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DESENSITIZATION OF MUSCARINIC M_1 RECEPTORS OF MURINE NEUROBLASTOMA CELLS (CLONE N1E-115) WITHOUT RECEPTOR DOWN-REGULATION AND PROTEIN KINASE C ACTIVITY

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Abstract—Acute desensitization of M_1 muscarinic receptor-mediated responses (cyclic GMP formation and inositol phosphate release) was studied in murine neuroblastoma cells (N1E-115 clone). After a 45-min incubation at 37° of N1E-115 cells either in monolayer or in suspension, with the muscarinic agonist carbachol (1 mM), the receptor-mediated cyclic GMP response to carbachol was nearly completely lost. This loss was associated with >80% loss of carbachol-mediated inositol phosphate release. The protein kinase C activator phorbol 12-myristate 13-acetate (PMA) inhibited both responses with similar potencies. Carbachol or PMA reduced by 30–40% the number of muscarinic receptor sites for antagonist and agonist on intact cells (determined in binding assays using [^{3}H]*N*-methylscopolamine) only for cells in monolayer and not for those in suspension. PMA but not carbachol pretreatment of cells in monolayer or in suspension caused a translocation of [^{3}H]phorbol 12,13-dibutyrate binding and protein kinase C activity. In addition, desensitization to carbachol occurred in cells largely depleted of protein kinase C by chronic exposure to PMA. Thus, agonist-mediated down-regulation is not needed for muscarinic M_1 receptor desensitization, which may be a result of the activation of a receptor-activated kinase different from protein kinase C.

Desensitization is defined as the loss of tissue sensitivity to an agonist after prolonged exposure to the agonist (tachyphylaxis). Despite years of research, the mechanism (which likely varies with the receptor–effector type) has not been elucidated fully [1–4]. Binding of agonist to the receptor is needed for this phenomenon to occur since antagonists, or agonists in the presence of an antagonist, do not cause desensitization. One hypothesis for the molecular basis of desensitization is rapid receptor down-regulation, a phenomenon that occurs with many types of receptors after prolonged exposure of cells to agonists.

We have studied receptor desensitization using a murine neuroblastoma clone (N1E-115) as a model system. The focus of this work is the muscarinic and other receptors that mediate cyclic GMP formation in a calcium-dependent manner. Clone N1E-115 cells have two distinct pharmacological subclasses of muscarinic receptors [5, 6]. The M_2 receptor displays high affinity for agonist and inhibits receptor-mediated cyclic AMP formation, whereas the M_1 receptor, with a low affinity for agonist, mediates cyclic GMP synthesis and the release of inositol phosphates and arachidonic acid. Both sites can be directly identified with the use of intact cells in competition binding assays between agonist and [^{3}H]*N*-methylscopolamine ([^{3}H]NMS)‡‡ [5, 6], a muscarinic antagonist that is relatively impermeable to cells.

Desensitization of M_1 muscarinic receptors of clone N1E-115 cells in suspension is a rapid process that depends on time, concentration of agonist, and temperature [3, 4]. It is clearly independent of the formation of second messengers (for example, cyclic GMP), since desensitization occurs under conditions which either do not permit or markedly reduce the formation of these messengers (for example, absence of extracellular Ca^{2+}). Although desensitization is complete after several minutes in the presence of high concentrations of carbachol, no change in receptor number is measurable with the use of the lipophilic radioligand [^{3}H]quinuclidinyl benzilate [3]. It is with [^{3}H]NMS, which binds to cell-surface receptors, that a clear loss in binding sites is seen.

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‡‡ Abbreviations: NMS, *N*-methylscopolamine; diC_8, *sn*-1,2-dioctanoylglycerol; EC_{50}, concentration causing 50% of maximal response; IC_{50} concentration causing 50% inhibition; IP, inositol phosphate; K_d, equilibrium dissociation constant; K_i, inhibition constant; 4α-PD, 4α-phorbol 12,13-didecanoate; PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; SNP, sodium nitroprusside; EGTA, ethyleneglycolbis (aminoethylether)tetraacetate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and DTT, dithiothreitol.

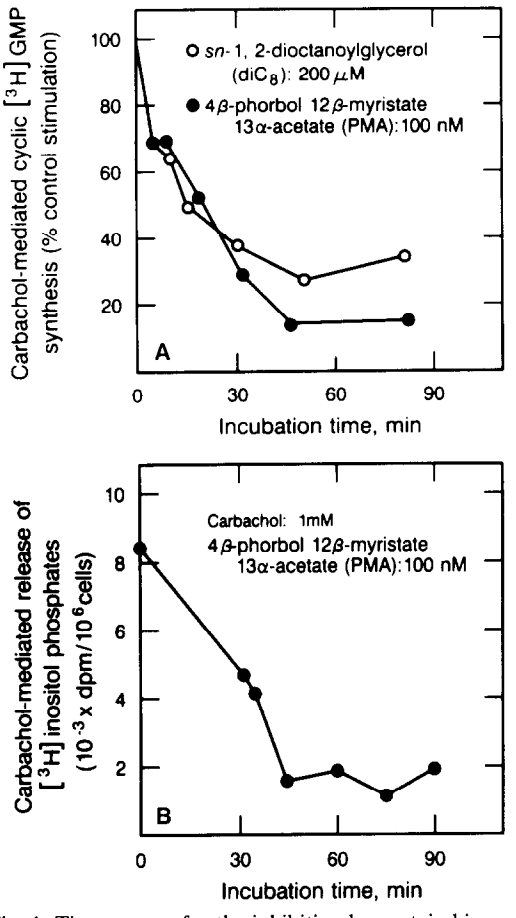

Fig. 1. Time–course for the inhibition by protein kinase C activators of carbachol-mediated responses in clone N1E-115 cells in suspension. (A) Carbachol-mediated cyclic GMP synthesis. The concentration of diC_8 (○) was 200 μM and that of PMA (●) 100 nM. Cells were subculture 20, 13 days after subculture. There were about 100,000 cells/well. At t = 0, basal release was 1.9×10^4 dpm/10^6 cells, and stimulated release was 9.7×10^4 dpm/10^6 cells. (B) Carbachol-mediated IP release. The concentration of PMA was 100 nM. Cells were subculture 15, 15 days after subculture. There were about 80,000 cells/well. Basal release averaged 0.99×10^4 dpm/10^6 cells, and stimulated release averaged 1.8×10^4 dpm/10^6 cells. In panels A and B, cells were stimulated with 1 mM carbachol for 30 sec and 30 min, respectively, after various times of preincubation with agents. Results presented are representative of three independent experiments.

