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Binding of Rat α_1-Inhibitor-3-Methylamine to the α_2-Macroglobulin Signaling Receptor Induces Second Messengers

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Abstract Binding of receptor-recognized forms of tetrameric human α_2-macroglobulin (α_2M*) to a macrophage signaling receptor induces cAMP synthesis, increases in inositol 1,4,5-triphosphate (IP_3) synthesis, and a concomitant rise in cytosolic free calcium ($[Ca^{2+}]_i$). The α_2M* signaling receptor is coupled to a pertussis-toxin insensitive G protein. Binding of α_2M* also occurs to the low density lipoprotein receptor-related protein/α_2M receptor (LRP/α_2MR), but this binding does not induce signal transduction. Rat α_1-inhibitor-3 (α_1I_3) is a monomeric member of the α-macroglobulin/complement superfamily. Like α_2M, it can react with proteinases or methylamine which induces a conformational change causing activated α_1I_3 to bind to LRP/α_2MR. We now report that α_1I_3-methylamine binds to the macrophage α_2M* signaling receptor inducing a rapid rise in the synthesis of IP_3 with a subsequent 1.5- to 3-fold rise in $[Ca^{2+}]_i$. α_1I_3-methylamine binding to macrophages also caused a statistically significant elevation in cAMP. Native α_1I_3, like α_2M, was unable to induce signal transduction. α_1I_3 forms a complex with α_1-microglobulin, which has a distinct conformation from α_1I_3 and is recognized by LRP/α_2MR. This complex also induces an increase in $[Ca^{2+}]_i$ comparable to the effect of α_1I_3-methylamine on macrophages. It is concluded that activation of α_1I_3 by methylamine or binding of α_1-microglobulin causes similar conformational changes in the inhibitor, exposing the receptor recognition site for the α_2M* signaling receptor, as well as for LRP/α_2MR.

Key words: α_2M*, cAMP synthesis, IP_3 synthesis, α_1I_3, conformational changes

The α-macroglobulin/complement superfamily includes complement components and proteinase inhibitors. Both complement components, such as C3 and C4, and most proteinase inhibitory α-macroglobulins, such as human α_2-macroglobulin (α_2M)[1], contain β-cysteinyl-γ-glutamyl thioesters, which become highly reactive after proteinase attack on the α-macroglobulin subunits [Swenson and Howard, 1979; Sottrup-Jensen et al., 1980; Howard 1981; see also Sottrup-Jensen, 1987; Chu and Pizzo, 1994; Salvesen and Pizzo, 1994, for review]. Reaction of human α_2M with proteinases or methylamine, the latter of which directly attacks the thioesters [Swenson and Howard, 1979; Barrett et al., 1979; Sottrup-Jensen 1980; Howard 1981], induces a conformational change in the inhibitor [Barrett et al., 1979; Gonias et al., 1982], exposing receptor recognition sites present in each subunit [Debanne et al., 1975, 1976; Van Leuven et al., 1978, 1986; Kaplan and Nielsen, 1979a,b; Willingham et al., 1980; Dickson et al., 1981; Imber and Pizzo, 1981; Maxfield et al., 1981; Kaplan et al., 1981]. These so-called activated forms of α_2M (α_2M*) bind to two types of receptors on macrophages; namely, low density lipoprotein-related protein/α_2M receptors (LRP/α_2MR) and a more recently described α_2M*

Abbreviations used: α_1I_3, α_1-inhibitor-3; α_1-m, α_1-microglobulin; α_2M, α_2-macroglobulin; α_2M*, receptor-recognized forms of α_2M; cAMP, cyclic adenosine monophosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; DAG, diacyl glycerol; Fura-2 AM, 1-[2(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl-2-(2′amino-5′-methylphenox y)-ethane,N,N,N′,N′-tetracetic acid acetoxymethyl ester; GPI, glycerol phosphoryl inositol; GTPγS, guanosine 5′-3-0-(thio)triphosphate; GDPβS guanyl 5′yl thiophosphate; Gpp(NH)p, guanosine 5′-(β-C-imido) triphosphate; IP_3, inositol 1,4,5-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperozine-ethanesulfonic acid; LRP/α_2MR, low-density lipoprotein receptor-related protein/α_2M receptors; α_2M*SR, the α_2M* signaling receptor; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; PDBu, phorbol dibutyrate; PKC, proteinase kinase C; RBF, the cloned and expressed receptor recognition fragment from rat α_1M; SEM, standard error of the mean.

