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POLYENE ANTIBIOTICS INCREASE THE IONIC PERMEABILITY OF SYNAPTOSOMAL PLASMA MEMBRANES

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Abstract—The effects of antifungal heptaene antibiotics candicidin and amphotericin B were investigated in isolated cerebral cortical nerve terminals (synaptosomes). The synaptosomes were incubated with candicidin or amphotericin B in the presence or absence of external Ca^{2+}. Candicidin (0.4–0.8 I.U./mL) increased intrasynaptosomal free Ca^{2+} significantly. This increase was not significantly suppressed by 30 μM verapamil or 2 μM nifedipin. In the absence of extrasynaptosomal Ca^{2+} intrasynaptosomal free Ca^{2+} was not changed by candicidin. Amphotericin B increased intrasynaptosomal free Ca^{2+} as well. Candicidin (0.05–0.6 I.U./mL) increased the respiration rate up to 3.5-fold above the basal rate. This response was not affected by the absence of extracellular Ca^{2+}. Ouabain completely blocked the increase of respiration caused by candicidin, whereas tetrodotoxin was ineffective. The plasma membrane depolarized in a dose-dependent manner after candicidin (0.2–0.8 I.U./mL). The mitochondrial membrane potential was little affected and only at the highest concentrations. The results indicate that heptaene polyenes increase synaptosomal ionic permeability, which is reflected in increased Ca^{2+}-influx and accelerated respiration. The increment in synaptosomal free calcium takes place probably as a nonspecific leak via typical polyene–cholesterol channels. The respiration is accelerated by increased Na^+-permeability through the plasma membrane which stimulates the function of Na^+, K^+-ATPase and thus increases the energy demand.

Polyene antifungal antibiotics are used in the chemotherapy of fungal infections. The antifungal effect of polyenes is due to binding of the molecule with the sterol components (predominantly ergosterol) in the yeast cell membranes [1, 2]. Polyenes bind also to the membranes containing cholesterol, which is the main sterol component in mammalian cells. The binding to plasma membrane induces the collapse of ionic gradients of cells which has usually been demonstrated with yeast or red blood cells by monitoring the release of K^+-ions [1, 2].

Increased permeability of Ca^{2+} has been demonstrated in the nystatin treated *Saccharomyces cerevisiae* yeast cells [3] and amphotericin B (AmB) has been shown to induce Ca^{2+} influx across sterol containing unilamellar liposomes [4]. The pentaene macrolide, filipin, was used as a probe in the measurement of plasma membrane cholesterol of synaptosomes isolated from alcohol treated rat brains [5]. This may indicate that antibiotics of pentaene type polyenes interact with synaptosomal membranes. The effects of polyenes on the ionic permeability of isolated nerve terminals have not been characterized.

In the present study we have examined the mechanism of action of aromatic heptaene polyene candicidin on synaptosomal plasma membrane by measuring the concentration of cytosolic free calcium, $[Ca^{2+}]_c$,¶ oxygen uptake and membrane potentials. Candicidin was used because of our other studies with candicidin, and because it is a more potent fungicide than amphotericin B. For comparison the calcium permeability was also tested by amphotericin B, a non-aromatic heptaene, which is used as a therapeutic agent in systemic infections. The results show that candicidin and amphotericin B increase cytosolic free Ca^{2+} and energy demand which are secondary to enhanced ionic permeability of the synaptosomal plasma membrane *in vitro*.

MATERIALS AND METHODS

Preparation of synaptosomes and incubations. Synaptosomes from the cerebral cortices of Dunkin–Hartley strain guinea pigs (aged 3–6 weeks) from the National Laboratory Animal Centre, University of Kuopio, were prepared in a Ficoll density gradient as previously described [6]. The synaptosomal pellets were stored on ice before incubations and the experiments were completed within 4 hr of preparation. Protein content in synaptic preparations was measured with the biuret method. Synaptosomes were incubated in a medium containing 122 mM

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¶ Abbreviations: $\Delta\psi_p$ and $\Delta\psi_m$, plasma membrane and mitochondrial electrical potentials, respectively; Cl-CCP, carbonylcyanide-*m*-chlorophenylhydrazone; $[Ca^{2+}]_c$, cytosolic or intrasynaptosomal free calcium; DiS-C_2-(5), 3,3′-diethylthiadicarbocyanine.

NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Na–TES, 10 mM D-glucose and 16 μM bovine serum albumin, pH 7.4 at 37°. $CaCl_2$ was added into tubes containing buffer and tissue to give final concentration of 1.2 mM after 5 min preincubation.

Cytosolic free calcium, $[Ca^{2+}]_c$. The concentration of free calcium ions, $[Ca^{2+}]_c$, in synaptosomes was determined spectrofluorimetrically (Hitachi F-4000 Fluorescence Spectrophotometer, Hitachi, Tokyo, Japan) using intrasynaptosomally-trapped fura-2 as an indicator as previously described [8, 9]. Synaptosomes (2 mg of protein/mL) were suspended in buffer containing 5 μM fura-2/AM and incubated for 30 min, centrifuged at 10,000 rpm for 1 min. The pellet was resuspended in the incubation medium to a protein concentration of 1 mg/mL. The fluorescence of intrasynaptosomal fura-2 was recorded 1 min before and after addition of the antibiotics or control (0.1% dimethyl sulfoxide (DMSO), v/v). When indicated, synaptosomes were preincubated with verapamil (0.03 mM) for 5 min before addition of polyenes. Increase in the fluorescence signal due to extrasynaptosomal fura-2 was corrected by addition of 20 μM $MnCl_2$ immediately before recording the fluorescence [9].

Respiration. Synaptosomal respiration was measured using a Clark-type oxygen electrode (Hansa-Tech, King's Lynn, U.K.) as described previously [7]. $CaCl_2$ or Na-ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) was added after 5 min preincubation to give final concentration of 1.2 mM. Respiration rates are expressed as nmol of oxygen consumed per minute per mg synaptosomal protein. The stimulation of oxygen consumption is presented as the relation of respiration rate measured after drug addition to the respiration measured before drug addition. The value of 1.0 indicates no change in the respiration.

Optical monitoring of membrane potential changes. The changes of mitochondrial membrane potential $\Delta\psi_m$ in the synaptosomes were monitored using cell-permeant dye, safranine O [10]. Briefly, 10 μM of safranine O was added after 1 min preincubation and after another 15 min, fluorescence of the dye was monitored with excitation at 537 nm and emission at 580 nm in a Hitachi F-4000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). Collective membrane potentials of synaptosomes (without distinguishing between plasma membrane potential $\Delta\psi_p$ and mitochondrial membrane potential $\Delta\psi_m$) were monitored using 1 μM DiS-C_2-(5) (3,3'-diethylthia-dicarbocyanine) as an indicator [11].

Polyene antibiotics. Amphotericin B was obtained from the Sigma Chemical Co. (St Louis, MO) and candicidin (1366 international units of heptaene/mg) from Dumex Ltd, Denmark. Antibiotics were dissolved in dimethylsulfoxide, DMSO (Merck p.a.) in a concentration 0.3–1 mg/mL. The maximum concentration of DMSO was 0.2% (v/v) which was ineffective in all experiments. The minimum inhibitory concentration (MIC) values against *Candida albicans* wild strain was 0.08–0.16 μg/mL corresponding to 0.1–0.2 I.U./mL (N = 3) for candicidin and 0.31–0.62 μg/mL (N = 3) for amphotericin B as tested with broth dilution method in buffered peptone–glucose (Sabouraud–Glucose 2%-Bouillon, Merck).

RESULTS

Free intrasynaptosomal calcium $[Ca^{2+}]_c$

Candicidin (0.4–0.8 I.U./mL) increased $[Ca^{2+}]_c$ after 1 min exposure (Table 1, Table 2). Amphotericin B increased $[Ca^{2+}]_c$ only slightly (Table 1). Incubation times with candicidin (0.2–0.8 I.U./mL) longer than 1 min (9 min) increased the $[Ca^{2+}]_c$ (Table 2). In the absence of external calcium, $[Ca^{2+}]_c$ elevations by candicidin and amphotericin B were severely reduced (Table 1). Thus the rise in $[Ca^{2+}]_c$ is most probably due to increased entry of Ca^{2+} into synaptosomes. Indeed, verapamil (30 μM), a blocker of a class of voltage-dependent Ca^{2+} channels did not significantly decrease the candicidin (0.8 I.U./mL) induced increase in $[Ca^{2+}]_c$ (Table 1). Nifedipin (2 μM), another type of calcium channel blocker, also did not decrease the candicidin (0.8 I.U./mL) induced increase in $[Ca^{2+}]_c$ at 2 μM concentration.

