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# Lack of Modulation by Phenobarbital of Cyclic AMP Levels or Protein Kinase A Activity in Rat Primary Hepatocytes

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**ABSTRACT.** Results of previous studies have substantiated a negative modulatory role for cyclic AMP (cAMP) and protein kinase A (PKA) dependent processes on the phenobarbital (PB) induction response in hepatocytes. The current study was conducted to further examine the potential role of second messenger pathways in the initial phases of induction, specifically addressing the effects of PB on the expression of intracellular cAMP levels and associated PKA activity. Using a highly differentiated primary rat hepatocyte system, cells were exposed to PB for various intervals (30 sec to 48 hr), and levels of intracellular cAMP and subsequent PKA activity were determined. Although PB markedly induced CYP2B expression, exposure to this agent produced no detectable increases in cAMP levels and PKA activity at any of the times examined. These results demonstrated that the initial events stimulated by PB in rat hepatocytes do not include alterations of cAMP levels or associated PKA activities. BIOCHEM PHARMACOL **58**;7:1109–1114, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** phenobarbital; cAMP; protein kinase A; primary hepatocyte; CYP2B; cytochrome P450

CYP† enzymes metabolize a broad range of endogenous and exogenous lipophilic compounds including drugs, steroids, and polyaromatic hydrocarbons [1]. Molecular mechanisms and the roles that signaling intermediates may play in determining PB induction of the *CYP2B* genes are not well understood, although it is well established that this process involves activation of *de novo* transcription [2, 3].

The effects of PB on the liver are diverse and include cellular hypertrophy and tumor promotional effects in rodents, as well as gene induction [4, 5]. In general, most liver processes are controlled by a variety of intracellular messengers and receptors that act as transducers for extracellular stimuli. Activation of protein kinases, through cyclic nucleotide stimulation, underscores many of the events regulating cellular function [6]. Earlier studies [7–9] examining the effects of PB on CYP gene induction and tumor promotion, suggested roles for the involvement of cAMP-dependent pathways in these processes. However, measures of nonspecific endpoints have made interpretation of these data difficult. Our laboratory has established a primary hepatocyte culture system that is highly permissive for differentiated hepatocyte function, CYP expression, and PB induction [10–12]. Using this defined system, dissection of signaling pathways is possible, and a striking inhibition of PB-mediated CYP gene induction by cAMP and PKA activators has been demonstrated [13]. Other investigators have also begun to investigate the effects of signaling intermediates on PB induction [14, 15].

Our goal in the present study was to examine changes in second messenger systems that occur upon initial exposure to PB. Although recent reports [13–18] suggested a requirement for phosphorylation and dephosphorylation events in controlling PB-mediated CYP expression, these studies induced cells in the presence of agents that modulate signal transduction processes. Alterations in signaling pathways immediately following cellular exposure to PB have not been investigated. In this study, we examined cAMP levels and PKA activities in time–course experiments subsequent to exposure of primary rat hepatocytes to PB and demonstrate that this signaling pathway was not activated in the early phases of the PB induction response.

## MATERIALS AND METHODS

### *Materials*

PB was obtained from the University of Washington Hospital Pharmacy Services. dBcAMP and dexamethasone were purchased from the Sigma Chemical Co. Forskolin and Ro 20–1724 were purchased from the Alexis Corp. All cell culture materials and Trizol™ were obtained from Life Technologies. Matrigel, ITS+, and NuSerum were obtained from Collaborative Biomedical Products.

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† *Abbreviations*: cAMP, cyclic AMP; CYP, cytochrome P450; dBcAMP, dibutyryl cAMP; DTT, dithiothreitol; ECM, extracellular matrix; PB, phenobarbital; PKA, protein kinase A; PKC, protein kinase C; and PKG, protein kinase G.

