Regulation of Protein Synthesis in Ventricular Myocytes by Vasopressin

THE ROLE OF SARCOPLASMIC/ENDOPLASMIC RETICULUM Ca\(^{2+}\) STORES*

(Received for publication, September 23, 1997, and in revised form, November 7, 1997)

Barbara A. Reilly, Margaret A. Brostrom‡, and Charles O. Brostrom
From the Department of Pharmacology, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Protein synthesis in H9c2 ventricular myocytes was subject to rapid inhibition by agents that release Ca\(^{2+}\) from the sarcoplasmic/endoplasmic reticulum, including thapsigargin, ionomycin, caffeine, and arginine vasopressin. Inhibitions were attributable to the suppression of translational initiation and were coupled to the mobilization of cell-associated Ca\(^{2+}\) and the phosphorylation of eIF2α. Ionomycin and thapsigargin produced relatively stringent degrees of Ca\(^{2+}\) mobilization that produced an endoplasmic reticulum (ER) stress response. Translational recovery was associated with the induction of ER chaperones and resistance to translational inhibition by Ca\(^{2+}\)-mobilizing agents. Vasopressin at physiologic concentrations mobilized 60% of cell-associated Ca\(^{2+}\) and decreased protein synthesis by 50% within 20–30 min. The inhibition of protein synthesis was exerted through an interaction at the V1 vascular receptor, was imposed at physiologic extracellular Ca\(^{2+}\) concentrations, and became refractory to hormonal washout within 10 min of treatment. Inhibition was found to attenuate after 30 min, with full recovery occurring in 2 h. Translational recovery did not involve an ER stress response but rather was derived from the partial repopulation of intracellular Ca\(^{2+}\) stores. Longer exposures to vasopressin were invariably accompanied by increased rates of protein synthesis. Translational inhibition by vasopressin, but not by Ca\(^{2+}\)-mobilizing drugs, was both preventable and reversible by treatment with phorbol ester, which reduced the extent of Ca\(^{2+}\) mobilization occurring in response to the hormone. Larger and more prolonged translational inhibitions occurred after down-regulation of protein kinase C. This report provides the first compelling evidence that hormonally induced mobilization of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) stores is regulatory upon mRNA translation.

Ca\(^{2+}\) sequestered by the endoplasmic reticulum (ER)\(^1\) is essential for optimal rates of protein synthesis occurring in nucleated mammalian cells (reviewed in Ref. 1). This control involves coupling of the rates of mRNA translation to those of protein translocation into the organelle for subsequent processing or folding. Depletion of ER sequestered Ca\(^{2+}\) to slow glycoprotein processing and transport competence, or introduction of a reducing environment to suppress ER processing of proteins with disulfide cross-links, results in the activation of the double-stranded RNA-activated protein kinase, the phosphorylation of eIF2α, the inhibition of eIF2B, and the slowing of translational initiation (2–6). An adaptive response, characterized by translational accommodation to continued depletion of ER Ca\(^{2+}\) stores by drugs such as ionomycin or thapsigargin or to the continued presence of a thiol-reducing agent, occurs over several hours. This adaptive response is dependent on increased expression of the ER resident chaperone, GRP78/BiP (1, 4, 7, 8). Inductions of this chaperone and of recovery from translational inhibition depend on activation of grp78 transcription and, in some cell types, a growth-promoting factor. The acute suppression of translational initiation by agents that inhibit ER protein processing, the induction of the ER chaperones GRP78 and GRP94, and the recovery of translational activity are characteristic of the “ER stress response” (1, 9, 10). Translational suppression, however, is not required for expression of the later events. Both GRP78 and translational tolerance can be induced by Ca\(^{2+}\)-mobilizing or thiol-reducing agents at concentrations that do not suppress protein synthesis (7).

The physiologic significance of translational suppression by conditions provoking the ER stress response is unclear. However, Ca\(^{2+}\)-mobilizing hormones, including epinephrine, angiotensin, and vasopressin, have been found to slow protein synthesis over several minutes in isolated hepatocytes (11–13). Inhibitions were reduced during incubations in Ca\(^{2+}\)-depleted media and overturned at supraphysiologic Ca\(^{2+}\) concentrations (11). Corresponding reductions were observed in the polysomal contents of excised portions of perfused rat liver in response to hormones and manipulations of the Ca\(^{2+}\) content of the perfusing medium (14). It was proposed that hormonally induced changes in intracellular Ca\(^{2+}\) homeostasis provide a mechanism for regulating the rate of protein synthesis in normal hepatocytes. Given the transient viability and low synthetic rates of dispersed hepatocytes and the technical limitations associated with perfused rat liver, it has not been possible to correlate translational rates with changes in cell-associated Ca\(^{2+}\) or eIF2α phosphorylation, to examine the reversibility of hormonally imposed inhibitions, or to ascertain whether an adaptive response occurs during continuous hormonal treatments. Although cultured cells are better suited to such studies, cell lines exhibiting translational suppression in response to hormones that mobilize Ca\(^{2+}\) from the ER to the cytosol have not been identified.

We now report that H9c2 ventricular myocytes respond to drugs that deplete the sarcoplasmic/endoplasmic reticulum (S/E/R) of Ca\(^{2+}\) and to physiologic concentrations of arginine

* This work was supported by a research award from the American Diabetes Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Department of Pharmacology, Robert Wood Johnson Medical School, 675 Hoes Ln., Piscataway, NJ 08854. Tel.: 732-235-4086; Fax: 732-235-4073; E-mail: brostrom@umdnj.edu.

1 The abbreviations used are: ER, endoplasmic reticulum; S(E)R, sarcoplasmic/endoplasmic reticulum; [Ca\(^{2+}\)], cytosolic free Ca\(^{2+}\) concentration; eIF, eukaryotic initiation factor; GRP, glucose-regulated stress protein; GRP78/BiP, glucose-regulated stress protein 78 or immunoglobulin heavy chain binding protein; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.

