Maitotoxin activates cation channels distinct from the receptor-activated non-selective cation channels of HL-60 cells

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We investigated whether maitotoxin activates non-selective cation channels, as was recently proposed [Soergel, Yasumoto, Daly and Gusovsky (1992) Mol. Pharmacol. 41, 487–493]. Stimulation of dibutyryl cyclic AMP-differentiated HL-60 cells with the chemotactic peptide N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP; 0.1 μM), the Ca²⁺-ATPase inhibitor thapsigargin (0.1 μM) or maitotoxin (25 ng/ml) resulted in an increase in cytoplasmic free calcium concentration ([Ca²⁺]i). Unlike fMLP and thapsigargin, maitotoxin produced no increase in [Ca²⁺], in the absence of extracellular Ca²⁺. The increase in [Ca²⁺], induced by fMLP was blocked by pretreatment with pertussis toxin (100 ng/ml for 24 h) but not that induced by maitotoxin. Similarly, the increase in [Ca²⁺], produced by fMLP but not that produced by maitotoxin was inhibited by pretreatment with phorbol myristate acetate (100 ng/ml). Both fMLP- and maitotoxin-induced increases in [Ca²⁺], were blocked by 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1H-imidazole hydrochloride (SKF 96365) in a concentration-dependent manner. However, the maitotoxin-induced increase in [Ca²⁺], was more sensitive to inhibition by SKF 96365 than the fMLP-induced increase. fMLP-induced increases in [Ca²⁺], were blocked by cations with Gd³⁺ being more effective than Cd²⁺, whereas for maitotoxin Cd²⁺ was more effective than Gd³⁺. Both fMLP and thapsigargin stimulated quenching of Fura-2 fluorescence in the presence of extracellular Mn²⁺, whereas maitotoxin produced no Mn²⁺ quenching. Taken together these results suggest that maitotoxin does not stimulate the non-selective cation channel activated by fMLP, but instead activates Ca²⁺ influx by a different mechanism.

INTRODUCTION

Maitotoxin is a high-molecular-mass polyether from the marine organism Gambierdiscus toxicus. Maitotoxin produces many responses in a wide variety of mammalian cells, including stimulation of hormone (Schettini et al., 1984) and neurotransmitter (Takahashi et al., 1983) secretion, contraction of cardiac (Kobayashi et al., 1985) and smooth (Ohizumi and Yasumoto, 1983) muscle, and stimulation of inositol phosphate (Berta et al., 1986) and arachidonic acid release (Choi et al., 1990) (for further references and reviews see Hamilton and Perez, 1987; Gusovsky and Daly, 1990). All these diverse actions are critically dependent on stimulation of Ca²⁺ entry caused by maitotoxin (see Hamilton and Perez, 1987; Gusovsky and Daly, 1990). The mechanism by which maitotoxin stimulates Ca²⁺ entry is poorly understood. Gusovsky and Daly (1990) have suggested that while maitotoxin stimulates voltage-dependent Ca²⁺ channels in excitable cells, this is secondary to a Ca²⁺- dependent depolarization and that maitotoxin first activates some other Ca²⁺ entry mechanism. Furthermore, maitotoxin also induces Ca²⁺ entry in non-excitable cells (Gusovsky et al., 1990; Columbo et al., 1992; Murata et al., 1992; Wanatabe et al., 1993). Maitotoxin is by itself unlikely to be an ionophore (Takahashi et al., 1983; Sladeczek et al., 1988; Murata et al., 1992), so a possibility is that maitotoxin directly stimulates a widely distributed Ca²⁺ permeant channel. Soergel et al. (1992) have reported that maitotoxin-induced Ca²⁺ entry and inositol phosphate turnover were blocked in rat insulina (RIN) and human insulinoma (HIT) cells by SKF 96365, a blocker of voltage-dependent Ca²⁺ channels and non-selective cation (NSC) channels (Merritt et al., 1990), but not by nifedipine, a blocker of voltage-dependent Ca²⁺ channels.

