Effects of cycloheximide and tunicamycin on cell surface expression of pancreatic muscarinic acetylcholine receptors

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The importance of glycosylation in cell surface expression of muscarinic receptors in cultured guinea pig pancreatic acini was investigated. Recovery of the muscarinic receptor population after carbachol-induced down regulation was blocked by cycloheximide but not by tunicamycin, although tunicamycin reduced [^{3}H]mannose incorporation into acinar macromolecules by up to 90%. Tunicamycin treatment also failed to alter carbachol stimulation of amylase secretion from cultured acini. These results indicate that glycosylation of the glandular subtype of muscarinic receptor in the pancreatic acinar cell is not necessary for its insertion in the plasma membrane or for its functional activity.

Carbachol; Muscarinic acetylcholine receptor; Pancreatic acini; Cycloheximide; Tunicamycin; Glycosylation

1. INTRODUCTION

Cholinergic agonists are potent stimulators of digestive enzyme secretion in the exocrine pancreas, an effect mediated by muscarinic acetylcholine receptors [1,2]. Recent pharmacological and molecular biological studies have indicated that glandular muscarinic receptors constitute a separate subtype (M3) that is distinct from both the cardiac M2 subtype and the M1 subtype that predominates in the central nervous system [3-5]. Both neuronal and myocardial muscarinic receptors have been shown to be glycoproteins [6,7], and sequence information predicts the presence of at least 4 sites for N-linked glycosylation in the M3 receptor subtype [5]. Liles and Nathanson [8] have shown that glycosylation of muscarinic receptors in mouse NE-115 neuroblastoma cells is important for maintenance of the plasma membrane receptor population. Comparable studies have not been carried out on exocrine gland cells. We therefore have examined the effects of tunicamycin, an inhibitor of N-glycosylation, as well as cycloheximide, on the recovery of muscarinic receptors that follows down regulation elicited by the cholinergic agonist carbachol in cultured guinea pig pancreatic acini when the agonist is withdrawn.

2. MATERIALS AND METHODS

Suspensions of dispersed acini were prepared from the pancreases of fasted male guinea pigs as described previously [9]. Dispersed acini were cultured at 37°C for up to 48 h in a defined medium consisting of DME/F12 supplemented with insulin, transferrin, selenium, bovine serum albumin, trypsin inhibitor, epidermal growth factor, ascorbate, Hepes (pH 7.4) and antibiotics [9]. In most experiments, acini were cultured first for 18-24 h with 0.1 mM carbachol to down regulate the muscarinic receptor population, rinsed, and recultured for an additional 24 h as above in the absence of drugs or in the presence of carbachol, tunicamycin, or cycloheximide. At the end of this second culture period, acini were collected and assayed for secretory responsiveness, [^{3}H]mannose and [^{3}H]leucine incorporation, and binding of the muscarinic antagonist [^{3}H]*N*-methylscopolamine (NMS).

For [^{3}H]NMS binding, acini were resuspended in 5 ml of a Hepes-buffered Ringers solution (pH 7.4) containing 0.1% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 0.5 nM [^{3}H]NMS with or without 10 μM atropine. Acinar suspensions were incubated with the labelled antagonist for 60 min at 37°C, collected on Whatman GF/A glass fiber filters, rinsed, and counted as previously described [9]. Specific [^{3}H]NMS binding was calculated as the difference in binding in the absence and presence of atropine. Specific binding of the antagonist was related to DNA content, as determined by the diphenylamine procedure [10].

Effects of tunicamycin, carbachol, and cycloheximide on incorporation of [^{3}H]mannose and [^{3}H]leucine into acinar macromolecules was determined during the second culture period. Acini were cultured in the presence of 2 μCi/ml of [^{3}H]mannose or 0.2 μCi/ml of [^{3}H]leucine in the absence of drugs, in the presence of 0.1 mM carbachol or cycloheximide, or in the presence of 0.01-10 μg/ml of tunicamycin. After 24 h of culture, acini were collected, rinsed, and resuspended and sonicated in distilled water. An equal volume of cold 20% trichloroacetic acid was added and after 10 min at 40°C, precipitated protein was pelleted by centrifugation. After a second resuspension and rinse in cold 10% trichloroacetic acid, precipitated material was solubilized and counted. Incorporation of the two labelled precursors was normalized to DNA values.

To assess possible effects of deglycosylation of acinar muscarinic receptors on cellular secretory responsiveness to carbachol, acini were incubated in the absence and presence of 3 μg/ml of tunicamycin during the second 24 h culture period. At the end of this period, both groups of acini were resuspended in Ringers solution as above containing a range of carbachol concentrations and incubated for 30 min

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at 37°C. Amylase released during this period was determined using Procian yellow starch [11] and expressed as a percentage of total cellular amylase content.

