MECHANISM OF AGONIST-EVOKED DOWN REGULATION OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN CULTURED RAT PANCREATIC ACINAR CELLS

By

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ABSTRACT

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Exposure of pancreatic acinar cells to cholinergic agonists causes down regulation of muscarinic receptors. To study this response, rat pancreatic acini were cultured with carbachol and its effects on binding of [^{3}H]N-Methylscopolamine (NMS) and [^{3}H]Scopolamine were examined. [^{3}H]NMS labels only cell surface muscarinic receptors, while [^{3}H]Scopolamine also labels intracellular muscarinic receptors. Exposure of acini to carbachol for 24 h caused disappearance of 90% of [^{3}H]NMS and [^{3}H]Scopolamine binding sites. Of 13 compounds tested, only four affected this carbachol-induced down regulation. The protein kinase inhibitors staurosporine and W-7 each slightly inhibited agonist-evoked decreases in binding sites for both radioligands. The lysosomotropic agents, methylamine and ammonium chloride, each inhibited disappearance of [^{3}H]Scopolamine binding sites by 80%, but only slightly inhibited disappearance of [^{3}H]NMS binding sites, suggesting that an endosomal/lysosomal pathway is involved in the degradation of internalized pancreatic muscarinic receptors.

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I would like to dedicate this work to the memory of my paternal grandmother, Virginia Rose Kovalcik.

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BACKGROUND

Pancreatic Anatomy

The pancreas is one of the major accessory organs of the digestive tract, lying in the abdomen caudal to the stomach and juxtaposed between the duodenum on the right and the spleen on the left. It consists of two functional portions; the exocrine pancreas, which secretes digestive enzymes and bicarbonate into the duodenum, and the Islets of Langerhans, which produce several hormones including insulin and glucagon.

The exocrine portion of the pancreas consists of two cell types; acinar cells and ductal cells. Together they make up about 85% of the cellular volume of the gland; with the ductal cells comprising only a small fraction of this total (2-4%). The acinar cells secrete 25-30 digestive enzymes in conjunction with an isotonic plasma ultrafiltrate. These enzymes include chymotrypsinogen, trypsinogen, proelastase, procarboxypeptidase, amylase, phospholipase A2 and ribonuclease. The main functions of the epithelial cells of the excretory duct system are to serve as a conduit for acinar secretions and to modify the initial acinar plasma ultrafiltrate by the addition of bicarbonate and water (Gorelick and Jamieson, 1987).

The Islets of Langerhans comprise only about 2% of the cell volume of the entire gland. These islets are distributed throughout the pancreas and are comprised of three different cell types: beta cells, delta cells and alpha cells. The beta cells produce insulin and are located innermost within the islets. The delta cells produce somatostatin and pancreatic polypeptide and are juxtaposed between the beta and alpha cells, which manufacture and secrete glucagon and are located on the periphery of the islets (Pelletier, 1977).

The anatomy of the exocrine pancreas is functionally related to its primary physiological role, the secretion of pancreatic juice into the duodenum. It's smallest functional unit is the acinus, which consists of up to 100 acinar cells polarized about a common luminal space. Emerging from each acinus is an intercalated duct. The duct cells forming the head of the intercalated duct and lining the acinar lumen are termed centroacinar cells. Intercalated ducts anastomose to form intralobular ducts, which in turn anastomose to form a larger conduit called an interlobular duct. Several interlobular ducts join to form the main pancreatic duct which runs the entire length of the pancreas. The latter joins the common bile duct in the region of the pancreas in close proximity to the duodenum. Here the two structures join to form a new structure termed the Ampulla of Vater, which empties into the duodenum of the small intestine at a site refered to as the duodenal papilla of Vater. This latter structure is located a short distance distal to the pyloric

sphincter (Bockman, 1986).

The pancreas receives most of its blood supply from two principal arteries, the celiac and superior mesenteric, which arise from the abdominal aorta. A smaller portion of the supply comes from the hepatic artery. The venous return of blood from the pancreas is via the superior mesenteric, splenic and pancreaticodoudenal veins, which anastomose to form the hepatic portal vein (Bockman, 1986). All the returning blood supplied to the Islets of Langerhans, which compromises about 11-23% of the entire arterial supply, drains into capillaries perfusing the acini before exiting through the pancreatic veins (Lifson, et. al., 1980).The existence of this islet-to-acinus portal system has led to the concept of a functional paracrine regulatory axis between the endocrine and exocrine portions of the pancreas (Williams and Goldfine, 1986).

Pancreatic Innervation

The pancreas receives innervation from the autonomic nervous system. The sympathetic division of the autonomic nervous system originates within the splanchnic nerve fibers. The innervation from the parasympathetic portion of the autonomic nervous system is via the vagus nerves. A large proportion of the nerve fiber tracks entering the pancreas follow the initial routes laid down by the vasculature supplying the gland. Both of the divisions of the autonomic nervous system send their preganglionic nerve fibers through

the celiac plexus.

All of the preganglionic sympathetic nerves synapse with neurons within this plexus. The majority of the post-ganglionic sympathetic nerve fibers emerging from the celiac plexus then form secondary perivascular plexuses surrounding the vasculature (Richins, 1945). The remaining postganglionic sympathetic fibers synapse in intra-pancreatic ganglia with noncholinergic-nonadrenergic nerves (Alm, et. al., 1967). While none of these postganglionic adrenergic fibers have been shown to innervate acinar cells, they have been identifed in close proximity with the ductal network of the gland (Legg, 1968), and with arterioles supplying pancreatic capillary beds (Barlow, et. al., 1971).

The preganglionic parasympathetic nerves contained in the vagal nerve tracks do not synapse with neurons within the celiac plexus, but rather pass through this plexus, where most of their number synapse with postganglionic cholinergic neurons that lie within the intrapancreatic ganglia. The remainder synapse with noncholinergic-nonadrenergic neurons within these ganglia. The postsynaptic parasympathetic nerves then innervate acini and ducts within the pancreas (Richins, 1945).

The noncholinergic-nonadrenergic nerves issue from the intrapancreatic ganglia and terminate on ducts and arterioles suppling pancratic capillaries. These nerve terminals have been demonstrated to contain vasoactive intestinal peptide (VIP), a putative neurotransmitter (Holst, et. al., 1979;

Fahrenkrug, 1979). Still other nerve terminals of this kind are located in close proximity to acini and contain gastrin-releasing peptide, another putative neurotransmitter (Holst, 1986).

Recently, an entero-pancreatic nerve network has been demonstrated (Kirchgessner and Gershon, 1990). This system contains afferent nerves arising from myoenteric neurons within the antrum and pyloric regions of the stomach and from the duodenum that synapse in the intrapancreatic ganglia as well. These nerves may represent the afferent pathway of stimulation during the gastric phase of pancreatic secretion and also may be responsible for a portion of the stimulus signaling during the intestinal phase of secretion. This nerve complex also may serve in the regulatory feedback on the pancreas during the circulatory-humoral phase of postprandial secretion.

Phases of Pancreatic Secretion

Exocrine pancreatic secretion occurs both during the interdigestive period and postprandially. The interdigestive phase of secretion occurs only when all chyme has been digested and vacated from the stomach, and has been absorbed and removed from the small intestine. This basal level of secretion *in vivo* is dependent on parasympathetic input from cholinergic nerve fibers, since administration of atropine inhibits nearly all enzyme secretion during this phase (Defillipi, et. al., 1982). This atropine blockade is

probably effected at the level of muscarinic acetylcholine receptors located on acinar cells. These muscarinic receptors appear to be tonically stimulated, due to the release of acetylcholine from postsynaptic parasympathetic nerve fibers. The basal level of bicarbonate secretion from the ductal network of the pancreas is correlated to circulating basal plasma levels of the hormone secretin and is likewise potentiated by cholinergic input. This neural contribution to basal levels of secretion of bicarbonate has been shown by the significant decrease in the rate of secretion elicited by atropine even in the presence of secretin (Osnes, et. al., 1979). Basal secretory periods are cyclic in nature and occur repetitively every one-to-two hours as long as the fasting state continues (Dimagno, et. al., 1979; Owyang, et. al., 1983). Each repetitive secretory burst of activity has a relatively short duration and conicides with the occurance of the interdigestive migrating myoelectric complex (Dimagno, et. al., 1979). In the intact organism, administration of atropine or other muscarinic receptor antagonists causes an inhibition of these repetitive phases of basal secretion during the fasting state (Magee and Naruse, 1983).

The exocrine pancreas also exhibits an integrated postprandial period of secretion. This period is necessary for the efficient digestion of food into its basic components of amino acids, short chain saccharides, triglycerides and fatty acids. This integrated response to ingestion of a meal is traditionally divided into three phases; cephalic, gastric

and intestinal. There is a developing body of evidence for a fourth phase (circulatory-humoral), in which there may be feedback regulation on pancreatic secretion due to the presence of absorbed nutrients and hormones in the plasma.

The cephalic phase of pancreatic secretion results from processed stimuli within the central nervous system, such as visualization of food, olfaction, and taste of the food to be ingested, as well as physical mastication and swallowing. This includes direct feedback information from mechano- and chemoreceptors located within the mouth, tongue, pharynx, esophagus and nasal passages, as well as visual receptors in the retina. The combined efferent neural pathways for responses to these stimuli apparently lie within the vagus nerve fiber tracks previously described. This view is supported by several lines of evidence, including augmentation of pancreatic secretion by direct electrical stimulation of the vagus nerve fibers (Kaminski, et. al., 1975). Administration of muscarinic receptor agonists induces secretion of pancreatic juice at a level similar to that seen in sham fed animals (Lin and Grossman, 1956; Brommelaer, et. al., 1981). Further administration of cholinergic antagonists such as atropine blocks pancreatic secretion in response to sham feeding (Anagostides, et. al., 1984).