Leakage of intrasynaptosomally trapped fura-2 can be used as an indicator of plasma membrane integrity [8]. In controls, (0.1% DMSO, v/v) and candicidin (0.4 I.U./mL) treated nerve terminals, extrasynaptosomal fura-2 fluorescence did not exceed 3% of total signal being at the same level both in candicidin treated (1.8 ± 1.3%; SEM, N = 3) and control synaptosomes (2.0 ± 0.5%; SEM, N = 3). Thus candicidin did not make the synaptosomal plasma membrane permeable to large molecules such as fura-2 (MW 1000).

Synaptosomal respiration

Candicidin stimulated the respiration of synaptosomes in the presence and absence of Ca^{2+} (Fig. 1). Stimulation of synaptosomal respiration by candicidin was linear ($r = 0.921$), reaching the value of about 3.5 (1.0 corresponds no stimulation) at concentration of 0.7 I.U./mL (Fig. 2). Amphotericin B also similarly stimulated synaptosomal respiration (data not shown).

Veratridine, which opens the voltage- and tetrodotoxin sensitive Na^+ channel thus allowing continuous influx of Na^+, caused a 3-fold [12] activation of synaptosomal oxygen uptake (data not shown). Ouabain which inhibits the function of Na^+, K^+-ATPase, totally inhibited the stimulation of respiration by candicidin (Figs 1 and 3). Thus it appears that candicidin increased the oxygen consumption by stimulating the energy dissipation at the Na^+ pump.

Puffer fish toxin tetrodotoxin (TTX, 2 μg/mL) which blocks the voltage-dependent Na^+ channel, caused a noticeable decrease in unstimulated respiration but did not prevent candicidin from stimulating synaptosomal respiration regardless of whether it was added before or after candicidin (Fig. 3). Verapamil (30 μM) did not affect the candicidin induced respiration (Fig. 1C). Nifedipin (2 μM) was also ineffective (data not shown).

Synaptosomal membrane potentials

Candicidin did not affect the fluorescence of safranine O below concentrations of 0.6 I.U./mL. DiS-C_2-(5) fluorescence showed a dose-dependent

Table 1. Free calcium concentration in synaptosomes treated with polyenes. Cortical synaptosomes (1 mg protein) loaded with fura-2 were incubated with or without polyene in the presence or absence of external Ca^{2+} (1.2 mM)

	External Ca^{2+} present		External Ca^{2+} absent	
	Basal level (nM)	Increment	Basal level (nM)	Increment
Control	258 ± 26	17 ± 8	135.0 ± 6.2	−3.5 ± 5.7
0.1% DMSO	(7)	(7)	(4)	(4)
Candicidin	278 ± 39	154 ± 34*	125.5 ± 6.5	18.8 ± 2.2*
0.4 I.U./mL	(7)	(7)	(4)	(4)
Candicidin	310 ± 34	203 ± 38*		
0.8 I.U./mL	(6)	(6)		
Verapamil 30 μM + candicidin	303 ± 18	167 ± 12*		
0.8 I.U./mL	(5)	(5)		
Nifedipin 2 μM + candicidin	311 ± 18	266 ± 16*		
0.8 I.U./mL	(4)	(4)		
AmB	211 ± 19	75 ± 5*	117.7 ± 5.4	3.3 ± 1.4
0.5 μg/mL	(4)	(4)	(3)	(3)

Data represents mean ± SE for number of determinations shown in parentheses.
AmB denotes amphotericin B.
* $P < 0.05$ compared with DMSO treated controls (Mann–Whitney U). No significant effect with verapamil (30 μM) or nifedipin (2 μM) pretreatment (Mann–Whitney U and *t*-test).

