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Activation of glucose transport during simulated ischemia in H9c2 cardiac myoblasts is mediated by protein kinase C isoforms

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Abstract

Glucose transport into cells may be regulated by a variety of conditions, including ischemia. We investigated whether some enzymes frequently involved in the metabolic adaptation to ischemia are also required for glucose transport activation. Ischemia was simulated by incubating during 3 h H9c2 cardiomyoblasts in a serum- and glucose-free medium in hypoxia. Under these conditions 2-deoxy-D-[2,6-^{3}H]-glucose uptake was increased (57% above control levels, $p<0.0001$) consistently with GLUT1 and GLUT4 translocation to sarcolemma. Tyrosine kinases inhibition via tyrphostin had no effect on glucose transport up-regulation induced by simulated ischemia. On the other hand, chelerythrine, a broad range inhibitor of protein kinase C isoforms, and rottlerin, an inhibitor of protein kinase C delta, completely prevented the stimulation of the transport rate. A lower activation of hexose uptake (19%, $p<0.001$) followed also treatment with Gö6976, an inhibitor of conventional protein kinases C. Finally, PD98059-mediated inhibition of the phosphorylation of ERK 1/2, a downstream mitogen-activated protein kinase (MAPK), only partially reduced the activation of glucose transport induced by simulated ischemia (31%, $p<0.01$), while SB203580, an inhibitor of p38 MAPK, did not exert any effect. These results indicate that stimulation of protein kinase C delta is strongly related to the up-regulation of glucose transport induced by simulated ischemia in cultured cardiomyoblasts and that conventional protein kinases C and ERK 1/2 are partially involved in the signalling pathways mediating this process.

Keywords: Glucose transport; Simulated ischemia; H9c2 cardiomyoblasts; Protein kinase C; Mitogen-activated protein kinases

Introduction

Glucose uptake has been shown to be modulated by a variety of stimuli which are involved in the adaptive response to ischemia and reperfusion, such as increased adrenergic tone, oxidative stress and inhibition of oxidative phosphorylation (Lopaschuk and Stanley, 1997). Hexose transport into cardiomyocytes is facilitated by members of the glucose transporter family, mainly the basal glucose transporter GLUT1, and the insulin-sensitive GLUT4 (Abel, 2004). The rate of glucose uptake is generally slower than the catalytic activity of hexokinase, which seems not to be inhibited by glucose-6-P in the heart (Manchester et al., 1994). Therefore, the rate of glucose transport itself represents a limiting step in the overall process of glucose utilization in cardiomyocytes. The mechanisms underlying the acute activation of glucose transport by ischemia are mainly related to translocation of GLUTs from intracellular membrane stores to the sarcolemma. A more prolonged exposure to ischemia results also in enhanced transcription of GLUT1 isoforms, with little or no effect on the expression of the other GLUT genes (Brosius et al., 1997). Catecholamines were shown to be involved in this process in the isolated and perfused rat heart, mainly through α-adrenoceptor stimulation (Egert et al., 1999a). Other factors produced under ischemic conditions modulate glucose transport, such as adenosine (Wyatt et al., 1989), eicosanoids (Dransfeld et al., 2002), ATP (Kim et al., 2002) and endothelin (Wu-Wong et al., 2000). However, the molecular components of the signalling pathway mediating GLUTs translocation are poorly understood. It has been demonstrated that, differently

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from insulin stimulation, ischemia-induced GLUT translocation does not depend on phosphatidylinositol-3 kinase (PI-3K) (Egert et al., 1999b; Medina et al., 2002; Russell et al., 1999). Myocardial ischemia stimulates AMP-activated protein kinase (AMPK), which may be considered as a fuel gauge signalling the need to turn on energy-generating metabolic pathways (Young et al., 1999). Russell et al. (Russell et al., 1999) suggest that this kinase plays an important role in augmenting glucose entry into the cell, because its pharmacological stimulation increases cardiac glucose uptake and transporter translocation. In the present research we evaluated the involvement in glucose transport activation of protein kinases, other than AMPK, that are as well modulated by an ischemic insult. The model used consisted in cardiomyoblasts subjected to hypoxic glucose/serum-free conditions in order to simulate ischemia in vitro (Bonavita et al., 2003). Here we suggest that the stimulation of protein kinase C (PKC), mainly the delta isoform (PKC-δ), is needed to activate glucose transport, and that conventional PKCs (cPKCs) and extracellular-regulated kinase ERK 1/2 could be partially involved in this process.

