G-Protein Mediates Voltage Regulation of Agonist Binding to Muscarinic Receptors: Effects on Receptor–Na^+ Channel Interaction

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ABSTRACT: Our previous experiments in membranes prepared from rat heart and brain led us to suggest that the binding of agonists to the muscarinic receptors and to the Na^+ channels is a coupled event mediated by guanine nucleotide binding protein(s) [G-protein(s)]. These in vitro findings prompted us to employ synaptoneurosomes from brain stem tissue to examine (i) the binding properties of [^{3}H]acetylcholine at resting potential and under depolarization conditions in the absence and presence of pertussis toxin; (ii) the binding of [^{3}H]batrachotoxin to Na^+ channel(s) in the presence of the muscarinic agonists; and (iii) muscarinically induced $^{22}Na^+$ uptake in the presence and absence of tetrodotoxin, which blocks Na^+ channels. Our findings indicate that agonist binding to muscarinic receptors is voltage dependent, that this process is mediated by G-protein(s), and that muscarinic agonists induce opening of Na^+ channels. The latter process persists even after pertussis toxin treatment, indicating that it is not likely to be mediated by pertussis toxin sensitive G-protein(s). The system with its three interacting components—receptor, G-protein, and Na^+ channel—is such that at resting potential the muscarinic receptor induces opening of Na^+ channels; this property may provide a possible physiological mechanism for the depolarization stimulus necessary for autoexcitation or repetitive firing in heart or brain tissues.

Muscarinic receptors mediate various responses in the peripheral and central nervous system [for review see Sokolovsky (1984) and McKinney and Richelson (1984)]. A possible relationship between muscarinic receptors and Na^+ channels was implied by studies showing that muscarinic stimulation triggers Na^+ fluxes in several systems [e.g., Keryer et al. (1979), Kato et al. (1983), and Korth and Kuhlkamp (1985)]. Our recent experiments in membranes prepared from rat heart and brain led us to suggest that the binding of agonists to the muscarinic receptors and to the Na^+ channels (in the open state) is a coupled event mediated by guanine nucleotide binding protein(s) [G-protein(s)] (Cohen-Armon et al., 1985; Cohen-Armon & Sokolovsky, 1986). These in vitro findings prompted us to investigate whether muscarinic agonist binding induced Na^+ uptake in synaptoneurosomes. Using synaptoneurosomes from brain stem tissue, we then examined (i) the binding properties of [^{3}H]acetylcholine (AcCh)[1] at resting potential and under depolarization conditions in the absence and presence of pertussis toxin; (ii) the binding of [^{3}H]batrachotoxin ([^{3}H]BTX) to Na^+ channel(s) in the presence of the muscarinic agonists; and (iii) muscarinically induced $^{22}Na^+$ uptake in the presence and absence of tetrodotoxin (TTX), which blocks Na^+ channels. The results reported here clearly indicate that depolarization induces conversion of binding sites for muscarinic agonists from the high-affinity to the low-affinity state and that the process is mediated by G-proteins. Binding of the muscarinic agonist carbamylcholine to the muscarinic receptor induces $^{22}Na^+$ uptake via sodium channels. The system with its three interacting components—receptor, G-protein, and Na^+ channel—is such that at resting potential (i.e., when the receptors are in the high-affinity state for agonist binding) the muscarinic receptor induces opening of Na^+ channels. We suggest that this capacity of muscarinic receptors to induce sodium uptake at resting potential may provide a possible physiological mechanism for the depolarization stimulus necessary for autoexcitation or repetitive firing in heart or brain tissues.

Materials and Methods

[^{3}H]AcCh (86 Ci/mmol, 98% purity) was purchased from Amersham Corp. (Buckinghamshire, England). Small aliquots of the radiochemical in ethanol/water (1:1 v/v) were kept at −70 °C and subjected to drying by a gentle stream of nitrogen prior to use. [^{3}H]NMPB (70 Ci/mmol, 97% purity) was prepared by catalytic tritium exchange as described elsewhere (Kloog et al., 1979). [^{3}H]BTX (50 Ci/mmol, >99% purity) and [α-^{32}P]NAD^+ (1000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). BTX was kindly supplied by Dr. J. W. Daly (Laboratory of Bioorganic Chemistry, National Institutes of Health, Bethesda, MD). BTX was dissolved in ethanol (10^{-3} M) and added to the assay mixture immediately before use. The storage period did not exceed 3 weeks. DFP and Gpp(NH)p were purchased from Sigma (St. Louis, MO). Pertussis toxin was purchased from List Biological Labs Inc. (Campbell, CA). Affinity-purified RV/3 (prep 638/22) rabbit antibodies vs the α-subunit of G_0 were kindly supplied by Dr. Allen M. Spiegel, NIH (Bethesda, MD). Affinity-purified horseradish peroxidase conjugated goat IgG directed against rabbit IgG (H+L) was obtained from Bio-Yeda (Rehovoth, Israel).

