Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 1627–1636
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Azaspiracid-4 inhibits Ca^{2+} entry by stored operated channels in human T lymphocytes

Amparo Alfonso [a], Yolanda Román [a], Mercedes R. Vieytes [b], Katsuya Ofuji [c], Masayuki Satake [c], Takeshi Yasumoto [d], Luis M. Botana [a,*]

[a] *Departamento de Farmacología, Facultad de Veterinaria, USC, 27002 Lugo, Spain*
[b] *Departamento de Fisiología, Facultad de Veterinaria, USC, 27002 Lugo, Spain*
[c] *Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*
[d] *Japan Food Research Laboratories, Tama, Tokyo 206-0025, Japan*

Received 13 January 2005

Abstract

Azaspiracids (AZs) are a new group of phycotoxins discovered in the Ireland coast that includes the isolated analogues: AZ-1, AZ-2, AZ-3, AZ-4 and AZ-5 and the recently described AZ-6–11. Azaspiracid toxic episodes show gastrointestinal illness, but neurotoxic symptoms are also observed in mouse bioassay. Despite their great importance in human health, so far its mechanism of action is largely unknown. In this report, we present the first data about the effect of AZ-4 on cytosolic calcium concentration $[Ca^{2+}]_i$ in freshly human lymphocytes. Cytoslic Ca^{2+} variations were determined by fluorescence digital imaging microscopy using Fura2 acetoxymethyl ester (Fura2-AM). AZ-4 did not modify cytosolic Ca^{2+} in resting cells. However, the toxin dose-dependent inhibited the increase in cytosolic Ca^{2+} levels induced by thapsigargin (Tg). AZ-4 decreased Ca^{2+}-influx induced by Tg but did not affect the Ca^{2+}-release from internal stores induced by this drug. The effects of AZ-4 on Ca^{2+}-influx induced by Tg were reversible and not regulated by adenosine 3′,5′-cyclic monophosphate (cAMP) pathway. When AZ-4 was added before, after or together with nickel, an unspecific blocker of Ca^{2+} channels, the effects were indistinguishable and additive. AZ-4 also inhibited maitotoxin (MTX)-stimulated Ca^{2+}-influx by 5–10%. Thus, AZ-4 appeared to be a novel inhibitor of plasma membrane Ca^{2+} channels, affecting at least to store operated channels, showing an effect clearly different from other azaspiracid analogues.

Keywords: Lymphocytes; Azaspiracids; Cytosolic calcium; Calcium channel; Calcium pools

1. Introduction

Azaspiracids (AZs), a new class of phycotoxins, have been implicated in several human intoxications from 1995. Recently, the dinoflagellate *Protoperidinium* has been identified as the progenitor of these phycotoxins [1]. The first confirmed incident of AZs intoxication occurred in The Netherlands following the consumption of mussels (*Mytilus edulis*) from Killary Harbour, Ireland [2]. The symptoms included nausea, vomiting, severe diarrhoea and stomach cramps. Moreover, after mouse bioassay neurotoxic symptoms were also observed [3,4]. AZs differ significantly from other marine phycotoxins, they have unique structural features characterized by a tri-spiro assembly, an azaspiro ring fused with a 2,9-dioxabicyclo [3.3.1]nonane and a terminal carboxylic acid group [2,5,6]. The analogues AZ-1 (azaspiracid), AZ-2 (8-methylazaspiracid) and AZ-3 (22-demethylazaspiracid) are the predominant AZs in nature [3,7]. AZ-4 and AZ-5 derivatives are hydroxy analogues of AZ-3 not abundant in shellfish [8]. Recently, the existence of six more different analogues have been reported in shellfish, AZ-6 is a positional isomer of AZ-1 and AZ-7–11 are hydroxyl analogues of AZ-1 [9].

Toxicological studies about the AZ-1 derivative showed multiple organ damage in mice, with several effects in the

Abbreviations: AZ, Azaspiracid; $[Ca^{2+}]_i$, cytosolic calcium concentration; cAMP, adenosine 3′,5′-cyclic monophosphate; dbcAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; DG, 1,2 diacylglycerol; FSK, forskolin; MTX, maitotoxin; PSS, physiological saline solution; SOC, channels store-operated Ca^{2+} channels; SQ22,536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; Tg, thapsigargin

* Corresponding author. Tel.: +34 982 252 242; fax: +34 982 252 242.
E-mail address: Luis.Botana@lugo.usc.es (L.M. Botana).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.03.022

small intestine and liver and necrosis of T and B lymphocytes in the spleen, thymus and Peyer's patches [4]. However, the intracellular target of these toxins is still unclear. In previous papers, we studied the effect of AZ-1, AZ-2 and AZ-3 derivatives on human lymphocytes [10,11], since these cells showed modifications after AZs intoxications. In these previous works, the intracellular signals calcium concentration $[Ca^{2+}]_i$, cyclic adenosine monophosphate (cAMP) levels and pH were studied and in the presence of toxins, both cAMP and cytosolic Ca^{2+} levels were increased.

Changes in the $[Ca^{2+}]_i$ are one of the main pathways by which information is transferred from extracellular signals to intracellular responses [12]. When Ca^{2+} signalling is stimulated in a cell, cytosolic Ca^{2+} levels are increased either by release from intracellular stores or by entry across plasma membrane channels [13]. In human lymphocytes, two different intracellular Ca^{2+} channels involved in stores depletion have been described: inositol trisphosphate receptors [14] and ryanodine receptors [15–17]. In addition, nicotinic acid adenine dinucleotide phosphate has recently been described as an essential intracellular Ca^{2+} mobilizing agent [18]. On the other hand, Ca^{2+} entry across plasma membrane can be mediated through different Ca^{2+} channels in lymphocytes. The most commonly observed mechanism is capacitative Ca^{2+} entry through store-operated Ca^{2+} channels (SOC channels), in which the emptying of stores is necessary and sufficient to activate Ca^{2+} entry. Despite the wealth of electrophysiological data on the properties of these channels, neither the mechanism that links stores depletion and Ca^{2+}-influx nor the molecular identity of the channels is understood. In addition, in human lymphocytes numerous reports suggest the presence of other types of Ca^{2+} channels such as inositol 1,4,5-trisphosphate receptors in the plasma membrane and channels related to classical L-type voltage Ca^{2+} channels. Inositol receptors, originally described in sarcoplasmic reticulum, are also expressed in the lymphocytes plasma membrane [14,19]. At last, non-voltage-gated Ca^{2+} channels related to classical L-type voltage Ca^{2+} channels in excitable cells have also found expressed in lymphocytes where they play a significant role in the Ca^{2+}-influx pathways [14,20,21]. There are several drugs commonly used to modulate these Ca^{2+} channels, some of them specific as ruthenium red to ryanodine receptors or L-type Ca^{2+} channel antagonists and some unspecific as lanthanum, nickel or SK&F96365 [22].

In this context, the aim of this work is to check and characterize AZ-4 effect on cytosolic Ca^{2+} levels in human lymphocytes. These cells were selected according to toxicological studies and also because they are stable cells that provide direct information from healthy human donors. Moreover, we compare the effect of AZ-4 with other AZ analogues also studied in our laboratory in the same cellular model.

2. Materials and methods

2.1. Chemicals and solutions

AZ-4 and MTX were obtained and isolated by M. Satake, K. Ofuji and T. Yasumoto. Fura2 acetoxymethyl ester (Fura2-AM) was from Molecular Probes. N^6,2′-*O*-Dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP) was from Sigma. Percoll® was from Pharmacia. Thapsigargin (Tg), forskolin (FSK) and [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine] (SQ22,536) were from Alexis. All the other chemicals were reagent grade and purchased from Sigma and Merck.

