RELEASE OF UNSATURATED VITAMIN B_{12} BINDING CAPACITY FROM HUMAN GRANULOCYTES BY THE CALCIUM IONOPHORE A23187

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SUMMARY: The ionophore A23187, in the presence of calcium ions, was capable of eliciting the release of granule-associated unsaturated vitamin B_{12} binding capacity from human granulocytes. The ionophore-induced extrusion of unsaturated vitamin B_{12} binding capacity was concentration, time and temperature-dependent. The release was blocked by 2-deoxyglucose and was unaccompanied by cytotoxicity (trypan-blue uptake and lactate dehydrogenase release). The unsaturated vitamin B_{12} binding capacity release was accompanied by the release of lysozyme, a specific granule marker enzyme.

INTRODUCTION

There is considerable evidence that unsaturated vitamin B_{12} binding capacity (UBBC)* is released from human granulocytes (PMN's) (1-4). UBBC is a measure of the degree of unsaturation of vitamin B_{12}-binding proteins. Corcino et al (1) suggested that vitamin B_{12}-binding proteins were particle-bound within the neutrophilic granulocytes. Recently, Kane and Peters (5) reported that UBBC was almost exclusively localized in the specific granules of human PMN's. The release of UBBC was stimulated by lithium, inhibited by sodium fluoride, and unaffected by other metabolic inhibitors, such as potassium cyanide, sodium azide, 2,4-dinitrophenol and 2-deoxyglucose (3). Stebbins and Herbert (6) concluded that UBBC release was not mediated primarily through changes in intracellular cyclic AMP levels. Although it is well known that the secretion of a number of granule-associated substances from human PMN's is Ca^{++}-dependent (7-12), there is no evidence that establishes the involvement of Ca^{++} in the

*Abbreviations: UBBC, unsaturated vitamin B_{12} binding capacity; PMN's, polymorphonuclear leukocytes; ACD, acid citrate dextrose; PBS, phosphate buffered saline; LDH, lactate dehydrogenase.

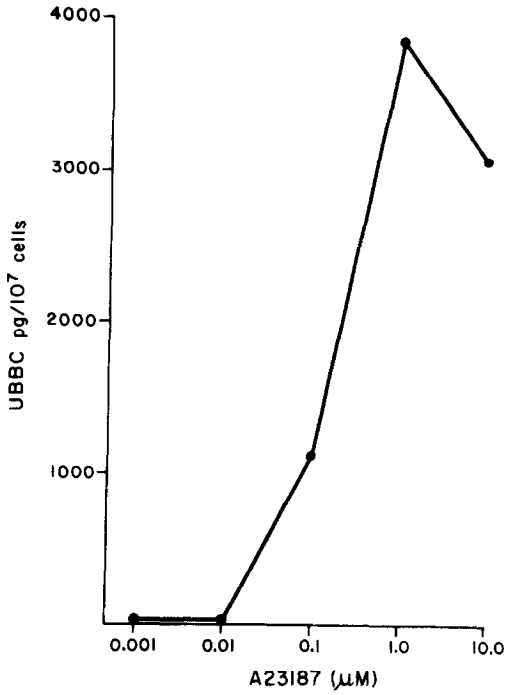

Fig. 1. Ionophore A23187 dose response curve with human PMN's incubated in PBS complete medium for 30 minutes at 37°C.

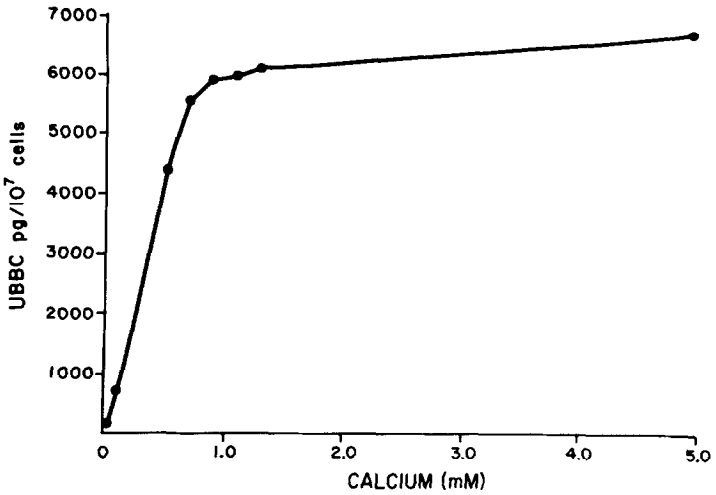

Fig. 2. Ca^{++}-induced release of UBBC from PMN's incubated for 30 minutes at 37°C. Ionophore concentration was 1.0 μM. Ca^{++} and Mg^{++}-free medium was supplemented with Ca^{++} at the indicated concentrations.

release of UBBC. We now report that the ionophore A23187-induced release of UBBC from human PMN's is a Ca^{++}-dependent process.

