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# Agonistic Activities of Histamine-Albumin Conjugates at Histamine $H_2$ Receptors on Human HL-60 Promyelocytic Leukemia Cells

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## SUMMARY

We previously identified functional histamine $H_2$ receptors on human HL-60 promyelocytic leukemia cells [*J. Biol. Chem.* **264**:18356–18362 (1989)]. In the present study, we have compared the action of histamine-albumin conjugates on $H_2$ receptor activation with that of histamine alone. Both histamine and conjugates increased intracellular levels of $Ca^{2+}$ in an $H_2$ receptor-specific manner. However, binding of fluoresceinated histamine-albumin conjugates to HL-60 cells was not dissociated by $10^{-4}$ M unlabeled histamine, although this concentration of histamine significantly desensitized conjugate responses. These data suggest that histamine-albumin conjugates not only activate $H_2$ receptors but also bind to HL-60 cells nonspecifically or in an $H_2$ receptor-unrelated manner. Moreover, histamine-induced $Ca^{2+}$ mobilization was transient, whereas conjugate-induced $Ca^{2+}$ mobilization was sustained for more than 10 min, as a result of the influx of extracellular $Ca^{2+}$. Therefore, the functional difference between histamine and conjugates may provide a good model for the further understanding of the activation mechanisms of receptor-operated $Ca^{2+}$ influx.

HA has been shown to play a role in various immunosuppressive functions *in vivo* and *in vitro* (1). Extensive pharmacological analyses using HR-specific agonists and antagonists demonstrated that immunosuppressive effects of HA are apparently mediated by the $H_2R$. In order to directly identify the $H_2R$ in immune cells, classical radiolabeled ligand binding using [$^3$H]HA or [$^3$H]cimetidine was carried out (2, 3). However, because of low affinity binding sites for the $H_2R$ and high nonspecific binding, the specificity of these radioligand binding assays was controversial (4, 5). Melmon *et al.* (6) have also demonstrated HR-bearing leukocytes, using agarose beads that were coated with HA conjugated to a protein carrier. In their report, HA was covalently conjugated to albumin, and HA-albumin conjugates were coupled to Sepharose beads, which were then incubated with human peripheral leukocytes. Using this method, it was demonstrated that the majority of HA-coated beads were bound by leukocytes. Kedar and Bonavida (7) have also identified HR-bearing leukocytes, by means of rosette formation using erythrocytes coated with HA-albumin conjugates. Furthermore, Osband *et al.* (8) utilized HA conjugated to fluoresceinated albumin, and HR-bearing cells were analyzed by flow cytometry. However, Muirhead *et al.* (9) reported that the levels of HA-albumin conjugate binding did not parallel the ligand potencies of the classical $H_2R$. Because high concentrations of HA or HA antagonists were required to displace HA-albumin conjugates from cells, binding characteristics of HA-albumin conjugates may be different from those of HA. If HA-albumin conjugates are acting noncompetitively or irreversibly, or bind to the $H_2R$ with much higher affinity than other known classical agonists and antagonists, the binding analysis of HA-albumin conjugates is difficult to interpret. Moreover, if HA-albumin conjugates not only bind to $H_2R$ but also bind to other cell surface components, the binding analysis is not a good system with which to characterize $H_2R$.

In our previous studies, we showed that human HL-60 promyelocytic leukemia cells expressed functionally active $H_2R$ (10). Upon stimulation with HA, these cells increased $[Ca^{2+}]_i$ in a $H_2R$-specific and dose-dependent manner. This cell system provides a good model for the molecular analysis of $H_2R$-ligand interaction and $H_2R$-mediated signal transduction mecha-

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**ABBREVIATIONS:** HA, histamine; BSA, bovine serum albumin; $[Ca^{2+}]_i$, levels of intracellular $Ca^{2+}$; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; FACS, fluorescence-activated cell sorter; FITC, fluorescein-5-isothiocyanate; fMLP, *N*-formyl-Met-Leu-Phe; HBSS, Hanks' balanced salt solution; HR, histamine receptor; $H_1R$, histamine $H_1$ receptor; $H_2R$, histamine $H_2$ receptor; PMA, phorbol 12-myristate 13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

nisms. In the present study, we have extended our previous studies to test whether HA-BSA specifically recognizes the $H_2R$ on HL-60 cells and activates intracellular $Ca^{2+}$ mobilization pathways. Here we show that HA-BSA recognizes and activates $H_2R$ on HL-60 cells with approximately 10-fold higher affinity than that of HA.