This paper is available on line at http://www.jbc.org
vasopressin with a rapid inhibition of mRNA translation at initiation. Inhibitions are coupled to the mobilization of cell-associated Ca$^{2+}$ and to the phosphorylation of eIF2a. Ionomycin and thapsigargin produced relatively stringent degrees of Ca$^{2+}$ mobilization in which translational recovery was associated with the induction of ER chaperones. Translational recovery from inhibition by vasopressin did not involve an ER stress response but derived instead from the partial repletion of intracellular Ca$^{2+}$ stores. Inhibition of protein synthesis by vasopressin became refractory to hormonal washout within 10 min of treatment. These findings support the validity of hormonally mediated regulation of mRNA translation involving mobilization of Ca$^{2+}$-sequestered within the S/E/R in ventricular myocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—H9c2(2–1) rat embryonic ventricular myocytes were obtained from the American Type Culture Collection at passage number 12. Arginine vasopressin, lysine vasopressin, desmopressin, endothelin-1, angiotensin II, phenylephrine, caffeine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin and thapsigargin were from Calbiochem. Monoclonal antibody to eIF2a was the gift of Dr. Lynn O’Brien, University of Rochester. Muscarinic antagonist leucine incorporation was measured as described previously (5). Films were scanned by an image analyzer as described previously (18).

**General Methodology**—Stock H9c2 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and subcultured before confluence. For experiments, monolayers were allowed to reach confluence after which cultures were maintained for 1 day in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum to promote differentiation of myoblasts into myotubes (15). Before treatments, cells were equilibrated for 5–15 min with serum-free Ham’s F-10 medium modified to contain 25 mM leucine and 0.2 mM Ca$^{2+}$. Leucine pulse incorporation was measured as described previously (16) for monolayers in multiwell trays (2.4 cm$^2$/well) and for 15-min incubations. Incubations were conducted in triplicate and results are presented as the mean ± S.E. of values obtained. Findings were reproduced on at least two separate occasions.

**Materials**—H9c2(2–1) rat embryonic ventricular myocytes were obtained from the American Type Culture Collection at passage number 12. Arginine vasopressin, lysine vasopressin, desmopressin, endothelin-1, angiotensin II, phenylephrine, caffeine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin and thapsigargin were from Calbiochem. Monoclonal antibody to eIF2a was the gift of Dr. Lynn O’Brien, University of Rochester. Muscarinic antagonist leucine incorporation was measured as described previously (5). Films were scanned by an image analyzer as described previously (18).

**General Methodology**—Stock H9c2 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and subcultured before confluence. For experiments, monolayers were allowed to reach confluence after which cultures were maintained for 1 day in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum to promote differentiation of myoblasts into myotubes (15). Before treatments, cells were equilibrated for 5–15 min with serum-free Ham’s F-10 medium modified to contain 25 mM leucine and 0.2 mM Ca$^{2+}$. Leucine pulse incorporation was measured as described previously (16) for monolayers in multiwell trays (2.4 cm$^2$/well) and for 15-min incubations. Incubations were conducted in triplicate and results are presented as the mean ± S.E. of values obtained. Findings were reproduced on at least two separate occasions.

**Materials**—H9c2(2–1) rat embryonic ventricular myocytes were obtained from the American Type Culture Collection at passage number 12. Arginine vasopressin, lysine vasopressin, desmopressin, endothelin-1, angiotensin II, phenylephrine, caffeine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin and thapsigargin were from Calbiochem. Monoclonal antibody to eIF2a was the gift of Dr. Lynn O’Brien, University of Rochester. Muscarinic antagonist leucine incorporation was measured as described previously (5). Films were scanned by an image analyzer as described previously (18).

**General Methodology**—Stock H9c2 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and subcultured before confluence. For experiments, monolayers were allowed to reach confluence after which cultures were maintained for 1 day in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum to promote differentiation of myoblasts into myotubes (15). Before treatments, cells were equilibrated for 5–15 min with serum-free Ham’s F-10 medium modified to contain 25 mM leucine and 0.2 mM Ca$^{2+}$. Leucine pulse incorporation was measured as described previously (16) for monolayers in multiwell trays (2.4 cm$^2$/well) and for 15-min incubations. Incubations were conducted in triplicate and results are presented as the mean ± S.E. of values obtained. Findings were reproduced on at least two separate occasions.

**Materials**—H9c2(2–1) rat embryonic ventricular myocytes were obtained from the American Type Culture Collection at passage number 12. Arginine vasopressin, lysine vasopressin, desmopressin, endothelin-1, angiotensin II, phenylephrine, caffeine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin and thapsigargin were from Calbiochem. Monoclonal antibody to eIF2a was the gift of Dr. Lynn O’Brien, University of Rochester. Muscarinic antagonist leucine incorporation was measured as described previously (5). Films were scanned by an image analyzer as described previously (18).

**General Methodology**—Stock H9c2 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and subcultured before confluence. For experiments, monolayers were allowed to reach confluence after which cultures were maintained for 1 day in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum to promote differentiation of myoblasts into myotubes (15). Before treatments, cells were equilibrated for 5–15 min with serum-free Ham’s F-10 medium modified to contain 25 mM leucine and 0.2 mM Ca$^{2+}$. Leucine pulse incorporation was measured as described previously (16) for monolayers in multiwell trays (2.4 cm$^2$/well) and for 15-min incubations. Incubations were conducted in triplicate and results are presented as the mean ± S.E. of values obtained. Findings were reproduced on at least two separate occasions.

**Materials**—H9c2(2–1) rat embryonic ventricular myocytes were obtained from the American Type Culture Collection at passage number 12. Arginine vasopressin, lysine vasopressin, desmopressin, endothelin-1, angiotensin II, phenylephrine, caffeine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin and thapsigargin were from Calbiochem. Monoclonal antibody to eIF2a was the gift of Dr. Lynn O’Brien, University of Rochester. Muscarinic antagonist leucine incorporation was measured as described previously (5). Films were scanned by an image analyzer as described previously (18).

