Involvement of \( \text{GABA}_A \) receptors containing \( \alpha_6 \) subtypes in antisecretory factor activity on rat cerebellar granule cells studied by two-photon uncaging

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**Abstract**
The antisecretory factor (AF) is an endogenous protein that counteracts intestinal hypersecretion and various inflammation conditions in vivo. It has been detected in many mammalian tissues and plasma, but its mechanisms of action are largely unknown. To study the pharmacological action of the AF on different \( \text{GABA}_A \) receptor populations in cerebellar granule cells, we took advantage of the two-photon uncaging method as this technique allows to stimulate the cell locally in well-identified plasma membrane parts. We compared the electrophysiological response evoked by releasing a caged GABA compound on the soma, the axon initial segment and neurites before and after administering AF-16, a 16 amino acids long peptide obtained from the amino-terminal end of the AF protein. After the treatment with AF-16, we observed peak current increases of varying magnitude depending on the neuronal region. Thus, studying the effects of furosemide and AF-16 on the electrophysiological behaviour of cerebellar granules, we suggest that \( \text{GABA}_A \) receptors, containing the \( \alpha_6 \) subunit, may be specifically involved in the increase of the peak current by AF, and different receptor subtype distribution may be responsible for differences in this increase on the cell.

**KEYWORDS**
2PE uncaging, antisecretory factor, cerebellar granule cells, \( \text{GABA}_A \) receptor, patch-clamp technique, RuBi-GABA

**INTRODUCTION**

The antisecretory factor (AF) is an endogenous 41-kDa protein expressed in different mammalian tissues and plasma (Johansson et al., 1995). It was identified, cloned and sequenced in the 1980s by Lange and Lönnroth and presents an inhibitory function against hypersecretion.
induced by enterotoxins, especially cholera toxin, or
due to chronic inflammatory diseases (Lange &
Lönnroth, 2001).

The antisecretory site, in the region between the
amino acids 35 and 42, has the sequence (IVCHSKTR).
Four cysteine groups form disulphide bridges in the N-
terminal region to guarantee the antisecretory activity
(Jennische et al., 2008; Johansson et al., 1997).

Several peptides and fragments were synthesized from
the recombinant protein; the AF peptide, formed by
16 amino acids (AF-16), is derived from amino acids 36–
51. AF-16 is chemically the most stable and was conclu-
sively selected for laboratory experiments.

Using the AF concept, clinical treatment of various
human diseases has significantly improved patients’ cli-
nical outcomes (Lange & Lönnroth, 2001). Such diseases
include Ménéire’s disease (Hanner et al., 2004, 2010),
diarrhoeal diseases of various origins (Björck et al., 2000;
Laurenius et al., 2003; Zaman et al., 2018), Mb Crohn
(Eriksson, Shafazand, Jennische, Lönnroth, & Lange,
2003), ulcerative colitis (Eriksson, Shafazand, Jennische,
& Lange, 2003) and suppression of elevated intracranial
pressure after head trauma (Gatzinsky et al., 2020). The pathophysiology behind these clinical
conditions varies widely, but they display various forms of
transport derangement of the water and ion transport
across the cellular membrane. These diseases represent a
multitude of pathological mechanisms, and consequently,
there is a general demand for a better understanding of a
more detailed ‘mechanism of action’ of the AF protein.
Such investigations must also include the mode of action
of the smaller, various biologically active AF-peptides,
which all emanate from the amino-terminal end of the
original, full-length AF protein. These studies should pri-
marily be accomplished by evaluating the influence on the
cellular reactivity to AF administration in various in vitro
test systems.

Previous works considered AF effect in GABAergic
transmission (Bazzurro et al., 2018; Kim et al., 2005;
Strandberg et al., 2014). GABA represents the principal
inhibitory neurotransmitter of the mammalian central
nervous system (CNS) and also acts on GABA_A receptors
in the enteric nervous system (ENS), albeit the enteric
GABAergic neurons are principally interneurons
releasing GABA as an excitatory neurotransmitter
(Galligan, 2002; Krantis, 2000; Seiffi et al., 2014).