## Experimental Procedures

**Materials.** HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). Media for tissue culture were obtained from the Cell Culture Facility (University of California, San Francisco, CA). The acetoxymethyl esters of indo-1 and fura-2, FITC-BSA, FITC-HA (Molecular Probes, Junction City, OR), $^{45}CaCl_2$ (2 mCi/ml), and [$^3$H]HA (50 Ci/mmol) (Amersham, Arlington Heights, IL) were obtained from the designated suppliers. 1,25-Dihydroxy-vitamin $D_3$ was obtained from Dr. M. Uskokovic (Hoffman-LaRoche, Nutley, NJ). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). HA-BSA or HA-(FITC-BSA) was prepared by the procedure of Melmon *et al.* (6). In brief, various amounts (3–300 mg) of HA dihydrochloride, 30 mg of BSA or FITC-BSA, and 0.3 g of EDC were mixed in phosphate-buffered saline, pH 7.2. The solution was incubated for 1–2 hr in the dark at room temperature, with intermittent shaking, and then dialyzed against phosphate-buffered saline for 2 days. In parallel experiments, trace amounts (1–10 $\mu$Ci) of [$^3$H]HA were added to the reaction mixture, in order to calculate the molar ratio of HA coupled to BSA.

**Cell culture.** HL-60 cells were grown in RPMI 1640 containing 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 10% fetal calf serum, at 37° in 5% $CO_2$/95% air, and were differentiated into granulocytes or monocytes by incubation with 1.2% DMSO, $10^{-8}$ M 1,25-dihydroxy-vitamin $D_3$, or 2 nM PMA for more than 5 days, as previously described (10).

**Measurement of fura-2 $Ca^{2+}$ signals.** HL-60 cells were suspended in BSA-HBSS, at $2 \times 10^7$ cells/ml, and loaded with fura-2 (2.5 $\mu$M) for 60 min at 37° in the dark, as described previously for the analysis of $H_1R$ and $H_2R$ activation (10, 11). After three washes with BSA-HBSS, cell pellets were resuspended in fresh BSA-HBSS at $5 \times 10^6$ cells/ml. The intracellular fura-2 signals were measured at 340-nm excitation and 510-nm emission, by using a fluorimeter (model 650-40; Perkin Elmer, Norwalk, CT).

**FITC-HA and HA-(FITC-BSA) binding.** DMSO-differentiated HL-60 cells, suspended in phenol red-free HBSS containing 0.1% BSA (BSA-HBSS), were pretreated with or without $3 \times 10^{-4}$ M HA at 37° for 5 min and then incubated with FITC-HA or HA-(FITC-BSA) at 4° for 1 hr. Cells were resuspended in fresh BSA-HBSS, and FITC fluorescence of individual cells was analyzed in a FACS (FACS IV; Becton Dickinson, Sunnyvale, CA) at 501-nm excitation and 525-nm emission.

**Two-color FACS analysis.** DMSO-differentiated HL-60 cells were suspended in fresh RPMI 1640 containing 0.1% BSA (BSA-RPMI), at $0.5–2 \times 10^7$ cells/ml, and loaded with indo-1 (3 $\mu$M) for 40 min at 37° in the dark, as described previously for the analysis of $H_1R$ and $H_2R$ activation (10, 12). After one wash with BSA-RPMI, cell pellets were resuspended in fresh BSA-RPMI at $5 \times 10^6$/ml and analyzed in a FACS equipped with an argon ion laser emitting 600 mW at 351–364 nm. In order to analyze the changes in $[Ca^{2+}]_i$ in individual cells, blue (485/22-nm) and violet (404/20-nm) band pass filters were used to collect indo-1 fluorescence emission. The ratio of violet to blue fluorescence is directly proportional to $[Ca^{2+}]_i$. FITC fluorescence of individual cells was also determined simultaneously, at 501-nm excitation and 525-nm emission.

