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THE ROLE OF TRANSGLUTAMINASE IN HUMAN ERYTHROCYTE ENDOCYTOSIS

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Summary: Receptor mediated endocytosis appears to depend on the action of a transglutaminase (TGase). Endocytosis can be induced in intact human RBC by the action of several classes of drugs. We tested the hypothesis that drugs acted by stimulating TGase activity. Of the endocytosis inducing drugs tested, neither primaquine nor vinblastine nor chlorpromazine enhanced TGase activity. We next tested the hypothesis that TGase activity was required for drug endocytosis in RBC by adding known TGase inhibitors. Paradoxically, m-Dansyl cadaverine, the most potent TGase inhibitor, produces endocytosis in human RBC. Therefore despite apparent striking morphologic similarities, drug induced endocytosis in RBC appears to proceed via different mechanisms from those involved in receptor mediated endocytosis in other cells.

In the receptor-mediated endocytosis of some hormones and growth factors, it appears that the receptor-ligand complex forms clusters over clathrin coated pits which are then internalized as endocytic vacuoles. Both the clustering and internalization of ligands are inhibited by a variety of agents shown to inhibit transglutaminase (TGase) and it is therefore proposed that TGase participates in receptor-mediated endocytosis (1-3). Human erythrocytes undergo endocytosis when exposed to drugs like primaquine, chlorpromazine, and vinblastine (4), all of which are amphipathic cations (4). However, the mechanism of drug action is not known nor is it clear that this is a form of receptor-mediated endocytosis (4). Furthermore, clustering

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of receptors can occur in neonatal but not adult human RBC (5). TGase has been measured in human red cells (6) although its physiologic role is unknown. Like all TGases, it is calcium dependent (6,7), and primaquine induced red cell endocytosis is enhanced by Ca^{++} addition (8). Therefore, we tested the hypothesis that TGase participates in drug induced endocytosis in intact human red cells.

Methods

Fresh heparinized venous blood was obtained from normal volunteers according to protocols approved by the Stanford Committee on Human Experimentation. Endocytosis was induced in intact human red cells by exposing them to primaquine, chlorpromazine, and vinblastine for 90 minutes at 37°C (4,8,9,10). The [^{57}Co]Co-vitamin B_{12} method for quantifying intact RBC endocytosis was used (9,10) and was confirmed by phase microscopic evaluation of all samples by a blind observer. TGase was measured at the beginning and the end of incubation essentially as described (6,7) using the filter paper method (11). At least two calcium concentrations were always used, and no TGase activity was detected in the absences of added Ca^{++}. The reaction measured is the Ca^{++} dependent binding of [^{14}C]- putrescine to dimethyl casein mediated by a frozen-thawed lysate of red cells. The assay mixture, contained in a volume of 140 µl, consistent of 60 µl of frozen-thawed lysate of human RBC suspension of known hematocrit, 40 µl of freshly made 2% N,N-dimethylated casein (Calbiochem), 50 mM Tris-HCl pH 7.5, and 20 µl of a stock solution of 1,4-[^{14}C]-putrescine (122 mCi/mmole, Amersham). Incubation was carried out at 37°C for 15 minutes and over this period of time, experiments showed that the reactions were linear. Duplicate 5 µl samples were removed at 0, 7.5, and 15 minutes and spotted on filter papers which were then dropped into ice cold 10% TCA at a volume of 5 ml per paper. The cold TCA was then stirred rapidly for 30 minutes and the papers were then passed through 3 changes of ice cold 5% TCA followed by a 5 minute wash with 1:1 ethanol/acetone, and then a final wash with acetone alone after which the papers were dried in a 60° oven and counted in Econofluor (NEN) in a Packard TriCarb-460CD liquid scintillation system. The inducers and inhibitors of RBC endocytosis were added exactly as described, before the addition of the red cells that initiated the 90 minute incubation period (9,10). The inhibitors bacitracin, m-Dansyl cadaverine, and histamine dihydrochloride were obtained from the Sigma Chemical Co., and tolbutamide sodium was obtained from Upjohn. The effect of these four inhibitors of TGase was tested (1) (Table 1). Only the 90 minute points from two experiments are shown because the "0" time values were essentially the same as the 90 minute vaues.

Results

The first hypothesis tested was that the endocytosis inducing drugs may act by increasing the activity of RBC transglutaminase. In no experiment (of the six performed) did exposure to primaquine, chlorpromazine and vinblastine cause an increase in TGase activity (Table 1). This observation stands in contrast to the 15-fold increase in TGase activity which is seen in lymphocytes incubated with concanavalin A (12). Dansyl cadaverine and histamine were very effective inhibitors of RBC TGase activity (Table 1).

