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BINDING OF [^{3}H]QUINUCLIDINYL BENZILATE TO INTESTINAL MUCUS

AN ARTIFACT IN IDENTIFICATION OF EPITHELIAL CELL MUSCARINIC RECEPTORS

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Abstract—The widely used muscarinic receptor ligand [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) was found to bind in a site-specific but artifactual manner to rat intestinal mucus, obscuring specific binding to muscarinic receptors on intestinal epithelial cells. Atropine inhibited [^{3}H]QNB binding to mucus with an apparent IC_{50} of 2.1×10^{-7} M, compared to an IC_{50} of 1.4×10^{-8} M obtained with a homogenate of intestinal epithelial cells. Unlabeled QNB also inhibited binding of [^{3}H]QNB to mucus but the apparent IC_{50} (4×10^{-7} M) was about 300-fold greater than the IC_{50} determined with a control tissue, heart muscle (IC_{50}, 1.2×10^{-9} M). [^{3}H]QNB binding was saturable over the concentration range of 1–7 nM in the heart, with an apparent K_D of 0.76 nM. As expected from the high IC_{50} for QNB in the mucus binding experiments, binding to mucus was not saturable over the 1–15 nM concentration range. Based on pH profiles and temperature dependency of binding, it seems unlikely that mucin, the primary component of mucus, was responsible for [^{3}H]QNB binding to the mucus. The findings have implications for studies which involve binding of [^{3}H]QNB in particular and other ligands in general to mucus-secreting epithelial tissues.

[^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) has been widely used as a ligand to identify muscarinic receptors in various tissues including rat brain [1], heart [2–4], and the digestive tract [5–7]. Muscarinic agonists stimulate ion secretion in isolated intestinal mucosa [8–10], suggesting that [^{3}H]QNB might also be useful in demonstrating the presence of muscarinic receptors in intestinal epithelial cells.

However, unlike brain and heart tissue, crude suspensions of intestinal cells are contaminated with mucus. Although we have purified a cell suspension and provided sound evidence for the presence of muscarinic receptors on colonocyte membranes [11], we observed in early work the apparent receptor-related (site-specific) binding of [^{3}H]QNB to intestinal mucus.

The present report characterizes this binding relative to that in a tissue known to contain muscarinic receptors and to be free of mucus. Because of the similarities in the characteristics of binding of [^{3}H]QNB to mucus and epithelial cell receptors, caution is advised in interpreting binding data obtained with this ligand in any cell preparation (e.g. salivary, tracheal, gastric, intestinal) that might be contaminated with mucus.

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MATERIALS AND METHODS

Preparation of tissue for binding studies. The following procedure was followed for isolation of ileal and colonic epithelial cells. Male Sprague–Dawley rats (four per experiment), obtained from the Laboratory Supply Co., Indianapolis, IN, and weighing between 200 and 350 g, were killed by a sharp blow to the head. Immediately after killing the rats, 30 cm of distal ileum or the entire colon was resected and cleaned of fat and mesentery, and the luminal contents were flushed three times with 10 ml of 0.9% (w/v) sodium chloride (normal saline) at room temperature. Segments were allowed to stand in normal saline at room temperature for 10 min and once again flushed before being everted over an aluminium vibrating coil (rod diameter 0.5 cm). The rods with the tissues were placed in 200 ml of isolation buffer in a plastic container for 10 min. The isolation buffer (pH 7.4 at 22°) had the following composition (mM): NaCl, 115.5; KCl, 4.6; NaH_2PO_4, 1.2; $NaHCO_3$, 21.9; $MgSO_4$, 1.2; glucose, 11.5; and EDTA, 5. The everted segments were vibrated at high frequency and low amplitude for 30 min (22°) in 250 ml of fresh isolation buffer with a Vibro-Mixer, model E-1 (Chemapec, Inc., Hoboken, NJ) to harvest cells. The cell suspension was filtered through a nylon mesh (250 μm) to trap mucus and subsequently centrifuged at 1500 *g* for 5 min (22°) to sediment the isolated cells. Cells were homogenized on ice for 20 sec in the isolation buffer with a model PT-10 Brinkmann Polytron (Westbury, NY) at setting 7. This cellular homogenate was then used in the binding assay.

