Receptor-recognized α_2-macroglobulin–methylamine elevates intracellular calcium, inositol phosphates and cyclic AMP in murine peritoneal macrophages

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Human plasma α_2-macroglobulin (α_2M) is a tetrameric proteinase inhibitor, which undergoes a conformational change upon reaction with either a proteinase or methylamine. As a result, a receptor recognition site is exposed on each subunit of the molecule enabling it to bind to its receptors on macrophages. We have used Fura-2-loaded murine peritoneal macrophages and digital video fluorescence microscopy to examine the effects of receptor binding on second messenger levels. α_2M–methylamine caused a rapid 2–4-fold increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) within 5 s of binding to receptors. The agonists induced a focal increase in $[Ca^{2+}]_i$ that spread out to other areas of the cell. The increase in $[Ca^{2+}]_i$ was dependent on the α_2M–methylamine concentration and on the extracellular $[Ca^{2+}]$. Both sinusoidal and transitory oscillations were observed, which varied from cell to cell. Neither α_2M nor boiled α_2M–methylamine, forms that are not recognized by the receptor, affected $[Ca^{2+}]_i$ in peritoneal macrophages under identical conditions of incubation. The α_2M–methylamine-induced rise in $[Ca^{2+}]_i$ was accompanied by a rapid and transient increase in macrophage inositol phosphates, including inositol tris- and tetrakis-phosphates. Native α_2M did not stimulate a rise in inositol phosphates. Finally, binding of α_2M–methylamine to macrophages increased cyclic AMP transiently. Thus receptor-recognized α-macroglobulins behave as agonists whose receptor binding causes stimulation of signal transduction pathways.

INTRODUCTION

Human α_2-macroglobulin (α_2M) belongs to a class of plasma proteinase inhibitors which is unique in its ability to inhibit proteinases of all four mechanistic classes (see Sottrup-Jensen et al., 1985, for a review). Proteinase inhibition is the result of steric interference with the access of larger substrates to the proteinase active site (Barrett and Starkey, 1973). This inhibitory mechanism is initiated when the proteinase attacks the 'bait region', a stretch of polypeptide that is particularly susceptible to proteolysis. A major conformational change in the inhibitor then occurs which can be detected as an altered migration on native polyacrylamide gels (the slow to fast conformational change).

Most members of the α-macroglobulin family of proteinase inhibitors possess reactive thiolesters. With only a few exceptions, reaction of this thiolester with ammonia or methylamine triggers a conformation change similar to that elicited by proteinase attack (Imber and Pizzo, 1981; Pizzo, 1988; Enghild et al., 1989). Concomitant with this conformational change, a receptor-binding epitope appears on α_2M. This receptor recognition site, which is responsible for the high-affinity binding of α-macroglobulins to macrophages and other cells, has been found in α-macroglobulins purified from a diverse group of organisms including representatives of the amphibian, avian and mammalian classes of the chordate phylum (see Pizzo, 1988, for review). In each case, the binding of α-macroglobulin–proteinase complexes to murine peritoneal macrophages occurs with extremely high affinity with K_d values of < 1 nM at 4 °C and about 10 nM at 37 °C (Pizzo, 1988).

Binding of human α_2M fast forms to macrophage receptors is followed by a number of events, including enhanced locomotion and chemotaxis (Forrester et al., 1983), down-regulation of proteinase synthesis (Johnson et al., 1982) and suppression of the respiratory burst (Hoffman et al., 1983). Binding of human α_2M fast forms to murine peritoneal macrophages also results in rapid secretion of prostaglandin E_2 (Uhing et al., 1991) and prevents interferon-γ-induced cell rounding (Roche et al., 1990). Binding of either α_2M–proteinase complexes or α_2M–methylamine to macrophages produces similar effects, as expected from the fact that both of these fast forms bind to the α_2M receptor with the same affinity (Imber and Pizzo, 1981; Pizzo, 1988).

Many of the macrophage functional responses which are affected by α_2M fast forms are also affected by agents which alter the levels of intracellular second messengers (Danø et al., 1985; Chonaib et al., 1987; O'Shea et al., 1987; Plaut, 1987; Yamamoto and Suzuki, 1987; Hoffman et al., 1988; Kammer, 1988; Lerner et al., 1988; Snyderman and Uhing, 1988; Takemura and Putney, 1989; Figueiredo et al., 1990). We hypothesized that the reported modulations of various macrophage functions by α_2M–proteinase complexes or by α_2M–methylamine may be mediated by second messengers generated during ligation of the α_2M receptor. We have, therefore, studied the effect of receptor-recognized α_2M–methylamine on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse peritoneal macrophages using digital fluorescent imaging of Fura-2-loaded macrophages. Our results show that human α_2M–methylamine produces a dose-dependent increase in $[Ca^{2+}]_i$. Furthermore, the binding of α_2M–methylamine to cell surface receptors on mouse peritoneal macrophages also results in transiently elevated levels of inositol phosphates and cyclic AMP (cAMP).

Abbreviations used: α_2M, α_2-macroglobulin; cAMP, cyclic AMP; $[Ca^{2+}]_i$, intracellular calcium concentration; Fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*NNN′N′*-tetra-acetic acid acetoxymethyl ester; IBMX, 3-isobutyl-1-methylxanthine; IP_1, inositol monophosphate, IP_2, inositol bisphosphate, IP_3, inositol 1,4,5-trisphosphate, IP_4, inositol 1,3,4,5-tetrakisphosphate; HHBSS, Hanks' balanced salt solution containing 10 mM Hepes, pH 7.4, and 3.5 mM $NaHCO_3$.

