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Pentobarbital Enhances Cyclic Adenosine Monophosphate Production in the Brain by Effects on Neurons but Not Glia

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Background: **Cyclic adenosine monophosphate (cAMP) is an important regulator of neuronal excitability. The effects of barbiturates on cAMP production in intact neurons are not known. This study used cultures of cortical neurons, cultures of glia, and slices of cerebral cortex from the rat to study the effects of barbiturates on cAMP regulation in the brain.**

Methods: **Primary cultures of cortical neurons or glia were prepared from 17-day gestational Sprague-Dawley rat fetuses and were used after 12–16 days in culture. Cross-cut slices (300 μm) were prepared from cerebral cortex of adult rats. Cyclic AMP accumulation was determined by measuring the conversion of [^{3}H]adenosine triphosphate (ATP) to [^{3}H]cAMP in cells preloaded with [^{3}H]adenine.**

Results: **Pentobarbital enhanced isoproterenol- and forskolin-stimulated, but not basal, cAMP accumulation in cultures of cerebral neurons. Cyclic AMP production was enhanced by pentobarbital in a dose-dependent fashion up to a concentration of 250 μM; This concentration of pentobarbital increased cAMP production by 40–50% relative to that in controls without pentobarbital. At 500 μM pentobarbital, the magnitude of the enhancement was less. Pentobarbital had no effect on isoproterenol-stimulated cAMP production in cultures containing only glia. Pentobarbital also enhanced isoproterenol-stimulated, but not basal, cAMP production in slices of cerebral cortex by ~30% at concentrations of 62.5–250 μM and by almost 100% at 500 μM.**

Conclusions: **Pentobarbital enhances stimulated cAMP accumulation in cultured preparations from brain and fresh cortical slices. Neurons are required for this effect. Because cAMP modulates neuronal excitability, this effect of pentobarbital may be an important mechanism by which this anesthetic influences brain function. (Key words: Anesthetics, intravenous: pentobarbital. Brain, neurons: primary cultured. Brain: glia; slices. Neuronal regulation: adenylyl cyclase, cyclic adenosine monophosphate).**

THE mechanisms of action of anesthetic drugs are not well understood at the cellular or molecular levels. Many effects of anesthetics, although, appear to be caused by influences on the regulation of transmembrane signaling and intracellular messengers. Barbiturates, for example, enhance $GABA_A$-mediated neuronal inhibition[1–3] and inhibit responses to the excitatory amino acids.[2,4–6] Barbiturates also affect the function of several types of ion channels including the nicotinic acetylcholine receptor,[7,8] calcium channels,[9,10] sodium channels,[11] and the adenosine triphosphate-K^+ channel.[12] Furthermore, barbiturates inhibit protein kinase C[13] and phosphatidylinositol hydrolysis[14,15] and are antagonists at A_1 adenosine receptors.[16] It therefore is apparent that the ultimate effect of barbiturates on neuronal function is an integrated response of the cell in response to many influences.

Adenosine 3′,5′-monophosphate (cyclic adenosine monophosphate [cAMP]) is an intracellular messenger that is ubiquitous in the regulation of many aspects of cellular function, including the regulation of neuronal excitability. We have shown previously that anesthetic barbiturates enhance isoproterenol-stimulated cAMP production in intact S49 wild type mouse lymphoma cells,[17] but we observed no effect of barbiturates on adenylyl cyclase activity in homogenates or membrane preparations from S49 cells. However, Dan'ura *et al.*[18–20] have shown that barbiturates inhibit adenylyl cyclase activity stimulated by guanine nucleotide, NaF-

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$AlCl_3$, or forskolin in preparations of membranes from rat brain, although these effects occurred at higher concentrations than those required for the effects we reported in intact S49 cells. The effects of barbiturates on cAMP accumulation in intact neurons are not known; hence, the purpose of this study was to determine the effects of barbiturates on cAMP production in intact neurons in both cultured preparations and fresh slices from cerebral cortex from the rat.

