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Stimulation-enhanced 3-*O*-methylglucose efflux from the frog sartorius: kinetics and properties of the system

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The characteristics of the process by which contraction enhances glucose transport in the frog sartorius were studied. Electrical stimulation increased the permeability of muscles to 3-*O*-methylglucose (3-*O*-MeGlc), a nonmetabolizable glucose analogue, increasing efflux as well as uptake. Enhanced efflux was due to an increase in V_{max} of the efflux process. A lactacidosis had no effect on basal 3-*O*-MeGlc efflux, and replacement of media Na^+ with Li^+ did not affect stimulation-induced uptake. Also, basal and stimulated uptake was not affected by 1 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator. Lastly, *N*-carbobenzoxy-glycyl-L-phenylalaninamide, which inhibits insulin-enhanced, but not basal, glucose uptake in adipocytes, inhibited both basal and stimulated 3-*O*-MeGlc fluxes in the frog sartorius. From these findings, we conclude: (1) contraction and exercise enhance glucose transport in muscle by increasing the number of transporters in the plasma membrane, or their turnover, by an unknown process; and (2) basal glucose transport of muscle, unlike that of adipocytes, can not be distinguished from stimulated transport on the basis of its insensitivity to *N*-carbobenzoxyglycyl-L-phenylalaninamide.

Introduction

In skeletal muscle, insulin and exercise enhance uptake of glucose or its nonmetabolizable analogue, 3-*O*-methylglucose (3-*O*-MeGlc), through an increase in V_{max} of the uptake process with no change in K_m [1–4]. An increase in V_{max} for uptake has traditionally been interpreted as either an increase in the number of active transporters in the plasma membrane, or an increase in the intrinsic activity of transporters already in the membrane [4]. In adipocytes, both actions have been ascribed to insulin's enhancement of glucose uptake [4]. Using rat hindlimb skeletal muscle, Hirshman et al. [5] and Klip et al. [6], recently showed that insulin increases the number of plasma membrane transporters, via recruitment from an intracellular pool, and Sternlicht et al. [7] showed that insulin increases both the number and intrinsic activity of plasma membrane transporters.

The situation with regard to the enhancement of glucose uptake by exercise is less well established. While exercise increased the number of glucose plasma membrane transporters in rat hindlimb muscle [8,9], no reciprocal reduction was seen in the insulin-sensitive intracellular pool of transporters [8]. Other studies reported an increase in transporter activity with no change in total number of plasma membrane transporters in rat hindlimb muscles following exercise [10,11]. Recently, King et al. [12] reported an increase following exercise in the rat in the number of glucose transporters in skeletal muscle plasma membrane vesicles, as well as an increase in vesicle glucose transport activity, measured under equilibrium exchange conditions. The increase in activity exceeded the increase in transporter number, indicating an increase in transporter turnover as well. Interpretation of these results, however, are complicated by several factors: (1) if the muscle glucose transporter is characterized by significant asymmetry in K_m between the two faces of the membrane, changes in affinity at one side of the membrane may not be detected by equilibrium exchange measurements [12]; (2) the sidedness of the sarcolemmal vesicles was not defined; (3) because exercise was used to stimulate glucose transport, rather than electri-

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Abbreviations: 3-*O*-MeGlc, 3-*O*-methylglucose; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

cal stimulation of isolated muscle, the contribution of other factors, such as insulin [13], can not be discounted; and (4), in theory, an increase in equilibrium exchange activity could occur independently of any change in net directional fluxes [14]. In red cells, differential effects of sulfhydryl blocking agents and insulin were noted on exchange and net fluxes of glucose, raising the possibility that the two processes are distinct [15].

Widdas [14] has proposed an alternative hypothesis for the increased V_{max} for glucose uptake produced by stimulation, which does not require an increase in the number of transporters in the membrane or their intrinsic activity (except possibly an increase in equilibrium exchange activity). The central feature of this hypothesis is that insulin or contraction results in a reversal of a natural bias of the glucose transporters (i.e., the glucose binding site) of muscle for an inward-facing orientation. The model predicts that stimulation should decrease glucose efflux from muscle through a reduction in V_{max} for efflux, although glucose efflux might be enhanced at less than saturating concentrations of intracellular glucose, due to a reduction in K_m for efflux as well. Application of this model to muscle is supported by the observation, made with the frog sartorius [1], that the glucose transporter of muscle is asymetric in its kinetic parameters, with the efflux process exhibiting a lower affinity, but greater V_{max}, than the uptake process. In the present study, we tested the model of Widdas by asking: (1) does stimulation enhance 3-*O*-MeGlc efflux from muscle?; and (2), if so, is this due to a change in V_{max} or K_m? These questions can only be answered by using whole muscle or muscle bundles. Whole muscle, the frog sartorius, was chosen for this study because of ease of preparation and because there is no damage from dissection to some fibers. The frog sartorius is eminently suited for studying the kinetics of transport, as it is a relatively thin muscle that remains functionally intact for hours following dissection [2]. In addition, the frog sartorius consists entirely of fast twitch fibers (types 1 and 2, with type 1 predominating, Ref. 16). Studies cited previously that employed vesicles from rat hindlimb muscle to examine effects of exercise on glucose transport dealt with a diverse mix of fiber types. Conceivably, different muscle fiber types might employ different mechanisms to regulate glucose transport, or the glucose transport process of different fibers might show different degrees of responsiveness to exercise. We also examined the possibility that one of several agents might act as mediators of (electrical) stimulation-enhanced 3-*O*-MeGlc fluxes. Finally, we examined whether basal and stimulated 3-*O*-MeGlc fluxes in the frog sartorius differed in their sensitivity to the dipeptide Cbz-Gly-Phe-NH_2 (*N*-carbobenzoxyglycyl-L-phenylalaninamide), as reported for adipocytes [17]. In these cells, Cbz-Gly-Phe-NH_2, a metalloendoproteinase inhibitor, was shown to interact directly with the glucose transporter(s) following insulin stimulation.

