Evidence for glucose-6-phosphate transport in rat liver microsomes

Isabelle Gerin[1], Emile Van Schaftingen*

Laboratory of Physiological Chemistry, ICP and Université Catholique de Louvain, Avenue Hippocrate 75, B-1200 Brussels, Belgium

Received 17 January 2002; revised 22 March 2002; accepted 24 March 2002

First published online 4 April 2002

Edited by Judit Ovádi

Abstract The existence of glucose-6-phosphate transport across the liver microsomal membrane is still controversial. In this paper, we show that S3483, a chlorogenic acid derivative known to inhibit glucose-6-phosphatase in intact microsomes, caused the intravesicular accumulation of glucose-6-phosphate when the latter was produced by glucose-6-phosphatase from glucose and carbamoyl-phosphate. S3483 also inhibited the conversion of glucose-6-phosphate to 6-phosphogluconate occurring inside microsomes in the presence of electron acceptors (NADP or metyrapone). These data indicate that liver microsomal membranes contain a reversible glucose-6-phosphate transporter, which furnishes substrate not only to glucose-6-phosphatase, but also to hexose-6-phosphate dehydrogenase.

Key words: Glucose-6-phosphate transport; Hexose-6-phosphate dehydrogenase; Glucose-6-phosphatase; 6-Phosphogluconate; Chlorogenic acid

1. Introduction

Glucose-6-phosphatase is a transmembrane protein [1] present in the endoplasmic reticulum of gluconeogenic organs (reviewed in [2,3]). It is almost specific for glucose-6-phosphate in intact microsomes, but loses this specificity after treatment of the microsomes with detergents, acting then equally well on mannose-6-phosphate. According to the substrate-transport model [4], glucose-6-phosphatase is a phosphatase with rather broad specificity, which has its catalytic site oriented towards the lumen of the endoplasmic reticulum. Translocases for glucose-6-phosphate, glucose and Pi would allow the exchange of substrate and products with the cytosol. This model is supported by several findings, including direct evidence for the transport of glucose-6-phosphate in liver microsomes and the demonstration that this transport is defective in glycogen storage disease type Ib [5], a disease due to a functional glucose-6-phosphatase deficiency [6]. Furthermore, the gene mutated in this disease encodes a 46 kDa transmembrane protein that belongs to the same family as the bacterial organophosphate transporters [7]. Finally, chlorogenic acid and its derivatives inhibit glucose-6-phosphatase in intact but not in detergent-treated microsomes, and do not inhibit the pyrophosphatase activity of microsomes [8–10]. It is therefore thought that they act by inhibiting glucose-6-phosphate transport. This interpretation was confirmed by the finding that one of these compounds, S3483, inhibits the activity of yeast phosphoglucose isomerase when this enzyme has been trapped inside microsomes [11].

The substrate-transport model is, however, not unanimously accepted. Using a filtration technique, some investigators found that only a small fraction of the glucose and Pi formed from glucose-6-phosphate appear to transit through the lumen of liver microsomes [12] and that there is no apparent transport of glucose-6-phosphate when glucose-6-phosphatase is inactive or if it is inhibited by vanadate [13–15]. Accordingly, a model (combined conformational-flexibility substrate-transport model) was proposed in which the catalytic site of glucose-6-phosphatase is inside the endoplasmic reticulum membrane and is connected to the cytosol by a channel that is permeable to glucose-6-phosphate, glucose and Pi, and to the lumen of the organelle by a channel permeable to glucose and Pi, but not to glucose-6-phosphate [15,16].

In the present paper we provide new evidence for the presence of glucose-6-phosphate transport by showing that, in intact microsomes, the chlorogenic acid derivative S3483 inhibits the oxidation of glucose-6-phosphate by microsomal hexose-6-phosphate dehydrogenase and that it also inhibits the exit of the glucose-6-phosphate that is formed from glucose and carbamoyl-phosphate.

