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Azaspiracid-1 inhibits bioelectrical activity of spinal cord neuronal networks

Nadezhda V. Kulagina [a,*], Michael J. Twiner [b], Philipp Hess [c], Terry McMahon [c], Masayuki Satake [d], Takeshi Yasumoto [e], John S. Ramsdell [b], Gregory J. Doucette [b], Wu Ma [a], Thomas J. O'Shaughnessy [a]

[a] *Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, 4555 Overlook Avenue, SW, Code 6900, Washington, DC 20375, USA*

[b] *Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, NOAA/National Ocean Service, 219 Fort Johnson Road, Charleston, SC 29412, USA*

[c] *Biotoxin Chemistry, Marine Environment and Food Safety Services, Marine Institute, Galway Technology Park, Parkmore West, Galway, Ireland*

[d] *Graduate School of Agricultural Science, Tohoku University, Sendai, Japan*

[e] *Japan Food Research Laboratory, Tama Laboratory, Nagayama, Tama, Tokyo, Japan*

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Abstract

Azaspiracid-1 (AZA-1) is a recently identified phycotoxin that accumulates in molluscs and can cause severe human intoxications. For this study, we utilized murine spinal cord and frontal cortex neuronal networks grown over 64 channel microelectrode arrays (MEAs) to gain insights into the mechanism of action of AZA-1 on neuronal cells. Extracellular recordings of spontaneous action potentials were performed by monitoring mean spike rate as an assay of the efficacy of AZA-1 to alter the bioelectrical activity of neurons in the networks. Via slow onset, AZA-1 decreased the mean spike rate of the spinal cord neurons with an IC_{50} of ca. 2.1 nM, followed by partial recovery of original activity when toxin was removed. Pre-treatment with the $GABA_A$ receptor antagonist bicuculline led to an increased response of the neuronal networks to AZA-1 exposure and resulted in an irreversible inhibition of spike rate. AZA-1 did not cause any changes in frontal cortex networks upon drug exposure. In addition, whole-cell patch clamp recordings from spinal cord neurons showed that AZA-1 had no significant effect on the voltage-gated sodium (Na^+) or calcium (Ca^{2+}) currents, suggesting that the toxin affected synaptic transmission in the neuronal networks through a mechanism independent of these voltage-gated channels.

Keywords: Biosensor; Azaspiracid-1; Extracellular recording; Phycotoxin; Microelectrode array; Primary neuronal cultures; Voltage-gated channels; Action potential

1. Introduction

In 1995, a novel marine phycotoxin, azaspiracid (AZA), was identified following cases of human intoxication in the Netherlands resulting from the consumption of mussels cultivated in Killary Harbour, Ireland (McMahon and Silke, 1996). The illness was subsequently termed azaspiracid shellfish poisoning (AZP). Since then cases of AZA intoxication and/or contamination of shellfish have been documented in several other European countries including UK, Norway, France, Spain and Italy (Satake et al., 1998; James et al., 2002; Magdalena et al., 2003).

* Corresponding author. Fax: +1 202 767 9594.
E-mail address: kulagina@cbmse.nrl.navy.mil (N.V. Kulagina).

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AZA-1, the most abundant of the 11 identified congeners (James et al., 2003), has been found in multiple shellfish species such as mussels, oysters, scallops, clams, cockles, and razor fish (Hess et al., 2001, 2003; Furey et al., 2003).

Human symptoms of AZA poisoning are gastrointestinal with early onset of nausea, vomiting, diarrhea, and stomach cramps (McMahon and Silke, 1996, 1998). Symptoms closely resemble those of diarrhetic shellfish poisoning caused by okadaic acid and dinophysistoxins, which have been documented to co-occur with AZAs in shellfish (Hess et al., 2001). Mice that were chronically administered oral AZA-1 (5–50 μg/kg) experienced weight loss and extreme fatigue, while subsequent histological analysis revealed a diverse range of abnormalities including accumulation of fluid, necrosis and edema in the lamina propria of the midintestinal tract and fused, shortened villi. Necrosis of T and B lymphocytes was also documented in the spleen and thymus, as well as fatty changes in the liver, hyperplasia of the epithelial lining in the stomach, and tumors in the lungs (Ito et al., 2000, 2002). Intraperitoneal injection of crude extracts of AZA-1 in mice revealed that many of the symptoms were similar to those of the paralytic shellfish poisoning (PSP) toxins, suggesting that AZA-1 could be a neurotoxin (McMahon and Silke, 1996). Early and progressive onset of paralysis, fatigue, labored breathing, and subsequent death were observed as soon as 35 min following injection (McMahon and Silke, 1998). Although it was observed in the mouse bioassay that AZA-1 caused neurotoxic symptoms, no histological results on the brain tissues have been reported.

