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The role of cell swelling in the stimulation of glycogen synthesis by insulin

Molham AL-HABORI, Matthew PEAK, Trevor H. THOMAS and Loranne AGIUS*
Department of Medicine, Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K.

In hepatocyte cultures, insulin stimulates cellular accumulation of K^+, partly (~ 20%) by net replacement of cell Na^+, but largely (~ 80%) by increasing the cell $K^+ + Na^+$ content, with a consequent increase in cell volume. An increase in cation content occurred within 5 min of exposure to insulin and was not secondary to metabolic changes. Insulin also increased the cation content, by increasing the Na^+ content, in a K^+-free medium or when K^+ uptake was inhibited with 1 mM-ouabain. However, insulin did not increase the cation content in a Na^+-free medium. The stimulation of glycogen synthesis by insulin, like the increase in cation content, was blocked in a Na^+-free medium, but not when K^+ uptake was inhibited. Hypo-osmotic swelling restored the stimulation of glycogen synthesis in a Na^+-free medium, indicating that the lack of effect of insulin in the iso-osmotic Na^+-free medium was not due to a direct requirement for Na^+ for glycogen synthesis, but to a secondary mechanism, dependent on Na^+ entry, that can be mimicked by hypo-osmotic swelling. Quinine increased cell volume further and caused a further increase in glycogen synthesis. The hypothesis that cellular uptake of K^+ may be part of the mechanism by which insulin controls metabolism was discounted, because inhibition of K^+ uptake does not block the metabolic effects of insulin [Czech (1977) Annu. Rev. Biochem. **46**, 359–384]. The present results support the hypothesis that an increase in cell cation content, and thereby cell volume, rather than K^+ uptake, is part of the mechanism by which insulin stimulates glycogen synthesis in hepatocytes.

INTRODUCTION

Glycogen synthesis in hepatocytes is stimulated by amino acids [1] and by insulin [2]. The stimulation of amino acids can be explained, at least in part, by cell swelling [3]. The rate of glycogen synthesis in hepatocyte cultures and its stimulation by insulin are influenced by the extracellular $[CO_2]$ and $[HCO_3^-]$ through secondary ionic changes [4]. These observations raise the question whether cell swelling may also be involved in the stimulation of glycogen synthesis by insulin.

Cellular ionic changes are among the most rapid effects of insulin [5–7]. Insulin increases membrane potential in muscle within less than 1 s [8], and it causes rapid uptake of K^+ by splanchnic tissues [9] and skeletal muscle *in vivo* [10]. It inhibits loss of K^+ by the perfused liver [11] and stimulates K^+ accumulation by muscle and adipose tissue preparations *in vitro* [5]. From a theoretical standpoint, net cellular accumulation of K^+ in response to insulin *in vivo* or *in vitro* could occur either by net replacement of cell Na^+, with an increase in the cell K^+/Na^+ ratio, or by an increase in the cell cation content (K^+ plus Na^+), with a consequent increase in cell volume [12]. Although several studies have determined the effects of insulin on the rates of Na^+ and K^+ flux [6,7,13,14], there is little information on the effects of insulin on the steady-state content of K^+ plus Na^+.

The present study had two aims: first, to establish whether insulin causes accumulation of K^+ by net replacement of cell Na^+ or by an increase in the cell cation ($K^+ + Na^+$) content; secondly, to investigate whether changes in cell K^+ and/or Na^+ content caused by insulin are essential for stimulation of glycogen synthesis. We used hepatocyte cultures for these studies because we have established that this is a suitable model to study the short-term stimulation of glycogen deposition by insulin [2]. We show that insulin stimulates accumulation of K^+ in hepatocyte cultures partly by net replacement of cell Na^+, but largely by an increase in the cell cation content with a consequent increase in cell volume, and that insulin stimulates glycogen synthesis only in situations where it increases the cation content. Although a Na^+-free medium blocks both the increase in cation content and the stimulation of glycogen synthesis by insulin, hypo-osmotic swelling restores the stimulation of glycogen synthesis in a Na^+-free medium, suggesting that an increase in cell volume is part of the mechanism by which insulin stimulates glycogen synthesis in Na^+-containing medium.

MATERIALS AND METHODS

Materials

$H^{36}Cl$, 3-*O*-methyl-D-[^{14}C]glucose, [U-^{14}C]glucose and UDP-[^{3}H]glucose were from Amersham International (Amersham, Bucks., U.K.), and [^{14}C]inulin was from New England Nuclear (Boston, MA, U.S.A.). Ouabain, quinine hydrochloride, 3-*O*-methyl-D-glucose, phloretin, insulin and glucagon were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sources of other reagents were as reported previously [2].

Hepatocyte culture

Hepatocytes were isolated by collagenase perfusion of whole liver of male Wistar rats (270–340 g body wt.) fed on standard rat chow *ad libitum* [2]. They were cultured in Minimum Essential Medium (MEM) supplemented with 5% (v/v) neonatal-calf serum in multiwell plates at a density of 6.4×10^4 cells/cm^2 [2]. After cell attachment (4–6 h) they were cultured in serum-free MEM containing 10 nM-dexamethasone without insulin, except where indicated. All experiments were performed after culture in serum-free medium for at least 16 h [2].

