Ligation of the α_2 -macroglobulin signalling receptor on macrophages induces protein phosphorylation and an increase in cytosolic pH

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We have recently described an α_2 -macroglobulin (α_2 M) signalling receptor which is distinct from the low-density lipoprotein-related protein/ α_2 M receptor (LRP/ α_2 MR). Ligation of the macrophage signalling receptor by α_2 M-methylamine stimulates production of several second messengers and involves a pertussis toxin-insensitive G-protein. We now report that binding of α_2 M-methylamine, or the cloned $M_r = 20000$ receptor-binding fragment from rat α_1 M, to macrophage α_2 M signalling receptors induces protein phosphorylation. By use of a monoclonal antibody to phospholipase C γ 1 (PLC γ 1) we were able to identify it as one target for protein phosphorylation. Phosphorylation was time and concentration dependent, being optimal at about 60 s of incubation and a 100–200 nM ligand concentration. By use of a second monoclonal antibody directed against phosphotyrosine, we were able to demonstrate that at least a portion of the label was incorporated into one or more tyrosine residues. PLC γ 1 phosphorylation was then studied in membrane preparations at

4 °C in order to minimize serine or threonine modification. Preincubation of macrophage membranes with genistein, a tyrosine kinase inhibitor, drastically reduced phosphorylation of PLC γ 1. Receptor-associated protein, which blocks α_2 M binding to LRP/ α_2 MR but not to the α_2 M signalling receptor, had no effect on α_2 M-methylamine-induced tyrosine phosphorylation of PLC γ 1. Binding of lactoferrin to LRP/ α_2 MR failed to induce phosphorylation of PLC γ 1, further supporting the hypothesis that the α_2 M signalling receptor and LRP/ α_2 MR are distinct entities. Growth factors which induce tyrosine phosphorylation typically cause a rise in cytosolic pH. Binding of α_2 M-methylamine to macrophages also gradually increased the intracellular pH in a concentration-dependent manner, being optimal at a 200 nM ligand concentration. The increase in pH was amiloride sensitive. We propose that receptor-recognized forms of α_2 M may function like growth factors with regard to macrophage regulation.

INTRODUCTION

α_2 -Macroglobulin (α_2 M) is a member of a large superfamily which also includes the complement components (for review see [1]). α_2 M reacts with proteinases of every mechanistic class and undergoes a major conformational change which exposes receptor recognition sites on each of its four subunits (for review see [2–4]). Like other members of its superfamily, each α_2 M subunit possesses an internal β -cysteinyl- γ -glutamyl thiolester which is directly attacked by small nucleophiles such as methylamine. This reaction also results in a major conformational change in the inhibitor, exposing the receptor recognition sites. Low-density lipoprotein-related protein/ α_2 M receptor (LRP/ α_2 MR) has been identified as one cellular receptor which can bind α_2 M (for review see [5]). This receptor functions as a scavenger receptor and binds many diverse ligands, including α_2 M-proteinase or -methylamine derivatives, plasminogen activators alone or in complex with inhibitors, lactoferrin, lipoprotein lipase and *Pseudomonas* exotoxin A. Generally, these ligands do not compete with each other for binding to LRP/ α_2 MR, presumably because they bind to independent sites on the receptor. However, the $M_r = 39000$ LRP/ α_2 M receptor-associated protein (RAP) blocks the binding of all known ligands to this receptor [6]. Recently, we have identified another distinct α_2 M receptor on

macrophages whose ligation induces a signalling cascade resulting in the production of *myo*-inositol 1,4,5-trisphosphate (IP $_3$) and an increase in intracellular Ca $^{2+}$ concentration ([Ca $^{2+}$] $_i$) [7–9]. RAP does not block the ability of α_2 M-methylamine to induce signalling by this ligand [8,9]. Further studies of LRP/ α_2 MR, however, demonstrated that it too is a receptor involved in regulating a signalling pathway in macrophages [9]. There are, however, significant differences in the mode of induction of the two pathways. Ligation of LRP/ α_2 MR by lactoferrin, lipoprotein lipase and *Pseudomonas* exotoxin A, but not α_2 M-methylamine or RAP, causes a rapid increase in macrophage synthesis of IP $_3$ with a concomitant increase in [Ca $^{2+}$] $_i$ [8,9]. Moreover, while the α_2 M signalling receptor is coupled to a pertussis toxin-insensitive G-protein, the LRP/ α_2 MR signalling pathway is pertussis toxin-sensitive [8,9].

Binding of α_2 M-proteinase or -methylamine to macrophages is known to regulate a number of properties of these cells, including their ability to secrete prostaglandins, generate a respiratory burst, secrete proteinases and kill tumour cells [10–12]. Other reports have also suggested that α_2 M may regulate growth and have mitogenic activity [13,14]; however, whether it is a direct property of α_2 M or results from its known ability to bind growth factors is unclear (for review see [15]). In the present report, we have examined the question of whether ligation of the α_2 M

Abbreviations used: α_2 M, α_2 -macroglobulin; LRP/ α_2 MR, low-density lipoprotein-related protein/ α_2 M receptor; RAP, $M_r = 39000$ LRP/ α_2 M receptor-associated protein; IP $_3$, inositol 1,4,5-trisphosphate; [Ca $^{2+}$] $_i$, intracellular Ca $^{2+}$ concentration; RBF, receptor-binding fragment of rat α_1 M; BCECF, [bis(carboxyethyl)-carboxy-fluorescein]; EGF, epidermal growth factor; TG, thioglycolate; PMSF, phenylmethanesulphonyl fluoride; IFN- γ , interferon- γ ; PDGF, platelet-derived growth factor; PLC γ 1, phospholipase C γ 1; HHBSS, Hank's balanced salt solution containing 10 mM Hepes, pH 7.4, and 3.5 mM NaHCO $_3$.

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signalling receptor, like binding of many growth factors to their receptors, induces phosphorylation of intracellular proteins. We present evidence that ligation of the α_2 M signalling receptor by α_2 M-methylamine or our cloned and expressed receptor-binding fragment (RBF) from rat α_1 M [16], induces phosphorylation of a number of proteins including phospholipase C γ 1 (PLC γ 1). Moreover, we demonstrate that ligation of the α_2 M signalling receptor, analogous to binding of a number of growth factors to their receptors, increases the intracellular pH of macrophages. These studies suggest that receptor-recognized forms of α_2 M may share some of the properties of classic growth factors such as epidermal growth factor (EGF).

