Inhibitory effect of ganglioside GD1b on K^+ current in hippocampal neurons and its involvement in apoptosis suppression

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Abstract **Gangliosides are endogenous membrane components enriched in neuronal cells. They have been shown to play regulatory roles in many cellular processes. Here, we show for the first time that ganglioside GD1b plays an antiapoptotic role in cultured hippocampal neurons. GD1b inhibited the voltage-dependent outward delayed rectifier current (I_K) but not the transient outward A-type current in a dose-dependent manner, with an IC_{50} value of 15.2 μM. This effect appears to be somehow specific, because GD1b, but not GM1, GM2, GM3, GD1a, GD3, or GT1b, was effective in inhibiting I_K. Intracellular application of staurosporine (STS; 0.1 μM) resulted in rapid activation of I_K, which was partially reversed upon addition of the K^+ channel blocker tetraethylammonium (TEA; 5 mM) and GD1b (10 μM). Furthermore, GD1b (10 μM) attenuated STS-induced neuronal apoptosis by nearly the same amount as 5 mM TEA. In addition, GD1b suppressed the apoptosis-associated caspase 3 activation that was activated by STS. Collectively, these findings suggest that GD1b plays an antiapoptotic role in cultured hippocampal neurons through its inhibitory effect on the I_K and caspase activity.**—Chen, X., S. Chi, M. Liu, W. Yang, T. Wei, Z. Qi, and F. Yang. **Inhibitory effect of ganglioside GD1b on K^+ current in hippocampal neurons and its involvement in apoptosis suppression.** ***J. Lipid Res.*** **2005.** 46: **2580–2585.**

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Gangliosides are particularly abundant in the nervous system (1).They have been shown to have regulatory roles in many physiological processes, such as nerve growth and differentiation (2, 3), audiogenic seizure (4), induction of apoptosis (5), tumor onset and progression (6), cell signaling (7), modulation of plasma membrane Ca^{2+}-ATPase (8), and involvement in the neurodegenerative gangliosidosis (9). They have also been shown to have protective roles in neuronal apoptosis. For example, mice deficient in complex ganglioside biosynthesis have a high incidence of apoptosis in the nervous system during embryonic development (3); cerebellar neurons from knockout mice lacking complex gangliosides undergo apoptosis easily (10); and predominant expression of b-series complex gangliosides in developing neuroprogenitor cells is relevant to a reduction of ceramide-induced apoptosis (11). These findings suggest that some species of complex gangliosides may play an antiapoptotic role in the nervous system.

On the other hand, a link between K^+ channels and apoptosis has become increasingly recognized (12–14). It has been demonstrated that excessive K^+ efflux promotes apoptosis in cortical (15), hippocampal (16), basal forebrain cholinergic (17), and cerebellar granule neurons (18), whereas a reduction of K^+ efflux by K^+ channel blockers suppresses apoptosis (13, 15). Therefore, in the present study, we attempt to test whether a single component of the ganglioside could inhibit the excessive outward K^+ current and consequently suppress cellular apoptosis in cultured hippocampal neurons. Among the gangliosides tested, we found that only ganglioside GD1b could inhibit the voltage-dependent outward delayed rectifier K^+ current (I_K) and reduce the activity of caspase 3, which in turn resulted in the suppression of neuronal apoptosis induced by staurosporine (STS). These findings indicate that ganglioside GD1b plays an antiapoptotic role in hippocampal neurons and suggest an interrelationship between gangliosides, K^+ channels, and neuronal apoptosis.

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Abbreviations: DEVD-MCA, acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide); DIV, days in vitro; I_A, transient outward A-type current; I_K, voltage-dependent outward delayed rectifier K^+ current; STS, staurosporine; TEA, tetraethylammonium; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

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MATERIALS AND METHODS

Cell culture

Hippocampal neurons were acutely dissociated according to a previous method (19) with slight modification. Briefly, the hippocampi were dissected from neonatal Sprague-Dawley rats (aged within 24 h; Weitonglihua Animal Center, Beijing, China), and neurons were dissociated by incubation (7 min, 37°C) in trypsin-EDTA (GIBCO) and triturated in DMEM (Life Technologies) supplemented with 10% bovine serum (Hyclone). The resulting hippocampal cells, at a density of 2×10^5 cells/cm^2 on poly-L-lysine (Sigma)-coated coverslips, were cultured in a humidified incubator in 5% CO_2 at 37°C. The medium was replaced 7 h later with Neurobasal Medium, B-27 (GIBCO), and 0.5 mM glutamine without antibiotic solution. After 48 h, the medium was changed to Neurobasal Medium and B27.

