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Phenylarsine oxide and hydrogen peroxide stimulate glucose transport via different pathways in isolated cardiac myocytes

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The aim of this study was to investigate the stimulating effects of sulfhydryl reagents on glucose transport in isolated rat heart muscle cells and to compare them with the action of insulin. Low concentrations of the sulfhydryl oxidants hydrogen peroxide (H_2O_2) and diamide (5–100 μM), but also of phenylarsine oxide (PAO) (0.5–3 μM), that is known to specifically react with vicinal SH-groups, stimulated the rate of 2-deoxy-D-glucose uptake by a factor of 4 to 8 in these cells, while higher concentrations were inhibitory. The stimulating effects of H_2O_2 or diamide, and, to a significantly lesser extent, those of PAO or insulin, were depressed in cells pretreated with the sulfhydryl-alkylating agent *N*-ethylmaleimide (56–100 μM). H_2O_2 raised the V_{max} and lowered the K_m of 3-*O*-methyl-D-glucose uptake, while PAO or insulin solely increased V_{max}. The increase in glucose transport caused by H_2O_2 was antagonized by the β-adrenergic agonist isoprenaline (1 μM) or by a membrane-permeant cyclic AMP analog, whereas the effects of PAO or insulin were not altered. The action of H_2O_2 was additive with the stimulation induced by the protein phosphatase inhibitors okadaic acid (1 μM) or vanadate (6 mM), whereas the responses to PAO or insulin were reduced in the presence of these agents. Finally, H_2O_2 and PAO, but not insulin, acted additively with the protein kinase C ligand phorbol myristate acetate (0.8 μM) and with phospholipase C (0.03 units/ml). We conclude that, in cardiac myocytes, H_2O_2, on the one hand, and PAO (and possibly insulin), on the other hand, stimulate glucose transport via at least two distinct, SH-dependent pathways. These pathways, in turn, differ from a protein kinase C- and from a phospholipase C-mediated mechanism.

Introduction

Regulatory pathways involved in the control of glucose uptake and metabolism were repeatedly suggested to contain essential sulfhydryl groups [4,5]. First evidence in favour of this idea originated from studies on isolated fat cells and muscle preparations in which the stimulating effect of insulin on glucose transport (i) could be inhibited by preincubating cells with agents such as *N*-ethylmaleimide, that specifically block SH-groups [6–8], and (ii) could be mimicked with sulfhydryl oxidants such as diamide or hydrogen peroxide, that promote the formation of disulfides [6–11]. However, the cellular site(s) mediating these effects were not identified.

More recently, M.D. Lane and colleagues observed that phenylarsine oxide (PAO), an arsenical compound known to form stable ring structures with *vicinal* sulfhydryls [12], specifically prevents the insulin-induced increase in glucose transport in 3T3-L1 adipocytes [13]. This inhibiting effect of PAO was confirmed in other tissues including isolated rat adipocytes [14], BHK-21 cells [15] and rat soleus muscle [16].

On the other hand, we and others have recently found that PAO does not only display inhibiting properties, but that it is per se also a potent stimulator of glucose transport in isolated cardiac myocytes [2], in 3T3-L1 adipocytes [17], in skeletal muscle [18] and in cultured mouse fibroblasts [19].

PAO was reported to inhibit phosphotyrosyl protein phosphatases [20], or to stimulate a tyrosine kinase activity [19], and to promote the accumulation of cellular proteins phosphorylated on tyrosine residues

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Abbreviations: dGlc, 2-deoxy-D-glucose; MeGlc, 3-*O*-methyl-D-glucose; H_2O_2, hydrogen peroxide; PAO, phenylarsine oxide; NEM, *N*-ethylmaleimide; PMA, 4β-phorbol 12β-myristate 13-acetate; PLC, phospholipase C; PKC, protein kinase C; 8-Br-cAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate.

[19,21,22]. However, it remains to be established whether such events are related to the stimulating effect of PAO on glucose transport. Nor is it known whether transport regulation by PAO involves elements of the insulin signal cascade. A further issue that needs to be elucidated is whether the stimulating effects of sulfhydryl oxidants described earlier [6–11], on the one hand, and the newly reported increase in glucose uptake upon PAO treatment [2,17–19], on the other hand, can be ascribed to the activation of one single cellular site.

We therefore decided to further characterize this effect of PAO in isolated cardiomyocytes and to compare it with that of insulin and of sulfhydryl oxidants. Moreover, we considered the possibility of a relationship with cellular events that have been proposed to play a role in the stimulation of glucose transport in insulin-sensitive tissues, such as processes involving protein phosphatases [23,24], protein kinase C [25–28], or phospholipase C [25–27].

The isolated rat cardiomyocytes used in this study were obtained by a modified isolation procedure recently developed in this laboratory. They are characterized by a very low basal hexose uptake rate that corresponds to values measured in intact perfused hearts [2]; consequently, these cells are highly responsive to stimuli of glucose transport and thus represent a particularly sensitive model in this respect [2,3].

