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MICROFILAMENTS REGULATE THE RATE OF EXOCYTOSIS IN RAT BASOPHILIC LEUKEMIA CELLS

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Disruption of microfilaments in rat basophilic leukemia (RBL) cells by exposure to cytochalasin B is observed to potentiate the rate of antigen-stimulated secretion from these cells. Under these conditions, cytochalasin B is without effect on the antigen-stimulated production of inositol phosphates or $^{45}Ca^{2+}$-influx. In streptolysin-O-permeabilized RBL cells, cytochalasin B is observed to potentiate the rate of secretion in response both to guanosine 5'-(2-thio)-O-triphosphate (GTPγS) and to Ca^{2+} (buffered between 0.1 and 10 μM). However, under these conditions, cytochalasin B does not affect to antigen-stimulated production of inositol phosphates. Consistent with these data, microfilaments are proposed to regulate a terminal step in exocytosis, in a physiologically relevant manner.

Recent studies utilizing permeabilized cell systems have uncovered a role for guanine nucleotide-binding proteins in the terminal steps of exocytosis. In studies using saponin-permeabilized platelets [1], Sendai virus-permeabilized neutrophils [2] and digitonin-permeabilized mast cells [3], GTPγS was shown to diminish the concentration of Ca^{2+} required for exocytosis. Recent studies by Gomperts and colleagues [4,5] have used mast cells permeabilized with streptolysin-O, a bacterial cytolysin [6], to observe stimulated secretion of granule contents upon the addition of non-hydrolysable analogs of GTP, including GTPγS . These studies showed that micromolar concentrations of free calcium synergize with GTPγS in causing secretion in this permeabilized cell system [7]. Millimolar concentrations of calcium are also sufficient to cause maximal release of granule contents in

Abbreviations

RBL: rat basophilic leukemia; GTPγS: Guanosine 5'-(2-thio)-O-triphosphate; GDPβS: Guanosine 5'-(2-thio)-O-diphosphate; DNP_{24}-BSA: dinitrophenyl-conjugated bovine serum albumin; BSS: buffered salt solution; 5-HT: 5-hydroxytryptamine.

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the absence of added guanine nucleotides. Less rapidly metabolized analogs of GDP such as GDPβS can specifically block the actions of GTPγS [4]. Taken together, these data have been interpreted to suggest the ability of a G-protein to modulate a calcium-dependent terminal step in exocytosis [7,8].

This report presents data to show that microfilament assembly may play an important role in regulating the rate of terminal steps in exocytosis, and suggests a general physiological role for the control of actin assembly in stimulus-secretion coupling.

Materials and Methods

Materials: $^{45}CaCl_2$, and [^{3}H] 5-HT (5-[1,2-^{3}H(N)]-hydroxy tryptamine binoxalate) were from Dupont-NEN (Boston, MA). myo-[2-^{3}H(N)]-Inositol was obtained from American Radiolabelled Chemicals (St. Louis, MO). Monoclonal anti-2,4-dinitrophenyl mouse IgE (H1.26.82; [9]) was purified using an affinity chromatography procedure as described elsewhere [10]. The antigen used (DNP_{24}-BSA) was bovine serum albumin (Cohn fraction IV, Sigma Chem. Co.) to which an average of 24 dinitrophenyl groups had been conjugated per 68 kDa BSA molecule [11]. Cytochalasin B and D were from Sigma Chem Co. (St. Louis, MO). All tissue culture reagents were from Gibco (Grand Island, NY). Streptolysin O was from Wellcome Diagnostics (Research Triangle, NC).

Cell preparation: RBL cells were maintained in monolayer culture and used 5-6 days after passage [12]. Cells were sensitized with IgE and harvested as described elsewhere [12].

[^{3}H] 5-HT release: [^{3}H] 5-HT secretion from intact cells was measured by release of incorporated [^{3}H] 5-HT, as detailed previously [13]. Inexperiments involving intact cells, Cytochalasin B was added for a period of 10 minutes at 37°C prior to exposure to antigen. In experiments performed with permeabilized cells, RBL cells were prepared as described for secretion assays with intact cells [13], washed once and resuspended in GAME buffer (137 mM K-glutamate, 5 mM ATP, 7 mM $MgCl_2$, 10 mM HEPES pH 7.6) with [Ca^{2+}] adjusted to the indicated levels, and permeabilized in suspension by incubation with 0.1 I.U./ml streptolysin O at 37°C for 5 min. In experiments involving the use of cytochalasin B-treated permeabilized cells, the intact cells were exposed to cytochalasin B for 5 minutes, the cells were then permeabilized as described above in GAME buffers containing cytochalasin B. In order to minimize the loss of intracellular components the cells were not washed following permeabilization. Permeabilized cells were aliquoted with appropriate concentrations of nucleotides or buffer and treated as described. Buffering of free Ca^{2+} levels was performed by addition of suitable amounts of EGTA, and free Ca^{2+} concentrations were determined using a calcium-selective mini-electrode.