**General Methodology**—Stock H9c2 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and subcultured before confluence. For experiments, monolayers were allowed to reach confluence after which cultures were maintained for 1 day in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum to promote differentiation of myoblasts into myotubes (15). Before treatments, cells were equilibrated for 5–15 min with serum-free Ham’s F-10 medium modified to contain 25 mM leucine and 0.2 mM Ca$^{2+}$. Leucine pulse incorporation was measured as described previously (16) for monolayers in multiwell trays (2.4 cm$^2$/well) and for 15-min incubations. Incubations were conducted in triplicate and results are presented as the mean ± S.E. of values obtained. Findings were reproduced on at least two separate occasions.

**Materials**—H9c2(2–1) rat embryonic ventricular myocytes were obtained from the American Type Culture Collection at passage number 12. Arginine vasopressin, lysine vasopressin, desmopressin, endothelin-1, angiotensin II, phenylephrine, caffeine, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin and thapsigargin were from Calbiochem. Monoclonal antibody to eIF2a was the gift of Dr. Lynn O’Brien, University of Rochester. Muscarinic antagonist leucine incorporation was measured as described previously (5). Films were scanned by an image analyzer as described previously (18).

**General Methodology**—Stock H9c2 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and subcultured before confluence. For experiments, monolayers were allowed to reach confluence after which cultures were maintained for 1 day in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum to promote differentiation of myoblasts into myotubes (15). Before treatments, cells were equilibrated for 5–15 min with serum-free Ham’s F-10 medium modified to contain 25 mM leucine and 0.2 mM Ca$^{2+}$. Leucine pulse incorporation was measured as described previously (16) for monolayers in multiwell trays (2.4 cm$^2$/well) and for 15-min incubations. Incubations were conducted in triplicate and results are presented as the mean ± S.E. of values obtained. Findings were reproduced on at least two separate occasions.
8–10-fold. Activation of the V1 vascular receptor has been established to signal an increase in 
$[\text{Ca}^{2+}]$, which is dependent, in part, on the release of ER sequestered cation to the

8–10-fold. Activation of the V1 vascular receptor has been established to signal an increase in $[\text{Ca}^{2+}]$, which is dependent, in part, on the release of ER sequestered cation to the cytosol by inositol triphosphate (27). The $\text{Ca}^{2+}$ dependence of the vasopressin inhibition of protein synthesis was examined as a function of increasing extracellular concentrations of the cation (Fig. 2). Vasopressin had no effect on leucine incorporation at low extracellular $\text{Ca}^{2+}$ concentrations but reduced incorporation at 1 mM free cation by half. Supraphysiologic (2–3 mM) extracellular $\text{Ca}^{2+}$ concentrations did not reverse the inhibition attributable to vasopressin.

The time dependence of the inhibition of protein synthesis by vasopressin was determined for cells incubated for 2 h with 0.2 mM Ca$^{2+}$, which is sufficient to maintain rates of leucine incorporation in untreated cells (Fig. 3). Within 20–30 min of hormone addition, maximal inhibition of leucine incorporation was observed. Thereafter, pulse incorporation rates were found to rise, with full recovery of activity observed at 2 h. The addition of EGTA in excess of $\text{Ca}^{2+}$ produced declining pulse incorporation rates that plateaued at 50% of control values from 1–2 h. Vasopressin added in combination with the chelator provoked larger inhibitions of leucine incorporation (67% at 30 min), which were sustained through 2 h of incubation.

Activation of phospholipase C in response to occupation of V1 vascular receptors is associated with the intracellular generation of both inositol triphosphate and diacylglycerol (27). The potential involvement of protein kinase C in translational regulation by vasopressin was therefore explored utilizing PMA as an activator of the enzyme. In control experiments, exposure of H9c2 cells to PMA for up to 30 min did not affect either leucine incorporation or cell-associated $\text{Ca}^{2+}$ (data not shown). By 45 min, however, leucine incorporation tended to rise modestly. The effects of vasopressin on leucine incorporation were examined in cells that had been either pretreated for 15 min with 1 mM PMA or carried as untreated controls (Table II). PMA pretreatment was found to abolish the inhibition of leucine incorporation that occurred in untreated cells in response to the subsequent addition of vasopressin. PMA also fully reversed (within 5 min) pre-existing inhibitions of leucine incorporation attributable to vasopressin (data not shown). In contrast, suppression of leucine incorporation occurring in H9c2 cells treated with ionomycin, thapsigargin, sodium arsenite, or di-thiothreitol or with heat shock (43 °C for 20 min), was neither prevented nor reversed by PMA (data not shown). $\text{Ca}^{2+}$ mobilization in response to vasopressin was reduced, but not abolished, by PMA (Table II). Vasopressin mobilized approximately 37% of cell-associated $\text{Ca}^{2+}$ in PMA-pretreated cells as compared with 50% release in non-treated cells; this degree of mobilization was completed within 10 min.
Chronic exposure to phorbol ester was also employed to investigate the effects of protein kinase C down-regulation on vasopressin signaling. Conditions were chosen that are reported to produce down-regulation of the kinase in neonatal cardiomyocytes (28). H9c2 cells were pretreated for 16 h in complete growth medium with or without the addition of PMA. The time dependence of vasopressin inhibition of protein synthesis was then examined (Fig. 4). Non-pretreated preparations responded to hormone with a rapid suppression of protein synthesis that maximized at 20 min. Thereafter, pulse incorporation rates increased such that the 2-h values exceeded those of untreated controls. In preparations exposed chronically to PMA, vasopressin evoked larger (80%) inhibitions of translational initiation and the release of cell-associated Ca2+ within 30 min by indeterminant mechanisms (data not shown).

