5-Hydroxytryptamine stimulates glucose transport in cardiomyocytes via a monoamine oxidase-dependent reaction

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This study deals with the effect of 5-hydroxytryptamine (5-HT; serotonin) on glucose transport in isolated rat cardiac myocytes. In these cells, 5-HT (10–300 μM), as well as tryptamine, 5-methoxytryptamine and dopamine, elicited a 3–5-fold increase in glucose transport, as compared with control. This effect was maximal after 90 min, and was concomitant with a 1.8- and 1.5-fold increase in the amounts of glucose transporters GLUT1 and GLUT4 at the cell surface of the cardiomyocytes, as determined by using the photoaffinity label ^{3}H-2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yl)propyl-2-amine (^{3}H-ATB-BMPA). In contrast, 3–3000 μM of the selective 5-HT receptor agonists 5-carboxyamido-tryptamine, α-methyl-serotonin, 2-methyl-serotonin or renzapride failed to stimulate glucose transport. The effect of 5-HT was not affected by (i) the 5-HT receptor antagonists methysergide (1 μM), ketanserin (1 μM), cyproheptadine (1 μM), MDL 72222 (1 μM) or ICS 205-930 (3 μM), nor by (ii) the adrenergic receptor antagonists prazosin (1 μM), yohimbine (1 μM) or propranolol (5 μM), nor by (iii) the dopaminergic antagonists SCH 23390 (1 μM) or haloperidol (1 μM). The monoamine oxidase inhibitors clorgyline (1 μM) and tranylcypromine (1 μM) completely suppressed the effect of 5-HT, whereas the control and insulin-stimulated rates of glucose transport were unaffected. Addition of catalase or glutathione diminished the 5-HT-dependent stimulation of glucose transport by 50%; these two factors are known to favour the degradation of H_2O_2 (which can be formed during the deamination of amines by monoamine oxidases). Glutathione also depressed the stimulatory action of exogenously added H_2O_2 (200 μM) by 30%. Furthermore, in cells treated with 5-HT, a time-dependent accumulation of 5-hydroxy-1*H*-indol-3-ylacetic acid (a product of 5-HT metabolism via monoamine oxidases) was observed, which paralleled the changes in glucose transport. In conclusion, the stimulation of glucose transport by 5-HT in cardiomyocytes is not mediated by a 5-HT_1, 5-HT_2, 5-HT_3 or 5-HT_4 receptor, nor by an adrenergic or dopaminergic receptor, but is likely to occur through the degradation of 5-HT by a monoamine oxidase and concomitant formation of H_2O_2.

INTRODUCTION

Many investigations performed *in vivo* suggest that 5-hydroxytryptamine (5-HT; serotonin) may play a role in the regulation of blood glucose. Thus, injection of tryptophan, 5-hydroxytryptophan (both precursors of 5-HT), or 5-HT itself, has been reported to result in hypoglycaemia in rodents [1–6]. Moreover, 5-HT receptor antagonists were shown to inhibit this hypoglycaemic effect [3,4], or themselves to have hyperglycaemic properties [7]. On the other hand, hyperglycaemia has also been observed upon administration of 5-HT [1] or 5-HT receptor agonists [8–11]. The picture is further complicated by the fact that some of these effects seem to be related to changes in the plasma level of insulin [4,6,8,9,11], but some are not [1,3,9]. In addition, central-nervous-system events appear to be involved in some instances [2,8–10], whereas in other cases evidence for peripheral mechanisms was presented [1,3,5,11].

Taken together, these investigations indicate that the influence of serotonergic mechanisms on glucose metabolism depends on the complex interplay of neuronal and hormonal factors operating *in vivo*. To our knowledge, no approach *in vitro* has been attempted to identify the possible site(s) of action of 5-HT. In particular, the question of which metabolic processes are directly involved in the reported effects on blood glucose is unsolved. The pattern of changes in liver metabolites observed by Smith and Pogson upon tryptophan administration seem to exclude an involvement of hepatic gluconeogenesis [3].

The purpose of the present work was to examine the possibility of a direct effect of 5-HT on muscle glucose utilization, since glucose disposal by muscle tissue is a determinant factor in the control of glycaemia. To this end, we used isolated cardiac myocytes, which have proved to be a useful model to investigate the control of glucose metabolism at the cellular level [12–14]. In particular, we focused on the action of 5-HT on the uptake of glucose across the plasma membrane, because glucose transport is known to be a major rate-limiting and highly regulated step of hexose utilization in heart and skeletal muscle [15–17].

EXPERIMENTAL

Chemicals

All chemicals were of highest purity grade available. H_2O_2, the detergent Thesit, as well as all chemicals for media used for

Abbreviations used: 5-HT, 5-hydroxytryptamine (serotonin); 5-HIAA, 5-hydroxy-1*H*-indol-3-ylacetic acid; ^{3}H-ATB-BMPA, ^{3}H-2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine; cAMP, cyclic AMP; cGMP, cyclic GMP; MAO, monamine oxidase; 6,7-ADTN, (±)-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; PMA, 4β-phorbol 12β-myristate 13α-acetate.

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cell isolation and glucose transport assays were from Merck (Darmstadt, Germany); 4β-phorbol 12β-myristate 13α-acetate (PMA), prazosin, propranolol, yohimbine, ketanserin, (−)-isoprenaline, dibutyryl cyclic AMP, dopamine, tryptamine, 5-HT, clorgyline, tranylcypromine, antipain, aprotinin, pepstatin and leupeptin were obtained from Sigma (Deisenhofen, Germany); sodium nitroprusside was from Fluka Chemie AG (Buchs, Switzerland); BSA (fraction V, fatty-acid-free), glutathione (oxidized) and catalase (from bovine liver) were from Boehringer (Mannheim, Germany); methysergide, cyproheptadine, haloperidol, *R*(+)-SCH-23390, MDL 72222 (3-tropanyl-3,5-dichlorobenzoate), 5-carboxyamido-tryptamine, α-methyl-serotonin, 2-methyl-serotonin, (±)-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN), pertussis toxin and cholera toxin were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.); 2-deoxy-D-[^{3}H]glucose and radioimmunoassay kits for the quantification of cyclic AMP (cAMP) and cyclic GMP (cGMP) were purchased from Amersham (Braunschweig, Germany); the photoaffinity label ^{3}H-2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine was kindly provided by Professor G. D. Holman (Department of Biochemistry, University of Bath, Bath, U.K.); purified bovine insulin was kindly given by Professor Axel Wollmer (Aachen, Germany); renzapride (BRL 24924) was kindly given by SmithKline Beecham (Betchworth, Surrey, U.K.); ICS 205-930 was kindly provided by Sandoz Pharma (Basel, Switzerland); (−)-deprenyl (= L-deprenyl or selegiline) was kindly given by Orion Corporation Farmos (Turku, Finland). Concentrated stock solutions of 4β-phorbol 12β-myristate 13α-acetate (in DMSO) and of insulin (in assay medium; see below) were stored at −20 °C in appropriate batches, and diluted just before addition to the isolated cardiomyocytes. Final concentrations of DMSO were typically 0.02–0.1 % in the transport assays and did not affect basal transport activity. All other chemicals were freshly prepared immediately before use.

