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MECHANISMS OF MEDIATOR RELEASE FROM HUMAN SKIN MAST CELLS UPON STIMULATION BY THE BRADYKININ ANALOG, [DArg^0-Hyp^3-DPhe^7]BRADYKININ*

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Abstract—We have used the bradykinin analog, [DArg^0-Hyp^3-DPhe^7]-bradykinin, as a model stimulus with which to examine peptide-induced mediator release from human skin mast cells (SMC) and to compare it with IgE-mediated release from the same cells. The bradykinin analog was an effective histamine secretagogue, inducing a comparable maximal level of release to that observed for anti-IgE. By contrast to anti-IgE, however, [DArg^0-Hyp^3-DPhe^7]-bradykinin did not stimulate marked release of prostaglandin D_2 (PGD_2) from these cells. In experiments where cells were exposed to both stimuli simultaneously, histamine release was additive, while PGD_2 release was the same as that observed for anti-IgE alone. The kinetics of [DArg^0-Hyp^3-DPhe^7]-bradykinin-stimulated histamine release were rapid, with 50% of maximal release being achieved within 30 sec, compared to 2–3 min for anti-IgE. Interestingly, when both stimuli were applied simultaneously, the kinetics of release were intermediate between those of either stimulus alone. Studies of the signal transduction pathways that may be involved in [DArg^0-Hyp^3-DPhe^7]-bradykinin-induced histamine release revealed striking differences to results obtained with anti-IgE. While agents that increase intracellular cyclic AMP have a pronounced inhibitory effect on IgE-mediated release, forskolin, isobutylmethylxanthine and isoproterenol were all totally ineffective at inhibiting histamine release induced by the bradykinin analog. Similarly, staurosporine, a relatively selective inhibitor of protein kinase C, and the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) an activator of this enzyme, both have pronounced effects on IgE-mediated histamine release from SMC but were completely inactive with regard to [DArg^0-Hyp^3-DPhe^7]-bradykinin-stimulated release. SMC stimulated with this peptide showed characteristic changes in intracellular free calcium levels, as assessed by digital video microscopy. This response differs from that induced by anti-IgE in that it had a more rapid onset, achieved a lower peak, and decayed much more rapidly. Analysis at the single cell level showed that cells that responded in this fashion upon exposure to the bradykinin analog were capable of showing an additional response upon subsequent exposure to anti-IgE. We conclude that histamine release from SMC in response to [DArg^0-Hyp^3-DPhe^7]-bradykinin occurs via a completely different mechanism from that in response to IgE-mediated stimuli. Peptide-induced release is rapid and is not susceptible to pharmacologic manipulation of intracellular cyclic AMP or protein kinase C but utilizes a rapid transient shift in intracellular calcium concentrations as part of its signal transduction pathway.

The human skin mast cell (SMC), unlike its counterparts in the lung, intestine, adenoid, and tonsil, is capable of releasing inflammatory mediators in response to a number of specific non-immunologic peptide secretagogues, as well as to IgE-dependent stimuli, such as anti-IgE [1–3]. The characteristics of skin mast cell histamine release induced by secretagogues such as compound 48/80, poly-L-lysine, substance P, vasoactive intestinal peptide (VIP) and somatostatin appear to be distinct from those for IgE-dependent stimuli [1–5]. Thus, it is possible that peptide-induced histamine release may also be regulated differently by pharmacologic agents.

Although the nonapeptide bradykinin is a secretagogue in rodent mast cells [6, 7], it is unable to stimulate mediator release from any human histamine-containing cells tested to date [8, 9]. We have shown recently, however, that analogs of bradykinin with as few as one amino acid substitution can act as secretagogues in human SMC. One such compound, [DArg^0-Hyp^3-DPhe^7]-bradykinin, was found to be a particularly effective stimulus of histamine release from SMC, while having no effect on lung mast cells or basophils [9]. Histamine release from SMC induced by bradykinin analogs, such as [DArg^0-Hyp^3-DPhe^7]-bradykinin, is not mediated via bradykinin receptors [9]. Moreover, stimulation of SMC with [DArg^0-Hyp^3-DPhe^7]-bradykinin is not strictly dependent on the presence of extracellular calcium and yields kinetic release curves characteristic of those previously described for substance P and morphine sulfate [1] as well as for fast univalent stimuli in other secretory cells [10, 11].

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The exact structural properties necessary to confer secretagogue activity upon peptides are unknown but have been suggested to include the presence of a cluster of basic amino acids at the N-terminal end, an abundance of hydrophobic residues in the C-terminal portion [12, 13], and aromaticity [9]. Moreover, it has been postulated that peptides satisfying such structural requirements induce noncytotoxic release of histamine from mast cells by interacting with a common binding site, rather than via interaction with a specific receptor in each case.

