Simultaneous Inhibitions of Inositol Phospholipid Breakdown, Arachidonic Acid Release, and Histamine Secretion in Mast Cells by Islet-activating Protein, Pertussis Toxin

A POSSIBLE INVOLVEMENT OF THE TOXIN-SPECIFIC SUBSTRATE IN THE Ca**+-MOBILIZING RECEPTOR-MEDIATED BIOSIGNALING SYSTEM*

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Incubation of rat mast cells with compound 48/80 resulted in transient breakdown of phosphatidylinositol 4,5-bisphosphate, rapid generation of inositol polyphosphates, 45Ca inflow, and the arachidonic acid liberation mainly from phosphatidylcholine, eventually leading to histamine secretion. All of these processes of signaling from Ca-mobilizing receptors to degranulation were markedly inhibited by prior 2-h exposure of cells to islet-activating protein (IAP), pertussis toxin. A23187 caused 45Ca inflow and releases of arachidonic acid and histamine without inducing breakdown of inositol phospholipids. The effects of A23187, in contrast to those of compound 48/80, were not altered by the exposure of cells to IAP. Incubation of the supernatant fraction of mast cell homogenates with the active component of IAP caused the transfer of the ADP-ribosyl moiety of added [α-35S]NAD to a protein with M, = 41,000. The IAP-catalyzed ADP-ribosylation of this protein was prevented by guanosine 5′-(3-O-thio)triphosphate, indicating that this IAP substrate resembles, in character, the α-subunit of the guanine nucleotide regulatory protein (Ni) involved in inhibition of adenylate cyclase. The degree of ADP-ribosylation of this IAP substrate was prevented progressively by pre-exposure of the homogenate-donor cells to increasing concentrations of IAP. The half-maximally effective concentrations of the toxin were 0.2 to 0.6 ng/ml for all the IAP-sensitive processes studied. Thus, the ADP-ribosylation of the M, = 41,000 protein occurring during exposure of cells to IAP appears to be responsible for the inhibition of signaling observed. It is proposed that the α-subunit of Ni, or a like protein, mediates signal transduction arising from Ca-mobilizing receptors, probably prior to Ca**+ gating.

Evidence has been provided by Ui and his colleagues for selective interaction of IAP1 with Ni that is involved in inhibition of adenylate cyclase (2-5). The function of Ni, to communicate between membrane receptors and the catalytic unit of adenylate cyclase is completely lost as a result of IAP-catalyzed ADP-ribosylation of an M, = 41,000 peptide, a GTP-binding subunit of Ni (2-7). Receptor-mediated (or GTP-dependent) inhibition of adenylate cyclase or decrease in cellular cAMP was enhanced concomitantly (4, 9, 12), probably reflecting an involvement of Ni in the adenylate cyclase activation system as well as the inhibition system (3). In any event, the cAMP content should be much higher in IAP-treated cells than in control nontreated cells after stimulation of receptors linked to adenylate cyclase.

Since cAMP is inhibitory to histamine release from mast cells presumably due to inhibition of calcium channels (see Refs. 13 and 14 for review), it would be possible to assume that IAP-induced inhibition of the histamine secretory response of mast cells arises from cAMP accumulation in the toxin-treated cells. This possibility was, however, denied by the recent data (1); the cAMP content was never higher in IAP-treated mast cells than in control cells even after stimulation of cells by compound 48/80 or β-adrenergic agonists.

The purpose of the present paper is to study an alternative possibility. We will show that incubation of rat mast cells with compound 48/80 gave rise to rapid breakdown of phosphatidylinositol 4,5-bisphosphate, generation of inositol phosphates, release of arachidonic acid, and increases in cell-bound 45Ca, indicating that Ca**+-mobilizing receptors (15, 16) were stimulated under these conditions. These effects of compound 48/80 were markedly inhibited by prior treatment of the cells with IAP. Since the IAP-induced inhibition was well correlated with the ADP-ribosylation of an M, = 41,000 peptide, it is suggested that a protein analogous to a subunit of Ni is somehow involved in receptor-mediated phospholipid metabolism leading to calcium gating.