Table 2. Effect of incubation time on the candicidin induced increment of free intrasynaptosomal calcium

	Basal level (nM)	1 min increment (nM)	9 min increment (nM)
0.2 I.U./mL	312 ± 22	92 ± 20	127 ± 31†
0.4 I.U./mL	311 ± 25	197 ± 15*	247 ± 41*†
0.6 I.U./mL	284 ± 31	224 ± 11*	342 ± 37*†
0.8 I.U./mL	298 ± 35	274 ± 25*	364 ± 50*†

Cortical synaptosomes (1 mg protein/mL) loaded with fura-2 were incubated with 0.2–0.8 I.U./mL candicidin for 1 or 9 min before determination of fura-2 fluorescence (no manganese correction). The external Ca^{2+} was 1.2 mM. The data represents mean ± SE of three determinations.
* $P < 0.05$ when compared to the treatment with 0.2 I.U./mL (*t*-test).
† $P < 0.05$ when compared to 1 min increment (*t*-test).

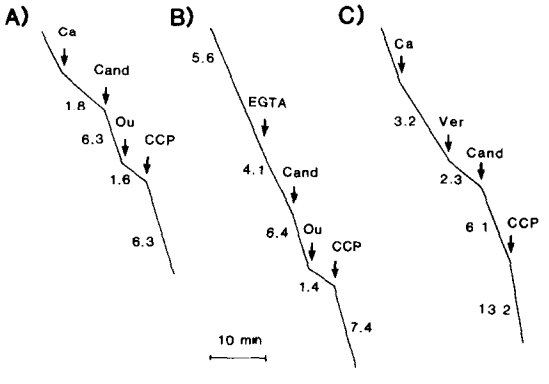

Fig. 1. Effect of candicidin on synaptosomal respiration. Synaptosomes (2 mg protein/mL) were incubated with (A) or without (B) calcium and with calcium in the presence of verapamil (C). The following additions were made as indicated: 1.2 mM $CaCl_2$ (Ca); 1.0 mM EGTA (EGTA); 30 μM verapamil (Ver); 0.8 I.U./mL candicidin (Cand); 0.1 mM ouabain (Ou) and 1.0 μM Cl-CCP (CCP). Values indicated are oxygen uptake in nmol/min (mg protein). Cl-CCP = carbonylcyanide-*m*-chlorophenylhydrazone.

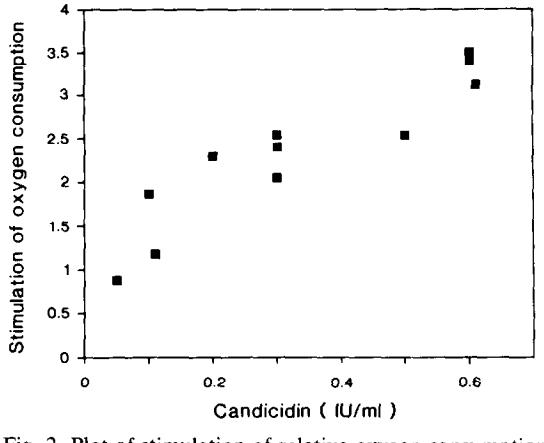

Fig. 2. Plot of stimulation of relative oxygen consumption (Y) by increasing candicidin concentrations (X). The O_2-consumption before candicidin addition was taken as basic level (1.0). The least squares equation for the correlation is $Y = 3.61\ X + 1.14$ ($r = 0.921$).

