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Role of Intracellular Ca^{2+} Mobilization in Muscarinic and Histamine Receptor-Mediated Activation of Guanylate Cyclase in N1E-115 Neuroblastoma Cells: Assessment of the Arachidonic Acid Release Hypothesis

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SUMMARY

This study evaluates the role of intracellular levels of Ca^{2+} $[Ca^{2+}]_i$ in cyclic GMP formation mediated by muscarinic and histamine receptors in the mouse neuroblastoma clone N1E-115. Muscarinic agonists activated the turnover of phosphoinositides with a relative maximal response similar to that observed previously for cyclic GMP formation. Carbamylcholine induced a transient increase in inositol trisphosphate with a time course similar to that of cyclic GMP formation. In cells loaded with the fluorescent Ca^{2+} probe fura-2/acetoxymethyl ester, carbamylcholine as well as histamine induced a rapid and transient rise in $[Ca^{2+}]_i$. The time course of the changes in $[Ca^{2+}]_i$ induced by agonists as well as by ionomycin closely paralleled that of cyclic GMP formation. Chelation of $[Ca^{2+}]_i$ by loading of N1E-115 cells with quin 2/acetoxymethyl ester inhibited cyclic GMP formation induced by agonists in a dose-dependent manner. When cyclic GMP formation induced by agonists was assayed after the cells were exposed to 3 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) for 2 min, the formation of cyclic GMP was not inhibited significantly; however, it was completely abolished after 30-min exposure to EGTA. Treatment of cells with phospholipase A_2 had no effect on resting $[Ca^{2+}]_i$ and only slightly increased cyclic GMP formation, in spite of the induction of a marked release of [^{3}H]arachidonate. Moreover, the formation of cyclic GMP induced by ionomycin was inhibited by the addition of phospholipase A_2. Melittin contaminated with phospholipase A_2 activity induced a rapid and sustained increase in cyclic GMP formation, as well as unesterified [^{3}H]arachidonate release. However, after inactivation of the phospholipase A_2 activity of melittin, its ability to stimulate cyclic GMP formation was enhanced. Our data indicate that receptor agonists stimulate cyclic GMP formation in N1E-115 cells by activating the formation of inositol trisphosphate, which is followed by the release of Ca^{2+} from intracellular stores. The evidence obtained does not support a major role for arachidonate release in receptor-mediated activation of guanylate cyclase. Conversely, it is consistent with an inhibitory role for arachidonic acid or its metabolites in this process.

Cyclic GMP has been identified in various mammalian tissues and in other organisms (1). It has been shown that muscarinic cholinergic agonists, as well as other neurotransmitters and hormones, increase the intracellular cyclic GMP content in a wide variety of tissues by activating a soluble guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] (2). Soluble guanylate cyclase has been found to be stimulated directly by nitric oxide and by agents that can spontaneously generate this activator (3, 4). However, the mechanisms by which activation of membrane-bound receptors stimulates this enzyme remain largely unknown, because its activity is not affected by receptor agonists in broken cell preparations or in purified enzyme preparations (5, 6), indicating that there is no direct coupling between the receptor and guanylate cyclase. Receptor-mediated cyclic GMP formation in intact cells has been consistently shown to require extracellular Ca^{2+} (7–9); thus, it has been suggested that Ca^{2+} influx or mobilization from intracellular stores might be involved in the activation of guanylate cyclase (10). However, the actual role of Ca^{2+} in the stimulation of soluble guanylate cyclase activity is far from being clear. The findings that Ca^{2+} neither activates the enzyme nor is required when the enzyme activity is assayed in broken cell preparations have led to the conclusion that its role is an

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3, inositol trisphosphate; fura-2/AM, fura-2 acetoxymethyl ester; quin2/AM, quin2 acetoxymethyl ester; CBC, carbamylcholine; PI, phosphoinositide; IPs, inositol phosphates; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TLC, thin layer chromatography.

indirect one (11). As a plausible alternative, it has been suggested that Ca^{2+} might enhance the release of polyunsaturated fatty acids, through activation of Ca^{2+}-sensitive enzymes such as phospholipase A_2 (12). In support of this contention stands the evidence that arachidonic acid or its oxidized products activate soluble guanylate cyclase in both crude and purified enzyme preparations (13–15). Moreover, because lipoxygenase inhibitors have been reported to inhibit cyclic GMP synthesis induced by receptor agonists in intact cells, it has been speculated that the products of this pathway could play an active role in receptor-mediated cyclic GMP formation (16–19).

In the present study, we have examined the relative importance of Ca^{2+} mobilization and arachidonate accumulation in cyclic GMP synthesis induced by muscarinic agonists, histamine, and Ca^{2+} ionophores in cultured mouse neuroblastoma cells (clone N1E-115). We provide evidence that strongly suggests that raising $[Ca^{2+}]_i$ is a necessary condition for receptor-mediated activation of guanylate cyclase and that the release of arachidonic acid by itself is not sufficient to activate cyclic GMP formation. In fact, arachidonic acid appears to play an inhibitory role in this process.

Experimental Procedures

Cell culture conditions. Mouse neuroblastoma clone N1E-115 cells (passage 10–15) were grown in 20 ml of Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum, in 75-cm^2 Corning culture flasks. Cells were incubated at 37° in a humidified atmosphere consisting of 10% CO_2 and 90% air. Cell culture procedures were similar to those described previously (20). Cells were utilized for experiments 15–20 days after subculture.

Cyclic GMP measurements. Cells were harvested using Puck's D_1 solution (20) and collected by low speed (250 × *g*) centrifugation for 1 min at 4°. The pellet was washed once with 20 mM HEPES buffer containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM glucose, pH 7.35–7.4, adjusted with sucrose to 334–340 mOsm. Cells were labeled with 10 μCi/ml [^{3}H]guanosine in 2 ml of HEPES buffer at 37° for 60 min. Labeled cells were washed, diluted with HEPES buffer (37°), distributed into multiwell trays (0.1–0.2 × 10^6 cells/well), and then preincubated for 15 min (or as indicated otherwise in the text) at 37°. Cells were stimulated with receptor agonists or activators for the times specified in the text, in a final volume of 0.3 ml at 37°. The reaction was terminated by using 5% trichloroacetic acid and cyclic [^{3}H]GMP was separated by ion exchange chromatography as described previously (20), using cyclic [^{14}C]GMP as internal standard. CBC, histamine, and sodium azide were prepared in HEPES buffer, whereas A23187 and ionomycin were dissolved in dimethyl sulfoxide (final concentration, <0.1%). Melittin was heated at 110° for 3 hr at pH 6 before use, in order to inactivate contaminating phospholipase A_2 activity (21).