Materials and Methods

Chemicals

3-*O*-Methyl-D-glucose, phloretin and cytochalasin B were purchased from Serva (Heidelberg, Germany); hydrogen peroxide and all chemicals for media used for cell isolation and glucose transport assays were from Merck (Darmstadt, Germany); diamide, phenylarsine oxide, *N*-ethylmaleimide, 4β-phorbol 12β-myristate 13-acetate, phospholipase C (*Clostridium perfringens*) and 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP) were obtained from Sigma (Deisenhofen, Germany); bovine serum albumin, fraction V, fatty acid-free, and adenosine desaminase from calf intestinal muscosa were from Boehringer (Mannheim, Germany); okadaic acid was purchased from Research Biochemicals (Cologne, Germany); purified bovine insulin was a kind gift from Barbara Rannefeld and Prof. Axel Wollmer (Aachen, Germany); 3-*O*-[^{3}H]methyl-D-glucose and 2-deoxy-D-[^{3}H]glucose were purchased from Amersham (Braunschweig, Germany). Concentrated stock solutions of the phorbol ester, cytochalasin B or PAO (in dimethylsulfoxide) and of insulin (in assay medium, see below) were stored at −20°C in appropriate aliquots, and diluted just prior to addition to the isolated cardiomyocytes. Final concentrations of dimethylsulfoxide were typically 0.02–0.1% in the transport assays and did not affect basal transport activitiy. Orthovanadate stock solutions (0.14 M) were prepared by the method of Goodno [1] and diluted immediately prior to use. All other chemicals were freshly prepared immediately before use.

Isolation of cardiomyocytes

Cardiomyocytes from adult Sprague-Dawley rats (180–220 g, fed ad libitum) were obtained by an improved method recently developed in this laboratory [2]. The cells obtained by this procedure display a very low basal glucose uptake rate (approx. 3 pmol 3-*O*-methyl-D-glucose/s per mg protein at 1 mM sugar), as compared with the values reported by other authors and react to a stimulation by insulin (10 nM) with an 8- to 20-fold increase in hexose transport activity [2].

Determination of 2-deoxy-D-glucose uptake

The uptake of 2-deoxy-D-[^{3}H]glucose (dGlC) was assayed (in medium A: 6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM Hepes, 1 mM $CaCl_2$ and 2% bovine serum albumin, fatty acid-free, pH 7.4 at 37°C, equilibrated with oxygen) as previously described [2]. In brief, 1 ml of cell suspension (corresponding to approx. 1.5 mg cell protein) was incubated with 0.5 ml of medium (control) or with an appropriate dilution of one or several agents to be assayed, at 37°C for the times indicated (typically 15–30 min). The samples were then incubated in the presence of dGlc for an additional 30 min before sugar uptake was stopped by adding phloretin (400 μM final concentration). Specific, i.e., glucose-carrier-mediated dGlc uptake was estimated by subtraction of uptake rates monitored in the presence of 400 μM phloretin from values measured in control or stimulated cells (note that saturating concentrations of phloretin or of cytochalasin B, another potent transport inhibitor, are equally effective in inhibiting glucose transport in these cells; sugar uptake in phloretin-treated cells averaged 17% of control values vs. 15–20% in myocytes exposed to 20 μM cytochalasin B [2]).

Kinetics of 3-O-methyl-D-glucose uptake

The kinetics of *zero-trans* 3-*O*-methyl-D-glucose (MeGlc) uptake was determined in an assay described elsewhere [3]. In brief, 175 μl cardiomyocyte suspension was incubated in the absence (control) or the presence of hydrogen peroxide (H_2O_2, 30 μM final concentration), phenylarsine oxide (PAO, 1.8 μM) or insulin (10 nM) in a total volume of 275 μl for 30 min at 37°C, before MeGlc (1.1 μCi 3-*O*-[^{3}H]methyl-D-glucose and an appropriate amount of unlabelled sugar) was added. Upon addition, the samples were quickly and vigorously vortexed to achieve an effective mixing and then shaken in a water bath at 37°C, before the uptake reaction was stopped by rapidly adding phloretin

(400 μM final concentration). In preliminary experiments, the *zero-trans* uptake rate of MeGlc (at 3 mM extracellular concentration) had proved to be linear for at least 120 s in control cells, 30 s in H_2O_2- or PAO-treated cells, and 10 s in insulin-stimulated cells. Consequently, total transport assay times (in the presence of MeGlc) were chosen as follows: 90 s for control cells, 25 s for H_2O_2- or PAO-treated cells and 8 s for insulin-treated cells. The extent of cell-bound radioactivity at zero time was measured in samples pretreated with the specific glucose transport inhibitor cytochalasin B (20 μM), in which the assay was stopped immediately upon MeGlc addition. The carrier-mediated, initial transport velocities were calculated by subtracting the uptake rates measured in the presence of cytochalasin B (0–420 s) from the values obtained in the absence of inhibitor. Cell protein was determined by the biuret method.

Statistics

The significance of differences between paired sets of values was assessed with a paired Student's *t*-test (Table II, Fig. 4). When a set of data was used for simultaneous comparison with several other sets of values, significance was evaluated by one-way analysis of variance (Table I, Fig. 3). For the comparison of K_m values of 3-*O*-methyl-D-glucose uptake under different experimental conditions (Fig. 2), linear regression data transformed in a Hanes plot were analyzed using an *F*-test.

