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$GABA_A$ receptor modulation by the Antisecretory Factor on cerebellar granule cells

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Summary. — The Antisecretory Factor (AF, Mw 41kD) is an endogenous protein, found in all mammalian tissues. AF acts *in vivo* by counteracting intestinal hypersecretion and various forms of inflammations. The detailed *in vitro* AF action on the cellular level is unknown. Using an *in vitro* neuronal model (granular cells from rat small brain) we studied AF action on ionotropic $GABA_A$ receptors. A facilitatory activity, due to increased $GABA_A$ receptors expression, was demonstrated on the neuronal plasma membrane in response to AF influence. This effect may result in inhibition of intestinal secretomotor cells.

1. – Introduction

The antisecretory factor (AF), a 41 kDa endogenous protein produced and stored in the pituitary gland, was initially described by Stefan Lange and Ivar Lonnroth in the mid 1980s. The AF protein has been cloned and sequenced by [1]. The discovery of AF was the result of searching for a substance that improved the intestinal immune defence to diarrhoea caused by cholera toxin [2-4]. In fact endogenous AF secretion increases after exposure to bacterial toxins and an increase in AF secretion in combination with an inflammatory reaction may be a part of normal defense against the secretory and inflammatory component in diarrhoea and other pathological processes involving membrane leakage and inflammation. AF is excreted in plasma and other tissue fluids in mammals, its immunohistological distribution suggests a role in the immune system [5]. The AF protein has a powerful ability to counteract different types of secretion. The clinical importance has been studied in different diseases where a disturbed fluid homeostasis is of vital importance, *e.g.* different bowel diseases, diarrhoea and Meniere's syndrome. The active site of AF (16 amino acids derived from amino acids no. 36-51 of the full length AF protein) is located in the amino terminal part of the AF protein [6]. The detailed, cellular mechanism of AF action *in vivo* remains to be described, but some

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of previous experimental work tentatively suggests either a direct block of cell volume sensitive anionic channels [7] or an action via the enteric nervous system [8, 9].

On the other hand, influences of AF on $GABA_A$ receptor mediated inhibition have been demonstrated in the rat hippocampus [10, 11]. The possibility is that this protein may exert effects on the central nervous system as well, via interaction with neuronal $GABA_A$ receptors. Thus, it is of interest to investigate the effects of AF on native neuronal $GABA_A$ receptors of a defined subunit composition. The aim of the present study was to describe the effects of AF-16 on the $GABA_A$ receptors present on rat cerebellar granule cells. In particular, the patch clamp technique is used and the effect of AF-16 on the chloride current activated by the neurotransmitter is determined. GABA neurons and $GABA_A$ receptors are abundantly present in the enteric nervous system [12, 13]. However, in contrast to the central nervous system, the enteric nervous system $GABA_A$ receptor activation often gives rise to excitation of the target neurons [12-14]. For this reason and for the rather intricate circuit connections within the enteric plexuses [15, 16], it is not straightforward to translate modifications of enteric $GABA_A$ receptor activity into final, functional effects on the intestinal secretion. It is, however, important to verify the effects of AF-16 on $GABA_A$ receptors of defined subtypes in the enteric nervous system, in order to formulate plausible hypotheses of its final, regulative action on the water and ion transport in the small intestine.

In any case, the positive or negative modulating activity on the $GABA_A$ receptors might result in different end effects concerning inhibition or excitation of neurons, since the final influence is determined by the electrochemical gradient for chloride ions in these cells.

2. – Methods

AF-16 peptide production. – The AF-16 peptide consisting of the amino acid sequence 36-51 (VCHSKTRSNPENNVGL) of the AF protein, was synthesized with solid phase synthesis by Ross-Petersen AS, Copenhagen, Denmark, as previously described [7].

Granule cell cultures. – Granule cells were prepared from eight-day-old Sprague-Dawley rats as described previously [17]. The cells were studied from the 6th to the 12th day *in vitro*. Experimental procedures and care of the animals were according to the EU Parliament and Council of September 22nd 2010 (2010/63/EU), they were approved by the Italian Ministry of Health (protocol number 2207) according to D.M. 116/1992. All efforts were made in order to minimize animal suffering and the number of animals necessary in order to obtain reliable results.

Whole-cell patch-clamp studies. – The membrane currents were measured with the standard whole-cell patch-clamp as previously described [17].

