

REGULATION OF ADIPOSE TISSUE LIPOLYSIS: EFFECTS OF NORADRENALINE AND INSULIN ON PHOSPHORYLATION OF HORMONE-SENSITIVE LIPASE AND ON LIPOLYSIS IN INTACT RAT ADIPOCYTES

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1. Introduction

In [1] we have demonstrated that hormone-sensitive lipase is phosphorylated in intact rat adipocytes, but the effects of hormones on the extent of this phosphorylation was not studied. The [^{32}P]phosphorylated enzyme protein migrated in a small [^{32}P]phosphopeptide band (1.5% of total) when proteins from adipocytes, incubated with $^{32}\text{P}_i$, were separated with SDS-PAGE. During the isolation of the [^{32}P]phosphorylated lipase we obtained no indication of heterogeneity of this 84 000 dalton [^{32}P]phosphopeptide band, which suggested that it mainly consisted of [^{32}P]hormone-sensitive lipase [1].

Based on these observations we have derived a method for quantitation of [^{32}P]hormone-sensitive lipase in adipocyte protein extracts. We had developed a technique for continuous monitoring of FFA release from adipocytes by pH-stat titration as a measure of hormone-sensitive lipase activity [2]. In combination, these techniques have enabled us to correlate the time-course of effects of noradrenaline and insulin on phosphorylation and activity of the enzyme, in intact adipocytes. The results suggest that these hormones can regulate lipolysis in the intact cell by altering the extent of phosphorylation of hormone-sensitive lipase.

Abbreviations: ATP, adenosine 5'-triphosphate; cyclic AMP, cyclic 3',5'-adenosinemonophosphate; Hepes, 2-[4-(2-hydroxyethyl)-piperazinyl(1)]-ethane sulfonic acid; FFA, free fatty acid; PCV, packed cell volume; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis

2. Methods

2.1. Quantitation of [^{32}P]hormone-sensitive lipase in adipocyte protein extracts

Rat adipocytes [1] were incubated under conditions given in the figure legends. Adipocytes were separated from incubation medium by floatation ($10\,000 \times g$ for 5 s, Beckman Microfuge B, 0.4 ml tubes) through dinonylphthalate (BDH) [3]. In <15 s the floated cells were dissolved, in the tubes, in 50 μl solution containing 3% SDS, 0.1 M 2-mercaptoethanol, 50 mM NaP_i , 10 mM EDTA, 10 mM NaF, and 10% (v/v) ethanol (pH 8.5). The tubes were stored at -20°C . For analysis by SDS-PAGE tubes were sliced and cell proteins (100–150 μg) precipitated in ice-cold acetone, extracted with organic solvents [4], and dissolved in 100 μl electrophoresis sample solution (1.25% SDS, 1 mM EDTA, 63 mM Tris-HCl (pH 8.6), 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 20 mg/l bromphenol blue) by repeated heating to 96°C for 1.5 min, sonication for 10 min and vortex mixing for 30 s.

SDS-PAGE was done following [5] but with 2 mM EDTA in all solutions [6], electrode buffer (pH 8.7) and acrylamide at $8 \times 2.4\%$ (T \times C). Kenacid blue (BDH) stained gels were dried and autoradiographed using Osray M3 films (Agfa-Gevaert). Gels and autoradiographs were scanned with a soft laser densitometer at 633 nm (model SL 504, Biomed Instr., Chicago, IL). Radioactivity in the hormone-sensitive lipase band was quantitated by liquid scintillation counting. After localization of the band, with the autoradiograph, a gel piece (2.5×12 mm) was punched out from the dried gel, dissolved in

1 ml 30% H_2O_2 , and Instagel (Packard) added. Background radioactivity was measured, in gel pieces of equal size from an area where no phosphopeptide was visible, and subtracted. Variation in amount of protein applied to the gels was $<10\%$ and not corrected for.

