

Characteristics of the free cytosolic calcium timelag following IgE-mediated stimulation of human basophils: significance for the nonreleasing basophil phenotype

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Abstract: These studies examine characteristics of the quiescent period (timelag) of the free cytosolic calcium ($[Ca^{++}]_i$) elevation that follows stimulation of human basophils through the IgE receptor. Previous studies established that the $[Ca^{++}]_i$ timelag was sensitive to the rate of ligand binding, but little else is known about this response characteristic. The $[Ca^{++}]_i$ timelag could be lengthened using antigenic stimulation that is rapid but only weakly induces secretion: tenfold differences in the “strength” of the stimulus, as assessed by histamine release, are associated with threefold differences in the timelag. Inhibiting p53/56lyn kinase with low concentrations of the specific inhibitor, PP1, lengthened the $[Ca^{++}]_i$ timelag dramatically. PP1 was also found to delay the onset of syk phosphorylation and histamine release. Staurosporine and genistein, which are known to inhibit early tyrosine kinases, had, at best, only modest effects on the $[Ca^{++}]_i$ timelag. Specific inhibitors of protein kinase C (PKC) had no effect on the $[Ca^{++}]_i$ timelag, and direct activation of PKC with PMA had only very modest effects on the timelag. Contrary to expectations, basophils with the so-called nonreleasing phenotype demonstrated an IgE-mediated $[Ca^{++}]_i$ response at the single-cell level. However, the length of $[Ca^{++}]_i$ timelag in nonreleasing basophils was threefold longer than normally found in releasing basophils. Furthermore, the $[Ca^{++}]_i$ response was significantly more asynchronous than in releasing basophils and lacking in a sustained $[Ca^{++}]_i$ elevation. These studies indicate that the $[Ca^{++}]_i$ timelag following stimulation through the IgE receptor is sensitive to inhibition of lyn kinase but not other agents that have been demonstrated to inhibit early tyrosine kinases previously. However, only one characteristic of the $[Ca^{++}]_i$ response phenotype of nonreleasing basophils—the $[Ca^{++}]_i$ timelag but not the absence of a sustained $[Ca^{++}]_i$ elevation—could be mimicked by inhibition of lyn kinase with PP1. *J. Leukoc. Biol.* 69: 224–232; 2001.

Key Words: mediator release · histamine release · protein kinase C · syk and lyn kinases

INTRODUCTION

Immunoglobulin (Ig)E-mediated activation of basophils and mast cells is central to the allergic response. The cytosolic free calcium elevation ($[Ca^{++}]_i$) that follows activation through the high-affinity IgE receptor is generally considered a key element among the signaling events responsible for secretion from these cells. At the single-cell level, the IgE-mediated $[Ca^{++}]_i$ response in human basophils is characterized by a quiescent period immediately following the addition of stimulus followed by a rapid transition to a higher cytosolic calcium level [1]. Often, the initial transition is transient, and the response may spike one or many times before assuming a generally higher level for a long period. The mechanisms responsible for the generation of calcium signaling in basophils may be similar to those found in many other cell types, but the details in any hematopoietic cell stimulated through an immunoreceptor are still imprecisely defined. Therefore, we have been interested in the characteristics of the quiescent period and what signaling events regulate its duration. There have been a variety of theoretical models of the early signaling events that lead to the $[Ca^{++}]_i$ response, and these models replicate many of the features observed in mast cells or basophils [2, 3]. One aspect of these models that appears important for explaining the observed characteristics of the $[Ca^{++}]_i$ response is the nonlinear nature of the inositol trisphosphate receptor [4]. Therefore, the signaling elements responsible for generating and maintaining inositol trisphosphate levels should influence the characteristics of the $[Ca^{++}]_i$ response heavily. Because human basophils are difficult to study, there is little known about the early steps that contribute to the first release of internal stores of calcium in this cell.

Our interest in developing a better understanding of the characteristics of this quiescent period was piqued by observations about the length of the quiescent period (what we call the timelag of the $[Ca^{++}]_i$ response) in basophils that have been called nonreleasers (the observations of which will be presented) [5, 6]. Nonreleasing basophils have been shown to possess cell-surface densities of IgE and receptor that are

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equivalent to releasing basophils but do not release mediators in response to stimulation with anti-IgE antibodies, anti-receptor, antibodies, or antigens (on cells passively sensitized with antigen-specific IgE) [5]. It can be shown that these aggregating stimuli bind to the cell surface and even induce changes in basophil function that indicate that at least a partial signal has occurred. Understanding the causes of this phenotype may lead to an appreciation of processes that regulate the expression of basophil function. The precise nature of the defect is not yet fully understood, although recent studies have suggested that these cells are deficient in the expression the tyrosine kinase, p72syk [7, 8]. A variety of studies in other related cell types have demonstrated the critical importance of p72syk as an early tyrosine kinase in the IgE-mediated signaling cascade [9–18], and its absence could explain the nonreleaser phenotype. However, there are aspects to the $[Ca^{++}]_i$ response in nonreleasing cells that raise questions about whether such a deficiency can fully explain this phenotype. Furthermore, the characteristics of the $[Ca^{++}]_i$ response in nonreleasing cells also raise questions about the factors regulating the quiescent (timelag) period of the $[Ca^{++}]_i$ response. The following studies were designed to characterize this quiescent period using a pharmacological approach and then to explore the characteristics of the response in releasing and nonreleasing basophils.

