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EFFECT OF OKADAIC ACID ON HUMAN BASOPHIL SECRETION

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Abstract—We examined the effects of a phosphatase inhibitor, okadaic acid, on mediator secretion from human basophils. These cells are known to respond to a number of stimuli that appear to utilize distinct biochemical pathways converging on mediator release. Okadaic acid was found to inhibit IgE-mediated release (histamine release inhibited 80 ± 12% and leukotriene release inhibited 100% following a 10-min preincubation with okadaic acid and stimulation with an optimal concentration of anti-IgE antibody) at a concentration of 1 μM. The concentration–response curve to okadaic acid was steep, with 0.1 μM yielding only 20 ± 10% inhibition of either mediator. Secretion following stimulation with the univalent stimulus, fMet-Leu-Phe peptide, was not inhibited by okadaic acid. Unlike cAMP-active agents that inhibit cytosolic Ca^{2+} elevations following IgE-mediated stimulation, the increased state of cellular protein phosphorylation, which presumably results from treatment with 1 μM okadaic acid, had no effect on the elevations in free cytosolic Ca^{2+} that follow stimulation with anti-IgE antibody of fMet peptide.

Human basophils, like tissue mast cells, are activated to secrete mediators involved in allergic reactions. Activation of these cells to secrete can be accomplished by a number of different stimuli. In general, two types of stimuli can be considered, namely univalent stimuli, such as the bacterial peptide fMet-Leu Phe (fMet peptide) [1, 2] or the complement peptide C5a [3, 4], and multivalent stimuli that act by causing the aggregation of cell surface IgE antibody and its corresponding high-affinity receptor. However, the signal transduction pathway following the ligand-induced reaction is largely unknown for any of the stimuli. Recent studies have implicated increases in cytosolic calcium levels and a change in protein kinase C activity [5, 6] in human basophils, mirroring similar results for mast cells of other species. More recently, studies in rat basophilic leukemia cells have also suggested that tyrosine kinase activity is crucial to the development of a secretory response [7, 8]. Thus, all known pathways of activation involve the activity of several classes of kinases: calcium presumably acting through calmodulin-dependent kinases, protein kinase C and tyrosine kinases directly acting on unknown substrates. It is likely that for each of these kinases, there are corresponding phosphatases to regulate the reaction. Indeed, phosphorylation of the IgE receptor is regulated by a phosphatase that rapidly dephosphorylates tyrosine residues [9]. In addition, monoclonal antibodies to CD45, a known plasma membrane bound phosphatase, inhibit IgE-dependent release in human basophils [10]. We have observed recently that the effect of the phosphorylation mediated by cAMP may be related to inhibition of influx of calcium during the IgE-mediated calcium response in basophils while not showing any effect on the fMet peptide-mediated calcium influx.†

Okadaic acid is a potent inhibitor of the soluble cytosolic serine and threonine phosphatases types 1 and 2A (for review see Ref. 11). Its effect generates a hyperphosphorylation of substrates within the cell, since the phosphates incorporated on the substrates by specific kinases are continuously acting in the presence of the phosphatase inhibitor. Since the phosphorylation of unknown substrates, either through protein kinase C, calcium/calmodulin-dependent kinase or other kinases, is a key step in the regulation of secretion in basophils, we examined the effect of okadaic acid in basophils stimulated with the nonspecific cross-linking stimulus, anti-IgE antibody, or the univalent stimulus, fMet peptide.

MATERIALS AND METHODS

Buffers. (PAG‡) 25 mM piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (PIPES) (Sigma Chemical Co., St. Louis, MO); 140 mM NaCl, 5 mM KCl, 0.003% human serum albumin (Miles Laboratories, Inc., Elkhart, IN); 0.1% glucose. (PAGCM) PAG with 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Reagents. 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 [12]. Fura-2AM and fura-2 (potassium salt) were obtained from Molecular Probes (Eugene, OR). Okadaic acid was purchased from LC Services (Boston, MA) and formyl-methionyl-leucyl-phenylalanine from Sigma. Okadaic acid was dissolved in 10% dimethyl sulfoxide

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‡ Abbreviations: PAG, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid; PAGCM, PAG with $CaCl_2$ and $MgCl_2$.