Although a variety of effects have been demonstrated in organisms exposed to AZA-1, the mechanism of action of this highly potent phycotoxin remains to be determined. AZA-1 toxicity and mode of action have been studied on several types of cells. These studies have demonstrated that AZA-1 is cytotoxic in the low nanomolar range (Flanagan et al., 2001; Twiner et al., 2005) and induces elevations in cytosolic calcium and cAMP in human T lymphocyte cells (Roman et al., 2002). Roman et al. (2002) has also shown that AZA-1 caused a reduction in cellular F-actin content of excitable neuroblastoma cells, and similarly, Twiner et al. (2005) has shown a reorganization of F-actin concurrent with distinct morphological changes in human T lymphocyte cells following exposure to AZA-1.

Neuroblastoma cells are the only neuron-relevant cells that have been tested for AZA-1 effect. The primary objective of this study was to investigate pharmacological effects of AZA-1 on spinal cord neuronal networks grown on microelectrode arrays (MEAs). Cultured neuronal networks are in vitro systems with pharmacological behavior resembling paternal tissue (Gross et al., 1997). The networks demonstrate high sensitivity to various environmental contaminants including the phycotoxins brevetoxin and saxitoxin (Kulagina et al., 2004, 2006) and show overall good correlation with in vivo experiments (Shaffer et al., 2004). Extracellular recordings, such as those employed in this study, are used for monitoring bioelectrical activity as represented by action potentials. These recordings are non-invasive and have the potential for long-term measurements. Neuronal networks, in combination with a portable recording system developed by the Naval Research Laboratory (NRL), allow for off-site testing (O'Shaughnessy et al., 2004; Kulagina et al., 2006), in addition to laboratory-based experiments such as that employed for this study. Our data demonstrate that AZA-1 inhibits bioelectrical activity of spinal cord neuronal networks at low nanomolar concentrations without the involvement of voltage-gated sodium and/or calcium channels. The possible involvement of the GABA neurotransmitter system in AZA-1 toxicity in spinal cord neuronal networks is discussed.

2. Materials and methods

2.1. AZA toxin

AZA-1 was extracted from 2 kg of mussels (*Mytilus edulis*) that were collected in 1996 from Killary Harbour, on the west coast of Ireland and in 1999 from Bantry Bay, on the southwest coast of Ireland. Toxins were extracted, with slight modifications, as previously described (Satake et al., 1998b; Ofuji et al., 1999). Stock AZA-1 was determined to be $>93\%$ pure by NMR and showed $<1\%$ impurity of other AZA subtypes/congeners by liquid chromatography–mass spectrometry (LC–MS). The secondary stock solution of AZA-1 (19 mM) contained 10% aqueous methanol. No effect of methanol was detectable with the neuronal networks at the concentrations used in this work.

2.2. MEAs, cell culture and recording

Cultured spinal cord and frontal cortex networks were purchased from Applied Neuronal Network Dynamics, Inc. (ANND, Dallas, TX). Techniques used to fabricate and prepare cultures over MEAs with 64 recording sites (10 μm in diameter) have been described previously (Gross et al., 1985). Cells were dissociated from either the spinal cord or frontal cortex tissue of embryonic Hsd:ICRb mice. The neuronal networks were grown from neuron/glia cell co-culture and maintained at 37 °C in Dulbecco's minimum essential medium in a 10% CO_2 incubator until use. Upon request, networks were loaded into stainless steel recording chambers and shipped by overnight commercial service from ANND to either the NRL (Washington, DC) or NOAA (Charleston, SC) laboratories where the experiments were performed. Arrived neuronal networks were ca. 30–50 days old and were used within 1 week of receipt.

