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Glycogen synthesis by the direct or indirect pathways depends on glucose availability: In vivo studies in frog oocytes

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Abstract **Besides the classic direct route, frog oocytes incorporate glucosyl units into glycogen by the so-called indirect pathway. The operation of both pathways depends on glucose availability. Below 0.5 mM glucose (calculated intracellular concentration), the indirect route accounts for 90% of polysaccharide formation, while the direct pathway supports 70% of total glucose incorporation when administered glucose is above 1.5 mM. A sigmoidal curve was obtained for the direct pathway with $n_H = 2.04$, and half saturation was reached at 2.6 mM glucose. The curve for the indirect route presented an n_H of 1.15 and an $S_{0.5}$ of 0.9 mM glucose.**

Keywords: Glycogen synthesis; In vivo metabolism; Frog oocyte

1. Introduction

In addition to the classic direct route, glycogen may be also synthesized from glucose by an indirect pathway which involves prior degradation of glucose to trioses, followed by gluconeogenesis to resynthesize hexose phosphates, which are then commited to glycogen formation [1–3]. The operation of the indirect pathway has been observed in several species and cells [1,4,5], but rat liver has been the most studied system. A vexing problem has been the quantitation of the relative contributions of the direct and indirect pathways to glycogen formation from a glucose load, and widely ranging estimates of the percentage of glucose carbon that follows the indirect pathway have been reported. Most of the variations surely stem from limitations of the methods employed and also from differences in the experimental conditions used (for a review see [6]). However, the influence of the nutritional state as a major factor involved in the indirect route contribution has been recognized in several studies. As Newgard and coworkers already noted [2], the administration of a large glucose bolus to rats resulting in a portal glucose concentration of 15 mM causes preferential glycogen synthesis through the direct pathway. Also, Landau and Warren [7] reported that the contribution of the pathways appears to be determined by the size of the glucose load, with larger contributions of the indirect route occurring with smaller loads. A study conducted in rats showed a higher contribution of the indirect pathway in 48 h fasted animals as compared with fed ones [8]. In agreement with these results, hepatocyte cultures derived from fasted-refed and fasted rats showed increased contribution of the indirect pathway compared with hepatocytes from fed rats [9].

Although experimental evidence for the operation of the indirect pathway in vivo was reported two decades ago, the regulatory mechanisms underlying the alternative or simultaneous operation of the two metabolic routes for glycogen synthesis remain largely unknown. This prompted us to search for a metabolic condition that could signal the preferential operation of one pathway over the other, and for an appropriate experimental system in which accurate measurements of the contribution of each pathway could be obtained. We have used the full grown stage VI amphibian oocyte as a model system for the in vivo study of glucose metabolism, its organization and regulation. The advantages of this system have been described elsewhere [10–12]. Glycogen is the main end product of glucose metabolism in oocytes [13,14]. A minor portion of the glucose microinjected into the cells (around 5%) is metabolized through the pentose phosphate pathway [15]. We have shown that, in vivo, oocytes incorporate glucosyl units into glycogen both by the direct and indirect routes [5]. This means that the Embden–Meyerhof pathway is operative in these cells, as also shown by Kessi et al. [5]. Furthermore, we developed a novel approach to estimate the contributions of both routes to glycogen synthesis in vivo based on the inhibition of gluconeogenesis by fructose 2,6-bisphosphate (fructose-2,6-bisP). The aim of the present work was to study the influence of glucose concentration in the operation of the two pathways. The results obtained show that the contribution of one pathway or the other to glycogen formation depends indeed on the amount of glucose microinjected into the oocytes. The indirect pathway is the only route operating at low glucose concentrations, while the direct route becomes predominant when intracellular glucose rises above 1 mM.

2. Materials and methods

2.1. Materials

Labeled compounds and mixtures for scintillation counting were from New England Nuclear, Boston, MA. Non-radioactive compounds and metabolites were mostly from Sigma.

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Abbreviations: Fructose-2,6-bisP, fructose 2,6-bisphosphate

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2.2. Animals

Freshly excised stage VI oocytes from the chilean frog *Caudiverbera caudiverbera* were used. The animals were obtained from a local dealer, fed and maintained in the laboratory until used.

2.3. Microinjection procedures and metabolic labeling

Before the experiments, aliquots of the radioactive compounds to be injected were dried in a Univapo concentrator centrifuge, unlabeled solutions were added and the samples were resuspended in Barth saline [16] in order to achieve the desired concentrations. High pressure liquid chromatography using a Dionex CarboPac PA1 column was used to check for purity of commercial compounds. By means of a Narishige automatic injector, individual oocytes were microinjected with 50 nL of saline containing the desired compounds. Then, groups of six to eight oocytes were incubated in 75 μL saline at 22° under 100% O_2.

