# Neurotransmitter receptors mediate cyclic GMP formation by involvement of arachidonic acid and lipoxygenase

(muscarinic/histaminergic/intracellular Ca$^{2+}$)

R. MICHAEL SNIDER*, MICHAEL MCKINNEY*, CARLOS FORRAY*, AND ELLIOTT RICHELSON*†‡

Departments of †Psychiatry and Psychology and *Pharmacology, Mayo Clinic and Foundation, Rochester, MN 55905

ABSTRACT    Evidence is presented that has led us to abandon the hypothesis that receptor-mediated cyclic GMP formation in cultured nerve cells occurs via the influx of extracellular calcium ions and an increase in the cytosolic free calcium ion concentration. While the cyclic GMP response is absolutely dependent on the presence of Ca$^{2+}$, there is no increase in free intracellular Ca$^{2+}$ subsequent to agonist stimulation. Instead, we have found that muscarinic or histamine H$_1$ receptor stimulation elicits the release of arachidonic acid through a quinacrine-sensitive mechanism, possibly phospholipase A$_2$. Inhibition of the release or metabolism of arachidonate by the lipoxygenase pathway prevents receptor-mediated cyclic GMP formation. We hypothesize that neurotransmitter receptors that mediate cyclic GMP synthesis function by releasing arachidonic acid and that an oxidative metabolite of arachidonic acid then stimulates soluble guanylate cyclase.

Activation of certain classes of neurotransmitter receptors stimulates the synthesis of cyclic GMP within certain cells. In virtually every system studied, this cyclic GMP response is dependent on the presence of extracellular Ca$^{2+}$ (1, 2). Murine neuroblastoma clone N1E-115 cells possess muscarinic and histamine H$_1$ receptors that mediate cyclic GMP formation (3–6). These cells, derived from a neuroblast, display many of the properties of normal differentiated neurons (7) and, as such, have proven to be an ideal model system for the study of the mechanisms of neurotransmitter interactions with nerve cells, including receptor activation, desensitization, and psychotherapeutic drug effects (3–14).

Many of the same receptor types that elicit a cyclic GMP response also stimulate the turnover of inositides, including phosphatidylinositol (PtdIns), resulting in the formation of phosphatidic acid (PtdOH) via 1,2-diacylglycerol (15). Receptor-mediated PtdIns turnover is hypothesized to cause an increase in the plasma membrane permeability to Ca$^{2+}$, resulting in an increase in the intracellular free calcium ion concentration or [Ca$^{2+}$]$_i$ (15). Muscarinic receptor stimulation of N1E-115 cells elicits a large increase in the incorporation of $^{32}$P into PtdIns (16). Receptor-mediated phospholipid labeling or the "phosphatidylinositol effect" is well documented and occurs in response to numerous agonists in a variety of cellular and tissue preparations (15, 17).

The hypothetical mechanism for receptor-mediated Ca$^{2+}$-dependent cyclic GMP formation is PtdIns hydrolysis with subsequent appearance of PtdOH, which acts to translocate Ca$^{2+}$ in sufficient quantities to increase [Ca$^{2+}$]$_i$, thus stimulating guanylate cyclase. In support of this mechanism, exogenous PtdOH is reported to stimulate $^{45}$Ca$^{2+}$ uptake and cyclic GMP formation in N1E-115 cells (18). However, we have gathered direct evidence that receptor activation does not cause an increase in [Ca$^{2+}$]$_i$. Instead, agonist-induced

cyclic GMP formation appears to result from release of arachidonic acid by a quinacrine-sensitive mechanism with subsequent metabolism of arachidonic acid via the lipoxygenase pathway.

## METHODS

**Cell Culture Conditions.** Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco's modified Eagle's medium (GIBCO) without antibiotics and supplemented with 10% (vol/vol) newborn calf serum (GIBCO; medium I). Cells (passage number, <16) were grown in 20 ml of medium I in 75-cm$^2$ Corning tissue culture flasks (250 ml; Corning) in an atmosphere of 10% CO$_2$/90% humidified air at 37°C. Cells were subcultured by removing growth medium and incubating for 10 min in a modified Puck's D$_1$ solution (ref 6; medium II) and resuspending in 10 ml of medium I. The cells were then inoculated into flasks (4–8 × 10$^5$ per flask) on day 0. The culture medium was changed on days 4, 6, and on each day thereafter by adding 10 ml of fresh medium I and removing 10 ml of medium I. Cells were negative for mycoplasma by bacteriologic criteria.

