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Short communication

Clotrimazole and efaroxan inhibit red cell Gardos channel independently of imidazoline I_1 and I_2 binding sites

Isabelle Coupry [a], Carrie C. Armsby [b], Seth L. Alper [b], Carlo Brugnara [b], Angelo Parini [a,*]

[a] *INSERM U388, Institut Louis Bugnard, CHU Rangueil, 31054 Toulouse, France*

[b] *Departments of Pathology and Clinical Laboratories, the Children's Hospital and the Molecular Medicine and Renal Units, Beth Israel Hospital, Harvard Medical School, Boston, MA 02115, USA*

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Abstract

In the present report, we investigated the potential involvement of imidazoline I_1 and I_2 binding sites in the inhibition of the Ca^{2+}-activated K^+ channel (Gardos channel) by clotrimazole in human red cells. Ca^{2+}-activated ^{86}Rb influx was inhibited by clotrimazole and efaroxan but not by the imidazoline binding site ligands clonidine, moxonidine, cirazoline and idazoxan (100 μM). Binding studies with [^{3}H]idazoxan and [^{3}H]*p*-aminoclonidine did not reveal the expression of I_1 and I_2 binding sites in erythrocytes. These data indicate that the effects of clotrimazole and efaroxan on the erythrocyte Ca^{2+}-activated K^+ channel may be mediated by a 'non-I_1/non-I_2' binding site.

Keywords: K^+ channel; Erythrocyte; Clotrimazole; Efaroxan; Imidazoline binding site

1. Introduction

Recent reports by our group have shown that clotrimazole is a potent and specific inhibitor of the Ca^{2+}-activated K^+ channel (Gardos channel) of sickle erythrocytes and prevents Ca^{2+}-dependent dehydration of sickle cells in vitro (Brugnara et al., 1993). In addition, studies in a transgenic mouse model for sickle cell disease have indicated that oral administration of clotrimazole is associated with inhibition of the red cell Gardos channel, increased red cell K^+ content and decreased mean corpuscular hemoglobin concentration (De Franceschi et al., 1994). The Gardos channel, either alone or in conjunction with K^+-Cl^- cotransport, plays a major role in dehydration of sickle cells (Glader and Nathan, 1978; Brugnara et al., 1993). As sickle cell dehydration is central to the pathophysiology of sickle cell disease, prevention of red cell dehydration appears to be one of the possible therapeutic strategies for sickle cell disease.

Clotrimazole is the most active imidazole studied so far in the inhibition of the Gardos channel. However, this specific effect is also reproduced by other imidazole derivatives. At present, the mechanisms responsible for inhibition of the Gardos channel by imidazole derivatives are not definitively understood.

Imidazoline binding sites, a class of membrane-bound proteins recently described in a variety of tissues and species (Bricca et al., 1989; Coupry et al., 1990; Mallard et al., 1992; Langin and Lafontan, 1989), could be the potential receptors mediating the effects of imidazole derivatives on the erythrocyte Gardos channel. Indeed, the major characteristic of these binding sites is represented by their recognition of a series of imidazole, imidazoli(di)ne and guanidinium derivatives (Michel and Ernsberger, 1992). In addition, the possible involvement of these binding sites in the regulation of the K^+ channel is further supported by three observations: (1) ligand binding to imidazoline binding sites is highly sensitive to K^+ and Rb^+ (Coupry et al., 1990), (2) imidazoline derivatives stimulate insulin secretion by inhibiting the ATP-sensitive K^+ channel in pancreatic β cells (Chan et al., 1994), and (3) imidazole derivatives which display weak inhibition of the Gardos

* Corresponding author. INSERM U388, Institut Louis Bugnard – CHU Rangueil – Bât. L3, 31054 Toulouse Cedex, France. Tel.: 33.61.32.26.22; fax: 33.62.17.25.54.

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channel also display a low affinity for imidazoline binding sites (Coupry et al., unpublished results).

Based on their pharmacological characteristics, imidazoline binding sites have been classified as I_1 (high affinity for clonidine) and I_2 (low affinity for clonidine) (Michel and Ernsberger, 1992).

To investigate the potential involvement of imidazoline binding sites in inhibition of the erythrocyte Gardos channel by antifungal imidazoles, we studied the effects of various imidazoline derivatives on Ca^{2+}-activated ^{86}Rb influx and we attempted to characterize imidazoline I_1 and I_2 binding sites in human red cells by using [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan as radioligands.

2. Materials and methods

2.1. Measurement of ^{86}Rb influx in human red cells

Venous blood was collected from normal subjects after obtaining informed consent. Blood was passed through cotton to decrease the number of leukocytes and then centrifuged in a Sorvall refrigerated centrifuge (RC5B, Du Pont Instruments, Sorvall Biomedical Div., Newtown, CT, USA) at 5°C for 10 min at $3000 \times g$. Cells were washed 5 times with a washing solution containing 152 mM choline chloride, 1 mM $MgCl_2$, 10 mM Tris (Tris(hydroxymethyl)aminomethane)-MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.4 at 4°C.