2.2. Hydroxylapatite chromatography of SDS-protein complexes

Autoradiographs (Kodak X-Omat R film, Du Pont Cronex intensifying screen, 3 h exposure) of wet, unstained SDS-PAGE gels were used to localize the 84 000 dalton [^{32}P]phosphopeptide band. This band was cut out from the gel and the [^{32}P]phosphopeptides eluted electrophoretically into a dialysis bag as in [7]. The eluates (1–2 ml) were dialysed for 2 h against 10 mM NaP_i , 0.1% SDS, and 1 mM dithio-

erythritol (pH 6.4) and fractionated on hydroxylapatite (HTP, Bio-Rad) according to [8].

2.3. Analytical procedures and materials

Intracellular ATP was determined on perchloric acid extracts of floated cells by an enzymatic fluorometric method [9]. [^{32}P]ATP in the same extracts was determined by liquid scintillation counting after thin-layer chromatography on poly(ethyleneimine)-cellulose (Merck) [10]. L-Noradrenaline bitartrate and bovine serum albumin, fraction V (extensively dialysed before use) were from Sigma Chemical Co., insulin (porcine, monocomponent, 26.3 units/mg) was a gift from Novo, acrylamide from Bio-Rad and carrier-free [^{32}P]orthophosphate from The Radiochemical Centre (Amersham).