Incubation media

The effects of insulin on the hepatocyte ion content and glycogen synthesis were determined either after incubation in MEM (which contains amino acids [15]) or after incubation in

Abbreviations used: MEM, Minimum Essential Medium.
* To whom all correspondence should be addressed.

balanced salts or modified salts solutions without amino acids. The balanced salts solution contained: 116 mM-NaCl, 12.5 mM-$NaHCO_3$, 20 mM-NaHepes, 5.1 mM-KCl, 1.23 mM-NaH_2PO_4, 1.23 mM-$CaCl_2$, 1.18 mM-$MgCl_2$, 0.8 mM-Na_2SO_4, pH 7.4, equilibrated with 2.5% CO_2. The hypo-osmolar medium was a similar composition, except that [NaCl] was 76 mM. The K^+-free solution was of similar composition to the balanced salts solution, except that KCl was omitted. The Na^+-free medium contained: 284 mM-sucrose, 12.5 mM-$KHCO_3$, 1.23 mM-KH_2PO_4, 1.23 mM-$CaCl_2$, 1.18 mM-$MgCl_2$, 0.8 mM-$MgSO_4$. The hypo-osmolar Na^+-free medium was of similar composition, except that [sucrose] was 204 mM. The media were supplemented with the substrates indicated.

Determination of K^+ and Na^+

For determination of the short-term (5–60 min) effects of insulin on the K^+ and Na^+ content, insulin was added, either to the medium in which the cells had been precultured (MEM containing dexamethasone), or after the medium was changed either to fresh MEM without dexamethasone (without or with added 10 mM-glucose), or with the salts solutions indicated containing 10 mM-glucose. On termination of the incubations the hepatocyte monolayers were washed four times with 100 mM-$MgCl_2$/10 mM-Tris, pH 7.0, and extracted in 1.5 mM-CsCl containing 0.1% (w/v) Triton X-100. The extracts were sonicated for 5 s, a sample was taken for determination of protein, and K^+ and Na^+ were determined by flame photometry.

Preliminary experiments established that there was no detectable loss of K^+ during the washing. These experiments involved two sets of triplicate wells: one was drained on termination of the incubation and extracted without washing, and the other was washed as described above. In the former set the cell K^+ content was determined by assuming that the total Na^+ content represented extracellular Na^+, and from this extracellular K^+ was estimated (from the Na^+/K^+ ratio of the medium) and subtracted from the total K^+. The values for cell K^+ measured in the washed cells were similar to the values estimated in the unwashed cells.

Determination of Cl^-

The hepatocytes were preincubated for a minimum of 2 h in MEM or in balanced salts solution containing $^{36}Cl^-$ (1–2 μCi/ml). They were then incubated with the additions indicated for 60 min. On termination of the final incubation, the hepatocytes were washed four times with 280 mM-sucrose/5 mM-Tris, pH 7.0, extracted in water, and the radioactivity was determined.

Determination of cell water

The hepatocytes were incubated for 60 min in balanced salts medium without added glucose and containing 10 mM-3-*O*-methyl-D-[^{14}C]glucose (1 μCi/ml) as described previously [16], except that incubations were terminated with three or four washes with 150 mM-NaCl containing 0.2 mM-phloretin and 0.1% (v/v) ethanol. It was established in preliminary studies that: (i) accumulation of 3-*O*-methyl[^{14}C]glucose had reached equilibrium (usually after 10–15 min); (ii) there was no detectable (< 1%) incorporation or irreversible sequestration of ^{14}C radioactivity, as determined from a 60 min wash-out period in cells preloaded with 3-*O*-methyl[^{14}C]glucose for 60 min. Cell K^+ was also determined in parallel incubations containing unlabelled 10 mM-3-*O*-methyl-D-glucose (without glucose) and washed with 100 mM-$MgCl_2$/10 mM-Tris, pH 7.0, as described above.

Glycogen synthesis

This was determined during either a 3 h incubation (MEM) or a 2 h incubation (salts solutions, without amino acids). The media contained 15 mM-[U-^{14}C]glucose (1.5 μCi/ml) and 5 mM-pyruvate. The incubations were terminated as described previously [2], and glycogen synthesis was determined from the incorporation of [^{14}C]glucose into glycogen, isolated by ethanol precipitation [2], and glycogen deposition was determined from the incremental increase in cellular glycogen, determined enzymically [2], and are expressed as nmol of glucose incorporated or deposited as glycogen per time interval per mg of protein.

Glycogen synthase (EC 2.4.1.11) activity

This was determined with either 0.1 mM-glucose 6-phosphate (synthase *a*) or with 4 mM-glucose 6-phosphate (total activity) as in [17], except that Na_2SO_4 was omitted. The activity of synthase *a* is expressed as a percentage of total activity.

Protein was determined by an automated Lowry method [18]. For determination of ion or water content incubations were in triplicate for each experimental condition, and for metabolic studies they were in duplicate for each condition. Ion content is expressed as nmol/mg of protein and water content, which represents the glucose-permeant water space [16], as μl/mg of protein. Values are means ± S.E.M. for the numbers of cell-culture preparations (experiments) indicated. Statistical analysis was by Student's paired *t* test.

RESULTS

Effects of insulin on the hepatocyte K^+ and Na^+ content in media with or without amino acids

Hepatocytes pre-cultured for 16 h with 10 nM-insulin had a higher K^+ content per mg of protein than controls cultured without insulin (287 ± 15 versus 213 ± 10 nmol of K^+/mg of protein; means ± S.E.M., $n = 5$, $P < 0.0025$). Table 1 shows the effects of culture for 16 h with 1 nM-, 10 nM- or 100 nM- insulin on the cell K^+ content and $K^+ + Na^+$ content. When insulin (10 nM) was added during the last 60 min, it increased the K^+ content and the total K^+ and Na^+ content in cells pre-cultured without insulin or with 1 nM-insulin (Table 1).

