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Introduction

IGF I stimulates proliferation and differentiation of osteoblastic cells [1–3] whereas insulin has been considered the physiological regulator of glucose metabolism in bone cells. For example, insulin stimulates glucose uptake in rat osteoblastic osteosarcoma cells [4, 5]. Besides insulin, also PTH enhances glycogen synthesis in calvaria cells but not in fibroblasts derived from newborn rats [6–8].

Cellular uptake of glucose is mediated by a family of facilitative glucose transporter (GLUT) isoforms [9]. Three of them are high-affinity transporters: GLUT1 (erythrocyte/Hep G2) which is found in most tissues (including tumors) and cell lines; GLUT3 (brain/placenta) which has the highest affinity for glucose and which is particularly abundant in brain (neurons); and GLUT4 (muscle/fat) which is exclusively expressed in insulin-sensitive tissues such as striated (skeletal and cardiac) muscle and adipose tissue [10–17]. In bone cells, insulin stimulates the transport of the glucose analogue 2-deoxy-D-glucose (2DG) [4], but glucose transport could not be attributed to a specific GLUT, although more recent findings indicate that both GLUT1 and GLUT3 (mRNA and protein) are expressed in rat osteogenic sarcoma cells [18]. GLUT3 was also detected in bone, and it was suggested that insulin regulates GLUT3-mediated glucose uptake in bone cells. Since insulin receptors are abundant (∼80,000/cell; [5]) in UMR 106-01 cells (University of Melbourne cell line, cloned from ^{32}P-induced rat osteogenic sarcoma), these have often been used as a model to study insulin actions on the osteoblast. IGF I mimics the stimulatory effect on glycogen

synthesis from [^{14}C] glucose in rat osteoblast-like cells [1, 6] and is more potent than insulin in this respect. Since the effects of insulin and IGF I on glucose transport have not been directly compared in the same bone cell system, the authors performed the current studies in osteoblastic PyMS cells, a cell line derived from rat bone (not osteosarcoma). Osteoblasts are typical target cells for calcium-regulating hormones, PTH and vitamin D (its active form, 1α,25-dihydroxyvitamin D$_3$/1α,25-dihydroxycholecalciferol; subsequently called vitamin D). Vitamin D and PTH share inhibitory effects on alkaline phosphatase and citrate decarboxylation in osteoblast-like cells [19], i.e., both may affect the citrate cycle, but have distinct effects on cAMP formation and phosphorylation patterns. The PyMS cell line is not only responsive to IGF I and insulin but also to vitamin D (increasing phosphate transport [20] and osteopontin expression), prostaglandin E$_2$ [21], PTH [22, this report], and to an increase in extracellular calcium [23]. The latter findings indicate that PyMS cells may be a valid bone cell model to study hormonal effects on glucose metabolism. PTH has been shown more than 50 years ago to affect glucose handling by bone cells [24, 25]. The major portion of glucose taken up by bone cells is metabolized to lactate and CO$_2$. PTH was found to increase lactate production and to decrease citrate decarboxylation by bone and osteoblastic cells [19, 24–27]. Moreover, it stimulated 2DG transport in UMR 106-01 rat osteogenic sarcoma cells after 1 h, and inhibited 2DG transport after 16 h [28]. PTH increased [^{14}C] glucose incorporation into glycogen in cultured rat calvaria cells but not in fibroblasts [6].

The authors have compared the potencies of IGF I, insulin, and PTH in stimulating 2DG uptake, the time courses of this stimulation, and their effects on glycogen synthesis in PyMS cells. It was found that GLUT1 and GLUT3 mRNA, and GLUT1 and GLUT3 protein, but not GLUT4 mRNA, were expressed in these osteoblastic cells, and that IGF I stimulates 2DG uptake more potently than insulin.

Materials and methods

Materials

Fetal calf serum (FCS), cell culture media, gentamicin, glutamine, and trypsin were purchased from Life Technologies (Grand Island, NY, USA). Bovine serum albumin was obtained from Serva and treated with charcoal; recombinant human (rh) IGF I was obtained from Ciba-Geigy, Basel; human insulin was gifted by Novo Nordisk. Bovine parathyroid hormone (PTH, 1-34) was purchased from Bachem, Torrence, CA.

Anti-glucose transporter 1 and 3 (GLUT1 and GLUT3) antibodies were purchased from several companies: Alpha Diagnostic (San Antonio, TX, USA), Biogenesis (Poole, England, UK), Santa Cruz Biotechnology (Santa Cruz, CA, USA), and FabGennix (Frisco, TX, USA) tested under a variety of conditions, the latter appeared to be the most useful in our hands.

Cell culture

Newborn rat calvaria cells and fibroblasts were prepared as previously described [6, 29]. Both cell types were grown to confluence on 10-cm-diameter plates in 5% FCS, detached with trypsin and replated as described below for PyMS cells. Secondary cultures were used. PyMS cells, a rat osteoblastic cell line, were gifted by Dr. A. Lichtler, University of Connecticut. The cells were passaged in Falcon tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 μg/ml), glutamine (2 mmol/l), and fetal calf serum (5%) and kept at 37°C in an atmosphere of 5% CO$_2$ in air. Cultures between passages 13 and 35 were used.

