Transmembrane Signaling by the B Subunit of Cholera Toxin: Increased Cytoplasmic Free Calcium in Rat Lymphocytes

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Abstract. It has previously been shown that the B subunit of cholera toxin, which binds solely to the plasma membrane ganglioside G_{M1}, stimulates the proliferation of rat thymic lymphocytes (Spiegel, S., P. H. Fishman, and R. J. Weber, 1985, *Science [Wash. DC]*, 230:1285–1287). The purpose of this study was to identify which transmembrane signaling system(s) are activated by the B subunit of cholera toxin. We compared the effects of B subunit and concanavalin A (Con A), a potent mitogenic lectin, on a number of second messenger systems that are putative mediators of T cell activation. Changes in the fluorescence of quin2-loaded cells revealed that mitogenic doses of either B subunit or Con A induced rapid and sustained increases in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). Within 5 min, $[Ca^{2+}]_i$ increased from a basal level of 69 ± 4 to 136 ± 17 and 185 ± 24 nM, respectively. The effects of B subunit and Con A were additive and

largely dependent on the presence of extracellular Ca^{2+}, though release of Ca^{2+} from intracellular stores could be detected for Con A, but not B subunit, using indo-1. The B subunit had no effect on either inositol phosphate levels or on the distribution of protein kinase C, indicating that, unlike Con A, the B subunit does not activate phosphoinositide hydrolysis. Fluorimetric measurements on cells loaded with bis(carboxyethyl)-5,6-carboxyfluorescein revealed that Con A induced a rapid cytoplasmic alkalinization via activation of Na^+/H^+ exchange, whereas B subunit had no effect on intracellular pH. Finally, by monitoring bis-oxonol fluorescence, we found that Con A induced a small hyperpolarization of the membrane potential, whereas B subunit had no acute effect. These data suggest that the biological effects of B subunit are mediated by an increase in $[Ca^{2+}]_i$ resulting from a net influx of extracellular Ca^{2+}.

GANGLIOSIDES, sialic acid–containing glycosphingolipids, are ubiquitous components of the plasma membrane of mammalian cells. Several recent studies have demonstrated that exogenously added gangliosides can alter lymphocyte proliferation. On one hand, exogenous gangliosides suppress the proliferation of lymphocytes induced by lectins, antigens, and interleukin 2 (21, 35, 50). In contrast, when hapten-modified gangliosides are inserted into thymic lymphocytes (thymocytes), the cells proliferate in response to multivalent hapten-binding proteins (39, 41, 44). These findings have led to the suggestion that gangliosides may play a role in the regulation of lymphocyte activation. Recently it was demonstrated that the B or binding subunit of cholera toxin, which is pentavalent and binds exclusively to G_{M1} ganglioside on the cell surface (8, 19), is mitogenic for rat thymocytes (39, 42).

The purpose of the present study was to investigate the mechanism whereby B subunit binding to rat thymocytes is transduced into a biological signal capable of stimulating proliferation. Unlike many other mitogens such as con-

canavalin A (Con A), which binds to a number of cell surface glycolipids and glycoproteins, B subunit interacts exclusively with a single glycolipid species, making it potentially very useful as a probe for studying lymphocyte activation. In this study we examined the effects of the B subunit of cholera toxin on several transmembrane signaling systems that are stimulated by other mitogens and have been proposed to play a role in lymphocyte activation. These included acute changes in the concentration of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$)[1] (22, 23, 48), stimulation of phosphoinositide hydrolysis (45), activation of Na^+/H^+ exchange (17), and changes in transmembrane potential (5, 48). The effects of Con A, which has been shown to stimulate all of these pathways (17, 45, 48), were also determined in order to validate the measurements and to confirm the responsiveness of the cells. A preliminary report of this work has appeared in abstract form (6).

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1. *Abbreviations used in this paper*: BCECF, bis(carboxyethyl)-5,6-carboxyfluorescein; bis-oxonol, bis-(1,3-diethylthiobarbiturate)trimethineoxonol; $[Ca^{2+}]_i$, concentration of cytoplasmic free Ca^{2+}; CT, whole cholera toxin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Materials and Methods

Reagents

Gramicidin, monensin, Con A, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), phenylmethylsulfonylfluoride, phosphatidylserine, and histone type III-S were from Sigma Chemical Co. (St. Louis, MO). The B subunit of cholera toxin was obtained from List Biological Laboratories, Inc. (Campbell, CA) or Schwarz/Mann Biotech (Cleveland, OH). Bis-(1,3-diethylthiobarbiturate)-trimethineoxonol (bis-oxonol) and the acetoxymethyl esters of bis(carboxyethyl)5,6-carboxyfluorescein (BCECF) and indo-1 were obtained from Molecular Probes, Inc. (Junction City, OR). Quin2 acetoxymethyl ester was from Lancaster Synthesis Ltd. (Morecambe, United Kingdom). Nigericin and ionomycin were from Calbiochem-Behring Corp. (La Jolla, CA). Amiloride was a gift of Merck Frosst Canada. RPMI 1640 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were from Gibco (Grand Island, NY). Inositol-free RPMI 1640 was prepared by the University of Toronto media preparation services. [³H]Myoinositol was from New England Nuclear (Boston, MA) and γ-[³²P]ATP and [³H]thymidine were from ICN Biomedicals, Inc. (Irvine, CA). Na⁺ solution contained (in mM): 140 NaCl, 1 KCl, 1 MgCl, 1 CaCl₂, 10 glucose, and 20 Hepes-Na, pH 7.3. Where indicated, CaCl₂ was omitted and 0.5–1 mM EGTA was added to the medium.

Cells

Thymic lymphocytes (thymocytes) were obtained from 150–180-g male Wistar rats as described previously (12). Unless otherwise specified, cell suspensions were maintained for up to 8 h at room temperature in nominally HCO_3^--free RPMI 1640 medium supplemented with 20 mM Hepes, pH 7.3.