Whole-cell patch-clamp recordings

Hippocampal neurons between 6 and 10 days in vitro (DIV) were cultured for whole-cell recording using an EPC-9 patch-clamp amplifier (HEKA). Current was activated by rectangular voltage steps from −90 to +70 mV for 200 ms. The program package PULSE+PULSEFIT (HEKA) was used for data acquisition and analysis. HBSS (Sigma) was taken as extracellular solution (in mM): 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2PO_4, 10 D-glucose, and 4.2 $NaHCO_3$. The intracellular solution contained (in mM) 140 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 2 Na_2ATP, and 10 HEPES at pH 7.3. 4-Aminopyridine (4-AP) was a gift from Dr. X. L. Wang (Institute of Materia Medica, Beijing, China). Methyl-β-cyclodextrin, gangliosides GM1, GM2, GM3, and GD1b, and other chemicals were obtained from Sigma unless stated otherwise. Potassium current rundown is apparent at first 10 min after formation of the whole-cell patches. To overcome this problem, each of the chemicals was added to judge its effect on the potassium current only after the current was stable for 3 min period, which usually takes ∼15–20 min. All experiments were performed at room temperature (22–25°C). All data are means ± SEM for at least three experiments.

Neuronal cell death assay

For the apoptosis assay, cells between 6 and 10 DIV were fixed in 95% ethanol for 5 min, washed with PBS (Sigma), and then stained with 5 μM 4′,6-diamino-phenylindole (Roche) for 15 min. Two hundred to 300 nuclei were counted in 12–16 randomly chosen subfields in one neuronal culture to determine the percentage of apoptotic cells on the basis of the appearance of apoptotic bodies. At least five different cultures were used for each experiment. In addition, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay kit (Roche) was applied to evaluate the apoptosis of different treatments according to the instructions provided by the maker. Images of the cells were obtained by a digital camera (Nikon) with a fluorescence microscope (IX71; Olympus).

Measurement of caspase 3-like activity

The activity of caspase 3 was measured by fluorogenic assay using acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA) as a specific substrate (20). Protein extracts were prepared from 5×10^6 cells by Dounce homogenization on ice. The homogenization buffer contained (in mM) 25 HEPES (pH 7.5), 5 EDTA, 1 EGTA, 5 $MgCl_2$, 5 dithiothreitol, and 10 μg/ml each of pepstatin, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride. After centrifugation at 12,000 *g* for 15 min, the supernatants were collected and protein concentrations were determined by the Bradford method. Volumes of protein extracts containing 100 μg of protein were incubated for 1 h at 37°C with reaction buffer (25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol, and 5 mM EDTA) in a total volume of 150 μl containing 25 μM DEVD-MCA. The enzyme-catalyzed release of 4-methylcoumaryl-7-amide was measured by a fluorescence microplate reader at excitation of 355 nm and emission of 460 nm.

