Calcium Ion Requirement in Lymphocyte Stimulation

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Accepted August 22, 1988

Lymphocyte stimulation is usually quantitated by the incorporation of ³H-thymidine into lymphocyte DNA. There are, however, great gaps between initial mitogenic stimulation and DNA synthesis which is the final outcome. The extracellular signals such as mitogenic stimulation are transmitted from membrane to nucleus by the intracellular messengers. Calcium ion (Ca²⁺) is one of such messengers. The requirement for Ca²⁺ in lymphocyte proliferation by mitogens has been reported by many investigators. However, there is disagreement concerning the stages at which Ca²⁺ is required. Whitney and Sutherland (1972) found that ethyleneglycol bis-(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA) caused complete inhibition of DNA synthesis only if added within a few hours of phytohemagglutinin (PHA) stimulation, and they concluded that there was a single, early, Ca²⁺ requiring period. On the other hand, Diamantstein and Ulmer (1975) suggested that Ca²⁺ is required for a step preceding DNA synthesis but not for the early phase of transformation induced by PHA. Nakabayashi (1979) indicated that, by using Ca²⁺ channel blockers, the need for Ca²⁺ is more pressing during the phase of commitment (the phase just before the onset of DNA synthesis) of lymphocyte activation than it is during the initiation phase. Bard *et al.* (1978) have reported that Ca²⁺ may well prove to play a part in the early signal but this cannot be its only role and the activation of lymphocytes by PHA has a sustained requirement for Ca²⁺ for at least 20 hours.

The present study represents the necessity of Ca²⁺ for RNA and protein synthesis which occur in the early phase and for the commitment of lymphocytes to enter S-phase.

Materials and methods

Human lymphocytes were obtained from resected tonsils of children 4 to 12 years old by means of Ficoll-Paque density gradient centrifugation (Komada 1979). The cells were suspended at 1×10^6 cells/ml in TC199 medium (Nakarai Chemicals, Ltd.) supplemented with 20% calf serum (Nakarai Chemicals, Ltd.). The cells were stimulated by PHA (Difco Laboratories, 3.3 μ g/ml, an optimal dose for DNA synthesis), and then EGTA and CaCl₂ were added at varying times. RNA, protein and DNA synthesis were determined by using ³H-uridine, ³H-phenylalanine and ³H-thymidine (Amersham International), respectively, and the radioactivity was counted (Komada 1979). The amount of Ca²⁺ taken up by lymphocytes was determined with ⁴⁵CaCl₂ and ³H-water (New England Nuclear) by separating cells from the isotope containing medium by centrifuging cells through an oil layer which was composed of di-n-butyl phthalate and corn oil (100: 33) (Komada *et al.* 1987).

Results and discussion

As preliminary experiments we determined the concentration of EGTA required for the inhibition of the incorporation of ³H-thymidine for the period 69–72h after PHA-stimulation.

Fig. 1. Effect of early 12 h Ca^{2+} depletion on RNA (a) and protein (b) synthesis. EGTA (1.7 mM) was added to the culture at the time of PHA addition, CaCl_2 (1.7 mM) was added at 12 h, and RNA and protein synthesis was determined at 14, 23 and 36 h. Little time lag was observed.

Fig. 2. Effect of early Ca^{2+} depletion (■) and Ca^{2+} depletion at the time of commitment (□) on DNA synthesis. EGTA (1.7 mM) was added at the time of PHA addition and CaCl_2 (1.7 mM) at 12 h. EGTA (3 mM) was added at 20 h, CaCl_2 (3 mM) at 32 h, and DNA synthesis was determined at various times.

Table 1. Ca^{2+} uptake during first 30 min, 12–12.6 h and 20–20.5 h after PHA stimulation

No. of experiments	0–30 min	12–12.5 h	20–20.5 h
1	1.57	1.43	1.13
2	1.63	1.43	1.63
3	1.40	1.79	1.32

The values are expressed as the ratio of $^{45}\text{Ca}^{2+}$ uptake of PHA stimulated cells to that of non-stimulated cells.

When EGTA was added to the cell culture at the time of the PHA addition, the DNA synthesis was completely inhibited by 1.7 mM EGTA. But DNA synthesis was completely recovered by the addition of equimolar CaCl_2 (1.7 mM) at the time of PHA and EGTA addition (data not shown). After 20h of PHA stimulation (just before the onset of DNA synthesis), DNA synthesis was inhibited by 3 mM EGTA, and was also completely recovered by the addition of equimolar CaCl_2 (3 mM) at the time of EGTA addition (data not shown). We used these doses of EGTA and CaCl_2 for the following experiments.

We first examined the effect of the early 12h Ca^{2+} depletion on RNA, protein and DNA synthesis, because the onset of RNA and protein synthesis was around 12h after PHA stimulation (Komada 1979). EGTA (1.7 mM) was added at the time of PHA addition, CaCl_2 (1.7 mM) was added after 12h, and RNA, protein and DNA synthesis were determined at various times. As shown in Fig. 1a, b and Fig. 2, RNA, protein and DNA synthesis were recovered by the addition of CaCl_2 after 12h Ca^{2+} depletion. There was little time lag on RNA and protein synthesis as compared with DNA synthesis.

To investigate the effect of Ca^{2+} depletion just before the onset of DNA synthesis, EGTA (3 mM) was added 20h after PHA stimulation. After 12h of EGTA addition (32h after PHA-stimulation), CaCl_2 (3 mM) was added and DNA synthesis was determined at various time. Fig. 2 shows that DNA synthesis was recovered by Ca^{2+} and that a time lag was observed.

The requirement for Ca^{2+} at both the stages was found, then we assayed the Ca^{2+} uptake by lymphocytes in each stage. As shown in Table 1, the enhancement of Ca^{2+} uptake was observed at the initiation, 12h and 20h after PHA-stimulation.

These results indicate that the Ca^{2+} depletion in the early phase does not delay the early biochemical events such as RNA and protein synthesis. This does not mean that Ca^{2+} is not necessary for the initiation, because the initiation occurs at the time of the mitogen addition if there is Ca^{2+} in the culture medium or at the time of Ca^{2+} addition when mitogen has already been present, and because the enhancement of Ca^{2+} uptake occurred at the initiation by PHA. Taken together with these findings and the results of Nakabayashi (1979), it was suggested that Ca^{2+} is required for both the initiation and commitment of lymphocytes to DNA synthesis, and the necessity of Ca^{2+} is more pressing during the time of commitment than during the early phase of lymphocyte stimulation.

Summary

The requirement for Ca^{2+} and Ca^{2+} uptake were determined both at the initial step and during the phase of commitment. The onset of RNA and protein synthesis, which occurred around 12h after stimulation, were not delayed by the early 12h Ca^{2+} depletion. On the other hand, DNA synthesis was delayed by both early and late Ca^{2+} depletion. The enhancement of Ca^{2+} uptake was observed at each stage. We concluded that Ca^{2+} is required for both the stages and that Ca^{2+} is more necessary for the time of commitment than it is for the early phase of lymphocyte stimulation.

Acknowledgement

We thank Dr. Taro Maeda for his kindness in supplying the surgically resected human tonsils.

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