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Regulation of Protein Synthesis in Isolated Hepatocytes by Calcium-Mobilizing Hormones

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SUMMARY

The incorporation of leucine into protein was studied in Ca^{2+}-depleted and Ca^{2+}-restored preparations of normal liver cells isolated from fed, adult male rats. Ca^{2+}-restored cells incorporated amino acid 5–10-fold more rapidly than did Ca^{2+}-depleted cells for incubation periods up to 1 hr. Readdition of Ca^{2+} at supraphysiologic concentrations (3 mM) to depleted cells restored the rate of incorporation within 8–10 min, whereas lesser concentrations of the cation acted more slowly. Vasopressin and α-adrenergic agonists rapidly (in minutes) inhibited amino acid incorporation to variable degrees in liver cells, with pronounced inhibitions (40–75%) occurring at moderate (0.1–1 mM) extracellular Ca^{2+} concentrations and smaller inhibitions (10–30%) occurring at supraphysiologic concentrations of the cation. Hormonally produced inhibitions were more intense at acid pH than at alkaline pH. The effects of epinephrine were mediated through α_1-adrenergic receptors and were not additive with those of vasopressin at saturating concentrations. It is proposed that these hormones, which are known to mobilize sequestered Ca^{2+} within liver cells, inhibit amino acid incorporation by influencing a Ca^{2+} requirement associated with protein synthesis.

Calcium ion, through its interactions with various intracellular receptor proteins such as calmodulin, is thought to function as a major regulator and coordinator of processes maintaining cellular functionality and responsiveness to external stimuli. Numerous humoral substances have been established to exert their actions by influencing intracellular free Ca^{2+} concentrations by mobilizing internally sequestered Ca^{2+} and/or by stimulating plasmalemmal transport of the cation. In perfused rat liver and isolated hepatocytes, for example, epinephrine and other α-adrenergic agonists, vasopressin, and angiotensin have been established to mobilize Ca^{2+} from intracellular sites (see Ref. 1 for review). The resultant increase in cytosolic free Ca^{2+} is thought to stimulate glycogenolysis via an allosteric activation of phosphorylase *b* kinase and to promote a rapid efflux of Ca^{2+} to the extracellular fluid. Isolated hepatocytes have been found to undergo a depletion of cellular Ca^{2+} of approximately 30% within 5 min of exposure to α-adrenergic agonists (2, 3).

Recently, Brostrom *et al.* (4) have reported that protein synthesis is sharply depressed in a variety of eukaryotic cell types exposed to conditions depleting Ca^{2+} but not Mg^{2+}. Protein synthesis was returned to maximal rates by restoration of Ca^{2+} to the cells. Although a Ca^{2+} requirement was shown for protein synthesis, it was not established whether the cation could serve as a physiologic regulator of the process. Several hormones, including cholecystokinin in isolated pancreatic acini (5) and corticotropin in adrenal cortex (6), have been reported in stimulate protein synthesis in a Ca^{2+}-requiring manner. Thyrotropin-releasing hormone and phorbol myristate acetate recently have been found to increase both prolactin synthesis and overall protein synthesis to a significantly greater extent in Ca^{2+}-restored than in Ca^{2+}-depleted GH_3 pituitary cells (7). In contrast, epinephrine was reported by Mandl *et al.* (8) to inhibit protein synthesis in isolated mouse hepatocytes through an α-adrenergic receptor-mediated action. This inhibition was not observed in hepatocytes depleted of Ca^{2+} by EGTA. Protein synthesis in rat submandibular cells has been reported by Takuma *et al.* (9) to be inhibited by α_1-adrenergic agonists in a manner thought to involve the manipulation of internal Ca^{2+} pools. The present report confirms and extends these observations in a study of protein synthesis in rat hepatocytes. Angiotensin, vasopressin, and α-adrenergic agonists, at concentrations established to mobilize hepatocyte Ca^{2+} stores, inhibited protein synthesis, with high extracellular Ca^{2+} concentrations reversing the inhibition. It is proposed that hormonally induced changes in intracellular Ca^{2+} homeostasis provide a mechanism for regulating the rate of protein synthesis.

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ABBREVIATIONS: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Experimental Procedures

Materials. TES, angiotensin II, vasopressin (grade 1–5), isoproterenol, dopamine, norepinephrine, vasoactive intestinal polypeptide, angiotensin II, histamine, glucagon, somatostatin, insulin (bovine), epinephrine, and yohimbine were purchased from Sigma Chemical Co. Propranolol from Ayerst, prazosin from Pfizer, and phenylephrine from Sterling-Winthrop were gifts. Collagenase (type CLSII) was purchased from Worthington. L-[3,4,5-^{3}H]Leucine (58 Ci/mmol) was purchased from International Chemical and Nuclear Corp. L-[^{35}S]Methionine was purchased from New England Nuclear.

Preparation of rat liver cells. Collagenase-dispersed hepatocytes were isolated by a modification of the method of Seglen (10). Male Sprague-Dawley rats (350 g) were anesthetized with pentobarbital intraperitoneally. Livers were perfused and collagenase treated as described by Seglen (10), except that the collagenase-containing buffer was adjusted to 1 mM Ca^{2+} instead of 5 mM. Following a 30-min collagenase digestion at 37°, the liver was dispersed by shaking into 180 ml of cold 138 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM EGTA, 25 mM TES, pH 7.6 (measured at 20°). The cell suspension was filtered through nylon mesh of 250 and 105 μm and centrifuged at 100 × *g* for 5 min. The supernatant fluid was discarded, and the cells were suspended in 80 ml (total volume) of a modified Ham's F-10 medium with the following composition (mM): KCl, 3,8; KH_2PO_4, 1.6; $MgSO_4$, 0.6; NaCl, 106; $NaHCO_3$, 4.8; Na_2HPO_4, 1; glucose, 25; hypoxanthine, 0.03; sodium pyruvate, 5; thymidine, 0.003; Ala, 0.1; Arg, 1; Asn, 0.11; Asp, 0.1; Cys, 0.2; Gln, 1; Glu, 0.1; Gly, 0.1; His, 0.1; Ile, 0.02; Lys, 0.16; Met, 0.01; Phe, 0.03; Pro, 0.1; Ser, 0.1; Thr, 0.03; Trp, 0.003; Tyr, 0.01; Val, 0.03; TES, 20; EGTA, 1; Eagle's basal medium vitamins (Gibco); 0.05 mg/ml of streptomycin sulfate, 0.03 mg/ml of penicillin G; 0.5 mg/ml of bovine serum albumin (Sigma), pH 7.6 at 37°.

