Coupling of Hormonal Stimulation and Transcription via the Cyclic AMP-Responsive Factor CREB Is Rate Limited by Nuclear Entry of Protein Kinase A

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Received 8 March 1993/Returned for modification 4 May 1993/Accepted 12 May 1993

Cyclic AMP (cAMP) regulates a number of eukaryotic genes by mediating the protein kinase A (PKA)-dependent phosphorylation of the CREB transcription factor at Ser-133. In this study, we test the hypothesis that the stoichiometry and kinetics of CREB phosphorylation are determined by the liberation and subsequent translocation of PKA catalytic subunit (C subunit) into the nucleus. Using fluorescence imaging techniques, we observed that PKA was activated in a stimulus-dependent fashion that led to nuclear entry of C subunit over a 30-min period. The degree of CREB phosphorylation, assessed with antiserum specific for CREB phosphorylated at Ser-133, correlated with the amount of PKA liberated. The time course of phosphorylation closely paralleled the nuclear entry of the catalytic subunit. There was a linear relationship between the subsequent induction of the cAMP-responsive somatostatin gene and the degree of CREB phosphorylation, suggesting that each event—kinase activation, CREB phosphorylation, and transcriptional induction—was tightly coupled to the next. In contrast to other PKA-mediated cellular responses which are rapid and quantitative, the slow, incremental regulation of CREB activity by cAMP suggests that multifunctional kinases like PKA may coordinate cellular responses by dictating the kinetics and stoichiometry of phosphorylation for key substrates like CREB.

Many growth factors and hormones regulate cellular activity through second messengers which correspondingly induce multifunctional protein kinases. For example, the cyclic AMP (cAMP)-dependent protein kinase A (PKA) regulates a number of cellular processes, including motility, metabolism, neurotransmitter release, and transcription, by the reversible phosphorylation of key substrates. Remarkably, these pathways converge on the same regulatory kinase, PKA, yet they display distinct kinetics and sensitivities to cAMP-mediated stimuli. Adrenaline stimulates glycogen metabolism, for example, through a cAMP-driven cascade which allows the initial hormonal signal to be amplified about 1,000-fold at each enzymatic step (3). This signal amplification promotes rapid on-off responses, as illustrated by the maximal activation of glycogen phosphorylase which occurs within 5 to 8 s of hormonal stimulation.

In contrast to the rapid induction seen with glycogen metabolism, cAMP-stimulated transcription proceeds at a far slower pace, with peak rates of transcription occurring only after 30 min (8, 11). cAMP regulates the expression of specific genes by mediating the PKA-dependent phosphorylation of the CREB transcription factor at Ser-133 (6). To explain the discrepancy between these two events, we developed a polyclonal antiserum directed against the phosphorylated form of CREB (phosphorylated at Ser-133). This antiserum could readily discriminate between phosphorylated and dephosphorylated forms of the protein, thereby enabling us to assess the kinetics and stoichiometry of

CREB phosphorylation in response to extracellular signals. Instead of the all-or-none response expected for a signal-amplifying mechanism, we found a linear relationship between the degree of CREB phosphorylation and the degree of hormonal stimulation. Unlike the rapid induction of glycogen-metabolizing enzymes by cAMP, the activation of CREB was very slow.

Microscopic imaging analysis of cells microinjected with fluorescently labeled PKA revealed that the nuclear translocation of C subunit was slow enough to be the rate-limiting step in the transcriptional response pathway. The amount of C-subunit activity translocated to the nucleus correlated with the strength of the hormonal stimulus. Our results suggest that the subcellular distribution of key PKA substrates may determine their relative sensitivities to hormonal stimulation.

MATERIALS AND METHODS

Antibody preparation. A CREB peptide containing residues 128 to 141 was synthesized and then purified by reversed-phase high performance liquid chromatography. The purified peptide was coupled to keyhole limpet hemocyanin by the method described by Vaughan et al. (16) and phosphorylated by incubation in a solution containing 10 μ g of C subunit of PKA per ml, 1 mM ATP, 2 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.0) at 30°C for 3 h. The coupled phosphopeptide (100 μ g) was emulsified in Freund's complete adjuvant and injected subcutaneously into rabbits on a biweekly schedule. Rabbits were bled 6 weeks after the initial injection. Immunoglobulin G was precipitated from

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serum with 50% saturated ammonium sulfate and partially purified by DEAE-cellulose chromatography to remove protein phosphatases in serum. The antibody against unphosphorylated CREB was adsorbed with unphosphorylated CREB peptide coupled to an Affi-Gel 10 resin. An antibody specific for phosphorylated CREB, named 5322, was further purified by affinity chromatography with phosphopeptide resin.