Materials and methods

Chemicals

Standard chemical compounds, phloretin, 2-deoxy-D-glucose (DOG), phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma Chemical (St. Louis, MO, USA). 2-Deoxy-D-[2,6-^{3}H]-glucose (DOG*) was from Amersham (UK). Nitrocellulose paper (BA 83) was obtained from Schleicher and Schuell (Keene, NH, USA). Rabbit polyclonal antiserum against rat GLUT1, GLUT3, and GLUT4, antirabbit IgG conjugated to horseradish peroxidase, and Western blotting luminol reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin), streptavidin-agarose beads and Micro BCA protein assay reagent were purchased from Pierce (Rockford, IL, USA). All pharmacological inhibitors of the enzymatic activities tested were from Calbiochem, CA, USA except for tyrphostin A 23 (Alexis biochemicals, CA, USA). H9c2 embryonic ventricular cell line was purchased from the European Collection of Cell Cultures (ECACC).

Glucose transport assay

Simulated ischemia was performed by incubating subconfluent H9c2 embryonic rat cardiac cells in DMEM buffer without fetal calf serum (FCS), glucose and pyruvate, in a gas-tight culture cabin where severe hypoxia ($pO_2 < 1\%$) was maintained with N_2/CO_2 (95/5) by an automatized supply control (BugBox-M, Ruskinn Technology, UK). The rate of glucose transport was evaluated in duplicate 60-mm culture plates, after 1–3 h of simulated ischemia or after deprivation of either oxygen, glucose, or serum. During simulated ischemia, the following enzymatic inhibitors were individually dissolved in the culture medium (target enzyme in brackets): 10 μM SB203580 (p38 MAPK), 50 μM PD98059 (ERK 1/2 kinase = MEK), 10 μM tyrphostin A 23 (tyrosine kinases = TKs), 20 μM LY294002 (PI-3K), 5 μM chelerythrine (PKCs), 10 μM rottlerin (PKC-δ), 2 μM Gö6976 (cPKCs). Subconfluent cells in 30 mm plates were washed twice with ice-cold PBS in order to discard all compounds dissolved in the medium, together with dead cells and cellular debris. Then the adherent cells were soaked in 1 ml of PBS and treated with a mixture of DOG* (0.4 μCi/assay) and 1.0 mM unlabeled glucose analogue for 10 min at 37 °C under conditions where the uptake was linear at least for 20 min. After this time, the uptake was stopped by adding phloretin (final concentration 0.2 mM), a potent inhibitor of glucose transport. The cells were washed with PBS and scraped after the addition of 1 ml of lysis buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS and 0.1% Triton), followed by freezing/thawing. Sample radioactivity was measured by liquid scintillation counting.

Crude membrane preparation

H9c2 cells from 24 plates were suspended in Tris buffer containing 1 mM PMSF, pH 7.4, and sonicated for 5 min. After removal of cellular debris, crude membranes were prepared by centrifugation at 100,000 ×*g* for 75 min and solubilized in Tris buffer containing 0.5% Triton X-100 and 1 mM PMSF, pH 7.4.