**Measurement of Relative Changes in Cyclic GMP Production.** The details of the use of radioactively labeled precursor for measuring relative change in cyclic GMP formation in intact cells are described elsewhere (6). In brief, the cells were harvested for assay by aspiration of medium I and incubation for 10 min in medium II, followed by isolation and low speed centrifugation (250 × $g$) for 90 sec at 5°C. The pellet was washed twice with a phosphate-buffered sodium chloride solution (medium III) containing 110 mM NaCl/5.3 mM KCl/1.8 mM CaCl$_2$/1.0 mM MgCl$_2$/2.0 mM Na$_2$HPO$_4$/25 mM glucose/70 mM sucrose (adjusted to pH 7.3–7.4; osmolality, 335–340 mOsm). The cells were suspended by trituration in 2 ml of medium III, and 20 μl of this suspension was removed and used for enumeration of cells (Coulter Counter). Precursor labeling was achieved by adding [$^3$H]guanine to the cell suspension (10 μCi/ml; 1 Ci = 37 GBq) (final concentration, 1.1 μM) and rotating cells at 37°C for 45 min at 80 rpm (Shaking Incubator GCA/Precision Scientific, Chicago, IL). Cells were briefly centrifuged, the radioactive supernatant was discarded, and then the cells were resuspended in medium III. This cell suspension was then distributed into wells of a multiwell tray (Linbro Plate, Flow) in 270-μl aliquots containing ≈1 × 10$^5$ cells. After equilibration of cells at 37°C for 15–20 min in the shaker bath at 80 rpm, agonist was added in a 30-μl vol for the times specified. Basal cyclic [$^3$H]GMP levels were determined by adding 30 μl of medium III. The reaction was terminated by the addition of 30 μl of 50% (wt/vol) trichloroacetic acid. After adding to each well 0.6 nCi (1400 dpm) of cyclic [$^{14}$C]GMP as an internal standard, the contents of each well were applied to an AG50 W-

Abbreviations: PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid.
‡To whom reprint requests should be addressed.

X2 ion exchange column (0.8 × 8.0 cm) that had been equilibrated with 0.1 M HCl. The columns were then washed with 4.4 ml of 0.1 M HCl (eluate discarded) and 1.0 ml of $H_2O$ (eluate discarded), and finally with 1.5 ml of $H_2O$, which was collected in plastic Microfuge tubes. To this eluate, equal volumes (30 $\mu$l) of 2.67 M $ZnSO_4$ and 2.67 M $Na_2CO_3$ were added to precipitate any residual [$^3H$]GTP or [$^3H$]GDP. The contents of the tubes were then mixed and centrifuged in a Beckman Microfuge for 2 min (setting 11). The supernatant was transferred to a scintillation vial containing 7 ml of scintillation fluid, and the radioactivity was determined in a Searle Isocap/300 liquid scintillation counter. All samples were corrected for the recovery of cyclic [$^{14}C$]GMP (60%–80%) and the quench was corrected by using the external standard ratio technique.

**Measurement of Light Emission from Aequorin-Loaded Neuroblastoma Cells.** Murine neuroblastoma clone N1E-115 cells were washed free of medium I and loaded in medium II in 2-ml polypropylene centrifuge tubes (3 × $10^6$ cells per ml). The cells were gently pelleted, the supernatant was removed, and the cells were resuspended sequentially in three solutions (A, B, and C) for 10 min each at 4°C. Solution A renders the cells reversibly hyperpermeable, solution B introduces aequorin into the cells, and solution C causes cellular resealing (19, 20). The composition of the solutions were as follows: solution A, 10 mM EGTA; 120 mM KCl; 3 mM ATP; 1 mM GTP; 2 mM $MgCl_2$; 2 mM $Na_2HPO_4$; 25 mM glucose: solution B, 0.1 mM EGTA; 120 mM KCl; 3 mM ATP; 1 mM GTP; 2 mM $MgCl_2$; 2 mM $Na_2HPO_4$; 25 mM glucose; aequorin, 0.3 mg/ml: solution C, 0.1 mM EGTA; 120 mM KCl; 3 mM ATP; 1 mM GTP; 10 mM $MgCl_2$; 2 mM $Na_2HPO_4$; 25 mM glucose (for all solutions, pH = 7.3 and osmolality = 340 mOsm). The cells were slowly warmed to room temperature in solution C, and medium III was gradually added over a period of 10–15 min to slowly increase the [$Ca^{2+}$]$_o$. The cells were then replated in complete growth medium and incubated for 10–12 hr in incubator conditions (37°C; 10% $CO_2$/90% humidified air). The cells were then harvested and transferred to the light-collecting apparatus (21) for recording of light signals. All experiments were conducted at 37°C by superfusion of the cell suspension with warm medium III. Calcium ionophore X537A, melittin, carbachol, or histamine at the indicated concentrations was added to the preparation by superfusion for the times indicated and then washed out with medium III.

