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Phosphorylation of Lymphocyte Nuclear Acidic Proteins: Regulation by Cyclic Nucleotides

Abstract. *Guanosine 3′,5′-monophosphate (cyclic GMP) and cholinergic agents stimulate incorporation of phosphate into specific nuclear acidic proteins of horse peripheral blood lymphocytes. Agents that raise intracellular adenosine 3′,5′-monophosphate (cyclic AMP) inhibit nuclear acidic protein phosphorylation. The opposing effects of cyclic GMP and cyclic AMP upon nuclear protein phosphorylation parallel the effects of the cyclic nucleotides upon induction of lymphocyte proliferation.*

Chromatin protein phosphorylation is an early event preceding the extensive gene activation occurring upon induction of lymphocyte proliferation by mitogens (*1*, *2*). Certain of the effects of exogenously added mitogens upon chromatin structure and activity may be mediated by guanosine 3′,5′-monophosphate (cyclic GMP). The mitogens phytohemagglutinin and concanavalin A (Con A) have both been observed to elevate intracellular levels of cyclic GMP more than tenfold within minutes of addition to human lymphocyte cultures (*3*, *4*). On the basis of evidence obtained with a number of cell types induced to proliferate (*5*), it has been hypothesized that cyclic GMP mediates the intracellular effects of mitogens acting at the cell surface, and that, conversely, adenosine 3′,5′monophosphate (cyclic AMP) mediates actions antagonistic to the mitogenic process (*3*). We now provide evidence that opposing influences of cyclic GMP and cyclic AMP upon lymphocyte proliferation may be mediated through the effects of these agents on phosphorylation of specific nonhistone chromatin proteins.

Lymphocytes were purified from horse peripheral blood by centrifugation over a cushion of Ficoll-Isopaque (*2*). Cells prepared as described were >90 percent lymphocytes, the remaining cells consisting primarily of monocytes, which were removed by glass adherence. Lymphocytes, cultured for 24 hours prior to use in experiments, were treated with [^{32}P]-phosphate as described in the legend to Fig. 1. After cell activity was stopped by rapidly freezing at −70°C, nuclei were isolated and nuclear acidic proteins were extracted (*2*, *6*, *7*). The protein fraction contained only 10 to 20 percent of the total lymphocyte nuclear protein (*2*). These proteins constitute a class of phosphoproteins that are species specific, tissue specific, and that have been observed to stimulate transcription from isolated DNA in vitro (*6*). These phenol-extracted acidic phosphoproteins were subjected to sodium dodecyl sulfate disc-gel electrophoresis, and [^{32}P]phosphate was assayed in individual electrophoretic protein bands (*7*).

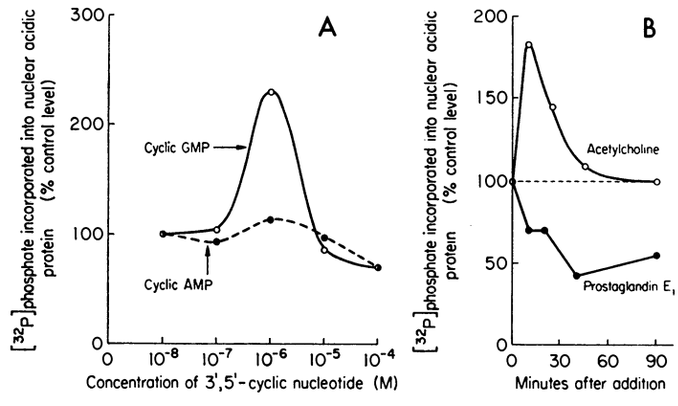
The differing effects of cyclic GMP and cyclic AMP on the amount of [^{32}P]phosphate incorporated into lymphocyte nuclear acidic proteins are shown in Fig. 1A. At a concentration of $10^{-6}M$ cyclic GMP stimulates phosphorylation of the total phenol-extracted protein fraction about twofold 10 minutes after addition to the culture medium. In contrast to this stimulatory effect of cyclic GMP, cyclic AMP, at any concentration tested, does not increase phosphate incorporation into the total nonhistone chromatin protein fraction. Both cyclic GMP and cyclic AMP inhibit nuclear acidic protein phosphorylation at concentrations $>10^{-5}M$. The 8-bromo derivative of cyclic GMP stimulates nuclear acidic protein phosphorylation at lower concentrations than does cyclic GMP. One-and-a-half– to twofold stimulation of phosphate incorporation into total nuclear acidic proteins was obtained at concentrations of 8-bromo cyclic GMP ranging from $10^{-7}M$ to $10^{-9}M$ in different experiments. 8-Bromo cyclic GMP does not inhibit nuclear acidic protein phosphorylation at concentrations as high as $10^{-5}M$. Monobutyryl derivatives of both cyclic GMP and cyclic AMP have no effect when used at low concentrations and are strongly inhibitory to phosphate incorporation at concentrations $>10^{-5}M$.

