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# A Novel Cell-Permeable Cromoglycate Derivative Inhibits Type I $Fc_\epsilon$ Receptor Mediated $Ca^{2+}$ Influx and Mediator Secretion in Rat Mucosal Mast Cells†

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ABSTRACT: Type I $Fc_\epsilon$ receptor ($Fc_\epsilon RI$) mediated $Ca^{2+}$ uptake and secretion of rat serosal mast cells have been shown to be inhibited by disodium 1,3-bis[(2′-carboxylatochromon-5′-yl)oxy]-2-hydroxypropane (disodium cromoglycate, DSCG), which is widely employed in the treatment of allergic asthma [Foreman et al. (1977) *Br. J. Pharmacol. 59*, 473P–474P; Cox (1967) *Nature (London) 216*, 1328–1329]. This drug was also found to modify the protein phosphorylation pattern of these mast cells [Theoharides et al. (1980) *Science 207*, 80–82]. We have isolated by affinity chromatography on a water-insoluble cromoglycate-carrying matrix a cytosolic enzyme recently identified as a nucleoside 5′-diphosphate kinase. In order to examine a possible intracellular activity of the drug, a cell-permeant cromoglycate derivative, 1,3-bis[[2′-[[(acetoxymethyl)oxy]carbonyl]chromon-5′-yl]oxy]-2-hydroxypropane [bis(acetoxymethyl) cromoglycate, CG/AM], has been synthesized, and its uptake and effect on the $Fc_\epsilon RI$-mediated exocytosis of mast cells was investigated. A tritium-labeled CG/AM derivative, used as radioactive tracer, was found to permeate mucosal mast cells of the rat line RBL-2H3 and accumulate intracellularly up to 40-fold its extracellular concentration following hydrolysis by cytoplasmic hydrolases. A CG/AM dose dependent inhibition of the $Fc_\epsilon RI$-induced mediator secretion was observed in RBL-2H3 cells loaded with this compound ($I_{50} \approx 40$ μM extracellular CG/AM). A similar dose-dependent inhibition was observed for both the $Fc_\epsilon RI$-mediated transient rise in the concentration of cytosolic free $Ca^{2+}$ ions ($[Ca^{2+}]_i$) and the net $Ca^{2+}$ influx, as monitored by the fluorescent indicator Quin2 and the radioactive tracer $^{45}Ca^{2+}$, respectively. These results clearly show that cell-permeant cromoglycate inhibits the $Fc_\epsilon RI$-mediated $Ca^{2+}$ influx into the cell and further underscore the dominant role of this process in the coupling of stimulus to secretion in RBL cells. Furthermore, with the identification of nucleoside 5′-diphosphate kinase as a potential intracellular target for CG activity, distinct mechanisms of action may be inferred for cell-permeant and nonpermeant forms of CG.

Mast cells and basophils express the type I cell surface receptors ($Fc_\epsilon RI$)[1] for the Fc domains of class E immunoglobulin (IgE). Aggregation of the $Fc_\epsilon RI$ either by multivalent antigen and IgE or by other agents that bind receptor–IgE complex epitopes initiates a cascade of processes which culminates in the release of inflammatory mediators (Ishizaka & Ishizaka, 1981). In certain serosal mast cell subtypes,

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[1] Abbreviations: CG, cromoglycate; CG/AM, cromoglycate bis(acetoxymethyl) ester; DSCG, disodium cromoglycate; IgE, immunoglobulin class E; $Fc_\epsilon RI$, type I cell surface receptor for the Fc domain of IgE; RBL, rat basophilic leukemia cells of the line 2H3.

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secretion can also be induced by alternative pathways to that initiated by $Fc_\epsilon RI$ aggregation, e.g., by different polycations such as compound 48/80 (Cochrane et al., 1982; Douglas & Ueda, 1973), polylysine (Ennis et al., 1980), or substance P (Mazurek et al., 1980b). Both types of stimuli (by $Fc_\epsilon RI$ aggregation or polycations) lead to a transient elevation in the cytosolic free calcium ion concentration ($[Ca^{2+}]_i$). Using $Ca^{2+}$ ionophores, it has been shown that an increase in $[Ca^{2+}]_i$ is by itself necessary and sufficient to induce exocytosis (Foreman et al., 1973). However, the coupling of the former two different stimuli apparently proceeds along distinct pathways: while $Fc_\epsilon RI$ aggregation causes primarily $Ca^{2+}$ influx from the cells' exterior (Beaven et al., 1984; Foreman & Mongar, 1972), stimulation by polycations produces a transient elevation of $[Ca^{2+}]_i$ also in the absence of external calcium, most probably by the release of these ions from intracellular stores (Cochrane et al., 1982; Gomperts & Fewtrell, 1985). The very early biochemical transduction events triggered by either stimulus take place in the plasma membrane, are energy requiring, and involve coupling elements such as G-proteins (Chabre, 1987; Sieghart et al., 1978) and the production of second messengers like inositol phosphates (Penner et al., 1988). Protein phosphorylation has also been implicated in the control of $Fc_\epsilon RI$-mediated secretion and other cellular responses (Miles & Huganir, 1988; Sagi-Eisenberg, 1989; Sagi-Eisenberg et al., 1985; Benhamou et al., 1990).

Disodium 1,3-bis[(2′-carboxylatochromon-5′-yl)oxy]-2-hydroxypropane (commonly known as cromolyn, DSCG, Intal, or Lomudal) is a drug widely used for the prophylactic treatment of allergic asthma (Cox, 1967; Cox et al., 1970). In serosal mast cells cromolyn has been shown to interfere with the biochemical cascade coupling the $Fc_\epsilon RI$-mediated stimulus with secretion. By contrast, most other antiallergic drugs exert their effects distal to the mediator release from mast cells. For example, cromoglycate (CG) has been found to inhibit in rat peritoneal mast cells, both the antigen-induced net $^{45}Ca^{2+}$ uptake (Foreman et al., 1977a) and mediator secretion (Cox, 1967; Garland & Mongar, 1974). In the same cell type, CG was also shown to markedly increase the phosphorylation of a 78-kDa polypeptide that was suggested to modulate the $Fc_\epsilon RI$-operated $Ca^{2+}$ influx (Theoharides et al., 1980; Wells & Mann, 1983). However, in mast cells of the rat line RBL-2H3, which closely resemble mucosal mast cells (Seldin et al., 1985) and are widely used as a model for the study of $Fc_\epsilon RI$-mediated exocytosis, secretion is not inhibited by treatment with up to 10 mM cromolyn. Rat intestinal mucosal mast cells were also found nonresponsive to the drug (Pearce et al., 1982).

On the basis of some of these observations, Mazurek et al. (1980) pursued the possibility that a membrane target exists for cromolyn, which can modulate the transient rise in $[Ca^{2+}]_i$ caused by $Fc_\epsilon RI$ aggregation. More recently, we have synthesized several cromolyn derivatives and conjugated them to macromolecules and insoluble supports, which were then employed in binding and affinity-based isolation experiments. Using one particular matrix of a number synthesized, we were able to isolate a novel glycoprotein (gp110) that in reconstituted planar lipid bilayers exhibited $Fc_\epsilon RI$-gated conductances, with high selectivity for $Ca^{2+}$ versus monovalent cations (Corcia et al., 1988; Hemmerich & Pecht, 1988). However, the structural requirements for the affinity of gp110 toward its isolating matrix were shown to involve more than just the bischromone nucleus. The nature of both the macromolecular carrier and in particular the linking spacer was shown to be crucial for the binding and isolation of this glycoprotein. In the course of the above studies we have found that a 72-kDa protein with kinase activity (p72) can be isolated from the cytoplasm of RBL cells, using matrices that have CG linked to agarose via a linear peptide spacer (Hemmerich and Pecht, unpublished observations). In contrast to gp110, this kinase exhibits affinity for the bischromone itself and could be eluted quantitatively with DSCG as a narrow band from the CG–agarose matrix. More recently, having sequenced the cDNA coding for the 18-kDa subunit of p72, it was identified by its homology and activity to be a nucleoside 5′-diphosphate kinase (S. Hemmerich, Y. Yarden, and I. Pecht, manuscript in preparation). Furthermore, the autophosphorylation of this enzyme, known to yield an intermediate of its catalytic cycle, was shown to be inhibited by CG. These observations raised the possibility that targets for CG, such as p72, may be present in the mast cell cytoplasm. In order to investigate this possibility, we designed a CG derivative that is able to passively traverse the plasma membrane and accumulate in the cell. Here we report the synthesis of such a diester of CG and its marked inhibitory effect on $Fc_\epsilon RI$-mediated stimulation of mucosal type mast cells of the line RBL-2H3.