In the present paper, we have used the analog [DArg^0-Hyp^3-DPhe^7]-bradykinin as a model stimuli to further explore peptide-induced mediator release from SMC. We have expanded our earlier studies by examining both preformed and newly generated mediators, and by exploring possible modes of signal transduction in response to peptide activation. We now report that pharmacologic modulation of intracellular cyclic AMP or protein kinase C did not alter [DArg^0-Hyp^3-DPhe^7]-bradykinin-induced mediator release from SMC. Cells releasing histamine in response to incubation with this peptide, however, did display a rapid transient increase in levels of intracellular calcium. Moreover, single cells that showed such a response to this stimulus showed an additional response upon a subsequent exposure to anti-IgE, further indicating that [DArg^0-Hyp^3-DPhe^7]-bradykinin is not lytic.

MATERIALS AND METHODS

Reagents. The following were purchased: synthetic bradykinin (Peninsula Laboratories, Belmont, CA); substance P (UCB Bioproducts, Brussels, Belgium); isoproterenol, isobutylmethylxanthine (IBMX), piperazine-*N*-*N*′-bis 2-ethanesulfonic acid (PIPES), hyaluronidase, EDTA, dimethyl sulfoxide (DMSO), silicon, and phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, MO); Percoll (Pharmacia, Uppsala, Sweden); human serum albumin (HSA) (Miles Laboratories, Inc., Elkhart, IN); deoxyribonuclease I and staurosporine (Calbiochem, San Diego, CA); fura-2AM (Molecular Probes, Eugene, OR); RPMI 1640 medium (Whitaker, M.A. Bioproducts, Walkersville, MD); calcium/magnesium free Hanks' balanced salt solution, penicillin/streptomycin, L-glutamine, and 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (Gibco, Grand Island, NY); and Nitex cloth (Tekko, Elmford, NY). The bradykinin analog, [DArg^0-Hyp^3-DPhe^7]-bradykinin, was provided by the Nova Pharmaceutical Corp., Baltimore, MD.

Isolation of human skin mast cells. A modification of the procedure of Lawrence *et al.* [2] was used. Briefly, adult skin obtained from mastectomies or cosmetic surgery procedures was placed in cold calcium/magnesium-free Hanks' balanced salt solution and used within 1 hr. After removal of fat by blunt dissection, the skin specimen was chopped to a size of 1 mm and washed once in PAG (25 mM PIPES, 110 mM NaCl, 6 mM KCl, 0.1% dextrose, and 0.003% human serum albumin, pH 7.35) at 4°. The fragments were washed again in PAG at 23° and incubated in PAG (1 g tissue/10 mL buffer) containing 1.5 mg/mL collagenase type II (Worthington Biochemical Co., Freehold, NJ), 0.4 mg/mL hyaluronidase (Sigma) and 1000 units deoxyribonuclease I for 4–6 hr at 37°. The partially digested tissue was filtered through Nitex cloth (150 μm pore size) at 2-hr intervals. Isolated cells were washed three times with PAG at 23°. Mast cell numbers were determined by light microscopy after staining with Alcian blue at pH 1.0 [14].

Short-term culture. Isolated cells were resuspended in RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine and 1% penicillin–streptomycin. Cells were cultured at 2×10^5 mast cells/mL in 25 cm^2 tissue culture flasks (BELLCO Biotechnology, Vineland, NJ) in humidified 95% air, 5% CO_2 at 37° for 12–16 hr. Cells were washed twice in PAG at 23° and counted with Alcian blue stain prior to experimental use.

Density gradient purification of SMC. Isotonic Percoll density medium was made by addition of 10% final volume of a 10× concentration of PIPES. Individual layers of Percoll were prepared by dilution in 1× PIPES. Dispersed skin cells were resuspended in 90% isotonic Percoll and placed at the bottom of each 12-mL polypropylene tube (Sarstedt, Newtown, NC). Discontinuous density gradients were prepared by sequential overlayering of 2 mL each of 80%, 70%, 60%, and 40% isotonic Percoll. Gradients were immediately centrifuged (1200 rpm × 15 min). Cells were collected at the interfaces between Percoll density layers with silanized glass pasteur pipettes. SMC (purity ranging from 70 to 92%) were found at the 70–80% interface. Cell suspensions were washed three times in PAG and counted with Alcian blue stain.

Drug preparation. The following drugs were freshly prepared as 10^{-2} M stocks: isoproterenol (dissolved in 0.9% NaCl containing sodium metabisulfite), IBMX (dissolved in hot distilled water), and forskolin (dissolved in EtOH). Stocks of staurosporine (100 μM) and the phorbol ester TPA (5 mg/mL) were dissolved in DMSO. Drugs were diluted to appropriate concentrations in PAGCM (PAG plus 1 mM $CaCl_2$ and 1 mM $MgCl_2$).