Liles *et al.* [7] reported that preincubation of N1E-115 cells in monolayer with carbachol induces rapid receptor down-regulation and simultaneous translocation of protein kinase C. The protein kinase C activator phorbol 12-myristate 13-acetate (PMA) also causes these effects on the receptor and kinase within the same time frame as agonist-mediated desensitization. These interesting data suggested that receptor internalization is caused by the activation of protein kinase C. This finding is supported by a large body of evidence showing that stimulation of the M_1 muscarinic receptor increases phosphatidylinositol hydrolysis by activation of phospholipase C. This results in the formation of diacylglycerol and inositol triphosphate. Inositol triphosphate mobilizes Ca^{2+} from intracellular storage sites, and this ion, together with diacylglycerol, activates protein kinase C with concomitant translocation of the enzyme from the cytosol to the membrane [8–10]. In addition, in many different tissues, protein kinase C is reported to play an important role in negative feedback functions for those receptors which when stimulated release inositol phosphates [10].

In a preliminary study, we found that PMA inhibits muscarinic M_1 receptor-mediated cyclic GMP formation and inositol phosphate release in cells treated in suspension [11]. However, we did not detect rapid, carbachol-induced, receptor down-regulation when cells were exposed for many minutes to carbachol. In contrast, Liles *et al.* [7] and others [12] have demonstrated before such receptor down-regulation in this same clone. In checking this apparent contradiction, we found that there was a difference in technique: we used cells in suspension, whereas these other workers [7, 12] used cells in monolayer. So, we studied the effects of carbachol and phorbol esters on muscarinic receptor desensitization and down-regulation in cells under both conditions. In our experiments with detached cells, these agents caused desensitization without altering binding. This suggests that desensitization occurs at a site distal to the receptor.

MATERIALS AND METHODS

Materials. Reagents and materials were bought from the following sources: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum from the Grand Island Biological Co. (Grand Island, NY); [^{3}H]*N*-methylscopolamine (88.4 Ci/mmol), *myo*-[^{3}H]inositol (15 Ci/mmol) and L-*myo*-[^{14}C]inositol 1-phosphate (55 mCi/mmol) from Amersham (Arlington Heights, IL); [^{3}H]guanosine (5 Ci/mmol) from ICN Radiochemicals (Irvine, CA); [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu, 10.2 Ci/mmol) and [γ-^{32}P]ATP from New England Nuclear Research Products (Boston, MA); 3-isobutyl-1-methylxanthine from the Aldrich Chemical Co. (Milwaukee, WI); aprotonin, bovine albumin (product No. A 7906), carbamylcholine chloride, diolein, γ-globulin, histone III-S, leupeptin, lithium chloride, Nonidet P40, *sn*-1,2-dioctanoylglycerol (diC_8), 4α-phorbol 12,13-didecanoate (4α-PD), PDBu, phosphatidylserine, PMA, polyethyleneglycol (mol. wt ≈ 8000), polyethyleneimine, and sodium nitroprusside (SNP) from the Sigma Chemical Co. (St. Louis, MO); Dowex AG 1-X8 (100–200 mesh) from Bio-Rad Laboratories. (Richmond, CA); DEAE Sephacel columns from Pharmacia, Inc. (Piscataway, NJ); Whatman GF/B filters and P-81 phosphocellulose paper from Whatman, Inc. (Clifton, NJ); and Safety-Solve from Research Products International Corp. (Mt. Prospect, IL).

Cell culture. Clone N1E-115 cells were grown as described before [13] in DMEM with 10% (v/v) fetal bovine serum (Medium I) as monolayers in flasks (75 cm^2, Corning Glass, Corning, NY). Confluent cells (days 10 to 21 after subculture) were harvested

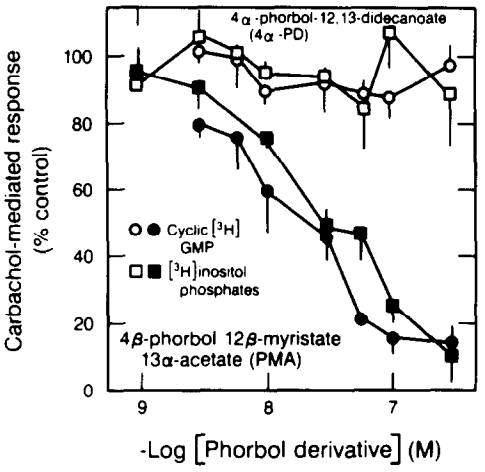

Fig. 2. Concentration dependence of the effects of phorbol esters on carbachol-mediated responses in clone N1E-115 cells in suspension. 4α-PD (○,□) and PMA (●,■) were incubated with cells in suspension at the concentrations shown for 45 min. Cells were then stimulated by carbachol 1 mM for 30 sec and 30 min for measurement of cyclic GMP synthesis (circles) and IP release (squares) respectively. The inactive phorbol, 4α-PD, lacked significant effects on carbachol-mediated responses. Results are means ± SE from three independent experiments.

by incubation in modified Puck's D_1 solution, disruption of the monolayer by agitation of the flask, and collection of the cells by low speed centrifugation. Cells were washed in an iso-osmolar phosphate-buffered saline (solution I) containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM Na_2HPO_4, 25 mM glucose and 75 mM sucrose with pH adjusted to 7.4 and osmolality adjusted to 335 ± 5 mOsmol with sucrose. All experiments were performed using early subcultures (passages 9 to 19).

