Control of Endogenous Phosphorylation of the Major cAMP-dependent Protein Kinase Substrate in Adipocytes by Insulin and β-Adrenergic Stimulation*

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(Received for publication, March 2, 1990)

In isolated, ¹²⁵I-P-, loaded, rat adipocytes, we have examined phosphorylation of the major cAMP-dependent protein kinase (A-kinase) substrate, a protein that appears to be associated with the lipid storage droplet and migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a 65–67-kDa doublet. In control cells, a strong phosphorylation signal is detected by autoradiography (A-kinase) A-kinase activity ratios range from ≈0.1 to ≈0.3–0.4 with increasing isoproterenol concentrations. By contrast, insulin-treated cells exhibiting A-kinase activity ratios over the range of 0.1–0.25 contain less ³²P in the 65–67-kDa protein than control cells exhibiting identical A-kinase activity ratios. At higher activity ratios (>0.3), this reduction in phosphorylation of the 65–67-kDa protein by insulin disappears. It is concluded that insulin stimulates a phosphatase activity that acts on the 65–67-kDa protein.

Insulin actions aside, these studies reveal two interesting phenomena. 1) Whereas elevated, steady-state A-kinase activities are established rapidly (1–2 min) upon isoproterenol stimulation, phosphorylation of the 65–67-kDa substrate proceeds through a burst, followed by a decline to a steady-state level by 10–12 min. An “adaptation” mechanism, providing for a constant response to a constant stimulus, may underlie this lack of parallelism between the time course of phosphorylation and A-kinase activity. 2) Removal of [³²P]orthophosphate immediately before isoproterenol stimulation leads to a rapid (t = 10 min) loss in labeling of the 65–67-kDa protein, whereas the phosphorylation state of other phosphoproteins is not changed. These data suggest that elevation of A-kinase activity leads to a rapid exchange of external P, with an ATP pool that is used by A-kinase.

Previously, by simultaneously monitoring steady-state levels of cAMP-dependent protein kinase (A-kinase) activity

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The abbreviations used are: A-kinase, cAMP-dependent protein kinase; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MOPS, 3-butoxy-4-methoxyaminooimido-1-propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MOPS, 3-butoxy-4-methoxysulfate-polyacrylamide gel electrophoresis as a 65–67-kDa doublet. In control cells, a strong phosphorylation signal is detected by autoradiography (A-kinase) A-kinase activity ratios range from ≈0.1 to ≈0.3–0.4 with increasing isoproterenol concentrations. By contrast, insulin-treated cells exhibiting A-kinase activity ratios over the range of 0.1–0.25 contain less ³²P in the 65–67-kDa protein than control cells exhibiting identical A-kinase activity ratios. At higher activity ratios (>0.3), this reduction in phosphorylation of the 65–67-kDa protein by insulin disappears. It is concluded that insulin stimulates a phosphatase activity that acts on the 65–67-kDa protein.

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and glycerol release in isolated rat adipocytes, we found that insulin exerted both cAMP related and cAMP independent antilipolytic actions (1). The latter effect was revealed by conditions under which insulin suppressed lipolysis despite the fact that A-kinase remained elevated at a level which, in the absence of insulin, led to substantially higher lipolysis. Moreover, the kinetic features of the relationship between lipolysis and the steady-state A-kinase activity led us to speculate that, in addition to lowering cAMP, insulin stimulated a protein phosphatase activity that dephosphorylated and inactivated the hormone-sensitive lipase, the rate-limiting enzyme of lipolysis (1).

A direct demonstration of this putative insulin-stimulated dephosphorylation of hormone-sensitive lipase requires isolation and biochemical dissection of the enzyme to determine the phosphate content of the "regulatory" phosphorylation site within the enzyme (2). As an alternative approach, we sought other, more abundant A-kinase substrates in fat cells, in order to probe the question of insulin-stimulated dephosphorylations. The goal was to compare directly the phosphorylation state of such proteins with the steady-state levels of A-kinase activity in order to determine if insulin stimulated the removal of phosphates by mechanisms independent of its ability to decrease cAMP, and therefore, lower A-kinase activity. In this paper we examine the phosphorylation state of the major adipocyte substrate for A-kinase, a 65–67-kDa protein that is apparently associated with the lipid storage droplet. Out findings indicate that insulin decreases the phosphorylation of this protein by a cAMP-independent process.

