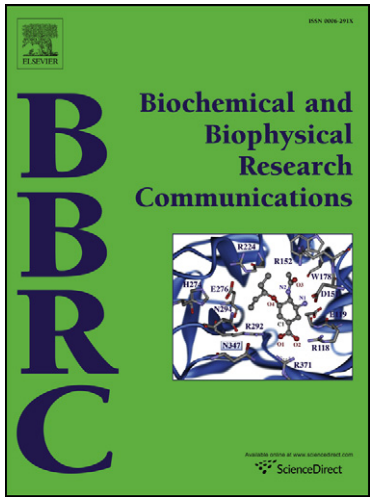

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Tracking of fast moving neuronal vesicles with ageladine A

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Keywords: sponge, ageladine A, fluorescence, alkaloid, imaging, patch clamp, marine natural product, hippocampus

Abstract

Ageladine A is a marine natural product that can be used to fluorescently stain living tissues and cells. Its fluorescence is highly pH dependent with the highest intensities under acidic conditions. We have used ageladine A to stain acidic vesicles in cells and found the compound especially useful for tracking transport vesicles in cultured nerve cells. Inward as well as outward ionic currents appear not to be influenced by ageladine A at concentrations of 10 μM or less. Higher concentrations than 30 μM reduce whole cell voltage dependent outward currents whereas inward currents remain unchanged up to 100 μM ageladine A (PC12 cells). Incubation with ageladine A (10 μM) in cultured hippocampal neurons does not alter miniature excitatory postsynaptic currents (mEPCS) amplitudes, frequency, rise or decay times. Fast moving vesicles are stained the brightest, suggesting they are the most acidic and likely to be Golgi derived and endocytotic vesicles for the fast anterograde and retrograde transport of proteins and other compounds needing an acidic environment.

Introduction

Many secondary metabolites (natural products) display biological activity and about half of the human pharmacopeia is derived from natural products chemistry [1]. This pre-eminence over synthetic chemicals is probably derived from evolutionary pressures in Nature to develop chemicals that bind selectively to specific biomolecules thus giving the producing organism some advantage over competitors [2, 3, 4]. Terrestrial natural products have been used as dyes since ancient times [5]. However, drugs and dyes derived from marine sources are relatively rare probably because of the difficulties associated with supply and availability. None the less, marine alkaloids and other marine natural products probably fulfil similar manifold ecological roles as their terrestrial counterparts. For example, sponges are well known to produce a large number of bioactive compounds such as brominated pyrrole imidazoles as feeding deterrents against predatory fishes [6, 7, 8]. Some brominated pyrrole imidazole alklaloids are known to be cytotoxic [9] and to interact with ion channels and be involved in cellular signalling in mammalian and invertebrate cells [10, 11, 12].

Ageladine A (4-(4,5-dibromo-1*H*-pyrrol-2-yl)-1*H*-imidazo[4,5-c]pyridin-2-amine) is a unique brominated pyridoimidazole alkaloid first isolated from the Japanese marine sponge *Agelas nakamuri* by Fusetani and co-workers in 2003 [13]. Subsequently, we have isolated the same compound from a Caribbean sponge *Agelas wiedenmayeri* [14]. Ageladine A has been reported to be a moderate inhibitor of matrix metalloproteases and to be intensely fluorescent [13]. This fluorescence is pH dependent as it is stronger under acidic conditions and barely detectable in alkaline solutions [15]. The synthesis of ageladine A has been described by several groups [16, 17, 18, 19], which provides a ready source of this compound and a viable alternative to isolation from natural sources. As ageladine A is pH sensitive and a useful dye for isolated cells as well as transparent whole animals [15] we postulated that the dye might be useful for staining acidic vesicles inside cells.

While lysosomes are known to have a very low pH (~4.8) there are possibly many other acidic vesicles. For example, the accumulation of dopamine, histamine, adrenaline, acetyl choline, tyramine etc. (neurotransmitters) into a transport vesicles of nerve cells requires the efflux of two protons, suggesting that these vesicles should also be highly acidic [20].

Nerve cells are the longest cells in mammals and can reach up to a metre in length thus requiring effective communication between the cell body and the dendritic arbors, axons and presynaptic termini [21, 22]. Consequently, neurons require reliable and timely transport of vesicles containing neurotransmitters, other signaling molecules, synaptic proteins, growth factors and ion channels. Organelles such as endosomes and mitochondria are also continuously transported within axons [22].

In the present work we describe the application of ageladine A in staining of living cultured cells and especially imaging of fast-moving vesicles in neurones.

