Enrichment, Solubilization, and Partial Characterization of Digitonin-Solubilized Muscarinic Receptors Derived from Canine Ventricular Myocardium

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SUMMARY. The subcellular distribution of cardiac muscarinic receptors was defined in canine ventricular myocardium, and receptors were solubilized from subcellular fractions enriched in muscarinic receptor content. The subcellular location of muscarinic receptors in cardiac tissue was determined by measurement of the distribution of \([1^H](\pm)\text{quinuclidinyl benzilate}-\text{binding activity in particulate fractions isolated from canine ventricular myocardium. Based upon excellent correlation between \([1^H](\pm)\text{quinuclidinyl benzilate binding and activity of the sarcolemmal Na}^+,K^+\text{-ATPase throughout the subcellular fractions, muscarinic receptors appeared to be localized to sarcolemma in canine ventricular myocardium. Therefore, membrane fractions enriched in sarcolemma were used as a source of cardiac muscarinic receptors for solubilization. Treatment of membrane vesicle fractions with digitonin (0.6%) resulted in solubilization of \([1^H](\pm)\text{quinuclidinyl benzilate-binding activity with an extraction yield of 23–35%. Criteria of pharmacological specificity and stereospecificity established the identity of the solubilized binding activity of muscarinic receptors. Solubilization of muscarinic receptors was documented by demonstration of hydrodynamic behavior consistent with molecularly dispersed material. Upon glycerol gradient centrifugation, digitonin-solubilized muscarinic receptors from cardiac tissue sedimented with an apparent sedimentation coefficient of 9.5. Pharmacological characterization of the digitonin-solubilized receptors revealed 8- to 39-fold reductions in affinities for muscarinic antagonists compared to the affinities exhibited by receptors in the membrane-bound state. Substantially greater reductions in agonist affinities (reduction of at least 700-fold for all agonists studied) suggested selective loss of ability of the digitonin-solubilized receptors to exhibit high affinity agonist interactions. In contrast to membrane-bound receptors, digitonin-solubilized receptors also demonstrated a loss of guanine nucleotide regulation, as well as steep agonist:radioisogand competition curves with slope factors of 1.0, suggesting a homogeneous population of agonist-binding sites. Interpreted within the context of a model of state interconversion for membrane-bound receptors, the results suggested that either muscarinic receptors of a single state were selectively solubilized, or that solubilization induced conversion of all receptors to a single low affinity state, possibly by removal of constituents necessary for assumption of a high affinity agonist conformation. (Circ Res 52: 664–676, 1983)
Therefore, sarcolemmal-enriched fractions have been employed as sources of cardiac muscarinic receptors for solubilization. Use of these enriched sources of cardiac muscarinic receptors for the preparation of solubilized receptors has afforded the opportunity to characterize both physical and pharmacological properties of digitonin-solubilized muscarinic receptors.

Methods

Preparation of Cardiac Membrane Vesicles

The two procedures used to prepare crude cardiac membrane vesicles selectively enriched in sarcoplasmic reticulum (procedure I) or sarcolemma (procedure II) are described in detail by Jones et al. (1979). Briefly, Procedure II differed from procedure I essentially in that several brief homogenization steps were first used to fragment selectively sarcoplasmic reticulum vesicles. Following consecutive low speed centrifugations, supernatant containing the sarcoplasmic reticulum vesicles was removed. Subsequent vigorous homogenization of particulate residue was used to generate membrane vesicles enriched in sarcolemma.

Ca++ loading and sucrose gradient centrifugation of cardiac membrane vesicles prepared by procedure I were performed as recently described (Jones and Cala, 1981). To facilitate separation of vesicles derived from sarcoplasmic reticulum from those derived from sarcolemma, procedure I membrane vesicles were incubated in the presence of ATP, Ca++, and oxalate to increase selectively the density of sarcoplasmic reticulum vesicles. The calcium-loaded vesicles suspended in 0.25 m sucrose were layered over a discontinuous sucrose gradient consisting of consecutive layers of 0.6 m, 0.8 m, 1.0 m, and 1.5 m sucrose. Centrifugation was performed for 120 minutes at 27,000 rpm in an SW27 rotor (Beckman Instruments). Fraction A was collected at 0.25 m:0.6 m interface, fraction B at the 0.6 m:0.8 m interface, fraction C at the 0.8 m:1.0 m interface, fraction D at the 1.0:1.5 m interface and fraction E was the pellet that sedimented through 1.5 m sucrose. Highly purified sarcoplemma was isolated from procedure II vesicles as recently described (Jones et al., 1980; Manalan and Jones, 1982). Based on analysis of marker enzymes, the highly enriched sarcolemmal vesicles contained less than 5% contamination by sarcoplasmic reticulum (Jones et al., 1980; Manalan and Jones, 1982). All membrane vesicle fractions were suspended in 0.25 m sucrose, 30 mM histidine and stored frozen at −20°C until use.

ATPase Assays

Na+,K+-ATPase activities were measured as previously described (Jones et al., 1977). To reveal total Na+,K+-ATPase activity, membrane vesicle preparations (0.4–0.8 mg/ml) were preincubated with sodium dodecyl sulfate at the optimal concentration of 0.3 mg/ml. Na+,K+-ATPase activity then was assessed in a medium containing 15–25 μg/ml of membrane protein, 50 mM histidine, 3 mM MgCl₂, 1 mM Tris/EGTA, 100 mM NaCl, 10 mM KCl, and 3 mM Tris/ATP, pH 7.4. Na+,K+-ATPase activity was taken as that activity inhabitable by 1 mM ouabain.

