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Interaction of the B Subunit of Cholera Toxin with Endogenous Ganglioside GM1 Causes Changes in Membrane Potential of Rat Thymocytes

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Summary. The fluorescent anionic dye, bisoxonol, and flow cytometry have been used to monitor changes in the membrane potential of rat thymocytes exposed to the B subunit of cholera toxin. The B subunit induced a rapid hyperpolarization, which was due to activation of a Ca^{2+}-sensitive K^+ channel. Reduction of extracellular Ca^{2+} to <1 μM by the addition of [ethylenebis(oxyethylenenitrilo)]tetraacetic acid immediately abolished the hyperpolarization caused by the B subunit. Cells treated with quinine and tetraethylammonium lost their ability to respond to the B subunit, whereas 4-aminopyridine did not have any effect. Thus, calcium-sensitive and not voltage-gated K^+ channels appeared to be responsible for the hyperpolarization. The results of ion substitution experiments indicated that extracellular Na^+ was not essential for changes in membrane potential. Further studies with ouabain, amiloride and furosemide demonstrated that electrogenic Na^+/K^+ ATPase, Na^+/H^+ antiporter and $Na^+/K^+/Cl^-$ cotransporter, respectively, were not involved in the hyperpolarization process induced by the B subunit. Thus, crosslinking of several molecules of ganglioside GM1 on the cell surface of rat thymocytes by the pentavalent B subunit of cholera toxin modulated plasma membrane permeability to K^+ by triggering the opening of Ca^{2+}-sensitive K^+ channels. A role for gangliosides in regulating ion permeability would have important implications for the function of gangliosides in various cellular phenomena.

Key Words B subunit of cholera toxin · membrane potential · hyperpolarization · thymocytes

Introduction

Gangliosides were first found in nervous tissue, where they are highly abundant, but are now known to be ubiquitous components of the plasma membrane of all vertebrate cells (Hakomori, 1981; Wiegandt, 1982; Svennerholm, 1984). Gangliosides have been implicated in a variety of important biological processes including the control of cell growth and differentiation (Fishman & Brady, 1976; Hakomori, 1985). Exogenously added gangliosides, in particular GM1, have neuritogenic and neurotrophic effects both in vivo and in vitro (Ledeen, 1984). Recently, the B subunit of cholera toxin has been used as a ganglioside-specific probe to investigate the role of plasma membrane ganglioside GM1 in the regulation of cell growth. The B subunit was found to be mitogenic for rat thymocytes (Spiegel, 1984; Spiegel, Fishman & Weber, 1985) and also to stimulate DNA synthesis and cell division in quiescent nontransformed mouse fibroblasts (Spiegel & Fishman, 1987). Although the underlying basis for this effect is not yet known, it involves the ability of the B subunit, which is pentavalent, to bind specifically to several GM1 molecules on the cell surface and thus to perturb the plasma membrane.

The present study focuses on the possibility that crosslinking of endogenous GM1 molecules by the multivalent B subunit of cholera toxin can induce changes in the permeability of the plasma membrane to small ions. Recently, Wilson and Chused (1985) demonstrated that flow cytometric recording of oxonol dye fluorescence permits quantitative determinations of lymphocyte membrane potential. This method provides a description of ion permeability and pharmacological characteristics of various ion channels. Using this technique, we found that binding of the B subunit of cholera toxin to endogenous gangliosides induced a rapid hyperpolarization of the membrane. This response was due to alterations in K^+ fluxes. A potential role for endogenous gangliosides in regulating membrane permeability has important implications, not only for the function of gangliosides in various phenomena of cell growth and differentiation, but also for their possible role in electrical excitability of neurons.

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Materials and Methods

Materials

Myo-[2-^{3}H]inositol (15 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Quin2-acetoxymethyl ester was from Lancaster Synthesis (Morecambe, U.K.). Ionomycin and A23187 were from Calbiochem-Behring (Bedford, MA). Bisoxonol (bis(1,3-dibutylbarbituric acid) trimethine oxonol) was obtained from Molecular Probes (Eugene, OR). The B subunit of cholera toxin was from List Chemical (Campbell, CA). All other reagents were from Sigma Chemical (St. Louis, MO). Medium 199 and RPMI 1640 were from Gibco Laboratories (Grand Island, NY). When alterations in inorganic salts were needed, the appropriate salts (dissolved in sterile deionized water) were added to medium 199 concentrate (Gibco), which lacked inorganic ions, to produce media of the desired composition.