Isolation of cardiomyocytes and determination of 2-deoxy-D-glucose uptake

Cardiomyocytes from adult Sprague–Dawley rats (180–220 g, fed *ad libitum*) were obtained as previously described [18]. The uptake of 2-deoxy-D-[^{3}H]glucose was assayed in medium containing 6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM Hepes, 1 mM $CaCl_2$ and 2 % BSA (fatty-acid-free), pH 7.4, 37 °C, equilibrated with oxygen, as described [18]. In brief, 1 ml of cell suspension (corresponding to approx. 1.5 mg of cell protein) was incubated with 0.5 ml of medium (control) or with an appropriate dilution of one or several agents to be assayed, in a shaking water bath at 37 °C for the times indicated. The samples were then incubated in the presence of 2-deoxy-D-[^{3}H]glucose (1 μM) for an additional 30 min at 37 °C (or, in the experiments shown in Figures 1 and 8, for 2.5 min) before sugar uptake was stopped by adding phloretin (400 μM final concn.). Specific, i.e. glucose-carrier-mediated, 2-deoxy-D-glucose uptake was calculated by subtraction of uptake rates monitored in the presence of 400 μM phloretin from total uptake values (carrier-mediated 2-deoxy-D-glucose uptake was ~ 80 % and ~ 95–98 % of total uptake in control and stimulated cells, respectively). Each experiment was done in triplicate. Under these assay conditions (30 min incubation in the presence of 1 μM 2-deoxy-D-glucose), the transport of glucose across the plasma membrane, and not its intracellular phosphorylation, limits the overall uptake of the sugar [14,18].

Quantification of cAMP and cGMP

Cardiomyocytes were exposed to 5-HT (100 μM), isoprenaline (1 μM), or sodium nitroprusside (100 μM) for the times indicated at 37 °C, before samples of the cell suspension (corresponding to ~ 70000 cells) were rapidly spun down (45 s, 14 *g*). The pellet from this centrifugation was immediately dissolved in 500 μl of cold ethanol (4 °C, 70 %) and sonicated for 15 s (Sonifier B-12, Branson). The samples were then centrifuged for 5 min at 10000 *g*, and the supernatants from this centrifugation were evaporated at 70 °C under nitrogen gas. The dry material obtained was dissolved in 250 μl of buffer (50 mM Tris/HCl, 4 mM EDTA, pH 7.5) and its content of cAMP or cGMP was determined by radioimmunoassay with commercial kits (Amersham) according to the manufacturer's instructions. The average recovery of 0.3 pmol of non-radioactive cAMP or 0.04 pmol of cGMP added to 500 μl of ethanol extract of cardiomyocytes was 93.3 ± 10.0 % (3 independent experiments in duplicate) and 97.5 ± 15.0 % (4 experiments) respectively. The calculated concentrations of cyclic nucleotides found in our control cells (~ 0.6 μM cAMP, ~ 75 nM cGMP) are in the range of values previously reported for heart and/or cardiomyocytes [19–21].

Measurement of 5-HT metabolites by HPLC

Cardiomyocytes were incubated for the indicated times in the presence of 5-HT at 37 °C, and were rapidly centrifuged (45 s, 30 *g*). The pellet was resuspended in 500 μl of cold $HClO_4$ (4 °C, 0.3 M), and homogenized for 2 × 3 s with a sonifier (Branson). The samples were then centrifuged for 3 min at 10000 *g*, and the supernatants were neutralized with $KOH/KHCO_3$ before samples thereof (typically 10 μl) were subjected to analysis by paired-ion reversed-phase HPLC by a slightly modified version of a previously described method [22]. To identify the major peaks detected in the cell extracts (Figure 7, peaks 1–4), standards of 5-HT, 5-hydroxy-1*H*-indol-3-ylacetic acid (5-HIAA), 5-hydroxytryptophol, as well as ADP and ATP, were separated by HPLC under the same elution conditions, either as external standards (i.e. in the absence of cell extract), or as internal standards (i.e. mixed with portions of extracts). The elution times of all standards were found to be identical in both cases. For quantification of the cellular content of 5-HIAA (in cells treated with 5-HT), the area of peak 2 (e.g. Figure 7b) was compared with that of an internal standard (50 μM final concentration) added to extracts from control samples (Figure 7c). The concentrations are expressed in mmol/litre of cellular water space (see also below, under 'Calculations and statistics').

Photoaffinity labelling of glucose transporters GLUT1 and GLUT4 at the surface of cardiomyocytes

The labelling of glucose transporters present at the surface of cardiomyocytes was adapted from a method previously described [23,24]. Cardiomyocytes (approx. 1.5×10^6 cells per sample in a total volume of 6 ml) were incubated in assay medium (see above) for 90 min at 37 °C in the absence (control) or in the presence of 5-HT (300 μM) or of insulin (10 nM, for the last 30 min of the incubation). Parallel samples were used for the determination of glucose transport, as described above. The cells allotted to the photoaffinity labelling were then washed twice (in a total time of approx. 10 min) with assay medium, containing either 300 μM 5-HT or 10 nM insulin, or no addition for control cells. The last pellet was resuspended in 500 μl of medium (with the same additions); 60 μl of the non-permeant photoreactive bismannose compound ^{3}H-2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)-benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine (^{3}H-ATB-

BMPA; 300 μCi) was added immediately before the samples were irradiated for 3 min with a mercury light (Hanau; wavelength cut-off below 300 nm). After irradiation the cells were washed four times with assay medium and then solubilized for 20 min on ice in 1 ml of phosphate buffer (5 mM, pH 7.2) containing 2% Thesit and the proteinase inhibitors antipain, aprotinin, pepstatin and leupeptin (1 μg/μl each). After centrifugation at 20000 *g* for 20 min at 4 °C, the supernatants were subjected to sequential immunoprecipitation with 75 μl of rabbit anti-GLUT4 serum and then with anti-GLUT1 serum, each coupled to 5 mg of Protein A–Sepharose. For this purpose, the samples were incubated for 2 h at 4 °C with the first antibody, and then spun down for 1 min at 140 *g*. The supernatants from this centrifugation were incubated for another 2 h with the second antibody, and the precipitates were immediately washed six times with phosphate buffer containing 1% Thesit and the proteinase inhibitors and once with phosphate buffer without detergent. The Sepharose from the second immunoprecipitation was also washed seven times with phosphate buffer, as described above. The Sepharose beads (with the adsorbed glucose transporters) were then incubated for 15 min in 10% (w/v) SDS/6 M urea/10% mercaptoethanol containing Bromophenol Blue, and the supernatant was quantitatively loaded on to a 10%-polyacrylamide gel. After the electrophoresis, each lane of the gel was cut into 15 pieces (6 mm each), the pieces were dissolved in 30% H_2O_2 containing 2% NH_3, and the radioactivity was measured in a liquid-scintillation counter (Wallac 1409). To quantify the amount of labelled glucose transporters in each sample, the background of the gel slices was subtracted from the (only) peak appearing in the lanes; this peak has an apparent molecular mass of 45 kDa (in anti-GLUT1 precipitates) or 50 kDa (in anti-GLUT4 precipitates), corresponding to the known molecular masses of these two proteins.

