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Enhancement of Glucose Transport in Clone 9 Cells by Exposure to Alkaline pH: Studies on Potential Mechanisms

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Summary. Incubation of a nontransformed rat liver cell line, Clone 9, at pH 8.5 resulted in an ~16-fold stimulation of cytochalasin B-inhibitable 3-O-methylglucose (3-OMG) transport, an effect that was independent of the presence of serum. Exposure to 100 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated 3-OMG uptake, and the enhancement was not additive to that produced by incubation at pH 8.5. In cells "depleted" of protein kinase C activity by a 20-hr exposure to TPA, however, the stimulation of 3-OMG transport in response to incubation at alkaline pH was still fully demonstrable. In control and alkaline pH-exposed cells, the inhibition of 3-OMG uptake by cytochalasin B was consistent with a single-site ligand binding model (K_i ~10^{-7} M). Northern blot analysis demonstrated the presence of only the human erythrocyte/rat brain/HepG2 cell glucose transporter-mRNA isoform (EGT), and the abundance of this mRNA was unchanged following exposure to alkaline pH. Immunoblot analysis, using polyclonal antibodies directed against the carboxy-terminal dodecapeptide of EGT, demonstrated an ~2.0-fold increase in the abundance of transporters in partially purified plasma membrane fractions following incubation at pH 8.5, while EGT abundance was unchanged in whole-cell extracts. It is concluded that the stimulation of glucose transport in response to incubation of Clone 9 cells at alkaline pH does not require the presence of serum or activation of protein kinase C, and that the response is at least in part mediated by an increase in the number of glucose transporters in the plasma membrane.

Key Words serum · phorbol ester · glucose transporter abundance · EGT · glucose transporter-mRNA

Introduction

Facilitated transport of glucose across plasma membranes in mammalian cells is mediated by specific glycoprotein glucose transporter (GT) molecules, several forms of which have recently been cloned and characterized [4, 5, 10, 22, 31, 39, 42, 50]. Certain organs, including kidney and intestine, additionally express Na^+/glucose carriers, cotransporters that bear no homology with the above glucose transporters [26]. Facilitative glucose transporters are functionally distinguishable by their affinity for glucose and their sensitivity to inhibition by cytochalasin B [16, 42, 54]. The human erythrocyte GT exhibits a K_i for cytochalasin B of ~10^{-7} M [35, 42], is widely distributed, and is closely homologous with the transporters expressed in rat brain and HepG2 cells [5, 39, 42].

In a previous study we reported that the rate of carrier-mediated glucose transport in a nontransformed rat liver cell line (Clone 9) is markedly stimulated by incubation at pH 8.5, achieved by reducing the CO_2 tension in a bicarbonate-containing medium [29]. In contrast to adult rat liver, these cells are characterized by internal glucose concentrations less than 10% that of the external medium, suggesting that in these cells under basal conditions glucose transport is rate limiting for glucose consumption [8, 38]. Incubation of Clone 9 cells at alkaline pH results within 15 min in a progressive stimulation of glucose transport such that following 2–3 hr of incubation at pH 8.5, the enhancement of transport is sufficient to render cell ATP content resistant to maximally effective concentrations of cyanide, presumably because near-normal rates of ATP synthesis are maintained by glycolysis alone [29]. This stimulation of glucose transport moreover is attributable to an increase in the V_{max} rather than to a change in the K_m for the transport process, and ongoing protein synthesis does not appear to be required for the response [29].

A stimulation of glucose transport by incubation at alkaline pH has also been reported in other cell systems, including rat adipocytes, and an apparent translocation of GTs to plasma membranes has been suggested as a possible mechanism [30, 32, 47, 51]. In the present study we show that the presence of serum with its associated factors is not a necessary condition for the glucose transport response, that in cells functionally "depleted" of protein kinase C activity by prolonged incubation in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA) maximal stimulation of glucose transport upon exposure to alkaline pH is fully preserved, and that the K_i

for cytochalasin B inhibition of glucose transport is unchanged despite an ~12-fold increase in the rate of transport. Consistent with the observed K_I for cytochalasin B (~10^{-7} M), Clone 9 cells are shown to express only the GT-mRNA encoding the human erythrocyte/rat brain/HepG2 cell glucose transporter (EGT), the abundance of which remains unchanged upon incubation at the alkaline pH. Finally we show, by Western blot analysis, that the EGT content in whole-cell extracts remains unchanged while its abundance is increased in a partially purified plasma membrane fraction isolated from cells incubated at alkaline pH.

Materials and Methods

Materials

Clone 9 cells [53] were obtained from American Type Culture Collection (Rockville, MD) and used between the 25th and 40th passages. [^{3}H]3-O-methyl-D-glucose (3-OMG; 5.1–8.5 Ci/mmol) and α-[^{32}P]TTP (3,000 Ci/mmol) were purchased from Amersham (Chicago, IL). Phloretin, cytochalasin B, bovine serum albumin, 12-O-tetradecanoylphorbol 13-acetate (TPA), and dimethyl sulfoxide (Me_2SO) were purchased from Sigma Chemical (St. Louis, MO). Nitrocellulose paper for Northern and Western blots were obtained from Schleicher and Schuell (Keene, NH) and BIO-RAD. (Richmond, CA), respectively. Acrylamide and other reagents necessary for Western blotting were obtained from BIO-RAD. Rabbit polyclonal antibody directed against the C-terminal dodecapeptide of EGT [5, 24] was purchased from East Acres Biologicals (Southbridge, MA). Goat anti-rabbit serum was purchased from Boehringer Mannheim (Indianapolis, IN), and rabbit peroxidase anti-peroxidase was obtained from Organon Teknika (Durham, NC). Calf serum and powdered culture medium were purchased from GIBCO (Grand Island, NY), and plastic culture dishes were obtained from Corning Glass Works (Medfield, MA).

