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A Possible Role of Calcium in the Action of Glucagon, cAMP and Dibutyryl cAMP on the Metabolism of Free Fatty Acids by Rat Hepatocytes

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SUMMARY. The effects of calcium (Ca^{++}) on the actions of glucagon, cAMP and dibutyryl cAMP on triacylglycerol synthesis, ketogenesis and output of glucose were studied using isolated hepatocytes. Glucagon, cAMP and Bt_2cAMP decreased the incorporation of [1-^{14}C] oleate into triacylglycerol and increased synthesis of ketone bodies and output of glucose. Basal rates of triacylglycerol synthesis, ketogenesis, and output of glucose, and the responses to glucagon and the cyclic nucleotides were diminished by depletion of calcium. Triacylglycerol synthesis was restored toward control values by treatment with calcium. Ionophore A 23187, mimicked the effects of glucagon on triacylglycerol biosynthesis provided calcium was present in the medium. Uptake of [1-^{14}C] oleate was impaired when the hepatocytes were depleted of calcium. These observations suggest that calcium plays an important role in hepatic triacylglycerol synthesis and in ketogenesis and on the actions of glucagon and adenylic cyclic nucleotides on these metabolic pathways.

We reported previously that glucagon and Bt_2cAMP[1], decreased output of triacylglycerol by the perfused rat liver and stimulated ketogenesis and output of glucose (1-3). Bu_2cAMP depressed the proportion of [1-^{14}C] oleate converted to triacylglycerol but increased the fraction converted to ketone bodies and CO_2 (2). In addition, the biosynthesis of phosphatidic acid, diacylglycerol and triacylglycerol from sn-[U-^{14}C]-glycerol-3-phosphate by microsomes isolated from livers perfused *in vitro* with Bt_2cAMP was less than that by microsomes isolated from livers perfused in the absence of the nucleotide (3). Since glucagon and cAMP were reported to participate in regulation of heptatic cellular homeostasis of calcium (4-6), and since uptake of calcium

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[1]Abbreviations used are: Bt_2cAMP, N^6-$O^{2'}$ dibutyryl adenosine 3',5'-monophosphate; cAMP, adenosine 3',5'-monophosphate; EGTA, ethylene glycos bis (β-aminoethyl ether) N,N'-tetraacetic acid; DG, diacylglycerol; TG, triacylglycerol.

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by hepatic microsomes resulted in a calcium dependent decrease in biosynthesis of phosphatidate and other glycerolipids (7), it is conceivable that calcium may mediate effects of glucagon and cAMP on triacylglycerol synthesis and ketogenesis. The purposes of the present investigation were to examine the effects of glucagon, cAMP and Bt_2cAMP on the incorporation of [1-^{14}C] oleate into glycerolipids and ketone bodies by isolated rat liver cells, and to evaluate th possibility that calcium might be required for the actions of glucagon, cAMP and Bt_2cAMP on hepatic triacylglycerol biosynthesis and ketogenesis. The data reported here present evidence for such a role of calcium.

MATERIALS AND METHODS.

Preparation and incubation of hepatocytes. Hepatocytes were prepared fro 250-300 g ad libitum fed male Sprague-Dawley rats (Charles River Breeding Labo tories, Wilmington, MA) by the liver perfusion technique of Berry and Friend (as modified by Berry and Werner (9). During the preparation of the hepatocyte glucose was added to the perfusion medium (final concentration, 15 mM) to mini mize hepatic glycogenolysis. The hepatocytes were washed and resuspended in calcium-free Krebs-Henseleit bicarbonate buffer (10).

Prior to incubation for measurement of the rates of incorporation of [1-^{14}C] oleate into glycerolipids and ketone bodies, and for estimation of glucose output, cells were incubated in normal Krebs-Henseleit buffer or were depleted of calcium by washing and incubating in calcium-free buffer containir 1 mM EGTA (11). When indicated, repletion of calcium-depleted cells with calcium was carried out by resuspension and incubation in normal buffer (11). In all these incubations 20 mM glucose was added to the buffer to limit glycogenolysis. The vessels (Erlenmeyer flasks) were gassed with a mixture of 95% O_2-5% CO_2, stoppered, and incubated at 37°C in a Dubnoff shaker at 60 oscillations per minute.