In preliminary experiments, an irradiation time of 3 min had been found to induce ~ 80% photolysis without affecting the viability of cardiomyocytes. The amount of 75 μl of each antiserum was chosen because it caused a maximal precipitation of GLUT1 or GLUT4. Moreover, further control experiments showed that no detectable amount of glucose transporters could be recovered from a second immunoprecipitation with the same antiserum. Finally, the radioactive peak detected on gel electrophoresis was confirmed to represent labelled glucose transporters, since labelling was abolished by the specific glucose-transport inhibitor cytochalasin B (20 μM, added to insulin-stimulated cells before irradiation).

Calculations and statistics

Cell protein was measured by the biuret method. For the determination of cellular concentrations (cAMP and cGMP, see above; 5-HIAA, see Figure 8), the water distribution space of cardiomyocytes was calculated by using a previously determined factor of 2.07 μl/mg of protein [18]. For the calculation of the EC_{50} from the data shown in Figure 2, a logit–log transformation of the original values was performed with a computer program (Prism) from GraphPad Software (San Diego, CA, U.S.A.). A paired Student's *t* test was used for statistical comparison of data sets shown in Tables 1, 2, 3, and Figure 6.

RESULTS

Effects of 5-HT, and of 5-HT receptor agonists and antagonists, on glucose transport

When isolated cardiac myocytes were exposed to 5-HT (300 μM), the rate of 2-deoxy-D-glucose transport slowly increased to a level about 5 times that measured in control cells, a maximal stimulation being reached after approx. 90 min (Figure 1). The concentration-dependence of this effect is illustrated in Figure 2: the EC_{50} of glucose transport stimulation by 5-HT is ~ 35 μM, as calculated from linearized values corresponding to the data shown.

To investigate which receptor may mediate the response of the glucose transport system to 5-HT, selective agonists to the major 5-HT receptor types were tested. As shown in Figure 3, all these agents, namely 5-carboxyamido-tryptamine (5-HT_1-specific [25,26]), α-methyl-serotonin (5-HT_2-specific [27,28]), 2-methyl-serotonin (5-HT_3-specific [27–29]) or renzapride (= BRL 24924; 5-HT_4-specific [30,31]), failed to increase (or even decreased) the rate of glucose transport in cardiomyocytes when added for 90 min in a concentration range (3–3000 μM) in which 5-HT had

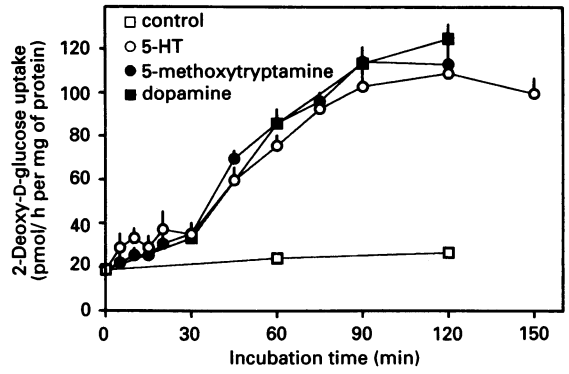

Figure 1 Time-dependence of the effects of 5-HT, 5-methoxytryptamine and dopamine on the rate of 2-deoxy-D-glucose transport in cardiomyocytes

The cells were incubated at 37 °C for the times indicated in the absence (control) or in the presence of 5-HT, 5-methoxytryptamine, or dopamine (300 μM each), before the rate of 2-deoxy-D-glucose uptake was measured over a period of 2.5 min, as described in the Experimental section. The dopamine samples contained, in addition, 1 μM prazosin and 5 μM propranolol, to prevent an interaction of dopamine with α_1- and β-adrenergic receptors. Data shown represent means ± S.E.M. of 3–6 independent experiments

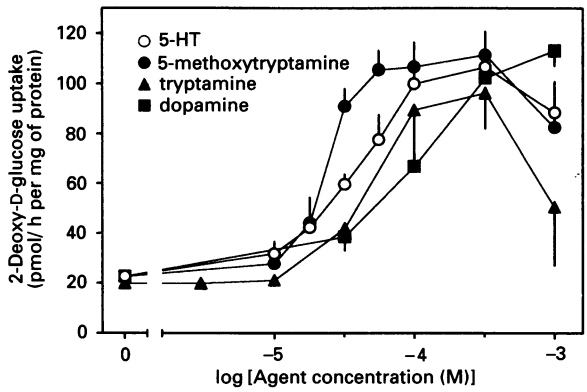

Figure 2 Concentration-dependence of the effects of 5-HT, 5-methoxytryptamine, tryptamine and dopamine on 2-deoxy-D-glucose transport

Cardiomyocytes were exposed for 90 min to 5-HT, 5-methoxytryptamine, tryptamine or dopamine at the indicated concentrations before the rate of 2-deoxy-D-glucose transport was determined over a period of 30 min as described in the Experimental section. As in the experiments shown in Figure 1, prazosin (1 μM) and propranolol (5 μM) were added to the dopamine samples. Results shown are means ± S.E.M. of 3 independent experiments.

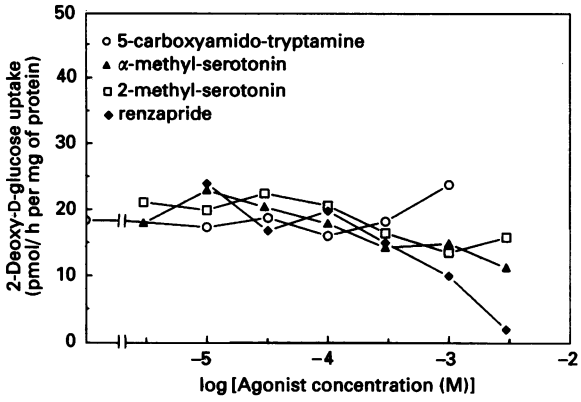

Figure 3 Effect of 5-HT-receptor agonists on 2-deoxy-D-glucose transport

Cardiomyocytes were incubated for 90 min in the presence of 5-carboxyamido-tryptamine, α-methyl-serotonin, 2-methyl-serotonin or renzapride at the indicated concentrations before the rate of 2-deoxy-D-glucose transport was measured over a period of 30 min. Data shown are means of 3–6 independent experiments. S.E.M. (not shown) was in each case less then 15% of mean values.

