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DIFFERENTIAL SENSITIVITY OF PHOSPHOINOSITIDE AND CYCLIC GMP RESPONSES TO SHORT-TERM REGULATION BY A MUSCARINIC AGONIST IN MOUSE NEUROBLASTOMA CELLS

CORRELATION WITH DOWN-REGULATION OF CELL SURFACE RECEPTORS*

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Abstract—Short-term agonist-induced loss of cell surface muscarinic receptors and desensitization of receptor-mediated cyclic GMP (cGMP) formation and phosphoinositide hydrolysis were examined in mouse neuroblastoma cells (clone N1E-115) in suspension. This treatment resulted in a time-dependent reduction of approximately 40% of the specific binding of the hydrophilic antagonist [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) with a $T_{1/2}$ of down-regulation of 4.83 min. Scatchard analysis revealed that brief exposure to the agonist resulted in a significant reduction in the B_{max} with no change in the K_d. Agonist-induced cGMP formation decreased in a similar time-dependent manner with an average $T_{1/2}$ of 4.79 min. However, desensitization of muscarinic receptor-stimulated accumulation of inositol phosphates demonstrated a much slower time-course and was accompanied by a reduction in the maximal response with no change in the EC_{50}. In addition, there was rapid partial recovery of cell surface receptors and desensitized cGMP response, with no apparent resensitization of phosphoinositide hydrolysis. Thus, there was a differential rate of short-term desensitization and resensitization of these two muscarinic receptor-mediated responses. Moreover, desensitization of cGMP formation, but not phosphoinositide hydrolysis, closely paralleled loss of cell surface muscarinic receptors.

Desensitization is a phenomenon whereby tissue responsiveness is decreased as a result of prolonged exposure to agonist. Activation of muscarinic receptors is associated with inhibition of adenylate cyclase, increased cyclic GMP (cGMP)‖ synthesis, and stimulation of phosphoinositide hydrolysis [1]. The sensitivity of muscarinic receptors in triggering these responses is subject to regulation by prior prolonged interaction of agonists with the receptor [1]. It has been theorized that desensitization of some muscarinic receptor-mediated responses in various tissues is due to a loss of cell-surface muscarinic receptors which mediate these events. In fact, when ligand binding to muscarinic receptors was examined utilizing the hydrophilic muscarinic antagonist [^{3}H]*N*-methylscopolamine ([^{3}H]NMS), a loss of binding sites was demonstrated following short-term agonist treatment in several tissues [2, 3].

Mouse neuroblastoma cells (clone N1E-115) have proven to be a good model for studying the regulation of muscarinic receptor density as well as the desensitization of muscarinic receptor-mediated responses. For instance, Richelson [4] demonstrated rapid desensitization of muscarinic receptor-mediated cGMP formation in intact mouse neuroblastoma cells in suspension following short-term agonist treatment. Also, brief incubation of these cells in monolayer with carbamylcholine results in a concentration-dependent down-regulation of [^{3}H]NMS binding sites, with a maximum of about 50% [5, 6]. We have compared the muscarinic receptor subtypes present in control and desensitized cells and reported a selective loss of the agonist low-affinity/pirenzepine high-affinity receptor conformation following short-term agonist treatment [7]. Activation of this specific receptor subtype is associated with stimulation of cGMP synthesis in mouse neuroblastoma cells [8]. While desensitization of muscarinic receptor-mediated phosphoinositide hydrolysis has not been examined in this cell line, Fisher and Snider [9] have shown that the pirenzepine high-affinity receptor subtype is also coupled to inositol lipid turnover in N1E-115 cells. This would

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‖ Abbreviations: cGMP, cyclic GMP; [^{3}H]NMS, [^{3}H]*N*-methylscopolamine; [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate, B_{max}, maximal binding capacity; K_d, equilibrium dissociation constant; EC_{50}, concentration producing a half-maximal response; $T_{1/2}$, half-life; and HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid.

suggest that cGMP formation and hydrolysis of phosphoinositides are both mediated by the M_1 receptor subtype which is sensitive to rapid regulation upon brief exposure to muscarinic agonists. In the present study, the detailed time–course of desensitization and recovery of these two muscarinic receptor-mediated responses was examined in intact mouse neuroblastoma cells and compared with the rate of agonist-induced decrease in cell surface muscarinic receptors determined under similar experimental conditions.

EXPERIMENTAL PROCEDURES

Chemicals. [^{3}H]NMS (87 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and [^{3}H]guanosine (5 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). [^{14}C]cGMP (52 mCi/mmol) and *myo*-[^{14}C]inositol-1-phosphate (55 mCi/mmol) were purchased from Amersham (Arlington Heights, IL), and *myo*-[2-^{3}H]inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St Louis, MO). Penicillin-G potassium, streptomycin sulfate, carbamylcholine chloride and 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) were supplied by the Sigma Chemical Co. (St Louis, MO). All other chemicals were of reagent grade and were supplied by the Sigma Chemical Co.