Tissue Preparation. Adult male rats of the CD strain were obtained from Levinstein's Farm, Yokneam, Israel, and maintained in an air-conditioned room at 24 ± 2 °C for 14

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[1] Abbreviations: AcCh, acetylcholine; BTX, batrachotoxin; NMPB, *N*-methyl-4-piperidyl benzilate; QNB, L-3-quinuclidinyl benzilate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; [^{3}H]BTX, batrachotoxin A 20-α [*benzoyl*-2,5-^{3}H]benzoate; DFP, diisopropyl fluorophosphate; [^{3}H]TPP^+, tetra[^{3}H]phenylphosphonium bromide; IgG, immunoglobulin G; Tris-HCl, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TTX, tetrodotoxin.

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h under fluorescent illumination and in darkness for 10 h. Food from Assia Maabarot (Tel Aviv, Israel) and water were supplied ad libitum. Rats aged 3–4 months weighing 190–250 g were decapitated and the brains rapidly removed. Brain stems from seven rats were dissected and synaptoneurosomes were prepared according to the method of Hollingsworth et al. (1985). The synaptosomes were resuspended in Krebs–Henseleit buffer containing 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgCl_2$, 2.5 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, and 1.18 mM KH_2PO_4 in an atmosphere of 95% O_2/5% CO_2 at 25 °C.

[^3H]AcCh Binding Assay. [^{3}H]AcCh binding to the muscarinic receptors was measured according to the method of Gurwitz et al. (1985), in *Ca^{2+}-free* Krebs–Henseleit buffer, i.e., $CaCl_2$ was replaced by NaCl (final concentration 120 mM) ([Ca^{2+}] < 30 μM). Aliquots (20 μL) of synaptosomes were added to tubes containing 20 μL of Ca^{2+}-free buffer, 150 μM DFP, and the indicated concentration of [^{3}H]AcCh. After 1 h of incubation with gentle shaking at 25 °C, 6 mL of ice-cold buffer was added, and the contents of the tubes were filtered under high pressure through GF/C filters (Whatman, 25-mm diameter). The filters were washed with an additional 4 mL of buffer within less than 3 s. Filters were counted for tritium by using a scintillation mixture (Hydroluma) and a scintillation spectrometer (LKB 1218) at 48% efficiency. Specific binding was determined by calculating the difference between the total binding and the nonspecific binding, the latter in the presence of 0.1 μM atropine. All determinations were carried out in triplicate and were found to vary by no more than 15%.

K^+ depolarization was achieved by the addition of 50 mM KCl and 75.2 mM NaCl throughout the incubation period (50 min) or during the last 5 or 10 min. Incubation was terminated by the addition of cold high-K^+ buffer. BTX depolarization was achieved by preincubation of the synaptoneurosomes with 1 μM BTX in the presence and absence of 1 μM TTX for 30 min at 36 °C in 4.7 mM K^+ buffer solution and in an atmosphere of 95% O_2/5% CO_2.

Measurement of [^3H]TPP^+ Accumulation. Depolarization was monitored by the decrease in [^{3}H]TPP^+ accumulation (Pauwels & Laduron, 1986). Synaptoneurosomes (5 mg of protein/mL) were incubated with 7×10^{-8} M [^{3}H]TPP^+ at 25 °C (40-μL volume) for 20 min (TPP^+ accumulation reached saturation after 15 min). Each sample was filtered on a Whatman GF/C filter and washed (8 × 2 mL) with the buffer used (at 4 °C). The filters were counted for tritium by using a scintillation mixture (Hydroluma). In parallel, samples incubated under identical conditions were washed on the filters by distilled water, in order to lyse the synaptosomes and provide a measure for the adsorption of [^{3}H]TPP^+ to the membranes. The values obtained in the latter procedure were subtracted from those obtained without lysis to yield the specific [^{3}H]TPP^+ accumulation. In a representative experiment following results were obtained. Data (in cpm) are given in pairs (without and with pertussis toxin treatment): (a) at rest, 413 100 vs 372 700; (b) with 50 mM K^+, 69 800 vs 59 500; (c) with 1 μM BTX, 33 900 vs 27 100.

[^3H]NMPB Binding. Aliquots (50 μL) of the preparation were incubated for 30 min at 25 °C with various concentrations of [^{3}H]NMPB (0.1–10 nM) in 1 mL of Ca^{2+}-free buffer. Binding was terminated by filtration through GF/C filters and washing 3 times with 3 mL of ice-cold buffer. Nonspecific binding was determined with 1 μM atropine. These assays were carried out in parallel with [^{3}H]AcCh binding assays in the same preparations.

[^3H]NMPB Displacement. Aliquots (50 μL) of the synaptosomes were added to tubes containing 190 μL of Ca^{2+}-free Krebs–Henseleit buffer containing 2 nM [^{3}H]-NMPB and 10 μL of agonist (10^{-9}–10^{-3} M). After 30 min of incubation with gentle shaking at 25 °C, binding was terminated by filtration according to the procedure described for the [^{3}H]AcCh binding assay.

Pertussis Toxin Treatment. Synaptoneurosomes were pretreated for 2 h in Ca^{2+}-free Krebs–Henseleit buffer containing 200 ng/mL pertussis toxin at 36 °C and in an atmosphere of 95% O_2/5% CO_2. Control and pretreated synaptoneurosomes were then washed in the same buffer and subjected to depolarization induced either by the addition of 50 mM K^+ during the last 5 min, during the last 15 min, or throughout the incubation period (50 min) with the agonist or by pretreatment of the synaptoneurosomes with 1 μM BTX for 30 min at 36 °C.

