The Calcium-Independent Stimulation of Thymic Lymphoblast DNA Synthesis by Low Cyclic GMP Concentrations (37687)

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In 1971, we (24) reported that exposure of rat thymic lymphocyte populations to either very low (5×10^{-12}–10^{-10} *M*) or very high (10^{-6}–5×10^{-6} *M*) cyclic GMP (cyclic guanosine 3′,5′-monophosphate) concentrations raised the cellular cyclic AMP (cyclic adenosine 3′,5′-monophosphate) content and stimulated the proliferation of the populations' actively "cycling" lymphoblasts. Intermediate (10^{-9}–10^{-7} *M*) cyclic GMP concentrations, on the other hand, did not by themselves affect either cyclic AMP metabolism or lymphoblast proliferation (24). However, it was subsequently reported (21) that, depending on the extracellular calcium concentration, these seemingly inert intermediate cyclic GMP concentrations can stop, or strongly promote, the progress of already stimulated lymphoblasts from the S(DNA-synthetic) phase into mitosis.

Calcium mediation of the complex actions of the intermediate cyclic GMP concentrations (21), together with its known abilities to raise the cellular cyclic AMP level, promote DNA synthesis, and stimulate lymphoblast proliferation (9, 11, 25, 26), suggested that the ion might mediate the stimulatory actions of the very low cyclic GMP concentrations. Thus, provided there is sufficient calcium in the medium, a low cyclic GMP concentration should stimulate lymphoblast proliferation by promoting the initiation of DNA synthesis. In the present communication, it will be shown that a low cyclic GMP concentration does "trigger" the initiation of DNA synthesis, but, contrary to expectation, extracellular calcium is not involved in this process. Indeed, we will suggest the possibility that instead of calcium-mediating cyclic GMP action, cyclic GMP may be one of the mediators of calcium's proliferative action.

Materials and Methods. Thymus glands were taken from male albino ("specific-pathogen-free") rats weighing between 180 and 200 g. Thymic lymphocyte populations (consisting of actively "cycling" lymphoblasts and postmitotic lymphocytes) were suspended (at a final concentration of about 1.0×10^7/ml) in a complex serum- and plasma-free tissue culture medium (MAC-1) (22, 25), and incubated at 37° while revolving at 40 rpm. Calcium was omitted from the medium, and in some experiments, the specific calcium chelator EGTA (ethylene glycol-*bis*-[β-aminoethyl ether]*N*-tetraacetic acid from Sigma Chemical Co., St. Louis, MO) was included to inactivate all traces of ionic calcium. The experiments began (*i.e.*, the nucleotides were added) only after a preincubation for 30 min to allow the cells to recover from the shock of being taken from the animal.

To study the effects of cyclic GMP and other guanosine mononucleotides (from Sigma Chemical Co.) on the flow of the actively "cycling" lymphoblasts into, and through, the DNA-synthetic phase of their cycle, we determined the changes with time of the proportion of cells which incorporate [^{3}H]thymidine into their nuclei in populations exposed to 0.06 m*M* colchicine. It should be noted that this colchicine concentration does not affect the entry of lymphoblasts into the S phase of their cycle, but it stops their progression through mitosis and prevents the initiation of another DNA-division cycle. For 0-time values, [^{3}H]thymidine (specific activity 18 Ci/mmole, New England Nuclear Corp., Boston, MA) was added 15 min before the colchicine and nucleotides, and then

at 0.75, 1.75, 2.75, etc., hr of incubation afterwards, to give a final radioactivity level of 10.0 μCi/ml in each suspension. Fifteen minutes later (at 0, 1, 2, or 3, etc., hr), the "flash"-labeled cells were fixed (while still suspended) in phosphate-buffered formalin (pH 7.0), washed twice (at room temperature) with a 10 m*M* unlabeled thymidine solution, mounted on slides, and finally covered with a layer of the nuclear track emulsion NTB-2 (Eastman Kodak, Rochester, NY). The final preparation of the autoradiographs and the validity of the method have been discussed previously (25).

In these short-term experiments, we have studied the effects of guanine nucleotides on the first stimulated cycle of proliferation of the lymphoblasts which constitute from 20 to 25% of thymic lymphocyte populations from our strain of rat, and are responsible for the continuous production of the larger subpopulation of postmitotic small lymphocytes in the thymus gland (3, 13). The proliferative activity of the lymphoblasts was determined using colchicine. In the presence of 0.06 m*M* colchicine *in vitro,* lymphoblasts continued to flow into mitosis as rapidly as in the thymus gland of the colchicine- (or colcemide)-treated rat (11, 14, 26), and it was assumed that their proliferative activity was proportional to the rate of accumulation of cells in colchicine metaphase.

It is important to note that none of the changes to be reported arose from alterations of the total cell concentration in the colchicine-containing suspensions. Neither the nucleotides nor EGTA significantly changed the total cell concentration as determined with a Coulter model B electronic cell counter (Coulter Electronics, Hialeah). Furthermore, most (85–90%) of the small lymphocytes stayed alive as indicated by their normal nuclear structure (20).