Measurement of PI hydrolysis. Harvested cells were labeled with *myo*-[^{3}H]inositol (50 μCi/2 ml) in HEPES buffer for 60 min at 37°. Labeled cells were washed once with HEPES buffer containing 10 mM LiCl, by low speed centrifugation. The pellet was resuspended in HEPES, 10 mM LiCl buffer, distributed into glass tubes (0.1–0.2 × 10^6 cells/tube), and equilibrated for 15 min at 37°. Cells were incubated with agonists or activators and the reaction was stopped by addition of 1 ml of chloroform/methanol/HCl (2:1:0.01, v/v), followed by vortex mixing and placement in an ice bath for 15 min. The phases were separated by the addition of 250 μl of water containing 0.5 nCi of [^{14}C]inositol-1-phosphate and 250 μl of chloroform, followed by centrifugation at 500 × *g* for 10 min at 4°. Aliquots (700 μl) of the aqueous phase were transferred to Dowex AG1-X8 (formate form) columns in order to isolate [^{3}H]IPs, according to the procedure of Berridge *et al.* (22). The columns were washed with 10 ml of water and 20 ml of 5 mM sodium tetraborate, 60 mM ammonium formate. Total [^{3}H]IPs were eluted with 4 ml of 1 M ammonium formate, 0.1 M formic acid, and collected into 20-ml scintillation vials. Radioactivity as ^{3}H and ^{14}C was determined and corrected for counting efficiencies and recovery of [^{14}C]inositol-1-phosphate. In IP_3 measurements, experiments were performed as above, except that columns were washed sequentially with 20 ml of 5 mM sodium tetraborate, 60 mM ammonium formate, 5 ml of 0.1 M ammonium formate, 0.1 M formic acid, and 10 ml of 0.4 M ammonium formate, 0.1 M formic acid, followed by collection of IP_3 in 5 ml of 1.0 M ammonium formate, 0.1 M formic acid.

Determination of cytosolic free Ca^{2+} concentration. The cytosolic free Ca^{2+} concentration in N1E-115 cells was quantitated by using the Ca^{2+}-sensitive fluorescent probe fura-2, according to the method of Grynkiewicz *et al.* (23). Detached N1E-115 cells at a density of 2 × 10^6 cells/ml were incubated in HEPES buffer with 5 μM fura-2/AM, for 60 min at 37° in the dark. Cells were centrifuged, washed twice with buffer to remove extracellular dye, and then resuspended in HEPES buffer at a concentration of 10^6 cells/ml. Two milliliters of cell suspension were transferred to a quartz cuvette in a Perkin Elmer LS-5 spectrofluorometer. In order to stabilize resting signals, the cell suspension was constantly stirred and maintained at 37° for at least 5 min before fluorescence measurement. Fluorescence changes were monitored continuously after stimulation of the cells, using an excitation wavelength of 340 nm and an emission wavelength of 510 nm with slit widths of 5 and 10 nm, respectively. Because the spectrofluorometer is not set up for simultaneous excitation at dual wavelengths, $[Ca^{2+}]_i$ levels are shown as a relative change in fluorescence units above baseline. It is to be noted, however, that the same cell concentrations and instrument sensitivity settings were used within each set of experiments. In some cases, the absolute resting $[Ca^{2+}]_i$ level was monitored by determination of the ratio between the fluorescence emission intensities of the probe (at 510 nm) excited at two different wavelengths, 340 and 380 nm, being switched manually. From the ratio $R = F_{340}/F_{380}$, basal $[Ca^{2+}]_i$ was calculated to be ~100 nM, according to the equation described by Grynkiewicz *et al.* (23), $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$, where K_d is the dissociation constant of the Ca^{2+}/fura-2 complex, estimated to be 225 nM in the cytosolic environment (23); R is the ratio of the fluorescence intensities obtained at 340 and 380 nm, determined at saturating Ca^{2+} (R_{max}) and low Ca^{2+} concentrations (R_{min}) by using 10 μM ionomycin and 5 mM $MnCl_2$, respectively; and β is $F_{380_{min}}/F_{380_{max}}$. In order to avoid the leakage of fura-2 from loaded cells, each sample was incubated separately with fura-2/AM, washed, and resuspended in fura-2-free buffer immediately before the beginning of fluorescence measurement. There was no measurable leakage of fura-2 during the time scale of the experiments (up to 15 min after washing of extracellular fura-2/AM). Each set of experiments was performed with cells from the same tissue culture flask, in order to minimize bias resulting from variability of the Ca^{2+} response.

Assay of arachidonate release. Confluent stationary phase N1E-115 cells were labeled with 10 μCi of [^{3}H]arachidonic acid for 18–24 hr, in 10 ml of complete growth medium. After being harvested from the flask with D_1 solution and washed once with HEPES buffer containing 1 mg/ml fatty acid-free bovine serum albumin, cells were pelleted, resuspended, and distributed into glass tubes (0.1–0.2 × 10^6 cells/tube). After equilibration for 15 min at 37°, the reaction was initiated by the addition of 30 μl of agonists or activators, in a final volume of 0.3 ml. At the specified time, the assay was terminated by the addition of 1 ml of 2,2,4-trimethylpentane/isopropanol/H_2SO_4 (10:40:1, v/v), vortex mixing, and placement in an ice bath. Unlabeled arachidonic acid and 1,2-dioleoyl-*rac*-glycerol were added as carriers for visualization of the lipids on TLC plates. To separate the phases, 500 μl of 2,2,4-trimethylpentane was added to each tube, followed by vortexing and centrifugation at 500 × *g* for 10 min at 4°. The organic phase (600 μl) was transferred to a new glass tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in chloroform and applied to 10 × 10 cm high performance TLC silica gel 60 plates. The TLC plates were developed in hexane/diethyl ether/acetic acid (70:30:3.5, v/v) and the lipids were visualized with iodine vapors.

Radioactivity was determined by scraping of regions of TLC plates into scintillation vials, addition of 5 ml of Budget-Solve, and quantitation by liquid scintillation counting.

Data analysis. Cell counts were obtained using a Coulter Counter (Model Zm). Dose-response curves of CBC-induced cyclic GMP formation and PI hydrolysis were fitted according to a four-parameter logisitic function by nonlinear regression analysis, using the GraphPAD computer program. Free extracellular Ca^{2+} concentrations after the addition of EGTA were calculated using the computer program SKINla, provided by Dr. Chi-Ming Hai (University of Virginia School of Medicine).

Materials. Dulbecco's modified Eagle's medium and newborn calf serum were purchased from GIBCO. [8-^{3}H]Guanosine (8 Ci/mmol) and [^{3}H]arachidonic acid (91 Ci/mmol) were purchased from DuPont-New England Nuclear. [8-^{14}C]Guanosine 3′,5′-cyclic monophosphate, ammonium salt (52 mCi/mmol), was obtained from Amersham Corp. *myo*-[^{14}C]Inositol-1-phosphate (250 mCi/mmol) and *myo*-[2-^{3}H]inositol (15 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. Ionomycin, quin 2/AM, fura-2/AM, and phospholipase A_2 from porcine pancreas were obtained from Calbiochem. High performance TLC-silica gel 60 plates were obtained from Merck. Lipid standards, 1,2-dioleoyl-*rac*-glycerol, arachidonic acid, melittin, and all other chemicals were purchased from Sigma Chemical Co.

Results

Correlation between muscarinic agonist-induced cyclic GMP formation and PI hydrolysis. CBC caused a time- and concentration-dependent increase in both the intracellular content of cyclic GMP and total IPs in N1E-115 cells (Fig. 1). The time course of CBC-mediated cyclic GMP formation was very rapid and transient, with a peak between 30 and 45 sec followed by a return to basal levels within 4 min, whereas accumulation of IPs peaked at 10–15 min and remained elevated up to 30 min (Fig. 1). However, the addition of 1 mM CBC in the absence of LiCl induced the formation of [^{3}H]IP_3 with a time course similar to that of cyclic GMP formation (Fig. 1, *inset*). Moreover, CBC-stimulated [^{3}H]IPs accumulation and cyclic GMP formation occur at a similar concentration range in N1E-115 cells, with an EC_{50} of 202 ± 17 and 153 ± 38 μM (means ± standard errors, five experiments), respectively (data not shown). These values are not significantly different ($P > 0.05$).