Methods

Animals and sartorius preparation. Young, male frogs (*Rana pipiens*), weighing 25 ± 6 g (mean ± S.D.) (n = 79), were obtained from West Jersey Biological (Wenonah, NJ). Frogs ($\leq$ 12) were housed in tanks (32 cm × 51 cm and 25 cm in height) with equal surface areas of water and dry land. The volume of water was 10 l. Experiments of Fig. 2 were carried out between June and October, using frogs that were forced fed one meal worm every other day to ensure equal nutritional status. Other experiments were done between August and March using unfed frogs. Frogs were used within 2 weeks. Frogs were anesthetized by being placed in the cold (4°C) for 1 h, and killed by double pithing. Except for experiments in Fig. 2, all muscles were incubated for 3 h following dissection or electrical stimulation: (1) to ensure that basal 3-*O*-MeGlc fluxes were not enhanced by the trauma of dissection, and (2) to load muscles with 3-*O*-MeGlc before efflux. Before stimulation, muscles (including nonstimulated controls) were preincubated for 90 min. Incubations were carried out in a shaking water bath (110 cycles/min).

Solutions. The standard physiological salt solution (PSS) consisted of (in mM): Na^+, 111; Cl^-, 105.5; K^+, 2.5; Ca^{2+}, 1; Mg^{2+}, 1; phosphate, 1; Hepes, 10; gassed with 100% O_2 during electrical stimulation, otherwise 100% air (pH 7.2). Modifications were as follows: (a) Ca^{2+}-free PSS: NaCl replaced $CaCl_2$, addition of 0.1 mM EGTA; (b) lactate PSS: equivalent exchange of NaCl with 40 mM sodium lactate. Li^+ PSS contained (in mM): Li^+ (or Na^+, control), 111; Cl^-, 102.5; K^+, 2.7; Ca^{2+}, 1.3; Mg^{2+}, 1.2, phosphate, 1.2; HCO_3^-, 15; gassed with 95% O_2/5% CO_2 (pH 7.2). This gas mixture was used by others [1–3] in studying glucose transport in the frog sartorius, and was used in our initial studies to compare our findings with theirs. Although 3% CO is physiological for frogs, and higher CO_2 might lower intracellular pH, comparisons were between muscle pairs, both of which were gassed with the higher CO_2. All PSS contained streptomycin (1 mg/ml) and penicillin G (100 μU/ml). Cbz-Gly-Phe-NH_2, TPA, and *N*-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide (W-7) were added from stock solutions in dimethyl sulfoxide, an equivalent amount (< 0.3%, v/v) of which was added to PSS of control muscle.

Electrical stimulation. Muscles were mounted under 1 g tension in a chamber maintained at 19°C. Stimulation was with 4 ms long rectangular pulses and a voltage that was supramaximal for contraction. Taking into account the output impedance of the stimulator,

as well as the impedance of PSS, the actual voltage between the stimulating electrodes was 1.2–1.6 V. Intensities of stimulation that produce a submaximal increase in 3-*O*-MeGlc uptake by the frog sartorius were employed [3].

Initial rates of 3-O-MeGlc uptake. According to the procedure of others [1], the extracellular space was first loaded by incubating muscles 20 min at 0°C. No significant 3-*O*-MeGlc uptake into the intracellular space occurred during this time. Afterwards, 3-*O*-MeGlc uptake into the intracellular space was permitted for 15 min at 19°C. Uptake media contained 5 mM 3-*O*-[^{3}H]MeGlc (0.5 μCi/ml), with 5 mM [^{14}C]mannitol (0.1 μCi/ml) as extracellular space marker. Muscles were then blotted (Whatman 40 filter paper slightly dampened with PSS), weighed, and placed in distilled water (2.0 ml) at 95°C for 10 min to facilitate homogenization. After cooling, muscles were homogenized, the homogenate centrifuged, and two 0.8 ml aliquots of supernatant assayed for radioactivity. Rates of 3-*O*-MeGlc penetration into the intracellular space are expressed as μmol/g wet weight per h. For testing the effect of Cbz-Gly-Phe-NH_2, the extracellular space was not filled first because this compound is less soluble at 0°C, and thus uptake under these conditions (control and treated) was not a measure of initial rates. In this study, muscles were dried (as below) and rates of uptake into the intracellular space expressed as μmol/ml intracellular water per 20 min.

3-O-MeGlc efflux. Muscles were incubated with 5 mM 3-*O*-[^{3}H]MeGlc (1–2 μCi/ml) and 5 mM [^{14}C]mannitol (0.2 μCi/ml), with muscles being transferred to fresh media after 2 h. After 3 h, muscles were blotted, and washout of labeled compounds followed using a series of flasks containing PSS (2 ml). Transfers between flasks were made after 2, 5, 15, 30, 45 and 60 min. Two 0.75 ml aliquots of each efflux PSS were sampled for radioactivity. Washout curves for each muscle were constructed and analysed, as described [18]. Studies comparing the sum of radioactivity in efflux washes and remaining in the muscle, to total radioactivity in the paired muscle that did not undergo efflux, showed that all radioactivity could be accounted for after efflux. Intracellular concentrations of 3-*O*-MeGlc given in Tables I, II and V were determined by subtracting the 3-*O*-MeGlc in the extracellular space, from the total amount of 3-*O*-MeGlc taken up by the muscle. Total water content of muscles, measured under identical conditions was 0.801 ± 0.001 ($n = 20$) ml/g wet weight, which agrees with what others have reported [1,19].

