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STUDIES OF THE INTRACELLULAR Ca^{2+} LEVELS IN HUMAN ADULT SKIN MAST CELLS ACTIVATED BY THE LIGAND FOR THE HUMAN c-*kit* RECEPTOR AND ANTI-IgE*

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Abstract—The human c-*kit* receptor ligand, rhSCF, is the only cytokine known to be active on human mast cells, but its intracellular signal transduction pathway is still unknown. We compared the effect of rhSCF on intracellular Ca^{2+} levels in purified (>70% pure) adult skin mast cells with two other immunologic stimuli, namely, anti-IgE and substance P. Both rhSCF (1 μg/mL) and anti-IgE (3 μg/mL) induced a rapid (<20 sec) and sustained ($T_{1/2}$ for decay >10 min) increase in free cytosolic Ca^{2+} concentration. In contrast, substance P (5 μM) elicited a very rapid (<1 sec) and transient ($T_{1/2}$ for decay ≈5 sec) rise in intracellular Ca^{2+} levels. Intracellular cAMP levels were then increased by pharmacologic means to examine the role of the cyclic nucleotide in controlling the Ca^{2+} response in skin mast cells. A combination of the general phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) (200 μM) and the adenylate cyclase activator, forskolin (30 μM) was effective in inhibiting the Ca^{2+} response induced by rhSCF or anti-IgE (82 and 68% inhibition, respectively), while IBMX and forskolin alone were much less effective. The phosphodiesterase isozyme IV inhibitor, rolipram (10 μM), variably affected the increase in Ca^{2+} levels induced by anti-IgE, but it exerted a significant inhibitory activity on anti-IgE- or rhSCF-induced response in the presence of forskolin (30 μg/mL) (33 and 67%, respectively). Two different protein kinase C (PKC) activators TPA (200 nM) and bryostatin 1 (200 nM) similarly inhibited rhSCF- (22 and 32%, respectively) and anti-IgE-induced (24 and 32%) Ca^{2+} response. Finally, the kinase inhibitor genistein (30 μg/mL) was a somewhat more effective inhibitor of the rise in intracellular Ca^{2+} induced by rhSCF (100%) than that activated by anti-IgE (54%) ($P < 0.05$). These data indicate that rhSCF and anti-IgE may act on human mast cells through a common pathway to increase free cytosolic Ca^{2+} levels and this effect is similarly modulated by various drugs.

Key words: mast cells; calcium; rhSCF; anti-IgE, pharmacology

The gene product of the steel locus of the mouse is a ligand for the c-*kit* proto-oncogene receptor, a receptor with tyrosine kinase activity, and a growth factor for murine mast cells [1]. Three groups of investigators independently cloned the cDNA for this novel cytokine that is produced by 3T3 fibroblasts and other cell types [2–8]. The activity of this factor on mast cell growth and differentiation may, at least partially, explain the property of 3T3 fibroblasts of inducing differentiation of mast cells from human cord blood cells [9]. The c-*kit* receptor ligand, which we will report here as SCF§ [2], promoted the development of mast cells when administered *in vivo* to mice [4, 10, 11], and induces maturation and proliferation of murine mast cells *in vitro* [2, 3, 11]. In addition, SCF causes degranulation of murine mast cells both *in vivo* and *in vitro* [12, 13]. We have recently reported that the rhSCF induces degranulation of mast cells isolated from human skin tissue at high (nanomolar) concentrations, whereas it potentiates IgE-mediated histamine release from these cells at lower (picomolar) concentrations [14]. Studies performed with human lung mast cells have suggested that the rhSCF potentiates anti-IgE-induced histamine secretion while lacking a direct pro-degranulatory activity [15]. In contrast, we found that rhSCF can directly induce release of mediators from this mast cell type as well [16]. Interestingly, rhSCF represents the only cytokine known to activate

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§ Abbreviations: anti-IgE, goat IgG anti-Fc fragment of human IgE; DNase, deoxyribonuclease; HSA, human serum albumin; IBMX, isobutylmethylxanthine; IP_3, inositol triphosphate; PAG, pipes buffer containing 0.003% HSA and 1 g/L glucose; PAGCM, pipes buffer containing 0.003% HSA, 1 g/L glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$; PKC, protein kinase C; rhSCF, recombinant human stem cell factor; SCF, stem cell factor; TPA, tetradecanoyl phorbol acetate.

human mast cells. Therefore, it appears that rhSCF may play a significant role in the pathophysiology of inflammatory and allergic reactions. However, very little is known about the signal transduction pathway by which rhSCF acts on the cells. Results obtained in murine mast cells suggest that SCF induces autophosphorylation of the c-*kit* receptor on tyrosine and binding to phosphatidylinositol 3′ kinase and phospholipase C [17]. Another report showed that the activation of a chimeric receptor by SCF causes modification of phospholipase C without affecting inositol phosphate production [18]. We have provided preliminary evidence that rhSCF induces a rise in intracellular Ca^{2+} levels in purified human cutaneous mast cells that is accompanied by histamine secretion [14]. In the current studies, we compared changes in the intracellular Ca^{2+} levels induced by rhSCF in human skin mast cells with two other stimuli which, as rhSCF, can act on these cells under *in vivo* conditions, namely, anti-IgE and substance P. In addition, we sought to compare the effects of various pharmacologic agents on the Ca^{2+} responses which follow activation with rhSCF and anti-IgE.

MATERIALS AND METHODS

Materials. The following were purchased: pipes, substance P, isobutylmethylxanthine, forskolin and hyaluronidase (Sigma Chemical Co., St Louis, MO, U.S.A.); genistein (GIBCO, Gaithersburg, MD, U.S.A.); type 1 deoxyribonuclease (DNase), fura-2AM, and crystallized HSA (Calbiochem Co., La Jolla, CA, U.S.A.); RPMI-1640 with 25 mM HEPES and L-glutamine, and penicillin/streptomycin solution (Gibco, Grand Island, NY, U.S.A.); type 2 collagenase (Worthington, Freehold, NJ, U.S.A.); nytex cloth (Tetko, Elmsford, NJ, U.S.A.); Percoll (Pharmacia, Piscataway, NY, U.S.A.); 60% perchloric acid (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.). Goat anti-human IgE was prepared as previously described [19]. Recombinant human c-*kit* receptor ligands were kindly donated by Dr K. M. Zsebo (Amgen, Thousand Oaks, CA, U.S.A.). Rolipram was a gift from Dr T. Torphy (Smith Kline & Beecham, King of Prussia, PA, U.S.A.).