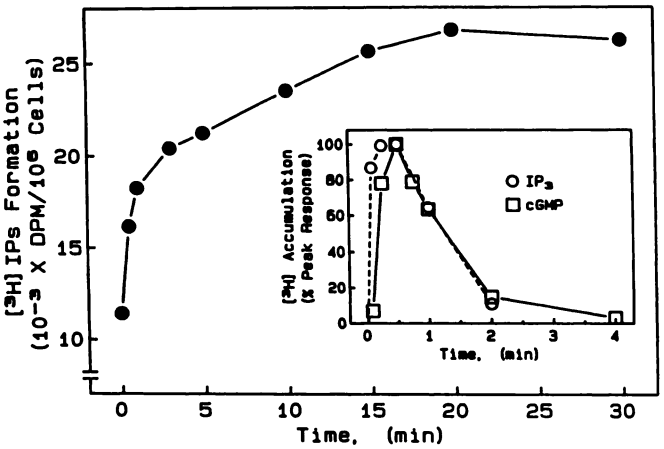

Fig. 1. Time course of CBC-stimulated [^{3}H]IPs and cyclic [^{3}H]GMP formation and [^{3}H]IP_3 accumulation (*inset*) in N1E-115 cells. Cells were prepared and incubated with [^{3}H]guanosine (for cyclic GMP assay) or *myo*-[^{3}H]inositol (for [^{3}H]IPs assay) at 37° for 60 min. CBC (1 mM) was added for the specified times and cyclic [^{3}H]GMP (□) or total [^{3}H]IPs in the presence of 10 mM LiCl (●) were assayed as described in Experimental Procedures. (○) time course of CBC-induced accumulation of [^{3}H]IP_3 assayed in the absence of LiCl. Data shown are representative of three independent experiments, performed in triplicate.

Furthermore, it has been shown that muscarinic agonists differ in their maximal response in activating cyclic GMP formation in N1E-115 cells (24). As shown in Table 1, a close correlation was found when the magnitude of the stimulation of cyclic GMP formation by these agonists at maximally effective concentrations was compared with the magnitude of maximal [^{3}H]IPs accumulation.

Relationship between Ca^{2+} mobilization and cyclic GMP formation. Due to the observed close association between the cyclic GMP and the PI responses, experiments were designed to examine the extent to which Ca^{2+} mobilization is related to cyclic GMP synthesis in N1E-115 cells. The $[Ca^{2+}]_i$ levels were raised using the Ca^{2+} ionophore ionomycin and cyclic GMP formation was measured. As shown in Fig. 2A, ionomycin caused a significant increase in cyclic GMP synthesis, in a time-dependent manner. After N1E-115 cells were loaded with the fluorescent Ca^{2+} indicator fura-2/AM, ionomycin induced a profound rise in $[Ca^{2+}]_i$, which was sustained up to 10 min (Fig. 2C). This time course corresponded well to that of cyclic GMP accumulation. The prolonged Ca^{2+} signal induced by ionomycin was not due to an effect of the ionophore on fura-2 leakage. On the other hand, A23187 elicited a transient cyclic GMP response, with a time course similar to that of CBC (not shown). Unfortunately, it was not possible to measure the effects of A23187 on $[Ca^{2+}]_i$, due to its interference with the fluorescence measurements.

Additionally, CBC and histamine also induced significant rapid and transient increases in $[Ca^{2+}]_i$ (Fig. 2C). Again, there was a good correspondence between the time course of agonist-induced changes in cyclic GMP levels and that of increased $[Ca^{2+}]_i$ accumulation (Fig. 2B). It should be noted, however, that when we tested the effects of each activator on the two responses the rise in $[Ca^{2+}]_i$ always took place earlier than the increase in cyclic GMP formation, suggesting a possible cause and effect relationship.

Source of Ca^{2+} involved in cyclic GMP synthesis. To further investigate the source of Ca^{2+} involved in cyclic GMP synthesis, we chelated intracellular Ca^{2+} by loading N1E-115 cells with quin2/AM and measured the activation of cyclic GMP formation. As shown in Fig. 3A, loading of N1E-115 cells

TABLE 1

Relative maximal responses of muscarinic agonists in inducing PI hydrolysis and cyclic GMP formation in N1E-115 neuroblastoma cells

Cells were incubated with the agonists in the presence of 10 μM physostigmine for 20 min or 30 sec for the PI and cyclic GMP measurements, respectively. Data are expressed as a percentage of the response to acetylcholine (means ± standard error; three experiments).

Agonist	Maximal response	
	PI hydrolysis	Cyclic GMP formation[a]
	%	
Acetylcholine (0.1 mM)	100	100
Carbamylcholine (10 mM)	111 ± 2	99 ± 2
Methacholine (1 mM)	107 ± 7	96 ± 3
Bethanechol (1 mM)	24 ± 2	18 ± 2
Arecoline (1 mM)	14 ± 1	6 ± 1
Pilocarpine (1 mM)	11 ± 3	2 ± 1
McN-A-343 (1 mM)	1 ± 1	2 ± 1

[a] Data were obtained from Ref. 24.

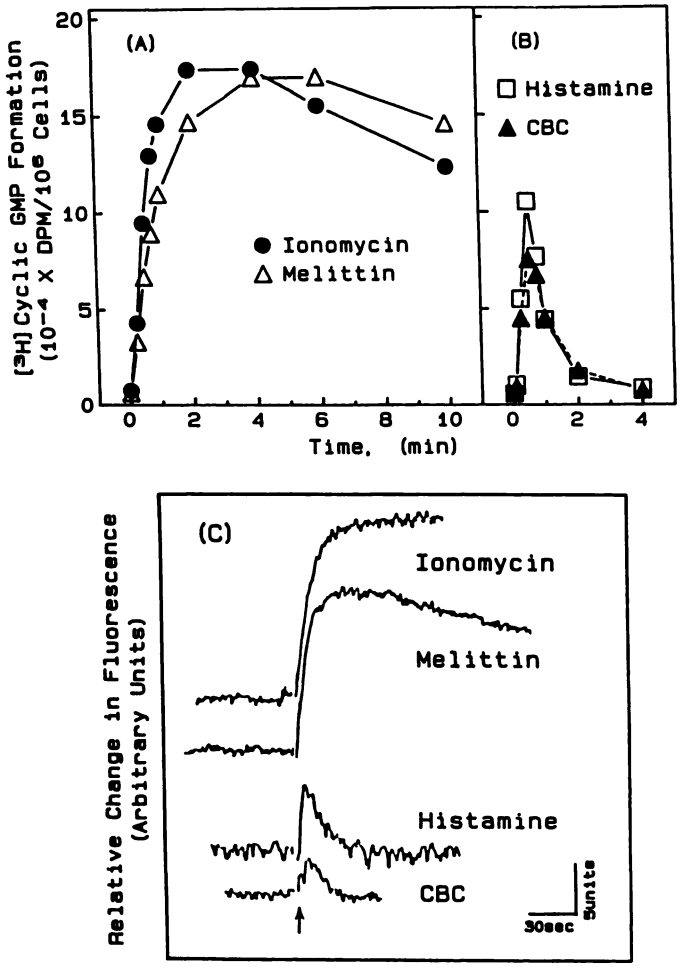

Fig. 2. Time course of cyclic [^{3}H]GMP formation (A and B) and increased [Ca^{2+}]$_i$ (C) induced by various activators in N1E-115 cells. For cyclic GMP measurements (A and B), cells were incubated with [^{3}H]guanosine for 60 min at 37° and then activated by ionomycin (10 μM), heated melittin (10 μg/ml), histamine (0.1 mM), or CBC (1 mM) for the indicated time. For intracellular free Ca^{2+} measurements (C), cells were incubated with 5 μM fura-2/AM for 60 min at 37° and then washed twice. After equilibration for 5 min at 37°, cells were stimulated with the various activators at the same concentrations as in A and B. *Vertical arrow*, time of addition of the activators. Data shown are representative of at least three independent similar experiments.