Kinetics of 3-O-MeGlc efflux. Muscles were incubated with various concentrations of 3-*O*-[^{3}H]MeGlc (1–50 mM) and [^{14}C]mannitol. To correct for osmolarity differences, the mannitol concentration was varied so that the sum of 3-*O*-MeGlc and mannitol was 55 mM. Mannitol in efflux PSS was 55 mM. To ensure loading of up to 20 mM, loading was carried out for 8 h at 26°C, with fresh PSS after 2.5 and 5 h. One muscle was stimulated 30 min at 1 Hz; the contralateral muscle served as nonstimulated control. For half the frogs, stimulation was done before efflux; for others, stimulation was done after dissection. Over a given range of intracellular 3-*O*-MeGlc, efflux rates were the same for both groups, an observation consistent with the finding that 30 min stimulation of the frog sartorius at 1 Hz causes a sustained (> 5 h) increase in initial rates of 3-*O*-MeGlc uptake, with no recovery [3]. Efflux of label was carried out for 45 min at 19°C with transfers at 2, 5, 15 and 30 min. Muscles were then blotted, weighed, and dried to constant weight (15 h) in a vacuum desiccator (80°C). It was established that no radiolabel was lost by this procedure, and no additional water was lost by heating for 24 h at 100°C. Dried muscles were homogenized in 1 ml of 5% TCA, the homogenate centrifuged at $1500 \times g$, and two 0.25 ml aliquots (brought to neutral pH and a final volume of 0.75 ml) of the supernatant sampled for radioactivity. Muscle water content and mannitol water volume were used to calculate intracellular water space.

Efflux over the first 15 min was discounted, as greater than 90% of the extracellular space washes out during that time, making accurate assessment of cellular efflux impossible. Efflux rates over two subsequent 15-min time intervals, i.e., over 15–30 min and 30–45 min, were measured from 3-*O*-MeGlc appearing in the PSS beyond residual washout of the extracellular space. Initial intracellular 3-*O*-MeGlc concentration for each washout interval was calculated from the 3-*O*-MeGlc in the washout PSS and in the mannitol-inaccessable compartment at the end of the interval. Compared with intracellular 3-*O*-MeGlc, the 3-*O*-MeGlc concentration in washout PSS was negligible. Rates were corrected for diffusion by subtracting out the initial phloretin-insensitive rate of 3-*O*-MeGlc efflux, determined by multiplying the initial intracellular 3-*O*-MeGlc concentration by the rate constant for 3-*O*-MeGlc efflux in the presence of 100 μM phloretin [18]. The rate constant in the presence of phloretin, determined under identical experimental conditions, was 0.0023 ± 0.0007 ($n = 6$) min^{-1}. Stimulation did not affect this value.

Materials. D-[U-^{14}C]Mannitol and 3-*O*-[^{3}H]methyl-D-glucose were purchased from either ICN Biomedicals (Costa Mesa, CA) or New England Nuclear (Boston, MA). Cbz-Gly-Phe-NH_2, TPA, and W-7 were obtained from Sigma (St. Louis, MO). Other chemicals were standard analytical grade.

Liquid scintillation spectroscopy. Radioactivity was measured with a Beckman LS1801 programed for simultaneous ^{3}H/^{14}C determination and automatic quench compensation.

Statistics. Results are reported as mean ± S.D. for (n) muscles or frogs. Comparisons were between muscle pairs, and the significance of differences between means determined by the paired Student's t-test. The computer software GraphPAD InPlot (GraphPAD Software, CA) was used to generate Figs. 2 and 3.

Results

Stimulation enhances both influx and efflux of 3-O-MeGlc

Uptake of 3-*O*-MeGlc by the frog sartorius was markedly enhanced by stimulation at 1 Hz for 30 min. With a medium concentration of 5 mM, initial rates of 3-*O*-MeGlc uptake were nearly 3-fold higher in stimulated muscle, 3.70 ± 1.89 μmol/g per h, compared to the nonstimulated, contralateral muscle, 1.32 ± 0.22 μmol/g per h ($n = 5$, $P < 0.01$). Muscles stimulated at 1 Hz exhibit a distinctive pattern of contraction marked by fatigue [20], but do contract for 30 min. An initial increase of some 10% in force is followed by a decline over 15 min to a new steady state level of some 20% of twitch force.

Efflux of 3-*O*-MeGlc from the sartorius occurred from three compartments. An initial compartment emptied within 2 min, and an intermediate compartment within 20 min. In nonstimulated muscles, the rate constants for 3-*O*-MeGlc efflux from these two compartments, and their combined volume of distribution, 0.26 ± 0.04 ($n = 31$) ml/g wet weight (WW), were identical to those for mannitol. The presence of two extracellular compartments in the sartorius is consistent with observations of others [21]; the first representing efflux from the space between fibers, the second, efflux from transverse tubules. The third compartment represented efflux from the mannitol-inaccessible, i.e., intracellular water space. The rate constant k for 3-*O*-MeGlc efflux from this compartment was calculated from the slope of the best straight line to the last 3 to 5 time points ($r^2 = 0.977 \pm 0.022$, $n = 80$). In muscle that had been electrically stimulated, 3-*O*-MeGlc efflux from the third compartment was markedly enhanced (Table I). Comparing muscle pairs, stimulation increased the rate constant k for 3-*O*-MeGlc efflux from the intracellular compartment nearly 4-fold (Table I). Since stimulation enhanced 3-*O*-MeGlc uptake, stimulated muscles contained some 5-fold as much 3-*O*-MeGlc after loading in PSS with 5 mM sugar than the nonstimulated, contralateral muscles (Table I). However, this 5-fold increase in intracellular 3-*O*-MeGlc can not explain increased efflux observed in stimulated muscles, as initial efflux rates for 3-*O*-MeGlc were some 14-fold higher in stimulated muscles, 7.83 ± 1.79 μmol/ml per h, as compared to nonstimulated muscles, 0.41 ± 0.23 μmol/ml per h ($n = 9$).

