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Pharmacology of IgE-Mediated Desensitization of Human Basophils: Effects of Protein Kinase C and Src-Family Kinase Inhibitors

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ABSTRACT. IgE-mediated down-regulation of secretion from basophils and mast cells is an important component of the overall cellular response that determines the ultimate extent of mediator release. The down-regulatory process that occurs during active secretion has also been associated with the methodological phenomenon called desensitization, but the mechanisms underlying desensitization are not understood. A variety of studies have suggested that activation of protein kinase C (PKC) results in down-regulation of IgE-mediated secretion so we have examined the effect of the PKC inhibitors Ro-31–8220 (3-[1-[3-amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide) and bis-indolylmaleimide II on desensitization in human basophils. At concentrations that have been shown previously to inhibit PKC-mediated functions in basophils completely, these two drugs had no effect on IgE-mediated desensitization. We did find, however, that the src-family kinase inhibitors PP1 [4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine] and PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine] inhibited desensitization as well as secretion. These data suggest that PKC has little role in down-regulating the IgE-mediated basophil response. However, like the activation signaling cascade, the desensitization process is dependent on the activation of src family kinases. BIOCHEM PHARMACOL **60**;11:1717–1727, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. human basophils; desensitization; protein kinase C; histamine release; src kinases; syk kinase

IgE-mediated secretion of mediators from human basophils results from a balance of reactions that both drive secretion or serve to limit its extent. For human basophils, the reactions that serve to limit secretion generally have been associated with the experimental phenomenon known as desensitization [1]. This phenomenon is demonstrated operationally by stimulating basophils with antigen under conditions suboptimal for secretion, usually in the absence of extracellular calcium [2]. The longer this is done, the more poorly the cells secrete when calcium is returned to the medium. When desensitized, basophils cease secreting all three major classes of mediators (granule contents such as histamine, rapidly formed lipid mediators such as LTC_4,† and slowly formed mediators such as IL-4) [3, 4]. Desensitization with one antigen may affect secretion induced by another non-cross-reacting antigen (in cells displaying IgE with more than one antigenic specificity) [5], but the degree to which other antigens are affected depends on the density of IgE specific for the antigen used to desensitize the cell [6]. Thus, desensitization may be specific or nonspecific in nature relative to all the IgE specificities displayed on the cell surface. However, while desensitization induced through the IgE receptor may affect other IgE receptor-mediated signaling, it has no inhibitory effect on signaling induced by a variety of other secretagogues. This set of observations suggests that specific pathways relevant only to IgE-mediated release are altered during desensitization. It also appears that desensitization alters internal signaling since expression of the receptor (and its associated IgE antibody) is not altered in desensitized human basophils [7]. The nature of this alteration in signaling remains unknown, although recent studies indicate that events upstream of syk phosphorylation are altered in cells that experience only specific desensitization, whereas events downstream of syk phosphorylation are altered during nonspecific desensitization.‡ Over the years, several hypotheses have been advanced to explain the desensitization process although most have not yet been tested in human cells.

One of these hypotheses concerns the role that PKC might play in limiting the secretion reaction. Studies in the mid-1980s indicated that activation of PKC with phorbol

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† *Abbreviations*: LTC_4, leukotriene C_4; IL-3, interleukin-3; IL-4, interleukin-4; PKC, protein kinase C; BPO, benzylpenicilloyl; EACA, ε-aminocaproic acid; PMA, phorbol 12-myristate 13-acetate; BIS I and II, bis-indolylmaleimide I and II; fMLP, formyl-methionyl-leucyl-phenylalanine; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; HSA, human serum albumin; FBS, fetal bovine serum; ECL, Enhanced Chemiluminescence; and OVA, ovalbumin.

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esters would inhibit the IgE-mediated elevation in cytosolic calcium [8]. This was shown subsequently to be true using human basophils and mast cells [9, 10]. These studies suggested that PKC might play a down-regulatory role during secretion and consistent with this hypothesis were observations in rat basophilic leukemia (RBL) cells that PKC is active during IgE-mediated secretion and that specific isozymes of PKC appeared to participate in both stimulatory and inhibitory signaling cascades [11–14]. Early studies in human basophils also indicated that a PKC-like activity was elevated during IgE-mediated secretion [15]. More recent studies also demonstrated that desensitization results in a loss in the cytosolic calcium elevation that normally occurs during secretion [4]. However, recent studies of human basophils have provided a different perspective on the role of PKC isozymes. There has been an inability to detect translocation of PKC isozymes during IgE-mediated stimulation, and newer PKC inhibitors—those not displaying inhibition of other kinases—were found to have little effect on mediator release. While these studies did suggest that PKC has little role in activating the basophil, there have been indications that PKC might still participate in down-regulation of secretion [16]. The same PKC inhibitors that did not inhibit mediator release did cause modest enhancement of mediator release at concentrations only slightly higher than the IC_{50} for inhibition of PMA-induced histamine release. While there are concerns about the specificity of these drugs at higher concentrations, the data suggested that desensitization might be inhibited by these drugs. Therefore, we examined the effects of selective PKC inhibitors on desensitization in basophils.

The earliest known events that follow aggregation of FcεRI ($\alpha\beta\gamma 2$) involve the phosphorylation of FcεRI β and γ subunits and the phosphorylation/activation of two receptor-associated tyrosine kinases [17–26]. The src-family kinase, lyn, has been implicated in the initial phosphorylation of the FcεRI β and γ subunits, which, in turn, leads to the recruitment of syk kinase to the immunotyrosine activation motifs of the γ2 subunits. Phosphorylation of syk ensues. Some of these steps have been examined in human basophils. A study by Benhamou *et al.* [27], using basophils from patients with acute basophilic leukemia or derived from bone marrow cultures, supported the basic model of lyn and syk phosphorylation early in the IgE-mediated response. More recently, studies by Kepley *et al.* [28] have provided further evidence that lyn and syk kinases are present and activated in normal peripheral blood basophils. We also have examined the kinetics of syk and lyn phosphorylation under a variety of conditions and find support for the basic model of secretion demonstrated in non-human cell lines. Recent studies in RBL cells have provided new hypotheses regarding regulation of secretion mediated through FcεRI. In one model, activation of early tyrosine kinase activity also leads to the recruitment, phosphorylation, and activation of phosphatases that may down-regulate the reaction. For example, SHIP (SH2 containing inositol phosphatase) has been shown to be recruited to the β subunit of FcεRI following activation, and its activity is dependent upon the activity of lyn and syk kinases [29]. Therefore, we also examined the ability of lyn kinase inhibitors to inhibit the process of desensitization (as well as activation) in human basophils.

MATERIALS AND METHODS

Reagents

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, human IgG, PMA, β-mercaptoethanol, Nonidet P-40 (Sigma); crystallized HSA (Miles Laboratories); FBS and RPMI 1640 containing 25 mM HEPES (GIBCO-BRL); Percoll (Pharmacia); 4α-PMA (LC Laboratories); and ionomycin (Calbiochem). BPO–HSA and BPO–EACA were synthesized as previously described [27]. gp120(HIV)–OVA conjugate was the gift of Dr. Frances Davis of the Tanox Corp. BIS I and II and Ro-31–8220 (3-[1-[3-amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide) were purchased from Calbiochem. The tyrosine kinase inhibitors PP1 and PP2 were purchased from Biomol and Calbiochem, respectively. All other reagents used were of the highest grade available.