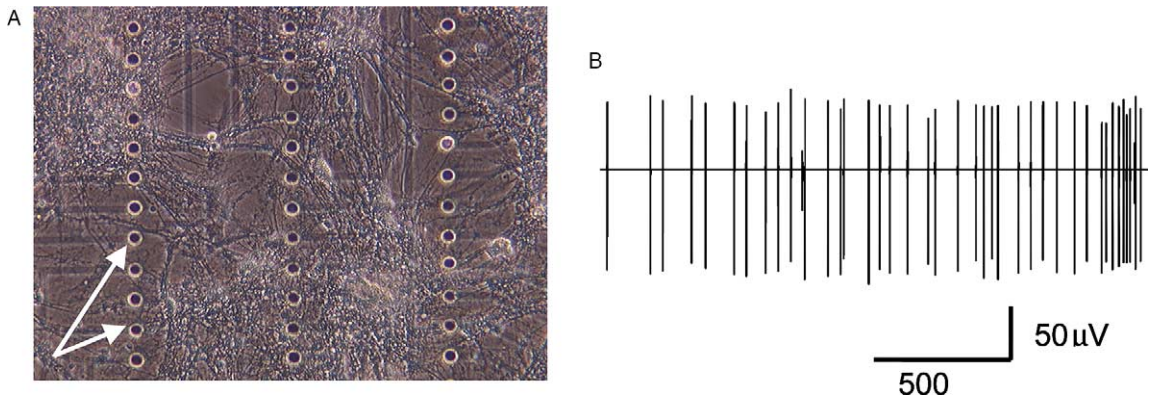

Fig. 1. (A). Phase contrast image of spinal cord neuronal network (ca. 30 days old) cultured over a MEA substrate. Arrows show microelectrode contacts that are ca. 10 μm in diameter. Neurons are phase dark, while glial cells appear bright. (B). Representative extracellular recording of bioelectrical activity represented by spikes on one active channel from a typical spinal cord network using the MEA approach.

Fig. 1A shows a portion of typical spinal cord neuronal network ca. 30 days old on a MEA. In addition, 35 mm plastic dishes prepared via the same protocol as those used for MEA experiments were plated with spinal cord neurons purchased from CellEnSys Biotechnology, LLC (Germantown, MD) for the whole-cell patch clamp experiments.

Electrophysiologic measurements were performed using the portable cell-based recording system with on-line monitoring of neuronal extracellular potentials (Pancrazio et al., 2003). Extracellular recordings were performed in a solution (HEPES-MEM) consisting of minimum essential medium (MEM) supplemented with 25 mM glucose, 40 mM *N*-[2-hydroxyethyl]-piperazine-*N*-[2-ethanesulfonic acid] (HEPES) and 26 mM $NaHCO_3$ at pH 7.4. The recording chamber temperature was maintained at 37 °C. A total recirculating HEPES-MEM volume of 20 ml was perfused across the networks at a flow rate of 1 ml/min. Before any toxin introduction, control HEPES-MEM perfusion was performed for at least 30–40 min until a stable baseline of bioelectrical activity was established for each network. Stock solution of AZA-1 (19 mM) in 10% methanol (vehicle control experiments) was then added to the medium reservoir in a concentration-increasing manner. Bicuculline was added to the reservoir as a single 5 μM concentration prior to AZA-1 addition. The networks were perfused with the chosen treatment solution until a new stable baseline for the mean spike rate was established (usually requiring at least 40 min). The networks were perfused with fresh HEPES-MEM during the wash phase.

2.3. Neuronal network metrics

Spike activity from neuronal networks was quantified using the mean spike rate. The sampling rate and threshold for spike detection were 40 kHz and 40 μV, respectively. Mean spike rate across all active channels was calculated with 1 min sampling intervals by first computing the spike rate per channel. Some data are reported as a normalized mean spike rate. In this case, spike rates of all active channels were normalized independently with respect to a control baseline before the mean spike rate was calculated.

