Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 127-133

Ca^{2+}-DEPENDENT STIMULATION OF 3-O-METHYLGLUCOSE TRANSPORT IN MOUSE FIBROBLAST SWISS 3T3 CELLS INDUCED BY PHORBOL-12,13-DIBUTYRATE

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Received February 20, 1985

SUMMARY: Binding of phorbol-12,13-dibutyrate (PDBu), a tumor promoter, to quiescent Swiss 3T3 cells increased the number of hexose carriers, resulting in stimulation of membrane transport of 3-O-methylglucose (3MeGlu) in a Ca^{2+}-dependent fashion. Extracellular Ca^{2+} was necessary to initiate the binding of PDBu to its receptor, and intracellular Ca^{2+} was required to maintain it. The loss of PDBu-binding, caused by elimination of Ca^{2+}, was accompanied by a loss of stimulation of hexose transport. These results indicated that Ca^{2+}-dependent, continuous binding of PDBu to its receptor was essential to induce the stimulation of hexose transport.

Tumor promoters have been shown to interact with plasma membrane to induce many biological events which seem to play an essential role in the process of tumor promotion (1,2). Among these events, stimulation of hexose transport induced by tumor promoters is one of the typical phenomena that has been demonstrated in various types of cell culture systems (3-6). In these studies, 2-deoxy-D-glucose was usually used as a substrate to measure the activity of hexose transport. However, 2-deoxy-D-glucose uptake does not represent true activity of hexose transport, since it is rapidly phosphorylated in cytoplasm after transport across plasma membrane (7,8). Therefore, in this study we used a non-metabolizable analogue of D-glucose, 3-O-methylglucose (3MeGlu) (9), as a substrate to re-evaluate the effect of tumor promoters on membrane transport of hexose. Although

ABBREVIATIONS: PDBu, phorbol-12,13-dibutyrate; 3MeGlu, 3-O-methylglucose; SV3T3 cells, Simian virus 40-transformed 3T3 cells; EGTA, ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline.

0006-291X/85 $1.50

the process of 3MeGlu transport in whole cells was generally too fast to measure the initial uptake rate (10,11), we found that in Swiss 3T3 cells and Simian virus 40-transformed 3T3 cells (SV3T3 cells), linearity of the uptake is maintained for several minutes.

Using this system, we analyzed the effect of a tumor promoter, PDBu, on hexose transport. This agent has been demonstrated to be as effective as 12-O-tetradecanoylphorbol-13-acetate in inducing the stimulation of hexose transport in many types of cells (4). Also its specific binding to the membrane receptor can be assayed using whole cells (3,12,13), which enables us to analyze the relationship between the binding phenomenon and the subsequent stimulation of hexose transport. This paper reports that the stimulation of 3MeGlu transport in quiescent 3T3 cells is accomplished by continuous binding of PDBu to its receptor, which is regulated by Ca^{2+}.

MATERIALS AND METHODS

Chemicals:

[^{3}H]-3-O-Methylglucose (5Ci/mmol), [^{3}H]-L-glucose (10.7Ci/mmol) and [^{3}H]-phorbol-12,13-dibutyrate (30.8Ci/mmol) were purchased from New England Nuclear. PDBu was obtained from P-L Biochemical Inc. and A23187 was from Calbiochem. Ethylene glycol bis (β-aminoethylether)-N,N,N',N',-tetraacetic acid (EGTA) was obtained from Nakarai Chemical Co. Inc. All other chemicals were obtained from commercial sources in either reagent grade or highest purity available.

Cell cultures:

Swiss 3T3 cells (14) and SV3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplement with 10% fetal calf serum. Cells were grown in plastic tissue culture plates (35 mm in diameter) in a humidified CO_2 incubator at 37°C. After 3 days of seeding at the density of 3×10^5 cells/2 ml, the culture became confluent, and these dishes were used for experiments.