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EXPERIMENTAL

Reagents

Sterile distilled water was obtained from Abbott Laboratories (Chicago, IL, U.S.A.). Brewer's thioglycollate broth and proteose peptone were purchased from Difco Laboratories (Baltimore, MD, U.S.A.). Casein was purchased from EM Chemicals (Elmsford, NY, U.S.A.). Culture media were purchased from Cellgro (Herndon, VA, U.S.A.) and Gibco Laboratories (Grand Island, NY, U.S.A.). 3-Isobutyl-1-methylxanthine (IBMX), BSA, Tes, adenosine deaminase, ATP, GTP and trichloroacetic acid were obtained from Sigma (St. Louis, MO, U.S.A.). 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*NNN′N′*-tetra-acetic acid acetoxymethyl ester (Fura-2/AM), Fura-2 pentapotassium salt and calcium–EGTA buffers were obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A). Methylamine (Gold Label) was from Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Other reagents used were of analytical grade.

Cell culture

Pathogen-free C57BI/6 mice (6 weeks old) were obtained from Charles River Laboratories (Raleigh, NC, U.S.A.). Thioglycollate-, casein- and proteose peptone-elicited macrophages were routinely obtained by peritoneal lavage with Hanks' balanced salt solution containing 10 mM Hepes, pH 7.4, and 3.5 mM $NaHCO_3$ (HHBSS). The cells were washed once with HHBSS, suspended in RPMI 1640 medium containing 2 mM glutamine, 12.5 units/ml penicillin, 6.25 μg/ml streptomycin and 5% fetal calf serum, and plated at a cell density of 1.0×10^6 cells/cm^2 glass coverslips kept in a 35 mm Petri dish. The macrophages were incubated for 2 h at 37 °C in a humidified CO_2 (5%) incubator. The cells were washed three times with HHBSS to remove non-adherent cells. The macrophages were then cultured in RPMI 1640 medium as above for 16–18 h.

Preparation of α_2M

Human α_2M was purified using chelate affinity chromatography as previously described (Imber and Pizzo, 1981). To obtain endotoxin-free material, the column matrix was washed with 8 M urea followed by extensive washing with deionized water. All buffers used to wash and elute the column were prepared with pyrogen-free water. The purity of the α_2M was demonstrated by SDS/PAGE (5–15% gels) in a glycine/2-amino-2-methyl-1,3-propanediol/HCl system (Bury, 1981). Methylamine derivatives were prepared as previously described (Imber and Pizzo, 1981; Enghild et al., 1989). The resultant reaction products were dialysed extensively against HHBSS at room temperature. The conversion to receptor-recognized forms was demonstrated to be complete by non-denaturing 4–20% pore limit gel electrophoresis in a Tris/EDTA/boric acid buffer system (Manwell, 1977). The concentrations of the ligands were determined spectrophotometrically at 280 nm using $A^{1\%}_{1cm} = 8.93$ for α_2M (Hall and Roberts, 1978).

Measurement of intracellular calcium levels

$[Ca^{2+}]_i$ in adherent macrophages was measured using the fluorescent indicator Fura-2/AM (Grynkiewicz et al., 1985). Macrophages incubated overnight in RPMI 1640 medium on glass coverslips were used. The Petri dish was removed from the incubator and cooled to room temperature. Fura-2/AM (1–1.5 μM) was added and the dish incubated at room temperature for 30 min in the dark. The monolayers of macrophages were then washed twice with low-calcium HHBSS (containing 75 μM Ca^{2+}). Glass coverslips bearing the macrophage monolayers in HHBSS were placed on the fluorescent microscope stage. $[Ca^{2+}]_i$ was measured by a digital video imaging technique employing a Carl Zeiss (Thornwood, NY, U.S.A.) model IM 35 microscope with a 100 × NA 1.4 UVF objective (Nikon, Garden City, NY, U.S.A.). After collecting baseline data, α_2M or α_2M–methylamine was added to the coverslip. Excitation light for fluorescence was provided by a 75 W xenon lamp. The temperature was maintained at 37 °C using an air curtain incubator. A digitized video image was obtained by averaging up