2.4. Glycogen isolation

After incubation glycogen was isolated from individual cells by repeated ethanol precipitation as described [17].

2.5. Quantitative estimation of the direct and indirect pathways

2.5.1. Comparison between the $^3H/^{14}C$ ratio of the administered doubly labeled glucose and that of the newly formed glycogen. Groups of six oocytes were microinjected with 0.5 or 6 nmol of [U-^{14}C, 5-^{3}H]glucose, with ^{3}H/^{14}C ratios (dpm/dpm) of 2.82 and 2.56, respectively. After 20 min incubation, each oocyte was individually processed for glycogen isolation and further radioactivity counting. Then, the relative ^{3}H/^{14}C was calculated by dividing the ^{3}H/^{14}C ratio in glycogen by the ^{3}H/^{14}C in the injected glucose and percent detritiation was estimated.

2.5.2. Inhibition of the indirect pathway by fructose-2,6-bisP. Groups of 8 oocytes were microinjected with 50 nL of variable amounts of [U-^{14}C]glucose (60 000 cpm). Other oocytes received the same solution plus 0.3 nmol (0.1 mM intracellular calculated concentration) of unlabeled fructose-2,6-bisP. After 15 min incubation, cells were individually processed for glycogen isolation and radioactivity counting. Substracting the values for glucose incorporation by the direct route (data obtained in the presence of fructose-2,6-bisP) from the total incorporation values (in the absence of fructose-2,6-bisP) the amount of glucose metabolized through the indirect pathway was obtained (total − direct = indirect).

3. Results and discussion

One of the most employed techniques for estimating the contributions of both the direct and indirect metabolic pathways to glycogen synthesis has been the comparison between the ^{3}H/^{14}C ratio of the doubly labeled administered glucose and that of the newly formed glycogen. However, it has been recognized that ^{3}H/^{14}C ratios are relatively insensitive to pathways contributions (for an extended discussion see [5]). This situation prompted us to search for a metabolic condition that could signal the preferential operation of one pathway over the other, and for an appropriate experimental system in which accurate measurements of the contribution of each pathway could be obtained. In previous work, we have shown that coinjection of [U-^{14}C]glucose (0.5 nmol) together with unlabelled fructose-2,6-bisP into oocytes drastically inhibits label incorporation into glycogen in vivo [5]. Fifty per cent inhibition was obtained with 2 μM fructose-bisP, a value identical to the K_i for the hexose-bisP of the purified oocyte fructose bisphosphatase [18]. Thus, fructose-2,6-bisP may be used as a potent inhibitor of gluconeogenesis and therefore of the indirect route allowing an accurate estimation of the contributions of the direct and indirect pathways for glycogen synthesis.

The dependence of glycogen labeling on glucose concentration after [U-^{14}C]glucose microinjection is shown in Fig. 1A.

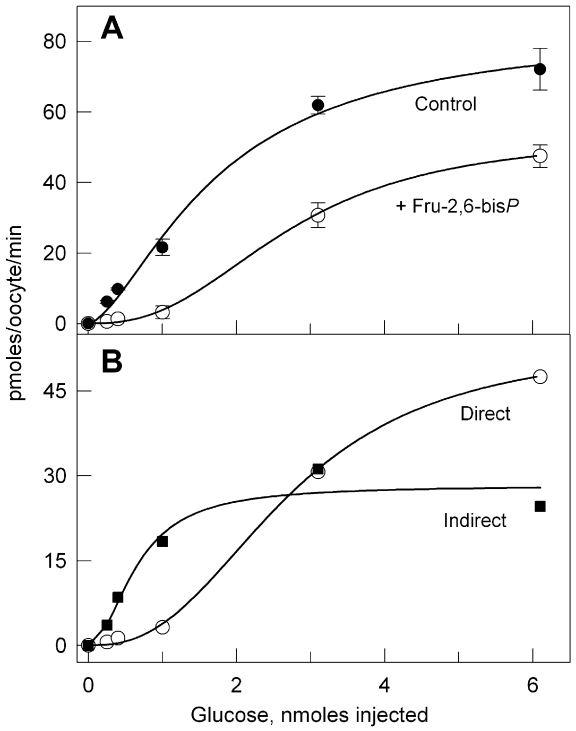

Fig. 1. Effect of glucose concentration on glycogen labeling. (A) Groups of 8 oocytes were microinjected with 50 nL of variable amounts of [U-^{14}C]glucose (60 000 cpm). Other oocytes received the same solution plus 0.3 nmol (0.1 mM intracellular calculated concentration) of unlabeled fructose-2,6-bisP. After 15 min incubation, cells were individually processed for glycogen isolation and radioactivity counting. (B) Quantitative contributions of the direct and indirect pathways to glycogen synthesis. Values were obtained as described in the text.

