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^{3}H-methyl scopolamine binding to dispersed pancreatic acini* ***

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Summary. Maximal amylase release occurred with 10^{-5} M carbachol and slightly greater than half maximal response occurred with 3×10^{-7} M carbachol in dispersed pancreatic acini. The preparation released more than 45% of its initial amylase content after 60 min of maximal carbachol stimulation. Electron microscopy revealed depletion of zymogen granules and the presence of secretory material in the ductules after carbachol stimulation. At 37° C, maximal binding of methyl scopolamine occurred in about 45 min with 3×10^{-10} M ^{3}H-methyl scopolamine. The dissociation constant for ^{3}H-methyl scopolamine was 6.8×10^{-10} M and saturation occurred at 109 pm/g protein. The I.C.$_{50}$ for ^{3}H-methyl scopolamine inhibition of carbachol-induced amylase secretion was 7×10^{-10} M.

Key words: Pancreas – ^{3}H-methyl scopolamine – Amylase release

Several previous investigators (Christophe et al. 1978; Jensen et al. 1978; Robberecht et al. 1978; Morisset et al. 1979) reported on the use of radioactively labeled agonists and antagonists for the study of pancreatic receptors. Agonists showed poor correspondence between their binding properties and biological activity (Jensen et al. 1980). Antagonists that compete with the binding of peptide hormones to the pancreatic receptors responsible for exocrine secretion are not yet available. The lack of correspondence of the binding of a ligand with its biological activity does not preclude its use in receptor assay studies. However, the compatibility of affinity constants for binding with pharmacologic activity is one criterion indicating that the ligand binding does, in fact, involve a receptor interaction. For this reason, when receptor assays are performed in a new system, it is important to combine studies of biologic activity with those of chemical binding if

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it is possible to do so. Although a relatively large number of muscarinic antagonists are available, few studies on muscarinic receptors of the pancreas have been reported. Morisset et al. (1977, 1979) and Larose et al. (1979, 1981) published a series of reports in which ^{3}H-quinuclidinyl benzilate (QNB) was used to study pancreatic muscarinic receptors. They reported that the binding of this compound to pancreatic tissue requires 90–120 min and that the non-specific binding is almost equal to the specific binding. While these studies indicate that QNB is useful in the study of pancreatic muscarinic receptors, it would seem important to determine whether other muscarinic antagonists might be more desirable for determining receptor densities in pancreatic acinar cells.

Studies on the binding of ^{3}H-methyl scopolamine to dispersed pancreatic acini were undertaken because previous investigations involving the use of ^{3}H-scopolamine in other tissues (Kloog and Sokolovsky 1978) indicated that it has the desirable characteristics of faster association time and less non-specific binding than QNB.

Materials and methods

Male Sprague-Dawley rats (150–200 g) were allowed free access to food and water until sacrificed by decapitation. The pancreases were removed, trimmed of excess fat and connective tissue, and digested according to the method of Jamieson and associates (Schultz et al. 1981). The digestion and incubation of acini were conducted in Leibovitz L-15 medium containing 10% newborn calf serum.

Amylase release was determined according to Gardner and Jackson (1977). Acini were incubated with the appropriate carbachol and methyl scopolamine concentrations in Leibovitz L-15 medium containing 10% newborn calf serum. Theophylline and trypsin inhibitor were not added to the incubation medium. At the end of the incubation period the cells were centrifuged for 30 sec at 8000 × g in a Beckman model 152 Microfuge. The amylase in the supernatant was determined on the autoanalyzer by a modification of the method of Harms and Camfield (1966).

