Calcium-Mediated Effects of Cyclic GMP on the Stimulation of Thymocyte Proliferation by Prostaglandin E_1[1] (36244)

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Exposure of rat thymic lymphocytes (thymocytes) to low (10^{-11} to 10^{-10} M) or high (10^{-6} to 5×10^{-6} M) concentrations of cyclic guanosine 3′,5′-monophosphate (cyclic GMP) raises the cell cyclic adenosine 3′, 5′-monophosphate content, and thereby stimulates their proliferation (12). However, intermediate (10^{-9} to 10^{-7} M) cyclic GMP concentrations do not affect the intracellular cyclic AMP level or cell proliferation (12). This striking multiphasic response suggests that, depending on its concentration, cyclic GMP can initiate at least two different reactions (3, 12).

In the present study, we show that the intermediate, nonmitogenic cyclic GMP concentrations have strong, calcium-mediated effects on the mitogenic action of the adenylate cyclase stimulator, prostaglandin E_1 (PGE_1) (4). These cyclic GMP concentrations do not affect PGE_1 ability to raise the cellular cyclic AMP content or to stimulate the initiation of deoxyribonucleic acid (DNA) synthesis, but depending on the extracellular calcium level, they can promote or totally inhibit the eventual progression of these stimulated cells into mitosis.

Materials and Methods. Thymocytes from the thymuses of albino, male (gnotobiotic) rats (180–200 g) were suspended (2×10^8 cells/ml) in a serum-free tissue culture medium, MAC-1 (10), and then incubated at 37° while revolving at 40 rpm. Full details of this procedure have already been presented (8, 10). The proliferative activity of the mitotically competent lymphoblasts, which constitute only 20% of a thymocyte population, was determined by adding the metaphase-blocking agent, colchicine (0.06 mM) to the medium and then measuring the subsequent accumulation, in the numerically constant cell population, of cells in colchicine metaphase (6–13).

Changes in the cellular cyclic AMP content were mainly determined using a method, which has already been thoroughly described and evaluated (8, 13). Briefly, the cyclic AMP-yielding adenosine triphosphate (ATP) pool was labeled by incubating thymocytes in medium containing ^{3}H-adenine. The labeled cells were then removed from the ^{3}H-adenine-containing medium, exposed to the guanine nucleotides (from Sigma Chemical Co., St. Louis) and/or prostaglandin E_1 (from Dr. J.E. Pike, Upjohn Co., Kalamazoo) and their ^{3}H-cyclic AMP content was determined at various times thereafter (8). This method did not measure the absolute intracellular cyclic AMP concentration (8, 12, 13). However, since the total cellular ATP content remained constant throughout the period of observation, the increases in the ^{3}H-cyclic AMP recorded in Fig. 2 could not have been artifacts due to fluctuations in the ^{3}H-ATP content. In some experiments, we measured the total cellular cyclic AMP content by extracting it (at 100°) from sonically disrupted thymocytes with a 50 mM sodium acetate (pH 4.0) solution; and determining its level in the extract, using a specific, cyclic AMP-binding protein from bovine muscle (5). The identity of the extracted substance was confirmed by treatment of the samples with a standard preparation of cyclic AMP-phosphodiesterase isolated from rat brain (2). This enzyme degraded all of the cyclic AMP in the samples.

The proportion of DNA-synthesizing cells

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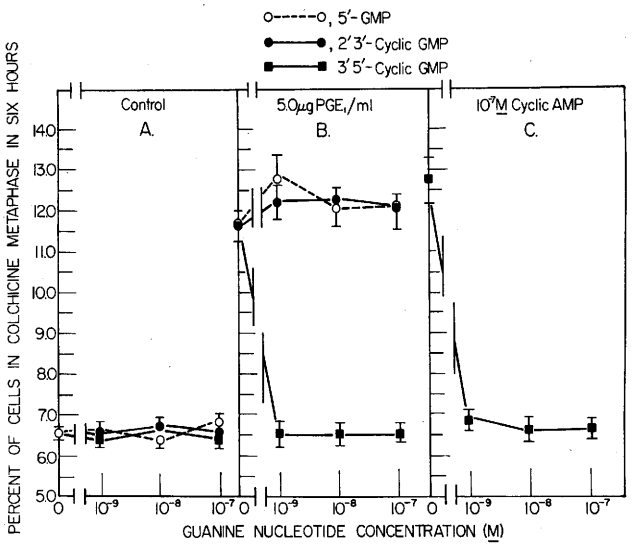

