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Methylxanthines inhibit glucose transport in rat adipocytes by two independent mechanisms

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The glucose transport system of the fat cell is regulated by both antilipolytic and lipolytic agents. Insulin stimulates glucose transport by a translocation of transporter molecules from an intracellular pool to the plasma membrane [1, 2]. More recent observations suggest that glucose transport is negatively regulated by lipolytic hormones and drugs [3–8]. Since this effect counteracts the activation of glucose transport by insulin, it might explain, in part, the reduced responsiveness to insulin observed in patients with type II-diabetes. Lipolytic hormones (catecholamines, ACTH, glucagon) presumably reduce the intrinsic activity of the hexose transporter by a temperature-sensitive and time-dependent mechanism [4–7]. Initially the rise in cellular cAMP was postulated as the underlying mechanism for glucose transport inhibition by all lipolytic agents. However, subsequent studies suggest that some lipolytic agents display inhibitory properties that differ from the transport inhibition elicited by the lipolytic hormones which is characterized by a lag phase of 1–2 min [5, 6]. The lipolytic agents forskolin [5, 8] and isobutylmethylxanthine (IBMX) [5, 6] inhibit glucose transport in intact adipocytes immediately following addition. In addition, forskolin and IBMX but not catecholamines inhibit glucose transport in membrane vesicles and compete with cytochalasin B for its D-glucose-suppressible binding sites on the adipocyte membrane [5, 8]. Therefore, the site of action of forskolin and IBMX seems to be closer to the glucose transporter than that of ACTH and the catecholamines. Lipolytic hormones might inhibit glucose transport via one or more intermittent nucleotide regulatory proteins or a signal cascade ending in the activation of a kinase, e.g. as is the case in lipolysis activation. These steps do not seem to be important for the immediate inhibitory effect by forskolin and IBMX.

In the present study we investigated the effects of the methylxanthines, caffeine and theophylline, on glucose transport in insulin-stimulated rat adipocytes. These observations were compared to the inhibitory effect of the lipolytic hormones. The results of this study suggest that the inhibition of glucose transport by caffeine and theophylline is the result of two mechanisms since these agents behave to some extent like lipolytic hormones and also very similar to the more direct acting drug forskolin. Therefore glucose transport inhibition by theophylline and caffeine does not necessarily represent a single mechanism but may best be interpreted as the consequence of at least two different inhibitory mechanisms.

Materials and Methods

Methyl-1-[^{3}H]D-glucose and [^{3}H]cytochalasin B were obtained from Amersham Buchler (Braunschweig, F.R.G.). 8-Phenyltheophylline was purchased from Aldrich (Steinheim, F.R.G.). Enprofylline was a generous gift from AB Draco (Sweden). All other reagents were from Merck AG (Darmstadt, F.R.G.).

Fat cells from the epididymal adipose tissue of fed Wistar rats (160–200 g) were isolated by collagenase digestion [9]. Cells were incubated at 37° in a Krebs–Ringer–Hepes buffer supplemented with 1% bovine serum albumin and glucose (1 mM). Glucose transport was measured with methylglucose [10] as described [6]. The uptake was stopped after 3 sec by the addition of phloretin (0.33 mM; pH 7.4). Initial uptake rates were calculated from the uptake values U_t and U_{max} according to the equations $-kt = \ln(U_{max} - U_t) - \ln(U_{max})$ and $v_i = k^* U_{max}$ [11]. Usually the tested agents were added immediately (less than 5 sec) prior to the transport measurement. In some experiments this preincubation time was varied as indicated.

Plasma membranes from insulin-stimulated adipocytes were prepared by differential centrifugation [1]. Cytochalasin B binding was assayed in the presence of either 200 mM D-sorbitol (total binding) or 200 mM D-glucose (D-glucose-insensitive binding).