It appears of interest to study in model systems with
well-characterized GABA_A receptors the effects of the AF
in order to build a framework of knowledge allowing to
conceive and test hypotheses about the roles of GABA
in the AF anti-hypersecretory activity via GABA_A receptors
of enteric ganglia. Based on a long experience with
GABA_A receptors of cerebellar granule cells (CGCs) in
primary cultures (Cupello et al., 2013; Robello
et al., 1993), we studied the effects of AF on those
GABA_A receptors. In addition, in this context, the CGC
model is interesting from another point of view. In fact,
AF induction in patients, who suffer from Ménéire’s dis-
ease, has been shown to counteract the symptoms of that
disease, especially vertigo (Hanner et al., 2004, 2010).
Granule cells have an essential role in controlling the cer-
ebellar output via Purkinje cells’ innervation and, in par-
cular, the inhibitory actions of Purkinje cells on lateral
vestibular nuclei of the brain stem (Hydén et al., 2000;
Ito, 1984; Ito et al., 1966). Thus, it appears interesting to
investigate further the AF action on the GABA_A recep-
tor-mediated inhibitory brake of cerebellar granule neurons.

With the use of the patch-clamp technique, we dem-
onstrated that AF-16 affects neuronal signalling in the rat
CGCs in vitro (Bazzurro et al., 2018). Specifically, the
results showed an increase in the peak current induced
by GABA after AF-16 pretreatment. The effect depended
on peptide concentration and incubation time: the higher
the concentration and longer incubation time, the more
the effect on GABA_A receptors until saturation.

Here, we use a new approach to better understand
the AF effect on GABA_A receptors and precisely follow
the receptor activation after AF-16 treatment, in a limited
cell area, thanks to caged GABA photorelease; this tech-
nique allows selecting a neuronal region of interest to
investigate the variations in the receptor modulation of
that specific region.

This approach offers the possibility of studying
GABA_A receptors of specific sub-neuronal districts such as
axons and potential axo-axonic synapses in situ in the
actual tissue (Nathanson et al., 2019; Schmid et al., 1996).

Caged compounds are chemically modified neuro-
transmitters whose biological activity is inhibited until
light activates them when and where needed. Once
uncaged, they become active effectors and interact with
their target inducing a response (Shi et al., 2014).

We chose RuBi-GABA, a commercial caged neuro-
transmitter described by Rial Verde et al. (2008), for
studying the specific GABA_A receptors modulation by
AF-16. After characterizing the uncaging process both for
1- (1PE) and 2-photon excitation (2PE) by using electro-
physiological measurements in CGCs (Cozzolino
et al., 2020), we decided to investigate the role of AF in
the GABA_A receptor modulation by taking advantage of
2PE uncaging RuBi-GABA (Diaspro et al., 2005). This
strategy allows photoreleasing femtoliter volumes of
GABA in specific and confined cell regions to stimulate
GABA_A receptors only in that portion of the cell
membrane.

We selected three regions on cerebellar granules (cell
body, axon initial segment and neurite) and studied how
AF-16 affects receptors’ response to GABA in those specific areas.

The rationale of the experiments was to understand which GABA_A subtypes were involved in the receptor response after AF-16 incubation, considering that α_6 subunits mainly characterize CGCs GABA_A receptors (Cupello et al., 2013; Gatta et al., 2009).

Because of this assumption, we focused on the α_6 subunit, analysing how furosemide, a pharmacological molecule that selectively blocks GABA_A receptors containing this subunit (Korpi et al., 1995; Korpi & Lüddens, 1997), affects the electrophysiological response if administered with AF-16.

This strategy allowed us to infer AF-16 action on a selected GABA_A receptor population.

2 | MATERIALS AND METHODS

2.1 | AF-16 peptide production

The AF-16 peptide formed by AF amino acid sequence 36–51 (VCHSKTRSNPENNVGL) was synthesized with solid-phase synthesis by Ross-Petersen AS, Copenhagen, Denmark, as described by Rapallino et al. (2003).