### Table II

| Time (min) | Leucine incorporation | Cell-associated Ca2+ |
|-----------|-----------------------|---------------------|
| +PMA      | +PMA                  |                     |
| 0         | 3.78 ± 0.11           | 4.68 ± 0.07         |
| 10        | 2.93 ± 0.01           | 3.77 ± 0.18         |
| 20        | 2.87 ± 0.09           | 3.66 ± 0.04         |
| 30        | 2.69 ± 0.06           | 4.61 ± 0.05         |

Inhibition of Translational Initiation and Phosphorylation of eIF2α by Vasopressin—The polysomal contents of H9c2 cells were examined after treatments with thapsigargin, vasopressin, PMA, and PMA in combination with vasopressin (Fig. 5). As anticipated, polysomes almost completely disappeared in response to thapsigargin, which slows initiation relative to peptide chain elongation in non-muscle cells (17). Vasopressin also reduced polysomal content, but not as dramatically as thapsigargin. Polysomes were preserved in incubations with PMA alone, and polysome disaggregation in response to vasopressin was reduced after brief pretreatment with the phorbol ester. To correct for potential differences in loading of gradients, the amounts of 80 S monosomes, small polysomes, and large polysomes were quantitated by absorbance at 254 nm and related to each other. For each treatment condition, the ratio of 80 S monosome:small polysome:large polysome was determined to be: control preparation, 30:10:10; thapsigargin, 67:3:0; vasopressin, 45:11:5; PMA, 24:10:10; PMA + vasopressin, 35:12:12. By this analysis, vasopressin caused 80 S monosomes to increase and polysomes to decrease, typical of a slowing of initiation. Large polysomes were reduced preferentially. By contrast, PMA, which stimulates amino acid incorporation, caused polysomes to accumulate at the expense of 80 S monosomes, consistent with increased rates of initiation. Effects of vasopressin were largely overturned by PMA, such that ribosomal profiles under this condition were similar to those of controls. Thapsigargin produced almost complete initiation blockade.

Suppression of translational initiation in non-muscle cells in response to drugs that deplete ER Ca2+ stores is attributable to the phosphorylation of the α-subunit of eIF2 by double-stranded RNA-activated protein kinase (1–4). The phosphorylated form of eIF2α is known to inhibit the GTP/GDP exchange factor, eIF2B, thereby preventing eIF2 recycling (19). Because eIF2B is usually present at much lower concentrations than eIF2, phosphorylations of 20–30% of the eIF-2α pool cause
substantial inhibitions of eIF2B activity in most cell types (5, 6, 13, 31, 32). Addition of Ca\(^{2+}\)-mobilizing drugs or vasopressin to H9c2 cells resulted in phosphorylation of eIF2\(\alpha\) corresponding closely with the degree of inhibition of leucine incorporation (Fig. 6). In untreated preparations, 94–100% of eIF2\(\alpha\) was present in the non-phosphorylated form (A, lanes 1 and 5; B, lanes 1 and 8; C, lanes 1 and 6). The strongest phosphorylations of eIF2\(\alpha\) and concomitant suppressions of leucine incorporation occurred in response to 1 \(\mu\)M thapsigargin (A, lane 2). Caffeine at 5 \(\mu\)M (A, lane 3) and vasopressin at 10 \(\mu\)M (A, lane 4) were each less effective than thapsigargin in promoting eIF2\(\alpha\) phosphorylation and inhibiting leucine incorporation. Increasing degrees of eIF2\(\alpha\) phosphorylation and accompanying inhibitions of protein synthesis were observed in response to increasing concentrations of ionomycin (B, lanes 2–6). Within the same cell sampling, eIF2\(\alpha\) was phosphorylated and protein synthesis was inhibited to comparable extents by 30 \(\mu\)M ionomycin (B, lane 4) and by 10 \(\mu\)M vasopressin (B, lane 7). Vasopressin produced eIF2\(\alpha\) phosphorylation and inhibition of leucine incorporation at 15 and 30 min (C, lanes 2 and 3) that dissipated by 120 min (C, lane 4) of treatment. Brief exposure to PMA abolished eIF2\(\alpha\) phosphorylation and translational suppression in response to 30 min of treatment with vasopressin (C, lane 5 as compared with lane 3).

Translational Recovery from Inhibition by Vasopressin—Neither the acute inhibition of protein synthesis by hormone nor recovery from this inhibition was altered by actinomycin D at concentrations inhibitory to gene transcription, by rapamycin at concentrations that inhibit the ribosomal p70 S6 kinase (33), or by cAMP-elevating agents and analogs at concentrations that inhibit signal transduction dependent on Ras (34, 35) (data not shown). Earlier findings (see Table II and Figs. 3 and 4) were consistent with roles for protein kinase C activation and Ca\(^{2+}\) in overturning the inhibition of leucine incorporation by vasopressin. The degree to which H9c2 cells replenish their Ca\(^{2+}\) stores during recovery from translational inhibition by vasopressin was therefore examined. Leucine incorporation and cell-associated Ca\(^{2+}\) were measured after varying times of treatment with vasopressin (Fig. 7). Inhibition of incorporation paralleled the decline in cell-associated Ca\(^{2+}\) during the first 30 min of hormonal treatment. At 20–30 min, 60% of Ca\(^{2+}\) stores were mobilized and protein synthesis was inhibited 50%. Protein synthesis steadily recovered from inhibition thereafter, with full recovery being observed by 100 min. At 2 h of treatment, pulse incorporation rates were 20% higher than those in untreated controls. During the recovery period (30–100 min) cell-associated Ca\(^{2+}\) also increased but at slower rates than observed for leucine incorporation. Full recovery of pulse incorporation was associated with the restoration of approximately one-fourth of the cation initially mobilized.

