Characterization of antilipolytic action of polyamines in isolated rat adipocytes

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The interactions of polyamines with the lipolytic system were studied in isolated rat adipocytes. Spermine, spermidine and putrescine significantly inhibited adenosine deaminase-stimulated lipolysis. An antilipolytic effect of spermine was detectable at a concentration of 0.25 mM ($P < 0.05$). At a concentration of 10 mM all three polyamines inhibited the stimulated lipolysis by 50–60% ($P < 0.001$). In addition, spermine enhanced the antilipolytic sensitivity of insulin. Spermine (1 mM) decreased the half-maximal inhibitory concentration of insulin from 320 ± 70 pM to 56 ± 20 pM ($P < 0.01$). The antilipolytic effects and the cyclic-AMP-lowering effects of the polyamines were almost completely prevented in the presence of different phosphodiesterase (PDE) inhibitors (3-isobutyl-1-methylxanthine and RO 20-1724) and, in addition, polyamines had no effect on lipolysis stimulated by dibutyl cyclic AMP, indicating that polyamines may inhibit lipolysis by activating the PDE enzyme. This latter suggestion was confirmed by demonstrating that spermine (5 mM) significantly enhanced the low- K_m PDE enzyme activity ($P < 0.01$). Finally, the amounts of polyamines present in isolated adipocytes were measured, and the estimated cytoplasmic concentrations were 0.02 mM (putrescine), 0.86 mM (spermidine), and 1.0 mM (spermine). It is concluded that polyamines may possibly be involved in the physiological regulation of triacylglycerol mobilization in adipocytes.

INTRODUCTION

The polyamines spermidine and spermine are organic polycations essential for cellular growth and function (Heby, 1981). Their exact physiological roles and mechanisms of actions are, however, poorly understood (Tabor & Tabor, 1984). In mammalian cells spermidine and spermine are usually present in millimolar concentrations (Russell, 1983). Their levels are elevated in rapidly proliferating cells such as regenerating liver, embryonic tissue and tumour cells (Tabor & Tabor, 1984; Pegg, 1986). Polyamines have been found to interfere with the activity of many enzyme systems, by mechanisms that are not fully understood (Tabor & Tabor, 1984). In addition, it has been demonstrated that polyamines may modulate the receptor binding of some hormones (D'Abronzio *et al.*, 1985; Swift & Dias, 1986), including the insulin-receptor binding in isolated adipocytes (Pedersen *et al.*, 1989). Using fluorescence cytochemistry and immunocytochemistry, we have recently found strong reactivity for spermidine and spermine in the thin layer of cytoplasm in rat adipocytes, which demonstrates that these polyamines are present in adipocytes (Pedersen *et al.*, 1989).

In order to examine further the possible biological function of polyamines in adipose tissue we have investigated the effect of polyamines on lipolysis, cyclic AMP (cAMP) accumulation and phosphodiesterase (PDE) activity in isolated adipocytes.

MATERIALS AND METHODS

Adenosine, adenosine deaminase (ADA), isoprenaline (isoproterenol), 3-isobutyl-1-methylxanthine (IBMX), polyamines, prostaglandin E_2 (PGE_2), dibutyl cAMP and human serum albumin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Crude collagenase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Insulin was kindly donated by Novo (Copenhagen, Denmark). D-4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidione (RO 20-1724) was kindly given by Boehringer (Mannheim, Germany). A radioimmunoassay kit for cAMP was obtained from New England Nuclear (Dreieich, Germany). [3H]cAMP (sp. radioactivity 33 Ci/mmol) was from Amersham (U.K.).

Preparation of isolated fat-cells

The cells were prepared from epididymal adipose tissue of male Wistar rats (175–225 g) fasted overnight as previously described (Richelsen & Pedersen, 1985). Minced adipose tissue was digested with collagenase (0.5 mg/ml) for 45–60 min at 37 °C by gently shaking. Moreover, as proposed by Honnor *et al.* (1985), 200 nM-adenosine was included in the isolation buffer. The adipocytes were filtered and washed four times in the incubation buffer. The size and concentration of adipocytes were determined as previously described (Richelsen *et al.*, 1984).

Abbreviations used: cAMP, cyclic AMP; PDE, phosphodiesterase; ADA, adenosine deaminase; IBMX, 3-isobutyl-1-methylxanthine; PGE_2 , prostaglandin E_2 .