The suspension was filtered through 62-μm nylon mesh. Cell counts and viabilities were determined by trypan blue exclusion, and aliquots were diluted in saline, centrifuged, and resuspended in 1 N NaOH for determination of protein by the method of Lowry *et al.* (11).

Determination of amino acid incorporation. Hepatocyte suspensions were diluted 1:10 with the modified Ham's F-10 supplemented with 10 μM leucine and 1.6 mM $MgCl_2$ unless otherwise indicated. Incubations were normally conducted at 37° for 30 min under 95% oxygen. Incubations contained 0.5 ml of cells, 0.02 ml of [^{3}H]leucine (0.002 mCi/tube), and 0.03 ml of additives in a total volume of 0.55 ml. Cell proteins were 0.5–1 mg/tube. The determination of the incorporation of radioactive amino acid into trichloroacetic acid-precipitable, alkali-stable [^{3}H]leucine was conducted as described previously (4), except that three washing cycles were performed and the samples were dissolved for 5 min in hot formic acid before the addition of scintillation fluid. Since standard errors for the assay were normally within 5% and seldom exceeded 10%, incubations were conducted for most assays in duplicate and the values were averaged. Standard errors from incubations with four replicates are, however, included in various tables where such information was considered helpful. The major findings illustrated in each table or figure were reproduced at least twice prior to conducting the final design illustrated for publication.

Determination of amino acid uptake. Suspensions of cells (10 ml) which had been preequilibrated at 37° at pH 7.6 for 30 min as described above, with or without Ca^{2+} in excess of EGTA, were adjusted with 10 μM [^{3}H]leucine (2 × 10^6 cpm/ml). At various times of incubation, aliquots (1 ml) were withdrawn in triplicate into tubes (16 × 100 mm), immediately diluted with 10 volumes of ice-cold buffer containing 1 mM leucine, and centrifuged at 2000 × *g* for 30 sec. The supernatant fluid was removed by aspiration and the pellet of cells was precipitated with 1 ml of cold 10% trichloroacetic acid and 1 mM leucine. The protein precipitate was pelleted by centrifugation at 2000 × *g* for 5 min. Aliquots (0.5 ml) of the supernatant fluid were analyzed for trichloroacetic acid-soluble radioactivity. The contribution of extracellular-associated radioactivity to these values was negligible as determined from similarly treated controls exposed to radioactivity on ice. Values are presented as the averages of triplicate samplings with corresponding standard errors.

Gel electrophoresis and fluorography. Studies involving electrophoresis and fluorography were conducted using detergent-solubilized extracts of washed hepatocytes incubated with [^{35}S]methionine in place of [^{3}H]leucine. Single-dimensional electrophoretic studies were conducted in 10% acrylamide gels as described by Laemmli (12). Two-dimensional gel electrophoresis of hepatocyte proteins labeled with [^{35}S]methionine was conducted by the method of O'Farrell (13) as modified by Garrels (14), using 4 parts of ampholine of pH range 5–7 and 1 part of pH range 3–10 ampholine for the first dimension. Molecular mass markers were phosphorylase *b*, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor, and lactalbumin derivatized with 4-dimethylaminoazobenzene-4′-sulfonyl chloride as described by Tzeng (15). Fluorography was performed as described by Skinner and Griswold (16).

Miscellaneous procedures. Nucleotide determinations were conducted for K_2CO_3-neutralized perchloric acid cell extracts with a Beckman series 344 high pressure liquid chromatography system equipped with a model C-RIA integrator recorder and a 25-cm Ultrasil AX column. The column was developed with a linear gradient of H_2KPO_4 ranging from 0.005 to 0.6 M. Calcium and magnesium solutions were standardized by atomic absorption spectrophotometry. Nucleotide and bovine serum albumin solutions were standardized by UV spectrophotometry.

Results

Effect of Ca^{2+} depletion and restoration on protein synthesis by isolated hepatocytes. Previous work (4) had confirmed the 1969 observation by Burton *et al.* (17) that Ca^{2+} addition to isolated hepatocytes in low Ca^{2+} medium stimulated amino acid incorporation into protein 5–6-fold. This effect was investigated in more detail using hepatocytes prepared by collagenase digestion of perfused livers from adult male rats and subsequently pretreated in a modified Ham's F-10 medium supplemented with 1 mg/ml of bovine serum albumin, 1 mM EGTA, and various concentrations of added Ca^{2+} for 15–30 min at 37°. Typically, cells pretreated without Ca^{2+} incorporated [^{3}H]leucine into trichloroacetic acid-precipitable material at approximately 10–15% of the rate of cells restored at 1 mM Ca^{2+} at a variety of unlabeled leucine concentrations (Table 1). Incorporation of amino acid by either Ca^{2+}-depleted cells or cells pretreated at 0.3 mM Ca^{2+} proceeded at linear rates for 60 min (Fig. 1). The addition of Ca^{2+} to Ca^{2+}-depleted cells simultaneously with [^{3}H]leucine provided an increasing rate of incorporation commencing at approximately 15 min of incubation

TABLE 1

Effect of unlabeled leucine on the incorporation of [^{3}H]leucine by isolated liver cells

Cells were equilibrated for 30 min at 1 mM EGTA or with 1 mM EGTA and 3 mM $CaCl_2$ in modified Ham's F10 medium at pH 7.4 under O_2 in 15 × 48-mm glass vials. A total of 1.3 × 10^6 cpm [^{3}H]leucine was added per incubation at the indicated concentrations of leucine. The incubations were conducted for 30 min with four replicates per determination.

