The effects of exogenous GM₁ ganglioside on depolarization and ligand-induced Ca²⁺ signaling were investigated in PC12 cells. Cellular responses to K⁺ depolarization and bradykinin application in control and GM₁-treated cells were examined with respect to: 1) changes in the intracellular Ca²⁺ concentration ([Ca²⁺]) measured using fura-2 fluorescence in single cells, and 2) changes in Ca²⁺-dependent protein kinase activity as assayed by two-dimensional phosphopeptide analysis of the site-specific phosphorylation of tyrosine hydroxylase. Pretreatment of cells with GM₁ (10 or 100 μM) enhanced K⁺ depolarization-stimulated increases in [Ca²⁺], and in 3²P₀₄ incorporation into tyrosine hydroxylase phosphopeptide T₂, a Ca²⁺/calmodulin-dependent protein kinase II substrate. In contrast, GM₁ treatment had no effect on the transient increases in [Ca²⁺] evoked by bradykinin or on bradykinin-induced increases in the site-specific phosphorylation of tyrosine hydroxylase. The depolarization-induced and GM₁-enhanced increases in [Ca²⁺]; and T₂ phosphorylation were prevented by removal of external Ca²⁺ or pretreatment with 1 μM nitrendipine, suggesting that these increases result from Ca²⁺ entry through dihydropyridine-sensitive Ca²⁺ channels. The ability of exogenous gangliosides to potentiate increases in [Ca²⁺]; may underlie their diverse neurotrophic and neurotropic actions in the nervous system.

Gangliosides constitute a diverse family of sialic acid-containing glycosphingolipids, which are components of nearly all vertebrate cell surface membranes (1, 2). These molecules are found in relative abundance in nervous tissue where the cell surface location and the temporal and spatial regulation of the expression of specific ganglioside species (3–5) has prompted investigation into the roles that these molecules play in nervous system development and function.

Two functional properties of gangliosides have emerged from studies in which gangliosides have been injected into animals or supplied exogenously to cells in culture (2). First, gangliosides enhance neuronal survival and regeneration in experimental models of central nervous system and peripheral nervous system damage (6, 7). Second, gangliosides promote neurite outgrowth from a variety of clonal cell lines (2), including PC12 cells (8, 9), and in primary cultures of central nervous system neurons (10, 11). The molecular basis of the neurotrophic effects of exogenous gangliosides applied either alone or in combination with growth factors such as nerve growth factor (NGF) is unknown.

Recent work has focused attention on gangliosides as modulators of membrane-associated protein kinases and transmembrane signal transduction events. For example, gangliosides GM₂ and GM₃ inhibit growth factor receptor tyrosine kinase activity (12, 13) and several ganglioside species can inhibit the activity of the Ca²⁺/phospholipid-dependent protein kinase (C kinase) (14). In addition, gangliosides can stimulate a Ca²⁺-dependent protein kinase activity (15) and several other kinases associated with brain (16, 17). In PC12 cells, GM₁ together with NGF stimulates a Ca²⁺-dependent protein kinase that is not normally activated in the NGF signal transduction cascade (18). Thus, the modulation of Ca²⁺-dependent signaling events by gangliosides may underlie their neurotrophic properties in neural cells. The relationships between ganglioside effects on Ca²⁺ signaling pathways and changes in intracellular calcium concentration ([Ca²⁺];) known to be important in the control of neurite extension and growth cone motility (19) have not been examined.

To explore the basis of ganglioside actions on Ca²⁺ signaling pathways in PC12 cells, the effects of exogenous gangliosides on [Ca²⁺];, were examined during K⁺ depolarization, which increases [Ca²⁺];, by promoting Ca²⁺ influx (20–22), and during bradykinin treatment, which causes Ca²⁺ release from internal stores (23, 24). Here we show that short term treatment of PC12 cells with micromolar concentrations of GM₁ ganglioside can enhance depolarization-induced entry of extracellular Ca²⁺ through nitrendipine-sensitive Ca²⁺ channels. The increase in [Ca²⁺];, after ganglioside treatment is sufficient to modulate Ca²⁺-dependent processes in these cells, as GM₁ treatment enhances K⁺ depolarization-induced increases in the activity of a Ca²⁺-dependent protein kinase. In contrast, ganglioside treatment had no effects on the activation by bradykinin of signaling pathways dependent upon the release of Ca²⁺ from intracellular, inositol 1,4,5-trisphosphate (IP₃)-sensitive stores. The ability of exogenous gangliosides to enhance depolarization-induced entry of Ca²⁺ may underlie their diverse trophic and neurotrophic effects on neuronal cells.