**EXPERIMENTAL PROCEDURES**

Materials—IAP was purified from the 2-day culture supernatant of Bordetella pertussis as described elsewhere (17, 18). Its A-protomer

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1 The abbreviations used are: IAP, islet-activating protein; Ni, the guanine nucleotide regulatory protein responsible for inhibition of adenylate cyclase; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GTP·S, guanosine 5′-(3-O-thio)triphosphate; Gpp(NH)p, guanyly-5′-β,γ-imino)triphosphate.
was isolated and purified by means of a column of carboxymethyl-

Sepharose CL-6B (19). IAP and its A-protomer were stored in a
vehicle consisting of 2 M urea and 0.1 M potassium phosphate buffer
immediately before use. The following reagents were obtained from commercial sources: bovine serum albumin (Fraction

V, E. Merck); 3-isobutyl-1-methylxanthine (Aldrich); A23187 (Calbi-
occhem); [1-32P]arachidonic acid (54.5 mCi/mmol) and [3-32P]adenosine triphosphate (23.2 mCi/mg) (New England Nuclear); [32P]NAD (50 Ci/mmol), myo-
[2-32P]inositol (15.6 Ci/mmol) and carrier-free [32P]Pi (11 mCi/ml) (Amer-

sham Corp.); Eagle's minimum essential medium (Flow Laborato-
ries). Other reagents were of the best grade available from commercial
sources.

**IAP Treatment and the Subsequent Incubation of Mast Cells.**—Mast
cells from the peritoneal cavity of male Wistar rats (250-300 g, body
weight) were isolated by differential centrifugation in bevine serum albumin as described previously (1). In most of the experiments in
this study, the cells were exposed to IAP (or its vehicle as control) at
the density of 10^6 cells/ml at 37°C in the Hepes-buffered solution (pH
7.4) containing 10 mM inositol, 0.1 mM CaCl_2, and 0.1 mM EGTA,
further supplemented with 0.2% bovine serum albumin under a gas mixture of 95% O_2/5% CO_2. The effect of IAP was the same in either medium. When
[32P]Pi was included in the medium for IAP treatment as described below, KH_2PO_4 was omitted from the Hepes-buffered solution to increase the specific radioactivity of pre-
cursor [32P]Pi. The time of the cell exposure to IAP was 2 h.
The (control) washing (consisting of several washings with Ringer's
solution) being washed several times, were submitted to incubation under various conditions as specified below in a total volume of 0.5 ml. The medium used for cell washing and incubation was the Hepes-buffered solution (pH 7.4)
of the same composition as that used for the above-mentioned IAP
treatment, except that the concentration of Hepes was reduced to 5
mM. Where indicated, histamine release into the medium (at a time
indicated after the addition of compound 48/80) as well as its content
in cells (before incubation) was determined fluorometrically as de-
scribed previously (1).

**Incorporation of [32P]Pi into Phospholipid Fractions.**—IAP treated (or control) radiolabeled cells in 0.5 ml of the Hepes-buffered solution containing no KH_2PO_4 were incubated with 5 μCi of [32P]Pi for 90 min.
Compound 48/80 was added at 30 min after the start of incubation.
The reaction was terminated by the addition of 1 ml of 15% trichlo-
roacetic acid. The precipitate was extracted with 2 ml of a 2:1 mixture of chloroform-methanol for 30 min. After vigorous stirring with 2 ml KCl, two phases were separated by centrifugation, and the lower phase was washed three times with methanol, 19 mM KH_2PO_4, and chloroform (48:47:3). An aliquot (1 ml) of the chloroform layer was evaporated to dryness, dissolved in 50 μl of the mixture of chloroform-
methanol, and spotted on a glass plate coated with Silica Gel H
prepared by mixing 40 g of silica gel with 1.2 g of Mg acetate.

Phospholipids were separated by two-dimensional chromatography
employing chloroform/methanol/28% NH_4OH/H_2O (65:35:3.2, v/v)
in the first dimension and chloroform/acetone/methanol/glacial
acetic acid/H_2O (10:4:2:2:1) in the second dimension (20). The re-
spective Rf values for the first and the second 90-min developments
were: phosphatidic acid 0.10 and 0.60, phosphatidylycerine 0.16 and
0.45, phosphatidylglycerol 0.18 and 0.33, phosphatidylcholine 0.31 and
0.55, phosphatidylinositol 0.24 and 0.67. The plates were dried and
the spots of phospholipids visualized by exposure to iodine vapor.
Phospholipids were impregnated with 1% potassium oxalate containing 2 mM EDTA.

**Analysis of [32P]Inositol Phosphates.**—The Hepes-buffered solution used for IAP treatment of mast cells was supplemented with
myo-[2-32P]inositol (100 μCi) to label the cells with [32P]inositol during
2-h exposure to IAP (or its vehicle). The labeled cells were washed
three times and then incubated for 30 min in Hepes-buffered solution
containing no KCl, the cells were again washed once, resuspended in
the fresh Hepes-buffered solution (4 × 10^6 cells in 0.2 ml), and incubated for 5 min without addition and then with the quick
addition of compound 48/80 (0.5 μg/ml). The incubation with compound 48/80 was continued for 5, 10, 20, 30, 45, and 60 min in Fig. 5
and 10 s in Fig. 6 and was stopped by the addition of 5% trichloroacetic
acid.