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signaling receptor (α_2M*SR). LRP/α_2MR is a scavenger receptor that binds diverse ligands including α_2M*, activated α_1I_3, *Pseudomonas* exotoxin A, lactoferrin, lipoprotein lipase, tissue factor pathway inhibitor, thrombospondin or plasminogen activators alone or in complex with their inhibitors [Van Leuven et al., 1986; Beisiegel et al., 1989; Kowal et al., 1989; Moestrup and Gliemann, 1989; Ashcom et al., 1990; Kristensen et al., 1990; Strickland et al., 1990; Bu et al., 1992; Chappell et al., 1992; Herz et al., 1992; Kounnas et al., 1992a,b; Nykjær et al., 1992; Orth et al., 1992; Williams et al., 1992; Willnow et al., 1992; Chappell et al., 1993; Kounnas et al., 1993a,b; Moestrup et al., 1993; Nykjaer et al., 1993; Warshawsky et al., 1994; Mikhailenko et al., 1995; see also Krieger and Herz, 1994, for review]. By contrast, α_2M*SR appears to be highly selective. It has been shown to bind human α_2M* or a cloned and expressed receptor recognition fragment from the homologous proteinase inhibitor rat α_1M [Salvesen et al., 1992], but none of the other known ligands for LRP/α_2MR [Misra et al., 1994a,b]. Ligation of α_2M*SR causes a very rapid increase in macrophage IP_3 synthesis followed by an increase in $[Ca^{2+}]_i$ [Misra et al., 1993, 1994a,b]. Ligation of α_2M*SR also increases cellular levels of cAMP [Misra et al., 1993].

Proteinase inhibitory α-macroglobulins are generally dimeric or tetrameric, while complement components are monomeric [Hall and Roberts, 1978; Feldman and Pizzo, 1984, 1985, 1986; Quigley and Armstrong, 1985; Armstrong and Quigley, 1987; Hudson et al., 1987; Spycher et al., 1987; Carlsson-Bostedt et al., 1988; Hergenhan et al., 1988; Gettins and Sottrup-Jensen, 1990; Enghild et al., 1990; see also Chu and Pizzo, 1994, for review]. Thus, human α_2M and rat α_1M are tetrameric proteinase inhibitors. When these inhibitors bind to a proteinase, the enzyme becomes physically entrapped in a cage-like structure that constitutes the inhibitor [Feldman et al., 1985; Delain et al., 1992]. The proteinases within such traps remain active against small substrates but are sterically hindered from access to large, macromolecular substrates. Several unusual monomeric members of the α-macroglobulin family have been described that behave like proteinase inhibitors not complement components [Saito and Sinohara, 1985; Suzuki and Sinohara, 1986; Lonberg-Holm et al., 1987; Rubenstein et al., 1993]. These inhibitors are able to block the active site of the proteinase by some form of steric hindrance despite their inability to form a cage around the proteinase [Enghild et al., 1989; Rubenstein et al., 1993].

A domain structure has been proposed for both the human α_2M subunits [Thomsen and Sottrup-Jensen, 1993] and α_1I_3 [Rubenstein et al., 1991]. These structures are fairly similar, except in one crucial respect; namely, the C-terminal receptor recognition domain of human α_2M consists of a 20-kDa polypeptide chain [Thomsen and Sottrup-Jensen, 1993], while this same 20-kDa region appears to be a part of a larger 40-kDa domain in α_1I_3 [Rubenstein et al., 1991]. This suggests potentially significant differences in folding of this region of the molecule. Nevertheless, most forms of proteinase inhibitory α-macroglobulins, including α_1I_3, are able to bind to LRP/α_2MR when activated by proteinase or methylamine [Enghild et al., 1989; Strickland et al., 1990; Kristensen et al., 1990; Moestrup and Gliemann, 1991; Nimpf et al., 1994; see also Moestrup, 1994, for review]. Rat α_1I_3 is also peculiar in that it covalently binds the immunoregulatory protein α_1-microglobulin (α_1-m) [Falkenberg et al., 1990], blocking the proteinase inhibitory activity of α_1I_3 and exposing receptor recognition sites for LRP/α_2MR [Falkenberg et al., 1995]. A monomeric α-macroglobulin is also present in the plasma of the American bullfrog [Rubenstein et al., 1993]. This protein completely lacks the ability to bind to cellular receptors whether or not it has been reacted with proteinases or methylamine [Rubenstein et al., 1993]. Whether this lack of cellular binding is a result of the sequence divergence or the domain structure of this protein is unknown; however, a tetrameric α-macroglobulin from the frog binds to cellular receptors with the same affinity as human α_2M* [Feldman and Pizzo, 1985].

Because of these unusual properties of α_1I_3, we investigated the ability of α_1I_3-methylamine and α_1I_3 in complex with α_1-m to induce signal transduction in macrophages. These studies demonstrate that either of these forms, but not native α_1I_3, induce signal transduction.

MATERIALS AND METHODS

Reagents

Sterile distilled water was obtained from Abbott Laboratories (Chicago, IL). Brewer's thioglycollate broth and proteose-peptone were purchased from Difco Laboratories (Baltimore, MD).

Casein was purchased from EM Chemicals (Elmsford, NY). Culture media were purchased from Cellgro (Herndon, VA) and Gibco Laboratories (Grand Island, NY). Bovine serum albumin (BSA), adenosine deaminase, ATP, and trichloroacetic acid were obtained from Sigma Chemicals (St. Louis, MO). Fura-2 AM, Fura-2 pentapotassium salt, and calcium-EGTA buffers were obtained from Molecular Probes (Eugene, OR). Methylamine (Gold Label) was from Aldrich Chemical (St. Louis). [^{3}H]PDBu (10–20 Ci/mmole) was purchased from NEN (Wilmington, DE) [^{32}P]-γ-ATP (6,000 Ci/mmole) was from Amersham (Arlington Heights, IL), and [^{33}P]-γ-ATP (2,000 Ci/mmole) was from NEN. 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). Thioglycollate-, casein-, and proteose-peptone-elicited macrophages were routinely obtained by peritoneal lavage with Hank's 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 U/ml penicillin, and 6.25 μg/ml streptomycin, and 5% fetal calf serum, and plated at a cell density of 1.0×10^6 cells/cm^2 on 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 nonadherent cells. The macrophages were then cultured in RPMI 1640 medium with the additions listed above for 16–18 h.

