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EFFECTS OF CHELATING AGENTS ON THE INITIAL INTERACTION OF PHYTOHEMAGGLUTININ WITH LYMPHOCYTES AND THE SUBSEQUENT STIMULATION OF AMINO ACID UPTAKE

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SUMMARY

The chelating agents, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) and EDTA, had no effect on the initial interaction of phytohemagglutinin with lymphocytes at concentrations which have been shown previously to inhibit the development of the phytohemagglutinin response completely. However, they had a marked inhibitory effect on uptake of the amino acid analog, α-aminoisobutyric acid in both unstimulated and phytohemagglutinin-stimulated cells. The inhibition of amino acid uptake by EGTA could be reversed by adding Ca^{2+} but not Mg^{2+}. These results demonstrated that Ca^{2+} is not essential to the initial interaction of phytohemagglutinin with the cell, but does influence amino acid transport which may be a critical preparatory event for later increased protein synthesis.

INTRODUCTION

Human lymphocytes which are normally non-dividing, can be transformed to lymphoblasts and mitotic cells by a variety of agents including phytohemagglutinin a plant mucoprotein[1]. The initial interaction of phytohemagglutinin with lymphocytes seems to involve a binding to membrane receptors[2–4]. Many alterations in cellular metabolism have been reported to occur subsequently. These changes include such early membrane-associated events as an activation of adenylate cyclase and increased production of cyclic AMP[5], enhanced uptake of Ca^{2+} (refs 6–8), amino acids[4,9,10] and K^+ (ref. 11) and alterations in membrane phospholipids[12]. In addition to, or as a result of these membrane events there is an early stimulation of histone acetylation[13], RNA and protein synthesis[14] and carbohydrate metabolism[15]. Some or all of these events certainly contribute to the eventual initiation of DNA synthesis about 18 h after phytohemagglutinin treatment.

It has been shown previously that medium Ca^{2+} is essential to the initiation

Abbreviation: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*, *N*'-tetraacetic acid.

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of the phytohemagglutinin response, and it is particularly required during the first few h after phytohemagglutinin treatment[16]. However, no exact function could be ascribed to Ca^{2+}. The purpose of the present work was to determine the influence, if any, of Ca^2 on the initial interaction of phytohemagglutinin with lymphocytes and on amino acid transport which is rapidly stimulated by phytohemagglutinin[4,9,10].

MATERIALS AND METHODS

The method used to determine whether the initial interaction of phytohemagglutinin with lymphocytes was modified by the chelating agents, EDTA and ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) did not directly measure phytohemagglutinin binding. Studies of binding using radioactively-labeled phytohemagglutinin are subject to the criticism that a considerable portion of the measured binding could be due to non-specific attachment to the cells at sites which do not contribute to activation of the cells. Hence, an inhibition of labeled phytohemagglutinin binding may not necessarily result in a corresponding inhibition of the final response.

Instead, lymphocyte suspensions which had been treated with phytohemagglutinin alone or with phytohemagglutinin and a chelating agent were centrifuged and resuspended in fresh medium (washed) to remove any phytohemagglutinin which was not bound to the cells. After several days in culture DNA synthesis in these lymphocyte cultures was assessed and compared. Thus, this procedure indirectly measures changes in the initial interaction of phytohemagglutinin with the specific surface sites involved in producing the later stimulation of DNA synthesis.

The procedures used for separating and culturing human venous blood lymphocytes and for assessing DNA synthesis have been described in detail previously[16]. In order to assess the effects of the chelators on the initial interaction of lymphocytes with phytohemagglutinin the appropriate chelating agent was preincubated with the cells for 30 min at 37 °C in complete basal Eagle medium *plus* 20% homologous AB serum before phytohemagglutinin was added at a concentration of 11.4 μg of protein/ml which produced maximum stimulation. The cultures were then incubated at 37 °C for 4 h. At the end of this period they were washed two times by centrifugation with 2 ml basal Eagle medium without serum at 4 °C and $150 \times g$ and were resuspended in 0.7 ml of medium containing 20% AB serum without additional chelator or phytohemagglutinin except where indicated to the contrary. The cultures were then incubated for an additional 64 h with [*Me*-^{3}H]thymidine present for the final 16 h. The chelators are non-toxic to the cells at the concentrations used even after 64 h exposure[16].

Lymphocytes for amino acid uptake studies were prepared by the method of Mendelsohn *et al.*[4] using a ficoll-hypaque centrifugation step to remove the erythrocytes. The lymphocytes were diluted to a concentration of $3.33 \cdot 10^6$/ml with basal Eagle medium *plus* 20% serum and were dispensed in 0.3-ml volumes (10^6 cells) to 17 mm × 100 mm disposable plastic tubes (Falcon). Phytohemagglutinin was added at a concentration of 22.8 μg/ml for various lengths of time.

