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Specific inhibition of Ca-activated K channels in red cells by selected dihydropyridine derivatives

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1 Thirty two dihydropyridine derivatives were screened as potential inhibitors of the Ca-activated K-channel in human red cells.

2 Three derivatives (26, 29, 32 see Tables 1 and 2) with high activity were then characterized in detail, and also tested against the smooth muscle Ca-channel and shown to have varying potencies.

3 One of the more potent derivatives (32) and nitrendipine were also tested on the Ca-activated K-channel, Maxi-K channel, from mouse pancreatic beta-cells.

4 We conclude from our results that it may be possible to develop selective Gardos-channel inhibitors based on these molecules, which may be of benefit in the treatment of sickle cell disease.

Keywords: Dihydropyridine; gardos channel; maxi-K channel; L-type Ca-channel; erythrocyte; β-cell; ileal smooth muscle

Introduction

It has been shown recently that dihydropyridines (DHP) not only inhibit L-type calcium channels, but are potent antagonists of the Ca-activated K-channel (Gardos-channel, Gardos, 1958) in human red cells (Kaji, 1990; Ellory *et al.*, 1992). In particular, nitrendipine is effective at 100 nM as a blocker of this channel. This finding is of potential therapeutic importance in sickle cell disease, where repeated oxygenation and deoxygenation of HbSS red cells raise intracellular calcium and activate the Gardos-channel causing significant K^+ loss, cell shrinkage, an increase in MCHC, and an enhanced probability of the formation of irreversibly sickled cells.

An obvious disadvantage of DHP derivatives in sickle cell therapy is their direct inhibition of L-type Ca^{2+}-channels in other tissues, especially heart and vascular smooth muscle. We have therefore investigated the potency of a number of DHP derivatives against the red cell Gardos-channel, the Maxi-K channel in pancreatic β-cells and the L-type Ca^{2+}-channel in smooth muscle in the hope of identifying specific structural characteristics which would allow a greater functional separation of activity of these derivatives. Initially 32 molecules based on the substituted DHP nitrendipine were assayed at a single dose (5 μM) against the red cell Gardos-channel. Subsequently, three active molecules (**26, 29, 32**) were selected and their IC_{50} measured both as inhibitors of Ca-dependent smooth muscle contraction (ileal muscle), and as inhibitors of the red cell Gardos channel. Finally, the ability of both nitrendipine (10 μM) and molecule number **32** (10 μM) to block the classical Maxi-K channel in pancreatic β-cells was tested. The results indicate selective differences in the pharmacology which may form a basis for the development of more specific Gardos-channel inhibitors in the future.

Methods

Erythrocytes

Blood (HbAA) from three healthy donors was taken into heparin and then washed three time by centrifugation (5 min at 3,000 *g*) in MOPS-buffered saline (MBS) composition (mM): NaCl 134.4, KCl 5, $MgCl_2$ 0.4, MOPS 15, and glucose 10, pH 7.4).

K^+ ($^{86}Rb^+$) flux measurements

The flux measurements were performed in the low-K^+ washing solution (as above) as previously described (Ellory *et al.*, 1992). Briefly, the final samples had a volume of 1.0 ml and a haematocrit of approximately 5%. Each sample contained ouabain (0.1 mM), bumetanide (0.1 mM) and $CaCl_2$ (0.4 mM). The nitrendipine derivatives were added to give the required final concentration of 5 μM, and the samples incubated at 37°C for 10 min. The Gardos K^+-channel was activated by the addition of the calcium ionophore, ionomycin and for experimental convenience, K^+ influx was estimated from the uptake of $^{86}Rb^+$ over a 60s incubation period. The flux was terminated by the addition of 300 μl of quinine (0.87 mM in ice-cold MBS), then four 300 μl aliquots were transferred immediately to microcentrifuge tubes containing quinine (0.1 mM in MBS) layered over dibutylphthalate. The tubes were then centrifuged (14000 *g* for 10 s) to form a pellet of red cells below the oil and the supernatant removed. The tubes were then rinsed three times with water and the dibutylphthalate aspirated. The red cells were then lysed with 0.5 ml Triton-X-100 (0.1% v/v) and deproteinised with 0.5 ml TCA (5% w/v). The $^{86}Rb^+$ in the supernatant was then measured by Cerenkov counting.