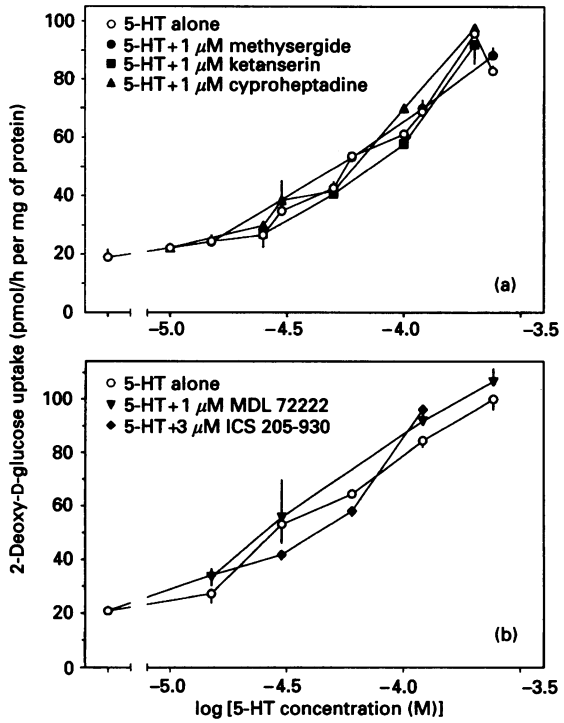

Figure 4 Influence of 5-HT-receptor antagonists on the stimulation of 2-deoxy-D-glucose transport by 5-HT

Cardiomyocytes were exposed to methysergide (1 μM), ketanserin (1 μM), cyproheptadine (1 μM), MDL 72222 (1 μM) or ICS 205-930 (3 μM) for 15 min, before 5-HT was added for an additional 90 min. 2-Deoxy-D-glucose transport was then measured as described in the Experimental section. The data illustrated represent means ± S.E.M. of 3 independent experiments.

been observed to be stimulatory (Figure 2). Only the less selective or non-selective 5-HT analogues 5-methoxytryptamine and tryptamine [32], but also dopamine, caused a stimulation of glucose uptake of the same magnitude and in a similar range of concentrations as that seen on 5-HT treatment (Figure 2). The

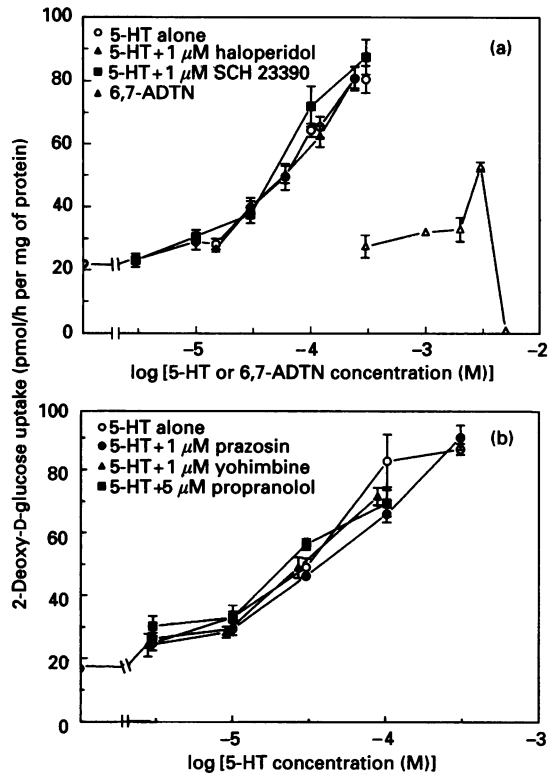

Figure 5 Influence of dopaminergic- and adrenergic-receptor antagonists on the stimulation of 2-deoxy-D-glucose transport by 5-HT

Cardiomyocytes were incubated for 15 min with dopaminergic antagonists (**a**: 1 μM haloperidol or 1 μM SCH 23390), or with adrenergic antagonists (**b**: 1 μM prazosin, 1 μM yohimbine or 5 μM propranolol) before 5-HT addition at the indicated concentrations for another 90 min, before 2-deoxy-D-glucose transport was measured. The dopamine (D_2) receptor agonist 6,7-ADTN (**a**) was added to parallel samples that were incubated for 90 min (in the absence of 5-HT and antagonists) before the transport measurement was carried out. Results shown are means ± S.E.M. of 3 independent experiments.

time-dependence of these effects was similar to that found for 5-HT (Figure 1). In addition, glucose transport stimulation by 5-methoxytryptamine, tryptamine or dopamine (used at saturating concentrations for 90 min) was not additive with the effect of 5-HT (results not shown).

Conversely, none of the 5-HT receptor antagonists tested had an influence on the concentration–response curve of the stimulating action of 5-HT: thus, methysergide ($5\text{-HT}_1/5\text{-HT}_2$ antagonist [32]), ketanserin or cyproheptadine (5-HT_2 antagonists [33,34]), MDL 72222 (5-HT_3 antagonist [29,32]) or ICS 205-930 ($5\text{-HT}_3/5\text{-HT}_4$ antagonist [27,31,35,36]) were all ineffective, even at concentrations exceeding their reported pK_i or IC_{50} values (Figure 4). Moreover, antagonists to other types of monoamine receptors also failed to alter the extent or the concentration-dependence of the glucose transport increase induced by 5-HT. In particular, this applies to the dopaminergic agents SCH 23390 (D_1-selective; 1 μM) or haloperidol (D_2-selective; 1 μM) (Figure 5a), and to the adrenergic antagonists prazosin (1 μM; α_1-adrenergic), yohimbine (1 μM; α_2-adrenergic) and propranolol (5 μM; β-adrenergic) (Figure 5b). Note that the dopaminergic D_2-agonist 6,7-ADTN [37] brought about a relatively small (2.5-fold) increase in glucose transport (in the absence of 5-HT), but only at millimolar concentrations (Figure 5a).

Table 1 Influence of pertussis toxin, cholera toxin, isoprenaline and dibutyryl cAMP on the stimulation of 2-deoxy-D-glucose transport by 5-HT

Cardiomyocytes were incubated in the absence ('No addition') or in the presence of isoprenaline (1 μM) for 15 min, or dibutyryl cAMP (0.1 mM) for 30 min, or pertussis toxin (1 μg/ml) for 120 min, or cholera toxin (1 μg/ml) for 120 min, before a sample of medium ('Control') or 5-HT (300 μM) was added for another 90 min. The rate of 2-deoxy-D-glucose transport was then determined. Data shown represent means ± S.E.M. of 4–6 independent experiments. Statistical significance of differences was assessed with a paired Student's t test: n.s., $P > 0.05$; *$P < 0.05$; ***$P < 0.001$, compared with values measured in the presence of 5-HT alone.

