Rat Basophilic Leukemia Cells Stiffen When They Secrete

Zi-Yao Liu, Jei-In Young, and Elliot L. Elson

Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. RBL cells provide a useful model of the IgE and antigen-dependent stimulus-secretion coupling of mast cells and basophils. We have measured cellular deformability to investigate the participation of cytoskeletal mechanical changes. Cross-linking cell-surface IgE–receptor complexes with multivalent ligands not only triggered secretion but also caused the cells to stiffen, i.e., to become more resistant to deformation. This mechanical response required receptor cross-linking, had a time course similar to that of secretion, and was reversed by DNP-L-lysine, a competitive inhibitor of antigen binding. Hence the same stimulus seems to elicit both stiffening and secretion. Cytochalasin D, which inhibits actin filament assembly, prevented or reversed stiffening, thereby implicating the cytoskeleton in the mechanical response. Increasing intracellular calcium ion concentration with the ionophore A23187 stiffened cells and stimulated secretion. Activation of protein kinase C with a phorbol ester also stiffened cells and enhanced both the stiffening and secretion caused by the ionophore. Yet cytochalasin D enhances secretion whereas activation of protein kinase *c* alone is insufficient for secretion. Therefore stiffening is neither necessary nor sufficient for secretion. These results characterize a cytoskeletal mechanical response triggered by the same receptor-dependent stimulus that elicits secretion and by second messengers that are thought to mediate between the receptor signal and secretion. The function of the mechanical response, however, remains to be determined.

EXCITATION and contraction in muscle are coupled by an increase in the concentration of Ca^{2+} ions in the sarcoplasm. An apparently similar intermediary role for Ca^{2+} in adrenal chromaffin cells motivated the development of the stimulus-secretion coupling model (Douglas and Rubin, 1961). In its original formulation this model did not explicitly include a mechanical response of the secretory cell. It has been suggested, however, that the cytoskeleton may be involved in secretion in several cell systems (e.g., Lacy et al., 1968; Allison, 1973). One possibility is that a cytoskeletal contraction accompanies and enhances secretion, for example, by increasing the pressure on secretory granules. Our aim was to investigate whether a cytoskeletal contraction contributes to stimulated secretion and thus that stimulus–secretion coupling might have an excitation–contraction component.

Our approach was based on the presumption that a contraction would increase the cytoskeletal tension developed in the cell and thereby increase its resistance to deformation (cf. Pasternak and Elson, 1985). We have therefore investigated whether the deformability of the 2H3 subline of rat basophilic leukemia (RBL)[1] cells changes in response to stimulation. The RBL-2H3 cells respond to cross-linking their surface Fc_ε receptors by secreting histamine, serotonin, and other granule components in close analogy with normal mast cells and basophils. These cells are sufficiently similar to their normal counterparts to provide a good model for the mechanism of stimulus–secretion coupling, but they respond more slowly and so are more accessible to our deformability measurements (Fewtrell and Metzger, 1981). Furthermore many molecular details of the early phases of the stimulus–secretion process in mast cells and RBL cells have been characterized (e.g., Gomperts et al., 1986; Gomperts, 1986; Beaven et al., 1984; Maeyama et al., 1986; Sagi-Eisenberg and Pecht, 1985; Metzger, 1986). There is also a morphological component of the response which includes increasing total F-actin, spreading over the substratum, and transforming the surface from a microvillous to a lamellar topography (Pfeiffer et al., 1985; Phillips et al., 1985). Although these morphological studies indicate a role for cytoskeletal processes, we considered that a more direct and quantitative assessment of the change in the mechanical state of the cytoskeleton would be informative.

We have carried out this investigation using an instrument called the Cell Poker, which measures the force with which a cell adherent to a glass substratum resists indentation of its exposed surface by a fine glass probe (Petersen et al., 1982; Pasternak and Elson, 1985). This yields a numerical value for the stiffness of each cell. The method is therefore both quantitative and objective, avoiding the qualitative interpre-

1. *Abbreviations used in this paper:* Anti-DNP-IgE, mouse monoclonal IgE specific for dintrophenyl; Con A, concanavalin A; Cyto D, cytochalasin D; DNP-BGG, bovine gamma globulin conjugated with ~16 DNP groups; [^{3}H]serotonin, 5-[1,2-(^{3}H)*N*]-hydroxytryptamine binoxalate; IgE-R, complex between IgE and Fc_ε receptor; poly[HEMA], poly(2-hydroxyethylmethacrylate); RBL, rat basophilic leukemia cell.

tation and the subjective selection inherent in morphological methods (Nunnally et al., 1980). This approach also has an advantage over biochemical analyses performed on a large pool of cells, because each cell is measured separately. The measurements can be performed rapidly on many individual cells to yield the distribution of stiffness values. This is especially important in this work because the mechanical response of the RBL-2H3 cells is very heterogeneous.

Our measurements detected an increased resistance to deformation, which for the most part occurs in parallel with secretion as predicted by our initial working hypothesis. Nevertheless, we found that under some conditions this mechanical response was neither necessary nor sufficient for secretion. Hence, the contribution of the cytoskeletal response to secretion, if any, is more complex than we had originally supposed and remains to be determined.

Materials and Methods

Reagents

Mouse monoclonal anti-DNP IgE (anti-DNP-IgE) (Liu et al., 1980) and DNP-conjugated bovine gamma globulin (DNP-BGG) were kindly provided by Dr. B. Baird and Dr. D. Holowka. There are approximately 16 DNP groups per BGG molecule (D. Holowka, personal communication). Cytochalasin D (cyto D), colchicine, ionophore A23187, PMA, and sodium azide were obtained from Sigma Chemical Co., St. Louis, MO; concanavalin A (Con A) was from Vector Laboratories, Burlingame, CA; and 5-[1,2-(^{3}H)-*N*]-hydroxytryptamine binoxalate ([^{3}H]serotonin; 21 Ci/mmol) was from New England Nuclear, Boston, MA.

Cells

Samples of the RBL-2H3 line developed by Siraganian et al. (1982) were generously supplied by Drs. H. Metzger, C. Fewtrell, and R. Siraganian. The cells were maintained and harvested as described (Barsumian et al., 1981; Taurog et al., 1979; Menon et al., 1984). The cells were typically grown in 75-cm^2 culture flasks. Adherent cells were selected for passage and for experiments and were released from the substratum by treatment for 5–7 min with trypsin-EDTA (Washington University Cancer Center). The trypsin activity was quenched by addition of an equal volume of Eagle's minimum essential medium (MEM) with Earle's balanced salt solution, 20% fetal bovine serum, 2 mM L-glutamine, and penicillin (100 U/ml) and streptomycin (100 μg/ml). To prepare for measurements the harvested cells were washed and resuspended in MEM with 20 mM Hepes, pH 7.4, and adjusted to a concentration of 2 × 10^6/ml.

