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Photolabeling of erythrocyte and adipocyte hexose transporters using a benzophenone derivative of bis(D-mannose)

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The benzophenone derivative of 1,3-bis(D-mannos-4-yloxy)-2-propylamine (BB-BMPA) has been tested as an exofacial photoaffinity label for the sugar transport systems of human erythrocytes and rat adipocytes. The half-maximal inhibition constants for the reagent are 971 μM in erythrocytes and 536 μM in basal and 254 μM in insulin-treated adipocytes. The photolabelling of erythrocyte membranes is very specific for the 50 kDa transporter peptide and is completely displaced by D-glucose. The exofacial photoaffinity labelling of adipocytes also shows labelling of a 50 kDa transporter peptide, which is displaced by cytochalasin B, but extensive nonspecific labelling of a 75 kDa plasma membrane peptide occurs. The transporter is labelled in insulin-treated cells but not in basal cells which indicates that this in situ labelling technique selectively reveals only those transporters that visit and are active in the plasma membrane during the labelling period. This also indicates that in basal cells transporters do not turn over rapidly. Subcellular redistribution of transporters after the labelling period has been studied. Following incubation and washing at 37°C in the presence of insulin, 30% of the transporters photolabelled at the plasma membrane are internalised and are found in the light microsome fraction of the cell. The proportion of transporter that is observed to be internalised is much greater than can be accounted for by a contamination of the light microsome fraction by plasma membrane. The labelled 50 kDa transporter peptide in the light microsomes is enriched when compared with the carry-over of the 75 kDa nonspecifically labelled plasma membrane peptide. Thus we have obtained direct evidence for transporter translocation.

Introduction

We have previously shown that the aryl-azide derivative of bis(D-mannose), 2-*N*-(4-azido-salicoyl)-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ASA-BMPA), can be successfully used for photoaffinity labelling the hexose transporter of human erythrocytes. We have used this compound to probe the location of the exofacial hexose binding site in the transporter amino acid sequence [1] and in studying conformational changes in the transporter [2]. The problem of exofacially photolabelling the insulin-dependent adipocyte transporter is much more difficult for the following reasons: (1) The transporter is much less abundant than in the erythrocyte [6]. (2) The cell density (cells per ml) that one can obtain with adipocyte suspensions is very small because of the large size of the adipocyte. (3) Membrane isolation and recovery of the photolabelled transporter is much more demanding and difficult than for the erythrocyte syster.

Abbreviations: BMPA, 1,3-bis(D-mannos-4-yloxy)-2-propylamine; BGPA, 1,3-bis(D-glucos-4-yloxy)-2-propylamine; ASA, 4-azido-salicoyl; BB-BMPA, 2-*N*-(4-benzoyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine.

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(4) Compounds which have good affinity for the erythrocyte system can have quite poor affinity for the adipocyte system so that the erythrocyte transporter cannot be used to rapidly screen compounds which would be potentially useful in adipocytes.

We have now tested the photolabelling of adipocytes by more than ten aryl-azide derivatives of bis(hexoses) but the results have been poor. The unsuitability of aryl azide derivatives for the difficult problem of adipocyte transporter photolabelling has now led us to an investigation of the suitability of photoreactive ketones. The present paper reports our findings using the highly reactive benzophenone derivative 2-*N*-(4-benzoyl)benzoyl-BMPA. Photoactivation of benzophenone-substituted compounds is thought to proceed through an entirely different mechanism to that involved in azide activation. The mechanism involves hydrogen abstraction from the protein which produces two radicals, one on the protein and one on the ligand. The radicals then combine in a reaction that can be highly effective and selective. This improvement in photochemical selectivity combined with a new method for synthesising large quantities of tritiated derivative has given some improvement in erythrocyte transporter labelling but more importantly has now enabled exofacial photolabelling of the adipocyte system to be realised. Thus the cell physiology of insulin activation and recruitment of transporters can be studied following the in situ labelling of a discrete plasma membrane pool of transporters.

Materials and Methods

Materials

PMSF, pepstatin, cytochalasin B and albumin (fraction 5) were from Sigma. Phloretin was from K and K laboratories. Collagenase was from Worthington. Insulin was from Novo. Electrophoresis chemicals were from B.D.H.