Cell lines and transfections. PC12 and NIH 3T3 cells were maintained in Dulbecco's minimal essential medium as described previously (6). Cells were transfected by calcium phosphate coprecipitation with a somatostatin CRE-CAT reporter gene plus RSV- β Gal internal control plasmid. For experiments with 8-bromo-cAMP (8Br-cAMP) and secretin, cells were transfected 20 h prior to treatment and harvested 4 to 5 h after stimulation with inducer.

FIG. 1. Activation and nuclear translocation of PKA following forskolin stimulation of PC12 cells. (Top) Photomicrographs of resting (A and B) and forskolin-treated (C and D) PC12 cells microinjected with doubly labeled PKA (FICRhR). Blue color indicates UV emission from holoenzyme (>560-nm wavelength), whereas yellow indicates presence of free C subunit (515-nm wavelength). (Bottom) Line graphs indicating time course (in minutes) of PKA dissociation (bottom graph) and nuclear translocation (top graph) in response to forskolin (10 μ M). The dotted vertical lines show when forskolin was applied to the cells. The top graph shows the ratio of PKA fluorescence intensity in the nucleus (N) versus that in cytoplasm (C), as determined by scanning confocal microscopy. The bottom graph shows the ratio of free C subunit to PKA holoenzyme, expressed as an emission ratio (515-nm wavelength/>560-nm wavelength) plotted against time.

Phosphorylation assay. PKA activity was determined in a standard 5-min assay (50 μ l) at 30°C, and reactions were performed in a solution containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 2.5 mg of CREB peptide per ml, 50 mM NaF, 1 mM Na vanadate, various concentrations of [γ -³²P]ATP, and the indicated amounts of the enzyme. The incorporation of ³²P into the CREB peptide trapped on phosphocellulose papers was measured by scintillation counting after extensive washing with 10% trichloroacetic acid.

Fluorescence labeling and microinjection of PKA. Doubly labeled PKA (FICRHR) was prepared as described previously (1), except that 1 mg of recombinant RII form of the regulatory subunit per ml labeled with 100 μ M 5'-(6')-carboxy tetramethylrhodamine succinimidyl ester (TROSU; Molecular Probes, Eugene, Oreg.) was used. Proteins were separated on 3-ml columns of Sephadex G-25 preequilibrated with a buffer solution containing 25 mM potassium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 5% glycerol and dialyzed against the same buffer overnight at 4°C. The protein was concentrated in injection buffer with Amicon Centricon concentrators.

For microinjection, PC12 cells were seeded on glass coverslips (no. 1) at least 24 h before use in experiments. FICRHR was introduced into cells by microinjection with glass needles as described previously. The protein concentration in the needle was approximately 20 μ M; the final intracellular protein concentrations were between 200 nM and 2 μ M, assuming an injection volume of 1 to 10% of the cell volume. All proteins were injected into the cytoplasm.

For fluorescence measurements, cells were imaged on a custom-built video rate scanning confocal microscope. Energy transfer was measured by monitoring the ratio of fluorescence intensities at the emission wavelengths of fluorescein (515 $\pm$ 14 nm) and rhodamine (>560 nm) compared with the 488-nm line on an argon ion laser. Each image was analyzed by drawing a circular spot over the area of interest and averaging the intensities as described previously (1). Both the fluorescein and rhodamine emission intensities were collected, permitting us to determine the nuclear brightness/cytoplasmic brightness ratios of fluorescently labeled C subunit as well as fluorescein emission intensity/rhodamine emission intensity ratios at fluorescein excitation wavelengths. Emission ratios were normalized to reflect the fraction of active kinase in cells. Maximum kinase activity was obtained by treating cells with 10 μ M forskolin.

