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Amylin and epinephrine have no direct effect on glucose transport in isolated rat soleus muscle

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Abstract Amylin and epinephrine did not significantly affect insulin stimulated, or basal, 3-*O*-methylglucose transport in isolated rat soleus muscle, as measured by the release of 3-*O*-methylglucose from pre-loaded tissue. Both amylin and epinephrine inhibited insulin-stimulated 2-deoxyglucose uptake (by 25% and 38%, respectively) in soleus muscle from fed rats but not from fasted rats. The latter results are consistent with amylin and epinephrine stimulating glycogenolysis and inhibiting hexokinase activity by intracellular accumulation of glucose 6-phosphate. We conclude that amylin, like epinephrine, does not specifically inhibit glucose transporters in skeletal muscle.

Key words: Amylin; Epinephrine; Soleus; Skeletal muscle; Glucose transport; 2-Deoxyglucose; 3-*O*-Methylglucose

1. Introduction

Amylin, a 37 amino acid hormone, is synthesized in the pancreatic β cells and co-secreted with insulin [1,2]. The first reported biological activity of amylin was the inhibition of insulin stimulated incorporation of labeled glucose into glycogen in soleus muscle preparations, an effect which is now well documented [1,3–5]. However, the literature of amylin's effects on glucose *transport* in muscle is confusing and contradictory. Some authors report an inhibition of glucose transport [4,6,7], whilst others report no effect [7,8]. Part of the confusion appears to originate from the interpretation of results showing reduced uptake of 2-deoxyglucose, since its uptake does not distinguish transport and phosphorylation. In other reports using 3-*O*-methylglucose, a glucose analog that is transported but not metabolized, either no effect [4] or an inhibitory effect of amylin [7] was demonstrated. These experiments are more difficult to perform in isolated tissues such as skeletal muscle than they are in isolated cellular preparations, in part because the contribution made by the interstitial extracellular space to tissue 3-*O*-methylglucose accumulation is large and unknown, and diffusional barriers to 3-*O*-methylglucose flux prevent us from knowing concentrations in the extracellular space that are needed for initial influx rate calculations [9]. A technique reported by Clausen [10] utilizing the ability of glucose transporters to transport 3-*O*-methylglucose out of preloaded cells, overcomes most of these issues. We used Clausen's method to reexamine the effects of amylin on basal and insulin-stimulated glucose transport in skeletal muscle, comparing these effects with effects of epinephrine, which acts via receptor-activated stimulation of cAMP production, as amylin is believed to do [11–13] We also investigated the effects of amylin on 2-deoxyglucose accumulation so they could be compared with effects on 3-*O*-methyl glucose flux.

2. Materials and methods

2.1. Animals and soleus assay

Soleus muscle strips were obtained from male Sprague–Dawley Rats (approximately 170 g), as described [5]. The muscle strips were incubated in the relaxed state. Most of the experiments used fed animals which were allowed food and water ad libitum up to the time of sacrifice, which was within four hours after the beginning of the light cycle. In some experiments animals were fasted for approx. 18 h by the removal of food and a change of bedding the evening before use. All incubations of soleus muscles were performed in 6 well culture plates in 2 ml of buffer/well. Culture plates were placed in a Modular Incubation Chamber (Billups-Rothenberg, Del Mar, CA) which was flushed with 95% 0_2–5% CO_2 for 30 s before placing on an orbital rotator inside a 37°C incubator. The incubation chamber was re-flushed each time the chamber was opened.

2.2. Incubations

2.2.1. 2-Deoxyglucose uptake. 2-Deoxyglucose is transported into muscle cells and is phosphorylated by hexokinase into 2-deoxyglucose 6-phosphate, but is not metabolized further and so can accumulate to measurable levels. 2-deoxyglucose uptake experiments were based on the method described by Kreutter [8]. Briefly, muscles were incubated for 15 min in a modified Krebs–Ringer bicarbonate (KRB) buffer (118.5 mM NaCl, 5.94 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4 and 25 mM $NaHCO_3$), containing 1 mM [^{14}C]L-glucose (0.1 μCi/ml), 2 mM pyruvate and 0.2% BSA. Insulin 7.1 nM (Humulin R, Lilly, Indianapolis In), amylin 100 nM (BaChem, Torrence Ca), or epinephrine 1 μM (Sigma, St Louis Mo), was then added and the muscles incubated for another 15 min after which 1 mM [^{3}H]2-deoxyglucose (0.2 μCi/ml) was added and the muscles incubated for an additional 30 min. At the end of the incubation period, muscles were removed from the incubation buffer, rinsed with saline, tendons were then removed, the remaining tissue blotted in tissue paper and the samples immediately frozen in liquid N_2. Soleus muscle samples were digested in NaOH and then added to 4 ml of Scintillation fluid (Ecolite, ICN) for radioactivity determination by scintillation spectroscopy.

