Title
The involvement of nuclear protein kinase from small dense nuclei of mouse brain in morphine tolerance-dependence

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THE INVOLVEMENT OF NUCLEAR PROTEIN KINASE FROM SMALL DENSE NUCLEI OF MOUSE BRAIN IN MORPHINE TOLERANCE-DEPENDENCE

by

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THE INVOLVEMENT OF NUCLEAR PROTEIN KINASE FROM SMALL DENSE NUCLEI OF MOUSE BRAIN IN MORPHINE TOLERANCE-DEPENDENCE

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ABSTRACT

Recent studies (17) show that chromatin template activity is significantly increased in small dense nuclei of mouse brain during morphine tolerance-dependence and is accompanied by an increase in phosphorylation of acidic chromatin proteins. To deduce whether or not the nuclear protein kinase activity may be responsible for the change in phosphorylation of chromatin proteins during morphine tolerance-dependence, the nuclear protein kinases from small dense nuclei of mouse brain have been partially purified by ammonium sulfate fractionation and phosphocellulose column chromatography. Two peaks of cyclic AMP-independent nuclear protein kinase activity are eluted from the phosphocellulose column by a linear NaCl gradient. After 72 hours of chronic morphine treatment, the specific activity of peak I, but not of peak II, is significantly increased. This increase in nuclear protein kinase activity may partially account for the elevated chromatin protein phosphorylation in small dense nuclei of mouse brain seen during morphine tolerance-dependence.

In addition to the nuclear protein kinases, the homologous RNA polymerases have also been found to be altered during chronic morphine treatment (27,28). Studies in many tissue sources suggest that nuclear protein kinase may be able to modify RNA synthesis through phosphorylation of the RNA polymerase enzyme (29,30,31,32). In view of the finding that both the nuclear protein kinase and RNA polymerase enzyme activities are altered during chronic morphine treatment, 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.

However, nuclear protein kinase regulation of RNA polymerase activity has not yet been demonstrated in brain tissue. Evidence is presented illustrating a correlation between phosphorylation and activation of RNA polymerase I and II activities by homologous nuclear protein kinase from small dense nuclei of mouse brain. Further studies show that modification of RNA polymerase II through phosphorylation can modify its Mg^{++} optimum in the same manner as chronic morphine treatment alone.

Existing evidence overwhelmingly indicates that cAMP is involved in morphine action (34,35,37,38,39,41) but the exact mechanism of cAMP involvement is unknown at the present time. Cyclic AMP has been shown to accelerate the development of morphine tolerance-dependence. Thus, if the increase in nuclear protein kinase activity is related to the development of morphine tolerance-dependence, injection of cAMP should enhance the chronic morphine-induced increase in nuclear protein kinase activity. Such investigations are described in this report and provide evidence suggesting that the degree of increase in nuclear protein kinase specific activity may be related to the degree of morphine tolerance-dependence developed, and that cAMP may be involved in the induction of nuclear protein kinase activity.

Finally, based on evidence in this report and from the current literature, two hypotheses are presented in an attempt to explain what biochemical mechanisms may be occurring during morphine tolerance-dependence. These are: (1) that cAMP appears to play a role in the

stimulation of nuclear protein kinase activity which may involve translocation of protein kinase to the nucleus during the development of morphine tolerance-dependence, and (2) that altered specific activities and modified co-factor requirements of key regulatory enzymes signify that a biochemical adaptation may be occurring during morphine tolerance-dependence.

I. INTRODUCTION

The narcotic alkaloid morphine has been historically used as a medicinal remedy for ages. The psychological effects of opium may have been known to the ancient Sumerians, whose ideograph for the poppy was hul ("joy") plus gil ("plants"), as early as 4000 B.C. (1). But the first undisputed reference to poppy juice is found in the writings of Theophrastus in the third century B.C. (1). Arabian physicians were well versed in the uses of opium and the drug was introduced to the Orient by Arabian traders (1). Despite its widespread use throughout the centuries as an analgesic, even today the mechanisms by which morphine exerts its effects remain unknown. Although morphine is probably the best agent used for the relief of pain, a major drawback and disadvantage to its continued use (for example, for chronic pain) is the development of morphine tolerance and physical dependence.

Chronic morphine treatment, either continuous or intermittent, results in tolerance-dependence development. In the tolerant animal a much greater dose of morphine must be administered to produce a given level of analgesia, the drug response, compared with the non-tolerant animal. With repeated administration of morphine the animal also becomes physically dependent during which the animal functions normally with the continued presence of the drug, but when the drug is removed or a narcotic antagonist is given, severe withdrawal symptoms are manifested.

Morphine tolerance and physical dependence almost always (2,3,4) occur simultaneously and the two conditions are rarely separable. The morphine tolerant-dependent state is generally considered (5) to be a consequence of adaptive homeostatic mechanisms which have developed

to allow the animal to behave normally under the new condition of continued presence of morphine in the body.

Ideally, it would be of tremendous advantage to be able to design a synthetic narcotic analgesic which lacks the addictive and dependence liabilities of morphine, but which remains as potent as morphine in its pain-killing effect. With this ultimate goal in mind, the mechanism of morphine tolerance-dependence must be understood at the biochemical and molecular level; this knowledge may allow an appropriate strategy to be developed for the design of an analgesic without addictive and dependence liabilities.

If it is believed that morphine tolerance-dependence is a consequence of adaptive homeostatic mechanisms, then it follows that during this state a whole spectrum of key regulatory enzymes are now operating at a new level to maintain the original cellular function and metabolism under different conditions. This would imply that (1) different types of enzymes may be required, or (2) relative ratios of different classes of enzymes may be required, or (3) different isozymes may be needed which can function more optimally in the new environment. These possibilities suggest that during morphine tolerance-dependence the synthesis of new proteins in cells may be essential. Studies in many laboratories have indicated that RNA and protein synthesis are involved in morphine tolerance-dependence and may involve alterations in gene expression. The genetic information stored in the nucleus of each cell is expressed by the complex mechanisms of transcription of DNA to RNA and of translation of RNA into protein. Inhibitors of RNA synthesis such as actinomycin D (6,7,8,9) and 8-azaguanine (10,11)

block the development of morphine tolerance in rats and mice. Other inhibitors of RNA synthesis, 6-mercaptopurine and 5-fluorouracil, reduce the level of tolerance developed during morphine infusion (12,13). Cycloheximide, an inhibitor of protein synthesis, also blocks tolerance and physical dependence development (14,15,16). However, a drawback to the use of these drugs is that they produce many other effects in the animal besides inhibition of RNA and protein synthesis. Therefore, if RNA and protein synthesis are involved in morphine tolerance-dependence, the individual steps in these processes of transcription and translation during chronic morphine treatment must be studied.

At the level of transcription Lee et al. (17) have found that chromatin template activity, measured by following ^{3}H-UTP incorporation into newly synthesized RNA, is increased by 54% in small dense nuclei of mouse brain during morphine tolerance-dependence. Further studies by Oguri et al. (18) indicate that this population of small dense nuclei is 97% morphologically homogeneous. They are described as being small, dense, and darkly staining and, on the basis of morphology, they are identified as being primarily oligodendroglial in origin (18,33).

Because phosphorylation of the nuclear proteins is thought to play a role in positive gene regulation (19,20,21), Oguri et al. (18) studied the phosphorylation of chromatin proteins in small dense nuclei of mouse brain during chronic morphine treatment. The phosphorylation of acidic chromatin proteins is increased by 60% during morphine tolerance-dependence, and this change is not due to a decrease in phosphoprotein phosphatase activity. The increase in phosphorylation may be due to a change in nuclear protein kinase enzyme activity or to a change in some

property of the chromatin phosphoproteins themselves.

To deduce whether the nuclear protein kinase activity is indeed responsible for the change in phosphorylation of chromatin proteins during morphine tolerance-dependence, purified fractions of nuclear protein kinase enzyme(s) from small dense nuclei must be studied. Numerous investigators have reported on the purification of multiple forms of nuclear protein kinases from several tissue sources (22,23,24); however, nuclear protein kinase(s) from brain tissue have not been extensively studied. This study describes the solubilization and partial purification of nuclear protein kinases from small dense nuclei of mouse brain and the effect of chronic morphine treatment on these purified protein kinases. Briefly, two peaks of nuclear protein kinase activity are resolved by phosphocellulose column chromatography. During morphine tolerance-dependence the specific activity of one peak but not the other is significantly increased relative to placebo controls.

Both the nuclear protein kinases and RNA polymerases have been suggested as important sites in the regulation of gene expression (25,26). In addition to the alteration of nuclear protein kinase activity during morphine tolerance-dependence, investigations by Stokes et al. (27,28) indicate that the homologous RNA polymerases are also altered by chronic morphine treatment. Not all properties of the RNA polymerases are altered, indicating that the RNA polymerases are altered specifically by this drug treatment. The specific activity of RNA polymerase I is decreased, and the optimum Mg^{++} concentration for RNA polymerase II and the Mn^{++}/Mg^{++} 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 (29,30,31,32). In view of the finding that both the nuclear protein kinase and RNA polymerase enzyme activities are altered during chronic morphine treatment, 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.

However, 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 studies presented show that modification of RNA polymerase II through phosphorylation by nuclear protein kinase can modify its Mg^{++} optimum in the same manner as chronic morphine treatment alone.

Numerous studies indicate that cAMP (cyclic adenosine 3',5'monophosphate) may play a significant role in morphine action. I.K. Ho et al. (34,35) have shown that a single icv. injection of cAMP or a phosphodiesterase inhibitor accelerates the rate of morphine tolerance-dependence development in mice. However, this treatment with cAMP or theophylline antagonizes acute analgesia (36). Sharma and Nirenberg's (37) model of cAMP involvement in acute and chronic morphine-treated

neuroblastoma-glioma hybrid cells also suggests a role for cAMP in morphine action. Furthermore, Collier (38), Sharma et al. (39,40), Blume (41) and others (42,43,44,45) have shown that morphine can regulate adenylate cyclase activity, the membrane-bound enzyme synthesizing cAMP. Many other investigators (46,47,48) have also found changes in cAMP levels in various brain areas during acute or chronic morphine treatment.

Existing evidence overwhelmingly indicates that cAMP is involved in morphine action but the exact mechanism of cAMP involvement is unknown at the present time. If the increase in nuclear protein kinase activity is related to the development of morphine tolerance-dependence, one would predict that an icv. injection of cAMP, which is known to accelerate the rate of morphine tolerance-dependence ( ), would enhance the chronic morphine-induced increase in nuclear protein kinase activity. These investigations are described in this report and provide evidence suggesting that the degree of increase in nuclear protein kinase specific activity may be related to the degree of morphine tolerance-dependence developed, and that cAMP may be involved in the induction of nuclear protein kinase activity.

Finally, based on evidence in this report and from the current literature, two hypotheses are presented in an attempt to explain what biochemical mechanisms may be occurring during morphine tolerance-dependence. These are: (1) that cAMP may be responsible for the increased nuclear protein kinase activities during morphine tolerance-dependence which may involve a translocation of protein kinase from the cytosol to the nucleus of the cell, and (2) that altered specific

activities and modified co-factor requirements of key regulatory enzymes signify that a biochemical adaptation may be occurring during morphine tolerance-dependence.

II. METHODS AND MATERIALS

Protein Kinase Assay

Nuclear protein kinase activity is measured by following ^{32}P-phosphate incorporation from (γ-^{32}P)-ATP into exogenously added casein as protein substrate. An aliquot of protein kinase sample is assayed in a total volume of 0.20 ml with the final concentrations: 50 mM potassium-phosphate buffer, pH 6.5; 0.3 mM 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/mmol specific activity, ICN, Irvine, California). All assay tubes are kept on ice before incubation. The reaction is started with the addition of (γ-^{32}P)-ATP, incubated for 5 minutes at 30° C, and stopped with the addition of 2 ml ice cold 5% TCA - 1.5% Na-PP_i (5% trichloro-acetic acid - 1.5% sodium pyrophosphate). Bovine serum albumin, 0.10 mg/tube, is added, and the mixture is vortexed and is allowed to stand on ice for 15 minutes. The precipitate is washed on Whatman GFC filters with 4 x 8 ml 5% TCA - 1.5% $NaPP_i$ and rinsed with 1 ml cold 95% ethanol. Each dried filter is placed in a Nalge filmware bag and counted with 1.5 ml scintiverse in a Beckman LS-100 scintillation counter for 5 minutes.

To determine the cAMP (cyclic adenosine 3',5'-monophosphate) dependence of the protein kinase, an aliquot of the enzyme fraction is pre-incubated with and without 10^{-6} M cAMP with the above specified buffer for 1 minute at 30° C, and immediately placed on ice. The phosphorylation reaction is started with the addition of (γ-^{32}P)-ATP and the mixture is incubated for an additional 5 minutes at 30° C.

Each assay is run in triplicate, using an incubation mixture containing the TCA-precipitated protein kinase preparation as the blank.

Purification of Nuclear Protein Kinase

Isolation of small dense nuclei from mouse brain. ICR mice (25 ± 2 g) from Simonsen Laboratories, Gilroy, California, are killed by decapitation. Brains are immediately removed and placed in ice cold 0.25 M sucrose TKM buffer (50 mM Tris HCl, pH 7.4 @ RT; 25 mM KCl; 5 mM $MgCl_2$). Brains are rinsed once with 0.25 M sucrose TKM buffer to remove the blood. One hundred fifty mice are usually used in each preparation unless otherwise indicated; this represents approximately 60 g of brain tissue, wet weight.

Small dense nuclei are purified according to the method described by Oguri et al. (18). Brains are homogenized in 2 volumes 0.25 M sucrose TKM buffer with 10 passes in a teflon-glass (Potter-Elvehjem) tissue grinder. The tissue is nearly completely homogenized by the fifth pass. The homogenate is filtered through 4 layers of cheesecloth into a graduated cylinder. The volume of the homogenate is measured and 2.3 M sucrose TKM is added to 2 times the volume of the homogenate and thoroughly mixed.

The brain homogenate mixture is then subjected to discontinuous sucrose density centrifugation. Approximately 27 ml of the mixture is layered over 9 ml 2.3 M sucrose TKM; 12 tubes are required for homogenate from 150 brains. The sucrose gradient is centrifuged in a Beckman SW 27 rotor for 100 minutes at 27 K rpm (130,000 g). The resultant pellet, which represents the small dense nuclear fraction,

is suspended in 5 ml 0.25 M sucrose TKM per tube, and nuclei from 6 tubes are then combined into one sample (2 samples per 150 brains). The two samples are centrifuged in a Sorvall Superspeed RC2-B centrifuge for 20 minutes at 18 K (40,000 g). The pellet (small dense nuclei) from each sample is resuspended in 5 ml TEMDG + 30 (50 mM Tris HCl pH 7.4 @ RT; 0.1 mM EDTA; 4 mM $MgCl_2$; 1 mM dithiothreitol (DTT); 25% glycerol v/v; 30 mM $(NH_4)_2SO_4$).

Solubilization of nuclear protein kinase. The nuclear protein kinase is solubilized from the small dense nuclei essentially according to the method described by Stokes et al. (49) for the solubilization of RNA polymerase. Briefly, the pellet of small dense nuclei from 75 mice is resuspended in 5.0 ml TEMDG + 30 buffer and homogenized with 7 to 8 passes in a teflon-glass tissue grinder. The suspension is adjusted to 0.32 M $(NH_4)_2SO_4$ by adding 555 µl 3 M $(NH_4)_2SO_4$, pH 7.4, to the 5.0 ml suspension, and sonicated 4 x 15 seconds using a Model W-220F Sonicator (Heat Systems-Ultrasonics, Inc., Plainview, New York). The suspension is centrifuged in a Beckman SW 65 rotor for 50 minutes at 45 K rpm (220,000 g) and the supernatant is collected for $(NH_4)_2SO_4$ precipitation.

Ammonium sulfate precipitation. The supernatant from the sonicated nuclei is subjected to a 40% ammonium sulfate precipitation. After addition of $(NH_4)_2SO_4$ crystals to the supernatant, the solution is stirred for 1 hour at 4° C. The precipitate is collected by centrifugation for 20 minutes at 18 K rpm (40,000 g) in a Sorvall centrifuge and resuspended in 11 ml TEMD buffer (50 mM Tris HCl, pH 7.4 @ RT;

0.1 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT) per sample from 150 mice. The resultant suspension is dialyzed against 200 volumes TEMD buffer overnight and applied to the phosphocellulose column.

Phosphocellulose column chromatography. The phosphocellulose resin (Whatman P 11 cellulose phosphate) is suspended in H_2O and washed with 0.1 M NaOH, H_2O, 0.1 M HCl, H_2O, 0.1 M NaOH, and H_2O to prepare the resin in the Na^+ form. The resin is then washed and equilibrated with TEMD buffer. The dialyzed 40% $(NH_4)_2SO_4$ fraction is applied to the phosphocellulose column (0.9 x 9 cm for 150 mice) and washed with 5 column volumes of TEMD buffer. Using a Pharmacia GM-1 gradient maker, the column is eluted with a linear NaCl gradient from 0 to 1.0 M NaCl in TEMD in 60 ml and fractions of 1 ml are collected. To measure the linearity of the NaCl gradient, the NaCl concentration in each fraction is measured by reading its conductivity using a Radiometer brand (Copenhagen) conductivity meter; known NaCl concentrations in TEMD buffer are used for the standard. Because NaCl was found to interfere with nuclear protein kinase activity, each 1 ml fraction is dialyzed against 100 volumes of TEMD buffer before assaying for protein kinase activity. Two major peaks of nuclear protein kinase activity are eluted from the phosphocellulose column; these peaks are referred to as nuclear protein kinase peaks I and II. All purification procedures are performed at 4° C.

Protein concentrations are measured by the method of Lowry et al. (50) after the samples have been dialyzed against water. Bovine serum albumin is used as protein standard.

Phosphoprotein Phosphatase Assay

Phosphoprotein phosphatase activity in fractions containing protein kinase activity is measured by first assaying the protein kinase in the manner described above. At the end of the 5 minute incubation at 30° C, the samples are placed on ice and nonradioactive ATP (15 µl of 25 mM ATP) is added, and the samples are incubated for an additional 5 to 20 minutes at 30° C. Phosphoprotein phosphatase activity is measured by following the disappearance of ^{32}P-phosphate-labeled casein after the addition of nonradioactive ATP.

Slab Gel Electrophoresis of Nuclear Protein Kinase

Denaturing condition. Slab gel electrophoresis of nuclear protein kinase peaks I and II is performed under denaturing conditions essentially according to the method of Maizel (51) on a 5-15% polyacrylamide gradient in 0.1% SDS (sodium dodecyl sulfate) using the Hoeffer slab gel apparatus - SE 500 series. The resolving gel is prepared by first preparing the 5% and 15% acrylamide gel solutions. The 5% acrylamide solution contains, in final concentrations, 1% glycerol v/v, 0.375 M Tris HCl pH 8.8 @ RT, 0.1% SDS, 5% polyacrylamide, 0.13% bis-acrylamide, 0.027% ammonium persulfate, 0.025% TEMED. The 15% acrylamide solution contains, in final concentrations, 4.6% glycerol v/v, 0.375 M Tris HCl pH 8.8 @ RT, 0.1% SDS, 15% acrylamide, 0.4% bisacrylamide, 0.03% ammonium persulfate, 0.025% TEMED. Six ml each of the 5% and 15% solutions are placed in a gradient maker and the 5% to 15% polyacrylamide slab gel gradient is prepared with a total slab gel volume of 12 ml. After

pouring the 5-15% gradient, 0.1% SDS solution is layered over the resolving gel. Polymerization of the resolving gel is complete by approximately one hour.