Antibodies

Goat anti-human IgE was prepared as described previously; the antibody used for these studies represented the IgG fraction of goat serum prepared by DE-52 chromatography [30]. BPO-specific IgE was purified as described previously from the serum of penicillin allergic patients [7], and anti-gp120 IgE antibody was the gift of Dr. Frances Davis of the Tanox Corp. Mouse anti-human p72syk was purchased from Santa Cruz Biotechnology Inc., while mouse anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology. Sheep anti-mouse Ig horseradish peroxidase, streptavidin horseradish peroxidase conjugate, ECL western blotting detection agents, and ECL hyperfilm were all purchased from Amersham.

Buffers and Media

PIPES–albumin–glucose (PAG) buffer contained 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. Negative selection was performed in 1x PIPES buffer containing 2 mM EDTA and 0.5% BSA. Lysis buffer contained 20 mM Tris (pH 7.8), 150 mM sodium chloride, 1% Nonidet P-40, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM benzamidine, and 1 μg/mL of aprotinin. In the electrophoresis studies, 2x SDS sample buffer contained 0.5 M Tris–HCl (pH 6.8), 10% (w/v) SDS, 0.1% bromophenol blue, 20% glycerol,

and 5% mercaptoethanol; TBST buffer contained 12 mM Tris base (pH 7.5), 150 mM NaCl, and 0.05% Tween-20; running buffer contained 25 mM Tris base, 192 mM glycine, and 0.1% SDS; transfer buffer contained 12 mM Tris base, 96 mM glycine, and 20% methanol; stripping buffer contained 62.5 M Tris–HCl (pH 6.7), 100 mM mercaptoethanol, and 2% SDS.

Basophil Purification

For the studies requiring immunoblots, basophils were purified from residual cells of normal donors undergoing leukapheresis as previously described [31]. The leukocytes were partially purified by Percoll density gradient and by countercurrent elutriation. Basophils are placed into culture (RPMI 1640 with 2% FBS and 20 mg/mL of gentamicin) for 1 hr after elutriation and one subsequent Percoll separation on a two-step gradient (1.066/1.079). After culturing overnight in RPMI-1640 supplemented with 2% FBS, gentamicin, and 30 pM IL-3 (a concentration predetermined to only maintain better viability but with no measurable priming effect on basophil function), the cells were purified further by negative selection using Miltenyi reagents (basophil isolation kit, containing anti-CD3, -CD7, -CD14, -CD15, -CD16, -CD36, -CD45RA, and HLA-DR) and columns (Miltenyi Biotec GmbH). The reagents from the Miltenyi kit were used at one-fifth their recommended concentrations, and the cells were incubated on ice (rather than at 10°) for 30 min at each step. Basophil purities were $> 99\%$.

Histamine Release and Sensitization

For the pharmacological and desensitization experiments, basophils were obtained by venipuncture and partially enriched over a single-step Percoll gradient. The cells were challenged, and supernatants were harvested for analysis by automated fluorimetry [32]. LTC_4 was measured by radioimmunoassay [3]. Histamine release is expressed as a ratio of sample to total histamine, obtained by lysis of an equivalent number of cells with perchloric acid, after subtracting spontaneous release. For the desensitization experiments using specific antigens, the basophils first were sensitized with a mixture of two specific IgE antibodies, anti-penicillin (BPO) at a concentration of 2 μg/mL and anti-gp120 at 2 μg/mL for 30 min at 37° in RPMI buffer containing 0.1 mM BPO–EACA, 10 μg/mL of heparin, and 1 mM EDTA. After washing, the cells were used for the experiments described. For the data analysis in these experiments, the relevant analysis within each experiment required expressing the amount of desensitization as a fraction of the control histamine release (i.e. stimulated release from non-desensitized cells). After expressing the data this way, the results were averaged for the replicate experiments, and the data were converted back to absolute histamine release by multiplying the averaged ratios by the average control histamine release. With this approach, control histamine release initially had a value of 1.0 (other data is expressed as a fraction of 1.0) times the actual average release; therefore, there are no standard error bars in Figs. 1 and 2 or Table 1 for these control values.

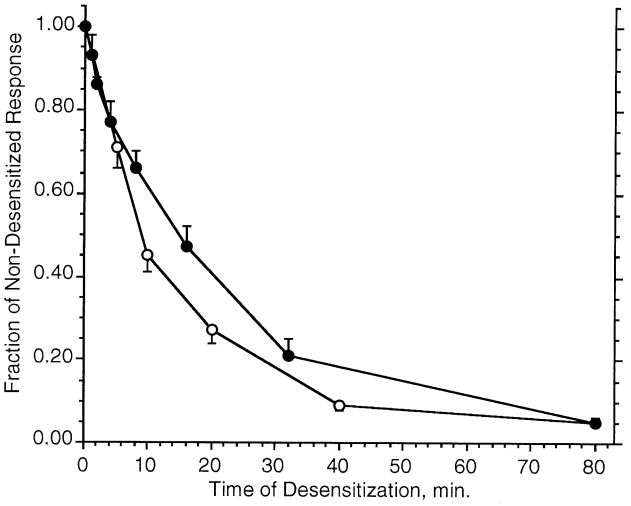

FIG. 1. Kinetics of desensitization. Cells were stimulated with either anti-IgE antibody at 0.1 μg/mL (●, N = 24) or BPO [11]–HSA at 0.5 μg/mL (○, N = 5) in PAG buffer for the times shown on the abscissa, after which calcium was added to the reaction and supernatants were harvested 45 min after the addition of extracellular calcium. Basophils stimulated with BPO–HSA were first sensitized with penicillin-specific IgE (see Materials and Methods). Error bars indicate SEM.

Desensitization in the Presence of Drugs

If a compound can be shown to effectively wash out of the cell with either a large dilution or a washing step (using centrifugation), the influence of the compounds on desensitization alone can be isolated. The experimental design was to stimulate the cells in the absence of extracellular calcium (desensitization phase) ± drug, wash the cells, and re-challenge them in the presence of extracellular calcium and the absence of drug. In several pilot experiments, BIS II, Ro-31–8220, and PP1 were first shown to be removed from the cells by a washing step. For example, if cells were incubated with 400 nM BIS II for 15 min, washed once, and resuspended for challenge with PMA without the further addition of BIS II, there was little or no inhibition of the PMA-induced release (compared with controls not treated with BIS II and in contrast to controls where BIS II was also included during stimulation with PMA) (data not shown). Therefore, BIS II and Ro-31–8220 could be included in the desensitization phase of the desensitization protocol without significantly influencing activation in the final challenge phase of the protocol. Similarly, preincubation of cells with PP1 for 15–60 min followed by washing resulted in cells that responded to IgE-mediated stimulation.