Measurement of hexose uptake:

Swiss 3T3 cells and SV3T3 cells were incubated for a designated period of time, and rinsed twice with 2 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The uptake was initiated by addition of 1 ml of PBS containing [^{3}H]-3MeGlu (4 μM, 5 μCi/ml) at 20°C. After a designated period of time, hexose uptake was stopped by washing the plates three time with 2 ml of ice-cold PBS. Cells were denaturated by addition of 0.1 ml of 5% trichloroacetic acid and then dissolved in 0.9 ml of 0.1 M NaCl/0.1% sodium dodecylsulfate, and aliquots of the lysate were taken for assay of radioactivity and for protein determination. Carrier-mediated uptake was calculated after correction of non-specific uptake determined with [^{3}H]-L-glucose. Results were expressed as pmoles of hexose per mg protein per min or normalized to a basal value of 100.

Measurement of Km and Vmax of hexose uptake:

Kinetic parameters Km and Vmax were determined using weighted fits of Lineweaver-Burk plots with the indivisual mean rates of triplicate experiments weighted by the factor $V^4/(SE)^2$ and S.E. for Km and Vmax were calculated as described by Wilkinson (15). Significant differences were assessed using the 2-tailed Student *t*-test.

Measurement of specific binding of PDBu:

The specific binding of PDBu were determined by incubation confluent 3T3 cells with 100 nM [^{3}H]PDBu in serum-free medium for a designated period of time at 37°C. The reaction was terminated by washing the cells with 2x5 ml of PBS of 2 min. Specific binding (expressed as d.p.m./10^6cells) is defined as the difference between [^{3}H]PDBu found in the absence and presence of 50 μM PDBu.

Determination of protein concentrations:

Concentration of protein were assayed by the modified Lowry's methods (16).

RESULTS

Exposure of 3T3 cells in the stationary phase to 100 nM PDBu induced a marked stimulation of 3MeGlu uptake (Fig.1). On the pther hand, in 3T3 cells in the log phase, hexose transport activity was scarcely affected by the treatment with PDBu (Table 1). In SV3T3 cells, the initial rate of hexose uptake was hardly affected by culture period or treatment with PDBu.

Fig.2 shows the kinetic analysis of PDBu-stimulated hexose uptake. PDBu-induced stimulation of hexose uptake was observed in the entire range of hexose concentrations tested in this study (0.125-2.0 mM). The Vmax value for hexose

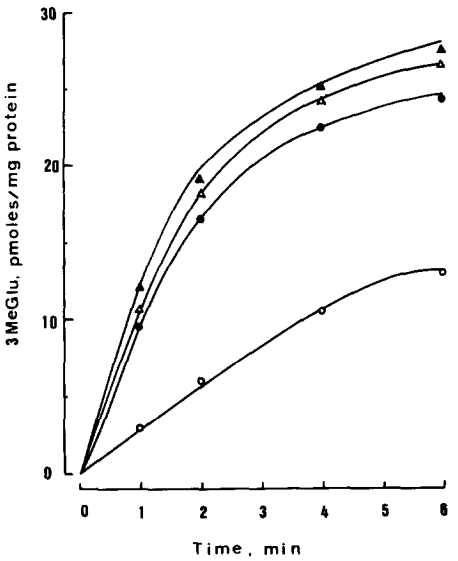


Figure 1. Time cources of 3MeGlu transport in 3T3 cells (○ and ●) and SV3T3 cells (△ and ▲). After 4 days of seeding 3x10^5cells/2 ml in a plastic Petri dish, cells were treated with 100 nM PDBu (● and ▲) or 0.2% ethanol (solvent control, ○ and △) for 3 h at 37°C. The cultures were assayed for the activity of 3MeGlu uptake for up to 6 min as described in MATERIALS AND METHODS. Data are expressed as the mean of triplicate experiments.