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Studies on lipolysis

Duplicate incubations were carried out with 175 μ l of adipocyte suspension (lipocrit 10%) in 10 mM-Hepes buffer (Richelsen & Pedersen, 1985) containing 2.5% (w/v) human serum albumin and ions as previously described together with adenosine (200 nM) (Honnor *et al.*, 1985). Adipocytes were preincubated for 15 min with the polyamines together with the indicated agents such as IBMX, RO 20-1724, PGE₂ and insulin. At the end of the preincubation period the lipolysis was stimulated by ADA (0.5 unit/ml) or isoprenaline as indicated. The incubation was continued for another 90 min at 37 °C with gentle shaking. The incubations were terminated by removing the adipocytes by centrifugation through silicone oil (Richelsen *et al.*, 1984). The release of glycerol was taken as an index of lipolysis. Glycerol was enzymically determined by a fluorimetric method (Peridochrom; Boehringer Mannheim). In later experiments it was found that the actions of polyamines were similar whether or not a 15 min preincubation period was used.

cAMP accumulation

The adipocyte incubations were performed essentially as previously described for human adipocytes (Richelsen & Sørensen, 1987). Briefly, the adipocytes were preincubated with spermine for 15 min in the same buffer as for glycerol release, then lipolysis was stimulated with ADA (0.5 unit/ml) and isoprenaline. The incubations were terminated exactly 10 min after addition of isoprenaline. cAMP extracted from the cells and medium was measured by radioimmunoassay (Richelsen & Sørensen, 1987).

PDE assay

Adipocytes were as described above incubated in the absence or the presence of 5 mM-spermine for 90 min in a parallel experiment. The adipocytes were then homogenized in Tris/HCl buffer (Manganiello & Vaughan, 1973). When the fat-cake was removed, the homogenate was sonicated for 10 s and centrifuged at 2000 *g* (4 °C) for 10 min. The upper liquid phase was used in the PDE assay.

The activity of the PDE enzyme was measured with [³H]cAMP in a two-step radioisotopic procedure involving measuring the end product [³H]adenosine after conversion of 5'-AMP into adenosine by the nucleotidase in snake venom (*Crotalus atrox*) (Beavo *et al.*, 1970; Wells *et al.*, 1975). Briefly, 25 μ l of PDE enzyme preparation was added to an assay mixture containing 50 mM-Tris, 3 mM-MgCl₂, 50–200 nM-[³H]cAMP and unlabelled cAMP at increasing concentrations (50–1000 nM) for 30 min at 30 °C. Then snake venom (2 mg/ml) was added, and the mixture incubated for a further 10 min at 30 °C. This reaction was terminated by addition of 750 μ l of 0.1 mM-adenosine. The [³H]adenosine formed during the reaction was separated from other nucleotides on QAE-Sephadex A25 (formate form). The column was eluted with ammonium formate (pH 7.4), and samples of the effluent were counted for radioactivity by liquid scintillation.

Polyamine measurements

Chemical analysis for polyamines was performed on 800 μ l of adipocyte suspension (lipocrit 20%) incubated

for 5 or 90 min. The incubations were terminated by adding 8 ml of ice-cold saline and 1.5 ml of silicone oil to the samples. The cells were separated from the medium by centrifugation into the chilled silicone oil. Subsequently, 500 μ l of ice-cold 0.2 M-HClO₄ containing a known amount of 1,6-diaminohexane (internal standard) was added to the cells, and the samples were shaken for 1 h at 4 °C before they were defatted with iso-octane and light petroleum (b.p. 35–60 °C). After centrifugation, 200 μ l samples of the aqueous phase were dansylated and assayed for polyamines by h.p.l.c. as previously described (Hougaard *et al.*, 1986).

The HClO₄ precipitates were resuspended in 1 M-NaOH and assayed for protein, with bovine serum albumin as standard (Lowry *et al.*, 1951).

Statistics

Data are given as means $\pm$ 1 S.E.M. In comparative studies significance was determined by using Student's paired *t* test.

RESULTS AND DISCUSSION

The polyamines inhibited ADA-stimulated lipolysis in adipocytes in a dose-dependent manner (Fig. 1). In the lower concentration range a significantly antilipolytic effect of spermine was observed at concentrations of 0.25 mM ($n = 5$, $P < 0.05$). Spermine and spermidine were more antilipolytic than their precursor, putrescine. At high concentrations (5–10 mM) both spermine and spermidine inhibited the ADA-stimulated lipolysis by 50–60% ($n = 8$, $P < 0.001$).

It has previously been suggested that polyamines may have antilipolytic actions (Lockwood *et al.*, 1971). The effect was, however, later ascribed to H₂O₂, produced by amine oxidase-catalysed oxidation of polyamines in the incubation buffer (Livingston *et al.*, 1977; Little & de Häen, 1980). The replacement of bovine serum albumin with human serum albumin in the incubation buffer abolished the production of H₂O₂ (Livingston *et al.*,

Fig. 1. Action of polyamines on ADA-stimulated lipolysis

Adipocytes were preincubated for 15 min with spermine (●), spermidine (○) or putrescine (□); lipolysis was then stimulated by ADA (0.5 unit/ml) and the incubation continued for further 90 min at 37 °C. Results are means $\pm$ 1 S.E.M. for 5–8 experiments.