2.4. Whole-cell patch clamp recordings

Whole-cell patch clamp experiments were conducted on spinal cord neuronal cells grown on 35 mm dishes. Whole-cell patch clamp methods were employed as described by Hamill et al. (1981). Patch pipettes were pulled with a Narishige PP-83 two-stage puller and heat-polished with a CPM-2 microforge (Adams List Associates, Westbury, NY) to a resistance of 5–10 MΩ when filled with the pipette solution. After achieving the cell-attached patch clamp configuration, whole-cell recording was attempted only when the seal resistance exceeded 10 GΩ. An Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) coupled with pClamp (v9; Axon Instruments) data acquisition software was used for recordings. Signals were filtered at 5 kHz with the patch amplifier's built-in four pole, Bessel low-pass filter and digitized at 10 kHz. Ohmic leak currents were subtracted using the P/N4 leak subtraction of pClamp. The patch electrode solution contained: 130 mM tetraethylammonium chloride, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, 5 mM MgATP at pH 7.4. The extracellular bathing solution was the same HEPES-MEM used in the extracellular recording experiments (see above). These solutions effectively blocked potassium currents allowing for the measurement of sodium and calcium currents. Sodium currents were measured from the peak of the rapidly inactivating inward current at the beginning of a trace and were completely abolished by 1 μM tetrodotoxin (TTX). For calcium current measurements, 1 μM TTX was added to the bath to inhibit sodium currents and calcium currents were measured as the average inward current from

30 to 50 ms after the step potential. While no pharmacologic assessment was done on these currents, they showed the typical IV relationship expected for calcium currents in neurons (Fig. 5B). All patch clamp experiments were conducted at room temperature. As the calcium current experiment compared the average currents between populations of cells, control and treatment, current densities were used to normalize for differences in cell size. Current densities were computed by dividing the current (pA) by the measured membrane capacitance (pF) which is proportional to cell size.

2.5. Data analysis and statistics

Concentration-dependent effects of the AZA-1 on mean spike rate are presented as a percent of control baseline signal. Spike rate values were averaged across the number of networks ('n') and channels ('m') as specified. For results with multiple points, an $n \geq x$ indicates that not all data points were taken for each network and the minimum number of networks included for any point was at least 'x'. IC_{50} (concentration giving 50% of the maximum inhibition) was calculated by a curve fit (Sigma Plot version 8.0; SPSS Inc., Chicago, IL) to a three-parameter logistic function $A/Y = (1 + X/X_0)^b$ where 'A' is the baseline signal, Y is the signal following the treatment, X is the concentration of toxin used, X_0 is the IC_{50}, and 'b' is the Hill coefficient. Calculation of the AZA-1 detection limit was based on statistically significant changes in the mean spike rate. Significance of the changes ($P < 0.05$) was identified by t-test (Sigma Plot, version 8.0; SPSS Inc., Chicago, IL).

3. Results

3.1. Extracellular recordings from cultured spinal cord neuronal networks

The spinal cord neuronal networks exhibited spike activity with amplitudes ranging from 70 to 650 μV and spike rates averaging 65 ± 22 Hz (mean ± SD, $m = 19$ channels). The change in mean spike rate during ca. 4 h of control HEPES-MEM perfusion was statistically insignificant as compared with the original baseline. Fig. 1B shows typical spontaneous spike activity for a single channel recorded from a spinal cord network.

Fig. 2A shows the concentration-dependent effect of AZA-1 on mean spike rate from a single experiment. Exposure to AZA-1 caused inhibition of bioelectrical activity represented by spike rate as concentrations increased between 0.2 and 10 nM. Inhibition of bioelectrical activity induced by AZA-1 resulted in gradual decline and usually at least 30–40 min (longest time was 90 min) was required to establish a new baseline after the introduction of additional concentration of AZA-1. Fig. 2 (inset) shows a partial trace of the response of a different network to the introduction of small concentrations of AZA-1, which demonstrates some degree of variability between the networks. The effect of AZA-1 was only partially reversible

