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Isolation and Purification of an Fc_ϵ Receptor Activated Ion Channel from the Rat Mast Cell Line RBL-2H3†

Stefan Hemmerich and Israel Pecht*
Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel
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ABSTRACT: Derivatives of the antiallergic drug cromolyn [disodium 5,5′-[(2-hydroxy-1,3-propanediyl)-bis(oxy)]bis[4-oxo-(4*H*-1-benzopyran)-2-carboxylate]], which can be conjugated covalently at the propane 2-position to macromolecules and to insoluble matrices, were synthesized. Conjugates of these derivatives with macromolecules were examined for their binding to cells of the rat basophilic leukemia line RBL-2H3, which is widely employed as a model for immunologically induced mast cell degranulation. Only those drug–protein conjugates in which the cromolyn analogue with an amino group at the propane 2-carbon instead of the hydroxyl was linked to the carrier by glutaraldehyde were found to exhibit specific and saturable binding to these cells. Analysis of the binding data for these conjugates yielded an apparent binding constant of $3.8 \pm 0.2 \times 10^8$ M^{-1} and an apparent number of binding sites for the probe of 4000–8000 per cell. The conjugates found to bind specifically to the cells were also immobilized on agarose matrices and employed in an affinity-based isolation of the membrane component responsible for the observed binding. A single labeled polypeptide was eluted from these columns, onto which either whole cell lysates or solubilized purified plasma membranes of surface-radioiodinated RBL-2H3 cells had been adsorbed. This membrane protein appears on autoradiograms of nonreducing SDS–PAGE as a single broad band of ~110 000 daltons (Da) apparent molecular mass. On autoradiograms of reducing gels, the only band detected has an apparent mass of ~50 000 Da and appears narrower. Elution of the columns with the drug and disulfide-reducing agents or with the latter alone resulted in significantly higher yields of the 50-kDa polypeptide. Both the intact and reduced proteins bind strongly to immobilized concanavalin A and less so to immobilized wheat germ agglutinin, suggesting that the isolated intact protein is probably a dimer of two glycosylated subunits of similar molecular mass. Treatment of the reduced protein with endoglycosidase F leads to a decrease in its apparent molecular mass by ~12 kDa, suggesting that the extent of glycosylation of this polypeptide is ~25%. As shown in the following paper, the intact protein constitutes a Ca^{2+} channel that is activated upon IgE–Fc_ϵ receptor aggregation.

The nature and sequence of biochemical processes, which couple the IgE-mediated stimulus with mediator secretion from mast cells, are topics of considerable research interest and activity (Ishizaka & Ishizaka, 1984; Gomperts & Fewtrell, 1985). One of the early events in this cascade that has been intensively studied and discussed is the transient increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mast cells of different origins (White et al., 1984; Beaven et al., 1984; Pervin et al., 1985; Sagi-Eisenberg et al., 1985). The requirement for millimolar concentrations of Ca^{2+} in the extracellular medium for the immunologically triggered secretion had been widely documented already in early studies (Mongar & Schild, 1958; Lichtenstein & Osler, 1964; Greaves & Mongar, 1968). Furthermore, different experiments provided indications for a net influx of these ions down their concentration gradient into the cells, probably via ion channels (Foreman & Mongar, 1972; Foreman et al., 1977a). In recent

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years, the capacity of inositol trisphosphate to release sequestered Ca^{2+} ion from intracellular depots (Downes & Michell, 1982; Berridge & Irvine, 1984) led several investigators to propose that the source of the transient rise in $[Ca^{2+}]_i$ is intrinsic rather than due to a channel-mediated influx (Gomperts & Fewtrell, 1985). The rat leukemia line RBL-2H3[1] (Barsumian et al., 1981) homologous to mucosal mast cells (Seldin et al., 1985) is widely employed for examination of the processes involved in IgE-mediated secretion and is a major source for identification and isolation of the cellular components involved in the above events (Froese, 1984; Holowka et al., 1980; Metzger et al., 1984). IgE-mediated secretion from RBL-2H3 cells was found to have an obligatory requirement for extracellular calcium (~0.7 mM half-saturation; Fewtrell & Metzger, 1981), and examination of the influx of these ions with $^{45}Ca^{2+}$ as tracer (Crews et al., 1981; Kanner & Metzger, 1984; Mohr & Fewtrell, 1987) further supported the notion of a channel opening being the main source of the rise in $[Ca^{2+}]_i$ in this cell line.

1,3-Bis[(2-carboxychromon-5-yl)oxy]-2-hydroxypropane disodium salt [5,5′-[(2-hydroxy-1,3-propanediyl)bis(oxy)]-bis[4-oxo-(4*H*-1-benzopyran)-2-carboxylate], commercially known as cromolyn, disodium cromoglycate, DSCG, Intal, or Lomudal] has been found to be an effective drug for treatment of allergic asthma (Cox et al., 1970; Kumagai & Tomioka, 1978). Whereas many antiallergic drugs exert their effect distal to the histamine release, cromolyn has been proposed to interfere with the mechanism leading to a transiently elevated $[Ca^{2+}]_i$ upon antigenic stimulation of the cells (Foreman & Garland, 1976). In rat peritoneal mast cells, cromolyn has been shown to inhibit to a certain degree the antigen-induced degranulation (Cox, 1967) and $^{45}Ca^{2+}$ influx (Foreman et al., 1977b). Recently, a modulatory effect of cromolyn has been demonstrated in RBL cells (Ran & Rivnay, 1987). This led to the idea that the drug may interact with a membranal component, which is involved in the Fc_ϵ receptor mediated calcium influx into mast cells and basophils, and has raised the possibility of isolating this component by affinity procedures based on such interactions.

Earlier studies in this laboratory suggested that a membrane component can be isolated from RBL cells, which can form ion channels when reconstituted into lipid bilayers (Mazurek et al., 1984). This component was isolated by using matrices assumed to be carrying the drug. The procedure employed at that stage for conjugating commercially available cromolyn to carriers involved alkylation of this drug with the bifunctional reagent 2-aminoethane-1-sulfate to yield an amino derivative that could be cross-linked to amine residues of carrier proteins by glutaraldehyde (Mazurek et al., 1982). We later established that following this protocol the desired conjugation could not be achieved. Nevertheless, an affinity isolation procedure employing the product of this reaction indeed yielded protein samples, which when assayed in reconstituted planar lipid bilayers exhibited IgE–Fc_ϵ receptor gated ion channel activity (Pecht et al., 1986). However, due to its low concentration in these preparations, the identity and nature of the active component could not be determined. This called for the development of an improved isolation procedure for this channel-forming protein that would provide it in a functional form and in amounts and purity that would allow its chemical characterization.

To this end, it was our first objective to prepare well-defined cromolyn analogues, which could be covalently attached to different carriers in a stable and well-controlled stoichiometry. Such tailor-made conjugates would enable carrying out binding measurements to the RBL-2H3 cells and the development of a satisfactory isolation procedure for the protein of interest.

Here we describe the synthesis of such cromolyn analogues, the preparation of different cromolyn-carrying macromolecules and matrices, and their application for binding measurements and for the isolation of a membranal protein from the RBL-2H3 cells. Incorporation of this isolated and purified protein together with the isolated IgE–Fc_ϵR complex into artificial membranes indeed yields antigen-induced specific calcium permeabilities across reconstituted micropipet-supported bilayers as shown in the accompanying paper (Corcia et al., 1988). However, our results imply that the interaction of this protein with the described cromolyn conjugates is not merely due to an affinity of the protein toward cromolyn but that the chemical nature of the spacer attaching the drug to the carrier seems to be crucial as well. Furthermore, the use of multivalent conjugates that provide higher avidity due to multiple interactions leads to amplification of the low affinity of cromolyn itself.

