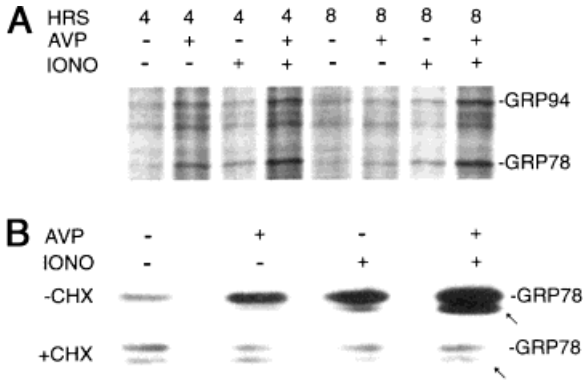

Fig. 9. Induction of GRP78 and GRP94 expression during treatments with vasopressin in combination with ionomycin; effects of cycloheximide on GRP78 content and modification during treatments. **A:** Methionine pulse incorporation into GRP78 and GRP94. H9c2 cells were treated for either 4 or 8 h without additions, with vasopressin (AVP, 1 μM), with ionomycin (IONO, 5 nM) or with both agents. Pulse labeling with [^{35}S]methionine was then performed, and lysates were analyzed by SDS–PAGE and autoradiography. **B:** Forms and content of GRP78. Additional preparations were treated for 8 h in the absence or presence of cycloheximide (CHX, 20 μM), vasopressin (AVP, 1 μM), and ionomycin (IONO, 5 nM). Lysates were then subjected to slab gel isoelectric focussing, followed by Western blotting for GRP78. Arrows indicate the migration position of ADP-ribosylated chaperone.

or the increase in GRP78 content due to vasopressin (not shown).

Vasopressin and Stress Protein Synthesis in Response to Ionomycin and Sodium Arsenite

H9c2 cells incubated for 4 or 8 h without additions, with vasopressin, with ionomycin, or with both agents were pulse labeled with methionine and analyzed by SDS/PAGE and autoradiography (Fig. 9A). The low concentration (5 nM) of ionomycin applied to the cells in this experiment produces moderate (50%) initial inhibition of protein synthesis while still supporting the subsequent induction of GRP78 accompanied by recovery of amino acid incorporation. Pulse incorporation into proteins was enhanced by either agent at 4 and 8 h. Incorporation following joint treatment with both agents was greater than that following treatment with either agent alone. As measured by pulse labeling, GRP78 synthesis in response to ionomycin was equivalent at 4 and 8 h whereas that in response to vasopressin was greater at 4 than at 8 h. In combination, however, the two agents produced profound stimulations of GRP78 and GRP94 synthesis at both 4 and 8 h of incubation. For comparison purposes total GRP78 content was determined by isoelectric focussing and Western blotting for cells incubated for 8 h without additions, with vasopressin, with ionomycin, or with both agents (Fig. 9B). Comparable increases in content were observed with vasopressin or ionomycin alone. Additive increases were observed when these agents were used in combination, with approximately half of the new chaperone converting to the modified form. Treatment with cycloheximide for 8 h did not affect the GRP78 content of untreated preparations but did abolish the increases in GRP78 content due to treatments with vasopressin, ionophore, or both agents. Approximately 40–50% of the chaperone was found to be modified after 8 h of incubation with cycloheximide, regardless of the presence of vasopressin or ionomycin during the incubations.

The effects of vasopressin on expression of the heat shock proteins during incubations with sodium arsenite was also examined. Cells were treated for 9 h without additions, with vasopressin, with 3 or 10 μM arsenite, and with vasopressin in combination with 3 or 10 μM arsenite. Proteins were then pulse labeled with methionine and analyzed by SDS/PAGE (Fig. 10). Up-regulation of overall protein synthesis and labeling of GRP94 were apparent in preparations treated with vasopressin alone.

Preferential synthesis of HSP90, HSP70, and HSP30 was observed after treatments with 3 or 10 μM arsenite. HSP110 was selectively synthesized in response to 10 μM arsenite. Vasopressin further enhanced synthesis of each of these HSPs when present concurrently with 3 or 10 μM arsenite.