EXPERIMENTAL PROCEDURES

Materials

Sterile water was obtained from Abbott Laboratories (Chicago, IL, U.S.A.). Brewer's thioglycolate (TG) broth was purchased from Difco Laboratories (Baltimore, MD, U.S.A.). Culture media were purchased from Life Technologies, Inc. (Grand Island, NY, U.S.A.). BSA, pertussis toxin, genistein, leupeptin, phenylmethanesulphonyl fluoride (PMSF), lactoferrin, sodium orthovanadate, sodium fluoride, protein G-Sepharose and EGF were purchased from Sigma (St. Louis, MO, U.S.A.). [32 P]Orthophosphate and [γ - 32 P]ATP were purchased from DuPont NEN (Boston, MA, U.S.A.). [2- 3 H]myo-Inositol (specific activity 10–20 Ci/mmol) was purchased from ARC, Inc. (St. Louis, MO, U.S.A.). Fura-2/AM and [bis(carboxyethyl)-carbonyl fluorescein] (BCECF) were purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Anti-PLC γ 1-specific monoclonal antibody was purchased from Life Technologies, Inc. Monoclonal antibodies directed against phosphotyrosine were produced from a hybridoma cell line, FB2, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). All other chemicals used were of reagent grade.

Preparation of α_2 M, RBF and RAP

Human α_2 M was prepared and reacted with methylamine as previously described [17]. RBF was cloned and expressed in *Escherichia coli* and purified to apparent homogeneity as previously reported [16]. α_2 M and RBF were assayed for endotoxin as previously described [18]. The final endotoxin levels never exceeded 10 pg/ml in a 1.3 μ M stock solution. RAP was a gift from Dudley Strickland (American Red Cross, Rockville, MD, U.S.A.).

Cell culture

The cell culture procedures have been described in detail previously [7–9]. In brief, pathogen-free C57b1/6 mice (6 weeks old) were obtained from Charles River Laboratories (Raleigh, NC, U.S.A.). TG-elicited macrophages were 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 and suspended in RPMI 1640 medium containing 2 mM glutamine, 12.5 units/ml penicillin, 6.5 μ g/ml streptomycin and 0.1 % BSA. The cells were plated on glass coverslips (4.0×10^6 cells), six-well plates [$(1.5\text{--}2.0) \times 10^6$ cells/well] or 35 mm Petri dishes [$(6\text{--}6.5) \times 10^6$ cells/dish] and incubated for 2 h at 37 °C in a humidified CO $_2$ (5 %) incubator. The monolayers were washed three times with HHBSS to remove non-adherent cells and cultured in RPMI 1640 medium as above for 16–18 h.

Cell labelling treatment, lysis, immunoprecipitation and Western blotting

Macrophage monolayers incubated overnight were washed twice with phosphate-free RPMI 1640 medium containing 0.1 % BSA and antibiotics as above and a volume of this medium was then added to the monolayers. The monolayers were labelled with [32 P]orthophosphate (0.5 mCi/dish) for 90 min at 37 °C in a humidified CO $_2$ (5 %) incubator. Monolayers were treated with EGF (200 ng/ml), α_2 M-methylamine (200 nM) or buffer for specific periods and immediately washed twice with a 10-fold volume of ice-cold Ca $^{2+}$ - and Mg $^{2+}$ -free PBS and lysed with cold solubilization buffer containing 1 % Triton X-100, 10 % glycerol, 50 mM Hepes, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 10 μ g/ml leupeptin and 1 mM PMSF, pH 7.5 [19]. The lysates were incubated on ice for 60 min and clarified by centrifugation at 27000 g for 30 min at 4 °C. Protein G-Sepharose was added and the lysate was incubated for 30 min at 4 °C and centrifuged for 30 min at 12000 g at 4 °C. PLC γ 1 in the lysate was immunoprecipitated with anti-PLC γ 1 monoclonal antibody and protein G-Sepharose added. The lysates were incubated for 2 h at 4 °C and centrifuged as above. The PLC γ 1 immune complexes were washed five times with 50 mM Tris-HCl containing 1 % Triton X-100, 1 % deoxycholate, 0.1 % SDS and 150 mM NaCl, pH 8.5 [19]. Immunoprecipitated proteins were dissolved by the addition of SDS sample buffer followed by boiling for 5 min and separated by SDS/PAGE (7.5 %), as described by Laemmli [20]. The proteins were transferred to nitrocellulose paper and the paper blocked with 3 % BSA in Tris-buffered saline, pH 8.0, containing 1 % Triton X-100 for 2 h at room temperature. The nitrocellulose paper was then incubated with phosphotyrosine monoclonal antibody (10 mg/10 ml) for 2–4 h at room temperature. Blots were developed with lactoperoxidase-linked secondary antibodies and visualized with 4-chloro-1-naphthol. The 32 P radioactivity in the immunoprecipitated proteins containing phosphotyrosine residues was quantified by volume integration in a Phosphor-imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Tyrosine phosphorylation of PLC γ -1 in membrane preparations treated with [γ - 32 P]ATP

PLC γ 1 may exist in the cytosol, however, a substantial fraction is associated with cellular membranes [21–26]. Phosphorylation of PLC γ 1 is most easily studied in membrane preparations where phosphorylation of a number of cytosolic proteins is eliminated. The enzyme of $M_r \sim 145000\text{--}150000$ [22] can be located by use of specific monoclonal antibodies to this protein. Macrophage monolayers incubated overnight [$(6\text{--}6.5) \times 10^6$ cells] were washed with cold PBS buffer, scraped into chilled tubes in PBS and centrifuged at 800 g at 4 °C for 5 min. To the pellet a volume of cold hypotonic buffer containing 20 mM Hepes, 3 mM KCl, 3 mM MgCl $_2$, leupeptin (50 μ g/ml) and 1 mM PMSF, pH 7.2, was added. Plasma membranes were prepared by breaking the cells by N $_2$ cavitation [600 lb/in 2 /20 min at 4 °C (1 lb/in 2 = 6.9 kPa)]. The homogenate was centrifuged at 600 g for 5 min at 4 °C and supernatant centrifuged at 100000 g for 45 min at 4 °C to obtain a plasma-membrane enriched preparation. The pellet was washed twice with hypotonic buffer and suspended gently in a volume of 50 mM Hepes containing 0.1 mM EDTA, 10 % glycerol, leupeptin (50 μ g/ml) and 1 mM PMSF, pH 7.2. The protein concentration was measured in the membrane preparations after solubilization in NaOH, as previously described [27]. Phosphorylation was performed in 40 μ l of 50 mM Hepes containing 30 mM MgCl $_2$, 2 mM dithiothreitol, 0.32 mM EGTA, 100 mM sodium orthovanadate, 15 mM ATP, leupeptin