Experimental Procedures

Materials

2,6-Dihydroxyacetophenone was synthesized from resorcinol as described (Russell & Frye, 1955). Later samples were obtained from Fluka (Buchs, Switzerland). β,β′-Dihydroxyisopropylamine (serinol) was synthesized in a three-step synthesis starting from formaldehyde and nitromethane, essentially as described (Schmidt & Wikendorff, 1918). All chemicals employed were from Merck, Darmstadt, FRG, or BDH, Poole, England, and of the best available commercial grade. Solvents were from Frutarom Ltd., Haifa, Israel. Dry Me_2SO was from Riedel de Haen, Seelze (Hannover), FRG. Bovine serum albumin (BSA), lactoperoxidase, Triton X-100, soy bean lipids (phosphatidylcholine type IV-S), proteolytic inhibitors, and dithiothreitol (DTT) were purchased from Sigma, St. Louis, MO. Commercial cromolyn and [^{3}H]cromolyn were obtained from Fisons plc, Loughborough, England. Sepharose 4B and molecular weight markers were purchased from Pharmacia. Lectin-agarose conjugates were obtained from Bio-Yeda, Rehovot, Israel. $Na^{125}I$ solution, Bolton and Hunter reagent, and endoglycosidase F (800 units/mL) were purchased from NEN, Boston, MA. Materials for polyacrylamide gel electrophoresis were from Sigma and Bio-Rad, Richmond, CA. IgA specific for 2,4-dinitrophenyl residues, which is secreted by the murine plasma cytoma MOPC-315, was purified in our laboratory from ascitic fluid essentially as described (Goetzl & Metzger, 1970). The multichain copolymer of alanine and lysine [A-L, Sela et al. (1956), M_r >100 000, [Ala]/[Lys] = 22 as determined by amino acid analysis of the hydrolysate] was a kind gift of I. Yacobson, Dept. of Biophysics, The Weizmann Institute of Science. Tissue culture media were obtained from Bio-Lab Ltd., Je-

[1] Abbreviations: A-L, poly(alanine-lysine); A-$L_{n/pb}$, A-L having attached *n* moieties cromolyn per peptide bond of the polymer via 6-(thiourea)caproamide spacers: BBS, borate-buffered saline; BSA, bovine serum albumin; BSA-(ga-cro)$_n$, BSA having attached *n* cromolyn moieties per protein molecule via 1,5-pentanediamine spacers; BSA-(ga-NH_2)$_n$, BSA derivatized with 1,5-pentanediamine spacers only; BSA-(itc-cro)$_n$, BSA having attached *n* cromolyn moieties per protein molecule via 6-(thiourea)caproamide spacers; ConA, concanavalin A; DNP, 2,4-dinitrophenyl; DTT, dithiothreitol; Fc_ϵR, the high-affinity receptor for IgE; IgE–Fc_ϵR, the complex of the receptor for IgE with its ligand IgE; FDNB, 1-fluoro-2,4-dinitrobenzene; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; OA, ovalbumin; PBS, phosphate-buffered saline; RBL-2H3, rat basophilic leukemia, subline 2H3; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

rusalem, Israel. Cells of the rats basophilic leukemia subline 2H3 (Barsumian et al., 1981) were grown in stationary flasks and detached by brief exposure to EDTA.

Analytical Procedures

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. NMR spectra were measured in $CDCl_3$ on a Varian FT 80A NMR spectrometer. Ultraviolet (UV) spectra were obtained with a Cary 118 UV/visible spectrophotometer and quartz cuvettes transparent for UV up to $\lambda < 200$ nm. The optical path width was 10 mm. Infrared (IR) spectra were obtained with a Signus 2000 spectrophotometer and were made in KBr pellets ($\sim$1 mg of compound in a 100-mg pellet). Thin-layer chromatography (TLC) was performed on silica gel F_{254} plates (Merck). Spots were usually detected by exposure to short-wavelength UV light. In the case of compounds carrying free amino groups, these chromatograms were stained with ninhydrin. SDS gel electrophoresis was done with the discontinuous buffer system described by Laemmli (Laemmli, 1970). Prior to electrophoresis, samples were boiled in either nonreducing or reducing sample buffer containing 3% SDS, 0.625 M Tris-HCl, pH 6.8, 10% glycerol, 0.6 mg of bromophenol blue, and 0.25 mM of either ethylene glycol or β-mercaptoethanol, respectively (final concentrations). Nonreduced samples were always run in a 7–10% polyacrylamide gradient gel with the following molecular mass standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa). Reduced samples were run in a 10–15% gradient gel with the following standards: phosphorylase B (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The gels were fixed in MeOH/AcOH/H_2O (50/12/38 v/v) and silver-stained as described (Goldmann et al., 1981). The R_f values of the markers were plotted versus the logarithm of their molecular mass, thus yielding a calibration curve relating the position of any observed protein band to its apparent molecular mass. The stained gels were later dried in vacuo and exposed on preflashed Kodak XAR-5 film at −70 °C.

Synthesis of Aminocromolyn and Its Derivatives

1,3-Bis[(2-carboxychromon-5-yl)oxy]-2-aminopropane (aminocromolyn) was prepared in a multistep synthesis outlined in Figure 1 and described in detail in the supplementary material. In short, 1 equiv of 1,3-dibromo-2-(benzoxycarbonylamido)propane (II), which had been synthesized from serinol via β,β'-dibromoisopropylammonium bromide (I), was reacted with 2 equiv of 2,6-dihydroxyacetophenone to give 1,3-bis[(6-hydroxyacetophenon-2-yl)oxy]-2-(benzoxycarbonylamido)propane (III). Cyclization of this compound with diethyl oxalate via the oxo ester IV yielded the diethyl ester of 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-(benzoxycarbonylamido)propane (V). Hydrolysis of this compound in a mixture of glacial acetic acid and concentrated hydrochloric acid gave 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-aminopropane hydrochloride (aminocromolyn, VI), while catalytic hydrogenation of the same compound yielded the diethyl ester of aminocromolyn (VII). From compound VII were obtained 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-[6-(*N*-tBoc-amino)caproamido]propane (IX) and 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-[6-[(2,4-dinitrophenyl)amino]caproamido]propane (DNP-cromolyn, X) by acylation with *N*ϵ-tBoc-aminocaproic acid or *N*ϵ-2,4-DNP-aminocaproic acid, respectively, and subsequent mild alkaline hydrolysis of the acylated diesters. The tBoc protection group was then cleaved from IX by

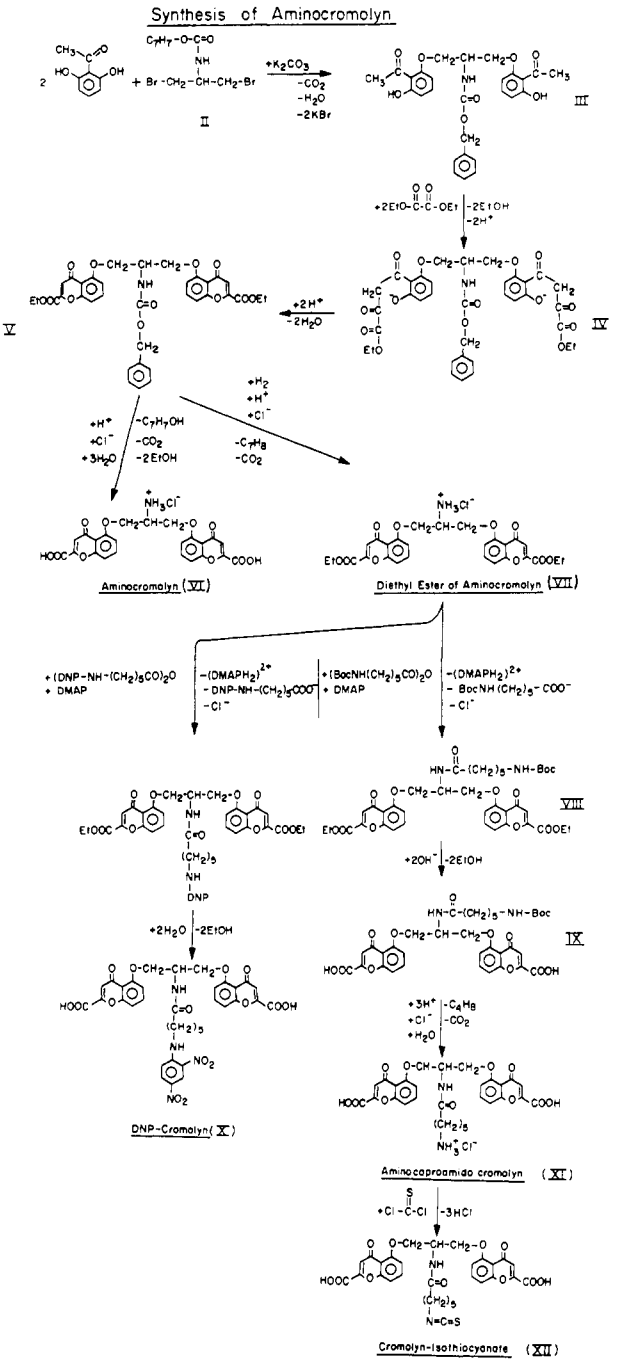

FIGURE 1: Flow chart describing synthesis of aminocromolyn and related ligands employed in affinity measurements and protein isolation experiments. 2,6-Dihydroxyacetophenone and 1,3-dibromo-2-(benzoxycarbonylamido)propane (II) were prepared as described under Experimental Procedures. DMAP, *p*-(*N*,*N*-dimethylamino)pyridine; $(DMAPH_2)^{2+}$, *p*-(*N*,*N*-dimethylammonio)pyridinium dication.

treatment with HCl in dioxane to yield 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-(6-aminocaproamido)propane hydrochloride [(aminocaproamido)cromolyn, XI]. Finally, reaction of this compound with thiophosgene gave 1,3-bis[(2′-carboxychromon-5′-yl)oxy]-2-(6-isothiocyanatocaproamido)-propane (cromolyn isothiocyanate, XII).

