Biochemical Pharmacology, Vol. 41, No. 12, pp. 1941–1948, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

DIFFERENTIAL EFFECTS OF PROPRANOLOL ON THE IgE-DEPENDENT, OR CALCIUM IONOPHORE-STIMULATED, PHOSPHOINOSITIDE HYDROLYSIS AND CALCIUM MOBILIZATION IN A MAST (RBL 2H3) CELL LINE

PEIYUAN LIN, GLORIA A. WIGGAN, ANN F. WELTON and ALASDAIR M. GILFILLAN*
Allergy and Inflammation Research, Department of Pharmacology, Hoffmann-La Roche, Nutley, NJ 07110, U.S.A.

(*Received* 18 *June* 1990; *accepted* 4 *January* 1991)

Abstract—Our previous studies demonstrated that propranolol, an inhibitor of phosphatidic acid phosphohydrolase (PAPase) (EC 3.1.3.4) blocks the IgE-dependent mediator release from a rat mast (RBL 2H3) cell line. To continue these studies, we examined the ability of propranolol to inhibit the IgE-dependent or ionomycin-mediated phosphoinositide hydrolysis and calcium mobilization in RBL 2H3 cells. RBL 2H3 cells, sensitized with mouse monoclonal anti-trinitrophenol IgE (anti-TNP IgE), were stimulated to release both histamine and peptidoleukotrienes (LT) in response to a suboptimal concentration of trinitrophenol-ovalbumin conjugate (TNP-OVA) or ionomycin. Preincubation of the cells with *d,l*-propranolol (300 μM) significantly ($P < 0.05$) inhibited the effects of both TNP-OVA and ionomycin on histamine and LT release. There was no difference in potency for the different isomers of propranolol, indicating that these effects were not a consequence of an effect on β_2-adrenergic receptors. TNP-OVA produced a rapid hydrolysis of phosphoinositides resulting in a time-dependent increase in mono- (IP_1), di- (IP_2), tri- (IP_3), and total inositol phosphate production. Ionomycin also produced a rapid increase in total inositol phosphate production; however, this largely reflected an accumulation of IP_1. Both secretagogues produced a rapid elevation in cytosolic free calcium ($[Ca^{2+}]_i$); however, the effect of ionomycin maximized within a much shorter time frame than the effect of TNP-OVA. The effects of TNP-OVA on phosphoinositide hydrolysis and increase in $[Ca^{2+}]_i$ were inhibited by propranolol over exactly the same concentration range as the effects of this compound on TNP-OVA-stimulated mediator release. In contrast, propranolol had no effect on the increase in $[Ca^{2+}]_i$ and phosphoinositide hydrolysis in response to ionomycin. Taken together, these results suggest that PAPase/phospholipase D (PLD) (EC 3.1.4.4) activation may be a prerequisite for both IgE-dependent and ionomycin-stimulated mediator release from RBL 2H3 cells. Although other explanations are possible, the data further suggest that receptor-mediated, but not ionophore-stimulated, phosphoinositide hydrolysis and $[Ca^{2+}]_i$ in RBL 2H3 cells may be regulated by a propranolol-sensitive pathway involving possible activation of PAPase.

Activated mast cells and basophils release a number of important inflammatory mediators which are capable of producing much of the symptomatology associated with immediate-type hypersensitivity allergic reactions [1]. These mediators can be resolved into two broad categories: (1) pre-synthesized mediators, such as histamine and serotonin, that are stored in discrete granules which are released by the process of exocytosis; and (2) *de novo* synthesized mediators such as leukotrienes and prostaglandins, that are synthesized as a consequence of cell activation, and then subsequently released. Mediator release is generally initiated when an appropriate antigen binds to and cross-links immunoglobulin E (IgE) occupying specific IgE receptors ($FC_\varepsilon RI$) on the surface of mast cells or basophils. The signal transduction pathway linking cross-bridging of the IgE receptors to degranulation appears to involve G protein [2–4] mediated activation of phospholipase C with subsequent phosphoinositide hydrolysis [5, 6]. The resulting production of inositol phosphates and diacylglycerol increases cytosolic calcium levels [7] and activates protein kinase C, respectively [8, 9]. The role for these processes in the IgE-dependent arachidonic acid metabolite release is unclear; however, studies indicate that phospholipase A_2 may be under G protein regulation [10].

Recent studies have demonstrated that phospholipase D (PLD) may also play an obligatory role in the signal transduction cascade leading to IgE-dependent mediator release [11–14]. This enzyme acts at the terminal phosphodiester groups of phospholipids, primarily phosphatidylcholine (PC), to yield phosphatidic acid (PA). The PA produced by the action of PLD can be subsequently dephosphorylated by the action of phosphatidic acid phosphohydrolase (PAPase) to form diacylglycerol (DAG). This increase in cellular DAG results in the activation of protein kinase C [15] and ultimately exocytosis [16].

Our earlier studies demonstrated that *d,l*-propranolol, an inhibitor of PAPase [17], prevents the IgE-dependent formation of DAG from PA in RBL 2H3 cells and that this results in the inhibition

* Author to whom correspondence should be addressed.

of IgE-dependent mediator release [14]. To further explore the role of the PLD/PAPase pathway in the signal transduction cascade linking IgE receptors to mediator release from mast cells and basophils, we examined the effect of propranolol on IgE-dependent or ionomycin-stimulated phosphoinositide hydrolysis and calcium mobilization in RBL 2H3 cells. These properties were compared to the ability of propranolol to inhibit mediator release in response to both secretagogues. This study has been presented previously, in part, in abstract forms [13, 18].