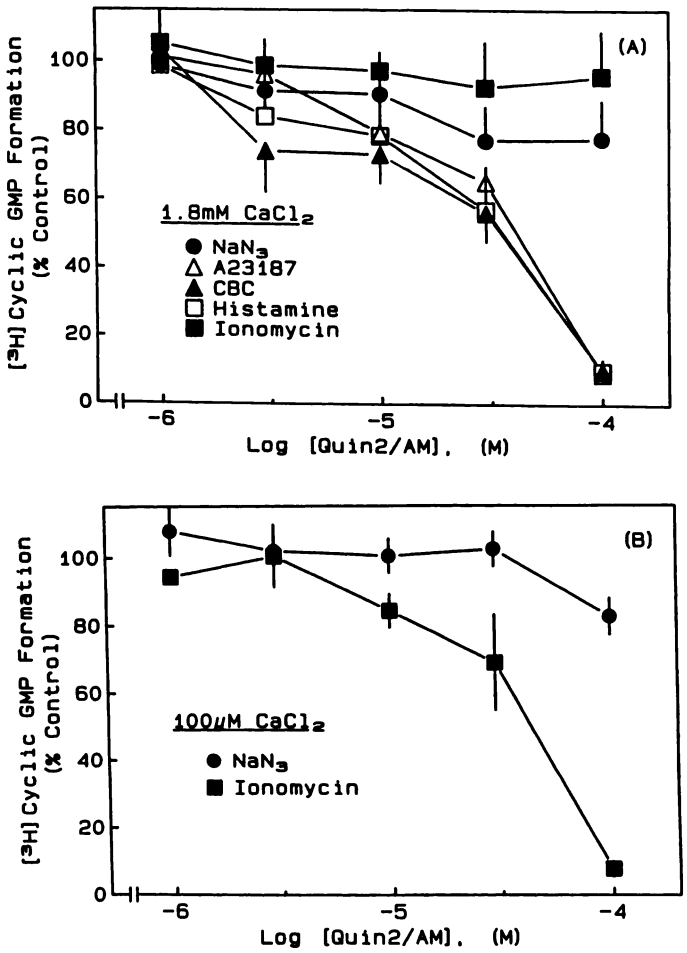

Fig. 3. Effect of chelation of intracellular Ca^{2+} by quin2/AM on cyclic [^{3}H]GMP responses induced by various activators in N1E-115 cells. Cells were prepared for cyclic [^{3}H]GMP assays as described in Experimental Procedures and then pretreated with or without increasing concentrations of quin2/AM for 60 min at 37° in the dark. A, Cells were washed and incubated with NaN_3 (5 mM, 5 min), A23187 (10 μM, 30 sec), ionomycin (10 μM, 3 min), CBC (1 mM, 30 sec), or histamine (0.1 mM, 30 sec) in normal extracellular free Ca^{2+} concentration (1.8 mM). B, Cells were incubated in low extracellular free Ca^{2+} concentration (~100 μM) that was adjusted by the addition of 1.7 mM EGTA to normal buffer at pH 7.4 and were then stimulated with 10 μM ionomycin for 3 min or 5 mM NaN_3 for 5 min. The results are means ± standard errors of three or four independent experiments performed in triplicate.

with quin2/AM caused a dose-dependent attenuation of the cyclic GMP response to CBC, histamine, and A23187 but not to ionomycin, at physiological extracellular free calcium concentrations (1.8 mM). These results can be accounted for by the fact that ionomycin produces a massive elevation in [Ca^{2+}]$_i$, due to both increased influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular pools, resulting in saturation of Ca^{2+} binding sites of quin2 (see below). Therefore, experiments with ionomycin were repeated at low extracellular concentrations of Ca^{2+} (~100 μM, obtained by addition of 1.7 mM EGTA to buffer containing 1.8 mM Ca^{2+} at pH 7.4). Under these conditions, quin2/AM loading induced a reduction of the cyclic GMP response to ionomycin in a concentration-dependent manner (Fig. 3B). At both calcium concentrations, loading with quin2/AM did not interfere with the activation of cyclic GMP formation by sodium azide (Fig. 3), indicating that this Ca^{2+} chelator does not cause direct inhibition of guanylate cyclase. Moreover, agonist-mediated PI hydrolysis was not attenuated by 100 μM quin2/AM (Table 2). These data suggest that the

TABLE 2

Lack of effect of intracellular Ca^{2+} depletion by quin2/AM on agonist-induced [^{3}H]IPs formation

Cells were prepared, incubated with *myo*-[^{3}H]inositol for 60 min at 37°, and then incubated without (control) or with 100 μM quin2/AM for another 60 min in the dark. Cells were washed and stimulated with 1 mM CBC or 0.1 mM histamine for 20 min at 37°. The results are represented as means ± standard errors from four independent experiments. The values obtained after treatment with quin2 were not significantly different from those obtained in control cells ($p > 0.05$).

	[^{3}H]IPs formation
	% of control
Carbamylcholine	120.14 ± 29.44
Histamine	116.22 ± 13.49

observed effects of quin2 on cyclic GMP formation are most likely due to its Ca^{2+}-chelating properties, which decrease the intracellular concentration of Ca^{2+} and, thus, interfere with guanylate cyclase activation.

To probe further into the subcellular source of Ca^{2+} required

for guanylate cyclase activation by agonists, we measured the cyclic GMP response under reduced extracellular concentrations of Ca^{2+}. This was achieved by adding a final concentration of 3 mM EGTA to normal buffer (1.8 mM Ca^{2+}) for 2 min, to adjust the extracellular free calcium to close to the resting intracellular free Ca^{2+} level (~100 nM) in these cells. CBC- and histamine-induced elevation in $[Ca^{2+}]_i$ under these low extracellular Ca^{2+} conditions was similar in both magnitude and time profile to the corresponding controls in normal Ca^{2+} (Fig. 4, D and E), suggesting that these receptors activated the release of Ca^{2+} from intracellular pools. However, the $[Ca^{2+}]_i$ rise induced by these agonists was abolished by pretreatment with 3 mM EGTA for 30 min (Fig. 4, D and E), probably due to a depletion of intracellular Ca^{2+} pools. Furthermore, using the same protocol to assay the formation of cyclic [^{3}H]GMP, preexposure of cells to 3 mM EGTA for 2 min caused only a slight decrease in the response induced by CBC and histamine, when compared with their controls, without altering the time course of the response (Fig. 4, A and B). Again, agonist-mediated cyclic GMP formation was almost abolished in cells pretreated with 3 mM EGTA for 30 min (Fig. 4, D and E).