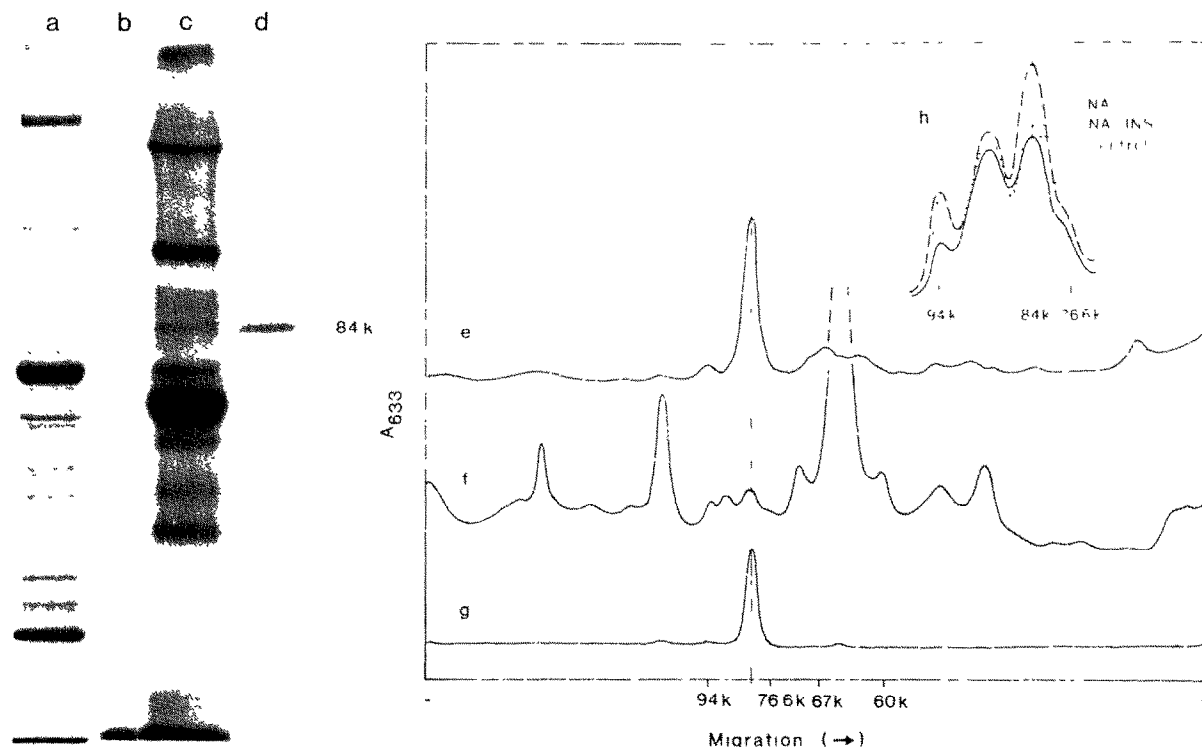


Fig.1. Adipocyte proteins and [^{32}P]phosphopeptides fractionated by SDS-PAGE. Adipocytes (50 μl PCV/ml) were incubated in Krebs-Ringer buffer with low phosphate (50 μM) containing 3.5% (w/v) bovine serum albumin, 5 mM glucose and 100 $\mu\text{Ci}/\text{ml}$ carrier-free $^{32}\text{P}_i$ for 40 min and, when indicated, with noradrenaline or noradrenaline followed by insulin. Delipidated cell protein (100–150 μg) was separated with SDS-PAGE on slab gels. (a) Protein stain of adipocyte protein extract. Autoradiograph and densitometric trace of; (b,e) purified, in vitro [^{32}P]phosphorylated hormone-sensitive lipase [11]; (c,f) [^{32}P]phosphopeptides from adipocytes exposed to noradrenaline (50 ng/ml = 2.95×10^{-7} M for 5 min); (d,g) [^{32}P]hormone-sensitive lipase isolated from cells incubated with $^{32}\text{P}_i$ [1]; (h) 84 000 dalton region of densitometric traces as in (c,f) where control = incubation without hormones, NA = addition of noradrenaline (50 ng/ml) for 5 min, and NA + INS = addition of insulin (100 $\mu\text{units}/\text{ml}$) for 10 min after the 5 min incubation with noradrenaline. Migrating positions of molecular weight marker proteins [1] and 84 000 dalton [^{32}P]phosphopeptide band have been indicated in the figure ($k = 10^3$).

3. Results

3.1. Quantitation of [^{32}P]hormone-sensitive lipase in adipocyte protein extracts

Protein from intact adipocytes, incubated with $^{32}\text{P}_i$ and noradrenaline, was separated with SDS-PAGE and the gels autoradiographed. The 84 000 dalton [^{32}P]phosphopeptide band, containing [^{32}P]hormone-sensitive lipase (identified in fig.1c,f), had the same electrophoretic mobility as a very small protein band (order of magnitude: 0.1% of total) on the protein-stained gel (fig.1a). Hormone effects on the height of the 84 000 dalton [^{32}P]phosphopeptide peak were observed (fig.1h).

To demonstrate that the hormone effects were due to changes in the extent of phosphorylation of hormone-sensitive lipase the [^{32}P]phosphopeptide-SDS complexes of the 84 000 dalton band were further fractionated by hydroxylapatite chromatography. As reference proteins [^{32}P]hormone-sensitive lipase, after purification from rat adipose tissue and phosphorylation in vitro [11], or after isolation from intact adipocytes incubated with $^{32}\text{P}_i$ [1], were used. The former (fig.2A) and the latter (not illustrated) reference [^{32}P]proteins were eluted at 0.48 M phosphate, coinciding with the major [^{32}P]phosphopeptide peak from the 84 000 dalton band (fig.2B-D). This peak was thus identified as [^{32}P]hormone-sensitive lipase. Changes of ^{32}P in the 84 000 dalton band, due to exposure of the adipocytes to hormones, were found almost entirely in this [^{32}P]hormone-sensitive lipase peak. These results verify that most of the 84 000 dalton [^{32}P]phosphopeptide SDS-PAGE band from adipocyte protein extracts consists of [^{32}P]hormone-sensitive lipase, that the [^{32}P]enzyme can be approximately

quantitated by determination of the ^{32}P of this band and, thus, that hormone alterations of ^{32}P in this band reflect changes in the extent of [^{32}P]phosphorylation of hormone-sensitive lipase.