PyMS cells grown to confluence were detached from the dishes with 0.25% trypsin and replated in Falcon multiwell tissue culture plates (35 mm diameter) at a density of 2×10^5 cells per well in DMEM medium containing 5% FCS. Confluent monolayers formed 3 days after seeding. Except for the experiments shown in Table 1 (6-h tests carried out and stopped 3 days after plating), test incubations were stopped 4 days after seeding the cultures, as shown in the flow diagram (Fig. 1). 24 h before take down, cell cultures were rinsed with serum-free medium and then kept in serum-free Ham's F12 medium containing gentamicin (50 μg/ml), glutamine (2 mmol/l) and charcoal-treated bovine serum albumin (BSA, 1 g/l, from Serva). Aliquots of test agents were added directly to the media.

Glucose transport studies

After culture (see above) and different treatments, monolayers were washed at room temperature with 1 ml transport buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1.5 mM CaCl$_2$, 15 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, adjusted to pH 7.4 with Tris–HCl). Transport buffer containing choline chloride instead of NaCl was used for measuring Na-independent [1-^{14}C] DG uptake. Uptake studies were initiated by adding 1 ml of transport buffer containing 0.1 mM cold DG and 2-deoxy-D-[1-^{14}C] glucose (1 μCi/ml; 58 mCi/mmol, Amersham, UK). 10 min later (after 5, 10, and 20 min in the time course experiments shown in Fig. 3), the buffer was removed, and the dishes were quickly rinsed three times with

Table 1 Effects of IGF I and PTH on 2-deoxyglucose uptake and glycogen synthesis in rat fibroblasts, calvaria and PyMS cells

Cells-treatment (6 h)	2-Deoxyglucose uptake (pmol/mg protein × 10 min)	^{14}C-glucose → glycogen (cpm/mg protein × 6 h)
Fibroblasts		
Control	2,157 ± 113	11,835 ± 301
IGF I, 10 nmol/l	3,110 ± 217*	26,345 ± 1,276*
PTH, 10 nmol/l	2,052 ± 161	12,774 ± 816
Calvaria cells		
Control	1,368 ± 62	18,345 ± 2,065
IGF I, 10 nmol/l	1,965 ± 92*	41,523 ± 5,276*
PTH, 10 nmol/l	1,817 ± 156*	29,200 ± 2,716*
PyMS cells		
Control	3,840 ± 186	13,442 ± 122
IGF I, 1 nmol/l	6,348 ± 172*	37,424 ± 1,792*
PTH, 10 nmol/l	4,457 ± 188*	16,041 ± 681

Cells were grown for 3 days in FCS-containing medium, rinsed and incubated for 6 h in serum-free medium with IGF I and PTH. Cells were rinsed and switched to room temperature for measurement of deoxyglucose uptake or they were pulsed during the 6-h test period at 37°C with 1 μCi D-[U-^{14}C]-glucose. Incorporation into glycogen was measured as described in Materials and methods. Data from 3 separate experiments (carried out in triplicate) are given
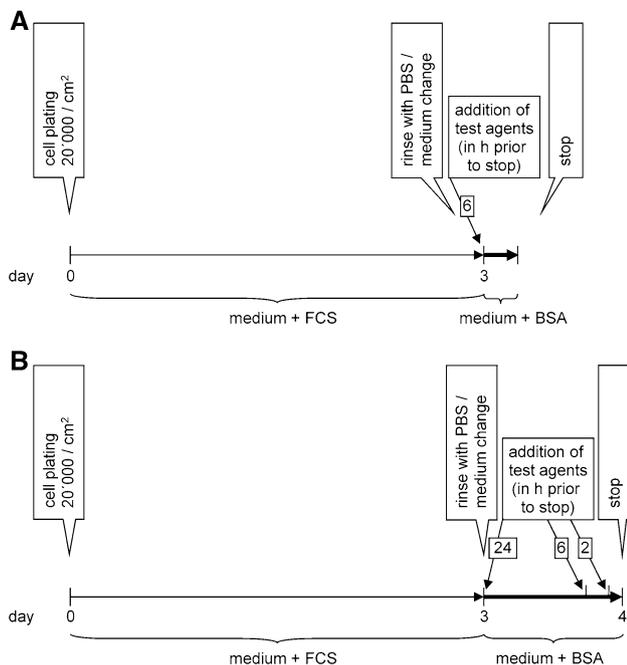
* $P < 0.05$ for comparison of hormone-treated vs. control

Fig. 1 Flow diagram of treatment protocols. The experiments with three distinct cell types summarized in Table 1 were performed according to the three day protocol (**A**) while all other experiments were performed only with PyMS cells, with a 4-day protocol (**B**)

1 ml of ice-cold transport buffer. The cells were then solubilized with 1 ml of 2% sodium dodecyl sulfate (SDS), and the radioactivity of a 0.5 ml aliquot was counted in a liquid scintillation counter.

[U-^{14}C] glucose incorporation into glycogen

After 3 days in culture (see above) in 5% FCS containing DMEM, cell layers were washed with serum-free Ham's F12 medium containing 1 g/l BSA and exposed for 6 h to IGF I or PTH and 1 μCi/dish of D-[U-^{14}C] glucose (Amersham, 270 mCi/mmol) in serum-free medium (Table 1). Alternatively, cells cultured for 3 days as above were washed and kept in serum-free medium for 24 h, and PTH, IGF I and D-[U-^{14}C] glucose were added for the times indicated in Fig. 2 and Table 3. To measure net glycogen synthesis from [^{14}C] glucose, cells were washed with ice-cold phosphate-buffered saline (PBS), and glycogen was precipitated and ^{14}C counted in a β-counter as described [6, 29].