Cell Culture and Exposure to Alkaline pH

Cells were maintained in Dulbecco's modified Eagle's medium containing 5.6 mM glucose and 44 mM $NaHCO_3$ (DME medium), supplemented with 10% calf serum, at 37°C in a 9% CO_2-humidified atmosphere (pH 7.45), as described previously [29, 53]. At confluence (~4 days following passage) the medium was replaced with fresh medium containing 10% calf serum or medium alone, and experiments were performed 20 hr later. All studies were carried out under control and experimentally modified conditions in parallel. Incubation at alkaline pH was carried out by placing the culture plates in an atmosphere containing 0.7% CO_2, yielding an extracellular pH of 8.5 [29]. Where indicated, plates were returned to the normal CO_2 tension for 30 min prior to study.

Measurement of [^{3}H]3-OMG Uptake

Confluent cells on 60-mm culture plates in triplicate were used for uptake measurements as previously described [29]. Uptake was terminated after 1 min of incubation. The accumulation of [^{3}H]3-OMG at 1 min in either control or stimulated cells did not exceed 30% of its corresponding steady-state value. Cytochalasin B-inhibitable 3-OMG uptake was calculated as the difference between the uptake in each of three plates in the absence of cytochalasin B and the mean uptake in three plates in the presence of the inhibitor assayed in parallel.

To examine the kinetics of inhibition of [^{3}H]3-OMG transport by cytochalasin B, triplicate culture plates were preincubated in the presence of various concentrations of cytochalasin B added in 10 μl of Me_2SO or Me_2SO alone for 30 min prior to measurement of uptake. Uptake was initiated by removal of the medium and incubation with uptake medium containing the corresponding concentration of cytochalasin B that had been employed in the preincubation period. Total cytochalasin B-inhibitable uptake was calculated as the difference between the uptake in the absence and presence of 50 μM cytochalasin B and normalized to 1.0. The uptake at each concentration of cytochalasin B was then calculated as the fraction of the uptake at the inhibitor concentration to the total cytochalasin B-inhibitable uptake. The data were fitted to either a one-site or two-site ligand binding computer model using nonlinear regression and "explicit weighing" of the SE of each of the experimental points in deriving the best fit (Enzfitter, Biosoft, Milltown, NJ).

Isolation of RNA and Northern Blots

RNA was isolated from brain (cerebrum), ventricular myocardium, and liver of 250-g male rats by ultracentrifugation through CsCl [13]. Clone 9 cell RNA was isolated as previously described [11]. Quantitation of RNA and fractionation in 1% agarose gels were performed as reported previously [11]. Following blotting and prehybridization, blots were hybridized with ~20 × 10^6 cpm of full-length rat brain glucose transporter-cDNA [5], EGT-cDNA, or with ~1200 and ~900 bp DNA fragments encoding the rat skeletal muscle/adipocyte GT (MGT) and rat liver GT (LGT), respectively [10, 50], [^{32}P]-labeled by "nick-translation" to ~5 × 10^8 cpm/μg DNA. Blots were washed as described [11], and following autoradiography the intensity of the bands determined by densitometry. Ethidium bromide staining of ribosomal RNA bands was monitored to ensure equivalent loading of the lanes and to control for completeness of transfer to nitrocellulose paper.

Cell Fractionation and Western Blots

Confluent cells on 6–8 plates were rinsed with ice-cold Hank's balanced salt solution followed by two rinses for 1 min each with ice-cold 1 mM sodium bicarbonate. Cells were then scraped into 1.5 ml of the bicarbonate solution containing 0.1 mM phenylmethylsulfonyl fluoride and an aliquot saved for analysis. The remainder was centrifuged at 660 × g for 10 min and the "nuclear" pellet discarded. In preliminary experiments it was found that this pellet fraction contained ~$\frac{1}{3}$ of the cell protein and small amounts of EGT and 5'-nucleotidase activity. The supernatant was then centrifuged at 10,000 × g for 22 min and the resulting membrane pellet suspended in ~150 μl of the lysis buffer. In repeated experiments it was found that this fraction contained ~$\frac{1}{2}$ of the pelletable 5'-nucleotidase activity and had a 2.5- to 3.0-fold higher specific activity than whole-cell lysates. Samples of whole-cell lysates and the above membrane fraction isolated from control and alkaline pH-exposed cells containing equivalent amounts of protein (40–100 μg protein) were fractionated by SDS-PAGE according to reported methods [24, 25]. Following electroblotting,

the blots were incubated with rabbit serum containing polyclonal antibodies directed against the carboxy-terminal dodecapeptide of EGT [5, 24] at 1:750 dilution, and then developed by the peroxidase method [17]. The relative intensity of the resulting bands was determined by densitometry. To ensure that the staining of the bands was in the linear range of the assay, a standard curve was generated employing 0.025, 0.05, 0.1, 0.2, and 0.4 μl of human erythrocyte lysate. The resulting intensities on the Western blot yielded a linear plot that passed through the origin ($r = 0.98$), and the intensities of the bands obtained from Clone 9 cell preparations were in the midrange of the curve.

To control for the degree of enrichment of plasma membranes in the membrane fractions isolated from cells incubated at pH 7.45 and 8.5, 5′-nucleotidase activity was measured as previously described in whole-cell lysates and membrane fractions in each experiment [28].

Protein Determination

Protein was determined by the method of Lowry et al. [36] using bovine serum albumin as a standard.

Statistical Methods

All values are expressed as means ± SE. For experiments involving multiple groups, the probability that a difference existed between the means of the groups was demonstrated by analysis of variance using the least significant difference for multiple comparisons [46]. $P < 0.05$ was taken as significant. In experiments involving two groups, Student's unpaired two-tailed t test was used to calculate the P values [46].