Added leucine	Leucine incorporation		Fold stimulation by Ca^{2+}
	EGTA	EGTA + Ca^{2+}	
μM	$cpm \cdot mg^{-1} \cdot 10^{-3}$		
None	2.4 ± 0.1	22.1 ± 1.1	9
3	2.3 ± 0.3	20.4 ± 1.2	9
10	2.0 ± 0.0	18.8 ± 0.9	9
30	1.5 ± 0.1	15.2 ± 0.3	10
100	0.8 ± 0.0	8.7 ± 0.3	11

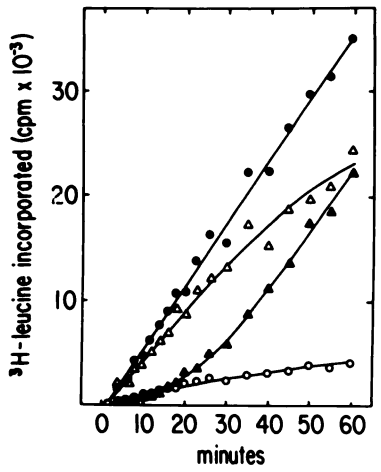

Fig. 1. Effect of Ca^{2+} depletion and repletion of hepatocytes on the incorporation of [^{3}H]leucine as a function of time. Cells were equilibrated in modified Ham's F10 medium, pH 7.6, at 37° in plastic Erlenmeyer flasks under O_2 with either 1 mM EGTA (○, ▲) or 1 mM EGTA + 1.3 mM $CaCl_2$ (●, △). After 30 min, the following additions were made: [^{3}]leucine, 4 μCi/ml (○), [^{3}H]leucine + 1.3 mM Ca^{2+} (final) (▲), [^{3}H]leucine (●), and [^{3}H]leucine + 3 mM EGTA (final) (△). Aliquots (0.5 ml) were removed at the indicated times and assayed for trichloroacetic aicd-insoluble radioactivity.

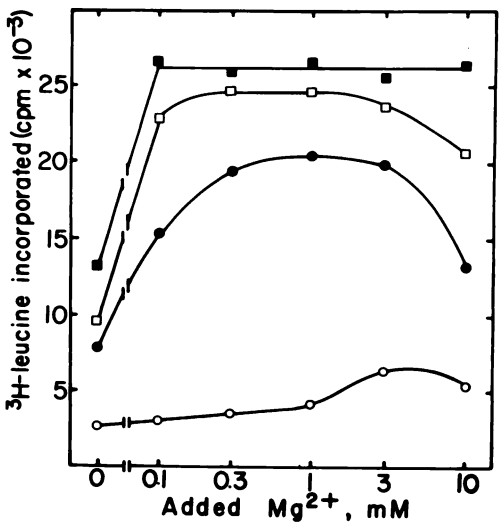

Fig. 2. Effect of Mg^{2+} concentration on the Ca^{2+} concentration dependence of leucine incorporation. Experimental procedures were similar to those of Table 1 except that the incubations (0.5 ml) were conducted at pH 7.6. Cells were pretreated for 30 min at 37° with $CaCl_2$ and $MgCl_2$ and then were incubated for 30 min with 10 μM [^{3}H]leucine (2 μCi/ml). Both pretreatments and incubations were conducted with 1 mM EGTA and the indicated $MgCl_2$ concentrations without added $CaCl_2$ (○) and at 0.1 (●), 0.3 (□), and 2 mM (■) added $CaCl_2$.

and becoming maximal at about 25 min of incubation. This maximal rate of incorporation was identical to that of hepatocytes restored with Ca^{2+} prior to the addition of radiolabeled amino acid. In contrast, Ca^{2+}-restored cells which were exposed to high EGTA simultaneously with the addition of [^{3}H]leucine exhibited decreasing rates of incorporation throughout the 1-hr incubation period. As the concentration of Mg^{2+} (0.6 mM) in this experiment (Fig. 1) was lower than that of EGTA in the medium, the possibility existed that this Mg^{2+} concentration was not optimal for hepatocyte protein synthesis. The effects of Mg^{2+} concentration on [^{3}H]leucine incorporation were therefore investigated separately (Fig. 2). Increasing concentrations of Mg^{2+} in incubations conducted at 1 mM EGTA without added Ca^{2+} were found to promote overall incorporation, up to a doubling at 3 mM Mg^{2+}. Higher concentrations of Mg^{2+} (10 mM) were inhibitory. Large increases in incorporation were found with additions of 0.1–3 mM Ca^{2+} in excess of EGTA at all Mg^{2+} concentrations tested, with the higher Ca^{2+} concentrations being the most stimulatory. At each Ca^{2+} concentration, Mg^{2+} concentrations from 0.1–3 mM were equally effective in maintaining incorporation. The inhibitory effect of 10 mM Mg^{2+} was overturned by free Ca^{2+} above 1 mM. Although Mg^{2+} at this range of concentrations did not appear to exert prominent effects on protein synthesis, subsequent experiments were conducted at 1.6 mM Mg^{2+}, a concentration comfortably in excess of chelator.

As shown earlier (Fig. 1), 300 μM free Ca^{2+}, when added simultaneously with [^{3}H]leucine to Ca^{2+}-depleted cells, required approximately 15 min to stimulate amino acid incorporation. This lag phase was considerably longer than that observed previously for C6 cells (4). [^{3}H]Leucine uptake was not affected by Ca^{2+} depletion (Fig. 3). Also, as determined by high pressure liquid chromatography, both Ca^{2+}-depleted and restored hepatocytes retained the same ATP content (16 nmol/mg) and an ATP:GTP ratio of approximately 7 after 30 min of incubation at 37° (not shown). To reliably assess the Ca^{2+} concentration

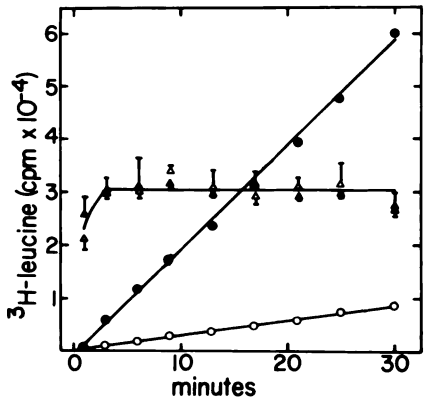

Fig. 3. [^{3}H]Leucine uptake by Ca^{2+}-depleted and Ca^{2+}-restored liver cells. Equilibration procedures and conditions were the same as described in the legend of Fig. 1. Hepatocytes were pretreated for 30 min at 1.6 mM $MgCl_2$, pH 7.6, and 1 mM EGTA with or without 2 mM $CaCl_2$. Uptake of 10 μM [^{3}H]leucine for Ca^{2+}-depleted (△) and Ca^{2+}-restored cells (▲) was determined as trichoroacetic acid-soluble radioactivity as described under Experimental Procedures. [^{3}H]Leucine incorporation into protein (alkali-stable trichloroacetic acid-insoluble material) was determined for the same incubations of Ca^{2+}-depleted (○) and Ca^{2+}-restored (●) cells and is plotted on the same scale.