EXPERIMENTAL PROCEDURES

RESULTS

Identification of Major Adipocyte Phosphoproteins and A-kinase Substrates—For reasons described in detail previously (4, 5), in order to obtain a strong phosphorylation signal upon elevation of A-kinase with isoproterenol, control cells were incubated with adenosine or the potent adenosine receptor agonist, PIA (10). Fig 1 shows an autoradiograph of [³²P]proteins in the supernatant, membrane, and fat "cake" fractions which were separated by SDS-PAGE. Clearly, the most dramatic changes upon isoproterenol stimulation were seen in proteins associated with the fat fraction, especially those at 84, 65–67, 45, and 36 kDa. Among this group, there was copious incorporation of radioactivity into the protein(s) that migrate at 65–67 kDa, the species we selected as a model
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Isolated adipocytes were loaded with $^{32}$P, as described under "Experimental Procedures" for loading at high specific radioactivity. After 2 h of incubation with $75 \mu$Ci/ml $^{32}$P, the cells were washed, resuspended in medium containing 0.1% BSA, and incubated in either the inhibited state (200 nM adenosine, Control) or stimulated state (1 nM isoproterenol plus 0.5 units/ml of adenosine deaminase) for 5 min. Subsequently, the adipocytes were homogenized, fractionated, and prepared for SDS-PAGE as described under "Experimental Procedures." The figure shows an autoradiograph of supernatant (S), membranes (M), and extracted fat cake (F) fractions that were chromatographed by SDS-PAGE on 10% polyacrylamide gels. Each lane contains material derived from approximately 10 µl of packed intact adipocytes.

The overall distribution of proteins, as determined by silver staining, among supernatant, membranes, and fat cake fractions did not mirror the distribution of radiolabeled proteins (data not shown). Despite the preponderance of phosphoproteins in the fat, especially in the stimulated cells, less than 20% of the stainable proteins were found in this fraction. Some of the fat-associated phosphoproteins may be membrane proteins which adhered to or were trapped within the fat cake, as evidenced by their distribution between the gross membrane and fat cake fractions, such as the 75- and 56-kDa species that are prominent phosphoproteins in both resting and stimulated cells. However, several of the major phosphoproteins appeared to be located exclusively in the fat fraction, including those at 45-, 65-67-, and 84-kDa protein, which is hormone-sensitive lipase [11, 12]. Similarly, the 62-kDa protein in unstimulated cells was found only in the fat cake.

Rapid Termination of Cell Incubations and Recovery of $^{32}$P-Proteins—The reasons for terminating incubations of $^{32}$P-loaded cells by acid precipitation and the methodology for separating cellular proteins from exogenous BSA are described under "Experimental Procedures." Fig. 2 presents a comparison of the phosphoproteins harvested after extraction of BSA with the phosphoproteins present in whole adipocytes that had been incubated briefly in a BSA-free medium. The results demonstrate that most, if not all, of the major detectable phosphoproteins were recovered with this procedure, and the dramatic increase in phosphorylation of the 65-67-kDa protein seen in the extracts of trichloroacetic acid precipitates reflected that seen in whole cells washed free of BSA. Scanning densitometry (data not shown) revealed that >80% of all radiophosphate incorporated into all adipocyte proteins was found in this single, fat-associated (Fig. 1) species in isoproterenol-stimulated cells. Note that the prominent 62-kDa phosphoprotein in control cells, also a fat-associated protein (Fig. 1), disappeared upon stimulation with isoproterenol (Fig. 2).

