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LY-83583 stimulates glucose transporter-1-mediated glucose transport independent of changes in cGMP levels

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Abstract

Exposure of Clone 9 cells, a nontransformed rat liver cell line expressing only the Glut1 glucose transporter isoform, to the guanylyl cyclase inhibitor LY-83583 was found to stimulate the rate of glucose transport (~ 7- to 8-fold in 1 h). A similar response to LY-83583 was found in NIH 3T3 fibroblasts, 3T3-L1 pre-adipocytes, and C_2C_{12} myoblasts. Neither the rate of glucose transport in cells under control conditions nor the effect of LY-83583 on glucose transport was altered by 10, 50, or 100 μM 8-bromo-cGMP or by addition of cGMP phosphodiesterase inhibitors, zaprinast, or dipyridamole suggesting that glucose transport and the response to LY-83583 is independent of cGMP levels. In addition, the effect of LY-83583 on glucose transport was not mediated by inhibition of oxidative phosphorylation, since exposure to the agent resulted in no increase in lactate production. Incubation of Clone 9 cells in the presence of the phospholipase C inhibitor U73122, however, attenuated the glucose transport response to LY-83583. Moreover, exposure to LY-83583 resulted in a rise in cell diacylglycerol content, and preincubation with U73122 significantly diminished this rise as well as the glucose transport response to LY-83583. The stimulatory effect of LY-83583 on glucose transport was significantly blocked by thapsigargin. Down-regulation of protein kinase C activity, resulting from 24 h pre-incubation in the presence of 160 nM phorbol-12-myristate 13-acetate, did not attenuate the glucose transport response to LY-83583. It is concluded that the stimulation of glucose transport in response to LY-83583 is independent of changes in cGMP levels, is not mediated by inhibition of oxidative phosphorylation, and is mediated, at least in part, through stimulation of the phospholipase C pathway.

Keywords: LY-83583; cGMP; Phosphodiesterase; Zaprinast; Dipyridamole; Phospholipase C; U73122; Thapsigargin; Nitric oxide (NO); Sodium nitroprusside; Protein kinase C

1. Introduction

Facilitative transport of glucose across plasma membranes is mediated by members of the Na^+-independent family of glucose transporter molecules (Glut) which are expressed in a tissue-specific manner (Muekler, 1990; Pessin and Bell, 1992; Ismail-Beigi, 1993). Among the glucose transporters, glucose transporter-1 is present in varying amounts in most tissues and is expressed at high levels in the brain, retina, placenta, human erythrocytes, and a variety of cells in culture (Muekler et al., 1985; Birnbaum et al., 1986; Pessin and Bell, 1992). In previous studies, we have reported that the rate of glucose transport in Clone 9 cells, a nontransformed rat liver cell line expressing only the glucose transporter-1 isoform, is markedly augmented in response to inhibition of oxidative phosphorylation by sodium azide or cyanide (Mercado et al., 1989; Shetty et al., 1992; Becker et al., 1996; Behrooz and Ismail-Beigi, 1997). It has also been shown that azide and other inhibitors of oxidative phosphorylation increase nitric oxide (NO) levels (Schulz et al., 1997). Since NO donors, such as sodium nitroprusside, have been reported to stimulate the rate of glucose transport (Balon and Nadler, 1997; Young et al., 1997), we tested the possibility that the stimulation of glucose transport in response to inhibition of oxidative phosphorylation is mediated through NO signalling mechanisms.

Results of initial studies on Clone 9 cells indicated that glucose transport was enhanced 2- to 3-fold by sodium nitroprusside (Prasad and Ismail-Beigi, unpublished obser-

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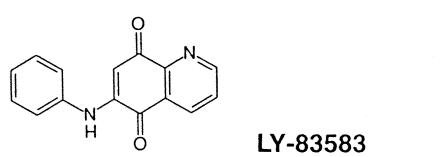
vations), presumably through generation of NO. Since it is well-known that many actions of NO are mediated by stimulation of guanylyl cyclase activity resulting in increased cGMP levels, we tested the effect of LY-83583—a well documented and specific inhibitor of guanylyl cyclase activity (Mulsch et al., 1988; Stuart-Smith et al., 1998)—on the stimulation of glucose transport in response to sodium nitroprusside. The experimental results yielded an unexpected finding, namely that exposure to LY-83583 alone resulted in a marked (~7 fold) stimulation of glucose transport. Studies were hence performed to further characterize the glucose transport response to LY-83583. A preliminary report of some of the findings has been presented (Prasad et al., 1998).

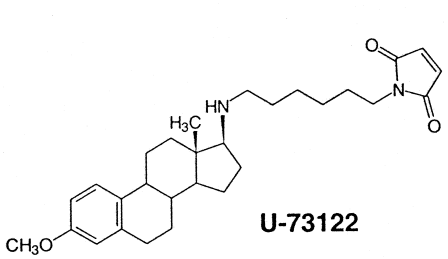
2. Materials and methods

2.1. Materials

Clone 9, C_2C_{12}, NIH 3T3, and 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), calf serum, and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). Plastic culture dishes were obtained from Corning Glass Works (Medfield, MA). 3-*O*-Methyl-D-[^{3}H] glucose ([^{3}H]3-*O*-methyl glucose) (3.4 mCi mmol^{-1}), and *sn*-1,2-diacylglycerol assay kit were purchased from Amersham (Arlington Heights, IL). [^{32}P]γ-ATP (4500 Ci mmol^{-1}) was purchased from ICN Biochemicals (Irvine, CA). LY-83583 and U73122 (Fig. 1), dipyridamole, zaprinast, 8-bromo-cGMP, and phorbol-12-myristate 13-acetate (PMA) were purchased from CalBiochem (La Jolla, CA). Lactate assay kit, sodium azide, cytochalasin-B, sodium nitroprusside, trichloroacetic acid, gamma globulin, thapsigargin, phloretin, thin-layer chromatography plates (silica gel), and standard chemical compounds were purchased from Sigma (St. Louis, MO).



LY-83583



U-73122

Fig. 1. Chemical structures of LY-83583 and U73122.

Horse anti-cGMP antibody and [^{125}I]cGMP were obtained as a gift from Dr. Chung-Ho Chang (Chang and Song, 1993). Dimethyl sulfoxide (DMSO), and hydrochloric acid were purchased from Fisher Scientific (Fairlawn, NJ).