Test tube cell challenges. Polystyrene tubes (Sarstedt) containing approximately 2×10^4 SMC in PAGCM each were equilibrated to 30° for 10 min. It has been demonstrated previously that histamine release from SMC occurs optimally at 30° [2]. All reactions were carried out as duplicates in 1.0 mL final volumes. All anti-IgE challenges were carried out with a final concentration of 3 μg/mL goat anti-human IgE (provided by Dr. Kimishige Ishizaka, Johns Hopkins University School of Medicine), a concentration previously determined to yield optimal mediator release from these cells [2].

For pharmacology experiments, cells were incubated with specific drugs for 10 min before challenge with anti-IgE or the peptide, [DArg^0-Hyp^3-DPhe^7]-bradykinin. For co-incubation studies, cells were incubated with the first stimulus for 5 min prior to challenge by the second stimulus. After a 30-min incubation, samples were centrifuged (1000 *g* for 5 min) and supernatants were removed and processed for histamine analysis. For kinetics experiments, a 100-μL aliquot of cell suspension was

removed at approximate time intervals from a common pot containing cells and buffer, cells and anti-IgE, cells and [DArg^0-Hyp^3-DPhe^7]-bradykinin or cells with anti-IgE plus [DArg^0-Hyp^3-DPhe^7]-bradykinin and placed in tubes containing 0.9 mL cold PAG containing 5 mM EDTA on ice to stop the reaction. Test tubes were then spun (1000 *g* for 5 min) and supernatants removed and analyzed as below.

Histamine release assay. All histamine release assays were carried out as duplicates in final volumes of 1.0 mL. The concentration of histamine in cell free supernatants was determined by the automated fluorometric technique of Siraganian [15]. Histamine release was calculated from the percentage of total histamine content, determined by lysis of cells in 2% perchloric acid, corrected for spontaneous release by buffer controls. Total histamine content was usually in the range of 50–70 ng/tube. Test drugs (in the absence of cells) did not interfere with this assay.

Prostaglandin D_2 (PGD_2) analysis. In test tube experiments, PGD_2 was measured in 100 μL cell free supernatants precipitated in 500 μL HPLC grade ethyl alcohol. The samples were spun (1000 *g* for 10 min) to remove precipitates and supernatants were removed to polypropylene tubes for storage at −20°. At the time of analysis, supernatants were evaporated and resuspended in 0.1% gelatin-PBS. A competitive radioimmunoassay using dextran-coated charcoal to separate bound from free ligand was performed according to the method of Schulman *et al.* [16]. All determinations were run in duplicate against a standard curve run in triplicate. The assay was sensitive to 20 pg/mL.

Calcium measurements. Measurements of intracellular free calcium were made by digital video microscopy as described previously [17]. Purified SMC (70–92%) were incubated with fura-2AM (1 μM) in 200 μL RPMI 1640 medium supplemented with 0.32 μM EDTA and 2% FCS at 37° for 20 min. The cells were washed once in PAG and resuspended at 1×10^6 SMC/200 μL PAG. In each experiment, a 15-μL cell suspension was allowed to settle in the center of a silanized cover slip in a modified Dvorak–Stotler observation chamber [17] for 10 min. The cells were then overlayed with 1 mL of buffer, and the chamber was placed on the microscope scanning stage. To be consistent with test tube challenge experiments, the temperature, measured by a probe placed in a chamber buffer, was brought to 30°. Calcium measurements were made before and after addition of the stimulus by ratio imaging analysis of the cells taken at two excitation wavelengths (352 and 380 nm). Cell responses were comparable at the beginning and end of a given series of experiments.

Experiments generally required 2–3 hr to complete (5–10 kinetic curves) and the intensity of fluorescence was not found to decrease during this period, indicating that there was little leakage of the fura-2 dye. There was also no noticeable photobleaching during the course of an experiment. At a gross level, the fura-2 dye appeared uniformly distributed in the cell. For human lung mast cells, we have found that fura-2 does not label the granules and that the cells labeled uniformly. Since there was no unexpected change in the fluorescent intensity of the cells excited at 352 nm during the course of the experiment, we conclude that fura-2 does not significantly label the granules of SMC (although this was not explicitly measured by analyzing the buffers of challenged cells for released fura-2).

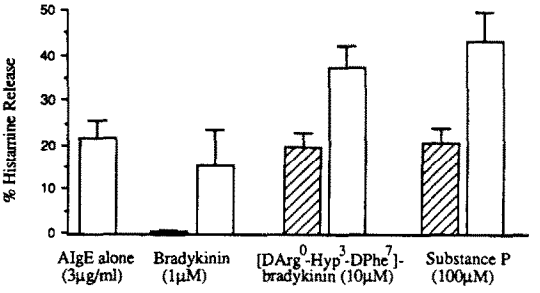

Fig. 1. Histamine release from human SMC challenged with anti-IgE alone or with bradykinin, [DArg^0-Hyp^3-DPhe^7]-bradykinin, or substance P either alone (shaded bars) or in the presence of anti-IgE (open bars). Results are the means ± SEM for 3–8 experiments.

