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Palytoxin-induced increase in cytosolic-free Ca^{2+} in mouse spleen cells

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Abstract

The effect of palytoxin ($C_{129}H_{223}N_3O_{54}$) on Ca^{2+} homeostasis in immune cells has not been studied. Therefore, we investigated the effect of palytoxin on the cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse spleen cells using a fluorescence Ca^{2+} indicator, fura-2. Palytoxin (0.1–100 nM) increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The palytoxin-induced increase in $[Ca^{2+}]_i$ was abolished by the omission of extracellular Ca^{2+} or 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SKF-96365, 100 μM), and was greatly inhibited by Ni^{2+} (2 mM). Ouabain (0.5–1 mM) partially inhibited the palytoxin-induced response. There was no effect of decreased extracellular Na^+ (6.2 mM), tetrodotoxin (1 μM), verapamil (10 μM), nifedipine (10 μM), ω-agatoxin IVA (200 nM), ω-conotoxin GVIA (1 μM), ω-conotoxin MVIIC (500 nM), or La^{3+} (100 μM). These results suggest that palytoxin increases $[Ca^{2+}]_i$ in mouse spleen cells by stimulating Ca^{2+} entry through an SKF-96365-, Ni^{2+}-sensitive pathway.

Keywords: Palytoxin; Ca^{2+}, cytosolic, free; Spleen cell; Fura-2

1. Introduction

Palytoxin ($C_{129}H_{223}N_3O_{54}$), isolated from coelenterates of some zoanthid species (genus *Palythoa*), is one of the most potent marine toxins known (Moore and Scheuer, 1971; Hashimoto, 1979). It produces a massive Ca^{2+} influx in many cell types, although the exact mechanisms by which this effect is generated remain obscure. At least three Ca^{2+} uptake mechanisms are involved in the actions of palytoxin in excitable cells: (i) voltage-dependent Ca^{2+} channels, such as L-type Ca^{2+} channels, that are activated by depolarization of the membrane; (ii) Na^+/Ca^{2+} exchanger, which drives Ca^{2+} inside the cells in response to an intracellular Na^+ load and membrane depolarization; and (iii) an as yet unidentified permeation pathway that is independent of changes in membrane potential or changes in pH_i (Frelin and Van Renterghem, 1995).

There are a few reports on the effect of palytoxin on Ca^{2+} homeostasis in non-excitable cells. Palytoxin induces a rise in the cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in fibroblasts (Wattenberg et al., 1989) and osteoblast-like Saos-2 cells (Monroe and Tashjian, 1995), but does not cause an influx of Ca^{2+} in erythrocytes (Chhatwal et al., 1983) or an increase in $[Ca^{2+}]_i$ in megakaryocytes (Ichida et al., 1999). Palytoxin decreases the number of surviving lymphocytes in vitro (Falciola et al., 1994) and in vivo (Ito et al., 1997). Altered intracellular cation concentrations, in particular increased Ca^{2+}, are generally associated with cell death, as illustrated by accelerated apoptosis when ionomycin, a Ca^{2+}-ionophore, was added to cultures of virus-infected human lymphocytes (Gaugeon et al., 1991). To our knowledge, however, the effect of palytoxin on $[Ca^{2+}]_i$ has not been studied in immune cells. Therefore, we investigated the effect of palytoxin on $[Ca^{2+}]_i$ in mouse spleen cells.

2. Materials and methods

2.1. Chemicals and media

Palytoxin (molecular weight, 2680), isolated from *Palythoa tuberculosa*, was purchased from Wako (Osaka, Japan). The toxin was dissolved in distilled water at a concentration of 100 μM and kept frozen as a stock solution at −20 °C. Bovine serum albumin (0.1%) was added as a protective

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colloid. The following materials were used: fura-2 acetoxymethyl ester (fura-2-AM), HEPES, EGTA, and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetomethyl ester (BAPTA-AM) (Dojindo Laboratories, Kumamoto, Japan); verapamil hydrochloride and nifedipine (Sigma, St. Louis, MO); ω-agatoxin IVA, ω-conotoxin GVIA, and ω-conotoxin MVIIC (Peptide Institute, Osaka, Japan); choline chloride and thapsigargin (Wako); 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SKF-96365) (Calbiochem, La Jolla, CA) and ouabain octahydrate (Aldrich, Milwaukee, WI). Sankyo (Tokyo, Japan) generously donated the tetrodotoxin. Other chemicals were of reagent grade or the highest quality available.