Assay of cyclic GMP formation in intact cells. Harvested cells were resuspended in 2.0 mL of solution I at 37° (3–6 × 10^6 cells/mL) and transferred to a 50-mL Erlenmeyer flask to which 20 μL (20 μCi) of [^{3}H]guanosine was added. Cells were placed in a water bath at 37° and incubated for 45 min after which time the excess unincorporated [^{3}H]guanosine was removed by low speed centrifugation and aspiration of the supernatant fraction. The pelleted cells were resuspended in solution I to a density of 3–4 × 10^5 cells/mL and distributed into the wells of a multiwell plate in 240-μL aliquots. Thirty microliters of PMA or diC_8 (at the concentrations shown) was added, and cells were incubated for 30 min (except where noted) before the addition of the stimulatory agents (30 μL) for the times shown. The assay was stopped with the addition of 30 μL of 50% trichloroacetic acid. The synthesized cyclic [^{3}H]GMP was isolated using ion exchange chromatography as described before [13]. For the desensitization experiments, cells were incubated with carbachol at 1 mM for 45 min in a flask at 37°, collected by low speed centrifugation, and washed three times with solution I at 15° to prevent resensitization. Before the addition of the stimulatory agents, the cells were warmed to 37° for 10 min.

Assay of the release of [^{3}H]*IPs.* Cells harvested as described above were resuspended in 2.0 mL of solution I at 37° and transferred to a 50-mL Erlenmeyer flask to which 50 μL (50 μCi) of [^{3}H]*myo*-inositol was added. This suspension was then incubated for 60 min at 37° in a shaking water bath. At the end of this incubation period, the cells were centrifuged, and the supernatant fraction was aspirated to remove excess radiolabel. The cells were then resuspended in solution I to give about 3–4 × 10^5 cells/mL and distributed into glass tubes in 210-μL aliquots. Thirty microliters of 100 mM lithium chloride was added, and cells were incubated further for 45 min (except the desensitization experiments in which LiCl was added just before the addition of agonist). Thirty microliters of PMA or diC_8 (at the concentrations shown) was added, and cells were incubated for 45 min before the addition of the stimulatory agents (in 30 μL). The reaction was stopped with the addition of 750 μL of chloroform/methanol (1:2). Each tube was then vortex-mixed and placed on ice. Two phases were formed by the addition of 250 μL of chloroform and 250 μL of an aqueous solution containing L-*myo*[^{14}C]inositol 1-phosphate as an internal standard. After further mixing, the tubes were centrifuged at about 400 *g* for 5 min. A portion (600 μL) of the upper phase was transferred to a polystyrene tube that contained 2 mL of water. This solution was then applied to a Dowex AG 1-X8 column (100–200 mesh), and the inositol phosphates were eluted as described elsewhere [14]. The methods for the desensitization experiments were as described above for the cyclic GMP assays.

Binding assays. [^{3}H]NMS binding was performed by procedures given in previous publications [5, 6]. The binding parameters for NMS and carbachol in competition with [^{3}H]NMS were determined as described, using intact N1E-115 cells at 15°. This temperature is needed to prevent receptor resensitization. The binding curves were analyzed by an iterative nonlinear computer method as described [5]; this method allows the determination of the equilibrium dissociation constants and capacities for the multiple muscarinic receptor subtypes to which carbachol binds in N1E-115 cells. [^{3}H]PDBu binding to intact cells was performed with cells diluted with solution I, containing 0.4% (w/v) bovine serum albumin (BSA) to give 2–3 × 10^5 cells/400 μL. To the 400-μL cell suspension in a polypropylene tube was added 50 μL of unlabeled PDBu or PMA. The reaction was started by adding 50 μL of 30 nM [^{3}H]PDBu and incubated for 15 min at 37°. The incubation mixture was diluted with 3 mL of cold isotonic saline solution (0.9%, w/v) and filtered through glass fiber filters (Whatman GF/B) pre-soaked in a 0.2% (w/v) polyethylenimine solution for more than 1 hr just before use. Each tube was washed three times with 5 mL of cold isotonic saline solution. The radioactivity on the filters was measured using liquid scintillation spectrometry. Nonspecific binding was defined as the binding in the presence of 3 μM PDBu (10–15% of the total binding). Data were analyzed by the program LIGAND [15]. For [^{3}H]PDBu binding to membranal and cytosolic fractions, cells (4 × 10^6 cells/mL) in homogenization buffer (20 mM Tris–HCl, pH 7.5, at 37°, 2 mM EDTA, 0.5 mM

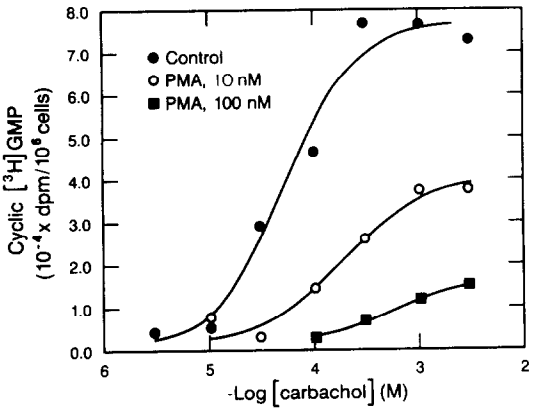

Fig. 3. Effects of PMA on the concentration–response curve for carbachol-mediated cyclic GMP formation by clone N1E-115 cells in suspension. PMA, at 10 nM (○) and 100 nM (■), was incubated with cells in suspension for 45 min before the stimulation of cells with carbachol for 30 sec at the concentrations shown. In this experiment, the EC_{50} and the maximum response for the control (●) was 5 μM and 7.8×10^4 dpm/10^6 cells respectively. Basal synthesis averaged 1.8×10^4 dpm/10^6 cells. The EC_{50} values shifted to 200 and 470 μM and the maximum responses were lowered to 4.1 and 1.6×10^4 dpm/10^4 cells, respectively, in the presence of 10 and 100 nM PMA, respectively. Cells were subculture 15, 12 days after subculture. There were about 100,000 cells/well. Similar results were obtained in four independent experiments.