The ratio information was converted to free calcium concentrations by the formula described by Grynkiewicz and coworkers [18]. The constants for this formula were obtained from intensity information on cells incubated in the presence of EDTA, EDTA + ionomycin (2–5 μg/mL) and ionomycin + calcium. The assumption was that these conditions would closely approximate the two extremes of free calcium needed to calibrate the fura-2 labeling. Under these conditions, the constants for skin mast cells were $R_{max} = 2.9$, $R_{min} = 0.45$ and $\beta = 6.9$.

Histamine release was determined by removing 1 mL of the supernatant from the microscope chamber after the completion of each observation period. Samples were analyzed for histamine release as described above.

Statistical methods. Results are expressed as the mean ± standard error of the mean (SEM). An analysis of variance test was used to compare kinetic curves. All other data were analyzed by comparing individual groups with a Student's *t*-test.

RESULTS

Peptide-induced SMC mediator release. SMC were exposed to buffer, bradykinin, the bradykinin antagonist [DArg^0-Hyp^3-DPhe^7]-bradykinin or substance P, alone or in combination with anti-IgE. The resulting histamine release is presented in Fig. 1. Bradykinin itself, at a maximal physiologic concentration (1 μM), did not induce significant histamine release above buffer controls (0.6 ± 0.3%) from SMC. This is consistent with our earlier observations that, even at nonphysiologic concentrations up to 1 mM, bradykinin is not a secretagogue for human histamine containing cells [9]. In contrast, SMC released 21 ± 3, 20 ± 2, and 18 ± 4% histamine when challenged with concentrations of anti-IgE (3 μg/mL), the bradykinin antagonist, [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM), or substance P (100 μM)

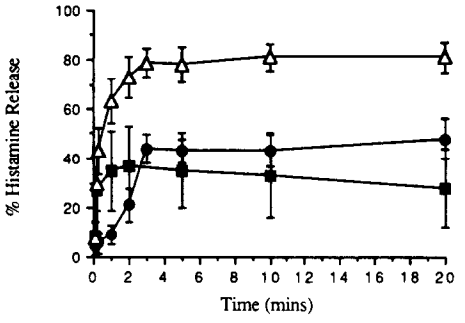

Fig. 2. Kinetics of histamine release from human SMC challenged with anti-IgE (●), [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) (■) or [DArg^0-Hyp^3-DPhe^7]-bradykinin plus anti-IgE (△). Values are the means ± SEM for 3 experiments.

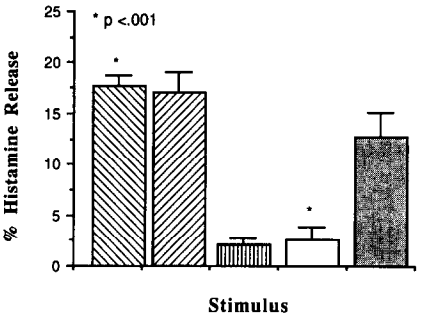

Fig. 3. Effects of short-term exposure to TPA on SMC release. Isolated SMC from four individuals were incubated with anti-IgE (3 μg/mL) alone (▨), [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) alone (▨), TPA (100 ng/mL) alone (▥), or with anti-IgE (□) or [DArg^0-Hyp^3-DPhe^7]-bradykinin (▨) following a 5-min preincubation with TPA. Results are means ± SEM.

respectively. Lower concentrations of [DArg^0-Hyp^3-DPhe^7]-bradykinin (1 μM), or substance P (10 μM) resulted in histamine release of only 3 ± 1 and 7 ± 2% respectively.

When SMC were incubated concurrently with [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) and anti-IgE, an additive response (38 ± 5%) significantly different from anti-IgE alone ($P < 0.01$) was seen. In a similar fashion, co-stimulation of SMC with anti-IgE and substance P (100 μM) released 43 ± 7% of the total histamine present ($P < 0.05$). No enhancement was seen when subthreshold concentrations of the peptides were added to anti-IgE.

In accordance with previous studies, we found that peptidergic stimuli failed to induce a marked release of PGD_2 [19]. SMC stimulated by anti-IgE released 124 ± 20 ng PGD_2/million SMC (N = 9), while in contrast, SMC challenged with [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) or substance P (100 μM) released only 10 ± 3 (N = 16) and 22 ± 13 (N = 3) ng PGD_2/million SMC respectively. Maximal PGD_2 release to anti-IgE was unaltered by co-stimulation of SMC with [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) and anti-IgE (3 μg/mL).