METHODS

Cell culture conditions. RBL 2H3 cells were maintained attached to 175 cm^2 tissue culture flasks (Falcon, Becton Dickinson, Lincoln Park, NJ) in culture medium consisting of Eagle's Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY) in a humidified atmosphere of 95% air, 5% CO_2 at 37°. For secretion experiments, the cells were plated in the flasks at a density of between 1 and 25 $\times$ 10^6 cells in 40 mL using the above conditions. These cells were grown to confluence and then dislodged from the plastic using trypsin (0.05%) and EDTA (0.02%) solution (Gibco). After rinsing, the cells were replated at a density of 1 $\times$ 10^6 cells in 1.5 mL of medium in 35-mm diameter tissue culture dishes (Falcon, Becton Dickinson).

Secretion experiments. The cells were incubated in the above medium containing mouse monoclonal anti-trinitrophenol IgE (anti-TNP IgE, 0.5 μg/mL) for 17–20 hr and then rinsed thoroughly with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 5.6 mM glucose, 10 mM HEPES, 1.8 mM $CaCl_2$, and 1.3 mM $MgSO_4$; pH 7.4). This was followed by the addition of 1 mL of this buffer to each dish and after a 5-min preincubation, the cells were triggered by the addition of TNP-ovalbumin conjugate (TNP-OVA). The inhibitors were routinely added 2–5 min prior to the addition of TNP-OVA. All compounds were added in 10 μL volumes of solutions 100-fold more concentrated than the final required concentrations, and an appropriate volume of the compound vehicle was added to the control dishes. After 30 min, the incubation medium was carefully removed and spun at 200 *g* for 5 min to remove any free floating cells. Distilled water was added to the cells remaining attached to the dish, and the cellular material was removed by freeze/thawing and then scraping with a rubber policeman.

Samples to be analyzed for histamine release were initially diluted with distilled water and then mixed with equal volumes of perchloric acid (0.8%). Samples for leukotriene assay were removed prior to processing the medium samples for histamine release and were stored at −20° until assayed.

Calcium studies. Cells to be examined for their cytosolic free calcium ($[Ca^{2+}]_i$) content were plated at a density of 25 $\times$ 10^6 cells in 40 mL of medium in 175 cm^2 flasks and incubated overnight in medium containing anti-TNP IgE as previously described. The cells were then dislodged from the plastic by the procedure outlined in the previous section. Next, the dislodged cells were rinsed twice with phosphate-free HEPES buffer containing 0.1% bovine serum albumin. The cells were then resuspended at a density of 1 $\times$ 10^6 cells/mL in this buffer containing fura-2 AM (10 μM) and incubated for 45 min at 37° in a shaking water bath. The cells were then rinsed three times and resuspended at a density of 1 $\times$ 10^6 cells/mL in the HEPES buffer. These cells were kept at 4° until conducting each experiment.

The fluorescence of the cell suspensions was examined using a Perkin-Elmer LS-5B spectrofluorometer using an emission wavelength of 505 nm and an excitation wavelength of 340 nm with data points examined at 8-sec intervals. During the experiments, the cells were maintained at 37° by means of circulating warmed water through a heated jacket in the cuvette chamber of the spectrofluorometer. At this temperature, there was a gradual increase in the fluorescence due to leaking of fura-2 from the cells into the bathing medium. We corrected for this leak by establishing a resting intracellular calcium level for each experiment by quenching the external fura-2 by the addition of Mn^{2+} (0.1 mM). After chelating the Mn^{2+} by the addition of calcium diethylenetriaminepentaacetic acid (0.5 mM), we determined the rate of the fura-2 leak by calculating the linear regression of the slope prior to the addition of the stimulating agent. This procedure was carried out for each experiment and the data were corrected for this leak accordingly.

The cells were preincubated for at least 5 min to allow the cells to equilibrate to 37° prior to the addition of the test compounds. When the effects of propranolol were examined, it was added 2 min prior to TNP-OVA. All compounds were added in 20 μL volumes of 100-fold more concentrated solutions than the final required concentration. The experiments were terminated 2–5 min later, at which time we determined the fluorescence due to a saturating calcium concentration (F_{max}) by permeabilizing the cells with digitonin (50 μM) in the presence of 10 mM calcium. The fluorescence due to zero calcium (F_{min}) was determined by quenching the fura-2 with Mn^{2+} (5 mM) to determine the autofluorescence of the system (F_{auto}). F_{min} was then calculated from the formula:

$$F_{min} = F_{auto} + (0.27 \cdot (F_{max} - F_{auto})).$$

This was determined to be the relationship between F_{auto} and F_{min} under our experimental conditions.

Cytosolic free calcium was then determined using the formula:

$$[Ca^{2+}]_i = 224\ nM \cdot (F_{340} - F_{min})/(F_{max} - F_{340})$$

where 224 nM is the K_d for calcium for fura-2 [19] and F_{340} is the fluorescence observed at 340 nm under our experimental conditions.