Proliferation Assay

The effect of the B subunit of cholera toxin on [³H]thymidine incorporation was determined to confirm that B subunit stimulated proliferation of the thymocytes used in these studies. Wistar thymocytes (2×10^6/well) were cultured in flat-bottomed microtiter trays in 0.2 ml RPMI 1640 medium supplemented with 5% FBS, 1 mM sodium pyruvate, nonessential amino acids, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). [³H]Thymidine incorporation was measured as described previously (42).

To assess the possible role of accessory cells in B subunit–induced proliferation, thymocytes were depleted of macrophages essentially as described by Rosenfelder (37). Cells not adhering to the plastic surface were gently removed and then assayed for [³H]thymidine incorporation as described above.

Binding of ¹²⁵I-Cholera Toxin to Thymocytes

Binding of ¹²⁵I-labeled cholera toxin (¹²⁵I-CT) was assayed as described previously (43), with some modifications. Thymocytes (3.9×10^6) were incubated with 0.5 nM ¹²⁵I-CT (1,120 cpm/fmol) in 0.2 ml of buffer containing 25 mM Tris-HCl (pH 7.4), 128 mM NaCl, 1 mM EDTA, 3 mM NaN₃, and 0.1% BSA. Where indicated, whole cholera toxin (CT), B subunit or antibodies to cholera toxin (anti–CT) were present. After 1 h at 37°C with constant shaking, the cells were washed three times with 3 ml of an ice-cold solution containing 5 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 3 mM NaN₃, and 0.2% BSA by centrifugation at 1,875 g for 10 min. The washed cells were then counted for bound ¹²⁵I-CT in a gamma counter (model 4000; Beckman Instruments, Inc., Palo Alto, CA).

Accumulation of AMP

Thymocytes were incubated in the absence or presence of B subunit (25 μg/ml) or CT (1 μg/ml) and assayed for cAMP accumulation as described previously (42).

Cytoplasmic Free Ca²⁺ Determinations

$[Ca^{2+}]_i$ was measured fluorimetrically using quin2 essentially as described previously (49). Briefly, thymocyte suspensions (5×10^7 cells/ml) in Hepes-buffered RPMI 1640 were loaded at 37°C for 30 min with 10 μM quin2 acetoxymethyl ester. Cells were then sedimented and resuspended in the indicated medium at 5×10^6 cells/ml. Fluorescence was monitored with excitation at 339 nm (3 nm slit) and emission at 495 nm (15 nm slit). Calibration of fluorescence versus $[Ca^{2+}]_i$ was obtained using 1 μM iono-

mycin and 2 mM Mn²⁺ as described previously (33). For indo-1 determinations, cells were loaded at 37°C for 20 min with 1 μM indo-1 acetoxymethyl ester. Fluorescence was monitored with excitation at 331 nm (3 nm slit) and emission at 410 (15 nm slit). As for quin2, calibration was obtained using ionomycin and Mn²⁺ (10).

Analysis of Inositol Phosphates

Thymocytes (40×10^6 cells/ml) were labeled with [³H]myoinositol (6 μCi/ml) by incubation at 37°C for 18 h in inositol-free Hepes-buffered RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were then sedimented and resuspended in fresh medium without antibiotics at 120×10^6 cells/ml. Aliquots (200 μl) were preincubated with 10 mM LiCl for 15 min in a shaking bath at 37°C. This was followed by incubation in the presence of Con A (10 μg/ml), B subunit (10–25 μg/ml), or vehicle for 5–10 min. Cells were then sedimented and [³H]inositol phosphates analyzed as described previously (14). Briefly, cells were extracted with chloroform/methanol, inositol phosphates separated on Dowex 1X-8 anion-exchange columns (Bio-Rad Laboratories, Richmond, CA), and samples counted by liquid scintillation.

Soluble Protein Kinase C Assay

Thymocytes suspended in Hepes-buffered RPMI 1640 (50×10^6 cells/ml) were incubated with B subunit (10 μg/ml), Con A (10 μg/ml), TPA (10⁻⁷ M), or vehicle for 10 min at 37°C. The cells were then washed, resuspended at 1.5×10^9 cells/ml in 140 mM Tris-HCl, 7 mM EDTA, pH 7.5, and subsequently lysed by addition of 6 vol ice-cold water with 1 mM phenylmethylsulfonylfluoride. After 10 min at 4°C, the samples were centrifuged at 48,000 g for 30 min at 4°C and the supernatants tested for protein kinase C activity. Supernatants were passed through a DE-52 (DEAE-cellulose) column to remove endogenous lipid, diacylglycerol, kinase inhibitors, and unrelated kinases as described (7). Protein kinase C was then eluted from the columns with 70 mM NaCl. Phosphotransferase activity was determined by measuring incorporation of ³²P from γ-[³²P]ATP into histone protein. The assay mixture contained in a volume of 250 μl/20 mM magnesium acetate, 1 mM CaCl₂, 100 μM ATP, 1 μCi γ-[³²P]ATP, 50 μg histone type III-S, 10⁻⁷ M TPA, and 24 μg phosphatidylserine. The reaction was started by addition of 5–20 μg of thymocyte protein and after a 3-min incubation at 35°C, terminated by the addition of 1 ml ice-cold 25% TCA. The precipitated histone was then separated by filtration on 0.45 μm HA filters, (Millipore/Continental Water Systems, Bedford, MA) followed by four washes with 3 ml 5% TCA each. Filters were then counted by liquid scintillation. All assays were performed in triplicate. Initial assays were performed in the presence and absence of TPA and phosphatidylserine to define protein kinase C–mediated phosphorylation. It was found that, when using the fraction eluted from the column with 70 mM NaCl, essentially all (>90%) the phosphorylation was mediated by protein kinase C.