The supernatant of the deproteinized cells was supplemented with
0.2 mM NaOH/0.1 M formic acid for 1 h under the same conditions as for the IAP

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acid.

The supernatant of the deproteinized cells was supplemented with
0.2 mM NaOH/0.1 M formic acid for 1 h under the same conditions as for the IAP treatment.

The cells were then washed twice with the Hepes-buffered solution and
again incubated in the fresh Hepes solution for 30 min at the same

cell density. The cells thus labeled with [32P]arachidonic acid (4 ×
10^6 cells in 0.5 ml) were further incubated with compound 48/80 (or
A23187) to cause the release of [32P]arachidonic acid.

In experiments shown in Table I, the 30-min incubated labeled
cells were again washed twice with and further incubated with com-
 pound 48/80 or A23187 for 10 min in the ordinary Hepes-buffered
solution (Ca^2+ free) and 0.1 mM EGTA-added Hepes solution (Ca^2+ free). The reaction was terminated by the addition of 1
ml of ice-cold Tris buffer and the supernatant obtained by 2-min
centrifugation at 4 °C was analyzed for the [32P]content and histamine.
The supernatant of phospholipid fractions of the pelleted cells was
determined by the procedure of Tolbert et al. (23).

**Release from Mast Cells.**—Mast cells treated or not with 10 ng/
ml of IAP for 2 h as described above were washed twice and suspended in the Hepes-buffered solution containing 4 μCi of [32P]Pi (× 10^6
in 0.5 ml). At 5 min of incubation at 37°C compound 48/80 (or
A23187) was added, and incubation was continued for 1 to 20 min.
The reaction was terminated by the addition of 4.5 ml of L-Methionine
solutions (25 mM Tris-Cl, pH 7.5, 0.9% NaCl, and 100 mM
HCl, pH 7.0). The supernatant separated by centrifugation

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RESULTS

Compound 48/80-induced Incorporation of $^{32}$P into Phosphatidylcholine, Phosphatidylinositol, and Phosphatidylethanolamine Fractions and Their Susceptibility to IAP Treatment—Fig. 1 shows time courses for $^{32}$P incorporation into the phosphatidylcholine, phosphatidylinositol, phosphatic acid, and phosphatidylethanolamine fractions during incubation of rat mast cells. Addition of compound 48/80 to the incubation medium gave rise to a rapid increase in the radioactivity in phosphatic acid and slower increases in phosphatidylinositol and phosphatidylethanolamine. It did not increase significantly, however, the rate of $^{32}$P incorporation into the phosphatidylethanolamine fraction.

The same experiments were repeated with mast cells that had been exposed to 10 ng/ml of IAP for 2 h. This IAP treatment of cells was without effect on the incorporation of $^{32}$P into the phospholipid fractions when cells were incubated without compound 48/80. The striking effect of IAP was observed, however, when cells were stimulated by the compound. The increase in the $^{32}$P content of phosphatidylcholine and phosphatic acid in response to compound 48/80 was much smaller in IAP-treated cells than in nontreated control cells at 30 and 60 min after the addition of the compound (Fig. 1, A and C), though there was no significant difference in $^{32}$P content of phosphatidylinositol between treated and control cells under the same conditions (Fig. 1B). The incorporation of $^{32}$P into phosphatidylethanolamine was not altered by prior treatment of cells with IAP either in the presence or absence of compound 48/80 (Fig. 1D).

The inhibition by IAP of compound 48/80-induced $^{32}$P labeling of phospholipid fractions was further studied in Fig. 2 in which the concentration of IAP was increased from 0.1 to 10 ng/ml. The effect of IAP on histamine release from the same cells was also studied (Fig. 2D). Increases in the IAP concentration gave rise to progressive decreases in the compound 48/80-induced $^{32}$P incorporation into phosphatidylcholine; the maximal inhibition caused by IAP over 3 ng/ml was

![Fig. 1. Time courses for the incorporation of $^{32}$P into mast cell phospholipid fractions in response to compound 48/80.](image)

![Fig. 2. Concentration-dependent inhibition by IAP of $^{32}$P labeling of phospholipid fractions and histamine release in mast cells.](image)
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79% (Fig. 2A). Similarly progressive decreases in $^{32}$P content were observed in phosphatidic acid with the maximal inhibition being 53% (Fig. 2C). No significant inhibition was, however, provoked by IAP in the $^{32}$P incorporation into phosphatidylinositol (Fig. 2B). IAP was also inhibitory to compound 48/80-induced histamine secretion from these cells in accordance with our previous results (1). The maximal inhibition attained 81% over 48/80-induced histamine secretion from these cells in accord-