Cell Preparation

Thymocytes were prepared from Sprague-Dawley rats (Food and Drug Administration colony) as described previously (Spiegel et al., 1985). The cells were maintained in RPMI 1640 or medium 199 ($1–2 \times 10^6$/ml) supplemented with 25 mM HEPES at 37°C in 5% CO_2/95% air in a fully humidified atmosphere. The 199 medium contained the following inorganic salts (in mM): 1.8 $CaCl_2$, 5.4 KCl, 0.8 $MgSO_4$, 116 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4. The RPMI 1640 medium contained the following salts (in mM): 0.6 $Ca(NO_3)_2$, 5.4 KCl, 0.4 $MgSO_4$, 103 NaCl, 24 $NaHCO_3$, 10.7 Na_2HPO_4. When a low extracellular Ca^{2+} was required, $CaCl_2$ was omitted from medium 199 and 2 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA) was added. Sodium-free (1 mM Na^+) medium was prepared by substituting choline chloride and choline bicarbonate for the corresponding sodium salts and replacing sodium N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid with 10 mM tris(hydroxymethyl)amino aminoethane hydrochloride (Tris-HCl). KCl and $KHCO_3$ were substituted for the corresponding sodium salts in calibration experiments (Wilson & Chused, 1985). The cells were assayed within 2 hr of removal from the thymus because the cells slowly depolarized spontaneously.

Flow Cytometry

Cytofluorometric determination of the relative membrane potential of individual thymocytes was performed using the procedure of Wilson and Chused (1985), with some modifications. Studies were performed using an EPICS V flow cytometer (Hialeah, FL) equipped with an argon laser emitting 250 mW at 488 nm. Histograms were collected on linear mode and fluorescence intensity represents 0–255 channels. Thymocytes were gated on low angle forward light scatter to exclude debris and dead cells, and the fluorescence emission intensity was used to measure the plasma membrane potential with bisoxonol. Excitation and emission wavelengths were 488 and 510–540 nm, respectively. The bisoxonol, which was dissolved in dimethyl sulfoxide, was added directly to the thymocyte suspension in medium 199 (or RPMI 1640) to a final dye concentration of 250 nM. The final dimethyl sulfoxide concentration was <0.25% (vol/vol), and the resulting volume changes were <1%. All samples were run at 37°C. Reproducible bisoxonol fluorescence measurements require pre-equilibration of the cytometer fluid tubing with the bisoxonol in medium 199 (or RPMI 1640) for 15 min before the assay. This permits stable fluorescence measurements for 10 min. After the pre-equilibration of the cytometer fluid tubing, a baseline was obtained with the cell suspension alone. Test reagents were added to the cell suspension and another histogram was recorded. Fluorescence values were then compared to baseline measurements.

Measurement of Cytoplasmic Free Ca^{2+} Concentration

Changes in cytoplasmic free Ca^{2+} following application of mitogenic concentration of the B subunit were determined by the fluorescent calcium-sensitive dye quin2-tetra(acetoxymethyl)ester, quin2/AM (Rink et al., 1983). Levels and variations of cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ were measured essentially as described (Dixon et al., 1987) except that thymocytes from Sprague-Dawley rats were suspended in HEPES-buffered RPMI 1640 (10^7 cells/ml) and were loaded at 37°C for 30 min with 5 μM quin2/AM. The cells were washed and resuspended in HEPES-buffered saline (10 mM HEPES at pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 5 mM glucose). Where indicated, $CaCl_2$ was omitted and 1.8 mM EGTA was added. Fluorescence of quin2 was monitored spectrofluorometrically with excitation at 339 nm and emission at 495 nm. Calibration of fluorescence was carried out using ionomycin and Mn^{2+} (Rink et al., 1983).

Measurement of Inositol Phosphates Levels

Thymocytes (10^8 cells/ml) were labeled with myo-[2-^{3}H]inositol (10 μCi/ml) by incubation at 37°C for 24 hr in inositol-free HEPES-buffered RPMI 1640. Cells were sedimented and resuspended for 15 min in fresh medium that contained 10 mM LiCl, an inhibitor of inositol phosphate phosphatase (Berridge, 1984), and the various mitogens. At the end of the stimulation period, the cells were sedimented, extracted with methanol/chloroform and the inositol phosphates were separated on Dowex AG 1X8 ion-exchange resin exactly as described previously (Spiegel & Panagiotopoulos, 1988).

Results

Effect of B Subunit and Concanavalin A (Con A) on Membrane Potential

When bisoxonol was added to a suspension of rat thymocytes, there was an immediate increase in 510–540 nm fluorescence, which reached a constant level within 10 min. When mitogenic concentrations of either the B subunit or Con A were added to the cell suspension, the fluorescence intensity rapidly decreased (Fig. 1), indicating that the cells were hyperpolarized and that the negatively charged bisoxonol was expelled from the cell. The hyperpolarization was observed within 1–2 min (limit of mea-

