Stimulating role of potassium ions and ouabain on glycogen synthesis in adipose tissue

Francisco SOBRINO, Guadalupe RUIZ and Raimundo GOBERNA
Department of Biochemistry, Faculty of Medicine, University of Sevilla, Sevilla, Spain

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1. Exposure of fat-pads to increasing concentrations of K^+ in the presence of insulin stimulates the incorporation of labelled glucose into glycogen. In the absence of hormone, only a slight incorporation of glucose into glycogen and slight glucose oxidation were detectable. 2. Ouabain alone, up to $100\ \mu M$, had no effect on synthesis of glycogen. Ouabain reinforced the effect of insulin on the conversion of glucose into glycogen in a Na^+ medium and in a equimolar Na^+-K^+ medium, but not in a K^+ medium. In addition, ouabain modified the optimal K^+/Na^+ ratio for glycogen synthesis. 3. The proportion of glycogen synthase in the active form was increased in a K^+ medium, and a faster rate of conversion of synthase *b* into *a* was observed under these conditions. No difference was detected in the rate of inactivation of phosphorylase in a K^+ or a Na^+ medium. 4. Even though these results, taken together, are consistent with the proposed role of phosphorylase *a* in the regulation of synthase activation, the molecular mechanism of action of K^+ in adipose tissue in increasing synthesis of glycogen cannot be explained simply by a faster inactivation of phosphorylase *a*. It is concluded that some undetermined effector(s) or signal could itself be a primary determinant for the greater activation of synthase observed in a K^+ medium.

Glycogen metabolism in adipose tissue is regulated by dietary and hormonal factors (Tuerkischer & Wertheimer, 1942; Wertheimer, 1945; Shafir *et al.*, 1965). It is controlled, as in other tissues, by the activity of glycogen synthase and glycogen phosphorylase (Gutman & Shafir, 1964; Jungas, 1966), which possess kinetic characteristics very similar to those described for hepatic and muscular enzymes. Some differences have been pointed out for the concentration of phosphorylase *a*, which regulates the activity of glycogen synthase phosphatase in fat-pads (Sobrinho & Hers, 1980).

It has been reported that K^+ has a stimulating effect on glycogen synthesis both in liver slices (Cahill *et al.*, 1958) and in hepatocytes (Hue *et al.*, 1975). On the other hand, ouabain, which inhibits active transport of K^+ , stimulates glycogen synthesis and glucose uptake in adipocytes, and inhibits hormone-dependent glycogenolysis (Ho & Jeanrenaud, 1967). In addition, incubation of rat diaphragm in a K^+ -free bicarbonate buffer (Clausen, 1966*b*) or with ouabain (Clausen, 1966*a*) has been shown to induce stimulation of glycogen synthesis. No such effect of ouabain has been seen in minced liver of the frog *Rana catesbeiana* (Blatt *et al.*, 1972).

No direct effect of increased K^+ concentration on the incorporation of glucose into glycogen has been described in adipose tissue. Consequently, the present study was undertaken to determine both the effect of K^+ on glycogen synthesis and the changes that occur in the activity of glycogen synthase and of glycogen phosphorylase under these ionic conditions.

Materials and methods

Chemical

Glucose, glucose 1-phosphate, glucose oxidase, peroxidase and salts were obtained from Merck, Darmstadt, Germany. Tris (Trizma base), AMP, caffeine, ouabain, UDP-glucose and shellfish glycogen (type II) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin and amylo-(α -1,4- α -1,6)-glucosidase were from Boehringer G.m.b.H., Mannheim, Germany. Insulin was from Novo Industri (Copenhagen, Denmark). [U - ^{14}C]Glucose, [U - ^{14}C]glucose 1-phosphate and UDP-[U - ^{14}C]glucose were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Hyamine was from New England Nuclear Corp., Boston, MA, U.S.A.

Animals, preparation and incubation of fat-pads

Male Wistar rats (180–200g) were starved for 5 days and then re-fed for 2 days with a standard diet. Water was given *ad libitum* and contained 10% of glucose during re-feeding. The rats were killed by stunning and cervical dislocation. Each experiment included five rats.