Agents known to elevate cyclic GMP concentrations in lymphocytes have effects on nuclear acidic protein phosphorylation opposite to those of agents known to elevate cyclic AMP. Cholinergic agents selectively elevate cyclic GMP in several tissues including lymphocytes (*8*). The data in Fig. 1B show that acetylcholine ($10^{-6}M$) stimulates the rate of phosphate incorporation into lymphocyte nuclear acidic proteins. The rate of protein phosphorylation is stimulated maximally within 10 minutes of acetylcholine addition and returns to control values within 90 minutes. Prostaglandin E_1 (PGE_1), an agent observed to raise cyclic AMP in lymphocytes (*9*), has effects on nuclear protein phosphorylation opposite to those exerted by the cholinergic agents (Fig. 1B). At $10^{-4}M$, PGE_1 inhibits nonhistone chromatin protein phosphorylation 60 percent within 45 minutes of addition to lymphocyte cultures. At the concentrations used, neither acetylcholine nor PGE_1 affects the viability of lymphocytes up to 24 hours after addition. Acetylcholine stimulated phosphorylation maximally at concentrations ranging from $10^{-6}M$ to $10^{-8}M$ in different experiments, while maximal inhibition by PGE_1 was observed from $10^{-4}M$ to $10^{-6}M$. The cyclic nucleotide phosphodiesterase inhibitors theophylline and papaverine were also examined for

their effects on nuclear acidic protein phosphorylation. Both theophylline ($10^{-3}M$) and papaverine ($10^{-4}M$) strongly inhibit phosphorylation induced by Con A. When added alone to cultures of resting lymphocytes, $10^{-4}M$ papaverine inhibits phosphate incorporation into nuclear acidic proteins 60 percent within 80 minutes of addition. Papaverine has been observed to selectively inhibit hydrolysis of cyclic AMP (*10*).

In Fig. 2 are shown the contrasting effects of the acetylcholine analog carbachol (carbamylcholine) at $10^{-7}M$ and PGE_1 at $10^{-6}M$ upon phosphate incorporation into individual nonhistone chromatin proteins separated by sodium dodecyl sulfate disc-gel electrophoresis. Carbachol selectively stimulates phosphate incorporation into specific lymphocyte chromatin proteins. Its effects are at a maximum at concentrations of $10^{-7}M$ to $10^{-9}M$. Effects of carbachol are blocked by inclusion of a tenfold excess of atropine in the lymphocyte culture medium (data not shown). PGE_1 selectively inhibits chromatin protein phosphorylation (Fig. 2). Carbachol and PGE_1 have opposing effects on phosphorylation of a gel band having a molecular weight of about 52,000. Phosphorylation of this particular protein band is also preferentially stimulated early in the induction of lymphocyte proliferation by Con A (*2*).

None of the agents examined alters the specific activity of incorporation of [^{32}P]phosphate into adenosine triphosphate (ATP) pools in lymphocytes. Since ATP is the donor of phosphate incorporated into nuclear proteins, this finding indicates that cyclic nucleotides regulate nonhistone chromatin protein phosphorylation by affecting the activity of protein kinases or protein phosphatases. Cyclic GMP–dependent protein kinases have been identified in several mammalian tissues (*11*), and cyclic GMP–dependent phosphorylation of specific mammalian smooth muscle membrane proteins has been observed (*12*). While no lymphocyte cyclic GMP–dependent protein kinase has yet been reported, our results suggest that such a kinase may be active in the horse lymphocyte nucleus.