There were two experimental protocols used to test for an effect of BIS II or Ro-31–8220 on desensitization, one designed to examine the effect on desensitization mediated

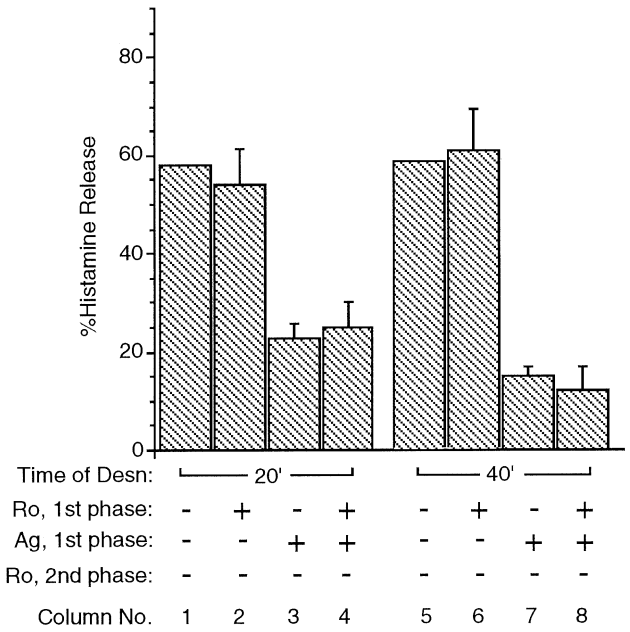

FIG. 2. No reversal of desensitization in cells treated with the PKC inhibitor Ro-31–8220 (N = 3). Cells were desensitized with anti-IgE antibody (0.2 μg/mL) for times that do not result in complete desensitization. The rows containing plus/minus indicate the presence or absence of drug in the desensitization phase of the experiment (row 1, labeled "Ro, 1st phase"), the presence of anti-IgE antibody in the desensitization phase of the experiment (row 2, labeled "Ag, 1st phase"), or drug in the re-challenge phase of the experiment (row 3, labeled "Ro, 2nd phase"). The PMA concentration in the re-challenge phase was 30 ng/mL. The drugs were incubated with the cells for 10 min in PAG buffer containing 5 μM EDTA, then anti-IgE antibody (0.1 μg/mL final concentration) was added, and the reaction allowed to proceed for 20 or 40 min with the total volume at this stage being 50 μL. The reaction was continued by adding 0.95 mL of buffer containing calcium/magnesium at 1 mM (PAGCM) and, where appropriate, enough anti-IgE antibody to result in a final concentration of 0.1 μg/mL. The reaction was continued for 45 min, and the supernatants were harvested for the determination of histamine. As noted in Materials and Methods, PMA was used to assess the effectiveness of the dilution. In cells incubated without drug, histamine release to PMA was 71 ± 14%. In cells incubated first with drug and then diluted, PMA induced 48 ± 10% release. In cells incubated with drug and then diluted with an equivalent concentration of drug and challenged with PMA, histamine release was 2 ± 2%. Error bars in the figure indicate SEM.

by polyclonal anti-IgE antibody and the other to examine both specific and nonspecific desensitization. For either protocol, a number of controls were included. The basic design was to first incubate cells suspended in PAG/5 μM EDTA ± 500 nM Ro-31–8220 for 10 min, add buffer ± anti-IgE antibody, and after 20–60 min, the cells either were diluted 20-fold with PAGCM with or without anti-IgE antibody or were washed once in PAG/5 μM EGTA to wash out the drug prior to re-challenge with antigen in the presence of extracellular calcium. In these experiments, it was useful to know that (i) the drug effectively inhibited PKC, and (ii) the wash or dilution step effectively removed

TABLE 1. Washout controls for PP1 desensitization experiments

		% Histamine release		
		Conditions		
Stimulus	Concentration	No PP1	+PP1	+PP1 (both)
anti-IgE	0.2 μg/mL	39	30 ± 2	2 ± 1
	0.05 μg/mL	35	28 ± 3	
	0.025 μg/mL	24	20 ± 2	
	0.0125 μg/mL	15	11 ± 1	
gp120=OVA	50 ng/mL	40	37 ± 3	1 ± 1
	15 ng/mL	38	32 ± 3	
	5.0 ng/mL	25	22 ± 2	
	1.5 ng/mL	15	11 ± 3	
BPO2	50 nM	36	36 ± 2	1 ± 1
	5.0 nM	41	36 ± 3	
	0.5 nM	36	34 ± 2	
	0.05 nM	26	23 ± 2	

Data show the effectiveness of the washing steps to remove PP1 used during the desensitization phase of the experiments in Fig. 6 and prior to re-challenge with stimulus. The column labeled "no PP1" refers to cells incubated without PP1 during the 30- to 50-min desensitization, washed, and challenged with indicated concentrations of stimulus. The column labeled "+PP1" refers to cells for which PP1 at 10 μM was included in the desensitization phase but washed away prior to stimulation. The final column labeled "PP1 (both)" indicates the response of these cells when PP1 was included in both the desensitization phase and the re-challenge phase of the experiment. Errors are only included for the "+PP1" and columns "+PP1 (both)" because the data were first calculated as a fraction of the non-treated response before converting these fractional values back to histamine release, as described in Materials and Methods. Errors indicate SEM, N = 5 for anti-IgE, N = 3 for gp120-OVA, and n = 3 for BPO2.

or diluted the drug prior to challenge in the presence of extracellular calcium. Since these two PKC inhibitors have marginal effects on IgE-mediated release, their effectiveness was examined by challenging cells with PMA. These two conditions were tested by: (i) maintaining the drug concentration at 500 nM for a portion of the cells throughout the challenge phase and stimulating with 30 ng/mL of PMA to assess the drug's inhibition of this stimulus, or (ii) challenge the washed or diluted cells with PMA to assess whether there remained some inhibition of PMA-induced release.

The protocol for Fig. 6 experiments did not need to include PMA stimulation. For each desensitization condition, enough cells were incubated during the desensitization phase so that after washing three tubes were set up for the re-challenge, one that was lysed for total histamine content and two that received the re-challenge stimulus (or buffer alone in the case of spontaneous release determinations). In this way, the total histamine content for each pot was used as a check against differences in the cell contents of each tube (although, in practice, the total histamine content for each pot was very similar). For a typical experiment, there were six conditions: no stimulus in either phase (for the spontaneous release determination during re-challenge), cells ± desensitizing stimulus, with and without drug, and cells with drug in both desensitization and re-challenge phase but no stimulus during the desen-

sitization phase (to determine the effectiveness of the drug). In the desensitization phase, cells were incubated in PAG buffer containing 5 μM EDTA, washed in the same buffer twice, and resuspended in PAGCM for re-challenge. For two conditions, cells ± drug but without stimulus during the desensitization phase, re-challenge consisted of four concentrations of stimulus in order to determine the effectiveness of the drug washout for a range of stimulations. The number of cells for these two conditions was adjusted to accommodate the larger number of re-challenge tubes needed.

Immunoprecipitation

Basophils (1.5 to 2.0 × 10^6) were lysed in 1 mL of lysis buffer, were pre-cleared with 20 μL of protein G sepharose beads for 30 min, and then were incubated with 1 μg/mL of p72syk prebound to 20 μL of protein G sepharose beads. After gentle rotation for 1 hr at 4°, the beads were washed once in ice-cold complete lysis buffer. The immunoprecipitated proteins then were eluted from the beads by boiling in 2x SDS sample buffer.

