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Photoaffinity Labeling of the Solubilized, Partially Purified Muscarinic Acetylcholine Receptor from Porcine Atria by *p*-Azidoatropine Methyl Iodide†

Christine Cremo and Michael I. Schimerlik*

ABSTRACT: The synthesis of a tritiated photoaffinity analogue of the muscarinic antagonist atropine, [^{3}H]-*p*-azidoatropine methyl iodide, is described. The compound appeared to bind to a single class of sites in membrane-bound, solubilized, and partially purified preparations of muscarinic receptor from porcine atria with a dissociation constant (determined by competition vs. [^{3}H]-L-quinuclidinyl benzilate) of about 1.0×10^{-7} M. This value was in agreement with the apparent dissociation constant (8.5×10^{-8} M) determined by measuring the concentration dependence of covalent incorporation into a partially purified receptor preparation. Competition experiments indicated that the specific covalent labeling could be blocked by the muscarinic agonist carbamylcholine and the antagonists L-quinuclidinyl benzilate and atropine. An apparent molecular weight of 75 000 ± 5000 was found for specifically labeled peptide(s) in a solubilized, partially purified receptor preparation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Although ligand interactions with mAcChR[1]-rich preparations from many tissues have been well characterized (Ehlert et al., 1981), structural studies have been limited due to difficulties in solubilization and, thus, purification. The techniques of affinity and photoaffinity labeling have been extremely useful, both in structural studies and as probes to characterize receptors at various stages of purification (Fewtrell, 1976; Heilbronn & Bartfai, 1978). Only two covalent probes for the mAcChR have been reported (Gill & Rang, 1973; Burgen et al., 1974; Amitai et al., 1982). Photoaffinity labeling of soluble mAcChR preparations from any tissue has not been reported to date.

The purpose of this investigation is to report the synthesis and characterization of a photoaffinity label, [^{3}H]-*p*-azidoatropine methyl iodide, specific for the mAcChR L-QNB binding site(s). Photolabeled digitonin/cholate-solubilized partially purified mAcChRs from porcine atria were characterized by gel electrophoresis to determine an apparent subunit molecular weight of the ligand binding peptide(s). The specificity of the probe for the mAcChR was verified, upon the basis of the agreement between reversible and covalent interaction with muscarinic antagonist and agonist binding sites. A preliminary report of this work has been previously published (Cremo & Schimerlik, 1983).

Experimental Procedures

Materials. Digitonin (lot no. 92F-0661) was purchased from Sigma Chemical Co., 2.4-cm DEAE (DE-81) filters were from Whatman, the preparative and analytical TLC plates were from Merck (silica gel 60 F254). (*p*-Aminophenyl)acetic acid and tropine were obtained from Aldrich, and [^{3}H]methyl iodide was from Amersham. [^{3}H]L-QNB (33.1 Ci/mmol), purchased from New England Nuclear, cochromatographed with a nonradiolabeled standard (the generous gift of Dr. W. E. Scott, Hoffman-La Roche Inc.) on TLC plates in solvent systems A (R_f 0.58) and D (R_f 0.38) with 95% of the radioactivity in the QNB spot. All other reagents were the highest purity commercially available. TLC plates were visualized by UV light at 254 nm and an iodoplatinate spray solution (Krebs et al., 1969).

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[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; nAcChR, nicotinic acetylcholine receptor; WGA, wheat germ agglutinin; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; L-QNB, L isomer of quinuclidinyl benzilate; [^{3}H]L-QNB, tritiated L isomer of quinuclidinyl benzilate; EDTA, ethylenediaminetetraacetic acid; [^{3}H]PrBCM, tritiated propylbenzilylcholine mustard; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran. TLC solvent systems were (A) chloroform–methanol–acetic acid–water (65:25:5:5), (B) chloroform–methanol (4:1), (C) 1-butanol–acetic acid–water (66:17:17), and (D) acetone–methanol–diethanolamine (10:10:0.3).

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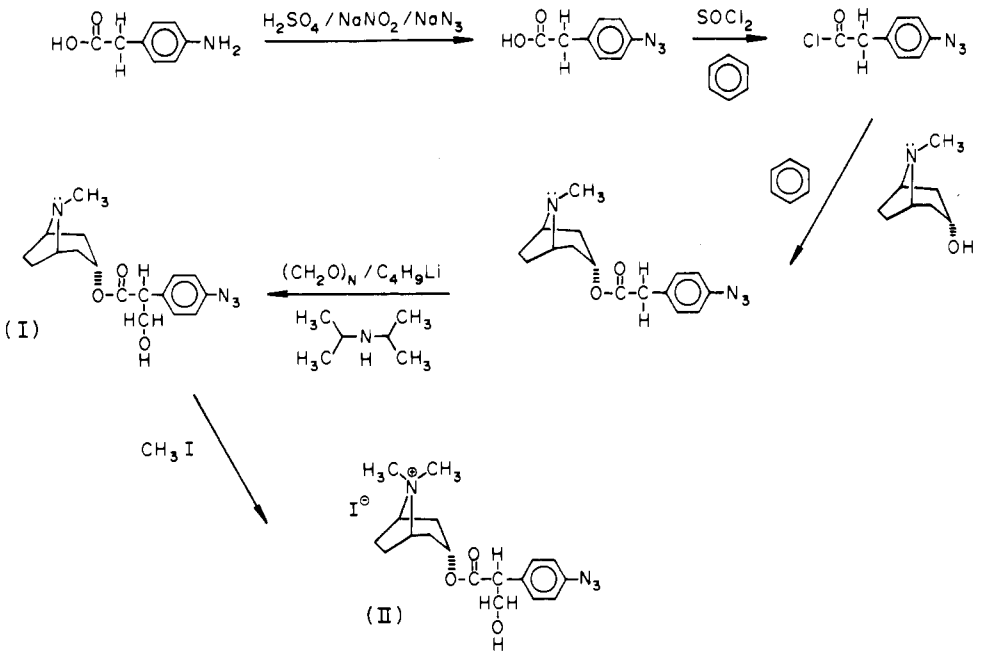

FIGURE 1: Synthesis of *p*-azidoatropine (I) and *p*-azidoatropine methyl iodide (II). Details of the synthesis are described under Experimental Procedures.

Synthesis of Azide Derivatives of Atropine (*Figure 1*). All light-sensitive reactions were done in the dark or with reaction vessels covered with foil unless otherwise noted.

