Transmembrane Signaling in P815 Mastocytoma Cells by Transfected IgE Receptors*

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In order to delineate structural-functional relationships of the mast cell receptor for IgE (Fc,RI) by molecular-genetic analysis, a transfectable cell must be identified which resembles mast cells except for being deficient in receptors. We have found that the well known murine mastocytoma P815 is suitable. These cells express no Fc,RI, lack mRNA for the α and β subunits of the receptor, but contain some mRNA for γ chains. After transfection with the cDNA for each of the subunits, stable clones could be isolated which expressed several hundred thousand normal Fc,RI and synthesized large amounts of mRNA for α, β, and γ, the last at 3-fold higher levels than in the untransfected cells. Aggregation of the transfected receptors led to opening of presumptive calcium channels and to activation of phospholipase C, phospholipase A2, and protein kinase C. The kinetics and other characteristics of the signals were similar to those observed after stimulation of the rat tumor mast cells from which the cell line was derived but were smaller in magnitude. These weaker signals most likely result from an overall reduced reactivity exhibited by the P815 cells since stimulation by other ligands led to weaker or even no responses. The cells failed to degranulate after either receptor aggregation or reaction with ionophores with or without phorbol ester. Both the transfected and untransfected P815 cells express Fc receptors for IgG (Fc,RII) which, interestingly, independently triggered similar responses despite their apparently simpler subunit structure.

Aggregation of the high affinity IgE receptors (Fc,RI)† on mast cells and basophils causes these cells to degranulate and to release a variety of preformed and newly synthesized mediators (1). Although many early biochemical events have been described which follow aggregation of the receptors (2, 3), none of the molecules that are responsible for these phenomena has been unambiguously identified. It is even unclear which if any of such events are due to the direct action of the aggregated receptors themselves.

The receptor is composed of three types of polypeptides: an IgE-binding α chain, a β chain, and two disulfide-linked γ chains. Each chain appears to be a transmembrane protein, and we have proposed a model in which there are altogether seven transmembrane segments, five cytoplasmic extensions, three extracellular extensions, two extracellular loops, and one cytoplasmic loop (4).

We plan to investigate which of these parts of the receptor are necessary to initiate one or more of the early biochemical signals. Such information could be used to identify critical "postreceptor" molecules (see "Discussion"). As a first step toward this goal, we have prepared various mutant cDNAs of each of the subunits and showed by virtue of their IgE binding that the mutant receptors could be expressed when transfected into COS 7 cells (49). However, upon aggregation, even the wild-type transfected receptors on such cells failed to initiate some of the principal early biochemical events, such as hydrolysis of phosphoinositides and a rise in intracellular Ca²⁺, which are induced by the endogenous receptors on mast cells.²

We now describe experiments in which such signals (and others) were observed when the unmutated cDNAs were transfected into the receptorless mouse mastocytoma P815. Therefore, functional IgE receptor complexes can be formed after transfecting receptor cDNAs into the P815 cells. In addition, this cell line appears to be a suitable host for future work employing mutant receptors.

MATERIALS AND METHODS

Cells—The 9H3 subline of rat basophilic leukemia (RBL) cells was cultured in monolayers in stationary flasks (5). Nonadherent mouse mastocytoma P815 cells, HTR subline (6), were a gift of B. Askonas, John Radcliffe Hospital, Headington Oxford, United Kingdom. Cells were cultured in upright flasks in Dubecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD) including 25 mM HEPES and 16% heat-inactivated fetal bovine serum (Biofluids) at 37 °C in 8% CO₂, 92% air in a humidified cell incubator.

Immunoglobulins—Antidinitrophenyl (DNP) monoclonal mouse IgE was from the hybridoma II-DNP:20.02 (7). Rat IgE of unknown specificity was from the immunocytoma IR162 (8). IgEs were purified as described by Holowka and Metzger (9). When required, proteins

² L. Miller, P. Millard, T. Ryan, C. Fewtrall, and H. Metzger, unpublished observations.

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1 The abbreviations used are: Fc,RI, receptor(s) with high affinity for immunoglobulin E; Fc,RII, low affinity receptor for immunoglobulin G; DMEM, Dulbecco's modified eagle's medium; DNP, 2,4-dinitrophenyl; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid]; HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; NECA, 1'-(N-ethylcarboxamido)adenosine; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); Quin 2, 2-[2-(2-bis(carboxymethyl)amino-3-methoxyphenoxymethyl]-6-methoxy-6-bis-[carboxymethyl]aminoquinoline); RBL, rat basophilic leukemia; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.
were radioiodinated using the chloramine-T method of McNabney and Dixon (10). Anti-Fc,RI receptor antibody 2.4G2 (11) was a gift of M. Sandor (University of Iowa). The anti-Fc,RI receptor 5.14 (12) was a gift from Zelig Eshhar (Weizmann Institute of Science, Rehovot, Israel).

Production of F(ab’)2 and Fab’ Fragments—F(ab’)2 fragments were produced from monoclonal antibody 5.14 basically as described by Rivera et al. (13). The Fab’ fragments were removed using a CNBr-activated Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) column using the Monoclonal Antibody Purification System II buffer system (BioRad). F(ab’)2 fragments were found to be free of intact IgG by gel electrophoresis. Fab’ fragments were prepared directly by reduction of F(ab’)2 with 10 mM cysteine for 30 min at 37°C and then alkylated with 12 mM iodoacetic acid for 30 min at room temperature. Proteins were concentrated and dialyzed versus borate-buffered saline (0.2 M H3BO3, 0.1 M NaCl, pH 8.0) overnight at 4°C. Purity of Fab’ fragments was verified using gel electrophoresis.