Cell culture conditions. Mouse neuroblastoma clone N1E-115 cells (passage 11–16) were grown in tissue culture flasks (75 cm^2/250 ml, Corning Glass Works) in 20 ml of Dulbecco's modified Eagle's medium with high glucose (Grand Island Biological Co., Grand Island, NY) containing streptomycin (20 μg/ml) and penicillin (100 units/ml) supplemented with 10% (v/v) newborn calf serum. The cells were incubated at 37° in an atmosphere consisting of 10% CO_2 and 90% humidified air and were grown for 12–20 days before starting the experiments. The cells were subcultured by incubation in a modified Puck's D_1 solution [10] and resuspension in culture medium as described previously [7]. The medium was changed on days 3 and 5 and everyday thereafter by the addition of 10 ml of fresh medium followed by removing 10 ml of medium. A medium change was performed at least 24 hr prior to starting the experiments.

Receptor binding assays. Intact cells were harvested for assay by aspirating the culture medium and incubating with Puck's D_1 solution. The dissociated cells were collected by low-speed centrifugation (250 *g*) for 2 min at 4°. The pellet was washed once with 10 ml of iso-osmotic HEPES physiological salt buffer solution consisting of (mM): NaCl, 110; KCl, 5.3; $CaCl_2$, 1.8; $MgSO_4$, 1.0; glucose, 25; and HEPES, 20 (pH 7.4, adjusted to 335–340 mOsM with sucrose). The pellet was resuspended in HEPES buffer warmed to 37° and aliquots were distributed into 50-ml plastic conical tubes. The cells were equilibrated at 37° for 20 min before being incubated with 1 mM (final concentration) carbamylcholine. Desensitization was stopped by rapidly diluting the cells with ice-cold HEPES buffer before centrifugation. The cells were then washed with 25 ml of buffer and centrifuged again. The washed pellet was resuspended in 5 ml of HEPES buffer, and 100-μl aliquots (~ 1.0 mg protein) were added to each binding tube in a final volume of 1 ml. For saturation experiments, intact cells were incubated with 0.2 to 1.0 nM [^{3}H]NMS in the absence (total binding) or the presence (nonspecific binding) of 2 μM atropine. All incubations were for 120 min at 15 ± 1° to prevent resensitization [5, 11]. The incubation was terminated by filtering the suspension under vacuum through Whatman GF/B glass fiber filters using a cell harvester (Brandel, Inc., Gaithersburg, MD). Unbound radioactivity was washed four times with 5 ml of ice-cold 0.9% (w/v) NaCl solution. Filters were put into scintillation vials, and 4.5 ml of Budget Solve scintillation fluid was added. Radioactivity was determined at least 6 hr later in a Beckman LS-6800 liquid scintillation counter which corrected for the counting efficiency of each individual sample (averaging 45%).

[^{3}H]cGMP measurements. The changes in cGMP formation in intact N1E-115 cells were measured by a method which involves utilizing a radioactively-labeled precursor to label intracellular stores of GTP and chromatographically isolating [^{3}H]cGMP [12]. The cells were harvested for assay by aspirating the culture medium and incubating with Puck's D_1 solution, followed by low-speed centrifugation (250 *g* for 2 min at 4°). The cells were washed once with 10 ml of HEPES buffer and centrifuged again. The cells were then resuspended in 2 ml of buffer, incubated with 10 μCi/ml of [^{3}H]guanosine and rotated at 37° for 50 min at 35 oscillations/min (Precision Scientific shaker bath). For desensitization experiments, the radioactive-labeled cell suspension was diluted with HEPES buffer, and aliquots were placed into 15-ml plastic conical centrifuge tubes. The cells were equilibrated at 37° for 20 min before being exposed to 1 mM carbamylcholine for different time periods. After incubation with the agonist at 37°, the reaction was stopped by the addition of ice-cold HEPES buffer and the cells were sedimented at 250 *g* for 1 min at 4°, washed with 10 ml of ice-cold buffer, and centrifuged again. The cell suspension was diluted with HEPES buffer warmed to 37° and 240-μl aliquots of this suspension were distributed into the wells of a multi-well plate (~ 0.2×10^6 cells/well). Following a 5-min equilibrating period at 37° in the shaker bath, the cells were stimulated with 30 μl carbamylcholine (1 mM final concentration) for 30 sec at 80 oscillations/min. Basal [^{3}H]cGMP levels were determined by adding 30 μl of HEPES buffer. For recovery experiments, the cells were allowed to recover in the wells for various times at 80 oscillations/min prior to stimulation. The reaction was terminated by the addition of 30 μl of 50% (w/v) trichloroacetic acid. To each well, ~ 0.6 nCi of [^{14}C]cGMP was added as an internal standard, and then the contents of the wells were passed through a (0.8 × 8 cm) AG50W-X2 (200–400 mesh) ion-exchange column that had been equilibrated with 0.1 N HCl. Each well was then washed with 0.5 ml of 5% trichloroacetic acid and this rinse was transferred to the columns. The columns were washed with 4.4 ml of 0.1 N HCl, 0.8 ml water (eluates discarded) and then 1.7 ml of water which was collected in plastic microfuge tubes. To the last eluate, 40 μl of both 2.7 M $ZnSO_4$ and 2.7 M Na_2CO_3 were