Results and Discussion

Caffeine and theophylline inhibited the uptake of [^{3}H]methylglucose in insulin-treated rat adipocytes in a concentration dependent manner (Fig. 1A). Both agents displayed this effect immediately following addition whereas in a previous study the inhibition of hexose transport by lipolytic hormones (ACTH, catecholamines) developed after a 1–2 min lag phase with a half-time of 4 min [6]. Glucose transport was inhibited by 90–95% at a concentration of 10 mM of either theophylline or caffeine. The IC_{50} values were 1.1 ± 0.1 mM (theophylline) and 1.4 ± 0.1 mM (caffeine). We next compared the transport inhibition produced by caffeine after 5 sec and after 10 min (Fig. 1B). The inhibitory potency of caffeine increased significantly after the 10-min treatment (IC_{50} 1.4 ± 0.1 mM vs 0.45 ± 0.08 mM after 10 min) suggesting that a second delayed inhibitory effect contributed to the inhibition after the 10-min treatment. Theophylline and caffeine were

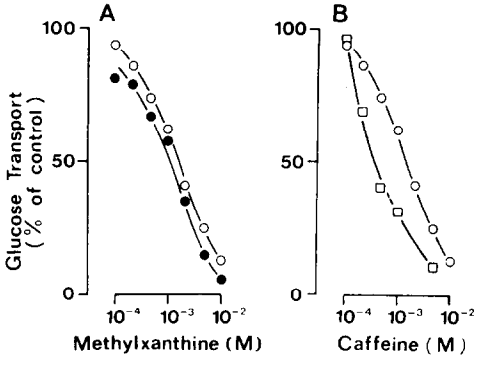

Fig. 1. (A) Concentration-dependent effect of theophylline (●) and caffeine (○) on glucose transport. Insulin-stimulated adipocytes were exposed to the indicated concentrations of the methylxanthines for 5 sec followed by labeled methylglucose (0.5 μCi, 0.15 mM) for a further 3 sec. Transport rates derived from seven experiments are expressed as a percentage of the control rate. (B) Comparison of the transport inhibitory effects of caffeine treatment for 5 sec (○; as in panel A) vs inhibition 10 min after caffeine addition (□; N = 6).

present for about 8 sec (5 sec preincubation, 3 sec transport) when the so-called immediate inhibition (Fig. 1A) was tested. The transport inhibition observed at that timepoint might either reflect a situation during the development of a time-dependent decrease of the transport rate or a state of inhibition that is established immediately. To test these two possibilities we next investigated the initial uptake of glucose during even shorter periods of theophylline treatment (3–9 sec). When theophylline (1 mM) was added 0, 3 and 6 sec prior to the tracer, the inhibition observed after the treatment periods of 3, 6 and 9 sec were identical (45%). Thus, the so-called immediate inhibition reflects a fully established level of glucose transport inhibition.

The development of the delayed inhibition was investigated further with caffeine and theophylline at a concentration of 1 mM at time points 1, 2, 3, 5 and 10 min. Glucose transport was inhibited by 45% in the immediate phase. A further 25% inhibition of glucose transport was observed after 5–10 min of incubation. This is shown in Fig. 2 for cells treated with theophylline (filled circles). Identical results were observed with caffeine (data not shown). The delayed or second inhibitory effect developed after a lag phase of about 1 min with a half-time of 3 min. The maximal inhibition (70%) was observed after 5 min. The time-course of this second component of methylxanthine action on hexose transport is very similar to the development of the inhibition observed with the lipolytic hormone ACTH [6]. The simultaneous addition of the antilipolytic hormone prostaglandin E_2 completely antagonized the inhibitory effect of ACTH [6]. Therefore, we tested PGE_2 for its effect on the inhibition of glucose transport by caffeine and theophylline. When added together with either methylxanthine PGE_2 did not significantly affect the immediate component of the transport inhibition (Fig. 2; circles at timepoint 0). On the other hand, the prostaglandin completely prevented the development of the second component elicited by theophylline (Fig. 2; open circles) and caffeine (data not shown). Glucose transport was maximally inhibited by 70% in the absence of PGE_2 and by 50% in the presence of the prostaglandin.

