Requirement for bivalent cations in the actions of insulin and sodium nitroprusside on metabolism in rat adipocytes

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The requirement for Ca^{2+} and Mg^{2+} in the actions of insulin and sodium nitroprusside on rat adipocyte metabolism was investigated: (i) sodium nitroprusside, but not insulin, increased cGMP levels in cells incubated in the absence of Ca^{2+} and/or Mg^{2+}; (ii) sodium nitroprusside and insulin are unable to increase the incorporation of [^{14}C]glucose into triglycerides and [^{14}C]leucine into proteins in the absence of Ca^{2+} and Mg^{2+}; (iii) sodium nitroprusside and insulin showed antilipolytic actions in Ca^{2+}- and Mg^{2+}-free medium. We conclude that in the absence of Ca^{2+} and Mg^{2+}, sodium nitroprusside and insulin have very similar regulatory properties on triglyceride, protein synthesis and adrenaline-stimulated lipolysis, but not on cGMP levels in rat adipocytes. This could provide evidence that omission of bivalent cations was inhibitory at more than one site, or that sodium nitroprusside mimics insulin's actions by another mechanism that does not involve cGMP.

Ca^{2+} Mg^{2+} cyclic GMP Insulin Sodium nitroprusside (Rat adipocyte)

1. INTRODUCTION

We have shown that sodium nitroprusside can mimic several of the effects of insulin on the metabolism of rat isolated adipocytes [1]. Both agents raise cyclic GMP levels [2–8], stimulate conversion of glucose to triacylglycerol, inhibit adrenaline-stimulated lipolysis, and stimulate the incorporation of leucine into protein [1]. However, the two agents differ in their effects on glucose transport into adipocytes. This is stimulated by insulin but sodium nitroprusside has no effect [1]. There is also considerable evidence that the effects of insulin are influenced by the bivalent cation composition of the incubation medium [7–11]. For instance, we have shown that insulin does not express many of its usual metabolic effects in adipocytes when these cells are incubated in a (Ca^{2+} + Mg^{2+})-free medium [10,11]. The purpose of the present study was to investigate the similarity between the effects of insulin and sodium nitroprusside on adipocyte metabolism by comparing their actions in normal and Ca^{2+}- and/or Mg^{2+}-depleted media. Such a study might also indicate where bivalent cations are required in the sequence of events by which insulin stimulates fat cell metabolism.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Glycerol kinase, pyruvate kinase, lactate dehydrogenase, glucose oxidase, peroxidase, nucleotides, and other fine chemicals were purchased from Boehringer (London); collagenase (from *Clostridium histolyticum*) was obtained from Worthington through Millipore (Park Royal, London). [^{14}C]Glucose (2–3 mCi/nmol), and [^{14}C]leucine (59 mCi/mmol), were obtained from Amersham International. All other chemicals were of Analar grade or the purest grade available.

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2.2. *Isolation and incubation of adipocytes*

Adipocytes were isolated from the epididymal adipose tissue of male albino Wistar rats fed ad libitum, weighing 150–200 g, as described [1]. The isolated cells were then washed 4 times with $(Ca^{2+} + Mg^{2+})$-free Krebs-Ringer-bicarbonate buffer containing 2% (w/v) purified bovine serum albumin which had been treated with EDTA to remove endogenous bivalent cations [10]. All media were gassed with O_2/CO_2 (19:1) to pH 7.35–7.40 before use, and maintained at 37°C under an atmosphere of O_2/CO_2 (19:1).

2.3. *Cell stability*

Cell stability was assessed by measuring the leakage of the cytoplasmic enzyme lactate dehydrogenase into the incubation medium [12].

2.4. *Cyclic GMP assay*

Cyclic GMP concentration was determined in isolated adipocyte suspensions incubated for 2 min using the cyclic GMP radioimmunoassay kit from Amersham, as described in [1]. Results are expressed as pmol cyclic GMP per g adipocytes dry wt.