2. Materials and methods

[^{14}C]Glucose-6-phosphate was synthesized from [^{14}C]glucose and ATP with yeast hexokinase and purified by chromatography on Dowex AG1-X8 (chloride form). Microsomes were prepared from overnight-fasted male Wistar rats by ultracentrifugation and washed once [17]. S3483 was kindly made available to us by Dr. H.J. Burger (Aventis Pharma, Germany)

For the measurement of glucose-6-phosphate formation, the microsomes were incubated at 30°C at a final concentration of 2.8 mg protein/ml in the presence of 20 mM HEPES, pH 7.1, 100 mM KCl, 2% polyethyleneglycol (added to allow rapid sedimentation of the microsomes [18]), 200 mM glucose, with 50 μM S3483 or with solvent (DMSO at a final concentration of 1%). Carbamoyl-phosphate was added after 5 min of preincubation. Samples of 0.4 ml were taken at the indicated times thereafter, mixed with 4 μl 100 mM vanadate and centrifuged at 10 000 × *g* for 15 s. The supernatant and pellet were immediately separated and mixed with 0.2 ml 10% $HClO_4$, the whole arrest procedure taking about 30 s. The perchloric acid extracts were neutralized with K_2CO_3 and centrifuged; the supernatants were diluted three-fold with water and applied on Dowex AG1-X8 columns (1 ml bed volume, in Pasteur capillary pipettes) to separate glucose from glucose-6-phosphate. The columns were washed with 2 ml H_2O; glucose-6-phosphate was eluted with 1.7 ml 0.5 M NaCl and was assayed spectrophotometrically [19] using *Leuconostoc*

*Corresponding author. Fax: (32)-2-7647598.
E-mail address: vanschaftingen@bchm.ucl.ac.be (E. Van Schaftingen).

[1] Present address: Department of Physiology, University of Michigan Medical School, Ann Arbor, MI, USA.

0014-5793/02/$22.00
PII: S0014-5793(02)02640-6

mesenteroides glucose-6-phosphate dehydrogenase in an Aminco Chance spectrophotometer.

For the measurement of the uptake of radioactive glucose-6-phosphate, microsomes (final concentration 7 mg/ml) were incubated with 20 mM HEPES, pH 7.1, 100 000 cpm [^{14}C]glucose-6-phosphate, 10 or 50 μM glucose-6-phosphate, 1 mM $MgCl_2$, 1 mM vanadate (to block glucose-6-phosphatase activity [20]) and with the indicated concentrations of electron acceptor in a final volume of 50 μl. At the indicated times, the sample was mixed with 1 ml of an ice-cold buffer containing 10 mM HEPES, pH 7.1, 100 mM KCl and 0.25 M sucrose, and rapidly filtered onto a Supor®-200 filter (25 mm diameter; 0.2 μm pores), which was washed with 10 ml of the HEPES/KCl/sucrose buffer and counted for radioactivity in the presence of 5 ml Hisafe II scintillation cocktail (Packard). The whole arrest procedure took about 30 s.

To identify the radioactive compound accumulating in microsomes, the experiment was terminated by filtration, as above, and the filter was immediately transferred into 1 ml 10% $HClO_4$, with which it was vigorously shaken. The perchloric acid extract was neutralized and 500 μl of it was incubated in a medium containing 20 mM HEPES, pH 7.1, 1 mM $MgCl_2$, 1 mM NADP with or without 0.5 U/ml 6-phosphogluconate dehydrogenase (from *Torula* yeast, type V, from Sigma) in a final volume of 1 ml. After 30 min at 30°C, the samples were loaded onto AG1-X8 columns (Cl^- form, 1 ml bed volume, in a Pasteur capillary pipette), which were washed successively with 5 ml water, 5 ml 150 mM NaCl and 5 ml 300 mM NaCl.