[^{32}P]ADP-Ribosylation with Pertussis Toxin. Synaptoneurosomes treated or untreated with toxin as described above were first washed in Ca^{2+}-free Krebs–Henseleit buffer. Homogenization was performed in buffer containing 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM DTT (pH 7.4). The preparation was centrifuged at 1000*g* for 10 min; the membranes were pelleted from the supernatant at 30000*g* for 20 min and were suspended to 200 μg of protein/50 μL of the same buffer. Samples containing 200 μg of protein were treated for 1 h at 37 °C with 1 μg of activated pertussis toxin (330 mM Tris-HCl, 14 mM DTT, 1 h at 37 °C) in buffer containing 2 mM $MgCl_2$, 2.3 mM ATP, 75 mM Tris-HCl, and 1 μCi [^{32}P]NAD^+. The final volume was 110 μL. Membranes were pelleted, resuspended in sample buffer (Laemmli, 1980), boiled for 2 min, and subjected to SDS–PAGE (8% polyacrylamide). Dried gels were then exposed to AGFA-CURIX RP2 X-ray film with enhancing screen for 5 days at −70 °C.

Immunoblotting. SDS gels were electroblotted overnight at 10 °C onto nitrocellulose paper with constant current of 150 mA as described by Towbin et al. (1979). Dried nitrocellulose strips were first immersed in 3% gelatin and then in anti-α_0 (1/20 dilution) in 1% gelatin according to the procedure of Dr. A. M. Spiegel. The bands with bound antibodies were visualized by a peroxidase-conjugated second antibody (Towbin et al., 1979).

[^3H]BTX Binding Assay. Aliquots of synaptosomes (20 μL) were added to tubes containing 20 μL of Ca^{2+}-free Krebs–Henseleit buffer (pH 7.8) containing 0.6 μM [^{3}H]BTX in the presence of various concentrations of carbamylcholine ± 0.1 μM atropine. For determination of nonspecific binding, 300 μM veratridine was added to the synaptosomes. After 30 min of incubation (which is sufficient for reaching equilibrium) with gentle shaking at 36 °C, 4 mL of buffer was added, and the contents of the tubes were filtered, washed, and counted for tritium as described for the [^{3}H]AcCh binding assay. All determinations were carried out in duplicate and varied by no more than 15%.

Acetylcholine Content. Acetylcholine content in synaptoneurosomes was measured by the chemiluminescence method (Israel & Lesbats, 1981) as described earlier in detail (Egozi et al., 1986).

$^{22}Na^+$ Uptake. Pertussis toxin treated synaptoneurosomes were washed twice and suspended in a solution containing 20 mM Tris-HCl, 1.2 mM $MgCl_2$, 10 mM glucose, and 264 mM sucrose (<0.5 mM Na^+). Aliquots were mixed with carrier-free $^{22}Na^+$ (final concentration 1.5 μCi/mL, $\sim 10^{-7}$ M ^{22}NaCl) ± TTX (1 μM). After 30 s, the radioactive mixtures

received carbamylcholine (10^{-9}–10^{-4} M) or carbamylcholine plus atropine (10^{-7} M). $^{22}Na^+$ uptake was monitored by applying 150-μL aliquots (~240 μg of protein) to a short Dowex column (50WX8 100–200 mesh Tris form) and eluting the synaptoneurosomes into counting vials with 1.5 mL of isotonic sucrose solution (Garty et al., 1983). More than 99.9% of the external radioactivity was trapped on the column (Garty et al., 1983), while the intrasynaptoneurosomal $^{22}Na^+$ was eluted through it. Dowex columns were prewashed with synaptoneurosomes to minimize their adsorption to the resin. Kinetic measurements have shown that $^{22}Na^+$ uptake in the presence of 1 μM BTX was completed within 15 s, where as TTX-blockable $^{22}Na^+$ uptake induced by carbamylcholine showed a linear time course up to 2 min (for example, in the presence of 10^{-6} M carbamylcholine, $^{22}Na^+$ uptake after 15, 30, 60, and 120 s was 50, 139, 305, and 617 pmol/mg of protein, respectively). Thus, for convenience the synaptoneurosomes were sampled 1 min after the agonist was added. In contrast, $^{22}Na^+$ uptake in the presence of BTX (which was not investigated in the present work) was already saturated after 15 s (the shortest sampling time possible). Therefore, the Na^+ uptake results in the present study cannot be directly compared with those obtained in the presence of BTX. The much faster uptake rate in the presence of BTX may be due to the fact that BTX keeps the Na^+ channel open; on the other hand, carbamylcholine could induce transient opening of the channel. This suggestion is supported by the observation that carbamylcholine-induced $^{22}Na^+$ uptake is observed only when $^{22}Na^+$ is added prior to or concomitantly with the agonist (as described above). Addition of $^{22}Na^+$ *after* the agonist results in no apparent uptake.

Data Analysis. Values for maximal binding capacity (B_{max}) and dissociation constants (K_d) were obtained by nonlinear regression analysis of binding isotherms. the competition curves were analyzed by either one or two binding sites (Kloog et al., 1980). Theoretical competition curves were fitted to experimental data points by using the nonlinear least-squares regression computer program BMDPAR (November 1978 revision), developed at the Health Science Computing Facility (University of California, Los Angeles, CA).