Biotinylation of plasma membranes

Subconfluent cells in 100-mm plates (3 plates per experimental condition) were rinsed twice with 5 ml of ice-cold PBS, pH 8.0 followed by the addition of 1.5 ml of cold biotinylation buffer (120 mM NaCl, 30 mM $NaHCO_3$ and 5 mM KCl, pH 8.5) containing 0.1 mg/ml freshly added NHS-LC-biotin. After 30 min of gentle swirling at 4 °C, the medium was aspirated and the plates were washed three times with 5 ml of buffer containing NaCl 140 mM, 20 mM Tris and 5 mM KCl, pH 7.5. Cells were then scraped and pooled in 1 ml of hypotonic homogenization buffer containing 10 mM $NaHCO_3$ and 100 μM each of TPCK, TLCK and PMSF. After 10 min on ice, the cells were homogenized in a Potter homogenizer with 20 strokes, and 100 μl of buffer containing 1.5 M NaCl and 100 mM Tris, pH 7.0 were added. The homogenates were then spun for 15 s at 18,000×*g* in a Eppendorf Microfuge to sediment nuclei. The resulting postnuclear supernatants were added to 1.5-ml Eppendorf Microfuge tubes containing 50 μl of streptavidin-agarose beads that had been sedimented following a pre-equilibration with 1 ml of homogenization buffer, and an additional 5 μl of 20 mM PMSF was added to each mixture. After gentle mixing of the samples by repeated inversion at 4 °C for 30 min, the beads were pelleted and surnatant fractions – containing intracellular proteins ("intracellular fraction") – were collected. Pelleted beads were washed three times with 1 ml of homogenization buffer containing freshly added protease inhibitors. The final pellets were resuspended in 80 μl of 1.2× Laemmli buffer lacking both mercaptoethanol and bromophenol blue and incubated at 65 °C for 30 min. The beads were

once again briefly pelleted and the supernatants containing solubilized plasma membrane were removed and frozen overnight prior to use. Because of the presence of high concentrations of SDS in some of the samples, the protein content of all fractions was determined by means of a micro-BCA protein assay kit using bovine serum albumin (in the presence or absence of appropriate concentrations of SDS) as a standard.

SDS-PAGE and Western blot analysis

Cell fractions containing equal amounts of protein (20 μg of protein/lane) were separated on 10% SDS-polyacrylamide gels, immunoblotted, revealed and quantified as previously described (Zanella et al., 2004).

Statistical analysis

Data are expressed as mean ± S.E. The statistical analysis was performed by one-way or two-way ANOVA, followed by Bonferroni's multiple comparison test or by Student's *t*-test (Fig. 2). Differences were considered significant for $p < 0.05$.

Results

A preliminary characterization of glucose transporters was performed in H9c2 cell preparations. The Western blot of total membrane extracts obtained from H9c2 cells revealed the presence of both GLUT1 and GLUT4 isoforms, while GLUT3 was not detectable (Fig. 1, upper panel). The kinetic parameters were evaluated in intact H9c2 cells in the presence of increasing concentration of DOG (Fig. 1, lower panel). The time-course of DOG transport was linear throughout the tested

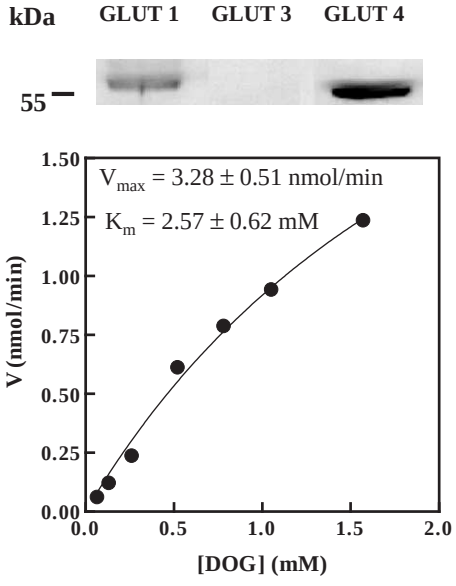

Fig. 1. Western blot and kinetic analysis of glucose transporters. Upper panel: H9c2 total crude membrane preparations were tested in duplicate with antibodies against either GLUT1, GLUT3 or GLUT4. Lower panel: H9c2 cells were incubated with different concentrations of DOG up to 2.0 mM for 10 min at 37 °C. Kinetics parameters were calculated through hyperbolic regression analysis of data obtained in duplicate.

