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Alkaline pH activates the transport activity of GLUT1 in L929 fibroblast cells

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Abstract

The widely expressed mammalian glucose transporter, GLUT1, can be acutely activated in L929 fibroblast cells by a variety of conditions, including glucose deprivation, or treatment with various respiration inhibitors. Known thiol reactive compounds including phenylarsine oxide and nitroxyl are the fastest acting stimulators of glucose uptake, implicating cysteine biochemistry as critical to the acute activation of GLUT1. In this study, we report that in L929 cells glucose uptake increases 6-fold as the pH of the uptake solution is increased from 6 to 9 with the half-maximal activation at pH 7.5; consistent with the pKa of cysteine residues. This pH effect is essentially blocked by the pretreatment of the cells with either iodoacetamide or cinnamaldehyde, compounds that form covalent adducts with reduced cysteine residues. In addition, the activation by alkaline pH is not additive at pH 8 with known thiol reactive activators such as phenylarsine oxide or hydroxylamine. Kinetic analysis in L929 cells at pH 7 and 8 indicate that alkaline conditions both increases the Vmax and decreases the Km of transport. This is consistent with the observation that pH activation is additive to methylene blue, which activates uptake by increasing the Vmax, as well as to berberine, which activates uptake by decreasing the Km. This suggests that cysteine biochemistry is utilized in both methylene blue and berberine activation of glucose uptake. In contrast a pH increase from 7 to 8 in HCLE cells does not further activate glucose uptake. HCLE cells have a 25-fold higher basal glucose uptake rate than L929 cells and the lack of a pH effect suggests that the cysteine biochemistry has already occurred in HCLE cells. The data are consistent with pH having a complex mechanism of action, but one likely mediated by cysteine biochemistry.

Keywords

GLUT1; glucose uptake; pH effects; L929 fibroblast cells; acute activation; membrane transport

1. Introduction

Proper regulation of glucose uptake and metabolism are critically important to human health. The human body has a variety of intricate mechanisms designed to maintain a

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relatively constant blood glucose concentration. Dysregulation of this exquisite control system contributes to several human diseases, including diabetes, metabolic syndrome and cancer. The central hallmark of diabetes and metabolic syndrome is a glucose transport system that has been either fully or partially compromised, thus rendering systemic uptake inefficient. In contrast to diabetes, cancer is more often characterized by hyperactive glucose uptake and extensive glycolytic metabolism. Accumulating evidence supports the notion that many types of cancer overexpress GLUT1 as a means to obtain the carbon required to meet the high anabolic demands of constitutive cellular replication [1–5]. As such, GLUT1 has been identified as a potential target for cancer therapy [1, 6]. The relationship of GLUT1 to both diabetes and cancer highlights the critical need to understand how its activity is regulated in mammalian cells under normal and pathological circumstances. A more complete understanding of GLUT1 biology will be helpful in identifying potential therapeutic strategies for patients who suffer from either of these devastating metabolic diseases.

GLUT1 is a member of the SLC2A family of passive hexose transporters with an identifying feature of 12 transmembrane α -helical domains. These transporters are found in virtually all mammalian cells, with different members expressed at varying levels in different cell types. GLUT1 is expressed highest in embryonic tissues, the blood-tissue interfaces, astrocytes, muscle tissue, and erythrocytes where it can make up to 10–20% of the membrane protein [7]. Historically GLUT1 has been viewed as the transporter responsible for supplying a basal level of glucose to all cells. However, there is accumulating evidence that GLUT1 can be quickly activated by a wide variety of reagents without an increase in either GLUT1 expression or total GLUT1 membrane concentration. Stimulants include cell stressors such as osmotic stress [8, 9], azide [10, 11], berberine [12, 13], methylene blue [14], and glucose deprivation [15, 16], as well as treatment with thiol reactive compounds such as phenylarsine oxide [17], cinnamaldehyde [18], nitroxyl [19], and hydroxylamine [20] all significantly activate GLUT1 within minutes. In addition, peptide C has shown to activate GLUT1 in erythrocytes [21]. The acute activation of GLUT1 is not well understood and most studies attribute the activation to an ‘unmasking’ of GLUT1 already present in the membrane [10, 14, 22, 23]. However, a recent study suggests that the acute activation of GLUT1 in blood-brain barrier endothelial cells is accompanied by an increase in the membrane concentration of GLUT1 [24]. Therefore, it appears that the acute activation of GLUT1 is complex and may differ from tissue to tissue.