Binding assays were conducted by a modification of the method of Fields et al. (1978). Leibovitz L-15 medium buffered at pH 7.4 and containing 10% newborn calf serum was used to incubate the cells. The ^{3}H-methyl scopolamine had a specific activity of 53.5 Ci/mmol and was obtained from New England Nuclear Corporation. The incubation of acini for the time course studies of ^{3}H-methyl scopolamine binding was conducted in 100 ml siliconized flasks which were capped and gassed at 10 min intervals with 100% oxygen. The incubation volumes were 20 ml and 15 ml for the total and non-specific binding respectively. Aliquots of 1 ml were taken at the appropriate times for binding assays. Determinations of total binding were done in triplicate, and non-specific binding determinations in duplicate. The bound ligand was separated from the free by filtration of the suspended acini through Whatman GF/B glass fiber filters positioned over a Millipore vacuum manifold. The filters were washed 4 times with ice cold phosphate buffered isotonic saline containing 0.5% albumin at a pH of 7.4. The filtration was completed within 10 sec. The ^{3}H-methyl scopolamine retained on the filters was allowed to extract for at least 24 h in Amersham PCS II scintillation fluid. Counting was performed on a Beckman LS-335 liquid scintillation counter. Typically the counts for total binding ranged from 350 cpm to 1500 cpm. The amount of acini used for binding studies was limited so that the total binding was less than 5% of the radioactivity in the medium. Incubations for equilibrium binding were conducted in siliconized 25 ml Erlenmeyer flasks which were capped and gassed with 100% oxygen at 10 min intervals. Incubations were carried out at 37° C for 45 min unless otherwise stated. The incubation volumes used to determine total and non-specific binding were 4 ml and 3 ml respectively. Triplicate 1 ml aliquots for the determination of the total binding and duplicate 1 ml aliquots for the determination of non-specific binding. Total binding was defined as the binding that occurred in the absence of atropine sulfate. Non-specific binding was defined as the binding that occurred in the presence of 10^{-4} M atropine sulfate. Specific binding was designated as the difference between total and non-specific binding. The protein content of the preparation was determined by centrifugation and resuspension of the preparation of acini 3 times in cold isotonic saline. The protein was solubilized by boiling of the acini for 10 min in 1 N NaOH and determined by the method of Lowry et al. (1951).

Preparations of dispersed acini were taken at the beginning and end of the time they were incubated in the presence of carbachol and prepared for transmission electron microscopy. The acini were centrifuged at 500 g for 2 min and immersed in 3% glutaraldehyde fixative in 0.2 M cacodylate buffer, pH 7.2, according to the method of Sabatini et al. (1963). After fixation the acini were rinsed 3 times in 0.2 M cacodylate buffer (Hayat 1970) at pH 7.2 containing 8.6% sucrose, and then post-fixed in s-collidine buffered osmium tetroxide (Bennett and Luft, 1959) for 1 h. Following a veronal-acetate buffer rinse (0.14 M, pH 7.2) (Palade, 1952) the acini were stained en bloc in 2% aqueous uranyl acetate (Hayat 1970), infiltrated in a one-to-one mixture of Spurr epoxy and acetone, followed by 100% epoxy (Spurr 1969), and embedded in Spurr at 60° C overnight.

Sections of approximately 1000 Å (pale gold to silver interference color) were spread with xylene vapor, placed on 200-mesh copper grids, dried and stained for 5 min in lead citrate (Reynods, 1963). Grids were viewed in a Phillips EM 300 transmission electron microscope at an accelerating voltage of 60 kV. Images were recorded on Kodak 4463 film at original magnifications between 1361 × and 8090 ×. Final magnifications are as noted in each Figure.

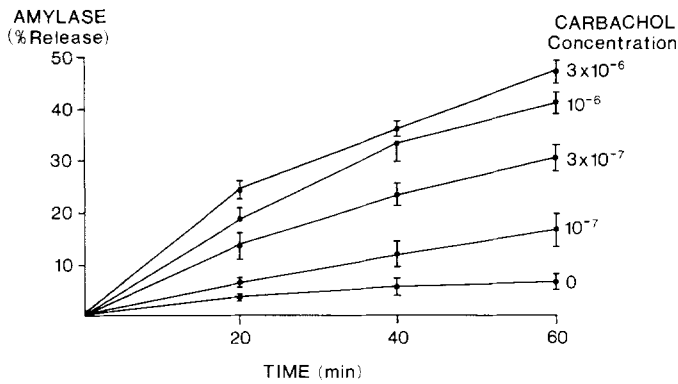


Fig. 1. Time course of amylase release by dispersed acini during carbachol stimulation. Mean and ± standard deviation of the mean for 6 experiments. Maximal amylase release occurred at 3×10^{-6} M carbachol concentrations, and the $E.D._{50}$ for carbachol induced amylase release was approximately 3×10^{-7} M. Most preparations released more than 45% of initial amylase content at end of 1 h