(DMSO), and the final concentration of DMSO (lower than 0.1%) did not affect mediator release.

Cell preparation. Basophils were purified from buffy coat cells, obtained from normal donors undergoing hemapheresis, using counter current elutriation and Percoll density gradients [5] or by affinity chromatography [13], as previously described. In the experiments presented, basophil purities were greater than 85%. For the anti-IgE experiments shown in Fig. 1, impure basophils were obtained from whole blood by separation on a single-step Percoll gradient as previously described.

Basophil counting. Basophils were stained with Alcian Blue [5] and counted in a Spiers Levy hemocytometer.

Fura-2AM labeling. Basophils were labeled with 1 μM fura-2AM for 20 min at 37° in RPMI-1640 (Gibco) also containing 0.32 mM EDTA and 2% fetal bovine serum (300,000–500,000 cells in 200 μL). After washing once with 200 μL PAG, the cells were resuspended in 200 μL PAG for loading in the microscopic stage.

Calcium measurements. Cytosolic calcium changes were determined by digital video microscopy using techniques described previously in detail [6, 14]. Briefly, a 15-μL sample of cells (20,000–30,000 cells) was loaded onto the siliconized cover slip of the microscope chamber and, after settling, overlaid with 1 mL of PAGCM buffer. After warming to 37°, monitoring of the cells was begun and after 4 frames (each frame is a single ratio measurement of a field of 30–100 cells) of pre-challenge calcium levels were acquired, the cells were challenged with 1 mL of stimulus in buffer and 50–150 frames of data acquired to determine the subsequent calcium response. Note that for the cells under observation, the area observed represents 0.02% of the total coverslip area. For the macro-event of adding 1 mL of stimulus, the cells in this area experience the same concentration of stimulus at all time points and this concentration reaches its final state within 10 sec [14]. Calibration of the calcium changes was described previously [6], and the same values for the constants were applied to these studies. The plots in this manuscript represent the average of kinetic curves obtained from several preparations of basophils, which themselves were the average of the 30–100 cells viewed under the microscope. We also have found that challenge of basophils with fMet peptide in RPMI-1640 (Gibco), where the pH is buffered by HCO_3/CO_2, leads to the same first and second phase calcium responses as observed in PAGCM buffers. This indicates that the second phase calcium response we observed is not an artifact of how the pH is buffered or the presence of bicarbonate ions as has been suggested in other studies [15, 16].

Histamine release measurements. Histamine release measurements in the test tube were made as described previously [14]. Histamine was determined by the automated method of Siraganian [17], and histamine release was calculated as the ratio of released histamine to total histamine after subtracting spontaneous release from each value. Spontaneous release ranged from 5 to 10%. The vehicle for okadaic acid, DMSO, was used in the controls at an equivalent final concentration (<0.1%). At the concentrations used, it had no effect on release.

Histamine release was also directly determined following calcium measurements by removing 1 mL of the supernatant from the microscope chamber (which contained a total of 2 mL after challenge) after the observation period was complete. Treatment with okadaic acid was accomplished by preincubating the cells for 10 min with the drug, which in the microscopic measurements was included in the buffer initially overlaid on the 15-μL cell drop (as described above). For microscopic studies, the total histamine content was obtained by treating 7.5 μL of cells with 200 μL of 8% perchloric acid and bringing the volume to 1 mL with PAGCM. In the test tube, cells were also lysed with perchloric acid.

Leukotriene (LT) release was made in the same manner except that 100-μL aliquots were analyzed by an LTC_4-specific radioimmunoassay as described previously [18, 19].

The data shown in the figures and in the text are expressed as means ± SEM.