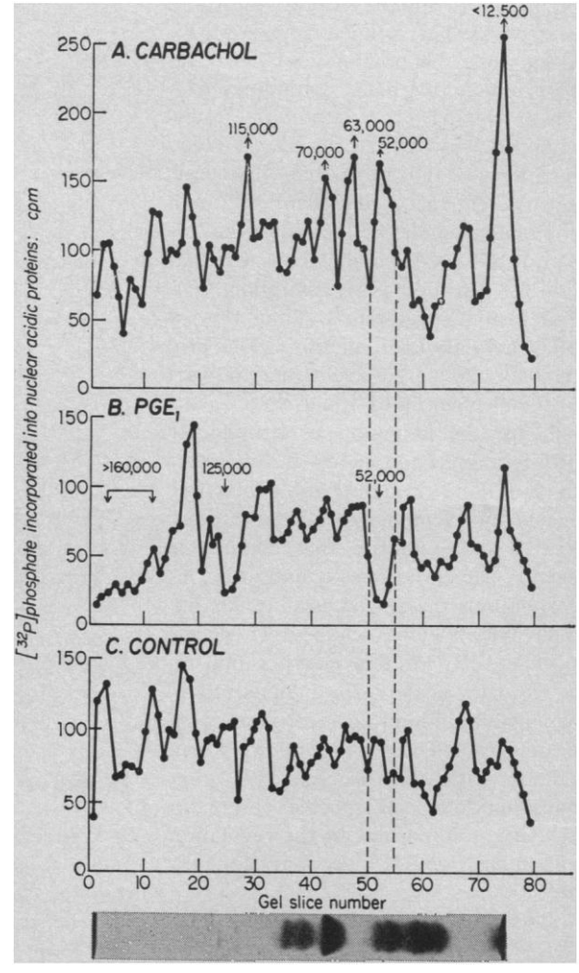


Fig. 1 (left). (A) Effects of cyclic GMP and cyclic AMP upon the amount of [^{32}P]phosphate incorporated into lymphocyte nuclear acidic proteins. Lymphocytes (5 ml; 2×17^7 cells per milliliter) were incubated for 20 minutes at 37°C with 5 mc of [^{32}P]NaH_2PO_4 in phosphate-free Eagle's minimum essential medium with 10 percent fetal calf serum. After addition of 0.5 ml of cyclic nucleotide to each portion of 10^8 cells, incubation was continued for 10 minutes. Cells were centrifuged, and the cell pellet was frozen by contact with acetone at −70°C. Nuclear acidic proteins were extracted and analyzed for radioactivity (*2*). The control incorporation of ^{32}P was 32,000 count/min per milligram of nuclear acidic protein. (B) Contrasting effects of acetylcholine and prostaglandin E_1 (PGE_1) on the rate of phosphate incorporation into nuclear acidic proteins of lymphocytes. Portions of cultured lymphocytes as described in (A) were incubated for the indicated times in the presence of either acetylcholine ($10^{-6}M$) or PGE_1 ($10^{-4}M$). Portions of 10^8 cells were treated with 5 mc of [^{32}P]NaH_2PO_4 for 30 minutes just prior to stopping cell activity by freezing at −70°C at the indicated times after the addition of pharmacologic agents. Nuclear acidic proteins were extracted, and radioactive phosphate was assayed (*2*, *7*). Because of differences in the magnitude of the response obtained in different experiments, data were not averaged, and representative experiments are shown. The control incorporation was 29,000 count/min per milligram of nuclear acidic protein. Fig. 2 (right). Contrasting effects of carbachol and PGE_1 on phosphate incorporation into individual nuclear acidic proteins separated by sodium dodecyl sulfate disc-gel electrophoresis. Lymphocytes were incubated for 20 minutes with [^{32}P]phosphate as in Fig. 1A. After addition of carbachol or PGE_1, incubation was continued for 10 minutes. Cell activity was stopped by freezing at −70°C, and nuclear acidic proteins were isolated and subjected to electrophoresis on 8.75 percent polyacrylamide gels (*2*, *7*). Each gel contained protein extracted from 10^7 lymphocytes. At concentrations used, neither carbachol nor PGE_1 altered the patterns of electrophoretic distribution of the nuclear acidic proteins. Gels were sliced transversely and slices were assayed for [^{32}P]phosphate (*2*). Effects of $10^{-7}M$ carbachol (A) and $10^{-6}M$ PGE_1 (B) are compared with phosphate incorporation into control lymphocytes (C). Arrows pointing upward denote stimulation; those pointing downward denote inhibition of phosphorylation at the indicated molecular weights. The opposing effects of carbachol and PGE_1 on phosphate incorporation into a protein fraction having a molecular weight of about 52,000 are indicated by the dotted lines.

