

NON-HORMONAL BURST IN THE LEVEL OF cAMP CAUSED BY A 'TEMPERATURE SHOCK' TO MOUSE THYMOCYTES

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1. Introduction

In the course of some studies related to the effect of various stimulants and hormones on isolated viable thymocytes [1,2] we obtained variable results in the levels of intracellular cAMP and the state of activation of cAMP-dependent protein kinase (EC 2.7.1.37). A systematic attempt to clarify the cause of this variability led us to the finding that the 'temperature shock' caused to thymocytes upon transfer from 4–37°C triggers the cells, elevates their cAMP levels, and activates their cAMP-dependent protein kinase. We wish to describe here this non-hormonal stimulation of cells and show how it can lead to erroneous results in monitoring and interpreting the consequences of hormonal stimuli *in vivo*.

2. Materials and methods

Thymus glands were removed from 4–6 week old C3HeB/BL F1 mice and transferred immediately to an RPMI-1640 buffer supplemented with 25 mM Hepes

(buffer A), kept at 4°C. All the procedure hereafter were carried out at 4°C. The glands were washed with the same medium, blood clots were carefully removed, and the free thymocytes were obtained by pressing the thymus glands through a metal mesh plate. The aggregated material was allowed to settle for 1 min and the homogeneous cell suspension was transferred to another tube and washed 3 times by centrifugation (5 min) at 900 × *g* and resuspension in buffer A. Finally the cell suspension was adjusted to ~ 1 × 10⁷ cells/ml. This preparation contained > 95% viable cells as judged by the standard Trypan Blue staining.

Intracellular levels of cAMP were measured by rupture of the cells (3 min in 0.1 N HCl, 100°C [3]) then determination of the cyclic nucleotide using the assay kit provided by Amersham which is based on:

- (i) The competition between unlabeled cAMP and a fixed quantity of [³H]cAMP for binding to a protein with high specificity for cAMP [4].
- (ii) The separation of the protein-bound cAMP from unbound nucleotides as in [5].

The 'activity ratio' of cAMP-dependent protein kinase (i.e., the activity measured in the absence of cAMP divided by the activity in the presence of 5 μM cAMP) was determined after rupture of the thymocytes and release of the enzyme by the following procedure: Samples of the cells to be tested (1 × 10⁷) were spun down (15 s at 12 000 × *g*) the pellet was then suspended for 2 min at 4°C in 0.5 ml buffer composed of Tris (10 mM), Mg (CH₃COO)₂

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethyl-piperazine *N'*-2-ethane sulfonic acid; PGE₁, prostaglandin E₁; THF, thymus humoral factor

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(3 mM), theophylline (1 mM), pH 7.2 (buffer B)*. An aliquot (50 μ l) 1.5 M NaCl in buffer B was then added and the incubation at 4°C was continued for an additional minute. The hypotonized cell suspension was then centrifuged (15 s at 12 000 $\times$ g) and the supernatant was assayed.

The cAMP-dependent protein kinase activity was assayed in reaction mixtures (final vol. 220 μ l) composed of 2-(*N*-morpholino)ethanesulfonic acid (45 mM), Mg (CH₃COO)₂ (9 mM) theophylline (1.14 mM), EGTA (90 μ M), histone H2b (1.8 mg/ml) and [γ -³²P]ATP (200–300 cpm/pmol, 115 μ M). Assay mixtures used for the determination of enzyme activity in the presence of cAMP contained also this nucleotide in final conc. 5 μ M. The pH of all assay mixtures was adjusted to 6.5 with NaOH. The assay was started by addition of the enzyme (50 μ l 12 000 $\times$ g supernatant mentioned above) to the assay mixture. The reaction was allowed to proceed for 10 min at 30°C and was terminated by pipetting out 100 μ l reaction mixture onto a filter paper (Whatman no. 3) and dropping it into ice-cold 10% trichloroacetic acid. The filter paper was then washed 5 more times with 10% trichloroacetic acid, rinsed in 100% ethanol, dried in ether and counted (in 10 ml toluene-based scintillation fluid) using a Packard Model 3003 Tri-Carb liquid scintillation spectrometer.

