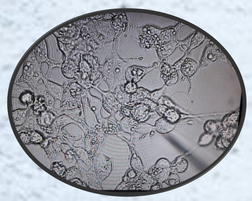

Activation of anion channels in human cells after long term exposure to the marine toxin azaspiracid

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Introduction

Azaspiracids (AZAs) include a group of marine toxins initially documented in the Netherlands after ingestion of contaminated mussels, harvested in Ireland coasts, by the end of the last century [1-3]. Azaspiracids are produced by dinoflagellates of the genera *Azadinium* and *Amphidoma* [4]. The presence of AZA toxins in fishery products has been regulated in Europe establishing a limit of 160 μg kg^{-1} AZA equivalents [5]. Thereafter, several acute *in vitro* studies were performed to elucidate its cellular targets, but the results obtained showed great controversy regarding the possible mechanism of action of AZAs that could contribute to the symptomatology elicited in humans after ingestion of contaminated fishery products. Our group has recently described that these toxins partially blocked sodium entry into the cells and caused cytoskeletal alterations [6]. Nevertheless, the effect of azaspiracid on anion channels remains almost completely unexplored [7]. Therefore, the main aim of our study was to gain more insight on the effects of AZA on ionic homeostasis using several pharmacological approaches.

Methods

Chemicals and toxin used

Azaspiracid-1 analogue was purchased from CIFGA (Lugo, Spain) and channel blockers were acquired from Sigma and Tocris.

HEK293 cells expressing the human $Na_v1.7$ sodium channel subunit

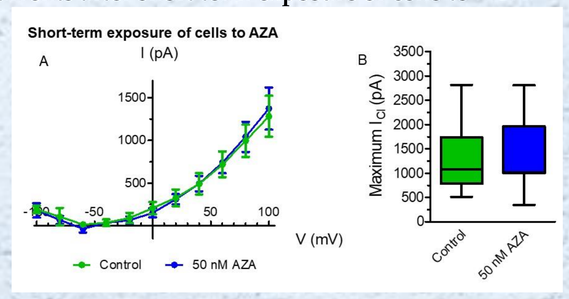
Immortalized human embryonic kidney cells (HEK293) transfected with the human $Na_v1.7$ alpha subunit of the sodium channels were kindly provided under a material transfer agreement with Dr Andrew Powell (GlaxoSmithKline R&D, Stevenage, UK).

Electrophysiological recordings

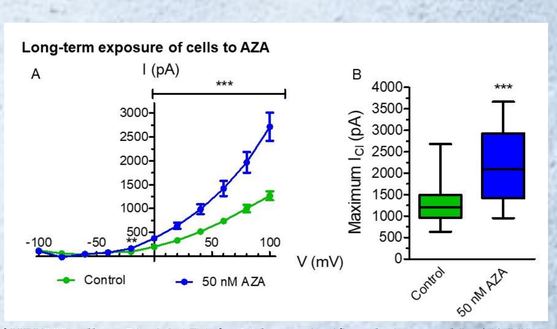
Electrophysiological measurements of chloride currents were obtained in whole-cell configuration on $Na_v1.7$ HEK cell line as previously described [6]. Chloride currents (I_{Cl}) were recorded by application of a voltage step protocol from -100 to +100 mV with 20 mV step increases and 400 ms duration.

Results

Exposure of HEK293 cells to AZA increases voltage-regulated chloride currents (I_{Cl}) after long-term treatments but had no effect on I_{Cl} currents after short term exposure of cells to AZA



Activation properties of chloride currents evoked from HEK293 cells. (A) Acute exposure of HEK293 cells to 50 nM AZA did not affect the maximum I_{Cl}. I-V curve for the chloride current activation in the absence and presence of toxin. I_{Cl} were obtained 5 minutes after bath application of solvent or AZA. Data are mean ± SEM of three different cells (B) Box and whiskers graph representing the median I_{Cl} and maximum and minimum values at 100 mV.



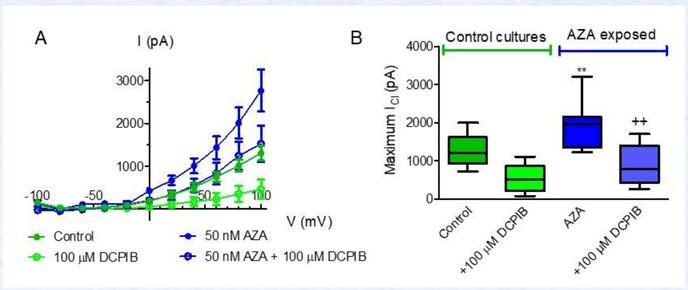
*Exposure of HEK293 cells to 50 nM AZA for 18 hours significantly increased I_{Cl} (A) I-V curve for the activation of chloride currents in the absence of azaspiracid and after exposure of the cells to AZA for 18 hours in culture. Data are mean ± SEM of 27 cells from 9 independent cultures. (B) Boxplot graphs showing the median I_{Cl} and maximum and minimum values recorded at the holding potential of 100 mV. ** p < 0.01 vs control, *** p < 0.001 vs control currents.*

Compilation of channel blockers used to modulate the AZA-induced I_{Cl} increase in HEK293 cells