As Fig. 1 shows, 3-*O*-MeGlc efflux from both resting and stimulated muscles was reduced by phloretin. Phloretin reduced basal and stimulated efflux by 53%

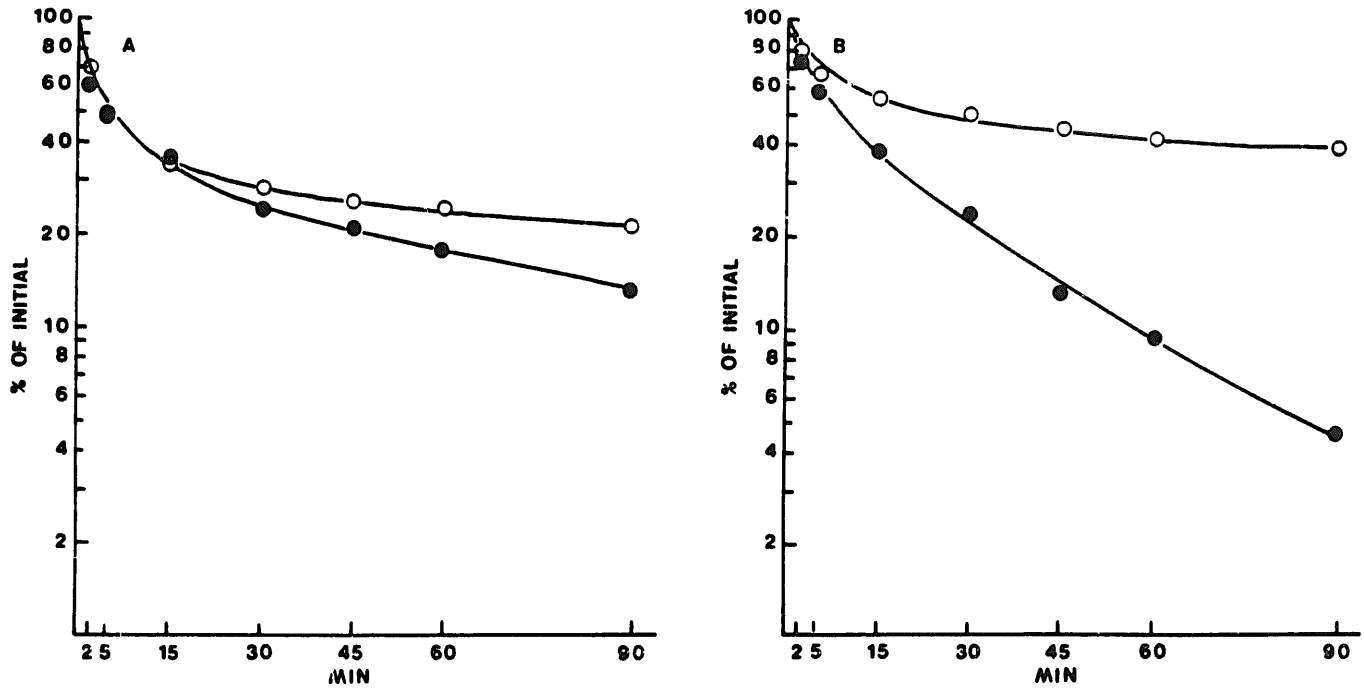

Fig. 1. Efflux of 3-*O*-MeGlc from stimulated and nonstimulated frog sartorius in the presence or absence of phloretin. The two muscles of a frog were incubated for 3 h in PSS containing 3-*O*-[^{3}H]MeGlc (1 μCi/ml) and 5 mM [^{14}C]mannitol (0.2 μCi/ml). Efflux was then followed over 90 min in the presence (open circles) or absence (closed circles) of 0.1 mM phloretin. (A) Efflux from nonstimulated muscles. (B) Efflux from muscles stimulated at 1 Hz for 30 min prior to being loaded with 3-*O*-MeGlc. Efflux is given as % of total sugar remaining in the muscle as a function of time.

TABLE I

Effect of electrical stimulation on 3-O-MeGlc efflux from the frog sartorius

Muscles were incubated 3 h in PSS with 5 mM 3-*O*-[^{3}H]MeGlc (1 μCi/ml) and 5 mM [^{14}C]mannitol (0.2 μCi/ml). One muscle of each frog was stimulated beforehand at 1 Hz for 30 min. The contralateral muscle served as nonstimulated control. Efflux was followed over 90 min into PSS. Shown is the rate constant k for efflux from the intracellular compartment and the concentration of 3-*O*-MeGlc in the intracellular compartment at the start of efflux. Values are means ± S.D. (n = 9).

	Paired muscles	
	nonstimulated	stimulated
k (min^{-1})	0.0070 ± 0.0021	0.0271 ± 0.0056 *
[3-*O*-MeGlc]$_i$ (mM)	0.94 ± 0.38	4.84 ± 0.60 *

* $P < 0.01$ compared with nonstimulated, contralateral muscle.

and 89%, respectively (Table II). The two residual rate constants were of similar magnitude. These data are consistent with 3-*O*-MeGlc efflux proceeding by the parallel pathways of carrier-mediated transport and simple diffusion, and with the relative contribution of simple diffusion being greater in the basal state. As shown by Fig. 1B, efflux from the intracellular pool was often rapid enough to partly obscure the intermediate compartment for 3-*O*-MeGlc in stimulated muscles with an initial intracellular 3-*O*-MeGlc concentration of less than 10 mM.