EGTA, 0.33 M sucrose, 100 μg/mL leupeptin) were disrupted on ice with 30 strokes in a Dounce homogenizer. After the homogenates were centrifuged at 100,000 *g* for 35 min at 4°, the small phospholipid layer was discarded, the cytosol was removed and diluted 1 to 5 with buffer (20 mM Tris–HCl, 12.5 mM $MgCl_2$, 75 mM NaCl, 4.375 mM $CaCl_2$), and the membranal fraction was diluted to 0.8 to 1.4 mg protein/mL with the assay buffer (20 mM Tris–HCl, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 60 mM NaCl, 66 mM sucrose) followed by homogenization (15 strokes in a Dounce tissue homogenizer). Binding was determined in duplicate in a total volume of 0.5 mL and was started by the addition of 0.25 mL homogenate to tubes containing 10 nM [^{3}H]PDBu prepared in 4% (w/v) bovine albumin and non-radioactively labeled PDBu ranging from 1 to 300 nM. The cytosolic assay buffer contained 100 μg/mL phosphatidylserine for optimal binding. Nonspecific binding was determined in the presence of 3 μM unlabeled PDBu. The homogenates were incubated for 30 min at 37° in 12×75 mm^2 polypropylene tubes. Separation of bound from free [^{3}H]PDBu in the membranal fraction was done by the addition of 4 mL of ice-cold 20 mM Tris–HCl to each tube followed by three successive 4-mL filtrations through a Whatman GF/B filter presoaked in 0.2% (w/v) polyethyleneimine. For the cytosolic fraction, each sample was placed on ice, and 200 μL γ-globulin (12 mg/mL) and 500 μL polyethyleneglycol (15%, w/v) were added to each tube to precipitate the bound [^{3}H]PDBu. After a 15-min incubation, each sample was filtered through a Whatman GF/B filter presoaked with 0.2% (w/v) polyethyleneimine on a Brandel apparatus. Then each test tube was washed quickly four times with 4 mL of 20 mM Tris–HCl containing 8.5% (w/v) polyethyleneglycol and each time the wash solution was passed through the filter. Before being analyzed for radioactivity, all filters stood for 5 hr in scintillation vials containing 14 mL Safety Solve to make soluble the radioactivity on the filters. Nonspecific binding for the cytosolic and membranal fractions averaged 28 and 17% of total binding respectively. Data were fitted as described above with the use of LIGAND [15].

Assay for protein kinase C activity. The isolation of protein kinase C was performed as described by Liles *et al.* [7]. Growth medium was removed from confluent cells in monolayer, and attached cells were washed with 10 mL of solution I. To 20 mL of fresh solution I in the flask was added phorbol ester (at the concentrations shown) in 200 μL of additional solution I, and cells were incubated for the specified times at 37°. After incubation with the phorbol ester or carbachol, cells were harvested either with the use of D_1 as described above or by scraping the flask with a Teflon coated scraper (Costar, Cambridge, MA). The latter method proved much faster, though no clear difference in enzyme activity was seen. Cells were then centrifuged at about 200 *g* at 4° for 1.5 min, and the supernatant fraction was removed. Cells were then washed once with ice-cold solution II (20 mM HEPES, 10 mM EGTA, 2 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, pH 7.5) [7], centrifuged for 2 min, and resuspended in 2.0 mL of ice-cold solution II (with or without 100 μg/mL leupeptin and 10 units/mL aprotonin). In several experiments, we scraped cells in isosmotic solution I containing 100 μg/mL leupeptin and collected cells by centrifugation for 2 min at 2°. Then cells were lysed with 2×15 sec bursts using an Ultrasonic Cell Disrupter (Kontes Glass Co., Vineland, NJ), and the sonicate was transferred to ultracentrifuge tubes and centrifuged at 100,000 *g* for 60 min at 4°. The supernatant or cytosolic fraction was applied to DEAE Sephacel columns, 0.6 mL bed volume, previously equilibrated in solution III (20 mM HEPES, 2 mM EGTA, 2 mM EDTA and 2 mM DTT, pH 7.5). The columns were washed with 5 mL of solution III which was discarded, and the cytosolic enzyme eluted with solution III containing 100 mM NaCl. The pellet from the first ultracentrifugation was sonicated briefly in solution III containing 1% Nonidet P40 and incubated for 60 min at 4° before centrifugation for 60 min at 4°. The supernatant fraction from the second centrifugation, consisting of soluble membrane fraction, was applied to similar columns and the enzyme eluted as described above.

The assay for protein kinase C activity in cytosolic and membrane fractions (Liles *et al.* [7]) involved ^{32}P incorporation into histone III-S substrate in the presence of Ca^{2+}, phosphatidylserine and diolein. To summarize, 15 μL of isolated enzyme preparation was added to a 1.5-mL conical plastic centrifuge tube containing 35 μL of a solution composed of 10 mM $MgCl_2$, 0.6 mM EDTA, 0.6 mM EGTA, 30 mM NaCl, 5.6 mM DTT, 50 μM ATP, 1 mg/mL histone III-S, 1.5 mM $CaCl_2$, 60 μg/mL phosphatidylserine, 6 μg/mL diolein and 0.5 μCi [γ-^{32}P]ATP. For measurement of basal enzyme activity, the phos-

Table 1. Binding of [^{3}H]NMS to intact clone N1E-115 cells after treatment in suspension or in monolayer with PMA or carbachol

	K_d (nM)	B_{max} (fmol/10^6 cells)
In suspension		
Control	0.17 ± 0.02	133 ± 18 (100%)
PMA (100 nM, 45 min)	0.20 ± 0.02*	142 ± 18* (107%)
Carbachol (1 mM, 45 min)	0.33 ± 0.12*	149 ± 33* (112%)
In monolayer		
Control	0.47 ± 0.17	134 ± 6 (100%)
PMA	0.42 ± 0.07*	97 ± 6† (72%)
Carbachol	0.30 ± 0.04*	78 ± 15† (58%)

Each value is the mean ± SE from at least three independent experiments. Numbers in parentheses indicate the percent of the control value.

