A ROLE FOR CYTOCHALASIN-SENSITIVE PROTEINS IN THE REGULATION OF CALCIUM TRANSPORT IN ACTIVATED HUMAN LYMPHOCYTES

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SUMMARY : Acute cytochalasin (B, A, and E) effects on $^{45}Ca^{++}$ uptake in purified human lymphocytes were studied. By themselves the cytochalasins failed to alter $^{45}Ca^{++}$ uptake. However, they produced a 1.5-4.0 fold augmentation of the early increase in Ca^{++} transport in response to PHA, con A or periodate. Ca^{++} uptake responses to Hg^{++} and Zn^{++} were unaffected. The enhancement of lectin stimulated $^{45}Ca^{++}$ uptake was very rapid (within 2 minutes) and was seen over a broad range of lectin and cytochalasin concentrations. Taken together with the recent demonstration that cytochalasin B potentiates lectin induced mitogenesis these results suggest that actomyosin-like proteins help modulate lymphocyte responses to mitogens.

Recently there has been considerable interest in the possible role of actomyosin-like proteins in the mechanical and contractile properties of mammalian cell surfaces. Spectrin, a protein with actin and myosin-like components has been found in large quantities in human erythrocyte membranes (1,2). In a variety of cell types including lymphocytes, 50 A° diameter microfilaments or structures with the immunological reactivity of actomyosin have been seen in close association with the plasma membrane (3,4,5). While there is much information about the early changes in plasma membrane function that lymphocytes undergo in response to mitogenic stimuli, the possible role of actomyosin-like proteins in these responses has not been investigated. In the present study the cytochalasins, a recently discovered family of drugs which have high binding affinity for contractile proteins (6,7), diminish cellular motility (8) and inhibit micropatch and cap formation in lymphocytes (9), have been used in an attempt to elucidate this question. We have studied cytochalasin effects on mitogen stimulated Ca^{++} uptake, since this is

one of the earliest physiological responses to mitogens and appears to be important in the ultimate development of a mitogenic response (10-15). Evidence will be presented that cytochalasins markedly augment the early ^{45}Ca response to mitogenic lectins and periodate.

MATERIALS AND METHODS

Concanavalin A (con A, Miles), $HgCl_2$ (Fisher), $ZnCl_2$ (Fisher), $NaIO_4$ (Fisher), colchicine (Sigma), vinblastine (Sigma) and α-methyl mannoside (Sigma) were dissolved and diluted in 0.1 M NaCl on the day of the experiment. P-PHA (Difco), E-PHA (erythroagglutinating PHA, Burroughs-Welcome) and wheat-germ agglutinin (WGA, Miles) were reconstituted with 0.1 M NaCl to give stock 1 mg/ml solutions and stored at -20°C or -80°C for not more than 14 days. Cytochalasins A, B and E (Aldrich) were dissolved in dimethyl sulfoxide (DMSO) to give 10^{-2} M stock solutions and stored at 4°C. All stored reagents were freshly diluted in 0.1 M NaCl on the day of the experiment.

Isolation of Lymphocytes. Human peripheral lymphocytes were isolated from heparinized venous blood of normal volunteer donors as described previously by sedimentation in 1.2% dextran for 45 minutes followed by isopycnic centrifugation of the leukocyte rich plasma layer on a Ficoll-Hypaque gradient (16). The cells were suspended in Eagle's Minimal Essential Medium (Gibco) containing 2% (vol:vol) heat inactivated human AB serum. The final pH of the medium was adjusted to 7.4 with 95% O_2 - 5% CO_2. Lymphocyte purity was routinely 92-98% as judged by microscopic examination. Cell viability was 95-98% as determined by dye exclusion.

Ca^{++} Transport Studies. Measurements of $^{45}Ca^{++}$ transport were made in purified lymphocytes suspended in nutrient medium at a cell density of $1\text{-}2X10^7$/ml as described previously (10). Briefly, the cells were preincubated for 15 minutes at 37°C; 0.3 ml aliquots were added to glass test tubes containing various dilutions of cytochalasin, lectin, other stimulatory agent or 0.1 M NaCl in a total volume of 0.37 ml. Ten µl of $^{45}CaCl_2$ in 0.1 M NaCl ($6X10^6$ CPM) were added and the cells were incubated at 37°C in a 5% CO_2 atmosphere for 2-60 minutes. Cells were processed for measurement of cell associated $^{45}Ca^{++}$ as described previously (10).