added to precipitate any residual GDP or GTP [12]. The tubes were then centrifuged in a Beckman microfuge for 2.5 min at 12,000 *g*. The supernatant fraction was transferred to scintillation vials, 10 ml of 3a70B complete counting fluid was added, and the radioactivity was determined by liquid scintillation spectrometry. All samples were corrected for the recovery of [^{14}C]cGMP, which was usually 50–70%. Basal [^{3}H]cGMP levels were subtracted from all carbamylcholine-stimulated values.

Phosphoinositide hydrolysis assays. After detaching from the flasks with D_1 solution and one wash with HEPES buffer, the cells were suspended in 2 ml of HEPES buffer and incubated with 50 μCi of *myo*[2-^{3}H]inositol at 37° for 60 min at 45 oscillations/min. The cell suspension was then centrifuged at 250 *g* for 2 min and the pellet resuspended in HEPES buffer. Aliquots of tissue were placed into 15-ml plastic conical centrifuge tubes and allowed to equilibrate for 15 min before incubation with 1 mM carbamylcholine at 37° for various time periods. Desensitization was stopped by rapidly diluting the cells with ice-cold HEPES buffer followed by centrifugation and one wash. The pellet was resuspended in HEPES buffer containing 11 mM LiCl in a concentration of $\sim 0.5 \times 10^6$ cells/ml and 270-μl aliquots of this suspension were distributed into 12 × 75 mm glass tubes. Following a 5-min adaptation period in a shaker bath, the cells were stimulated with 30 μl of carbamylcholine (1 mM final concentration, unless otherwise noted) for 15 min at 80 oscillations/min at 37°. The release of basal [^{3}H]inositol phosphates was determined by adding 30 μl of HEPES buffer. For recovery experiments, the cells were allowed to resensitize in assay tubes for various time periods at 80 oscillations/min prior to stimulation. The reaction was terminated by the addition of 750 μl of ice-cold chloroform–methanol (1:2, v/v). To each tube, ~ 0.6 nCi of [^{14}C]inositol-1-phosphate (in 250 μl of water) was added as internal standard, followed by 250 μl of chloroform. The samples were vortexed and the phases were separated by centrifugation at 600 *g* for 10 min. A 600-μl aliquot of the aqueous phase of each tube was diluted with 2 ml of water and transferred to a Dowex AG1-X8 column (100–200 mesh, formate form, 0.8 cm × 1.9 cm). The columns were washed with 10 ml of water and 20 ml of 5 mM disodium tetraborate/60 mM ammonium formate. Total [^{3}H]inositol phosphates were eluted with 4 ml of 1 M ammonium formate/0.1 M formic acid and collected into scintillation vials. Sixteen milliliters of 3a70B counting fluid was added to the vials and radioactivity was determined. All samples were corrected for the recovery of [^{14}C]inositol-1-phosphate, which was usually ~ 50%. Basal levels of [^{3}H]inositol phosphate release were subtracted from all carbamylcholine-stimulated values.

Data analysis. Cell numbers were counted using a Coulter Counter, model Z_M (Coulter Electronics). Student's *t*-test was used to determine statistical significance, and proteins were measured according to the method of Lowry *et al.* [13] using bovine serum albumin as standard. Saturation isotherms were analyzed according to the Scatchard transformation [14] using least-squares linear regression analysis and

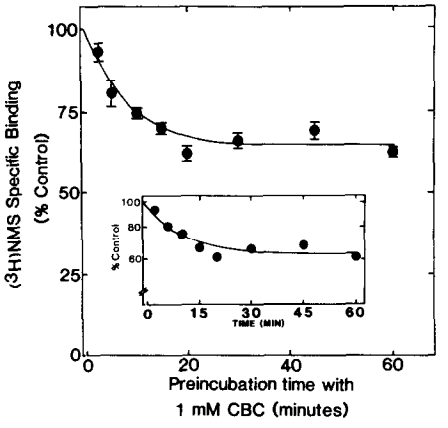

Fig. 1. Time–course of agonist-induced loss of [^{3}H]NMS binding sites. Intact mouse neuroblastoma cells in suspension (passage 14–16, 12–20 days) were incubated with 1 mM carbamylcholine (CBC) for various time periods at 37° at a cell concentration of 0.9 mg protein/ml. The cells were then washed twice and incubated with 0.2 nM [^{3}H]NMS for 120 min at 15°. The assay was terminated by rapid filtration through GF/B filters. Data are expressed relative to control cells which were not preincubated with the agonist and are presented as the means ± SE of five independent experiments, each determined in triplicate. Inset is the semilogarithmic plot of the data. Control specific binding averaged 22.5 fmol/mg protein.

the concentration of the unbound ligand, in order to calculate the values of the maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d). Desensitization data were analyzed according to the following equation:

$$\ln (B_e/(B_e - B_t)) = K \cdot t$$

where B_e and B_t are the magnitudes of desensitization at steady state and at time t, respectively, and K is the rate constant.