The composition of saline solution (PBS) used to lymphocyte purification was (mM): Na^+ 145.2, K^+ 4.7, HPO_4^{2-} 8.2, $H_2PO_4^-$ 1.5, Cl^- 141.2 and ethylenediaminetetracetic acid (EDTA) 2. The composition of physiological saline solution (PSS) used for microscopy experiments was (mM): Na^+ 142.3, K^+ 5.94, Ca^{2+} 1, Mg^{2+} 1.2, Cl^- 126.1, CO_2^- 22.85, $H_2PO_4^-$ 1.2 and SO_4^{2-} 1.2, and glucose 1 mg/mL, giving a final osmotic pressure of 300 ± 5 mOsm/kg H_2O. Ca^{2+}-free PSS was done by omitting Ca^{2+} from the PSS. In all the experiments, the incubation PSS was equilibrated with CO_2 and the final pH was adjusted to 7.2.

Stock solutions of drugs and toxins were made in dimethylsulfoxide (DMSO). DMSO effect is always checked at the concentration used and no effect was reported. Tg was used at the concentration 2 μM. Two hundred and fifty micrometers was the stock solution of AZ-4. Serial dilutions of AZ-4 were done when different toxin concentrations were required.

2.2. Human lymphocytes isolation

Peripheral human lymphocytes were isolated from fresh human blood from healthy donors. The blood was diluted 1:1 with PBS plus EDTA 2 mM. Four millilitre of diluted blood was carefully placed over 3 mL of 57.5% isotonic percoll. After centrifugation (25 min, $1000 \times g$), percoll was eliminated by washing three times with PBS plus EDTA 2 mM (5 min, $400 \times g$). Lymphocyte purity was always higher than 85%.

2.3. Cell labelling and determination of $[Ca^{2+}]_i$. image processing

Purified lymphocytes were loaded with the Ca^{2+} sensitive fluorescent dye Fura2-AM 2 μM for 10 min at 37 °C. After incubation, loaded cells were washed three times and allowed to attach to poly-L-lysine-coated 22 mM glass coverslips for 10 min. The glass coverslips were inserted into a thermostated chamber at 37 °C (Life Science Resources) and cells were viewed with a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40×-inmersion UV-Fluor objective). The thermostated chamber was used in the open bath configuration and

additions were made by aspiration and addition of fresh PSS.

$[Ca^{2+}]_i$ was obtained from the images collected by excitation fluorescence with a Life Science Resources equipment. The light source was a 175-W xenon lamp and light reaches the objective with optic fiber. The excitation wavelengths for Fura2 were 340 and 380 nM, with emission at 510 nM. The calibration of the fluorescence versus intracellular calcium was made by the method of Grynkiewicz et al. [23].

2.4. Statistical analysis

$[Ca^{2+}]_i$ of all cells observed in each experiment were averaged. All the experiments were carried out at least three times by duplicate. Results were analysed using the Student's *t*-test. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± S.E.M.

3. Results

The effect of AZ-4 on Ca^{2+} homeostasis is first examined in human lymphocytes bathed in a Ca^{2+} containing PSS. Hundred, 200, 500 and 1000 nM AZ-4 were checked and as Fig. 1A shows the toxin does not modify cytosolic Ca^{2+} levels at any concentration studied after 20 min incubation. Next (Fig. 1B), we studied the effect of AZ-4 on cells initially bathed in a Ca^{2+}-free PSS, and after being for 10 min under those conditions, 1 mM Ca^{2+} was added. Results show that AZ-4 does not significantly modify cytosolic Ca^{2+} levels in these conditions.

Fig. 2 shows the effect of AZ-4 when Ca^{2+}-influx is activated by Tg. This drug passively releases Ca^{2+} from

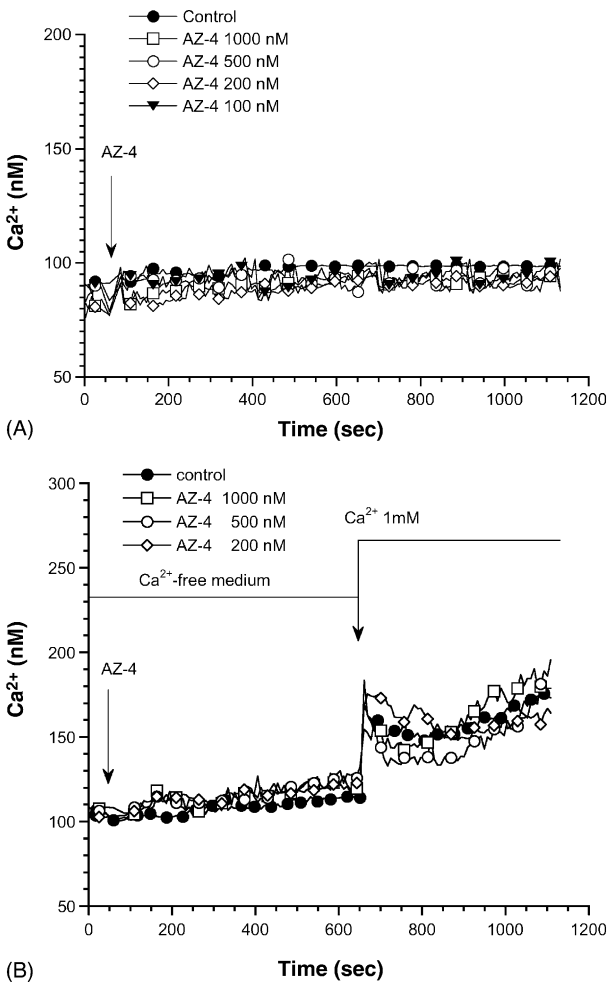


Fig. 1. Effect of different concentrations of AZ-4 on the $[Ca^{2+}]_c$ in human lymphocytes. (A) Cells bathed in a Ca^{2+} containing PSS. The arrow indicates the addition of different AZ-4 concentrations. (B) Cells are first bathed in a Ca^{2+}-free PSS and after 10 min, 1 mM Ca^{2+} is added. The first arrow indicates AZ-4 addition (different concentrations) and the second arrow indicates 1 mM Ca^{2+} addition. Mean ± S.E.M. of approximately 700 cells observed in seven experiments. To have a cleaner graphic, only one out of seven data were plotted.

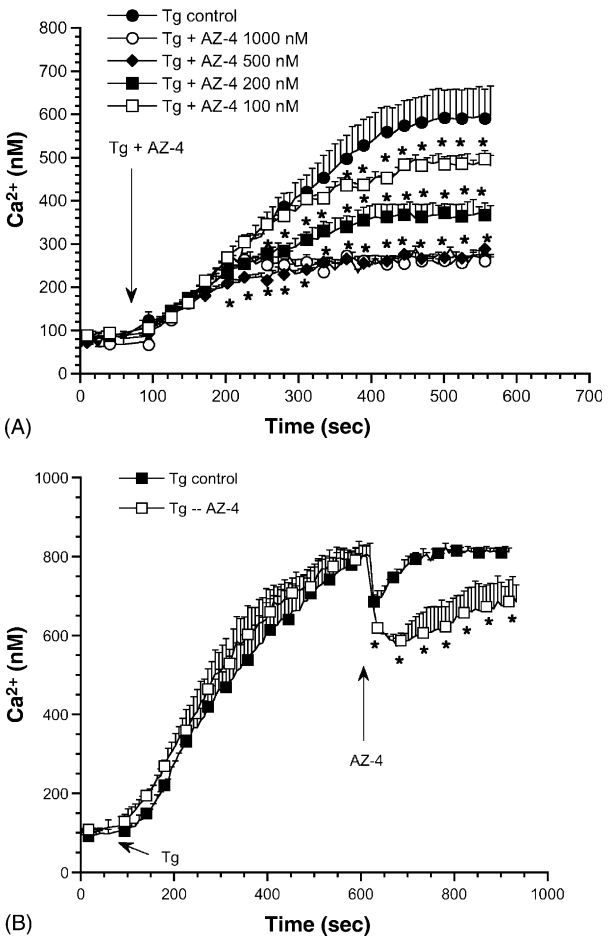


Fig. 2. Effect of AZ-4 on Tg-induced Ca^{2+}-influx in human lymphocytes. The cells are bathed in a Ca^{2+} containing PSS. (A) Different concentrations of AZ-4 and 2 μM Tg are added simultaneously. The arrow indicates drugs addition. Asterisk (*) indicates significant differences with respect to Tg control. (B) Two micrometers Tg (first arrow) is added and 10 min later 200 nM AZ-4 (second arrow). Asterisk (*) indicates significant differences with respect to Tg control. Mean ± S.E.M. of approximately 520 cells observed in six experiments. To have a cleaner graphic, only one out of seven data were plotted. Error bars of all points are shown.