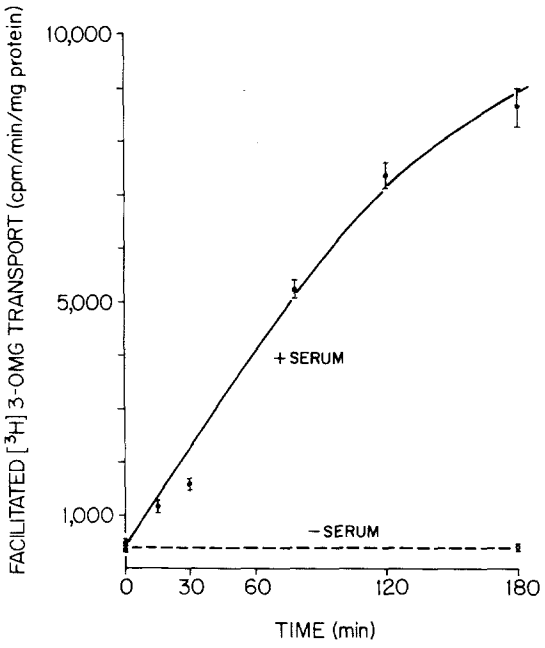

Fig. 1. Effect of addition of calf serum on the rate of cytochalasin B-inhibitable [^{3}H]3-OMG uptake in Clone 9 cells. Confluent cells were rinsed once in 4 ml of DME medium and preincubated for 20 hr in DME medium devoid of serum. At the initiation of the experiment, 0.44 ml of calf serum (sold line) or DME medium (dashed line) was added to the cells and [^{3}H]3-OMG transport measured at the times indicated in triplicate plates in uptake medium containing 7.5 μCi/ml of [^{3}H]3-OMG in the presence and absence of 50 μM cytochalasin B. Results of cytochalasin B-inhibitable uptake are shown as means ± SE. The stimulation of transport at 15 min and at later time points was significant. Similar results were obtained in a second independent experiment

Results

Previous studies in a variety of cell systems have demonstrated that the addition of serum and purified growth factors results in a dramatic stimulation of glucose transport [2, 18, 27, 44]. To examine the effect of addition of serum, confluent Clone 9 cells were preincubated for 20 hr in medium devoid of serum, and serum was then added. Addition of serum resulted in a marked stimulation of cytochalasin B-inhibitable 3-OMG transport that was demonstrable within 15 min and increased to >16-fold by 3 hr (Fig. 1). In contrast, cytochalasin B-insensitive 3-OMG transport remained unchanged during the 3-hr incubation (*data not shown*). Experiments were next performed to determine whether the stimulation of transport resulting from exposure to alkaline pH necessitates the prior presence of serum, as might be the case if incubation at alkaline pH serves to enhance or amplify serum- and growth factor-mediated stimulation of glucose transport. To test this possibility, the stimulatory effect of exposure to alkaline pH was tested in cells preincubated in medium devoid of serum for 20 hr, a period that would be expected to cause degradation and depletion of any remaining growth factors; cells preincubated in serum-containing medium served as controls (Table 1). In cells preincubated in medium-containing serum, exposure to alkaline pH resulted in a large (~16-fold) stimulation of cytochalasin B-inhibitable 3-OMG transport. Notably, in cells preincubated in the absence of serum, exposure to the alkaline pH likewise stimulated the rate of cytochalasin B-inhibitable 3-OMG transport by 19-fold. It thus appears that, although the acute addition of serum with its associated growth factors can markedly stimulate glucose transport in Clone 9 cells preincubated for prolonged periods in the absence of serum (Fig. 1), the presence of these factors is not necessary for the transport response induced by exposure to alkaline pH (Table 1). It should be noted that the rate of 3-OMG transport under basal conditions at pH 7.45 was nearly equal in cells preincubated in the absence and presence of serum for 20 hr indicating that the stimulatory effect of serum on glucose transport is no longer present after prolonged incubation in serum-containing medium.

Table 1. Effect of preincubation of Clone 9 cells in the presence or absence of serum for 20 hr on the rate of [^{3}H]3-OMG transport following exposure to alkaline pH

Cytochalasin B	+ Serum		− Serum	
	Control	Alkali treated	Control	Alkali treated
−	480 ± 20	5,970 ± 40	550 ± 15	6,430 ± 100
+	115 ± 5	160 ± 5	220 ± 15	270 ± 20
Difference	365 ± 20	5,810 ± 40	330 ± 20	6,160 ± 100

Confluent cells were rinsed twice with 4 ml of DME medium and then preincubated for 20 hr in DME medium containing 10% calf serum or medium alone. Cells were then incubated for 2 hr at either 9 or 0.7% CO_2 (pH 7.45 and 8.5, respectively), and, following an additional 30-min incubation at 9% CO_2, [^{3}H]3-OMG uptake was determined in the absence or presence of 50 μM cytochalasin B in triplicate plates (*see* Materials and Methods for details). Uptake medium contained 7.5 μCi [^{3}H]3-OMG per ml. Results of uptake (cpm/min/mg protein) are expressed as means ± SE. Similar results were obtained in a second independent experiment.

The possibility that substances capable of stimulating glucose transport are released into the medium by cells incubated at alkaline pH was next examined. Confluent cells were incubated at control or alkaline pH (9 and 0.7% CO_2, respectively), for 2 hr following which the medium bathing the cells ("conditioned" medium) was removed and the alkaline medium rapidly titrated back to pH 7.45 with CO_2. New plates of confluent cells were then exposed to these "conditioned" media for 30 min and the rate of cytochalasin B-inhibitable 3-OMG transport determined. Exposure of cells to alkaline pH-"conditioned" medium for 30 min resulted in no change in the rate of 3-OMG transport (*data not shown*), a time at which the rate of transport is stimulated some threefold following incubation at alkaline pH [29].