RESULTS

Inhibitory effect of GD1b on the I_K

To investigate whether a specific component of the complex gangliosides plays a role in antiapoptosis by inhibiting the outward K^+ current, we tested the effect of gangliosides GM1, GM2, GM3, GD1a, GD1b, GD3, and GT1b on whole-cell outward K^+ current of pyramidal neurons in hippocampus using the patch-clamp technique. The pyramidal neurons were identified based on the presence of a major apical dendrite, basal dendrites, and a relatively small soma that had whole-cell capacitance of ∼20 pF (21). Cultured hippocampal neurons between 6 and 10 DIV were studied. Outward K^+ currents that were consistent with I_K and transient outward A-type current (I_A) were present simultaneously in most of the cells tested (**Fig. 1A**). The steady-state outward current (corresponding to I_K) activated at a voltage of +70 mV was 495 ± 57 pA (n = 31 cells; mean ± SEM). This value is similar to that of cortical neurons (15). Addition of 12 μM GD1b into the bath solution reduced the current to 56% of the control value (0.56 ± 0.06; n = 13). From the dose-dependent curve, the IC_{50} value for GD1b inhibition of the current is ∼15.2 μM (Fig. 1A, B). In contrast to the significant effect of GD1b on the I_K, the same concentration (12 μM) of monosialogangliosides GM1, GM2, GM3, disialoganglioside GD1a, and trisialoganglioside GT1b only slightly inhibited the I_K (Fig. 1E). Disialoganglioside GD3 showed a biphasic effect on the I_K: I_K increased to 130% after a 3–10 min addition of GD3 to the bath solution (1.30 ± 0.08; n = 4), then decreased to 71% (0.71 ± 0.11). These effects of the gangliosides should not be caused by the rundown of the current, because the I_K almost did not change in the control group (Fig. 1E), for which the procedure was the same except that no drug was added. Furthermore, GD1b clearly inhibited the I_K when I_K was isolated using 4-aminopyridine to eliminate I_A (Fig. 1C). In contrast, addition of GD1b had almost no effect on the 4-aminopyridine-sensitive I_A that was isolated by tetraethylammonium (TEA) to eliminate I_K (Fig. 1D), indicating that GD1b specifically acts on I_K but not on I_A. Therefore, we focused our study on the inhibitory effect of GD1b on the I_K.

Antiapoptotic effect of GD1b on STS-induced apoptosis of hippocampal neurons

The critical role of K^+ efflux in apoptosis (12–15) suggests that GD1b may play an antiapoptotic role for neurons through its inhibitory effect on I_K, as other K^+ channel blockers do. **Figures 2**, **3A** show that treatment of neurons with 0.3 and 1 μM STS for 24 h induced apoptosis in ∼27% and 32% of the cells, respectively, when judged by the appearance of apoptotic bodies. In contrast, <3% of the untreated control (sham wash) cells showed such apoptotic