2.5. *Glucose uptake and metabolism*

Two methods have been used to assess the effect of bivalent cations on glucose uptake and triglyceride synthesis in the absence or presence of insulin or sodium nitroprusside. Total glucose removal from the incubation medium was used as a measure of the overall effect of insulin and sodium nitroprusside on glucose transport and metabolism in the cells. For this, the glucose remaining in the medium after incubation was assayed by a modification of the glucose oxidase method [13] using 2,2′-azinodi(3-ethylbenzthiazoline-sulphonate) as the chromogen. The uptake and retention within the cells of ^{14}C from [^{14}C]glucose was also used to follow glucose transport into and subsequent metabolism within the cells under these conditions. At the end of incubation, adipocytes were separated from the incubation medium using the oil (dinonyl phthalate) flotation method [14]. To measure incorporation of [^{14}C]-glucose into triacylglycerol, adipocytes were incubated in Krebs-Ringer-bicarbonate buffer containing 3 mM glucose and 0.1 μCi/ml [^{14}C]glucose. Triacylglycerol was extracted from the cells using chloroform/methanol/water (2:4:1.5, by vol.) [15]. Thin-layer chromatography of the chloroform-soluble extract showed that 85–90% of the label incorporated was in the triacylglycerol fraction. Results are expressed as nmol D-glucose taken up from the external medium, or as μgatom carbon incorporated into triacylglycerol/100 mg cells dry wt per 2 h.

2.6. *Incorporation of [^{14}C]leucine into adipocyte protein*

Incorporation of [^{14}C]leucine into material precipitable with 10% (w/v) trichloroacetic acid was taken to measure the rate of protein synthesis within the adipocytes [16]. After incubation with 0.1 Ci/ml [L-^{14}C]leucine, adipocytes (0.1 ml) were pipetted onto 30-mm diameter filter discs (Whatman no.41) and dried in air before being placed onto ice-cold 10% (w/v) trichloroacetic acid. The discs containing precipitated protein were washed 3 times with fresh trichloroacetic acid and then extracted with ethanol and diethyl ether to remove residual trichloroacetic acid and lipids. Recovery of ^{125}I-labelled albumin taken through this procedure was 102 ± 3%. Results are expressed as nmol [^{14}C]leucine incorporated/100 mg cell dry wt per 2 h.

2.7. *Lipolysis*

Adipocyte lipolysis was assessed by measurement of the glycerol [17] and the non-esterified fatty acids [18] produced during incubation of the cells.

2.8. *Statistics*

The results presented are representative experiments, each of which has been repeated at least 3 times. Results are expressed as the mean ± SE and the significance of any difference observed was assessed using Student's *t*-test.

3. RESULTS

The results in table 1 show the effects of insulin (7×10^{-10} M) and sodium nitroprusside (10^{-4} M) on the cyclic GMP content of adipocytes in the absence or presence of 2 mM $CaCl_2$. A significant reduction in basal cyclic GMP levels, from 5.3 to 1.8 pmol, was seen when calcium was omitted. The action of insulin to increase the concentration of

Table 1

Effect of extracellular Ca^{2+} on the concentration of cyclic GMP in rat isolated fat cell suspensions

Additions	Cyclic GMP concentration (pmol per g dry wt cells)	
	2 mM Ca^{2+}	No Ca^{2+}
None	5.3 ± 0.2	1.8 ± 0.5
Insulin (7×10^{-10} M)	30 ± 0.9	3.5 ± 0.5
Sodium nitroprusside (10^{-4} M)	29 ± 2.1	30 ± 1.2

Isolated rat fat cells were incubated in (Ca^{2+} + Mg^{2+})-free medium or in medium containing 2 mM calcium with 10^{-4} M sodium nitroprusside or 7×10^{-10} M insulin, as indicated, for 2 min. Assays of cyclic GMP were then performed as described in section 2. Values are means ± SE of 4 experiments performed in triplicate

cyclic GMP was dependent on the presence of Ca^{2+}. However, sodium nitroprusside increased cyclic GMP to the same level (29–30 pmol) whether Ca^{2+} was present or not.

The dependence on Ca^{2+} and Mg^{2+} for stimulation of glucose uptake by insulin and sodium nitroprusside in adipocytes is shown in table 2. Omission of Ca^{2+} and Mg^{2+} completely inhibited the ability of insulin to stimulate glucose uptake. Sodium nitroprusside had no significant effect under any of the conditions tested.