In order to determine if maitotoxin is indeed activating NSC channels, we compared the efficacies of SKF 96365, Cd²⁺ and Gd³⁺ in inhibiting Ca²⁺ entry induced by maitotoxin and the chemotactic peptide N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP) in differentiated HL-60 cells. HL-60 cells were used as a model, as they are devoid of voltage-dependent Ca²⁺ channels, and have a well-characterized NSC channel (Krautwurst et al., 1992, 1993), which is activated by fMLP and thapsigargin (Demaurex et al., 1992). We report here, on the basis of sensitivity to SKF 96365 and inorganic blockers and permeability to Mn²⁺, that maitotoxin does not activate the fMLP-activated NSC channel of HL-60 cells, but activates Ca²⁺ influx through a different mechanism.

MATERIALS AND METHODS

Materials

fMLP, 4β-phorbol 12-myristate 13-acetate, 4α-phorbol 12,13-didecanoate and thapsigargin were obtained from Sigma Chemie (Deisenhofen, Germany). Fura-2 acetoxyethyl ester (Fura-2/AM) was purchased from Calbiochem (Frankfurt/Main, Germany). Maitotoxin was obtained from Wako Pure Chemicals Industries Ltd. (Wako, Japan). SKF 96365 was a gift from Dr. J. E. Merritt, SmithKline Beecham (Welwyn, Herts., U.K.). All other reagents were of analytical grade or the best available commercial grade and obtained from standard commercial suppliers. All buffers were adjusted to the stated pH with 1 M NaOH. fMLP, SKF 96365, 4β-phorbol 12-myristate 13-acetate

Abbreviations used: [Ca²⁺], cytosolic free Ca²⁺ concentration; db-cyclic AMP, dibutyryl cyclic AMP; DMSO, dimethyl sulfoxide; fMLP, N-formyl-l-methionyl-l-leucyl-l-phenylalanine; Fura-2/AM. Fura-2 acetoxyethyl ester; NSC channels, non-selective cation channels; SKF 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1H-imidazole hydrochloride. *To whom correspondence should be addressed.
and thapsigargin were dissolved in dimethyl sulphoxide (DMSO) to give stock solutions of 10 mM. Fura-2/AM was dissolved in DMSO to give a 2 mM stock. Heavy metals were dissolved in deionized water to give 10 mM stock solutions. Maitotoxin was dissolved in deionized water to give a stock solution of 50 µg/ml. Stock solutions were stored as aliquots at -20 °C. Further dilutions were made in deionized water.

Cell culture

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) horse serum, 1% (v/v) non-essential amino acids, 2 mM l-glutamine, 50 units of penicillin/ml and 50 µg of streptomycin/ml in a humidified atmosphere with 7% CO₂ at 37 °C. To induce differentiation, HL-60 cells were seeded at a density of 1 x 10⁶/ml and were cultured for 48 h in the presence of 0.2 mM dibutyryl cyclic AMP (db-cyclic AMP) (Chaplinski and Niedel, 1982; Seifert and Schächtele, 1988). Cells were harvested by centrifugation for 10 min at 250 g. Cell preparations contained more than 90% viable cells as judged by Trypan Blue exclusion.

Measurement of cytosolic free Ca²⁺ concentration ([Ca²⁺]ᵢ)

