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Effects of Cyclic Nucleotides on Lipid Biosynthesis in Mouse Mammary Gland Explants[1] (41647)

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Abstract. The effects of dibutyryl cAMP, 1-methyl-3-isobutyl xanthine (MIX), cGMP, dibutyryl cGMP, and 8-bromo cGMP on the rate of lipid synthesis in mouse mammary gland explants were studied. Dibutyryl cAMP at 10^{-4} *M* selectively inhibited the effect of prolactin on the rate of [^{14}C]acetate incorporation into lipids. At 10^{-3} *M*, dB-cAMP inhibited the effects of insulin, insulin plus cortisol, and prolactin. The phosphodiesterase inhibitor, MIX, inhibited both basal and prolactin-stimulated incorporation rates in a concentration-dependent fashion. These data suggest an inhibitory role for cAMP in the regulation of lipogenesis in the mammary gland. Cyclic GMP, db-cGMP, and 8-bromo cGMP were all without effect on either basal or prolactin-stimulated incorporation rates. Therefore, it appears that cGMP, by itself, is not involved in the regulation of lipogenesis in the mammary gland.

The cyclic nucleotides have been implicated as possible second messengers in mediating the actions of several hormones (1). Several investigators have reported that the onset of lactation in rats and mice is accompanied by increased levels of cGMP and reduced levels of cAMP in mammary cells (2, 3). The actions of prolactin in cultured mammary tissues have also been extensively examined with regard to the possible functions of the two major cyclic nucleotides, 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′5′-cyclic guanosine monophosphate (cGMP).

The effects of exogenously added cyclic nucleotides and inhibitors of cyclic nucleotide phosphodiesterase, such as 1-methyl-3-isobutyl xanthine (MIX) and theophylline, have been tested in attempts to assign a role for these mediators in prolactin's actions. Loizzi *et al.* found that dibutyryl cAMP (dB-cAMP) inhibited lactose synthesis in slices of lactating guinea pig mammary glands (4). Speake and co-workers demonstrated that dB-cAMP and theophylline reduced the activity of fatty acid synthetase in rabbit mammary glands (5). Similar effects have been shown in the mammary glands of rats, where dB-cAMP reduced the rate of synthesis of DNA, RNA, and fatty acids (6); in mice, dB-cAMP reduced the rate of synthesis of RNA and casein (7, 8). These observations, combined with the changes in cAMP levels during pregnancy and lactation, suggest an inhibitory role for this mediator on lactogenesis during pregnancy, which is removed at the onset of lactation.

Cyclic GMP, on the other hand, has been implicated as a possible mediator in the stimulation of lactation. Cyclic GMP has been shown to mimic the prolactin stimulation of RNA synthesis in mouse mammary glands (7). Other investigators have demonstrated that cGMP enhances the rate of RNA synthesis in isolated nuclei from lactating rabbit mammary glands (9). Cyclic GMP has no effect, however, on casein synthesis in mice (8), although it has been reported to increase casein mRNA levels in rat mammary tissues (10), albeit to a small extent.

Prolactin has been shown to cause an increase in the rate of [^{14}C]acetate incorporation into lipids in mammary gland explants (11). The present studies were performed to examine the effects of cyclic nucleotides, their analogs, and inhibitors of phosphodiesterase on the prolactin stimulation of lipid biosynthesis in the mouse mammary gland.

Materials and Methods. Midpregnant (10–14 days of pregnancy) Swiss–Webster mice, used in all experiments, were purchased from Harlan Laboratories, Inc., Indianapolis, Indiana. Day zero of pregnancy was the day sperm were found in the vagina. Ovine pro-