3. RESULTS

3.1. *Inhibition of recovery of [^{3}H]N-methylscopolamine binding to acini*

We previously showed that the size of the muscarinic receptor population in cultured guinea pig pancreatic acini was decreased by 90% after 18 h of exposure to 0.1 mM carbachol [9]. Removal of the agonist from the culture environment allowed the receptor population to recover, so that by 24 h after carbachol was withdrawn the number of cell surface receptors present was increased several-fold. In the present work, we determined the effects of cycloheximide and tunicamycin on this recovery. Inhibitory effects of cycloheximide on the increase in [^{3}H]NMS binding seen after removal of carbachol were first noticeable at 10 nM and appeared to be maximal at 0.1 mM (Fig. 1). The IC_{50} for this effect was approximately 0.1 μM. By contrast, tunicamycin caused only a small reduction in recovery of [^{3}H]NMS binding sites (Fig. 2), even at quite high concentrations.

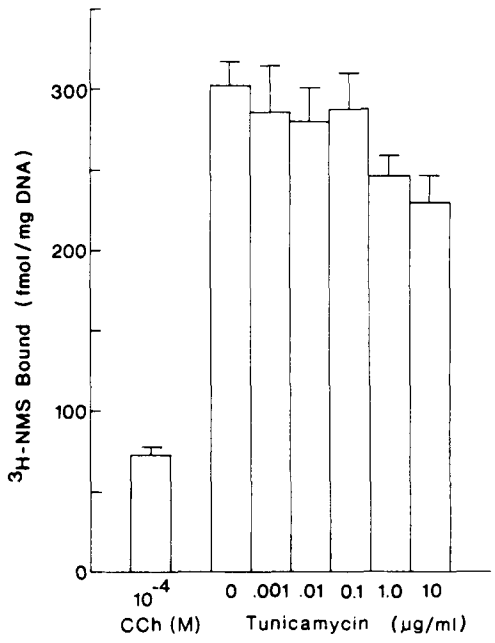

Fig. 2. Effect of tunicamycin on recovery of muscarinic receptors in pancreatic acini after carbachol-induced down regulation. Acini were cultured and [^{3}H]NMS binding was determined as in Fig. 1. Results represent means ± SE of 4 experiments.

3.2. *Effects of tunicamycin on [^{3}H]mannose and [^{3}H]leucine incorporation*

Since tunicamycin caused only a small inhibition of the recovery of [^{3}H]NMS binding sites, we assessed its effects on both mannose and leucine incorporation into acinar cell macromolecules (Fig. 3). Tunicamycin had a dramatic inhibitory effect on [^{3}H]mannose incorporation, reducing it by 85–90% at a concentration of 10 μg/ml. By contrast, incorporation of [^{3}H]leucine was only decreased by 20–25% at this concentration. These results clearly demonstrate the inhibitory specificity of

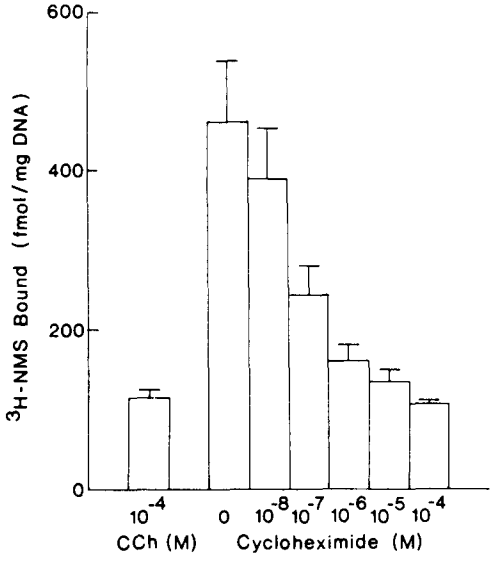

Fig. 1. Effect of cycloheximide on recovery of muscarinic receptors in pancreatic acini after carbachol-induced down regulation. Acini were cultured for 18 h with 0.1 mM carbachol, then for 24 h in the continued presence of the agonist carbachol (CCh), in the absence of drugs, and in the presence of various concentrations of cycloheximide. [^{3}H]NMS binding to acini was determined at the end of the second culture period. Results represent means ± SE of 3 experiments.

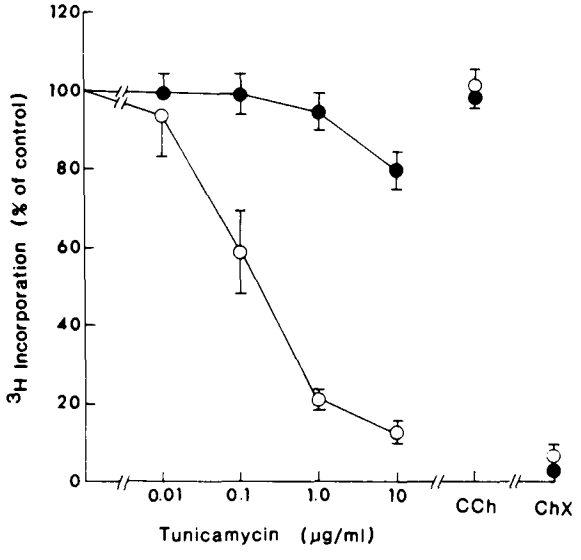

Fig. 3. Effects of tunicamycin, chcloheximide (ChX), and carbachol (CCh) on [^{3}H]mannose (○) and [^{3}H]leucine (●) incorporation into macromolecules in pancreatic acini. Acini were cultured as in Fig. 1 for 18 h with 0.1 mM carbachol, then for 24 h with 0.1 mM carbachol, 0.1 mM cycloheximide, or varying concentrations of tunicamycin. [^{3}H]mannose or [^{3}H]leucine was included in the culture medium during the second culture period. Results represent means ± SE of 4 experiments.