Biochemical Labeling and Purification of Fc from Transfected Cells—Twenty-five million stably transfected P815 HTR cells (below) were cultured in cysteine-free DMEM (NIH media laboratory) with 16% dialyzed fetal bovine serum (GIBCO) for 4 h in spinner flasks at 37°C in 8% CO2, 92% air. All plasmids were reacted with heat-treated RNase A before transfection (16). After the final purification, the plasmids were hydrated in sterile high pressure liquid chromatography grade water. In some cases, pSVL plasmids containing α, β, and γ chains were linearized before transfections with Psil (Pharmacia). Fab’ from these and pSV2 neo were linearized with EcoRI (New England Biolabs). Transfections—Transfections were carried out basically as described by Van Pel et al. (6). Briefly, 1.4 × 10⁵ P815 HTR cells were washed twice and resuspended in DMEM (plus 25 mM HEPES) free of fetal bovine serum. Cells were allowed to attach to 60-mm tissue culture dishes (Corning Glass, Corning, NY) for 15 min at 37°C. One ml of the culture medium was added. The supernatant was removed with heat-treated RNase A, and the cells were incubated overnight for biochemical labeling. Receptors were purified as described by Rivera et al. (13).

Expression Vectors—cDNAs coding for each of the subunits of the rat high affinity IgE receptor were subcloned separately into the eukaryotic expression vector pSVL (Pharmacia) as described previously (4). Transformed bacteria containing the selective marker plasmid pSV2 neo (14) were obtained from American Tissue Type Culture Collection (Rockville, MD). The plasmids were prepared in large chloride gradients. All plasmids were reacted with heat-treated RNase A. DNA was isolated using a Pharr&&ia Celiphect kit. Precipitates were formed for 30 min at 68°C and the precipitate was then plated in the same selective medium at 0.5 and 1 cell/well. The DNA was heated at 65°C for 3 min to free the cut ends.

Selection and Cloning of Transfectants—Forty-eight h after transfection, the cells were resuspended in selective medium containing 1.1 mg/ml G418 (GIBCO) and incubated at 37°C in 5% CO2, 95% air. For transfections in which chloramphenicol was used, the DNA was heated at 65°C for 3 min to free the cut ends.

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removed, 1 ml of 0.1% Triton X-100 was added, and the pellet of cells was incubated for 10 min at room temperature. The lysis of cells was used to calculate total incorporation of $^{[3]H}$arachidonic acid and $^{5}[^{3}H]$hydroxytryptamine. $^{3}H$ and $^{14}C$ counts were determined with a Tracer Analytic MII scintillation counting system set to a narrow window $^{3}C/^{14}C$ program.

**Phosphorylation of Myosin in Transfected and RBL Cells**—Two days before use, RBL-2H3 cells were trypticized and set to grow in suspension cultures at a density of $1 \times 10^{6}$ cells/ml. Cells were washed twice in phosphate-free buffered salt solution (buffer A in Ref. 22) and then resuspended at $5 \times 10^{6}$ cells/ml. Cells were labeled by incubation with $[^{32}P]orthophosphoric acid (250 $\mu$Ci/ml) at $37^\circ$C for 2 h in 10 ml of buffer A, with the inclusion of DNP-specific IgE at 1 $\mu$g/ml to sensitize the cells. P815 cells were resuspended and radio-labeled similarly at $4 \times 10^{6}$ cells/ml. After labeling, cells were centrifuged and washed twice with buffer A. The cells were then resuspended in aliquots of 0.3 ml ($8.3 \times 10^{6}$ cells/ml) for RBL-2H3 and 1.5 $\times 10^{6}$ cells/ml for P815 cells. The cells were stimulated at $37^\circ$C for various times by the addition of either specific antigen (DNP$_{2}$-BSA) or IgE-loaded cells or anti-FcRI antibody 5.14. At each time point, 1 ml of ice-cold buffer A was added to the tubes and cells pelleted in a microcentrifuge for 10 s (this time had been predetermined to be enough to pellet all the cells but not to cause any cell damage). An aliquot of the supernatant was retained to determine the secretion of histamine or $[^{3}H]$hydroxytryptamine, but the remainder was separated via an acid buffer (22) was added to the cell pellet and the tubes vortexed. The cell lysate was then treated exactly as described previously (22) to immunoprecipitate myosin and analyze the phosphorylation of the light chains.

However, an alternative method of mapping the myosin tryptic peptides was utilized. Following digestion by trypsin and lyophilization, the residue was resuspended in 50 mM ammonium bicarbonate and applied to a urea-polyacrylamide isoelectric focusing gel on LKB GeBond FAG film (23). The Amphotole solutions were 0.0 ml of pH 2.5-4, 0.5 ml of pH 5.5-7, and 0.5 ml of pH 8-10. The peptides were separated by electrophoresis (LKB 2117 Multiphor) at 25 watts/1250 V for 1 h at 4 $^\circ$C, with 1 M H$_2$PO$_4$ as the anode buffer and 1 M NaOH as the cathode buffer. The gels were stained in a heat vacuum, dried and subjected to autoradiography as described previously (22). The radioactive tryptic phosphopeptides were identified by phosphorylating myosin light chains in vitro, separating the tryptic phosphopeptides by two-dimensional peptide mapping, and then scraping them off the silica gel plates for separation by one-dimensional isoelectric focusing.

