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POSSIBLE NUCLEAR PROTEIN KINASE REGULATION OF HOMOLOGOUS RIBONUCLEIC ACID POLYMERASES FROM SMALL DENSE NUCLEI OF MOUSE BRAIN DURING MORPHINE TOLERANCE-DEPENDENCE

INVOLVEMENT OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE

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Abstract—A correlation between phosphorylation and stimulation of RNA polymerases I and II by homologous nuclear protein kinase peak I from small dense nuclei of mouse brain was demonstrated. Incubation of RNA polymerase II with nuclear protein kinase peak I lowered the optimum Mg^{2+} concentration of the polymerase in the same manner as chronic morphine treatment alone did, suggesting that the change in Mg^{2+} optimum of RNA polymerase II seen during morphine tolerance-dependence may occur through changes in phosphorylation as a result of increased nuclear protein kinase activity. Experiments investigating the involvement of cyclic 3′,5′-adenosine monophosphate (cAMP) in morphine tolerance-dependence demonstrated that dibutyryl-cAMP enhanced the degree of morphine tolerance developed, as reported previously [I. K. Ho, H. H. Loh and E. L. Way, *J. Pharmac. exp. Ther.* **185**, 347 (1973); I. K. Ho, H. H. Loh, H. N. Bhargava and E. L. Way, *Life Sci.* **16**, 1895 (1975)], and also enhanced the chronic morphine-induced increase in nuclear protein kinase specific activity. Alterations in the regulation of the nuclear protein kinase activity may have subsequently affected RNA polymerase activity through phosphorylation. These results suggest that, during morphine tolerance-dependence development, cAMP may be involved in the possible nuclear protein kinase regulation of homologous RNA polymerase.

Nuclear protein kinase and homologous RNA polymerase activities from small dense nuclei of mouse brain are both altered during morphine tolerance-dependence [1, 2]. Partial purification of the nuclear protein kinases from small dense nuclei results in the resolution of two major peaks of cAMP-independent nuclear protein kinase activity; these have been termed peaks I and II. After 72 hr of chronic morphine treatment, the specific activity of peak I, but not of peak II, is increased significantly by approximately 25 per cent [1]. Investigations on the homologous RNA polymerases during morphine tolerance-dependence [2] indicate that the specific activity of RNA polymerase I is decreased, and the optimum Mg^{2+} concentration for RNA polymerase II and the Mn^{2+}/Mg^{2+} ratios of RNA polymerases II and III are altered by chronic morphine treatment.

Studies in many tissue sources suggest that nuclear protein kinase may be able to modify RNA synthesis through phosphorylation of the RNA polymerase enzyme [3–6]. In view of the finding that both the nuclear protein kinase and RNA polymerase enzyme activities are altered during morphine tolerance-dependence and the hypothesis that protein kinase may regulate RNA polymerase activity, the close functional interaction between the nuclear protein kinase and homologous RNA polymerase may be altered during morphine tolerance-dependence. This interaction between the two enzymes may be an important factor involved in the increased chromatin template activity seen during chronic morphine treatment [7].

Nuclear protein kinase regulation of RNA polymerase activity has not yet been demonstrated in brain tissue. Evidence is presented in this study illustrating a correlation between phosphorylation and activation of RNA polymerase activity by homologous nuclear protein kinase from small dense nuclei of mouse brain. Further evidence is presented suggesting that modification of RNA polymerase II through phosphorylation can modify its optimum co-factor requirement in the same manner as chronic morphine treatment alone does.

Because morphine *in vitro* has no effect on nuclear protein kinase and RNA polymerase activities [1, 2], the modification of these two enzyme systems during chronic morphine treatment is probably an effect several steps removed from the initial site of morphine action involved in the development of morphine tolerance-dependence. Numerous studies [8–11] indicate that cyclic 3′,5′-adenosine monophosphate (cAMP) may be involved in morphine tolerance-dependence. With regard to the induction of the tolerant-dependent state, investigations by Ho *et al.* [8, 9] show that a single injection of cAMP

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accelerates the rate of morphine tolerance-dependence development. Collier [12], suggests that an increase in the concentrations in the cAMP system may be an explanation for opiate dependence. Thus, it is possible that cAMP may be involved in the chronic morphine-induced increase in nuclear protein kinase activity. Such evidence, presented in this report, suggests that cAMP may also be involved in the nuclear protein kinase regulation of homologous RNA polymerase during morphine tolerance-dependence.

