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PHOSPHORYLATION OF A MAST CELL PROTEIN IN RESPONSE TO TREATMENT WITH ANTI-ALLERGIC COMPOUNDS

IMPLICATIONS FOR THE MODE OF ACTION OF SODIUM CROMOGLYCATE

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Abstract—Challenge of rat peritoneal mast cells with anti-rat IgE induces a similar pattern of protein phosphorylation to that already reported for compound 48/80. Rapid phosphorylation of a mast cell protein, mol. wt 78,000, is induced by sodium cromoglycate and several chemically related anti-allergic agents in the absence of any challenge. Phosphorylation of this protein reflects their potency in inhibiting anti-IgE-induced histamine release. Compounds which inhibit histamine release by elevating intracellular cAMP levels do not induce phosphorylation. However, dibutyryl-cGMP induces phosphorylation of the 78,000 mol. wt protein in the absence of any challenge, at concns which inhibit IgE-dependent histamine release. Sodium cromoglycate appears to activate an endogenous control mechanism for switching off mediator release using a mechanism mediated by cGMP.

Since its introduction in 1968, sodium cromoglycate has been widely used in the prophylatic treatment of bronchial asthma. Experimentally, sodium cromoglycate has been shown to inhibit the release of mediators from rat mast cells induced by a variety of immunologic and non-immunologic stimuli [1, 2], and antigen-induced mediator release from chopped human lung [3]. Since secretion of mediators by mast cells is believed to be of clinical importance in allergic diseases [4], as well as being a good model for stimulus–secretion coupling, much work has been undertaken in an attempt to elucidate the mechanism of sodium cromoglycate action on mast cells.

It has been reported [5] that when rat mast cells are challenged with compound 48/80 or the Ca^{2+}-ionophore A23187, phosphorylation of three proteins of mol. wt 68,000, 59,000 and 42,000 was evident within 10 sec. Phosphorylation of a fourth protein, mol. wt 78,000, was not evident until 30–60 sec after challenge with compound 48/80. Many biochemical processes are regulated by protein phosphorylation [6] and it was suggested that the early rapid phosphorylation of the three proteins might be involved in the initiation of secretion, while the late phosphorylation of the 78,000 mol. wt protein might be associated with the termination of secretion and restabilisation of the cell.

A subsequent publication [7] showed that treatment of rat mast cells with sodium cromoglycate, even in the absence of challenge, similarly induced phosphorylation of a 78,000 mol. wt protein, suggesting that cromoglycate may stabilise the mast cell by activation of a natural secretion control mechanism.

Phosphorylation of the 78,000 mol. wt protein by sodium cromoglycate is of great importance since it provides a basis for the activity of the compound at the molecular level. We have further investigated these phosphorylation events, looking at the effect of immunologically-stimulated secretion and the effects of some anti-allergic compounds and biochemical modulators.

MATERIALS AND METHODS

Sodium cromoglycate, FPL 55618 [5-(3-methylbutyloxy)-4-oxo-8-prop-2-enyl-4*H*-1-benzopyran-2-carboxylic acid] and FPL 57787 (6,7,8,9-tetrahydro - 5 - hydroxy - 4 - oxo - 10 - propyl - 4*H* - 1-naphtho[2,3-b]pyran-2-carboxylic acid) were synthesised at Fisons plc, Pharmaceutical Division (Loughborough, U.K.). These compounds were tested as the sodium salts.

[^{32}P]Orthophosphate, carrier-free in aqueous solution, was obtained from Amersham International; cyclic nucleotide analogues, phosphatidylserine (PS)† and IMX from Sigma; and salbutamol from Allen & Hanburys.