Determination of protein content

In order to correct for interassay differences in protein content, identically treated dishes were used for protein analysis. After 3 days (Table 1) or 4 days of culture (24 h in serum-free medium), cells from parallel dishes were lysed into 1 ml of 0.1% Triton X-100 for the determination of protein content by a modification of the Lowry procedure [20] or by the BCA method [22]; alkaline phosphatase activity was also measured [20].

[^{3}H] thymidine incorporation into DNA

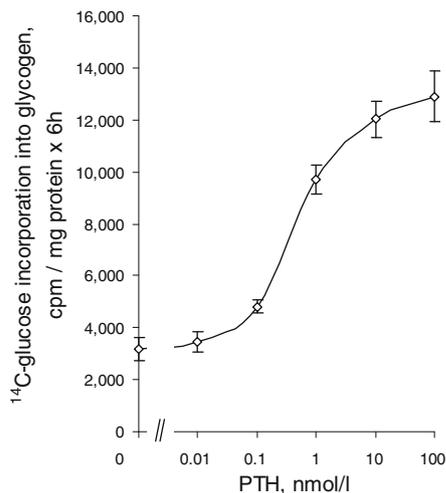Cells were plated and grown for 3 days in 5% FCS-containing medium, then washed and exposed to serum-free

Fig. 2 Effect of parathyroid hormone on D-[U-^{14}C] glucose incorporation into glycogen in rat osteoblastic PyMS cells. Cells were grown for 3 days in FCS-containing medium, rinsed with serum-free medium and then exposed to serum-free medium for 24 h. PTH and D-[U-^{14}C] glucose were added for the last 6 h. Glycogen synthesis was determined as described in Materials and methods (3 experiments: 2 in triplicate and 1 in quadruplicate)

test medium (containing 1 g/l BSA and hormones as indicated) for 18 h, and subsequently pulsed with [methyl-^{3}H] thymidine (Amersham, 80 Ci/mmol; 1 µCi/dish) for 3 additional h. After rinsing with cold PBS, DNA was precipitated and washed three times with 10% trichloroacetic acid. The precipitate was dissolved in 1 N KOH, and incorporated radioactivity was measured in a liquid scintillation counter [20–23].

Total RNA isolation and Northern blot analysis for GLUT1, GLUT3 and GLUT4 mRNA

Cells were plated at a density of 10^6 cells/10 cm diameter dish (Falcon) in medium containing 5% FCS and grown for 3 days. Confluent monolayers were kept in serum-free media for 24 h with hormones added as indicated. The culture medium was sucked off and the cell layer was washed 3 times with ice-cold PBS and scraped into ice-cold PBS. Cells were collected and then lysed in 4 M guanidine isothiocyanate containing 5 mmol/l sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, and 0.5% sarcosine. Total RNA from rat osteoblast-like PyMS cells, newborn rat calvarial bone, rat heart and rat brain was obtained by high-speed sedimentation through a cesium chloride cushion, and concentrations were determined spectrophotometrically; RNA was stored at −80°C until assayed [30].

Denatured total RNA (20 µg) from tissues and cells was electrophoresed on a 1% agarose gel (stained with ethidium bromide) containing 2 M formaldehyde, transferred onto a

nylon membrane (Hybond-XL, Amersham, UK) by capillary blotting, and fixed by UV cross-linking according to standard procedures [31]. Prehybridization and hybridization were performed as described earlier [32]. cDNA probes of GLUT1 and GLUT3 were constructed using RNA from rat tissues and RT-PCR, with the following set of forward and reverse primers: 1A/1B (1A:5′-GCGGGA GAAGAAGGTC-3′; 1B: 5′-AGGAGAGTGGCTGATAA AAA-3′) for GLUT1 (GenBank accession no. M13979) and U/L (U: 5′-TGGAAGAGCGGTTGGA-3′; L: 5′-GGC AGCGAAGATGATAAAAA-3′) for GLUT3 (GenBank accession no. U17978), corresponding to products of 1010 bp (GLUT1) and 1155 bp (GLUT3) on 1–1.5% agarose gels. The cDNA probe for GLUT4 has been previously described [33]. The cDNA probes were labeled by random primer extension using a commercial kit (Roche Diagnostics) and [α-^{32}P] deoxy-CTP (3,000 Ci/mmol; Amersham) to specific activities of 2–4×10^9 cpm/µg DNA. After 14–16 h of incubation at 42°C, the membranes were washed twice for 15 min at room temperature in $2\times$ SSPE–0.1% SDS and then at 49–52°C twice for 10 min in $0.1\times$ SSPE–0.1% SDS. Membranes were then exposed at −80°C to a BioMax film (Kodak, Rochester, NY, USA) in cassettes equipped with intensifying screens to visualize ^{32}P-labelled cDNA-mRNA hybrids. mRNA levels were quantitated by scanning densitometry using a Bio-Rad (Hercules, CA, USA) video densitometer. Variations of gel loading were corrected against 18S ribosomal RNA levels.