**$^{45}Ca$ influx.** The influx of $^{45}Ca^{2+}$ was determined by the method of Brown *et al.* (13). In brief, HL-60 cells were resuspended in $Ca^{2+}/Mg^{2+}$-free BSA-HBSS, at $2 \times 10^7$ cells/ml, and stimulated with either HA or HA-BSA in the presence of 1 $\mu$Ci/ml $^{45}CaCl_2$, for 2 min at 37°. Cells were then harvested onto Whatmann GF/C glass fiber filters and were immediately washed six times with HBSS. The radioactivity of individual filters was determined in a liquid scintillation counter (LS 5801; Beckman Instruments, Irvine, CA).

## Results

**HA-BSA-induced mobilization of intracellular $Ca^{2+}$.** In order to calculate the amount of bound HA in HA-BSA and HA-(FITC-BSA) preparations, a fixed amount (30 mg) of BSA was mixed with 3, 30, and 300 mg of HA in the presence of a trace amount of [$^3$H]HA (1–10 $\mu$Ci), as described in Experimental Procedures. As a result, the molar ratio of HA to BSA was approximately 1:1, 3:1, and 5:1, respectively.

In order to investigate whether HA-BSA mobilizes intracellular $Ca^{2+}$ in a $H_2R$-specific manner, we prepared EDC-treated BSA and histidine-BSA conjugates, under identical conditions as HA-BSA, and compared them with HA-BSA. As shown in Fig. 1, HA-BSA significantly increased $[Ca^{2+}]_i$, whereas both EDC-treated BSA and histidine-BSA failed to mobilize intracellular $Ca^{2+}$, even at 10–20-fold higher concentrations of BSA. The effect of HA-BSA was dose dependent, with an $EC_{50}$ of approximately $1 \times 10^{-6}$ M HA, based on the incorporation of [$^3$H]HA into HA-BSA preparations (Fig. 1), which was one-tenth that of HA alone (10). Furthermore, the $H_2R$-specific antagonist cimetidine inhibited HA-BSA-induced mobilization of intracellular $Ca^{2+}$ more significantly than did the $H_1R$-specific antagonist pyrilamine (Fig. 2). The $IC_{50}$ of cimetidine was approximately $10^{-5}$ M, which was 10-fold higher than that against HA-induced $Ca^{2+}$ mobilization in HL-60 cells (10).

HA and HA-BSA increased $[Ca^{2+}]_i$ in both undifferentiated and DMSO-differentiated neutrophilic HL-60 cells (Fig. 3). In contrast, both HA and HA-BSA responses disappeared when cells were differentiated into monocytic cells by incubation with vitamin $D_3$ or PMA (Fig. 3).

In our previous studies, we have shown that HA specifically desensitized a subsequent response to HA but not to the chem-

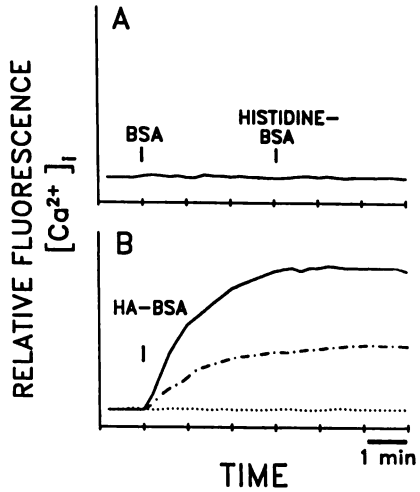


**Fig. 1.** HA-BSA-induced increases in $[Ca^{2+}]_i$ in HL-60 cells. HL-60 cells were suspended in BSA-HBSS, at $2 \times 10^7$ cells/ml, and loaded with fura-2/acetoxymethyl ester (2.5 $\mu$M) for 60 min at 37° in the dark. Cell pellets were resuspended in fresh BSA-HBSS, at $5 \times 10^6$ cells/ml, and then stimulated with 200 $\mu$g/ml EDC-treated BSA or 200 $\mu$g/ml histidine-BSA conjugate (A) or HA-BSA at 10 $\mu$g/ml (BSA concentration) (——), 1 $\mu$g/ml (–·–·–), or 0.1 $\mu$g/ml (···) (B), at the indicated points, and the intracellular fura-2 signals were measured at 340-nm excitation and 510-nm emission by using a fluorimeter. Shown is a typical experiment, which was reproduced three times.