internal stores by inhibition of sarcoendoplasmatic reticulum Ca^{2+} ATPases and consequently, it induces Ca^{2+}-influx through SOC channels. As Fig. 2A shows, simultaneous application of AZ-4 and Tg inhibits Tg-induced Ca^{2+} rise in human lymphocytes bathed in a Ca^{2+} containing PSS. The effect increases with the toxin concentration, 20% inhibition in the presence of 100 nM AZ-4, 50% inhibition in the presence of 200 nM AZ-4 and 70% in the presence of either 500 or 1000 nM AZ-4. From these results, 200 nM AZ-4 was selected to study Ca^{2+} increase inhibition. The increase in $[Ca^{2+}]_i$ induced by Tg is initially due to the empty of intracellular Ca^{2+} pools and lately to the Ca^{2+} entry from the extracellular media [24]. Therefore, we studied the effect of AZ-4 when Ca^{2+}-influx induced by Tg is already activated. In this case, the toxin is added 10 min after Tg that is after Ca^{2+}-influx activation. Fig. 2B shows that cells treated with Tg increase their cytosolic Ca^{2+} levels to 800 nM, but when AZ-4 is added, Tg-stimulated $[Ca^{2+}]_i$ increase is significantly reduced (25%).

In order to investigate if AZ-4 effect on Tg-stimulated $[Ca^{2+}]_i$ increase is related to stores release, AZ-4 and Tg were added in a Ca^{2+}-free solution; with this approach it is possible to establish the difference between Ca^{2+}-release and Ca^{2+}-influx. As Fig. 3 shows, when Tg is applied to cells in a Ca^{2+}-free PSS, a Ca^{2+} rise of approximately 300 nM, due to stores depletion, is observed. Subsequent restoration of Ca^{2+} to the solution induces a rapid elevation of $[Ca^{2+}]_i$ due to influx through opened SOC channels. When AZ-4 and Tg are added simultaneously, stores depletion induced by Tg are not modified, although Ca^{2+}-influx is reduced by approximately 40% (Fig. 3A). With the aim of testing if the AZ-4 absence effect over stores depletion is due to a longer onset time for AZ-4 than for Tg in a new experiment, AZ-4 is added 5 min before Tg. As Fig. 3B shows after AZ-4 pre-incubation the empty of Ca^{2+} stores induced by Tg is not affected, even though Ca^{2+}-influx is again reduced. In a similar experiment, the reversibility of AZ-4 effect was investigated by washing AZ-4 from the extracellular PSS 10 min later its addition. As Fig. 4 shows, when the toxin is washed from the

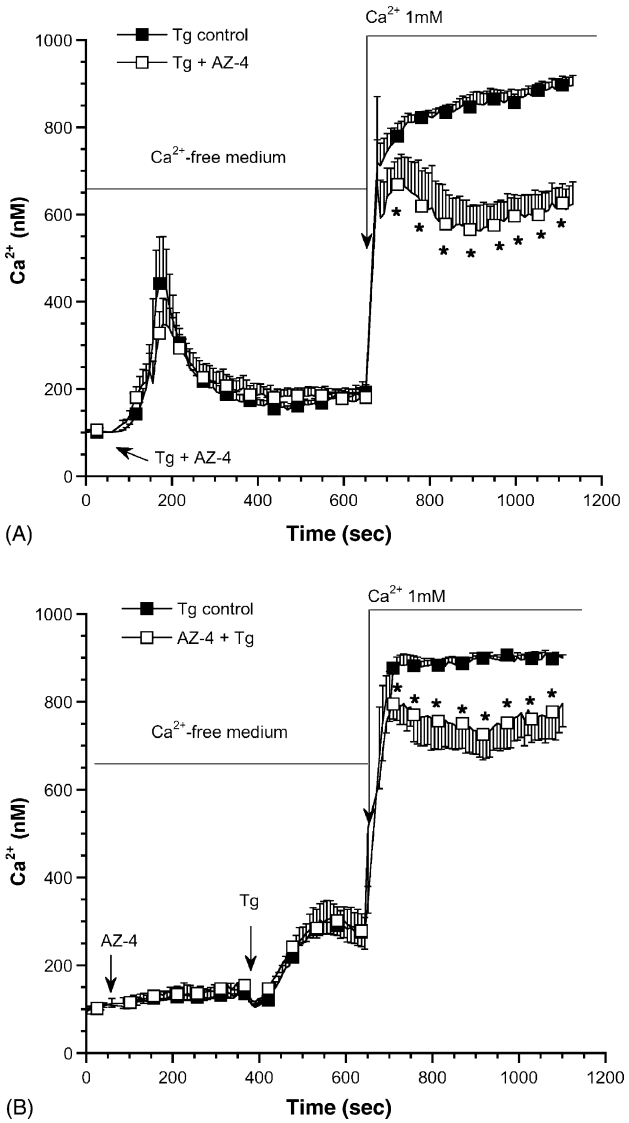


Fig. 3. Effect of AZ-4 on Tg-induced Ca^{2+}-influx in human lymphocytes. Cells are first bathed in a Ca^{2+}-free PSS and after 10 min, 1 mM Ca^{2+} is added. (A) Two hundred nanometers AZ-4 and 2 μM Tg are added simultaneously (first arrow). Asterisk (*) indicates significant differences with respect to Tg control. (B) AZ-4 (first arrow) is added 5 min before Tg addition (second arrow). Asterisk (*) indicates significant differences with respect to Tg control. Mean ± S.E.M. of approximately 340 cells observed in three experiments. To have a cleaner graphic, only one out of seven data were plotted. Error bars of all points are shown.