2.2. Cell culture

Clone 9 cells were maintained in Dulbecco's modified eagle medium (DMEM; 5.6 mM glucose) containing 10% calf serum at 37°C with 8% CO_2 and reached confluence after ~4 days. Cells were used between passages 27 and 53. NIH-3T3 cells, 3T3-L1 cells, and C_2C_{12} cells were grown in DMEM containing 10% fetal bovine serum. After confluence, the medium was replaced with serum-free DMEM 24 h prior to initiation of all experiments. Sodium azide and 8-bromo-cGMP dissolved in 40 μl of DMEM, and LY-83583, thapsigargin, and U73122 dissolved in 10 μl DMSO were added directly to culture dishes at appropriate times.

2.3. Measurement of [^{3}H]3-O-methyl glucose uptake

Cells were grown on 60-mm culture dishes. Uptake assay was performed in replicate dishes for a 1.0 to 1.5 min interval, as described previously, in the absence and presence of 50 μM cytochalasin B (Mercado et al., 1989). Cytochalasin B-inhibitable [^{3}H]3-*O*-methyl-D-glucose ([^{3}H]3-OMG) uptake was calculated as the difference in uptake in the absence and presence of cytochalasin B assayed in parallel.

2.4. Measurement of cGMP

Cells grown on 100-mm culture dishes were exposed to various reagents for indicated times in the presence of 10 μM dipyridamole, a cGMP phosphodiesterase inhibitor (Beavo and Reifsnyder, 1990). Immediately following exposure to the indicated stimuli, cells in each dish were washed four times with 10 ml of ice-cold phosphate-buffered saline. Following complete drainage, 250 μl of ice-cold 10% trichloroacetic acid was added to each dish and dishes were immediately placed in −80°C for 1 h. After thawing, cells were scraped and centrifuged at 14,000 × *g* for 10 min at 4°C and the resulting supernatants were placed in glass tubes. 50 μl of 1 M HCl was added to each tube, and the aqueous phase was extracted 3 times with 2 ml of ethyl ether. The aqueous phase was placed in uncapped tubes for 16 h at room temperature to allow the remaining ether to evaporate. Next, 50 μl of 1 M sodium acetate, 40 μl of 1 M NaOH, and 10 μl of triethanolamine:acetic anhydride (2:1, v/v) were sequentially added to each tube with brief mixing after each addition. 100 μl of antibody against cGMP and 100 μl of [^{125}I] cGMP was added, and the tubes were gently shaken for 4 h at 4°C. Next, 50 μl of rabbit gamma globulin was added to each sample. Following the addition of 2 ml

isopropanol to each sample, tubes were centrifuged at $2700 \times g$ for 30 min at 4°C. Supernatants were decanted and pellets were dried for 1 h at room temperature. Finally, samples were counted in a gamma counter. cGMP content in samples was determined using cGMP standards assayed in parallel (Chang and Song, 1993).

2.5. Measurement of lactate

Lactate concentration in the medium was measured in cells grown in 60-mm dishes that had been washed once with 4 ml of Hank's balanced salt solution and incubated in the presence of 1 ml of DMEM for 1 h in the presence or absence of sodium azide or LY-83583. Samples of the medium were analyzed for lactate as described previously and in the kit's instruction guide (Becker et al., 1996).

2.6. Assay of sn-1,2-diacylglycerol

After addition of the indicated reagents to cells, plates were washed twice with ice-cold phosphate-buffered saline (PBS), harvested in 5 ml of ice-cold PBS, placed in 15 ml Falcon Blue Max Jr. tubes, and centrifuged for 5 min at $720 \times g$. Pellets were resuspended in 0.8 ml of 1 M NaCl and 3 ml of a chloroform:methanol (1:2, v/v) was added, and the tubes were shaken vigorously. Then, 1 ml of 1 M NaCl and 1 ml of chloroform were then added, tubes were capped, vortexed, and centrifuged for 2 min at $2350 \times g$. Next, 200 μl of the chloroform phase was transferred to 2 ml microfuge tubes, and samples were evaporated to dryness in a speed-vac for 1 h. Subsequent steps, including measurement of diacylglycerol by its quantitative conversion to [^{32}P]phosphatidic acid in the presence of [^{32}P]-γ-ATP, were according to the kit's instructions. Following the reaction and extraction procedures, radioactivity in phosphatidic acid was determined by thin layer chromatography using the manufacturer's protocol. Bands corresponding to [^{32}P]phosphatidic acid (detected by autoradiography) were cut-out and counted. Diacylglycerol content in samples were determined using diacylglycerol standards assayed in parallel.

2.7. Statistical methods

All values are expressed as means ± S.E. Student's unpaired two-tailed *t*-test was used, and a *P* value of < 0.05 was taken to be significant (Snedecor and Cochran, 1976).

3. Results

3.1. Enhancement of glucose transport in Clone 9 and other cells in response to LY-83583

Exposure of Clone 9 cells to 10 μM LY-83583 for 1 h stimulated the rate of cytochalasin B-inhibitable 3-*O*-methyl glucose transport 7.4 ± 0.3 fold (Fig. 2). As a comparison, treatment of cells with 10 mM sodium azide for 1 h, an inhibitor of oxidative phosphorylation known to stimulate glucose transport in the cells (Shetty et al., 1992), resulted in a 4.1 ± 0.3-fold increase in the rate of glucose uptake. To determine the concentration–response relationship to LY-83583, Clone 9 cells were exposed to 0, 1, 3, 10, and 33 μM LY-83583 for 1 h prior to measurement of cytochalasin B-inhibitable [^{3}H]3-*O*-methyl-D-glucose ([^{3}H]3-OMG) uptake; rates of uptake were half-maximally and maximally stimulated at ~0.8 and ~10 μM LY-83583, respectively (Fig. 3A). The time-course of the stimulatory effect of LY-83583 on glucose transport was also determined (Fig. 3B). Rate of [^{3}H]3-OMG uptake was significantly stimulated after 10 min exposure to 10 μM LY-83583, and reached near-maximal levels at 90 min.

