Cyclic AMP stimulates dephosphorylation of specific proteins in intact S49 mouse lymphoma cells

Zoltán Kiss and Robert A. Steinberg*, +

Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, 6701 Szeged, Hungary, and *+Biological Sciences Group, U-44, The University of Connecticut, Storrs, CT 06268, USA*

Received 22 August 1984; revised version received 4 December 1984

Two-dimensional gel electrophoresis of proteins labeled with $^{32}P_i$ in S49 mouse lymphoma cells revealed five phosphoproteins that were rapidly and reversibly dephosphorylated in response to elevation of cyclic AMP (cAMP). Under basal conditions, labeling of at least two of these proteins was limited by slow turnover of protein-bound phosphate. The rapid cAMP-mediated dephosphorylation of these species was attributable, therefore, to stimulation of a specific phosphoprotein phosphatase.

Cyclic AMP *Dephosphorylation* *Protein kinase* *Protein phosphatase* *Two-dimensional gel*

1. INTRODUCTION

In animal systems the primary function of cAMP is activation of cAMP-dependent protein kinases and concomitant stimulation of specific protein phosphorylations [1–3]. Cellular protein phosphorylation can also be regulated indirectly by cAMP. Several cAMP-independent protein kinases are substrates for the cAMP-dependent enzyme, and their activities are either stimulated or depressed by cAMP-dependent phosphorylation [4–6]. Furthermore, a specific inhibitor of protein phosphatase can be activated by cAMP-dependent phosphorylation [7,8]. In previous reports from this laboratory, high resolution two-dimensional gel electrophoresis was used to study cAMP-stimulated protein phosphorylations in intact S49 mouse lymphoma cells [9,10]. Mutants lacking catalytic activity of cAMP-dependent protein kinase [11] were deficient in these responses [9]. We show here that elevated cAMP also results in kinase-dependent dephosphorylation of several proteins in S49 cells. For at least two phosphoproteins, cAMP enhances rates of dephosphorylation. Therefore, it appears that elevation of cAMP can cause stimulation of a specific phosphoprotein phosphatase.

Abbreviations: cAMP, cyclic AMP; DME, Dulbecco's modified Eagle's medium

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Materials*

D,L-Isoproterenol, D,L-propranolol, and 3-isobutyl-1-methyl xanthine were from Sigma (St. Louis, MO), and [^{32}P]orthophosphoric acid (carrier-free in water) was from ICN (Irvine, CA). X-ray film (type XAR) and photographic chemicals were from Eastman Kodak (Rochester, NY), and intensifying screens ('Lightning-plus') were from DuPont (Wilmington, DE). Chemicals for two-dimensional gel electrophoresis were obtained as in [12].

2.2. *Methods*

S49 mouse lymphoma cells were grown in suspension culture in DME supplemented with 10% heat-inactivated horse serum as in [12]. Cells were labeled with $^{32}P_i$ in DME prepared with either

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

1 mM orthophosphate (figs 1,2) or no phosphate (fig.3) and supplemented with 10% heat-inactivated horse serum that had been dialyzed extensively against 0.15 M sodium chloride [13].

Cells were harvested by centrifugation, washed, and resuspended at 2×10^6 cells/ml in labeling media as in [9,13]. For the experiments of figs 1 and 2, $^{32}P_i$ was added to 0.5 mCi/ml and cultures were labeled for 3.5 (fig.1) or 3 h (fig.2). For the experiment of fig.3 cells were preincubated for 30 min at 37°C before adding $^{32}P_i$ to 4.4 mCi/ml and labeling for 4 min. Drug treatments are described in the figure legends. After labeling, cells were diluted 5-fold with ice-cold phosphate-buffered saline, harvested by centrifuging 5 s at $10000 \times g$ and lysed with gel sample buffer containing approx. 9.9 M urea as in [9,13]. Samples of extracts containing equal amounts of acid-precipitable radioactivity were subjected to two-dimensional gel electrophoresis, and labeled proteins were detected by direct autoradiography of dried gels [9,13]. For the experiment of fig.3, intensifying screens were used for enhancement. All gel patterns are shown with acidic ends of isoelectric focusing gel dimensions to the right.