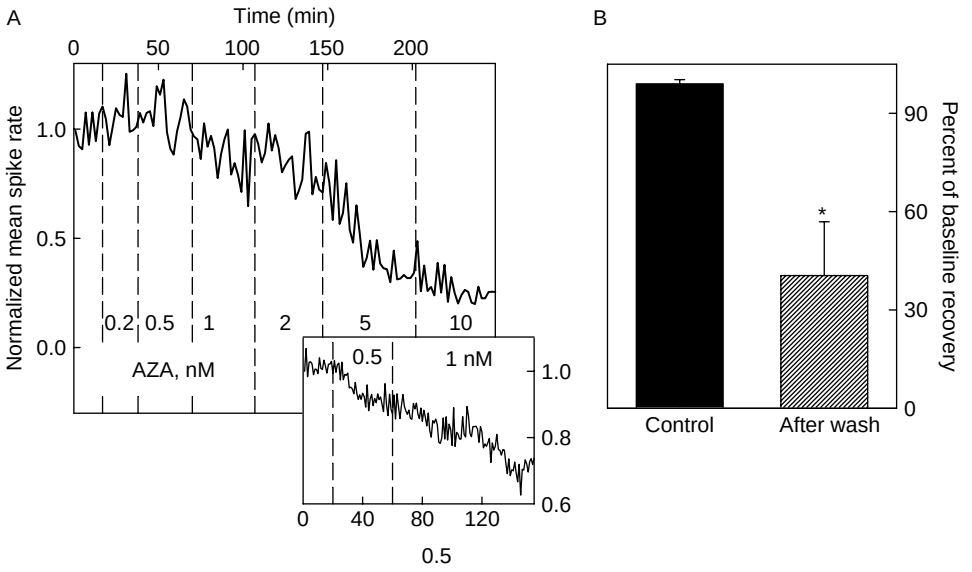

Fig. 2. (A). Representative recording of mean spike rate after the introduction of increasing concentrations of AZA-1; inset shows partial trace of a second network responding to introductions of small concentrations of AZA-1. (B). Baseline recovery (mean ± SD, $n = 4$) after exposure to 10 nM AZA-1 (highest concentration used in this study). The wash was performed by perfusion of HEPES-MEM recording medium without recirculation for at least 4 h (*$P < 0.01$).

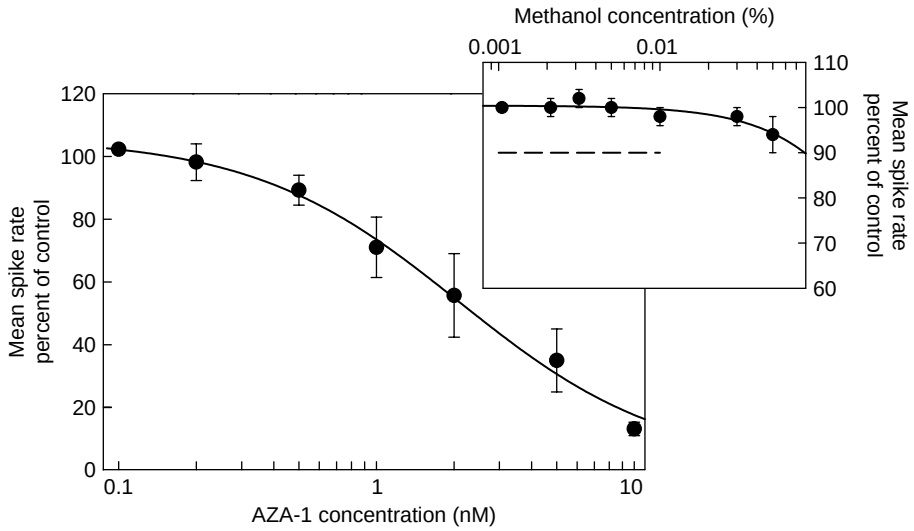

Fig. 3. Concentration-dependent effect of AZA-1 on spinal cord neuronal network mean spike rate (mean ± SD, $n \geq 3$). Inset: Control concentration-dependent effect of methanol (AZA-1 vehicle) on spinal cord network spike rates (mean ± SD, $n = 3$). Dashed horizontal line shows concentration range of methanol used with AZA-1 in this study. Curve fitting was to three-parameter logistic function.

after washing the network with fresh HEPES-MEM for at least 2 h. Recovery corresponded to 40 ± 32% (mean ± SD, $n = 4$) relative to the original baseline prior to AZA-1 introduction (Fig. 2B).

