histone distribution throughout the gel since both proteins enter only the outer parts during the penetration. Here, a compromise between protein elution from the gel (i.e. of inhibitor) and degree of protein penetration (i.e. of substrate and enzyme) had to be approached. Protamine can be used as well as histone as the substrate. For this purpose, however, the gel should be adjusted to pH 7.8, which is the optimum for the protamine phosphorylation with rat muscle enzyme[6]. Taking into account the contamination with other small proteins it was estimated that about 5 µg of pure PK-inhibitory protein should be easily detectable by this procedure. Preliminary experiments have shown that some inhibitor preparations from rat muscle contain 2 closely located activities inhibitory for the catalytic subunit with regard to histone phosphorylation. The use of other PK-substrates as well as inhibitor preparations from different tissues should help to elucidate the physiological significance of these small proteins.

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Evidence against the involvement of cyclic GMP in the insulin-stimulation of lipoprotein lipase activity in fat cells[1]

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Summary. Under in vitro experimental conditions in which insulin increases adipose tissue lipoprotein lipase, cyclic GMP or dibutyryl cyclic GMP has no effect on this enzyme in rat adipose tissue fragments, or on either the intra- or extracellular forms of this enzyme in isolated fat cells. These results do not support the involvement of cyclic GMP in the insulin-stimulation of lipoprotein lipase in adipose tissue.

In a recent report, Vydelingum et al.[2] confirmed previous findings[3,4] by showing the ability of insulin to increase the 3′-5′ cyclic guanosine monophosphate (cyclic GMP) content and to enhance the lipoprotein lipase (LPL) activity of adipose tissue in vitro. Although the kinetics of these effects were markedly different[2], these authors suggested, that cyclic GMP might mediate the insulin stimulation of LPL activity in adipose tissue.

As the direct effects of cyclic GMP on adipose tissue LPL were not investigated in these studies[2], we have tested this hypothesis by studying, under the experimental conditions used by Vydelingum et al.[2], the influence of different concentrations of cyclic GMP or its stable analog, dibutyryl cyclic GMP (dcGMP), on the LPL activity of adipose tissue. Moreover, the same investigations were carried out on isolated fat cells to determine whether cyclic GMP might affect selectively the intracellular or the extracellular form[5,6] of LPL. The present data clearly indicate that, with experimental conditions under which insulin increases adipose tissue LPL, cyclic GMP and dcGMP have no effect on this enzyme, a result which therefore does not support the involvement of cyclic GMP in the mechanism of the insulin-mediated stimulation of LPL activity in adipose tissue.

Materials and methods. Before sacrifice male Wistar rats (120–140 g) were fasted overnight but given 5% (w/v) glucose in the drinking water. After decapitation, epididymal fat pads were excised and isolated fat cells prepared as previously described[7]. In studies using adipose tissue, fragments from one pad from each animal served as control, while fragments from the other pad were used as the test material. Adipose tissue (200 mg/ml) or isolated fat cells (500 µl packed cells/ml) were incubated under O_2/CO_2 (95/5, v:v) in the absence or presence of the compounds to be tested in Krebs-Ringer[8] -bicarbonate buffer (pH 7.4) containing 1.25 mM calcium, 5 mM glucose, 20 mg/ml dialysed bovine albumin (fraction V) and an amino-acid mixture (final concentration 390 nM)[9], the composition of which corresponded to the amino-acid concentration of rat plasma[9]. In experiments using fat cells, the incubation buffer was added with fresh rat serum (3.8%, v/v).

Adipose tissue fragments were incubated for 2 h at 37 °C in the absence or presence of the compounds to be tested. Fat cells were first preincubated fro 30 min at 26 °C after which the compounds to be tested were added and the incubation further extended for an additional 90-min period. Fat cells and adipose tissue fragments were then separated from the

Influence of various concentrations of dibutyryl cyclic GMP on the intra- and extracellular forms of lipoprotein lipase in isolated rat adipocytes in vitro

Concentration of dibutyryl cyclic GMP added to the incubation medium (M)	Lipoprotein lipase activity (% of basal activity)	
	Remaining in the cells	Released from the cells into the incubation medium
0	100 ± 17	100 ± 3
10^{-11}	101 ± 22	87 ± 15
10^{-9}	126 ± 11	90 ± 9
10^{-7}	97 ± 12	102 ± 4
5×10^{-4}	114 ± 13	92 ± 6

Isolated fat cells were incubated at 26 °C as described under material and methods section. After a preincubation period of 30 min, different concentrations of dibutyryl cyclic GMP were added; 90 min later, fat cells were separated from the medium and lipoprotein lipase activities determined in both the fat cells and the incubation medium as described. Lipoprotein lipase activity is expressed as percentage of the basal values obtained in cells incubated without dibutyryl cyclic GMP. Each value is the mean ± SEM of 2 experiments performed in triplicate.