Total membrane preparation and Western immunoblot analysis for GLUT1 and GLUT3

Cells were plated at a density of 3×10^6 cells/15 cm diameter dish (TPP) in medium containing 5% FCS and grown for 3 days. Confluent monolayers were kept in serum-free media for 24 h with hormones added as indicated. Medium was removed, and the cell layers were rinsed twice with ice-cold PBS; cells were scraped into 4 ml PBS and collected by centrifugation in a tube and the pellet dissolved in 5 ml homogenization buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 255 mM sucrose, and containing 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 200 µM PMSF as proteinase inhibitors), then glass Dounce-homogenized by 15 moves/strokes, transferred back to tubes and centrifuged for 10 min at 700 g and 4°C; the resulting supernatant was then centrifuged at 19,500 g for 75 min, and the pellet redissolved in 0.8 ml. An aliquot was used for determination of the protein concentration by the BCA method, and the tubes were frozen at −80°C. Thirty µg samples were mixed with loading buffer, heated to 56°C and applied per lane on SDS–PAGE (10%), and transferred to a 0.2-µm nitrocellulose membrane (Trans-Blot, BioRad 162-0147) for Western analysis. Equal

loading was confirmed by Ponceau S staining. Filters were incubated overnight at 4°C in (affinity-purified) primary antibody (rat Glut1 (FabGennix Inc; Glut-101 AP) and rat Glut3 (FabGennix Inc; Glut-301 AP)), both at 1:4,000, were then washed three times with TBS-T (Tris-buffered saline-Tween-20) and incubated for 1 h in secondary antibody (goat anti-rabbit HRP, BioRad 170-6515, at 1:3,000) at room temperature. After washing, proteins were detected using the enhanced chemiluminescence (ECL) detection reagents (Amersham 1059250) applied as recommended by the manufacturer (Amersham).

Statistical analysis

Results were obtained from 3 to 8 independent experiments. Data are expressed as means ± SEM. Statistical significance was assessed by one-way ANOVA with repeated measures and Bonferroni's multiple comparison test correction using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). A P value <0.05 was considered to be statistically significant. Lineweaver–Burk trend lines were plotted with double inverse value axes for uptake (y-axis) as function of 2DG concentrations (x-axis) and used for estimation of K_m and v_{max}.

Results

Characterization of PyMS cell responses to PTH and IGF I

Preliminary experience with studies on glucose uptake in PyMS cells and previous data on glycogen synthesis in rat calvaria cells [6] had indicated that the treatment protocol (serum withdrawal, medium change) had a marked influence on basal activities (glucose uptake and glycogen synthesis) and hormone responsiveness; treatment protocols are, therefore, outlined in Fig. 1. PTH-stimulated [^{14}C] glucose incorporation into glycogen (Fig. 2) in a dose-dependent manner, significantly at 0.1 nmol/l and maximally at 10 nmol/l; the latter concentration was used for the experiments shown in Tables 1, 2, 3. Vitamin D had no effect (not shown) when tested under the same conditions. The data summarized in Table 1 show 2DG uptake and [^{14}C] glucose incorporation into glycogen of PyMS cells, as compared to rat fibroblasts and rat calvaria cells after 6-h exposure to IGF I or PTH. IGF I exposure increased both subsequent 2DG uptake and glycogen synthesis during the test incubation in all cell types (a lower concentration of IGF I was used in the case of PyMS cells since this cell line is particularly sensitive to IGF I) [23]. PTH stimulated 2DG uptake and [^{14}C] glucose incorporation into glycogen

only in calvaria and PyMS cells but not in fibroblasts, i.e., only in osteoblastic cells (Table 1). Only PyMS cells were used for the following studies. Comparison of Fig. 2 and Table 3 with Table 1 shows that PyMS cells incorporated more glucose into glycogen when tested shortly after serum deprivation than when kept under serum-free conditions overnight before the 6-h test. On the other hand, PTH, was relatively more effective in stimulating glycogen synthesis under the latter conditions (serum-deprived for 18 h, Fig. 2, Table 3) than immediately after serum deprivation (Table 1).

Characterization of 2-deoxyglucose transport

2DG uptake was the same in sodium-containing and sodium-free buffer, i.e., Na-independent. While previous exposure to IGF I increased subsequent 2DG uptake by the cells, replacing sodium by choline in the transport buffer had no impact on 2DG uptake which increased linearly with incubation time in control- and IGF I-treated cells for at least up to 20 min (Fig. 3). Kinetic parameters of the glucose transport system were obtained from Lineweaver–Burk plots for which a series of experiments with 2DG concentrations ranging from 0.02 to 2 mmol/l were carried out. The apparent affinity constant (K_M) of the glucose transport was calculated from curves, and was similar in PyMS (2.2 mmol/l) and calvaria cells (1.6 mmol/l) (mean of 2 exp.); IGF I did not affect the K_M of the transport system but increased the maximal velocity (v_{max}) of the glucose transport system when assessed in 6-h tests (as in Tables 1 and 2).