Preparation of α_1I_3

Rat α_1I_3 was purified using chelate affinity chromatography as previously described [Enghild et al., 1989a]. To obtain endotoxin-free material, the column matrix was washed with 8M 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_1I_3 was demonstrated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (5–15% gels) in a glycine/2-amino-2-methyl-1,3-propandiol/HCl system [Enghild et al., 1989a,b]. Methylamine derivatives were prepared as previously described [Enghild et al., 1989a]. The resultant reaction products were dialyzed extensively against HHBSS at room temperature. The conversion to receptor-recognized forms was demonstrated to be complete by nondenaturing 4–20% pore limit gel electrophoresis in a Tris/EDTA/boric acid buffer system [Enghild et al., 1989a,b]. $\alpha_1I_3 \cdot \alpha_1$-m complexes were the kind gift of Dr. Bo Åkerström, (University of Lund, Lund, Sweden). α_1I_3 forms a 1:1 covalent complex with α_1-m of $M_r \sim 220{,}000$.

Measurement of Intracellular Calcium Levels

$[Ca^{2+}]_i$ in adherent macrophages was measured using the fluorescent indicator Fura-2 AM as previously described [Misra et al., 1993]. 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 HHBSS containing 75 μM Ca^{2+}. Glass coverslips bearing the macrophage monolayers in this buffer were placed on the fluorescent microscope stage. $[Ca^{2+}]_i$ was measured by a digital video imaging technique employing a Carl Zeiss (Thornwood, NY) model IM 35 microscope with a 100× NA 1.4 UVF objective (Nikon, Garden City, NY). After collecting baseline data, the α_1I_3, α_1I_3-methylamine or $\alpha_1I_3 \cdot \alpha_1$-m 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 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, Inc., Michigan City, IN) and then computed with 16-bit precision, using an IC 300-Workstation (Inovision, Research Triangle Park, NC). Routinely, excitation intensity was attenuated 100- to 1,000-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 to values obtained with the same equipment using Fura-2 containing EGTA–Ca^{2+} buffers.

Quantification of Inositol Phosphates

Peritoneal macrophages were plated at 1.5–2 × 10^6 cells/4.5 cm^2 in inositol-free RPMI 1640

medium containing 5% fetal bovine serum (FBS), 2 mM glutamine, 125 U/ml penicillin and 6.25 μg/ml streptomycin and incubated for 2 h at 37°C in a CO_2 (5%) humidified incubator. The nonadherent cells were removed by washing three times with HHBSS and inositol-free RPMI 1640 medium added to monolayers. Cells were radiolabeled with 8 μCi/ml of 2-[^{3}H]myo-inositol for 16 h at 37°C in CO_2 (5%)-humidified incubator. Monolayers were washed five times with HHBSS containing 10 mM $LiCl_2$, 1 mM $MgCl_2$, pH 7.4. The cells were exposed to a α_1I_3 or α_1I_3-methylamine in 1 ml of the above medium for varying 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 and transferred to tubes containing 5 mM EDTA and 1 ml of octylamine:freon (1:1, v/v) and the tubes centrifuged at 2,800 rpm for 20 min. The upper phase was applied to a 1-ml packed Dowex resin column (AG1-X8, formate form, BioRad, Richmond, CA) and sequentially eluted in a batch fashion with H_2O, 50 mM, 200 mM, 400 mM, 800 mM, 1.2 M, and 2.0 M ammonium formate containing 0.1 M formic acid, respectively, as previously described [Misra et al., 1993]. An aliquot was used for determining radioactivity in a liquid scintillation counter.

Effect of GTPγS and Gpp(NH)p on IP_3 in Macrophages Stimulated with α_1I_3-Methylamine

Thioglycollate-elicited macrophages (2×10^6 cells/4.5 cm^2) were radiolabeled with 2-[^{3}H]myo-inositol and, after overnight incubation, the monolayers were washed three times with HHBSS containing 10 mM LiCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The cells were permeabilized with saponin (20 μg/ml) for 10 min at 30°C in 1 ml of buffer containing 110 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 3 mM Na_2ATP, 8 mM creatine phosphate, 6 U/ml creatine phosphokinase 20 mM Hepes, 10 mM LiCl, 4 mM $MgCl_2$, 1 mM EGTA, and 0.317 mM $CaCl_2$, pH 7.0 (final concentrations of Ca^{2+} and Mg^{2+} 0.1 mM and 1.25 mM, respectively). The cells were washed four times with HHBSS containing 10 mM LiCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The washed monolayers were incubated with GTPγS (20 μM) or Gpp(NH)p(100 μM) for 10 min at 37°C before adding buffer or α_1I_3-methylamine. IP_3 was quantified as described above.