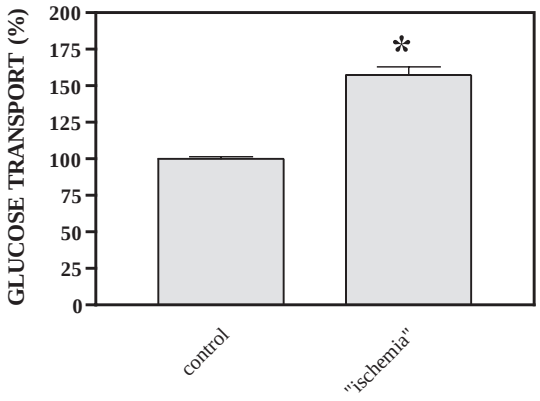

Fig. 2. Glucose transport under conditions of oxygen, serum and glucose deprivation (simulated ischemia). H9c2 cells were subjected to a protocol of simulated ischemia ("ischemia") for 3 h and then incubated for 10 min at 37 °C with DOG as described in the Materials and Methods section. Values are expressed as percent of control (2250 cpm/μg protein = 100%) and are represented as mean ± S.E. of several experiments ($n = 14$). *$p < 0.0001$ vs. control value.

period of 20 min (data not shown). The overall K_m value, obtained by the regression analysis of the data, was in accordance with that expected from the resulting affinity of GLUT1 and GLUT4 interaction, since their individual K_m values are 1.5 mM and 2.0–5.0 mM, respectively, as reported in other studies (Depre et al., 1998; Eckel et al., 1983).

Glucose transport was then assessed under conditions of simulated ischemia that elicited a significant increase in glucose transport (57%, $p < 0.0001$) after 3 h (Fig. 2). Isolation of plasma membranes, by means of biotinylation, and following immunoblotting probing for both glucose transporters, allowed to evaluate the effects of 3 h of simulated ischemia on GLUTs translocation from intracellular stores. The semi-quantitative analysis of GLUT1 showed that it was already present in the plasma membrane under control conditions and that its translocation from the intracellular membrane compartments was slightly activated by simulated ischemia (Fig. 3A). On the other hand, GLUT4 markedly translocated to the cellular surface as a consequence of simulated ischemia, with a simultaneous decrease on intracellular immunoreactive GLUT4 (Fig. 3B).

The mechanism through which simulated ischemia activated glucose transport was studied by using specific inhibitors of some enzymes early involved in the signal transduction processes, such as PKC, PI-3K, and TKs. Chelerythrine abolished the increase in glucose transport rate induced by simulated ischemia. LY294002 and tyrphostin did not affect this process (Fig. 4A). These data show that PKCs are involved in the activation of glucose transport due to simulated ischemia, but PI-3K and TKs are not. To investigate about the isoforms of PKC involved in glucose transport, H9c2 cells subjected to simulated ischemia were incubated with rottlerin or Gö6976 which are inhibitors of PKC-δ (Geschwendt et al., 1994; Wang et al., 2003) and conventional PKCs (Wang et al., 2003), respectively. Rottlerin has also been described to inhibit other PKC isoforms (Geschwendt et al., 1994) but at more elevated concentrations