(*1*) *p-Azidoatropine* (*I*). The (*p*-azidophenyl)acetate ester of tropine was synthesized by a modification of the method of Moreno-Yanes & Mahler (1980). Eleven grams (159 mmol) of $NaNO_2$ in 130 mL of H_2O was added dropwise to a stirred suspension of 20 g (132 mmol) of (*p*-aminophenyl)acetic acid in 227 mL of 2.1 M H_2SO_4 maintained at 0 to −5 °C with an ice–salt bath. After this was stirred for an additional 20 min, 5 drops of a saturated urea solution was added. After a 10-min stirring, 17.2 g (265 mmol) of NaN_3 in 130 mL of H_2O was added dropwise. The resulting reddish foam was collected by filtration, washed with cold H_2O, and air-dried, prior to crystallization from hot 95% ethanol. After this was dried over P_2O_5, the yield was 15.1 g (68%). The product migrated as a single spot in solvent system B, R_f 0.79. A second crop of crystals from 95% EtOH may be obtained to increase the overall yield. Seventeen grams (112 mmol) of (*p*-azidophenyl)acetic acid was refluxed for 1 h with a 10-fold molar excess of thionyl chloride. The solution was flash evaporated, and the dark red-brown oil was evaporated 4 times from dry benzene to remove remaining thionyl chloride. Fifteen grams of tropine (112 mmol) was dissolved in 80 mL of dry benzene and added dropwise into the acid chloride refluxing in 120 mL of dry benzene. The reaction was refluxed 0.5 h after tropine addition and stirred overnight at room temperature. The brown precipitate was collected by filtration and dissolved in H_2O. Na_2CO_3 was added to pH 9, and the ester product was extracted with 3 times 100-mL aliquots of ether. The ether was then back-extracted with a saturated NaCl solution, filtered, and flash evaporated to dryness. The resulting dark brown oil was dried over P_2O_5 for 2 days and gave one major spot, R_f 0.29, when analyzed by TLC in solvent system B (yield 35%). The hydroxymethylation was performed in a three-neck round-bottomed flask equipped with a stir bar. A separate round-bottomed flask containing 10 g of paraformaldehyde (dried overnight over P_2O_5) was connected by Teflon tubing to one neck of the reaction flask. All connections were sealed with rubber septum stoppers. The sealed reaction flask with paraformaldehyde connection in place was cooled to 0 °C and flushed continuously with N_2 gas. One equivalent (4.8 mL) of diisopropylamine and 50 mL of dry THF were added by syringe to the reaction flask and allowed to cool on ice with stirring. One equivalent (20.6 mL) of *n*-butyl lithium was slowly added, and the solution was cooled on ice for 15 min and then on a dry ice–acetone bath for 15 min. Ten grams of the (*p*-azidophenyl)acetate ester of tropine dissolved in a minimum volume of dry THF was added by syringe, washed in with an additional 150 mL of dry THF, and allowed to sit for 20 min. The paraformaldehyde flask was heated to 150 °C in an oil bath. The resulting formaldehyde gas was bubbled through the Teflon tube into the reaction for 1–2 h, until a film of paraformaldehyde formed over the surface of the reaction and the color changed from dark red-brown to lighter red. The paraformaldehyde was removed from the heat and the reaction mixture was equilibrated to room temperature overnight. Na_2CO_3 was then added to pH 9, and the solution was extracted with 3 times 100-mL aliquots of ether. The ether was back-extracted with saturated NaCl solution and dried over Na_2SO_4. TLC analysis in solvent system A showed the crude product to be an approximately equimolar mixture of the (*p*-azidophenyl)acetate ester of tropine (R_f 0.54) and its hydroxymethylated derivative (R_f 0.40) and small amounts of unknown products. The ether was flash evaporated, and the product was purified by preparative TLC in solvent system A. The product was eluted from the gel with the same solvent solution, flash evaporated to dryness, dissolved in ether, and dryed over Na_2SO_4; TLC analysis showed a single spot in solvent system A (R_f 0.40) and C (R_f 0.33). The ether was flashed to dryness, and the compound (red-brown powder) was stored at −20 °C over Ca_2SO_4 (yield 40%): ^1H NMR ($CDCl_3$) δ 7 (4 H, q, aromatic protons), 4.9 (1 H, t, C_3 methine in tropane skeleton), 4.1 (1 H, s, OH), 3.5–4.0 (3 H, m, $-CH-CH_2-OH$), 2.9 (2 H, br s, C_1 and C_5 methines in tropane skeleton), 2.18 (3 H, s, $>N-CH_3$), 1–2 (10 H, m, four methylenes in tropane skeleton).

(*2*) *p-Azidoatropine Methyl Iodide* (*II*). The free base of *p*-azidoatropine (I) was methylated in $CHCl_3$ by stirring with equilmolar CH_3I for 3 days at 4 °C. The white precipitate was collected by filtration, washed with $CHCl_3$, and dissolved in 95% EtOH and a small amount of H_2O. The quaternized

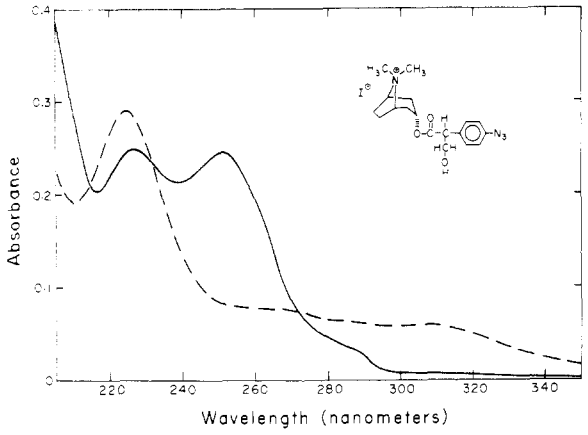

FIGURE 2: Effect of photolysis on the absorption spectrum of II. A 21.5 μM solution of II in H_2O (1-cm path length) was irradiated for 0 (solid line) and 10 (dashed line) min as described under Experimental Procedures: λ_{max}, 227 nm and 252 nm; ϵ, 11 700 and 11 400 cm^2/mol.

product was separated from the unreacted tertiary compound by preparative TLC in solvent system A (R_f 0.15): mp = 183–188 °C dec; IR_{max}^{KBR} 3400 (br, H bonding), 2100 (sharp, azide), 1735 (sharp, ester), 1575 (sharp, phenyl ring) cm^{-1}. Anal. (M.H.W. Laboratories, Phoenix, AZ; ±0.3%). Calcd for $C_{18}H_{25}O_3N_4I$: C, 45.8; H, 5.3; 0, 10.2; N, 11.9; I, 26.9. Found: C, 45.6; H, 5.5; O, 10.3; N, 11.8; I, 26.8. UV–vis spectrum (Figure 2) showed a characteristic phenylazide absorption maximum at 252 nm, which was diminished after exposure to UV light.