The internal and external solutions for electrophysiological measurements were performed as previously reported [18]. In all experiments, the peak currents evoked by $10\,\mu$M GABA, according to the various experiments, in the presence of AF-16 were referred to those activated by plain GABA in the same cell. Thus, the averages reported in the figures represent the percent changes of currents in the presence of AF-16 at various concentrations ($1\,$pM–$10\,\mu$M) in comparison with the control (plain GABA) currents. In particular, the enhancement $E\%$ were expressed as

$$E\% = 100\frac{I_{AF,GABA} - I_{GABA}}{I_{GABA}}, \tag{1}$$

where $I_{AF,GABA}$ represents the chloride current in the presence of AF-16 plus GABA and I_{GABA} is the one with plain GABA.

Dose response curves were fitted to the following equation:

$$E\%(C_{AF}) = \frac{E\%_{Max} \cdot C_{AF}^n}{(C_{AF}^n + k^n)}, \tag{2}$$

where C_{AF} is AF-16 concentration and $E\%_{Max}$ represents the saturation level of $E\%$.

In the experiments, AF-16 was preincubated 3 min before application of GABA. Evaluation of the control chloride current peak due to plain GABA was before AF-16 application and after its washout. Experiments were taken into account when the two were consistent.

AF-16 was always co-applied with GABA after the preincubations. In some experiments, however, 3 min 0.01 μM AF-16 preincubation was followed by 2 min washout of AF-16, and after this washout, application of plain GABA took place. In a few experiments, AF-16 was in the intracellular solution at a concentration of 1 μM in order to test whether it had any effect on GABA activated current from the cell's inside.

All chemicals, apart from AF-16, were purchased from Sigma Chemicals Co., St Louis, MO, USA.

Immunocytochemistry. – After incubation with 1 μM AF-16 at 37 °C for 1 h the cells, seven *days in vitro*, 7 DIV, were fixed in 4% paraformaldehyde in PBS buffer from Sigma Aldrich (Milano, Italy) for 15 min at room temperature, washed three times with PBS solution, permeabilized with 0.1 Triton X-100 from Sigma Aldrich in PBS + BSA (Bovine Serum Albumine, from Sigma Aldrich, Milano, Italy) for 5 min and incubated overnight at 4 °C with primary antibody and successively with immunofluorescent labeling as previously described [18].

Fluorescence image acquisition was performed by a multi-channel Leica TCS SP5 laser-scanning confocal microscope, equipped with 458, 476, 488, 514, 543 and 633 nm excitation lines. Images were taken through a plan-apochromatic oil immersion objective 63 × /1.4. Leica "LAS AF" software package was used for acquisition, storage and visualization.

The density of subunit $\gamma 2$ of GABA$_A$ receptor in control and treated samples was evaluated by immunocytochemistry using the same concentrations and time of incubation of primary and secondary antibodies for both samples and analyzed with Leica TCS SP5 microscopy.

Many images of different granule cells of control and treated with AF peptide were taken for each sample.

Acquired images were analysed with Fiji Is Just Imagej software.

The receptor density was evaluated by fluorescence average, estimated in two different ways: choosing regions of interest (ROIs) that include the whole granule cells or selecting marginal plasma membrane neuronal regions.

Statistical analysis. – Final data are given as mean ± SEM. Statistical comparisons were made by Students t-test.

3. – Results

Electrophysiological results: peak component of the GABA activated current. – Pretreatment of the cells with AF-16 for 3 min caused a statistically significant increase

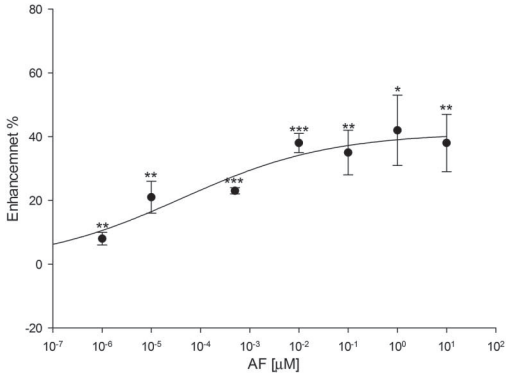

Fig. 1. – Dose-response curve for AF-16 enhancement ($E\%$) of 10 μM GABA induced peak chloride current. The closed circles represent effects after 3 min perfusion of AF-16. Vertical bars represent SEM's. Number of experiments was at least three in all cases. Statistical significance of $E\%$ increase is reported as asterisks: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Adapted from [18].

of the peak chloride current at all the concentrations studied in the 1 pM–10 μM range (always: $p < 0.01$ or $p < 0.001$; only for 1 μM AF-16: $p < 0.05$, fig. 1). No effect was found on the basal (GABA independent) chloride current.