RESULTS

Effects of short-term agonist exposure on muscarinic receptor binding. Preincubation of intact mouse neuroblastoma N1E-115 cells in suspension with 1 mM carbamylcholine for various times at 37° resulted in a time-dependent loss of approximately 40% of [^{3}H]NMS binding sites with a steady state occurring about 15 min following treatment with the agonist (Fig. 1). Linear transformation of the data in Fig. 1 showed that the time–course of [^{3}H]NMS down-regulation was monophasic ($r = 0.90$–0.99). The average $T_{1/2}$ for down-regulation of specific [^{3}H]NMS binding was 4.83 ± 0.7 min (mean ± SE, N = 5).

To examine the characteristics of this loss of [^{3}H]NMS binding in desensitized cells, intact N1E-115 cells in suspension were preincubated with or without carbamylcholine for 15 min at 37°, washed free of agonist, and incubated with increasing concentrations of [^{3}H]NMS. Saturation isotherms of specific [^{3}H]NMS binding demonstrated saturability and showed that the ligand labeled significantly less receptors in desensitized cells when compared to the

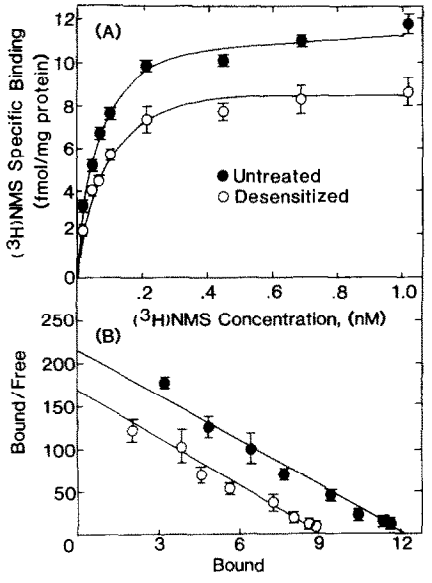

Fig. 2. Effect of preincubation of intact mouse neuroblastoma cells in suspension with carbamylcholine on the saturation isotherms of [^{3}H]NMS. Neuroblastoma cells (passage 16, 12–15 days) were harvested, resuspended in HEPES buffer as detailed in "Experimental Procedures," and incubated with or without 1 mM carbamylcholine for 15 min at 37° (~ 1.0 mg protein/ml). Intact cells were washed and then incubated with increasing concentrations of [^{3}H]NMS in the presence and absence of 2 μM atropine for 120 min at 15°. The data presented were determined in triplicate and are shown as the means ± SE from three independent experiments. (A) Saturation isotherms of [^{3}H]NMS binding in control and desensitized N1E-115 cells; (B) Scatchard plots.

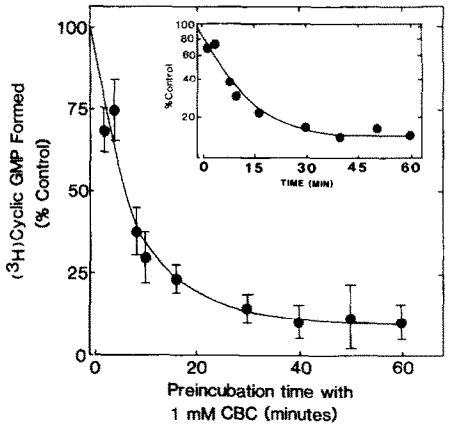

Fig. 3. Time–course of carbamylcholine-induced desensitization of muscarinic receptor-mediated [^{3}H]cGMP formation in intact mouse neuroblastoma cells. N1E-115 cells, following prelabeling with [^{3}H]guanosine, were incubated in suspension (~1.0 mg protein/ml) with or without 1 mM carbamylcholine (CBC) for various times at 37°. Following removal of the agonist, cells were resuspended in buffer, distributed into wells (~0.2 × 10^6 cells/well) and stimulated with 1 mM carbamylcholine for 30 sec. Data are means ± SE (N = 5) relative to control cells which were preincubated in parallel in the absence of the agonist (average absolute response of 99,000 dpm/10^6 cells). Basal levels (19,000 dpm/10^6 cells) were subtracted from all values. Inset is the semilogarithmic transformation of the data.

corresponding control cells (Fig. 2A). The Scatchard plot from these data demonstrated an average B_{max} of 12.1 ± 0.7 fmol/mg protein and a mean K_d value of 58 ± 1.2 pM for the untreated group (Fig. 2B). However, brief exposure to the agonist resulted in a significant reduction ($P < 0.05$) in the B_{max} without a significant change ($P > 0.05$) in the equilibrium dissociation constant of the ligand (Fig. 2B). Analysis of the saturation isotherms for the desensitized group revealed average B_{max} and K_d values of 8.8 ± 0.5 fmol/mg protein and 51 ± 3 pM respectively.