Rabbit anti-rat IgE, immunoglobulin fraction, was supplied by Dr D. I. Pritchard of the Department of Immunology. Antiserum to rat IgE was prepared by immunising rabbits with rat monoclonal IgE (IR162) kindly supplied by Dr H. Bazin (University of Louvain, Belgium). The antiserum, which was shown to have some cross-reactivity for normal rat

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† Abbreviations: Bu_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate; Bu_2cGMP, $N^{2'}$,$O^{2'}$-dibutyrylguanosine 3′:5′-cyclic monophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; IMX, 3-isobutyl-1-methylxanthine; PS, phosphatidylserine; SDS, sodium dodecyl sulphate; THG, HEPES-buffered Tyrode containing gelatin (0.1% w/v).

serum was rendered specific for rat IgE by affinity chromatography using rat immunoglobulin, obtained by ammonium sulphate precipitation, coupled to cellulose carbonate [8]. An immunoglobulin preparation was obtained by DEAE-cellulose chromatography [9].

The buffer used throughout was THG of the following composition: NaCl 137 mM, KCl 2.7 mM, NaH_2PO_4 0.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, glucose 5.6 mM, HEPES 10 mM, adjusted to pH 7.4 with NaOH and containing gelatin (1 mg/ml). All compounds under test were made up in a Ca^{2+}, Mg^{2+}-free THG at 4 times the required final concn. Anti-IgE was four-fold conc. in THG.

Purification of rat peritoneal mast cells. Male Sprague–Dawley rats (250–300 g) were killed in an atmosphere of CO_2 and the peritoneal cavity lavaged with 20 ml ice-cold THG containing heparin (5 units/ml). Washings were pooled and centrifuged (200 *g*, 5 min, 4°). The cells were resuspended in THG and the mast cells isolated on a Percoll gradient [10] as follows. Ninety per cent Percoll in isoosmotic THG was produced by mixing 9 vols of Percoll with 1 vol. of 10-fold conc. phosphate-free THG. Ninety per cent Percoll and cell suspension were mixed to give a final density of 1.09; typically 4 ml of 90% Percoll to 1.6 ml cells. The Percoll was overlayed with 1 ml THG. After centrifugation (200 *g*, 15 min, 4°) the cells at the interface were discarded, the mast cell pellet resuspended in THG, and washed by centrifugation to remove residual Percoll (200 *g*, 5 min, 4°).

Mediator release from rat peritoneal mast cells. Purified mast cells were resuspended to a working dilution of 0.3×10^6 cells/ml in THG containing PS (30 μg/ml). PS is added to potentiate IgE-dependent histamine release [11, 12]. Inhibitors and rabbit anti-rat IgE (IgG fraction) were placed in a tube in a combined vol. of 0.1 ml. Tubes and cells were prewarmed to 37° and the release process started by the addition of 0.1 ml cells. After incubating for 12 min at 37°, the process was terminated by the addition of 0.8 ml ice-cold THG. After centrifugation (200 *g*, 5 min, 4°), 0.6 ml of supernatant was removed, acidified with an equal vol. of 0.8 M $HClO_4$ and histamine determined by an automated fluorometric method (Technicon AAII Industrial Method No. 164-73E). The total histamine in the cells was measured after the addition of 0.1 ml cells to 0.9 ml H_2O and acidification with 1 ml, 0.8 M $HClO_4$.

The effect of inhibitor on histamine release was expressed as release in the presence of the inhibitor as a percentage of the release in the absence of inhibitor, both being corrected for spontaneous release (generally 1–2.5% of the total). All determinations were performed in duplicate or triplicate.

The inhibition of anti-rat IgE induced release by cromoglycate showed a certain amount of variation between experiments, particularly in regard to the maximum inhibition attained. This variation tended to depend on the severity of the challenge required to give a particular response (i.e. the sensitivity of the cells) and on the extent of the unblocked response. The level of the anti-rat IgE challenge was therefore maintained so as to give a release of approximately 20% of the total histamine in the absence of inhibitor.

Phosphorylation of mast cell proteins. After centrifugation through the Percoll gradient, the pelleted mast cells were washed by centrifugation (200 *g*, 5 min, 4°) in 10 ml of phosphate-free THG.

