

A secondary metabolite, 4,5-dibromopyrrole-2-carboxylic acid, from marine sponges of the genus *Agelas* alters cellular calcium signals

Ulf Bickmeyer^{a,*}, Michael Assmann^b, Matthias Köck^b, Christian Schütt^a

^a Alfred-Wegener-Institut für Polar- und Meeresforschung in der Helmholtz-Gemeinschaft, Biologische Anstalt Helgoland, Kurpromenade, D-27498 Helgoland, Germany

^b Alfred-Wegener-Institut für Polar- und Meeresforschung in der Helmholtz-Gemeinschaft, Am Handelshafen 12, D-27570 Bremerhaven, Germany

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Abstract

A secondary metabolite from sponges of the genus *Agelas*, 4,5-dibromopyrrole-2-carboxylic acid, which is well known as feeding deterrent, was investigated for effects on the cellular calcium homeostasis in PC12 cells. 4,5-Dibromopyrrole-2-carboxylic acid did not change intracellular calcium levels if applied alone without cell depolarization. During depolarization of the cellular membrane using high potassium solution, a dose dependent reduction of intracellular calcium elevation was revealed utilizing Fura II as calcium indicator. Significant reduction was seen at concentrations higher than 30 μ M in a series of experiments, but in single experiments a concentration of 300 nM was still reversible effective. In the same concentration range, the onset of depolarization induced calcium elevations was significantly delayed by 4,5-dibromopyrrole-2-carboxylic acid. Dose dependent reduction and delay of depolarization evoked calcium elevations are probably due to a reduction of calcium entry via voltage operated calcium channels. One cellular mode of action of the feeding deterrent potential of 4,5-dibromopyrrole-2-carboxylic acid to fishes may be an interaction with the cellular calcium homeostasis of exposed cells.

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1. Introduction

Marine organisms represent an excellent source for bioactive compounds. Especially sponges are known to be a rich font for interesting secondary metabolites, for example the genus *Agelas* (Agelasidae) is well known to contain a variety of bromopyrrole alkaloids (reviewed in Braekman et al., 1992; Lindel et al., 2000). Recently, detailed studies have shown both at laboratory and field conditions, that brominated pyrrole alkaloids like 4,5-dibromopyrrole-2-carboxylic acid were the principal fish antifeedant metabolites within the sponge tissue of *Agelas* sponges at natural concentrations (Pawlik et al., 1995; Chanas et al., 1996; Assmann et al., 2000, 2004). However, the chemosensory and cellular mechanisms of brominated pyrrole alkaloids to function as a feeding deterrent are not investigated so far. Chemo-receptive cells are exposed to the environment and cellular signaling

may be modulated by substances interacting with the basic physiology of the cell, not necessarily by binding to specific taste receptors. Therefore, we tested 4,5-dibromopyrrole-2-carboxylic acid, a prominent secondary metabolite of *Agelas* sponges, for cell physiological effects in an established cell line. PC12 cells stem from a rat pheochromocytoma of the adrenal gland. These cells can be differentiated into neuron-like cells and produce catecholamines as adrenalin, noradrenalin and dopamine like their organ counterpart, the chromaffin cells from the adrenal gland. Chromaffin cells and PC12 cells have been widely used for pharmacological and toxicological investigations of heavy metals (Weinsberg et al., 1995; Westerink and Vijverberg, 2002a), polychlorinated biphenyls (Messeri et al., 1997; Westerink and Vijverberg, 2002b) and alkaloids (Lee and Kim, 1996; Gafni et al., 1997; Bickmeyer et al., 1998; Kim et al., 2001; Smith et al., 2002). Hormone secretion is triggered by influx of extracellular calcium through voltage dependent calcium channels, which can be monitored using calcium sensitive dyes (Gryniewicz et al., 1985; Deitmer and Schild, 2000). The

* Corresponding author. Tel.: +49 4725 819 224; fax: +49 4725 819 236.
E-mail address: ubickmeyer@awi-bremerhaven.de (U. Bickmeyer).

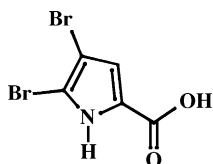


Fig. 1. Structure of 4,5-dibromopyrrole-2-carboxylic acid.

intracellular calcium level is crucial for numerous cellular processes as calcium is a ubiquitous second messenger following ligand binding of G-protein coupled receptors. Several plant alkaloids, for example cannabinoids (Khasabova et al., 2002) and tetrandrine (Bickmeyer et al., 1998) as well as caffeine (Bennett et al., 1998), affect calcium influx and calcium levels in neurons and secretory cells.

We decided to test one prominent bromopyrrole alkaloid, 4,5-dibromopyrrole-2-carboxylic acid (Fig. 1), for pharmacological effects on cellular signaling, focusing on intracellular Ca^{2+} signals in the well established cell line PC12.