METHODS

Purification of protein kinase and RNA polymerase. Briefly, small dense nuclei were prepared from male, ICR mice (Simonsen Laboratories, Gilroy, CA) according to the method of Oguri *et al.* [13]. The nuclei were sonicated, to solubilize the chromatin proteins, and were centrifuged. For the purification of the nuclear protein kinases, the resulting supernatant fraction was subjected to ammonium sulfate fractionation and phosphocellulose column chromatography as described by Hook *et al.* [1]. For the purification of the RNA polymerases the supernatant fraction was subjected to DEAE-Sephadex column chromatography according to the method of Stokes *et al.* [2[.

Assay of protein kinase activity. Protein kinase was assayed as described by Hook *et al.* [1], in a total volume of 0.20 ml, with the following final concentrations: 50 mM potassium–phosphate buffer, (pH 6.5), 0.3 mM ethyleneglycol bis-(aminoethylether)tetra-acetate (EGTA), 10 mM NaF, 10 mM $MgCl_2$, 25 μg/ml casein, 10 μM ATP, and 1 μCi [γ-^{32}P]ATP per tube (10–25 Ci/mmole, ICN Pharmaceuticals, Irvine, CA). The reaction mixture was incubated for 5 min at 30°, the reaction was stopped with the addition of cold 5% TCA–1.5% Na-PP_i (5% trichloroacetic acid–1.5% sodium pyrophosphate). The samples were washed on Whatman GFC filters with 4 × 8 ml of 5% TCA–1.5% Na-PP_i, rinsed with 1 ml of cold 95% ethanol, and counted in a Beckman LS-100 scintillation counter.

Assay of RNA polymerase activity. RNA polymerase activity was assayed as described by Stokes [14] in total volume of 0.5 ml with the following final concentrations: 100 mM Tris–HCl (pH 8.9 at room temperature), 35 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol (DTT), 1 mM EDTA, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM bentonite, 1 mM ATP, 1 mM GTP, 1 mM CTP, 0.1 mM UTP, 30–40 μCi/tube [^{3}H]UTP (24–40 mCi/mmole, ICN Pharmaceuticals), and 1 mg/ml calf thymus DNA. The samples were incubated for 30 min at 37°; the incubation was stopped with the addition of 10% TCA—3% PP_i. The samples were washed on Whatman GFC filters with 7 × 8 ml of 5% TCA–3% PP_i, rinsed with 1 ml of cold 95% ethanol, and counted in a Beckman LS-100 scintillation counter.

Nuclear protein kinase and RNA polymerase assay. Approximately 10 μg protein from the RNA polymerase fraction was preincubated with various amounts of nuclear protein kinase in final concentrations of 50 mM potassium–phosphate buffer, (pH 7.4), 0.1 mM EGTA, 10 mM NaF, 10 mM $MgCl_2$, 20 μM ATP, and 2 μCi [γ-^{32}P]ATP per tube for 5 min at 30°. The tubes were then placed on ice and the RNA polymerase assay medium was immediately added to each tube to attain the final concentrations as described for the RNA polymerase assay. Samples were incubated and prepared for scintillation counting as described for the polymerase assay.

All enzyme assays were run in triplicate, using an incubation mixture containing the TCA-precipitated enzyme sample as the blank [1]. Protein concentrations were measured by the method of Lowry *et al.* [15] after the samples had been dialyzed against water.

Dibutyryl-cAMP injection and chronic morphine treatment. Morphine pellet implantation has been demonstrated [7] to render mice with a high degree of morphine tolerance-dependence. A 75-mg morphine pellet was implanted under the skin and a placebo pellet containing no drug was implanted for the control group. One hour later, the mice were injected intracerebroventricularly (i.c.v.) with 28 μg dibutyryl-cAMP in 4 μl saline. The mice were killed 24 hr later and the nuclear protein kinases were purified.

Measurement of tolerance to morphine analgesia by mouse tail-flick assay. Mice were implanted with the morphine or placebo pellet and injected i.c.v. 1 hr later with 28 μg dibutyryl-cAMP; the pellets were removed 24 hr later. The measurement of morphine tolerance by the mouse tail-flick method was performed 6 hr after pellet removal [16]. Mice were injected s.c. with 5–100 mg/kg morphine sulfate in saline, and tail-flick latencies were measured 45 min later; 40 sec was used as the maximum latency time indicating analgesia [16].

All experiments were repeated at least three times unless otherwise indicated.