**Release of [$^3H$]Arachidonic Acid from Intact Murine Neuroblastoma Cells.** Labeling of cells with [$^3H$]arachidonic acid was achieved by adding 15 $\mu$Ci of [$^3H$]arachidonic acid (87.4 Ci/mmol) to a flask of stationary phase N1E-115 cells (≈6 × $10^6$ cells per flask) and incubating for 18 hr in complete growth medium. Greater than 95% of the [$^3H$]arachidonate becomes esterified by the cells by this procedure. Cells were harvested for assay and washed twice by suspension in medium III and low speed centrifugation (1000 rpm for 1 min). The final cell suspension (4 × $10^5$ cells per ml) was in medium III containing bovine serum albumin at 1 mg/ml (essentially fatty acid free; Sigma). Cells were gently resuspended by trituration and distributed in 240-$\mu$l aliquots into plastic 10 × 75 mm tubes (1 × $10^5$ cells per tube). Assays were done at 37°C with antagonists preincubated for 20–30 min prior to challenge with agonists. After a 30 sec incubation with carbachol (final concentration, 1.0 mM) or histamine (0.1 mM) the assay was terminated by adding 3 ml of ice-cold 10 mM EGTA (pH 7.0). [$^3H$]Arachidonic acid remaining in membranes was separated from released or free [$^3H$]arachidonic acid by centrifuging the mixture at 15,000 × $g$ for 15 min at 4°C. Assays were conducted in sextuplets with triplicates used for total $^3H$ release and for [$^3H$]arachidonic acid release. Total $^3H$ release was determined by carefully transferring the aqueous supernatant to scintillation vials for determination of radioactivity. [$^3H$]Arachidonic acid release was determined by adding to the 2.8-ml aqueous supernatant 50 $\mu$l of 1 M HCl/500 mg of KCl/16 $\mu$g of unlabeled arachidonate (as a carrier) and 1500 dpm of [1-$^{14}C$]arachidonic acid for calculation of recovery, and by extracting twice with 3 ml of 2,2,4-trimethylpentane/ethyl acetate (1:2, vol/vol). Extraction efficiency was >90%. The organic extracts were combined, evaporated to dryness under a stream of $N_2$ gas, reconstituted in 30 $\mu$l of chloroform, and spotted onto lanes of a 20 × 20 cm Silica Gel G TLC plate (with preabsorbent layer; Analtech, Newark, DE). The TLC plates were developed with ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (90:50:20:100, vol/vol). Individual spots of arachidonic acid were visualized with iodine vapors (compared with standard arachidonic acid run on the same TLC plate), scraped into scintillation vials, and [$^3H$]- and [$^{14}C$]arachidonic acid were quantitated in a Beckman 7800 counter. All samples were corrected for quench and for recovery of [$^{14}C$]arachidonate (60%–75%).

**Materials.** Carbamoylcholine chloride, histamine dihydrochloride, nordihydroguaiaretic acid, quinacrine hydrochloride, atropine, and pyrilamine malate were obtained from Sigma; arachidonic acid was from Nu Chek Prep; [$^3H$]arachidonic acid and [$^{14}C$]arachidonic acid were from New England Nuclear; [$^3H$]guanine was from Schwarz/Mann Radiochemicals; [$^{14}C$]guanosine 3',5'-monophosphate was from Amersham. Calcium ionophore X537A and 5,8,11,14-icosatetraynoic acid were provided by Hoffmann-LaRoche. Aequorin and melittin were provided by J. R. Blinks and F. G. Prendergast, respectively (Department of Pharmacology, Mayo Foundation). Human $\alpha$-thrombin was provided by J. W. Fenton II (Center for Laboratories and Research, New York State Department of Health).