Cells in phosphate-free buffer were suspended in a small vol. (0.15–0.3 ml) at 0.5×10^7–1×10^7 mast cells/ml, and incubated with carrier-free [^{32}P]orthophosphate for 15 min at 37° at a final radionucleotide concn of 2 mCi/ml. The cells were washed by centrifugation in 10 ml phosphate-free THG to remove excess [^{32}P]phosphate and the pelleted cells resuspended to a final cell density of approximately 0.5×10^6 cells/ml. In experiments involving treatment with anti-rat IgE, a final cell density of 0.2×10^6 was used to allow comparison with histamine release experiments. Buffer or test compound (0.025 ml) was placed in a 1.5 ml polypropylene conical microcentrifuge tube and the tubes and cells prewarmed to 37°. Cells (0.075 ml) were added to the tube and incubated at 37° for 30 sec (unless stated otherwise), after which time the reaction was stopped by the addition of 0.025 ml of five-fold conc. gel sample buffer [13] containing 5% (v/v) 2-mercaptoethanol, 12.5% (w/v) SDS and 25% (v/v) glycerol. Tubes were capped and placed in a boiling water bath for 2 min. Samples were stored overnight at −20° before analysis by SDS–polyacrylamide gel electrophoresis.

SDS–polyacrylamide gel electrophoresis. Electrophoresis, in the presence of SDS, was carried out in a slab gel consisting of a gradient of polyacrylamide and a stacking gel, according to the method of Laemmli [13]. The resolving gel was $140 \times 120 \times 0.75$ mm and, unless otherwise stated, consisted of an exponential gradient, 7.5–10% (w/v) in acrylamide with 1/37.5 cross-linker. The gradient was formed by underlaying, using a gradient maker with a mixing chamber (light solution) with a cross-sectional area 2.9-fold that of the second chamber (heavy solution). Samples (0.025 ml) were applied by underlaying to wells cast in the stacking gel using a 20-well comb. Gels were calibrated using a commercial standard kit covering the subunit mol. wt range 14,100–94,000 (Pharmacia). After staining with Coomassie brilliant blue R, gels were dried and autoradiographed using Industrex C X-ray film (Kodak), generally for 2–4 days. To provide a graphical representation, autoradiographs were scanned with a Vitatron densitometer using a 0.25 mm slit and the peak height used as an arbitrary unit of radioactivity.

RESULTS

Mast cells were purified to > 98% on Percoll gradients ($\rho = 1.09$). Recovery varied, but normally exceeded 80%.

A proportion of red cells penetrated the gradient and lavage fluid contaminated with blood was therefore discarded. Increasing the gradient density to 1.1 reduced red cell contamination of the pellet at the expense of mast cell recovery.

The time-course of the phosphorylations induced in rat mast cells by challenge with compound 48/80

was similar to that previously reported [5]. Phosphorylation of proteins of mol. wts similar to those observed after 48/80 treatment were seen 45 sec after challenge with anti-rat IgE (Fig. 1).

When cells were treated with sodium cromoglycate (10^{-5} M), in the absence of any challenge, phosphorylation of a 78,000 mol. wt polypeptide was induced. The onset of this phosphorylation was very rapid, reaching 75% of the maximum within 10 sec, and decreasing slowly after 30 sec (Fig. 2). Appearance of this phosphorylated protein occurs within the time for the onset of IgE-dependent histamine release [14, 15]. An autoradiograph of a gel showing a dose response of the sodium cromoglycate-induced phosphorylation is shown in Fig. 3. Reference to Fig. 1 shows that the level of induction of this phosphorylation by sodium cromoglycate is more pronounced than the phosphorylation during challenge. Two other chromones, FPL 57787 and FPL 55618, which were more potent than sodium cromoglycate at inhibiting anti-IgE-induced histamine release *in vitro* [Fig. 4(a)] were similarly more potent at inducing phosphorylation of the 78,000 mol. wt protein [Figs 3 and 4 (b)].