2.2 | Animals

Sprague–Dawley rats were housed in the Department of Pharmacy’s animal unit, Section of Pharmacology and Toxicology of Genoa University. Animals were treated according to the E.U. Parliament and Council Directive of 22 September 2010 (2010/63/E.U.) and approved by the Italian Ministry of Health (COD. 75FI1.N.6DX) D.M. 116/1992. We made all efforts to minimize animal suffering and reduce the number of animals used.

2.3 | CGC primary cultures

CGCs were prepared from 6- to 8-day-old Sprague–Dawley rats, following procedures described previously (Robello et al., 1993).

The cells were plated at a density of 1 × 10^6 per dish on 24-mm poly-L-lysine-coated glass coverslip and maintained in 90% Basal Medium Eagle, 10% Fetal Calf Serum (Sigma-Aldrich, St. Louis, MO, USA), 25-mM KCl, 2.0-mM glutamine, 100 μg/ml gentamicin at 37°C in a humidified 95% air 5% CO_2 atmosphere.

A 10 μM cytosine arabinoside (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium to prevent glial cell growth at 18–24 h from plating. The medium was renewed at 48 h, and 10 μM cytosine arabinoside was added again. We studied the cells, in vitro, from the fifth to the 10th day.

2.4 | Electrophysiology

The membrane currents were measured with the whole-cell patch-clamp technique, setting the holding potential at −80 mV and using an Axopatch 200 B (Axon Instruments, Burlingame, CA, USA) as previously described in Robello et al. (1993).

Pipettes were pulled from borosilicate glass capillaries type TW 150-3 (OD 1.5 mm ID 1.12 mm World Precision Instruments Inc., Sarasota, FL, USA) and using a P-30 puller (Sutter Instruments Co., Novato, CA, USA). We recorded the currents with a Labmaster D/A, A/D converter driven by pClamp 10 software (Axon Instruments, Burlingame, CA, USA). We analysed the experimental data with pClamp and SigmaPlot 12 (SYSTAT Software, San Jose, CA, USA).

We maintained the cells in the recording bath with the standard external solution (pH 7.4 with NaOH) containing (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl_2, 1 MgCl_2, 5 HEPES, 10 glucose; we filled the glass pipettes with the internal solution that contained (in mM) 142 KCl, 5 HEPES, 10 glucose, 2 M_2 Cl_2, 3 ATP; we added tris base to adjust the pH to 7.3.

In some experiments, 5.0-μM biocytin conjugate with CF®640R (Biotium Inc., Fremont, CA, USA) was included in the internal solution to highlight the cell morphology.

In all experiments, the caged neurotransmitter RuBiGABA (Tocris Cookson Ltd, Bristol, UK) and AF-16 were diluted in the external solution to reach the needed final concentration.

The solutions were applied to the cell bath by continuous perfusion (~3 ml/min gravity flow).

2.5 | Confocal imaging and two-photon uncaging

The optical set-up used for performing the experiments consists of an inverted three-channel laser-scanning confocal microscope (Leica TCS SP5) equipped with 458-, 476-, 488-, 514-, 543- and 633-nm excitation lines and a plan-apochromatic oil immersion objective ×63/1.4. To check the cell’s position, we acquired images in transmitted light, using a 633-nm laser source, before and after the uncaging process using the ‘LAS AF’ software package (Leica Microsystems, Germany).
We performed two-photon excitation with a Ti: Sapphire laser (Chameleon II, Coherent Inc., Santa Clara, CA, USA), operating 100 fs pulses at 80 MHz of repetition frequency. We measured the average beam power at the objective focal point with a PM100A power metre (Thorlabs Inc., Newton, NJ, USA) (Diaspro & Robello, 2000).

We set up the bleach points' coordinates, laser power and exposure time using the FRAP Wizard Leica module, as previously described (Cozzolino et al., 2020).

3 | RESULTS

To evaluate the AF’s effect in different and specific neuronal regions, we chose to study the AF-16 action on the soma, the axon initial segment (AIS) and neurites (Figure 1). We administered AF-16 at a concentration of 1.0 μM for 3 min to work with the lowest concentration granting the maximum enhancement effect on the peak current component as demonstrated in a previous work by Bazzurro et al. (Bazzurro et al., 2018).