RESULTS

Recent studies from this laboratory have shown that various lymphocyte mitogens (P-PHA, E-PHA, con A, Hg^{++}, Zn^{++}, pokeweed, trypsin and periodate) all produce early increases in Ca^{++} uptake in these cells (10). In the present report lymphocytes exposed to P-PHA underwent the expected Ca^{++} response. When cytochalasin B (CB) and P-PHA were used in combination the cells underwent a 1.5-4.0 fold greater increase in $^{45}Ca^{++}$ uptake than cells treated with P-PHA alone (Figure 1). Two other cytochalasins, A and E, produced a similar response. Amplification of the P-PHA response was consistently seen at cytochalasin concentrations of 10^{-5} - 10^{-6} M. In approximately 50% of experiments similar effects were obtained at 10^{-7} or even 10^{-8} M

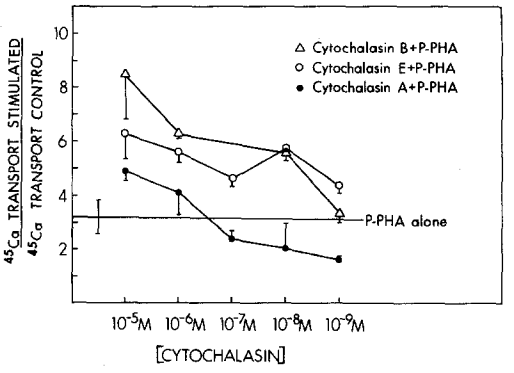

Figure 1: Enhancement of P-PHA stimulation of $^{45}Ca^{++}$ uptake by 3 different cytochalasins. Cells were incubated for 10 minutes at 37°C at a final concentration of 3.3 µg P-PHA/ml. The statistical bars represent one half of the standard deviation determined on triplicate values.

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cytochalasin concentrations. The fall in $^{45}Ca^{++}$ uptake seen at low concentrations of cytochalasin A in Figure 1 has not been observed in other experiments. By themselves, the cytochalasins did not have a statistically significant effect on $^{45}Ca^{++}$ uptake although modest (30% or less) stimulation was occasionally seen. The cytochalasin effect was not due to traces of the DMSO used to dissolve the cytochalasins (maximal final concentration 0.1%, vol:vol) although stimulation of Ca^{++} transport by DMSO alone was observed in some experiments.

The time course of CB enhancement of P-PHA responsiveness is seen in Figure 2. CB effects on $^{45}Ca^{++}$ uptake were observed within 2 minutes. The rapidity of the CB effect and the relative insensitivity of P-PHA stimulation of Ca^{++} uptake to metabolic inhibitors (14) make it likely that CB is acting directly on cytochalasin sensitive proteins rather than through secondary changes in intracellular metabolism.

Enhancement with CB was seen over a broad range of P-PHA concentrations including concentrations sufficiently high that the response to lectin alone had already plateaued (Figure 3). This would suggest that the effects of CB

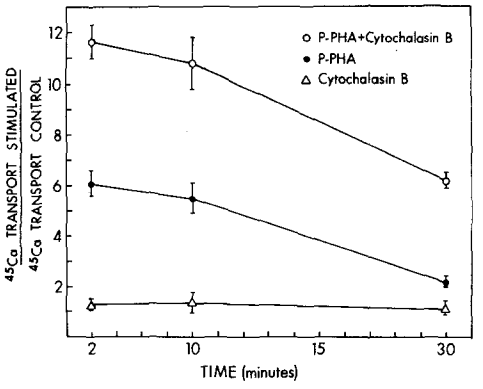

Figure 2: Time course of CB (10^{-5} M) enhancement of P-PHA (3.3 µg/ml) stimulation of $^{45}Ca^{++}$ uptake.

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are not being mediated through changes in lectin binding. In accord with this view, short time course ^{125}I-con A binding studies currently being conducted in our laboratory indicate that 10^{-5} - 10^{-9} M CB has no effect whatsoever on con A binding (not shown).

Other agents which stimulate early Ca^{++} uptake and mitogenesis also were studied (Figure 4). Interestingly, while the Ca^{++} uptake response to con A, E-PHA and periodate was enhanced by CB, the response to the heavy metal mitogens, $ZnCl_2$ ($2X10^{-4}$ M) or $HgCl_2$ ($5X10^{-5}$ M), was unaffected. This suggests that the metals are altering Ca^{++} transport by a different mechanism than the other agents. The response to the con A-CB combination was completely blocked by 50 mM α-methyl mannoside, a hapten inhibitor of con A binding (27), but not by other sugars indicating that it is initiated through specific carbohydrate receptors (not shown). Cells treated with the nonmitogen, WGA, and CB did not accumulate more Ca^{++} than the 0.1 M NaCl controls.

In contrast to the results with the cytochalasins, vinblastine (10^{-4} M - 10^{-8} M) and colchicine (10^{-3} M - 10^{-8} M), agents which interact selectively with microtubules, did not exert marked or consistent effects on Ca^{++} uptake in mitogen stimulated cells (not shown).