## RESULTS

We sought to gather direct evidence to test the hypothesis that neurotransmitter receptor-mediated cyclic GMP formation occurs as the result of an increase in [$Ca^{2+}$]$_i$. One of the most satisfactory tools for directly monitoring changes in [$Ca^{2+}$]$_i$ in living cells is the bioluminescent protein aequorin, which changes the intensity of its light emission in response to variations in [$Ca^{2+}$]$_i$ within the physiological range (21). A population of murine neuroblastoma cells (clone N1E-115) was "loaded" with aequorin by making them reversibly hyperpermeable by incubation with EGTA as described by McClellan and Winegrad (19) and modified by Morgan and Morgan (ref. 20; see *Methods* for details). Subsequently, these cells were challenged with receptor agonists and other agents that stimulate cyclic GMP formation. Cells rendered hyperpermeable and loaded with aequorin by this method produced cyclic GMP in response to neurotransmitter agonists and calcium ionophore X537A in an identical manner as untreated cells (83%–112% of untreated cell response). $Ca^{2+}$ ionophore X537A and melittin, a polypeptide isolated from bee venom, caused an immediate increase in light emission (i.e., an increase in [$Ca^{2+}$]$_i$) from aequorin-loaded neuroblastoma cells (Fig. 1). By contrast, neurotransmitter receptor agonists carbachol or histamine at maximal doses repeatedly and consistently failed to alter [$Ca^{2+}$]$_i$, as measured by the aequorin technique (Fig. 1). The sensitivity of aequorin to changes in [$Ca^{2+}$]$_i$ dictates that an increase in the light emission should occur if [$Ca^{2+}$]$_i$ increases above ≈0.1 $\mu$M (21). Thus, it was clear that neurotransmitter agonists did not increase [$Ca^{2+}$]$_i$ above "resting glow" levels.

In marked contrast to the time course of light emission from aequorin-loaded neuroblastoma cells (Fig. 1) is the time course of cyclic GMP formation stimulated by carbachol, histamine, ionophore X537A, and melittin (Fig. 2). Carbachol and histamine, neurotransmitter receptor agonists that failed to augment [$Ca^{2+}$]$_i$, caused a rapid increase in cyclic

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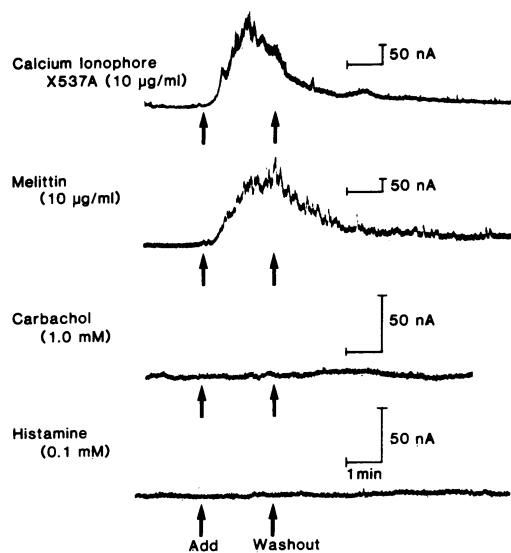
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FIG. 1. Light emission from aequorin-loaded neuroblastoma cells. Experiments were conducted with a population ($1-2 \times 10^6$ cells) of intact cells loaded with the calcium indicator. Calcium ionophore X537A, melittin, carbachol, or histamine at the indicated concentration was added to the preparation by superfusion of the cell suspension, for the times indicated, with drug in medium III at 37°C and washed out with drug-free medium III. This figure depicts representative results obtained in one of 10–15 separate experiments conducted under identical conditions.