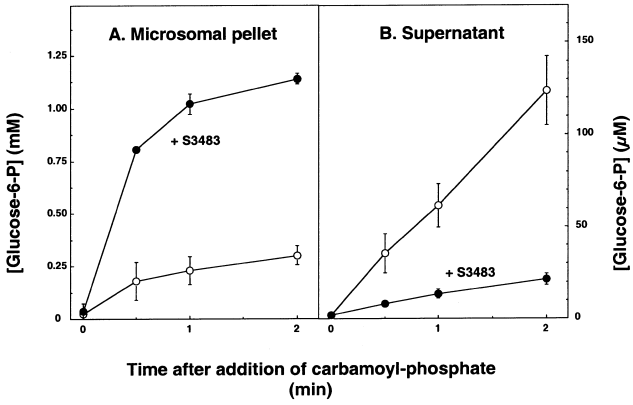

Fig. 1. Effect of S3483 on the formation of glucose-6-phosphate by intact microsomes. Liver microsomes were preincubated for 5 min with 200 mM glucose with or without 50 μM S3483 before the addition of 10 mM carbamoyl-phosphate. At the indicated times the samples were rapidly mixed with vanadate and centrifuged; perchloric extracts were prepared from the supernatants and pellets. The concentration in the microsomal lumen was calculated by assuming a luminal water content of 3.2 μl/mg protein [22].

3. Results and discussion

3.1. Inhibition of the efflux of glucose-6-phosphate by S3483

Glucose-6-phosphatase is able to catalyze the synthesis of glucose-6-phosphate from glucose and carbamoyl-phosphate [21]. According to the substrate-transport model, this synthesis should involve the entry of glucose and carbamoyl-phosphate in the microsomes, the synthesis of glucose-6-phosphate in the luminal compartment and its exit from the microsomes through the glucose-6-phosphate transporter. If such is the case, S3483 should cause the intramicrosomal accumulation of glucose-6-phosphate.

In the experiment shown in Fig. 1, microsomes were incubated in the presence of glucose and carbamoyl-phosphate with or without S3483. At the indicated incubation times, the microsomes were rapidly centrifuged and perchloric acid extracts were prepared from the pellets and the supernatants. The figure shows that S3483 inhibited the appearance of glucose-6-phosphate in the medium but that it caused a marked increase in the intravesicular concentration of this phosphate ester which reached ≈1 mM. The effect of S3483 to cause the appearance of a gradient between the intravesicular and the extravesicular concentrations of glucose-6-phosphate indicates that it inhibits glucose-6-phosphate transport out of the microsomes. Due to this inhibition, the intramicrosomal concentration of glucose-6-phosphate increases until the rate of hydrolysis matches the rate of synthesis, explaining the appearance of a steady state and the fact that S3483 decreases the total formation of glucose-6-phosphate.

That the inhibition exerted by S3483 is not complete is due to the fact that part of the microsomes are not latent (≈5–10%) and that disrupted microsomes catalyze much more rapidly the synthesis of glucose-6-phosphate from carbamoyl-phosphate and glucose than intact microsomes [21], owing probably to the rate-limiting aspect of carbamoyl-phosphate transport.

On a theoretical basis, the following possibilities can be envisaged to account for the inhibitory effect of S3483 and other chlorogenic acid derivatives in the context of the combined conformational-flexibility substrate-transport model: that they interact directly with glucose-6-phosphatase (i), or that they block the hypothetical internal (ii) or external (iii) channel. Explanations (i) and (ii) are not compatible with the observation of intramicrosomal accumulation of glucose-6-phosphate. Blocking of the external channel could cause the accumulation of a few molecules of glucose-6-phosphate, but not as much as observed here. Assuming that glucose-6-phosphatase represents 0.1% of microsomal protein and has a molecular mass of 35 000 Da [23], the microsomal accumulation of glucose-6-phosphate that we observe corresponds to ≈100 molecules per molecule of glucose-6-phosphatase.