MATERIALS AND METHODS

Materials

Human IgG, piperazine *N,N'* bis 2 ethane sulphonic acid (PIPES), glucose, ethyleneglycol-bis-*N,N,N',N'*-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), fetal calf serum (FCS), bovine serum albumin (BSA), human serum albumin (HSA), perchloric acid, sodium orthovanadate, benzamidine, aprotinin, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, 2-mercaptoethanol, and Nonidet P-40 (NP-40) were all purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 supplemented with 25 mM HEPES and L-glutamine was bought from BioWhittaker (Walkersville, MD), and gentamicin was obtained from Gibco BRL (Grand Island, NY). Sodium dodecylsulphate (SDS) and Tris were purchased from Bio-Rad Laboratories (Philadelphia, PA). Protein G sepharose and Percoll were purchased from Pharmacia Biotech (Piscataway, NJ). Tris-glycine gels (10%) and 2× sample buffer were bought from Novex (Carlsbad, CA), and biotinylated molecular weight markers were purchased from New England Biolabs (Beverly, MA). The antibody cocktail and columns used in the negative selection of human basophils were purchased from Miltenyi Biotec GmbH (Auburn, CA). Mouse anti-human p72syk (4D10) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the mouse anti-phosphotyrosine monoclonal antibody (mAb), 4G10, was purchased from Upstate Biotechnology (Lake Placid, NY). Sheep anti-mouse Ig horseradish peroxidase (HRP), streptavidin HRP conjugate, enhanced chemiluminescence (ECL) Western blotting detection agents, and ECL hyperfilm were all purchased from Amersham (Arlington Heights, IL). Drugs for these studies were obtained from various sources: PPI, Biomol (Plymouth Meeting, PA); phorbol 12-myristate 13-acetate (PMA), Sigma; forskolin and rolipram, gifts of Dr. Bradley Udem, Johns Hopkins University, Asthma & Allergy Center (Baltimore, MD); bis-indolylmaleimide II, LY294002, genistein, and staurosporine, Calbiochem (La Jolla, CA). 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 [19]. Benzylpenicilloyl (BPO)-HSA (11 BPO/HSA) and BPO₂ (bivalent BPO-octamine) were synthesized as previously described [20]. BPO-specific IgE was purified as described previously from the serum of penicillin allergic patients [20].

Buffers

PIPES buffer contained 25 mM PIPES, 110 mM NaCl, and 5 mM KCl, adjusted to pH 7.4 with 1 N HCl; PIPES-albumin-glucose (PAG) contained 0.003% (w/v) HSA and 0.1% (w/v) glucose; PAGCM was PAG with 1 mM CaCl₂ and 1 mM MgCl₂; and elutriation buffer for the purification of human basophils contained PIPES buffer, 0.1% (w/v) glucose and 0.25% (w/v) BSA. Lysis buffer contained 20 mM Tris (pH 7.8), 150 mM sodium chloride, 1% NP-40, 5% glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM benzamidine, and 1 μg/ml aprotinin. In the electrophoresis studies, 2× 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; and stripping buffer contained 7 M guanidine hydrochloride.

Isolation of human basophils

Basophils were purified from buffy coat cells obtained from normal donors undergoing leukapheresis as previously described [21]. The leukocytes were partially purified by Percoll density gradients and counter-current elutriation. The basophils were then placed into culture (RPMI 1640 with 2% FCS and 20 μg/ml gentamicin) for 1 h after elutriation and then further purified on a subsequent, two-step Percoll density gradient (1.066/1.079). After culturing overnight in RPMI 1640 supplemented with 2% FCS, gentamicin and in some later experiments, 30 pg/ml interleukin (IL)-3 (a concentration pre-determined to only maintain better viability but with no measurable priming effect on basophil function). For most of these experiments, the cells were purified further by an additional, two-step Percoll gradient (1.0067/1.079 g/ml). However, in more recent experiments, the cells were purified further by negative selection using Miltenyi reagents and columns (Miltenyi Biotec GmbH). We have not detected a difference in the behavior of the cells purified by the two methods. For these studies, basophil purities were >95% and most often nearly 100%.

For the studies of releasers and nonreleasers, donors consisted of adult subjects who were selected from the personnel of the Department of Clinical Immunology. The donors were nonatopic as assessed by the questionnaire. For the purpose of this study, histamine release from human basophils in response to anti-IgE (0.2 μg/ml) antibody was determined on at least three different occasions. A “nonreleaser” was defined as a donor whose basophils released <5% histamine, and a “releaser” was a donor whose basophils released >10% histamine on all three occasions. Donors were asked if they had any medical problems, especially any known history of atopy (asthma, allergic rhinitis, chronic urticaria, atopic dermatitis, etc.), and if they were receiving any medication. Blood was drawn by venipuncture, and the cells were separated using a two-step, Percoll density gradient. Briefly, the leukocytes were partially purified in accuspin tubes on a two-step, Percoll density gradient (1.070/1.082 g/ml). The interface containing the basophils (10–20% pure) was removed and washed in PAG. The basophils were then further purified by negative selection using the Miltenyi basophil isolation kit (which contains a cocktail of hapten-conjugated CD3, CD7, CD14, CD15, CD16, CD36, CD45RA, and HLA-DR antibodies and MACS microbeads coupled to an anti-hapten-conjugated mAb) and glycophorin A microbeads. Any remaining contaminating cells consisted mainly of monocytes and lymphocytes. This procedure required 2–3 h of preparation, and purities ranged 90–98%.

Cell counting

Basophils were stained with Alcian blue [22] and counted in a Spiers-Levy hemocytometer.

Histamine release and sensitization

All reactions were carried out in PAGCM buffer. Cell concentration depended on the assay; simple histamine release used 2×10^4 /ml, and assessment of syk phosphorylation used $7.5\text{--}10 \times 10^6$ basophils/ml. For the kinetics of histamine release, the reactions were stopped with ice-cold PAG buffer containing 10 mM EDTA. The supernatants were harvested after centrifugation. In each experiment, perchloric acid at a 1.6% final concentration was added to some tubes to determine total histamine content. Histamine was assayed by an automated fluorometric technique [23]. The percentage of histamine release

was calculated from the ratio of sample to total histamine after spontaneous release was subtracted from both. For sensitization with BPO-specific IgE, the cells were incubated for 25 min at 37°C with 5 µg/ml purified, BPO-specific IgE in RPMI medium containing 10 µg/ml heparin and 1 mM EDTA. Cells were used after three washes with PAG buffer. Previous studies established that the 5 µg/ml concentration of BPO-specific IgE was just sufficient to fully sensitize available unoccupied FcεRI during the time used for passive sensitization. Previous studies also demonstrated that measured cell-surface density of BPO-specific IgE roughly titrated according the concentration of BPO-specific IgE used for sensitization [24].