(3) [^{3}H]-p-Azidoatropine Methyl Iodide. A solution of 3.6 mg (11 μmol) of free base in 450 μL of $CHCl_3$ was added to 10 μmol of [^{3}H]methyl iodide (10.0 Ci/mmol) cooled with liquid N_2. The reaction was sealed after it reached room temperature. After 6 days at 4 °C, the reaction mixture was applied to a preparative TLC plate and chromatographed in solvent system A as described for the synthesis of the unlabeled compound. The radioactive product was eluted with solvent system A, flash evaporated, brought to a volume of 7 mL with 95% EtOH, and stored in a foil wrapped vial at −20 °C over $CaSO_4$. Analytical TLC analysis indicated that the radioactive product cochromatographed with the cold compound in solvent system A (R_f 0.17) and B (R_f 0.15) with 92% of the radioactivity in the single spot, yield 12%. The product was very stable under the storage conditions, no change in the TLC analysis was observed over 1 year.

Preparation of Membrane-Bound and Solubilized mAcChR. Atrial membranes were prepared as described by Peterson & Schimerlik (1984) in 25 mM imidazole, 1 mM EDTA, and 0.1 mM PMSF, pH 7.4 Digitonin at 0.4% (w/v) and 0.08% (w/v) cholate were used together to solubilize atrial membranes by a modification of the method of Cremo et al. (1981) as described by Peterson & Schimerlik (1984). The solubilized receptor was further purified in the same detergent system by a preparative WGA affinity chromatography method described by Herron & Schimerlik (1983). Dialysis or gel filtration chromatography was used to change the buffer to 0.04% (w/v) digitonin, 0.008% (w/v) cholate, 10 mM PO_4, 1 mM Na_2EDTA, and 0.1 mM PMSF, pH 7.4 (buffer A), for equilibrium titrations and photoaffinity labeling.

[^{3}H]L-QNB Binding Assays and Equilibrium Titrations. Solubilized mAcChR concentration was quantitated in terms of [^{3}H]L-QNB sites by using a DEAE filter disk assay developed by Schmidt & Raftery (1973) for the nAcChR and modified in this laboratory for use with the digitonin/cholate mixed-detergent system (Cremo et al., 1981). The DEAE disk assay was also used for equilibrium titrations. Nonspecific binding was measured in the presence of 10 μM atropine. Protein was measured by the method of Peterson (1977).

Equilibrium titration curves measuring [^{3}H]L-QNB displacement were analyzed by using weighted least-squares fit to eq 1. The value of the dissociation constant (K_i) for a given

$$\frac{[I_0]}{[R_0]\left[1 - \frac{\overline{RQ}}{\overline{RQ_0}}\right]} - 1 = \bar{j} = \frac{[Q]}{[RQ]}\frac{K_i}{K} \qquad (1)$$

competitive inhibitor was calculated from the slope of the plot of $\bar{j}$ function (Best-Belpomme & Dessen, 1973) vs. [Q]/[RQ], where [Q] equals the free [^{3}H]L-QNB at fixed levels of total inhibitor at differing [I_0], [RQ] equals specifically bound [^{3}H]L-QNB, $\overline{RQ}$ and $\overline{RQ_0}$ equal the fractional saturation of L-QNB sites in the presence of total inhibitor concentration [I_0] and in the absence of inhibitor, respectively. Thus, from the slope of the plot of $\bar{j}$ vs. [Q]/[RQ] and the value of K, the dissociation constant for L-QNB, the K_i values could be computed for the inhibitor.

Photoaffinity Labeling. All photolabeling experiments were done in buffer A. Samples were preequilibrated at room temperature for 0.5 h in the presence or absence of competing reagents before equilibrating for 24 h in the dark on ice with given concentrations of [^{3}H]-*p*-azidoatropine methyl iodide. Samples (0.5 mL) were equilibrated on ice with stirring in a 2 × 1.3 cm plastic beaker. A UVSL-25 Mineralight lamp (Ultraviolet Products, Inc.; λ_{max} = 254 nm, about 650 μW/cm^2 at 3 cm) positioned 3 cm above the solution was used to irradiate the samples for 5 min. The photolyzed samples were then denaturated immediately with SDS and β-mercaptoethanol in electrophoresis sample buffer according to Laemmli (1970), unless otherwise specified.

Denaturing Gel Electrophoresis. Electrophoresis with 7.0% polyacrylamide containing 0.1% SDS was performed as described by Laemmli (1970). Gels were poured in tubes 1-cm wide and various lengths between 80 and 120 cm with a 2.0-cm, 3% stacking gel and electrophoresed at 4 °C. Gels with photolabeled samples were sliced into 1-mm pieces (only the running gels were sliced). Each slice was oxidized in a 10-mL glass scintillation vial with 0.90 mL of 30% H_2O_2 at 75 °C overnight before counting in 6.5 mL of toluene–Triton fluor. Identical gels used to visualize protein patterns of photolabeled samples and molecular weight standards were marked at the dye front and stained for 2 h with Coomassie blue, destained with 7.5% HAc–5% MeOH, and scanned densitometrically at 600 nm with a Cary 219 spectrophotometry gel scanning accessory. Gels that were sliced and counted for tritium were not stained and destained because control experiments using analytical TLC indicated that the *p*-azidoatropine methyl iodide ester linkage was unstable to destaining conditions (7.5% HAc, 5% MeOH, 21 °C, 2–3 days). Molecular weight standards were prepared as other samples with respect to detergent content and volumes loaded.