Ca++-ATPase activity was determined in a medium containing 15–25 μg/ml of membrane protein, 50 mM histidine, 3 mM MgCl₂, 100 mM NaCl, 3 mM Tris/EGTA, and 3 mM Tris/ATP, pH 7.4 (Jones et al., 1977). Ca++-ATPase activity was taken as that activity inhabitable by 1 mM Tris/EGTA. In all assays of Ca++-ATPase, the divalent cation ionophore A23187 (3 μg/ml) was included in the medium to eliminate calcium accumulation spaces (Jones et al., 1977).

[3H]-QNB Filtration Assay

Assay of membrane-bound muscarinic receptors was performed using a [3H]-QNB binding assay, by methods analogous to those described previously for [3H]-QNB (Yamamura and Snyder, 1974; Fields et al., 1976; Mirro et al., 1980). Membrane vesicles (5–50 μg protein) were incubated in 1 or 5 ml of medium containing either 50 mM Tris (pH 7.5 at 25°C) or 50 mM sodium phosphate buffer, pH 7.4, indicated concentrations of [3H]-QNB, guanine nucleotides, and relevant drugs. Dithiothreitol (0.1 mM), PMSF (0.1 mM), and leupeptin (10 μM) were without effect on binding parameters in the membrane system and were therefore omitted from the assay mixtures. Incubations were conducted at 37°C for 60 minutes, a time which allowed complete equilibration of [3H]-QNB binding. Incubations were terminated by rapid filtration through Whatman GF/C filters. Alternatively, following the 60-minute incubations, tubes were placed on ice prior to filtration to provide a control for comparison with results for solubilized receptors (see below). Identical results were obtained with either method. Filters were rinsed with four 5-ml aliquots of ice cold incubation buffer, air dried, and suspended in 10 ml Aquasol. Retained radioactivity was determined by liquid scintillation counting. All determinations of total and non-specific binding were performed in triplicate. Specific binding was taken as that binding which was inhibited by 1 μM atropine.

Muscarinic Receptor Solubilization

The solubilization procedure was performed by modification of the method described by Hurko (1978) for solubilization of muscarinic receptors from brain. All procedures were performed at 4°C. Membrane vesicles (approximately 3 mg protein) were suspended in 8 ml of buffer A, consisting of 150 mM sodium phosphate, pH 7.4, 0.1 mM dithiothreitol, 0.1 mM PMSF, and 10 mM leupeptin, and centrifuged at 33,000 g for 30 minutes. The supernatant was discarded and the pellet resuspended in 1.5 ml of identical buffer containing 0.6% digitonin. This suspension was incubated for 30 minutes at 4°C with occasional stirring and then subjected to centrifugation at 82,000 g for 60 minutes to sediment residual unsolubilized material. The supernatant containing solubilized receptors was collected, and assayed for protein. The supernatant was diluted accordingly in the above-described buffers to achieve a protein concentration of 0.75 mg/ml and a final digitonin concentration of 0.3%. All preparations of solubilized receptors were used immediately. Recovery of solubilized muscarinic receptors, assessed by the [3H]-QNB charcoal adsorption assay, was in the range of 25% to 35%.

Protein Determinations

All protein determinations used in the analysis of receptor solubilization were performed utilizing Coomasie brilliant blue as described by Bradford (1976). Protein determinations during the course of preparation and characterization of the membrane vesicle fractions were performed by the method of Lowry et al. (1951). As previously noted...
(Hurko, 1978; Gorissen et al., 1981), the presence of digitonin affects results of both of these protein assays. In the case of the assay of protein using Coomassie blue (Bradford, 1976), the presence of digitonin results in a systematic overestimation of the protein present (Gorissen et al., 1981). Similarly, in the present study, inclusion of digitonin in protein standards used for calibration curves did not completely correct for digitonin effects on protein assays in the membrane system, as reflected by protein recoveries in excess of 100% as a result of treatment of membranes with digitonin. In the absence of a completely satisfactory method of correction, protein determinations have been calibrated against a standard curve performed in the presence of digitonin, and results were used without additional correction in the calculation of receptor concentrations.

**[3H]-QNB Charcoal Adsorption Assay**

Assay of solubilized muscarinic receptors was performed using a [3H]-QNB charcoal adsorption assay. Development of this assay based on the high affinity ligand [3H]-QNB was patterned after a [3H]dextemide/[3H]levetimide charcoal assay described by Gorissen et al. (1981). Solubilized receptors (approximately 30 μg protein) were incubated in 0.4 ml of buffer A, 0.03% digitonin, indicated concentrations of [3H]-QNB, guanine nucleotides, and relevant drugs. Incubations were conducted at 37°C for 60 minutes to allow complete equilibration of [3H]-QNB binding. Following incubation, tubes were placed on ice, 0.5 ml of a cold (4°C) 10% suspension of activated charcoal containing 4% bovine serum albumin was added, and the resulting mixtures vigorously agitated. Mixtures containing activated charcoal were subsequently incubated for exactly 20 minutes on ice prior to centrifugation for 5 minutes at 4°C in a Beckman microfuge model B. A 0.6-ml aliquot of supernatant was removed from each tube, suspended in 10 ml of Aquasol, and radioactivity determined with a Beckman liquid scintillation counter (counting efficiency 56%). All determinations of total and nonspecific binding were performed in duplicate. Specific binding was taken as that binding which was displaceable by 10 μM atropine. The concentration of solubilized muscarinic receptors in the incubations was in the range of 30-60 μM, a concentration which minimized changes in free radioligand concentration during the course of the binding reaction. Even at the low receptor concentrations used, specific [3H]-QNB binding was linearly related to protein concentration over the entire range of protein concentrations encountered in the present study.