Buffers. Pipes (P) contains 25 mM piperazine-*N,N′*-bis(2-ethane-sulfonic acid), 110 mM NaCl, and 5 mM KCl, pH 7.4. PAG has in addition 0.1% dextrose and 0.003% HSA. PAGCM is PAG containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Isolation and purification of human skin mast cells. Skin mast cells were isolated from human skin tissues by a modification of a technique previously described [20]. Briefly, adult skin from subjects undergoing mastectomies or cosmetic surgery was placed in PAG, the subcutaneous fat removed, and the tissue chopped finely into pieces of approximately 1 mm. The fragments were washed with PAG at 4°, then at room temperature, and dispersed by 2 hr incubation at 37° in a solution (10 mL PAG/g of tissue) containing (per g of skin tissue): 2500 U collagenase, 4 mg hyaluronidase, 1 mg DNAse, and 1% penicillin/streptomycin solution. The remaining tissue was filtered through nitex cloth (150 μ pore size) and the dispersed cells washed three times with PAG at room temperature. The mast cells were identified by alcian blue staining at pH 1.0 [21]. The cell-free supernatant and undigested tissue were recombined and incubated at 37° for an additional 2 hr. The cycle was eventually repeated in order to improve the mast cell yield. Dispersed human skin mast cells (1–5% pure) were then partially purified (>70% purity) by a multidensity Percoll gradient, as previously described [22].

Histamine release. Histamine release was determined 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 the different compounds was accomplished by preincubating the cells for 10 min with the drug, which was included in the buffer initially overlaid on the 15 μL cell drop (see next paragraph). The total histamine content was obtained by treating 7.5 μL of the cells with 200 μL

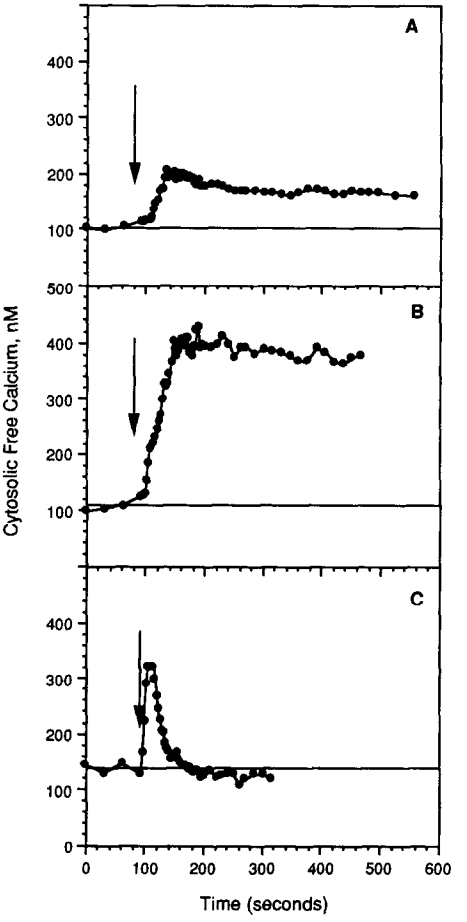

Fig. 1. Effect of different stimuli on the intracellular Ca^{2+} levels in purified (>70% pure) skin mast cells. Panels A, B and C show the average Ca^{2+} response of cells challenged by rhSCF (1 μg/mL) (N = 5), anti-IgE (3 μg/mL) (N = 6), and substance P (5 μM) (N = 2), respectively. The mast cell purity was 80 ± 4, 82 ± 4 and 88 ± 6% for the experiments with rhSCF, anti-IgE, and substance P, respectively. The arrows indicate the addition of the stimulus.