Cell Treatments

The RBL cells were sensitized by incubating with anti-DNP-IgE at a concentration of 5 μg/ml at 37°C for 1 h. This saturates the cell surface Fc_ε receptors with IgE (Mendoza and Metzger, 1976). Secretion and cellular mechanical changes were triggered by cross-linking the cell surface IgE with DNP-BGG (10 μg/ml).

Cellular responses were also triggered by means not involving IgE. These include incubation with the following materials at 37°C: the ionophore A23187 at various concentrations and pH 7.4 and 8.0 in the presence of 2.0 mM calcium ions for specified times; the lectin Con A at 30 μg/ml for 30 min; the activator of protein kinase C, PMA (16 nM) for specified times; the cytoskeletal inhibitors cyto D (2 μM) and colchicine (10 μM) for 30 min; and the metabolic inhibitor sodium azide (10 mM) for 45 min. Measurements of cellular deformability were carried out in the absence of Con A but in the presence of A23187, PMA, colchicine, cyto D, and NaN_3 at the concentrations listed.

Assay of Serotonin Secretion

RBL cells harvested from monolayer cultures were resuspended in MEM (without bicarbonate and glutamine) containing 20% FBS and buffered to pH 7.4 with 10 mM Hepes (MEM-Hepes). These cells (at 2 × 10^6 cells/ml) were sensitized as described above with anti-DNP-IgE at 5 μg/ml for 1 h at 37°C and at the same time were incubated with [^{3}H]serotonin (2 μCi/ml). The sensitized cells were then washed twice and resuspended as described by Fewtrell et al. (1981) except that measurements were made both at pH 7.4 and at pH 8.0. Up to 2 ml of this cell suspension was incubated at 37°C with DNP-BGG or another releasing agent, aliquots were removed after 5, 15, 30, 45, or 60 min, and the reaction was terminated by cooling the tubes to 4°C and adding an equal volume of ice-cold PBS. Then the cells were sedimented for 10 min at 1,700 *g*. Aliquots (200 μl) of the supernatant were added to 10 ml Aquasol (New England Nuclear), and the activity of ^{3}H was determined by scintillation counting. Results were expressed as the percent of the total cell-associated [^{3}H]serotonin released into the supernatant. The total [^{3}H]serotonin content of the cells before release was determined by adding 3% triton X-100 to a 200-μl cell suspension and scintillation counting as above. The stimulated release was the difference between the percent of total serotonin released in the absence (spontaneous release) and presence of DNP-protein or other releasing agent. Cell viability was determined by trypan blue exclusion. Measurements were considered valid only if 95% or more of the cells excluded the dye.

Measurements of Cellular Deformability

The design and principles of operation of the Cell Poker have been described previously (Petersen et al., 1982; Pasternak and Elson, 1985). A suspension of cells (2 × 10^6 cells/ml) was placed on an 18-mm diameter round glass coverslip (No. 2, Taylor Chemical Co., St. Louis, MO), and the cells were allowed to settle and attach to the glass for 3–5 min at room temperature. Then the coverslip was mounted in the Cell Poker so that the cells were immersed in MEM-Hepes, pH 7.4. The Cell Poker chamber contained 10–12 ml of this solution and was thermostated at 23°C (room temperature) for all measurements. The Cell Poker motor was programmed to execute a single triangular waveform with a velocity of 5.2 μm/s and a total amplitude of 2.6 μm.

We observed that the responsiveness of cells to IgE cross-linking, as measured both by serotonin release and increase in stiffness, decreased over time at different rates for different batches of cells. We also had difficulty in recovering responsive cells from frozen samples. This was a major cause of variability among our experiments. Therefore we found it necessary to renew our cultures of RBL-2H3 cells with fresh samples obtained from the sources listed above at various times throughout the course of this work. There was also some variability in responsiveness among these different cultures and therefore among the experiments we describe below. We have, however, taken care that data cited for a particular experiment were obtained within a short period of time using cells from the same source. Furthermore we have remeasured the response to cross-linking IgE for each experiment to provide an index of the responsiveness of the cells for that experiment.

Data Analysis

The Cell Poker provides a measurement of the force exerted on the probe tip by the cell as a function of the displacement of the tip. Before coming into contact with the cell surface the force is zero. Once the tip contacts the cell the force exceeds zero and increases as the tip further indents the cell surface. When the direction of the motor reverses, the tip is withdrawn and the force exerted on it by the cell decreases. Typically at corresponding depths of indentation the force exerted during withdrawal is less than that exerted while indentation increases. The hysteresis loop thus formed results from cellular viscosity. Ideally the plot of force versus indentation should provide a quantitative estimation of the elasticity modulus and viscosity of the cell (Daily, 1985). The interpretive scheme for this continuum mechanics analysis is, however, not yet available. Therefore, for present purposes as in previous applications (Pasternak and Elson, 1985) we use the initial slope of the ingoing branch of the plot of force versus indentation as an empirical measure of cellular viscoelasticity. This slope or "stiffness" in units of millidyne per micrometer increases whenever either the cellular elastic or viscous resistance to indentation increases. For most of our measurements the ingoing branch of the force/deformation plot was approximately linear, thereby yielding an unambiguous determination of the stiffness. Statistical analyses were carried out using the RS/1 data analysis system (BBN Research Systems, Cambridge, MA).

Measurements of Cells on Poly[HEMA]-coated Substrata

We have investigated the effects of changes of cell shape (Pfeiffer et al., 1985) by measuring deformability of cells on substrata coated with poly(2-hydroxyethylmethacrylate), poly[HEMA], a surface which diminished cell

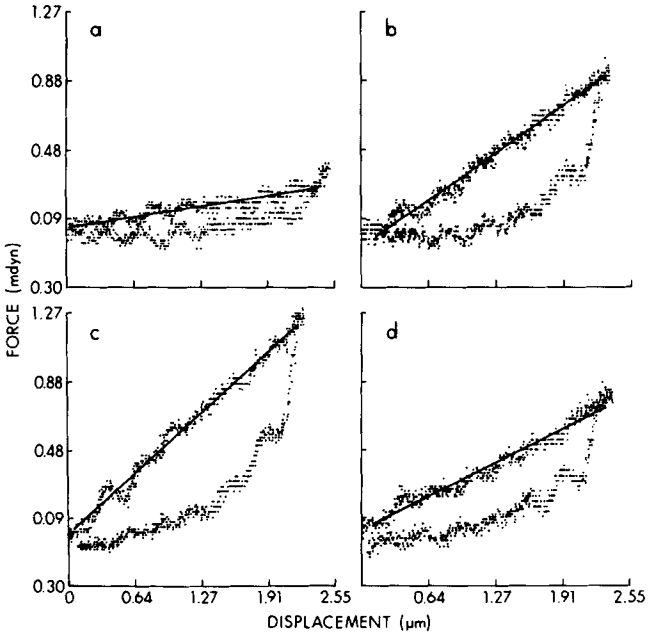

Figure 1. Dependence of RBL cell stiffness on time of incubation with the cross-linking ligand DNP-BGG. Representative plots of force versus displacement are given for the incubation times 0, 15, 60, and 120 min, in panels *a*, *b*, *c*, and *d*, respectively. The linear fit to the first branch of the plot, which determines the stiffness value, is superimposed. The stiffness values for each plot, which are close to the means for the respective populations, are 0.10, 0.40, 0.55, and 0.30 mdyn/μm, respectively.