A plausible mechanism for the acute activation of GLUT1 comes from extensive work with erythrocytes. The data suggest that GLUT1 can assemble in the membrane as a monomer, as dimers, or as homotetramers each capable of transporting glucose, but with the tetramer as the most active form [7, 25–27]. Additionally, evidence indicates that the tetramer is stabilized by the formation of a disulfide bond within each GLUT1 subunit of the tetramer. This potential model for the acute activation of GLUT1 has not been directly demonstrated, though, we have generated significant data that suggests that thiols may be critical to the activation of GLUT1 in L929 fibroblast cells. This cell line expresses GLUT1 as the exclusive glucose transporter [28] and glucose uptake can be maximally activated within two minutes by thiol reactive compounds such as phenylarsine oxide, nitroxyl, and

hydroxylamine, which is believed to be converted to nitroxyl [17, 19, 20]. Pretreatment of cells with iodoacetamide to react with free thiols, blocks the activation of these compounds.

If cysteine residues are involved in the acute activation of glucose uptake in L929 cells, we hypothesized that alkaline conditions should increase the concentration of the thiolate and might be expected to enhance disulfide bond formation and glucose uptake. The effects of pH on GLUT1 activity have not previously been reported. Therefore, the purpose of this study was to measure the effects of pH on GLUT1 transport activity in L929 cells and determine how pH changes interact with other activators of glucose uptake.

2. Materials and Methods

2.1 Chemicals

Phenylarsine oxide (PAO), methylene blue (MB), hydroxylamine (HA), sodium azide (Az), cinnamaldehyde (CA), iodoacetamide (IA), berberine, 2-deoxy-D-glucose-[1,2-³H] (2DG) and D-mannitol-1-¹⁴C were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2 Cell culture

L929 mouse fibroblast cells were obtained from the American Type Culture Collection. The immortalized human corneal–limbal epithelial (HCLE) cell line was obtained from Dr. Ilene Gipson (Department of Ophthalmology, Harvard Medical School) and maintained as monolayer cultures in Keratinocyte-Serum Free medium (K-SFM) (Invitrogen, Carlsbad, CA), as previously described [29]. To initiate each experiment, approximately 1.5×10^5 L929 fibroblast cells were plated into each well of a 24-well culture-treated plate in 1.0 mL of low glucose (5.5 mM) DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were grown overnight at 37 °C in an incubator supplied with humidified room air with 5% CO₂. Since the magnitude of stimulatory or inhibitory effects varies somewhat depending on the confluency of the cells, experiments were typically done with cells near confluency (3.2×10^5 cells/well for a 24-well plate).

2.3 General experimental design

To initiate an experiment, the media from cells in 24-well plates were removed and cells were then incubated in 0.4 mL of fresh treatment media consisting of either low-glucose DMEM alone (0% FBS) or low-glucose DMEM plus the chemical of interest (see figure legends) or glucose-free DMEM (activation by glucose deprivation). Cells were maintained at 37 °C for the times indicated. Reagents were added to the media or 2DG uptake solution from 100–200× aqueous (HA), or ethanol (CA) or DMSO (PAO, berberine) stock solutions. Ethanol and DMSO at the concentrations added have no effect on glucose uptake [17, 18].