Inositol phosphate production. To assess the effect of propranolol on inositol phosphate production, the cells were plated as described above at a density of 25 $\times$ 10^6 cells/mL in medium containing [^{3}H]*myo*-inositol (10 μCi/mL). After incubating overnight, the cells were rinsed thoroughly and then incubated in medium containing cold *myo*-inositol (10 mM) for

1 hr. The cells were then dislodged from the tissue culture flasks as described above and rinsed thoroughly with HEPES buffer containing lithium chloride (10 mM). Next, the cells were resuspended at a density of 1×10^6 cells/mL in the HEPES buffer containing lithium and incubated for 5 min at 37° before TNP-OVA or ionomycin was added. Propranolol was added routinely 2 min prior to the addition of TNP-OVA or ionomycin. At predetermined times, the reaction was terminated by the addition of methanol/chloroform (2:1). The inositol phosphates were extracted and then separated by sequential elution from Dowex formate (Bio-Rad, Richmond, CA) anion exchange columns with increasing concentrations of ammonium formate solution as described previously [20].

The lipid fraction was extracted [21] after acidification, and the inositol phosphates were calculated as a percentage of total radioactivity.

Viability studies. The viability of the treated cells was assessed by measuring the rate of lactate dehydrogenase release in these cultures compared to non-treated cells.

Assays. The histamine content of the perchloric acid precipitated samples was assessed by initially centrifuging the samples at 500 *g* for 10 min and then measuring the histamine content of the resulting supernatant by an automated spectrofluorometric assay [22]. LT was assayed by a radioimmunoassay kit (New England Nuclear, Wilmington, DE), and lactate dehydrogenase (EC 1.1.1.27) was assayed by following the disappearance of NADH at 340 nm [23].

Statistics and data presentation. Histamine release was presented as the amount in the medium as a percentage of the total (i.e. medium + cellular content). Leukotriene release was presented as nanograms of leukotriene found in the medium per 10^6 cells. Unless otherwise indicated, the results were presented as means ± SEM and were analyzed by Student's two-tailed *t*-test for paired samples.

Materials. RBL 2H3 cells were obtained from Dr. Hydar Ali (Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) and mouse monoclonal anti-TNP IgE and TNP-OVA were obtained from Dr. John Hakimi, Department of Immunopharmacology, Hoffmann-La Roche, Nutley, NJ. Fura-2 AM was obtained from Molecular Probes (Eugene, OR). Dowex anion exchange resin (formate form, 100–200 mesh) was obtained from Bio-Rad and [^{3}H]*myo*-inositol from Amersham, Arlington Heights, IL. Other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

Effect of propranolol on histamine and leukotriene release from RBL 2H3 cells. In cells sensitized by pre-exposure to anti-TNP IgE, a suboptimal concentration of TNP-OVA (5 ng/mL) [24] resulted in a significant increase in both histamine (29.26 ± 4.57% of cellular content, N = 4; $P < 0.05$) and LT (3.26 ± 1.17 ng/10^6 cells, N = 5; $P < 0.05$) release from basal levels of 3.29 ± 0.39% of cellular content for histamine and an undetectable level (<0.1 ng/10^6 cells) for LT. Maximal release from these cells was determined previously to be 49.2% for histamine and 12.4 ng/10^6 cells for LT at 10 ng/mL of TNP-OVA [24]. The calcium ionophore, ionomycin (1 μM), significantly enhanced both histamine (39.4 ± 6.62% of cellular content, N = 4, $P < 0.05$) and leukotriene release (3.12 ± 0.52 ng/10^6 cells, N = 0.05, $P < 0.05$) from a basal level of 3.38 ± 0.67% of cellular content for histamine and an undetectable level (<0.1 ng/10^6 cells) for LT.

When cells were pretreated with *d,l*-propranolol, there was a concentration-dependent inhibition of

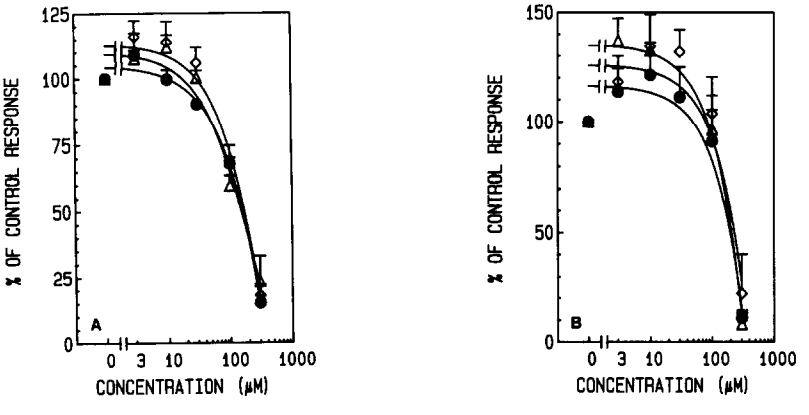

Fig. 1. Effects of *l*-, *d*-, and *d,l*-propranolol on the IgE-dependent release of (A) histamine and (B) leukotrienes (LT) from RBL 2H3 cells. Key: (◇) *l*-propranolol; (△) *d*-propranolol; and (●) *d,l*-propranolol. The results are means ± SEM of 4–5 experiments, and the values represent the percentage of the responses obtained with TNP-OVA (5 ng/mL) in the absence of propranolol. These values were 29.2 ± 4.5% of cellular content for histamine release and 3.26 ± 1.17 ng/10^6 cells for LT release above a basal release of 3.29 ± 0.39% of total cellular content and <0.1 ng/10^6 cells for histamine and LT release, respectively. Propranolol was added 2 min prior to the addition of TNP-OVA, and the experiments were terminated 30 min later. Other experimental details are outlined in Methods.