Measurement of Cytoplasmic pH

pH_i was measured fluorimetrically using BCECF as described previously (12). Thymocyte suspensions (5×10^7 cells/ml) in Hepes-buffered RPMI 1640 were loaded at 37°C for 20 min with 2 μM BCECF acetoxymethyl ester. Cells were then sedimented and resuspended in the indicated medium at 3×10^6 cells/ml. BCECF fluorescence was monitored with excitation at 495 nm (5 nm slit) and emission at 525 nm (10 nm slit). Calibration of fluorescence versus pH_i was obtained using the K⁺-nigericin method as described previously (46).

Measurement of Membrane Potential

Membrane potential was measured fluorimetrically using bis-oxonol essentially as described previously (32). Bis-oxonol (0.2 μM, final) was added to cells (3×10^6 cells/ml) suspended in the indicated medium. Bix-oxonol fluorescence was monitored with excitation at 540 nm (3 nm slit) and emission at 580 nm (10 nm slit). Calibration of fluorescence versus membrane potential was obtained by adding gramicidin (0.1 μM, final) to cells suspended in medium containing varying ratios of Na⁺ and the impermeant cation N-methyl-D-glucamine⁺. Membrane potential (E_m) was then calculated as $E_m = 60 \log ([Na^+]_o/[Na^+ + K^+]_i)$ assuming that Na⁺ and K⁺ are equally permeable through gramicidin.

Other Methods

Fluorescence measurements were made using fluorescence spectrophotom-

eters (model F-4000; Hitachi Ltd., Tokyo, Japan; or models 650-40 or LS-5; Perkin-Elmer Corp., Pomona, CA). Protein was determined by the method of Bradford (2). All experiments were performed at 37°C. Unless otherwise indicated, results are presented either as traces representative of at least three experiments, or as the mean ± the standard error of the number of determinations specified.

Results

[^{3}H]Thymidine Incorporation

As previously reported (42), the B subunit of cholera toxin was found to stimulate the proliferation of the thymocytes used in these studies (Table I). Proliferation was observed at concentrations of B subunit as low as 25 ng/ml (data not shown) and maximum mitogenic stimulation was achieved at 1–2.5 μg/ml. Removal of accessory cells by adherence to plastic did not affect this response (Table I). As has been previously shown for Sprague-Dawley rats from Zivic-Miller Labs (Zellenople, PA) (42), the mitogenic response of Wistar thymocytes to the B subunit is a result of its specific binding to the cell surface receptor for cholera toxin (ganglioside G_{M1}), since preincubation of the B subunit with antibodies to cholera toxin abolished the increase in thymidine incorporation induced by B subunit (Table I) and also completely blocked the binding of ^{125}I-cholera toxin to the cells (Table I). Unlabeled cholera toxin or B subunit also blocked this binding (Table I).

cAMP Accumulation

The A subunit of cholera toxin directly activates adenylate cyclase and increases intracellular cAMP levels (8). To rule out the possibility that the mitogenic effects of the B subunit on Wistar thymocytes arose from contamination by the A subunit, we measured the ability of the B subunit to elevate cAMP in Wistar thymocytes. The B subunit, even at high concentrations (25 μg/ml) had no effect on cAMP levels: the intracellular levels of cAMP in B subunit–treated cells were 19 ± 2 compared with untreated control levels of 16 ± 2 pmol/mg protein. In contrast, a much lower concentration of CT (1 μg/ml) caused a pronounced increase in cAMP levels to 401 ± 30 pmol/mg (data are means ± SD of triplicate determinations from a single experiment representative of four individual experiments). Thus, the mitogenic effects of the

B subunit on Wistar thymocytes must be unrelated to increases in cAMP.

Cytoplasmic Free Ca^{2+}

Using the fluorescent Ca^{2+}-sensitive dye, quin2, it was found that application of the B subunit of cholera toxin to thymocytes induced a rapid and sustained increase in $[Ca^{2+}]_i$ (Fig. 1 a). $[Ca^{2+}]_i$ increased from basal levels of 69 ± 4 nM ($n = 24$) to 136 ± 17 nM ($n = 14$). Con A induced larger sustained increases in $[Ca^{2+}]_i$ to levels of 185 ± 24 nM ($n = 10$) (Fig. 1 b). It was found consistently that the onset of the rise in $[Ca^{2+}]$; induced by maximally mitogenic doses of B subunit was virtually immediate. In contrast, there was a lag of ~30 s before $[Ca^{2+}]_i$ increased in response to equivalent doses of Con A (Fig. 1, a and b).

A similar final level of $[Ca^{2+}]_i$ was attained by cells treated with the B subunit over the range of doses (0.25 –25 μg/ml), which has been shown to be mitogenic for thymocytes (42). The rate of rise in $[Ca^{2+}]_i$ was, however, dose dependent, increasing at larger concentrations of B subunit (cf. Fig. 1, a and d). Once $[Ca^{2+}]_i$ had stabilized after addition of either B subunit or Con A, further application of the same ligand had no additional effect on $[Ca^{2+}]_i$. If however, Con A was applied to cells originally exposed to B subunit, a further increase in $[Ca^{2+}]_i$ was observed having a time course and magnitude similar to the initial response to the lectin (final $[Ca^{2+}]_i = 264 ± 36$ nM, $n = 11$). Conversely, addition of B subunit to Con A-treated cells resulted in a second wave of $[Ca^{2+}]_i$ increase (final $[Ca^{2+}]_i = 240 ± 44$ nM, $n = 5$)^2. These observations suggest that B subunit and Con A modulate $[Ca^{2+}]_i$ via two different mechanisms, or that different subpopulations of cells respond to each ligand.

Increases in $[Ca^{2+}]_i$ can arise from an influx of extracellular Ca^{2+} or a release of Ca^{2+} from intracellular stores. To distinguish between these sources, we tested the effects of B subunit and of Con A on quin2-loaded cells suspended in Ca^{2+}-free medium containing EGTA. Under these conditions, there was no detectable change in $[Ca^{2+}]_i$ in response

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2. In contrast to the additive effect of B subunit and Con A on $[Ca^{2+}]_i$, CT antagonizes the effect of Con A on $[Ca^{2+}]_i$ (48). This antagonism is a cAMP-dependent process and can be mimicked by other agents that raise intracellular cAMP levels.