Results

Reversible Interaction of p-Azidoatropine Methyl Iodide with mAcChR [^{3}H]L-QNB Binding Sites. The titration curve of specifically bound [^{3}H]L-QNB vs. *p*-azidoatropine methyl iodide for WGA partially purified mAcChRs (Figure 3) was consistent with competition for a single class of L-QNB sites. the K_i for [^{3}H]-*p*-azidoatropine methyl iodide calculated from the average of two experiments was 1.0 ± 0.4) × 10^{-7} M. The photoaffinity label also appeared to displace [^{3}H]L-QNB from a single class of sites for the membrane-bound and deter-

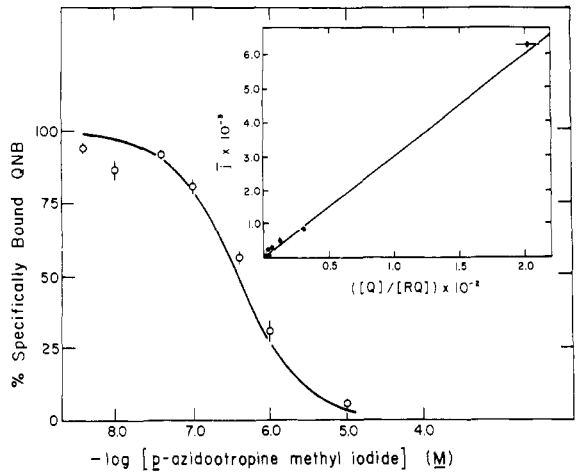

FIGURE 3: *p*-Azidoatropine methyl iodide (II) titration of specifically bound [^{3}H]L-QNB to WGA partially purified mAcChR. The azide concentration was varied at constant mAcChR (1.7 nM L-QNB sites) and [^{3}H]L-QNB (1.0 nM) concentrations and equilibrated in the dark for 2 h at room temperature. After 100-μL aliquots were removed to measure total [^{3}H]L-QNB concentration, total bound [^{3}H]L-QNB was measured by the DEAE disk assay. Data were evaluated by a weighted least-squares fit to eq 1 (inset) to give a value of (7.6 ± 1.2) × 10^{-8} M for the K_d of II. The curve through the data points was calculated from the law of mass action by using (2.5 ± 0.4) × 10^{-10} M as the K_d for L-QNB, the above value for II, and the experimentally determined concentrations of free [^{3}H]L-QNB at each data point.

gent-solubilized mAcChR preparations (data not shown), with a K_i not significantly different from that reported here for the WGA partially purified mAcChR.

Covalent Labeling of mAcChR with p-Azidoatropine Methyl Iodide. Denaturing SDS–polyacrylamide gel electrophoresis was used to assay for covalent interaction of [^{3}H]-*p*-azidoatropine methyl iodide with the mAcChR. Figure 4A shows the distribution of radioactive label in a typical gel of [^{3}H]-*p*-azidoatropine methyl iodide labeled partially purified mAcChR. A densitometric scan of an identical sample stained with Coomassie blue is shown in Figure 4B. Nonspecific labeling was measured in the presence of 110 nM L-QNB. Under the experimental conditions in Figure 4 (K_d for L-QNB binding to the WGA partially purified fraction equaled 250 pM and L-QNB binding sites equaled 40 nM as measured by DEAE disk assay), saturation of L-QNB binding sites was predicted at 110 nM L-QNB. Background levels (approximately 100 cpm) of radioactivity were observed between R_f 0.0 and 0.95 in control experiments (data not shown), where either the protein was omitted, the tritiated probe was added without photolyzing, or the tritiated probe was prephotolyzed before incubating and rephotolyzed in the presence of the receptor preparation. If samples were not dialyzed before electrophoresis, a component of the free photolyzed probe was detected as radioactivity migrating in a broad peak at the top of the gel (R_f 0.0–0.2). Dialysis prior to electrophoresis reduced this component to background levels as seen in Figure 4A. This treatment, however, did not alter the distribution of radioactivity in the R_f 0.2–0.95 region. A large amount of radioactivity comigrating with the dye front (R_f 1.0) to the leading schleiren line (region of gel not shown) was present in experiments such as in Figure 4A and the three control experiments (prephotolysis, protein omitted, and no photolysis) and could only be partially reduced by dialyzing samples prior to electrophoresis. L-QNB or atropine preincubations in the nanomolar to micromolar range did not reduce this labeling, indicating its probable lack of specificity for the mAcChR. Labeling at the dye front to the leading schleiren line may represent covalently and noncovalently labeled lipids, detergent