To further establish that increased efflux of 3-*O*-MeGlc with stimulation was not due to a difference in concentration, we examined 3-*O*-MeGlc efflux as a function of intracellular concentration, using muscle pairs, one of which was stimulated (Fig. 2). Under the conditions employed, it was possible to attain intracellular levels of 3-*O*-MeGlc in nonstimulated muscles at the start of efflux as high as 20 mM. As Fig. 2 shows,

TABLE II

Effect of phloretin on nonstimulated and stimulated 3-O-MeGlc efflux from the frog sartorius

Muscles were incubated 3 h in PSS with 5 mM 3-*O*-[^{3}H]MeGlc (1 μCi/ml) and 5 mM [^{14}C]mannitol (0.2 μCi/ml), following dissection (nonstimulated) or electrical stimulation at 1 Hz for 30 min. At the start of efflux, the intracellular 3-*O*-MeGlc concentrations were (in mM): nonstimulated, 1.38 ± 0.19 (n = 6); stimulated, 4.19 ± 0.39 (n = 6). Efflux was followed over 90 min into PSS without any addition or with 100 μM phloretin. Paired muscles were used. Shown is the rate constant k for efflux from the cellular compartment. Values are means ± S.D. (n = 3).

	k (min^{-1}) for paired muscles	
	no phloretin	100 μM phloretin
Nonstimulated	0.0093 ± 0.0005	0.0044 ± 0.0003 *
Stimulated	0.0281 ± 0.0021	0.0031 ± 0.0006 *

* $P < 0.025$ with contralateral muscles (i.e., no compared phloretin).

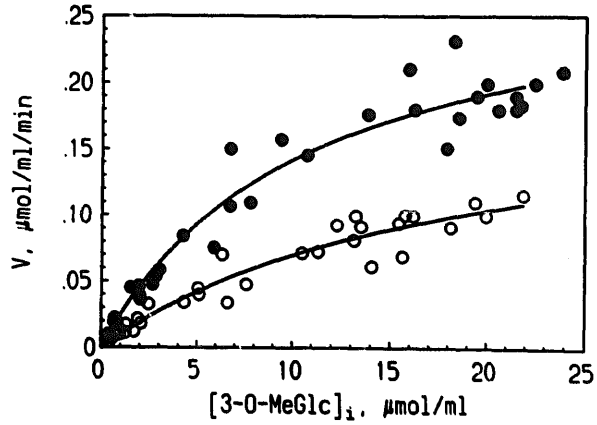

Fig. 2. 3-*O*-MeGlc efflux from stimulated (closed circles) and nonstimulated (open circle) muscles as a function of intracellular concentration, [3-*O*-MeGlc]$_i$. Muscles were incubated for 8 h at 26°C with various concentrations of 3-*O*-[^{3}H]MeGlc (1–50 mM, 1.0 μCi/ml) and [^{14}C]mannitol (0.2 μCi/ml). The sum of mannitol and 3-*O*-MeGlc was 55 mM. One muscle of each pair was stimulated at 1 Hz for 30 min following dissection or before efflux. Efflux was followed over 45 min at 19°C into 2 ml of PSS, with transfer to new PSS at 2, 5, 15 and 30 min. During the first 15 min, > 90% of the extracellular space washed out from the muscles. Rates of 3-*O*-MeGlc efflux (μmol/ml per min) from the intracellular compartment were determined for two subsequent 15-min intervals, from the amount of 3-*O*-MeGlc appearing in efflux PSS beyond residual washout of the extracellular space. Rates were corrected for 3-*O*-MeGlc diffusion by subtracting out the initial phloretin-insensitive rate of efflux. Initial intracellular 3-*O*-MeGlc for each washout interval was calculated from the amount of 3-*O*-MeGlc which appeared in the washout medium, and the amount left in the mannitol-inaccessible compartment of the muscle at the end. Shown are 33 data points for both stimulated and nonstimulated muscles from 18 frogs. For 15 frogs, two determinations of efflux rate were made; for the others, one. Saturation binding curves were fitted to the data by nonlinear regression: r^2 = 0.932 and 0.950 for nonstimulated and stimulated muscles, respectively.

both stimulated and basal effluxes showed saturation-type kinetics, and stimulation enhanced 3-*O*-MeGlc efflux at all intracellular concentrations. Lineweaver-Burk analysis of these data indicates that stimulation increased the V_{max} for efflux from 0.1399 to 0.3298 μmol/ml per min, with no effect on K_m, which was 11.1 and 12.9 mM for nonstimulated and stimulated muscles, respectively (Fig. 3).

Role of various agents in stimulating 3-O-MeGlc fluxes

The question of what factor or agent links stimulation and glucose transport was next addressed. Glucose transport and Na^+, K^+-ATPase activity are coordinately regulated in smooth muscle [22]. Whether this occurs in skeletal muscle was examined by substitution of Li^+ for Na^+. In nonstimulated muscle, 3-*O*-MeGlc uptake was not affected by incubation in Li^+ PSS. In two muscles, initial rates of 3-*O*-MeGlc uptake in Li^+ PSS were 0.84 and 1.68 μmol/g per h, while rates of uptake by their respective pairs incubated in Na^+ PSS were 1.06 and 1.77 μmol/g per h. (Muscles were

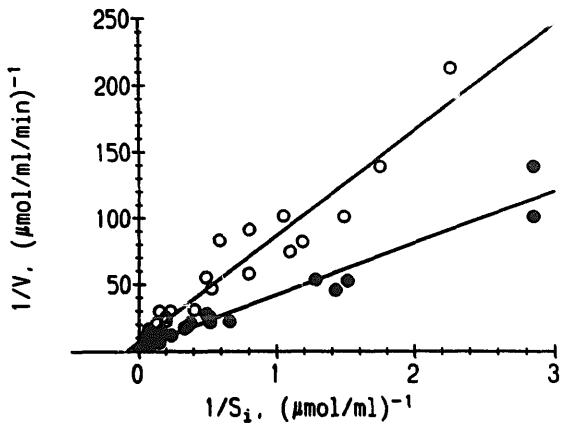

Fig. 3. Lineweaver-Burk plot of 3-*O*-MeGlc efflux from nonstimulated (open circles) and stimulated (closed circles) frog sartorius muscle (inverse rate of efflux, V^{-1}, vs. inverse intracellular 3-*O*-MeGlc concentration, S^{-1}). Data are from the study shown in Fig. 2. Lines were fitted to the data by the least-squares method of linear regression ($r^2 = 0.971$ and 0.958 for nonstimulated and stimulated muscles, respectively). Apparent K_m for 3-*O*-MeGlc efflux is 11.1 mM for nonstimulated, and 12.9 mM for stimulated muscles. V_{max} is 0.140 and 0.330 μmol/ml per min for nonstimulated and stimulated muscles, respectively.

pre-equilibrated for 60 min in the appropriate PSS). Li^+ substitution for Na^+, which produced only subtle differences in the pattern of contraction seen with 1 Hz stimulation, did not prevent stimulation-enhanced 3-*O*-MeGlc uptake. For muscles stimulated 30 min in Li^+ PSS, uptake was 5.42 ± 0.09 μmol/g per h, as compared to 5.01 ± 0.37 μmol/g per h for the paired controls, stimulated in Na^+ PSS ($n = 3$).