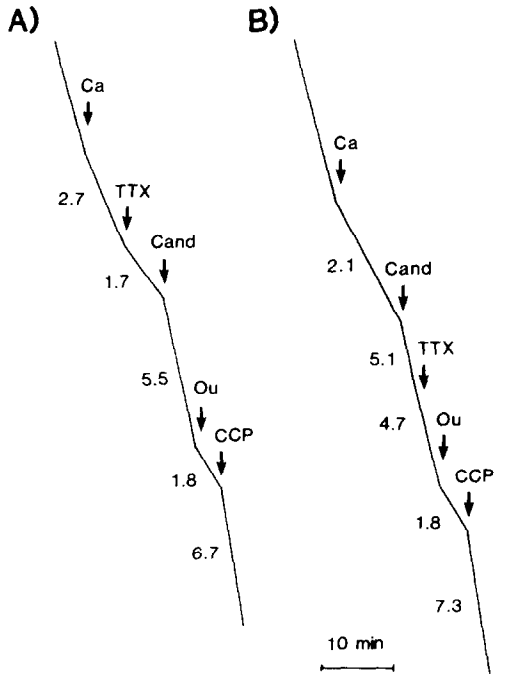

Fig. 3. Effect of TTX (2 μg/mL) on the candicidin stimulated oxygen consumption. TTX was added before (A) and after (B) candicidin was added. The other additions were as in Fig. 1. The plot is one of three different experiments.

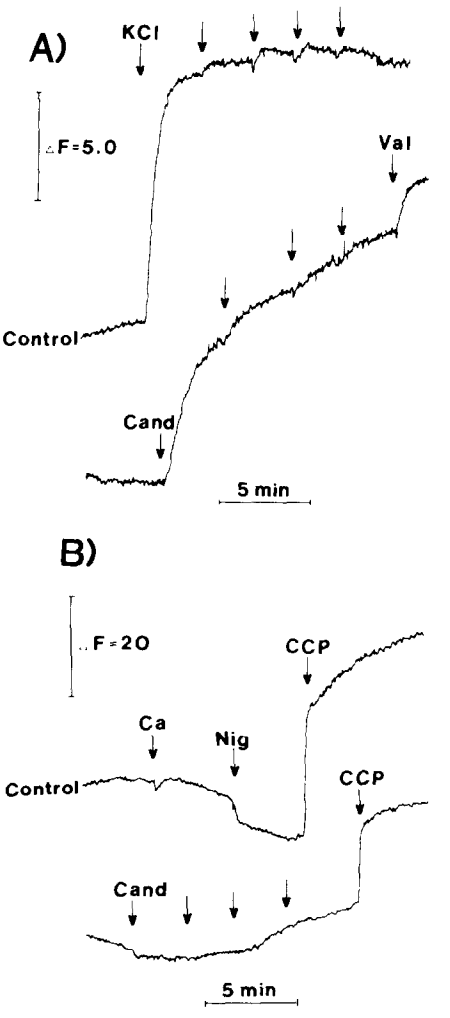

Fig. 4. Effects of candicidin on the DiS-C_2-(5) (A) and safranine O fluorescences (B). Consecutive additions to final concentrations of candicidin (Cand, 0.2, 0.4, 0.6, 0.8 I.U./mL) or KCl (10.6, 18.1, 33.1, 55.6, 78.1 mM) were made as indicated. Valinomycin (Val, 2.5 μM) was added to achieve the maximal depolarization of synaptosomal membranes. Nigericin (Nig) was added to hyperpolarize mitochondrial membranes and CCP to collapse $\Delta\psi_m$. The fluorescence signal is presented as arbitrary units.

increase (indicative to plasma membrane depolarization) upon addition of candicidin (Fig. 4A). Valinomycin (2.5 μM), a K^+ ionophore, added after candicidin induced the maximal DiS-C_2-(5) fluorescence (Fig. 4A).

For reference, effects of an ionophore nigericin and a protonophore CCP on safranine O fluorescence are shown in Fig. 4B. The effect of nigericin is due to H^+/K^+ exchange across the inner mitochondrial membrane leading to hyperpolarization of $\Delta\psi_m$ whereas CCP collapses $\Delta\psi_m$. Safranine O fluorescence was not affected by candicidin below concentration of 0.6 I.U./mL but a slow depolarization of mitochondria was evident at higher concentrations than these. These observations indicate that candicidin depolarized $\Delta\psi_p$ at concentrations where it also stimulated respiration.

DISCUSSION

Synaptosomes maintain high ionic gradients across the plasma and mitochondrial membranes by means of aerobic metabolism [12, 13]. Unstimulated respiration of nerve terminals reflects collective energy dissipation due to leakage of Na^+ at the plasma membrane and to protons (H^+) at the mitochondrial membrane [13]. Coupling between the mitochondrial respiration and Na^+ conductance is demonstrated when either veratridine alkaloid [12], allowing Na^+ entry through voltage dependent Na^+-channels, or removal of external divalent cations, which decrease the Na^+ electrochemical potential, activate oxygen uptake of synaptosomes [7].