## Materials and Methods

*Materials.* Disodium 1,3-bis[(2′-carboxylatochromon-5′-yl)oxy]-2-hydroxypropane and disodium [$^3$H]cromoglycate were generous gifts of Fisons plc, Loughborough, England. Bromomethyl acetate and *N,N*-diisopropylamine were obtained from Aldrich, Milwaukee, WI. [$^3$H]Serotonin (27 800 Ci/mol) and $^{45}CaCl_2$ were purchased from New England Nuclear, Boston, MA. *p*-Nitrophenyl β-D-2-acetamido-2-deoxyglucopyranoside for the β-hexosaminidase assay and compound 48/80 were obtained from Sigma, St. Louis, MO. Quin2/AM was purchased from Molecular Probes, Junction City, OR. Ionomycin was from Calbiochem, La Jolla, CA. Salts and other chemicals were from either Merck, Darmstadt, FRG, or BDH, Poole, England, and of best available grade. DNP$_{11}$BSA prepared by derivatization of bovine serum albumin (BSA, Sigma A-4503) with 1-fluoro-2,4-dinitrobenzene (Merck) was used for antigenic stimulations. Murine monoclonal IgE-class (2,4-dinitrophenyl)-specific antibodies, secreted by hybridoma cells (HI-DNP-ε-26.82) (Liu et al., 1980), were isolated as detailed elsewhere (Holowka & Metzger, 1982). Rat monoclonal IgG specific for determinants on the Fc portion of murine IgE (Baniyash & Eshhar, 1984) was a kind gift of Dr. Z. Eshhar, Department of Chemical Immunology, The Weizmann Institute of Science. Lumax and Permafluor III scintillation liquids were from Lumac, Landgraaf, The Netherlands, and from Packard, Downers Grove, IL, respectively. Tissue culture media were obtained from Bio-Lab, Ltd., Jerusalem, Israel.

*Analytical Procedures.* Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. NMR spectra were measured in $(CD_3)_2SO$ using a Bruker WH 270 NMR spectrometer. Fluorescence emission spectra were measured in a continuously stirred quartz cuvette (7 × 7 × 14 mm; sample volume, 1 mL) by use of a Perkin-Elmer fluorometer, PM4. For determination of β-radiation the respective samples were mixed with a 20-fold volume of Lumax/xylene (1/3 v/v) and counted in a Kontron BETAmatic liquid scintillation counter.

*Buffers.* Tyrode was 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $NaH_2PO_4$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4, and 0.2% BSA. Quenching buffer was 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. Buffer TM was 20 mM Tris-HCl and 10 mM $MgCl_2$, pH 8.0. BSSA was 154 mM NaCl, 2.7 mM KCl,

0.68 mM $CaCl_2$, 10 mM $NaH_2PO_4$, 10 mM $KH_2PO_4$ pH 7.2, and 0.1% BSA.

*Cells and Cell Culture.* Mast cells of the rat basophilic leukemia secreting line 2H3 (RBL-2H3) have been cultured in our laboratory for the last decade and originate at the National Institutes of Health, Bethesda, MD (Barsumian et al., 1981). The high-secretor subclone H24 selected from our cells was a kind gift of Dr. S. Ran, Department of Membrane Research, The Weizmann Institute of Science. The cells were grown in stationary flasks using minimal essential medium supplemented with 10% fetal calf serum, 2 mM sodium glutamate, and antibiotics (MEM 10% FCS). Upon reaching confluency, cells were detached by a 5-min exposure to 10 mM EDTA and either used for experiments or passed for expansion into larger flasks. The experiments described in the following were all performed with the H24 subclone, but the parental cells as well as a RBL-2H3 cells of different origins yielded qualitatively identical results.

*Organic Synthesis. (A) Bis(acetoxymethyl) Cromoglycate (CG/AM).* 1,3-Bis[(2′-carboxychromon-5′-yl)oxy]-2-hydroxypropane (cromoglycic acid) was obtained by titration of an aqueous solution of DSCG with 6 N HCl to pH <2. The voluminous precipitate was collected by centrifugation, washed three times with water, ethanol, and ether, and dried in vacuo over $P_2O_5$. The yield was quantitative: mp 254–256 °C; $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 7.71 [t, 2 H, benzoaromatic C(7′)H], 7.18 [d, 2 H, benzoaromatic C(6′)H], 7.1 [d, 2 H, benzoaromatic C(8′)H], 6.66 [s, 2 H, pyranoid C(3′)H], 4.6 (m, 1 H, CH of propane-2), 4.3 (d, 4 H, $CH_2$ of propane-1,3).

A 1020-mg (2.72-mmol) sample of cromoglycic acid (MW 468) was dissolved with 704 mg (5.45 mmol) of *N,N*-diisopropylethylamine and 1 g (6.54 mmol) of bromomethyl acetate in 10 mL of dry ethanol-free $CHCl_3$. This solution was stirred 24 h at 25 °C, followed by addition of 1 mL of MeOH and loading onto a 540 mm × 22 mm i.d. column of silica gel 60 (Merck, Darmstadt, FRG) equilibrated in $CHCl_3$/MeOH, 10/1 (v/v). The column was eluted with the same solvent system, and 5-mL fractions were collected. The diester eluted very close to the void volume, while monoester and unreacted diacid were strongly retarded. The fractions containing the diester were pooled and evaporated to near dryness at 25 °C. The oily residue was redissolved in 2 mL of $CHCl_3$ and added dropwise to 100 mL of ether with constant stirring. This yielded an almost colorless crystalline precipitate, which was collected on a glass filter, washed twice with cold ether, and dried in vacuo over $P_2O_5$. Yield, 800 mg (60%); mp 88–92 °C; TLC, $R_f$ = 0.54 in $CHCl_3$/MeOH, 25/1 (v/v); $^1H$ NMR [$(CD_3)_2SO$] $\delta$ 7.71 [t, 2 H, benzoaromatic C(7′)H], 7.18 [d, 2 H, benzoaromatic C(6′)H], 7.1 [d, 2 H, benzoaromatic C(8′)H], 6.66 [s, 2 H, pyranoid C(3′)H], 5.95 (s, 4 H, $OCH_2O$ of acetoxymethyl), 4.6 (m, 1 H, CH of propane-2), 4.3 (d, 4 H, $CH_2$ of propane-1,3), 2.16 (s, 4 H, $CH_3$ of acetoxymethyl).

*(B) [$^3H$]CG/AM.* A 3.5-$\mu$g (6.8-nmol) sample of disodium [$^3H$]cromoglycate (specific activity, 4.25 × $10^7$ dpm/nmol ≅ 16000 Ci/mol) was diluted with 10 mg (19.5 $\mu$mol) of unlabeled DSCG and converted into the bis(acetoxymethyl) ester essentially as described above. Yield, 5.1 mg (42.5%); specific activity 27300 dpm/nmol ≅ 10.3 Ci/mol.

*Uptake of [$^3H$]CG/AM by RBL Cells.* [$^3H$]CG/AM was dissolved in dry DMSO at 100 times the indicated final extracellular concentrations, and 20-$\mu$L aliquots of these stock solutions were diluted into 1 mL of Tyrode. The resulting solutions, which appeared opaque at [CG/AM] ≥ 100 $\mu$M, were added to 1 mL of cell suspension (2 × $10^6$ cells/mL) in the same buffer, either immediately (I) or following 1.5-h hydrolysis at 25 °C (II). This yielded suspensions of $10^6$ cells/mL containing 1% DMSO and [$^3H$]CG/AM at the indicated final concentrations. Following 30-min incubation with gentle shaking (25 °C), cells were sedimented, washed three times in Tyrode, and lysed in 100 $\mu$L of 10% aqueous Triton X-100, and the $\beta$-radiation in 50-$\mu$L lysate was determined. The counts measured in sample II (3% of those in the corresponding sample I) were assumed to represent adsorption of [$^3H$]cromoglycate to the cells and therefore subtracted. The resulting net counts were converted into intracellular CG concentrations by using a total cell volume of $10^{-6}$ $\mu$L (Fewtrell & Sherman, 1987).