Conjugation of Aminocromolyn and Cromolyn Isothiocyanate to Macromolecules

Conjugation of Aminocromolyn to Proteins with Glutaraldehyde. A solution of globular protein (BSA or ovalbumin) in 0.1 M $NaHCO_3$ (10 mg/mL) was adjusted to pH 9.5 with aqueous NaOH. To the stirring sample were added as solids the appropriate amount of aminocromolyn hydrochloride

Table I: Controlled Conjugation of Cromolyn to Macromolecules

(a) [AC]:[BSA]:[$CH_2(CH_2CHO)_2$] in reaction mixture[a]	degree of derivatization[b]
13:1:167	3.8
26:1:167	9.7
33:1:167	12.5
40:1:167	15.9
53:1:167	17.5
130:1:167	21
40:1:33	5
(b) [CITC]:[BSA] in reaction mixture[c]	**degree of derivatization[b]**
4.3	4.3
10.6	6.5
21.3	8.8
42.6	13.5
107	25
(c) [CITC]:[CONH] in reaction mixture[d]	**degree of derivatization[e]**
0.02	0.02
0.038	0.036
0.076	0.066
0.19	0.104

[a] Conjugation of cromolyn to bovine serum albumin by glutaraldehyde and subsequent treatment with $NaBH_4$. [b] The degree of derivatization was expressed as the number of conjugated cromolyn molecules per mole of protein. [c] Conjugation of cromolyn isothiocyanate with BSA. [d] Reaction of cromolyn isothiocyanate with the synthetic amino acid copolymer A-L [$(Ala_{22}Lys)_n$]. [CONH] is the concentration of peptide bonds in the reaction mixture. [e] Because the molecular mass of this polymer is an average value (>100 000 as observed on SDS–PAGE), the degree of derivatization is defined here as the number of conjugated cromolyn moieties per peptide bond of the polymer.

(varied from 2 to 20 mM) and 2 equiv of $NaHCO_3$. For a structural control–conjugate 5 mM NH_4Cl was added instead. Glutaraldehyde (either 5 or 25 mM) was added dropwise in form of a 5% solution in 80 mM $NaHCO_3$. The reaction mixture, which developed a deep yellow color, was stirred for 5 h at room temperature; then it was cooled in ice and treated with 70 mM $NaBH_4$. The sample was stirred on ice for more 4 h, upon which time the yellow color disappeared. Then it was dialyzed extensively against repeated changes of PBS. The protein concentration in the dialyzed solution was calculated from the amount of protein weighed in and the volume of this solution. The degree of derivatization, namely, n = [cromolyn]/[protein], was determined by light absorption of the dialyzed solution at 325 nm [ϵ_{325nm}(cromolyn) = 8500 mol^{-1} cm^{-1}]. The degrees of derivatization obtained were found to be dependent on the employed concentrations of aminocromolyn and glutaraldehyde as presented under Results and Table Ia. These conjugate solutions were either used as such for all purposes or lyophilized for long-term storage.

Conjugates of Cromolyn Isothiocyanate with Globular Proteins. A 1% solution of protein in 0.1 M $NaHCO_3$ was adjusted to pH 10.5 with aqueous NaOH. To the stirring sample was added the appropriate amount of solid cromolyn isothiocyanate (varied from 0.65 to 16 mM) together with 2 equiv of $NaHCO_3$. The solution was stirred for 4 h at 25 °C. Then it was dialyzed extensively against repeated changes of PBS. The degrees of derivatization of the different conjugates were determined as described above. Its dependence on the employed concentration of cromolyn isothiocyanate is presented under Results and in Table Ib.

Conjugation of Cromolyn Isothiocyanate to Poly(alanine-lysine). Cromolyn isothiocyanate was reacted with the synthetic branched copolymer of alanine and lysine $(Ala_{22}Lys)_n$ [A-L, Sela et al. (1956), [A-L] = 3.75 mg/mL = 0.084 M peptide bond] under the same conditions employed for the reaction with globular proteins. The concentration of cromolyn isothiocyanate in the reaction mixture was varied from 1.6 to 16 mM. The different conjugates obtained are presented under Results and in Table Ic.

Derivatization of the Conjugates with FDNB. The dialysates containing cromolyn–protein conjugate in PBS (10 mg of conjugate per mL) were adjusted to pH 8.5 by addition of aqueous Na_2CO_3. These solutions were treated with 16 mM FDNB. The samples were stirred for 3 h at 25 °C and then they were dialyzed extensively against repeated changes of PBS. The degree of derivatization was determined by the differential absorption of the dialysates at 400 and 354 nm: ϵ_{400nm}(DNP) = 7200 M^{-1} cm^{-1}, ϵ_{400nm}(cromolyn) $\approx$ 0, $\rightarrow A_{400nm}$ = [DNP] × 7200 M^{-1}; ϵ_{354nm}(DNP) = 17 400 M^{-1} cm^{-1}, ϵ_{354nm}(cromolyn) = 3390 M^{-1} cm^{-1}, $\rightarrow A_{354nm}$ = [DNP] × 17 400 M^{-1} + [cromolyn] × 3390 M^{-1} $\rightarrow\rightarrow$ [cromolyn] = (A_{354nm} − [DNP] × 17 400 M^{-1})/3390 M^{-1}. With BSA–cromolyn conjugates, 10 ± 2 DNP residues were found to be attached per conjugate monomer under the described conditions.

Preparation of the Affinity Matrices

Derivatization of Agarose with the Drug–Protein Conjugates. The aminocromolyn–macromolecule conjugates were covalently attached to CNBr-activated Sepharose 4B essentially as described (Cuatrecasas, 1970). Typically, 3 mL of dialyzed conjugate solution containing ~30 mg was adjusted to pH 8.5 with 5 M Na_2CO_3 and added to 4 mL of activated gel. A degree of derivatization of ~5 mg of conjugate/mL of gel was usually achieved.

DNP-Specific Immunoadsorbent (MOPC-315-Sepharose). Affinity-purified, DNP-specific IgA secreted by the murine plasmacytoma MOPC-315 was immobilized on Sepharose 4B by the CNBr method (Cuatrecasas, 1970). The degree of derivatization was found to be 4.4 mg of MOPC-315/mL of gel, as determined by measuring the unbound protein spectrophotometrically ($\epsilon^{1\%}_{280\ nm}$ = 14) in the filtrate.

Binding Assays

Measurements of the conjugate binding to RBL-2H3 cells were done at 4 °C in disposable polystyrene test tubes (70 mm × 8.5 mm) on cell suspensions at a concentration of 1.6 × 10^7 cells/mL in a modified Tyrode's balanced salt solution (NaCl 137 mM, KCl 2.7 mM, NaH_2PO_4 0.4 mM, HEPES 10 mM, pH 7.4, glucose 5.6 mM, $CaCl_2$ 0.3 mM, $MgCl_2$ 0.5 mM, BSA 0.2% w/v). The ^{125}I-labeled conjugate at a specific activity of 5 × 10^{17} cpm/mol was employed in a concentration range of 10^{-10}–10^{-7} M. Incubation of the samples was carried out with shaking on ice for 30 min. (In the competition measurements, the cells were incubated with the respective unlabeled conjugate for 30 min on ice prior to treatment with the labeled probe.) Aliquots of 125 μL of cell suspension (2 × 10^6 cells) were layered on top of 200 μL of ice-cold fetal calf serum placed at the bottom of elongated conical microcentrifuge tubes (total capacity 400 μL). The cells were separated from their supernatants by centrifugation for 1 min through the serum in a Beckman 152 microfuge and formed a pellet at the bottom of the test tubes. These pellets were cut off and their γ-radiation was measured in a Kontron GAMMAmatic counter. The rest of the tubes containing the unbound conjugate were counted as well. Thus, [bound conjugate] and [free conjugate] were both determined independently. The standard error σ_n in the determination of $[conjugate]_{bound}$ ranged from ±15% for $[conjugate]_{total} < 10^{-9}$ M to ±3% when $[conjugate]_{total} \approx 10^{-8}$ M. Scatchard plots were constructed by linear least-squares fit of the data taking into account only

those obtained for $[\text{conjugate}]_{\text{total}} < 2 \times 10^{-8}$ M, since at higher concentrations nonspecific binding became dominant. From the binding parameters derived by Scatchard analysis, theoretical saturation curves with a linear term accounting for nonspecific binding were calculated. The value of this term (0.01) was obtained from the binding following preincubation with 100 times molar excess of the unlabeled probe, which competitively inhibited the specific component of the binding completely (cf. Figure 3). These curves were fitted to the experimental data by iterative variation of the affinity constant and the number of binding sites toward a minimum of the mean squared standard deviation of the experimental data from the curve. This procedure altered the affinity constant by less than 5%; however, the number of binding sites was diminished by up to 30%.

