

# Modulation of the expression of GABA<sub>A</sub> receptors in rat cerebellar granule cells by protein tyrosine kinases and protein kinase C

Raffaella Balduzzi<sup>a</sup>, Aroldo Cupello<sup>b</sup>, Mauro Robello<sup>a,\*</sup>

<sup>a</sup>Unità INFM, Dipartimento di Fisica, Università di Genova, Via Dodecaneso 33, 16146 Genoa, Italy

<sup>b</sup>Centro di Neurofisiologia Cerebrale, C.N.R., Genoa, Italy

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## Abstract

The expression of GABA<sub>A</sub> receptors in rat cerebellar granules in culture has been studied by  $\beta_{2/3}$  subunit immunocytochemistry and fluorescence confocal microscopy. These cells show labeling all over the cell bodies' plasma membrane and dendrites. Treatment with the protein tyrosine kinase (PTK) inhibitor genistein results in a decrease of the labeling associated with the  $\beta_{2/3}$  subunit in both cell bodies and dendrites. No effect was found with an inactive genistein analogue, daidzein. A similar effect was found with a protein kinase C (PKC) activator, phorbol myristate acetate (PMA). The effects of genistein and PMA are additive. The interpretation of the results is that PTK inhibition blocks exocytotic deposit of newly synthesized GABA<sub>A</sub> receptors onto the neuronal plasma membrane. On the other hand, PKC activation speeds up endocytotic removal of GABA<sub>A</sub> receptors. © 2002 Published by Elsevier Science B.V.

**Keywords:** GABA<sub>A</sub> receptor; Granule cell; Immunocytochemistry; Protein tyrosine kinase; Protein kinase C

## 1. Introduction

A phenomenon repeatedly described in the literature is the so called “run-down” of the GABA<sub>A</sub> receptor function when neurons in culture are registered in the whole-cell configuration of the patch-clamp technique in the absence of ATP in the pipette medium [1–3]. This phenomenon consists in a gradual decrease of the amplitude of the chloride currents activated by the application of GABA. This phenomenon, in the case of GABA<sub>A</sub> receptors of cerebellar granule cells in culture, is completed within around 30 min from the obtainment of the whole cell configuration [3]. Since this phenomenon is prevented by including ATP–Mg<sup>2+</sup> in the recording pipette, it has been suggested that a phosphorylation/dephosphorylation event is involved [2].

In a previous report, our group suggested that in cerebellar granules in culture, this phenomenon is due to removal of phosphate groups from protein tyrosine residues [4]. These residues are not necessarily part of the GABA<sub>A</sub> receptors subunits; they may be part of proteins interacting with those receptors. In another study, we demonstrated that also in recombinant rat GABA<sub>A</sub> receptors expressed in

*Xenopus* oocytes, the protein tyrosine kinase (PTK) inhibitor genistein caused a reduction of the plasma membrane expression of such receptors [5].

Here we report that in cerebellar granules in culture, genistein causes a reduction of the expression of GABA<sub>A</sub> receptors in both dendrites and the cell body plasma membrane. A similar effect is caused by phorbol myristate acetate (PMA), an activator of protein kinase C (PKC). The two effects are additive and proceed independently.

## 2. Materials and methods

### 2.1. Cells culture

Granule cells were prepared from cerebella of 8-day-old Wistar rats following the procedure of Levi et al. [6], as previously described [3]. Briefly, the minced tissue was first suspended in trypsin (0.25 mg/ml, Type III Sigma) for 15 min at 37 °C in a shaking water bath, and then in deoxyribonuclease and trypsin inhibitor. Finally, it was dispersed by gently drawing it into a fire-polished Pasteur pipette. Cells were resuspended in basal Eagle's medium with Earle's salts supplemented with 10% fetal calf serum (Gibco Bio-Cult, UK), 25 mM KCl, 2 mM glutamine and

\* Corresponding author. Tel.: +39-10-3536316; fax: +39-10-314218.