The present results also show that depolarization of synaptosomal plasma membrane by heptaenes is followed by an increase in energy dissipation. The increase in synaptosomal oxygen consumption is probably due to enhanced plasma membrane Na^+-permeability and subsequent activation of Na^+,K^+-ATPase as indicated by sensitivity to ouabain. In yeast cells heptaene polyenes interact with membrane sterol components forming a channel-like pore in the plasma membrane making it leaky to mono- and divalent cations [1, 2]. Using intact red blood cells it has been demonstrated that the total flux of K^+ across the plasma membrane induced by tetraene polyene nystatin was partially inhibited by 0.1 mM ouabain indicating that polyenes activate Na^+,K^+-ATPase [14]. It is possible that heptaene polyenes

act on the synaptosomal plasma membrane in the same way, i.e. make the membrane leaky to cations like K^+ and Na^+. On the other hand, amphotericin B has been shown to inhibit Na^+,K^+-ATPase at concentrations above 5 μM [15]. This inhibitory effect of heptaenes on the synaptosomal Na pump could not be eliminated by the experimentation used in this study.

KCl-depolarization of synaptosomes has been shown to cause a poor or negligible increase in oxygen consumption [12]. Therefore the increase in Na^+ leakage across the synaptosomal plasma membrane by heptaene polyenes as reflected by activation of respiration might be due to the formation of a cholesterol–polyene ion conducting pore similar to that described for yeast cells or unilamellar sterol containing liposomes [4]. This pathway may be different from voltage-dependent Na^+ channel because TTX did not prevent the effect of candicidin.

Increase of $[Ca^{2+}]_c$ by candicidin occurred at the concentration where only DiS-C_2-(5) fluorescence indicated depolarization (Fig. 4) suggesting that the rise in $[Ca^{2+}]_c$ is associated with a drop of $\Delta\psi_p$. Verapamil (30 μM) and nifedipin (2 μM), blockers of voltage-dependent Ca^{2+} channels, did not significantly decrease the candicidin induced increase of $[Ca^{2+}]_c$. They did not affect the synaptosomal respiration either. By the experimentation used we conclude, however, that main influx of Ca^{2+} is due to the cholesterol–polyene channel. The total increase of $[Ca^{2+}]_c$ was of the same order of magnitude as previously reported for KCl-depolarization of synaptosomal preparation [8, 9].

Of the optical probes used to assess the synaptosomal membrane potentials safranine O detects the polarization of intrasynaptosomal mitochondria without significant contribution of $\Delta\psi_p$ [16]. A potential-dependent dye, DiS-C_2-(5), has been shown to monitor both $\Delta\psi_p$ and $\Delta\psi_m$ of isolated nerve terminals [11]. Thus the use of both safranine O and DiS-C_2-(5) allows a sufficient discrimination between $\Delta\psi_p$ and $\Delta\psi_m$. The experimentation of this study could not eliminate the possibility that both membranes are affected at concentrations 0.6 I.U./mL and more. The effect of candicidin on the $\Delta\psi_m$, however, is probably secondary. Because the stimulation of respiration by candicidin was due to increased Na^+ permeability, the K^+ equilibrium potential (using $^{86}Rb^+$ as an indicator) would be expected to overestimate the $\Delta\psi_p$ [13] and was not used in this study.

The results suggest that candicidin and amphotericin B *in vitro* bind to synaptosomal plasma membrane not only forming a channel which is leaky to monovalent cations such as K^+ and Na^+ but making the plasma membrane permeable also to Ca^{2+}. The binding increases the synaptosomal respiration (at least at the concentrations used in this study) probably by activated function of Na^+,K^+-ATPase. The polyene–cholesterol channels increase the ionic permeability of synaptosomal plasma membrane leading to the depolarization of the plasma membrane.

Hence, the mechanism of action of polyenes in mammalian cells include the depolarization of plasma membrane and the influx of Ca^{2+} but further studies are indicated to confirm details of the altered Ca^{2+} metabolism and its significance to the toxicity of polyene antibiotics.

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