Intestinal mucus was isolated from three gut seg-

ments (25 cm in length) from each of four male rats (200–300 g). After removing fat and mesentery, and flushing the lumen with normal saline, segments were everted (both ends were tied with silk sutures to prevent contamination by serosal tissue) and placed in 350 ml of normal saline at 37° for 30 min. The segments were removed and discarded, and the mucus that was secreted into the medium was collected by centrifugation at 3000 *g* for 5 min (22°). Pellets were homogenized (in the appropriate buffer) for 20 sec (Polytron at setting 7) and used in the binding assay. For the purpose of this paper, therefore, mucus is defined as the viscous secretion obtained from intestinal segments, which is collectable by centrifugation after a 30-min incubation in normal saline. These secretions of the small intestinal and colonic goblet cells are composed principally of high molecular weight glycoproteins (mucins), ions and water [12]. The pellet may also contain denuded epithelial cells, cellular debris, and bacteria [12].

To obtain an estimate of the amount of mucus in the homogenates used for the binding assay, mucin content was determined by radioimmunoassay specific for rat goblet cell mucin (performed by Dr. J. Forstner; [13]).

Heart tissue from male rats (200–300 g) was prepared for the binding assay in the following manner. The heart was removed, blotted free of blood, minced and homogenized in ice-cold buffer [0.25 M sucrose, 50 mM tris (hydroxymethyl) aminomethane (Tris), pH 7.4 at 37°] with a Polytron at setting 5. The homogenate was filtered through three layers of cheesecloth and centrifuged at 30,000 *g* for 15 min at 4° in a Sorvall centrifuge (model RC 2-B). The pellet was collected and washed once in 50 mM Tris buffer (pH 7.4 at 37°) and used for binding experiments.

Protein was determined by the method of Lowry *et al.* [14] using bovine serum albumin as the standard.

[^{3}H]*Quinuclidinyl benzilate binding.* In general, tissue or mucus was added to 2 ml polystyrene cups (Kew Scientific, Columbus, OH) and incubated at 37° for 30–40 min in a Dubnoff incubator with constant shaking (100 cycles/min), with 0.5 to 1.5 nM [^{3}H]QNB (12.0 or 29.4 Ci/mmole) and specific drugs (where indicated) in a total volume of 1.5 ml of 50 mM Tris buffer (pH 7.4 at 37°). After incubation, samples were filtered under vacuum through Tris-prewetted Whatman GF/B glass fiber filters and processed as previously described [11]. Radioactivity bound to the tissue was determined using a Beckman LS-345 liquid scintillaton spectrometer. Appropriate corrections were made for quench which was monitored by an automatic external standard. Counting efficiencies for tritium were routinely between 35 and 40%.

[^{3}H]QNB bound in the presence of 1×10^{-5} M atropine was operationally defined as nonspecific binding. Nonspecific binding was subtracted from total binding to obtain "specific" binding. The IC_{50} is defined as the molar concentration of drug which inhibited total [^{3}H]QNB binding to tissue by 50%. IC_{50} Values were determined from a log-probit plot, using four to five different concentrations of the inhibitor. Binding to the filters (~1% of total radioactivity added) accounted for most of the nonspecific binding when [^{3}H]QNB was present at ~0.5 nM. There was no detectable binding to the plastic incubation cups.

Sources of drugs. DL-[Benzilic-3-^{3}H]quinuclidinyl benzilate (12.0 Ci/mmole) was obtained from the Amersham Corp. (Arlington Heights, IL) and DL-[benzilic-4,4'-^{3}H]quinuclidinyl benzilate (29.4 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). We routinely checked the radiochemical purity of [^{3}H]QNB by thin-layer chromatography using silica gel-GF plates (New England Nuclear) and a solvent system of chloroform–acetone–diethylamine (60:30:10). The percent radiochemical purity was found to be never less than 95%.

