Potassium channel blockers quinidine and caesium halt cell proliferation in C6 glioma cells via a polyamine-dependent mechanism

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Abstract

Potassium channels are ubiquitous in cells and serve essential functions in physiology and pathophysiology. Potassium channel blockers have been shown to block tumour growth by arresting cells at the G0/G1 checkpoint of the cell cycle. We investigated the effect of quinidine and caesium (Cs⁺) on cell proliferation, LDH (lactate dehydrogenase) release, free internal calcium, membrane potential, polyamine concentration, ODC (ornithine decarboxylase) activity and polyamine uptake in C6 glioma cells. The EC₅₀ for reducing cell proliferation was 112 µM for quinidine, whereas Cs⁺ was less effective with an EC₅₀ of 4.75 mM. KCl or sucrose did not affect proliferation. LDH release was augmented by quinidine. Quinidine caused a transient increase in free internal calcium but decreased calcium after a 48 h incubation period. Further 300 µM quinidine depolarized the cell membrane in a similar range as did 30 mM KCl. Quinidine decreased cellular putrescine beyond detection levels while spermidine and spermine remained unaffected. ODC activity was reduced. Addition of putrescine could not override the antiproliferative effect owing to a reduced activity of the polyamine transporter. Our study indicates that the antiproliferative effect of quinidine is not due to a simple membrane depolarization but is caused by a block of ODC activity.

Introduction

Potassium channels are found ubiquitously in animal and plant cells. They control the membrane potential as well as electrical signalling and are known to be involved in cell proliferation. Potassium channel blockers such as quinidine, 4-AP (4-aminopyridine), Cs⁺ or TEA (tetraethyl-ammonium) have been shown to stop cell proliferation and tumour growth in a variety of cell types via an arrest in the G0/G1 transition during the cell cycle (for reviews, see [1,2]). However, the cellular mechanism by which potassium channel activity contributes to cell division is not clear. Polyamines not only modulate potassium channels [3,4], but also are crucial for cells to pass through the cell cycle [5]. We investigated the effects of Cs⁺, which is used in cancer therapy [6,7], and quinidine, a drug used in the clinic for treatment of malaria [8] and heart arrhythmias [9], on polyamine homeostasis, membrane potential, mitochondrial activity, calcium levels and cytotoxicity in C6 glioma cells.

Materials and methods

Cell culture

C6-glioma cells were kept at 37°C, 5% CO₂ and 90% humidity in Ham’s F-10 medium supplemented with 9% FCS (fetal calf serum) and 1% penicillin/streptomycin. In the case of polyamine substitution experiments, FCS was replaced by HS (horse serum). For experiments cells were plated on poly(D-lysine)- or fibronectin-coated 96-well microtitre plates, flasks or coverslips. At 5 h after seeding, control medium was changed to experimental medium containing either quinidine or Cs⁺.

Cell proliferation assay

Cell numbers were measured with a Crystal Violet assay as described previously [10,11]. Net proliferation was determined by subtracting the number of initially seeded cells. Controls were set to 100% unless mentioned otherwise.

Analysis of dose–response relationships

Data were fitted according to the equation:

\[ Y = 1 \left( \frac{[c]}{[c] + EC_{50}} \right) \]

where \( Y \) is normalized response (values from control experiments were set to 1), \( c \) is concentration and \( b \) is Hill coefficient.

Key words: cell cycle, cell proliferation, mitochondria, ornithine decarboxylase (ODC), potassium channel, quinidine.

Abbreviations used: 4-AP, 4-aminopyridine; BrDU, 5-bromo-2’-deoxyuridine; FCS, fetal calf serum; HS, horse serum; LDH, lactate dehydrogenase; NF2, neurofibromatosis type 2; ODC, ornithine decarboxylase; TEA, tetraethyl-ammonium; XTT, sodium 3,3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulphonic acid hydrate.

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Cytotoxicity
Cytotoxicity was determined 48 h after seeding by LDH (lactate dehydrogenase) release using a colorimetric detection kit from Boehringer Mannheim according to the manufacturer’s instructions.

