Volume 225, number 1,2, 16–20 FEB 05346 December 1987

Human T lymphocyte mitogenesis in response to the B oligomer of pertussis toxin is associated with an early elevation in cytosolic calcium concentrations

Colette F. Strnad and Richard A. Carchman

Department of Pharmacology and Toxicology, Medical College of Virginia, Richmond, VA 23298-0001, USA

Received 8 October 1987

Pertussis toxin was found to serve as a mitogen in the human T lymphocyte, an effect which could be mimicked by its resolved binding component, the B oligomer. The mechanism of action of this component appeared to involve a rapid and sustained elevation of cytosolic calcium levels, as monitored by fura-2 fluorescence. The source of mobilized calcium was predominantly extracellular, suggesting that the binding of the B oligomer to the T cell plasma membrane in some way elicited calcium channel activation. Notably, the influx of calcium was not observed with cholera toxin, an AB toxin lacking mitogenic effects on the human T lymphocyte.

T lymphocyte; Ca^{2+}; Pertussis toxin; Mitogen

1. INTRODUCTION

The AB toxins, cholera and pertussis, have gained considerable popularity over the last decade as a result of their ability to ADP-ribosylate and so modify the function of certain guanine nucleotide-binding proteins (G-proteins) involved in the transduction of hormonal signals. Both toxins consist of a catalytic A subunit and a B, or binding, component. The A subunit is responsible for the ADP-ribosylation of G-protein substrates by virtue of its ability to catalyze the transferral of an ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to certain residues on the target proteins. The B component is instrumental in providing the contact between the toxin and the cell plasma membrane which is necessary for the transfer of the A subunit into the cell.

Correspondence address: C.F. Strnad, Department of Pharmacology and Toxicology, Medical College of Virginia, Box 613 MCV Station, Richmond, VA 23298-0001, USA

Pertussis toxin, produced by the bacterium *Bordetella pertussis*, first gained recognition for its ability to produce certain in vivo responses including sensitization to histamine [1], hypoglycemia [2], and a leukocytosis associated with elevated levels of circulating lymphocytes [3,4]. The toxin consists of six subunits [5], the largest (28 kDa) of which is a catalytic moiety (the A subunit), while the other five, ranging in molecular mass from 9.3 to 23 kDa, are associated to form a pentameric binding component (the B oligomer). The suppression of inhibitory input to the adenylate cyclase system [6], as well as excitatory input to the phosphoinositide-phosphodiesterase system in certain cell types [7–9], are among the best documented effects of pertussis toxin-mediated ADP-ribosylation. The efficacy of the A subunit in intact cells is dependent on the presence of the B oligomer, which appears to undergo bivalent binding to certain, currently unidentified receptors on the cell surface, so facilitating transfer of the A subunit across the plasma membrane. However, evidence that, in certain cell systems, the

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

B oligomer may play a more active role in the modulation of cellular functions has been provided by Tamura et al. [10] who found that the resolved B oligomer of pertussis toxin stimulated mitosis in murine splenocytes and enhanced glucose oxidation in rat adipocytes. Despite the important implications of these findings with respect to the lymphocytosis-promoting [3,4] and hypoglycemic [2] physiological effects of the toxin, the mechanism of action of B-oligomer-mediated responses has remained undefined.

Recent evidence from our laboratory suggests that pertussis toxin-induced proliferation in the human T lymphocyte is attributable to an action of the B oligomer and occurs by means of a calcium-dependent transduction process. This finding raises interesting questions regarding the nature of the pertussis toxin receptors and their possible significance in normal proliferative events.

2. MATERIALS AND METHODS

The following materials were purchased from the sources indicated: pertussis toxin and cholera toxin from List Biological Laboratories (Campbell, CA), fura-2 acetoxy methyl ester from Calbiochem-Behring (San Diego, CA), [^{3}H]thymidine from New England Nuclear (Boston, MA), OKT3 from Ortho Diagnostics (Rahway, NJ) and interleukin 2 from Cetus (Emeryville, CA). The resolved B oligomer of pertussis toxin was a gift from Dr Drusilla Burns of the Division of Bacterial Products, Food and Drug Administration [11].

T lymphocytes were isolated from human peripheral blood obtained from normal healthy volunteers. The diluted whole blood was subjected to centrifugation on a Ficoll-Hypaque density gradient. T lymphocytes were separated from contaminating monocytes and B cells by a plastic adherence procedure followed by the passage of cells through a nylon wool column.