Inhibition by IAP of Compound 48/80-induced Generation of Inositol Phosphates—The incubation of $[3H]$inositol-labeled mast cells with compound 48/80 gave rise to increases in inositol 1,4,5-trisphosphate (Fig. 5). LiCl, an inhibitor of inositol 1-phosphatase, was not included in the incubation medium, since it did not increase or maintain significantly the cellular level of inositol phosphates in mast cells under these conditions (not shown). The compound 48/80-induced increase in inositol 1,4,5-trisphosphate was very rapid and attained its highest level in 5 s (Fig. 5C), being followed by the increase in inositol 1,4-bisphosphate with the peak at 10 s (Fig. 5B). Inositol 1-monophosphate increased more gradually up to 20 s (Fig. 5A). The time courses for these inositol phosphate generations were coincident with rapid breakdown of phosphatidylinositol 4,5-bisphosphate in Fig. 3; inositol 1,4,5-trisphosphate should be a direct product of the break-

**Fig. 2. Time courses for rapid changes in $^{32}$P-labeled inositol phospholipids and phosphatidic acid in response to compound 48/80.** $^{32}$P-labeled control (O) and IAP-treated (E) cells were incubated with 0.5 mg/ml of compound 48/80, and changes in the $^{32}$P content in phosphatidic acid (Panel A), phosphatidylinositol (Panel B), phosphatidylinositol 4,5-bisphosphate (Panel C), and phosphatidylinositol 4-monophosphate (Panel D) are plotted as percentages (means ± S.E. from 3 separate experiments) of the initial (zero time) values obtained immediately before the addition of compound 48/80. The concentration of IAP was 10 mg/ml. The effect of IAP was significant (* indicates $<0.05$; ** indicates $p<0.01$). The initial values (cpm/10$^5$ cells) for control and IAP-treated cells, respectively, are: phosphatidic acid, 450 ± 70 and 471 ± 50; phosphatidylinositol, 445 ± 25 and 464 ± 27; phosphatidylinositol 4,5-bisphosphate, 394 ± 35 and 348 ± 35; phosphatidylinositol 4-monophosphate, 1981 ± 212 and 2037 ± 227. There was no significant difference in these initial values between control and IAP-treated cells.

**Fig. 4. Concentration-dependent reversal by IAP of compound 48/80-induced decreases in $^{32}$P-labeled phosphatidylinositol 4,5-bisphosphate.** The mast cells exposed to increasing concentrations of IAP and labeled with $^{32}$P were incubated with 0.5 mg/ml of compound 48/80 for 5 s as described under "Experimental Procedures." The $^{32}$P content of phosphatidylinositol 4,5-bisphosphate is shown as percentages (means ± S.E. from 3 separate experiments) of the initial values obtained immediately before addition of compound 48/80. The effect of IAP was significant (* indicates $p<0.05$; ** indicates $p<0.01$).
down, and it must be hydrolyzed to inositol 1,4-bisphosphate and inositol 1-monophosphate in succession.

The increases in inositol phosphates in response to compound 48/80 were markedly prevented by prior treatment of cells with IAP (Fig. 5). The prevention was dependent on the concentration of IAP used (Fig. 6) with the maximal inhibition being 71, 86, and 65% for inositol 1-monophosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate, respectively. The half-maximally inhibitory concentration of IAP was between 0.3 and 1 ng/ml for either case. Thus, breakdown of phosphatidylinositol 4,5-bisphosphate directly responding to receptor stimulation by compound 48/80 was strongly inhibited by the exposure of mast cells to low concentrations of IAP.

Arachidonic Acid Release from Mast Cell Phospholipids in Response to Compound 48/80 and Its Inhibition by Prior Treatment of Cells with IAP—Mast cells were labeled with [14C]arachidonic acid by preincubation for 1 h. In the representative experiments, [14C] content of the cellular phospholipid fraction, after twice washing of cells, was 100,000–130,000 cpm/10^6 cells, and 2.0, 7.2, 16.9, and 64.2% of this total radioactivity were found in phosphatidic acid, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylcholine fractions, respectively. The radioactivity in these phospholipid fractions was not altered by adding IAP into the 14C-labeling medium.

A short-term incubation of the thus labeled mast cells with compound 48/80 gave rise to 14C release; the release was dependent on concentrations of the compound (Fig. 7B).
usually observed after 10-min incubation with 1 μg/ml of compound 48/80. Thus, most of the [14C] discharged in response to compound 48/80 originated from phosphatidylcholine. The potency of compound 48/80 to induce [14C]arachidonic acid release was comparable with its potency to elicit histamine release (Fig. 7).