To determine whether the observed stimulation of glucose transport in response to LY-83583 is specific to Clone 9 cells, we measured the effect of LY-83583 on the rate of [^{3}H]3-OMG uptake in three additional cell types, all of which express only the glucose transporter-1 isoform (Harrison et al., 1991; Kotliar and Pilch, 1992; Ismail-Beigi, unpublished observations); the results are shown in Table 1. In response to exposure to 10 μM LY-83583, cytochalasin B-inhibitable [^{3}H]3-OMG uptake was stimulated 4.3-, 5.0-, and 3.7-fold in NIH 3T3 fibroblasts,

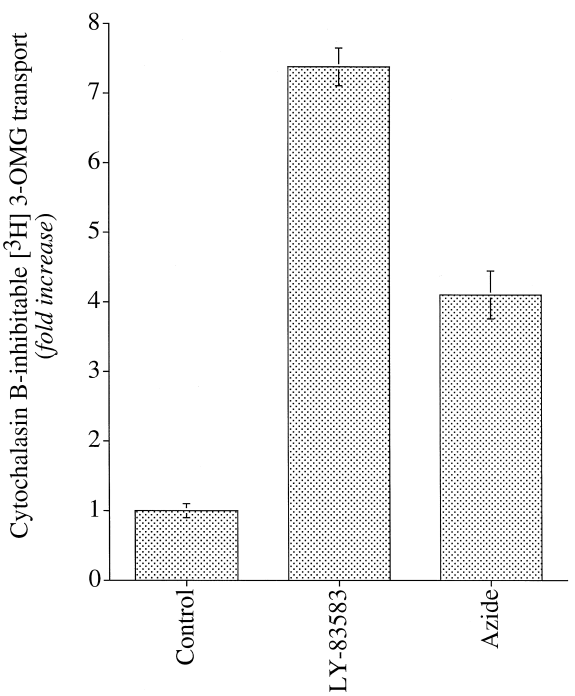


Fig. 2. Effect of LY-83583 and azide on cytochalasin B-inhibitable [^{3}H]3-*O*-methyl-D-glucose ([^{3}H]3-OMG). Clone 9 cells were exposed to 10 μM LY-83583 or 10 mM sodium azide for 1 h, and [^{3}H]3-OMG uptake was determined for a 1 to 1.5 min interval in triplicate dishes in the absence or presence of 50 μM cytochalasin B, as described under Section 2. Carrier-mediated transport in control plates averaged 760 ± 50 cpm min^{-1} (mg protein)$^{-1}$. The experiment was repeated three times, and the results were averaged. The data are expressed as mean ± S.E.

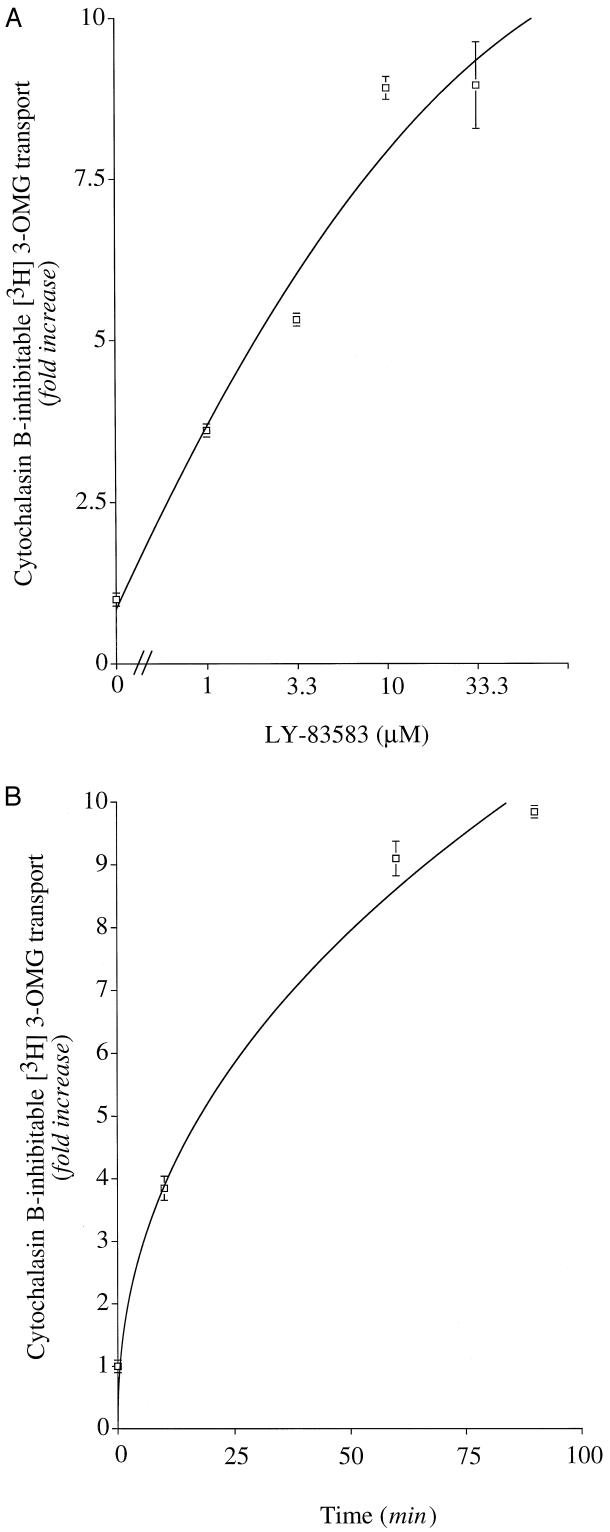


Fig. 3. Time course and dose response of the effect of LY-83583 on cytochalasin B-inhibitable [^{3}H]3-OMG uptake. (A) Clone 9 cells were exposed to 0, 1, 3, 10, and 30 μM LY-83583 for 1 h. (B) Cells were exposed to 10 μM LY-83583 for 10, 60, and 90 min. The rate of [^{3}H]3-*O*-methyl glucose uptake was determined, as detailed under Section 2. The experiments were repeated a second time and the results averaged; data are expressed as means ± S.E.

Table 1
Effect of LY-83583 on cytochalasin B-inhibitable [^{3}H]3-OMG uptake in Clone 9, NIH 3T3, 3T3-L1, and C_2C_{12} Cells

Cell type	LY-83583	[^{3}H]3-OMG uptake (fold increase)
Clone 9	−	1.0 ± 0.1
	+	7.5 ± 0.4[a]
NIH 3T3	−	1.0 ± 0.1
	+	4.3 ± 0.1[a]
3T3-L	−	1.0 ± 0.2
	+	5.0 ± 0.2[a]
C_2C_{12}	−	1.0 ± 0.1
	+	3.7 ± 0.2[a]

Clone 9 cells, NIH 3T3 fibroblasts, 3T3-L1 pre-adipocytes, and C_2C_{12} myoblasts were exposed to 10 μM LY-83583 for 1 h. The rate of cytochalasin B-inhibitable [^{3}H]3-OMG uptake was determined as detailed under Section 2; means ± S.E. The experiment was repeated a second time with similar results. Uptake under control conditions was 772 ± 52, 320 ± 26, 112 ± 25, and 493 ± 32 cpm min^{-1} (mg protein)$^{-1}$ in Clone 9, NIH 3T3, 3T3-L1, and C_2C_{12} cells, respectively.
[a] $P < 0.05$ as compared to the respective values in the absence of LY-83583.