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lactin (NIH-P-S14) and bovine growth hormone (NIH-GH-B16) were gifts from the National Institute of Arthritis and Metabolic Diseases. Other substances were purchased from the following sources: Cortisol from Charles Pfizer Company; Nystatin from E. R. Squibb and Sons, Inc.; Medium 199 Earle's salts, from K. C. Biological Inc.; [1-^{14}C]acetate (57.7 Ci/mmole) from the New England Nuclear Corporation; porcine insulin, penicillin, and streptomycin from the Eli Lilly Company; N^6-2′-dibutyryl cyclic adenosine 3′,5′-monophosphate (dB-cAMP), guanosine-3′,5′-cyclic monophosphate (cGMP), 8-bromo guanosine-3′-5′-cyclic monophosphate (8-bromo cGMP), N^6-2′-*O* dibutyryl cyclic guanosine monophosphate (dB-cGMP), and 1-methyl-3-isobutyl xanthine (MIX) from Sigma Chemical Company.

Methods used to prepare and culture mammary explants were described earlier (11, 12). Briefly, mice were killed by cervical dislocation and the caudal pair of mammary glands removed aseptically and placed in Medium 199 Earle's salts. The glands were cut into pieces weighing 3–5 mg, and four pieces, one from each of four mice, were placed in each vial on siliconized lens paper floating on 2 ml Medium 199 Earle's salts. Each vial of four explant pieces were treated as one observation. All incubations of these explants were carried out in polypropylene vials maintained in a 37°C water bath in an atmosphere of 95% O_2–5% CO_2. Explants were incubated in media containing insulin (1 μg/ml) plus cortisol (0.1 μg/ml) for 24 hr. Prolactin and/or various other agents were then added and incubations continued for 10 hr. [^{14}C]acetate was added for the terminal 2 hr of incubation for the pulse-labeling of lipids.

Following pulse-labeling, explants were removed, blotted, and weighed. They were then homogenized in 2 ml distilled water and the lipids extracted by the method of Bligh and Dyer (13), all at 4°C. One-hundred microliter aliquots of the organic layer were evaporated by dryness in scintillation vials. They were subsequently resuspended in 10 ml Triton X-100 scintillation fluid (52.5 mg 1,4-bis-(5-phenyloxyzol-2yl) benzene (POPOP), 4.2 g 2,5-diphenyloxyzol (POP), 900 ml toluene, and 300 ml Triton X-100 and the radioactiv-

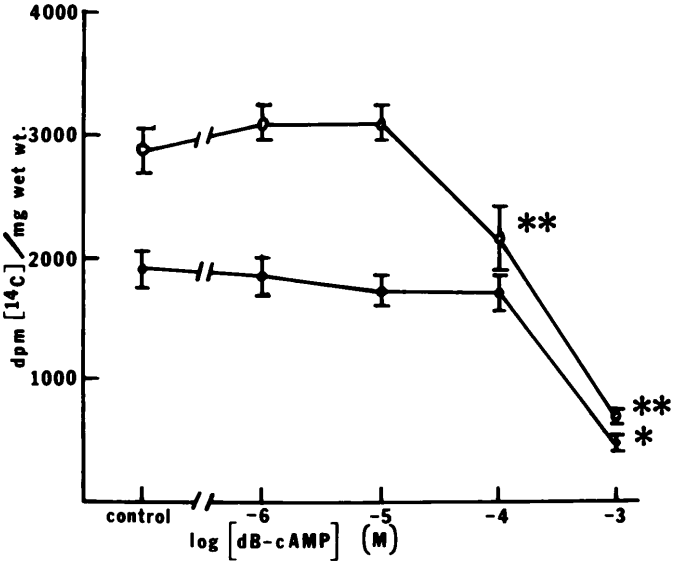

FIG. 1. Effect of dB-cAMP on prolactin stimulation of [^{14}C]acetate incorporation into lipids. Explants were incubated in media containing insulin (1 μg/ml) plus cortisol (0.1 μg/ml) for 24 hr. Dibutyryl-cAMP (●) or dB-cAMP plus prolactin in 1 μg/ml (○) were then added and incubation continued for 10 hr. [^{14}C]acetate (0.5 μCi/ml) was added to all groups for the terminal 2 hr of incubation. Values represent the mean ± standard error of 12 observations. *Significantly less than control at $P < 0.05$. **Significantly less than prolactin at $P < 0.05$.