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**Northern Blots**—Total RNA was prepared from $1 \times 10^{6}$ untransfected P815 and transfected clone 6 cells by guanidine isothiocyanate lysis followed by purification by centrifugation through CsCl gradients (16). Northern blots were carried out basically as described by Davis et al. (16). Fifteen $\mu$g of RNA was loaded onto each lane of a 1% agarose gel containing 2% formaldehyde to inhibit RNases. Transfected and untransformed RNA were loaded overnight onto 0.45-$\mu$m nitrocellulose and then blotted onto 0.45-$\mu$m Trans-Blot transfer medium (Bio-Rad). $[^{32}P]$-Labeled probes were prepared by nick translation of cDNAs corresponding to the full-length coding sequences for the $\alpha$, $\beta$, and $\gamma$ chains of the rat FcRI as well as the mouse $\gamma$ chain (24). Radioactive labelings were carried out using the Boehringer Mannheim nick translation kit. The relative amount of RNA bound to each lane of the nitrocellulose membrane was determined after washing the blots for 15 min in boiling H$_2$O and then reactivating them with a probe corresponding to the rat muscle glyceraldehyde-3-phosphate dehydrogenase sequence from the plasmid pRGAPDH-13 (25). The probe was radiolabeled using the Boehringer Mannheim random priming kit. The plasmid was a gift of Dr. John Cav. (NIH). Probes were denatured as described (15), and blots reacted overnight at 42 $^\circ$C with $1 \times 10^{7}$ cpn of each probe. For high stringency, filters were washed four times (20 min each) at room temperature with 0.1% SDS, 2 $\times$ SSC (16), and then twice (20 min each) at 55 $^\circ$C with 0.1% SDS, 0.1 $\times$ SSC. For low stringency, filters were washed at room temperature with 0.1% SDS and then washed once in 50% SSC as above and then twice 20-min washes with 0.1% SDS, 1 $\times$ SSC at 50 $^\circ$C. Autoradiographs were carried out with Kodak XAR-5 film at -80 $^\circ$C.

**Release of $\beta$-Glucuronidase**—Release of $\beta$-glucuronidase after stimulation of transfected P815 and RBL cells was measured according to Fishman et al. (26). Cells (1 $\times 10^{6}$ cells/ml) were loaded with 10 $\mu$Ci/ml of $[^{3}H]$hydroxytryptamine and then washed twice in culture medium and once in stimulation buffer (26). Cells were diluted in stimulation buffer to $2 \times 10^{6}$ cells/ml and reacted with 100 ng/ml DNP$_{2}$-BSA. Alternatively, non-IgE-loaded cells were stimulated directly with 4 $\mu$g/ml anti-FcRI antibody. After a 1-h reaction with the stimulus at 37 $^\circ$C, an aliquot of the cell suspension was sonicated 3 min for determination of total $\beta$-glucuronidase content. Another aliquot was centrifuged at 1000 $\times g$ for 10 min. Two hundred $\mu$l of the supernatant was combined with an equal volume of substrate (30 mM phenolphthalein mono-$\beta$-glucuronide, pH 4.5; Sigma) and 800 $\mu$l of 0.1 M sodium acetate, pH 4.5. The solution was incubated at 37 $^\circ$C for 1 h and the reactions stopped with 800 $\mu$l of 0.2% SDS containing 0.1 M 2-amino-2-methylpropanol-1, pH 11. Relative amounts of $\beta$-glucuronidase released were determined by measuring the absorbance of the solution at 560 nm.

**RESULTS**

**Stable Transfectants**

We have introduced cDNAs coding for each of the subunits of the rat high affinity IgE receptor into P815 HTR cells, a P815 subline selected to be highly transfec"table by calcium phosphate precipitation (6). Stable transfectants arose after selection in G418-containing medium (see "Materials and Methods"). Two sets of transfected cells were isolated. The first was derived from transfections using linearized pSVL and pSV2 neo plasmids. Using the rosetting assay described previously for detecting high affinity receptors for IgE (4), about 15% of selected cells scored positive. These cells were cloned at 0.5 and 1.0 cell/well and were again assessed by the rosetting assay. This procedure yielded four clones in which 100% of the cells scored positive. Two clones were characterized (clones 4 and 5) and continued to bind IgE for over 4 months, after which only frozen cell stocks were retained. A second set of clones was isolated from transfections using nonlinearized plasmids. For these transfections, around 65% of transfected and selected cells scored positive in the rosetting assay. After identical cloning steps, eight clones were isolated in which 100% of cells scored positive. Three clones were retained for characterization (clones 6, 7, and 8).

**Properties of P815 Cells**

The P815 cells used in our work were originally derived from a transplantable neoplasm that arose in a male DBA/2 mouse that had been repeatedly painted on the skin with methylcholanthrene (27). The solid tumor was converted to an ascites form and contained high histamine and serotonin levels as well as exhibiting metachromatic granules characteristic of mast cells. However, these malignantly transformed cells had a much lower number of these granules than normal mast cells. After adaptation of the ascites tumor to continuous cell culture, the cells continued to have high histamine and serotonin levels (28).

We have chosen a subline of the P815 cells which had been selected to be highly transfec"table by calcium phosphate precipitation (6). The cells grow in suspension in culture but will attach weakly to tissue culture dishes after a 10-15 min incubation in medium containing no serum (6). The cells have a diameter of $3.5 \pm 1.0 \mu$m, as measured by light microscopy. Transfection with FcRI cDNAs and selection of stably transfected cell clones did not alter the size, shape, or the general appearance of the cells. P815 cells are smaller than RBL cells, which were measured to be $14.7 \pm 1.0 \mu$m in diameter.

Whereas the original P815 cell line, after adaptation to growth in culture, had histamine levels as high as 540 nmol/10$^{6}$ cells (28), our cells contained a negligible amount of histamine as measured by radioimmunoassay (29). We have not measured the endogenous serotonin level. However, plasma membrane transport mechanisms appear to be functional since the cells take up $[^{3}H]$hydroxytryptamine from the cell culture medium. A second difference is that substantial

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1 J. R. Sellers, unpublished observations.
granules could not be discerned either by Wright or by acid toluidine blue staining. Therefore, the P815 cell line we used for our work has lost certain properties characteristic of both mast cells and the neoplasm from which it was derived. However, these cells are still suitable for our studies since, as described below, the cells retain the ability to respond to aggregation of the transfected IgE receptors. Other P815 sublines also lack visible granules after staining and contain very low levels of histamine and serotonin (30, 31).