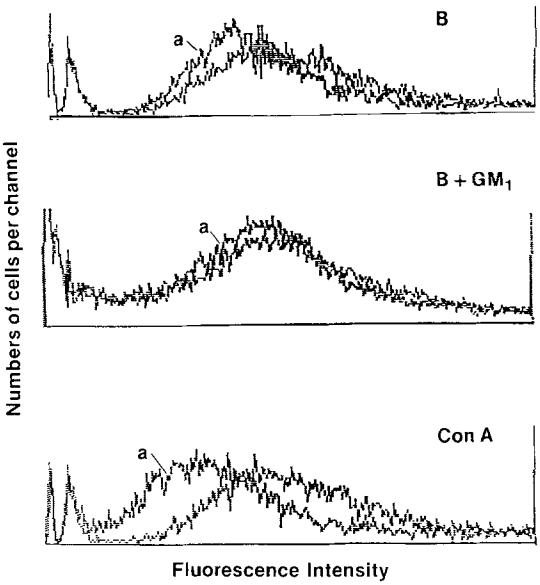

Fig. 1. Effect of Con A and the B subunit of cholera toxin on bisoxonol fluorescence of rat thymocytes. Thymocytes were equilibrated with 250 nM bisoxonol and fluorescence profiles were recorded by flow cytometry as described in Materials and Methods. Mitogenic concentrations of either Con A (2 μg/ml) or B (1 μg/ml) or B equilibrated with free GM1 were added to the sample and 1 min later a second fluorescent profile (*a*) was recorded. In each case, from 10,000 to 25,000 cells were analyzed. Histograms with and without mitogen are overlaid. Histograms are representative of 10 and 23 separate determinations

surement of the cell sorter) after addition of either mitogen. A similar effect was noted over the concentration range of 1–20 μg/ml of B (*data not shown*). The hyperpolarization in response to the B subunit was due to the binding of the B subunit to the thymocytes, as preincubation of the B subunit with ganglioside GM1 blocked the effect (Fig. 1). Addition of the buffer in which the B subunit was reconstituted or of bovine serum albumin did not cause any change in the membrane potential. Furthermore, it had been shown previously that the B subunit does not elevate adenylate cyclase in rat thymocytes (Spiegel et al., 1985). Thus, the effect cannot be due to any contaminating A subunit.

Although it has been well-documented that changes in oxonol fluorescence in cell suspension reflect changes in cell membrane potential (Rink et al., 1980; Tsien, Pozzan and Rink, 1982), calibrating the dye response is difficult. Conventional methods for calibrating membrane potential and dye fluorescence intensity rely on the use of ionophores such as valinomycin. However, because bisoxonol interacts with valinomycin, we were unable to use this method and instead used a new calibration procedure developed by Wilson and Chused (1985). This method utilizes the observation that the mean oxonol fluorescence value for unmanipulated cells increases linearly with the log of $[K^+]_o$ when $10 \leq [K^+]_o \leq 120$ mM, as would be expected from a K^+-dominated diffusion potential. Although the fluorescence is unchanged between 2 and 10 mM $[K^+]_o$, the linear correlation can be extended downward to this range by the addition of 50 nM of the ionophore A23187. The A23187 activates Ca^{2+}-sensitive K^+ channels so that the standard curve can be extended below 10 mM K_o (Fig. 2). Using this correlation procedure and assigning an intracellular K^+ concentration of 130 mM (Wilson & Chused, 1985; Rink et al., 1980; Damjanovich et al., 1987), the electrical potential difference across the plasma membrane for resting thymocytes is −70 mV. Con A and the B subunit induced hyperpolarization of 9.1 ± 4.3 ($n = 10$) and 3.8 ± 2.1 ($n = 23$), respectively.[1] Tsien et al. (1982) observed a 20-mV hyperpolarization of mouse thymocytes within a few minutes after treatment with Con A. We have confirmed this effect, although our observations suggest a smaller hyperpolarization of 9.1 mV, which is consistent with the effect observed by Felber and Brand (1983) in mouse thymocytes (10 mV), and Tatham, O'Flynn and Linch (1986) in human T-lymphocytes (4.5 mV).

Inhibition of B Subunit-Induced Alterations in Membrane Potential by EGTA

Several lines of evidence suggest that Con A induced hyperpolarization by causing a rise in cytoplasmic free Ca^{2+}, which in turn activates a plasma membrane K^+ conductance of the sort described in red cells (Lew & Ferreira, 1978; Tsien et al., 1982; Tatham et al., 1986). To determine if such channels are involved in the hyperpolarization induced by the B subunit, the thymocytes were exposed to the B subunit immediately after being transferred into a calcium-free buffer containing 2 mM EGTA. The hyperpolarization was significantly reduced or abolished completely. In some cases, depolarization occurred (Fig. 3). In agreement with previous reports (Tatham & Delves, 1984; Tatham et al., 1986), Con A induced a depolarization in the absence of Ca^{2+} (Fig. 3).

Effect of the B Subunit of Cholera Toxin on Cytoplasmic Free Ca^{2+}

The B subunit induced a rapid and profound increase in intracellular free Ca^{2+} as measured with

[1] It is unlikely that these small changes in the membrane potential play a significant role in the initiation of the proliferative response.