RESULTS

A correlation between nuclear protein kinase peak I phosphorylation and stimulation of homologous RNA polymerase activity from small dense nuclei of mouse brain is shown in Fig. 1. Each RNA polymerase—I, II and III—was incubated with increasing amounts of nuclear protein kinase peak I enzyme. The nuclear protein kinase and RNA polymerase enzymes were each added to the assay medium at respective protein concentrations which have been shown previously [1, 2] to be linear with respect to the activity of each enzyme when assayed alone. Although the nuclear protein kinase activity was found to be optimal at pH 6.5 to pH 7.4, the RNA polymerases expressed their optimal activities at more basic pH [14]; therefore, the RNA polymerases were preincubated with protein kinase at pH 7.4.

RNA polymerase I and II activities were stimulated with increasing amounts of protein kinase and, at the same time, phosphorylation was also increased. It is seen that a given relative increase in phosphorylation was not associated with the same relative increase in RNA synthesis. The RNA polymerase I activity increased by 240 per cent when incubated with nuclear protein kinase, but the phosphorylation increased by only 64 per cent. For RNA

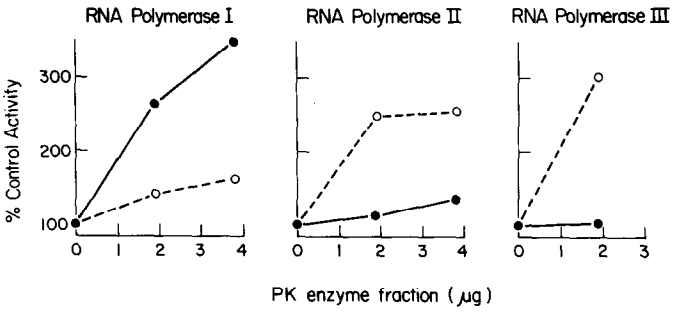

Fig. 1. Protein kinase peak I phosphorylation and stimulation of homologous RNA polymerases. Each RNA polymerase was incubated with increasing amounts of protein kinase peak I. [^{3}H]UMP incorporation (●——●) and [^{32}P]phosphate incorporation (○--○) were measured.

polymerase II, RNA synthesis increased by 35 per cent, while phosphorylation increased by 150 per cent. Furthermore, it appears that RNA polymerase I activity was stimulated to a much greater extent than RNA polymerase II for a given relative increase in phosphorylation. With regard to RNA polymerase III, although phosphorylation was increased, no corresponding change in RNA polymerase activity was seen.

Because the protein kinase and RNA polymerase fractions are only partially pure [1, 14], it is not known if the RNA polymerase molecule itself was phosphorylated or if some other molecule was phosphorylated which, in turn, was then able to stimulate RNA synthesis. Nevertheless, these data suggest that the nuclear protein kinase may have been regulating RNA polymerase I and II activities through a phosphorylation mechanism.

Because both the nuclear protein kinase and RNA polymerases are extremely labile [1, 2] once their partial purification is achieved, it was not possible to investigate the reversibility of nuclear protein kinase phosphorylation and stimulation of RNA polymerase activity with the use of phosphoprotein phosphatase, or to investigate whether the RNA polymerase enzyme itself or some other molecule is phosphorylated.

The presence of protein kinase activity in the RNA polymerase I fraction made it difficult to study further the correlation between phosphorylation and stimulation of RNA polymerase I activity. Therefore, these investigations continued with the study of RNA polymerase II and its stimulation by nuclear protein kinase.

Modification of enzymes through phosphorylation has been shown in many systems [17, 18] to result in altered enzyme activities and modified co-factor requirements for the enzymatic reaction being catalyzed. Because chronic morphine treatment alters the optimum Mg^{2+} concentration for RNA polymerase II [2], and nuclear protein kinase may be able to modify RNA polymerase II activity through a phosphorylation mechanism, the ability of nuclear protein kinase to change the optimum Mg^{2+} concentration required for maximum RNA polymerase II activity was investigated.