Because of the similarity between the time courses of the stimulation of 3-OMG transport following exposure to alkaline pH [29] and that following the addition of serum (Fig. 1), as well as the comparable degree of enhancement of 3-OMG transport in response to both of these stimuli, we explored the possibility that the alkaline pH-induced effect is mediated at a step distal to the interaction of growth factors with their specific receptors. A large number of growth factor-induced effects in a variety of cells can be attributed to activation of protein kinase C [1, 6, 40]. In initial experiments the effect of activation of protein kinase C by TPA on the rate of 3-OMG transport in Clone 9 cells preincubated for 20 hr in medium devoid of serum was examined (Fig. 2). Addition of TPA (100 ng/ml) resulted in a stimulation of cytochalasin B-inhibitable 3-OMG transport that was significant at 15 min and reached ~10-fold by 3 hr (Fig. 2, *inset*); half-maximal stimulation at 3 hr was observed at ~1 ng TPA/ml (Fig. 2). In contrast, TPA produced no change in the rate of cytochalasin B-insensitive uptake (*data not shown*).

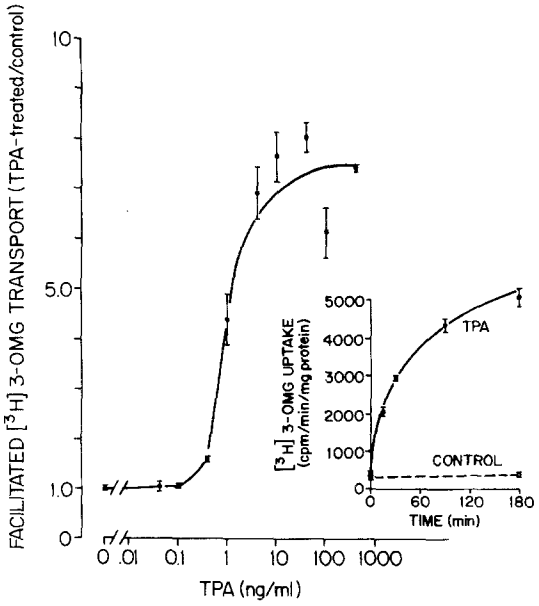

Fig. 2. Effect of TPA on cytochalasin B-inhibitable [^{3}H]3-OMG transport in Clone 9 cells. Confluent cells were preincubated in DME medium devoid of serum for 20 hr, following which 10 μl of Me_2SO containing the indicated final concentrations of TPA was added to the plates. After 3 hr of incubation, [^{3}H]3-OMG uptake was measured in triplicate plates in the absence and presence of 50 μM cytochalasin B in uptake medium containing 5 μCi/ml of [^{3}H]3-OMG. The experiment was performed twice, and the results of cytochalasin B-inhibitable uptake were averaged and expressed as the ratio to the rate of uptake observed after the addition of Me_2SO alone. Cytochalasin B-inhibitable uptake was 350 ± 20 cpm/min/mg protein (mean ± SE; n = 6) in the absence of TPA. *Inset:* Cells were preincubated as described above. TPA in 10 μl Me_2SO to yield a final concentration of 100 ng/ml or 10 μl Me_2SO alone was added to the plates. Results of cytochalasin B-inhibitable uptake are presented as means ± SE. The stimulation of transport at 15 min and at later time points was significant. Similar results were obtained in a second independent experiment

Table 2. Effect of incubation of Clone 9 cells at alkaline pH in the absence or presence of TPA on the rate of cytochalasin B-inhibitable [^{3}H]3-OMG uptake

pH	TPA	Cytochalasin B-inhibitable uptake (cpm/min/mg protein)
7.45	−	400 ± 25
8.5	−	5,520 ± 100
7.45	+	5,140 ± 220
8.5	+	6,430 ± 220

Confluent cells were incubated for 20 hr in 4 ml of DME medium devoid of serum following which 10 μl of either Me_2SO alone or Me_2SO containing TPA (final concentration 100 ng/ml) was added and incubation continued for 2.5 hr at pH 7.45 or 8.5. Following an additional 30-min incubation at pH 7.34 [^{3}H]3-OMG uptake was measured in triplicate plates in the absence and presence of 50 μM cytochalasin B in uptake medium containing 7.5 μCi [^{3}H]3-OMG/ml. Results of cytochalasin B-inhibitable uptake are expressed as means ± SE. Similar results were obtained in a second independent experiment.

We next performed experiments to determine whether the stimulatory effects of TPA and of incubation at alkaline pH on the rate of 3-OMG transport are independent of each other. Confluent cells preincubated for 20 hr in medium devoid of serum were incubated for 2.5 hr in the presence of 100 ng/ml TPA or diluent alone at either control or alkaline pH. Following an additional 30-min incubation at control pH, the rate of cytochalasin B-inhibitable 3-OMG transport was measured (Table 2). Exposure to either alkaline pH alone or to TPA at control pH resulted in ~13-fold stimulation of 3-OMG transport. Although exposure to the two stimuli together resulted in a somewhat larger degree of enhancement (~16-fold) than did exposure to either stimulus alone, the combined effect of the two stimuli was considerably less than additive.