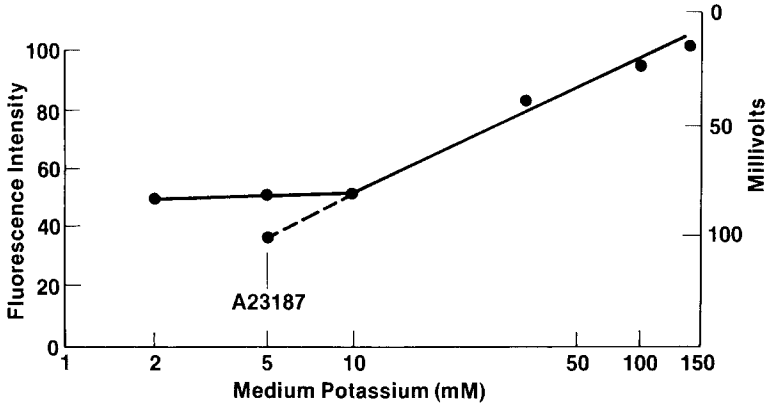

Fig. 2. Calibration of bisoxonol fluorescence. The bisoxonol fluorescence of rat thymocytes was measured as a function of extracellular K^+ concentration by isotonic substitution of Na^+ for K^+ as described in Materials and Methods. The data are presented as the mean fluorescence peak channel *vs.* extracellular K^+ concentration (—). Fifty nM A23187 was added to samples of <10 mM KCl (--). Representative of at least three separate determinations

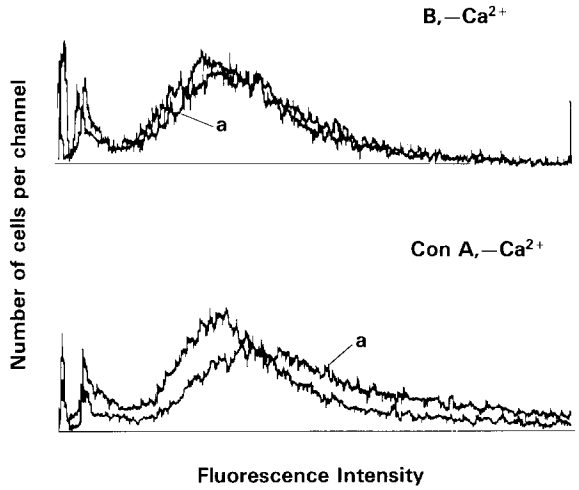

Fig. 3. Effect of Ca^{2+} depletion on hyperpolarization induced by the B subunit and Con A. Cells suspended in Ca^{2+}-free medium supplemented with 2 mM EGTA were equilibrated with bisoxonol and a baseline histogram was collected. One min after the addition of the B subunit or Con A, another histogram (*a*) was collected. In each case, the fluorescence profiles from 10,000 to 25,000 cells were recorded. Histograms with and without mitogen are overlaid. Histograms are representative of at least eight separate determinations

Table 1. Effect of B subunit of cholera toxin on intracellular free Ca^{2+} and the level of inositol phosphates

Addition[a]	$[Ca^{2+}]_i$ (nM)	Level of [^{3}H]inositol phosphates		
		IP_1	IP_2 (cpm per well)	IP_3
None	75 ± 5	1836 ± 178	303 ± 85	258 ± 27
ConA	190 ± 25	3766 ± 166	740 ± 43	378 ± 11
B	155 ± 22	1733 ± 231	360 ± 26	250 ± 25
EGTA + B	72 ± 6	ND[b]	ND	ND

[a] The concentrations of the indicated agents were as follows: B, 1 μg/ml; ConA, 2 μg/ml; EGTA, 2 mM.
[b] ND, not determined.

the fluorescent Ca^{2+}-sensitive dye quin2/AM. Intracellular free Ca^{2+} increased from a basal level of 75 ± 5 mM (n = 5) to 155 ± 22 mM (n = 5) (Table 1). As has been shown previously for Wistar rat thymocytes (Dixon et al., 1987), the increase in $[Ca^{2+}]_i$ induced by a maximally mitogenic concentration of the B subunit of cholera toxin was virtually immediate. Removal of external Ca^{2+} completely inhibited the B-induced Ca^{2+} signal, while re-addition of Ca^{2+} rapidly restored the response. This inhibitory effect is not due to a lack of binding of the B subunit to the cells since the binding of the B subunit to GM1 is a Ca^{2+}-independent process (Spiegel et al., 1985). These findings indicate that the increase in $[Ca^{2+}]_i$ induced by the B subunit arises from a net influx of Ca^{2+} from extracellular sources and not from a release of Ca^{2+} from intracellular stores and are consistent with the observation that inositol trisphosphate, the second messenger that mediates release of Ca^{2+} from intracellular stores (Berridge & Irvin, 1984), was not elevated in response to the B subunit (Table 1). In contrast, Con A induced a significant increase in the level of inositol phosphates (IP_1, IP_2, IP_3) and also induced a larger $[Ca^{2+}]_i$ rise than the B subunit.