Figure 2 shows the typical traces of chloride current evoked by RuBi-GABA photolyzed with 2PE at about 2 μm from the soma. During experiments, we kept on measuring the current while applying the following procedure. We perfused 10-μM RuBi-GABA in the petri-dish with CGCs, uncaged the compound (Figure 2a) and washed it away with the external solution. Then, we perfused 1.0-μM AF-16 for 3 min (Figure 2b), administered a 10-μM RuBi-GABA and 1.0-μM AF-16 solution and activated the 2PE beam again (Figure 2c). We repeated the same procedure in different neuron regions. We reported typical currents in Figure 3.

The comparison of the currents evoked by RuBi-GABA before and after the treatment with AF-16, on the same cell, shows a significant increase in the peak current; thus, AF may facilitate the activation of GABA_A receptors. We have quantified the AF action, in terms of percentage effect (E%), by applying the formula below to peak current data:

\[ E% = \left( \frac{I_{RuBi-GABA+AF} - I_{RuBi-GABA}}{I_{RuBi-GABA}} \right) \times 100, \]

where \( I_{RuBi-GABA+AF} \) is the chloride peak current induced by uncaging RuBi-GABA in the presence of AF-16 and \( I_{RuBi-GABA} \) is the one evoked by just photolyzing RuBi-GABA. The E% values, reported in Table 1, derive from averaging the E% calculated on n cells; data are reported as mean ± SEM and compared with controls by using Student’s t test. In Table 2, we compared AF effect on the three neuronal regions under study (soma, AIS and neurite) by analysis of variance (ANOVA) test and on couples of regions (soma vs. AIS, soma vs. neurite, AIS vs. neurite) by post hoc Student’s t test.

To understand which receptor subtypes may be involved in the AF mechanism of action, we performed experiments using furosemide, a selective blocker of α6 subunit-containing GABA_A receptors; we reported typical traces of current for the cell body in Figure 4.

We followed this protocol: We measured the current evoked by uncaging 10-μM RuBi-GABA (Figure 4a, trace [A]); after a 3-min incubation with 1.0-μM AF-16 (Figure 4a, trace [B]), we administered and photolyzed RuBi-GABA with 1.0-μM AF-16 (Figure 4a, trace [C]); then, after a 1-min wash-out with the external solution (Figure 4b, trace [B]), we perfused and uncaged a solution of RuBi-GABA with 1.0-mM furosemide (Figure 4b, trace [C]). We observed a significant decrease of the current peak, probably due to furosemide blocking of α6 receptor subtypes.

Finally, after a second 3-min treatment with AF (Figure 4c, trace [B]), we dispensed RuBi-GABA with 1.0-μM AF-16 and 1.0-mM furosemide, uncaged it by activating a 2PE beam and registered a current peak (Figure 4c, trace [C]) comparable with the one acquired with only 1.0-mM furosemide in addition to uncaged RuBi-GABA (Figure 4b, trace [C]). Data indicate that in the presence of 1.0-mM furosemide, no effect by AF-16 is present.

![Figure 1](image1.png)

**Figure 1** The image illustrates different uncaging points on soma (1), axon initial segment (AIS) (2) and neurite (3) of a cerebellar granule cell that has been processed to reveal biocytin conjugate with CF640R during patch-clamp recording.
In Table 3, we reported the data as mean ± SEM, statistical significance versus control was calculated by Student’s *t* test.

In Table 2, we reported the data as mean ± SEM, statistical significance versus control was calculated by Student’s *t* test.

In the past decades, various molecules, named caged neurotransmitters, have been developed, and now they are
widely used to study neurobiological processes such as synaptic transmission and plasticity.

Caged neurotransmitters like RuBi-GABA are valuable tools for neurophysiology studies because light activation allows controlling the timing, location and amplitude of neurotransmitter release.