Kinetics of mediator release. To clarify the mechanism by which [DArg^0-Hyp^3-DPhe^7]-bradykinin affects IgE-stimulated mediator release, a series of kinetic analyses were performed. Figure 2 shows the kinetics of histamine release from SMC challenged with anti-IgE, [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) and [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) plus anti-IgE. As seen in the previous series of experiments, maximal percent histamine release was comparable for anti-IgE and [DArg^0-Hyp^3-DPhe^7]-bradykinin alone and additive when the two stimuli were used together. At 1 min post-challenge, SMC stimulated with [DArg^0-Hyp^3-DPhe^7]-bradykinin alone reached 95% of their maximal response, while those cells stimulated with anti-IgE alone reached only 20% of their maximal response. Interestingly, the rate of mediator release in the co-stimulated cells was intermediate between that for either stimulus alone (75% of maximal response). The rate of histamine release by SMC to anti-IgE plus [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) was significantly different ($P < 0.01$) than the rate for anti-IgE alone. No significant difference was seen in the rate of histamine release between anti-IgE-treated cells and cells treated with anti-IgE and suboptimal concentrations of peptide (data not shown).

These results suggest, as did previous observations, that [DArg^0-Hyp^3-DPhe^7]-bradykinin induces histamine release through a different mechanism than anti-IgE. In an attempt to better define this non-immunologic mechanism of mediator release, we next examined the effects of various pharmacologic agents on [DArg^0-Hyp^3-DPhe^7]-bradykinin-induced mediator release and accompanying free cytosolic calcium changes.

Effects of altering cyclic AMP and protein kinase C on SMC mediator release. It has long been established that drugs that increase intracellular cyclic AMP significantly inhibit mediator release from human basophils [20, 21]. While their potencies differed markedly, agents that elevate intracellular cyclic AMP inhibit anti-IgE-induced histamine release from SMC by 42–51% [22]. In this study, we examined these inhibitors of anti-IgE directed histamine release for effects on [DArg^0-Hyp^3-DPhe^7]-bradykinin peptide-induced SMC mediator release. Control histamine release to [DArg^0-Hyp^{3}DPhe^7]-bradykinin (10 μM) ranged from 33 to 38%. This release was not altered significantly by incubation of SMC with IBMX (1–100 μM), forskolin (0.1–10 μM), or isoproterenol (0.01–1 μM), with maximal inhibition of 1 ± 3 (N = 2), 8 ± 8 (N = 3), and 2 ± 5% (N = 3) respectively.

Previous studies have shown that short-term incubation of human basophils with the phorbol ester, TPA, upregulates protein kinase C (PKC) activity and results in mediator release [23]. SMC from four donors were incubated with TPA (100 ng/mL) for 5 min prior to challenge with either buffer, anti-IgE, or [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM). As shown in Fig. 3, although TPA alone has no effect on histamine release from SMC, pre-exposure to TPA completely inhibited anti-IgE-induced histamine release ($P < 0.001$), while having no significant effects on [DArg^0-Hyp^3-DPhe^7]-bradykinin-induced histamine release.

We next examined the effects of staurosporine, a PKC inhibitor [24]. Staurosporine treatment has been shown previously to inhibit anti-IgE-induced mediator release from skin mast cells [25]. Preincubation of SMC with staurosporine (0.1 to 100 nM) prior to challenge with [DArg^0-Hyp^3-DPhe^7]-bradykinin (10 μM) only inhibited the response 3 ± 5%. Thus, neither pharmacologically-induced up- or down-regulation of PKC activity in SMC appears to affect the peptide-induced release process.

This pharmacologic evidence suggests that activation of SMC by [DArg^0-Hyp^3-DPhe^7]-bradykinin can occur independently of mobilization of cyclic AMP, or PKC-dependent processes. Direct measurements of cyclic AMP and PKC changes will only be possible after we improve our digestion and purification techniques to yield greater numbers of purified SMC.

Changes in cytosolic calcium. Information regarding intracellular levels of free calcium ($[Ca^{2+}]_i$) in SMC has not been previously available, but should provide interesting insights into the regulation of mediator release from these cells. Human SMC (70–92% purity) were labeled with fura-2AM and loaded into the microscope chamber for analysis of cytosolic calcium changes. Baseline levels of cytosolic calcium in SMC were 104 nM and remained unchanged when SMC were incubated with PAGCM alone over the course of the experiment. As is typical for these experiments, baseline histamine release to PAGCM was somewhat high (18 ± 6%, N = 4). [We have found that the mechanical aspects of obtaining the supernatant from the microscope chamber and its subsequent centrifugation lead to some additional spontaneous release, either through cell disruption or to pelleting of purified cells to the walls of the centrifuge tube, with their subsequent inclusion in the supernatant rather than the pellet.]