Results. The proportion of cells making DNA (*i.e.,* which incorporated [^{3}H]thymidine into their nuclei) in thymic lymphocyte populations in calcium-free MAC-1 medium rose slightly (1.2 times) during the first 2–3 hr after the addition of colchicine (Fig. 1). However, the proportion had returned to the starting value by 5 hr (Fig. 1). Throughout the incubation period, lymphoblasts flowed steadily into mitosis and accumulated at the colchicine-blocked metaphase stage of the mitotic sequence (Fig. 1).

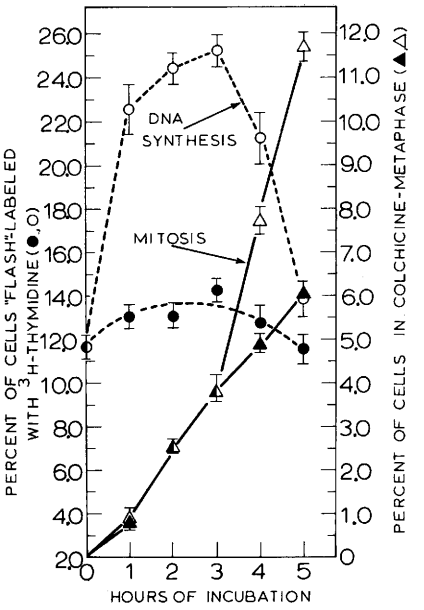

FIG. 1. The ability of a very low concentration of cyclic GMP (cyclic guanosine 3′,5′-monophosphate) to stimulate DNA synthesis and proliferation of lymphoblasts in thymic lymphocyte populations which were isolated and preincubated for 30 min in calcium-free MAC-1 medium. Colchicine and cyclic GMP were added at 0 hr: (●, ▲) control suspensions; (○, △) suspensions containing 5×10^{-11} *M* cyclic GMP. The points are means ± SEM of 9 separate determinations.

Addition of 5×10^{-11} *M* (the most effective of the mitogenic low concentrations) cyclic GMP to the medium along with colchicine caused a large fraction (about half) of the population's lymphoblasts to start making DNA, and during the next 2–3 hr, the proportion of cells incorporating [^{3}H]thymidine reached a peak value which was over twice the initial value (Fig. 1). After 3 hr, the stimulated lymphoblasts rapidly left the S phase and began to surge into mitosis soon afterwards (Fig. 1). The same concentration of another cyclic guanine nucleotide, 2′,3′-cyclic GMP (cyclic guanosine 2′,3′-monophosphate), or guanosine 5′-monophosphate did not significantly affect DNA synthesis or proliferation (Fig. 2).

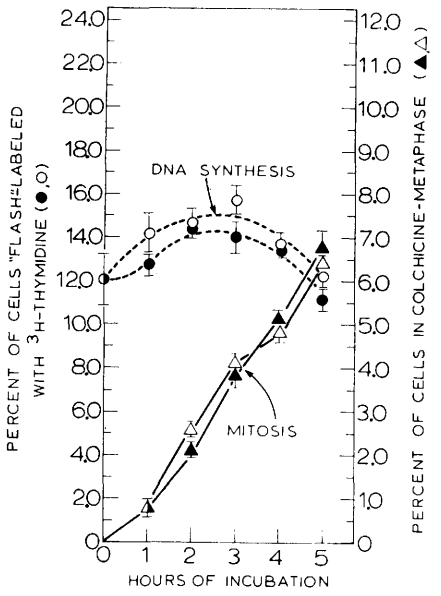

FIG. 2. The inabilities of very low concentrations of cyclic guanosine 2′,3′-monophosphate (2′,3′-cyclic GMP) and guanosine 5′-monophosphate (5′-GMP) to significantly affect DNA synthesis and proliferation of lymphoblasts in thymic lymphocyte populations which were isolated and preincubated for 30 min in calcium-free MAC-1 medium. Colchicine and nucleotides were added at 0 hr: (●, ▲) suspensions exposed to 5×10^{-11} *M* 5′-GMP; (○, △) 5×10^{-11} *M* 2′,3′-cyclic GMP. The points are means ± SEM of 6 separate determinations.

Since no calcium was added to the MAC-1 medium, these observations strongly suggested, but did not prove, that extracellular calcium plays no role in cyclic GMP's stimulatory action. Proof of calcium independence was provided by the observation that 5×10^{-11} *M* cyclic GMP "triggered" DNA synthesis even in the presence of EGTA concentrations (0.2 and 0.4 m*M*) which were far higher than needed to inactivate the 0.02 m*M* calcium normally present in the extracellular fluid of thymic lymphocyte populations in calcium-free MAC-1 medium (22).

