Vol. 90, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 12, 1979 Pages 810-818

EFFECT OF CALCIUM ON THE CYCLIC GMP ELEVATION INDUCED BY THYMOSIN FRACTION 5

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Received August 23,1979

SUMMARY: Thymosin fraction 5 induces an increase in cyclic GMP but not cAMP in murine thymocytes. Calcium (0.6 mM) is necessary for an optimal response in both phosphate buffered saline and hepes-buffered RPMI 1640 media. The calcium dependence of the cGMP response was most pronounced in a minimal salts medium (PBS) and higher concentrations (greater than 0.8 mM) caused a lessening of the cGMP elevation induced by thymosin. Basal cGMP levels of thymus and spleen lymphocytes vary with increasing concentrations of calcium (0-1 mM) and to a lesser extent, the levels of cAMP also are increased. Calcium uptake was measured both at mitogenic levels of Con A and at thymosin concentrations which were similar to those necessary for the increase in cGMP. The results suggest that calcium and cGMP play an important role in T cell differentiation under the influence of thymosin.

Thymosin, a family of polypeptides isolated from bovine thymus glands, has proven useful in the restoration of immunity in children with demonstrated thymic deficiencies and has been found to improve immune function in cancer patients and is effective as an adjuvant to chemotherapeutic treatment of certain cancers (1-3). Recent investigations, however, have suggested that changes in second messenger levels may act as important signals in molecular events involved in T cell maturation (4-11). These studies, while not conclusive, do suggest that thymus factors may exert their effect on differentiation of various populations via a diverse set of mechanisms. Thus both cyclic AMP and cyclic GMP mediated events have been proposed. This is based on experiments suggesting that 1) the addition of exogenous cyclic nucleotides mimics some responses of lymphocytes to thymus factors and 2) thymus factors increase cyclic AMP or cyclic GMP in target lymphocytes (for review, see 4).

Although calcium has been known to play an essential role in lymphocyte activation and differentiation, only recently has calcium been classified as a second messenger (12,13). In this communication we report that the cyclic GMP elevation induced by thymosin in murine thymocytes is calcium dependent. An influx of calcium under conditions in which an increase in cyclic GMP can be demonstrated is also

ABBREVIATIONS:

PBS, phosphate buffered saline; HRPMI, Hepes buffered Roswell Park Memorial Institute medium.

0006-291X/79/190810-09$01.00/0

reported. Finally, experiments suggesting conditions under which basal cyclic nucleotide levels vary with the calcium concentration of the media also indicate an importance of calcium.

MATERIALS AND METHODS

Animals. C57Bl/6 mice were obtained from Jackson Laboratories, Bar Harbor, Me. Nude mice (BALB/c background) were from our own colony. Mice used were from 6-12 weeks of age.

Media. Dulbecco's phosphate buffered saline (calcium-magnesium free) and Hepes buffered RPMI (HRPMI) were purchased from Gibco, Grand Island, N.Y. Calcium-magnesium free HRPMI was prepared according to the published formula in the Gibco catalog and Hepes buffer added with a reduction in the amount of NaCl to maintain osmolarity.

Preparation of Lymphoid Cell Suspensions. The lymphoid cells were prepared in the buffers indicated without calcium and were treated with ACT (0.15 mM ammonium chloride buffered with 0.17 M Tris, pH, 7.2) to lyse the red cells, as previously described (9). Incubations (37°, 5% CO_2/air) were 30 minutes in the calcium concentration indicated to allow the levels of cyclic nucleotides to stabilize before the addition of agents for the 5 minute incubation. The reactions were terminated by quick freezing the tubes containing the incubation mixtures in a dry ice-ethanol bath for one minute, followed by three minutes in a boiling water bath.

Cyclic Nucleotide Assays. Cyclic AMP and cyclic GMP were assayed as previously described (9,14,15). In short, cAMP was assayed in 250 x g supernatants after addition of ^{125}I-cAMP and cAMP specific antisera prepared by Schwarz-Mann, Vineland, N.J. via the procedure of Steiner et al. (16). Cyclic GMP was assayed in a similar manner except that the cGMP in the sample was acetylated by the addition of 0.005 ml of a mixture of triethyl amine and acetic acid (2:1) to the 0.1 ml aliquots of sample (17).

Thymosin. Thymosin fraction 5 was prepared for us by Hoffman-LaRoche, Nutley, N.J. and was the same endotoxin free material as used in previous publications (9, 14,15). Spleen fraction 5 was prepared exactly as thymosin fraction 5 except from spleen tissue.