The subcellular distribution of this cell-associated CG was examined in cells loaded with 2 mM [$^3H$]CG as follows: The washed cells were centrifuged once through a cushion of 15% sucrose in Tyrode (5 min, 1500 rpm), then washed twice in Tyrode, taken up in 0.5 mL of 0.25 M sucrose in Tyrode, and sonicated briefly (5 s) by means of a Kontes Micro Ultrasonic tip sonicator. The sonicate was layered on top of a discontinuous sucrose gradient in Tyrode (bottom, 40%; top, 15%) and spun for 45 min at 35 krpm. Thereafter, the top layer (cytosol), 15/40 interphase (microsomes), and pellet (organelles and cytoskeleton) were counted separately.

*Secretion Assays.* Secretion from RBL cells was assayed in duplicate or triplicate by quantitation of released granular mediators: either [$^3H$]serotonin or $\beta$-hexosaminidase. Though the standard deviations of the measured values from the mean were usually smaller in the determination of labeled serotonin (±2%) than of $\beta$-hexosaminidase activity (±5%), the results of both methods were similar.

The serotonin secretion assay was based on the protocol reported by Taurog et al. (1977): RBL cells were grown overnight to confluency in MEM 10% FCS supplemented with [$^3H$]serotonin (27800 Ci/mol, 2 $\mu$Ci/mL) and the monoclonal IgE-class antibody specific for the 2,4-dinitrophenyl (DNP) hapten (2 $\mu$L of ascites/mL). Then, cells were harvested, washed twice in Tyrode, and resuspended in this buffer to a concentration of 2 × $10^6$ cells/mL. Aliquots of these suspensions were treated with equal volumes of CG/AM freshly diluted into Tyrode (1 part 100-fold concentrated CG/AM stock solution in DMSO into 50 parts buffer), thus yielding suspensions of $10^6$ cells/mL in Tyrode containing 1% DMSO and CG/AM at the indicated final concentrations. Controls were done in Tyrode/1% DMSO without CG/AM. Following 30-min incubation with gentle shaking (25 °C), cells were sedimented, washed three times in Tyrode, resuspended in this buffer to a concentration of $10^6$ cells/mL, and dispensed as 100-$\mu$L aliquots into the wells of a 96-well microtiter plate.

Alternatively, RBL cells were plated into 96-well microtiter plates ($10^5$ cells/well) and grown overnight to confluency in MEM 10% FCS supplemented with [$^3H$]serotonin (27800 Ci/mol, 2 $\mu$Ci/mL) and monoclonal DNP-specific IgE (2 $\mu$L of ascites/mL). On the following morning, the medium in the wells was discarded and adherent cell monolayers were washed three times with 0.25-mL portions of Tyrode. Then 100 $\mu$L of CG/AM, freshly diluted from the 100-fold concentrated DMSO stock solution into Tyrode to the indicated final concentration, was added to each well. Following incubation for 30 min at 25 °C, supernatants were aspirated and monolayers washed three times with 0.25-mL portions of Tyrode.

The suspensions or adherent monolayers of CG-loaded cells were now challenged (in the microtiter plate wells) with the indicated final concentrations of either specific polyvalent antigen ($DNP_{11}BSA$) or monoclonal IgG specific for murine $\epsilon$-chain or ionomycin (added in 50- or 150-$\mu$L aliquots, re-

spectively, to the cell suspensions or monolayers). Following 30-min incubation at 37 °C, plates were centrifuged, and 100-μL aliquots of supernatants were taken for determination of their β-radiation. Total [$^3$H]serotonin content of the cells was determined following lysis with 1% Triton X-100, and all results are presented as percent of the total content.

β-Hexosaminidase activity was determined in these supernatants essentially as described elsehwere (Schwartz et al., 1979): 20-μL portions of the supernatants were transferred into the wells of a separate microtiter plate. To these samples was added 50 μL of substrate solution (1.3 mg/mL *p*-nitrophenyl β-D-2-acetamido-2-deoxyglucopyranoside in 0.1 M sodium citrate, pH 4.5), and the plates were incubated for 90 min at 37 °C. The reaction was stopped with 150 μL of 0.2 M glycine, pH 10.7. The *p*-nitrophenolate formed upon substrate hydrolysis was measured at 405 nm in an ELISA reader. The measured optical densities were converted into percent of total β-hexosaminidase content by comparison with the optical density produced by Triton X-100 lysates of the cells.

*Measurement of Intracellular Free Calcium Concentrations.* RBL cells sensitized with DNP-specific monoclonal IgE were washed and resuspended in Tyrode to a concentration of $2 \times 10^6$/mL. Following addition of 4 μM Quin2/AM (as 1 mM solution in DMSO) cells were incubated 30 min at 37 °C. Then, 2.5-mL aliquots of this suspension were treated with 2.5 mL of CG/AM freshly diluted into Tyrode (1 part 100-fold concentrated CG/AM stock solution in DMSO into 50 parts buffer), thus yielding 5 mL of cell suspension ($10^6$ cells/mL) in Tyrode containing 1% DMSO and CG/AM at the indicated final concentrations. After 15-min incubation with gentle shaking at 25 °C, cells were sedimented, washed three times with 10-mL portions of Tyrode, and resuspended in this buffer to $10^6$/mL. For each measurement, 1 mL of such a suspension was placed into a continuously stirred quartz cuvette (7 × 7 × 14 mm) thermostated at 37 °C in a PM4 Perkin-Elmer fluorometer. Following thermal equilibration, cells were stimulated with either specific antigen ($DNP_{11}BSA$ 10 ng/mL) or ionomycin (1 μM). Quin2 fluorescence intensity $F$ (excitation 339 nm, emission 492 nm) was recorded as function of time. Cells were lysed after the antigen-induced rise in fluorescence has leveled off (3–4 min) by addition of 0.5% Triton X-100 to obtain $F_{max}$. Then, the intrinsic non-Quin2-associated fluorescence $F_{min}$ was determined following addition of 150 μM $MnCl_2$. In order to examine possible Quin2 fluorescence quenching by CG, emission spectra were measured between 400 and 550 nm on resting cells loaded with Quin2 only and compared to those of cells loaded with both Quin2 and 4 mM (intracellular) CG. The observed practically identical spectra excluded any significant quenching. The concentration of the cytosolic free calcium ions ($[Ca^{2+}]_i$) was calculated as before (Gertler & Pecht, 1988) by using the equation $[Ca^{2+}]_i = [K_d(\text{Quin2})]\{(F - F_{min})/(F_{max} - F)\}$, with $K_d(\text{Quin2}) = 115$ nM.

*Measurements of $^{45}Ca^{2+}$ Uptake.* Experiments were performed on RBL monolayers in 24-well Costar plates ($3.5 \times 10^5$ cells/well) essentially as described (Fewtrell & Sherman, 1987). Following 30-min preincubation at 37 °C either with 100 μM Quin2/AM in Tyrode/1% DMSO or with this buffer only, the monolayers were incubated as described above with either 200 μM CG/AM in Tyrode/1% DMSO or buffer for 15 min at 25 °C. Following three washings with Tyrode, cells were challenged with specific antigen ($DNP_{11}BSA$) at the indicated concentrations in Tyrode supplemented with 20 μCi/mL $^{45}Ca^{2+}$ (prewarmed to 37 °C). After 4 min at 37 °C supernatants were aspirated. Monolayers were rapidly washed three times with 2-mL portions of ice-cold quenching buffer and lysed in 1 mL of 0.1% Triton X-100. Then the β-radiation of 0.5-mL lysate samples was determined, and the measured cpm were converted into nmol of $Ca^{2+}/10^6$ cells. All experiments were done in duplicate.

