# IN VIVO ACTIVATION OF cAMP-DEPENDENT PROTEIN KINASE BY AMINOPHYLLINE AND 1-METHYL, 3-ISOBUTYLXANTHINE

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SUMMARY

Aminophylline and 1-methyl, 3-isobutylxanthine, inhibitors of phosphodiesterases, caused an increase in the cyclic AMP level and an elevation in the activity of cyclic AMP-dependent protein kinase in the liver of the rat within 10-20 min of administration. The activation of cyclic AMP-dependent protein kinase appeared to be a more accurate measure of cyclic AMP-dependent events than cyclic AMP concentration and was statistically more reliable. After Sephadex chromatography, the protein kinase yielded the same activation pattern as measurable in the crude supernatant of liver.

Methylxanthine derivatives have been shown to inhibit phosphodiesterase activity in vitro and in vivo (1,2). The inhibition of cellular phosphodiesterases causes an elevation of cAMP in the cell, and thus should activate cAMP-dependent protein kinase. Further, the efficacy of the inhibitor would be expected to relate to the amount of the activation of cAMP-dependent protein kinase.

Increases in cellular cAMP have been demonstrated to activate cAMP-dependent protein kinase (3,4,5), although the activity of the kinase has not been studied after the administration of methylxanthine derivatives. The activation mechanism involves a reaction of cAMP with the R subunit of the R-C complex and the release of the active C subunit (4). Present evidence indicates that all the effects of cAMP are mediated through its ability to control the level of the free C subunit (4,5), although this is still open to question.

In this paper, we report for the first time the activation of cAMP-dependent

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Abbreviations used: cAMP, cyclic AMP; MIX, 1-methyl, 3-isobutylxanthine; R-C, regulatory catalytic form of cAMP-dependent protein kinase; R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase.

protein kinase in rat liver in response to methylxanthine derivatives and that the extent of activation of cAMP-dependent protein kinase correlates with the potency of the methylxanthine derivatives used. Further, it has been difficult in the liver to stabilize the C unit of the kinase because the liver contains a large amount of rapidly reassociating kinase. We have been able to stabilize the protein kinase utilizing G-100 Sephadex chromatography in the presence of 0.1M KCl and 2 mg/ml of bovine serum albumin. Because of the high degree of reproducibility and reliability of cAMP-dependent protein kinase assays as opposed to the wide variation in reliability of cAMP measurements, it would appear that the extent of activation of cAMP-dependent protein kinase is a better measure of a cAMP-dependent mechanism than the changes in the cellular concentration of cAMP.

## MATERIALS AND METHODS

Sprague-Dawley rats (male, 100-125g) were used in all experiments. Rats were injected with aminophylline in 0.9% saline (i.p., 200 μmol/kg) or MIX in 5:1 0.9% saline-ethanol (v:v, i.p., 40 μmol/kg). Controls were injected with the appropriate solvent. All rats were maintained in a room with a 0600-1800 hour photoperiod. To avoid the possibility of diurnal enzyme changes, all rats were killed at 1030 hours ± 30 min.

To assay cAMP concentration, a section of each liver (200-250 mg) was removed and frozen solid on dry ice within 10 sec of sacrifice. The liver tissue was weighed rapidly and homogenized in 4 ml/g of 0.4N perchloric acid. The cAMP was separated from the other acid-soluble nucleotides by chromatography on aluminum oxide and Dowex columns as described by Mao and Guidotti (6). The concentration of cAMP was determined by its ability to activate a cAMP-dependent protein kinase isolated from bovine heart muscle (7).

In order to measure the activity of cAMP-dependent protein kinase, a 500-mg portion of each liver was homogenized rapidly in 3 ml/g of 0.01M sodium-potassium phosphate buffer, pH 6.5, containing 10mM EDTA, 10mM aminophylline, and 5mM NaF. The homogenate was centrifuged for 20 min at 50,000 x g; the supernatant solution was removed and used as the source of enzyme in the following procedure. Cyclic AMP-dependent protein kinase activity was determined by measuring the protein kinase activity with and without the addition of exogenous cAMP (8). The assay mixture contained 100 μg of calf thymus histone (Schwarz/Mann), 0.01M phosphate buffer, pH 6.5, 0.025M $Mg(Ac)_2$, 5mM NaF, 4.14 x $10^{-6}$M [$\gamma$-$^{32}$P] ATP (6 Ci/mM, New England Nuclear), 0.025 ml of a 1:5 dilution of the original enzyme extract, and either 0.01 ml of $H_2O$ or 0.01 ml of (2 x $10^{-5}$M) cAMP solution, in a total volume of 0.2 ml. The reaction was allowed to proceed for 5 min at 30 °C and then was terminated by the addition of 50 μl of 50% trichloroacetic acid containing 5mM ATP. The precipitate was collected on a cellulose nitrate filter (HAWPO 2412, Millipore) and washed with cold 5% trichloroacetic acid. The filters were dried and then counted in an Omnifluor-toluene cocktail. The activity of protein kinase was expressed by the ratio of $^{32}$P incorporated into histones in the absence of cAMP(-cAMP) and in the presence of cAMP(+cAMP).