RESULTS

We first examined the concentration-dependent effect of okadaic acid, with a fixed 10-min preincubation, on the release induced by anti-IgE antibody and fMet peptide; both stimuli were used at concentrations that caused optimal secretion. As shown in Fig. 1, 1 μM okadaic acid completely inhibited both histamine and LTC_4 release induced by anti-IgE, histamine release being 56 ± 10% with

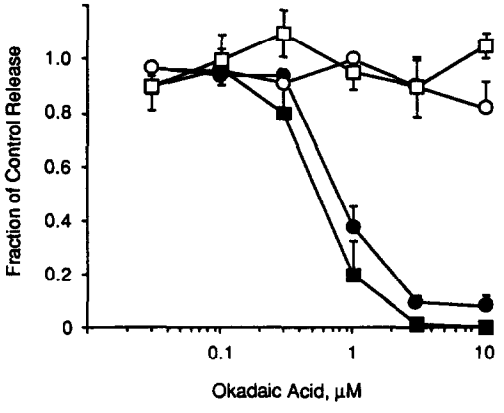

Fig. 1. Inhibition of mediator release in human basophils by okadaic acid. Basophils were challenged in the presence of various concentrations of okadaic acid following a 10-min preincubation with the drug. Histamine release (●) and leukotriene C_4 release (■) were measured following stimulation with an optimal concentration of anti-IgE antibody (0.1 μg/mL) (control histamine release = 56 ± 10% and control leukotriene release was 23 ng/μg histamine). Histamine release (○) was determined following an optimal concentration of fMet peptide (1 μM) (control histamine release was 72 ± 13%, N = 3). Leukotriene C_4 (□) was studied in purified basophils (control leukotriene C_4 release = 19.2 ng/μg histamine, N = 3). Values are means ± SEM.

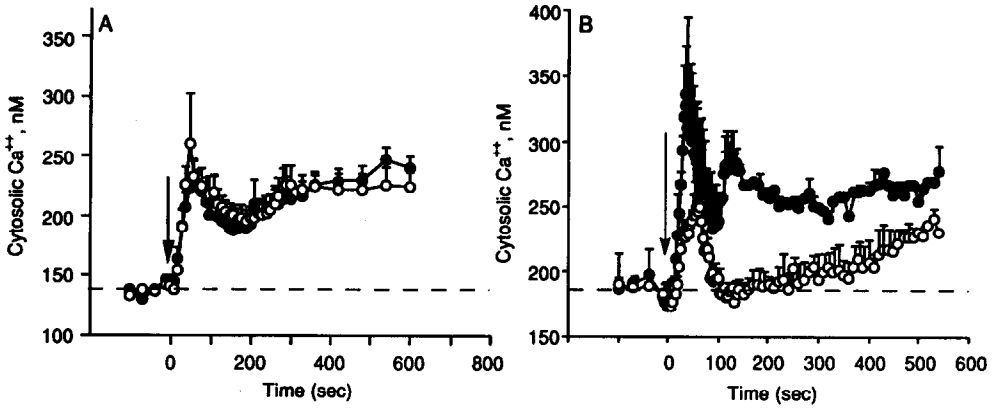

Fig. 2. Effect of okadaic acid on the cytosolic Ca^{2+} response following stimulation with anti-IgE antibody (panel A, 1 μM okadaic acid; panel B, 10 μM okadaic acid). Panel A: Purified basophils were preincubated with 1 μM okadaic acid for 10 min prior to the beginning of monitoring and the addition of anti-IgE antibody (0.7 μg/mL). The addition of the stimulus is marked by an arrow. Key: (●) control cells, and (○) cells treated with okadaic acid. The curves represent the average of seven experiments. Histamine release in untreated cells was 43 ± 5%. Panel B: Purified basophils were preincubated with 10 μM okadaic acid for 10 min prior to the beginning of monitoring and the addition of anti-IgE antibody (0.7 μg/mL); the curves represent the average of three experiments. Histamine release in untreated cells was 40 ± 12%. Values are means ± SEM.