Further experiments were undertaken to assess the importance of bivalent cations for glucose metabolism in isolated adipocytes and for cellular integrity (table 3). In the absence of insulin or sodium nitroprusside, isolated adipocytes take up and incorporate [^{14}C]glucose into triacylglycerol at a basal rate that proved to be independent of the presence or absence of Ca^{2+} and Mg^{2+}. The uptake and retention of [^{14}C]glucose gave consistently lower values than did the glucose oxidase method even with the same preparation of cells, although the overall pattern of responses was always the same. The most probable explanation for this is the metabolism of some of the [^{14}C]glucose taken up by the cells into products that were released from the cells. Insulin (7×10^{-10} M) stimulated triacylglycerol synthesis (4-fold) and glucose uptake (3-fold) only when extracellular Ca^{2+} and/or Mg^{2+} were present. Sodium nitroprusside stimulated incorporation into triacylglycerol 2-fold when the medium contained Ca^{2+} and Mg^{2+}, but had no effect in the absence of these ions. Sodium nitroprusside did not affect total glucose uptake under any incubation conditions. The (Ca^{2+} + Mg^{2+})-free medium led to the appearance of 22–25% of the cellular lactase dehydrogenase activity in the medium. Control cells leaked only 4–9% of the enzyme. Insulin routinely caused a small decrease in the amount of lactate dehydrogenase leaked, but only in the presence of Ca^{2+} and Mg^{2+}. Sodium nitroprusside had no effect on cell viability under any conditions.

Table 2

Effect of calcium and magnesium on D-glucose uptake in isolated rat adipocytes in the presence or absence of insulin and sodium nitroprusside

Additions (mM)		D-Glucose uptake (μmol/100 mg dry wt cell per 2 h)		
Ca^{2+}	Mg^{2+}	None	Insulin (7×10^{-10} M)	Sodium nitroprusside (10^{-4} M)
–	–	1.79 ± 0.19	1.97 ± 0.1	1.62 ± 0.09
2	–	1.92 ± 0.2	7.11 ± 0.06	1.97 ± 0.06
–	2	1.62 ± 0.11	7.25 ± 0.08	1.99 ± 0.17
2	2	1.94 ± 0.99	7.5 ± 0.14	2.15 ± 0.17

Rat isolated fat cells were prepared in normal medium but washed and incubated at 37°C in (Ca^{2+} + Mg^{2+})-free Krebs bicarbonate buffer medium for 2 h. Glucose uptake was assayed by the glucose oxidase method [13]. Results are the means ± SE of 4 experiments performed in triplicate

Table 3

Effects of (Ca^{2+} + Mg^{2+})-free medium on insulin- and sodium nitroprusside-stimulated triacylglycerol synthesis, glucose uptake and lactate dehydrogenase release in rat isolated adipocytes

Additions	Ca^{2+},Mg^{2+} at 2 mM	Glucose uptake (μmol/100 mg cell dry wt per 2 h)	[^{14}C]Glucose uptake (μmol/100 mg dry wt per 2 h)	Triacylglycerol synthesis (μgatom C incorporated/100 mg cell dry wt per 2 h)	LDH leakage
None	–	2.23 ± 0.12	1.72 ± 0.11	3.06 ± 0.72	23 ± 4
	+	2.52 ± 0.14	1.78 ± 0.17	3.12 ± 0.54	8 ± 1
Insulin	–	6.95 ± 0.2	5.75 ± 0.08	12.66 ± 1.44	4 ± 1
	+	2.38 ± 0.21	1.61 ± 0.17	3.72 ± 0.42	22 ± 5
Sodium nitroprusside	–	2.24 ± 0.09	1.52 ± 0.06	2.94 ± 0.54	25 ± 4
	+	2.54 ± 0.11	1.76 ± 0.07	7.02 ± 0.66	9 ± 2

Cells were prepared as described in section 2, washed in the (Ca^{2+} + Mg^{2+})-free medium and incubated for 2 h with Ca^{2+},Mg^{2+} (2 mM), insulin (7×10^{-10} M) and sodium nitroprusside (10^{-4} M). Glucose uptake was measured by the glucose oxidase method, and triacylglycerol synthesis or ^{14}C glucose uptake by the oil flotation method. Results are the means ± SE of 4 experiments

The results in table 4 show that the incorporation of [^{14}C]leucine into adipocyte protein depended on the presence of bivalent cations under basal conditions and in the presence of insulin or sodium nitroprusside. No stimulation of protein synthesis in adipocytes was seen when Ca^{2+} and Mg^{2+} were omitted. Basal rates of incorporation were also reduced in the absence of these ions. The addition of both Ca^{2+} and Mg^{2+} allowed a stimulation of leucine incorporation by insulin or sodium nitroprusside that was little greater than that seen when Mg^{2+} alone was added.