Calcium imaging
Intracellular-free calcium was measured on a Bio-Rad MRC-600 confocal microscope equipped with Time Course software as described previously [10]. Cells were loaded with the calcium indicator Fluo-3/AM (acetoxymethyl ester) and calcium concentrations were calculated according to the method of Kao et al. [12].

Cell cycle analysis
Flow cytometry
The percentage of cells in G0/G1 phase was estimated using flow cytometry as described in [10]. Samples were examined on a FACStar flow cytometer and analysed with FACStar plus software (Becton Dickinson). A total of 10^4 cells were counted for each sample. The percentage of cells in G0/G1 phase was calculated: (counts G0 + G1)/(counts G0 + G1 + S + G2 + M-phase).

BrdU (5-bromo-2′-deoxyuridine) incorporation
Cells entering S-phase were investigated using a kit from Boehringer Mannheim to detect BrdU [13]. After seeding, cells were grown for 24 h and subsequently exposed to 10 µM BrdU for another 24 h. After a total of 48 h, cells were processed according to the manufacturer’s protocol. For each sample, a total of 100 cells were counted and the percentage of BrdU-positive cells, indicating progression into S-phase, was calculated.

Intracellular polyamine concentration
Cells were grown under control or experimental conditions and intracellular polyamine concentration was measured as described previously [14].

Mitochondrial activity
Mitochondrial activity was assayed with an XTT {sodium 3,3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate} EZ4U kit from Biomedica. Tetrazolium salt reduced by mitochondrial activity to formazan was measured photometrically [15]. This reaction can be used for direct monitoring of mitochondrial function [16]. In parallel, Crystal Violet tests were performed to assay the exact cell number of each individual experiment.

Electrophysiology
Electrophysiological recordings were done in the whole-cell patch–clamp mode [17]. In short, signals were amplified with a List EPC-7 amplifier. Data were sampled online and analysed with pClamp software (Axon Instruments). Medium supplemented with FCS and 10 mM Hepes, pH 7.2, was chosen as bathing solution to simulate conditions as present in proliferation experiments. The pipette solution was 140 mM KCl, 2 mM MgCl₂, 11 mM EGTA/KOH, 10 mM glucose, 10 mM Hepes and 1 mM CaCl₂, pH 7.2.

Determination of ODC (ornithine decarboxylase) activity
Cells were incubated for 6 h with 300 µM quinidine. ODC activity was estimated according to the method of Janne and Williams-Ashman [18].

Polyamine uptake
Cells were pre-incubated for 18 h with 300 µM quinidine. The medium was changed to HBSS (Hanks balanced salt solution) supplemented with 300 µM quinidine and 0.1 µCi of [14C]putrescine (100 µM). Uptake was allowed for 45 min.

Statistical analysis
Significance levels were determined either with Student’s t test or, where appropriate, with a one-way ANOVA followed by Dunnett’s multiple comparison test as post test. Outliers were removed by application of Grubb’s test for outliers. All data are given as means ± S.E.M.

Results
Quinidine and Cs⁺ were found to reduce proliferation of C6 glioma cells after 48 h of treatment in a dose-dependent manner. The EC₅₀ was 112 ± 7.75 µM (n = 4) with a Hill coefficient of 2.15 for quinidine and 4.75 ± 0.32 mM with a Hill coefficient of 1.64 for Cs⁺ (Figure 1). Further experiments were performed with the more effective drug quinidine. To test whether the effect of quinidine was reversible, cells were incubated with 300 µM quinidine for 48 h then washed twice with control medium and set in a quinidine-free environment for another 48 h. As a result of the quinidine release, net proliferation increased again by 32.4 ± 3.5% (n = 6), indicating a reversible mechanism of action. To test whether the antiproliferative effect of quinidine is due to
membrane depolarization, cells were incubated for 48 h with 30 mM KCl. No change of proliferation [100.2 ± 0.07% of control proliferation (100%), n = 3] was observed at high potassium concentrations. To exclude any osmotic effects, we incubated C6 glioma cells for 48 h with 60 mM sucrose. Proliferation remained unchanged under this hyperosmolar condition (98.9% of control proliferation, n = 5).