[Ca²⁺], was determined with the dye Fura-2/AM as previously described (Schwaner et al., 1992; Seifert et al., 1992) with minor modifications. Briefly, HL-60 cells were suspended at 1 x 10⁷ cells/ml in loading buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 1 mM Na₂HPO₄, 5 mM NaHCO₃, 5.5 mM glucose and 20 mM Hepes, pH 7.4, supplemented with 1% (w/v) BSA. Fura-2/AM was added to give a final concentration of 4 µM, and cells were incubated for 30 min at 37 °C. After this incubation period, the cells were diluted to 0.5 x 10⁶ cells/ml and were centrifuged for 10 min at 250 g to remove extracellular dye. The cells were then resuspended at 2 x 10⁶ cells/ml in measurement buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 5.5 mM glucose and 20 mM Hepes, pH 7.4, supplemented with 100 µM CaCl₂, and kept at room temperature until measurement of [Ca²⁺]ᵢ. This phosphate- and bicarbonate-free buffer was required for experiments with heavy metals, and was used throughout for purposes of consistency (Merritt et al., 1989). A sample (0.5 ml) of cell suspension was added to acryl fluorescence cuvettes (Sarstedt, Nümbrecht, Germany), further diluted with 1.5 ml of the measurement buffer and CaCl₂ added to give a final concentration of 1 mM. Fluorescence was determined at 37 °C under constant stirring at 1000 rev./min, using a LS 50B dual-wavelength spectrofluorimeter (Perkin-Elmer, Beaconsfield, Bucks., U.K.). The excitation wavelengths were 340 nm and 380 nm and the emission wavelength was 510 nm. In some experiments, a Ratio II spectrofluorimeter (Amino, Silver Spring, MD, U.S.A.) was used. The excitation wavelength was 340 nm, and the emission wavelength was 510 nm. Cells were incubated for 3 min at 37 °C before addition of stimuli. Fluorescence signals were calibrated after lysis of the cells with 0.1% (w/v) Triton X-100 (maximal fluorescence) and subsequent addition of 20 mM EGTA (minimal fluorescence). [Ca²⁺]ᵢ was calculated according to eqn. 5 (dual wavelength) or eqn. 6 (single wavelength) in Grynkiewicz et al. (1985). Ca²⁺ entry was estimated by subtracting increases in [Ca²⁺]ᵢ observed in the absence of extracellular Ca²⁺ from responses in the presence of extracellular Ca²⁺. The value so obtained appears to be mainly due to entry of extracellular Ca²⁺ with negligible contributions from other sources (see Krautwurst et al., 1992 and the Discussion).

Measurement of Mn²⁺ quenching

Mn²⁺ quenching experiments were performed in nominally Ca²⁺-free measurement buffer on the Ratio II spectrofluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 510 nm (Merritt et al., 1989; Krautwurst et al., 1992).
experiments, Mn$^{2+}$ was added to nominally Ca$^{2+}$-free measurement buffer 1 min before the stimulus (forward method), in other experiments Mn$^{2+}$ was added to nominally Ca$^{2+}$-free measurement buffer 1 min after the stimulus (reverse method, see Merritt et al., 1989). The reverse methodology is more suitable for demonstrating slowly developing NSC-channel opening, such as that produced by thapsigargin. Traces were calibrated against quenching of Fura-2 fluorescence by known concentrations of Mn$^{2+}$ in cell suspensions permeabilized by Triton X-100.

RESULTS

In the presence of extracellular Ca$^{2+}$, fMLP (0.1 µM) produced a rapid increase in [Ca$^{2+}$], which remained elevated for several min (Figure 1). Both thapsigargin (0.1 µM) and maitotoxin (25 ng/ml) produced more slowly developing increases in [Ca$^{2+}$], in the presence of extracellular Ca$^{2+}$. In the absence of extracellular Ca$^{2+}$, fMLP (0.1 µM) produced a transient increase in [Ca$^{2+}$], thapsigargin (0.1 µM) produced a longer-lived increase in [Ca$^{2+}$], which returned to basal levels after 3–4 min (see Figure 1). In contrast, maitotoxin produced no increase in [Ca$^{2+}$], in the absence of extracellular Ca$^{2+}$.

The fMLP-induced increase in [Ca$^{2+}$], [404 ± 39 nM (n = 2); mean ± S.D. (n, independent experiments performed in duplicate)] was almost abolished by pretreatment with pertussis toxin [100 ng/ml; 24 h, 25 ± 2 nM (n = 2)] while the maitotoxin-induced rise was unaffected [704 ± 64 nM (n = 2) versus control 587 ± 66 nM (n = 2), measured 80 s after stimulus addition], suggesting that maitotoxin does not act by stimulating pertussis toxin-sensitive G-proteins. This is in agreement with the results of Gusovsky et al. (1990). Similarly, the fMLP-induced increase in [Ca$^{2+}$], [108 ± 6 nM (n = 2)] was substantially inhibited by pretreatment with 4β-phorbol 13-myristate 12-acetate [100 ng/ml; 28 ± 13 nM (n = 2)] whereas the maitotoxin-induced rise was unaffected [776 ± 342 nM (n = 3) versus control.