The stacking gel contains, in final concentrations, 0.125 M Tris HCl pH 6.8 @ RT, 0.1% SDS, 3% acrylamide, 0.08% bis-acrylamide, 0.03% ammonium persulfate and 0.025% TEMED. The 0.1% SDS solution layered above the resolving gel is removed and the stacking gel is layered above the resolving gel. The slot former is set in its proper position and polymerization of the gel is allowed to proceed and is usually completed by one hour.

After determining which fractions from the phosphocellulose column contain protein kinase activity, nuclear protein kinase peaks I and II are collected and concentrated 10-fold. To concentrate the samples, they are placed in dialysis bags and placed on dry Sephadex G-200 resin at 4° C. After concentration, the samples are mixed with "sample buffer" (2:1, sample:sample "buffer") and boiled for 5 minutes. The final concentrations of the components of the boiled sample are 3% glycerol v/v, 20 mM Tris HCl pH 6.8, 2.2% mercaptopropanol, and 50 µg/ml bromophenol blue. For each sample, 25-50 µg protein in 50-80 µl is applied to each slot of the stacking gel. Cytochrome c (12,500 mw) and BSA (68,000 mw) are used as molecular weight markers (52).

The electrode buffer contains, in final concentrations, 0.025 M Tris-glycine pH 8.9 and 0.1% SDS. Electrophoresis is run at a constant voltage of 200 V with refrigeration. Electrophoresis is usually completed in approximately 4 hours. The slab gels are then removed and stained for 1 hour in 0.2% Coomasie Brilliant Blue, 50% methanol, 7%

acetic acid. The gel is destained in 25% methanol, 7% acetic acid overnight.

Non-denaturing condition. Slab gel electrophoresis of nuclear protein kinase peaks I and II is performed under non-denaturing conditions on a 4-10% polyacrylamide gradient. 4% and 10% acrylamide solutions are prepared for the resolving gel. The 4% acrylamide solution contains, in final concentrations: 1% glycerol v/v, 0.375 M Tris HCl pH 8.8 @ RT, 4% acrylamide, 0.10% bisacrylamide, 0.027% ammonium persulfate, 0.025% TEMED. The 10% acrylamide solution contains, in final concentrations, 4.6% glycerol v/v, 0.375 M Tris HCl pH 8.8 @ RT, 10% acrylamide, 0.27% bis-acrylamide, 0.03% ammonium persulfate, 0.025% TEMED. Six ml each of the 4% and 10% solutions are placed in a gradient maker and the 4% to 10% polyacrylamide slab gel gradient is prepared. The stacking gel is prepared in the same manner here as that for the denaturing gel, except that no SDS is included in the gel.

Protein samples are concentrated by dialysis against dry Sephadex G-200 resin. Glycerol and bromophenol blue are added to the sample to give final concentrations of 10% glycerol and 60 µg/ml bromophenol blue, respectively.

The electrode buffer contains 0.025 M Tris-glycine, pH 8.9. Electrophoresis is run at a constant voltage of 200 V with refrigeration. The slab gels are stained and destained as described for the gels run under denaturing conditions.

Estimate of Molecular Weight of PK I and II by Gel Exclusion

To estimate the molecular weight of nuclear protein kinase peaks I and II, each purified protein kinase fraction is passed through Sephadex (Pharmacia) gel filtration resins with varying molecular weight exclusion limits. The gel filtration resins are swollen in TEMD buffer at room temperature with appropriate swelling times for the different sizes of resin as indicated in the Pharmacia handbook for Sephadex gel filtration (53). Columns of Sephadex G-25, -50, -75, -100, -150, and -200 (0.9 x 5.0 cm) were equilibrated by washing with 10 column volumes of TEMD buffer at 4° C. The void volume is measured by passing 100 µl of 0.1% blue dextran (Sigma) through the column and is found to be approximately 2.0 ml for a column 0.9 x 5.0 cm packed with the various sizes of gel filtration resins. 150 µl (5-10 µg protein) of the nuclear protein kinase sample is applied and the column is then eluted with 11 ml (1 void volume + 3 column volumes) TEMD buffer and fractions of 0.5 ml are collected. A 50 µl aliquot from each fraction is assayed for protein kinase activity.

Chronic Morphine Treatment

Mice are rendered morphine tolerant-dependent by pellet implantation according to the method developed by Way *et al.* (54). A 75 mg morphine pellet is implanted under the skin, and the mice are killed 72 hours later. This procedure has been shown to produce an extremely high degree of morphine tolerance-dependence. A placebo pellet containing no drug is implanted for the control group.

The morphine and placebo pellets are prepared by the U.C. Pharmacy. The morphine pellet contains 75 mg morphine sulfate and 75 mg calcium stearate. The placebo pellet contains 75 mg lactose and 75 mg calcium stearate.

To investigate whether the effects produced by chronic morphine treatment are naloxone-reversible, a 10 mg naloxone pellet is implanted with the 75 mg morphine pellet. The naloxone pellet is implanted 2 hours before the morphine pellet and the mice are killed 72 hours later. In this experiment there are four drug treatment groups: placebo-placebo, placebo-morphine, naloxone-placebo, and naloxone-morphine.

For the chronic morphine and naloxone experiments, 75 mice are implanted for each drug treatment group. The phosphocellulose column chromatography is performed as previously described for the purification of nuclear protein kinase from 150 mice. With 75 mice, the size of the phosphocellulose column is reduced to 0.9 x 4.5 cm and eluted with the same linear NaCl gradient (0 to 1.0 M NaCl in 60 ml).

RNA Polymerase Assay

RNA polymerase activity from small dense nuclei of mouse brain is assayed according to the method described by Stokes *et al.* (49). RNA polymerase activity is followed by measuring ^{3}H-UTP incorporation into RNA. An aliquot of RNA polymerase enzyme is assayed in a total volume of 0.5 ml with the final concentrations: 100 mM Tris HCl pH 8.9 @ RT; 35 mM $(NH_4)_2SO_4$; 1 mM 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 (30 mCi/mmole, ICN, Irvine, California); 0.1 mg/ml calf thymus DNA.

The assay tubes are incubated for 30 minutes at 37° C and the reaction stopped with the addition of 4 ml ice cold 10% TCA - 3% PP_i. Bovine serum albumin, 0.30 mg/tube, is added and the reaction mixtures are vortexed and allowed to sit on ice for 30 minutes. The reaction mixture from each assay tube is washed on Whatman GFC filters with 7 x 8 ml 5% TCA - 3% Na PP_i and rinsed with ice cold 95% ethanol. Each dried filter is placed in a Nalge filmware bag and counted with 1.5 ml Scintiverse in a Beckman LS-100 scintillation counter for 5 minutes.

Purification of RNA Polymerase

RNA polymerase from small dense nuclei of mouse brain is purified according to the method developed by Stokes *et al*. (49). The small dense nuclei are isolated and sonicated in the same manner as described in the procedure for the purification of nuclear protein kinase. The supernatant of the sonicated nuclei is dialyzed overnight against 200 volumes TEMDG + 30. This dialyzed fraction is applied to a DEAE-Sephadex column (0.9 x 14 cm) which has been equilibrated in TEMDG + 30 buffer and then washed with two column volumes TEMDG + 30 buffer. Three peaks of RNA polymerase activity are eluted by a discontinuous ammonium sulfate gradient. RNA polymerases I, II and III are eluted stepwise with buffer (TEMDG) containing 225, 275 and 1000 mM $(NH_4)_2SO_4$, respectively. Column fractions are dialyzed against 100 volumes TEMDG + 30 buffer before assaying for RNA polymerase activity.

Nuclear Protein Kinase Peak I and RNA Polymerase Assay

The effect of nuclear protein kinase peak I on homologous RNA poly-

merase activities from small dense nuclei of mouse brain is investigated by preincubating each partially purified RNA polymerase fraction with nuclear protein kinase to allow the phosphorylation reaction to occur, and subsequently, conditions of the reaction mixture are adjusted for the assay of RNA polymerase activity. Phosphorylation is followed by measuring ^{32}P-phosphate incorporation and RNA polymerase activity is measured by following ^{3}H-UTP incorporation.

Nuclear protein kinase peak I and RNA polymerase I, II and III are partially purified from small dense nuclei of mouse brain according to the procedures described previously. Approximately 10 µg protein of RNA polymerase fraction are preincubated with varying amounts, 0-4 µg protein, of nuclear protein kinase peak I with the final concentrations of 50 mM potassium phosphate buffer pH 6.5 or 7.4, 0.3 mM EGTA, 10 mM NaF, 10 mM $MgCl_2$, 20 µM ATP, and 2 µCi (γ-^{32}P)-ATP per tube for 5 minutes at 30° C. At the end of the 5 minutes preincubation, the tubes are placed on ice, and the RNA polymerase assay medium is immediately added to each tube to attain the final concentrations: 0.1 M Tris HCl pH 7.4 at 37° C, 0.1 mg/ml calf thymus DNA, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.02 mg/ml bentonite, 2 mM $MnCl_2$, 1 mM EDTA, 1 mM DTT and 30-40 µCi/tube ^{3}H-UTP. The samples are incubated for 30 minutes at 37° C and the reaction is stopped with 5 ml of 10% TCA-3% $NaPP_i$. The samples are washed and counted as described for the RNA polymerase assay.

Studies on the effect of protein kinase on the Mg^{++} dependence of the RNA polymerases are performed in the absence of Mn^{++} under identical polymerase assay conditions as described by Stokes *et al.* (27).

Separation of Nuclear Protein Kinase Peak I and RNA Polymerase II by Phosphocellulose Column Chromatography

Because the nuclear protein kinase peak I and RNA polymerase II fractions are only partially pure, during the incubation of protein kinase and polymerase to allow phosphorylation to occur (measured by ^{32}P-phosphate incorporation) it is not known whether the RNA polymerase enzyme molecule itself is being phosphorylated, or some other molecule is phosphorylated which, in turn, is then able to modify the RNA polymerase activity. In an attempt to find some evidence which can suggest whether or not the RNA polymerase molecule is phosphorylated, the nuclear protein kinase peak I and RNA polymerase II fractions are incubated to allow phosphorylation to occur and this mixture is then subjected to phosphocellulose column chromatography to separate the protein kinase from the RNA polymerase. ^{32}P-phosphate, indicative of phosphorylation, will be followed on the phosphocellulose column and fractions will be assayed for both protein kinase and RNA polymerase activities. The elution profile of ^{32}P-phosphate relative to RNA polymerase activity can suggest if the RNA polymerase or some other protein may be phosphorylated.

More specifically, 1.0 ml of nuclear protein kinase peak I and 1.5 ml RNA polymerase II are incubated with protein kinase assay medium, which includes 100 μCi (γ-^{32}P)-ATP, in a total volume of 5.0 ml for 5 minutes at 30° C. The protein kinase reaction is stopped by placing the reaction mixture on ice. To remove the (γ-^{32}P)-ATP, the 2 x 2.5 ml of the reaction mixture are applied to two PD-10 prepacked columns (Sephadex G-25, from Pharmacia, these columns are generally used for

desalting purposes) which have been washed and equilibrated with TEMDG + 30 buffer. After the reaction mixture has entered the PD-10 column, 3.5 ml TEMDG + 30 buffer is applied and the eluate collected. The Whatman P-11 phosphocellulose resin is prepared by washing in 0.1 M $(NH_4)_2SO_4$, H_2O, 0.1 M H_2SO_4, H_2O, 0.1 M $(NH_4)_2SO_4$, H_2O and TEMDG + 30 buffer to prepare the resin in the NH_4^+ form. The 7.0 ml eluate from the PD-10 column is applied to the phosphocellulose column (0.9 x 4.5 cm) and washed with 20 ml TEMDG + 30 buffer. A stepwise gradient of 600 mM $(NH_4)_2SO_4$ in TEMDG buffer is applied to the phosphocellulose column. Fractions of 1.5 ml are collected for the wash and stepwise $(NH_4)_2SO_4$ gradient. Fractions eluting off the column after the $(NH_4)_2SO_4$ gradient has been applied are dialyzed against 200 volumes of TEMDG + 30 buffer. ^{32}P is measured in each fraction and all fractions are assayed for protein kinase and RNA polymerase activity. ^{3}H-ATP (28 Ci/mmol, ICN, Irvine, California) is also applied to the phosphocellulose column as a standard to find the elution position of ATP.

Slab Gel Electrophoresis and ^{32}P-autoradiography of PK I + RNA Polymerase II

To find which proteins are being phosphorylated during the incubation of nuclear protein kinase peak I with RNA polymerase II, this reaction mixture is subjected to slab gel electrophoresis under denaturing conditions on a 5-15% polyacrylamide gradient in 0.1% SDS, and ^{32}P-autoradiography of the gel is subsequently performed.

Nuclear protein kinase peak I and RNA polymerase II are purified as described previously. Each enzyme fraction is concentrated 2-fold by dialysis against dry Sephadex G-200 resin. One ml of nuclear protein kinase peak I and 1.5 ml RNA polymerase II are incubated with protein kinase assay medium, containing 1 mCi (γ-^{32}P)-ATP, in a total volume of 5.0 ml for 10 minutes at 30° C. The phosphorylation reaction is stopped with the addition of 1% SDS and 0.15 ml 3-mercapto-1,2,propanediol. The mixture is boiled for 5 minutes and then concentrated to approximately 0.5 ml by dialysis against dry Sephadex G-200 resin. Glycerol and 5 µl bromophenol solution (Biorad) are added to the concentrated sample to give the final concentrations 10% glycerol v/v and 40 µg/ml bromophenol blue.

The resolving gel consists of a 5-15% polyacrylamide gradient, 0.1% SDS. The slab gel is prepared as described previously in this section. Electrophoresis is run at a constant voltage of 200 V with refrigeration. The slab gel is stained with Coomasie Brilliant Blue and destained as described previously.

The slab gel is dried using the Hoeffer Slab Gel Dryer. The slab gel is placed on a sheet of filter paper wetted with water and one layer of Saran Wrap is placed over the slab gel. This preparation is positioned on the slab gel dryer and dried for one hour.

^{32}P-autoradiography of the slab gel is accomplished by exposing X-ray film (Kodak) to the dried slab gel for 4-8 hours (55). The film is developed by immersing the film in developer (Kodak RP X-Omat Developer) for 5 minutes. The film is then rinsed with 1% acetic acid, placed in the fixer solution (Kodak RP X-Omat Fixer) for 2 minutes, and rinsed

under cold running tap water for 15 minutes. All procedures for ^{32}P-autoradiography are performed in a photographic darkroom.

Brain Subcellular Fractionation and PK Activity Ratio -cAMP/+cAMP

Mouse brains are fractionated essentially according to the method of Gurd et al. (56) and Cotman et al. (57) into four subcellular fractions: P_1, which contains primarily nuclei, whole cells, and other such large particles; P_2, which contains nerve-ending particles, mitochondria, myelin, and microsomes; S, which contains microsomes and other undefined particles; M, which contains primarily microsomes. Mouse brains are homogenized in 9 volumes of 0.32 M sucrose - 1 mM HEPES pH 7.0 with 8 to 10 excursions in a teflon-glass tissue grinder. The homogenate is centrifuged for 10 minutes at 3 K rpm (1000 g) in a Sorvall Superspeed centrifuge. The resultant pellet, P_1, is resuspended in 1 ml 0.32 M sucrose - 1 mM HEPES pH 7.0 buffer per mouse brain. The resultant supernatant is centrifuged for 10 minutes at 12 K rpm (17,000 g); the supernatant is referred to as the S fraction and the pellet, P_2, is washed 3 times in the original 9 volumes of sucrose-HEPES buffer per brain. The supernatant of the first wash of the P_2 fraction is taken as the M fraction. The flow-chart diagram (Figure 1) more clearly illustrates this fractionation procedure.

Protein kinase activity is measured in the four brain subcellular fractions - P_1, P_2, S, M - in the presence and absence of cAMP. An aliquot of enzyme sample is incubated in a total volume of 0.2 ml for 5 minutes at 30° C with, in final concentrations, 50 mM potassium-phosphate buffer pH 6.5, 10 mM NaF, 3 mM EGTA, 10 mM $MgCl_2$, 0.3 mg/ml his-

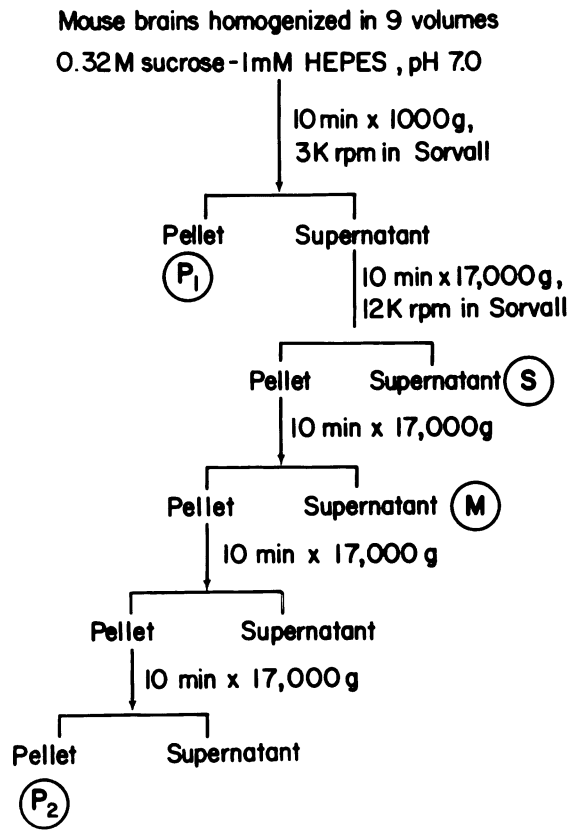


FIGURE 1. Brain Subcellular Fractionation

tone, 25 µg/ml casein, ± 5 x 10^{-6} M cAMP. The reaction is stopped with the addition of 2 ml 5% TCA - 1.5% $NaPP_i$; the samples are washed and counted as described previously for the protein kinase assay. All subcellular fractions are assayed for protein kinase activity in the linear protein concentration range.

The protein kinase activity ratio -cAMP/+cAMP is calculated by the ratio: $\frac{\text{protein kinase activity in absence of cAMP}}{\text{protein kinase activity in presence of cAMP}}$

Dibutyryl cAMP icv. Injection and Chronic Morphine Treatment

To investigate whether the dibutyryl cAMP injection and chronic morphine treatment can potentiate one another's effect on nuclear protein kinase peaks I and II specific activities, mice are implanted with the morphine pellet and then 1 hour later injected intracerebroventricularly with 28 µg dibutyryl cAMP. A piece of narrow plastic tubing is placed over the needle of a 10 µl Hamilton syringe, so that the distance from the edge of the tubing to the beginning of the opening of the tip of the syringe is equal to 2.5 mm. Thus, the solution is injected into the ventricle of the brain at 2.5 mm away from the skull. 28 µg dibutyryl cAMP in 4 µl saline solution (0.9% NaCl, sterile) is injected icv. and the mice are killed 24 hours after injection. Nuclear protein kinase peaks I and II are purified as previously described.

Measurement of Tolerance to Morphine Analgesia by Mouse Tail-Flick Assay

Measurement of mouse tail-flick latencies has been shown to be a reliable method for assessing the degree of analgesia produced by

morphine in mice (54). In this method, 45 minutes after injection (s.c.) of various doses of morphine, the tail of the mouse is placed over an intense beam of light and the time required for the mouse to flick his tail away from the beam of light is recorded as the latency time. The intensity of the beam of light is adjusted so that the latency time of mice injected with saline is approximately 2-4 seconds. To measure the degree of tolerance to morphine produced by certain drug treatments on the animal, mouse tail-flick latencies are measured at different doses of morphine; in the more tolerant group the dose-response curve of latency time versus dose of morphine should be shifted to higher doses of morphine.

