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Imidazole antimycotics inhibitors of cytochrome P_{450} increase phosphatidylserine synthesis similarly to K^+-channel blockers in Jurkat T cells

Claude Aussel and Jean Philippe Breittmayer

INSERM U343, Interactions cellulaires en Immunologie, Faculté de Médecine, 06107 Nice Cédex, France

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The imidazole antimycotics, miconazole, econazole and triclomazole as well as α-naphtoflavone, known as powerful inhibitors of cytochrome P_{450} and previously recognized as K^+ channel blockers are shown to be potent activators of the base exchange enzyme system responsible for the biosynthesis of phosphatidylserine in Jurkat T cells. The inhibition of CD3-induced Ca^{2+} influx by antimycotics but not by K^+ channel blockers, demonstrated that the rise in phosphatidylserine synthesis caused by the two classes of drugs, was independent of Ca^{2+} influx in the cells. In addition, we show that the action of these drugs on phosphatidylserine synthesis was not mimicked by modifications of membrane potential. The regulation of both K^+ channels and the base exchange enzyme system thus occurs through a similar (or common) pathway that is independent of Ca^{2+}-influx and membrane potential.

K^+ channel; Ca^{2+} channel; Cytochrome P_{450}; Antimycotics; Phosphatidylserine; Membrane potential

1. INTRODUCTION

The imidazole antimycotics, miconazole, econazole and clotrimazole, have been demonstrated to be potent inhibitors of plasma membrane Ca^{2+} channels in rat thymocytes [1,2]. Since imidazole antimycotics were also inhibitors of cytochrome P_{450} and/or closely related proteins, it was suggested that a cytochrome P_{450} may regulate Ca^{2+} permeability of plasma membrane [1,2]. Recently, Alvarez et al. [3] have demonstrated that the drugs known as cytochrome P_{450} inhibitors were also able to block K^+ channels in red cell, Ehrlich ascites tumor cells as well as in rat thymocytes suggesting that Ca^{2+}-channels and K^+-channels might have similar regulatory mechanisms. Previous reports from our group have shown in one hand, that in Jurkat T cells [4] and in the monocytic cell line, THP_1 [5], the classical K^+-channel blockers, quinine, quinidine, 4-aminopyridine, tetraethylammonium and clofilium [6] increased specifically phosphatidylserine (PtdSer) synthesis. It was thus of interest to study: (1) whether cytochrome P_{450} inhibitors, as other K^+ channel blockers, modify PtdSer synthesis in Jurkat T cells; and (2) to see whether the regulation of PtdSer synthesis involves changes in Ca^{2+} permeability of the plasma membrane or modifications of the membrane potential.

Correspondence address: C. Aussel, INSERM U343, Interactions cellulaires en Immunologie, Faculté de Médecine, 06107 Nice Cédex, France. Fax: (33) (92) 03 5470.

2. MATERIALS AND METHODS

2.1. *Cells*

Jurkat D. The human T cell line Jurkat was kindly supplied by Dr. A.M. Schmitt-Verhulst (Centre d'Immunologie, Marseille-Luminy, France). Cells were cloned by limiting dilution. Clone D was selected on the base of its IL-2 production when activated with phytohaemagglutinin and the phorbolester, TPA. Cells were cultured in RPMI 1640 (Seromed, Lille, France) supplemented with 5% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate and 0.1 μM β-mercaptoethanol.

2.2. *Monoclonal antibodies*

The anti-CD3 mAb, secreted by the hybridoma X35 was kindly provided by Dr. D. Bourrel (CRTS, Lille, France). This mAb was purified on protein A-Sepharose and used at 2 μg/ml.

2.3. *Chemicals*

[^{3}H]Serine (10–30 Ci/mmol) was purchased from Amersham, UK. The potassium ionophore, valinomycin was from Calbiochem (Meudon, France). The sodium ionophore, gramicidin, and the K^+ channel blockers, quinine, quinidine, 4-aminopyridine and tetraethylammonium, were from Sigma Chemical Co. (La Verpillére, France). The cytochrome P_{450} inhibitors, econazole, miconazole, clotrimazole and α-naphtoflavone were from Sigma. Clofilium was purchased from RBI, Natick, MA, USA.