the TNP-OVA-stimulated histamine release (Fig. 1). When analyzed statistically using Student's *t*-test for paired samples, propranolol at 300 μM significantly reduced the release of histamine to 6.27 ± 1.03% of total cellular content above basal release ($P < 0.05$; $N = 4$). The effect of propranolol was not dependent on its isomeric form as the potencies of the *l*-, *d*-, and the racemic form were identical (Fig. 1A). The effect was also not a consequence of cell damage as there was no evidence of increased release of lactate dehydrogenase (a cytosolic marker) in response to the agents (data not shown). In addition to the effect on degranulation, the release of LT was also inhibited by *d,l*-propranolol (Fig. 1B). The release of LT was reduced to 0.42 ± 0.14 ng/10^6 cells ($P = 0.57$; $N = 5$). The concentration range for this phenomenon was identical to that on histamine release.

We examined the effects of propranolol on ionomycin-stimulated mediator release. This calcium ionophore bypasses the IgE receptor and any G protein(s) associated with the IgE receptor. *d,l*-Propranolol inhibited the secretion of both histamine and LT (Fig. 2) in response to ionomycin. When analyzed statistically using Student's *t*-test for paired samples, propranolol at 300 μM significantly reduced the release of histamine to 10.4 ± 4.64% of total cellular content above basal release ($P < 0.05$; $N = 4$) and LT to 0.32 ± 0.20 ng/10^6 cells ($P < 0.05$; $N = 5$). The concentrations of *d,l*-propranolol required to inhibit the responses of ionomycin-mediated secretion were identical to the concentrations required to inhibit the TNP-OVA-mediated secretion.

Effect of propranolol on $[Ca^{2+}]_i$ *in RBL 2H3 cells.* Both ionomycin and TNP-OVA resulted in a rapid increase in cytosolic free calcium levels. The responses due to ionomycin generally maximized within 10–30 seconds after addition to the cells (Fig. 3). The responses observed with TNP-OVA were slower in nature and maximal response was generally observed between 2 and 3 min after the addition of this secretagogue (Fig. 4). The maximal responses of both agents, however, were similar.

Propranolol, when added to the cuvette 2 min prior to the addition of TNP-OVA, inhibited the TNP-OVA-stimulated increase in $[Ca^{2+}]_i$ (Fig. 4). The inhibitory effect of propranolol on the TNP-OVA-stimulated increase in $[Ca^{2+}]_i$ was concentration dependent (Fig. 5). Propranolol (300 μM) significantly reduced the effect of TNP-OVA on $[Ca^{2+}]_i$ from 294.7 ± 30.8 to 98.64 ± 7.0 nM above the resting levels of 119.0 ± 7.8 and 116.1 ± 4.6 nM, respectively ($P < 0.05$; Student's *t*-test for paired samples). Similar concentrations of propranolol were required to produce these results as were required to inhibit TNP-OVA-stimulated mediator release. In contrast to the effects on TNP-OVA-stimulated increase in $[Ca^{2+}]_i$, propranolol (300 μM) had no effect on the increase in $[Ca^{2+}]_i$ produced by ionomycin ($P > 0.05$; Student's *t*-test for paired samples (Figs. 3 and 5).

Effect of propranolol on inositol phosphate generation. In cells prelabeled with [^{3}H]*myo*-inositol, TNP-OVA produced a rapid increase in the formation of mono-, di-, tri-, (Fig. 6) and total inositol phosphates (Fig. 7). TNP-OVA-stimulated IP_2 and IP_3 levels essentially maximized within 10 min of application of the stimulus. The TNP-OVA-stimulated IP_1 levels, however, continued to increase with time for at least 30 min after the addition of the stimulus. Ionomycin (1 μM) also stimulated total inositol phosphate production

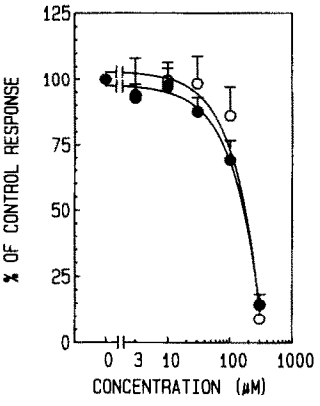

Fig. 2. Effect of *d,l*-propranolol on the ionomycin-stimulated release of histamine and leukotrienes from RBL 2H3 cells. Key: (●) histamine release; and (○) LT release. The results are means ± SEM of 4–5 experiments, and the values represent the percentage of the responses obtained with ionomycin (1 μM) in the absence of the propranolol. These values were 36.0 ± 6.8% of total cellular content for histamine and 3.12 ng/10^6 cells for LT above the basal levels of 3.12 ± 0.82% of total cellular content and <0.1 ng/10^6 cells for histamine and LT release, respectively. Propranolol was added 2 min prior to the addition of ionomycin, and the experiments were terminated 30 min later. Other experimental details are outlined in Methods.