As expected from the histamine release kinetics, $[Ca^{2+}]_i$ changes in SMC challenged with either anti-IgE or [DArg^0-Hyp^3-DPhe^7]-bradykinin exhibited strikingly different patterns of response (Fig. 4). Anti-IgE challenge of SMC induced significant increases in cytosolic calcium with a net peak of 230 ± 40 nM (N = 7). Calcium levels then declined slightly (decay $T_{1/2}$ = 1200 sec), but never returned to baseline levels during the observation period of 15–20 min (Fig. 4). Histamine release, measured from samples removed directly from the chamber was 37 ± 9% (N = 6). Although the rate of histamine release was never measured directly in these assays, based on previous observations in our laboratory the calcium response appears to occur prior to the release of histamine.

SMC challenged with [DArg^0-Hyp^{3}DPhe^7]-bradykinin (10 μM) reached a maximum net $[Ca^{2+}]_i$ of 160 ± 50 nM (N = 5) within 120 sec (Fig. 4). This transient peak decayed with a $T_{1/2}$ of 230 sec to a level 100 nM above pre-challenge calcium levels. The later phase of this response had a much slower decay characteristic. Histamine release was 30 ± 20% (N = 4). Challenge of SMC with a lower concentration (1 μM) of [DArg^0-Hyp^3-DPhe^7]-bradykinin led to a more modest rise in cytosolic calcium levels (net peak = 116 nM).

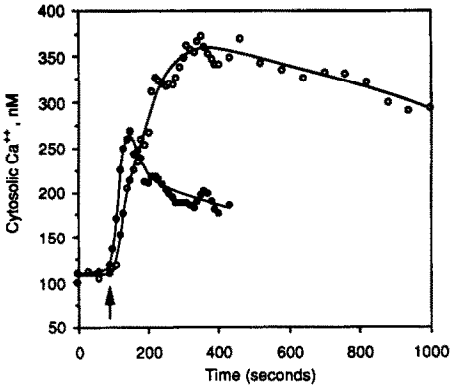

Fig. 4. Kinetics of $[Ca^{2+}]_i$ changes in SMC (mean purity = 80%) challenged with 3 μg/mL anti-IgE (○) or 10 μM [DArg^0-Hyp^3-DPhe^7]-bradykinin (●) in PAGCM. In the case of anti-IgE, each point is the average normalized data for 7 preparations of SMC. In the case of [DArg^0-Hyp^3-DPhe^7]-bradykinin, each point is the average normalized data for 5 preparations of SMC. Arrow represents introduction of stimulus.

The peak free calcium response following the simultaneous addition of [DArg^0-Hyp^3-DPhe^7]-bradykinin and anti-IgE antibody was found to be essentially additive. In two experiments, the [DArg^0-Hyp^3-DPhe^7]-bradykinin-induced response was 117 nM (net), the anti-IgE response was 206 nM (net), and the combined addition resulted in a 315 nM net change. Histamine release was somewhat less than additive (47% for anti-IgE, 40% for [DArg^0-Hyp^3-DPhe^7]-bradykinin, and 68% for both stimuli together). It should be noted that the histamine release in these experiments was high for each independent stimulus making additivity more difficult to achieve.

The use of digital video microscopy allowed us to measure $[Ca^{2+}]_i$ in single cells. With this analysis we were able to determine whether the observed additivity in calcium response to anti-IgE and [DArg^0-Hyp^3-DPhe^7]-bradykinin was due to the triggering of two separate populations of SMC. In addition, this approach allowed us to assess whether [DArg^0-Hyp^3-DPhe^7]-bradykinin was lytic for a subpopulation of cells or in some way alters the response of the cells to anti-IgE antibody.

The changes in $[Ca^{2+}]_i$ were measured for each cell in the field of view (30–40 cells/field). In the experiment shown in Fig. 5, the peak $[Ca^{2+}]_i$ responses following the sequential addition of [DArg^0-Hyp^3-DPhe^7]-bradykinin and anti-IgE antibody (10 min after the addition of [DArg^0-Hyp^3-DPhe^7]-bradykinin) are plotted for each cell. The cell preparation used for this experiment was 84% pure. Thus, in a field of thirty-nine cells it may be anticipated that approximately six cells may be non-mast cells. It seems reasonable to assume, therefore, that the four cells that were unresponsive to either stimuli may represent contaminating cells. Several cells responded to only one stimulus (N = 7 [18%] to anti-IgE alone, N = 7 [18%] to [DArg^0-Hyp^3-DPhe^7]-bradykinin alone). The majority of the cells (56%) were found to respond to both stimuli. The