3.2. Inhibition of hexose-6-phosphate dehydrogenase by S3483

If S3483 inhibits glucose-6-phosphate transport across the endoplasmic reticulum membrane, it should slow down the oxidation of glucose-6-phosphate by hexose-6-phosphate dehydrogenase, an enzyme present in the lumen of the endoplasmic reticulum [24]. The system used to test this effect is based on the observation that metyrapone is a substrate for a reductase present in the endoplasmic reticulum [25]. This agent is therefore expected to stimulate the conversion of glucose-6-phosphate to 6-phosphogluconate in intact microsomes by allowing re-conversion of microsomal NAD(P)H to $NAD(P)^+$.

As shown in Fig. 2, metyrapone caused a dose-dependent increase in the accumulation of radioactivity in microsomes incubated with [^{14}C]glucose-6-phosphate together with 1 mM vanadate, which completely inhibits glucose-6-phosphatase activity. The accumulated radioactivity elutes from an anion-exchanger column at higher concentrations of NaCl than glucose-6-phosphate (Fig. 3), suggesting that the formed compound corresponds to 6-phosphogluconate. This conclusion was confirmed by the observation that incubation of the neutralized perchlorate extract with 6-phosphogluconate dehydrogenase displaced the peak of radioactivity. Since hexose-6-phosphate dehydrogenase is not a transmembrane protein, but is present in the lumen of microsomes [24,26], the formation of 6-phosphogluconate inside microsomes indicates that

glucose-6-phosphate has access to the lumen of microsomes, in support of the substrate-transport model.

NADP was also found to stimulate the accumulation of radioactivity in intact microsomes, but to a lesser extent than metyrapone (Fig. 2). The accumulated product also corresponded to 6-phosphogluconate (not shown). These results suggest that NADP can slowly penetrate into liver microsomes, in agreement with the observation that hexose-6-phosphate dehydrogenase activity is not completely latent at 37°C [26].

Fig. 4 shows that S3483 inhibited the accumulation of radioactive 6-phosphogluconate in microsomes incubated with metyrapone or NADP, but that it had no effect in the absence of electron acceptor. It had no effect on the accumulated radioactivity, when added just before filtration, indicating that it did not decrease the retention of microsomes on the filters. Other experiments showed that 50 μM S3483 had no effect on hexose-6-phosphate dehydrogenase activity (measured spectrophotometrically with 0.1 mM glucose-6-phosphate and 0.5 mM NADP) in detergent-treated microsomes (not shown).

These experiments and previous work show therefore that three different enzymes that use glucose-6-phosphate (glucose-6-phosphatase [8–10], hexose-6-phosphate dehydrogenase (this paper) and yeast phosphoglucose isomerase, when this enzyme has been artificially entrapped into liver microsomes [11]) are inhibited by S3483 when they are present in intact microsomes, but not when they are free, or when the microsomes have been treated with detergents. The conclusion is therefore that S3483 acts on a common step for the utilization of glucose-6-phosphate by these enzymes, i.e. the transport of this phosphate ester across the microsomal membrane. This transport, which is deficient in glycogen storage disease type Ib, is carried out by the putative transporter that is mutated in this disorder [7], as confirmed by in vitro experiments in which wild-type and mutated proteins have been expressed in COS cells [27]. Furthermore, the protein that binds chlorogenic acid derivatives is apparently absent from the liver of at least one patient with glycogen storage disease type Ib [28].

Our experiments support nonetheless the conclusion of Xie et al. [16] that glucose-6-phosphate is not exchanged with Pi when it crosses the microsomal membrane: Pi crosses the membrane in the same direction as glucose-6-phosphate in the experiments with carbamoyl-phosphate, whereas no Pi is formed in the second type of experiments due to inhibition of glucose-6-phosphatase by vanadate. These data, as well as the finding that glucose-6-phosphatase is still active when Pi efflux from microsomes is blocked through the formation of a complex with Pb^{2+} [11], indicate that the mammalian glucose-6-phosphate transporter behaves as a uniport, contrasting with its bacterial homolog uhpT, which behaves as a hexose-6-phosphate/Pi antiport [29]. This dissimilarity is not surprising, considering the low degree of identity between these proteins (≈20%) and the fact that they belong to a superfamily comprising uniporters, symporters and antiporters [30].

