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Maitotoxin, a potent, general activator of phosphoinositide breakdown

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Maitotoxin (MTX), a potent marine toxin, elicits a calcium-dependent activation of cells that can be inhibited by calcium channel blockers like nifedipine. MTX also stimulates phosphoinositide breakdown in smooth muscle cells, NCB-20 cells and PC12 cells through a nifedipine-insensitive mechanism. We now report that MTX stimulates phosphoinositide breakdown in a wide variety of cells, and appears to repesent the first general activator of this second messenger-generating system. MTX-induced stimulation of phosphoinositide breakdown is dependent in every cell line on the presence of extracellular calcium. In differentiated HL60 cells, in which a chemotactic peptide (fMLP) activates phosphoinositide breakdown via a pertussis toxin-sensitive mechanism, MTX-induced stimulation is not affected by pertussis toxin treatment. A phorbol ester has no effect on the response to MTX. Thus, MTX stimulates phosphoinositide breakdown through a calcium-dependent mechanism that at least in three cell lines (PC12, NCB20 and HL60) is not mediated by a pathway that involves a pertussis toxin-sensitive guanine nucleotide-binding protein.

Maitotoxin; Phosphoinositide breakdown; Ca^{2+} channel

1. INTRODUCTION

Maitotoxin (MTX), isolated from the dinoflagellate *Gambierdiscus toxicus* [1], elicits the release of neurotransmitters and hormones from secretory cells [2–5], stimulates the uptake of calcium [1,2,4–7] and induces the contraction of smooth muscle and cardiac tissue [6,8,9]. In every case, the effects of MTX are dependent on the presence of extracellular calcium. In most cells calcium channel blockers inhibit the effects of MTX [1,6] and it has been proposed that MTX is a direct activator of voltage-dependent calcium channels [1].

At lower concentrations than those required to elicit calcium channel activation, MTX elicits phosphoinositide breakdown in rat aortic myocytes [10], neuroblastoma hybrid NCB-20 cells [11] and pheochromocytoma PC12 cells [12]. Stimulation of phosphoinositide breakdown by MTX depends on the presence of extracellular calcium [11]. However, in contrast to the effects of MTX on voltage-dependent calcium channels, the stimulation of phosphoinositide breakdown by MTX is not affected by a variety of organic and inorganic blockers of calcium channels [10–12].

We now show that MTX stimulates the breakdown of phosphoinositides in a wide variety of cells and in brain synaptoneurosomes, as assessed by the accumulation of [^{3}H]inositol mono-, bis- and trisphosphates. The response is insensitive to pertussis toxin or phorbol esters.

2. MATERIALS AND METHODS

2.1. *Cells*

Cells were generously provided as follows: NCB-20, Dr D.M. Chuang (NIMH, Washington, DC); C6 rat glioma, Dr P. Fishman (NINCDS, Bethesda, MD); P2E rabbit kidney cell line, Dr L. Arendt (Michigan State University, East Lansing, MI); L fibroblasts, Dr P. Torrence (NIDDK, Bethesda); RBL2H3, Dr M. Beaven (NHLBI, Bethesda); PC12 pheo-

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Table 1

Maitotoxin (MTX)-induced stimulation of phosphoinositide breakdown in cultured cell lines and guinea pig synaptoneurosomes