Immunoprecipitation

For syk phosphorylation studies, $1.5\text{--}2 \times 10^6$ -purified basophils/condition were resuspended in PAGCM and stimulated with anti-IgE antibody at a final concentration of 0.2 µg/ml. At various times after stimulation, the cell suspension was centrifuged at 14,000 *g* for 10 s, the supernatant was removed, and the lysis buffer (1 ml) was added to the cell pellet. After sitting on ice for 10 min, the tubes were centrifuged for 3 min at 14,000 *g*. Centrifuged lysates were pre-cleared by incubation with protein G sepharose beads for 30 min at 4°C. The pre-cleared lysates were then incubated with 1 µg/ml anti-p72syk pre-bound to protein G sepharose beads. After gentle rotation for 1 h at 4°C, the beads were washed, and the immunoabsorbed proteins were eluted from the beads by boiling in 2× SDS sample buffer. Control experiments revealed that an irrelevant IgG antibody or mouse anti-human p72syk in the absence of lysate did not pull down syk in the immunoprecipitates.

Blotting of proteins

Proteins were separated in 10% tris-glycine gels under reducing conditions and electrotransferred on to a nitrocellulose membrane. The free binding sites were blocked by incubating the membrane overnight at 4°C with 3% BSA in TBST. The nitrocellulose membranes were then incubated with 0.5 µg/ml anti-phosphotyrosine mAb, 4G10, in 1% BSA/TBST for 1 h at room temperature. The membrane was then washed with TBST prior to the addition of an anti-mouse HRP conjugate (1:3000 dilution) for 1 h at room temperature. After further washing of the membranes with TBST, the phosphoproteins were visualized using ECL. The nitrocellulose membrane was exposed to ECL hyperfilm for 15 s–10 min. Following exposure, the nitrocellulose membrane was stripped for 30 min at room temperature with stripping buffer and re-probed with 0.2 µg/ml mouse anti-human p72syk. After exposure to chemi-

luminescence-detection agents, the intensity of each band was determined using densitometric analysis using a Kodak DC120 digital camera and acquisition software. In pilot experiments, we determined from twofold dilutional analysis that ECL detection was linear over the range of detection required.

[Ca⁺⁺]_i measurements

Basophils were labeled with 1 µM fura-2AM for 20 min at 37°C in RPMI 1640 containing 2% FCS (300,000–500,000 cells in 200 µl). After washing once with 200 µl PAG, the cells were resuspended in PAG for loading in the microscope stage [25, 26]. [Ca⁺⁺]_i was determined by digital video microscopy using techniques previously described in detail [25, 27]. Briefly, 15 µl cells (20,000–30,000) were loaded onto the siliconized coverslip of the microscope chamber and, after settling, overlaid with 1 ml PAGCM buffer. After warming to 37°C, monitoring of the cells was begun and after several frames (each frame is a single-ratio measurement of a field of 30–100 cells) of pre-challenge [Ca⁺⁺]_i levels were acquired, the cells were challenged with 1 ml stimulus in buffer. Data were then acquired for 50–150 frames at intervals of 1–10 s to determine the subsequent [Ca⁺⁺]_i response. For studies of basophil response in the microscope chamber (resting on the siliconized coverslip), the concentration of anti-IgE antibody that is optimal for histamine release occurs at a slightly higher concentration than is typical for experiments in test tubes (0.5 µg/ml vs. 0.2 µg/ml, respectively). The [Ca⁺⁺]_i timelag is the difference between the time of stimulus addition and the first statistically significant elevation in [Ca⁺⁺]_i. (See Figure 2A for an example of two single cells and their associated timelag intervals.)

Statistics

For the measurements of net average [Ca⁺⁺]_i increase and the [Ca⁺⁺]_i timelag, the errors noted in Table 1 represent the errors associated with averaging the results from separate experiments (where the net elevation in [Ca⁺⁺]_i for any one condition is itself an average of 30–70 cells), which we think better reflects the biological variation inherent in using basophils from different donors. Error bars in other plots represent the SE.

RESULTS

Our previous studies of the timelag phenomenon in human basophils indicated that the timelag could be lengthened sim-

TABLE 1. Effect of pharmacological agents known to inhibit the IgE-mediated cytosolic calcium response in human basophils on the timelag characteristic

Drug	Concentration	<i>n</i>	Avg Ca ⁺⁺ (nM)		Timelag (secs)	
			–	+	–	+
PP1	0.8 µM	2	41 ± 8	24 ± 3 (0.59 ± 0.04)	66 ± 4	254 ± 10 (3.8 ± 0.51)
Staurosporine	30 nM	5	79 ± 23	43 ± 11 (0.55 ± 0.13)	46 ± 8	76 ± 25 (1.65 ± 0.53)
Genistein	10 µM	4	46 ± 10	29 ± 8 (0.65 ± 0.18)	45 ± 8	81 ± 16 (1.79 ± 0.36)
PMA	10 ng/ml	3	75 ± 8	36 ± 13 (0.46 ± 0.11)	43 ± 11	75 ± 24 (1.67 ± 0.25)
Bis II	400 nM	3	50 ± 12	64 ± 3 (1.28 ± 0.20)	60 ± 2	60 ± 3 (1.00 ± 0.05)
FK + RP	30 µM/10 µM	3	111 ± 6	76 ± 11 (0.69 ± 0.10)	32 ± 4	32 ± 3 (0.98 ± 0.06)
LY294002	10 µM	2	53 ± 4	19 ± 2 (0.36 ± 0.02)	56 ± 6	77 ± 2 (1.36 ± 0.15)

Basophils were pre-incubated with the listed drugs for 10 min prior to the addition of 0.5 µg/ml anti-IgE antibody (0.2 µg/ml in the case of PP1). Concentrations were chosen to produce approximately 50% inhibition of the [Ca⁺⁺]_i response for the period of observation (usually 10–15 min), where the drug was known to inhibit the IgE-mediated response, and other choices were based on parameters described in Results. The number of experiments for each drug (e.g., different preparations of basophils for which 30–60 single cells were analyzed for each *n*) is shown; the average net elevation in [Ca⁺⁺]_i is shown with (+) and without (–) drug for the period of observation, and the average timelags with and without drug are shown. The ratio of the parameter for cells incubated with drug/parameter without drug is shown in parentheses. Statistical significance was achieved for PP1 (*p* = 0.042, *n* = 2) and genistein (*p* = 0.024, *n* = 4); the other drugs did not result in statistically significant increases in the [Ca⁺⁺]_i timelag.