**EXPERIMENTAL PROCEDURES**

Materials—Ganglioside GM₁, bradykinin, and nitrendipine were obtained from Sigma. H₃⁻²PO₄ (HCl-free, carrier-free) was obtained

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from ICN Radiochemicals. Fura-2-AM and 4Br-A23187 were purchased from Molecular Probes (Eugene, OR). Gangliosides GD1a and GT1b were a gift of R. K. Yu (Dept. of Biochemistry and Molecular Biophysics, Medical College of Virginia). Omega conotoxin was a gift from Dr. P. Adams (Howard Hughes Institute, Stony Brook, NY). All other reagents and chemicals were of the highest grade commercially available.

**Cell Culture Conditions**—PC12 cells (25) were cultured in DMEM containing 10% horse serum and 5% fetal calf serum (JRH, Lenexa, KS). Cells were incubated at 37 °C in an atmosphere of 90% air, 10% CO2 and were grown to a density of 2 x 10^6 cells/100-mm dish and passed every 5-7 days.

**Fura-2 Cell Loading Conditions**—PC12 cells were cultured in 10% horse serum and 5% fetal calf serum in DMEM on poly-L-lysine/ laminin-coated glass coverslips (22 x 22 mm) at a density of 1-2 x 10^6 cells/cover slip. Measurements were made on cells 2-3 days after plating. Cells were incubated for 2 h at 37 °C in serum-free DMEM with or without G_{M1}, and the cells were loaded with the membrane-permeable form of the Ca^{2+} indicator fura-2 (26) by incubation with 1-2.5 mM fura-2 acetoxymethyl ester in Hepes-buffered DMEM for 30 min at 25 °C. Loading was performed at 25 °C to minimize compartmentalization of the trapped dye. Cells were washed several times with Hepes-buffered DMEM and were incubated in media without fura-2-AM for an additional 15 min to allow for complete destereification of the dye.

**Microfluorimetric Ca^{2+} Measurements**—The equipment, microscope modifications, and software were essentially as described (27). Fluorescence intensities were measured from single cells after providing illumination with alternating excitation wavelengths of 360 and 380 nm. Fluorescence emitted at 505 nm was monitored by photon counting, and [Ca^{2+}], was calculated as described (26, 28). Calibration of fura-2 fluorescence was done using PC12 cells loaded with fura-2 by intracellular patch pipette, and the calibration constants used were those as described (26). To verify the Ca^{2+} sensitivity of the fura-2 fluorescence, Mn^{2+} was loaded into fura-2-containing cells using the Ca^{2+} ionophore 4Br-A23187. The resulting fluorescence signal after Mn^{2+} loading was reduced by 92% (n = 4). For recording, cells were kept at 25 °C and bathed in Hepes-buffered saline, pH 7.2, containing 5.4 mM KCl, 20 mM NaCl, 20 mM Hepes, 25 mM glucose, 1 mM NaH_{2}PO_{4}, 1.5 mM MgSO_{4}, and 5 mM CaCl_{2}. Cells were depolarized by bath exchange of normal Hepes-buffered saline with saline containing concentrations of KCl between 20 and 60 mM with a corresponding reduction in NaCl to maintain isosmolarity. Responses were quantitated by measuring the peak or maximal rise in [Ca^{2+}] i, evoked by each treatment. This method was chosen since the kinetics of the responses of single cells varied due to the time lag required for a complete change in bathing solutions.