The gastric phase of pancreatic secretion is due primarily to the distension of the gastric wall of the stomach due to the physical presence of ingested food. This response is mediated by a vagovagal reflex and a gastro-

pancreatic reflex located within the entero-pancreatic local nerve network previously described (Kirchgessner and Gershon, 1990; Blair, et. al., 1966; Debas and Yamagishi, 1978). Support for a vagovagal reflex is shown by administering atropine or by truncal vagotomy, which both abolish the stimulation of pancreatic juice flow in response to gastric wall distension (White, et. al., 1963; White, et. al., 1962). Afferent nerves also exist between the stomach and the pancreas, and this entero-pancreatic complex has been implicated in the gastro-pancreatic reflex (Kirchgessner and Gershon, 1990; Debas and Yamagishi, 1978). The other component of gastric phase stimulation is hormonal in nature and is thought to be dependent on gastrin. Gastrin is released from G cells located in the pyloric region of the stomach in reponse to the presence of amino acids or peptides in the stomach lumen and to direct vagal stimulation activated in response to gastric wall distension. Local distension of the distal portion of the stomach of a vagotomized cat or the placement of solutions of amino acids and peptides in the stomach elicits the release of gastrin and stimulates pancreatic secretion (Blair, et. al., 1966). Administration of purified gastrin <u>in vivo</u> also produces a pancreatic level of secretion that is significantly increased above the basal level (Preshaw, et. al., 1965). However, normal circulating levels of gastrin during this phase of secretion do not appear to be sufficient under physiological conditions to produce the levels of pancreatic secretion

recorded These effects of gastrin seem to be acting through a weak interaction with cholecystokinin (CCK) receptors located on acinar cells (Solomon, 1987).

The intestinal phase of pancreatic secretion accounts for the greatest percentage of the total volume of both bicarbonate and enzyme secretion in response to a meal. In the duodenum, acidic chyme acts as the major stimulus for the release of secretin from S cells. Secretin chiefly appears to stimulate the pancreatic ductal cells to secrete a bicarbonate-rich fluid (Schaffalitzky de Muckadell, et. al., 1981; Meyer, et. al., 1970; Faichney, et. al., 1981). Also within the duodenum, fats, amino acids, and peptides act as the major stimulants for the release of cholecystokinin from I cells. This secretagogue interacts with receptors located on acinar cells to induce pancreatic enzyme secretion (Harper and Raper, 1943; Petersen and Grossman, 1977). Cholecystokinin also has been shown to potentiate bicarbonate secretion from duct cells induced by secretin, although the mechanism of this effect is unknown (Konturek, et. al., 1971a; Fink, et. al., 1982).

There also may be a negative feedback mechanism regulating pancreatic enzyme secretion during the intestinal phase. This concept is supported by studies in rats where stimulation of pancreatic enzyme flow was evoked upon removal of pancreatic enzymes from the small intestinal lumen and upon infusion of proteolytic inhibitors into the lumen (Green and Lyman, 1971; Green and Lyman, 1972).

There also is mounting evidence for a direct role of the entero-pancreatic nerve network in the intestinal phase of pancreatic secretion. This phase of pancreatic secretion is inhibited by infusion of muscarinic receptor anatagonists, even in the presence of luminal HCl (Konturek, et. al., 1971b; Singer, et. al., 1981). Infusion of the latter also inhibits pancreatic enzyme and bicarbonate secretion in the presence of infused secretin. Further, vagal efferents, when electrically stimulated, potentiate ductal bicarbonate secretion in the presence of administered secretin. Atropine prevents this potentiation and abolishes the pancreatic enzyme secretory response to amino acid infusion into the intestinal lumen (Chey, et. al., 1979; Beglinger, et. al., 1984). Truncal vagotomy duplicates the effects of atropine administration just described (Dembinski, et. al., 1974). Truncal vagotomy and muscarinic receptor antagonists also inhibit pancreatic enzyme secretion in response to intralumenal infusion of amino acids, peptides and fats into the small intestine (Konturek, et. al., 1974; Thomas, 1964). By contrast, vagotomy and cholinergic antagonist administration don't seem to interfere with either the release of cholecystokinin, or with it's effects when infused to elicit enzyme secretion (Solomon and Grossman, 1979; Singer, et. al., 1980). These examples of the inhibitory effects of vagotomy and cholinergic antagonist administration point to a necessary integral entero-pancreatic neural reflex and probably to a vagovagal reflex arc in the secretion

response in duct cells. These studies also point to a co-mediated pancreatic enzyme secretory response in acinar cells involving both a humoral input (cholecystokinin), and a cholinergic neural input targeted to muscarinic receptors on the plasma membranes of both exocrine cell types in the gland.

The final phase of pancreatic secretion in the postprandial state is the circulatory-humoral phase. This phase has as it's primary characteristic feedback regulation resulting from circulating nutrients in the plasma. These nutrients, fats in the form of fatty acids and triglycerides, amino acids, and divalent cations all cause an increase in pancreatic secretion rates when administered intraluminally into the small intestine and intravenously into the blood stream (Matsuno, et. al., 1981; Edelman and Valenzuela, 1983; Konturek, et. al., 1979; Inoue, et. al., 1985). The mechanisms for this feedback regulation are not yet known in detail, but chemoreceptors in higher centers of the central nervous system may be involved in afferent signaling to the parasympathetic nervous system which, in turn, carries the efferent signal to cholinergic receptors in the pancreas.

Intracellular Signaling in the Exocrine Pancreas

From the preceding discussion it is clear that parasympathetic cholinergic pathways are instrumental in the stimulation of all phases of interdigestive and postprandial secretion of bicarbonate and digestive enzymes in the

exocrine pancreas. Both of the exocrine cell types in the pancreas contain many secretagogue receptors, including muscarinic cholinergic receptors. The various secretagogue receptors, when activated by their respective agonists, transduce the chemical signal of the hormone or neurotransmitter through one or more guanine nucleotide binding proteins, which in turn activate one of two different functional intracellular messenger systems (Gardner and Jensen, 1981). One of these systems involves stimulation of an enzyme termed phospholipase C, which catalyzes the rapid turnover of a minor phospholipid in the plasma membrane; phosphatidylinositol-4,5-bisphosphate (PIP2) (Halenda and Rubin, 1982). The catalysis of PIP2 causes a rapid increase in two intracellular messengers, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3, acting through a putative receptor on the endoplasmic reticulum (Dormer and Williams, 1981) causes the mobilization of intracellular calcium. DAG, in conjunction with calcium, activates the serine/threonine kinase, protein kinase C. Secretagogues that stimulate this system include cholecystokinin, acetylcholine (ACh), gastrin and bombesin (an Amphibian analogue of GRP) (Gardner and Jensen, 1987).

The other intracellular messenger system involves activation of adenylate cyclase. This enzyme catalyzes the formation of cyclic adenosine monophosphate (cAMP) from ATP. The latter functions as an intracellular messenger and activates cyclic AMP-dependent protein kinase, another

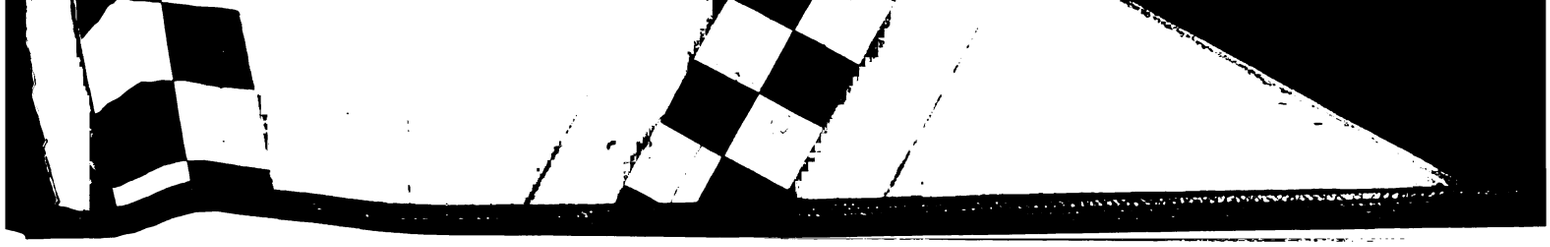
serine/threonine kinase (Rutten, et. al., 1972; Jensen and Gardner, 1978). Secretagogues that stimulate cyclic AMP production include secretin, vasoactive intestinal peptide (VIP), somatostatin and glucagon (Gardner and Jensen, 1987).

The various agonists that stimulate either one or the other of the two intracellular messenger systems usually don't directly activate the other and vice versa.

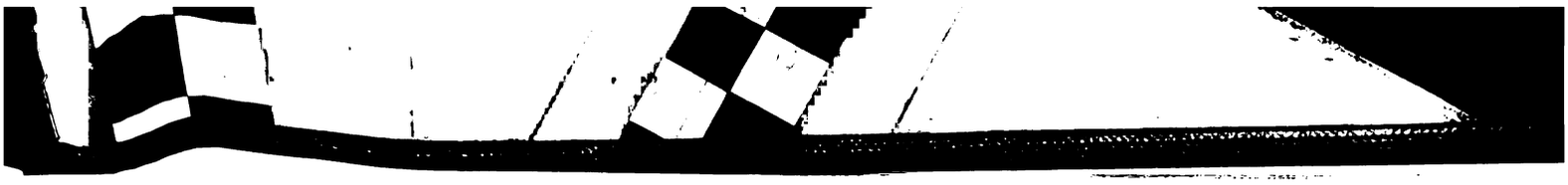
In addition to the two systems discussed above, there appears to be, at least in acinar cells, a third intracellular messenger system involving tyrosine kinase activation. Insulin, insulin-like growth factors, and epidermal growth factor stimulate the tyrosine kinase activity of their respective receptors. Although these substances appear to be important regulators of metabolic and trophic responses, they are not direct secretagogues of digestive enzyme release (Logsdon and Williams, 1983; Williams and Goldfine, 1985).