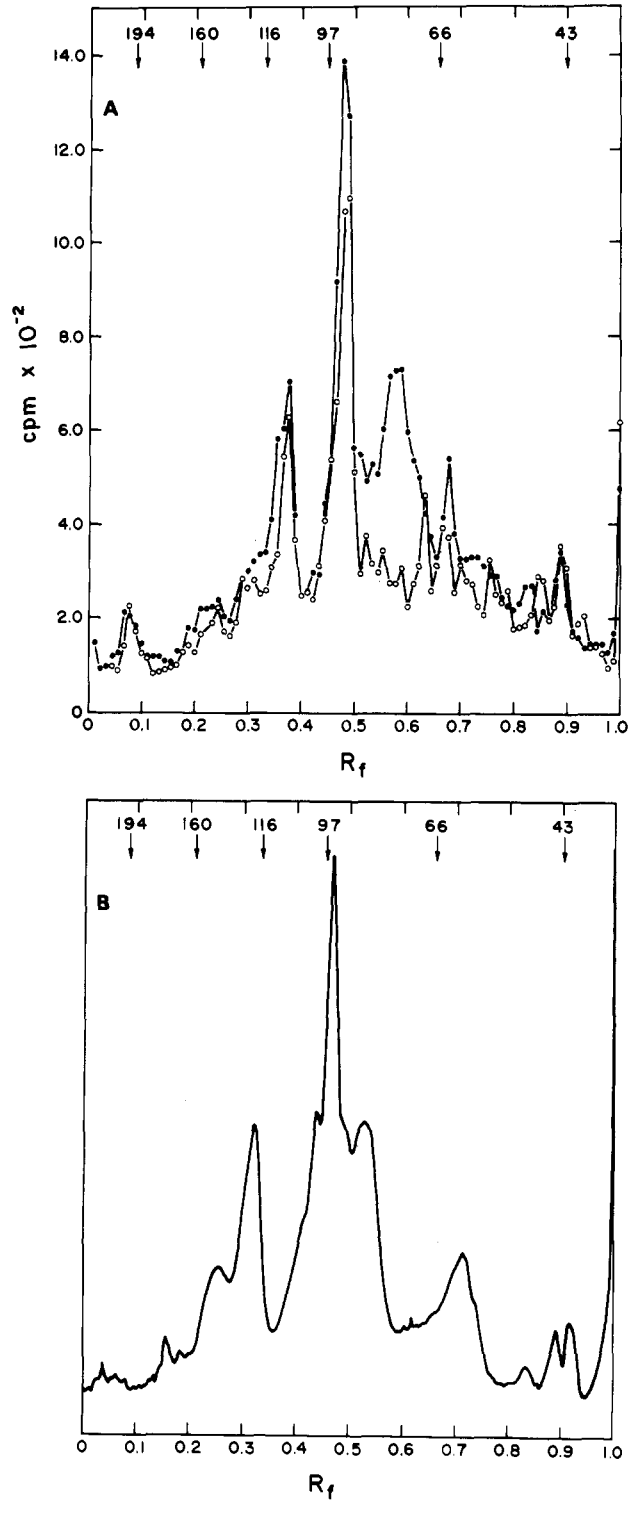

FIGURE 4: (A) SDS–PAGE of WGA partially purified mAcChR covalently labeled with 150 nM [^{3}H]-p-azidoatropine methyl iodide. Aliquots of 0.5 mL of receptor (0.6 mg of protein/mL; 15 nM L-QNB sites) were labeled in the absence (●) or presence (○) of 110 nM L-QNB and prepared for electrophoresis as described under Experimental Procedures except that samples were dialyzed against buffer A after photolysis to remove free probe. Molecular weight standards (indicated by arrows) were ovalbumin (43K), bovine serum albumin (66K), phosphorylase A (97K), β-galactosidase (116K), debranching enzyme (160K), and myosin (194K). The dye front corresponds to R_f 1.0. (B) Scan at 590 nm of a Coomassie blue stained SDS–PAGE of WGA partially purified mAcChR. The protein sample was identifical with that described above.

and low molecular weight proteins, and some nonphotolyzed probe. The mAcChR preparations appeared to be stable under the conditions of photolysis. No loss in [^{3}H]L-QNB binding

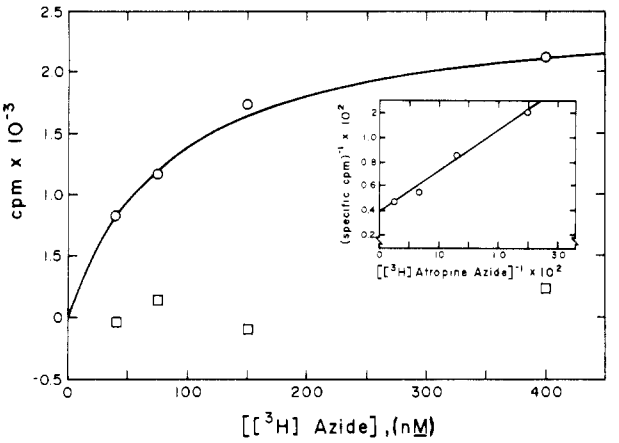

FIGURE 5: Dependence of [^{3}H]-*p*-azidoatropine methyl iodide specific labeling in the M_r 70 000–79 000 (circles) and 90 000–92 000 (squares) region on [^{3}H]-*p*-azidoatropine methyl iodide concentration. Aliquots of 0.5 mL (0.6 mg of protein/mL, 44 nM [^{3}H]L-QNB binding sites) of WGA partially purified mAcChR were labeled at given [^{3}H]-*p*-azidoatropine methyl iodide concentrations and prepared for electrophoresis as described under Experimental Procedures. Specific labeling was calculated by subtracting the cpm across the M_r 70 000–79 000 region in the presence of 110 nM L-QNB from that value in the absence of L-QNB for each data point. The data were analyzed (see inset) by using $C = C_0[L_0]/(K_{app} + [L_0])$, where C = specific cpm measured at total ligand concentration $[L_0]$ and C_0 = specific cpm observed at saturation with L. C_0 = 2560 cpm and K_{app} = 8.5 × 10^{-8} M were determined from the slope and y intercept of a least-squares fit of the plot of $1/C$ vs. $1/[L_0]$. The solid line drawn through the data points was calculated from these values.

activity was measured after 10 min of photolysis. Control gel experiments indicated that photolysis did not induce protein cross-linking.

The results in Figure 4A indicated that 110 nM L-QNB significantly decreased the labeling of one or more proteins migrating in the broad M_r range of 70 000–79 000 (R_f 0.53–0.62). Similar results were found with 10 μM atropine, indicating that the parent compound also protects against covalent incorporation. Therefore, it seemed reasonable to attribute the covalently incorporated radioactivity in this region of the gel to proteins specifically labeled by photolysis in the presence of [^{3}H]-*p*-azidoatropine methyl iodide. A large amount of radioactivity was also incorporated into one or more proteins migrating in the M_r 90 000–92 000 region; however, incorporation was only partially blocked in the presence of 110 nM L-QNB. The specificity of covalent labeling, as measured by gel electrophoresis, for the M_r 90 000–92 000 and 70 000–79 000 regions was demonstrated by the two experiments shown in Figure 5 and 6. The plot of specific covalent labeling in the M_r 70 000–79 000 region as a function of [^{3}H]-*p*-azidoatropine methyl iodide concentration (Figure 5) showed saturable labeling at higher azide concentrations. This behavior was consistent with the formation of a reversible azide–protein complex prior to nitrene formation and covalent attachment of the analogue. The dissociaton constant for the azide calculated from these data (Figure 5, insert), 85 nM, agreed well with the value calculated by competition with [^{3}H]L-QNB, 100 nM (Figure 3). The specific labeling in the M_r 90 000–92 000 region does not saturate over the concentration range of tritiated probe examined.

Figure 6 shows the specific covalent labeling in the M_r 70 000–79 000 region as a function of added L-QNB. Under the experimental conditions (L-QNB sites ≫ K_d for L-QNB), the law of mass action for simple competitive inhibition predicts that L-QNB binds in a linear manner (decreasing ^{3}H-labeled azide incorporation) until a breakpoint equal to the

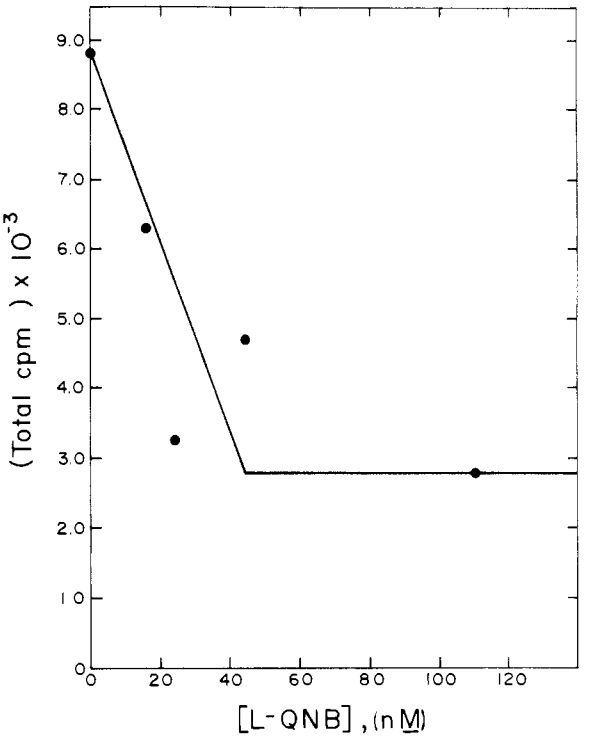

FIGURE 6: Titration of [^{3}H]-*p*-azidoatropine methyl iodide labeling by L-QNB in the M_r 70 000–79 000 region. Samples were as described in Figure 5 and labeled at 150 nM [^{3}H]-*p*-azidoatropine methyl iodide. Gel electrophoresis was as described in Figure 5. The solid line was calculated from the data for 0 (maximal labeling) and 110 nM L-QNB (equal to background labeling) and an L-QNB site concentration of 44 nM (see text).