ply by reducing the concentration of the stimulus [1]. Although this represented one clue as to the nature of the early signaling, it could simply be argued that the rate in which the cross-linking stimulus formed aggregates determined the length of the timelag, which was not surprising. We wished to know whether a rapidly established aggregate that nevertheless resulted in a weaker signal would show longer timelags. In addition, given equal binding rates, we were interested in what difference in signal strength could lead to twofold or greater increases in the timelag (the relevance of which is found in the nonreleaser experiments described below). To address this issue, we compared the timelags following stimulation of sensitized cells with a simple bivalent form of penicillin (BPO2) or a penicillin-HSA conjugate (BPO-HSA). Previous studies have indicated that BPO2 equilibrates very rapidly [28, 29], with equilibrium aggregates established in <1 s, and binding studies of BPO-HSA indicate that tens of seconds are required for maximum binding [24]. Conversely, BPO2 is a weak stimulus, presumably because of the more evanescent nature of the cross-links that are formed [28, 30–32]. **Figure 1A** shows the relative strength of these two stimuli. This figure shows that for these two donors, there was an eight–tenfold difference in the amount of IgE required to obtain equivalent histamine release when stimulated with BPO-HSA or BPO2 (which should translate to an eight–tenfold difference in cell-surface density of BPO-specific IgE). Figure 1, B and C, compares the timelags for cells stimulated with BPO-HSA or BPO2. The timelags (see Fig. 3 or 4 for examples of how these timelags appear for single cells) were approximately threefold longer for BPO2 than for BPO-HSA (a median of 17 s for BPO-HSA and 54 s for BPO2), suggesting that a signal-strength difference of approximately tenfold translates to a timelag difference of approximately threefold.

We next explored how various pharmacological agents that are suspected to influence some of the steps thought to be involved in calcium signaling alter the duration of the timelag. The activation of lyn kinase is thought to represent the first enzymatic step in signaling through FcεRI. Others have demonstrated that PP1 and PP2 are potent inhibitors of lyn kinase [33, 34], and we found that either drug effectively inhibits phosphorylation of lyn and syk kinases during stimulation of basophils with anti-IgE antibody [35]. **Figure 2A** shows the effects of 0.8 μM PP1 on the timelag in basophils stimulated with anti-IgE antibody. In data not shown, PP1 at 10 μM had no effect on the characteristics of calcium response following stimulation with formyl-Met-Leu-Phe (fMLP; which operates through a G-protein-linked receptor). **Figure 3** shows the frequency histogram of the cytosolic calcium response timelag for all the cells examined. The timelag was increased approximately fourfold at this concentration of PP1. It was remarkable that the timelag could be extended to as long as 10 min with PP1 at this concentration. Despite these long timelags, the ultimate response appeared otherwise normal for most cells. Not as evident in the single-cell traces, the average calcium response late in the reaction was equivalent or greater than in cells not treated with PP1. For the two experiments that form the basis of the histograms in Figure 3, the net cytosolic calcium elevation from 5–10 min averaged 33 ± 2 nM for cells not treated with PP1 and 38 ± 6 nM for cells treated with PP1,

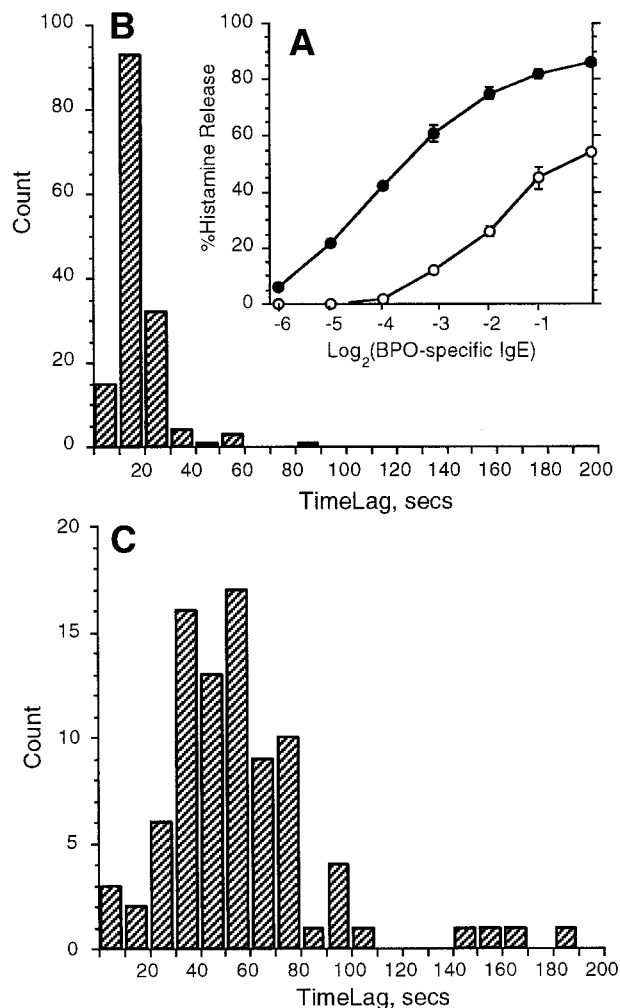


Fig. 1. Differences in the Ca timelag in basophils stimulated with bivalent penicillin (BPO2) or multivalent penicillin [BPO(11.5)-HAS]. Sensitized basophils were challenged with optimal concentrations of BPO2 (50 nM; ○) or BPO(11.5)-HSA (0.5 μg/ml, ~1 nM; ●). (A) Relative histamine release response for cells sensitized with serial twofold dilutions of BPO-specific IgE. (B and C) Frequency histograms for the $[Ca^{++}]_i$ timelags for fura-2-labeled cells stimulated with BPO(11.5)-HSA or BPO2, respectively.

and the average (see Table 1) for the entire period of observation was lower for the cells treated with PP1 because of the delayed onset of the response. We also noted that at concentrations below 1 μM, the kinetics of histamine release reflected the fact that the calcium response was delayed but otherwise normal, i.e., at 0.8 μM PP1, and release did not occur for 8 min compared with 2 min in cells not treated with PP1 but then progressed to the same final amount of histamine release as the untreated cells (Fig. 2B). It should be noted that the average timelag could be adjusted over a large range by adjusting the concentration of PP1. Concentrations above 1 μM resulted in many cells not responding within a timeframe of 15–20 min. Figure 2C also demonstrates that phosphorylation of syk kinase was delayed and blunted in cells treated with 0.8 μM PP1.