2.4 Glucose uptake assay

Glucose uptake was measured using the radiolabeled glucose analog 2-deoxyglucose (2DG) as previously described. Briefly, the media was replaced with 0.2 mL of glucose-free HEPES buffer (140 mM NaCl, 5 mM KCl, 20 mM HEPES/Na (varied pH), 2.5 mM MgSO₄,

1 mM CaCl₂, 2 mM NaPyruvate, 1 mM mannitol) supplemented with 1.0 mM (0.3 µCi/mL) 2-DG (1,2-³H) and 1.0 mM (0.02 µCi/mL) mannitol (1-¹⁴C). Uptake media was supplemented with additional compounds, such as PAO or HA, as indicated in the figure legends. For the kinetics experiment, the concentration of 2DG was varied as indicated in the figure legends. After a 10-minute incubation, cells were washed twice with cold glucose-free HEPES. The cells were lysed in 0.3 mL of 0.3M NaOH and the ³H-2 DG and ¹⁴C-mannitol were measured using scintillation spectrometry. Mannitol is not normally taken up by cells, therefore the inclusion of ¹⁴C-mannitol in the uptake media allows us to account for any surface binding, or to account for excess radioactivity that might remain after the washes. It also allowed us to monitor potential toxic effects of the experimental treatments that would compromise the cell membrane.

2.5 Statistical analysis

Data reported represent means of 4–8 samples and glucose uptake was measured as nmol/10 min/well ± standard error, and normalized to uptake at pH=6.0. Statistical significance was determined by a two-tailed t-test. Statistical significance is reported at P< 0.01.

3. Results

3.1 GLUT1 transport activity is enhanced in alkaline conditions

Evidence from erythrocytes suggests that the formation of an internal disulfide bond stabilizes a homotetramer, which is a more active transporter [7]. While not have direct evidence for the formation of homotetramers in L929 cells, we have observe that some thiol reactive compounds activate GLUT1 within minutes implicating that cysteine residues play a key role in the acute activation of GLUT1 in these cells. Thiol chemistry should be enhanced under alkaline conditions, due to the enhanced nucleophilicity of the thiolate. To determine if pH could alter the activity of GLUT1, we measured glucose uptake in L929 cells at a variety of pHs ranging from 4–10. The results shown in Figure 1 indicate that glucose uptake increases six-fold over that range. The significant change in uptake occurs between pH=6–8 with the half-maximal activation at pH=7.5. Therefore, the remaining experiments focused on pHs 6, 7 and 8.

3.2 Glucose uptake recovers quickly from the effects of pH

The effects of pH have a rapid onset, occurring within the 10 minutes required for measuring 2DG uptake. If the alkaline pH does stimulate the formation of a disulfide bond, we might expect that the activation of pH 8 might be maintained for some time after the pH returns to 7. In order to understand how quickly the cells recovered from the effects of pH, L929 cells were incubated at pH 6, 7, or 8 for 10 minutes followed by the immediate measurement of glucose uptake at pH=7. For comparison, uptakes were also measured at the pH matching the incubation pH. The results, shown in Figure 2, again demonstrate a strong pH effect when uptakes are measures at 6,7 and 8, but clearly this effect is quickly reversed. While there is a trend for cells pretreated at pH 8 to be higher than cells maintained at pH 7 (P=.08), there is no statistically significant residual effects of the pH 6 or 8 pretreatment.

3.3 Thiol reactive compounds mute the pH effect

If the pH effect is thiol dependent, we would predict that a pretreatment of cells with thiol reactive compounds should mute the pH effect. To test this, L929 cells were pretreated for 20 minutes with, either 1.0 mM iodoacetamide (IA), a compound that forms a covalent adduct with free thiols by a nucleophilic substitution reaction, or 2.0 mM cinnamaldehyde (CA), a compound forms a covalent adduct with thiols by a Michael addition reaction. Following incubation with these compounds, uptakes were measured at pH 6, 7, and 8. As seen in Figure 3, CA significantly flattens the pH effects with small increase from pH 6 to 7, but no further enhancement of uptake at pH 8. Pretreatment with IA completely abolishes the pH effect.