Effect of GDPβS on GTPγS-Stimulated IP_3 Formation in Macrophages Exposed to α_1I_3-Methylamine

Radiolabeled, permeabilized thioglycollate-elicited macrophages were prepared as described above. To each well, 0.5 ml of HHBSS buffer containing 10 mM LiCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ was added followed by 500 μM of GDPβS. The cells were incubated at 37°C as above for 15 min followed by the addition of GTPγS (20 μM). The cells were incubated at 37°C for 10 min before adding α_1I_3-methylamine. Other details of incubation and quantification of IP_3 are described above. The effect of pertussis toxin on IP_3 synthesis in thioglycollate-induced macrophages exposed to α_1I_3-methylamine was also assessed as previously described [Misra et al., 1994a,b].

cAMP Measurements

Macrophages were isolated from mice treated with thioglycollate, washed, and suspended 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 1 vol of HHBSS for 5 min at 37°C in 5% CO_2 and treated with either buffer or the α_1I_3 forms in the presence of 75 μM Ca^{2+} for varying periods of time and incubated as above. The reactions were stopped with 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 water, boiled for 5 min and centrifuged in a microcentrifuge at 12,000 rpm for 90 s as previously described [Misra et al., 1993]. The supernatant was assessed for cAMP levels using Amersham (Arlington Heights, IL) RIA kits for cAMP. The pellet was used for quantitation of protein by Bradford's method [1976].

PKC Measurements

Thioglycollate-elicited murine peritoneal macrophages were plated at a density of 6×10^6 cells in 35-mm Petri dishes containing RPMI 1640 medium as described under Cell Culture. After 16–18 h, the medium was aspirated and the cells washed three times in HHBSS. Native α_1I_3, α_1I_3-methylamine or buffer each containing 75 μM Ca^{2+} was then added. The cells were incubated for 20 min at 37°C in a humidified incubator

under 5% CO_2. The reaction was terminated by aspirating the buffer and addition of a volume of buffer containing 20 mM Hepes, 10 mM EGTA, 2 mM EDTA, 5 mM DTT, 20 μg/ml leupeptin, 1 mM PMSF, 0.25 M sucrose, 1% nonidet P40, pH 7.4. The cells were scraped, transferred to tubes, and sonicated on ice (five 10-s bursts with 30-s intervals). The sonicate was left on ice for 20 min and then centrifuged at 100,000*g* for 60 min at 4°C. The supernatant was then applied to a DE 52 column preequilibrated with 20 mM Hepes, 10 mM EGTA, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, 20 μg/ml leupeptin, pH 7.4. The column was eluted with the same buffer containing 300 mM NaCl at a flow rate of 8 ml/h. The PKC activity was determined by histone IIIs phosphorylation using [^{32}P]-γ-ATP [Sahyoun et al., 1989] and by [^{3}H]-PDBu binding [Misra and Sahyoun, 1987]. In some experiments, staurosporine (20 nM) an inhibitor of PKC, was added to the cell culture medium for a period of 8 h prior to addition of α_1I_3 or α_1I_3-methylamine. Histone III phosphorylation was studied as above except that [^{33}P]-γ-ATP was employed to replace [^{32}P]-ATP.

RESULTS

Effect of α_1I_3 and α_1I_3-Methylamine on Macrophage Inositol Phosphates

In many cell types, agonist-induced changes in $[Ca^{2+}]_i$ are associated with receptor-coupled production of IP_3 and DAG via stimulation of phospholipase C [see Nishizuka, 1984, 1992; Berridge, 1987; Putney et al., 1989; Berridge, 1993, for review]. We therefore studied the effect of α_1I_3-methylamine on macrophage IP_3 production. Exposure of thioglycollate-elicited macrophages to α_1I_3-methylamine caused a significant increase in IP_3 (Fig. 1), but not mother inositol phosphates (data not shown). The kinetics and maximal effect of α_1I_3-methylamine on IP_3 synthesis are essentially identical to results obtained when α_2M* binds to its signaling receptor [Misra et al., 1993, 1994a,b].

α_1I_3-Methylamine-Induced $[Ca^{2+}]_i$ Increases in Single Macrophages

In view of the effects of α_1I_3-methylamine on macrophage IP_3 synthesis, we next employed digital imaging fluorescence microscopy to study changes in $[Ca^{2+}]_i$ at the single cell level in monolayers of cells loaded with Fura-2. Murine macrophages elicited by intraperitoneal injection of either thioglycollate, casein, or proteose-peptone were employed. Previous studies demonstrate that cells elicited by any of these agents have the same number of α_2M* receptors and that the affinity for human α_2M-methylamine is also identical (K_d at 4°C ~ 1.0 nM) [Imber et al., 1982].