Isolation of a 110-kDa Protein from RBL-2H3 Cells

Surface Radioiodination of RBL Cells. RBL-2H3 cells (2.5×10^8) were harvested from tissue culture bottles or plates and washed 3 times in PBS. Lactoperoxidase (90 μL of 0.5 mg/mL) in PBS, 40 μL of $Na^{125}I$ solution (100 mCi/mL, specific activity 13.0 Ci/μg iodine), and 20 μL of 0.03% H_2O_2 were added to the intensely shaken cells. After 4, 8, 12, and 16 min, 90 μL of the lactoperoxidase solution and 20 μL of 0.03% H_2O_2 were added. Finally, after 20 min the reaction was terminated by addition of 50 mL of PBS and sedimentation of the cells followed by three further washings with PBS.

Preparation of Cell Lysates. The surface-radiolabeled and washed cells were lysed in 0.2 M borate, 0.16 M NaCl, and 2 mM $CaCl_2$, pH 7.4 (BBS-Ca^{2+}), supplemented with 0.5% Triton X-100, leupeptin (0.75 μg/mL), pepstatin (2.25 μg/mL), and aprotinin (0.6 TIU/mL). Two milliliters of this lysis buffer was employed per 10^8 cells. The crude lysate was centrifuged for 1 h at 18 000 rpm. The supernatant was immediately processed further, while the pellet was discarded.

Preparation of Solubilized Purified RBL Plasma Membranes. RBL plasma membranes were isolated from surface-labeled cells on a discontinuous sucrose density gradient essentially as described elsewhere (Sagi-Eisenberg & Foreman, 1984) for rat peritoneal mast cells. The purified membranes were washed once with BBS-Ca^{2+} and solubilized in the above-described lysis buffer. Two milliliters of lysis buffer was employed per quantity of membranes equivalent to 10^8 cells. The detergent-insoluble components were sedimented at 18 000 rpm, and the supernatant was processed further immediately.

Affinity Chromatography: Method 1. A Polyprep column (Bio-Rad) was packed with 0.5 mL of the matrix gel and washed with BBS-Ca^{2+} containing 0.5% Triton X-100. Cell lysate or solubilized membranes obtained from 2.5×10^8 cells (5 mL) were passed over this column during 3 h. The column was washed overnight with BBS-Ca^{2+} containing 0.5% Triton X-100 and 0.15% soybean lipids (buffer I) at 4 °C at a delivery rate of 10 mL/h. The column was first eluted with 20 mM commercial cromolyn in buffer I at 4 °C at an elution rate of 0.33 mL/h. Ten fractions of 0.5 mL each (first fraction 0.33 mL) were collected. Thereafter, the column was further eluted with 20 mM cromolyn and 10 mM DTT in buffer I at the same slow rate. Again, 10 fractions (first fraction 0.33 mL, fractions 2–10 each 0.5 mL) were collected. Each fraction collected was analyzed by SDS–PAGE as described above.

Method 2. MOPC-315-Sepharose (0.5 mL, 4.4 mg of protein/mL of gel) was washed with BBS on a sintered glass filter and packed in a Polyprep column. A dilute (~0.1% in BBS) solution of a 2,4-dinitrophenylated cromolyn–BSA conjugate carrying ~10 DNP residues per BSA was passed through this column until the effluent turned yellow, thus indicating saturation of the immunoadsorbent. The gel was then washed thoroughly, first with BBS-Ca^{2+} and then followed by BBS-Ca^{2+} containing 0.5% Triton X-100. The cell lysate or solubilized membrane fraction was loaded on this column, and it was washed overnight with buffer I at 4 °C at a delivery rate of 10 mL/h. The column was first eluted with 5 mM $N\epsilon$-DNP-lysine in buffer I at 4 °C at an elution rate of 1 mL/h. Ten 0.5-mL fractions (first fraction 0.33 mL) were collected. Thereafter, the column was further eluted with alkaline buffer I (pH raised to pH 10.5 with NaOH). Again, 10 fractions (first 0.33 mL, followed by 0.5 mL for each further fraction) were collected. Each fraction collected was analyzed by SDS–PAGE as described above. Finally, the column was washed with 0.1 M Tris containing 0.5% Triton X-100, pH 10.5, then alternatingly with acetate buffer (pH 4) and borate buffer (pH 9), and finally with BBS-Ca^{2+}. Following this treatment, the column was found to be free from conjugate or other proteins, as judged by SDS–PAGE analysis of an extract of matrix beads made by reducing sample buffer, and could be reused.

Interaction with Lectins. Samples of the isolated protein in either its intact or reduced form were examined for their binding to the following five lectins conjugated to agarose by 2-h incubation with these matrices at 4 °C: concanavalin A (ConA-agarose), soybean agglutinin (SBA-agarose), lentil lectin (LL-agarose), wheat germ agglutinin (WGA-agarose), and fucose binding protein (FBP-agarose). One milliliter of each of the gels was packed on Polyprep columns. Following washing with buffer I, the columns were each eluted with their respectively specific saccharide at a concentration of 5% (w/v) in buffer I (methyl α-mannoside, GalNAc, methyl α-mannoside, GlcNAc, and L-fucose respectively). Following this elution, a sample of the matrix was taken and boiled in reducing sample buffer. The effluent, eluate, and sample buffer extract of each column were analyzed by SDS–PAGE and autoradiography.

Purification of the Intact and Reduced Protein by Preparative SDS–PAGE and Electroelution. A 1.5-mL aliquot of an eluate containing either the intact protein or the 50-kDa subunit was treated with 0.75 mL of 3-fold concentrated nonreducing or, respectively, reducing SDS–PAGE sample buffer at room temperature and loaded onto either a 7–10% or, respectively, 10–15% SDS–PAGE gradient gel (gel height 200 mm). Following the electrophoresis, both the left and right margins of the slab gel, which contained eosin-labeled molecular weight markers, were cut off. From the core of the gel, the upper and lower margins were removed at positions 20 mm above and below the respective position of the band of interest as estimated by its R_f value (~0.3 for the intact protein on a 7–10% gradient or 0.33 for the reduced form on a 10–15% gradient). The central gel piece was then sliced horizontally into 20 slices of 2-mm thickness each. Each of these slices was soaked overnight at 4 °C in 30 μL of 50% (w/v) glycerol, 50 mM Tris–base, 0.1% SDS, and 2 mg/mL bromophenol blue. The radioactivity of each slice was then measured, and the two slices containing the activity peak were pooled and placed into an electroelution cell. This cell was filled with the usual electrophoresis buffer and subjected to a potential of 100 V for 30 min and 50 V for a further 30 min. The layer above the positive membrane was aspirated and processed further for reconstitution into planar lipid bilayers as described under Experimental Procedures in the accompanying paper (Corcia et al., 1988). In the case of the reduced form (50-kDa subunit), the respective electroeluate was de-

pleted of low molecular weight compounds by repeated concentration and dilution of the sample on a Centricon (Amicon) ultramembrane (washing buffer: PBS containing 0.1% SDS). This sample was hydrolyzed in 6 N HCl, and the hydrolysate was subjected to amino acid analysis in a Biotronic LC 6001 amino acid analyzer.

Enzymatic Cleavage of the Protein-Bound Carbohydrate. Endoglycosidase F treatment was carried out in a reaction mixture containing 0.1 M sodium phosphate, pH 6.1, 0.05 M EDTA, 1% Triton X-100, 0.1% SDS, and 1% β-mercaptoethanol as described elsewhere (Elder & Alexander, 1982). A sample of the 50-kDa subunit was incubated at 37 °C with 2.7 units/mL of the enzyme in the reaction mixture. An additional 2.7 units/mL was introduced following 6 h and again after 12 h. In parallel, an identical sample was incubated at 37 °C in the same reaction mixture, however, without enzyme. After 24 h, both samples were boiled in reducing sample buffer for 5 min and analyzed by SDS–PAGE and autoradiography.