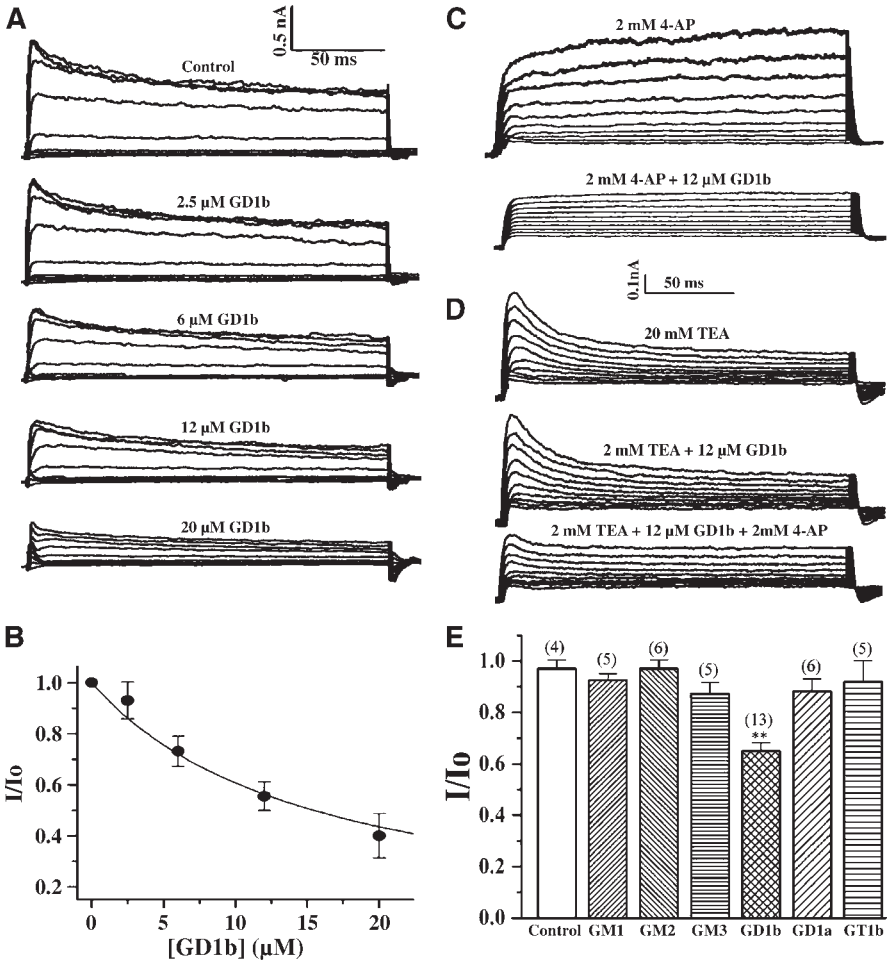

Fig. 1. Inhibitory effect of GD1b on the voltage-dependent outward delayed rectifier K^+ current (I_K). A: Current traces showing the inhibitory effect of different concentrations of GD1b on the I_K from the same cell. Current was activated by rectangular voltage steps from −90 to +70 mV for 200 ms. B: Dose-response curves of GD1b on I_K. The steady-state I_K at +70 mV was measured as a mean value in a range from 85% to 100% of the current trace. Each point represents the average value ± SEM from at least seven experiments. The solid line is the best fit to relative whole-cell currents (I/I_o) against the concentrations of GD1b ([GD1b]) according to the equation $I/I_o = \{(1 + [GD1b]/ IC_{50})^{-1}\}$, where IC_{50} = 15.2 μM. GD1b on I_K. C: 4-AP, 4-aminopyridine. D: GD1b on transient outward A-type current (I_A). TEA, tetraethylammonium. To get I_A, current was activated by 10 successive 200 ms rectangular voltage steps from a holding potential of −80 mV to potentials between −50 and +40 mV in both C and D. E: Ratio values of whole-cell I_K before (I_o) and after (I) application of different gangliosides at the same concentration (12 μM) as that of GD1b. Each column represents average values ± SEM, with the number of experiments performed shown in parentheses. ** Significant differences between the effect of GD1b and GM1, GM2, GM3, GD1b, and GT1b on I_K ($P < 0.01$; one-way ANOVA).

characteristics. Preincubation of hippocampal neurons with 10 μM GD1b could significantly reduce STS-induced apoptosis like that of TEA. TUNEL assay reconfirmed the inhibitory effect of GD1b on the STS-induced apoptosis. As shown in Fig. 2C, most of the cells treated with GD1b and STS show normal morphology, such as smooth cell body and clear dendrites, whereas cells treated with STS alone do not. To further confirm that GD1b plays an antiapoptotic role in neuronal cells, we measured the activity of caspase 3, a main executioner caspase (20), after treating the cells with STS in the presence and absence of GD1b. The results shown in Fig. 3B demonstrated that the activity of caspase 3 was gradually increased with time after STS treatment and that such an increase could be diminished by treatment with 10 μM GD1b.

Inhibitory effect of GD1b on the apoptosis-associated enhancement of I_K

The antiapoptotic effect of K^+ channel blockers has been attributed to their ability to inhibit the enhanced I_K (17, 22). Therefore, we tested whether GD1b could reduce the enhanced outward K^+ currents associated with neuronal apoptosis. STS has been well established and widely used to promote apoptosis through the intracellular pathway in different cell types (22). To accelerate the effect of STS in inducing enhancement of the I_K, STS was applied directly intracellularly by backfilling STS solution into the patch pipette. In 6–10 DIV neurons, the steady-state current density of I_K at +70 mV was 17 ± 4.6 pA/pF (**Fig. 4A**, **C**). Half an hour after backfilling of 0.1 nM STS, I_K at +70 mV increased from 17 to 31 pA/pF. Similar results were

