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Ageladine A, a pyrrole–imidazole alkaloid from marine sponges, is a pH sensitive membrane permeable dye

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ABSTRACT

The alkaloid ageladine A, a pyrrole–imidazole alkaloid isolated from marine *Agelas* sponges shows fluorescence in the blue–green range during excitation with UV light with the highest absorption at 370 nm. The fluorescence of this alkaloid is pH dependent. Highest fluorescence is observed at pH 4, lowest at pH 9 with the largest fluorescence changes between pH 6 and 7. Ageladine A is brominated, which facilitates membrane permeation and therefore allows for easy staining of living cells and even whole transparent animal staining. To calculate the exact pH in solutions, cells, and tissues, the actual concentration of the alkaloid has to be known. A ratiometric measurement at the commonly used excitation wavelengths at 340/380 nm allows pH measurements in living tissues with an attenuated influence of the ageladine A concentration on calculated values. The fluorescence changes report small intracellular pH changes induced by extracellular acidification and alkalization as well as intracellular alkalization induced by ammonium chloride.

The pyrrole–imidazole alkaloid ageladine A was first isolated and described by Fujita et al. [1] using bioassay guided fractionation of extracts of the marine sponge *Agelas nakamurai*.

The alkaloid showed biological effects such as the inhibition of matrix metallo-proteinases and the inhibition of cell migration of bovine endothelial cells. In 2006 the total synthesis of ageladine A was completed by the groups of Weinreb and Karuso [2,3] and later optimized [4,5].

Brominated pyrrole–imidazole alkaloids are known to be fish feeding deterrent against the reef fish *Thalassoma bifasciatum* [6,7] and demonstrate antibiotic activity [8] even against pathogens like *Helicobacter pylori* [9]. Especially, the degree of bromination and the guanidine moieties have shown to be important for the alkaloids efficacy to disturb cellular calcium ion entry via voltage operated channels in neuroendocrine cells [10–12], which possess mainly L- and N-type calcium channels common in neuronal cells. During these fluorescence measurements of cellular effects by pyrrole–imidazole alkaloids, ageladine A was noticed to show fluorescence during UV excitation [12], which was also described earlier by Fujita et al. [1]. We demonstrate here other surprising properties of ageladine A such as its sensitivity to pH changes covering a wide range and because of its high membrane permeability an easy whole animal pH sensitive staining.

Material and methods

Culture methods. PC12 cells from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were kept in culture medium containing RPMI 1640, 10% fetal calf serum, 5% horse serum, and 100 U penicillin/streptomycin per milliliter. Cells were cultivated in an incubator at 37 °C, 90% humidity and 5% CO_2 with regular medium changes twice a week or when additionally necessary. Cells grew in culture flasks and 1–2 days prior to the experiments were seeded into petri dishes coated with collagen. Shrimps of *Macrobrachium argentinum* were raised at the laboratory facilities of the Biologische Anstalt Helgoland, Germany, by Dr. Klaus Anger.

Fluorimetric measurements. Fluorescence was monitored by an imaging system (Visitron) and a CCD camera mounted on an inverted microscope (Zeiss Axiovert 100). Fluorescence was obtained through an UV objective (Zeiss NeoFluar 20×). For optical excitation a monochromator (Visichrome, Visitron Systems) and for generation of emission spectra a fluorospectrometer (NanoDrop ND-3300, PeqLab) was used. Twenty to 30 PC12 cells were measured simultaneously and separated using "the region of interest" function of the software (Metafluor, Meta Imaging Series) (Fig. 3). For image documentation (Fig. 2) a confocal laser scanning microscope (Leica TCS SP2) was applied.

Results are presented as the mean ± SEM, unless stated otherwise (Fig. 3). Statistics and calculations were performed using computer software Prism (Graphpad) and Igor (WaveMetrics).