In the rest of this study, short-term (5–60 min) effects of insulin on K^+ and Na^+ were determined in cells pre-cultured without insulin. The increase in cell K^+ content on addition of insulin occurred within the first 5–15 min and approached a plateau after about 15 min (Fig. 1). The increment in K^+ content during 60 min incubation with insulin (Table 2) was 11–17%, and the increment in the total K^+ plus Na^+ content was 9–14% in experiments where insulin was added either directly to the medium in which the cells had been pre-cultured (Table 2, *a*: MEM containing 10 nM-dexamethasone); or when insulin was added after the medium was changed to fresh MEM without dexamethasone and either without or with added 10 mM-glucose (Table 2, *b* and *c*). Since there is no deposition of glycogen during incubation in MEM without added glucose [2], these results indicate that the increase in cation content was not secondary to glycogen deposition.

An increase in cation content was also observed in a balanced salts medium without amino acids (Table 2, *d*), indicating that it is not secondary to uptake of amino acids. The K^+/Na^+ ratio was increased by insulin in MEM and in the balanced salts medium (Table 2). In molar terms the decrease in the Na^+ content could account for only about 20% of the increment in K^+, indicating that the increment in K^+ is largely due to the increase in total cation (K^+ and Na^+) content.

Effects of insulin on the hepatocyte K^+ and Na^+ content in the presence of ouabain and in K^+-free or Na^+-free media

Three mechanisms have been proposed to explain the stimulation of cellular uptake of K^+ by insulin: Moore [6] proposed

Table 1. Effects of long-term (16 h) or short-term (1 h) culture with insulin on the cell K^+ and Na^+ content of hepatocytes

Hepatocytes were cultured for 16 h with 10 nM-dexamethasone and 1 nM-, 10 nM- or 100 nM-insulin as indicated. During the last 60 min of the incubation 10 nM-insulin was added to one set of cultures; the other set received saline. Cell K^+ or K^+ plus Na^+ are expressed as nmol/mg of cell protein. Values are means ± S.E.M. for triplicate wells for five cultures.

[Insulin] (nM) during 16 h culture	Insulin during 60 min...	K^+ content (nmol/mg of protein)		$K^+ + Na^+$ content (nmol/mg of protein)	
		None	Insulin	None	Insulin
0		222±4	258±5	244±2	276±9
1		252±6	323±3	269±7	338±5
10		318±4	324±10	335±3	354±11
100		323±12	318±9	341±10	337±12

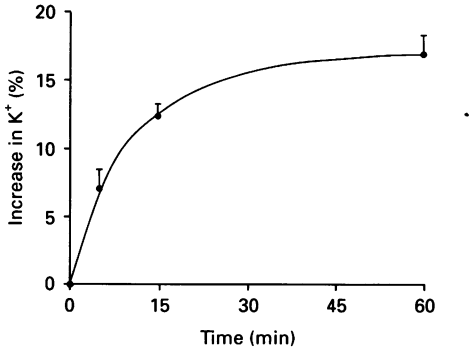

Fig. 1. Time course of the effects of insulin on the hepatocyte K^+ content

Hepatocytes were pre-cultured for 16 h in MEM without insulin. The medium was then changed with fresh MEM supplemented with 10 mM-glucose (without dexamethasone). The hepatocytes were incubated with 10 nM-insulin for the indicated time intervals. The increase in the cell K^+ content caused by insulin is expressed as a percentage of controls incubated without insulin. Values are means ± S.E.M. for five cultures.

that this occurs by activation of Na^+/K^+-ATPase; Zierler [7] proposed that it occurs by inhibition of K^+ efflux [7]; and Fehlmann & Freychet [13] suggested primary activation of Na^+/H^+ exchange with secondary Na^+/K^+ exchange as a result of increased Na^+ entry. The latter proposal was based on the finding that amiloride, an inhibitor of Na^+/H^+ exchange, inhibited the stimulation of Rb^+ influx by insulin [13]. However, amiloride also inhibits the tyrosine kinase activity of the insulin receptor [19]. It is therefore not a sufficiently specific inhibitor to establish the role of Na^+/H^+ exchange in insulin action. To establish whether activation of Na^+/K^+-ATPase and/or stimulation of Na^+-entry mechanisms are part of the mechanism by which insulin increases the cell cation content, we determined the effects of ouabain, an inhibitor of Na^+/K^+-ATPase [6], and of K^+-free and Na^+-free media on the effects of insulin on the cell cation content in media without amino acids (Table 2, *d–g*).

During incubation with 1 mM-ouabain, the cell K^+ content decreased and the Na^+ content increased (Table 2, *e*), consistent with inhibition of Na^+/K^+-ATPase, which maintains the transcellular K^+ and Na^+ gradients. Insulin increased the Na^+ content further ($P < 0.01$) and slightly counteracted the decrease in K^+ caused by ouabain. The increase in Na^+ is consistent with direct stimulation by insulin of Na^+ entry. The diminished decrease in K^+ could be due to inhibition of K^+ efflux [7]. Insulin caused a similar increase (15–20 nmol/mg) in the cation content ($K^+ + Na^+$) in the presence of ouabain as in its absence (Table 2, *e* versus *d*). Similar changes in K^+ and Na^+ were observed in a K^+-free medium (Table 2, *f*), suggesting that the effects of ouabain are due to inhibition of K^+ uptake. When hepatocytes were incubated in a Na^+-free medium, insulin did not significantly affect the total cell K^+ plus Na^+ content. This contrasts with the 9–14% increase in the K^+ plus Na^+ content by insulin in the other incubation media (Table 2, *a–f*). The increase in cation content caused by insulin in the presence of Na^+, but not in its absence, is consistent with a primary effect of insulin on Na^+-entry mechanisms [20]. However, there was an increase in the K^+/Na^+ ratio by insulin in the control medium, but not when K^+ entry was inhibited, owing to a decrease in Na^+ as well as an increase in K^+. This suggests that, in addition to increased Na^+/K^+ exchange because of stimulation of Na^+ entry, there is also independent stimulation by insulin of ouabain-sensitive K^+/Na^+ exchange, as suggested by Moore [6].