FIG. 1. The effects of 2',3'-cyclic guanosine monophosphate (2',3'-cyclic GMP), 3',5'-cyclic guanosine monophosphate (cyclic GMP) and guanosine 5'-monophosphate (5'-GMP) on the abilities of prostaglandin E_1 (PGE_1) and cyclic AMP to stimulate the proliferation of thymocytes suspended in medium containing 0.6 m*M* calcium. Cell populations were incubated for 6 hr in colchicine-containing medium. The proportions of cells which had flowed into mitosis during this period and accumulated at metaphase were then determined. The values are the means ± SEM of determinations on 10 separate cultures.

in a thymocyte population was determined by suspending the cells in medium containing 6.0 μCi/ml of ^{3}H-thymidine (sp act, 18.0 Ci/mmole; New England Nuclear Corp., Boston). After a 1 hr incubation, the maximum number of cells had entered the DNA-synthetic phase; and the suspended cells were fixed in phosphate-buffered formalin (pH 7.0), washed twice with a cold (4°) solution of 10 m*M* unlabeled thymidine, mounted on slides, and finally covered with a layer of the nuclear track emulsion NTB-2 (Eastman Kodak, Rochester). The subsequent preparation of the autoradiographs and the adequacy of this method for measuring DNA synthesis have already been fully discussed (8, 10, 13).

Results. Exposure of thymocyte populations (in medium containing 0.6 m*M* calcium) to 5.0 μg of prostaglandin E_1 (PGE_1)/ml strongly increased the flow of members of the lymphoblast subpopulation into mitosis (Fig. 1B). PGE_1 action was completely inhibited by exogenous cyclic GMP concentrations between 10^{-9} and 10^{-7} *M*, but it was unaffected by 2',3'-cyclic GMP and guanosine 5'-monophosphate (Fig. 1B). None of the guanine mononucleotides affected the proliferation of untreated cells (Fig. 1A).

Since PGE_1 stimulatory action is mediated by cyclic AMP (4, 13), the inhibitory cyclic GMP concentrations should also inhibit the mitogenic action of cyclic AMP itself (6). As expected, 10^{-9} to 10^{-7} *M* cyclic GMP totally blocked the stimulation by 10^{-7} *M* cyclic AMP of the proliferation of thymocytes suspended in medium containing 0.6 m*M* calcium (Fig. 1C).

Since the intermediate concentrations of cyclic GMP can stimulate one type of cyclic AMP-hydrolyzing phosphodiesterase in broken cell preparations (3), the inhibition of cell proliferation might be thought to be due to a prevention of PGE_1 from raising the cellular cyclic AMP content. However, this was not the case; PGE_1 raised the cyclic AMP level to the same extent in the presence, or absence, of 10^{-7} *M* cyclic GMP (Fig. 2). In the presence of 0.6 m*M* calcium,

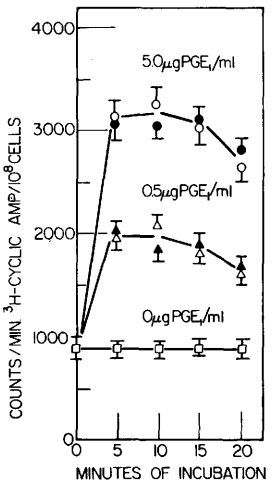

FIG. 2. The effects of prostaglandin E_1 (PGE_1) alone and PGE_1 plus cyclic GMP on the ^{3}H-cyclic AMP level of cells which had been suspended in medium containing 0.6 m*M* calcium and prelabeled with ^{3}H-adenine: (□, △, ○) suspensions without cyclic GMP; and (▲, ●) suspensions containing 10^{-7} *M* cyclic GMP. The values are the means ± SEM of 10 separate determinations.