IgE Binding Properties of Transfected Cells

To determine if the binding characteristics of the transfected receptors were similar to those of native receptors, we measured the dissociation rate of 125I-labeled mouse IgE since it is the slow rate of dissociation rather than an unusually fast rate of association which accounts for the high affinity of this receptor (19). Clone 6 was chosen for study because it proved to be the most active in signal transduction (below). The dissociation constant (k-1) at 37 °C calculated from the data presented in Fig. 1 (1.7 ± 0.3 × 10^{-5} s^{-1}) is in good agreement with the value of 1.6 × 10^{-5} s^{-1} reported by Kulczycki and Metzger (19) for endogenous receptors on RBL cells. When cell-bound IgE was measured after adding buffer but no excess unlabeled rat IgE to 125I-labeled loaded cells (Fig. 1, A), the cells continued to bind additional IgE. A similar trend was observed with transfected COS 7 cells but not with RBL cells. Since the growth rates of both COS 7 and P815 cells are greater than that of RBL, this result may reflect cell growth over the 5-h period of the experiment.

We also used 125I-IgE binding assays to determine the number of Fc,RI expressed by each transfected P815 clone. Clones 4 and 5, which were derived from transfections using linearized DNA, expressed 2–3 × 10^6 receptors/cell (Table I). However, clones 6, 7, and 8, which were derived from transfections using circular plasmid DNA, expressed 2.7–3.8 × 10^6 receptors/cell (Table I). The number of receptors expressed by the clones decreased 10–20% during the first 6 weeks after transfection. After that time, these values stabilized. Entries reported in Table I were measured 8 weeks after transfections.

Structural Characterization of Transfected Receptors

We also wished to verify that the transfected receptors had the expected subunit structure. Receptor-IgE complexes were purified from biosynthetically labeled P815 clones 6 and 8 and analyzed on polyacrylamide gels (Fig. 2A). The transfected receptors from both clones were composed of α, β, and γ chains, although those from the transfected cells showed a slightly lower content of γ chains. Possibly the dissociability of the transfected receptors is even greater than that observed normally (32). However, the decrease in γ in the receptor complex is small, and we have not made a sufficient number of measurements to quantify this phenomenon. The α chain band appears somewhat broader in the transfected cells than in RBL (Fig. 2B), which suggests heterogeneous glycosylation of this subunit in the P815 cells.

Northern Blotting of Transfected Cells

Recently, Ra et al. (33) showed that mRNA apparently identical to that of the γ chain of the rat Fc,RI is expressed in a variety of murine cell lines that do not express Fc,RI. Among the positive lines was the mastocytoma P815 used in our work. All γ chain positive lines (and only such lines) also
expressed mRNA for the α chain of the Fc,RII. It was of interest to compare the levels of γ mRNA (as well as mRNA for the other subunits) in the transfected and untransfected cells. Fig. 3 shows Northern blots of total RNA purified from clone 6 and from nontransfected P815 cells. Clone 6 RNA hybridized strongly with cDNA for the rat α and β chains, but no reactivity was observed with RNA from the nontransfected P815 cells. For the α chain, we found both a 1.6- and a 2.6-kilobase RNA species and for the β chain, 1.7- and 2.8-kilobase species. We have also observed multiple species in RNA purified from transfected COS 7 cells (not shown).

We used three different probes to compare the relative amounts of γ message in transfected and nontransfected cells. Fig. 3 shows the results of hybridization with a probe corresponding to the full coding sequence of the mouse γ chain. Even though the homology between mouse and rat γ chains is 94% at the DNA level, we washed the blots at low stringency to minimize the preferential reactivity of the endogenous mouse sequence. By quantitative analysis of the blots, transfected cells appeared to express over three times more γ message than the nontransfected cells. Similar results were obtained with a probe for the rat γ chain and with a 37-base oligonucleotide corresponding to a DNA sequence that is identical between mouse and rat γ. The rat γ mRNA in clone 6 cells transfected with pSVL plasmids is around 1 kilobase and appears to be larger than the message for the endogenous γ chain (Fig. 3). These experiments show that clone 6 cells express substantially more transfected rat γ mRNA than endogenous mouse γ message.

Early Responses

Calcium Response in Transfected Cells—One of the most extensively characterized early responses of RBL cells after stimulation via IgE receptors is a rapid rise in intracellular calcium. We assessed whether a similar calcium rise could be induced by aggregating the IgE receptors on transfected P815 cells. Clone 6 cells were loaded with DNP-specific IgE, and the intracellular free calcium was monitored using the fluorescent calcium indicator Quin 2. Upon addition of DNP24-BSA a 50-100 nM rise in cytoplasmic free Ca2+ occurred (Fig. 4, top, trace B). Addition of free DNP hapten (10-8 M) caused a rapid degradation of this response, which demonstrates the requirement for aggregation in order to effect the antigen-induced activation of these cells. Nonreceptor-mediated effects of the antigen could be ruled out since no response could be measured if antigen was added to transfected cells that had not been incubated with IgE. The response was similar over a broad range of antigen concentrations (10-100 ng/ml).