Effects of insulin on the hepatocyte Cl^- content

The two most abundant permeant anions in the cell are Cl^- and HCO_3^-. We determined the effects of insulin (10 nM) on the cell Cl^- content in MEM and in balanced salts media, without or with 12.5 mM-HCO_3^- (replaced by equimolar NaHepes). The Cl^- content of hepatocytes incubated in MEM was increased by 12% with insulin (control 33.5±3.9, insulin 37.4±3.6 nmol/mg of protein; means ± S.E.M., $n = 4$, $P < 0.01$). In molar terms, this increment (3.88±0.65 nmol of Cl^-/mg of protein) was smaller than the increment in K^+ (32.3±3.8 nmol of K^+/mg of cell protein). In the balanced salts medium the Cl^- content was higher in the absence than in the presence of HCO_3^- and showed a larger increase by insulin in the bicarbonate-free medium (control 39.5±3.2, insulin 51.5±6.3; means ± S.E.M., $n = 6$) than in the bicarbonate-containing medium (control 22.0±2.0, insulin 25.5±1.6), suggesting that in the presence of a CO_2/HCO_3^- system an increase in cell HCO_3^- may contribute to the increase in anions.

Determination of cell water

Various techniques involving use of extracellular markers such as inulin [21], have been used to measure cell volume of hepatocyte cultures. We found substantial uptake of [^{14}C]inulin by hepatocytes (equivalent to 40% of cell volume) during 5 min incubation with 1 mg of inulin/ml, consistent with previous studies [22]. We therefore measured the cell water content from the accumulation of 3-*O*-methyl[^{14}C]glucose [16], because this technique does not involve use of extracelluar markers. The main limitation of this technique, which measures the glucose-permeant water space, is that it can only be used in a glucose-free medium [16]. Consequently, cell water could not be determined under parallel conditions as for the metabolic studies. However, in additional

Table 2. Effects of insulin on the K^+ and Na^+ content of hepatocytes

Hepatocytes were pre-cultured for 16 h in MEM containing 10 nM-dexamethasone and were then incubated for 60 min without or with 10 nM-insulin either added directly to the medium in which the cells had been pre-cultured (*a*) or after the medium was replaced with fresh MEM (*b*, *c*) or with balanced salts (*d*, *e*) or modified salts media (*f*, *g*) without amino acids (*d*–*g*). The cell K^+ and Na^+ contents are expressed as nmol/mg of cell protein or as the percentage increase caused by insulin relative to the respective controls. Values are means ± S.E.M. for the numbers of cell cultures indicated in parentheses. Statistical analysis (paired *t* test), insulin versus respective control: $*P < 0.05$, $**P < 0.01$.

	Content (nmol/mg of protein)			
	K^+	Na^+	$K^+ + Na^+$	K^+/Na^+
(*a*) No change of medium (3)	215±13	20±2	235±15	10.95±0.80
+ insulin	241±18*	15±1	256±19*	16.55±0.84*
% increase	11.9±2.5		8.8±3.6	
(*b*) MEM (4)	214±9	24±4	238±9	9.32±1.43
+insulin	247±8*	16±2	262±10*	15.61±1.59*
% increase	15.7±3.3		10.1±1.9	
(*c*) MEM + 10 mM-glucose (4)	216±19	19±2	235±18	11.50±1.87
+insulin	246±15*	18±2	264±14*	13.82±2.00
% increase	14.2±3.6		13.6±3.2	
(*d*) Balanced salts (4)	202±11	19±2	221±12	11.28±0.45
+insulin	225±11*	16±1	239±13*	14.52±0.78*
% increase	11.4±0.9		9.1±1.1	
(*e*) +1 mM-ouabain (4)	141±9	70±2	210±9	2.03±0.15
+insulin	148±12	83±2**	230±11*	1.80±0.15
% increase	4.9±2.8		9.6±1.2	
(*f*) K^+-free medium (3)	120±3	74±2	193±4	1.63±0.04
+insulin	127±3	81±2*	208±4*	1.56±0.03
% increase	5.8±0.9		7.8±0.6	
(*g*) Na^+-free medium (4)	219±15	6±2	225±16	44.7±9.8
+insulin	222±11	4±1	224±9	67.3±22.7

Table 3. Effects of insulin on the water and K^+ content of hepatocytes

Hepatocytes were cultured for 16 h in MEM containing 10 nM-dexamethasone and the concentrations of insulin indicated. They were then incubated for 60 min in balanced salts medium without glucose and containing 10 mM-3-*O*-methyl-D-[^{14}C]glucose. Cell water and K^+ were determined as described in the Materials and methods section. Cell water is expressed as μl/mg of cell protein and cell K^+ as nmol/mg of cell protein or nmol/μl of cell water. Results are means ± S.E.M. for five cultures. Statistical analysis (paired *t* test), insulin versus respective control: $*P < 0.05$, $**P < 0.005$. The values in parentheses show the effect of insulin expressed as a percentage of the respective controls.