MATERIALS AND METHODS

Preparation of granulocyte suspensions. Human PMN's from normal donors were isolated from buffy coats of freshly drawn ACD blood using Ficoll Isopaque according to the method of Boyum (13). Red blood cells were lysed with ammonium chloride by the procedure of Dioguardi et al (14) with minor modifications. Cells were washed twice with saline and suspended in Dulbecco's phosphate buffered saline (PBS), pH 7.2 (Associated Biomedic Systems, Inc., Buffalo, N.Y.) with or without Ca^{++} and Mg^{++} containing 0.1% bovine serum albumin. PBS complete medium contained Ca^{++} and Mg^{++} concentrations of 0.9 mM and 0.4 mM, respectively. Ca^{++} and Mg^{++}-free medium was supplemented with $CaCl_2$ and $MgCl_2$ salts, as indicated in the individual experiments.

Measurement of UBBC, lysozyme and lactate dehydrogenase (LDH) release. Approximately 1 x 10^7 PMN's were incubated with shaking in 3 ml of buffer in 17 x 100 mm cotton-stoppered plastic tubes. Cells were preincubated 5 minutes in the buffer before addition of the Ca^{++} ionophore A23187 (generously supplied by Dr. Robert L. Hamill, Eli Lilly Co., Indianapolis, Ind.) Following various periods of incubation, the PMN suspensions were placed in an ice bath, centrifuged at 1120 x g for 10 minutes at 4°C, and the cell-free supernatants decanted for assay. The UBBC was determined by the method of Gottleib et al (15), lysozyme (EC 3.2.1.17) according to the procedure of Litwack (16) using *Micrococcus lysodeikticus* as substrate and LDH (EC 1.1.1.27) by the method of Wacker et al (17). Release of UBBC was expressed as picograms released per 1 x 10^7 cells after correction for untreated controls. Lysozyme and LDH release were expressed as a percentage of the total enzyme released by 1 x 10^7 PMN's after incubation for 30 minutes in medium containing 0.2% Triton X-100.

RESULTS

The release of UBBC from human PMN's was ionophore A23187 and Ca^{++} dose-dependent (Figs. 1 and 2). Maximum release of UBBC from PMN's suspended in complete PBS medium occurred at an ionophore concentration of 1.0 μM (Fig. 1). Cells in Ca^{++} and Mg^{++}-free medium containing 1.0 μM A23187 released UBBC with the addition of as little as 0.1 mM $CaCl_2$ (Fig. 2). Maximum release, without apparent cellular damage, occurred with Ca^{++} concentrations of approximately 1.0 mM. Concentrations of Ca^{++} higher than 2.0 mM resulted in macroscopic changes in the appearance of the cell pellet and distorted Wright-stained cells observed by light microscopy. These alterations suggested cellular damage and could account for slightly higher release observed at a concentration of 5.0 mM Ca^{++}.

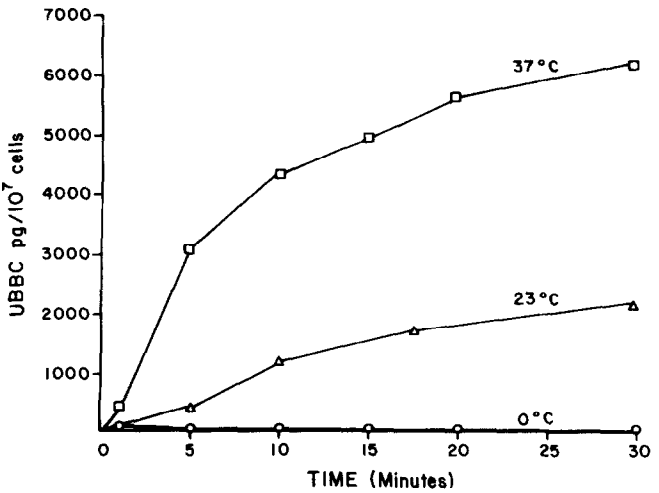

Fig. 3. Time and temperature dependence of UBBC release from PMN's. Ionophore concentration was 1.0 μM in complete PBS medium.

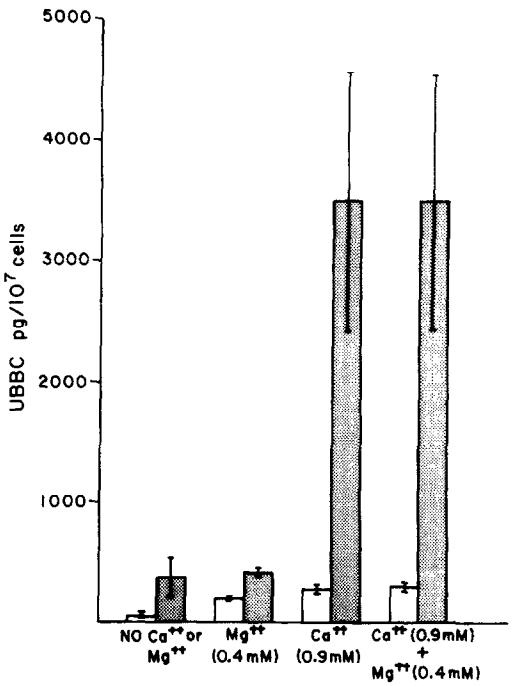

Fig. 4. Requirement of Ca^{++} for the ionophore-induced release of UBBC from PMN's incubated for 30 minutes at 37°C. Ionophore concentration was 1.0 μM. Ca^{++} and Mg^{++}-free medium was supplemented with $CaCl_2$ and $MgCl_2$ salts to the indicated concentrations. The open and shaded bars represent reaction mixtures without and with ionophore, respectively. Each bar represents the mean of three experiments ± S.E.M.