Quantitation of ^{3}H-inositol phosphates: Intact [^{3}H]-myo-inositol-labelled RBL cells were prepared as described elsewhere [13]. The cells were then treated with the indicated concentrations of cytochalasin B for 10 min at 37°C. In experiments with intact cells, the cells were exposed to 0.2 ml BSS (135mM NaCl, 5mM KCl, 2mM $MgCl_2$, 1.8mM $CaCl_2$, 5mM glucose, pH 7.4) containing antigen, and the inositol phosphates were extracted and quantified by anion-exchange chromatography on Dowex AG-1-X8 resin [14]. For experiments using permeabilized cells, the [^{3}H]myo-inositol-labelled intact cells were resuspended in GAME buffer and permeabilized in

suspension as described above. Following exposure to GTPγS (10 μM) for 5 min, inositol phosphate production in the permeabilized cells was measured as described [14].

^{45}Ca Influx: Measurements were performed as previously described [13]. Briefly, cells were sensitized, replated in 24-well plates, and washed twice with 1 ml BSS (warmed to 37°C). Cells were exposed to either cytochalasin B (5 μM) or vehicle (DMSO) for 10 min at 37°C. Cells were then incubated at 37°C, for varying periods of time (0-5 min), in BSS (0.5 ml/well) containing antigen (1 μg/ml) and ^{45}Ca (10 μCi/ml). Spontaneous influx was measured in the absence of added antigen. The wells were quenched and washed, and ^{45}Ca uptake was measured by determining cell-associated $^{45}Ca^{2+}$ following solubilization of cells with 0.1% Triton X-100 (0.5 ml/well).

Results

Cytochalasin B enhances the initial rate of antigen-stimulated [^{3}H] 5-HT secretion from intact RBL cells. As shown in Figure 1, the pretreatment of intact RBL cells with 5 μM cytochalasin B for 10 min enhances the initial rate of antigen-stimulated [^{3}H] 5-HT secretion from these cells by about 3-fold. Under these conditions, the effect of cytochalasin B pretreatment is entirely on the rate of secretion while the maximal extent and spontaneous levels of secretion remains unchanged. Higher concentrations of cytochalasin B (20 μM) show a greater enhancement of the rate of antigen-stimulated secretion (~ 4-5 fold). However, at these higher concentrations of cytochalasin B substantial spontaneous release was observed (15% over 30 min). At lower concentrations (0.5-2 μM), cytochalasin B causes an approximately 2-fold potentiation of the initial rate of antigen-stimulated secretion. Cytochalasin D was found to have identical effects to cytochalasin B at similar concentrations (data not shown).

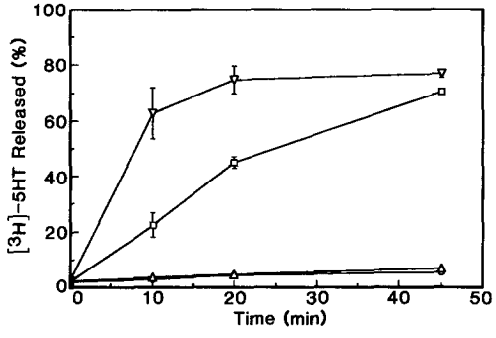

Figure 1: Cytochalasin B pretreatment of RBL cells enhances the rate of antigen-stimulated secretion. Intact RBL cells were treated with vehicle (○, □) or 5 μM cytochalasin B (△, ▽) for 10 min at 37°C. The cells were then exposed to either vehicle (○, △) or 1 μg/ml DNP_{24}-BSA (□, ▽) for the indicated times. Secretion of [^{3}H]-5HT was monitored as described in Experimental Methods.

Cytochalasin B does not affect the antigen-stimulated production of inositol phosphates or $^{45}Ca^{2+}$ uptake in RBL cells. As shown in Table 1(A), the treatment of intact RBL cells with cytochalasin B does not alter the antigen-stimulated production of inositol phosphates at times when the enhancement of secretion by cytochalasin B is evident (i.e. 5 min in Figure 1). Also shown in Table 1(A) is the lack of any effect of cytochalasin B on the antigen-stimulated influx of $^{45}Ca^{2+}$ into RBL cells.

Previous studies have revealed a GTPγS-stimulatable phospholipase C activity in streptolysin-O-permeabilized RBL cells [ref. 15, and unpublished observations). The effect of cytochalasin B on this pathway was investigated, and the results are summarized in Table 1B. Under the conditions of these experiments, there is no detectable effect of cytochalasin B on the GTPγS-stimulated production of inositol phosphates.