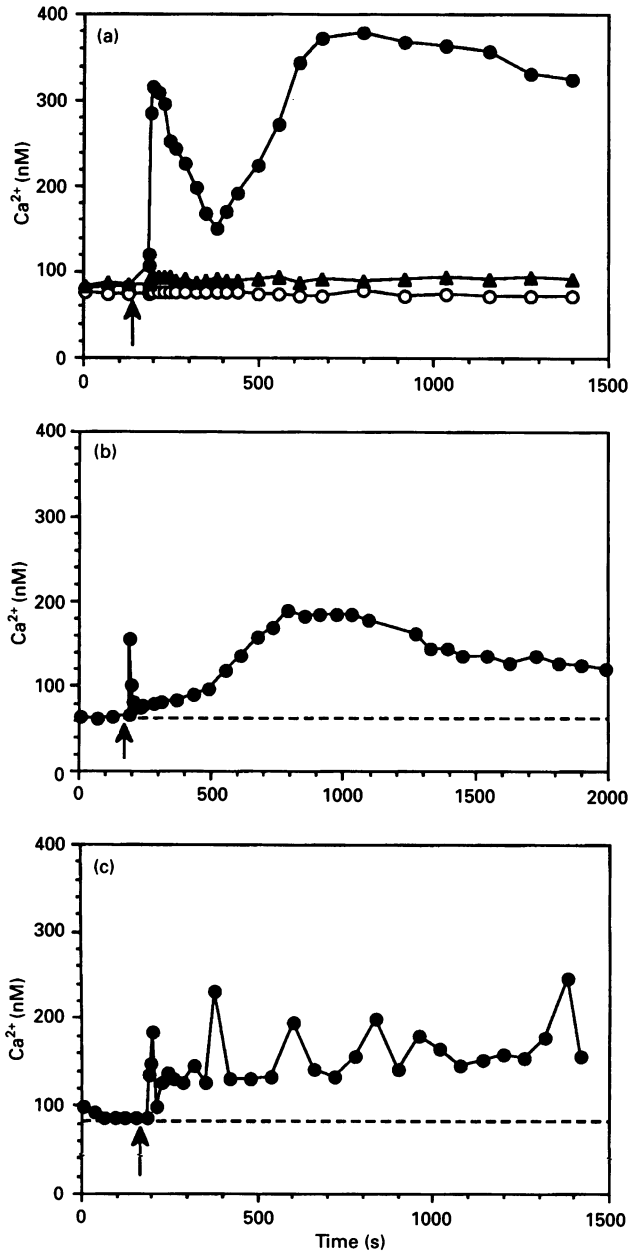

Figure 1 Heterogeneity in $[Ca^{2+}]_i$ responses by thioglycollate-elicited peritoneal macrophages exposed to α_2M–methylamine

Changes in $[Ca^{2+}]_i$ were monitored using the fluorescent Ca^{2+} indicator dye Fura-2 as described in the Experimental section. The arrows indicate the time at which α_2M–methylamine was added. The patterns shown in panels (**a**), (**b**) and (**c**) are representative of typical $[Ca^{2+}]_i$ responses elicited in macrophages upon exposure to α_2M preparations. (**a**) ●, α_2M–methylamine (114 nM); ○, native α_2M (300 nM); ▲, boiled α_2M–methylamine (230 nM). (**b**) and (**c**) ●, α_2M–methylamine (86.7 nM); ----, baseline $[Ca^{2+}]_i$.