**In Situ Protein Kinase Activity Assay**—In situ ^{32}P0_{4} labeling of proteins, peptide mapping procedures, and quantitation of ^{32}P0_{4} incorporation into tyrosine hydroxylase phosphopeptides were as described (29, 30). For labeling experiments, PC12 cells were grown to 10^6 cells/60-mm tissue culture dish. Cells were incubated in a low phosphate DMEM (0.09 mM Na_{2}HPO_{4}) containing 2.0 mM/ml ^{32}P0_{4} for 2 h at 37 °C. Labeling was terminated by removing the culture media and resuspending cells in ice-cold lysis buffer (0.01 M Na_{2}HPO_{4}, pH 7.1, 0.1 M NaF, 0.24 M sucrose, and 0.01 M EDTA). Cells were lysed in the same buffer containing 10% Nonidet P-40, and non-nuclear proteins were extracted and subjected to one-dimensional electrophoresis on 10% SDS-polyacrylamide slab gels (30). ^{32}P-Labeled tyrosine hydroxylase was visualized by autoradiography, extracted from the gel, and subjected to two-dimensional trypptic peptide mapping.

**RESULTS**

**Effects of G_{M1} Pretreatment on K^{+} Depolarization-induced Increases in [Ca^{2+}]**—The effects of K^{+} depolarization on [Ca^{2+}], were first characterized in untreated PC12 cells. As shown in Fig. 1 and Table I, depolarization of cells by bath application of saline containing between 30 and 60 mM KCl resulted in graded increases in [Ca^{2+}]. Changes in [Ca^{2+}], were observed within 30 s of application of elevated K^{+}. This lag can be accounted for by the time required for a complete exchange of the bathing solution, which is estimated at between 15 and 30 s. Depolarization induced an initial peak in [Ca^{2+}], which declined to a lower level that was maintained for several seconds, as shown in Fig. 1A. As described in the Experimental Procedures, the data for peak change in [Ca^{2+}], were obtained after the time lag required for complete exchange of the bathing solution, which is estimated at between 15 and 30 s. A summary of the data for peak increase in [Ca^{2+}], is shown in Table I. Essentially no difference was observed between untreated cells and those treated with 10 or 100 mM G_{M1}, for 2 h immediately before the dye loading. Cells were depolarized by bath exchange of normal saline with saline containing 30, 35, and 60 mM KCl. Solid bar indicates the time period of treatment with high K^{+} saline. Dashed line represents zero Ca^{2+} level. B, effects of removal of [Ca^{2+}], during the depolarization. C, effects of nitrendipine treatment on depolarization-induced increases in [Ca^{2+}]. Calculated [Ca^{2+}], greater than 2 mM are not accurate due to dye saturation. The scales differ between the traces.

**TABLE I**

| Condition | [Ca^{2+}], rest (mM) | [K^{+}] (mM) | Peak increase in [Ca^{2+}] | n |
|-----------|---------------------|-------------|--------------------------|---|
| Un treated | 120 ± 6             | 30          | 195 ± 15                 | 28 |
| G_{M1}-treated | 123 ± 5       | 30          | 263 ± 16                 | 30 |
| Un treated | ND                  | 30          | 1328 ± 216               | 6  |
| G_{M1}-treated | 40                | 30          | 1496 ± 158               | 6  |
| Un treated | ND                  | 60          | 1521 ± 438               | 5  |
| G_{M1}-treated | 60                | 60          | 1720 ± 423               | 5  |

* Significance value = p < 0.005 (t test). Where not indicated, values are not significantly different.  
* ND, not determined.
throughout the exposure to elevated K+. Washout of K+ with normal saline (5.4 mM KCl) caused [Ca2+]i to return to its resting value within 15–30 s. The kinetics of the response varied with [K+]o, as the initial peak rise in [Ca2+]i became more pronounced and occurred with a shorter time lag upon treatment of cells with higher concentrations of [K+]o. Table I gives the peak or maximal rise in [Ca2+]i, over a range of [K+]o. The K+ depolarization-induced increases in [Ca2+]i, were dependent on extracellular Ca2+ as removal of Ca2+ during the exposure to elevated K+ caused [Ca2+]i to return to basal levels (Fig. 1B). Similarly, if the cells were depolarized in Ca2+-free saline, there was no depolarization-induced increase in [Ca2+]i. (data not shown). The pathway of Ca2+ entry during K+ depolarization was sensitive to the dihydropyridine family of channel blockers (Fig. 1C) as application of 1 μM nitrendipine for 5 min prior to depolarization inhibited the peak rise in [Ca2+]i, by 87 ± 5% (mean ± S.E., n = 5). Thus, as reported previously (21), K+ depolarization of PC12 cells results in the rapid and sustained elevation of [Ca2+]i, due to the entry of extracellular Ca2+ through a dihydropyridine-sensitive pathway.