Preparation of [2-^{3}H]BMPA

Since large quantities of this material were required a new synthetic route was used. This involved synthesis of a bis sugar with an oxime at the 2-position of the propane bridge. The strategy then involved reduction of this by tritium using Raney nickel as catalyst. 500 mg 1,3-bis(1,6-anhydro-4-deoxy-2,3-*O*-isopropylidene-β-D-mannopyranose-4-yloxy)-2-propanone [14] plus 500 mg hydroxylamine in 8 ml pyridine and 4 ml methanol were heated at 70°C for 3 h. The product was extracted into 100 ml of chloroform. This was washed with 2 × 50 ml water. The water layer was re-extracted with 50 ml chloroform. The combined chloroform extract was purified on a 10 ml silica gel column using ethyl acetate/light petroleum ether (4:5, v/v) as eluent. Yield 450 mg. The purified product 1,3-bis(1,6-anhydro-4-deoxy-2,3-*O*-isopropylidene-β-D-mannopyranose-4-yloxy)-2-propanoxime was crystallised from ethanol. Mass spectrum showed $m/z = 474$ (c.i.) $(M+1)$ and $m/z = 458$ (e.i.) $(M - Me)$. 270 MHz ^{1}H-NMR. (C^2HCl_3) showed δ 8.60 (s, 1H, NH), 5.35 (d, 2H, $J_{1,2}$ 2.7 Hz, H-1), 4.66 (dd, 2H, $J_{5,6}$ 6.0 Hz, H-5), 4.58, 4.34 (d,s 2H each, CH_2-C-CH_2), 4.23 (m, 2H, H-3), 4.08 (dd, 2H, $J_{2,3}$ 6.0 Hz, H-2), 3.94 (dd, 2H, $J_{6,6}$, 6.5 Hz, H-6), 3.73, 3.68 (2s, each 2H, H-4), 1.53, 1.34 (2s, each 6H, $2CMe_2$).

15 mg of the oxime were reduced with 5 Ci of tritium gas by Amersham International (TR 3) using Raney nickel as catalyst and methanol as solvent. This gave 100 mCi of the tritiated amine. 5 mg of carrier amine were added. This was purified on silica gel using ethyl acetate/light petroleum ether followed by ethyl acetate/methanol as described previously [14,37] and the product in 1 M HCl was then heated at 100°C for 90 min. This solution was then neutralised with Amberlite IRA-93 (HO^-) resin and stored at 1 mCi/ml in ethanol/water (1:1, v/v) at −20°C, yield 73 mCi. Greater than 85% pure on paper chromatography in butanol/ethanol/water (49:11:19, v/v).

Preparation of (4-benzoyl)benzoyl-[2-^{3}H]BMPA

16.3 mg 4-benzoylbenzoate plus 19 mg hydroxybenzotriazole were dissolved in 400 μl dimethylethyleneglycol in a microfuge tube. 29.6 mg dicyclohexylcarbodiimide in 200 μl dimethylethylene glycol were then added and the reaction was left at 4°C overnight. The tube was centrifuged briefly to pellet the dicyclohexylurea and 200 μl of the clear supernatant was added to a solution containing 1 mCi of BMPA in 50 μl methanol and 3 μl of *N*-methylmorpholine as base. After 2 h at

room temperature TLC analysis in ethyl acetate/methanol/water (9:2:1, v/v) showed greater than 80% conversion to give BB-[2-^{3}H]BMPA. The product was separated from minor radiolabelled impurities by paper-chromatography in butanol/ethanol/water (49:1:19, v/v). Complete removal of hydroxybenzotriazole was not achieved since this compound has only a slightly higher mobility than BB-BMPA. For the preparation of the small quantity of BB-BMPA required as chromatographic standard and for transporter affinity determinations direct coupling using ethyl chloroformate was studied. 15 mg 4-benzoyl-benzoate and 5 μl pyridine and 3 μl ethylchloroformate in 300 μl dimethylethylene glycol were mixed in a microfuge tube. After 10 min the tube was centrifuged and the clear supernatant was added to 4 mg of BMPA in 300 μl methanol plus 3 μl of *N*-methylmorpholine. After 2 h the mixture was concentrated and applied to a 5 ml silica gel column which was equilibrated with ethyl acetate/methanol (3:1, v/v) and then eluted with ethyl acetate/methanol/water (12:2:1, v/v). Combined fractions containing the product were rechromatographed to give pure product. Yield 1.4 mg. Mass spectrum (positive ion f.a.b.) showed $m/z = 624$ $(M+1)$. Chromatography of radiolabelled and unlabelled samples showed that radioactivity, ultraviolet density and sugar stain cochromatographed.