The curve obtained in the absence of fructose-2,6-bisP (control curve) corresponds to total label incorporation, i.e., direct plus indirect pathways. Label incorporation into glycogen in the presence of fructose-2,6-bisP corresponds to glycogen synthesized through the direct pathway. An amount of 0.3 nmol fructose-2,6-bisP was microinjected into the oocytes, which is equivalent to 0.1 mM intracellular concentration and is 50 times the K_i. The extent of the inhibition produced by fructose-2,6-bisP depends on the amount of glucose injected into the oocytes. The inhibition is maximal (about 90%) at low glucose loading, and reaches a plateau of 30% inhibition around 6 nmol glucose. Substracting the values for glucose incorporation by the direct route from the total incorporation values, the amount of glucose metabolized through the indirect pathway (total − direct = indirect) was obtained (Fig. 1B). Inspection of the curves of Fig. 1B shows that the indirect pathway exhibits a michaelian type response (Table 1) with a half saturation at 0.9 mM glucose and a Hill coefficient of 1.15. On the other hand, the direct pathway shows a distinctive sigmoid behavior with a Hill coefficient of 2.04 and half saturation at 2.6 mM glucose (Table 1). We propose that the direct pathway behaves as an ultrasensitive system with a built-in threshold operating as a bioswitch [19,20] triggered by glucose or a derived metabolite. The calculated percent contribution of both pathways to glycogen synthesis is depicted in Fig. 2. It is clearly seen that the indirect pathway accounts for around 90% of polysaccharide formation when glucose availability is low (below

Table 1
Kinetic constants for the two pathways for glycogen synthesis in frog oocytes

Pathway	n_H	$S_{0.5}$ (mM)
Direct	2.04	2.60
Indirect	1.15	0.93

Values for the direct pathway were obtained by linearization of the corresponding curve in Fig. 1B by the method of Lineweaver–Burk. The constants for the indirect pathway were obtained by transformation of the data presented in Fig. 1B to a Hill plot.

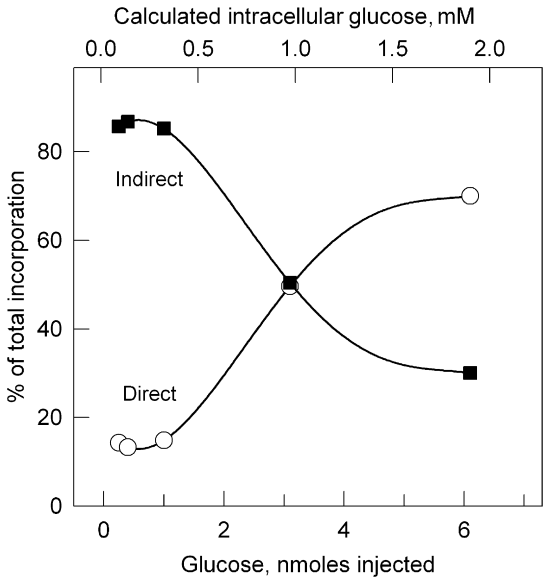

Fig. 2. Estimated percentage contribution of the direct and indirect pathways for glycogen synthesis in frog oocytes. Values were estimated by assigning 100% incorporation to each experimental point in the control curve in Fig. 1A. Calculated intracellular glucose for each amount of glucose injected into the oocytes is shown.

0.5 mM), while the direct route supports approximately 70% of total glucose incorporation at glucose levels above 1.5 mM. We have never observed less than 30% operation of the indirect pathway, even if glucose injected was raised up to 9 nmol (3 mM intracellular). This observation suggests that the indirect pathway is the route operating by default, while the direct pathway requires a critical glucose concentration in order to become activated. Both pathways are equally active at about 1 mM intracellular glucose.