Pancreatic Muscarinic Receptors

Muscarinic acetylcholine receptors in the pancreas were first characterized through the use of radiolabelled antagonists such as [^{3}H]quinuclidinyl benzilate (QNB) and [^{3}H] N-Methylscopolamine (NMS) and through studies of the effects of cholinergic agonists and antagonists on digestive enzyme secretion from *in vitro* preparations of acinar cells. One of the first *in vitro* studies using dispersed acini showed that QNB selectively inhibited amylase release in a



dose-dependent manner in the presence of a physiological muscarinic agonist (Morisset, et. al., 1977). Later studies showed that [^{3}H]QNB bound specifically to pancreatic acinar homogenates (Larose, et. al., 1979) and to intact acini (Larose, et. al., 1981). This binding was saturable, of very high affinity, and exhibited a stereo selectivity that is common to all pharmacological antagonists. Scatchard plots of the saturation binding of [^{3}H]QNB were linear, a characteristic of a population of homogenous binding sites. The K_D for QNB in this study was 64 pM and the binding capacity (B_{max}) was 2605 fmol/mg of DNA. Correlation between receptor saturability and the ability of QNB to inhibit amylase release in a dose-dependent fashion established that the sites of QNB binding were the receptors that are involved physiologically with cholinergically-stimulated pancreatic enzyme release (Larose, et. al., 1981). This study showed that QNB bound to a single muscarinic binding site (Hill coefficient of 1.0), while cholinergic agonists like carbachol bound to at least two sites of different affinity (Hill coefficients less than unity) (Larose, et. al., 1979). In accordance with previous studies of muscarinic agonist binding in other tissues (Birdsall, et. al., 1978; Birdsall and Hulme, 1976), the authors used a computer-generated two-site binding model to fit their carbachol-binding data and found a high degree of correlation with these other published works. Their results suggest that carbachol can bind to both a high and a low affinity site on the muscarinic



acetylcholine receptor. This study also showed that binding of the agonist to the high affinity class of sites is responsible for pancreatic release of amylase, while further binding to the low affinity sites at high agonist concentrations results in the inhibition of digestive enzyme secretion. They suggested that the low affinity class of receptors may reflect a desensitized form of the high affinity binding site for carbachol (Larose, et. al., 1981).

Two other studies used [^{3}H]NMS and obtained similar results (Appert, et. al., 1981; Dehaye, et. al., 1984). Their results correlated well with the initial QNB binding study, showing a single class of binding sites for NMS and a two-site binding profile for the cholinergic agonists carbachol, oxotremorine, and muscarine. Dehaye, and coworkers also showed that the cholinergic agonist pilocarpine was not as effective as carbachol at eliciting pancreatic enzyme release and antagonized the effects of this agonist. Pilocarpine also seemed to bind only to a single class of medium-to-low affinity binding sites.

While these pharmacological studies were being carried out, other research attempting to elucidate the molecular structure of the muscarinic receptor was underway. Birdsall, et. al. (1979) and later Venter (1983) pioneered the use of [^{3}H]Propylbenzilylcholine mustard (PBCM) to determine the molecular weight of the muscarinic receptor protein molecule. This antagonist covalently labels the receptor in an irreversible fashion. In these initial studies, muscarinic

receptors in a wide variety of tissues including brain, cardiac muscle, and intestinal smooth muscle were labelled with [^{3}H]PBCM. These tissues were taken from several mammalian species including dogs, rats, humans, and guinea pigs. The labelled proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. These studies indicated that muscarinic receptors consist of a single glycosylated polypeptide with an approximate molecular weight of 80 kDa, a result supported by another group who reported a range of molecular weights from 75-85 kDa for porcine cardiac muscle (Herron and Schimerlik, 1983). However, two years later Hootman, et. al. (1985) using the same techniques previously described, showed that muscarinic receptors in the rat parotid and pancreas glands had molecular weights of 105 and 120 kDa, respectively. These authors suggested that muscarinic receptors in exocrine glands are either more extensively glycosylated then those in smooth and cardiac muscle and in the central nervous system or that the amino acid sequence of the glandular form of the receptor was longer, suggesting the expression of an entirely different gene product. It would be another two years however, before the question of the significance of molecular heterogeneity of muscarinic receptors would be definitively resolved.

A major advancement in understanding of the structure of the muscarinic acetylcholine receptor was provided by the cloning and sequencing of the entire receptor family from rat

and human genomic libraries by two separate research groups (Bonner, et. al., 1987; Peralta, et. al., 1987). These studies revealed that there are at least four subtypes of muscarinic acetylcholine receptors representing four unique gene products (m1-m4). The four subtypes differ from one another in their primary amino acid sequence and in their tissue specific expression. More recently, a fifth subtype (m5) has been described (Bonner, et. al., 1988). Table 1. summarizes these findings.

Table 1. Characteristics of muscarinic acetylcholine receptor subtypes

Subtypes	Amino Acids	Molecular Weights*	mRNA Distribution
m1	460	51,400	brain, glands
m2	466	51,700	brain, heart, smooth muscle
m3	589	66,200	brain, glands, smooth muscle
m4	478	53,000	brain
m5	531	60,100	?

* Unglycosylated

Analysis of the amino acid sequence of these receptor subtypes using hydrophobicity profiles and other predicted structural determinants suggested that the family of muscarinic receptors belonged to yet a larger superfamily of membrane receptors which also contains the adrenergic receptors. This larger family has several distinguishing characteristics. These receptors all contain seven membrane spanning domains and have their amino terminus located on the external face of the plasma membrane, while their carboxyl

terminus is located on the cytoplasmic face of the membrane. The various members of this superfamily of receptors all interact with guanine nucleotide-binding proteins at the cytoplasmic surface of the plasma membrane (Venter, et. al., 1989).

Over the last several years, pharmacological studies have identified a number of muscarinic antagonists that appear to discriminate among the five receptor subtypes. Two different groups have used these ligands to determine the muscarinic receptor subtype present in acinar cells of the pancreas (Korc, et. al., 1987; Ackerman, et. al., 1989; Louie and Owyang, 1986). These studies used [^{3}H]NMS, which is not subtype-selective, and the selective antagonists in a simple competition binding paradigm. In these studies, the non-selective antagonist atropine and the three subtype selective antagonists, pirenzepine (m1), AF-DX 116 (m2), and 4-DAMP (m3) were tested for their ability to compete with [^{3}H]NMS for binding to pancreatic acinar muscarinic receptors. All three studies showed that the muscarinic receptor subtype present in acinar cells had the greatest affinity for atropine followed closely by 4-DAMP. Pancreatic muscarinic receptors had the lowest affinity for AF-DX 116 and an intermediate affinity for pirenzepine. In each of these studies, the ability of each selective antagonist to inhibit [^{3}H]NMS binding was correlated with inhibition of carbachol-stimulated pancreatic amylase release as proof of selectivity of binding to the physiologically relevant receptor sites.

The only two antagonists tested that were able to inhibit amylase release in a biologically relevant dose range were atropine and the m3 subtype selective probe 4-DAMP.

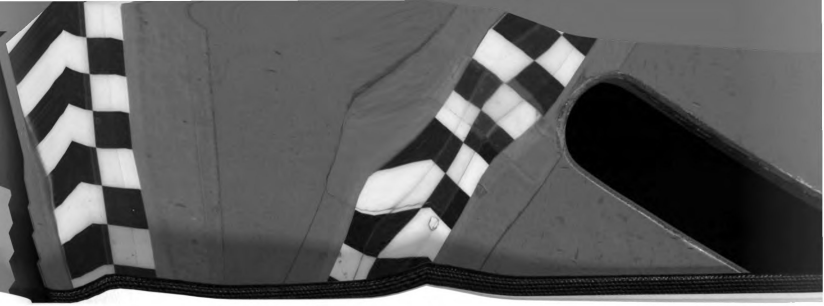
These studies supported the view that pancreatic acinar cells express the m3 form of muscarinic acetylcholine receptor and that this form is responsible for mediating cholinergically-activated pancreatic enzyme release.

While only a handful of *in vivo* studies on pancreatic muscarinic receptors have been carried out, some intriguing data has appeared regarding regulation of the growth and maturation of muscarinic receptor populations in pancreatic acinar cells from birth through weaning and into senility (Morisset, et. al., 1984; Dumont, et. al., 1981a, 1981b, 1982). Using [^{3}H]QNB, Morisset, Dumont, and coworkers showed that specific binding sites for the antagonist were present in the fetal pancreas and that the maximal level of binding sites for the ligand occurred at approximately one month of age. With senility (over one year of age), the level of binding sites decreased significantly. Although the total pancreatic muscarinic receptor number waxed and waned during the animals life time, the affinity of the receptors for the radioligand did not change with age. The cholinergic responsiveness of acini prepared from pancreases of rats of different ages correlated well with the size of the muscarinic receptor population, increasing after weaning and declining with senility. Responsiveness to carbachol was first noticeable on the third day after birth and was

directly inhibited by QNB in a dose-dependent manner. Agonist binding to muscarinic receptors of the neonatal rat pancreas showed the characteristic heterogenous population of binding sites previously seen in adult animals. In addition, the dose-response curve for carbachol stimulation of amylase release from acini was unchanged with age, indicating that the receptors maintained the same affinity for the agonist throughout the life span of the animal.

Other studies carried out by this group examined the effects of altering weaning period on the maturation of the muscarinic receptor population. In these experiments, the length of the weaning period was changed from a normal value of 21 days to 12,14,16,23,25 or 28 days. In both delayed and premature weaning schemes, the relative affinity of the receptor population for [^{3}H]QNB was unaffected. However, the premature transfer of suckling rats to a solid food diet greatly accelerated the upregulation of the receptor population, while a total milk diet maintained beyond the normal weaning date suppressed this upregulation.

Two other <u>in vivo</u> rat developing pancreatic model studies are worth noting. These studies focused on the fetal rat before birth. The first study indicated that fetal pancreatic tissue was unresponsive to cholinergic stimulation prior to birth. Unstimulated amylase release at this time period was above the basal level of secretion but does not reach the agonist-regulated secretory rate seen on the day of birth and at other postnatal time points in the life cycle of the rat



(Doyle and Jamieson, 1978). The second study confirmed the results of this earlier study, showing that neonatal pancreatic tissue is unresponsive to cholinergic-induced amylase release until one day after birth (Werlin and Stefaniak, 1982). These results are in slight disagreement with the above studies of Morisset, et. al. (1984), which suggested that one day old neonatal rat pancreatic tissue is unresponsive to cholinergic stimulation.