3T3-L1 preadipocytes, and C_2C_{12} myoblasts, respectively. Taken together, these data suggest that LY-83583 evokes a marked and rapid stimulation of glucose transport that is manifest in the variety of cell types examined.

3.2. Effect of LY-83583 on intracellular cGMP levels and glucose transport

The observed stimulatory effect of LY-83583 on the rate of glucose transport in conjunction with its known inhibitory action on guanylyl cyclase activity leading to a reduction of cellular cGMP levels (Diamond and Chu, 1985; Schmidt et al., 1985), raised the possibility that a decrease in cell cGMP levels mediates the enhancement of glucose transport. If this were the case, then an increase in cellular cGMP levels should abrogate the glucose transport response to LY-83583. To test this possibility, Clone 9 cells were exposed to the membrane-permeant cGMP analog, 8-bromo-cGMP, in the presence and absence of LY-83583 (Fig. 4). In comparison with control cells, and as already noted, exposure to 10 μM LY-83583 alone for 1 h resulted in 7.1 ± 0.3-fold increase in the rate of [^{3}H]3-OMG uptake. 10 μM LY-83583 in the presence of 10 μM 8-bromo-cGMP also resulted in a 7.0 ± 0.2-fold increase in the rate of uptake, while exposure to 10 μM 8-bromo-cGMP alone had no effect on the rate of glucose transport. In additional experiments, exposure to 50 or 100 μM 8-bromo-cGMP had no effect on either the basal or LY-83583-stimulated rate of glucose transport (data not shown). As an alternative method of increasing cellular cGMP levels, zaprinast or dipyridamole, cGMP phosphodiesterase inhibitors, were employed (Beavo and Reifsnyder, 1990). The stimulatory effect of 10 μM LY-83583 was largely unaffected by the presence of zaprinast or

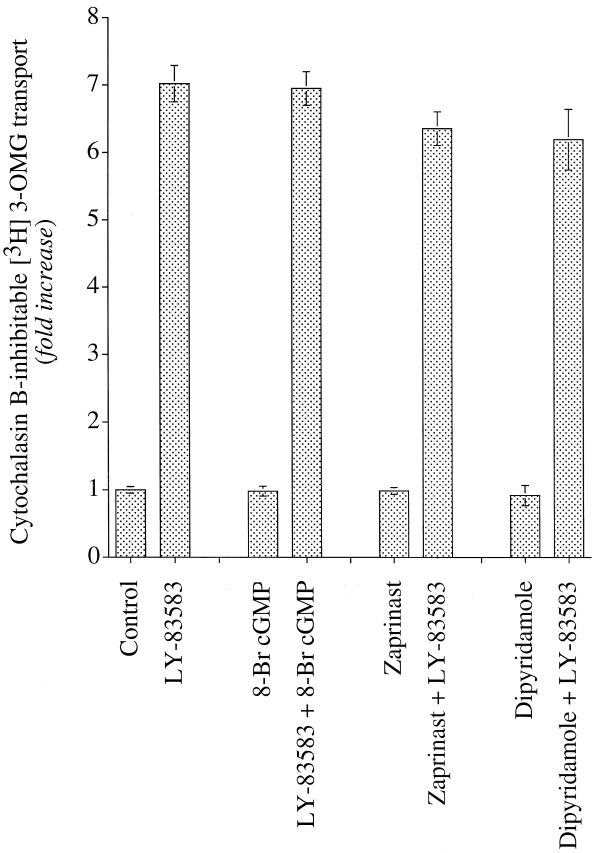


Fig. 4. Effect of LY-83583, 8-bromo-cGMP, zaprinast, and dipyridamole on cytochalasin B-inhibitable [^{3}H]3-OMG uptake. Clone 9 cells were exposed for 1 h to LY-83583, 8-bromo-cGMP, zaprinast, or dipyridamole, (all 10 μM), and their combination, as indicated. The rate of [^{3}H]3-*O*-methyl glucose uptake was then determined. Results represent the average of two to three independent experiments; means ± S.E. The rate of transport in the presence of 8-bromo-cGMP, zaprinast, or dipyridamole alone was not significantly different than that in control cells.

dipyridamole, and zaprinast and dipyridamole alone had no significant effect of the basal rate of glucose uptake (Fig. 4).

To verify that addition of 8-bromo-cGMP to the culture dishes resulted in elevated intracellular cGMP levels, cell cGMP content was measured by radioimmunoassay (Fig. 5). To obtain measurable levels of cGMP, all cells were exposed to 10 μM dipyridamole in addition to the test agents. Addition of 1 μM or 10 μM 8-bromo-cGMP resulted in significant increases in cGMP content of Clone 9 cells. (It should be noted that cells were washed four times to remove all traces of external 8-bromo-cGMP prior to lysis of the cells, and in preliminary experiments, cellular cGMP levels were the same after two versus four washes prior to harvest.) cGMP levels remained unchanged in the presence of 10 μM LY-83583 in conjunction with dipyridamole. As a positive control, exposure of cells to 5 mM sodium nitroprusside resulted in a significant increase in cGMP levels, and this effect was partially blocked by LY-83583. Taken together, the above results suggested that the stimulation of glucose transport in response to LY-83583 is independent of changes in intracellular cGMP levels.

3.3. Effect of LY-83583 on lactate production in Clone 9 cells

Other potential pathways that could mediate the glucose transport response to LY-83583 were examined. We have previously shown that inhibition of oxidative phosphorylation by cyanide or azide results in a rapid stimulation of glucose transport in these cells (Mercado et al., 1989; Shetty et al., 1992; Becker et al., 1996). In addition, inhibition of oxidative phosphorylation leads to an increase in cytosolic $NADH/NAD^+$ and lactate/pyruvate ratios and results in a marked increase in the rate of lactate production (LaNoue and Schoolwerth, 1979; Becker et al., 1996). To discern whether LY-83583's stimulatory effect