Resolution of 65-67-kDa Phosphoprotein Doublet—For studies in which the phosphorylation state of proteins in metabolically regulated cells (see below) was investigated, all cell incubations were terminated and processed by the extraction method described above. Quantitation of the phosphorylation state of the 65-67-kDa protein was performed by scanning densitometry of autoradiographs. Although not immediately apparent in the overexposed autoradiographs in the above figures, there was a clear doublet of phosphoproteins in the 65-67 kDa region. To test if the 65- and 67-kDa bands were a single protein whose migration in gels changed as a result of increased phosphate incorporation, a partially purified fraction of adipocyte protein from unlabeled cells was phosphorylated with exogenous A-kinase. As seen in Fig. 3A, upon phosphorylation of this fraction with a tracer concentration of [γ-32P]ATP, the 65-kDa phosphoprotein was detected initially. However, within 3 min of addition of an excess of non-radioactive ATP, the sharp phosphoprotein band at 65 kDa became diffuse over the 65-67 kDa region, and with...
were purified from cytosolic fractions of homogenates according to a minor contaminant of hormone-sensitive lipase preparations which adipocyte homogenates, a small fraction of this protein appeared as proteins: effect of cold ATP chase on migration of 32P-labeled protein(s) are found predominately in the fat cake of centrifuged containing approximately 5 μg of protein, was incubated at 30 °C recently developed procedure." An aliquot of the lipase preparation, with 10 pmol units of A-kinase that was purified according to the procedure of Beavo et al. (13). The reaction was initiated with A-kinase activity ratios over this intermediate range, insulin-replete incubations than in control incubations in order to achieve a given A-kinase activity ratio (see legend to Fig. 4). Fig. 5B) Fig. 5 presents a comparison of time courses of phosphorylation of the 65-67 kDa protein in 32P-preloaded adipocytes that were incubated subsequently with varying isoproterenol concentrations in the continued presence of [32P]orthophosphate (Fig. 5A) or in the absence of [32P]orthophosphate (Fig. 5B). Fig. 5, A and B reveal two different phenomena. We showed previously (5) that under our incubation conditions, for any given isoproterenol concentration, an elevated A-kinase activity is established within 1–2 min and remains constant for up to 25 min. However, in the 32P-replete incubations (Fig. 5A), labeling of the 65-67 kDa protein proceeded through an initial spike, followed by a decline, and steady-state phosphorylation of the protein was not established until 8–12 min. This phenomenon was evident for all intermediate levels of stimulation; maximal or supramaximal isoproterenol concentrations led to a rapid and invariant level continued incubation nearly all of the radioactivity was found at the 67 kDa region (see densitometric scans, Fig. 3B). A reasonable interpretation of this experiment is that progressive phosphate incorporation after 7 min of incubation, not visible because of the lowered ATP specific radioactivity, altered the migration of the 65-kDa phosphoprotein such that it migrated as a 67-kDa species. Based on these results, we proceeded on the assumption that the 65- and 67-kDa species represented different phosphorylation states of a single polypeptide.

Comparison of Lipolysis, Activation State of A-kinase, and Phosphorylation State of the 65–67-kDa Protein: Effects of Insulin—Isolated fat cells were subjected to finely graded increases in isoproterenol concentration in order to produce finely graded increases in the A-kinase activity ratio. As found previously by this laboratory under identical incubation conditions (5), glycerol release approached a maximum as the activity ratio approached approximately 0.4 in the non-radioactive cells (data not shown). Similarly, phosphorylation of the 65–67-kDa polypeptide in the parallel, 32P-loaded cells was acutely sensitive to A-kinase activity ratios (Fig. 4A), and in numerous experiments, incorporation of 32P ranged from nil to maximal as the A-kinase activity ratio ranged from <0.1 to approximately 0.3–0.4 in the absence of insulin. As with the experiment in Fig. 2, increased incorporation of 32P into the 65–67-kDa species was accompanied by a parallel loss of the 62-kDa phosphoprotein (Fig. 4A). The reciprocal relationship between phosphorylation of the two species, 62 and 65–67 kDa, was maintained in incubations in which cells stimulated by removal of adenosine (4, 5) were progressively inhibited with increasing concentrations of PIA (data not shown). That is, upon stimulation by adenosine removal, over the range of PIA concentrations that lowered A-kinase activity from approximately 0.35 to <0.1, phosphorylation of the 65–67-kDa protein was diminished whereas the 62-kDa species appeared.

In order to view possible insulin effects not related to decreases in A-kinase activity, autoradiographs and corresponding densitometric scans of the 65–67-kDa protein from control and insulin-replete incubations are aligned according to A-kinase activity (Fig. 4A). Note that since insulin decreases cAMP and thus, A-kinase activity, slightly higher isoproterenol concentrations were required in the insulin-replete incubations than in control incubations in order to decrease in vitro phosphorylation of the 65-67-kDa species, insulin-replete incubations incorporated less phosphate into the 65-67-kDa protein. Finally, as A-kinase activity exceeded 0.3–0.35, the inhibition of phosphate incorporation by insulin was overcome.