Vasopressin and Tolerance to ER Stress

As is typical of cultured mammalian cells, H9c2 myocytes exposed to ionomycin for several hours develop translational resistance to subsequent rechallenge with Ca^{2+}-mobilizing agents [Reilly et al., 1998]. This resistance is due, at least in part, to the suppression of eIF2 kinase by continuously synthesized GRP78 during persistent ER stress [Brostrom and Brostrom, 1998]. Vasopressin-treated cells were examined for development of similar tolerance. Cells were treated for 16 h without additions, with vasopressin, or with ionomycin. Preparations were then washed, equilibrated in fresh medium, and rechallenged briefly with ionomycin at varying concentrations, followed by measurements of leucine pulse incorporation into proteins (Fig. 11). Incorporation into naive preparations was suppressed 50% by 5 nM and 73% at 10 nM ionomycin. Higher concentrations of ionophore were required to suppress incorporation into ionomycin-treated preparations (e.g., 50% inhibition at 20 nM drug). Although vasopressin-treated cells had faster rates of leucine incorporation than did control and ionomycin-treated cells, tolerance to inhibition by ionomycin was not expressed.

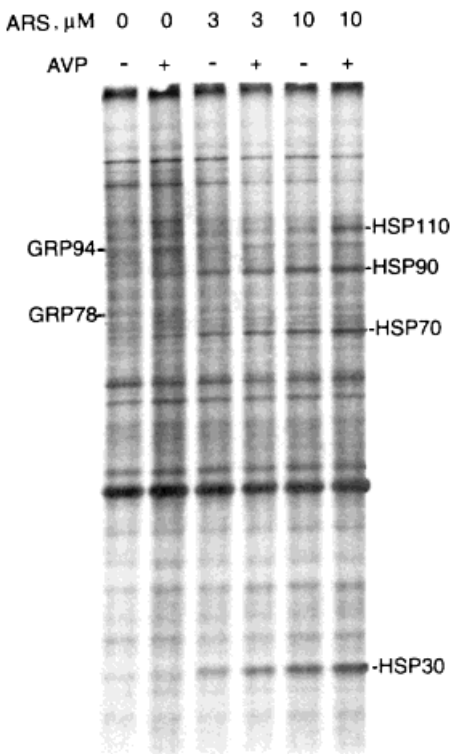

Fig. 10. Expression of the heat shock proteins during treatments with vasopressin in combination with sodium arsenite. H9c2 cells were incubated for 9 h without additions, with 1 μM vasopressin (AVP), with 3 or 10 μM sodium arsenite (ARS), or with both vasopressin and 3 or 10 μM sodium arsenite. Pulse labeling with [^{35}S]methionine was then performed, and lysates were analyzed by SDS–PAGE and autoradiography. The migration positions of GRP94 and GRP78 are indicated on the left and the migration positions of heat shock proteins of 110, 90, 70, and 30 kDa are indicated on the right.

DISCUSSION

Induction of *grp78* and *grp94* gene expression is characteristically associated with the accumulation of unfolded protein within the ER lumen during chronic ER stress. Stressful conditions established to signal increased expression of these chaperones include persistent depletion of ER Ca^{2+} such that protein folding or assembly is impaired, disturbance of the redox potential of the ER such that appropriate disulfide bonds are not formed, and incorrect or incomplete glycoprotein processing. Increased expression of GRP78 is tightly associated with an increased capacity to survive

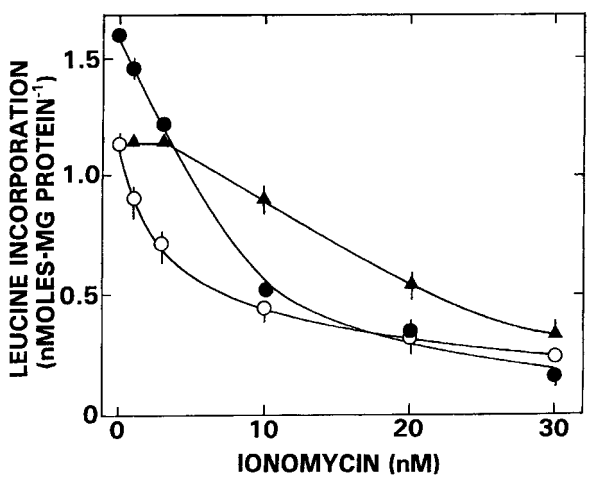

Fig. 11. Inhibition of leucine pulse incorporation by ionomycin after extended incubations in the absence or presence of vasopressin or ionomycin. H9c2 cells were pretreated for 16 h without additions (○), with 1 μM vasopressin (●), or with 10 nM ionomycin (). Preparations were washed twice, equilibrated in fresh medium for 30 min, and challenged for 30 min with ionomycin at the concentrations indicated. Pulse incorporation of [^{3}H]leucine into proteins was then determined.

