Effects of weakly basic amines on proteolytic processing and terminal glycosylation of secretory proteins in cultured rat hepatocytes

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We examined the effects of weakly basic amines on the secretion and post-translational modifications of secretory proteins in cultured rat hepatocytes. Weakly basic amines such as methylamine, chloroquine and NH_4Cl strongly inhibited not only protein secretion, but also the proteolytic conversion of a proform of complement C3, allowing the precursor to be released into the medium. The amines, however, had no effect on the proteolytic conversion of prohaptoglobin into its subunits. Since available evidence indicates that the conversion of pro-C3 occurs at the Golgi complex while that of prohaptoglobin takes place in the endoplasmic reticulum, it is most likely that the weak bases specifically affect the proteolytic event occurring at the Golgi complex. Electron microscopic observations confirmed that the amines caused morphological changes of the Golgi complex, consisting of dilated cisternae and swollen vacuoles. When the glycosylation of α_1 -protease inhibitor and haptoglobin was examined, it was found that the amines caused a marked accumulation in the cells of both glycoproteins corresponding to the mature secreted forms. Neuraminidase digestion demonstrated that the glycoproteins accumulating in response to the amines had acquired terminal sialic acid. The results indicate that the amines do not significantly affect terminal glycosylation, in contrast with their definite effect on proteolytic processing, despite the fact that both modifications take place in the Golgi complex.

INTRODUCTION

Weakly basic amines such as chloroquine, methylamine and NH_4Cl are often referred to as lysosomotropic agents. At the time when the name was coined by de Duve and coworkers [1], only lysosomes were known as an acidic compartment in cells. However, there is accumulating evidence to show that not only lysosomes but also endosomes, coated vesicles and the Golgi complex have their own ATP-dependent proton pumps to maintain an acidic internal milieu [2–10]. A functional importance of acidic compartments has been demonstrated in the endocytic pathway with the aid of lysosomotropic amines. These amines are believed to get into acidic compartments and accumulate there as protonated forms, perturbing many processes such as lysosomal enzyme delivery, endocytosis of ligands and entry of some viruses [11,12].

In contrast with the detailed analysis of their effects on the endocytic pathway, the effects of weak amines on the secretory pathway have not been studied well. In a recent paper we have demonstrated that the proteolytic conversion of proalbumin in cultured hepatocytes was inhibited and proalbumin was released into the medium in the presence of weakly basic amines, and suggested the possibility that weakly basic amines could enter the Golgi complex and perturb its function [13]. Our results are compatible with recent findings by Anderson & Pathak [14] who have clearly shown that the *trans* Golgi cisternae and associated secretory vesicles are acidic.

In the present study we further examined the effects of

weak amines on the secretory process of newly synthesized proteins in cultured rat hepatocytes. We demonstrate that weak bases cause the swelling of the Golgi complex and block the proteolytic conversion of secretory protein precursors occurring within the Golgi complex.

MATERIALS AND METHODS

Materials

L-[4,5- ^3H]Leucine (58.4 Ci/mmol) and L-[^{35}S]methionine (1150 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.); chloroquine from Sigma Chemicals (St. Louis, MO, U.S.A.); methylamine, NH_4Cl and neuraminidase (*Arthrobacter ureafaciens*) from Nakarai Chemicals (Kyoto, Japan) and Immusorbin (fixed *Staphylococcus aureus* cells) from Wako Junyaku (Tokyo, Japan). Goat anti-(rat C3) serum was obtained from Cappel Laboratories (West Chester, PA, U.S.A.). Antibodies against rat α_1 -proteinase inhibitor [15] and haptoglobin [16] were raised in rabbits as described previously. Methylamine, NH_4Cl and chloroquine were dissolved in distilled water and adjusted to neutral pH with 1 M-NaOH before use (1 M for methylamine and NH_4Cl , and 5 mM for chloroquine).

Culture and labelling of hepatocytes

Hepatocytes were isolated from adult Wistar rats, weighing 200–250 g, by the collagenase perfusion

Abbreviations used: C3, the third component of complement; MEM, minimum essential medium; endo H, endo- β -N-acetylglucosaminidase H.