Kinetic Features of Phosphorylation of 65–67-kDa Protein— Fig. 5 presents a comparison of time courses of phosphorylation of the 65–67-kDa protein in 32P-preloaded adipocytes that were incubated subsequently with varying isoproterenol concentrations in the continued presence of [32P]orthophosphate (Fig. 5A) or in the absence of [32P]orthophosphate (Fig. 5B). Fig. 5, A and B reveal two different phenomena.

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**Fig. 4.** Isoproterenol-dependent activation of A-kinase and phosphorylation of the 65–67-kDa protein in $^{32}$P-loaded adipocytes: evidence for A-kinase-independent decrease in phosphorylation by insulin. Isolated adipocytes were loaded with $^{32}$P at 10 μCi/ml and maintained in the presence of $^{32}$P, during the incubation with stimulatory and inhibitory ligands, such as isoproterenol and insulin. Cells were incubated with receptor ligands for 20 min. Incubations of radioactive cells were terminated with trichloroacetic acid, and the proteins were extracted as described under “Experimental Conditions.” Non-radioactive cells were incubated in parallel for determinations of A-kinase activity ratios and glycerol content as described under “Experimental Procedures.” Panel A shows autoradiographs of the 65–67 kDa region of SDS-PAGE gels of extracts of $^{32}$P-loaded fat cells that were subjected to a range of isoproterenol concentrations as indicated below, as were the non-radioactive cells shown in panel A. The $^{32}$P-loaded cells were incubated with the β-adrenergic agonist both in the absence (Control) and presence (+ Insulin) of 2000 microunits/ml of insulin. The 65–67-kDa band is noted with the arrow at the right of the autoradiograph strips. The arrow at the left of the autoradiograph strips denotes the 62-kDa band. Densitometric tracings over the 65 and 67 kDa region, as well as the area of each tracing in arbitrary absorbance units, are shown directly below the autoradiographs. Also shown below each scan is the A-kinase activity ratio for the identical incubation in the non-radioactive cells. The autoradiographs and densitometric scans are aligned to match incubations with and without insulin according to A-kinase activity ratios. Since insulin lowers cAMP and, thus, A-kinase activity ratios, slightly higher isoproterenol concentrations were necessary to achieve approximately equivalent A-kinase activities in the presence than in the absence of insulin. Panel B, derived from the data in panel A, is a plot of the combined densitometric scanning area of the 65–67 kDa region of the autoradiograph versus the A-kinase activity ratios for incubations conducted in the absence (○—○) and presence (●—●) of insulin. Isoproterenol concentrations (nM) reading from left to right, were (+insulin) 1, 2, 4, 6, 9, 12, 24, 32, 64, and 128; (+insulin) 1, 2, 12, 18, 24, 32, 64, 128, and 1000. The arrow on the horizontal axis denotes the A-kinase activity ratio and corresponding area of the 65–67-kDa band for cells incubated without isoproterenol or insulin but in the presence of 200 nM adenosine.

of phosphorylation over the incubation time course. Although the early time course of labeling was not studied in detail, a number of different experiments revealed that the steadystate levels of phosphorylation achieved by 15–20 min were generally 20–40% lower than peak levels seen at 3–8 min. Thus, concomitant with a stable, unchanging A-kinase activity ratio, steady-state phosphorylation of the 65–67 kDa proceeds through a “burst,” or overshoot, prior to declining to a steady-state level.

In the incubations conducted without readdition of $^{32}$P (Fig. 5B), phosphorylation of the 65–67-kDa doublet by all isoproterenol concentrations at 4 min was approximately 10–
presence or absence of 32Pi. Thus, the time-dependent loss of presence or absence of radiophosphate during the second there was a marked, time-dependent decrease (t = 10 min) in upon removal of the radioisotope from the medium (Fig.

washed and resuspended in medium in which "Pi was replenished at for 2 h with 10 &i/ml of 32P, as described under “Experimental

diately, the cells were incubated 0.5 units/ml adenosine deaminase, 3 substrates (data not shown).