At the level of accuracy of the experimental data and parameters fitting procedures, the binding of AcCh and carbamylcholine to this preparation was well described by a model of two noninteracting sites. We therefore performed the analysis employing the simple two-sites model; while this does not imply that there are indeed only two affinity states in the synaptosomes, this approach is still valid for detecting changes in the agonist binding characteristics due to altered conditions (e.g., depolarization).

Results and Discussion

Prior to the depolarization experiments described below, we characterized the binding of muscarinic ligands to synaptosomes prepared from brain stem tissue. Binding of the highly specific antagonist [^{3}H]NMPB yielded B_{max} of 370 ± 50 fmol/mg of protein (n = 5), with K_d = 1.8 ± 0.2 nM. Direct binding of [^{3}H]AcCh to the high-affinity sites (Gurwitz et al., 1985) yielded a maximal binding capacity of 150 ± 25 fmol/mg of protein (n = 10). These results, as well as the results of competition experiments given below, are in excellent agreement with previous binding data obtained for rat brain stem homogenates (Gurwitz et al., 1985), indicating that the binding properties of the muscarinic receptors in synaptoneurosomes and in membrane preparations are very similar.

In order to investigate the effects of depolarization on the binding properties of muscarinic ligands to synaptoneurosomes,

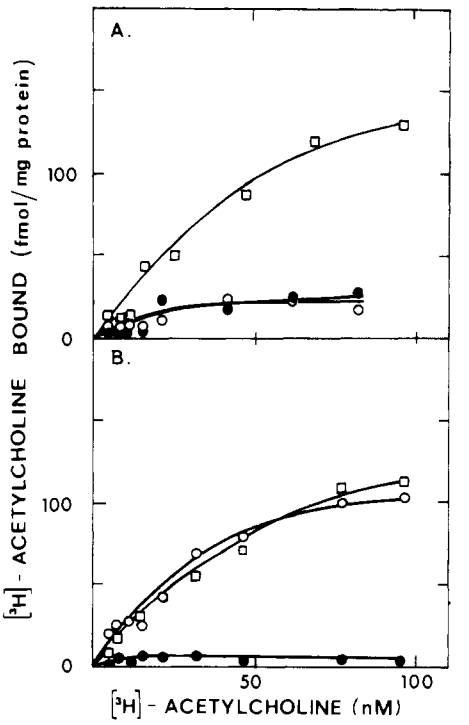

FIGURE 1: [^{3}H]AcCh binding to synaptoneurosomes under resting conditions (□) and following depolarization and in the absence (●) and presence (○) of the Na^+-channel blocker TTX (1 μM). (A) Depolarization was achieved by the addition of 50 mM K^+ during the last 5 min of incubation with the agonist. The results presented here are from a representative experiment, 1 of 10 performed. Each data point was determined in duplicate. The SD derived from separate experiments was below 15%. (B) Depolarization was achieved by pretreatment of the synaptoneurosomes with 1 μM BTX. The pretreated synaptoneurosomes were then incubated with [^{3}H]AcCh. The results presented here are from a representative experiment, one of five performed. Each data point was determined in duplicate. The SD was as in (A).

the latter were depolarized either by exposure to high concentrations of K^+ (usually 50 mM) or by pretreatment with 1 μM batrachotoxin, which interacts with a site located on the voltage-sensitive Na^+ channel and induces persistent activation (Catterall, 1975; Daly, 1982; Khodorov, 1985). To obviate possible interference caused by the effects of acetylcholine released during depolarization, Ca^{2+}-free buffers known to prevent AcCH release [e.g., Drapeau and Blaustein (1983)] were used ([Ca^{2+}] < 30 μM). Indeed, depolarization had no significant effect on the acetylcholine content of synaptoneurosomes, which was 28.6 ± 7 and 27.4 ± 4 pmol/mg of protein before and after depolarization by 50 mM K^+ (10–30 min), respectively. Depolarization of the synaptoneurosomes was accompanied by a decrease in binding of [^{3}H]AcCh to the high-affinity sites of the muscarinic receptor: maximal [^{3}H]AcCh binding in the control preparation was 150 ± 25 fmol/mg of protein. Addition of buffer containing 50 mM K^+ resulted in reduction of the maximal binding to 40 ± 6 fmol/mg of protein (n = 10), and addition of 100 mM K^+ reduced the binding to zero. A typical experiment is illustrated in Figure 1A. Similar effects on [^{3}H]AcCh binding were observed when the synaptoneurosomes were pretreated with 1 μM BTX (n = 5) (Figure 1B). Repolarization of the synaptoneurosomes following K^+ depolarization by transfer to the original buffer regenerated maximal [^{3}H]AcCh binding (155 ± 25 fmol/mg of protein).