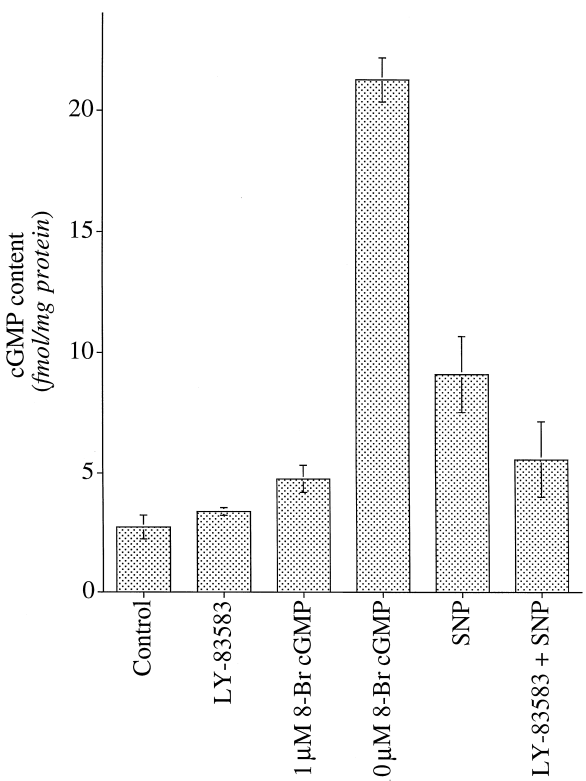


Fig. 5. Effect of LY-83583, sodium nitroprusside, and 8-bromo-cGMP on intracellular cGMP levels. Clone 9 cells were exposed to 10 μM dipyridamole for 10 min prior to addition of all the test agents. Cells were treated with diluent, 10 μM LY-83583, 1 μM 8-bromo-cGMP, or 10 μM 8-bromo-cGMP, or with 5 mM sodium nitroprusside (SNP) in the presence or absence of 10 μM LY-83583 for 1 h. Intracellular cGMP content was measured by radioimmunoassay, as described under Section 2. Results represent the average of three independent experiments, each performed in duplicate culture plates; data are expressed as mean ± S.E. cGMP levels were not significantly changed in the presence of LY-83583 alone as compared to control; all other values were different that control cells ($P < 0.05$).

Table 2
Effect of LY-83583 and azide on the rate of lactate production in Clone 9 cells

Stimulus	Lactate production (nmol (mg protein)$^{-1}$ h^{-1})
None	2.52 ± 0.18
LY-8583	2.12 ± 0.15
Azide	6.97 ± 022[a]

Confluent cells were exposed to diluent, 10 μM LY-83583, or 10 mM sodium azide for 1 h, and lactate was measured in the medium. The experiment was performed three times in duplicate, and results averaged; means ± S.E.
[a] $P < 0.05$ as compared to control.

on glucose transport is in response to a potential inhibition of oxidative phosphorylation by the agent, we measured the rate of lactate production in Clone 9 cells exposed to LY-83583 (Table 2); lactate produced by cells treated with azide served as a positive control. In cells treated with 10 μM LY-83583 for 1 h, the rate of lactate production averaged 2.1 ± 0.15 nmol (mg protein)$^{-1}$ h^{-1} which was not different than the rate in control cells.

3.4. Role of activation of phospholipase C in the stimulation of glucose transport in response to LY-83583

The possibility that activation of phospholipase C, a well characterized upstream mediator of many signalling pathways, is involved in LY-83583's stimulation of glucose transport was explored. We first investigated the effect of U73122 on the glucose transport response to LY-83583; U73122 is a potent inhibitor of phospholipase C with an IC_{50} of ~10 μM for phospholipase C in many cell types (De Moel et al., 1995; Wang et al., 1997), although lower IC_{50} values have also been reported (Zheng et al., 1995). In the presence of 10 μM U73122, the stimulatory effect of LY-83583 on glucose transport in Clone 9 cells was reduced by 45% (Fig. 6); cells treated with 10 μM U73122 alone exhibited a slight (1.3 ± 0.1 fold) increase in transport, which was not significant.

A presumed stimulation of phospholipase C activity by LY-83583 would be predicted to increase cellular levels of *sn*-1,2-diacylglycerol, a product of the reaction catalyzed by phospholipase C. The effect of LY-83583 on cellular diacylglycerol content was hence assayed by measuring the production of [^{32}P] phosphatidic acid in the presence of exogenously added ^{32}P-labeled ATP and diacylglycerol kinase (see *Methods*). Clone 9 cells exposed to 10 μM LY-83583 for 30 min exhibited 3.0 ± 0.1-fold increase in diacylglycerol content compared to untreated control cells (473 ± 37 to 1438 ± 13 pmol (mg protein)$^{-1}$) (Table 3). In contrast, cells preincubated with 10 μM U73122 for 10 min prior to the 30 min exposure to LY-83583 manifest only a 1.4 ± 0.2-fold increase in diacylglycerol content. Exposure to 10 μM U73122 alone also increased cell

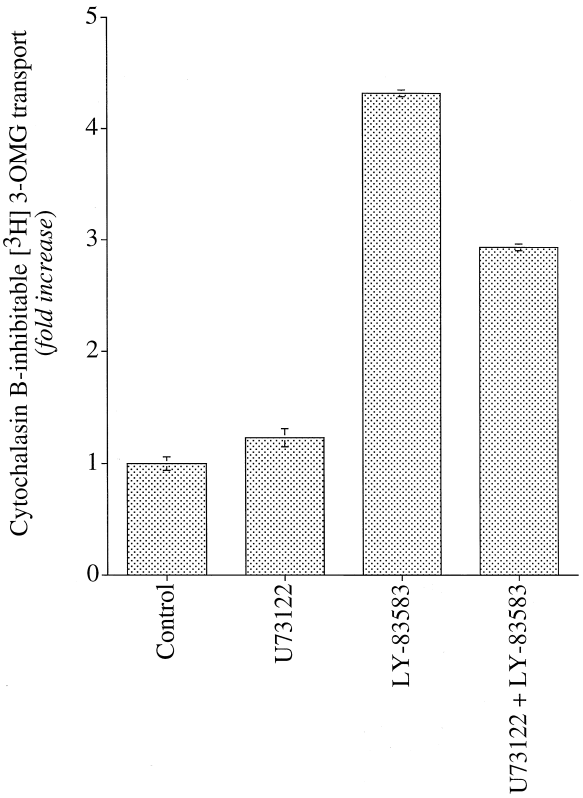


Fig. 6. Effect of U73122 on the stimulation of cytochalasin B-inhibitable [^{3}H]3-OMG uptake. Clone 9 cells were exposed to 10 μM U73122 for 35 min, LY-83583 for 30 min, or the combination of the agents; in the latter case, U73122 was added 5 min prior to addition of LY-83583. The rate of [^{3}H]3-*O*-methyl glucose uptake was then determined. Results represent the average of five independent experiments performed in duplicate; means ± S.E. The slight increase in the rate in the presence of U73122 alone was not significant; the rate of transport was significantly reduced by LY-83583 plus U73122 as compared to LY-83583 alone ($P < 0.05$).

diacylglycerol content; the reasons for this unexpected finding is not known.