To determine whether GM1 can modulate K+ depolarization induced increases in [Ca2+]i, the peak increases in [Ca2+]i, were compared between cells from untreated and GM1-treated cultures as described under “Experimental Procedures.” Since K+ depolarization-induced increases in [Ca2+]i display considerable variability at [K+]o ≥ 40 mM (Table I), GM1 effects on [Ca2+]i, were examined at a submaximal level of [K+]o (30 mM), such that peak increases in [Ca2+]i could be easily quantitated. Fig. 2A shows [Ca2+]i measurements taken from fura-2-loaded cells treated in the absence or presence of GM1 (10 or 100 μM) for 2 h immediately prior to dye loading. Pretreatment of cells with GM1 enhanced the increase in [Ca2+]i, caused by 30 mM KCl compared to untreated control cells (Table I).

A comparison of the peak increases in [Ca2+]i, resulting from depolarization with 30 mM KCl in untreated and GM1-treated cells is shown in Fig. 2B. The peak increases in [Ca2+]i, in response to 30 mM KCl for 1–3 min ranged from 122 to 410 nM in untreated cells and from 114 to 486 nM in GM1-treated cells, indicating that a similar range of increases in [Ca2+]i, can be achieved in both populations. However, the mean peak increase in [Ca2+]i, in response to 30 mM KCl differed significantly between these treatment groups (untreated cells, 195 ± 15 nM; 10 μM GM1-treated cells, 285 ± 21 nM; 100 μM GM1-treated cells, 276 ± 25 nM; mean ± S.E.). These differences cannot be attributed to effects on the resting [Ca2+]i, as ganglioside pretreatment did not change the resting [Ca2+]i, compared to untreated cells (Table I). These data demonstrate that GM1 pretreatment causes a shift in the mean peak increase in [Ca2+]i, in response to a depolarization with 30 mM KCl without significantly altering either the resting level of [Ca2+]i, or the maximal level of [Ca2+]i, that can be achieved.

The results shown in Fig. 2B reveal a broad range in the magnitude of the increases in [Ca2+]i, evoked in response to 30 mM KCl in both treatment groups, suggesting that these cells are heterogeneous in terms of their ability to respond to a given depolarization. The cell-to-cell variability in these responses was examined by first comparing responses between cells of the same treatment group and then by comparing the responses of a single cell after repeated depolarization with 30 mM KCl. Increases in [Ca2+]i, were determined in several cells from the same culture dish by taking [Ca2+]i, measurements from single cells during depolarization with 30 mM KCl for 90 s with 3–5 min intervals between selection of new cells.

The increase in [Ca2+]i, in response to 30 mM KCl in the untreated group was 213 ± 72 nM; in the GM1-treated group, 282 ± 86 nM (mean ± S.D.), representing a variation of 34% in the response within each group. The variation in [Ca2+]i, in response to a given depolarization in an individual GM1-treated cell was measured during repeated exposure to 30 mM KCl for 90 s with 3-min intervals between applications. The resulting peak change in [Ca2+]i, varied by less than 10% from the mean (304 ± 27 nM, mean ± S.D., four determinations). These results demonstrate that although an individual cell responds in a highly reproducible manner to a given depolarization, comparison of the responses of cells within the same culture dish shows a significant degree of variability. Thus, comparison of responses between cells from GM1-treated or untreated cultures would likely reveal a degree of overlap in the peak increases in [Ca2+]i, in response to a depolarization and this overlap may account, in part, for the lack of effect of GM1 pretreatment in some cases.