Discussion When thymic lymphocyte populations are suspended in a calcium-free medium, only those lymphoblasts which have started making DNA before isolation can proceed afterwards to synthesize DNA and progress into mitosis (11, 26). The rest of the lymphoblasts stay in the pre-DNA-synthetic G1 phase of their cycle unless calcium is added to a concentration greater than 1.0 m*M* (11, 26). However, as we have seen, exposure to a very low cyclic GMP concentration (5×10^{-11} *M*) can replace calcium completely. Like calcium (9), this cyclic nucleotide briefly raises the cellular cyclic AMP level (24) and causes the blocked lymphoblasts to enter rapidly the S phase of their cell cycle. The present observations show that extracellular calcium is not involved in cyclic GMP's stimulatory action. Parenthetically, this calcium independence of the action of the very low cyclic GMP levels must not be confused with the previously reported (21) mediation by calcium of the entirely different actions of much higher (and possibly nonphysiological) cyclic GMP concentrations on the progression of already stimulated (*e.g.*, by prostaglandin E_1) (5, 22, 23) lymphoblasts through the G2 phase of their cycle.

Whether the proliferative action of the very low cyclic GMP concentration is physio-

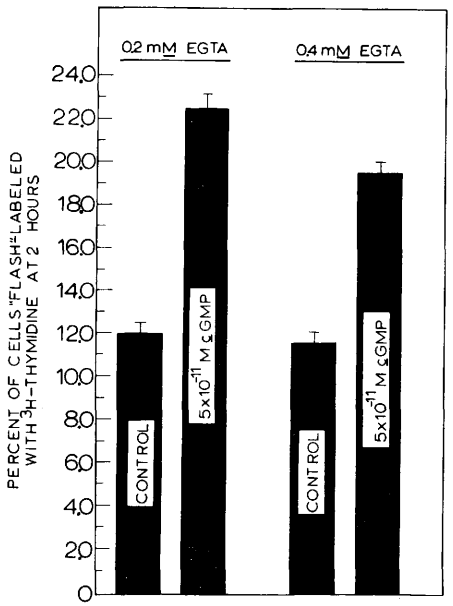

FIG. 3. A demonstration of the lack of involvement of calcium in the cyclic GMP-induced stimulation of lymphoblast DNA synthesis. Thymic lymphocyte populations were isolated in calcium-free MAC-1 medium containing the specific calcium chelator EGTA and preincubated for 30 min. Colchicine and cyclic GMP were added at 0 hr. The values are means ± SEM of 11–13 separate determinations.

logically important remains to be determined. The cyclic nucleotide may activate the same pre-S reactions as extracellular calcium which, either alone or in concert with the parathyroid hormone, normally maintains the proliferation of such cells as thymic lymphoblasts (*in vivo* and *in vitro*), erythroid and lymphoid bone marrow cells (*in vivo*), hepatocytes during liver regeneration, and normal fibroblasts (*in vitro*) (1, 9, 11, 14–17, 26). In view of the stimulatory efficacy of very low cyclic GMP concentrations, fluctuations of the nucleotide's intracellular level should considerably modify calcium's physiologically important regulatory action.

On the other hand, it is cyclic AMP which is known to directly mediate calcium's proliferative action on thymic lymphoblasts (26). This conclusion is based on the facts that mitogenic calcium concentrations raise the cellular cyclic AMP content (9), and low (10^{-8}–10^{-6} *M*) cyclic AMP concentrations completely mimic calcium's stimulatory actions on DNA synthesis and proliferation both *in vivo* and *in vitro* (4, 11, 12, 18, 22–26). However, calcium's cyclic AMP-elevating ability seems to be entirely inconsistent with its equally well-demonstrated ability to *lower* thymocyte adenylate cyclase activity (25). The present observations might contribute to the eventual resolution of this puzzling contradiction. Since a low cyclic GMP concentration mimics all of calcium's actions on thymic lymphocytes, including the elevation of the cellular cyclic AMP level (24), cyclic GMP might be calcium's first intracellular messenger, and cyclic AMP would then be cyclic GMP's mediator. So far, using a sensitive radioimmune assay technique, we have been unable to show that calcium raises the average cellular cyclic GMP content in thymocyte populations. However, it would be expected from the present observations that stimulatory increments in the cellular cyclic GMP content would be very small, and these small changes might well occur in only half of the lymphoblast subpopulation. Thus, this important possibility merits further testing with other techniques, since calcium is known to affect the cyclic GMP content of several other tissues (19) and to increase guanylate cyclase activity (2, 7). Furthermore, a burst of cyclic GMP formation (6) seems to be associated with the very much increased flow of calcium into human lymphocytes caused by phytohemagglutinin (27, 28).

Summary. A very low cyclic GMP concentration (5×10^{-11} *M*) causes the actively "cycling" lymphoblasts in isolated rat thymic lymphocyte populations to start synthesizing DNA by a calcium-independent process. These stimulated cells are able to progress subsequently through the DNA-synthetic phase and enter mitosis. The possible significance of the cyclic GMP-induced stimulatory action in the regulation of lymphoblast proliferation is discussed. An attempt is also made to interrelate the similar actions of cyclic GMP and cyclic AMP.

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