Fig. 2. Interaction of spermine with isoprenaline-induced lipolysis

Adipocytes were preincubated for 15 min with (●) and without (○) spermine (5 mM), and then lipolysis was stimulated by increasing concentrations of isoprenaline for 90 min. Results are means $\pm$ 1 S.E.M. ($n = 5$); $*P < 0.01$.

1977; Cascieri *et al.*, 1979). Consequently, all our studies were performed in buffer containing human serum albumin. Moreover, our results were unaffected by the presence of the amine oxidase inhibitor aminoguanidine (2 mM) and catalase (0.02 mg/ml) (results not shown). Thus the present effects of polyamines cannot be ascribed to H_2O_2 formation.

Different pharmacological tools were used to determine at which step(s) the polyamines act on the lipolytic cascade. It was found that spermine (1–5 mM) was without antilipolytic properties when lipolysis was stimulated by the cAMP analogue dibutyryl cAMP (results not shown). In contrast, it was found that spermine (5 mM) had a small but significantly inhibitory effect on isoprenaline-induced lipolysis, except at very high concentrations of isoprenaline (10 μ M) (Fig. 2). Moreover, it was found that spermine had no effects on lipolysis stimulated with the PDE inhibitor IBMX (results not shown). Interestingly, IBMX and another PDE inhibitor, RO 20-1724, almost completely prevented the antilipolytic effect of spermine on the ADA-stimulated lipolysis (Fig. 3). In the absence or presence of RO 20-1724 (200 μ M), the antilipolytic effect of spermine (5 mM) was $51 \pm 10\%$ and $12 \pm 3\%$ respectively ($P < 0.01$). These findings could be extended to the action of spermine on cAMP accumulation. In these experiments IBMX (200 μ M) was also able to prevent the observed inhibitory effect of spermine on isoprenaline-stimulated cAMP accumulation. Spermine (10 mM) inhibited cAMP accumulation by $64 \pm 5\%$ (control) or $11 \pm 3\%$ (IBMX) ($P < 0.01$; Fig. 4). On the other hand, IBMX had no effects on the cAMP-lowering effect of PGE_2 , indicating that the action of IBMX was specific for the antilipolytic properties of polyamines.

All these findings suggest that polyamines presumably affect lipolysis by interacting with the PDE activity.

Fig. 3. Effects of PDE inhibition on the action of spermine

Adipocytes were preincubated with spermine in increasing concentrations with and without IBMX (200 μ M) (a) or with and without RO 20-1724 (200 μ M) (b). Lipolysis was then stimulated by ADA (0.5 unit/ml). Results are means $\pm$ 1 S.E.M. (6–8 parallel experiments); $*P < 0.01$.

Fig. 4. Effect of IBMX on the interaction between spermine and cAMP

Adipocytes were incubated with spermine in increasing concentrations in the presence (●) or absence (○) of IBMX (200 μ M). cAMP accumulation was stimulated by ADA (0.5 unit/ml) and isoprenaline (50 nM) for 10 min. These data were compared with the inhibitory action of 1 mM- PGE_2 in the presence (■) or absence (□) of IBMX (200 μ M). cAMP was measured by radioimmunoassay. Results are means $\pm$ 1 S.E.M. ($n = 5$); $*P < 0.01$.

Fig. 5. Effect of spermine on PDE activity (Lineweaver-Burk plots)

Adipocytes were incubated in the presence (●) or absence (○) of 5 μ M-spermine for 90 min. The PDE enzyme was extracted and assayed as described in the Materials and methods section. Spermine was also present in these latter procedures ($n = 5$).

Accordingly, the effect of spermine on the PDE activity was also determined. As shown in Fig. 5, spermine (5 mM) had a clear stimulatory effect on the low- K_m PDE activity. This action was detected when intact adipocytes were incubated together with spermine, but also if spermine was first added during the enzyme-extraction procedure. From linearization of the data it was observed that spermine especially enhanced the V_{max} of the enzyme from 3.2 (control) to 4.9 pmol/min per 10^6 cells (spermine) ($P < 0.01$) without affecting the K_m value (0.48 μ M to 0.46 μ M). Accordingly, these data confirm the assumption that polyamines may decrease the cAMP accumulation and inhibit lipolysis through their direct stimulatory effects on the low- K_m PDE activity.

That polyamines had effects on the PDE enzyme in rat adipocytes might not be surprising, in light of previous findings showing that polyamines can affect several enzymes such as protein kinases (Walton & Gill, 1983; Tabor & Tabor, 1984; Toolsee & Huang, 1985) and certain protein phosphatases (Thomas *et al.*, 1986). However, to our knowledge no action of polyamines on PDE enzymes has been published. The more specific interactions of the polyamines with the PDE enzyme have still not been identified.