Materials and Methods

Materials

Chemicals used in the current study were obtained from the following sources: isoproterenol, pentobarbital, cytosine-β-D-arabino-furanoside, 3-isobutyl-1-methylxanthine (IBMX), Hank's balanced salt solution, Dulbecco's modified Eagle's medium with 25 mM hydroxyethylpiperazineethane sulfonic acid (HEPES) (DMEM-H), poly-D-lysine, and trypsin from Sigma (St. Louis, MO); Ham's F-12 and penicillin/streptomycin from JRH Biosciences (Lenexa, KS); and fetal calf serum from Biocell Laboratories (Rancho Dominguez, CA).

Cell Culture

Cultures of cerebral cortical neurons and glia were prepared from 17-day gestational Sprague-Dawley rats using protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania, as described previously.[21,22] Whole cerebral hemispheres were collected in Hank's balanced salt solution from fetuses of 17-day gestational Sprague-Dawley rats. They were washed twice in phosphate buffered saline, then digested using trypsin ($5\ mg \cdot ml^{-1}$) in DMEM-H, washed three times in Hank's balanced salt solution, then triturated with a "fire-polished" Pasteur pipette into DMEM-H supplemented with 10% Ham's F12 media, 10% heat inactivated fetal calf serum, $100\ \mu g \cdot ml^{-1}$ streptomycin, and 100 units $\cdot ml^{-1}$ penicillin. Cells were plated at a density of 4×10^5 cells per 1.2-cm diameter tissue culture well that had been pretreated with $10\ \mu g \cdot ml^{-1}$ poly-D-lysine. Cultures were grown in a 37°C humidified incubator containing 5% CO_2 in air, and were fed approximately every 2 or 3 days with fresh medium. On day 5, neuronal cultures were treated with $10\ \mu g \cdot ml^{-1}$ cytosine-β-D-arabino-furanoside for 24 h to stop nonneuronal cell proliferation. Glial cell cultures were prepared by dispersing the mixed cultures by digestion with trypsin at day 5 and replating them at a density of 8×10^4 cells per 1.2-cm diameter well. These cultures were also fed every 2 or 3 days but were not treated with cytosine-β-D-arabino-furanoside. This procedure resulted in preparations that were essentially devoid of neuronal cells. For both types of cultures, feedings after day 5 were with the aforementioned media, with the exception of Ham's F-12, which was removed to exclude glutamate from the feeding. Cultures were used for experiments after 12–16 days in culture.

Slice Preparation

The brains of female Sprague-Dawley rats (mothers of fetuses used for preparations of primary cultures) were removed after anesthesia with carbon dioxide and death by decapitation. The brains were rinsed in ice-cold DMEM-H that was previously equilibrated with 95% O_2/5% CO_2 and the cerebral cortex was dissected and prepared as 300 μm cross-cut slices using a McIlwain tissue chopper (Mickle, Gomshall, Surrey, England) as has been described by others.[22] Slices were dispersed in fresh, ice-cold DMEM-H that was previously equilibrated with 95% O_2/5% CO_2.

Measurement of Cyclic Adenosine Monophosphate Accumulation

Cyclic AMP accumulation in cultured cells was determined in cells preloaded with radiolabeled adenine as described by Atkinson and Minneman.[22] Neuronal cultures were incubated with 0.5 μCi [2,8-^{3}H]adenine in 0.5 ml growth media for 120 min at 37°C, then washed twice with serum-free DMEM-H. When included, IBMX (0.5 mM) and pentobarbital were added to the cultures in a final reaction volume of 0.5 ml for a 20-min preincubation period. Cyclic AMP accumulation was initiated by the addition of control vehicle or medium containing isoproterenol, clonidine, or forskolin. The reaction was stopped after 10 min by the addition of trichloroacetic acid for a final concentration of 5% in a final volume of 1.0 ml. The contents of the wells were collected into glass tubes, samples were centrifuged at 500*g* for 10 min after which the [^{3}H]cAMP and [^{3}H]adenosine triphosphate were isolated by sequential chromatography over Dowex and alumina columns as described previously.[17] Cyclic AMP accumulation was calculated as the percentage of [^{3}H]adenosine triphosphate initially present in the cultures that was converted to [^{3}H]cAMP. Cyclic AMP production was linear up to at least 20 min in this model (data not shown).