	Control	5-HT
No addition	26.6 ± 2.0	131.3 ± 5.6
+ Pertussis toxin	25.3 ± 5.2	118.8 ± 5.1 (n.s.)
+ Cholera toxin	26.0 ± 5.1	96.4 ± 4.0 (*)
+ Isoprenaline	22.9 ± 3.0	88.0 ± 8.2 (***)
+ Dibutyryl cAMP	25.8 ± 7.3	113.2 ± 8.5 (*)

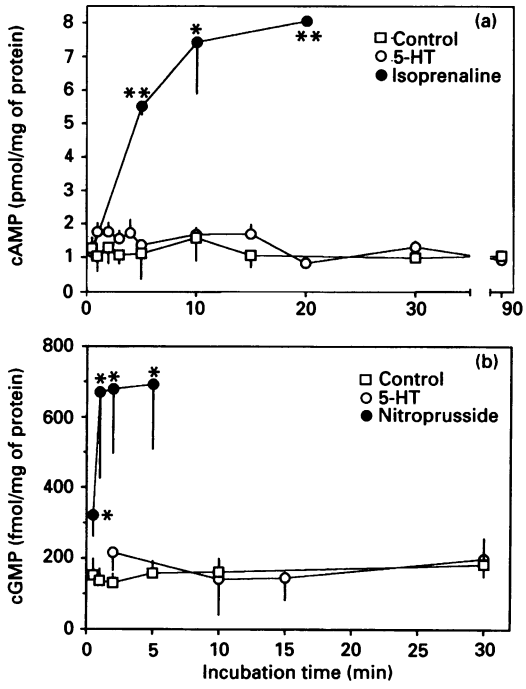

Figure 6 Effect of 5-HT on the content of cAMP and cGMP in cardiomyocytes

Cardiomyocytes were incubated in the absence (control) or in the presence of 5-HT (100 μM), isoprenaline (1 μM) or sodium nitroprusside (100 μM) for the times indicated, before the cellular content of cAMP (**a**) or cGMP (**b**) was measured as described in the Experimental section. Results shown are means ± S.E.M. of 3–4 independent experiments (each experiment was done in duplicate): *$P < 0.05$; **$P < 0.01$, compared with time-matched control values by paired Student's t test.

Table 2 Influence of MAO inhibitors, catalase and glutathione on the stimulation of 2-deoxy-D-glucose transport by 5-HT, insulin and H_2O_2

Cardiomyocytes were incubated with (−)-deprenyl (1 μM), clorgyline (1 μM), tranylcypromine (1 μM) or catalase (10000 units/ml), either for 15 min before 5-HT (100 μM) was added for another 90 min, or for 75 min before insulin (30 nM) was added for another 30 min. Glutathione (1 mM, oxidized form) was added simultaneously with 5-HT for 90 min, or for 30 min before H_2O_2 addition (200 μM) for another 30 min. Data are means ± S.E.M. of 3–4 independent experiments: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus 5-HT alone; †$P < 0.05$ versus H_2O_2 alone; n.s., $P > 0.05$ versus insulin alone (paired Student's t test).

	Rate of 2-deoxy-D-glucose transport (pmol/h per mg of protein)
Control	20.0 ± 1.4
+ (−)-deprenyl	18.9 ± 1.6
+ clorgyline	18.4 ± 0.7
+ tranylcypromine	18.4 ± 4.1
+ catalase	16.2 ± 3.8
+ glutathione	21.8 ± 2.6
5-HT	79.3 ± 6.0
+ (−)-deprenyl	77.1 ± 4.2
+ clorgyline	17.9 ± 2.3 (***)
+ tranylcypromine	16.1 ± 4.2 (***)
+ catalase	54.1 ± 12.6 (**)
+ glutathione	59.2 ± 8.9 (*)
+ catalase + glutathione	25.6 ± 4.6 (**)
Insulin	300.4 ± 16.2
+ (−)-deprenyl	303.3 ± 1.1
+ clorgyline	345.9 ± 56.0 (n.s.)
+ tranylcypromine	298.7 ± 32.4
+ catalase	298.2 ± 10.9
H_2O_2	93.3 ± 5.0
+ glutathione	72.0 ± 7.7 (†)

Glucose transport stimulation by 5-HT and intracellular signalling

The next approach used to define the pathway by which 5-HT acts on the uptake of glucose in cardiomyocytes was to examine the possible involvement of G-proteins and of the second-messenger systems that have been reported to mediate other effects of 5-HT. As shown in Table 1, pre-treatment of myocytes with cholera toxin (1 μg/ml) for 120 min depressed the effect of 5-HT by 33%, whereas pertussis toxin (1 μg/ml) had no significant influence. However, cholera toxin is known to raise the cAMP concentration in this and other cell types [19]. Since the 5-HT-dependent increase in glucose transport was inhibited to a similar extent by the β-adrenergic agonist isoprenaline (1 μM) and by the membrane-permeant cAMP analogue dibutyryl cAMP (0.1 mM) (Table 1), the toxin's effect is likely to involve an elevated concentration of the cyclic nucleotide, rather than the inhibition of a cholera-toxin-sensitive G-protein activatable by 5-HT.

It is well known that 5-HT receptors of the 5-HT_1 subtypes are negatively coupled to the adenylate cyclase [28,32]. Since the effect of 5-HT on glucose transport was antagonized by interventions elevating the cAMP level in cardiomyocytes (see Table 1), it is conceivable that 5-HT exerts its stimulating effect by lowering the cAMP level in these cells. However, when cardiomyocytes were incubated in the presence of 5-HT (100 μM) for 0.5–90 min, no significant change in their cAMP content was found (Figure 6a). Note that isoprenaline led, as expected, to a rapid and large increase in cAMP in these cells (Figure 6a). The lack of effect of 5-HT on the cAMP content also indicates that 5-HT_4 receptors are not likely to be involved in the observed glucose-transport stimulation, since these receptors are positively linked to the adenylate cyclase in heart [36,38] and other tissues [28,32].