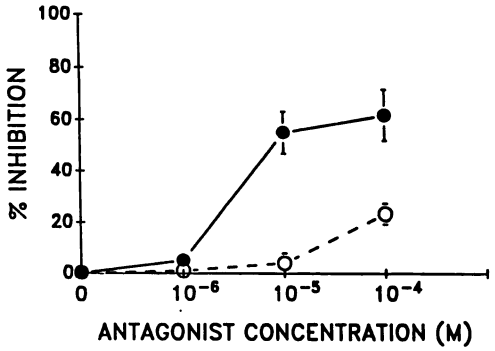


**Fig. 2.** Antagonist selectivity of HA-BSA-induced intracellular $Ca^{2+}$ mobilization. Fura-2-loaded HL-60 cells were pretreated with $10^{-6}$ to $10^{-4}$ M cimetidine (●) or pyrilamine (○) for 5 min at 37° and then stimulated with 10 μg/ml HA-BSA. Results were expressed as a percentage of inhibition {(untreated HA-BSA response − treated HA-BSA response)/ untreated HA-BSA response × 100}. Each data point was the mean ± standard error from three separate experiments.

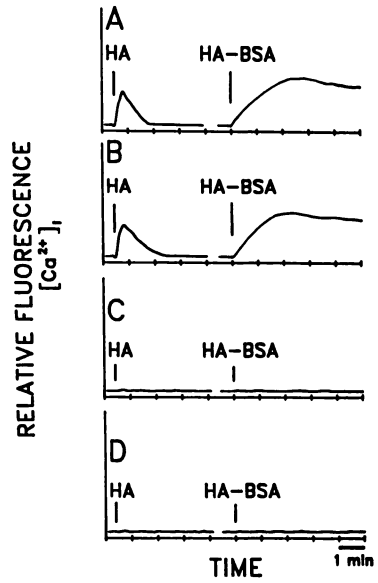


**Fig. 3.** Cell type specificity of HA-BSA-induced intracellular $Ca^{2+}$ mobilization. HL-60 cells were differentiated into the granulocytic cell type by incubation with 1.2% DMSO (B) or differentiated into the monocytic cell type with $10^{-8}$ M 1,25-dihydroxy-vitamin $D_3$ (C) or 2 nM PMA (D) for 5 days, and HA- or HA-BSA-induced intracellular $Ca^{2+}$ mobilization was compared with that of undifferentiated HL-60 cells (A).

otactic peptide fMLP (10). Therefore, receptor-specific desensitization/down-regulation is an alternate method to characterize the $H_2R$ specificity, without using any agonists and antagonists. The result was that a previous exposure to $10^{-4}$ M HA significantly inhibited the secondary HA response and also inhibited the subsequent HA-BSA response (inhibition = 82.2 ± 1.2%, three experiments) (Fig. 4). HA-BSA also inhibited the secondary HA response (inhibition = 83.4 ± 9.6%, three experiments) as well as the subsequent HA-BSA response (Fig. 4). However, fMLP failed to desensitize the HA-BSA response, and HA-BSA failed to inhibit the secondary fMLP response, although $10^{-4}$ M HA increased $[Ca^{2+}]_i$ to a similar degree as did $10^{-6}$ M fMLP (Fig. 4).

Previous exposure to HA desensitized HA-BSA-induced intracellular $Ca^{2+}$ mobilization (Fig. 4). However, under identical conditions, HA-(FITC-BSA) binding (approximately $10^{-8}$ M HA, based on the incorporation of $[^3H]$HA into HA-BSA prep-