Table 1. Effect of culture periods on hexose uptake in 3T3 and SV3T3 cells

Culture periods	3MeGlu uptake (pmoles/mg protein/min)	
	None-treated	PDBu-treated
(A)		
2 day cultured cells	8.20 ± 1.23	9.56 ± 1.01
4 day cultured cells	2.74 ± 0.28	8.23 ± 1.64*
(B)		
2 day cultured cells	9.88 ± 0.97	10.12 ± 0.98
4 day cultured cells	9.25 ± 0.67	9.95 ± 1.02

3T3 cells (A) and SV3T3 cells (B) were inoculated as a density of 3×10^5 cells/2 ml of medium per Petri dish. After 2 days (log phase), or 4 days (confluent phase), PDBu or vehicle was added, and incubation was continued for 3 h. Ethanol was added as a vehicle for test compound. After the dish was washed with PBS, hexose uptake at 20°C was measured. Data are expressed as mean ± S.E. (n=3 , $p<0.01$* vs other values).

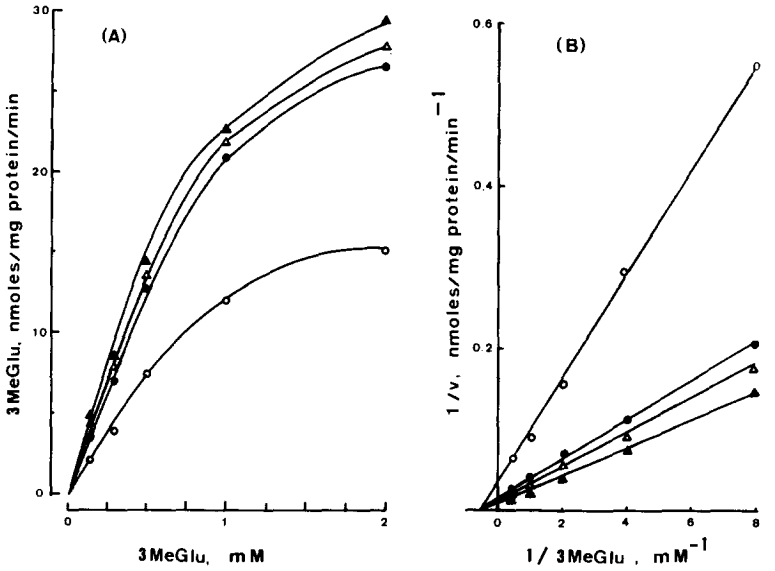
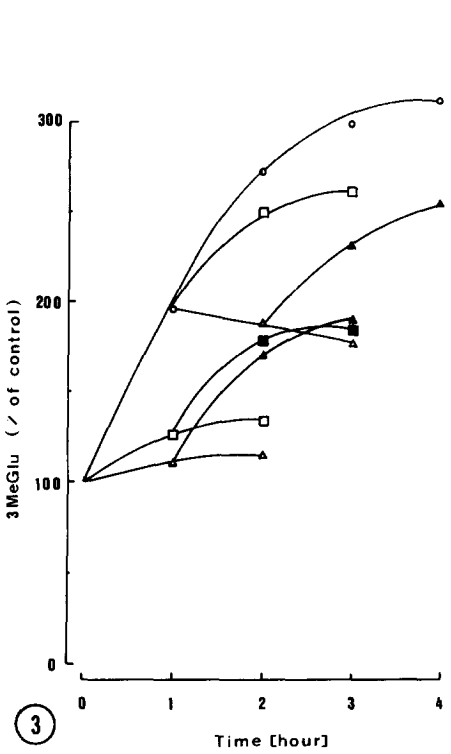


Figure 2. Kinetic analysis of 3MeGlu transport in PDBu-treated 3T3 cells and SV3T3 cells. A Lineweaver-Burk plot of the initial rate of 3MeGlu transport induced by PDBu in 3T3 cells (O and ●) and SV3T3 cells (Δ and ▲) was employed. Four day-cultured cells were tested. PDBu (● and ▲) or vehicle (O and Δ) was added, and 3 h later 3MeGlu uptake was assayed. 3MeGlu uptake for 30sec at a concentration between 0.125 mM and 2 mM was determined. The linearity of the transport was maintained for at least 30 sec at a higher concentration of 3MeGlu (2 mM). Data are expressed as the mean of triplicate experiments.

uptake in PDBu-treated cells was significantly increased about 3.5-fold above the control (109.6 ± 12.5 vs 26.5 ± 3.8 pmoles/mg protein/min ; n=5 , $p<0.01$), whereas the Km value was not affected (2.75 ± 0.30 vs 2.73 ± 0.48 mM).