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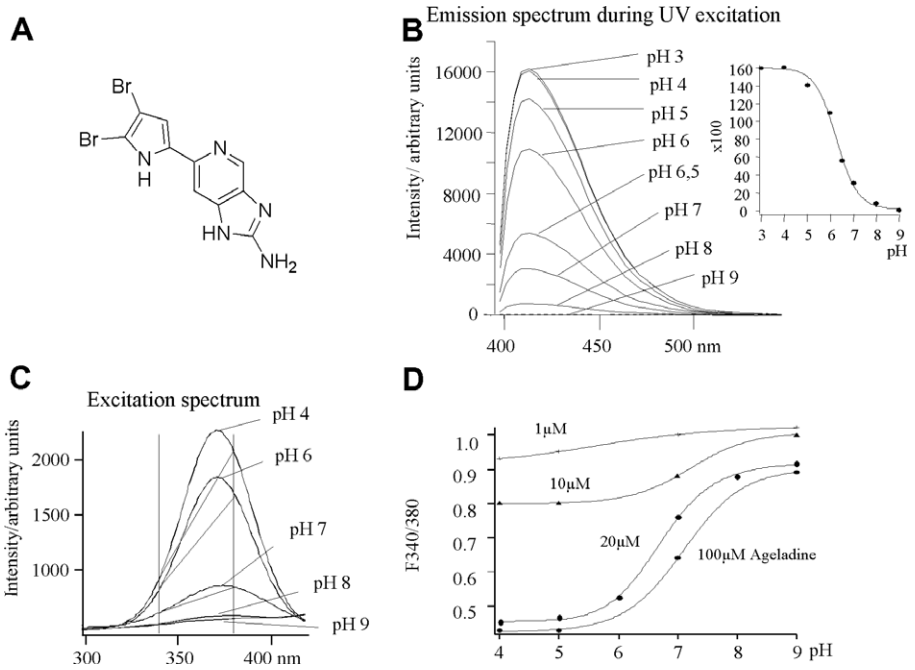

Fig. 1. (A) Chemical structure of ageladine A and its fluorescence properties shown in (B) the emission during excitation with 365 nm UV light increases with decreasing pH. The emission peaks at 415 nm. The half maximal pH reported is at 6.26 U (inlet). (C) Excitation spectrum at different pH values as indicated. The lines at 340 and 380 nm mark excitation wavelengths during ratiometric measurements shown in (D). (D) To reduce the influence of dye concentration on pH measurement, at excitation at 340 and 380 nm and division of both obtained values F340/380 was calculated. Between 20 and 100 µM ageladine A, the obtained values allow an initial appraisal of the actual pH.