The antilipolytic effect of insulin and sodium nitroprusside has also been investigated in the absence and presence of extracellular Ca^{2+} and Mg^{2+} (table 5). In the unstimulated state there is a

Table 4

Effect of extracellular calcium and magnesium on insulin- or sodium nitroprusside-stimulated [^{14}C]leucine incorporation into protein in rat isolated adipocytes

Cation added (mM)		[^{14}C]Leucine incorporation (nmol/100 mg dry wt cells per 2 h)		
Ca^{2+}	Mg^{2+}	None	Insulin (7×10^{-10} M)	Sodium nitroprusside (10^{-4} M)
–	–	0.02 ± 0.002	0.02 ± 0.0001	0.02 ± 0.001
2	–	0.21 ± 0.06	0.46 ± 0.04	0.32 ± 0.04
–	2	0.29 ± 0.03	0.62 ± 0.06	0.44 ± 0.05
2	2	0.32 ± 0.05	0.69 ± 0.09	0.45 ± 0.01

Rat isolated fat cells were prepared in normal Krebs-Ringer bicarbonate, washed and incubated in the (Ca^{2+} + Mg^{2+})-free medium with the additions shown above. [^{14}C]Leucine incorporation into protein was estimated at the end of 2 h incubation as described in section 2. Values are the means ± SE of 4 experiments

Table 5

Effect of extracellular calcium and magnesium on the antilipolytic effect of insulin: comparison with the effect of sodium nitroprusside on glycerol release induced by adrenaline in rat isolated adipocytes

Ca^{2+} (2 mM)	Mg^{2+} (2 mM)	Adrenaline (3 μM)	None	Insulin (7×10^{-10} M)	Sodium nitroprusside (1×10^{-4} M)
−	−	−	0.19 ± 0.07	0.21 ± 0.07	0.19 ± 0.08
−	−	+	0.57 ± 0.09	0.37 ± 0.08	0.41 ± 0.07
+	−	+	1.23 ± 0.26	0.61 ± 0.09	0.62 ± 0.08
−	+	+	0.84 ± 0.09	0.47 ± 0.08	0.48 ± 0.08
+	+	+	1.31 ± 0.21	0.78 ± 0.09	0.72 ± 0.09

Rat isolated fat cells were prepared in normal Krebs Ringer bicarbonate, washed and incubated in the (Ca^{2+} + Mg^{2+})-free medium with the additions shown above. Medium was analysed for glycerol release after 1 h incubation, as described [17]. Results are the means ± SE of 4 experiments

slow release of glycerol from the adipocytes incubated in the absence of bivalent cations, and this rate was unaltered by insulin or sodium nitroprusside. In the absence of extracellular Ca^{2+} or Mg^{2+}, adrenaline (3 μM) stimulated lipolysis, but it was less effective than in the presence of a bivalent cation. Addition of a bivalent cation increased the response to adrenaline, at equimolar concentrations Ca^{2+} being more effective than Mg^{2+}. The adrenaline response seen in the presence of Ca^{2+} alone was almost as great as that seen in the presence of both Ca^{2+} and Mg^{2+}. Under all conditions of added Ca^{2+} and Mg^{2+}, either singly or together, both insulin and sodium nitroprusside significantly inhibited glycerol release. Similar effects were seen when the production of nonesterified fatty acids was monitored under these experimental conditions (not shown).

4. DISCUSSION

Our results indicate that many of the metabolic effects of insulin on isolated adipocytes are expressed only in the presence of Ca^{2+} and/or Mg^{2+} in the intracellular medium. The one exception to this is its antilipolytic effect (table 5) since insulin reduced glycerol production by adipocytes even in the (Ca^{2+} + Mg^{2+})-free medium. One of the major actions of insulin is to increase the rate of glucose transport and its intracellular metabolism. Both of these actions are expressed only in the presence of an extracellular bivalent cation (tables 2,3; and [10,11]). Other studies have suggested that Ca^{2+} may regulate glucose transport in adipose tissue and muscle [19–21]. In contrast to the effect of insulin, the basal uptake and metabolism of glucose is insensitive to bivalent cations and this is consistent with previous studies [10,22]. Sodium nitroprusside could not be investigated in this system since it does not affect glucose transport [1].

The stimulatory effects of insulin and sodium nitroprusside on the incorporation of ^{14}C from [^{14}C]glucose into triacylglycerol were not seen in the absence of extracellular Ca^{2+} and Mg^{2+} (table 3). Since sodium nitroprusside increased cyclic GMP in adipocytes in the (Ca^{2+} + Mg^{2+})-free medium (table 1), this implies that Ca^{2+} and/or Mg^{2+} is required for cyclic GMP to act on lipogenesis in these cells.