* Not significantly different from control ($P > 0.05$) by one-way analysis of variance.

† Significantly different from control ($P < 0.01$) by one-way analysis of variance.

Table 2. Binding of carbachol to intact clone N1E-115 cells after treatment in suspension with carbachol

	B_H (fmol/10^6 cells)	B_L	K_H (μM)	K_L (μM)	$\%B_H$
Control	45 ± 7	80 ± 20	0.90 ± 0.20	18 ± 3	35
Carbachol-treated	33 ± 6	60 ± 20	0.70 ± 0.40	18 ± 7	34

B_H and B_L represent the B_{max} values of the high- and the low-affinity sites respectively. K_H and K_L represent K_d values of the high- and low-affinity sites respectively. Each value is the mean ± SE from five independent experiments. The cells were desensitized by incubation with carbachol (1 mM) for 45 min. Carbachol was removed by washing the cells before the binding assay was performed with the intact cells at 15° for 45 min. Details of the data analysis were described in a previous publication [5].

* None of the carbachol-treated values were significantly different from the control ($t = 1.23$, $df = 8$, $P > 0.10$).

phatidylserine, diolein and Ca^{2+} were omitted and the EGTA concentration was raised to 5 mM. The reaction was started by the addition of the enzyme and incubated for 5 min at 30°. The assay was ended by spotting 30 μL of the 50 μL reaction volume on 2 × 2 cm squares of P-81 phosphocellulose paper which were immersed in 75 mM phosphoric acid. The papers were removed after a minimum of 45 min, washed three times with water, once in acetone and finally in petroleum ether. The amount of ^{32}P bound was determined by liquid scintillation spectrometry. Protein kinase C activity was calculated by subtracting the amount of ^{32}P bound without Ca^{2+}, phosphatidylserine, and diolein from the amount of ^{32}P bound in the presence of these necessary cofactors.

Several changes in the protocol were tried, with similar results, and included the following: adding two protease inhibitors, leupeptin (100 μg/mL) and aprotonin (10 units/mL) to the lysis buffer to prevent any possible degradation of the enzyme during cell lysis; using a Dounce homogenizer or using a cell sonicator (2 × 15 sec bursts) to disrupt cells; varying the time and speed of both configurations; changing the elution volume; and using a Tris buffer system in the assay [16]. In addition, because solution II used by Liles *et al.* [7] to wash cells is hypotonic, we also used isosmotic solution I with leupeptin (100 μg/ml) to wash cells.

Statistical analyses of data. Student's *t*-test was employed to determine whether group means were significantly different. These tests were considered significant if $P < 0.05$.

RESULTS

Effects of PMA on muscarinic M_1 receptor-mediated cyclic GMP formation and IP release by N1E-115 cells in suspension. PMA or a synthetic diacylglycerol, diC_8 [17], at 100 nM and 200 μM, respectively, inhibited carbachol-stimulated cyclic GMP formation by N1E-115 cells in a time-dependent manner. The maximum inhibition was achieved after 45 min of preincubation with the cells (Fig. 1A). PMA (100 nM) inhibited carbachol-stimulated IP release with a similar time–course (Fig. 1B). PMA added to cells for 45 min inhibited carbachol-stimulated cyclic GMP formation and inositol phosphate release in the same concentration-dependent manner (Fig. 2). Concentration-dependency was also seen for diC_8 ($IC_{50} \approx 10$ μM, data not shown), whereas 4α-PD lacked significant effect (Fig. 2). Thus, protein

kinase C activators can inhibit putative second messenger formation mediated through the muscarinic M_1 receptor of clone N1E-115 cells.

The IC_{50} values (± SE, N = 4) for PMA and its inhibition of carbachol-stimulated cyclic GMP formation and IP release were 27 ± 9 and 32 ± 6 nM, respectively, values that are reasonably close to the reported activation constants for PMA and protein kinase C (range 5–10 nM) [8] and the K_i (10.2 ± 0.4 nM) of PMA obtained in our binding assays with [^{3}H]PDBu, as discussed further below.

For receptor-mediated cyclic GMP formation (Fig. 3), but not for receptor-mediated inositol phosphate release (data not shown), PMA shifted the EC_{50} for agonist to the right. For both second messenger responses, PMA lowered the maximum response. Cells desensitized to carbachol showed these same changes, respectively, in the concentration-response curves for agonist when either cyclic GMP formation [3] or inositol phosphate release (data not shown) was measured. Thus, PMA treatment of cells mimicked the desensitization of the muscarinic M_1 receptor, implicating protein kinase C activation in desensitization, as suggested by Liles *et al.* [7].

Muscarinic receptor binding of [^{3}H]*NMS to N1E-115 cells treated in suspension or in monolayer.* Although preincubation of clone N1E-115 cells in monolayer with carbachol [7, 12] or PMA in the presence of the calcium ionophore A23187 [7] induces a rapid decrease in [^{3}H]NMS binding sites, we have been unable to show a significant change in the number or the affinity of binding sites for [^{3}H]NMS for cells in suspension exposed to these agents (Table 1). In addition, preincubation of cells in suspension with 1 mM carbachol for 45 min had no statistically significant effect on the B_{max} or K_d of either the low- or the high-affinity agonist binding sites of the muscarinic receptor (Table 2).