further stress [Little et al., 1994; Gething, 1999; Hampton, 2000] and with the development of translational resistance to the stress following an acute suppression of translational initiation via phosphorylation of eIF2α [Brostrom and Brostrom, 1998]. This report provides the first evidence that physiological concentrations of a Ca^{2+}-mobilizing hormone signal increased expression of the GRPs. Although vasopressin stimulated the incorporation of amino acids into H9c2 cell proteins over 4–24 h of incubation, the GRPs were selectively labeled at early treatment times. By the techniques of pulse labeling, SDS–PAGE and autoradiography, these chaperones were the only proteins that clearly experienced preferential synthesis at any given time of exposure to vasopressin. Findings were incompatible, however, with the induction of an authentic UPR by vasopressin. Although increased GRP78 expression during hormonal treatment correlated with the degree to which cellular Ca^{2+} stores were depleted, translational tolerance to drugs that deplete these stores did not develop. Selective GRP78 synthesis in vasopressin-treated cells was sustained for shorter periods than those observed during challenge with Ca^{2+}-mobilizing drugs such as ionomycin or thapsigargin. Preferential synthesis peaked at 4–6 h and declined thereafter, with optimal increases in GRP78 content attained at 4–8 h. Upon cessation of preferential synthesis GRP78 was post-translationally modified, as is generally observed when the unmodified form is present at concentrations in excess of processing needs [Ledford and Leno, 1994; Laitusis et al., 1999]. Finally, expression of GRP78 in vasopressin-treated H9c2 cells was neither potentiated by okadaic acid, a protein phosphatase inhibitor, nor blocked by SB 203580, a specific inhibitor of p38 MAP kinase. By contrast, *trans*-activation of *grp78* transcription during various stresses is reported to be enhanced by okadaic acid [Cao et al., 1995; Hsieh et al., 1996; Chen et al., 1997] and inhibited by SB 203580 [Chen et al., 1998; Chen et al., 2000].

Several observations support the hypothesis that increased expression of GRP78 by vasopressin-treated H9c2 cells is contingent on release of Ca^{2+} sequestered by the ER. First, mobilization of cell-associated Ca^{2+} stores preceded appearance of new GRP78 molecules, with degree of mobilization correlating with degree of induction as a function of vasopressin concentration [Brostrom et al., 2000] (Figs. 1 and 4). Second, preferential synthesis of GRP78 and the increase in GRP78 content due to vasopressin treatment were both suppressed at supraphysiologic extracellular Ca^{2+} concentrations (Figs. 3 and 4). Such high Ca^{2+} concentrations are expected to diminish ER Ca^{2+} depletion during hormonal challenge. In contrast the induction of GRP78 was unaffected during blockade of plasmalemmal Ca^{2+} entry with nifedipine (Fig. 8), which lowers the Ca^{2+} content of H9c2 cells by 30% [Brostrom et al., 2000]. Third, treatment with vasopressin in combination with PMA, which lessens the amount of Ca^{2+} mobilized in response to the hormone [Reilly et al., 1998], resulted in slower inductions of GRP78 as compared to vasopressin treatment alone (Fig. 7B and C). Fourth, pretreatment with PMA altered both vasopressin-dependent Ca^{2+} mobilization and GRP78 expression similarly. As compared with non-pretreated controls, PMA-pretreated cultures displayed more prolonged depletion of Ca^{2+} stores (Fig. 7A) and more intense methionine incorporation into GRP78 at comparable times of vasopressin treatment (Fig. 7C).

Direct-acting inhibitors of mRNA translation, such as cycloheximide, that depress protein flow to the ER produce the ADP-ribosylation of GRP78 [Ledford and Leno, 1994; Laitusis et al., 1999] (Fig. 9). This observation is explicable in terms of the modification and inactivation of GRP78 following its release from proteins completing processing in the ER. Under conditions where new protein influx is suppressed at mRNA translation, ER processing requirements for GRP78 are sharply reduced. It is also clear that the UPR, characterized by increased *grp78* gene transcription, cannot be signaled in the absence of protein influx [Kim et al., 1987; Price et al., 1992; Brostrom et al., 1995]. By contrast, the induction of *grp78* mRNA occurs specifically when protein processing capacity is lower than synthetic capability, as is observed during treatments that inhibit processing or folding. The induction is also observed when the flow of new processible polypeptides through the ER lumen is increased relative to processing capacity. For example, *grp78* mRNA is rapidly induced following release of translational activity from an extended cycloheximide block [Brostrom et al., 1995]. The increased expression of GRP78, observed at specific times of vasopressin treatment, may be coupled to

changes in protein synthetic rate. Amino acid incorporation into cellular proteins increases by approximately 50% within 2–3 h of exposure to vasopressin, concurrent with selective GRP78 synthesis. The possibility that the increased flow of processible protein through the ER lumen during this time signals a need for additional chaperone molecules cannot be excluded. Additionally the cessation of GRP78 induction after 8 h, followed by post-translational modification, may indicate that rates of protein processing/folding and of protein synthesis achieve longer term balance.