In order to confirm that conversion of the agonist-binding state from high to low affinity had actually occurred and that the observed reduction in [^{3}H]AcCh binding was not due

instead to unavailability of part of the receptor population, [^{3}H]antagonist/agonist competition experiments were performed. Binding of the antagonist [^{3}H]NMPB was not affected by either high [K^+] (B_{max} = 390 ± 40 fmol/mg of protein; n = 3) or by pretreatment with BTX (B_{max} = 402 ± 55 fmol/mg of protein; n = 3); thus, [^{3}H]NMPB could be used in displacement experiments aimed at measuring the effect of depolarization on the binding of unlabeled agonists. Analysis of the displacement curves of carbamylcholine and acetylcholine employing a model of two noninteracting sites with high and low affinities for agonist binding indicated that depolarization induces a substantial reduction in the fraction of agonist high-affinity sites. The average values obtained from five competition experiments for carbamylcholine binding were 54 ± 6% prior to depolarization at resting potential [K_H = (1.4 ± 0.4) × 10^{-7} M; K_L = (12 ± 5) × 10^{-5} M] as compared to 20 ± 4% (in the presence of 50 mM K^+) and 14 ± 5% (in the presence of 1 μM BTX). For acetylcholine binding the corresponding values were 55 ± 5% [K_H = (4.2 ± 0.9) × 10^{-8} M; K_L = (9 ± 3) × 10^{-6} M], 24 ± 5%, and 16 ± 3% (n = 3). Depolarization did not affect the values of K_H or K_L for these agonists. It therefore follows that depolarization induced conversion of the muscarinic receptors from a state of high affinity to a state of low affinity for agonist binding.

Experiments conducted at resting potential showed that the sodium channel blocker TTX (1 μM) did not significantly affect [^{3}H]AcCh binding to membranes, nor did it inhibit [^{3}H]BTX binding to the Na^+ channel at 36 °C (McNeal et al., 1985). The temperature is of extreme importance in these experiments, since it has been shown that at lower temperatures TTX inhibits [^{3}H]BTX binding (Brown, 1986). Measurement under depolarization conditions showed that the reduction in binding of [^{3}H]AcCh was prevented when TTX (1 μM) was added during pretreatment of synaptoneurosomes with BTX (Figure 1B) but not when TTX was added during synaptosomes exposure to high K^+ concentration (Figure 1A). It thus appears that the change in membrane potential and not the induction of a sodium current caused the depletion in high-affinity binding sites of the muscarinic receptor. It should be noted that previous experiments on depolarization-induced changes in muscarinic receptors in rat cerebral cortex synaptosomes (Luqmani et al., 1979) indicated that electrical pulses and veratrine treatment affect antagonist binding. Depolarization with 56 mM K^+ produced no change in the number of antagonist binding sites. The latter results are in agreement with the results reported here.

Since interconversion from high- to low-affinity state is known to be induced by guanine nucleotides [reviewed in Sokolovsky (1984) and McKinney and Richelson (1984)], we attempted to determine whether a G-protein is involved in inducing the interconversion brought about by depolarization. As shown in Figure 2, the depolarization-induced conversion of agonist binding sites from the high- to low-affinity state was prevented by pretreatment of the synaptoneurosomes with pertussis toxin (200 ng/mL for 2 h at 36 °C), which is known to modify G-protein(s) by ADP-ribosylation (Ui, 1984; Florio & Sternwise, 1985; Pfaffinger et al., 1985; Liang et al., 1986; Haga et al., 1986). Pertussis toxin treatment itself (without depolarization) had only a minor effect (at most a 10% reduction) on the B_{max} of [^{3}H]AcCh in the synaptoneurosomes. It also had no effect on [^{3}H]NMPB binding (370 ± 50 fmol/mg of protein in the control vs 350 ± 45 fmol/mg of protein in the toxin-treated preparation). To confirm that the effect induced by the pertussis toxin is in fact ADP-ribosylation of the G-protein, we subjected the synaptoneurosomes to two

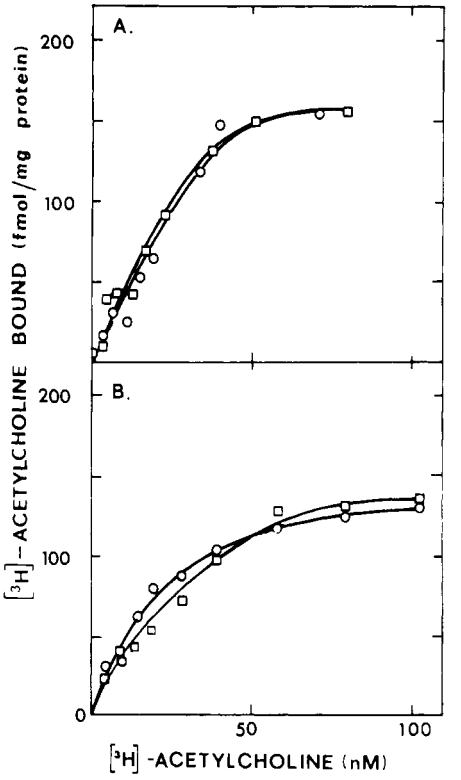

FIGURE 2: Effect of pertussis toxin on the binding of [^{3}H]AcCh to synaptoneurosomes obtained from brain stem tissue at resting potential (□) and at depolarization (○) [50 mM K^+ (A) and 1 μM BTX (B)]. The results shown are of a representative experiment, one of six (A) or five (B) performed. Each data point was determined in duplicate. The SD derived from separate experiments was below 15%.