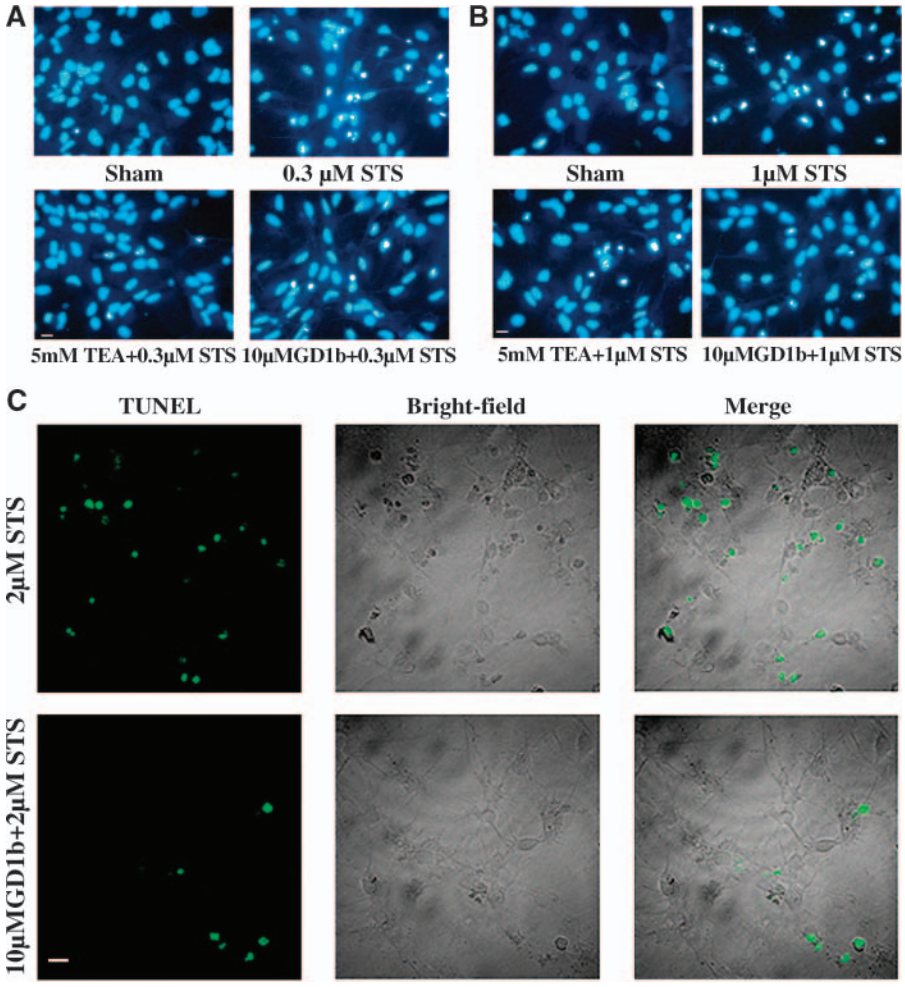

Fig. 2. Inhibitory effect of GD1b and TEA on staurosporine (STS)-induced apoptosis. Antiapoptotic effect of GD1b and TEA on 0.3 μM (A) and 1 μM (B) STS-induced apoptosis. Cells were stained with 5 μM 4′,6-diamino-phenylindole after 24 h of treatment with STS at the indicated concentrations. C: TUNEL stain of neuronal cells treated with STS alone or in the presence of GD1b. Bar = 12.5 μm. TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

obtained by bath application of a higher concentration of STS (10 nM): the current density increased from 17 to 28 pA/pF (Fig. 4B, C). This result is consistent with previous reports that the apoptosis inducer STS greatly augmented I_K (15, 23). Application of 10 μM GD1b inhibited the current by ~20% for both intracellular application of 0.1 nM and extracellular application of 10 nM STS (Fig. 4B, D). The enhancement in I_K induced by STS was also blocked by TEA (Fig. 4A, B, D), suggesting that the enhanced current was mainly I_K.

DISCUSSION

Gangliosides and K^+ channels have been shown to play regulatory roles in neuronal apoptosis. Our findings indicate that there exist inherent interrelationships between gangliosides, K^+ channels, and apoptosis. Four lines of evidence suggest that ganglioside GD1b plays an antiapoptotic role in neuronal cells. First, GD1b inhibited I_K in a dose-dependent manner. Second, GD1b attenuated the enhanced I_K evoked by the apoptosis inducer STS. Third, GD1b suppressed apoptosis as judged by TUNEL assay and the appearance of an apoptotic body. Fourth, GD1b suppressed the caspase 3 activation induced by STS. Activation of the outward K^+ current has been shown to be essential to apoptosis in almost all cell types (23). Thus, the first two lines of evidence suggest that GD1b may prevent the intracellular K^+ loss and consequently suppress apoptosis. Collectively, these results demonstrate that a single species of complex ganglioside, GD1b, plays an antiapoptotic role in hippocampal neurons through its inhibitory effect on I_K.