*Histamine Secretion from Rat Peritoneal Mast Cells.* Mast cells were harvested from the peritoneal cavity of Wistar rats and purified according to Lagunoff and Rickard (1987). For sensitization, the cells were incubated with monoclonal DNP-specific IgE (2 μL of ascites/mL) in buffer BSSA at 25 °C for 30 min. They were washed, resuspended in BSSA to $10^6$ cells/mL, and dispensed as 100-μL aliquots into a 96-well microtiter plate. CG/AM was added to yield the indicated final concentrations in 100 μL of BSSA/1% DMSO. Following 15-min incubation at 25 °C cells were sedimented, washed once with BSSA, resuspended in 50 μL of the same buffer, and incubated for 15 min at 37 °C. Cells were then challenged with either concanavalin A and phosphatidylserine, (5 and 50 μg/mL, respectively), compound 48/80 (5 μg/mL), or 3 μM ionomycin (added in 50 μL of BSSA). For determination of the cells' total histamine content, 50 μL of 2% aqueous Triton X-100 was added instead. After 15 min at 37 °C, cells were sedimented and 85-μL aliquots of the supernatants were taken for histamine determination by the *o*-phthaldialdehyde method of Shore et al. (1959). All experiments were done in duplicate, and results are given as percent of total histamine content.

*Inhibition of p72 Autophosphorylation by CG.* p72 was purified from RBL cytosol by affinity chromatography on Sepharose–poly(*N*-succinimidyl caproate) (activated CH-Sepharose, Pharmacia 17-0490-01) derivatized with 1,3-bis-[(2′-carboxycromon-5′-yl)oxy]-2-(6″-aminocaproamido)-propane [(aminocaproamido)cromolyn, 18 μmol of ligand/mL of gel matrix; Hemmerich & Pecht, 1988]. This procedure and the detailed structural and biochemical characterization of p72 as a nucleoside diphosphate kinase will be described elsewhere (S. Hemmerich, Y. Yarden, and I. Pecht, manuscript in preparation). Formation of a phosphoprotein intermediate has been shown to be characteristic for this enzyme (Parks & Agarwal, 1973). Inhibition of p72 autophosphorylation by CG was measured as follows: In each assay 1 μM p72 was incubated with the indicated concentrations of CG in 40 μL of buffer TM for 15 min at 25 °C, followed by addition of 100 μM [$^{35}$S]ATPγS and further incubation for 30 min at 25 °C. Each sample (40 μL) was then diluted with 1 mL of ice-cold TM and filtered rapidly through 25 mm i.d. nitrocellulose filters (Schleicher & Schüll BA85). The filters were washed four times with 2-mL portions of cold TM and dried. They were placed into 10 mL of Permafluor III/xylene (1/9 v/v) and counted (the counter had been calibrated by measuring the cpm of filters soaked with defined amounts of [$^{35}$S]ATPγS). Each data point was done in triplicate and its average radioactivity converted into $[\text{thiophosphate}]_{bound}$.

## Results

*Synthesis of Bis(acetoxymethyl) Cromoglycate.* By analogy to the series of cell-permeant fluorescent $Ca^{2+}$ or pH indicators developed by Tsien (Tsien, 1981; Tsien et al., 1982), we chose to neutralize the negative charges of the two carboxylates in the CG molecule by esterification with acetoxymethyl residues. These have been shown to be promptly hydrolyzed by cytoplasmic esterases (Thomas et al., 1979). Bis(acetoxymethyl) cromoglycate (CG/AM, Figure 1) was prepared by alkylation of cromoglycic acid with bromomethyl acetate as described

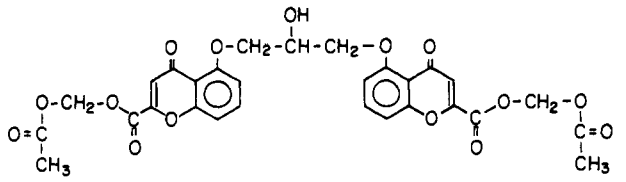

FIGURE 1: Structural formula of bis(acetoxymethyl) cromoglycate (CG/AM).

under Materials and Methods. The compound is soluble in DMSO and DMF yet has only a limited solubility in ethanol and practically none in water. Its NMR spectrum is consistent with the structure depicted in Figure 1. Hydrolysis of the compound at pH 7.4 was monitored by measuring fluorescence emission spectra of a $10^{-4}$ M solution in Tyrode buffer at 25 °C between 340 and 600 nm (excitation wavelength, 325 nm) at the time intervals indicated in Figure 2: Unlike CG/AM, the product of its hydrolysis, namely, the CG dianion, exhibits a fluorescence emission with a maximum at 480 nm (Figure 2a). The increase in fluorescence as a function of time (Figure 2b) could be fitted well to an exponential function yielding a hydrolysis rate constant of 0.026 ± 005 $min^{-1}$ under these conditions.

*Accumulation of CG/AM in RBL Cells.* A radioative derivative of CG/AM, in which two hydrogens at each benzoaromatic nucleus are replaced by tritium, was synthesized from the respectively labeled cromoglycic acid, and its uptake by RBL cells (incubation time, 30 min) was monitored. Cytoplasmic CG concentrations were measured as a function of the extracellular CG/AM concentrations. The dose dependence was linear with no saturation observed within the examined (5–100 μM) concentration range yielding 40-fold higher cytosolic CG concentrations than the extracellular [CG/AM]. Lysis and fractionation of these [$^3$H]CG-loaded cells showed that 95% of the cell-associated radioactivity is in the cytosol, while 2% and 3% were found in the microsomal and organelle/cytoskeleton fractions, respectively.

The uptake of CG/AM by RBL cells was independently monitored by comparing the fluorescence emission spectra of cell suspensions that had been incubated with $10^{-4}$ M of either CG/AM or CG for 30 min, and subsequently washed, with those of untreated controls. As evident in Figure 3, only CG/AM-treated cells displayed the CG-associated fluorescence emission maximum at 480 nm. Since CG/AM itself is hardly fluorescent at this wavelength (Figure 2a), this result is further proof that the compound is trapped in the cells due to its conversion to the (fluorescent) CG dianion.

*Effect of CG/AM on Cell Viability.* Immediate effects of CG/AM on the viability of RBL cells were determined by Trypan Blue exclusion: Cell suspensions were counted in the presence of the dye before and after a 30-min incubation with CG/AM at 25 °C. Viability of the cells was always found >98% following exposure to extracellular CG/AM concentrations up to 200 μM. In order to assess possible delayed cytotoxicity, CG-loaded cells were grown overnight in monolayer culture and, on the following morning, both their viability and secretion capacity were examined (by Trypan Blue exclusion and antigen-induced β-hexosaminidase release, respectively). While cells treated with up to 50 μM CG/AM grew as fast as the untreated control cells, those having been exposed to 100 μM CG/AM multiplied only half as fast and cell death amounted to about 10%. Their secretion was found to be identical with that of cells treated with CG/AM for short terms (with respect to both its antigen dependence and maximal value). However, in cells loaded with CG to a higher extent (incubated with ≥200 μM CG/AM) the observed death rate at this time was >90%.