Amino acid uptake was assessed using α-[^{3}H]aminoisobutyric acid, a non metabolizable amino acid analog which shares a common transport mechanism with several naturally occurring amino acids[17–19]. It was added to cultures at various

times at a concentration of 0.1 mM (1 μCi) for a 20-min incubation period to measure the initial rate of uptake. At the end of this period the cells were washed 3 times by centrifugation at 4 °C and 1000 × *g* with 6 ml of ice-cold saline containing 0.1 mM non-radioactive α-aminoisobutyric acid. After the final wash the cells were resuspended in 1 ml of saline, and aliquots were placed on 22-mm filter-paper discs (No. 3 Whatman qualitative), dried thoroughly with a heat lamp and counted in a Nuclear Chicago liquid scintillation counter.

EDTA and EGTA were obtained from Fisher Scientific Co., Montreal, Quebec. [*Me*-^{3}H]Thymidine (6.0 Ci/mmole) and α-[^{3}H]aminoisobutyric acid (0.2–0.4 Ci/mmole) came from Schwarz Bioresearch, Orangeburg, N.Y., and New England Nuclear, Boston, Mass., respectively. The medium and supplements were products of Grand Island Biological Co., Grand Island, N.Y. and the phytohemagglutinin (PHA-P) was obtained from Difco Laboratories, Detroit, Mich.

RESULTS

EGTA at 1.4 or 1.5 mM completely inhibited phytohemagglutinin-stimulated isotope incorporation when present for the entire culture period as was expected from previous results[16] (Table I, middle column). Washing the cells after 4 h incubation with phytohemagglutinin alone did not significantly reduce the final response indicating that the initial interaction of phytohemagglutinin with the cells which led to stimulation of DNA synthesis was complete[2,3]. The presence of EGTA at 1.4 mM, which completely inhibits the development of nucleic acid synthesis[16], or even at 1.5 mM, which was slightly toxic (10%) to the cells in long-term cultures, had no effect on this initial 4-h interaction since after washing and adding only complete fresh medium the final thymidine incorporation was not significantly different from that where no EGTA was present for the 4-h period (Table I, right-hand column).

TABLE I

EFFECT OF EGTA ON THE INITIAL INTERACTION OF PHYTOHEMAGGLUTININ WITH LYMPHOCYTES

Lymphocyte cultures were incubated in the presence of phytohemagglutinin with or without EGTA for 4 h. Cells were then either washed and resuspended in complete fresh medium or not washed. Cultures were then incubated for 64 h with [*Me*-^{3}H]thymidine present for the final 16 h. Other experimental details appear in Materials and Methods. Results are expressed as the mean cpm ± S.E. of 3–6 replicate cultures.

EGTA concn (mM)	*[Me-^{3}H]Thymidine incorporation (cpm)*	
	Not washed	*Washed*
0	16 400 ± 1130	14 900 ± 1640
1.4	116 ± 5	15 700 ± 2130
1.5	87 ± 2	15 100 ± 1880
No phytohemagglutinin	123 ± 8	129 ± 7

EDTA also completely inhibits the phytohemagglutinin response when present for the entire culture period at concentrations slightly above 1 mM (refs 16 and 20). Incubation of the phytohemagglutinin-treated cells in medium containing 1.4 mM EDTA for the entire experimental period (not washed) completely abolished the normal increase in DNA synthesis as expected (Table II). The higher concentrations of 5 and 10 mM were also fully inhibitory. Similar to the EGTA experiments, washing of cells treated only with phytohemagglutinin for 4 h did not alter the final response. Incubation of the cells with 1.4 mM EDTA in addition to phytohemagglutinin for the 4 h prior to washing caused a 22% inhibition of the normal increase in DNA synthesis. When additional phytohemagglutinin was added to washed cells, no enhancement of the normal phytohemagglutinin response (cultures without EDTA) occurred. However, the extra phytohemagglutinin restored the inhibited responses to normal showing that the cells had remained capable of responding. Cell death was not a factor in these experiments since it was established previously that the chelators were non-toxic at the concentrations used[16].

TABLE II

EFFECT OF EDTA ON THE INITIAL INTERACTION OF PHYTOHEMAGGLUTIN WITH LYMPHOCYTES

Lymphocyte cultures were incubated in the presence of phytohemagglutinin with or without EDTA for 4 h. Cells were then either washed and resuspended in complete fresh medium, washed and resuspended in complete fresh medium+phytohemagglutin (11.4 μg/ml), or not washed. Cultures were then incubated for 64 h with [*Me*-^{3}H]thymidine present for the final 16 h. Other experimental details appear in Materials and Methods. Results are expressed as the mean cpm ± S.E. of 3–6 replicate cultures.