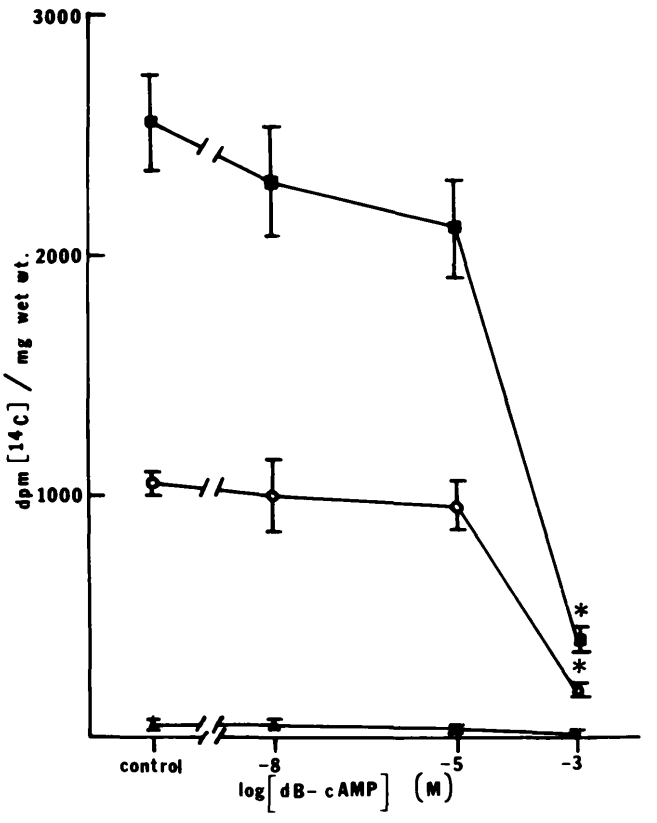

FIG. 2. Effect of dB-cAMP on basal levels of [^{14}C]acetate incorporation into lipids. Explants were incubated in media containing no hormones (●), insulin at 1 μg/ml (○), or insulin plus cortisol at 0.1 μg/ml (■) for 24 hr. Dibutyryl-cAMP was then added to certain groups and incubations continued for 10 hr. [^{14}C]acetate (0.5 μCi/ml) was added for the terminal 2 hr of incubation. Values represent the mean ± standard error of six observations. *Significantly less than control at $P < 0.05$.

ity determined in a liquid scintillation spectrometer. The rate of [^{14}C]acetate incorporation into lipids was expressed as DPM/milligram wet tissue weight, and used as an index of the rate of lipid biosynthesis.

Statistical comparisons were made with Student's *t* test or Dunnet's test.

Results. The effect of dB-cAMP on [^{14}C]acetate incorporation into lipids is shown in Fig. 1. These data show no effect of dB-cAMP on the basal rate of [^{14}C]acetate incorporation until 10^{-3} *M*, where a sharp decrease occurs. Dibutyryl-cAMP at 10^{-4} *M* does, however, attenuate prolactin's action on [^{14}C]acetate incorporation, and attenuates it even further at 10^{-3} *M*. Butyrate, by itself, at concentrations up to 1 m*M* had no effect on the prolactin response. Figure 2 shows the effect of dB-cAMP on the rate of [^{14}C]acetate incorporation into lipids in the presence or absence of insulin, and/or cortisol. Although having no effect on the rate of [^{14}C]acetate incorporation in the absence of hormones in the incubation media, dB-cAMP at 10^{-3} *M* causes marked inhibition of the actions of insulin or insulin plus cortisol on the rate of incorporation of [^{14}C]acetate into lipids.

The effect of 1-methyl-3-isobutyl xanthine (MIX), a potent phosphodiesterase inhibitor is shown in Fig. 3. Although MIX at 0.01 m*M* attenuates the prolactin response without changing basal activity, higher concentrations of the inhibitor decrease both basal and prolactin-stimulated rates of [^{14}C]acetate incorporation. A stimulation over baseline by prolactin is seen at all concentrations of MIX employed.