A variety of cell types respond to protracted ER Ca\(^{2+}\) mobilization with an ER stress response that includes the induction of GRP78 and the development of translational tolerance to Ca\(^{2+}\)-mobilizing agents. H9c2 cells that had recovered from translational suppression by vasopressin over 2 h and corresponding untreated controls were examined for the development of such tolerance upon challenge with various concentrations of ionomycin, with thapsigargin at 1 \(\mu\)M, or with vasopressin at 10 \(\mu\)M (Table III). Comparable rates of leucine incorporation were observed for the hormonally pretreated and non-treated cells before challenge. Leucine incorporation in both preparations was reduced in a dose-dependent manner in response to increasing concentrations of ionomycin and was inhibited extensively in response to thapsigargin. A brief challenge with vasopressin, however, suppressed leucine incorporation in the non-treated controls by 68% but had no effect on...
incorporation in the vasopressin-pretreated preparations. Cell-associated Ca\(^{2+}\) was also determined for non-treated and vasopressin-pretreated preparations before and after challenge with Ca\(^{2+}\)-depleting drugs (Table III). The Ca\(^{2+}\) contents of vasopressin-pretreated cells were 45% of those of non-treated controls. Ionomycin mobilized Ca\(^{2+}\) in a dose-dependent fashion from both the non-treated and the vasopressin-pretreated preparations. The Ca\(^{2+}\) contents of both preparations were reduced to comparably low values by 150 nM ionomycin and by 1 \(\mu M\) thapsigargin. After brief challenge with vasopressin, 65% of cell-associated Ca\(^{2+}\) was mobilized from the non-treated controls but no Ca\(^{2+}\) was released from the hormone-pretreated preparations.

To verify that H9c2 cells are capable of expressing the ER stress response, myocytes were incubated for 8 h at varying concentrations of ionomycin or with 0.5 \(\mu M\) thapsigargin. Preparations were washed to remove ionophore, and proteins were pulse-labeled with \(^{35}S\)methionine and sampled for SDS-PAGE (7.5%) and autoradiography (Fig. 8). Preferential labeling of GRP78 and GRP94 was observed in response to treatments with 10–100 nM ionophore, with optimal labeling occurring at 30 nM (lanes c–f as compared with lane a). A modest increase in labeling of other proteins was also apparent in the treated preparations. Incubation with 5 \(\mu M\) ionomycin did not promote increased expression of the GRPs or stimulation of overall protein synthesis (lane b). The GRPs were also labeled preferentially in thapsigargin-pretreated preparations (lane g). Thapsigargin was not removed by the washing procedure, however, as evidenced by the decrease in overall protein labeling. Additional samples were pretreated for 2 h in the absence and presence of vasopressin before incubations in the absence or presence of ionomycin (30 nM). Preparations were washed and proteins were pulse-labeled and analyzed by SDS-PAGE and autoradiography as above (Fig. 8). Although labeling of most cellular proteins was modestly increased in the vasopressin-treated sample, expression of the GRPs was not selectively increased by the hormone (lane k as compared with lane i). No selective labeling of the GRPs was observed during shorter incubations with vasopressin (data not shown). However, pretreatment with vasopressin followed by incubation with ionomycin resulted in greater pulse-labeling of GRP78 and GRP94 than was observed in samples incubated with ionophore alone (lane l as compared with lane j).

To ascertain whether translational tolerance to inhibition by vasopressin accompanies induction of the GRPs, H9c2 cells were pretreated for 8 h in the absence or presence of ionomycin or thapsigargin (irreversible). Preparations were washed and then challenged with ionomycin at increasing concentrations, with thapsigargin, or with vasopressin. After 30 min of treatments, samples were analyzed by measurement of leucine pulse incorporation (Table IV). Incorporation in the control preparations was inhibited in the typical fashion by ionophore, thapsigargin, and vasopressin. By contrast, protein synthesis in ionomycin-pretreated preparations was markedly resistant to suppression by these agents. No inhibition by ionophore was observed except at the highest (150 nM) concentration tested, and 10 \(\mu M\) vasopressin was ineffective. Although thapsigargin suppressed incorporation in ionomycin-pretreated preparations, the extent of this suppression was not as great as in controls. Samples pretreated with thapsigargin had somewhat lower synthetic rates as compared with untreated controls, but protein synthesis was completely unaffected after challenge with ionomycin at all concentration tested, with thapsigargin, or with vasopressin.

Reversibility of Translational Inhibition by Vasopressin—Physiologic responses to vasopressin are known to persist despite receptor internalization, which, depending on cell type, at 37 °C occurs within 3–20 min of receptor occupation (36–38). It was nonetheless unclear whether the translational inhibition imposed by vasopressin in H9c2 cells was reversible or persisted upon removal of the hormone. Cells were therefore treated with vasopressin for 0, 2, 5, 10, 15, or 20 min, and the preparations were washed three times with medium lacking hormone. Washed preparations were then incubated for 30 min in fresh medium lacking or containing vasopressin, followed by measurements of leucine pulse incorporation (Table V). Inhibitions of protein synthesis attributable to vasopressin ranged from 55% at 2 min to 69% at 20 min of treatment. These inhibitions were reversible by removal of hormone at 2 and 5 min of treatment, but thereafter were irreversible.
and molecular weight markers is indicated on radiography. The migration position of analyzed by SDS-PAGE (7.5%) and autoradiography. The migration position of molecular weight markers is indicated on the ordinate in kDa.

### TABLE IV

| Tolerance to translational inhibition by vasopressin and other Ca\(^{2+}\)-mobilizing agents after expression of the GRPs |
|---------------------------------------------------------------|
| H9c2 cells were incubated for 8 h in serum-free F-10 medium in the absence of drug or with 100 nM ionomycin or 1 \(\mu\)M thapsigargin. Cells were washed twice with medium lacking drugs and containing 2 mg/ml fatty acid free bovine serum albumin. Proteins were then pulse-labeled by incubation for 30 min in medium containing 5 \(\mu\)M methionine and 20 \(\mu\)Ci/ml \(^{35}\)S) methionine, and lysates were analyzed by SDS-PAGE (7.5%) and autoradiography. The migration position of molecular weight markers is indicated on the ordinate in kDa. |