**Electrophysiology**

Control membrane resting potential of C6 glioma cells was $−46.28 ± 3.86$ mV (n = 5). Application of 300 µM quinidine to the bath solution caused a depolarization of $+16.53 ± 3.4$ mV (n = 5). A depolarization in the same range (+25.8 ± 3.3 mV, n = 4) was evoked by adding 30 mM KCl to the bath solution.

**Cell cycle**

C6 glioma cells start cycling under control conditions 12 h after seeding, as indicated by a significant reduction of cells in G2/G1 phase compared with 2 h after seeding. In contrast, cells treated with 300 µM quinidine did not significantly enter other phases of the cell cycle until 18 h after plating. Furthermore, quinidine-treated cells were always found to a higher extent in G2/G1 than in controls. To confirm this G2/G1 arrest, BrdU incorporation was studied. At 24 h after seeding cells, BrdU was added for another 24 h. Under control conditions, 93.25 ± 1.45% (n = 4) of the cells were BrdU-positive compared with 40.1 ± 6.9% (n = 4) for cells treated with 300 µM quinidine (Figure 2). This was even more pronounced when cells were kept in culture for 72 h with a BrdU boost during the last 24 h of the experiment [control 94.0 ± 1.08% (n = 4) BrdU-positive, 300 µM quinidine 22.5 ± 3.92% (n = 4) BrdU-positive].

In order to examine the reason for the slowing of cell cycle kinetics, we investigated the concentration of intracellular polyamines and free internal calcium, as both are known to be essential factors for cells to progress through the cell cycle [5,19]. Intracellular polyamines were measured 6, 12, 18, 24 and 48 h after incubation of the cells with 300 µM quinidine. Putrescine was reduced or not detectable in the presence of quinidine. This effect was observed from 6 h and continued during the whole experiment. However, spermidine and spermine were not reduced significantly by 300 µM quinidine at any time point. After 6 h of incubation with quinidine, ODC activity was reduced significantly (control: 1603 ± 112.7 pmol of [14]CO₂/h per mg of protein; 300 µM quinidine: 66.0 ± 23.56 pmol of [14]CO₂/h per mg of protein; n = 4). Addition of 100 µM putrescine could not counteract the antiproliferative effect of 112 µM quinidine (97.9 ± 13.06%, n = 4, of the proliferation as seen with quinidine alone). Since this might be due to a deficient polyamine uptake, we studied the function of the polyamine transporter. We found quinidine to reduce significantly the uptake of putrescine (control: 745 ± 50.9 c.p.m./mg of protein, n = 4; 300 µM quinidine: 416.5 ± 7.6 c.p.m./mg of protein n = 4), indicating a direct effect of quinidine on the polyamine transporter in C6 glioma cells.

**Figure 2 | BrdU staining**

(A) In control cells almost all nuclei are BrdU-positive, indicating a passage through the S-phase of the cell cycle. (B) Cells treated with 300 µM quinidine for 48 h. Note that only a few nuclei were stained with BrdU, suggesting that most cells did not enter the S-phase.

To test whether free intracellular calcium concentration is also affected by quinidine, cells were grown in the presence or absence of 300 µM quinidine. After 48 h, the internal free calcium concentration in control cells was 137.3 ± 6.6 nM (n = 27), while cells treated with quinidine showed a marked reduction in free internal calcium (98.6 ± 5.7 nM; n = 15). In contrast, cells incubated for 48 h with 30 mM KCl exhibited the same internal free calcium concentration as controls did (136.6 ± 14.47 nM; n = 11). However, application of quinidine resulted in a transient increase in calcium.