Several compounds, which act by elevating intracellular levels of cAMP, were tested for their ability to inhibit anti-rat IgE induced histamine release and to phosphorylate the 78,000 mol. wt protein in comparison with sodium cromoglycate. Dibutyryl-cAMP (an analogue of cAMP which is capable of entering the cell), salbutamol (a β_2-adrenoceptor agonist) and IMX (a phosphodiesterase inhibitor) all inhibited anti-IgE-induced histamine release from rat mast

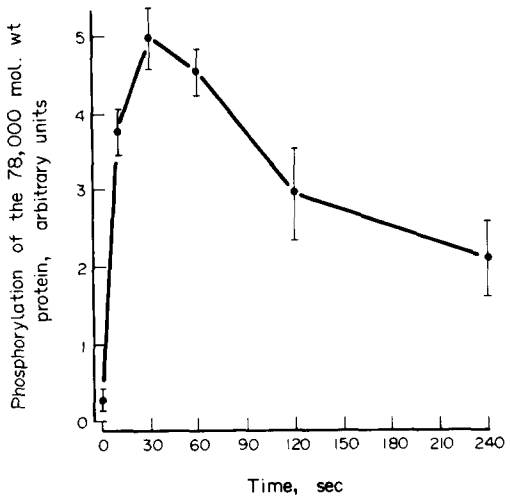

Fig. 2. Time-course of phosphorylation of the 78,000 mol. wt protein induced by sodium cromoglycate (10^{-5} M) (± range of duplicates about the mean).

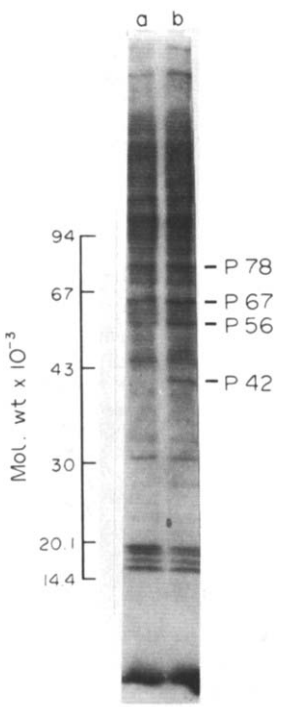

Fig. 1. Autoradiograph showing anti-rat IgE induced phosphorylation of mast cell proteins. (a) Control. (b) Challenge with anti-rat IgE. The incubation period was 45 sec. The gel contained a linear gradient, 7.5–15% (w/v) in acrylamide. Mol wts × 10^{-3} of phosphorylated proteins are shown on the right and the position of calibration standards are shown on the left of the autoradiograph. The challenge was equivalent to a release of 40% of the total histamine.

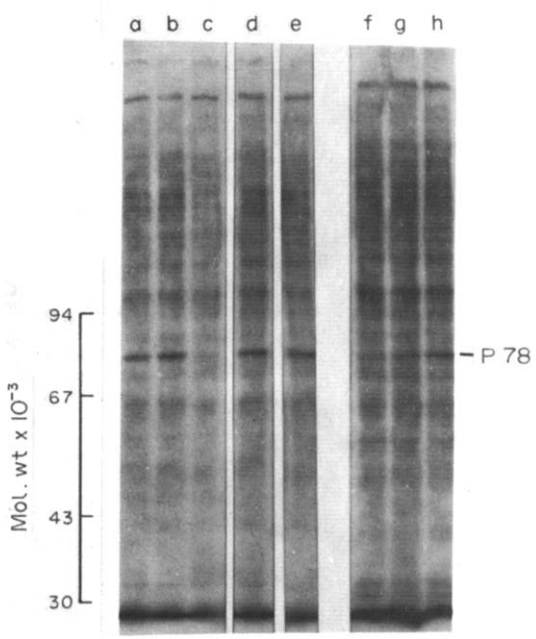

Fig. 3. Autoradiographs showing the effect of anti-allergic chromones and dibutyryl-cAMP upon phosphorylation of the 78,000 mol. wt protein. (a) 10^{-5} M sodium cromoglycate, (b) 10^{-4} M sodium cromoglycate, (c) control, (d) 10^{-7} M FPL 55618, (e) 10^{-6} M FPL 57787, (f) 10^{-5} M Bu_2cGMP, (g) 10^{-4} M Bu_2cGMP and (h) 10^{-3} M Bu_2cGMP. a–e are from the same autoradiograph, while f–h are taken from a different experiment. Mol. wts × 10^{-3} of calibration standards are shown on the left and the phosphorylated protein on the right.