Methods

Cell culture methods

Dissociated hippocampal neurones were prepared as previously described [23, 24] from embryonic day 18 hippocampi maintained for 18–28 days. Hippocampal neurons cells were grown at 37 °C in 5% CO_2 in DMEM GlutaMax (Invitrogen, Carlsbad CA, USA) medium supplemented with 10% FBS, 100 µg/mL Pen-Strep, 25 µg/mL zeocin, 5 µg/mL blasticidin and 25 µg/mL hygromycin. PC12 cells from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were kept in culture medium containing RPMI 1640, 10% FBS, 5% horse serum 100 U penicillin/streptomycin/mL. Cells were cultivated in an incubator at 37 °C, 90% humidity and 5% CO_2 with regular medium changes twice a week or additionally when necessary. Cells

grew in culture flasks and 1-2 days prior to the experiments were seeded into Petri dishes coated with collagen.

Measurement of mEPSCs in hippocampal neurons

The extra cellular medium contained: 145 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 10 mM D-Glucose, with the pH adjusted to 7.4. In order to block spontaneous network activity, NMDA receptors and $GABA_A$ receptors were blocked with 1 µM tetrodotoxin (TTX), 10 µM APV and 50 µM picrotoxin in the extracellular medium. The bath temperature was kept at 20-25 °C (room temperature). Borosilicate pipettes were used to produce patch electrodes with resistances of 3-5 MΩ. The intracellular solution contained: 140 mM K-gluconate, 2 mM $MgCl_2$, 4 mM NaATP, 0.1 mM EGTA (ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*,*N´*,*N´*-tetraacetic acid), 10 mM HEPES. The pH was adjusted to 7.25 with 1 M KOH.

Recordings of mEPSCs were performed with an EPC-10 double patch-clamp amplifier (HEKA Electronics, Lambrecht, Germany). Data were acquired and stored using Patchmaster software version 2.0 (HEKA Electronics, Lambrecht, Germany) and analysed with Mini-Analysis (Synaptosoft Co., Decatur, USA).

Ionic currents in PC12 cells

Recordings were done using the EPC-7 patch clamp amplifier (List electronics) and analysed with the computer program Signal 3 (CED). All experiments were carried out one or two days after plating cells in collagen coated dishes (see above). The physiological bath solution for measurement of in- and out-ward currents comprised: 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 30 mM D-glucose, 26 mM HEPES. The pH was adjusted to 7.2 with NaOH. The physiological pipette solution contained: 140 mM K-

gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_2ATP, 10 mM EGTA, 10 mM HEPES, 0.4 mM Na_2GTP, adjusted to pH 7.2 with NaOH. The bath solution to measure calcium channel currents contained: 135 mM tetraethylammonium chloride (TEA-Cl), 10 mM HEPES, 1.2 mM $MgCl_2$, 10 mM $BaCl_2$, 2 µM TTX (pH adjusted to 7.2 with TEA-OH). The pipette solution comprised: 135 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM Na_2ATP (adjusted to pH 7.2 with TEA-OH). Currents were recorded with patch pipettes of approximately 5 MΩ resistance. Currents evoked from a holding potential of –70 mV to +10 mV for 200 ms every 30 s or by increasing voltage steps (+10 mV, 100 ms, every 10 s) starting from −90 mV up to +60 mV for current-voltage (IV) relationship. Current traces for IV relationships were leak subtracted. Some experiments were performed using the Port-a-Patch system (Nanion) equipped with an EPC10 amplifier using the same solutions as in conventional patch clamp experiments.

Imaging of vesicular mobility in primary neuronal cultures

Hippocampal cultures 10 DIV were incubated for 15-30 min in 10 µM ageladine A added to the culture medium. After the incubation period, cells were transferred into an open chamber and the culture medium was replaced by extracellular solution as described for electrophysiological recordings. Cells were imaged at 35-37 °C mounted onto an inverted microscope (Axioobserver Zeiss Jena, Germany) equipped with a 63× objective (NA = 1.45, Zeiss Jena, Germany) or 100× objective (NA = 1.3, Zeiss Jena, Germany).

Ageladine A loaded cells were excited with a xenon lamp at 365-395 nm using a Polychromator V (Till Photonics, Germany) and fluorescence was detected by the use a bandpass emission filter 447/±30 nm (Semrock, USA). Fluorescent images were acquired using the time-lapse protocol of the Meta-View software (Analytical technology, USA) with an integration time of 30 ms and an acquisition rate of 3 Hz for up to 600 consecutive frames.