[^{32}P]Hormone-sensitive lipase could be quantitated either by determination of densitometric peak height,

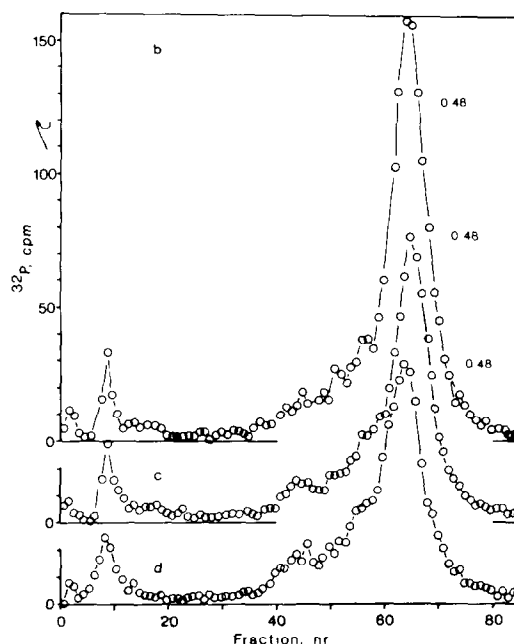
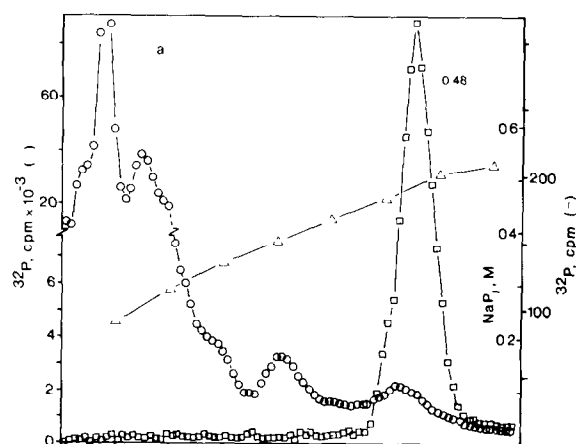


Fig.2. Hydroxylapatite chromatography of [^{32}P]phosphopeptide-SDS complexes. [^{32}P]Phosphopeptides, eluted electrophoretically from SDS-PAGE gel slices or in an adipocyte protein extract, were dialyzed against 10 mM sodium phosphate buffer (pH 6.5) containing 0.1% SDS, and 1 mM dithioerythritol. They were then applied to hydroxylapatite columns (10 \times 0.7 cm), pre-equilibrated in the dialyzing buffer, and eluted at $27 \pm 1^\circ\text{C}$ with a 0.20–0.50 M sodium phosphate gradient (pH 6.5) with SDS and dithioerythritol as above. Flowrate was 7.0 ml/h and fraction vol. 0.7 ml. Delipidated adipocyte protein (100 μg) was added to obtain an equal amount of protein in each sample applied. Sodium phosphate concentration was determined by conductivity, and ^{32}P radioactivity as Cerenkov-radiation. (a) (\circ — \circ) delipidated adipocyte protein (100 μg) extract; (\square — \square) [^{32}P]hormone-sensitive lipase after SDS-PAGE, the enzyme had been phosphorylated in vitro after purification from adipose tissue [11]; (b) 84 000 dalton [^{32}P]phosphopeptide band from protein extract of adipocytes, incubated as in fig.1, noradrenaline 50 ng/ml for 5 min; (c) as in (b) but cells incubated with noradrenaline (50 ng/ml, 5 min) followed by insulin, 100 $\mu\text{units/ml}$ for 10 min; (d) as in (b) but cells incubated without hormones. Phosphate gradient is plotted for adipocyte protein extract only. Values in the figure denote phosphate molarity in peak fractions. Fraction numbers are not exactly related to phosphate concentration.