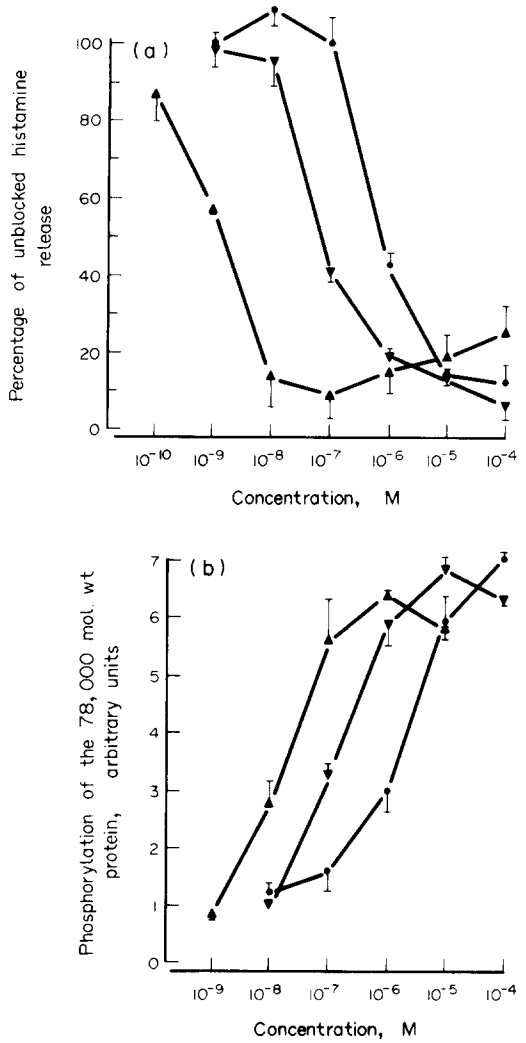

Fig. 4. (a) Inhibition of anti-IgE-induced histamine release by anti-allergic chromones (± S.D.). Similar responses were obtained on a minimum of two occasions for each of the compounds. Unblocked release 14.6%. (b) Phosphorylation of the 78,000 mol. wt protein. The responses shown are typical of those obtained on four different occasions (± range of duplicate samples about the mean). Sodium cromoglycate (●), FPL 57787 (▼) and FPL 55618 (▲).