Table I. Effect of B Subunit and Anti–Cholera Toxin Antibodies on Thymocyte Proliferation and Binding of ^{125}I-Cholera Toxin to Thymocytes

Addition	[^{3}H]Thymidine incorporation*		^{125}I-Cholera toxin bound‡
	Minus B subunit	Plus B subunit	
	cpm	cpm	fmol/10^6 cells
None	3,615 ± 850	18,150 ± 145	69.5 ± 5.4
Anti-CT	4,290 ± 920	5,210 ± 810	—
Macrophage-depleted thymocytes	6,630 ± 580	28,170 ± 2,710	—
Cholera toxin	—	—	0.43 ± 0.04
B subunit	—	—	0.46 ± 0.03
Anti-CT	—	—	0.24 ± 0.02

Thymocytes from Wistar rats were prepared and maintained as described in Materials and Methods. Values are means ± SD of triplicate determinations from representative experiments. Similar results were obtained in at least three additional experiments.
* Thymocytes (2 × 10^6 in 0.2 ml medium) were cultured in the absence or presence of B subunit (2.5 μg/ml). Where indicated B subunit was preincubated with anti–cholera toxin antiserum (anti-CT, 1:10 dilution) for 1 h before addition to the cells (42). Macrophage depletion was performed as described in Materials and Methods. The amount of [^{3}H]thymidine incorporated into cells treated with Con A (2.5 μg/ml) was 78,500 ± 6,200 cpm.
‡ Thymocytes were incubated in the presence of B subunit (25 μg/ml), whole cholera toxin (25 μg/ml), or anti–cholera toxin antiserum (1:10 dilution) and assayed for ^{125}I-cholera toxin binding as described in Materials and Methods.
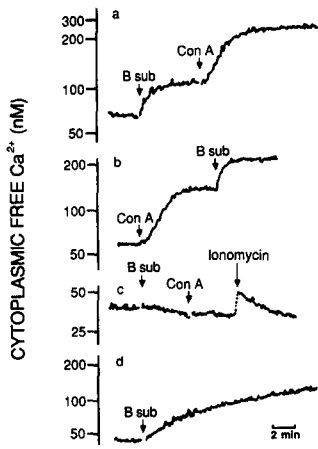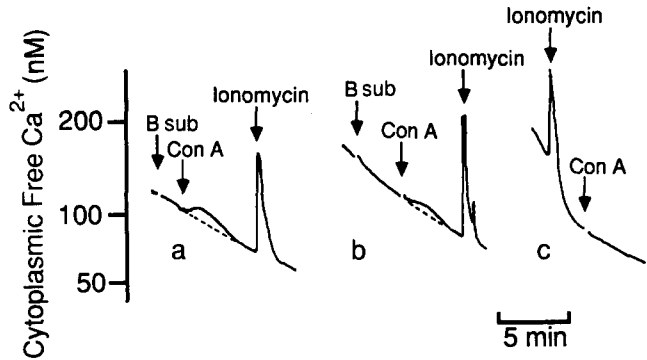
Figure 1. Quin2 measurements of the effects of the B subunit of cholera toxin and Con A on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ of rat thymocytes was monitored fluorimetrically using quin2 as described in Materials and Methods. (*a* and *b*) Cells were suspended in Na^+ solution. The B subunit of cholera toxin (*B sub*) and Con A were added where indicated to a final concentration of 10 μg/ml. (*c*) Cells were suspended in Ca^{2+}-free Na^+ solution and EGTA (1 mM) was added at the beginning of the trace. B subunit (10 μg/ml), Con A (10 μg/ml), and ionomycin (1 μM) were added where indicated. (*d*) Cells were suspended in Na^+ solution and B subunit (0.25 μg/ml) was added where indicated.

to either B subunit or Con A. In contrast, ionomycin, a divalent cation ionophore, induced a small transient rise in $[Ca^{2+}]_i$, presumably by releasing Ca^{2+} from intracellular stores (Fig. 1 *c*). These observations suggest that the increases in $[Ca^{2+}]_i$ induced by B subunit and Con A arise largely, if not entirely, from net influx of extracellular Ca^{2+}. However, the relatively high intracellular concentrations of quin2 required for reliable fluorescence determinations are potentially capable of buffering small Ca^{2+} transients. Therefore, using this dye, the possibility cannot be excluded that a portion of the response arises from release of a limited pool of intracellular Ca^{2+}. To circumvent this problem, we also performed measurements on cells lightly loaded with a second dye, indo-1, which is effective as a Ca^{2+} indicator at much lower cytoplasmic concentrations than quin2 (15). Essentially the same results were obtained with both dyes in Ca^{2+}-containing medium, i.e., Con A and B subunit induced sustained increases in $[Ca^{2+}]_i$ (not illustrated). However, using indo-1-loaded cells in Ca^{2+}-free medium, we were able to detect a small transient increase in $[Ca^{2+}]_i$ (peak magnitude ~10 nM) in response to Con A, but not B subunit (Fig. 2). Partial depletion of intracellular Ca^{2+} stores by prolonged incubation in Ca^{2+}-free medium reduced this response (Fig. 2 *b*) and elimination of these stores by prior exposure to ionomycin abolished the response (Fig. 2 *c*). These data suggest that part of the Con A-induced Ca^{2+} response arises from the release of intracellular Ca^{2+}, whereas the response to B subunit solely involves influx from the external medium.

Inositol Phosphates and Protein Kinase C

It is well established that mitogens such as Con A and phytohemagglutinin activate phospholipase C (45), which in turn hydrolyses polyphosphoinositides liberating the intracellular second messengers diacylglycerol and inositol trisphosphate. Diacylglycerol is thought to activate protein kinase C (28), and inositol 1,4,5-trisphosphate to mediate the release of Ca^{2+} from intracellular stores (1). To determine whether B subunit also activates these pathways, we assayed its effects on the levels of inositol phosphates and on the distribution of protein kinase C.