Results

Effect of hydrogen peroxide, diamide and phenylarsine oxide on 2-deoxy-D-glucose uptake

As shown in Fig. 1, low concentrations (5–30 μM) of the oxidant hydrogen peroxide (H_2O_2) enhance the transport of 2-deoxy-D-[^{3}H]glucose (dGlc) in isolated cardiomyocytes, while higher concentrations are inhibitory. At these relatively low concentrations, H_2O_2 is likely to oxidize exclusively the most reactive groups present in living cells, i.e., SH-groups. The effects of the peroxide could indeed be mimicked by the SH-specific oxidant diamide at similar concentrations (Fig. 1). H_2O_2 and diamide stimulated dGlc transport to a comparable maximal extent (about 4- to 6-fold, as compared to control values, maximal stimulation being reached after 10 to 15 min (not shown).

Since diamide is likely to induce the formation of disulfides from neighbouring SH-groups [29], we also tested the effects of phenylarsine oxide (PAO), an agent known to bridge between vicinal sulfhydryls [12]. In cardiomyocytes treated with PAO (0.5–3 μM), dGlc uptake was markedly increased, while higher concentrations were inhibitory (Fig. 1), in analogy to the effects observed in the presence of diamide or H_2O_2.

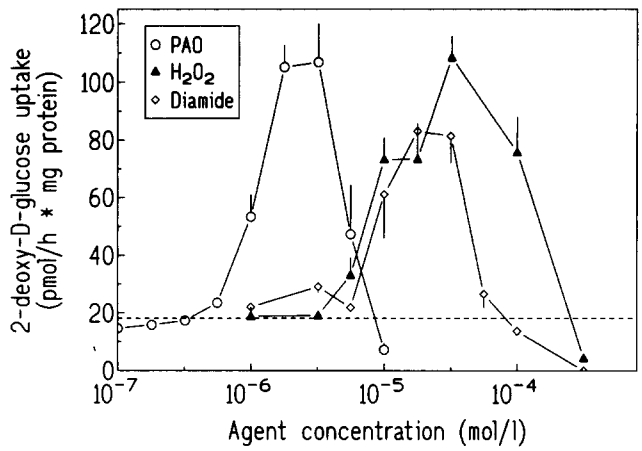

Fig. 1. Concentration-dependence of the effects of H_2O_2, diamide and PAO on dGlc uptake. Cardiomyocytes were exposed to H_2O_2, diamide or PAO at the indicated concentrations for 30 min at 37°C, before the dGlc transport assay was performed, as described in Materials and Methods. Each point represents the mean ± S.E. of at least two independent experiments (n = 6–9). The dotted line represents the level of dGlc uptake in control cells.

The stimulation of dGlc transport by PAO (1.8 μM) was complete after 15 min (not shown) and reached 5- to 8-times the control level.

Phloretin (0.4 mM) or cytochalasin B (20 μM), completely suppressed the glucose uptake activity not only of control cells, but also of cells stimulated with H_2O_2 (30 μM), diamide (20 μM) or PAO (1.8 μM) (not shown), indicating that transport stimulation by these SH-reagents involves a glucose carrier-mediated process.

Stimulating concentrations of PAO (1.8 μM) or of diamide (30 μM) did not change, and H_2O_2 (30 μM) even tended to increase the free energy of ATP-hydrolysis from −65 to −68 kJ/mol, as determined by a method previously described [30]. This observation shows that, at the concentrations used, none of these agents has a toxic effect on energy metabolism, which is a very sensitive indicator of an intact intermediary metabolism in these cells.

When cardiomyocytes are treated with a combination of PAO or H_2O_2 (or diamide) with insulin, the resulting dGlc transport stimulation is markedly lower than with insulin alone (and is similar to that observed with PAO or H_2O_2 alone) (Table II).

Influence of a pretreatment with N-ethylmaleimide on the effects of hydrogen peroxide, diamide, phenylarsine oxide and insulin

The question now arises as to whether (i) H_2O_2, diamide and PAO interact with a common cellular site, and (ii) whether one (or several) of these agents may share their stimulating pathway with insulin. In a first approach aiming at this issue, we investigated whether the effects of these four stimuli can be prevented by preincubating isolated cardiomyocytes with *N*-ethyl-

TABLE I

Influence of a pretreatment of cardiomyocytes with NEM on the stimulation of dGlc uptake induced by H_2O_2 diamide PAO or insulin

The cells were incubated for 15 min at 37°C in the absence or in the presence of NEM at the indicated concentrations, before H_2O_2 (30 μM), diamide (20 μM), PAO (1.8 μM), or insulin (10 nM) were added. The incubation was continued for an additional 15 min and the dGlc uptake assay was performed as described in Materials and Methods. Results are mean values from at least two or three independent experiments (±S.E.).