GMP formation, which peaked at 30 and 45 sec, respectively. Melittin and X537A, which caused a striking and immediate increase in light emission (thus, $[Ca^{2+}]_i$; Fig. 1), produced a relatively slow increase in cyclic GMP levels, which peaked only after more than 2 min (Fig. 2). Thus, a clear dissociation was evident between an increase in $[Ca^{2+}]_i$ and cyclic GMP accumulation. This suggests that while increased $[Ca^{2+}]_i$ is capable of stimulating cyclic GMP formation, neurotransmitter agonists do not act in this manner.

Having provided evidence that an increase in the $[Ca^{2+}]_i$ was not the mechanism that couples receptor activation with guanylate cyclase, an alternative working hypothesis was formulated. In some experimental systems, most notably
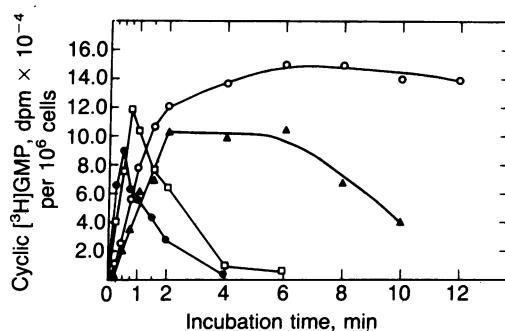


FIG. 2. Time course for the effect of neurotransmitter agonists carbachol (●) and histamine (□) or calcium ionophore X537A (▲) and melittin (○) on cyclic GMP formation in neuroblastoma cells. Clone N1E-115 cells (subculture 10) were grown and assayed for relative changes in cyclic [³H]GMP formation with the use of a radioactively labeled precursor in intact cells. After incubation of cells at 37°C for 15–20 min in the shaker bath at 80 rpm, carbachol (final concentration, 1.0 mM), histamine (0.1 mM), X537A (10 µg/ml), or melittin (10 µg/ml) was added in a 30-µl vol for the specified times. Basal cyclic [³H]GMP levels were determined by adding 30 µl of phosphate-buffered saline. This figure depicts a representative result from one of three separate experiments.

Table 1. Effects of inhibitors of arachidonic acid metabolism on agonist-stimulated cyclic [³H]GMP formation in intact murine neuroblastoma cells

| | Cyclic [³H]GMP, dpm per $10^6$ cells | | |
| --- | --- | --- | --- |
| | Carbachol (1.0 mM, 30 sec) | Histamine (0.1 mM, 45 sec) | Thrombin (10 nM, 1 min) |
| Control | 48,300 | 55,400 | 39,100 |
| Quinacrine | | | |
| 40 µM | 33,000 (68%) | 44,300 (80%) | 32,700 (84%) |
| 120 µM | 2,730 (6%) | 25,400 (46%) | 9,710 (25%) |
| 200 µM | 130 (<1%) | 13,300 (24%) | 3,430 (9%) |
| Control | 32,800 | 48,300 | 33,000 |
| ETYA | | | |
| 5 µM | 28,400 (87%) | 44,600 (92%) | 25,400 (77%) |
| 15 µM | 15,500 (47%) | 30,500 (63%) | 17,400 (53%) |
| 30 µM | 4,470 (14%) | 19,900 (41%) | 4,100 (12%) |

Experiments were conducted as described in Fig. 2 legend except that a 30-min preincubation in the presence of antagonist preceded challenge with agonist. Similar results were obtained in three and six separate experiments with 5,8,11,14-icosatetraynoic acid (ETYA) and quinacrine, respectively. Each value was determined in duplicate and replicates varied by 10% or less. Numbers in parentheses represent percent of control response.

platelets stimulated with thrombin, receptor-mediated PtdIns turnover and formation of diacylglycerol and PtdOH is followed by activation of phospholipase $A_2$ and/or diglyceride lipase, which mediates the release of arachidonic acid from membrane lipids (22–25). We recently reported that α-thrombin stimulates cyclic GMP synthesis in neuroblastoma N1E-115 cells (26). This cyclic GMP response requires catalytically active α-thrombin for maximal activity, and the concentration dependence in neuroblastoma cells and platelets is virtually identical. The cyclic GMP response of thrombin in N1E-115 cells is $Ca^{2+}$ dependent, with nearly the same time course as that produced by carbachol or histamine, suggesting that neurotransmitter agonists may share with thrombin a common mechanism of action for stimulation of cyclic GMP formation.