Inositol phosphate levels were measured in thymocytes

Figure 2. Indo-1 measurements of the effects of the B subunit of cholera toxin and Con A on $[Ca^{2+}]_i$. To detect the release of Ca^{2+} from intracellular stores, rat thymocytes were lightly loaded with indo-1 and $[Ca^{2+}]_i$ monitored fluorimetrically as described in Materials and Methods. Cells were suspended in Ca^{2+}-free Na^+ solution and EGTA (0.5 mM) was added at the beginning of each trace. The baseline fluorescence of indo-1-loaded thymocytes in Ca^{2+}-free medium always exhibited a steady decrease. B subunit (*B sub*; 10 μg/ml), Con A (10 μg/ml), and ionomycin (1 μM) were added where indicated. B subunit had no effect on $[Ca^{2+}]_i$ (*a* and *b*), whereas Con A induced a small transient increase in $[Ca^{2+}]_i$ (*a*). The magnitude of this Con A-induced increase was reduced with longer times of exposure to EGTA (*b*). Prior addition of ionomycin abolished the effect of Con A (*c*).

equilibrated overnight with [^{3}H]myoinositol followed by incubation with or without B subunit or Con A for 5 and 10 min. To prevent the rapid hydrolysis of inositol phosphates to inositol, experiments were performed in the presence of lithium, an inhibitor of inositol-1-phosphate and inositol bis-phosphate phosphatases (1). Whereas Con A induced a significant increase in the levels of inositol phosphates, the B subunit of cholera toxin caused no changes even at concentrations up to 25 μg/ml (Fig. 3).

We next assayed cytoplasmic protein kinase C levels after stimulation by B subunit. It has been previously shown that both phorbol esters and physiological stimuli, which elevate
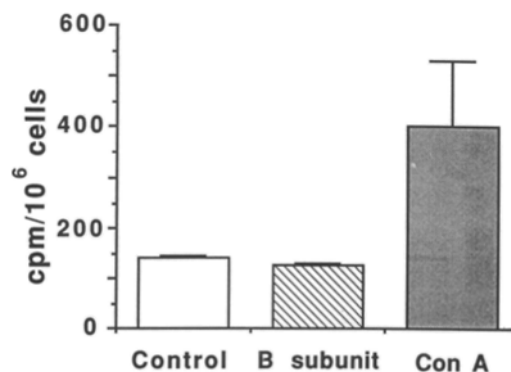
Figure 3. Effects of the B subunit of cholera toxin and Con A on the formation of inositol phosphates. Rat thymocytes were incubated for 10 min with either B subunit (10 μg/ml), Con A (10 μg/ml), or vehicle (*Control*) at 37°C in the presence of 10 mM LiCl. Inositol phosphate levels were then determined as described in Materials and Methods. Data are presented as the total radioactivity (cpm) incorporated into inositol phosphate, bisphosphate, and trisphosphate per 10^6 cells. Values are means ± SE from two experiments with duplicate determinations.
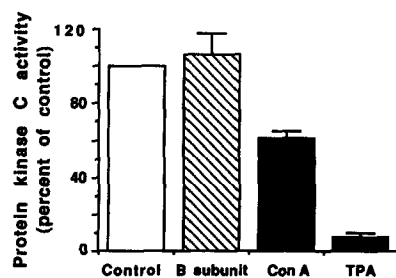
the cytoplasmic levels of diacylglycerol, cause a redistribution of protein kinase C: a decrease in cytoplasmic kinase C levels and a corresponding increase in membrane-associated kinase activity (7, 27). Therefore, the intracellular location of protein kinase C can be used as an indirect measure of the activation of phospholipase C. To determine whether application of B subunit leads to redistribution of protein kinase C, we assayed cytoplasmic protein kinase C levels after a 10-min incubation with B subunit, Con A, or the phorbol ester, TPA. As expected, both Con A and TPA decreased soluble protein kinase C activity. In contrast, cytoplasmic levels of the kinase in B subunit–treated cells were not significantly different from controls (Fig. 4). Taken together, these data indicate that the B subunit of cholera toxin has no effect on phosphoinositide hydrolysis and does not activate protein kinase C in rat thymocytes.
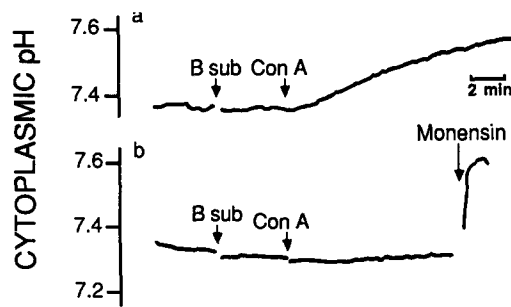
Cytoplasmic pH

It has been suggested that activation of Na^+/H^+ exchange is a key event in the initiation of the mitogenic response (29). Accordingly, Con A has been shown to induce a Na^+-dependent cytoplasmic alkalinization in mouse thymocytes, presumably reflecting the activation of Na^+/H^+ exchange (17). Using the fluorescent pH-sensitive dye BCECF, we found that Con A also induces cytoplasmic alkalinization of rat thymocytes (Fig. 5). The dependence of this phenomenon on extracellular Na^+ (data not shown) and its sensitivity to amiloride (Fig. 5) suggest that it arises from activation of Na^+/H^+ exchange. In contrast to Con A, the B subunit was found to have no effect on pH_i (Fig. 5). The failure of the B subunit to alter pH_i in the presence of amiloride (Fig. 5) or in the absence of Na^+ (not shown) precluded the possibility that, in addition to activating Na^+/H^+ exchange, B subunit was simultaneously activating a second process that offset the effects of Na^+/H^+ exchange on pH_i. These observations indicate that the B subunit of cholera toxin has no acute effect on Na^+/H^+ exchange or other pH_i-modulating systems.