As Table III shows, stimulation-induced efflux of 3-*O*-MeGlc was not prevented by the protein synthesis inhibitor, cycloheximide, nor by leupeptin, a Ca^{2+}-dependent extralysosomal proteinase inhibitor [23]. Although stimulation of the frog sartorius enhances Ca^{2+}

TABLE III

Effect of cycloheximide, leupeptin and Ca^{2+}-free PSS on enhancement of 3-O-MeGlc efflux from the frog sartorius by electrical stimulation

Muscles were incubated 3 h in PSS with 5 mM 3-*O*-[^{3}H]MeGlc (1–2 μCi/ml), following stimulation (1 Hz, 30 min) in the presence (treated) or absence (nontreated) of cycloheximide, leupeptin, or Ca^{2+}-free PSS. Before stimulation, treated muscles were exposed to either cycloheximide or Ca^{2+}-free PSS for 90 min, or leupeptin for 3–5 h (also present during the 3 h loading). 3-*O*-MeGlc efflux was followed over 90 min. Shown are rate constants k for efflux from the intracellular compartment. Values are means ± S.D. ($n = 3$). None of the treatments had a significant effect.

Treatment	k (min^{-1}) for paired muscles	
	nontreated	treated
Cycloheximide (0.5 mM)	0.0291 ± 0.0029	0.0319 ± 0.0030
Leupeptin (100 μM)	0.0355 ± 0.0095	0.0324 ± 0.0047
Ca^{2+}-free media	0.0308 ± 0.0059	0.0271 ± 0.0075

TABLE IV

Effects of TPA and W-7 on initial rates of 3-O-MeGlc uptake by the frog sartorius

Initial rates of 3-*O*-MeGlc uptake into the intracellular compartment were determined in muscle incubated 15 min in PSS with 5 mM 3-*O*-[^{3}H]MeGlc (0.8 μCi/ml) and 5 mM [^{14}C]mannitol (0.15 μCi/ml), following a 20 min incubation at 0°C to load the extracellular space. Exposure to TPA (1 μM) and W-7 (25 μM) was for 90 and 60 min, respectively, including during stimulation. Where noted, stimulation was at 1 Hz for 30 min. Values are means ± S.D. Treatment with TPA or W-7 had no effect on uptake.

	Initial uptake rate (μmol/g per h) paired muscles	
	nonstimulated	stimulated
No TPA	1.23 ± 0.63	2.87 ± 1.09
1 μM TPA	1.22 ± 0.40	3.22 ± 0.86
(n)	(6)	(9)
No W-7	-	2.91 ± 0.24
25 μM W-7	-	2.86 ± 0.51
(n)		(3)

fluxes across the sarcolemma [20], this event is unlikely to trigger glucose transport, as enhancement of efflux was not affected by stimulation in Ca^{2+}-free PSS (Table III). To examine if acidification enhanced 3-*O*-MeGlc efflux, muscles were preincubated 15 min in lactate PSS at pH 6.4, then loaded 3 h in lactate PSS at pH 7.2, conditions where intracellular pH of the sartorius was observed to fall 0.36 units and where full recovery was prevented [24]. No effect of a lactacidosis was seen on the rate constant k for 3-*O*-MeGlc efflux from the intracellular compartment, which was 0.0066 ± 0.0015 min^{-1} and 0.0057 ± 0.0009 min^{-1}, for control and treated muscles, respectively ($n = 3$). None of the manipulations in Table III nor a lactacidosis affected how much 3-*O*-MeGlc was taken up by muscles during loading.

Electrical stimulation enhances phospholipase C activity in frog muscle, thus generating diacylglycerols, a physiological activator of protein kinase C [25]. To assess whether protein kinase C was involved in regulating 3-*O*-MeGlc transport, the effect of TPA on both basal and stimulated 3-*O*-MeGlc uptake was tested. A 90 min exposure to TPA did not affect initial rates of uptake by nonstimulated muscle (Table IV). No effect was observed when exposure to TPA was increased to 3 h (data not shown). TPA (1 μM) also had no effect on uptake, when it was present during stimulation at 0.3 Hz for 30 min ($n = 4$). At 1 Hz, TPA (1 μM) overall had no effect in 9 muscles on 3-*O*-MeGlc uptake (Table IV), though uptake was enhanced by 31.0 ± 17.3% ($P < 0.01$) in seven of these muscles. Since the TPA effect was modest and variable, it is unlikely that stimulation activates glucose transport by increasing protein kinase C activity.

The intracellular Ca^{2+} receptor, calmodulin, was reported to be involved in insulin's action in enhancing glucose transport in adipocytes [26]. Table IV shows that W-7 at 25 μM, a concentration expected to produce half maximal inhibition of calmodulin [27], did not prevent stimulation-enhanced uptake of 3-*O*-MeGlc. At 100 μM, W-7 also did not prevent stimulation-enhanced uptake, although uptake was $32 \pm 19\%$ less than in nontreated, stimulated muscles ($n = 3$, $P < 0.025$). However, the reduction over time in twitch force, normally seen with stimulation at 1 Hz [20], was $29 \pm 19\%$ greater in muscle treated with 100 μM W-7 ($n = 3$, $P < 0.05$).