![Fig. 3. Northern blots of clone 6 and nontransfected P815 cells.](image-url)

**Fig. 3.** Northern blots of clone 6 and nontransfected P815 cells. Fifteen μg of total RNA per lane from nontransfected P815 cells (lanes 2) and clone 6 cells (lanes 1) was electrophoresed and blots carried out as described under “Materials and Methods.” Blots were probed with nick-translated cDNAs corresponding to the full-length coding sequences of the rat Fc,RII α chain (αr), the Fc,RII β chain (βr), or the mouse γ chain (γm).
Activation of RBL cells occurs when IgE receptors are brought into close proximity by a variety of means. We were interested in finding if different methods of aggregation would also activate transfected P815 cells. Incubation of cells with IgE, followed by anti-IgE, stimulated transfected cells, although the calcium rise was less (28 nm in the experiment shown in Fig. 4, top, trace C). We have also tested for activation of cells after directly cross-linking the transfected receptors with an antireceptor antibody. Mouse monoclonal antibody 5.14 described by Baniyash et al. (12) binds to (or close to) the IgE-binding site on the a chain of the rat receptor and induces mast cell degranulation. Reaction of transfected cells with this antibody proved to be the most efficient method for stimulating calcium responses (Fig. 4, top, trace A). The antibody was effective over a broad range of concentrations (0.2–20 µg/ml), with all clones bearing large numbers of receptors (Table IIA). However, the antireceptor antibody had no effect on calcium levels in untransfected P815 cells (Fig. 4, top, trace D; Table IIA) or on transfected clones 4 and 5 having low numbers of receptors (Table IIA). These studies further support the correlation between aggregation of IgE receptors and stimulation of cellular calcium responses. Note that the resting levels of free intracellular calcium in P815 cells (approximately 100 nm) are very similar to the resting levels in RBL cells but that the receptor-mediated rise in calcium is substantially less in the case of the transfected P815. It should also be noted that, as in the case of RBL cells, the absolute increase in calcium after cell activation with the stimulus varied with each cell preparation (between 50 and 200 nm). Therefore, relative stimulations were always compared using cells from the same preparation with measurements conducted on the same day.

The calcium response in RBL cells is exquisitely sensitive to temperature (34). We tested whether the transfected cells exhibited a similar dependence on temperature in order to determine the optimal conditions for stimulation. The temperature optimum was 37 °C, similar to the value observed with RBL cells (34). At higher temperatures, calcium responses were weaker and occurred more rapidly. At lower temperatures, weaker and slower responses were observed. These trends in the calcium responses suggest mechanistic similarities in the signaling pathways between the transfected P815 and RBL cells. The pH optimum for the calcium response in the transfected cells was pH 7.2.

We attempted to enhance the response by inclusion of additives that augment the responses of normal mast cells, basophils, and various cell lines such as the RBL cells. Using medium made up with 30% D2O (20), the response of clone 6 cells to IgE plus antigen was increased in only one of four experiments, the others showing either no difference or even a reduction. Similarly, incubating the cells with 5 µg/ml cytochalasin B did not enhance their Ca2+ response in two experiments.

**Mechanism of Rise in Ca2+**—The increase in intracellular free calcium which follows aggregation of IgE receptors on RBL cells results from release derived from intracellular stores as well as influx of extracellular Ca2+ by activation of a putative calcium channel (35). We tested to see if the rise in Ca2+ in the transfected P815 cells was due to similar mechanisms. When cells were stimulated in the absence of extracellular calcium, a rise in calcium could be measured, but this was very small compared with that seen when calcium was added to the medium (Fig. 4, middle; Table IIB). Similarly, addition of 1 mM EGTA to cells stimulated in a calcium-containing buffer brought about a rapid reduction of the response (not shown). These results suggest that extracellular calcium plays as important a role in the calcium response for transfected P815 cells as it does for RBL cells.

**Relationship between Receptor Number and Calcium Signal**—That clones 4 and 5 bearing only about 30,000 receptors/cell failed to respond with a detectable Ca2+ signal was not altogether surprising since using similar experimental protocols, the calcium response of RBL cells appeared to be roughly proportional to the number of receptors that were cross-linked (20). Thus, a response only 10% as large as that seen with clone 6 might have been difficult to detect. However, when we tested this experimentally, an anomaly became apparent. Cells of clone 6 were first incubated with nonspecific rat IgE plus antigen was increased in only one of four experiments, the others showing either no difference or even a reduction. Similarly, incubating the cells with 5 µg/ml cytochalasin B did not enhance their Ca2+ response in two experiments.

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**Mechanism of Rise in Ca2+**—The increase in intracellular free calcium which follows aggregation of IgE receptors on RBL cells results from release derived from intracellular stores as well as influx of extracellular Ca2+ by activation of a putative calcium channel (35). We tested to see if the rise in Ca2+ in the transfected P815 cells was due to similar mechanisms. When cells were stimulated in the absence of extracellular calcium, a rise in calcium could be measured, but this was very small compared with that seen when calcium was added to the medium (Fig. 4, middle; Table IIB). Similarly, addition of 1 mM EGTA to cells stimulated in a calcium-containing buffer brought about a rapid reduction of the response (not shown). These results suggest that extracellular calcium plays as important a role in the calcium response for transfected P815 cells as it does for RBL cells.
number of aggregatable receptors was below the number observed on the unresponsive cells of clones 4 and 5. These results suggest that the apparent unresponsiveness of the latter clones is not due simply to the insensitivity of our assay.

Role of Fc, Receptors—The antireceptor antibody, which is a mouse IgG of isotype 1 (12), stimulated the transfected cells most actively. Therefore, it was important to exclude the possibility that activation of endogenous Fc, receptors via possible synergistic combination of Fc, receptors and the transfected Fc,RI was not contributing to the response. An analogous phenomenon has been observed on mast cells and has been dubbed “allogetic degranulation” (36). In that case, an IgE antihistocompatibility antibody is able to trigger mast cell degranulation by engaging the Fc,RI via its Fc region while at the same time being bound to a histocompatibility fragment that are almost as effective as the intact antibody when equivalent amounts (on a molar basis) were used. As can be seen from Table IIID, these fragments were totally ineffective (Table IIB). Furthermore, when the anti-Fc,RI antibody 2.4G2 was used to block the potential binding of the Fc,RI to the Fc,RI, no inhibition of the response by Fc,RI was observed (Fig. 4, bottom).