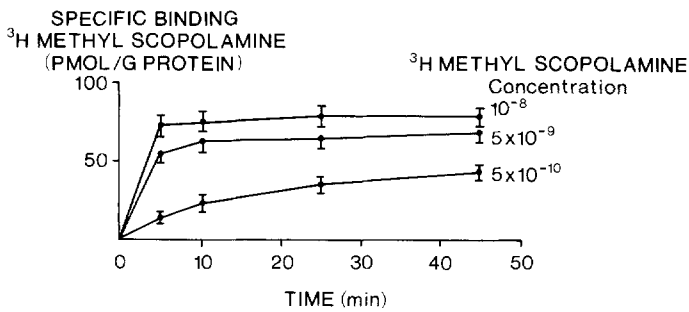


Fig. 2. Time course for ^{3}H-methyl scopolamine binding. Mean and ± standard deviation of mean for 6 experiments. Non-specific binding approximately 10% of specific binding. Range of protein concentrations of preparation 0.4 to 0.9 mg/ml. Incubations conducted at 37° C

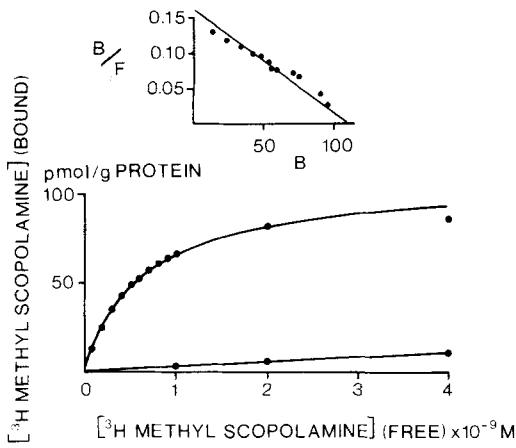


Fig. 3. Saturation binding studies of ^{3}H-methyl scopolamine to dispersed pancreatic acini. Specific binding indicated by upper curve, non-specific binding by lower line. Points on each curve represent the mean of 4 experiments. Preparation incubated at 37° C for 45 min. Scatchard plot derived from specific binding data in the upper part of Fig. Regression line fitted to the points in the Scatchard plot by the least squares method. Maximum binding 109 p mol/g of protein. Dissociation constant (K_D) 0.68×10^{-9} M. Line connecting specific binding points in saturation binding curves fitted based on equation: $B=(B_{max} \times F)/(K_D+F)$. B amount of ligand bound; F concentration of free ligand

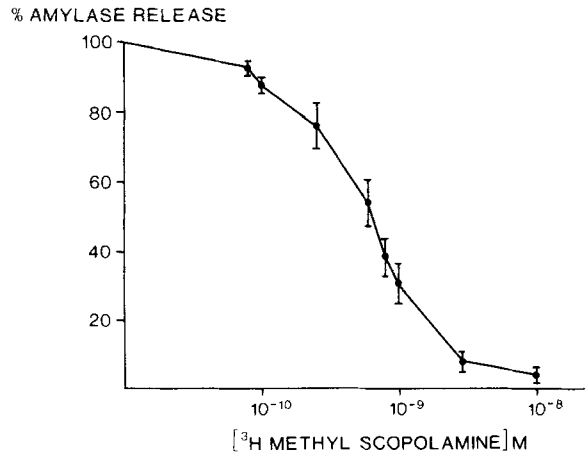


Fig. 4. Effects of ^{3}H-methyl scopolamine on carbachol stimulated amylase release by dispersed acini. Mean ± S.D. for 4 experiments. Carbachol concentration 3×10^{-7} M, incubation time 45 min. Curve corrected for baseline amylase release of carbachol stimulation. $I.C._{50}$ for ^{3}H-methyl scopolamine inhibition of carbachol-stimulated amylase release approximately 0.7×10^{-9} M

Results

Time course for amylase release by dispersed acini during carbachol stimulation. The effects of carbachol on amylase release observed in 6 experiments is shown in Fig. 1. The maximal rate of amylase release occurred with 3×10^{-6} M carbachol. Concentrations of 10^{-5} M carbachol produced the same or slightly less amylase