Effect of Cbz-Gly-Phe-NH_2 on 3-O-MeGlc fluxes

The dipeptide Cbz-Gly-Phe-NH_2, which inhibits insulin-enhanced but not basal glucose uptake by adipocytes [17], had comparable effects on basal and electrically enhanced 3-*O*-MeGlc uptake by the frog sartorius. At a concentration (1.5 mM) that produces half-maximal inhibition of glucose uptake by human erythrocytes [28], Cbz-Gly-Phe-NH_2 reduced basal 3-*O*-MeGlc uptake (μmol/ml per 20 min) by 53%, from 0.95 ± 0.23 to 0.45 ± 0.10 ($n = 4$, $P < 0.025$), and stimulated uptake by 46%, from 1.48 ± 0.26 to 0.78 ± 0.31 ($n = 3$, $P < 0.025$).

Cbz-Gly-Phe-NH_2 (1.5 mM) was also effective in reducing efflux of 3-*O*-MeGlc from the intracellular compartment (Table V). Basal efflux was reduced by 36.6%, while the enhanced efflux that resulted from electrical stimulation was reduced by 66.0%. Insulin also enhanced 3-*O*-MeGlc efflux (Table V), and this enhanced efflux was inhibited by Cbz-Gly-Phe-NH_2 as well, by 74.5%. However, when compared relative to the maximum effect of phloretin (Table II), the effect of 1.5 mM Cbz-Gly-Phe-NH_2 in inhibiting 3-*O*-MeGlc efflux was similar in basal and stimulated muscles.

TABLE V

Effect of Cbz-Gly-Phe-NH_2 on nonstimulated and stimulated 3-O-MeGlc efflux from the frog sartorius

Muscles were incubated 3 h in PSS with 5 mM 3-*O*-[^{3}H]MeGlc (1 μCi/ml) and 5 mM [^{14}C]mannitol (0.2 μCi/ml), following dissection (basal or insulin) or electrical stimulation at 1 Hz for 30 min. Where indicated, 0.4 U/ml insulin (with 1% bovine serum albumin) was present during incubation. At the start of efflux, the intracellular 3-*O*-MeGlc concentrations were (in mM): nonstimulated, 1.24 ± 10.47 ($n = 6$); stimulated 4.25 ± 0.31 ($n = 6$); insulin, 4.22 ± 0.57 ($n = 6$). Efflux was followed over 90 min into PSS without any addition (control) or with 1.5 mM Cbz-Gly-Phe-NH_2. Paired muscles were used. Shown is the rate constant k for efflux from the cellular compartment. Values are mean ± S.D. ($n = 3$).

	k (min^{-1}) for paired muscles	
	control	Cbz-Gly-Phe-NH_2
Nonstimulated	0.0082 ± 0.0016	0.0053 ± 0.0008 *
Simulated	0.0215 ± 0.0019	0.0073 ± 0.0020 *
Insulin	0.0239 ± 0.0073	0.0061 ± 0.0031 *

* $P < 0.025$ compared with paired controls.

Discussion

Kinetics of stimulation-enhanced 3-O-MeGlc efflux

Recent studies have established that facilitative glucose transport is mediated by a family of structurally related proteins [4]. Five isoforms of the transporter identified to date by cDNA cloning are expressed to varying degrees in different tissues, and include a unique isoform that is expressed only in insulin-responsive tissues, such as adipocytes and muscle. Glucose transporters are glycosylated integral membrane proteins showing considerable homology in their primary sequences [4]. Hydropathy analysis indicates that the transporters consist of 12 transmembrane helices which form a pore or channel through which glucose passes [4]. Although the precise details of how the transporters function is not known, much work on red cells has given rise to a model in which a binding site for glucose exists within the channel of the transporter, with the channel constricted beyond that site [4,14]. The binding of glucose is proposed to induce a conformational change in the channel, such that the constriction moves to the opposite side of the binding site. Thus, glucose transport through the membrane is accomplished by exposure of the binding site to alternative sides of the membrane.

Recently, Widdas [14] suggested that a reversal of natural bias of the glucose transporters (i.e., the glucose binding site) of muscle for an inward-facing orientation in the basal state could explain either insulin- or contraction-induced increases in glucose uptake. Giving credence to this proposal is the observation that the V_{max} for glucose and 3-*O*-MeGlc efflux from the frog sartorius is at least 2- to 8-fold greater than the corresponding V_{max} for influx [1]. Putative modifier sites on the cytosolic surface of the transporter, or an increased affinity of the inward-facing carrier for glucose, would explain why stimulation does not lead to a net accumulation of glucose or glucose analogue. Following stimulation, an increase in intracellular glucose might act via the modifier sites to lock the transporter in an inward-facing conformation, while possibly enhancing net exchange activity [14]. We tested this proposal by examining the effect of stimulation on 3-*O*-MeGlc efflux from the frog sartorius, since the model predicts that stimulation should decrease 3-*O*-MeGlc efflux through a decrease in the V_{max} for efflux, or enhance efflux at less than saturating concentrations of 3-*O*-MeGlc, due to a reduction in K_m as well. These ideas can only be examined with whole muscle, given the shortcomings of vesicle studies listed previously

(Introduction), as well as the inherent difficulties in counting glucose transporters with cytochalasin B.

Our results show that stimulation does enhance 3-*O*-MeGlc efflux from the sartorius (Figs. 1 and 2) and, further, that this increase is due to an increased V_{max} (Figs. 2 and 3). Thus, we conclude that the model of Widdas does not explain contraction-enhanced glucose transport in (fast twitch) muscle fibers. Rather, stimulation must increase the number of transporters in the membrane, or the turnover of transporters already in the membrane.