RESULTS

To evaluate PKA activation and nuclear translocation in response to cAMP, we microinjected PC12 cells with FICRHR, fluorescently labeled PKA holoenzyme (Fig. 1). As described previously (1), FICRHR consists of a catalytic (C) subunit labeled with fluorescein and a regulatory (R) subunit labeled with rhodamine. Fluorescence resonance energy transfer between labeled R and C subunits, readily detectable in the holoenzyme state, is eliminated upon dissociation of the catalytic subunits. By monitoring the fluorescence emission in microinjected cells, we could measure the degree of PKA activation and the governing concentration of free cAMP in living cells.

Free catalytic subunit activity, as assessed by microscopic imaging, was very low in unstimulated PC12 cells. Almost no PKA holoenzyme could be seen in the nucleus by this technique (Fig. 1). Upon stimulation with forskolin (10 μ M), nearly 100% of the holoenzyme dissociated within 2 min. Following an initial lag period of 3 to 5 min, nuclear

FIG. 2. Production of phosphorylated-CREB-specific antibody. (A and B) Immunoblots of recombinant CREB (WT) and CREB-M1 (M1) proteins. CREB-M1 contains the Ser-133 $\rightarrow$ Ala-133 substitution which destroys the PKA phosphorylation site. The absence (-) or presence (+) of ATP in reaction mixtures containing CREB (or M1) protein plus the catalytic subunit of PKA is indicated above each lane. (A) Immunoblot with CREB antibody 220 (220-Ab) raised against a synthetic peptide spanning residues 136 to 150. (B) Immunoblot with affinity-purified 5322 antibody. The arrow points to 43-kDa CREB band. Smaller protein bands are digestion products of CREB and CREB-M1. Fainter bands at 80 kDa correspond to dimeric forms of CREB. (C and D) Immunoblots of phosphorylated CREB from rat brain tissues. Nuclear extracts (10 μ g of protein) prepared from rat hypothalamus (Hypo.) and hippocampus (Hipp.) were incubated with the C subunit of PKA (0.1 μ g) in the presence (+) or absence (-) of 100 μ M ATP and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (C) Immunoblot with antibody 220, showing the same amount of CREB in each sample. (D) Immunoblot with 5322 antibody, recognizing only phosphorylated CREB from brain tissue. Recombinant CREB protein (CREB) is shown as a positive control. The positions (in kilodaltons) of molecular size markers (Mr) are shown to the left of gels.

translocation of C subunits proceeded steadily and reached a plateau at 30 min after stimulation. Under these conditions, the C subunit appeared to be concentrated 1.4-fold in the nucleus relative to the cytoplasm. The kinetics of C-subunit entry into the nucleus coincided with the previously described rates of CREB phosphorylation and CRE-dependent transcription in response to forskolin (8, 11), with each becoming maximal after 30 min. The level of nuclear C subunit did not decrease in parallel with the drop in CREB phosphorylation which we previously observed after 1 h (8) but remained elevated for at least 50 min after forskolin stimulation.

To determine the amount of PKA available for phosphor-

FIG. 3. CREB phosphorylation in response to forskolin. (A and B) Immunoblot analysis of nuclear extracts prepared from control (C) and forskolin-treated (F) PC12 cells using antiserum that was specific for phosphorylated CREB (5322 Ab) or that was nondiscriminating (244 Ab). PK-A, PC12 extract treated with purified PKA in vitro to obtain 100% CREB phosphorylation. MW, molecular size (in kilodaltons). The arrow points to CREB protein. Increasing amounts of untreated (U-CREB) or phosphorylated CREB (P-CREB) are indicated by the solid triangles, with the largest amount indicated by the thickest part of the triangle and with the smallest amount indicated by the tip. (C and D) Standard curves obtained with recombinant proteins given in the blots in panels A and B. The amount of CREB (in nanograms) is plotted against relative autoradiographic intensity (in arbitrary units). Open circles represent protein standards. Closed circles show intensity of control (C), forskolin (F)-, and PKA-treated samples from PC12 extracts given in the blots above. C/F indicates that the values for control and forskolin-treated cells were identical.

ylation of CREB in response to hormonal stimulation, we measured PKA activity in PC12 cell extracts, using a CREB peptide (amino acids [aa] 128 to 141) as the test substrate (not shown), and calculated the level of cAMP-dependent PKA activity in PC12 cells to be 1.2 μ M, which is consistent with estimates for other cell types (3). On the basis of the nuclear intensity/cytoplasmic intensity measurements we obtained with fluorescently labeled PKA, the concentration of free catalytic subunit in the nucleus should approach 1.2 to 1.7 μ M after 30-min exposure to 10 μ M forskolin.