Cell or tissue	$[Ca^{2+}]$ (mM)	cpm/10000 cpm in lipids		
		$[^3H]IP_1$	$[^3H]IP_2$	$[^3H]IP_3$
NCB-20 neuroblastoma hybrid	4.5			
Control		280 ± 95	50 ± 15	120 ± 50
MTX		2160 ± 200 (780%)	280 ± 60 (590%)	300 ± 50 (250%)
C6 rat glioma cells	4.5			
Control		290 ± 160	90 ± 10	130 ± 20
MTX		2820 ± 470 (980%)	350 ± 50 (410%)	300 ± 20 (230%)
P2E kidney cells	4.5			
Control		710 ± 110	110 ± 10	140 ± 10
MTX		1600 ± 140 (220%)	280 ± 40 (250%)	280 ± 20 (200%)
L fibroblasts	1.5			
Control		1420 ± 80	260 ± 60	270 ± 60
MTX		4350 ± 620 (310%)	1100 ± 200 (430%)	1290 ± 200 (480%)
RBL2H3 basophils	4.5			
Control		600 ± 100	140 ± 30	200 ± 20
MTX		1230 ± 340 (200%)	170 ± 5 (120%)	200 ± 5 (100%)
PC12 pheochromocytoma cells	1.5			
Control		330 ± 50	40 ± 5	60 ± 5
MTX		1660 ± 5 (510%)	190 ± 30 (450%)	160 ± 20 (260%)
HL60 human leukemic cells	2			
Control		960 ± 60	170 ± 30	230 ± 30
MTX		3350 ± 340 (350%)	1120 ± 100 (670%)	310 ± 20 (130%)
Mouse pituitary cells	1.5			
Control		260 ± 40	80,80	470,380
MTX		770 ± 150 (300%)	1080,310	710,340
Guinea pig synaptoneurosomes	1.5			
Control		720 ± 80		
MTX		1300 ± 250 (180%)		

Cells were labelled with [^{3}H]inositol (10 μCi/ml) for 12–16 h in multiwell plates (36 h for HL60 cells), while synaptoneurosomes were labelled for 60 min (see [15] for details). After washing, cells or synaptoneurosomes were exposed to MTX (0.5 ng/ml) for 30 min at 37°C. Incubations were stopped by aspirating the buffer and adding 1 ml of 6% trichloroacetic acid. [^{3}H]Inositol phosphates were anayzed by anion-exchange chromatography as described [15]. For HL60 cells the method of Brandt et al. [14] was followed. The Ca^{2+} concentration was that which allowed for maximal stimulation of phosphoinositide breakdown in each cell. Values in parentheses are percent of respective control

chromocytoma, Dr G. Guroff (NINCDS, Bethesda); HL60, Dr J. Gutkind (NIDR, Bethesda); mouse anterior pituitary primary cultures, Dr M.G. Castro (NICHD, Bethesda).

2.2. *Cell culture*

Culture media for cells were as follows: NCB-20 and L fibroblasts, Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and penicillin (100 U/ml) and streptomycin (100 μg/ml); C6 rat glioma cells, DMEM with 5% FCS; P2E cells, DMEM with 10% FCS and 1 μM dexamethasone; RBL2H3, Eagle's medium with Earle's salts and with 15% FCS; PC12 cells, DMEM with 6% fetal calf serum, 6% horse serum and penicillin (100 U/ml) and streptomycin (100 μg/ml); HL60 cells, RPMI 1640 with 10% FCS; mouse anterior pituitary cells, DMEM with 10% FCS.

2.3. *Phosphoinositide breakdown*

Experiments in NCB-20, C6 glioma, P2E cells, L fibroblasts, RBL2H3, PC12 cells and mouse anterior pituitary cultures were determined as described [11]. Briefly, on the day before the experiment, cells were transferred from 150-cm^2 culture flasks and subcultured in 12-well dishes with medium containing 10 μCi/ml [^{3}H]inositol (14–17 Ci/mmol). [^{3}H]Inositol-labelled cells were washed twice with buffer A (118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 20 mM Hepes; pH 7.4). Cells were then incubated in buffer A, containing 60 mM LiCl (osmolarity maintained by reducing NaCl to 58 mM), for 20 min. Agents were then added and incubations continued for 30 min at 37°C. Cells were scraped from the plates and transferred to 1.5-ml microfuge tubes. After centrifugation for 1 min the supernatant was discarded and 1 ml of 6% trichloroacetic acid was added. The tubes were vortex-mixed, centrifuged for 1.5 min and the supernatant was applied to anion-exchange columns (Bio Rad AG 1X8, 100–200 mesh, formate form). Separation and elution of [^{3}H]inositol phosphates by anion-exchange chromatography was performed as described by Berridge et al. [13]. For HL60 cells, the labeling, washing and phosphoinositide breakdown procedure was as described by Brandt et al. [14]. For guinea pig synaptoneurosomes our described procedure was followed [15].