(50 $\mu\text{g/ml}$), PMSF (1 mM), pH 7.0, and suspended plasma membranes (40 μg of protein). The membranes were incubated with EGF, $\alpha_2\text{M}$ -methylamine, RBF, RAP or lactoferrin at specified concentrations for 15 min at 4 °C before addition of [γ - ^{32}P]ATP. When genistein (20 μM) or pertussis toxin (20 $\mu\text{g/ml}$) was used, the membranes were pre-incubated for 20 min at 4 °C before the addition of the ligands. Genistein was employed as an inhibitor of tyrosine kinases [28,29]. Pertussis toxin was used to evaluate the role of G-proteins (for review see [30]) involved in $\alpha_2\text{M}$ -methylamine- or EGF-induced tyrosine phosphorylation of PLC $\gamma 1$. Pertussis toxin was employed as previously described [8].

The various reactions were initiated by the addition of [γ - ^{32}P]ATP (8 $\mu\text{Ci/assay}$) and incubations were performed at 4 °C for specified time periods. At this temperature Ser and Thr phosphorylation are significantly decreased [31–35]. The reactions were terminated by the addition of SDS sample buffer. The tubes were boiled for 5 min and phosphorylated proteins separated by SDS/PAGE (7.5 % gel) as described by Laemmli [20]. The gels were stained with Coomassie Blue, destained and dried and phosphorylation of PLC $\gamma 1$ was quantified by means of a Phosphorimager.

Measurement of inositol phosphates

The generation of inositol phosphates in TG-elicited macrophages stimulated with EGF (200 ng/ml) or $\alpha_2\text{M}$ -methylamine (200 nM) in the absence or presence of genistein was quantified according to methods published previously [7,8] except that the cells were incubated in inositol-free RPMI 1640 medium which contained penicillin (12.5 units/ml), streptomycin (6.5 $\mu\text{g/ml}$) and 0.1 % BSA [8,9]. Genistein (20 μM) was added to monolayers 12 h before stimulation with agonists. In preliminary experiments, we observed that incubation of macrophages with 20 μM genistein for 2–16 h caused no loss in cell viability as judged by Trypan Blue exclusion assay.

Measurements of $[\text{Ca}^{2+}]_i$

The effects of treatment of TG-elicited macrophages incubated in serum-free RPMI 1640 medium with EGF (200 ng/ml) and $\alpha_2\text{M}$ -methylamine (200 nM), in the presence and absence of genistein, on $[\text{Ca}^{2+}]_i$, were measured as described previously in Fura-2/AM-loaded cells [7–9]. Genistein (20 μM) was added to monolayers 12 h before stimulation with EGF or $\alpha_2\text{M}$ -methylamine.

Measurements of cytosolic pH

TG-elicited peritoneal macrophages were incubated overnight in serum-free RPMI 1640 medium on glass coverslips as above and then washed with HHBSS with sodium bicarbonate, pH 7.1. A volume of the buffer was added to the monolayer followed by the addition of 2 μM BCECF. The monolayers on glass coverslips were incubated at room temperature for 20 min, washed with the buffer three times and the coverslips placed on the fluorescent microscope stage. Cytosolic pH was measured by a digital video imaging technique in cells stimulated with agonists which were added after obtaining a stable baseline as previously described [36]. To study the effect of pertussis toxin, the macrophage monolayers were pretreated with pertussis toxin (20 $\mu\text{g/ml}$) for 2 h before addition of the ligands to the cells. To study the effect of amiloride on pH changes induced by ligands, BCECF-loaded macrophage monolayers were pretreated with amiloride (5 mM) for 5 min before addition of the ligands to the cells.

RESULTS AND DISCUSSION

$\alpha_2\text{M}$ -methylamine induces tyrosine phosphorylation of PLC $\gamma 1$

The stimulation of cellular proliferation and differentiation by peptide growth factors involves the activation of signalling pathways that are initiated by specific receptors at the cell surface [37–49]. A key event in the stimulation of cells by these polypeptide growth factors, such as EGF, is the activation of their specific receptor tyrosine kinases following dimerization and autophosphorylation of the receptor at one or more Tyr residues. It is believed that tyrosine-phosphorylated substrates of these growth factor receptor kinases are essential mediators of signal transduction processes that lead to cellular responses [42,44–47,50–53]. Protein phosphorylation represents a major mechanism of signal transduction and the state of phosphorylation determines the functioning of various cellular proteins. Mutations that eliminate the tyrosine kinase activity of the peptide-specific receptor also abolish the biological function of the ligated receptor [54–57]. The study of EGF has provided a framework for understanding the cellular and molecular events that underlie the biological effects of a number of growth factors [42]. The results presented in this communication demonstrate that like EGF, stimulation of murine peritoneal macrophages with receptor-recognized forms of $\alpha_2\text{M}$, $\alpha_2\text{M}$ -methylamine and the 20 kDa RBF, rapidly induces tyrosine phosphorylation of PLC $\gamma 1$.