EDTA concn (mM)	*[Me-^{3}H]Thymidine incorporation (cpm)*		
	Not washed	*Washed*	*Washed + phytohemagglutin*
0	19 500 ± 2120	18 200 ± 594	19 200 ± 795
1.4	125 ± 12	14 000 ± 1702	16 700 ± 1620
5.0	107 ± 7	1 550 ± 160	17 500 ± 2520
10.0	102 ± 3	94 ± 9	16 900 ± 1130
No phytohemagglutin	119 ± 6	132 ± 9	—

After the initial interaction of phytohemagglutinin with the lymphocyte membrane many cellular processes are stimulated, including an increased uptake of the amino acid analog, α-aminoisobutyric acid, which has been observed 30 min after phytohemagglutinin treatment[4]. EGTA inhibited α-aminoisobutyric acid uptake in both resting and phytohemagglutinin-stimulated lymphocytes (Table III). It reduced the phytohemagglutinin-enhanced uptake to unstimulated levels. The inhibition could be reversed by Ca^{2+} but not Mg^{2+} which was also the case with nucleic acid synthesis[16]. EDTA also strongly inhibited α-aminoisobutyric acid uptake (Table III, last line).

The initial rates of α-aminoisobutyric acid uptake were measured at different times up to 5 h after adding phytohemagglutinin, and the effects of EGTA on these

TABLE III

EFFECT OF CHELATING AGENTS ON α-AMINOISOBUTYRIC ACID UPTAKE BY LYMPHOCYTES

The procedure for assessing α-[^{3}H]aminoisobutyric acid uptake appears in Materials and Methods. Lymphocytes were cultured for 3 h with or without phytohemagglutinin and the indicated chelating agents before isotope uptake was determined with a 20 min pulse. Results are expressed as the mean uptake in cpm ± S.E. of 3 or 4 replicate cultures.

Treatment	*α-[^{3}H]Aminoisobutyric acid uptake (cpm)*	
	Phyto-hemagglutinin	*Control*
None	604 ± 17	147 ± 11
1.4 mM EGTA	141 ± 9	42 ± 6
1.4 mM EGTA + 3.0 mM Ca^{2+}	565 ± 21	125 ± 12
1.4 mM EGTA + 3.0 mM Mg^{2+}	157 ± 18	33 ± 8
1.4 mM EDTA	117 ± 7	35 ± 1

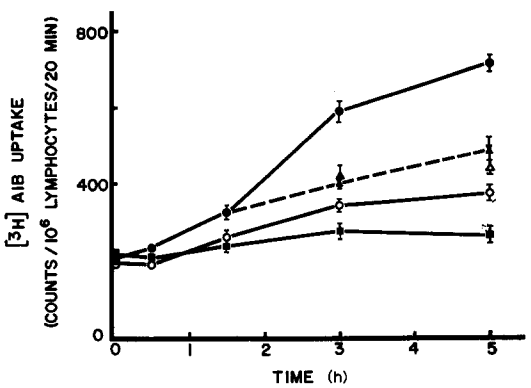

Fig. 1. Effect of EGTA on the initial rate of phytohemagglutinin-stimulated α-aminoisobutyric acid (AIB) uptake. ■—■, unstimulated; ●—●, phytohemagglutinin only; ○—○, phytohemaglutinin + 1.4 mM EGTA added 15 min before phytohemaglutinin; × – – ×, phytohemagglutinin + 1.4 mM EGTA added at 1.5 h; ▲, phytohemagglutinin + 1.4 mM EGTA added at 3 h; △, phytohemagglutinin + 1.4 mM EGTA added at 5 h. α-[^{3}H]Aminoisobutyric acid uptake was determined with a 20-min incubation with isotope initiated at the indicated times. Results are expressed as the mean uptake in counts/10^6 lymphocytes/20 min ± S.E. of 3 or 4 replicate cultures.

rates of uptake were observed (Fig. 1). There was no significant increase in rate of uptake of α-aminoisobutyric acid by unstimulated cells during the experiment. Phytohemagglutinin caused an accelerated uptake after 30 min as was reported before[4], and the rate of uptake increased rapidly thereafter. When EGTA was present from the start of the culture, phytohemagglutinin did not stimulate uptake at all for 1.5 h. At 3 and 5 h with EGTA present from the start there was a slight significant increase ($P < 0.05$) in uptake above unstimulated levels.