Other drugs were obtained as indicated: atropine sulfate, bovine albumin (Fraction V), hexamethon-

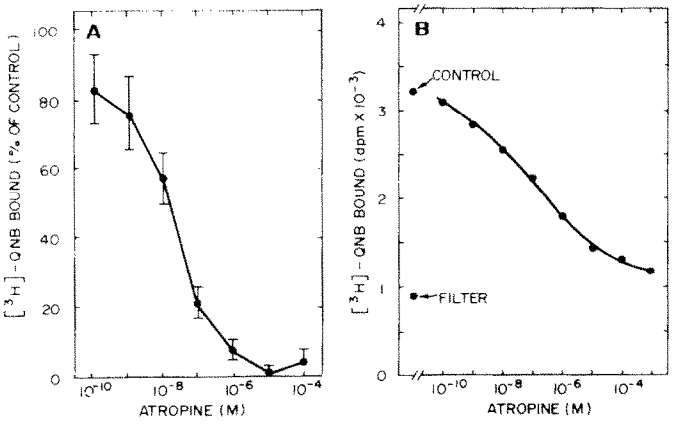

Fig. 1. Inhibition of [^{3}H]QNB binding by atropine. Incubations were performed at 37° for 35 min in the isolation buffer described in Materials and Methods. [^{3}H]QNB was present at ~1.0 nM with ~ 400–500 μg protein. (A) Colonic epithelial cells isolated by vibration. Points represent total [^{3}H]QNB binding minus filter binding and are the mean ±S.E. from three experiments each in triplicate. An IC_{50} of 1.4×10^{-8} M for atropine was obtained for binding to isolated cells. (B) Intestinal mucus. Data are from a representative experiment performed in duplicate. Control indicates incubations without atropine and filter refers to filter binding. An IC_{50} of 2.1×10^{-7} M for atropine was obtained for binding to mucus.

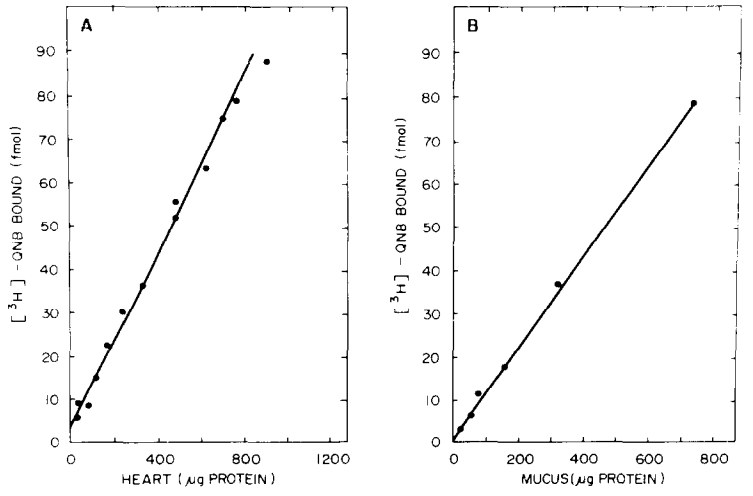

Fig. 2. Effect of protein concentration on [^{3}H]QNB binding. Incubations were performed at 37° for 35 min in Tris buffer as described in Materials and Methods. (A) Total binding to heart. Data are from two experiments each in triplicate. (B) Total binding to mucus. Data are from two experiments each in triplicate.

ium bromide, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulfate, mucin (crude, type II from porcine stomach), and Trizma HCl, Trizma base (Sigma Chemical Co., St. Louis, MO). Unlabeled (±)-QNB HCl was provided by Hoffmann–La Roche Inc. (Nutley, NJ).