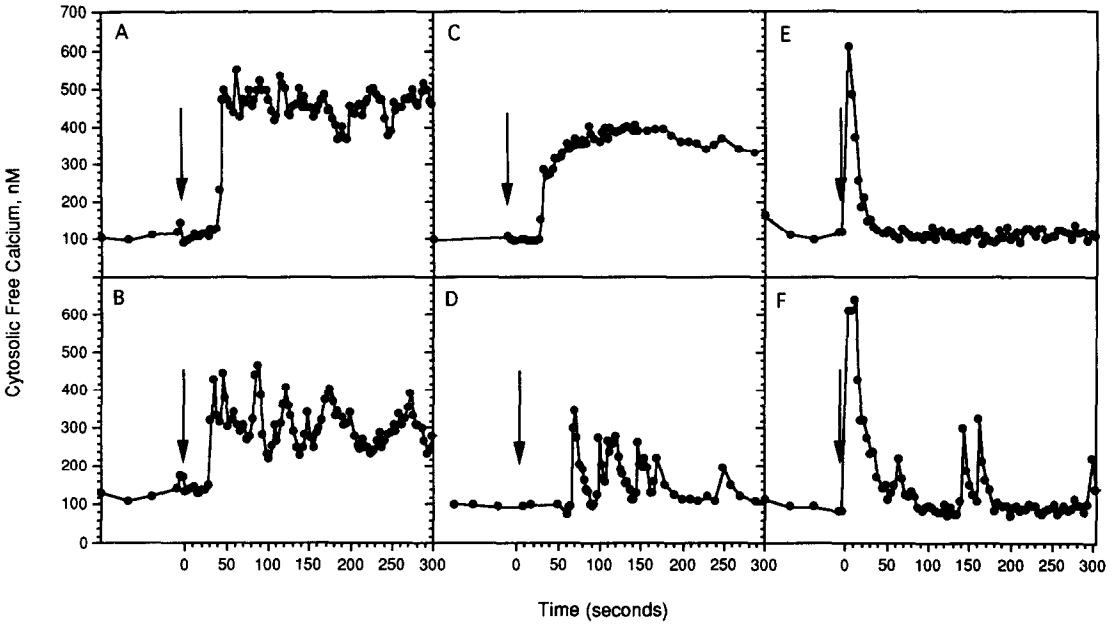

Fig. 2. Kinetics of the Ca^{2+} response in single cells stimulated by anti-IgE (3 μg/mL) (panels A and B), rhSCF (1 μg/mL) (panels C and D), and substance P (5 μM) (panels E and F). For each stimulus results obtained in 2 single cells are shown. The cells were specifically chosen to show two features: (a) lag time and (b) response with and without oscillations. The arrows indicate the addition of the stimulus. See text for further details.

of 8% perchloric acid and bringing the volume to 1 mL with PAGCM. Histamine was measured by the automated fluorometric technique described by Siraganian [23].

Measurement of intracellular Ca^{2+}. Cytosolic Ca^{2+} measurements were made using a Zeiss Axiovert microscope equipped with epifluorescence as previously described [24]. Purified mast cells were first labeled with 1 μM fura-2AM for 20 min at 37° in RPMI-1640 also containing 0.32 mM EDTA and 2% fetal calf serum (3–5) × 10^5 cells in 200 μL. The cells were then washed in PAG and resuspended in PAG buffer for loading into the microscope observation chamber. For each experiment, 15 μL of suspended cells (≈25,000 cells) were placed in the center of a siliconized cover slip which made up the base of the observation chamber and allowed to settle. The cells were overlaid with 1 mL of PAGCM buffer or of the drug at 37° and the chamber was placed on the microscope scanning stage. Four ratio images, 30 sec apart, were taken to establish a baseline and then the stimulus was added in 1 mL of PAGCM. Typically, the Ca^{2+} response was followed with a series of 40–80 images, 2–10 sec apart followed by ratio images taken at progressively longer intervals. Each frame represents a single ratio measurement of a field of 20–80 cells.

We have previously established that fura-2 did not affect the release of histamine from mast cells stimulated with anti-IgE antibody [24]. Mast cells were generally labeled uniformly with the fura-2 dye and there was no gross compartmentalization of the fura-2 observed in resting cells. Ratio measurements were converted to free calcium concentrations using the equation described by Grynkievicz *et al.* [25], with parameters previously established for human mast cells [24].

Statistical analysis. The results are expressed as the mean ± SEM. Data were analysed by the two-tailed Wilcoxon signed-rank non-parametric analysis.

RESULTS

Comparison between the effect of rhSCF, anti-IgE and substance P on intracellular Ca^{2+} *levels in human skin mast cells*

In a first series of experiments we compared the effect of rhSCF on the intracellular Ca^{2+} levels in partially purified (>70% pure) human cutaneous mast cells with that of anti-IgE and substance P. Figure 1 (panel A) shows the average Ca^{2+} response from 5 mast cell preparations stimulated by rhSCF (1 μg/mL). rhSCF induced a rapid and sustained ($T_{1/2}$ for decay of the Ca^{2+} signal was >10 min) elevation of intracellular Ca^{2+} levels.

Anti-IgE (3 μg/mL)-induced stimulation also elicited a relatively rapid and sustained ($T_{1/2}$ for decay >10 min) rise in intracellular Ca^{2+} levels (Fig. 1, panel B).

In contrast with rhSCF and anti-IgE substance P (5 μM) induced a very rapid (<1 sec) and transient ($T_{1/2}$ for decay ≈5 sec) elevation in intracellular Ca^{2+} levels (Fig. 1, panel C).

Figure 2 shows examples of single cell responses. These data are relevant since we have previously shown that anti-IgE causes a single cell response that differs from the average population response [26]. Specifically, there is a substantial and variable

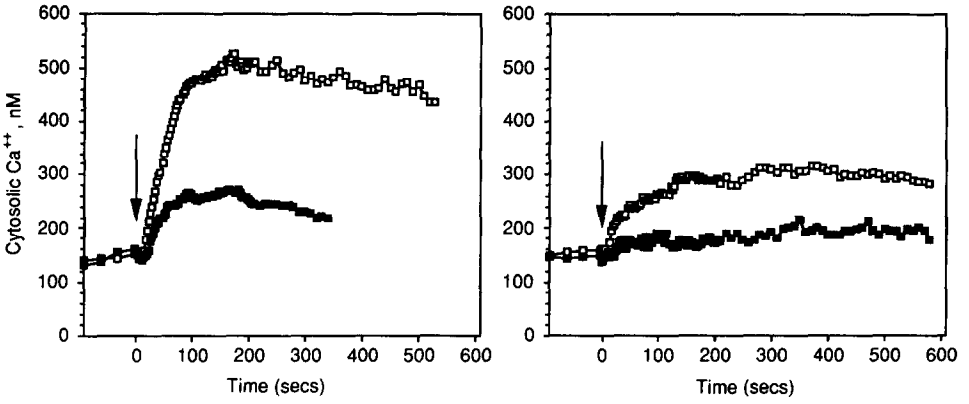

Fig. 3. Effect of a 10 min cell preincubation with the combination of IBMX (200 mM) and forskolin (30 μM) on the Ca^{2+} response induced by 3 μg/mL anti-IgE (left panel) and 1 μg/mL rhSCF (right panel). The curves represent the average response from 3 (anti-IgE) and 5 (rhSCF) experiments, respectively. Mast cell purity was 81 ± 5% for anti-IgE- and 84 ± 3% for rhSCF-activated Ca^{2+} response. The per cent inhibition of anti-IgE-induced histamine release in these experiments was 68 ± 6%. The arrows indicate the addition of the stimulus.

lag between the time one stimulus is added and the subsequent rapid transition to a new, essentially steady state signal (see panel A of Fig. 2). We found that, like IgE-mediated release, stimulation with rhSCF showed the same characteristics (panel C). Again, these characteristics were in contrast to those which followed stimulation with substance P (panel E). We were somewhat surprised to find that skin mast cells occasionally show significant oscillations in cytosolic Ca^{2+}. These were not a feature of mast cells purified from lung and uterus, although we speculated that the right conditions might lead these cells to oscillate [26]. Oscillations were more common following stimulation with rhSCF, occurring in 30–50% of the cells (panel D), while occurring only in 5–10% of the cells stimulated by anti-IgE (panel B, the variation in panel A only marginally suggests oscillations and is more likely to represent noise in the instrumentation). However, the difference may be due to different levels of activation with the two stimuli [26]. Finally, ≈10% of mast cells responding to substance P also showed a small degree of oscillation (panel F).