For proliferation studies, cells were incubated at 5×10^5 cells per well in the presence of the indicated concentrations of the stimuli in question. After 48–72 h, the cells were pulsed for 4 h with 1 μCi/well of [^{3}H]thymidine. At the end of the pulse, cells were collected on glass fibre filter paper and counted for radioactivity in a Beckman LS 1801 liquid scintillation spectrometer.

For the purposes of intracellular calcium measurement, cells were loaded with the fluorescent calcium chelator, fura-2 [12], by means of a 60 min preincubation at 37°C with the membrane-permeant acetoxy methyl derivative of the dye at 1 mM. At the end of the incubation, cells were washed, resuspended in RPMI 1640 and stored on ice in 1 ml aliquots of approx. 20×10^6 cells. Immediately prior to use, each sample was sedimented in a microfuge and resuspended in 2 ml of a simplified saline solution, pre-warmed to 37°C. Calcium measurements were conducted in a Spex model CM-2 cation measurement system, with dual excitation at 340 and 380 nm. Emission was monitored at 505 nm. Fluorescence intensity ratios were converted to calcium concentrations using a revised version of the Spex CMPROG.

3. RESULTS AND DISCUSSION

Pertussis toxin was found to induce the proliferation of human T lymphocytes at concentra-

Table 1

T lymphocyte proliferation in response to AB toxins or their components

Condition (g/ml)	[^{3}H]Thymidine incorporation (cpm)
Control	432 ± 32
Pertussis toxin	
AB (5×10^{-7})	2817 ± 11
AB (5×10^{-8})	456 ± 33
AB (5×10^{-9})	380 ± 9
B (5×10^{-7})	6905 ± 53
B (5×10^{-8})	298 ± 9
B (5×10^{-9})	326 ± 17
Control	431 ± 19
Cholera toxin	
AB (5×10^{-7})	264 ± 19
AB (5×10^{-8})	268 ± 35
AB (5×10^{-9})	557 ± 56

T lymphocytes were treated with the indicated concentrations of the pertussis toxin holotoxin (AB), the cholera toxin holotoxin (AB), and the B oligomer of pertussis toxin (B) over a 72 h time interval. The data shown represent the means ± SD for triplicate determinations from a single experiment which is representative of at least three

tions of 0.5 μg/ml (table 1). Lower concentrations proved to be non-mitogenic. In view of reports of a pertussis toxin-mediated ADP-ribosylation of a 41 kDa protein in the T lymphocyte [13], it was desirable to determine whether or not the mitogenic properties of the holotoxin could be unequivocally dissociated from ADP-ribosylation events. This was achieved through use of the purified B oligomer. The observation that the B oligomer, alone, at concentrations of 0.5 μg/ml was efficacious as a mitogenic stimulus obviated a role for ADP-ribosylation in pertussis toxin-mediated proliferation (table 1). Notably, the proliferative properties of pertussis toxin and its B oligomer could not be mimicked by cholera toxin.

In an effort to characterize the mechanism of action of pertussis toxin and its B oligomer as mitogens, cytosolic calcium levels in T lymphocytes were monitored by means of the intracellular calcium indicator, fura-2. Exposure of cells to mitogenic concentrations of either pertussis toxin or its resolved B oligomer resulted in a gradual and sustained elevation of cytosolic calcium concentrations (fig.1). The calcium mobilization response appeared to be minimally dependent on intracellular calcium, being for the most part abolished upon chelation of extracellular calcium by 1 mM EGTA. EGTA was added to T cell suspensions for 1 min prior to administration of the stimuli so as to achieve depletion of extracellular calcium without appreciably affecting the intracellular stores. In accordance with the proliferation data, fairly high concentrations of pertussis toxin were required for an appreciable calcium signal with approx. 0.1 μg/ml of the toxin proving to be the threshold and 5 μg/ml providing the maximal response (fig.2). Notably, calcium mobilization was observed for the mitogenic per-