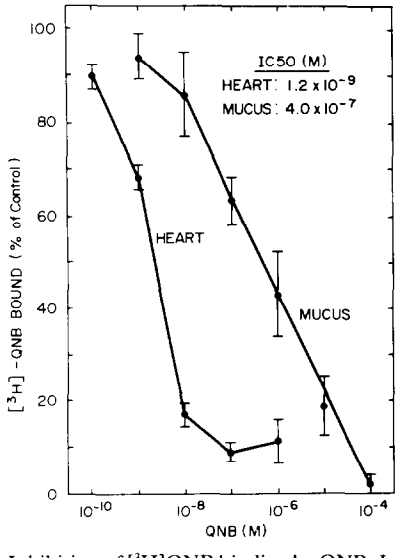

Fig. 3. Inhibition of [^{3}H]QNB binding by QNB. Incubations were performed at 37° for 35 min in Tris buffer as described in Materials and Methods. [^{3}H]QNB was present at ~1.0 nM with ~400–600 μg protein. Points represent total [^{3}H]QNB binding minus filter binding are and the mean ±S.E. from three experiments each in duplicate. For each experiment, heart and mucus were isolated from the same rat, and binding assays were performed simultaneously. The IC_{50} values of 1.2×10^{-9} M and 4.0×10^{-7} M were obtained for QNB in heart and mucus respectively.

RESULTS

Estimation of mucus content. Initial experiments were designed to obtain an estimate of the relative amount of mucus contained in the isolated epithelial cell and mucus pellets. When samples were assayed for goblet cell mucin by radioimmunoassay [13], twice as much mucin (per μg protein) was found in the mucus as compared to the cellular pellet (13.4 vs 7.2 ng mucin/μg protein). There was no measureable mucin in tissues (e.g. skeletal muscle) devoid of mucus.

Characteristics of [^{3}H]QNB binding to ileal and colonic epithelial cells and mucus. Preliminary experiments were designed to test the ability of atropine to inhibit [^{3}H]QNB binding to a homogenate of isolated colonic epithelial cells. Atropine inhibited the binding of [^{3}H]QNB in a concentration-dependent manner (Fig. 1A), reaching maximum inhibition at 5×10^{-6} M to 1×10^{-5} M, with an IC_{50} of 1.4×10^{-8} M. Similar results were obtained for ileal cells (data not shown). Surprisingly, atropine also inhibited [^{3}H]QNB binding to mucus. However, the curve for binding to mucus was more shallow and was shifted to the right; IC_{50} was 2.1×10^{-7} M, about 15-fold greater than for the cell homogenate (Fig. 1B).

We chose to compare binding of [^{3}H]QNB to mucus and heart muscle (as a "positive" control), in an attempt to elucidate the differences and/or similarities in the characteristics of the binding. Both heart and mucus bound [^{3}H]QNB, as a function of protein concentration, in a similar manner (Fig. 2). Mucus did not pass through the filter as evidenced by the linearity of binding. Unlabeled QNB inhibited the binding of [^{3}H]QNB to heart in a concentration-dependent manner (Fig. 3), reaching maximum inhibition at about 1×10^{-7} M with an IC_{50} of 1.2×10^{-9} M. In contrast, a much more shallow curve was obtained for binding to mucus, with an apparent IC_{50} that was 300-fold higher at 4.0×10^{-7} M (Fig. 3).

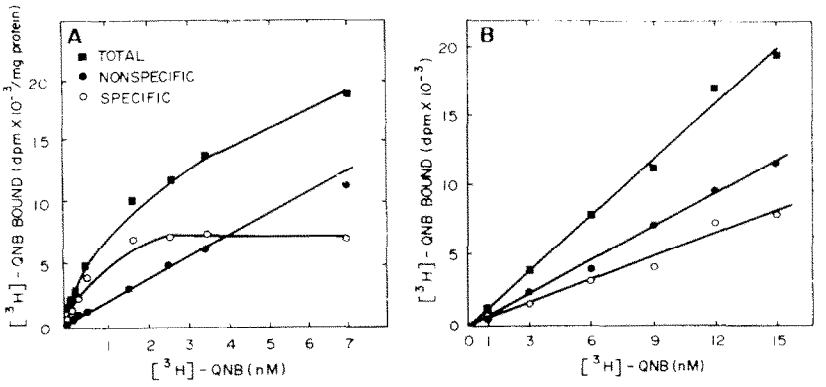

Fig. 4. Binding as a function of increasing concentrations of [^{3}H]QNB. Tissue homogenates were incubated, as described in Materials and Methods, with various concentrations of [^{3}H]QNB. Nonspecific binding was measured in the presence of 1×10^{-5} M atropine. (A) Binding to heart. Data are from a representative experiment performed in triplicate. Specific binding approached saturation at a [^{3}H]QNB concentration of about 2 nM; half-maximal saturation occurred at about 0.5 nM. The apparent dissociation constant (K_D) and maximum number of binding sites (B_{max}) obtained from a Scatchard plot of specifically bound [^{3}H]QNB were 0.76 nM and 136 fmoles/mg protein respectively. (B) Binding to mucus. "Specific" binding to mucus, operationally defined as the difference between total and nonspecific binding, although displaceable by atropine was not saturable.