Several studies also have focused on the effects of prolonged exposure of pancreatic acinar cells to cholinergic agonists on subsequent secretory responsiveness and size of the acinar muscarinic receptor population. Exposure of isolated pancreatic acini to carbachol causes a maximal rate of digestive enzyme secretion within 5 min. However, this rate is not maintained indefinitely, but declines after the first 30 min, the reponse becoming desensitized. If acini are preincubated with a maximally effective concentration of carbachol for 30 to 60 min, the dose-response curve for the agonist determined in a subsequent incubation is shifted three-to-six fold to the right (Asselin, et. al., 1987; Blanchard, et. al., 1990; Hootman, et. al., 1986). This reduced potency of a cholinergic agonist to elicit pancreatic enzyme release after a previous exposure to the agonist is a classic case of desensitization. This response, which is complete within 60 min, does not result from a large reduction in the size of the overall acinar cell muscarinic receptor population (Hootman, et. al., 1986, 1989). However,

if agonist exposure is prolonged over a time scale of hours rather then minutes, a decrease in the actual size of the muscarinic receptor population is observed (Hootman, et. al., 1986, 1989). Up to 90% of [^{3}H]NMS binding sites have disappeared after 24 h of exposure of pancreatic acini to carbachol (Hootman, et. al., 1986). Pancreatic enzyme release in response to cholecystokinin in these studies was only slightly affected by carbachol preincubation. Since both carbachol and cholecystokinin utilize the same intracellular messenger systems (Hootman and Williams, 1987), Hootman, Asselin, and Blanchard and their coworkers concluded that cholinergic agonist-induced desensitization was mostly homologous, occuring at the muscarinic receptor level. By contrast, in more detailed studies Vinayek, et. al. (1990) showed that preincubation of guinea pig pancreatic acini with high concentrations of carbachol also caused a heterologous desensitization of digestive enzyme secretion in response to cholecystokinin and to bombesin, although the time-and dose-dependency differed from that of homologous desensitization. This desensitization was accompanied by a decrease in the percentage of high affinity binding sites for cholinergic agonists and a proportional increase in the percentage of low affinity binding sites.

Changing the Ca^{2+} concentration of the bathing medium to a value significantly greater then or less than the physiological range during the period of cholinergic agonist pre-exposure has no effect on the level of desensitization

exhibited by these cells (Asselin, et. al., 1987). However, pre-exposure of acini to the phorbol ester TPA does cause a time-and dose-dependent desensitization of subsequent carbachol-induced pancreatic enzyme release (Blanchard et. al., 1990; Vinayek, et. al., 1990), although exposure of acini to TPA after pre-exposure to carbachol had no further effect on digestive enzyme release. These results led to the conclusion that phorbol esters desensitize pancreatic muscarinic receptors by a different subcellular mechanism that is at least partially different from that involved in cholinergic agonist-evoked desensitization.

The question of whether desensitization is a necessary prerequisite for down regulation of the muscarinic receptor population also was addressed by Hootman, et. al. (1989). In this study, rat pancreatic acini were preincubated with carbachol and with the partial agonist pilocarpine, which Dehaye and coworkers (1984) had shown previously to be a less effective secretagogue than carbachol. When acini pre-exposed to carbachol for 30 min were incubated subsequently with a range of concentrations of pilocarpine, no stimulation of amylase secretion was observed. By contrast, when acini pre-incubated with pilocarpine were subsequently exposed to a range of carbachol concentrations, their secretory responsiveness was unaltered. However, when acini were cultured with either carbachol or pilocarpine for up to 24 hours, each agonist caused a time-and dose-dependent down regulation of the muscarinic receptor population. These

results indicate that cholinergic desensitization and down regulation are caused by two distinct subcellular events.

INTRODUCTION

Cholinergic agonist-induced down regulation of muscarinic receptors has been observed not only in pancreatic acinar cells, but in many other cell types including neuroblastoma cells (Siman and Klein, 1979; Taylor, et. al., 1979), chick retina and heart cells (Galper, et. al., 1982; Siman and Klein, 1983), thyroid epithelial cells (Champion and Mauchamp, 1982), astrocytoma cells (Harden, et. al., 1985), and cerebellar granule cells (Xu and Chuang, 1987). The widespread occurance of the observed effect of chronic cholinergic agonist occupancy on muscarinic acetylcholine receptor population size suggests that this phenomonen constitutes a common physiological process for all of the muscarinic receptor subtypes. Despite this fact, little is currently known of the molecular mechanisms that underly the selective removal of cholinergic agonist-stimulated muscarinic receptors from the cell surface or of their intracellular processing once they are sequestered in the cytoplasmic domain of the cell. To address these questions, previous studies have monitored the effects of chronic cholinergic agonist exposure on muscarinic receptor population size using both the membrane impermeant antagonist [^{3}H]NMS and the lipophillic tertiary antagonist [^{3}H]QNB.

Galper, et. al. (1982) showed that carbachol induced a rapid decrease in the density of [^{3}H]NMS binding sites in cultured chick heart cells, but a much slower disappearance of cellular sites for [^{3}H]QNB binding. The lag in disappearance of binding sites for QNB was interpreted as the time required for intracellular transport of the internalized receptors to their final sites of degradation. However, in other studies (Buyse, et. al., 1989; Gossuin, et. al., 1984), the high degree of cytoplasmic trapping of [^{3}H]QNB constituted a serious hindrance to its use as an effective probe for muscarinic receptors in intracellular compartments. In 1988, Fisher showed that the muscarinic antagonist [^{3}H]Scopolamine is membrane permeant and exhibits low non-specific binding. He has since demonstrated (Thompson and Fisher, 1990) that this ligand can be utilized to gain an accurate assessment of the total cell population of muscarinic receptors and, in combination with [^{3}H]NMS, can be used to assess shifts in receptor distribution among various cellular compartments.

In the present study, these two tritiated radioligands were utilized to study the effect of prolonged exposure of rat pancreatic acini to carbachol on muscarinic receptor sequestration and degradation and to assess the effects of various agents on these processes. Results of these studies suggest that degradation of muscarinic receptors occurs within the first two hours after their agonist-evoked removal from the cell surface and that this takes place through an endosomal/lysosomal pathway.

MATERIALS AND METHODS

Materials

Antibiotic/antimycotic mixture, bovine serum albumin (BSA, fraction V), carbamylcholine chloride (carbachol), cytochalsin-D, L-glutamine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), methylamine, minimal Eagle's medium amino acids, nocodazole, ouabain, scopolamine, soybean trypsin inhibitor (SBTI, type 1-S), and 12-O-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma Chemical Co., St. Louis, MO; chromatographically purified collagenase (400-600 U/mg) from Worthington Biochemicals, Freehold, NJ; bombesin, secretin, and vasoactive intestinal peptide (VIP) from Bachem Biosciences, Philadelphia, PA; ionophore A23187 from CalBiochem-Behring, La Jolla, CA; staurosporine from Kyowa Haako USA, New York, NY; W-7 from Seikagaku America, Inc., St. Petersburg, FL; and CMRL 1066 culture medium, epidermal growth factor (EGF), and ITS+ cell growth supplement from Collaborative Research, Waltham, MA. Cholecystokinin octapeptide (CCK8) was a gift from the Squibb Institute for Medical Research, Princeton, NJ. [N-methyl-3H]scopolamine chloride (NMS, 71.3 Ci/mmol) was purchased from Du Pont-New England Nuclear, Boston, MA. [3H]Scopolamine chloride (74.9 Ci/mmol) was custom

synthesized by Amersham, Arlington Heights, IL. Male outbred rats (250-400 gm) were obtained from Sprague-Dawley, Indianapolis, IN.

Preparation of pancreatic acini

Acini were prepared from the pancreases of fasted male rats as previously described by Hootman, et. al. (1985), 1989) by digestion with purified collagenase (60-70 U/ml) in a Ringers solution consisting of (in mM) 118 NaCl, 4.7 KCl. 1.0 Na_2PO_4, 1.1 $MgCl_2$, 5.5 glucose, 2.0 L-glutamine, 0.5 $CaCl_2$, and 10.0 HEPES (adjusted with 2.0 N NaOH to pH 7.4) with minimal Eagle's medium amino acids, 0.1% BSA, and 0.1% SBTI.

Culture of pancreatic acini

Acini from a single pancreas were suspended after isolation in 80-120 ml of a solution consisting of CMRL 1066 culture medium supplemented with 10mM HEPES, 0.2% SBTI, 0.1 mM ascorbic acid, 5 nM EGF, 1% ITS+ (consisting of 6 ug/ml of insulin, 6 ug/ml of transferrin, 6 ng/ ml of selenium, 2.0% BSA, and 5 ug/ml of linoleic acid), 100 u/ml of penicillin, 10 ug/ml of streptomycin, and 25 ug/ml of amphotericin. This suspension was distributed in 10 ml aliquots into 15 X 100 mm plastic Petri dishes. Carbachol and various agents were added to selected dishes and acini were cultured for up to 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Radioligand binding assays

At selected times, cultured pancreatic acini were collected, rinsed twice with the previously described Ringers solution, and resuspended in 5.5 ml of the same. Duplicate 1.0 ml aliquots of these suspensions were each added to 4.0 ml of the same Ringers solution containing (final concentrations) either 1.0 nM [^{3}H]NMS or 2.0 nM [^{3}H]Scopolamine with or without 10 uM unlabelled scopolamine. These suspensions were incubated at 37°C for 60 min. Acini were collected on Whatman GF/A glass fiber filters, rinsed three times with 5.0 ml of ice-cold 0.9% NaCl, and extracted and counted as described previously by Hootman, et. al. (1986). A 1.0 ml aliquot of each acinar suspension was sonicated and assayed for DNA content by the diphenylamine procedure (Croft and Lubran, 1965). In some instances, acini were sonicated prior to the binding assay. In others, the binding assay was carried out at 4°C for 24 h. When sonicated acini were assayed for radiolabelled antagonist binding, Whatman GF/B glass fiber filters were utilized.

Data Analysis

Results are presented as means ± SEM for the number of experiments carried out on individual preparations of acini. Statistical differences between mean values were assessed through the use of Student's two-tailed *t* tests.

RESULTS

Saturation binding studies with both [^{3}H]NMS and [^{3}H]Scopolamine were carried out on several suspensions of freshly isolated acini to assess levels of binding for each antagonist. Scatchard analyses of these experiments are shown in Figure 1. Acini maximally bound 1.98 $\pm$ 0.07 fmol/ug DNA (n = 5) of [^{3}H]NMS with a mean KD of 0.18 $\pm$ 0.03 nM. Corresponding values for [^{3}H]Scopolamine binding were 1.93 $\pm$ 0.18 fmol/ug DNA and 0.68 $\pm$ 0,08 nM, respectively. Although the K_D for [^{3}H]NMS binding was significantly lower ($P < 0.05$) than that for [^{3}H]Scopolamine, there was no significant difference between the mean maximal binding levels determined for the two antagonists.