Calcium Assay. Beckman microfuge tubes (0.4 ml, Beckman Instruments, Fullerton, Ca.) were prepared by layering 0.05 ml of formic acid below a layer of 0.1 ml of silicon oil (Versilube 50, General Electric Silicone Division, Waterford, N.Y.) (18, 19). Cells (0.5 ml prepared as above in HRPMI), agents (0.05 ml at 3x concentration in HRPMI) and ^{45}Calcium (0.5 ml of a 1 mCi/ml HRPMI) were then mixed and incubated for the desired times (5 minutes in the experiments reported) at 37°C. The uptake at the times and concentrations desired was then determined by layering 0.1 ml of incubation mixture on top of the silicon oil in the microfuge tubes and centrifuging for 3 minutes at 10,000 x g in a Brinkman Microfuge (Model 52, Brinkman Instruments, Canterbury, N.Y.) to separate the cells from the medium. The microfuge tubes were frozen and the tip (containing the cells) was removed by cutting at the oil-acid interface. The tips were then placed in Aquasol for counting.

Mitogen Assays. The mitogen response of thymus lymphocytes was assayed as outlined previously (14) utilizing the MASH harvester. Cells were pulsed (1 μCi of ^{3}H-thymidine/cell (1.9 Ci/mMole) was then added for 8 hours) on day 2, harvested and counted.

RESULTS

The cyclic AMP and cyclic GMP assays were independent of both the calcium concentration (from 0 mM to 1.2 mM) and the buffer utilized (PBS or HRPMI). For example, binding to the cAMP specific antibody in the presence of 50 pmoles of cAMP was

TABLE 1

CALCIUM DEPENDENCE OF THE CYCLIC GMP RESPONSE OF THYMOCYTES TO THYMOSIN FRACTION 5

Calcium[a] (mM)	Cyclic AMP (pmoles/10^7 cells) BASAL	+ THYMOSIN[b]	Cyclic GMP(fmoles/10^6 cells) BASAL	+ THYMOSIN	Increase in cGMP[c]	SI corr.[d]
a) Phosphate buffered saline						
0	1.80 ± 0.02	1.91 ± 0.05	3.37 ± 0.34	8.92 ± 0.37	(5.55)	0.75
0.1	2.30 ± 0.02	2.58 ± 0.26	3.00 ± 0.28	10.21 ± 0.36	(7.21)	1.27
0.3	2.75 ± 0.12	3.00 ± 0.18	11.07 ± 0.31	19.37 ± 0.14	(8.30)	1.17
0.6	4.51 ± 0.07	4.44 ± 0.30	33.21 ± 1.57	59.01 ± 4.36	(25.80)	1.58
0.8	4.12 ± 0.12	4.90 ± 0.33	40.91 ± 0.58	52.06 ± 4.65	(11.15)	1.11
b) Hepes buffered RPMI						
0	1.46 ± 0.11	1.26 ± 0.07	1.52 ± 0.41	2.53 ± 0.19	(1.01)	0.06
0.1	1.52 ± 0.04	1.63 ± 0.04	1.38 ± 0.52	2.10 ± 0.75	(0.72)	-0.23
0.2	1.82 ± 0.05	1.56 ± 0.13	1.32 ± 0.50	3.84 ± 0.53	(2.52)	1.06
0.5	2.40 ± 0.22	2.18 ± 0.18	4.09 ± 0.23	7.75 ± 0.20	(3.66)	1.30
0.8	1.70 ± 0.11	1.70 ± 0.16	1.18 ± 0.27	4.20 ± 0.08	(3.02)	1.50
1.0	1.90 ± 0.24	1.85 ± 0.11	1.39 ± 0.21	3.72 ± 0.24	(2.33)	0.92

[a] Calcium was added as $CaCl_2$ to give the concentration indicated. Visible precipitates were formed when concentrations of 0.8 mM were reached.

[b] Incubation with thymosin was as indicated in the text (100 μg/ml for 5 min at 37°C).

[c] Increase is the value of cGMP in thymocytes incubated with thymosin minus the value of the media control for that calcium concentration (expressed as fmoles/10^6 cells).

[d] SI corr. is the value after thymosin incubation-crossreacting material measured in thymosin without cells/media control. In PBS the crossreacting material was 6.38 ± 0.25 fmoles/aliquot; in HRPMI it was 2.43 ± 0.28.

35.35 ± 0.64% in calcium-free PBS and 36.31 ± 0.43% in 1.2 mM $CaCl_2$. At acetylated cyclic GMP values similar to those in the assay (e.g. 10 fmoles/0.1 ml) binding was 28.61 ± 1.95% and 27.73 ± 0.09%, respectively.