3. RESULTS AND DISCUSSION

Fig.1 shows that cAMP has both positive and negative effects on protein phosphorylation in intact S49 mouse lymphoma cells. Isoproterenol, a β-adrenoreceptor agonist, and propranolol, a β-

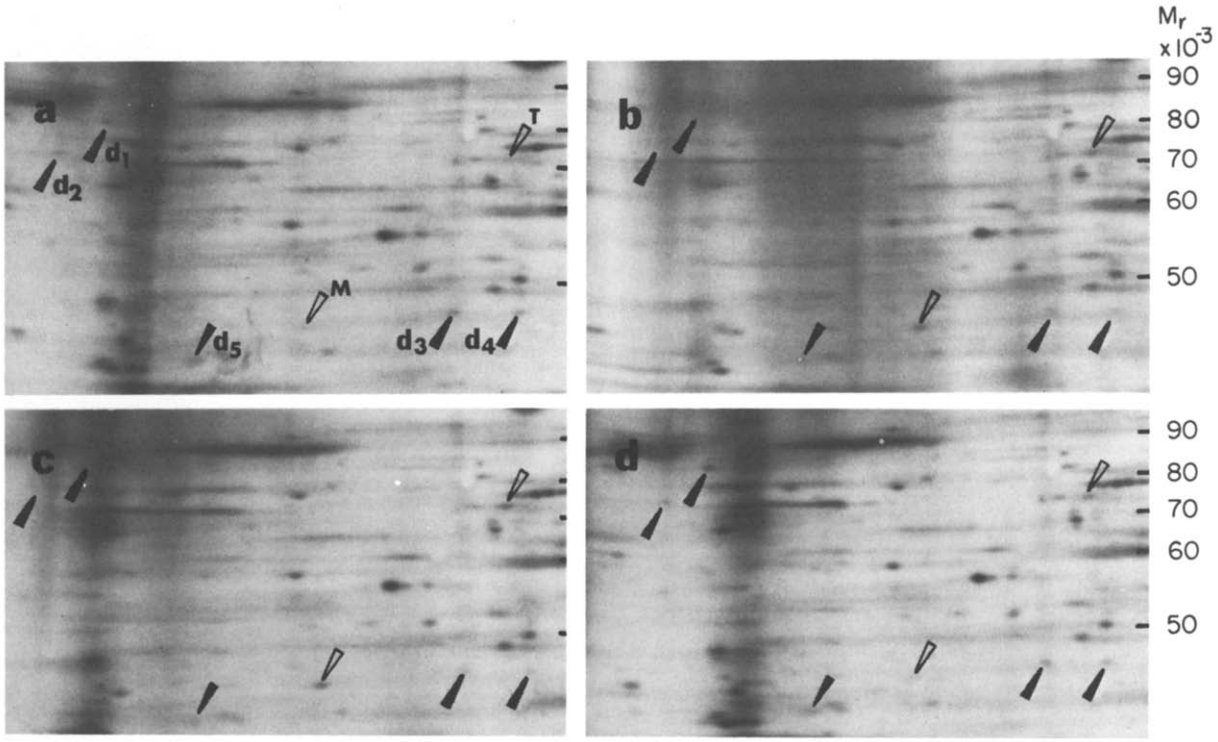

Fig.1. Isoproterenol stimulates both phosphorylation and dephosphorylation of S49 cell proteins. S49 cells were labeled for 3.5 h with $^{32}P_i$ and either left untreated (a), treated with isoproterenol (final concentration, 5 μM) for the final 15 min of labeling (b), treated with isoproterenol for the final 30 min of labeling (c), or treated with isoproterenol for the final 30 min of labeling and with propranolol (final concentration, 20 μM) for the final 15 min of labeling (d). After labeling, cells were harvested and lysed, and portions of extracts containing 1.1×10^6 cpm of acid-precipitable radioactivity were subjected to two-dimensional gel electrophoresis. Patterns shown are from 6-day autoradiographic exposures and contain proteins with p*I*'s between about 6.2 and 7.0. Filled arrowheads indicate positions of cAMP-inhibited phosphoproteins; open arrowheads indicate positions of cAMP-stimulated phosphoproteins. Letter and number designations are used in the text to refer to the species indicated. Approximate molecular masses of these species are 80 kDa for d_1, 73 kDa for d_2, 46 kDa for d_3 and d_4, 41.5 kDa for d_5, 71 kDa for T, and 44 kDa for M.