To determine the half-life of cell surface muscarinic receptors in the cultured rat pancreatic acinar cell, acini were cultured for up to 24 h in the absence and presence of 0.1 mM carbachol and 0.1 mM cycloheximide, a potent protein synthesis inhibitor, and assayed for [^{3}H]NMS binding at six hour intervals (Figure 2). This concentration of carbachol is somewhat supramaximal as far as the agonist's ability to stimulate digestive enzyme secretion is concerned (Hootman, et. al., 1989). In suspensions of acini cultured in the absence of either compound, the level of [^{3}H]NMS binding

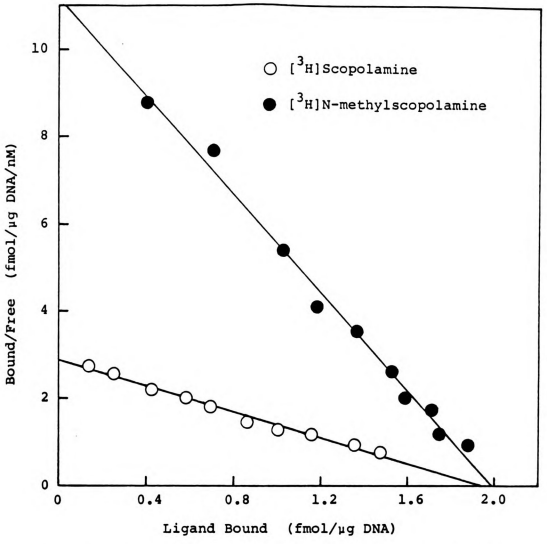


Figure 1. Scathard analysis of the specific binding of [^{3}H]Scopolamine (O) and [^{3}H]N-methylscopolamine (●) to muscarinic acetylcholine receptors on rat pancreatic acini. Acini were incubated with concentrations of each antagonist from 0.05 - 2.0 nM for 120 min at 37°C. Non-specific binding was determined by the inclusion of 10 uM unlabelled scopolamine in duplicate samples. Results shown are means of five experiments. For [^{3}H]N-methylscopolamine binding, B_{max} = 1.98 fmol/ug DNA and K_D = 0.18 nM. For [^{3}H]Scopolamine binding, B_{max} = 1.93 fmol/ug DNA and K_D = 0.68 nM.

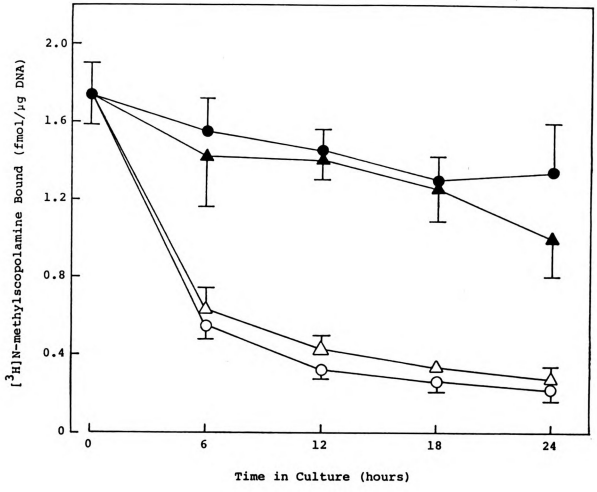


Figure 2. Effects of carbachol and cycloheximide on levels of binding of [^{3}H]N-methylscopolamine in suspensions of cultured rat pancreatic acini. Acini were cultured for 24 h in the absence of drugs (●) or in the presence of 0.1 mM carbachol (○) or 0.1 mM cycloheximide (▲) or both compounds (△). Results represent the means ± SEM of three experiments.

sites declined by approximately 25% over the first 18 h and then rebounded by about 5% over the next six hours.

Acini cultured in the presence of carbachol exhibited a decline in [^{3}H]NMS binding relative to control values. By 24 h, [^{3}H]NMS binding to acini incubated with carbachol was only 12% of the original value. Cycloheximide did not appreciably alter the level of [^{3}H]NMS binding over this same 24 h period, either in suspensions of control acini or in suspensions of acini cultured in the presence of carbachol except over the last six hour period in the former, where instead of exhibiting a slight rebound, receptor levels continued to decline slowly.

The time course of carbachol-induced down regulation of both cell surface muscarinic receptors and total cellular muscarinic receptors also was determined through simultaneous measurements of [^{3}H]NMS and [^{3}H]Scopolamine binding (Figure 3). Addition of 0.1 mM carbachol to the culture medium caused a rapid decline in [^{3}H]NMS binding with a half-time of 3.9 $\pm$ 0.4 h ($n = 3$). [^{3}H]Scopolamine binding sites declined more slowly over the 24 h culture period, with a half-time of 5.7 $\pm$ 0.7 h. The difference in half-times for disappearance of binding sites for each antagonist was significant ($P < 0.05$), and indicates that in the presence of carbachol, muscarinic receptors are removed from the acinar cell surface and sequestered for an average of one to two hours before being degraded internally.

The estimate of the time dependency for carbachol-induced

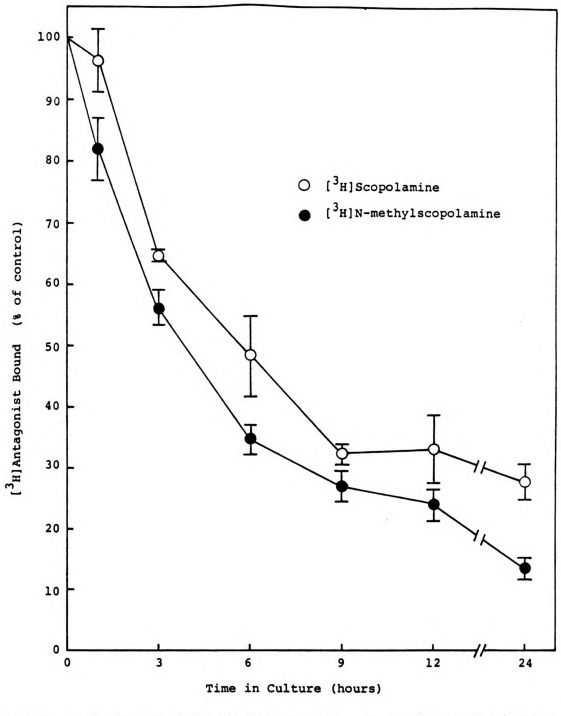


Figure 3. Effect of carbachol exposure on levels of binding sites for [^{3}H]N-methylscopolamine (●) and [^{3}H]Scopolamine (O) in suspensions of cultured rat pancreatic acini. Mean half-times for the agonist-induced decrease in binding sites for the two antagonists are 3.9 h and 5.7 h, respectively. Results shown represent ± SEM of three experiments.

down regulation of [^{3}H]NMS binding sites noted above does not take into account the 60 min incubation period of the binding assay itself. As recently demonstrated by Fisher (1988), in some cell types, internalized muscarinic receptors can quickly recycle to the cell surface once the cholinergic agonist is withdrawn. If such a salvage pathway were present in the pancreatic acinar cell, the extent of internalization of cell surface muscarinic receptors induced by carbachol would be underestimated at each time point examined due to rebound occuring during the binding assay. To examine this question, acini were cultured in the absence and presence of carbachol for six hours and the [^{3}H]NMS and [^{3}H]Scopolamine binding assays were conducted under three sets of conditions; intact acini at 37°C for 60 min, intact acini at 4°C for 24 h, and sonicated acini at 37°C for 60 min. Incubation of cells at the lower temperature has been shown in many previous studies to prevent shuttling of materials to and from the plasma membrane (Fisher,1988). The long incubation period was necessary at 4°C to allow binding to reach an equilibrium, which is normally attained at 37°C within 40-50 min for these two radiolabeled antagonists (data not shown). Binding to sonicated acini also was examined, as the resulting disruption of cellular integrity was expected to likewise disrupt any membrane recycling pathways, although there is the potential complication here that muscarinic receptors may become inaccessible to [^{3}H]NMS by entrapment in closed vesicular compartments induced by sonication. Results

Table 2. Effect of assay conditions on estimation of carbachol-induced down regulation of [^{3}H]NMS and [^{3}H]Scopolamine binding sites in cultured rat pancreatic acini.

Assay Condition	Carbachol Pre-exposure	Antagonist Bound (fmol/μg DNA) [^{3}H]NMS	[^{3}H]Scopolamine
Intact acini; 60 min, 37°C	No	1.56 ± 0.11	1.34 ± 0.13
	Yes	0.54 ± 0.05	0.59 ± 0.06
Intact acini; 24 h, 4°C	No	1.37 ± 0.18	1.42 ± 0.20
	Yes	0.45 ± 0.04	0.56 ± 0.09
Sonicated acini; 60 min, 37°C	No	1.25 ± 0.07	1.05 ± 0.05
	Yes	0.52 ± 0.03	0.49 ± 0.02

Acini were cultured for 6 h in the absence or presence of 0.1 mM carbachol prior to the antagonist binding assay. Results represent the means ± SEM of 4-5 experiments.

of these studies are shown in Table 2. Intact acini, when assayed for [^{3}H]NMS binding at 37°C after six hours of exposure to carbachol, bound 0.54 ± 0.05 fmol/ug DNA, 34.6% of the paired control value of 1.56 ± 0.11 fmol/ug DNA. Intact acini assayed at 4°C bound 0.45 ± 0.04 fmol/ug DNA, 32.8% of their control binding level, while sonicated acini bound 0.52 ± 0.03 fmol/ug DNA, 41.6% of their control. The close correspondence of the degree of down regulation induced by carbachol measured under all three assay conditions mediates against the presence of an active recycling pathway for internalized muscarinic receptors in the pancreatic acinar cell. The measured degree of down regulation of [^{3}H]Scopolamine binding sites, as expected, also was similar when the three assay conditions were compared in the same manner.

