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Differential effects of maitotoxin on ATP secretion and on phosphoinositide breakdown in rat pheochromocytoma cells

Fabian Gusovsky, John W. Daly, Takeshi Yasumoto[+] and Eduardo Rojas*

*Laboratories of Bioorganic Chemistry and *Cell Biology and Genetics, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA* and [+] *Faculty of Agriculture, Tohoku University, Sendai, Japan*

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Maitotoxin (MTX) induced exocytotic secretion of ATP from PC12 rat pheochromocytoma cells. The threshold for stimulation of secretion was at concentrations of about 2 ng/ml of MTX. Maximal release occurred at 40 ng/ml. MTX-induced ATP release required the presence of calcium in the extracellular medium and could be inhibited by nifedipine, a specific blocker of voltage-dependent calcium channels. In addition to the effects on ATP secretion from PC12 cells, MTX stimulated the breakdown of phosphoinositides, as measured by the accumulation of [^{3}H]inositol phosphates. Maximal stimulation of phosphoinositide breakdown was reached at only 0.5–1.0 ng/ml MTX. MTX at concentrations required to evoke ATP release (>2 ng/ml) had lesser or no effect on phosphoinositide breakdown. Although stimulation of phosphoinositide breakdown by MTX was dependent on extracellular calcium, it was insensitive to the calcium channel blockers nifedipine, D-600 and cobalt ions. The different concentration range required to elicit these responses and the varying sensitivity to calcium channel blockers indicate that MTX-evoked secretion and MTX-stimulated phosphoinositide breakdown are independent phenomena in PC12 cells.

ATP secretion; Phosphoinositide breakdown; Maitotoxin; (PC12 cell)

1. INTRODUCTION

Maitotoxin (MTX) is a potent toxin present in the dinoflagellate *Gambierdiscus toxicus* [1]. MTX increases resting tension in cardiac muscle [2], causes contraction of smooth muscle [3], induces insulin release [4] and stimulates quantal release of neurotransmitters [1,5,6]. These effects of MTX require the presence of calcium in the extracellular medium. Furthermore, MTX-induced release of neurotransmitters is inhibited by dihydropyridine calcium channel blockers [1,6]. Thus, it has been proposed [1] that MTX is a direct calcium channel activator.

Here, we demonstrate the inhibitory effects of the dihydropyridine nifedipine on MTX-evoked release of ATP from PC12 cells. However, MTX-induced calcium-dependent phosphoinositide breakdown in the same cells is not affected by nifedipine or other calcium channel blockers. Moreover, lower concentrations of MTX are required to stimulate phosphoinositide breakdown than those required to evoke ATP release from PC12 cells.

Correspondence address: F. Gusovsky, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

2. MATERIALS AND METHODS

2.1. *Materials*

MTX was isolated from *G. toxicus* as described [1]. Culture media and sera were from Gibco (Grand Island, NY). Nifedipine and tetrodotoxin were from Sigma (St. Louis, MO), [^{3}H]inositol (12–17 Ci/mmol) from New England Nuclear (Boston, MA), methoxyverapamil (D600) from Knoll (Ludwigshafen am Rhein, FRG) and luciferin-luciferase mixture from Analytical Luminescence Laboratory (San Diego, CA).

2.2. *Cell culture*

PC12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, were kindly provided by Dr G. Guroff (NIH, Bethesda, MD). PC12 cells were grown in Dulbecco's

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modified Eagle's medium with 6% fetal calf serum, 6% horse serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown at 37°C in an atmosphere enriched in CO_2.

2.3. *Phosphoinositide breakdown*

The day before the experiment the cells were transferred from 150-cm^2 culture flasks and subcultured in 6-well dishes (35 mm diameter wells) and incubated with 10 μCi/ml [^{3}H]inositol (14–17 Ci/mmol) for 14–16 h. The procedure was essentially as described by Chuang [7] for NCB-20 cells. [^{3}H]Inositol-labelled cells were washed twice with Hepes buffer containing 118 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose and 20 mM Hepes; pH 7.4. The cells were then incubated in Hepes buffer 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 (1 min) the supernatant was discarded and 1 ml 6% trichloroacetic acid was added. The tubes were vortex-mixed, centrifuged for 15 min and the supernatant was applied to anion-exchange columns (Bio Rad AG 1X8, 100–200 mesh formate form). Separation and elution of inositol phosphates were performed according to Berridge et al. [8]. Results are expressed as cpm in [^{3}H]inositol phosphate per 10000 cpm in lipids, or as percent of control.

2.4. *Release of ATP in PC12 cells*

ATP release from PC12 cells was measured as described elsewhere for bovine chromaffin cells [9]. Briefly, the luminescent oxidation of luciferin catalyzed by luciferase was used to detect ATP secreted by the cells. The contents of one vial of luciferin-luciferase mixture was dissolved in 5 ml Krebs Hepes solution (in mM: 135 NaCl, 10 $NaHCO_3$, 10 NaHepes, 5 KCl, 2.6 $CaCl_2$, 2 $MgCl_2$, 10 glucose; pH 7.4). The assay was carried out by mixing 140 μl Krebs Hepes solution containing freely suspended PC12 cells, plus 50 μl luciferin-luciferase solution. Agents were added in 10–20 μl in Krebs Hepes solution. Light was measured with a custom-made light detector [9].