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Fig. 1. Dose effects of weakly basic amines on protein secretion

Rat hepatocytes (2×10^6 cells/dish) were preincubated for 30 min in the absence or presence of chloroquine (a), methylamine (b) or NH_4Cl (c). The cells were labelled for 10 min with $20 \mu\text{Ci}$ of $[^3\text{H}]$ leucine and chased. At the indicated times aliquots of media were taken and used for determination of the radioactivity in trichloroacetic acid-insoluble materials. Concentrations of each amine were as follows. In (a), 0 ($\circ$), $25 \mu\text{M}$ ($\triangle$), $50 \mu\text{M}$ ($\square$), $100 \mu\text{M}$ ($\bullet$) and $500 \mu\text{M}$ ($\blacktriangle$). In (b) and (c), 0 ($\circ$), 10 mM ($\triangle$), 20 mM ($\square$), and 50 mM ($\bullet$).

method [17]. Isolated hepatocytes (2×10^6 cells/dish) were cultured in 60-mm Falcon dishes pretreated with 0.1 mg of collagen/dish. Each dish contained 4 ml of Eagle's MEM [18], supplemented with 5% newborn calf serum, $0.1 \mu\text{M}$ -insulin, $1 \mu\text{M}$ -dexamethasone, and $60 \mu\text{g}$ of kanamycin/ml [19]. All the following experiments were performed using the cells after a 2-day culture.

The cells were preincubated at 37°C for 30 min in MEM lacking serum and unlabelled methionine in the absence or presence of amines at the indicated concentrations. The cells were then labelled for 2 h with $[^35\text{S}]$ methionine ($25 \mu\text{Ci}/1.5 \text{ ml}$ per dish). Media were collected and centrifuged at $3000 g$ for 10 min. The cells were washed three times with cold phosphate-buffered saline (pH 7.4) and lysed in 0.5 ml of 10 mM-Tris/HCl (pH 7.5) containing 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 150 mM-NaCl and 5 mM-EDTA, followed by centrifugation at $15000 g$ for 10 min. A mixture of protease inhibitors (leupeptin, antipain, elastinal, pepstatin A, chymostatin, phosphoramidon, and aprotinin) was added to cell lysates and media to a concentration of $10 \mu\text{g}$ of each/ml. In pulse-chase experiments, the cells were pulse-labelled for 10 min with $[^3\text{H}]$ leucine ($20 \mu\text{Ci}/2 \text{ ml}$ per dish) or with $[^35\text{S}]$ methionine ($100 \mu\text{Ci}/1.5 \text{ ml}$ per dish), and chased in fresh MEM containing the respective unlabelled amino acid. Amines, when indicated, were present throughout the pulse-chase periods. Cell lysates and media were prepared as above.

Immunoprecipitation

Cell lysates and media were pretreated with $30 \mu\text{l}$ of Immusorbin (10%, v/v) at 4°C for 30 min and centrifuged at $10000 g$ for 2 min. To each supernatant was added $4 \mu\text{l}$ of the indicated antiserum and the mixture was incubated at 4°C for 1 h, followed by additional

incubation with $100 \mu\text{l}$ of Immusorbin for 30 min. The samples were centrifuged at $10000 g$ for 2 min. Immusorbin pellets were washed five times with 10 mM Tris/HCl (pH 7.5) containing 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 150 mM-NaCl and 2 mM-EDTA, and once with 62.5 mM-Tris/HCl (pH 6.8). The immunocomplex was dissolved in $50 \mu\text{l}$ of 62.5 mM-Tris/HCl (pH 6.8) containing 1% SDS, 1% (v/v) 2-mercaptoethanol and 10% (w/v) glycerol by heating at 100°C for 3 min and frozen at -20°C until use (for SDS/polyacrylamide gel electrophoresis). For treatment with neuraminidase, the immunocomplex was dissolved in 1 mM-Tris/HCl (pH 7.5) containing the same other components as above.