[Insulin] (nM)	Cell water (μl/mg)	Cell K^+ (nmol/mg)	$[K^+]$ (nmol/μl)
Control	1.71±0.18 (100)	190±17 (100)	114±4
1	1.80±0.21 (105)	203±13* (107)	115±7
10	1.92±0.21* (112)	219±15** (115)	116±7
100	2.05±0.19** (120)	223±17** (117)	110±5

experiments we determined the cation content under similar conditions as for the water determinations.

The water content of hepatocytes pre-cultured for 16 h without or with insulin was determined after incubation for 60 min in glucose-free balanced salts medium containing 10 mM-3-*O*-methyl-D-[^{14}C]glucose [16]. Hepatocytes cultured with insulin (10–100 nM) had a higher water content (12–20%) in relation to protein than controls cultured without hormone (Table 3). In these experiments the K^+ content per mg of protein showed a similar percentage increment with insulin (15–17%) to the water content (Table 3), and consequently there was no significant difference in $[K^+]$ (nmol/μl of water) between control and insulin-cultured cells (Table 3). The difference in K^+ content (nmol/mg of protein) between cells cultured with insulin (10 nM and 100 nM) and controls was lower after the 60 min incubation with 3-*O*-methyl-D-glucose (10% and 13% increases respectively at 10 nM- and 100 nM-insulin) than after termination of the 16 h culture, before incubation with 3-*O*-methyl-D-glucose (16% and 29% increases respectively). This indicates that incubation with 3-*O*-methyl-D-glucose in a glucose-free medium diminishes, but does not abolish, the increment in K^+ content between insulin-cultured and control cells.

When hepatocytes pre-cultured without insulin were incubated with 3-*O*-methyl-D-glucose for 60 min (in glucose-free medium) without or with 10 nM-insulin, the water content was higher in the presence of insulin by 4% (control 1.74±0.09, insulin 1.81±0.10 μl/mg; means ± S.E.M., $n = 13$, $P < 0.001$) and the K^+ content by 5% (control 211±10, insulin 222±10 nmol/mg; means ± S.E.M., $n = 4$, $P < 0.005$), indicating that insulin increases the cell water content during short-term incubations with 3-*O*-methyl-D-glucose by approximately the same extent as the increment in K^+. When hepatocytes were preincubated with 10 mM-3-*O*-methylglucose for 60 min before addition of insulin and incubations were terminated after either 5 min or 60 min, an increase in cell volume was also observed after 5 min (control 1.86±0.02, 5 min insulin 1.93±0.05, 60 min insulin 1.99±0.02 μl/mg of protein; means ± S.E.M., $n = 3$).

Effects of ouabain and of K^+-free or Na^+-free media on the stimulation of glycogen synthesis by insulin

Since insulin increased the cell cation content in MEM and in balanced salts medium (by increasing the K^+ content) and in the presence of ouabain and in the K^+-free medium (by increasing the Na^+ content) but not in the Na^+-free medium (Table 2), we

Table 4. Effects of insulin and ouabain on glycogen synthesis

Hepatocytes pre-cultured for 16 h in MEM without insulin were incubated for 3 h in fresh MEM containing 15 mM-[U-^{14}C]glucose and 5 mM-pyruvate and either 12.5 mM- or 25 mM-HCO_3^- and equilibrated with either 2.5% or 5% CO_2 in either the absence or presence of 10 nM-insulin or 1 mM-ouabain as indicated. Glycogen synthesis is expressed as nmol of [^{14}C]glucose incorporated into glycogen/3 h per mg of cell protein. Values are means ± S.E.M. for four cultures. Statistical analysis was by the paired *t* test: $^aP < 0.05$, $^bP < 0.005$, 5% CO_2 versus 2.5% CO_2; $*P < 0.05$, insulin versus respective control.

			Glycogen synthesis (nmol/3 h per mg of protein)			
			Control		Ouabain	
$[CO_2]$ (%)	$[HCO_3^-]$ (mM)	Insulin...	−	+	−	+
2.5	12.5		43±5	62±12*	43±5	65±11
2.5	25		26±4	34±7	33±3	49±5*
5	12.5		73±3^b	100±11*,^{a}	32±6^a	50±6*
5	25		59±8^a	80±15^a	32±4	48±6*

Table 5. Effects of Na^+, K^+ and hypo-osmolarity on the stimulation of glycogen synthesis by insulin

Hepatocytes pre-cultured for 16 h in MEM without insulin were incubated for 2 h in balanced salts medium (without amino acids) without or with Na^+ or K^+ as indicated. The hypo-osmolar Na^+-containing and Na^+-free media were prepared as described in the Materials and methods section. All media contained 15 mM-glucose and 5 mM-pyruvate without or with insulin as indicated. Glycogen synthesis is expressed as nmol of glucose units deposited as glycogen/2 h per mg of cell protein. Values are means ± S.E.M. for five cultures. Insulin versus respective control: $*P < 0.05$, $**P < 0.005$.

		Glycogen synthesis		
Media	Insulin	Balanced	Na^+-free	K^+-free
Iso-osmolar	−	29±2	15±2	31±3
	+	44±2**	14±3	42±3*
Hypo-osmolar	−	51±1	41±6	
	+	60±2*	64±3*	
Hypo-osmolar + 0.1 mM-quinine	−	62±3	50±3	
	+	70±9	67±5*	

determined the effects of ouabain (Table 4) and of K^+-free and Na^+-free media (Table 5) on the stimulation of glycogen synthesis by insulin to establish whether changes in glycogen synthesis are associated with changes in cell K^+ and/or Na^+ content. Because both the basal rate of glycogen synthesis and the insulin-stimulated rate are influenced by the CO_2 and HCO_3^- concentrations, probably through changes in cell volume [4], we determined the effects of ouabain in MEM equilibrated with different concentrations of CO_2 and HCO_3^- to establish whether effects of pH changes and insulin on glycogen synthesis are dependent on K^+-uptake mechanisms (Table 4).