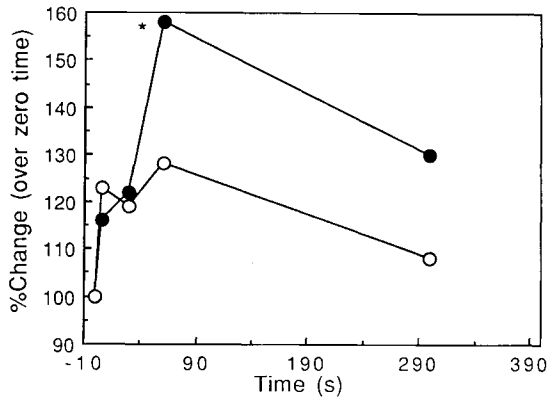

Fig. 1. Formation of IP_3 in macrophages stimulated with native and α_1I_3-methylamine. Monolayers of cells were stimulated with either α_1I_3 (200 nM) (○) or α_1I_3-methylamine (200 nM) (●) for varying periods of time. Water-soluble inositol phosphates were fractionated (see Materials and Methods) and radioactivity determined on an aliquot. Values are percent change compared to the zero time value, which is taken as 100%, and are representative of at least three different experiments. The actual radioactivity values at t = 0 were 520 ± 45 cpm (α_1I_3-methylamine) and 439 ± 66 CPM (α_1I_3). *, significantly different from the buffer control ($P < 0.05$).

Table I summarizes the data from all cells studied. Exposure of macrophages to native α_1I_3 did not cause a change in $[Ca^{2+}]_i$ as compared to a buffer control. However, macrophages obtained by each of the three standard eliciting agents and exposed to α_1I_3-methylamine showed a statistically significant increase in $[Ca^{2+}]_i$, as compared to α_1I_3 or the buffer control.

We recently demonstrated that covalent complexes of α_1I_3 and α_1-m do not bind proteinases and are receptor recognized by LRP/α_2MR, targeting them for clearance from the murine circulation [Falkenberg et al., 1995]. Only limited amounts of $\alpha_1I_3 \cdot \alpha_1$-m were available, and it was not possible to study the effects of this complex on IP_3 production. Since responses in $[Ca^{2+}]_i$ are more easily quantified, we chose to use the available $\alpha_1I_3 \cdot \alpha_1$-m to study the effects of several concentrations on macrophage $[Ca^{2+}]_i$ (Fig. 2). Figure 2 shows a typical response of a single cell exposed to $\alpha_1I_3 \cdot \alpha_1$-m, as observed by digital imaging microscopy. Comparisons of cells treated with α_1I_3-methylamine and $\alpha_1I_3 \cdot \alpha_1$-m (25 nM)

TABLE I. $[Ca^{2+}]_i$ in Macrophages Exposed to α_1I_3 and α_1I_3-Methylamine

Elicitant	$[Ca^{2+}]_i$ (nM)		Cell No.
	α_1I_3	α_1I_3-methylamine	
Thioglycollate	82.15 ± 0.17[a]	168.95 ± 1.07*	20
Casein	91.52 ± 0.27	319.25 ± 4.63*	25
Proteose-Peptone	80.85 ± 0.27	141.00 ± 2.14*	18

[a]The values reported are the mean ± SEM. The cells were exposed to α_1I_3 or α_1I_3-methylamine at a concentration of 200 nM.

*Significantly different from their respective buffer controls ($P < 0.05$ by Student's t-test). The values observed with native α_1I_3 are comparable to the buffer control and not significantly different.

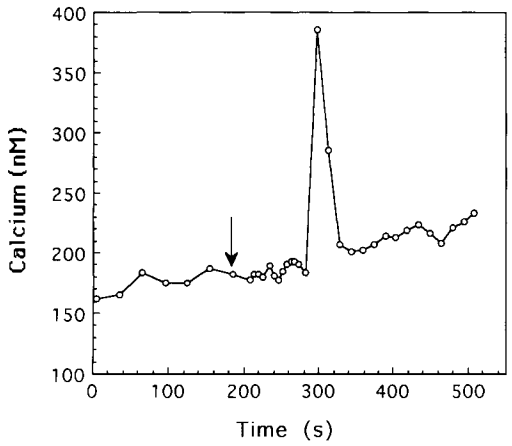

Fig. 2. Single cell response of macrophages exposed to $\alpha_1I_3 \cdot \alpha_1$-m. Thioglycollate-elicited macrophages were used in this study. Monolayers of Fura-2 loaded cells were stimulated with $\alpha_1I_3 \cdot \alpha_1$m (200 nM). *Arrow,* time of addition of the complex.

were performed on the same day to minimize day to day variation in cellular responses. The responses observed for α_1I_3-methylamine were 248.49 ± 18.23 (nM) basal, 357.49 ± 32.74 (nM) stimulated and for $\alpha_1I_3 \cdot \alpha_1$-m 211.21 ± 7.73 (nM) basal, 303.27 ± 6.59 (nM) stimulated. While the responses to α_1I_3-methylamine were generally greater, the responses to $\alpha_1I_3 \cdot \alpha_1$-m were statistically significant.

Effect of GTP Analogues on IP_3 Synthesis in Permeabilized Macrophages Exposed to α_1I_3-Methylamine

We previously demonstrated that the α_2M*SR is coupled to a pertussis toxin-insensitive G protein [Misra et al., 1994a,b]. Consistent with these previous studies, incubation of permeabilized macrophages with either of the nonhydrolyzable GTP analogues, GTPγS, or Gpp(NH)p significantly augmented IP_3 synthesis in cells exposed to α_1I_3-methylamine (Fig. 3). GDPβS, which maintains the α_2M*SR-coupled G protein in the inactive state [Misra et al., 1994a], significantly inhibited GTPγS-induced potentiation of IP_3 production in permeabilized macrophages exposed to α_1I_3-methylamine. Pertussis toxin treatment of macrophages did not effect the generation of IP_3 in macrophages exposed to α_1I_3-methylamine (data not shown). Taken together, these studies are consistent with our previous observations that ligation of the α_2M*SR by human α_2M* activates a pertussis toxin-insensitive G protein.