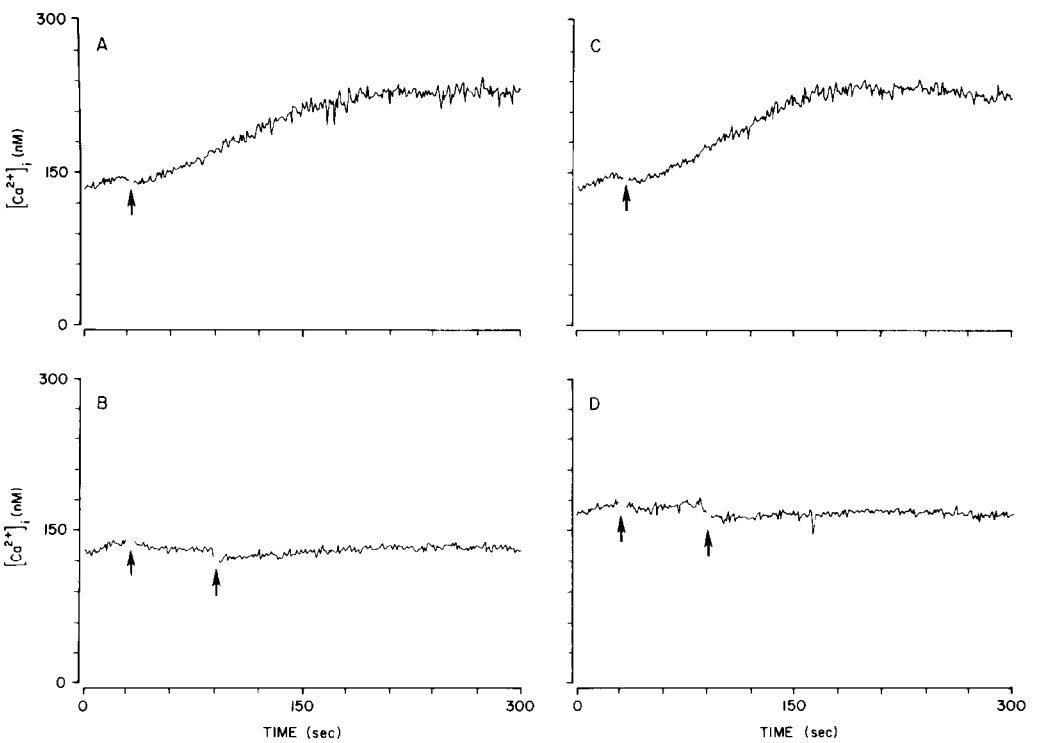


Fig.1. Fluorescence tracing obtained by stimulation of fura-2-loaded T lymphocytes with (A) 0.5 μg/ml pertussis toxin, (B) 0.5 μg/ml pertussis toxin following a 1 min preincubation with 1 mM EGTA, (C) 0.5 μg/ml B oligomer, and (D) 0.5 μg/ml B oligomer following a 1 min preincubation with 1 mM EGTA. The arrows indicate the time of addition of the agents in question. The tracings shown were obtained from a single experiment which was representative of at least three.

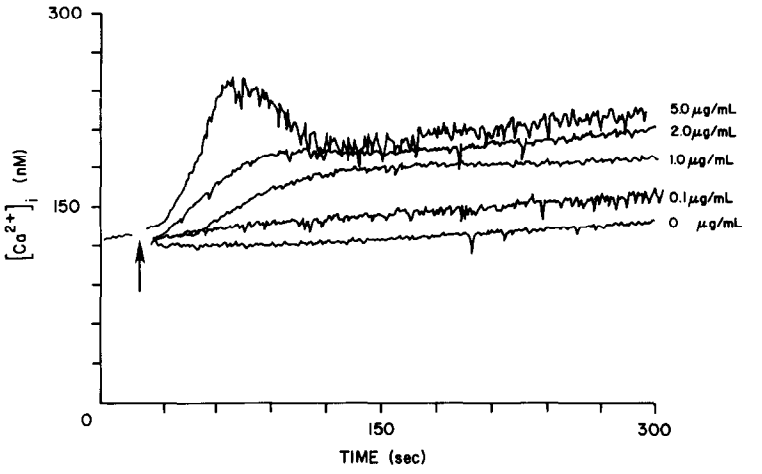


Fig.2. Fluorescence tracings obtained by exposure of fura-2-loaded T lymphocytes to a range of concentrations of the B oligomer of pertussis toxin. The arrow indicates the time of addition of the stimulus. The tracings shown were obtained from a single experiment which was representative of at least three.

tussis holotoxin and its B oligomer, but not for the non-mitogenic AB toxin, cholera toxin (fig.3).