Fig. 3 shows the concentration–response curve compiled from four networks for the effect of AZA-1 on mean spike rate. The maximum concentration used was 10 nM AZA-1. Although this concentration did not abolish neuronal activity completely (ca. 85%), higher concentrations were not used due to limited AZA-1 availability. Fitting to a three-parameter logistic function curve yielded an $IC_{50} = 2.1 \pm 0.5$ nM (mean ± SD, $n \geq 4$). Based on statistically significant changes from the original baseline, the detection limit for AZA-1 in spinal cord neuronal networks was calculated to be 0.5 nM. Fig. 3 (inset) shows data from control experiments with methanol, which served as the vehicle for AZA-1. Although methanol had some effect on neuronal networks at higher concentrations, it did not cause any significant change in mean spike rate at the concentrations used for the administration of AZA-1 (see dashed line on Fig. 3 inset). The highest concentration of methanol in recording solutions corresponded to 0.01%.

In this work, we also exposed frontal cortex neuronal networks to the same concentration range of AZA-1 (up to 10 nM, $n = 2$ networks) as was used for spinal cord networks. Interestingly, AZA-1 did not cause any significant changes in frontal cortex network baseline activity, which remained completely flat throughout the introduction of the toxin (data not shown).

Fig. 4 shows the effect of AZA-1 on networks pre-treated with 5 μM bicuculline, a $GABA_A$ receptor antagonist. Inhibition of the GABA neurotransmitter system by this receptor antagonist enhanced neuronal activity by ca. 40% as demonstrated by the increase in mean spike rate (Fig. 4A). Subsequent addition of 2 nM AZA-1 (corresponding to the previously calculated IC_{50} value) in the presence of bicuculline induced almost complete inhibition of spike activity to 6.5 ± 3.5% (mean ± SD, $n = 2$ networks, $m = 9$ channels). It is important to note that in the bicuculline pre-treatment experiments (mean ± SD) were calculated for 'm' number of channels (from $n = 2$ networks). However, the introduction of 2 nM AZA-1 in the absence of bicuculline irreversibly inhibited bioelectrical activity of spinal cord networks to only 54 ± 13% (mean ± SD, $n = 4$) of the original baseline (Fig. 4B). The difference between the effects in the presence and absence of bicuculline was statistically significant ($P < 0.01$). It should be noted that when present by itself, the bicuculline effect was completely reversible upon removal of the chemical. Treatment of networks with 5 μM bicuculline after exposure to 10 nM AZA-1 did not cause any significant changes in neuronal activity (data not shown).

3.2. Whole-cell patch clamp recordings on spinal cord neuronal cells

Fig. 5 shows the effect of AZA-1 on voltage-gated sodium and calcium currents. Evoked sodium currents were measured in two neurons before and 4 min after the addition of 10 nM AZA-1. No statistically significant changes were noted in the sodium currents evoked by 75 ms step depolarization from a resting potential of −70 mV to potentials ranging from −60 to 60 mV; moreover, results