"Specific" binding to mucus, operationally defined as the difference between total and nonspecific binding (in the presence of 1×10^{-5} M atropine), although inhibitable by atropine was not saturable over the concentration range of [^{3}H]QNB tested (Fig. 4B). On the other hand, specific binding to heart approached saturation at a [^{3}H]QNB concentration of about 2 nM, with half-maximal saturation occurring at ~0.5 nM (Fig. 4A). The apparent dissociation constant (K_D) and maximum number of binding sites (B_{max}) obtained from a Scatchard plot of specifically bound [^{3}H]QNB for heart were 0.76 nM and 136 fmoles/mg protein respectively.

Atropine (10 μM) inhibited [^{3}H]QNB binding to heart by 72% and to mucus by 35%, while hexamethonium, serotonin and histamine (all 10 μM) had no effect on [^{3}H]QNB binding to either preparation.

Characteristics of [^{3}H]QNB *binding to mucin.* Mucus secretions of the intestine are composed principally of high molecular weight glycoproteins known as mucins. We therefore measured [^{3}H]QNB binding to isolated mucin in an effort to determine if binding to this component could account for the observed binding to mucus. [^{3}H]QNB bound to mucin in a linear fashion (Fig. 5A). However, much more mucin (per mg protein) was required to bind the same amount of ligand as compared to heart and mucus (see Fig. 2). The [^{3}H]QNB binding to mucin was not inhibited by atropine (Fig. 5B), hexamethonium, serotonin or histamine (all 10 μM).

Effect of pH and temperature on [^{3}H]QNB *binding.* Binding was also measured as a function of pH and temperature. There was a broad pH optimum for binding to the heart, with specific binding remaining

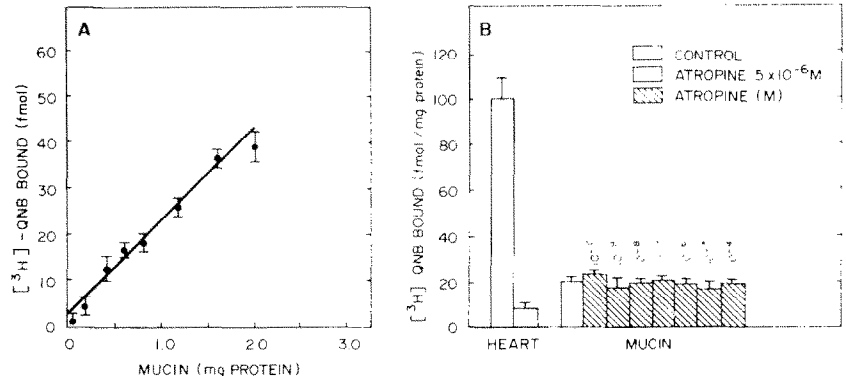

Fig. 5. [^{3}H]QNB binding to mucin. Incubations were performed at 37° for 35 min in Tris buffer as described in Materials and Methods. [^{3}H]QNB was present at ~1.0 nM. (A) Effect of protein concentration on [^{3}H]QNB binding. Points represent total minus filter binding and are the mean ± S.E. from three experiments each in duplicate. (B) [^{3}H]QNB binding to mucin as a function of atropine concentration. Data represent the mean ±S.E. from three experiments each in triplicate or duplicate. Filter binding has been subtracted. Binding to the heart is presented for comparison.

relatively stable between pH 5.5 and 7.5. In contrast, binding to mucus and mucin exhibited a pH optimum of 6.5. At their pH optima, specific binding to heart, mucus and mucin were 70, 30 and 0% of total binding respectively.