First addition	Second addition	dGlc uptake (pmol/h per mg protein)	% of maximal effect [a]
None	none	19.8± 1.6	
NEM 56 μM	none	19.2± 2.0	
NEM 75 μM	none	20.6± 0.8	
NEM 100 μM	none	20.2± 3.0	
None	PAO	139.0± 2.6	100
NEM 56 μM	PAO	138.8± 5.0	99.7
NEM 75 μM	PAO	104.9±10.4	72.1
NEM 100 μM	PAO	79.8± 5.7	51.4
None	H_2O_2	118.3± 1.3	100
NEM 56 μM	H_2O_2	85.6± 6.0	64.7 **
NEM 75 μM	H_2O_2	56.4± 3.7	33.0 **
NEM 100 μM	H_2O_2	23.5± 7.1	3.7 **
None	diamide	124.5± 3.4	100
NEM 56 μM	diamide	100.2± 7.6	75.3 **
NEM 75 μM	diamide	80.9± 6.0	55.7 **
NEM 100 μM	diamide	50.9±11.2	25.1 **
None	insulin	303.1± 5.2	100
NEM 56 μM	insulin	275.2± 5.2	90.3 *
NEM 75 μM	insulin	235.9±12.6	76.7 n.s.
NEM 100 μM	insulin	122.6±16.4	37.3 n.s.

[a] Percentages are calculated from the arithmetical difference between stimulated and control values. * $P < 0.05$; ** $P < 0.01$; n.s. not significant, compared to the corresponding percentage of the PAO effect, i.e., to the percentage values obtained in samples pretreated with the corresponding NEM concentration and then incubated with PAO (the comparison was done by one-way analysis of variance).

maleimide (NEM), an agent that specifically alkylates (and thus blocks) SH-groups. NEM (56–100 μM), which did not affect the free energy of ATP-hydrolysis (not shown), nor the rate of basal glucose transport, clearly reduced the stimulation brought about by H_2O_2, diamide, PAO or insulin (Table I). However, when these stimulants were administrated before NEM, the agent failed to inhibit their effects (data not shown). It should be noted that the stimulation by H_2O_2 or diamide is significantly more sensitive to a pretreatmernt with NEM than the stimulation by PAO or insulin (Table I).

Kinetics of 3-O-methyl-D-glucose transport

To further characterize the stimulatory effects of H_2O_2 and PAO, and to compare them with the action of insulin, we determined the kinetic parameters of the cytochalasin B-sensitive uptake of 3-*O*-methyl-D-glucose (MeGlc) in isolated cardiomyocytes after treatment with these agents. In accordance with previously published data [31,32], insulin (10 nM) increased V_{max} (from 45 to 825 pmol MeGlc per s per mg protein, without affecting the affinity of the transport system (Fig. 2). PAO (1.8 μM) acted similary, but caused a smaller change in V_{max} than insulin (up to 242 pmol MeGlc per s per mg protein. In contrast, H_2O_2 (30 μM) did not solely raise V_{max} (to 120 pmol MeGlc per s per mg protein, but it also lowered the K_m (from 12.2 to 7.2 mM; $p < 0.001$ as compared by *F*-test analysis of linear regression data).

Influence of a β-adrenergic stimulation on the effects of hydrogen peroxide, phenylarsine oxide, and insulin

The next approach designed to compare the effects of H_2O_2, PAO, and insulin was to examine the influence of a β-adrenergic agonist on the increase in glucose transport induced by these agents. It is well established that, in rat adipocytes, the basal and insulin-stimulated glucose transport is largely depressed by the β-adrenergic agent isoprenaline via a cyclic AMP-dependent mechanism [33–35]. In our cardiac myocytes, however, neither isoprenaline (1 nM–1 μM) nor the cell membrane-permeant cAMP analog 8-Br-cAMP (1 mM) altered the basal or insulin-stimulated rate of dGlc uptake (Fig. 3). Note that low isoprenaline concentrations (1–10 nM) even tended to slightly increase the rate of insulin-stimulated uptake, whereas higher concentrations (1 μM) decreased the basal transport values, but these effects were not statistically significant (not shown). Similary, the stimulating action

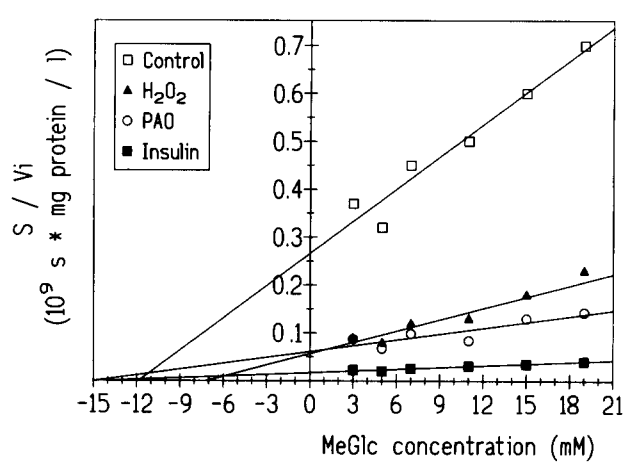

Fig. 2. Hanes plot of 3-*O*-methyl-D-glucose uptake measured in control, H_2O_2-, PAO-, or insulin-treated cardiomyocytes. Cardiomyocytes were preincubated in the absence (control) or in the presence of 30 μM H_2O_2, 1.8 μM PAO or 10 nM insulin for 30 min at 37°C. The initial rate of MeGlc uptake velocity was then determined as described in Materials and Methods. Each point represents the mean of 2–4 independent experiments ($n = 14–28$). The regression coefficients (r) of the linearized data are: 0.97 (control), 0.98 (H_2O_2), 0.87 (PAO) and 0.95 (insulin).