2.4. *Materials*

Maitotoxin was purified to homogeneity from *G. toxicus* as in [1]. [^{3}H]Inositol (12–17 Ci/mmol) was obtained from Dupont, NEN (Boston, MA); pertussis toxin (IAP) from List Biological (Campbell, CA); formylmethionylleucylphenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO); ionomycin and A23187 from Calbiochem (La Jolla, CA); and culture media and sera from Gibco (Grand Island, NY).

3. RESULTS

In every cell line and in synaptoneurosomes, MTX-elicited stimulation of phosphoinositide breakdown is eliminated when the incubation medium lacks calcium ([11,12] and not shown). Results were similar in media containing 10 mM LiCl (not shown). MTX-elicited stimulation of phosphoinositide breakdown increases with increasing extracellular calcium concentrations ([12] and not shown). The requirement of calcium for MTX action varies from cell to cell ([12] and not shown). There seem to be two different groups in terms of calcium threshold for MTX activity. In one group (PC12, P2E, L fibroblasts, RBL, HL60 and mouse pituitary), MTX stimulation of phosphoinositide breakdown can be observed at calcium concentrations as low as 50–100 μM. In the other group (NCB20, C6, synaptoneurosomes) MTX action can only be detected at calcium concentrations of 1–1.5 mM and higher. As previously noted for NCB-20 and PC12 cells [12], the concentration of extracellular calcium at which maximal MTX-elicited stimulation of phosphoinositide breakdown occurs differs markedly in different cell lines: The percent stimulation of formation of [^{3}H]inositol phosphates listed in table 1 corresponds in each case to the maximal stimulation by MTX at the calcium concentration that is optimal for each cell line. In most cell lines calcium concentrations $\geq$9 mM result in a reduction of MTX-stimulated phosphoinositide breakdown (not shown). But in a rat glioma cell line (C6) and

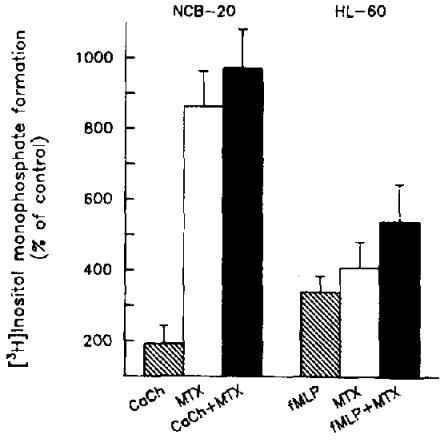

Fig.1. Effect of combination of maitotoxin and receptor agonists on phosphoinositide breakdown. NCB-20 cells differentiated with sodium butyrate for 3 days and HL60 cells differentiated with dibutyryl cyclic AMP for 36 h were labelled with [^{3}H]inositol as described [14]. Cells then were washed and incubated with carbamylcholine (CaCh, 1 mM), maitotoxin (MTX, 0.5 ng/ml), or fMLP (1 μM) alone or in combination for 30 min. [^{3}H]Inositol monophosphate was analyzed as in [14]. Values are from three or more experiments performed in triplicate. Values are expressed as percent of respective controls.

in guinea pig cerebral cortical synaptoneurosomes no reduction of MTX-mediated stimulation of phosphoinositide breakdown occurs even with 9.5 mM calcium. The calcium ionophores ionomycin (1 μM) and A23187 (1 μM) have no or minimal effects on [^{3}H]inositol phosphate formation in NCB-20 or PC12 cells (not shown), while MTX elicits about 8- and 5-fold stimulation in these two cell lines, respectively (table 1).

Combination of carbamylcholine with MTX in differentiated NCB-20 cells or of fMLP with MTX in differentiated HL60 cells results in formations of [^{3}H]inositol phosphates that are additive or somewhat less than additive, respectively, to the responses obtained with MTX and receptor agonists alone (fig.1).