Photolabelling of erythrocytes

Erythrocytes from 3-week-old transfusion blood were washed five times in phosphate saline buffer (154 mM NaCl/12.5 mM sodium phosphate (pH 7.2)). The inhibition constant for BB-BMPA inhibition of D-galactose transport was determined using previously described methods [37] but using only a single inhibitor concentration of 1.4 mM. For the photolabelling, 400 μl of erythrocyte suspension (20% cytocrit) were mixed with 10 μCi of BB-BMPA in 400 μl of a solution containing D-mannitol or D-glucose and were irradiated for the indicated times in 1-mm pathlength cuvettes. The cuvettes were covered with a 10 mm pathlength quartz cell containing a chemical filter of 1% cumene in 2,2,4-trimethylpentane. The light source was a Rayonet RPR-100 photoreactive using RPR-3000 lamps. After irradiation the cells were washed five times in phosphate saline buffer. The membranes were then isolated [3] and subjected to polyacrylamide gel electrophoresis.

Photolabelling of adipocytes

Rat adipocytes were isolated as previously described [4–7] and after washing in a 1% albumin in Hepes buffer (140 mM Na^+; 4.7 mM K^+; 2.5 mM Ca^{2+}; 1.25 mM Mg^{2+}; 142 mM Cl^-, 2.5 mM $H_2PO_4^-/HPO_4^{2-}$; 1.25 mM SO_4^{2-}; 10 mM Hepes (pH 7.4 at 37°C)) were resuspended at 25% cytocrit in 0.1% albumin Hepes buffer. Half-maximal inhibition constants (K_i values) for BB-BMPA inhibition of 3-*O*-methyl-D-glucose transport were determined using previously described methods [37]. Inhibition was determined at 0.25, 0.5, 0.75 and 1 mM and the K_i values were calculated by nonlinear regression. For the photolabelling 250 μCi of BB-BMPA was added to 10 ml of adipocyte suspension (25% cytocrit) in a 10 cm Petri dish and irradiated in the Rayonet photoreactor for 3 × 30 s with manual resuspension of the cells between the irradiation intervals. Usually two such samples were used in each experiment; seven rats provided adequate numbers of cells for such experiments. Throughout the cell washing, irradiation and subsequent membrane isolation the two samples were treated in parallel. Following irradiation cells were immediately washed in a 1% albumin Hepes buffer at 37°C. Generally the cell washing was repeated four times and with appropriate additions (as specified) in the wash buffer. Following the washing steps the adipocytes were resuspended in Tris/EDTA/sucrose buffer. (10 mM Tris HCl; 0.5 mM EDTA; 250 mM sucrose (pH 7.2)) and homogenised at approx. 18°C with seven strokes in a Potter-Elvejhem homogeniser. This had an estimated clearance of 0.15–0.25 mm and rotation speed of 1600 rpm. Isolation of a light microsomal and plasma membrane fraction then followed the method of Cushman and Wardzala [6] and Simpson et al. [9] except for the use of a 15% Ficoll step gradient instead of sucrose. 2 ml of the resuspended plasma membrane plus mitochondria pellet were layered onto 3 ml of the Ficoll and centrifuged at 50000 × *g* for 45 min. The plasma membrane fraction at the interface was collected and diluted in the Tris/EDTA/sucrose buffer and then pelleted at

30000 × *g* for 30 min. The yield and purity of the fractions were similar to those reported by other groups [6,8–11]; each half of an experiment generally gave approx. 400 μg of both plasma membrane and light microsomes. The 5′-nucleotidase activities in these two fractions were 620 ± 41 and 25.6 ± 0.7 nmol/h per mg. The 5′-nucleotidase activity in the high density microsome fraction was 100 ± 16 nmol/h per mg. Characterizations of additional marker enzymes, electron micrographs and D-glucose transport rates was carried out by Rees [12]. The D-glucose transport rate in plasma membranes from insulin-treated cells was 5-times higher than in plasma membranes from basal cells [12].