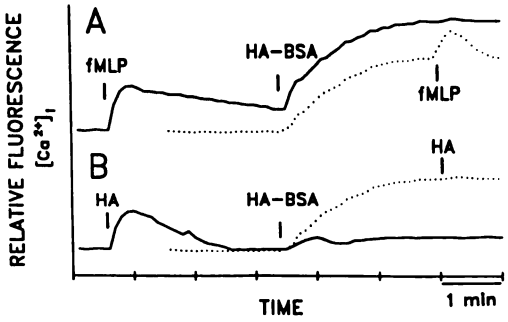


**Fig. 4.** Receptor-specific desensitization. Fura-2-loaded DMSO-differentiated HL-60 cells were suspended in BSA-HBSS, and $[Ca^{2+}]_i$ was measured in a fluorimeter, as described in Experimental Procedures and Fig. 1. Cells were then stimulated with $10^{-6}$ M fMLP, $10^{-4}$ M HA, or HA-BSA (10 μg/ml BSA concentration) at the indicated points. Shown is a typical paired experiment (——; · · · · · · ·), which was reproduced three times.

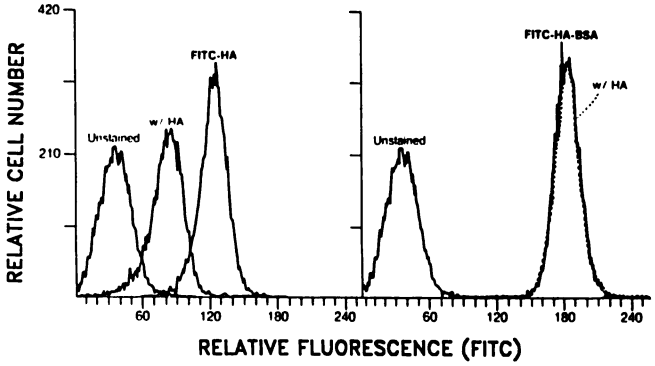


**Fig. 5.** FITC-HA and HA-(FITC-BSA) binding. DMSO-differentiated HL-60 cells were suspended in phenol red-free BSA-HBSS, at $2 \times 10^7$ cells/ml, and pretreated with $10^{-4}$ M unlabeled HA for 5 min at 37° (*w/HA*). Cells were then incubated with $10^{-7}$ M FITC-HA (A) or HA-(FITC-BSA) (approximately $10^{-8}$ M HA concentration) (B) at 4° for 1 hr. Cells were resuspended in fresh BSA-HBSS, and FITC fluorescence of individual cells was analyzed in a FACS at 501-nm excitation and 525-nm emission. Shown is a typical experiment, which was reproduced twice.

arations) was not dissociated by pretreatment with $10^{-4}$ M unlabeled HA (Fig. 5). In contrast, FITC-HA binding ($10^{-7}$ M) was significantly inhibited by $10^{-4}$ M unlabeled HA (Fig. 5).

**Two-color FACS analysis.** In order to analyze the relationship between the cell population recognized by HA-BSA and intracellular $Ca^{2+}$ mobilization, DMSO-differentiated HL-60 cells were loaded with indo-1, and then $[Ca^{2+}]_i$ and the relative intensity of cell surface labeling with HA-(FITC-BSA) in individual cells were simultaneously monitored by two-color fluorescence in a FACS. As shown in Fig. 6A, the unstimulated cell population was mainly located in domain V (unstimulated), in which both $[Ca^{2+}]_i$ and relative FITC intensity were low. We have previously demonstrated that approximately 30–40% of cells moved to domain VI (stimulated), a cell population with high $[Ca^{2+}]_i$ and low FITC intensity, when cells were exposed to $10^{-5}$ M unlabeled HA (10). When cells were exposed to FITC-BSA, cells were located in domain III (nonspecific BSA binding), in which intracellular $[Ca^{2+}]_i$ was low but relative FITC intensity was higher than that of the unstimulated cell population (Fig. 6B). Furthermore, after stimulation with HA-(FITC-BSA), >90% of cells moved to domain II (specific HA-BSA binding with high $[Ca^{2+}]_i$), a cell population with high $[Ca^{2+}]_i$ and higher FITC intensity than that of FITC-BSA alone (Fig. 6C). However, in domain II, this cell population was