Cytochalasin B enhances the initial rate of GTPγS-stimulated secretion from streptolysin-O permeabilized RBL cells. The addition of 10μM GTPγS significantly increased the rate of [^{3}H] 5-HT release from permeabilized cells that were buffered at 0.1μM free Ca^{2+}. GDPβS (100 μM) was found to inhibit the GTPγS (5 μM)-stimulated secretion by more than 90% (data not shown).

Table 1: Effect of Cytochalasin B on (A) antigen-stimulated[1] inositol phosphate production and $^{45}Ca^{2+}$ influx in intact RBL cells and (B) GTPγS-stimulated[2] inositol phosphate production in permeabilized RBL cells

Addition	Inositol phosphates[3] (dpm/5 min/10^7cells)	$^{45}Ca^{2+}$ influx (pmoles/3 min/10^6cells)
(A)		
Antigen	5800 (400)	850 (40)
Antigen + Cytochalasin B	5700 (500)	860 (40)
(B)		
GTPγS	6600 (300)	-
GTPγS + Cytochalasin B	6400 (400)	-

[1]Antigen used was DNP_{24}-BSA at 1μg/ml. In the absence of antigen the basal production of inositol phosphates (about 550dpm/5min./10^7cells) and the basal rate of $^{45}Ca^{2+}$-influx (about 50 pmoles/3min./10^6cells) was similar in cytochalasin-treated and control cells.

[2]GTPγS (10 μM) was added following permeabilization. The basal production of inositol phosphates in the absence of added GTPγS was similar in cytochalasin-treated and control cells (about 600dpm/5min./10^7cells).

[3] Total [^{3}H] label incorporated into cellular phospholipids was ~70,000 dpm/10^7 cells. LiCl (5 mM) was present during incubations. Numbers in parentheses refer to standard deviations from two independent experiments each performed in triplicate.

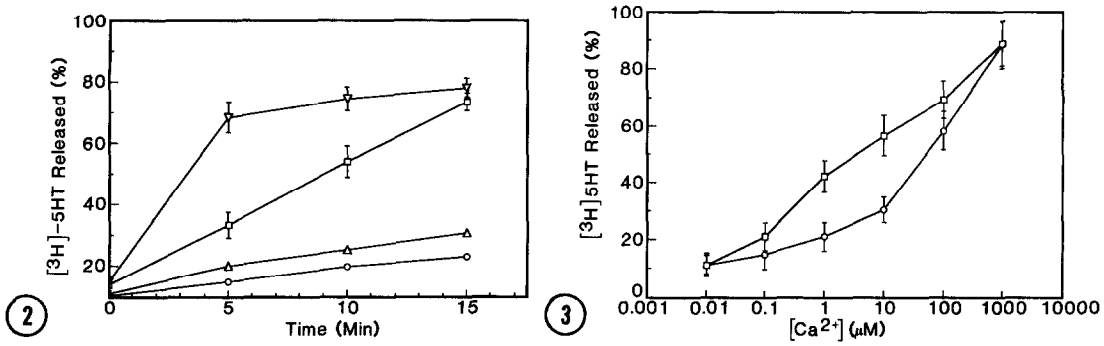

Figure 2: Cytochalasin B pretreatment enhances the rate of GTPγS-stimulated secretion from streptolysin-O permeabilized RBL cells. Permeabilized RBL cells were prepared in the presence of vehicle (○ , □) or 5 μM cytochalasin B (△,▽) as described in Materials and Methods with the free ionized calcium level buffered at 100nM. The permeabilized cells were then exposed to either vehicle (○ , △) or 10 μM GTPγS (□ , ▽) for the indicated times. Secretion of [^{3}H]-5HT was monitored as described in Experimental Methods.

Figure 3: Cytochalasin B pretreatment enhances the rate of calcium-stimulated secretion from streptolysin-O permeabilized RBL cells. Permeabilized RBL cells were prepared in the presence of vehicle (○) or 5 μM cytochalasin B (□) with $[Ca^{2+}]_i$ buffered at the indicated levels as described in Materials and Methods. The release of [^{3}H]-5HT label was monitored 5 minutes after the addition of streptolysin-O as described in Experimental Methods.

Importantly, cytochalasin B pretreatment was found to enhance the initial rate of GTPγS-stimulated secretion approximately 4-fold compared to permeabilized cells stimulated with GTPγS in the absence of cytochalasin B (Figure 2). The enhancement of secretion from permeabilized cells by cytochalasin B was also observed in response to Ca^{2+} alone in the concentration range of 1-10 μM (Figure 3). As seen in Figure 3, there was no observable effect of cytochalasin B on the secretion of [^{3}H] 5-HT from permeabilized RBL cells upon exposure to millimolar levels of calcium.