Effect of α_1I_3 and α_1I_3-Methylamine on Macrophage Protein Kinase C Activity

The above observations suggest that α_1I_3-methylamine stimulates phospholipase C with production of IP_3 and DAG. Since increased $[Ca^{2+}]_i$ and DAG typically stimulate PKC activity [see Nishizuka, 1984, 1992; Berridge, 1987; Putney et al., 1989; Berridge, 1993, for review], the effect of α_1I_3-methylamine on macrophage PKC activity was studied. PKC activity was evaluated by studying both [^{3}H]PDBu binding to macrophage preparations and the incorporation of ^{32}P or ^{33}P into histone IIIs from radiolabeled ATP. [^{3}H]PDBu binding by macrophages was two- to threefold greater in cells exposed to α_1I_3-methylamine as compared to buffer or α_1I_3 (Fig. 4). The extent of histone IIIs phosphorylation was also increased two- to threefold in thioglycollate-elicited macrophages treated with α_1I_3-methylamine, as compared to buffer or α_1I_3 (Fig. 5). Preaddition of staurosporine caused a significant decrease in histone IIIs phosphorylation. These studies, when taken together, indicate that binding of α_1I_3-methylamine to macrophage receptors stimulates the activity of PKC.

Effect of α_1I_3 and α_1I_3-Methylamine on Macrophage cAMP

Thioglycollate-elicited macrophages were exposed to native α_1I_3 or α_1I_3-methylamine for varying periods of time and cAMP pools quantified (Fig. 6). While native α_1I_3 did not cause a significant increase in cAMP, exposure of macrophages to α_1I_3-methylamine resulted in a statistically significant increase in cAMP at 10–30 min of exposure. After 30-min exposure to α_1I_3-methylamine, macrophage cAMP levels returned to basal levels.

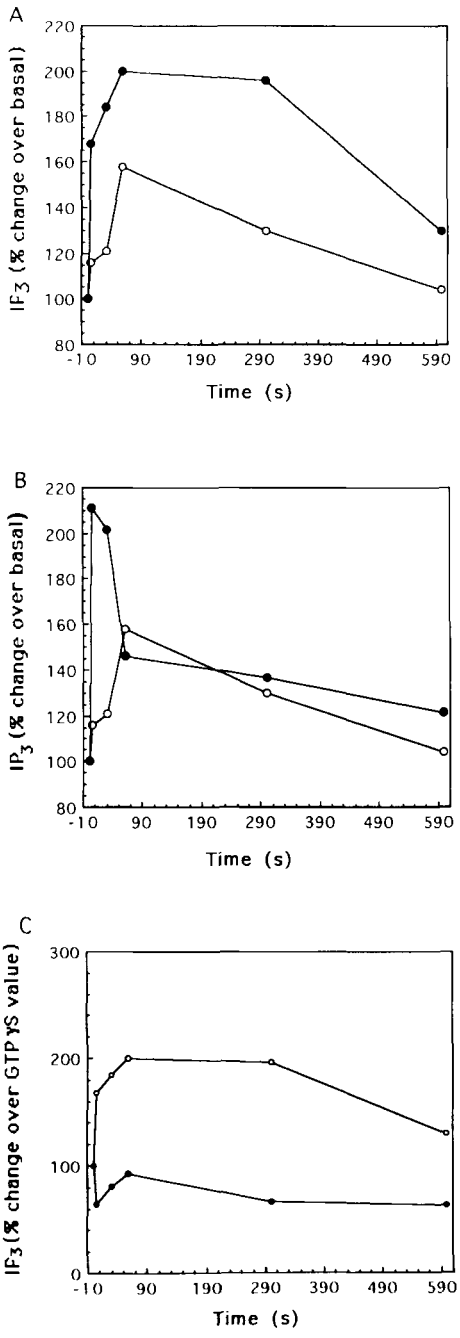

Fig. 3. Effect of GTP analogues on IP_3 synthesis in permeabilized macrophages exposed to α_1I_3-methylamine. **A:** Cells incubated with (●) or without (○) GTPγS (20 μM) prior to addition of α_1I_3-methylamine (200 nM). **B:** Cells incubated with (●) or without (○) Gpp(NH)p (100 μM) prior to addition of α_1I_3-methylamine (200 nM). **C:** Cells were pretreated with GDPβS (500 μM) (●) or buffer (○) before addition of GTPγS and subsequent addition of α_1I_3-methylamine (200 nM). **A–C:** Changes in IP_3 synthesis in respective controls and experimentals are represented as percent change over basal value at zero time, taken as 100%. In a representative experiment, the radioactivity of IP_3 at zero time in different groups was: α_1I_3-methylamine 560 ± 70 cpm; GTPγS ± α_1I_3-methylamine 600 ± 35 cpm; Gpp(NH)p ± α_1I_3-methylamine 700 ± 67 cpm and GDPβS ± GTPγS ± α_1I_3-methylamine 819 ± 51 cpm, respectively.