Increasing the extracellular CO_2/HCO_3^- ratio decreases extracellular and intracellular pH, whereas increasing $[CO_2]$ at a constant CO_2/HCO_3^- ratio decreases intracellular pH, since cells are more permeable to CO_2 than to HCO_3^- [23]. In the absence of ouabain, at each $[HCO_3^-]$, rates of glycogen synthesis were higher ($P < 0.005$) at 5% CO_2 than at 2.5% CO_2, and at a constant CO_2/HCO_3^- ratio rates of glycogen synthesis were also higher ($P < 0.001$) at 5% CO_2 (25 mM-HCO_3^-) than at 2.5% CO_2 (12.5 mM-HCO_3^-), suggesting that extracellular and intracellular acidification increase glycogen synthesis. Ouabain abolished the stimulation by 5% as compared with 2.5% CO_2, but it did not abolish the stimulation by insulin (Table 4).

Insulin increased glycogen synthesis in the balanced salts medium and in the K^+-free medium, but not in the Na^+-free medium (Table 5). Insulin binding determined with A_{14}-mono[^{125}I]iodoinsulin [24] was not impaired in the Na^+-free medium when determined with tracer alone or with 1 nM- or 10 nM-insulin (results not shown), indicating that the lack of stimulation of glycogen synthesis in the Na^+-free medium is not due to impaired insulin binding.

Effects of hypo-osmolarity on glycogen synthesis, glycogen synthase and cell volume

Since Na^+ is involved in the co-transport of various ions, including H^+, HCO_3^-, Ca^{2+} and possibly also Mg^{2+} and PO_4^{3-}, the suppression of the effect of insulin in a Na^+-free medium could be due to secondary ionic changes. To distinguish between impaired cell swelling in a Na^+-free medium as distinct from secondary ionic changes as a result of lack of Na^+, we determined the effects of hypo-osmotic swelling on glycogen synthesis (by decreasing [NaCl] in the Na^+-containing medium by 40 mM and by decreasing [sucrose] in the Na^+-free medium by 80 mM). When hepatocyte suspensions are incubated in hypo-osmolar media, they initially swell osmotically (within 2 min) and subsequently (5–10 min) partially down-regulate their volume [25,26]. This regulated decrease in volume can be inhibited by quinine [25]. We therefore examined the effects of hypo-osmolarity in the absence and presence of 0.1 mM-quinine (Table 5). Preliminary experiments showed that the effects of quinine on cell volume and glycogen synthesis were biphasic, with maximum effects in the range 0.1–0.2 mM. Higher concentrations of quinine (1 mM) caused increased leakage of lactate dehydrogenase.

Glycogen synthesis was increased by hypo-osmolarity in both the Na^+-containing medium ($P < 0.001$) and in the Na^+-free medium ($P < 0.01$), and quinine caused a further increase in glycogen synthesis in the hypo-osmolar media ($P < 0.05$; Table 5). Although insulin did not increase glycogen synthesis in the iso-osmolar Na^+-free medium ($P > 0.4$), it caused significant stimulation in the hypo-osmolar Na^+-free medium (Table 5).

The activity of glycogen synthase determined after 15 min exposure to insulin was increased ($P < 0.05$) in the balanced salts medium (control, 23±2%; insulin, 30±2%, synthase *a* as % total activity; means ± S.E.M., $n = 4$), but not in the Na^+-free medium (control, 26±4%; insulin, 22±3%). The activity was significantly increased ($P < 0.005$) after 15 min incubation in the

hypo-osmolar Na^+-containing medium (control, 23±2%; hypo-osmolar, 27±1%), indicating that the stimulation of glycogen synthesis by hypo-osmotic swelling (Fig. 5) was associated with activation of glycogen synthase.

Cell volume determined after 60 min incubation in medium containing 10 mM-3-*O*-methylglucose was increased ($P < 0.005$) in the hypo-osmolar media by 15% in the Na^+-containing medium and by 10% in the Na^+-free medium. Insulin increased cell volume in both the balanced salts medium (control 2.19±0.05, insulin 2.29±0.04 μl/mg of protein; means±S.E.M., $n = 4$) and in the hypo-osmolar Na^+-containing medium (2.52±0.09; insulin, 2.64±0.16) but not in the iso-osmolar Na^+-free medium (1.97±0.05; insulin, 1.96±0.06) or in the hypo-osmolar Na^+-free medium (2.17±0.08; insulin, 2.15±0.08).