A comparison of the effect of chronic morphine treatement *in vivo* and the effect of nuclear protein kinase peak I *in vitro* on the Mg^{2+} optimum of RNA polymerase II from small dense nuclei of mouse brain is shown in Fig. 2. In this figure, panels A and B represent two different experiments. In panel A, RNA polymerase II was purified from chronic morphine- and placebo-treated mice. In panel B, RNA polymerase II was purified from naive mice and incubated in the absence and presence of nuclear protein kinase peak I. RNA polymerase activity was assayed at different Mg^{2+} concentrations in both experiments. In panel A, the optimum Mg^{2+} concentration for RNA polymerase II from placebo-treated mice was 8.5 mM and was shifted to 6 mM Mg^{2+} in the chronic morphine group. In panel B, the optimum Mg^{2+} concentration for RNA polymerase II incubated without protein kinase was approximately 8.5 mM Mg^{2+}. However, when incubated with nuclear protein kinase peak I, the optimum Mg^{2+} concentration was lowered to 6 mM. These data indicate that incubation of RNA polymerase II with protein kinase peak I shifted the optimum Mg^{2+} concentration in the same manner as chronic morphine treatment alone. Furthermore, the shapes of the curves in the two experiments are surprisingly similar. It appears that the *in vivo* effect of chronic morphine treatment on the Mg^{2+} optimum for RNA polymerase II can be produced *in vitro* by merely incubating the polymerase with homologous nuclear protein kinase. This suggests that the increased nuclear protein kinase peak I activity during chronic morphine treatment [1] may, in part, be responsible for the modified Mg^{2+} optimum of RNA polymerase II from tolerant-dependent mice.

Because the nuclear protein kinase and RNA polymerases are not modified by morphine *in vitro* [1, 2], the chronic morphine-induced changes in these enzymes probably occur several steps away from the initial site of morphine action. Evidence presented here suggests that the altered Mg^{2+} optimum of RNA polymerase II during chronic morphine treatment may occur through changes in phosphorylation as a result of increased nuclear protein kinase peak I activity. But how may chronic morphine treatment result in altered nuclear protein

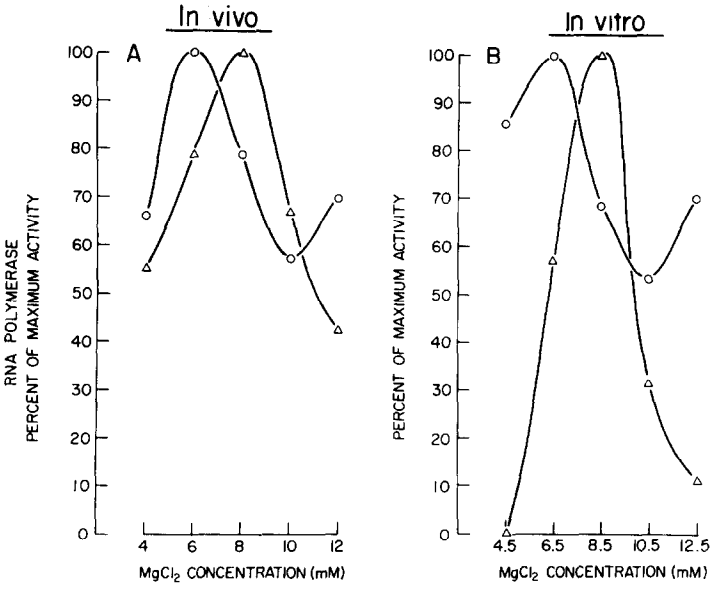

Fig. 2. Comparison of the effect of chronic morphine treatment *in vivo* and nuclear protein kinase peak I *in vitro* on the Mg^{2+} optimum of RNA polymerase II. Panel A: RNA polymerase II is purified from placebo- (△——△) and chronic morphine- (○——○) treated mice. Polymerase activity was measured for both drug treatment groups at different Mg^{2+} concentrations. Panel B: RNA polymerase II was purified from naive mice and assayed at different Mg^{2+} concentrations in the absence (△——△) and presence (○——○) of nuclear protein kinase peak I.

kinase activity? Because cAMP has been shown to accelerate the development of morphine tolerance-dependence [8] and is believed to be involved in the control of cellular functions through regulation of protein kinases [18–20], it is possible that the increased activity of nuclear protein kinase peak I may be related to the involvement of cAMP in morphine tolerance-dependence. If this is the case, injection of cAMP during chronic morphine treatment should potentiate the increase in nuclear protein kinase peak I seen during morphine tolerance-dependence. To investigate this possibility, mice were implanted with placebo or morphine pellets and injected i.c.v. 1 hr later with dibutyryl-cAMP, a more lipid soluble analog of cAMP. The mice were killed 24 hr later and the activity of the partially purified nuclear protein kinase was measured.