| Agent | Leucine incorporation |
|-------|-----------------------|
|       | Control | Pretreated with ionomycin | Pretreated with thapsigargin |
|       | nmol/mg protein | nmol/mg protein | nmol/mg protein |
| Control | 2.88 ± 0.15 | 3.85 ± 0.05 | 1.54 ± 0.12 |
| Ionomycin (5 nM) | 2.03 ± 0.07 | 3.97 ± 0.09 | 1.40 ± 0.05 |
| Ionomycin (15 nM) | 1.97 ± 0.11 | 3.80 ± 0.06 | 1.57 ± 0.10 |
| Ionomycin (25 nM) | 1.77 ± 0.07 | 3.83 ± 0.00 | 1.50 ± 0.00 |
| Ionomycin (50 nM) | 1.18 ± 0.13 | 3.78 ± 0.09 | 1.50 ± 0.06 |
| Ionomycin (150 nM) | 0.62 ± 0.05 | 3.52 ± 0.12 | 1.48 ± 0.09 |
| Thapsigargin (1 \(\mu\)M) | 0.31 ± 0.06 | 0.83 ± 0.02 | 1.50 ± 0.09 |
| Vasopressin (10 \(\mu\)M) | 1.39 ± 0.05 | 3.75 ± 0.06 | 1.60 ± 0.02 |

### TABLE V

| Reversibility of translational inhibition by vasopressin |
|----------------------------------------------------------|
| H9c2 cells were treated with vasopressin (100 nM) for the indicated times. Medium was then removed, and the cells were washed three times with medium lacking vasopressin. Medium lacking or containing hormone was then added as indicated. After 30 min, medium was again replaced with fresh medium lacking or containing vasopressin, and pulse incorporation of \(^{35}\)H leucine into proteins was determined. |

| Initial incubation with vasopressin | Leucine incorporation |
|-----------------------------------|-----------------------|
| min                               | nmol/mg protein       |
| 0                                 | 4.88 ± 0.21           |
| 2                                 | 4.65 ± 0.17           |
| 5                                 | 4.51 ± 0.15           |
| 10                                | 2.78 ± 0.07           |
| 15                                | 2.38 ± 0.11           |
| 20                                | 2.14 ± 0.04           |

### DISCUSSION

Although Ca\(^{2+}\) sequestration by the ER has been previously established to maintain optimal rates of translational initiation in mammalian cells, the drugs utilized to mobilize the cation invariably led to the development of an ER response. This report provides the first compelling evidence that hormonally induced mobilization of S(E)R Ca\(^{2+}\) stores is regulatory upon translation in that it is receptor-mediated, observable at physiological extracellular Ca\(^{2+}\) concentrations, and reversible without the generation of a stress response. Translational inhibitions coinciding with mobilization of S(E)R Ca\(^{2+}\) stores of H9c2 ventricular myocytes were imposed by arginine vasopressin at concentrations that raise [Ca\(^{2+}\)], in normal cardiomyocytes (39, 40). Inhibitions were exerted as a consequence of a hormonal interaction at the V1 vascular receptor (39, 40), the receptor expressed in normal cardiac tissue (27). Vasopressin exerted a persistent, albeit decreasing, inhibition of translation for periods of 1–2 h. This result was not surprising in that vasopressin receptors are known to undergo internalization within 3–20 min in various tissues after hormonal binding. The physiologic effects of the hormone persist after internalization (27). The ability of the hormone to signal translational inhibition in H9c2 cells was reversible by washing for only several minutes. Circulating vasopressin has a relatively long half-life (approximates 20 min) and undergoes degradation in the liver and kidney but not in the pulmonary circulation (41).

Inhibition of the translational process in H9c2 cells by treatment with vasopressin involved imposition of the same mechanisms as those exerted by ionomycin, which destroys intracellular Ca\(^{2+}\) gradients, and thapsigargin, which blocks the Ca\(^{2+}\)-ATPase of the S(E)R. Vasopressin, like ionomycin and thapsigargin (1–7, 17), slowed translation at the initiation step and caused eIF2\(\alpha\) to become phosphorylated. Phosphorylation of this initiation factor correlated closely with hormonal inhibitions of protein synthesis under all experimental conditions tested. As has been observed invariably with agents that inhibit translational initiation by mobilizing ER Ca\(^{2+}\) stores (1, 4, 5, 7, 8, 17), protein synthesis in cells expressing increased concentrations of ER chaperones was tolerant to inhibition by vasopressin. The mechanism through which vasopressin caused Ca\(^{2+}\) stores of H9c2 cells to be mobilized was not explored. Unlike depletion of Ca\(^{2+}\) stores by thapsigargin or ionophores, Ca\(^{2+}\) depletion in response to vasopressin began to attenuate after 30 min. Attenuation presumably involved either decreased production of, enhanced degradation of, or declining responsiveness to intracellular messenger(s) that mobilize Ca\(^{2+}\) from the S(E)R to the cytosol. Vasopressin acting at V1 vascular receptors of various tissues is established to signal the activation of phospholipase C and the generation of inositol trisphosphate during the first few min of treatment (27). Inositol trisphosphate is widely documented as an intracellular mediator of hormone-dependent Ca\(^{2+}\) mobilization, although cyclic ADP-ribose has also been advanced as a putative physiologic regulator of ryanodine-sensitive Ca\(^{2+}\)-dependent release.
processes in intact mammalian systems, including heart (20, 42, 43). Mediation of the Ca\(^{2+}\)-mobilizing effects of vasopressin by cyclic ADP-ribose in H9c2 cells would be consistent with the actions of caffeine, a pharmacologic activator of the cardiac ryanodine receptor (20), in promoting Ca\(^{2+}\) release, translational suppression, and eIF2\(\alpha\) phosphorylation. Caffeine affects these parameters in a quantitatively similar manner to vasopressin. Eicosanoids have also been proposed to mediate the Ca\(^{2+}\)-mobilizing actions of vasopressin in smooth muscle cells (27).