Stimulation of 2DG transport by IGF I, insulin, and PTH

IGF I was similarly effective in stimulating 2DG uptake shortly following serum deprivation (Table 1) and after being kept (for 18 h without hormones) in serum-free medium (Tables 2 and 3). The stimulatory effect of 1 nmol/l IGF I on 2DG uptake was significant in cells which had been exposed to IGF I for 30 min to 24 h (Table 2). The (preexposure) effects of both IGF I and insulin were more pronounced after 2 and 6 h than after 30 min, and their effects were compared by dose–response curves. IGF I was effective at lower concentrations than insulin (Fig. 4). Despite its pronounced effect on glycogen synthesis (Fig. 2, Table 3), PTH was much less potent than IGF I in stimulating 2DG uptake (Tables 1, 2, 3), a consistent finding with the treatment protocol used in the current experiments. Similar to previous findings in calvaria cells, PTH had no immediate (within the first 2 h) stimulatory effect on 2DG uptake in PyMS cells; an effect of PTH was observed only after a 6-h exposure

Table 2 Effects of IGF I and PTH on 2DG uptake (A) and protein content and alkaline phosphatase activity in PyMS cells: time course

Treatment	2-Deoxyglucose uptake (pmol/mg protein × 10 min)			
Time:	30 min	2 h	6 h	24 h
A				
Control	704 ± 24	752 ± 46	652 ± 37	679 ± 57
IGF I, 1 nmol/l	914 ± 35*	1,295 ± 35*	1,228 ± 107*	919 ± 54*
PTH, 10 nmol/l	603 ± 42*	819 ± 57	760 ± 33	811 ± 63*

Treatment (24 h)	Protein content (mg/dish)	Alkaline phosphatase activity (μmol/h x mg protein)
B		
Control	0.105 ± 0.003	3.36 ± 0.56
IGF I, 1 nmol/l	0.125 ± 0.007*	3.23 ± 0.45
PTH, 10 nmol/l	0.100 ± 0.002	3.68 ± 0.67

Cells were grown for 3 days in FCS-containing medium, rinsed with serum-free medium and kept in serum-free medium for the last 24 h. IGF I (final concentration 1 nmol/l) and PTH (final concentration 10 nmol/l) were added for the indicated times before assessment of 2DG uptake (A) and determination of protein content and alkaline phosphatase activity (B) per dish, as described in Materials and methods. Data from 3 separate experiments (carried out in triplicate) are shown

* $P < 0.05$ for comparison of hormone-treated vs. control

Table 3 Effects of IGF I and PTH on 2DG uptake and glycogen synthesis in PyMS cells

Treatment (6 h)	2-Deoxyglucose uptake (pmol/mg protein × 10 min)	^{14}C-glucose → glycogen (cpm/mg protein × 6 h)
Control	773 ± 88	280 ± 40
PTH, 10 nmol/l	930 ± 90	1,044 ± 61*
IGF I, 1 nmol/l	1,781 ± 164*	870 ± 40*
IGF I + PTH	1,588 ± 178*	2,203 ± 157**

Cells were grown for 3 days in FCS-containing medium and kept in serum-free medium for the following (last) 24 h. IGF I (final concentration 1 nmol/l) or PTH (final concentration 10 nmol/l) were added 6 h before assessment of 2DG uptake or of glycogen synthesis from D-[U-14C] glucose. Data from 3 separate experiments (carried out in triplicate) are shown

* $P < 0.05$ for comparison of hormone-treated vs. control

** $P < 0.05$ for comparison of IGF I- + PTH-treated vs. IGF I- or PTH-treated alone

(Tables 1, 2, 3). Dose–response experiments revealed a consistent and significant stimulatory effect of PTH on 2DG uptake in 6-h tests (Fig. 4, treated/control $P < 0.01$ for all PTH concentrations versus control; conditions as in Table 2, where the 6-h point shows only a 1.2-fold stimulation), even at low doses (half-maximal stimulation at ∼10 pmol/l), but the effect was minor, also at higher doses of PTH (Table 1, Fig. 4), and much less impressive than its effect on glycogen synthesis (Table 3, Fig. 2). PTH and IGF I were also tested in combination. Their effects on glycogen synthesis, but not on 2DG uptake, were additive (Table 3).

Effects of PTH, IGF I, and insulin on DNA synthesis

PTH caused a dose-dependent inhibition of [3H]thymidine incorporation into DNA, significant at 1 nmol/l and above, whereas vitamin D stimulated DNA synthesis in a dose-dependent manner (significantly at 10 pmol/l and above) as previously reported [20, 22]. Both IGF I and insulin increased [3H]thymidine incorporation into DNA. IGF I was effective at lower doses than insulin (Fig. 4).