Prior treatment of mast cells with IAP was very effective in inhibiting compound 48/80-induced [14C]arachidonic acid release as well as histamine release from the cells (Fig. 7). In the representative experiments, challenge with compound 48/80 produced 20.2% decrease in control cells and 6.7% decrease in IAP-treated cells in the [14C] content of phosphatidylcholine. Changes in [14C] in other phospholipid fractions were small and variable. It is likely, therefore, that discharge of [14C]arachidonic acid from phosphatidylcholine was suppressed by IAP treatment of cells.

The inhibition by IAP of [14C]arachidonic acid release was so striking that compound 48/80 was no longer stimulatory on the release in mast cells that had been exposed to 1 ng/ml or more of IAP (Fig. 8). Since the conditions for arachidonate release experiments differed somewhat from those used for the previous 32P incorporation experiments in Fig. 2 (see "Experimental Procedures"), the effect of IAP on compound 48/80-induced histamine secretion was again studied with [3H]arachidonate-labeled mast cells (Fig. 8). The concentration-response relationship for the inhibitory action of IAP was very similar between [14C]arachidonic acid and histamine releases (Fig. 8), and the degrees of inhibition elicited by various concentrations of IAP were also well correlated with each other. The half-maximally inhibitory concentration of IAP was around 0.3 ng/ml for either case. Thus, the inhibition by IAP of compound 48/80-induced arachidonic acid release from mast cells appears to be closely related to the toxin-specific inhibition of histamine release from the cells.

A Possible Involvement of Phospholipase A2 in Compound

48/80-induced Arachidonate and Histamine Releases—Both [14C]arachidonate and histamine releases from prelabeled mast cells elicited by compound 48/80 were inhibited by mepacrine or p-bromophenacyl bromide progressively, as the concentrations of the inhibitors were increased (Fig. 9). The inhibition of [14C]arachidonate release was associated with an inhibition of phosphatidylcholine breakdown, e.g. compound 48/80 provoked 38% decrease in control cells and only 16% decrease in cells treated with 100 μM mepacrine in [14C] in the phosphatidylcholine fraction. Probably mepacrine and p-bromophenacyl bromide inhibit phospholipase A2-catalyzing hydrolysis of phosphatidylcholine. It is also probable that breakdown of phospholipids to liberate arachidonic acid may play an important role in compound 48/80-induced histamine release from mast cells.

Extracellular Ca2+-dependent Releases of Histamine and [14C]Arachidonate and Their Insusceptibility to IAP—Incubation of mast cells with A23187, a calcium ionophore, resulted in marked histamine secretion in a concentration-dependent manner (Fig. 10A). This Ca2+-induced histamine release was associated with [14C]arachidonate acid release (Fig. 10B) and [14C]-labeled phosphatidylcholine breakdown (not shown). The dose-response relationship for A23187-induced [14C]arachidonate release was almost identical with the relationship for the histamine release, indicating that hydrolysis of phospholipids is closely related to A23187-induced histamine release. This action of A23187 on [14C]arachidonate and histamine releases differed strikingly from the similar action of compound 48/80 in that it was not inhibited by prior treatment of cells with IAP at all (Fig. 10).

A23187 functioned actually as a Ca ionophore in mast cells; the amount of cell-bound 45Ca increased progressively, probably reflecting increased Ca inflow, when the cells were incubated in the 45Ca2+-containing medium further supplemented with A23187 (Fig. 11). Compound 48/80 was more
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Table I

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Ca (1 mM)</th>
<th>PtdIn-P2</th>
<th>Histamine release</th>
<th>Arachidonate release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells not treated</td>
<td>% of control</td>
<td>% of total</td>
<td>cpm/10^6 cells</td>
<td></td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>-</td>
<td>56 ± 5.5</td>
<td>68 ± 0.8</td>
<td>107 ± 3.5</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>+</td>
<td>70 ± 1.0</td>
<td>81 ± 1.4</td>
<td>105 ± 4.1</td>
</tr>
<tr>
<td>A23187</td>
<td>-</td>
<td>104 ± 10.5</td>
<td>0 ± 0.2</td>
<td>5 ± 1.7</td>
</tr>
<tr>
<td>A23187</td>
<td>+</td>
<td>98 ± 5.7</td>
<td>94 ± 3.0</td>
<td>120 ± 10.5</td>
</tr>
<tr>
<td>Cells treated with IAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>-</td>
<td>79 ± 1.0</td>
<td>47 ± 0.2</td>
<td>29 ± 6.0</td>
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<tr>
<td>Compound 48/80</td>
<td>+</td>
<td>82 ± 1.0</td>
<td>30 ± 1.5</td>
<td>13 ± 2.0</td>
</tr>
<tr>
<td>A23187</td>
<td>-</td>
<td>88 ± 11.3</td>
<td>0 ± 0.1</td>
<td>2 ± 1.0</td>
</tr>
<tr>
<td>A23187</td>
<td>+</td>
<td>102 ± 3.5</td>
<td>91 ± 2.2</td>
<td>128 ± 18.7</td>
</tr>
</tbody>
</table>