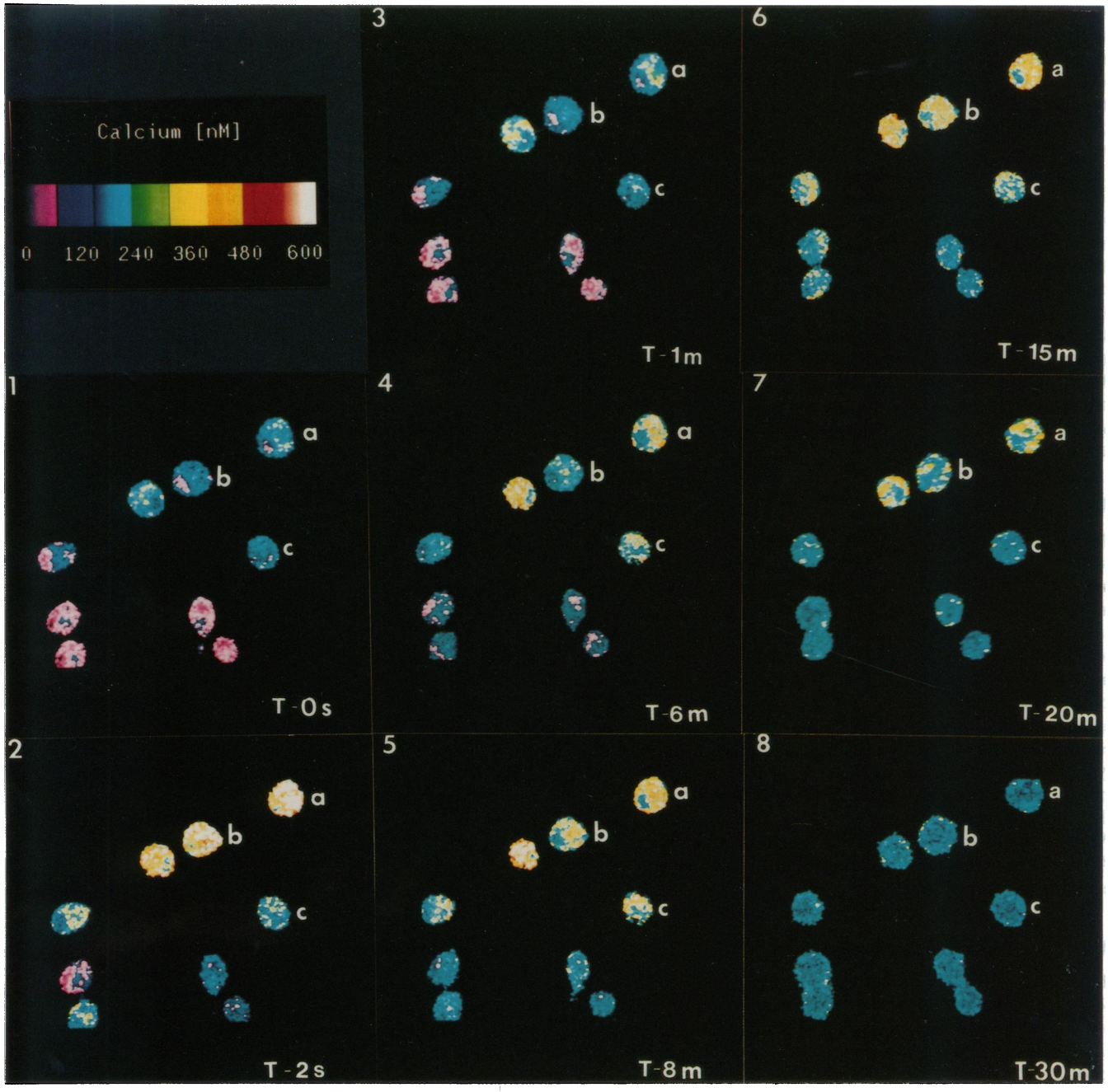

Figure 2 A representative photochart showing sequential changes of $[Ca^{2+}]_i$ with time ($t = 0$ s to 30 min) of individual macrophages exposed to α_2M–methylamine

Cells a, b and c show asynchronous increase in $[Ca^{2+}]_i$ and are analogous to panels (a), (b) and (c) of Figure 1. These cells exhibit sustained and transitory oscillations. The increase in $[Ca^{2+}]_i$ is focal and subsequently appears to propagate throughout the cell.

to 256 frames with the following filter combinations: Fura-2 excitation 340 and 380 nm; emission > 450 nm. Video frames were collected using an ISIT-66 camera (DAGE-MTI, Michigan City, IN, U.S.A.) and then computed with 16-bit precision using an IC 300-Workstation (Inovision Corp., Research Triangle Park, NC, U.S.A.). Routinely, excitation intensity was attenuated 100–1000-fold before reaching the cell and the background images were obtained. The $[Ca^{2+}]_i$ was measured by subtracting the background from images on a pixel basis. To obtain $[Ca^{2+}]_i$ for an individual cell, the mean value of the pixel ratio for the cell was compared with values obtained with the same equipment using Fura-2 containing EGTA/Ca^{2+} buffers (Grynkiewicz et al., 1985).

Quantification of inositol phosphates

Peritoneal macrophages were plated at 2×10^6 cells/4.5 cm^2 in inositol-free RPMI 1640 medium containing 5% fetal bovine serum, 2 mM glutamine, 125 units/ml penicillin and 6.25 μg/ml streptomycin and incubated for 2 h at 37 °C in a CO_2 (5%)-

humidified incubator. The non-adherent cells were removed by washing three times with HHBSS and inositol-free RPMI 1640 medium was added to monolayers. Cells were radiolabelled with 8 μCi/ml *myo*-[2-^{3}H]inositol for 16 h at 37 °C in a CO_2 (5%)-humidified incubator. Monolayers were washed five times with HHBSS containing 10 mM LiCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4. The cells were exposed to α_2M or α_2M–methylamine in 1 ml of the above medium for various periods of time at 37 °C in a CO_2 (5%)-humidified incubator. The reaction was terminated by aspirating the medium and adding 6.25% ice-cold perchloric acid. The cells were scraped off and transferred to tubes containing 5 mM EDTA and 1 ml of octylamine/Freon (1:1, v/v), and the tubes were centrifuged at 5600 *g* for 20 min. The upper phase was applied to a 1 ml packed Dowex resin column (AG1-X8, formate form; Bio-Rad, Richmond, CA, U.S.A.) and sequentially eluted in a batch fashion with water and then 50 mM, 200 mM, 400 mM, 800 mM, 1.2 M and 2.0 M ammonium formate containing 0.1 M formic acid (Berridge, 1983). An aliquot was used for determining radioactivity in a liquid scintillation counter.

cAMP measurements

Macrophages were isolated from mice treated with thioglycollate, washed and suspended in RPMI 1640 medium as described under 'Cell culture'. Macrophages were plated at 2×10^6/4.5 cm^2 or 16×10^6/28.2 cm^2. Macrophages cultured overnight were washed twice with HHBSS, preincubated in a volume of HHBSS for 5 min at 37 °C in 5% CO_2 and treated with either buffer or the α_2M forms for various periods of time and incubated as above. The reactions were stopped by addition of ice-cold methanol. The plates were kept on ice, while macrophages were removed by scraping and transferred to tubes for lyophilization. Lyophilized cells were resuspended in 1 ml of water, boiled for 5 min and centrifuged in a Microfuge at 12000 rev./min for 90 s. The supernatant was assessed for cAMP levels using Amersham (Arlington Heights, IL, U.S.A.) radioimmunoassay kits for cAMP. The pellet was used for quantification of protein by the method of Bradford (1976).