Membrane Potential

We next examined the effect of the B subunit on the membrane potential of rat thymocytes. Some authors have suggested that changes in membrane potential play a crucial role in the activation of B lymphocytes (3). Because an increase
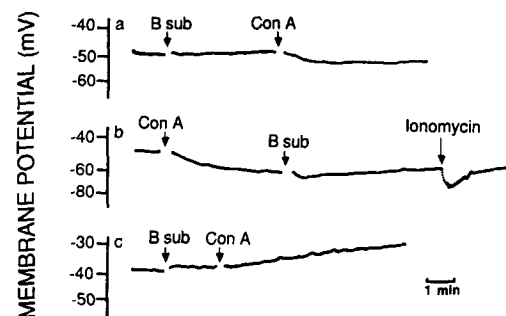
Figure 5. Effects of the B subunit of cholera toxin and Con A on cytoplasmic pH. Rat thymocytes were loaded with BCECF and pH_i monitored as described in Materials and Methods. (*a*) Cells were suspended in Na^+ solution. B subunit (*B sub*; 10 µg/ml) and Con A (10 µg/ml) were added where indicated. (*b*) Cells were suspended in Na^+ solution containing 200 µM amiloride, an inhibitor of the plasma membrane Na^+/H^+ exchanger. B subunit (10 µg/ml) and Con A (10 µg/ml) were added where indicated. Monensin (4 µM), a Na^+/H^+-exchanging ionophore that is insensitive to amiloride, was added to confirm the responsiveness of the cells and the sensitivity of the assay.

in $[Ca^{2+}]_i$ was detected after treatment with B subunit, it was conceivable that nonspecific cation channels or voltage-sensitive Ca^{2+} channels were being activated. By monitoring bis-oxonol fluorescence, we compared the acute effects of B subunit and Con A on membrane potential. There was no detectable change in fluorescence in response to B subunit (Fig. 6 *a*). In contrast, mitogenic concentrations of Con A induced a small hyperpolarization (Fig. 6 *b*). This response was variable, sometimes being sustained and occasionally returning to the resting potential within minutes. When B subunit was applied after a prior application of Con A, an additional small hyperpolarization was sometimes detected (Fig. 6 *b*). Since the magnitude and kinetics of these changes in membrane potential paralleled the changes in $[Ca^{2+}]_i$ described above, it seemed possible that these hyperpolarizations arose from activation of a Ca^{2+}-dependent K^+ conductance. The presence of Ca^{2+}-dependent K^+ channels could be demonstrated independently using ionomycin (Fig. 6 *b*). In Ca^{2+}-containing media, the ionophore (25–50 nM) in-

Figure 4. Effects of the B subunit of cholera toxin and TPA on soluble protein kinase C levels. Rat thymocytes were incubated for 10 min at 37°C with B subunit (10 µg/ml), Con A (10 µg/ml), TPA (10^{-7} M), or vehicle (*Control*). Cells were then homogenized and the protein kinase C activity of the soluble fraction assayed as described in Materials and Methods. Data are means ± SE of three experiments with triplicate determinations.

Figure 6. Effects of the B subunit of cholera toxin and Con A on membrane potential. The membrane potential of rat thymocytes was monitored fluorimetrically using bis-oxonol as described in Materials and Methods. B subunit (*B sub*; 10 µg/ml), Con A (10 µg/ml), and ionomycin (100 nM) were added where indicated. (*a* and *b*) Cells were suspended in Na^+ solution. (*c*) Cells were suspended in Ca^{2+}-free Na^+ solution supplemented with 1 mM EGTA.

Table II. Effect of Phorbol Ester on Proliferation Induced by the B Subunit of Cholera Toxin

Addition	[³H]Thymidine incorporation
	cpm
None	4,210 ± 320
TPA	2,055 ± 430
B subunit	17,400 ± 3,130*
TPA + B subunit	47,775 ± 9,510

Cells (5 × 10⁶/ml) were incubated in RPMI 1640 medium for 2 h at 37°C with B subunit (10 µg/ml) or the phorbol ester TPA (1 nM). Where indicated, B subunit (10 µg/ml) was added after 1 h to TPA-treated cells for an additional 1 h at 37°C. Cells were then washed twice with RPMI containing defatted BSA and twice with RPMI. Aliquots (2 × 10⁶ cells) were then incubated in the culture medium (10⁷ cells/ml) as described for the proliferation assay for 72 h and thymidine incorporation measured (42). Data are means ± SD of quadruplicate determinations from a single experiment representative of two independent experiments.
* When B subunit (2.5 µg/ml) was present for the entire 72-h incubation period, thymidine incorporation was 20,320 ± 2,790 cpm.

duced a clear hyperpolarization. These concentrations of ionomycin produced elevations of $[Ca^{2+}]_i$ of the same order as those induced by the lectin (not illustrated). Consistent with the opening of Ca^{2+}-sensitive K^+ channels, Con A, which depends primarily on external Ca^{2+} to elevate $[Ca^{2+}]_i$, did not induce a hyperpolarization in Ca^{2+}-free medium (Fig. 6 c). It did, however, induce a slow steady increase in fluorescence which was found to be artifactual, presumably resulting from an interaction between bis-oxonol and Con A occurring only in the absence of Ca^{2+} and in the presence of EGTA. Experiments in Ca^{2+}-free medium failed to demonstrate any Ca^{2+}-independent effects of the B subunit on membrane potential (Fig. 6 c).

Potentiation of B Subunit-induced Proliferation by Phorbol Ester

As described above, the B subunit of cholera toxin induces a pronounced increase in intracellular Ca^{2+} but does not activate protein kinase C. It has been reported that the phorbol ester TPA, which directly activates protein kinase C, is by itself a weak mitogen that acts synergistically with calcium ionophore in promoting the proliferation of T lymphocytes (24). We therefore investigated whether TPA would potentiate the effect of B subunit on lymphocyte proliferation. We found that preincubation of thymocytes with TPA for 1 h followed by the addition of B subunit produced a greater proliferative response than observed in cells not preincubated with TPA (Table II). The increase in $[Ca^{2+}]_i$ after addition of B subunit was similar with or without TPA pretreatment (data not shown).