Effects of short-term agonist treatment on muscarinic receptor-mediated responses. Following preincubation of intact mouse neuroblastoma cells in suspension with carbamylcholine, agonist-induced [^{3}H]cGMP formation was diminished in a time dependent manner, reaching a maximal steady state of about 80% reduction of response following 30 min of exposure to the agonist (Fig. 3). When the data in Fig. 3 were transformed linearly, the rate of desensitization of muscarinic receptor-mediated [^{3}H]cGMP synthesis was monophasic (r = 0.90–0.99) and exhibited an average $T_{1/2}$ of 4.79 ± 1.4 min (mean ± SE, N = 5). It should be noted, however, that a small fraction of the cGMP response appeared to be more resistant to desensitization (Fig. 3). The significance of this observation is not clear at present. Under similar conditions, there was also a time-

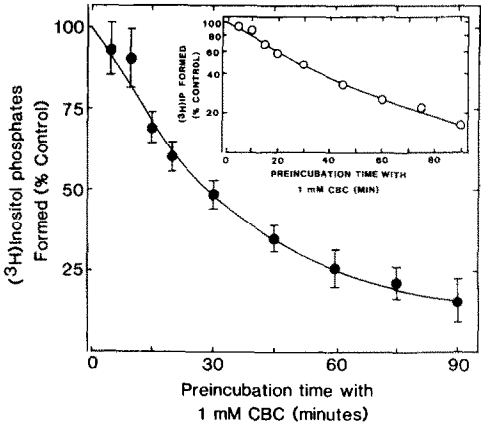

Fig. 4. Time–course of agonist-induced desensitization of muscarinic receptor-mediated accumulation of [^{3}H]inositol phosphate. N1E-115 cells were harvested and labeled with [^{3}H]*myo*-inositol as described under "Experimental Procedures." Intact cells were then resuspended in HEPES buffer (~1.0 mg protein/ml) and incubated with 1 mM carbamylcholine (CBC) for the indicated time points. Following washing and distribution into assay tubes (~0.5 × 10^6 cells/tube), the cells were stimulated with 1 mM carbamylcholine for 15 min at 37°, followed by determination of [^{3}H]inositol phosphate accumulation. Data are expressed as a percentage of control cells which were not pre-exposed to the agonist but otherwise incubated under identical conditions (10,400 dpm/10^6 cells). Basal levels (averaging 4,000 dpm/10^6 cells) were subtracted from all values. Results are means ± SE of nine experiments each determined in triplicate. Inset is the semilogarithmic transformation of the data.

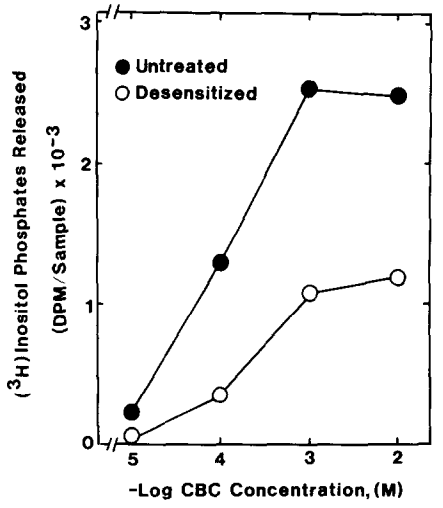

Fig. 5. Dose–response curves of muscarinic agonist-induced accumulation of [^{3}H]inositol phosphates in control and desensitized N1E-115 cells. Intact mouse neuroblastoma cells were prepared for assay as described in "Experimental Procedures" and preincubated with or without 1 mM carbamylcholine for 30 min at 37° at a protein concentration of 1.0 mg/ml. Following washing and distribution into individual tubes (~0.8×10^6 cells/tube), intact cells were stimulated with increasing concentrations of carbamylcholine for 15 min at 37° in the presence of 10 mM LiCl. The data shown represent typical results from one of seven independent experiments. Basal levels were subtracted from all values.