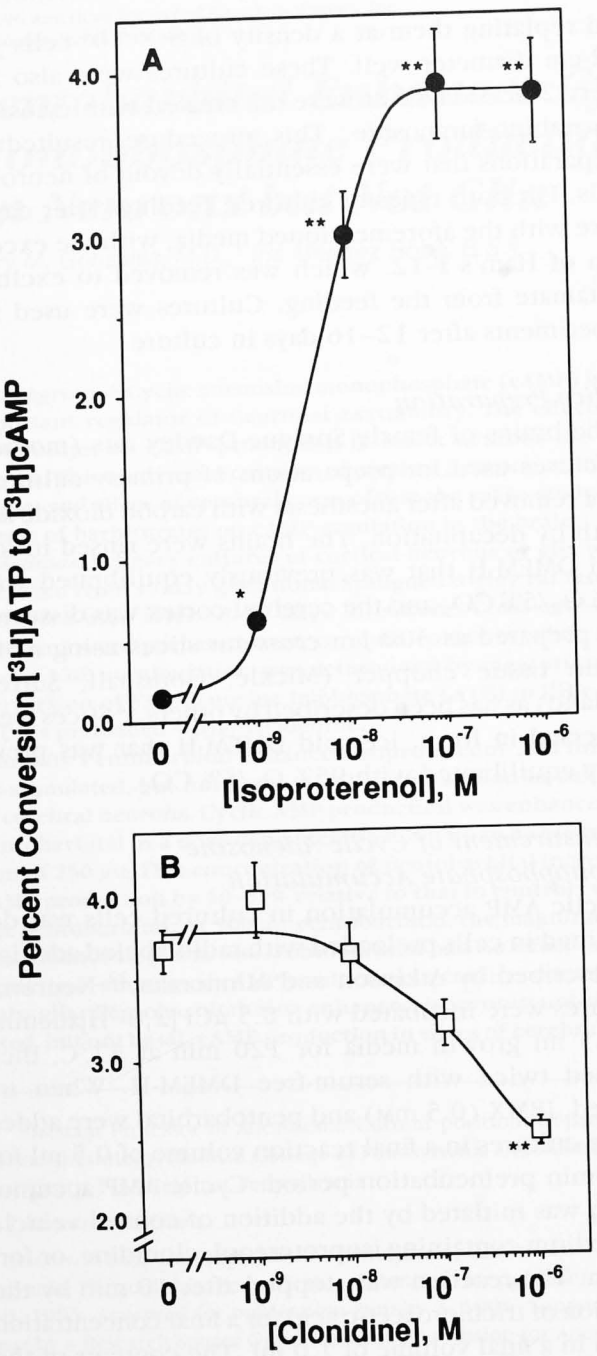

Fig. 1. Effect of stimulatory and inhibitory agonists on cyclic adenosine monophosphate accumulation in cultures of cortical neurons. Cultures were exposed to 0.5 mM 3-isobutyl-1-methylxanthine for a 20 min preincubation period before the addition of agonist. (*A*) Increasing concentrations of isoproterenol were added to cultures to stimulate the production of cyclic adenosine monophosphate. Data are presented as the percentage of [^{3}H]adenosine triphosphate that was converted to [^{3}H]cyclic adenosine monophosphate and represent the mean ± SEM values for four experiments. Where error bars are not seen, the variance is less than the dimension of the symbol for the data point. *$P < 0.05$ and **$P < 0.01$ compared to the value in the absence of isoproterenol by analysis of variance and Dunnett's *post hoc* correction. (*B*) Increasing concentrations of clonidine were added to cultures to inhibit forskolin-stimulated (5 μM) cyclic adenosine monophosphate production. Data are presented as above in (*A*) for four experiments. **$P < 0.01$ compared to value in the absence of clonidine by analysis of variance and Dunnett's *post hoc* correction.