The degree of tolerance to morphine produced by morphine pellet implantation and dibutyryl-cAMP injections are measured for the four treatment groups: morphine-dbcAMP, morphine-saline, placebo-dbcAMP, and placebo-saline. Mice are implanted with the morphine (75 mg) or placebo pellet and then injected icv. one hour later with 28 μg dibutyryl-cAMP in 4 μl saline. The pellets are removed 24 hours later and the measurement of tolerance by the mouse tail-flick method is performed 6 hours after pellet removal. The mouse tails are painted black with a felt pen and the mice are injected subcutaneously with 5-100 mg/kg morphine sulfate in saline. Tail-flick latencies are measured 45 minutes later and 40 seconds is used as the maximum latency time indicating analgesia. Dose-response curves are plotted as the $\log_{10}$ (latency time) versus morphine dose.

All experiments are repeated at least two to three times.

MATERIALS

Casein, histone, EGTA, EDTA, BSA - fraction V, cAMP, dibutyryl cAMP, Folin and Ciocalteau Phenol Reagent, theophylline, SDS, TEMED, Coomasie Brilliant Blue, HEPES, E. coli alkaline phosphatase and bentonite were from Sigma Chemical Company, St. Louis, Missouri. Tris, ATP, CTP, GTP, UTP, dithiothreitol and calf thymus DNA were from Calbiochem, San Diego, California. NaF, $MgCl_2$, $MnCl_2$, trichloroacetic acid, $NaPP_i$, KCl, acetic acid, sucrose, glycerol, NaCl, $(NH_4)_2SO_4$, NaOH, HCl and $(NH_4)OH$ were all of analytical grade from Mallinckrodt, St. Louis, Missouri. Potassium phosphate, $CuSO_4 \cdot 5\ H_2O$ and Na-tartrate were from Baker Reagents, Phillipsburg, New Jersey. Acrylamide, bis-acrylamide, glycine, ammonium persulfate and bromophenol blue were from Biorad, Richmond, California. Mercaptopropanol and methanol were from Aldrich Chemical Company, Milwaukee, Wisconsin. Dialysis tubing with average pore radius permeability of 24 angstroms was from VWR, San Francisco, California. The 75 mg morphine sulfate, 10 mg naloxone, and placebo pellets were made by the School of Pharmacy, University of California, San Francisco. Scintiverse was from Fisher Scientific, Fair Lawn, New Jersey. Sephadex G-25, -50, -75, -100, -150, -200 and DEAE-Sephadex resins were from Pharmacia, Piscataway, New Jersey. Cheesecloth, 95% ethanol and saline solution (0.9% NaCl, sterile) were from the Storehouse, University of California, San Francisco.

III. RESULTS

A. Purification of Nuclear Protein Kinase from Small Dense Nuclei of Mouse Brain

Because Oguri *et al.* (18) found that it is the phosphorylation of the acidic chromatin proteins and not the histones which is altered during morphine tolerance-dependence, throughout the development of the purification procedure the nuclear protein kinase activity is assayed with exogenous casein, an acidic protein, as protein substrate. The flow chart diagram (Figure 2) illustrates the procedure developed for the purification of nuclear protein kinase from small dense nuclei of mouse brain. In a usual preparation, brains from 150 mice are homogenized and the small dense nuclei are isolated by discontinuous sucrose density centrifugation. The nuclei are sonicated and then centrifuged. The resultant supernatant is then subjected to a 40% ammonium sulfate precipitation and phosphocellulose column chromatography.

The solubilized nuclear protein kinase from small dense nuclei is quite sensitive to NaCl. Figure 3 shows the sensitivity of nuclear protein kinase activity from the supernatant of the sonicated nuclei. It is seen that the protein kinase activity is greatly decreased by NaCl. Because NaCl interferes with nuclear protein kinase activity, all fractions eluted from the phosphocellulose column by the NaCl linear gradient are dialyzed against buffer without NaCl before assaying for protein kinase activity.

The purification factors and the activity recoveries obtained at each step of the procedure developed for the partial purification of

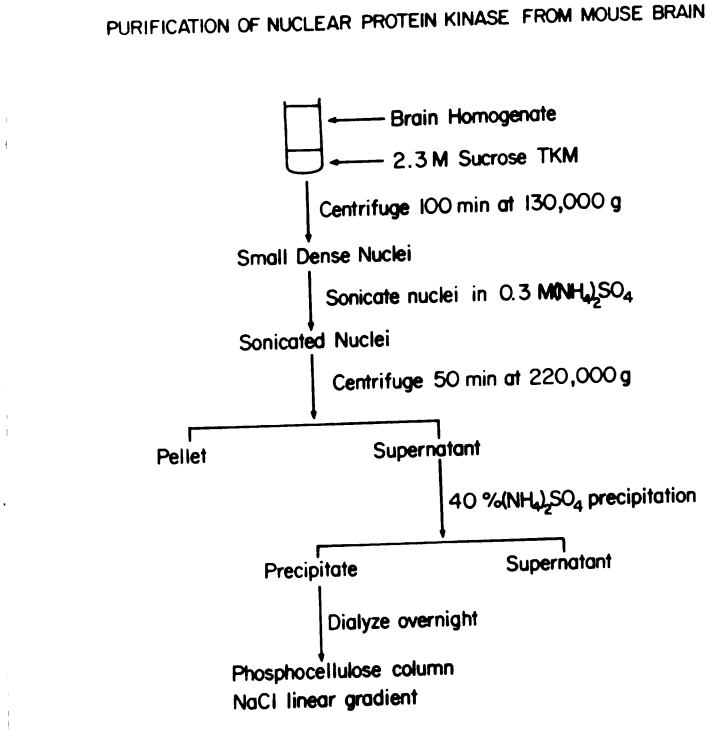


FIGURE 2. Flow-chart diagram of procedure developed for the purification of nuclear protein kinase from small dense nuclei of mouse brain.

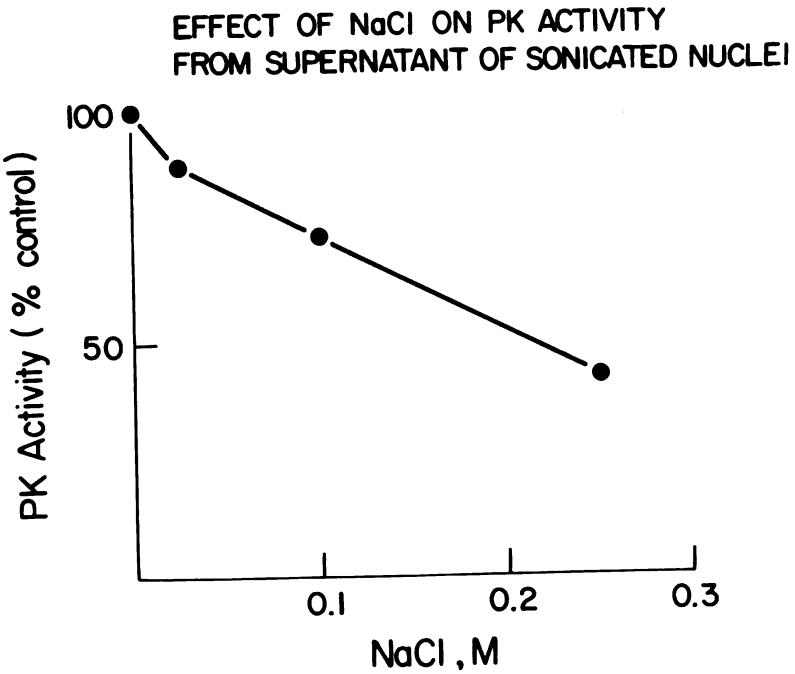


FIGURE 3. Sensitivity of nuclear protein kinase to NaCl.

the nuclear protein kinase from small dense nuclei of mouse brain are shown in Table 1. The 40% $(NH_4)_2SO_4$ precipitation of the supernatant fraction from the sonicated nuclei results in a purification factor of approximately 5-fold and a recovery of 85% of the total protein kinase activity from the sonicated whole nuclei. The phosphocellulose column chromatography results in the resolution of two major peaks of protein kinase activity. These two peaks are referred to as nuclear protein kinase peaks I and II. The specific activities of peaks I and II represent a purification factor of 26- and 22-fold, respectively, and the sum of the total activities in both peaks represents an activity recovery of 26% relative to sonicated whole nuclei.

The elution profile of nuclear protein kinase specific activity on the phosphocellulose column is shown in Figure 4. Elution of the column by a linear NaCl gradient from 0 to 1.0 M NaCl in TEMD buffer results in two major peaks of protein kinase activity. Peaks I and II are eluted at approximately 0.45-0.50 M and at 0.56-0.63 M NaCl, respectively. Using known NaCl concentrations in TEMD buffer as standard, conductivity measurements of the phosphocellulose column fractions show the NaCl gradient to be quite linear. Protein concentration is measured for each fraction and no definitive peaks of protein are eluted from the phosphocellulose column.

The nuclear protein kinase peaks I and II are both found to be cAMP-independent (Figure 5). However, there are two minor peaks of cAMP-dependent nuclear protein kinase, which elute at higher NaCl concentrations than peaks I and II. Without cAMP, there is little activity in these two fractions; but when assayed in the presence of 10^{-6} M cAMP,

TABLE 1

Partial Purification of Nuclear Protein Kinase from Small Dense Nuclei of Mouse Brain

	Specific activity (pmol phosphate incorp/mg prot/5 min)	Purification factor (x)	Activity recovery (%)
Sonicated whole nuclei	88	1	100%
40% $(NH_4)_2SO_4$ precipitate	479	5.3	85%
Phosphocellulose column			
Peak I	2420	26	12%
Peak II	2060	22	14%

The purification factor and activity recovery are expressed relative to sonicated whole nuclei. All fractions are assayed with exogenous casein as protein kinase substrate.

Specific activities of peaks I and II represent fractions of maximum specific activity within each peak.

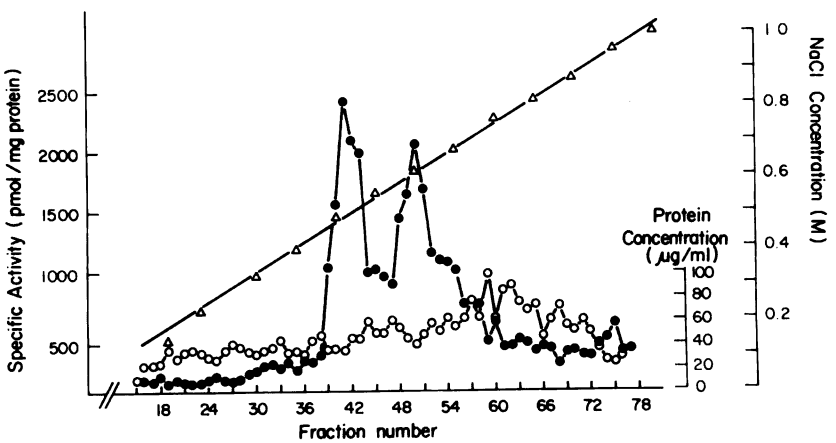


FIGURE 4. Phosphocellulose column chromatography of nuclear protein kinases from small dense nuclei of mouse brain. The phosphocellulose column is eluted by a linear NaCl gradient (Δ—Δ) and fractions of 1 ml are collected. Protein kinase specific activity (●—●) with exogenous casein as substrate and protein concentration (O—O) are measured for each fraction.

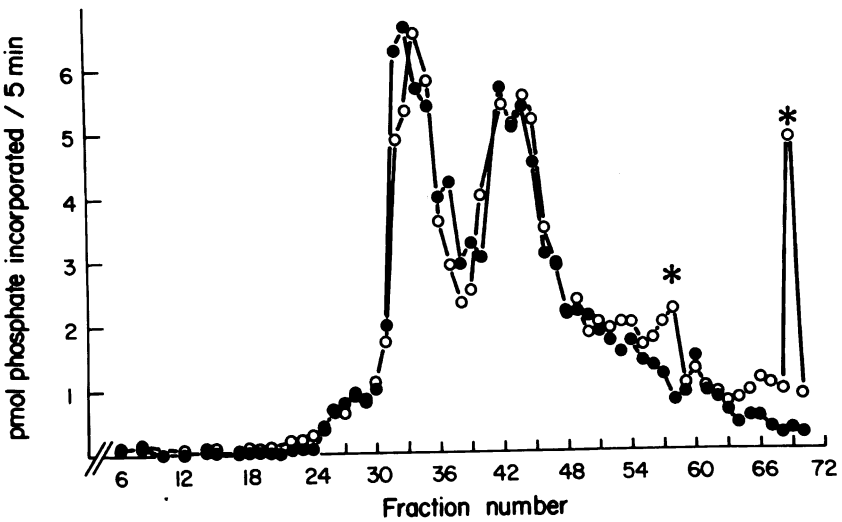


FIGURE 5. cAMP dependence of nuclear protein kinase eluted from phosphocellulose column. The column is eluted as in Figure 4 and each fraction is assayed for protein kinase activity (pmol phosphate incorporated/5 min) in the absence (●—●) or presence (○—○) of 10^{-6} M cAMP. Asterisks indicate fractions whose activities are significantly increased with 10^{-6} M-cAMP.

the activities increase 3-fold and 16-fold, respectively. The specific activities of these two peaks of cAMP-dependent nuclear protein kinase could not be measured since the protein concentration is so small that it is measured as zero protein by the Lowry protein assay.

When assayed without exogenously added protein, the activities of nuclear protein kinase peaks I and II are reduced to one-third and two-thirds, respectively, of the activity assayed with exogenous casein. Both peaks are found to prefer casein, an acidic protein, over histones, which are basic proteins, as protein substrate.

The protein and incubation time dependence of nuclear protein kinase peaks I and II are shown in Figure 6. Protein kinase activity is linear up to 3 μg protein, and 1-2 μg of protein is normally used in the assays. With regard to incubation time, the activity is linear up to 10 minutes, and an incubation time of 5 minutes is routinely used. Protein kinase activity is optimum at a temperature of 30-45° C (Figure 7) and is normally incubated at 30° C. These two peaks of nuclear protein kinase show no definitive pH optimum between pH 6.5 and pH 8.0 (Figure 7); the enzyme is normally incubated at pH 6.5.

The apparent K_m values determined by the Lineweaver-Burk plot (Figure 8) for nuclear protein kinase peaks I and II are found to be 3 and 4 μM ATP, respectively. Thus, although these two peaks are eluted from the phosphocellulose column at different NaCl concentrations, they show no significant difference in their apparent K_m values for ATP.

The partially purified nuclear protein kinase peaks I and II are relatively unstable when kept at 4° C. The phosphocellulose column fractions are assayed on the third day after the mice have been sacrificed

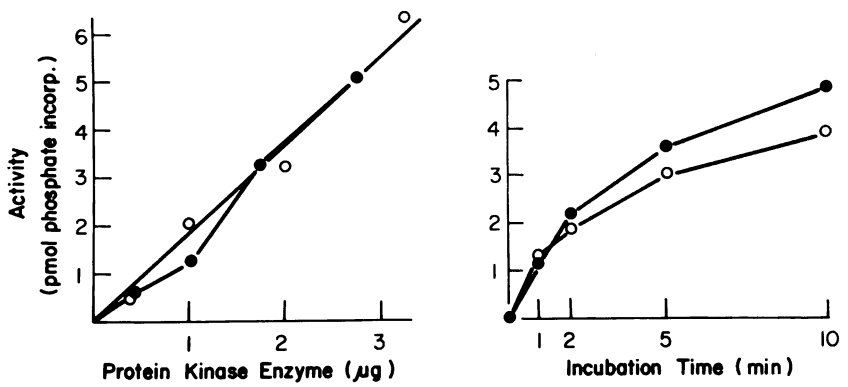


FIGURE 6. Protein and time dependence of nuclear protein kinase; peak I (●—●) and peak II (o—o).

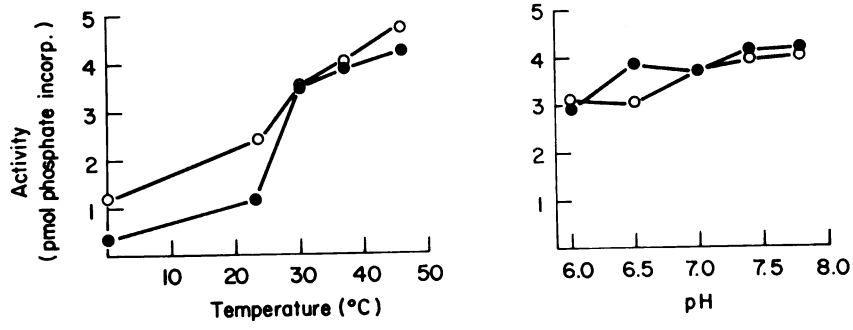


FIGURE 7. Temperature and pH dependence of nuclear protein kinase; peak I (●—●) and peak II (o—o).

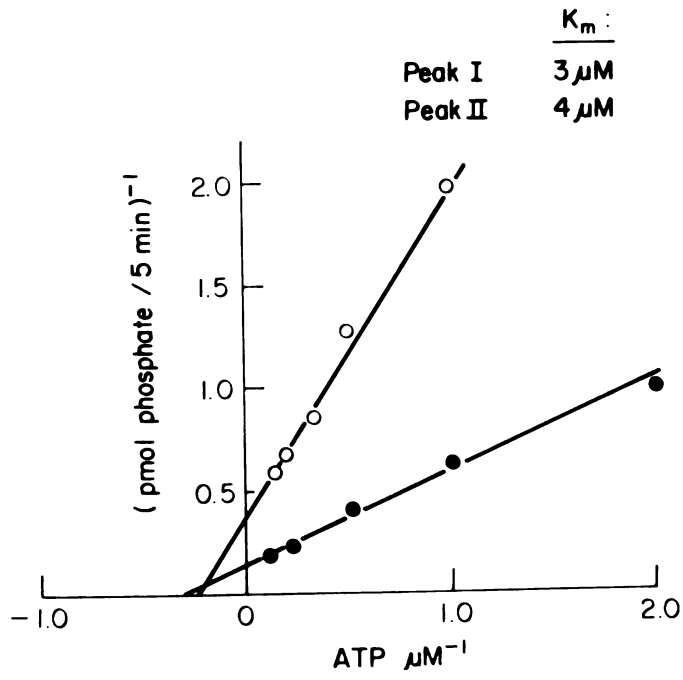


FIGURE 8. Determination of K_m for ATP of nuclear protein kinase by the Lineweaver-Burk plot; peak I (●—●) and peak II (○—○).

(day 1 = day mice are sacrificed). By the fourth day the protein kinase activity is decreased by approximately 50% relative to the respective activity on the third day. Therefore, in all experiments nuclear protein kinase activity is assayed on the third day after the mice have been sacrificed.