spreading and flattening. Cells were cultured on glass coverslips coated with poly[HEMA] as described by Folkman and Moscona (1978). Poly[HEMA] (7.2 g) was dissolved in 50 ml 95% ethanol and the mixture was rotated slowly overnight at 37°C to dissolve the polymer completely and then was centrifuged at 760 *g* for 30 min. The supernatant was used as a stock solution and was diluted 1,000-fold with 95% ethanol. Then the 18-mm diameter round coverslips to be used for measurements of cellular deformability were coated with this solution in a depth of ~1 mm. The coverslips were dried while placed level in a sterile hood free of vibrations. A thin, hard sterile film of optically clear polymer tightly bonded to the glass surface formed after the alcohol evaporated. RBL cells were plated on the coated coverslips and cultured for 24 h before measurement of deformability.

Results

Response to Aggregation of Surface-bound IgE

Our measurements demonstrate a striking increase in the stiffness of RBL cells in response to cross-linking cell surface receptor–IgE complexes (IgE-R) (Fig. 1). In almost every measurement the first branch of the plot (deformation and force increasing) was essentially linear. Hence the "stiffness" was well defined as the slope of this branch. Not only the slope but also the hysteresis area enclosed by the two branches of the plot increased (Fig. 1). Because the hysteresis area is proportional to the energy dissipated by viscous "friction," not only the elastic but also the viscous resistance to deformation increased as a result of cross-linking IgE-R. The stiffness, which we use empirically to measure cellular deformability, registers changes in both elastic and viscous resistance.

The increase in stiffness triggered by cross-linking IgE-R is readily discernable in typical measurements of single living cells (Fig. 1, *A–D*). The response varied from one cell to another, however. Therefore we measured many cells to characterize each population statistically. Fig. 2, *A–E*, presents the distributions of stiffness values for control cells and cells responding to IgE-R cross-linking. Table I, which summarizes these data, presents not only the mean stiffness, the SD (to characterize the breadth of each population), and the SEM (to facilitate comparison of populations), but also the median stiffness for each population. As the histograms demonstrate, the distributions of stiffness are sometimes asymmetric, not normal, and can include values that lie well beyond the major grouping of stiffness measurements. Hence the median provides a conservative way to compare differences in stiffness between populations. The relatively soft control RBL cells did not substantially increase stiffness when monomeric IgE was bound to their surfaces (Table I). Cross-linking the bound IgE with DNP-BGG did, however, strongly increase stiffness. As the proportion of soft cells diminished, the proportion of cells with stiffness distributed over a wide range of higher values increased (Fig. 2).

The stiffness increased fairly rapidly at first and then more slowly to a maximum value sixfold greater than the control

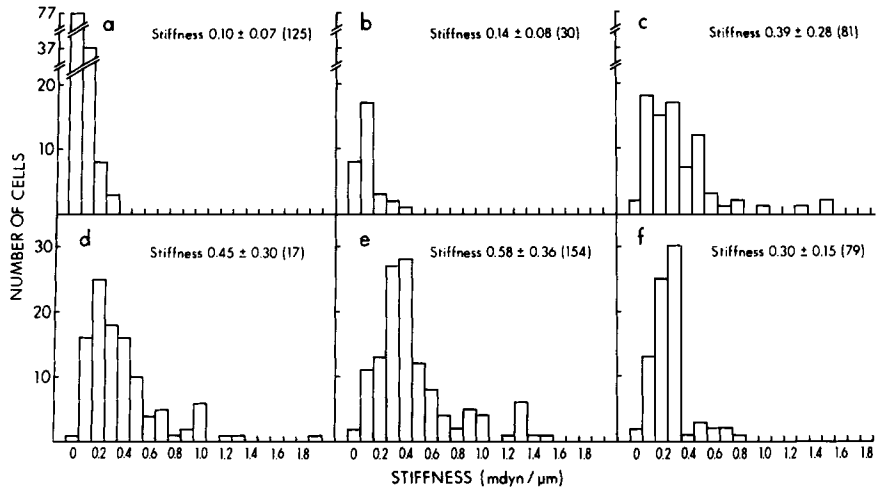

Figure 2. Distribution of stiffness values for RBL cells incubated with DNP-BGG for various times. (*a*) Control. (*b*) Exposed to IgE but not to DNP-BGG. (*c–f*) Cells sensitized by incubation with IgE and then incubated with DNP-BGG for 15 min (*c*), 30 min (*d*), 60 min (*e*), and 120 min (*f*). Mean stiffness ± SD (No. of measurements) is given in upper right corner of each panel.

Table I. Stiffening of RBL Cells by Cross-linking Bound IgE

Condition	Stiffness*				No. cells measured	% >1.0
	Mean	Median	SD	SEM		
	mdyn/μm	*mdyn/μm*	*mdyn/μm*	*mdyn/μm*		
Control	0.10	0.08	0.07	0.006	125	0
After binding IgE	0.14	0.12	0.08	0.02	30	0
Incubated with DNP-BGG for						
15 min‡	0.39	0.33	0.28	0.03	81	5
30 min	0.45	0.36	0.30	0.03	107	8
60 min	0.58	0.48	0.36	0.03	154	13
120 min	0.30	0.29	0.15	0.02	79	0
Con A (30 μg/ml, 30 min)	0.16	0.15	0.09	0.02	35	0
IgE + Con A (30 μg/ml, 30 min)	0.21	0.19	0.14	0.01	96	0

* Stiffness is defined as the initial slope of the plot of force versus extent of deformation as described under Methods. All measurements of deformability were performed at 23°C. The last column provides the percentage of the population with stiffness values >1.0 mdyn/μm.
‡ The cells were incubated with IgE for 60 min at 37°C and then with DNP-BGG or Con A at 37°C for the time periods specified. In addition to the incubation at 37°C there was also a variable period lasting from 5 to 30 min at room temperature after excess ligands had been removed during which the cells were allowed to attach to the substratum (5–10 min) and during which the measurements of deformability were actually being performed (up to 20 min). Because cell stiffness does increase in response to cross-linking surface bound IgE at room temperature, the stated periods of incubation with DNP-BGG or Con A should be considered nominal rather than precisely defined response times.

level during the first hour of incubation with DNP-BGG (Table I, Fig. 3). By 2 h after the addition of DNP-BGG, stiffness began to diminish. As explained in Table I, the stated incubation periods should be considered nominal response times. Addition of 15–20 min to these nominal times might provide a more realistic estimate of the measured response times. Therefore stiffness may begin to increase much more rapidly than we can determine from these measurements. In a subsequent experiment with a different batch of cells we attempted to improve our time resolution. In this measurement the cells, with IgE bound on their surface as before, were incubated with DNP-BGG for 5 min at 37°C and then allowed only 2 min to adhere to the glass coverslip. Stiffness measurements were then obtained quickly over the next 10 min at 23°C. For this sample of 14 cells the mean and median stiffness values were 0.37 and 0.32 mdyn/μm, respectively, with SD = 0.21 and SEM = 0.06 mdyn/μm. Hence the cells attained approximately half their maximum stiffness increase within 10–15 min.