Results

Conjugation of Cromolyn to Macromolecules

The carbon-2 of the propane linking the two chromone heterocycles was chosen as the point of conjugating cromolyn to different carriers. The choice of this site was made because it was assumed to have no role in the pharmacological activity of the drug (Cairns et al., 1972) and because it is the center of symmetry of the molecule. In the original drug, a hydroxyl is present at this position. Alkylation of this hydroxyl is not possible, because the chromone heterocycle is readily opened by the strong bases required for the reaction (Ellis, 1977). An alternative route, namely, acylation of this hydroxyl, yielded products that undergo hydrolysis in slightly alkaline solutions. Therefore, it was decided to synthesize a derivative with an amine at this position. The 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-aminopropane (aminocromolyn) was prepared in a multistep synthesis starting from 2,6-dihydroxyacetophenone (Figure 1). Two further low molecular weight derivatives of aminocromolyn were also synthesized, both of which carry a C_6 spacer with different functional groups at its end. These are 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-[6-[(2,4-dinitrophenyl)amino]caproamido]propane (DNP-cromolyn) and 1,3-bis[(2-carboxychromon-5-yl)oxy]-2-(6-isothiocyanatocaproamido)propane (cromolyn isothiocyanate). These three compounds were used for the preparation of cromolyn–macromolecule conjugates and cromolyn-carrying insoluble matrices and for probing the structural requirements for their use in affinity studies.

Two different protocols were employed in the derivatization of proteins or synthetic amino acid polymers with aminocromolyn:

(1) Glutaraldehyde was used to conjugate aminocromolyn to globular proteins (e.g., BSA, ovalbumin). The dialdehyde forms azomethine groups with the amine of the ligand on one end and that of lysyl residues of the protein on the other. These Schiff bases are subsequently stabilized by reduction with $NaBH_4$. Such drug–protein conjugates having BSA as carrier were termed BSA-(ga-cro)$_n$. A high extent of protein–protein di- and multimer formation during the derivatization was observed. The monomeric drug conjugate was also isolated from this mixture by size-exclusion chromatography on Sephadex G-150 or G-200. In order to account for the effect of glutaraldehyde-treated protein alone, appropriate control conjugates were prepared with an equivalent amount of NH_4Cl instead of aminocromolyn in the conjugation procedure, thus yielding reference macromolecules [BSA-(ga-NH_2)$_n$] carrying the 1,5-diaminopentane side chains.

(2) Cromolyn isothiocyanate was reacted with proteins or synthetic poly(amino acids) at pH 10.5, where the isothiocyanate group reacts with primary amines of the macromolecules to form thiourea bridges. Such conjugates having BSA as carrier were termed BSA-(itc-cro)$_n$. This method also allows derivatization of synthetic amino acid polymers, which were found difficult to derivatize by dialdehydes, since they tend to precipitate under these conditions. The polymer employed was the branched multichain copolymer of alanine and lysine (Ala$_{22}$Lys)$_n$ [A-L, Sela et al. (1956)]. Since the molecular weight of this polymer is only an average one, the degree of derivatization of these conjugates was defined by the number of aminocromolyn residues bound per peptide bond of this polymer. Therefore, these conjugates were termed A-L-cro$_{n/pb}$ (n molecules of aminocromolyn conjugated per peptide bond).

Using both protocols, we could tune the reactions by an appropriate choice of the ratio of the reactants to yield any degree of derivatization desired (Table I). The maximal degree of derivatization achieved by either procedure was similar (a maximum of ~25 molecules of aminocromolyn or cromolyn isothiocyanate could be conjugated to 1 molecule of BSA).

Binding of the Conjugates to RBL-2H3 Cells

With the range of different polyvalent cromolyn–macromolecule conjugates, we proceeded to investigate their binding to the RBL-2H3 cells. The conjugates were radiolabeled with the Bolton and Hunter reagent (Bolton & Hunter, 1973), yielding radioactive probes with a specific activity in the range of $\sim 5 \times 10^{17}$ cpm/mol of conjugate. The binding of cromolyn itself to the cells was examined by using ^{3}H-labeled commercial cromolyn with a specific activity of 10 μCi/nmol, generously made available by Fisons plc, Loughborough, England. These different probes were employed in binding assays, which gave the following results:

With the available specific activity no binding of [^{3}H]cromolyn to RBL-2H3 cells could be resolved.

A-L, derivatized with cromolyn isothiocyanate to different degrees (A-L-cro$_n$), did not exhibit any specific and saturable element in its binding to the cells. This can be seen in Scatchard plots constructed from these experiments in which the data produce a practically horizontal line (Figure 2). Therefore, this conjugate was later also used as a reference for nonspecific binding.

All different BSA–cromolyn isothiocyanate conjugates were consistently found to show a rather low degree of specific binding (less than 2% of the total introduced radioactivity), which was therefore also poorly reproducible.

The cromolyn conjugates prepared by glutaraldehyde attachment were found to behave distinctly different from those obtained with cromolyn isothiocyanate. Already the structural control conjugate [BSA-(ga-NH_2)$_n$], accounting only for the effect of glutaraldehyde-treated BSA, showed a specific component in its binding, albeit with low affinity and a relatively large mean standard deviation of the experimental data from the calculated Scatchard plot (Figure 2). The respective aminocromolyn–protein conjugates, namely, BSA or ovalbumin, derivatized with aminocromolyn by glutaraldehyde attachment [BSA-(ga-cro)$_n$ or OA-(ga-cro)$_n$], showed reproducibly specific and saturatable binding in the large number (>20) of separate experiments that were carried out. Data from three typical independent binding assays with the same conjugate BSA-(ga-cro)$_{10}$ have been plotted together in Figure 2. As seen in the inset, the specific element of the binding is saturable.

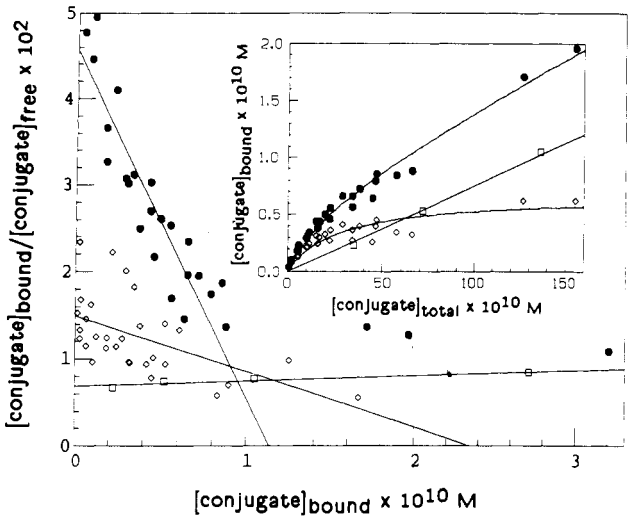

FIGURE 2: Scatchard plots derived from the binding of drug–macromolecule conjugates to RBL-2H3 cells. Binding experiments were carried out and analyzed as described under Experimental Procedures. The binding of BSA-(ga-cro)$_{10}$ (●) and BSA-(ga-NH$_2$)$_n$ (◇) presented in the figure are averages of triplicates and were obtained in three independent experiments. The molecular mass of conjugates having BSA as carrier was set to 67 kDa [10^{-10} M BSA-(ga-cro)$_{10}$ ≅ 10^{-9} M conjugated aminocromolyn]. The binding of A-L-(itc-cro)$_{0.066/pb}$, which is A-L derivatized with cromolyn isothiocyanate to a degree of 0.066 conjugated aminocromolyn moieties per peptide bond of the polymer, does not show any specific element (□). In order to enable comparison between the behavior of this conjugate and that of BSA-(ga-cro)$_{10}$, its concentration is given as [conjugated aminocromolyn]/10. Inset: Theoretical binding isotherms calculated as described under Experimental Procedures and presented along with their respective experimental data. (●) Total binding of BSA-(ga-cro)$_{10}$, (□) total binding of A-L-(itc-cro)$_{0.066/pb}$, (◇) specific binding of BSA-(ga-cro)$_{10}$ obtained by subtraction of the nonspecific component from the total binding of this conjugate.