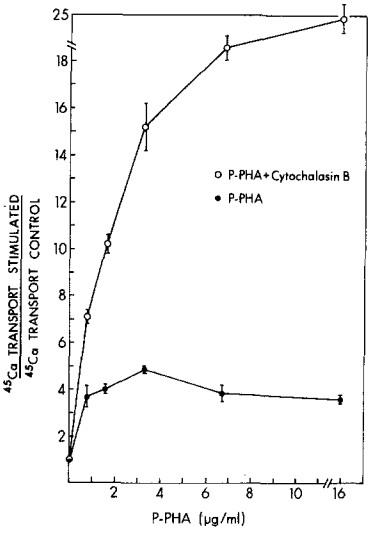

Figure 3: Relationship between P-PHA dose and CB enhancement of $^{45}Ca^{++}$ uptake. Cells were incubated for 10 minutes at 37°C in the presence and absence of 10 µM CB. CB alone had no effect on $^{45}Ca^{++}$ uptake (not shown).

DISCUSSION

The results of the present study indicate that CB and its congeners markedly increase the early Ca^{++} transport response in human lymphocytes to a number of mitogenic agents. As possible explanations for the cytochalasin effect, a direct action through actomyosin-like proteins or a secondary action through changes in lectin binding, carbohydrate transport (17,18) or energy utilization (19) all need to be considered. As already discussed, we are presently studying the binding of ^{125}I labeled con A with CB treated lymphocytes and there is no evidence that con A binding is altered (not shown). An effect through secondary changes in glucose transport or intracellular metabolism also is unlikely since: 1) CB enhancement of P-PHA stimulated Ca^{++} transport is demonstrable within 2 minutes, at low concentrations of CB; 2) it occurs in simple salt solutions without glucose as well as in complex tissue culture media (not shown); 3) under short incubation conditions similar to those used in our experiments Ca^{++} transport in human lymphocytes is

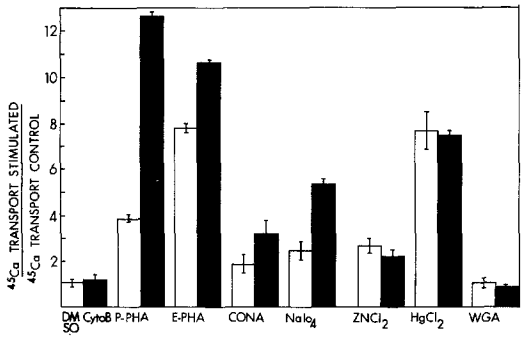

Figure 4: CB enhancement of $^{45}Ca^{++}$ uptake in cells stimulated with a variety of mitogenic and nonmitogenic agents. The cells were incubated for 10 minutes at 37°C. Open bars, CB absent; closed bars, CB ($1X10^{-5}$ M) present. P-PHA 3.3 µg/ml; E-PHA 20 µg/ml; con A 25 µg/ml; $NaIO_4$ $5X10^{-4}$ M; $ZnCl_2$ $2X10^{-4}$ M; $HgCl_2$ $5X10^{-5}$ M; WGA 15 µg/ml.

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largely unaffected by changes in temperature and energy metabolism (14); 4) early Ca^{++} transport responses to Hg^{++} and Zn^{++} and spontaneous Ca^{++} uptake are not stimulated by CB, suggesting that CB is acting in a highly selective manner. While none of these features proves that the effect of CB is being exerted through the actomyosin system per se, taken together they are strongly suggestive.

Assuming that actomyosin-like proteins are indeed involved since the increase in Ca^{++} transport is very rapid and is seen in association with agents which act at the external cell membrane,it seems logical to assume that the elements of the actomyosin system through which the cytochalasins are acting are also physically associated with the plasma membrane. Durham has proposed that filaments of actin are inserted directly into the plasma membrane of mammalian cells and help modulate movements of the cell surface in response to external stimuli (20). There is evidence to indicate that myosin also is present in this location (21), although this has not yet been conclusively demonstrated in lymphocytes. Since actomyosin complexes tend to be highly sensitive to Ca^{++} and Ca^{++} uptake is increased in response to lectin, we presume that one of the early effects of mitogenic lectin binding

is to activate actomyosin contraction. This would most likely occur through local increases in Ca^{++} concentration. Direct perturbation of actomyosin molecules by attached lectin receptor molecules also is possible. Since CB inhibits actomyosin function and enhances Ca^{++} uptake we would further suggest that when actomyosin contraction cannot occur, the disorganization of membrane structure normally associated with lectin binding is qualitatively altered or accentuated thereby increasing the physiological response. According to this scheme, actomyosin contraction acts as a negative feedback mechanism decreasing the response of the cells to surface perturbation.

Two additional observations provide further support for a role of cytochalasin sensitive structures in the modulation of lymphocyte activation: 1) we have recently found that CB augments early increases in cAMP and amino-isobutyric acid (AIB) transport in lectin stimulated human lymphocytes (22,23); 2) the mitogenic response to lectins in rabbit and human lymphocytes is markedly enhanced by the cytochalasins (23,24,25). Since cytochalasins increase Ca^{++} uptake and Ca^{++} appears to play an important role in AIB transport (26) and mitogenesis (see above), the enhancement of these responses is not surprising but further studies are needed to establish a direct relationship.

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