Preliminary experiments suggested that extracellular Ca^{2+} was not essential for the stiffening response. For example, in one experiment cells were incubated with IgE for 30 min at 4°C for 30 min in MEM lacking Ca^{2+} ions, then exposed to DNP-BGG for 30 min in the same medium, and then deformability was measured over the next 20 min at 23°C. For a population of 35 cells the mean and median stiffness values were 0.36 and 0.30 mdyn/μm, respectively, with SD and SEM of 0.20 and 0.03 mdyn/μm, respectively. The unstimulated control value for mean stiffness was 0.12 mdyn/μm under the same conditions. In the presence of 2 mM Ca^{2+} this protocol yielded a comparable stiffness response.

The plant lectin Con A also provokes secretion by RBL cells, principally by cross-linking the IgE receptor via bound IgE (Fewtrell et al., 1979). Table I also shows that Con A causes only a small but significant ($P < 0.05$) stiffening of RBL cells in the absence of bound IgE and a slightly but significantly ($P < 0.05$) greater stiffening in its presence. The extent of this stiffening is substantially less than that triggered by IgE and DNP-BGG.

Stiffening was also slightly but significantly increased by exposure of cells to 10 mM NaN_3 for 45 min at 37°C. A population of 77 cells yielded mean and median stiffness values of 0.17 and 0.14 mdyn/μm with SD and SEM of 0.10 and 0.01 mdyn/μm, respectively. Azide ion elicits a stronger stiffening response in mouse spleen lymphocytes (Pasternak et al., 1985).

Serotonin Secretion

Cross-linking IgE-R with DNP-BGG caused a substantial release of [^{3}H]serotonin throughout the 1-h measuring period, in agreement with previous results (Fewtrell et al., 1979; Pfeiffer et al., 1985) (Fig. 4). The calcium ionophore A23187, 0.5 μM, in the presence of 2 mM Ca^{2+} ions elicited a slightly lower secretion response, but augmentation of the ionophore with 16 nM PMA increased secretion to the level achieved by DNP-BGG. A still further increase in secretion was obtained by adding cyto D (2 μM) to cells which had been treated with A23187 and PMA. Cyto D also enhanced

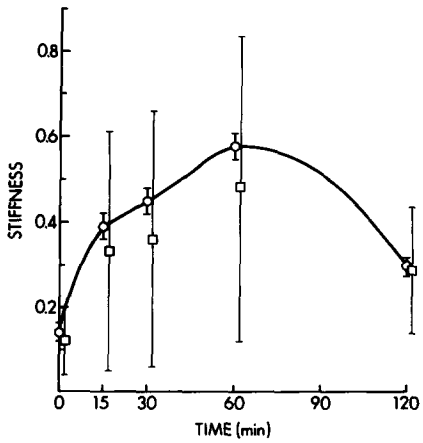

Figure 3. Stiffness of RBL cells versus time of incubation with DNP-BGG. (○) Mean stiffness; error bars indicate SEM. (□) Median stiffness; error bars indicate SD. Median values are displaced to slightly longer times for clarity.

secretion elicited by DNP-BGG between 1.5- and fivefold in different measurements (data not shown). Enhancement of antigen-induced secretion by cytochalasins has been previously observed by Menon et al. (1986) and was characterized in greater detail by Seagrave et al. (1987).

Relationship of Changes in Stiffness to Changes in Cell Thickness

The measured changes in cell stiffness coincided with changes in cell shape. Both control cells and cells bearing monomeric IgE appeared round with numerous microvilli distributed over their surfaces. As previously described (Pfeiffer et al., 1985; Phillips et al., 1985), cross-linking surface-bound IgE increased the extent of membrane ruffling and stimulated spreading of the cells. The number of cells undergoing this morphological change increased over at least an hour after cross-linking bound IgE.

Our previous work has shown that deformability depends on cell shape. As cells spread over a substratum and their thickness diminishes, we expect both from theory and from experimental observation that the stiffness should increase due to the influence of the rigid substratum (Daily, 1985). To minimize this influence we selected for deformability measurements cells which had largely retained the round shape typically observed before activation. Nevertheless it was possible that the thickness of cells that we measured varied substantially as a result of stimulation by antigen and IgE.

We tested the possibility that the deformability changes which we saw might have been due simply to changes in cell shape and thickness rather than to changes in cellular material properties (i.e., to changes in elastic or viscous parameters) by culturing cells on coverslips coated by poly[HEMA]. It has previously been shown that this coating inhibits cell spreading to an extent that depends on the thickness of the poly[HEMA] layer (Folkman and Moscona, 1978; Daily, 1985). RBL cells cultured on poly[HEMA]-coated coverslips retained their spherical shape even after responding to IgE and DNP-BGG. Nevertheless the response to cross-linking IgE did cause the cells to appear to have a more villous surface under the relatively low magnification (32× objective) of the Hoffman modulation contrast optics used in the Cell Poker microscope. The thickness of cells on normal and poly[HEMA]-coated coverslips was measured in the Cell Poker as described previously (Pasternak and Elson, 1985). In agreement with expectations from morphological studies (Pfeiffer et al., 1985; Phillips et al., 1985), cross-linking IgE-R caused a substantial reduction (35%) in the thickness of cells on uncoated coverslips (Table II). Control cells on poly[HEMA]-coated substrata were on the average ~2 μm thicker than those on uncoated coverslips. After cross-linking IgE-R there was only a 20% reduction in average thickness so that the treated cells on poly[HEMA] were only ~0.5 μm thinner than the control cells on uncoated coverslips. Finally, the absolute stiffness values and the changes in stiffness due to cross-linking IgE were even greater on poly[HEMA]-coated than on uncoated coverslips (Table II). Hence we conclude that the changes in cell stiffness due to IgE cross-linking are not due to changes in cell shape or thickness but rather must have resulted from changes in cellular viscoelastic properties. Moreover the increase in stiffness that results from cross-linking IgE-R was entirely reversed by cyto D in cells on poly[HEMA] (Table II) as it was in cells on normal substrata.