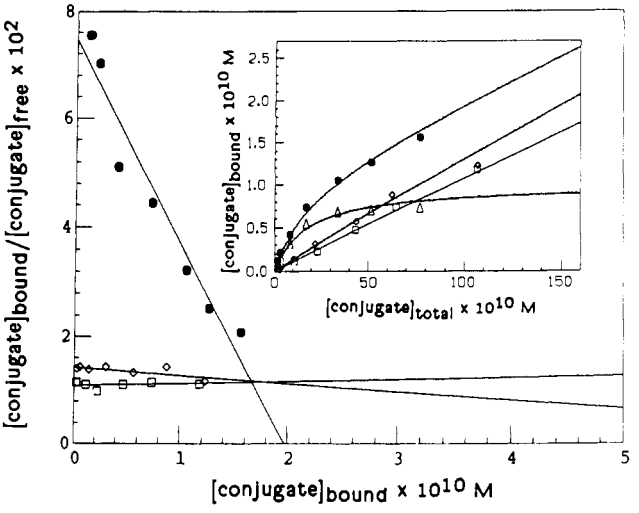

FIGURE 3: Competition between radiolabeled and unlabeled BSA-(ga-cro)$_{10}$ conjugate presented as Scatchard plots and binding isotherms (inset). (●) Binding of labeled conjugate without competition → affinity constant, 3.8 × 10^8 M^{-1}, number of binding sites, 7000 per cell; (◇) preincubation with 10^{-7} M and (□) with 10^{-6} M unlabeled conjugate. (△) (in inset) Specific contribution to the binding obtained by subtraction of the fully competed binding of BSA-(ga-cro)$_{10}$ from its binding without competition.

Employing Scatchard analysis, these data were found to fit well a straight line (Figure 2, main panel), yielding an apparent binding constant of 3.8 ± 0.2 × 10^8 M^{-1}. The apparent number of binding sites was calculated from this series of experiments to be 4200 binding sites for conjugate per cell.

For conjugates carrying averages of 5 up to ~18 aminocromolyn groups per BSA, the apparent binding constant

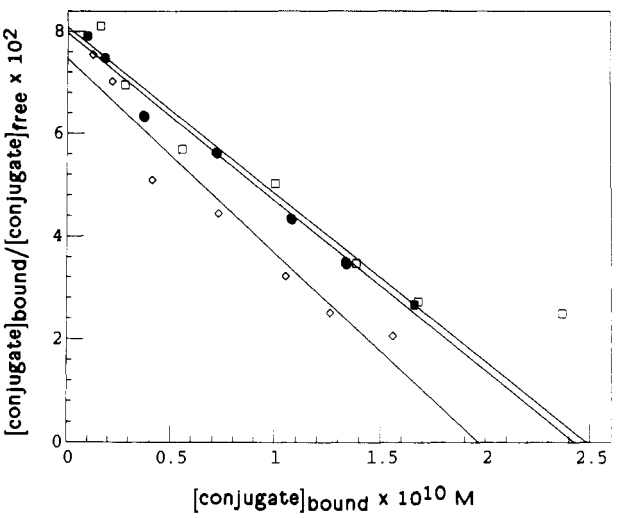

FIGURE 4: Binding of BSA–cromolyn conjugates with different degree of derivatization. Superimposed Scatchard plots of the binding of BSA-(ga-cro)$_5$ (●), BSA-(ga-cro)$_{10}$ (◇), and BSA-(ga-cro)$_{17}$ (□).

appears to be independent of the degree of derivatization (Figure 4). With higher degrees of derivatization (~25) a large increase in the nonspecific component of the binding was observed, from which the specific component could not be resolved reliably. The apparent number of binding sites per cell showed a broader spread in the different experiments. However, it was always found to be in the range between 4000 and 8000 binding sites for conjugate per cell. Preincubation with 100 μM commercial cromolyn did not inhibit the binding of these conjugates; however, the unlabeled multivalent conjugates competed effectively with the labeled ones as illustrated in Figure 3.

Isolation of a 110-kDa Membranal Protein from RBL-2H3 Cells

We proceeded to identify and isolate the membrane component(s) responsible for the binding of the above described conjugates to RBL-2H3 cells. To this end, both the binding and nonbinding conjugates were immobilized on CNBr-activated agarose and packed into columns. RBL-2H3 cells were surface radioiodinated and lysed with the nonionic detergent Triton X-100 as described under Experimental Procedures. The detergent-insoluble fraction was sedimented, and the supernatant was loaded onto the specified columns. Following extensive washing with working buffer containing Triton X-100 and phospholipids (buffer I, cf. Experimental Procedures), the columns were eluted with 20 mM cromolyn in the same buffer. Then, a matrix sample was boiled in reducing sample buffer in order to release the still-retained proteins. Samples of the eluted fractions and the sample buffer extract of the matrix were subjected to SDS–PAGE. Labeled protein was detected by autoradiography. In several dozens (>36) of independent experiments carried out so far, the following results were consistently obtained: Matrices carrying nonbinding conjugates did not retain labeled protein. Matrices carrying the cromolyn–protein conjugates, prepared by glutaraldehyde attachment and shown to bind specifically to the cells, always yielded the same labeled polypeptide in the eluates. On nonreducing gels it appeared as a broad band centered at an apparent molecular mass of 110 kDa and spanning the range of ±10 kDa. On reducing gels, a single radioactive band centered at ~50 kDa was observed that appears narrower, spanning the range of ±3 kDa (Figure 5, lane B). We have also observed traces of the same protein employing matrices carrying the structural control conjugate BSA-(ga-NH$_2$)$_n$,

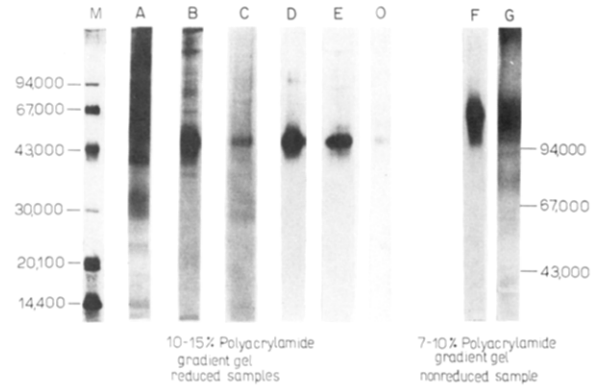

FIGURE 5: SDS–PAGE of the isolated proteins. (A) Total lysate of surface radioiodinated RBL-2H3 cells. (B) Protein sample prepared by absorption of total cell lysate onto $(cro\text{-}ga)_{10}$-BSA-Sepharose and elution with 20 mM cromolyn and 1 mM dithiothreitol (DTT) in buffer I. (C) Protein sample prepared by absorption of total cell lysate onto Sepharose-BSA-$(ga\text{-}NH_2)_n$ and elution with 20 mM cromolyn and 10 mM DTT. (D) Protein sample prepared by absorption of total cell lysate onto an α-DNP immunoadsorbent (MOPC315-Sepharose) that had been previously saturated with $DNP_8BSA\text{-}(ga\text{-}cro)_{10}$. Elution buffer, 5 mM $N\epsilon$-DNP-lysine. (E) Protein sample prepared by absorption of solubilized fractionated plasma membranes of surface-labeled RBL cells onto $(cro\text{-}ga)_{10}$-BSA-DNP_8-MOPC315-Sepharose and elution with 5 mM $N\epsilon$-DNP-lysine. A–E: reducing 10–15% polyacrylamide gradient gels. F and G: nonreducing SDS–PAGE of the same sample shown in D and in E, respectively (7–10% polyacrylamide gradient gels). Lanes A–G are autoradiograms, while lane O presents a silver-stained 10–15% gel of the 50-kDa polypeptide obtained by electroelution of this band following electrophoresis of sample B. (M) Molecular weight markers visualized by silver stain.

which is assumed to account for the effect of the 1,5-pentanediamine spacer (Figure 5, lane C). The yield of the intact protein eluted with cromolyn was relatively low: Only <20% of the labeled polypeptide could be released from the matrix. This could be overcome by addition of 10 mM β-mercaptoethanol or 1 mM DTT to the elution buffer or elution with either of these reducing agents alone, which caused essentially the quantitative release of this protein from the matrix. However, these agents always led to leakage of the immobilized conjugates, which were found along with the reduced protein in the eluate. The silver-stained sample in Figure 5, lane O, which is free of conjugate, was obtained by electroelution of the 50-kDa band from a nonfixed SDS gel, as described under Experimental Procedures.