The standard incubation medium contained the following: 125 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 6 mM glucose, 1 mM $CaCl_2$, and 25 mM HEPES. The final pH was adjusted to 7.4 with NaOH. Ca^{2+}-free medium was made by omitting Ca^{2+} from the standard medium. For low-Na^+ medium, NaCl was replaced with equimolar choline chloride or isotonic sucrose, keeping the HEPES buffer unchanged.

2.2. Spleen cells

The spleens were isolated from *ddY* strain male and female mice (30–35 g) under ether anesthesia. The spleen cells were treated with pH 7.65 buffer (17 mM Tris–HCl and 0.83% ammonium chloride) to lyse erythrocytes and washed three times in the standard medium. The cells were resuspended in standard medium, Ca^{2+}-free medium, or low-Na^+ medium for the measurement of $[Ca^{2+}]_i$. A heterogeneous spleen cell population consists mostly of lymphocytes, which are T-cells and B-cells (Arora et al., 1981).

2.3. $[Ca^{2+}]_i$

Intracellular $[Ca^{2+}]$ levels were measured by monitoring the intensity of fura-2 fluorescence as described by Spinozzi et al. (1995). In brief, a spleen cell suspension (10^7 cell/ml) was incubated in 5 μM fura-2-AM dissolved in dimethyl sulfoxide at 37 °C for 30 min. The cells were washed three times and then resuspended in the standard medium (5 ml). Aliquots (0.5 ml) of cell suspension were placed in a cuvette and pre-incubated for 10 min before the addition of agents. Measurement of fluorescence was started 4 min before the agents were added and continued for 10 min thereafter. Samples in the cuvette were maintained at 37 °C and mixed with a magnetic stirrer. Fluorescence of fura-2 was measured with a spectrofluorometer (model CAF-100; Jasco, Tokyo, Japan) using the ratio mode. Excitation wavelengths were 340 and 380 nm, and the emission wavelength was 500 nm. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (1985). After the $[Ca^{2+}]_i$ measurement, cell viability was determined to be greater than 80% using the Trypan blue exclusion test. This value was consistent with that of human lymphocytes reported by Falciola et al. (1994).

2.4. Experimental animals

All procedures pertaining to the care and use of experimental animals were approved by the Animal Research Committee, Obihiro University, and conducted in accordance with both the Guidelines for Animal Experiment in Obihiro University and the Guiding Principles for the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989.

2.5. Statistical analysis

The statistical significance of differences was assessed using the Student's *t*-test. A two-tailed *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of palytoxin on $[Ca^{2+}]_i$

Palytoxin increased $[Ca^{2+}]_i$ in mouse spleen cells in a concentration-dependent manner. The response became apparent at a concentration of 0.1 nM and reached a peak at 10 nM (Fig. 1). $[Ca^{2+}]_i$ reached a sustained level approximately 10 min after the addition of palytoxin (Fig. 1). The palytoxin-induced increase in $[Ca^{2+}]_i$ was abolished by preloading with an intracellular Ca^{2+} chelator, BAPTA-AM (500 μM), or by the post-addition of a Ca^{2+} chelator, EGTA (2 mM) (data not shown).

