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Hormonal regulation of hormone-sensitive lipase in intact adipocytes: Identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin

(cyclic AMP/protein kinase/corticotropin/noradrenaline/propranolol)

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ABSTRACT **In isolated adipocytes, fast-acting lipolytic hormones and insulin have been shown previously to control lipolysis by regulating the activity of hormone-sensitive lipase, the rate-limiting enzyme, through an increase or decrease, respectively, of the extent of phosphorylation of the enzyme. Here, we demonstrate that exposure to lipolytic hormones (corticotropin, noradrenaline) led to phosphorylation at two sites on the M_r 84,000 lipase subunit. One, designated "basal site," was phosphorylated also in the absence of any hormonal stimulation, its phosphorylation apparently not being influenced by hormones. The second, designated "regulatory site," was identical to that phosphorylated by cyclic AMP-dependent protein kinase on the isolated lipase. The regulatory site was not appreciably phosphorylated in the absence of hormones, but exposure of the cells to noradrenaline increased its phosphorylation extent to that of the basal site. Insulin or the β-adrenergic antagonist propranolol decreased the extent of phosphorylation of the regulatory site to the low level before stimulation, apparently without effect on the basal site. Phosphoserine was the only phosphorylated amino acid residue at both sites. Limited proteolytic digestion indicated that the two sites were separated by less than about 170 amino acid residues. Thus, control of adipose tissue lipolysis by fast-acting lipolytic hormones and by insulin is exerted through the regulation of the phosphorylation state of a single phosphoserine residue in the hormone-sensitive lipase.**

The mobilization of fatty acids from adipose tissue is largely regulated by neural and hormonal control of the activity of the hormone-sensitive lipase, the rate-limiting enzyme that catalyzes the hydrolysis of the stored triacylglycerol (for review, see refs. 1 and 2). Although discovered as a hormonally regulated enzyme activity more than 20 years ago (3–5), the regulatory mechanisms only recently have become amenable to examination on the molecular level, by the recent identification of the hormone-sensitive lipase protein (6). It was suggested early on, in analogy with the well-established glucagon activation of liver glycogen phosphorylase, that the control of the lipase activity could be mediated through cyclic AMP, and some evidence for this was found (7). Following the realization that cyclic AMP-dependent protein kinase was the mediator of cyclic AMP action (8), this enzyme also was shown to enhance the lipase activity in adipose tissue homogenates (9). Subsequent work during the 1970s (summarized in refs. 10 and 11) led to the formulation of the "lipolytic activation cascade" hypothesis to describe the cyclic AMP-mediated hormonal activation of lipolysis.

The purification of the hormone-sensitive lipase has made it possible to directly demonstrate that the enzyme is phosphorylated on a single serine residue per M_r 84,000 subunit by the cyclic AMP-dependent protein kinase and that its activity is thereby enhanced (12, 13). In intact adipocytes, fast-acting lipolytic hormones have been shown to increase the extent of phosphorylation and activity of the enzyme, and insulin has been shown to reverse these effects (14, 15). Incorporation of phosphate into the enzyme also in the absence of hormonal stimulation has indicated that more than one site was phosphorylated after exposure of the fat cells to the lipolytic hormones (15). The purpose of the present work was to identify these postulated sites and to analyze how these hormones affected their phosphorylation state in order to establish that the hormone-sensitive lipase was indeed controlled hormonally through cyclic AMP-mediated phosphorylation. In addition, to gain further insight into the mechanism of the antilipolytic action of insulin, in particular the possible role of cyclic AMP reduction, we wanted to identify the site(s) whose phosphorylation state was affected by this hormone.

In this communication we show that two discrete sites are phosphorylated in the hormone-sensitive lipase subunit in intact adipocytes. The phosphorylation state of only one of these, identified with that previously (13) found to be phosphorylated *in vitro* by cyclic AMP-dependent protein kinase, was affected by exposure of the cells to fast-acting lipolytic hormones, insulin, or propranolol. Thus, the lipolytic hormones stimulate adipose tissue lipolysis through cyclic AMP-mediated phosphorylation of this regulatory site, and these effects are inhibited by propranolol. Insulin exerts its antilipolytic action specifically by decreasing the phosphorylation state of the same regulatory site.