Pretreatment with pertussis toxin reduces fMLP-mediated stimulation by about 50%, but has no effect on MTX-elicited stimulation of phosphoinositide breakdown in differentiated HL60 cells (table 2). Pertussis toxin-pretreatment also does not affect significantly MTX-elicited phosphoinositide breakdown in NCB-20 or PC12 cells (table 2). A protein kinase C activator, phorbol diacetate, which in some systems can inhibit receptor-mediated (see [16]) and NaF-mediated [17] stimulation of phosphoinositide breakdown, does not affect MTX-elicited stimulation in NCB-20 or PC12 cells (table 2).

Table 2

Effects of pertussis toxin and phorbol esters on phosphoinositide breakdown

(A) Effects of pretreatment with pertussis toxin on MTX and fMLP stimulation of phosphoinositide breakdown in HL60 cells[a]

	Saline	Pertussis toxin
	(cpm in [^{3}H]inositol monophosphate fraction)	
Control	930 ± 70	770 ± 110
fMLP (1 μM)	3130 ± 230 (340%)	1660 ± 430 (220%)
MTX (0.5 ng/ml)	3650 ± 310 (390%)	3470 ± 410 (450%)

(B) Lack of effect of pertussis toxin and phorbol diacetate on MTX-induced stimulation of phosphoinositide breakdown in NCB-20, PC12 and C6 cells[b]

	Pertussis toxin[c]	Phorbol diacetate[d]
	(% of response to MTX alone)	
NCB-20 neuroblastoma hybrid	100	120,90
PC12 pheochromocytoma	90	100
C6 glioma	90	ND

[a] HL60 cells were differentiated and labelled with [^{3}H]inositol as described [14]. Cells were pretreated with saline or pertussis toxin (100 ng/ml) for 2 h at 37°C. Cells were then washed and treated with fMLP or MTX as indicated for 30 min at 37°C and [^{3}H]inositol phosphates were analyzed as in [14]. Results are averages of three experiments performed in triplicate. Values in parentheses are percent of respective control

[b] Cells were labelled with [^{3}H]inositol for 14–16 h. After washing cells were incubated with MTX (0.5 ng/ml) for 30 min and [^{3}H]inositol phosphates were analyzed as described [14]. Results are expressed as percentage of response obtained in the absence of pertussis toxin pretreatment or incubation with phorbol diacetate. Values are from single experiments performed in triplicate

[c] Pertussis toxin (100 ng/ml) was present during the 14–16 h labeling with [^{3}H]inositol

[d] Phorbol diacetate (1 μM) was present during incubation with MTX

4. DISCUSSION

MTX appears to be a general activator of phosphoinositide breakdown having elicited a marked calcium-dependent accumulation of [^{3}H]inositol phosphates in all cell lines as yet studied (table 1). Both the threshold concentration of calcium at which MTX first elicits accumulation of [^{3}H]inositol phosphates (see section 3) and the concentration of calcium at which a maximal response to maitotoxin occurs (table 1) differ in different cell lines. In every cell studied, the absence of extracellular calcium eliminates MTX responses.

In the differentiated NCB-20 cells, muscarinic stimulation of phosphoinositide breakdown occurs in the absence of extracellular calcium, whereas MTX stimulated measurable formation of [^{3}H]-inositol phosphates only at calcium concentrations $\geq$1.5 mM (not shown). Thus, extracellular calcium is a requirement for MTX-mediated, but not receptor-mediated responses. In this cell line, however, the calcium ionophores ionomycin and A23187 (1 μM) cause only a slight increase in [^{3}H]inositol phosphate formation (not shown) at the calcium concentration at which MTX induces the response indicated in table 1. Thus, MTX

clearly must act in a manner different from calcium ionophores. Previous data on calcium influx in liposomes indicated no ionophore activity for MTX [18].

Calcium could be required for MTX binding to an effector site, or alternatively, MTX-elicited stimulation of phosphoinositide breakdown could be elicited through a calcium uptake mechanism that is activated by MTX. If so, such an uptake mechanism must be present in all cells as yet studied, and must be insensitive to blockade by calcium channel blockers as shown in studies with NCB-20, PC12 cells and aortic myocytes [10–12.