successive processes of ADP-ribosylation, first on the intact synaptoneurosomes (containing unlabeled NAD) and then on membranes prepared from them (by use of [^{32}P]NAD^+). No labeling was observed in membranes from synaptoneurosomes preincubated for 2 h with pertussis toxin (200 ng/mL), while in the absence of such preincubation, labeling was detected. The inability of the membranes from pertussis toxin treated synaptoneurosomes to undergo ADP-ribosylation with [^{32}P]-NAD^+ indicates that pertussis toxin sensitive G-protein(s) present in this preparation had already been completely ADP-ribosylated. Quantitative [^{32}P]ADP-ribosylation of the synaptoneurosomal membranes revealed the presence of a 39-kDa labeled protein (Figure 3, lane A), most likely the G_o-protein, as indicated by electroblotting followed by labeling with anti-α_o antibodies (Figure 3, land D). Also, in the presence of Gpp(NH)p (200 μM) no change was observed in the binding of [^{3}H]AcCh to membranes prepared from synaptoneurosomes pretreated with pertussis toxin, whereas in membranes from untreated preparations [^{3}H]AcCh binding was reduced from 180 ± 27 to 100 ± 17 fmol/mg of protein ($p < 0.0001$) (n = 6).

Since pretreatment with pertussis toxin had no effect on (i) the [^{3}H]NMPB binding to synaptneurosomes, (ii) the membrane potential, and (iii) the ability of high [K^+] to induce membrane depolarization as determined by [^{3}H]TPP^+ accumulation experiments (n = 3) (see Materials and Methods for results of [^{3}H]TPP^+ measurements), we conclude that the depletion in high-affinity receptor sites normally caused by depolarization was prevented by pertussis toxin induced ADP-ribosylation. It seems that an unmodified G-protein is essential for the translation of the potential signal into a change in the muscarinic receptor with its associated alteration in properties.

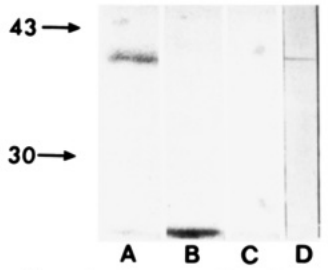

FIGURE 3: Autoradiography (lanes A–C) and immunoblotting (lane D) of synaptoneurosomal membranes ADP-ribosylated with pertussis toxin in the presence of [^{32}P]NAD$^+$. Each lane contains 200 μg of protein as determined by the Lowry method. Molecular weights were determined by plotting the distance migrated for standard proteins (94, 67, 43, 30, 20, and 14 kDa) stained with Coomassie Blue vs the logarithm of their molecular weights. The part of the gel in the front to 50 kDa is shown. (Lane A) Ribosylation of synaptosomal membranes that were not subjected to any pretreatment. (Lane B) Ribosylation of synaptosomal membranes pretreated with pertussis toxin. (Lane C) Same as in (A) except that ribosylation is carried out in the absence of toxin (control). (Lane D) Immunoblot of synaptosomal membranes labeled with anti-α_0. Calculated molecular weight for the band was 39 000 (n = 5).

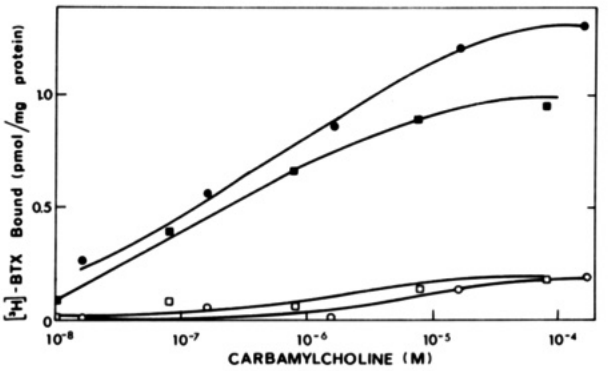

FIGURE 4: Binding of [^{3}H]BTX (0.6 μM) to synaptoneurosomes in the presence of carbamylcholine (●, ■) and carbamylcholine plus 0.1 μM atropine (○, □) following pretreatment with pertussis toxin (circles) or in the presence of 1 μM TTX (squares). The results presented here are from a representative experiment, one of three performed. Each data point was determined in duplicate.

Previous studies of the effects of pertussis toxin on other preparations have indicated that the toxin treatment itself abolished high-affinity agonist binding sites [e.g., Kurose et al. (1983)]. In the present study, we found specific experimental conditions under which pertussis toxin treatment by itself did not alter agonist binding but prevented Gpp(NH)p or depolarization-induced conversion of high-affinity agonist binding sites to the low-affinity state, indicating that the effect of the toxin on the receptor–G-protein interaction differs from that of, e.g., Gpp(NH)p. When we employed higher toxin concentrations or longer incubation times, we found toxin-mediated abolition of high-affinity sites (not shown). This indicates that there are two distinct phases in the effect of the toxin on the system, a phenomenon that might be usefully exploited in attempts to elucidate the mode of G-protein–receptor interaction.