Activation of phospholipase C by LY-83583 would be expected to results in an inositol 1,4,5-trisphosphate-in-

Table 3
Effect of LY-83583 in the presence or absence of U73122 on the production of *sn*-1,2-diacylglycerol in Clone 9 cells

Stimulus	Diacylglycerol content (pmol (mg protein)$^{-1}$)
none	473 ± 37
LY-83583	1438 ± 13[a]
U73122	1000 ± 201[a]
U73122 + LY-83583	643 ± 133

Growth arrested cells in triplicate culture dishes were exposed to 10 μM LY-83583 for 30 min in the absence or presence of 10 μM U73122 added 5 min prior to addition of LY-83583. Diacylglycerol content was assessed by measuring the amount of [^{32}P]phosphatidic acid produced following conversion of diacylglycerol to phosphatidic acid; see Section 2 for further details. The experiment was repeated two more times with similar results; means ± S.E.
[a] $P < 0.05$ as compared to control.

duced release of Ca^{2+} from microsomal stores. Moreover, the presumed release of Ca^{2+} induced by LY-83583 could be linked to the LY-83583-induced stimulation of glucose transport. To examine this possibility, we employed thapsigargin, a potent inhibitor of microsomal Ca^{2+}-ATPase which results in a rapid and near-complete depletion of microsomal Ca^{2+} stores in a variety of cells (Moore et al., 1991; Tsukamoto and Kaneko, 1993). The stimulatory effect of LY-83583 on glucose transport was significantly reduced in cells preincubated for 10 min with thapsigargin (Fig. 7); 10 μM thapsigargin alone caused a slight increase in the rate of transport.

We also explored the possibility that stimulation of protein kinase C activity (perhaps secondary to phospholipase C activation) mediates the glucose transport response to LY-83583. Accordingly, cells were preincubated for 24 h in the presence of diluent (DMSO) or 160 nM phorbol-12-myristate 13-acetate (PMA) to 'down-regulate' protein kinase C activity. Following this treatment, cells were exposed to diluent, fresh PMA, or LY-83583 (Fig. 8). The results showed that exposure to LY-83583 was associated with a large stimulation of glucose transport in both control cells and in cells pre-exposed to PMA.

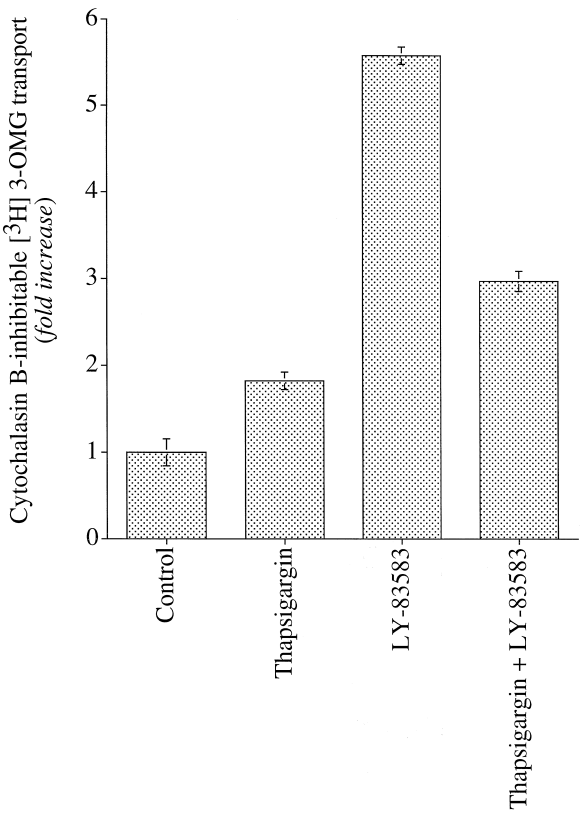


Fig. 7. Effect thapsigargin on the stimulation of cytochalasin B-inhibitable [^{3}H]3-OMG uptake by LY-83583. Clone 9 cells were exposed to 10 μM LY-83583 for 30 min, 10 μM thapsigargin for 40 min, or 10 μM LY-83583 for 30 min following a 10 min preincubation with 10 μM thapsigargin. The experiment, performed in duplicate culture dishes, was repeated two additional times with similar results; means ± S.E. All values are significantly different than control; the rate in the presence of thapsigargin and LY-83583 is significantly lower than that in the presence of LY-83583 alone ($P < 0.05$).

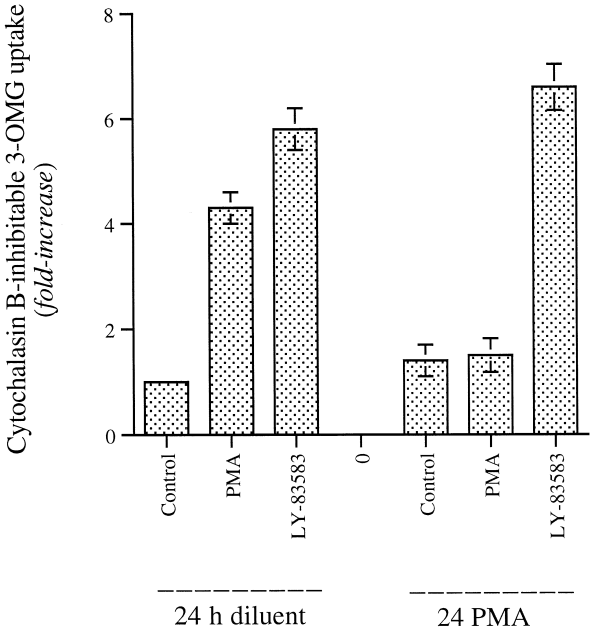


Fig. 8. Effect of 'down-regulation' of protein kinase C on the stimulation of cytochalasin B-inhibitable [^{3}H]3-OMG uptake by LY-83583. Clone 9 cells incubated in the presence of diluent (DMSO) or 160 nM PMA for 24 h prior to a 30 min exposure to diluent, 160 nM fresh PMA, or 10 μM LY-83583. The rate of cytochalasin B-inhibitable [^{3}H]3-OMG uptake was then determined. The experiment was repeated a second time and the results averaged; means ± S.E. The stimulatory effect of fresh PMA on glucose transport was nearly eliminated in cells preincubated with PMA ('down-regulation' of protein kinase C). Exposure to LY-83583 stimulated the rate of glucose transport to an equivalent degree in cells preincubated in the presence or absence of PMA for 24 h.