Bacitracin produced barely detectable inhibition and tolbutamide none. The entry of bacitracin and tolbutamide into red cells was not measured. Mixtures of TGase inhibitors and endocytosis inducing drugs gave the same TGase values as the inhibitors alone (data not shown). The requirement for

Table 1

TGase activity

(nanomoles/min/ml packed RBC)

	0.5 mM Ca^{++}	1.0 mM Ca^{++}	2.5 mM Ca^{++}
Inhibitors of TGase			
Experiment 1			
Control	32.0	35.6	
Histamine	0.7 (2)*	0.6 (2)	
Experiment 2			
Control	23.0	33.0	42.0
m-Dansyl cadaverine, 0.1 mM	6.0 (26)	8.0 (24)	10.0 (24)
1.0 mM	1.0 (4)	1.9 (3)	2.0 (5)
Tolbutamide, 20 mM	25.0 (109)	34.0 (103)	43.0 (102)
Bacitracin, 10 mM	20.0 (87)	27.0 (82)	31.0 (74)
Endocytosis Inducers			
Experiment 2			
Control	23.0	33.0	42.0
Primaquine 2 mM	19.0 (83)	29.0 (88)	39.0 (93)
3 mM	19.0 (83)	28.0 (85)	35.0 (83)
Vinblastine 0.5 mM	21.0 (91)	30.0 (91)	34.0 (81)
1.0 mM	22.0 (96)	30.0 (91)	37.0 (88)
Chlorpromazine 0.6 mM	23.0 (100)	32.0 (97)	39.0 (93)
0.9 mM	23.0 (100)	30.0 (91)	36.0 (86)

*Numbers in parantheses refer to % control

Table 2

RBC endocytosis* (% control)

	PRIMAQUINE 2 mM	CHLORPROMAZINE 0.6 mM	VINBLASTINE 0.5 mM
TGase INHIBITOR			
Histamine, 5 mM	78 ± 14	105 ± 19	107 ± 7
10 mM	58 ± 13 (p=.02)	100 ± 21	116 ± 7
20 mM	39 ± 9 (p<.001)	95 ± 25	121 ± 19
Bacitracin, 10 mM	59 ± 3 (p<.01)	146 ± 84	176 ± 80
20 mM	6 ± 2 (p<.02)	52 ± 7	9 ± 9 (p=.05)
Tolbutamide, 20 mM	141 ± 19	126 ± 27	112 ± 87
m-Dansyl cadaverine			
0.1 mM	111 ± 7	135 ± 24	109 ± 18
0.5 mM	210 ± 75	126 ± 29	
1.0 mM	1577 ±104# (p<.05)	422 ± 93# (p<.05)	250 ± 37# (p<.05)

*In each experiment, RBC were incubated in duplicate with endocytosis drug alone. The trapped [^{57}Co]Co-vitamin B_{12} values for the RBC plus drug samples were arbitrarily set at 100 and the values obtained for red cells incubated with endocytosis drug plus TGase inhibitor were then related to that value. Values shown are Mean ± SEM. P values were determined by paired t analysis and are shown in parentheses where there were significant differences between values observed and the values obtained with drug alone.

#The results in these experiments were subsequently confirmed (Table 3, Figure 1).

Ca^{++} and the clear cut inhibition by histamine and Dansyl cadaverine indicated that TGase was the activity being measured.

A second hypothesis was that while the endocytosis inducing drugs might produce their effect by other mechanisms, TGase activity is still a necessary but not sufficient condition for endocytosis in human red cells. This hypothesis was explored by attempting to block endocytosis with the known TGase inhibitors (Table 2). Histamine inhibited only primaquine endocytosis while 20 mM bacitracin produced a significant inhibition of

Table 3

RBC endocytosis induced by m-Dansyl cadaverine
([^{57}Co]Co-vitamin B_{12} method)

	Experiment 1		Experiment 2	
	cpm/ml packed RBC	% control	cpm/ml packed RBC	% control
Control (no additions)	1525		619	
m-Dansyl cadaverine,				
0.5 mM	1525	100	---	--
1.0 mM	3056	200	---	--
2.0 mM	---	--	3706	599
5.0 mM	75,603	4900	---	--

primaquine and vinblastine induced endocytosis. Surprisingly, m-Dansyl cadaverine, the most potent TGase inhibitor, did not block endocytosis (Table 1). In fact, at concentrations in excess of 1 mM, m-Dansyl cadaverine seemed to enhance all forms of drug induced endocytosis (Table 2). As part of these experiments, RBC had been incubated with TGase inhibitors alone, and these studies showed that while 20 mM histamine, bacitracin, and tolbutamide produced no endocytosis, m-Dansyl cadaverine alone produced substantial endocytosis, the extent of which varied from experiment to experiments but was usually detectable at levels above 1-2 mM (Table 3, Figure 1).