In Table 1, it is seen that i.c.v. injection of dibutyryl-cAMP alone increased the specific activity of nuclear protein kinase peak I by 74 per cent. In addition, the mean specific activity in the morphine saline group was larger than that of the placebo–saline group by 65 per cent. It must be pointed out that in this study mice were treated for only 24 hr with chronic morphine, whereas the previously reported [1] increase in protein kinase peak I activity was found in mice treated with chronic morphine for 72 hr. Most importantly, it was seen that animals treated with both chronic morphine and dibutyryl-cAMP displayed the greatest increase in nuclear protein kinase specific activity compared with those treated with only chronic morphine or dibutyryl-cAMP alone. The specific activity of nuclear protein kinase peak I from the morphine–dibutyryl-cAMP group increased by approximately 100 per cent relative to the morphine-saline group.

Table 1. Effect of dibutyryl-cAMP and chronic morphine treatment on nuclear protein kinase peak I*

Treatment	Protein kinase specific activity	% Change
Placebo–saline	800 ± 100	100
Placebo–dibutyryl-cAMP	1394 ± 67†	174
Morphine–saline	1317 ± 207†	165
Morphine–dibutyryl-cAMP	1902 ± 32†	270

* Mice were implanted with placebo or morphine pellets, injected i.c.v. with dibutyryl-cAMP, and killed 24 hr later. Each value is the average specific activity (±S.E.M.) from two experiments and each experiment consisted of seventy-five mice per treatment group.

† $P < 0.05$ (*t*-test), statistically significant relative to the placebo–saline group.

To investigate whether the degree of tolerance developed in the morphine–dibutyryl-cAMP group was indeed greater than in the other groups, 24 hr after pellet implantation and i.c.v. injection of dibutyryl-cAMP, the degree of morphine tolerance development was measured for each group. The dose–response curves in Fig. 3 show that the morphine–dibutyryl-cAMP group displayed the highest degree of morphine tolerance compared with the other groups treated with only morphine or dibutyryl-cAMP alone. The morphine–saline group displayed an intermediate degree of morphine tolerance. The placebo–dibutyryl-cAMP group appears to have developed no morphine tolerance when compared with the control group, placebo–saline. The morphine–dibutyryl-cAMP group displayed the highest degree of morphine tolerance and at the same time exhibited the greatest increase in nuclear

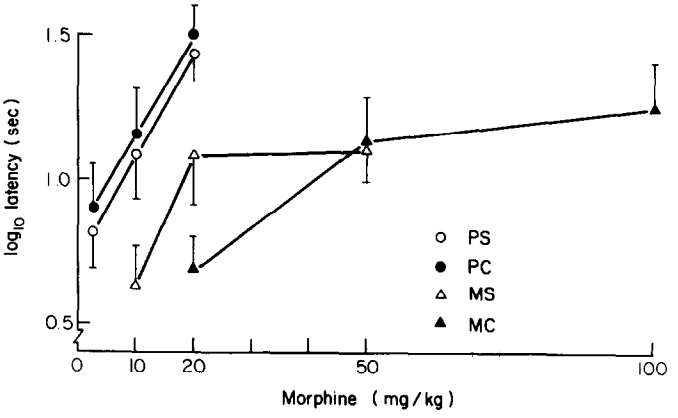

Fig. 3. Effect of dibutyryl-cAMP on morphine tolerance. Mice were injected i.c.v. with dibutyryl-cAMP or saline and implanted with a placebo or morphine pellet; the degree of morphine tolerance was measured 24 hr later by mouse tail-flick latencies. Each point is the mean from ten mice. The treatment groups are: placebo–saline (○——○); placebo–dibutyryl-cAMP (●——●); morphine–saline (△——△); and morphine–dibutyryl-cAMP (▲——▲).

protein kinase specific activity. These results suggest that the increase in nuclear protein kinase specific activity may be correlated with the degree of morphine tolerance and, furthermore, that cAMP may be involved in the biochemical mechanism responsible for the increased nuclear protein kinase activity during morphine tolerance-dependence.

DISCUSSION

RNA polymerase I and II activities are stimulated when the enzymes are incubated with increasing amounts of nuclear protein kinase peak I and, at the same time, phosphorylation is also increased. These results suggest a correlation between phosphorylation and stimulation of RNA polymerase I and II activities by homologous nuclear protein kinase. It should be noted that it is not known if the RNA polymerase molecule itself is phosphorylated or if some other molecule is phosphorylated which, in turn, is then able to stimulate RNA synthesis. The data suggest, however, that the nuclear protein kinase may be regulating RNA polymerase I and II activities through a phosphorylation mechanism.