cAMP production in isolated membranes

Macrophages (1×10^7 cells) were cultured overnight and incubated under the conditions described for cAMP measurements. At the end of the incubation the plates were quickly placed on ice, the medium was removed and a volume of 10 mM Tes/0.25 M sucrose, pH 7.0, containing leupeptin (20 μg/ml) and phenylmethanesulphonyl fluoride (50 μg/ml) was added to each well. The cells were quickly scraped off, transferred to a tube and sonicated (Vibra Cell VC50; Sonics & Materials, Danbury, CT, U.S.A.) on ice (3×10 s bursts at the maximal setting with 30 s intervals). The crude membrane preparation was obtained by centrifuging the sonicate at 40000 *g* for 5 min at 4 °C. The supernatant was aspirated, and the crude membrane preparation was resuspended in 50 mM Tes, 12.5 mM $MgCl_2$ and adenosine deaminase (2 units/ml), pH 7.4, incubated for 10 min at 37 °C and centrifuged at 40000 *g* for 20 min at 4 °C. The pellet was suspended in a volume of the above buffer and assayed for cAMP, a measure of adenylate cyclase activity, at 37 °C in 50 mM Tes buffer, pH 7.4, containing 4 mM $MgCl_2$, 1 mM ATP and 1 μM GTP as described by Okonogi et al. (1991). The reaction was stopped with 50 μl of 25% ice-cold trichloroacetic acid followed by the addition of 650 μl of 50 mM sodium phosphate buffer, pH 7.4. The tubes were centrifuged in a Microfuge for 2 min at 12000 rev./min and the supernatant was removed for cAMP assay as described above. Membranes were assayed for protein content (Bradford, 1976).

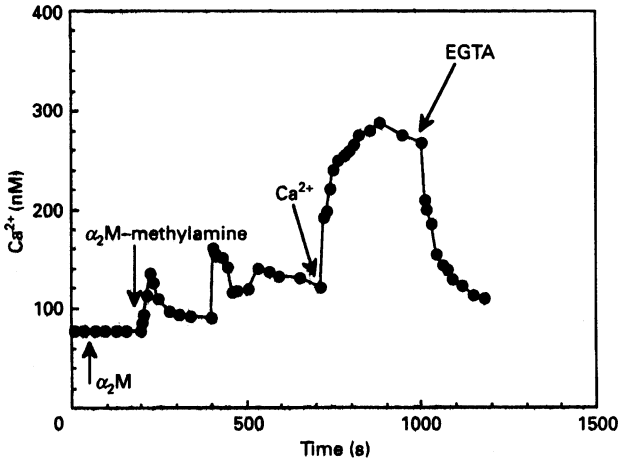

Figure 3 Contribution of intracellular and extracellular Ca^{2+} to the macrophage $[Ca^{2+}]_i$ response

A representative response as shown of $[Ca^{2+}]_i$ in Fura-2-loaded macrophages ($n = 18$) exposed to α_2M–methylamine (114 nM), extracellular Ca^{2+} (1 mM) and EGTA (2 mM) included in the incubation medium at these final concentrations. Additions were made as indicated by the arrows.

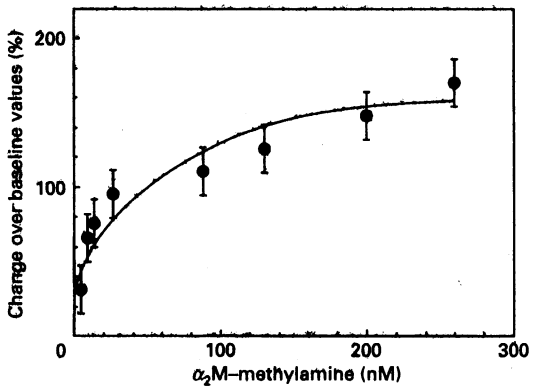

Figure 4 Dose–response of $[Ca^{2+}]_i$ in macrophages

Various amount of α_2M–methylamine (4–250 nM) were added to Fura-2-loaded macrophages. Each point represents the mean ± S.E.M. from three to six separate experiments involving 25–40 cells in each experiment. Increases in $[Ca^{2+}]_i$ were quantified using digital fluorescence microscopy as outlined in the Experimental section. For each experiment, the increase in $[Ca^{2+}]_i$ was determined at 2 min.

RESULTS

α_2M–methylamine-induced $[Ca^{2+}]_i$ increases in single macrophages

Digital imaging fluorescence microscopy was employed to study changes in $[Ca^{2+}]_i$ at the single-cell level in monolayers of Fura-2-loaded peritoneal macrophages, which were elicited in mice by intraperitoneal injections of thioglycollate, casein or proteose peptone. Representative results from thioglycollate-elicited macrophages are shown in Figures 1–4. Before examining the effect of α_2M–methylamine treatment, several control experiments were performed. Native α_2M does not bind to the macrophage receptor (Pizzo, 1988). Exposure of macrophages ($n = 45$) to concentrations of native α_2M from 4 to 250 nM caused

little or no change in $[Ca^{2+}]_i$ (Figure 1). Endotoxins, which may contaminate protein preparations, can significantly affect $[Ca^{2+}]_i$ (Prpic et al., 1987; Letari et al., 1991). While every care was taken to prevent endotoxin contamination, studies were performed to control for this potential problem. Native α_2M and α_2M–methylamine preparations which were boiled for 3 min had no effect on the fluorescence ratios in peritoneal macrophages (Figure 1). Since endotoxins are stable to this treatment and α_2M is not, it was concluded that the preparations were endotoxin-free.