Thrombin-stimulated arachidonic acid release in platelets is blocked by quinacrine and is reportedly due to phospholipase $A_2$ inhibition (23, 27). In our studies, quinacrine antagonized the cyclic GMP response in N1E-115 cells elicited by carbachol, histamine, and thrombin in a concentration-dependent manner (Table 1) with approximately equal potency against all three agonists. The $IC_{50}$ (concentration that inhibits by 50%) values for quinacrine versus carbachol, histamine, and thrombin were 65, 102, and 90 µM, respectively. Quinacrine failed to inhibit sodium nitroprusside-induced cyclic GMP formation at concentrations as high as 750 µM, far above those that markedly antagonized receptor-stimulated cyclic GMP formation, ruling out the possibility that quinacrine acts nonspecifically.

Finally, we obtained data that directly implicate the release of arachidonic acid in receptor-mediated cyclic GMP formation. Intact neuroblastoma cells were radiolabeled with [³H]arachidonic acid, which was quantitatively incorporated into phospholipids, and subsequently these cells were challenged with agonists and other agents (X537A and melittin) that cause cyclic GMP formation. Carbachol, histamine, X537A, and melittin increased [³H]arachidonic acid release under the same conditions in which these agonists cause cyclic GMP formation (Table 2). Carbachol- and histamine-induced release of [³H]arachidonic acid was antagonized by quinacrine and by the receptor antagonists atropine and pyrilamine, respectively, in the same concentration range that is known to block cyclic GMP accumulation.

After receptor-mediated arachidonic acid release, arachi-

Table 2. Release of [³H]arachidonic acid from intact murine neuroblastoma N1E-115 cells

| | ³H released | | | [³H]Arachidonic acid released | | |
|---|---|---|---|---|---|---|
| | Total, dpm per $10^6$ cells ($\pm$SEM) | Net, dpm per $10^6$ cells | % of control | Total, dpm per $10^6$ cells ($\pm$SEM) | Net, dpm per $10^6$ cells | % of control |
| Basal release | 60,370 ± 2,160 | | | 8,785 ± 430 | | |
| Carbachol (1 mM; 30 sec) | 72,900 ± 1,850 | 12,500 | 100 | 10,700 ± 165 | 1,890 | 100 |
| With atropine (0.1 μM) | 61,600 ± 610 | 1,250 | 10 | 9,300 ± 580 | 500 | 26 |
| With quinacrine (350 μM) | 60,800 ± 1,210 | 460 | 3.7 | 7,900 ± 320 | 0 | <0 |
| Histamine (0.1 mM; 30 sec) | 77,800 ± 2,780 | 17,500 | 100 | 13,900 ± 930 | 5,110 | 100 |
| With pyrilamine (1 μM) | 63,900 ± 50 | 3,520 | 20 | 10,600 ± 30 | 1,800 | 35 |
| With quinacrine (350 μM) | 63,000 ± 2,790 | 2,600 | 15 | 8,900 ± 330 | 150 | 3 |
| X537A (10 μg/ml; 3 min) | 87,200 ± 2,760 | 26,800 | | 12,500 ± 395 | 3,700 | |
| Melittin (10 μg/ml; 6 min) | 404,000 ± 10,200 | 344,000 | | 230,100 ± 21,965 | 22,200 | |

Experiments were conducted using cells prelabeled with [³H]arachidonate. Results represent mean (± SEM) values from triplicate determinations within a single experiment with similar results obtained in six separate experiments.

donate can undergo oxidative metabolism via cyclooxygenase or lipoxygenase and form prostaglandins or hydroxyicosatetraenoic acids, respectively. We examined the possible involvement of arachidonic acid metabolites in the cyclic GMP response produced by carbachol, histamine, and thrombin by using selective antagonists of cyclooxygenase and lipoxygenase. 5,8,11,14-Icosatetraynoic acid, a lipoxygenase inhibitor, produced a concentration-dependent blockade of carbachol-, histamine-, and thrombin-induced cyclic GMP formation (Table 1). The IC$_{50}$ for 5,8,11,14-icosatetraynoic acid antagonism of cyclic GMP formation ranged from 14 to 20 μM and was noncompetitive in nature. Similar results were obtained with nordihydroguaiaretic acid, another inhibitor of the lipoxygenase metabolism of arachidonic acid. IC$_{50}$ values for nordihydroguaiaretic acid ranged from 7 to 24 μM for inhibition of agonists' responses. Cyclooxygenase inhibition with indomethacin (concentrations up to 10 μM) failed to cause any consistent modification of agonist-stimulated cyclic GMP synthesis. Interestingly, in numerous experiments with indomethacin (0.1–1 μM) a 10%–40% increase in agonist responses was observed. This finding could be explained by the shunting of arachidonic acid to the lipoxygenase pathway.