Analysis of GM1 Effects on [Ca2+]i, in Individual Cells—The effects of GM1 on K+ depolarization-induced changes in [Ca2+]i, in the same cell were analyzed by comparing peak increases in [Ca2+]i, prior to and immediately after a 10-min
exposure to GM1. Fig. 3 shows [Ca2+]i measurements taken from fura-2-loaded cells during depolarization with 30 mM KCl followed by washout with normal saline and application of either normal saline (Fig. 3A) or saline containing 10 (Fig. 3B) or 100 µM GM1 (Fig. 3C) for 10 min. Comparison of the increases in [Ca2+]i, revealed that GM1 application potentiated the response to 30 mM KCl by ≈30-35% when compared to the response obtained prior to ganglioside treatment in the same cell (Table II). Enhanced responses to GM1 were observed in all cells examined. No differences in depolarization-induced increases in [Ca2+]i were observed in cells treated for 10 min with normal saline (Fig. 3A and Table II). Treatment of cells with the di- and trisialogangliosides GDb4 and GTb4 for 10 min caused a similar enhancement in K+ depolarization-induced increases in [Ca2+]i, in these assays, whereas treatment with other charged or uncharged glycolipids (sulfatide and galactocerebroside) had no effect on depolarization-induced Ca2+ entry (data not shown). These results demonstrate that short term treatments with exogenous ganglioside are sufficient to enhance depolarization-induced increases in [Ca2+]i, when measured in the same cell.

We examined the effects of nitrendipine on the GM1-enhanced entry of Ca2+. Cells were initially depolarized with 30 mM K+, treated with 100 µM GM1 for 10 min, and then depolarized again with 30 mM K+ to measure the ganglioside-induced enhancement of [Ca2+]i (18). Following a 5-min treatment with nitrendipine, the same cell was again depolarized with 30 mM K+. As shown in Fig. 4, treatment with nitrendipine prevented the increases in [Ca2+]i due to depolarization. In 8 separate experiments, the increase in [Ca2+]i in GM1-treated cells was inhibited by 90 ± 4% (mean ± S.E.) by nitrendipine. In this same experimental paradigm, ω-conotoxin (10 µM, 5 min) was ineffective in blocking the depolarization-induced increases in [Ca2+]i, (data not shown). Thus, the enhanced response to K+ depolarization in GM1-treated cells is pharmacologically indistinguishable from the responses in untreated PC12 cells.

Effects of GM1 Pretreatment on K+ Depolarization-induced Phosphorylation of Tyrosine Hydroxylase—The results presented above demonstrate that GM1 treatment can potentiate depolarization-induced increases in [Ca2+]i. To determine whether ganglioside effects on Ca2+ are sufficient to modulate Ca2+-dependent processes in the cells, we examined the site-specific phosphorylation of tyrosine hydroxylase using the same treatment paradigms as in the fura-2 measurements. Short term depolarization with K+ (30 s) resulted in the specific phosphorylation of serine 19 within phosphoprotein T2 (31). Peptide T2 has been identified as a specific substrate for Ca2+-calmodulin-dependent protein kinase II (CaM kinase II) in vitro (31, 32); thus, changes in the phosphorylation state of peptide T2 can be used to assess relative changes in Ca2+-dependent protein kinase activity in intact cells.

Exposure of PC12 cells to 30 mM KCl for 30 s caused a specific increase in the phosphorylation of peptide T2 compared to untreated, control cells (Fig. 5 and Table III). The phosphorylation states of peptides T1, T3, and T4 were not affected by K+ depolarization. Pretreatment of the cells with GM1 for 2 h potentiated K+ depolarization-induced increases in peptide T2 phosphorylation when compared to treatment with K+ alone (Fig. 5 and Table III). Similar results were obtained with a 10-min GM1 treatment (data not shown). Treatment with GM1 alone was previously shown to have no effects on the phosphorylation of tyrosine hydroxylase phosphopeptides (18). The ability of GM1 pretreatment to potentiate K+ depolarization-induced increases in peptide T2 phosphorylation is not likely to be due to an inhibition of phosphatase activity as the rate of loss of [32P]PO4 from pulse-labeled tyrosine hydroxylase did not differ between K+-treated and GM1/K+-treated cells (data not shown). Treatment of the cells with 1 µM nitrendipine for 15 min prior to depolarization