3. RESULTS

MTX (2–20 ng/ml) elicited ATP release from freely suspended PC12 cells (fig.1). Concentrations of MTX below 2 ng/ml did not induce measurable secretion of ATP in PC12 cells. MTX-induced secretion of ATP from PC12 cells was apparent 2 s after the addition of MTX and reached a maximum level after 30–60 s. Release of ATP was observed with concentrations of MTX of 2 ng/ml, while the maximal response was reached at about 40 ng/ml. MTX-induced release of ATP was markedly inhibited in the absence of extracellular calcium (not shown) and in the presence of 10 μM nifedipine (fig.2).

MTX induced the formation of [^{3}H]inositol phosphates in PC12 cells in the presence of LiCl,

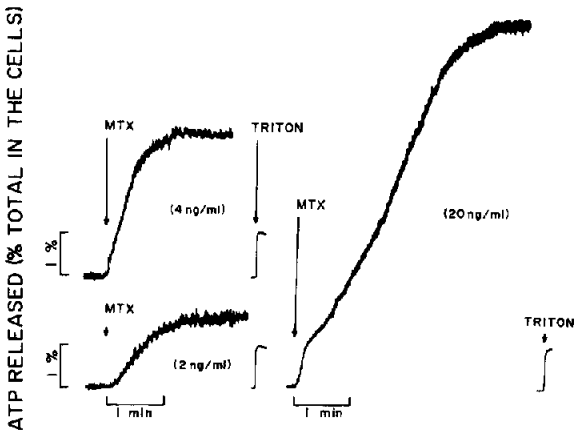

Fig.1. MTX-evoked ATP release from freely suspended PC12 cells. Chart records of the photomultiplier output representing the time integral of the ATP released in the reaction chamber. About 2.5 × 10^5 cells were added to the reaction mixture. Arrows indicate the addition of 20 μl Krebs solution containing increasing amounts of MTX (final concentration in parentheses). At the end of each experiment, the amplifier gain was attenuated by a factor of 100 and 10 μl of a 1% Triton solution was added as indicated by the arrows. The signal gave the total ATP remaining in the cells.

a measure of phosphoinositide breakdown (fig.3). A maximal response was observed at 0.5–1.0 ng/ml MTX. Higher concentrations of MTX had smaller or no effect on phosphoinositide breakdown (fig.3). The effects of MTX on phosphoinositide breakdown were abolished when calcium was omitted from the incubation buffer, but were unaffected in sodium-free medium (table 1). Blockers of calcium channels, such as

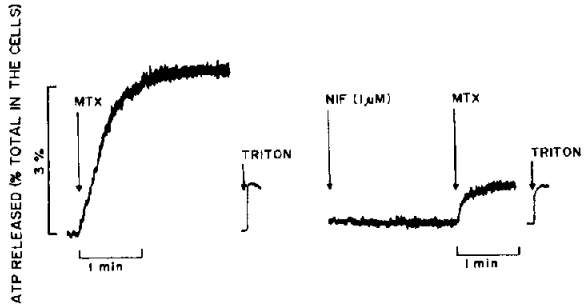

Fig.2. Inhibition of MTX-evoked ATP release by nifedipine. Recordings of release of ATP evoked by MTX (4 ng/ml) in the absence (left) and presence (right) of 1 μM nifedipine. See details for calibration in legend to fig.1.

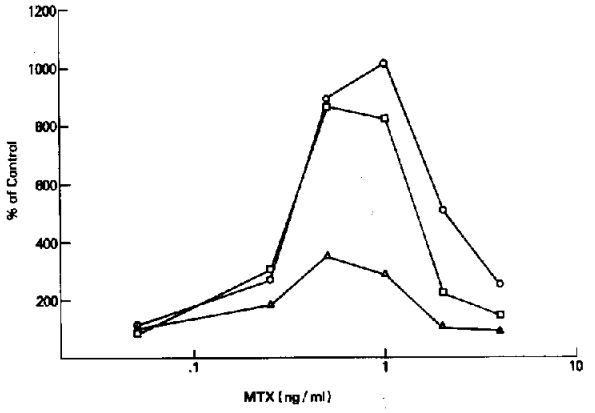

Fig.3. Dose-dependent stimulation of phosphoinositide breakdown by MTX. PC12 cells labelled with [^{3}H]inositol were preincubated with Hepes buffer with LiCl for 20 min. MTX was added and incubations were carried out for 30 min at 37°C. [^{3}H]Inositol phosphates were separated and analyzed as described in section 2. (○) [^{3}H]Inositol monophosphate, (□) [^{3}H]inositol bisphosphate, (△) [^{3}H]inositol trisphosphate. Results are from a representative experiment repeated three times.

nifedipine and D600, or cobalt ions, did not inhibit MTX-elicited phosphoinositide breakdown (table 1, and not shown).