Neuraminidase digestion

Frozen samples were thawed, boiled again for 3 min and centrifuged at $10000 g$ for 2 min. Each supernatant was diluted 10-fold with 50 mM-acetate buffer (pH 5.0). Digestion with neuraminidase (0.2 unit/ml) was carried out at 37°C for 24 h in the presence of 0.5% Triton X-100, 0.04% NaN_3 , 1 mM-phenylmethanesulphonyl fluoride and the mixture of protease inhibitors ($10 \mu\text{g}$ of each/ml). Proteins were precipitated with cold acetone containing 0.1M-HCl as described before [20] and dissolved in the buffer used for SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis

Frozen samples were thawed, boiled again for 3 min and centrifuged at $10000 g$ for 2 min. Samples were analysed by electrophoresis on SDS/polyacrylamide gels with the following concentrations (w/v): 7.5% for C3, 10% for α_1 -proteinase inhibitor and 12.5% for haptoglobin [21]. Gels were fixed and processed for fluorography as described previously [19].

Fig. 2. Effects of basic amines on the proteolytic conversion of pro-C3

Cells (2×10^6 cells/dish) were preincubated in the absence (lanes 1 and 2) or presence of 100 μ M-chloroquine (lanes 3 and 4) and 10 mM-methylamine (lanes 5 and 6), and labelled for 2 h with 25 μ Ci of [35 S]methionine. Cell lysates (lanes 1, 3 and 5) and media (lanes 2, 4 and 6) were prepared and used for immunoprecipitation of C3. The immunoprecipitates were analysed by electrophoresis on SDS/polyacrylamide gels (7.5%), followed by fluorography. Pro-C3, α and β denote the proform and α and β subunits of C3. M_r values of marker proteins are indicated at the left side of the gels; β' (180000), β (140000), α (100000) and α (42000) subunits of RNA polymerase B, and rat serum albumin (68000).

Electron microscopy

Hepatocytes cultured in the presence or absence of amines on collagen-coated glass slides were fixed in 2% (w/v) OsO_4 with Millonig's buffer (pH 7.3) for 2 h at 4 $^{\circ}\text{C}$. The specimens were then stained *en bloc* for 1 h at room temperature with 2% (w/v) uranyl acetate in distilled water. The specimens were dehydrated through an ascending series of ethanol and embedded in Spurr's resin [22]. Thin sections were cut, stained with lead citrate and uranyl acetate and examined in a Hitachi H-700 electron microscope.

RESULTS

Effects of weakly basic amines on protein secretion

Fig. 1 depicts the time course of protein secretion in the presence or absence of amines in cultured rat hepatocytes which were pulse-labelled with [^3H]leucine. All three weak amines (methylamine, chloroquine and NH_4Cl) retarded the secretion of the newly synthesized proteins in a dose-dependent manner. Chloroquine at 500 μ M apparently inhibited the secretion to an extent comparable with that with methylamine at 20 mM; however, protein synthesis was severely inhibited, causing detachment of some cells from the substratum. We therefore used chloroquine at 100 μ M in the following experiments. Protein synthesis was inhibited by 30% of the control level at 50 mM-methylamine or 50 mM- NH_4Cl . The

results indicate that all the amines used strongly inhibit protein secretion.

Effects of amines on the proteolytic conversion of pro-C3

Fig. 2 shows the effect of amines on the proteolytic processing of C3. As previously reported [23], C3 is synthesized as a single polypeptide chain (pro-C3) with M_r 180000 in rat hepatocytes (Fig. 2, lane 1), and proteolytically converted to the α (M_r 115000) and β (M_r 65000) subunits, which are secreted into the medium (lane 2). In the presence of either chloroquine or methylamine a new band which had the same mobility as that of the precursor in the cells appeared in the medium (lanes 4 and 6), indicating that the proteolytic conversion of pro-C3 was inhibited by these amines and the proform itself was released into the medium. Release of the precursor was confirmed at higher concentrations of NH_4Cl (results not shown). At the same concentration methylamine was more potent than NH_4Cl in inhibiting the conversion of pro-C3. This difference seems to be closely related to their potency to retard secretion (Fig. 1).