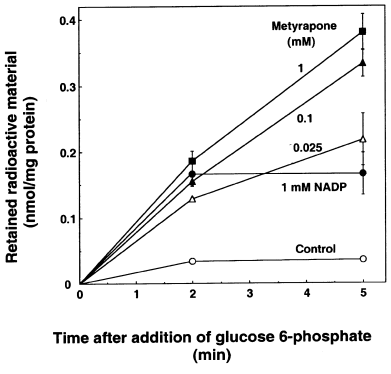

Fig. 2. Effect of metyrapone and NADP on the accumulation of radioactivity in microsomes incubated with [^{14}C]glucose-6-phosphate. Microsomes were incubated with [^{14}C]glucose-6-phosphate, 50 μM glucose-6-phosphate, 1 mM vanadate and with the indicated concentrations of electron acceptor. Samples were filtered at the indicated times (2 and 5 min).

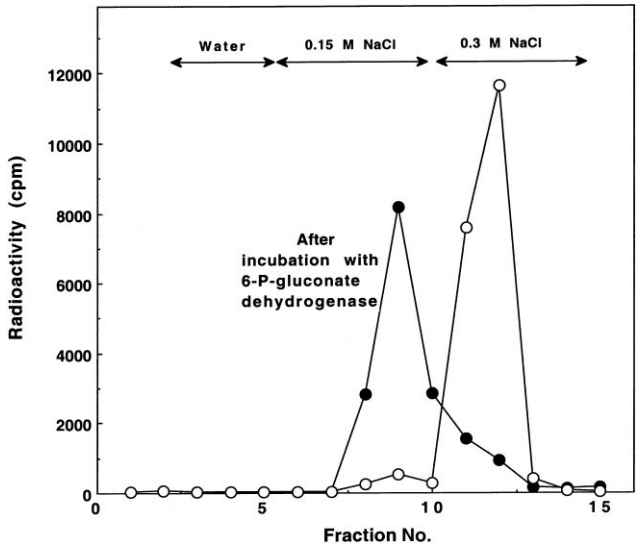

Fig. 3. Identification of the accumulated radioactive product as 6-phosphogluconate. Microsomes were incubated for 10 min with 100 000 cpm [^{14}C]glucose-6-phosphate, 50 μM glucose-6-phosphate, 1 mM vanadate and 1 mM metyrapone before being filtered. The radioactive material retained on the filter was eluted and treated or not with 6-phosphogluconate dehydrogenase and NADP before being chromatographed on AG1-X8.

4. Conclusion

This work adds two new pieces of evidence for the presence of a glucose-6-phosphate transporter in liver microsomes. The fact that S3483 and other chlorogenic acid derivatives inhibit glucose-6-phosphate hydrolysis in intact microsomes, in hepatocytes [8–10] and in vivo [31,32] indicates that glucose-6-phosphate transport is a mandatory step for its hydrolysis by glucose-6-phosphatase. The apparent absence of glucose-6-phosphate uptake reported by Xie et al. [16] is probably due to the fact that the filtration technique used by these authors is not rapid enough to prevent the exit of glucose-6-phosphate during the washings, particularly when no transport inhibitor is included in the washing solution. By contrast, glucose-6-phosphate transport could be demonstrated, even in the presence of vanadate, in an experimental setting in which DIDS, an inhibitor of glucose-6-phosphate translocase [33], was included in the washing buffer [34]. By the same token, the apparent release of glucose and Pi from the catalytic site of glucose-6-phosphatase directly into the medium is due to the fact that the membrane of most microsomal vesicles is readily permeable to glucose and Pi [35,36]. This is supported by the observation that about 80% of the Pi that is formed from