Table 1

Effects of nifedipine, calcium-free and sodium-free media on maitotoxin-stimulated phosphoinositide breakdown in PC12 cells

	cpm in [^{3}H]inositol phosphate per 10000 cpm in lipids	% control
Normal media		
Control	550 ± 86	100
MTX (0.5 ng/ml)	2140 ± 480	390
+ nifedipine (10 μM)	1750 ± 350	320
Calcium-free medium		
Control	270 ± 9	100
MTX (0.5 ng/ml)	300 ± 17	110
Sodium-free medium		
Control	370	100
MTX (0.5 ng/ml)	1030	280

PC12 cells labeled with [^{3}H]inositol were preincubated with media containing LiCl for 20 min. Incubations were then carried out for 30 min. [^{3}H]Inositol phosphates were extracted and separated as indicated in section 2. Results are means of 3 independent experiments (± SE) or from a single experiment performed in triplicate

4. DISCUSSION

Application of MTX to PC12 cells at concentrations of 2–40 ng/ml causes the release of ATP co-stored with catecholamines in the secretory granules (fig.1). The kinetics of release are consistent with the release of ATP from the vesicular pool. These results are in agreement with previous work [1] in which MTX evoked the release of [^{3}H]norepinephrine in PC12 cells. The observed inhibitory effect of the calcium channel blocker nifedipine (fig.2) is also consistent with results [1] showing inhibition by nifedipine of MTX-evoked release of norepinephrine in PC12 cells. Furthermore, stimulatory effects of MTX on release of neurotransmitters and hormones are also inhibited in other cell types by calcium channel blockers [6,10]. Thus, it appears that at concentrations >2 ng/ml, used in the present study and in other reports [1,5,6], MTX interacts directly with the L-subclass of calcium channels that are sensitive to dihydropyridines, and thereby triggers calcium-dependent release processes.

MTX at lower concentrations (0.5–1 ng/ml) markedly stimulates phosphoinositide breakdown in PC12 cells (fig.3). Stimulatory effects of MTX on phosphoinositide breakdown have been observed in aortic myocytes [11], NCB-20 cells [12] and synaptoneurosomes [13]. Higher concentrations of MTX have less or no effect on phosphoinositide breakdown in PC12 cells (fig.3). Similar dose-response relationships pertained for MTX-elicited stimulation of phosphoinositide breakdown in NCB-20 cells [12]. Stimulation of phosphoinositide breakdown by MTX in PC12 cells is not sensitive to calcium channel blockers, including nifedipine, but it is eliminated in the absence of extracellular calcium (table 1). MTX had been previously shown to elicit calcium-dependent phosphoinositide breakdown, resistant to inhibition by calcium channel blockers in NCB-20 cells [12] and in aortic myocytes [11].

The concentration of MTX required to elicit release of ATP in PC12 cells is at least 4-fold greater than that required to stimulate phosphoinositide breakdown in the same cells (cf. figs 1,3), suggesting that two independent mechanisms are involved in these responses. Indeed, prior literature would also indicate that systems involved in phosphoinositide breakdown

[11,12] are much more sensitive to MTX than are systems involved in stimulation of calcium uptake, muscle contraction and neurotransmitter release [1,5,6]. However, the range of concentrations of MTX that have been reported to be required to elicit biological responses is quite broad. Thus, in PC12 cells MTX can evoke secretion at concentrations in the range 1–100 ng/ml [1]. In cultured neurons release is seen only at higher concentrations, ranging between 100 and 1000 ng/ml [5]. In NG108-15 cells MTX-induced calcium uptake is observed at concentrations in the range 30–100 ng/ml [6], while in pancreatic islet cells MTX stimulates calcium uptake and evokes insulin release at relatively low concentrations of 0.03–1 ng/ml [4]. The chemical structure of MTX is as yet unknown, and it is uncertain whether the purity and activity of preparations of MTX from different laboratories are identical. However, in the present study the same MTX preparation was used with the same cell type. Thus, the observation that MTX elicited the two biological responses at markedly different ranges of concentration provides strong evidence that MTX affects ATP release through a less sensitive pathway, involving activation of nifedipine-sensitive calcium channels, while affecting phosphoinositide breakdown through a more sensitive pathway, not involving calcium channels sensitive to nifedipine and other channel blockers. It is, however, possible that the primary event is stimulation of phosphoinositide breakdown and that at concentrations of >2 ng/ml MTX, second messengers generated initially through phosphoinositide breakdown result in activation of L-type calcium channels, thereby triggering release of neurotransmitters.

MTX stimulates phosphoinositide breakdown in a wide variety of cell types (unpublished). Although the molecular mechanism of stimulation of phosphoinositide breakdown by MTX remains to be determined, MTX may prove to be a general activator of phospholipase (s) C involved in phosphoinositide breakdown either directly or in some way by enhancing calcium input to the system.

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