Red cells were incubated at room temperature in a medium containing 18 mM NaCl, 2 mM KCl, and 10 mM Tris-HCl, pH 8.0, at a concentration of 1×10^7 cells/ml, in the presence of 100 nM ouabain, 10 μM bumetanide, 10 μM rauwolscine and the desired amount of drug to be tested for inhibition. At the end of the incubation the cell suspension was spun at $3000 \times g$ for 10 min, the supernatant removed, and a smaller volume of medium containing A23187 (60 μmol/l cells), 50 μM $CaCl_2$, and ^{86}Rb was added to a final hematocrit of 4–5%. Aliquots of this cell suspension were taken at specified times (1, 3, and 5 min) and spun in Eppendorf tubes containing 0.4 ml butylphthalate oil and 0.8 ml of medium with 5 mM EGTA (*N*,*N*,*N*′,*N*′-tetracetic acid). The supernatant and the upper layer of oil were carefully removed, and the cell pellet was counted in a gamma counter.

2.2. Binding studies

For binding studies, blood samples were collected in Na-citrate 3.8% and then centrifuged at $3000 \times g$ for 10 min at 4°C. Cells were washed twice in buffer A containing 150 mM NaCl, 5 mM Na-phosphate 5 mM, pH 8. Cells were centrifuged 5 times at $10000 \times g$ for 30 min at 4°C. The final cell pellet was resuspended and sonicated in incubation buffer, containing Tris-HCl 50 mM, pH 7.4, EGTA 2 mM, $MgCl_2$ 1 mM.

Membranes (150–500 μg of protein) were incubated in a final volume of 250 or 500 μl of incubation buffer with increasing concentrations of [^{3}H]idazoxan or [^{3}H]*p*-aminoclonidine (0.5–40 nM), for 30–90 min at room temperature in the presence of 10 μM rauwolscine to mask α_2-adrenoceptors. Non-specific binding was defined in the presence of 10 μM cirazoline and 10 μM rilmenidine for [^{3}H]idazoxan and [^{3}H]*p*-aminoclonidine binding respectively. For competition studies, membranes were incubated with increasing concentrations of competitors (10^{-9} to 10^{-5} M). Bound radioligand was separated from the free by vacuum filtration over glass fiber filters as previously described (Coupry et al., 1990). Radioactivity was quantitated by liquid scintillation spectroscopy.

3. Results

As previously reported, Ca^{2+}-activated ^{86}Rb influx in human red cells was inhibited by clotrimazole in a dose-dependent manner (IC_{50} of 89 ± 26 nM). Among the different ligands of imidazoline binding sites, only efaroxan decreased Ca^{2+}-activated ^{86}Rb influx with an IC_{50} of 111.5 ± 25 μM (Fig. 1). In contrast, in the same experimental conditions, moxonidine and clonidine (I_1 ligands), cirazoline (I_1/I_2 ligand) and idazoxan (I_2 ligand) did not affect ^{86}Rb influx at concentrations up to 100 μM.

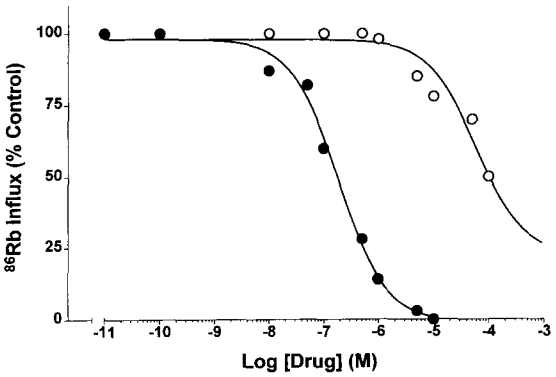

Fig. 1. Dose-response curve for inhibition of Ca^{2+}-activated ^{86}Rb influx by clotrimazole and efaroxan. ^{86}Rb influx was measured in washed human red cells resuspended in saline medium with various concentrations of clotrimazole (closed circles) and efaroxan (open circles). The saline medium contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-MOPS, pH 7.40 at 37°C, and 10 mM glucose. The final concentration of A23187 was 60 μmol/l cells in saline. Results are the mean ± S.D. of three separate experiments. Curves were fitted with Ultrafit. IC_{50} was calculated from the fitted curves.