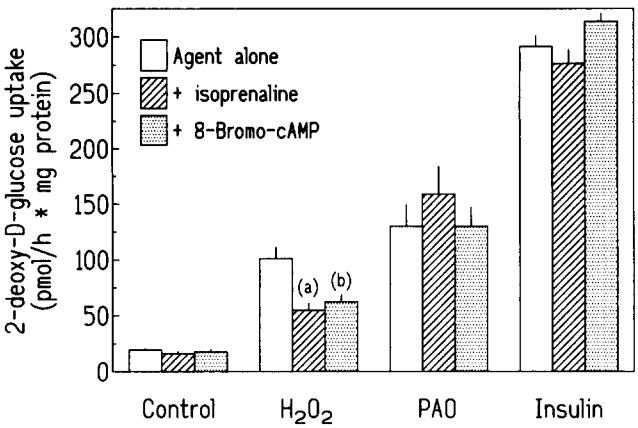

Fig. 3. Influence of isoprenaline and 8-Br-cAMP on the effects of H_2O_2, PAO, and insulin on dGlc uptake. Cardiomyocytes were incubated for 30 min in assay medium at 37°C (open bars) or they were exposed (i) to adenosine desaminase (1.5 unit/ml) for 15 min and to isoprenaline (1 μM) for an additional 15 min (diagonal bars) or (ii) to 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) for 30 min (speckled bars), before H_2O_2 (30 μM), PAO (1.8 μM), or insulin (10 nM) were added for another 30 min at 37°C. Data are means of 2–9 independent experiments (each done in triplicate) ±S.E. Statistical significance was assessed by one-way analysis of variance; (a) $P < 0.01$ and (b) $P < 0.05$ vs. value measured in the presence of H_2O_2 alone.

of PAO was not influenced by isoprenaline (10 nM–1 μM) or by 8-Br-cAMP (Fig. 3). In contrast, the effect of H_2O_2 was reduced by about 50% in the presence of the adrenergic agonist (with an IC_{50} of approx. 10 nM) or of the cAMP analog (Fig. 3). Analog results were obtained when cardiomyocytes were pretreated with cholera toxin (1 μg/ml) for 120 min (which is known to rise the cAMP levels in these cells: Ref. 36): transport stimulation by H_2O_2 was depressed, whereas the action of insulin remained unchanged (not shown).

Hydrogen peroxide or phenylarsine oxide and intracellular signalling elements

Finally, we addressed the question whether the effects of PAO and H_2O_2 described above may be related to the action of other glucose transport stimulators known to be effective in fat and muscle tissues: vanadate, okadaic acid, the phorbol ester 4β-phorbol 12β-myristate 13-acetate (PMA) and phospholipase C (PLC).

Vanadate (a phosphotyrosyl protein phosphatase inhibitor [37]) and okadaic acid (a serine/threonine phosphatase inhibitor [38]) were shown to mimic the action of insulin in adipocytes (e.g., Refs. 23 and 39) and skeletal muscle (e.g., Refs. 18 and 24); in addition to stimulating glucose transport, okadaic acid prevented the (considerably larger) transport stimulation induced by insulin in adipose cells [39].

In our experiments with cardiomyocytes, both okadaic acid and vanadate also proved to be potent stimulators of glucose uptake when added alone (Fig. 4), with EC_{50} values of approx. 0.3 μM or 0.6 mM, respectively (not shown). On the other hand, when either agent was added prior to insulin, the effect of the hormone was reduced by approx. 20% (okadaic acid, 1 μM) or by 30% (vanadate, 6 mM) (Fig. 4). Similarly, a combined treatment of cardiomycytes with vanadate and PAO (1.8 μM) resulted in a weaker transport stimulation than with PAO alone; a combination of okadaic acid and PAO failed to increase glucose transport to a larger extent than with okadaic acid or PAO alone (Fig. 4). By contrast, okadaic acid or vanadate, on the one hand, and H_2O_2 (30 μM), on the other hand, clearly acted in an additive manner in stimulating glucose uptake in these cells (Fig. 4).

The stimulating effects of the protein kinase-C activator PMA and of exogenously added PLC on glucose transport have been documented in a number of investigations (see e.g., Refs. 25–28). In cardiomyocytes, both PMA (0.1–1 μM) or PLC (from *C. perfringens*, 0.03 unit/ml) caused a 2- or 2.5-fold increase in dGlc-transport, respectively (Table II).