This study takes advantage of the multiphoton uncaging method to determine how pharmacological molecules, particularly the AF-16 antisecretory peptide, modulate GABA_\text{A} receptors in different regions of CGCs. The advantage of this method is in photoreleasing small volumes (in the order of fl) (Ellis-Davies, 2007) of neurotransmitter (i.e., GABA) in well-defined positions and thus stimulating small portions of the cell at a time. We applied 2PE uncaging to RuBi-GABA, a commercial caged-neurotransmitter, to study the variations of the chloride current associated with the activation of GABA_\text{A} receptors in the proximity of the neuron soma, AIS and neurites (Figure 1).

We were interested in studying the AIS considering recent reports about the physiological importance of GABA_\text{A} inhibitory synapses in the neuronal axonal initial segment (AIS) (Koike et al., 2013; Kriebel et al., 2011; Nathanson et al., 2019).

### Table 3

Percentage effect (E%) of 1-μM AF-16 (first row), 1-mM furosemide (second row) and 1-mM furosemide + 1-μM AF-16 (third row) on 10-μM uncaged RuBi-GABA induced current after the incubation with AF-16 for 3 min

|                        | E% ± SEM | n  | p values |
|------------------------|----------|----|----------|
| 10-μM RuBi-GABA + 1-μM AF-16 | 45 ± 5   | 8  | p < 0.05 |
| 10-μM RuBi-GABA + 1-mM furosemide | −65 ± 2  | 9  | p < 0.001 |
| 10-μM RuBi-GABA + 1-mM furosemide + 1-μM AF-16 | −64 ± 2  | 9  | p < 0.001; n.s.* |

Note: For each data series, the number of experiments (n) and the statistical significance (p values) of the results are reported. The data are given as mean ± SEM; statistical comparisons were analysed with Student’s t test versus the control (no AF-16, no furosemide): p values. The last column reports the comparison between the 10-μM RuBi-GABA + 1-mM furosemide + 1-μM AF-16 and the 10-μM RuBi-GABA + 1-mM furosemide case (n.s.*: not significant). Abbreviation: AF, antisecretory factor.

### Figure 4

Example of chloride current traces evoked by uncaging 10-μM RuBi-GABA (750 nm, 100 ms, 30 mW, −80 mV) on the soma of the same cell. (a) and (c): (A) Current trace recorded after the photorelease of 10-μM RuBi-GABA, (B) incubation for 3 min with 1-μM AF-16 and (C) current measured after the uncaging of 10-μM RuBi-GABA in combination with 1-μM AF-16 and, only for trace c, 1 mM furosemide. (b): (A) Current trace recorded after the photorelease of 10-μM RuBi-GABA, (B) wash-out with external solution for 1 min and (C) current measured after the uncaging of 10-μM RuBi-GABA in combination with 1 mM furosemide.
Schmid et al., in a previous work (Schmid et al., 1996), suggested the presence of axo-axonic synapses in the cerebellar granule neuron terminals. Such synapses act on GABA_A receptors with the pharmacological characteristics of granule cells: For example, they are not sensitive to classical benzodiazepines. We thus thought it would be interesting to test whether the AF peptides could affect axonal GABA_A receptors.

Even if the AF mechanism of action is still partly unknown, AF-16 seems to facilitate the GABAergic transmission in the cerebellar granule neuron (Bazzurro et al., 2018). With this in mind, we investigated how AF-16 works in different cell regions and analysed the GABA activated chloride currents, and related the variations to differences in GABA_A receptors distribution.

At first, to study the effect of AF-16 on electrophysiological behaviour, we compared data acquired by uncaging RuBi-GABA with 2PE with previous experimental data obtained by perfusing GABA in the petri dish with cerebellar granules (Bazzurro et al., 2018).

In both cases, AF-16 increases the peak current evoked by the neurotransmitter. The peaks determined with this new approach are lower than the peaks achieved with GABA only. The lower peaks might reflect the release of relatively few uncaged neurotransmitter molecules, which activates a lower number of receptors on the cell membrane.

The data suggest that AF-16 may act differently on the considered areas, implying a different effect on the receptor subtypes of GABA_A: In fact, the results showed significant differences in the effects of AF-16 in the AIS compared with the soma and neurites (Tables 1 and 2); in particular, the action seems more effective in the cell body and neurites, suggesting that AF-16 interacts with a specific subtype of GABA_A receptors.