Blotting of Proteins

Proteins were separated in a 10% Tris-glycine gel under reducing conditions and electrotransferred onto a nitrocellulose membrane. The free binding sites were blocked by incubating the membrane overnight at 4° with 4% BSA in TBST. Then the nitrocellulose membranes were incubated with 0.5 μg/mL of the anti-phosphotyrosine monoclonal antibody, 4G10, in 1% BSA/TBST for 1 hr at room temperature. The membrane was washed thoroughly with TBST prior to the addition of an anti-mouse horseradish peroxidase conjugate (1:3000 dilution) for 1 hr at room temperature. After further extensive washing of the membranes with TBST, the phosphoproteins were visualized using ECL. The nitrocellulose membrane was exposed to ECL hyperfilm for 15 sec to 15 min. Then the nitrocellulose membrane was stripped for 1 hr at 50°, and reprobed with 0.2 μg/mL of mouse anti-human p72syk. After exposure to chemiluminescence detection agents, the intensity of each band was determined using densitometric analysis. In all experiments, equal protein loading was confirmed by both the level of syk in each lane (the second blotting) and Coomassie Blue staining. Pilot experiments established the relative linearity of the western blot procedure for a modest range of gel loading. ECL exposures were chosen to best work within the linear range of the assay.

RESULTS

PKC Inhibitors

As noted previously, desensitization is operationally demonstrated by stimulating basophils in the absence of extracellular calcium for various periods of time. The extent of desensitization is assessed by adding calcium to the extracellular buffer to initiate the remaining secretory reaction—in these studies, the release of histamine. The kinetics of the desensitization process are shown in Fig. 1 for both anti-IgE antibody and for stimulation with antigen, in this case represented by the multivalent penicillin–albumin conjugate (with cells sensitized with penicillin-specific IgE). The kinetics of this process suggest that it is useful to examine the effects of drugs on desensitization after significant desensitization has occurred, usually at times greater than 15 min.

The modest enhancement of IgE-mediated release by BIS II, or especially Ro-31–8220, as previously reported [16], suggested that these compounds may inhibit PKC activity that is involved in down-regulation of the IgE-mediated response. Figure 2 shows results for cells desensitized with anti-IgE antibody for two time periods, 20 or 40 min (two time points were examined to exclude the possibility that there may have been differential effects of the drugs at different time points). The non-desensitized response to anti-IgE antibody in the absence of drug was 58% (columns 1 and 5; cells incubated for 20 or 40 min in the absence of drug or anti-IgE during the calcium-free phase). Desensitization (treatment with anti-IgE antibody in the calcium-free phase) for 20 and 40 min in the absence of drug decreased release to 23 and 15%, respectively (columns 3 and 7). The release was not different in samples containing drug during the desensitization phase (columns 4 and 8). The PMA control data are included in the figure legend. The effect of dilution was nearly as expected; the 20-fold dilution should have returned the PMA response to within 60–75% of the response in cells not exposed to drug.* These data suggest that Ro-31–8220 had no effect on the rate or extent of desensitization measured this way. A similar experiment with BIS II resulted in the same conclusion (data not shown).

Desensitization has two components: the desensitization may be specific for the antigen used to desensitize the cells or it may alter the response of the cells to other non-cross-reacting antigens [5, 6]. To examine both specific and nonspecific desensitization, two additional experimental elements were added to the protocol (see Materials and Methods). The cells were sensitized with two non-cross-reacting antigen-specific IgE antibodies (penicillin-specific and gp120-specific IgE), and the residual effects of the desensitizing antigen (BPO–HSA) present during the re-challenge with the non-cross-reacting antigen were reduced by inclusion of the monovalent form of the desensitizing antigen (BPO–EACA) during the re-challenge (although it was found to be minimal). This protocol also differs from that for the experiments shown in Fig. 2 in that the effective dilution of the drug should have been greater since the cells were centrifuged to remove the initial desensiti-

* Previous studies of the concentration-dependence of BIS II and Ro-31–8220 on PMA-induced release indicated that 20 nM PKC inhibitor would inhibit PMA-induced release 10–30%. However, concentrations below 100 nM have neither enhancing nor inhibitory effects on IgE-mediated secretion.

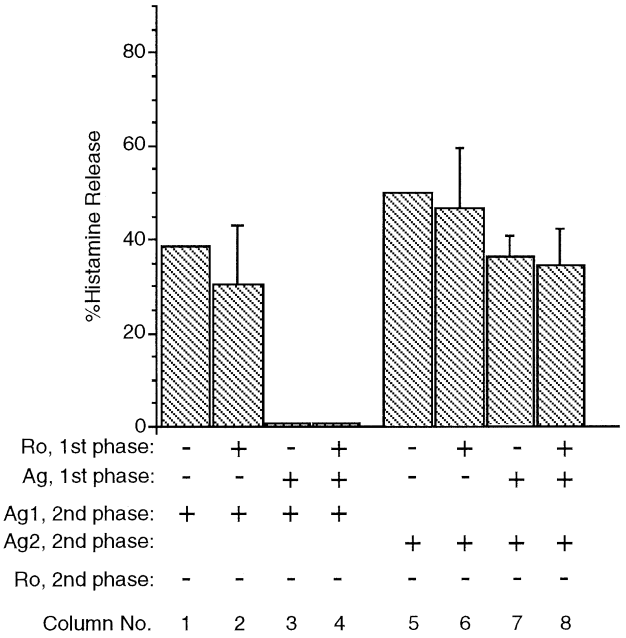

FIG. 3. No inhibition of specific or nonspecific desensitization by Ro-31–8220 (N = 3). The cells were sensitized with both BPO-specific and gp120-specific IgEs, desensitized with 0.5 μg/mL of BPO–HSA in the presence or absence of Ro-31–8220, and re-challenged with either BPO–HSA (0.5 μg/mL) or gp120–OVA (50 ng/mL). The rows containing plus/minus indicate the presence or absence of drug in the desensitization phase of the experiment (row 1, labeled "Ro, 1st phase"), the presence of BPO–HSA in the desensitization phase of the experiment (row 2, labeled "Ag, 1st phase"), BPO–HSA in the re-challenge phase of the experiment (row 3, labeled "Ag1, 2nd phase"), gp120–OVA (+ BPO–EACA) in the re-challenge phase of the experiment (row 4, labeled "Ag2, 2nd phase"), or drug in the re-challenge phase of the experiment (row 5, labeled, "Ro, 2nd phase"). The PMA concentration in the re-challenge phase was 30 ng/mL. The sensitized cells, suspended in PAG/5 μM EDTA were split into four pots, two of which were not desensitized ± Ro-31–8220 (500 nM) and two of which were desensitized with BPO–HSA (500 ng/mL) ± Ro-31–8220 (500 nM). All four pots were incubated for 60 min. The reactions then were centrifuged (4 min), and the cell pellets were resuspended in PAG and distributed to tubes for challenge. The cells that were to be challenged with gp120–OVA, PMA, or PAGCM (spontaneous release controls) were first treated for 5 min with 0.1 mM BPO–EACA to dissociate any bound BPO–HSA, and then the stimuli were added in buffer containing enough calcium/magnesium to result in 1 mM final concentrations. For cells re-challenged with BPO–HSA, the monovalent hapten was not included. As noted in Materials and Methods, PMA was used to assess the effectiveness of the washing step. In cells incubated without drug, histamine release to PMA was 68 ± 8%. In cells incubated first with drug and then diluted, PMA induced 51 ± 10% release. In cells incubated with drug and then diluted with an equivalent concentration of drug and challenged with PMA, histamine release was 3 ± 3%. Error bars in the figure indicate SEM.

zation buffer. It can be seen in Fig. 3 that the response to BPO–HSA was ablated completely following 60 min of desensitization (compare column 3 with column 1). Ro-31–8220 had no effect on this desensitization (compare column 4 with column 3). Thus, Ro-31–8220 had no effect on specific desensitization. Likewise, there was no effect of Ro-31–8220 on nonspecific desensitization. Comparing column 7 with column 5, it can be seen that there was partial nonspecific desensitization ($P = 0.021$ for inhibition), i.e. pretreatment with BPO–HSA caused partial inhibition of the response of the cell to gp120–OVA (≈25%). Ro-31–8220 did not change the extent of nonspecific desensitization (compare columns 7 and 8). The PMA control data are included in the figure legend and indicate that there was a reasonably effective washout of the drug.