NCB-20 cells after treatment with sodium butyrate express a muscarinic receptor coupled to phospholipase C [19]. The HL60 human leukemic cells after differentiation with dibutyryl cyclic AMP express a chemotactic peptide (fMLP) receptor coupled to phospholipase C [14,20]. Since combinations of MTX with the receptor agonists in these cells result in responses that are larger than those induced by MTX or agonists alone (fig.1), different mechanisms for the action by MTX and the receptor agonists appear likely.

Inhibition of fMLP stimulation of phosphoinositide breakdown by pertussis toxin indicates that a pertussis toxin-sensitive guanine nucleotide-binding protein mediates between fMLP receptor and phospholipase C activation in HL60 cells ([14], see also [16]). The lack of effect of pertussis toxin on responses to MTX in HL60 and other cells (table 2) suggests that MTX-elicited stimulation of phosphoinositide breakdown is not mediated by a receptor coupled to a guanine nucleotide-binding protein, although it is conceivable that a different G-protein insensitive to pertussis toxin might be involved in MTX-elicited responses.

Inhibition of receptor-mediated phosphoinositide breakdown by phorbol esters has been proposed to be mediated through phosphorylation of guanine nucleotide-binding proteins by protein kinase C [17,21]. The lack of effect of phorbol esters on MTX-elicited stimulation of phosphoinositide breakdown (table 2), again in contrast to inhibition of receptor-mediated responses by phorbol esters, provides further evidence for a different transduction mechanism for responses to MTX.

The possibility that MTX could directly activate a guanine nucleotide-binding protein involved in signal transduction to phospholipase C seems unlikely. Thus, guanine nucleotide-binding proteins involved in phospholipase C activation seem to differ between cells; in some systems they are pertussis toxin-sensitive and in others they are not. Since MTX is a general activator for generation of inositol phosphates in all cells as yet tested, then one would have to postulate MTX to be a general activator of both pertussis toxin-sensitive and -insensitive guanine nucleotide-binding proteins. MTX does not affect directly other guanine nucleotide-binding protein-mediated phenomena, like stimulation or inhibition of adenylate cyclase (not shown). Thus, MTX is not a general activator of the guanine nucleotide-binding proteins.

It appears probable that activation of phospholipase C by MTX occurs either directly or by making calcium readily available for enzyme activation. If the latter is true, then MTX must interact with an as yet undescribed calcium uptake mechanism, which is present in most cells and is in every case closely related to phospholipase C function. A distinction between these two mechanisms, direct activation of phospholipase C or stimulation of a calcium uptake system closely associated with phospholipase C by MTX, cannot be made as yet. The very high potency of MTX (EC_{50} 150 pM), the stimulation by MTX of phosphoinositide breakdown in cells in which calcium ionophores have marginal effects (see section 3) and the lack of calcium ionophore activity for MTX in liposomes [18], all indicate that MTX does not stimulate phospholipase C simply by acting as a calcium ionophore.

MTX elicits increases in intracellular calcium that appear in PC12 and NCB-20 cells to parallel stimulation of inositol 1,4,5-trisphosphate formation [12]. MTX also has effects on cyclic AMP accumulation elicited by receptor agonists or forskolin that parallel those seen with phorbol esters in PC12 and NCB-20 cells [12]; i.e. both MTX and phorbol esters enhance forskolin-stimulated accumulation of cyclic AMP in PC12 cells and both inhibit prostaglandin E_2-stimulated accumulation of cyclic AMP in NCB-20 cells [12], probably in both cases through activation of protein kinase C by diacylglycerides generated during phosphoinositide breakdown. Thus, MTX, like receptors coupled to phospholipase C, generates functionally active second messengers; i.e.,

inositol 1,4,5-trisphosphate and diacylglycerides; the inositol trisphoshate then promotes release of calcium from intracellular stores, while diacylglycerides then stimulate protein kinase C.

In conclusion, MTX appears to be a general and useful activator of the phospholipase C involved in phosphoinositide breakdown. The use of MTX allows investigation of effects of phosphoinositide breakdown in cells that possess no known receptors coupled to phospholipase C. Such a general activator also allows for the investigation of possible receptor-mediated inhibitory inputs to phospholipase C.

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