adrenoreceptor antagonist, were used to effect rapid increases or decreases of cAMP levels in cells prelabeled with $^{32}P_i$; high resolution two-dimensional gel electrophoresis was used to separate labeled phosphoprotein species. Cells were incubated with $^{32}P_i$ for 3 h before adding drugs so that intracellular ATP pools would be equilibrated with isotope. When cells were treated with isoproterenol for 15 or 30 min (fig.1b,c), labeling of some species was stimulated (M and T in portions of gel patterns shown), and labeling of several other species was depressed (d_1–d_5). For phosphoproteins d_1 and d_2 depression of labeling was virtually complete, but for proteins d_3–d_5 decreases were to about 30–50% of basal labeling. As shown for stimulatory effects [9], the inhibitory effects of isoproterenol on protein phosphorylation appeared to result from activation of cAMP-dependent protein kinase: they could be elicited in wild-type cells by analogs or inducers of cAMP, but they could not be elicited in mutant cells lacking catalytic subunit of cAMP-dependent kinase (not shown). After incubating cells with isoproterenol for 15 min, treatment with propranolol for another 15 min reversed the effects of isoproterenol on proteins M and d_1–d_5 (fig.1d). (Dephosphorylation of protein T was incomplete after 15 min of treatment with propranolol). Since the isoproterenol-mediated depression of phosphoprotein labeling could be reversed with propranolol, it reflected dephosphorylation rather than degradation of proteins d_1–d_5.

Fig.2 shows a time course for isoproterenol-stimulated dephosphorylations. A cell culture was labeled for 3 h with $^{32}P_i$, and portions were treated with isoproterenol (and isobutyl methylxanthine, an inhibitor of phosphodiesterases) for times indicated in the figure legend. Phosphorylation of proteins d_1, d_3, and d_4 was decreased maximally within 2.5 min of isoproterenol addition, while dephosphorylation of protein d_2 (and d_5, not shown) was apparently slower. In similar experiments, samples were taken after adding propranolol to $^{32}P_i$-labeled cultures that had been treated with isoproterenol; rephosphorylation of proteins d_1, d_3, and d_4 was complete within 2 min (not shown).

Net dephosphorylation of proteins could result from either inhibition of a protein kinase or stimulation of a phosphoprotein phosphatase.

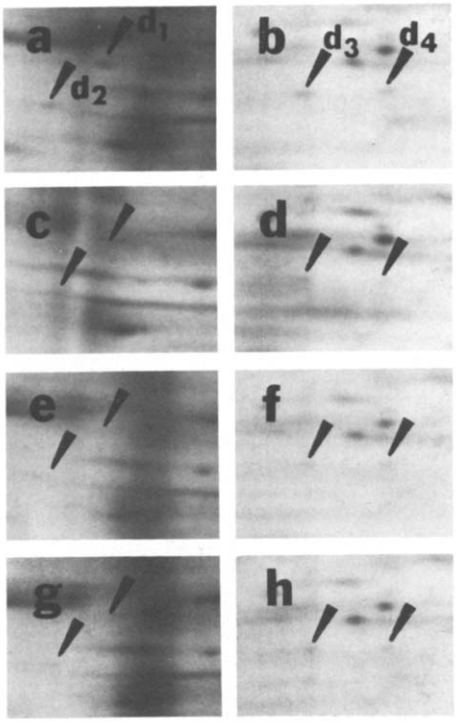

Fig.2. Time courses for isoproterenol-stimulated dephosphorylations of proteins d_1–d_4. Cells were labeled with $^{32}P_i$, extracts were prepared, and gels were run as described in section 2. Cultures were either untreated (a,b) or treated with 10 μM isoproterenol and 50 μM 3-isobutyl-1-methylxanthine for the final 2.5 min (c,d), 8 min (e,f), or 20 min (g,h) of labeling; 8.7×10^5 cpm of acid-precipitable radioactivity were loaded onto gels, and autoradiograms were exposed for 8 days. Portions of gel patterns are shown containing proteins d_1 and d_2 (a,c,e,g) or proteins d_3 and d_4 (b,d,f,h).

Comparison of half-times for dephosphorylation under conditions of basal and elevated intracellular cAMP might distinguish between these two mechanisms. The experiment of fig.2 shows that half-times for dephosphorylation of proteins d_1, d_3, and d_4 were much less than 2 min under conditions of elevated intracellular cAMP. The slow equilibration of phosphate and ATP pools precluded measurement of basal rates of dephosphorylation by label-chase procedures using $^{32}P_i$, but the rapid relabeling of phosphoproteins d_1, d_3, and d_4 following addition of propranolol to isoproterenol-treated cells suggested that basal dephosphorylation rates could be estimated from pulse labeling experiments. Since proteins whose label-

ing was inhibited by isoproterenol should be phosphorylated in basal cells, their labeling with $^{32}P_i$ would require exchange of protein-bound phosphate. Therefore, the extent to which $^{32}P_i$ could be incorporated into these proteins during a short pulse would be determined by rates of their dephosphorylation.