Inhibitors of Early Tyrosine Kinases

The inability of PKC inhibitors to inhibit desensitization suggests that PKC does not have a role in this process. More recent studies have suggested alternative mechanisms for desensitization that may not require the activity of the early tyrosine kinases normally associated with the first steps in the IgE-mediated signaling cascade. We and others have demonstrated recently that normal peripheral blood basophils show early tyrosine phosphorylation of both lyn and syk following IgE-mediated stimulation [28, 33]. The src-family kinase inhibitors PP1 and PP2 have been found to be relatively specific inhibitors of lyn kinase [34, 35]. Figure 4 shows that both PP1 and PP2 inhibited IgE-mediated histamine release with an IC_{50} value of approximately 2 μM. Interestingly, at lower concentrations there was modest enhancement of release, especially release of LTC_4. The data in Fig. 4 show inhibition of IgE-mediated release but not inhibition of release following stimulation with the bacterial tripeptide, fMLP (1 μM) or PMA (10 ng/mL) (in data not shown, PP1 or PP2 also did not affect release induced by ionomycin). Activation of lyn kinase should lead to the next step in the signaling cascade, the activation/phosphorylation of syk kinase, which we use here as a marker of lyn kinase activation. Figure 5A demonstrates that activation through FcεRI/IgE showed the expected phosphorylation of syk kinase, whereas fMLP did not. Figure 5 also shows that PP1 and PP2 at concentrations of 10 μM inhibited the phosphorylation of syk kinase completely.

We therefore examined the ability of PP1 to inhibit IgE-mediated desensitization. The requirements for being able to wash out the drug used during the desensitization phase of the experiment were more strict since PP1 is a good inhibitor of secretion and, therefore, would interfere with the ability to examine the extent of desensitization by measuring subsequent histamine release. Hence, cells were centrifuged twice following the desensitization phase. As noted in Table 1, cells first treated for 30–45 min with PP1, washed, and challenged with various concentrations of anti-IgE or antigen released nearly as well as those not treated with PP1 for the same period of time. Nevertheless, there remained a residual and subtle effect on the subsequent release. This small effect precluded the use of higher concentrations of PP1 or PP2 during the desensitization

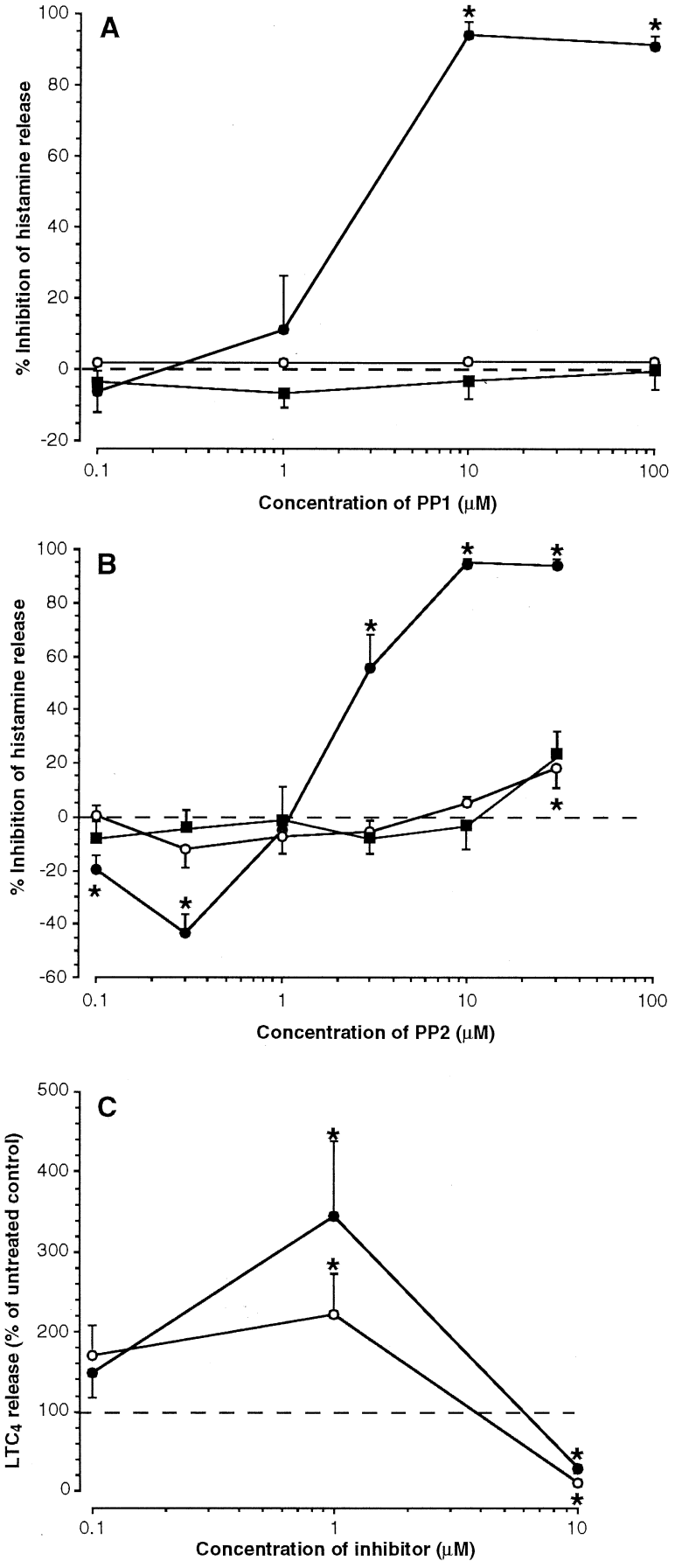

FIG. 4. Concentration-dependence of PP1 and PP2 on IgE-mediated mediator release from basophils. Mixed leukocytes were preincubated with PP1 at the concentrations indicated for 10 min prior to the addition of stimulus. Anti-IgE antibody (●), 0.2 μg/mL, fMLP (○), 1 μM, or PMA (■), 10 ng/mL, was added, and histamine and LTC_4 were measured in the harvested supernatants 45 min after their addition. Panels A and B show results as a percent inhibition for PP1 (N = 5) and PP2 (N = 4), respectively. Histamine release without PP1 averaged 50 ± 12% for cells stimulated with anti-IgE antibody and 78 ± 13% for fMLP. Panel C shows data for LTC_4, expressed as percent of control release (N = 5), PP1 (○), or PP2 (●) with anti-IgE antibody (0.2 μg/mL) as the stimulus. LTC_4 release in the absence of PP1 or PP2 was 23 ± 12 pmol/10^6 basophils. Statistical significance was achieved at the points labeled with an asterisk ($P < 0.05$). Error bars in the figure indicate SEM.