Epididymal adipose tissue was excised rapidly and was preincubated for 20 min at 37°C in 40 ml of Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4, containing 3% (w/v) bovine serum albumin. The tissue was then cut into small pieces (20–40 mg), distributed into plastic vessels and incubated with labelled glucose (0.33 μ Ci/ml), 40 munits of insulin/ml and additions as indicated for each experiment.

Analytical procedure

To measure incorporation of [U- 14 C]glucose into glycogen, the fat-pads were immediately heated at 100°C in 2 ml of 30% (w/v) KOH for 30 min. Radioactive glycogen was isolated and measured by the method of Hue *et al.* (1975), except that the labelled glycogen was finally dissolved in 0.5 ml of 50 mM-sodium acetate buffer, pH 5, and incubated for 60 min at 37°C in the presence of 0.1 mg of amylo-(α -1,4- α -1,6)-glucosidase. Its radioactivity was counted in a liquid-scintillation spectrometer with Biofluor (New England Nuclear, Dreieichenhain, Germany) as scintillant.

For measurement of enzyme activities, the fat-pads were rapidly homogenized with a Potter–Elvehjem homogenizer in 0.5 ml of ice-cold 100 mM-NaF/10 mM-EDTA/25 mM-Tris/HCl, adjusted to pH 7.4 at 4°C. The extracts were centrifuged at 8000g for 5 min in a Beckman B Microfuge and the supernatant was used immediately for assays of enzyme activities. Glycogen phosphorylase and glycogen synthase were assayed as described by Sobrino & Hers (1980).

Oxidation of [U- 14 C]glucose was measured after collecting $^{14}\text{CO}_2$ in 0.2 ml of Hyamine placed in the centre well of an Erlenmeyer flask. After the incubation, the fat-pads were transferred to 30% (w/v) KOH, the incubation medium was acidified with 0.5 ml of 20% (v/v) HClO_4 , and the CO_2 thus liberated was collected during 60 min at 37°C. The radioactivity recovered in Hyamine was counted with Biofluor as scintillant.

Media

In all experiments Krebs–Henseleit bicarbonate buffer containing 3% bovine serum albumin, pH 7.4, was utilized. The gas phase was O_2/CO_2 (19:1). The buffer had the following constant composition (mM): Ca^{2+} , 1.25; Mg^{2+} , 1.18; H_2PO_4^- , 1.18; SO_4^{2-} , 1.18; HCO_3^- , 25; Cl^- , 125. The Na^+ and K^+ concentrations were as follows: Na^+ medium, Na^+ 143, K^+ 5.9; K^+ medium, Na^+ 32.4, K^+ 116.5; Na^+/K^+

medium, Na^+ 74, K^+ 75. In the experiments shown in Fig. 1, these solutions were mixed and supplemented with salts to obtain the concentration indicated.

Experiments with a cell-free system

The extracts of adipose tissue were filtered on Sephadex G-25 and incubated as described by Sobrino & Hers (1980). The protein concentration was measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Effect of K^+ and ouabain on synthesis of glycogen

Fig. 1 shows the effect on the incorporation of glucose into glycogen in fat-pads when Na^+ is replaced by K^+ as the prominent ion in the incubation medium. In the presence of insulin, increase in the K^+ concentration gradually increased the synthesis of glycogen, up to a measured K^+/Na^+ ratio of 117:32. As other authors have observed in studies of adipocytes (Touabi & Jeanrenaud, 1970), there was also a daily variation between our experiments, but the maximum values of the observed increase in incorporation of glucose varied from 2 to 4 times the values found in Na^+ medium. A greater increase in the K^+/Na^+ ratio produced an inhibition of glycogen synthesis. The stimulating effect of high K^+ concentration on glycogen synthesis, previously observed in hepatocytes (Hue *et al.*, 1975), was also apparent in our experiments, although in liver there was an optimal K^+/Na^+ ratio of 140:0, obviously different from that found here in adipose tissue. Addition of 25 μM -ouabain in the presence of insulin potentiated the effect of K^+ at certain concentrations of the ion, and in the presence of ouabain there was a lesser requirement for K^+ for reaching maximal stimulation. Subsequent confirmation of this stimulatory effect of ouabain is shown in Table 1. Under the ionic conditions normally used (Na^+ medium), ouabain (plus insulin) had a 30–40% stimulating effect compared with insulin alone. The same result was found in a medium containing equimolar Na^+ and K^+ (Na^+/K^+ medium), in which the maximum synthesis of glycogen in the presence of ouabain was observed (Fig. 1). In contrast, in K^+ medium ouabain had no effect. The effect of ouabain in Na^+ medium is in agreement with the effect previously described in adipocytes (Ho & Jeanrenaud, 1967), although in this last preparation the effect was observed in the absence of insulin. In our experimental conditions with fat-pads, the effect of K^+ and Na^+ concentrations and of ouabain on the conversion of glucose into glycogen was only observed in the presence of insulin.