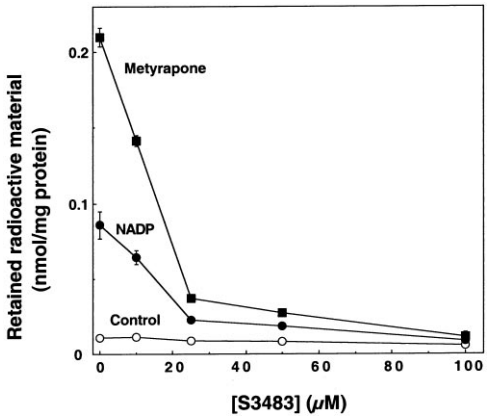

Fig. 4. Inhibition of the accumulation of radioactive 6-phosphogluconate by S3483. Microsomes were preincubated for 5 min with the indicated concentrations of S3483, 1% DMSO (used to solubilize S3483), and 1 mM metyrapone or 1 mM NADP, before the addition of 10 μM [^{14}C]glucose-6-phosphate. The incubations were arrested 5 min later and the samples were filtered.

glucose-6-phosphate is found inside microsomes when incubations are run in the presence of Pb^{2+}, which traps Pi as a lead phosphate precipitate [11].

Acknowledgements: This work was supported by the Concerted research action program of the Communauté Française de Belgique; the Belgian Federal Service for Scientific, Technical and Cultural affairs; and by the Juvenile Diabetes Foundation International. The authors thank G. Noël for her help in the experimental work and H.G. Hers and M. Veiga-da-Cunha for their comments.