It is well established that 5-HT_2 receptors are coupled to the phosphatidylinositol cascade, and thus trigger the release of inositol trisphosphate and diacylglycerol [28]. We have previously reported that, in cardiomyocytes, the diacylglycerol analogue and protein kinase C activator PMA increases glucose transport about 2-fold [13]. However, when cardiomyocytes were exposed

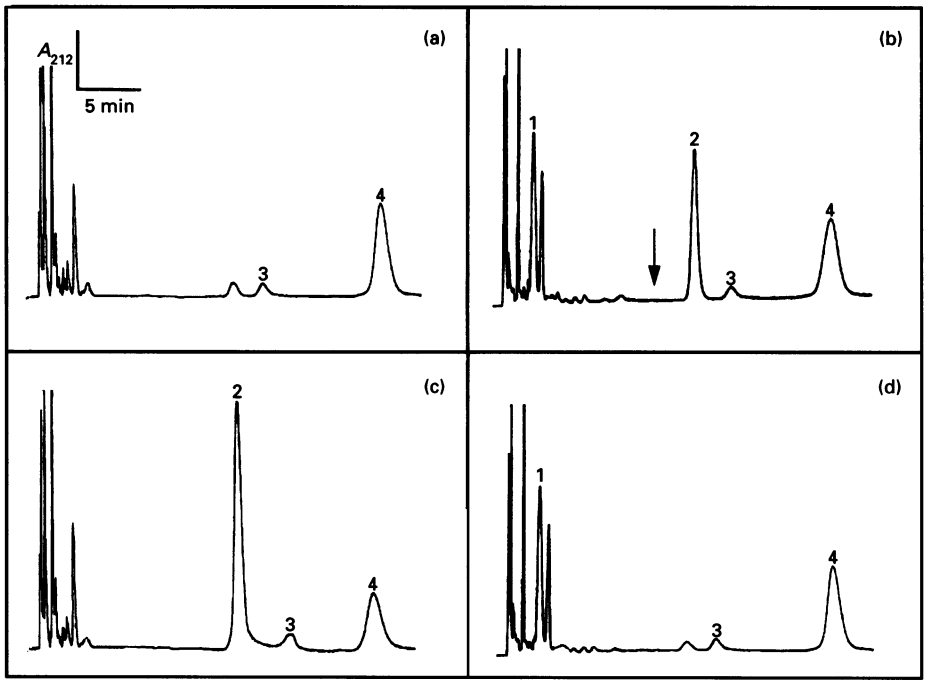

Figure 7 HPLC analysis of cellular extracts from cardiomyocytes treated with 5-HT

Cardiomyocytes were incubated in the absence (**a**, **c**) or in the presence of 300 μM 5-HT (**b**, **d**) for 90 min at 37 °C before they were homogenized and analysed by paired-ion reversed-phase HPLC, as described in the Experimental section. Results shown are representative elution patterns of (**a**) extract from control cells (**b**) extract from cells treated with 5-HT (**c**) extract from control cells, to which a 5-HIAA standard (final concentration 50 μM) had been added before HPLC analysis, and (**d**) extract from cells exposed to the MAO inhibitor tranylcypromine (20 μM) for 15 min before 5-HT (300 μM) was added for another 90 min. External 5-HT and 5-HIAA standards (in the absence of cell extract) were eluted at the positions corresponding to peaks 1 and 2 respectively (not shown). Peaks 3 and 4 represent ADP and ATP respectively. The arrow in (**b**) indicates the time at which 5-hydroxytryptophol would be eluted under the separation conditions used. The traces show the A_{212} of the eluted material.

for 90 min to a combined treatment with PMA (0.8 μM) and 5-HT (300 μM), the resulting rate of glucose transport clearly exceeded that measured in the presence of either agent alone (PMA, 57.5±3.8; 5-HT, 77.8±2.1; PMA+5-HT, 150.3±9.1 pmol/h per mg of protein; 2 experiments, each in triplicate). This observation indicates that the protein kinase C branch of the phosphatidylinositol signalling system, at least, is not responsible for the stimulation of glucose transport by 5-HT.

Finally, 5-HT has been reported to promote the formation of cyclic GMP in neuronal cell lines, both by a fast process (20 s) mediated by 5-HT_3 receptors [39–41], and by a slower process (10 min, with an EC_{50} of 10–30 μM) that is insensitive to antagonists of known 5-HT receptors [39,41]. We have therefore examined the possibility that the rise in glucose transport induced by 5-HT in cardiomyocytes may be preceded or correlated with a release of cGMP. As illustrated in Figure 6(b), 5-HT (100 μM) failed to influence the cGMP concentration over a period of 30 min, i.e. in a time span in which glucose-transport stimulation becomes apparent (Figure 1). It is noteworthy that nitroprusside (100 μM), which caused a marked rise in cGMP in cardiomyocytes (Figure 6b), had no effect on glucose transport when added to these cells for either 15 or 45 min (results not shown).

Involvement of 5-HT metabolism in glucose-transport stimulation

In view of (i) the slow onset of glucose-uptake activation by 5-HT (Figure 1), (ii) the relatively high 5-HT concentrations required (Figure 2), and, (iii) the fact that no 'classical' 5-HT receptor type, nor an adrenergic or dopaminergic receptor, appears to be involved (see the previous section and Figures 3–5 and Figure 6a), we considered the hypothesis that the observed effect of 5-HT may be elicited by some product of 5-HT metabolism in cardiomyocytes. Since 5-HT is known to be degraded by monoamine oxidases (MAO) in various organs, including the heart [42,43], we assessed the influence of potent MAO inhibitors on the action of 5-HT on glucose transport. As illustrated in Table 2, preincubation of myocytes with the MAO-A-selective inhibitor clorgyline (1 μM), as well as the non-selective (MAO-A and MAO-B) inhibitor tranylcypromine (1 μM), completely prevented the stimulation of glucose uptake by 5-HT (Table 2), indicating that a MAO-dependent step may indeed be essential for the stimulating process. In contrast, the MAO-B- specific inhibitor (−)-deprenyl (1 μM) was ineffective (Table 2). Only supra-micromolar concentrations of (−)-deprenyl diminished the response of cardiomyocyte glucose transport to 5-HT, with a pK_i of 5.5±0.2, whereas the pK_i of clorgyline's action was 7.8±0.3, as determined in three independent experiments (results not shown).

Considering, on the one hand, that the oxidation of monoamines also generates H_2O_2, and on the other, that H_2O_2 is a potent stimulator of glucose transport in cardiomyocytes ([13] and Table 2), one may suspect that this agent may mediate the effect of 5-HT on glucose transport. In line with this idea, addition of catalase to the incubation medium depressed the stimulatory action of 5-HT by 50% (Table 2). Higher catalase activities (> 10000 units/ml) did not result in a stronger inhibition (results not shown). Furthermore, exogenous glutathione (which has been shown to be taken up by cardiomyocytes and to

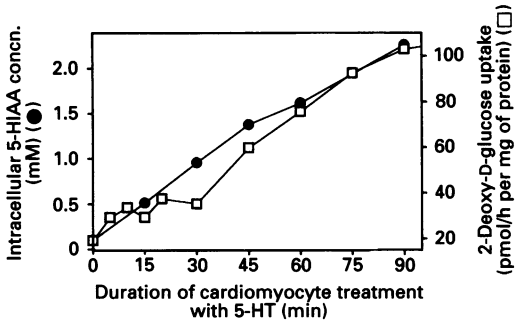

Figure 8 Comparison of the time-dependence of 5-HIAA accumulation and glucose-transport stimulation in cardiomyocytes treated with 5-HT

Cardiomyocytes were exposed to 5-HT (300 μM) for the times indicated before the rate of 2-deoxy-D-glucose transport (□) or the cellular concentration of 5-HIAA (●) was determined as described in the Experimental section. The glucose-transport data are the same as those shown in Figure 1. The 5-HIAA cellular-concentration data are means of two independent experiments (each experiment was done in duplicate).