Electrophoresis

Membranes were solubilised at room temperature and were run on 10% acrylamide gels using a Tris/borate buffer (50 mM Tris; 40 mM boric acid; 2.5 mM SDS (pH 8.3)). The gels were stained with Coomassie blue, and then destained and sliced (three slices per cm). The slices (in scintillation vials were dried at 60°C for 6 h and then dissolved in 0.5 ml of alkaline hydrogen peroxide at 60°C for a further 3–4 h. Scintillant was added and the radioactivity counted [13].

Results

Irradiation of intact erythrocytes in the presence of BB-[2-^{3}H]BMPA results in the incorporation of radiolabel into the hexose transporter. Irradiation times of 2–3 min give similar levels of incorporation (Fig. 1). Incorporation of radiolabel into the transporter is entirely blocked by D-glucose (Fig. 2) indicating that the label is highly selective for the transporter and gives no incorporation into any other red cell protein. This can be contrasted with the previously studied aryl-azide derivative ASA-BMPA which gives some 10–15% of the total incorporation into non-transporter proteins. The affinity for BB-BMPA was assessed by measuring the inhibition of D-galactose transport. The K_i was 971 ± 42 μM. The affinity is thus much lower than for the azidosalicoyl derivative (K_i = 60–100 μM) [37] and this suggests that the increased selectivity is entirely due to the difference in mechanism for photoactivation.

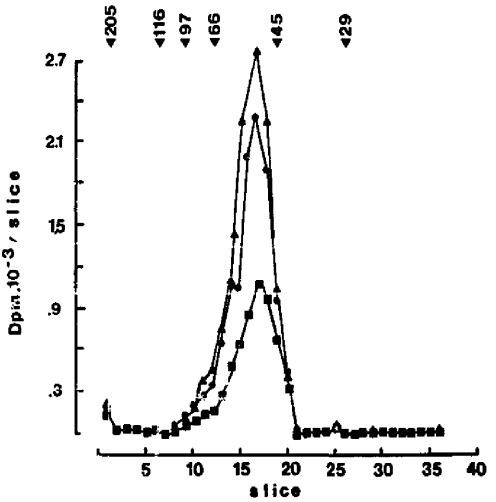


Fig. 1. Exofacial photolabelling of erythrocytes with BB-BMPA. 800 μl of an erythrocyte suspension (10% cytocrit) was irradiated for 1 (■) 2 (●) or 3 (△) min in the presence of 10 μCi of [2-^{3}H]BB-BMPA. Membranes were isolated and subjected to electrophoresis on 10% acrylamide gels.

The activation mechanism involves hydrogen abstraction to give two radicals, one on the ligand and one on the protein. The radicals then com-

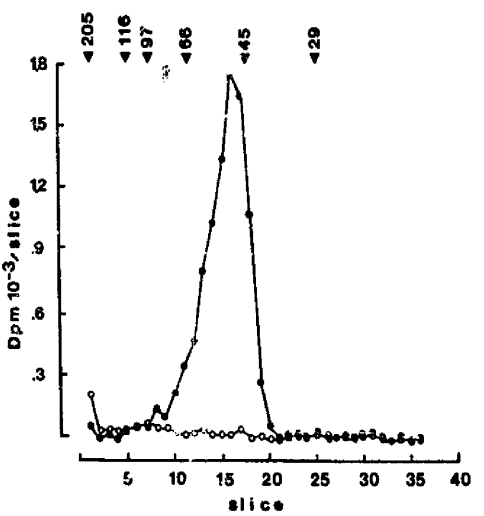
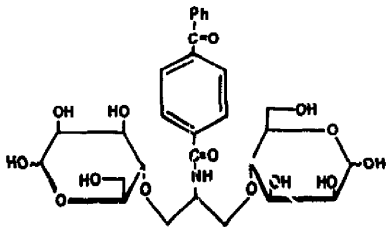


Fig. 2. Displacement of BB-BMPA photolabelling by D-glucose. 800 μl of erythrocyte suspensions (10% cytocrit) were photolabelled by irradiation for 3 min in the presence of 10 μCi [2-^{3}H]BB-BMPA and either 320 mM D-glucose (○) or 320 mM D-mannitol (●).