*CG/AM Inhibition of the $Fc_\epsilon RI$-Mediated Secretion from RBL Cells.* Secretion was assayed either in suspensions or with adherent monolayers of CG-loaded RBL cells as described under Materials and Methods. The results of a typical set of experiments done on cell suspensions are presented in Figure 4. While CG/AM at an extracellular concentration of 50 μM inhibited the release by ~70%, increasing extracellular CG/AM concentration to ≥100 μM blocked secretion completely. DSCG at these and also higher concentrations (up to 10 mM) was found to be ineffective under these conditions. When adherent cell monolayers were employed in such experiments, the extracellular CG/AM concentrations required in order to achieve the same degree of inhibition were about 1.5-fold higher (Figure 5; extracellular [CG/AM] concentration required for 50% of maximal inhibition, $I_{50}$, ≈ 60 μM). In contrast, the secretion elicited by the calcium ionophore ionomycin from CG/AM-loaded cells was shown to be be-

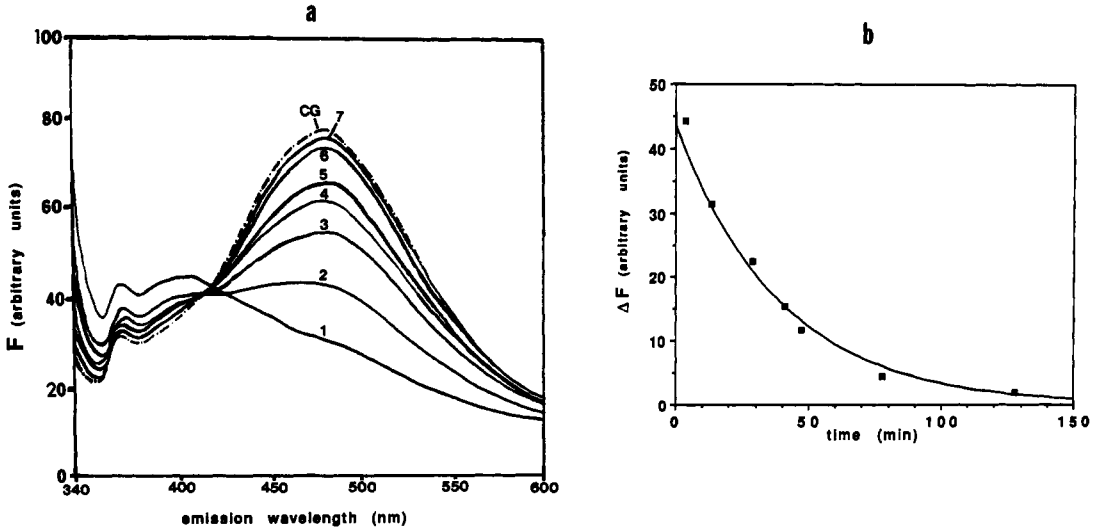


FIGURE 2: Nonenzymatic hydrolysis of CG/AM at pH 7.4. (a) CG/AM (10 mM in DMSO) was diluted at time zero into Tyrode buffer (pH 7.4) to $10^{-4}$ M final concentration. Then fluorescence emission spectra (excitation wavelength, 325 nm) were recorded following 3.8 min (1), 13.5 min (2), 29.0 min (3), 41 min (4), 47 min (5), 78 min (6), and 128 min (7). The emission spectrum of $10^{-4}$ M DSCG in Tyrode was recorded as well (CG). (b) $\Delta F$ (defined as $F_{480\,nm}$ of CG minus $F_{480\,nm}$ of CG/AM at pH 7.4) as a function of time. The best-fit exponential function yielded a hydrolysis rate constant of 0.026 $min^{-1}$.

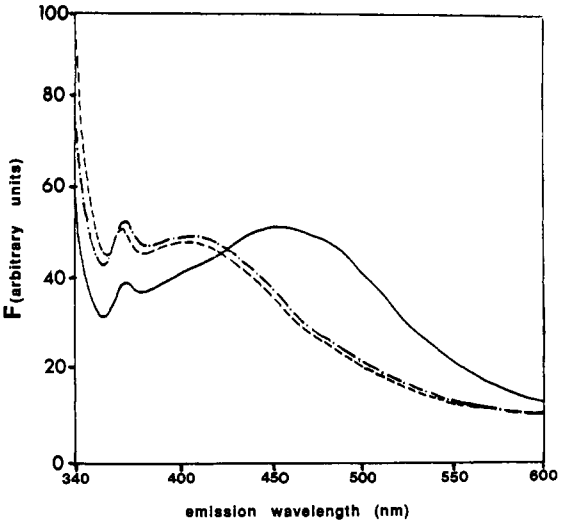


FIGURE 3: Uptake and hydrolysis of CG/AM by RBL cells monitored by fluorescence. Cell suspensions ($10^6$ cells/mL) were incubated with 100 $\mu$M extracellular CG/AM or CG in Tyrode/1% DMSO for 30 min at 25 °C. Cells were then washed three times and resuspended in Tyrode and their emission spectra recorded. Fluorescence emission spectra (excitation wavelength, 325 nm) of the cells, incubated with 100 $\mu$M of either CG/AM (—) or CG (---) as compared to untreated cells (-·-), were recorded.

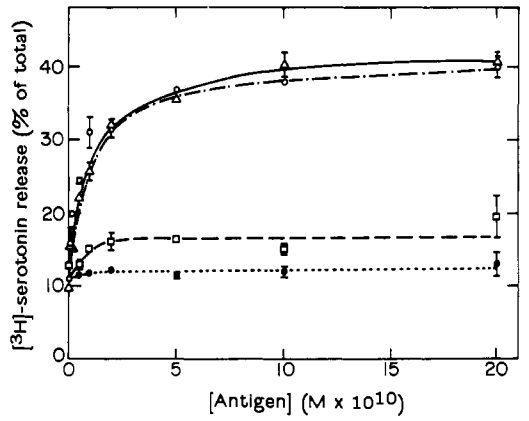


FIGURE 4: Inhibition by CG/AM of the $Fc_\epsilon RI$-mediated [$^3$H]serotonin release from RBL cell suspensions. Cells loaded with [$^3$H]serotonin and sensitized with IgE specific for the DNP hapten were suspended in Tyrode/1% DMSO ($10^6$ cells/mL) and incubated for 30 min with the indicated concentrations of CG/AM at 25 °C. Then, cells were washed and challenged with the indicated molar concentrations of specific polyvalent antigen ($DNP_{11}BSA$, $M_r \sim 67K$). Following 30-min incubation at 37 °C the cells were sedimented, and [$^3$H]-serotonin content in the supernatants was determined and normalized to the total serotonin content of the cells as determined in Triton X-100 lysates. The data presented are averages of duplicates and were obtained in a typical experiment out of a series of four independent ones. (▲) Control; (○) 200 $\mu$M DSCG; (□) 50 $\mu$M CG/AM; (■) 200 $\mu$M CG/AM.

tween 2- and 3-fold higher than that observed with untreated control cells (Figure 6).

*CG/AM Inhibition of both the $Fc_\epsilon RI$-Mediated Quin2 Signal and Net $^{45}Ca^{2+}$ Uptake in RBL Cells.* In order to examine whether the intracellular CG interferes with the stimulus transduction at a stage prior to or after the $Fc_\epsilon RI$-mediated transient rise of the cytosolic free calcium ion concentration, $[Ca^{2+}]_i$ was monitored in cells loaded with both CG and the fluorescent $Ca^{2+}$ indicator Quin2 upon stimulation with either specific polyvalent antigen ($DNP_{11}BSA$) or calcium ionophore (ionomycin). Prior to stimulation, fluorescence emission spectra of such cells were measured (treated with 100 $\mu$M CG/AM) and compared to those of cells loaded with

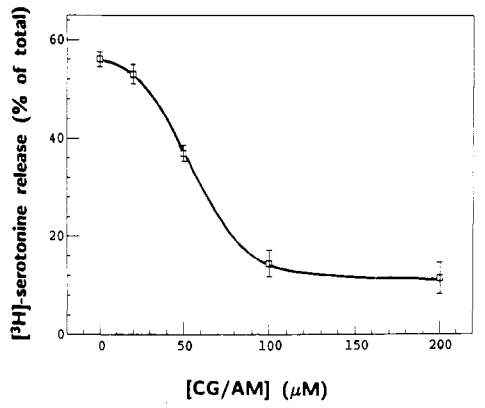


FIGURE 5: Inhibition of the $Fc_\epsilon RI$-mediated [$^3$H]serotonin release from RBL adherent monolayers. Confluent monolayers of cells loaded with [$^3$H]serotonin, sensitized with DNP-specific IgE, and adherent to the flat bottoms of the wells of a microtiter plate ($\sim 10^5$ cells/well) were incubated 30 min with the indicated concentrations of CG/AM at 25 °C. Then, monolayers were washed and challenged with the 67 ng/mL (1 nM) $DNP_{11}BSA$ (30 min, 37 °C). [$^3$H]Serotonin released into the supernatants was determined as described in the legend to Figure 3. The data presented are averages of duplicates and were obtained in a typical experiment out of a series of three independent ones.