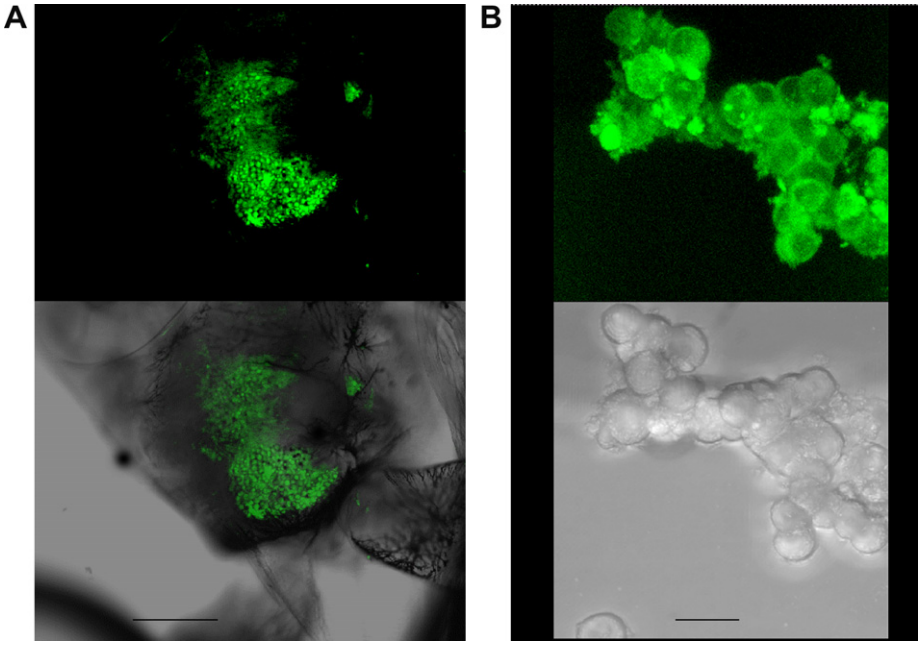

Fig. 2. (A) Whole animal staining of a shrimp (*Macrobrachium argentinum*) reveal low pH in tissues. To demonstrate the position of the stained midgut gland, the image was merged with a transmission image showing the anterior part of the carapx (dorsal view) (lower panel, scale bar 300 µM). (B) PC12 cells stained with ageladine A during UV excitation and the transmission image of the cells (lower panel, scale bar 16 µM).

Sponge secondary metabolites and chemicals. Ageladine A (Fig. 1A) was isolated from dichloromethane/methanol extracts of the Caribbean sponge *Agelas wiedenmayeri* using previously reported methods of collection and isolation [13]. The structure was identified by comparing the 1D NMR und MS data to previously described data [1]. Other chemicals were obtained from Sigma, Merck, Fluka and Molecular Probes.

Results

Ageladine A is a brominated pyrrole–imidazole alkaloid (Fig 1A), which can be protonated at the guanidine moiety and stabilized in two forms. The maximum of fluorescence is at 415 nm during excitation with UV light (365 nm) with a broad emission spectrum up to more than 500 nm (Fig. 1B). pH values in the range between 4 and 9 are reported by fluorescence changes

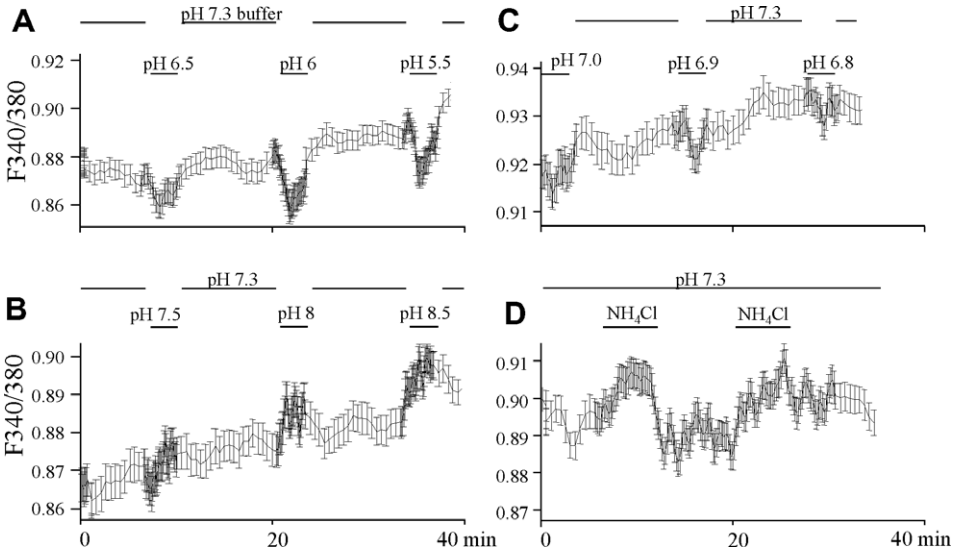

Fig. 3. (A) Ratiometric measurement of intracellular pH during acidification of the buffer. The ratio follows the acidification to lower values (about 20 cells were measured simultaneously indicated as ±SEM). (B) Alkalization of the buffer induces intracellular pH changes measured by an increase of ratio. (C) Small changes to lower pH values are reported by ratio changes. (D) The pH of the buffer solution was adjusted to pH 7.3. NH_4Cl (20 mM) was applied twice accompanied by intracellular pH changes.