2.2.2. 3-O-Methylglucose efflux. 3-*O*-Methylglucose is transported into muscle cells but is not phosphorylated by hexokinase. However, because glucose can be transported in either direction (into or from cells), inward and outward 3-*O*-methylglucose fluxes equalize and intracellular concentration approaches that in the incubation medium so that changes in content can no longer measure flux into muscle. However efflux of label from loaded cells into a label-free medium can be used to measure transport with good sensitivity, and effects due to exchange with the interstitial extracellular space can be taken into account.

Methods employed were based on those described by Clausen [10]. In the first series of experiments, results shown in Fig. 1, muscles were preincubated for 60 min in a modified Krebs-Ringer bicarbonate buffer (loading buffer) containing 1.68 mM $CaCl_2$, 0.2% BSA, 1 mM pyruvate, 1 mM [^{3}H]3-*O*-methylglucose (1 μCi/ml) and 1 mM [^{14}C]L-glucose (1 μCi/ml). Muscles were then transferred to 5 ml of the same buffer without glucose or label (wash buffer) and rinsed for 2 min by orbital rotation at room temperature before being transferred to 2 ml of fresh wash buffer, with or without insulin, to measure efflux of label. At the

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end of the efflux incubation, muscle samples were removed, rinsed, trimmed, and frozen as described above. The muscle content of [^{14}C]L-glucose and [^{3}H]3-*O*-methylglucose was determined as described above.

In a second series of experiments muscles were first incubated for 60 min in loading buffer containing 4.5 μCi/ml of [^{3}H]3-*O*-methylglucose and were then briefly washed as described above. Muscle pieces were then transferred to fresh wash buffer (1 soleus muscle strip/well in 2 ml of buffer). Hormones were added after 40 min of efflux incubation. Efflux of label from the muscle into the incubation buffer was followed by taking duplicate 50 μl samples of the efflux buffer every 10 min for 30 min. At the end of the incubation period muscle pieces were rinsed, blotted and frozen as described above to determine ^{3}H content.

2.3. Numerical methods

Specific uptake of 2-deoxyglucose was calculated by subtracting the extracellular trapping of L-glucose. The rate of 2-deoxyglucose uptake is expressed as nmol 2-deoxyglucose/g wet weight/min. Statistical significance was determined by use of the Student's *t*-test (Instat).

In Fig. 1, the glucose content of the muscle is expressed as nmol/g wet wt. The rate of 3-*O*-methylglucose appearance in the efflux buffer appeared to be constant between 40 and 70 min (results not shown). Scintigraphy of bathing media and digested muscles at the end of experiments enabled calculation of the total radiography that had been loaded into muscle before efflux was observed. The rate of efflux in Fig. 2 was expressed as the percent of this initial content that was released into the efflux buffer per min [10] between 40 and 70 min of the incubation.

3. Results and discussion

Fig. 1 shows a rapid efflux of both 3-*O*-methyl glucose and L-glucose from pre-loaded tissue. It can be seen that after 40 min of incubation, the rate of L-glucose efflux from muscle approaches zero whilst 3-*O*-methylglucose in muscle continues to decline at an essentially linear rate. The results suggest that efflux during the initial 40 min of incubation represents diffusion of label from the interstitial space and that after 40 min efflux mostly represents transport from intracellular compartments. The results also repeat a previous observation [10] that insulin rapidly stimulates the efflux of 3-*O*-methylglucose glucose transport (which is transported by specific carriers) but has no effect on the efflux of L-glucose (which diffuses like D-glucose, but is not transported).

In the following series of experiments hormone additions

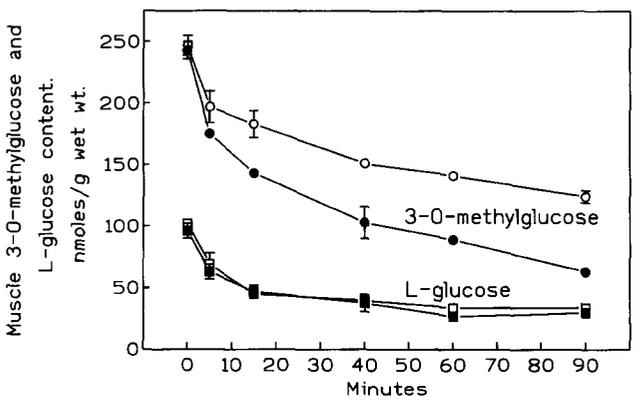

Fig. 1. Release of preloaded glucose from isolated rat soleus muscle. Isolated soleus muscle strips were incubated with [^{3}H]3-*O*-methylglucose (● ○) and [^{14}C]L-glucose (■, □) for 60 min prior to being incubated in label-free buffer in the presence (●, ■) or absence (○, □) of insulin. The glucose content of the muscles at the times indicated was calculated as described in the methods. Results are means ± S.E. of quadruplicate muscle strips from a representative experiment.