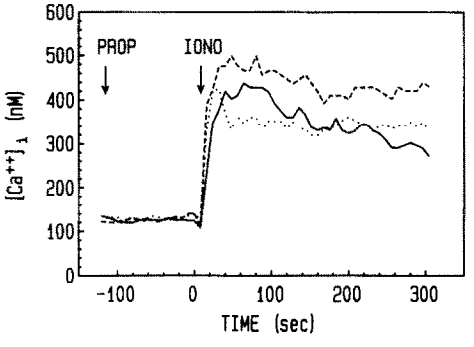

Fig. 3. Effect of ionomycin on $[Ca^{2+}]_i$ in the absence and presence of *d,l*-propranolol. Key: PROP, propranolol; IONO, ionomycin; (——) 1 μM ionomycin alone; (– – –) 1 μM ionomycin + 100 μM propranolol; and (·····) 1 μM ionomycin + 300 μM propranolol. The results are typical of at least $N = 4$, the means and standard errors of which are shown in Fig. 5. The cells were loaded with fura-2 AM (10 μM) for 45 min as described in Methods. The fluorescence of the cells and hence the cytosolic free calcium levels were then determined before and after the addition of the test compounds (arrows), again as detailed in Methods.

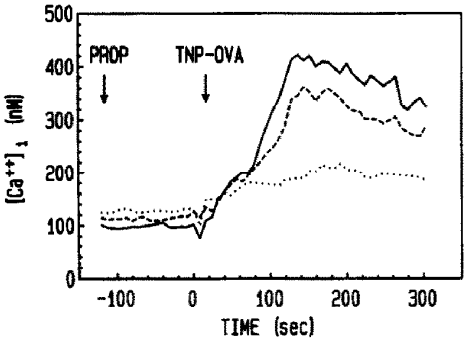

Fig. 4. Effect of TNP-OVA on $[Ca^{2+}]_i$ in the absence and presence of *d,l*-propranolol. Key: PROP, propranolol; (——) 5 ng/mL TNP-OVA alone; (– – –) 5 ng/mL TNP-OVA + 100 μM propranolol; and (·····) 5 ng/mL TNP-OVA + 300 μM propranolol. The results are typical of at least N = 4, the means and standard errors of which are shown in Fig. 5. The cells were sensitized with anti-TNP IgE (0.5 μg/mL) and then loaded with fura-2 AM (10 μM) for 45 min as described in Methods. The fluorescence of the cells and hence the cytosolic free calcium levels were then determined before and after the addition of the test compounds (arrows), again as detailed in Methods.

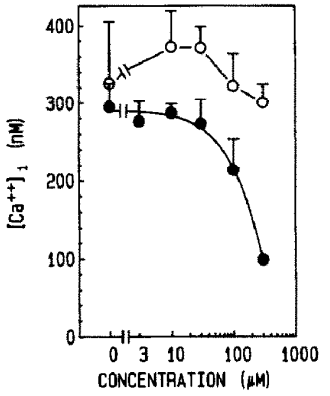

Fig. 5. Concentration–effect relationship for propranolol on the increase of $[Ca^{2+}]_i$ in response to TNP-OVA and ionomycin. Key: (●) 5 ng/mL TNP-OVA + propranolol; and (○) 1 μM ionomycin + propranolol. The results are means ± SEM of 4 experiments and represent the increase in cytosolic free calcium in response to TNP-OVA above the resting pretreatment levels of between 96.9 and 160.0 nM. The experiments were conducted as described in the legends to Figs. 3 and 4.

(control = 2.44% of total radioactivity, treated = 3.28% of total radioactivity: $P < 0.05$, N = 4). This increase in phosphoinositide hydrolysis, however, primarily reflected an increase in IP_1 accumulation. The initial increases in phosphoinositide hydrolysis in response to both stimuli were similar during the first 120 sec. After this time the response to ionomycin leveled off (data not shown) as the response to TNP-OVA continued to rise to a maximum at 15 min (Fig. 7).

We examined the effect of *d,l*-propranolol on the

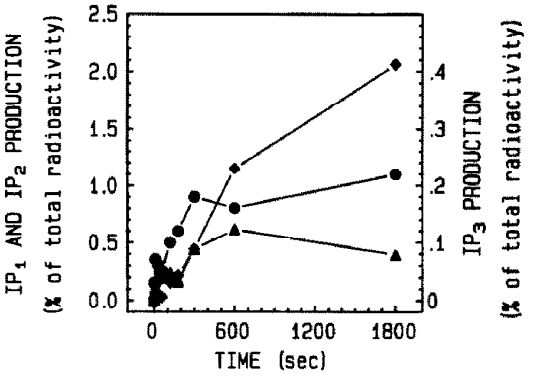

Fig. 6. Effect of TNP-OVA on the formation of IP_1, IP_2, and IP_3 in RBL 2H3 cells. Key: (◆) IP_1; (▲) IP_2; and (●) IP_3. The results are the means of duplicate experiments for IP_1 and IP_2 and a representative observation for IP_3. The time courses of these experiments are typical of several such observations. The values represent the percentage of total radioactivity above the resting levels of 1.2 ± 0.05, 0.30 ± 0.06, and 0.14% for IP_1, IP_2, and IP_3, respectively. The total labeling in the control cells was 108,000 cpm per culture (mean of 2), and this was unaffected by TNP-OVA. The experiments were conducted as described in the legend to Table 1 and terminated at the times indicated on the graph. The inositol phosphate levels were then determined as described in Methods. The concentration of TNP-OVA was 10 ng/mL.