Ionic Basis for Induced Alteration in Membrane Potential

To further characterize the ion channels involved in this hyperpolarization response to the B subunit, we used a pharmacological approach. Experiments were carried out in the presence of a series of channel blockers. The blockers used and the channels they block are given in Table 1. K^+ channels of T-lymphocytes are blocked reversibly in a dose-dependent manner by tetraethylammonium (TEA), quinine and 4-aminopyridine (4-AP) (De Coursey et

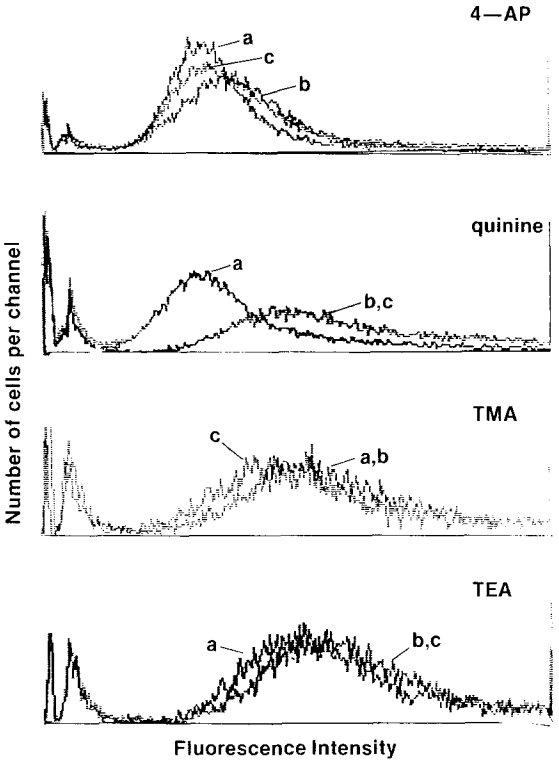

Fig. 4. Effects of 4-AP, quinine, TEA and TMA on resting potential and B subunit-induced hyperpolarization of rat thymocytes. Cells are equilibrated with bisoxonol in the absence (*a*) or presence (*b*) of 10 mM 4-AP, 100 μM quinine, 40 mM TMA or 40 mM TEA. The B subunit was added to each sample and another histogram (*c*) was collected 1 min later. Histograms in the absence (*a*) and presence of blockers (*b*) and B subunit (*c*) are overlaid. Histograms are representative of five different determinations

al., 1984). Quinine is believed to block Ca^{2+}-activated K^+ channels, but at the high concentrations used in these studies voltage-gated K^+ channels are also blocked (Lew & Ferreira, 1978). TEA also blocks both Ca^{2+} and K^+ voltage-gated K^+ channels, whereas 4-AP has been reported to specifically block voltage-gated K^+ channels (Schwartz & Passow, 1983). Therefore, the aminopyridines can distinguish between the Ca^{2+}-activated K^+ channels and the voltage-gated K^+ channels. 4-AP (10 mM) depolarized rat thymocytes, suggesting a blockage of the voltage-gated K^+ channels. However, cells depolarized by 4-AP repolarized after addition of the B subunit (Fig. 4). In contrast, cells depolarized by quinine (100 μM) or TEA (40 mM) did not repolarize after the addition of the B subunit. Tetramethylammonium (TMA) (40 mM), an inactive analogue of TEA, did not block the response to the B subunit. Thus, the inhibition by TEA cannot be attributed to an increase in osmolarity of the medium, but to its ability to specifically block K^+ channels.

Table 2. Effect of inhibitors on hyperpolarization of thymocytes induced by the B subunit of cholera toxin

Inhibitor	Concentration	Target	Effect
Ouabain	1 mM	Na^+/K^+ ATPase	None
Amiloride	100 μM	Na^+/H^+ antiport	None
Furosemide	1 mM	$Na^+/K^+/Cl$ exchange	None
Quinine	100 μM	Ca^{2+}-gated K^+ channels	Blocked
TEA	40 mM	K^+ channels	Blocked
4-AP	10 mM	Voltage-gated K^+ channels	None

The hyperpolarization induced by the B subunit could be blocked even at a low quinine concentration (20 μM), which had no effect on the resting membrane potential (*data not shown*). These experiments with the K^+ channel blockers indicate that only Ca^{2+}-activated K^+ channels are involved in the hyperpolarization of thymocytes by the B subunit.

Since mitogens, such as Con A, induce changes in K^+ fluxes and also increase plasma membrane permeability to Na^+ (Tatham et al., 1986; Kiefer, Blume & Kaback, 1980), we investigated the dependence of the hyperpolarization that we observed on external Na^+. Replacement of Na^+ by the poorly permeant cation choline had no significant effect on the hyperpolarization response to the B subunit (*data not shown*). We also examined the effects of inhibitors of Na^+ transport (Table 2). The hyperpolarization induced by the B subunit persisted in the presence of 1 mM ouabain (Fig. 5), indicating that stimulation of the electrogenic Na^+/K^+ pump was not responsible for the observed hyperpolarization. Ouabain alone had no effect immediately after addition to the cells and caused only a slight increase in fluorescence after a 5-hr incubation. The hyperpolarization caused by the B subunit was also unaffected by the addition of amiloride (Fig. 5), thus implying that the Na^+/H antiporter was not involved in this response. Similarly, the diuretic furosemide (1 mM), which inhibits the $Na^+/K^+/Cl^+$ cotransporter, did not block the hyperpolarization induced by the B subunit (Fig. 5).