However, in confirmation of the previous studies, cells in monolayer incubated with carbachol (1 mM, 45 min) had a decrease of 40% in the B_{max} for [^{3}H]NMS binding with no change in the K_d (Fig. 4 and Table 1) and cells in monolayer incubated with PMA (100 nM, 45 min) had a decrease of 30% in this B_{max} with no change in the K_d (Fig. 4 and Table 1). These results showed that preincubation of cells in suspension with carbachol or PMA does not induce rapid down-regulation, even though causing the reduction of receptor-mediated responses.

Either cell to cell contact or an intact cytoskeleton afforded cells in monolayer may be critical to produce the rapid internalization. Since cellular contact is known to elevate intracellular cyclic AMP levels [18], we incubated cells in suspension with cyclic AMP (1 mM) plus 3-isobutyl-1-methylxanthine (1 mM), a phosphodiesterase inhibitor, before exposure to either carbachol or PMA. However, this pretreatment had no significant effect on [^{3}H]NMS binding.

Effects of carbachol desensitization and PMA on sodium nitroprusside-stimulated cyclic GMP formation. Since the evidence suggested that changes at the muscarinic receptor itself were not responsible for desensitization, we determined if the activity of guanylate cyclase was changed by desensitization. Sodium nitroprusside (SNP) can stimulate the

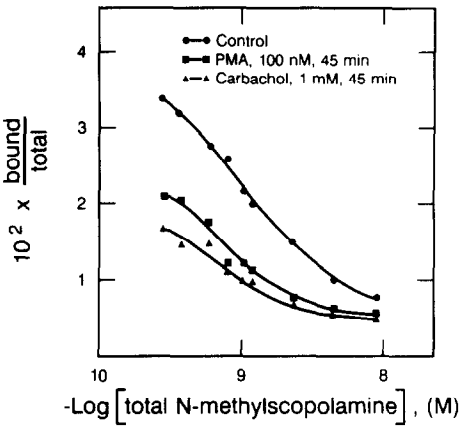

Fig. 4. [^{3}H]NMS binding to intact clone N1E-115 cells after treatment in monolayer with carbachol. Cells in monolayer were treated with 1 mM carbachol or 100 nM PMA for 45 min in solution I at 37°. After cells were harvested by scraping or by incubation with D_1 solution, they were resuspended in solution I, washed three times with this solution, and then incubated with [^{3}H]NMS (0.5 nM) and unlabeled NMS (0.1 to 10 nM) for 45 min at 15°. In the representative experiment shown, the K_d values of the control (●), PMA- (■) and the carbachol-(▲) treated cells were 0.67, 0.28, and 0.37 nM, and the B_{max} values were 144, 99, and 85 fmol/10^6 cells respectively. Results of all experiments are summarized in Table 1.

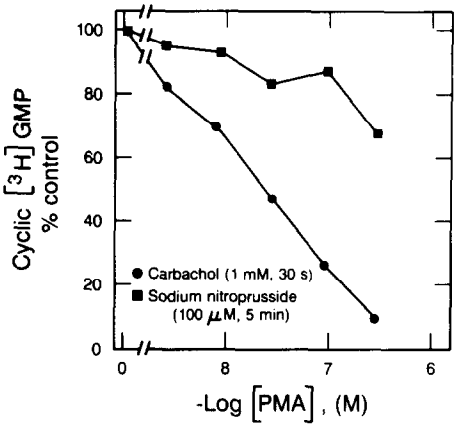

Fig. 5. Effects of PMA on sodium nitroprusside- and carbachol-mediated cyclic GMP synthesis by N1E-115 cells in suspension. Cells were incubated with PMA at the concentrations shown for 45 min and then stimulated with sodium nitroprusside (■, 100 μM, 5 min) or carbachol (●, 1 mM, 30 sec). Results are averages from at least three independent experiments.

enzyme by an oxidative process largely independent of extracellular Ca^{2+} [19]. Pretreatment with PMA did not have a major effect on SNP-stimulated cyclic GMP formation at concentrations that markedly inhibited carbachol-medicated cyclic GMP responses (Fig. 5). In cells desensitized to carbachol (1 mM for 45 min), the cyclic GMP response to carbachol was abolished completely, IP release was 18% of control (data not shown), but the response to SNP did not

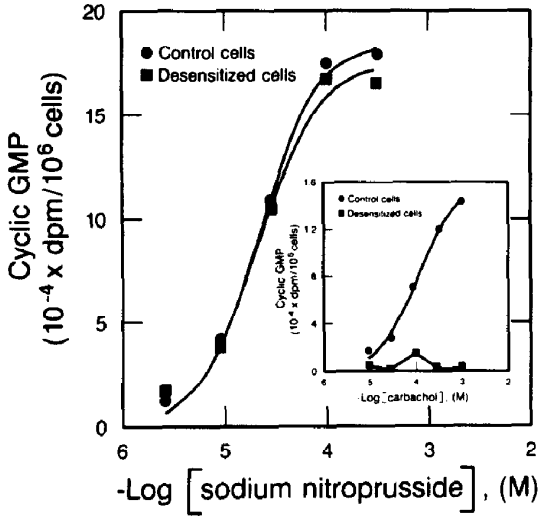

Fig. 6. Effects of preincubation of clone N1E-115 cells in suspension with carbachol on sodium nitroprusside- and carbachol-mediated cyclic GMP concentration–response relationships. Cells (subculture 20, 13 days after subculture) were preincubated with 1 mM carbachol for 45 min to desensitize the muscarinic M_1 receptor. Cells were then washed three times with solution I, distributed into wells at about 100,000 cells/well, and stimulated by carbachol or SNP at the concentrations shown for 30 sec and 5 min respectively. Basal values averaging 0.56×10^4 dpm/10^6 cells were subtracted from the results which are from one of three independent experiments.

change significantly (Fig. 6). These results suggest that neither guanylate cyclase nor cyclic GMP phosphodiesterase is involved in causing desensitization or in the action of PMA.