To monitor CREB phosphorylation in response to cAMP, we developed an antiserum specific for CREB phosphorylated at Ser-133 raised against a PKA-phosphorylated synthetic CREB peptide (aa 128 to 141). Immunoblot analysis (Fig. 2) revealed that the affinity-purified antiserum 5322 could discriminate between dephosphorylated and PKA-phosphorylated CREB protein, whereas CREB antiserum 220 (7), raised against a synthetic CREB peptide spanning aa 136 to 150, could not. Antiserum 5322 could not detect PKA-treated CREB-M1 protein which contains a Ser-133→Ala-133 substitution, suggesting that phosphorylation of Ser-133 was indeed critical for immunoreactivity.

To determine the selectivity of antiserum 5322 for phosphorylated CREB protein versus other cellular substrates of PKA, we examined crude extracts of rat hippocampus and hypothalamus by Western blot (immunoblot) analysis (Fig. 2). It was remarkable that 5322 antiserum could indeed distinguish a single 43-kDa CREB band after but not before in vitro phosphorylation with PKA. In contrast, the nondiscriminating 244 CREB antiserum showed no preference for either form of the protein. Moreover, other CREB homologs, such as ATF-1 (39 kDa) and CREM (21 kDa) (5, 9), apparently did not cross-react with this antiserum, although we cannot exclude the possibility that the closely related cAMP-responsive factor CREM τ (43 kDa) is not recognized by this antiserum.

To test whether the 5322 antiserum could detect CREB phosphorylation after induction by cAMP in living cells, we compared CREB immunoreactivity prior to and following treatment of PC12 pheochromocytoma cells with forskolin (Fig. 3). A single 43-kDa immunoreactive CREB band was specifically enhanced within 30 min of treatment and was accompanied by a four- to fivefold induction in run-on transcription from the cAMP-responsive somatostatin gene

FIG. 4. Effect of PKA phosphorylation on salt elution and DNA-binding profiles of CREB. (A) Western blot analysis of cytoplasmic (CYTO) and nuclear fractions from PC12 cells using nondiscriminating 244 CREB antiserum. Results with phosphorylated-CREB-specific 5322 antibody (not shown) were identical. The molarity of KCl used to wash nuclei is shown above the lanes. The arrow points to the position of immunoreactive CREB protein. The positions (in kilodaltons) of molecular size (MW) markers are shown to the left. (B) Gel retardation assay of CREB and Δ CREB proteins using double-stranded somatostatin CRE oligonucleotide. U and P denote unphosphorylated or PKA-phosphorylated proteins, respectively. (C) DNA-binding curve of CREB and phosphorylated CREB proteins determined by filter-binding assay. P-CREB, phosphorylated CREB. K_d values for both CREB forms are indicated.

(8, 11) (not shown). The increase in phosphorylated CREB immunoreactivity after 30 min showed a strong correlation with the peak phosphorylation of Ser-133, determined previously by two-dimensional tryptic mapping studies (8). To determine the stoichiometry of phosphorylation after forskolin treatment, we performed quantitative Western blot analysis. To quantitate the levels of phosphorylated CREB and total CREB, we obtained standard curves for phosphorylated-CREB-specific (5322 Ab) and nondiscriminating (244 Ab) CREB antisera with purified recombinant CREB protein. For each amount of CREB or phosphorylated CREB loaded onto a polyacrylamide gel (0, 1, 2.5, 5, and 10 ng), we plotted the autoradiographic intensity corresponding to 125 I-labeled goat anti-rabbit secondary antibody (Fig. 3C and D). Detection of PKA phosphorylated CREB was linear from 0 to 10 ng for both antisera. Unphosphorylated CREB was recognized equally well by the nondiscriminating antiserum 244, but no signal was observed with the phosphorylated-CREB-specific antiserum 5322, as expected.