Measurement of cAMP accumulation in slices was determined in cells preloaded with radiolabeled adenine as described by Atkinson and Minneman.[22] Buffer was decanted from the slices prepared as described earlier and the tissue was incubated in fresh DMEM-H that was previously equilibrated with 95% O_2/5% CO_2 and containing 1.0 μCi [2,8-^{3}H]adenine per milliliter for 120 min at 37°C while being bubbled with 95% O_2/5% CO_2, then washed twice with DMEM-H. Aliquots of slices containing approximately 25 mg wet weight of brain tissue were transferred into reaction tubes containing 0.5 mM IBMX and pentobarbital in a final reaction volume of 0.5 ml for a 20-min preincubation period. The reactions were performed and the samples were analyzed as described earlier for cultured cells.

Data Analysis

Data from each type of experiment were analyzed using a paired Student's *t* test, one-sample *t* test with correction for multiple comparisons, or analysis of variance with *post hoc* corrections using Dunnett's test, as indicated in the figure legends. A value of $P < 0.05$ was considered significant.

Results

Before beginning experiments with pentobarbital, the function of receptor-modulated cAMP production by both stimulatory and inhibitory G-protein-linked pathways was tested in cultured neurons. The β-adrenergic agonist, isoproterenol, significantly stimulated cAMP production in cultures of neurons, with a maximal increase of approximately tenfold over basal at concentrations of 0.1 and 1 μM (fig. 1A). The α_2-adrenergic agonist, clonidine, inhibited forskolin-stimulated cAMP production by ~30% at a concentration of 1 μM (fig. 1B).

The effect of pentobarbital on cAMP accumulation was tested in these cultures. Isoproterenol (1 μM), as

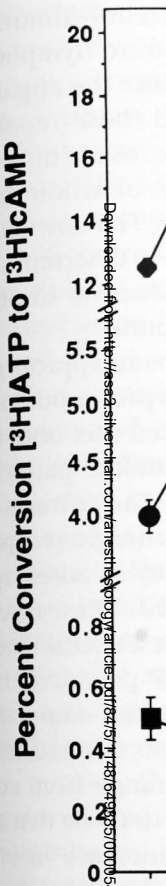

**Fig. 2. Effect of pen
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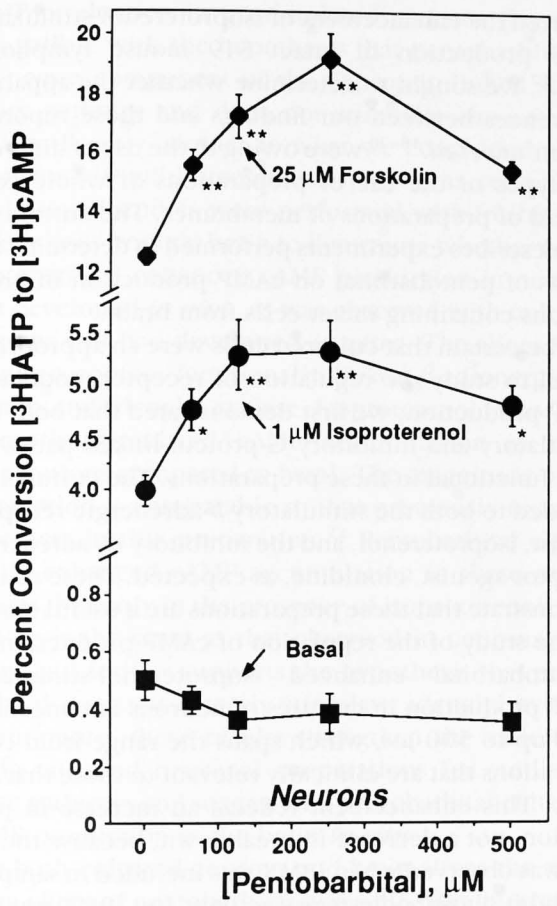

Fig. 2. Effect of pentobarbital on basal (unstimulated), isoproterenol-stimulated (1 μM) and forskolin-stimulated (25 μM) cyclic adenosine monophosphate accumulation in cultures of cortical neurons. Samples were exposed to 0.5 mM 3-isobutyl-1-methylxanthine and the indicated concentration of pentobarbital for a 20 min preincubation period before the addition of isoproterenol or forskolin. Results are presented as the mean ± SEM values for 8–12 experiments. *$P < 0.05$ and **$P < 0.01$ compared to the value in the presence of isoproterenol or forskolin, but in the absence of pentobarbital by analysis of variance and *post hoc* test using the Dunnett's test.