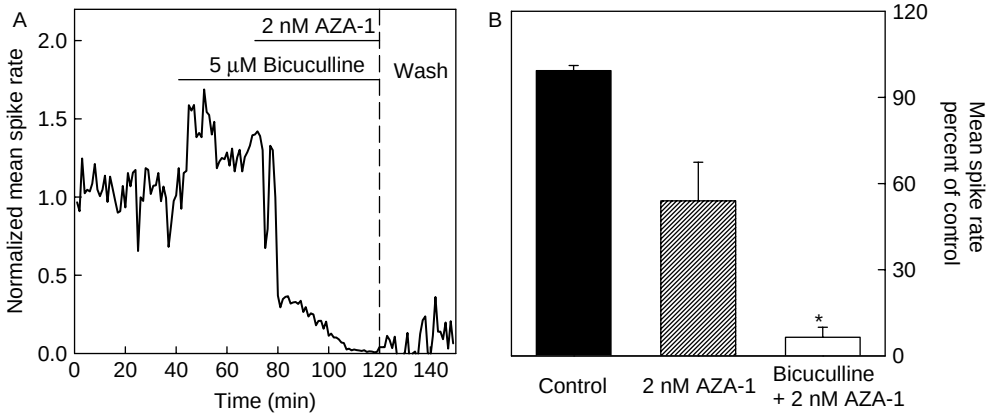

Fig. 4. Effect of pre-treatment with the $GABA_A$ receptor antagonist bicuculline on network sensitivity to AZA-1 exposure. (A). Representative recording of mean spike rate after the introduction of bicuculline (5 μM) and AZA-1 (2 nM). Solid horizontal lines represent exposure periods for each treatment. (B). Inhibition of mean spike rate due to exposure to AZA-1 alone versus AZA-1 exposure following bicuculline pre-treatment (mean ± SD, $n=3$ (control), $n=4$ (AZA-1 treatment); mean ± SD, $m=9$ (bicuculline pre-treatment)). It is important to note that in the bicuculine pre-treatment experiments (mean ± SD) were calculated for 'm' number of channels (from $n=2$ networks). The difference between the effects in the presence and absence of bicuculline was significant (*$P<0.01$).

were similar to three control neurons where only the methanol carrier (0.01%) was added to the bath solution (Fig. 5A). Because of significant calcium current rundown in the neurons, the effect of AZA-1 on voltage-gated calcium currents was determined by comparing the mean peak calcium current densities between eight cells treated with 10 nM AZA-1 to eight cells treated only with the methanol carrier. In this case, currents were evoked from a holding potential of −80 mV to potentials ranging from −70 to 60 mV. The results were a maximum evoked mean current density of −20.4 ± 4.6 pA/pF (mean ± SD, $n=8$ cells) for control versus −15.7 ± 6.7 pA/pF (mean ± SD, $n=8$ cells) for AZA-1 treated cells (Fig. 5B). There was no significant difference ($P=0.127$) as determined by Student's t-test.

4. Discussion

In this study, we used spinal cord and frontal cortex neuronal networks cultured over MEAs for pharmacological evaluation of AZA-1. This recently identified phycotoxin

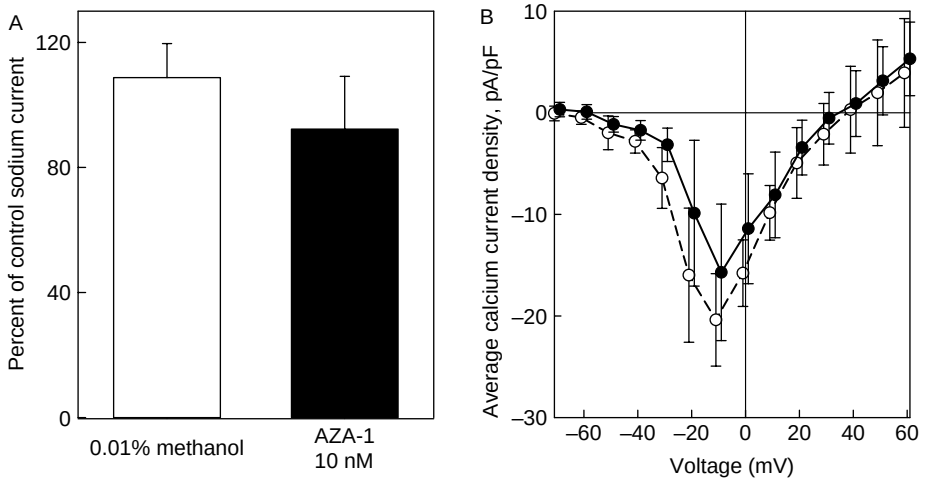

Fig. 5. Effect of AZA-1 on voltage-gated sodium (A) and calcium (B) currents in murine spinal cord neurons. (A) There were no significant changes in sodium current (mean ± SD) 4 min after the addition of either 0.01% methanol (vehicle) ($n=3$) or 10 nM AZA-1 ($n=2$). (B). No significant change in calcium current densities (mean ± SD) was found when comparing results from eight control (○, dashed line) neurons treated with 0.01% methanol (vehicle) and eight neurons exposed to 10 nM AZA-1(●, solid line).

has caused severe cases of human intoxication following consumption of contaminated seafood and induced neurological symptoms in mice (McMahon and Silke, 1996). Although it is known that AZA-1 affects different cell lines including neuroblastoma cells (Flanagan et al., 2001; Roman et al., 2002; Twiner et al., 2005), this is the first report that demonstrates that AZA-1 affects neuronal cells prepared from primary cultures.