**TABLE II**

*Effects of GM1 treatment on K+ depolarization-induced increases in [Ca2+]i, in single PC12 cells*

| Pretreatment | Posttreatment | Mean ± S.E., n |
|--------------|--------------|----------------|
| Normal saline| 203 ± 29 (3)  |                |
| GM1 (10 µM, 10 min) | 193 ± 30 |                |
| Pretreatment | 236 ± 18 (7) |                |
| Posttreatment| 312 ± 17 (5) |                |
| GM1 (100 µM, 10 min) | 217 ± 38 (5) |                |
| Pretreatment | 331 ± 42 (6) |                |

*Significance value = p < 0.004 (t test). Significance value = p < 0.04 (t test). Where not indicated, values are not significantly different.
FIG. 5. Phosphopeptide mapping analysis of the effects of GM₁ on K⁺ depolarization-induced increases in tyrosine hydroxylase phosphorylation. Top panel (−NTP) shows phosphopeptide maps from cells labeled with ³²P₀₄ alone (control) or incubated in the absence or presence of 10 μM GM₁ for 2 h and treated with 30 mM KCl for 30 s immediately prior to cell harvest and lysis. Bottom panel (+NTP) shows phosphopeptide maps from cells labeled and incubated in the absence or presence of GM₁ for 2 h then pretreated with 1 μM nitrendipine (NTP) for 15 min prior to addition of KCl. Tyrosine hydroxylase was subjected to two-dimensional trypsinic peptide mapping as described under “Experimental Procedures.” Phosphopeptides T₁–T₄ are labeled in the control sample, and the directions of electrophoresis and chromatography are indicated.

Table III
Effects of GM₁ on K⁺ depolarization and bradykinin-induced phosphorylation of tyrosine hydroxylase phosphopeptides

| Condition          | Relative ³²PO₄ incorporation (% of control; mean ± S.E.) |
|--------------------|-------------------------------------------------------|
| 30 mM KCl (30s)    | T₁ 130 ± 18 588 ± 124 94 ± 14 30 mM KCl + GM₁ (30s) | T₂ 126 ± 22 923 ± 188⁶ 107 ± 17 30 mM KCl + GM₁ (100 nM, 1 min) | T₃ 160 ± 33 148 ± 22 99 ± 8 30 mM KCl + GM₁ (190 nM, 5 min) | T₄ 132 ± 27 139 ± 16 107 ± 13 30 mM KCl + GM₁ (100 nM, 2 h) | T₅ 140 ± 36 340 ± 28 425 ± 71 30 mM KCl + GM₁ (100 nM, 3 h) | T₆ 98 ± 21 330 ± 60 330 ± 57

*p < 0.05 ANOVA. The effects of GM₁ pretreatment on the bradykinin samples are not significant.

FIG. 6. Comparison of the effects of GM₁ treatment on K⁺ depolarization and bradykinin-induced increases in [Ca²⁺]

The effects of GM₁ treatment on [Ca²⁺] were compared after K⁺ depolarization and bradykinin application to fura-2-loaded cells as shown in Fig. 6. The cells were treated consecutively with 30 mM KCl, 40 mM KCl, and 100 nM bradykinin with a 3-min washing interval between treatments. GM₁ pretreatment had no effect on bradykinin-induced increases in [Ca²⁺]. In 5 separate experiments, the increase in [Ca²⁺] after bradykinin treatment in control, untreated cells was 508 ± 122 nM (mean ± S.E.). For cells that were pretreated for 2 h with 10 μM GM₁, the peak increase in response to bradykinin was 488 ± 124 nM (mean ± S.E.). In the experiment shown in Fig. 6, GM₁ enhanced the rise in [Ca²⁺] evoked by depolarization with 30 mM KCl, but had no significant effect on the response to 40 mM KCl. The results demonstrate that GM₁ pretreatment can act differentially to enhance the peak increase in [Ca²⁺], produced in response to a submaximal K⁺ depolarization while having no effect on the change in [Ca²⁺] induced by application of bradykinin. Thus, the effects of GM₁ may be limited to a potentiation of increases in [Ca²⁺], occurring via Ca²⁺ entry rather than by agonist-induced Ca²⁺ release from IP₃-sensitive intracellular stores.