MATERIALS AND METHODS

Materials. Carrier-free $^{32}P_i$ was from Amersham. [γ-^{32}P]ATP was prepared as described (16). Silicic acid thin-layer plates (0.25 mm) used for phosphopeptide mapping and silicic acid high-performance thin-layer chromatography plates used for phosphoamino acid analysis were obtained from Merck; Sephadex G-25 superfine was from Pharmacia; L-noradrenaline bitartrate and D,L-propranolol were from Sigma; corticotropin, residues 1–24 [ACTH-(1–24); adrenocorticotropic hormone], was a gift from CIBA–Geigy; and insulin (porcine, monocomponent) was a gift from Novo (Copenhagen, Denmark). Hormone-sensitive lipase from rat adipose tissue was purified to ≈5% protein purity, up to and including the hydroxyapatite step after the second gradient sievorptive chromatography (12). The catalytic subunit of cyclic AMP-dependent protein kinase was purified to homogeneity (17). *Staphylococcus aureus* V8 protease was from

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Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); PCV, packed cell volume.

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Miles, and trypsin treated with diphenylcarbamyl chloride was from Sigma.

Preparation of Adipocytes. Intact adipocytes (male Sprague–Dawley rats, 100–140 g) were prepared by collagenase digestion, and their viability and sensitivity to hormones were evaluated as described (18, 19). Amounts of cells are given as packed cell volume (PCV) (20).

Purification of Hormone-Sensitive Lipase from Cells. Adipocytes (PCV, 21 ml) from 65 rats (epididymal and perirenal fat) were incubated (PCV, 100 μl/ml) in Krebs–Ringer buffer with reduced phosphate concentration (100 μM) containing 20 mM Hepes, 3–5% (wt/vol) bovine serum albumin (fraction V, Sigma), and 5 mM glucose at pH 7.40 and 37°C. Carrier-free [^{32}P]orthophosphate was added to a final concentration of 0.25 mCi/ml (1 Ci = 37 GBq). After a 50-min incubation, ACTH (10 ng/ml) was added for an additional 5 min.

At the end of the incubation, the adipocytes were immediately separated from the incubation medium by floating and resuspending twice (within 10 min) in 0.25 M sucrose/1 mM dithioerythritol/1 mM EDTA/2 mM ATP, pH 7.4, containing the protease inhibitors leupeptin, pepstatin, and antipain (Peptide Institute, Osaka, Japan) at 5 μg/ml each and ACTH at 10 ng/ml. The cells were homogenized in 1 vol of the above solution containing 20 mM NaF. Hormone-sensitive lipase was purified from the cell homogenate as in ref. 14 up to and including the QAE-Sephadex gradient sievorptive chromatography and hydroxyapatite chromatography. Homogenous lipase protein (M_r, 84,000) was then isolated by NaDodSO$_4$/PAGE (21) and electrophoretic elution from gel slices (22).

Monitoring of Hormone-Sensitive Lipase Activity and Phosphorylation in Intact Adipocytes. Adipocytes (PCV, 1 ml) were incubated (PCV, 50-μl/ml) in Krebs–Ringer phosphate buffer with low phosphate (100 μM) containing 3–5% bovine serum albumin, 0.5 mM glucose, and 0.7 mCi of $^{32}P_i$ per ml for 50 min and, as indicated, with noradrenaline (150 nM) followed by insulin (700 pM) or propranolol (1 μM).

The activity of hormone-sensitive lipase in the intact cells was monitored continuously by pH-stat titration of free fatty acid release (18, 19). The extent of phosphorylation of the enzyme was determined by densitometry of the M_r 84,000 ^{32}P-labeled phosphopeptide band on autoradiographs after NaDodSO$_4$/PAGE of total, delipidated cell proteins; 0.25-ml aliquots of cell incubations were treated as described (15), and delipidated cell protein corresponding to 0.5 ml of the cell incubation was subjected to NaDodSO$_4$/PAGE (21).