Discussion

The response of lymphocytes to polyclonal mitogens such as Con A or phytohemagglutinin is a convenient model for the study of cell activation and growth. The binding of mitogens to the surface of lymphocytes leads to a pleiotropic response that ultimately results in cell proliferation. Since the initiation of DNA synthesis is a late event, occurring 48 h after exposure to mitogenic agents, attention has been focused on initial cellular responses in the expectation that early events will provide clues as to the primary regulatory mechanisms.

Thus, increases in $[Ca^{2+}]_i$, stimulation of phosphoinositide hydrolysis, activation of Na^+/H^+ exchange, and changes in membrane potential have all been implicated as essential early events in the mitogenic response (17, 22, 23, 45, 48).

It is well established that Con A, a potent polyclonal mitogen, induces a sustained increase in the $[Ca^{2+}]_i$ of thymocytes (17, 48; Fig. 1), a portion of which arises by the release of Ca^{2+} from intracellular stores (17; Fig. 2). The putative signal inducing the release from internal stores is inositol 1,4,5-trisphosphate. Indeed, it has also been reported that Con A induces the rapid formation of inositol phosphates in mouse thymocytes (45; see also Fig. 3), likely resulting from the activation of phospholipase C. Diacylglycerol, the other product of the hydrolysis of phosphoinositides by phospholipase C, or its structural analogs, the phorbol esters have been shown to be comitogenic in lymphocytes and their effects are potentiated by elevated $[Ca^{2+}]_i$ (24). The effect of Con A on the pH_i of thymocytes is more controversial: earlier reports suggested that the lectin had no effect on pH_i (34, 36), whereas a more recent report (17) demonstrated, as we have found, a rapid cytoplasmic alkalinization arising from activation of Na^+/H^+ exchange. In any event, it is becoming increasingly clear that activation of the Na^+/H^+ antiport and the resulting cytoplasmic alkalinization are not essential for the initiation of cellular proliferation in lymphocytes (25). Considerable confusion also exists regarding the nature of the membrane potential changes associated with mitogenesis, the mechanism underlying these changes, and their significance to the proliferative response. It has been suggested that lectin binding induces a negative shift in the potential sensitivity of the voltage-dependent K^+ channels of T cells, and that opening of these channels is essential for mitogenesis (5). However, both the reproducibility of the measurements (38) and the validity of the interpretation (10) have been questioned. Our results are in agreement with those of Tsien and co-workers (48), who found that Con A induced a membrane potential hyperpolarization in thymocytes. This hyperpolarization possibly resulted from activation of a Ca^{2+}-dependent K^+ conductance, which has been documented to exist in lymphocytes (31), though changes in the voltage-sensitive K^+ conductance cannot be ruled out. Although the presence of a normal resting membrane potential is essential for lymphocyte proliferation (9), there is no evidence to suggest that the comparatively small hyperpolarization elicited by Con A plays a role in the initiation of mitogenesis.

The simultaneous activation of a number of transmembrane-signaling systems possibly reflects the relative lack of specificity of Con A, which binds to a number of cell surface glycoproteins and glycolipids. In contrast, the B subunit of cholera toxin binds only to G_{M1} ganglioside and stimulates only the influx of extracellular Ca^{2+}. That B subunit causes an increase in $[Ca^{2+}]_i$ was demonstrated directly by measurement of quin2 and indo-1 fluorescence, and indirectly by measurement of membrane potential. The prolonged nature of the $[Ca^{2+}]_i$ changes recorded with quin2 is not due to the buffering characteristics of the probe. This is indicated by two findings. First, in quin2-loaded cells, sustained elevations in $[Ca^{2+}]_i$ were observed for up to 15 min without decreasing detectably. Second, a similar sustained Ca^{2+} response to B subunit was observed using cells loaded lightly with indo-1, which has a much smaller Ca^{2+}-buffering capacity than quin2 (15). The fluorescence changes

seen in response to B subunit were not artifactual inasmuch as B subunit had no effect in Ca^{2+}-free medium, and in Ca^{2+}-containing medium the response occurred in the range where B subunit is mitogenic and it was saturable, as expected of a receptor-mediated biological response. Moreover, although application of B subunit per se did not have an acute effect on membrane potential (Fig. 6 a), addition of the B subunit subsequent to Con A application caused a small hyperpolarization (Fig. 6 b). These observations can be explained by assuming that the $[Ca^{2+}]_i$ levels attained with B subunit alone are insufficient to trigger the K^+ conductance, but that the added effects of both mitogens exceed the threshold required to open the channels.

The lack of effect of B subunit on $[Ca^{2+}]_i$ in Ca^{2+}-free medium suggests that the B subunit does not induce release of Ca^{2+} from intracellular stores. This was observed even in cells loaded lightly with indo-1 under conditions where a release of intracellular Ca^{2+} could be detected in response to Con A (Fig. 2). These data are consistent with the observation that inositol trisphosphate, the second messenger that mediates release of Ca^{2+} from intracellular stores in a variety of cell types (1), was produced in response to the lectin but not to the B subunit. That B subunit does not stimulate phosphoinositide hydrolysis was further indicated by measurements of the distribution of protein kinase C. The cytoplasmic levels of the kinase were not affected by B subunit, indicating that diacylglycerol was not liberated. Consistent with this conclusion, it was found that B subunit did not affect Na^+/H^+ exchange activity, which is thought to be modulated by protein kinase C, the putative target of diacylglycerol. Phorbol esters, exogenous activators of protein kinase C, are known to activate Na^+/H^+ exchange in thymocytes (13), and it has been suggested that activation of Na^+/H^+ exchange by growth factors and mitogens such as Con A is mediated by the release of diacylglycerol and subsequent activation of protein kinase C (17).