Signals were recorded with a back-illuminated thinned CCD camera (QuantEM, Roper Scientific, USA). Randomly selected dendritic regions were imaged over a total of 20 min. The tracking of individual vesicles was performed with the track object option in the MetaMorph software (Analytical technology, USA). Mean velocity was calculated from the displacement per frame for reconnected trajectories of at least 50 frames. Alternatively fluorescence was monitored by an imaging system (Visitron) which comprised a CCD camera (Coolsnap) mounted on an inverted microscope (Zeiss Axiovert 100). For optical excitation a Polychromator with a xenon flash tube (λ_{ex} 370nm, λ_{em} 500nm, Visichrome, Visitron Systems) was used.

Ageladine A

Ageladine A was synthesised from 2-aminohistamine [16] or obtained from the sponge *Agelas wiedenmayeri* [14].

Results

Ageladine A (fig. 1A) was found to possess a pH dependent fluorescence with an apparent pK_a of 6.3 (fig. 1B). While the nature of the pH induced fluorescence is not known, this behaviour correlates exactly with the calculated charge state of ageladine A (fig. 1C) [25] suggesting that protonation of the guanidine moiety is required for fluorescence. The fluorescence changes are completely reversible and the compound stable under all tested pH conditions. As the predicted charge state is zero at physiological pH (7.2), ageladine A easily permeates biological membranes but tends to accumulate in acidic environments (fig. 1D).

Ageladine A was also found to accumulate in lipids (e.g. a drop of microscopy immersion oil) and if the pH of the surrounding buffer (ACSF) is lowered the fluorescence increases inside

the droplet but not in the surrounding buffer (fig. 2). This was a surprising result because ageladine A is a highly polar, water-soluble alkaloid that should not have too much affinity for lipids such as a droplet of oil. However, as the charge calculations showed, at physiological pH, the neutral form predominates (fig. 1C) and could be partially partitioned into oil droplets with the hydrophilic part of the molecules (guanidine moiety) outside of the droplet. This is supported by a larger increase in fluorescence at the edge of the lipid droplets (23%) compared to the centre of the droplet (16%) (fig. 2) even though the sample thickness is much greater at the centre of the droplet. The increase in fluorescence inside the oil droplet is not surprising and is typical of ICT fluorophores that are more fluorescent in hydrophobic environments [26].

As cellular membranes are lipids, we assumed a similar situation might occur in living cells. Using confocal microscopy, we found that ageladine A does accumulate in cell membranes during incubation reaching target sites within 10 min. and continuing to accumulate over time (data not shown). Usually 10-30 min of incubation is sufficient to obtain satisfying fluorescence images using 1-10 µM ageladine A.

Electrophysiological controls

At a concentration of 10 µM, the highest concentration we used for staining of living cells, there was no measurable change in mEPSCs for hippocampal cultures. This indicated an absence of any disturbance to synaptic transmission. We found no alterations in distribution of mEPSCs amplitudes neither in rise time nor decay time of mEPSCs (fig. 3).

To test if ageladine A interacts with membrane properties at higher concentrations or influences ion channel activity as has been seen with other pyrrole-imidazole alkaloids [18] we tested for this in PC12 cells. Whole cell patch-clamp measurements of cellular properties revealed no change in outward currents or inward currents after incubation with 10 µM or lower concentrations of ageladine A. Outward currents are reduced by ~20% at 30 µM and by

~80% at 100 µM. Inward currents, in (undifferentiated) PC12 cells, are calcium ion currents, which are not altered at concentration up to 100 µM ageladine A (see Supplementary Data).

Vesicle tracking with a CCD camera

Incubation of hippocampal cultures with 10 µM ageladine A for 30 min. leads to profound staining of fast moving vesicles located in the neuronal processes and axons. In video imaging experiments (see Supplementary Data), single vesicles could be tracked inside dendrites and axons. These vesicles moved long distances with an average speed of 0.46 µm/s, with some vesicles moving at speeds of 4 µm/s (fig. 4). Taking into account the properties of ageladine A, these vesicles should be acidic such as lysosomes, endosomes or transport vesicles. The exact type of vesicles that we observed remains to be elucidated, but as the number of stained fast moving vesicles seems to be small they are unlikely to be lysosomes.

Discussion

Fast axonal transport in neurons is required to deliver cargo over the distance of several metres in large animals with a speed of several µm/s [27]. The nature of transported compounds is manifold. Beside lysosomes there are other acidic vesicles transporting neurotransmitters or neuromodulators [20]. Myosins, kinesins and dyneins are the molecular motor proteins responsible for the fast and slow axonal transport [28].