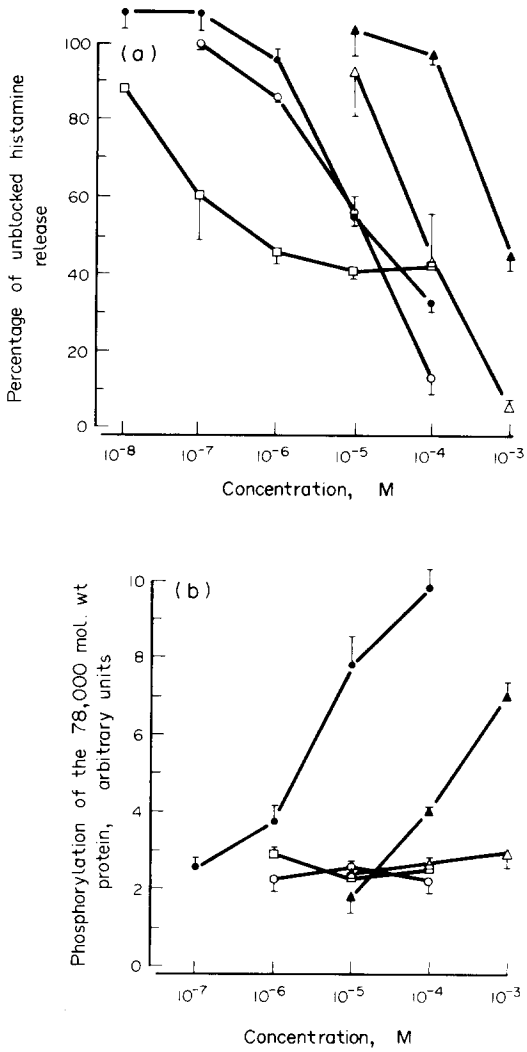

Fig. 5. (a) Inhibition of anti-IgE-induced histamine release from rat mast cells by compounds known to elevate cyclic nucleotide levels compared to sodium cromoglycate (± S.D.). All results were obtained in the same experiment (unblocked release 18.5%) except dibutyryl-cAMP which is from an experiment with a similar sodium cromoglycate response (unblocked release 12.7%). All responses observed on a minimum of four occasions, except IMX (3). (b) Phosphorylation of the 78,000 mol. wt protein. The responses shown are typical of those obtained on a minimum of three separate occasions, except IMX (2) (± range of duplicate samples about the mean). Sodium cromoglycate (●), salbutamol (□), IMX (○), dibutyryl-cAMP (△) and dibutyryl-cGMP (▲).

cells, as did sodium cromoglycate [Fig. 5(a)]. However, only sodium cromoglycate induced phosphorylation of the 78,000 mol. wt protein above background levels [Fig. 5(b)].

An analogue of cGMP which is capable of penetrating the cell membrane, dibutyryl-cGMP, also inhibited anti-IgE-induced mediator release [Fig. 5(a)]. Like sodium cromoglycate, it also induced phosphorylation of the 78,000 mol. wt protein at concns which inhibited histamine release [Figs 3 and 5(b)].

DISCUSSION

The use of Percoll gradients to purify rat peritoneal mast cells enables high-purity preparations (> 98%) to be used. This is necessary to minimise background phosphorylations contributed by contaminating cells. The use of Percoll is considered to be preferable to many of the density media used previously. It has a very low osmolarity, permitting the use of isoosmotic gradients, thus minimising hyperosmotic shock to the cells.

Forty-five seconds after challenging mast cells with anti-rat IgE (Fig. 1), a similar pattern of phosphorylation is observed to that obtained with compound 48/80. However, there are some well-docu-

mented differences between the two stimuli. Their requirements for extracellular Ca^{2+} [12, 14] and exogenous PS [11, 12] differ, as does the time-course of histamine release [14]. Also, there is a sharp peak of cAMP 15 sec after challenge with anti-IgE [15, 16], but a decrease after challenge with 48/80 [17]. Despite these differences in the initial biochemical events, our results show that both stimuli induce similar phosphorylation patterns, suggesting similar control mechanisms at this level.

The ability of sodium cromoglycate to induce phosphorylation of the 78,000 mol. wt protein correlates with its ability to inhibit histamine release (Fig. 4). This correlation extends to other sodium cromoglycate like compounds, covering a 500-fold potency range as inhibitors of histamine release. Also, phosphorylation of the 78,000 mol. wt protein by sodium cromoglycate demonstrates tachyphylaxis similar to that observed in inhibition of 48/80 challenge [7]. The connection between the degree of phosphorylation of the 78,000 mol. wt protein and the ability to inhibit histamine release, therefore, is strong. The rapid onset of the phosphorylation induced by sodium cromoglycate (Fig. 2) is consistent with its ability to inhibit anti-IgE-induced mediator release when this compound is added at the same time or just prior to challenge.

Intracellular protein phosphorylations are catalysed by protein kinases (2.7.1.37). These enzymes require binding of a specific ligand, a second messenger, for activity. The second messenger is commonly cAMP, cGMP or Ca^{2+}, the concn of which is elevated by the action of a primary messenger [6]. We therefore investigated the possible role of some second messengers in mediating the sodium cromoglycate induced phosphorylation of the 78,000 mol. wt protein.