Therefore, we postulate that two independent mechanisms contribute to the inhibition of glucose transport by theophylline and caffeine. The second, delayed component of the inhibitory effect resembled the negative regulation of glucose transport by the lipolytic hormones (catecholamines, ACTH). This effect is thought to be elicited either by the activation of rat fat cell adenylate cyclase [12] or the interaction with nucleotide regulatory proteins (G-proteins) that might be part of the regulation of glucose transport in adipose tissue [13]. Methylxanthines may mimic both of these effects by their antagonism at the A_1-adenosine receptor [14, 15]. In order to determine the contribution of this site of action we tested the more specific adenosine receptor antagonist 8-phenyltheophylline for its effect on insulin-stimulated glucose transport. In contrast to caffeine and theophylline this alkylxanthine did not inhibit glucose transport immediately following its addition (Fig. 3). However, 8-phenyltheophylline inhibited the transport rate in a concentration dependent manner when tested after a 10-min incubation (Fig. 3; IC_{50} 1.1 ± 0.4 μM; maximal inhibition 35%; N = 3). The time-course of glucose transport inhibition by a maximally effective concentration of 8-phenyltheophylline (20 μM, data not shown) is identical to that of the development of the delayed inhibition seen with theophylline (Fig. 2). Next we tested enprofylline, an alkylxanthine that is a less potent adenosine receptor antagonist than theophylline [16], for its effect on glucose transport. This agent produced an immediate inhibition of glucose transport with the same efficacy but an even higher potency when compared to theophylline and caffeine (IC_{50} of enprofylline 0.4 and 0.6 mM in two separate experiments vs 1.1 ± 0.1 mM theophylline, N = 7, Fig. 1A). The data obtained with enprofylline and 8-phenyltheophylline indicate that the adenosine antagonistic properties of alkylxanthines are not involved in the mechanism mediating the immediate inhibition of glucose transport by lipolytic methylxanthines. On the other hand, these properties seem to be mediating the second component of the inhibition seen with caffeine and theophylline.

The immediate inhibitory action accounts for about 65–75% of the total inhibition observed with theophylline and caffeine at a concentration of 1 mM. It resembles the transport inhibition produced by agents like forskolin [8], furosemide [17] and dipyridamole [18] which are suggested to interact more directly with the hexose transporter. A characteristic of these transport inhibitors supporting this hypothesis is their competition with the ligand of the glucose transporter, cytochalasin B, for its D-glucose-suppressible binding sites. Lipolytic hormones have not been reported to affect cytochalasin B binding to the adipocyte membrane. To test whether the immediate phase of glucose transport inhibition can be attributed to such a direct interaction with the glucose transporter we tested theophylline and caffeine for their effect on cytochalasin B binding. Both agents reduced the binding of [^{3}H]cytochalasin B

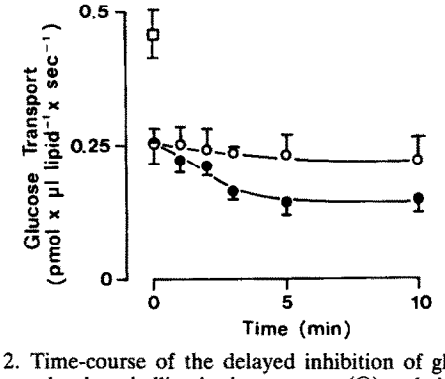

Fig. 2. Time-course of the delayed inhibition of glucose transport by theophylline in the presence (○) and absence (●) of PGE_2 (2.8 μM). Insulin-pretreated adipocytes were exposed to the two treatment regimes for the indicated time periods. Glucose transport was subsequently measured. The square (□) represents the transport rate of untreated cells. The values of the treated cells at timepoint 0 represent the immediate inhibitory effect. Data are means ± SE of eight experiments.

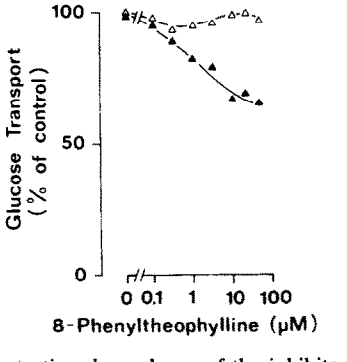

Fig. 3. Concentration-dependence of the inhibitory effect of 8-phenyltheophylline on insulin-stimulated glucose transport. After pretreatment with insulin cells were treated with 8-phenyltheophylline either for 5 sec (△, N = 2) or 10 min (▲, N = 3). The transport rates are presented as a percentage of the respective control rates.