Ageladine A is found in *Agelas* spp where it plays an unknown role in the biology and ecology of the sponge. Other brominated pyrrole alkaloids, especially some of the dimeric molecules, are known to have antibiotic, antiparasitic and cytotoxic effects [9, 14, 29], interact with membrane ion channel function [30] and therefore ionic trans-membrane transport processes [11] and inhibit cell motility in a variety of cancer cell lines [31]. As sensory and olfactory neurones of predators are primarily exposed to sponge compounds [11]

and are equipped with numerous ion channel and cellular signal chains it seems plausible that these alkaloids serve as feeding deterrents as described by several authors [6, 7, 8].

Ageladine A is a monomeric pyridoimidazole alkaloid that displays a relatively low toxicity and can be used for whole animal live imaging without apparent effects on behaviour and mobility (unpub. observation). Rather surprisingly ageladine A readily enters cells, probably because of its low charge at physiological pH of 7.3 and accumulates in acid vesicles and its membranes being protonated and charged. It is thus well suited to studying these acid vesicles under physiological conditions. Endosomes and lysosomes are known to have low pH values and synaptic transmitter vesicles can have low pH's during certain phases of their life cycle, where protons can be used for transport processes across the membrane [20, 32]. From our results, the very fast moving vesicles are brightly stained with ageladine A, suggesting these are also the most acidic. The small number and rapid speed of some of these vesicles suggests that they could be a subcategory of transport vesicles for either proteins or neurotransmitters; which need very rapid relocation within the neuron. The brightly stained vesicles are few in number and move with high speeds. These data suggest we are observing specialised and acidic vesicles. Mitochondria, microfilaments and neurofilamentes can be excluded based on the high velocities observed [28]. The kinesin motor (KIF1A) in hippocampal neurons may be responsible for fast axonal transport processes involved in vesicular transport [33].

Conclusions

At concentrations used for cellular staining, ageladine A showed no evidence of toxic effects in either PC12 cells or cultured hippocampal neurones. The ability of ageladine A to specifically stain fast-moving vesicles in live nerve cells suggests that this compound could find applications in fluorescence microscopy.

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Figure 1: Chemical structure of the major fluorescent form of ageladine A (A), pH dependency of fluorescence (B) correlates with the predicted charge state of the compound (C). Staining of hippocampal neurones (82 × 82μm) with ageladine A (10 μM) with excitation at 370 nm and emission at >447 nm showing acidic vesicles (D)

Figure 2: Fluorescence intensity of oil droplets suspended in water (pH 7.3) containing 10 μM ageladine A (A). The solution was acidified to pH 1 at ~14 min., resulting in an increase in fluorescence inside the oil droplet (~0.2 mm). An increase in fluorescence was observed inside the oil droplet (red line) and no increase was observed in the water (green line). The

largest increase in fluorescence after acidification was observed at the oil-water interface (blue line). Panel B shows the areas used to generate the graph in Panel A.

Figure 3: Original data trace (A) and distribution of mEPSC amplitudes in the presence and absence of ageladine A (10 µM) (B). Neither rise time of single events nor decay time was affected at the concentration used for the described experiments (C).

Figure 4: Normarski phase contrast image of neuronal processes (A) and movements of single stained vesicles over time (B). Distribution of velocities of vesicles in the indicted number of intervals indicate a mean velocity of 0.46 µm/s with a maximum speed of 4 µm/s. (C).

Supporting Information:

Results for charge and pKa calculations for ageladine A and time lapse photographs, movies of vesicle movements and electrophysiological data are available in the Supporting Data.

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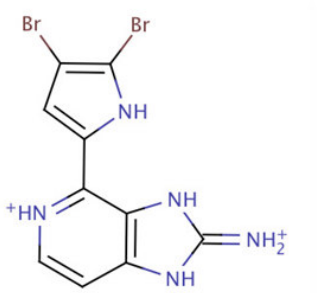

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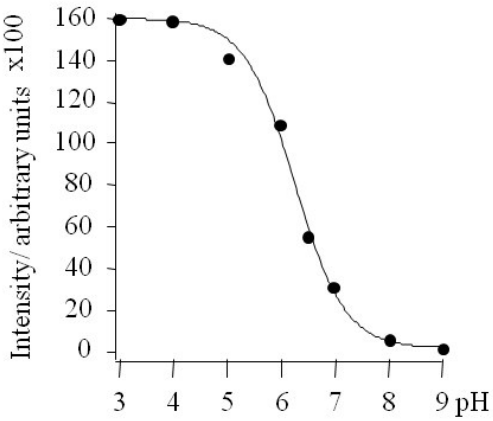