Neuronal networks resemble paternal tissue, and are extremely sensitive to various environmental contaminants. Exposure to AZA-1 induced a slow inhibition of bioelectrical spike activity at low nanomolar concentrations. The effective concentration range established in this study has been shown to cause cytotoxic and cytoskeletal changes in different mammalian cell lines as well as teratogenic effects and growth impairment in fish embryos (Twiner et al., 2005; Colman et al., 2005). Although AZA-1 produced inhibition of neuronal activity, its effect is different from that observed with saxitoxin and brevetoxin (Kulagina et al., 2006). The effects of these two phycotoxins, both of which are voltage-gated sodium channel modulators, occurred on a shorter time scale and were completely reversible. This is not the case upon removal of AZA-1, where mean spike rate only partially recovered after several hours of washing with fresh perfusion medium. Several factors such as strong binding affinity of AZA-1 to the site of action, activation of a second messenger cascade (G protein-coupled receptors) that opens ion channel via intermediaries, or cell death could contribute to the slow decline and partial recovery of the baseline. All attempts to quantify neuron viability in the networks in the presence of AZA-1 were inconclusive due to the heavy population of glia cells present in co-culture. On the other hand, when the effect of AZA-1 (10 nM) was studied on mouse neural precursor cells, at least 24 h exposure to the toxin was required to demonstrate a statistically significant change in cell viability from the control (unpublished observation). This idea is in agreement with the observations by Twiner et al. (2005) where it was demonstrated for seven cell types that unusually long time (>24 h) of AZA-1 exposure was required for complete cytotoxicity and necrotic cell lysis.

The effect of AZA-1 appears to be tissue specific, as frontal cortex neuronal networks were not affected by AZA-1 at concentrations up to 10 nM. This is in contrast to saxitoxin and brevetoxin, which affect both types of neuronal networks, although some tissue-specific differences have been observed (Kulagina et al., 2004). The tissue- specific effect of AZA-1 suggests that the toxin acts on a system that is present or dominates in only one of the two cell culture types and complements the idea that voltage-gated sodium channels can be ruled out as the mechanism of AZA-1 action due to their presence in both tissue types used herein.

The patch clamp data supports the idea that the action of AZA-1 on the neuronal network activity was not mediated by voltage-gated sodium channels. While no statistically significant change in calcium currents was noted, involvement of voltage-gated calcium channels cannot be completely ruled out as the scarcity of AZA-1 allowed for only a small number of cells to be tested, which would preclude detection of subtle changes in calcium currents.

A synergistic effect between the AZA-1 and $GABA_A$ receptor antagonist bicuculline was observed in this study. Our data show that blocking the $GABA_A$ neurotransmitter system with bicuculline prior to AZA-1 application not only altered the concentration–response characteristics of AZA-1, but also resulted in complete irreversibility of AZA-1 action. These results may be indicative for the mode of action of AZA-1. It is possible that the effect of AZA-1 on bioelectrical activity is through action on one of the neurotransmitter systems, either directly or indirectly, that is modulated by the $GABA_A$ system. It is also possible that the elevated activity in the presence of bicuculline makes the neurons more susceptible to the action of AZA-1. The irreversible nature of the inhibition of networks treated with bicuculline followed by AZA-1 could be due to neuronal death; however, residual action potential activity suggests that at least a portion of the neurons were still alive. Ultimately, it is impossible to determine the mechanism behind the AZA-1 effect from the limited data set collected here. It does, however, provide a strong starting point for additional experiments into the mode of action of AZA-1 on neurons when toxin supplies become available.

In conclusion, this study is a first demonstration of the effect of AZA-1 on bioelectrical activity of neuronal networks prepared from primary cultures. Although some modes of AZA-1 action have been ruled out, the task of elucidating the mechanism by which this new phycotoxin alters neuronal activity remains to be determined.

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