4. Discussion

The experimental results obtained in this study present several novel findings. *First*, LY-83583 was found to dramatically enhance the rate of glucose transport, and the response was observed in several cell systems examined; *Second*, LY-83583's effect on glucose transport appeared to be independent of its inhibitory actions on guanylyl cyclase or a reduction in cellular cGMP levels. This important inference is based on the finding that the stimulatory effect of LY-83583 on glucose transport was not blocked by 10 to 100 μM 8-bromo-cGMP. Moreover, addition of 8-bromo-cGMP (up to 100 μM) did not alter the basal rate of transport; *Third*, LY-83583's effect on glucose transport was shown not to be secondary to inhibition of oxidative phosphorylation; and *Fourth*, the results suggest that the stimulation of transport in response to LY-83583 is mediated, at least in part, by activation of phospholipase C.

The present studies were initiated to better define the mechanisms underlying stimulation of glucose transport in response to inhibition of oxidative phosphorylation by

azide (Shetty et al., 1992). Potential signalling pathways considered were those linked to nitric oxide generation, because exposure of cells to azide has been shown to stimulate NO production (Schulz et al., 1997), and NO has been proposed to stimulate the rate of glucose transport (Balon and Nadler, 1997; Young et al., 1997). To test the possibility that stimulation of guanylyl cyclase activity mediates the stimulation of glucose transport in response to LY-83583, we measured the effect of a known inhibitor of guanylyl cyclase activity (LY-83583) on sodium nitroprusside-induced enhancement of glucose transport. The experimental results identified an unexpected and novel finding, namely that exposure to LY-83583 alone resulted in a dramatic stimulation of glucose transport.

It is well-documented that LY-83583 is a potent and specific inhibitor of guanylyl cyclase (Diamond and Chu, 1985; Schmidt et al., 1985; Mulsch et al., 1988; Stuart-Smith et al., 1998). If a reduction in cell cGMP level mediates the transport response to LY-83583, then elevation of cGMP levels by addition of 8-bromo-cGMP to the external medium, or prevention of endogenous cGMP hydrolysis by cGMP-specific phosphodiesterase inhibitors (such as zaprinast or dipyridamole), would be expected to significantly attenuate the response. It was found, however, that addition of exogenous 8-bromo-cGMP (up to 100 μM) or zaprinast or dipyridamole did not inhibit the glucose transport response to LY-83583. To verify that the addition of 8-bromo-cGMP resulted in increased intracellular cGMP levels, assays were performed to measure cGMP levels directly. The results indicated that exposure to 10 μM 8-bromo-cGMP resulted in elevated cell 8-bromo-cGMP levels, as compared to control cells. In this series of experiments, we could not demonstrate a reduction of cGMP levels in the presence of LY-83583 alone. While this latter finding remains unexplained, it is possible that the addition of dipyridamole (a cGMP phosphodiesterase inhibitor) prior to exposure to LY-83583 may have obscured the expected decrease in intracellular cGMP concentration. (The very low levels of cGMP in cells under basal conditions in the absence of dipyridamole were unreliable by the assay employed.) Taken together, the above results suggest that the stimulation of glucose transport by LY-83583 is independent of changes in intracellular cGMP concentration.

It has been reported that in addition to LY-83583's well-known inhibitory actions on guanylyl cyclase activity, LY-83583 also stimulates intracellular superoxide formation (Baas and Berk, 1995). Reactive oxygen species such as superoxide and hydrogen peroxide have been shown not only to stimulate glucose transport (Fischer et al., 1993; Kozlovsky et al., 1997), but also to enhance phospholipase C activity (Kontos and Hess, 1983). We hence explored the possibility that the stimulatory effect of LY-83583 on glucose transport is mediated through activation of phospholipase C. The following experimental results are consistent with the above possibility, namely: (i) exposure to LY-83583 resulted in a significant rise in cell diacylglycerol content, and the increase in diacylglycerol was diminished by U73122, an inhibitor of phospholipase C activity; (ii) U73122 attenuated the glucose transport response to LY-83583; and (iii) the stimulatory effect of LY-83583 on glucose transport was significantly blocked by thapsigargin, a specific inhibitor of microsomal Ca^{2+}-ATPase, which leads to a depletion of Ca^{2+} stores from the microsomal compartment. This latter result is consistent with other reports suggesting that the downstream effects of phospholipase C activation and the attendant increase in cell inositol-1,4,5-triphosphate levels require the presence of normal microsomal calcium stores (Daniell and Harris, 1988). The observation that thapsigargin only partially attenuated the glucose transport response to LY-83583 suggests that other sources of calcium (perhaps from the extracellular compartment) are also involved in the response. Further studies are required to delineate the relative importance of various calcium sources in the stimulation of glucose transport by LY-83583.

Stimulation of phospholipase C activity and the resultant increase in diacylglycerol and intracellular Ca^{2+} would be expected to lead to stimulation of protein kinase C activity. It is also well established that prolonged exposure of cells to phorbol esters (such as PMA) results in 'down-regulation' of protein kinase C activity (Bandyopadhyay et al., 1997; Formisano et al., 1998). Results of experiments reported herein indicate that LY-83583's effect to stimulate glucose transport is unaffected in cells pre-exposed to PMA. However, since not all protein kinase C isoforms are 'down-regulated' by this treatment (Bandyopadhyay et al., 1997; Formisano et al., 1998) it is possible that the effect of phospholipase C is mediated by these diacylglycerol-insensitive protein kinase C isoforms. Further studies are required to verify the mediating role of the phospholipase C pathway in the glucose transport response to LY-83583, and to determine the intermediate steps linking phospholipase C activation to the observed stimulation of glucose transport.

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References

Baas, A.S., Berk, B.C., 1995. Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2^- in vascular smooth muscle cells. Circ. Res. 77, 29–36.

Balon, T.W., Nadler, J.L., 1997. Evidence that nitric oxide increases glucose transport in skeletal muscle. J. Appl. Physiol. 82, 359–363.

Bandyopadhyay, G., Standaert, M.L., Galloway, L., Moscat, J., Farese,

R.V., 1997. Evidence for involvement of protein-kinase C (PKC)-ζ and noninvolvement of diacylglycerol-sensitive PKCs in insulin-stimulated glucose transport in L6 myotubes. Endocrinology 138, 4721–4731.

Beavo, J.A., Reifsnyder, D.H., 1990. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharmacol. Sci. 11, 150–155.