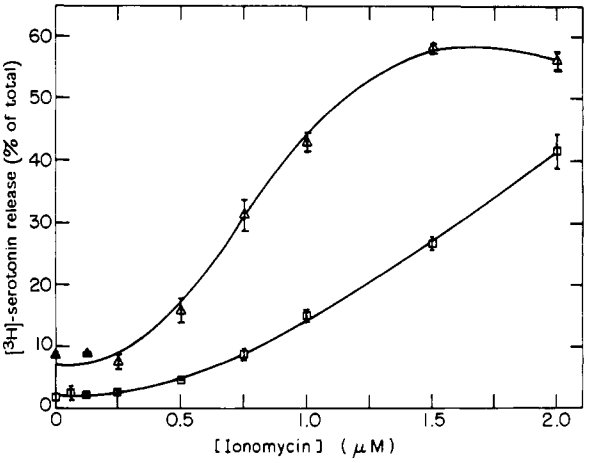


FIGURE 6: Ionophore-induced secretion of CG/AM-loaded RBL cells compared with untreated control cells. Ionomycin-induced [$^3$H]-serotonin release was measured in confluent RBL monolayers as described above for the antigen-induced release (legend to Figure 5). These data are averages of duplicates from a typical experiment out of a series of three independent ones. (▲) Cells incubated 30 min at 25 °C with 200 $\mu$M CG/AM; (□) untreated cells.

Quin2 only. In both samples, quantum yields at the emission maximum of Quin2 (492 nm) were found identical within a range of ±5%. As illustrated in Figure 7, the antigen-induced transient elevation of $[Ca^{2+}]_i$ as monitored by Quin2 was found to be markedly suppressed by CG/AM in a dose-dependent manner. Inhibition was already observed at 10 $\mu$M and complete abrogation was reached at ≥50 $\mu$M extracellular CG/AM concentration. Significantly, the half-time required for the antigen-induced Quin2 signal to reach its maximal value was also found to be prolonged in CG-loaded cells (Figure 7, insert). In cells treated with higher CG/AM concentrations (>50 $\mu$M) the ionomycin-induced transient rise in $[Ca^{2+}]_i$ was suppressed too. However, as evidenced by data shown in Figure 6, this inhibition was considerably less pronounced and was never complete under these conditions.

The $Fc_\epsilon RI$-mediated net $^{45}Ca^{2+}$ uptake was measured on adherent monolayers of CG-loaded and compared to control cells according to reported protocols (Crews et al., 1981; Kanner & Metzger, 1984) as described under Materials and Methods. In parallel experiments, these cells were loaded with

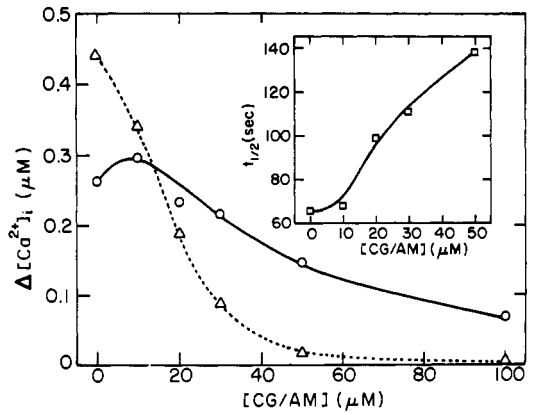


FIGURE 7: Inhibition of the transient rise in $[Ca^{2+}]_i$ by CG/AM. RBL cells sensitized with DNP-specific monoclonal IgE were incubated 30 min with 4 μM Quin2/AM, and afterward for another 15 min (25 °C) with the indicated CG/AM concentrations ([CG/AM]) in Tyrode/1% DMSO. Following three washes, cells were resuspended to $10^6$/mL in Tyrode. For each measurement 1 mL of such a suspension was placed into a continuously stirred quartz cuvette (7 × 7 × 14 mm) thermostated at 37 °C in a Perkin-Elmer fluorometer, MPF4. Following thermal equilibration, cells were stimulated with either specific antigen ($DNP_{11}BSA$, 10 ng/mL) (Δ) or ionomycin (1 μM) (O). Quin2 fluorescence, *F* (excitation 339 nm, emission 492 nm), was recorded as function of time and translated into concentration of cytosolic free calcium as described under Materials and Methods. In the main panel the the net rise of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) observed after addition of stimulant is plotted versus [CG/AM]. In the insert, the time required for $\Delta[Ca^{2+}]_i$ to reach 50% of its maximal level ($t_{1/2}$) is plotted also versus [CG/AM]. Pooled data of two independent experiments were used to construct these plots.

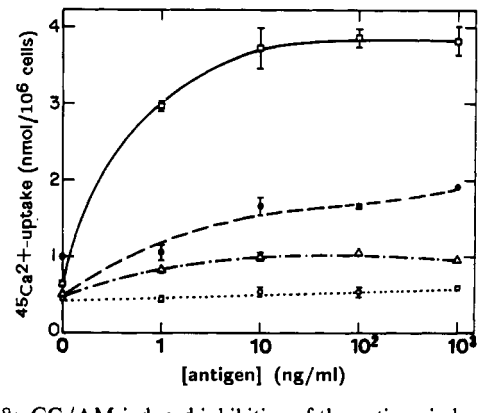


FIGURE 8: CG/AM-induced inhibition of the antigen-induced $^{45}Ca^{2+}$ uptake by RBL cells. Adherent cell monolayers grown in 24-well Costar plates (3.5 × $10^5$ cells/well), sensitized with IgE specific for the DNP hapten, and either loaded with 4 mM intracellular Quin2 (□, ●) or untreated (Δ, O) were incubated with 200 μM CG/AM in Tyrode/1% DMSO (●, O) or with buffer only (□, Δ) for 15 min at 25 °C. Then cells were washed and challenged with specific antigen ($DNP_{11}BSA$) at the indicated concentrations in Tyrode supplemented with 20 μCi/mL $^{45}Ca^{2+}$ (prewarmed to 37 °C). After 4 min at 37 °C supernatants were aspirated. Monolayers were washed rapidly with ice-cold quenching buffer and lysed in 0.1% Triton X-100. Lysate aliquots were counted, and the measured cpm were converted into nmol of $Ca^{2+}/10^6$ cells. The data shown are averages of duplicates measured in a typical experiment out of a series of three independent ones.

Quin2 to intracellular concentrations which cause buffering of $[Ca^{2+}]_i$ [100 μM extracellular Quin2/AM, 30 min, 37 °C, resulting in 4 mM intracellular Quin2 (Fewtrell & Sherman, 1987)]. In Figure 8 a comparison is shown between the $^{45}Ca^{2+}$ uptake in CG-loaded and untreated RBL cell monolayers either with or without buffering of $[Ca^{2+}]_i$ by Quin2. Preincubation for 15 min with 200 μM CG/AM caused a practically complete elimination of the antigen-induced contribution to the $^{45}Ca^{2+}$ uptake.

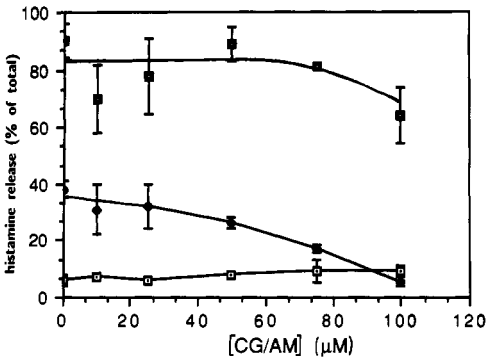


FIGURE 9: CG/AM inhibition of histamine secretion by rat peritoneal mast cells. Suspensions ($10^6$ cells/mL) of purified rat peritoneal mast cells sensitized with a monoclonal IgE (specific for the DNP hapten) were incubated for 15 min (25 °C) with the indicated final concentrations of CG/AM in Tyrode/1% DMSO. Cells were then washed once, incubated for 15 min at 37 °C, and challenged with either concanavalin A (Con A) and phosphatidylserine (◆) (5 and 50 μg/mL, respectively) or compound 48/80 (□) (5 μg/mL). Basal histamine release was also determined (⊡). Following 15 min at 37 °C cells were sedimented, and histamine content in the supernatants was determined and normalized to the total histamine content of the cells as measured in Triton X-100 lysates. The depicted data are averages of duplicates and were obtained all from the same rat peritoneal mast cell preparation.