0.5 and 5.0 μg of PGE/ml raised the ^{3}H-cyclic AMP content of cells (prelabeled with ^{3}H-adenine) by about 100 and 200%, respectively, in 5 min (Fig. 2). In terms of the total intracellular cyclic AMP concentration, 0.5 μg of PGE_1/ml raised the level from 20 ± 1 × 10^{-8} to a maximum of 149 ± 6 × 10^{-8} pmoles/cell, while 5.0 μg of PGE_1/ml raised the level to 262 ± 18 × 10^{-8} pmoles/cell at 5 min.

Cyclic GMP effectiveness in blocking the action of PGE_1 was very strongly influenced by the extracellular calcium concentration. PGE_1 (5.0 μg/ml) maximally stimulated the proliferation of thymocytes suspended in calcium-free medium, but it became progressively less effective as the calcium concentration was raised above 0.2 m*M* (Fig. 3C). Calcium inhibitory effect, like that of cyclic GMP, was not due to an inhibition of cyclic AMP production; the stimulation of cyclic AMP formation by PGE_1 is unaffected by extracellular calcium concentrations between 0 and 1.0 m*M* (13). Inclusion of 10^{-7} *M* cyclic GMP in the calcium-free medium did *not* inhibit the mitogenic action of 5.0 μg of PGE_1/ml; but addition of as little as 0.5 m*M* calcium to the medium enabled cyclic GMP to completely block the action of PGE_1 (Fig. 3C). Varying the extracellular calcium concentration between 0 and 1.0 m*M* in the presence or absence of 10^{-7} *M* cyclic GMP did not affect the proliferation of untreated cells (Fig. 3A). Thus, the intermediate cyclic

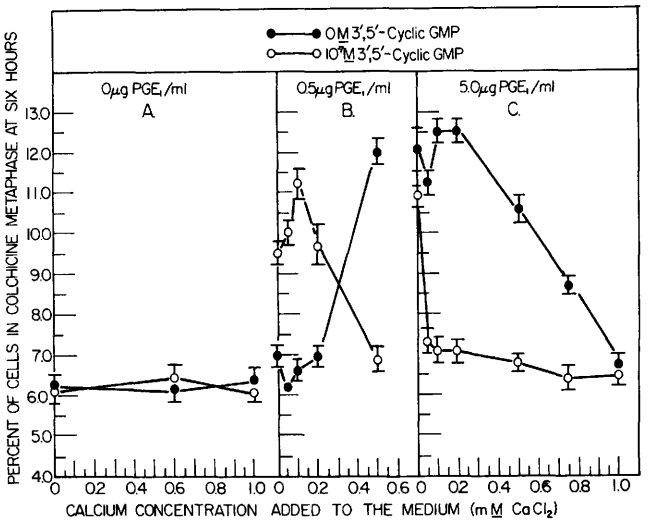

FIG. 3. The effects of calcium and cyclic GMP on the ability of two concentrations of prostaglandin E_1 to stimulate the flow of thymic lymphoblasts into mitosis. The values are the means ± SEM of 8 to 12 separate determinations.

GMP concentrations inhibit the mitogenic action of PGE_1 only when calcium is present. Calcium may therefore be the actual mediator of the cyclic nucleotide action.

Unlike 5.0 μg of PGE_1/ml, 0.5 μg of PGE_1/ml could not stimulate cell proliferation in the absence of calcium, but required an extracellular calcium concentration in excess of 0.2 m*M* to do so (Fig. 3B). Surprisingly, 10^{-7} *M* cyclic GMP actually enabled 0.5 μg of PGE_1/ml to *stimulate* cell proliferation in medium without added calcium (Fig. 3B). The addition of 0.1 m*M* calcium and 10^{-7} *M* cyclic GMP to the medium enabled 0.5 μg of PGE_1/ml to nearly maximally stimulate cell proliferation (Fig. 3B). However, when the calcium level was raised to 0.5 m*M*, the cyclic nucleotide again completely inhibited the action of PGE_1 (Fig. 3B). Thus, since 0.5 μg of PGE_1/ml induces a smaller increase in the cellular cyclic AMP content than 5.0 μg of PGE_1/ml (149×10^{-8} as opposed to 262×10^{-8} pmoles/cell), the actual effect (promotion or inhibition) of cyclic GMP on proliferation depends on the intracellular cyclic AMP level as well as the extracellular calcium concentration.