2.4. *Phosphatidylserine synthesis*

Jurkat cells (2×10^6) were maintained in 0.5 ml of a buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 2.5 mM glucose, 20 mM HEPES, 0.1% bovine serum albumin, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at 37°C in the presence of 4 μCi/ml [^{3}H]serine and effectors (see concentrations in the figure legends). After an incubation period varying from 0 to 2 h the cells were rapidly sedimented in an Eppendorf centrifuge, the supernatants were discarded and the cell phospholipids were extracted using chloroform–methanol according to Bligh and Dyer [7]. The lipid extracts were analyzed by thin-layer chromatography in a solvent system composed of chloroform/methanol/acetic acid/

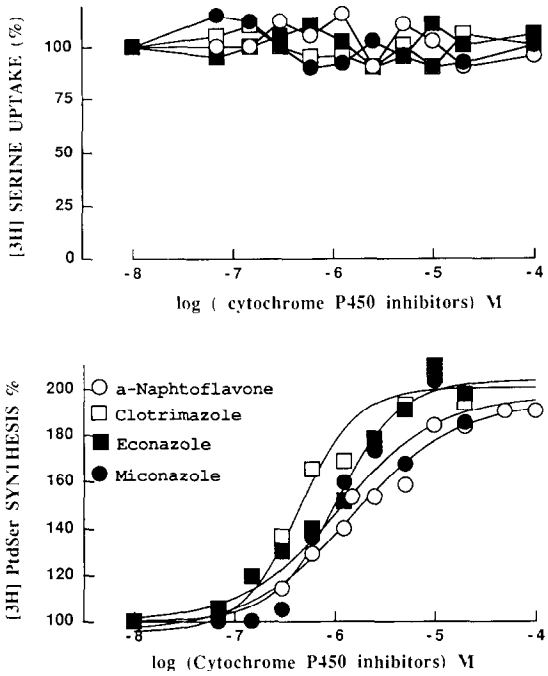

Fig. 1. Effect of inhibitors of cytochrome P_{450} on [^{3}H]serine uptake in Jurkat cells (higher panel) and incorporation of [^{3}H]serine into phosphatidylserine (lower panel). Cells were incubated for 3 h in the presence of various concentrations of the different drugs. Results are expressed as % change versus control cells representing 100%. Other details as in section 2.

water (75:45:12:3). Authentic phospholipids standards (Sigma Chemical Co., St. Louis, MO) were run in parallel and detected with iodide vapors. Radioactivity in lipid spots was determined by using an automatic linear radiochromatography analyzer (Berthold).

2.5. *Measurements of changes in Ca^{2+}*

The assay of cytosolic Ca^{2+} was performed using Indo-1 (Calbiochem) [8]. Cells (5×10^6/ml) were loaded using 5 μM Indo-1 at 37°C in the dark for 1 h, then washed and resuspended in medium containing the different effectors. The analyses were performed on a fluorescence-activated cell sorter (FACStar plus, Becton Dickinson). The fluorescence intensity at 480 nm corresponding to the free Indo-1 concentration as well as the fluorescence at 400 nm corresponding to the complex Ca^{2+}–Indo-1 were measured. The ratio fluorescence at 400 nm/480 nm allows the evaluation of changes in cytosolic free Ca^{2+} concentration, independently of the cell size and the intracellular Indo-1 concentration.

Table I

The EC_{50} obtained when measuring [^{3}H]serine incorporation into phosphatidylserine in the presence of different cytochrome P_{450} inhibitors (see Fig. 1) or different classical K^+ channel blockers are compared to the IC_{50} previously published for their action on K^+ channels

Effectors	EC_{50} PtdSer	IC_{50} K^+ channels	Refs.
Cytochrome P_{450} inhibitors			
Clotrimazole	0.4 μM	0.5 μM	3
Econazole	1.15 μM	1.8 μM	3
Miconazole	1.07 μM	1.5 μM	3
α-Naphtoflavone	1.5 μM	26 μM	3
Potassium channel blockers			
Quinine	26 μM	22 μM	4,14,15
Quinidine	18 μM	–	14,15
Clofilium	50 μM	60–80 μM	6.14
4-Aminopyridine	2 mM	3 mM	4,14.15,16
Tetraethylammonium	40 mM	30 mM	4,14.15,16

3. RESULTS

3.1. *Effect of cytochrome P_{450} inhibitors on PtdSer synthesis*

Clotrimazole, econazole and miconazole, known as antimycotics, but recently recognized both as inhibitors of cytochrome P_{450} and inhibitors of K^+ channels in thymocytes were tested in Jurkat T cells to see whether they modified PtdSer synthesis. As shown in Fig. 1, the three drugs were able to markedly increase PtdSer in a concentration dependent mode. Measuring [^{3}H]serine uptake by the cells showed (Fig. 1) that the drugs do not significantly change the transport of the amino acid, indicating that the increase in PtdSer synthesis was not a consequence of an increased uptake of [^{3}H]serine but was most likely due to an increase in the activity of the serine base exchange enzyme system. The EC_{50} calculated from the concentration–response curves are depicted in Table I and compared to other PtdSer inducers such as the classical K^+ channel blockers, quinine, quinidine, 4-aminopyridine, tetraethylammonium and clofilium. Kinetics of PtdSer synthesis in the absence or presence of 25 μM clotrimazole (Fig. 2) showed that the drugs increased PtdSer synthesis very rapidly. Similar results (not shown) were obtained when using econazole or miconazole.