In order to determine if EGTA had a direct effect on the stimulated α-aminoisobutyric acid accumulation as opposed to an indirect effect due to an inhibition of an essential prior event, the chelator was also added at various times after phytohemagglutinin. The broken line in Fig. 1 shows the effect of adding EGTA 1.5 h after phytohemagglutinin uptake. This late addition caused a highly significant inhibition (about 50%) of the subsequent uptake although it did not reduce it to the levels observed when the chelator was present from the start of the experiment (75–100% inhibition). The even later addition of EGTA at 3 of 5 h was also effective in causing an immediate 50% inhibition of phytohemagglutinin-stimulated α-aminoisobutyric acid uptake (▲ and △ in Fig. 1, respectively).

DISCUSSION

Previous work from this laboratory has established that lymphocytes treated with phytohemagglutinin do not transform when the medium Ca^{2+} concentration is below about 10 μM, and that maximum transformation occurs only above about 0.6 mM (ref. 16). However, it was also found that removal of Ca^{2+} with EGTA after 12 h in culture had no effect on the ultimate stimulation of DNA synthesis[16]. Thus, Ca^{2+} had to be exerting its influence on some early event(s) in the process. The early development of RNA synthesis, during the first 6 h after phytohemagglutinin exposure, was inhibited by EGTA which further indicated the influence of calcium on very early events[16]. It could not be determined from these results whether calcium had a direct effect on RNA synthesis or whether it acted on earlier events.

The transformation process is most likely initiated by phytohemagglutinin binding to membrane receptors[2–4]. The interaction of phytohemagglutinin, a negatively charged glycoprotein, with the membrane could quite conceivably involve Ca^{2+} since Ca^{2+} is readily available at the cell surface[21]. Indeed, Kay[22] and Lindahl-Kiessling[3] observed that EDTA inhibited the binding of phytohemagglutinin to lymphocytes. The present data agreed with their findings in that EDTA at concentrations greater than 5 mM inhibited the initial interaction of phytohemagglutinin with the cells. However, we found only a marginally significant 22% inhibition of the initial interaction at 1.4 mM, a concentration which was sufficient to completely inhibit stimulation of nucleic acid sysnthesis when present for the entire culture period[16,20]. The other chelator investigated, EGTA, had no effect on the interaction even at 1.5 mM which is well above the concentrations required to completely inhibit transformation[16].

The implication from the prior studies of the influence of EDTA on phytohemagglutinin binding[3,22] was that divalent cations, particularly Ca^{2+}, influenced the binding of phytohemagglutinin to lymphocytes thereby providing the main explanation for the inhibitory effect of EDTA on the transformation process. However, it has been shown that the effect of EDTA on lymphocyte transformation is not due solely to a reduction of the Ca^{2+} level since reversal of the EDTA effect cannot be achieved by adding Ca^{2+} only[16,20]. Further, the concentrations of EDTA used to modify binding were far above those required to completely inhibit the response[16,20]. The present results using EGTA, a chelator which exerts its inhibitory effect primarily by reducing the Ca^{2+} level[16], clearly demonstrated that Ca^{2+} is not essential to the initial interaction of phytohemagglutinin with lymphocytes. The

possibility remains that some other divalent cations may affect this interaction, but since there was little inhibition of the initial interaction at lower EDTA levels which still blocked DNA synthesis, it seems likely that they do not have an essential role. It should be noted that even if transformation requires the uptake of phytohemagglutinin, the same conclusion can be drawn, *i.e.* that Ca^{2+} is not required for the initial interaction of phytohemagglutinin with lymphocytes. Hence, Ca^{2+} must exert its critical effect(s) between this initial interaction and the development of RNA synthesis during the first several h after phytohemagglutinin treatment.

It is well-known that Ca^{2+} influences membrane transport of water[23], monovalent cations[24] and glucose[25]. Therefore, the effect of reducing the medium Ca^{2+} level with EGTA on α-aminoisobutyric acid uptake was studied. The results observed here demonstrated that calcium influences transport of this amino acid analog by lymphocytes. Phytohemagglutinin-stimulated uptake was reduced to control levels by EGTA when present from the start of the culture, and addition of the chelator even several hours after phytohemagglutinin still caused a marked and immediate reduction of uptake.

This paper provides evidence that Ca^{2+} is not required for the initial interaction of phytohemagglutinin with the lymphocyte, and it indicates one point where Ca^{2+} influences the transformation response, amino acid uptake. Further studies will quite likely establish additional Ca^{2+} functions. One event which may be particularly important to the process is the reported increased production of cyclic AMP[5]. It has recently been shown that calcium influences cyclic AMP production in rat thymocytes treated with parathyroid hormone[26], and it may prove worthwhile to look for a similar regulatory role in the transformation process.

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