The early enzymatic steps during stimulation through FcεRI involve tyrosine kinases. Following activation of lyn is the activation of syk kinase. As will be discussed below, we could not use piceatannol, a putative, selective, syk kinase inhibitor,

but staurosporine and genistein are now known to inhibit several tyrosine kinases potently. Each of these drugs has been previously shown to be effective inhibitors of secretion [36, 37] from human basophils. (Previous studies established the following IC₅₀ values for inhibition of the histamine release for each of the drugs: PP1, 2 μ M; genistein, 10 μ M; staurosporine, 30 nM. The best combination of forskolin and rolipram rarely causes >50% inhibition of the calcium signal in basophils, so these drugs were used at concentrations considered near maximal without causing nonspecific effects. PMA inhibits the

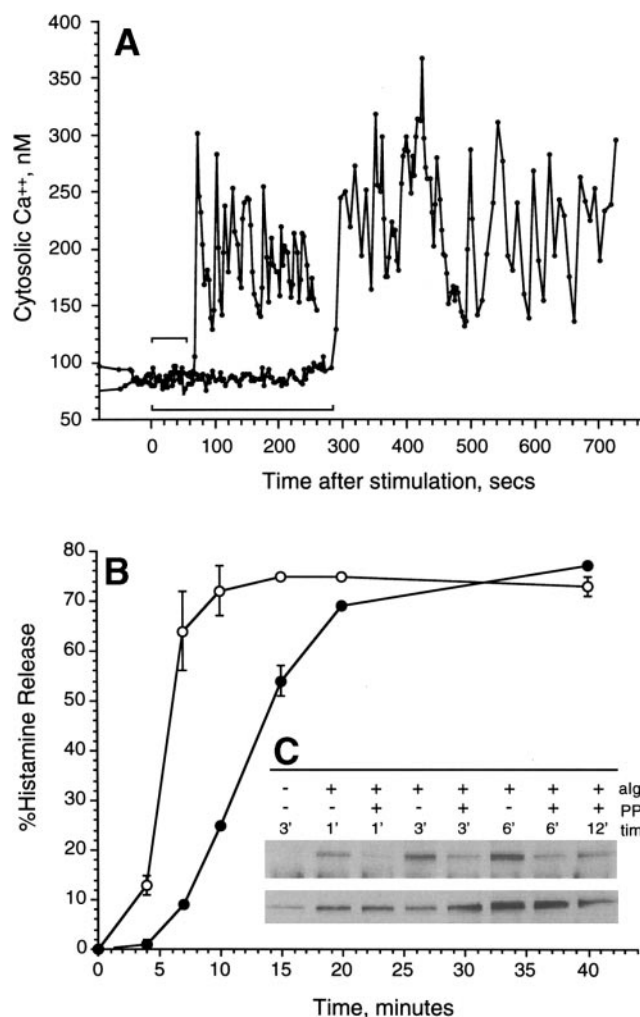


Fig. 2. Effect of PP1 on the basophil calcium and histamine release. (A) Two examples of single-cell $[Ca^{2+}]_i$ measurements for cells stimulated with anti-IgE antibody (0.2 μ g/ml). The kinetic timecourse for cells stimulated without PP1 (tracing with a short timelag interval) is representative of this population of cells, and the tracing with the longer time interval is representative of cells stimulated in the presence of 0.8 μ M PP1. The cells were incubated $\pm$ 0.8 μ M PP1 for 10 min prior to the addition of stimulus. (B) Kinetics of histamine release in the presence (●) and absence (○) of 0.8 μ M PP1 ($n=2$). Cells were incubated for 10 min with or without PP1 and stimulated with an optimal concentration of anti-IgE antibody (0.2 μ g/ml). At the times shown, ice-cold PAG containing 10 mM EDTA was added to stop the reaction, the tubes centrifuged, and the supernatants harvested for analysis of histamine content. (C) Western blot data for cells incubated $\pm$ PP1 (0.8 μ M) for 10 min prior to the addition of anti-IgE at 0.2 μ g/ml ($n=1$). Cells were harvested at the times shown, and syk was immunoprecipitated and analyzed by Western blot. The top blot shows the anti-phosphotyrosine blot, and the bottom blot shows the anti-syk blot [the top blot stripped and reblotted with anti-syk (4D10)].

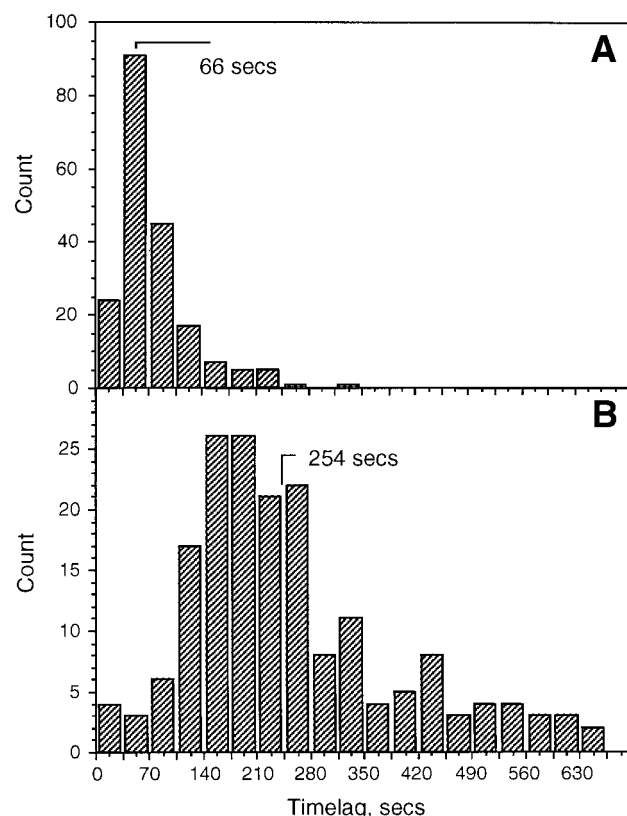


Fig. 3. Frequency histograms for the $[Ca^{2+}]_i$ timelags for cells challenged in the presence or absence of PP1. A combination of results from two preparations of basophils, panel A shows the histogram for cells stimulated with 0.2 μ g/ml anti-IgE antibody, and panel B shows the results for cells stimulated in the presence of 0.8 μ M PP1 (in both instances, there was a 10-min preincubation period without or with drug).