We also measure glucose uptake in the presence of thiol-reactive compounds known to activate glucose uptake. If the pH effects are activating thiol chemistry we would predict that the thiol reactive compounds would not be additive to the effects at pH 8. Glucose uptake results at pH 6, 7 or 8 in the presence and absence of PAO, a compound that reacts with vicinal thiols, or HA, a compound that is thought to be converted to nitroxyl in L929 cells and stimulate formation of disulfide bonds, are shown in Figure 4. The data indicate that PAO and HA both significantly activate glucose uptake at pH 6 and 7, but have no additional activation at pH=8.

3.4 Combined effects of pH with other activators are variable

We were curious to understand what effect pH would exert on cells that were activated prior to exposure to changes in pH. Glucose uptake in L929 cells was activated by glucose deprivation, or treatment with maximally effective concentrations of either MB or berberine. Briefly, cells were treated for 30 minutes with media without glucose (NG), or low glucose media containing either 50 μ M MB, or 50 μ M berberine. Glucose uptake was then measured at pHs 6, 7, and 8. The results, shown in Figure 5, indicate that pH effects essentially additive to the effects of MB or berberine, but not to the effects of glucose deprivation. The relative magnitudes of the effects of these three activators at pH 7 were consistent with previously published studies [13, 14, 16].

3.5 Glucose uptake is not activated at pH 8 in HCLE cells

Previous work has shown that HCLE cells express GLUT1 as the primary glucose transporter and have much higher glucose uptake rates than L929 cells [30]. The enhanced uptake was caused by an increase expression combined with an activation of the transporter. It was observed that compounds which activated glucose uptake in L929 cells either had no effect in HCLE cell, or in the case of thiol reactive compounds, actually inhibited glucose uptake [30]. If GLUT1 in HCLE cells is already activated by involvement of the key thiols, we hypothesized that alkaline conditions would not further activate glucose uptake. To investigate this, we measured glucose uptake in HCLE cells at pHs ranging from 6–9 and the results are shown in Figure 6. The data are normalized to uptake at pH=6 and full activation of a 2.5-fold increase occurs between pH 6 and 7, but no further activation after pH=7. This differs significantly from L929 cells where an overall 6-fold activation occurs between 6 and 9 with the major increase occurring between 7 and 8.

3.6 Kinetics of glucose uptake at pH 7 and 8

Previous work in L929 fibroblast cells has shown that the kinetics of activation of GLUT1 can vary depending on the stimulant. Glucose deprivation and berberine treatment decrease the K_m of transport with little effect on the V_{max} , while methylene blue treatment increases the V_{max} with little effect on K_m [13, 14, 16]. Since the greatest effects of pH occur between pH 7 and 8, we measured the kinetics of uptake at these two pHs. 2DG uptakes at various concentrations of 2DG were measured in L929 cells at either pH 7 or pH 8 and the results are shown in Figure 7. The fitted data indicate that pH 8 increases the V_{max} of transport from 7.5 to 11.5 nmol/10min/well and decreases the K_m from 5.5 to 3.8 mM.

4. Discussion

GLUT1 is widely expressed in mammalian systems and responsible for basal glucose uptake in most cells. However, accumulating studies have clearly demonstrated that this transporter can also be quickly activated at the membrane either with or without a concomitant increase in membrane concentration of GLUT1. In cases where activity of GLUT1 increases without a change in its membrane concentration, the mechanism of activation is not well understood. One possibility is that the level of GLUT1 activity is associated with a migration to or away from lipid rafts [10, 15, 22, 23]. A second possibility is that GLUT1 can form homotetramers, which are more active than the monomer or dimer forms. This potential model for activation has been generated from extensive kinetic analysis and biochemical isolation of GLUT1 from erythrocytes where GLUT 1 accounts for 10–20% of the membrane protein [7]. The data suggest that the more active tetramer form is more stable with oxidized GLUT1 monomers where a disulfide bond has formed between cysteine residues 347 and 421 [26, 31]. The GLUT1 interactions that stabilize the tetramer appear to primarily involve transmembrane helix 9 [32]. The reversible formation of tetramers as a mechanism for acute activation of GLUT1 has not been directly confirmed. However, we have generated significant data in L929 fibroblast cells that demonstrate that GLUT1 can be acutely activated by a variety of stimulants and the fastest acting compounds are thiol reactive compounds.