Calcium added to thymocytes isolated in calcium free buffer, caused an increase in cGMP (10-fold; Table 1). Basal cyclic AMP values were also elevated but only by about 2-fold (Table 1). This increase was rapid (detectable at 5 minutes) and stabilized after 30 minutes (data not shown). Thymosin did not increase cAMP values at any concentration of calcium in either buffer (Table 1) when compared to the appropriate control. Thymosin did increase cGMP (above the value due to the cross-reacting material) at all calcium concentrations. This increase was optimal at 0.6 mM $CaCl_2$ in PBS and between 0.5 and 0.8 mM $CaCl_2$ in HRPMI. The calcium dependence of the elevation of cyclic GMP was greatest in PBS whether the effect of calcium alone or the addition of thymosin were measured (Table 1). Spleen fraction 5 (although it also contained some crossreactivity in the cyclic GMP assay) did not cause an elevation in cGMP (above the crossreacting material value) at any calcium concentration in any cell population (Table 2). Thymosin fraction 5 elevated cGMP in thymocytes at the optimal calcium concentration but not in nude mouse spleen lymphocytes (Table 2). As previously reported (15), normal spleen lymphocytes responded with an increase in cGMP but the increase was not statistically significant.

To confirm the calcium dependence of the response to thymosin, calcium uptake was measured (18,19). Con A induced an increase in calcium influx at concentrations which were mitogenic for thymocytes and this response was taken as an indication of the validity of our procedure (19) (Table 3). Utilizing these conditions, thymosin fraction 5 caused an influx of calcium when measured at 5 minutes post addition of fraction 5 (Figure 1). When spleen lymphocytes were utilized, the influx noted was not statistically significant (data not shown).

DISCUSSION

Although the importance of calcium in lymphocyte responses (e.g. activation, proliferation, differentiation) has been recognized, the specific mechanisms through which calcium influences such events remains unclear (12,13). Our studies support the recent suggestions that calcium and cyclic GMP interact in lymphocytes (12,13, 19) and indicate for the first time a calcium involvement possibly via cGMP in the differentiation of lymphocytes under the influence of thymosin. The thymosin induced increase of cyclic GMP in target thymocytes was enhanced by the addition of calcium and was most pronounced when the media in which the cells were incubated was Dulbecco's phosphate buffered saline. Cell viability was not significantly effected by the lack of calcium (90-95%) after the 30 minutes at 37°C) and the addition of glucose did not effect the response. As previously reported, LPS increased both cAMP and cGMP in thymocytes (14) and the cGMP increase was calcium dependent (un-

TABLE 2

CALCIUM DEPENDENCE OF CYCLIC GMP RESPONSE OF LYMPHOCYTES TO THYMOSIN FRACTION 5 AND SPLEEN FRACTION 5[a]

	Basal cAMP (Fmole/10^7 cells)	Basal cGMP (Fmole/10^6 cells)	Increase in Cyclic GMP + Thymosin Fr. 5	(Fmoles/10^6 cells)[d] +Spleen Fr. 5
Thymus[b]				
0.0[c]	1.31 ± 0.05	1.98 ± 0.21	(4.06 ± 0.46)[d]	(2.00 ± 0.20)
0.4	2.93 ± 0.05	37.31 ± 0.27	18.14 ± 0.90 ($P<.005$)	(1.86 ± 0.08)
0.8	2.76 ± 0.06	34.48 ± 1.85	7.44 ± 0.37 ($P<.05$)	(2.06 ± 0.16)
Spleen				
0.0	3.07 ± 0.35	4.09 ± 0.13	(3.96 ± 0.12)	0.59 ± 0.04 NS
0.4	4.69 ± 0.33	34.26 ± 2.20	10.23 ± 0.67 NS	(2.16 ± 0.13)
0.8	6.75 ± 0.12	26.79 ± 2.15	-5.37 ± 0.42 NS	(3.60 ± 0.28)
Nude Spleen				
0.0	4.26 ± 0.15	6.72 ± 0.23	(2.97 ± 0.08)	0.16 ± 0.01 NS
0.4	5.00 ± 0.12	35.51 ± 2.42	(4.00 ± 0.27)	(3.57 ± 0.74)
0.8	7.32 ± 0.30	36.21 ± 2.08	(-1.11 ± 0.21)	(3.58 ± 0.18)

[a] See text for details of assay.

[b] Thymus and spleen were from C56Bl/6J mice, nude mice spleens were from nu/nu Balb/c.

[c] Calcium was added as $CaCl_2$ to calcium-magnesium free PBS in the concentrations indicated.

[d] Significance was determined through the use of student's t test. Values in parenthesis indicate that the value is not significantly different from the crossreacting material (thymosin = 4.95 ± 0.30, spleen fr. 5 = 1.96 ± 0.21.) Significance in all cases is compared to the appropriate control without subtraction for crossreacting material (NS = $P>.05$).