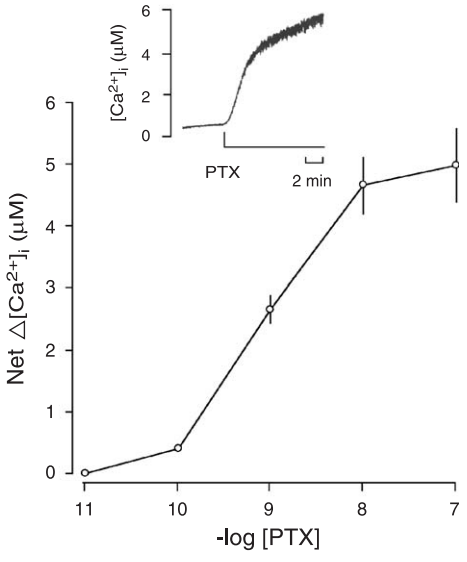

Fig. 1. Dose-dependent palytoxin-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded spleen cells were incubated for 10 min with or without palytoxin (PTX). The net $\triangle[Ca^{2+}]_i$ is the difference in the increase in $[Ca^{2+}]_i$ 10 min after the addition of palytoxin and vehicle. Each point represents the mean ± S.E.M. of values from 5 to 10 experiments. Insets: time course of changes in $[Ca^{2+}]_i$ in response to palytoxin (10 nM).

3.2. Effect of removal of external Ca^{2+} or pre-addition of Ca^{2+} channel antagonists on palytoxin-induced increase in $[Ca^{2+}]_i$

The palytoxin-induced increase in $[Ca^{2+}]_i$ was abolished by the removal of external Ca^{2+} or the addition of SKF-96365 (a Ca^{2+} channel blocker, 100 μM) (Fig. 2). Ni^{2+} (a Ca^{2+} channel blocker, 2 mM) also greatly inhibited the palytoxin-induced response (Fig. 2). In Ca^{2+}-free medium, the resting $[Ca^{2+}]_i$ was lower than that in the standard medium (data not shown). Verapamil (an L-type Ca^{2+} channel blocker, 10 μM), nifedipine (an L-type Ca^{2+} channel blocker, 10 μM), ω-agatoxin IVA (a P-type Ca^{2+} channel blocker, 200 nM), ω-conotoxin GVIA (an N-type Ca^{2+} channel blocker, 1 μM), ω-conotoxin MVIIC (a P/Q-type Ca^{2+} channel blocker, 500 nM), and La^{3+}(a Ca^{2+} channel blocker, 100 μM) failed to inhibit the palytoxin-induced increase in $[Ca^{2+}]_i$ (Fig. 2).

3.3. Effect of low-Na^+ media and tetrodotoxin on palytoxin-induced increase in $[Ca^{2+}]_i$

When NaCl was replaced with equimolar choline chloride or isotonic sucrose, the effect of palytoxin was not affected (Fig. 3). In the low-Na^+ solution (6.2 mM), the resting $[Ca^{2+}]_i$ was higher than that attained in the presence of NaCl (data not shown). Tetrodotoxin (a voltage-dependent Na^+ channel blocker, 1 μM) also had no effect (Fig. 3).