*CG/AM Inhibition of Mediator Secretion in Rat Peritoneal Mast Cells.* Histamine secretion from sensitized rat peritoneal mast cells preincubated with CG/AM was measured as described under Materials and Methods. Following 15-min preincubation with the drug at 25 °C, cells were challenged either by concanavalin A (Con A) together with phosphatidylserine (5 and 50 μg/mL), by compound 48/80 (5 μg/mL), or by ionomycin (3 μM). The histamine release observed at various extracellular CG/AM concentrations is shown in Figure 9. The 48/80-induced secretion, known to be independent of extracellular calcium ions (Gomperts & Fewtrell, 1985), was only moderately inhibited (≤20%). A similar slight inhibition of their secretion was also observed in CG-loaded mast cells stimulated by ionomycin (data not shown). By contrast, secretion elicited by ConA, which is assumed to cause IgE–$Fc_\epsilon RI$ aggregation, was found to be markedly inhibited and was completely abolished at extracellular [CG/AM] ≥ 100 μM. The dose–response function of this inhibition ($I_{50}$ ≈ 60 μM extracellular CG/AM) was similar to that observed in RBL cells. Within a series of five independent experiments, the measured extent of ConA-induced histamine secretion ranged between 20% and 40%, probably reflecting variations in the quality of the different mast cell preparations. However, the CG/AM-induced inhibition was qualitatively always similar. Figure 9 presents the data obtained with the mast cell preparation that yielded the highest ConA-elicited histamine release.

*Inhibition of p72 Autophosphorylation by CG at Millimolar Concentrations.* We have recently cloned and sequenced the cDNA coding for the 18-kDa subunit of p72 (S. Hemmerich, Y. Yarden, and I. Pecht, manuscript in preparation). The sequence showed very extensive homology with those of the nucleoside diphosphate kinases (EC 2.7.4.6) isolated from a bacterium as well as with analogous human and mouse proteins (Rosengard et al., 1989; Munoz-Dorado et al., 1990). As part of the functional characterization of p72, we examined the effect of CG on the autophohorylation of purified p72 with ATPγS as substrate. Nucleoside diphosphate kinases are known to undergo autophosphorylation as part of their catalytic cycle (Parks & Agarwal, 1973). At 100-fold molar substrate excess, p72 was shown to incorporate 2 mol of

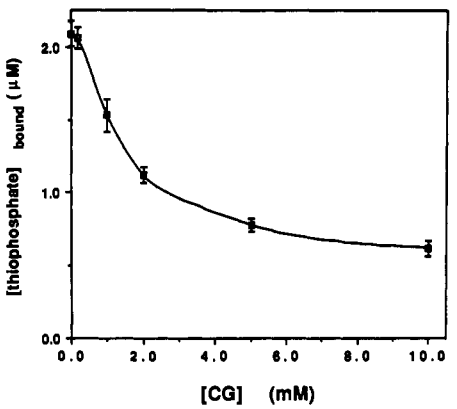


FIGURE 10: Inhibition of p72 autophosphorylation by CG. Affinity-purified p72 (1 μM) was preincubated with DSCG (30 min, 25 °C) at the indicated concentrations and then reacted with 100 μM [$^{35}$S]ATPγS (1550 Ci/mol). The thiophosphorylated protein was subsequently adsorbed on nitrocellulose filters and its β-radiation determined. The figure presents concentrations of protein-bound thiophosphate as function of [CG]. The data are averages of triplicates with standard deviations as indicated.

thiophosphate/mol of protein, requiring no additional cofactors but $Mg^{2+}$ ions. The results, summarized in Figure 10, show that this phosphorylation is inhibited in a dose-dependent manner by CG. The observed $I_{50}$ (2 mM) is similar to that observed for the inhibition of $Fc_\epsilon R$-mediated secretion by intracellular CG.

## Discussion

Identification of the cellular components that couple $Fc_\epsilon$ receptor aggregation with the resultant transient increase in $[Ca^{2+}]_i$ is essential for an understanding of the mast cell stimulus–secretion coupling cascade in molecular terms. This goal requires the design and preparation of appropriate reagents that interact and distinguish between the effector molecules mediating the various, often parallel, signal transduction pathways in mast cells. The central role of a transient rise in $[Ca^{2+}]_i$ in $Fc_\epsilon RI$-mediated exocytosis is amply documented (Beaven et al., 1984; Foreman et al., 1983, 1977a). This so-called "$Ca^{2+}$ signal" is considered to be produced both by influx of calcium from the exterior through $Fc_\epsilon RI$-gated ion channels and by inositol triphosphate mediated release of these ions from intracellular stores (Gomperts & Fewtrell, 1985). In the widely studied RBL-2H3 line of rat mucosal mast cells, the contribution of $Ca^{2+}$ influx to the rise in $[Ca^{2+}]_i$ was shown to be essential and dominant (Beaven et al., 1984; Kanner & Metzger, 1984; Mohr & Fewtrell, 1987).

We have examined the antiallergic drug cromolyn as a potential ligand probing for components involved in the coupling of $Fc_\epsilon RI$ aggregation to the $Ca^{2+}$ influx process, because of its well-established inhibition of the antigen-induced net $Ca^{2+}$ uptake and secretion in rat peritoneal mast cells (Cox, 1977; Foreman et al., 1977b; Sagi-Eisenberg, 1985). In RBL cells as in other mucosal-type mast cells, this drug failed to show an inhibitory effect on secretion (Pearce, 1986). Recently, a cytoplasmic nucloside 5′ diphosphate kinase has been isolated from RBL-2H3 cells utilizing CG as an affinity chromatography element (S. Hemmerich, Y. Yarden, and I. Pecht, in preparation). This observation raised the possibility that intracellular targets may exist for cromolyn that are inaccessible for the membrane-impermeant drug. We have therefore synthesized bis(acetoxymethyl) cromoglycate (CG/AM), shown it to be a membrane-permeant cromolyn derivative, and studied its upake by and modulation of mediator secretion from RBL cells. The compound accumulated in these cells due to its conversion into the membrane-impermeant CG dianion, yielding cytoplasmic concentrations 40-fold higher than in the extracellular medium. This accumulation factor is practically the same as that reported for the $Ca^{2+}$ indicator Quin2/AM [0.1 mM extracellular Quin2/AM results in 4 mM cytoplasmic Quin2 (Fewtrell & Sherman, 1987)], the membrane permeation properties of which are also provided by esterification of carboxylates with acetoxymethyl moieties.

The $Fc_\epsilon RI$-mediated secretion of RBL-cells was effectively inhibited by CG/AM, in a dose-dependent manner ($I_{50} \approx 40$ μM extracellular CG/AM, yielding ~1.6 mM cytoplasmic CG). In cell monolayers, the observed $I_{50}$ was higher (60 μM), probably reflecting limited accessibility of the drug to these cells. By contrast, the ionophore-induced secretion was not inhibited but rather augmented. This suggests that the exocytotic coupling cascade distal to the increase in $[Ca^{2+}]_i$ remains unperturbed at cytoplasmic [CG] as high as 8 mM.