Effects of weak amines on the proteolytic conversion of prohaptoglobin

We carried out pulse-chase experiments in the presence or absence of methylamine at 20 mM to determine the effect on the proteolytic processing of prohaptoglobin (Fig. 3). After a 5 min pulse period, the detectable form was a proform with M_r 45000 (lane 1). However, the β subunit, with M_r 33000, appeared in the cells after 10 min pulse (lane 2), supporting the notion that this particular processing occurs at an earlier stage of intracellular transport before the protein reaches the Golgi complex [16]. Since the α subunit contains no methionine, it was not identified here. The terminal glycosylation of the β subunit, which was detected by increase of its M_r to 36000, became evident after 30 min of chase (lanes 3–5), suggesting that the β subunit with M_r 33000 had not yet arrived at the Golgi complex. In the presence of 20 mM-methylamine prohaptoglobin was still cleaved and the β subunit appeared at the same time schedule (lanes 8 and 9) as in the absence of the amine. Similar results were obtained at 50 mM-methylamine, which almost completely inhibited secretion (Fig. 1), or in the presence of chloroquine or NH_4Cl (results not shown). The results indicate that the proteolytic conversion of prohaptoglobin is not inhibited by these amines, in contrast with their inhibitory effect on the conversion of pro-C3 (Fig. 2) and proalbumin [13].

Effects of methylamine on the glycosylation of α_1 -protease inhibitor and haptoglobin

Rat α_1 -protease inhibitor is a glycoprotein containing 13.2% carbohydrate by weight [15]. In a previous report [19] we showed that α_1 -protease inhibitor is initially synthesized as a M_r 51000, endo H sensitive form and processed to a M_r 56000, terminally glycosylated mature form, which is the only form released into the medium. Fig. 4(a) shows the dose effect of methylamine on the glycosylation of α_1 -protease inhibitor. In the control culture, we could detect two forms, a major band with M_r 51000 and a minor band with M_r 56000, in the cells (lane 1) and only the latter form in the medium (lane 2). The intracellular M_r 56000 form has a relatively short

Fig. 3. Effects of methylamine on the proteolytic conversion of prohaptoglobin

Cells were preincubated in the absence (lanes 1–6) or presence (lanes 7–12) of 20 mM-methylamine, pulse-labelled with 100 μ Ci of [35 S]methionine for 5 min or 10 min and chased up to 2 h. Cell lysates (lanes 1–5 and 7–11) and media (lanes 6 and 12) were prepared and used for immunoprecipitation of haptoglobin. The immunoprecipitates were analysed by electrophoresis on SDS/polyacrylamide gels (12.5%), followed by fluorography. Lanes 1 and 7, cell lysates after 5 min pulse; lanes 2 and 8, cell lysates after 10 min pulse; lanes 3 and 9, cell lysates at 30 min chase; lanes 4 and 10, cell lysates at 60 min chase; lanes 5 and 11, cell lysates at 120 min chase; lanes 6 and 12, media at 120 min chase. M_r values of marker proteins at the left side are: rat serum albumin (68000), ovalbumin (46000), α (42000) and β (38000) subunits of RNA polymerase B, porcine trypsin (23000) and soya-bean trypsin inhibitor (21000). Pro and β denote the proform and β subunit of haptoglobin.

Fig. 4. Effects of methylamine on the glycosylation of α_1 -protease inhibitor

(a) Cells were preincubated in the absence (lanes 1 and 2) or presence of 10 mM- (lanes 3 and 4) and 20 mM- (lanes 5 and 6) methylamine, and labelled with [35 S]methionine for 2 h. Cell lysates (lanes 1, 3 and 5) and media (lanes 2, 4 and 6) were prepared and used for immunoprecipitation. The immunoprecipitates were analysed by electrophoresis on SDS/polyacrylamide gels (10%), followed by fluorography. (b) The samples were incubated in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of neuraminidase as described in the Materials and methods section, and analysed as above. Cell lysates (lanes 1 and 2) and media (lanes 3 and 4) from the control culture; cell lysates (lanes 5 and 6) and media (lanes 7 and 8) from the culture in the presence of 20 mM-methylamine. Marker proteins are: rat serum albumin (68000), the heavy chain (55000) of IgG and ovalbumin (46000).

half time, and once formed it is rapidly released into the medium [19]. In the presence of 10–20 mM methylamine we found a marked accumulation of the M_r 56000 form in the cells (lanes 3 and 5). Although the intracellular accumulation of this form was also observed in the presence of microtubule-affecting agents such as colchicine [19] and taxol [24], it was more prominent in the presence of the amine.