calcium response with an IC₅₀ of 10 ng/ml but causes histamine release itself, so this value is only relevant to the calcium response. Bis II does not inhibit histamine release but maximally inhibits PMA-induced release at 400 nM. For LY294002, there is some discordance in the inhibition of histamine release—IC₅₀=1–2 μ M—and the $[Ca^{2+}]_i$ response—IC₅₀=8–10 μ M. Historically, the IC₅₀ for inhibition of histamine release by staurosporine has been increasing steadily for unknown reasons. Our first studies placed the IC₅₀ at 1 nM [36]. By the early 1990s, it was found to be between 5 and 10 nM, and by 1996, the IC₅₀ had reached a stable value of $\sim$ 30 nM, the same IC₅₀ found for its close analog, Go-6936 [38]. The IC₅₀ for inhibition of the calcium response by PMA has increased slightly from our original study [36], possibly because of changes in how we purify and handle basophils.)

We found that 100 nM staurosporine inhibited syk kinase phosphorylation, determined 5 min after the addition of anti-IgE antibody by 57 $\pm$ 15% (unpublished results). The effects of genistein on IgE-mediated syk phosphorylation haven't been examined. **Table 1** summarizes the results for the effects of these drugs on the timelag characteristic. The concentration of the drugs was chosen to result in only partial inhibition ($\sim$ 50%) of the calcium response to determine the effects on the timelag characteristic. Although each of the drugs inhibited the average cytosolic calcium response effectively (net average

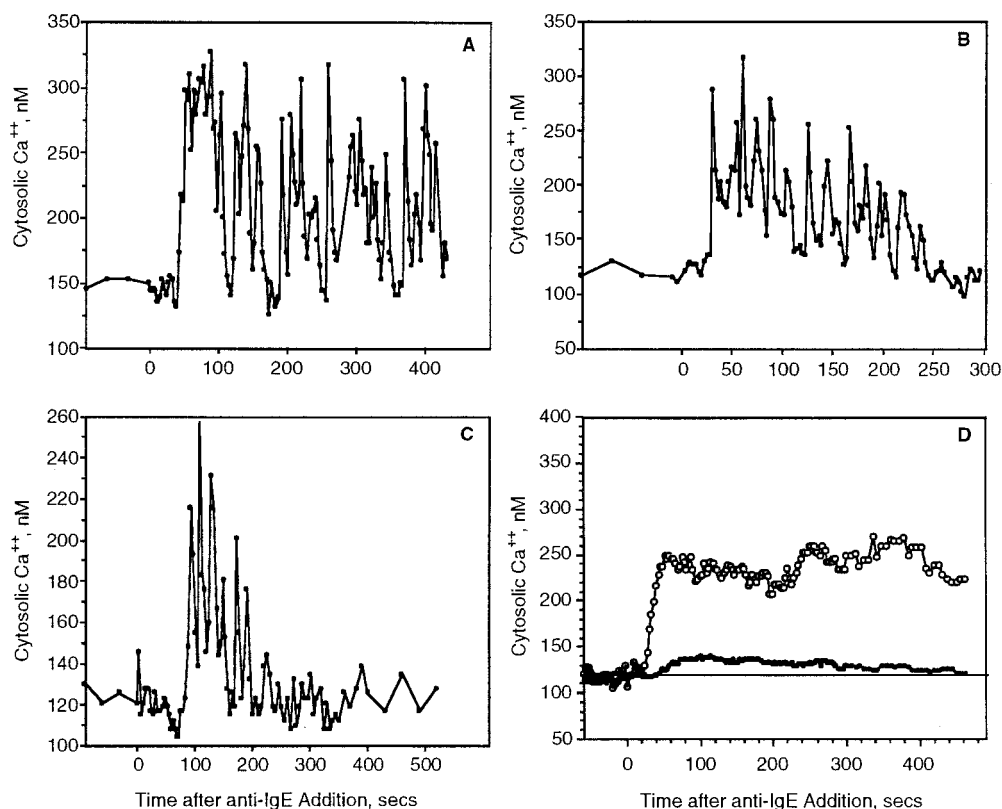


Fig. 4. Characteristics of the single-cell $[Ca^{++}]_i$ response in basophils from releasing or nonreleasing donors. Selected examples of the single-cell response are shown. (A) A representative result for a cell from a releasing donor. (B) A releasing basophil stimulated in the presence of 5 mM EGTA. (C) A basophil obtained from a nonreleasing donor. (D) Average $[Ca^{++}]_i$ response for the two groups of donors, releasers ($\circ$; $n=2$) and nonreleasers ($\bullet$; $n=3$). In all cases, the cells were stimulated with a concentration of anti-IgE antibody considered optimal for secretion in the microscope chamber, 0.5 μ g/ml.

elevation for the 0–15 min timeframe), the drugs did not always result in lengthening the timelag. Even the increases caused by genistein were modest when compared with the ability of PP1 to increase the timelag. At higher concentrations, these drugs completely inhibited the $[Ca^{++}]_i$ response; i.e., there was no timelag to measure. We examined three other classes of agents known to inhibit the cytosolic calcium response in basophils [39], drugs which elevate cAMP levels (a combination of forskolin and rolipram), protein kinase C (PKC) modulators, and a PI3 kinase inhibitor (Table 1). The combination of cAMP-elevating agents had no effect on the $[Ca^{++}]_i$ timelag but did inhibit the average $[Ca^{++}]_i$ response. The results with PMA were somewhat more variable, but there remained no statistically significant increase in the timelag. We also examined the reasonably selective PKC inhibitor, bis-indolylmaleimide II [38], but found no effect of this drug on the timelag. We have not found a drug that shortened the timelag. Finally, we have found that LY294002, the selective PI3 kinase inhibitor, inhibits the IgE-mediated calcium response in human basophils [40]. However, there was only a modest increase in the timelag that was evident in both experiments (but not statistically significant for $n=2$).