It was indicated that exogenously administered gangliosides are taken up by cells in three different modes: loosely associated micelles, trypsin-labile fraction, and membrane-inserted monomers (24). We do not have strong evidence to exclude the first and the second mode. But we think that at least some amount of the gangliosides could insert into the membrane and interact with the ion channel that nested in the membrane. Gangliosides are a kind of sphingolipid, which together with cholesterol constitute a ma-

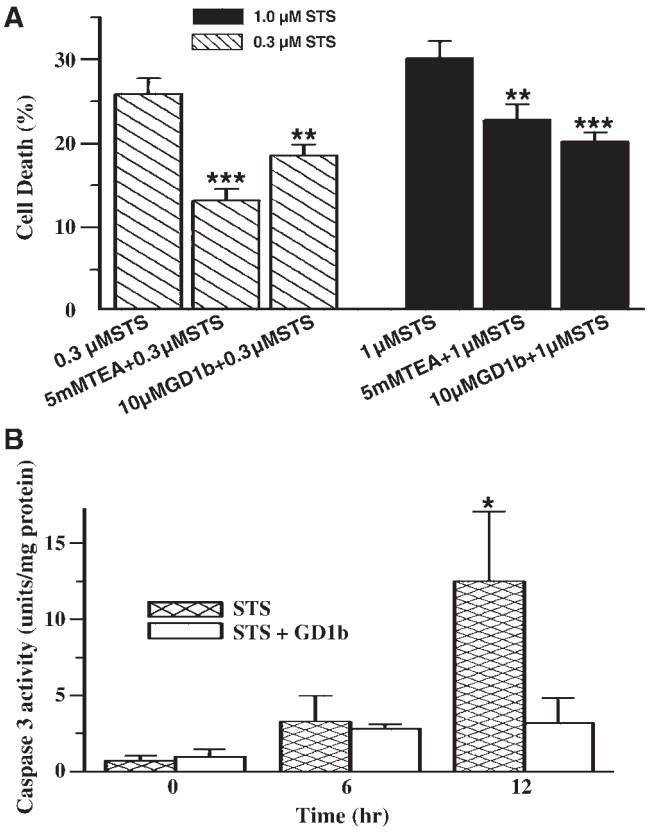

Fig. 3. Summarized data for the inhibitory effect of GD1b on neuronal apoptosis and caspase 3 activity. A: Antiapoptotic effect of GD1b and TEA on STS-induced apoptosis. Neuronal apoptosis was induced by 24 h of exposure to 0.3 or 1 μM STS. Each column represents average values ± SEM from five experiments. Asterisks indicate differences from control (** $P < 0.01$, *** $P < 0.001$; one-way ANOVA). B: Inhibitory effect of GD1b on STS-induced caspase 3 activity. The caspase 3 activity after 0, 6, and 12 h of treatment with STS in the presence or absence of GD1b was measured by determining the ability of cell extracts to cleave the colorimetric substrate acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide). One unit of caspase 3-like activity was defined as releasing 1 pmol of 4-methylcoumaryl-7-amide per minute at 37°C at saturating substrate concentrations. Data are presented as means ± SEM (n = 3). * $P < 0.05$ (one-way ANOVA).