0.1 μg/mL anti-IgE in the absence of okadaic acid, and 3 ± 2 with 0.1 μg/mL anti-IgE and 10 μM okadaic acid. The inhibitory profile for both histamine and LTC_4 was the same, LTC_4 release being 23 ng/μg histamine in the absence of okadaic acid and undetectable with 10 μM okadaic acid. Histamine and leukotriene release induced by fMet peptide were unaffected with concentrations of okadaic acid ranging from 1 nM to 10 μM (the LTC_4 release results were generated with purified basophils; in impure basophils or mixed mononuclear cells, we obtained 50% inhibition with 3 μM okadaic acid, which appeared to be attributable to the effect of fMet peptide on other cell types).

Because we have observed inhibition of the second phase of the calcium response, that phase related to the influx of external calcium, by cAMP active agents during IgE-mediated release, we examined the effect of okadaic acid on the calcium response. Figure 2 shows the effect of two concentrations of okadaic

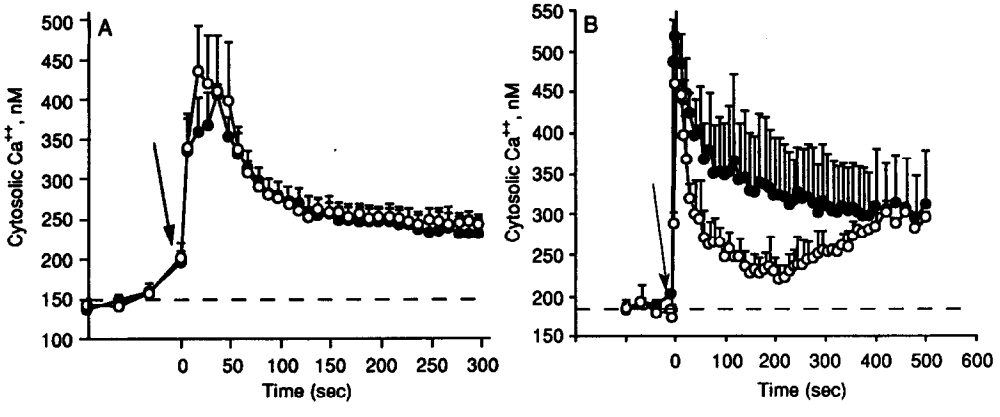

Fig. 3. Effect of okadaic acid on the cytosolic Ca^{2+} response following stimulation with fMet peptide (panel A, 1 μM okadaic acid; panel B, 10 μM okadaic acid). Panel A: Purified basophils were preincubated with 1 μM okadaic acid for 10 min prior to the beginning of monitoring and the addition of fMet peptide (0.1 μM). The addition of the stimulus is marked by an arrow. Key: (●) control cells, and (○) cells treated with okadaic acid. The curves represent the average of seven experiments. Histamine release in untreated cells was 52 ± 9%. Panel B: Purified basophils were preincubated with 10 μM okadaic acid for 10 min prior to the beginning of monitoring and the addition of fMet peptide (1 μM); the curves represent the average of three experiments. Histamine release in untreated cells was 72 ± 9%. Values are means ± SEM.

acid (1 and 10 μM) on cytosolic calcium levels in basophils stimulated with anti-IgE antibody. With 1 μM okadaic acid there was no observable effect in calcium levels as compared to the control, whereas in the presence of 10 μM okadaic acid there was some inhibition of the first phase of the calcium response (that attributable to the release of internal stores of calcium [20, 21]) and a marked inhibition of the second phase. It should be noted, however, that mediator release (histamine and LTC_4) followed the profile indicated in Fig. 1, i.e. at 1 μM okadaic acid; IgE-mediated histamine and leukotriene release were inhibited 80–100%.

Figure 3 shows that 1 μM okadaic acid had no effect on the calcium response following stimulation with fMet peptide. In the presence of 10 μM okadaic acid, there was a non-statistically significant inhibition of the second phase of the calcium response. As with IgE-mediated release experiments, we measured histamine release in the same sample used on the microscope, with results similar to Fig. 1 (see figure legends).