It has been reported that rat isolated adipocytes require millimolar concentrations of Ca^{2+} in the extracellular medium to sustain maximal rates of protein synthesis in the presence of insulin [23]. An absolute requirement for extracellular bivalent cations for basal incorporation of [^{14}C]leucine into adipocyte protein and for the stimulation by insulin is demonstrated in table 4. The inhibition of basal incorporation into protein in the (Ca^{2+} + Mg^{2+})-free medium is in contrast to the lack of effect of this medium on basal glucose uptake and metabolism. The stimulation of

[^{14}C]leucine incorporation into protein by sodium nitroprusside was also abolished by incubation of the cells in the (Ca^{2+} + Mg^{2+})-free medium, in contrast to the lack of effect of this medium on basal glucose uptake and metabolism. Like the effect of insulin, the stimulation by sodium nitroprusside was slightly greater with Mg^{2+} alone than with Ca^{2+} alone, although neither condition was as effective as the presence of both Ca^{2+} and Mg^{2+}.

The results in table 5 show that the stimulation of lipolysis by a maximal concentration of adrenaline was reduced but not abolished in adipocytes incubated in the (Ca^{2+} + Mg^{2+})-free medium. Magnesium alone partially and Ca^{2+} or (Ca^{2+} + Mg^{2+}) fully restored the stimulation. Ca^{2+} is known to be required for adrenocorticotrophin to stimulate lipolysis in adipose tissue [24], and adrenaline has been reported to stimulate release of fatty acids from adipose tissue incubated in a (Ca^{2+} + Mg^{2+})-free medium [24,25]. The removal of Ca^{2+} has been shown to reduce the effectiveness of submaximal concentrations of insulin but the maximal was antilipolytic in the absence of Ca^{2+} and Mg^{2+}. Under these conditions insulin had little effect on cyclic GMP levels in the adipocytes (table 1), suggesting that the antilipolytic action of insulin is, at least in part, independent of cyclic GMP. On the other hand, sodium nitroprusside was also antilipolytic in the (Ca^{2+} + Mg^{2+})-free medium, but this agent increased cellular cyclic GMP levels. Thus, the effects of sodium nitroprusside and insulin clearly cannot be related solely to changes in the cyclic GMP concentration. The small rise in cyclic GMP seen in response to insulin in the (Ca^{2+} + Mg^{2+})-free medium is unlikely to be sufficient to bring about the observed inhibition of adrenaline-stimulated lipolysis, since the concentration of cyclic GMP produced was still below that present in the unstimulated state in the normal medium. Thus, cyclic GMP may modulate lipolysis, and may even play a role in the antilipolytic effect of insulin under normal conditions when the hormone can raise cyclic GMP levels in the cells, but it is unlikely to be the only mechanism involved. It is possible that in the (Ca^{2+} + Mg^{2+})-free medium, where adrenaline itself is a much less effective stimulus, the ability of insulin to lower intracellular cyclic AMP levels may be more significant, although in the normal state it has proved difficult to correlate quantitatively the fall in cyclic AMP levels in response to insulin and the inhibition of hormone-stimulated lipolysis observed [28]. A mechanism similar to that demonstrated in liver plasma membranes where insulin inhibits adenylate cyclase [29] and activates a membrane-bound cyclic nucleotide phosphodiesterase [30], perhaps through a specific guanine nucleotide regulatory protein, might be important here.

The present results show that insulin and sodium nitroprusside had very similar effects on triacylglycerol synthesis, protein synthesis and hormone-stimulated lipolysis in both the normal and the (Ca^{2+} + Mg^{2+})-free medium, but their effects on cyclic GMP levels differed markedly. In the (Ca^{2+} + Mg^{2+})-free medium nitroprusside increased cyclic GMP levels as usual while the stimulatory effect of insulin was very much reduced. This could imply that the (Ca^{2+} + Mg^{2+})-free medium was inhibitory at more than one site – in the case of insulin it blocked the stimulation of cyclic GMP levels by the hormone, while in the case of sodium nitroprusside, it blocked the effects of cyclic GMP on metabolism. An alternative explanation might be that sodium nitroprusside mimicks insulin's actions on metabolism, but through a mechanism, as yet unknown, that does not involve cyclic GMP.

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