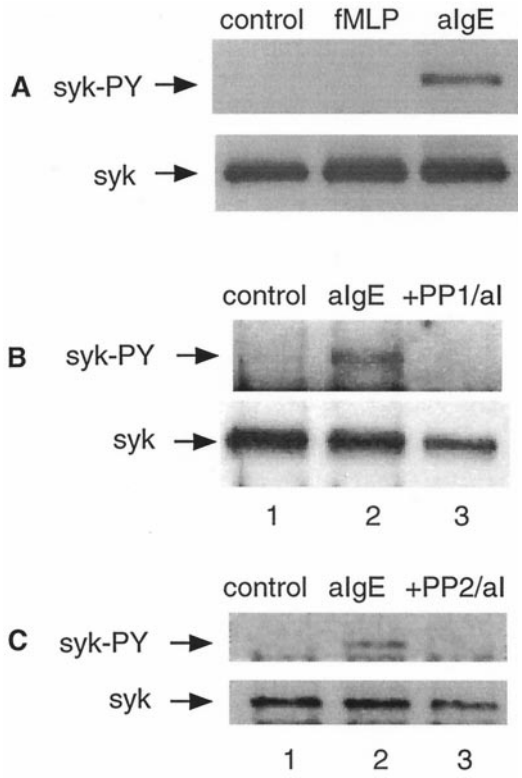

FIG. 5. Western blots of syk phosphorylation following stimulation with anti-IgE antibody in the presence or absence of PP1 or PP2 or stimulation with fMLP. Panel A shows that syk phosphorylation follows stimulation with 0.2 μg/mL of anti-IgE antibody (aIgE) but not 1 μM fMLP. Cells were harvested at 1 min following fMLP and at 5 min following anti-IgE antibody. In panels B and C, purified basophils were preincubated with PP1 or PP2 at 10 μM for 10 min prior to the addition of 0.2 μg/mL of anti-IgE antibody. Five minutes after the addition of anti-IgE antibody, the reactions were stopped by centrifugation, and the cell pellet was lysed, immunoprecipitated with anti-syk, and analyzed for syk phosphorylation by western blotting. Panel B shows results for PP1 and panel C for PP2. Scanning the band densities resulted in the following data: for panel B, 100, 1810, and 20 (arbitrary units) for anti-phosphotyrosine bands in lanes 1, 2, and 3, respectively, and 6310, 5700, and 3680 for anti-syk bands. For panel C, 0, 525, and 20 for anti-phosphotyrosine bands in lanes 1, 2, and 3, respectively, and 3450, 3390, and 2450 for anti-syk bands, respectively.

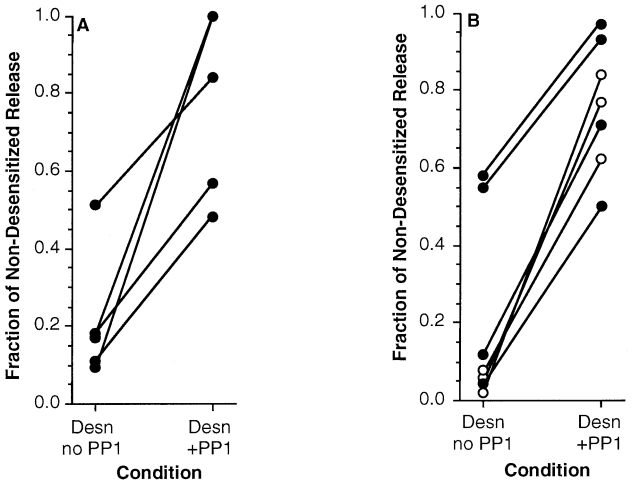

FIG. 6. Inhibition of desensitization in cells co-incubated with PP1. Panels A and B separate the results into preparations that were desensitized with (0.2 μg/mL) anti-IgE antibody or preparations that were first sensitized with antigen-specific IgE and desensitized with the relevant antigen. The results in panel B represent a mix of experiments where cells were sensitized with either BPO-specific IgE or gp120-specific IgE. For those experiments where the cells were sensitized with BPO-specific IgE, some included desensitization with BPO2 and others used BPO–HSA. BPO2 concentrations were 0.5 nM, BPO–HSA concentrations were 0.5 μg/mL, and gp120–OVA concentrations were 50 ng/mL. The open circles in panel B represent the results for experiments where desensitization was done with BPO2. Otherwise, the results are not distinguished by different symbols. Histamine release from non-desensitized cells averaged 39% for anti-IgE antibody (N = 5), 50% for BPO–HSA (N = 1), 41% for BPO2 (N = 3), and 40% for gp120–OVA (N = 3).

phase of the experiment, although we did attempt to use higher concentrations (data not shown). Figure 6A demonstrates that PP1 could inhibit desensitization induced by anti-IgE antibody partially. Similar experiments are shown in Fig. 6B except that the basophils were desensitized with specific antigens. Basophils that had been sensitized with either BPO-specific IgE were stimulated with BPO2, and cells that were sensitized with gp120-specific IgE were stimulated with gp120–OVA. When these stimuli were used, PP1 also caused inhibition of desensitization, and in some instances desensitization was nearly ablated. For example, desensitization in the absence of PP1 resulted in cells responding at 10% of the non-desensitized response, while cells desensitized in the presence of PP1 responded at 90% of the non-desensitized response. On average, however, reversal of desensitization remained partial with no clear pattern of differences among the various stimuli. In data not shown, with a more limited series of experiments, similar results were obtained for PP2.

DISCUSSION

These data provide two useful insights. First, treatment by drugs that can be demonstrated to effectively inhibit a variety of PKC-mediated events has no effect on any form of desensitization. Second, effective inhibition of early tyrosine kinases inhibits desensitization. Such data suggest that the mechanism of desensitization is dependent on the activity of these early tyrosine kinases. An important caveat to this interpretation is the absence of knowledge regarding the absolute specificity of the drugs in question. The results for the PKC inhibitors were negative, indicating that there should be little concern about selectivity in these experiments. However, it remains possible that these agents are too selective for specific isozymes of PKC, leaving open the possibility that an unusual PKC isoform is acting in a down-regulatory role in basophils. Mitigating this concern is evidence that Ro-31–8220 broadly inhibits many PKC isozymes [36], and these drugs have been shown previously to alter six different functional endpoints of PMA-induced

activation of basophils [16]. With respect to PP1/PP2, thus far they have been found to be relatively selective inhibitors of the src family kinases [34, 35], but further study will be needed to make a strong conclusion regarding their absolute selectivity. However, with respect to the desensitization studies, PP1 was only functionally present during the desensitization phase of the experiment, so that the possibility of nonselective effects is lessened by the more restricted domain of its use (i.e. not the whole secretory reaction). With respect to its effects on secretion, there are probably more potential sites of action that could confound a clear interpretation of the results. However, the inhibitory action of PP1 or PP2 was restricted to IgE-mediated stimulation. Neither stimulation with another receptor-dependent secretagogue, fMLP, nor non-physiological stimuli, such as PMA and ionomycin, was inhibited by PP1/PP2. This suggests that there are later signaling events involved in degranulation of the cells that are not affected by PP1 or PP2. For example, we recently found that there was convergence of signaling for anti-IgE antibody and fMLP at p21ras but, prior to activating this important signaling element, the two pathways differed.* PP1 only inhibits the IgE-mediated pathway and ras activation but does not inhibit ras activation induced by fMLP. Together, the signaling studies and the functional studies with non-IgE-dependent secretion suggest that PP1 and PP2 do not inhibit late signaling.