α-Naphtoflavone, known as a specific effector of cytochrome P_{450}, was also studied. The results obtained with this drug were also included in Fig. 3 and Table I. α-Naphtoflavone appeared as a strong inducer of PtdSer synthesis as expected if this drug blocks K^+ channels as described by Alvarez et al. [3].

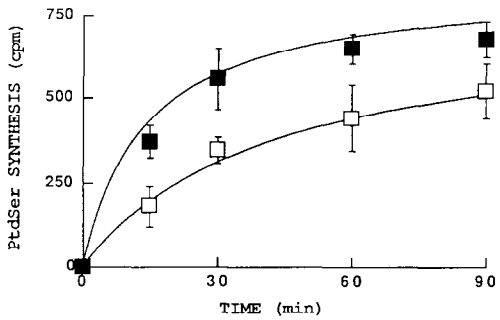

Fig. 2. Kinetics of clotrimazole induced incorporation of [^{3}H]serine into phosphatidylserine. Jurkat cells were incubated as a function of time in the presence of 25 μM clotrimazole. Phospholipids were extracted and separated on thin-layer chromatography plates. Results are expressed as cpm ± S.D. (n = 6). □, represents control cells; and ■, clotrimazole treated cells.

3.2. *Effect of membrane polarization on serine uptake and PtdSer synthesis*

Hyperpolarization of Jurkat cells by using the K^+-ionophore, valinomycin at 5×10^{-6} M markedly increased [^{3}H]serine uptake by the cells (Table II) and concomitantly increased the synthesis of PtdSer as measured by the incorporation of [^{3}H]serine into PtdSer.

When Jurkat cells were depolarized by using the Na^+ ionophore gramicidin at 2.5×10^{-6} M, a large decrease in [^{3}H]serine uptake was observed. This decrease was accompanied by a marked inhibition of PtdSer synthesis. In order to confirm these results, we depolarized the cells by replacing NaCl with KCl in the incubation medium. As shown in Table II, the depolarization process was accompanied both by a decreased incorporation of [^{3}H]serine by the cells and a decreased synthesis of PtdSer. The specificity of the results obtained, with either valinomycin or gramicidin, was tested by using different compounds known as modifiers of the ionic status of the cells such as nigericin, ouabain, amiloride and tetrodotoxin. Each drug used at the concentration of 5×10^{-6} M caused no significant change on both [^{3}H]serine uptake by Jurkat cells and PtdSer synthesis.

3.3. *Changes in intracellular Ca^{2+}*

Since PtdSer is exclusively synthesized by a base exchange enzyme system that requires Ca^{2+} for its activity [9,10], and since in activated Jurkat cells [11] as in glioma C_6 cells [12] the synthesis of PtdSer is strongly inhibited as the result of emptying intracellular Ca^{2+} stores, we have studied whether cytochrome P_{450} inhibitors and the more classical K^+ channel blockers could modify Ca^{2+} movements induced after activation of Jurkat T cells with CD3 mAbs. As shown in Fig. 4, quinine, a classical K^+ channel blocker does not modify the mobilization of Ca^{2+} from intracellular stores and was without effect on Ca^{2+} influx induced by CD3 mAb. Identical results (not shown) were obtained when using other K^+ channel blockers such as quinidine, clofilium and 4-aminopyridine. In contrast, it was found that clotrimazole inhibits Ca^{2+} influx in CD3 activated cells while the mobilization of Ca^{2+} from intracellular compartments was not affected by this drug. Miconazole, econazole and α-naphtoflavone displayed properties similar to that we have described herein for clotrimazole (not shown).

Table II

Effect of membrane potential on [^{3}H]serine uptake by Jurkat cells and incorporation of [^{3}H]serine into phosphatidylserine

Effectors	[^{3}H]Serine uptake	PtdSer synthesis
None	26,990 ± 1,768	9,681 ± 255
Valinomycin	34,574 ± 3,636	13,343 ± 1,178
Gramicidin	3,233 ± 250	834 ± 125
KCl	4,253 ± 375	326 ± 132

The hyperpolarizing K^+ ionophore valinomycin was used at 5×10^{-6} M, the depolarization was obtained either with the Na^+ ionophore, gramicidin at 5×10^{-6} M or by replacing NaCl by an equivalent amount of KCl in the incubation medium. Cells were incubated for 2 h in the presence of [^{3}H]serine. [^{3}H]Serine uptake is expressed as cpm ± S.D. ($n = 6$) corresponding to a 50 μl sample and radioactivity determined by scintillation counting while PtdSer synthesis corresponds to chloroform/methanol extracts analysed after thin-layer chromatography and radioactivity determined by scanning chromatography plates.