Fig. 5. Time course of isoproterenol-stimulated phosphorylation of the 65–67-kDa protein: comparison of the maintenance and removal of exogenous 32P, on steady-state 32P incorporation into protein. Isolated adipocytes were loaded with 10 μCi/ml of 32P, as described under “Experimental Procedures” for leading to low specific radioactivity. The cells were washed and resuspended in medium in which 32P was replenished at 10 μCi/ml (panel A) or in which 32P was omitted (Panel B). Immediately, the cells were incubated 0.5 units/ml adenosine deaminase, 3 mM PIA, and isoproterenol at the following concentrations (nM): 0, •, 0.5, ○—○; 1, ×—×; 2, ■—■ and 4, ▲—▲. The arrow on the vertical axis notes the phosphorylation state of cells incubated without isoproterenol.

20% lower than in the 32P, replete incubations. Remarkably, however, over 8–16 min after introduction of isoproterenol, there was a marked, time-dependent decrease (t = 10 min) in the radioactivity incorporated into the 65–67-kDa protein upon removal of the radioisotope from the medium (Fig. 5A). Note that the only difference between Fig. 5, A and B, is the presence or absence of 32P. Thus, the time-dependent loss of 32P incorporation into the 65–67-kDa protein occurred under conditions of active phosphorylation, presumably with ATP of rapidly decreasing specific radioactivity. By contrast, over the relatively brief time course of these experiments, the presence or absence of radiophosphate during the second incubation had little or no effect on the phosphorylation state of other major phosphoproteins, all of which are not A-kinase substrates (data not shown).

DISCUSSION

A major goal of the present investigation was to examine the effects of insulin on the phosphorylation state of A-kinase substrates in intact adipocytes. Based on the arguments developed below, we conclude that insulin promotes the dephosphorylation of the major adipocyte A-kinase substrate by a mechanism separate from insulin-mediated lowering of cAMP and A-kinase activity.

As a model A-kinase substrate we chose the protein that migrates at 65–67 kDa on SDS-PAGE, by far the most abundant phosphoprotein in adipocytes in which A-kinase is elevated. Remarkably, the vast majority of 32P phosphates transferred to all adipocyte proteins by A-kinase are inserted into this single, yet unidentified, protein. Indeed, as suggested by Garrison (14), we have found that examining phosphorylation changes of this protein is the most sensitive indicator of hormone action in intact cells. The protein is multiply phosphorylated by A-kinase, both in vitro and in vivo, resulting in reduced migration during SDS-PAGE, i.e. apparent molecular mass shift from 65 to 67 kDa. Preliminary analysis of proteolytic digests of the 65- and 67-kDa species provides further evidence that these bands are phosphorylation variants of the same protein (data not shown). Phosphorylation of the 65–67-kDa doublet is correlated closely with insulin A-kinase activity ratios over the range of 0.1 to approximately 0.35, the same range over which lipolysis is increased. However, the 65–67 kDa is not hormone-sensitive lipase, which is an 84-kDa A-kinase substrate (11, 12), and polyclonal antibodies against the lipase do not recognize the 65 67 kDa protein.7 With few exceptions (15, 16), this abundant phosphoprotein has been overlooked, primarily because it partitions into the fat cake, a fraction frequently discarded in adipocyte studies.

If insulin were to decrease the phosphorylation of A-kinase substrates by merely decreasing the A-kinase activity, the relationship between A-kinase activity and phosphorylation state would be identical in the presence and absence of insulin. However, this is clearly not the case (Fig. 4B). In the presence of insulin, at intermediate A-kinase activity ratios (approximately 0.15–0.25), there is substantially less phosphate in the 65–67 kDa protein than in the absence of insulin. Moreover, this difference in phosphorylation between the insulin-replete and insulin-deficient condition disappears as the A-kinase activity ratio is increased to approximately 0.4. Previously, with highly similar findings on the relationship between A-kinase activity and glycerol release in the presence and absence of insulin, we surmised that insulin stimulated the dephosphorylation, and thus the inactivation, of hormone-sensitive lipase (1). Recently, Strafors and Honnor (17) confirmed our earlier speculation (1) with their findings that insulin reduced the phosphate content of hormone-sensitive lipase by a mechanism not explained by the cAMP-lowering effect of insulin. Our findings with the 65–67-kDa protein indicate that this insulin effect might apply generally to A-kinase substrates. Moreover, as was argued previously for the effects of insulin on lipolysis (1), the ability of increasing A-kinase activity to overcome the insulin-induced reduction in 65–67 kDa phosphorylation suggests that insulin activator a phosphatase. Thus, at the intermediate A-kinase activity levels, phosphatase activity prevails, whereas further increases in A-kinase activity overcome the effects of the phosphatase.