C3HeB/BL F1 mice were obtained from the Experimental Animal Unit of the Weizmann Institute of Science. cAMP, ATP and PGE₁ were purchased from Sigma, RPMI-1640 buffer supplemented with 25 mM Hepes was purchased from Bio-Lab Lab., Israel. 2-(*N*-morpholino)ethanesulfonic acid was obtained from Serva Feinbiochemica, [γ -³²P]ATP and the cAMP assay kit (TRK 432) were bought from The Radiochemical Center, Amersham. Cholera enterotoxin was purchased from Schwartz Mann, and THF (prepared as in [6]) was a gift of Y. Yakir and Professor N. Trainin.

* Exposure to these hypotonic conditions brings about mild rupture of the thymocytes (keeping the cell nuclei morphologically intact) with concomitant quantitative release of the enzyme into the supernatant. The subsequent adjustment of the salt concentration to an isotonic concentration reverses the massive cytosol-to-nucleus translocation of the free catalytic subunit of the enzyme which occurs under hypotonic conditions (Y. Z., R. C. and S. S., submitted)

3. Results and discussion

The transfer of viable thymocytes from 4–37°C triggers the cells in a way which is reminiscent of hormonal stimulation, causing a rise and then an exponential drop in the level of intracellular cAMP. Following the kinetics of the process (fig.1) it can be seen that the elevation of cAMP levels occurs concomitantly with an in vivo activation of cAMP-dependent protein kinase, as indicated by the parallel changes in the activity ratio (i.e., the state of activation) of the enzyme. This non-hormonal burst in the level of cAMP caused by the 'temperature shock' (which occurs to some extent also when the thymocytes are isolated and kept at 20°C, then transferred to 37°C) may give rise to erroneous results when monitoring the molecular consequences of hormonal stimuli in vivo. For example, depending on the conditions of incubation of the cells with the hormone, one can obtain dramatically different types of responses. As seen in fig.2A, addition of PGE₁ to thymocytes which had been pre-equilibrated at 37°C for 90 min results in an immediate activation of the kinase. Within 15 s, the activity ratio of the enzyme rises from a basal level of ~ 0.4 (measured before addition of the hormone) up to a value of ~ 0.8 , then

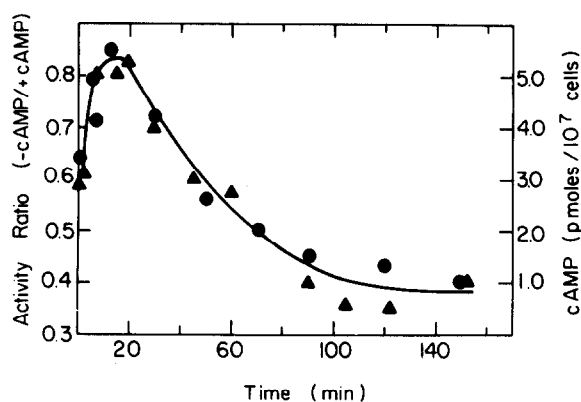


Fig.1. Kinetics of the elevation in cAMP and of the activation of cAMP-dependent protein kinase in mouse thymocytes when transferred from 4–37°C. Viable thymocytes (1×10^7 cells/ml buffer A) were isolated at 4°C as in section 2, then transferred (time 0) to 37°C. At the indicated times, samples were withdrawn and their cAMP content (Δ) as well as the state of activation of the enzyme (activity ratio, $\bullet$) were determined.