In order to substantiate the reliability of this ratio as an index of cAMP-

dependent protein kinase activity, Sephadex chromatography was employed to separate the subunits (3). An aliquot (250 µl) of the supernatant solution was applied to a G-100 Sephadex chromatography column (2 cm x 32 cm) equilibrated with 0.05M phosphate buffer containing 5mM NaF, 10mM EDTA, 0.1M KCl, and 2 mg/ml bovine serum albumin (Armour Pharmaceutical Co.). Seventy fractions (0.85 ml each) were collected. Fractions were assayed for cAMP-dependent protein kinase activity as described above.

## RESULTS AND DISCUSSION

Aminophylline administration resulted in an almost 2-fold increase in the cAMP concentration within 1 hr (Fig. 1). The administration of MIX resulted in a more rapid and greater increase, and the cAMP levels remained elevated longer than after administration of aminophylline. This is in agreement with previous reports that MIX is a more potent inhibitor of phosphodiesterases than aminophylline (1). The increases in the cAMP level corresponded well with the increases in cAMP-dependent protein kinase activity (Fig. 2). Within 10 min of MIX administration, cAMP-dependent protein kinase was 152% of control, whereas 20 min after administration of aminophylline, cAMP-dependent protein kinase was only 120% of control. Again, protein kinase was activated more rapidly, attained a higher catalytic activity, and remained elevated longer following administration of MIX than with aminophylline.

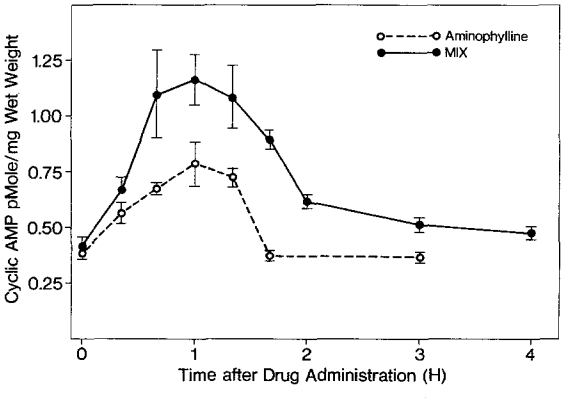


Figure 1. Changes in hepatic cAMP levels in the rat following the administration of aminophylline (200 µmol/kg, i.p.) and 1-methyl, 3-isobutylxanthine (40 µmol/kg, i.p.). Cyclic AMP levels were determined by measuring the ability of the purified nucleotide (6) to activate a cAMP-dependent protein kinase (7). Each point represents the mean ± S.E.M. of 4 different animals.

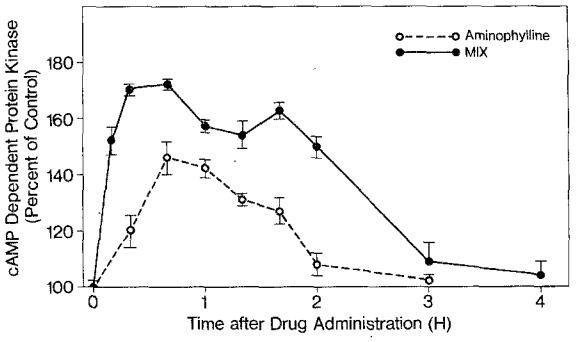


Figure 2. Changes in cAMP-dependent protein kinase activity in rat liver following the administration of aminophylline (200 µmol/kg, I.P.) and 1-methyl, 3-isobutylxanthine (40 µmol/kg, i.p.). Cyclic AMP-dependent protein kinase activation was determined by measuring the activity with and without the addition of cAMP. The change in this ratio (-cAMP/+cAMP) with time was then expressed as percent of control (see Table 1 for control values). Each point represents the mean ± S.E.M. for duplicate determinations of 4 or more animals.

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Figure 3 shows the elution profile of cAMP-dependent protein kinase 40 min after either the administration of MIX or ethanol saline solution only. In Figure 3A, about 50% of the cAMP-dependent protein kinase was in the R-C form (fractions 27-37) and 50% in the C form (fractions 39-59). The relative amounts of R-C and C in the control directly correlate with the -cAMP/+cAMP ratio of 0.54 (Table 1). Within 40 min of MIX administration, there was a change in the profile (Fig. 3B). There was less cAMP-dependent protein kinase in the R-C form and a corresponding increase in the C form. These changes are due to an elevation in endogenous cAMP which binds to the R unit of the R-C form of the kinase and results in the release of C (4). Therefore, the addition of exogenous cAMP to the assay is incapable of further dissociating the small amount of R-C form that remains after MIX administration. This profile correlates well with the -cAMP/+cAMP ratio of 0.93 (Fig. 3, Table 1). This indicates that the use of the proper salt concentration as well as the addition of a small amount of bovine serum albumin to the buffer system (see Methods) prevents the C unit from reassociating during the chromatography procedure. This is an important point since liver cAMP-dependent protein kinase rapidly reassociates.