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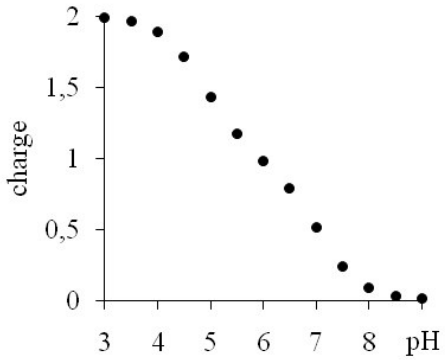

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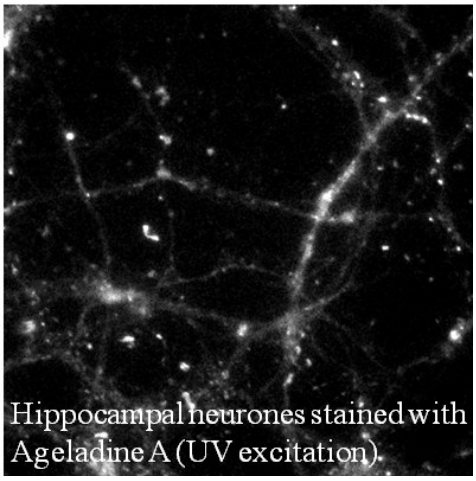
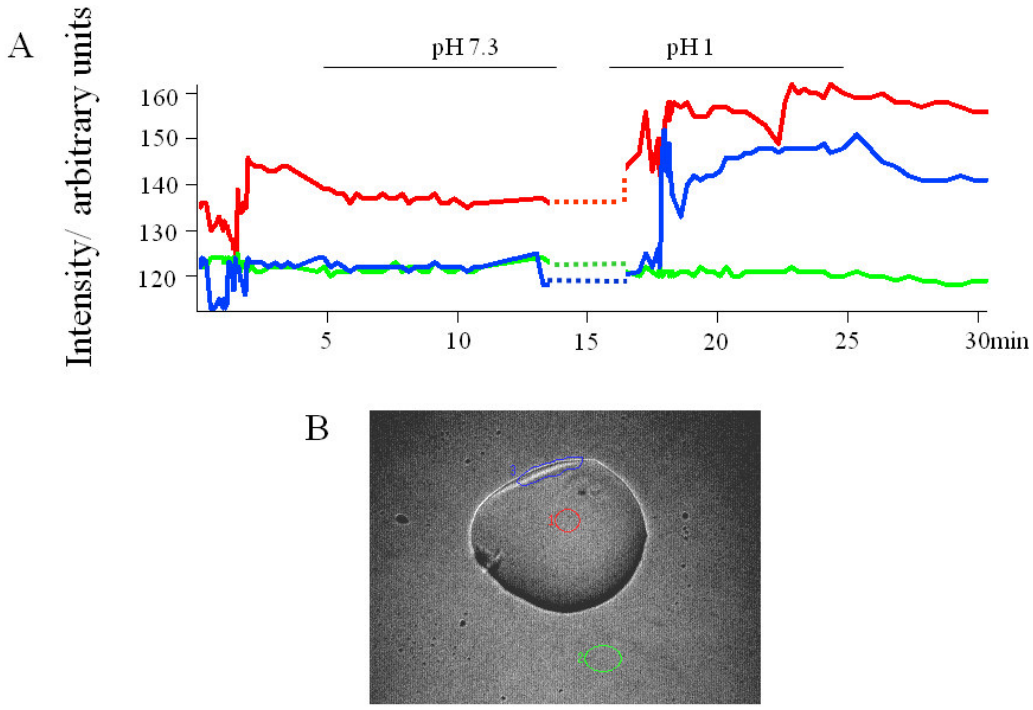
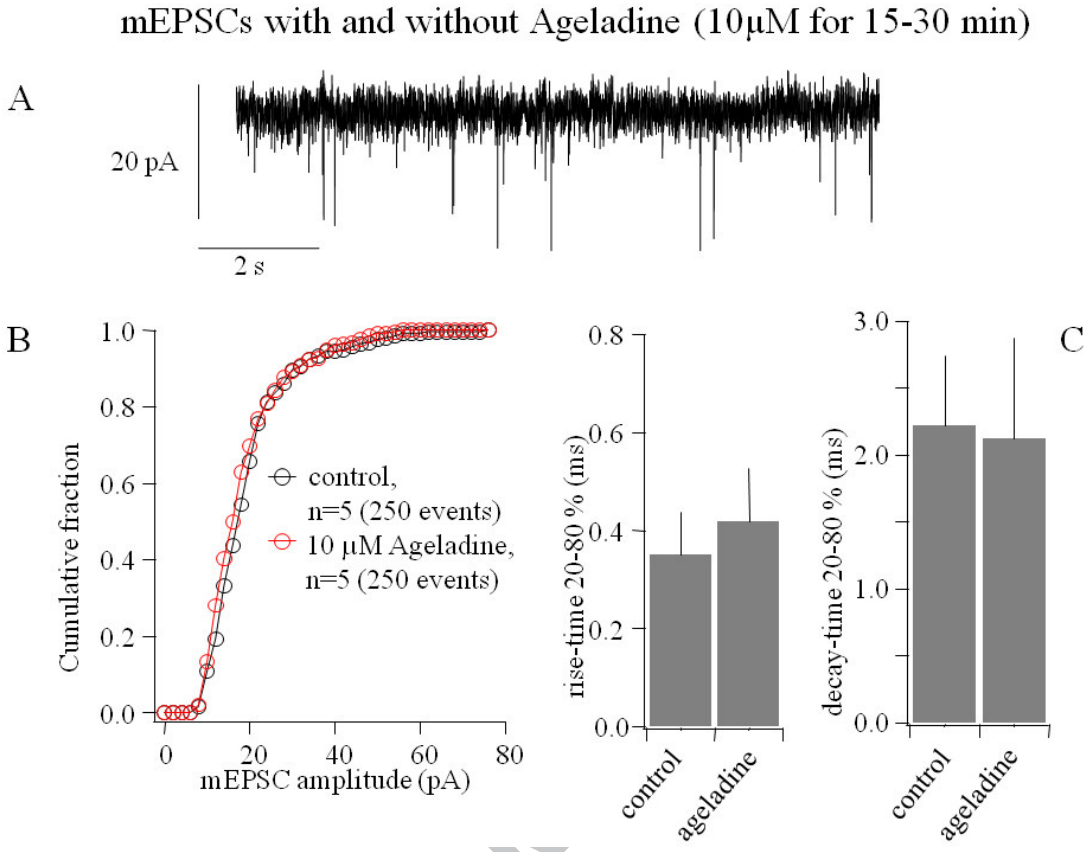
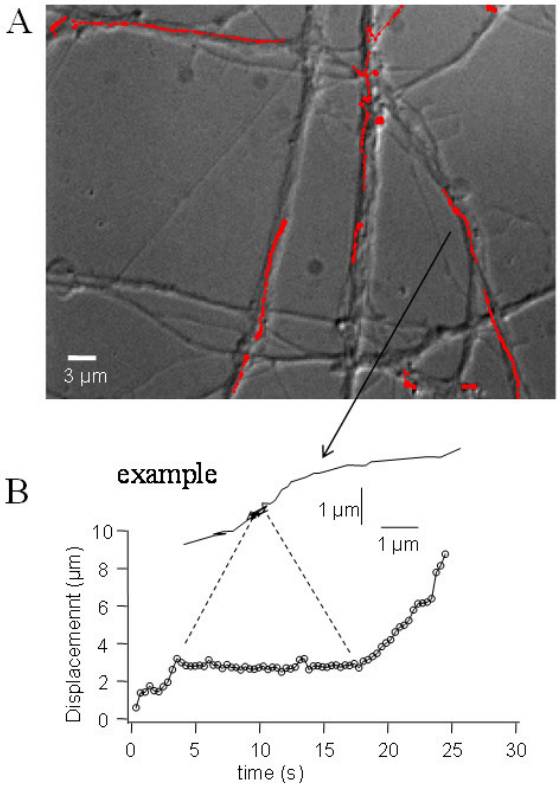


Immersion oil in water with ageladine during acidification







Movement of stained vesicles in neuronal processes. Median vesicle speed was 0.46µm/s

Vesicle tracking in cultured hippocampal neurons is shown using the alkaloid ageladine A derived marine sponges.
Ageladine A is pH sensitive and predominantly stains acidic vesicles.
Ageladine A seems to not disturb cellular signaling in concentrations used (1-10μM).
The calculated charge of the molecule mirrors its pH dependent fluorescence.