Increased cyclic AMP in lymphocytes is associated with an inhibition of phosphorylation of chromatin proteins (*13*). Whether inhibition of phosphorylation by cyclic AMP represents an effect of cyclic AMP on phosphatase activity remains to be determined.

Several recent experiments support the hypothesis that cyclic GMP and cyclic AMP exert opposing influences on the induction of lymphocyte proliferation. Results obtained with sensitized rat spleen lymphocytes indicate that cyclic GMP and cholinergic agents enhance the cytotoxic function of T (thymus-derived) lymphocytes while agents which raise intracellular concentrations of cyclic AMP are inhibitory (*14*). Both 8-bromo cyclic GMP ($10^{-6}M$ to $10^{-7}M$) and carbachol ($10^{-10}M$ to $10^{-13}M$) enhance the rate of labeled thymidine incorporation into graft-versus-host primed mouse splenocytes while theophylline and PGE_1 inhibit uptake of labeled thymidine (*14*). These findings correlate with results obtained with human peripheral blood lymphocytes indicating that cholinergic agents enhance phytohemagglutinin-stimulated uptake of labeled thymidine (*8*), while PGE_1 inhibits this stimulation of thymidine incorporation (*9*). Our results suggest that stimulation of phosphorylation of nuclear acidic proteins by cholinergic agents may be part of the mechanism by which these agents enhance the induction of lymphocyte proliferation. Experiments are necessary to determine the effects of cholinergic agents upon lymphocyte RNA synthesis. Recent data indicate that cholinergic agents can influence lymphocyte incorporation of labeled uridine. In human lymphocytes, cholinergic agents stimulate [^{14}C]uridine incorporation into RNA (*8*) while PGE_1 inhibits phytohemagglutinin-induced incorporation of uridine (*9*). These observations are in accord with the proposal (*2*) that phosphorylation of specific chromatin proteins is important in the regulation of synthesis of RNA necessary for proliferation.

Phosphorylation of a protein fraction having a molecular weight of about 52,000 is stimulated by cholinergic agents and inhibited by PGE_1 (Fig. 2). Phosphorylation of this same chromatin protein fraction is stimulated preferentially early after mitogenic stimulation of horse lymphocytes with Con A (*2*). Our data suggest that this protein plays a regulatory role in the process of induction of lymphocyte proliferation and indicate that the phosphate content of this chromatin protein is regulated by cyclic nucleotides. The evidence supports the hypothesis that control of phosphorylation of specific chromatin proteins is an important mechanism regulating early events in the induction of lymphocyte proliferation.

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Transforming Potential of the Anticancer Drug Adriamycin

Abstract. *A Fischer rat embryo cell system in vitro, which had been shown to be highly accurate in identifying chemical carcinogens and to have application in the study of chemicals having anticancer properties, was used to study the anticancer drug adriamycin. At a nontoxic dose adriamycin not only did not protect the cells from transformation by the carcinogen 3-methylcholanthrene, but was found in two separate experiments to act on its own as a transforming agent.*

We have described a sensitive in vitro Fischer rat embryo cell system (F1706) for identifying chemical carcinogens (*1*). Using a clone of this culture, which we call H43, we report here that the anticancer compound adriamycin (*2*), at a nontoxic dose, not only does not protect the cells from transformation by 3-methylcholanthrene (3MC) but was found in two separate experiments to act on its own as a transforming agent.

Using the reduction in plating efficiency and the number of cells per