Effects of hypo-osmolarity and quinine on the cation and Cl^- contents of hepatocytes

The above volume changes (10–15%, increase) caused by hypo-osmolarity are lower than the theoretical increase in cell volume (36%) if only osmotic changes occurred, suggesting efflux of solute. We determined the effects of hypo-osmolarity, quinine, insulin and glucagon on the cell cation and Cl^- contents to establish to what extent cell volume changes are associated with net loss or gain of solute. Table 6 shows the effects of hypo-osmolarity (−80 mosM) and quinine (0.1 mM) on the cation and Cl^- contents determined after 60 min. During incubation of hepatocytes in a hypo-osmolar Na^+-containing medium, the K^+ content declined by 9% and the Cl^- content by 28% (Table 6). These changes occurred within 5–10 min and then reached a plateau. Values for cellular K^+ and Cl^- contents were similar at 10 min, 30 min and 60 min, but were slightly lower at 5 min (results not shown). In molar terms, the decrease in Cl^- content in the hypo-osmolar medium was smaller than the decrease in cation ($K^+ + Na^+$) content (Table 6). Quinine (0.1 mM) increased the Cl^- content in both the iso-osmolar and hypo-osmolar media (Table 6). Cell volume in the hypo-osmolar medium was higher in the presence of quinine. Whereas insulin increased the K^+ content, the cation ($K^+ + Na^+$) content and the Cl^- content, glucagon increased the K^+ content but not the total cation content, and it decreased the Cl^- content (Table 6). This shows that insulin, glucagon and hypo-osmolarity have different effects on the cation and Cl^- contents of hepatocytes.

DISCUSSION

Effects of insulin on the steady-state content of K^+ and Na^+ in hepatocytes

Studies *in vivo* [9] and *in vitro* [6] have established that insulin stimulates the rapid transfer of K^+ from extracellular to intracellular fluid, but it is not established to what extent this occurs by net replacement of cell Na^+, as distinct from an increase in cell cation content and thereby cell swelling.

We demonstrate in this study that hepatocytes pre-cultured with insulin have higher K^+ and water contents per unit amount of protein than do cells pre-cultured without hormone (Fig. 1, Table 2), and that insulin causes a rapid increase in the K^+ content of hepatocytes pre-cultured without hormone (Fig. 1), which can be accounted for in part (20%) by net replacement of cell Na^+, but largely (80%) by an increase in the cell cation content with an increase in cell volume (Tables 2, 3 and 6). This effect of insulin on the cation and water content was also observed in the absence of amino acids and/or glycogenic substrates (Tables 2 and 6), suggesting that it is not secondary to stimulation of amino acid uptake or stimulation of glycogen synthesis and is therefore probably, at least in part, a primary event.

The increase in cation content caused by insulin was associated with an increase in the Cl^- content, which was smaller in molar terms than the increment in cations, indicating that the content of other anions must be increased to balance the charges. The alkalinization of the cytosol caused by insulin [6,20] may be a contributing factor to the increase in anion content through an increase in $[HCO_3^-]$ or the dissociation of weak organic acids.

The increase in the Na^+ content caused by insulin in the presence of ouabain and in the K^+-free medium suggests that insulin increases the K^+ content in the balanced medium by primary stimulation of Na^+ entry and secondary Na^+/K^+ exchange. Stimulation of Na^+/H^+ exchange is presumably one mechanism whereby insulin stimulates Na^+ entry [6,20]. However, other mechanisms, such as Na^+/HCO_3^- symport, cannot be excluded. The increase in the cell K^+/Na^+ ratio caused by insulin when K^+ uptake is not inhibited suggests that, in addition to secondary activation of Na^+/K^+ exchange as a result of increased Na^+ entry, insulin also independently activates Na^+/K^+-ATPase, as suggested for other tissues [6]. The time course of the effects

Table 6. Effects of hypo-osmotic swelling and quinine on the K^+, Na^+ and Cl^- contents of hepatocytes

Hepatocytes pre-cultured for 16 h in MEM without insulin were pre-equilibrated for 3 h in balanced salts medium without added substrates. Incubations for subsequent determination of Cl^- contained $^{36}Cl^-$ (1 μCi/ml). After the 3 h equilibration, incubations for determination of cell water received 10 mM-3-*O*-methyl[^{14}C]glucose (1 μCi/ml) and other incubations received 10 mM-3-*O*-methylglucose. Quinine (0.1 mM), insulin (10 nM) and glucagon (100 nM) were added at the concentrations indicated, and the medium was made hypo-osmolar (−80 mosM) where indicated by addition of water. Incubations were continued for 60 min and were terminated as described in the Materials and methods section. The ion content is expressed as nmol of ion/mg of cell protein and the water content as μl/mg of cell protein. Values are means±S.E.M. for triplicate incubations from each of three cultures (ion content) or two cultures (water content) and are representative of at least six cultures. Values expressed as a percentage of controls are shown in parentheses.

	Content (nmol/mg)				Water content (μl/mg)
	K^+	Na^+	$K^+ + Na^+$	Cl^-	
Control	195±5 (100)	11.8±1.3 (100)	206 (100)	22.5±0.5 (100)	1.44±0.05 (100)
Hypo-osmolar	178±4 (92)	9.5±1.1 (81)	188 (91)	16.3±0.6 (73)	1.63±0.05 (114)
Quinine	187±12 (96)	12.5±2.7 (106)	191 (93)	28.0±0.2 (125)	1.52±0.05 (106)
Hypo-osmolar + quinine	176±7 (91)	6.9±2.2 (59)	183 (89)	19.9±1.4 (88)	1.75±0.03 (122)
Insulin	208±7 (107)	8.8±1.9 (75)	217 (105)	25.7±0.5 (114)	1.51±0.05 (105)
Glucagon	199±7 (102)	4.8±0.2 (41)	204 (99)	15.4±0.5 (68)	1.47±0.02 (102)

of insulin on cell K^+ (Fig. 1) agrees with the rapid uptake of K^+ by splanchnic tissues *in vivo* during an insulin infusion [9].