Figure 1 shows the results of an experiment in which intact macrophages were prelabelled with ^{32}P after which they were exposed to $\alpha_2\text{M}$ -methylamine or EGF, the latter employed as a positive control. Compared with the buffer control, a number of

Figure 1 Immunoprecipitation of PLC $\gamma 1$ in ^{32}P -prelabelled macrophages

Lane 1 shows a Coomassie Blue stain of proteins in solubilized macrophages. Lanes 2 and 3 are gel scans obtained from the Phosphorimager of $\alpha_2\text{M}$ -methylamine (200 nM) and buffer-exposed macrophages respectively. The protein load in lanes 2 and 3 was identical. These studies were performed at least 17 times at various ligand concentrations with similar results. Lanes 4–6 are from a typical Western blot obtained in a two-step procedure as described in the Experimental procedures section. In brief, a monoclonal antibody was used to precipitate PLC $\gamma 1$. The precipitate was then subjected to SDS/PAGE and, after transfer to nitrocellulose paper, probed with a second monoclonal antibody specific for phosphotyrosine residues. Lane 4 shows the buffer control. Lane 5 shows the results of cell treated with $\alpha_2\text{M}$ -methylamine (200 nM) for 60 s while lane 6 is the positive control of cells exposed to EGF (200 ng/ml) for 60 s. The molecular mass markers indicate a phosphorylated protein of $M_r \sim 145\,000$ – $150\,000$, consistent with the expected molecular mass of PLC $\gamma 1$ [22].

Figure 2 Tyrosine phosphorylation of PLC γ 1 in macrophage preparations treated with α_2 M-methylamine, EGF and lactoferrin

Membrane preparations at 4 °C (40–50 μ g of total protein) were stimulated with α_2 M-methylamine or EGF as detailed in the Experimental procedures section. In order to compare the concentration dependence of PLC γ 1 phosphorylation, 60 s was chosen as the incubation time for both EGF and α_2 M-methylamine (based on data presented in Table 1). The images of gels from six separate experiments were quantified by use of the Phosphorimager. The SDS/PAGE gel scan was analysed in the mobility region around $M_r \sim 145\,000$ –150 000 where PLC γ 1 migrates, as seen in Figure 1. 32 P-labelled PLC γ 1 is expressed as the percentage increase over the buffer control. The concentration scale is in nM for α_2 M-methylamine (●), pM for lactoferrin (■) and ng/ml for EGF (▲). The concentrations for α_2 M-methylamine and lactoferrin were chosen based on our previous studies of signal transduction by these ligands [7–9].

proteins showed increased incorporation of 32 P when α_2 M-methylamine was employed as a ligand. Because PLC γ 1 is a known target for growth factor-induced phosphorylation and after receptor ligation (see for example [37–39]), we focused on identifying this protein. PLC γ 1 ($M_r \sim 145\,000$ –150 000) was immunoprecipitated by employing a specific monoclonal antibody. The precipitate was subjected to SDS/PAGE and Western blotting employing a monoclonal antibody to phosphotyrosine. The resultant gel (Figure 1) was then analysed and quantified by a Phosphorimager. Compared with the buffer control, both α_2 M-methylamine (200 nM) and EGF (200 ng/ml) induced a significant increase in 32 P-labelled PLC γ 1 of $178.56 \pm 13.50\%$ and $270.02 \pm 21.32\%$ respectively. Since these studies were performed with intact cells at 37 °C, 32 P-incorporation into PLC γ 1 may involve modification of Tyr, Ser or Thr residues. However, the results of the blotting study with a monoclonal antibody directed against phosphotyrosine (Figure 1) clearly indicate that at least part of this phosphorylation involves modification of one or more Tyr residues. Since PLC γ 1 is not itself a tyrosine kinase, these studies suggest that ligation of the α_2 M signalling receptor induces tyrosine kinase activity.

Tyrosine phosphorylation of PLC γ 1 in membrane preparations by α_2 M-methylamine, RBF, lactoferrin and RAP

The studies described above were pursued further using macrophage membrane preparations and were performed at 4 °C, conditions which minimize phosphorylation of Ser or Thr residues [31–35]. EGF was used as positive control for these experiments. RBF was studied to rule out the possible role of growth factors which might contaminate α_2 M preparations. Lactoferrin and RAP were included in these experiments to probe the possible role of LRP/ α_2 MR in phosphorylating PLC γ 1. The effects of α_2 M-methylamine, EGF and lactoferrin on 32 P-incorporation from [γ - 32 P]ATP into PLC γ 1 are compared in

Table 1 Effect of time of incubation on α_2 M-methylamine- and EGF-induced tyrosine phosphorylation of PLC γ 1 in membrane preparations at 4 °C

Time (s)	α_2 M-methylamine	EGF
0	5.96 $\pm$ 0.75*	4.89 $\pm$ 0.94
15	16.77 $\pm$ 1.35	15.41 $\pm$ 0.76
30	22.19 $\pm$ 2.63	20.39 $\pm$ 2.22
60	24.74 $\pm$ 1.68	35.08 $\pm$ 3.62
300	26.34 $\pm$ 2.66	21.93 $\pm$ 2.26

* Arbitrary units were calculated by volume integration of the image of SDS/PAGE gels as described in the Experimental procedures section. The data are from six separate gel studies $\pm$ S.E.M. Data analysis was by Phosphorimager of the gel scan in the mobility region around $M_r \sim 145\,000$ –150 000 where PLC γ 1 migrates as seen in Figure 1.

Table 2 Modulation of EGF-, α_2 M-methylamine- and RBF-induced tyrosine phosphorylation of PLC γ 1 in membrane preparations at 4 °C by genistein and pertussis toxin

For these studies, the basal value in the presence of buffer was taken as 100%. The values shown represent four separate experiments $\pm$ S.E.M. Tyr phosphorylation of PLC γ 1 was assessed in ligand-exposed cells 60 s after addition of [γ - 32 P]ATP. PTx indicates the addition of pertussis toxin (20 μ g/ml) for 2 h before addition of the ligands.