Incubation of a variety of cells at high concentrations of phorbol esters for 16–24 hr has been shown to functionally "deplete" the cells of protein kinase C activity [6, 7, 43]. Accordingly, to further explore the potential mediating role of protein kinase C activation in the glucose transport response, we examined the effect of prolonged preincubation with TPA on the stimulation of glucose transport in response to exposure to alkaline pH. In other studies we have observed that exposure of Clone 9 cells to 100 ng/ml TPA for 20 hr completely "depletes" them of high-affinity phorbol-dibutyrate binding sites (A. Bhutada & F. Ismail-Beigi, *unpublished observations*). Accordingly confluent cells were preincubated with either TPA (100 ng/ml) or diluent alone for 20 hr in medium devoid of serum and then exposed for an additional 2.5 hr to 100 ng/ml of TPA or diluent at either pH 7.45 or 8.5, as indicated (Table 3). In cells incubated in the absence of TPA during the 20-hr preincubation period, both the acute addition of TPA and exposure to alkaline pH resulted in large stimulations of cytochalasin B-inhibitable 3-OMG transport. In contrast to the acute stimulatory effect of TPA on 3-OMG transport (Fig. 2), in cells pre-exposed to TPA for 20 hr the rate of 3-OMG transport had returned to near-basal levels, and further addition of TPA to these cells resulted in no stimulation of transport, consistent with the premise that the preincubation had functionally "depleted" the cells of protein kinase C activity. In striking contrast was the finding that cells pre-exposed to TPA for 20 hr fully retained the capacity to respond to subsequent incubation at alkaline pH with a marked stimulation of [^{3}H]3-OMG transport.

It was previously shown that the increase in the rate of glucose transport following exposure to alkaline pH is attributable to an increase in the V_{max} for transport rather than to a change in the K_m for glucose, consistent with an increase in the number or catalytic turnover rate of glucose transporters in the plasma membrane [29]. Because of the potential heterogeneity of glucose transporters present in certain cells, reflected in part in differing affinities for cytochalasin B [12, 19, 23, 48, 56], and the possibility that the enhanced rate of 3-OMG transport following exposure to alkaline pH might be mediated by a set of glucose transporters different from those operative under control conditions, we examined the inhibition kinetics of 3-OMG transport by cytochalasin B in control cells and in cells preincubated at alkaline pH (Fig. 3). Exposure to alkaline pH resulted in ~12-fold increase in the rate of cytochalasin B-inhibitable 3-OMG transport. The experimental results obtained at varying cytochalasin B concentrations were fitted on one-site and two-site ligand binding (inhibition) models. Neither set of data obtained for control and alkaline pH-exposed cells could be fitted to a two-site inhibition model where a minor site represented 5% or more of the total transport activity. In contrast, both sets of data conformed well to a one-site inhibition model characterized by K_I of 1×10^{-7} and 1.9×10^{-7} M cytochalasin B for control cells and for cells preincubated at alkaline pH, respectively. The marked stimulation of 3-OMG transport thus takes place in the absence of any major change in the inhibition characteristics of glucose transport by the specific inhibitor.

The finding that glucose transport in Clone 9 cells was half-maximally inhibited by $\sim 10^{-7}$ M cytochalasin B suggested that these cells express either the EGT or MGT isoforms [5, 35, 39, 42]. To determine which isoform is expressed in these cells, Northern blot analysis was performed on samples of

Table 3. Effect of preincubation of Clone 9 cells for 20 hr in the presence and absence of TPA on the rate of cytochalasin B-inhibitable [^{3}H]3-OMG uptake in response to exposure to alkaline pH

TPA at $t = -20$ hr	TPA at $t = 0$	pH	Cytochalasin B-inhibitable uptake, (cpm/min/mg protein)
−	−	7.45	250 ± 10
−	−	8.5	4,990 ± 190
−	+	7.45	3,220 ± 120
+	−	7.45	330 ± 10
+	−	8.5	5,560 ± 160
+	+	7.45	315 ± 20

Confluent cells were incubated in DME medium devoid of serum in the presence of TPA in 10 μl Me_2SO (final concentration 100 ng/ml) or Me_2SO alone for 20 hr (TPA at $t = -20$ hr). At the start of the experiment ($t = 0$), 10 μl of Me_2SO alone or Me_2SO containing TPA (to yield a final concentration of freshly added TPA of 100 ng/ml) was added to the plates and incubation continued at either pH 7.45 or 8.5 as indicated. The plates were then incubated at pH 7.45 for 30 min and [^{3}H]3-OMG uptake was measured in triplicate plates in the absence and presence of 50 μM cytochalasin B in uptake medium containing 5 μCi of [^{3}H]3-OMG/ml. Results of cytochalasin B-inhibitable uptake are expressed as means ± SE. Similar results were obtained in two additional independent experiments.

total RNA isolated from Clone 9 cells; RNA isolated from rat brain, heart, and liver served as controls. Following fractionation of the four RNA samples, replicate blots were probed with the EGT-, MGT-, and LGT-cDNAs (Fig. 4). The expression of the GT isoforms in the three rat tissues conforms to previous reports [5, 10, 50]. As can also be seen from the figure, Clone 9 cells appear to express the EGT-mRNA isoform exclusively. To examine the possibility that the stimulation of glucose transport in response to incubation at alkaline pH is mediated at a pretranslational level, RNA isolated from control cells and cells incubated at pH 8.5 for 30 min was fractionated and probed with the EGT-cDNA. No major change in the abundance of the EGT-mRNA occurred following exposure to alkaline pH (Fig. 5, lanes *A* and *B*) and, in four independent experiments, the abundance of this mRNA in cells incubated at pH 8.5 averaged 0.95 ± 0.10 that of control cells ($P > 0.05$); similar results were obtained in cells incubated at pH 8.5 for 2 hr (*data not shown*). As a positive control, RNA was isolated and fractionated from cells incubated for 20 hr in DME medium devoid of serum and from similar cells 1 hr following the addition of calf serum at a final concentration of 10% (Fig. 5, lanes *C* and *D*). In keeping with previous reports [27, 44], addition of serum markedly increased (by ~sixfold) the abundance of EGT-mRNA.