Figure 2 Effects of SKF 96385 on the fMLP- and maitotoxin-induced increases in [Ca$^{2+}$], in db-cyclic AMP-differentiated HL-60 cells

The figure shows original results. Cells were loaded with Fura-2 as described in the Materials and methods section and incubated in measurement buffer containing either 1 mM Ca$^{2+}$ or 1 mM EGTA (0 Ca$^{2+}$) with either solvent (Control) or SKF 96385 (SKF) at 37 °C for 3 min before the addition of the stimulus [fMLP, 0.1 µM (a); maitotoxin (MTX), 25 ng/ml (b)]. Fluorescence was monitored on the LS 50B spectrophotometer. The broken line indicates basal Ca$^{2+}$ values in the presence of 1 mM Ca$^{2+}$. Similar results were seen in at least two further experiments done in duplicate.

Figure 3 Effects of heavy metals on the fMLP- and maitotoxin-induced increases in [Ca$^{2+}$], in db-cyclic AMP-differentiated HL-60 cells

The figure shows original results. Cells were loaded with Fura-2 as described in the Materials and methods section and incubated in measurement buffer containing either 1 mM Ca$^{2+}$ or 1 mM EGTA (0 Ca$^{2+}$) with either deionized water (Control) or metal ion at 37 °C for 3 min before the addition of the stimulus [fMLP, 0.1 µM (a); maitotoxin (MTX), 25 ng/ml (b)]. Fluorescence was monitored on the LS 50B spectrophotometer. Gd$^{3+}$ and Cd$^{2+}$ were both at a concentration of 10 µM. Similar results were seen in at least three further experiments done in duplicate or triplicate.
DISCUSSION

Maitotoxin has been reported to stimulate Ca\(^{2+}\) entry in all cells reported so far except 3T3 fibroblasts (see review by Gusovsky and Daly, 1990), including both excitable and non-excitatory cells. In agreement with a previous report (Gusovsky et al., 1990), we report here that maitotoxin increases [Ca\(^{2+}\)]\(_i\) in db-cyclic AMP-differentiated HL-60 cells. The question is, how is maitotoxin producing this increase in [Ca\(^{2+}\)]? As maitotoxin neither acts to permeabilize liposomes (Takahashi et al., 1983; Murata et al., 1992) or mitochondria (Takahashi et al., 1983) nor forms pores in black-lipid membranes (Sladecek et al., 1988), maitotoxin is unlikely to be acting as an ionophore. Furthermore, in the present

Figure 4 Effects of SKF 96365 or heavy metals on the fMLP- and maitotoxin-induced increases in Ca\(^{2+}\) entry in db-cyclic AMP-differentiated HL-60 cells

The figure shows the mean ± S.D. from three separate experiments performed in duplicate. Cells were loaded with Fura-2 as described in the Materials and methods section and incubated in measurement buffer containing 1 mM Ca\(^{2+}\) with either solvent (control) or blocker at 37 °C for 3 min before the addition of the stimulus [fMLP (0.1 μM ( [] ; maitotoxin (MTX), 25 ng/ml ( [] )]. Fluorescence was monitored on the LS 50B spectrofluorimeter. (a) fMLP-induced Ca\(^{2+}\) entry values were calculated either 20 s (peak) or 80 s (shoulder) after addition of stimulus. By 80 s after the addition of fMLP, the release of intracellular Ca\(^{2+}\) had returned to basal. Maitotoxin-induced Ca\(^{2+}\) entry values were calculated 80 s after addition of maitotoxin, by which time plateau values had been reached. (b) Differences between Ca\(^{2+}\) entry in the presence of Gd\(^{3+}\) or Cd\(^{2+}\) (10 μM). Mean Ca\(^{2+}\) entry measured at 80 s was 214 ± 112 nM (n = 6) for fMLP (0.1 μM) and 980 ± 499 nM (n = 6) for maitotoxin (25 ng/ml).