GLUT1 and GLUT3 mRNA in rat bone and osteoblastic cells

Total RNA was analyzed for GLUT-specific transcripts by Northern blot hybridization. RNA was prepared from PyMS cells and from newborn rat bone; for comparison, RNA was prepared from newborn rat liver, brain, and heart which served as positive controls for GLUT1, -3 and -4. RNA from parietal bone (and from calvaria osteoblasts, not shown) and from PyMS cells were positive for GLUT1 (mRNA size ∼3.0 kb) but gave a stronger signal with the GLUT3 cDNA probe (mRNA size ∼4.0 kb) (Fig. 5, left), and no signal at all with the GLUT4 probe (not shown, heart and cultured heart muscle cells were positive for GLUT4 [33]).
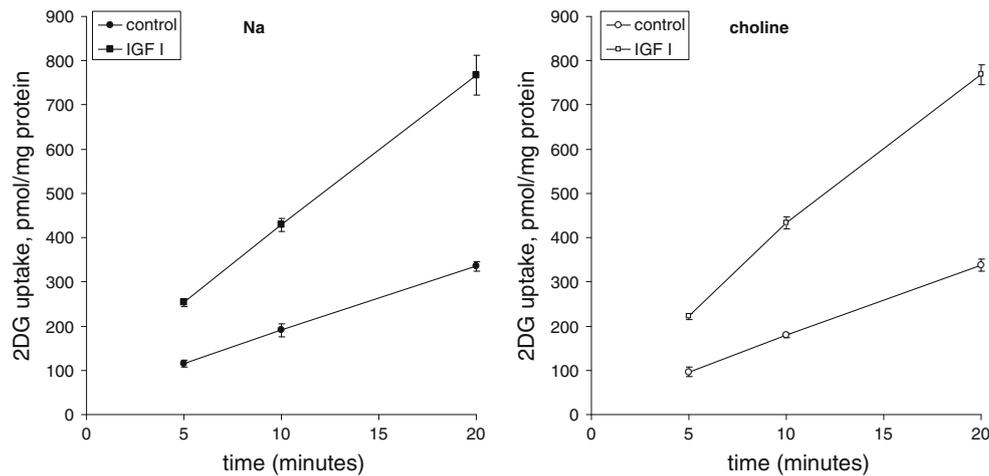
Fig. 3 Effects of different incubation times (for the uptake assay), sodium-containing and sodium-free (choline-containing) transport buffer on 2DG uptake in osteoblastic cells exposed to hormone-free control medium or to IGF I for the last 6 h. Cells were grown for 3 days in FCS-containing medium, rinsed with serum-free medium and then exposed to serum-free medium for 24 h. IGF I (final concentration 1 nmol/l) was added for the last 6 h before assessment of 2DG uptake which was measured over different times in sodium-containing (*left*) and in sodium-free (choline-containing, *right*) buffer, as described in Materials and methods. Data from 2 separate experiments (carried out in triplicate) are shown

GLUT1 and GLUT3 in rat osteoblastic cells

Total membranes were analyzed for GLUT1, and GLUT3 protein by Western blot analysis (the authors had tried a variety of commercially available antibodies, and finally found the ones given in the Materials and methods section to be the most suitable); for comparison, total membranes were prepared from rat brain which served as positive control for GLUT1 and GLUT3 (Fig. 5, right). The strongest signals with GLUT1 and GLUT3 antibodies were found in membranes prepared from brain (at ~ 44 and at ~ 45–48 kDa), and bands were also detected in membranes prepared from PyMS cells, at apparent molecular masses of ~ 48–50 kDa for GLUT1 and of ~ 45–49 kDa for GLUT3. The small observed differences in the MW could be attributed to tissue-specific differences (between brain and bone cells) in the glycosylation patterns of the proteins [34].