The absence of an alkaline pH-induced increase in the abundance of EGT-mRNA, the stimulation of glucose transport in the absence of any change in its sensitivity to cytochalasin B, and the observation that the glucose transport response is not blocked by cycloheximide [29], suggest that the enhancement of glucose transport in response to incubation at alkaline pH occurs at the post-translational level. Such findings would be consistent with either an increase in the number of functional transporters in the plasma membrane (perhaps through translocation) or an activation of pre-existing transporters. To distinguish between these possibilities, the abundance of glucose transporters was measured by Western blotting of whole-cell extracts and partially purified plasma membrane fractions isolated from control cells and from cells incubated at alkaline pH using a rabbit polyclonal antibody directed against the carboxy-terminal dodecapeptide of the EGT [5, 24]. Results of determinations of protein, 5′-nucleotidase activity and EGT abundance from an experiment in control cells are shown in Table 4. The membrane fraction contained ~15% of the total cell EGT content and had a 2.7-fold higher 5′-nucleotidase activity per unit protein that the total-cell lysate. Calculation of total EGT abundance was based on the assumption that the final yield of color resulting from a given quantity of EGT does not vary with the differing composition of the fractions with respect to other components employed in the Western blot analysis.

The antibody recognized a protein band with an apparent M_r of 45,000 to 50,000 (Fig. 6). To control for the degree of enrichment of plasma membranes in the membrane fractions derived from control cells and from cells exposed to alkaline pH, 5′-nucleotidase activity was measured in these samples. In three independent experiments it was found that the specific activity of 5′-nucleotidase was identical in whole-cell lysates of control and alkaline pH-exposed cells (averaged of 0.65 μmol P_i/hr/mg pro-

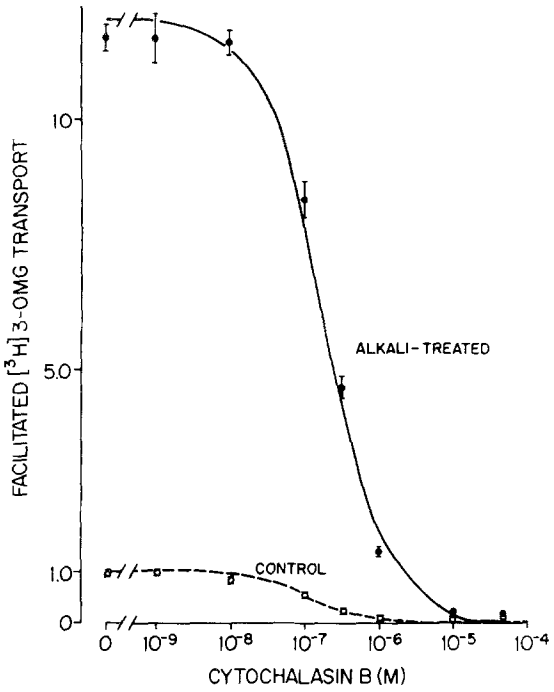

Fig. 3. Inhibition of factilitated [^{3}H]3-OMG transport by cytochalasin B in control Clone 9 cells and cells pre-exposed to alkaline pH. Confluent cells in DME medium containing 10% calf serum were exposed to 9 or 0.7% CO_2 for 2 hr, at which time 10 μl Me_2SO containing cytochalasin B to yield the indicated final concentrations was added to triplicate plates. The cells were then incubated at 9% CO_2 for an additional 30 min prior to measurement of [^{3}H]3-OMG uptake. Uptake medium containing 10 μCi [^{3}H]3-OMG/ml and the corresponding concentrations of cytochalasin B was then added to the plates. Total cytochalasin B-inhibitable uptake, calculated as the difference in uptake between plates containing Me_2SO alone and those containing 50 μM cytochalasin B, was normalized to 1.0 for the control cells. Uptake at each cytochalasin B concentration was then calculated as the fraction of the total cytochalasin B-inhibitable uptake. The total rate of cytochalasin B-inhibitable uptake in cells incubated at alkaline pH was calculated as the ratio to that of control cells. The experiment was performed twice employing triplicate culture plates at each concentration of cytochalasin B, and the averaged normalized data expressed as means ± SE. Total cytochalasin B-inhibitable uptake rates were 720 ± 40 and 8,350 pH, respectively (mean ± SE; n = 6). The data were fitted to a one-site ligand binding inhibition model using nonlinear regression and "explicit weighing" of the SE at each experimental point; the derived best-fit curve is shown

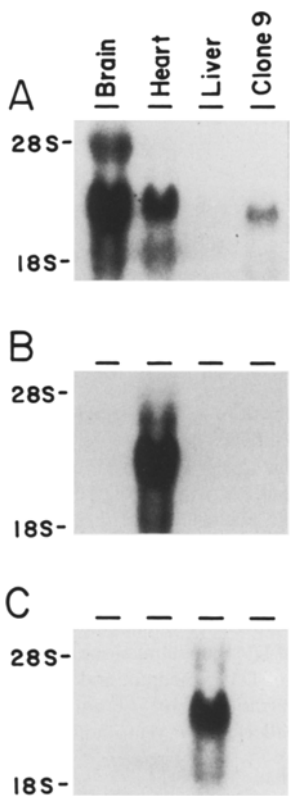

Fig. 4. Expression of glucose transporter isoforms in rat brain, heart, liver, and Clone 9 cells. RNA was isolated from rat cerebrum (brain), ventricular myocardium (heart), and liver as well as from Clone 9 cells. Forty μg of RNA was loaded in each of the lanes. The resulting blots were probed with EGT-cDNA (*A*), MGT-cDNA (*B*), and LGT-cDNA (*C*). Autoradiography was performed for 24 hr. The positions of 28S and 18S ribosomal RNA bands are shown