Therefore, on a qualitative level, the Ca^{2+} response to rhSCF and to anti-IgE antibody were similar, although the response to anti-IgE was generally of greater magnitude than rhSCF. These two responses were qualitatively different from the Ca^{2+} response to substance P.

Effect of drugs increasing intracellular cAMP levels on the changes in cytosolic Ca^{2+} induced by rhSCF and anti-IgE

Substance P-induced increase in cytosolic Ca^{2+} was transient and different from both rhSCF- and anti-IgE-activated Ca^{2+} responses, which, in turn, were quite similar to each other. Therefore, all subsequent experiments were performed in order to try to differentiate the Ca^{2+} signals elicited by rhSCF and anti-IgE. Our general strategy was to determine whether agents which were known to modify the exposure to anti-IgE would have similar effects on rhSCF-induced release. We tested three unrelated sets of inhibitors of which cAMP elevators were first examined. From previous experience, we knew that inhibition of histamine release and Ca^{2+} changes required relatively large increases in cAMP [27]. Therefore, we first treated cells with a combination of forskolin and IBMX and later examined more subtle combinations of drugs to raise cAMP levels.

The combination of the general phosphodiesterase inhibitor IBMX (200 μM) and the adenylate cyclase activator, forskolin (30 μM) was the most effective in inhibiting intracellular Ca^{2+} changes induced by rhSCF and anti-IgE (82 and 68% inhibition, respectively) (Fig. 3); IBMX or forskolin alone were much less effective than their combination (for anti-IgE 28 and 47% inhibition, respectively).

Since preliminary evidence suggests that the major phosphodiesterase isozyme present in human inflammatory cells is isotype IV, we used the specific phosphodiesterase isozyme IV inhibitor rolipram to evaluate its role in controlling the rise in cytosolic Ca^{2+} levels induced by rhSCF or anti-IgE. Rolipram (10 μM) exerted a variable effect on the activation of mast cells by anti-IgE, but coincubation with forskolin (30 μM) caused a significant inhibition of Ca^{2+} levels induced by anti-IgE or rhSCF (33 and 67% inhibition, N = 3–4, respectively).

Effect of protein kinase C activators on the changes in intracellular Ca^{2+} levels induced by rhSCF and anti-IgE

Because the activation of PKC may be an important component of the response of human inflammatory cells, including anti-IgE-stimulated human mast cells [28], we evaluated the effect of PKC activation on the Ca^{2+} levels in skin mast cells challenged by rhSCF and anti-IgE. We used TPA and bryostatin 1, which appear to activate PKC through distinct mechanisms [29]. TPA (200 nM) and bryostatin 1 (200 nM) caused a similar inhibition

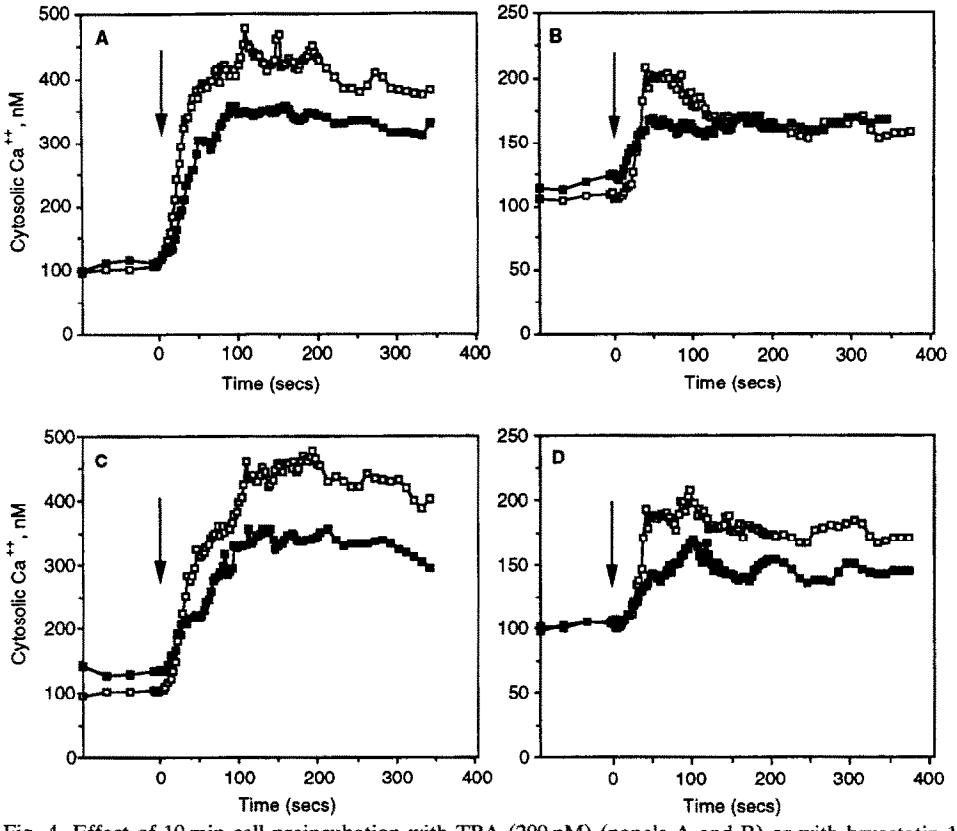

Fig. 4. Effect of 10 min cell preincubation with TPA (200 nM) (panels A and B) or with bryostatin 1 (200 nM) (panels C and D) on the Ca^{2+} response induced by 3 μg/mL anti-IgE (panels A and C) and 1 μg/mL rhSCF (panels B and D). The curves represent the average response from three (TPA + rhSCF) or four experiments. Mast cell purity ranged from 71 to 93%. The arrows indicate the addition of the stimulus.

of rhSCF- (22 and 32% inhibition, respectively) and anti-IgE- induced (24 and 32% inhibition, respectively) elevations in cytosolic Ca^{2+} levels (Fig. 4).