We next studied the effects of α_2M–methylamine on $[Ca^{2+}]_i$ in macrophages. Of 165 cells examined, 140 (85%) demonstrated an increase in $[Ca^{2+}]_i$ (Figures 1 and 2). Changes in $[Ca^{2+}]_i$ followed one of several well-described patterns which have been observed with other receptor-mediated signal transduction responses (for reviews, see Berridge and Irvine, 1989; Berridge, 1990; Putney, 1990; Meyer and Stryer, 1991; Irvine, 1992). Changes in $[Ca^{2+}]_i$ in individual cells generally consist of sinusoidal oscillations and/or spikes (Woods et al., 1986, 1987; Monck et al., 1988; Prentki et al., 1988; Hallam et al., 1989; Kawanishi et al., 1989; Muallem et al., 1989; Rooney et al., 1989, 1990; Hajjar and Bonventre, 1991; Hansen et al., 1991). The most common pattern of response ($n = 45$) is shown in Figure 1(a). Upon binding α_2M–methylamine, these cells demonstrated a large increase in $[Ca^{2+}]_i$ from basal levels of about 80 nM to as much as 320 nM within 1 min of exposure to ligand. Following a return to near-baseline levels, a sustained increase to levels near 400 nM was observed for up to 15 min.

The next most common pattern ($n = 40$) is shown in Figure 1(b). These cells showed a very rapid (5 s) increase in $[Ca^{2+}]_i$ as a sharp spike up to a level of 160 nM. This returned to basal levels before gradually increasing to a level of about 200 nM and then again declining to baseline after about 15 min. In an additional 30 cells, the spike at 5 s was absent but the more sustained response reached similar levels before return to baseline in about 15 min (results not shown). In 25 cells, binding of α_2M–methylamine resulted in transient increases in $[Ca^{2+}]_i$ of moderate amplitude and irregular frequency for a period of about 15 min (Figure 1c). Colour photographs taken from the screen of the instrument are shown in Figure 2. Single cells responding in these respective patterns, marked a, b and c, can be observed. The increase of $[Ca^{2+}]_i$ is asynchronous, originating from a single loci and spreading to other areas of the cell. Casein- or peptone proteose-elicited macrophages responded in similar ways when treated with α_2M–methylamine (results not shown). These studies were performed in medium containing 75 μM Ca^{2+}, essential for the binding of α_2M–methylamine to its receptor (Imber and Pizzo, 1981).

In order to determine whether extracellular Ca^{2+} contributes to the α_2M–methylamine-induced increase in $[Ca^{2+}]_i$, the studies shown in Figure 3 were performed. After stimulating macrophages with α_2M–methylamine, the extracellular $[Ca^{2+}]$ in the medium was increased by addition of Ca^{2+} to achieve a final concentration of 1 mM. This resulted in a rapid increase in $[Ca^{2+}]_i$ which could be rapidly abolished by addition of EGTA (2 mM). By contrast, addition of the same concentration of Ca^{2+} to the medium in the absence of α_2M–methylamine resulted in a less than 10% increase in $[Ca^{2+}]_i$ (results not shown). These studies suggest that increases in $[Ca^{2+}]_i$ in response to α_2M–methylamine may involve external sources as well as intracellular stores of Ca^{2+}. However, the data obtained in these studies (Figures 1 and 3) indicate that the maximal $[Ca^{2+}]_i$ response is very similar whether the external $[Ca^{2+}]$ is 75 μM or 1 mM. Subsequent studies were, therefore, performed with an external $[Ca^{2+}]$ of 75 μM.

The response of thioglycollate-elicited macrophages to α_2M–methylamine was dose-dependent, as shown in Figure 4. The EC_{50} calculated from this Figure is 15 nM, which is approximately equivalent to the K_d of 10 nM for α_2M–methylamine binding to the receptor at 37 °C (Pizzo, 1988).

Effect of α_2M and α_2M–methylamine on cellular inositol phosphates

Thioglycollate-elicited macrophages were exposed to α_2M or α_2M–methylamine for various lengths of time. As shown in Figure 5 and Table 1, a significant rise in inositol 1,4,5-trisphosphate (IP_3) was observed in α_2M–methylamine-exposed cells within 5 s of treatment. Levels returned to baseline within about 1 min. A significant increase also occurred in levels of inositol 1,3,4,5-tetrakisphosphate (IP_4) over the same time interval (Table 1). By contrast, macrophages exposed to native α_2M showed no rise in IP_3, and an actual reduction compared

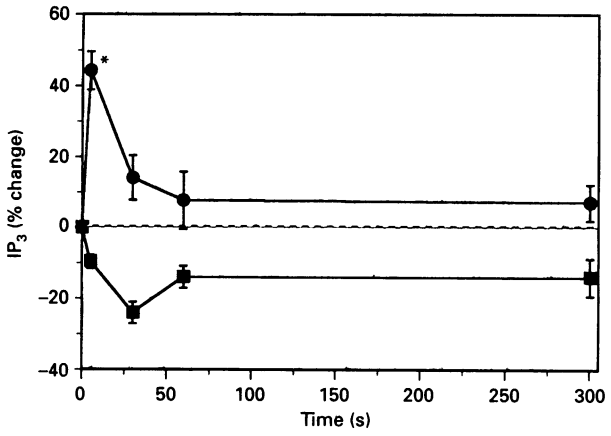

Figure 5 IP_3 formation in macrophages exposed to α_2M–methylamine

Macrophages were exposed to native α_2M (160 nM) (■) or α_2M–methylamine (155 nM) (●) and incubated for various period of time. The values are the means ± S.E.M. for three experiments run in duplicate. The value which is significantly different ($P < 0.05$) from buffer-exposed macrophages is denoted by *. A one-tailed Student's t test was used to analyse the data.