In this study we report that glucose uptake increases six-fold from low to high pH in L929 cells. The onset of this effect is fast and quickly reverses when cells are returned to pH 7. The uptake assay measures both the transport of glucose via GLUT1 and its phosphorylation by hexokinase. Previous studies have shown that the activity of hexokinase does not change from pH 6–8 [33, 34] and we do not observe the pH effect under all conditions (see Figures 3–5). Therefore, it seems very likely that the increasing pH is enhancing the transport activity of GLUT1 itself rather than activating hexokinase.

This study does not reveal the molecular change in GLUT1 that accounts for its increased activity at alkaline pH, but the data are consistent with cysteine residues, with a pK_a of about 7.5, playing a key role. The major rationale for this conclusion is the observation that thiol reactive compounds mute the pH effect. The pH effects are significantly curtailed by the pretreatment of cells with CA, a good Michael acceptor of thiols, and completely negated by pretreatment with IA. Also, thiol reactive compounds such as PAO and HA, which fully activate glucose uptake in L929 cells within 2 minutes, are additive to the pH

effects at submaximal levels (pH 6 and 7), but not at the maximally activating pH conditions of 8. The nonadditivity of the maximally effective conditions of pH 8 with maximally active concentrations of either PAO or HA suggests that pH, PAO and HA share a similar mechanism of activation.

This study does not provide direct evidence for the formation of a disulfide bond under alkaline conditions. However, there is chemical logic to the notion that the more nucleophilic thiolate would stimulate the oxidative chemistry of disulfide formation. However, the fast recovery suggests that either this reversible oxidation is very fast or that a stabilized disulfide bond may actually not form; rather it may simply be that the thiolate itself triggers a conformational change that enhances GLUT1 activity. Subsequent work will be needed to help sort this out.

The effects of CA reported in this study appear to be in conflict with an earlier study from this lab. This study reports the CA activates uptake at pH 6 and 7 and inhibits uptake at pH 8 with the break-even point just above pH 7 (Figure 3), while a previous study shows an activating effect for CA at a reported pH of 7.4 [18]. However, the effects of pH that we report here actually help explain some puzzling observations that we have made subsequent to that study. We have occasionally observed that CA either did not elicit an activation of glucose uptake or it actually was inhibitory. This variability result can now be attributed a variable pH of the uptake media. While our stock HEPES buffer is pH 7.4, the final pH of our uptake buffers was not rigorously checked. We now recognize that the pH of our final uptake buffers are varied and can be significantly lower. At a lower pH, CA activates glucose uptake as previously reported [18].

The enhanced GLUT1 activity that occurs between 7 and 8 in L929 cells is not observed in HCLE cells (see Figure 6). HCLE cells have a much higher concentrations of GLUT1 and glucose uptake rates and activators effective in L929 cells, including berberine, MB, and the thiol active compounds, do not further active glucose uptake [30]. This suggests that the thiol chemistry that may be initiated by the change from pH 7 to 8 has already occurred in HCLE cells. Interestingly, the same 2-fold decrease in activity from pH 7 to 6 is observed in both cell lines. Thus, it is not clear if the pH changes observed over the full pH range (Figure 1) is the continuation of a single effect or if pH is actually having multiple effects on glucose uptake.

Previous work has suggested that there may be multiple mechanisms for the activation of GLUT1 in L929 cells. The clearest evidence for this comes from kinetic analysis of glucose uptake in L929 cells which has shown that glucose deprivation and treatment with berberine decrease the K_m of uptake without a significant change in the V_{max} , while treatment with methylene blue increase the V_{max} without significant change in the K_m [13, 14]. The data presented here suggests that the pH effects tap into both mechanisms of activation. First, the kinetics of glucose uptake at pH 7 and 8 indicate that alkalinity both increases the V_{max} of uptake and decreases the K_m . Second, the pH effects are additive to the effects of both berberine and MB suggesting that pH is contributing additional activating potential to the two pathways represented by berberine and MB. The pH effects are not additive to glucose deprivation. However, glucose starvation is the most robust activator and may simply

maximize the activation potential in L929 cells. Clearly, more work needs to be done to understand the mechanism or mechanisms for the acute activation of GLUT1.