To determine whether recovery of cell surface muscarinic receptors in the acinus occured over a longer time interval, acini also were cultured for four hours in the presence of 0.1 mM carbachol, the agonist was rinsed away, and the acini were re-cultured for an additional four hours. Binding of [^{3}H]NMS was determined at hourly intervals after removal of carbachol (Figure 4). Binding assays were carried out at 4°C to prevent further membrane and receptor recycling during the assay period. Over the second four hour culture period, the level of acinar [^{3}H]NMS binding sites increased only a small amount, suggesting that recovery of the plasma membrane population of muscarinic receptors following agonist-induced

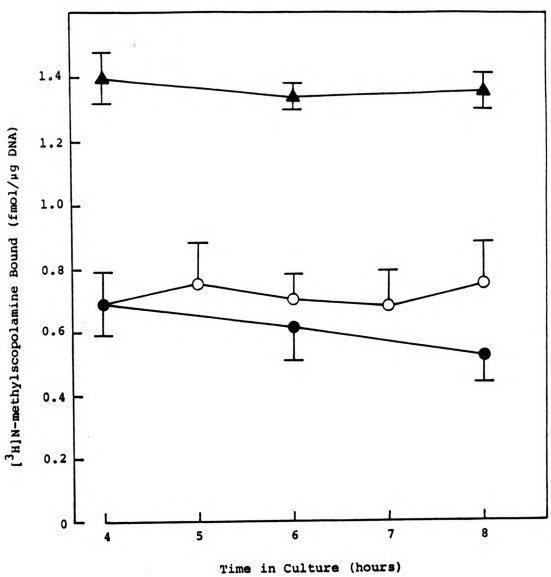


Figure 4. Recovery of [^{3}H]NMS binding sites on cultured rat pancreatic acini after agonist-induced down regulation. Acini were cultured either in the absence (▲) or presence (●) of 0.1 mM carbachol for 4 h. Part of the acini that had been cultured with carbachol were then rinsed and re-cultured in its absence (○) for an additional 4 h. Acini were collected at the indicated times and assayed for [^{3}H]NMS binding at 4°C to prevent further membrane recycling. Results represent means ± SEM of three experiments.

internalization is very slow. This observation is in accord with that made previously on the recovery of muscarinic acetylcholine receptors in guinea pig pancreatic acini following carbachol-induced down regulation (Hootman, et. al., 1986).

To assess the possible involvement of other cellular organelles or processes in the observed agonist-induced disappearance of acinar muscarinic receptors, acini were incubated in the presence of a variety of agents along with carbachol for six hours prior to determination of levels of binding for both tritiated antagonists (Table 3). In these studies, all agents were added to the culture medium 30 min prior to the addition of carbachol. None of the agents tested in the absence of carbachol had any significant effect on the levels of [^{3}H]NMS and [^{3}H]Scopolamine binding over the six hour test period. Carbachol alone reduced [^{3}H]NMS binding to 35.9 $\pm$ 1.8% of the control value and [^{3}H]Scopolamine binding to 40.4 $\pm$ 2.4% of control. Culture of acini at 4°C for six hours completely prevented the agonist-evoked disappearance of [^{3}H]NMS binding sites. Addition to the culture medium of the pancreatic secretagogues bombesin, cholecystokinin, secretin, and VIP did not, however, either diminish or increase the effect of carbachol exposure on binding sites for either of the radiolabelled ligands. Nor did the Ca^{2+} ionophore A23187, the protein kinase C activator TPA, the Na,K-ATPase inhibitor ouabain, the microfilament inhibitor cytochalasin-D, or the microtubule depolymerizing agent

Table 3. Effect of various agents on carbachol-induced down regulation of [^{3}H]NMS and [^{3}H]Scopolamine binding sites in cultured rat pancreatic acini.

Treatment	Antagonist Bound (% of control)	
	[^{3}H]NMS	[^{3}H]Scopolamine
None (carbachol alone)	35.9 ± 1.8 (24)	40.4 ± 2.4 (17)
4°C	100.3 ± 10.0 (4)*	———
Bombesin (10 nM)	33.4 ± 4.0 (4)	36.6 ± 3.6 (3)
CCK-8 (1 nM)	37.5 ± 6.4 (4)	38.1 ± 3.2 (3)
Secretin (100 nM)	38.2 ± 3.9 (4)	34.9 ± 2.1 (3)
VIP (10 nM)	33.7 ± 4.0 (4)	39.6 ± 0.9 (3)
A23187 (3 μM)	42.2 ± 3.3 (3)	46.1 ± 7.7 (4)
W-7 (50 μM)	45.2 ± 3.1 (7)*	55.5 ± 6.2 (4)*
TPA (1 μM)	39.5 ± 4.8 (4)	38.3 ± 2.6 (4)
Staurosporine (1 μM)	53.8 ± 4.9 (5)*	48.4 ± 4.7 (4)
Cytochalasin-D (10 μg/ml)	36.8 ± 1.2 (5)	45.8 ± 3.8 (5)
Nocodazole (10 μg/ml)	36.4 ± 3.4 (5)	42.1 ± 4.9 (5)
Methylamine (30 mM)	47.9 ± 4.3 (5)*	85.2 ± 3.3 (7)*
Ammonium chloride (30 mM)	49.8 ± 7.5 (4)*	87.6 ± 17.1 (4)*
Ouabain (0.5 mM)	41.9 ± 2.9 (4)	40.3 ± 3.3 (4)

Acini were incubated for 6 h in the absence or presence of 0.1 mM carbachol and the indicated compounds prior to the antagonist binding assay. The numbers of individual experiments are indicated in parentheses. *, values significantly different ($P < 0.05$) from value of carbachol alone.

nocodazole. The calmodulin inhibitor W-7 slightly diminished the regulatory effect of carbachol on levels of binding sites for both antagonists, as did staurosporine, a potent inhibitor of protein kinase C. The two lysosomotropic agents, methylamine and ammonium chloride, also caused a slight inhibition of the carbachol-induced disappearance of [^{3}H]NMS binding sites. However, unlike any of the other agents tested, both ammonium chloride and methylamine caused a dramatic decrease in the ability of carbachol to down regulate binding sites for [^{3}H]Scopolamine. Methylamine reduced the degree of down regulation of the total cell muscarinic receptor population from 59.6% to 14.8%. Ammonium chloride reduced down regulation to only 12.4%. These results are shown graphically in (Figure 5). One other lysosomotropic agent, monensin, also was tested, but it had severe deleterious effects on cellular viability over the six hour culture period. Chloroquine, which also inhibits lysosomal function, was not tested in these studies, since it exhibits potent antimuscarinic properties (Habara, et. al., 1986).

These studies suggested that lysosomotropic agents inhibit the degradation of sequestered and internalized muscarinic receptors in response to cholinergic agonist-evoked down regulation, thus trapping them intact in an intracellular compartment. To determine whether these receptors are still able to bind [^{3}H]NMS, in four experiments, acini were incubated for six hours in culture medium containing 30 mM methylamine or 30 mM methylamine plus

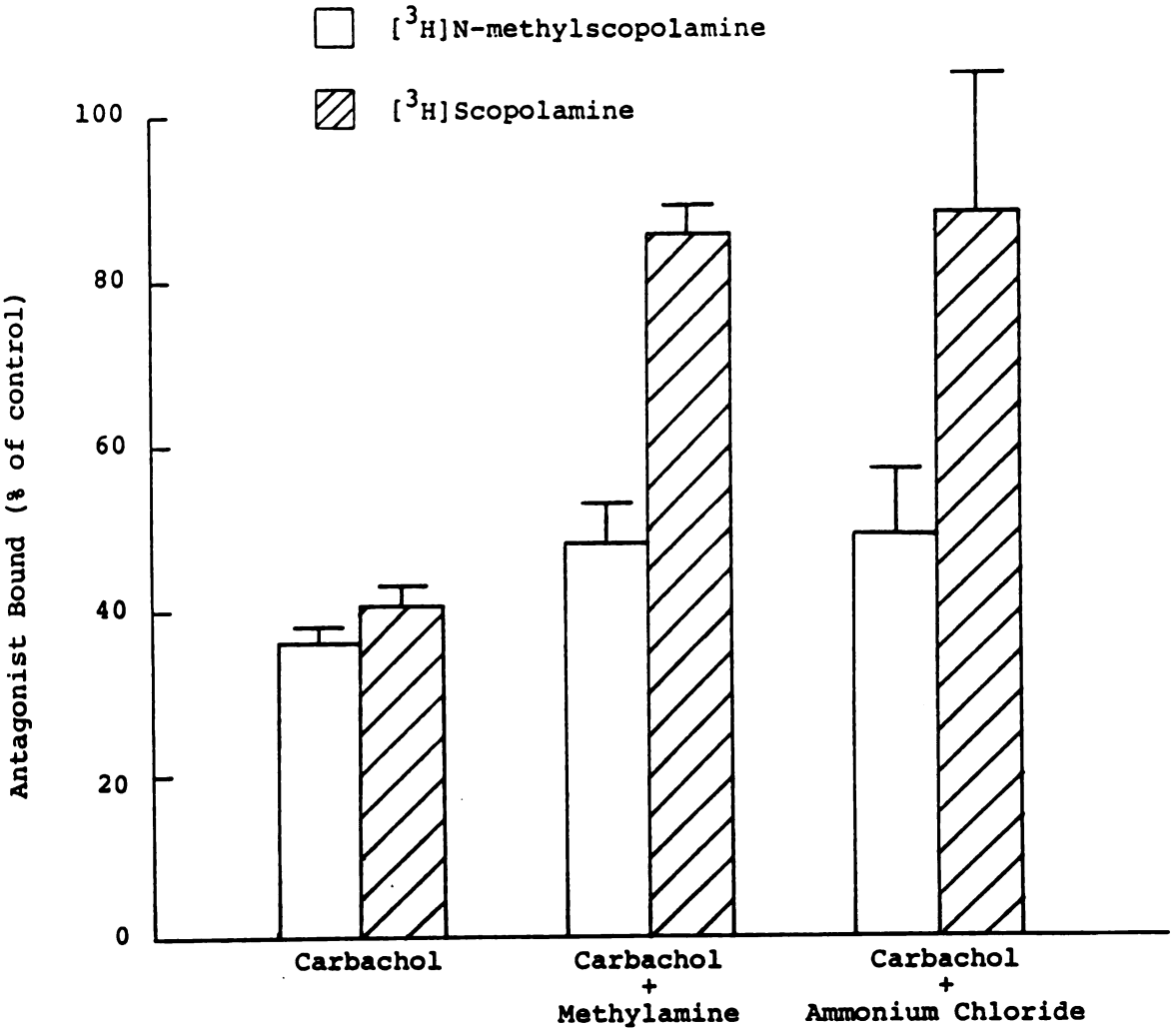


Figure 5. Effects of methylamine and ammonium chloride on carbachol-induced disappearance of [^{3}H]Scopolamine and [^{3}H]N-methylscopolamine binding sites in rat pancreatic acini. Results represent the means ± SEM of 4-7 experiments.