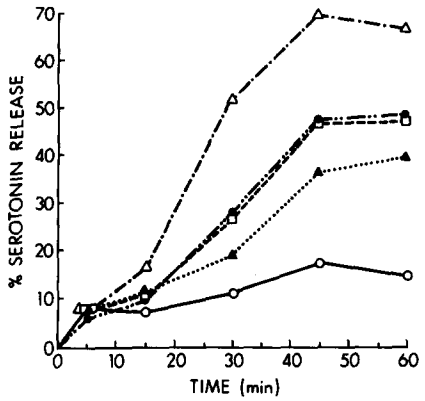

Figure 4. Secretion of [^{3}H]serotonin by RBL cells. (○) Control. (▲) A23187 (0.5 μM). (□) A23187 (0.5 μM) + PMA (16 nM). (△) A23187 (0.5 μM) + PMA (16 nM) + Cyto D (2.0 μM). (●) DNP-BGG (5 μg/ml). Concentration of Ca^{2+} was 2 mM for all samples. Before addition of DNP-BGG cells were sensitized by incubation with IgE (5 μg/ml) for 60 min at 37°C, and all other incubations were at that temperature. The concentration of DNP-BGG used in our experiments was well above the level required to elicit a maximal response (Pfeiffer et al., 1985). PMA, A23187, and cyto D were added simultaneously. In this experiment the cells in all samples were 100% viable according to their impermeability to trypan blue.

Table II. Thickness and Stiffness of RBL Cells on Glass and Poly[HEMA]-coated Substrata

On glass		On poly[HEMA]	
Cell thickness	Stiffness*‡	Cell thickness	Stiffness
μm	*mdyn/μm*	*μm*	*mdyn/μm*
Control:			
10.56 ± 1.59 (28)	0.10 ± 0.05 (28)	12.44 ± 0.82 (29)	0.10 ± 0.04 (47)
Responding:‡			
6.90 ± 1.39 (38)	0.35 ± 0.22 (38)	9.94 ± 1.14 (14)	0.54 ± 0.19 (14)

* Mean stiffness ± SD (No. of cells).
‡ Cells were triggered to respond by exposing to IgE (60 min) and then DNP-BGG (30 min), both at 37°C. Cells on poly[HEMA] which had been triggered to respond in this way and then exposed to cyto D (2 μM, 30 min) had a mean stiffness of 0.09 ± 0.05 mdyn/μm (17 measurements).

We also tested the dependence of cell stiffness on thickness by inquiring whether there is a relationship between these properties within a measured population. One approach was to compute the correlation coefficient, R, for the dependence of stiffness on thickness (Hoel, 1971). The value of R can vary from −1.0 to +1.0. For thin cells stiffness is expected to be a single valued function of thickness and to increase as thickness decreases (Daily, 1985). Hence R should approach −1 if cell thickness were an important determinant of stiffness in our measurements. If thickness were not an important determinant, then R should approach 0. For responding cells (for which there is a relatively wide range in stiffness values) on uncoated glass R = −0.08 and on poly[HEMA] R = −0.13. In both cases these values are consistent with the supposition that thickness plays little if any role in determining stiffness in our measurements. This test is best applied to

Table III. Inhibition of Stiffening by DNP-L-Lysine and by Cromolyn

Condition	Stiffness* ± SD (No.)
	mdyn/μm
Control	0.12 ± 0.02 (16)
IgE	0.16 ± 0.10 (26)
IgE, DNP-BGG (30 min)	0.28 ± 0.14 (23)
IgE, DNP-BGG (30 min) + DNP-L-Lys‡	0.19 ± 0.10 (14)
IgE, DNP-BGG (60 min)	0.40 ± 0.19 (23)
IgE, DNP-BGG (60 min) + DNP-L-Lys‡	0.11 ± 0.09 (18)
Control	0.15 ± 0.05 (20)
IgE, DNP-BGG (30 min)	0.61 ± 0.38 (19)
IgE, DNP-BGG (30 min) + Cromolyn§	0.25 ± 0.19 (19)

Cells were incubated with IgE for 60 min in each instance and then with DNP-BGG for the cited times. Temperature was 37°C during all incubations.
* Mean stiffness ± SD (No. of cells).
‡ Incubations with DNP-L-Lys (20 μM) were for 2 min.
§ Incubation with Cromolyn (100 μM) was for 30 min.

Table IV. Effects of Cytoskeletal Inhibitors

Condition	Stiffness ± SD (No.)
	mdyn/μm
Control	0.11 ± 0.05 (37)
Control, Cyto D (30 min)	0.07 ± 0.04 (69)
IgE, DNP-BGG (30 min)	0.36 ± 0.19 (54)
IgE, Cyto D (10 min), DNP-BGG (30 min)	0.03 ± 0.02 (7)
IgE, DNP-BGG (60 min)	0.30 ± 0.23 (35)
IgE, DNP-BGG (60 min), Colchicine (30 min)	0.21 ± 0.13 (70)
IgE, DNP-BGG (60 min), Cyto D (30 min)	0.11 ± 0.09 (35)
IgE, DNP-BGG (60 min), (Cyto D + Colchicine) (30 min)	0.08 ± 0.05 (31)

Cells were incubated with IgE for 60 min and then with DNP-BGG and cytoskeletal inhibitors in the order and for the time periods listed. Concentrations of cytochalasin D and colchicine were 2.0 and 10 μM, respectively. All incubations at 37°C.

normally distributed samples exhibiting a linear dependence of stiffness on thickness. Because some of our samples deviate fairly widely from a normal distribution and because stiffness may depend nonlinearly on thickness we have also used a curve fitting approach to test the dependence of stiffness on thickness. We have fitted stiffness to thickness both as a straight line and as a second order polynomial. For the linear fit the slopes of plots of stiffness, S, versus thickness, h, for responding cells were $dS/dh = -0.014$ and -0.022 mdyn/μm² on glass and poly[HEMA] respectively, indicating little if any dependence of stiffness on thickness. Furthermore in both cases there was no evidence for a statistically significant linear relationship between stiffness and thickness (significance level, $P > 0.5$). Fits to a second order polynomial did not improve the significance level and also showed a negligible dependence of stiffness on thickness. Hence allowing for a nonlinear dependence of stiffness on thickness does not change our conclusions. Therefore statistical analysis of the distribution of measured values for responding RBL cells on glass and on poly[HEMA] confirms our previous conclusion that changes in cell thickness have little if any influence on the reported stiffness measurements.

Inhibition of Signal Transduction

Cross-linking Fc_ε receptors constitutes the primary signal for secretion by RBL cells. When RBL cells with receptors aggregated by anti-DNP-IgE and DNP-BGG were exposed to the monovalent competitive hapten DNP-L-lysine, antigen-induced secretion ceased (Pfeiffer et al., 1985; Kanner and Metzger, 1983). Similarly the stiffening response was substantially reversed by a brief exposure of the triggered cells to DNP-L-lysine (Table III). Hence, dispersal of the cross-linked receptor aggregates rapidly returned the cell toward its original mechanical state.