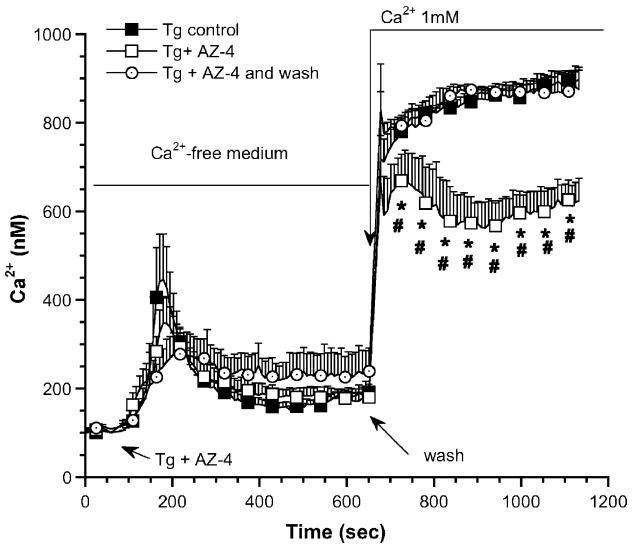


Fig. 4. Effect of AZ-4 removal from the extracellular PSS after AZ-4 inhibition on Tg-induced Ca^{2+}-influx in human lymphocytes. The cells are first bathed in a Ca^{2+}-free PSS and later 1 mM Ca^{2+} is added. Cells are simultaneously treated with 2 μM Tg and 200 nM AZ-4 (first arrow). AZ-4 is present all the time (open square) or it is washed from the extracellular PSS 10 min after its addition (open circles). Mean ± S.E.M. of approximately 340 cells observed in three experiments. Asterisk (*) indicates significant differences with respect to Tg control. Hash (#) indicates significant differences with respect to Tg plus AZ-4 and wash. To have a cleaner graphic, only one out of seven data were plotted. Error bars of all points are shown.

extracellular solution the inhibitory effect of AZ-4 disappears. Thus, AZ-4 (200 nM) reversibly inhibits the increase of $[Ca^{2+}]_i$ induced by Tg, regardless of the order of addition (simultaneously, after or before Tg), without interference with the ability of Tg to release Ca^{2+} from internal stores.

Nickel (Ni^{2+}) has been commonly used as an unspecific inhibitor of plasma membrane Ca^{2+} channels [25]. Several experiments were performed in order to compare the effects of this drug and AZ-4. Fig. 5A shows that after the simultaneous addition of AZ-4 and Tg, subsequent addition of 1 mM Ni^{2+} inhibits the Ca^{2+} rise induced by Tg. The percentage of inhibition, calculated over the value of $[Ca^{2+}]_i$ observed at 600 s, after 1 mM Ni^{2+} addition was 40% in the absence and 75% in the presence of AZ-4. Final cytosolic Ca^{2+} levels after Ni^{2+} addition were from 800 to 520 nM and 200 nM for Tg and Tg + AZ-4, respectively. Thus, Ni^{2+} reduces $[Ca^{2+}]_i$ even when AZ-4 has inhibited the entry of Ca^{2+} induced by Tg. Then the effect of AZ-4 after Ni^{2+} inhibition was checked. As Fig. 5B shows, the addition of Ni^{2+} inhibits Tg-induced Ca^{2+} increase. When in these conditions AZ-4 is added a new decrease in Ca^{2+} levels is observed. Final cytosolic Ca^{2+} values after AZ-4 addition fall from 800 to 600 nM and 300 nM in the presence of Tg and Tg + Ni^{2+}, respectively. Since AZ-4 and Ni^{2+} have effect when they are added sequentially, these drugs were finally added alone and combined after Tg. As Fig. 5C shows, the Tg-induced Ca^{2+} increase was inhibited 25% by AZ-4, 35% by Ni^{2+} and 45% by AZ-4 plus Ni^{2+}, since cytosolic calcium levels decrease

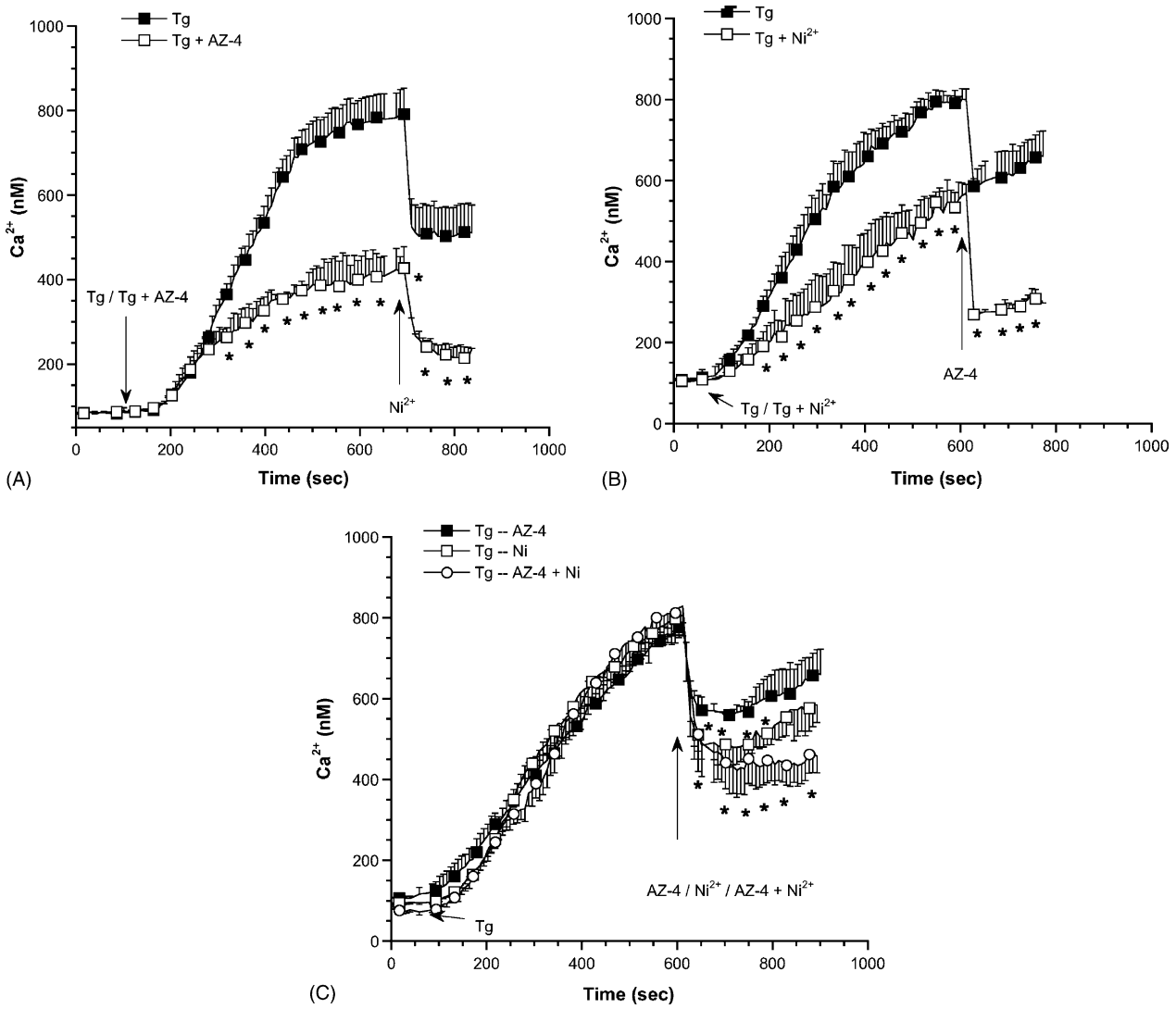


Fig. 5. Interactions between Ni^{2+} and AZ-4 as inhibitors of Tg-induced Ca^{2+}-influx in human lymphocytes. (A) Two micrometer Tg (closed squares) or 2 μM Tg plus 200 nM AZ-4 (open squares) are added simultaneously (first arrows) and 10 min later, 1 mM Ni^{2+} is added (second arrow). Asterisk (*) indicates significant differences with respect to Tg control. (B) Two micrometers Tg (closed squares) or 2 μM Tg plus 1 mM Ni^{2+} (open squares) are added simultaneously (first arrows) and 10 min later, 200 nM AZ-4 is added (second arrow). Asterisk (*) indicates significant differences with respect to Tg control. (C) Cells are treated with 2 μM Tg (first arrow) and 10 min later 200 nM AZ-4 (closed squares), 1 mM Ni^{2+} (open squares) or 200 nM AZ-4 plus 1 mM Ni^{2+} (open circles) are added. Asterisk (*) indicates significant differences with respect to Tg control. Mean ± S.E.M. of approximately 560 cells observed in five experiments. To have a cleaner graphic, only one out of seven data were plotted. Error bars of all points are shown.

approximately from 800 nM in the presence of Tg to 620, 550 and 450 nM when AZ-4 and/or Ni^{2+} are added.