Although increased phosphatase activity has been invoked frequently to explain some physiological effects of insulin (see Ref. 18 for review), direct demonstrations of increased protein phosphatase activity are few, including early observations with fat cells (19), and recent studies with 3T3 cells (20, 21). Additional support is derived from findings on insulin-mediated changes in the phosphorylation state of phosphatase inhibitors (22, 23). Further insulin has been shown to decrease the phosphorylation of a number of proteins at sites not subject to phosphorylation by A-kinase, and thus not attributable to decreases in cAMP. In most cases, phosphatase activity has been the preferred explanation for these insulin-induced dephosphorylations of, among others, glycogen synthase (24), phosphofructokinase-2 (25), acetyl-CoA carboxylase (26), pyruvate dehydrogenase (27), and glycogen phosphorylase (28). However, changes in phosphorylation may result from changes in kinases, phosphatases, or both. In none of the above examples was it possible to measure simultaneously the active state of the kinase responsible for phosphorylating those sites which were dephosphorylated by insulin, and thus conclusions must be tempered by this lack of information. Indeed, an insulin-mediated decrease in multifunctional protein kinase has been demonstrated by Ramakrishna.

J. J. Egan, A. S. Greenberg, M.-K. Chang, and C. Londos, unpublished observations.
and Benjamin (29). In the studies described herein, cell incubations were terminated in a manner which maintained both the endogenous activation state of A-kinase as well as the concomitant phosphorylation state of the major cell target of A-kinase action. As such, we have had the distinct advantage of knowing the intrinsic activation state of A-kinase under all conditions for which insulin promotes dephosphorylation of this major 65–67-kDa phosphoprotein.

Insulin actions aside, there are two intriguing findings in these studies on the temporal relationships between A-kinase activity and phosphorylation of the 65–67-kDa protein. First, a gradient of increasing isoproterenol concentrations results in a gradient of increasing steady-state A-kinase activities, matched by a gradient of increasing steady-state protein phosphorylation states. Under our cell incubation conditions, isoproterenol-stimulated increases in A-kinase activity achieve steady-state by 1–2 min and remain elevated over the time course of these experiments (4, 5), a finding confirmed by others (30). Similarly, phosphorylation of the 65–67-kDa protein occurs rapidly, albeit with an initial "overshoot" (see below). Given the rapid elevations of both A-kinase and phosphorylation, one might expect, in the absence of a compensatory phosphatase, all levels of stimulation to lead ultimately to maximal phosphorylation. Clearly, this does not occur (Fig. 5A). Thus, it may be assumed that the cells have the capacity to adjust, temporally, to prolonged stimulation. It should be emphasized that this is not classical desensitization at the receptor level, since A-kinase activity, presumably a reflection of the steady-state cAMP concentration, is unchanged over the time course of these experiments. The second interesting kinetic phenomenon in response to isoproterenol stimulation is the initial spike of phosphorylation prior to establishment, by 12–16 min, of an elevated steady-state level of phosphorylation. Concomitant with an unchanging steady-state A-kinase activity level, there is an initial overshoot of 32P incorporation into the A-kinase substrate. This phenomenon might be explained by a rapid, but temporally limited, inhibition of a phosphatase, which would account for the initial burst of phosphorylation. For example, transient phosphorylation and modification of a phosphatase inhibitor might occur (31). Alternatively, the declining phase of the spike might represent a delayed activation of a phosphatase. The simultaneous activation of both kinase and phosphatase activities would provide the cell a strategy for producing graded responses to graded stimuli and for generating a constant response to a constant concentration of stimulant. By mechanisms as yet unknown, it would appear that the fat cell "adapts" to constant stimulation to produce a constant response, akin to the response repertoire of unicellular organisms (32).