demonstrated earlier, and forskolin (25 μM) significantly stimulated cAMP production compared to basal cAMP production. Pentobarbital enhanced isoproterenol- and forskolin-stimulated cAMP production in these preparations with a biphasic dose-response curve (fig. 2). Cyclic AMP production was enhanced by pentobarbital in a dose-dependent fashion up to a concentration of 250 μM; This concentration increased cAMP production by 40–50% relative to that in controls without pentobarbital. At 500 μM pentobarbital, the magnitude of the enhancement was less. Similar experiments performed in the presence of isoproterenol but in the absence of IBMX showed a similar response but the signal was smaller (data not shown).

To determine whether the effect of pentobarbital required neurons, experiments were performed with cultures of glia. Isoproterenol significantly stimulated cAMP production, compared to basal, at concentrations of 5 nM and 1 μM (data not shown). Cyclic AMP production stimulated by 1 μM isoproterenol was greater in these preparations than in cultures of neurons. Pentobarbital had no effect on cAMP production in the presence of either concentration of isoproterenol (fig. 3).

Experiments were performed with slices prepared from cerebral cortexes of adult Sprague-Dawley rats to determine whether or not pentobarbital enhanced cAMP production in neurons that had developed *in vivo* as well as those that had developed in culture. Cyclic AMP production in these preparations was significantly stimulated by isoproterenol (fig. 4). Pentobarbital enhanced isoproterenol-stimulated cAMP production by ~30% at concentrations of 62.5, 125, and 250 μM, and by nearly 100% at a concentration of 500

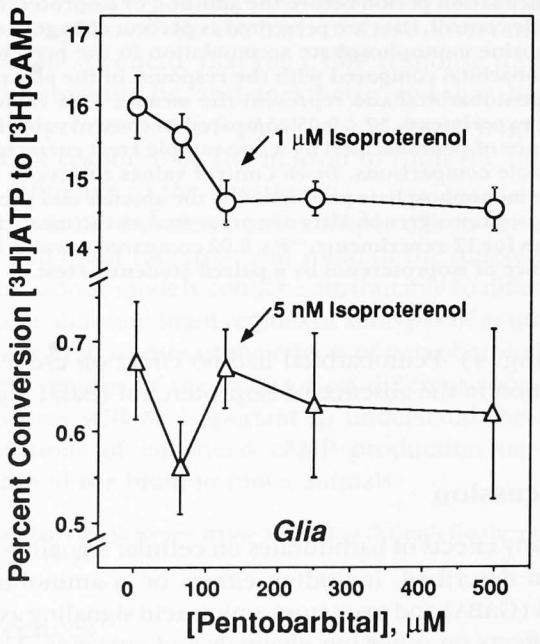

Fig. 3. Effect of pentobarbital on isoproterenol-stimulated (5 nM and 1 μM) cyclic adenosine monophosphate accumulation in cultures of cortical glia. Samples were exposed to 0.5 mM 3-isobutyl-1-methylxanthine and the indicated concentration of pentobarbital for a 20-min preincubation period before the addition of isoproterenol. Results are presented as the mean ± SEM values for 7–9 experiments.