Previous studies suggested that the IgE-mediated calcium response in nonreleasing basophils might be nonexistent [6, 36]. These results were based on an extrapolation of results for poorly releasing basophils or relied on data examining the whole population response under conditions of optimal stimu-

lation. We have found that the details are more subtle. At the single-cell level, nonreleasing basophils do, in fact, show a $[Ca^{++}]_i$ response. **Figure 4** shows the characteristics of this response under conditions of optimal stimulation. Figure 4A shows a typical $[Ca^{++}]_i$ response in “releasing” basophils and demonstrates the sustained nature of an oscillating signal. Figure 4B shows an example of the response of “releasing” basophils when challenged with an optimal concentration of anti-IgE antibody with the simultaneous addition of a chelating concentration of EGTA. This addition allows the continued expression of the immediate $[Ca^{++}]_i$ response as a result of the release of internal stores of Ca^{++} but suppresses the influx phase of the response. This can be seen in single cells as the steadily decreasing magnitude of the oscillations until the response is dampened out. Figure 4C shows the response characteristics of “nonreleasing” basophils (see Materials and Methods). It is evident that there can be initial responses with a magnitude similar to releasing cells that rapidly dampen in a manner similar to that seen in the middle panel. (It appears unlikely that this is a response to some sort of contaminant in our anti-IgE preparation. Thus far, there are two types of calcium response observed in human basophils, those receptors using heterotrimeric GTP-binding proteins—e.g., fMLP, C5a, PAF—and those represented by Fc ϵ RI—with no others currently known. Only activation through Fc ϵ RI is known to be inhibited by PP1 currently; this anti-IgE-antibody response can be inhibited completely with 10 μ M PP1. Therefore, any

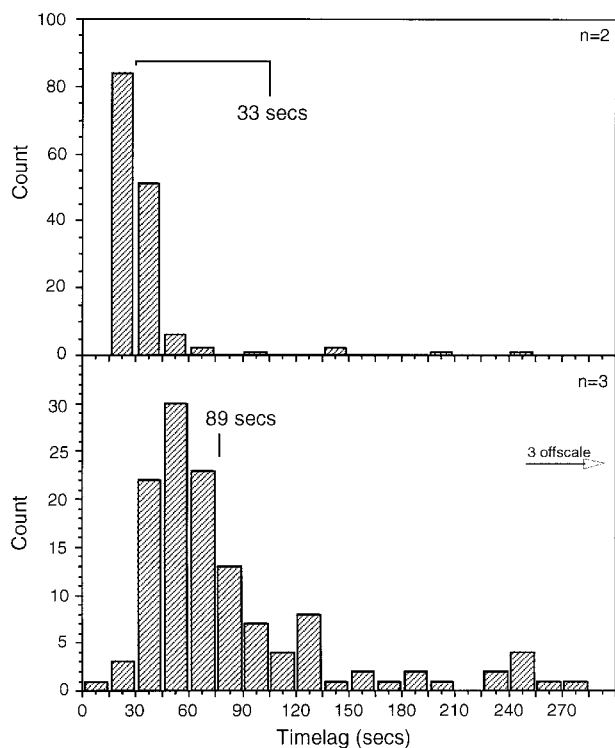


Fig. 5. Frequency histograms for the $[Ca^{++}]_i$ timelag in basophils obtained from releasing and nonreleasing donors. The top panel shows the histogram composite for basophil preparations obtained from two donors with the releasing phenotype. The bottom panel shows the histogram composite for basophil preparations from three donors classified as having a nonreleasing basophil phenotype. The mean timelag values are shown.

contaminant would have to utilize a PP1-sensitive pathway. In addition, the general characteristics of the calcium response are also consistent with an IgE-mediated response.)

It should also be noted that there was a modest difference in the fraction of cells that don't appear to respond to stimulation, 0% in the releasers and $20 \pm 5\%$ in the nonreleasers. The average elevation in $[Ca^{++}]_i$ for the 0–15-min observation period was 13 nM for the nonreleasers and 113 nM for the releasers (Fig. 4D). A second observation derived from these studies is that the quiescent timelag period is longer for basophils derived from “nonreleasers”. **Figure 5** shows frequency distributions for single-cell timelags for cells challenged with an optimal concentration of anti-IgE antibody. On average, the nonreleaser timelag was approximately three times longer than the response in releasing basophils.

In previous studies, we found that supraoptimal concentrations of polyclonal goat anti-IgE antibody induced a more synchronized response [41]. Although this concentration of stimulus leads to poorer histamine release, the magnitude of the first phase of the response, the release of calcium from internal stores, is actually greater (the second phase is weaker as discussed in [41]). We interpret this result to mean that not only is there synchronization of the response among different cells but also that there is synchronization of the signal within individual cells, resulting in a stronger initial response within individual cells. This result suggested that we attempt to accentuate the nonreleaser calcium response by challenging

with supraoptimal, anti-IgE antibody (3 μ g/ml). When this is done, a modest average population response is observed (average net elevation in the 0–2-min timeframe was 128 nM compared with 312 nM in releasing cells), in contrast to the nearly absent average response observed following stimulation with lower concentrations of anti-IgE antibody as a result of asynchronous responses among individual cells.

DISCUSSION

Previous studies established that slower antigen-binding led to long timelags, a result that is not surprising given that antigen-binding studies show that even optimal concentrations of antigens require tens of seconds to reach maximal binding [1, 24]. The current studies using BPO2 and BPO-HSA demonstrated that ligand-binding rates are not the sole determinant of longer times lags. Although the binding rate of BPO2 can only be indirectly assessed or calculated from theoretical models [29], there is strong supportive evidence that such bivalent ligands bind rapidly and establish an approximate equilibrium of IgE aggregates rapidly [28, 42]. Recent studies using paucivalent ligands to stimulate rat basophilic leukemia (RBL) cells demonstrate that rapid binding, but presumably short-lived aggregates, leads to the effect known as kinetic proofreading [32]. Initial signaling elements may be quite active, but signaling elements further downstream are only poorly activated. Our results support such a view. The longer timelag with BPO2 indicates that the steps leading to the initial release of calcium from internal stores are only weakly established by this ligand. The data also suggest that a tenfold change in the “strength” of the stimulus, measured at the distant endpoint of histamine release, translates as a threefold increase in the timelag, at least for these two types of aggregating molecules.