tein), whereas the enzyme activity in membrane fractions was 2.8 ± 0.1-fold that of whole-cell extracts in both groups of cells, consistent with a lack of preferential recovery of plasma membranes in either treatment group; the fractional yield of membrane protein from both groups of cells was similarly unchanged (*data not shown*). Exposure of cells to pH 8.5 for 2 hr followed by 30 min of incubation at pH 7.45 produced no significant change in the intensity of the obtained EGT band in whole-cell extracts as compared to that observed for control cells (alkaline pH-treated/control of 1.1 ± 0.1, n = 3). In contrast, the intensity of the EGT band in the lane containing membranes isolated from cells exposed to pH 8.5 was more intense than that in the lane containing an equivalent amount of membrane protein from control cells (Fig. 6). In three independent experiments incubation at alkaline pH increased the relative abundance of the transporter in the membrane fraction by 2.0 ± 0.2-fold ($P < 0.05$). The relative abundance of EGT in the supernatant fraction remaining after separation of the above membranes appeared to decrease after incubation at alkaline pH and averaged 75 ± 3% that of supernatants derived from control cells ($P < 0.05$).

Discussion

Glucose transport is markedly stimulated by insulin in target cells including cardiac and skeletal muscle

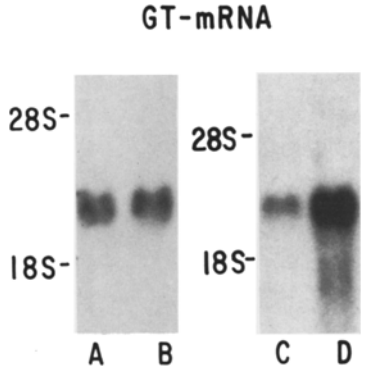

Fig. 5. Effect of exposure to alkaline pH and to calf serum on the expression of EGT-mRNA in Clone 9 cells. Left panel: Confluent cells on 100-mm plats were incubated for 30 min at 9% CO_2 (*A*) or 0.7% CO_2 (*B*) and RNA then isolated. Thirty-five μg of total RNA was loaded in each lane and the resulting Northern blot hybridized with EGT-cDNA. Autoradiography was performed for 48 hr. The positions of 28S and 18S ribosomal RNA are shown. Right panel: Confluent cells in 100-mm plates were preincubated for 20 hr in 8 ml of DME medium devoid of serum and, following the addition 4 ml of DME medium alone (*C*) or 4 ml of a solution containing 2.8 ml of DME medium and 1.2 ml of calf serum to yield a final concentration of 10% serum (*D*), RNA was isolated after 1 hr and probed as above. Autoradiography was carried out for 24 hr

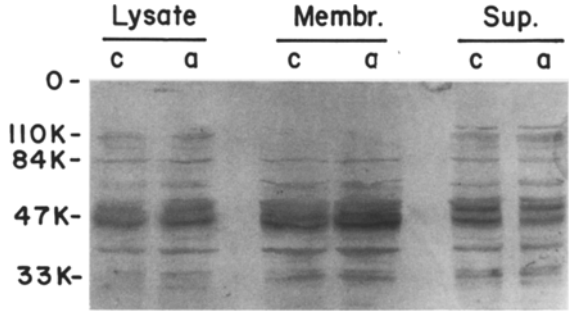

Fig. 6. Immunoblot of glucose transporters in whole-cell lysates, membrane fractions, and remaining supernatants of Clone 9 cells incubated at control and alkaline pH. Confluent cells were incubated at either pH 7.45 or 8.5 for 2 hr. Following a 30-min incubation at pH 7.45, cells were rinsed, harvested, and fractionated as described in Materials and Methods. Cells from eight 100-mm plates were pooled in each case. Samples of whole-cell lysates (*Lysate;* 80 μg protein), membrane fractions (*Membr.;* 140 μg of protein), and the supernatant (*Sup.;* 55 μg protein) were fractionated in 10% polyacrylamide gels and then transferred to nitrocellulose paper; *c* and *a* denote control and alkaline pH-exposed cells, respectively. The resulting blot was treated with rabbit antiserum directed against carboxy-terminal dodecapeptide of EGT at a 1:750 dilution. Molecular weight markers are shown on the left, and *o* denotes the origin

Table 4. Protein content, 5′-nucleotidiase activity, and EGT abundance in Clone 9 cell fractions

Fraction	Protein, mg	5′-nucleotidase,		Total EGT, relative units
		μmol P_i/hr	μmol P_i/hr/mg protein	
Lysate	4.9	3.1	0.63	70
Membrane	0.7	1.2	1.73	10
Supernatant	2.7	1.4	0.50	55

Whole-cell lysates were prepared from cells on 10 confluent 100-mm plates in 1 mM bicarbonate buffer as described in Materials and Methods. After centrifugation at 660 × *g* for 10 min the "nuclear" pellet was discarded. Samples were then centrifuged at 10,000 × *g* for 22 min to obtain membrane and supernatant fractions. In the calculation of relative EGT abundance, equivalent amounts of protein were loaded per lane in the Western blots and the resulting intensity of the EGT band of whole-cell lysates was taken as 1.0.

myocytes and adipocytes, and the stimulatory effect is at least in part mediated by translocation of specific transporters from intracellular sites to the plasma membrane [4, 10, 15, 22, 49]. In addition, glucose transport is enahnced in a variety of cells by a host of conditions and agents including transformation, deprivation of glucose, exercise, addition of serum and growth factors, phorbol esters, calcium ionophores, and adenosine, as well as in response to inhibition of oxidative phosphorylation or incubation at alkaline pH [2, 3, 18, 20, 21, 25, 27, 29, 34, 37, 38, 41, 44, 45, 47, 51, 52]. The enhancement of glucose transport in some of the above states is mediated by translocation of GTs while, in others, an increased abundance of GTs mediated at transcriptional as well as post-translational levels underlies the response [27, 34, 41, 44, 45, 52]. In the present study we have examined some of the potential mechanisms mediating the marked stimulation of glucose transport in Clone 9 cells in response to incubation at alkaline pH.