Treatment of PC12 cells with bradykinin increases the activity of CaM kinase II within 1 min of application (33) and increases the phosphorylation of tyrosine hydroxylase phos-
phopeptides T2 and T3 when measured after 10 min of exposure to the drug (34). To investigate the effects of GM1 treatment on bradykinin-induced increases in Ca²⁺-dependent protein kinase activity, the site-specific phosphorylation of tyrosine hydroxylase was assayed after 1- and 5-min exposure to the ligand. The effects of GM1 were determined in combination with 100 nM bradykinin, a dose determined to be sub-maximal for bradykinin-induced increases in the phosphorylation of tyrosine hydroxylase (data not shown). As shown in Table III, pretreatment of cells with 10 µM GM1 for 2 h did not alter the site-specific phosphorylation of tyrosine hydroxylase produced by either a 1- or 5-min application of bradykinin. Thus, in contrast to the stimulatory effects of GM1 on Ca²⁺-dependent protein kinase activity during depolarization, GM1 does not enhance Ca²⁺-dependent protein kinase activity after bradykinin treatment.

**DISCUSSION**

The results presented in this study demonstrate that short term exposure of PC12 cells to GM1 ganglioside leads to a potentiation of the cellular responses to depolarization with elevated [K⁺]o. Depolarization-induced increases in [Ca²⁺], resulting from Ca²⁺ influx through dihydropyridine-sensitive Ca²⁺ channels were enhanced significantly in GM1-treated cells compared to control cells. Cellular responses to agents that raise [Ca²⁺], via promoting the release of Ca²⁺ from internal stores were unaffected by GM1 pretreatment. The enhanced levels of [Ca²⁺], achieved after ganglioside treatment may mediate the neuritogenic and neurotrophic properties of gangliosides.

The ability of gangliosides to amplify increases in [Ca²⁺], during depolarization provides a basis for the potentiating effects of gangliosides on a Ca²⁺-dependent protein kinase activity in PC12 cells. CaM kinase II is a likely candidate for this kinase since it can phosphorylate tyrosine hydroxylase in *vitro* at serine 19 (32), which is contained within phophopeptide T2 (31). Depolarization with varying levels of [K⁺], brings about a graded phosphorylation of tyrosine hydroxylase phosphopeptide T2, which is maximal at [K⁺]o ≥ 50 mM (18). A similar dose-response relationship was seen in the [Ca²⁺], measurements reported here. Both types of responses were enhanced when ganglioside pretreatment was combined with sub-maximal depolarization. The simplest explanation of these data is that ganglioside incorporation leads to increased Ca²⁺ entry after sub-maximal depolarization and that the increased levels of [Ca²⁺], achieved by ganglioside treatment enhance the activation of CaM kinase II.

Greater than 90% of the increase in [Ca²⁺], in untreated or GM1-treated cells was abolished by nitrendipine pretreatment implicating L-type Ca²⁺ channels in the responses measured here. The observations that L-type Ca²⁺ channels appear to be responsible for >90% of K⁺ depolarization-induced Ca²⁺ entry (35) and carry >90% of depolarization-induced Ca²⁺ current (36) support with this hypothesis. Additional support for this hypothesis comes from our analysis of the effects of GM1, pretreatment on bradykinin-induced Ca²⁺ signaling events. Bradykinin binding to PC12 cells causes a rapid and transient increase in [Ca²⁺], as a result of IP3-induced release of Ca²⁺ from intracellular stores and of Ca²⁺ entry through a nitrendipine-insensitive pathway (21, 24). Treatment of cells with GM1 ganglioside had no effect on levels of [Ca²⁺], or on Ca²⁺-dependent protein kinase activity stimulated in response to bradykinin. GM1 also had no effect on tyrosine hydroxylase phosphorylation when assayed in combination with the nicotinic cholinergic agonists carbachol and 1,1-dimethyl-4-phenylpiperazine and or with muscarine, all of which stimulate phosphatidylinositol turnover and cause increases in [Ca²⁺], in part via IP3-induced Ca²⁺ release (data not shown). Thus, it is likely that GM1 exerts its effects on cellular physiology by an interaction with a Ca²⁺ signaling pathway involving influx of Ca²⁺ through dihydropyridine-sensitive, L-type Ca²⁺ channels.