Calcium channel antagonist assay

The calcium channel antagonist activities of molecules **26, 29** and **32** were determined as the concentration required to produce 50% of the muscarinic receptor-mediated (carbachol, 1.6×10^{-7} M) Ca^{2+}-dependent contraction of guinea-pig ileal longitudinal smooth muscle. This was measured according to the procedure described by Dagnino *et al.* (1987).

Measurement of block of Maxi-K channels from pancreatic β-cells

Pancreatic β-cells possess Ca^{2+}-activated K^+-channels with biophysical properties highly similar to the classical Maxi-K type of K^+-channel (Latorre *et al.*, 1989; Ashcroft & Rors-

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man, 1989). The actions of molecule **32**, nitrendipine and nifedipine were tested on the activity of Maxi-K channels by the patch-clamp technique. Single-channel activity of the Maxi-K channel was recorded in outside-out membrane patches excised from pancreatic β-cells, isolated from mouse pancreas, using the methods described by Bokvist *et al.* (1990a,b). The pipette (intracellular) solution contained (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 0.2, MGATP 3 and K-HEPES 10 (pH 7.2). The bath (extracellular) solution contained (in mM) NaCl 138, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.6 and Na-HEPES 10 (pH 7.4). The membrane potential was held at 0 mV and experiments conducted at room temperature. Under these conditions only potassium currents flowing through the Maxi-K type of K-channels are recorded (Bokvist *et al.*, 1990b). The effect of the dihydropyridine on channel activity was determined by normalizing the channel activity in the test solution (NP) to the average of its value in the control solution before and after exposure to the dihydropyridine.

Materials

$^{86}RbCl$ was supplied by NEN (UK). Ouabain, nifedipine and bumetanide were from Sigma Chemical Co. Nitrendipine was a gift from Bayer. The 1,4-dihydropyridyl derivatives (**1–32**) were prepared using the literature procedures as follows: **8–10**, **16** (Dagnino *et al.*, 1986); **1–5**, **18**, **20**, **22** (Akula *et al.*, 1989); **26–27**, **29**, **31–32** (Wynn *et al.*, 1988); **6**, **11–13** (Akula *et al.*, 1990a); **7**, **14** (Akula *et al.*, 1990b); **19**, **21** (Ramesh *et al.*, 1987); **15**, **17**, **23–25**, **28**, **30** (Knaus & Wolowyk, unpublished results). Ionomycin and charybdotoxin were from Calbiochem Corporation (CA, U.S.A.). All other reagents were of analytical grade.

Results

Table 1 presents the results of experiments screening 32 dihydropyridine derivatives at a single dose (5 μM) as inhibitors of the Ca-activated K^+ efflux from normal (HbAA) red cells. Molecules **26**, **29**, **32** were then selected for detailed study, and dose-response curves generated for inhibition of both the red cell Gardos-channel, and an ileal smooth muscle preparation. The results of these experiments are presented in Table 2. It can be seen that differences in potency against the two systems were found. Thus, **29** was more potent against the Gardos-channel than the L-type Ca-channel; conversely **32** was a much more effective inhibitor of the L-type Ca-channel, than against the red cell Gardos-channel.