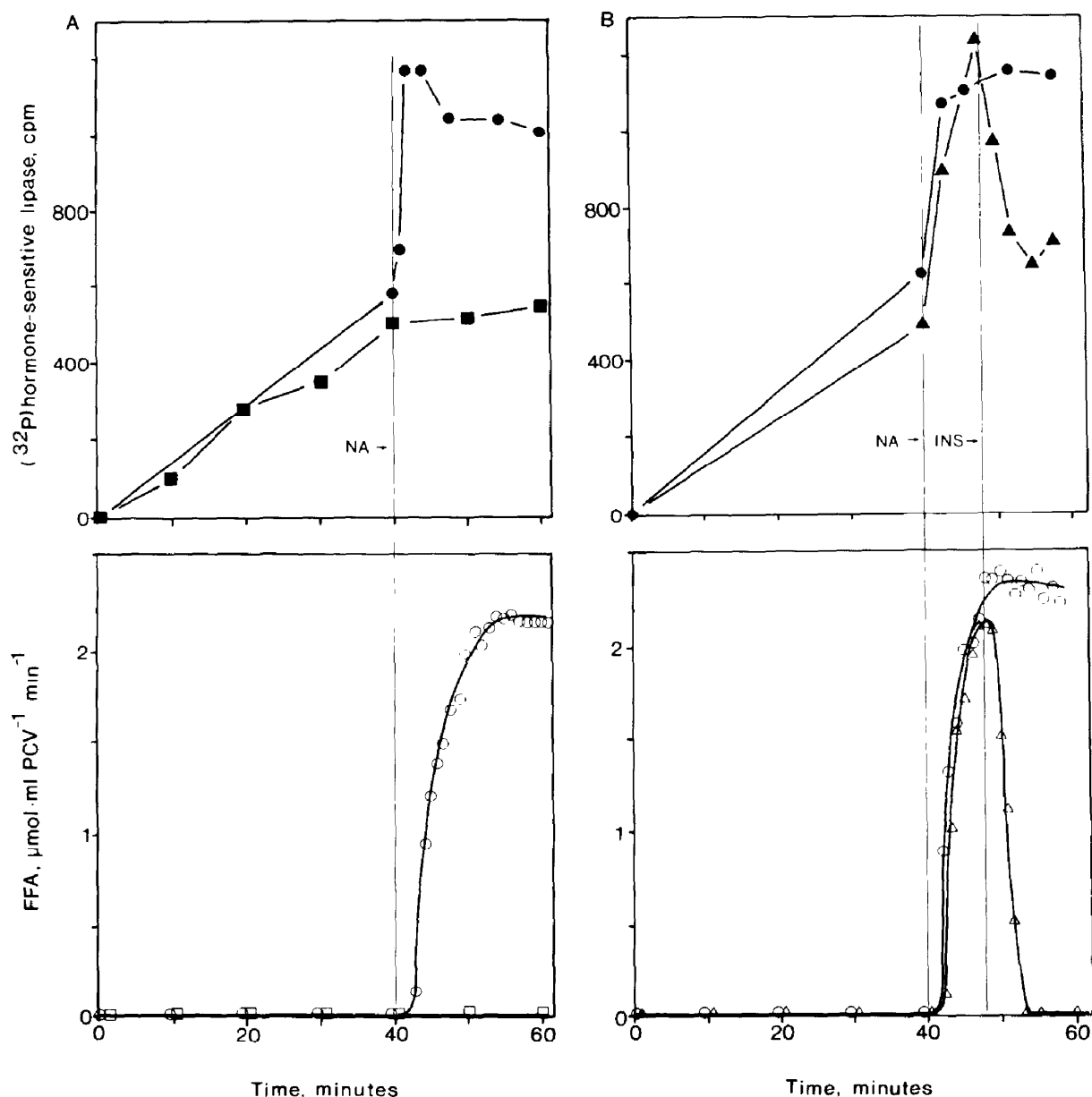


Fig. 3. Effect of noradrenaline and insulin on the time-course of $[^{32}\text{P}]$ phosphorylation of hormone-sensitive lipase and on lipolysis rate. Adipocytes were incubated as in fig. 1. Lipolysis, measured as FFA release, was monitored continuously by pH-stat titration [2] and lipolysis rate derived over 1 min intervals. Duplicate samples (0.5 ml) of the adipocyte suspension were taken at different times and the $[^{32}\text{P}]$ phosphopeptides separated by SDS-PAGE. ^{32}P radioactivity in hormone-sensitive lipase was determined by liquid scintillation counting; each point is the mean of 2 values. (A) Effect of noradrenaline, 50 ng/ml (NA; \bullet , \circ) compared to control without hormone (\blacksquare , \square). (B) Effect of insulin, 100 $\mu\text{units/ml}$ (INS; \blacktriangle , \triangle) on noradrenaline-stimulated (50 ng/ml) adipocytes compared to noradrenaline-stimulated control (\bullet , \circ). Closed symbols refer to $[^{32}\text{P}]$ phosphorylation of hormone-sensitive lipase, open symbols to FFA release rate.

or by direct ^{32}P counting as in section 2. The latter method was used since it gave higher reproducibility.