For phosphopeptide analysis, the ^{32}P-labeled lipase protein was isolated from total delipidated cell protein by NaDodSO$_4$/PAGE (21) and electrophoretically eluted from gel slices (22).

Phosphopeptide Mapping by Two-Dimensional Thin-Layer Chromatography/Electrophoresis. Hormone-sensitive lipase was isolated after purification from cells or directly from total delipidated cell proteins by NaDodSO$_4$/PAGE. The lipase protein was electrophoretically eluted from gel slices (22), alkylated, dialyzed, and lyophilized as described (13). The enzyme protein (≈0.1 μg) was redissolved in 0.5% NH_4HCO_3 and digested with V8 protease (100 μg/ml for 6 hr at 37°C) and, after boiling for 1 min, with trypsin (20 μg/ml in 2 mM $CaCl_2$ for 15 hr at 37°C).

After lyophilization, the whole digest was redissolved in 33% (vol/vol) pyridine and spotted on a silicic acid thin-layer plate (10 × 10 cm). Electrophoresis in the first dimension was performed at pH 3.4 in pyridine/acetic acid/H_2O, 1:10:89 (vol/vol), at 500 V for 60 or 90 min (as indicated) at 5°C. Ascending chromatography in a second dimension perpendicular to electrophoresis was in 1-butanol/pyridine/acetic acid/H_2O, 15:10:3:12 (vol/vol).

RESULTS

Identification of Phosphorylation Sites in Hormone-Sensitive Lipase. ^{32}P-labeled hormone-sensitive lipase was purified from isolated intact adipocytes, which had been preincubated with $^{32}P_i$ and briefly exposed to ACTH. The enzyme protein was isolated in a homogenous form by NaDodSO$_4$/PAGE. Peptide mapping after digestion with V8 protease and trypsin produced two phosphopeptides (Fig. 1*A*). These phosphopeptides define two discrete phosphorylation sites in hormone-sensitive lipase isolated from intact cells. The consistently lower extent of phosphorylation of the more acidic phosphopeptide after purification of the enzyme from the cells compared to the direct isolation by NaDod-