The molar amounts of $H^+$ and formaldehyde produced upon intracellular hydrolysis of Quin2/AM are twice those produced from CG/AM. Still, cells loaded with as much as 7 mM intracellular Quin2 were found to secrete normally (Beaven et al., 1984). Cytotoxicity of CG/AM was observed in cells loaded with >4 mM cytoplasmic CG only after >12 h in culture. It is relevant to compare the effects of Quin2/AM in RBL-2H3 cells and in other cell types. Uptake of this indicator by RBL-2H3 cells yielding intracellular concentrations as high as 20 mM was reported to be noncytotoxic (Fewtrell & Sherman, 1987). In the latter study, however, viability was apparently monitored over short time periods (by Trypan Blue exclusion and leakage of lactate dehydrogenase). Thus, although we observe long-term decrease in viability effected by a high CG load, our experiments certainly involve less perturbation than studies of secretion from permeabilized mast cells (Howell et al., 1987). Exocytosis of the latter cells has recently been reported to occur in the presence of metabolic inhibitors even without an ATP requirement (Tatham & Gomperts, 1989). These results suggest that cell viability is not prerequisite for the function of at least part of the secretory cascade.

The observed rise in secretion of CG-loaded cells in response to ionophores suggested that the drug affects a step prior to the antigen-induced transient rise in $[Ca^{2+}]_i$ in the stimulus–secretion coupling. Therefore, by use of Quin2, $[Ca^{2+}]_i$ levels were measured as function of time following stimulation in CG-loaded cells and compared with unloaded control cells. The close proximity of the CG absorption maximum at 325 nm to the Quin2 excitation band (339 nm) raised the possibility of quenching Quin2 fluorescence by the chromone. However, the quite similar quantum yields of Quin2 emission observed in both resting CG-loaded cells and control cells apparently rule out this possibility. The $Fc_\epsilon RI$-mediated transient $[Ca^{2+}]_i$ elevation was found to be inhibited by the drug with a dose–response pattern very similar to that observed for the inhibition of mediator release.

Several different causes may be considered for the observed suppression of the $Fc_\epsilon RI$-mediated $[Ca^{2+}]_i$ rise in CG-loaded RBL: (1) blockade of $Ca^{2+}$ influx; (2) inhibition of $Ca^{2+}$ release from internal depots; (3) buffering of $[Ca^{2+}]_i$ by CG, earlier shown to have an affinity for $Ca^{2+}$ ions; (4) combinations of these processes. In order to examine these possibilities, we measured $^{45}Ca^{2+}$ uptake rates during the first 4 min following antigen stimulation in CG/AM-loaded cells and compared these with the rates observed in unloaded cells. $^{45}Ca^{2+}$ uptake has routinely been used to monitor changes in

$Ca^{2+}$ permeability of the plasma membrane, and initial uptake rates are generally assumed to represent the true unidirectional $Ca^{2+}$ influx into the cells (Wollheim & Sharp, 1981) probably unaffected by $[Ca^{2+}]_i$. However, the measured uptake is usually found to be small in terms of cpm and superimposed on a substantial basal contribution. Extrusion of $^{45}Ca^{2+}$ by calcium pumps and exchangers also tends to diminish the experimentally measured uptake, in particular, at longer incubation periods. Therefore, in a series of $^{45}Ca^{2+}$ uptake measurements we made use of the protocol designed by Fewtrell and Sherman (1987) and buffered $[Ca^{2+}]_i$ by loading the RBL cells with the high-affinity chelator Quin2 ($K_d \approx 150$ nM). In Quin2-loaded cells, the net $Ca^{2+}$ uptake required for causing the same rise in $[Ca^{2+}]_i$ is markedly higher than in unbuffered cells, with a respective reduction in the error caused by $^{45}Ca^{2+}$ extrusion. Thus we measured, at maximal antigen stimulation, a net uptake of $3.3 \pm 0.2$ nmol of $Ca^{2+}/10^6$ cells in RBL loaded with 4 mM Quin2. This value is similar to that reported in the above-mentioned study (Fewtrell & Sherman, 1987). Without Quin2 buffering, we observed a net uptake of $0.45 \pm 0.085$ nmol of $Ca^{2+}/10^6$ cells $\cong 4.5 \times 10^{-16}$ mol of $Ca^{2+}$/cell, resulting in a $[Ca^{2+}]_i$ rise of 0.45 $\mu$M [cell volume $\sim 10^{-6}$ $\mu$L (Fewtrell & Shermann, 1987)]. Practically the same increase in $[Ca^{2+}]_i$ was determined independently by the Quin2 measurements (Figure 7), indicating that $Ca^{2+}$ influx accounts for a considerable fraction of all the $Fc_\epsilon RI$-mediated calcium signal. This observation is consistent with the manifold evidence that, in RBL cells, $Ca^{2+}$ influx across the plasma membrane through $Fc_\epsilon RI$-gated channels is a major transduction element of the immunological stimulus–secretion coupling (Beaven et al., 1984; Mohr & Fewtrell, 1987). Exposure of both Quin2-loaded and unloaded cell monolayers to 200 $\mu$M CG/AM for 15 min at 25 °C resulted in complete abrogation of the antigen-induced contribution to the $^{45}Ca^{2+}$ uptake (Figure 8).

The above results, in particular those of the $^{45}Ca^{2+}$ uptake experiments, indicate that intracellular CG interferes with process(es) that couple the $Fc_\epsilon$ receptor aggregation with the $Ca^{2+}$ uptake. The action of this drug at a step prior to the elevation of $[Ca^{2+}]_i$ is clearly suggested by the parallel dose–response patterns observed for both the Quin2 signal and secretion. Moreover, the observed inhibition of the $Fc_\epsilon RI$-mediated net $^{45}Ca^{2+}$ uptake, even in cells heavily loaded with the $Ca^{2+}$ buffer Quin2, underscores the notion that the drug impairs the antigen-induced influx of $Ca^{2+}$ ions. The failure of CG/AM to inhibit the polycation stimulation of rat peritoneal mast cells, which does not require external $Ca^{2+}$ (Gomperts & Fewtrell, 1985), is also in line with the above rationale.

The cDNA coding for the 18-kDa subunit of p72 isolated by afffinity chromatography on a CG matrix has very recently been cloned and sequenced (S. Hemmerich, Y. Yarden, and I. Pecht, manuscript in preparation). The determined nucleotide sequence was found to have extensive homology with all nucleoside diphosphate kinase sequences known so far (Munoz-Dorado et al., 1990; Rosengard et al., 1989). Autophosphorylation of this protein has also been established as an intermediate in the catalytic cycle of this type of enzyme (Parks & Agarwal, 1973), and as reported here, cromolyn is indeed found to be an inhibitor of that autophosphorylation activity of p72 with an $I_{50}$ of 2 mM. This falls within the range of the cytoplasmic cromolyn concentrations shown above to produce the inhibition of $Fc_\epsilon RI$-mediated stimulation of RBL-2H3 cells. This enzyme could thus be a potential cytosolic target for CG activity. Nucleoside diphosphate kinase is assumed to be a major element in the synthesis of nucleotides triphosphates (Parks & Agarwal, 1973; Kimura & Shimada, 1988). Inhibition of this kinase may in turn affect the cellular GTP/GDP balance and therefore the function of G-proteins. Hence it is not surprising that such marked interference with the $Fc_\epsilon RI$-mediated stimulation is observed.

The significant differences among serosal mast cells (both in subtypes and among species) in terms of their pharmacological sensitivity to (extracellularly applied) DSCG were mentioned earlier. Maximal inhibition of $Fc_\epsilon RI$-mediated secretion by the drug ranges from 80% in rat peritoneal mast cells to none in all murine serosal subtypes (Pearce, 1986). Moreover, the DSCG inhibition of rat peritoneal mast cells appears to be strongly dependent on details of the stimulation protocol (Krüger & Bloom, 1979). In clear contrast, mucosal type mast cells are apparently always nonresponsive to the drug (Pearce et al., 1982). Thus, results of the experiments described in this report suggest that the cell-permeant CG/AM interferes with $Fc_\epsilon RI$-mediated secretion by inhibiting the nucleoside 5′-diphosphate kinase activity. As stated above, this would probably affect the cellular nucleotide triphosphate concentrations, and hence such activities as GTP-dependent pathways would be impaired (Howell et al., 1987; Penner et al., 1988). The mechanism by which the nonpermeant DSCG inhibits $Fc_\epsilon RI$-mediated secretion in serosal mast cells is most probably not related to the former and still remains unknown.

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