Fig.3 shows results from an experiment in which cells were labeled for only 4 min with $^{32}P_i$. Because of nonlinear incorporation, most labeling under this procedure occurred during the final 2 min of incubation (unpublished). One sample was untreated (fig.3a); a second sample was pretreated with isoproterenol and labeled in the presence of this agonist (fig.3b); and a third sample was pretreated with isoproterenol and then treated with propranolol during the labeling period (fig.3c). Labeling of proteins d_1 and d_3 was barely detectable in gel patterns from basal or isoproterenol-treated cells (fig.3a,b), but it was relatively strong in patterns from cells that were treated sequentially with isoproterenol and propranolol (fig.3c); phosphoproteins d_2, d_4, and d_5 were not detected in any of these patterns. Since pretreatment with isoproterenol causes extensive dephosphorylation of proteins d_1 and d_3 (fig.2), their labeling in fig.3c reflects an amount attributable to rephosphorylation. Labeling of proteins d_1 and d_3 under basal conditions requires phosphate turnover; the relatively poor labeling of these species in fig.3a thus reflects slow rates of dephosphorylation (i.e., half-times greater than 2 min). We conclude that elevated levels of intracellular cAMP stimulate dephosphorylation rates for phosphoproteins d_1 and d_3, and therefore, that elevated cAMP can stimulate activity of a specific phosphoprotein phosphatase.

The isoproterenol-stimulated phosphoprotein M provided a positive control for the results discussed above. Labeling of this species was strong in isoproterenol-treated cells (fig.3b) but relatively weak in basal or propranolol-treated cells (fig.3a, c). Since pretreatment with isoproterenol was sufficient to stimulate essentially complete phosphorylation of protein M (unpublished), its labeling in the presence of isoproterenol depended on phosphate exchange. (Complete isoproterenol-dependent phosphorylation of protein T required more than 15 min of exposure to the drug, unpublished, so labeling of this protein in fig.3b resulted, in part, from de novo phosphorylation). The appearance of protein M in fig.3b showed that it was feasible to label proteins by phosphate exchange and that dephosphorylation of protein M was fast

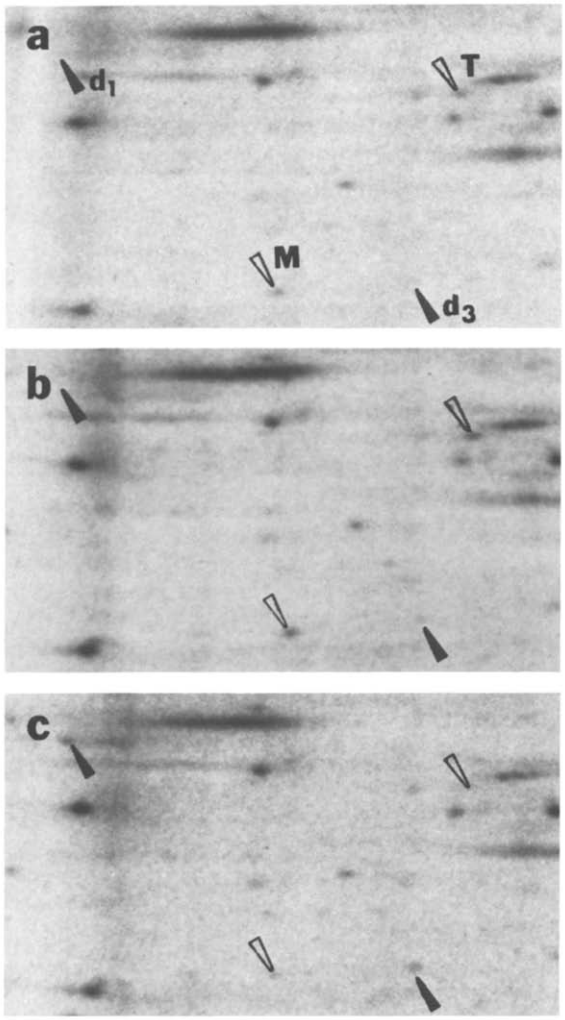

Fig.3. $^{32}P_i$-labeling of cAMP-regulated phosphoproteins in a short pulse. Cells were washed, resuspended in phosphate-free medium, and preincubated for 30 min at 37°C before labeling with $^{32}P_i$ for 4 min. Cells for (a) were left untreated, and those for (b) and (c) were treated with 5 μM isoproterenol for 5 min before adding label. For (c), 20 μM propranolol was added simultaneously with label. Extracts were prepared, and samples containing 10^5 cpm of acid-precipitable radioactivity were subjected to two-dimensional gel electrophoresis. Autoradiography (with intensifying screens) was for 14 days at −70°C.