Similar experiments with ionomycin showed a very rapid and transient rise in $[Ca^{2+}]_i$ when cells were exposed to 3 mM EGTA for 2 min, whereas this ionophore showed a more sustained response in cells under normal extracellular Ca^{2+} concentrations (1.8 mM Ca^{2+}) (Fig. 4F). These data suggest that the $[Ca^{2+}]_i$ elevation response to ionomycin is the result of both Ca^{2+} influx across the plasma membrane (a sustained response)

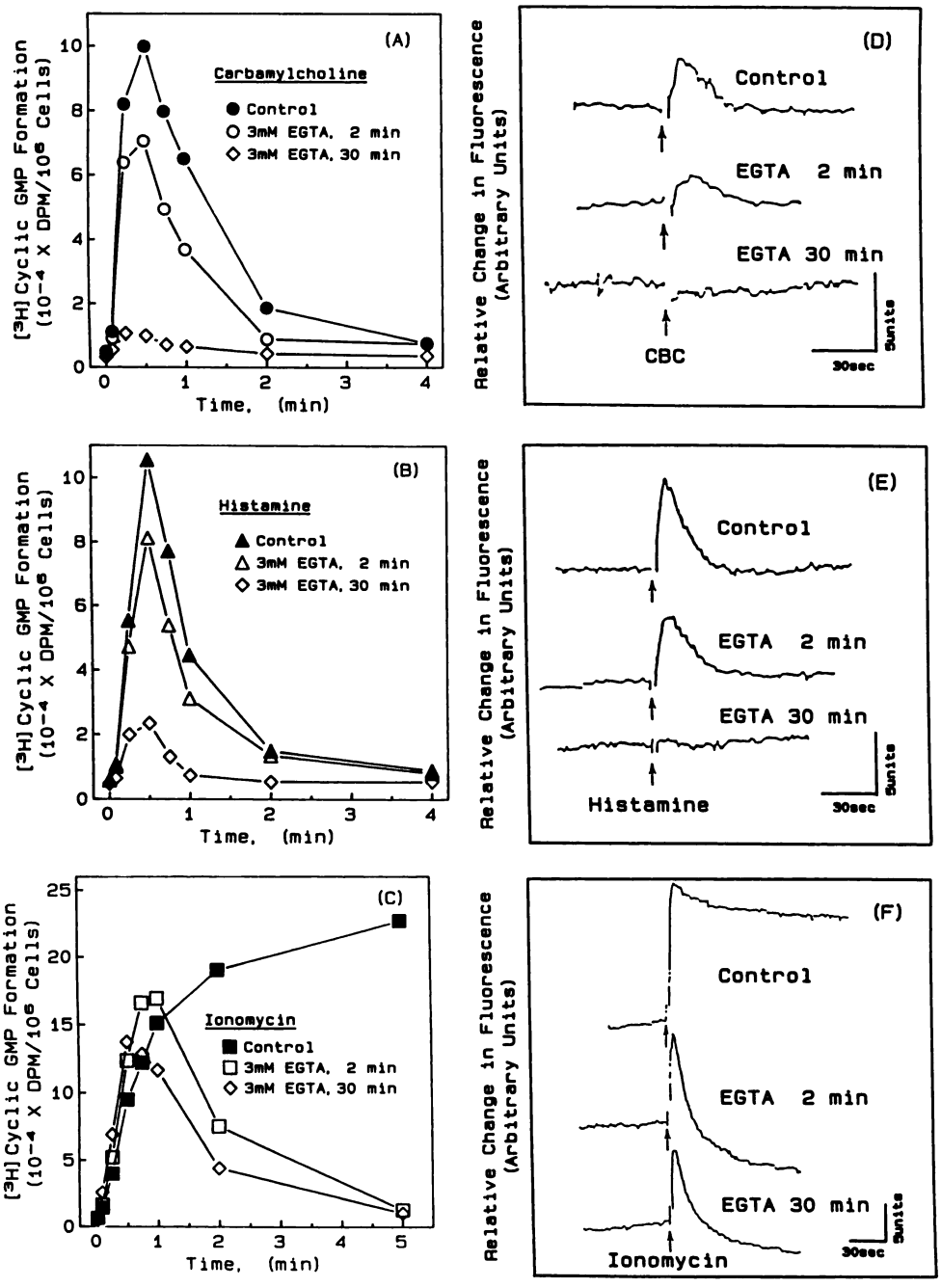

Fig. 4. Effects of short and long term preincubation with 3 mM EGTA on CBC-, histamine-, and ionomycin-induced cyclic [^{3}H]GMP formation and $[Ca^{2+}]_i$ elevation in N1E-115 cells. After incubation with [^{3}H]guanosine for 60 min at 37°, cells were pretreated without or with 3 mM EGTA for 2 or 30 min and then stimulated with 1 mM CBC (A), 0.1 mM histamine (B), or 10 μM ionomycin (C) for the specified time. Cyclic [^{3}H]GMP was isolated as described in Experimental Procedures. For the $[Ca^{2+}]_i$ response, cells were loaded with 5 μM fura-2/AM for 60 min at 37° in the dark, washed twice, equilibrated for 5 min at 37°, and then pretreated without or with 3 mM EGTA for 2 or 30 min. Cells were stimulated with 1 mM CBC (D), 0.1 mM histamine (E), or 10 μM ionomycin (F). Data shown are representative of three similar experiments.

and redistribution from intracellular pools (a transient response). There was an excellent correspondence between the time course of cyclic GMP formation and the changes in intracellular Ca^{2+} in response to ionomycin at both extracellular Ca^{2+} concentrations. It should be noted, however, that the intracellular Ca^{2+} pools that are sensitive to ionomycin and the cyclic GMP response to this agent are less sensitive to pretreatment with EGTA for 30 min (Fig. 4, C and F). In contrast, short term treatment with EGTA did not influence the time course or maximal cyclic GMP synthesis induced by A23187 (data not shown). These results suggest that this Ca^{2+} ionophore might increase $[Ca^{2+}]_i$ by its mobilization from intracellular pools.

Testing of the role of arachidonate release in cyclic GMP synthesis. In an attempt to determine the relative importance of arachidonate in the activation of guanylate cyclase in N1E-115 cells, we studied the effects of exogenously added phospholipase A_2 on the intracellular levels of cyclic GMP. However, phospholipase A_2 (0.1–320 units/ml) produced only a 2-fold increase in cyclic GMP formation in intact N1E-115 cells (see Fig. 8 and data not shown). This is in contrast to the profound increases in the levels of this cyclic nucleotide induced by either CBC and histamine (5–10-fold) or ionomycin and A23187 (30–40-fold). This effect of phospholipase A_2 showed a time course that varied significantly from one experiment to another and was not concentration dependent (data not shown). On the other hand, phospholipase A_2 induced a significant release of arachidonate in a time- and concentration-dependent manner (Fig. 5). No changes in $[Ca^{2+}]_i$ were observed after incubation of cells with phospholipase A_2 (data not shown).