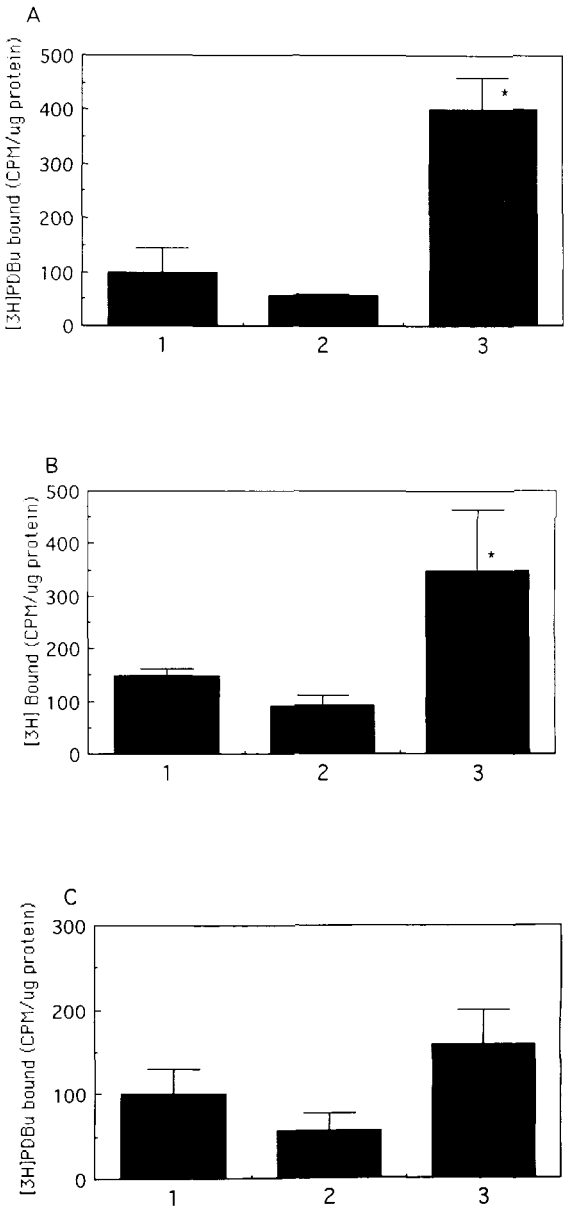

Fig. 4. [^{3}H]PDBu binding by macrophages stimulated with α_1I_3-methylamine. Peritoneal macrophages elicited with thioglycollate (**A**), casein (**B**), and proteose-peptone (**C**) were stimulated with buffer, α_1I_3 (200 nM) and α_1I_3-methylamine (200 nM) for 20 min and processed for binding assays (see Materials and Methods for details). Values are the mean ± SEM from four different experiments. *, significantly different from the buffer control ($P < 0.05$). *Column 1* is the buffer control. *Column 2* indicates addition of α_1I_3 and *column 3* of α_1I_3-methylamine.

These studies suggest that binding of α_1I_3-methylamine to macrophages activates adenylyl cyclase in addition to its effects on phospholipase C-mediated pathways described above. These results are consistent with observations made when α_2M^* is employed as a ligand for the α_2M^*SR [Misra and Pizzo, 1994].