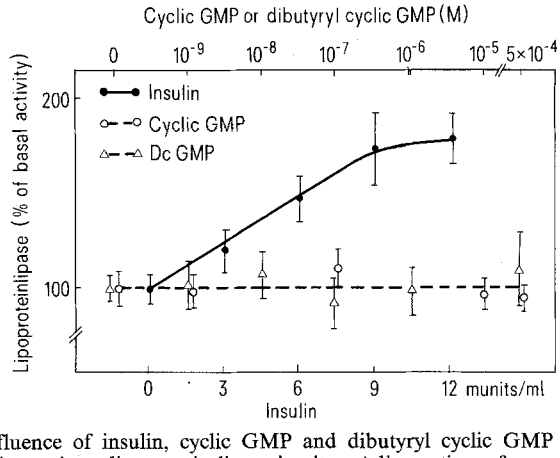
buffer, homogenized at 4 °C in 50 mM NH_4OH - NH_4Cl buffer (pH 8.6), centrifuged at 4 °C and the clear supernatant used for the assay of LPL.

Assay of LPL was performed according to a modification of the procedure of Greten and Walter[10], as follows: in a total volume of 1 ml, each vial contained 0.6 ml of substrate emulsion consisting of 1.2 nmoles tri[-1-^{14}C]oleylglycerol (sp. act. 55 mCi/mmole), 21.8 µmoles of unlabeled trioleylglycerol, 40 mg bovine albumin, 50 µl of a 1:100 diluted Triton X-100 solution all in 0.2 M Tris-HCl buffer (pH 8.6) and 150 µl of rat serum as cofactor and 0.4 ml of enzyme solutions. Incubations were performed for 60 min at 37 °C. Lipids were then extracted[11] and [^{14}C]-labeled free fatty acids isolated by the ion exchange procedure of Kelley[12]. LPL activity is calculated as µmoles FFA released per gram fat pad or gram cell lipid per hour and usually expressed as a percentage of the control. To ensure reproducibility each experiment was repeated at least 3 times.

Cyclic GMP and dcGMP were purchased from Sigma and tri[-1-^{14}C]oleylglycerol from the Radiochemical Centre, Amersham.

Results and discussion. As shown in the figure, insulin between 3 and 12 mU/ml increased, as expected from previous reports[4], the LPL activity in rat adipose tissue. The magnitude of this effect which was 50% lower than the one reported by Vydelingum et al.[2], was in agreement with that usually found under similar experimental conditions by most authors[4,6,13].

The same experiments were performed with various concentrations (1 nM-0.5 mM) of cyclic GMP in place of insulin. This rather broad range of concentrations was chosen to be compatible, at least for the lower concentrations (1-100 nM), with the molar levels of cyclic GMP usually reported in the fat cell water space[14,15]. As shown in the figure, none of these concentrations induced any significant rise in the LPL activity. As this lack of effect could be linked to a degradation of the nucleotide by phosphodiesterase, additional experiments were undertaken in the presence of theophylline (5×10^{-3} M), a potent inhibitor of phosphodiesterase[16]. Under these conditions, addition of theophylline alone reduced LPL activity by 50%, an effect which would be an additional argument in favour of the existence of a negative control of LPL by cyclic AMP[17]; however, this inhibitory effect of theophylline was not reversed by cyclic GMP (data not shown).



Influence of insulin, cyclic GMP and dibutyryl cyclic GMP on adipose tissue lipoprotein lipase in vitro. Adipose tissue fragments were incubated as described under materials and methods, in the presence of different concentrations of insulin, cyclic GMP or dibutyryl cyclic GMP. After 2 h, adipose tissue fragments were removed and the lipoprotein lipase activity determined. Lipoprotein lipase activity is expressed as a percentage of the basal value for tissue incubated without insulin, cyclic GMP or dibutyryl cyclic GMP. Each point is the mean of 5 determinations from 1 representative experiment repeated 3 times and the vertical lines represent 2 SEM.

Another possible explanation for the inability of exogenous cyclic GMP to modify adipose tissue LPL activity could be a poor penetration of this nucleotide into the fat cell. To overcome this possibility, incubations were performed with various concentrations of the lipophilic and stable analog of cyclic GMP, dcGMP[18], and, here again, no significant modification of the adipose tissue LPL activity could be observed (figure). Moreover, when isolated fat cells were incubated in place of adipose tissue fragments with dcGMP (10 pM-0.5 mM), this nucleotide did not have any more significant influence on LPL activity. Indeed, as indicated by the data in the table, neither the LPL activity released from the cell into the medium, nor the LPL activity remaining intracellularly were found to be altered after 90-min exposure at 26 °C to dcGMP.

From these negative results, it seems highly unlikely that cyclic GMP may be involved in the mechanism(s) responsible for the insulin-induced activation of LPL activity in adipose tissue[4] and in isolated adipocytes[6,19], contrary to the attractive suggestion recently put forward by Vydelingum et al.[2].

Besides, this suggestion is also inconsistent with the observations in a recent report[20]showing an increase in the fat cell cyclic GMP level after exposure to catecholamines, a group of hormones which have been reported to inhibit rather than to stimulate the LPL activity of rat adipose tissue[17] and, as recently shown, to inhibit both the extra- and intracellular forms of this enzyme in isolated rat fat cells[21].

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