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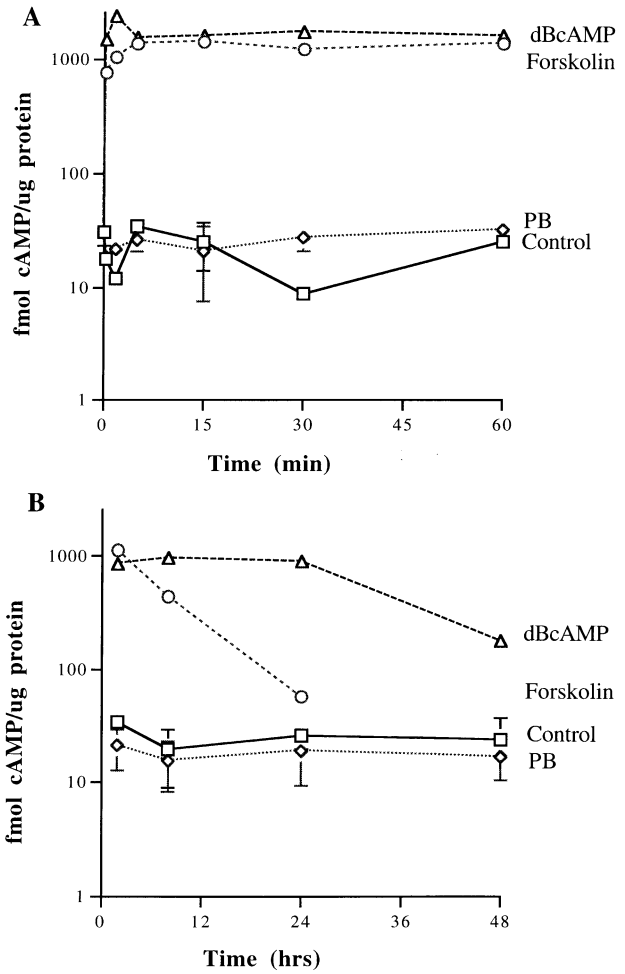


**FIG. 1. Effects of dBcAMP (25 μM), forskolin (100 μM), and PB (500 μM) on intracellular cAMP levels in primary rat hepatocytes. Cells were seeded for 48 hr before treatments began. Panel A represents the effects on intracellular cAMP after brief treatment intervals, 30 sec to 1 hr. Panel B represents the effects of longer treatment periods, 2–48 hr. As the dBcAMP and forskolin treatments functioned as positive controls in these experiments, the phosphodiesterase inhibitor Ro 20-1724 (200 μM) was added to these treatments. Each value represents the average ± SD from at least 3 independent hepatocyte preparations. Intracellular cAMP analysis was performed in duplicate for each sample evaluated.**

### *Primary Hepatocyte Isolation and Culture*

Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion *in situ* [19] and cultured with modifications [10, 18] of the protocols as described previously. A dilute concentration (233 μg/mL final concentration) of ECM (Matrigel) was added as an overlay 16 hr after initial plating of the cells. Medium changes were conducted thereafter on a daily basis, and a 25 nM dexamethasone concentration was maintained in all media.

### *Treatments*

PB (1.0 M) and dBcAMP (100 mM) were dissolved in tissue culture grade water as stock solutions and stored in aliquots at −20°. Forskolin (100 mM) and Ro 20-1724 (500 mM) were dissolved in DMSO as stock solutions and also stored at −20°. Cells were cultured for 48 hr before the addition of any treatments. Final concentrations of each agent were: PB, 500 μM; dBcAMP, 25 μM; forskolin, 100 μM; and Ro 20-1724, 200 μM, unless otherwise stated. For all analyses, representative data are shown from multiple studies performed independently with different hepatocyte preparations.

### *cAMP Analysis*

Intracellular cAMP was measured using the Biotrak™ cAMP enzyme immunoassay system from Amersham. Cells were stimulated for the indicated times, and cell extracts were prepared. Briefly, cells were washed twice with cold PBS and then lysed and scraped in 800 μL of ice-cold 70% ethanol. Cell debris was pelleted at 2000 *g*, and the resulting supernatant was subsequently lyophilized and stored at −20°. For analysis, resulting pellets were resuspended in 500 μL of the required assay buffer. Protein concentrations were determined using bovine serum albumin as a standard with the BCA protein assay reagent (Pierce).

### *Measurement of PKA Activity*

PKA activity was assessed by measuring the transfer of $^{32}$P-labeled phosphate to a biotinylated Kemptide (LRRASLG) substrate using the Promega's Signa-TECT™ PKA assay system. At specified time points after treatments, cells were washed twice and then scraped with cold PBS. Cells were pelleted at 500 *g* at 4° and resuspended in 100 μL of ice-cold buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$). The resulting cell suspension was lysed by brief (7 sec) sonication on ice, and the cell debris was pelleted at 15,000 *g* at 4°. Five microliters of supernatant was assayed for 5 min at 30° with: 5 μL PKC assay buffer (Promega), 0.1 mM ATP, 5 μL [γ-$^{32}$P]ATP (6000 Ci/mmol), and 0.1 mM PKA biotinylated Kemptide substrate, with or without activation by 5 μM cAMP. Mixtures without exogenous cAMP measured endogenously activated PKA, while mixtures with added cAMP measured the total available PKA in the cells. Reaction termination, spotting onto membranes, and washing were carried out as directed by the manufacturer's protocol. Protein concentrations were determined using bovine serum albumin as a standard with the Bio-Rad Protein assay.