The effect of cGMP on [^{14}C]acetate incor-

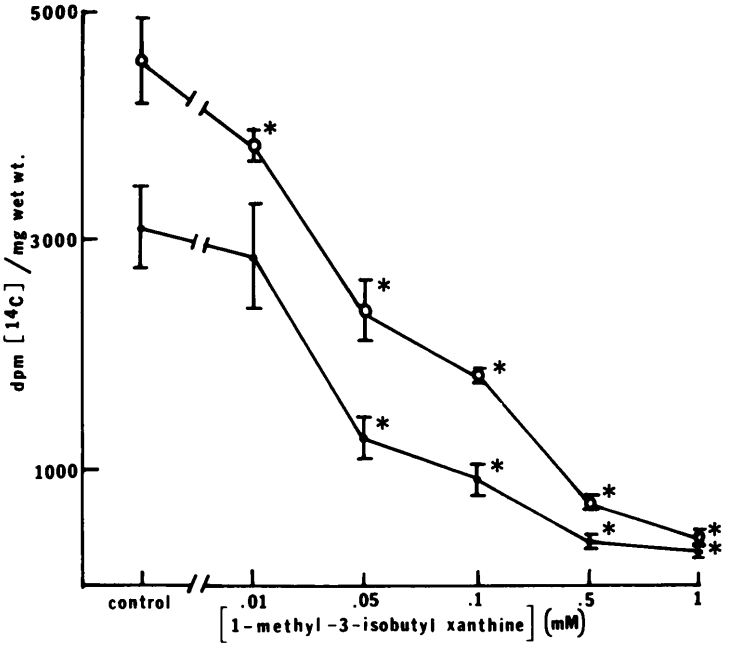

FIG. 3. Effect of 1-methyl-3-isobutyl xanthine (MIX) on [^{14}C]acetate incorporation into lipids. Explants were incubated in media containing insulin (1 μg/ml) plus cortisol (0.1 μg/ml) for 24 hr. Prolactin (1 μg/ml) and/or MIX were then added to certain groups and incubation continued for 10 hr. [^{14}C]acetate (0.5 μCi/ml) was added for the terminal 2 hr of incubation. Values represent the mean ± standard error of six observations. Control (●), prolactin (○). *Significantly less than control at $P < 0.05$.

poration into lipids is shown in Table I. Cyclic GMP from 10^{-6} to 5×10^{-3} *M* has no effect on either the basal rate of [^{14}C]acetate incorporation, or the prolactin response. Table II shows the effect of two cGMP analogs, dB-cGMP, and 8-bromo-cGMP, on the rate of [^{14}C]acetate incorporation. Again, no effect of these compounds is observed on either the basal rate or prolactin-induced rate of [^{14}C]acetate incorporation.

Discussion. Dibutyryl-cAMP at 10^{-4} *M* inhibits the prolactin-induced rate of [^{14}C]-

TABLE I. EFFECT OF CYCLIC GMP ON [^{14}C]ACETATE INCORPORATION INTO LIPIDS[a]

cGMP conc. (*M*)	*N*		dpm ^{14}C incorporated into lipids (dpm/mg wet wt)		*P*	
	C	PRL	C	PRL	C	PRL
0	6	5	1737 ± 222[b]	2975 ± 202	—	—
10^{-6}	6	5	1437 ± 213	3235 ± 422	N.S.*	N.S.**
10^{-5}	6	5	1529 ± 62	2571 ± 92	N.S.	N.S.
10^{-4}	6	6	1535 ± 214	2494 ± 270	N.S.	N.S.
10^{-3}	6	6	1587 ± 189	2581 ± 291	N.S.	N.S.
5×10^{-3}	6	6	1349 ± 195	2363 ± 179	N.S.	N.S.