We then examined the interaction between muscarinic receptors and Na^+ channels at resting and depolarization conditions. The specific binding of [^{3}H]BTX to rat brain stem synaptoneurosomes is difficult to measure because of the high nonspecific binding. Only 10–15% of the total binding fraction was specific. In order to overcome this difficulty and to enhance the specific binding, previous studies have employed scorpion venom (Catterall et al., 1981; Creveling et al., 1982; McNeal et al., 1985) in the binding assay. As we have shown earlier with rat atrial membranes (Cohen-Armon & Sokolovsky, 1986), we find here that carbamylcholine under certain experimental conditions markedly enhances the binding of [^{3}H]BTX to the synaptoneurosomes; the resulting specific binding was 40–50% of the total, thus allowing more accurate measurements. It should be noted that in the presence of 1 μM scorpion venom (36 °C for 60 min) carbamylcholine affected only slightly [^{3}H]BTX binding. Thus, at 100 nM [^{3}H]BTX, a concentration at which maximal enhancement of binding is observed with scorpion toxin, carbamylcholine enhanced binding by only 50% (see below).

[^{3}H]BTX binding to the synaptoneurosomes was increased in the presence of carbamylcholine only when the depolarization caused by inward Na^+ current was prevented by the sodium channel blocker TTX (1 μM) or when the effect of depolarization on the muscarinic receptor was prevented by ADP-ribosylation (Figure 4). Under either of the latter conditions, raising the concentration of carbamylcholine from 10^{-8} to 10^{-4} M was accompanied by a marked increase in [^{3}H]BTX binding (9.5-fold higher than [^{3}H]BTX binding of 0.1 ± 0.05 pmol/mg of protein in the control) (n = 3). Previous experiments (Catterall et al., 1981) on the binding of [^{3}H]BTX to synaptic nerve-end particles demonstrated a binding capacity of 2.1 ± 0.2 pmol/mg of protein in the presence of 1 μM scorpion toxin; since the latter enhances [^{3}H]BTX binding to the synaptosomes 10–20-fold, it follows that in the absence of scorpion toxin [^{3}H]BTX binding should be around 0.1–0.2 pmol/mg of protein—rather close to the value observed here for brain stem synaptosomes and the previously reported value of 0.17 pmol/mg of protein detected in rat atrial membranes (Cohen-Armon & Sokolovsky, 1986). The increase in [^{3}H]BTX binding did not occur in the presence of atropine (10^{-7} M) or QNB (10^{-7} M), thus confirming the muscarinic nature of the system. The dose–response curves shown in Figure 4 are shallow, most likely due to the allosteric nature of the interaction between the muscarinic high-affinity agonist binding sites and the [^{3}H]BTX sites, as previously observed in membrane preparations (Cohen-Armon et al., 1985; Cohen-Armon & Sokolovsky, 1986). [^{3}H]TPP$^+$ accumulation experiments (performed as described under Materials and Methods) demonstrated that pretreatment of the synaptoneurosomes with pertussis toxin did not interfere with the ability of BTX to induce depolarization (see [^{3}H]TPP$^+$ accumulation under Materials and Methods).

We then proceeded to investigate the effect of carbamylcholine on TTX-blockable Na^+ uptake to the synaptoneurosomes. This was done by measuring the effect of carbamylcholine on $^{22}Na^+$ uptake under conditions that prevent the depolarization-induced conversion of high-affinity sites to the low-affinity state in the receptor. It was necessary to remove sodium ions from the medium while the osmolarity remained constant; substitution of Na^+ by choline and other quaternary nitrogen cations was not possible because of their effect on agonist binding to muscarinic receptors (Murphy & Sastre, 1983; Hosey, 1983), so sucrose was used instead. As shown in Figure 5, carbamylcholine induces TTX-sensitive uptake of $^{22}Na^+$ into synaptoneurosomes pretreated with pertussis toxin, i.e., where the muscarinic receptor is maintained in the high-affinity state. The effect of carbamylcholine on $^{22}Na^+$ uptake was completely blocked by atropine (10^{-7} M). These experiments were obviously performed in the absence of BTX, since they were aimed to explore $^{22}Na^+$ uptake induced by the muscarinic agonist and not by the toxin, which would induce by itself the open state of the channel. As expected, 1 μM carbamylcholine did not further increase the $^{22}Na^+$ uptake induced by pretreatment with 1 μM BTX (data not shown).

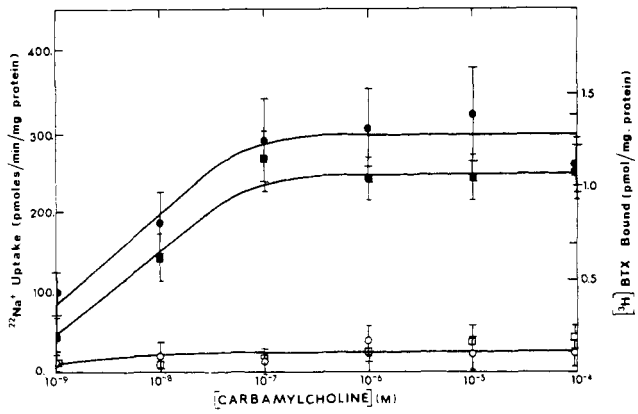

FIGURE 5: TTX-blockable $^{22}Na^+$ uptake (circles) and [^{3}H]BTX binding to pertussis toxin treated synaptosomes (squares) as a function of carbamylcholine concentration in the presence (□, ○) and absence (■, ●) of atropine (10^{-7}–10^{-6} M) under low ionic strength conditions necessary for the uptake experiment. The points are the mean ± SD of three to six separate experiments. Following the addition of, e.g., 10^{-6} M carbamylcholine, typical values obtained for $^{22}Na^+$ uptake after 1 min were as follows (in pmol/mg of protein): control, 642; +1 μM TTX, 625; +carbamylcholine, 1040; +carbamylcholine + TTX, 642; +carbamylcholine + atropine, 604; the latter + TTX, 628.