Treatment	PLC γ 1 Tyr phosphorylation (% change over basal values)
EGF (200 ng/ml)	245.09 $\pm$ 21.32
EGF + PTx	63.22 $\pm$ 4.69
EGF + Genistein	115.46 $\pm$ 35.39
α_2 M-methylamine (200 nM)	179.21 $\pm$ 14.18
α_2 M-methylamine + PTx	154.48 $\pm$ 10.23
α_2 M-methylamine + genistein	105.44 $\pm$ 12.58
α_2 M-methylamine (boiled)	77.61 $\pm$ 10.67
RBF (40 nM)	166.31 $\pm$ 13.33
RBF + PTx	165.88 $\pm$ 16.70
RBF + genistein	83.90 $\pm$ 19.38
Lactoferrin (20 pM)	82.94 $\pm$ 6.92

Figure 2 and Tables 1 and 2. Following SDS/PAGE, the gels were analysed with a Phosphorimager. α_2 M-methylamine (200 nM) and EGF (200 ng/ml) induced time-dependent increases in phosphorylation of PLC γ 1 which were essentially maximal at 60 s (Table 1). While EGF and α_2 M-methylamine treatment of the membranes both induced concentration-dependent increases in PLC γ 1 phosphorylation (Figure 2), neither lactoferrin (20 pM) (Figure 2) nor RAP (results not shown) induced phosphorylation of PLC γ 1. RBF (40 nM) induced a comparable increase in phosphorylation of PLC γ 1 as compared with α_2 M-methylamine (200 nM) (Table 2). RAP did not block the ability of α_2 M-methylamine or RBF to induce phosphorylation of PLC γ 1 (results not shown). The increase in 32 P-incorporation into PLC γ 1 over the buffer control, seen when membranes were exposed to α_2 M-methylamine (Figure 2) at 4 °C, is comparable to results obtained with studies of whole cell labelling at 37 °C (Figure 1). These results suggest that the bulk of the 32 P-labelling observed in these experiments performed at 37 °C is incorporated into one or more Tyr residues.

The ligand concentrations chosen for RBF and lactoferrin are based on previous studies of the effects of these ligands on signal

Figure 3 The effect of genistein on IP₃ generation by macrophages

[2-³H]Myo-inositol-labelled macrophages were incubated for 12 h with genistein (20 μ M) before being stimulated with ligand. (a) Cells exposed to α_2 M-methylamine (200 nM) alone (●) or after genistein exposure (○). (b) Cells exposed to EGF (200 ng/ml) alone (▲) or after genistein exposure (△). The values reported are from two separate experiments performed in duplicate. The data at each time-point were averaged and the mean $\pm$ S.E.M. calculated.

transduction in macrophages as well as the known K_d values for binding of these ligands to macrophage cellular receptors [7–9]. The ligand concentration chosen for the study of α_2 M-methylamine is based on the dose curve in Figure 2. This concentration (200 nM) was also shown to induce maximal changes in IP₃ synthesis and [Ca²⁺]_i in previous studies [7–9]. In those studies, RBF (40 nM) and α_2 M-methylamine (200 nM) had comparable effects of the induction of IP₃ synthesis and increases in [Ca²⁺]_i in macrophages [7–9].

It is concluded that ligation of LRP/ α_2 MR by RAP or lactoferrin is not associated with a change in tyrosine kinase activity. Furthermore, while α_2 M binds to both LRP/ α_2 MR and its own signalling receptor [8,9], these studies indicate that only ligation of its specific receptor by α_2 M-methylamine is associated with phosphorylation of PLC γ 1. These results are consistent with our previous reports that binding of α_2 M-methylamine to its specific signalling receptor, but not to LRP/ α_2 MR, induces increases in IP₃ and a rise in [Ca²⁺]_i [8,9].

We next performed experiments to determine whether genistein or pertussis toxin affected phosphorylation of PLC γ 1 macrophage membranes at 4 °C by the various ligands (Table 2). Genistein, an inhibitor of tyrosine kinase activity [28,29], completely blocked phosphorylation of PLC γ 1 by EGF, α_2 M-methylamine and RBF. By contrast, pertussis toxin had no effect on α_2 M-methylamine- or RBF-induced phosphorylation; however, it does block the effect of EGF on this reaction. The effect of pertussis toxin on EGF-induced phosphorylation of PLC γ 1 has been reported

Figure 4 The effect of genistein on [Ca²⁺]_i levels in single macrophages exposed to α_2 M-methylamine or EGF

Macrophages were preloaded with the Ca²⁺ fluorescent indicator Fura-2/AM. The procedure is described in detail elsewhere [7]. After preincubation with genistein (20 μ M) for 12 h, the cells were exposed to ligand. (a) Cells exposed to α_2 M-methylamine (200 nM) alone (●) or after genistein exposure (○). (b) Cells exposed to EGF (200 ng/ml) alone (▲) or after genistein exposure (△). In each panel, the arrow indicates the time of ligand addition. The experiment was performed on four separate occasions and 18–25 cells were analysed by the digital imaging microscope in each of the separate studies. The response rate ranged from 78 to 88% in the four studies. The results of α_2 M-methylamine are comparable to our previous report of 85% responsiveness [7]. The cells shown in (a) and (b) are representative of responding cells with respect to the amplitude of response.

previously [19,29]. The lack of a pertussis toxin effect on the ability of α_2 M-methylamine or RBF to stimulate phosphorylation of PLC γ 1 can be explained by one of two mechanisms. Either the ability of the α_2 M signalling receptor to stimulate phosphorylation of PLC γ 1 does not involve a G-protein or if a G-protein is involved, it is pertussis toxin insensitive. While our previous studies demonstrate that the α_2 M signalling receptor is linked to a pertussis toxin-insensitive G-protein [8,9], the current studies cannot distinguish between these two models.

Inhibition of α_2 M-methylamine and EGF-induced generation of IP₃ by genistein

Figure 3 shows the effect of genistein on IP₃ generation by macrophages exposed to either α_2 M-methylamine or EGF. When the cells were treated with α_2 M-methylamine, IP₃ generation increased by 80% over the buffer control (Figure 3a). Pre-treatment with genistein reduced the peak to about 45% over the control. These studies suggest that IP₃ generation by cells stimulated with α_2 M-methylamine involves both PLC γ 1 and other isoforms of PLC, presumably PLC β , which do not depend

Figure 5 The effect of α_2 M-methylamine on macrophage cytosolic pH

Macrophages were preloaded with BCECF, a fluorescent indicator sensitive to pH changes [36], before exposure to α_2 M-methylamine (200 nM). The experiment was performed on four separate occasions and 20–25 cells were analysed per study by the digital imaging analyser. Numbers on the photograph indicate the time in seconds. An increase in macrophage cytosolic pH after exposure to α_2 M-methylamine was noted in more than 95% of the cells. RBF (40 nM) and EGF (200 ng/ml) were also studied and the results were similar to those obtained with α_2 M-methylamine (cells not shown). Boiled α_2 M-methylamine and buffer caused no pH change.

on tyrosine phosphorylation for activity (for review see [40]). These results are consistent with our previous observation that ligation of the α_2 M signalling receptor results in IP_3 generation via a G-protein-linked process [8,9]. In the case of EGF, IP_3 generation was stimulated by about 35% over the baseline buffer control (Figure 3b). Pretreating the cells with genistein reduced IP_3 generation to nearly the baseline level. These studies, as expected, indicate that EGF-stimulated IP_3 production occurs primarily as a result of the hydrolysis of phosphatidylinositol 4,5-bisphosphate by $\text{PLC}\gamma 1$ which is activated after tyrosine kinase-induced phosphorylation of the enzyme.