The question remains of how the B subunit stimulates a net flux of extracellular Ca^{2+} into the cell. One possibility is that B subunit binding to or cross-linking of G_{M1} ganglioside activates directly a preexisting Ca^{2+} channel. Such a mechanism would be consistent with the rapidity of the Ca^{2+} response observed at high doses of B subunit (Fig. 1 a). In addition, evidence also exists that B subunit-G_{M1} complexes may interact with integral membrane proteins or protein complexes. G_{M1} ganglioside does not span the plasma membrane, yet once bound and cross-linked by cholera toxin or B subunit, it can apparently interact with the cytoskeleton. This proposal is based on the observation that the B subunit reduces the susceptibility of G_{M1} to detergent extraction (16), and that B subunit-G_{M1} complexes on lymphocytes laterally redistribute to form caps (30, 39, 43). Furthermore, the ability of cytoskeletal disrupting agents to inhibit cholera toxin–induced capping (30) and the demonstration of co-capping of the toxin and α-actinin (19), clearly indicate that G_{M1} ganglioside is associated with transmembrane protein(s) which in turn interact with the cytoskeleton. If there is also an association between G_{M1} and protein components of an ion channel, then it is conceivable that clustering of G_{M1} by the B subunit could cause a change in the gating properties of the ion channel. The presence of a distinct mitogen-regulated Ca^{2+} channel in T lymphocytes was recently demonstrated by Kuno and co-workers (20).

It is possible that instead of activating preexisting Ca^{2+} channels, B subunit induces the *de novo* formation of Ca^{2+}-permeable pores. In this regard, it has been shown that either whole cholera toxin or B subunit can induce the release of trapped glucose from liposomes containing G_{M1} (26). Similarly, it has been shown that cholera toxin induces the formation of relatively nonselective ion channels in artificial lipid bilayers containing G_{M1} (47). However, it is unlikely that B subunit induced such channels in lymphocytes, inasmuch as addition of the toxin subunit did not lead to a depolarization of the membrane potential. Lastly, we cannot exclude the possibility that, rather than stimulating influx of extracellular Ca^{2+}, B subunit is reducing Ca^{2+} efflux from the cell by an inhibitory interaction with the plasma membrane Ca^{2+}-ATPase or Na^+/Ca^{2+} exchanger.

Consistent with the results of a previous study on Sprague-Dawley thymocytes (42), we have found that B subunit does not increase cAMP levels in Wistar thymocytes. This observation rules out the possibility that the biological response to B subunit arises from contamination by the A subunit of cholera toxin, which can activate adenylate cyclase by ADP-ribosylation of the stimulatory G protein (4).

The lack of effect of depletion of accessory cells on B subunit-induced mitogenesis (Table I) is consistent with a mechanism involving direct mitogenic stimulation of the thymocytes. Also in this regard, it has been reported that B subunit stimulates proliferation of quiescent mouse fibroblasts, a process in which accessory cells do not play a role (40). We conclude that thymocytes both increase $[Ca^{2+}]_i$ and proliferate directly in response to B subunit. Lymphoproliferation induced exclusively by elevation of $[Ca^{2+}]_i$ has been repeatedly demonstrated using divalent cation ionophores such as A23187 and ionomycin (23, 24). On the other hand, the maximum mitogenic effect of the B subunit was only a fraction ($\leqslant 20\%$) of that obtained with Con A. The greater mitogenic potency of the lectin is consistent with its multiple effects on a variety of signaling pathways. Thus, the concomitant effects of Con A on $[Ca^{2+}]_i$ and phosphoinositide hydrolysis are likely to contribute synergistically to the ensuing mitogenic response. This conclusion is consistent with the reported synergistic effects of calcium ionophores and phorbol esters on lymphocyte proliferation (24). The comparatively smaller mitogenic effect of B subunit could therefore be explained by assuming that only the Ca^{2+}-sensitive pathway is activated. Consistent with this hypothesis, the maximal levels of proliferation obtained with calcium ionophores are also only a fraction of the response produced by mitogenic lectins. Moreover, an effect of B subunit solely on $[Ca^{2+}]_i$ is consistent with the observed potentiation by phorbol ester of B subunit–induced proliferation (Table II). Recently, an additional, as yet unidentified, mitogenesis-signaling pathway has been suggested to exist in lectin-stimulated lymphocytes (11, 18). The possibility that in addition to increasing $[Ca^{2+}]_i$, B subunit also activates an unidentified pathway cannot be excluded. Furthermore, it should be noted that we have examined only the acute effects of B subunit on a number of well-characterized transmembrane signaling systems. We cannot exclude the possibility that B subunit may have delayed effects on these systems differing from the short-term effects of other mitogens such as Con A.

In conclusion, we have demonstrated that the B subunit of

cholera toxin induces a sustained increase in the $[Ca^{2+}]_i$ of rat thymocytes, but does not have acute effects on phosphoinositide hydrolysis, cytoplasmic pH, or membrane potential. Preliminary results have shown that B subunit induces similar increases in the $[Ca^{2+}]_i$ of quiescent mouse fibroblasts (Spiegel, S., unpublished data) and human B lymphocytes (Dixon, S. J., unpublished observations), suggesting that this may be a general phenomenon. Consequently, studies in which whole cholera toxin is used to determine the biological role of adenylate cyclase will have to be interpreted cautiously to distinguish between the effects of the A subunit on G_s and the B subunit on $[Ca^{2+}]_i$. These results also suggest that the B subunit of cholera toxin may be useful as an agent for specifically increasing $[Ca^{2+}]_i$ without perturbing cytoplasmic pH, intracellular Ca^{2+} stores, or mitochondrial function as do conventional Ca^{2+} ionophores such as A23187 and ionomycin, which function as $Ca^{2+}/2H^+$ exchangers. Lastly, our results indicate that G_{M1} ganglioside may play a role in regulation of $[Ca^{2+}]_i$ and perhaps functions as a receptor for physiological ligands.

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