In order to circumvent the observed difficulty in elution of the intact protein, a different isolation procedure was developed. The cromolyn conjugates, shown to be effective in retaining the 110-kDa protein, were derivatized further with 2,4-dinitrofluorobenzene (FDNB), thus yielding conjugates carrying both aminocromolyn and 2,4-dinitrophenyl (DNP) residues. Under the employed conditions, typically 8–12 DNP moieties were found to be attached to such conjugates with BSA as carrier. These bifunctional conjugates were immobilized on a DNP-specific immunoadsorbent (MOPC-315-agarose; Goetzl & Metzger, 1970), and RBL cell lysates were adsorbed onto these matrices. Elution of the columns with a monovalent hapten, namely, $N\epsilon$-DNP-lysine caused the practically quantitative elution of the intact protein along with the conjugate (Figure 5, lane F; reduced form, lane D). Finally, when solubilized fractionated RBL plasma membranes, which had been prepared on a discontinuous sucrose density gradient (Sagi-Eisenberg & Foreman, 1984), were used in the latter isolation procedure instead of whole cell lysates, the very same labeled protein was also obtained (Figure 5, lane G; reduced form, line E). Although this isolation protocol using fractionated membranes as the starting material yielded less of the desired protein, contamination of these preparations by other polypeptides was however markedly reduced.

Table II: Amino Acid Composition of the 50-kDa Subunit of the Channel-Forming Protein[a]

amino acid	% w/w[b]	% mol/mol	amino acid	% w/w[b]	% mol/mol
Asp	9.32	8.78	Ile	6.23	5.96
Thr	4.20	4.53	Leu	7.47	7.15
Ser	5.99	7.43	Tyr	1.85	1.24
Glu	15.37	12.89	Phe	3.89	2.86
Gly	6.17	11.71	His	7.96	6.28
Ala	5.31	8.08	Lys	8.95	7.78
Val	8.40	9.19	Arg	8.02	5.57
Met	0.99	0.78			

[a] The protein sample was prepared for amino acid analysis as described under Experimental Procedures. [b] Percent weight per weight not taking into account the carbohydrate content.

Initial Biochemical Characterization of the 110-kDa Protein

Amino Acid Analysis. The 20 mM β-mercaptoethanol eluate from a $(cro\text{-}ga)_{10}$-BSA-Sepharose column, which contained the reduced (50-kDa) form of the protein obtained from 2.5×10^8 cells, was purified further by preparative SDS–PAGE and electroelution as described under Experimental Procedures. Following removal of low molecular weight compounds by repetitive ultrafiltration, the protein sample was hydrolyzed overnight in 6 N HCl. The hydrolysate was subjected to amino acid analysis. All common amino acids have been found in the hydrolysate, with a relatively low content of methionine and tyrosine and a relatively high content of glutamate (Table II). By integration of the molar quantity detected of each amino acid, the amount of protein was calculated to be 0.5 μg.

Interaction with Lectins. The intact (110-kDa) protein as well as its reduced form (50 kDa) was found to bind to immobilized ConA and WGA. The intact protein could not be eluted from either of these matrixes by 0.25 M of their respectively specific saccharides, namely, methyl α-mannoside or GlcNAc. However, the reduced form was eluted from the latter matrix quantitatively with GlcNAc, while its release from the ConA matrix by methyl α-mannoside was incomplete (Figure 6). Because the conjugate present in the samples of the reduced protein was partly retained by the WGA column and also eluted with GlcNAc, purification of the 50-kDa polypeptide toward a single band on silver-stained SDS gels was not achieved by this procedure.

Extent of Glycosylation. In order to examine the relative amount of carbohydrate carried on the 50-kDa polypeptide, a sample of this component was further affinity purified on WGA-agarose. This sample was then treated exhaustively with endoglycosidase F (Elder & Alexander, 1982), as described in detail under Experimental Procedures, followed by SDS–PAGE analysis along with an untreated control. In the untreated sample, a second band with an apparent mass of ~42 kDa was observed on autoradiograms (Figure 7, lane II) along with the 50-kDa band, while in the deglycosylated sample a similar doublet was seen, which is however deleted by 12 kDa with respect to that of the control (Figure 7, lane I). These findings suggest that the 50-kDa polypeptide carries carbohydrate residues of ~12 kDa corresponding to an extent of glycosylation of ~25%, while the lower band observed in both the enzyme-treated sample and untreated control is most probably a product of proteolytic cleavage having occurred during the prolonged incubation of both samples at 37 °C.

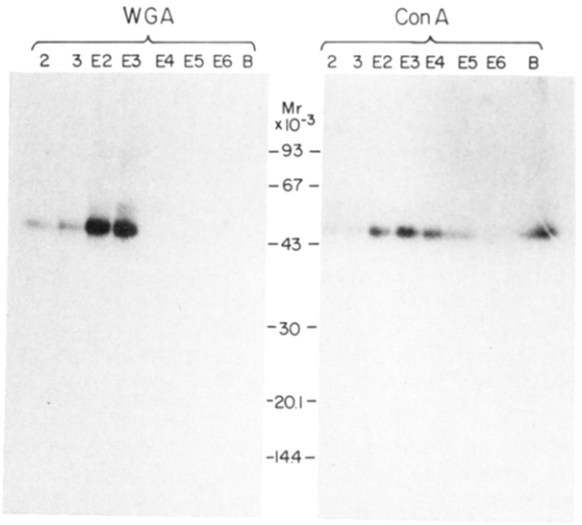

FIGURE 6: Binding of the 50-kDa subunits to lectins. Samples of the purified protein in its reduced form were adsorbed onto columns of either wheat germ agglutinin agarose (WGA) or concanavalin A-agarose (ConA). Following extensive washing, the columns were eluted with 5% *N*-acetylglucosamine (WGA) or 5% methyl α-mannopyranoside (ConA). The figure presents SDS gel autoradiograms of samples of the effluents (1, 2) and the eluate fractions (E2–E6). Lines B: β-mercaptoethanol/SDS extracts of the eluted matrices.

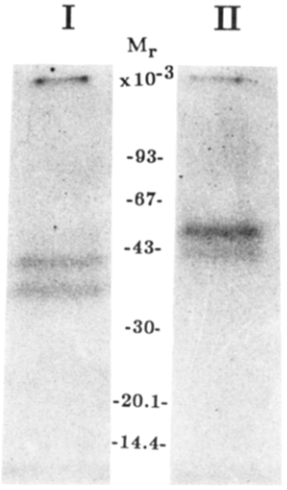

FIGURE 7: Enzymatic cleavage of the protein-bound carbohydrate. Endoglycosidase F treatment of the 50-kDa subunit was carried out as described under Experimental Procedures. The figure presents SDS gel autoradiograms of the cleaved sample (I) and the untreated control (II).

Preparation of Samples for the Functional Characterization of the 110-kDa Protein

In order to establish the function of the isolated protein, several samples containing the described protein were reconstituted together with the purified IgE–$Fc_\epsilon R$ complex into lipid planar bilayers for conductance measurements. The results of these experiments are presented in the subsequent paper (Corcia et al., 1988). However, to ascertain that the observed conductances are indeed due solely to the described 110-kDa protein and not to other potential contaminants, a 20 mM cromolyn eluate from a $(cro\text{-}ga)_{10}$-BSA-Sepharose containing the intact 110-kDa protein was subjected to preparative SDS–PAGE. Then, only the (110 ± 5)-kDa band was electroeluted as described under Experimental Procedures. The electroeluate was processed further for reconstruction together with IgE–$Fc_\epsilon R$ into liposomes as detailed in the subsequent paper (Corcia et al., 1988). These reconstituted liposomes were employed in the same conductance measurements already performed using the purified but not electrophoresed protein samples. The results in both cases were very similar. Furthermore, control samples were prepared by electroelution from either above or below the 110-kDa band. No activity was observed with these samples.

Discussion

In this study we aimed at the identification and isolation of the plasma membrane component of RBL-2H3 cells that is responsible for the Fc_ϵ receptor gated Ca^{2+} conductances across coreconstituted lipid planar bilayers reported previously (Mazurek et al., 1984; Pecht et al., 1986). Therefore, well-defined reagents that would interact with this protein were developed on the basis of the original working hypothesis assuming an affinity of this protein for the antiallergic drug cromolyn. In order to ascertain the covalent conjugation of the assumed ligand cromolyn, several derivatives of the drug were synthesized, all with an amino group at position 2 of the propane bridge linking the two chromone heterocycles. This amino group was employed in covalently conjugating these derivatives to macromolecular carriers by several different spacers. Conjugation protocols for the aminocromolyn derivatives were developed so as to control the number of drug molecules attached per carrier. Stability of these covalent conjugates as well as their extent of derivatization was quantitatively monitored via the characteristic UV absorption band of the chromone nucleus.