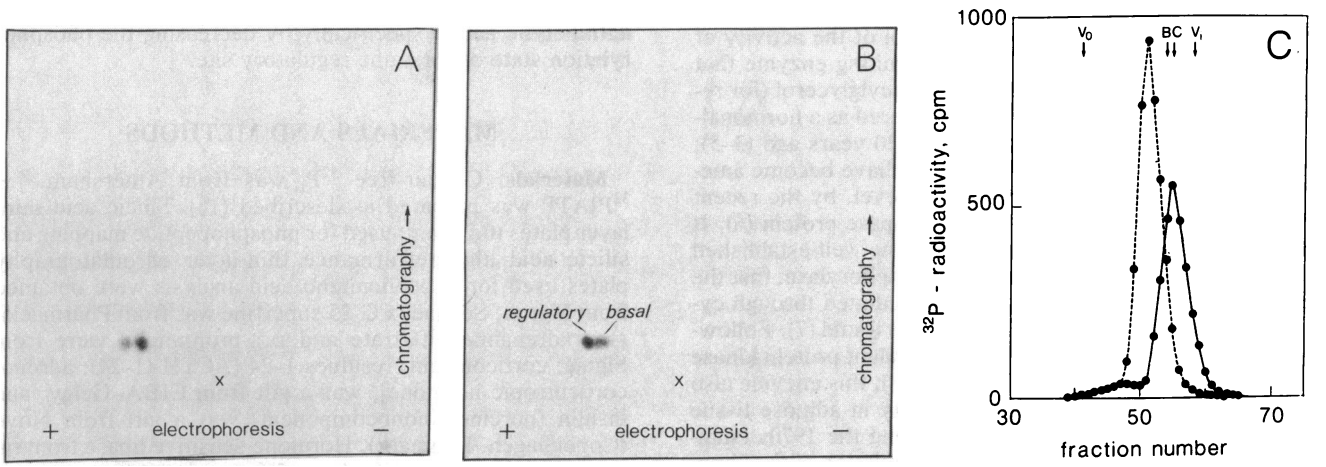

FIG. 1. Identification of hormone-sensitive lipase phosphorylation sites. (*A*) ^{32}P-labeled hormone-sensitive lipase was purified from isolated, intact adipocytes exposed to ACTH and then digested with *Staphylococcus aureus* V8 protease and trypsin before phosphopeptide mapping by electrophoresis for 60 min and chromatography. (*B*) ^{32}P-labeled hormone-sensitive lipase purified from adipocytes as in *A* was mixed with ^{32}P-labeled hormone-sensitive lipase phosphorylated by cyclic AMP-dependent protein kinase *in vitro*, and the mixture was digested for phosphopeptide mapping as in *A*. The "×" indicates the point of application. For explanation of "regulatory" and "basal" see text. (*C*) The regulatory and the basal peptides were separately extracted from the silicic acid with 10% (vol/vol) acetic acid and lyophilized. The phosphopeptides were redissolved in 0.25 M pyridine/acetic acid, pH 6.5, subjected separately to gel chromatography in Sephadex G-25 superfine (3 × 180 mm), and equilibrated in the sample buffer; fractions of about 20 μl were collected. V_0, void volume (determined with blue dextran or myoglobin); V_i, inner volume (determined with $^{32}P_i$ or [^{3}H]glycerol); B, elution position for bacitracin (M_r, 1411); C, elution position for cyanocobalamin (M_r, 1355). —, Regulatory peptide; ---, basal peptide.

SO_4/PAGE (below) was probably due to protein phosphatase-catalyzed dephosphorylation during the purification of the enzyme.

The more acidic phosphopeptide was identical with the single phosphopeptide previously identified (13) after phosphorylation of purified hormone-sensitive lipase with cyclic AMP-dependent protein kinase *in vitro*, as evidenced by the following. ^{32}P-labeled hormone-sensitive lipase purified from intact adipocytes and hormone-sensitive lipase ^{32}P-phosphorylated by cyclic AMP-dependent protein kinase *in vitro* were mixed, digested with the proteases, and subjected to phosphopeptide mapping (Fig. 1*B*). This resulted in a selective relative increase of the radioactivity of the more acidic phosphopeptide (hereafter referred to as regulatory peptide or site) (Fig. 1*B*) when compared to the control digest consisting only of enzyme protein phosphorylated in the intact cell (Fig. 1*A*). Moreover, a mixture of regulatory peptide from *in vitro* and *in vivo* phosphorylated lipase protein subjected to gel chromatography (Fig. 1*C*) was eluted as a single symmetrical peak with the same K_{avg} as cyanocobalamin (M_r, 1300). [The same K_{avg} was previously found for the phosphopeptide obtained after phosphorylation of isolated hormone-sensitive lipase by cyclic AMP-dependent protein kinase (13).] The regulatory peptide was clearly separated from the basal peptide, which eluted during the gel chromatography with a slightly larger apparent M_r (≈2000).

After partial acid hydrolysis of hormone-sensitive lipase phosphorylated in either the basal site or in both the basal and regulatory sites, the only phosphorylated amino acid detected was phosphoserine (Fig. 2).

Proximity of the Two Phosphorylation Sites in the Primary Structure. The patterns of phosphopeptides separated by NaDodSO$_4$/PAGE after limited digestion with V8 protease (23) were identical for hormone-sensitive lipase phosphorylated either in the basal site only or in both sites (Fig. 3). Both phosphorylation sites were contained in the limit peptide of M_r ≈ 17,000 that was obtained (Fig. 3); thus, they are separated by at most ≈170 residues.

Effects of Hormones on the Extent of Phosphorylation of the Two Sites. The effects of noradrenaline on the total extent of phosphorylation of hormone-sensitive lipase and on the enzyme activity in intact rat adipocytes are shown in Fig. 4.