## DISCUSSION

We demonstrated, by using aequorin preloaded neuroblastoma cells, that a large change in the [Ca$^{2+}$]$_i$ did not occur after receptor activation. Previous work has clearly shown that the presence of extracellular Ca$^{2+}$ is absolutely necessary for receptor-mediated cyclic GMP formation. Moreover, agents that increase [Ca$^{2+}$]$_i$, such as melittin, Ca$^{2+}$ ionophores, depolarization with high K$^+$ or veratridine (28), or

exogenous PtdOH (18) are capable of triggering cyclic GMP formation. The finding that thrombin stimulates cyclic GMP formation in N1E-115 cells (26) is pivotal to our formulation of a hypothesis of neuronal receptor-mediated cyclic GMP formation. To account for all our results, we propose a scheme of biochemical events that couple neurotransmitter agonists with cyclic GMP formation (Fig. 3). This scheme draws on some parallel observations made in other systems, most notably in platelets. In platelets stimulated with thrombin, the initial event is the activation of a phospholipase C, which stimulates PtdIns turnover, resulting in the formation of PtdOH via 1,2-diacylglycerol (22–25). Muscarinic receptor stimulation greatly enhances PtdIns turnover (increased by 680%) in murine neuroblastoma clone N1E-115 cells (16). The formation of PtdOH in membrane phospholipids is hypothesized to be a calcium-gating mechanism (15, 29), and increase in [Ca$^{2+}$]$_i$ was thought to be the stimulus for guanylate cyclase activation (8). In platelets, however, PtdIns breakdown is also associated with release of arachidonic acid by phospholipase A$_2$ (22, 23) or by diglyceride lipase (24, 25). We propose that an arachidonate release mechanism that operates in concert with the PtdIns cycle such as occurs in platelets also underlies neuroblastoma receptor-mediated cyclic GMP responses. As we have shown, muscarinic and histaminergic stimulation of cells elicits [³H]arachidonic acid release, which is blocked by receptor antagonists (Table 2). These data demonstrate such an effect produced by carbachol or histamine. The stimulation of guanylate cyclase by arachidonic and other free fatty acids or by their oxidative derivatives and by hydroxyl radicals is well established in numerous experimental systems (1, 30–35), and the data presented here further implicate receptor-mediated re-
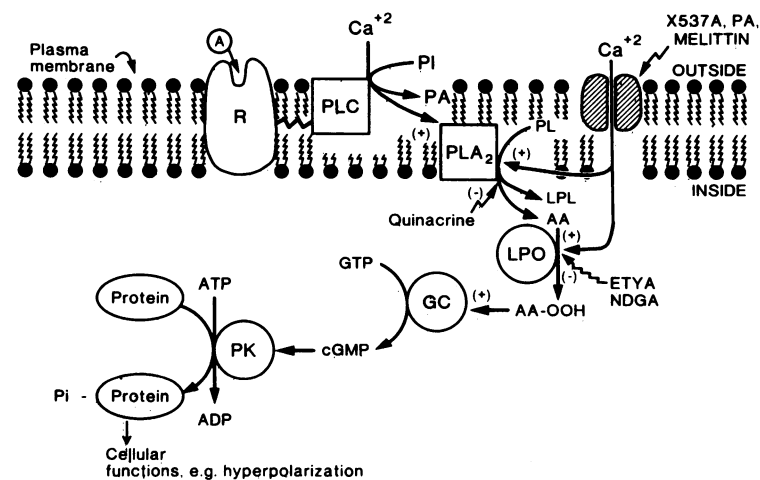


FIG. 3. Hypothetical scheme for the molecular mechanism of neurotransmitter-stimulated cyclic GMP formation. A, agonist; R, receptor; PLC, phospholipase C; PLA$_2$, phospholipase A$_2$; PI, PtdIns; PA, PtdOH; PL, phospholipid; LPL, lysophospholipid; AA, arachidonic acid; AA-OOH, hydroperoxyarachidonic acid; LPO, lipoxygenase; ETYA, 5,8,11,14-icosatetraynoic acid; NDGA, nordihydroguaiaretic acid; GC, guanylate cyclase; PK, protein kinase.