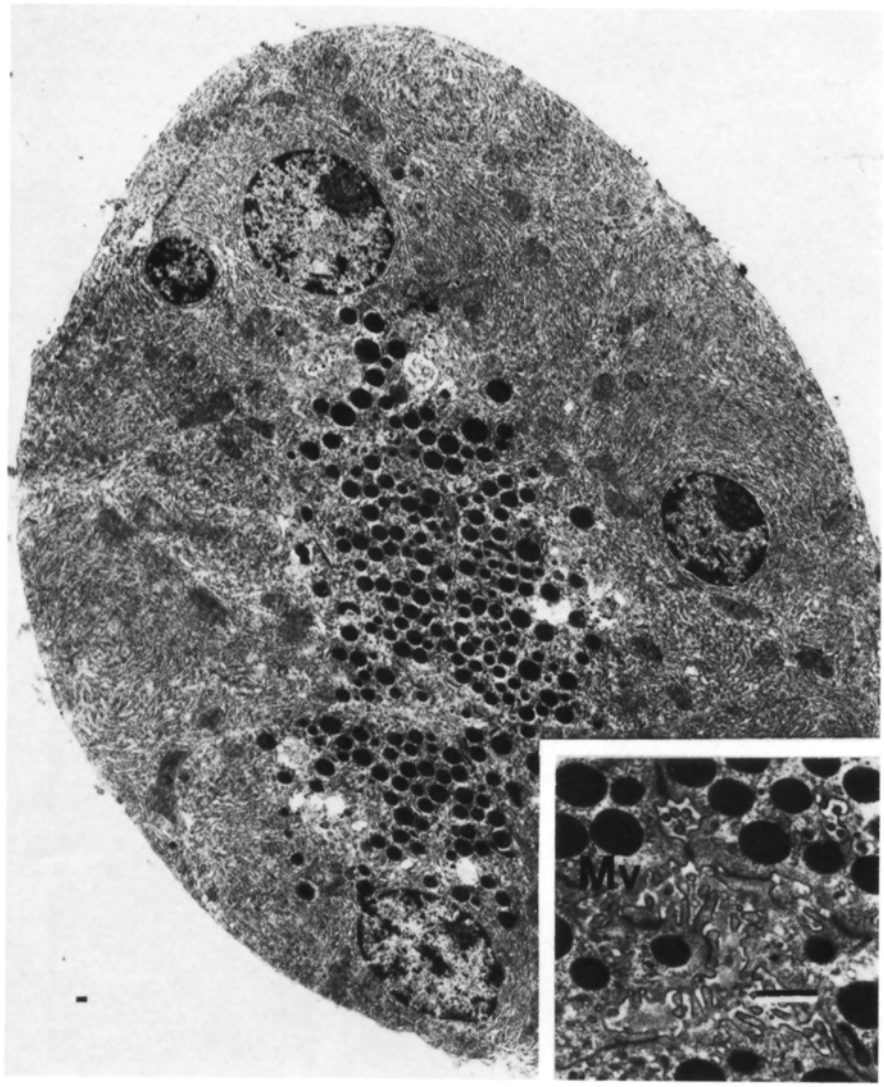


Fig. 5. Freshly isolated pancreatic acinus. No carbachol treatment. Ductule lumina show no swelling but slight amount of secretory material within (*inset*). Microvilli Mv are evident. Bar represents 1 μm. ×3,466. *Inset:* ×9,257

release. With 3×10^{-6} M carbachol, 23% (± 2.4 S.D.), 35.5% (± 2.4 S.D.) and 46.8% (± 1.6 S.D.) of the initial amount of amylase in the acini were released at 20, 40 and 60 min respectively. The rate of amylase release was not constant with time at any of the carbachol concentrations studied but decreased with time. Slightly greater than half-maximal amylase Release occurred with 3×10^{-7} M carbachol.