The intracellular and secreted forms were characterized by their response to neuraminidase digestion (Fig. 4b). Upon digestion of the intracellular forms from the

control culture, the M_r 56000 form disappeared and the M_r 51000 form increased its intensity (lanes 1 and 2), demonstrating that the M_r 56000 form was shifted to the same position as the latter after its desialylation. The M_r 56000 form accumulated intracellularly (lanes 5 and 6) as well as secreted (lanes 7 and 8) in the presence of methylamine were also completely sensitive to neuraminidase. These results indicate that the terminally sialylated α_1 -protease inhibitor was caused to accumulate intracellularly by the amine and finally secreted into the medium.

Fig. 5. Effects of methylamine on the glycosylation of haptoglobin

Cells were preincubated in the absence (lanes 1–4) or presence of 20 mM-methylamine (lanes 5–8), and labelled with [35 S]methionine for 2 h. Immunoprecipitates were prepared from cell lysates (lanes 1, 2, 5 and 6) and media (lanes 3, 4, 7 and 8). The samples were further incubated in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of neuraminidase as described in the Materials and methods section, and analysed by electrophoresis on SDS/polyacrylamide gels (12.5%), followed by fluorography.

Fig. 5 shows the effect of methylamine on the glycosylation of haptoglobin. In the absence of the amine, two forms of the β subunit, a major one with M_r 33000 and a minor with M_r 36000, were found intracellularly (lane 1), and only the M_r 36000 form was secreted into the medium (lane 3). In the presence of 20 mM-methylamine, the mature form with M_r 36000 markedly accumulated within the cells (lane 5), and was finally secreted (lane 7). Both the intracellular and secreted M_r 36000 forms were found to be sensitive to the neuraminidase treatment (lanes 6 and 8) as observed for those obtained from the control culture (lanes 2 and 4). Taken together, these results indicate that the terminal glycosylation of haptoglobin as well as α_1 -protease inhibitor proceeds almost normally and the sialylated glycoproteins accumulated intracellularly in the presence of 20 mM-methylamine. A similar accumulation of the mature glycoproteins was observed in the presence of chloroquine and NH_4Cl (results not shown).

Effect of weak bases on the morphology of the Golgi complex

Fig. 6 shows an electron micrograph of hepatocytes incubated with 10 mM-methylamine. As early as 30 min of the treatment, there appeared many large vacuoles and distended cisternae in the Golgi region. Although it is difficult to tell exactly from which organelles these vacuoles arose, many vacuoles must be derived from the Golgi elements, since some vacuoles were found to be associated with the dilated cisternae, possibly the *trans* elements of the Golgi complex. Another type of vacuoles containing remnants of membrane structures, which were easily identified as those derived from the secondary lysosomes, were observed only at a later stage of the treatment. A similar morphological alteration with many vacuoles and partially dilated Golgi cisternae was also observed in the cells treated with other weak amines, chloroquine and NH_4Cl (results not shown).

Fig. 6. Electron micrograph of the methylamine-treated cells

Hepatocytes were incubated at 37 °C with 10 mM-methylamine for 30 min, and processed for electron microscopic observation. Detail of the Golgi region is shown in high magnification ($\times 22500$).

DISCUSSION

Weakly basic amines have been successfully used as a perturbant of lysosomes and endosomes, highlighting the physiological importance of acidic compartments in the endocytic pathway [12,25]. We took the same strategy in order to show that the Golgi complex is another acidic compartment in cells and maintenance of its acidity is also important in the secretory process. Recently, by use of elegant techniques Anderson & Pathak [14] and Schwartz *et al.* [26] have demonstrated evidence that amines enter the cisternae and vesicles at the *trans* region of the Golgi complex, which favours our approach in the present study.