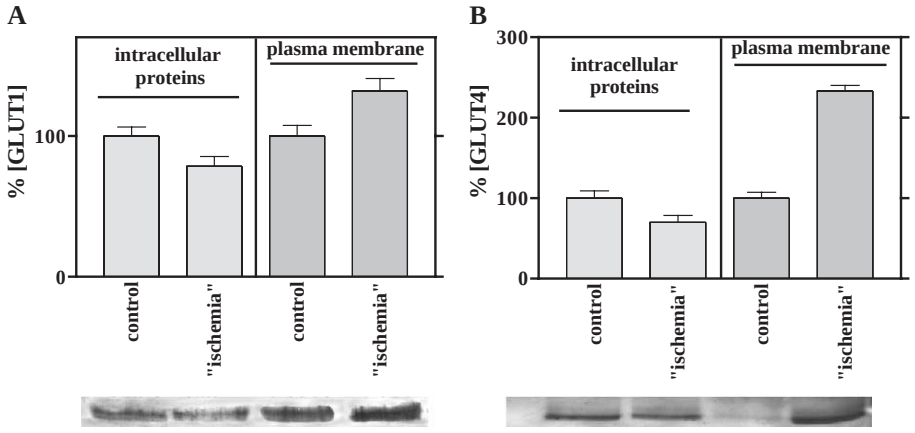

Fig. 3. Relative GLUT1 and GLUT4 distribution in plasma membranes and intracellular fractions from H9c2 cells subjected to simulated ischemia ("ischemia"). Plasma membrane-enriched fractions were isolated by means of biotinylation (see "Methods") from control cells and cells subjected to 3 h of "ischemia". Specific GLUT isoforms were revealed alternatively using anti-GLUT1 and -GLUT4 antibodies. Lower panels: blots representative of repeated experiments are shown. Equal amounts of proteins were loaded in all lanes. Upper panels: Results of scanning densitometric analysis are presented as mean ± S.D. Relative amounts are in arbitrary units, normalized with respect to controls (100%).

with respect to those utilised in the present work. It can also affect negatively CaM Kinase III activity, nevertheless this enzyme, specific for the elongation factor 2, has not been reported as involved in glucose transport regulation. The stimulation of glucose transport was blocked in the presence of rottlerin and was reduced (19%, $p < 0.01$) during incubation with Gö6976 (Fig. 4B). Immunoblotting analysis of the membrane-enriched fraction was performed to further investigate the effect of PKC-δ inhibition on GLUT4 membrane translocation. Rottlerin abolished GLUT4 translocation to membrane induced by "ischemic" conditions while it did not exert any effect on aerobic control (Fig. 5). Under the same approach, rottlerin did not induce any significant change in GLUT1 membrane distribution under "ischemic" conditions (data not shown). Therefore, under these conditions, PKC-δ seems to be necessary for glucose transport activation.Although a less specific effect of Gö6976 on PKC-δ cannot be ruled out, these data indicate for a partial involvement of cPKCs in response to ischemia. Since the MAPK family is activated during ischemia, glucose uptake modulation was also evaluated in the presence of inhibitors of either p38 MAPK or ERK 1/2 kinase (MEK). Fig. 6 shows that SB203580, an inhibitor of p38 MAPK, did not affect the increase in glucose uptake induced by simulated ischemia, while PD98059, an inhibitor of MEK, partially counteracted the effect (only 31% increase, $p < 0.01$). All tested pharmacological inhibitors exerted none or minimal effects on glucose uptake under control aerobic conditions.

Discussion

H9c2 embryonic myoblasts are a cell line derived from rat heart ventricle and are extensively used as a cellular cardiac model because they share most of the molecular and functional features of adult cardiomyocytes (Hescheler et al., 1991). Glucose uptake has been already investigated under different experimental conditions in H9c2 cells differentiated into multinucleated myotubes, thus expressing characteristics of the skeletal, but not cardiac, muscle (Tsiani et al., 1998; Yu et al., 1999, 2000). A major difference relative to glucose

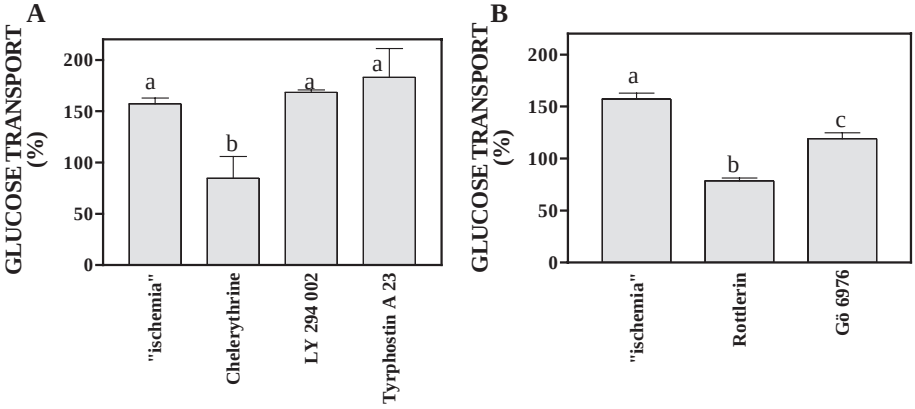

Fig. 4. Effect of various inhibitors of signal transduction pathways on glucose transport. H9c2 cells were incubated during 3 h of "ischemia" with 5 μM chelerythrine, 20 μM LY294002 or 10 μM tyrphostin (panel A) and with 10 μM rottlerin or 2 μM Gö6976 (panel B). Glucose uptake was then determined as described in the Materials and Methods section. The results are expressed as percent of aerobic control (100%) and represent means ± S.E.; $n \geq 3$ for all the tested inhibitors. Values with the same superscript are not significantly different.