dependence of hepatocyte protein synthesis, it was first necessary to establish the time dependency of the response of various Ca^{2+} concentrations (Fig. 4). Ca^{2+}-depleted cells responded to the addition of 3 mM free Ca^{2+} with increased rates of [^{3}H] leucine incorporation within 8 min, achieving linear maximal rates of incorporation by 15 min. In contrast, the addition of lower concentrations of Ca^{2+} required longer periods of exposure for detectable effects on protein synthesis to become apparent. Linear rates of amino acid incorporation occurred at each added Ca^{2+} concentration, but the rates were slower at lower concentrations of the cations. Based on these data, the cells were routinely equilibrated with the desired Ca^{2+} concen-

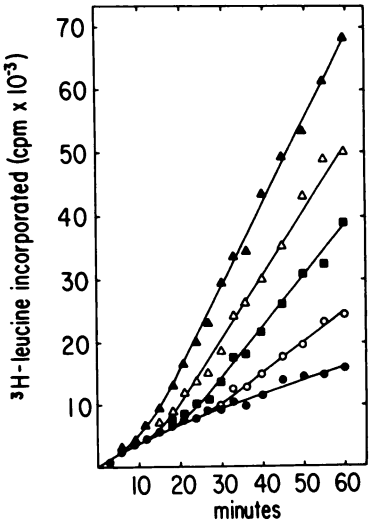

Fig. 4. Time of onset of the stimulation of leucine incorporation by various Ca^{2+} concentrations. Equilibration procedures and conditions were the same as those described in the legend of Fig. 1. Hepatocytes were pretreated for 30 min in medium with 1.6 mM Mg^{2+} and 1 mM EGTA. Incubations were initiated by the simultaneous addition of [^{3}H]leucine and $CaCl_2$. $CaCl_2$ was added at 0 (●), 1 mM (○), 1.1 mM (■), 1.3 mM (△), and 4 mM (▲).

tration for 30 min at 37° prior to use in subsequent experiments.

Ca^{2+} alone, of a series of polyvalent cations tested, was fully effective in restoring the rate of protein synthesis in Ca^{2+}-depleted hepatocytes. In incubations with 1.6 mM Mg^{2+} and 300 μM test cations in excess of EGTA, Ca^{2+} stimulated protein synthesis 5-fold and Sr^{2+}, 2-fold; Co^{2+}, Ni^{2+}, Zn^{2+}, Ba^{2+}, La^{3+}, and Mn^{2+} stimulated synthesis 10–50%. Cu^{2+}, Fe^{2+}, and Fe^{3+} were inhibitory (data not shown).

Hormonal effects on protein synthesis. Various hormones including α-adrenergic agonists, vasopressin, and angiotensin II have been found to provoke a rapid mobilization of Ca^{2+} in liver cells (2, 3). Effects of these and other agents were therefore sought on [^{3}H]leucine incorporation into protein at pH 7.4 (Table 2). None of the agents tested produced marked effects on incorporation in Ca^{2+}-depleted cells. At 1 mM free Ca^{2+}, reproducible inhibitory effects on incorporation were observed with the α-adrenergic agonists, epinephrine, norepinephrine, and phenylephrine, and with vasopressin. Angiotensin II was less inhibitory than these agents; isoproterenol, dopamine, vasoactive intestinal polypeptide, histamine, glucagon, and insulin were not inhibitory. Insulin at concentrations (2 milliunits/ml) higher than those tested in Table 2 stimulated incorporation 10–20%. Also tested, but without pronounced effects, were various combinations of the adenylate cyclase activator, forskolin (1 μM), with isoproterenol or glucagon (not shown). More complete Ca^{2+} concentration curves were conducted with and without the addition of 1 μM epinephrine as a typical inhibitory agent (Fig. 5). Epinephrine increased the concentration of Ca^{2+} required to restore protein synthesis, with approximately 3 mM Ca^{2+} in excess of EGTA almost fully reversing the inhibition by the catecholamine in this experiment. Such relatively complete reversal of hormonal action by supraphysiologic Ca^{2+} concentrations was found for approximately one-third of the cell preparations. Most preparations were reversed 85% or more and some were reversed as little as 70% under comparable conditions. The inhibition of amino

TABLE 2

Effects of various agents on [^{3}H]leucine incorporation by isolated liver cells

Cells were equilibrated for 25 min in medium at pH 7.4 adjusted to 1 mM EGTA or to 1 mM EGTA + 2 mM $CaCl_2$. After 5 min exposure to the indicated hormones, [^{3}H]leucine (1.3×10^6 cpm) and 10 μM leucine were added and the incubation was conducted for 30 min.