We used the phosphorylation of syk kinase as an indicator of the ability of PP1 and PP2 to inhibit lyn kinase. The current paradigm of activation through FcεRI suggests that lyn kinase activity would be required to observe syk phosphorylation. However, some loss of syk phosphorylation could occur if PP1 or PP2 was able to inhibit syk kinase activity as well because this enzyme engages in some self-phosphorylation once activated. Previous studies of these two inhibitors have shown that they do not inhibit syk kinase [34, 35]. Therefore, it seems likely that the inhibitory effect on syk phosphorylation results from the action of PP1 on lyn kinase. Another indicator would be phosphorylation of either the β or γ subunits of FcεRI, but neither we nor others have yet established the characteristics of phosphorylation of these two subunits in human basophils.

As noted previously, desensitization can be specific or nonspecific in character. In other words, restricted to the domain of IgE-mediated release, desensitization can be specific for the desensitizing antigen or affect stimulation by other non-cross-reacting antigens. Past experience indicates that sensitization of cells with an antigen-specific IgE does not typically lead to cells that experience extensive nonspecific desensitization when challenged with specific antigen [6]. In contrast, desensitization with anti-IgE antibody does often lead to nonspecific desensitization [7]. Although this is to be expected because this stimulus is capable of aggregating all cell surface IgE, it was also found to be true when the re-challenge stimulus was an antigen that binds to a mouse IgE† that had been used to sensitize the basophil to which our polyclonal anti-IgE antibody did not bind. The fact that we did not produce different results when cells were desensitized with antigen or anti-IgE antibody, when examining the effects of PP1, suggests that lyn kinase is a requisite step for either form of desensitization. Furthermore, challenge or desensitization of cells with a simple bivalent hapten like BPO2 should not result in large scale aggregation of cell surface BPO-specific IgE [37–39] as often appears to occur when multivalent haptenated proteins are used [40, 41]. The observation that PP1/PP2 inhibited desensitization induced by this type of stimulus to an extent similar to either anti-IgE antibody or multivalent gp120–OVA further suggests that all forms of desensitization rely on activation of early signaling events. Inhibition of desensitization was not complete in most cases, but we were not able to test higher concentrations of PP1 since there was too much residual effect on secretion during the re-challenge phase of the experiment.

The observation that PP1 and PP2 do cause modest enhancement of secretion at concentrations below 1 μM is consistent with their ability to inhibit desensitization. Similar results were found for diisopropylfluorophosphate (a serine esterase inhibitor), where concentrations greater than 1 mM markedly inhibited secretion while a very narrow concentration range between 0.2 and 0.5 mM was found to enhance secretion markedly and inhibit desensitization completely [1]. The balance of activation and de-activation is such that it is possible that a single drug could result in enhancement in one restricted concentration range while inhibiting in another. The ability to observe such an effect presumably would depend on the differential sensitivity of the activating and de-activating processes to the drug in question. If there was little differential sensitivity, then enhancement would probably not be observed. In the case of PP1 and PP2, the enhancement was at best modest, suggesting only a slight difference in sensitivity of the two processes to inhibition of lyn kinase.

In summary, these studies indicate that PP1 and PP2 are selective inhibitors of IgE-mediated release and, as expected from studies in both human basophils and other mast cell models, probably cause inhibition by reducing the activity of the earliest step in signaling, the activation of lyn kinase. Inhibition of this kinase also inhibits the process of desensitization, suggesting that like activation, the signaling elements that participate in desensitization are also dependent on the earliest steps in signaling. Unless there is a PKC isozyme present in basophils that is resistant to inhibition with BIS II or Ro-31–8220, these studies also suggest that PKC does not participate in the process of desensitization as previously suspected.

* Miura K and MacGlashan DW Jr, Manuscript submitted for publication.

† Dintrophenyl-specific mouse IgE; data presented in Lavens-Phillips S and MacGlashan DW Jr, Manuscript submitted for publication.