Pharmacological studies indicated that inhibiting the earliest known step in the Fc ϵ RI signaling cascade, the activation of lyn kinase, with PP1 resulted in profound increases in the $[Ca^{++}]_i$ timelag. Although the concentration of 0.8 μ M PP1 could be considered arbitrary, it was chosen because it resulted in increases in the timelag similar to those found for the nonreleasers. In this context, the characteristics of the late calcium response in the presence of PP1 were interesting. At these concentrations of PP1, the net average increase in calcium for the period of observation was decreased, because the initial phase of the response was delayed significantly in onset. The actual elevation, once established, was very similar to the response without PP1 present. A similar characteristic was observed for histamine release kinetics. This observation places the results with PP1 in contrast to the characteristics of the nonreleaser calcium response (see below).

We did examine other tyrosine kinase inhibitors but were surprised to find that they only poorly increased the duration of timelag. Indeed, staurosporine's effects were not statistically significant. However, an increase in duration of the timelag did occur in the presence of genistein ($p=.024$), albeit an increase that was quite modest when compared with the effects of PP1. The lengthening occurring in the presence of staurosporine could have been mitigated, because this drug is also a potent inhibitor of PKC, which has been implicated in down-regula-

tion of immunoreceptors and G-protein-linked, 7-transmembrane-type receptors [43–47]. Therefore, we examined the effect of PMA on the timelag and found only modest increases with low concentrations (higher concentrations completely inhibit the calcium response) that were quite variable, which only bordered on statistical significance ($p=.057$) in these weakly powered studies. (The ratio of timelags in the presence or absence of drug was variable among basophil preparations, although within individual preparations, the distribution of timelags for the two conditions, composed as they were from 30–70 single-cell measurements each, was statistically different for some experiments.) Furthermore, inhibition with potent and reasonably selective PKC inhibitors had no measurable effect on the timelag. Taken together, these data indicate that there is little evidence that PKC plays a role in regulating the duration of the timelag and thus suggest that the variable effect of staurosporine is not easily explained by its potential to inhibit PKC.

The last set of inhibitors we examined were those that elevate cAMP levels or inhibit PI3 kinase. We have shown previously that marked elevations in cAMP cause reasonable decreases in the second phase of the cytosolic calcium response without altering the peak of the first phase of the response [39]. Here, we find that not surprisingly, these drugs did not alter duration of the timelag. These results reinforce the previous conclusion that in basophils, the two phases of the calcium response can be regulated independently by cAMP-dependent signaling elements. PI3 kinase has also been implicated in regulating the sustained phase of the $[Ca^{++}]_i$ response [48, 49], although we have not seen clear evidence for preferential effects in the human basophil. Wortmannin, another PI3 kinase inhibitor, has been demonstrated to inhibit the $[Ca^{++}]_i$ response in basophils [50] and LY294002, shown to inhibit secretion and extracellular signal-regulated kinase phosphorylation [51]. Studies of wortmannin in RBL cells suggested that there was an increased $[Ca^{++}]_i$ timelag [52]. With more experiments, the trend to lengthening that we observed might become statistically significant, but compared with PP1 or even genistein, the increase in our studies was quite modest.

Previous studies would have suggested that nonreleasing basophils would show no calcium response following stimulation with anti-IgE antibody. However, our own previous studies did not examine previously characterized, nonreleasing basophils specifically. Results for nonreleasers could only be extrapolated from linear regressions of results using cells that had been handled previously for 24 h. Furthermore, the correlation plot in our former studies was based on time-average calcium elevations, which as noted below, mask asynchronous transient elevations (indeed, even a simple linear regression of the former data would predict a small, nonzero, average elevation for nonreleasers). In studies by Knol *et al.* [6], nonreleasing basophils were examined directly by flow cytometry to measure changes in $[Ca^{++}]_i$. This technique does not follow the response of a single cell through time so transient oscillatory elevations that occur asynchronously are not easily identified. (When observing tens of cells/time interval, a slight haze of noise above the baseline may be expected to be observed, a characteristic of the calcium responses from nonreleasing ba-

sophils in the Knol *et al.* [6] studies. The problem is that for these points above the baseline, it is unknown if they are indeed above their own baseline.) In our current studies, there was an increase in the duration of the timelag and a transient elevation in calcium that mimicked the calcium response observed in basophils from “releaser” subjects stimulated in the presence of EGTA to suppress the influx phase of the response. In our own studies of the nonreleaser phenotype, we have not only found markedly reduced expression of syk kinase but also markedly reduced expression of lyn kinase [8]. The donors used in these $[Ca^{++}]_i$ studies were the same donors we characterized for lyn and syk kinase expression—lyn and syk kinase were expressed ten–15-fold less in the nonreleasers, and we found an ~ninefold lower average $[Ca^{++}]_i$ response. In the PP1 experiments, basophils would experience reduced lyn activity and therefore reduced syk activity, but the absence of syk in the nonreleasers might alter the characteristics of the response further. It is possible that the distinguishing difference between nonreleasers and releasing cells treated with PP1 is the absence of syk. In this context, it would be helpful if we could examine the consequences of syk kinase inhibition. However, the only available putative syk-selective inhibitor is piceatannol, and we have found that piceatannol alters basophil responses in unexpected ways for a syk kinase inhibitor. For example, it inhibits fMLP-induced LTC4 release with an IC50 lower than required for inhibition of IgE-mediated histamine release. Furthermore, piceatannol does not inhibit the $[Ca^{++}]_i$ response or the phosphorylation of Shc in human basophils, an immediate downstream substrate of syk in a variety of other cell types (unpublished results).

In summary, these studies showed that weak signaling resulted in prolonged timelags and that the absence of syk and lyn kinase led to the expected increased duration of the timelag in nonreleasing basophils. However, the full character of the calcium response in nonreleasing basophils could not be mimicked by inhibition of lyn kinase using PP1. Surprisingly, other tyrosine kinase inhibitors resulted in little, or at best modest (relative to PP1), increases in the timelag. PKC activation had inconsistent effects, and its inhibition had no effect on the timelag.

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