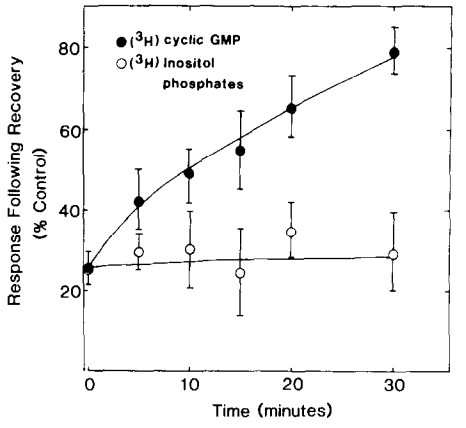

Fig. 6. Time–course of recovery of desensitized muscarinic receptor-mediated [^{3}H]cGMP formation and accumulation of [^{3}H]inositol phosphates. Intact mouse neuroblastoma cells were prelabeled with [^{3}H]guanosine or [^{3}H]*myo*-inositol, resuspended in HEPES buffer and incubated in suspension (~4.0 mg protein/ml) with or without 1 mM carbamylcholine for 30 min at 37°. The cells were then washed twice with cold buffer, distributed into wells (~0.2×10^6 cells/well) or glass tubes (~0.5×10^6 cells/tube) as described under "Experimental Procedures" and allowed to recover at 37° in a shaker bath at 80 oscillations/min. The cells were stimulated with 1 mM carbamylcholine at various time points of recovery. Basal levels were subtracted from all values. Results are expressed relative to control cells which were treated identically except for the absence of preincubation with the agonist (averaging 4,000 and 45,000 dpm/10^6 cells above basal for the phosphoinositide and cGMP responses respectively). Data shown for the recovery of [^{3}H]cGMP formation represent the average of six separate experiments, whereas the results presented for recovery of [^{3}H]inositol phosphate accumulation are the average of three independent experiments; the bars represent ± SE.

dependent decrease in muscarinic receptor-stimulated accumulation of [^{3}H]inositol phosphates (Fig. 4). Transforming the data linearly according to a monophasic model yielded an average $T_{1/2}$ of desensitization of 29.9 ± 4.8 min (mean ± SE, N = 9). Attempts were also made to analyze the data nonlinearly. However, only three of nine experiments could be analyzed by a biphasic model with average $T_{1/2}$ values for desensitization of 20.1 ± 11.3 min (63.2 ± 1.1% of total) and 62.6 ± 16.9 min (mean ± SE, N = 3).

We also investigated if the subsensitivity of muscarinic receptors to mediate phosphoinositide hydrolysis was due to a change in the maximal response or the agonist potency. Upon preincubation of intact mouse neuroblastoma cells with 1 mM carbamylcholine for 30 min at 37°, there was a reduction in the maximal response in desensitized cells when compared to the corresponding control group (Fig. 5). The basal release of [^{3}H]inositol phosphate was not affected by preincubation with the agonist. The EC_{50} (concentration producing a half-maximal response) for carbamylcholine-induced accumulation of [^{3}H]inositol phosphates was similar in control (92 ± 12 μM, mean ± SE, N = 7) and desensitized cells (124 ± 51 μM, N = 6). This suggests that desensitization of phosphoinositide hydrolysis following short-term incubation with carbamylcholine is a result of a decrease in the maximal response with no change in the agonist potency.

Recovery of muscarinic receptor-mediated responses following short-term desensitization. Experiments were performed to investigate the recovery of muscarinic receptor-stimulated [^{3}H]cGMP formation and accumulation of [^{3}H]inositol phosphates. Intact N1E-115 cells in suspension were incubated with 1 mM carbamylcholine for 30 min at 37° and the recovery of these two responses was examined at 37° following removal of the agonist. There was a time-dependent resensitization of the muscarinic receptor-mediated [^{3}H]cGMP response, reaching 80% of control values following a 30-min recovery period (Fig. 6). However, the muscarinic receptor-stimulated accumulation of [^{3}H]inositol phosphates showed little, if any, recovery under similar experimental conditions (Fig. 6).

DISCUSSION

The present results demonstrated a time-dependent down-regulation of specific [^{3}H]NMS binding following short-term desensitization in intact mouse neuroblastoma N1E-115 cells in suspension. Moreover, desensitization of the muscarinic receptor-mediated cGMP response followed a similar time course as the loss of cell surface muscarinic receptors,

whereas a considerably slower time course was observed for the desensitization of muscarinic receptor-stimulated phosphoinositide hydrolysis in N1E-115 cells. Following removal of the desensitizing agonist, there was a rapid partial recovery of cell surface muscarinic receptors and the cGMP response without significant resensitization of phosphoinositide hydrolysis.