The antiasthma drug cromolyn (1,3-bis [2-carboxychromon-5-yloxy]-2-hydroxy propane) is supposed to bind to and inhibit a calcium channel in RBL cell membranes which is activated by aggregation of the Fc_ε receptor and is essential for secretion (Mazurek et al., 1984). Incubation of RBL cells for 30 min with 100 μM cromolyn, a concentration which inhibits mast cell degranulation (Fewtrell and Gomperts, 1977), substantially reduced the increase in stiffness resulting from cross-linking IgE-R (Table III).

Effects of Cytoskeletal Disruption

Cytoskeletal inhibitors demonstrate the involvement of microfilaments in the observed stiffness changes and provide another way of reversing or preventing these changes. Cyto

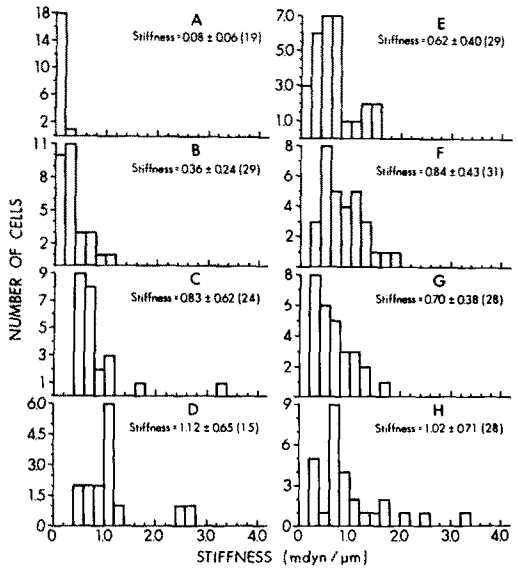

Figure 5. Distribution of stiffness values of RBL cells exposed to A23187 and to PMA. Cells were in 2 mM Ca^{2+} at pH 7.4. (*A–D*) Cells were incubated for 30 min with A23187 at 0.01 (*A*), 0.50 (*B*), 1.0 (C), and 6.0 μM (*D*). (*E*) Incubation with PMA (16 nM) for 30 min. (*F*) Incubation with PMA (16 nM) and A23187 (0.01 μM) for 30 min. (*G*) Incubation with PMA (16 nM) and A23187 (0.5 μM) for 5 min. (*H*) Incubation with PMA (16 nM) and A23187 (0.5 μM) for 30 min. The mean stiffness ± SD (No. of measurements) is given in upper right corner of each panel.

Table V. Effects of A23187 and PMA

Condition	Stiffness				No. cells measured	% >1.0*
	Mean	Median	SD	SEM		
	mdyn/μm	*mdyn/μm*	*mdyn/μm*	*mdyn/μm*		
Treatments at pH 8.0:						
Control	0.14	0.15	0.04	0.01	21	0
A23187‡						
5 min	0.37	0.33	0.20	0.03	36	0
15 min	0.32	0.27	0.17	0.03	28	0
30 min	0.28	0.27	0.08	0.02	25	0
A23187‡ + cyto D§	0.13	0.11	0.10	0.02	37	0
Treatments at pH 7.4:						
Control	0.07	0.07	0.03	0.006	25	0
A23187 (30 min)						
0.01 μM	0.08	0.06	0.06	0.01	19	0
0.50 μM	0.36	0.33	0.24	0.04	29	3
1.0 μM	0.83	0.64	0.62	0.13	24	22
6.0 μM‖	1.12	1.01	0.65	0.17	15	60
PMA¶	0.62	0.56	0.40	0.07	29	17
PMA + A23187 (0.01 μM)¶	0.84	0.68	0.43	0.08	35	35
PMA + A23187 (0.50 μM)**	0.70	0.60	0.38	0.07	28	21
PMA + A23187 (0.50 μM)¶	1.02‡‡	0.76	0.71	0.13	28	32
PMA + Cyto D + colchicine§§	0.10	0.10	0.03	0.01	15	0

For all experiments with A23187 Ca^{2+} concentration = 2 mM. In all experiments cells were treated at 37°C; deformability was measured at 23°C.
* The last column provides the percentage of the population with stiffness values greater than 1.0 mdyn/μm.
‡ For experiments at pH 8 the concentration of A23187 was 0.5 μM.
§ After a 20-min incubation with A23187 the cells were incubated with cyto D (3 μM) for an additional 30 min before measuring stiffness. The first branch of the force vs. deformability measurement seemed to be consistently biphasic in these measurements.
‖ Cells experienced a large change of shape under this condition.
¶ PMA concentration was 16 nM for all experiments in which it was present; cells were incubated for 30 min before measuring stiffness.
** Duration of treatment before measurement of stiffness = 5 min.
‡‡ Average excludes one outlying value of 5.19 mdyn/μm.
§§ Cyto D (2 μM) and colchicine (10 μM) were present with PMA (16 nM) for 30 min before measuring stiffness.

D marginally softens control cells (Table IV) (statistically significant at $P < 0.05$ according to Student's t test). Preincubation of IgE-sensitized cells with cyto D for 10 min before exposure to DNP-BGG entirely prevented the stiffening response and caused the cells to soften relative to control cells. Colchicine had an insignificant effect on stiffening. (The median stiffness values for the responding and colchicine-treated responding cells were 0.22 and 0.21 mdyn/μm, respectively.) Exposure to cyto D or to colchicine and cyto D together did, however, completely reverse the stiffening triggered by cross-linking IgE-R and restored the cells to the control level. Hence the integrity of microfilaments was essential for receptor stimulated stiffening of RBL cells. The stiffening caused by Con A was also reversed by cyto D (data not shown).

Response to Calcium Ion and Phorbol Ester

Both mast cells and RBL cells secrete serotonin when exposed to A23187 at a sufficiently high Ca^{2+} concentration (Foreman et al., 1973; Fewtrell et al., 1981). PMA, which activates protein kinase C (Nishizuka, 1984), is not an effective stimulus of serotonin secretion by itself but does enhance secretion stimulated by A23187 and Ca^{2+} (Heiman and Crews, 1985; Sagi-Eisenberg et al., 1985; Maeyama et al., 1986; cf. Fig. 4). Distributions of stiffness values in cells responding to A23187 and PMA are shown in Fig. 5. In some responding populations there are a few cells with very high stiffness values and a high fraction of cells with stiffness >1. Hence Table V provides median as well as mean stiffness values. The stiffness increased with increasing ionophore concentration and was reversed by cyto D (Table V). The ionophore was effective at both pH 7.4 and pH 8. At 6.0 μM A23187 there was a substantial change in cell shape (and possibly some cell damage) which was not detectable at lower concentrations (cf. Pfeiffer et al., 1985). Exposure of cells to A23187 at 0.5 μM for 30 min elicited comparable increases in stiffness at pH 7.4 and at pH 8.0. PMA (16 nM) by itself also caused a substantial increase in stiffness. Furthermore, PMA and A23187 acted synergistically at low ionophore concentration. At a concentration of 0.01 μM, A23187 had no detectable effect on the stiffness of RBL cells after a 30-min incubation. In the presence of 16 nM PMA, however, the stiffness increased to a value that was significantly higher than elicited by PMA alone ($P < 0.05$). Also at higher concentrations of A23187 (0.50 μM), which were sufficient to increase stiffness, the presence of PMA and A23187 together increased stiffness to a value beyond that elicited by either agent separately.