Fig. 1. Effect of Na^+ and K^+ concentrations and ouabain on the incorporation of $[U-^{14}C]$ glucose into glycogen in isolated fat-pads

Fat-pads were incubated in Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 3% (w/v) bovine serum albumin, for 1 h in the presence of various concentrations of Na^+ and K^+ , and in the presence of: ●, 25 μ M-ouabain and 40 munits of insulin/ml; ○, 40 munits of insulin/ml; △, without additions. The glucose concentration was 20 mM in all conditions. Values shown are means $\pm$ S.E.M. (vertical bars) for eight observations on three different preparations of fat-pads. Values for insulin + ouabain that are statistically different from the corresponding values with insulin alone, under the same ionic conditions, are indicated by * $P < 0.01$.

The effects of insulin and ouabain on the oxidation of glucose are shown in Table 2. Ouabain alone had no detectable effects on this process, and the production of $^{14}CO_2$ was the same in Na^+ or K^+ medium. In the presence of 40 munits of insulin/ml, about 3.5 μ mol of glucose was oxidized/h of incubation per g of tissue, when 20 mM-glucose was the substrate. These values are in good agreement with those previously reported (Jeanrenaud & Renold, 1959). With insulin and ouabain (under

different ionic conditions) there were no significant changes in glucose oxidation compared with that for insulin alone.

The kinetics of incorporation of glucose into glycogen seemed to follow a non-linear pattern in either Na^+ medium or K^+ medium, though the greatest synthesis was obtained with the latter ion (Fig. 2). In adipocytes the incorporation is linear with time (Lawrence & Lerner, 1978). It is possible that the treatment with collagenase might modify the sensitivity of the cell membrane for insulin-dependent glucose transport (Avruch *et al.*, 1972).

Table 3 shows the dependence of glycogen synthesis on the glucose concentration with altered ionic conditions. These results confirm the previous observation of a non-linear increase in incorporation of glucose into glycogen on changing from 1.25 mM- to 5 mM-glucose in fat-pads (Jeanrenaud & Renold, 1959), and they can reflect the interstitial diffusion barrier that exists in the adipose tissue for low glucose concentrations (Avruch *et al.*, 1972).

Activity of glycogen phosphorylase and glycogen synthase in Na^+ or K^+ medium

Fig. 3 shows that in a K^+ medium there was a faster activation of synthase and a greater proportion of the enzyme in the active form. At the beginning of the incubation 60–90% of the phosphorylase was found in the *a* form. The rate of conversion of phosphorylase *a* was the same under both ionic conditions, and at 20 min of incubation 25% of the phosphorylase remained in the *a* form. In a Na^+ medium a clear delay can be observed in the activation of synthase *b* in all experiments shown. In the presence of K^+ , the delay in the activation of synthase can also be observed, but only when the incubation is done for a short period of time. This delay might be related, as discussed previously (Hers, 1976), to the time required for the conversion of phosphorylase *a* into *b*. The inverse correlation between the activities of synthase *a* and phosphorylase *a* is shown in Fig. 4. The synthase was in the active form when phosphorylase *a* was below 30–40% of the total activity.

Table 4 shows the effects of 50 μ M-ouabain and insulin on synthase *a* and phosphorylase *a* activity, either in Na^+ or in Na^+ – K^+ medium. With insulin more synthase was in the active form in the last medium; this corresponded to a greater incorporation of labelled glucose into glycogen. Nevertheless, the addition of ouabain caused only a small (about 14–17%) increase in the synthase *a* activity, when compared with insulin alone. Perhaps the powerful effect of insulin can mask the ouabain effect on enzyme activation. The phosphorylase *a* activity was about 26–30% of the total activity after 40 min incubation under all conditions studied.