### *RNA Analysis*

Total RNA was isolated [20] using Trizol as described by the supplier (Life Technologies). For slot blot evaluation, 5 μg of total RNA was applied directly to a Genescreen Plus nylon membrane (DuPont) under vacuum and denaturing conditions using a Minifold II apparatus (Schleicher &

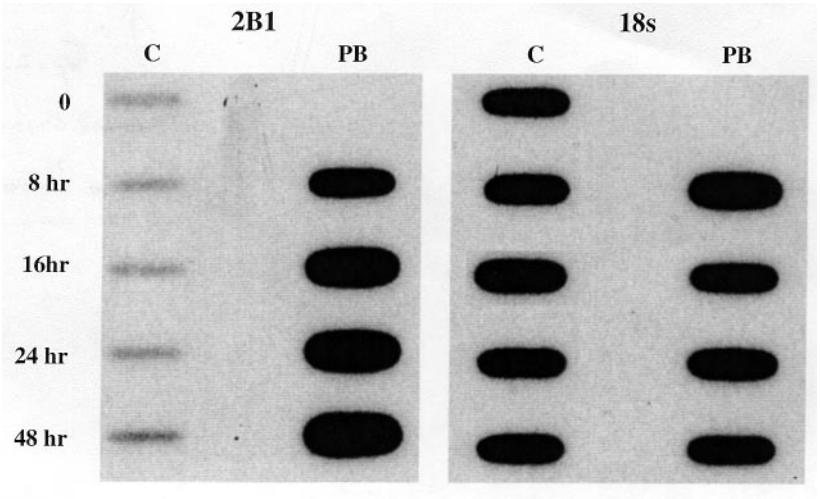


**FIG. 2. Effects of PB upon the induction of CYP2B1 in primary rat hepatocytes. Cultures were seeded for 48 hr before the addition of PB (500 μM). Total RNA was isolated and evaluated as stated under Materials and Methods. Ribosomal 18S RNA hybridization levels were used to demonstrate equal loading between samples.**

Schuell). The membranes were hybridized with specific $^{32}$P-radiolabeled oligonucleotides for CYP2B1 and 18S rRNA, as described previously [18, 21].

## RESULTS

In the experiments conducted here, we examined the effects of PB on intracellular cAMP levels and subsequent PKA activity in primary rat hepatocytes. In Fig. 1, data are presented demonstrating that treatment of primary rat hepatocytes with either forskolin or dBcAMP resulted in up to 50-fold stimulation of intracellular cAMP levels relative to control (untreated) cells. For dBcAMP, this increase was sustained over a 24-hr period and maintained the elevated level even at 48 hr. The increase in cAMP produced by forskolin was more transient and began to approach basal levels within 24 hr. These results appear consistent with the relative resistance to phosphodiesterase degradation offered by the dibutyryl moiety of dBcAMP. Unlike the cAMP analog or the known adenylate cyclase activator forskolin,

**TABLE 1. Activity of cAMP-dependent protein kinase in treated rat hepatocytes***

| Time | Treatment | −cAMP† (pmol ATP/min) | +cAMP† (pmol ATP/min) | % of Total activated‡ |
|---|---|---|---|---|
| 30 sec | Control | 0.708 ± 0.078 | 9.710 ± 1.018 | 7.294 ± 0.448 |
| | PB | 0.660 ± 0.169 | 10.765 ± 1.795 | 6.446 ± 2.600 |
| | Forskolin | 1.843 ± 0.163 | 7.129 ± 2.054 | 27.129 ± 6.144 |
| | dBcAMP | 3.461 ± 0.298 | 11.245 ± 3.007 | 31.979 ± 6.004 |
| 5 min | Control | 0.901 ± 0.227 | 12.592 ± 1.632 | 7.084 ± 0.993 |
| | PB | 0.721 ± 0.155 | 12.574 ± 0.212 | 5.718 ± 1.142 |
| | Forskolin | 2.482 ± 0.376 | 8.508 ± 2.121 | 29.761 ± 3.275 |
| | dBcAMP | 5.189 ± 1.669 | 12.672 ± 3.477 | 40.528 ± 2.255 |
| 15 min | Control | 0.851 ± 0.054 | 14.477 ± 1.556 | 5.906 ± 0.366 |
| | PB | 0.636 ± 0.159 | 15.775 ± 2.582 | 3.988 ± 0.363 |
| | Forskolin | 2.126 ± 0.494 | 7.090 ± 1.256 | 29.778 ± 2.063 |
| | dBcAMP | 4.564 ± 1.762 | 12.770 ± 5.349 | 36.190 ± 1.756 |
| 1 hr | Control | 0.349 ± 0.078 | 13.486 ± 2.084 | 2.700 ± 0.990 |
| | PB | 0.603 ± 0.100 | 17.179 ± 6.398 | 4.079 ± 2.065 |
| | Forskolin | 0.973 ± 0.085 | 7.843 ± 2.291 | 13.423 ± 4.766 |
| | dBcAMP | 4.126 ± 1.792 | 11.066 ± 2.958 | 36.041 ± 6.784 |
| 8 hr | Control | 0.452 ± 0.042 | 13.610 ± 2.145 | 3.390 ± 0.674 |
| | PB | 0.523 ± 0.089 | 15.852 ± 3.440 | 3.512 ± 1.305 |
| | Forskolin | 0.439 ± 0.198 | 6.573 ± 1.604 | 7.513 ± 4.846 |
| | dBcAMP | 2.798 ± 1.497 | 7.431 ± 1.503 | 35.71 ± 13.00 |
| 24 hr | Control | 0.412 ± 0.049 | 14.510 ± 1.334 | 2.847 ± 0.355 |
| | PB | 0.456 ± 0.113 | 15.328 ± 0.828 | 3.012 ± 0.888 |
| | Forskolin | 0.235 ± 0.173 | 9.338 ± 0.226 | 2.487 ± 1.795 |
| | dBcAMP | 3.116 ± 0.836 | 8.221 ± 0.233 | 38.14 ± 11.24 |