Effect of kinase inhibitor genistein on the changes in intracellular Ca^{2+} induced by rhSCF and anti-IgE

It is known that rhSCF binds to a receptor with tyrosine kinase activity, the c-*kit* receptor [1], and that the IgE-mediated cell activation is accompanied by the phosphorylation of different proteins on tyrosine [30]. Therefore, we evaluated the effect of the kinase inhibitor, genistein, on the activation of mast cells induced by rhSCF and anti-IgE. Pretreatment of mast cells with genistein (30 μg/mL) caused a complete inhibition of the rhSCF-induced rise in cytosolic Ca^{2+} levels (Fig. 5). Genistein also caused an inhibition (54%) of the anti-IgE-elicited Ca^{2+} response that appeared to be less than this drug's inhibitory effect on the activation induced by rhSCF ($P < 0.05$) (Fig. 5). However, this was due to the magnitude of the activation by anti-IgE since in individual experiments in which the Ca^{2+} responses were more equivalent, genistein was nearly as effective on both stimuli.

DISCUSSION

In the human mast cell, as well as in other cell types, IgE-mediated cell activation is usually accompanied by a rise in intracellular Ca^{2+} levels [24]. We recently found that there was an elevation in the levels of intracellular Ca^{2+} following a challenge of human skin mast cells with rhSCF [14]. Because of the lack of information about the intracellular signal transduction pathway activated by the c-*kit* ligand, particularly in human cells, we studied in more detail this aspect of the activity of rhSCF in human skin mast cells. In this study, we were looking for qualitative differences in the Ca^{2+} responses to three different stimuli as well as whether pharmacologic agents could distinguish between these stimuli.

In these studies we confirmed that the Ca^{2+} response that follows stimulation with anti-IgE or rhSCF is qualitatively similar at both the population and individual cell levels. These two stimuli were distinguished from substance P which causes only a transient Ca^{2+} response in these cells. The only difference between rhSCF and anti-IgE was quantitative, with anti-IgE causing a much larger response in these cells, both in terms of Ca^{2+} response

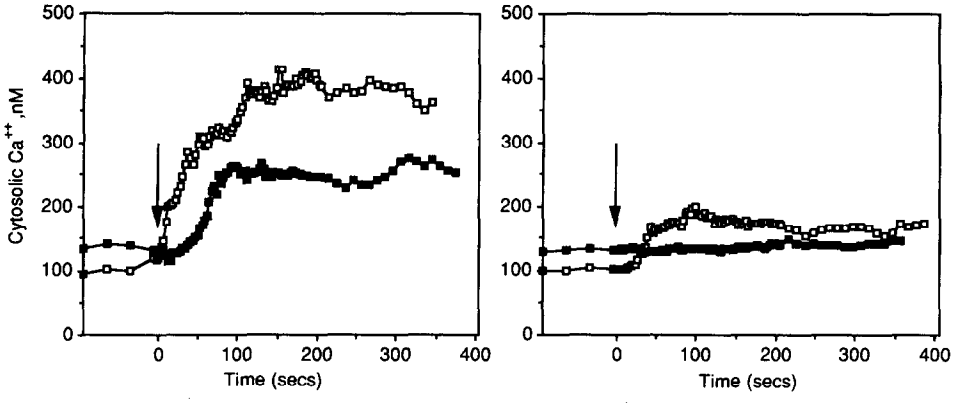

Fig. 5. Effect of a 10 min cell preincubation with genistein (30 μg/mL) on the Ca^{2+} response induced by 3 μg/mL anti-IgE (left panel) and 1 μg/mL rhSCF (right panel). The curves represent the average response from 6 (anti-IgE) and 5 (rhSCF) experiments, respectively. Mast cell purity was 82 ± 4% for anti-IgE- and 84 ± 4% for rhSCF-activated Ca^{2+} response. The per cent inhibition of anti-IgE-induced histamine secretion by genistein in these experiments was 64 ± 16%. The arrows indicate the addition of the stimulus.

and histamine release. Higher concentrations of rhSCF may have resolved these differences, but were impractical for these studies. It is generally accepted that Ca^{2+} responses in many cells result from the release of internal stores of Ca^{2+} and the influx of Ca^{2+} from external sources. Experiments in mast cells and basophils stimulated with anti-IgE indicate that both these phases exist following challenge, while for stimuli like substance P in mast cells or C5a and platelet-activating factor in basophils [31, 32], only the release of internal Ca^{2+} is dominant. Accordingly, the degranulation of mast cells activated by substance P is only partially dependent on the presence of extracellular Ca^{2+} [33 and M. Columbo, unpublished observations]. In addition, purified human skin mast cells challenged by substance P do not release newly synthesized mediators (prostaglandin D_2) [33]. This suggests that the second component of the Ca^{2+} response, as we have observed in human basophils [31], may play a role in the neosynthesis of mediators by these cells. Unfortunately, we do not yet know why some stimuli initiate only the early phase of the Ca^{2+} response and therefore cannot easily speculate on the mechanisms which distinguish substance P from anti-IgE and rhSCF: we can only note that anti-IgE and rhSCF are qualitatively similar.