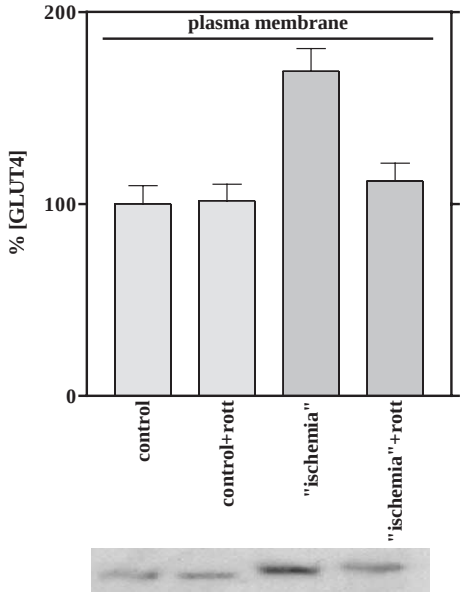

Fig. 5. Effect of rottlerin on GLUT4 distribution in plasma membranes and intracellular fractions from H9c2 cells subjected to simulated ischemia ("ischemia"). Plasma membrane-enriched fractions were isolated by means of biotinylation (see "Methods") from control cells and cells subjected to 3 h of "ischemia". The effect of 10 μM rottlerin on GLUT4 translocation in response to 3 h of "ischemia" are presented. Lower panel: a blot representative of repeated experiments is shown. Equal amounts of proteins were loaded in all lanes. Upper panel: results of scanning densitometric analysis are presented as mean ± S.D. Relative amounts are in arbitrary units, normalized with respect to controls (100%).

transport between skeletal and cardiac cells is that the former utilize also the GLUT3 isoform of glucose transporter, that is not expressed in rodent myocardium (Guillet-Deniau et al., 1994; Matsumoto et al., 1995; Zorzano et al., 1997). According to these findings, we did not reveal GLUT3 protein in the H9c2 cardiomyoblasts used throughout this study.

Few reports have described glucose transport in isolated cardiac cells under conditions of energetic imbalance (Colston and Wheeler, 2001). The use of cultured homogeneous myocardial cells instead of the whole heart allows to exclude that other cells with different carbohydrate metabolism could participate in the process of glucose uptake. Some conditions simulating ischemia have been already proposed for cultured H9c2 cells (Conde et al., 1997; He et al., 1999), also in our previous works (Bonavita et al., 2003). When H9c2 cells underwent simultaneous deprivation of oxygen, glucose and serum to simulate in vitro ischemic conditions, we observed about 57% increase in glucose transport after three hours. The mechanisms underlying a rapid increase in the rate of glucose transport in response to energetic failure include the activation of GLUT1 molecules located into the plasma membrane together with the translocation to the plasma membrane of additional GLUT1 and GLUT4 molecules from the intracellular membrane compartments (Zhang et al., 1999; Zorzano et al., 1997). Only after a more prolonged exposure to these conditions, enhanced transcription and translation of the gene encoding for GLUT1 can occur, without involvement of the other GLUT genes (Remondino et al., 2000; Walker et al., 1989). Consistently with other reports (Brosius et al., 1997; Egert et al., 1999b), also our model of simulated ischemia provoked the translocation of both transporters to the sarcolemma, with a greater effect for GLUT4. Since the increase in sarcolemmal amount of glucose transporter molecules induced by simulated ischemia was similar to the magnitude of glucose uptake stimulation (about two-fold), we suggest that the process of translocation largely accounts for the activation of the transport of the hexose.