Discussion

We have demonstrated that when the B subunit of cholera toxin binds to the cell surface ganglioside GM1 of rat thymocytes,[2] a hyperpolarization of the

[2] It was previously established that GM1 is the only receptor for the B subunit of cholera toxin in rat thymocytes (Spiegel et al., 1985).

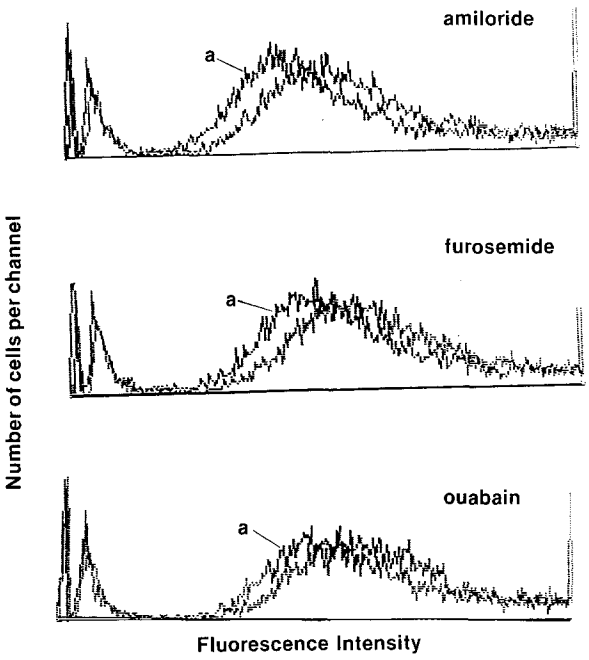

Fig. 5. Effect of amiloride, furosemide and ouabain on membrane hyperpolarization induced by the B subunit. Cells were equilibrated with bisoxonol in the presence of 10 μM amiloride, 1 mM furosemide, or 1 mM ouabain and analyzed on the cell sorter. The B subunit was then added to the sample and another histogram (*a*) was collected 1 min later. Histograms in the absence and presence of the B subunit are overlaid. Histograms are representative of five different determinations

plasma membrane occurs. The hyperpolarization is probably due to a K^+ conductance since, of the main intracellular ions, only K^+ has an equilibrium potential more negative than the resting potential(s). In addition, the hyperpolarization remained essentially unaffected in a low Na^+ medium (with choline substituted for Na^+), showing that extracellular Na^+ was not essential for the changes in membrane potential. Further studies in the presence of ouabain, amiloride and furosemide demonstrated that the B subunit did not activate the electrogenic Na^+/K^+ ATPase, the Na^+/H^+ antiporter or the $Na^+/K^+/Cl^-$ cotransporter; and also that these transporters did not play a role in the hyperpolarization process. The lack of activation of the Na^+/H^+ exchange is supported by the findings that the B subunit had no effect on intracellular pH as revealed by flow cytometric measurements on Sprague-Dawley rat thymocytes loaded with biscarboxyethyl-5,6-carboxyfluorescein (S.A. Mulhern, *unpublished observations*) and also by spectrofluorometric measurements on Wistar rat thymocytes (Dixon et al., 1987). The fact that we did not observe a change in membrane potential in our previous studies (Dixon et al., 1987) was probably due to the use of the less sensitive spectrofluorometer.

The fact that the hyperpolarization was diminished or abolished in the absence of extracellular Ca^{2+} suggests the existence of a Ca^{2+}-sensitive process that tends to generate the hyperpolarization. This effect was further identified as an activation of a Ca^{2+}-sensitive K^+ channel by a pharmacologic approach. Hence, TEA and quinine, which block Ca^{2+}-activated K^+ channels, inhibited the hyperpolarization response. In contrast, 4-AP, which blocks voltage-sensitive K^+ channels, did not. These data suggest that the B subunit of cholera toxin facilitates Ca^{2+} entrance into the cells, which, in turn, triggers the opening of Ca^{2+}-sensitive K^+ channels, leading to membrane hyperpolarization. Support for this view comes from direct measurements of cytoplasmic free Ca^{2+} with the fluorescent Ca^{2+}-sensitive dye, quin2, on Sprague-Dawley (Table 1) and Wistar rat thymocytes (Dixon et al., 1987). The fluorescence of quin2-loaded rat thymocytes increases immediately after the addition of the B subunit to the thymocyte suspension, indicating a rapid increase in intracellular free Ca^{2+}. This increase in cytoplasmic free Ca^{2+} arises entirely from a net influx of extracellular Ca^{2+}. The timing of the membrane responses to the B subunit appears to be similar to that of the rise in $[Ca^{2+}]_i$. Also, both effects are abolished when low-Ca^{2+} medium is used. Thus, our data suggest that crosslinking of several molecules of GM1 by the pentavalent B subunit of cholera toxin increases $[Ca^{2+}]_i$, which triggers the opening of Ca^{2+}-sensitive K^+ channels, results in the hyperpolarization of the plasma membrane.