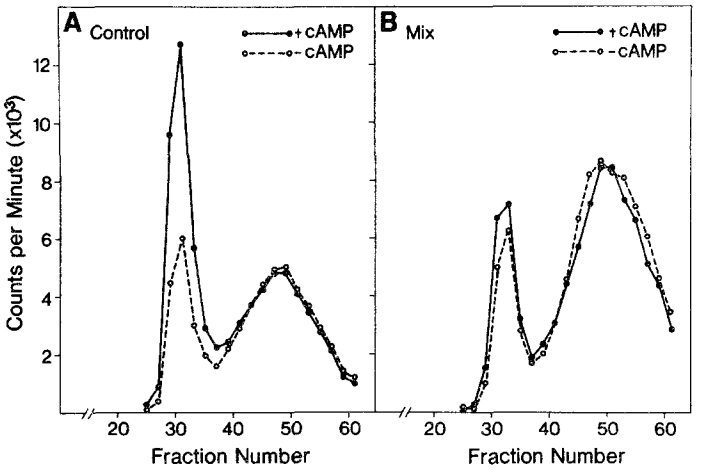


Figure 3. Sephadex G-100 chromatography of rat liver protein kinase 40 min after the administration of either ethanol/0.9% saline (1:5, i.p.) [control, A] or of 1-methyl, 3-isobutylxanthine (40 μmol/kg, i.p.) [MIX, B]. 250 μl of the pooled supernatant solution (see Materials and Methods) was applied to a Sephadex column (32 cm x 2 cm) equilibrated with 0.05M sodium-potassium phosphate buffer containing 10mM EDTA, 5mM NaF, 0.1M KCl, and 2 mg/ml of bovine serum albumin. The fractions were assayed in the absence and presence of exogenous cAMP. The regulatory-catalytic form of cAMP-dependent protein kinase is found in fractions 27-35, and the catalytic form of protein kinase is found in fractions 39-57.

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This technique should be useful in the further study of the properties of liver protein kinases.

From these results, it is clear that aminophylline and MIX are useful in studying the activation of cAMP-dependent protein kinase(s). Further, the activation of cAMP-dependent protein kinase appears to be a better measure of cAMP-dependent events since the protein kinase was activated maximally within 30 min of MIX (Fig. 2), whereas cAMP following MIX was not maximal until 60 min. The possibility exists that the continued rise in cAMP may serve to maintain protein kinase in the C form.

Measurement of cAMP-dependent protein kinase is more reproducible than cAMP determinations as the standard error was much larger for cAMP determinations than for protein kinase. This may be due to the rapid turnover of cAMP. This cyclic nucleotide has been shown to increase rapidly in the blood after

TABLE 1. Effects of Aminophylline and 1-methyl, 3-isobutylxanthine on cyclic AMP Concentration and on the Activity of cyclic AMP-dependent Protein Kinase of Rat Liver.

| | AMINOPHYLLINE | | | 1-METHYL, 3-ISOBUTYLXANTHINE | | |
|---|---|---|---|---|---|---|
| Time (min) | cAMP (pmol/mg, wet weight) | cAMP-Dependent Protein Kinase $\frac{-cAMP}{+cAMP}$[a] | % of Control | cAMP (pmol/mg, wet weight) | cAMP-Dependent Protein Kinase $\frac{-cAMP}{+cAMP}$[a] | % of Control |
| 0 | 0.421±0.014 | 0.45±0.01 | 100±2 (N=11) | 0.384±0.033 | 0.54±0.01 | 100±2 (N=22) |
| 10 | --- | --- | --- | --- | 0.82±0.04 | 152±5 (N=6) |
| 20 | 0.573±0.044 | 0.54±0.03 | 120±6 (N=6) | 0.662±0.051 | 0.92±0.01 | 170±2 (N=6) |
| 40 | 0.675±0.022 | 0.66±0.04 | 146±6 (N=9) | 1.100±0.200 | 0.93±0.02 | 172±2 (N=8) |
| 60 | 0.788±0.100 | 0.64±0.02 | 142±3 (N=9) | 1.170±0.130 | 0.85±0.01 | 157±2 (N=13) |
| 80 | 0.725±0.032 | 0.54±0.01 | 131±2 (N=4) | 1.080±0.150 | 0.83±0.04 | 154±5 (N=9) |
| 100 | 0.375±0.013 | 0.57±0.03 | 127±5 (N=6) | 0.895±0.040 | 0.88±0.03 | 163±3 (N=8) |
| 120 | --- | 0.49±0.02 | 108±4 (N=5) | 0.617±0.038 | 0.81±0.03 | 150±4 (N=14) |
| 180 | 0.325±0.01 | 0.46±0.01 | 102±2 (N=4) | 0.519±0.022 | 0.59±0.04 | 109±7 (N=6) |
| 240 | --- | --- | --- | 0.478±0.026 | 0.56±0.03 | 104±5 (N=6) |

[a] $\frac{-cAMP}{+cAMP}$ ratio is the cpm of $^{32}P$-phosphate incorporated into histone by cAMP-dependent protein kinase with and without the addition of exogenous cAMP.

tissue stimulation (9) and is metabolized by several forms of phosphodiesterases (10). Variability in the assay for cAMP could also result from the complicated purification procedure required to assess its concentration.

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