We have previously shown that agents which elevate intracellular cAMP levels inhibit IgE-mediated histamine secretion from human basophils and mast cells [34–36]. The inhibition of mediator release from inflammatory cells by elevated cAMP levels has been associated with inhibition of cytosolic Ca^{2+} or phospholipid turnover [37–40]. In the present study, we found that elevated cAMP levels inhibit anti-IgE- and rhSCF-induced rises in intracellular Ca^{2+}. The rank of potency for inhibition by the different compounds (or combinations of compounds) on the Ca^{2+} signal was similar to that shown for human basophils [27], where it correlates with the ability of these agents to elevate intracellular cAMP levels. We have recently found that, in anti-IgE-stimulated human basophils, cAMP-active agents inhibit only the second phase (extracellular Ca^{2+}-dependent) of the Ca^{2+} response, without affecting the initial phase (inositol triphosphate, IP_3-dependent) [27 and Botana and MacGlashan, unpublished observations]. In contrast to basophils, human mast cells usually do not show an obvious transition between the first and second phase of the intracellular Ca^{2+} signal. This makes it difficult to assess the effect of the cAMP-active agents on the two phases of the Ca^{2+} response without designing experiments to produce only a first phase response with EGTA. Meanwhile, we note that when we used a stimulus whose activity on cytosolic Ca^{2+} is characterized by only the first, transient phase such as substance P, the cAMP-active drugs failed to affect its Ca^{2+} signal as well as histamine release [Columbo, unpublished observations]. This would suggest that in human mast cells the inhibiting effect of the Ca^{2+}-active compounds may also be related to their activity on the second phase of the Ca^{2+} response.

Interestingly, we found that the activity of cAMP-active agents on rhSCF-induced Ca^{2+} signal was very similar to that of anti-IgE: rhSCF activates human skin mast cells by a pathway that is inhibitable by increasing intracellular levels of cAMP.

We have previously demonstrated that a prolonged (18 hr) pretreatment of human skin mast cells with the PKC activator TPA causes an inhibition of the subsequent release of mediators induced by anti-IgE [28]. In the present study, we found that a brief preincubation (10 min) with TPA causes an inhibition of the IgE-mediated Ca^{2+} response. Therefore, in human skin mast cells, PKC appears to modulate the Ca^{2+} signal, as previously shown in other cell systems [41, 42]. The fact that TPA causes inhibition of the Ca^{2+} response is not surprising since PKC activation has been shown to have both positive and negative effects on cell activation [41, 43]. In

addition, we have shown that a brief (10 min) pretreatment with TPA completely abolishes the Ca^{2+} signal induced by anti-IgE in human basophils [42].

Bryostatins are macrocyclic lactones that act on PKC similarly to the phorbol esters (such as TPA) in many systems [44]. The differences in the effects of bryostatins and TPA have been hypothesized to be due to an activity on different PKC isozymes [29]. PKC exists, in fact, as a family of at least eight isozymes differing in cellular localization [45], substrate specificity [46], activation requirement [45–49], and tissue distribution [46]. More recently, however, it has been suggested that bryostatins differ from TPA in their binding affinity and slow rate of release [50].

We found that the activation of PKC by TPA or bryostatin 1 causes an inhibition of the Ca^{2+} signal induced by rhSCF that is quantitatively similar to that induced by anti-IgE. These results underscore another similarity between the activation pathways utilized by rhSCF and anti-IgE to activate human skin mast cells. This also represents, to our knowledge, the first evidence, although indirect, that a c-*kit* ligand acts through a pathway which can be modulated by PKC-dependent events in any human or non-human cell type.

rhSCF is the ligand for a receptor with tyrosine kinase activity, the c-*kit* receptor [1]. It has been also shown in basophilic cell lines that the IgE-mediated stimulation of these cells causes a phosphorylation of several proteins on tyrosine [30]. Genistein, a kinase inhibitor, significantly inhibits the Ca^{2+} response induced by rhSCF or anti-IgE. It has to be underscored that genistein is not a specific tyrosine kinase inhibitor but it also affects other kinases, such as PKC [51]. The inhibiting effect of genistein is not a general phenomenon in the human skin mast cells since the activation of these cells elicited by substance P is unaffected by cell pretreatment with genistein [Columbo, unpublished observations]. It is also worth noting that our finding that genistein inhibits anti-IgE-induced Ca^{2+} response appears to contrast recent studies with rat basophilic leukemia cells showing that genistein does not affect IgE-mediated IP_3 generation [52]. It seems likely that the initial elevation in cytosolic Ca^{2+} following anti-IgE antibody or rhSCF is mediated by IP_3, since the rate of the transition is very abrupt (<2 sec) and this agrees with studies on the mobilization of Ca^{2+} from internal stores by IP_3 [53]. Therefore, the inhibition of the initial change in Ca^{2+} by genistein in mast cells may indicate that IP_3 generation is altered.

In conclusion, our studies demonstrate that the activation of human skin mast cells by rhSCF and anti-IgE utilizes a common pathway that is sensitive to increases in intracellular cAMP, to PKC activation, and to the inhibition of tyrosine kinase and, possibly, other kinases. Our results are the first to begin to clarify the intracellular signal transduction pathway utilized by the c-*kit* ligand in the activation of human mast cells. It is evident, however, that additional studies will be necessary to elucidate the latter issue and to uncover other similarities (or differences) between the activity of rhSCF and anti-IgE in mast cells.