Time course studies of ^{3}H-methyl scopolamine binding. The time course for ^{3}H-methyl scopolamine binding to dispersed pancreatic acini at 37° C

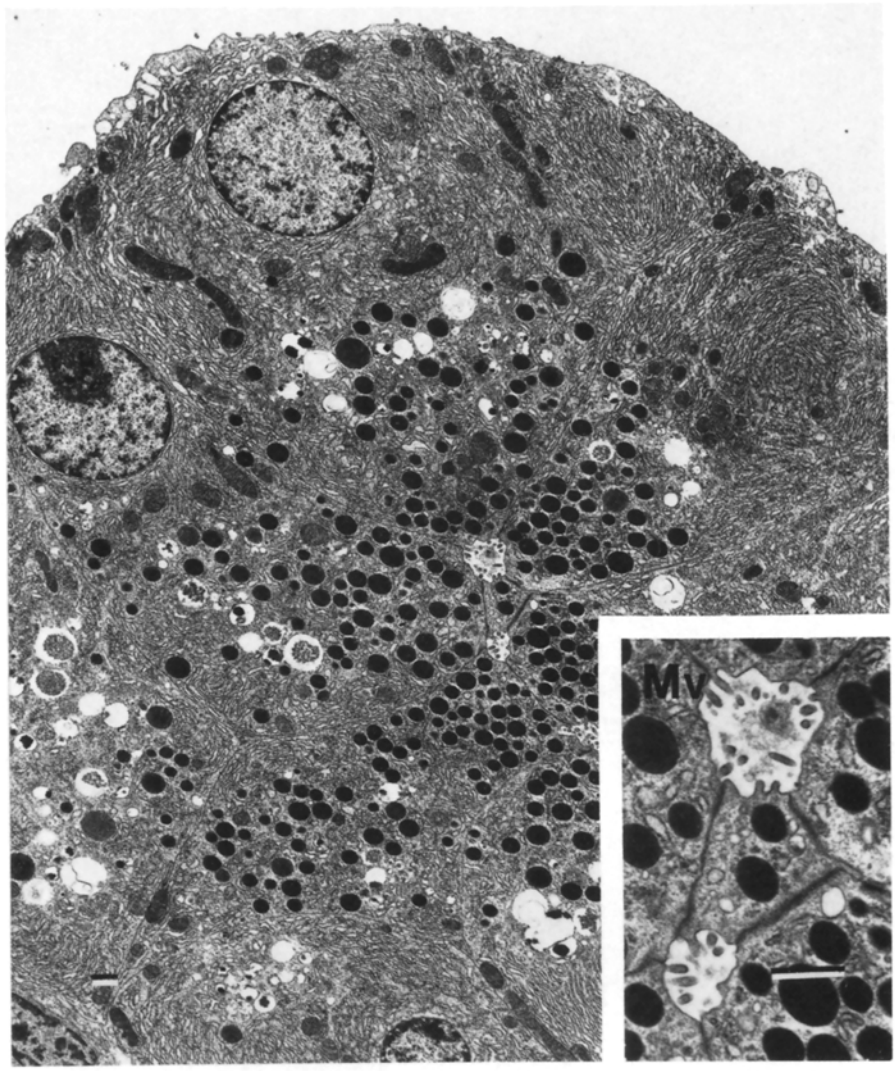
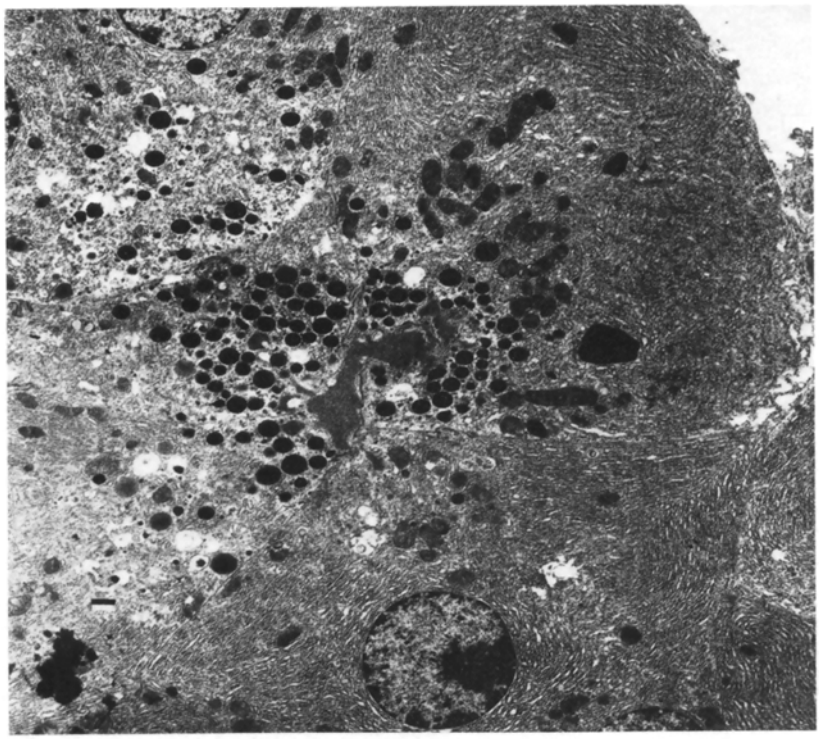