**Millipore Filtration**

To document solubilization of muscarinic receptors, aliquots of digitonin extracts were incubated at 37°C for 60 minutes in 0.4 ml of buffer A, 0.03% digitonin, 1 μM [3H]-QNB, in the absence or presence of 100 μM atropine. Following incubation, tubes were placed on ice. Aliquots of 0.2 ml were suspended in 5 ml of ice cold buffer A containing 0.03% digitonin and rapidly filtered through Millipore 0.22-μm filters. Filters were rinsed with three 5-ml aliquots of identical buffer, dried, and retained radioactivity determined by liquid scintillation counting. The [3H]-QNB charcoal adsorption assay was conducted on duplicate incubation mixtures to allow comparison of binding activity in the digitonin extracts with that retained by the 0.22-μm Millipore filters.

**Gel Filtration**

Solubilization of muscarinic receptors was established by gel filtration of digitonin-extracted material on Sepharose 4B-CL. Sarcolemmal enriched membrane vesicles (SL, 1.3 mg protein) were sedimented in a total volume of 300 μl of medium containing 50 mM sodium phosphate, pH 7.4, 0.125 M sucrose, and 15 mM histidine by centrifugation at 33,000 g for 30 minutes at 4°C. Supernatant was removed, and pelleted material resuspended in 200 μl of buffer A with 0.6% digitonin. Solubilization was conducted as described, except that the resultant supernatant was not diluted further prior to loading onto the column. The digitonin-solubilized material (200 μl) was applied to a 0.5 x 20 cm column of Sepharose 4B-CL equilibrated at 4°C with buffer A with 0.03% digitonin. The column was eluted with identical buffer at a flow rate of 2 ml/hour, collecting fractions of 80-100 μl dropwise. Aliquots were taken for protein determination. Every three consecutive fractions were pooled and assayed for soluble muscarinic receptor content using the charcoal adsorption assay. Assays contained 1.25 nm [3H]-QNB in the absence or presence of 10 μM atropine. Specific binding was taken as the difference between single determinations of total and nonspecific binding. Comparable results were obtained when receptors were radiolabeled prior to solubilization, and radioactivity present in each column fraction was determined by liquid scintillation counting. Elution of [3H]-QNB-labeled sarcolemmal vesicles was determined in the same Sepharose 4B-CL column equilibrated in buffer A without digitonin. Elution of free [3H]-QNB and protein calibration markers catalase, yeast alcohol dehydrogenase, and bovine serum albumin (Stokes radii 52, 46, and 35, respectively) were determined in the presence and absence of 0.03% digitonin. Elutions of free [3H]-QNB and protein standards were identical in the presence or absence of 0.03% digitonin.

**Glycerol Gradient Centrifugation**

Highly enriched preparations of sarcolemmal vesicles (SL) were used as a source of muscarinic receptors for centrifugation in the glycerol gradients. Muscarinic receptors were radiolabeled prior to solubilization, a modification which facilitated removal of the bulk of free [3H]-QNB prior to glycerol gradient centrifugation. Sarcolemmal enriched membrane vesicles (SL, 0.5-0.6 mg protein) were incubated for 30 minutes at 37°C in 200 μl of medium containing 0.125 M sucrose, 15 mM histidine, and 1 μM [3H]-QNB, in the absence or presence of 10 μM atropine. Incubation mixtures were cooled to 4°C and subjected to centrifugation at 45,000 g for 30 minutes. Supernatants containing free [3H]-QNB were removed and pelleted membranes containing radiolabeled receptors were resuspended in 0.5 ml of buffer A with 0.6% digitonin. Solubilization was performed as described above, except that resulting supernatants containing solubilized receptors were not diluted further. Supernatants (0.45 ml) containing solubilized muscarinic receptors which had been radiolabeled in the absence or presence of 10 μM atropine were layered onto 10-40% linear glycerol gradients (4.6-ml volume) resting on 0.3-ml cushions of 60% glycerol. Gradients were buffered with buffer A with 0.2% digitonin. Both internal calibration standards and external calibration standards applied to identical gradient fractions labeled sarcolemmal vesicles 11.3 S (Summer and Gralen, 1938); yeast alcohol dehydrogenase, 7.4 S (Hayes and Velick, 1954); bovine serum albumin, 4.35 (Creeth, 1952). Glycerol gradients were subjected to centrifugation at 2°C for 16 hours in a Beckman SW55 rotor at 50,000 rpm. Approximately 30 160-fl fractions were collected from the bottom of each gradient. From each fraction, 50-μl aliquots were removed for assay of protein profiles of solubilized material, as well as internal and external protein.
calibration standards. Aliquots (100 μl) of each fraction were suspended in 10 ml Aquasol for quantitation of [3H]-QNB by liquid scintillation counting. The glycerol gradient centrifugations were quite reproducible, as determined by internal and external standardization. Accordingly, specific [3H]-QNB binding to solubilized receptors was taken as the difference between counts present in corresponding gradient fractions prepared from material labeled in the absence and presence of 10 μM atropine.

Analysis of Data

Data from radioligand competition curves were subjected to mathematical transformation to obtain linearized plots of log [RQ/RQₘₐₓ - RQ] vs. log A, where RQₘₐₓ is the amount of [3H]QNB bound specifically in the absence of competing drug, RQ is the specific [3H]QNB binding in the presence of competing drug, and A is the concentration of competing drug. Linear regression analysis of these derived data provided values for the slope factor ("pseudo-Hill coefficient") and x-intercept [equivalent to log (IC₅₀)]. Kᵢ for competing drug was then calculated from the IC₅₀ by the method of Cheng and Prusoff (1973). Guanine nucleotide-induced changes in Kᵢ for competing muscarinic agonists were analyzed for statistical significance by use of paired t-tests of Kᵢ's derived from paired competition curves generated in the absence or presence of added guanine nucleotides.