Histamine secretion from mast cells may be inhibited by elevation of intracellular cAMP [18]. Three types of compound that were expected to elevate intracellular cAMP levels were tested. Dibutyryl-cAMP, a permeable cAMP analogue; salbutamol, a β_2-adrenoceptor agonist; and IMX, a phosphodiesterase inhibitor, all inhibited histamine release but did not induce phosphorylation of the 78,000 mol. wt protein (Fig. 5). Thus, it is very unlikely that the protein kinase which catalyses this phosphorylation is cAMP-dependent. A previous report [19] has suggested poor sensitivity of rat peritoneal mast cells to β-adrenoceptor stimulants. More recently however, isoprenaline has been reported to block histamine release from rat mast cells only when it produces a rise in cAMP, with the additional observation that this cAMP elevation is variable between different cell preparations [20]. Our results support this latter observation in that we find that histamine release from rat mast cells can be inhibited by salbutamol. It is possible that the receptor–adenylate cyclase (4.6.1.1.) coupling may be susceptible to differences in manipulation of the cells.

In other systems a control function by cGMP has been observed. Associated with the activation of platelets by thrombin is a series of protein phosphorylations and a rise in cGMP [21, 22]. Treatment with 8-bromo-cGMP inhibits platelet activation, while inducing phosphorylation of a 50,000 mol. wt protein in the presence or absence of thrombin [22, 23]. It has been suggested that the rise in cGMP during activation is a negative feedback control and that independent elevation of cGMP stabilizes the platelet [23]. Further, it has been proposed that cGMP acts as feedback inhibitor, rather than a second messenger, of muscarinic cholinergic smooth muscle contraction in the rat ductus deferens [24] and in bovine coronary smooth muscle [25]. It was of interest therefore to see whether cGMP could be producing a feedback inhibition of secretion in rat mast cells via the late phosphorylation of the 78,000 mol. wt protein. In keeping with this hypothesis, Lewis *et al.* [15] have reported a transient rise in mast cell cGMP 30 sec after challenge with anti-rat $F(ab')_2$.

Treatment of mast cells with the permeable cGMP analogue, dibutyryl-cGMP, inhibited anti-IgE-induced histamine release when added at the time of challenge [Fig. 5(a)]. A pronounced phosphorylation of the 78,000 mol. wt protein was observed at the inhibitory concns [Fig. 5(b)]. 8-Bromo-cGMP gave similar results (data not shown). This contrasts with an earlier report of potentiation of immunologic histamine release from human lung tissue by 8-bromo-cGMP [26]. It is, however, difficult to compare results obtained from such a heterogenous mixture of cells with these from a pure cell population.

It seems probable, therefore, that phosphorylation of the 78,000 mol. wt protein which is induced by sodium cromoglycate and which also occurs as the putative switch off event during secretion is mediated through the action of cGMP-dependent protein kinase. Sodium cromoglycate like compounds would therefore be expected to elevate intracellular cGMP. Attempts to measure changes in cGMP after sodium cromoglycate treatment have, as yet, been unable to detect the predicted rise, or background levels of cGMP. If these changes are restricted to a particular pool of cGMP, any elevation may be below a detectable level.

Sodium cromoglycate has been reported to be a cGMP phosphodiesterase (3.1.4.35) inhibitor in a variety of cell-free systems at high concns ($\sim 10^{-4}$ M) [27, 28]. Sodium cromoglycate is a very hydrophilic compound with a pK_a of 1.66 and, therefore, unlikely to enter the cell at anything like the concn required for phosphodiesterase inhibition. Also, IMX, a more potent inhibitor of cGMP phosphodiesterase than sodium cromoglycate [28] and more capable of entering the cell, does not induce phosphorylation. This would suggest that IMX inhibits mediator release by an action on cAMP phosphodiesterase (3.1.4.17), rather than on cGMP phosphodiesterase. It is improbable, therefore, that sodium cromoglycate acts by direct inhibition of cGMP phosphodiesterase. A more logical alternative would be a stimulation of guanylate cyclase (4.6.1.2). This enzyme has soluble and membrane-bound forms in a variety of tissues [29] so that direct activation by sodium cromoglycate at the plasma membrane is a possibility.

In conclusion, sodium cromoglycate appears to activate, in a unique manner, a natural mechanism for switching off histamine release mediated through

cGMP. Agents such as salbutamol and IMX which are believed to inhibit histamine release from rat mast cells by elevating intracellular cAMP do not activate the same control mechanism.

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