The results of various combined treatments with H_2O_2 (30 μM), PAO (1.8 μM), PMA (0.8 μM), PLC (0.03 units/ml), and insulin (10 nM) are also presented in Table II. These data can be summarized as follows: (i) the effects of PMA and PLC are partially additive; (ii) neither agent, however, causes a further change in dGlc uptake activity in cells that are stimulated with insulin; (iii) the stimulatory action of H_2O_2 or PAO,

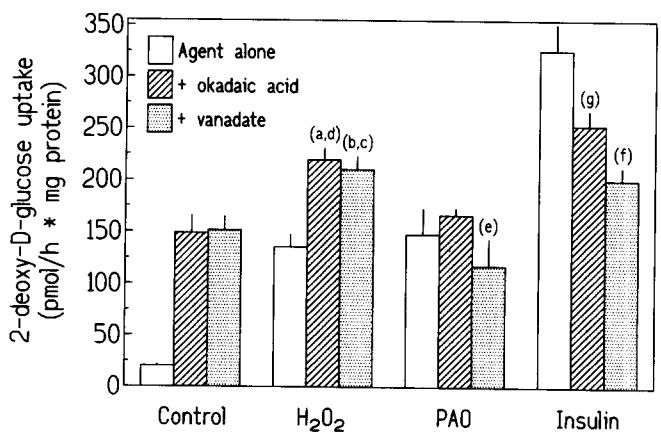

Fig. 4. Effect of combined treatments with okadaic acid or vanadate, and with H_2O_2, PAO, or insulin on dGlc uptake. Cardiomyocytes were exposed to assay medium (open bars) or to okadaic acid (1 μM) for 15 min (diagonal bars) or to vanadate (6 mM) for 60 min (speckled bars) at 37°C before H_2O_2 (30 μM), PAO (1.8 μM), or insulin (10 nM) were added for another 30 min prior to dGlc uptake determination. Data are means of 2–5 independent experiments ±S.E. (each experiment was done in triplicate). (a) $P < 0.05$ vs. okadaic acid alone; (b) $P < 0.05$ vs. vanadate alone; (c) $P < 0.01$ and (d) $P < 0.05$ vs. H_2O_2 alone; (e) $P < 0.05$ vs. PAO alone; (f) $P < 0.01$ and (g) $P < 0.05$ vs. insulin alone (paired *t*-test).

TABLE II

Effect of combined treatments with H_2O_2 (30 μM), PAO (1.8 μM), PMA (0.8 μM), phospholipase C (C. perfringens, 0.03 unit/ml or B. cereus), and insulin (10 nM) on dGlc uptake

Cardiomyocytes were exposed to one or two agents simultaneously, as indicated, for 30 min at 37°C. For the insulin-containing samples, the cells were treated for 10 min with the first agent before insulin was added, and then incubated for another 20 min. The dGlc transport rate was then determined as described in Materials and Methods. Data are mean values (±S.E.) from 2–8 independent experiments (each experiment done in triplicate).

Additions	dGlc uptake (pmol/h per mg protein
None (control)	20.1 ± 1.4
Insulin	291.7 ± 12.3
H_2O_2	84.1 ± 10.4
+ PMA	130.2 ± 17.0 [a]
+ PLC	132.4 ± 21.1 [a]
+ insulin	89.0 ± 2.3 [c]
PAO	114.0 ± 11.9
+ PMA	162.7 ± 21.8 [a]
+ PLC	151.6 ± 26.4 [a]
+ insulin	136.6 ± 18.9 [c]
PMA	40.6 ± 1.9
+ PLC	65.1 ± 1.7 [b,d]
+ insulin	278.3 ± 14.3 [n.s.]
PLC	53.0 ± 5.4
+ insulin	287.5 ± 16.1 [n.s.]

[a] $P < 0.05$ and [b] $P < 0.01$ vs. values measured in the presence of the first agent alone; [c] $P < 0.001$ vs. insulin alone; [d] $P < 0.01$ vs. PLC (*C. perfringens*) alone; [n.s.] not significant vs. insulin alone (paired *t*-test).

on the one hand, and of PMA or PLC, on the other hand, are at least partially additive.

Discussion

The present study provides evidence for the involvement of cellular sulfhydryl groups in the regulation of glucose transport in cardiac tissue: thus, (i) we found that a mild oxidant (H_2O_2 at micromolar concentrations), but also the SH-specific oxidant diamide, or phenylarsine oxide (PAO), an arsenical compound that reacts with vicinal sulfhydryls [12], markedly stimulate the uptake of glucose in isolated cardiomyocytes (Fig. 1), i.e., to an extent that reaches approx. 30% to 50% of the effect elicited by insulin; and (ii) moreover, pretreatment of heart muscle cells with micromolar concentrations of the SH-blocking agent *N*-ethylmaleimide (NEM) depresses or prevents the increase in glucose transport induced by H_2O_2, diamide, PAO or insulin (Table I).

Furthermore, four lines of evidence emerging from this work indicate that at least two different pathways containing essential SH-groups and leading to glucose transport stimulation are expressed in cardiac myocytes.