Although the endogenous Fc,RI do not contribute to the response mediated by the transfected Fc,RI, the former receptors can themselves activate the P815 cells. Using radiolabeled rat antibody 2.4G2 we observed approximately 3.7 × 10^6 antibody molecules bound to the P815 cells. Addition of this antibody alone failed to initiate a calcium response (Fig. 4, bottom). However, after addition of a goat anti-rat IgG antibody, a response about two-thirds as large as that observed by aggregating Fc,RI was observed. The response of transfected and untransfected cells was equivalent, and prior blocking of Fc,RI on the transfected cells with monomeric IgE did not inhibit the effectiveness of 2.4G2 plus goat anti-rat IgG. Thus, Fc,RI on the P815 cells appear to be capable of mediating a response independent of Fc,RI. These findings are interesting in view of the apparent differences in the subunit structure of these receptors (see “Discussion”).

Comparison with Other Cell Activators—The response of the P815 cells to aggregation of the IgE receptors was considerably smaller than the response observed with RBL cells. As we have already noted, aggregation of the endogenous Fc, receptors on the untransfected and transfected cells led to an intracellular calcium rise only two-thirds of that induced by the transfected Fc,RI. Since the mechanism by which Fc, receptors stimulate the cells is likely to resemble the action of the Fc,RI, these results suggest that the modest rise in Ca^2+ reflects the general metabolic capacity of the cells rather than some defect in the transfected Fc,RI. In order to explore this possibility further, we incubated the cells with a number of reagents that are known to stimulate RBL cells or other rodent mast cells. The results are collected in Table III.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>P815 (clone 6)</th>
<th>RBL</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionomycin (0.1 μM)</td>
<td>73 ± 5.7</td>
<td>325 ± 20</td>
<td>0.21</td>
</tr>
<tr>
<td>NECA (10 μM)</td>
<td>426</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NECA (10 μM) + dexamethasone (10 μM)</td>
<td>358 ± 93</td>
<td>426</td>
<td>0.83</td>
</tr>
<tr>
<td>Leukotriene D4 (1 μM)</td>
<td>255 ± 17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leukotriene E4 (1 μM)</td>
<td>214 ± 8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin*</td>
<td>358 ± 0.5</td>
<td>101 ± 7.2</td>
<td>0.19</td>
</tr>
<tr>
<td>Fc,RI</td>
<td>53.6 ± 6.7</td>
<td>320 ± 15</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*Response of clone 6 cells relative to RBL cells.

In contrast to the response of RBL cells, addition of dexamethasone was without effect. In the P815 cells the response was much lower than that occurring in RBL cells. The minimal dose to obtain the maximal response was 0.1 unit/ml for clone 6; 0.5 unit/ml for RBL cells.

Values are means ± S.D. of at least triplicate experiments.

This value is comparable to the value of 41 ± 1 given by untransfected P815 cells.

Finally, the results with the nonspecific stimulator ionomycin (Table III) provide further evidence that the P815 cells have a general reduced responsiveness. Indeed, their responsiveness to the transfected receptors relative to their responsiveness to other stimuli is comparable to or better than the corresponding responses in the RBL cells.

Phosphoinositide Turnover—Hydrolysis of phosphoinositides is a prominent early event when RBL-2H3 cells are activated upon aggregation of their IgE receptors (2), and, as in other systems, this response may constitute a critical signal in the activation pathway. To see if the transfected receptors could initiate a similar response, we measured turnover of inositides in the transfected P815 cells. Clones 4 and 5 showed little or no increase in hydrolysis upon stimulation whereas clones 6, 7, and 8 showed a readily measurable effect upon stimulation with the antireceptor antibody (Fig. 6, inset). As also shown in the figure, much of the response occurs within 30 s after adding the stimulus. These kinetics are similar to those for the rise of intracellular calcium studied in parallel on the same cell preparations. These data suggest that as for RBL cells, IgE receptor-mediated activation of P815 cells involves a coupled calcium and phosphatidylinositol response.

Effect of Cholera and Pertussis Toxin—GTP-binding proteins are implicated in the coupling of certain receptors to activation of phospholipase C (41). However, whether such proteins are involved in the activation of RBL cells and related mast cells via aggregation of the IgE receptor is unclear (3). What is known is that none of the early signals or later events is suppressed by pertussis toxin (42) whereas there is a variable increase upon addition of cholera toxin (43). We tested the role of toxin sensitive G proteins during activation of P815 clone 6 by measuring hydrolysis of phosphoinositides.

G. Alber and H. Metzger, manuscript in preparation.
and the [3H]inositol phosphates were measured as described under "Materials and Methods." Data points represent the mean of triplicate values + SE. [3H]Inositol phosphates generated in nonstimulated cells (see inset) at each time point were subtracted from the data to calculate net inositol phosphate production. Inset, hydrolysis of phosphoinositides by five transfected P815 cell clones. P815 clones 4, 5, 6, 7, and 8 (5 × 10^6 cells/ml) were stimulated for 30 min at 37 °C with 1 μg/ml cholera toxin or 10 μg/ml pertussis toxin. The truces shown in the figure are representative of several experiments. Note difference in scale for the ordinate.

**Fig. 5.** Time course for changes in intracellular calcium after stimulation of RBL and clone 6 cells with thrombin or via the receptor for IgE. The doses of thrombin used for stimulating the RBL and clone 6 cells were the minimal doses that gave a maximum response. Similarly, the reagent used to aggregate the FcRI was that which gave the maximal stimulation. Fluorescence intensity was measured in Quin 2-loaded cells and analyzed as described under "Materials and Methods." Top, RBL cells (1 × 10^6 cells/ml) were incubated with IgE and then reacted with 10 ng/ml DNP-28-BSA (---) or with 0.1 unit/ml rat thrombin (-----). The stimulant was added at the time indicated by the arrow. Bottom, clone 6 cells (1 × 10^6 cells/ml) were stimulated with 2 μg/ml antireceptor antibody 5.14 (-----) or with 0.1 unit/ml mouse thrombin (-----). The traces shown in the figure are representative of several experiments. Note difference in scale for the ordinate.