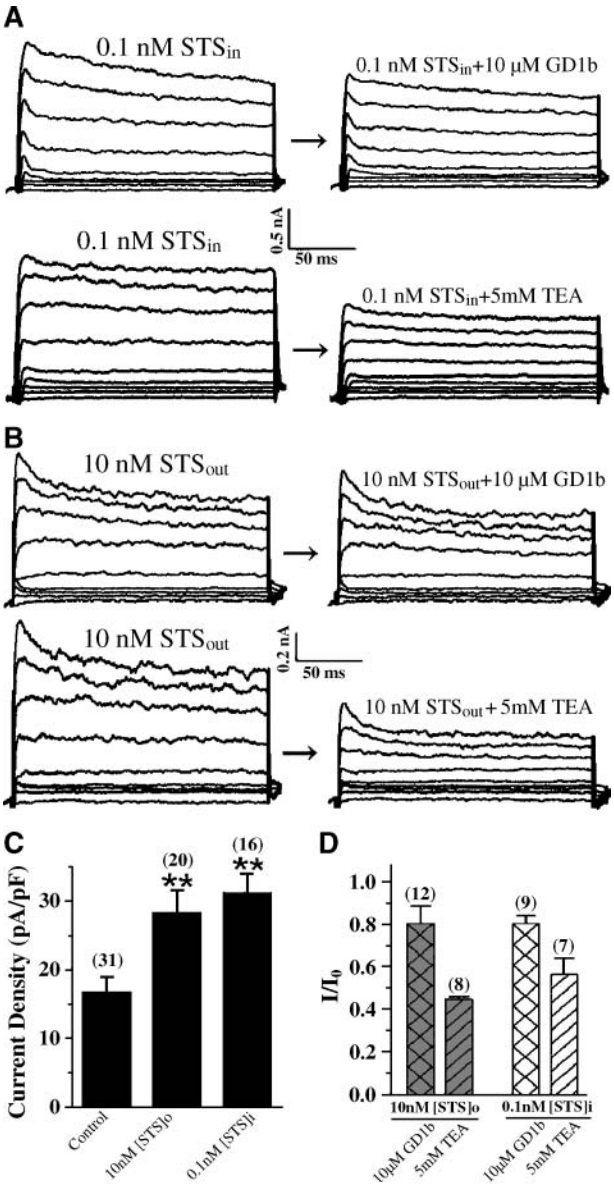

Fig. 4. Inhibitory effect of GD1b on the STS-induced I_K. Inhibitory effect of 10 μM GD1b and 5 mM TEA on enhanced I_K induced by intracellular application of 0.1 nM (A) or extracellular application of 10 nM (B) STS. Current was activated by rectangular voltage steps from −90 to +70 mV for 200 ms. C: Summarized data showing the enhancement of I_K at +70 mV by extracellular and intracellular application of 10 and 0.1 nM STS, respectively. $[STS]_o$ and $[STS]_i$ represent extracellular and intracellular concentrations of STS, respectively. Each column represents average values ± SEM, with experimental times in parentheses. ** Difference from control ($P < 0.01$; one-way ANOVA). D: Percentage of STS-enhanced current blocked by the application of GD1b and TEA (mean ± SEM), with the number of experiments performed in parentheses.

jor lipid component of lipid rafts in animal cell membranes (25). Indeed, ganglioside itself has been believed to be a component or even a marker of lipid rafts (26–28). Recently, it was shown that lipid rafts play important roles in regulating ion channels (29–32). Therefore, it is reasonable to speculate that GD1b regulated the channels responsible for I_K in the lipid rafts. In support of this speculation, the steady-state I_K activated at a voltage of +70 mV was 329 ± 39 pA (n = 3) after treatment with 10 mM cyclodextrin, an agent known to deplete membrane cholesterol (26). This value was much lower than that in untreated cells (495 ± 57 pA; n = 31 cells), suggesting that ion channels responsible for I_K are located in lipid raft microdomains. These data suggest that lipid rafts may provide a platform for GD1b to regulate the I_K and play the antiapoptotic role in neuronal cells. Additional work is needed to elucidate the mechanism of how ganglioside GD1b regulates the I_K and how it plays the antiapoptotic role in neuronal cells.

Excessive apoptotic nerve cell death is implicated in the pathogenesis of several devastating neurodegenerative disorders, including Alzheimer's disease (33–35). An early prerequisite for apoptosis is the cell shrinkage that is largely dependent on the outward K^+ current. Potent K^+ channel

blockers, such as TEA, have been shown to suppress apoptosis by inhibiting outward K^+ current. However, their toxic effects for cells suggest that an endogenous molecule specifically targeting K^+ channels is needed to block proapoptotic excessive K^+ efflux and apoptotic death (36). Gangliosides are sialic acid-containing glycosphingolipids present mainly on the outer leaflet of the plasma membrane of vertebrate cells (7). Therefore, as an endogenous membrane component, the inhibitory effect of ganglioside GD1b on neuronal apoptosis has intuitive appeal and its antiapoptotic effect should have minimal side effects, for a potential therapeutic value in attenuating neuronal apoptosis in neurodegenerative disorders caused by excessive apoptosis.

In conclusion, our results suggest that GD1b plays an antiapoptotic role in neurons through its inhibitory effect on the I_K and caspase 3 activity.

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