Becker, M., Newman, S., Ismail-Beigi, F., 1996. Stimulation of GLUT-1 glucose transporter expression in response to inhibition of oxidative phosphorylation: role of reduced sulfhydryl groups. Mol. Cell Endocrinol. 121, 165–170.

Behrooz, A., Ismail-Beigi, F., 1997. Dual Control of Glut-1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation. J. Biol. Chem. 272, 5555–5562.

Birnbaum, M.J., Haspel, H.C., Rosen, O.M., 1986. Cloning and characterization of a cDNA encoding the rat brain glucose-transporter protein. Proc. Natl. Acad. Sci. USA 83, 5784–5788.

Chang, C.H., Song, D.L., 1993. Melittin potentiates guanylate cyclase activation stimulated by atrial natriuretic factor and ATP. J. Biol. Chem. 268, 4908–4911.

Daniell, L.C., Harris, R.A., 1988. Inositol phosphates, GTP and caffeine release calcium from brain microsomes. Soc. Neurosci. 14, 84.

De Moel, M.P., Van de Put, F.H., Vermegen, T.M., De Pont, J.H., Willems, P.H., 1995. Effect of the aminosteroid, U73122, on Ca^{2+} uptake and release properties of rat liver microsomes. Eur. J. Biochem. 234, 626–631.

Diamond, J., Chu, E., 1985. A novel cGMP-lowering agent, LY-83583, blocks carbachol-induced cyclic GMP elevation in rabbit atrial strips without blocking the negative inotropic effect of carbachol. Can. J. Physiol. Pharmacol. 63, 908–911.

Fischer, Y., Rose, H., Thomas, J., Deuticke, B., Kammermeier, H., 1993. Phenylarsine oxide and hydrogen peroxide stimulate glucose transport via different pathways in isolated cardiac myocytes. Biochim. Biophys. Acta 1153, 97–104.

Formisano, P., Oriente, F., Miele, C., Caruso, M., Auricchio, R., Vigliotta, G., Condorelli, G., Beguinot, F., 1998. In NIH-3T3 fibroblasts, insulin receptor interaction with specific protein kinase C isoforms controls receptor intracellular routing. J. Biol. Chem. 273, 13197–13202.

Harrison, S.A., Buxton, J.M., Czech, M.P., 1991. Suppressed intrinsic catalytic activity of GLUT1 glucose transporters in insulin-sensitive 3T3-L1 adipocytes. Proc. Natl. Acad. Sci. USA 88, 7839–7843.

Ismail-Beigi, F., 1993. Metabolic regulation of glucose transport. J. Membr. Biol. 135, 1–10.

Kontos, H.A., Hess, M.L., 1983. Oxygen radicals and vascular damage. Adv. Exp. Med. Biol. 161, 365–375.

Kotliar, N., Pilch, P.F., 1992. Expression of the glucose transporter isoform GLUT 4 is insufficient to confer insulin-regulatable hexose uptake to cultured muscle cells. Mol. Endocrinol. 6, 337–345.

Kozlovsky, N., Rudich, A., Potashnik, R., Bashan, N., 1997. Reactive oxygen species activate glucose transport in L6 myotubes. Free Radic. Biol. Med. 23, 859–869.

LaNoue, K.F., Schoolwerth, A.C., 1979. Metabolite transport in mitochondria. Annu. Rev. Biochem. 48, 871–922.

Mercado, C.L., Loeb, J.N., Ismail-Beigi, F., 1989. Enhanced glucose transport in response to inhibition of respiration in Clone 9 cells. Am. J. Physiol. 257, C19–C28.

Moore, W.C., Hargrove, H.M., Salama, A.I., Patel, J., 1991. Calcium mobilization and entry by thapsigargin in neuronal tumor cell line, SK-N-SH. NeuroReport 2, 124–126.

Muekler, M., 1990. Family of glucose-transporter genes: implication for glucose homeostasis, and diabetes. Diabetes 39, 6–11.

Muekler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E., Lodish, H.F., 1985. Sequence and structure of a human glucose transporter. Science 229, 941–945.

Mulsch, A., Busse, R., Sabine, L., Forstermann, U., 1988. LY 83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. J. Pharmacol. Exp. Ther. 247, 283–288.

Pessin, J.E., Bell, G.I., 1992. Mammalian facilitative glucose transporter family: structure and molecular regulation. Annu. Rev. Physiol. 54, 911–930.

Prasad, R.K., Behrooz, A., Ismail-Beigi, F., 1998. Stimulation of glucose transport by LY-83583: potential mechanisms. FASEB J. 825A.

Schmidt, M.J., Sawyer, B.D., Truex, L.L., Marshall, W.S., Fleisch, J.H., 1985. LY-83583: an agent that lowers intracellular levels of cyclic guanosine 3′,5′-monophosphate. J. Pharmacol. Exp. Ther. 232, 764–769.

Schulz, J.B., Matthews, R.T., Klockgether, T., Dichgans, J., Beal, M.F., 1997. The role of mitochondrial dysfunction and neuronal nitric oxide in animal models of neurodegenerative diseases. Mol. Cell. Biochem. 174, 193–197.

Shetty, M., Loeb, J.N., Ismail-Beigi, F., 1992. Enhancement of glucose transport in response to inhibition of oxidative metabolism: pre- and post-translational mechanisms. Am. J. Physiol. 262, C527–C532.

Snedecor, G.W., Cochran, W.G., 1976. Statistical Methods. Iowa Univ. Press, Ames, IA.

Stuart-Smith, K., Warner, D.O., Jones, K.A., 1998. The role of cGMP in the relaxation to nitric oxide donors in airway smooth muscle. Eur. J. Pharmacol. 341, 225–233.

Tsukamoto, A., Kaneko, Y., 1993. Thapsigargin, a Ca^{2+}-ATPase inhibitor, depletes the intracellular Ca^{2+} pool and induces apoptosis in human hepatoma cells. Cell Biol. Int. 17, 969–970.

Wang, J.P., Hsu, M.F., Kuo, S.C., 1997. Inhibition by abruquinone A of phosphoinositide-specific phospholipase C activation in rat neutrophils. Eur. J. Pharmacol. 319, 131–136.

Young, M.E., Radda, G.K., Leighton, B., 1997. Nitric oxide stimulates glucose transport and metabolism in rat skeletal muscle in vitro. Biochem. J. 322, 223–228.

Zheng, L., Paik, W.Y., Cesnjaj, M., Balla, T., Tomic, M., Catt, K.J., Stojilkovic, S.S., 1995. Effects of the phospholipase-C inhibitor, U73122, on signalling and secretion in pituitary gonadotrophs. Endocrinology 136, 1079–1088.