Materials

[3H]-QNB (44 Ci/mmol) was purchased from Amer sham. Unlabeled and tritiated (±)QNB (29 Ci/mmol) were obtained from New England Nuclear. Digitonin, guanosine triphosphate (GTP), S'-guanylylimidodiphosphate [Gpp(NH)p], atropine sulfate, oxotremorine, methacholine, carbamylcholine, bovine serum albumin, and yeast alcohol dehydrogenase were purchased from Sigma Chemical Company. Catalase was from Boehringer-Mannheim. Sepharose 4B-CL was a product of Pharmacia Fine Chemicals. Coomassie brilliant blue G-250 was from BioRad.

Results

Subcellular Distribution of Cardiac Muscarinic Receptors

Selection of a suitable membrane source from which to prepare solubilized cardiac muscarinic receptors was facilitated by the examination of the distribution of muscarinic receptors in subcellular fractions from canine ventricular myocardium. Initially, muscarinic receptor distribution was examined in subcellular fractions prepared by differential centrifugation of a crude ventricular homogenate (procedure I). Na⁺,K⁺-ATPase activity, unmasked by the ionophore A23187, was used as a marker of sarcoplasmic reticulum content. Partial separation of the membranes derived from sarcolemma from those derived from sarcoplasmic reticulum was confirmed by analysis of unmasked ATPase activities (Fig. 1). The activity of the sarcolemmal marker Na⁺,K⁺-ATPase was enriched in subfraction A, the subfraction of lightest buoyant density, by 3- to 7-fold relative to the corresponding activity of the crude membrane vesicle fraction. Similar enrichment of [3H]-QNB binding activity in subfraction A was demonstrated. Conversely, activity of the sarcoplasmic reticulum marker Ca++-ATPase was decreased in subfraction A relative to the corresponding activity in the crude membrane vesicle fraction. In the denser fractions (B through E), Ca++-ATPase activity was increased, whereas all other activities decreased in parallel fashion. Subfraction E contained vesicles of sarcoplasmic reticulum relatively free of sarcolem mal contamination. As shown in Figure 1, subfraction E exhibited minimal SDS unmasked Na⁺,K⁺-ATPase activity (4.3 μmol Pi/mg per hr), representing 6% of the activity of subfraction A. The [3H]-QNB-binding activity present in subfraction E, representing only 6% of the corresponding activity of subfraction A, could thus be accounted for on the basis of sarcolem mal contamination of this subfraction.

To obtain a membrane fraction containing the highest possible muscarinic receptor content, a highly enriched preparation of sarcolemmal vesicles was isolated by sucrose density gradient centrifugation of cardiac membrane vesicles obtained by procedure II, as recently described (Jones et al., 1980; Manalan and Jones, 1982). Based on analysis of unmasked ATPase activities (Na⁺,K⁺-ATPase ≥ 80 μmol Pi/mg protein per hour; Ca++-ATPase ≤ 5 μmol Pi/mg protein per
FIGURE 1. Distribution of \(^{3}H\) (±) QNB-binding activity (■) and the activity of marker enzymes \(Na^+,K^+\)-ATPase (□), and \(Ca^{++}\)-ATPase (■) in procedure I membrane vesicles (MV-I) and membrane vesicle subpopulations (A–E) isolated by discontinuous sucrose density gradient centrifugation. Partial separation of sarcolemma-enriched (A) and sarcoplasmic reticulum-enriched (E) subpopulations of membrane vesicles is confirmed by analysis of \(Na^+,K^+\)-ATPase and \(Ca^{++}\)-ATPase activities.

hour), this subpopulation represented the purest available preparation of sarcolemmal vesicles, relatively free of contamination by sarcoplasmic reticulum. Enrichment of \(^{3}H\) (±) QNB-binding activity once again correlated with enrichment of \(Na^+,K^+\)-ATPase activity in this fraction. This preparation of sarcolemmal vesicles exhibited the highest obtainable content of particulate muscarinic receptors from canine ventricular myocardium (\(^{3}H\) (±) QNB-binding activity above 4000 fmol/mg protein).

Based upon positive correlation of \(^{3}H\) (±) QNB-binding activity with activity of the sarcolemmal \(Na^+,K^+\)-ATPase in all of the subcellular fractions (Fig. 2), muscarinic receptors appeared to be localized to sarcolemma in canine ventricular myocardium. In addition, demonstration of a negative correlation of binding activity with the activity of \(Ca^{++}\)-ATPase-dissociated \(^{3}H\) (±) QNB binding from a location in sarcoplasmic reticulum (Fig. 1).

Throughout the fractionation process, the binding properties of the muscarinic receptors were quantitatively preserved (data not shown). The muscarinic receptor population remained saturable, without alteration in affinity for \(^{3}H\) (±) QNB or a series of unlabeled muscarinic agents. Preservation of the stereospecificity of interaction of the stereoisomers of benzetimide with muscarinic receptors was also demonstrated.

Solubilization of Cardiac Muscarinic Receptors

Muscarinic receptors were solubilized by incubation of cardiac membrane vesicle preparations with 0.6% digitonin. Application of this technique to solubilization of muscarinic receptors using crude cardiac membrane vesicles (procedure I) as a receptor source produced solubilized receptors in sufficient concentration to allow pharmacological characterization. The sarcolemma-enriched membrane vesicles (SL) were used as a receptor source for gel filtration

FIGURE 2. Correlation of \(^{3}H\) (±) QNB-binding activity with activity of the sarcolemmal \(Na^+,K^+\)-ATPase throughout subcellular fractionation. In all preparations from crude ventricular homogenate to sarcolemma-enriched membrane vesicles, \(^{3}H\) (±) QNB-binding activity demonstrated positive correlation with the \(Na^+,K^+\)-ATPase activity (\(r = 0.98\)), suggesting that muscarinic receptors are localized to sarcolemma in canine ventricular myocardium.
and sedimentation studies allowing the application of receptors in a minimal volume. For both membrane vesicle preparations, recovery of solubilized receptors, as assessed by the charcoal adsorption assay, was in the range of 25–35%, comparable to that reported by Hurko for digitonin solubilization of receptors from brain homogenates (1978).