5. Conclusions

This study reports that alkaline pH reversibly activates glucose uptake in L929 cells. The effect is effectively blocked by prior treatment with thiol reactive compounds such as IA and is not additive to thiol reactive activators at pH 8 suggesting that the effects of pH are mediate by key cysteine residues with a pKa of 7.5. In HCLE cells where GLUT1 is highly active under basal conditions, a change in pH from 6 to 7 doubles glucose uptake no further activation occurs between pHs 7 and 9.

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Research Highlights

- Glucose uptake increases 6-fold as pH increases from 6 to 9.
- pH activation is blocked by prior treatment of cells with iodoacetamide or cinnamaldehyde
- Thiol reactive activators, such as phenylarsine oxide and hydroxylamine, are additive to pH effects at 6 and 7, but not at pH 8
- pH effects are additive to activating effects of berberine and methylene blue, but not to effects of glucose deprivation
- Increase of pH from 7 to 8 increases the V_{max} and decreases the K_m of glucose uptake

Figure 1.

Alkaline pH activates glucose uptake. 2DG uptake was measured at various pH's ranging from 4 to 10 and the data are expressed as means $\pm$ S.E. and normalized to the uptake at pH=6. All uptakes measured above pH=6 were significantly greater at $P < 0.01$.

Figure 2.

Recovery from pH effects. Cells were treated for 10 minutes at either pH 6, 7 or 8 and 2DG uptake was measured at pH=7 (first three bars) or measured at a matching pH of 6, 7 or 8 (last three bars). Data are expressed as means $\pm$ S.E. and normalized to the uptake in cells maintained at pH 6. Uptakes measure at pH 7 are not different from each other.

Figure 3.

Pretreatment of cells with IA or CA mutes pH effects. Cells were treated for 20 minutes either with no additions (Con), 2.0 mM cinnamaldehyde (CA), or 1.0 mM iodoacetamide (IA). Uptakes were measured at pH 6, 7, or 8 and data are means $\pm$ S.E. normalized to control uptake at pH 6.

Figure 4.

Effects of PAO and HA are not additive to effects of pH 8. 2DG uptakes were measured at pH 6, 7, or 8 in the presence of either no additions (Con), or 5 μ M phenylarsine oxide (PAO), or 5 mM hydroxylamine (HA). Data are means $\pm$ S.E. normalized to control uptake at pH 6. PAO and HA treatments at pH 6 and 7, but not at pH 8, were significantly greater than control at $P < 0.01$.

Figure 5.

Effects of pH combined with other stimulants. Cells were treated for 20 minutes with maximally activating conditions of either media without glucose (NG), or 50 μ M berberine (Ber), or 50 μ M methylene blue (MB). 2DG uptakes were measured at pH 6, 7, or 8 and data are means $\pm$ S.E. normalized to control uptake at pH 6. All treatment conditions were greater than control at $P < 0.01$ at all three pH conditions. Only NG had no significant increase from pH 6–8.

Figure 6.

Alkaline pH activates HCLE cells only from pH 6 to 7. 2DG uptake was measured in HCLE cells at pH ranging from 6 to 9. Data are means $\pm$ S.E. normalized to control uptake at pH 6. All pH conditions were significantly greater than uptake at pH 6 at $P < 0.01$. There was no difference in uptakes at pH 7–9. Data from L929 cells are shown for comparison.

Figure 7. Effects of pH on the kinetics of 2DG uptake. 2DG uptakes were measured at varying concentrations of 2DG at pH 7 and 8. Data are means $\pm$ S.E. of quadruplicate samples from a representative experiment and expressed as nmol/10min/well. Lines are best fit lines using Michaelis-Menton kinetic analysis.