Fig. 6. Acinus fixed after 1 h incubation in carbachol free medium. Ductule lumen shows moderate amount of secretory material. Lumen not swollen, microvilli *Mv* resembling those in Fig. 5 (*inset*). Bar 1 μm. ×3,281. *Inset:* ×10,113

(mean ± standard deviation for 6 experiments) is shown in Fig. 2. The time required to reach equilibrium varied with the concentration of the ligand used. At 5×10^{-9} M concentrations of ^{3}H-methyl scopolamine that interval was about 10 min. On the other hand, it took 45 min to reach equilibrium with 5×10^{-10} M ^{3}H-methyl scopolamine.

Saturation binding studies. The results of the equilibrium binding studies performed with ^{3}H-methyl scopolamine in concentrations ranging from 0.1 to 4 nM are



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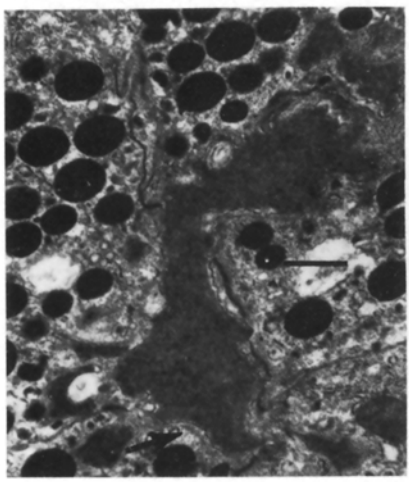

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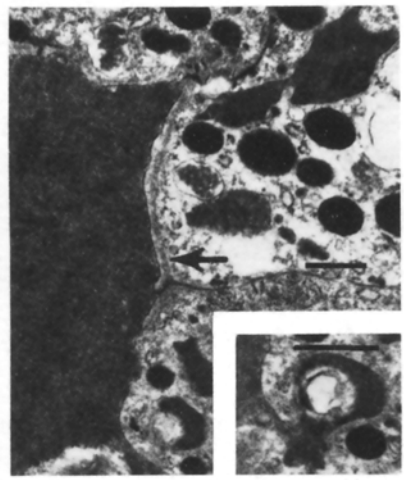

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Figs. 7–9. Acinus fixed after 1 h in medium containing 3×10^{-6} M carbachol. Ductule lumina expanded and filled with dense secretory product. Microvilli absent, × 3,283. Note apical web of microfilaments. Figs. 8 (*lower left*) × 8,235 and 9 (*lower right*) × 8,235 (*arrows*). Note also abnormally discharging secretory granules (Figs. 8, 9). *Inset* to Fig. 9 depicts one of the granules in an adjacent section. Its contents are continuous with material in lumen. Bar(s) 1 μm. × 11,172

shown in Fig. 3. Here the points represent the mean of 4 experiments. The specific ligand binding, shown in the upper curve of Fig. 3a, demonstrates a hyperbolic function typical for saturation phenomena. The non-specific binding, defined as that which occurs in the presence of 10^{-4} M atropine (lower curve of Fig. 3a) varies linearly with ligand concentration. A Scatchard plot derived from the specific binding data in Fig. 3a is shown in Fig. 3b. The dissociation constant for ^{3}H-methyl scopolamine binding was 0.68 nM and saturation occurred at 109 pmol/g protein.