Discussion

Insulin deficiency causes severe metabolic disturbances and diabetic bone disease [35, 36]. The latter may be due not only to indirect effects but also to insulin deficiency per se. Insulin increases collagen synthesis [37] and bone formation [38]. Insulin resistance and hyperinsulinemia are associated with increased bone mineral density; this positive association, however, has been found to be diminished after adjustment for body mass index [39]. Insulin receptors are present in UMR-106 rat osteoblastic osteosarcoma cells [4, 5, 40] and in rat bone [41] and therefore, it has been suggested that insulin may stimulate glucose uptake also in bone [42]. However, the reported insulin dose–response curves for 2DG uptake are distinct from those observed in fat cells, where insulin stimulates GLUT4-mediated glucose transport. Thus, previous studies reported an ED$_{50}$ of 9×10^{-10} mol/l [5] or of $\sim 10^{-8}$ mol/l [41] for insulin-stimulated glucose uptake in osteosarcoma cells (as compared to $\sim 5 \times 10^{-11}$ mol/l in adipocytes). Few studies have tested the effects of insulin on glucose transport in isolated non-malignant osteoblast-like cells, but IGF I has not been tested. The authors had previously found that IGF I was effective at lower concentrations than insulin in stimulating glycogen synthesis from [^{14}C] glucose in PyMS and primary rat calvaria cells [1, 6, 29]. Our current studies show that IGF I stimulates glucose transport in PyMS cells and that it is effective at much lower concentrations than insulin, not only regarding glycogen and DNA synthesis but also with regard to enhancing glucose uptake (Fig. 4): 10–20 times higher concentrations of insulin than of IGF I are required for significant stimulation of DNA synthesis and 2DG uptake; even 50–100 times higher concentrations of insulin than of IGF I are needed for half maximal stimulation of DNA synthesis and 2DG uptake. Since insulin binds type 1 IGF receptors with a K_d of $\sim 10^{-8}$ mol/l, whereas it binds to its own receptors with a much lower K_d ($\sim 10^{-10}$ mol/l), it is likely that insulin signals through type 1 IGF receptors to stimulate glucose uptake in osteoblastic, at least in PyMS cells. These cells are particularly sensitive to stimulation by IGF I since they have a high number of type 1 IGF receptors and since they do not produce significant amounts of IGF I [23]. According to our findings, the "insulin-sensitive" glucose
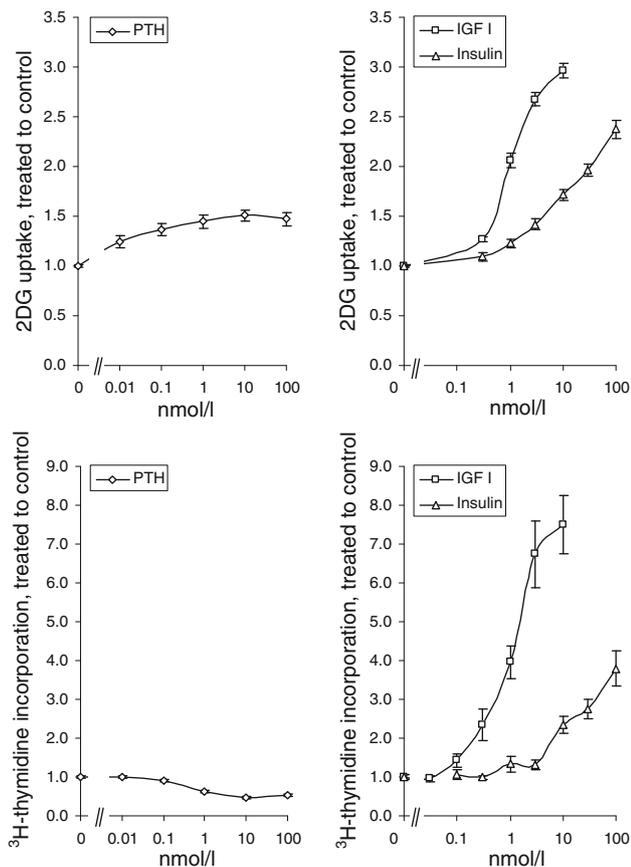
Fig. 4 Effects of PTH, IGF I, and insulin on 2DG uptake and DNA synthesis in rat osteoblastic cells. 2-deoxyglucose uptake (*upper panels*) and [^{3}H] thymidine incorporation into DNA (*lower panels*) in PyMS cells exposed to PTH (*left panels*) or to IGF I and insulin (*right panels*). Cells were grown for 3 days in FCS-containing medium, rinsed with serum-free medium, and exposed for 24 h to serum-free medium. PTH was added for the last 6 h; IGF I or insulin were added for the last 2 h. 2DG uptake was then measured at room temperature as described in Materials and methods (2DG uptake PTH (6 h): 8 experiments in triplicate; 2DG uptake IGF I/insulin (2 h): 6 experiments in triplicate), or exposed to serum-free test medium containing PTH, IGF I or insulin for 18 h and pulsed with 1 µCi [methyl-^{3}H] thymidine for additional 3 h (thymidine incorporation PTH: 5 experiments in triplicate; thymidine incorporation IGF I/insulin: 3 experiments in triplicate)

Fig. 5 Northern blot (*left panel*) and Western immunoblot (*right panel*) analysis for GLUT1 and GLUT3. Northern blot analysis for GLUT1 and GLUT3: Total RNA was prepared from rat brain and bone, and from PyMS cells (treated for the last 6 h with control or IGF I-containing test medium) and subjected to Northern. Twenty µg RNA was loaded per lane. *Bars* indicate the position of ribosomal RNA. GLUT1 and GLUT3 mRNA are particularly abundant in brain but also detectable in bone and in PyMS cells. Western immunoblot analysis for GLUT1 and GLUT3: Total membranes were prepared form rat brain and from PyMS cells (treated for the last 6 h with control or IGF I-containing test medium) and subjected to Western. Thirty µg protein was loaded per lane; bars indicate the position of marker proteins. GLUT proteins were less abundant in TM from PyMS cells than in TM from brain. GLUT1 is detected at an apparent molecular weight of about 50 kDa, GLUT3 at an apparent molecular weight of about 45–47 kDa

uptake system in osteoblastic cells may be rather an IGF I-sensitive glucose uptake system, and type 1 IGF receptors are likely to play a major role in mediating 2DG uptake by rat PyMS cells. It may be argued that PyMS cells represent a "preosteoblastic" cell line [20] and that the role of insulin may be more prominent in osteoblasts. Insulin was more effective with regard to stimulation of collagen synthesis in rat osteosarcoma UMR cell lines [40] than in "non-transformed" preosteoblastic UMR cell lines [43]; however, even in the former cells, 1 nmol/l of insulin was required to exert a significant effect. The authors have directly compared the potencies of insulin and IGF I and found that
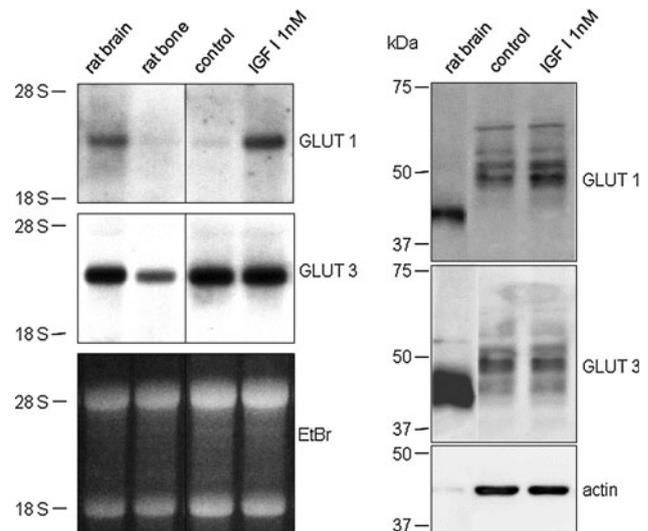
IGF I was effective at lower doses than insulin, in primary rat osteoblasts [29] and in PyMS cells, both regarding 2DG uptake (Fig. 4) and glycogen synthesis [1]. Glucose uptake by rat osteoblastic cells is a Na-independent process. By contrast, phosphate and alanine uptake in the same cell line are Na-dependent processes [20, 21, 23]. The apparent K_M for 2DG uptake in both primary rat calvaria and PyMS cells was ~ 1.6 and ~ 2.2 mmol/l, respectively, i.e., close to the published K_M of the high affinity glucose transporters, particularly GLUT3 [44]. For the latter, the K_M for 2DG was found to be lowest, i.e., at 1–2 mmol/l when tested in Xenopus oocyte expression system [44]. A similar K_M value for the 2DG transport system has been reported for UMR osteosarcoma cells (1.9 mmol/l) [4]. K_M values for the high affinity GLUTs vary according to the literature, being lowest for GLUT3 (1.4 mmol/l) and somewhat higher for GLUT1 (6.9 mmol/l) [16]. Our analysis with osteoblastic cells suggests a dominant role of high affinity glucose transporters but cannot give an accurate figure since it is likely that several GLUTs account for overall glucose uptake by the cells. Thomas and co-workers [28] found a stimulatory effect of PTH on 2DG uptake in UMR cells at 1 h (and