[a] Explants were incubated in media containing insulin (1 μg/ml) plus cortisol (0.1 μg/ml) for 24 hr. Prolactin (1 μg/ml) and/or cGMP were then added to certain vials and incubation continued for 10 hr. [^{14}C]acetate (0.5 μCi/ml) was added for the terminal 2 hr of incubation.

[b] Values represent the mean ± standard error of *N* observations.

* Significantly different from control (C) group without cGMP.

** Significantly different from prolactin (PRL) group without cGMP.

TABLE II. EFFECT OF 8-BROMO-cGMP AND DIBUTYRYL-cGMP ON [^{14}C]ACETATE INCORPORATION INTO LIPIDS[a]

Agent	N		dpm ^{14}C incorporated into lipids (dpm/mg wet wt)		P	
	C	PRL	C	PRL	C	PRL
None	5	6	2278 ± 142[b]	5969 ± 332	—	—
dB-cGMP (10^{-4} *M*)	5	6	1940 ± 54	5356 ± 294	N.S.*	N.S.**
dB-cGMP (10^{-3} *M*)	6	5	1980 ± 168	4238 ± 428	N.S.	N.S.
8-bromo-cGMP (10^{-5} *M*)	6	6	2239 ± 108	6195 ± 557	N.S.	N.S.
8-bromo-cGMP (10^{-4} *M*)	6	6	2127 ± 163	5126 ± 334	N.S.	N.S.
8-bromo-cGMP (10^{-3} *M*)	6	5	2057 ± 150	5369 ± 95	N.S.	N.S.

[a] Explants were incubated in media containing insulin (1 μg/ml) plus cortisol (0.1 μg/ml) for 24 hr. Prolactin and/or cGMP analogs were then added and incubation continued for 10 hr. [^{14}C]acetate (0.6 μCi/ml) was added for the terminal 2 hr of incubation.

[b] Values represent the mean ± standard error of *N* observations.

* Significantly different than control (C) group without agent.

** Significantly different than prolactin (PRL) group without agent.

acetate incorporation into lipids. This selective inhibition of the prolactin effect correlates well with the findings of numerous investigators examining various other parameters of lactogenesis (4–8, 14). These data suggest an inhibitory role for cAMP in the control of lipid metabolism in the mammary gland. At 10^{-3} *M*, dB-cAMP also inhibits the rates of [^{14}C]acetate incorporation due to insulin, or insulin plus cortisol. This inhibition is of unknown physiological significance.

The data on the effect of MIX support an inhibitory role for cAMP in regulating lipid metabolism in the mammary gland. MIX is reported to be a potent inhibitor of phosphodiesterases (15). Therefore, intracellular cyclic nucleotide levels would be expected to increase in proportion to the concentration of MIX. With the exception of 0.01 m*M*, MIX reduces both basal and prolactin-stimulated [^{14}C]acetate incorporation rates in a concentration-dependent manner. Although prolactin effects are seen at all concentrations of MIX tested, 0.01 m*M* MIX reduces the magnitude of the prolactin-induced [^{14}C]acetate incorporation with no effect on the basal incorporation rate. These data on dB-cAMP and MIX suggest an inhibitory role for intracellular cAMP in regulating lipogenesis in the mammary gland. This is in agreement with the inhibitory action of cAMP on the production of other milk products (4–8, 14). Combined with findings on changes in cAMP levels during pregnancy and lactation (2, 3), it seems likely that cAMP may function to block lipid synthesis in the mammary gland during pregnancy. The removal of this inhibition at parturition may therefore result in an increased rate of lipid synthesis during lactation.

The lack of effect of cGMP or its analogs, dB-cAMP and 8-bromo cGMP, on lipogenesis are in contrast to earlier findings showing a stimulation by cGMP of the synthesis of RNA (7, 9) and casein mRNA (10). However, cGMP alone was reported earlier to have no effect on casein synthesis (8). Cyclic GMP, by itself, apparently does not mediate prolactin's effects on lipid synthesis in the mammary gland.

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