Next, we check the effect of AZ-4 over Ca^{2+}-influx induced by other drugs. Maitotoxin (MTX) is a potent water-soluble phycotoxin that activates Ca^{2+}-influx through non-selective cation channels in different cellular models [26–31]. However, there is no report about the effect of MTX on intracellular Ca^{2+} stores; so, we study the effect of MTX on human lymphocytes initially bathed in a Ca^{2+}-free PSS. As Fig. 6A shows, MTX does not increase cytosolic Ca^{2+} levels in a Ca^{2+}-free solution; hence, no detectable Ca^{2+}-release from internal stores takes place.

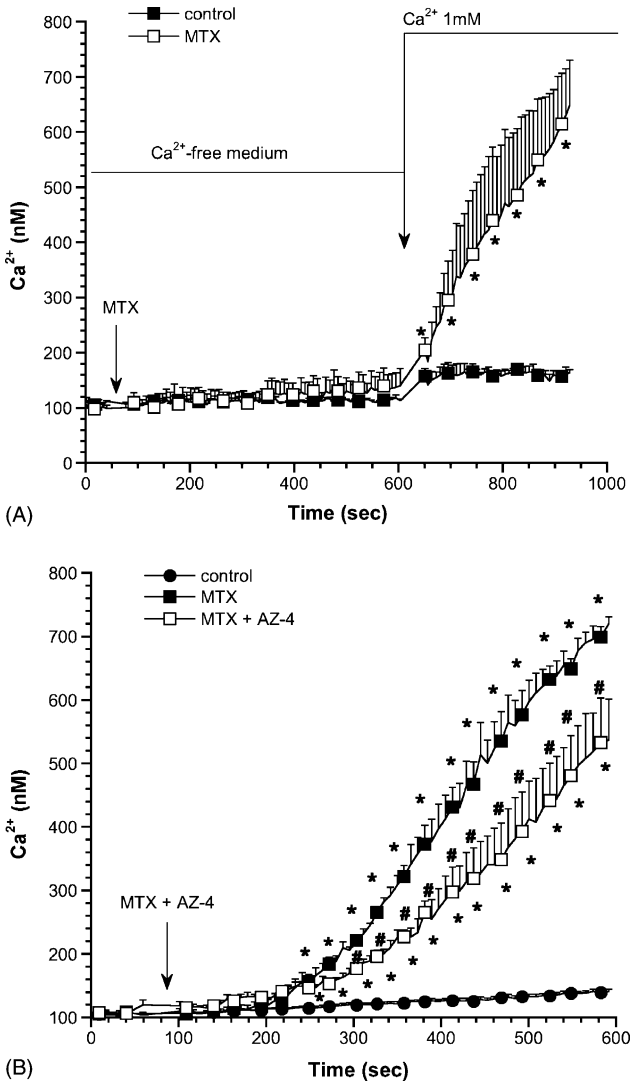


Fig. 6. Effect of AZ-4 over MTX-induced Ca^{2+}-influx in human lymphocytes. (A) Effect of 5 nM MTX on the cytosolic Ca^{2+} levels. Cells are first bathed in a Ca^{2+}-free PSS and after 10 min, 1 mM Ca^{2+} is added. The first arrow indicates MTX addition and the second arrow indicates 1 mM Ca^{2+} addition. Asterisk (*) indicates significant differences with respect to control cells. (B) Effect of 200 nM AZ-4 over 5 nM MTX-induced Ca^{2+} rise in cells bathed in a Ca^{2+} containing PSS. Asterisk (*) indicates significant differences with respect to control cells. Hash (#) indicates significant differences with respect to MTX. Mean ± S.E.M. of approximately 362 cells observed in three experiments. To have a cleaner graphic, only one out of seven data were plotted. Error bars of all points are shown.

However, when 1 mM Ca^{2+} is restored, an important increase of Ca^{2+} levels is observed, showing the cells final Ca^{2+} levels of 600 nM. When cells are bathed in a Ca^{2+} containing PSS (Fig. 6B), MTX induces a potent increase of $[Ca^{2+}]_i$ up to 800 nM. In these conditions, AZ-4 partially inhibits MTX-induced Ca^{2+} rise with values of 10–15% of inhibition.

Despite the dissimilar effects of AZ analogues over Ca^{2+} (Table 1) in previous experiments we had observed a common point: the modulation of cAMP pathway negatively regulates the effect of AZs over Ca^{2+} [10]. Therefore, finally the effect of cAMP pathway over AZ-4 Ca^{2+}-influx inhibition was checked. As Fig. 7 shows, neither the activation of adenylyl cyclase with FSK [32] nor its inhibition with SQ 22,536 [32] nor the direct increase of cAMP levels with the cAMP analogue dbcAMP, modulates the inhibitory effect of AZ-4.

4. Discussion

The present paper shows the effect of AZ-4 derivative over cytosolic Ca^{2+} levels in human lymphocytes. Surprisingly, AZ-4 has opposite effects to AZ-1, AZ-2 and AZ-3 analogues [10,11]. Since between these four analogues there are some structural differences, the results point to some important structure–activity relationship.

These results show that AZ-4 reduces the increase in $[Ca^{2+}]_i$ induced by Tg in human lymphocytes. Two mechanisms could explain the inhibitory effect of AZ-4, the increase of Ca^{2+} extrusion across the plasma membrane, or the inhibition of plasma membrane Ca^{2+} channels activated by Tg, the SOC channels [33,34]. The first mechanism does not seem to fix with our data since in a Ca^{2+}-free solution after Tg addition cytosolic Ca^{2+} levels are the same independently of toxin presence. Moreover, if AZ-4 acts by increasing Ca^{2+} extrusion, the inhibitory action of AZ-4 will be the same independently of the stimuli present, and AZ-4 should inhibit MTX-induced Ca^{2+} increase with the same intensity as Tg-induced Ca^{2+} increase. Therefore, this possibility could be ruled out. The second option means that AZ-4 inhibits SOC channels, an effect that could takes place in several direct or indirect ways: inhibition of Ca^{2+} release from internal stores, direct blocking of SOC channels, uncoupling stores and SOC channels [34], inhibition of mitochondrial Ca^{2+} uptake [35–38] or altering membrane potential [14,39]. However, we have demonstrated that AZ-4 does not exert any detectable effect on the Ca^{2+}-release from internal stores elicited by Tg; thus, we can reduce the possibilities to inhibit or blocking of SOC channels.