Incubations for measurement of incorporation of [1-^{14}C] oleate into neut lipids and ketone bodies were performed in 25 ml Erlenmeyer flasks. Each flask contained 2 ml of isolated liver cells (in amounts representing 0.2 - 0 mg DNA) and 2 ml of a complex of 3% bovine serum albumin with 0.5 mM [1-^{14}C] oleate (0.25 µCi). The purification of bovine serum albumin and the preparation of the oleate-albumin complex has been previously described (12). Glucag (10^{-9} and 10^{-7} M), cAMP (10^{-5} and 10^{-4} M) and Bt_2cAMP (10^{-5} and 10^{-4}) were added to the medium as indicated. At appropriate intervals, aliquots of cell suspension (cells + medium) were taken for analysis.

Extraction and separation of lipids. Aliquots of the incubation mixture were extracted for lipids and then lipid classes were fractionated by thin layer chromatography as described elsewhere (13).

Ba-Zn precipitation. Samples of the cell suspension were deproteinized with $Ba(OH)_2$-$ZnSO_4$ (14) and aliquots of the protein-free supernatant were analyzed for glucose (15) and for ketone bodies.

Measurement of the radioactivity. Radioactivity in the lipid fractions was measured from the silica gel that had been scraped off plates into vials

and counted in a liquid scintillation spectrometer (Beckman LS3155T) using diluted Permafluor as scintillation fluid. Measurement of ^{14}C into ketone bodies was carried out according to Bieberdorf, Chernick and Scow (16).

DNA content of hepatocytes. DNA content of isolated liver cells was determined by the diphenylamine method of Burton (17).

Chemicals. Sodium adenosine 3':5'-monophosphate and sodium $N^6,O^{2'}$-dibutyryl adenosine 3':5'-monophosphate were purchased from the Sigma Chemical Co., St. Louis, MO: Glucagon and ionophore A23187 were gifts from Eli Lilly and Co., Indianapolis, Indiana. Oleic acid (>99% purity) was obtained from Nu-Chek Prep., Elysian, Minnesota. Bovine serum albumin (Fraction V, powder) was obtained from Pentex Inc., Kankakee, IL [1-^{14}C] oleic acid (sp. act. 55.96 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Massachusetts. All other chemicals were obtained from standard sources.

RESULTS AND DISCUSSION. The effects of glucagon, cAMP and Bt_2cAMP on incorporation of [1-^{14}C] oleate into triacylglycerol and ketone bodies, and on output of glucose by isolated hepatocytes is shown in figure 1. In confirmation of the data obtained with the isolated perfused rat liver (1-3), glucagon, cAMP and Bt_2cAMP inhibited the synthesis of triacylglycerol and stimulated ketogenesis and output of glucose by hepatocytes with normal calcium levels. Stimulation by adenylic cyclic nucleotides and glucagon of ketogenesis (18-19) and glycogenolysis (6,11,20-22) by rat hepatocytes has also been reported by several laboratories. In the experiments reported here, these responses were reduced in magnitude in calcium depleted hepatocytes. Assimacopoulos-Jeannet et al. (11) reported that the effects of the 1 mM EGTA used for depletion of calcium from the hepatocytes were not attributable to any cell damage. The basal incorporation of radioactive fatty acid into triacylglycerol and ketone bodies by calcium depleted cells was reduced in comparison with that of normal calcium cells in the absence of glucagon or nucleotide. Glucose output was also depressed by calcium deficiency, in agreement with observations reported by Assimacopoulos-Jeannet et al. (11). Pointer et al. did not observe any differences in glucose output due to calcium depletion; however, in agreement with the data presented in figure 1, they observed that the glycogenolytic action of glucagon was inhibited slightly by the omission of calcium (21). Furthermore, absence of calcium from the incubation medium reduced the activation of glycogen phosphorylase by glucagon (6).