Table 1 Inositol phosphates in macrophages exposed to α_2M and α_2M–methylamine

Macrophages were incubated with α_2M (160 nM) or α_2M–methylamine (155 nM) for 5 s. Details are given in the Experimental section. The values reported are the means ± S.E.M. from three separate experiments in duplicate. * Significantly different from their respective buffer controls ($P < .0.05$ by Student's t test). The values for the buffer control were 572, 332, 281, 291 and 214 c.p.m. for IP_1, IP_2, IP_3, IP_4 and > IP_4 respectively.

	Inositol phosphates (c.p.m./1.5 × 10^6 cells)	
Inositol phosphates	α_2M	α_2M-Methylamine
IP_1	887.3 ± 104.8	1367.3 ± 94.6
IP_2	347.0 ± 33.1	452.3 ± 53.0
IP_3	267.7 ± 5.8	370.7 ± 8.2*
IP_4	221.7 ± 8.0	377.7 ± 46.5*
> IP_4†	220.3 ± 17.4	271.3 ± 0.3

† Denotes isomers containing five or more phosphate groups.

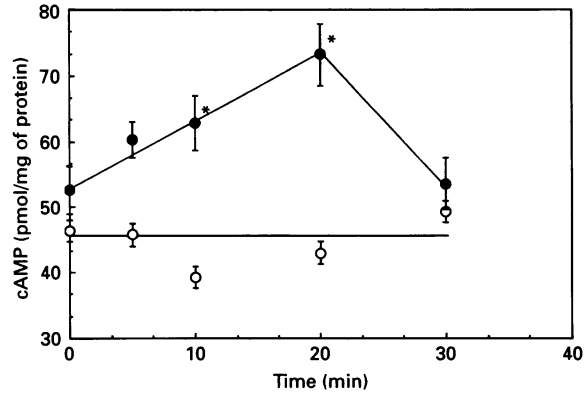

Figure 6 Stimulation of cAMP formation in macrophages exposed to α_2M–methylamine

Macrophages were exposed to native α_2M (○) or α_2M–methylamine (●) and incubated for various periods of time. cAMP was isolated and quantified using a radioimmunoassay. Values are means ± S.E.M. from five to seven experiments run in duplicate. Values which are significantly different ($P < 0.05$) from native α_2M-exposed macrophages are denoted by *. A one-tailed Student's t test was used to analyse the data.

Table 2 Generation of cAMP in crude membrane preparations

Macrophages were incubated with α_2M–methylamine (137 nM) at 37 °C for 10 min, and the membranes were isolated. The *de novo* generation of cAMP by these membrane preparations (20–40 μg of protein/assay) was assayed, reflecting the combined activities of adenylate cyclase and of phosphodiesterase. IBMX, an inhibitor of phosphodiesterase activity, was added to a final concentration of 100 μM. Values are the means ± S.E.M. from three different experiments with duplicate samples. * Significantly different from their respective controls ($P < 0.05$ by Student's t test).

Additions	cAMP (pmol/mg of protein)
Buffer only	56.91 ± 2.41
IBMX + buffer	61.01 ± 1.73
α_2M	57.10 ± 1.26
IBMX + α_2M	60.77 ± 1.98
α_2M–methylamine	70.09 ± 2.40*
IBMX + α_2M–methylamine	83.72 ± 0.91*

with a buffer control was noted between 5 and 60 s (Figure 5; Table 1). Similarly, exposure of macrophages to α_2M did not cause an increase in IP_4 (Table 1).

Effect of α_2M and α_2M–methylamine on cellular cAMP

Thioglycollate-elicited macrophages were exposed to different concentrations of native α_2M or α_2M–methylamine. Native α_2M had little effect on basal levels of cAMP (Figure 6). However, exposure of the cells to α_2M–methylamine caused a significant rise in cAMP for periods of up to 20 min. At any one time, the cellular cAMP pool is determined by the balance between synthesis and degradation. These two processes were evaluated by estimating the activities of adenylate cyclase in the presence or absence of the phosphodiesterase inhibitor IBMX. The production of cAMP by crude membrane preparations (pmol/mg of protein) was higher in macrophages stimulated with α_2M–methylamine than in those stimulated with either buffer or native α_2M for up to 10 min; however, at later times, the response to α_2M–methylamine declined to the baseline. In the presence of the phosphodiesterase inhibitor IBMX, the cAMP pool increased by about 25% (Table 2).

DISCUSSION

The receptor for α_2M fast forms is a large, multifunctional transmembrane complex which binds apolipoprotein E-containing lipoproteins in addition to a variety of α-macroglobulins from various species (Pizzo, 1988). The binding of all receptor-recognized α-macroglobulins occurs with very high affinity (K_d at 4 °C of ~ 1 nM). The modulating effects of human α_2M fast forms on macrophage functions (Johnson et al., 1982; Forrester et al., 1983; Hoffman et al., 1983; Pizzo, 1988; Roche et al., 1990; Uhing et al., 1991) suggest the possibility of second messenger involvement. Using human α_2M–methylamine, we have examined the $[Ca^{2+}]_i$ response to receptor occupancy at the single-cell level using digital imaging fluorescence microscopy. We also examined the effects of receptor ligation on levels of inositol phosphates and cAMP and on adenylate cyclase and phosphodiesterase activities.