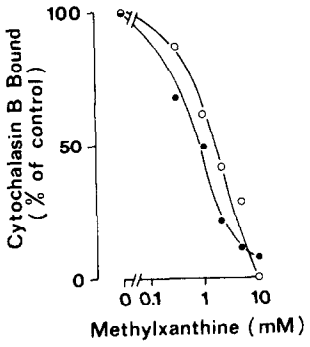

Fig. 4. Cytochalasin B binding to plasma membranes of insulin-treated adipocytes in the presence of theophylline (●, N = 3) or caffeine (○, N = 5). The binding of [^{3}H]cytochalasin B was measured either in the presence of D-sorbitol (200 mM) or D-glucose (200 mM). The amount bound to D-glucose-suppressible sites is expressed as a percentage of the respective binding to untreated membranes.

to its D-glucose-suppressible binding sites on the plasma membranes of insulin-treated fat cells in a concentration dependent manner (Fig. 4). The IC_{50} values for the competition were 1.1 mM (theophylline) and 1.6 mM (caffeine). These values are in good agreement with the IC_{50} values for hexose transport inhibition in the intact cell (Fig. 1A). This demonstrates that the site of the immediate action of theophylline and caffeine does not seem to involve the adenosine receptor and/or G-proteins but is located at or near the glucose transporter itself.

In summary, this study characterized the biphasic inhibition of fat cell glucose transport by the lipolytic agents caffeine and theophylline. Like the lipolytic drug forskolin, both methylxanthines produced an immediate inhibition of glucose transport that was not seen with 8-phenyltheophylline, a pure adenosine receptor antagonist. The immediate inhibition was therefore not mediated by the adenosine receptor antagonism but seems to be due to a direct interaction with the hexose transporter. This conclusion is supported by the immediate onset of the inhibition and additionally by the interference of theophylline and caffeine with the binding of cytochalasin B, a ligand of the glucose transporter that binds to an intracellular site of the transporter molecule [19]. In addition, a second, delayed inhibitory effect of theophylline and caffeine on glucose transport was observed. This portion shared many aspects of the inhibitory effect of lipolytic hormones. It developed over a period of about 5 min and was antagonized by the simultaneous addition of the antilipolytic hormone PGE_2. This component of transport inhibition could be attributed to the antagonistic effect of methylxanthines at the fat cell A_1-adenosine receptor since it was also seen with 8-phenyltheophylline. This conclusion is further supported by data showing that the removal of endogenous adenosine with adenosine deaminase resulted in a comparable 25–30% inhibition of insulin-stimulated glucose transport [4, 13]. In addition, the time course of glucose transport inhibition by the subsequent addition of adenosine deaminase [13] is similar to that of the delayed portion of the inhibition seen with theophylline and caffeine. Both treatments produced their maximal inhibition after 5 min.

In conclusion, the methylxanthines theophylline and caffeine inhibit glucose transport by a combination of two different modes of action. The immediate major component is mediated via a direct interaction with the hexose transporter whereas the delayed component involves adenosine receptor antagonism and thereby the interaction with G-proteins.