Table 1. *Effect of ouabain on incorporation of [$U\text{-}^{14}\text{C}$]glucose into glycogen in isolated fat-pads*

The compositions of the media are indicated in the Materials and methods section. The concentration of insulin was 40 munits/ml in all experiments. In Expts. 1, 2 and 3, the values are means $\pm$ S.E.M. for three different observations with a single preparation of fat-pads. Other values are means of two observations in a single preparation. Values for insulin + ouabain significantly different from their corresponding values of insulin are indicated by * $P < 0.01$ and ** $P < 0.001$.

Expt. no.	Glucose (mM)	Additions	Medium ...	Glucose incorporated into glycogen ($\mu\text{mol/h}$ per g of tissue)		
				Na^+	$\text{Na}^+ - \text{K}^+$	K^+
1	20	Insulin		0.65 ± 0.02	1.11 ± 0.03	—
		Insulin + 50 μM -ouabain		$0.83 \pm 0.03^*$	$1.76 \pm 0.08^{**}$	—
2	20	None		—	—	0.06 ± 0.03
		50 μM -ouabain		—	—	0.09 ± 0.02
		Insulin + 50 μM -ouabain		—	—	2.31 ± 0.27
3	10	Insulin		0.31 ± 0.01	—	1.05 ± 0.07
		100 μM -ouabain		0.04 ± 0.01	—	0.07 ± 0.01
		Insulin + 100 μM -ouabain		$0.49 \pm 0.01^*$	—	1.10 ± 0.10
4	10	Insulin		0.56	—	1.35
		Insulin + 25 μM -ouabain		0.77	—	1.29
		Insulin + 50 μM -ouabain		0.76	—	1.31
5	10	None		0.06	0.05	—
		Insulin		0.71	1.49	—
		25 μM -ouabain		0.07	0.10	—
		Insulin + 25 μM -ouabain		0.89	2.01	—

Table 2. *Effect of ouabain on the oxidation of [$U\text{-}^{14}\text{C}$]glucose in isolated fat-pads*

The compositions of the media are indicated in the Materials and methods section. The concentrations of insulin and ouabain were 40 munits/ml and 50 μM respectively. The glucose concentration was 20 mM in all conditions. Results shown are means $\pm$ S.E.M. for three different observations with a single preparation of fat-pads.

Additions	Medium ...	Glucose oxidized to CO_2 ($\mu\text{mol/h}$ per g of tissue)		
		Na^+	$\text{Na}^+ - \text{K}^+$	K^+
None		0.78 ± 0.16	0.64 ± 0.11	0.72 ± 0.18
Ouabain		0.75 ± 0.07	0.81 ± 0.19	—
Insulin		3.71 ± 0.30	3.98 ± 0.20	3.25 ± 0.34
Ouabain + insulin		3.50 ± 0.63	4.10 ± 0.51	3.10 ± 0.22

Table 3. *Effect of glucose concentration on the incorporation of [$U\text{-}^{14}\text{C}$]glucose into glycogen in a Na^+ or K^+ medium in isolated fat-pads*

General procedure was as in Fig. 2, except that the glucose concentration was varied. Values shown are means for two preparations of fat-pads with triplicate incubations.

Glucose (mM)	Medium ...	Glucose incorporated into glycogen (nmol/h per g of tissue)	
		Na^+	K^+
1		8.2	11.7
2		18.6	25.3
3		—	136.2
5		110.3	274.8
10		256.1	584.5

Ionic effect in a cell-free system

The inactivation of phosphorylase and the activation of synthase were studied in a series of experiments with extracts of adipose tissue previously filtered on Sephadex, and incubated under different ionic conditions. Fig. 5 shows one illustrative experiment of these. NaCl or KCl (150 mM) had no differential effects on these processes. In the experiment shown, these salts slightly inhibited the phosphorylase inactivation, although in other preparations the effect was more marked. Nevertheless, the most striking effect of salts was the potent inhibition of the synthase activation. Glucose partially overcomes the inhibition of the conversion of synthase *b* into *a*, but the activity of the synthase *a* after 60 min preincubation was very low com-

Table 4. *Effect of ouabain on synthase a and phosphorylase a activities in isolated fat-pads*

General procedure was as in Table 1. Fat-pads were incubated in 20mM-glucose and 40munits of insulin/ml, in the presence or absence of 50μM-ouabain, for 40min. The values represent the means of two observations in a single preparation. Values in parentheses are activities of synthase a expressed as a percentage of the activity with insulin.