Since PGE_1, like other cyclic AMP elevators, and cyclic AMP itself (6, 8–11, 13), rapidly (within 1 hr) stimulates the initiation of DNA synthesis (Table I), cyclic GMP and its mediator, calcium, may influence the prostaglandin mitogenic action by affecting this initial process. Despite the different elevations of the intracellular cyclic AMP level caused by 0.5 and 5.0 μg of PGE_1/ml, DNA synthesis was equally stimulated, and was completely unaffected by calcium (Table I). Furthermore, 10^{-7} *M* cyclic GMP also did not inhibit the initial stimulation of DNA synthesis (Fig. 4) under conditions in which it completely blocked the progression of the PGE_1-treated cells into mitosis (Fig. 3B, C). Therefore, intermediate levels of cyclic GMP (and their mediator calcium) only affect the operation of those reactions responsible for the progression of the PGE_1-stimulated, DNA-synthesizing lymphoblasts into mitosis.

TABLE I. A Comparison of the Effects of the Extracellular Calcium Concentration on the Stimulation of DNA Synthesis and Cell Proliferation by Prostaglandin E_1 (PGE_1).[a]

Calcium conc added to medium (m*M*)	Maximum proportion of cells labeled with ^{3}H-thymidine (at 1 hr)	Total percentage of cells which reached colchicine metaphase during 6 hr
	Control	
0	14.0 ± 0.4 (11)	6.6 ± 0.1 (17)
0.5	12.7 ± 0.4 (10)	6.5 ± 0.1 (18)
1.0	13.2 ± 0.2 (13)	6.6 ± 0.2 (19)
	PGE_1, 0.5 μg/ml	
0	22.3 ± 0.6 (15)	6.1 ± 0.3 (10)
0.5	22.0 ± 0.5 (10)	12.0 ± 0.3 (10)
1.0	21.7 ± 0.6 (13)	12.1 ± 0.3 (10)
	PGE_1, 5.0 μg/ml	
0	22.1 ± 1.1 (11)	12.1 ± 0.5 (10)
0.5	21.3 ± 0.4 (10)	10.6 ± 0.3 (10)
1.0	21.3 ± 0.5 (13)	6.7 ± 0.3 (10)

[a] The values are the means ± SEM of the number of separate determinations noted in parentheses.

Discussion. The additional cyclic AMP produced by adenylate cyclase in the membrane of a PGE_1-treated lymphoblast probably initiates the DNA-synthetic and mitogenic processes at an "activation site" which is also in the cell membrane (8, 13) (Fig. 5). This localization of the initiating reactions is based on the fact that exogenous cyclic AMP maximally stimulates DNA synthesis and cell proliferation without entering the cell (6, 7, 9, 10). Cyclic AMP (both endogenous and exogenous) may stimulate the activation site to produce intermediate compounds which enter the cell and start the calcium-insensitive DNA-synthetic reaction and the calcium-sensitive mitogenic process (Fig. 5).

An understanding of cyclic GMP influence on the mitogenic process requires an understanding of the complex effect on this process of its mediator, calcium. We start by considering the promotion of the mitogenic process by calcium in PGE_1-treated cells containing a lower stimulatory cyclic AMP level (149×10^{-8} pmoles/cell; Fig. 3B). Since calcium also powerfully increases the mitogenic effectiveness of exogenous cyclic AMP (13) which