Scheme I. Structure of 2-*N*-(4-benzoylbenzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (BB-BMPA).

bine. This type of mechanism may be particularly suitable for biological systems since close association of the ligand and protein radicals will occur in the protein active site [15]. Radical quenching by solvent is thought to be slower for the ketone derived carbenes than for nitrenes derived from aryl azides [15,16]. This may be advantageous as it will prolong the reactive lifetime of the carbene radical which can thus give high photochemical yields [15].

The difficulties with using aryl azides are particularly evident when trying to label a protein which is not very abundant in the membrane. The problems associated with photolabelling the adipocyte transporter were referred to in the introduction. Results showed that the following aryl-azide derivatives of bis(hexoses) (BMPA and the D-glucose equivalent BGPA) were not at all useful in the adipocyte. The compounds that were tested were 2-*N*-(4-azidosalicoyl)-BMPA and -BGPA, 2-*N*-(4-azido-2-nitrophenyl)-BMPA and -BGPA, 2-*N*-(3-hydroxy-4-azidobenzoyl)-BGPA, 2-*N*-(4-azidobenzoyl)-BGPA, 2-*N*-(5-azido-2-nitrobenzoyl)-BGPA, 2-*N*-(3-azido-4-methoxybenzoyl)-BGPA and 2-*N*-(2-propyloxy-4-azidobenzoyl)-BGPA. 2-*N*-(4-Azido-3,5-diiodobenzoyl)-BMPA and -BGPA were partially useful in that they showed half-maximal inhibition constants of 20 μM in insulin-treated adipocytes (which is a much better affinity than is shown by BB-BMPA) and also showed detectable amounts of transporter labelling (Rees, Karim and Holman, unpublished). However, they also showed large amounts of non-specific photolabelling.

In contrast to the above problems associated with aryl azides the BB-BMPA compound shows a considerable improvement in adipocyte transporter photolabelling even though its affinity constant was poor. The half-maximal inhibition constants (K_i values) for BB-BMPA inhibition of 3-*O*-methyl-D-glucose transport were 536 ± 158 μM ($n = 5$) in basal cells and 254 ± 68.5 μM ($n = 9$) in insulin-treated cells. This 2-fold difference in affinity between basal and insulin-treated cells is consistent with our previous study of a series of bis(hexoses) where 2–4-fold differences were shown. These changes can be considered minor when compared with the 60-fold differences in the 3-*O*-methyl-D-glucose transport rate (in the absence of inhibitor) which occur in these experiments on whole cells.

Nonspecific labelling of a nontransporter 75 kDa peptide by BB-BMPA is very evident on the SDS gels obtained from the photolabelling experiments but this is clearly separable from the transporter peak at 50 kDa (Fig. 3). The labelling of the 50 kDa transporter peptide was blocked by 50 μM cytochalasin B (Fig. 3) and by 3-*O*-methyl-D-glucose, but not by D-mannitol (not shown).

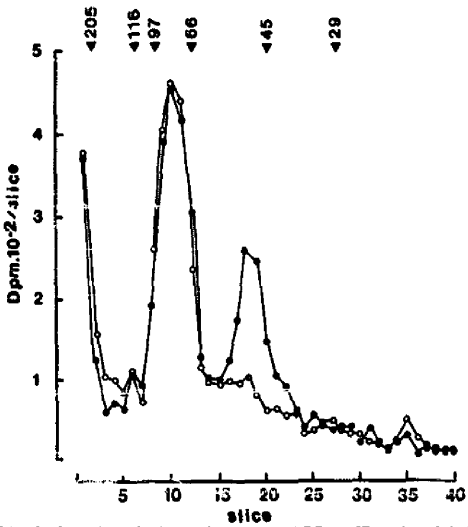


Fig. 3. Cytochalasin B displacement of BB-BMPA photolabelling of intact adipocytes treated with 10 nM insulin. A 25% adipocyte suspension was irradiated in the presence of 250 μCi [2-^{3}H]BB-BMPA for 90 s in the presence (○) or absence (●) of 50 μM cytochalasin B. Cells were washed and then plasma membranes were isolated and subjected to electrophoresis on 10% acrylamide gels.