[^{3}H]PDBu binding and protein kinase C activity. [^{3}H]PDBu binding to intact cells in suspension was time- and concentration-dependent and saturable. The K_d (± SE) was 16.4 ± 0.3 nM, within reasonable agreement with the reported K_d values in different tissues [20, 21]. The B_{max} (± SE) was 3.44 ± 0.07 pmol/10^6 cells. The calculated Hill coefficient (± SE) was 0.94 ± 0.09, a value compatible with the presence of non-cooperative, homogeneous binding sites. The K_i of PMA was 10.2 ± 0.4 nM, close to its IC_{50} values for carbachol-mediated responses and to its reported activation constant of PMA and protein kinase C (5–10 nM) [8].

In other experiments we determined the binding constants for [^{3}H]PDBu in cytosolic and membranal preparations of cells in monolayer. For membranes, the K_d and B_{max} values (± SE) were 22 ± 8 nM and 1.1 ± 0.5 pmol/mg protein (N = 5), respectively, and for the cytosol, 15 ± 6 nM and 3.9 ± 0.1 pmol/mg protein (N = 5), respectively. Exposure of cells in monolayer or suspension to carbachol (1 mM for 30 sec, 5 min, 15 min, 30 min, or 45 min) had no significant effect on these values (data not shown). However, treatment with PDBu (200 nM for 15 min; N = 3) caused a significant ($P < 0.05$) reduction in the cytosolic fraction (from a mean ± SE of 84 ± 2% of total binding to 66 ± 4%) and a significant increase in the membranal fraction (from 16 ± 2% of total binding to 34 ± 4%).

Consistent with these binding data were the findings of no significant shift in activity of protein kinase C from the cytosol to the membrane after incubation of cells with carbachol (1 mM from 1 to 30 min; cells in monolayer or in suspension; N = 19). We found no shift in protein kinase C activity no matter which technique was used for washing and harvesting cells, or for measuring the activity of this enzyme. However, PMA (100 nM, from 15 to 45 min; N = 19) consistently caused a shift in activity from the cytosol to the membrane for cells in monolayer or in suspension. For example, for control cells in suspension, the distribution of protein kinase C activity between cytosol and membrane was (mean ± SE; N = 10) 91 ± 2 and 9 ± 2% respectively; for cells treated in suspension with PMA (100 nM for 45 min; N = 10), it was 67 ± 6 and 32 ± 6 respectively. The change in distribution caused by PMA was significantly different from controls ($t = 26.05$, df = 18, $P < 0.0001$).

Effects of desensitization to PMA on desensitization to carbachol. If protein kinase C were involved in the process of rapid desensitization to carbachol, then desensitization of this enzyme by prolonged exposure of cells to phorbol ester would affect this process. Cells exposed for 24 hr to PMA (100 nM) had 2 and 7% of control protein kinase C activity in the cytosolic fraction ($t = 2.53$, df = 4, $P = 0.03$) and membranal fraction ($t = 1.87$, df = 4, $P = 0.067$) respectively. In addition, with cells so exposed, fresh PMA (100 nM) added to cells for 15 min before stimulation with carbachol no longer inhibited the formation of cyclic GMP. In fact, PMA now appeared to potentiate the effects of carbachol on cyclic GMP formation (Table 3, B.1). After prolonged exposure to PMA, cells had responsiveness to carbachol similar to that of cells not exposed previously to PMA.,The ability of carbachol to desensitize these cells was not affected. In cells not exposed to PMA for 24 hr and not entirely desensitized to carbachol, PMA for 15 min completely inhibited cyclic GMP formation mediated by carbachol (Table 3, A.4). However, for cells exposed to PMA for 24 hr and desensitized partly to carbachol, adding back PMA appeared to increase the responsiveness of the cells to this agonist (Table 3, B.4).

DISCUSSION

In confirmation of the work of Liles *et al.* [7], our studies appear to implicate protein kinase C in the desensitization of the muscarinic M_1 receptor of murine neuroblastoma clone N1E-115. However, a role for protein kinase C may be more apparent than real. We do not suggest, as others have [7, 12], that rapid receptor down-regulation is responsible for this desensitization.

In our studies PMA, an activator of the protein kinase C, had a very similar inhibitory effect on muscarinic receptor-mediated cyclic GMP formation and IP release with the IC_{50} in each receptor-mediated event being similar to one another and to the equilibrium dissociation and activation constants for PMA and this enzyme. The synthetic diacylglycerol, diC_8 [17], mimicked the effects of PMA on receptor-mediated cyclic GMP synthesis, whereas

Table 3. Effects of 24-hr pre-incubation with PMA on carbachol desensitization and PMA inhibition of muscarinic receptor-mediated cyclic GMP formation

	Preincubation I (24 hr) PMA (100 nM)	II (45 min) Carbachol (1 mM)	III (15 min) PMA (100 nM)	Net cyclic GMP response (dpm/10^5 cells)	% Control
A. Cells not preincubated with PMA					
1.	−	−	−	129,000 ± 5,000	
2.	−	−	+	103,000 ± 7,000†	80
3.	−	+	−	40,000 ± 2,000‡	31
4.	−	+	+	0§‖	0
B. Cells preincubated with PMA					
1.	+	−	−	117,000 ± 4,000¶	
2.	+	−	+	14,000 ± 7,000**	120
3.	+	+	−	13,000 ± 2,000††	11
4.	+	+	+	26,000 ± 2,000‡‡§§	22

Cells (passage 14, 14 days after subculture) were treated in monolayer without (−) or with (+) 100 nM PMA for 24 hr (I), harvested, and labeled with [^{3}H]guanosine as described under Materials and Methods. Cells from each group were divided in half and incubated without (−) or with (+) 1 mM carbachol for 45 min (II). Cells were washed twice with ice-cold solution I, resuspended in solution I to 6.25×10^5 cells/mL, and aliquoted (240 μL) to wells of a multiwell tray. PMA (100 nM final concentration) was added to one-half of the wells of each condition and cells were incubated for 15 min (III) before the addition of 5 mM carbachol for 30 secs. Results are averages from two independent experiments, each determined in triplicate. Similar results were obtained in two other independent experiments.