Table 3 Influence of 5-HT and insulin on the amount of glucose transporters GLUT1 and GLUT4 at the surface of cardiomyocytes

Cardiomyocytes were incubated for 90 min at 37 °C in the absence (control) or in the presence of 5-HT (300 μM) or insulin (10 nM, for the last 30 min of the incubation) before they were subjected to photoaffinity labelling with ^{3}H-ATB-BMPA, and the amount of cell-surface glucose transporters was quantified, as described in the Experimental section. Data shown represent means ± S.E.M. of 5–12 independent experiments, expressed as values normalized to control. The statistical significance of the differences from control was assessed with a paired Student's *t* test; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control.

	Relative amount of glucose transporters at the cell surface (compared with 1.00 in control cells)	
	GLUT1	GLUT4
5-HT	1.79 ± 0.54 (*)	1.54 ± 0.33 (**)
Insulin	2.94 ± 0.38 (***)	6.05 ± 0.72 (***)

reinforce, i.e. to shift their cellular glutathione system towards a reduced state, and thus to improve their anti-oxidative capacity [44]) also diminishes the impact of 5-HT, as well as that of exogenously added H_2O_2 on glucose transport (Table 2) (a concentration of 1 mM glutathione was chosen because it is saturating with respect to the cellular glutathione reduction in these cells [44]). It should be noted that none of these pre-treatments (with MAO inhibitors, catalase or glutathione) affected the basal or the insulin-stimulated rate of glucose transport (Table 2). Thus, these inhibitors seem to be devoid of general unspecific or even toxic effect at the concentrations used. Semicarbazide (1 mM), an inhibitor of the so-called semicarbazide-sensitive amine oxidase, which is distinct from MAO [43], did not alter the glucose-transport response to 5-HT (results not shown).

Finally, we examined the possible appearance of known (MAO-derived) products of 5-HT metabolism, namely 5-HIAA and 5-hydroxytryptophol [32], in extracts from cardiomyocytes previously treated with 5-HT. Analysis of these extracts by reversed-phase HPLC revealed two large peaks that were present in samples from cells treated with 5-HT (300 μM) (Figure 7b, peaks 1 and 2), but not in control samples (Figure 7a). Parallel HPLC runs with standards (without cell extracts) showed that peaks 1 and 2 exactly correspond to the elution times of 5-HT and 5-HIAA respectively (results not shown). In contrast, no peak corresponding to 5-hydroxytryptophol was detected in samples from 5-HT-treated cells (under the elution conditions used, 5-hydroxytryptophol would appear at the time indicated by the arrow in Figure 7b). Addition of a 5-HIAA standard to extracts from control cells resulted in the appearance of peak 2 (Figure 7c). Moreover, when cardiomyocytes were exposed to 5-HT in the presence of tranylcypromine (20 μM), peak 2 disappeared (Figure 7d).

The intracellular 5-HIAA concentration was calculated from the magnitude of peak 2 (by using internal standards, as in Figure 7c) and plotted as a function of the duration of cardiomyocyte treatment with 5-HT. As shown in Figure 8, 5-HIAA accumulates in these cells in a time-dependent manner, and this accumulation approximately parallels the observed increase in glucose transport.

Taken together, these findings suggest that 5-HT is taken up and metabolized to 5-HIAA by cardiomyocytes, and that the resulting H_2O_2 formation eventually leads to the stimulation of glucose transport in these cells.

Effect of 5-HT on glucose-transporter distribution

In a last set of experiments, we have addressed the question whether the stimulation of glucose transport by 5-HT may be explained by recruitment of glucose transporters from an internal storage site to the plasma membrane, as is known to be the case with insulin [45,46]. For this purpose, we used the specific photoaffinity label ^{3}H-ATB-BMPA. This non-permeant compound allows selective tagging, and thus quantification of the amount, of glucose transporters present at the cell surface [23,24]. As shown in Table 3, exposure of cardiac myocytes to 5-HT (300 μM) for 90 min led to a 1.54-fold increase in the content of GLUT4 (the 'insulin-sensitive', i.e. insulin-recruitable, glucose transporter of muscle and fat tissues). In addition, 5-HT recruits the other transporter isoform, GLUT1, to the cell surface by a factor of 1.79 (Table 3). By comparison, a saturating concentration of insulin (the most potent stimulus of glucose transport in these cells) caused a 6.05-fold increase in GLUT4 and a 2.94-fold increase in GLUT1. In the same experiments, 5-HT and insulin enhanced the rate of glucose transport by 3.1- and 12.9-fold respectively. Thus the translocation of glucose transporters to the plasma membrane appears to be, at least partially, responsible for the observed changes in glucose transport.

DISCUSSION

5-HT has been suggested to be involved in the control of glycaemia and glucose metabolism [1,3–11]. The aim of the present study was to examine the effect *in vitro* of 5-HT on glucose utilization in a peripheral tissue, namely cardiac muscle cells. In particular, glucose transport was considered because this process limits the overall utilization of glucose in muscle tissue and is known to be regulated by many hormonal and metabolic factors (such as insulin, contractile activity, hypoxia etc.).

In isolated cardiomyocytes, 5-HT caused a concentration- and time-dependent 4–5-fold enhancement in the rate of glucose transport (Figures 1 and 2). This effect can be partially explained, like that of insulin, by an increase in the amount of glucose transporters (GLUT1, GLUT4) in the plasma membrane of these cells (Table 3). However, several findings indicate that the

stimulation of glucose transport by 5-HT does not occur via the same pathway or signal as the effect of insulin. Thus the effect of 5-HT has a slow onset (Figure 1), whereas that of insulin is maximal after a few minutes [47]. Moreover, the insulin-dependent stimulation of glucose transport is not affected by agents that largely diminish or even suppress the action of 5-HT, such as cAMP-elevating substances ([13], and Table 1), inhibitors of MAOs, catalase or glutathione (Table 2).

Attempts to define the receptor type by which 5-HT impinges on the glucose-transport system of cardiac myocytes remained unsuccessful. Thus the action of 5-HT was not mimicked by selective agonists to known 5-HT receptors such as 5-carboxyamido-tryptamine (5-HT_1 [25,26]), α-methyl-serotonin (5-HT_2 [27,28]), 2-methyl-serotonin (5-HT_3 [27–29]) or renzapride (5-HT_4 [30,31]) (Figure 3). Neither was the concentration-dependence nor the extent of glucose-transport stimulation by 5-HT modified by the 5-HT-receptor antagonists tested: methysergide (5-HT_1/5-HT_2 antagonist [32]), ketanserin or cyproheptadine (5-HT_2 [33,34]), MDL 72222 (5-HT_3 [29,32]) or ICS 205-930 (5-HT_3/5-HT_4 [27,31,35,36]) (Figure 4). All these agonists and antagonists were administered at concentrations above their reported EC_{50} (or pA_2, $= -\log IC_{50}$) or IC_{50}(or pK_i) values (see references quoted above).