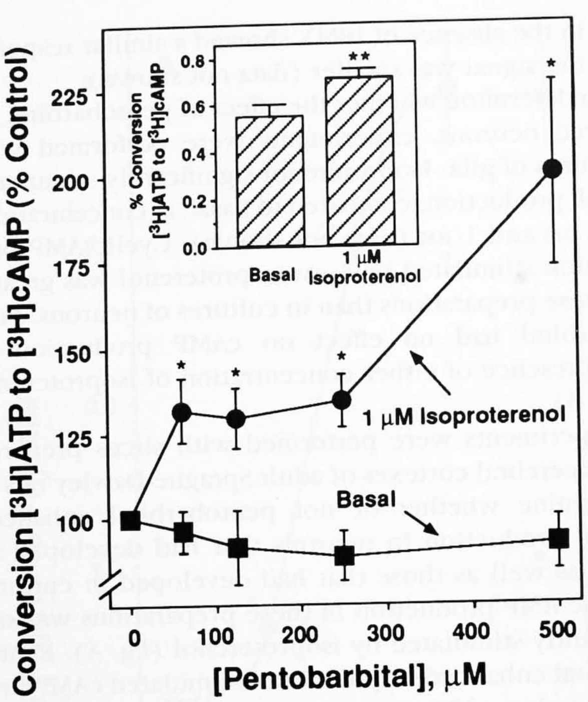

Fig. 4. Effect of pentobarbital on cyclic adenosine monophosphate accumulation in the presence and absence (basal) of 1 µM isoproterenol-stimulated in cortical slices from rat brain. Samples were exposed to 0.5 mM 3-isobutyl-1-methylxanthine and the indicated concentration of pentobarbital for a 20-min preincubation period before the addition of isoproterenol or vehicle control. Data are presented as percent change in cyclic adenosine monophosphate accumulation in the presence of pentobarbital compared with the response in the absence of the pentobarbital and represent the mean ± SEM values for 8–12 experiments. *$P < 0.05$ compared to control value in the absence of pentobarbital by a one-sample *t* test corrected for multiple comparisons. *Inset:* Control values for cyclic adenosine monophosphate production in the absence and presence of 1 µM isoproterenol. Data are presented as the mean ± SEM values for 12 experiments. **$P < 0.02$ compared to value in the absence of isoproterenol by a paired Student's *t* test.

µM (fig. 4). Pentobarbital had no effect on cAMP production in the absence of isoproterenol (basal, fig. 4).

Discussion

Many effects of barbiturates on cellular signaling have been described, including effects on γ-aminobutyric acid (GABA) and excitatory amino acid signaling as well as effects on other ion channels and enzymes. The reported effects on cAMP production have been variable. Dan'ura *et al.*[18–20] reported that barbiturates inhibit cAMP production stimulated by forskolin, 5′-guanylylimidodiphosphate (Gpp[NH]p), or NaF-$AlCl_3$ in membranes prepared from rat brain. However, we previously reported the enhancement of isoproterenol-stimulated cAMP production in intact S49 mouse lymphoma cells.[17] We sought to determine whether the apparent differences between our findings and those reported by Dan'ura *et al.*[18–20] were owing to the use of different cell types or the use of preparations of whole cells instead of preparations of membranes. The current report describes experiments performed to determine the effects of pentobarbital on cAMP production in preparations containing intact cells from brain.

To be certain that cultured cells were an appropriate model to study the regulation of receptor-modulated cAMP production, we first demonstrated that both the stimulatory and inhibitory G-protein-linked pathways were functional in these preparations. The cultures responded to both the stimulatory β-adrenergic receptor agonist, isoproterenol, and the inhibitory α_2-adrenergic receptor agonist, clonidine, as expected. These results demonstrate that these preparations are a useful model for the study of the regulation of cAMP production.

Pentobarbital enhanced isoproterenol-stimulated cAMP production in cultures of neurons at concentrations up to 500 µM, which spans the range from concentrations that are clinically relevant to those that are toxic. This enhancement reflects an increase in production, not a decrease in breakdown, because the effect was observed when IBMX was included in samples to inhibit phosphodiesterase activity, but IBMX was not required for the effect. Although these cultures predominantly contain neurons, some glia are present in these preparations to support the growth of the neurons. Based only on the data from these preparations, we could not rule out that the effect of pentobarbital is occurring in the glia, and because glia do contribute to the regulation of excitatory neurotransmission,[23] it is important to make this distinction. When cultures are dispersed and replated, essentially only glia grow, neurons do not survive this manipulation, as we[21] and others[22] have described. When cAMP production stimulated by 5 nM or 1 µM isoproterenol was measured in these preparations, pentobarbital had no effect. The production of cAMP by glia stimulated by 1 µM isoproterenol was much greater than that observed in preparations of neurons stimulated by 1 µM isoproterenol but was similar to that in neurons stimulated by 25 µM forskolin. These data suggest that the lack of effect of pentobarbital on glial preparations is not due to the absolute level of activity observed in the absence of pentobarbital, but rather that neurons are required for the effect. The lack of an effect of pentobarbital on

cAMP production in unstimulated neurons (basal) is compatible with the hypothesis that pentobarbital influences a component of the G-protein-linked stimulatory pathway and does not stimulate the catalytic moiety directly, similar to what we concluded previously for S49 wild type mouse lymphoma cells.[17]