Discussion

These studies demonstrate an enhancement of the antigen-stimulated secretion of 5-HT from RBL cells by treatment with the microfilament-disrupting agent cytochalasin B. The conditions for treatment of the cells with cytochalasin B or D and the concentrations at which these agents were used (5 μM for 10min) are consistent with their effect on cellular microfilaments assembly in RBL cells [16,17]. Using a streptolysin-O-permeabilized cell system, cytochalasin B is observed to enhance the rate of GTPγS-stimulated release of 5-HT 4-fold in a manner consistent with the

effects of cytochalasin B on antigen-stimulated secretion from intact RBL cells (Figures 1 and 2).

The GTPγS-dependent secretion from mast cells has been demonstrated to display a synergism with micromolar concentrations of free ionized calcium [4,5]. Cytochalasin B pretreatment enhances secretion in the permeabilized cell system in response to micromolar concentrations of calcium even in the absence of GTPγS (Figure 3). This is consistent with the hypothesis that cytochalasin B affects a step subsequent to that of the guanine nucleotide-binding protein. The lack of effect of cytochalasin B in the presence of millimolar concentrations of Ca^{2+} may reflect the difficulty in detecting an increase in the rapid secretion that is observed under these conditions (~90% within 5 min). Alternatively, millimolar levels of calcium may result in a loss of microfilament organization that would relieve the inhibitory effect of microfilaments on secretion and thus eliminate the effect of cytochalasin B [18].

Under conditions where the enhancement of GTPγS-stimulated secretion by cytochalasin B is evident (i.e. 5 min in Figure 2), we do not see any potentiation of GTPγS-stimulated inositol phosphate production (Table 1) or arachidonic acid production (data not shown) in permeabilized cells. These data suggest that the effect of cytochalasin B in permeabilized cells is not due to an enhancement of stimulated second messenger production that could lead to an activation of certain second messenger-dependent secretory pathways in the permeabilized cells.

Data presented in the present study are consistent with a model in which microfilament assembly in these cells, and perhaps others too, serves as a barrier to exocytosis. In this model the disruption of this meshwork of filaments by cytochalasins enhances the rate of exocytosis. A similar model has been proposed on the basis of studies of cell surface stiffening in response to antigen on RBL cells [17]. Using the membrane capacitance technique [19] to study exocytosis, Nube and Lindau [20] report the acceleration of GTPγS-stimulated exocytosis in neutrophils by the treatment with cytochalasin B. While their results are consistent with a role for microfilaments in controlling the rate of exocytosis, the possible effects of cytochalasin B on other GTPγS-activated second messenger pathways in the whole-cell patch clamp configuration they used was not ruled out. The studies reported here address this possibility more directly, by assaying the production of potential second messengers in intact as well as permeabilized cell preparations.

Our results are most consistent with the effect of cytochalasin B on the terminal steps of secretion in intact cells rather than on early events in antigen-stimulated signal transduction. Previous studies into the effect of cytochalasin B on RBL cells have suggested that disruption of the cellular microfilament meshwork potentiates the IgE receptor-mediated activation of calcium influx [21]. Under the present conditions we do not observe any potentiation of the influx of Ca^{2+}, as measured in $^{45}Ca^{2+}$-tracer studies (Table 1(A), and unpublished observations). One difference between those earlier studies [21] and those reported here is the nature of the antigen used to cause IgE receptor aggregation. In the previous studies aggregated ovalbumin was employed as antigen. This antigen has been shown to provide a weaker activation response that has different Ca^{2+} requirements from the multivalent antigen we have employed [22]. Recent studies from our laboratory suggest that cytochalasin D can potentiate the IgE receptor-mediated increase in the cytoplasmic free ionized calcium concentration in response to certain weaker receptor cross-linking ligands and low concentrations of more potent antigens, such as DNP_{24}-BSA used in this study (unpublished data). It is possible that there exist multiple pathways for IgE receptor-mediated Ca^{2+} homeostasis that differ in their sensitivity to cytochalasin B or D. The contribution of these pathways may vary depending on the nature of antigen employed.

The modulation of microfilament assembly as a means of regulating exocytosis has possible physiological relevance as mentioned above. Elson and co-workers [17] have reported that the plasma membrane of RBL cells undergoes a stiffening upon aggregation of the IgE receptors that has a time-course similar to the secretion response. This antigen-stimulated stiffening can be substantially blocked by cytochalasins, under conditions where secretion is enhanced, suggesting a role for the receptor-mediated regulation of microfilament assembly in membrane stiffening that is related to the secretion response. In other studies it has been observed that, following IgE receptor activation on RBL cells and mast cells, there is an increase in the cellular F-actin content [23]. Our results suggest that such a regulation of microfilament assembly might reflect the activation of cellular pathways that control the rate of exocytosis. Further studies will be aimed at elucidating the mechanism of this process.

Acknowledgments

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