References

[1] Pan, C.J., Lei, K.J., Annabi, B., Hemrika, W. and Chou, J.Y. (1998) J. Biol. Chem. 273, 6144–6148.
[2] van de Werve, G., Lange, A., Newgard, C., Méchin, M.C., Li, Y. and Berteloot, A. (2000) Eur. J. Biochem. 267, 1533–1549.
[3] Van Schaftingen, E. and Gerin, I. (2002) Biochem. J. 362, 513–532.
[4] Arion, W.J., Wallin, B.K., Lange, A.J. and Ballas, L.M. (1975) Mol. Cell. Biochem. 6, 75–83.
[5] Igarashi, Y., Kato, S., Narisawa, K., Tada, K., Amano, Y., Mori, T. and Takeuchi, S. (1984) Biochem. Biophys. Res. Commun. 119, 593–597.
[6] Narisawa, K., Igarashi, Y., Otomo, H. and Tada, K. (1978) Biochem. Biophys. Res. Commun. 83, 1360–1364.
[7] Gerin, I., Veiga-da-Cunha, M., Achouri, Y., Collet, J.-F. and Van Schaftingen, E. (1997) FEBS Lett. 419, 235–238.
[8] Arion, W.J., Canfield, W.K., Ramos, F.C., Schindler, P.W., Burger, H.J., Hemmerle, H., Schubert, G., Below, P. and Herling, A.W. (1997) Arch. Biochem. Biophys. 339, 315–322.
[9] Arion, W.J., Canfield, W.K., Ramos, F.C., Su, M.L., Burger, H.J., Hemmerle, H., Schubert, G., Below, P. and Herling, A.W. (1998) Arch. Biochem. Biophys. 351, 279–285.
[10] Hemmerle, H., Burger, H.J., Below, P., Schubert, G., Rippel, R., Schindler, P.W., Paulus, E. and Herling, A.W. (1997) J. Med. Chem. 40, 137–145.
[11] Gerin, I., Noël, G. and Van Schaftingen, E. (2001) Diabetes 50, 1531–1538.
[12] Xie, W., van de Werve, G. and Berteloot, A. (2001) J. Membr. Biol. 179, 113–126.
[13] St-Denis, J.F., Berteloot, A., Vidal, H., Annabi, B. and van de Werve, G. (1995) J. Biol. Chem. 270, 21092–21097.
[14] Lei, K.J., Chen, H., Pan, C.J., Ward, J.M., Mosinger, B., Lee, E.J., Westphal, H., Mansfield, B.C. and Chou, J.Y. (1996) Nat. Genet. 13, 203–209.
[15] Xie, W., van de Werve, G. and Berteloot, A. (2001) FEBS Lett. 504, 23–26.
[16] Schulze, H.U., Nolte, B. and Kannler, R. (1986) J. Biol. Chem. 261, 16571–16578.
[17] Niculescu, L. and Van Schaftingen, E. (1998) Diabetologia 41, 947–954.
[18] Banhegyi, G., Braun, L., Marcolongo, P., Csala, M., Fulceri, R., Mandl, J. and Benedetti, A. (1996) Biochem. J. 315, 171–176.
[19] Michal, G., 1988. In: Bergmeyer, H.U (Ed.), Methods of Enzymatic Analysis, 3d edn. Revised, Vol. 6, 191–198, VCH-Verlagsgeselchaft, Weinheim.
[20] Singh, J., Nordlie, R.C. and Jorgenson, R.A. (1981) Biochim. Biophys. Acta 678, 477–482.
[21] Lueck, J.D. and Nordlie, R.C. (1970) Biochem. Biophys. Res. Commun. 39, 190–196.
[22] Marcolongo, P., Fulceri, R., Giunti, R., Burchell, A. and Benedetti, A. (1996) Biochem. Biophys. Res. Commun. 219, 916–922.
[23] Speth, M. and Schulze, H.U. (1992) Eur. J. Biochem. 208, 643–650.
[24] Hori, S.H. and Takahashi, T. (1977) Biochim. Biophys. Acta 496, 1–11.
[25] Maser, E. and Netter, K.J. (1989) Biochem. Pharmacol. 38, 3049–3054.
[26] Takahashi, T. and Hori, S.H. (1978) Biochim. Biophys. Acta 524, 262–276.
[27] Chen, L.Y., Lin, B., Pan, C.J., Hiraiwa, H. and Chou, J.Y. (2000) J. Biol. Chem. 275, 34280–34286.
[28] Kramer, W., Burger, H.J., Arion, W.J., Corsiero, D., Girbig, F., Weyland, C., Hemmerle, H., Petry, S., Habermann, P. and Herling, A. (1999) Biochem. J. 339, 629–638.
[29] Maloney, P.C., Ambudkar, S.V., Anatharam, V., Sonna, L.A. and Varadhachary, A. (1990) Microbiol. Rev. 54, 1–17.
[30] Marger, M.D. and Saier, M.H. (1993) Trends Biochem. Sci. 18, 13–20.
[31] Herling, A.W., Burger, H.J., Schwab, D., Hemmerle, H., Below, P. and Schubert, G. (1998) Am. J. Physiol. 274, G1087–G1093.
[32] Herling, A.W., Burger, H.J., Schubert, G., Hemmerle, H., Schaefer, H. and Kramer, W. (1999) Eur. J. Pharmacol. 386, 75–82.
[33] Zoccoli, M.A. and Karnovsky, M.L. (1980) J. Biol. Chem. 255, 1113–1119.
[34] Banhegyi, G., Marcolongo, P., Fulceri, R., Hinds, C., Burchell, A. and Benedetti, A. (1997) J. Biol. Chem. 272, 13584–13590.
[35] Marcolongo, P., Banhegyi, G., Benedetti, A., Hinds, C.J. and Burchell, A. (1998) J. Clin. Endocrinol. Metab. 83, 224–229.
[36] Meissner, G. and Allen, R. (1981) J. Biol. Chem. 256, 6413–6422.