Discussion

We have characterized a cytoskeletal mechanical change which is stimulated in RBL cells for the most part in parallel with secretion. Our measurements of stiffening, although still phenomenological, provide a unique quantitative index

of this response. From the general perspective of nonmuscle cell mechanics we would like to know its structural basis and mechanism. The observed correlations with secretion prompt us further to consider the relationship of the observed stiffening to the stimulus and to secretion, and its function in the stimulus-secretion response.

Structural Basis of Stiffening

Changes in cell stiffness could result from changes in cell shape (Daily, 1985). The measurements of cells on poly-[HEMA] substrata (Table II) exclude that the observed changes in stiffness were due simply to changes of cell thickness. Indeed, it is likely that the shape changes previously observed (Pfeiffer et al., 1985; Phillips et al., 1985) are driven by the same cellular mechanical processes that increased stiffness.

Actin filaments participated in the observed increases of stiffness (Table IV). Cyto D marginally diminished the baseline stiffness of the RBL cells. When added after cross-linking, cyto D reversed the increase in stiffness whereas colchicine, an inhibitor of microtubule assembly, had an insignificant effect on stiffness of responding cells. Therefore, we conclude that the stiffening triggered by cross-linking the Fc_ε receptor requires the integrity of microfilaments. Also, when cells were preincubated with cyto D before cross-linking IgE-R, the stiffness did not increase but dropped to a level below that of untreated control cells. This suggests that some filaments that are resistant to cyto D before or after the response are sensitive to that inhibitor during the response.

Possible Mechanisms of Stiffening

The stiffening we have observed could have been caused by a cellular contraction as originally hypothesized, but it could also have resulted from increases in microfilament concentration, cross-linking, or degree of polymerization.

A contractile tension generated by actin–myosin interactions (cf. Volpe et al., 1985; Di Virgilio et al., 1986) could stiffen a cell by making its cortex (Bray et al., 1986) more difficult to stretch. Cytochalasin D could inhibit development of tension by promoting disassembly of the participating microfilaments. A symmetrical radial or circumferential tension would drive a cell toward a spherical shape. Therefore cells responding on adhesive glass surfaces must experience a net component of the tension directed toward the substratum to cause the observed spreading and flattening (Table II; Pfeiffer et al., 1985; Phillips et al., 1985). Our observations of cells on poly[HEMA] indicate that a strong adhesive interaction with the substratum is necessary to generate the anisotropic tension required for flattening and spreading. Yet this adhesive interaction is not necessary for stiffening because stiffness increases in lightly adherent cells which flatten only slightly.

The content of F-actin in RBL cells increases in response to cross-linking Fc_ε receptors (Pfeiffer et al., 1985). A greater density of microfilaments could make the cell more difficult to deform by increasing cytoplasmic viscoelasticity even in the absence of a contractile tension. Furthermore an increase in the extent of cross-linking of filaments (Flory, 1953) or in their degree of polymerization (Klotz and Zimm, 1972) would also increase cellular viscoelasticity even at constant total filament content. No data are yet available on these latter interesting possibilities.

Two other possibilities can be mentioned but seem unlikely. A change in intracellular osmotic (turgor) pressure could increase cell stiffness and could have some of the same mechanical effects as a cortical contraction. But the effects of cytoskeletal inhibitors both on stiffening and secretion and the observed spreading and flattening of cells argue against this mechanism. Cell spreading and flattening due to an adhesive interaction between the cell and substrate also could increase the internal pressure of the cell, but the measurements of cells on poly[HEMA] seem to rule out this explanation.

Relationship of Stiffening to Stimulus

A relatively natural stimulus, the cross-linking of IgE-R by a multivalent ligand, substantially increased cellular stiffness (Figs. 1–3, Table I). Our results indicate both similarities and some differences between the processes that trigger stiffening and secretion. (*a*) Simply binding IgE to its receptor was insufficient; both responses required the cross-linking of IgE-R (Tables I and III). (*b*) Both responses were quickly reversed by DNP-lysine, a competitive monovalent ligand for IgE which eliminates cross-linking (Table III; cf. Pfeiffer et al., 1985; Kanner and Metzger, 1983; Fewtrell, 1985). (*c*) The time dependence for stiffening (Fig. 3) was similar to that for secretion (Fig. 4; Fewtrell et al., 1979; Pfeiffer et al., 1985). Our measurements of the overall duration of serotonin release agree with those of earlier workers, but we observed a 15–20-min lag period which was not present in the earlier measurements. We cannot explain this discrepancy. Stiffness rose rapidly with no apparent lag seen in the nominal response times. (*d*) Although the stiffening produced by Con A was statistically significant (Table I), the response was much smaller than that produced by IgE and antigen. Therefore, Con A induces degranulation of RBL-2H3 cells relatively more effectively than it does stiffening (Fewtrell et al., 1979). (*e*) The stiffening was partly reversed by cromolyn, which is thought to block an IgE-dependent calcium channel involved in initiating secretion (Mazurek et al., 1983; Mazurek et al., 1984) (Table III). This result is, however, somewhat paradoxical and difficult to interpret. Cromolyn does not inhibit secretion in RBL cells under conditions in which secretion by mast cells is inhibited (Fewtrell and Metzger, 1981), and other data raise uncertainties about the role of the cromolyn-binding protein (Metzger, 1986; Lindau and Fernandez, 1986). Furthermore, as discussed above, preliminary experiments suggest that external Ca^{2+} is not essential for the stiffening response. Nevertheless, an increase of cytosolic Ca^{2+} by an ionophore does stiffen RBL cells (Table V). Thus whereas Ca^{2+} influx is sufficient, it is not necessary for stiffening. External Ca^{2+} and therefore presumably Ca^{2+} influx is necessary for secretion. This different requirement for external Ca^{2+} may indicate a significant difference in the pathways from stimulus to the stiffening and secretion responses. Interpretation of the effects of cromolyn requires more information about its mechanism of action, especially its effect on intracellular Ca^{2+} in RBL cells.

The heterogeneity of the stiffening response among individual cells is striking (Fig. 2) and is reminiscent of the heterogeneity in secretion seen in other systems (e.g., Salomon and Meda, 1986; Frawley et al., 1986). It would be in-

teresting to learn whether there is a similar heterogeneity in RBL cells. We are, however, unaware of data on the distribution of secretion responses among individual RBL cells. Although more remains to be done to explore the relationship between the triggering of stiffening and of secretion, the available evidence indicates that the same receptor-dependent stimuli can elicit both responses.