Our experiments examining signal-transduction pathways of known 5-HT receptors also indicate that no 'classical' 5-HT receptor mediates the effect of 5-HT on glucose transport. The finding that 5-HT was devoid of effect on the cAMP level in cardiomyocytes (Figure 6a) seems to exclude the involvement of 5-HT_1 receptors (that are negatively coupled to the adenylate cyclase [28]), as well as 5-HT_4 receptors (that are positively coupled to the adenylate cyclase [30]). It should be noted that 5-HT_4 receptors were found in human [38] and pig heart [36] preparations, but we are not aware of reports on 5-HT_4 (or 5-HT_1) receptors in the rat heart. Furthermore, the additivity of the stimulation by 5-HT, on the one hand, with that of the protein kinase C activator PMA, on the other hand, suggests that this kinase is not responsible for the effect of 5-HT on glucose transport; this indirectly suggests that no 5-HT_2 receptors, which are known to cause the breakdown of phosphatidylinositol and the release of the protein kinase C activator diacylglycerol [28], are mobilized in these cells under the experimental conditions used. Finally, this study rules out an involvement of cGMP (Figure 6b), which was shown to mediate a fast 5-HT_3-dependent response to 5-HT [39–41] and a slow response unrelated to classical 5-HT receptors [39,41] in neuronal cell lines.

Although the involvement of the more recently discovered 5-HT_5, 5-HT_6 and 5-HT_7 receptors was not assessed in the present study, the results of these pharmacological approaches, along with the slow onset of glucose transport stimulation by 5-HT and the requirement for relatively high concentrations of the monoamine, led us to consider the possibility that no receptor-derived signal, but rather a product of 5-HT metabolism, may induce the effect observed in cardiomyocytes. We indeed found experimental evidence supporting this hypothesis. First, the effect of 5-HT was completely suppressed by inhibitors of monoamine oxidases (Table 2). Second, the increase in glucose transport correlates with the appearance and accumulation in cardiomyocytes of 5-HIAA (Figures 7 and 8), a metabolite derived from the MAO-dependent degradation of 5-HT [32]. Third, H_2O_2, a known product of monoamine oxidation [43], is a potent stimulator of glucose transport in these cells ([13]; Table 2), and the effect of 5-HT is inhibited by interventions promoting the removal of cellular H_2O_2 and/or inhibiting the effect of exogenous H_2O_2 (addition of catalase or of glutathione; Table 2).

The finding that a MAO-A-selective inhibitor (clorgyline) exhibits a clearly higher efficiency ($pK_i = 7.8$) than does a MAO-B-selective agent [(−)-deprenyl; $pK_i = 5.2$] in depressing the glucose-transport response to 5-HT indicates that the amine is mainly degraded by a MAO-A in these cells. This is consistent with the well-known property of 5-HT to be preferentially deaminated by this MAO type [43]. In addition, MAO-A has been shown to be the predominant form of MAO in the adult rat heart [48]. Our experiments, however, leave the question unsolved by which mechanism 5-HT is taken up in cardiomyocytes. The data obtained only allow us to rule out a receptor-mediated endocytosis involving classical 5-HT_1–5-HT_4 receptors (Figure 4), dopaminergic (Figure 5a) or adrenergic receptors (Figure 5b). Recently, expression of specific 5-HT transporters (all encoded by a single gene) has been demonstrated in peripheral tissues, including lung, placenta, adrenal medulla and platelets [59], and it is conceivable that a similar protein may be responsible for the uptake of 5-HT in cardiac myocytes, although it is so far unknown whether this type of transporter is also present in the heart.

An additional observation of potential interest is that 5-HT analogues (tryptamine, 5-methoxytryptamine) and dopamine caused an increase in glucose transport, of the same extent, and with a similar time- and concentration-dependence, as that produced by 5-HT (Figures 1 and 2). This indicates that the MAO-dependent mechanism operating with 5-HT may be activated by a number of other monoamines which are known to be good substrates for MAOs [43]. In line with this idea, we recently found that the stimulation of cardiomyocyte glucose transport by adrenaline or noradrenaline, which is mediated by α_1-adrenoreceptors [49], also comprises a slow component and is partially counteracted by MAO inhibitors (Y. Fischer and J. Thomas unpublished work).

Further investigations will be required to clarify the possible relevance of the MAO-dependent stimulation of glucose uptake found in cardiomyocytes to the serotonergic effects on glycaemia observed *in vivo*. For instance, it should be established whether a similar mechanism exists in skeletal muscles, which represent a major site of glucose disposal. In this context, it is noteworthy that H_2O_2 also has a stimulatory action on glucose transport in skeletal muscle [50]. Furthermore, the question arises as to whether local concentrations of monoamines could be sufficient in heart (and muscle) tissue to sustain a high rate of H_2O_2 formation. This may, in particular, hold for situations of increased monoamine release, such as sympathetic stimulation, or platelet activation. However, we are not aware of studies in which changes in 5-HT content have been quantified in heart and muscles under such conditions. It is nevertheless noteworthy that the cardiac concentrations of other monoamines such as adrenaline and noradrenaline are in the micromolar range in rats [51]. On the other hand, the generation of H_2O_2 in the respiratory chain of animal mitochondria [52], and of heart mitochondria in particular, is well established and has been shown to be increased, depending on the energy substrates utilized [53]. Thus this additional source of H_2O_2 may lower the 5-HT or monoamine concentrations required to induce a significant enhancement of glucose uptake in heart and/or muscles.

Finally, beyond the regulation of glucose metabolism, our finding of a MAO-dependent effect of 5-HT may be of more general interest. For instance, one should consider the possibility that a similar pathway may mediate some other effects of 5-HT that were reported to necessitate relatively high concentrations of the amine, and/or that could not be ascribed to a classical 5-HT receptor type [39,41,54–58]. It is noteworthy that some of these effects impinge on the myocardial function, namely contractility [54,55].

To summarize, the stimulation of glucose transport induced by 5-HT in isolated cardiac myocytes does not appear to occur via a classical 5-HT receptor (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4), nor via a dopaminergic or aminergic receptor. Our data strongly suggest that 5-HT is metabolized by these cells through a MAO-dependent pathway, and that H_2O_2 produced in the course of this 5-HT degradation eventually causes the activation of the glucose transport system. The last step of this process consists of a recruitment of glucose transporters (GLUT1, GLUT4) to the plasma membrane.

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