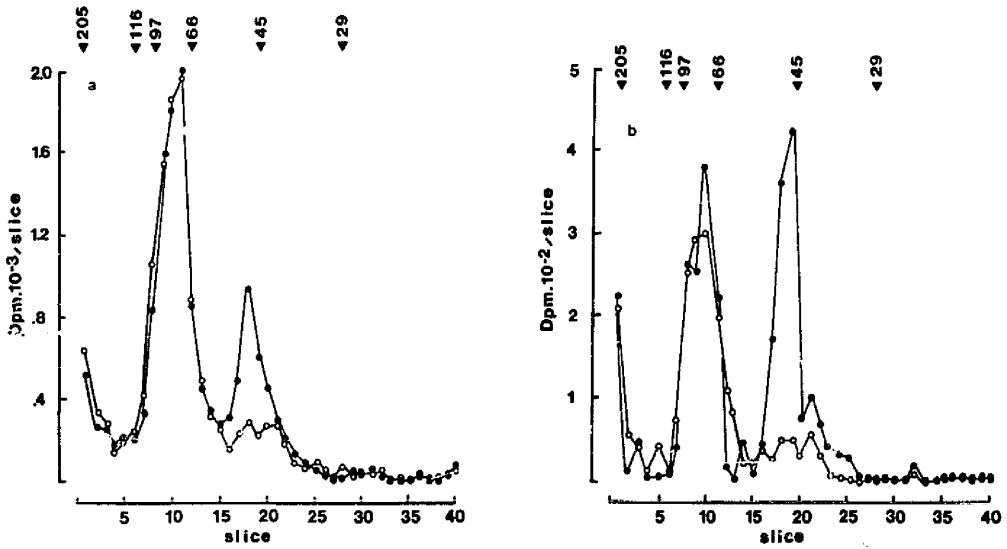


Fig. 4. (a) Photolabelling of basal and insulin-treated adipocytes. 25% adipocyte suspensions from either basal cells (○) or cells treated with 10 nM insulin (●) were irradiated for 90 s in the presence of 250 μCi [2-^{3}H]BB-BMPA. Cells were washed at 37°C for 40 min in either insulin-free or insulin-containing buffer, respectively. Plasma membranes were isolated and subjected to electrophoresis on 10% acrylamide gels. (b) Internalisation of photolabelled transporter. Light microsomes were isolated from the cell suspensions referred to in (a). Photolabelled membrane proteins of the light microsomes from insulin-treated cells (●) and from basal cells (○) are shown.

Having determined that the transporter is labelled by BB-BMPA we investigated the extent to which transporter labelling was insulin dependent. Fig. 4a compares photoincorporation into the plasma membranes derived from basal (non-stimulated) adipocytes and insulin-treated adipocytes. The incorporation into the transporter is at least 7-fold lower in the absence of insulin. The magnitude of this effect is large but is still less than the 60-fold changes in transport rates that are evident in 3-*O*-methyl-D-glucose transport assays on whole cells.

In this experiment cells were incubated and washed with insulin-containing buffer for 40 min at 37°C before homogenisation. This period was considered to be sufficient to allow recycling and equilibration with the intracellular pool of transporters. Fig. 4b shows the profile of radioactivity in the light microsomes. Internalisation of the transporter has occurred since the peak height of the 50 kDa peptide relative to the peak height of the 75 kDa plasma membrane marker peptide is much higher. This can only have happened if the transporter were selectively removed from the plasma membrane. 30% of the total transporter is internalised and transferred to the light microsome pool. The label in the 75 kDa band is due to a calculated 9.6% plasma membrane contamination of the light microsomes. The observed transporter peak in the light microsome fraction can be corrected for the same level of crossover from labelled plasma membrane transporter. This correction gives a value of 24.6% internalisation. However, contamination of plasma membrane by light microsomes is also likely. This cannot be estimated and so any correction of light microsomes by plasma membrane contamination is rather one-sided and so we have not generally used it. Its importance here is that it directly demonstrates that internalisation of transporter occurs to a level which is much greater than can be attributed to simple contamination.