TNP-OVA- and ionomycin-stimulated phosphoinositide production by exposing the cells to propranolol 2 min prior to the addition of the stimuli. The experiments were then terminated 15 min later. Under these conditions, *d,l*-propranolol (300 μM) inhibited the TNP-OVA-stimulated formation of

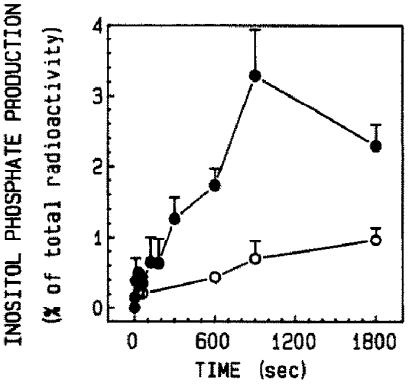

Fig. 7. Time courses of the effect of propranolol on the increase in inositol phosphate production in response to TNP-OVA in RBL 2H3 cells. Key: (●) (10 ng/mL) TNP-OVA alone; and (○) 10 ng/mL TNP-OVA + 300 μM *d,l*-propranolol. The results are the means ± SEM of up to 4 separate experiments and represent the total inositol phosphate production above the basal levels of 2.44 ± 0.27% (N = 6) of total radioactivity (89,100–174,000 cpm per culture). The experiments were conducted as described in the legend to Table 1 and were terminated at the times indicated on the graph. The inositol phosphate levels were then determined as described in Methods.

Table 1. Effect of *d,l*-propranolol on the production of inositol phosphates in response to TNP-OVA and ionomycin

Inositol phosphate	− Propranolol	+ Propranolol	P
TNP-OVA			
IP_1	2.25 ± 0.27	0.76 ± 0.16	<0.05
IP_2	0.89 ± 0.26	0.28 ± 0.10	<0.05
IP_3	0.25 ± 0.07	0.04 ± 0.04	<0.05
Total IPs	3.29 ± 0.66	0.70 ± 0.25	<0.05
Ionomycin			
IP_1	0.46 ± 0.12	0.76 ± 0.16	NS
Total IPs	0.56 ± 0.15	0.77 ± 0.19	NS

Results are means ± SEM of 4 experiments and were analyzed by Student's *t*-test for paired samples. The values represent the percent of total radioactivity found in the indicated fractions above that found in untreated cells. The basal levels were 2.25 ± 0.27, 0.89 ± 0.2, 0.28 ± 0.05, and 3.29 ± 0.66% of total radioactivity for IP_1, IP_2, IP_3, and total inositol phosphates, respectively. The cells were loaded with [^{3}H]*myo*-inositol, and the experiments were conducted as described in Methods and the legend to Fig. 6. Concentrations: TNP-OVA, 5 ng/mL; ionomycin, 1 μM; and *d,l*-propranolol, 300 μM. NS = not significant.

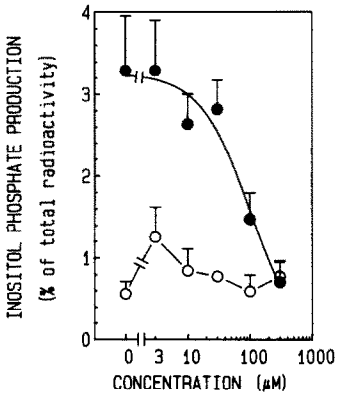

Fig. 8. Effect of *d,l*-propranolol on the inositol phosphate formation in response to TNP-OVA and ionomycin. Key: (○) 1 μM ionomycin + propranolol; and (●) 5 ng/mL TNP-OVA + propranolol. The results are the means ± SEM of 4 experiments and represent the total inositol phosphate production above the basal levels of 2.72 ± 0.3% of total radioactivity. The total radioactivity in the control cells was 154,000 ± 12,700 cpm per culture (N = 4), and this labeling was unaffected by TNP-OVA, ionomycin or propranolol. The experiments were conducted as described in the legend to Table 1 and terminated after 15 min. The inositol phosphate levels were then determined as described in Methods.

IP_1, IP_2, IP_3 (Table 1) and total inositol phosphates (Fig. 8). The inhibitory effect of propranolol on IgE-dependent inositol phosphate production was observed at all times (Fig. 7). In contrast to the above observations, the increase in IP_1 (Table 1) and total inositol phosphate production by ionomycin (Fig. 8) was unaffected by preincubation with *d,l*-propranolol.