As in various non-muscle cell types (1, 4, 5, 7, 8, 17), recovery of H9c2 cells from translational inhibition by Ca\(^{2+}\)-mobilizing drugs was contingent upon induction of expression of ER chaperones. In contrast, recovery from translational inhibition by vasopressin depended on partial re-accumulation of Ca\(^{2+}\) and, most probably, the activation of protein kinase C. Indirect evidence, including abolition of vasopressin inhibition in the presence of a phorbol ester and prolonged inhibition of translation under conditions wherein the enzyme is known to be down-regulated, supports a role for protein kinase C in this event. Both calphostin C and chelerythrine at concentrations used widely to investigate the involvement of protein kinase C in various processes, produced the mobilization of Ca\(^{2+}\) and the phosphorylation of eIF2\(\alpha\). It was not possible to determine whether the effects of these inhibitors derived from inhibition of protein kinase C activity or from actions at other sites. Any action through protein kinase C would imply that the enzyme is partially coupled to translational rates in H9c2 cells in the absence of hormonal influences. No other requirements for recovery of translational activity could be identified. Other protein kinases that are signaled in response to vasopressin, such as ribosomal S6 kinases (44) and mitogen-activated protein kinase (45, 46), could not be implicated. Although activation of V1 vascular receptors is known to signal increased transcription of the immediate early genes (27), transcriptional events were not required for translational recovery.

Recovery from translational inhibition by vasopressin was associated with partial, rather than full, restoration of cell-associated Ca\(^{2+}\), presumably at the S/E/R. Information is lacking regarding whether H9c2 or other cell types possess critical ER or S/E/R “pools” or subcompartments of Ca\(^{2+}\) supporting translation. Translational inhibition in GH\(_3\) and HepG2 cells occurs at somewhat greater degrees of ER Ca\(^{2+}\) depletion than are required to impede protein processing within the organelle (1, 7, 26). Inhibition of protein processing invariably appears to trigger the phosphorylation of eIF2\(\alpha\) in response to Ca\(^{2+}\) depletion. Ca\(^{2+}\) re-accumulation in H9c2 cells after 2 h of vasopressin treatment, therefore, may restore ER function sufficiently that protein synthesis can resume. PMA, presumably by activating protein kinase C, reduced the degree to which vasopressin lowered cell-associated Ca\(^{2+}\).

Although ionomycin (30 nM) and vasopressin (10 \(\mu\)M) generated comparable degrees of cation mobilization and eIF2\(\alpha\) phosphorylation over 30 min, only ionomycin produced a subsequent ER stress response. The ionophore at 30 nM clearly signaled comparable degrees of GRP78 induction and translational suppression in H9c2 cells to those observed in other cell types (1, 4, 5, 7–10, 17). Continued exposures to either ionomycin or thapsigargin elicited strong inductions of GRP78 and GRP94 and development of translational tolerance to ER stressors in H9c2 cells without additional requirements for auxiliary promoters (serum, PMA, growth factors, cAMP analogs) observed in GH\(_3\), NIH-3T3, and myeloma cells (1, 4, 5, 7, 17). Vasopressin treatment of H9c2 cells differs from other Ca\(^{2+}\)-mobilizing drugs by permitting partial recovery of cell-associated Ca\(^{2+}\) after 30 min. This recovery, which is accompanied by increasing rates of protein synthesis, may sufficiently restore S/E/R function as to ablate the development of an ER stress response.

Vasopressin exerts inotropic actions on cardiovascular performance that was until recently, attributed to hormonal effects at the vasculature and kidney. The hormone is now appreciated to stimulate lipid metabolism in perfused hearts (47) and H9c2 cells (24), to increase [Ca\(^{2+}\)]\(_{\text{cyt}}\) (1), in cardiomyocytes (39), to potentiate ventricular \(i\)-type currents via V1 vascular receptor stimulation (40), and to cause atrial natriuretic factor to be secreted from cardiomyocytes (48). The translational suppression observed in H9c2 cells in response to vasopressin provides additional evidence that the heart is directly targeted by this hormone. The functional significance of the short-term suppression of ventricular protein synthesis accompanying mobilization of cell-associated Ca\(^{2+}\) by the hormone remains to be clarified. Both muscle contraction, which is supported by [Ca\(^{2+}\)]\(_{\text{cyt}}\), and mRNA translation, which is supported by S/E/R sequestered Ca\(^{2+}\), are energy-intensive processes. Reduced translation accompanying release of S/E/R Ca\(^{2+}\) to the cytosol may therefore function to divert ATP production toward supporting increased contractile activity. In addition to its anti-diuretic actions, vasopressin serves broadly as a growth factor and, depending on cell type, can promote either hypertrophy or hyperplasia (27). Induction of specific gene expression and enhanced rates of protein synthesis are both required for growth responses to vasopressin. In support of a growth-promoting effect of vasopressin on H9c2 cells, prolonged exposure to the hormone was invariably accompanied by increased rates of amino acid incorporation into protein. It is anticipated that H9c2 cells should provide a highly useful model system for investigating both the acute and the chronic effects of vasopressin on the biochemistry and physiology of ventricular myocytes.

REFERENCES

1. Brostrom, C. O., and Brostrom, M. A. (1998) Prog. Nucleic Acids Res. Mol. Biol. 58, 79–125

2. Prostko, C. R., Dholakia, J. N., Brostrom, M. A., and Brostrom, C. O. (1995) J. Biol. Chem. 270, 6211–6215

3. Srivastava, S. P., Davies, M. V., and Kaufman, R. J. (1995) J. Biol. Chem. 270, 16619–16624

4. Brostrom, C. O., Prostko, C. R., Kaufman, R. J., and Brostrom, M. A. (1996) J. Biol. Chem. 271, 24965–25002

5. Prostko, C. R., Brostrom, M. A., Malaria, E. M., and Brostrom, C. O. (1992) J. Biol. Chem. 267, 16751–16754

6. Kimball, S. R., and Jefferson, L. S. (1992) Am. J. Physiol. 263, E958–E964

7. Brostrom, M. A., Prostko, C. R., Gmitler, D., and Brostrom, C. O. (1995) J. Biol. Chem. 270, 4127–4132

8. Morris, J. A., Dorner, A. J., Edwards, C. A., Hendershot, L. M., and Kaufman, R. J. (1997) J. Biol. Chem. 272, 4327–4334