As mentioned above, the comparison between the $^3H/^{14}C$ ratio of the doubly labeled administered glucose and the ratio in glycogen has been the preferred technique for estimating the contributions of both the direct and indirect metabolic pathways to glycogen synthesis. The rationale of this approach lies in the fact that if glucose is converted into glycogen exclusively by the direct pathway, then the $^3H/^{14}C$ ratio in glycogen should be identical to the ratio in the microinjected glucose. However, if glucose follows the indirect route, the $^3H/^{14}C$ ratio in glycogen should be lower because of detritiation at the triose-phosphate isomerase reaction. We have also used this approach in order to compare the results with the ones obtained by our method using fructose-2,6-bisP as inhibitor of the indirect route. With this purpose, oocytes were microinjected with different concentrations of [U-^{14}C, 5-^{3}H]glucose. The results obtained show that a significant detritiation (54%) of glycogen glucosyl units occurs in oocytes microinjected with 0.5 nmol of radioactive glucose (0.17 mM calculated intracellular concentration assuming a cell volume of 3 μL and homogeneous distribution of the sugar). In oocytes that received 6 nmol glucose (2 mM intracellular concentration), glycogen detritiation was only 18% (Table 2). We should stress the fact that the experimentally obtained value in the latter case was 36%, but the results obtained before by using fructose-2,6-bisP showed that, even under conditions where the direct pathway is the prefered one, around 30% of the indirect route is always operative. Thus, the experimental value of 36% involves the contribution of both pathways (100%), and therefore was corrected by discounting the contribution of the indirect route (30%). The results obtained by using the $^3H/^{14}C$ ratios indicate that, at low glucose concentrations, at least 50% of the microinjected glucose reached the step of the triose phosphates before beeing incorporated into glycogen, and that the contribution of the indirect pathway is significantly higher than that of the direct route at low glucose concentrations. These results are in agreement with the relative lack of sensibility of the $^3H/^{14}C$ ratios to pathways contributions. When glucose availability is low (0.5 nmol injected), the dual tracer technique showed 50% contribution of the indirect pathway, while the combined use of radioactive glucose with fructose-2,6-bisP allowed an estimation of 90% contribution of the pathway under the same conditions. It seems therefore that, compared with the dual tracer technique, our method allows improved accurate measurements of the contribution of each pathway and enables correct quantitative distinction between them.

The experimental system used in this study allows the control of the operation of the direct or indirect pathways by manipulating the amount of glucose microinjected into the oocytes in the presence or absence of fructose-2,6-bisP. Thus, we could induce cellular conditions in which glycogen deposition occurs solely by the indirect route. Although precursor concentration is one of the factors regulating the proportion of direct versus indirect pathways contribution to glycogen synthesis, the mechanism by which high glucose levels (or a derived metabolite) triggers the direct pathway still remains unclear. Glucose-6-P stands out as the best candidate, since it has been long recognized that the activity of glycogen synthase largely depends on the presence of the hexose-P. We have found that oocyte glycogen synthase is in fact allosterically activated by glucose-6-P under in vivo conditions [21]. Also, it has been shown that glucose-6-P stimulates the activity of protein phos-

Table 2
Relative $^3H/^{14}C$ ratios in glycogen from oocytes microinjected with 0.5 and 6 nmol doubly labeled glucose

Injected glucose	Relative $^3H/^{14}C$	Percent detritiation
0.5	0.46 ± 0.023	(54) – 54
6.0	0.64 ± 0.010	(36) – 18[a]

Groups of six oocytes, in duplicate, were injected with 50 nL of saline containing 0.5 or 6 nmol of [U-^{14}C]glucose plus [5-^{3}H]glucose. After 20 min incubation, each oocyte was individually processed for glycogen isolation and radioactivity counting. Results are given as the means ± S.E.M. of 12 individual observations. Relative $^3H/^{14}C$ is the $^3H/^{14}C$ ratio in glycogen divided by the $^3H/^{14}C$ in the injected substrate. Values of $^3H/^{14}C$ (dpm/dpm) for the 0.5 and 6 nmol solutions to be injected were 2.82 and 2.56, respectively.
[a]Corrected for 30% indirect pathway operation at 6 nmol glucose, see Section 3.

phatase 1, which activates glycogen synthase by dephosphorylation [22]. The switch-like response described above for the direct pathway may arise from the various phosphorylation-dephosphorylation steps involved in the regulation of glycogen synthase activity.

Finally, we would like to point out that at present we have no good explanations as to the physiological significance of the indirect pathway. Compared with the direct route, this is a circuitous and energy wasting process. It seems that glycogen synthesis has a higher level of complexity than previously believed, and answers to these and several other unresolved problems should wait for further work.

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