720 ± 275 nM (n = 3), measured 80 s after stimulus addition].

4z-Phorbol 12,13-didecanoate was without effect on the fMLP-induced increase in [Ca\(^{2+}\)] (data not shown). As phorbol ester pretreatment can interfere with receptor coupling to phospholipase C (Smith et al., 1987), this result suggests that maitotoxin acts at a site beyond phospholipase C.

Both fMLP- (0.1 μM) and maitotoxin- (25 ng/ml) induced increases in [Ca\(^{2+}\)] were blocked by SKF 96365 (Figure 2). SKF 96365 was less effective at blocking the peak component of the fMLP-induced increase in [Ca\(^{2+}\)], than the 'shoulder', i.e. the persistent response at the time when intracellular release had returned to basal values (Figure 3). SKF 96365 was more effective at blocking maitotoxin-induced [Ca\(^{2+}\)] increases than either component of the fMLP-induced increase in [Ca\(^{2+}\)], (Figure 4).

Gd\(^{3+}\) (10 μM) strongly inhibited fMLP-induced increases in [Ca\(^{2+}\)], while Cd\(^{2+}\) (10 μM) was virtually ineffective (see Figures 3 and 4). As with the effects of SKF 96365, Gd\(^{3+}\) was more effective on the shoulder than on the peak response. In contrast, Gd\(^{3+}\) (10 μM) had no effect on the maitotoxin-induced increases in [Ca\(^{2+}\)], while Cd\(^{2+}\) (10 μM) produced a moderate inhibition.

Both fMLP (0.1 μM) and thapsigargin (0.1 μM) produced substantial quenching of Fura-2 fluorescence at 360 nm in the presence of extracellular Mn\(^{2+}\) (Figure 5), presumably due to entry of Mn\(^{2+}\) through NSC channels (Merritt et al., 1989). In contrast, maitotoxin produced no quenching of fluorescence, using either the reverse (see Figure 5) or the forward method (data not shown). Using the reverse methodology with Ca\(^{2+}\) (i.e. adding the stimulus to cells suspended in nominally Ca\(^{2+}\)-free buffer, then adding Ca\(^{2+}\) 2 min later, excitation wavelength 340 nm) [Ca\(^{2+}\)], increased rapidly after maitotoxin application to the concentrations achieved using the forward method (data not shown). The concentration-response curves to fMLP for increasing [Ca\(^{2+}\)], and Mn\(^{2+}\) quenching were almost superimposable, and the sensitivities of stimulus-induced Ca\(^{2+}\) entry and Mn\(^{2+}\) quenching to SKF 96365 were similar (data not shown), suggesting that in these cells stimulus-induced fluorescence quenching by Mn\(^{2+}\) was an index of stimulus-induced Ca\(^{2+}\) entry.
study maitotoxin-induced [Ca++] increases cannot be secondary to inositol trisphosphate-induced emptying of intracellular Ca++ stores, as in the absence of extracellular Ca++, no increases in [Ca++], are seen, even though inositol trisphosphate-releasable stores are present, as shown by the responses to fMLP and thapsigargin (see Figure 1). This is in agreement with previous reports (Gusovsky et al., 1990; see also Gusovsky and Daly, 1990).

Soergel et al. (1992) have suggested that maitotoxin directly activates an NSC channel(s). In the present study the maitotoxin-induced Ca++ entry was inhibited by SKF 96365 (see Figures 2 and 4), which blocks both voltage-dependent Ca++ channels and NSC channels (Merritt et al., 1990). As HL-60 cells have an SKF 96365-inhibitable NSC channel and are devoid of voltage-dependent Ca++ channels (Krautwurst et al., 1992), maitotoxin could plausibly be activating NSC channels in these cells. This finding is in agreement with the report of Soergel et al. (1992) that SKF 96365, but not nifedipine, inhibits Ca++ entry in HIT and RIN cells.