Four different solubilization criteria were examined in order to document that muscarinic receptors were being studied in a solubilized state. First, the binding activity present in digitonin extracts prepared by this method remained in the supernatant fraction following recentrifugation at 105,000 g for 90 minutes, consistent with solubilization of receptors. Second, unlike binding activity present in the membrane vesicles, $[^3H]$QNB binding activity present in the digitonin extracts passed through 0.22-μm Millipore filters, providing further evidence of solubilization. Third, enriched preparations of digitonin-solubilized receptors derived from the SL fraction were subjected to gel filtration on Sepharose 4B-CL under conditions identical to that present in the charcoal adsorption assay (Fig. 3). Elution of a single symmetrical peak of $[^3H]$QNB-binding activity at an elution volume intermediate between that of unsolubilized receptors and free $[^3H]$QNB documented the existence of muscarinic receptors in solubilized form. Fourth, glycerol gradient sedimentation was utilized as an additional independent method to corroborate muscarinic receptor solubilization. Digitonin-solubilized muscarinic receptors labeled with $[^3H]$QNB sedimented through the glycerol gradients as a single symmetrical peak (Fig. 4). Based upon comparison with sedimentation of internal and external standards, a sedimentation coefficient for the muscarinic receptor–digitonin complex of approximately 9S was obtained (Fig. 4, inset).

Characterization of Digitonin-Solubilized Cardiac Muscarinic Receptors

Assay of solubilized muscarinic receptors was performed using a $[^3H]$QNB charcoal adsorption assay. The binding characteristics of the digitonin-solubilized $[^3H]$QNB receptors were systematically compared with the properties of the membrane-bound muscarinic receptors present in the cardiac membrane vesicle preparations. $[^3H]$QNB binding to both membrane-bound (Fig. 5A) and digitonin-solubilized receptors (Fig. 5B) was saturable. For both particulate and solubilized receptors, Scatchard plots (Scatchard, 1949) of data from saturation curves (insets) were linear, consistent with high affinity binding of $[^3H]$QNB to an apparently homogeneous population of receptors. Notably, the $K_D$ for $[^3H]$QNB binding to solubilized receptors derived by Scatchard analysis was $7.6 \times 10^{-10}$ M, a value reflecting an approximately

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Gel filtration of digitonin-solubilized cardiac muscarinic receptors on Sepharose 4B-CL. Chromatography was performed as described in Methods. Elution of cardiac muscarinic receptors solubilized from highly enriched sarcolemmal vesicles (○) was determined by assaying column fractions using the $[^3H]$QNB charcoal adsorption assay. Elution of $[^3H]$QNB-labeled receptors present in sarcolemmal-enriched membrane vesicles (△), as well as elution of free $[^3H]$QNB (●), was also determined. Arrows indicate elution position of marker proteins catalase, yeast alcohol dehydrogenase (ADH), and bovine serum albumin (BSA) from which a plot of Stokes radius vs. elution volume was constructed (inset).
The muscarinic receptors exhibited more than 1000-fold greater affinity for dextemizide than for levetimide (Fig. 6, Table 1). Moreover, solubilized muscarinic receptors demonstrated 8-fold lower affinity for dextemizide than did the receptors in their membrane-bound state. Similar data derived from atropine: \[^{3}H\]QNB competition curves demonstrated 24-fold reduction in muscarinic receptor affinity associated with solubilization in digitonin (Fig. 6, Table 1). For all these muscarinic antagonists, slope factors ("pseudo-Hill coefficients") derived from \[^{3}H\]QNB competition curves were approximately 1.0, consistent with a simple competitive interaction between unlabeled antagonist and radioligand for binding to membrane-bound or solubilized receptors. In addition, the order of potency of this series of muscarinic antagonists was identical for membrane-bound and solubilized preparations, thus documenting preservation of the pharmacological specificity typical of muscarinic receptors. Notably, although digitonin solubilization was accompanied by a decrease in receptor affinity for all antagonists examined, the extraordinarily high affinity interaction of QNB with receptors was the most significantly attenuated by this alteration in the receptor milieu (Table 1). Addition of guanine nucleotides had no effect on the position or shape of antagonist: radioligand competition curves for either preparation (data not shown).

Interaction of a series of muscarinic agonists with membrane-bound and digitonin-solubilized receptors was similarly explored. Agonist: \[^{3}H\]QNB competition curves describing interactions with membrane-bound receptors were shallow in contour, with slope factors in the range of 0.60 for all agonists studied (Fig. 7, Table 1). Addition of 0.1 mM GTP to incubation mixtures induced a rightward shift in agonist: radioligand competition curves (Fig. 7A), resulting in approximately 2-fold increases in calculated inhibition constants for all three agonists (Table 1).

Addition of the nonhydrolyzable analogue Gpp(NH)p (0.1 mM) induced quantitatively identical increases in inhibition constants (data not shown).