* Effect of Ca is significant (p < 0.01).
* Effect of Ca is not significant (p > 0.01).
* Effect of IAP is significant (p < 0.05).
* Effect of IAP is not significant (p > 0.05).

Arachidonate from mast cells was treated with IAP as shown in Table I, indicating that the inhibition of extracellular Ca^2+ was responsible for A23187-induced release reactions. This dependence on extracellular Ca^2+ of the A23187 action forms a sharp contrast with the compound 48/80-induced release reactions which were observed similarly regardless of whether or not Ca was present in the medium, though slightly less histamine was released in the absence of Ca^2+ than in its presence (Table I). The important findings in Table I were that there was no rapid breakdown of phosphatidylinositol 4,5-bisphosphate in response to A23187 even in the presence of extracellular Ca^2+ and that the degree of compound 48/80-induced breakdown of the polyphosphoinositide was not affected by Ca depletion. IAP was inhibitory to the breakdown in either the presence or absence of extracellular Ca^2+. Thus, receptor-mediated and IAP-susceptible breakdown of phosphatidylinositol 4,5-bisphosphate was unlikely to result from, or depend on, increases in cellular Ca^2+.

ADP-ribosylation of the M, = 41,000 Protein during Treatment of Mast Cells with IAP as the Mechanism for IAP-induced Inhibition of Histamine Release—Exposure of a variety of cells to IAP resulted in ADP-ribosylation of the α-subunit of N, the protein with M, = 41,000 (2-4, 6, 7, 28). Receptor-mediated inhibition of adenylate cyclase was no longer observed in these cells, since N, lost its function after being ADP-ribosylated (2-4, 28-30). In mast cells, too, M, = 41,000 protein was ADP-ribosylated by IAP as shown in Fig. 12.

The enzyme catalyzing ADP-ribosylation is the A-protomer moiety of IAP (28), entering intact cells by the aid of the B-oligomer moiety which binds to the cell surface (31). The A-protomer exhibits the enzymic activity after the intrapeptide disulfide bonds are reduced by a certain cellular oxidoreduc-
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Fig. 12. ADP-ribosylation of the $M_r = 41,000$ protein in the supernatant fraction of mast cells by the A-protomer of IAP. The membrane-rich pellet (lanes A and B) and the supernatant fraction (lanes C and D) were prepared from the mast cells not treated with IAP and incubated with $[\alpha-^{32}P]NAD$ in the presence (lanes B and D) or absence (lanes A and C) of the preactivated A-protomer of IAP, as described under "Experimental Procedures." The autoradiographic patterns of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown. The $M_r$ markers were electrophoresed in lane E: bovine serum albumin ($M_r = 68,000$), ovalbumin (43,000), chymotrypsinogen (25,700), myoglobin (17,200), and cytochrome c (11,700).

tase (26). We used, therefore, the A-protomer of IAP, after preactivation with dithiothreitol, for ADP-ribosylation of the mast cell protein; no ADP-ribosylation occurred with the native IAP itself in the cell-free preparation of this cell type (not shown) just as has been reported with the particulate fraction of rat heart tissues (32). Since our preliminary experiments not shown here revealed that an $M_r = 41,000$ protein was ADP-ribosylated by the preactivated A-protomer of IAP in a homogenate of mast cells but was not in the precipitated fraction, we compared the ADP-ribosylation between the membrane-rich pellet and the cytosol fractions in Fig. 12. A protein band corresponding to $M_r = 41,000$ was labeled when the cytosol was incubated with the preactivated A-protomer of IAP and $[\alpha-^{32}P]NAD$. Unexpectedly, no ADP-ribosylation occurred in the membrane preparation.