Increased expression of GRP78 and GRP94 is clearly insufficient for induction of hypertrophy in H9c2 cells. Although these chaperones are preferentially synthesized in response to ionomycin (Fig. 1B), cellular protein content is not increased and cell viability is not sustained. Furthermore, extended (18 h) incubations at physiologic to high extracellular Ca^{2+} concentrations were associated with increased GRP78 content (Fig. 4C) and with accelerated rates of protein synthesis [Brostrom et al., 2000], but hypertrophy did not develop during incubations at these Ca^{2+} concentrations (unpublished observations). In the presence of vasopressin, however, hypertrophy was observed over a broad range of Ca^{2+} concentrations. In H9c2 cells vasopressin signals multiple events including the activations of PKC, phospholipase C-β, phospholipase A_2, and the p42 MAP kinase (ERK2) [Tran et al., 1995; Chen and Chen, 1999] all of which are likely to participate in the trophic response. It is equally reasonable to propose that increased expression of GRP78, a chaperone essential to protein translocation, processing and folding, would be required at the early stages of cellular hypertrophy wherein protein synthetic rates rise and cell mass begins to expand.

Translational up-regulation and increased synthesis of GRP78 are observed at comparable times of vasopressin treatment and abolished in the presence of actinomycin D. Current evidence, however, does not support the hypothesis that newly synthesized GRP78 supports translational up-regulation. Several arguments underpin this assertion. First, translational up-regulation usually preceded by 1–2 h any notable increase in GRP78 content. Second, the increased synthesis and expression of GRP78 were abolished at high Ca^{2+} concentrations, whereas translational up-regulation was not [Brostrom et al., 2000]. Third, treatment with wortmannin at concentrations known to inhibit phosphoinositide 3-kinase in H9c2 cells [Tsiani et al., 1998] was found to hasten translational up-regulation, but selective synthesis of GRP78 occurred at comparable times in the absence and presence of the inhibitor. It is more likely that the two events are signaled through different transcriptional mechanisms. Translational up-regulation is blocked by the cell-permeable PKC pseudosubstrate inhibitor, peptide 19-27, whereas Ca^{2+} mobilization is unaffected [Brostrom et al., 2000].

A number of findings from this study favor the proposal that early translational up-regulation by vasopressin serves, in part, to support the synthesis of newly transcribed mRNAs, particularly those synthesized in abundance or that are capable of entering polysomes under special conditions. Examples of such mRNAs are those for GRP78 and for the heat shock proteins which are both synthesized at high concentrations and selectively translated during the relevant stress [Subjeck and Shyy, 1984; Panniers, 1994; Sierra and Zapata, 1994; Brostrom and Brostrom, 1998]. Synthesis of GRP78 by ionomycin-treated H9c2 cells was significantly greater in the presence as compared to absence of vasopressin (Fig. 9). Similarly, synthesis of the heat shock proteins by arsenite-treated cells was greater in the absence as compared to presence of the hormone (Fig. 10).

The selective synthesis of GRP78 in a variety cell types exposed to Ca^{2+} mobilizing agents including ionomycin is known to involve increased rates of *grp78* gene transcription [Little et al., 1994]. Selective synthesis of the chaperone in H9c2 cells treated with either ionomycin or vasopressin almost certainly derives from transcriptional considerations since it is abolished by low concentrations of actinomycin D [Reilly et al., 1998] (Fig. 8A). Regardless of the conditions under which selective synthesis of GRP78 was observed in this study, increases in cellular GRP78 content invariably followed. H9c2 myocytes should therefore provide an excellent system in which to explore regulation of *grp78* expression by Ca^{2+} and to characterize further the effects of vasopressin on expression of this gene at both the transcriptional and translational steps.

It is unclear whether normal cardiomyocytes, like H9c2 heart-derived myocytes, express the *grp* genes in response to vasopressin or whether

up-regulation of these genes should be considered a marker of cardiac hypertrophy. Expression of the *grp* genes in vasopressin-treated H9c2 cells clearly follows early markers of cardiac hypertrophy in these cells such synthesis of inositol trisphosphate, elevation of $[Ca^{2+}]_i$, and activations of PKC, MAP kinases, and ERKs, and precedes later markers including detectable increases in total protein content and suppression of DNA replication (unpublished observations). It remains to be established that re-expression of specific fetal genes, the hallmark of hypertrophy of neonatal and adult cardiomyocytes, need occur in vasopressin-treated H9c2 cells. H9c2 myocytes, which are embryonic in origin, may express such genes constitutively. Vasopressin-treated H9c2 cells are nonetheless predicted to provide a simple and reproducible model in which to explore the role(s) of increased expression of GRP78 and/or GRP94 in induction of cellular hypertrophy.

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