*Values are means ± SD from a minimum of 4 samples, taken from at least 2 individual hepatocyte preparations.
†Kinase activities are expressed as enzyme-specific activity, in pmol ATP incorporated into substrate/min.
‡The percent of total activated was determined by dividing the endogenous cAMP levels (−cAMP) by exogenously activated samples (+cAMP).

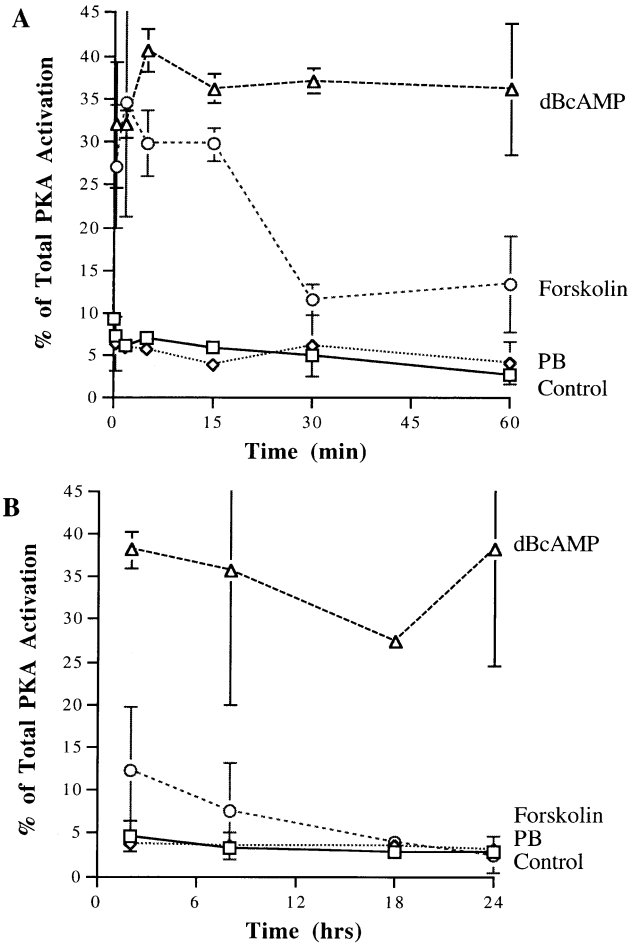


**FIG. 3. Effects of dBcAMP (25 μM), forskolin (100 μM), and PB (500 μM) on PKA activity levels in primary rat hepatocytes. Cells were seeded for 48 hr before treatments began. Panel A represents the effects on PKA activity over short-term treatments, 30 sec to 1 hr. Panel B represents the effects of longer treatment intervals, 2–48 hr. The phosphodiesterase inhibitor Ro 20-1724 (200 μM) also was added to the dBcAMP and forskolin treatments. The percentage of total PKA activation is the amount of endogenous PKA activity/the amount of total available PKA as determined by the addition of exogenous cAMP (5 μM) to the sample. The absolute values for these percentages (pmol ATP/min) are shown in Table 1. Each value represents the average ± SD from at least 3 independent hepatocyte preparations. PKA activity, in the absence of exogenously added cAMP, was determined in duplicate for each sample evaluated.**

PB exposures (500 μM) did not result in any detectable transient or sustained increase in intracellular cAMP levels. As shown in Fig. 2, the inductive effects of PB upon CYP2B1 expression are easily detected within an 8-hr period, yet PB-treated cells harvested as early as 30 sec and as late as 48 hr did not show any corresponding increases, or decreases, in intracellular cAMP levels as compared with control cells.