The dose response (Figure 5) confirmed that the TTX-blockable $^{22}Na^+$ uptake induced by carbamylcholine is characterized by high affinity toward the agonist (IC_{50} = 8 ± 3 nM). It should be pointed out that since the time-dependent increase in $^{22}Na^+$ uptake induced by carbamylcholine is still linear after 1 min (see Materials and Methods), the measured uptake should be proportional to the number of the open Na^+ channels.

The amount of carbamylcholine-mediated Na^+ uptake under our experimental conditions is lower than the values reported previously for neurotoxin-mediated uptake [e.g., Catterall (1975, 1977) and Tamkun and Catterall (1981)]. This could be due to (i) transient opening of Na^+ channels by carbamylcholine, as contrasted with the constant activation of the channels induced by, e.g., BTX, or (ii) different Na^+ concentrations—the amount of uptake is proportional to the Na^+ concentration (Tamkun & Catterall, 1981), which was lower in our experiments as compared to those quoted above. It is also noteworthy that we chose not to employ ouabain in the $^{22}Na^+$-uptake measurements since blockade of the Na^+ pump may significantly alter the physiological state of the synaptosomes, e.g., increase the internal Na^+/K^+ ratio, raise the ATP level, affect the membrane potential, etc.

Figure 5 also depicts the effects of carbamylcholine on [^{3}H]BTX binding to pertussis toxin treated synaptosomes under the low ionic strength conditions employed for the parallel uptake experiment ([^{3}H]BTX binding under normal ionic strength conditions is shown in Figure 4). The very good correlation between $^{22}Na^+$ uptake and the carbamylcholine-induced enhancement in [^{3}H]BTX binding to the Na^+ channel under the low ionic strength conditions employed for the uptake experiment (Figure 5) supports the contention that muscarinic agonists induce opening of Na^+ channels.

Alternatively, it could be argued that carbamylcholine opens a K^+ channel and that this hyperpolarizes the membrane, leading to higher negative membrane potential that increases Na^+ uptake. However, this possibility is contradicted by the following: (i) As discussed below, pertussis toxin treatment eliminates muscarinic agonist mediated opening of K^+ channels. (ii) Measurements of [^{3}H]TPP^+ accumulation (see Materials and Methods) in the absence and presence of 10^{-5} and 10^{-4} M carbamylcholine were 55 000, 49 200, and 55 700 cpm, respectively, clearly indicating that there is no hyperpolarization under our experimental conditions.

Recent electrophysiological studies (Sorota et al., 1985; Pfaffinger et al., 1985; Breitwieser & Szabo, 1985; Sasaki & Sato, 1987; Yatani et al., 1987; Logothetis et al., 1987) have indicated that the coupling of cardiac muscarinic receptors to specific K^+ channels is mediated by a pertussis toxin sensitive G-protein. In these cases guanine nucleotides induced opening of K^+ channels. This type of coupling is a mirror image of the interaction with the Na^+ channels described in the present paper; in the brain stem membrane preparations Gpp(NH)p induces decoupling of muscarinic receptors from the Na^+ channel—as was also observed by us in cardiac membrane preparations (Cohen-Armon & Sokolovsky, 1986). This notion gains further support by the recent observation (Sasaki & Sato, 1987) that pertussis toxin prevented AcCh-induced outward K^+ current but not inward Na^+ current in abdominal ganglion cells from aplysia.

In conclusion, we have shown here that (i) agonist binding to muscarinic receptors is voltage dependent, (ii) this process is mediated by G-protein(s), and (iii) muscarinic agonists induce opening of Na^+ channels. The latter process as well as the effects on [^{3}H]BTX binding persists even after pertussis toxin treatment, indicating that they are not likely to be mediated by pertussis toxin sensitive G-protein. In view of our previous results with brain stem and an atrial *membrane preparation*, the possible interaction of G-protein with the Na^+ channel cannot be discounted. As noted in the text we selected specific experimental conditions for ADP-ribosylation of the synaptoneurosomes. Different conditions as well as other experimental approaches will enable us in the future to elucidate the possible role of G-protein(s) in Na-channel function if it exists as speculated by us previously (Cohen-Armon et al., 1985; Cohen-Armon & Sokolovsky, 1986).

To the best of our knowledge, this is the first time that membrane potential has been shown to play a central role in controlling the affinity state of agonist binding to muscarinic receptors; further research may well reveal that this phenomenon is general and is detectable also in other receptor systems. Moreover, the system with its three interacting components—receptor, Na^+ channel, and G-protein—is such that only at resting potential, when the muscarinic receptors are in a state of high affinity for agonist binding, will they induce opening of Na^+ channels. This opening will lead to membrane depolarization, which in turn will induce conversion of high-affinity agonist binding sites to the low-affinity state, terminate the increased Na^+ permeability, and induce a return to resting potential. We suggest that the capacity of muscarinic agonists, through their binding with high affinity to the muscarinic receptor, to induce sodium currents at resting potential may serve as a depolarization stimulus for autoexcitation or repetitive firing in heart or brain tissues, where the muscarinic receptor is the main cholinergic receptor.

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