Pretreatment of mouse neuroblastoma cells in suspension with the muscarinic agonist carbamylcholine at 37° resulted in a rapid and time-dependent loss of approximately 40% of specific [^{3}H]NMS binding sites with a $T_{1/2}$ of 4.83 min (Fig. 1). Scatchard analysis of specific [^{3}H]NMS binding revealed that down-regulation of cell-surface muscarinic receptors was the result of a decrease in maximal receptor density without a change in affinity upon desensitization (Fig. 2). A loss of [^{3}H]NMS binding sites has been noted in several cell culture systems following short-term treatment with agonists [2, 3, 5, 7]. It has been suggested that short-term preincubation with muscarinic agonists leads to a rapid sequestration of muscarinic receptors which are no longer accessible to the hydrophilic ligand [^{3}H]NMS, and perhaps to the positively-charged muscarinic agonists [5]. In fact, Harden and his group have demonstrated the ability of several cholinergic agonists in induce the appearance of an internalized muscarinic receptor species in human astrocytoma cells [2]. Previous work from our laboratory has shown that short-term incubation of mouse neuroblastoma cells *in monolayer* with carbamylcholine for 30 min also results in a rapid reduction of cell-surface muscarinic receptors to about 50% of control, although a detailed time–course of this phenomenon was not studied [5, 7]. By utilizing similar experimental protocols, it is demonstrated in the present work that desensitization of agonist-induced cGMP synthesis follows an analogous time–course as the disappearance of cell surface muscarinic receptors in mouse neuroblastoma cells (Fig. 3). We have shown previously that the partial reduction of [^{3}H]NMS binding sites upon short-term desensitization could be accounted for by a selective loss of the agonist low-affinity/pirenzepine high-affinity muscarinic receptor conformation in mouse neuroblastoma cells [7]; this specific conformation is associated with the induction of cGMP synthesis [15]. This offers an explanation for the similar rates of agonist-induced desensitization of the cGMP response and down-regulation of cell-surface receptors observed in the present study.

Desensitization of agonist-induced phosphoinositide hydrolysis was clearly much slower than down-regulation of [^{3}H]NMS binding sites or desensitization of the muscarinic receptor-stimulated cGMP response (Fig. 4). This is an interesting finding considering the fact that recent studies by Fisher and Snider [9] have linked phosphoinositide turnover to the M_1 receptor subtype in mouse neuroblastoma N1E-115 cells, the same receptor subtype which mediates the cGMP response. Our present work represents the first attempt to examine short-term desensitization of muscarinic receptor-mediated accumulation of inositol phosphates in N1E-115 cells upon preincubation with a muscarinic agonist, although Cohen *et al.* [16] have demonstrated a decreased rate of ^{32}P incorporation into phosphatidylinositol during continuous short-term cholinergic stimulation in these cells. Reports from several laboratories have suggested that muscarinic receptor-mediated phosphoinositide hydrolysis does not desensitize rapidly. For instance, short-term pretreatment of human astrocytoma cells with carbamylcholine does not desensitize the muscarinic receptor-mediated phosphoinositide response [17]. It is only upon prolonged treatment with the agonist that this response begins to diminish in parallel with receptor degradation [17]. In addition, rapid desensitization of the response to other receptor agonists such as histamine [18] and substance P [19] has been noted without a concomitant carbamylcholine-induced loss in muscarinic receptor-stimulated accumulation of inositol phosphates. Recently, Xu and Chuang [20] demonstrated fast and slow components of desensitization of muscarinic receptor-mediated phosphoinositide hydrolysis in cerebellar granule cells. When we attempted to fit our own data to a biphasic model of desensitization, the half-life of 20.1 min for the fast component of desensitization was still considerably longer than the time course for both desensitization of the cGMP response ($T_{1/2}$ = 4.8 min) and down-regulation of cell surface receptors ($T_{1/2}$ = 4.8 min). If release of Ca^{2+} from intracellular stores plays a role in the induction of cGMP synthesis, it is probable that there is a faster desensitization of the Ca^{2+} signal as compared to the phosphoinositide response. Masters *et al.* [17] have provided evidence for such differential desensitization in human astrocytoma cells.

Muscarinic receptor-mediated inositol lipid hydrolysis continues at a time when the rapidly-regulated population of cell surface receptors is already down-regulated to a steady state. Although this fact could argue for the existence of spare receptors for phosphoinositide hydrolysis in N1E-115 cells, Fisher and Snider [9] have demonstrated the lack of receptor reserve for muscarinic receptor-stimulated phosphoinositide turnover in this neuronal clone. In support of this finding, the present studies illustrate that desensitization of inositol lipid hydrolysis is the result of a decreased maximal response with no change in the EC_{50} for the agonist (Fig. 5). In addition, internalized muscarinic receptors may still be capable of coupling to phospholipase C. However, it has been demonstrated in other tissues that complete blockade of cell surface muscarinic receptors totally abolishes the phosphoinositide signal [21]. Another possible explanation is that phosphoinositide hydrolysis in mouse neuroblastoma N1E-115 cells is mediated by more than one muscarinic receptor subtype. A strong argument sustaining this proposal is that a major portion of the phosphoinositide response remains intact under desensitizing conditions which are known to abolish the pirenzepine high-affinity binding sites in these cells [7]. In support of this notion, data from chick heart and 1321N1 astrocytoma cells [22] as well as neostriatum [23] revealed that the muscarinic receptor binding conformation that regulates phosphoinositide hydrolysis in these tissues exhibits low-affinity for pirenzepine. Furthermore, both the M_1 and M_2