These studies were then extended to examine the effects of genistein on ligand-induced increases in $[\text{Ca}^{2+}]_i$. Cells were preloaded with Fura 2/AM. After pretreatment with genistein, the macrophages were exposed to the ligands and examined in a digital imaging microscope (Figure 4). Genistein only partially blocked the increase in macrophage $[\text{Ca}^{2+}]_i$ stimulated by α_2 M-methylamine exposure (Figure 4a). By contrast, pretreatment of macrophages with genistein abolished the increase in $[\text{Ca}^{2+}]_i$ associated with treatment of macrophages with EGF (Figure 4b). These results are consistent with the studies presented above since the rise in intracellular $[\text{Ca}^{2+}]_i$ is dependent on increased synthesis of IP_3 (for review see [30]).

The particular cell exposed to genistein shown in Figure 4(a) responded in an oscillatory pattern to α_2 M-methylamine (two peaks). The significance of oscillatory versus sustained patterns of response is unclear. It does not appear to be directly related to the use of genistein since other cells did not show such a pattern

(not shown). This variation in the type of response is typically seen when cells are exposed to α_2 M-methylamine. Sustained responses and oscillatory responses occur with nearly equal frequency [7].

Stimulation of macrophages with α_2 M-methylamine raises cytosolic pH

Na^+/H^+ exchange is a ubiquitous transport process directly involved in a myriad of physiological processes such as intracellular pH regulation, cell volume regulation, transepithelial ion transport and cellular responses to mitogens [58–61]. In many cells, this transport system is quiescent and can be activated by growth factors, cytokines, phorbol esters, cell volume changes or changes in the electrochemical gradients of Na^+ and/or H^+ [59,61,62]. It has been reported that in astrocytes interferon- γ (IFN- γ), interleukin-1 and tumour necrosis factor- α activate both the Na^+/H^+ antiporter and induce cell proliferation [59–64]. Murine peritoneal macrophages possess a Na^+/H^+ antiporter which is stimulated by IFN- γ and is inhibited by the diuretic amiloride [36,65,66].

In addition to their ability to regulate tyrosine kinase activity, α_2 M-methylamine and RBF have effects on the macrophage Na^+/H^+ antiporter, further suggesting growth factor-like activity for these ligands. For these studies, cells were preloaded with the pH-sensitive fluorescent probe BCECF. After ligand exposure, the cells were examined in a digital imaging microscope. Figure 5 shows a series of colour photographs taken from the digital

Figure 6 The effect of α_2 M-methylamine at different concentrations on macrophage cytosolic pH

These studies were conducted as described in the legend to Figure 5. Macrophages were preloaded with BCECF before ligand exposure. The dose-response curve for the change in cytosolic pH as a function of α_2 M-methylamine concentration is based on four separate studies and 20–25 cells were analysed per study. The values reported are the mean $\pm$ S.E.M. for all responsive cells in the four experiments.

Table 3 Changes in cytosolic pH after exposure of macrophages to various ligands

The data are taken from four separate studies in which 20–25 cells were analysed by digital imaging microscopy for each experiment. The mean pH value $\pm$ S.E.M. is given for all cells analysed.

Ligand	pH		Δ pH/min
	Initial	After 5 min	
α_2 M-methylamine (200 nM)	7.22 $\pm$ 0.02	7.49 $\pm$ 0.02	0.05
α_2 M-methylamine (boiled)	7.15 $\pm$ 0.02	7.17 $\pm$ 0.02	< 0.01
Lactoferrin (20 pM)	7.34 $\pm$ 0.02	7.39 $\pm$ 0.02	< 0.01
RBF (40 nM)	7.17 $\pm$ 0.02	7.45 $\pm$ 0.01	0.06
EGF (200 ng/ml)	7.22 $\pm$ 0.02	7.59 $\pm$ 0.05	0.07

Table 4 Effect of pretreatment of cells with pertussis toxin on α_2 M-methylamine- and EGF-induced changes in pH

The data are taken from four separate studies in which 20–25 cells were analysed by digital imaging microscopy for each experiment. The mean pH value $\pm$ S.E.M. is given. PTx indicates addition of pertussis toxin (20 μ g/ml) for 2 h before addition of the ligands.

Additions	pH	
	Initial	After 5 min
EGF (200 ng/ml)	7.14 $\pm$ 0.007	7.47 $\pm$ 0.018
EGF + PTx	7.15 $\pm$ 0.004	7.16 $\pm$ 0.004
α_2 M-methylamine (200 nM)	7.17 $\pm$ 0.014	7.50 $\pm$ 0.015
α_2 M-methylamine + PTx	7.11 $\pm$ 0.014	7.33 $\pm$ 0.01

imaging microscope demonstrating exposure of macrophages to α_2 M-methylamine. These images demonstrate that stimulation of macrophages by α_2 M-methylamine raises cytosolic pH. This effect was dose-dependent, reaching a maximal effect at a 100–200 nM ligand concentration (Figure 6). This dose curve shows a comparable pattern to the effect of α_2 M-methylamine on IP $_3$

Table 5 Effect of amiloride on α_2 M-methylamine- and EGF-induced increase in pH

The data are taken from four separate studies in which 20–25 cells were analysed by digital imaging microscopy for each experiment. The mean pH value $\pm$ S.E.M. is given.