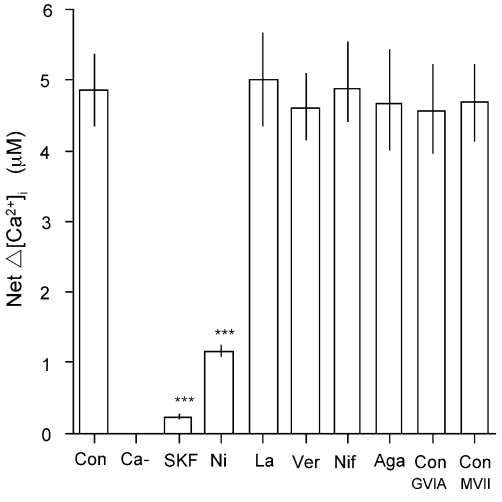

Fig. 2. Effects of the removal of external Ca^{2+} or various Ca^{2+} channel antagonists on the palytoxin-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded spleen cells were incubated for 10 min with or without palytoxin. The removal of external Ca^{2+} (Ca−) was achieved by omitting Ca^{2+} from the standard medium. SKF-96365 (SKF, 100 μM), Ni^{2+} (Ni, 2 mM), La^{3+} (La, 100 μM), verapamil (Ver, 10 μM), nifedipine (Nif, 10 μM), ω-agatoxin IVA (Aga, 200 nM), ω-conotoxin GVIA (Con GVIA, 1 μM), or ω-conotoxin MVIIC (Con MVII, 500 nM) was added 10 min before exposure to palytoxin (10 nM). The control (Con) is the palytoxin-induced response in the standard medium without Ca^{2+} channel antagonists. The net $\triangle[Ca^{2+}]_i$ is the difference in the increase in $[Ca^{2+}]_i$ 10 min after the addition of palytoxin and vehicle. Data are the mean ± S.E.M. of values from 5 to 10 experiments. Values significantly different from the control level are indicated: ***$P<0.001$.

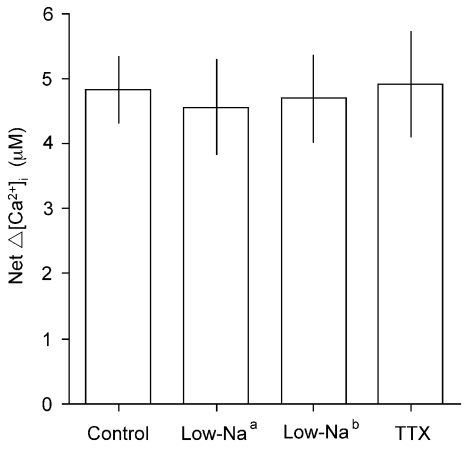

Fig. 3. Effect of low-Na^+ media or tetrodotoxin on the palytoxin-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded spleen cells were incubated for 10 min with or without palytoxin. For low-Na^+ medium (Low-Na), NaCl was replaced with equimolar choline chloride ([a]) or isotonic sucrose ([b]), keeping the HEPES buffer unchanged. Tetrodotoxin (TTX, 1 μM) was added 10 min before exposure to palytoxin (10 nM). The control is the palytoxin-induced response in the standard medium without TTX. The net $\triangle[Ca^{2+}]_i$ is the difference in the increase in $[Ca^{2+}]_i$ 10 min after the addition of palytoxin and vehicle. Data are the mean ± S.E.M. of values from 5 to 10 experiments.

3.4. Effect of ouabain on palytoxin-induced increase in $[Ca^{2+}]_i$

Ouabain (a specific inhibitor of Na^+,K^+-ATPase) inhibits the palytoxin-induced increase in $[Ca^{2+}]_i$ in human osteoblast-like Saos-2 cells (Monroe and Tashjian, 1995), rabbit

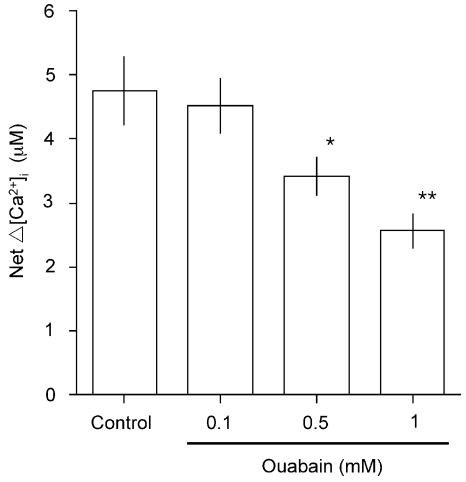

Fig. 4. Effect of ouabain on the palytoxin-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded spleen cells were incubated for 10 min with or without palytoxin. Ouabain (0.1–1 mM) was added 10 min before exposure to palytoxin (10 nM). The control is the palytoxin-induced response in the standard medium without ouabain. The net $\triangle[Ca^{2+}]_i$ is the difference in the increase in $[Ca^{2+}]_i$ 10 min after the addition of palytoxin and vehicle. Data are the mean ± S.E.M. of values from 5 to 10 experiments. Values significantly different from the control level are indicated: *$P<0.05$, **$P<0.01$.