Fig.3 shows the role of Ca^{2+} in the stimulation of hexose uptake in PDBu-treated 3T3 cells. When either 3 mM EGTA to chelate extracellular Ca^{2+} or 3 mM EGTA plus 5 μM A23187 to remove both intra- and extracellular Ca^{2+} (17) was added to the culture medium simultaneously with PDBu, the stimulatory effect of PDBu on hexose transport was completely suppressed. Addition of 3 mM EGTA plus 5 μM



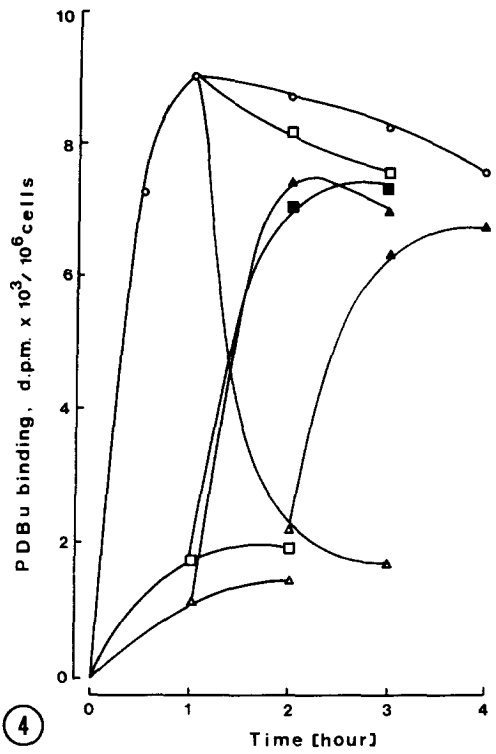


Figure 3. Effect of EGTA and A23187 on PDBu-stimulated 3MeGlu transport in 3T3 cells. The medium of confluent culture of 3T3 cells was replaced with DMEM containing PDBu (100 nM). At the same time or 1 h later, 3 mM EGTA (□), 3 mM EGTA plus A23187 (△) or vehicle alone (○) was added to each dish. In the case of Ca^{2+} restoration experiments, 3 mM Ca^{2+} was added to the medium (▲ and ■) after a designated period of incubation. The data have been normalized to a basal value of 100 for each experiment. Absolute basal uptake ranged from 4.40 to 9.08 pmoles/mg protein/min, under the conditions used.

Figure 4. Effect of EGTA and A23187 on PDBu binding in 3T3 cells. The medium of confluent culture of 3T3 cells was replaced with DMEM containing PDBu (100 nM). At the same time or 1 h later, 3 mM EGTA (□), 3 mM EGTA plus A23187 (△) or vehicle (○) was added to each dish. In the case of Ca^{2+} restoration experiments, 3 mM Ca^{2+} was added to the medium (▲ and ■) after a designated period of incubation. The data are expressed as the mean of triplicate experiments.

A23187 at 1 h after PDBu treatment also blocked PDBu-induced stimulation of hexose transport. However, adding 3 mM EGTA only at 1 h after PDBu treatment did not inhibit the stimulation of hexose transport induced by PDBu. In order to exclude the possibility that the inhibition of PDBu-induced stimulation of hexose transport by treatment with EGTA and A23187 was the result of their general cytotoxicity, the effect of Ca^{2+} restoration at 1 h after the addition of EGTA and A23187 was investigated. The results show that the inhibitory effect of these agents was completely reversed by restoring Ca^{2+} in the culture medium and occurred in just the same manner as that observed by the treatment with PDBu alone.