Additive	Leucine incorporation	
	Ca^{2+}-depleted cells	Ca^{2+}-restored cells
	$cpm \cdot mg^{-1} \cdot 10^{-3}$	
None (control)	3.6 ± 0.4	23.5 ± 1.2
Epinephrine (1 μM)	3.2 ± 0.2	10.2 ± 0.5
Isoproterenol (1 μM)	3.5 ± 0.2	20.9 ± 1.1
Norepinephrine (1 μM)	3.3 ± 0.1	10.5 ± 0.9
Phenylephrine (10 μM)	3.4 ± 0.5	11.2 ± 0.3
Dopamine (10 μM)	3.6 ± 0.1	23.1 ± 0.9
Histamine (10 μM)	3.9 ± 0.2	23.8 ± 1.6
Vasoactive intestinal polypeptide (1 μM)	4.1 ± 0.2	24.5 ± 0.9
Angiotensin II (25 nM)	3.7 ± 0.1	16.0 ± 0.6
Vasopressin (10 nM)	3.6 ± 0.2	10.7 ± 0.5
Glucagon (10 nM)	4.0 ± 0.2	22.3 ± 1.4
Insulin (0.2 milliunits/ml)	3.8 ± 0.2	25.0 ± 1.2
Somatostatin (0.1 μM)	3.7 ± 0.2	23.1 ± 1.1

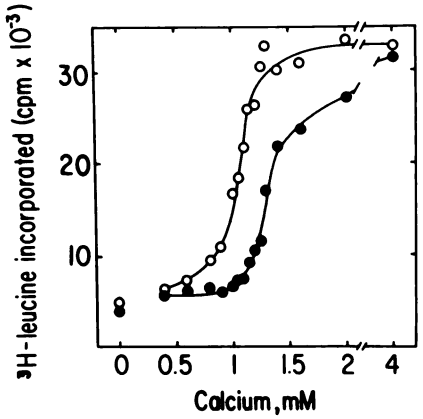

Fig. 5. Effects of epinephrine on the Ca^{2+} concentration dependence of leucine incorporation. Equilibration procedures and conditions were the same as those in Table 1. Epinephrine and 10 μM ascorbic acid were added 5 min before initiating the incorporation with [^{3}H]leucine. Incubations contained 1.6 mM $MgCl_2$, 1 mM EGTA, and the indicated concentrations of $CaCl_2$ with (●) or without (○) 1 μM epinephrine.

acid incorporation by vasopressin and α-adrenergic agonists was increased in intensity at more acidic pH ranges and less subject to reversal by supraphysiologic extracellular Ca^{2+} concentrations (Table 3).

The above experiments (Tables 2 and 3, Fig. 5) involved a 5-min exposure of the cells to hormone prior to addition of [^{3}H]leucine. It was not known, however, whether this period of exposure was optimal. The time dependency for humoral inhibition of protein synthesis was therefore examined at several Ca^{2+} concentrations (Fig. 6). Maximal inhibition at 100 μM Ca^{2+} in excess of EGTA occurred within 5 min after adding either 10 μM phenylephrine or 10 nM vasopressin (Fig. 6A). The rate of incorporation then remained linear and slightly higher than that of the EGTA control for the remainder of the incubation period. At 300 μM free Ca^{2+}, approximately 10 min of exposure were required for the full extent of inhibition to develop (Fig. 6B). At 3 mM free Ca^{2+}, only a small inhibition (~10%) occurred with either agent during the incubation period

TABLE 3

Hormonal effects on [^{3}H]leucine incorporation at various pH values

Cells were pretreated as described in Table 2 at either pH 7.2 or 7.6 and incubated with 10 μM leucine for 30 min. All incubations contained 1 mM EGTA and the indicated concentrations of total added Ca^{2+}.

pH	Total added $CaCl_2$	Leucine incorporation		
		No hormone	Vasopressin (10 nM)	Phenylephrine (10 μM)
	mM			*cpm·mg^{-1}·10^{-3}*
7.2	none	2.7 ± 0.2	2.4 ± 0.2	2.6 ± 0.3
	1.2	20.2 ± 0.9	5.5 ± 0.3	5.2 ± 0.3
	2.0	21.6 ± 1.0	9.3 ± 0.4	8.5 ± 0.2
	4.0	21.8 ± 2.0	13.2 ± 0.7	12.2 ± 0.5
7.6	none	2.7 ± 0.2	2.4 ± 0.1	2.5 ± 0.1
	1.2	22.9 ± 1.1	11.2 ± 0.5	11.8 ± 0.5
	2.0	22.3 ± 1.3	13.4 ± 0.3	13.5 ± 0.8
	4.0	22.0 ± 1.2	15.6 ± 0.9	15.6 ± 0.4

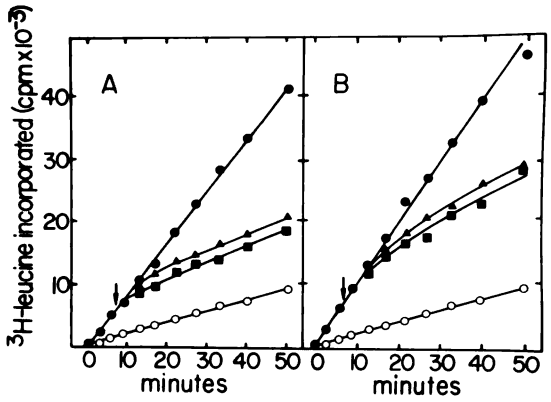

Fig. 6. Time dependence of the inhibition of leucine incorporation by vasopressin and phenylephrine. Equilibration procedures and conditions were the same as those in Fig. 1, with vasopressin and phenylephrine being added 7 min after initiation of incorporation by the addition of [^{3}H] leucine. Incubations conducted at 1 mM EGTA without added Ca^{2+} (Ca^{2+}-depleted cells) are represented by an *open symbol* (○). Incubations conducted with EGTA + 1.1 mM $CaCl_2$ (A) or 1.3 mM $CaCl_2$ (B) are represented by *solid symbols*: Ca^{2+}-restored cells without drug, ●; vasopressin (10 nM) added at *arrow*, ●; phenylephrine (10 μM) added at *arrow*, ▲.

(not shown). Subsequent experiments were conducted with a 5-min preexposure of the hepatocytes to hormone before the addition of radiolabeled amino acid. The concentration dependence for vasopressin and epinephrine was examined at pH 7.4 with 1 mM Ca^{2+} in excess of EGTA (Table 4). Vasopressin and epinephrine were approximately equal in inhibitory efficacy. Half-maximal effects occurred with the addition of approximately 1 nM vasopressin or 0.1 μM epinephrine. Combinations of various concentrations of vasopressin and epinephrine were tested to ascertain whether the two hormones provided additive inhibitions of protein synthesis in hepatocytes. At low concentrations of each agent, their inhibitory effects were additive. For example, at 30 nM epinephrine, more inhibition could be generated by the further addition of vasopressin. High concentrations of either agent provided a large inhibition that was only marginally increased by the further addition of the other agent. For example, at 1 μM epinephrine, no further increase in inhibition was generated by vasopressin. α-Adrenergic antagonists did not influence the inhibitory action of vasopressin (not shown).