The mechanism by which high concentrations of histamine block primquine endocytosis and high concentrati4ns of bacitracin block primaquine and vinblastine endocytosis remains to be detemined. TGase inhibition is not the mechanism because almost complete TGase inhibition, produced by m-Dansyl cadaverine, did not block drug induced endocytosis. In fact, addition of m-Dansyl cadaverine at concentrations which almost completely inhibited red cell TGase produced RBC endocytosis, perhaps because m-Dansyl cadaverine has characteristics of an amphipathic cation. The argument linking TGase

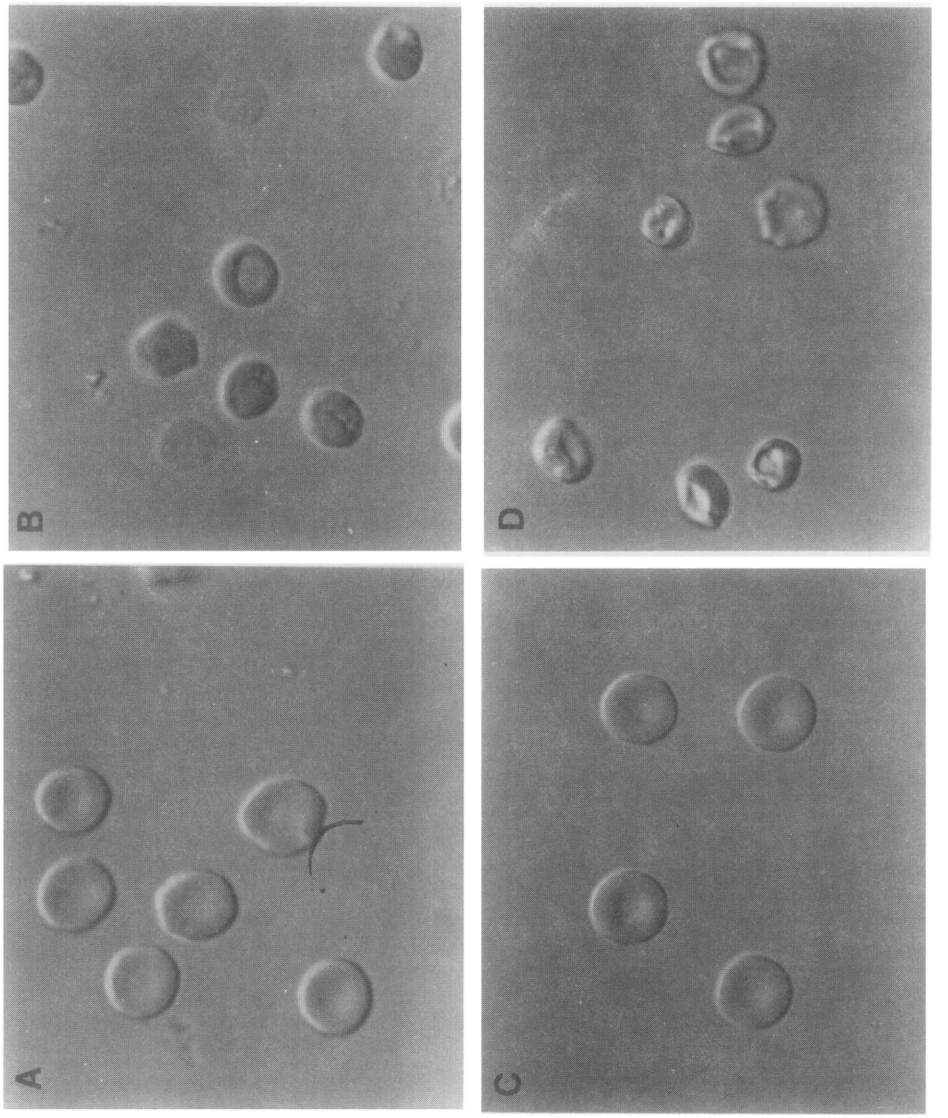

Figure 1: Nomarski interference microscopy of A) control RBC B) RBC plus 3 mM primaquine C) RBC plus 20 mM histamine D) RBC plus 1 mM m-Dansyl cadaverine. In Nomarski interference microscopy, endocytic vacuoles appear as "pocks" of sharply demarcated depressions.

and receptor-mediated endocytosis depends heavily on parallel observations where inhibitors of TGase also inhibit endocytosis (1-3). The parallelism between TGase inhibition and endocytosis is disrupted in the case of the human red cell, therefore TGase is not required for all forms of endocytosis. However, we do not wish to imply that TGase is not important for receptor-mediated endocytosis. Plasma membranes of adult human RBC differ from other plasma membranes as noted above (5). In fact, the proponents of the linkage between receptor-mediated endocytosis and TGase were careful to point out that there were other forms of endocytosis which did not require clustering of receptors of TGase activity (1). Human RBC drug-induced endocytsois probably represents one of these alternative forms of endocytosis.

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