The cytosolic fractions were prepared from mast cells that had been exposed to increasing concentrations of IAP for 2 h. They were then incubated with $[\alpha-^{32}P]NAD$ in the presence of a saturating concentration of the preactivated A-protomer of IAP. The ADP-ribosylation of the $M_r = 41,000$ protein in the cytosolic fraction decreased progressively as the concentration of IAP was increased from 0.1 to 2.5 ng/ml in the medium for the previous incubation of intact cells. During exposure of mast cells to IAP, the ADP-ribosyl moiety of intracellular NAD had been transferred to a fraction of the endogenous pool of the $M_r = 41,000$ protein, the residual fraction of which could serve as the substrate for the A-protomer of IAP added to the subsequent incubation of the cytosol with $[\alpha-^{32}P]NAD$. Thus, the decrease in the substrate protein for the A-protomer of IAP afforded evidence that the same protein was ADP-ribosylated during exposure of intact cells to IAP (3, 4, 7). The half-maximally effective concentration of IAP involved in this intact-cell ADP-ribosylation was between 0.25 and 0.5 ng/ml. The decrease in $^{32}$P-labeled $M_r = 41,000$ protein in the cytosol was correlated with the degree of IAP-induced inhibition of histamine secretion from the cytosol-donor cells (Fig. 13). The ADP-ribosylation of the $M_r = 41,000$ protein appeared to be responsible for the IAP-induced inhibition of histamine secretion from mast cells.

ADP-ribosylation of the mast cell $M_r = 41,000$ protein by the preactivated A-protomer of IAP was interfered with by the addition of GTPyS into the incubation medium (Fig. 14). The $\alpha$-subunit of N, was found to be ADP-ribosylated by IAP when it was associated with the $\beta\gamma$-subunit (30). The addition of GTPyS to the $\alpha\beta\gamma$-oligomer of N, resulted in its dissolution into the $\alpha$- and $\beta\gamma$-subunits (29); the thus isolated $\alpha$-subunit did not serve as the substrate of IAP-catalyzed ADP-ribosylation (30). Thus, the GTPyS-induced inhibition of ADP-ribosylation seen in Fig. 14 strongly suggested that the $M_r = 41,000$ protein ADP-ribosylated by IAP in mast cells is a GTP-binding protein somewhat analogous to the $\alpha$-subunit of N, in character.

DISCUSSION

The present results confirm our previous report (1) that prior treatment of mast cells with IAP resulted in marked inhibition of receptor-mediated histamine release from the

Fig. 13. ADP-ribosylation of the $M_r = 41,000$ protein during incubation of mast cells with increasing concentrations of IAP as correlated with the inhibition of histamine release from the same cells. Mast cells exposed to increasing concentrations of IAP for 2 h were divided into two batches. One batch of the cells was incubated with 0.5 $\mu$g/ml of compound 48/80 for 10 min to measure histamine secretion, while the other batch was homogenized to obtain the supernatant fraction, which was further incubated with $[\alpha-^{32}P]NAD$ and the preactivated A-protomer of IAP as described under "Experimental Procedures." The $M_r = 41,000$ protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and ADP-ribosylation of this protein was calculated based on its $^{32}$P content (O). The inhibition of histamine release by IAP is shown as a percentage of the control value obtained with the cells not treated with IAP (O). The data are means of the duplicate observations.
IAP-treated cells than in control cells upon stimulation of adenylylate cyclase-coupled receptors, as actually observed in a number of cell types (see Ref. 28 for review). In the case of rat peritoneal mast cells, however, prior exposure of the cells to IAP failed to enhance cAMP accumulation occurring upon the addition of compound 48/80 or β-adrenergic agonists (1). The IAP-specific substrate is thus unlikely to be coupled to these receptors as a component of the adenylylate cyclase system in mast cells. Instead, it would be more likely to play an important role in another signal transduction system communicating between the compound 48/80 receptor and exocytosis of histamine granules, as implied by the present observation that the signal transduction was blocked as a result of ADP-ribosylation of the \( M_0 = 41,000 \) protein.

A number of papers have recently been published which focus on a scheme of the reaction sequence responsible for signal transduction from Ca\(^{2+}\)-mobilizing receptors to the eventual cell responses (see Refs. 16 and 27 for review). The general scheme currently accepted is: (i) rapid breakdown of phosphatidylinositol 4,5-biphosphate following the gradual recovery with phosphorylation of diacylglycerol as the initial step; (ii) Ca\(^{2+}\) mobilization from the exterior or from a cell-associated store as a result of the Ca\(^{2+}\) ionophoretic action of the phospholipid breakdown product(s) including inositol 1,4,5-trisphosphate; (iii) Ca\(^{2+}\)-induced phospholipase A\(_2\) activation leading to arachidonic acid release from membrane phospholipids which is the precursor of prostaglandins and leukotrienes. An involvement of phospholipid methylation (36) or phospholi-pid- and Ca\(^{2+}\)-dependent protein kinase (37) is also proposed.