Finally, a further surprising finding is the rapid turnover of 32P content of the 65–67-kDa protein upon removal of radioactive orthophosphate from the medium (Fig. 5B), a phenomenon not seen with other proteins that are not A-kinase substrates. This finding, coupled with the maintenance of steady-state labeling of the 65–67-kDa species in the 32P replete medium (cf. Fig. 5, A and B), indicates that the phosphates in this protein are rapidly turning over. The enzyme is the requirement for the continued presence of 32P, in the medium to achieve steady-state 32P incorporation, especially since the half-time for disappearance of radioactivity in the protein (∼10 min) is considerably shorter than the half-time required to achieve steady-state labeling of cellular ATP in adipocytes, usually ∼40 min (24, 33).* These data suggest that phosphate of rapidly decreasing specific radioactivity is introduced into the protein upon removal of radiophosphate from the cell incubation medium. Loading of cells with 32P is performed under unstimulated conditions, whereas the above mentioned "unloading" of radiophosphate from protein occurs under isoproterenol-stimulated conditions. The precipitous decline in radiophosphate content of the 65–67-kDa protein may result from a rapid equilibration of the radioactive ATP pool with the external non-radioactive orthophosphate upon stimulation with isoproterenol. Preliminary experiments indicate that isoproterenol stimulation does not lead to a drastic lowering of the specific radioactivity of the total cellular ATP pool labeled in the gamma position. Perhaps a small fraction of the total ATP pool serves as the substrate for A-kinase, as suggested by the findings of Mayer and Krebs (34), who observed that phosphates incorporated into phosphorylase kinase in 32P-loaded skeletal muscle were of higher specific radioactivity than phosphates in total cellular ATP. Whatever the underlying reason for this phenomenon, these data indicate that optimal radiolabeling of proteins requires the continued presence of 32P in the medium during stimulation of cells with hormones.

Acknowledgments—We wish to thank Drs. J. C. Corbin (Nashville), J. C. Garrison (Charlottesville), and S. Shenolikar (Houston) for useful discussions and for providing materials prior to publication. We are grateful to Dr. R. O. Scow for advice on extraction of proteins from fat cakes, and to Dr. Ian A. Simpson for critical review of the manuscript.

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EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from Sigma Chemical Co.: adenosine triphosphate (GTP) and adenosine diphosphate (ADP) synthetase, ATP, 6-phosphogluconate oxidase, and 6-phosphogluconate dehydrogenase. All other chemicals were obtained from Sigma Chemical Co. The adenyl cyclase assay was performed as described previously (24). The synthesis of GTP was performed by the method of Smith and Bobko (25).

Isolation of cell organelles and purification of adenosine kinase. The isolation of the minimal organelle fraction was performed as described previously (26). The purification of adenosine kinase was performed by the method of Koshland (27). The purification of GTP was performed by the method of Ballou and Fischer (28).

For determination of the amount of adenosine kinase activity in whole-cell extracts, the following reference proteins were used: 1) the reference protein was obtained from Sigma Chemical Co. and was analyzed by the method of Koshland and Goldbeter (29). The activity of adenosine kinase in the presence of adenosine triphosphate (ATP) was measured in a buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mM MgCl2. The reaction was stopped by the addition of 5% trichloroacetic acid. The activity of adenosine kinase was measured by the method of Koshland and Goldbeter (30). The activity of adenosine kinase was measured by the method of Ballou and Fischer (31)

Transmission of phosphorylation and regulation of cell cycle. The results obtained in these experiments are in agreement with previous reports (32). The results obtained in these experiments are in agreement with previous reports (32).

SYNOPSIS

The isolation of the minimal organelle fraction and the purification of adenosine kinase were performed as described previously (24). The cloning of the minimal organelle fraction was performed by the method of Smith and Bobko (25). The cloning of the minimal organelle fraction was performed by the method of Koshland (27). The cloning of the minimal organelle fraction was performed by the method of Ballou and Fischer (28).

For determination of the amount of adenosine kinase activity in whole-cell extracts, the following reference proteins were used: 1) the reference protein was obtained from Sigma Chemical Co. and was analyzed by the method of Koshland and Goldbeter (29). The activity of adenosine kinase in the presence of adenosine triphosphate (ATP) was measured in a buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mM MgCl2. The reaction was stopped by the addition of 5% trichloroacetic acid. The activity of adenosine kinase was measured by the method of Koshland and Goldbeter (30). The activity of adenosine kinase was measured by the method of Ballou and Fischer (31).
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J. Biol. Chem. 1990, 265:18769-18775.

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