Additions	Medium	...	Activity (% of total)			
			Synthase a		Phosphorylase a	
			Na ⁺	Na ⁺ -K ⁺	Na ⁺	Na ⁺ -K ⁺
Insulin			22.6 (100)	41.2 (100)	30.6	27.2
Insulin + ouabain			25.8 (114)	48.3 (117)	27.0	26.8

Fig. 2. *Time-course of the effect of Na⁺ and K⁺ media on the incorporation of [U-¹⁴C]glucose into glycogen in isolated fat-pads*

Fat-pads were incubated in Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 3% (w/v) bovine serum albumin [O, Na⁺ medium; ●, K⁺ medium (with compositions as indicated in the Materials and methods section)], in the presence of 10mM-[U-¹⁴C]glucose and 40munits of insulin/ml for different times.

pared with that in control extracts. This could suggest a direct inhibition of the synthase a activity by high concentrations of salts.

Discussion

This work gives evidence for the potentiating effect of K⁺ on the insulin-dependent synthesis of glycogen in adipose tissue. No effect of K⁺ has been detected in this process in the absence of the

hormone. This is in accord with the classical view of the requirement for insulin for glycogen synthesis in fat-pads. Although this dependence is primarily related to the facilitation of glucose transport by insulin (Crofford & Renold, 1965), the insulin effect on glycogen synthesis in adipose tissue cannot be readily explained on this basis alone (Jeanrenaud & Renold, 1959).

On the other hand, the role of Na⁺ and K⁺ in mediating the action of insulin on the transport of glucose and its subsequent metabolism in adipocytes has not been clarified. Rodbell (1967) observed that K⁺ is required for the stimulation by insulin of the transport of glucose. In our experiments, however, the glucose oxidation remained unchanged under different ionic conditions, in the presence of insulin and ouabain. Previous studies have reported (Letarte & Renold, 1969) that, in the presence of insulin, replacement of Na⁺ by K⁺ strikingly decreases glucose metabolism. But it should be noted that in those conditions in which the replacement is not total there were no variations in the CO₂ production. Data of the present study (Table 2) are in keeping with such results.

As in previous studies with adipocytes (Ho & Jeanrenaud, 1967), the present paper shows a potentiating effect of ouabain on the synthesis of glycogen in fat-pads. The fact that we did not find an effect of ouabain by itself could be due to the adipose-tissue preparation used. An apparent discrepancy emerges from the fact that the same effect on glycogen synthesis was seen with ouabain or with increased concentration of K⁺ ions. Although it is difficult to explain these results, it should be noted that the inhibition of Na⁺ + K⁺-dependent ATPase produced by cardiac glycosides was also obtained by modifying the Na⁺/K⁺ ratios for the optimal activity of the enzyme *in vitro* (Skou, 1965; Dahl & Hokin, 1974). From these results it could be thought that under those ionic conditions, where an increase in the synthesis of glycogen has been observed, possibly the ATPase activity would be diminished or abolished.

The effect of K⁺ on the activities of glycogen

Fig. 3. Effect of Na^+ and K^+ media on synthase and phosphorylase interconversion in isolated fat-pads. General procedure was as in Fig. 2. Δ , $\blacktriangle$, Na^+ medium; $\circ$, $\bullet$, K^+ medium; $\blacktriangle$, $\bullet$, synthase a activity; Δ , $\circ$, phosphorylase a activity. The curves represent three typical experiments performed with different preparations of fat-pads, where the time of incubation was varied. The glucose concentration was 20 mM throughout.

Fig. 4. Correlation between the activities of phosphorylase a and synthase a in isolated fat-pads.