We have investigated the binding of the different conjugates prepared from several macromolecular carriers, with different spacers, as well as with different degrees of derivatization, to intact RBL-2H3 cells. Specific binding to the cells was consistently observed with those conjugates that had aminocromolyn attached to globular proteins (BSA, ovalbumin) by glutaraldehyde and subsequent reduction of the azomethine groups resulting in a pentane-1,5-diamine spacer. By contrast, those conjugates where cromolyn was conjugated to protein carriers or synthetic poly(amino acids) via a 6-(thiourea)-caproyl spacer [by direct reaction of (ϵ-isothiocyanatocaproamido)cromolyn with the carrier] did not exhibit any affinity. Notably, proteins derivatized in an analogous fashion with glutaraldehyde and ammonium followed by reduction to yield diaminopentane side chains did show a limited extent of binding to the cells. This may be either a reflection of an element of specificity for this alkanediamine moiety or of a hydrophobic binding. The reversibility of the interaction between the RBL-2H3 cells and the binding conjugates was established by competition experiments with the unlabeled conjugate, which has shown effective displacement (Figure 3).

We proceeded to examine the capacity of the different drug–macromolecule conjugates to serve in the isolation of the channel-forming protein from total lysates or fractionated plasma membranes of surface-labeled RBL-2H3 cells. Only those conjugates that exhibited specific binding to the cells were found to be effective in the isolation procedure and to retain always the same labeled protein. By contrast, Sepharose-poly(*N*-hydroxysuccinimide ester), which was derivatized directly with (*N*ϵ-aminocaproamido)cromolyn, was found ineffective. Thus, the structural requirement for the spacer observed in the binding measurements is indeed also maintained in the isolation procedure. This point became further evident in isolation experiments using Sepharose matrices that carried BSA derivatized with 1,5-diaminopentane groups and which enabled as well retaining of the same protein, albeit in very much smaller amounts.

From Scatchard analysis of the extensive binding assays carried out, it emerges that the number of bound conjugate

molecules per cell is within the range of 4000–8000. The binding constant was found to be $3.8 \pm 0.2 \times 10^8$ M^{-1}. Variation of the degree of carrier derivatization over the range from 5 up to 18 cromolyn groups per macromolecule (BSA) did not significantly alter the observed number of binding sites per cell and apparent binding constant (Figure 4). Higher derivatization (e.g., 25 mol of cromolyn/mol of BSA) leads to a large increase in nonspecific binding, while the binding of conjugates with an average of ca. two cromolyns was similar to that exhibited by the control conjugate of the protein derivatized only with glutaraldehyde and ammonium as mentioned above. Hence, the conjugates employed in the majority of these measurements carried an average of 10 cromolyn molecules per macromolecule.

The observed relative independence of the conjugate interaction with the cells on the extent of its derivatization could be a result of at least two situations:

(1) A requirement for di- or polyvalency in the binding of the conjugate to the channel-forming protein; i.e., assuming one binding site per 50-kDa subunit of the protein, an effective interaction with a given conjugate would require an appropriate spatial proximity of two drug residues on the carrier. The random derivatization of the carrier protein probably satisfies such a requirement in those conjugates carrying the intermediate number of drug molecules per carrier. Two observations support this notion: (a) No significant binding of ^{3}H-labeled cromolyn with the available specific activity could be detected, as probably the affinity of the nonderivatized drug is too low. (b) Disulfide reductants were found to be most effective in eluting the protein from its matrix-bound state. Since the intact protein is assumed to be a disulfide-linked dimer of two subunits of practically the same apparent mass (see below), reduction would loosen the structure and cause dissociation.

(2) The need for an appropriate microenvironment suited for the interaction between the conjugate and the protein is provided only by a limited number of the drug residues carried on a given macromolecule. Probability for such sites is optimized at the intermediate range of derivatization, hence the observed particular dependence of the interaction between the different macromolecular derivatives and the cells.

Presently we cannot resolve between the above two possibilities, although we designed experiments that could have provided some insight into this problem. For example, we tried to conjugate aminocromolyn via glutaraldehyde to synthetic poly(amino acids). Such carriers could yield a better defined environment for the drug. Unfortunately, treatment of these polymers with the dialdehyde yielded water-insoluble products due to extensive cross-linking. By contrast, direct conjugation of (ϵ-isothiocyanatocaproamido)cromolyn to this type of carrier yielded ineffective products, as already mentioned above.

As indicated above, analysis of the binding data of the conjugate BSA-(ga-cro)$_{10}$ enables us to set approximate limits on the number of sites per cell that are available for interaction with it. Assuming a random spatial distribution of drug residues on the conjugate surface and one binding site on the intact channel-forming protein, a lower limit for the number of copies of such protein per cell could fall in the above-mentioned range of $\sim 10^4$. The yield of isolated protein as determined by amino acid analysis of its purified fraction (50-kDa subunit) is ~ 0.5 μg from 2.5×10^8 cells, which allows an independent assessment of the number of copies of this protein per cell. Thus, one obtains 5×10^{12} molecules of the 50-kDa polypeptide (0.5 μg $\cong 10^{-11}$ mol) processing 2.5×10^8 cells. This yields a lower limit of ca. 10^4 copies of the intact 110-kDa protein per cell, which is in agreement with the number estimated above. As the total amount of protein content of fractionated plasma membranes of RBL-2H3 cells is found to be $\sim 1.5 \times 10^{-11}$ g per cell, this particular membrane component represents about 0.01% of the total plasma membrane protein. For comparison, the Fc_ϵ receptor constitutes ~0.3% of the total plasma membrane protein, as estimated by binding of labeled IgE to the intact cells.

Mazurek et al. (1982) reported earlier the isolation of a 60-kDa polypeptide from RBL cells using agarose matrices assumed to carry cromolyn moieties either directly or in the form of multivalent cromolyn–BSA conjugates. IgE–Fc_ϵ R-gated conductance was indeed observed first with lipid bilayers reconstituted with IgE–Fc_ϵR and the protein present in these preparations (Mazurek et al., 1984; Pecht et al., 1986). However, the 60-kDa protein was later shown to be a contamination of seral origin (V. Dulič, unpublished results). This finding led to the suggestion that the active component was present in these preparations in minute amounts, which nevertheless could be detected with the patch clamp methodology that, in principle, requires the presence of as little as a single active channel in the bilayer. Subsequent studies revealed that, due to the misdesigned conjugation procedure, the matrices employed in the earlier studies actually did not carry covalently attached cromolyn but probably only BSA derivatized with 1,5-diaminopentane spacers. However, we have shown in the present study that these matrices were also effective in the isolation protocol, yielding the 110-kDa polypeptide, albeit in very small amounts (cf. Figure 5, lane C). Thus, it is evident that the active component was present in these earlier preparations in rather low concentrations, though enriched relative to other RBL plasma membrane proteins.

Finally, several features of the isolated protein deserve recapitulation: Samples prepared in the presence of disulfide reductants such as DTT or β-mercaptoethanol yield in autoradiograms of SDS–PAGE a single band with an apparent molecular mass of ~50 kDa. Samples isolated under nonreducing conditions also exhibit a single, though broader and more diffuse band with an apparent mass of ~110 kDa. This band is shifted to 50 kDa upon reduction. As only the mentioned reductants, but not cromolyn alone, are able to quantitatively elute the protein from the conjugate column, its affinity for the conjugate is probably maintained in the intact state. These observations suggest that the native protein is a dimer of two polypeptide chains of similar, if not identical, size. The binding of the isolated protein to immobilized lectins, namely, to concanavalin A or to wheat germ agglutinin, clearly suggest that it is a glycoprotein. The specific elution with GlcNAc from the WGA column and the exhaustive deglycosylation with endoglycosidase F, which causes a reduction in the apparent mass of the 50-kDa subunit by 12 kDa, further substantiate this point and imply ~25% glycosylation of this polypeptide.

The isolation and purification procedure described in this paper is currently employed in the preparation of protein samples, which would enable the further chemical and functional characterization of this channel-forming protein.

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Supplementary Material Available

Description of the synthesis of the cromolyn derivatives as outlined in Figure 1 and the IR and UV spectra of these

compounds (4 pages). Ordering information is given on any current masthead page.

Registry No. I, 115705-74-3; II, 115705-75-4; III, 115705-76-5; IV, 115705-77-6; V, 115705-78-7; VI, 115705-79-8; VII, 115705-80-1; VIII, 115705-81-2; IX, 115705-82-3; X, 115705-83-4; XI, 115705-84-5; XII, 115705-85-6; EtOCOCOOEt, 95-92-1; $(DNP\text{-}NH(CH_2)_5CO)_2O$, 115705-86-7; $(BocNH(CH_2)_5CO)_2O$, 70604-37-4; 2,6-dihydroxyacetophenone, 699-83-2; Sepharose 4B, 9012-36-6.

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