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REFERENCES

1. Witte ON, Steel locus defines new multipotent growth factor. *Cell* **63**: 5–6, 1990.
2. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA, Mendiaz EA and Langley KE, Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* **63**: 195–201, 1990.
3. Martin FH, Suggs SV, Langley KE, Lu HS, Ting J, Okino KH, Morris CF, McNiece IK, Jacobsen FW, Mendiaz EA, Birkett NC, Smith KA, Johnson MJ, Parker VP, Flores JC, Patel AC, Fisher EF, Erjavec HO, Herrera CJ, Wypych J, Sachdev RK, Pope JA, Liesle I, Wen D, Lin C-H, Cupples RL and Zsebo KM, Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* **63**: 203–211, 1990.
4. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu R-Y, Birkett NC, Okino KH, Murdock DC, Jacobsen FW, Langley KE, Smith KA, Takeishi T, Cattanach BM, Galli SJ and Suggs SV, Stem Cell Factor (SCF) is encoded at the *Sl* locus of the mouse and is the ligand for the c-*kit* tyrosine kinase receptor. *Cell* **63**: 213–224, 1990.
5. Williams DE, Eisenman J, Baird A, Rauch C, Ness KV, March CJ, Park LS, Martin U, Mochizuki D, Boswell HS, Burgess GS and Lyman SD, Identification of a ligand for the c-*kit* proto-oncogene. *Cell* **63**: 167–174, 1990.
6. Copeland NG, Gilbert DJ, Cho BC, Donovan PJ, Jenkins NA, Cosman D, Anderson D, Lyman SD and Williams DE, Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell* **63**: 175–183, 1990.
7. Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, March C, Boswell HS, Gimpel SD, Cosman D and Williams DE, Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* **63**: 235–243, 1990.
8. Huang E, Nocka K, Beier DR, Chu T-Y, Buck J, Lahm H-W, Wellner D, Leder P and Besmer P, The hematopoietic growth factor KL is encoded at the *Sl* locus and is the ligand of the c-*kit* receptor, the gene product of the W locus. *Cell* **63**: 225–233, 1990.
9. Furitsu T, Saito H, Dvorak AM, Schwartz LB, Irani AA, Burdick JF, Ishizaka K and Ishizaka T, Development of human mast cells *in vitro*. *Proc Natl Acad Sci USA* **86**: 10039–10043, 1989.
10. Tsai M, Shih L, Newlands GFJ, Takeishi T, Langley KE, Zsebo KM, Miller HRP, Geissler EN and Galli SJ, The rat c-*kit* ligand, stem cell factor, induces development of connective tissue-type and mucosal mast cells *in vivo*. Analysis by anatomical distribution, histochemistry and protease phenotype. *J Exp Med* **174**: 125–131, 1991.
11. Tsai M, Takeishi T, Thompson H, Langley KE, Zsebo KM, Metcalfe DD, Geissler EN and Galli SJ, Induction of mast cell proliferation, maturation and heparin synthesis by the rat c-*kit* ligand, stem cell factor. *Proc Natl Acad Sci USA* **88**: 6382–6386, 1991.
12. Galli SJ, Tsai MT, Langley KE, Zsebo KM and Geissler EN, Stem cell factor (SCF), a ligand for c-*kit*, induces mediator release from some populations of mouse mast cells. *FASEB J (abstr)* **5**: A1092, 1991.