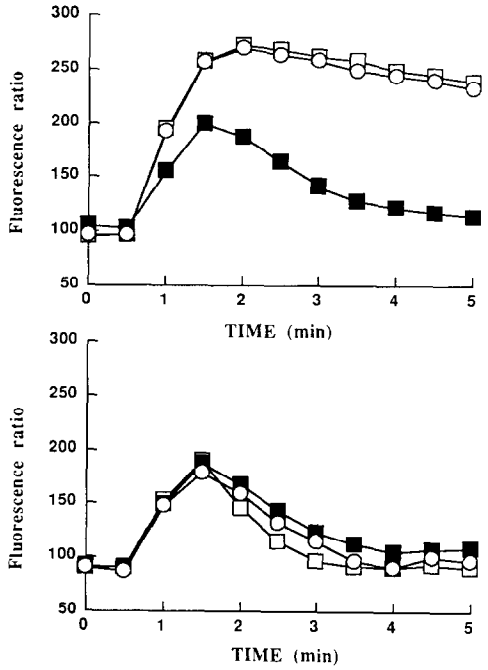

Fig. 3. Effect of quinine and clotrimazole on CD3-induced changes in cytosolic Ca^{2+} concentration. The higher panel represents cytosolic Ca^{2+} changes induced by CD3 mAb (2 μg/ml) in the absence (□) or presence of either 50 μM quinine (○) or 50 μM clotrimazole (■) in a medium containing 1 mM Ca^{2+}. The lower panel represents Ca^{2+} mobilization from intracellular stores, the experiment was done under the same conditions as above but in the absence of Ca^{2+} in the medium (2 mM EGTA added).

4. DISCUSSION

The base exchange enzyme system responsible for the biosynthesis of PtdSer in cells has been previously shown to be highly activated by K^+ channel blockers such as quinine, 4-aminopyridine, tetraethylammonium and clofilium in Jurkat T cells and in the monocyte/macrophage cell line THP_1 [4–6]. In the present report we demonstrate that some imidazole antimycotics (miconazole, econazole, clotrimazole) display similar properties. In a previous paper, Alvarez et al. [3] have shown that all these drugs are potent inhibitors of K^+ channels in red cells, Ehrlich ascites tumor cells and thymocytes. The results described herein, demonstrate that antimycotics strongly raise the incorporation of [^{3}H]serine into

PtdSer in the absence of significant modifications of the uptake of [^{3}H]serine by the cells, suggesting that the blockade of K^+ channel and activation of PtdSer synthesis are two closely related phenomena.

As described by Alvarez et al. [1,2] and in the present work, the drugs known as antimycotics also are able to strongly decrease the Ca^{2+} influx caused after stimulation of Jurkat cells by CD3 mAb without changes in Ca^{2+} mobilization from intracellular stores. On the contrary, the well-known K^+ channel blockers (quinine, 4-aminopyridine and clofilium) were unable to modify Ca^{2+} influx generated by this activation process. Since the classical K^+ channel blockers and the antimycotics increased similarly PtdSer synthesis, it can be concluded that the increase in PtdSer synthesis observed when using these drugs occurs independently of the presence or absence of a Ca^{2+} influx in Jurkat T cells. On the other hand, since K^+ channel blockers, are susceptible to changing membrane potential in Jurkat cells, we have studied whether modifying membrane potential (by replacing NaCl by KCl in the medium or by using gramicidin or valinomycin) induced changes in [^{3}H]serine uptake and PtdSer synthesis. It was shown that depolarization with KCl or gramicidin dramatically decreased [^{3}H]serine uptake by the cells and concomitantly decreased PtdSer synthesis. On the contrary, hyperpolarization induced by valinomycin induced an increase of [^{3}H]serine uptake by the cells and a modest increase in the formation of PtdSer (38%). Since K^+ channel blockers did not change [^{3}H]serine uptake and doubled the amount of PtdSer synthesized, it appears that the mode of action of K^+ channel blockers on the base exchange enzyme system is not caused by changes of the membrane potential. In addition, the use of nigericin, ouabain, amiloride and tetrodotoxin, indicated that PtdSer synthesis is not significantly regulated by either K^+/H^+ exchange, Na^+,K^+-ATPase, Na^+/H^+ exchange or Na^+ channels.

The data presented herein indicate that in Jurkat cells, antimycotics similarly to the classical K^+ channel blockers enhance PtdSer synthesis independently of Ca^{2+} movements and independently of membrane potential. Our results thus allow one to suggest that antimycotics block K^+ channel in Jurkat cells as previously demonstrated in other cellular systems [3]. This proposal, rather speculative at present, is susceptible to confirmation or dismissal by future research based on patch clamp analysis of K^+ channels in Jurkat T cells. Finally, by using antimycotics and α-naphtoflavone, we have demonstrated for the first time that the regulation of the base exchange enzyme system responsible for PtdSer synthesis may involve a cytochrome P_{450} or a closely related protein.

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