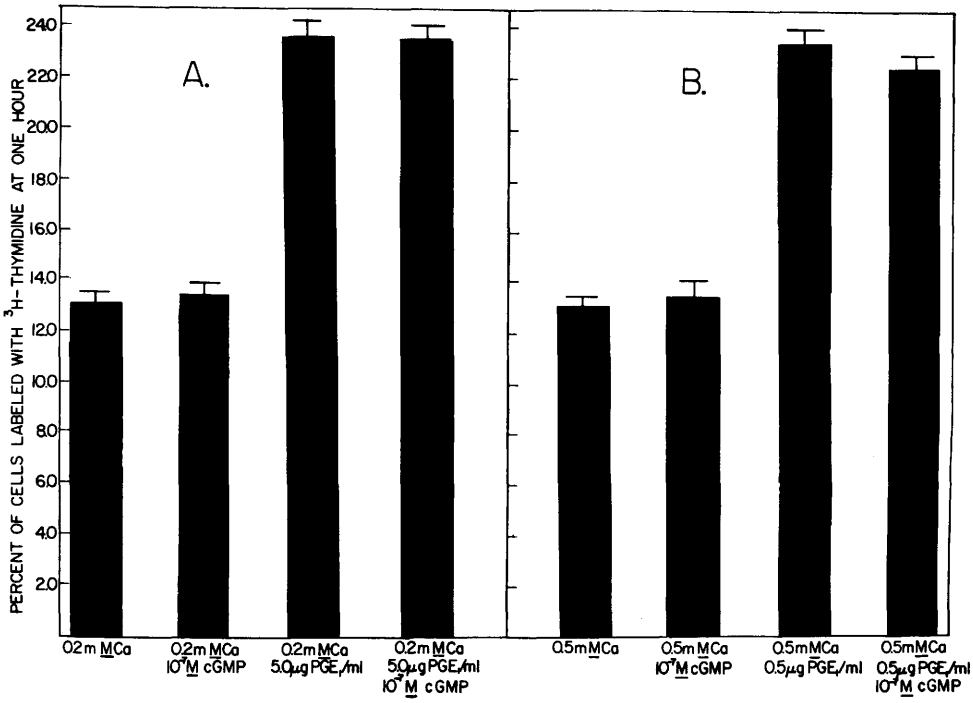

FIG. 4. The inability of 10^{-7} *M* cyclic GMP (cGMP) to affect the PGE_1-induced stimulation of DNA synthesis under conditions in which it completely inhibits the progression of the stimulated cells into mitosis (Fig. 3B, C): The values represent the maximum proportions of DNA-synthesizing cells obtained in the cell populations and are the means ± SEM of 10 separate determinations.

does not enter the cell (7), the promoting reaction may occur at the cell surface; the ion might enable low cyclic AMP levels to stimulate the production of the mitogenic initiator by the activation site (Fig. 5). However, extracellular calcium is not an obligatory component of this reaction since it is not needed when the exogenous (13) or endogenous (Fig. 3B) cyclic AMP level (as raised by PGE_1) is high enough.

The striking inhibition by calcium of the mitogenic process in PGE_1-treated cells containing a higher endogenous cyclic AMP concentration (260×10^{-8} pmoles/cell) indicates that calcium can also block some part of this process. The surface activation site (Fig. 5) is unlikely to be involved in this inhibitory action, since the maximum mitogenic effect of a high concentration (10^{-7} *M*) of the nonpenetrating exogenous cyclic AMP is *not* affected even by 1.0 m*M* calcium (13). This calcium effect may stem from the known ability of endogenous cyclic AMP to mobilize calcium from binding sites in intracellular, "ion-buffering" structures such as mitochondria (1). Therefore, if a large amount of cyclic AMP is produced in the cell membrane, enough of it might (unlike exogenous cyclic AMP) enter the cell and release sufficient calcium to affect the operation of the mitogenic process. When the binding sites contain small amounts of calcium as might be the case in PGE_1-treated (5.0 μg/ml) cells suspended in low-calcium medium, the cyclic AMP coming in from the membrane could not release enough calcium to affect the mitogenic process (Fig. 3C). However, if the medium has a high calcium content and the intracellular binding sites correspondingly contain more calcium, the same amount of incoming cyclic AMP could then release sufficient calcium to stop the mitogenic process (Figs. 3C and 5B).