number of L-QNB sites. The predicted theoretical curve, calculated from labeling at [L-QNB] equal to 0 and 110 nM (i.e., saturation) and the fractional occupation of L-QNB binding sites, fit reasonably well to the data in Figure 6 for the labeling of the M_r 70 000–79 000 region. This was consistent with the notion that L-QNB competitively inhibited azide binding to a site associated with the M_r 70 000–79 000 labeled peak.

A carbamylcholine titration of specifically bound [^{3}H]L-QNB measured by the DEAE disk assay is shown in Figure 7A. Carbamylcholine appeared to bind competitively to a single class of [^{3}H]L-QNB sites. A K_i for carbamylcholine calculated from the average of four experiments was (2.3 ± 1.3) × 10^{-4} M. Figure 7B shows a carbamylcholine titration of [^{3}H]-*p*-azidoatropine methyl iodide labeling in the M_r 70 000–79 000 region. The K_i for carbamylcholine calculated in Figure 7A, 230 μM, was used to calculate the theoretical curve (assuming simple competitive inhibition) drawn through the data in Figure 7b. The fit to the data indicated that the dissociation constant for carbamylcholine as measured by the two methods was in good agreement.

Discussion

The *p*-azido analogue of atropine methyl iodide has been synthesized in a tritiated form to a specific activity of 10 Ci/mmol. The results of IR, ^{1}H NMR, and UV–vis spectra and elemental analysis were consistent with the proposed structure (II; Figure 1). *p*-Azidoatropine methyl iodide behaved as a typical muscarinic antagonist displacing [^{3}H]L-QNB in a competitive manner from a single class of sites with a K_d of approximately 100 nM in the membrane-bound, solubilized extract (data not shown) and WGA partially purified receptor preparations (Figure 3). Direct reversible binding studies using the tritiated probe were avoided due to

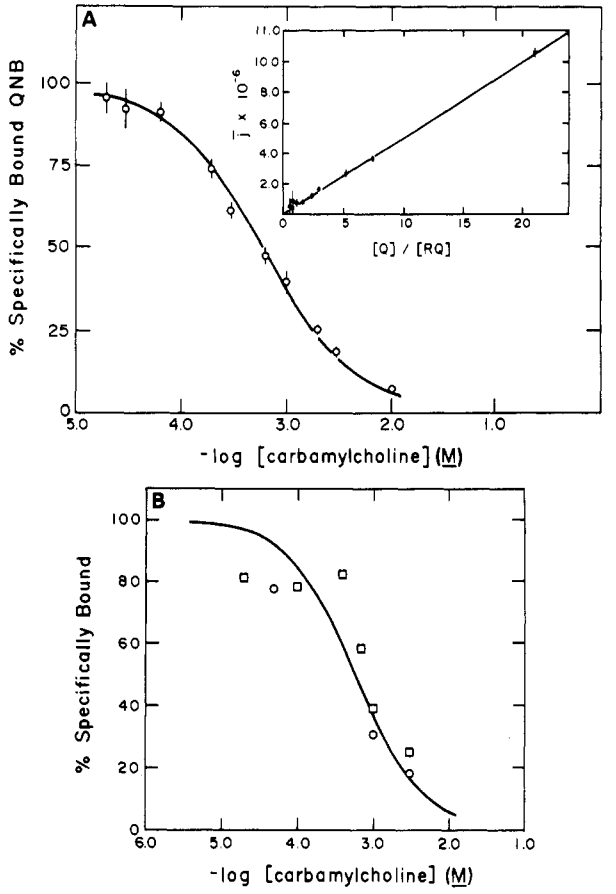

FIGURE 7: (A) Carbamylcholine titration of specifically bound [^{3}H]L-QNB to WGA affinity partially purified mAcChR. Specifically bound [^{3}H]L-QNB was measured as described in Figure 3 (1.0 nM L-QNB sites, 0.95 nM [^{3}H]L-QNB). Data were evaluated as in Figure 3 to give a value of $(1.3 \pm 0.2) \times 10^{-4}$ M for the K_d of carbamylcholine. (B) Dependence of [^{3}H]-*p*-azidoatropine methyl iodide specific covalent labeling in the M_r 70 000–79 000 region on carbamylcholine concentration. In two separate experiments, 0.5-mL aliquots [L-QNB sites: (circles) 36 nM; (squares) 43 nM] of WGA partially purified mAcChR were labeled at 150 nM probe and prepared for electrophoresis as described under Experimental Procedures. Nonspecific labeling, measured in the presence of 110 nM L-QNB, was subtracted from the total cpm in the M_r 70 000–79 000 region before normalizing to maximal labeling, measured in the absence of carbamylcholine. The curve through the data was calculated by assuming simple competitive inhibition, where R, the concentration of free L-QNB sites, varied over the titration, and where the K_i for carbamylcholine equals 2.3×10^{-4} M.

difficulties with assay procedures and cost considerations in the case of equilibrium dialysis. The dissociation rate constant was too fast to measure by the DEAE filter disk assay. A K_d of 1 nM for the parent compound, atropine, for the digitonin/cholate-solubilized (Herron & Schimerlik, 1982) and membrane-bound mAcChR (Schimerlik & Searles, 1980) has been determined by competition vs. [^{3}H]L-QNB from previous work in this laboratory. Therefore, if one assumes a synthetic yield of equal proportions of D and L isomers, substitution at the para position of the phenyl ring with an azide increases the K_d 75-fold. The K_d for the azide derivative described here was approximately 6-fold lower than that reported for (*p*-azidophenyl)acetate ester of tropine binding to rate brain membrane fractions (Moreno-Yanes & Mahler, 1980).