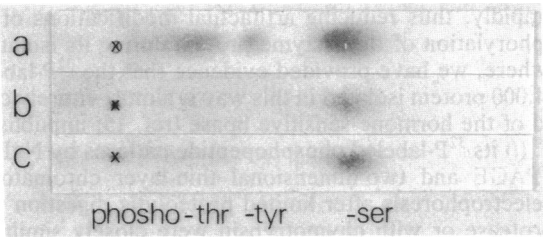

FIG. 2. Identification of phosphorylated amino acid residues. ^{32}P-labeled hormone-sensitive lipase, phosphorylated at the basal site or at the basal and regulatory sites, was isolated by NaDodSO$_4$/PAGE from cells (PCV, ≈0.1 ml) sampled before addition of hormones—i.e., basal conditions (step a in Fig. 4)—or after addition of 150 nM noradrenaline (step b in Fig. 4), respectively. The electrophoretically eluted protein was hydrolyzed in 6 M HCl under argon for 3 hr at 110°C. The solution was then evaporated to dryness and redissolved in water containing 50 nmol of phosphoserine, phosphotyrosine, and phosphothreonine (Sigma). The phosphorylated amino acids were separated by electrophoresis on silicic acid thin-layer plates (high-performance thin-layer chromatography, 10 × 10 cm) at pH 1.9 in 2.5% (vol/vol) formic acid and 15% acetic acid at 10 V/cm for 10 min and 55 V/cm for 140 min. The plates were dried, stained with ninhydrin, and autoradiographed. Rows: a, ninhydrin stain of the reference phosphoamino acids; b and c, autoradiographs of samples from hormone-sensitive lipase phosphorylated at the basal site (row b) and at the basal plus the regulatory site (row c). ×, Point of application.

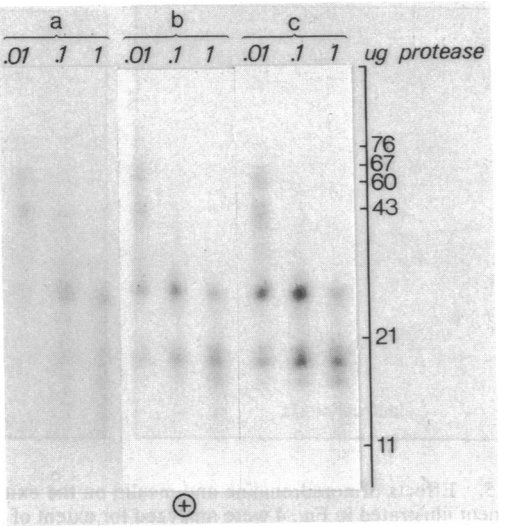

FIG. 3. Phosphopeptide pattern after NaDodSO$_4$/PAGE of proteolytic digests of hormone-sensitive lipase from cells. Gel slices containing hormone-sensitive lipase phosphorylated at the basal site or at the basal and regulatory sites from NaDodSO$_4$/PAGE of total cell protein of cells (PCV, 25 μl) sampled before addition of hormones [i.e., basal conditions (step a in Fig. 4)] (lanes a), after addition of 150 nM noradrenaline (i.e., step b in Fig. 4) (lanes b), and after addition of 700 pM insulin (step c in Fig. 4) (lanes c). The gel slices were digested with the indicated amounts of *Staphylococcus aureus* V8 protease and separated by NaDodSO$_4$/PAGE as described (23). The dried gel was autoradiographed. The M_rs × 10^{-3} of the following reference proteins are indicated: colipase (11,000), soybean trypsin inhibitor (21,000), ovalbumin (43,000), bovine serum albumin (67,000), catalase (60,000), and human transferrin (76,700).