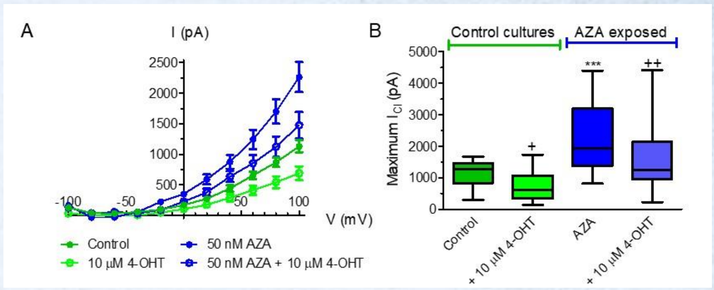
Channel inhibitor	Concentration	Channel selectivity
DCPIB	100 μM	VRAC
DIDS	500 μM	ClC-6 and 7, Maxi Cl^-, ClC-Ka and Kb, CaCC, VRAC
NPPB	100 μM	TRPA1, ClC-2 and 7, Maxi Cl^-, CaCC, VRAC
Hydroxytamoxifen (4-OHT)	10 μM	VRAC, Maxi Cl^-

Anion channels modulators employed to analyze the effect of azaspiracid on the I_{Cl} and their selectivity classified following the IUPHAR data base. VRAC: Volume Regulated Anion Channel, ClC: family of Chloride Channels, Maxi Cl^-: Maxi chloride channel TRP: Transient Receptor Potential Channels, CaCC: Calcium Activated Chloride Channel.

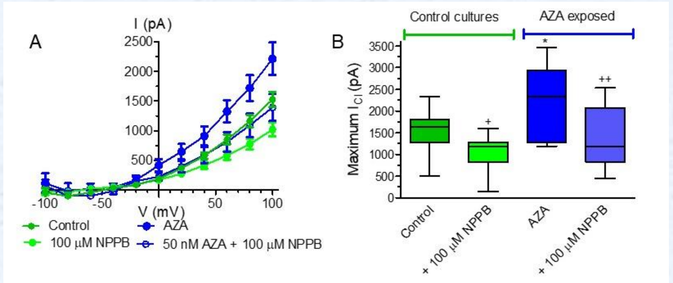
Effect of anion channel blockers on the AZA-induced I_{Cl} increase in HEK293 cells



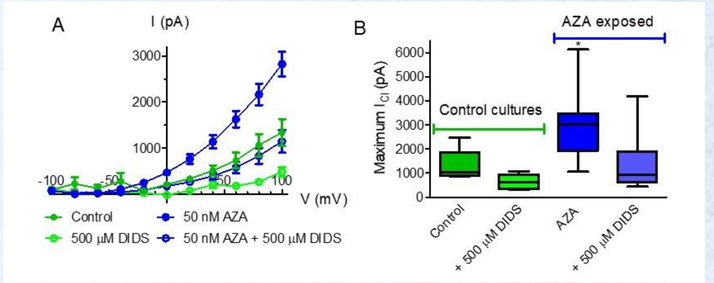
*DCPIB decreased chloride currents evoked from HEK cells in control conditions and in cells exposed to 50 nM AZA for 18 h. (A) I-V relationship for the activation of I_{Cl} currents in control cells and in AZA treated cells before and after application of 100 μM DCPIB. (B) Box and whiskers graph representing intensities of I_{Cl} currents at 100 mV in control cells and in cells exposed to AZA in absence and presence of 100 μM DCPIB. ** p < 0.01 vs control currents. ++ p < 0.01 vs currents without channel blocker.*



*Tamoxifen diminished chloride currents evoked in $Na_v1.7$ HEK cells in control conditions and in cells exposed to 50 nM of AZA for 18 h. (A) I–V curve for chloride current activation in the absence of 50 nM AZA and in the presence of the toxin before and after addition of 10 μM tamoxifen.(B) Representative boxplot graph of the maximum I_{Cl} current at the 100 mV voltage step in different experimental conditions. *** p < 0.001 vs control currents. + p < 0.05, +++ p < 0.001 vs currents without tamoxifen.*



*NPPB abolished the chloride currents increase observed in $Na_v1.7$ HEK293 cells after exposition to 50 nM AZA for 18 h. (A) Voltage-dependence of the chloride currents activation in control cells and in cells treated with AZA and the subsequent addition of 100 μM NPPB. (B) Box and whisker plot representing the I_{CL} current after a voltage step of 100 mV. * p < 0.05 vs control currents. ++ p < 0.01 vs currents without channel blocker.*



*DIDS reduced the chloride current increase elicited by azaspiracid. (A) I-V graph for the activation of I_{Cl} currents in control cells and in cells treated with 50 nM AZA, before and after application of 500 μM DIDS. (B) Maximum I_{Cl} currents at 100 mV of control cells before and after bath application of the channel blocker. * p < 0.05 vs control currents.*

Conclusions

1. Short term exposure of human cells to nanomolar concentrations of azaspiracids did not activate chloride anion channel.
2. Low nanomolar concentrations of the toxin led to a dramatic alteration in chloride homeostasis and to an increase in chloride efflux after long term exposure.
3. A complete inhibition of the increase in I_{Cl} current resulting from activation of VRAC channels after exposure of the cells to several channel blockers was not achieved.
4. The lack of specific inhibitors for each chloride channel family hinders the ability to make a conclusive statement on the identity of the anion channels involved on the cellular effect of azaspiracid.
5. Further studies should be pursued to analyze the role of voltage-gated channels in the cell morphology changes induced by azaspiracids and their involvement on the human poisonings elicited by the toxin.

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