To determine the stoichiometry of CREB phosphorylation

in PC12 cells, we analyzed 20 μ g of PC12 nuclear extract from forskolin-treated or control cells for total CREB and phosphorylated CREB content by Western blotting. The total amount of CREB in treated and control samples was estimated by measuring the signal intensity obtained with the nondiscriminating CREB 244 antiserum and plotting that value against the standard curve corresponding to recombinant CREB protein. Both treated and control samples contained 8 ng of total CREB per 20 μ g of PC12 nuclear extract protein (Fig. 3D).

The phosphorylated CREB-specific antiserum 5322 (Fig. 3C) detected less than 1 ng of phosphorylated CREB per 20 μ g of nuclear protein in control PC12 cells but recognized 3 to 4 ng of phosphorylated CREB per 20 μ g of nuclear extract protein from forskolin-treated cells. Since *in vitro* PKA-phosphorylated control nuclear extracts contained 8 ng of phosphorylated CREB per 20 μ g of nuclear extract protein, all of the CREB protein in PC12 cells appears to be phosphorylatable, but only about 40% of total CREB is actually modified in response to forskolin stimulation.

To compare the cellular distribution and DNA-binding characteristics of CREB versus phosphorylated CREB proteins, we prepared cytoplasmic and nuclear extracts from both unstimulated and forskolin-treated PC12 cells (Fig. 4). Neither phosphorylation-specific nor nondiscriminating antisera could detect CREB in cytoplasmic extracts from control or treated cells. In both cases, the vast majority of CREB immunoreactivity was eluted from isolated nuclei with a salt step of 0.42 M KCl, placing the theoretical concentration of CREB at 400 nM. The absence of any visible shift in salt elution profile upon phosphorylation suggested that CREB binds its cognate CRE sites on chromatin without regard for its phosphorylation state. *In vitro* DNA-binding curves for phosphorylated and dephosphorylated CREB protein were also superimposable, each having K_d values of 10^{-9} M (Fig. 4) on the somatostatin CRE, far

FIG. 5. Phosphorylation of wild-type CREB synthetic peptides by PKA *in vitro*. Lineweaver-Burke plot of CREB phosphorylation by PKA. The rate of 32 P incorporation into CREB peptide (aa 128 to 141) was determined in the presence of 10 nM PKA, 0.2 or 1 mM of ATP, and the indicated concentrations of the peptide. Data are expressed as the double reciprocal plot of the reaction velocity and the peptide concentration. The K_m and V_{max} values are shown on the graph.

FIG. 6. (A) cAMP stimulates CREB phosphorylation and gene expression in a dose-dependent manner. Effect of 8-Br-cAMP on CRE-CAT activity (top) and CREB phosphorylation (bottom) in NIH 3T3 cells. The bar graph shows absolute conversion (%Conversion) of [14 C]chloramphenicol to acetylated forms. All samples are from cells transfected with somatostatin CRE-CAT reporter plasmid and harvested 5 h after stimulation. Chloramphenicol acetyltransferase assays were performed on extracts normalized to cotransfected RSV- β Gal control plasmid. The concentration of 8-Br-cAMP employed is shown. Numbers in parentheses over each bar indicate fold induction over untreated cells. (Bottom) Western blot analysis of CREB phosphorylation using phosphorylated CREB-specific 5322 antibody. Each sample was normalized to total protein. The concentration (μ M) of 8-Br-cAMP is indicated over each lane. The arrow points to immunoreactive CREB bands. The faint lower band may represent CREB degradation product. The positions (in kilodaltons) of molecular size standards (Mr) are shown to the left of the gel. (B) Stimulus-dependent activation of PKA in NIH 3T3 cells. Proportion of C subunit liberated from PKA holoen-

zyme (%PK-A Activation) shown after 30-min stimulation with various concentrations of 8-Br-cAMP. Following microinjection of FICRhR, fluorescently labeled PKA holoenzyme, relative kinase activation was determined by comparing the ratio of fluorescence emission at 515 nm (free C subunit) versus that at 560 (holoenzyme).

less than the nuclear concentration of CREB. Thus, in contrast to a recent study reported by Nichols et al. (13), phosphorylation did not appear to regulate transcription of cAMP-responsive genes by recruitment or stabilization of CREB on DNA. The absence of detectable CREB or phosphorylated CREB in cytoplasm indicated that CREB phosphorylation must be initiated in the nucleus.