First, the stimulating action of H_2O_2 is clearly more sensitive than that of PAO or that of insulin towards a pretreatment with NEM (Table I). Second, H_2O_2 alters both the V_{max} and the K_m values of 3-*O*-methyl-D-glucose uptake, whereas PAO and insulin only increase V_{max} (Fig. 2). Third, the stimulation of dGlc uptake by H_2O_2 is considerably depressed by the β-adrenergic agent isoprenaline, probably via a cAMP-mediated process, since the same result was obtained with 8-Br-cAMP (Fig. 3), or with cholera toxin, which is known to increase the cAMP level in these cells [36] (not shown). In marked contrast, the effects of PAO and insulin are completely insensitive to these interventions (Fig. 3). Note that this lack of antagonism of a β-adrenergic treatment on insulin-stimulated glucose transport contrasts with the inhibition observed in fat cells [33–35], and in some [40], but not all [41] cardiac models.

Fourth, the response of the myocyte glucose transport system to H_2O_2 is additive with the stimulating action of protein phosphatase inhibitors of two types: okadaic acid (a specific inhibitor of serine/threonine phosphatases 1 and 2A [38]) and vanadate (an inhibitor of phosphatyrosyl protein phosphatases [37]). In contrast, the effects of PAO or insulin are inhibited by vanadate (and that of insulin, in addition, by okadaic acid) (Fig. 4). Thus, some protein phosphatase-dependent steps may modulate (or even be involved in) the stimulation of glucose transport by insulin and by PAO in these cells, while the stimulatory pathway triggered by H_2O_2 does not appear to contain the target proteins for vanadate and okadaic acid.

On the whole, the results obtained in these four sets of experiments clearly imply that, in heart muscle cells, H_2O_2, on the one hand, and PAO (and possibly insulin) on the other hand, elicit their stimulating effects on glucose transport via at least two distinct pathways. Furthermore, on the basis of similarities becoming evident here, it may be speculated that PAO and insulin share a common locus of action. This could, for instance, be a cellular protein becoming phosphorylated on tyrosine residues and playing an essential role in signal transmission; on the one hand, it is indeed well established that tyrosine phosphorylation represents an early and critical event in the signal chain triggered by insulin (e.g., Ref. 42), and, on the other hand, recent reports have shown that PAO treatment leads to the accumulation of several phosphotyrosyl-containing proteins [19,21,22]. However, the precise cellular site of PAO action with respect to glucose transport stimulation remains a matter of speculation, especially as most studies performed so far focused on the inhibiting effects of this compound.

As for the stimulating effect of H_2O_2 observed in cardiomyocytes, it is conceivable that it is related to that of contractile activity (which represents, besides insulin, a major stimulus of glucose uptake in muscle tissue). Consistent with this possibility is an earlier observation made in our laboratory, namely that in electrically stimulated, i.e., contracting, isolated rat cardiomyocytes, the K_m of MeGlc-uptake was reduced from 12.3 to 7.7 mM [43], i.e., to an extent very similar to the change we now observed upon H_2O_2 treatment. Further investigations will be required to clarify this issue.

The results presented in Table II show that the pathways activated by PAO and H_2O_2 must be, at least partially, distinct from a protein kinase C-dependent and from a phospholipase C-dependent mechanism which also lead to glucose transport stimulation in cardiomyocytes and in other insulin-sensitive tissues [25–28].

Finally, a comparison of data obtained in isolated adipocytes and muscle preparations with the results presented here for cardiac myocytes reveals striking differences with regard to the effects of sulfhydryl reagents: (i) the concentrations of H_2O_2 or of diamide required to stimulate the uptake of glucose in myocytes (5–100 μM, Fig. 1) are considerably lower than in adipocytes (1–20 mM [6,7,9] or skeletal muscle (5–10 mM [8,10,11]); (ii) in adipocytes from adult rats PAO has only inhibiting effects on glucose transport [14]; (iii) in isolated adipocytes, PAO was shown to prevent the rise in glucose transport induced by PMA [44], whereas in cardiac cells, PAO has a stimulatory effect on top of that of PMA (Table II). Interestingly, it was recently reported that PAO stimulates glucose transport in cultured 3T3-L1-adipocytes [17], in rat skeletal muscle [18], and in cultured mouse fibroblasts [19]. Taken together, these observations clearly show that the presumable SH-dependent events participating in the regulation of glucose transport exhibit tissue-specific peculiarities, or, alternatively, that they even involve different pathways in different cell types.

In conclusion, the present study shows that reactions involving SH-groups can largely modulate the function of essential proteins participating in the stimulation of glucose transport in cardiac myocytes. Importantly, at least two distinct SH-dependent pathways appear to be expressed in these cells: one pathway is triggered by H_2O_2 and the other by PAO (and possibly by insulin).