When we investigated the signal transduction pathways potentially involved in mediating the increase in glucose transport rate, we confirmed that PI-3K is not involved in the activation induced by simulated ischemia, as already documented in other studies where different ischemic conditions and cells were utilized (Egert et al., 1999b; Medina et al., 2002; Russell et al., 1999). In the present study the involvement of PKC in the glucose transport stimulation was clearly demonstrated, as shown by the lack of up-regulation of hexose uptake in the presence of chelerythrine. In agreement with our results, stimulation of PKC by phorbol esters has been reported to increase glucose transport and GLUT4 translocation in rat heart (Morabito et al., 2002). PKCs are involved in signalling pathways downstream to several agonists produced in the energy-deprived heart, such as adenosine. Adenosine production by both endothelial and myocardial cells increases several fold in the ischemic heart due to the breakdown of ATP and is reported to exert protective effects on cardiomyocytes (Ely and Berne, 1992). A1-receptors are present on the sarcolemmal surface of adult and neonatal cardiomyocytes and their stimulation causes several beneficial effects (Sommerschild and Kirkeboen, 2000), including activation of glucose transport (Wyatt et al., 1989). Moreover, the intracellular signalling pathway coupled to stimulation of A1-receptor involves PKC (Downey et al., 1994), the same enzyme shown to be involved in glucose transport activation in the model used in this study. Looking at which isoform of PKC was involved in the activation of glucose transport by simulated ischemia, we observed that rottlerin, a specific inhibitor of PKC-δ, completely blocked the up-regulation of this process, and that

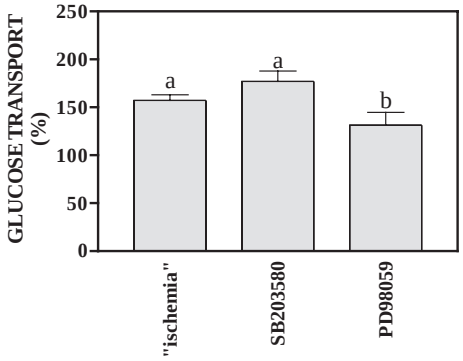

Fig. 6. Effect of MAPK inhibitors on glucose transport. H9c2 cells were subjected to 3 h of "ischemia" in the presence of either 10 μM SB203580 or 50 μM PD98059. Glucose uptake was then determined as described in the Materials and Methods section. Results are expressed as percent of aerobic control (100%) and represent means ± S.E.; $n = 6$. Values with the same superscript are not significantly different.

conventional PKCs could also possibly involved. Since PKC-δ represents a major isoform expressed in cardiomyocytes (Erdbrugger et al., 1997) we can stress its relevance in increasing the availability of glucose during ischemia.

It is also known that H_2O_2 production increases during ischemia (Werns and Lucchesi, 1989) and its elevated concentration results in a rapid stimulation of myoblast glucose transport through the activation of a tyrosine kinase-dependent PLC (Prasad and Ismail-Beigi, 1999). However, in our cellular model, tyrphostin, an inhibitor of tyrosine kinases, was not able to attenuate glucose transport activation. Conditions of energy-deprivation and oxidative stress are both responsible also for MAPKs activation (Bogoyevitch, 2000). It is also interesting to note that PKC, that was closely related to glucose transport in our experimental model, may activate ERK *via* Ras, even if this pathway is not the only able to converge to this subfamily of MAPKs (Liebmann, 2001). During ischemia ERK stimulation generally confers cardioprotection (Wang et al., 1998), while p38 MAPK activation more frequently causes apoptosis (Ma et al., 1999). According to these findings, we observed that also glucose transport was activated, at least in part, by ERK 1/2 stimulation, while p38 MAPK was not involved. By contrast, Tong et al. have recently reported that ischemic preconditioning enhanced glucose uptake by a p38 MAPK-mediated mechanism in Langendorff-perfused rat hearts (Tong et al., 2000). One possible explanation of these opposite results may be that in the whole heart the involvement of p38 MAPK in glucose transport could be referred also to cells different from cardiomyocytes.

We can conclude that these results indicate that stimulation of protein kinase C delta is strongly related to the up-regulation of glucose transport induced by simulated ischemia in cultured cardiomyoblasts and that conventional protein kinases C, and ERK 1/2 could participate to the signalling pathways mediating this process.

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