To determine the K_m of PKA for the CREB phosphorylation site at Ser-133, we employed both full-length CREB protein and a synthetic CREB peptide (aa 128 to 141) (Fig. 5). Remarkably, the K_m value for CREB was 10 μ M, far above the estimated nuclear concentration of the protein (400 nM). The same K_m value (10 μ M) was obtained with a 14-aa synthetic CREB peptide (aa 128 to 141), suggesting that this low K_m was caused by the PKA phosphorylation site itself and not to steric hindrance from other regions of CREB. The PKA phosphorylation site (aa 130 to 134; Arg Arg Pro Ser Tyr) is followed by two basic residues, Arg-135 Lys-136, which have previously been characterized as potent negative regulators of PKA when located at +2 and +3 positions relative to the Ser phosphoacceptor (10). These values are consistent with those reported by Colbran et al. (4).

Although the affinity of PKA for CREB was low, the V_{max} for CREB phosphorylation was quite high (800 μ mol/min/ μ mol of enzyme) (Fig. 5), placing the theoretical rate of CREB phosphorylation under physiological conditions at $V = V_{max}[S]/(K_m + [S])$ or 32 μ mol/min/ μ mol of PKA. Given this predicted rate of phosphorylation, CREB protein should therefore be quantitatively phosphorylated within seconds, suggesting that the nuclear transport of PKA is rate limiting. This estimate does not, however, take into account other nuclear substrates of PKA which might compete for the kinase and thereby reduce the rate and extent of CREB phosphorylation.

The high predicted rate of CREB phosphorylation prompted us to ask whether any stimulus might be sufficient to trigger near-quantitative phosphorylation of CREB. We examined CREB phosphorylation and CRE-CAT activity in NIH 3T3 cells treated with increasing concentrations of 8-Br-cAMP (Fig. 6A). Western blot analysis revealed that CREB protein was actually phosphorylated in a dose-dependent manner, and the degree of phosphorylation after 30 min coincided with the relative induction of the somatostatin promoter, as determined by a transient assay. The phosphatase inhibitor okadaic acid had no effect on peak levels of CREB phosphorylation after 30 min (not shown), suggesting that the previously described protein phosphatase 1 (PP-1) attenuation of CREB (8) must occur later. In the absence of detectable PP-1 activity, the incremental phosphorylation of CREB should therefore reflect a corresponding dose-dependent increase in free nuclear C subunit.

To test this possibility, we measured the proportion of PKA holoenzyme dissociated after treatment of NIH 3T3 cells with various concentrations of 8-Br-cAMP (Fig. 6B). Using microinjected FICRhR, we observed a graduated release of C subunit from the holoenzyme which coincided with similar changes in CREB phosphorylation and cAMP-

FIG. 7. Dose-dependent effect of secretin on CRE-CAT activity and CREB phosphorylation. (Top) CRE-CAT activity derived from PC12 cells treated with secretin at the indicated concentration and harvested 5 h after stimulation. Bars represent % [14 C]chloramphenicol conversion obtained from cells transfected with somatostatin CRE-CAT plasmid. All samples were assayed for chloramphenicol acetyltransferase activity after normalizing to cotransfected RSV- β -Gal control plasmid. (Bottom) Western blot of PC12 extracts treated with increasing doses of secretin (0, 1, 10, 30, 100, and 1,000 nM [indicated by the height of the solid triangle over the lanes]) using phosphorylated CREB-specific antibody 5322. The arrow points to immunoreactive phosphorylated-CREB band. The positions (in kilodaltons) of molecular size standards (Mr) are shown to the left of the gel.

dependent transcription. Thus, for example, 30% of maximal PKA activation (100 μ M 8Br-cAMP) was accompanied by about 30% of maximal CREB phosphorylation and CRE-CAT induction at the same level of inducer.