The nature of the covalent interactions of the photolyzed *p*-azidoatropine analogue with the WGA partially purified mAcChR L-QNB binding site(s) was examined by SDS gel electrophoresis (Figure 4). Only protein(s) in the M_r 70 000–79 000 range was (were) covalently labeled in a specific manner by the activated probe. The distribution of radioactivity on the gel was attributed to tritiated probe covalently bound to protein, upon the basis of the results of control experiments showing resistance of label to dialysis and lack of labeling with unphotolyzed or prephotolyzed probe. The prephotolysis control also indicated that covalent binding was probably not due to long-lived decomposition products resulting from photolysis of the azide but direct interaction of the photoactivated azide at the mAcChR binding site(s).

Labeling in the M_r 70 000–79 000 region was tentatively assigned to the mAcChR ligand binding peptide(s) on the basis of the agreement between the reversible binding data (Figures 3 and 7B) and the covalent binding data (Figures 5–7A) for this photoaffinity probe. The dependence of labeling in the M_r 70 000–79 000 range upon [^{3}H]-*p*-azidoatropine methyl iodide concentration (Figure 5) was saturable, consistent with a reversible protein–ligand complex formed prior to photoactivation. The azide concentration at half-maximal labeling, obtained from the double-reciprocal plot (Figure 5, inset), was 85 nM. This value agreed well with the K_i of 100 nM for the azide obtained by reversible inhibition of [^{3}H]L-QNB binding (Figure 3). It should be noted, however, that the method in Figure 5 cannot be used to measure a true K_d for the azide since at the high receptor concentrations used, free probe was not equivalent to the total probe concentration over the range of the titration. The concentration of azide at which half-maximal labeling was found could also be affected by the formation of a metastable intermediate that has a higher affinity for the mAcChR than the azide. This would shift the concentration of half-maximal labeling to lower azide concentrations. However, any metastable intermediates must have a lifetime shorter than seconds to minutes since no covalent incorporation was observed when the mAcChR was added to solutions of prephotolyzed probe. A labeling efficiency of 2.7% was calculated from the maximal specific cpm in Figure 5, assuming 20% overall counting efficiency and one azide site per L-QNB site (determined by DEAE disk assay).

The data in Figure 6 show that the labeling in the M_r 70 000–79 000 region can be titrated with increasing concentrations of L-QNB. Although the expected theoretical curve, assuming 1:1 stoichiometry, and the data points generally agree, this method was not sufficient to determine an exact break point to give the number of azide binding sites directly. Therefore, the stoichiometry of azide vs. L-QNB sites could not be rigorously determined from the data in Figure 6.

Figure 7B shows that specific labeling in the M_r 70 000–79 000 region can be titrated by the muscarinic agonist carbamylcholine. Again, the covalent labeling (Figure 7B) and reversible binding results (Figure 7A) are in good agreement. Carbamylcholine appears to interact with a single class of low-affinity sites (Figure 7A) as measured by [^{3}H]L-QNB competition. However, the data in Figure 7B alone were not sufficient to rule out a biphasic titration due to relatively large uncertainties in the data.

The results of the experiments in Figure 4–6 indicate that the labeling in the M_r 70 000–79 000 region represents a ligand-binding peptide(s) specific to the mAcChR. Both muscarinic antagonists (Atropine and L-QNB) and an agonist (Carbamylcholine) inhibited binding of a photoaffinity label in concentration ranges consistent with inhibition constants measured by an independent method. It seems reasonable to suggest that this (these) labeled peptide(s) is (are) a part of the binding site for L-QNB, carbamylcholine, and atropine and its analogue *p*-azidoatropine methyl iodide; however, the presence of additional regulatory sites cannot be ruled out by these experiments.

In some electrophoresis experiments where the receptor was prepared in the absence of PMSF (data not shown), the M_r 70000–79000 peak was resolved into a doublet. The reason for this is not known; however, proteolytic digestion is probable, especially in a muscle preparation. Benovic et al. (1983) have determined that apparent heterogeneity in photolabeled β-adrenergic receptors could be explained by proteolysis. In all experiments, we find that specific labeling is defined by a broad band often spanning six to seven, 1-mm gel slices (Figure 3), which seemed to be inconsistent with the resolution of the gel method. Venter (1983) and Birdsall et al. (1979) published very similar broad gel profiles in [^{3}H]PrBCM labeling of brain, heart, and guinea pig ileum smooth muscle membrane-bound mAcChRs. These results could be due to heterogeneously labeled peptides. Work in this laboratory indicated that WGA partially purified soluble mAcChRs from pig atria are sialoglycoproteins (Herron & Schimerlik, 1983). Therefore, broad bands on gels may be due to carbohydrate heterogeneity of the receptor. Although the 70–79K band comigrates with a peak in Figure 4B, at this level of purification (assuming a M_r of 75000 and one [^{3}H]L-QNB binding site per molecule) the mAcChR represents less than 1% of the total protein and cannot be visualized with Coomassie blue (Figure 4B).

As shown in Figure 3, a relatively large amount of label was also covalently, but nonspecifically incorporated into the M_r 90000–92000 range. The data for the M_r 90000–92000 region in Figure 5 showed no saturable dependence upon azide concentration and was good evidence for the nonspecific nature of this labeling. This labeling was also not protected in a saturable manner by L-QNB or carbamylcholine over the concentration ranges examined in Figures 6 and 7, respectively (data not shown). The nonspecific labeling in the M_r 90000–92000 range was not identified but comigrates with a major protein peak observed by Coomassie blue staining (Figure 4B). The possibility of specific labeling being masked by this large component of nonspecific labeling cannot be ruled out.

To this date, we have not detected specific covalent labeling in membrane-bound photolabeled preparations. Although the results are preliminary, experiments were hampered by the nonspecific labeling resulting from high tritiated probe concentrations (up to 40-fold over the K_d for the azide) required to obtain detectable labeling by the gel electrophoresis method. Studies with the GF/B filter assay (Yamamura & Snyder, 1974) for the membrane-bound receptor were also hampered by large amounts of nonspecifically bound label to the filters. Preliminary labeling experiments similar to that in Figure 3 with the solubilized membranes show specific labeling only in the M_r 70000–79000 region. However, the total labeling was only slightly above the background level of nonspecific labeling measured in the presence of L-QNB. The low signal to noise ratio in these experiments was probably due to low site concentration (5–10 nM) and high azide concentration (150 nM) needed to detect labeling. We have not been able to detect inhibition of [^{3}H]L-QNB binding by photolabeling the extract or membranes in the presence of cold probe by using the DEAE disk assay or GF/B assay. A labeling efficiency in the membranes and extract of 2–3% would then not be inconsistent with these results, as an inhibition of 2–3% would be difficult to reproducibly detect by either of these assays.