3.2. Time-course of effect of noradrenaline and insulin on [^{32}P]phosphorylation of hormone-sensitive lipase and on lipolysis rate

Intracellular concentration of ATP was 123 ± 4 (6) nmol/ml PCV (mean \pm SD, no. cell batches) and varied by $<9\%$ within each experiment. There was no significant change with time or by addition of hormones during the experiment. $^{32}\text{P}_i$ was incorporated into cellular [^{32}P]ATP, at a constant rate. After 40 min incubation [^{32}P]ATP had reached an approximately constant specific activity ($<7\%$ increase over 40–60 min incubation) with no significant effect of addition of noradrenaline or insulin.

In adipocytes incubated without added hormone, ^{32}P incorporation into hormone-sensitive lipase increased, concomitantly with [^{32}P]ATP specific activity, to a steady state level, with no change in lipolysis rate (fig.3A). Noradrenaline addition, in <2 min increased [^{32}P]phosphorylation and concomitantly, with a slight delay, the activity of the enzyme (FFA release) (fig.3A). In 11 expt the [^{32}P]phosphorylation increased to an average of $178 \pm 7.2\%$ (SEM) $p < 0.001$, of controls. Addition of insulin to maximally noradrenaline-stimulated adipocytes, in <4 min reduced the extent of [^{32}P]phosphorylation of the enzyme, approaching that obtained before noradrenaline-stimulation (fig.3B). This decrease in ^{32}P content was followed, with a slight delay, by a reduced rate of lipolysis. (This effect on FFA release was due to inhibition of lipolysis and not to an increased re-esterification, since it was not significantly affected by absence of glucose in the incubation.) In 6 similar experiments insulin addition reduced the ^{32}P content of the enzyme from $186 \pm 8.1\%$ (SEM) to a level of $118 \pm 3.3\%$ (SEM) of that obtained before noradrenaline addition. This reduction was statistically significant, $p < 0.01$ (Student's t -test for paired observations). The same extent of [^{32}P]phosphorylation, which was obtained after insulin, was not associated with any measurable lipolysis in the dose-response experiments (fig.4, below).

3.3. Effects of noradrenaline and insulin concentration on [^{32}P]phosphorylation of hormone-sensitive lipase and lipolysis rate