Furthermore, we also studied the effects of melittin on arachidonate release and cyclic GMP formation, because it was shown that this peptide induces a massive release of arachidonic acid as well as an increase in $[Ca^{2+}]_i$ in N1E-115 cells loaded with aequorin (18). However, melittin prepared from bee venom is often contaminated with phospholipase A_2; thus, it was necessary to inactivate this phospholipase A_2 activity by treatment at high temperature (21). Unheated melittin stimulated arachidonate release linearly with time up to 6 min (Fig. 6), whereas it caused a rapid increase in cyclic GMP that reached a steady state after 3–4 min (Fig. 7). In addition, although the stimulation of arachidonate accumulation by melittin was abolished by the heating process (Fig. 6), its effect on cyclic GMP formation was maintained and even increased in magnitude at all time points studied (Fig. 7). These results suggest that the effects of melittin on cyclic GMP formation are not related to its ability to release arachidonic acid. On the other hand, both unheated and heated melittin induced a similar increase in $[Ca^{2+}]_i$, with a time course that paralleled that of the increase in cyclic GMP formation (data not shown).

Because heated melittin caused a higher magnitude of the cyclic GMP response, when compared with that of unheated melittin, and due to the failure of phospholipase A_2 to signifi-

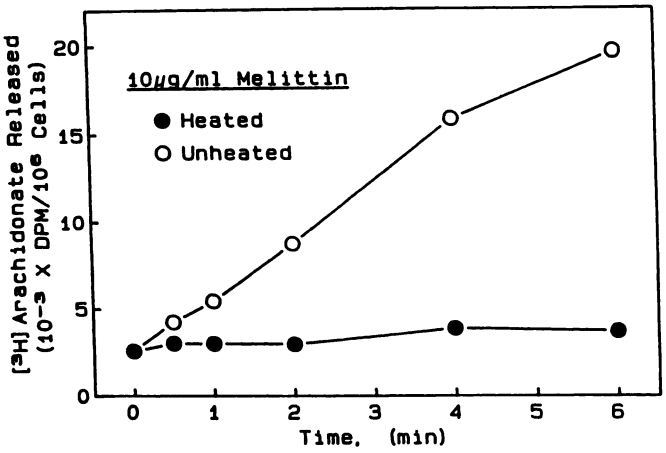

Fig. 6. Effect of heat inactivation on the time course of melittin-mediated [^{3}H]arachidonate release in N1E-115 cells. Cells were prepared for assay as in Fig. 5, followed by stimulation with 10 μg/ml of either unheated melittin or heated melittin for the time indicated. [^{3}H]Arachidonate release was assayed as described in Experimental Procedures. Data shown are representative of three similar experiments performed in duplicate.

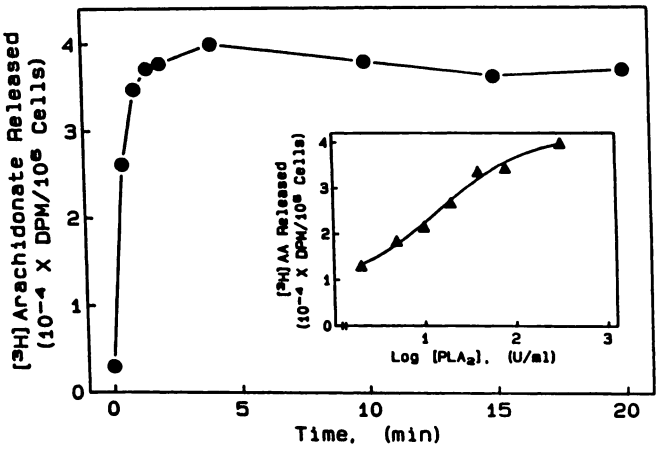

Fig. 5. Phospholipase A_2-induced [^{3}H]arachidonate accumulation in N1E-115 cells. Cells were incubated in monolayer with 10 μCi of [^{3}H]arachidonic acid in tissue culture medium for 18–24 hr at 37°. Cells were harvested, washed, and stimulated with 20 units/ml phospholipase A_2 for the specified time at 37°. [^{3}H]Arachidonate released was isolated by TLC, as described in Experimental Procedures. *Inset*, concentration dependence of phospholipase A_2-induced [^{3}H]arachidonic acid release measured after 4 min. The results shown are representative of three similar experiments performed in duplicate. *PLA_2*, phospholipase A_2.

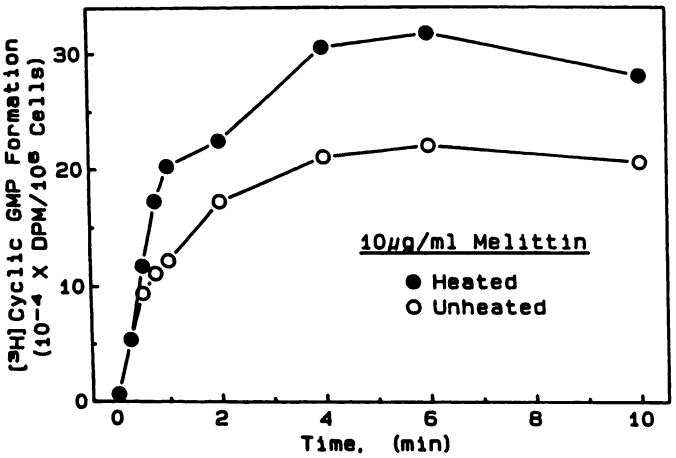

Fig. 7. Effect of heat inactivation on the time course of melittin-activated cyclic [^{3}H]GMP formation in N1E-115 cells. After incubation of cells with [^{3}H]guanosine for 60 min at 37°, cells were stimulated with 10 μg/ml of either unheated melittin or heated melittin for the specified time. The separation of cyclic [^{3}H]GMP was processed as described in Experimental Procedures. The results shown are representative of three independent experiments, performed in triplicate, with similar outcomes. The effects of unheated and heated melittin were significantly different from each other at all time points except at 15 sec ($p < 0.05$).

cantly increase cyclic GMP levels by itself, it was interesting to examine the effects of phospholipase A_2 on the cyclic GMP response induced by ionomycin. Unexpectedly, phospholipase A_2 added around the peak cyclic GMP response reduced the effects of ionomycin, as reflected by a significant shortening of the time course of the response (Fig. 8). In addition, when phospholipase A_2 was added to cells at different times before the addition of ionomycin, it caused a time- and concentration-dependent decrease in ionomycin-induced cyclic GMP synthesis (Fig. 9), in parallel with its ability to increase arachidonate release (compare Figs. 5 and 9).

Discussion

These studies provide evidence for a strong link between receptor-mediated increases in $[Ca^{2+}]_i$ and cyclic GMP formation. In addition, they demonstrate an inhibitory, but not a stimulatory, role of arachidonic acid or its metabolites in cyclic GMP synthesis. Stimulation of muscarinic receptors by their agonists causes an increase in both cyclic GMP formation and PI hydrolysis in N1E-115 neuronal cells (Fig. 1). These two responses are temporally associated when IP_3 synthesis is considered, and the receptor-mediated IP_3 formation takes place earlier than cyclic GMP accumulation. In addition, the EC_{50} of CBC and the maximal response to the different muscarinic agonists for both PI hydrolysis and cyclic GMP synthesis are closely related (Table 1; $r = 0.994$, with a slope of 0.96 for the regression line of correlation). This is consistent with the work of others, which showed that muscarinic agonists, histamine, and neurotensin induce the formation of IPs with the same potency and pharmacological profile as the cyclic GMP response in N1E-115 cells (24–26). Previous studies also have shown that addition of IP_3 to permeabilized N1E-115 cells induces an increase in cyclic GMP formation (27).