Addition	pH	
	Initial	After 5 min
EGF (200 ng/ml)	7.21 $\pm$ 0.023	7.54 $\pm$ 0.016
EGF + amiloride	7.37 $\pm$ 0.015	7.37 $\pm$ 0.010
α_2 M-methylamine (200 nM)	7.16 $\pm$ 0.04	7.55 $\pm$ 0.019
α_2 M-methylamine + amiloride	7.19 $\pm$ 0.03	7.26 $\pm$ 0.019

synthesis and the rise in [Ca $^{2+}$] $_i$, which plateau at similar ligand doses [7]. The pH response of macrophages exposed to α_2 M-methylamine, RBF, lactoferrin and EGF are compared in Table 3. Neither lactoferrin (20 pM) nor boiled α_2 M-methylamine affected intracellular pH. α_2 M-methylamine (200 nM), RBF (40 nM) and EGF (200 ng/ml) had comparable effects, causing a significant increase in pH. That RBF produces comparable effects to α_2 M-methylamine at a lower dose, as discussed above, was also observed with increases in IP $_3$ and [Ca $^{2+}$] $_i$, despite the fact that the K_d of α_2 M-methylamine is 10-fold better than that of RBF [8,9]. The reason for this difference is currently unknown.

Table 4 demonstrates that pertussis toxin pretreatment abolishes the ability of EGF to increase intracellular pH, while the ability of α_2 M-methylamine to increase cytosolic pH remained unaffected by pertussis toxin. Table 5 demonstrates that pretreatment of macrophages with an inhibitor of the Na $^+$ /H $^+$ antiporter amiloride, abolished the ability of both α_2 M-methylamine and EGF to stimulate an increase in intracellular pH. Taken together, these studies demonstrate that an amiloride-sensitive Na $^+$ /H $^+$ antiporter is involved in α_2 M-methylamine-induced changes in cytosolic pH and this channel appears to be pertussis toxin-insensitive.

In summary, the observations made with α_2 M-methylamine and RBF suggest that receptor-recognized forms of α_2 M have certain properties in common with growth factors. While this question requires further investigation, some previous studies support this hypothesis. Salomon et al. [13] reported that an α_2 M-like contaminant termed embryonin in fetuin preparations promoted cellular proliferation. Our laboratory then demonstrated that embryonin was identical with bovine α_2 M [67]. Subsequent studies have suggested that this growth promoting activity might be attributed to platelet-derived growth factor (PDGF) covalently bound to the α_2 M [68]. More recently, Bonner et al. [14] have presented data indicating that α_2 M receptor-recognized forms are synergistic to the actions of PDGF even when the latter growth factor is not bound to the α_2 M. In collaboration with the laboratory of Dr. Steven Gonias, we have recently demonstrated that α_2 M-methylamine stimulates a 5-fold increase in [3 H]thymidine incorporation by rat aortic smooth muscle cells (D. J. Webb, I. M. Hussaini, A. M. Weaver, T. L. Atkins, C. T. Chu, S. V. Pizzo, G. K. Owens, and S. L. Gonias, unpublished work). These studies also support the concept that α_2 M receptor-recognized forms possess properties typically seen with growth factors such as EGF.