Unlike the cholera toxin-binding site, which has been identified as the G_{M1} monosialoganglioside [14,15], the membrane components responsible for pertussis toxin binding remain to be defined. The steep dose-response requirements for the mitogenic effect may suggest the importance of the extensive aggregation of these receptor molecules for effective transduction of the mitogenic signal. The structural properties of the toxin are consistent with such a model. The pentameric B oligomer appears to be assembled from two dimeric entities joined through a connecting C subunit [10]. Interaction of each dimer with an independent recognition site would result in a bivalent binding phenomenon. Upon dissociation of the B oligomer into its constituent dimers in the presence of urea, the mitogenic properties of the toxin are lost, even though the dimers retain their ability to compete with the holotoxin for binding sites [10]. The divalent attachment of the intact B oligomer could conceivably result in the cross-linking of receptor sites in a manner analogous to that seen with plant lectins. Notably, lectin-induced mitogenesis is likewise associated with changes in cytosolic free calcium levels [16,17].

The near absolute dependency of pertussis toxin-induced calcium mobilization on the presence of extracellular calcium implicates the opening of calcium channels as an important event in the transduction process activated by the binding event. Whether this calcium influx is attributable to the generation of a second messenger substance derived from the phosphoinositide turnover cycle [18] or perhaps to more direct interactions between the toxin-binding sites and channel complexes is a matter currently under investigation. In an effort to elucidate further the molecular mechanisms involved in pertussis toxin-stimulated mitogenesis,

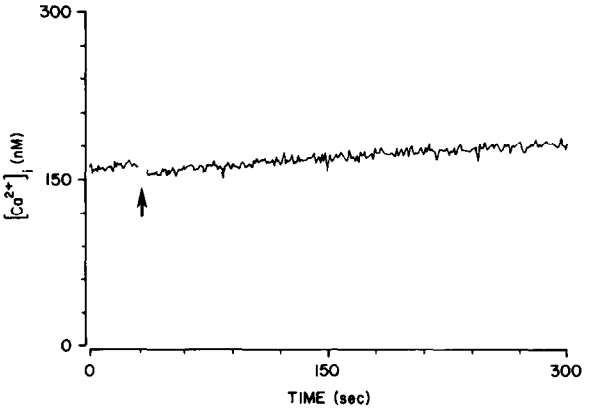


Fig.3. Fluorescence tracing obtained by exposure of fura-2-loaded T lymphocytes to 0.5 μg/ml cholera toxin. The arrow indicates the time of addition of the toxin. The tracing shown was obtained from a single experiment which was representative of at least three.

we are presently conducting studies of phosphoinositide turnover and protein kinase C activation in the pertussis toxin-stimulated T lymphocyte.

ACKNOWLEDGEMENTS

This work was supported by HHS grant ES03572. The authors would like to thank Dr Drusilla Burns of the Division of Bacterial Products, Food and Drug Administration for her generous gift of a sample of the B subunit of pertussis toxin.

REFERENCES

[1] Munoz, J.J. and Bergman, R.K. (1968) Bacteriol. Rev. 32, 103–126.
[2] Yajima, M., Hosoda, K., Kanbayashi, Y., Nakamura, T., Nogimori, K., Mizushima, Y., Nakase, Y. and Ui, M. (1978) J. Biochem. 83, 295–303.
[3] Morse, S.I. and Bray, K.K. (1969) J. Exp. Med. 129, 523–558.
[4] Morse, S.I. and Morse, J.H. (1976) J. Exp. Med. 143, 1483–1502.
[5] Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982) Biochemistry 21, 5516–5522.
[6] Murayama, T. and Ui, M. (1983) J. Biol. Chem. 258, 3319–3326.
[7] Moreno, G.J., Mills, I., Garcia-Sainz, J.A. and Fain, J.N. (1983) J. Biol. Chem. 258, 10938–10943.
[8] Nakamura, T. and Ui, M. (1983) Biochem. Pharmacol. 32, 3435–3441.
[9] Okajima, F., Katada, T. and Ui, M. (1985) J. Biol. Chem. 260, 6761–6768.
[10] Tamura, M., Nogimori, K., Yajima, M., Ase, K. and Ui, M. (1983) J. Biol. Chem. 258, 6756–6761.
[11] Burns, D.L. and Manclark, C.R. (1986) J. Biol. Chem. 261, 4324–4327.
[12] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[13] Evans, S.W., Beckner, S.K. and Farrar, W.L. (1987) Nature 325, 166–168.
[14] Cuatrecasas, P. (1973) Biochemistry 12, 3577–3581.
[15] Van Heyningen, W.E. (1974) Nature 249, 415–417.
[16] Greene, W.C., Parker, C.M. and Parker, C.W. (1976) Cell. Immunol. 25, 74–89.
[17] Tsien, R.Y., Pozzan, R. and Rink, T.J. (1982) Nature 295, 68–71.
[18] Kuno, M. and Gardner, P. (1987) Nature 326, 301–304.