DISCUSSION

In response to the appropriate stimulus, RBL 2H3 cells, and other models of mast cell function, release both preformed, granule-associated mediators such as histamine and serotonin, and *de novo* synthesized mediators such as leukotrienes and prostaglandins. The nature of the release of both groups of mediators from these cells is very similar, and it has been suggested that the signal transduction pathways leading to the release of these mediators may be linked [14, 25], at least in the initial stages. As discussed earlier, there is convincing evidence for a role for phosphoinositide hydrolysis with resulting elevation of $[Ca^{2+}]_i$ and activation of protein kinase C in these early stages. Recent evidence has also suggested an important role for PLD in conjunction with PAPase in both histamine [11] and arachidonic acid metabolite release [13, 14] from a number of mast cell models including RBL 2H3 cells. We previously demonstrated that *d,l*-propranolol, an inhibitor of PAPase, blocks IgE-dependent mediator release and formation of DAG from PA in RBL 2H3 cells [14]. We have extended these studies to examine the effects of propranolol on receptor and ionophore-stimulated calcium mobilization and phosphoinositide hydrolysis in RBL 2H3 cells and compared these effects to the actions of propranolol on mediator release.

Preincubation of RBL 2H3 cells with *d,l*-propranolol resulted in a concentration-dependent inhibition of the release of both histamine and LT in response to TNP-OVA. These responses were independent of the isomeric form of propranolol and were observed at higher concentrations than those required to block the β_2-adrenergic receptor. It is, therefore, unlikely that the responses we observed were a consequence of the ability of propranolol to act at this receptor. The concentrations, however, were similar to those that have been reported to block the IgE-dependent formation of DAG from PA in RBL 2H3 cells [14] and the actions of PAPase in human neutrophils [26]. The stimulatory effects of ionomycin on both histamine and LT release were also inhibited by preincubation with propranolol. The concentration of propranolol required for these effects was identical to those required for inhibition of the TNP-OVA responses on mediator release. Ionomycin circumvents the requirement of IgE receptor occupancy and G-protein activation for mediator release from RBL 2H3 cells. The effects of propranolol on IgE-dependent release were, therefore, unlikely to be due to a direct action on the IgE receptor or associated G protein. The effects of ionomycin, and related compounds such as A23187, are linked to their abilities to function as calcium ionophores. Recent studies have demonstrated that they share the ability of agents that cross-bridge IgE receptors to stimulate phosphoinositide hydrolysis [27] and activate PLD [28]. Our studies confirm that both $FC_\varepsilon RI$ cross-bridging and ionomycin treatment result in phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ in RBL 2H3 cells. The time courses of these responses were similar to those previously reported [5–7, 27].

When propranolol was added to the cells, there was a concentration-dependent inhibition of phosphoinositide hydrolysis in response to TNP-OVA. In contrast, preincubation of the cells with propranolol failed to inhibit the increase in phosphoinositide hydrolysis in response to ionomycin, despite the inhibitory effects on mediator release described above. These results indicate that the effect of propranolol on IgE-dependent phosphoinositide hydrolysis was not due to a direct action of propranolol on phospholipase C. It is possible, however, that ionomycin and IgE activate different isoforms of phospholipase C, with propranolol selectively inhibiting the isoform directly linked to the IgE receptor. The IgE-dependent increase in $[Ca^{2+}]_i$ was also inhibited by propranolol over the same concentration range as required for the inhibition of mediator release. Again, propranolol failed to inhibit the response to ionomycin. This is similar to the report of Drabikova *et al.* [29] who demonstrated that the effect of compound 48/80 but not A23187 on $[^{45}Ca^{2+}]$ uptake into rat peritoneal mast cells is inhibited by propranolol. The above results, therefore, demonstrate that the inhibitory effects of propranolol on mediator release are not dependent on the effects on phosphoinositide hydrolysis and calcium mobilization, but likely reflect its ability to inhibit the activation of PAPase.

In conclusion, the results of this study demonstrate that propranolol inhibited the release of inflammatory mediators from RBL 2H3 cells in response to TNP-OVA and ionomycin. The inhibitory effects of propranolol on mediator release in response to TNP-OVA were accompanied by inhibition of the TNP-OVA-stimulated increase in $[Ca^{2+}]_i$ and phosphoinositide hydrolysis. In contrast, the increases in these parameters in response to ionomycin were unaffected. Although other interpretations are certainly possible, the data suggest that receptor-mediated, but not ionophore-stimulated, phosphoinositide hydrolysis and $[Ca^{2+}]_i$ in RBL 2H3 cells may be regulated by a propranolol-sensitive pathway involving possible activation of PAPase.