Figure 2 presents an example of the recordings obtained with a 3 min long pre-treatment with 0.01 μM AF-16. After washout of the AF-16 peptide, the GABA current went back to its original level. This occurred in all experiments performed.

A saturating dose-response curve was obtained for the stimulatory effect of AF-16 in that range after a 3 min long incubation period and co-application with GABA (fig. 1). The fit of the data with eq. (2) yielded a low k of 41 pM, an $E\%_{Max}$ of 37% and $n = 0.64$. In part of the experiments, AF-16 was pre-incubated 30 s at concentrations of 0.01–10 μM before application of GABA. In this case there is a statistically significant increase of the peak chloride current starting from 0.1μM. However, this effect is much lower than the one with 3 min incubation ($p < 0.01$) (Data not shown).

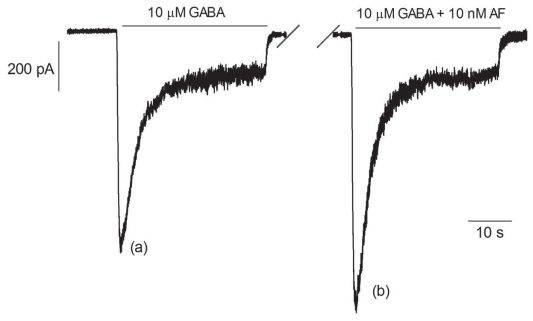

Fig. 2. – Example of the effect of preincubation of cells with 0.01 μM AF-16 for 3 min on the peak chloride current evoked by 10 μM GABA. The recording to the left is the current activated by plain 10 μM GABA (a); the recording to the right is that of application of AF together with GABA after 3 min perfusion with AF 0.01 μM AF-16 (b). Adapted from [18].

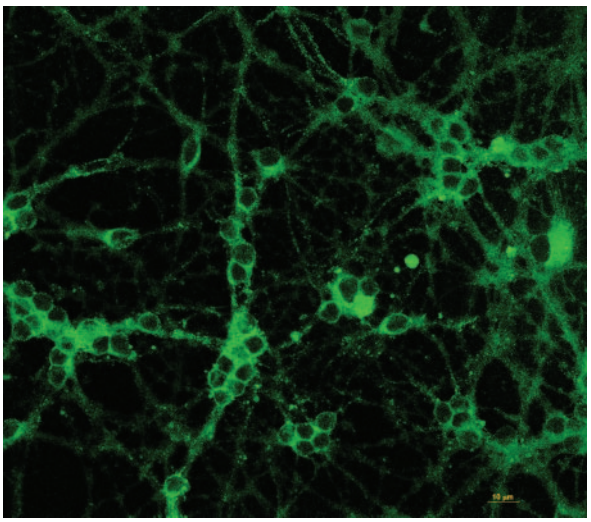

Fig. 3. – Confocal image showing the $\gamma 2$ subunit containing GABA$_A$ receptors distribution in granule cells permeabilized with 0.1% Triton X-100 for 5 min before incubating with the primary antibody. Bar = 10 μm.

In another set of experiments, 1 μM AF-16 was only added to the intracellular medium. After such an addition, the chloride current evoked by 10 μM GABA was recorded after breaking of the neuronal membrane in the whole cell configuration. This high AF-16 concentration was used so as to favour its rapid diffusion into the cell body. In these experimental conditions, no increase of the GABA current was found ($-3.0 \pm 2.0\%$, $n = 3$, ns). Furthermore, a 3 min long preincubation with external 0.01μM AF-16 followed by a 2 min washout did not result in a disappearance of the effect. Instead, the effect persisted ($+27.0 \pm 8.0\%$, $n = 5$, $p < 0.01$).

Immunocytochemical results. – Membrane expression of GABA$_A$ receptor $\gamma 2$ subunit was quantified by immunolabeling (fig. 3). The distribution of the $\gamma 2$ subunit in control granule cells and in cells pretreated with 1 μM AF-16 was quantified in terms of immunoreactivity density on cells' membranes. Indeed the membrane distribution of the $\gamma 2$ subunit of GABA$_A$ receptors yielded a highly significant ($p < 0.001$) average increase by $+17.4 \pm 0.4\%$ ($n = 32$) after AF-16 treatment. When the calculations were extended to comprehend also the cells' interior, the level of $\gamma 2$ subunit immunoreactivity resulted still increased by AF ($+16.2 \pm 0.3\%$, $n = 37$; $p < 0.001$) (Data not shown).

In fig. 4 immunofluorescence pictures demonstrate the expression of flotillin-1 in cerebellar granule cells: the distribution of patches of flotillin-1 localized in the plasma membrane.