2. Material and methods

2.1. Culture methods

PC12 cells from the DSMZ (German collection of microorganisms and cell cultures) were kept in culture medium containing RPMI 1640, 10% fetal calf serum, 5% horse serum, and 100 units penicillin/streptomycin per mL. Cells were cultivated in an incubator at 37 °C, 90% humidity and 5% CO_2 . Cells grew on collagen-coated cover slips and/or in collagen coated petri dishes. Cells were fed every three days and subcultivated when necessary.

2.2. Fluorimetric measurements of intracellular calcium levels

Cells were incubated with buffer (in mM: 125 NaCl, 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 1.3 NaH_2PO_4 , 30 glucose, 26 *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid Na-salt (HEPES)) containing 5 μM Fura II acetoxymethylester for 30 min at room temperature (20 ± 2 °C). The incubation buffer (with Fura II) was removed and cells were washed for 20 min (buffer without Fura II). Fluorescence of cells was monitored by an imaging system (Visitron, Puchheim) and a CCD camera mounted on an inverted microscope (Zeiss Axiovert 100). About 30 cells were measured simultaneously, separated using the region of interest function of the software. Fluorescence was obtained through an UV objective (Zeiss NeoFluar). Data were obtained by division of two images, one obtained at 340 nm, the other at 380 nm excitation. For determination of R-min and R-max, 50 μM Fura II diluted in calcium buffer was used for R-max, buffer without CaCl_2 but with additional 10 mM EGTA for determination of R-min in calibration experiments. The K_d value of Fura II at room temperature was taken from the literature (135 nM; Grynkiewicz et al., 1985), the values of Sf2 and Sb2 were determined

at 380 nm at calcium free and calcium saturated conditions obtaining a value Sf2/Sb2 of 3.23. Fluorescence ratios were converted into calcium concentrations by the formula given by Grynkiewicz et al., (1985). 4,5-Dibromopyrrole-2-carboxylic acid was stored as 100 mM stock solution in methanol at 4 °C. The addition of less than 1% methanol (as vehicle control) to the experimental buffer did not change calcium signal of PC12 cells and 4,5-dibromopyrrole-2-carboxylic acid did not interfere with Fura II in the concentration used in the experiments.

2.3. Experimental design

The recording chamber, mounted on a microscope had a volume of 2 mL and the peristaltic pump was adjusted to 3 mL/min exchanging the chamber volume in less than a minute. To depolarize the cells, 80 mM KCl (high K^+) was used supplemented for 80 mM NaCl in the experimental buffer. The depolarization of the cellular membrane potential therefore increased gradually in less than a minute during perfusion. Cells were depolarized three times for 1 min during the course of a single experiment of about 60 min. About 30 cells were measured simultaneously. By using a stock solution of 100 mM 4,5-dibromopyrrole-2-carboxylic acid much lower concentrations than 1% methanol were applied in most experiments.

Results are presented as mean \pm S.E.M. Statistics and calculations were performed using computer software Prism (Graphpad) and Igor (WaveMetrics). The *t*-test ($p < 0.05$, two tailed) was used for calculations of significance. Drug induced changes of intracellular calcium elevation were compared with the corresponding controls (1. K^+ depolarization) with paired *t*-test. One experiment using around 30 cells was considered as $N = 1$.

For analysis of the drug-induced delay, experiments were compared with a set of independent control experiments (*t*-test, unpaired). The spell to response (1. K^+ depolarization) was determined for each experiment and defined as zero delay, whereas the delay of 2. K^+ depolarization (with drug or as control) was reported.

2.4. Isolation and extraction of 4,5-dibromopyrrole-2-carboxylic acid

The investigated brominated pyrrole alkaloid (Fig. 1) was isolated from dichloromethane/methanol extracts of *Agelas wiedenmayeri* and *Agelas sventres* using previously reported methods for isolation and structural elucidation (Assmann et al., 1999).

3. Results

PC12 cells responded to repetitive depolarizations without a change in the amplitude or time course of the calcium response (Fig. 2).

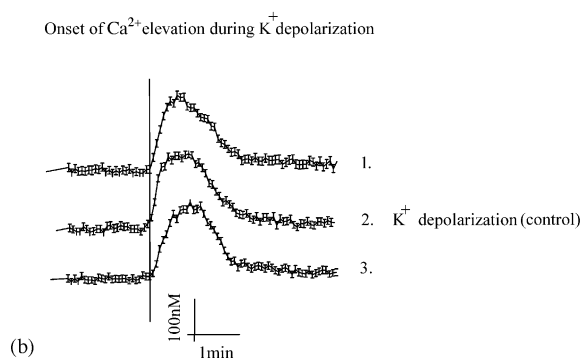
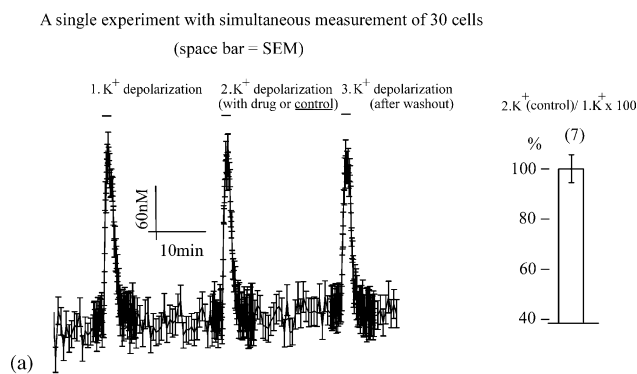


Fig. 2. (a) A typical experiment with three repetitive calcium elevations induced by 80 mM K^+ (control). The column shows the ratio $\times 100$ of the 2. K^+ depolarization to the 1. K^+ depolarization for seven different control experiments. (b) No delay of the onset of K^+ induced calcium elevations appears during repetitive stimulation.