Cyclic GMP calcium-mediated action

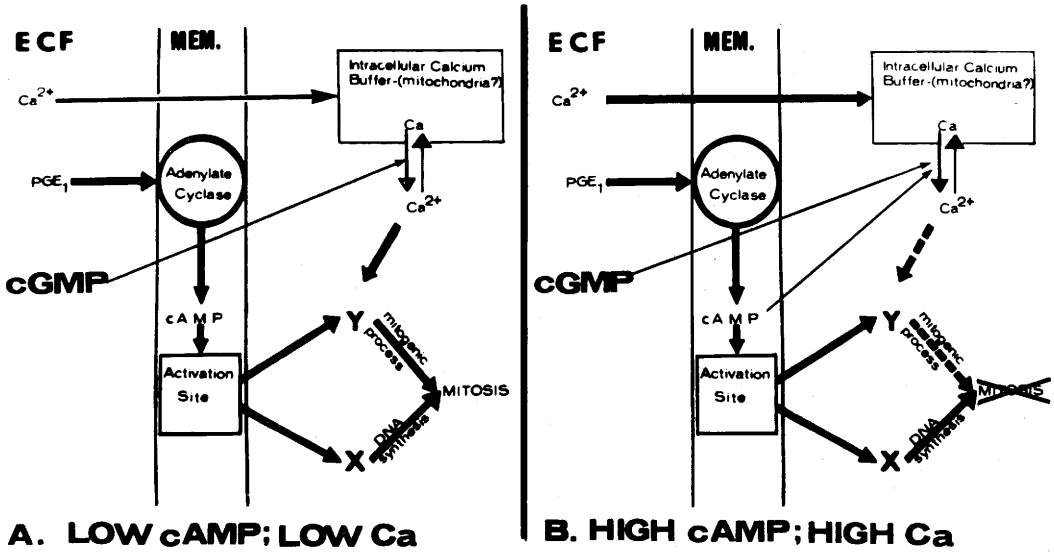

FIG. 5. A summary of mechanisms by which calcium and intermediate cyclic GMP concentrations might affect the operation of the mitogenic process in PGE_1-stimulated thymic lymphoblasts: (cAMP) cyclic AMP; (ECF) extracellular fluid; (cGMP) cyclic GMP; (MEM) cell membrane; (PGE_1) prostaglandin E_1; (→) stimulation; (- - →) inhibition.

could be explained by postulating that it also mobilizes calcium from intracellular binding sites. Thus, in PGE_1-treated cells which are in a medium containing a small amount of calcium and which have a lower cyclic AMP content (149×10^{-8} pmoles/cell), cyclic GMP could substitute for a lack of cyclic AMP coming from the membrane and mobilize enough calcium to *promote* the mitogenic process (Figs. 3B and 5A). Alternatively, if the PGE_1-treated cell contains a high level of endogenous cyclic AMP (260×10^{-8} pmoles/cell) cyclic GMP would add its calcium-mobilizing capacity to that of the incoming cyclic AMP, larger amounts of calcium would be released from the binding sites, and the apparent susceptibility of the mitogenic process to inhibition by calcium would be increased (Figs. 3C and 5B).

Summary. Low (10^{-11} to 10^{-10} M) and high (10^{-6} to 5×10^{-6} M) cyclic GMP concentrations raise the cellular cyclic AMP content and stimulate rat thymocyte proliferation, but intermediate (10^{-9} to 10^{-7} M) cyclic GMP concentrations have no effect on these activities. The intermediate cyclic GMP concentrations also do not affect the ability of prostaglandin E_1 (PGE_1) to raise the cellular cyclic AMP concentration and stimulate DNA synthesis. However, depending on the intracellular cyclic AMP content and the extracellular calcium concentration, the intermediate cyclic GMP concentrations can either strongly promote or completely inhibit progression of the PGE_1-stimulated, DNA-synthesizing cells into mitosis. It is concluded that calcium mediates the actions of cyclic GMP and an attempt is made to explain the complex actions of these agents on the mitogenic process.

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