Agonist: \[^{3}H\]QNB competition curves generated using digitonin-solubilized receptors documented a striking reduction in receptor affinities for agonists. The 700 to 1000-fold reductions in agonist affinities associated with digitonin solubilization were of much greater magnitude than the reductions encountered in the study of antagonist binding (Table 1). Comparison of agonist: radioligand competition curves suggested additional alteration in the nature of agonist: receptor interactions associated with the digitonin-solubilized state. As exemplified in Figure 7, oxotremorine radioligand competition curves for digitonin-solubilized receptors were relatively steep, with slope factors of approximately 1.0, consistent with a simple competitive interaction between oxotremorine and \[^{3}H\]QNB for binding to the solubilized receptor population (Fig. 7B). Guanine nucleotides (0.1 mM GTP or the nonhydrolyzable analogue Gpp(NH)p), whether

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**Figure 4.** Glycerol gradient sedimentation of digitonin-solubilized muscarinic receptors derived from highly enriched cardiac sarcolemmal vesicles. Receptors were labeled with \[^{3}H\]QNB, solubilized, and subjected to sedimentation in a 10–40% linear glycerol gradient, as described in Methods. Specific \[^{3}H\]QNB binding (0) was computed as the difference between counts present in corresponding gradient fractions prepared from receptors labeled in the absence and presence of 10 μM atropine. Arrows mark the positions of calibration standards catalase, yeast alcohol dehydrogenase (ADH), and bovine serum albumin (BSA), from which a plot of sedimentation coefficient (S) vs. fraction number was constructed (inset). Based upon three similar experiments, a sedimentation coefficient of 9 S was determined for digitonin-solubilized muscarinic receptors derived from canine ventricular sarcolemma.

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33-fold reduction in receptor affinity for \[^{3}H\]QNB when compared with that exhibited by the muscarinic receptors in their membrane-bound state (KD 2.3 × 10^{-11} M). Similar results were obtained in assays conducted in the presence of a 20-fold higher concentration of digitonin (0.6%), suggesting that the observed reductions in affinity were not the result of a simple competition between digitonin and radioligand for binding to the solubilized receptors. The lowered affinity of solubilized receptors for QNB was corroborated by comparison of unlabeled (±)QNB: \[^{3}H\]QNB competition curves generated using membrane-bound and digitonin-solubilized receptors (Fig. 6). Inhibition constants (Ki) for (±)QNB competition curves documented a quantitatively similar 39-fold reduction in affinity of the digitonin-solubilized receptors for (±)QNB (Table 1). For muscarinic receptors, both in membrane-bound and solubilized form, values of inhibition constants for (±)QNB were approximately twice that of the corresponding dissociation constants for \[^{3}H\]QNB, suggesting that equilibrium binding of (±)QNB reflected association of muscarinic receptors with the (−) stereoisomer. Stereospecificity of interaction of the stereoisomers of benzetamide with muscarinic receptors was also documented. Based on \[^{3}H\]QNB competition curves, both membrane-bound and digitonin-solubilized
FIGURE 5. Comparison of equilibrium binding of [3H]l-QNB to cardiac muscarinic receptors in membrane-bound (panel A) and digitonin-solubilized states (panel B). Membrane vesicles prepared by procedure I were employed as a source of muscarinic receptors for this comparison. Solubilization and binding assays were performed as described in Methods. Specific binding is plotted as a function of [3H]l-QNB concentration. Error bars are ± 1 SE of the difference, computed from triplicate determinations of total and nonspecific binding. Scatchard plots of bound radioligand/free radioligand (B/f) vs. bound radioligand (B) are shown in the insets. Based upon three similar experiments, dissociation constants (Kd) derived from Scatchard analysis of [3H]l-QNB binding are 23 ± 2 pm for membrane-bound receptors, and 760 ± 25 pm for muscarinic receptors in the digitonin-solubilized state.

Discussion

Muscarinic receptor content was determined in a series of subcellular fractions prepared from canine ventricular myocardium by a combination of differential centrifugation, calcium loading, and discontinuous sucrose gradient sedimentation. Based upon positive correlation between [3H](±)QNB binding and the activity of the sarcotlemmal Na⁺,K⁺-ATPase, muscarinic receptors appeared to be localized to sarcotlemma in canine ventricular myocardium. Accordingly, membrane vesicle fractions demonstrating enrichment of sarcotlemmal activities were used as enriched sources of cardiac muscarinic receptors to facilitate solubilization and characterization.

Cardiac muscarinic receptors were solubilized from sarcotlemmal-enriched canine ventricular membrane vesicle preparations using aqueous solutions of 0.6% digitonin. Development of a [3H]l-QNB charcoal adsorption assay facilitated characterization of the solubilized cardiac muscarinic receptors. Preservation of the expected pharmacological specificity, as well as stereospecificity, established that the solubilized [3H]l-QNB-binding activity actually represented solubilized muscarinic receptors.

Documentation of solubilization was provided by the demonstration that [3H]l-QNB-binding activity behaved as molecularly dispersed material upon both gel filtration and glycerol gradient sedimentation. The sedimentation coefficient for digitonin-solubilized muscarinic receptors from cardiac sarcolemma was 9S, in agreement with values for digitonin-solubilized receptors from bovine brain (Hurko, 1978) and rat brain (Gorissen et al., 1978). This value suggested a molecular complex somewhat larger than that of the single species of molecular weight 80,000 identified by SDS-polyacrylamide gel electrophoresis of brain membrane fractions covalently labeled with the muscarinic antagonist, [3H]propylbenzylcholine mustard (Birdsall et al., 1979). Based upon present results, it appeared possible that the 80,000 dalton monomer represented only one subunit of a functional multi-subunit receptor complex. However, the hydrodynamic properties presently reported represented behavior of a digitonin-receptor complex, and the contribution of digitonin molecules to the observed be-
FIGURE 6. Comparison of antagonist-[3H]-QNB competition curves for cardiac muscarinic receptors in membrane-bound (panel A) and digitonin-solubilized states (panel B). Membrane vesicles prepared by procedure 1 were employed as a receptor source for this comparative analysis. Filtration (panel A) and charcoal adsorption assays (panel B) were performed as described in Methods. Specific [3H]-QNB binding, expressed as a percentage of binding in the absence of competing drug, is plotted as a function of competing drug concentration. (±)QNB, (●); dexetimide, (◆); atropine, (▲); levetimide (○). [3H]-QNB concentration was approximately 80 pm for competition curves generated in the membrane preparations (panel A), and approximately 1000 pm for curves generated for solubilized preparations (panel B). Slope factors and inhibition constants derived from competition curves are presented in Table 1.

behavior must also be considered in interpretation of the data.