The first K^+ depolarization was established as first control, which was normalized to 100%, the second K^+ depolarization was under influence of the drug. The third K^+ depolarization was the washout control to reveal the reversibility of drug effects. The time between two K^+ depolarization was 20 min.

3.1. Delay of calcium elevation

4,5-Dibromopyrrole-2-carboxylic acid delayed the onset of the calcium elevation induced by a K^+ depolarization (Fig. 3). This delay was at least partially reversible by washout of the drug for 20 min. The maximum delay of calcium response induced by 4,5-dibromopyrrole-2-carboxylic acid was 23 s under the applied experimental conditions. Significant delays were obtained in a series of experiments using 30 μM 4,5-dibromopyrrole-2-carboxylic acid. However, in single experiments performed with 30 cells simultaneously, a reversible delay of calcium responses was observed at a concentration of 300 nM 4,5-dibromopyrrole-2-carboxylic acid. During application of 300 μM the delay was not significant, possibly by additional cellular effects of the substance at high concentrations counteracting the induced delay of calcium elevation. The delay can be correlated to the perfusion rate of the peristaltic pump to give additional 0.96 mL of the

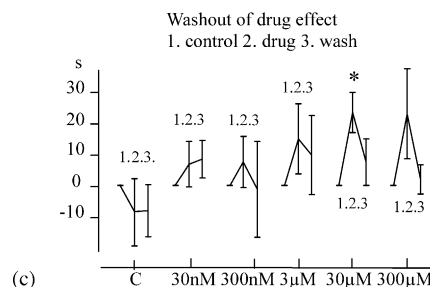
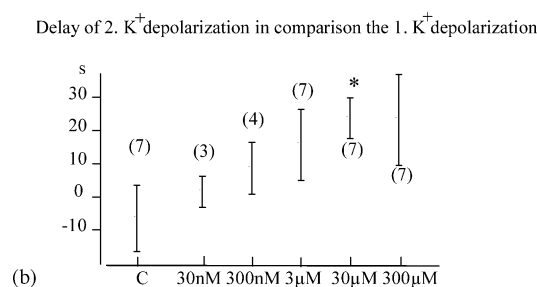
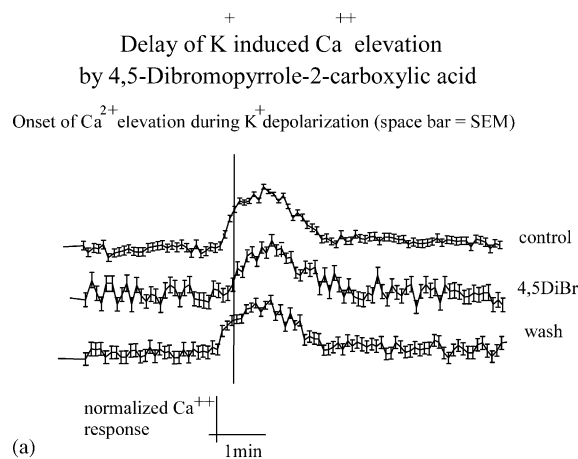


Fig. 3. (a) The onset of K^+ induced Ca^{2+} elevation is delayed by 4,5-dibromopyrrole-2-carboxylic acid. (b) Dose dependent delay of calcium elevations. (*) $p < 0.05$. (c) 4,5-Dibromopyrrole-2-carboxylic acid induced delay of calcium elevations is reversible by 20 min of washout. (*) $p < 0.05$ between 2. depolarization of the controls (C) and 2. depolarization with drug (30 μM). The cipher in () represents the number of experiments N .

K^+ solution in the perfusion chamber to induce a calcium elevation.

3.2. Reduction of calcium elevation

4,5-Dibromopyrrole-2-carboxylic acid dose dependently reduced intracellular calcium elevations induced by K^+ depolarization (Fig. 4). The reduction was visible after pre-incubation of the drug and was reversible by 20 min of washout. The highest concentration used was 1 mM 4,5-dibromopyrrole-2-carboxylic acid. We refused to apply higher concentrations to prevent from more unspecific cellular effects of the sponge secondary metabolite. The lowest concentration used was 30 nM whereas significant effects

related brominated pyrrole alkaloids. This will be one of our future approaches to investigate the pharmacological properties of 4,5-dibromopyrrole-2-carboxylic acid in detail.

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