One millimeter Ni^{2+} is a known inhibitor of Ca^{2+} channels in several cellular models including SOC channels [22,40,41]. Ni^{2+} results show that the effects of AZ-4 and Ni^{2+} are indistinguishable and additives. Furthermore, both drugs act one after other reducing $[Ca^{2+}]_i$ to approxi-

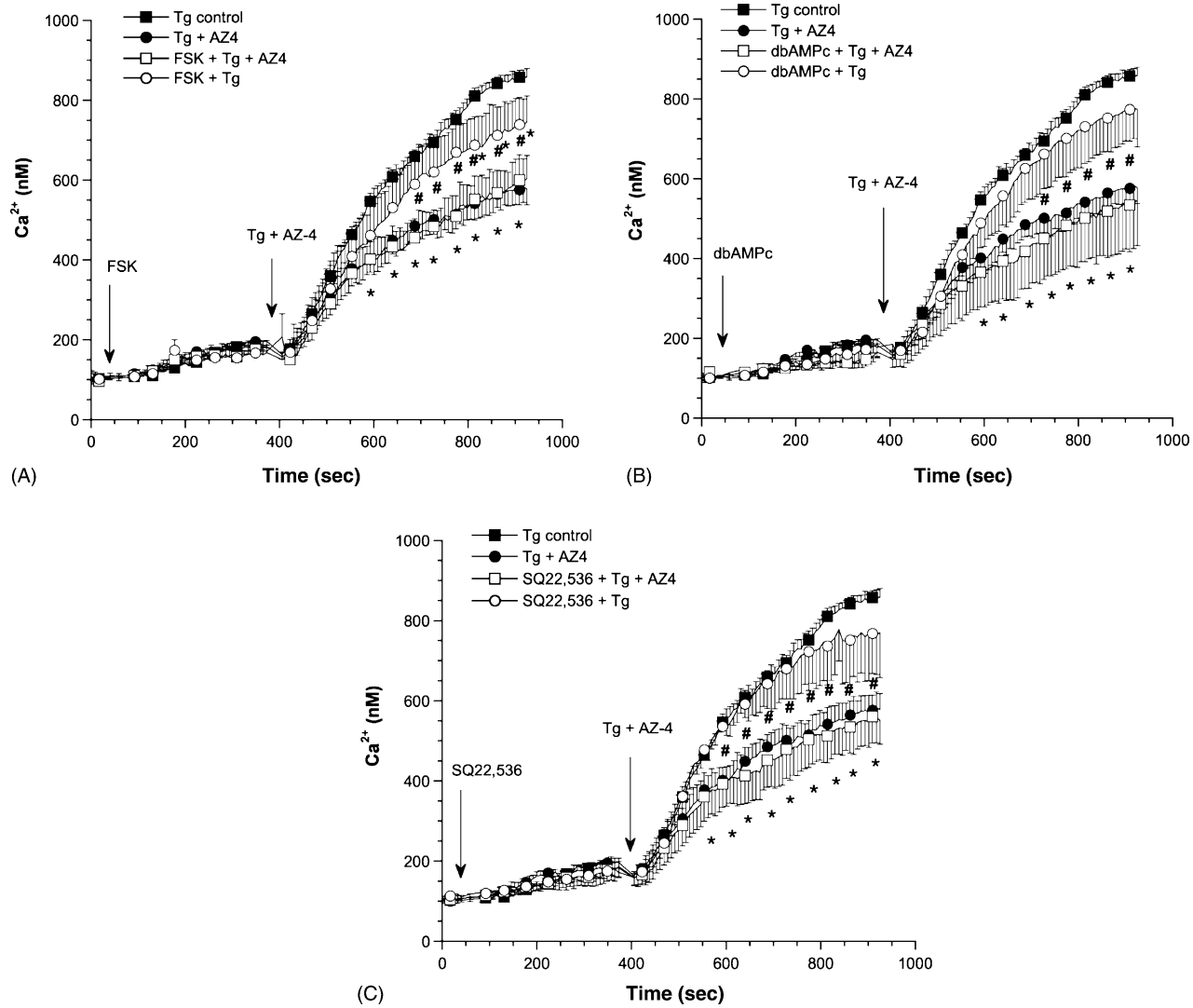


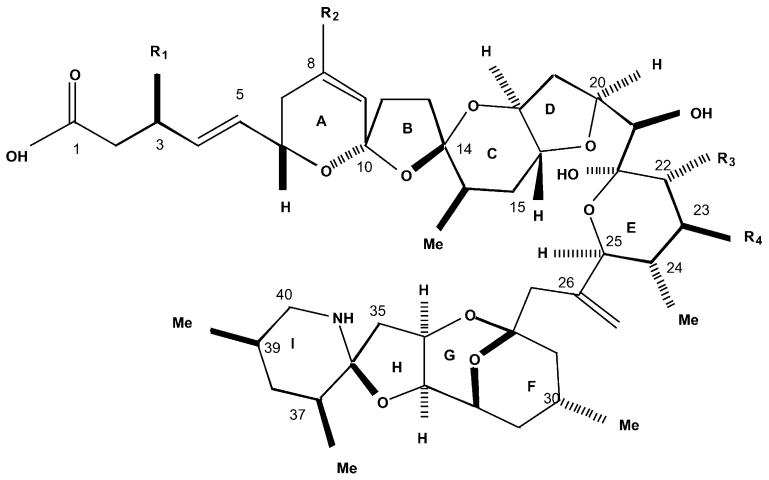
Fig. 7. Effect of previous up- and down-regulation of cAMP pathway on AZ-4 inhibition over Tg-induced Ca^{2+}-influx in human lymphocytes. Cells are bathed in a Ca^{2+} containing PSS. (A) The cells are pre-incubated with or without 30 μM FSK, and then 2 μM Tg plus 200 nM AZ-4 are added simultaneously. Asterisk (*) indicates significant differences with respect to Tg control. Hash (#) indicates significant differences with respect to FSK + Tg + AZ4. (B) The cells are pre-incubated with or without 250 μM dbcAMP, and then 2 μM Tg plus 200 nM AZ-4 are added simultaneously. Asterisk (*) indicates significant differences with respect to Tg control. Hash (#) indicates significant differences with respect to dbcAMP + Tg + AZ4. (C) The cells are pre-incubated with or without 10 μM SQ22,536 and then 2 μM Tg plus 200 nM AZ-4 are added simultaneously. Asterisk (*) indicates significant differences with respect to Tg control. Hash (#) indicates significant differences with respect to SQ22,536 + Tg + AZ4. Mean ± S.E.M. of approximately 315 cells observed in three experiments. To have a cleaner graphic, only one out of seven data were plotted. Error bars of all points are shown.

Table 1
Effects of different azaspiracids analogues added to human lymphocytes bathed by different solutions

Toxin	$[Ca^{2+}]_i$ significant variations of cytosolic Ca^{2+} levels (nM) over control induced by AZs in human lymphocytes				
	In a Ca^{2+}-free PSS		When 1 mM Ca^{2+} is restored to a Ca^{2+}-free PSS		In Ca^{2+} containing PSS + 2 μM Tg
	Effect (200 nM)	Maximal effective concentration (nM)	Effect (200 nM)	Maximal effective concentration (nM)	Effect (200 nM)
AZ-1	20.7 ± 10.5	1000	22.7 ± 10.2	1000	[a]
AZ-2	13.5 ± 2.6	200	66.2 ± 16	200	0
AZ-3	No effect		32 ± 3.8	1000	0
AZ-4	No effect		No effect		−368 ± 76.4
AZ-5	No effect		No effect		0

Each value is the result of subtracts cytosolic Ca^{2+} levels of azaspiracids-treated cells (200 nM) and cytosolic Ca^{2+} levels of control-cells. In each case concentrations between 50 and 1000 nM were checked. Each experiment represents the mean ± S.E.M. of approximately 320 cells observed in three different experiments.

[a] This condition was not checked.