An apparent M_r of 70000–79000 is in general agreement with values previously reported by using [^{3}H]PrBCM interactions with membrane-bound mAcChRs from brain and smooth muscle (Birdsall et al., 1979). Upon the basis of the target-size analysis, Venter (1983) has proposed that the muscarinic receptor in brain and smooth muscle is composed of an M_r 80000 monomer. Venter (1983) has reported M_r 81000 ± 3000 and 78000 ± 1800 for [^{3}H]PrBCM mAcChR labeling in canine and rat heart membranes, respectively, by gel electrophoresis. Recently, investigators in this laboratory (Peterson & Schimerlik, 1984) have reported [^{3}H]PrBCM labeling in the M_r 70000–80000 range for both membrane-bound and digitonin/cholate-solubilized pig atrial mAcChR preparations by gel electrophoresis. Andrē et al. (1983) recently reported an apparent M_r of 70000 for a radioiodinated protein eluted by PrBCM from a dexetimide affinity column. Avissar et al. (1983), using an arylazide derivative of the muscarinic antagonist [^{3}H]-*N*-methyl-4-piperidyl benzilate, reported a single labeled peak at M_r 86000 in their electrophoresis studies of membrane-bound rat cerebral cortex and two peaks at M_r 160000 and 86000 in rat atrial membranes. These authors have suggested that the M_r 86000 species represents the low-affinity agonist binding of the mAcChR and that antagonists do not distinguish between these forms. We did not detect specific labeling with our antagonist photolabel at M_r 160000 in the WGA partially purified solubilized preparation. However, since this preparation exhibited only low-affinity agonist sites, lack of labeling at higher molecular weight need not be inconsistent with the model presented by Avissar et al. (1983).

In conclusion, we have reported specific labeling of soluble mAcChR L-QNB binding sites, using a phenylazide analogue of atropine, [^{3}H]-*p*-azidoatropine methyl iodide. This is the first report of photoaffinity labeling of this receptor in soluble form. This atropine analogue can be easily synthesized in a radiolabeled form with high specific activity that exhibits excellent stability to storage. Reversible binding studies can be performed in the dark due to stability of the probe in the aqueous buffer systems. The good specificity and reasonable labeling efficiency should prove useful to further probe the structural and functional properties of more highly purified soluble receptor preparations.

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Photoaffinity Labeling of the Major Nucleosidetriphosphatase of Rat Liver Nuclear Envelope†

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ABSTRACT: We employed the photoaffinity probe 8-azidoadenosine 5′-triphosphate (aATP) to identify the nuclear envelope (NE) nucleosidetriphosphatase activity (NTPase) implicated in control of RNA transport. The photoprobe was hydrolyzed at rates comparable to those for ATP, with a Michaelis constant of 0.225 mM. Photolabeling was dependent upon UV irradiation (300-nm max) and was not affected by quercetin. Unlabeled ATP or GTP competed with [^{32}P]aATP in photolabeling experiments, and UTP was a less effective competitor, paralleling the substrate specificity of the NTPase. Incubation of NE with aATP led to a UV, time, and concentration dependent irreversible inactivation of NTPase. The inactivation could be blocked by ATP or GTP. Polyacrylamide gel electrophoresis and autoradiography of photolabeled NE showed selective, UV-dependent labeling of a 46-kDa protein with both [γ-^{32}P]aATP and [α-^{32}P]aATP. This band was not labeled with [γ-^{32}P]ATP. Since the NE NTPase implicated in RNA transport is modulated by RNA, we examined the effects of RNA on the labeling process. Removal of RNA from the NE preparations (by RNase/DNase digestion) reduced NTPase by 30–40% and eliminated photolabeling of the 46-kDa band. Addition of yeast RNA to such preparations increased NTPase activity to control levels and selectively reinstated photolabeling of the 46-kDa band. These results suggest that the 46-kDa protein represents the major NTPase implicated in RNA transport.

Transfer of prelabeled RNA from nuclei to a surrogate cytoplasm is an energy-dependent process (Ishikawa et al., 1969; Raskas, 1971; Racevskis & Webb, 1974; Clawson et al., 1978; Jacobs & Birnie, 1982). Current evidence suggests the energy is provided by phosphate bond cleavage of di- or triphosphate nucleotides by a nuclear envelope (NE) triphosphatase (Agutter et al., 1979b; Clawson et al., 1980a,b; Purrello et al., 1982; Murty et al., 1983; Baglia & Maul, 1983). Furthermore, the activity of this enzyme is modulated by RNA (Agutter et al., 1979b; Clawson et al., 1980a). The nuclear envelope is a unique double-membrane structure containing specialized pore lamin components (Dwyer & Blobel, 1974; Comings & Okada, 1976; Maul, 1977; Gerace et al., 1978; Krohne et al., 1978; Berezney, 1979; Shaper et al., 1979; Unwin & Milligan, 1982), and its preparations consists of a distinct and limited array of polypeptides (Franke, 1974; Lam & Kasper, 1979; Richardson & Maddy, 1980b; Shelton et al., 1980) when separated by polyacrylamide electrophoresis under denaturing conditions. However, the identity of the protein(s) responsible for the nucleosidetriphosphatase (NTPase) activity (activities) has not been established. We used 8-azidoadenosine 5′-triphosphate (aATP) as a selective photoaffinity agent (Haley & Hoffman, 1974; Koberstein et al., 1976; Wagenvoord et al., 1977; Shia & Pilch, 1983; Hollemans et al., 1983) to mark the enzyme(s). We observed photolabeling of a 46-kDa NE protein, which was modulated by RNA, and suggest that this band contains the major NTPase implicated in RNA transport.

Materials and Methods

Nuclei were prepared from male Sprague-Dawley rats by the technique of Blobel & Potter (1966): 0.5 mM phenylmethanesulfonyl fluoride (PMSF) was included in the 2.3 M sucrose buffer cushion and in all subsequent preparative steps. NE were isolated by a modification of the technique described by Monneron (1974), with PMSF included in the gradient. NTPase activity was determined as described (Clawson et al., 1980a). To remove RNA from NE preparations, purified NE were resuspended in TKM buffer (50 mM Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride] (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$) plus 0.5 mM PMSF, DNase I (Miles) was added to 10 μg/mL and RNase A was added to 10 μg/mL, and this suspension was incubated for 20 min at room temperature; the resulting suspension was layered over

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