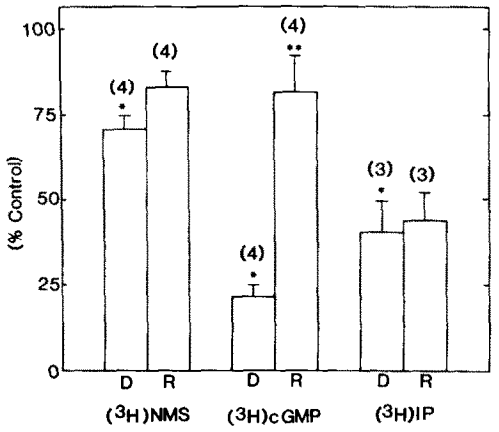

Fig. 7. Magnitudes of recovery of carbamylcholine-induced receptor down-regulation and desensitization of receptor-mediated responses. Following the preparation of cells for the different assays, cells (~4 mg protein/ml) were incubated with or without 1 mM carbamylcholine for 30 min at 37°. [^{3}H]NMS specific binding and the formation of cGMP and inositol phosphates (IP) were determined in desensitized cells (D) and in cells which were allowed to recover for 45 min at 37° (R). Data are expressed as the mean percentage ± SEM relative to control cells which were treated identically except for preincubation with the agonist. Stimulated phosphoinositide and cGMP responses in control cells were 5,884 and 117,290 dpm/10^6 cells above basal, respectively, while the corresponding values in cells that were not desensitized but incubated in parallel with desensitized cells during the recovery step were 6,006 and 105,810 dpm/10^6 cells. Specific [^{3}H]NMS binding values in these two groups of controls were 22.4 and 17.3 fmol/mg protein respectively. The number of independent experiments, each performed in triplicate or quadruplicate, is indicated in parentheses. Key: (*) significantly different from control, $P < 0.05$; and (**) significantly different from corresponding desensitized cells, $P < 0.05$.

receptor subtypes have been found to be linked to phosphoinositide hydrolysis in rat brain [24]. More recently, it has been shown that at least two of the four cloned muscarinic receptor subtypes can couple to phospholipase C when expressed in mammalian cells [25]. Therefore, it is possible that the slow time-course of desensitization of the phosphoinositide response (Fig. 4) is a result of different rates of desensitization of the phosphoinositide pools coupled to multiple subtypes of muscarinic receptors.

Our results indicate a rapid, time-dependent resensitization of the desensitized cGMP response in intact mouse neuroblastoma cells to 80% of control levels following a 30-min recovery period (Fig. 6). This recovery of the cGMP response correlates well with reappearance of cell surface receptors (Fig. 7). More interestingly, there is little, if any, recovery of the muscarinic receptor-mediated phosphoinositide response at a time when the cGMP response is almost fully recovered (Fig. 6). Although there is limited information regarding resensitization of desensitized phosphoinositide hydrolysis, it was reported recently that a lengthy period (2 days) is required for the recovery of this muscarinic response following short-term desensitization in astrocyte cultures [26]. A similar time-course was also noted for the recovery of alpha$_1$-stimulated phosphoinositide hydrolysis following short-term agonist treatment [27]. The specific mechanisms underlying the inability of the desensitized phosphoinositide hydrolysis to recover following desensitization are presently unknown. One possibility is that refractoriness of phosphoinositide turnover may involve a more permanent post-receptor mechanism of desensitization such as a change in the coupling of the muscarinic receptor to a guanine nucleotide binding protein. Another possible explanation is that the first exposure to the agonist depletes the radioactively labeled polyphosphoinositide pool which is coupled to muscarinic receptor activation. However, preliminary data showed that very little recovery takes place after 90 min when the phosphoinositide response is measured using a continuous labeling technique (i.e. by adding [^{3}H]*myo*-inositol to washed desensitized cells followed by adding the stimulating agonist in the continuous presence of the labeled precursor; C. L. Cioffi, unpublished data). In addition, a very slow recovery of desensitized muscarinic-mediated phosphoinositide hydrolysis has been reported even when labeling of polyphosphoinositides was performed after removal of the desensitizing agonist [26].

In summary, we have observed a differential rate of short-term desensitization of the muscarinic receptor-stimulated cGMP and phosphoinositide responses in N1E-115 cells. Only desensitization of the former response paralleled the decrease in the density of cell surface muscarinic receptors. More importantly, the number of cell surface receptors and desensitized cGMP formation partially recover at a time where there is no significant recovery of the desensitized phosphoinositide response under the same experimental conditions. These findings are important in terms of our understanding of muscarinic receptors–effector coupling mechanisms in neuronal tissue.

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