REFERENCES

1. Casale TB and Marom Z, Mast cells and asthma. The role of mast cell mediators in the pathogenesis of allergic asthma. *Ann Allergy* **51**: 2–6, 1987.
2. Penner R, Multiple signaling pathways control stimulus–secretion coupling in rat peritoneal mast cells. *Proc Natl Acad Sci USA* **85**: 9856–9860, 1988.
3. WoldeMussie E, Ali H, Takaishi T, Siraganian RP and Beaven MA, Identification of variants of the basophilic leukemia (RBL-2H3) cells that have defective phosphoinositide responses to antigen and stimulants of guanosine 5'-triphosphate-regulatory proteins. *J Immunol* **139**: 2431–2438, 1987.
4. Wilson BS, Deanin GG, Standefer JC, Vanderjagt D and Oliver JM, Depletion of guanine nucleotides with mycophenolic acid suppresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. *J Immunol* **143**: 259–265, 1989.
5. Cunha-Melo JR, Dean NM, Moyer JD, Maeyama K and Beaven MA, The kinetics of phosphoinositide hydrolysis in rat basophilic leukemia (RBL-2H3) cells varies with the type of IgE receptor cross-linking agent used. *J Biol Chem* **262**: 11455–11463, 1987.
6. Beaven MA and Cunha-Melo JR, Membrane phosphoinositide-activated signals in mast cells and basophils. *Prog Allergy* **42**: 123–184, 1988.
7. Beaven MA, Rogers J, Moore JP, Hesketh TR, Smith GA and Metcalfe JC, The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J Biol Chem* **259**: 7129–7136, 1984.
8. White JR, Pluznik DH, Ishizaka K and Ishizaka T, Antigen-induced increase in protein kinase C activity in plasma membrane of mast cells. *Proc Natl Acad Sci USA* **82**: 8193–8197, 1985.
9. Nagao S, Nagata K, Kohmura Y, Ishizuka T and Nozawa Y, Redistribution of phospholipid/Ca^{2+}-dependent protein kinase in mast cells activated by various agonists. *Biochem Biophys Res Commun* **142**: 645–653, 1987.
10. Narasimhan V, Holowka D and Baird B, A guanine nucleotide-binding protein participates in IgE receptor-mediated activation of endogenous and reconstituted phospholipase A_2 in a permeabilized cell system. *J Biol Chem* **264**: 1459–1464, 1990.
11. Gruchalla RS, Dinh TT and Kennerly DA, An indirect pathway of receptor-mediated 1,2-diacylglycerol formation in mast cells. I. IgE receptor-mediated activation of phospholipase D. *J Immunol* **144**: 2334–2342, 1990.
12. Kennerly DA, Diacylglycerol metabolism in mast cells. *J Biol Chem* **262**: 16305–16313, 1987.
13. Lin P, Wiggan GA, Welton AF and Gilfillan AM, Does diacylglycerol act as a source for arachidonic acid in rat basophilic leukemia (RBL 2H3) cells. *FASEB J* **4**: 494, 1990.
14. Lin P, Wiggan GA and Gilfillan AM, Activation of phospholipase D in a rat mast (RBL 2H3) cell line: A possible unifying mechanism for IgE-dependent degranulation and arachidonic acid metabolite release. *J Immunol* **146**: 1609–1616, 1991.
15. Rando RR, Regulation of protein kinase C activity by lipids. *FASEB J* **2**: 2348–2355, 1988.
16. Kennerly DA, Sullivan TJ, Sylvester P and Parker CW, Diacylglycerol metabolism in mast cells: A potential role in membrane fusion and arachidonic release. *J Exp Med* **150**: 1039–1044, 1979.
17. Koul O and Hauser G, Modulation of rat brain cytosolic phosphatidate phosphohydrolase: Effect of cationic amphiphilic drugs and divalent cations. *Arch Biochem Biophys* **253**: 453–461, 1987.
18. Lin P, Wiggan GA, Welton AF and Gilfillan AM, Possible role for phospholipase D (PLD) in leukotriene (LT) production in rat basophilic (RBL 2H3) cells. *FASEB J* **4**: A1939, 1990.
19. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
20. Gilfillan AM, Wiggan GA, Hope WC, Patel BJ and Welton AF, Ro 19-3704 directly inhibits the immunoglobulin E-dependent mediator release by a mechanism independent of its platelet-activating factor antagonist properties. *Eur J Pharmacol* **176**: 255–262, 1990.
21. Berridge MJ, Dawson RMC, Downes CP, Heslop JP and Irvine RF, Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**: 473–482, 1983.
22. Siraganian RP, An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* **57**: 383–391, 1974.
23. Wróblewski F and LaDue JS, Lactate dehydrogenase activity in blood. *Proc Soc Exp Biol Med* **90**: 210–213, 1955.
24. Gilfillan AM, Wiggan GA and Welton AF, Pertussis toxin pretreatment reveals differential effects of

adenosine analogs on IgE-dependent histamine and peptidoleukotriene release from RBL-2H3 cells. *Biochim Biophys Acta* **1052**: 467–474, 1990.

25. Gilfillan AM, Wiggan GA and Welton AF, The effects of inhibitors of protein kinase C activity on the IgE dependent release of histamine and leukotriene C_4 from RBL 2H3 cells. *Agents Actions* **30**: 418–425, 1990.
26. Billah MM, Eckel S, Mullmann TJ, Egan RW and Siegel MI, Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. *J Biol Chem* **264**: 17069–17077, 1989.
27. Lo TN, Saul W and Beaven MA, The actions of Ca^{2+} ionophores on rat basophilic (2H3) cells are dependent on cellular ATP and hydrolysis of inositol phospholipids. A comparison with antigen stimulation. *J Biol Chem* **262**: 4141–4145, 1987.
28. Gonzaga H and Beaven MA, A role for phospholipase D in the exocytotic response of a cognate (RBL 2H3) mast cell line to Ca^{2+}-ionophore, A23187. *FASEB J* **4**: A546, 1990.
29. Drabikova K, Pecivova J and Nosal R, Beta-adrenoceptor blocking drugs and calcium transport in isolated rat mast cells. *Agents Actions* **27**: 33–35, 1989.