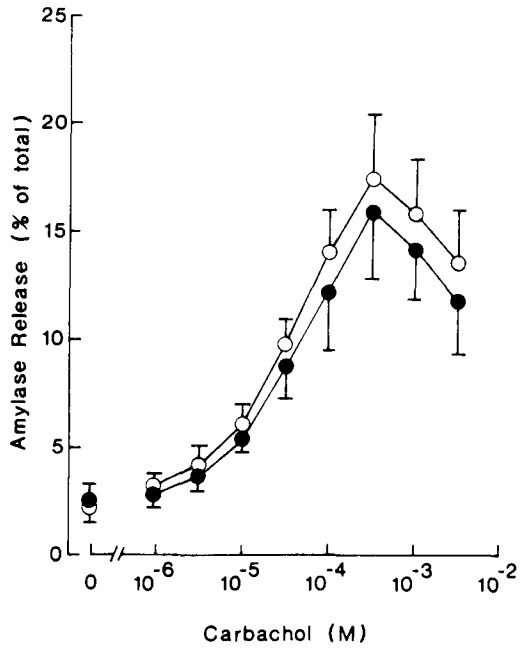

Fig. 4. Effect of tunicamycin treatment on secretory responsiveness of pancreatic acini. Acini were cultured as above with carbachol for 18 h and then for 24 h either with no drugs (●) or with 3 μg/ml of tunicamycin (○). At the end of this second culture period, the ability of both groups of acini to secrete amylase in response to a range of carbachol concentrations was assessed. Results represent means ± SE of 5 experiments.

tunicamycin and suggest that the small decrease in [^{3}H]NMS binding seen at high tunicamycin concentrations (Fig. 2) results from a general inhibitory effect on protein synthesis. As expected, 0.1 mM cycloheximide inhibited nearly all incorporation of both mannose and leucine, although carbachol had no effect on either.

3.3. *Effect of tunicamycin on carbachol-induced amylase secretion*

A possible effect of tunicamycin on the functional properties of acinar muscarinic receptors also was probed by comparing the ability of carbachol to stimulate digestive enzyme release from tunicamycin-treated and control acini (Fig. 4). In both groups of acini, carbachol evoked a biphasic release of amylase, from a basal level of 2–3% to 16–18% at a concentration of 0.3 mM. The EC_{50} for stimulation of amylase release from both groups of acini was approximately 30 μM.

4. DISCUSSION

This study demonstrates that in the pancreatic acinar cell, tunicamycin does not prevent the reappearance of muscarinic receptors at the cell surface during recovery of the receptor population from agonist-induced down regulation. Evidence that the increase in [^{3}H]NMS binding sites does in fact represent integration of new receptor protein into the acinar cell plasma membrane is two-fold. First, [^{3}H]NMS is a quaternary muscarinic antagonist of low lipid solubility and there is now considerable evidence that it labels only cell surface receptors [12]. A second line of evidence is illustrated in Fig. 4. When guinea pig pancreatic acini are cultured for 18–24 h in the presence of carbachol, their secretory responsiveness is virtually nil [9]. Yet after 24 h of subsequent culture in the absence of the agonist, they are again able to respond in a vigorous fashion, whether or not tunicamycin is included in the culture medium. The fact that these newly synthesized receptors can mediate carbachol stimulation of amylase release indicates that they are located in the plasma membrane and are in functional association with the complex of primary effectors that generates appropriate intracellular messengers. These results also suggest that exposure of acini to tunicamycin does not alter the agonist binding properties of the pancreatic muscarinic receptor, as is also the case for the reticulocyte beta adrenergic receptor [13].

Although it has been demonstrated [8] that disruption of normal glycosylation pathways in a mouse neuroblastoma cell line with tunicamycin also decreased cell surface expression of muscarinic receptors, this does not appear to be the case for pancreatic acinar cells. These observations may reflect cell-specific differences in processing pathways or differences characteristic of the particular muscarinic receptor subtypes expressed. The cell surface expression of some other integral plasma membrane proteins, including receptors for insulin [14], have been shown to be sensitive to tunicamycin, although Zamofing and colleagues [15] recently showed that deglycosylation of the nascent beta subunit of Na^+,K^+-ATPase in toad urinary bladder cells with tunicamycin does not prevent its insertion into the plasma membrane. Results presented in the present study indicate that glycosylation of the M3 muscarinic receptor subtype in the pancreatic acinar cell is not a prerequisite for its proper insertion in the plasma membrane and that glycosyl residues on the receptor do not play an important role in its ability to interact with agonists.

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