In previous studies based on the use of carbocyanin dyes to monitor the membrane potential, we found that rat lymphocytes depolarized in response to mitogenic concentrations of the B subunit or Con A (Spiegel, Fishman & Mulhern, 1986*a*). Although it seems that the data in this report are contradictory to those in our earlier report, this is not necessarily the case. This difference is due to the use of different membrane potential-sensing dyes in the two reports. The cyanin dye that we had used earlier has been shown to accumulate within cells and to concentrate in mitochondria where it affects respiration and ATP production (Montecucco, Pozzant & Rink, 1979; Wilson, Seligmann & Chused, 1985). A more likely explanation for this discrepancy is based on the observation that cyanin dyes appear to bind preferentially to open K^+ channels (Simons, 1976, 1979). Other studies (Rink et al., 1980) suggest that the cyanine dye diS-(3)-(5) blocked Ca^{2+}-activated K^+ channels in mouse lymphocytes. Since the B subunit and Con A induced the opening of these channels, the membrane depolarization ob-

served with the cyanin dyes is probably an artifact due to increased binding of the dye to open K^+ channels.

Recently, a lipid-layer crystallization technique was used for imaging the B subunit of cholera toxin bound to GM1 (Ludwig et al., 1986). Image processing of electron micrographs revealed a ring of five protein densities with a central hole of 20 nm diameter. These data are consistent with a pentameric doughnut-shaped organization of the bound B subunit that lies flat on a membrane surface. This observation might be interpreted to mean that the B subunit could cause pore formation in the membrane. The B subunit, when bound to GM1-containing liposomes, causes them to release trapped glucose (Moss et al., 1977). This is an unlikely explanation for the observed hyperpolarization because, in such a case, depolarization would be due to leakage of Na^+. Furthermore, the hyperpolarization should not exhibit the pharmacological characteristics of Ca^{2+}-gated K^+ channels. The possibility that such pores allow the selective influx of Ca^{2+} also seems unlikely.

In summary, our results imply that reorganization of the negatively charged gangliosides by the B subunit of cholera toxin can modulate both the net influx of Ca^{2+} and the efflux of K^+, resulting in membrane hyperpolarization. The possibility that gangliosides can modulate ion channels has been proposed previously, based on studies with cultured kidney epithelial cells (Spiegel, Handler & Fishman, 1986*b*). Insertion of specific gangliosides into the apical membrane of the epithelia increased Na^+ transport in response to vasopressin, cholera toxin and 8-bromo cAMP. Based on this observation, it was proposed that specific gangliosides can be modulators of Na^+ channels present in the apical membrane of epithelial cells. The well-established influence of gangliosides on neuronal growth and survival is due to their ability to enhance membrane ion permeability (Gorio et al., 1983). A role for gangliosides in the regulation of membrane permeability could also have important implications for the function of gangliosides in various cellular phenomena, such as growth, differentiation and electrical excitability of neurons. Neurons have a large number of gangliosides in their plasma membranes and since their electrical activity is an expression of ionic fluxes across their membranes, modulation of ion transport may be a likely function for gangliosides.

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References

Berridge, M.J. 1984. Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220:**345–360

Berridge, M.J., Irvine, R.F. 1984. Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature (London)* **312:**315–321

Damjanovich, S., Aszalos, A., Mulhern, S.A., Szollosi, J., Balazs, J., Tron, L., Fulwyler, M.J. 1987. Cyclosporin depolarizes human lymphocytes: Earliest observed effect on cell metabolism. *Eur. J. Immunol.* **17:**763–768

De Coursey, T.E., Chandy, K.G., Gupta, S., Cahalan, M.D. 1984. Voltage-gated K^+ channels in human T lymphocytes: A role in mitogenesis? *Nature (London)* **307:**465–468

Dixon, S.J., Stewart, D., Grinstein, S., Spiegel, S. 1987. Transmembrane signaling by the B subunit of cholera toxin: Increased cytoplasmic-free calcium in rat thymocyte. *J. Cell Biol.* **105:**1153–1161

Felber, S.M., Brand, M.D. 1983. Early plasma-membrane-potential changes during stimulation of lymphocytes by concanavalin A. *Biochem. J.* **210:**885–891

Fishman, P.H., Brady, R.O. 1976. Biosynthesis and function of gangliosides. *Science* **194:**906–915

Gorio, A., Carmignoto, G., Ferrari, G., Marini, P., Nunzi, M.G. 1983. Plasticity in neuronal regeneration: Implications for the role of exogenous gangliosides. *In:* Nervous System Regeneration. B. Haber, J.R. Perez-Polo, G.A. Hashim, A.M. Guiffrida-Stella, and N.W. Paul, editors. pp. 157–174. Alan R. Liss, New York

Hakomori, S. 1981. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *Annu. Rev. Biochem.* **50:**733–764

Hakomori, S. 1985. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: Overview and perspectives. *Cancer Res.* **45:**2405–2414