13. Wershil BK, Tsai M, Geissler EN, Zsebo KM and Galli SJ, The rat c-*kit* ligand, stem cell factor, induces c-*kit* receptor-dependent mouse mast cell activation *in vivo*. Evidence that signalling through the c-*kit* receptor can induce expression of cellular function. *J Exp Med* **175**: 245–255, 1992.
14. Columbo M, Horowitz EM, Botana LM, MacGlashan DW Jr, Bochner BS, Gillis S, Zsebo KM, Galli SJ and Lichtenstein LM, The recombinant human c-*kit* receptor ligand, rhSCF, induces mediator release from human cutaneous mast cells and enhances IgE-dependent mediator release from both skin mast cells and peripheral blood basophils. *J Immunol* **149**: 599–608, 1992.
15. Bischoff SC and Dahinden CA, c-*kit* ligand: a unique potentiator of mediator release by human lung mast cells. *J Exp Med* **175**: 237–244, 1992.
16. de Paulis A, Ciccarelli A, Cirillo R, de Crescenzo G, Columbo M and Marone G, Modulation of human lung mast cell function by the c-*kit* receptor ligand. *Int Arch Allergy Appl Immunol* **99**: 326–329, 1992.
17. Rottapel L, Reedijk M, Williams DE, Lyman SD, Anderson DM, Pawson T and Bernstein A, The *steel/W* transduction pathway: kit autophosphorylation and its association with a unique subset of cytoplasmic signaling proteins is induced by the steel factor. *Mol Cell Biol* **11**: 3043–3051, 1991.
18. Lev S, Givol D and Yarden Y, A specific combination of substrates is involved in signal transduction by the *kit*-encoded receptor. *EMBO J* **10**: 647–654, 1991.
19. Adkinson NF Jr, Measurement of total serum immunoglobulin E and allergen-specific immunoglobulin E antibody. In: *Manual of Clinical Immunology* (Eds. Rose NR and Friedman H), 2nd Edn, pp. 800–807. The American Society of Microbiology, Washington, DC, 1980.
20. Lawrence ID, Warner JA, Cohan VL, Hubbard WC, Kagey-Sobotka A and Lichtenstein LM, Purification and characterization of human skin mast cells. Evidence for human mast cell heterogeneity. *J Immunol* **139**: 3062–3069, 1987.
21. Gilbert HS and Ornstein L, Basophil counting with a new staining method using Alcian Blue. *Blood* **46**: 279–286, 1975.
22. Benyon RC, The human skin mast cell. *Clin Exp Allergy* **19**: 375–387, 1989.
23. Siraganian RP, An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* **57**: 383–394, 1974.
24. MacGlashan DW Jr, Single-cell analysis of Ca^{++} changes in human lung mast cells: graded vs. all-or-nothing elevations after IgE-mediated stimulation. *J Cell Biol* **109**: 123–134, 1989.
25. Grynkievicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J Biol Chem* **260**: 340–350, 1985.
26. MacGlashan DW Jr and Guo C-B, Oscillations in free cytosolic calcium during IgE-mediated stimulation distinguish human basophils from human mast cells. *J Immunol* **147**: 2259–2269, 1991.
27. Botana LM, Lichtenstein LM and MacGlashan DW Jr, Differential modulation of mediator release in human basophils. *J Allergy Clin Immunol* **87**: 240, 1991.
28. Massey WA, Cohan VL, MacGlashan DW Jr, Gittlen SW, Kagey-Sobotka A, Lichtenstein LM and Warner JA, Protein kinase C modulates IgE-mediated activation of human mast cells from lung and skin. I. Pharmacologic inhibition. *J Pharmacol Exp Ther* **258**: 824–829, 1991.
29. Hess AD, Silanskis MK, Esa AH, Pettit GR and May WS, Activation of human T lymphocytes by bryostatin. *J Immunol* **141**: 3263–3269, 1988.
30. Benhamou M, Gutkind JS, Robbins KC and Siraganian RP, Tyrosine phosphorylation coupled to IgE receptor-mediated signal transduction and histamine release. *Proc Natl Acad Sci USA* **87**: 5327–5330, 1989.
31. Warner JA, Peters SP, Lichtenstein LM, Hubbard W, Yancey KB, Stevenson HC, Miller PJ and MacGlashan DW Jr, Differential release of mediators from human basophils: differences in arachidonic acid metabolism following activation by unrelated stimuli. *J Leuk Biol* **45**: 558–571, 1989.
32. Columbo M, Casolaro V, Warner JA, MacGlashan DW Jr, Kagey-Sobotka A and Lichtenstein LM, The mechanism of mediator release from human basophils induced by platelet-activating factor. *J Immunol* **145**: 3855–3861, 1990.
33. Benyon RC, Robinson C and Church MK, Differential release of histamine and eicosanoids from human skin mast cells activated by IgE-dependent and non-immunological stimuli. *Br J Pharmacol* **97**: 898–904, 1989.
34. Lichtenstein LM and Margolis S, Histamine release *in vitro*: inhibition by catecholamines and methylxanthines. *Science* **161**: 902–903, 1968.
35. Lichtenstein LM, The mechanism of basophil histamine release induced by antigen and by the calcium ionophore A23187. *J Immunol* **114**: 1692–1699, 1975.
36. Peachell PT, MacGlashan DW Jr, Lichtenstein LM and Schleimer RP, Regulation of human basophil and lung mast cell function by cyclic adenosine monophosphate. *J Immunol* **140**: 571–579, 1988.
37. Foreman JC, Hallett MB and Mongar JL, The relationship between histamine secretion and ^{45}calcium uptake by mast cells. *J Physiol (London)* **271**: 193–211, 1977.
38. White JR, Ishizaka T, Ishizaka K and Sha'afi RI, Direct demonstration of increased intracellular concentration of free calcium as measured by quin-2 in stimulated rat peritoneal mast cell. *Proc Natl Acad Sci USA* **81**: 3978–3982, 1984.
39. Kennerly DA, Secosan CJ, Parker CW and Sullivan TJ, Modulation of stimulated phospholipid metabolism in mast cells by pharmacologic agents that increase cyclic 3′,5′adenosine monophosphate levels. *J Immunol* **123**: 1519–1524, 1979.
40. Undem BJ, Torphy TJ, Goldman D and Chilton FH, Inhibition by adenosine 3′,5′-monophosphate of eicosanoid and platelet-activating factor biosynthesis in the mouse PT-18 mast cell. *J Biol Chem* **265**: 6750–6758, 1990.
41. Berridge MJ, Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu Rev Biochem* **56**: 159–193, 1987.
42. Warner JA and MacGlashan DW Jr, Signal transduction events in human basophils. A comparative study of the role of protein kinase C in basophils activated by anti-IgE antibody and formyl-methionyl-leucyl-phenylalanine. *J Immunol* **145**: 1897–1905, 1990.
43. Nishizuka Y, Molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**: 661–665, 1988.
44. Pettit GR, Herald CL, Doubek DL, Herald DL, Arnold E and Clardy J, Isolation and structure of bryostatin 1. *J Am Chem Soc* **104**: 6846–6848, 1982.
45. Kikkawa U, Ogita K, Shearman MS, Ase K, Sekiguchi K, Naor Z, Kishimoto A, Nishizuka Y, Saito N, Tanaka C, Ono Y, Fujii T and Igarashi K, The family of protein kinase C: its molecular heterogeneity and differential expression. *Cold Spring Harbor Symp Quant Biol* **53**: 97–102, 1988.
46. Kikkawa U, Kishimoto A and Nishizuka Y, The protein kinase C family: heterogeneity and its implications. *Annu Rev Biochem* **58**: 31–44, 1989.
47. Huang K-P, Huang FL, Nakabayashi H and Yoshida

Y, Biochemical characterization of rat brain protein kinase C isozymes. *J Biol Chem* **263**: 14839–14845, 1988.

48. Schaap D and Parker PJ, Expression, purification, and characterization of protein kinase C-ε. *J Biol Chem* **265**: 7301–7307, 1990.
49. Leibersperger H, Gshwendt M and Marks F, Purification and characterization of a calcium-unresponsive, phorbol ester/phospholipid-activated protein kinase from porcine spleen. *J Biol Chem* **265**: 16108–16115, 1990.
50. Lewin NE, Dell'Aquila ML, Pettit GR, Blumberg PM and Warren BS, Binding of [^{3}H]bryostatin 4 to protein kinase C. *Biochem Pharmacol* **43**: 2007–2014, 1992.
51. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M and Fukami Y, Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262**: 5592–5595, 1987.
52. Stephan V, Benhamou M, Gutkind JS, Robbins KC and Siraganian RP, FcεRI-induced protein tyrosine phosphorylation of pp72 in rat basophilic leukemia cells (RBL-2H3). Evidence for a novel signal transduction pathway unrelated to G protein activation and phosphatidylinositol hydrolysis. *J Biol Chem* **267**: 5434–5441, 1992.
53. Finch EA, Turner TJ and Goldin SM, Calcium as coagonist of inositol 1,4,5-triphosphate-induced calcium release. *Science* **252**: 443–446, 1991.