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REFERENCES

- 1 Sottrup-Jensen, L. (1987) in *Plasma Proteins* (Putnam F. W., ed.), pp. 191–291, Academic Press, New York
- 2 Pizzo, S. V. (1988) *ISI Atlas Sci. Biochem.* **1**, 242–246
- 3 Borth, W. (1992) *FASEB J.* **6**, 3345–3353
- 4 Delain, E., Pochon, F., Barry, M. and Van Leuven, F. (1992) *Electron Microsc. Rev.* **5**, 231–281
- 5 Krieger, M. and Herz, J. (1994) *Annu. Rev. Biochem.* **63**, 601–638
- 6 Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M. and Argraves, W. S. (1990) *J. Biol. Chem.* **265**, 17401–17404
- 7 Misra, U. K., Chu, C. T., Rubenstein, D. S., Gawdi, G. and Pizzo, S. V. (1993) *Biochem. J.* **290**, 885–891
- 8 Misra, U. K., Chu, C. T., Gawdi, G. and Pizzo, S. V. (1994) *J. Biol. Chem.* **269**, 12541–12547
- 9 Misra, U. K., Chu, C. T., Gawdi, G. and Pizzo, S. V. (1994) *J. Biol. Chem.* **269**, 18303–18306
- 10 Johnson, W. J., Pizzo, S. V., Imber, M. J. and Adams, D. O. (1982) *Science* **218**, 574–576
- 11 Hoffman, M., Feldman, S. R. and Pizzo, S. V. (1983) *Biochim. Biophys. Acta* **760**, 421–423
- 12 Uhing, R. L., Martenson, C. H., Rubenstein, D. S., Hollenbach, P. W. and Pizzo, S. V. (1991) *Biochim. Biophys. Acta* **1093**, 115–120
- 13 Salomon, D. S., Bano, M., Smith, K. B. and Kidwell, W. R. (1982) *J. Biol. Chem.* **257**, 14093–14101
- 14 Bonner, J. C., Badgett, A., Osorio-Vargas, A. R., Hoffman, M. and Brody, A. R. (1990) *J. Cell. Physiol.* **145**, 1–8
- 15 LaMarre, J., Wollenberg, G. K., Gonias, S. L. and Hayes, M. A. (1991) *Lab. Invest.* **65**, 3–14
- 16 Salvesen, G., Quan, L. T., Enghild, J. J., Snipas, S., Fey, G. H. and Pizzo, S. V. (1992) *FEBS Lett.* **313**, 198–202
- 17 Imber, M. J. and Pizzo, S. V. (1981) *J. Biol. Chem.* **256**, 8134–8139
- 18 Chu, C. T. and Pizzo, S. V. (1993) *J. Immunol.* **150**, 48–58
- 19 Yang, L., Baffy, G., Rhee, S. G., Manning, D., Hansen, C. A. and Williamson, J. R. (1991) *J. Biol. Chem.* **266**, 22451–22458
- 20 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 21 Bennett, F. C. and Crooke, S. T. (1987) *J. Biol. Chem.* **262**, 13789–13797
- 22 Lee, K. Y., Ryu, S. H., Suh, P.-G., Chol, W. C. and Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5540–5544
- 23 Katan, M. and Parker, P. J. (1987) *Eur. J. Biochem.* **168**, 413–418
- 24 Banno, Y., Yada, Y. and Nozawa, Y. (1988) *J. Biol. Chem.* **263**, 11459–11465
- 25 Pawelczyk, T. and Lowenstein, J. M. (1993) *Biochem. J.* **291**, 693–696
- 26 Nakanishi, O., Shibasaki, F., Hidaka, M., Homma, Y. and Takenawa, T. (1993) *J. Biol. Chem.* **268**, 10754–10759
- 27 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 28 Akiyama, T. and Ogawara, H. (1991) *Methods Enzymol.* **201**, 362–370
- 29 Yang, L. J., Rhee, S. G. and Williamson, J. R. (1994) *J. Biol. Chem.* **269**, 7156–7162
- 30 Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- 31 Graff, J. M., Rajan, R. R., Randall, R. R., Nairn, A. C. and Blackshear, P. J. (1991) *J. Biol. Chem.* **266**, 14390–14398
- 32 Harlan, D. M., Graff, J. J., Stumpo, D. J., Eddy, R. L., Jr, Shows, T. B., Boyle, J. M. and Blackshear, P. J. (1991) *J. Biol. Chem.* **266**, 14399–14405
- 33 Misra, U. K. and Sahyoun, N. (1987) *Biochem. Biophys. Res. Commun.* **145**, 760–768
- 34 Sahyoun, N., LeVine, H., Bronson, D. and Cuatrecasas, P. (1984) *J. Biol. Chem.* **259**, 9341–9344
- 35 Wise, B. C., Glass, D. B., Chou Jen, C.-H., Raynor, R. L., Katoh, N., Schatzmann, R. C., Turner, R. S., Kibler, R. F. and Kuo, J. F. (1982) *J. Biol. Chem.* **257**, 8489–8495
- 36 Prpic, V., Yu, S.-F., Figueiredo, F., Hollenbach, P. W., Gawdi, G., Herman, B., Uhing, R. J. and Adams, D. O. (1989) *Science* **244**, 469–471
- 37 Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G. and Carpenter, G. (1992) *J. Biol. Chem.* **267**, 10447–10456
- 38 Park, D. J., Min, H. K. and Rhee, S. G. (1992) *J. Biol. Chem.* **267**, 1496–1501
- 39 Meisenhelder, J., Suh, P.-G., Rhee, S. G. and Hunter, T. (1989) *Cell* **57**, 1109–1122
- 40 Majerus, P. W., Ross, T. S., Cunningham, T. W., Caldwell, K. K., Jefferson, A. B. and Bansal, V. S. (1990) *Cell* **63**, 459–465
- 41 Carpenter, G. and Wahl, M. I. (1990) *Handb. Exp. Pharmacol.* **247**, 7612–7672
- 42 Carpenter, G. and Cohen, S. (1990) *J. Biol. Chem.* **265**, 7709–7712
- 43 Ullrich, A. and Schlessinger, J. (1990) *Cell* **61**, 203–212
- 44 Aaronson, S. A. (1991) *Science* **254**, 1146–1153
- 45 Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J. and Rhee, S. G. (1991) *Cell* **65**, 435–441
- 46 Cantley, L. C., Auger, K. R., Carpenter, G., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) *Cell* **64**, 281–302
- 47 Hunter, T. and Karin, M. (1992) *Cell* **70**, 375–387
- 48 Margolis, B. L., Rhee, S. G., Felder, S., Mervic, M., Lyall, R. M., Levitski, A., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1989) *Cell* **57**, 1101–1107
- 49 Rhee, S. G. (1991) *Trends Biochem. Sci.* **16**, 297–301
- 50 Cross, M. and Dexter, T. M. (1991) *Cell* **64**, 271–280
- 51 Lamy, F., Wilkins, F., Baptist, M., Posada, J., Roger, P. P. and Dumon, J. E. (1993) *J. Biol. Chem.* **268**, 8398–8401
- 52 Cadena, D. L. and Gill, G. N. (1992) *FASEB J.* **6**, 2332–2337
- 53 Johnson, G. L. and Vaillancourt, R. R. (1994) *Curr. Opin. Cell Biol.* **6**, 230–238
- 54 Herskovitz, J. S., Burgess, C. C., Obar, R. A. and Vollee, R. B. (1993) *J. Cell Biol.* **122**, 565–578
- 55 Mohammadi, M., Dionne, C. A., Li, W., Li, N., Spivak, T., Honneaggar, A. M., Jaye, M. and Schlessinger, J. (1992) *Nature (London)* **358**, 681–684
- 56 Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del-Rosario, M., Mirda, D. and William, L. J. (1992) *Nature (London)* **358**, 678–681
- 57 Sorokin, A., Mohammadi, M., Huang, J. and Schlessinger, J. (1994) *J. Biol. Chem.* **269**, 17056–17061
- 58 Rubin, R. A., O'Keefe, E. J. and Earp, H. S. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 776–780
- 59 Grinstein, S., ed. (1988) *Na⁺/H⁺ Exchange*, p. 359, CRC Press, Boca Raton, FL
- 60 Lukacs, G. L., Kapus, A., Nauda, A., Romanek, R. and Grinstein, S. (1993) *Am. J. Physiol.* **265**, C3–C14
- 61 Boron, W. F. (1992) in *The Kidney: Physiology and Pathophysiology* (Seldin, D. W. and Gieblich, G., eds.), pp. 219–263, Raven Press, New York
- 62 Benos, D. J., McPherson, S., Hahn, B. H., Chaikin, M. A. and Benveniste, E. N. (1994) *J. Biol. Chem.* **269**, 13811–13816
- 63 Rozengurt, E. (1986) *Science* **234**, 161–166
- 64 Moolenaar, W. H. (1986) *Annu. Rev. Physiol.* **48**, 363–376
- 65 Simchowicz, L. (1985) *J. Biol. Chem.* **260**, 13248–13255
- 66 Swallo, C. J., Grinstein, S. and Rotstein, O. D. (1990) *J. Biol. Chem.* **265**, 7645–7654
- 67 Feldman, S. R., Gonias, S. L., Ney, K. A., Pratt, C. W. and Pizzo, S. V. (1984) *J. Biol. Chem.* **259**, 4458–4462
- 68 Libby, P., Raines, E. W., Cullinane, P. M. and Ross, R. (1985) *J. Cell. Physiol.* **125**, 357–366