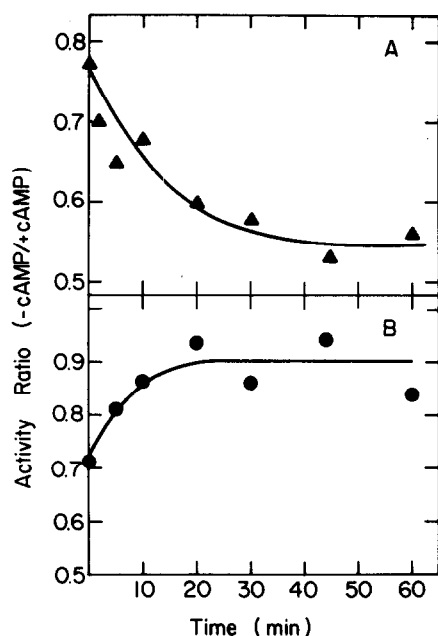


Fig.2. Effect of the conditions of preincubation on the response of viable thymocytes to PGE_1 , as monitored by the activation state (activity ratio) of cAMP-dependent protein kinase. (A) Viable mouse thymocytes (1.1×10^7 cells/ml buffer A) were prepared at 4°C as in section 2, then allowed to equilibrate at 37°C in samples of 0.9 ml. After 90 min of pre-equilibration, aliquots of $100 \mu\text{l}$ PGE_1 (1×10^{-4} M in buffer A) were added, and the incubation at 37°C was continued. At the indicated times of incubation with the hormone, the cells were spun down, subjected to hypotonic shock as section 2 and the state of activation of their cAMP-dependent protein kinase (activity ratio) was determined. (B) Viable thymocytes (1.1×10^7 cells/ml of buffer A) were prepared in 0.9 ml samples (at 4°C) as in section 2, then mixed with $100 \mu\text{l}$ PGE_1 (1×10^{-4} M in buffer A) which was also kept at 4°C , and transferred immediately to a water bath kept at 37°C . At the indicated times the cells were spun down and the state of activation of their cAMP-dependent protein kinase was determined as in (A).

it slowly drops down with time. On the other hand, if the hormone and the cells are mixed together while at 4°C and then transferred to 37°C , the activity ratio of the enzyme rises to a value of ~ 0.9 within 20 min and this high activation state is preserved for at least 60 min (fig.2B). It seems therefore that in the case of thymocytes isolated at 4°C , a preincubation period of 90–100 min is required for the cells to accommodate themselves to 37°C and bring down their cAMP level

and the activity ratio of their cAMP-dependent protein kinase to the basal values. Using shorter preincubation periods (cf. [7–10]) might superimpose considerable temperature effects on the hormone-triggered events and lead to wrong conclusions.

The molecular basis of the temperature-triggered effects on thymocytes is not clear yet. They might be associated with structural changes in the cell membrane similar to those induced by detergents [11] which give rise to the activation of adenyl cyclase. They might also be linked to a direct or indirect modulation of a cellular phosphodiesterase [11,12] or GTPase [13–15]. Another intriguing possibility is that these temperature effects might be mediated through a temperature-dependent polymerization and depolymerization of the microtubule or microfilament system [16,17]. In any case, the observations reported here are to be taken into consideration in the design of *in vivo* experiments with intact cells and in the interpretation of such results. Moreover, they might

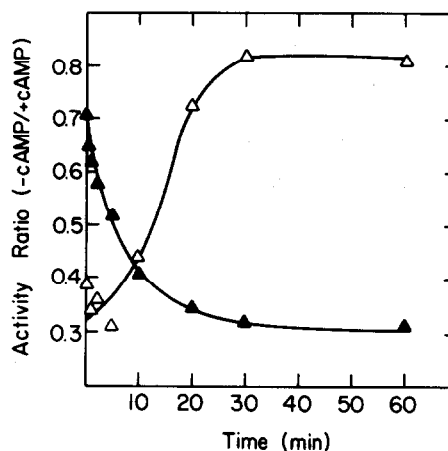


Fig.3. Effect of THF and of cholera enterotoxin on the state of activation of cAMP-dependent protein kinase. Viable mouse thymocytes (1.1×10^7 cells/ml of buffer A) were prepared at 4°C as in section 2 and allowed to pre-equilibrate in 0.9 ml samples for 90 min at 37°C . The cells were then stimulated by addition of $100 \mu\text{l}$ samples of either THF ($\blacktriangle$) (6×10^{-7} M in buffer A) or cholera enterotoxin ($\triangle$) (1.25×10^{-7} M in buffer A) to the various cell samples, and the incubation at 37°C was continued. At the indicated times of incubation with the stimulant the cells were spun down, ruptured by hypotonic shock as in section 2 and the state of activation of their cAMP-dependent protein kinase (activity ratio) was determined.

also provide us with a simplified model system for the study of mechanisms of hormonal stimulation, since the two types of response patterns depicted in fig.2 are also observed with specific hormones and natural stimulants. As seen in fig.3, the response of thymocytes to THF (a specific thymic humoral factor for which these cells constitute a target tissue [18]) resembles the response of these cells in fig.2A, while the response of thymocytes to cholera enterotoxin (even when the cells are preincubated for 90 min at 37°C before stimulation) is reminiscent of the type of response depicted in fig.2B.

In conclusion, the possibility of stimulating a hormonal response by a physical means alone (i.e., without exposure to any hormone or stimulant whatsoever), and the ability to induce at will different response patterns in a given cell to a given hormone, may provide us with important clues in unravelling the mechanisms of hormonal action *in vivo*.

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