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REFERENCES

1. Cushman SW and Wardzala LJ, Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. *J Biol Chem* **255**: 4758–4762, 1980.
2. Suzuki K and Kono T, Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc Natl Acad Sci USA* **77**: 2542–2545, 1980.
3. Green A, Glucagon inhibition of insulin-stimulated 2-deoxyglucose uptake by rat adipocytes in the presence of adenosine deaminase. *Biochem J* **212**: 189–195, 1983.
4. Smith U, Kuroda M and Simpson IA, Counter-regulation of insulin-stimulated glucose transport by catecholamines in the isolated rat adipose cell. *J Biol Chem* **259**: 8758–8763, 1984.
5. Kashiwagi A, Hueckstaedt TP and Foley JE, The regulation of glucose transport by cAMP stimulators via three different mechanisms in rat and human adipocytes. *J Biol Chem* **258**: 13685–13692, 1983.
6. Steinfelder HJ, Schramm S and Joost HG, Prostaglandin E_2 differentiates between two forms of glucose transport inhibition by lipolytic agents. *Naunyn-Schmiedebergs Arch Pharmacol* **336**: 105–110, 1987.
7. Joost HG, Weber TM, Cushman SW and Simpson IA, Insulin-stimulated glucose transport in rat adipose cells. Modulation of transporter intrinsic activity by isoproterenol and adenosine. *J Biol Chem* **261**: 10033–10036, 1986.
8. Joost HG and Steinfelder HJ, Forskolin inhibits insulin-stimulated glucose transport in rat adipose cells by a direct interaction with the glucose transporter. *Mol Pharmacol* **31**: 279–283, 1987.
9. Rodbell M, Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* **239**: 375–380, 1964.
10. Whitesell RR and Gliemann J, Kinetic parameters of transport of 3-*O*-methylglucose and glucose in adipocytes. *J Biol Chem* **254**: 5276–5283, 1979.
11. Foley JE, Cushman SW and Salans LB, Glucose transport in isolated rat adipocytes with measurements of L-arabinose uptake. *Am J Physiol* **234**: E112–E119, 1978.
12. Taylor WM, Mak ML and Halperin ML, Effect of 3′:5′-cyclic AMP on glucose transport in rat adipocytes. *Proc Natl Acad Sci USA* **73**: 4359–4363, 1976.
13. Kuroda M, Honnor RC, Cushman SW, Londos C and Simpson IA, Regulation of insulin-stimulated glucose transport in the isolated rat adipocyte. cAMP-independent effects of lipolytic and antilipolytic agents. *J Biol Chem* **262**: 245–253, 1987.
14. Londos C, Cooper DMF, Schlegel W and Rodbell M, Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP dependent process: basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. *Proc Natl Acad Sci USA* **75**: 5362–5366, 1978.

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15. Trost T and Schwabe U, Adenosine receptors in fat cells. Identification by (−)-N⁶-[³H]phenylisopropyladenosine binding. *Mol Pharmacol* **19**: 228–235, 1981.
16. Persson CGA, Andersson KE and Kjellin G, Effects of enprofylline and theophylline may show the role of adenosine. *Life Sci* **38**: 1057–1072, 1986.
17. Jacobs DB, Mookerjee BK and Jung CY, Furosemide inhibits glucose transport in isolated rat adipocytes via direct inactivation of carrier proteins. *J Clin Invest* **74**: 1679–1685, 1984.
18. Steinfelder HJ and Joost HG, Inhibition of insulin-stimulated glucose transport in rat adipocytes by nucleoside transport inhibitors. *FEBS Lett* **227**: 215–219, 1988.
19. Gorga FR and Lienhard GE, Equilibria and kinetics of ligand binding to the human erythrocyte glucose transporter. Evidence for an alternating conformation model for transport. *Biochemistry* **20**: 5108–5113, 1981.

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In vivo and *in vitro* inhibition of platelet aggregation by SV-IV, a major protein secreted from the rat seminal vesicle epithelium

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SV-IV* (seminal vesicle protein No. 4, according to its mobility in SDS–PAGE) is a basic, thermostable protein of low M_r (9758) synthesized and secreted under strict testosterone control from the rat seminal vesicle epithelium. The sequence of its 90 amino acids has been determined and much is known on the molecular biology of the SV-IV gene [1–8]. Computer and immunological analyses have shown that uteroglobin, a progesterone induced and binding protein widespread in many tissues and body fluids of rabbits, presents a significant degree of homology with SV-IV [9]. In addition, both uteroglobin and SV-IV were shown to possess anti-inflammatory properties [10–12] and have been found to effectively inhibit chemotaxis and phagocytosis of macrophages and neutrophils [10, 13, 14]. The mechanism of action at the basis of all these effects seems to be related, at least in part, to the inhibition of phospholipase A_2 (PLA_2, EC 3.1.1.4) [12, 15] and consequently to a block of the arachidonic acid (AA) cascade. More recently, it has been demonstrated that uteroglobin inhibits thrombin-induced platelet aggregation *in vitro* and it has been suggested that this effect was due to the inhibitory action of the protein on PLA_2 [16]. To determine whether SV-IV also possesses anti-thrombotic effects, we examined the ability of the protein to inhibit platelet aggregation induced by different agents (thrombin, ADP, collagen, platelet activating factor (PAF) and AA) both *in vivo* and *in vitro*.