The addition of compound 48/80 to mast cell suspensions gave rise to a rapid breakdown of \(^{32}\)P-labeled phosphatidylinositol 4,5-biphosphate (Fig. 3) and generation of labeled inositol tris-, bis-, and monophosphates (Fig. 5), which was followed by gradual increases in \(^{32}\)P-labeled phosphatic acid (Figs. 1 and 3) and phosphatidylinositol and phosphatidylcholine (Fig. 1). The inflow of \(^{44}\)Ca into the cells (Fig. 11) and releases of arachidonic acid (accompanied by phosphatidylcholine breakdown) and histamine (Fig. 7) were also observed. Arachidonate and histamine releases were induced by A23187 (Fig. 10) as well as by compound 48/80, and both were inhibited by mepacrine or \(\beta\)-bromophenacyl bromide (Fig. 9), in accordance with the scheme that arachidonic acid production by means of Ca\(^{2+}\)-induced phospholipase A\(_2\) activation is an indispensable process in compound 48/80-induced histamine release. Thus, the receptor for compound 48/80 in mast cells is one of the Ca\(^{2+}\)-mobilizing receptors, stimulation of which triggers the above-mentioned scheme of reaction sequence eventually leading to histamine secretion (38, 39). Compound 48/80 was as effective in causing phosphatidylinositol 4,5-bisphosphate breakdown and arachidonic acid release in the Ca\(^{2+}\)-free medium as in the Ca\(^{2+}\)-containing medium (Table I). This finding, together with the failure of A23187 to induce the polyphosphophatidylinositol breakdown (Table I), suggested that phosphatidylinositol 4,5-bisphosphate was first decomposed and then Ca\(^{2+}\) was mobilized principally from the intracellular stores upon stimulation of membrane receptors by compound 48/80.

All the phospholipid metabolisms, \(^{44}\)Ca inflow, and histamine secretion triggered by compound 48/80 receptor stimulation were strongly inhibited by prior 2-h exposure of mast cells to IAP. The concentration of IAP required for the half-maximal inhibition was 0.2 to 0.6 ng/ml for each of these processes of signaling and was roughly equal to the toxin concentration to cause the half-maximal ADP-ribosylation of...
the $M_r = 41,000$ protein in intact cells. It would be likely that an IAP-sensitive GTP-binding protein with $M_r = 41,000$ mediates the very early processes of signaling, i.e., those between receptors and phosphatidylinositol 4,5-bisphosphate breakdown. The IAP-catalyzed ADP-ribosylation of this protein may lead to the loss of its function as a signaling mediator.

Gomperts (40) suggested an involvement of a GTP-binding protein in Ca$^{2+}$-mediated histamine release from mast cells by showing that the cells became responsive to added Ca$^{2+}$ after being loaded with GTPyS or Gpp(NH)p. We have recently confirmed his observation and further found that IAP was partially inhibitory to the Ca$^{2+}$-dependent releases of histamine and arachidonic acid from Gpp(NH)p-loaded mast cells (41). This finding would appear to be consistent with the idea that the IAP-sensitive GTP-binding protein is involved in Ca$^{2+}$-dependent activation of phospholipase A$_2$ leading to histamine secretion. The IAP substrate might play an important role at more-than-one sites in the Ca$^{2+}$-mobilizing system.

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IAP exerted similar inhibitory influences on the chemotactic peptide-induced arachidonic acid release and $^{45}$Ca inflow in guinea pig neutrophils leading to the inhibition of O$_2$ generation (42). This IAP-induced inhibition was due to ADP-ribosylation of a membrane $M_r = 41,000$ protein (42). More recently, the degradation of phosphatidylinositol 4,5-bisphosphate, production of inositol 1,4,5-trisphosphate, and increases in intracellular $^{45}$Ca in quick response to the formyl peptide were found to be suppressed by the IAP treatment of the cells. In the case of mouse 3T3 fibroblasts, stimulation of membrane receptors by thrombin, bradykinin, platelet-activating factor, or angiotensin II resulted in releases of inositol and arachidonic acid and $^{45}$Ca inflow as well as in inhibition of adenylate cyclase (43, 44). These effects of receptor stimulation, except for inositol release, were abolished by prior exposure of cells to IAP which had been previously reported to cause ADP-ribosylation of a membrane $M_r = 41,000$ protein (2). Thus, the occurrence of IAP-sensitive Ca$^{2+}$-linked signaling system is not restricted to mast cells. The IAP substrate with $M_r = 41,000$ appeared to mediate signal transductions arising from Ca-mobilizing receptors as well as from adenylate cyclase-linked receptors in a number of cell types. Further studies are now in progress in our laboratory to identify the exact site(s) mediated by the IAP substrate.

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REFERENCES