After addition of $^{32}P_i$, hormone-sensitive lipase radioactivity increased, reaching steady state after 40–50 min (basal conditions, step a) with no increase of the basal lipolytic rate. In the absence of hormones, this steady-state level of ^{32}P-labeled hormone-sensitive lipase will be maintained (15). This

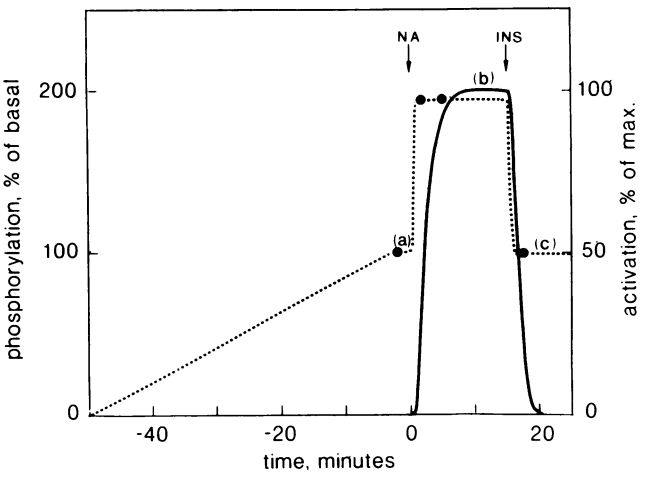

FIG. 4. Effects of noradrenaline and insulin on hormone-sensitive lipase activity and extent of phosphorylation in intact adipocytes. Adipocytes were prepared and incubated. Enzyme activity was continuously monitored in the incubation by pH-stat titration of free fatty acid release, and the extent of enzyme phosphorylation was analyzed after NaDodSO$_4$/PAGE on samples in triplicate (15). The idealized curve (····), which denotes enzyme phosphorylation, was estimated from the indicated points of experimental observations and from curves obtained from similar experiments performed previously (15, 24). NA, addition of noradrenaline (150 nM); INS, addition of insulin (700 pM).

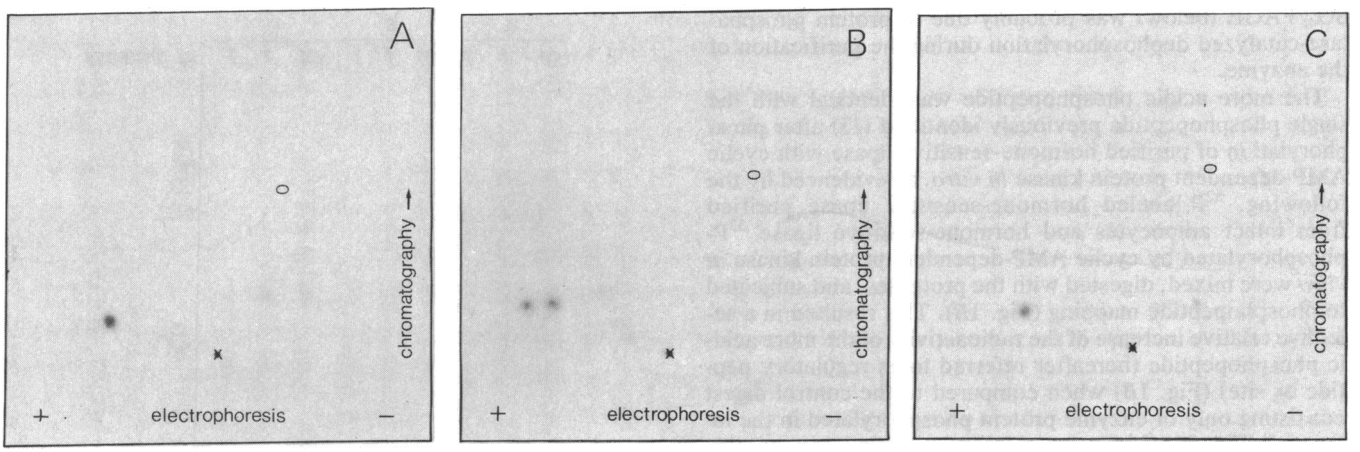

FIG. 5. Effects of noradrenaline and insulin on the extent of phosphorylation of the regulatory and basal sites. Samples of cells from the experiment illustrated in Fig. 4 were analyzed for extent of phosphorylation of hormone-sensitive lipase at the regulatory and basal sites. Cells (PCV, ≈0.1 ml) were sampled at step a (*A*), at step b (*B*), and at step c (*C*) of Fig. 4. ^{32}P-labeled hormone-sensitive lipase was isolated by NaDodSO$_4$/PAGE, digested with *Staphylococcus aureus* V8 protease and trypsin, and subjected to phosphopeptide mapping by electrophoresis for 90 min and chromatography. The "×" indicates the point of application, and the ring indicates the position of dinitrophenyllysine.