4. – Discussion

In the present study we observed an increase, induced by AF-16, of the peak chloride current evoked by GABA in cultured cerebellar granule cells. This effect of AF-16 is dependent on concentration and incubation time, and demonstrates a low k of 41 pM. The fact that the AF-16 stimulated action persists for some minutes after washout tentatively

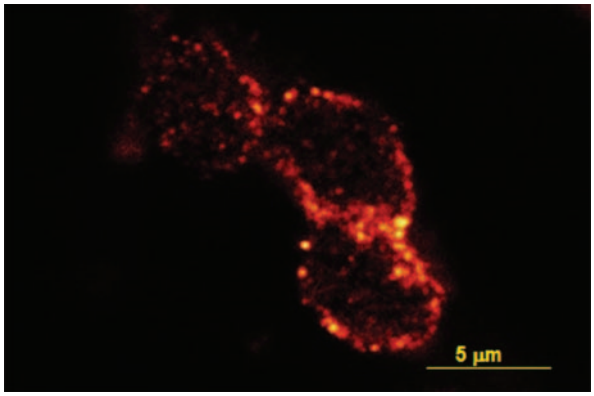

Fig. 4. – Confocal image showing flotillin-1 distribution in granule cells permeabilized with 0.1% Triton X-100 for 5 min before incubating with the primary antibody. Bar = 5 μm.

suggests that a biochemical cascade reaction is started, which results in an increased function or presence of $GABA_A$ receptors at the cellular membrane. The immunocytochemical data demonstrate an increased presence of the $GABA_A$ receptor $\gamma 2$ subunit in the cells, as receptors on their way to membrane expression but particularly as expressed receptors on the cell plasma membrane. This subunit is characteristic of the synaptic $GABA_A$ receptor population which generates the peak component of the GABA evoked current [19, 20]. Therefore there is evidence that the AF effect is due to an increased expression/function of $GABA_A$ receptors at the cellular membrane surface. This action most probably involves a biochemical cascade of events. A possibility to be investigated is the involvement of flotillin-1 which was shown to participate in neurotransmitter receptors' membrane insertion at synapses [21]. In fact, AF has been shown to interact with it [22] and indeed flotillin-1 is expressed in patches on the membranes of the cerebellar granule cells (fig. 4).

Flotillins have been implicated in myriad processes that include endocytosis, signal transduction and regulation of the cortical cytoskeleton, yet the molecular mechanisms that underlie flotillin function in these different cases are still poorly understood [23]. Our data also show that AF-16 does not seem to act on the cellular inside, but evidently interacts with membrane structures or proteins located on the external part of the plasma membrane. The level of increase by AF of the peak GABA activated current (around 40%) and the lower increase of the $\gamma 2$ subunit expression as evaluated by immunocytochemistry (16–18%) suggest that the increase of the maximal current evoked by GABA is partly due to increased membrane expression of the receptors and partly due to increased functionality.

The AF-16 mediated modulation of $GABA_A$ receptor activity in central nervous system neurons has been studied in hippocampal slices [10, 11].

Moreover in [10] evidence has been provided that AF could be a negative modulator of GABA ergic transmission in the hippocampus and that this protein may be involved in a gut-brain loop controlling intestinal secretion and inflammation.

It is becoming increasingly evident that bidirectional signalling exists between the gastrointestinal tract and the brain. This relationship, commonly dubbed the gut-brain axis involves various afferent and efferent pathways such as the vagus nerve and the hypothalamic-pituitary-adrenal pathway to regulate aspects of homeostasis such as

satiety and hunger, and inflammation. Disruption of the gut-brain axis has been shown to be involved in the pathogenesis of a diverse range of diseases, including Parkinson disease and irritable bowel syndrome. Although the majority of data to-date is from animal models, this emerging area of research is evolving quickly and with promising value.

In that case, as in ours, the possibility that these effects are aspects of a gut-brain axis cannot be neglected. There is a different end result by AF action resulting in less inhibition in hippocampal pyramidal cells and more inhibition in cerebellar granule cells. This may be explained in terms of different cellular mechanisms and function within the relevant circuits in which the two types of cells are involved. In conclusion, the AF system has been shown to counteract intestinal hypersecretion [24, 25], inflammatory events [26-28] and raises in intracranial pressure [29, 30]. It has also probably a role in counteracting endolymph hyperproduction in the inner ear as implied by results in clinical trials in Meniere's disease [31, 32]. In all cases a prominent feature is the control of fluid balance in different body organs. Further work is needed in order to establish whether the AF system [24] is also involved in brain activity modulation.