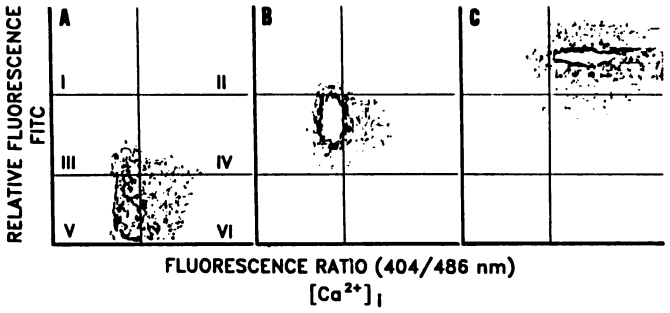


**Fig. 6.** Two-color FACS analysis of HA-(FITC-BSA) binding and the simultaneous measurement of intracellular $Ca^{2+}$ mobilization. Cells were suspended in BSA-RPMI, at 0.5–2 × $10^7$ cells/ml, and loaded with indo-1 (3 μM) for 40 min at 37° in the dark, as described in Experimental Procedures. After one wash with BSA-RPMI, cell pellets were resuspended in fresh BSA-RPMI, at 5 × $10^6$/ml, and analyzed in a FACS equipped with an argon ion laser emitting 600 mW at 351–364 nm. In order to analyze the changes in $[Ca^{2+}]_i$ in individual cells, blue (485/22-nm) and violet (404/20-nm) band pass filters were used to collect indo-1 fluorescence emission. The ratio of violet to blue fluorescence is directly proportional to $[Ca^{2+}]_i$. After stimulation with 10 μg/ml levels of either FITC-BSA (B) or HA-(FITC-BSA) (C) for 2 min, the relative intensity of fluorescence in individual cells was also determined simultaneously, with indo-1 signals at 501-nm excitation and 525-nm emission. Results were displayed as a function of relative intensity of FITC and $[Ca^{2+}]_i$ (404-nm/486-nm emission) on a logarithmic scale. Domains I, III, and V indicate low $[Ca^{2+}]_i$ and domains II, IV, and VI indicate high $[Ca^{2+}]_i$. Domains V and VI indicate the autofluorescence of cells, domains III and IV indicate the nonspecific binding of FITC-BSA, and domains I and II indicate the specific binding of HA-(FITC-BSA). Shown is a typical experiment, which was reproduced twice.

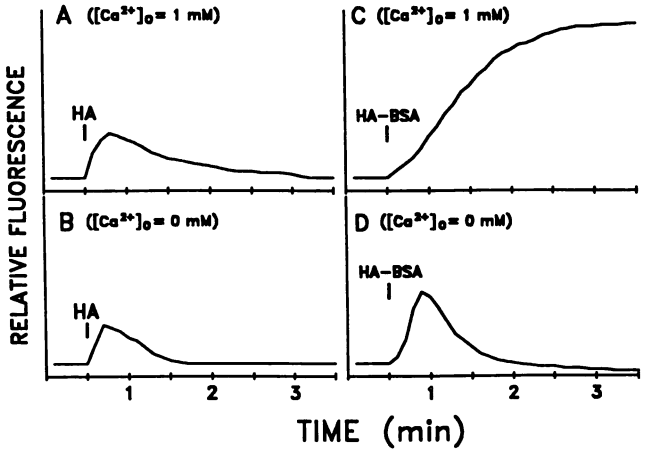


**Fig. 7.** Effect of extracellular $Ca^{2+}$ on HA- and HA-BSA-induced intracellular $Ca^{2+}$ mobilization. Fura-2-loaded DMSO-differentiated HL-60 cells were suspended in HBSS (A and C) or $Ca^{2+}$-free BSA-HBSS containing 1 mM EGTA (B and D), and $[Ca^{2+}]_i$ was measured in a fluorimeter, as described in Experimental Procedures and Fig. 1. Cells were then stimulated with $10^{-4}$ M HA (A and B) or $10^{-5}$ M HA-BSA (HA concentration) (C and D). Shown is a typical experiment, which was reproduced three times.

distributed horizontally, and the level of FITC-HA was not correlated with $[Ca^{2+}]_i$.