Materials and Methods

Materials. The protein SV-IV was purified to homogeneity from adult rat (Fisher–Wistar) seminal vesicle secretion as described by Ostrowski *et al.* [1], and its purity was evaluated by 15% PAGE, in denaturing and non-denaturing conditions, and by amino acid composition analysis [9].

Bovine plasma thrombin (285 NIH units/mg protein), equine muscle ADP sodium salt (grade IX), human placenta collagen (type VI), porcine liver AA free acid (approx. 99%), and PAF (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bovine pancrease ribonuclease A was from Pharmacia (Uppsala, Sweden); indomethacin (Liometacen) was from Chiesi Farmaceutici (Milan, Italy).

In vivo *experiments of platelet aggregation in rats*. Male Wistar rats (200–250 g) were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). Platelet aggregation was induced by i.v. (jugular vein) administration of 1 unit/kg of thrombin, 1 mg/kg of ADP, 2 mg/kg of collagen, 0.75 μg/kg of PAF, or 1.5 mg/kg of AA, essentially according to the method previously described by Pinon [17]. Blood samples were collected from the carotid artery 3 min after thrombin, collagen or AA injections, and 0.5 min after ADP or PAF injections, into two plastic disposable syringes (0.4 mL of blood/syringe). One syringe contained 1.6 mL of EDTA + formalin buffer (EDTA tetrasodium salt 24 mM, KH_2PO_4 1.3 mM, Na_2HPO_4 5.4 mM and formalin (40% sol) 2.5%) (sample A), whereas the second one contained 1.6 mL of EDTA buffer (EDTA 24 mM, KH_2PO_4 3.3 mM, Na_2HPO_4 13.4 mM) (sample B). After mixing, the samples were transferred to polystyrene tubes and allowed to stand for 10 min before centrifugation (350 *g* for 10 min). In sample A, aggregates were fixed by formalin, whereas in sample B they were dissociated and the platelets were made unaggregable by EDTA. Platelets were counted by light microscopy and platelet-count ratio was calculated as platelet count in the sample A/platelet count in the sample B. The anti-thrombotic effect of SV-IV was assayed by i.v. (jugular vein) injection of appropriate amount of protein dissolved in 0.2 mL of saline, 3 min before the administration of the aggregating agents. Controls were carried out by injecting saline or ribonuclease (1.5 mg/kg) under the same experimental conditions.

Platelet aggregation in vitro. Blood was collected from ether-anesthetized male rabbits (2.5–3 kg) by intracardiac puncture and anticoagulated with 0.38% trisodium citrate. The platelet-rich plasma was obtained by centrifuging the blood at 350 *g* for 10 min after dilution with platelet-poor plasma to obtain 4×10^8 cells/mL, and used for ADP aggregation studies. In addition, for platelet aggregation induced by thrombin, collagen, PAF and AA, the platelet-rich plasma was preliminarily washed with 0.38% trisodium citrate and centrifuged at 550 *g* for 15 min. The platelets contained in the pellet were then resuspended in Tyrode's solution without calcium (4×10^8 cells/mL). All aggregations were performed in aggregometer cuvettes containing 0.25 mL of platelet suspension. SV-IV was added in saline and preincubated with platelets for 3 min at 37°. Thrombogenic agents were then added and the aggregation measured at 37° in a ELVI 840 aggregometer. Control aggregation curves were obtained in the absence of SV-IV

* Abbreviations: SV-IV, seminal vesicle protein No. 4; PLA_2, phospholipase A_2; AA, arachidonic acid.