All of these lines of evidence are consistent with the hypothesis that receptor occupation by agonists is followed by the activation of an endogenous phospholipase C that breaks down phosphatidylinositol-4,5-bisphosphate. One of its products, IP_3, in turn releases Ca^{2+} from intracellular stores (28–30) to cause activation of guanylate cyclase. In the present study, we have demonstrated that CBC and histamine cause rapid and transient increases in cytosolic free Ca^{2+} levels (Fig. 2B) that were blocked completely by 100 nM atropine and 1 μM pyrilamine, respectively (data not shown), suggesting that these responses are specifically mediated by muscarinic and histamine receptors. This is in contrast to a previous report that muscarinic agonists cause no increase in $[Ca^{2+}]_i$ in N1E-115 neuroblastoma cells loaded with the Ca^{2+} indicator aequorin (18). A possible explanation for this discrepancy may be due to a high background luminescence because of cell injury during the particular loading process employed in the latter study, which might have obscured the agonist-induced $[Ca^{2+}]_i$ response, or due to the higher sensitivity of fura-2 relative to aequorin for the detection of discrete changes in $[Ca^{2+}]_i$. Other studies, however, have shown that histamine and CBC induce a transient increase in $[Ca^{2+}]_i$ in these cells. However, whereas Ohsako and Deguchi (31) reported a Ca^{2+} transient in response to receptor activation, with a peak at about 15 sec and a return to basal levels within 2 min, our data as well as those by Oakes *et al.* (32)

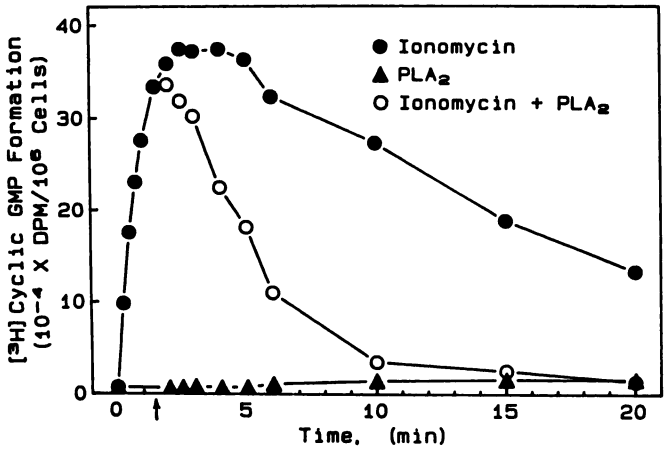

Fig. 8. Inhibitory effect of phospholipase A_2 on ionomycin-induced cyclic [^{3}H]GMP accumulation in N1E-115 cells. Cells were prepared for cyclic [^{3}H]GMP assay as described in Experimental Procedures. Cells were incubated with 10 μM ionomycin alone (●), with ionomycin for 1.5 min followed by addition of 20 units/ml phospholipase A_2 (○), or with buffer for 1.5 min and then with phospholipase A_2 (▲), for the specified times. *Arrow*, point of addition of phospholipase A_2. Data shown are representative of three similar experiments performed in duplicate. Phospholipase A_2 increased cyclic GMP formation 2-fold.

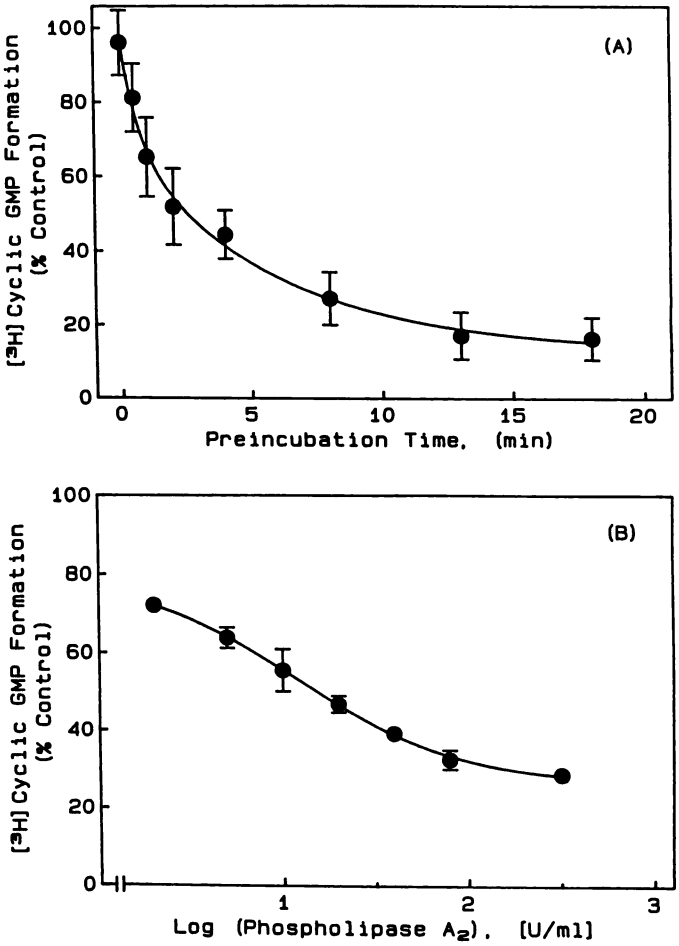

Fig. 9. Time- and concentration-dependent inhibitory effect of phospholipase A_2 on cyclic [^{3}H]GMP formation induced by ionomycin. Cells were prepared and labeled with [^{3}H]guanosine as described in Experimental Procedures. A, Labeled cells were preincubated with 20 units/ml phospholipase A_2 at the indicated times, followed by stimulation with 10 μM ionomycin for another 2 min. B, After incubation with increasing concentrations of phospholipase A_2 for 4 min at 37°, cells were activated by 10 μM ionomycin for 2 min. The results are shown as means ± standard errors of three independent experiments performed in triplicate.

demonstrate that the Ca^{2+} response peaks at 5 sec and declines to basal levels in 30 sec. This difference may be explained by the differences in Ca^{2+} measurement techniques (using fura-2 versus quin2) and experimental conditions (see below).

The time courses for receptor-mediated cyclic GMP and $[Ca^{2+}]_i$ elevations are comparable, with the $[Ca^{2+}]_i$ response preceding cyclic GMP formation stimulated by both receptor agonists and ionomycin (Fig. 2, A and B). Therefore, it is unlikely that the rise in $[Ca^{2+}]_i$ results from the increase in cyclic GMP synthesis. Furthermore, the results from quin2 experiments suggest that the activation of guanylate cyclase by agonists is prevented by chelation of intracellular free Ca^{2+} (Fig. 3). It is unlikely that the rise in $[Ca^{2+}]_i$ generates IP_3, because A23187 induces massive Ca^{2+} mobilization in N1E-115 cells (29) but it does not alter PI turnover (data not shown). In addition, quenching of $[Ca^{2+}]_i$ had no effect on [^{3}H]IPs accumulation induced by receptor agonists (Table 2). Therefore, these findings suggest that the sequence of receptor activation is as follows: PI hydrolysis, Ca^{2+} mobilization, and then guanylate cyclase activation to form cyclic GMP.