aortic valve endothelium (Amano et al., 1997), and porcine coronary artery (Ishii et al., 1997). Therefore, we examined whether ouabain affects the palytoxin-induced increase in $[Ca^{2+}]_i$ in mouse spleen cells. The palytoxin-induced increase in $[Ca^{2+}]_i$ was partially inhibited by ouabain (0.5–1 mM) (Fig. 4). Ouabain did not affect resting $[Ca^{2+}]_i$. Zimlichman et al. (1987) reported that ouabain (1 mM) had no effect on $[Ca^{2+}]_i$ in lymphocytes, platelets, or adrenomedullary cells.

4. Discussion

In mouse spleen cells, palytoxin (0.1–10 nM) induced an increase in fura-2 fluorescence in a concentration-dependent manner. The response to palytoxin was abolished by pre-loading with BAPTA-AM or by the post-addition of EGTA. Fura-2 fluorescence reached a sustained level approximately 10 min after the application of palytoxin. Thus, an increase in fura-2 fluorescence induced by palytoxin represents an increase in $[Ca^{2+}]_i$ in the mouse spleen cells.

Removal of external Ca^{2+} completely inhibited the palytoxin-induced increase in $[Ca^{2+}]_i$. Pre-application of palytoxin had no effect on the increase in $[Ca^{2+}]_i$ induced by thapsigargin in the absence of external Ca^{2+} (data not shown). These results suggest that palytoxin increases $[Ca^{2+}]_i$ by increasing the influx of external Ca^{2+} into spleen cells and that palytoxin has no effect on the release of Ca^{2+} from the endoplasmic reticulum. In excitable cells, palytoxin increases $[Ca^{2+}]_i$ through the activation of voltage-dependent Ca^{2+} channels following membrane depolarization due to an increased influx of Na^+ (Dubois and Cohen, 1977; Ito et al., 1979; Kudo and Shibata, 1980; Pichon, 1982; Tatsumi et al., 1984; Satoh and Nakazato, 1991; Ishii et al., 1997). It has been suggested that voltage-dependent Ca^{2+} channels are not expressed at a significant level in non-excitable cells such as lymphocytes (Lewis and Cahalan, 1995). There are two reports, however, of voltage-gated Ca^{2+} currents in whole-cell recordings from T-cell lines (Dupuis et al., 1989; Densmore et al., 1992). In the present study, the response to palytoxin was not modified in the low-Na^+ medium or inhibited by various types of voltage-dependent Ca^{2+} channel blockers. Therefore, palytoxin might increase $[Ca^{2+}]_i$ through activation of other pathway(s).

Ouabain, a specific inhibitor of Na^+,K^+-ATPase, inhibited the palytoxin-induced increase in $[Ca^{2+}]_i$. This is consistent with reports that ouabain inhibits the palytoxin-induced increase in $[Ca^{2+}]_i$ in human osteoblast-like Saos-2 cells (Monroe and Tashjian, 1995), rabbit aortic valve endothelium (Amano et al., 1997), and porcine coronary artery (Ishii et al., 1997). Therefore, it is suggested that palytoxin acts on or near the ouabain-binding site of Na^+,K^+-ATPase.

Palytoxin does not form pores in planar lipid bilayers and its action is clearly distinct from that of natural ionophores such as amphotericin B (Ahnert-Hilger et al., 1982; Frelin and Van Renterghem, 1995). Numerous investigations indicate that a channel, simultaneously open on both sides of the plasma membrane, can also be formed within the mammalian Na^+,K^+-ATPase upon interaction of the enzyme with palytoxin (Chhatwal et al., 1983; Scheiner-Bobis et al., 1994; Kim et al., 1995; Redondo et al., 1996; Hirsh and Wu, 1997; Scheiner-Bobis and Schneider, 1997). Monroe and Tashjian (1995) proposed that the interaction of palytoxin with Na^+,K^+-ATPase in osteoblast-like Saos-2 cells created a channel that allowed the influx of extracellular Na^+ and Ca^{2+}.