Previous studies have indicated that the stimulation of glucose transport following the addition of serum and specific growth factors is mediated by

an early stimulation of the rate of transport that is followed by enhanced biosynthesis of transporters by a transcriptionally mediated increase in GT-mRNA abundance [2, 18, 27, 44]. Because incubation at alkaline pH could potentially increase the sensitivity of Clone 9 cells to growth factors present in serum, as has been proposed for the increased sensitivity of adipocytes to insulin at alkaline pH [47], we determined whether the presence of serum was essential in the alkaline pH-induced stimulation of glucose transport. We found that while acute addition of serum can indeed markedly augment the rate of glucose transport in these cells, the stimulation obtained in response to exposure to alkaline pH remains unaltered in cells incubated for 20 hr in medium devoid of serum and growth factors (Table 1).

Activation of protein kinase C in response to addition of phorbol esters has been reported to stimulate glucose transport in a variety of cell systems [34, 41]. We thus examined the possibility that activation of protein kinase C mediates the observed stimulation of glucose transport in response to incubation at alkaline pH. TPA itself was found to markedly stimulate the rate of glucose transport in these cells, and the effect was not additive to that produced by incubation at alkaline pH alone (Table 2). This latter finding suggested either that the two stimuli share a common mechanism of action or that the response elicited by each stimulus alone is near maximal for these cells such that superimposition of the other stimulus has little further effect. To distinguish between these possibilities, we "depleted" the cells of protein kinase C activity by a 20-hr preincubation in the presence of 100 ng/ml TPA [6, 7, 43] and then examined the effect of incubation at alkaline pH on glucose transport. The stimulatory effect of incubation at pH 8.5 under these conditions was found to be fully preserved despite antecedent "depletion" of the cell protein kinase C activity by prolonged exposure to TPA (Table 3). It would thus appear that the alkaline pH response is not mediated by activation of protein kinase C *per se*. Whether steps beyond activation of this enzyme system underlie the glucose transport response remains to be explored in future studies.

Incubation of Clone 9 cells at alkaline pH increases the V_{max} for glucose transport [29] consistent with an increase in the number of functional transporters in the plasma membrane or activation of a fixed number of pre-existing sites. If the response is mediated by an increase in the number of transporters in the plasma membrane, then the "new" transporters might exhibit characteristics different from those of the pre-existing sites. In a previous study no change in the K_m for glucose transport was noted following incubation at alkaline pH [29]. To further explore this possibility, we examined the sensitivity of glucose transport to inhibition by cytochalasin B in control and stimulated cells (Fig. 4). The K_I for transport remained essentially unchanged despite a more than 10-fold stimulation of glucose transport in cells exposed to alkaline pH, a finding consistent with either a lack of functional heterogeneity in glucose transporters expressed in Clone 9 cells. The observed K_I of $1-2 \times 10^{-7}$ M, moreover, suggested that these cells express either the EGT or MGT isoforms [5, 35, 39, 42]. Northern blot analysis of Clone 9 cell RNA with cDNAs encoding EGT, MGT, and LGT showed that these cells express only the EGT-mRNA isoform (Fig. 4).

The lack of an increase in the abundance of EGT-mRNA upon incubation of Clone 9 cells at alkaline pH (Fig. 5), in conjunction with previous results demonstrating that ongoing protein synthesis is not required for the alkaline pH-induced response [29], suggests that the stimulation of glucose transport in response to alkaline pH is mediated primarily by posttranslational mechanisms. To distinguish between such mechanisms involving a translocation of transporters from intracellular sites to the plasma membrane from mechanisms involving an intrinsic activation of pre-existing sites, Western blotting was used to quantitate the abundance of GTs in partially purified plasma membrane fractions isolated from control and alkaline pH-stimulated cells (Fig. 6). Rabbit polyclonal antibodies directed against the carboxy-terminal dodecapeptide of EGT were used in these studies [5, 24]. The relative abundance of EGT in whole-cell extracts of cells incubated at pH 8.5 remained unchanged whereas EGT abundance in membrane fractions enriched in plasma membranes increased by ~2.0-fold, suggesting that the response is at least in part mediated by increased abundance of transporters (presumably by translocation) in the plasma membranes. Consistent with the above suggestion was the finding that relative EGT abundance in the supernatant fraction remaining after separation of the membrane fraction decreased in response to incubation at alkaline pH. Such a translocation mechanism has previously been suggested on the basis of observations on adipocytes incubated at alkaline pH [51]. The 2.0-fold increase in GT abundance in membrane fractions from Clone 9 cells appears modest in comparison to the more than 12-fold stimulation of transport in response to incubation at alkaline pH and raises the possibility of an activation of GT sites pre-existing in the plasma membrane in contributing to the response to incubation at alkaline pH. In this context, it has been recently suggested that the stimulation of glucose transport in adipocytes in response to insulin, in differentiated 3T3-L1 cells in response to cholera toxin or insulin, and in

skeletal muscle following exercise is mediated by a combination of the above two mechanisms [9, 14, 33, 48, 55]. Whether the dramatic stimulation of glucose transport induced by incubation of Clone 9 cells at alkaline pH also involves participation of both translocation and mechanisms leading to intrinsic activation of transporters remains a subject for future investigation.

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