To test whether receptor-mediated stimuli also regulate CREB activity through a slow, incremental mechanism, we treated PC12 cells with secretin. This hormone has previously been shown to stimulate tyrosine hydroxylase (TH) activity in PC12 cells through PKA-dependent phosphorylation of the enzyme within 8 to 10 s of treatment (14). In contrast with the rapid activation of TH, CREB phosphorylation in response to secretin was far slower (30 to 60 min) and proceeded in a dose-dependent manner (Fig. 7). CREB was phosphorylated at a threshold dose of 1 to 2 nM secretin and became maximal at 100 nM secretin. Somatostatin promoter activity, as measured by a transient assay over a 5-h period, increased in a parallel fashion, suggesting that cAMP-dependent transcription was also tightly coupled to the strength of the extracellular stimulus.

DISCUSSION

Although the signal cascade paradigm has been useful in explaining rapid, quantitative induction of metabolic events, this model may be inappropriate for transcriptional processes which often display graded responses to hormones and growth factors. Three features, a low concentration of substrate (CREB protein), delayed transport of C subunit to the nucleus, and a low affinity of PKA for CREB, appear to promote a slow, graded transcriptional response to extracellular inducers relative to other cellular events.

Like other nuclear regulatory factors, CREB is expressed at low levels compared with other cellular proteins. PKA is far more abundant than CREB in PC12 cells, suggesting that Ser-133 phosphorylation must be a low-efficiency event in order to be incrementally regulated. In contrast, phosphorylase kinase, the first step in the glycogen cascade, is present at about 20-fold-higher concentrations (3) and theoretically would be phosphorylated about 25 times faster than CREB.

The time required for nuclear entry of PKA also contributes to the kinetic differences between cytoplasmic and nuclear events. Glycogen metabolism and neurotransmitter synthesis occur within seconds of stimulation with cAMP, but transcriptional events become maximal only after 30 min. Our results with the fluorescently labeled PKA holoenzyme suggest that the kinetics for transcriptional induction coincide with the time required for nuclear entry of free catalytic subunit. The lag period between such cytoplasmic and nuclear events may thus favor a prioritized set of responses to cellular inducers.

Having ventured into the nucleus after stimulation, the free C subunit does not rapidly exit into the cytoplasm or become noticeably associated with R subunit. The dephosphorylation of CREB in face of persistent nuclear C subunit (8) probably reflects competition between phosphorylating and dephosphorylating activities arising from PKA and PP-1, with PP-1 prevailing at later times. Indeed, Buellens et al. (2) have recently identified a nuclear inhibitor of PP-1, termed NIPP-1, which releases active PP-1 in response to PKA.

Phosphorylation of CREB did not appear to trigger expression of the somatostatin gene by recruitment of CREB onto DNA. Indeed, CREB retains full PKA responsiveness when fused to heterologous DNA-binding domains, suggesting that phosphorylation specifically regulates the activation domain of this protein (8, 12, 15). Unlike the glycogen-metabolizing enzymes, however, PKA regulates CREB ac-

tivity through an incremental mechanism, and we attribute some proportion of this stimulus-dependent regulation to two basic residues C terminal to the PKA phosphorylation site, which have previously been shown to increase the apparent K_m of PKA for such substrates as much as 100-fold (10). Our results suggest that multifunctional kinases like PKA may coordinate cellular responses to extracellular stimuli by regulating key substrates with distinct affinities for the enzyme. The dynamics of PKA transport to cellular organelles after stimulation may order the series of events which follow hormonal stimulation.

ACKNOWLEDGMENTS

This work was supported by NIH grants GM37828 and CA54418, by the Howard Hughes Medical Institute, and by the Foundation for Medical Research, Inc. R.T. is a Howard Hughes Institute Investigator, and M.R.M. and W.V. are FMR investigators.

We thank T. Hunter for helpful discussions and critical review of the manuscript. We also thank C. Hoeger, T. Goedken for synthesis of CREB peptides, B. Coyne for secretarial assistance, and members of the Montminy Laboratory for numerous suggestions.

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