		R_1	R_2	R_3	R_4	8-Me	14-Me	22-Me	24-Me	26=CH$_2$	30-Me	37-Me	39-Me
Azaspiracid	(AZA1, 1)	H	H	Me	H		41	42	43	44	45	46	47
8-methylazaspiracid	(AZA2, 2)	H	Me	Me	H	41	42	43	44	45	46	47	48
22-demethylazaspiracid	(AZA3, 3)	H	H	H	H		41		43	44	45	46	47
3-hydroxy-22-demethylazaspiracid	(AZA4, 4)	OH	H	H	H		41		43	44	45	46	47
23-hydroxy-22-demethylazaspiracid	(AZA5, 5)	H	H	H	OH		41		43	44	45	46	47

Fig. 8. Chemical structure of azaspiracids [5,6].

mately the same final values, regardless of the order of addition the inhibition reaches close to 75%. However, this high inhibition is not reached if Ni^{2+} and AZ-4 are added after the activation of Ca^{2+}-influx by Tg. The effect is bigger when the inhibitors are added at the same time, but never 75%. From these results, we can conclude that AZ-4 and Ni^{2+} are blocking the same channels. As with lanthanum and 2-aminoethoxydiphenyl borate, no further effect in Ca^{2+}-influx is observed when they are added together [42]. But also, since the effects are additive both drugs might be acting in two different channels. In addition, the lower effect observed when inhibitors are added after Tg can be a consequence of a sterical impediment. But also, AZ-4 and Ni^{2+} effects are higher when they are added before SOC channels are opened. Therefore, when Ca^{2+}-influx is activated the inhibition is lower, probably in these conditions the competition Ca^{2+} AZ-4–Ni^{2+} is higher as well as the sterical impediment. In any case, from these results we can conclude that AZ-4 inhibits SOC Ca^{2+}-influx and probably other Ca^{2+} channels, that is, AZ-4 shows a similar effect as Ni^{2+}.

On the other hand, the effect of AZ-4 on Ca^{2+}-influx through SOC activated channels is reversible. The most likely explanation for these results is that AZ-4 inhibits SOC channels by direct interaction with the channel pore, with another region of channel protein or with a closely associated regulatory protein. However, uncoupling stores and SOC channels [34], the inhibition of mitochondrial Ca^{2+} uptake [35–38] or some alteration in membrane potential [14,39] is hypothesis that cannot be excluded at the moment.

MTX is an interesting activator of Ca^{2+}-influx. In a recent study, the Ca^{2+}-influx induced by MTX in human lymphocytes has been characterized. This study indicates that SK&F96365, a voltage-independent Ca^{2+} channel antagonist, blocks the MTX-induced Ca^{2+} rise, and that nifedipine, a L-type Ca^{2+} channel blocker, has no effect [43]. Thus, it seems that non-voltage-gated channels related to classical L-type channels are not implied in MTX-elicited Ca^{2+} entry in lymphocytes [14]. We have shown that MTX does not modify Ca^{2+} from intracellular stores, which suggests that SOC channels are not involved in Ca^{2+}-influx induced by this toxin. This is in accordance with results obtained in liver cells where 2-aminoethyl diphenylborate, a known inhibitor of Ca^{2+}-release activated Ca^{2+} current, does not exert observable effect on MTX-initiated Ca^{2+}-influx [44,45]. Consequently, the partial inhibition that AZ-4 exerts over MTX-induced Ca^{2+}-influx seems to indicate that AZ-4 also acts through another type of Ca^{2+} channels, probably some no selective cation channel usually activated by MTX. AZ-4 is a potentially useful inhibitor of Ca^{2+} channels, SOC and non-SOC channels. However, further experiments on the molecular underpinnings may give clues to known the mechanism of Ca^{2+} channels inhibition by AZ-4.

In order to compare the effect of AZ-4 with the other azaspiracid analogues we summarize in Fig. 8 the structure of AZ analogues and its effects over $[Ca^{2+}]_i$ in human lymphocytes. Table 1 shows the effects of AZ-1, AZ-2, AZ-3, AZ-4 and AZ-5, in the most relevant conditions studied and the maximal effective toxin concentration in each case. AZ-1 and AZ-2 increase $[Ca^{2+}]_i$ by activation of

Ca^{2+}-release from internal stores (shown in the table as Ca^{2+} levels in a Ca^{2+}-free PSS), and Ca^{2+}-influx (shown in table as Ca^{2+} levels when Ca^{2+} is restored) [10,11]. AZ-3 induces only Ca^{2+}-influx (shown in the table as Ca^{2+} levels when Ca^{2+} is restored) [11]. AZ-4 does not induce Ca^{2+}-influx and inhibits Tg-induced Ca^{2+} rise. And finally, AZ-5 does not modify intracellular Ca^{2+} homeostasis. All these results clearly distinguish AZ-4 from other AZ analogues. AZ-3, with the simplest structure, induces Ca^{2+} entry. AZ-1 and AZ-2, with methyl groups in C22 or C8, respectively, elicit Ca^{2+}-release from internal stores, besides Ca^{2+}-influx. Moreover, AZ-4 that only differs from AZ-3 in a hydroxyl group located in C3, presents a new an interesting feature, which is the inhibition of capacitative Ca^{2+} entry induced by Tg. Also interesting is the lack of effect of AZ-5, characterized by a hydroxyl group in C23. Finally, and opposite to another AZ analogues, the inhibitory effect of AZ-4 over Tg-induced Ca^{2+}-influx is not dependent of cAMP pathway. Therefore, the size and the complexity of radicals are important to azaspiracids effect, since a difference between a methyl or a hydroxyl group means activation or inhibition of Ca^{2+}-influx. These results are in agreement with previously reported in vivo toxicity experiments [8] showing that AZ-4 and AZ-5 are much less toxic than AZ-1–3. AZ-4 and AZ-5 are the only toxins of this group that do not increase calcium-influx. We believe this is an indication that calcium-influx is functionally linked to the toxic effects of this group of compounds. In summary, the results obtained so far point to the interesting observation that the chemical series of AZs might have different targets, which provides a very complex profile and implications to its toxicology and pharmacology.

Acknowledgments

We thank Dr. Carlos Areal, Centro de Transfusión de Galicia, for the supply of human blood from donors. This work was funded with grants MCYT BMC2000-0441, SAF2003-08765-C03-02, REN2001-2959-C04-03, REN2003-06598-C02-01, AGL2004-08268-02-O2/ALI, INIA CAL01-068, Xunta PGIDT99INN26101, PGIDIT03AL26101PR and PGIDIT04TAL261005PR, FISS REMA-G03-007, and FOOD-CT-2004-514055.

References

[1] James K, Moroney C, Roden C, Satake M, Yasumoto T, Lehane M, et al. Ubiquitous "benign" alga emerges as the cause of shellfish contamination responsible for the human syndrome, azaspiracid poisoning. Toxicon 2003;41:145–51.

[2] Satake M, Ofuji K, Naoki H, James KJ, Furey A, McMahon T, et al. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, Mytilus edulis. J Am Chem Soc 1998;120(38):9967–8.

[3] Ofuji K, Satake M, McMahon T, Silke J, James KJ, Naoki H, et al. Two analogs of azaspiracid isolated from mussels, Mytilus edulis, involved in human intoxication in Ireland. Nat Toxins 1999;7:99–102.

[4] Ito E, Satake M, Ofuji K, Kurita N, McMahon T, James K, et al. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. Toxicon 2000;38:917–30.

[5] Nicolaou KC, Koftis TV, Vyskocil S, Petrovic G, Ling T, Yamada YMA, et al. Structural revision and total synthesis of azaspiracid-1. Part 2. Definition of the ABCD domain and total. Angew Chem Int Ed 2004;43(33):4318–24.

[6] Nicolaou KC, Vyskocil S, Koftis TV, Yamada YMA, Ling T, Chen DYK, et al. Structural revision and total synthesis of azaspiracid-1. Part 1. Intelligence gathering and tentative proposal. Angew Chem Int Ed 2004;43(33):4312–8.

[7] James K, Lehane M, Moroney C, Fernandez-Puente P, Satake M, Yasumoto T, et al. Azaspiracid shellfish poisoning: unusual toxin dynamics in shellfish and the increased risk of acute human intoxications. Food Addit Contam 2002;19(6):555–61.