inhibition at 16 h). The authors found stimulatory effects of PTH on 2DG uptake after 6 and 24 h in the PyMS cells (Table 2), as well as an identical time course in primary calvaria cells. DNA synthesis is clearly decreased by PTH, as a consequence, glucose will not be increasingly used for growth in response to PTH stimulation. Therefore, unlike the stimulatory effect of IGF I, the effect of PTH on 2DG uptake and on glycogen synthesis may be relatively specific as it is restricted to osteoblastic cells (Table 1) and as it is not readily explained in the context of overall stimulation of cell proliferation.

Previous studies [18] indicated that rat osteogenic osteosarcoma cells express both GLUT1 and GLUT3. They found GLUT3 mRNA and protein in several UMR rat osteoblastic cell lines and GLUT3 mRNA in rat bone, suggesting that GLUT3 is expressed in osteoblastic cells. Our observations with a different (non-tumorigenic) cell line support this finding. The hybridization signal of GLUT3 transcripts is also present in rat bone and in rat calvaria cells (not shown). In agreement with findings in mammalian neuroblasts and trophoblasts, GLUT1, the isoform that is ubiquitously found in all proliferating cells, is replaced by GLUT3 when PyMS cells decrease their growth rate and begin differentiating to attain their mature functional form. Glucose uptake was the highest in serum-primed cells, i.e., when the cells were rapidly growing, and GLUT1 mRNA expression was relatively high; it is also known that GLUT1 is expressed at high levels by most tumor cells and may, therefore, serve to cover the particular requirement for rapid growth (allowing tumor visualization in vivo by 2-deoxy-2-[^{18}F]fluoro-D-glucose positron emission tomography [45]). GLUT1 mRNA was downregulated when PyMS cells were kept for a prolonged period of time in serum-free medium. Results from our laboratory (in preparation) have shown that under these conditions, (IGF I responsiveness is maintained and PTH responsiveness markedly increased, with a 3.4 ± 0.2-fold stimulation of 2DG uptake) overall (presumably "growth-related") 2DG uptake decreased, as also indicated by comparing 2DG uptake rates in Tables 1, 2, and 3, illustrating the effect of short-term serum deprivation. To avoid different degrees of residual serum effects, the time course experiments presented in Table 2 share a common culture period including the same time in serum-free medium. In contrast to tissues with insulin-regulated glucose uptake (adipose tissue, striated muscle), GLUT4 mRNA could not be detected in PyMS cells, primary calvaria-derived osteoblasts, or parietal bone. It cannot be ignored that additional members of the SLC2 family (GLUT6 to GLUT14) may be involved in the glucose uptake in PyMS cells, since several of these newer members of GLUTs are *bona fide* glucose transporters [46]. Thus far, it has not been possible to assign glucose transport in osteoblasts to a specific GLUT isoform,

but the findings of this study suggest that GLUT3 and GLUT1 remain the best candidates. GLUT3 and GLUT1 could be identified in PyMS cells and high affinity glucose transport would be consistent with GLUT3 playing a central role. IGF I rapidly increases 2DG uptake, well before marked changes in GLUT protein synthesis and cellular content occur, consistent with translocation of GLUTs from an intracellular membrane fraction to the plasma membrane. GLUT1 and GLUT4 facilitative glucose transporters translocate in response to IGF I or insulin stimulation [47], and GLUT3 is also redistributed in response to IGF I or insulin after short-term exposure, e.g., in L6 rat myotubes [48]. However, there is currently no proof to establish that the observed increased 2DG uptake by osteoblastic cells in response to acute IGF I stimulation can be attributed to a translocation of GLUT3 (or GLUT1)-containing vesicles from an intracytoplasmic pool to the plasma membrane. According to our findings, GLUT4 is absent in osteoblastic cells, and insulin is unlikely to play a physiological role in stimulating glucose transport in bone tissue.

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