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References

1. Kazimierczak W, Meier HL, MacGlashan DW Jr and Lichtenstein LM, An antigen-activated DFP-inhibitable enzyme controls basophil desensitization. *J Immunol* **132:** 399–405, 1984.
2. Lichtenstein LM and De Bernardo R, IgE mediated histamine release: *In vitro* separation into two phases. *Int Arch Allergy Appl Immunol* **41:** 56–71, 1971.
3. MacGlashan DW Jr, Peters SP, Warner J and Lichtenstein LM, Characteristics of human basophil sulfidopeptide leukotriene release: Releasability defined as the ability of the basophil to respond to dimeric cross-links. *J Immunol* **136:** 2231–2239, 1986.
4. MacGlashan DW Jr, Desensitization of IgE-mediated IL-4 release from human basophils. *J Leukoc Biol* **63:** 59–67, 1998.
5. Sobotka AK, Dembo M, Goldstein B and Lichtenstein LM, Antigen-specific desensitization of human basophils. *J Immunol* **122:** 511–517, 1979.
6. MacGlashan DW Jr and Lichtenstein LM, The transition from specific to nonspecific desensitization in human basophils. *J Immunol* **127:** 2410–2414, 1981.
7. MacGlashan DW Jr, Mogowski M and Lichtenstein LM, Studies of antigen binding on human basophils. II. Continued expression of antigen-specific IgE during antigen-induced desensitization. *J Immunol* **130:** 2337–2342, 1983.
8. Sagi ER, Lieman H and Pecht I, Protein kinase C regulation of the receptor-coupled calcium signal in histamine-secreting rat basophilic leukaemia cells. *Nature* **313:** 59–60, 1985.
9. Warner JA and MacGlashan DW Jr, Signal transduction events in human basophils. A comparative study of the role of protein kinase C in basophils activated by anti-IgE antibody and formyl-methionyl-leucyl-phenylalanine. *J Immunol* **145:** 1897–1905, 1990.
10. Massey WA, Cohen VL, MacGlashan DW Jr, Gittlen SW, Kagey-Sobotka A, Lichtenstein LM and Warner JA, Protein kinase C modulates IgE-mediated activation of human mast cells from lung and skin. I. Pharmacologic inhibition. *J Pharmacol Exp Ther* **258:** 824–829, 1991.
11. Ozawa K, Szallasi Z, Blumberg PM and Beaven MA, The role of Ca^{2+}-independent isozymes of protein kinase C (PKC) in exocytosis in antigen-stimulated rat basophil RBL-2H3 cells. *FASEB J* **6:** A1633, 1992.
12. Ozawa K, Szallasi Z, Kazanietz MG, Blumberg PM, Mischak H, Mushinski JF and Beaven MA, Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells: Reconstitution of secretory responses with Ca^{2+} and purified isozymes in washed permeabilized cells. *J Biol Chem* **268:** 1749–1756, 1993.
13. Rivera J and Beaven MA, Regulation of secretion from secretory cells by protein kinase C. In: *Protein Kinase* C (Eds. Parker P and Dekker L), pp. 131–164. Landes Co., Austin, TX, 1997.
14. Chang EY, Szallasi Z, Acs P, Raizada V, Wolfe PC, Fewtrell C, Blumberg PM and Rivera J, Functional effects of overexpression of protein kinase C-α, -β, -δ, -ε, -η in the mast cell line RBL-2H3. *J Immunol* **159:** 2624–2632, 1997.
15. Warner JA and MacGlashan DW Jr, Protein kinase C (PKC) changes in human basophils. IgE-mediated activation is accompanied by an increase in total PKC activity. *J Immunol* **142:** 1669–1677, 1989.
16. Miura K and MacGlashan DW Jr, Expression of protein kinase C isozymes in human basophils: Regulation by physiological and non-physiological stimuli. *Blood* **92:** 1206–1218, 1998.
17. Jouvin MH, Adamczewski M, Numerof R, Letourneur O, Valle A and Kinet JP, Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J Biol Chem* **269:** 5918–5925, 1994.
18. Yamashita T, Mao S-Y and Metzger H, Aggregation of the high-affinity IgE receptor and enhanced activity of $p53/56^{lyn}$ protein-tyrosine kinase. *Proc Natl Acad Sci USA* **91:** 11251–11255, 1994.
19. Pribluda VS, Pribluda C and Metzger H, Biochemical evidence that the phosphorylated tyrosines, serines, and threonines on the aggregated high affinity receptor for IgE are in the immunoreceptor tyrosine-based activation motifs. *J Biol Chem* **272:** 11185–11192, 1997.
20. Mao SY and Metzger H, Characterization of protein-tyrosine phosphatases that dephosphorylate the high affinity IgE receptor. *J Biol Chem* **272:** 14067–14073, 1997.
21. Torigoe C, Goldstein B, Wofsy C and Metzger H, Shuttling of initiating kinase between discrete aggregates of the high affinity receptor for IgE regulates the cellular response. *Proc Natl Acad Sci USA* **94:** 1372–1377, 1997.
22. Benhamou M, Stephan V, Gutkind SJ, Robbins KC and Siraganian RP, Protein tyrosine phosphorylation in the degranulation step of RBL-2H3 cells. *FASEB J* **5:** A1007, 1991.
23. Stephan V, Benhamou M, Gutkind JS, Robbins KC and Siraganian RP, FcεRI-induced protein tyrosine phosphorylation of pp72 in rat basophilic leukemia cells (RBL-2H3). Evidence for a novel signal transduction pathway unrelated to G protein activation and phosphatidylinositol hydrolysis. *J Biol Chem* **267:** 5434–5341, 1992.
24. Kihara H and Siraganian RP, Src homology 2 domains of Syk and Lyn bind to tyrosine-phosphorylated subunits of the high affinity IgE receptor. *J Biol Chem* **269:** 22427–22432, 1994.
25. Minoguchi K, Swaim WD, Berenstein EH and Siraganian RP, Src family tyrosine kinase $p53/56^{lyn,}$ a serine kinase and FcεRI associate with α-galactosyl derivatives of ganglioside GD_{1b} in rat basophilic leukemia RBL-2H3 cells. *J Biol Chem* **269:** 5249–5254, 1994.
26. Hutchcroft JE, Geahlen RL, Deanin GG and Oliver JM, FcεRI-mediated tyrosine phosphorylation and activation of the 72-kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc Natl Acad Sci USA* **89:** 9107–9111, 1992.
27. Benhamou M, Feuillard J, Lortholary O, Bourgeois C, Michel L, LeGoff L, Michel A, Mencia-Huerta J-M, Lejeune F, Casassus P, Debré P and Arock M, Protein tyrosine kinases in activation signal of human basophils through the immunoglobulin E receptor type I. *J Leukoc Biol* **59:** 461–470, 1996.
28. Kepley CL, Wilson BS and Oliver JM, Identification of the FcεRI-activated tyrosine kinases Lyn, Syk, and Zap-70 in human basophils. *J Allergy Clin Immunol* **102:** 304–315, 1998.
29. Kimura T, Sakamoto H, Appella E and Siraganian RP, The negative signaling molecule SH2 domain-containing inositol-polyphosphate 5-phosphatase (SHIP) binds to the tyrosine-phosphorylated β subunit of the high affinity IgE receptor. *J Biol Chem* **272:** 13991–13996, 1997.
30. Adkinson NF Jr, *Measurement of Total Serum Immunoglobulin E and Allergen-Specific Immunoglobulin E Antibody*, American Society of Microbiology, Washington, DC, 1980.
31. MacGlashan DW Jr, White JM, Huang SK, Ono SJ, Schroeder J and Lichtenstein LM, Secretion of interleukin-4 from human basophils: The relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J Immunol* **152:** 3006–3016, 1994.
32. Siraganian RP, An automated continuous-flow system for the

extraction and fluorometric analysis of histamine. *Anal Biochem* **57:** 383–394, 1974.
33. Lavens-Phillips SE and MacGlashan DW Jr, The role of p53/56lyn and p72syk in IgE-mediated signal transduction in human basophils. *FASEB J* **12:** A591, 1998.
34. Orlicek SL, Hanke JH and English BK, The src family-selective tyrosine kinase inhibitor PP1 blocks LPS and IFN-γ-mediated TNF and iNOS production in murine macrophages. *Shock* **12:** 350–354, 1999.
35. Amoui M, Dráber P and Dráberová L, Src family-selective tyrosine kinase inhibitor, PP1, inhibits both FcεRI- and Thy-1-mediated activation of rat basophilic leukemia cells. *Eur J Immunol* **27:** 1881–1886, 1997.
36. Goekjian PG and Jirousek MR, Protein kinase C in the treatment of disease: Signal transduction pathways, inhibitors and agents in development. *Curr Med Chem* **6:** 877–903, 1999.
37. Dembo M, Goldstein B, Sobotka AK and Lichtenstein LM, Histamine release due to bivalent penicilloyl haptens: Control by the number of cross-linked IgE antibodies on the basophil plasma membrane. *J Immunol* **121:** 354–358, 1978.
38. MacGlashan DW Jr, Dembo M and Goldstein B, Test of a theory relating to the cross-linking of IgE antibody on the surface of human basophils. *J Immunol* **135:** 4129–4134, 1985.
39. MacGlashan DW Jr, Schleimer RP and Lichtenstein LM, Qualitative differences between dimeric and trimeric stimulation of human basophils. *J Immunol* **130:** 4–6, 1983.
40. MacGlashan DW Jr and Lichtenstein LM, Studies of antigen binding on human basophils. I. Antigen binding and functional consequences. *J Immunol* **130:** 2330–2336, 1983.
41. Seagrave JC, Deanin GG, Martin JC, Davis BH and Oliver JM, DNP-phycobiliproteins, fluorescent antigens to study dynamic properties of antigen-IgE-receptor complexes on RBL-2H3 rat mast cells. *Cytometry* **8:** 287–295, 1987.