In the present study, the following novel observations were made: (i) the binding of α_2M–methylamine to peritoneal macrophages caused a rapid increase of $[Ca^{2+}]_i$ which exhibits both sinusoidal oscillations and transitory spikes; (ii) the magnitude of the Ca^{2+} response is dependent on agonist concentration; (iii) extracellular Ca^{2+} may contribute to the increase in $[Ca^{2+}]_i$; (iv) binding of α_2M–methylamine to macrophages causes a rapid, significant increase in IP_3 and IP_4; and (v) binding of α_2M–methylamine to the receptor causes a transient rise in cAMP.

The regulation of calcium homeostasis is of crucial importance to the maintenance of cellular functions. The increase of $[Ca^{2+}]_i$ in response to the interaction of an agonist with its membrane receptor is one of the most common second messenger responses to signal transduction events (Berridge, 1987; Berridge and Irvine, 1989; Meyer and Stryer, 1991). Ca^{2+} flux measurements in a variety of cells have shown that the initial rapid increase in $[Ca^{2+}]_i$ is largely due to release of Ca^{2+} from intracellular stores (Berridge, 1987, 1990; Berridge and Irvine, 1989; Meyer and Stryer, 1991; Hansen et al., 1991). The initial release of Ca^{2+} occurs on binding of IP_3 to its receptors on membrane-localized internal Ca^{2+} storage pools. Since IP_3 receptors do not exhibit desensitization, a decline in the release of Ca^{2+} from these stores is dependent on the rapid metabolism of IP_3 (Berridge and Irvine, 1989). The rapid and transient rise in IP_3 elicited by α_2M–methylamine probably regulates macrophage $[Ca^{2+}]_i$ in this manner.

Subsequently, Ca^{2+} may also enter the cells from the extracellular milieu (Penner et al., 1988; Hallam et al., 1989; Berridge, 1990; Putney, 1990; Meldolesi et al., 1991; Sage, 1992). Using α_2M–methylamine as an agonist, we also observed entry of extracellular Ca^{2+} into macrophages. However, the mobilization of intracellular Ca^{2+} is probably the first mechanism to be activated, since the various oscillatory patterns of $[Ca^{2+}]_i$ increase were observed with α_2M–methylamine bound to macrophages in tissue culture medium which contained a low $[Ca^{2+}]$. The various oscillatory patterns in $[Ca^{2+}]_i$ seen when α_2M–methylamine binds to macrophages are typical of the responses of many cells to agonists (Woods et al., 1986, 1987; Monck et al., 1988; Kawanishi et al., 1989; Rooney et al., 1989, 1990; Sage et al., 1989; Berridge, 1990; Hajjar and Bonventre, 1991; Hansen et al., 1991).

Binding of α_2M–methylamine to thioglycollate-elicited macrophages also caused a transient rise in cellular cAMP levels. The rise and fall of cAMP in macrophages exposed to α_2M–methylamine may result from several mechanisms. One possible explanation is that α_2M–methylamine binding to macrophages activates not only adenylate cyclase but also phosphodiesterase. Alternatively, the transient rise in cAMP in α_2M–methylamine-stimulated macrophages could be due to attenuation of the receptor-mediated stimulation of adenylate cyclase through phosphorylation of specific protein components of the receptor machinery (Hansdorff et al., 1990). By either mechanism, exposure of macrophages to α_2M–methylamine would result in a tight regulation of the cAMP pool so that cAMP-mediated events would occur only over a reasonably limited period of time.

The results of the present study may aid in clarifying the effects of α_2M receptor-recognized forms on macrophage immune function. BInding of α_2M–methylamine to peritoneal macrophages was originally reported to counteract the increased expression of Ia antigen induced by interferon-γ (Hoffman et al., 1987). More recently, Roche et al. (1990) have shown that α_2M–methylamine affected neither the average number of Ia molecules expressed per cell nor biosynthesis of Ia. However, the morphology of cells pretreated with α_2M–methylamine and then exposed to interferon-γ was drastically different from that of cells treated with interferon-γ alone (Roche et al., 1990). The original assay used for Ia expression on macrophages depended on visual observation of macrophages exposed to a fluorescenated antibody against Ia. Rounding up of cells may affect scoring as either Ia-positive or -negative without any change in Ia expression. The observation that α_2M–methylamine increases cAMP, yet does not affect Ia expression, is interesting in view of previous studies showing that increases in cellular cAMP suppress the Ia response (Prpic et al., 1987). However, in that study, cAMP levels were elevated for hours after exposure to agents which suppress Ia expression. In the present study, α_2M–methylamine binding to macrophages produced only a brief increase in cAMP. These studies suggest that there is a relationship between the period of time that cAMP is elevated following macrophage response to a ligand and the regulation of Ia expression.

In summary, binding of human α_2M–methylamine to macrophages rapidly modulates $[Ca^{2+}]_i$. Furthermore, the binding of α_2M–methylamine also affects levels of inositol phosphates and cAMP. Thus the binding of receptor-recognized α_2M to its cell-surface receptor rapidly activates signalling cascades which may modulate macrophage function.

This work was supported by the National Heart, Lung and Blood Institute (grant HL-24066) and the National Cancer Institute (grant CA-29589).

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Received 3 September 1992/2 November 1992; accepted 25 November 1992