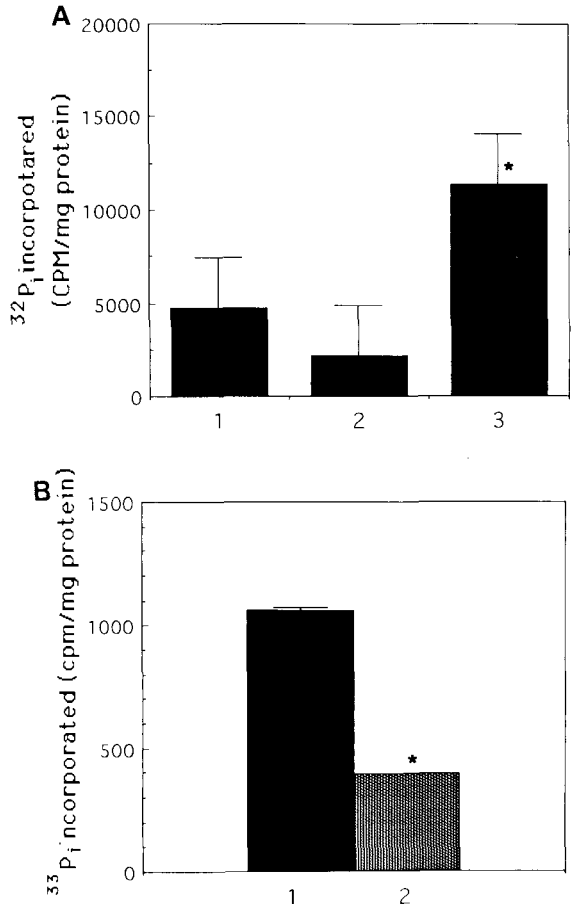

Fig. 5. Histone phosphorylation by macrophages stimulated with α_1I_3-methylamine. **A:** Peritoneal macrophages elicited with thioglycollate were stimulated with buffer (*column 1*), α_1I_3 (200 nM) (*column 2*), and α_1I_3-methylamine (200 nM) (*column 3*) for 20 min and processed for determining histone IIIs phosphorylating activity (see Materials and Methods for details). Values are the mean ± SEM from three different experiments. *, significantly different from controls ($P < 0.05$). **B:** Effect of staurosporine on histone IIIs phosphorylation. *Column 1* indicates the activity of PKC stimulated by α_1I_3-methylamine (200 nM), while *column 2* shows the effect of pretreating the macrophages with staurosporine for 8 h prior to addition of α_1I_3-methylamine (200 nM). Values are the mean ± SEM from three different experiments. The error bar for column 2 cannot be discerned from the top of the column. *, significant difference ($P < 0.05$) when staurosporine was present.

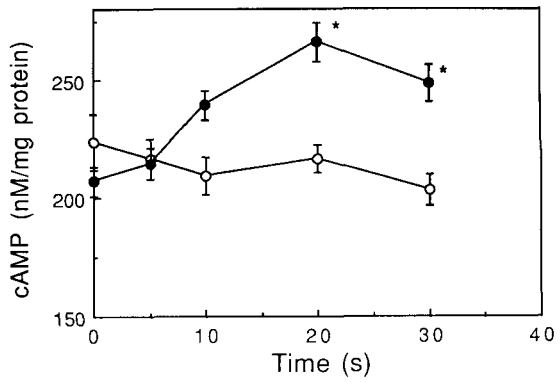

Fig. 6. Changes in cellular cAMP levels in macrophages stimulated with native and α_1I_3-methylamine. Monolayers of peritoneal macrophages were stimulated with native (200 nM) (○) or α_1I_3-methylamine (200 nM) (●) for varying periods of time and processed for cellular cAMP isolation and determination (see Materials and Methods for details). Values are ±SEM from five different experiments. *, significantly different from the buffer control ($P < 0.05$).

DISCUSSION

The subunits of human α_2M show about 58% homology to rat α_1I_3 [Braciak et al., 1988; Aiello et al., 1988]. Nevertheless, there is some dispute about the domain architecture of the carboxyl terminal region of α_2M subunits and monomeric α_1I_3 [Rubenstein et al., 1991; Thomsen and Sottrup-Jensen, 1993]. Since the C-terminal contains the binding site for all known receptor-recognized forms of α-macroglobulins [Enghild et al., 1989b; Salvesen et al., 1992; Holtet et al., 1994], this is an important consideration. While almost all forms of proteinase inhibitory α-macroglobulins appear to bind to LRP/α_2MR, data for the α_2M*SR are much more limited. However, both human α_2M* and a cloned and expressed C-terminal fragment (RBF) from the homologous rat α_1M [Salvesen et al., 1992] bind to the α_2M*SR [Misra et al., 1994a,b]. Both α_2M* and RBF induce comparable macrophage signaling responses after binding to α_2M*SR [Misra et al., 1994a,b] despite the fact that α_2M* shows a 25- to 100-fold better binding affinity to macrophage receptors [Enghild et al., 1989b; Salvesen et al., 1992]. These data suggest that ligand valency is not a major consideration in macrophage signaling responses when α_2M*SR is engaged. However, the available data do not address the issue of whether the presumed differences in the architecture of monomeric α_1I_3 would allow it to bind not only to LRP/α_2MR, but also to α_2M*SR. Moreover, as noted in the Introduction, the one other well characterized α-macroglobulin from the frog does not bind to cellular receptors [Rubenstein et al., 1993].

In the present study, we demonstrate that α_1I_3 when activated by methylamine is able to bind to α_2M*SR and induce changes in intracellular levels of cAMP, IP_3, and $[Ca^{2+}]_i$ all of which are comparable to effects observed when α_2M* or RBF are employed as ligands [Misra et al., 1993, 1994a,b]. Native α_1I_3 is unable to induce signal transduction, which is also comparable to observations made with human α_2M [Misra et al., 1993, 1994a,b]. Thus methylamine-induced conformational change in monomeric α_1I_3 ex-

poses the receptor recognition site for α_2M*SR in this protein, just as it does in tetrameric human α_2M.

Studies with $\alpha_1I_3 \cdot \alpha_1$-m indicate that this complex also binds to α_2M*SR, suggesting that the covalent linkage of α_1-m to α_1I_3 alters its conformation much as methylamine treatment or proteinase attack. This is a somewhat surprising result since complexes of α_1I_3 and α_1-m are present in the circulation at a concentration of about 40 μg/ml [Falkenberg et al., 1990]. Complexes of α_1I_3 and α_1-m must therefore be produced constantly and at a high rate, since LRP/α_2MR-induced cellular uptake and catabolism of $\alpha_1I_3 \cdot \alpha_1$-m is very rapid [Falkenberg et al., 1995]. The constant production of these complexes suggests that their presence plays an important role in normal physiology of rodents, perhaps in part through binding to α_2M*SR.

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