time course of lipase radioactivity closely parallels the specific radioactivity of the cellular [^{32}P]ATP (15). Addition of noradrenaline rapidly increased (approximately doubled) the extent of hormone-sensitive lipase phosphorylation (step b) and, after a short lag-time, the lipolytic rate [the [^{32}P]ATP specific radioactivity has been shown not to be affected (15, 24)] (Fig. 4). Addition of insulin rapidly decreased the extent of phosphorylation of the hormone-sensitive lipase to the basal level (step c) obtained before exposure to hormones. After a short lag-time, the rate of lipolysis also was reduced to basal conditions (Fig. 4).

Because of the obvious risk of changes in the extent of phosphorylation of the enzyme during its purification from the cells, as was indeed found (cf. Fig. 1*A*), a technique for the direct isolation of the enzyme protein from total cell protein by NaDodSO$_4$/PAGE was used. With this technique the cells were removed from the incubation medium, and enzyme-catalyzed reactions were stopped by sodium dodecyl sulfate within a few seconds (15). It previously has been shown that the ^{32}P-labeled M_r 84,000 polypeptide isolated in this way by NaDodSO$_4$/PAGE consists almost entirely of ^{32}P-labeled hormone-sensitive lipase (15, 24).

At basal conditions (Fig. 4, step a), before the addition of noradrenaline, only the basal peptide had incorporated ^{32}P$_i$ (Fig. 5*A*). Addition of the hormone (Fig. 4, step b) increased the phosphorylation of the regulatory peptide to about the same level as that of the basal peptide (Fig. 5*B*). Addition of insulin (Fig. 4, step c) caused the selective and apparently complete dephosphorylation of the regulatory site (Fig. 5*C*). [In addition to these two major phosphopeptides other, minor (<15% of total) spots were often found, which were of variable intensity and occurrence, probably representing partially digested material.]

The β-adrenergic antagonist propranolol inhibited the noradrenaline-stimulated lipolysis (Fig. 6). As expected, concomitant with the enzyme activity, the extent of phosphorylation was rapidly reversed to the basal level (Fig. 6*A*) through the almost complete dephosphorylation of the regulatory site (Fig. 6*B*).

DISCUSSION

The short-term hormonal control of lipolysis through the reversible phosphorylation of a single regulatory site of hormone-sensitive lipase was demonstrated. The identification of this regulatory site with that (13) phosphorylated *in vitro* by cyclic AMP-dependent protein kinase is strong evidence for cyclic AMP mediation of the effect of the fast-acting lipolytic hormones on fat-cell lipolysis.

Our previous demonstration that insulin inhibits lipolysis through net dephosphorylation of hormone-sensitive lipase (15, 24) could be shown to be entirely due to dephosphorylation of the single phosphoserine residue at the regulatory site. It remains to be established whether insulin causes this effect through inhibition of the cyclic AMP-mediated phosphorylation, promotion of the dephosphorylation [i.e., activation of a protein phosphatase(s)], or indeed through both processes. However, the dephosphorylation of a cyclic AMP-specific phosphorylation site argues for a role of cyclic AMP reduction (cf. ref. 2), especially since protein phosphatase 1 seems to be involved in the dephosphorylation (H. Olsson, personal communication).