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lease of arachidonic acid in the process of neurotransmitter-mediated cyclic GMP formation.

Invoking a role for phospholipase $A_2$/diglyceride lipase may also explain the mechanism by which agents such as calcium ionophores and melittin are capable of causing cyclic GMP formation in nerve cells. Specifically, $Ca^{2+}$ ionophores and melittin have been shown to markedly activate endogenous phospholipase $A_2$, thus causing arachidonate release in platelets (27) and in cultured murine fibroblasts (36), respectively. We also showed that X537A and melittin markedly stimulated the release of [$^3$H]arachidonate (Table 2), perhaps explaining the prolonged time course of cyclic [$^3$H]GMP levels produced by these agents (Fig. 2).

The question remains as to what is the function of $Ca^{2+}$ in receptor-mediated responses. It is clear that extracellular $Ca^{2+}$ is absolutely required for the response but that $[Ca^{2+}]_i$ does not change after receptor activation. We believe that some transmembrane event, initiated by the receptor, is catalyzed by $Ca^{2+}$ bound to intramembrane sites, perhaps to head groups of phospholipids. On receptor activation and PtdIns hydrolysis, the bound ions may cause the mobilization of $Ca^{2+}$ to other bound sites, perhaps onto phospholipase $A_2$. In this case, $[Ca^{2+}]_i$ would not change, as $Ca^{2+}$ remains in a bound state inaccessible to the cytosol. Alternatively, $Ca^{2+}$–phospholipid complexes could act to effect conformational changes in membrane proteins, thus activating them. The hypothesis we favor for receptor-mediated slow neurotransmission (cyclic GMP formation) is that the agonist–receptor interaction causes local changes in the PtdIns/PtdOH ratio as a result of phospholipase C action. Phospholipase $A_2$ and/or diglyceride lipase may be $Ca^{2+}$ accepting sites on the intracellular membrane surface because both are highly $Ca^{2+}$-dependent and, as shown here, may be intricately involved in the mechanism of the neurotransmitter receptor–cyclic GMP transduction mechanism.

Within the scheme outlined in the present hypothesis, it is possible that calmodulin and/or the polyphosphoinositides are involved. Calmodulin regulates many $Ca^{2+}$-dependent phenomena and has been found to stimulate phospholipase $A_2$ in human platelets (37) and in renal medullary preparations (38). In addition, more careful examination of PtdIns responses have revealed that phosphodiesteratic cleavage of polyphosphoinositides is the first response after receptor activation in numerous cellular and tissue preparations (17, 39), including those of the central nervous system (40). The possible involvement of these, as well as other $Ca^{2+}$-dependent mechanisms in neurotransmitter-mediated cyclic GMP responses remains to be evaluated.

Other areas of particular interest are the nature of the arachidonate metabolites formed in response to neuronal receptor stimulation and the mechanism by which one or more of these activate guanylate cyclase or elicit other neuronal responses. Recently, it was shown that platelet guanylate cyclase is directly activated by arachidonic acid and linoleic acid that is released from the membranes (41).

It has been previously hypothesized that because many of the same agonists that stimulate PtdIns turnover also cause cyclic GMP formation, the two events are interrelated (29, 42). The major points that the present results add to the current knowledge of this phenomenon are as follows: (*i*) that a large calcium transient does not occur after receptor activation and (*ii*) that neurotransmitter receptor agonists that stimulate cyclic GMP formation also cause arachidonic acid release. Phospholipase $A_2$ and diglyceride lipase are the two known enzymes that, when activated, cause arachidonic acid release. The fact that these enzymes are both stimulated by calcium may explain the $Ca^{2+}$ dependency of receptor-mediated cyclic GMP formation in intact cells.

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