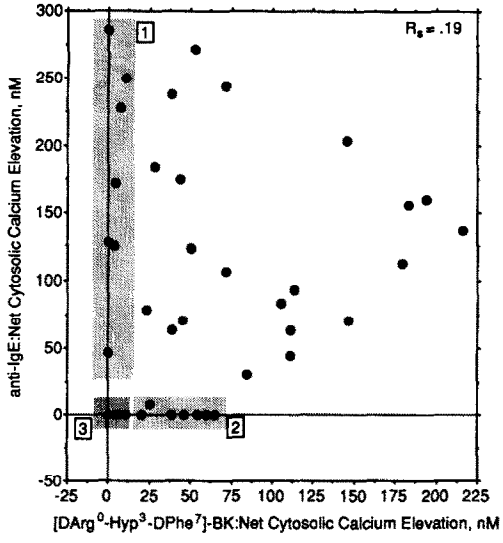

Fig. 5. Single cell analysis of $[Ca^{2+}]_i$ changes in SMC (N = 39 cells; purity = 84%) sequentially challenged with [DArg⁰-Hyp³-DPhe⁷]-bradykinin (10 μM) and anti-IgE (3 μg/mL) in PAGCM. The shaded regions indicate groups of cells where the response was restricted to one or the other stimulus, or neither (based on the known noise in the calcium determination); region 1 = response only to anti-IgE antibody; region 2 = response only to [D-Arg⁰-Hyp³-DPhe⁷]-bradykinin; region 3 = response to neither stimulus.

additivity in the average calcium and histamine release responses do not appear to be accounted for by distinctly responding populations. The [DArg⁰-Hyp³-DPhe⁷]-bradykinin-induced responses which decayed to resting free calcium levels could be followed by a response to anti-IgE antibody. The average anti-IgE response of cells that had responded to [DArg⁰-Hyp³-DPhe⁷]-bradykinin and then returned to baseline was the same as the average anti-IgE-induced calcium response observed in cells which had not first experienced a challenge with [DArg⁰-Hyp³-DPhe⁷]-bradykinin. There was no apparent correlation between the ability of cells to respond to the two stimuli ($r_s = 0.19$), further indicating a dissociation in the activation pathways used by these two stimuli.

DISCUSSION

The ability of certain peptides to cause histamine release from skin mast cells has been demonstrated not only from studies using isolated cells but also by the induction of wheal and flare reactions following intradermal injections with basic peptides such as compound 48/80, morphine, substance P, vasoactive intestinal peptide (VIP), and somatostatin [3–5, 9, 19]. Interestingly, a number of these peptide secretagogues for human SMC, including substance P, somatostatin and VIP, have been shown previously to induce histamine from rodent peritoneal mast cells [6, 26, 27].

The bradykinin analog, [DArg⁰-Hyp³-DPhe⁷]-bradykinin, is a useful model compound for the study of peptide-induced activation. It induces a similar, or greater, maximal level of histamine release than substance P and is about 10-fold more potent. Although [DArg⁰-Hyp³-DPhe⁷]-bradykinin is a secretagogue for human skin mast cells, it does not induce release from any other type of human histamine containing cell [9]. Thus, while this property may limit the intradermal application of this compound as a kinin antagonist, it has not prevented testing of this compound in the human respiratory tract [28]. Mediator release from SMC in response to [DArg⁰-Hyp³-DPhe⁷]-bradykinin is clearly quite distinct from that for IgE-dependent stimuli but is typical of that induced by peptide stimuli [19] in that it displays rapid kinetics, is only partially dependent on the presence of extracellular calcium and, as demonstrated here, is characterized by the release of histamine in the absence of any marked production of PGD_2. A further indication that the mechanism by which this peptide induces histamine release is different from that of IgE-dependent stimuli is provided by our data showing that co-incubation of SMC with [DArg⁰-Hyp³-DPhe⁷]-bradykinin and anti-IgE leads to release of an additive amount of histamine, while leaving anti-IgE-induced PGD_2 production unaffected. Similar results were obtained with co-incubations of anti-IgE and substance P.

In the present study we have begun to examine the signal transduction events that may be associated with [DArg⁰-Hyp³-DPhe⁷]-bradykinin-induced SMC activation and have compared our findings with those for IgE-mediated stimuli in the same cells. Previous studies in both humans [9] and rats [27] have suggested that histamine release activity induced by bradykinin analogs may not be mediated by a specific receptor mechanism. Since little is known of the activation–secretion coupling sequence for peptide stimuli, we used pharmacological tools known to interfere with pathways of signal transduction by anti-IgE to test for effects on peptide-induced release.

Elevation of intracellular cyclic AMP levels dramatically affects IgE-mediated secretion from all human histamine containing cells examined [21, 29]. It is not surprising, therefore, that in SMC, treatment with IBMX, forskolin, or isoproterenol, agents that act to increase intracellular cyclic AMP levels, prior to stimulation with anti-IgE, all significantly inhibit both histamine and PGD_2 release [22]. In marked contrast, however, our present results show that [DArg⁰-Hyp³-DPhe⁷]-bradykinin-induced histamine release was unaffected by pretreatment of SMC with comparable concentrations of these cyclic AMP active agents.