DISCUSSION

There are few well-established facts on the signal transduction pathways in human basophils. However, most studies in a wide variety of cell types indicate that the concerted action of kinases and phosphatases control secretory processes. This is likely to be true in basophils. In human basophils, we have noted that while all types of stimuli alter cytosolic calcium levels and protein kinase C activity [5, 6, 20, 22], the stimuli, in fact, follow distinct pathways which accomplish the same end result. We have most actively compared IgE-mediated stimulation and the action of univalent stimuli, fMet peptide and C5a. While the biochemical measurements (elevations in cytosolic calcium and protein kinase C activity) that follow activation with these stimuli do show differences in details whose significance is not yet understood, pharmacologic studies often differentiate these stimuli quite well. For example, fMet peptide-induced secretion is inhibited completely by pertussis toxin and unaffected by glucocorticosteroids, whereas the IgE-mediated release is inhibited profoundly by steroids and not affected by pertussis toxin [23, 24]. A qualitatively similar result has been observed in rat basophilic leukemia cells [25]. Staurosporine, a kinase inhibitor with some selectivity for PKC and tyrosine kinases [26], enhances fMet peptide-induced release while completely inhibiting IgE-mediated release [6]. The recent studies concerning the inhibition of human basophil secretion by anti-CD45 antibodies also indicated a stimulus-selective effect, with no inhibition of fMet peptide and significant inhibition of IgE-mediated release [10]. Agents that elevate cAMP also had little or no effect on fMet peptide-induced release while causing moderate inhibition of IgE-mediated release*. These last three differences highlight specific characteristics that may relate to the activity of kinases and phosphatases or, more generally, to the state of phosphorylation within the cell. In this context, it is interesting to note that okadaic acid inhibits IgE-mediated release while having no effect on fMet peptide-induced secretion.

There is discordance between the results with cAMP-active agents, okadaic acid and inhibition with staurosporine. The first two agents would be expected to elevate the state of phosphorylation (presumably serine and threonine phosphorylation), whereas staurosporine would inhibit phosphorylation. Yet, in each case, release is inhibited. This only reflects our lack of knowledge concerning the important details of the reaction. For IgE-mediated release, okadaic acid appears to have two effects on cellular activation. First, mediator release was inhibited with no detectable inhibition of calcium. Only at higher concentrations was the calcium response inhibited. We conclude that calcium levels are regulated by a phosphorylation pathway 10-fold less sensitive than the exocytosis machinery. It is important to bear in mind that the inhibition seems to affect both internal (IP3-dependent, first calcium phase) and extracellular (second phase) calcium. The 2-fold effect of okadaic acid may be explained by noting that the inhibition of PP1 (phosphatase 1), is 10-fold less sensitive than its inhibition of PP2A [11]. Therefore, it may be possible that mediator release is affected by PP2A and cytosolic calcium changes by PP1. As in any pharmacologic study, the strength of the observations depends on the specificity of the drug being used so while okadaic acid thus far appears to be a reasonably selective agent, there have been some reports describing other actions. For example, okadaic acid is known to stimulate the ATP-dependent interaction between actin and myosin of smooth muscle via a direct effect on myosin [27]. Nevertheless, this action is only observed at concentrations 100 times higher than those required to inhibit phosphatase activity. Therefore, it may be possible that the effects that we describe with 10 μM okadaic acid are less specific than with 1 μM, the concentration described to be effective in several cellular models [11], so that our results fit in the relevant concentration range.

The results with fMet peptide indicate that the internal mechanisms regulating the transduction of its signal are unaffected by okadaic acid although calcium elevations can be affected with high concentrations of okadaic acid in the absence of an effect on mediator release. If we accept that the calcium response represents a relatively early event during signal transduction and is mandatory for secretion, then okadaic acid may exert its effects later in the IgE-mediated pathway. Alternatively, okadaic acid alters a pathway that operates in parallel events that modulate calcium levels, but a pathway that is equally necessary for secretion of both types of mediators (preformed and non-preformed).

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