As the results of Table 4 indicated that epinephrine was acting at an α-adrenergic receptor, it was reasonable to characterize the receptor as being α_1 or α_2, since Ca^{2+} mobilization by the catecholamine has been reported to occur via an α_1 receptor (18). A study of amino acid incorporation with various selective antagonists for β, α_1-, and α_2-adrenergic receptors was therefore conducted at various epinephrine concentrations at pH 7.4 (Table 5). A low, free extracellular Ca^{2+} concentration (0.2 mM) was employed in this experiment to assume a strong hormonal response. In the absence of added epinephrine, antagonists in the low micromolar concentration range did not affect the rate of amino acid incorporation. At either 0.1 or 0.3 μM epinephrine, the α_1-selective adrenergic antagonist, prazosin, at low concentrations (0.01 μM) completely blocked the inhibitory effects of the hormone. Phentolamine, a nonselective α-adrenergic antagonist, was a poorly effective blocker of the inhibiton at 3 μM. Yohimbine, an α_2-selective adrenergic antagonist, was an effective blocker only at relatively high concentrations (10 μM). Propranolol, a nonselective β-adrenergic antagonist, did not affect the inhibitory actions of epinephrine. This spectrum of inhibitory actions and sensitivities would be predicted for an α_1-epinephrine receptor.

TABLE 4

Concentration dependence of epinephrine and vasopressin in inhibiting leucine incorporation

Cells were equilibrated for 25 min at pH 7.4 with 1 mM EGTA and 2 mM $CaCl_2$. Varying concentrations of vasopressin and epinephrine with 10 μM ascorbate were then added for 5 min. The incorporation with 10 μM [^{3}H]leucine was then conducted for 30 min.

Additives		Leucine incorporation
Epinephrine	Vasopressin	
μM	*nM*	*cpm·mg^{-1}·10^{-3}*
none	none	17.4 ± 0.4
0.01	none	16.9 ± 0.7
0.03	none	14.0 ± 0.9
0.1	none	12.0 ± 0.1
0.3	none	9.4 ± 0.1
1.0	none	8.8 ± 0.2
3.0	none	8.9 ± 0.2
none	1	13.7 ± 0.5
none	3	11.6 ± 0.3
none	10	11.3 ± 0.2
none	30	11.2 ± 0.5
0.03	1	11.8 ± 0.3
0.03	30	11.0 ± 0.3
1.0	1	8.8 ± 0.2
1.0	30	9.0 ± 0.2

TABLE 5

Ability of various adrenergic antagonists to block the inhibition of leucine incorporation by epinephrine

Cells were equilibrated for 20 min at pH 7.4 with 1 mM EGTA and 1.3 mM $CaCl_2$. Adrenergic antagonists were then added for 5 min and, finally, various concentrations of epinephrine were added for an additional 5 min. [^{3}H]Leucine with 10 μM unlabeled leucine was then added for 30 min.

Additive	Leucine incorporation[a]		
	Epinephrine Concentration (μM)		
	0	0.1	0.3
	cpm·mg^{-1}·10^{-3}		
None (control)	25.3 ± 0.8	14.4 ± 0.3	12.0 ± 1.2
Propranolol (10 μM)	23.1 ± 0.7	13.3 ± 0.2	11.4 ± 0.6
Phentolamine (3 μM)	25.6 ± 1.4	20.9 ± 0.6	16.1 ± 0.9
Prazosin (0.01 μM)	26.4 ± 1.3	26.8 ± 0.2	26.4 ± 0.9
Yohimbine (1 μM)	26.0 ± 0.5	20.5 ± 0.4	14.9 ± 0.5
Yohimbine (10 μM)	26.1 ± 1.0	25.9 ± 1.7	23.0 ± 1.5

[a] Incorporation in Ca^{2+}-depleted controls was 3.9 ± 0.2 cpm·mg^{-1}·10^{-3}.

Pulse labeling experiments with [^{35}S]methionine were conducted to ascertain whether the synthesis of most proteins or only that of a limited group was affected by these hormones and by Ca^{2+} depletion. Labeled proteins were separated by two-dimensional acrylamide gel electrophoresis and analyzed for radioactivity by fluorography. Radioactivity in all protein populations derived from Ca^{2+}-depleted cells was greatly reduced in contrast to proteins of cells restored with 200 μM free Ca^{2+} (Fig. 7). Vasopressin (20 nM) and epinephrine (0.3 μM) reduced labeling of all proteins markedly at 200 μM free Ca^{2+}. In additional experiments conducted with one-dimensional acrylamide gel electrophoresis (not shown), vasopressin and epinephrine were each found to reduce pulse labeling in all protein bands in a dose-dependent manner.

Throughout these experiments, EGTA was used to control the free Ca^{2+} concentration of the incubation. However, cells that were prepared and incubated without EGTA behaved similarly to those exposed to the chelator at comparable concentrations of free Ca^{2+} (Table 6). For example, at 1 mM free Ca^{2+}, the same maximal rate of incorporation was seen in incubations with or without EGTA. Also, vasopressin and epinephrine inhibited protein synthesis to the same extent with and without EGTA at comparable free Ca^{2+} concentrations. In incubations conducted without added Ca^{2+}, however, protein-synthetic activity was lower when chelator was added, as opposed to samples without chelator. Under this condition the free Ca^{2+} concentration of samples with EGTA would be expected to be much lower than for comparable incubations without chelator.