Fig. 4 shows the role of Ca^{2+} in specific binding of PDBu to its receptor. Addition of 3 mM EGTA plus 5 μM A23187 to the culture medium simultaneously with PDBu caused the complete suppresion of PDBu-binding to its receptor. Addition of 3 mM EGTA plus 5 μM A23187 at 1 h after PDBu addition caused PDBu, once bound to its receptor, to detach from it. On the other hand, addition of 3 mM EGTA alone at 1 h after PDBu addition did not remove PDBu from its receptor. However, PDBu binding was completely recovered by restoring 3 mM Ca^{2+} in the culture medium at 1 h after addition of EGTA and A23187.

DISCUSSION

The present study shows that PDBu is a stimulator of hexose uptake in quiescent Swiss 3T3 cells, but not in logarithmically growing 3T3 cells and SV3T3 cells. Kinetic analysis revealed that Vmax of hexose transport markedly increased without a change in the Km of the transport in PDBu-treated 3T3 cells. These phenomena observed in promoter-treated 3T3 cells resemble those seen after malignant transformation of 3T3 cells ; i.e., in both cases, the increase in hexose uptake seemed to be caused by changes in cell membrane, accompanying an increase in the number of functional carriers for hexose.

The results from measurement of specific binding of PDBu suggest that extracellular Ca^{2+} is very important to initiate binding of PDBu to its receptor. Influx of extracellular Ca^{2+} into the cells may be also important to initiate binding of PDBu. Intracellular Ca^{2+} appeared to be required to maintain binding.

The ability of Ca^{2+} to stimulate hexose uptake was completely coupled with PDBu binding to the cell surface receptor. In other words, continuous binding of PDBu, which is dependent on Ca^{2+}, is essential to increase the activity of hexose transport.

The precise mechanism of the stimulation of hexose transport is still unknown. In the case of insulin-induced stimulation of hexose transport, increased translocation of the hexose carrier from its cytoplasmic pool to plasma membrane has been proposed as a possible mechanism (18,19). We are now investigating whether this mechanism can also explain the action of tumor promoters.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan.

REFERENCES

1. Diamond, L., O'Brien, T.G. and Baird, W.M. (1980) Adv. Cancer Res. 32, 38-48.
2. Blumberg, P.M. (1980) CRC Crit. Rev. Toxicol. 8, 153-197.
3. Driedger, P.E. and Blumberg, P.M. (1977) Cancer Res. 37, 3257-3264.
4. Lee, L.S. and Weinstein, I.B. (1979) J. Cell Physiol. 99, 451-460.
5. Copley,M., Gindhart, T. and Colburn, N. (1983) J. Cell Physiol. 114, 173-178.
6. Nordenberg, J., Stenzel, K.H. and Norvogrodsky, A. (1983) J. Cell Physiol. 117, 183-188.
7. Renner, E.D., Plagewann, P.G.W. and Bernlohr, R.W. (1972) J. Biol. Chem. 247, 5765-5776.
8. Hatanaka, M., Augl, C. and Gilden, R.V. (1970) J. Biol. Chem. 245, 714-717.
9. Hatanaka, M. (1974) Biochem. Biophy. Acta 335, 77-104.
10. Whitesell, R.R. and Gliemann, J. (1979) J. Biol. Chem. 254, 5276-5283.
11. Vinten, J., Gliemann, J. and Østerlind, K. (1976) J. Biol. Chem. 251, 794-800.
12. Horowitz, A.D., Greenebaum, E. and Weinstein, I.B. (1981) Proc. Natl. Acad. Sci., USA 41, 2315-2319.
13. Jaken, S., Tashjian, A.H. and Blumberg, P.M. (1981) Cancer Res. 41, 2175-2181
14. Todaro, G.J. and Green, H. (1963) J. Cell Biol. 17, 299-313.
15. Wilkinson, G.N. (1961) Biochem. J. 80, 324-332.
16. Markwell, M.A., Hass, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206-210.
17. Yamanishi, K., Nishino, H. and Iwashima, A. (1983) Biochem. Biophys. Res. Commun. 117, 637-642.
18. Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758-4762.
19. Suzuki, K. and Kuno, T. (1980) Proc. Natl. Acad. Sci. USA 77, 2542-2545.