Polyacrylamide slab gel electrophoresis was performed on samples obtained at successive stages of the purification scheme developed for the partial purification of nuclear protein kinase peaks I and II. The slab gel can show the relative purity of protein kinase peaks I and II and the relative effectivenes of the purification procedure. As shown in Figure 9, samples of brain homogenate, sonicated small dense nuclei, 40%-$(NH_4)_2SO_4$ precipitate, and nuclear protein kinase peaks I and II are subjected to slab gel electrophoresis under denaturing conditions with a basic buffer system. The samples are applied to a 3% polyacrylamide stacking gel with a resolving gel of 5-15% polyacrylamide gradient, 0.1% SDS. The slab gels show that throughout the purification procedure numerous protein bands are enriched and deleted. The protein kinase peaks I and II are seen to contain fewer protein bands compared to the other fractions. However, both nuclear protein kinase peaks I and II contain many protein bands and are, therefore, only partially pure. Both peaks contain several small molecular weight protein bands. Protein kinase peak II is especially enriched in one particular small molecular weight protein band. In contrast to protein kinase peak II, peak I contains several high molecular weight protein bands which are absent in peak II. This difference between the two peaks is more clearly shown in Figure 10 where more protein has been applied to the

Slab Gel Electrophoresis of Purification Scheme for PK I and II

5-15 % polyacrylamide gradient, 0.1 % SDS

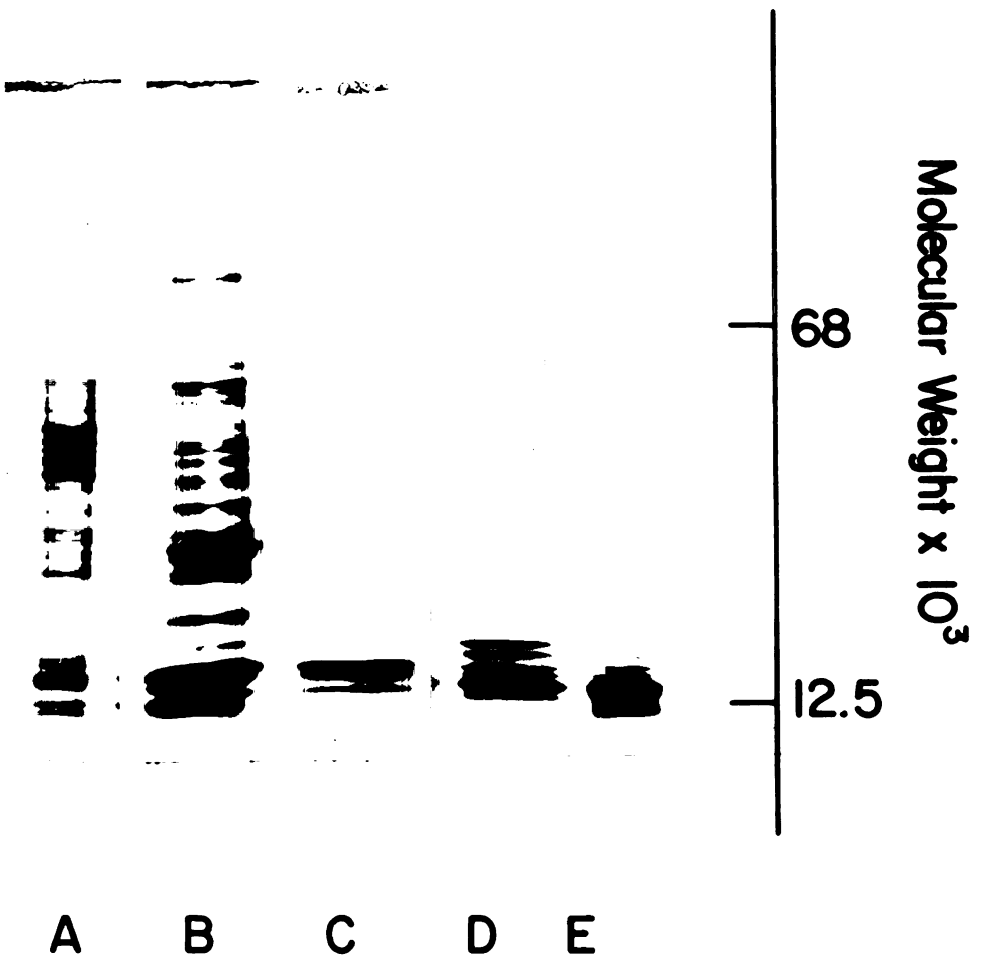


Figure 9.

Slab Gel Electrophoresis of Purification Schems for PK I and II

5-15 % polyacrylamide gradient, 0.1 % SDS

Figure 9.

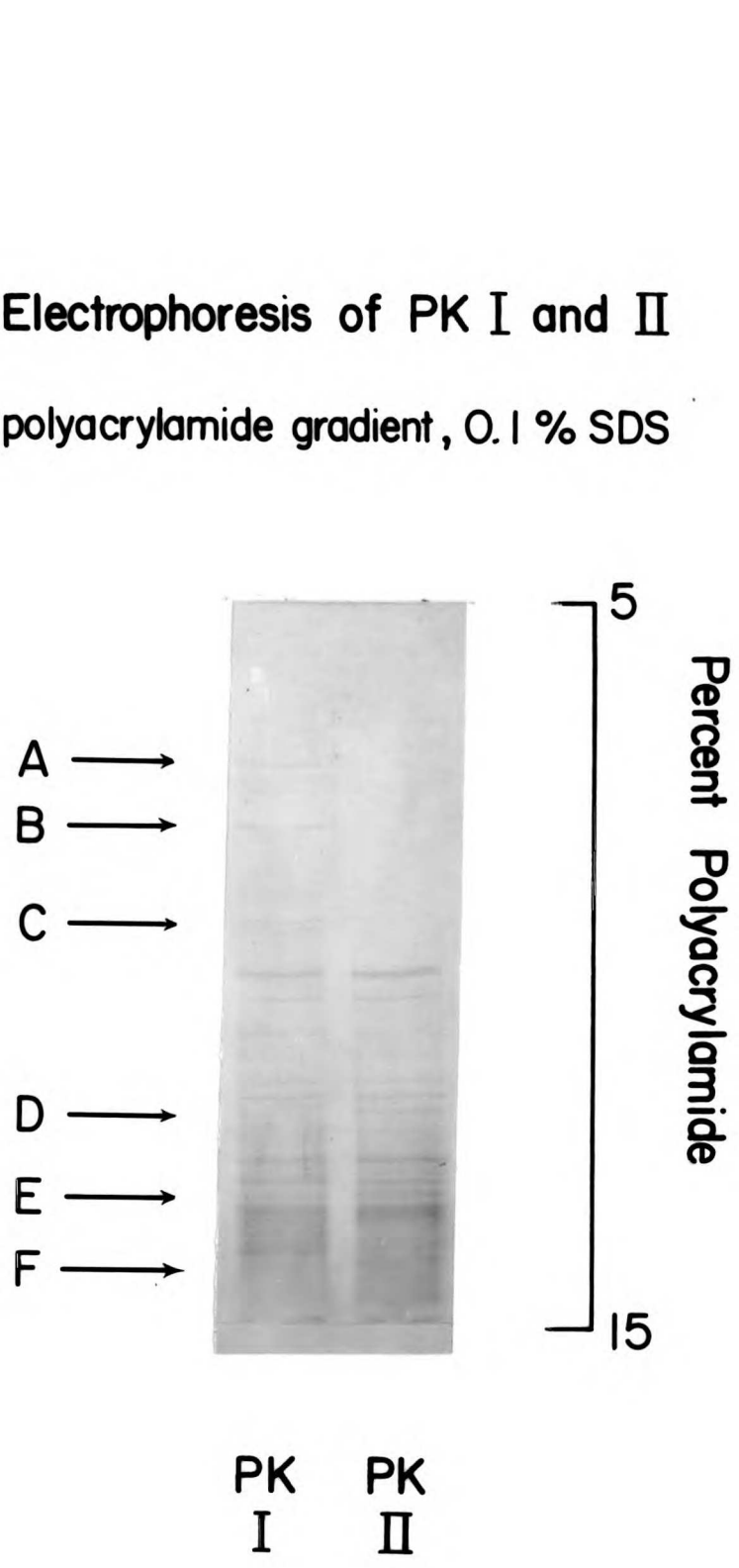

Slab Gel Electrophoresis of PK I and II
5-15 % polyacrylamide gradient, 0.1 % SDS
A
B
C
D
E
F
5
15
Percent Polyacrylamide
PK I
PK II

Figure 10.

Slab Gel Electrophoresis of PK I and II

5-15 % polyacrylamide gradient, 0.1 % SDS

Figure 10.

FIGURE 9. Polyacrylamide slab gel electrophoresis of the successive stages of the purification scheme for nuclear protein kinase from small dense nuclei of mouse brain. Brain homogenate (A), sonicated small dense nuclei (B), 40%-ammonium sulfate precipitate (C), nuclear protein kinase peak I (D), and nuclear protein kinase peak II (E) were applied to the stacking gel with the resolving gel consisting of a 5-15% polyacrylamide gradient, 0.1% SDS and run at a constant voltage of 200 V until the dye front reached the end of the gel. Approximately 15-20 µg protein were applied onto the gel for samples A, B, and C. Approximately 25 µg protein was applied to the gel for samples D and E. (See Methods for detailed procedure.)

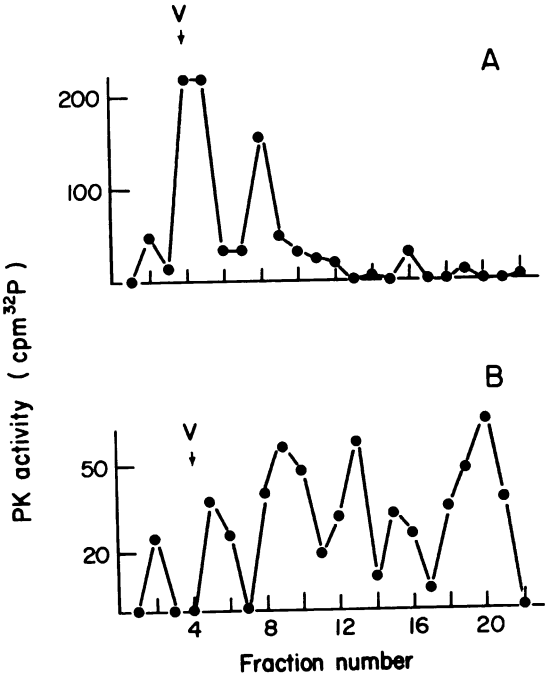
FIGURE 10. Polyacrylamide slab gel electrophoresis of nuclear protein kinase peaks I and II. 30-45 µg protein were applied to the stacking gel with a resolving gel of 5-15% polyacrylamide gradient. (See Methods for detailed procedure.)

gel than in Figure 9. At positions labeled A and B in Figure 10, there are two protein bands in the high molecular weight region in PK I which are absent in PK II. At position C, a band which appears as a doublet in PK I appears as a singlet in peak II. Furthermore, at positions D, E and F protein bands seen in PK II appear to be absent in PK I. Thus, the primary difference between PK I and PK II is that PK I contains several high molecular weight protein bands which are not seen in PK II, and PK II contains several small molecular weight protein bands not present in PK I.

This result leads one to speculate that the protein molecule(s) responsible for protein kinase activity may be of large molecular weight in PK I and of small molecular weight in PK II. Polyacrylamide gel electrophoresis under non-denaturing conditions and subsequent assay of the gel for protein kinase activity can demonstrate molecular weight differences in the protein kinase from PK I and PK II. Nuclear protein kinase peaks I and II were subjected to polyacrylamide (4-10% gradient) gel electrophoresis under non-denaturing conditions. However, none of the proteins entered the resolving gel; all of the Coomasie blue stain appeared at the top of the resolving gel. Even in samples with 0.1% Triton, most of the proteins remained at the top of the resolving gel and a very small amount entered the gel. Because the protein kinase peaks I and II must initially undergo considerable concentration in order to obtain enough protein to apply to the gel, it is possible that at the higher concentration the proteins may form aggregates and, therefore, are unable to enter the gel. Because the proteins do not enter the gel, no further resolution of protein kinase activity can be made by this method.

Determination of the approximate molecular weight of the protein kinase in peaks I and II can be made by molecular exclusion chromatography. Using Sephadex gel filtration resins with various molecular weight exlcusion limits, the molecular weight of the protein kinase enzyme can be estimated by following its activity on a gel filtration column and determining its elution volume. On a Sephadex G-150 column, the nuclear protein kinase peak I activity is eluted at the void volume; no activity is eluted after the void volume. This indicates that protein kinase peak I has a molecular weight larger than 150,000. Sephadex G-200 column chromatography of PK I resolves the fraction into two components of protein kinase activity as shown in Figure 11, panel A. One component elutes at the void volume indicating a molecular weight of larger than 200,000, and the other component elutes just after the void volume indicating a molecular weight of approximately 150,000-200,000.

Gel filtration column chromatography of nuclear protein kinase peak II on a Sephadex G-50 column indicates that its molecular weight is smaller than 30,000 since none of the protein kinase activity is eluted at the void volume. Sephadex G-25 column chromatography of PK II (Figure 11, panel B) resolves the fraction into several peaks of protein kinase activity, all of which elute after the void volume. These results indicate that the protein kinases contained in PK II have a molecular weight of less than 5,000. A molecular weight of 5,000 for PK II seems rather small. However, it must be noted that molecular weight determination by molecular exclusion chromatography assumes that the proteins are globular in shape. Thus, the actual molecular weight



A. Sephadex G-200 column chromatography of PK I

B. Sephadex G-25 column chromatography of PK II

FIGURE 11. Sephadex G-200 column chromatography of PK I (panel A) and Sephadex G-25 column chromatography of PK II (panel B). Protein kinase activity (●—●) is measured in each fraction (0.5 ml/fraction). V = void volume.

of PK II may be very different from 5,000.

The results obtained from the polyacrylamide gel electrophoresis and gel filtration experiments indicate that PK I contains primarily large molecular weight (above 150,000) protein kinases and PK II contains several small molecular weight (less than 5,000) protein kinases. (Note: The molecular weight exclusion limits of the Sephadex gel filtration resins are determined from the Pharmacia Handbook of Gel Filtration, theory and practice.)

III. B. Nuclear Protein Kinase during Morphine Tolerance-Dependence

The purpose of developing the procedures for the partial purification of nuclear protein kinase from small dense nuclei of mouse brain is to investigate whether or not the nuclear protein kinase activity may be responsible for the increased chromatin protein phosphorylation seen during morphine tolerance-dependence. After being rendered morphine tolerant-dependent by pellet implantation for 72 hours, the mice are decapitated and the nuclear protein kinases are purified as described; that is, by ammonium sulfate fractionation and phosphocellulose column chromatography. The nuclear protein kinase specific activities of peaks I and II are measured for both chronic morphine and placebo groups.

At 72 hours after morphine pellet implantation, the mice show a characteristic physical appearance which differs remarkably from their placebo controls. The morphine tolerant-dependent mice show slow, clumsy locomotor behavior and loss in body weight. Their fur is ungroomed and patchy-looking, and there are no feces on the bottom of their cages. In contrast, the placebo treated mice show normal locomotor behavior

and their fur is clean and groomed. There is much feces in the cages from the placebo group. Normally, a mortality rate of 1-2 per 100, and 0 per 100 mice occur in chronic morphine and placebo groups, respectively.

The effect of chronic morphine treatment on nuclear protein kinase from small dense nuclei of mouse brain is shown in Table 2. After chronic morphine treatment, the specific activity of nuclear protein kinase peak I is increased by an average of 26%. However, the specific activity of nuclear protein kinase peak II is unaltered after the drug treatment. The two peaks of protein kinase activity are eluted from the phosphocellulose column at the same NaCl concentration in both chronic morphine and placebo groups. Thus, the physical properties of peaks I and II which determine their elution profile on the phosphocellulose column are not altered during morphine tolerance-dependence.

Also, morphine *in vitro* at 10^{-7} to 10^{-3} M has no effect on the activities of peaks I and II isolated from naive mice. This indicates that the increase in nuclear protein kinase peak I specific activity during morphine tolerance-dependence is not a direct effect of the morphine molecule on the protein kinase enzyme; rather, the change in peak I activity is probably an effect several steps removed from the initial site of morphine action.

Up to this point it has been assumed that ^{32}P-phosphate incorporation into casein from (γ-^{32}P)-ATP represents protein kinase activity. However, it is actually a measure of the net result of protein kinase and phosphoprotein phosphatase enzyme activities. If the nuclear protein kinase peak I fraction contains any phosphoprotein phosphatase activity, it is possible that during morphine tolerance-dependence a

TABLE 2

Effect of Chronic Morphine Treatment on

Nuclear Protein Kinase from Mouse Brain

	Protein kinase specific activity ± S.E. (pmol phosphate incorp./mg protein)		
	Placebo	Morphine	% Change
Peak I	1360 ± 60	1720 ± 58	26*
Peak II	1180 ± 170	1190 ± 99	0

Each value is the average specific activity from three experiments and each experiment consists of 75 mice per treatment group.

* $P < 0.01$, two-tailed t-test

decrease in phosphatase activity could contribute to the increased incorporation of ^{32}P-phosphate into casein seen in the peak I fraction. Therefore, the phosphoprotein phosphatase activity in the nuclear protein kinase peak I fraction from chronic morphine and placebo treated groups is measured.

Phosphoprotein phosphatase activity is measured by first assaying the protein kinase as usual, and at the end of the 5 minutes incubation at 30° C the samples are placed on ice, nonradioactive ATP is added and the samples are incubated for an additional 5 to 20 minutes. Phosphoprotein phosphatase activity is measured by following the disappearance of ^{32}P-phosphate-labeled casein after the addition of the nonradioactive ATP.

The phosphatase activity in nuclear protein kinase peak I from placebo and chronic morphine treated mice is shown in Figure 12. The slope of these curves represents the rate of phosphatase activity. The two curves are quite parallel, indicating that the phosphatase specific activity in peak I is the same for both placebo and chronic morphine groups. The phosphatase activity is very low compared with the protein kinase activity; its specific activity is approximately one-tenth that of the protein kinase. Thus, the increased specific activity of the protein kinase peak I seen during morphine tolerance-dependence is not due to an alteration in phosphoprotein phosphatase activity.

Studies investigating co-factor optimums for RNA polymerase (27) and amino-acyl-tRNA transferases (58) indicate that certain co-factor optimums are modified after chronic morphine treatment. The optimum Mg^{++} concentration for RNA polymerase II and the optimum ratio Mn^{++}/Mg^{++}

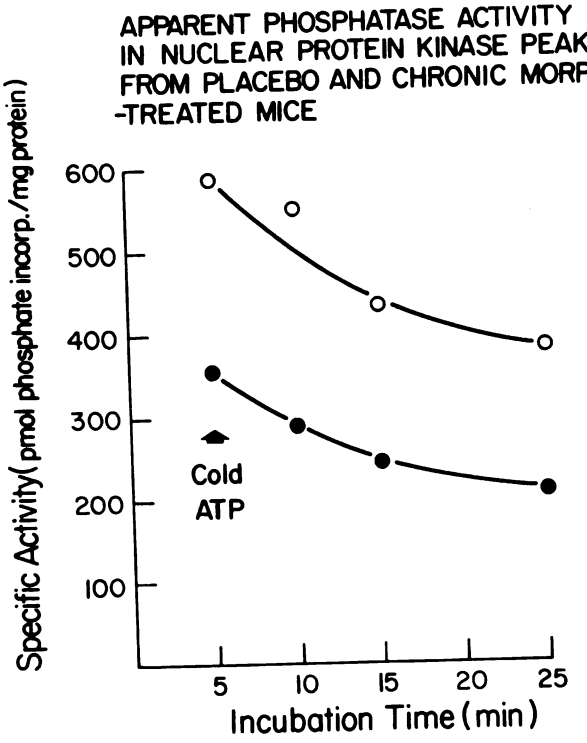


FIGURE 12. Phosphoprotein phosphatase activity in PK I from placebo (●—●) and chronic morphine-treated (○—○) mice.

for RNA polymerases II and III from small dense nuclei of mouse brain are altered during chronic morphine treatment. The optimum Mg^{++}/ATP ratio and temperature for some isozymes of amino-acyl-tRNA transferases from mouse brain are altered during morphine tolerance-dependence. Therefore, to find if the co-factor requirements of nuclear protein kinase peaks I and II are altered during morphine tolerance-dependence, the K_m for ATP, the Mg^{++} optimum concentration, and the cAMP-dependence are measured for peaks I and II isolated from chronic morphine and placebo treated mice. No significant differences between chronic morphine and placebo groups are observed (Table 3) for any of these parameters.