Kiefer, H., Blume, A.J., Kaback, R. 1980. Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes. *Proc. Natl. Acad. Sci. USA* **77:**2200–2204

Ledeen, R.W. 1984. Biology of gangliosides: Neuritogenic and neuronotrophic properties. *J. Neurosci. Res.* **12:**147–159

Lew, V.L., Ferreira, H.G. 1978. Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. *Curr. Top. Membr. Transp.* **10:**217–277

Ludwig, D.S., Ribi, H.O., Schoolnik, G.K., Kornberg, R.D. 1986. Two-dimensional crystals of cholera toxin B-subunit-receptor complexes: Projected structure at 17-Å resolution. *Proc. Natl. Acad. Sci. USA* **83:**8585–8588

Montecucco, C., Pozzant, T., Rink, T.J. 1979. Dicarbocyanine fluorescent probes of membrane potential block lymphocyte capping, deplete cellular ATP and inhibit respiration of isolated mitochondria. *Biochim. Biophys. Acta* **552:**552–557

Moss, J., Richards, R.L., Alving, C.R., Fishman, P.H. 1977. Effect of the A and B protomers of choleragen on release of trapped glucose from liposomes containing or lacking ganglioside GM1. *J. Biol. Chem.* **252:**797–798

Rink, T.J., Montecucco, C., Hesketh, T.R., Tsien, R.Y. 1980. Lymphocyte membrane potential assessed with fluorescent probes. *Biochim. Biophys. Acta* **595:**15–30

Rink, T., Sanchez, A., Grinstein, S., Rothstein, A. 1983. Volume restoration in osmotically swollen lymphocyte does not involve changes in free Ca^{2+} concentration. *Biochim. Biophys. Acta* **762:**593–596

Schwartz, W., Passow, H. 1983. Ca^{2+}-activated K^+ channels in

erythrocytes and excitable cells. *Annu. Rev. Physiol.* **45:**359–374

Simons, T.J. 1976. Carbocyanine dyes inhibit Ca-dependent K efflux from human red cell ghosts. *Nature (London)* **264:**467–469

Simons, T.J.B. 1979. Actions of a carbocyanine dye on calcium-dependent potassium transport in human red cell ghosts. *J. Physiol. (London)* **288:**481–507

Spiegel, S. 1984. Ganglioside involvement in lymphocyte stimulation. *In:* Cellular and Pathological Aspects of Glycoconjugate Metabolism. H. Dreyfus, R. Massarelli, L. Freysz, and G. Rebel, editors. Vol. 126, pp. 463–477. INSERM, Paris

Spiegel, S., Fishman, P.H. 1987. Gangliosides as bimodal regulators of cell growth. *Proc. Natl. Acad. Sci. USA* **84:**141–147

Spiegel, S., Fishman, P.H., Mulhern, S.A. 1986*a*. Cytoplasmic membrane potential of lymphocytes is decreased by the B subunit of cholera toxin. *Fed. Proc.* **45:**1882 *(Abstr)*

Spiegel, S., Fishman, P.H., Weber, R.J. 1985. Direct evidence that endogenous GM1 ganglioside can mediate thymocyte proliferation. *Science* **230:**1285–1287

Spiegel, S., Handler, J.S., Fishman, P.H. 1986*b*. Gangliosides modulate sodium transport in cultured toad kidney epithelia. *J. Biol. Chem.* **261:**15755–15760

Spiegel, S., Panagiotopoulos, C. 1988. Mitogenesis of 3T3 fibroblasts induced by endogenous ganglioside is not mediated by cAMP, protein kinase C, or phosphoinositides turnover. *Exp. Cell Res.* **177:**414–427

Svennerholm, L. 1984. Biological significance of gangliosides. *In:* Cellular and Pathological Aspects of Glycoconjugate Metabolism. H. Dreyfus, R. Massarelli, L. Freysz, and G. Rebel, editors. Vol. 126, pp. 21–44. INSERM, Paris

Tatham, P.E.R., Delves, P.J. 1984. Flow cytometric detection of membrane potential changes in murine lymphocytes induced by concanavalin A. *Biochem. J.* **221:**137–146

Tatham, P.E.R., O'Flynn, K., Linch, D.C. 1986. The relationship between mitogen-induced membrane potential changes and intracellular free calcium in human T-lymphocytes. *Biochim. Biophys. Acta* **845:**202–211

Tsien, R.Y., Pozzan, T., Rink, T.J. 1982. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature (London)* **295:**68–71

Wiegandt, H. 1982. The gangliosides. *Adv. Neurochem.* **4:**149–223

Wilson, H.A., Chused, T.M. 1985. Lymphocyte membrane potential and Ca^{2+}-sensitive potassium channels described by oxonol dye fluorescence measurements. *J. Cell. Physiol.* **125:**72–81

Wilson, H.A., Seligmann, B.E., Chused, T.M. 1985. Voltage-sensitive cyanine dye fluorescence signals in lymphocytes: Plasma membrane and mitochondrial components. *J. Cell. Physiol.* **125:**61–71

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