All the weak amines used here impaired protein secretion, resulting in an accumulation of the newly synthesized proteins in the cells. Electron microscopic observation revealed that the weak amines caused the dilation and swelling of the Golgi elements surrounded with many vacuoles (Fig. 6). Wibo & Poole [27] reported similar morphological changes of the Golgi complex in rat fibroblasts in response to chloroquine. These morphological changes suggest that the amines enter the Golgi complex, are protonated and accumulate there as in the case of lysosomes and endosomes, resulting in a rise of the luminal pH of the Golgi complex and eventually its swelling.

In the previous paper we reported that in the presence of amines the proteolytic conversion of proalbumin was inhibited and proalbumin itself was released into the medium [13]. The present study further demonstrated that the proteolytic processing of pro-C3 was also inhibited by the amines (Fig. 2). C3 is synthesized as a single polypeptide chain precursor, which is intracellularly cleaved into the subunits at the paired basic amino acid residues adjacent to the *N*-terminus of the α subunit [28]. This mode of processing is closely akin to many polypeptide hormones, neuropeptides and proalbumin, and is thought to occur in the Golgi complex [29,30]. In contrast, the weak amines failed to inhibit the proteolytic conversion of prohaptoglobin (Fig. 3) which presumably takes place in the endoplasmic reticulum

[16]. Taken together, these results strongly suggest that weakly basic amines specifically impede the proteolytic events occurring in the Golgi complex in the secretory pathway.

Another major change induced by the amines was the intracellular accumulation of the mature forms of α_1 -protease inhibitor and haptoglobin (Figs. 4 and 5). Digestion with neuraminidase demonstrated that the accumulated glycoproteins were sialylated, as were the secreted forms. Several lines of evidence indicate that sialylation of the asparagine-linked oligosaccharide chains occurs at the *trans* (outermost) cisternae of the Golgi complex [31,32]. Thus, it is most likely that these glycoproteins reach the *trans* acidic compartment of the Golgi complex, acquire the terminal sugars and accumulate there in the presence of the amines.

The question is why terminal glycosylation proceeds almost normally in the presence of the amines while proteolytic processing is strongly inhibited under the same conditions. Two possibilities may be considered to explain the discrepancy. Firstly, if the putative protease had a narrow optimal pH range as compared with those of glycosyltransferases, the proteolytic processing would be more sensitive to the alkalization caused by the amines. Secondly, if the secretory glycoproteins were loosely associated with the Golgi membranes, as suggested for the situation in the endoplasmic reticulum [33], then the dilution effect caused by the amines would be less for the glycoproteins but crucial for albumin. This would also account for the difference in sensitivity of the proteolytic conversion between proalbumin and the glycoprotein pro-C3; the conversion of the former was more sensitively inhibited by the amines than was that of the latter (results not shown).

The effects of the amines on the proteolytic conversion of the three different precursors are quite similar to those of the carboxylic ionophore monensin we previously reported [16,19,34,35]. These findings raise a possibility that the mechanism by which weakly basic amines cause morphological and functional perturbation of the Golgi complex might be the same as that considered for monensin. However, the molecular forms of α_1 -protease inhibitor and the haptoglobin β subunit secreted in the presence of monensin were found to be immature forms with M_r 51000 [19] and 33000 [16], respectively. Analyses of the oligosaccharide chains showed that their oligosaccharides were composed of rather heterogenous components largely devoid of sialic acid residues (S. Ogata, Y. Misumi & Y. Ikehara, unpublished results). Obviously further studies are required to understand the mechanism underlying the disparate effects of the weak amines and monensin on the terminal glycosylation of glycoproteins and differential effects of the weak amines on proteolytic processing and terminal glycosylation. Nevertheless, the use of weakly basic amines proves valuable for detailed analysis of the complex functions of the Golgi elements, as presented in this study.

Recently Strous *et al.* [36] have reported the effect of lysosomotropic amines on the secretory pathway in human hepatoma cells. In their report the secretion of albumin, orosomucoid and transferrin was shown to be impaired concomitant with the vacuolization of the Golgi complex in the presence of NH_4Cl and primaquine. These findings are in accordance with our results presented here.

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