Based on the appearance and behavioral characteristics of mice 72 hours after morphine pellet implantation, it was found that at different times of the year the mice seem to be sometimes less susceptible and sometimes more vulnerable to the chronic drug treatment. It is known that the ICR mice are an inbred strain of mice and periodically Simonsen Laboratories, the supplier, must change the breeding line of the mice several times a year. In most experiments, 72 hours after morphine pellet implantation the physical appearance of mice is distinctly different from that of the placebo controls, and the specific activity of the nuclear protein kinase peak I is usually increased by approximately 26%. However, when the mice appear less susceptible to chronic morphine treatment their physical appearance is not overwhelmingly distinct from that of the placebo controls, and the specific activity of peak I is increased by only 10%, which is not statistically significant. Furthermore, when the mice appear more vulnerable to the chronic morphine

TABLE 3

K_m for ATP, Mg^{++} Optimum, and Cyclic AMP Dependence of Peaks I and II after Chronic Morphine Treatment

	Placebo	Chronic Morphine
Peak I		
K_m (μM ATP)	4	5
Mg^{++} optimum (mM)	4	4
cAMP dependence	independent	independent
Peak II		
K_m (μM ATP)	4	2
Mg^{++} optimum (mM)	6	7
cAMP dependence	independent	independent

treatment, their physical appearance and mortality rate of 30% are very different from those of the placebo controls, and now the specific activity of peak I is increased by 60%. This comparison of the relative degree of severity of physical characteristics in mice 72 hours after morphine pellet implantation with the magnitude of peak I increase in specific activity is illustrated in Table 4.

Thus, by looking at the relative degree of severity of the physical characteristics in mice 72 hours after morphine pellet implantation, one can almost predict to what degree the specific activity of the nuclear protein kinase peak I is increased. If the physical appearance and characteristics of the mice after chronic morphine treatment are correlated with the degree of morphine tolerance-dependence developed, it would be attractive to propose the hypothesis that the degree of increase in peak I specific activity is correlated with the degree of morphine tolerance-dependence. However, the degree of morphine tolerance-dependence development is normally defined as the ratio of the morphine ED_{50} in the tolerant animal: morphine ED_{50} in the non-tolerant animal by mouse tail-flick assay (54), and is not usually based on the physical appearance of the tolerant-dependent animal. Because the ED_{50} in the tolerant-dependent mice was not measured in each experiment before purifying the nuclear protein kinase peak I, it cannot be concluded at this point that the degree of increase in peak I specific activity is correlated with the degree of morphine tolerance-dependence development, although this idea is suggested by the experimental observations.

To investigate if the effect of chronic morphine treatment on

TABLE 4

Comparison of the Relative Degree of Severity of Physical Characteristics in Mice 72 Hours after Morphine Pellet Implantation with the Magnitude of Nuclear Protein Kinase Peak I in Specific Activity

Relative degree of severity of physical characteristics 72 hr after morphine pellet implantation	% Increase in nuclear protein kinase peak I specific activity
1 - Locomotor behavior and appearance of fur similar to placebo group. Some feces on bottom of cage. Overall, mice do not appear much different from placebo mice.	10%
2 - Slow, clumsy locomotor behavior. Fur is ungroomed and patchy-looking. No feces in cage. Mortality rate 1-2%.	26% *
3 - Very slow, clumsy locomotor behavior. Fur is ungroomed and patchy-looking. No feces in cage. Mortality rate 30%.	60% *

* statistically significant, $p < 0.01$ two-tailed t-test

nuclear protein kinase peak I activity can be reversed by naloxone, an antagonist of morphine, mice are implanted with a 10 mg naloxone pellet 2 hours before implantation of the 75 mg morphine pellet. This procedure is repeated 24 hours later so that each mouse has a total of four implanted pellets. At 72 hours the mice are sacrificed and the nuclear protein kinase peak I is purified. Table 5 shows the specific activity of peak I obtained for each treatment group. It is seen that, as shown previously, morphine increases the peak I specific activity by 22%. Naloxone increases the peak I activity by 25%. When morphine and naloxone are implanted together the peak I activity is increased by 45%. These data suggest that morphine and naloxone can each increase peak I activity, and that when implanted together their effects appear to be additive. From these data, naloxone does not reverse the chronic morphine effect on peak I specific activity.

However, because of the extremely large placebo pellet effect, the results of the chronic morphine and naloxone experiment are questionable. At the time of this experiment two morphine pellets per mouse were required to produce the chronic morphine effect on nuclear protein kinase peak I activity. Therefore, two naloxone pellets were implanted with the two morphine pellets, making the total number of pellets per mouse equal to 4. As shown in Table 6, the implantation of four placebo pellets dramatically decreases the specific activity of peak I from 1400 to 430 pmol phosphate incorporated/mg protein/5 minutes, thereby reducing the activity by 70%. Due to the large placebo effect it is felt that the results of the chronic morphine and naloxone experiment are not conclusive and that the data only suggest that the chronic

TABLE 5

Effect of Chronic Morphine and Naloxone Treatment on Nuclear Protein Kinase Peak I Activity

Treatment group	Protein kinase peak I specific activity (pmol phosphate incorporated/ mg protein/5 min)	% Activity relative to placebo-placebo
Placebo-placebo	430	100%
Placebo-morphine	524	122%
Naloxone-placebo	537	125%
Naloxone-morphine	625	145%

A 10 mg naloxone pellet is implanted 2 hr before the 75 mg morphine pellet. This procedure is repeated 24 hours later. Mice are sacrificed at 72 hours and the nuclear protein kinase peak I is purified as described in the methods section. 75 mice are implanted for each treatment group.

TABLE 6

Placebo Pellet Effect on Nuclear Protein Kinase Peak I Activity

No. of placebo pellets implanted	PK I Specific Activity (pmol/phosphate incorp/mg prot/5 min)
0	1400
1	1360
4	430

The placebo pellet(s) is implanted and mice are sacrificed 72 hours later. 75 mice are implanted for each group. PK I is purified as described in the Methods section.

morphine effect on nuclear protein kinase peak I specific activity is not naloxone reversible.

This large placebo effect produced by 4 placebo pellets may be due to the stress induced on the animal each time the pellet is implanted or it may be due to the filler substance, Ca^{++} and lactose, in the tablet.

In the experiments investigating chronic morphine and placebo treatment on the specific activities of nuclear protein kinase peaks I and II, only one pellet was implanted per mouse. With one pellet the placebo effect on peak I activity is negligible (peak I activity being decreased by 3%) compared to the chronic morphine effect of a 26% increase in the specific activity of peak I. Therefore, the conclusion that chronic morphine treatment increases nuclear protein kinase peak I specific activity remains valid.

III. C. Nuclear Protein Kinase and Homologous RNA Polymerase

Chronic morphine treatment has been shown in the previous section to result in an increase in the specific activity of nuclear protein kinase peak I from small dense nuclei of mouse brain. In addition, studies by Stokes *et al.* (27) indicate that the homologous RNA polymerases are also modified by chronic morphine treatment. The specific activity of the major peak of nuclear protein kinase is increased by 26% while that of RNA polymerase I, believed to synthesize rRNA is decreased by 67%, both results being statistically significant relative to placebo controls. The optimum concentration of co-factors for RNA

polymerase II and III activities believed to synthesize hnRNA and tRNA, respectively, are also altered during morphine tolerance-dependence.

Nuclear protein kinases and RNA polymerases have been suggested as important sites in the regulation of gene expression (25,26). In particular, nuclear protein kinase may be able to modify RNA synthesis through phosphorylation of the RNA polymerase enzyme (29,30,31,32). In view of the finding that both the nuclear protein kinase peak I and RNA polymerase enzyme activities are altered after chronic morphine treatment, and the hypothesis that protein kinase may regulate RNA polymerase activity, the close functional interaction between these two enzymes may be modified during morphine tolerance-dependence. This interaction between the two enzymes may be involved in the increased chromatin template activity seen during morphine tolerance dependence (17).

However, nuclear protein kinase peak I regulation of RNA polymerase activities has not yet been demonstrated in brain tissue. 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 Figure 13. Each RNA polymerase, I, II and III, is incubated with increasing amounts of nuclear protein kinase peak I enzyme. The protein kinase and RNA polymerases are each added to the assay medium at respective protein concentrations which have been previously shown to be linear with respect to the activity of each enzyme when assayed alone. Phosphorylation is measured by following ^{32}P-phosphate incorporation from (γ-^{32}P)-ATP into TCA-precipitated protein. RNA polymerase activity is measured by following ^{3}H-UTP incorporation

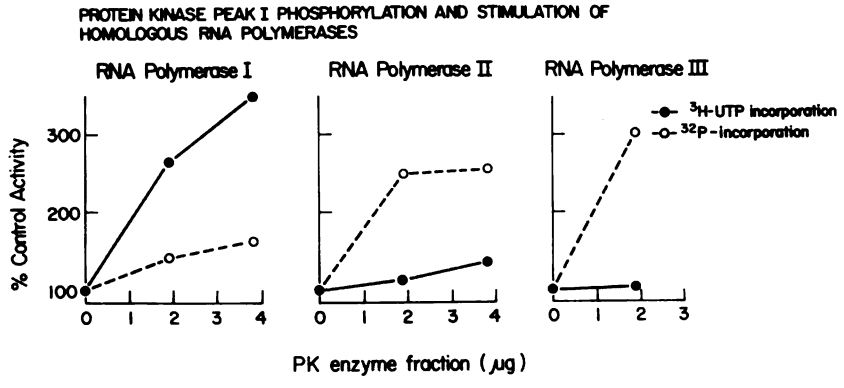


FIGURE 13. Protein kinase peak I phosphorylation and stimulation of homologous RNA polymerases. Each RNA polymerase is incubated with increasing amounts of protein kinase peak I. ^{3}H-UTP incorporation (●—●) and ^{32}P-phosphate incorporation (○—○) are measured.

into synthesized RNA. Although the nuclear protein kinase activity is optimum at pH 6.5 or pH 7.4, the RNA polymerases express their optimum activities at a more basic pH; therefore, all assays in this experiment are performed at pH 7.4.

RNA polymerase I and II activities are stimulated with increasing amounts of protein kinase and, at the same time, phosphorylation is also increased. However, a given relative increase in phosphorylation is not associated with the same relative increase in RNA synthesis. The RNA polymerase I activity increases by 240% when incubated with nuclear protein kinase, but the phosphorylation is only increased by 164%. For RNA polymerase II, RNA synthesis is increased by 35% while phosphorylation is increased by 50%. Furthermore, it appears that RNA polymerase I activity is 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 is increased, no corresponding change in polymerase activity is seen. These results suggest a correlation between phosphorylation and stimulation of RNA polymerase I and II activities by nuclear protein kinase. Because the protein kinase and RNA polymerase fractions are only partially pure, it is not known if the RNA polymerase molecule itself is phosphorylated or some other molecule is phosphorylated which, in turn, is then able to stimuate RNA synthesis. Nevertheless, this data suggests that the nuclear protein kinase may be regulating RNA polymerase I and II activities through a phosphorylation mechanism.

The presence of protein kinase activity in the RNA polymerase I fraction made it difficult to further study 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 peak I.

Modification of enzymes through phosphorylation has been shown in many systems (59,60) to result in altered enzyme activities and co-factor requirements for the enzymatic reaction being catalyzed. Because chronic morphine treatment alters the optimum Mg^{++} concentration for RNA polymerase II (27), the ability of nuclear protein kinase to change the optimum Mg^{++} concentration required for maximum RNA polymerase II activity was investigated.

A comparison of the effect of chronic morphine treatment *in vivo* and the effect of nuclear protein kinase peak I *in vitro* on the Mg^{++} optimum of RNA polymerase II from small dense nuclei of mouse brain is shown in Figure 14. In this figure, panels A and B represent two different experiments. In panel A, RNA polymerase II is purified from chronic morphine and placebo treated mice. In panel B, RNA polymerase II is purified from naive mice and incubated in the presence and absence of nuclear protein kinase peak I. RNA polymerase activity is assayed at different Mg^{++} concentrations in both experiments. In panel A, the optimum Mg^{++} concentration for RNA polymerase II from placebo treated mice is 8.5 mM and is shifted to 6 mM Mg^{++} in the chronic morphine group. In panel B, the optimum Mg^{++} concentration for RNA polymerase II incubated without protein kinase is approximately 8.5 mM Mg^{++}. However, when incubated with nuclear protein kinase peak I, the optimum Mg^{++} concentration is lowered to 6 mM. These data indicate that incubation of RNA polymerase II with protein kinase peak I shifts the

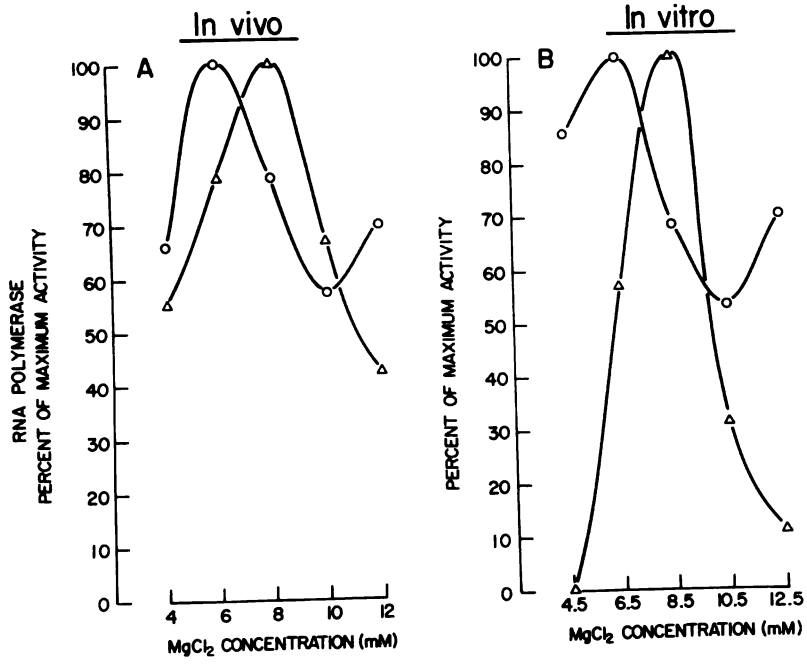


FIGURE 14. Comparison of the effect of chronic morphine treatment in vivo and nuclear protein kinase peak I in vitro on the Mg^{++} optimum of RNA polymerase II.

Panel A: RNA polymerase II is purified from placebo (Δ—Δ) and chronic morphine (o—o) treated mice. Polymerase activity is measured for both drug treatment groups at different Mg^{++} concentrations.

Panel B: RNA polymerase II is purified from naive mice and assayed at different Mg^{++} concentrations in the absence (Δ—Δ) and presence (o—o) of nuclear protein kinase peak I.

optimum Mg^{++} 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^{++} optimum for RNA polymerase II can be reproduced in vitro by merely incubating the polymerase with homologous nuclear protein kinase. This suggests that the change in Mg^{++} optimum of RNA polymerase II during chronic morphine treatment may be occurring through changes in phosphorylation as a result of altered nuclear protein kinase activity.

It is not known if the alteration of the Mg^{++} optimum of RNA polymerase II by nuclear protein kinase is specific for protein kinase peak I and not for peak II. Because the specific activity of protein kinase peak I and not of peak II is increased during morphine tolerance-dependence, it may be expected that protein kinase II should not lower the Mg^{++} optimum of RNA polymerase II. Figure 15 shows the effect of nuclear protein kinase peak II on the Mg^{++} dependence of RNA polymerase II. Although PK II stimulates RNA polymerase II activity and phosphorylation is increased 5-fold, PK II does not lower the Mg^{++} optimum of RNA polymerase II. The optimum Mg^{++} concentration of RNA polymerase II incubated without PK II is 10.5 mM Mg^{++} and when incubated with PK II, the Mg^{++} optimum appears to be 12.5 mM Mg^{++}. It is difficult to determine the exact Mg^{++} optimum in the presence of PK II because the RNA polymerase activity curve does not reach a plateau and does not decrease at higher Mg^{++} concentrations. It is not possible to measure polymerase activity at higher Mg^{++} concentrations because the Mg^{++} starts to precipitate at concentrations greater than 12.5 mM Mg^{++}. Nevertheless,

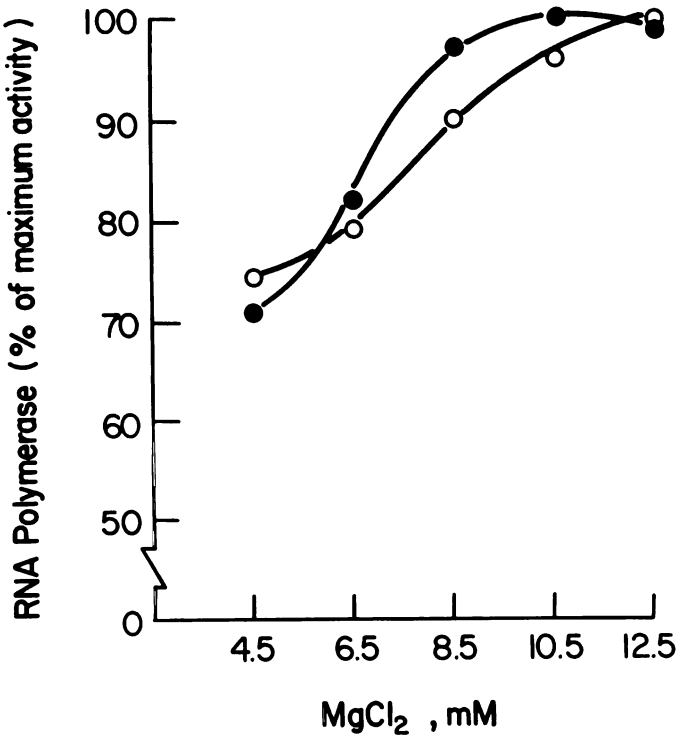


FIGURE 15. Effect of nuclear protein kinase peak II on the Mg^{++} dependence of RNA polymerase II. RNA polymerase II activity is measured at different Mg^{++} concentrations in the absence (●—●) and presence (o—o) of nuclear protein kinase peak II.

these results suggest that PK II may slightly increase the Mg^{++} optimum of RNA polymerase II. Thus, the lowering of the RNA polymerase II Mg^{++} optimum *in vitro* appears to be occurring selectively through PK I and not PK II.

It is noted that in Figure 15 the Mg^{++} optimum of RNA polymerase II incubated without protein kinase is 10.5 mM whereas in Figure 14 it is 8.5 mM; repeated experiments have shown that the Mg^{++} optimum of RNA polymerase II is normally in the range 8.5-10.5 mM mg^{++}. Furthermore, the shapes of the curves in Figures 14 and 15 are not exactly the same. The curve in Figure 15 shows a less definitive Mg++ optimum peak compared with that in Figure 14. However, the main point still holds that unlike peak I, protein kinase peak II does not appear to lower the Mg^{++} optimum of RNA polymerase II. In both Figures 14 and 15, the amount of phosphorylation did not change with Mg^{++} concentration, since a maximum amount of protein kinase is present.