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REFERENCES

[1] Johansson E., Lönnroth I., Lange S., Jonson J., Jennische E. and Lönnroth C., *J. Biol. Chem.*, **270** (1995) 20615.
[2] Lönnroth I. and Lange S., *FEBS Lett.*, **177** (1984) 104.
[3] Lönnroth I. and Lange S., *Biochim. Biophys. Acta.*, **883** (1986) 138.
[4] Johansson E., Jennische E., Lange S. and Lönnroth I., *Gut*, **41** (1997) 642.
[5] Lange S., Jennische E., Johansson E. and Lönnroth I., *Cell Tissue Res.*, **296** (1999) 607.
[6] Johansson E., Lange S. and Lönnroth I., *Biochim. Biophys. Acta.*, **1362** (1997) 177.
[7] Rapallino M. V., Cupello A., Lange S. and Lönnroth I., *Acta Physiol. Scand.*, **179** (2003) 367.
[8] Lönnroth I., Lange S. and Skadhauge E., *Comp. Biochem. Physiol.*, **90A** (1988) 611.
[9] Grøndahl M. L., Sørensen H., Unmack M. A., Holm A. and Skadhauge E., *J. Comp. Biochem. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.*, **188** (2002) 589.
[10] Kim M., Wasling P., Xiao M.-Y., Jennische E., Lange S. and Hanse E., *Regul. Pept.*, **129** (2005) 109.
[11] Strandberg J., Lindquist C., Lange S., Asztely F. and Hanse E., *Front. Cell Neurosci.*, **8** (2014) 1.
[12] Krantis A., *News Physiol. Sci.*, **15** (2000) 284.
[13] Galligan J. J., *Neurogastroenterol. Motil.*, **14** (2002) 61.
[14] Reis H. J., Vanden Berghe P., Romano-Silva M. A. and Smith T. K., *Neuroscience*, **139** (2006) 485.
[15] Gwynne R. M. and Bornstein J. C., *Curr. Neuropharmacol.*, **5** (2007) 1.
[16] Furness J. B., Callaghan B. P., Rivera L. R. and Cho H.-J., *Microbial Endocrinology: the Microbiota-Gut-Brain Axis in Health and Disease*, in *Microbial Endocrinology*, edited by Lyte M. and Cryan J. F. (Springer, New York) 2014, pp. 39–71.
[17] Robello M., Amico C. and Cupello A., *Neuroscience*, **53** (1993) 131.
[18] Bazzurro V., Gatta E., Cupello A., Lange S. and Robello M., *J. Mol. Neurosci.*, **64** (2018) 312.
[19] Farrant M. and Nusser Z., *Nat. Rev. Neurosci.*, **6** (2005) 215.

[20] Cupello A., Di Braccio M., Gatta E., Grossi G., Nikas P., Pellistri F. and Robello M., *Neurochem. Res.*, **38** (2013) 2453.
[21] Bodrikov V., Pauschert A., Kochlamazashvili G. and Stuermer C. A. O., *Exp. Neurol.*, **289** (2017) 31.
[22] Johansson E., Jonson I., Bosaeus M. and Jennische E., *Reg. Pept.*, **146** (2008) 303.
[23] Otto G. P. and Nichols B. J., *J. Cell. Sci.*, **124** (2011) 3933.
[24] Lange S. and Lönnroth I., *Int. Rev. Cytol.*, **210** (2001) 39.
[25] Zaman S., Aamir K., Lange S., Jennische E., Silfverdal S. A. and Hanson L. Å., *Acta Pediatr.*, **103** (2014) 659.
[26] Davidson T. S. and Hickey W. F., *J. Leukoc. Biol.*, **76** (2004) 835.
[27] Davidson T. S. and Hickey W. F., *Lab. Invest.*, **84** (2004) 307.
[28] Graber D. J., Harris B. T. and Hickey W. F., *J. Neuroinfl.*, **8** (2011) 122.
[29] Jennische E., Bergström T., Johansson M., Nystrm K., Tarkowski A., Hansson H. A. and Lange S., *Brain Res.*, **127** (2008) 189.
[30] Al-Olama M., Lange S., Lnnroth I., Gatzinsky K. and Jennische E., *Acta Neurochir.*, **157** (2015) 129.
[31] Hanner P., Rask-Andersen H., Lange S. and Jennische E., *Acta Otolaryngol.*, **130** (2010) 223.
[32] Leong S. C., Narayan S. and Lesser T. H., *Ann. Otol. Rhinol. Laryngol.*, **122** (2013) 619.