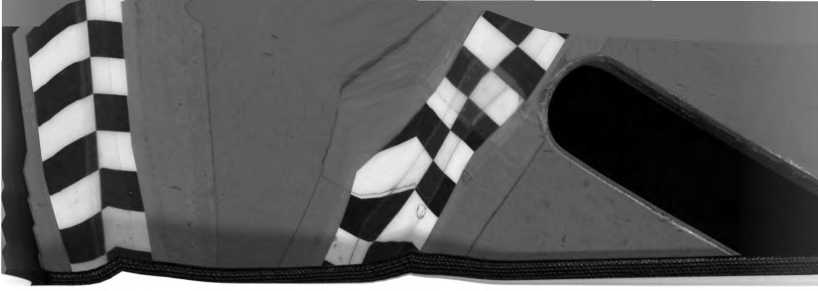
0.1 mM carbachol and either left intact or sonicated prior to assessment of [^{3}H]NMS binding. Intact acini cultured with carbachol as well as methylamine bound 52.4 ± 1.7% of the level of [^{3}H]NMS bound by intact acini that had been cultured with methylamine alone. Sonicates of the same preparations bound 72.8 ± 3.7% of control levels. These experiments demonstrate that sonication of acini causes some but not all of the internalized receptor molecules to again become accessible to [^{3}H]NMS. The remainder likely are still trapped in undisrupted vesicular compartments where they remain inaccessible to the hydrophillic antagonist.

In order to assure that the observed effects of carbachol, methylamine and ammonium chloride were not caused by changes in receptor afffinity for the two radiolabelled muscarinic antagonists, we also carried out saturation binding studies with acini incubated for six hours with each of these agents (Table 4). The B_{max} and K_D for binding of both [^{3}H]NMS and [^{3}H]Scopolamine were not significantly different among acini incubated in culture medium alone and acini cultured in medium containing either 30 mM methylamine or 30 mM ammonium chloride. Nor were these values different from those for freshly isolated acini (Figure 1). In contrast, six hour exposure of acini to 0.1 mM carbachol decreased the B_{max} for [^{3}H]NMS binding to 36.1% and that for [^{3}H]Scopolamine binding to 44.3% of their respective control values without altering the K_D for binding of either antagonist. These results demonstrate that the observed

Table 4. Effect of 6 h culture of rat pancreatic acini with carbachol, ammonium chloride, and methylamine on binding parameters for [^{3}H]NMS and [^{3}H]Scopolamine.

Culture Medium Additions	[^{3}H]NMS		[^{3}H]Scopolamine	
	K_D (nM)	B_{max} (fmol/µg DNA)	K_D (nM)	B_{max} (fmol/µg DNA)
None	0.19 ± 0.04	2.02 ± 0.11	0.72 ± 0.07	1.94 ± 0.11
Methylamine (30 mM)	0.20 ± 0.02	2.15 ± 0.14	0.78 ± 0.13	2.20 ± 0.25
Ammonium chloride (30 mM)	0.22 ± 0.04	2.04 ± 0.29	0.71 ± 0.14	2.09 ± 0.10
Carbachol (0.1 mM)	0.20 ± 0.02	0.73 ± 0.06 *	0.69 ± 0.13	0.86 ± 0.09 *

Results represent the means ± SEM of 3 experiments. *, values significantly different ($P < 0.05$) from control values.



decreases in labelled antagonist binding evoked by carbachol exposure result from a change in muscarinic receptor numbers and not receptor affinity for [^{3}H]NMS and [^{3}H]Scopolamine.

DISCUSSION

Results of this study confirm an earlier report by Hootman, et. al. (1989) that the cholinergic agonist carbachol causes down regulation of muscarinic receptors in cultured rat pancreatic acinar cells. In addition, through utilization of the lipophilic muscarinic antagonist [^{3}H]Scopolamine, it has been here shown that acinar cell muscarinic receptors, once internalized in response to agonist activation, are relatively quickly degraded through a presumably endosomal/lysosomal pathway.

As noted above, prolonged exposure of rat pancreatic acinar cells to cholinergic agonists results in a profound desensitization that consists of at least two phases. The first phase is a rapid homologous desensitization, which is complete within 30-60 min (Asselin, et. al., 1987; Hootman, et. al., 1986, 1989). This phase constitutes a decrease in secretory responsiveness that occurs without an appreciable decline in cell surface muscarinic receptor numbers. Activation of protein kinase C has been implicated in this phase (Blanchard, et. al., 1990; Vinayek, et. al., 1990). The second phase entails the disappearance of muscarinic receptors from the plasma membrane. Up to 24 h is required to effect the loss of the majority of the receptor population

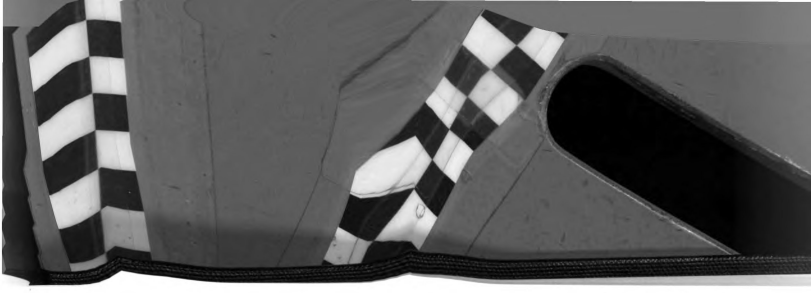
(Hootman, et. al., 1986, 1989). After 24 hours of cholinergic agonist exposure, acini no longer can respond to carbachol, although they can secrete digestive enzymes in response to other secretagogues such as cholecystokinin (Hootman, et. al., 1986). In the guinea pig pancreatic acinus, cholecystokinin pre-exposure also decreases cholinergic responsiveness by a heterologous desensitization mechanism (Vinayek, et. al., 1990), suggesting that at least some degree of cross-talk exists between these two secretagogue receptor systems. Since both muscarinic receptors and cholecystokinin receptors elicit intracellular signals through the same messenger systems, the existence of this type of heterologous desensitization is not surprising.

As discussed previously, Hootman and coworkers (1989) also earlier showed that the partial cholinergic agonist piilocarpine caused down regulation of acinar muscarinic receptors, but was ineffective in eliciting the early phase of homologous desensitization. This observation suggests that the rapidly occuring cholinergic desensitizaion of secretory responsiveness is caused by a change in receptor properties distinct from that which elicits the down regulation of activated receptors from the cell surface.

In the rat pancreatic acinus, [^{3}H]NMS and the lipophilic antagonist [^{3}H]Scopolamine label the same maximal number of muscarinic receptors at 37°C (Figure 1). This observation suggests that very few receptors are located in intracellular pools in unstimulated acini or that those within

intracellular compartments can rapidly equilibrate with the population of cell surface receptors. Since the rate of turnover of muscarinic receptors in rat pancreatic acinar cells appears to be quite slow (Figure 2), intracellular receptors would be expected to constitute only a small percentage of the whole at any one time. Data presented in Table 2 and Figure 4 also indicate that rapid recycling of internalized muscarinic receptors to the cell surface does not occur to any large extent in the rat pancreatic acinar cell. This is unlike the situation seen in SK-N-SH neuroblastoma cells (Thompson and Fisher, 1990) and 1321N1 astrocytoma cells (Harden, et. al., 1985), where withdrawal of carbachol after a period of exposure is followed by a rapid recovery of [^{3}H]NMS binding sites to near pre-exposure levels. The importance of this salvage pathway is unclear, although it may be important in the regulation of neuronal cell responses in the brain, affording the cells a rapid means of recovery of responsiveness to cholinergic stimulation upon removal of the agonist. This rapid recovery pathway may not be important biologically in the regulation of pancreatic function, where the response to cholinergic stimulation needs to be more prolonged while digestive metabolites remain in the duodenum and jejunum.

Other differences also exist between pancreatic acinar cells and neuronal cell types with respect to the kinetics of the regulatory effects of cholinergic agonist exposure on muscarinic receptors. In most cultured cell types of neuronal



origin, including cerebellar granule cells and neuroblastoma X glioma hybrids (Maloteaux, et. al., 1983), astrocytoma cells (Harden, et. al., 1985), neuroblastoma cells (Thompson and Fisher, 1990), and corticostriatal neurons (Eva, et. al., 1990), the carbachol-induced decrease in binding sites for [^{3}H]NMS is quite rapid, with half-times on the order of 15-30 min. In guinea pig and rat pancreatic acini, it is much slower, with measured half-times for the decrease in [^{3}H]NMS binding sites of three-to-four hours (Hootman, et. al., 1986, 1989; Figure 3 of this paper). This differential temporal regulation of receptor sequestration may be physiologically relevant. The rapid disappearance of [^{3}H]NMS binding sites from neuronal cell membranes may be another way that these cells regulate their responsiveness to cholinergic signaling in the brain and allow for a further complexity of neuronal pathway communication. This type of response may be important in neural habituation and adaptation to incoming signaling within the brain. As noted above, the prolonged time course of agonist-induced down regulation of muscarinic receptors in the pancreatic acinar cell may reflect the prolonged need for enzyme secretion in response to the presence of digestive nutrients in the stomach and small intestine.

Of the various agents tested (Table 3), only methylamine and ammonium chloride dramatically inhibited the carbachol-induced disappearance of binding sites for either muscarinic antagonist, and then only for [^{3}H]Scopolamine. These two compounds have been shown previously to block protein

degradation in lysosomes by preventing their acidification (Grinde and Seglen, 1980; Seglen, et. al., 1979). De Lisle and Williams (1987) recently demonstrated that incubation of pancreatic acini with 30 mM methylamine rapidly abolished all intracellular acidic compartments. Potau, et. al. (1984) earlier showed that methylamine did not block insulin internalization by pancreatic acini from diabetic mice, but interfered with its intracellular processing, leading to its accumulation within the acinar cell. Results in the current study thus implicate an acidic endosomal/lysosomal pathway in the processing of pancreatic muscarinic acetylcholine receptors internalized in response to cholinergic agonist exposure. They also indicate that microtubules and microfilaments do not play an essential role in these processes of sequestration, intracellular transport, and degradation, nor does it appear likely that phosphorylation of activated muscarinic receptors in the acinar cell by protein kinase C or Ca^{2+}, calmodulin-dependent protein kinase provides the signal for internalization.