In designing a suitable assay for solubilized preparations of muscarinic receptors, the problem of separation of bound radioligand from free radioligand has been approached previously in a variety of ways, including spotting on DEAE-cellulose discs, ammonium sulfate precipitation, polyethylene glycol 6000 precipitation, Sephadex G-50 gel exclusion, and charcoal adsorption, as well as equilibrium dialysis (Beld and Ariens, 1975; Aronstam et al., 1978; Gorissen et al., 1978; Hurko, 1978; Gorissen et al., 1981; Cremo et al., 1981). Recently, a rapid charcoal adsorption assay using [3H]dexetimide has been developed, characterized, and shown to give results quantitatively identical to those obtained using the Sephadex G-50 assay method in the study of digitonin-solubilized muscarinic receptors (Gorissen et al., 1981). In the present study, the charcoal adsorption method was applied in the development of an assay for solubilized receptors using the high affinity muscarinic antagonist [3H]-QNB. The result was a rapid, efficient assay method which has proven to be well suited to the study of digitonin-solubilized muscarinic receptors derived from cardiac tissue.

To provide functional assessment of the solubilized muscarinic receptors, equilibrium binding characteristics of digitonin-solubilized receptors were compared quantitatively with those present in cardiac membrane vesicles. Results obtained for membrane-bound canine cardiac muscarinic receptors demonstrated excellent agreement with those reported for rabbit heart homogenates (Fields et al., 1978). Analysis of [3H]-QNB saturation curves confirmed the extraordinary high affinity interaction between [3H]-QNB and cardiac muscarinic receptors, with apparent dis-
Cardiac membrane vesicles prepared by procedure I were used as a source of muscarinic receptors. Parameters derived from [3H]-QNB competition curves (see Methods) are presented as a mean ± SEM for the number of experiments shown in parentheses. For some agonists, results are means of duplicate experiments, expressed as a mean ± SD. P values are results of paired t-tests of inhibition constants derived for paired agonist: radioligand competition curves generated in the absence and presence of 0.1 mM GTP. Addition of guanine nucleotides had no effect on the position or configuration of competition curves for muscarinic antagonists (data not shown). The affinity ratio for each agent is the quotient of the inhibition constant for solubilized receptors divided by the inhibition constant for membrane-bound receptors. For both digitonin-solubilized preparations, affinity ratios for most agents were similarly examined in the membrane-bound and digitonin-solubilized states. Indeed, using crude homogenates of canine ventricular myocardium, we have documented up to 30-fold reductions of receptor affinity for oxotremorine induced by guanine nucleotides (unpublished observation).

Interactions of muscarinic receptors with agonists were similarly examined in the membrane-bound and digitonin-solubilized preparations. Muscarinic agonist: radioligand competition curves generated for membrane-bound receptors were shallow in contour, with slope factors (~pseudo Hill coefficients~) substantially less than one. In agreement with other studies (Berrie et al., 1979; Rosenberger et al., 1979), addition of 0.1 mM GTP induced a significant rightward shift in the position of radioligand competition curves for all agonists studied. These guanine nucleotide-induced alterations in agonist affinities were detectable in the various sarcosomal-enriched fractions, which have been shown to consist of membrane vesicles existing predominantly in a functionally sealed, right-side-out orientation (Jones et al., 1980; Manalan and Jones, 1982). Consequently, the observed magnitude of the guanine nucleotide effects may have been limited as a result of incomplete access of exogenous nucleotides to regulatory sites in the vesicular preparations used. Indeed, using crude homogenates of canine ventricular myocardium, we have documented up to 30-fold reductions of receptor affinity for oxotremorine induced by guanine nucleotides (unpublished observation).

Table 1

<table>
<thead>
<tr>
<th>Antagonists</th>
<th>Added GTP</th>
<th>Slope factor</th>
<th>Kᵢ (μM)</th>
<th>Slope factor</th>
<th>Kᵢ (μM)</th>
<th>Affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(±) QNB</td>
<td>0</td>
<td>0.65 ± 0.03</td>
<td>0.055 ± 0.009 (3)</td>
<td>0.69 ± 0.04</td>
<td>0.039 ± 0.001 (3)</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>0.70 ± 0.03</td>
<td>0.12 ± 0.02 (5)</td>
<td>0.97 ± 0.05</td>
<td>0.42 ± 0.07 (4)</td>
<td></td>
</tr>
<tr>
<td>Dexetimide</td>
<td>0</td>
<td>0.57 ± 0.02</td>
<td>0.48 ± 0.05 (3)</td>
<td>0.93 ± 0.04</td>
<td>1.3 ± 0.09 (3)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>0.64 ± 0.05</td>
<td>1.1 ± 0.2 (3)</td>
<td>1.02 ± 0.05</td>
<td>30 ± 2 (3)</td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>0</td>
<td>0.57 ± 0.02</td>
<td>0.58 ± 0.09 (3)</td>
<td>1.03 ± 0.04</td>
<td>1300 ± 300 (2)</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>0.69 ± 0.04</td>
<td>1.2 ± 0.2 (3)</td>
<td>0.90 ± 0.05</td>
<td>4900 ± 1300 (2)</td>
<td></td>
</tr>
<tr>
<td>Levetimide</td>
<td>0</td>
<td>0.57 ± 0.02</td>
<td>0.58 ± 0.09 (3)</td>
<td>1.03 ± 0.04</td>
<td>1300 ± 300 (2)</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>0.69 ± 0.04</td>
<td>1.2 ± 0.2 (3)</td>
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<td>4900 ± 1300 (2)</td>
<td></td>
</tr>
</tbody>
</table>