Fig. 4b also shows that in basal cells there is no labelled transporter present in the light microsome fraction. This rules out the possibility that any transporters from the internal pool visited the

plasma membrane and became active during the 2–3-min labelling period (this includes irradiation time and resuspension intervals) and so shows that transporters do not turn over rapidly in the basal state. However, the labelling experiment does not rule out the possibility that inactive transporters may be translocated rapidly.

Discussion

In recent years several new reagents have become available for photoaffinity labelling the human erythrocyte hexose transporter. Shanahan et al. [17] and Weber and Eichholz [18] used iodinated azidosalicoyl derivatives of 6-aminoglucose. We have developed a series of similar compounds but based on bis(D-mannos-4-yloxy)-2-propylamine (BMPA). The bis(hexose) compounds are impermeable and so can selectively label [3] and identify [1] the exofacial binding site on the transporter. The bis(hexoses) thus complement studies on photoactivatable cytochalasin B labelling as this compound binds to [19,20] and labels the internal site on the transporter [1,21,22]. Recently a forskolin photoaffinity label has been developed which is potentially very useful as it shows high affinity and can be radioiodinated to high specific activity [23].

The new reagent BB-BMPA that is described here also labels the human erythrocyte transporter well. The labelling pattern shows some improvement over the previously studied ASA-BMPA in that no other proteins are labelled. With ASA-BMPA we observed some labelling (about 10–15% of the total) in an additional band at about 75 kDa. The labelling by BB-BMPA is of particular interest as it shows that compounds containing ketone groups next to unsaturated carbon-carbon bonds are good photolabelling compounds for the hexose transporter and therefore further studies with additional compounds which undergo the same type of reaction mechanism would be of interest. Most of the photoreactive ligands used in biochemistry are aryl azides and there are relatively few reports on the use of ketones. Benzophenone derivatives of ATP [24] and the diuretic bumetamide [25] have been studied. Certain steroids with ketone groups are known to be good photoaffinity labelling reagents [26]. It is possible that cytochalasin B photoaffinity labelling shows a similar mechanism though the keto group here would be expected to be less reactive than that present in the highly conjugated benzophenone ring system. It has been suggested [22,27,28] that photoactivation of tryptophan or tyrosine groups is involved in the light-induced covalent binding of cytochalasin B. It is also possible that photoinduced fluorescence energy transfer between such residues and the ligand might participate in the photolabelling process as has been suggested for some other photoactivation systems [29].

Previous studies on the photolabelling of the rat adipocyte hexose transporter have also used cytochalasin B [30–34]. This has also been used to photolabel the 3T3 L1 adipocyte transporter [35]. May et al. [36] used a permeable phenylazide derivative of maltosylamine to label the transporter in the light microsome membrane fraction isolated form rat adipocytes.

We developed the bis(hexoses) with the particular problem of labelling the adipocyte carrier in view [37]. We showed that unlike the freely permeable compounds described above the hydrophilic bis(hexoses) are impermeable in adipocytes and thus are potentially capable of labelling just those transporters that reside in the plasma membrane. This advantage is somewhat adversely balanced by the compounds having low affinity when compared with the alternative labelling reagents such as cytochalasin B and forskolin. Thus many early attempts at photolabelling the adipocyte transporter with bis(hexoses) substituted with aryl azides gave poor results (see results section for a description of the unsuccessful compounds). However, the improvement in selectivity shown by the benzophenone substituted compound has now enabled us to clearly label this transporter. The labelled transporter runs as a band at 50 kDa in our gel system which is clearly separated from the 75 kDa nontransporter band. Others [30–34] who have used alternative labelling reagents have estimated the transporter molecular mass to be 46 kDa but this is probably because the transporter mobility is variable in different gel electrophoresis systems [38].