[8] Ofuji K, Satake M, McMahon T, James KJ, Naoki H, Oshima Y, et al. Structures of azaspiracid analogs, azaspiracid-4 and azaspiracid-5, causative toxins of azaspiracid poisoning in Europe. Biosci Biotech Biochem 2001;65(3):740–2.

[9] James KJ, Sierra MD, Lehane M, Brana Magdalena A, Furey A. Detection of five new hydroxyl analogues of azaspiracids in shellfish using multiple tandem mass spectrometry. Toxicon 2003;41(3):277–83.

[10] Román Y, Alfonso A, Louzao MC, de la Rosa LA, Leira F, Vieites JM, et al. Azaspiracid-1, a potent, nonapoptotic new phycotoxin with several cell targets. Cell Signal 2002;14:703–16.

[11] Román Y, Alfonso A, Vieytes MR, Ofuji K, Satake M, Yasumoto T, et al. Effects of azaspiracids 2 and 3 on intracellular cAMP, [Ca^{2+}], and pH. Chem Res Toxicol 2004;17:1338–49.

[12] Barrit GJ. Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. Biochem J 1999;37:153–69.

[13] Putney JWJ, Broad LM, Braun FJ, Lievremont JP, Bird GS. Mechanisms of capacitative calcium entry. J Cell Sci 2001;114(Pt 12):2223–9.

[14] Grafton G, Thwaite L. Calcium channels in lymphocytes. Immunology 2001;104(2):119–26.

[15] Schwarzmann N, Kunerth S, Weber K, Mayr GW, Guse AH. Knockdown of the type 3 ryanodine receptor impairs sustained Ca^{2+} signaling via the T cell receptor/CD3 complex. J Biol Chem 2002;277(52):50636–42.

[16] Hosoi E, Nishizaki C, Gallagher KL, Wyre HW, Matsuo Y, Sei Y. Expression of the ryanodine receptor isoforms in immune cells. J Immunol 2001;167(9):4887–94.

[17] Guse AH, Tsygankov AY, Weber K, Mayr GW. Transient tyrosine phosphorylation of human ryanodine receptor upon T cell stimulation. J Biol Chem 2001;276(37):34722–7.

[18] Berg I, Potter BV, Mayr GW, Guse AH. Nicotinic acid adenine dinucleotide phosphate (NAADP(+)) is an essential regulator of T-lymphocyte Ca(2+)-signaling. J Cell Biol 2000;150(3):581–8.

[19] Vazquez G, Wedel BJ, Bird GS, Joseph SK, Putney JW. An inositol 1,4,5-trisphosphate receptor-dependent cation entry pathway in DT40 B lymphocytes. EMBO J 2002;21(17):4531–8.

[20] Kotturi MF, Carlow DA, Lee JC, Ziltener HJ, Jefferies WA. Identification and functional characterization of voltage-dependent calcium channels in T lymphocytes. J Biol Chem 2003;278(47):46949–60.

[21] Savignac M, Badou A, Moreau M, Leclerc C, Guèry J, Paulet P, et al. Protein kinase C-mediated calcium entry dependent upon dihydropyridine sensitive channels: a T cell receptor-coupled signaling pathway involved in IL-4 synthesis. FASEB J 2001;15(9):1577–9.

[22] Taylor CW, Broad LM. Pharmacological analysis of intracellular Ca^{2+} signalling: problems and pitfalls. TIPS 1998;19:370–5.
[23] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{++} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[24] Thastrup O, Cullen P, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum ATPase. Proc Natl Acad Sci USA 1990;87:2466–70.
[25] Parekh AB, Penner R. Store depletion and calcium influx. Physiol Rev 1997;77(4):901–30.
[26] Daly JW, Harper J. Loperamide: novel effects on capacitative calcium influx. Cell Mol Life Sci 2000;57:149–57.
[27] Olivi L, Bressler J. Maitotoxin stimulates Cd influx in Madin–Darby kidney cells by activating Ca^{2+}-permeable cation channels. Cell Calcium 2000;27(4):187–93.
[28] Martínez-François JR, Morales-Tlalpan V, Vaca L. Characterization of the maitotoxin-activated cationic current from human skin fibroblasts. J Physiol 2002;538:79–86.
[29] Morales_Tlalpan V, Vaca L. Modulation of the maitotoxin response by intracellular and extracellular cations. Toxicon 2002;40(5):493–500.
[30] Nakahata N, Yaginuma T, Ohizumi Y. Maitotoin-induced phosphoinositide hydrolysis is dependent on extracellular but not intracellular Ca^{2+} in human astrocytoma cells. Jpn J Pharmacol 1999;81(2):240–3.
[31] Gregory RB, Sykiotis D, Barritt GJ. Evidence that store-operated Ca(2+) channels are more effective than intracellular messenger-activated non-selective cation channels in refilling rat hepatocyte intracellular Ca(2+) stores. Cell Calcium 2003;34(3):241–51.
[32] Hanoune J, Defer N. Regulation and role of adenylyl cyclase isoforms. Annu Rev Pharmacol Toxicol 2001;41:145–74.
[33] Prakriya M, Lewis RS. Potentiation and inhibition of Ca(2+) release-activated Ca(2+) channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP(3) receptors. J Physiol 2001;536(Pt 1):3–19.
[34] Mason MJ, Mayer B, Hymel LJ. Inhibition of Ca^{2+} transport pathways in thymic lymphocytes by econazole, miconazole, and SKF 96365. Am J Physiol 1993;264(3 Pt 1):C654–62.
[35] Hoth M, Fanger CM, Lewis RS. Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol 1997;137(3):633–48.
[36] Hoth M, Button DC, Lewis RS. Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes. Proc Natl Acad Sci USA 2000;97(19):10607–12.
[37] Lewis RS. Calcium signaling mechanisms in T lymphocytes. Annu Rev Immunol 2001;19:497–521.
[38] Glitsch MD, Bakowski D, Parekh AB. Store-operated Ca(2+) entry depends on mitochondrial Ca(2+) uptake. EMBO J 2002;21(24):6744–54.
[39] Fanger CM, Rauer H, Neben AL, Miller MJ, Rauer H, Wulff H, et al. Calcium activated potassium channels sustain calcium signaling in T lymphocytes. J Biol Chem 2001;276(15):12249–56.
[40] Killoran PL, Walleczek J. Inhibition of store-operated calcium entry in human lymphocytes by radiation: protection by glutathione. Radiat Res 1999;152(6):611–21.
[41] Alfonso A, De la Rosa LA, Vieytes MR, Botana LM. Dimethylsphingosine increases cytosolic calcium and intracellular pH in human T lymphocytes. Biochem Pharmacol 2003;65(3):465–78.
[42] Dobrydneva Y, Blackmore P. 2-Aminoethoxydiphenyl borate directly inhibits store-operated calcium entry channels in human platelets. Mol Pharmacol 2001;60(3):541–52.
[43] de la Rosa LA, Alfonso A, Vilarino N, Vieytes MR, Yasumoto T, Botana LM. Maitotoxin-induced calcium entry in human lymphocytes: modulation by yessotoxin, Ca(2+) channel blockers and kinases. Cell Signal 2001;13(10):711–6.
[44] Gregory RB, Rychkov G, Barritt GJ. Evidence that 2-aminoethyl diphenylborate is a novel inhibitor of store-operated Ca^{2+} channels in liver cells, and acts through a mechanism which does not involve inositol trisphosphate receptors. Biochem J 2001;354(Pt 2):285–90.
[45] Gregory RB, Barritt GJ. Evidence that Ca^{2+}-release-activated Ca^{2+} channels in rat hepatocytes are required for the maintenance of hormone-induced Ca^{2+} oscillations. Biochem J 2003;370:695–702.