Cardiac membrane vesicles prepared by procedure I were used as a source of muscarinic receptors. Parameters derived from [3H]-QNB competition curves (see Methods) are presented as a mean ± SEM for the number of experiments shown in parentheses. For some agonists, results are means of duplicate experiments, expressed as a mean ± SD. P values are results of paired t-tests of inhibition constants derived for paired agonist: radioligand competition curves generated in the absence and presence of 0.1 mM GTP. Addition of guanine nucleotides had no effect on the position or configuration of competition curves for muscarinic antagonists (data not shown). The affinity ratio for each agent is the quotient of the inhibition constant for solubilized receptors divided by the inhibition constant for membrane-bound receptors. For both digitonin-solubilized preparations, affinity ratios for most agents were similarly examined in the membrane-bound and digitonin-solubilized states. Indeed, using crude homogenates of canine ventricular myocardium, we have documented up to 30-fold reductions of receptor affinity for oxotremorine induced by guanine nucleotides (unpublished observation).

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Interactions of agonists with muscarinic receptors were profoundly altered upon receptor solubilization.
Complexity manifested by slope factors less than 1, agonist interaction with membrane-bound muscarinic receptors. Thus, characteristic properties of agonists were not demonstrable with digitonin-solubilized receptors. Notably, the magnitude of these reductions in agonist affinities was far greater than that observed for antagonists. Agonist radioligand competition curves exhibited steep contours, with slope factors of approximately 1.0. Furthermore, guanine nucleotide effects on agonist affinities were not demonstrable with digitonin-solubilized receptors. Thus, characteristic properties of agonist interaction with membrane-bound muscarinic receptors, including relatively high affinity, kinetic complexity manifested by slope factors less than 1, and regulation by guanine nucleotides were lost upon receptor solubilization in digitonin. Taken together, these observations suggested the presence of agonist-selective alterations in the behavior of muscarinic receptors following solubilization in digitonin.

Based upon quantitative analysis of the shallow agonist-binding curves exhibited by muscarinic receptors from brain, Birdsall et al. (1980a) have proposed that muscarinic receptors exist in three different interconvertible states, exhibiting different affinities for agonists. On the basis of agonist affinities, these receptor states have been termed super-high (SH), high (H), and low (L) affinity (Birdsall et al., 1980a). Within the context of this model, guanine nucleotides have been interpreted to induce a change in receptor state from H to L (Birdsall et al., 1980a). Such transitions have been proposed to reflect conformational changes attendant to receptor effector coupling (Birdsall et al., 1980a). The molecular mechanisms accounting for the interconversions between these states have not been established, but interactions involving either membrane "matrix elements" or specific receptor-associated subunits have been proposed to account for the experimental results (Birdsall et al., 1980b). In this regard, it is tempting to suggest possible analogy with the β-adrenergic receptor system, in which guanine nucleotide interaction with a membrane-associated nucleotide-binding protein appears to result in dissociation of this protein from the β-adrenergic receptor, with attendant conversion of the dissociation receptor to a state of low agonist affinity (Limbird et al., 1980; DeLean et al., 1980; Stadel et al., 1980). Thus, the characteristic shallow agonist radioligand competition curves have been interpreted to reflect the complex regulation of receptor units, subject to conformational constraints imposed by additional interacting membrane constituents.

In the present study, alterations in the characteristics of receptor:agonist interaction were demonstrated for cardiac muscarinic receptors subjected to solubilization in digitonin. In contrast to membrane-bound receptors, solubilized receptors exhibited reductions in agonist affinities, loss of guanine nucleotide regulation, and steep agonist:radioligand competition curves with slope factors of 1.0, suggesting a homogeneous population of agonist-binding sites. Based upon the model of state interconversion for membrane-bound receptors, present results suggested that muscarinic receptors of a single state were selectively solubilized, or that solubilization induced conversion of all receptors to a single low affinity state, possibly by removal of constituents necessary for assumption of a high-affinity agonist conformation. In either case, the subpopulation of muscarinic receptors solubilized fails to exhibit alterations in agonist affinities in response to guanine nucleotides, a phenomenon that has been thought to reflect consequences of receptor:effector coupling. It is possible that this functional deficiency results from the absence of essential molecular constituents in the digitonin-solubilized complex. Alternatively, required
Digitonin-Solubilized Muscarinic Receptors

constituents, although present, may be incapable of functional interactions in the suboptimal environment provided by digitonin molecules.

Physiologically, cholinergic stimulation of ventricular myocardium has been shown to exert a negative inotropic effect, which is accentuated by the presence of concomitant adrenergic stimulation (review see Levy, 1971). At the biochemical level, muscarinic agonists attenuate both GTP and isoproterenol-stimulated adenylyl cyclase in membrane fractions derived from ventricular myocardium (Watanabe et al., 1979). Recently, all of the individual components of a catecholamine responsive complex (β-adrenergic receptors, adenylate cyclase, cAMP-dependent protein kinase) have been identified in highly purified preparations of sarcolemma (Jones et al., 1979, 1980; Manalan and Jones, 1982). The present results document a sarcolemmal location for muscarinic receptors as well. Thus, it seems possible that muscarinic modulation of the adenylate cyclase complex is mechanistically confined to interactions which occur entirely within the sarcolemma. The solubilization of muscarinic receptors from canine ventricular myocardium provides a basis for reconstitution experiments directed toward understanding the molecular mechanisms accounting for muscarinic effects in cardiac tissue.

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cardiac tissue. Eur J Pharmacol 56: 179–180


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