**Fig. 6.** Production of [3H]inositol phosphates upon stimulation of transfected cells. P815 clone 6 cells were incubated with mvo [3H]inositol overnight and stimulated with 10 μg/ml antireceptor antibody (5 × 10^6 cells/ml). Reactions were stopped at various times, and the [3H]inositol phosphates were measured as described under "Materials and Methods." Data points represent the mean of triplicate values ± S.E. [3H]Inositol phosphates generated in nonstimulated cells (see inset) at each time point were subtracted from the data to calculate net inositol phosphate production. Inset, hydrolysis of phosphoinositides by five transfected P815 cell clones. P815 clones 4, 5, 6, 7, and 8 (5 × 10^6 cells/ml) were stimulated for 30 min at 37 °C with 1 μg/ml cholera or pertussis toxin (hatched bars) or 10 μg/ml cholera or pertussis toxin (filled bars) antireceptor antibody. [3H]Inositol phosphate production in nonstimulated cells (baseline value) is also shown for comparison (open bars).

after incubating the cells with cholera or pertussis toxin prior to stimulating them with antireceptor antibody. Cells preincubated with 1 μg/ml cholera toxin for 3 h hydrolyzed phosphoinositides about 45% more actively (from 0.9 to 1.3% net hydrolysis), but those incubated with 1 μg/ml pertussis toxin showed no change in their response. These results are qualitatively similar to those observed with the endogenous receptors on RBL cells (3).

**Release of Arachidonic Acid**—Another early event after the cross-linking of IgE receptors on RBL cells is the release of arachidonic acid from cellular phospholipids via activation of phospholipase A2 (21). The source of the released arachidonic acid appears to be primarily from phosphatidylinositol and phosphatidylserine (44). We found that transfected P815 cells also release arachidonic acid when IgE receptors are aggregated by IgE and antigen or by antireceptor antibody (Fig. 7). Also shown for comparison is the release of arachidonic acid from RBL and from nontransfected P815 cells stimulated with the same concentration of antireceptor antibody. As expected from studies on RBL cells (21), the time course for release of arachidonic acid is slower than that for phosphatidylinositol hydrolysis (Figs. 6 and 7).

**Activation of Protein Kinase C and Phosphorylation of Myosin**

Recently, a link has been established between the activation of the IgE receptor and phosphorylation of cellular myosin by protein kinase C (22). The significance of this phosphorylation during degranulation is still unknown. However, these studies provide an indirect method to monitor IgE receptor-linked activation of protein kinase C in transfected P815 cells. Fig. 8 is a representative autoradiogram of tryptic peptides of 32P-labeled 20-kDa myosin light chains separated on an isolectric focusing gel. As described previously (22), in unstimulated RBL-2H3 cells (control), there is a basal level of phosphorylation at sites known to be phosphorylated by myosin light chain kinase. Following 10 min of antigenic stimulation, there is little change in phosphorylation at these sites, but phosphorylation occurs at another site known to be phosphorylated by protein kinase C. At the same time the RBL cells release 35% of their total pool of histamine. In P815 cells, there is no measurable secretion of histamine following stimulation (below), but a similar pattern of phosphorylation is observed. In unstimulated cells, the only phosphopeptide apparent corresponds to the sites phosphorylated by myosin.
FIG. 8. Phosphorylation of myosin light chains upon stimulation of P815 cells (see “Discussion”). Phosphorylation of RBL-2H3 cells was determined by quantitative analysis of inositol phosphate turnover (12.5% polyacrylamide). The light chains were purified from the gels, subjected to tryptic digestion, and the peptide mixtures separated on an isoelectric focusing gel (see “Materials and Methods”). The phosphopeptides labeled 2 and 3 correspond to sites phosphorylated by myosin light chain kinase. Phosphopeptide 3 is monophosphorylated on serine 19 (19% of total), and phosphopeptide 2 is diphosphorylated at serine 19 and threonine 18. Phosphopeptides labeled 1 and 4 correspond to serine 1 or 2, phosphorylated by protein kinase C but believed to result from a different tryptic cleavage (22). Nonstimulated (control) cells (C) were incubated 10 min at 37°C in buffer A. In these control cells, one predominant phosphopeptide appears, derived from phosphorylation by myosin light chain kinase. This peptide appears as bands 2 and 3, corresponding to phosphorylation of both threonine 18 and serine 19 (band 2) or of serine 19 alone (band 3). Upon stimulation, a second phosphopeptide appears as a result of phosphorylation by protein kinase C. This peptide appears predominantly as band 4 for P815 cells and corresponds to phosphorylation of serine 1 or serine 2. For RBL cells, this peptide also appears as band 1 and probably represents variable trypsin proteolysis of the peptides at adjacent basic amino acid residues (22). Direction of isoelectric focusing toward the anode was toward the top of the figure.

light chain kinase. Upon stimulation the incorporation of phosphate at these sites is unchanged, but a new phosphopeptide, corresponding to that phosphorylated by protein kinase C, appears. The amount of phosphate at this site is greatest at 6 min and declines slowly thereafter. RBL-2H3 cells had shown an increase of protein kinase C phosphorylation after 1 min, with a peak of phosphorylation at 5–10 min (22), so the response of the P815 cells may be more rapid (or becomes attenuated more quickly).

The myosin heavy chain in RBL-2H3 cells shows three major phosphopeptides in unstimulated cells which, following stimulation, do not change. However, a new phosphopeptide, believed to be due to the activity of protein kinase C, appears (22). Preliminary studies suggest a small increase in phosphorylation at this site in the myosin heavy chain of P815 cells also (data not shown).