of ageladine A (Fig. 1B). The half maximal pH is 6.26 calculated using sigmoidal curve parameters (Fig. 1B, Igor, MaveMetrics). The excitation maximum is at 370 nm and UV excitation ranges from 325 nm up to 415 nm (Fig. 1C). Dye concentration influences fluorescence and therefore enables no exact calculation of pH without knowledge of the dye concentration using a calibration curve. To reduce the influence of the dye concentration, we tried to calculate pH from reported intensity values without knowledge of actual dye concentrations. By using excitations of 340 and 380 nm (emission from 470 nm upwards) it was tried to minimize the influence of dye concentration. At concentrations of ageladine A between 20 (half maximal pH 6.78) and 100 μM (half maximal pH 7.01) an estimation of the actual pH values in solutions can be done (Fig. 1D). This is necessary especially in cells where the actual concentration of ageladine A is unknown and can only be estimated by its concentration during incubation. The ratiometric measurement approach allows a rough estimation of pH values without the knowledge of the exact ageladine A concentration (Fig. 1D). Use of the excitation and filter settings fitting for the calcium chelating dye Fura 2 allowed this experimental approach.

The bromination of ageladine A facilitates membrane permeation, which enables short incubation times of 10 min to stain living cells and of 30–60 min (10 μM) for whole animals (Fig. 2). Whole animal incubation of transparent marine animals, in this case a decapod shrimp larva (*M. argentinum*), shows the tissues and cells with a low pH during UV excitation using a confocal microscope (Fig. 2A). The fluorescent tissue is the midgut gland of the crustacean which is known to synthesize and to release digestive enzymes. Depending on the specific function, the different cell types may contain huge numbers of lysosomes with high acid phosphatase activity and, thus, a low pH [15]. Additionally, the gastric fluid, which accumulates in the stomach and fills the extracellular space of the midgut gland tubules, is slightly acidic as well showing pH values between 4 and 6 [16]. The midgut gland is shown in the merger of UV excitation and transmission images (lower image Fig. 2A). Fig. 2B shows images of PC12 cells, which are stained with ageladine A (20 μM loading) and excited using an UV argon laser (upper image Fig. 2B); additionally the cells are shown as transmission image (lower image Fig. 2B). Ageladine A seems to accumulate in the cellular membrane and some bright dots inside the cell may be low pH cell organelles such as lysosomes.

Using transparent animals, cells and tissues with low pH are stained best because of the specific properties of ageladine A. It increases emission in the blue–green light range during UV excitation in dependency of the pH value. Changes in extracellular pH lead to variations in intracellular pH, which can be reported by ratiometric measurements (Fig. 3). Acidification reduces ratio values (Fig. 3A) whereas high pH increases ratio values (Fig. 3B). Even small changes in pH are reported (Fig. 3C). To alter intracellular pH without change of the pH of the extracellular solution we applied 20 mM NH_4Cl, which is known to alkalize cell interior followed by acidification [14]. This was done to rule out the possibility, that ageladine A, which seems to be located in the cell membrane and the cytosol (Fig. 2B), reports extracellular pH changes. NH_4Cl in pH neutral solution clearly increases the fluorescence ratio of ageladine A indicating its potency to report intracellular pH changes.

Discussion

Additionally to its pharmacological action as anti-angiogenic compound and metallo-protease inhibitor, ageladine A is a reliable and stable fluorescent pH sensor, and because of its membrane permeability, can be used for detection of intracellular pH changes. Especially the staining of transparent animals allows for a fast location of low pH tissues in organisms. This is probably promoted by the degree of bromination of ageladine A, allowing for fast and successful incubation of whole animals. Experiments can be performed with the often used filter setting for Fura 2 which is present in many physiology laboratories. For the exact calculation of pH values, the actual ageladine A concentration has to be known but a rough estimation can be done without this information by ratiometric measurements. This makes ageladine A a promising pH sensor with a high potency for widespread use. Especially staining of transparent animals allows a fast and easy approach for detection of acidic tissues and cells and calculation of its pH values.

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