Inhibition of amylase release by ^{3}H-methyl scopolamine. The effects of ^{3}H-methyl scopolamine on carbachol-stimulated amylase release by dispersed acini (mean ± S.D. for 4 experiments) is shown in Fig. 4. The carbachol concentration used was 3×10^{-7} M and the incubation time 45 min. The curve was corrected for the baseline amylase release that occurred during the 45 min incubation period in the absence of carbachol. The I.C.$_{50}$ for ^{3}H-methyl scopolamine was approximately 7×10^{-10} M.

Electron microscopic studies. The plasma membranes on the outer surfaces of the acini which were directly exposed to collagenase appeared to be undamaged when examined by transmission electron microscopy (Figs. 5–9). There was an abundance of zymogen granules in the apical portion of the cells. The acinar lumens were narrow, contained prominent microvilli and only a slight amount of secretory material. A few loose and dense condensing vacuoles were observed in the vicinity of the Golgi complex. After incubation for 1 h in carbachol-free media the appearance of the acini was not significantly changed relative to that observed in the preincubation specimens.

The acini observed after 1 h of incubation in 3×10^{-6} M carbachol were markedly depleted of zymogen granules. Condensing vacuoles and lysosomal organelles were observed in the region between zymogen granules and nucleus. The microvilli facing the acinar lumens were flattened and the lumens were distended by secretory material. The zymogen granules adjacent to luminal surface of the acinar cells occasionally appeared to fuse with other zymogen granules more distant from the lumen as they discharged their material into the ductular lumen. An apical web of microfilaments could be clearly seen in the acini after carbachol stimulation.

Discussion

Numerous reports have appeared concerning the effects of secretagogues on isolated pancreatic acini or acinar cells. In earlier studies in Jamieson's laboratory collagenase and other proteolytic enzymes were used in a Ca^{++}-free medium to dissociate pancreatic tissue into individual cells (Amsterdam and Jamieson 1974, 1974a). The low sensitivity and responsiveness of dispersed acinar cells relative to those observed in pancreatic lobules raised doubt about the usefulness of acinar cell preparations in the study of pancreatic secretion. Recently several laboratories (Jensen et al. 1978; Peikin et al. 1978; Williams et al. 1978; Schultz et al. 1981) reported that the secretory responses of dispersed acini are comparable to those in

situ. Most of these preparations were made in Ca^{++}-containing media, and proteolytic enzymes other than collagenase were omitted.

In addition to the reduced responsiveness to secretagogues, morphologic changes occur when pancreatic tissue is incubated in Ca^{++}-free media or in a medium that contains enzymes which attack proteins other than collagen. Meldolesi et al. (1979) incubated guinea pig acini in Ca^{++}-free medium and found that after 2 h most of the tight junctions were dissembled. They also found a loss of the normally asymmetric distribution of intramembranous particles on the P-fracture faces of the basolateral and luminal plasmalemma. Jamieson and associates (Schultz et al. 1981) studied the morphologic effects of crude collagenase and chymotrypsin on pancreatic acinar cell. These enzymes resulted in an aggregation of the intramembranous particles and a proliferation of non-cross linked sealing strands in many of the tight junctions. The mechanisms by which certain proteolytic enzymes and Ca^{++}-free media, which cause these morphologic changes, affect the responses to secretagogues have not yet been determined.

Jamieson and associates (Schultz et al. 1981) also found that gel filtration with G-75 Sephadex separates collagenase from other proteolytic enzymes that cause cellular damage. They found that, when Worthington Type III crude collagenase is subjected to this treatment, a peak of collagenase activity elutes from the G-75 Sephadex column shortly after the void volume which is low in non-collagenase proteolytic activity. This fraction of collagenase could be used in the preparation of highly responsive acini. In the present study we have confirmed the observations of Schultz and associates and have obtained consistently good results with this preparation of collagenase. In contrast to the findings of other laboratories (Peikin et al. 1978; Williams et al. 1978) we have obtained poor or inconsistent results using SIGMA Type VI chromatographically purified collagenase.

The ultrastructural appearance of the dispersed pancreatic acini of the rat in these experiments is comparable to that in a similar preparation of mouse acini (Williams 1978, 1980). Williams also reported that maximal doses of the ionophore A23187 cause a release of about 35% of the amylase content in 30 min in mouse acini and observed a similar amount of zymogen degranulation. The appearance of loose and tight condensing vacuoles and lysosomal degradation of organelles was also previously reported in dispersed pancreatic acinar cells by Amsterdam and Jamieson (1974a).