The values of synthase a plotted versus the corresponding values of phosphorylase a come from Fig. 3: Δ , $\blacktriangle$, (a); $\circ$, $\bullet$, (b); $\square$, $\blacksquare$, (c). Results of similar experiments are also included: ∇ , $\blacktriangledown$, and $\diamond$, $\blacklozenge$. Fat-pads were incubated in either Na^+ (open symbols) or K^+ (closed symbols) medium.

Fig. 5. Effect of NaCl , KCl and glucose on the interconversion of two forms of phosphorylase and glycogen synthase in a cell-free system from epididymal adipose tissue.

The extract was filtered on Sephadex G-25 and incubated at 25°C in the presence of 0.1% (w/v) glycogen, 5 mM- $(\text{NH}_4)_2\text{SO}_4$ and with the following additions: none ($\circ$, $\bullet$); 150 mM- NaCl or KCl (Δ , $\blacktriangle$); 20 mM-glucose, 75 mM- NaCl and 75 mM- KCl ($\square$, $\blacksquare$). Samples ($50\mu\text{l}$) were withdrawn at different times and assayed immediately for synthase a and phosphorylase a activities. Open symbols: phosphorylase a ; closed symbols: synthase a .

synthase and glycogen phosphorylase in adipose tissue showed some differences from that previously observed in hepatocytes (Hue *et al.*, 1975). Indeed, in fat-pads no differential effect of K^+ or Na^+ medium on the inactivation of phosphorylase in the presence of insulin has been observed; nevertheless,

in a K^+ medium, the activation of the synthase was greater (Fig. 3). In a Na^+ medium this activation was preceded by a clear delay, which was not so evident in a K^+ medium unless the measurements were made within a short period of time.

In a filtered cell-free extract from adipose tissue, the effect of salts ($NaCl$ or KCl) was more clear on the synthase than on the phosphorylase system. In liver extracts it has been previously reported that on addition of $10\text{ mM}-(NH_4)_2SO_4$ the activation of synthase started and was more rapidly complete; the same effect could be obtained with $100\text{ mM}-KCl$ (De Wulf *et al.*, 1970). In a filtrate of adipose tissue there was also a requirement for salts [$5\text{--}10\text{ mM}-(NH_4)_2SO_4$] for full activation of synthase phosphatase and for synthase activity (results not shown), but a greater concentration of salt (e.g. 150 mM) was clearly inhibitory (Fig. 5).

Although our observations suggest a sequential relationship between activation of synthase and prior inactivation of phosphorylase α , it must be recognized that in adipose tissue this link is much less efficient than in liver. In agreement with previous results (Sobrinho & Hers, 1980), the activation of synthase did not take place unless the amount of phosphorylase α was about 30–40% of the total phosphorylase. This latency in the conversion of synthase b into a was also reflected in the non-linear kinetics of the incorporation of glucose into glycogen, presumably related to the time required for phosphorylase α to reach a concentration non-inhibitory for the synthase phosphatase.

Although this research has shown a stimulating effect of K^+ on glycogen synthesis through a parallel activation of glycogen synthase, it does not offer an explanation for the molecular mechanism that regulates this process. It can be assumed that some other factors, not yet determined, intervene in this increased activity of the enzyme.

However, some possibilities can be suggested, such as that the effect of K^+ could be exercised through ATP availability in relation to the modification of ATPase activity (Bihler & Jeanrenaud, 1970), or that K^+ could affect the synthase activity, by modifying Ca^{2+} movement as demonstrated for glycogenolysis in perfused liver (Jacob *et al.*, 1980). In this last context, the inhibitory action of Ca^{2+} on synthase phosphatase in liver extracts is noteworthy (van de Werve, 1981). Another possibility that may be considered is that K^+ may affect the activity of phosphorylase kinase either directly or by a change in the intracellular Ca^{2+} concentration. K^+ is known to suppress completely the phenylephrine effect on the activation of phosphorylase in hepatocytes (van de Werve *et al.*, 1977).

Clearly further studies are necessary to establish the mechanism of the role of ions in the regulation of substrate metabolism and of the hormonal effect on

transport of Na^+ and K^+ across the membrane of fat-cells.

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