In a further set of experiments, LDH release was tested as an indicator of membrane disintegration. It was found that, during quinidine application, cytotoxicity doubled after 24 h (control: 8.4 ± 3.4%; 300 µM quinidine: 15.8 ± 3.1%, n = 3), with a further significant increase after 48 h (control: 9.02 ± 2.59%; 300 µM quinidine: 36.9 ± 2.84%, n = 3). To make sure that these changes are not underestimated by an interaction of quinidine with LDH, as described previously for mitochondrial LDH [20], we lysed C6 glioma cells with Triton X-100 and performed the assay in the presence or absence of 300 µM quinidine. Attenuance values were 1.297 ± 0.03 (n = 8) for control and 1.263 ± 0.02 (n = 8).
Discussion

We found the potassium channel blockers quinidine and Cs⁺ to reduce cell proliferation in a dose-dependent manner, with quinidine to be more effective drug. It prevented cell cycling of C6 glioma cells and diminished the intracellular putrescine concentration via a block of ODC. In addition, the polyamine transporter was obstructed, while the mitochondrial activity was reduced and LDH release was increased. Depolarization evoked by an increased extracellular potassium concentration had no effect on cell proliferation.

Our results are in agreement with other studies showing quinidine to reduce cell proliferation in a number of cell lines in the micromolar range [21–24]. The EC₅₀ of quinidine in our proliferation experiments is approx. 10-fold higher than the believed therapeutic concentration in humans (7–15 μM [24]). This might be due to a lesser sensitivity of the rat-derived C6 glioma cell line to quinidine than is observed in human cell lines. The Hill coefficient for quinidine as well as for Cs⁺ indicates a 2:1 stoichiometry between these drugs and potassium channels.

Interestingly, a simple membrane depolarization by an increased extracellular potassium concentration, which is in the same range as the depolarization caused by quinidine, does not affect cell proliferation. This indicates that the antiproliferative effect of potassium channel blockers may not be explained by a more depolarized membrane potential, but rather is related to qualitative or quantitative aspects of channel activation or the status of the channel. While potassium channels at a more depolarized membrane potential can be still active, blocked channels no longer transport potassium out of the cell and are considered to be inactive. This suggests that channel activity might be a key for the control of cell proliferation. In contrast, Utermark et al. [23] using two different strains of human NF2 (neurofibromatosis type 2)-deficient human malignant mesothelioma cells, with one being quinidine-sensitive and the other not, conclude that quinidine action in their system did not depend on potassium channel activity, but rather is attributed to a loss of the expression of the tumour-suppressor gene merlin. A similar result has been reported by Rosenbaum et al. [24] from human neurofibromatotic NF2 Schwann cells whose abnormal proliferation could be stopped by quinidine, whereas healthy control Schwann cells were unaffected. These two findings point to a potassium-channel-independent effect of quinidine. Another explanation could be that quinidine not only blocks potassium channels but also blocks calcium and sodium channels; however, these channels are affected by quinidine to a much lesser degree [25,26].

Quinidine depolarizes C6 glioma cells as expected by a potassium channel blocker and at the same time decreases putrescine concentrations in the cell, while intracellular spermidine and spermine concentrations remain unchanged. The reduction in putrescine is directly related to a block of ODC activity by quinidine. A reduced polyamine concentration is well known to inhibit the progression of cells into the S-phase of the cell cycle [27,28]. This is in agreement with our findings that C6 glioma cells enter the S-phase at a reduced rate after incubation with quinidine. Extracellular addition of polyamines cannot override this cell cycle halt, since quinidine in addition blocks the polyamine transporter in these cells.

Polyamines not only favour cell proliferation and development but also modulate ion-channel activity [3,4,29–31]. Any changes in polyamine concentrations will lead to a different activity in a number of ion channels. In this respect, quinidine may additionally affect channel activity via an indirect polyamine-dependent pathway, thus contributing to decreased cell proliferation. Quinidine further reduced mitochondrial activity, indicating a cytotoxic effect at higher concentrations which is confirmed by an elevated LDH release.

In conclusion, quinidine and Cs⁺ prevent cell cycling via a non-membrane-potential-dependent mechanism, by blocking potassium channels. While the direct link between the reduction in cell proliferation and the channel block remains to be elucidated, we could show that quinidine blocks ODC, resulting in reduced proliferation. This supports the fact that quinidine is so far the only clinically available and approved potassium channel blocker which may benefit tumour patients.

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References


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