This effect of PK II is in direct contrast with the effect of PK I and chronic morphine treatment on the Mg^{++} optimum of RNA polymerase II. Thus, it is appropriate that for the cell to lower the Mg^{++} optimum of RNA polymerase II during morphine tolerance-dependence, the activity of PK I and not of PK II should increase. Experimental evidence illustrates that only PK I and not PK II specific activity is increased during chronic morphine treatment.

To propose that nuclear protein kinase is modifying the homologous RNA polymerase activity through a phosphorylation mechanism, experiments should be performed to show that this phenomenon is reversible

with the use of phosphoprotein phosphatase. In addition, because it is known that the protein kinase peak I and RNA polymerase fractions are only partially pure, studies must be undertaken which can indicate whether the RNA polymerase molecule itself is being phosphorylated or some other molecule is being phosphorylated.

The ability of E. coli alkaline phosphatase to inhibit phosphorylation by nuclear protein kinase peak I is shown in Table 7. In panel A, when peak I is incubated with increasing amounts of alkaline phosphatase, its activity is decreased substantially. However, because the phosphatase is prepared in 2.6 M $(NH_4)_2SO_4$ solution by Sigma, it is possible that the $(NH_4)_2SO_4$ alone may affect the peak I activity. In panel B, it is seen that $(NH_4)_2SO_4$ alone decreases peak I activity. Therefore, the $(NH_4)_2SO_4$ in the phosphatase solution must be removed. When peak I is assayed with dialyzed phosphatase (panel C), no decrease in phosphorylation is seen. The failure of the phosphatase to reduce the amount of phosphorylation is probably due to the continued presence of highly active protein kinase. As soon as the phosphatase completes the dephosphorylation reaction, plenty of protein kinase is available to re-phosphorylate the same site; therefore, the ^{32}P-phosphate incorporated into the protein substrate is not decreased. On the contrary, it is seen (panel C) that as protein kinase is incubated with increasing amounts of phosphatase, the ^{32}P-phosphate incorporated does not remain constant, but is slightly increased. This result is probably due to endogenous phosphate being released from its protein site by the phosphatase and the subsequent re-phosphorylation, allowing new ^{32}P-phosphate to be incorporated, by the protein kinase.

TABLE 7

Alkaline Phosphatase and Nuclear Protein Kinase Peak I

	PK I activity (cpm ^{32}P)	% Change
A. Phosphatase-undialyzed (in 2.6 M $(NH_4)_2SO_4$ solution)		
0 µg	8514	100%
1	5073	59
5	3035	36
10	1406	16
B. $(NH_4)_2SO_4$		
0	6299	100%
0.12 M	5244	83
C. Phosphatase-dialyzed (in 10 mM K-P buffer, pH 7.8)		
0 µg	3162	100%
1	3637	113
5	4175	132
20	3883	122

Protein kinase peak I activity is incubated with different concentrations of phosphatase (undialyzed and dialyzed) and ammonium sulfate. Protein kinase activity is assayed with exogenously added casein as protein substrate in 50 mM potassium-phosphate buffer pH 7.8, 3 mM EGTA, 10 mM NaF, and 10 mM $MgCl_2$.

Because incubation of protein kinase with phosphatase does not decrease the amount of phosphorylation, to show that protein kinase phosphorylation and stimulation of RNA polymerase is a reversible phenomenon, the protein kinase and polymerase cannot together be incubated with phosphatase. The protein kinase enzyme must be removed from the phosphorylated RNA polymerase fraction before phosphatase is added to the polymerase to show that dephosphorylation results in a reduction of RNA polymerase activity.

The nuclear protein kinases and RNA polymerases have been purified by cation exchange (phosphocellulose) and anion exchange (DEAE Sephadex) column chromatography, respectively. The use of these dissimilar ion exchange resins for the purification of these two classes of enzymes exemplifies their differential ionic properties. Since the protein kinases and RNA polymerases probably have different adsorption properties on the same ion exchange resin, it is possible that a mixture of these two enzymes may be separated by phosphocellulose column chromatography. That is, the RNA polymerase may not be adsorbed onto the phosphocellulose resin (since it is known that the polymerase is adsorbed onto DEAE-Sephadex resin) and the protein kinase is known to be adsorbed onto this resin.

To find whether or not the RNA polymerase II molecule may be phosphorylated by nuclear protein kinase peak I, the protein kinase peak I and RNA polymerase II fractions are incubated with (γ-^{32}P)-ATP to allow phosphorylation to occur. This mixture is then subjected to phosphocellulose column chromatography in an effort to try to separate the protein kinase from the RNA polymerase. ^{32}P-phosphate, indicating

phosphorylation, is followed on the column and fractions are assayed for both protein kinase and RNA polymerase activities. The elution profile of ^{32}P-phosphate relative to polymerase activity can suggest if the RNA polymerase or some other protein may be phosphorylated.

After the protein kinase and RNA polymerase mixture is applied to the phosphocellulose column, the column is washed with TEMDG + 30 buffer and eluted with a discontinuous ammonium sulfate gradient of 600 mM. In panel A of Figure 16, the elution profile of ^{32}P and ^{3}H-ATP, as the ATP standard, is shown. It should be noted that the ^{3}H-ATP is run on a different column of the same size and under identical conditions. The ^{32}P and ^{3}H peaks elute at approximately the same position in the wash from the phosphocellulose column. Thus, this peak of ^{32}P probably represents ^{32}P-ATP. No peaks of ^{32}P or ^{3}H are eluted by the stepwise 600 mM $(NH_4)_2SO_4$ gradient. The elution profiles of the protein kinase and RNA polymerase activities are shown in panel B. The major peak of protein kinase is eluted from the column by the ammonium sulfate stepwise gradient. However, the total activity in this peak is much less than the total protein kinase activity applied onto the column, indicating that the protein kinase activity is unstable and has partially decayed. No RNA polymerase activity is found in any fraction from the phosphocellulose column. The RNA polymerase activity is normally very unstable and loses most of its activity by one day after its purification is achieved. Therefore, it is not possible to take the RNA polymerase through many manipulations before assaying its activity because of its highly unstable nature. Thus, the problem of whether the RNA polymerase molecule itself is phosphorylated remains unanswered.

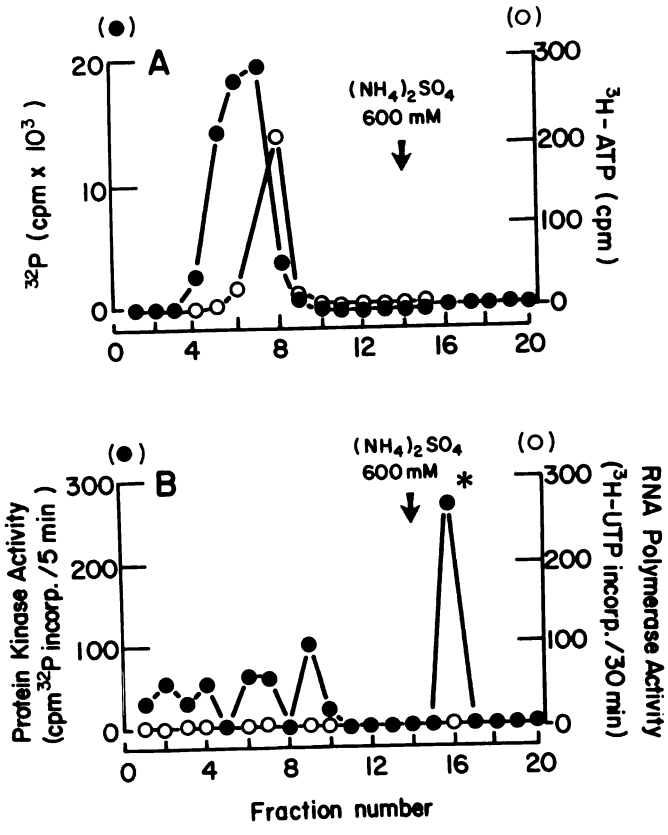


FIGURE 16. Phosphocellulose column chromatography of PK I + RNA polymerase II mixture. PK I and RNA polymerase II are incubated with (γ-^{32}P)-ATP under protein kinase assay conditions. The mixture is then subjected to phosphocellulose column chromatography.

Panel A. ^{32}P-phosphate (●—●) and ^3H-ATP (○—○) elution profile.

Panel B. Elution profile of protein kinase activity (●—●) and RNA polymerase activity (○—○).

Furthermore, investigations of the reversibility of protein kinase phosphorylation and stimulation of RNA polymerase II activity are impossible; by the time the polymerase fraction is phosphorylated and separated from the protein kinase so that phosphatase can be added, the RNA polymerase activity is totally decayed.

Polyacrylamide slab gel electrophoresis followed by ^{32}P-autoradiography of the phosphorylated protein kinase peak I and RNA polymerase II mixture can indicate how many proteins are being phosphorylated, the relative size of these phosphorylated proteins, and which fraction--the protein kinase or polymerase fractions--the phosphorylated proteins originate from. Although this procedure cannot show whether the RNA polymerase molecule itself is being phosphorylated, it can indicate if proteins in the polymerase fraction are phosphorylated. PK I and RNA polymerase II are incubated with (γ-^{32}P)-ATP under assay conditions suitable for protein kinase activity; the reaction is stopped with SDS. The mixture is then subjected to polyacrylamide gel electrophoresis with the resolving gel of 5-15% polyacrylamide gradient, 0.1% SDS. The gel is stained with Coomasie Brilliant blue and the ^{32}P-autoradiograph accomplished by exposing X-ray film to the dried slab gel. The stained slab gel and corresponding ^{32}P-autoradiograph are shown in Figure 17. The Coomasie Brilliant blue stained gel shows that both the PK I and RNA polymerase II fractions each contain a multitude of different protein bands. However, the ^{32}P-autoradiograph shows that there are only three main protein bands (indicated by A, B or C in Figure 17) and approximately 3-4 minor bands which are selectively phosphorylated. The predominant phosphorylated band (C) is a small molecular weight

Slab Gel Electrophoresis and ^{32}P Autoradiography of Protein Kinase peak I plus RNA polymerase II

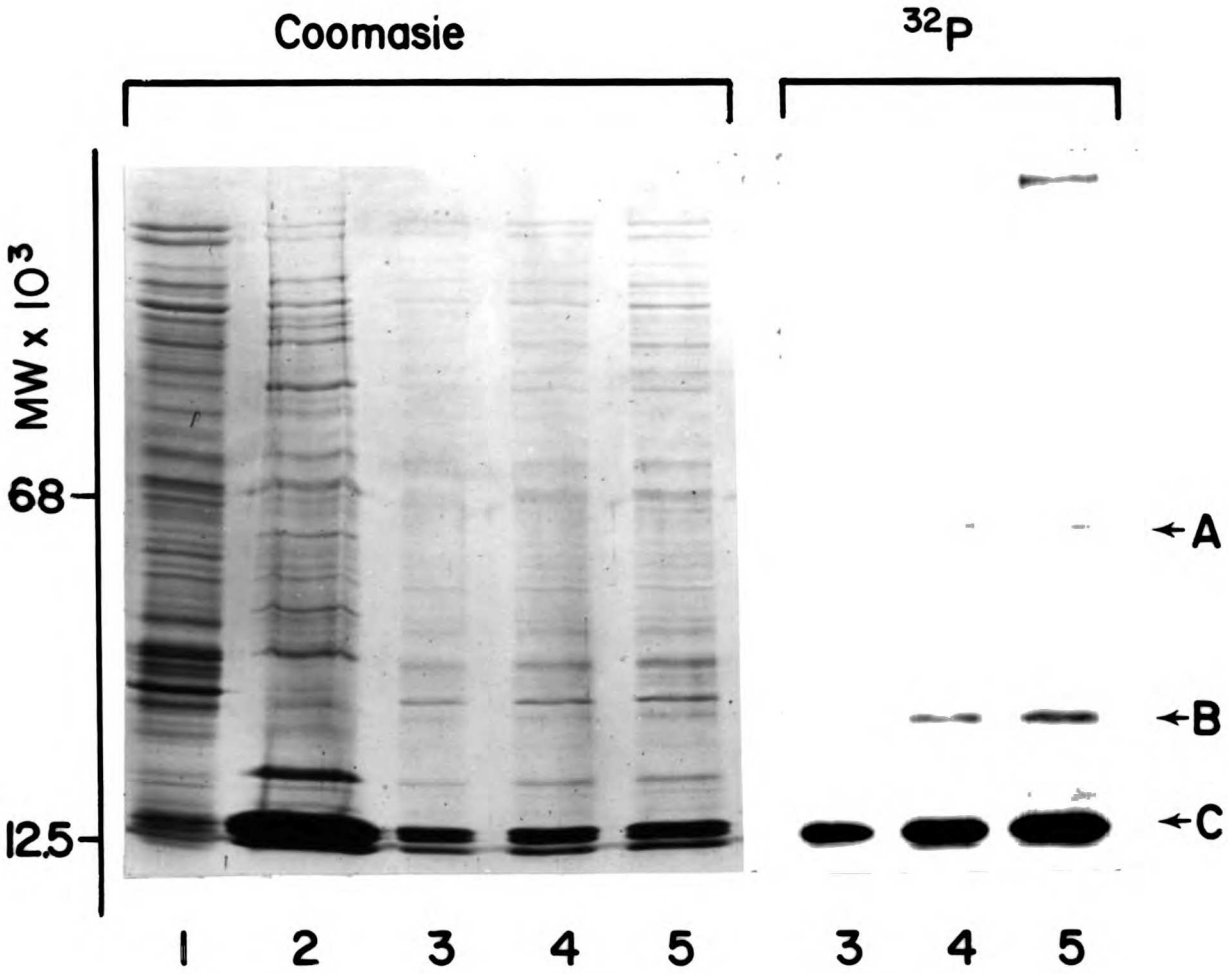


Figure 17.

Slab Gel Electrophoresis and ^{32}P Autoradiography of Protein Kinase peak I plus RNA polymerase II

Figure 17.

FIGURE 17. Polyacrylamide gel electrophoresis and ^{32}P-autoradiography of protein kinase peak I plus RNA polymerase II. The PK I + RNA polymerase II mixture is subjected to gel electrophoresis with the resolving gel 5-15% polyacrylamide gradient, 0.1% SDS and ^{32}P-autoradiography. The numbered slots represent: (1) PK I alone, (2) RNA polymerase alone, (3) PK I + RNA polymerase, 10 µl, (4) same as (3), 20 µl, (5) same as (3), 30 µl. Numbers (1) and (2) are non-radioactive samples; numbers (3), (4) and (5) contain ^{32}P. A, B and C indicate major phosphorylated protein bands.

protein (close to 12,000 mw) which probably originates from the RNA polymerase fraction. Due to the presence of multiple protein bands in both the protein kinase and RNA polymerase fractions in many regions of the slab gel, it is difficult to determine whether the remaining phosphorylated bands originate from the protein kinase or RNA polymerase fractions.

The RNA polymerase II enzyme has been shown in various tissue sources (61,62) to be composed of multiple subunits whose moleclar weights range from 11,000-200,000. Yamamoto and Takahashi (61) have found that the DNA-dependent RNA polymerase II from rat brain nuclei consists of nine subunits with molecular weights as low as 14,000 and 11,500. Based on this evidence it is possible that the low molecular weight (approximately 12,000) phosphorylated protein band seen in the ^{32}P-autoradiograph may be a subunit of the RNA polymerase II enzyme. However, this possibility cannot be determined until the RNA polymerase II enzyme is purified to homogeneity.

III. D. Involvement of cAMP in Chronic Morphine-Induced Increase in Nuclear Protein Kinase Peak I Activity

Existing evidence overwhelmingly indicates that cAMP is involved in morphine action, and in particular I.K. Ho *et al.* (34) have shown that a single icv. injection of cAMP accelerates the rate of morphine tolerance-dependence. If the increase in nuclear protein kinase peak I specific activity is related to the development of morphine tolerance-dependence, one would predict that an icv. injection of cAMP, which

accelerates the rate of morphine tolerance-dependence development, would also enhance the chronic morphine-induced increase in peak I activity. This result would also suggest that cAMP is involved in the increased protein kinase peak I activity seen during chronic morphine treatment. An investigation designed to test this conjecture was performed as follows. Mice were implanted with placebo or morphine pellets and injected icv. with 28 μg dibutyryl-cAMP, a more lipid soluble analog of cAMP. The mice were sacrificed 24 hours later and the nuclear protein kinases from the small dense nuclei were purified as described previously. The time of 24 hours of morphine pellet implantation was chosen because maximum morphine tolerance-dependence has not yet developed at this time point (5,54). The acceleration of tolerance-dependence by cAMP should be more easily demonstrated at 24 hours than at the time point of 72 hours when tolerance-dependence development is already nearly maximal. The results of this experiment are shown in Table 8.

It is seen that icv. injection of dibutyryl-cAMP alone increases the specific activity of both nuclear protein kinase peaks I and II by 74% and 65%, respectively. Chronic morphine treatment alone does not appear to alter the specific activity of peaks I and II. Although the mean specific activity in the morphine-saline groups is larger than than of the placebo-saline group, the difference between the two groups is not statistically significant due to the large variance in the morphine-saline group. Most importantly, the table shows that treatment with both chronic morphine and dbcAMP increases the specific activity of both peaks I and II by 170% and 105%, respectively. Treatment of

TABLE 8

Effect of Dibutyryl-cAMP and Chronic Morphine Treatment

on Nuclear Protein Kinase Peak I and II

Treatment Group	PEAK I Specific Activity ± S.E.M. (pmol phosphate incorp./ mg protein/5 min.)	PEAK I % Change	PEAK II Specific Activity ± S.E.M. (pmol phosphate incorp./ mg protein/5 min.)	PEAK II % Change
Placebo-saline	800 ± 100	100%	855 ± 100	100%
Placebo-dbcAMP	1394 ± 67*	174%	1454 ± 56*	170%
Morphine-saline	1317 ± 207	165%	1295 ± 198	151%
Morphine-dbcAMP	1902 ± 32*x	270%	1757 ± 17*x	205%

* $P < 0.05$, t-test, statistically significant relative to placebo-saline group.

x $P < 0.05$, t-test, statistically significant between the two groups indicated by the "x" symbol.

Mice were implanted with placebo or morphine pellets and injected icv. with dibutyryl cAMP, and sacrificed 24 hours later. Each value is the average specific activity from two experiments and each experiment consists of 75 mice per treatment group.

the animal with both chronic morphine and dibutyryl-cAMP raises the specific activity of peaks I and II to a much greater level than either treatment alone. Furthermore, simultaneous treatment of chronic morphine with dibutyryl-cAMP enhances peak I activity to a much larger extent than peak II activity; peak I activity is increased by 170% while that of peak II is increased by only 105%. The augmentation of peak II activity suggests that, in addition to peak I, peak II may possibly be involved in the development of the tolerant-dependent state. After 72 hours of chronic morphine treatment when maximum tolerance-dependence has developed (5,54), peak II shows no change in specific activity. However, after 24 hours of chronic morphine treatment with or without dibutyryl cAMP injection (Table 8), the specific activity of peak II appears to be increased. These data suggest that perhaps an alteration in peak II activity may be occurring at 24 hours chronic morphine treatment but by 72 hours its activity has returned to normal levels. Overall, these experiments indicate that chronic morphine and cAMP are synergistic and that they potentiate one another's effect on nuclear protein kinase specific activities. Thus, cAMP may be involved in the chronic morphine-induced increase in nuclear protein kinase activity.