The role of PKC in IgE-induced mast cell signal transduction schemes is complex. In human basophils, PKC levels have been shown to be elevated following stimulation by IgE-related mechanisms [23]. Little data are available with human mast cells, but in mouse bone marrow derived mast cells, PKC elevations also occur upon IgE-mediated stimulation [30]. Staurosporine, a selective inhibitor of PKC activity, completely inhibits histamine release to anti-IgE in human basophils at subnanomolar concentrations [25] and inhibits release from SMC at a concentration of 1–2 nM [25]. This

inhibition of SMC was more pronounced than that observed for lung or bronchoalveolar mast cells [25]. Yet, in contrast to its striking effects on anti-IgE induced histamine release, staurosporine had no significant effect on mediator release induced by [DArg^0-Hyp^3-DPhe^7]-bradykinin. This was not unexpected, since low concentrations of staurosporine, that inhibit histamine release during IgE-mediated stimulation of human basophils, have no effect on stimulation of these cells by univalent stimuli, such as the bacterial peptide, formyl-Met-Leu-Phe, or the anaphylatoxin, C5a. At higher concentrations, staurosporine actually enhances release induced by these two stimuli.

Short-term incubation with phorbol esters, such as TPA, is known to promote PKC activity [31]. In rodent histamine containing cells this pretreatment variably reduces IgE-mediated histamine release [7, 32, 33]. This study demonstrates a similar inhibitory effect of short-term TPA treatment on SMC stimulated with anti-IgE, while showing no significant effects on peptide-induced histamine release. The effects of acute TPA treatment and staurosporine on IgE-mediated release highlight the possible roles of PKC enzymes in signal transduction for this class of stimuli, while their lack of effect on the kinin analog suggests that PKC enzymes have little or no role in release induced by this stimulus. We have shown, in human basophils, a somewhat similar contrast between IgE-mediated release and release initiated by univalent stimuli [34]. In basophils, PKC enzymes appear to have multiple roles, and the release that follows stimulation with IgE-dependent agents may be a fine balance of both pro- and anti-degranulatory roles for PKC. These multiple actions of PKC lead to the apparently paradoxical effects of staurosporine and acute TPA treatment on IgE-mediated release in the basophil and, on the basis of the data presented here, in the skin mast cell as well. In human basophils, there appears to be some role for PKC only as a down-regulator of secretion following formyl-Met-Leu-Phe stimulation. In these skin mast cell studies, however, [DArg^0-Hyp^3-DPhe^7]-bradykinin appears to induce release independently of PKC.

Changes in intracellular free calcium levels in SMC responding to either IgE-mediated stimuli or univalent peptide secretagogues had not been examined previously. The kinetics and magnitude of response in cytosolic calcium levels observed by digital video microscopy during anti-IgE stimulation of SMC were similar to those previously observed for the human lung mast cell [17]. The changes in anti-IgE-induced cytosolic calcium were associated with histamine release.

In contrast, peptide-induced changes in intracellular calcium resulted in smaller peak responses and, consistent with the more rapid kinetics for histamine release, were characterized by a more rapid increase and a faster decay. The characteristics of this response are similar to those observed for other univalent stimuli in other cell types where the rapid transient peak has been attributed to the release of calcium from intracellular stores. The weak dependence of the secretory response to [DArg^0-Hyp^3-DPhe^7]-bradykinin on extracellular calcium suggests that the initial transient $[Ca^{2+}]_i$ seen with this stimulus is also derived from internal stores. Although this has not been specifically examined for [DArg^0-Hyp^3-DPhe^7]-bradykinin, substance P also induces a rapid transient calcium response in SMC incubated in the absence of extracellular calcium (Massey WA and MacGlashan DW, unpublished observations). Exposure to [DArg^0-Hyp^3-DPhe^7]-bradykinin does not interfere with the ability of an individual cell to mobilize cytosolic calcium in response to a subsequent challenge with anti-IgE. This result suggests that there is no cross-desensitization occurring between the stimuli and also strengthens the argument that [DArg^0-Hyp^3-DPhe^7]-bradykinin-induced release is not a lytic phenomenon. Moreover, these results illustrate the interesting point that there is indeed individual heterogeneity of activation within a single population of SMC from one donor.

In summary, it is known that, *in vitro*, human SMC release histamine, PGD_2 and small amounts of leukotriene C_4 in response to stimulation with anti-IgE. This release process requires the presence of extracellular calcium. It is accompanied by increases in intracellular calcium concentrations and is sensitive to alterations in PKC and cyclic AMP levels within the cells. In contrast, our present studies show that histamine is the predominant mediator released following stimulation by non-immunologic stimuli such as the bradykinin analog [DArg^0-Hyp^3-DPhe^7]-bradykinin. Histamine release in response to this peptide occurs independently of alterations in intracellular cyclic AMP or PKC levels. Peptide-induced histamine release is not dependent on extracellular calcium [9], but utilizes a rapid transient shift in intracellular calcium concentrations as part of its signal transduction pathway.

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