Table 3

CON A INDUCED CALCIUM UPTAKE AND PROLIFERATION[a]

Con A (ug/ml)	Calcium uptake (CPM) Experiment 1	Calcium uptake (CPM) Experiment 2	^{3}H-thymidine incorporation Experiment 2
0	331 ± 22	1782 ± 258	1749 ± 507
0.5	--	--	7224 ± 360
1	--	--	10295 ± 600
3	667 ± 94	2991 ± 146	18363 ± 928
5	759 ± 68	3150 ± 439	20503 ± 778
10	501 ± 34	3441 ± 490	24158 ± 2636
20	505 ± 22	2725 ± 352	21745 ± 1882
30	--	2459 ± 153	--

[a] See text for details of assays for calcium uptake at 5 minutes and mitogen response at two days.

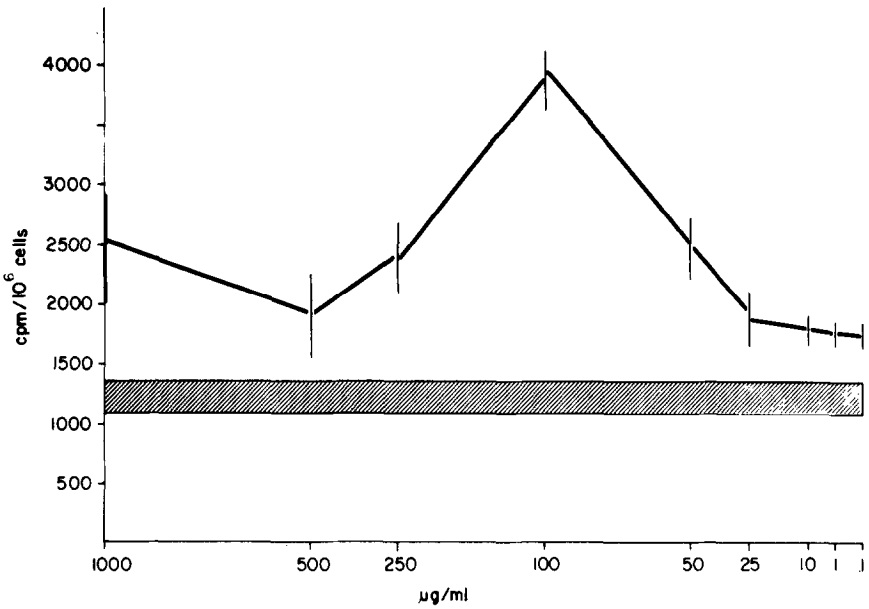

Figure 1. Thymosin induced calcium uptake. When assayed as described in text (37° for 5 minutes), thymosin fraction 5 induced uptake of ^{45}calcium in murine thymocytes in a dose dependent manner. The hatched line represents the values for 12 samples which contained no thymosin and each point is the mean ± standard error for samples in quadruplicate.

published observation). Whether complex media such as HRPMI contains material which inhibits the calcium induced increase or whether PBS affects cells such that they are more responsive, is not clear. Since other investigators often use Dulbecco's minimal essential media which is higher in calcium (1.2 mM) such media may not allow the demonstration of a cyclic GMP elevation by thymosin, or may reduce the response measured.

Calcium influx can be measured by several methods, but the advantage of the procedure of Freeman et al. (19) is that any local equilibrium which has been established around the cell is not disturbed and the backgrounds in many cases are lower than with filter paper or cell pelleting procedures (18, unpublished observations). Although this procedure measures "associated" calcium in addition to bound and influx calcium, recently Jensen and Rasmussin (21) have demonstrated that in lymphocytes only 30-40% of the counts are external and as many as 60-70% are actually internalized. In agreement with the results of other investigators, we demonstrated that Con A induced calcium uptake with a dose response similar to that for its mitogenic effect (19-23). Freeman et al. also reported that LPS

did not induce an influx of calcium (19). As seen in Figure 1, calcium influx in thymocytes occurred at concentrations of thymosin which resulted in the optimal increase in cyclic GMP.

Thymosin fraction (which contains several polypeptides) increases cGMP in a subpopulation of lymphocytes (15). This increase is not mimicked by spleen fraction 4 (Table 2, ref. 9) and is not found in nude mice spleen lymphocytes (15). The results of our experiments to date thus suggest that thymosin induces a calcium influx and cyclic GMP elevation in a target population of lymphocytes and that these events may serve as a signal to the precursor cell to differentiate into a more mature thymus dependent lymphocyte.

ACKNOWLEDGMENTS

We would like to thank Dr. H. Sheppard for his helpful suggestions during the course of this work. Portions of this study were supported by a McLaughlin pre-doctoral fellowship to PHN, and in part by grants from the National Cancer Institute (CA-16964 and CA-20667), Hoffman LaRoche, Inc., and the J.A. Hartford Foundation. We would like to thank Sandi Randazzo for her assistance in preparing the manuscript

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