* Net response was obtained by subtracting basal values of about 28,000 dpm/10^6 cells and 17,000 dpm/10^6 cells from values for cells in A and B respectively. These basal values were significantly different ($t = 8.77$, df = 22, P = 0.0001).

† Significantly different from A.1 ($t = 3.04$, df = 10, P = 0.012).
‡ Significantly different from A.1 ($t = 15.55$, df = 10, P = 0.0001).
§ Significantly different from A.1 ($t = 23.81$, df = 10, P = 0.0001).
‖ Significantly different from A.3 ($t = 19.93$, df = 10, P = 0.0001).
¶ Not significantly different from A.1 ($t = 1.75$, df = 10, P = 0.11).
** Significantly different from B.1 ($t = 2.94$, df = 10, P = 0.015).
†† Significantly different from B.1 ($t = 21.25$, df = 10, P = 0.0001).
‡‡ Significantly different from B.1 ($t = 18.11$, df = 10, P = 0.0001).
§§ Significantly different from B.3 ($t = 4.27$, df = 10, P = 0.002).

the inactive phorbol, 4α-phorbol 12,13-didecanoate, was without effect. PMA shifted the EC_{50} for carbachol to the right and lowered the maximal response for cyclic GMP synthesis. In IP assays, it only lowered the maximal response. These respective changes in the concentration–response curves for carbachol caused by PMA were very similar to those seen with carbachol-induced desensitization in this clone for cyclic GMP synthesis [3] and for IP release as presented here. In addition, the time–course for these effects of PMA was consistent with agonist-mediated desensitization.

Liles *et al.* [7] reported that carbachol translocates protein kinase C from cytosol to membrane with a time–course consistent with down-regulation and desensitization of the M_1 receptor. However, despite many experiments that followed the methods of Liles *et al* [7], as well as our own modifications, and those of others [16], we were unable to show a translocation of this enzyme by carbachol. We found consistent translocation with PMA. It is unclear why we were unable to reproduce the results of Liles *et al.* [7] with the use of carbachol. Similarly, we found no changes in the distribution of [^{3}H]PDBu binding between membranes and cytosol from cells pretreated with carbachol, although, once again, PMA caused a change. However, recently, Messing *et al.* [22] showed that nicotinic and muscarinic agonists stimulate rapid protein kinase C translocation in rat pheochromocytoma cells (PC12).

Despite our results, it is still possible that receptor stimulation leads to activation of protein kinase C, since Halsey *et al.* [23] have suggested that agonists which stimulate release of inositol phosphates may preferentially activate the membrane-associated protein kinase C. Suggesting a role for protein kinase C in this receptor desensitization is difficult, however, since not only was there no translocation of protein kinase C caused by carbachol, but also there was desensitization to carbachol occurring in cells largely lacking cytosolic protein kinase C activity (Table 3). Although protein kinase C activation can mimic in much detail the desensitization to a muscarinic agonist, our results strongly suggest that activation of this enzyme is not responsible for this process. Similar conclusions were reached for the β-adrenergic receptor on rat glioma cells [24, 25]. We hypothesize there is yet another kinase, a receptor-activated kinase, acting at the same or similar sites as that of protein kinase C, to cause receptor desensitization.

The muscarinic M_1 receptor of clone N1E-115 cells desensitizes without down-regulation of the receptor for cells in suspension, while for cells in monolayer, desensitization was accompanied by down-regulation. Further research is required to learn the reason for the lack of down-regulation of the muscarinic receptor in cells in suspension. However, receptor internalization may require a certain type of cell-to-cell contact or a special configuration of the cytoskeletal structure afforded cells in monolayer. Another possibility is that for cells in suspension, the receptor down-regulation may occur but at a much slower rate than that for cells in monolayer and thus would only be observed after extended exposure of cells to agonist.

However, if we assume that the mechanism of desensitization of the muscarinic receptor is the same for these cells under both conditions (monolayer and suspension), we conclude that the mechanism of desensitization occurs at a site distal to the receptor binding site. Thus, down-regulation of the receptor could be a result of desensitization.

Other sites that could possibly be involved in the mechanism of desensitization come to mind when we consider the steps involved between receptor activation and the biochemical responses elicited. To date all neurotransmitters that stimulate soluble guanylate cyclase also stimulate release of inositol phosphates [26]. A current hypothesis states that receptor-mediated cyclic GMP formation results from metabolism of polyphosphoinositides via action of phospholipase C, subsequent phospholipase A_2 activation, and release of arachidonic acid. Arachidonate or its lipoxygenase metabolite(s) would then stimulate guanylate cyclase [27, 28]. Desensitization does not affect SNP-stimulated soluble guanylate cyclase, also largely unaffected by PMA. Further, IP release desensitizes with similar characteristics to that of cyclic GMP synthesis. Thus, the site of desensitization likely involves a locus in common with both receptor responses.

Evidence suggests that a guanine nucleotide binding protein (G_0) and phospholipase C are involved in common with receptor-mediated release of inositol phosphates and cyclic GMP formation [19, 29–32]. Protein kinase C and phorbol esters inhibit guanine nucleotide-dependent inositol phosphate release in membranes of astrocytoma cells [33]. PMA inhibits the β-adrenergic receptor response by uncoupling the receptor from the GTP binding protein [34]. Thus, G_0 may be the site phosphorylated by a receptor-activated kinase (perhaps akin to that which phosphorylates receptors linked to adenylate cyclase [35]) to cause desensitization of receptors that mediates the release of inositol phosphates. This event, in turn, could cause the receptor to which G_0 was coupled to be internalized or altered in binding characteristics to occlude [^{3}H]NMS binding sites.

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