In the present study, the K_m for 3-*O*-MeGlc efflux was similar for both nonstimulated and stimulated muscles, i.e., 11 and 13 mM, respectively. In two other studies, we observed even lower K_m values for efflux from stimulated muscles (4.7 and 6.5 mM). In contrast, Narahara and Özand [1] reported that the K_m for 3-*O*-MeGlc efflux from the frog sartorius was 24 mM. This latter study is frequently cited (e.g., Ref. 4) as evidence for the marked asymmetry in kinetic parameters for glucose influx and efflux in skeletal muscle, as the K_m for uptake from resting and stimulated frog sartorius muscles was 4 mM [1,3], a value consistent with what has been reported for facilitative glucose uptake in adipocytes, red cells, as well as other muscle [4]. The discrepancy between the previous study and ours in the K_m for 3-*O*-MeGlc efflux might be due to differences in methods: (1) the previous study did not correct for the contribution of diffusion to 3-*O*-MeGlc efflux; and (2) muscles of the previous study were loaded with intracellular 3-*O*-MeGlc concentrations (11–18 mM) that were lower than the estimated K_m value, so that the previously reported K_m value must be considered an overestimate. The observation that the K_m for efflux of the erythroid transporter isoform is increased by ATP [4], suggests an alternative explanation. Muscles in the study of Narahara and Özand [1] were loaded by insulin-stimulation, for a shorter length of time (5 h) and at a lower temperature (19°C), (some were loaded at 4°C for 18 h), than muscles of our study, which were loaded at 26°C for 8 h. Our conditions may have resulted in a loss of some factor (a decrease in a membrane-associated ATP pool?) that reduces the affinity of the inward-facing transporter for glucose. However, the interaction of ATP or any factor with the muscle/adipocyte glucose transporter isoform has not been reported [4].

As Table V shows, insulin also enhanced 3-*O*-MeGlc efflux from the sartorius. Others [3] had shown that the effect of insulin and contraction in enhancing 3-*O*-MeGlc uptake by the sartorius are additive only to a maximum value, suggesting that the two act upon the same pool of transporters. However, the conclusion that contraction in the sartorius must have also caused recruitment of transporters into the membrane, as shown for insulin in other muscles [5,6], is not warranted: (1) recruitment of transporters has not been demonstrated in fast twitch muscle fibers; and (2) several important differences have been noted between insulin- and contraction-enhanced 3-*O*-MeGlc uptake in the sartorius [29]. In mammalian muscles, the effects of insulin and contraction on glucose transport were reported to be additive [4,30,31], although this conclusion has recently been challenged [10].

Possible causes of enhanced 3-O-MeGlc transport

Cycloheximide did not prevent stimulation-enhanced efflux, indicating that de novo protein synthesis did not contribute to enhancement of transport by contraction (Table III). Others [29] had shown that protein synthesis inhibitors prevent reversal of stimulation-enhanced glucose transport in the frog sartorius, suggesting that stimulation may cause breakdown of a putative inhibitor protein to the transporter. However, enhancement of efflux was not blocked by leupeptin, a Ca^{2+}-dependent extralysosomal proteinase inhibitor [23] (Table III). Removal of Na^+ or Ca^{2+} (Table III and text) from the media did not prevent stimulation-enhanced 3-*O*-MeGlc transport, indicating that enhanced flux of these ions across the sarcolemma during stimulation does not trigger enhanced glucose transport. In addition, we were unable to demonstrate a role for cellular acidification or calmodulin (Table IV) in the process by which stimulation enhances glucose transport.

The phorbol ester, TPA had no effect on basal or stimulated 3-*O*-MeGlc uptake (Table IV). After we completed our study, Sowell et al. [32] reported that TPA had little effect on basal glucose transport in several muscles of rat. In contrast, TPA and other phorbol esters enhance glucose transport in a number of other insulin-responsive cells, including adipocytes [33], heart cells [34] and myocytes [35]. Such cells have a unique glucose transporter isoform,that is recruited into the membrane in response to insulin [4]. However, a recent study [36] in adipocytes showed that phorbol esters do not cause incorporation into the membrane of this transporter isoform, but rather the erythroid transporter isoform, the importance of which to glucose transport in muscle (in particular the frog sartorius) is unknown. While mRNA encoding the erythroid transporter isoform has been detected, at very low levels, in muscle [37], at least one study using diaphragm muscle failed to detect any proteins that were immunoreactive with antibodies against the erythroid transporter isoform [38].

Effect of Cbz-Gly-Phe-NH_2 on 3-O-MeGlc transport

Cbz-Gly-Phe-NH_2 inhibited both basal and stimulated 3-*O*-MeGlc transport in the frog sartorius by about the same extent. These results indicate that there may be fundamental differences in the basal

transporters of adipocytes and muscle, as basal glucose transport by adipocytes was insensitive to Cbz-Gly-Phe-NH_2 [17]. Recent studies have shown that basal glucose uptake by adipocytes is mediated by the erythroid transporter isoform [39], whereas insulin-stimulated uptake by both adipocytes and muscle is mediated by another, unique transporter isoform [4,39]. As mentioned earlier, the importance of the erythroid transporter isoform to basal glucose uptake in muscle is not known. Our finding that Cbz-Gly-Phe-NH_2 inhibited both basal and stimulated hexose transport in the sartorius, does not rule out that possibility, as Cbz-Gly-Phe-NH_2 was found to inhibit the erythroid transporter as well [28]. It is likely that some additional factor(s) act upon the erythroid transporter isoform of nonstimulated adipocytes to modulate (i.e., dampen) its activity.

In summary, stimulation-enhanced glucose transport in the frog sartorius is unlikely to be mediated by protein kinase C or calmodulin activation. What the link is between stimulation and glucose transport is not known. The finding of others [3] that the increase is a function of the frequency of stimulation and not of the amount of work done, suggests it is some membrane related process. Our study shows that it does not involve the membrane transport systems for Na^+, Ca^{2+}, or for maintaining intracellular pH. Finally, we have shown that stimulation enhances 3-*O*-MeGlc efflux from muscle by enhancing the V_{max} of the efflux process. Taken together with the similar effect of stimulation on influx [3], we conclude that stimulation enhances glucose transport in muscle by increasing the number, or turnover, of transporters in the plasma membrane.

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