In the present study, we also provide evidence that, when the concentration of extracellular Ca^{2+} approximates that of $[Ca^{2+}]_i$, CBC and histamine could still increase $[Ca^{2+}]_i$ levels with a magnitude and time course similar to those observed in cells maintained under normal extracellular Ca^{2+} concentrations. This finding contradicts that reported by others that most of the Ca^{2+} response induced by receptor agonists in N1E-115 cells could be blocked by complexation of external Ca^{2+} with EGTA (32). This discrepancy is probably due to different experimental designs or culture conditions. For example, differentiated cells were used in the latter study but not in the present work. More importantly, we have shown here that the time of exposure of cells to EGTA is critical; it could probably alter the equilibrium between extracellular Ca^{2+} and intracellular Ca^{2+} stores, and this could finally lead to depletion of the latter. These results are consistent with those reported by Casteels and Raeymaekers (33), where exposure of *Taenia coli* to EGTA resulted in a rapid depletion of intracellular Ca^{2+} stores, probably by disturbing mechanisms important in maintaining Ca^{2+} homeostasis. Also, cyclic GMP formation induced by receptor agonists has been shown to be exclusively dependent on the presence of extracellular Ca^{2+} (7). However, our study clearly shows that cyclic GMP formation induced by receptor activation is not altered by a short term decrease in extracellular Ca^{2+} but is affected, similar to the Ca^{2+} response, by long term exposure to EGTA. These results suggest that receptor-mediated Ca^{2+} mobilization from intracellular pools can lead to activation of guanylate cyclase. On the other hand, reduction of extracellular Ca^{2+} changed the time course of the ionomycin-induced increase in $[Ca^{2+}]_i$ from being sustained to being transient, suggesting that ionomycin induces both Ca^{2+} influx and release from intracellular stores. However, it appears that the latter compartment is different from that sensitive to receptor activation, due to its insensitivity to prolonged treatment with EGTA. A similar dependence of ionomycin-induced cyclic GMP formation on extracellular Ca^{2+} was observed. Taken together, our results suggest that an increase of intracellular Ca^{2+} levels, regardless of its source, is an essential factor in the guanylate cyclase activation process. It should be noted, however, that agents that mobilize Ca^{2+} from intracellular stores (e.g., receptor agonists and A23187) elicit a transient increase in cellular cyclic GMP that parallels the transient Ca^{2+} signal. In contrast, agents that cause an additional sustained increase in the influx of extracellular Ca^{2+} produce a long-lasting cyclic GMP response (e.g., ionomycin).

Previous studies have suggested that lipoxygenase-derived metabolites of arachidonate might be important in coupling neurotransmitter receptors to cyclic GMP formation in N1E-115 neuroblastoma cells, based mainly on the ability of phospholipase A_2 and lipoxygenase inhibitors to attenuate receptor-mediated cyclic GMP synthesis (18). However, although quinacrine (a phospholipase A_2 inhibitor) and eicosatetraynoic acid (a lipoxygenase inhibitor) suppress the receptor-mediated cyclic GMP response (18, 19), they also interfere with the response to sodium azide at similar concentrations, suggesting direct inhibition of guanylate cyclase.[3] In addition, it has been shown that quinacrine interacts directly with muscarinic receptors (34). Thus, previous studies utilizing these inhibitors to investigate the role of arachidonic acid in the activation of guanylate cyclase should be interpreted with caution, due to their nonspecific effects. However, using a different approach to study the possible involvement of unesterified arachidonate, we found that, in spite of the marked release of [^{3}H]arachidonic acid caused by the addition of phospholipase A_2, it failed to significantly stimulate cyclic GMP synthesis in intact N1E-115 cells. It should be noted that other possible explanations for the small magnitude of phospholipase A_2-induced cyclic GMP synthesis include its mobilization of arachidonic acid from a pool different from that sensitive to receptor agonists or to the extracellular medium rather than intracellularly. However, we failed to demonstrate any significant increases in arachidonate release in N1E-115 cells by several efficacious activators of guanylate cyclase, including CBC, histamine, A23187, and ionomycin.[4] These results are at variance with those reported previously (18), and we have no explanation for this discrepancy.

In contrast, phospholipase A_2 exerted an inhibitory effect on ionomycin-induced cyclic GMP formation. There was no effect of phospholipase A_2 on cell viability under these conditions. In agreement with these observations, it has been shown that the cyclic GMP response to muscarinic receptor agonists in N1E-115 cells is inhibited by arachidonic acid, its air-oxidized products, and different hydroxyl derivatives of arachidonate (35, 36), suggesting that the observed inhibitory effects of phospholipase A_2 are not due to nonspecific effects. This is supported by the findings that there is a good correlation between the time course and the dose-response relationship in the ability of phospholipase A_2 to induce arachidonic acid release and to inhibit cyclic GMP formation and that the cyclic GMP response to melittin was potentiated by heating. Similar observations have been reported for the effects of arachidonate on nitroprusside-stimulated guanylate cyclase activity (15). It is unlikely that the inhibitory effects of phospholipase A_2 are related to its ability to disrupt the composition of the cell membrane, because guanylate cyclase in N1E-115 cells is mainly (>85%) cytosolic (11). Furthermore, exogenously added phospholipase C, which also disrupts the cell membrane milieu, increases intracellular Ca^{2+} and cyclic GMP levels in these cells.[5] Taken together, our data actually support an inhibitory

[3] W. Surichamorn, unpublished data.
[4] W. Surichamorn, C. Forray, and E. E. El-Fakahany, unpublished data.
[5] W. Surichamorn and E. E. El-Fakahany, manuscript in preparation.

role of arachidonic acid and its metabolites in the activation of guanylate cyclase in intact N1E-115 cells. However, the mechanisms underlying these inhibitory effects remain undefined, because previous evidence indicates that arachidonic acid or its metabolites activate soluble guanylate cyclase in other tissues (15), in sharp contrast to their effects in N1E-115 cells (35, 36).

Thus, the present studies provide several lines of evidence that clearly indicate that Ca^{2+} mobilization is a common intermediate step of receptor-mediated cyclic GMP formation. However, the mechanisms underlying guanylate cyclase activation by Ca^{2+} require further exploration, although consequent activation of cellular phospholipase A_2 to release arachidonic acid does not appear to play a major role. Most likely, the effect of Ca^{2+} on guanylate cyclase is an indirect one, because it has been shown that increasing concentrations of Ca^{2+} progressively inhibited guanylate cyclase activity in subcellular fractions of N1E-115 cells (11). The evidence gathered so far might be taken to suggest that the role of Ca^{2+} might involve the release or synthesis of an endogenous activator of guanylate cyclase in N1E-115 cells that is different from arachidonic acid. Recently, we have demonstrated that activation of muscarinic receptors in N1E-115 cells results in the formation of free radical species derived from L-arginine.[6] Additionally, L-arginine in the presence of Ca^{2+} is capable of fully activating cytosolic guanylate cyclase.[7] Similarly, muscarinic receptor-mediated relaxation of vascular smooth muscle involves the activation of guanylate cyclase by a factor released from the endothelium (37, 38). The tentative identification of this factor as nitric oxide, a direct activator of soluble guanylate cyclase derived from L-arginine, has been proposed (39). Moreover, recent evidence suggests that similar mechanisms of activation of guanylate cyclase are operative in neural tissue (40). Alternatively, activation of intracellular phosphorylation cascades by Ca^{2+} might also play a role. Future studies aimed at demonstrating the role of Ca^{2+} at the molecular level should be fruitful towards an understanding of the mechanisms of activation of soluble guanylate cyclase by neurotransmitters and hormones.

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