The methodology used here for determination of hormone-sensitive lipase phosphorylation, namely, isolation of the ^{32}P-labeled enzyme protein from the total cell protein by NaDodSO$_4$/PAGE, made it possible to isolate the enzyme protein rapidly, thus reducing artifactual modifications of the phosphorylation of the enzyme protein during its isolation. Elsewhere, we have provided evidence that the ^{32}P-labeled M_r 84,000 protein isolated in this way is almost entirely composed of the hormone-sensitive lipase (ref. 15; unpublished data): (*i*) its ^{32}P-labeled phosphopeptide patterns by NaDodSO$_4$/PAGE and two-dimensional thin-layer chromatography/electrophoresis after limited proteolytic digestion with V8 protease or with chymotrypsin were closely similar to those of ^{32}P-labeled hormone-sensitive lipase purified from cells (unpublished data); (*ii*) it cofractionated with enzyme activity during purification of hormone-sensitive lipase from fat cells (14); and (*iii*) its elution pattern from hydroxyapatite in NaDodSO$_4$ was identical with *in vitro* phosphorylated ^{32}P-labeled hormone-sensitive lipase (15). The results presented in this report verify the identification because the same two phosphopeptides were obtained after digestion of the enzyme protein purified from fat cells and after isolation by NaDodSO$_4$/PAGE from total cell protein.

The regulatory and basal phosphopeptides were clearly separated from each other by gel chromatography, with estimated M_rs of ≈1300 and 2000, respectively, which would indicate that they are not simply mono- and diphosphorylated forms of the same peptide. This is also strongly supported by phosphorylation of the lipase with different cyclic AMP-independent protein kinases. Cyclic AMP-dependent protein kinase phosphorylates only the regulatory site; a cyclic nu-

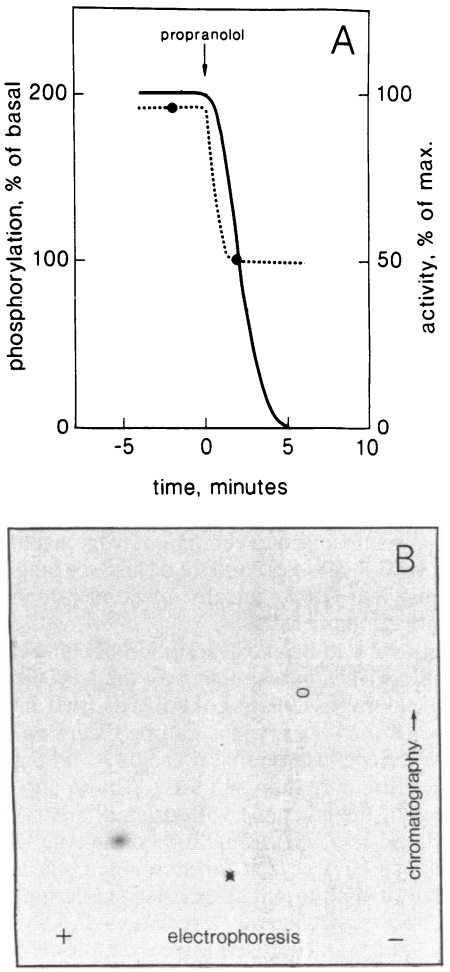

FIG. 6. Effects of propranolol on hormone-sensitive lipase activity and phosphorylation. Adipocytes, isolated and incubated, were exposed to noradrenaline (150 nM) for 10 min before propranolol (1 μM) was added. (*A*) Enzyme activity was continuously monitored in the incubation by pH-stat titration of free fatty acid release, and the extent of enzyme phosphorylation was analyzed after NaDodSO$_4$/PAGE on samples in triplicate (15). The idealized curve (····), which denotes enzyme phosphorylation, was estimated from the indicated points of experimental observations and from more detailed results of the effects of propranolol obtained previously (24). (*B*) Cells (PCV, ≈0.1 ml) sampled 7 min after the addition of propranolol were analyzed for the extent of phosphorylation at the regulatory and basal sites; electrophoresis was for 90 min. The "×" indicates the point of application, and the ring indicates the position of dinitrophenyllysine.

cleotide-independent protein kinase, glycogen synthase kinase-4 (25), phosphorylates only the basal site (unpublished data); and cyclic GMP-dependent protein kinase phosphorylates both sites at about the same rate (unpublished data).

The finding reported here that lipolysis is controlled through reversible phosphorylation of a single serine residue in hormone-sensitive lipase makes this enzyme and the isolated fat cell an attractive model for the study of the mechanisms of hormone action. This is made particularly true by the unique simplicity by which the enzyme activity can be continuously monitored in the intact cell, in combination with the determination of the phosphorylation state of the enzyme.

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