Activity of Maxi-K channels could be readily recorded, single-channel current amplitude 5 ± 0.2 pA, in outside-out patches held at a membrane potential of 0 mV. Application of 100 nM charybdotoxin slowly and reversibly reduced the activity of the channel to 46 ± 8% of control activity ($n = 6$). In all 9 patches tested, external 1 mM TEA produced a rapid, reversible and almost complete block of Maxi-K channel activity ($K_d = 140$ μM, Bokvist *et al.*, 1990b). Molecule **32** (10 μM) had no effect on the activity of the Maxi-K channel (activity 106 ± 7% of control, $n = 8$) whereas 10 μM nitrendipine increased the activity of the Maxi-K channel to 120 ± 7% of its control value ($n = 9$, $P = 0.02$). In two patches tested, 10 μM nifedipine which blocks the Gardos-channel with a K_d of 4 μM (Kaji, 1990) was without effect on the activity of the Maxi-K channel.

Drugs **29**, **32** and nitrendipine were also tested against HbSS cells and no significant differences in the IC_{50} between normal and HbSS cells were found.

Table 1 The inhibition of the Gardos channel in normal HbAA erythrocytes by derivatives of nitrendipine at a concentration of 5 μM

No.	R^1	R^2	R^3	*% inhibition*
1	ethyl	cyclohexyl	2-pyridyl	− 4.6 ± 2.6
2	cyclohexyl	cyclohexyl	2-pyridyl	13.7 ± 7.5
3	*t*-butyl	cyclohexyl	2-pyridyl	− 10.8 ± 4.1
4	methoxyethyl	cyclohexyl	2-pyridyl	− 3.8 ± 3.5
5	isopropyl	cyclopentyl	2-pyridyl	3.7 ± 4.0
6	isopropyl	4-chlorophenylethyl	2-pyridyl	8.4 ± 3.2
7	isopropyl	2-furanylmethyl	2-pyridyl	− 0.7 ± 5.9
8	isobutyl	methyl	2-pyridyl	3.1 ± 7.0
9	methyl	methyl	3-pyridyl	9.8 ± 6.8
10	isobutyl	isobutyl	3-pyridyl	− 0.2 ± 3.1
11	isopropyl	4-fluorophenylethyl	3-pyridyl	− 6.6 ± 7.4
12	isopropyl	4-chlorophenylethyl	3-pyridyl	8.1 ± 5.6
13	isopropyl	4-methoxyphenylethyl	3-pyridyl	2.6 ± 1.6
14	isopropyl	2-furanylmethyl	3-pyridyl	43.9 ± 3.5
15	ethyl	2-cyanoethyl	3-pyridyl	− 16.8 ± 13
16	isopropyl	isopropyl	3-pyridyl	− 14.9 ± 7.3
17	methyl	2-cyanoethyl	3-pyridyl	− 14.8 ± 17
18	cyclohexyl	cyclohexyl	3-pyridyl	− 14.1 ± 12.5
19	*t*-butyl	t-butyl	3-pyridyl	− 3.4 ± 6.5
20	methyl	cyclohexyl	3-pyridyl	12.8 ± 9.8
21	methyl	t-butyl	3-pyridyl	− 14.3 ± 17.9
22	cyclohexyl	cyclohexyl	4-pyridyl	− 6.3 ± 14.9
23	isopropyl	3-pyridylmethyl	2-nitrophenyl	− 15.9 ± 18.3
24	isopropyl	3-pyridylmethyl	3-nitrophenyl	− 4.8 ± 8.1
25	ethyl	ethyl	phenyl	43.2 ± 1.7
26	methyl	methyl	cyclohexyl	97.0 ± 0.8
27	methyl	methyl	methyl	2.5 ± 4.0
28	methyl[a]	methyl	H	0.5 ± 4.3
29	methyl	methyl	4-nitrophenyl	98.7 ± 0.7
30	methyl[b]	methyl	H	1.8 ± 3.5
31	methyl[c]	methyl	phenyl	24.4 ± 3.0
32	methyl	methyl	phenyl	82.5 ± 3.1

The compounds in this table have the general structure (1) except for (a) which is a mixture of compounds of general structure (1) and (2) (1:1 ratio, $R^1 = R^2 = Me$, $R^3 = H$), (b) has general structure (2) ($R^1 = R^2 = Me$, $R^3 = H$) and (c) has general structure (2) ($R^1 = R^2 = Me$, $R^3 = Ph$).