Hypotheses on the role of changes in K^+ and Na^+ uptake in the insulin-signalling mechanism

The early hypothesis [5] that cellular uptake of K^+ is part of the insulin-transduction mechanism was discounted because inhibition of K^+ uptake in muscle and adipose-tissue preparations *in vitro* does not abolish the metabolic effects of insulin on glucose and fatty acid metabolism. The present findings, that ouabain and K^+-free media do not block the stimulation of glycogen synthesis by insulin hepatocytes, are consistent with similar findings in muscle and adipose-tissue preparations [5]. The later hypothesis, that cytosolic alkalinization resulting from activation of Na^+/H^+ exchange [6,20] is part of the mechanism by which insulin stimulates glycolysis, was supported by two sets of observations [20]: first, that a Na^+-free medium blocks both the alkalinization of the cytosol and the stimulation of glycolysis by insulin; secondly, that alkalinization mimics the effects of insulin on glycolysis. This hypothesis for a role of cytosolic alkalinization in mediating the metabolic effects of insulin [6,20] has recently been extended to explain the activation of tyrosine hydroxylase in brain [27]. A new hypothesis is that activation of Na^+/H^+ exchange may mediate the metabolic effects of insulin through an increase in cell cation content and thereby cell volume, as distinct from an increase in cell pH [12].

Evidence supporting the hypothesis that an increase in cation content is essential for the stimulation of glycogen synthesis by insulin

The hypothesis that an increase in cation content, and thereby cell volume, is a component of the mechanism by which insulin stimulates glycogen synthesis is supported by the following evidence. (1) Insulin increases the cell cation content ($K^+ + Na^+$) in a balanced salts medium, in a K^+-free medium and when K^+ uptake is inhibited with ouabain, but not in a Na^+-free medium (Table 2). (2) The increases in cation and water content also occur in the absence of glycogenic substrates and amino acids (Tables 2 and 6), indicating that they are not secondary to stimulation of glycogen synthesis or uptake of amino acids. (3) Insulin stimulates glycogen synthesis in media in which it increases the cation content (in a balanced salts medium and in a K^+-free medium), but not in a Na^+-free medium (Table 5). (4) Hypo-osmotic swelling in a Na^+-containing or in a Na^+-free medium mimics the effects of insulin on glycogen synthesis (Table 5). (5) The cell cation and Cl^- contents are decreased by hypo-osmotic swelling, but increased by insulin (Table 6), indicating that the insulin-like effects of hypo-osmotic swelling cannot be explained by the ionic changes induced by swelling. (6) Hypo-osmotic swelling causes cell acidification [28]; however, the stimulation of glycogen synthesis cannot be explained by the acidification, because the stimulation of glycogen synthesis by CO_2-induced acidification was abolished by ouabain (Fig. 3), suggesting that it is not a direct pH effect, but is due to secondary ionic or volume changes. (7) Hypo-osmotic swelling restores the stimulation of glycogen synthesis by insulin in a Na^+-free medium (Table 5), indicating that the failure of insulin to stimulate glycogen synthesis in an iso-osmotic Na^+-free medium is due not to a direct requirement for Na^+, but to a secondary mechanism dependent on Na^+ that can be substituted for by hypo-osmotic swelling.

Interactions of volume changes with other mechanisms

The hypothesis that cell swelling is a component of the mechanism by which insulin stimulates glycogen synthesis does not imply the exclusion of other mechanisms. Since activation of Na^+/H^+ exchange by insulin increases cytosolic pH [6,20], this may have an additive or synergistic effect with swelling on carbohydrate metabolism [4].

Following the discovery that the insulin receptor has tyrosine kinase activity, research on the insulin-transduction mechanism has focused on two hypotheses: a phosphorylation cascade triggered by the tyrosine kinase of the insulin receptor, and formation of second messengers [29]. Since the Na^+/H^+ antiporter is regulated by phosphorylation [30], it is possible that the insulin-induced increase in cell cation content may be controlled in part by a phosphorylation mechanism.

Some effects of insulin, for example the activation of the mitochondrial enzyme pyruvate dehydrogenase, are difficult to explain by a phosphorylation cascade triggered by the receptor tyrosine kinase [31]. This suggests that at least some effects of insulin are not mediated via a cascade linked to the receptor tyrosine kinase. Whether a phosphorylation cascade can be sufficiently rapid to explain the hyperpolarization induced by insulin [8] is debatable, since this is induced within less than 1 s [8].

The stimulation of glycogen synthesis by amino acid-induced swelling [3] and by hypo-osmotic swelling are associated with an increase in the activation state of glycogen synthase. This raises the question how a morphological change can mimic the effect of insulin on the activation state of glycogen synthase, and possibly, therefore, on the phosphorylation state of the protein. The stimulation of glycogen synthesis and activation of glycogen synthase by hypo-osmotic swelling cannot be explained by changes in cyclic AMP or Ca^{2+}, because both are increased during swelling [32], and this would be expected to inhibit rather than stimulate glycogen synthesis. There is a precedent for a lack of correlation between glycogen metabolism and cyclic AMP levels [33], since stimulation of glycogen degradation by glucagon can be uncoupled from the increase in cyclic AMP by disruption of the microfilament or microtubule cytoskeleton. This uncoupling occurs at a site distal to activation of cyclic-AMP-dependent protein kinase [33], suggesting that a cytoskeletal mechanism could be an intermediate link in the control of glycogen degradation by glucagon. It is possible that a cytoskeletal mechanism is part of the sequence of events by which insulin regulates glycogen synthesis. Disruption of the microfilament cytoskeleton inhibits the stimulation by insulin of membrane ruffling, amino acid transport and gene expression [34,35], suggesting the involvement of the cytoskeleton in other effects of insulin.

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