Relationship of Stiffening to Secretion

In several stimulus-secretion systems early steps after the binding of a ligand to a membrane receptor (often with cross-linking) involve the hydrolysis of phosphatidylinositol phosphate to diacylglycerol and inositol triphosphate (Becker, 1986; Gomperts et al., 1986; Gomperts, 1986; Turk et al., 1987). The former activates protein kinase C; the latter releases Ca^{2+} from intracellular stores. We have tested the effects on stiffness of these two processes which are supposed to mediate between stimulus and secretion (Fig. 4; Fewtrell et al., 1981; Maeyama et al., 1986; Heiman and Crews, 1985; Sagi-Eisenberg et al., 1985; Metzger, 1986). Table V and Fig. 5 demonstrate that both increasing intracellular Ca^{2+} via the ionophore A23187 and treatment by PMA separately caused substantial cellular stiffening that could equal or exceed that produced by cross-linking IgE-R and that depended on microfilament integrity. Furthermore, the two treatments together caused still greater increases both in mean stiffness and in individual stiffness values that substantially exceeded those stimulated by cross-linking IgE-R. Thus the RBL-2H3 cells had a capacity for stiffening well beyond that activated by cross-linking Fc_ε receptors. Hence two processes thought to mediate between stimulus and secretion also increase stiffness.

Stiffness measurements provide an indicator of cytoskeletal function which differs in part from the morphological observations of Pfeiffer et al. (1985). They have reported that IgE-R cross-linking and PMA but not A23187 caused RBL-2H3 cells to spread and flatten. Our own observations of cell shape are consistent with these except that we observed a change in cell shape at a high concentration of A23187 (6 μM), beyond the range studied by Pfeiffer et al. Hence stiffness increased at elevated intracellular Ca^{2+} without substantial change in cell shape except at very high ionophore concentrations. Therefore the deformability measurements have revealed a cytoskeletal process caused by A23187 that was not detected by morphological observations.

Role of Stiffening in Secretion

To understand the role of stiffening in secretion it is necessary to take account of the effects of cyto D. An earlier study indicates that cytochalasins A and B inhibit mast cell secretion (Orr et al., 1972). More recent work (Menon et al., 1986; Seagrave et al., 1987) and our observations show that cyto D enhances secretion by RBL cells. Hence stiffening is not necessary for secretion by RBL cells with impaired actin filament systems. This might indicate that stiffening generally has no role in secretion. Stiffening could, however, have an important influence on secretion by cells with intact actin filaments. The strong correlation between stiffening and secretion discussed above encourages us to consider how stiffening might influence secretion.

Perhaps the simplest interpretation of the effects of cyto D is that stiffening corresponds to an inhibition of secretion, for example, by preventing transport of secretion granules to the plasma membrane. Then the same signal would trigger both secretion and simultaneously a process which diminished its rate (cf. Metzger, 1986). If the stiffening were regulated by other stimuli, this apparently paradoxical mechanism would confer an additional level of control on secretion rate. The inhibition could result from increased cortical contractile tension, which could impair access to the plasma membrane, or from increased actin filament density or cross-linking, which could immobilize granules in the cytoskeletal matrix. Disassembly of filaments by cyto D could attenuate both sorts of inhibitory effects and thereby enhance secretion. It is also possible, however, that stiffening could exert a positive effect on secretion. For example, tension applied to the cytoskeletal cortex might create holes in the filament mesh that separates granules from the plasma membrane (cf. Allison, 1973; Oster, 1985) and thereby facilitate granule-membrane contact. Cyto D might so attenuate the density of filaments and thereby facilitate approach of granules to the membrane that secretion could occur even more rapidly in its presence than with an intact but contracted filament network.

Stiffening could also be related to activities at the plasma membrane triggered by cross-linking Fc_ε receptors, such as increases in endocytosis by coated pits and fluid-phase pinocytosis (Pfeiffer, 1985) and the clustering (Menon et al., 1984) and immobilization (Menon et al., 1986) of IgE-R oligomers.

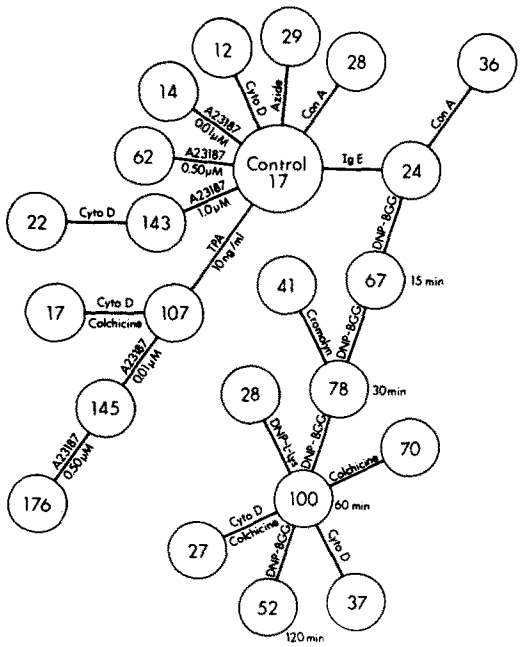

Figure 6. Summary of RBL cell stiffening responses. The maximum mean stiffening response to cross-linking Fc_ε receptors (0.58 mdyn/μm, Table I) was taken as 100%. This figure presents the percent mean stiffening relative to this value, calculated from data in the tables. In some instances the 100% value was chosen to account for differences in the response level for a given experiment. For example in the DNP-L-Lys and cromolyn measurements the 100% values were taken to be 0.40 of 0.61 mdyn/μm, respectively (Table III).

To understand the mechanism of stiffening and its role in secretion it is essential to obtain additional information, for example, on the effects of specific inhibitors of actin–myosin contraction or on the properties of mutant cells that display parallel defects in the two responses.

Generality of Stiffness Response

The changes in stiffness we have measured in responding RBL cells, summarized in Fig. 6, are similar in broad outline to those observed in lymphocytes responding to cross-linking surface Ig and Con A acceptors (Pasternak and Elson, 1985). Both microtubules and microfilaments participate in the mechanical response of lymphocytes, which occurs during capping. In lymphocytes as in RBL cells both contractile forces generated by actin–myosin interactions (cf. Bourguignon and Bourguignon, 1984) and increases in actin polymerization during capping (Laub et al., 1981) could contribute to the measured stiffening.

More recently we have observed an increase in the stiffness of rat pancreatic islets of Langerhans cells responding to an increase in glucose concentration (Schwab et al., 1986). This effect could be related to insulin secretion by these cells. In lymphocytes and islet cells it is thought that the primary signal from the extracellular stimulus is mediated through breakdown of phosphoinosotide phosphate to yield activators of protein kinase C and release of intracellular Ca^{2+} as described above for RBL cells (cf. Wolf et al., 1986; Paul et al., 1986). Hence the cytoskeletal changes that cause stiffening may contribute to different types of cellular activation processes.

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