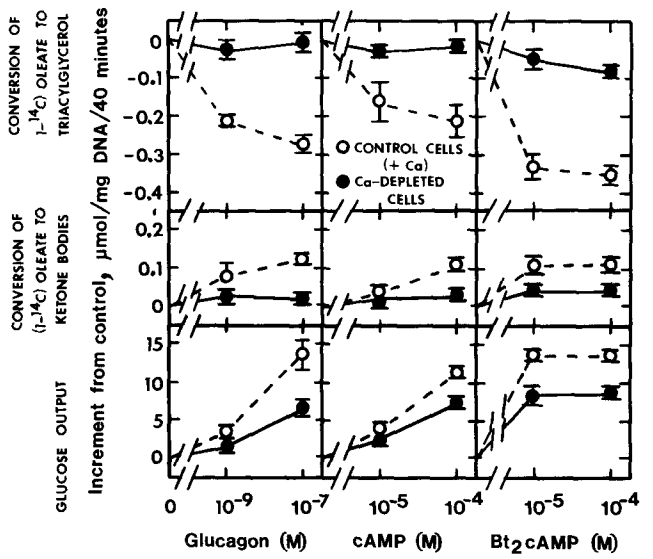


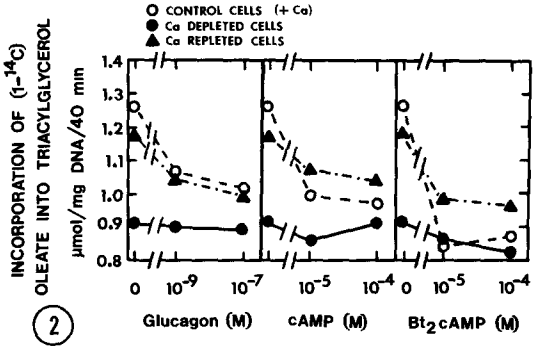
Fig. 1. Effects of depletion of calcium on the actions of glucagon, cAMP and Bt_2cAMP on incorporation of [1-^{14}C] oleate into triacylglycerol and ketone bodies, and on output of glucose by isolated hepatocytes.

Hepatocytes were isolated in calcium-free buffer and then washed with the buffer in which incubations were carried out. Cells were incubated in normal calcium buffer, or were depleted of calcium by washing and incubating for 20 minutes at 37°C in calcium-free buffer containing 1 mM EGTA. The cells were separated rapidly from the medium by centrifugation and resuspended in normal buffer or calcium-free buffer. Incorporation of [1-^{14}C] oleate into triacylglycerol and ketone bodies was measured during 40 minutes incubation with additions. Additional experimental details are given in the text. Data are means ± SE from three separate preparations of cells. All incubations were performed in duplicate. Basal values of incorporation of [1-^{14}C] oleate into triacylglycerol and ketone bodies, and for output of glucose were 1.34 ± 0.14, 0.38 ± 0.07 and 18.56 ± 2.75 µmol/mg DNA/40 minutes, respectively, for normal calcium cells. The corresponding values for calcium depleted cells were 0.91 ± 0.16, 0.25 ± 0.05 and 11.15 ± 3.51, respectively. These values were statistically different (by paired differences) from normal calcium cells ($p < 0.05$).

In a second series of experiments, cells depleted of Ca^{++} were repleted by preincubation of the cells in Ca^{++} containing buffer, the purpose being to determine the reversibility of effects of Ca^{++} depletion on hormone action. It was observed that basal rates of triacylglycerol synthesis as well as the responses to glucagon and cyclic nucleotides were restored toward normal by repletion with Ca^{++} (figure 2).

Ionophore A23187, which increases the rate of uptake and concentration $^{45}Ca^{++}$ in hepatocytes (11), mimics the effect of glucagon on incorporation of [1-^{14}C] oleate into triacylglycerol. Glucagon, like A23187, also increases the hepatic uptake of calcium from the medium (4,6,11). Glucagon (Figures 1-2) and the divalent cation ionophore (Figure 3) inhibited synthesis of triacylglycerol in cells incubated with normal calcium buffer. This inhibition by glucagon or ionophore A23187, however, was not observed when the hepatocytes were incubated in calcium-free buffer containing 1 mM EGTA, although basal rates of triacylglycerol synthesis were reduced by Ca^{++} deficiency. The response of the hepatocytes to glucagon and ionophore A23187 was restored partially by addition of 3.4 mM $CaCl_2$ to the EGTA-calcium free buffer. It is therefore possible that intracellular concentrations of calcium affect triacylglycerol synthesis in hepatocytes and that the effect of glucagon on the microsomal biosynthetic pathway may be calcium dependent (3).