9. Lee, A. S. (1992) Curr. Opin. Cell Biol. 4, 267–273

10. Little, E., Ramakrishnan, M., Roy, B., Gazi, G., and Lee, A. S. (1994) Crit. Rev. Eukaryotic Gene Exp. 4, 1–18

11. Brostrom, C. O., Boecskio, S. B., Brostrom, M. A., and Galuska, E. M. (1986) Mol. Pharmacol. 29, 104–111

12. Menaya, J., Parrilla, R., and Ayuso, M. S. (1988) Biochem. J. 254, 773–779

13. Kimball, S. R., and Jefferson, L. S. (1990) J. Biol. Chem. 265, 16794–16798

14. Chin, K.-V., Cade, C., Brostrom, M. A., and Brostrom, C. O. (1988) Int. J. Biochem. 20, 1313–1319

15. Schopf, G., Rumpfeld, H., and Muller, M. M. (1986) Biochim. Biophys. Acta 884, 319–325

16. Brostrom, C. O., Boecskio, S. B., and Brostrom, M. A. (1983) J. Biol. Chem. 258, 14390–14399

17. Wong, W. L., Brostrom, M. A., Kurnetsov, G., Gmitler-Yellen, D., and Brostrom, C. O. (1993) Biochem. J. 289, 71–79

18. Chin, K.-V., Cade, C., Brostrom, M. A., and Brostrom, C. O. (1987) J. Biol. Chem. 262, 16509–16514

19. Scorsone, K. A., Panniers, R., Rowlands, A. G., and Henshaw, E. C. (1987) J. Biol. Chem. 262, 14538–14543

20. Bakovev, S., Galime, A., Ashama, G., Potter, B. V., and Terrar, D. A. (1996) Biochim. Biophys. Acta 898, 989–996

21. Kanuro, I., and Yazaki, Y. (1993) Annu. Rev. Physiol. 55, 55–75

22. Decker, R. S., Cook, M. G., Behnke-Barclay, M., and Decker, M. L. (1995) Circ. Res. 77, 544–555

23. Bogyo, M. A., and Sugden, P. H. (1996) Int. J. Biochem. Cell. 28, 1–12

24. Tran, K., Zha, X., Chan, M., and Choy, P. C. (1995) Mol. Cell. Biochem. 151, 69–75

25. Tran, K., Man, R. Y., and Choy, P. C. (1996) Biochim. Biophys. Acta 1259, 283–290
26. Kuznetsov, G., Brostrom, M. A., and Brostrom, C. O. (1992) J. Biol. Chem. 267, 3932–3939
27. Thibonnier, M. (1992) Regul. Pept. 38, 1–11
28. Johnson, J. A., Adak, S., and Mochly-Rosen, D. (1995) Life Sci. 57, 1027–1038
29. Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989) Biochem. Biophys. Res. Commun. 159, 548–553
30. Jarvis, W. D., Turner, A. J., Povirk, L. F., Taylor, R. S., and Grant, S. (1994) Cancer Res. 54, 1707–1714
31. Safer, B. (1983) Cell 33, 7–8
32. Rowlands, A. G., Panniers, R., and Henshaw, E. C. (1988) J. Biol. Chem. 263, 5526–5533
33. Price, D. J., Grove, J. R., Calvo, V., Arruuch, J., and Bierer, B. E. (1992) Science 257, 973–977
34. Cook, S. J., and McCormick, F. (1993) Science 262, 1069–1072
35. Burgering, B. M. Th., Pronk, G. J., van Weeren, P. C., Chardin, P., and Bos, J. L. (1993) EMBO J. 12, 4211–4220
36. Fishman, J. B., Dickey, B. F., Bucher, N. L. R., and Fine, R. E. (1985) J. Biol. Chem. 260, 12641–12646
37. Lutz, W., Salisbury, J. L., and Kumar, R. (1991) Am. J. Physiol. 261, F1–F13
38. Briner, V. A., Williams, B., Tsai, P., and Schrier, R. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2854–2858
39. Za, Y. J., and Gopalakrishnan, V. (1991) Circ. Res. 69, 239–245
40. Zhang, S., Hirano, Y., and Hirai, S. (1995) Circ. Res. 76, 592–599
41. Culpepper, R. M., Hebert, S. C., and Andreoli, T. E. (1985) in Textbook of Endocrinology (Wilson, J. D., and Foster, D. W., eds) p. 619, W. B. Saunders, Philadelphia
42. Meszaros, L. G., Bak, J., and Chu, A. (1993) Nature 364, 76–79
43. Lee, H. C., Gallione, A., and Walseth, T. F. (1994) Vitam. Horm. 48, 199–257
44. Granot, Y., Erikson, E., Fridman, H., Van Putten, V., Williams, B., Schrier, R. W., and Maller, J. L. (1993) J. Biol. Chem. 268, 9564–9569
45. Nishioka, N., Hirai, S., Miruno, K., Osada, S., Suzuki, A., Kosaka, K., and Ohno, S. (1995) FEBS Lett. 377, 393–398
46. Aharonovitz, O., and Granot, Y. (1996) J. Biol. Chem. 271, 16494–16499
47. Palazzo, A. J., Malik, K. U., and Weis, M. T. (1991) Am. J. Physiol. 260, H604–H612
48. Van der Bent, V., Church, D. J., Vallotton, M. B., Meda, P., Kem, D. C., Capponi, A. M., and Lang, U. (1994) Am. J. Physiol. 266, H597–H605
Regulation of Protein Synthesis in Ventricular Myocytes by Vasopressin: THE ROLE OF SARCOPLASMIC/ENDOPLASMIC RETICULUM Ca2+ STORES
Barbara A. Reilly, Margaret A. Brostrom and Charles O. Brostrom

J. Biol. Chem. 1998, 273:3747-3755.
doi: 10.1074/jbc.273.6.3747

Access the most updated version of this article at http://www.jbc.org/content/273/6/3747

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 25 of which can be accessed free at http://www.jbc.org/content/273/6/3747.full.html#ref-list-1