Table 2 Comparison of calcium channel antagonist activity and Gardos channel inhibition of selected derivatives

No.	R^1	R^2	R^3	*Calcium channel antagonist activity* (ID_{50}, M)	*Gardos channel inhibition* (ID_{50}, M)
26	methyl	methyl	cyclohexyl	$3.05 \pm 1.07 \times 10^{-7}$	$3.22 \pm 0.74 \times 10^{-7}$
29	methyl	methyl	4-nitrophenyl	$1.21 \pm 0.20 \times 10^{-6}$	$2.29 \pm 0.90 \times 10^{-7}$
32	methyl	methyl	phenyl	$2.61 \pm 0.04 \times 10^{-8}$	$1.44 \pm 0.51 \times 10^{-6}$

The compounds in this table have the general structure (1) shown in footnote to Table 1.

Discussion

A number of structure-activity correlations were observed that produce superior Gardos-channel inhibition, these include: (1) The 1,4-dihydropyridine ring is essential since 3,5-dimethyl 1,4-dihydro-2,6-dimethyl-4-phenyl-3,5-pyridinedicarboxylate (**32**) is more active than the aromatic pyridine compound 3,5-dimethyl 2,6-dimethyl-4-phenyl-3,5-pyridinedicarboxylate **31**; (2) Increasing the size of the alkyl ester substituents decreases activity since the 3,5-dimethyl compound **32** ($R^1 = R^2 = Me$) was more potent than the 3,5-diethyl analogue **25** ($R^1 = R^2 = Et$); (3) Compounds having a C-4 cycloalkyl **26** (R^3 = cyclohexyl), aryl **29** (R^3 = 4-nitrophenyl) or phenyl substituent (R^3 = phenyl) as in **32** provided more potent activity than compounds having a pyridyl substituent such as **9** (R^3 = 3-pyridyl); (4) The nature of the C-4 substituent appears to be a selective determinant of both L-type Ca-channel and Gardos-channel antagonism since the 4-cyclohexyl compound **26** was equipotent in both assays, whereas the 4-nitrophenyl compound **29** was more active as a Gardos-channel inhibitor than as a L-type Ca-channel antagonist, whilst the 4-phenyl compound, **32**, was a more potent antagonist of the L-type Ca-channel then the Gardos-channel. Both compound **32** and nifedipine, potent antagonists of both the L-type Ca-channel and the Gardos-channel were without effect on the Maxi-K channel suggesting that nonspecific block of Maxi-K in other tissues by these compounds is unlikely. We have not attempted to explain the surprising ability of nitrendipine to activate the Maxi-K channel, a phenomenon that warrants further investigation.

These structure-activity correlations indicate that 3,5-dimethyl 1,4-dihydro-2,6-dimethyl-4-(4-nitrophenyl)-3,5-pyridinedicarboxylate (**29**) is a good 'lead component' for further structural manipulation in the design of a selective 1,4-dihydropyridine antagonist for the Gardos-channel.

Our original hypothesis in screening these molecules was that the nitro group may be less important in the context of Gardos-channel inhibition than in blocking the L-type Ca-channel. This idea, however, is not completely borne out by the results, since compound **29** with the 4-nitrophenyl group proved more useful than the simple unsubstituted phenyl compound **32**.

These results confirm earlier experiments indicating that DHP derivatives are effective Gardos-channel inhibitors. They also offer prospects for the development of modifications to these molecules to increase the efficacy of red cell Gardos-channel inhibition whilst avoiding significant effects on L-type Ca-channels and Maxi-K channels. The final goal of therapy for sickle cell disease relies on the development of more specific inhibitors of the Gardos-channel which would avoid the hypotensive effects of the DHP class of drugs.

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