Binding of [^{3}H]QNB to heart was temperature dependent; both total and specific binding increased when tissue was incubated at 0, 25 and 37°. Specific binding increased from 33% at 0° to 40% at 25° and 78% at 37°. Nonspecific binding stayed relatively constant. When tissue from heart was preincubated for 10 min at 90°, specific binding was completely abolished and nonspecific binding increased. "Specific" binding to mucus remained at 40% of total binding, and both total and nonspecific binding decreased, from 0 to 37°. When mucus was preincubated at 90° "specific" binding was abolished but total binding was twice that at 37°. Total and nonspecific binding to mucin were identical (no specific binding) and progressively decreased when incubated at 0, 25, 37 and 90° respectively.

DISCUSSION

The present study demonstrates that rat intestinal mucus binds [^{3}H]QNB in a displaceable fashion. The characteristics of this binding differ from binding to "true" muscarinic sites in heart. However, the binding closely resembles true receptor binding so that care should be taken when [^{3}H]QNB is used to identify muscarinic receptors in mucus-producing (containing) tissues.

Mucus bound [^{3}H]QNB nonspecifically to a large extent, but we were surprised to find that atropine and QNB inhibited the binding. However, the characteristics of binding to mucus and its inhibition by atropine and QNB were dissimilar to binding to heart (see Figs. 3 and 4). Since true receptor binding is saturable, we determined whether or not the binding of [^{3}H]QNB to mucus was saturable. It cannot be considered specific since we found it did not saturate over the [^{3}H]QNB concentration of 1–15 nM, certainly a range over which saturation to muscarinic receptors occurs when this ligand is used with other tissues [1, 2, 4, 11]. This was expected, since the IC_{50} values for QNB and atropine were about 100-fold higher in the mucus preparation than in the heart. Thus, at best the binding of [^{3}H]QNB to mucus can be considered to be nonspecific at a site where atropine and QNB effectively compete with [^{3}H]QNB. The only similarities we observed between [^{3}H]QNB binding to mucus and heart were the amount of binding as a function of protein concentration and the fact that non-muscarinic drugs had no effect on binding to either heart or mucus.

The goblet cells of the intestinal tract secrete mucus glycoproteins (mucins) which are the principal constituents of the mucus gel matrix that lines the surface of the intestinal mucosa [15–17]. We therefore measured the binding of [^{3}H]QNB to mucin, to test if binding to this component could account for the observed binding to mucus. Although mucin bound [^{3}H]QNB, atropine was unable to inhibit this binding; thus, binding to mucin can be defined as truly nonspecific in nature. Therefore, mucin *per se* does not account for the observed inhibition by atropine of [^{3}H]QNB binding to mucus. It must be pointed out that mucin isolated from porcine stomach was used in this study and it may bind QNB differently from isolated rat intestinal mucin, which was not available for purchase. Furthermore, the isolation procedure to obtain the mucin may have altered its binding characteristics.

It must be remembered that mucus consists of more than just the mucin glycoproteins. Although some of the displaceable binding of [^{3}H]QNB observed in the mucus preparation may have been to epithelial cells that are constantly being shed into the lumen at villous tips, the binding characteristics were not the same as those of isolated cells (see Fig. 1), or binding to a mucus-free plasma membrane preparation [11]. Binding could have also been to secreted mucin by-products altered in structure by enzymes intrinsic to the intestine or by bacterial enzymes [18]. It is also possible that [^{3}H]QNB became trapped in pores in the cross-linked mucopolysaccharides in a fashion similar to that reported for small antibiotic molecules [19–21].

In conclusion, our findings suggest that [^{3}H]QNB binds to mucus, but the characteristics of this binding are not those of true receptor-related binding. The implications of interference of binding by mucus are indeed broad since trachial, salivary, gastric, genitourinary and intestinal epithelia produce and secrete mucus. In any of these tissues in which muscarinic receptors are being investigated, [^{3}H]QNB in particular (and other ligands in a wider sense) may lead to wrong conclusions regarding the presence and characteristics of true receptor sites.

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