Increase in extent of [^{32}P]phosphorylation of the

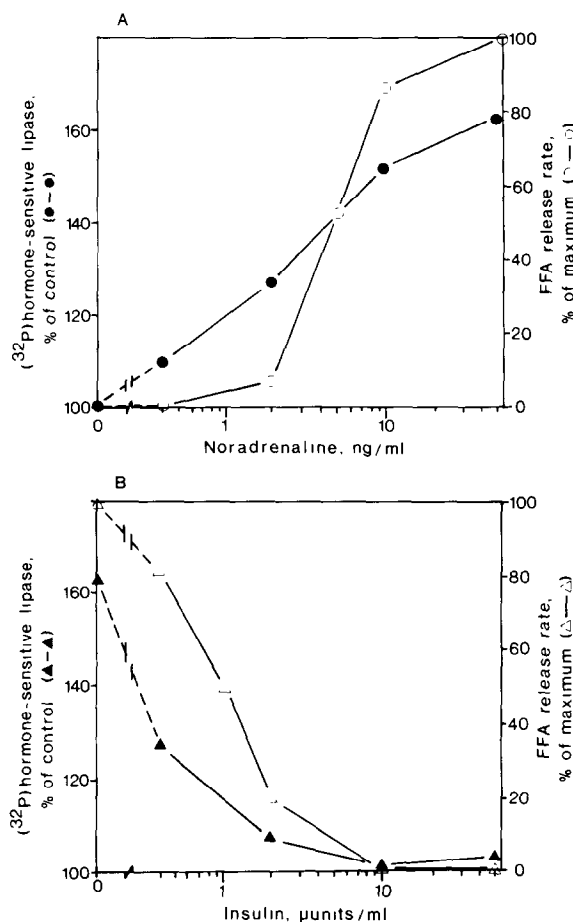


Fig.4. Effects of different concentrations of noradrenaline and insulin on lipolysis rate and [^{32}P]phosphorylation of hormone-sensitive lipase. Adipocytes were incubated as in fig.1, but with Hepes, 24 mM, in 1 ml final vol. (A) Exposure of the cells to the indicated concentrations of noradrenaline for 10 min, after 40 min preincubation with $^{32}\text{P}_i$. (B) Exposure of the cells to 10 ng noradrenaline/ml for 10 min as in (A) followed by insulin at the indicated concentrations for 10 min. Closed symbols refer to ^{32}P -phosphorylation of enzyme, expressed as % of controls without hormone (40 min values); open symbols to FFA release rate. Each value is the mean of 4.

enzyme and rate of lipolysis occurred over the same range of noradrenaline concentrations, with half-maximal effect at 3–5 ng/ml (fig.4A). Decrease in extent of [^{32}P]phosphorylation of the enzyme and rate of lipolysis, pre-stimulated with 10 ng noradrenaline/ml, occurred over the same range of insulin concentrations, with half-maximal effect at 0.5–1 μ unit/ml (fig.4B).

4. Discussion

These results suggest that the activity of hormone-sensitive lipase in intact adipocytes is regulated by hormonal alterations of the extent of phosphorylation of the enzyme protein. Most of the ^{32}P radioactivity present in the 84 000 dalton band in the SDS-PAGE gels was due to hormone-sensitive lipase, even though this band also contained some other ^{32}P phosphopeptides. Moreover, hormones affected mainly, if not exclusively, the ^{32}P phosphorylation of hormone-sensitive lipase. The enzyme was extensively ^{32}P -phosphorylated without any effect on lipolysis rate, when the fat cells were incubated in the absence of hormone (fig. 3A). This may reflect phosphate exchange without net increase in phosphorylation, but it cannot be excluded that the phosphorylation occurred at a different site not directly affecting enzyme activity (cf. [13]).

Since noradrenaline effects are presumed to be mediated by cyclic AMP-dependent phosphorylation, the present work indicates that the *in vitro* phosphorylation of the purified enzyme with a cyclic AMP-dependent protein kinase [11,12] is physiologically important. An important finding was that insulin inhibition of noradrenaline-stimulated lipolysis was associated with a decrease in the extent of phosphorylation of the enzyme. This suggests one possible mechanism for the action of insulin. It could, however, not be distinguished whether the effects of insulin were mediated by changes in protein kinase activity or phosphoprotein phosphatase activity. The sensitivity of lipolysis and enzyme phosphorylation to insulin concentration was in the same range as that found recently for both lipolysis and glucose uptake in rat adipocytes under similar conditions [14].

The presence of, and hormone effects on, a small ^{32}P phosphopeptide band, given the app. mol. wt 86 000 dalton, had been noticed, but its identity was not discussed [15]. The reason for not observing the hormone-sensitive lipase ^{32}P phosphopeptide band in the SDS-PAGE fractionations [16] may be that, at the ionic strength used, the enzyme was not separated from the fat, after homogenization of the adipocytes (cf. [11]).

The continuous monitoring of enzyme activity in intact cells, in combination with determinations of enzyme phosphorylation, makes this system particularly useful for studies of hormonal regulatory mechanisms. The effects of hormones on enzyme

activity and phosphorylation discussed here, certainly suggest a number of important aspects of their mechanism of action that are now being investigated further.

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