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References

1 Goodno, C. (1982) Methods Enzymol. 85, 116–123.
2 Fischer, Y., Rose, H. and Kammermeier, H. (1991) Life Sci. 49, 1679–1688.
3 Fischer, Y., Rose, H. and Kammermeier, H. (1990) FEBS Lett. 274, 127–130.
4 Gilbert, H.F. (1982) Biol. Chem. 257, 12086–12091.
5 Ziegler, D.M. (1985) Annu. Rev. Biochem. 54, 305–329.
6 Czech, M.P., Lawrence, J.C. and Lynn, W.S. (1974) Proc. Natl. Acad. Sci. USA 71, 4173–4177.
7 Czech, M.P. (1976) J. Biol. Chem. 251, 1164–1170.
8 Kozka, I.J. and Gould, M.K. (1984) Biochim. Biophys. Acta 797, 212–220.
9 Ciaraldi, T.P. and Olefsky, J.M. (1982) J. Cell. Physiol. 110, 323–328.
10 Sorensen, S.S., Christensen, F. and Clausen, T. (1980) Biochim. Biophys. Acta 602, 433–445.
11 Forsayeth, J. and Gould, M.K. (1983) Biochim. Biophys. Acta 759, 184–191.
12 Torchinsky, Y.M. (1984) in Sulfur in Proteins (Torchinsky, ed.), pp. 46–48, Pergamon Press, Oxford, New York, Paris.
13 Frost, S.C. and Lane, M.D. (1985) J. Biol. Chem. 260, 2646–2652.
14 Douen, A.G., Kacem, R. and Jones, M.N. (1988) Biochim. Biophys. Acta 944, 444–450.
15 Warren, A.P. and Pasternak, C.A. (1989) J. Cell. Physiol. 138, 323–328.
16 Sowell, M.O., Robinson, K.A. and Buse, M.G. (1988) Am. J. Physiol. 255, E159–E165.
17 Gould, G.W., Lienhard, G.E., Tanner, L.I. and Gibbs, E.M. (1989) Arch. Biochem. Biophys. 268, 264–275.
18 Henriksen, E.J. and Holloszy, J.O. (1990) Am. J. Physiol. 258, C648–C653.
19 Ballotti, R., Tartare, S., Chauvel, A., Scimeca, J.-C., Alengrin, F., Filloux, C. and Van Obberghen, E. (1991) Exp. Cell Res. 197, 300–306.
20 Liao, K., Hoffman, R.D. and Lane, M.D. (1991) J. Biol. Chem. 266, 6544–6553.
21 Bernier, M., Laird, D.M. and Lane, M.D. (1987) Proc. Natl. Acad. Sci. USA 84, 1844–1848.
22 Hoffman, R.D. and Lane, M.D. (1992) J. Biol. Chem. 267, 14005–14011.
23 Bernier, M., Laird, D.M. and Lane, M.D. (1988) J. Biol. Chem. 263, 13626–13634.
24 Tanti, J.F., Gremeaux, T., Van Obberghen, E. and Lemarchand-Brustel, Y. (1991) J. Biol. Chem. 266, 2099–2103.
25 Cherqui, C., Caron, M., Wicek, D., Lascols, O., Capeau, J. and Picard, J. (1986) Endocrinology 118, 1759–1769.
26 Obermaier-Kusser, B., Muehlbacher, C., Mushack, J., Seffer, E., Ermel, B., Machicao, F., Schmidt, F. and Haering, H.-U. (1989) Biochem. J. 261, 699–705.
27 Henriksen, E.J., Rodnick, K.J. and Holloszy, J.O. (1989) J. Biol. Chem. 264, 21536–21543.
28 Yu, B., Standaert, M., Arnold, T., Hernandez, H., Watson, J., Ways, K., Cooper, D.R. and Farese, R.V. (1992) Endocrinology 130, 3345–3355.
29 Kosower, E.M., Correa, W., Kinon, B.J. and Kosower, N.S. (1972) Biochim. Biophys. Acta 204, 39–44.
30 Kammermeier, H., Schmidt, P. and Jüngling, E. (1982) J. Mol. Cell. Cardiol. 14, 267–277.
31 Eckel, J., Pandalis, G. and Reinauer, H. (1983) Biochem. J. 212, 385–392.
32 Zaninetti, D., Greco-Perotto, R. and Assimacopoulus-Jeannet, F. (1988) Biochem. J. 250, 277–283.
33 Kashiwagi, A., Huecksteadt, T.P. and Foley, J.E. (1983) J. Biol. Chem. 258, 13685–13692.

34 Smith, U., Kuroda, M. and Simpson, I.A. (1984) J. Biol. Chem. 259, 8758–8763.
35 Joost, H.G., Weber, T.M., Cushman, S.W. and Simpson, I.A. (1986) J. Biol. Chem. 261, 10033–10036.
36 Eckel, J., Gerlach-Eskuchen, E. and Reinauer, H. (1990) Biochem. J. 272, 691–696.
37 Swarup, G., Cohen, S. and Garbers, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104–1109.
38 Cohen, P. (1989) Annu. Rev. Biochem. 56, 453–508.
39 Corvera, S., Jaspers, S. and Pasceri, M. (1991) J. Biol. Chem. 266, 9271–9275.
40 Shanahan, M.F., Edwards, B.M. and Ruoho, A.E. (1986) Biochim. Biophys. Acta 887, 121–129.
41 Rattigan, S., Edwards, S., Hettiarachchi, M. and Clark, M.G. (1986) Biochim. Biophys. Acta 889, 225–235.
42 Kahn, C.R. and White, M.F. (1988) J. Clin. Invest. 82, 1151–1156.
43 Rose, H., Schnitzler, N. and Kammermeier, H. (1987) Biomed. Biochim. Acta 46, 622–627.
44 Cherqui, G., Caron, M., Wicek, D., Capeau, J. and Picard, J. (1989) Mol. Cell. Endocrinol. 65, 13–25.