Because cAMP has been reported (34) to accelerate the rate of morphine tolerance-dependence development and cAMP also enhances the chronic morphine-induced increase in nuclear protein kinase specific activity it is attractive to suggest that the increase in nuclear protein kinase specific activity may be related to the development of morphine tolerance-dependence. Therefore, to investigate if the degree of tolerance developed in the morphine-dbcAMP group is indeed greater

than in the other three groups, analgesia measured by mouse tail-flick latencies is measured for each treatment group. The animals are treated for 24 hours with chronic morphine with or without the injection of dibutyryl cAMP and the dose-response curves for morphine analgesia are measured in each treatment group as shown in Figure 18. It is seen that the morphine-dbcAMP group displays the largest degree of morphine tolerance compared with the other groups treated with only morphine or dibutyryl cAMP alone. The morphine-saline group displays an intermediate degree of morphine tolerance. The placebo-dbcAMP group appears to have developed no morphine tolerance when compared with the control group, placebo-saline. The morphine-dbcAMP group displays the largest degree of morphine tolerance, and at the same time possesses the greatest increase in nuclear protein kinase peak I and II specific activities. These results highly suggest that the increase in nuclear protein kinase specific activities may be correlated with morphine tolerance.

The predominant site of action of cAMP in mammalian cells is widely believed to be the cAMP-dependent protein kinases (60,63,64,65). Cyclic AMP activates the protein kinase by binding to the regulatory subunit of the kinase and subsequently the regulatory and catalytic subunits are dissociated to produce active catalytic subunit. 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 kinase peaks I and II from small dense nuclei of mouse brain are cAMP-independent and, therefore, cannot be regulated directly by cAMP. An alternative explanation involves a translocation of catalytic subunits of

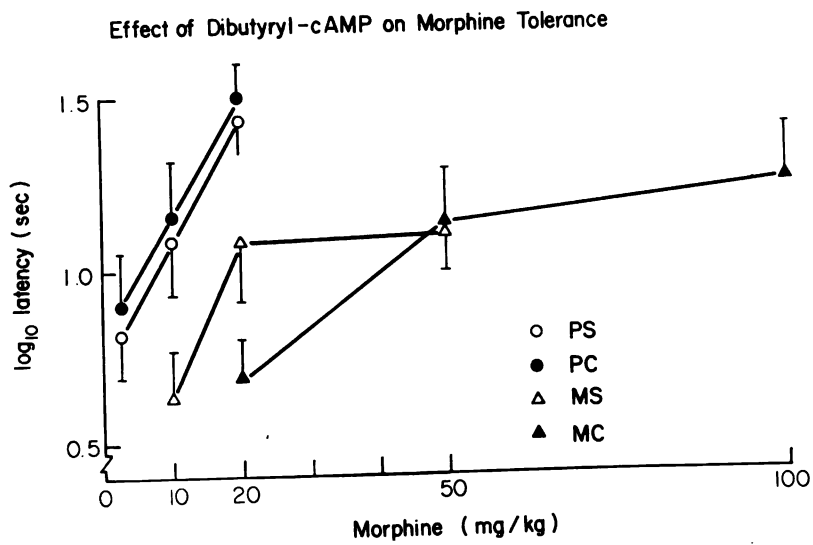


FIGURE 18. Effect of dibutyryl-cAMP on morphine tolerance. Mice were injected icv. with dibutyryl-cAMP or saline, implanted with a placebo or morphine pellet, and the degree of morphine tolerance measured 24 hours later by mouse tail-flick latencies. Each point represents the mean from 10 mice. The treatment groups are: placebo-saline (○—○), placebo-dbcAMP (●—●), morphine-saline (△—△), and morphine-dbcAMP (▲—▲).

protein kinase from the cytosol to the nucleus of the cell. This hypothesis of translocation of protein kinase has been proposed by Costa (66,67,68) and Jungman (69,70) and is schematically illustrated in Figure 19. When there is an elevation of cAMP level in the cell, it is 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 catalytic subunit is then translocated to the nucleus which results in augmentation of cAMP-independent nuclear protein kinase(s) and modification of gene expression through phosphorylation of nuclear proteins. Supportive evidence indicating that this phenomenon may be involved in morphine tolerance-dependence shows that icv. injections of dibutyryl-cAMP alone and in conjunction with chronic morphine treatment increase the specific activity of a cAMP-independent nuclear protein kinase from small dense nuclei of mouse brain.

Inhibition of the nuclear protein kinase activities by the heat stable protein kinase inhibitor characterized by Walsh et al. (89) would also support the idea that a translocation of protein kinase may be involved in morphine tolerance-dependence. A thermostable protein inhibitor which binds to and inhibits the activity of the catalytic subunits of cAMP-dependent protein kinase has been described in several tissues (89-92). Thus, if the nuclear protein kinases from mouse brain result from the translocation of catalytic subunits of cAMP-dependent protein kinase from the cytosol to the nucleus, the PK inhibitor should inhibit nuclear protein kinase activity. Nuclear protein kinases from mouse brain are assayed in the absence and presence of PK inhibitor

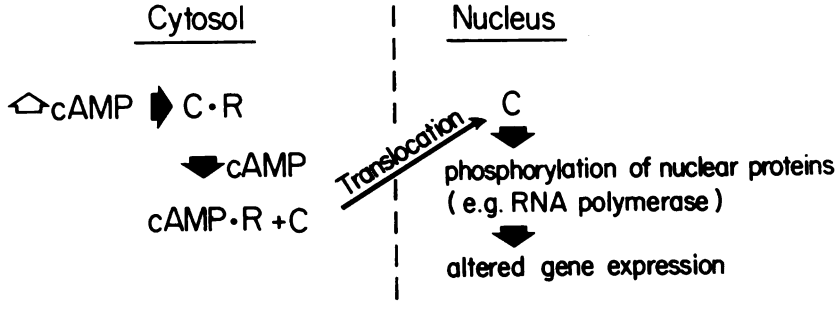


FIGURE 19. Translocation of Protein Kinase

from rabbit skeletal muscle. Cyclic AMP-dependent protein kinase from bovine heart is used as a positive control for the activity of the PK inhibitor. Results (Table 9) show that the PK inhibitor, at concentrations which inhibit the cAMP-dependent protein kinase from bovine heart, does not inhibit nuclear protein kinase peak I activity. However, nuclear protein kinase peak II activity is partially inhibited by the PK inhibitor. These data suggest that peak I is not a cAMP-dependent protein kinase and that a certain portion of the multiple forms of protein kinase which make up peak II may be cAMP-dependent protein kinase(s).

Investigations by Costa (66,67) demonstrating protein kinase translocation in rat adrenal medulla and C6 glioma cells show that basal nuclear protein kinase activity is not inhibited by the PK inhibitor. Only the increase in nuclear protein kinase activity during the new experimental condition which initiates protein kinase translocation is inhibited by the PK inhibitor. Thus, although basal nuclear protein kinase peak I activity is not inhibited by the PK inhibitor, it is not known if the increased activity seen during chronic morphine treatment may or may not be inhibited by PK inhibitor. Because it is not known if the isozyme responsible for the increased protein kinase activity is the same as those present in the unstimulated peak I fraction, it is possible that the stimulated peak I activity may respond differently to PK inhibitor compared to the unstimulated activity.

Inhibition of nuclear protein kinase peak II by the heat stable PK inhibitor and potentiation by dibutyryl cAMP of peak II activity 24 hours after chronic morphine treatment suggest the possibility that a translocation of protein kinase(s) in the peak II fraction may be

TABLE 9

Effect of Heat Stable PK Inhibitor on

Nuclear Protein Kinase Peaks I and II

Enzyme	PK Inhibitor (µl)	^{32}P-incorporated ± S.E.M. (cpm)	% Control Activity
cAMP-dependent protein kinase (from beef heart)	0	8863 ± 193	100%
	20	1309 ± 35	15%
	50	691 ± 55	8%
Nuclear protein kinase peak I	0	6394 ± 63	100%
	20	6349 ± 515	99%
	50	6778 ± 424	106%
Nuclear protein kinase peak II	0	1531 ± 179	100%
	20	1038 ± 19	68%
	50	1029 ± 14	67%

cAMP-dependent protein kinase from beef heart (Sigma) and nuclear protein kinase peaks I and II from mouse brain were assayed in the absence and presence of heat stable protein kinase inhibitor from rabbit skeletal muscle (provided by Dr. Ramachandran, Hormone Research Laboratory, University of California, San Francisco).

involved in the development of morphine tolerance-dependence. It is not known, however, if the increase in peak II activity is inhibited by PK inhibitor.

Further evidence showing a change in the ratio of cAMP-independent/ cAMP-dependent protein kinase activity as an index of activation of this enzyme (71) during chronic morphine treatment in different brain subcellular fractions would also support the idea of a translocation of protein kinase occurring during the development of morphine tolerance-dependence. Mice are chronically treated with morphine by pellet implantation for 0 to 60 hours. The PK activity ratio of

$$\frac{\text{cAMP-independent protein kinase activity}}{\text{cAMP-dependent protein kinase activity}}$$

is measured in four subcellular fractions from whole brain by assaying protein kinase activity in the absence and presence of 2×10^{-6} M cAMP. The protein kinase specific activity in the absence and presence of cAMP is also measured in each fraction. The four subcellular fractions are designated: P_1, which contains primarily nuclei, whole cells and other such large particles; P_2, which contains nerve-ending particles, mitochondria, myelin, and microsomes; S, which contains microsomes and other undefined particles; and M, which contains primarily microsomes. Presented in Figures 20 and 21, the results show that no significant differences occur in the PK activity ratio or the PK specific activity between the placebo and chronic morphine-treated groups at any time point. In Figure 21 there appears to be a placebo effect since the protein kinase specific activity from both placebo and morphine groups initially rises, decreases, and then levels off. However, the main

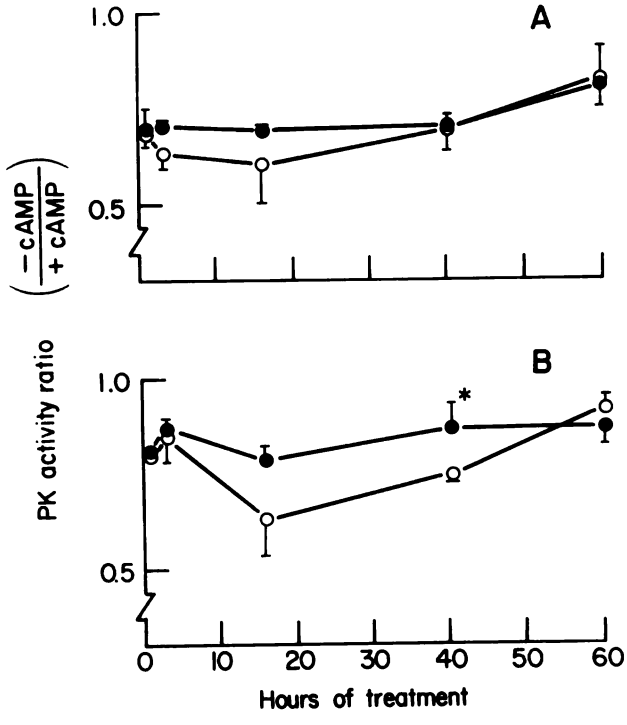


FIGURE 20. A, B. PK Activity Ratio in Different Brain Subcellular Fractions during Chronic Morphine Treatment.

The PK activity ratio is measured in P_1 (panel A) and P_2 (panel B) fractions from placebo (o—o) and chronic morphine-treated (●—●) mice.

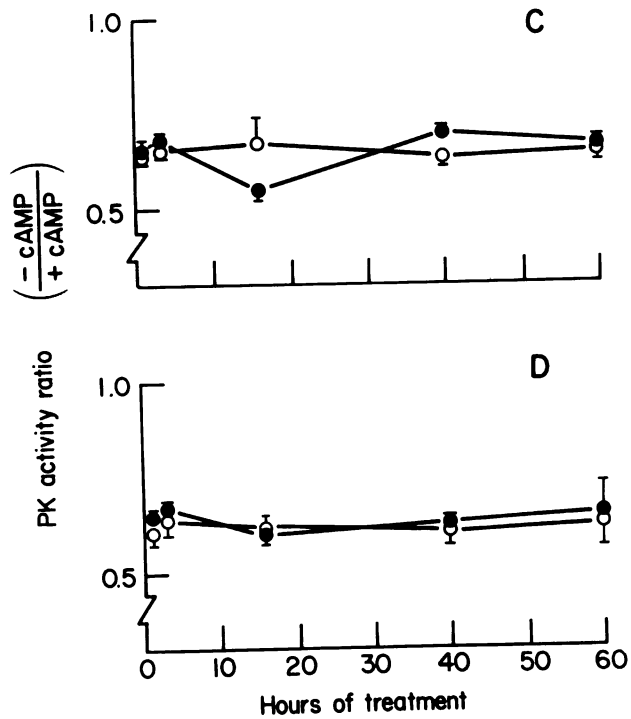


FIGURE 20. C,D. PK Activity Ratio in Different Brain Subcellular Fractions during Chronic Morphine Treatment.

The PK activity ratio is measured in S (panel C) and M (panel D) fractions from placebo (o—o) and chronic morphine-treated mice (●—●).

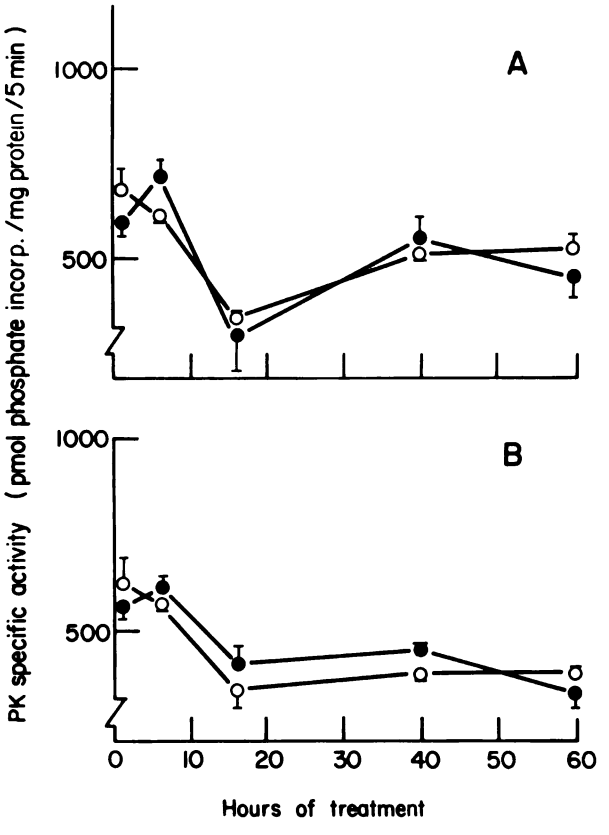


FIGURE 21. A, B. PK Specific Activity in Different Brain Subcellular Fractions during Chronic Morphine Treatment.

The protein kinase specific activity is measured in P_1 (panel A) and P_2 (panel B) fractions from placebo (o—o) and chronic morphine-treated mice (●—●).

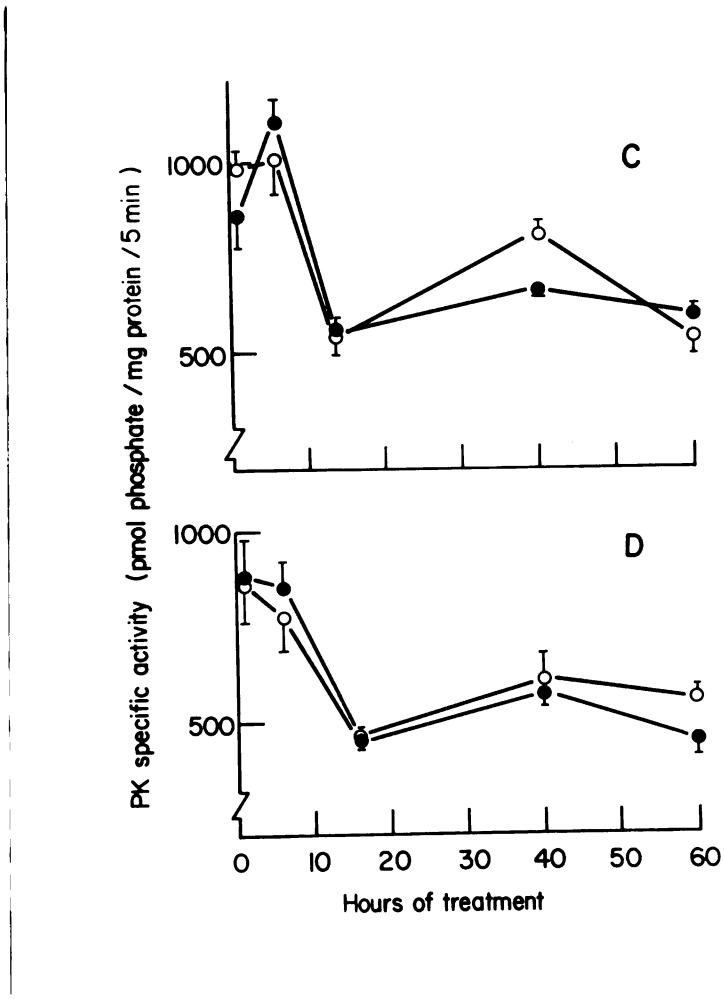


FIGURE 21. C, D. PK Specific Activity in Different Brain Subcellular Fractions during Chronic Morphine Treatment.

The protein kinase specific activity is measured in S (panel C) and M (panel D) fractions from placebo (○—○) and chronic morphine-treated mice (●—●).

point of the figure remains that no difference is seen between the two treatment groups.

It is not surprising that no differences are found in the protein kinase activity ratio or specific activity between the placebo and chronic morphine groups. The subcellular fractions are prepared from whole brain which is composed of a multitude of different cell types and different functional regions. A small significant change in the protein kinase activity in different regions of one particular cell type would probably be undetectable when measured in whole brain. Oligodendroglial cells comprise only approximately 17% of the cell population in most brain areas (84). The heterogeneity of the brain composition makes it impossible to study such a parameter as the protein kinase activity ratio in any cell population of the brain. Further study of the possible involvement of translocation of protein kinase in morphine tolerance-dependence development requires investigation in cell culture where a homogeneous population of cells can be utilized.

IV. DISCUSSION

Nuclear protein kinase from small dense nuclei of mouse brain has been partially purified by ammonium sulfate fractionation and phosphocellulose column chromatography. Elution of the phosphocellulose column by a linear NaCl gradient results in the resolution of two main peaks, peaks I and II, of cAMP-independent nuclear protein kinase activity. These peaks represent purification factors of 26- and 22-fold, respectively, relative to sonicated whole nuclei. This is believed to be the first partial purification of nuclear protein kinase from brain tissue.

Polyacrylamide slab gel electrophoresis under denaturing conditions of the purified nuclear protein kinase peaks I and II shows that both peaks contain numerous protein bands and, therefore, are only partially pure. It is seen that peak I contains several high molecular weight protein bands not seen in peak II, and peak II contains several low molecular weight protein bands not seen in peak I. The approximate molecular weight of protein kinase in peaks I and II is determined by molecular-exclusion chromatography. Sephadex G-200 column chromatography of PK I resolves the fraction into two components of protein kinase activity of molecular weights > 200,000 and 150,000-200,000. Sephadex G-25 column chromatography of PK II resolves the fraction into several peaks of activity all of which have a molecular weight of less than 5,000. In agreement with the results from the polyacrylamide gel electrophoresis, determination of protein kinase molecular weight shows that peak I has a much larger molecular weight than peak II.