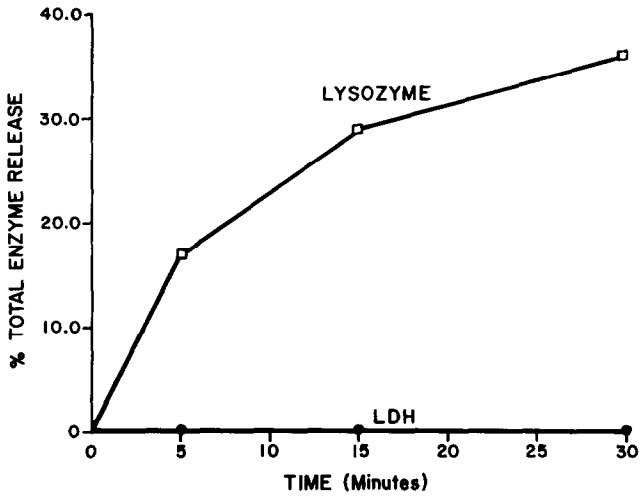

Fig. 5. Time dependence of lysozyme and LDH release from PMN's incubated at 37°C in complete PBS medium containing 1.0 μM ionophore.

The release of UBBC was temperature and time-dependent (Fig. 3). Maximum release occurred after incubation for 30 minutes at 37°C in complete PBS medium. Although not shown, UBBC release at 60 minutes was 14% less than that seen at 30 minutes. The ionophore-induced release of UBBC required Ca^{++}, but not Mg^{++} (Fig. 4). Mg^{++} alone, in the presence of ionophore, did not induce release of UBBC. Furthermore, it did not enhance the release observed with Ca^{++} plus ionophore. Mg^{++} at higher concentrations (up to 1.8 mM), in the presence of 1.0 μM ionophore and 0.9 mM Ca^{++}, gave the same results as those seen with 0.4 mM Mg^{++}.

Addition of glucose (1.0 mg/ml) to the complete PBS medium containing ionophore (1.0 μM) yielded the same amount of UBBC released in unsupplemented medium. However, UBBC release, induced by ionophore (1.0 μM) in complete PBS medium, was reduced about 87% by 1 mM 2-deoxyglucose. The mean ± standard error of the mean (n=3) for UBBC release per 1 x 10^7 PMN's incubated for 30 minutes at 37°C, was 547 ± 133 and 3570 ± 383 picograms for reaction mixtures with or without 2-deoxyglucose, respectively.

The release of UBBC was accompanied by the release of lysozyme,

a specific granule marker enzyme (Fig. 5). The kinetics of release were similar to those seen for UBBC. Approximately 37% of the total enzyme activity was released during 30 minutes of incubation. This compared to about 78% for UBBC release. Cell viability was not altered, as indicated by the lack of release of cytoplasmic LDH and by the absence of significant changes in the percentage (>98%) of cells which excluded trypan blue.

DISCUSSION

The role of Ca^{++} in the release of a number of substances from human PMN's is well established (7-12). In the presence of extracellular Ca^{++}, the ionophore A23187, which facilitates transmembrane fluxes of Ca^{++} and Mg^{++} (18), greatly enhanced the release of granule-associated enzymes (7-10,12), eosinophil chemotactic factor (11) and slow reacting substances of anaphylaxis (19) from human leukocytes and histamine from human basophils (20). This investigation stems from the observation that the release of lysozyme, an enzyme located in the PMN specific granules, is stimulated by Ca^{++} and the ionophore A23187 (7,12). Since it was recently reported that the specific granules of human PMN's contain nearly all of the UBBC (5), it was of interest to examine the role of Ca^{++} and A23187 in the release of UBBC.

This investigation demonstrated that Ca^{++} induced the release of UBBC from human PMN's. The phenomenon required the presence of A23187 at a concentration similar to that observed for the release of other PMN granule-associated substances (8,9,20). Although the release was Ca^{++}-dependent, no requirement for Mg^{++} was observed. Unlike the Mg^{++} inhibition of Ca^{++} ionophore-induced lysozyme release reported by Goldstein et al (7), we did not see any inhibition of UBBC release by Mg^{++}.

An intact glycolytic system for UBBC release was shown by the inhibition of the process by 2-deoxyglucose. However, these results

are in direct contrast to Li^+-stimulated release of UBBC which was unaffected by 2-deoxyglucose (3). A further difference between Ca^{++} ionophore and Li^+-induced release of UBBC is evident in the time required for maximum release. Whereas the ionophore produced its maximal effect in minutes, Li^+ required greater than 8 hours.

This report describes the first direct evidence of Ca^{++}-dependent ionophore release of UBBC from human PMN's. The mechanism of release and its physiological significance are under investigation.

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