In our experiments we found that 3×10^{-6} M carbachol causes maximal amylase release which is about 47% of the initial amylase content at the end of 1 h of stimulation. Carbachol concentrations of 1×10^{-5} M cause the same or less release as those of 3×10^{-6} M. Half-maximal amylase release by carbachol occurs at about 3×10^{-7} M. In contrast Gardner and associates (Peikin et al. 1978) found that maximal and half-maximal amylase release occurred at 10^{-5} and 10^{-6} M carbachol concentrations, respectively. The incubation media used in that study contained 5 mM theophylline. Korman and Gardner (1979), however, reported later that theophylline and other methyl xanthines cause the dose-response curve for the effect of carbachol on amylase release to shift to the right. Larose et al. (1981) recently reported that the $E.C._{50}$ for carbachol stimulation of amylase release was 4×10^{-7} M in pancreatic lobules incubated in the absence of theophylline. In our

study we omitted theophylline from the incubation since there was no apparent need for it. Trypsin inhibitor was also omitted from the incubation media in most instances because it did not enhance the responsiveness of the acini.

Kinetic studies of amylase release by dispersed acini versus that of single acinar cells were previously reported (Peikin et al. 1978). Those investigators found that optimal concentrations of cholecystokinin-octapeptide (CCK-OP) cause a release of 32 to 40 % of the total amylase initially present in their acinar preparation at the end of a 60 min period of stimulation. The amylase release with carbachol stimulation is similar to that with CCK-OP. The combined action of vasoactive intestinal peptide (10^{-8} M) and CCK-OP causes a release of about 50 % of the initial amylase content. About 6 % of the initial amylase content of the preparation is liberated after 1 h of incubation in the absence of any secretagogues. This baseline release of enzyme is comparable to that observed in our preparation.

Oliver (1981) observed that it was important to maintain her preparation in an atmosphere containing 5 % CO_2 at all times. Since the medium we use (Leibovitz L-15 medium) does not contain a bicarbonate buffering system, it is not necessary to maintain our preparation in a 5 % CO_2 environment. We found that the selection of the particular collagenase preparation used is the most important factor in maintaining the responsiveness of the acini to carbachol stimulation.

The time required for ^{3}H-methyl scopolamine to reach equilibrium in pancreatic tissue appears to be considerably shorter than that required for ^{3}H-QNB. Morisset and associates (Morisset et al. 1977, 1979; Larose et al. 1979, 1981) found that up to 2 h were required to attain equilibrium conditions with 40 pM QNB at 37° C. Other investigators, on the other hand, reported that maximal binding occurred in the guinea pig ileum with 900 pM QNB within 10–30 min at 35° C (Yamamura and Snyder 1974). In contrast to the findings of Yamamura and Synder, another report indicated that 600 pM QNB reached maximal binding in 40 min at 35° C while scopolamine required 23 min under similar conditions (Kloog and Sokolovsky 1978). The current study is consistent with the findings of previous investigators in that the time required for maximal methyl scopolamine binding in pancreatic tissue is shorter than that reported for QNB by Morisset and associates (Larose et al. 1979) under similar conditions. The current study also emphasizes the fact that lower ligand concentrations require longer times to reach equilibrium than higher ligand concentrations.

In the current study the $I.C._{50}$ for methyl scopolamine inhibition of carbachol induced enzyme secretion was approximately 7×10^{-10} M, and the dissociation constant for ^{3}H-methyl scopolamine binding was 6.8×10^{-10} M. This is a good indication that the binding observed did, in fact, represent a receptor interaction. Morisset and associates (Morisset et al. 1977, 1979; Larose et al. 1979, 1981) reported a similar correspondence between the dissociation constant for ^{3}H-QNB binding and its $E.D._{50}$ for inhibition of carbachol induced enzyme secretion. The incubation times used by those investigators, however, were approximately twice those used in the present study. Morisset and associates (personal communication) were unable to observe a good correspondence between biologic activity and tissue binding of ^{3}H-QNB when the incubation times were reduced to less than 90 min. They felt that this effect occurred because 90–120 min are required for maximal QNB binding.

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