However, the maitotoxin-induced increase in [Ca++] was more sensitive to SKF 96365 than the fMLP-induced increase in [Ca++], raising the possibility that the mechanisms activated by maitotoxin and fMLP are not identical. Further evidence that maitotoxin and fMLP activate different mechanisms for increasing [Ca++] comes from inhibition by heavy-metal ions. In electrophysiological studies the fMLP-activated NSC channel is differentially inhibited by heavy-metal ions, with Gd3+ being more potent than Cd++ (Krautwurst et al., 1993). This was confirmed in the present study, where the fMLP-induced increases in Ca++ entry are blocked by 10 μM Gd3+ but not by 10 μM Cd++ (see Figures 3 and 4). In contrast, the maitotoxin-induced increases in Ca++ entry were unaffected by 10 μM Gd3+, whereas 10 μM Cd++ produced a modest but significant block. Thapsigargin-induced increases in Ca++ entry have a sensitivity to heavy-metal ions similar to that of fMLP (Demaurex et al., 1992).

In the present study Ca++ entry was estimated by subtracting increases in [Ca++], measured in the absence of extracellular Ca++ from that measured in its presence, and it may be argued that the differences in sensitivity to blockers are an artefact of this procedure. However, the sensitivity of fMLP-induced Ca++ entry to SKF 96365, Gd3+ and Cd++ at 80 s after addition of stimulus, by which time release of Ca++ from the intracellular store had returned to baseline, agrees reasonably well with electrophysiological studies (Krautwurst et al., 1993).

We studied an index of Ca++ entry uncomplicated by Ca++ release from intracellular stores, i.e. stimulus-induced quenching of Fura-2 fluorescence (Merritt et al., 1989; Demaurex et al., 1992; Krautwurst et al., 1992). However, although fMLP and thapsigargin stimulated substantial quenching of Fura-2 fluorescence in the presence of extracellular Mn++, maitotoxin produced no quenching at all under a variety of protocols (see Figure 5). This suggests that the mechanism by which maitotoxin increases [Ca++], is poorly permeable to Mn++, especially since the thapsigargin-induced Mn++ quenching could be clearly seen, although the latter Ca++ entry develops even more slowly than that of maitotoxin and is also smaller in extent (see Figures 1 and 5). Murata et al. (1992) reported that maitotoxin induces an influx of 45Mn++ into HIT cells. However, the influx of 45Mn++ was more than 20 times smaller than 45Ca++-influx and this report is thus consistent with our results.

Does maitotoxin produce increases in [Ca++], in our HL-60 cells by blocking Ca++ efflux, rather than increasing Ca++ entry? This is unlikely since, as described above, the increase in [Ca++], is blocked by SKF 96365, which does not affect Ca++ efflux in the concentrations we have used (Merritt et al., 1990). Similarly, the inhibition of the effect of maitotoxin by low concentrations of Cd++ would also argue against an effect on the efflux mechanism. Finally, maitotoxin appears to open voltage-independent channels in HL-60 cells (D. Krautwurst, unpublished work). Taken together these results suggest that maitotoxin is indeed increasing Ca++ entry in db-cyclic AMP-differentiated HL-60 cells.

To summarize, maitotoxin and fMLP activate two separate Ca++ entry pathways, both of which are sensitive to SKF 96365 but have different sensitivities to heavy-metal ions and different permeabilities to Mn++. This suggests that the maitotoxin-activated Ca++ entry pathway in HL-60 cells is not the receptor-dependent NSC channel that is stimulated by fMLP and thapsigargin. Whether maitotoxin stimulates Ca++ entry via activation of a novel voltage-independent Ca++-permeant channel or some other mechanism, remains to be determined.

We wish to thank Evelyn Gläss for excellent technical assistance. This work was supported by funds from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. I.F.M. is an Australian NH&MRC Research Fellow.

REFERENCES


Received 25 October 1993/18 February 1994; accepted 23 February 1994