Discussion

The results of this report relate to three lines of research from the literature: first, the observation originally reported in

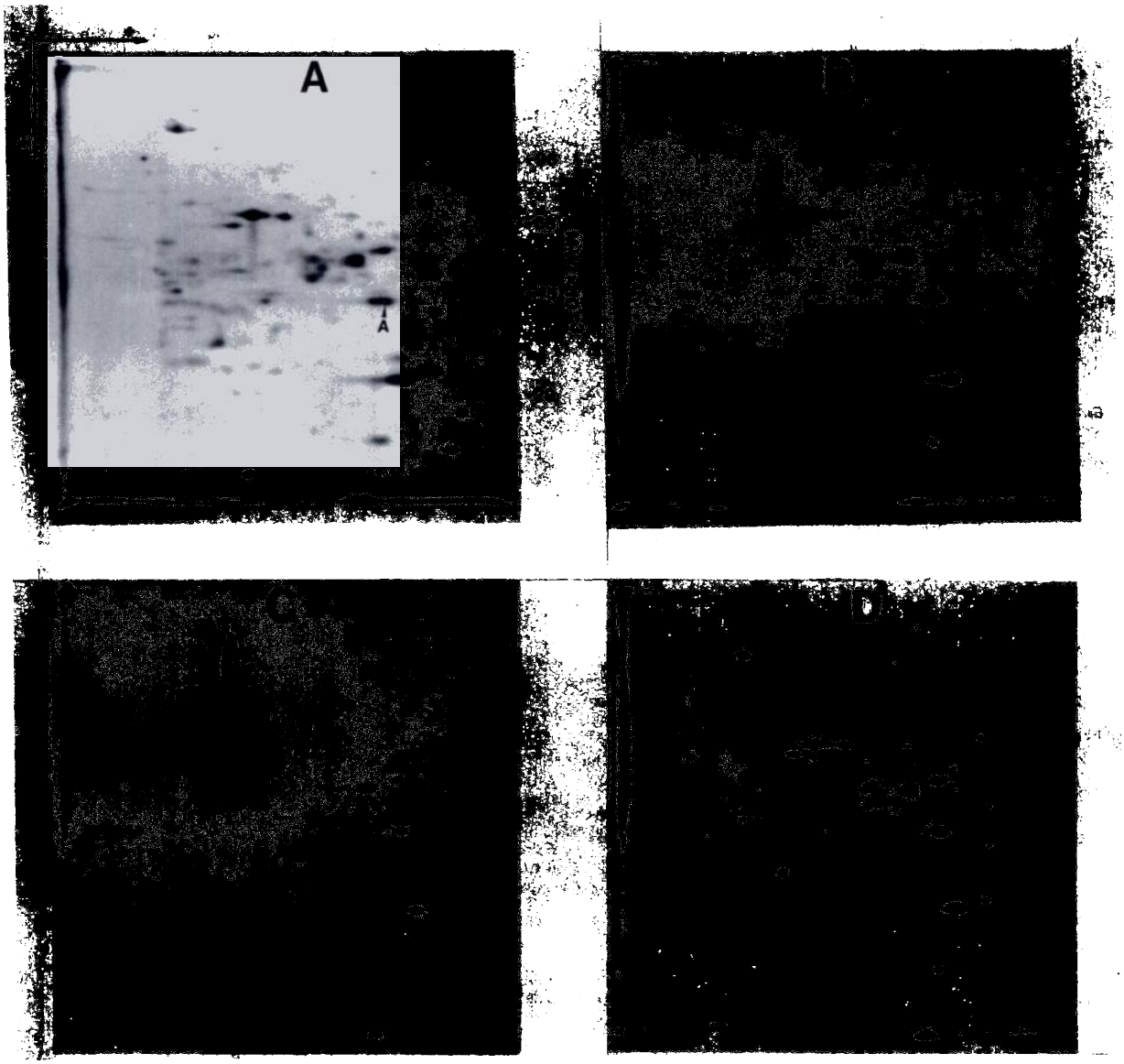

Fig. 7. Hormonal effects on [^{35}S]methionine incorporation into various protein populations as analyzed by polyacrylamide gel electrophoresis and fluorography. Incubation procedures were the same as in Fig. 2. Incubations were conducted for 30 min at 1.6 mM $MgCl_2$ and 10 μM (8 μCi/ml) [^{35}S] methionine. The cells were washed in buffer and aliquots were analyzed for incorporation of radioactivity into protein by two-dimensional acrylamide gel elecrtrophoresis and fluorography as described in Experimental Procedures. *Panels* represent incubations conducted at 1 mM EGTA and: A, 1.2 mM $CaCl_2$; B, 1.2 mM $CaCl_2$ and 2 μM epinephrine; C, no hormone or $CaCl_2$; and D, 1.2 mM $CaCl_2$ and 10 nM vasopressin. The position at which actin migrated is indicated in *panel* A by *A*.

TABLE 6

Hormonal inhibition of leucine incorporation at comparable free Ca^{2+} concentrations with and without EGTA

Liver cells were prepared without chelator. Cells were equilibrated for 30 min with or without 1 mM EGTA at various concentrations of added Ca^{2+} and then incubated with 10 μM [^{3}H]leucine for 30 min. In incubations without EGTA, total added Ca^{2+} was taken to be free Ca^{2+}. In incubations containing EGTA, Ca^{2+} added in excess of chelator was considered to be free Ca^{2+}.

Added Free Ca^{2+}	Leucine incorporation					
	No hormone		Vasopressin (10 nM)		Epinephrine (1 μM)	
	−EGTA	+EGTA	−EGTA	+EGTA	−EGTA	+EGTA
mM	$cpm \cdot mg^{-1} \cdot 10^{-3}$					
none	13.6 ± 0.5	3.4 ± 0.1	6.7 ± 0.1	2.9 ± 0.1	6.7 ± 0.2	3.0 ± 0.1
0.1	20.6 ± 0.9	22.0 ± 0.7	7.9 ± 0.0	9.7 ± 0.8	7.5 ± 0.5	9.8 ± 0.3
0.3	23.0 ± 1.2	22.5 ± 0.2	11.3 ± 0.7	12.1 ± 0.3	10.6 ± 0.1	11.9 ± 0.5
1.0	24.0 ± 1.0	24.4 ± 0.9	16.5 ± 0.6	17.1 ± 0.4	15.4 ± 0.9	16.0 ± 0.6