CGCs in primary culture are mainly composed of α6, β2/3, γ2 and δ subunits. Previous studies (Cupello et al., 2013; Gatta et al., 2009) showed that the GABA_A subtypes that contribute to the peak current are α6β2/3γ2, α6β2/3γ2 + α6α6β2/3γ2 and α6β2/3 incomplete receptors. So studying the peak current variations, induced by AF-16, means investigating its effects on these subtypes.

Because the AF-16 percentage effect is predominant in the neurites and in the cell body, the AF probably affects the receptor subtypes composed of the α6 subunit, which is mainly expressed in the plasma membrane of the soma and neurites (Merlo et al., 2004).

In order to test this hypothesis, we performed measurements with the pharmacological molecule furosemide, a selective blocker of GABA_A receptor subtypes composed of α6 subunit (Korpi et al., 1995; Korpi & Lüddens, 1997).

We used a furosemide concentration of 1.0 mM for blocking only α6β2/3γ2 containing GABA_A receptors, being the IC_{50} 10μM, as shown by Korpi et al. (Korpi et al., 1995). In fact, to block α6β2/3γ2 subtypes, higher concentrations are necessary, being the IC_{50} > 3 mM (Korpi et al., 1995). When furosemide 1.0 mM was perfused with GABA, the peak current decreased.

AF-16 increases the peak current evoked by the activation of GABA_A receptors after the photorelease of RuBi-GABA (Figure 4a), but furosemide almost entirely blocks the AF-16 effect (Figure 4b,c and Table 3).

The fact that AF selectively stimulates the activity of GABA_A receptors of composition α6β2/3γ2, and not that of GABA_A receptors of different subunit composition, is interesting. These receptors are specific for cerebellar granule neurons (Farrant & Nusser, 2005). They have specific pharmacology; they are not sensitive to 1,4-benzodiazepines. Particular pharmacology could be of limited interest if we think about the endogenous mechanisms of the nervous tissue in situ. However, it indicates a particular structure and biochemistry: In fact, the α6β2/3γ2 receptors have different characteristics from those α6β2/3γ2. They have a higher affinity for the endogenous ligand GABA (Sigel & Baur, 2000), different deactivation and different desensitization kinetics (Tia et al., 1996). This prevalence of the α6β2/3γ2 receptor in cerebellar granule neurons could be explained by the need for these receptors, which act by mediating phasic inhibition. Such receptors could act on top of the tonic inhibition mediated by α1(α6)β2/3δ, which are sensitive to very low extracellular concentrations of GABA (≤0.5 μM). This feature may be essential for the proper functioning of the glomeruli structure in the cerebellum.

We have recent evidence of the presence of the GABA_A receptor α6 subunit in enteric ganglia neurons (own unpublished results). A specific action of local GABA on such neurons may be part of the antisecretory activity of AF. This aspect must be a subject for our future investigations.

5 CONCLUSIONS

We applied the uncaging technique to study variations in the receptor responses in a region of interest (ROI) after treatment with AF.

The AF effect, previously tested in CGCs, facilitates the activity of GABA_A receptors.

The uncaging method validates the data obtained with the classical electrophysiological techniques and verifies the importance of AF in several neuronal areas. Moreover, it highlighted a different action on different
ROIs, revealing a specificity on the GABA$_A$ receptor subtypes containing the $\alpha_6$ subunit.

This topic could be extended to a deeper study on various receptor subtypes using a combination of other pharmacological molecules, for example, the benzodiazepine antagonist flumazenil. Such a combination might explain how the AF-16 effect may change in different parts of the neuron.

Moreover, advanced microscopy combined with selectively labeling GABA$_A$ receptor subunits could shed light on how AF modifies the distribution of receptors subtypes in different neuronal areas. Comparing the data acquired with electrophysiological measurements with high-resolution microscopy could significantly improve the comprehension of the AF-16 mechanism of action.

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CONFLICT OF INTEREST
The authors declare no competing financial interests.

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