Binding experiments in human red cell membranes were performed using either [^{3}H]*p*-aminoclonidine or [^{3}H]idazoxan, two radioligands of I_1 and I_2 imidazoline binding sites respectively. At different concentrations of [^{3}H]*p*-aminoclonidine or [^{3}H]idazoxan, 'specific binding', as defined using 10 μM of cirazoline or rilmenidine, accounted for 10–20% of total binding. However, specific binding was not saturable by increasing radioligand concentrations and incubation time. The binding parameters were not improved by modifying protein concentration, incubation volumes and buffer composition. Finally, competition studies showed that total binding was not displaced by idazoxan, cirazoline, guanabenz, clonidine or moxonidine at concentrations lower than 10 μM. Using similar experimental conditions, [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan allowed the identification and characterization of I_1 and I_2 binding sites in various tissues, including kidney and brain stem (Michel and Ernsberger, 1992). Our results indicate that binding of [^{3}H]idazoxan and [^{3}H]*p*-aminoclonidine to human red cell membranes does not satisfy the criteria to defining the interaction of a ligand to its specific receptor.

4. Discussion

The identification of imidazoline binding sites allowed to propose new mechanisms responsible for the pharmacological activity of imidazoline derivatives. Indeed, functional studies showed that the centrally mediated decrease in arterial blood pressure and the regulation of renal sodium excretion by imidazoline derivatives (i.e. clonidine and rilmenidine), which has been related to the stimulation of α_2-adrenoceptors, are due, in part, to the binding to imidazoline I_1 binding sites (Bousquet et al., 1984; Penner and Smyth, 1994). On the other hand, we have recently shown that the imidazoline I_2 binding site is a binding site located on monoamine oxidases A and B (Tesson et al., 1995), two mitochondrial enzymes involved in the oxidative deamination of neurotransmitters (catecholamines and serotonine) and exogenous amines. Enzyme assays performed in transformed yeast expressing monoamine oxidase A or B (Tesson et al., 1995), in rabbit kidney (Tesson et al., 1995) and in rat liver (Carpéné et al., 1995) suggested the involvement of the I_2 binding sites in the inhibition of monoamine oxidase activity.

As imidazoline binding sites have been identified in different tissues, it is conceivable that they could mediate a large variety of functional activities. The inhibition of the Gardos channel by clotrimazole is one of the potential effects mediated by imidazoline binding sites. However, the fact that, in our study, imidazoline binding site ligands did not inhibit Ca^{2+}-activated ^{86}Rb influx and radioligand binding studies revealed neither imidazoline I_1 nor I_2 binding sites in human red cell membranes indicates that inhibition of red cell Ca^{2+}-activated K^+ transport by clotrimazole does not take place via I_1 and/or I_2 binding sites.

Among different imidazoline ligands, only efaroxan, to a lesser degree and with lower potency than clotrimazole, inhibited Ca^{2+}-activated ^{86}Rb influx in erythrocytes. Recent studies have shown that this compound at equally high concentration stimulates insulin release in rat pancreatic islet β-cells (Chan et al., 1994) and RIN-5AH cells (Olmos et al., 1994) by lowering K^+ efflux through ATP-sensitive K^+ channels. The fact that the pharmacological profile of this effect was not that expected for imidazoline binding sites suggested the involvement of a 'non-I_1/non-I_2' binding site in stimulation of insulin secretion by efaroxan.

It is noteworthy that inhibition of erythrocyte Ca^{2+}-activated K^+ transport and stimulation of insuline secretion in pancreatic islet β-cells and in RIN-5AH cells by imidazoline derivatives share some common properties: first, both effects require an elevated concentration of efaroxan; second, they display a pharmacological profile different from that expected for imidazoline I_1 and I_2 binding sites and finally they are both related to the inhibition of K^+ channels. These observations suggest that the effects of imidazoline drugs in erythrocytes, pancreatic islet β-cells and in RIN-5AH may involve unknown subtypes of imidazoline binding sites associated with K^+ channels. The fact that some imidazoline derivatives stimulating insuline secretion in RIN-5AH cells are ineffective in red cells suggests that the ligand recognition properties of these 'non-I_1/non-I_2' may depend on their relationship with different K^+ channels. Indeed, the effects of imidazoline derivatives in pancreatic β-cells and erythrocytes involve two distinct K^+ channels, the ATP-sensitive and the Ca^{2+}-activated K^+ channel. Our results show that clotrimazole, as well as other imidazole derivatives (Brugnara et al., 1993) are much more potent than efaroxan in inhibiting red cell Ca^{2+}-activated K^+ channel. This suggests that the putative imidazoline 'non-I_1/non-I_2' binding site in red cells may preferentially recognize imidazole rather than imidazoline derivatives. Further experiments are necessary to confirm this hypothesis and to verify whether imidazole compounds behave as inhibitors of the ATP-sensitive K^+ channel in pancreatic islet β-cells and RIN-5AH cells.

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