Although the molecular mechanisms that mediate desensitization and down regulation of muscarinic receptors in the pancreatic acinar cell remain unclear, an analogy may be drawn to another better defined receptor system, the β-adrenergic receptors. These two regulatory processes have been studied in the β-adrenergic receptor system in considerable detail by several research groups, most notably that headed by Robert Lefkowitz. Receptor regulatory

mechanisms may be very similar in these two receptor families, since both belong to the larger superfamily of guanine nucleotide-binding protein coupled receptors (Venter, et. al., 1989).

In the β-adrenergic receptor system, chronic agonist exposure leads to rapid desensitization of the receptor by a homologous mechanism, attributable in part to phosphorylation of amino acids within the primary receptor sequence by two separate serine/threonine protein kinases [β-adrenergic receptor kinase (BARK) and protein kinase A (PKA)] (Hausdorff, et. al., 1990). This rapid desensitizing effect does not appear to induce sequestration and internalization of the receptor, but rather functionally uncouples the receptor from the cellular membrane signal transducing element, a stimulatory GTP-binding protein termed Gs (Hausdorff, et. al., 1990). Further, two distinct primary sequence domains of the receptor seem to be important for causing desensitization. These two sites are agonist dose-dependent and kinase selectively recognized. At low concentrations of agonist stimulation, only one site is preferentially phosphorylated by PKA. When higher doses of agonist are present the induced desensitization that occurs is due to a two site phosphorylation event involving both PKA and BARK (Hausdorff, et. al., 1989). This two site phosphorylation is at least partially responsible for a shift of the receptor high affinity agonist binding sites to the lower affinity state, thereby producing the desensitizing

effect. This suggests that only the agonist-occupied high affinity site on the receptor is the physiologically functional substrate for the protein kinases inducing the phosphorylation and covalent modification of the receptor protein.

Recently, Benovic, et. al. (1987) showed that a functional analog of the retinal protein, arrestin, may be responsible for part of the desensitization attributed to the phosphorylating events carried out by BARK. They suggest that the phosphorylation-induced modification of the receptor may be a signal for the binding of an analogous arrestin-like molecule, which would further insure uncoupling of the β-adrenergic receptor from its stimulatory GTP-binding protein in the membrane, possibly due to steric hindrance alone.

Additionally, Sibley, et. al. (1986) have shown that agonist-induced down regulation of the β-adrenergic receptor in turkey erythrocytes temporally follows the events leading to its desensitization. In response to prolonged agonist exposure, the receptor is sequestered and transported into the cytoplasm in a light vesicle fraction. This internalized form of the receptor can be purified by differential centrifugation due to its presence in these vesicles of lighter density. These receptors, when reconstituted with Gs are less effective in activating the guanine nucleotide-binding protein than control receptors purified from the plasma membrane fraction. This sequestered, desensitized form of the β-adrenergic receptor also can be recycled back to the

plasma membrane, if it is acted upon by a specific protein phosphatase found in association with these vesicles (Sibley, et. al., 1986). This recycling pathway may represent the same pathway described by Thompson and Fisher (1990) and others for muscarinic receptors in neuroblastoma cells and other neuronal cell types.

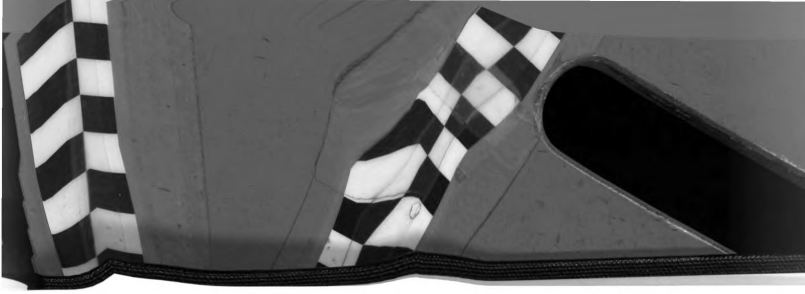
Many of the events which appear to be involved in desensitization and down regulation of β-adrenergic receptors also have been implicated in regulation of m1 and m2 subtypes of the muscarinic receptor family. Phosphorylation of the m2 subtype of the muscarinic receptor has been tied to desensitization in chick cardiac muscle cells (Kwatra, et. al., 1987). While PKC appears to be involved in the muscarinic desensitization response in some cell types (Blanchard, et. al., 1990; Vinayek,et. al., 1990) there exists the possibilty that another serine/threonine protein kinase similar to BARK, but specific for the muscarinic receptor family (a putative muscarinic acetylcholine receptor kinase or "MARK") may participate in these processes. This prospect also raises the possibility of an analogous arrestin-like molecule that could interact to bring about desensitization and internalization of muscarinic receptors. Harden, et. al. (1985) and Hoover and Toews, et. al. (1990) have shown that the m1 muscarinic receptors of astrocytoma cells are sequestered in a light membrane vesicle fraction following exposure of these cells to carbachol. These sequestered forms of the muscarinic receptor could

conceivably be acted on by one or more protein phosphatases associated with these vesicles in a fashion analogous to the β-adrenergic receptors, leading to their reinsertion in the plasma membrane. In the pancreatic acinar cell, a similar salvage pathway may not exist since it appears from results of the present study that sequestration of acinar m3 muscarinic receptors is followed inevitably by their degradation. It appears, therefore, that only newly synthesized receptors can restore the integrity of the initial cell receptor population in the acinar cell. However, there still exists the possibility that the m3 muscarinic receptors of the pancreatic acinar cell may be subject to a similar mechanism of sequestration with divergence only after the internalization process is completed.

Recently, Lechleiter, et. al. (1990) and Wess, et. al. (1990) have shown that short amino acid sequences at both ends of the third intracytoplasmic (I3) loop of the m2 and m3 subtypes of muscarinic receptors are important recognition sites for guanine nucleotide-binding proteins. Recent work by Shapiro and Nathanson (1989) has indicated that a specific sequence of amino acids within the I3 loop of the mouse m1 muscarinic receptor is necessary for agonist-induced down regulation. This work was supported by Maeda, et. al. (1990), who showed that when the amino terminus portion of the I3 loop of the muscarinic receptor is deleted or mutated, agonist-evoked sequestration is significantly inhibited. The mechanism through which the conformation of this sequence is

altered by agonist binding and how this relates to sequestration remain to be determined both for the m1 subtype and for other muscarinic receptor subtypes, including the m3 receptor present in the pancreatic acinar cell.

Additional very recent evidence from studies by Wang, et. al. (1990) has shown that chronic exposure to cholinergic agonists of CHO cells expressing transfected m1 muscarinic receptors also causes a decrease in steady state levels of the mRNA coding for the muscarinic receptor protein. This latest finding suggests that agonist-evoked down regulation of muscarinic receptors in at least some cell types may involve both an increase in receptor degradation and a decrease in receptor synthesis.



SUMMARY

1) There is no significant difference between the mean maximal levels of binding (B_{max}) for the two muscarinic antagonists, [^{3}H]N-methylscopolamine and [^{3}H]Scopolamine to unstimulated rat pancreatic acinar cells (Figure 1), suggesting that very few muscarinic receptors exist within intracellular compartments in unstimulated acini or that those existing in intracellular pools can rapidly equilibrate with the population of receptors located on the cell surface.

2) Protein synthesis appears not to be necessary for the process of cholinergic agonist-evoked receptor internalization and degradation in pancreatic acini (Figure 2). The turnover rate of muscarinic acetylcholine receptors in cultured rat pancreatic acini is very slow, with a half-life exceeding 24 h (Figure 2).

3) Cholinergic agonist-evoked down regulation of muscarinic receptors in rat pancreatic acini occurs over a period of hours. The T_{50} for disappearance of [^{3}H]NMS binding sites, a measure of receptor sequestration, is 3.9 $\pm$ 0.4 h and that for [^{3}H]Scopolamine binding sites, a measure of receptor degradation, is 5.7 $\pm$ 0.7 h (Figure 3). This difference between half-times for agonist-

induced sequestration and degradation indicates that 90-120 min elapses between internalization and destruction of activated muscarinic receptor molecules.

4) There does not appear to be a rapid salvage pathway for sequestered muscarinic receptors in rat pancreatic acini (Figure 4). The restoration of the plasma membrane muscarinic receptor population to levels corresponding to those found in previously unstimulated acinar cells is far slower than the progression of agonist-induced receptor removal from the cell surface.

5) The lysosomotropic agents, methylamine and ammonium chloride, significantly inhibit carbachol-evoked disappearance of [^{3}H]Scopolamine binding sites (Table 3, Figure 5), suggesting that the endosomal/lysosomal pathway plays an integral part in the degradation of muscarinic receptors internalized in response to cholinergic stimulation.

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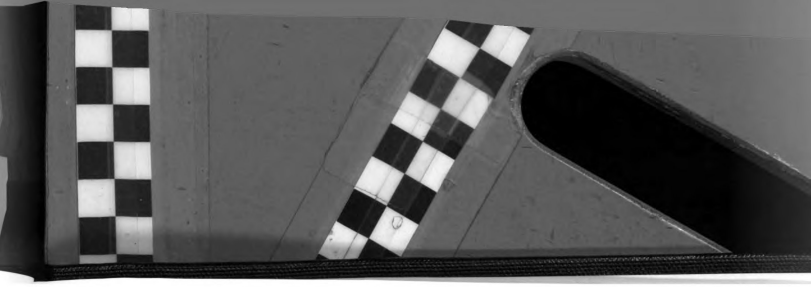

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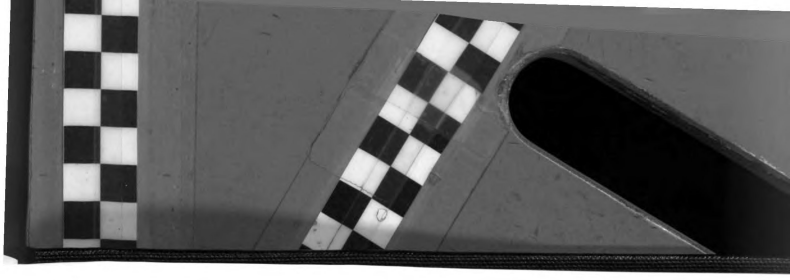

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