A particular use of the bis(hexoses) in experiments on the adipocyte transporter is to selectively label the plasma membrane pool of trans-

porters in intact cells. This in situ labelling allows cellular processing of the transporter to be followed. Thus the impermeable bis(hexoses) may be useful for studying the recruitment hypothesis for transporter activation by insulin. This hypothesis, described by Cushman and Wardzala [6] and by Susuki and Kono [39], has now been widely supported by several groups who have shown changes in the distribution of cytochalasin B binding sites between the plasma membrane and the light microsomes. Additional evidence for recruitment has also been obtained by studying in situ labelling by cytochalasin B [30] and by a cytochemical approach in 3T3 L1 adipocytes which uses antibodies to detect transporters in either the plasma membrane or in a post-Golgi membrane pool [40]. The disadvantage of most previously used techniques is that factors such as new transporter synthesis, loss of transporters by cellular degradation processes and cross-contamination of isolated membrane fractions can all influence the observed steady-state distribution. With the in situ labelling technique using the bis(hexose) BB-BMPA we have obtained direct evidence for at least a 7-fold insulin-dependent increase in the number of available transporters. Although there is a large pool of light microsome transporters in basal cells any contamination of the plasma membrane fraction by these transporters due to homogenisation is not measured using our technique as these transporters are not labelled. The estimated magnitude of the insulin effect on cytochalasin B binding to isolated plasma membrane fractions appears to vary between different laboratories. Cushman's group (who showed that the homogenisation protocol is critically important in estimating the transporter distribution) found conditions in which they observe greater than 6-fold insulin-dependent increases in both cytochalasin B binding and transport rates in their plasma membrane fractions [41]. Thus our estimate of the magnitude of the insulin effect on BB-BMPA photolabelling is consistent with their results on transporter number but involves a technique which is less dependent on homogenisation conditions. The low level of transporter labelling in plasma membranes and light microsome membranes derived from intact basal cells indicates that the transporter pool present in the light microsomes does not turnover rapidly – it does not translocate and become active during the labelling period. If translocation and activation are separate processes (as suggested by Muhlbacher et al. [42]) then it may be possible that inactive transporters translocate and recycle rapidly without becoming labelled.

With the in situ labelling technique we have obtained direct evidence that transporters which reside in the plasma membrane at the time of labelling are moved back (internalised) to the light microsome fraction. In this case the proportion of labelled transporters observed in the two fractions is influenced by the homogenisation conditions. We can correct for labelled transporters from plasma membrane which contaminate the isolated light microsomes but we cannot at present estimate the proportion of labelled and internalised transporters which contaminate the isolated plasma membrane fraction.

Quantifying the proportion of transporters in the plasma membrane which are subsequently internalised is now of considerable importance as Muhlbacher et al. [42] have shown that the phorbol ester TPA can mimic the insulin effect on transporter distribution but cannot mimic its full effect on transport [42,47,48]. Muhlbacher et al. [42] have suggested that translocation does not account for the full insulin effect on transport and that some additional activation and presumably inactivation mechanism operates. Thus methods are needed to quantify the proportion of plasma membrane and light microsome transporter isoforms which are (a) active, (b) inactive and (c) translocatable. Our own estimate of the proportion of transporters (30%) which equilibrate with the internal pool of membranes in the presence of insulin may seem low but the result is consistent with the equilibration with only one of the light microsome transporter isoforms. Horuk et al. [32] showed that in insulin-treated cells approx. 60% of cytochalasin B labelled transporters reside in the light microsomes but only about half of these are the pH 5.6 isoform. 30% of the total cellular transporters are present in the light microsomes as a pH 6.4 isoform in both basal and insulin-treated cells. It is therefore possible that transporter photolabelled with BB-BMPA at the plasma membrane is the pH 5.6 isoform and that only this form equilibrates with the light microsomes. The

presence in the plasma membrane of multiple transporter isoforms has been suggested by James et al. [43] who showed that there is a unique insulin-dependent transporter which is recognised by their monoclonal antibody (which was raised against an adipocyte derived transporter antigen) and also an additional transporter which is recognised by antibodies which are raised against human erythrocyte transporter [44–46].

In situ labelling with BB-BMPA may be useful in studying the transporter inactivation process which occurs on insulin removal. The technique would produce labelled transporters which could then be processed by the cell on insulin removal to give a modified transporter isoform. A proportion of this modified transporter would then be expected to be internalised.

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