**Influx of extracellular $Ca^{2+}$.** Although HA-BSA has been shown to increase $[Ca^{2+}]_i$ in HL-60 cells, the kinetics of HA-BSA-induced $[Ca^{2+}]_i$ mobilization were different from those of HA alone (Figs. 1, 3, and 4). HA-induced increases in $[Ca^{2+}]_i$ were maximal at 20–25 sec after stimulation and returned to basal levels within 2 min (Figs. 3 and 4), whereas HA-BSA-induced increases in $[Ca^{2+}]_i$ were maximal at 2.7 ± 0.2 min (nine experiments) after stimulation and did not return to basal levels even after 10 min (Figs. 1, 3, and 4). Furthermore, fura-2-loaded HL-60 cells were suspended in $Ca^{2+}$-free HBSS containing 1 mM EGTA, and the changes in $[Ca^{2+}]_i$ were analyzed in a fluorimeter. As shown in Fig. 7, both HA and HA-BSA mobilized intracellular $Ca^{2+}$, even in the absence of extracellular $Ca^{2+}$. However, the sustained phase of elevated $[Ca^{2+}]_i$ after exposure to HA-BSA was completely abolished when cells were suspended in a $Ca^{2+}$-free solution (Fig. 7).

In order to directly analyze whether HA-BSA additionally activates $Ca^{2+}$ influx in HL-60 cells, cells were suspended in a $Ca^{2+}$-free solution containing $^{45}Ca^{2+}$, and the influx of $^{45}Ca^{2+}$ was assessed as described in Experimental Procedures. As a result, HA-BSA significantly increased $^{45}Ca^{2+}$ influx into HL-60 cells 14.7 ± 2.1-fold (four experiments), whereas HA failed to increase $^{45}Ca^{2+}$ influx (0.83 ± 0.12-fold, four experiments) (Fig. 8).

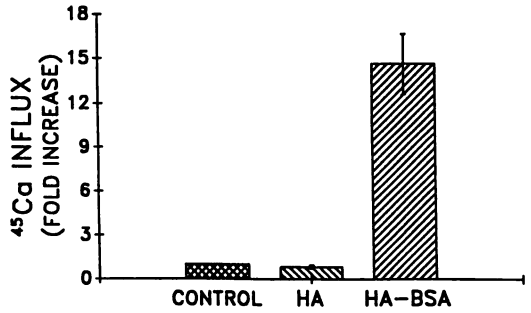


**Fig. 8.** HA-BSA-induced $^{45}Ca^{2+}$ influx. HL-60 cells were resuspended in $Ca^{2+}/Mg^{2+}$-free BSA-HBSS, at 2 × $10^7$ cells/ml, and stimulated with either HA or HA-BSA in the presence of 1 μCi/ml $^{45}Ca^{2+}$, for 2 min at 37°. Cells were then harvested onto Whatmann GF/C glass fiber filters and were immediately washed six times with ice-cold HBSS. The radioactivity of individual filters was determined in a liquid scintillation counter. Each value was the mean ± standard error from four separate experiments performed in duplicate. Matched paired *t* test indicated that the difference between control and HA-BSA was significant ($p < 0.001$).

## Discussion

HA-BSA has been extensively used, for many years, to identify HR-bearing cells (4–8). However, results of HA-BSA binding are controversial and, under certain circumstances, binding data did not parallel the ligand potencies of the classical HR (9). Therefore, in the present study, we have focused on the functional activity of HA-BSA, and an attempt was made to overcome the difficulty of HA-BSA binding. As a result, HA-BSA significantly increased $[Ca^{2+}]_i$ in undifferentiated and DMSO-differentiated granulocytic HL-60 cells (Figs. 1 and 3), which was more sensitive to the $H_2R$-specific antagonist cimetidine than to the $H_1R$ antagonist pyrilamine (Fig. 2). Interestingly, the $EC_{50}$ of HA-BSA was one-tenth that of HA alone (Fig. 1), whereas the $IC_{50}$ of cimetidine was 10-fold higher than that against HA-induced intracellular $Ca^{2+}$ mobilization (Fig. 2). This suggests that HA-BSA has approximately 10-fold higher affinity for $H_2R$ than does HA.