The palytoxin-induced increase in $[Ca^{2+}]_i$ was abolished by SKF-96365, which blocks both voltage-gated Ca^{2+} entry and receptor-mediated Ca^{2+} entry (store-operated Ca^{2+} entry) (Merritt et al., 1990). Such inhibition does not involve the action of SKF-96365 on voltage-dependent Ca^{2+} channels, because the response to palytoxin was not modified in the low-Na^+ medium and the various types of voltage-dependent Ca^{2+} channel blockers have no effect in a paradigm where SKF-96365 almost completely blocks the palytoxin-induced response. Furthermore, because palytoxin failed to increase $[Ca^{2+}]_i$ in Ca^{2+}-free medium, receptor-mediated Ca^{2+} entry does not contribute to the palytoxin-induced response. Thus, palytoxin might interact with the SKF-96365-sensitive Ca^{2+}-entry system rather than with voltage-gated Ca^{2+} entry and receptor-mediated Ca^{2+} entry. Ni^{2+}, a Ca^{2+} channel blocker, also greatly inhibited the palytoxin-induced increase in $[Ca^{2+}]_i$. Ni^{2+}, at the concentration used in the present experiment (2 mM), did not quench the fura-2 fluorescence excited at 360 nm in the presence or absence of palytoxin (data not shown), suggesting that it did not enter the cell. These data are consistent with those of Shibuya and Douglas (1992). These results suggest that palytoxin increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} entry through an SKF-96365-, Ni^{2+}-sensitive pathway.

In excitable cells, palytoxin causes Ca^{2+} influx through the Na^+/Ca^{2+} exchanger in a reverse mode (Frelin and Van Renterghem, 1995). Balasubramanyam et al. (1994) reported that an Na^+/Ca^{2+} exchanger exists on the plasma membrane of lymphocytes. To determine whether the palytoxin-induced increase in $[Ca^{2+}]_i$ is attributable to increased activity of the Na^+/Ca^{2+} exchanger, we examined the effect of a low-Na^+ medium. The palytoxin-induced increase in $[Ca^{2+}]_i$ was not dependent on external Na^+. Thus, it is suggested that an Na^+/Ca^{2+} exchanger in a reverse mode is not associated with the palytoxin-induced increase in $[Ca^{2+}]_i$.

Palytoxin decreases the number of surviving lymphocytes in vitro (Falciola et al., 1994) and in vivo (Ito et al., 1997). In vitro, palytoxin (100 nM) killed approximately 80% of the cells within 2 h. In the present study, $[Ca^{2+}]_i$ reached a maximal and sustained level approximately 10 min after the application of palytoxin (100 nM). Because ouabain protects lymphocytes from palytoxin, Falciola et al. (1994) reported that palytoxin acts via pores formed in the Na^+,K^+-ATPase molecule. Altered intracellular cation concentrations, in particular an increased $[Ca^{2+}]_i$, are generally associated with cell death, as illustrated by acceler-

ated apoptosis when ionomycin, a Ca^{2+}-ionophore, was added to cultures of virus-infected human lymphocytes (Gaugeon et al., 1991). Therefore, it is suggested that palytoxin increases $[Ca^{2+}]_i$ and then leads to cell death.

The Ca^{2+}-permeable channels and Ca^{2+} entry mechanisms of lymphocytes are still far from being completely understood (Guse, 1998; Lewis, 2001). Therefore, we believe that palytoxin can serve as a tool to study and clarify some aspects of Ca^{2+} entry in these cells.

In conclusion, the present study demonstrates that palytoxin increases $[Ca^{2+}]_i$ in mouse spleen cells by stimulating Ca^{2+} entry through an SKF-96365-, Ni^{2+}-sensitive pathway.

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