Since basal rates of both triacylglycerol synthesis and ketogenesis were reduced by calcium depletion, since the intracellular availability of free fatty acid or the CoA derivatives is an important regulatory factor in the partition of fatty acid between pathways of esterification and oxidation (13), and since it is quite likely that these same fatty acids are probable candidates for the primary signal for very low density lipoprotein (VLDL) synthesis by the liver, it was of interest to study any possible influence of calcium on hepatic uptake of free fatty acid. Depletion of calcium from hepatocytes was accompanied by a decrease in the rate of uptake of free fatty acid (Figure 4, panel A). This decrease in uptake may explain, at least in part, the depressed



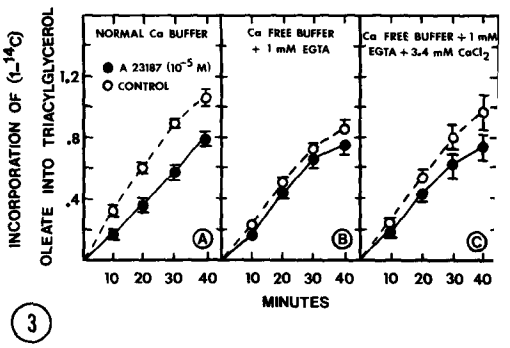


Fig. 2. Effects of calcium depletion and repletion on incorporation of [1-^{14}C] oleate into triacylglycerol by isolated hepatocytes incubated with glucagon, cAMP and Bt_2cAMP.

Normal calcium cells and calcium depleted cells were prepared as described in the legend for Fig. 1. Calcium repletion of hepatocytes was carried out by incubating calcium depleted cells for 20 minutes in normal calcium buffer. Each point represents the average of duplicate determinations from a single preparation of cells.

Fig. 3. Effect of calcium on the action of ionophore A23187 on the incorporation of [1-^{14}C] oleate into triacylglycerol by isolated hepatocytes.

Hepatocytes were incubated for 10 minutes in normal calcium buffer, or calcium-free buffer containing 1 mM EGTA. After 10 minutes preincubation, addition of 2 ml of [1-^{14}C] oleate - albumin complex was made and incorporation of radioactive fatty acid into triacylglycerol was measured at the time indicated. Ionophore A23187 (10^{-5}M) was dissolved in dimethylsulfoxide (11). Data are mean values ± SE of three incubations from two different cell preparations.

basal rates of triacylglycerol synthesis and ketogenesis with depletion of calcium. Incorporation of [1-^{14}C]-oleate into triacylglycerol was reduced by depletion of Ca^{++} whereas incorporation into diacylglycerol was unaffected (Figure 4, panel B) suggesting that the various metabolic steps in the synthesis of triacylglycerol are not uniformly affected by calcium depletion. It is

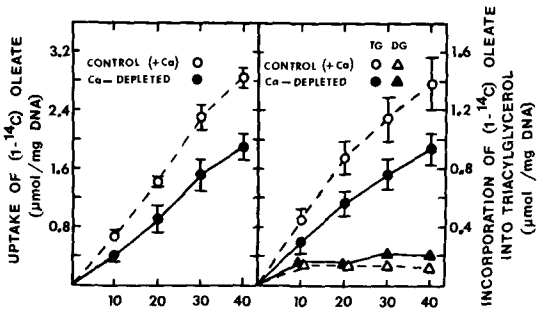


Fig. 4. Effect of depletion of calcium on uptake and incorporation of [1-^{14}C] oleate into triacylglycerol and diacylglycerol by isolated hepatocytes.

See experimental details in legend for Fig. 1 and "Experimental Procedures' section. Each value is the mean ± SE from three incubations from two different preparations of cells.

of interest in this regard that a reciprocal relationship exists between passive or MG-ATP dependent active accumulation of calcium in hepatic microsomes, and the microsomal biosynthesis of phosphatidic acid and other glycerolipids (7). It should be reemphasized that although calcium may be required for the mechanism of uptake of free fatty acid by the hepatocyte and, subsequently, affect basal rates of fatty acid to triacylglycerol, the actions of glucagon and the cyclic nucleotides in normal Ca^{++} media may, in part, be mediated through metabolic changes resulting from the redistribution of calcium among subcellular organelles. On the basis of experiments with the perfused liver, it was clear that glucagon (1) or Bt_2cAMP (1-3) did not affect the rate of FFA (in normal Ca^{++} containing media) but clearly affected the metabolic disposition of the fatty acid.

The data presented here provide evidence for a role for calcium as an additional messenger in the actions of glucagon and the cyclic nucleotides on hepatic metabolism of free fatty acids, as has been suggested for glycogenolysis and gluconeogenesis (4,6,11,21,23-26). Calcium, which may be released into cytosol from intracellular pools (membranes?), or may enter the cell from

extracellular sources, may then be taken up by subcellular organelles, to affect specific enzymes involved in the biosynthesis of glycerolipids or in ketogenesis.

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