We used a synthetic Kemptide substrate to assess increases in PKA activity following treatment with either dBcAMP, forskolin, or PB. As shown in Fig. 3, dBcAMP and forskolin treatments resulted in marked increases in PKA activity that were clearly detectable within 30 sec of stimulation. The increases produced by forskolin were more transitory than those elicited by the cAMP analog, returning to near basal levels within 30 min. In contrast, treatment with PB resulted in no detectable changes in PKA activity levels. The data provided in Table 1 further demonstrate the lack of PB effect upon the total cellular PKA activity or the fraction of PKA activated by endogenous levels of cAMP.

## DISCUSSION

The results presented in this study demonstrate that PB additions to primary hepatocytes produced no detectable alterations in either cAMP level or subsequent PKA activity. The experimental analyses were conducted using a highly defined primary hepatocyte culture system, a system that has been characterized previously to reflect *in vivo* induction, activity, and expression of multiple liver-specific enzymes [10–12, 21, 22], together with associated hepatic-specific responses to a variety of pharmacological and physiological agents, such as cyclic nucleotide analogs and glucagon, a hormonal agent that stimulates cAMP production in the liver *in vivo* [7, 13, 18, 23]. The PB induction response is highly conserved across animal species [3].

Investigators have attempted to examine the roles of specific signaling pathways in CYP gene regulation by either blocking or enhancing specific signal transduction pathways [13–15, 23, 24]. The activation of adenylate cyclase and the subsequent elevation of cAMP levels by forskolin, an agent used in the current experiments, have been demonstrated in essentially all cell types and tissues examined. For intact cells, it is largely accepted that forskolin activation results in pharmacological effects closely associated with cAMP [25, 26]. The rapid response and transient nature of these second messenger signals, occurring within seconds or minutes of agent exposure, make them difficult events to snapshot, especially in whole animal systems. Prior investigators have attempted to examine cAMP levels in the whole animal model but experienced considerable difficulty in detecting changes in cAMP levels subsequent to inducer treatment [7].

Results from some early *in vivo* experiments and studies with hepatocytes in suspension previously indicated that PB may exert its effects through the activation of a cAMP-dependent protein kinase that, in turn, might increase the levels of CYP gene expression [7, 8]. However, these studies suffer by their use of imprecise endpoints as well as their utilization of supra-physiologic doses of cAMP. Of interest from the tumor promotion standpoint, PB was reported to inhibit the phorbol ester-induced translocation of PKC to the plasma membrane in hepatocytes from rats that had ingested PB in their water [27]. In addition, it was suggested that PB inhibited PKC by occupying the diacylglycerol binding site [28].

However, studies from our laboratory and others have

yielded a different spectrum of conclusions. Using defined primary rat hepatocyte cultures, we have demonstrated that cAMP analogs and adenylate cyclase activators, such as forskolin, dramatically inhibit the PB induction response of *CYP2B* genes [13]. Furthermore, evidence was provided indicating that the second messenger pathway involved in these effects was, in fact, PKA, not PKG or PKC [13]. Additionally, it was determined that okadaic acid markedly potentiates the repressive effects of cAMP on the PB induction process [23], results that have been corroborated by other investigators using mouse hepatocyte cultures to examine PB-inducible expression of the *CYP2B10* gene [17]. Together, these data are strongly supportive of the involvement of cAMP-dependent PKA activation, together with protein phosphatase pathways, as modulators of PB responsiveness in mammalian hepatocytes.

The current investigation is unique in that it addresses the potential role of early signaling transients, specifically alterations in cAMP level or PKA activity, subsequent to exposure of hepatocytes to PB. In light of the recent results indicating that activators of cAMP pathways, or inhibitors of protein phosphatases, modulate the PB induction response [13, 15, 23, 24], we were surprised to find that PB itself did not alter cAMP levels and subsequent PKA activity in isolated hepatocytes. Thus, the issues of how PB is mediating its effects and what intracellular signaling pathways are involved remain unclear. However, despite their involvement in modulating the induction process, the results presented here demonstrate that the initial events stimulated by PB in rat hepatocytes do not include alterations of cAMP levels or associated PKA activities. Further studies are necessary to characterize alternative pathways through which PB may be signaling within these cells.

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