1969 by Burton *et al.* (17), that isolated liver cells in low Ca^{2+} medium incorporate amino acid less rapidly than those in high Ca^{2+} medium; second, the finding first noted in 1960 by Pryor and Berthet (19), that epinephrine inhibits protein synthesis in liver slices; and, third, the various reports that α-adrenergic agonists, vasopressin, and angiotensin affect liver cell Ca^{2+} homeostasis (1). Isolated, normal liver cells were found to behave comparably to tumor cells in tissue culture in terms of requiring Ca^{2+} for protein synthesis (4, 7). Exposure of liver cells to medium containing EGTA for 15–30 min at 37° resulted in an 80–90% decrease in the rate of protein synthesis, decreases similar to those found for C6 glioma (4) and GH_3 pituitary cells (7). Somewhat longer periods of exposure to Ca^{2+} were required for restoration of protein synthesis in Ca^{2+}-depleted liver cells as compared to tissue culture cells. The Ca^{2+} concentration dependence for restoration of protein synthesis, however, was not notably different for the various cell preparations, despite the use of rather different incubation media. As found with C6 and GH_3 cells, the ability to take up amino acid and maintain ATP and GTP content was not affected by Ca^{2+} depletion, and good restoration of protein synthesis was achieved only by the specific readdition of Ca^{2+} to the medium. Even without the addition of EGTA to the medium, liver cells incubated without Ca^{2+} experienced a two-thirds reduction in the rate of protein synthesis, with activity being restored by the addition of Ca^{2+} (Table 6). The enriched amino acid contents of the hepatocyte incubation medium and their rapid uptake ruled against any important requirement for protein catabolism in supplying amino acids for protein synthesis in these experiments. In previous studies with C6 and GH_3 cells (4, 7), protein catabolism was not significantly affected by Ca^{2+} depletion.

Experiments in this report were conducted with hepatocytes isolated from mature, fed, male rats. Liver is well known to be subject to many hormonal influences which vary to some degree with species, sex, age, and diet (1, 20). A number of agents in addition to epinephrine have been reported to lower the rate of protein synthesis. For example cAMP, cAMP analogs, and hormones such as glucagon which increase cAMP content reportedly inhibit protein synthesis in liver cells (21, 22) and in cell-free protein-synthesizing systems prepared from livers of younger rats (23). Epinephrine has been reported by Mandl *et al.* (8) to inhibit mouse hepatocyte protein synthesis in a manner which is blocked by the α-adrenergic antagonist, phenoxybenzamine, but not propranolol. This inhibition developed after 40 min in hepatocytes incubated at 2.5 mM Ca^{2+} but not in incubations conducted at 0.2 mM EGTA without Ca^{2+}. Controls with 2.5 mM Ca^{2+} were 2-fold more active than controls with EGTA. The results of the present report qualitatively confirm and extend those by Mandl *et al.* (8). Not only was a more extensive inactivation by Ca^{2+} depletion found, but also a more rapid inactivation by α-adrenergic agonists. It is likely, however, that the rapidity of the hormonal inactivation is determined in large part by the concentration of Ca^{2+} in the medium. Conditions which would have been expected to elevate hepatocyte cAMP content, such as incubation with glucagon or isoproterenol with or without forskolin, did not in our hands provide significant changes in the rate of protein synthesis for hepatocytes from large, adult males. Liver cells from such animals, however, have been reported to have a reduced ability to accumulate cAMP in response to various hormones such as β-adrenergic agonists (20). It should be noted that the present results do not exclude cAMP inputs into the hormonal regulation of protein synthesis but, rather, emphasize an important role for the Ca^{2+} in the process.

The present results support the hypothesis that a sequestered pool of intracellular Ca^{2+} is required for the maintenance of high rates of protein synthesis in liver cells. Either EGTA or low Ca^{2+} media, which with time deplete both bound and free intracellular Ca^{2+}, inhibited protein synthesis. Hormones, such as α-adrenergic agonists, vasopressin, and angiotensin II are reported to promote the production of *myo*-inositol 1,4,5-trisphosphate, which rapidly increases intracellular free Ca^{2+} concentrations in hepatocytes from sequestered stores (24, 25). The source of this Ca^{2+} is widely believed to be the endoplasmic reticulum rather than the mitochondria (26). Since these agents inhibit protein synthesis while increasing the intracellular free Ca^{2+} concentration of hepatocytes, it would appear likely that the inhibition involves mobilization of sequestered Ca^{2+} from the endoplasmic reticulum. The regulatory nature of the Ca^{2+} requirement for protein synthesis is emphasized by the occurrence of substantial (approximately 50%) inhibitions at physiologic pH and serologic concentrations of free extracellular Ca^{2+}, and at concentrations of hormones believed to occur *in vivo*. As extracellular Ca^{2+} was increased to supraphysiologic concentrations (3 mM), the hormonal inhibition of protein synthesis was attenuated, presumably because Ca^{2+} concentration gradients favored sequestration.

The mechanism by which Ca^{2+} influences protein synthesis is as yet undefined. The endoplasmic reticulum appears to represent a common focal point for the sequestration of Ca^{2+} subject to mobilization by α-adrenergic agonists and vasopressin and for the binding of a considerable fraction of the polysomes present in the cell. Although we have not attempted to correlate the calcium content of the endoplasmic reticulum with the rate of protein synthesis, it is attractive to speculate that the degree of saturation of this storage site with Ca^{2+} in some manner imposes translational control on peptide synthe-

sis. In this regard, regulation involving reversible protein phosphorylation, as, for example, of the ribosomal S6 protein or the eukaryotic initiation factor eIF-2, or by a Ca^{2+}-dependent protein kinase cannot be ruled out.

The physiological significance of a putative coupling of the rate of protein synthesis to the availability of Ca^{2+} for storage in the endoplasmic reticulum also remains to be defined. Protein synthesis is a complex, energy-intensive cellular process which through its products affects all aspects of cellular performance. It would seem mandatory that this process be closely integrated and coordinated with other cell processes. Ca^{2+}, which is an important stimulus-response coupling factor, provides a logical candidate for subordinating the rate of protein synthesis to the functional status of the cell. For example, one prime function of liver is the production and export of glucose in response to a variety of hormones activating glycogenolysis and gluconeogenesis. Hormones which mobilize Ca^{2+} from the endoplasmic reticulum activate glycogenolysis through increasing intracellular free Ca^{2+} and, hence, stimulating phosphorylase kinase activity. A concomitant inhibition of protein synthesis would presumably redirect the utilization of amino acids toward deamination and subsequent conversion to glucose by gluconeogenesis.

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