We can only speculate on how this specificity of site of action is achieved. The incorporation of negatively charged gangliosides into the outer leaflet of the plasma membrane may modestly increase the negative electrostatic or surface potential (37, 38). This increase in surface potential could effect the functioning of voltage-dependent ion channels in two ways. First, the voltage dependence of gating of channels could be shifted towards more negative potentials (39, 40), such that a sub-maximal depolarization would lead to more channel openings in ganglioside-treated cells as compared to control, untreated cells. Second, the increased surface potential could concentrate cations, including Ca²⁺, in the extracellular space immediately adjacent to the plasma membrane and increase the driving force of current flow through open channels. Consequently, a greater amount of Ca²⁺ would enter the cell. Alternatively, the added gangliosides might associate specifically with L-type Ca²⁺ channels via interactions between the channel and the ganglioside molecule and thereby alter channel function. Whatever the nature of the interactions between added ganglioside and L-type Ca²⁺ channels, the effects we have observed indicate that L-type Ca²⁺ channels are a major cellular target for ganglioside action.

While we cannot directly rule out the possibility that exogenous gangliosides interact with the active cellular processes that regulate calcium homeostasis, such as extrusion by Ca²⁺ ATPases or sequestration into organelles, it seems unlikely for several reasons. First, ganglioside treatment does not alter resting [Ca²⁺]; any effect of exogenous gangliosides on membrane pumps would require a voltage-dependent interaction with those pumps since gangliosides increase [Ca²⁺], only after depolarization. Second, the majority of the exogenously added gangliosides associate with the outer leaflet of the plasma membrane and less than 5% of the added ganglioside reaches the cytoplasm of the cell (41). Thus, it is unlikely that exogenously added gangliosides interact with intracellular organelles. A complete determination of the site of action of exogenous gangliosides will require either whole-cell or single-channel recordings from Ca²⁺ channels in PC12 cells, an approach that is beyond the scope of this study.

Previous studies from our laboratory have demonstrated that GM1 treatment can stimulate a Ca²⁺-dependent protein kinase in NGF-treated PC12 cells (18). Since GM1 can potentiate K⁺ depolarization-induced increases in [Ca²⁺], we examined whether GM1 might effect [Ca²⁺]; in combination with NGF. Using fura-2 measurements in single cells, we did not detect an increase in [Ca²⁺]; in response to NGF (50 ng/ml) in either control or GM1-treated cells (n = 22). These findings are consistent with a previous account which showed no effect of NGF on [Ca²⁺]; (42) but in are in disagreement with the work of others (43, 44) who found small changes in [Ca²⁺]; in single cells (±100 nm) and in populations of cells (25-50 nm). This discrepancy may be due to differences in the sensitivity of the method used for measuring [Ca²⁺]; or in assay conditions, or to variations existing among PC12 cells from different laboratories. Although the effects of NGF on [Ca²⁺]; remain controversial, a subset of NGF actions appear to require Ca²⁺ influx (45, 46). Although the [Ca²⁺]; measurement data reported here do not support a mechanism in which gangliosides potentiate Ca²⁺ entry in the presence of NGF, the synergistic action of these treatments on the activity of a
Ca2+-dependent protein kinase nevertheless requires the entry of extracellular Ca2+ through nitrendipine-sensitive Ca2+ channels.

Calcium appears to be a key second messenger involved in neuronal survival and process outgrowth (19). A number of recent observations implicate gangliosides as modulators of both intracellular Ca2+ levels and Ca2+-dependent processes in neurons. For example, the neurite-promoting effects of exogenous gangliosides on the Neuro-2a neuroblastoma cell line are both dependent upon and accompanied by increased Ca2+ entry (47, 48). The data presented here suggest that gangliosides may exert their actions by enhancing Ca2+ entry through dihydropyridine-sensitive Ca2+ channels. The changes in gene expression, cytoskeletal organization (49), and cell surface properties that occur after such an elevation of [Ca2+]i may be sufficient to enhance neurite outgrowth.

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