The interactions of spermine with conventional antilipolytic compounds such as insulin and PGE_2 were also studied. The half-maximal inhibitory concentration of insulin (IC_{50}), in the presence and absence of spermine (1 mM), was 56 ± 20 pM and 320 ± 70 pM respectively ($P < 0.01$) when lipolysis was stimulated by ADA (Fig. 6a). Similar results were obtained when lipolysis was stimulated by isoprenaline (50 nM). Then the IC_{50} value of insulin was decreased by spermine (1 mM) from 53 ± 20 pM to 13 ± 5 pM ($P < 0.05$) (Fig. 6b). However, spermine (1–5 mM) had no effects on the antilipolytic properties of PGE_2 , when lipolysis was stimulated by either ADA or isoprenaline (results not shown). Thus spermine potentiates the antilipolytic sensitivity of insulin by a factor of 4–5, without affecting the antilipolytic sensitivity of PGE_2 . The biochemical mechanism(s) behind this specific potentiating effect of spermine is unknown. However, since we have recently demonstrated

Fig. 6. Influence of spermine on the antilipolytic effect of insulin

Adipocytes were incubated in the presence (●) or absence (○) of spermine (1 mM) together with increasing concentrations of insulin. Lipolysis was stimulated by ADA for 90 min ($n = 8$) (a) or with ADA together with 50 nM-isoprenaline ($n = 6$) (b). Results are means ± 1 S.E.M.; * $P < 0.05$.

that polyamines enhanced insulin-receptor binding in adipocytes (Pedersen *et al.*, 1989), that effect of polyamines could probably, at least partially, explain the enhanced antilipolytic sensitivity of insulin. On the other hand, since the antilipolytic effect of insulin seems to be mediated by activation of some forms or subspecies of PDE enzymes (Houslay, 1986; Manganiello & Elks, 1986), it is likely that insulin and spermine act on different subspecies of the PDE enzyme, or spermine perhaps makes the PDE enzyme more sensitive to insulin.

In order to evaluate the physiological relevance of the antilipolytic effect of polyamines, the polyamine concentration was also determined in isolated adipocytes. The amounts of putrescine, spermidine and spermine in non-stimulated adipocytes incubated for 5 min were not significantly different from that found after incubation for 120 min (Table 1). As virtually all polyamines and proteins in adipocytes are localized within the thin layer of cytoplasm surrounding the lipid droplet (Pedersen *et al.*, 1989), a rough calculation of the cytoplasmic polyamine concentrations can be made. Assuming that the proteins constitute about 20% (w/v) of the adipocyte cytoplasm, the cytoplasmic concentrations, taken as a whole, will be as follows: putrescine 0.02 mM, spermidine 0.86 mM, and spermine 1.0 mM. Thus in the present study millimolar concentrations of spermidine and spermine were found in the cytoplasm of isolated adipocytes. If the polyamines are compartmentalized within the cytoplasm, regional concentrations may, however, be even higher.

Table 1. Concentrations of polyamines in non-stimulated isolated adipocytes

Samples (800 μ l) of isolated adipocytes [(0.5–1) $\times 10^6$ cells] were incubated for 5 or 90 min in incubation buffer and assayed for polyamines and protein as described in the Materials and methods section. Results are means $\pm$ S.D. of *n* experiments.

Time of incubation (min)	<i>n</i>	Content (nmol/mg of protein)		
		Putrescine	Spermidine	Spermine
5	8	0.1 $\pm$ 0.03	4.28 $\pm$ 0.6	5.05 $\pm$ 0.9
90	3	< 0.05	3.71 $\pm$ 0.5	5.42 $\pm$ 1.5

Thus polyamines presumably occur in sufficient amounts to affect adipocyte lipolysis.

In conclusion, it has been demonstrated that polyamines have antilipolytic effects in rat adipocytes. These actions seemed to be due to an activation of the PDE enzyme, resulting in decreases in cAMP. Furthermore, spermine enhanced the antilipolytic sensitivity of insulin through still unknown mechanisms. In light of the observations that polyamines are localized in rat adipocytes, it would be interesting in further studies to evaluate whether these endogenous polyamines may have a physiological role in modulating adipose-tissue triacylglycerol metabolism.

The technical assistance of P. Sonne, A. Stenderup and J. Søholt is gratefully acknowledged. This study was supported by the Novo Foundation, Nordic Insulin Foundation, the Danish Cancer Society, the Danish Diabetic Association and the Danish Medical Research Council.

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Received 30 December 1988/10 March 1989; accepted 28 March 1989