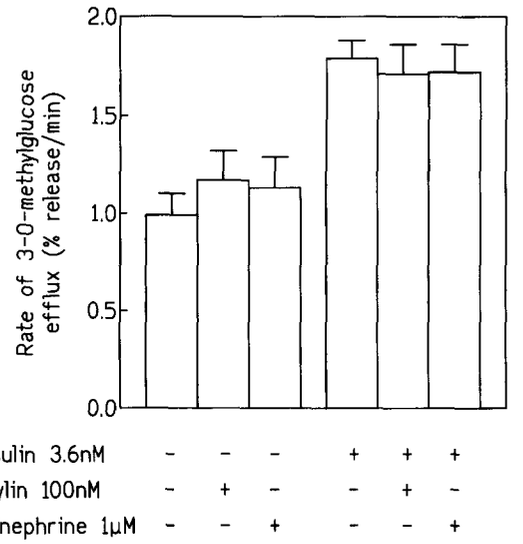

Fig. 2. Rate of 3-*O*-methylglucose efflux from preloaded isolated rat soleus muscle. Muscles were incubated with [^{3}H]3-*O*-methylglucose as described above and then further incubated with label-free buffer for 40 min after which hormones were added as indicated and incubations continued for an additional 30 min. The rate of efflux of label during this 30 min period was followed by taking samples of the incubation buffer and determining the label-content of the muscle at the end of the incubation, and is expressed as the percent of this initial content of label that was released into the efflux buffer per min. Results are means ± S.E. of 9–15 muscle strips from 3–4 independent experiments.

were made at the 40 min time point and incubations were continued for an additional 30 min. Fig. 2 shows the average rate of 3-*O*-methylglucose efflux from the tissue into the surrounding media during the 30 min. hormones were present. Insulin stimulated basal 3-*O*-methylglucose efflux by approximately 80%. Neither amylin nor epinephrine significantly affected either basal or insulin-stimulated glucose transport. Similar results were obtained using muscles from fed or fasted rats (results not shown). A tendency for amylin and epinephrine to stimulate the basal rate of 3-*O*-methylglucose efflux, as has been reported previously for epinephrine in rat skeletal muscle and [14] and human adipocytes [15], failed to reach significance in these experiments.

Fig. 3 shows 2-deoxyglucose uptake by isolated soleus muscles from both fed and fasted rats. Insulin stimulated 2-deoxyglucose accumulation by 244% and 141% in muscles from fed and fasted rats respectively, consistent with previously reported values [16,17]. Amylin or epinephrine did not affect basal 2-deoxyglucose uptake in muscles from fed or fasted rats and neither agent affected insulin stimulated 2-deoxyglucose accumulation in muscles from fasted rats. In muscles from fed rats, both amylin and epinephrine tended to decrease insulin stimulated 2-deoxyglucose accumulation (25% decrease, $P = 0.19$ and 38% decrease, $P = 0.09$, respectively). Leighton and Cooper [6] failed to show an effect of amylin on insulin stimulated 2-deoxyglucose accumulation, whereas Kreutter et al. [8] and Young et al. [7] reported that amylin inhibited insulin-stimulated 2-deoxyglucose accumulation by 65 and 45%, respectively.

The lack of effect of amylin and epinephrine on 3-*O*-methylglucose efflux indicates these agents did not directly affect glucose transport to any physiologically significant extent. Inhi-

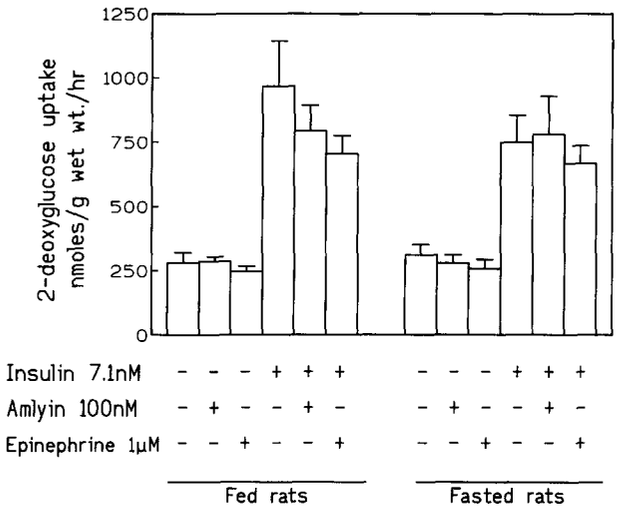

Fig. 3. Rate of 2-deoxyglucose uptake in isolated rat soleus muscle. Muscle strips were incubated with hormones as indicated for 15 min prior to the addition of [^{3}H]2-deoxyglucose for 30 min. Results are expressed as nmol 2-deoxyglucose uptake/min/mg wet wt. and are means ± S.E. of 8–12 muscle strips from 2–3 independent experiments.

bition of 2-deoxyglucose accumulation may result from the indirect inhibition of hexokinase activity [18] likely to follow elevated glucose 6-phosphate [7] concentrations observed after amylin (or epinephrine) stimulation of glycogenolysis. Compared to those in fed rats, muscles from fasted rats have less glycogen [19] which could be expected to produce a smaller G-6-P pool under glycogenolytic conditions and hence not inhibit hexokinase activity as much.

We conclude that amylin, like epinephrine, is unlikely to directly affect glucose transport in skeletal muscle. Our results are consistent with amylin (and epinephrine) decreasing glucose uptake indirectly by inhibiting hexokinase through activation of glycogenolysis and thus elevation glucose 6-phosphate levels.

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