**Discussion**

Upon aggregation of their surface-bound receptors for IgE, the transfected P815 cells responded with a variety of biochemical changes that resemble those observed when the endogenous receptors on RBL and related cells are stimulated (2, 9). Like the endogenous receptors, the transfected ones initiated these responses only when aggregated. The biochemical changes we observed included a rise in intracellular Ca²⁺, hydrolysis of phospholipids, and highly specific phosphorylations.

Where studied in detail, the rise in cytoplasmic Ca²⁺ in RBL and related cells appears to result from a recruitment of internal stores as well as from an influx of Ca²⁺ through a still poorly characterized mechanism (35). In the transfected P815 cells, the rise in intracellular Ca²⁺ was markedly diminished but not totally ablated in the absence of extracellular Ca²⁺. Thus, in these cells also, it is likely that both internal and external sources contribute to the increase. As in RBL and related cells, the rise in Ca²⁺ in transfected and stimulated P815 cells was sensitive to relatively small changes in temperature and occurred optimally at a value similar to that measured for RBL cells. RBL and similar cells, previously incubated with radioactive inositol, release labeled inositol phosphates when their IgE receptors are aggregated (2). The rate of this release is virtually indistinguishable from the rate of increase in cytoplasmic Ca²⁺ (20). The amount of release is resistant to inhibition by pertussis toxin (42) but is somewhat enhanced by exposure to choleragenoid (43). Aggregation of the receptors on the transfected P815 cells induced a qualitatively similar response. Likewise, the transfected receptors stimulated release of labeled fatty acids from P815 cells that had incorporated radiolabeled arachidonic acid, and again the kinetics were similar to those observed with RBL and similar cells (21).

A variety of experimental protocols have demonstrated that aggregation of the endogenous IgE receptors on RBL and similar cells activates protein kinase C (22, 45). Among the evidence for such activation is the phosphorylation of specific residues on the light chain of myosin (22). Aggregation of the transfected receptors on P815 cells stimulated phosphorylation of apparently the same residues.

The responses by the transfected cells also showed some differences compared with the RBL cells from which the cDNAs for the transfected receptors on the P815 cells were derived. The responses were diminished in magnitude about 3–10-fold. This was true of all the responses to aggregation of the FcRI that we examined. This concordance could result from a common pathway (and there are models that link all of the responses we studied (2, 31) or from some more general metabolic difference such as the availability of ATP which, where determined, appears to be required for all the responses we examined (2).

The likelihood of metabolic differences between P815 cells and RBL cells is also supported by the weak calcium signals observed after stimulation of the P815 cells with ionomycin, thrombin, and anti-Fc, R antibodies followed by anti-IgG. Furthermore, stimuli that elicit strong signals in RBL cells such as NECA and leukotrienes D₄ and E₄ had no affect on internal calcium levels in the P815 cells even though P815 cells have been shown to have [³H]NECA binding sites on their surface (46, 47). From the data summarized in Table III it appears that for each of those stimuli that initiate a rise in phosphate myristate acetate to stimulate release from P815 clone 6 whereas they are effective stimulators of secretion by RBL cells (2).

**Conclusions**

The responses by the transfected cells also showed some differences compared with the RBL cells from which the cDNAs for the transfected receptors on the P815 cells were derived. The responses were diminished in magnitude about 3–10-fold. This was true of all the responses to aggregation of the FcRI that we examined. This concordance could result from a common pathway (and there are models that link all of the responses we studied (2, 31) or from some more general metabolic difference such as the availability of ATP which, where determined, appears to be required for all the responses we examined (2).
calcium in both types of cells, the magnitude of the response in the P816 cells is reduced by a similar fraction (0.17-0.21). Thus, the small signals observed upon aggregation of the transfected Fc,RI are most likely derived from a reduced reactivity of the cells rather than from possible structural or functional defects in the transfected receptors. In fact, the largest of the signals that we observed in the P815 cells have been produced by aggregation of the transfected IgE receptors.

Some quantitative aspects relating to the aggregation of the receptors were unexpected. In the transfectants, antireceptor antibodies regularly led to a more robust response than did other aggregating stimuli. In RBL cells, if anything, the opposite is true. It is also strange that clones bearing small numbers of transfected receptors failed to respond whereas those with higher expression responded well even when the absolute number of receptors that were stimulated was smaller than the number on the resistant cells. We have no data that would allow us to choose among several possible explanations. Finally, unlike the RBL cells, the P815 cells are unable to degranulate. Since the transfected and untransfected cells alike failed to respond to ionophore plus phorbol ester, it is likely that they are missing the more distal components of the secretory response.

At this stage we do not plan to explore in detail the effect of numberless variables on each of the responses in the transfected cells. Instead, we plan to focus on changes in these responses which may result from alterations in the structure of the IgE receptor introduced by in vitro mutation. Providing such transfected subunits can be expressed in sufficient amounts, it may even be possible to study altered IgE chains even though the P815 cells carry endogenous IgE chains. In principle, this is no different from studying transfected insulin-like growth factor I receptors on Chinese hamster ovary cells that contain endogenous receptors (48).

If by such studies critical regions of the receptor can be identified, this information could be used to develop probes by which "postreceptor" components could be identified and ultimately isolated and characterized. Conceptually, we are looking for those parts of the receptor which, upon aggregation, act as ligands for such components. It is noteworthy that after aggregation, the endogenous Fc, receptors on the P815 cells stimulated responses similar to those stimulated by Fc,RI and vice versa. Some Fc, receptors are now known to contain IgE chains (33) as well as the immunoglobulin-binding α chains. However, no β or β-like subunits have yet been detected. It may be interesting to explore the responses stimulated by the alternative receptors in detail. Possibly such a comparison may yield clues about functions that relate to the β chains specifically.

REFERENCES