During morphine tolerance-dependence the specific activity of

nuclear protein kinase peak I is significantly increased at 24 and 72 hours chronic morphine treatment relative to placebo controls. However, the specific activity of peak II is increased at 24 hours chronic morphine treatment and appears to return to normal levels by 72 hours chronic morphine. Thus, chronic morphine treatment does not change the activities of all the nuclear kinases at once, but results in a preferential alteration of only certain isozymes at particular time points during the development of morphine tolerance-dependence.

Chronic naloxone treatment, an opiate antagonist, does not appear to reverse the chronic morphine-induced increase in nuclear protein kinase peak I activity, although the large number of pellets involved in this experiment created a large placebo effect. Current literature on the multiplicity of opiate receptors (72,73,74,75) creates some doubt as to whether the morphine receptor responsible for the acute pain-killing effects is the identical receptor responsible for the development of morphine tolerance-dependence. The relationship between the receptors responsible for the acute and tolerant-dependent effects of morphine is not known at the present time. Studies by Schulz _et al._ (75) show that vas deferentia from mice treated chronically with D-Ala^2-D-Leu^5-enkephalin (DADL) are not cross-tolerant with sufentanyl and vice versa. Lee _et al._ (74) have shown that leu-enkephalin and met-enkephalin affect morphine analgesia in different manners. If multiplicity of opiate receptors is defined in terms of multiple recognition sites whose activation by specific opioids cause independent responses, the naloxone reversibility of every opiate-induced effect is less likely.

Oguri *et al*. (18) originally reported that the phosphorylation of chromatin proteins from small dense nuclei of mouse brain is increased by 60% during morphine tolerance-dependence. However, this study reports that the activity of nuclear protein kinase peak I is increased by only 26% during chronic morphine treatment. It appears that the increase in the activity of peak I cannot account for the total increase in phosphorylation of chromatin proteins observed by Oguri *et al*. (18). There are several explanations for this apparent discrepancy. Firstly, the isolated protein kinases in this study are assayed with exogenous casein as protein substrate. Casein is not the natural protein substrate for these protein kinases *in vivo*. Therefore, with the endogenous chromatin proteins as protein substrate, the activity of protein kinase peak I may be much greater than with casein as substrate. Secondly, there is the possibility that the chromatin proteins themselves are modified during morphine tolerance-dependence. A change in the protein substrates, in addition to the increased nuclear protein kinase activity, could further alter the total phosphorylation of the chromatin proteins. Thus, during morphine tolerance-dependence the elevation of nuclear protein kinase peak I specific activity can partially account for the increase in phosphorylation of chromatin proteins.

In view of the findings that both the nuclear protein kinase and homologous RNA polymerases from small dense nuclei of mouse brain are altered during chronic morphine treatment, and the hypothesis that protein kinase may regulate RNA polymerase activity, the functional interaction between these two enzymes may be modified during morphine tolerance-dependence. However, protein kinase regulation of RNA poly-

merase activity in brain tissue must first be demonstrated. RNA polymerase I and II activities are stimulated with increasing amounts of nuclear protein kinase peak I, and phosphorylation is also increased. These results suggest a correlation between phosphorylation and stimulation of RNA polymerases I and II activities by nuclear protein kinase. However, for RNA polymerase III, although phosphorylation is increased, no corresponding change in polymerase activity is seen. These data suggest that the nuclear protein kinase may be regulating RNA polymerase I and II activities through a phosphorylation mechanism.

Because of the extreme lability of both the purified protein kinase and RNA polymerase, it was not possible to carry out further experiments which would indicate whether the RNA polymerase molecule itself is being phosphorylated or some other molecule is phosphorylated, and whether this phenomenon is reversible by phosphoprotein phosphatase.

Studies on the Mg^{++} requirement of RNA polymerase II show that incubation of RNA polymerase II with nuclear protein kinase peak I lowers the optimum Mg^{++} concentration of the polymerase in the same manner as chronic morphine treatment alone. It appears that an _in vivo_ effect of chronic morphine treatment can be reproduced _in vitro_ by merely incubating the polymerase with protein kinase peak I. Furthermore, the Mg^{++} optimum is not lowered by peak II. This suggests that the change in Mg^{++} optimum during chronic morphine treatment may be occurring through changes in phosphorylation as a result of increased nuclear protein kinase peak I activity. Thus, it is likely that during chronic morphine treatment the alterations in nuclear protein kinase and RNA polymerase activities are not occurring independently of one

another, but the functional interaction between these two enzymes may be modified.

Existing evidence (34,37,38,39,40,41) overwhelmingly indicates that cAMP is involved in morphine tolerance-dependence but the exact mechanism of cAMP involvement is unknown at the present time. Investigations described in this report show that intracerebroventricular injection of dibutyryl-cAMP enhances the chronic morphine (after 24 hours) induced increase in nuclear protein kinase peak I and II specific activities. 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 increases in nuclear protein kinase peak I and II activities seen at 24 hours chronic morphine treatment 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. These findings give important implications as to the possible biochemical mechanism of morphine tolerance-dependence development, which will be further discussed.

At the level of transcription, several biochemical alterations in small dense nuclei of mouse brain have been shown to occur during morphine tolerance-dependence: (1) chromatin template activity is increased, (2) nuclear protein kinase peak I specific activity is increased, (3) RNA polymerase I specific activity is decreased, the optimum Mg^{++} concentration for RNA polymerase II and the Mn^{++}/Mg^{++}

ratios of RNA polymerases II and III are altered, and (4) the increase in nuclear protein kinase peak I specific activity may result in a shift in the Mg^{++} optimum of RNA polymerase II through a phosphorylation mechanism. Modulation of nuclear systems implies some functional alteration in macromolecular synthetic processes, and these are indeed found during morphine tolerance-dependence. Protein synthesis is increased during chronic morphine treatment (58), although no unique "morphine tolerance-dependence protein" has been found. However, changes in the isozyme functions of the aminoacyl-tRNA transferases, an important class of enzymes involved in protein translation, are found during chronic morphine treatment. The optimum Mg^{++}/ATP ratio and temperature of the aminoacyl-tRNA transferases for phenylalanine, tryptophan, lysine and leucine are altered during morphine tolerance-dependence (58).

The appearance of these new isozymes of key regulatory enzymes involved in transcription and translation with modified specific activities and different optimum co-factor requirements suggests that they are being adapted in response to new conditions in their local environment during morphine tolerance-dependence. In this hypothesis of biochemical adaptation, certain biochemical systems are altered in specific manners during chronic morphine treatment, but the net effect of the total of these adaptations is the normal behavior of the animal in the continued presence of morphine, that is, the tolerant-dependent state. Upon removal of the drug, the previously altered conditions return to their pre-drug states. The biochemical systems which had adapted can no longer function normally and the withdrawal syndrome is observed.

As illustrated in Figure 22, discussion of changes in regulatory enzymes at the level of transcription and translation led to the hypothesis of a biochemical adaptation occurring during morphine tolerance-dependence. Alterations at the level of transcription can result in modified gene expression, and subsequently the synthesis of new proteins is achieved which provide the basis for the biochemical adaptation during morphine tolerance-dependence. However, none of the processes are affected by morphine in vitro, indicating that these changes are indirect effects of the drug. What possibly may be happening during the continued presence of the drug which leads to the alterations observed in transcription and translation? Or, in other words, what steps may be involved between the morphine-receptor and the manifestation of the changes at the level of transcription and translation? The potentiation by cAMP of the chronic morphine-induced increase in nuclear protein kinase peaks I and II specific activities after 24 hours chronic morphine treatment suggests that cAMP may be involved as a mediator between the morphine-receptor and alterations at the level of transcription seen during morphine tolerance-dependence. Cyclic AMP is generally believed to stimulate cAMP-dependent protein kinases by dissociating the regulatory and catalytic subunits to result in active catalytic subunit. 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 kinase peaks I and II are cAMP-independent and, therefore, cannot be regulated directly by cAMP. An alternative explanation may involve a translocation of catalytic subunits of protein kinase

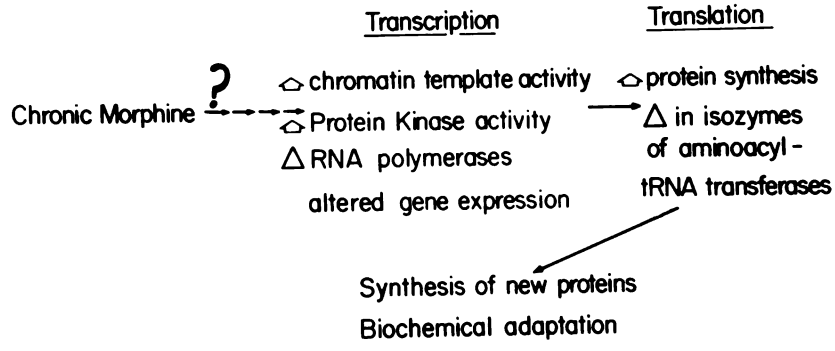


FIGURE 22. Alterations at the Level of Transcription and Translation during Morphine Tolerance-Dependence.

from the cytosol to the nucleus of the cell.

Costa's laboratory (66,67) has demonstrated the translocation of protein kinase to the nucleus of adrenal medulla cells during the induction of tyrosine hydroxylase promoted by cold stress. Jungman et al. (69) provide evidence indicating that gonadotropin induces a cAMP-dependent translocation of cytoplasmic protein kinase to nuclear acceptor sites in ovarian tissue. Basically, in this phenomenon of translocation when there is an elevation of cAMP in the cell, the cAMP binds to the regulatory (R) subunit of a cAMP-dependent cytosolic protein kinase and dissociates the regulatory (R) and catalytic (C) subunits of the kinase. The catalytic subunit is then translocated to the nucleus which results in modification of gene expression through phosphorylation of nuclear proteins. This scheme is illustrated in Figure 19.

This sequence of events is proposed to be initiated by cAMP and there is much evidence to indicate a role for cAMP in morphine tolerance-dependence. Intracerebroventricular injection of dibutyryl cAMP in mice has been shown to accelerate the development of morphine tolerance-dependence (34) and in the neuroblastoma-glioma hybrid cell a model of cAMP involvement in morphine tolerance-dependence is proposed by Sharma and Nirenberg (37). Morphine has also been shown to modify adenylate cyclase activity, the enzyme synthesizing cAMP (38,39,41,42,43).

With regard to the development of morphine tolerance-dependence, the acceleration of morphine tolerance-dependence by cAMP implies that during the development of tolerance-dependence there is an elevation of cAMP in certain brain regions. This new condition of elevated cAMP may

result in a number of responses, one of which may be the dissociation and translocation to the nucleus of catalytic subunits of protein kinase. If this scheme is operating during morphine tolerance-dependence development, one would predict (1) an increase in cAMP-independent nuclear protein kinase specific activity during morphine tolerance-dependence, (2) cAMP should enhance the chronic morphine-induced increase in nuclear protein kinase activity, and (3) the increase in nuclear protein kinase should correlate with the degree of tolerance-dependence developed. All three of these predictions have been borne out by experimental evidence presented in this dissertation. Specifically, nuclear protein kinase peak I specific activity is increased at 24 and 72 hours chronic morphine treatment. Peak II activity is increased at 24 hours chronic morphine treatment but returns to normal levels by 72 hours. Dibutyryl cAMP enhances the increases in the specific activities of peaks I and II. Furthermore, the increases in peak I and II specific activities appear to be correlated with the degree of morphine tolerance, as measured by mouse tail-flick latencies.

If nuclear protein kinase peaks I and II result from translocation of catalytic subunits of protein kinase from the cytosol to the nucleus of the cell, they should be inhibited by the heat stable PK inhibitor which has been demonstrated (89) to bind to and inhibit the activity of catalytic subunits of cAMP-dependent protein kinases. Results indicate that peak II but not peak I is inhibited by the PK inhibitor. Based on these findings, it is possible that peak II but not peak I may become associated with the nucleus through a translocation mechanism. It should be noted that at this point, it is not known if the increased

nuclear protein kinase activities are inhibited by the PK inhibitor.

Although these results support the idea of a translocation of protein kinase occurring during morphine tolerance-dependence, they do not prove that this is happening. Detailed studies on the dissociation of regulatory and catalytic subunits of protein kinase and subsequent directed translocation of catalytic subunits to the nucleus must be undertaken. However, because of the heterogeneity of cell types in the brain, these further studies should be performed in cell culture where the cellular homogeneity can allow more precise and detailed investigation of morphine-induced translocation.

Other possibilities may explain how cAMP may be involved in the increased nuclear protein kinase activity during the development of morphine tolerance-dependence. Cyclic AMP, either directly or indirectly, may stimulate protein synthesis resulting in the production of new complexes of protein kinase enzyme. Increased protein synthesis has been observed during morphine tolerance-dependence development (58). Changes in nuclear phosphoprotein phosphatase activities are probably not involved since phosphatase activity in chromatin from small dense nuclei of mouse brain is not altered during chronic morphine treatment (18). In addition, cAMP may increase nuclear protein kinase activity through an enhancement of the processing of possible protein precursors of the protein kinase enzyme.

Overall, a proposed biochemical scheme for the development of morphine tolerance-dependence which takes into account observed changes found at the level of transcription and translation and the involvement of cAMP and protein kinase is illustrated in Figure 23. It is proposed

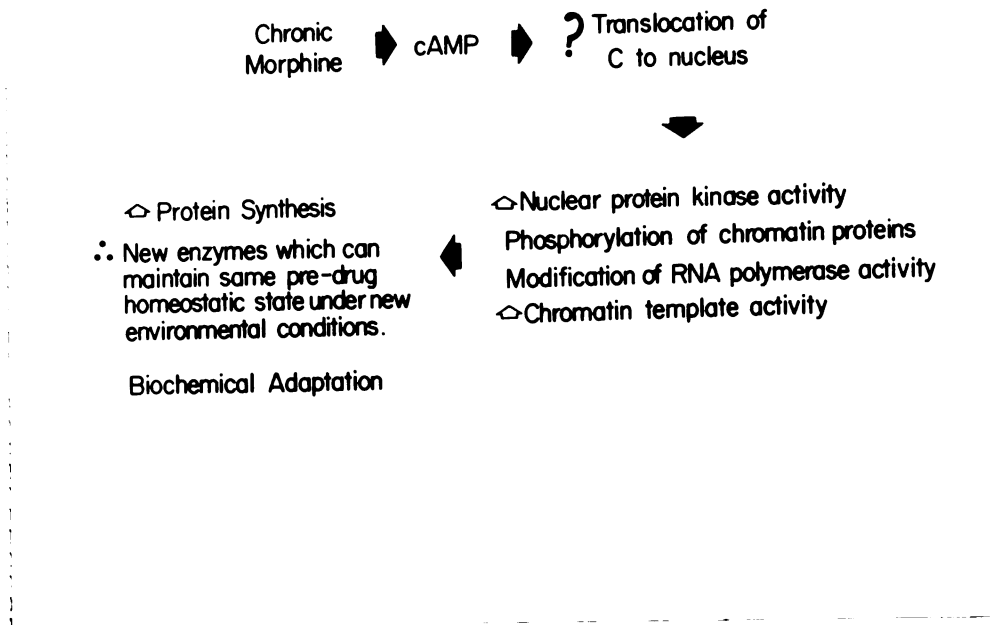


FIGURE 23. The Role of Nuclear Protein Kinase in the Development of Morphine Tolerance-Dependence

that during chronic morphine treatment an elevation of cAMP in some particular cell type(s) will induce an increase in nuclear protein kinase activities. Whether or not this induction involves a translocation of protein kinase remains to be discerned by further experiments. Other possibilities whereby cAMP may increase cAMP-independent nuclear protein kinase may involve induction of protein synthesis resulting in the production of new molecules of protein kinase or may involve enhanced processing of protein precursors of the protein kinase enzyme. Through phosphorylation of chromatin proteins, RNA polymerase activity is modified to increase chromatin template activity and results in altered gene expression. From the increase in synthesis of new RNA species, it follows that protein synthesis is increased. This results in the production of new enzymes which can maintain the same pre-drug homeostatic state under new environmental conditions, or, in other words, results in biochemical adaptation.

This sequence of events is proposed to be initiated by a chronic morphine-induced elevation of cAMP; however, many will argue that morphine inhibits the activity of adenylate cyclase and decreases the level of cellular cAMP (39-42,46). One must remember that these studies investigated the acute effects of morphine. The cell will try to compensate for this acute effect by raising its cAMP concentration to pre-drug levels, thereby adapting to the new condition of continued presence of morphine. It is during this compensatory rise in cAMP level when a stimulation of nuclear protein kinase activities may be initiated and when tolerance-dependence may be developing. In Sharma and Nirenberg's (37) model of cAMP involvement in morphine tolerance-

dependence, morphine treatment initially lowers the cellular level of cAMP but with continued drug treatment the cAMP level returns to normal, and they propose that this period of normal cAMP level represents the tolerant-dependent state. Thus, in their model it is during the compensatory increase in cAMP level when a stimulation of nuclear protein kinase may be initiated.

All effects of morphine are presumed to occur through interaction of the morphine drug molecule with the morphine receptor. The morphine-receptor complex is then believed to initiate events resulting in the physiological effects observed during morphine treatment. The induction of morphine tolerance-dependence is, thus, assumed to occur through the morphine receptor. Because the increase in nuclear protein kinase activity during morphine tolerance-dependence is demonstrated in small dense nuclei which are primarily oligodendroglial in origin, many will question the role of glial cells in tolerance-dependence when morphine receptor binding has only been demonstrated in neuronal (76,77,78,79) and not glial cell membranes. Biochemical isolation techniques involving subcellular fractionation of brain tissue have successfully produced a fraction enriched in synaptic plasma membranes (56) and specific morphine binding has been demonstrated in this fraction (77,80). However, no biochemical isolation procedure has been developed which can provide a fraction greatly enriched in glial membranes. Thus, in actuality, it is not known how much or what kind of morphine binding glial membranes may possess. Furthermore, recent evidence indicating the multiplicity of opiate receptors (72,73,74,75) produces some question as to whether the receptors involved in acute and chronic morphine effects may or may

not be the same. In addition, because glial cell function is believed to be required for the support and maintenance of brain homeostasis (81,82,83,84,85) it is more than likely that these cells are involved in morphine tolerance-dependence.

The proposed biochemical scheme (Figure 23) of the involvement of cAMP and protein kinase in the development of morphine tolerance-dependence provides clinical implications on the interactions of commonly ingested substances such as caffeine with heroine or morphine addiction and physical dependence. The common beverage coffee contains the compound caffeine which can raise cellular cAMP levels by inhibiting phosphodiesterase, the enzyme which metabolizes cAMP. Studies by Jones et al. (86) show that human subjects pretreated with caffeine displayed significantly more opiate withdrawal-like characteristics upon naloxone adminstration, suggesting that there appears to be a mild, naloxone precipitated, quasi-opiate withdrawal syndrome, as Collier (87) has demonstrated in rats. A result from these studies is the suggestion that heroin addicts should drink milk instead of coffee (86).

In summary, based on evidence presented in this dissertation and from the current literature, two hypotheses are presented in an attempt to explain what biochemical mechanism may be occurring during the development of morphine tolerance-dependence. These are (1) that cAMP may be responsible for the increased nuclear protein kinase activities during morphine tolerance-dependence which may involve a translocation of protein kinase from the cytosol to the nucleus of the cell, and (2) that altered specific activities and modified co-factor requirements

of key regulatory enyzmes suggest that a biochemical adaptation is occurring during morphine tolerance-dependence.

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