in the presence of elevated cAMP (i.e., half-time much less than 2 min).

Cyclic AMP-mediated decreases of phosphoprotein labeling have been reported in several other systems [14–19], but this is the first report in which the mechanism for these decreases has been explored. Our results show that cAMP-mediated decreases in phosphoprotein labeling result from net dephosphorylation of the proteins affected. For proteins d_2, d_4, and d_5, we could not distinguish whether cAMP-mediated effects were on a kinase or a phosphatase, but, for proteins d_1 and d_3, rates of dephosphorylation were enhanced. The simplest explanation for this latter result is that cAMP-dependent phosphorylation stimulates the activity of a phosphoprotein phosphatase specific for proteins d_1 and d_3, but we cannot exclude mechanisms by which cAMP indirectly activates a phosphatase. Whatever the mechanism, our results are the first to clearly implicate enhanced protein phosphatase activity among cellular responses to cAMP.

ACKNOWLEDGEMENTS

We thank Caroline Murphy for maintaining cell cultures, Marian Rettenmeyer for providing photographic assistance, and David Borst for offering critical comments on the manuscript. These studies were supported by Grant AM33977 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

REFERENCES

[1] Nimmo, H.G. and Cohen, P. (1977) in: Advances in Cyclic Nucleotide Res. (Greengard, P., and Robison, G.A. eds) vol. 8, pp. 145–266, Raven Press, New York.
[2] Greengard, P. (1978) Science 199, 146–152.
[3] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[4] Conti, M.A. and Adelstein, R.S. (1980) Fed. Proc. 39, 1569–1573.
[5] Krebs, E.G. (1981) Curr. Top. Cell. Regul. 18, 401–419.
[6] Cohen, P. (1981) in: Cellular Controls in Differentiation (Lloyd, C.W., and Rees, D.A. eds) pp. 81–103, Academic Press, New York.
[7] Huang, F.L., and Glinsmann, W.H. (1976) Eur. J. Biochem. 70, 419–426.
[8] Ingebritsen, T.S., and Cohen, P. (1983) Science 221, 331–338.
[9] Steinberg, R.A., and Coffino, P. (1979) Cell 18, 719–733.
[10] Steinberg, R.A. (1981) in: Protein Phosphorylation (Rosen, O.M., and Krebs, E.G. eds), Cold Spring Harbor Conferences on Cell Proliferation, vol. 8, pp. 179–193, Cold Spring Harbor Laboratory, New York.
[11] Steinberg, R.A., Van Daalen Wetters, T., and Coffino, P. (1978) Cell 15, 1351–1361.
[12] Steinberg, R.A., and Agard, D.A. (1981) J. Biol. Chem. 256, 10731–10734.
[13] Steinberg, R.A. (1983) in: Hormone Action: Protein Kinases (Corbin, J.D., and Hardman, J.G. eds), Methods in Enzymology, vol. 99F, pp. 233–243, Academic Press, New York.
[14] Koroscil, T.M., and Gallant, S. (1980) J. Biol. Chem. 255, 6276–6283.
[15] Baum, B.J., Freiberg, J.M., Ito, H., Roth, G.S., and Filburn, C.R. (1981) J. Biol. Chem. 256, 9731–9736.
[16] Drust, D.S., Sutton, C.A., and Martin, T.F.J. (1982) J. Biol. Chem. 257, 3306–3312.
[17] Garrison, J.C., and Wagner, J.D. (1982) J. Biol. Chem. 257, 13135–13143.
[18] Feinstein, M.B., Egan, J.J., and Opas, E.E. (1983) J. Biol. Chem. 258, 1260–1267.
[19] Spruill, W.A., Steiner, A.L., Tres L.L., and Kierszenbaum, A.L. (1983) Proc. Natl. Acad. Sci. USA 80, 993–997.