In order to further confirm whether HA-BSA increased $[Ca^{2+}]_i$ by acting through $H_2R$ on HL-60 cells, we have prepared EDC-treated BSA and histidine-BSA conjugates under identical conditions as HA-BSA. As shown in Fig. 1, both EDC-treated BSA and histidine-BSA failed to mobilize $[Ca^{2+}]_i$ at concentrations as high as 200 μg/ml BSA. Furthermore, HA-BSA increased $[Ca^{2+}]_i$ only in HA-responsive cells (undiffer-

entiated and DMSO-differentiated HL-60 cells), but not in HA-unresponsive cells (vitamin $D_3$- and PMA-induced monocytic cells) (Fig. 3). Moreover, we have also focused on the effect of receptor-specific desensitization/down-regulation, because $H_2R$ on HL-60 cells have been shown to be desensitized by previous exposure to HA but not the chemotactic peptide fMLP, although both $10^{-4}$ M HA and $10^{-6}$ M fMLP increased $[Ca^{2+}]_i$ to a similar degree and in the same inositol trisphosphate-dependent manner (10). As shown in Fig. 4, previous exposure to HA significantly inhibited the secondary HA-BSA response, and HA-BSA also inhibited the secondary HA response. However, HA-BSA was independent of the response to fMLP; HA-BSA failed to inhibit the secondary fMLP response, and fMLP failed to desensitize the HA-BSA response (Fig. 4). Therefore, these data strongly suggest that HA-BSA is capable of activating $H_2R$ on HL-60 cells.

In contrast to HA-BSA-induced intracellular $Ca^{2+}$ mobilization, HA-(FITC-BSA) binding did not reflect a direct interaction with $H_2R$, because HA-(FITC-BSA) binding was not dissociated by pretreatment with $10^{-4}$ M unlabeled HA (Fig. 5), although this concentration of HA completely desensitized HA-BSA-induced intracellular $Ca^{2+}$ mobilization (Fig. 4). Moreover, two-color FACS analysis demonstrated that the level of HA-(FITC-BSA) was not correlated with $[Ca^{2+}]_i$ in individual HL-60 cells (Fig. 6C), although HA-(FITC-BSA) bound to HL-60 cell more significantly than did FITC-BSA alone (Fig. 6B). These data suggest that the majority of HA-BSA binds to the surface of HL-60 cells nonspecifically (or in an $H_2R$-unrelated manner), and only a small fraction of HA-BSA binds to and activates $H_2R$. Therefore, the high nonspecific HA-BSA binding may mask the low specific binding to $H_2R$.

In the present studies, we have also found that, unlike HA, HA-BSA additionally induced $Ca^{2+}$ influx into HL-60 cells (Figs. 7 and 8). The sustained elevation of $[Ca^{2+}]_i$ after HA-BSA stimulation was also inhibited by previous exposure to HA (Fig. 4), suggesting that $Ca^{2+}$ influx is mediated by $H_2R$ but not by nonspecific binding to cell surface components of HL-60 cells. In the present study, in order to explore the possible mechanism of HA-BSA-induced $Ca^{2+}$ influx, we have constructed HA-BSA conjugates with various molar ratios of HA to BSA. The result was that HA-BSA with an equal molar ratio of HA to BSA could induce $[Ca^{2+}]_i$ mobilization similar to that of multivalent HA-BSA compounds (data not shown). However, because the calculation of HA-BSA ratios produces only average values, it is difficult to know the exact valency of HA-BSA involved in individual cellular responses. Interestingly, our preliminary experiments also show that HA-BSA alters the kinetics of inositol phosphate metabolic products and predominantly generates inositol 1,3,4,5-tetrakisphosphate (14). Because 1,3,4,5-tetrakisphosphate has been shown to activate $Ca^{2+}$ channels (15) and binding sites for 1,3,4,5-tetrakisphosphate were also reported in HL-60 cells (16), the altered metabolism of inositol phosphates induced by HA-BSA might be closely related to $Ca^{2+}$ channel activation.

Both HA-BSA and the chemotactic peptide fMLP induce similar intracellular $Ca^{2+}$ mobilization, with a sustained phase of elevated $[Ca^{2+}]_i$. However, fMLP-induced intracellular $Ca^{2+}$ mobilization is accompanied by intracellular actin polymerization (10), whereas HA-BSA-induced intracellular $Ca^{2+}$ mobilization is not (data not shown). The functional consequences of HA-BSA-induced intracellular $Ca^{2+}$ mobilization remain to be elucidated.

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