Studies on the Mg^{2+} requirement of RNA polymerase II show that incubation of RNA polymerase II with nuclear protein kinase lowers the optimum Mg^{2+} concentration of the polymerase in the same manner as chronic morphine treatment alone does. It appears that the *in vivo* effect of chronic morphine treatment on the Mg^{2+} optimum of RNA polymerase II can be reproduced *in vitro* by merely incubating the polymerase with nuclear protein kinase peak I. This suggests that the change in Mg^{2+} optimum of RNA polymerase II during chronic morphine treatment may occur through changes in phosphorylation as a result of increased nuclear protein kinase peak I activity. Thus, it is possible that, during morphine tolerance-dependence, the alterations in nuclear protein kinase and RNA polymerase activities do not occur independently of one another, but the functional interaction between these two enzymes may be modified.

Alterations in nuclear protein kinase and RNA polymerase activities from small dense nuclei of mouse brain are probably several steps removed from the initial site of morphine action since the activity of neither enzyme is modified when incubated with morphine *in vitro* [1, 2]. Changes in the Mg^{2+} optimum of RNA polymerase II during chronic morphine treatment may be occurring through changes in phosphorylation as a result of increased nuclear protein kinase activity. But, what biochemical mechanism is involved in the chronic morphine-induced increase in nuclear protein kinase? Existing evidence suggests that cAMP may be involved in morphine tolerance-dependence [8–11]. Investigations described in this report show that i.c.v. injection of dibutyryl-cAMP enhances the chronic morphine-induced increase in nuclear protein kinase specific activity. Furthermore, measurement of mouse tail-flick latencies indicates that animals given both cAMP and chronic morphine developed much more morphine tolerance than animals receiving either treatment alone. These results suggest that the degree of increase in nuclear protein kinase peak I activity may be related to the degree of morphine tolerance-dependence developed and, more importantly, that cAMP may be involved in the modification of nuclear protein kinase activity during chronic morphine treatment.

The action of cAMP is generally believed [19] to be mediated through regulation of cAMP-dependent protein kinases. Cyclic AMP activates the cAMP-dependent protein kinase by binding to the regulatory subunit of the kinase and subsequently the regulatory and catalytic subunits are dissociated to produce active catalytic subunits. Thus, the cAMP enhancement of the chronic morphine-induced increase in nuclear protein kinase may be thought to involve a direct action of cAMP on cAMP-dependent protein kinase in nuclei. However, the nuclear protein kinases partially purified from small dense nuclei of mouse brain are cAMP-independent and, therefore, cannot be regulated directly by cAMP.

An alternative explanation for the involvement of

cAMP in the chronic morphine-induced increase in cAMP-independent nuclear protein kinase involves a translocation of catalytic subunits of protein kinase from the cytosol to the nucleus of the cell. This hypothesis of translocation of protein kinase has been proposed by Costa [21–23] and Jungmann *et al.* [24, 25]. When there is an elevation of cAMP level in the cell, it has been proposed that the cAMP binds to the regulatory subunit of a cAMP-dependent cytosolic protein kinase and dissociates the regulatory and catalytic subunits of the kinase. The free catalytic subunit is then translocated to the nucleus which results in an augmentation of cAMP-independent nuclear protein kinase(s) and modification of gene expression through phosphorylation of nuclear proteins. Supportive evidence suggesting that this phenomenon may be involved in morphine tolerance-dependence shows that i.c.v. injection of dibutyryl-cAMP alone, and in conjunction with chronic morphine treatment, increases the specific activity of a cAMP-independent nuclear protein kinase or, more specifically, the nuclear protein kinase peak I from small dense nuclei of mouse brain. Furthermore, the morphine-induced increase in nuclear kinase peak I was inhibited by the protein kinase inhibitor [26] which inhibits the catalytic subunit from cAMP-dependent protein kinases (unpublished material).

Thus, a possible sequence of events in the development of morphine tolerance-dependence may occur as follows. The acceleration of morphine tolerance-dependence by cAMP [8] suggests that there is an elevation of cAMP in certain brain regions during the development of tolerance-dependence. This new condition of elevated cAMP could result in a number of responses, one of which may be the dissociation and translocation of protein kinase. Increased nuclear protein kinase may result in the increased chromatin phosphorylation and template activity as reported previously [7, 13], and may regulate RNA polymerase activities through a phosphorylation mechanism. Such a hypothesis will, no doubt, require modification and reconsideration as new findings develop. At the present time, however, it can explain how cAMP may be involved in the possibile nuclear protein kinase regulation of homologous RNA polymerases during morphine tolerance-dependence.

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