Additional studies were performed with freshly prepared slices from rat brain to determine whether or not pentobarbital enhanced cAMP production in neurons that developed *in vivo*, as was observed with cultured neurons that had developed *in vitro*. The slices were found to contain functionally linked β-adrenergic receptors and adenylyl cyclase. Isoproterenol (1 μM) significantly stimulated cAMP accumulation in these preparations compared to basal. The magnitude of the stimulation is comparable to that previously reported by others in this preparation.[22] Pentobarbital significantly enhanced cAMP accumulation in these preparations, but only in the presence of isoproterenol. This finding is the same as that observed in neuronal cultures, and further supports the hypothesis that pentobarbital does not directly stimulate adenylyl cyclase. Furthermore, these results strengthen the validity of using cultured neuronal preparations for studies of these pathways and suggest that pentobarbital enhances cAMP production in the brain *in situ*.

In both cultured neurons and brain slices the maximum effect of pentobarbital was a 30–50% increase in cAMP production. This measurement is an overall average increase in production in the entire sample. Because the effect is not seen in all cells, as demonstrated by the experiments with glial cultures, it is likely that the magnitude of the effect of pentobarbital is much greater in some subpopulation of cells, and therefore, the effect on cellular function would be expected to be quite significant in this group of cells.

Many investigators have demonstrated that cAMP can play a role in the depression of excitatory neurotransmission or enhancement of inhibitory neurotransmission. Activation of β-adrenergic receptors increases the frequency of inhibitory synaptic currents in rat cerebellar stellate and Purkinje cells, by a mechanism that probably involves intracellular cAMP.[24] Analogs of cAMP inhibit spontaneous firing of Purkinje neurons.[25] γ-Aminobutyric acid-mediated currents are potentiated by cAMP-dependent protein kinase in cerebellar Purkinje cells.[26,27] γ-Aminobutyric acid-mediated currents are potentiated by cAMP in cerebrocortical, cerebellar, and hypothalamic neurons.[28] Activation of β-adrenergic receptors leads to a reduced frequency of spontaneous excitatory spikes and a hyperpolarization of Purkinje cells.[29] Phosphorylation of inhibitory glycine channels expressed in xenopus oocytes by cAMP-dependent protein kinase results in an enhancement of glycine-evoked current.[30,31] Isoproterenol enhanced the inhibitory responses to GABA, mimicking the effect of benzodiazepines in neurons from the lateral hypothalamus.[32] Norepinephrine activation of β-adrenergic receptors enhanced GABA-activated inhibitory synaptic mechanisms in the cerebellum and cerebral cortex.[33] Enhancement of neurotransmitter-stimulated cAMP production by pentobarbital may, therefore, play a role in the depression of excitatory neurotransmission or enhancement of inhibitory neurotransmission by one or more of these mechanisms.

However, several other investigators have demonstrated an excitatory effect of cAMP on neuronal activity. Cyclic AMP or cAMP-dependent kinase enhanced responses to excitatory amino acids in several experimental models.[29,34–39] Cyclic AMP also has been shown to decrease the inhibitory response to GABA.[40–44] It also appears that in one specific brain region, the locus ceruleus, inhibition of adenylyl cyclase mediates the hypnotic effect of an α_2-adrenergic receptor agonist.[45] On the basis of these observations, one might speculate that the cAMP response to pentobarbital would be 'anti-anesthetic' and that the enhanced production of cAMP could be a negative feedback response by the neuron to limit the inhibitory influence of the anesthetic.

Cyclic AMP, therefore, has multiple effects on neuronal and brain function, and some of the differences seen in various models could be attributable to different effects on different brain regions or subtypes of neurons. More detailed studies of the effects of pentobarbital on specific regions of the brain or on different subtypes of neurons will be important to understand the full implications of enhanced cAMP production on the function of the brain in intact animals.

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