E-mail address: robello@ge.infm.it (M. Robello).

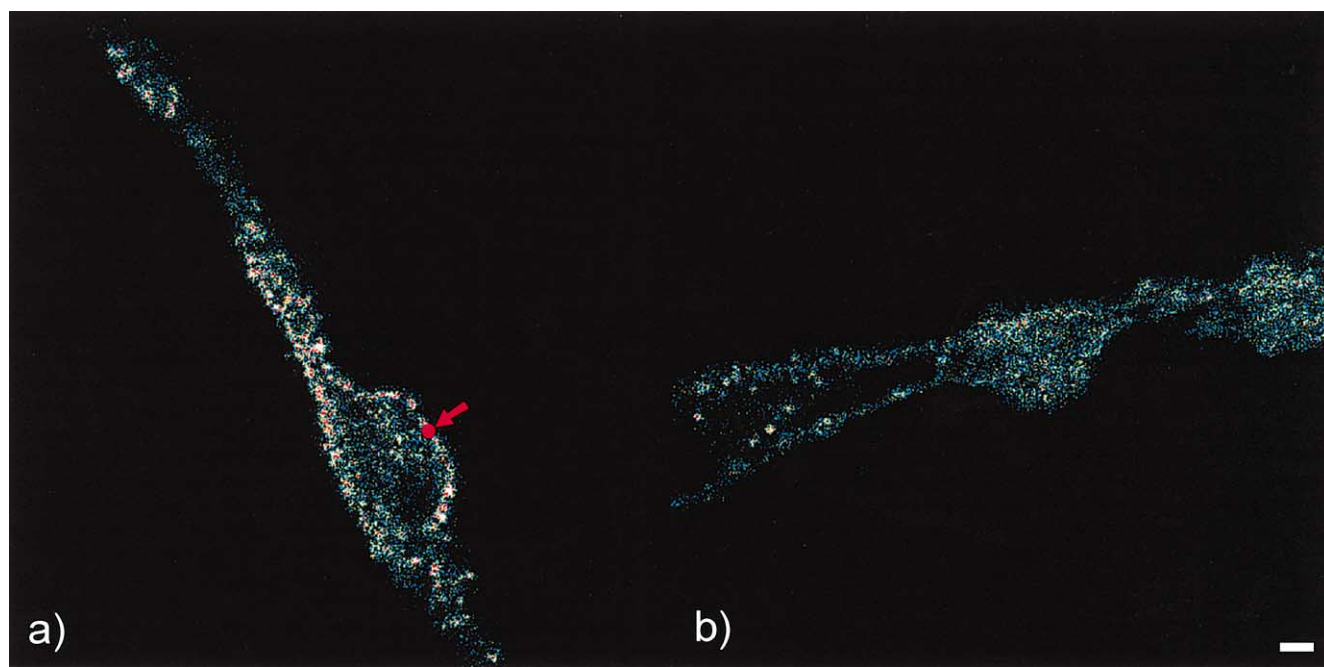


Fig. 1. Representative confocal images showing  $\beta_{2/3}$  subunit containing GABA<sub>A</sub> receptors distribution in a control cell (a), and in another cell incubated with genistein 200  $\mu$ M for 60 min (b). In image (a), the 52-pixel circle, indicated by the arrow, represents the typical placement of the ROI circles along the cell body membrane. Bar=1  $\mu$ m.

100  $\mu$ g/ml gentamicine and plated on poly-L-lysine-coated glass cover slips placed in 10 mm sterilized glass dishes at a density of  $1 \times 10^6$  per dish and kept at 37 °C in a humidified 95% air–5% CO<sub>2</sub> atmosphere. Experiments were performed 5 days after plating.

## 2.2. Treatments of granule cells

Five days after plating, cells were incubated at 25 °C in 100 or 200  $\mu$ M genistein for 20, 40 and 60 min. Genistein, that had been previously dissolved in DMSO as a 100-mM

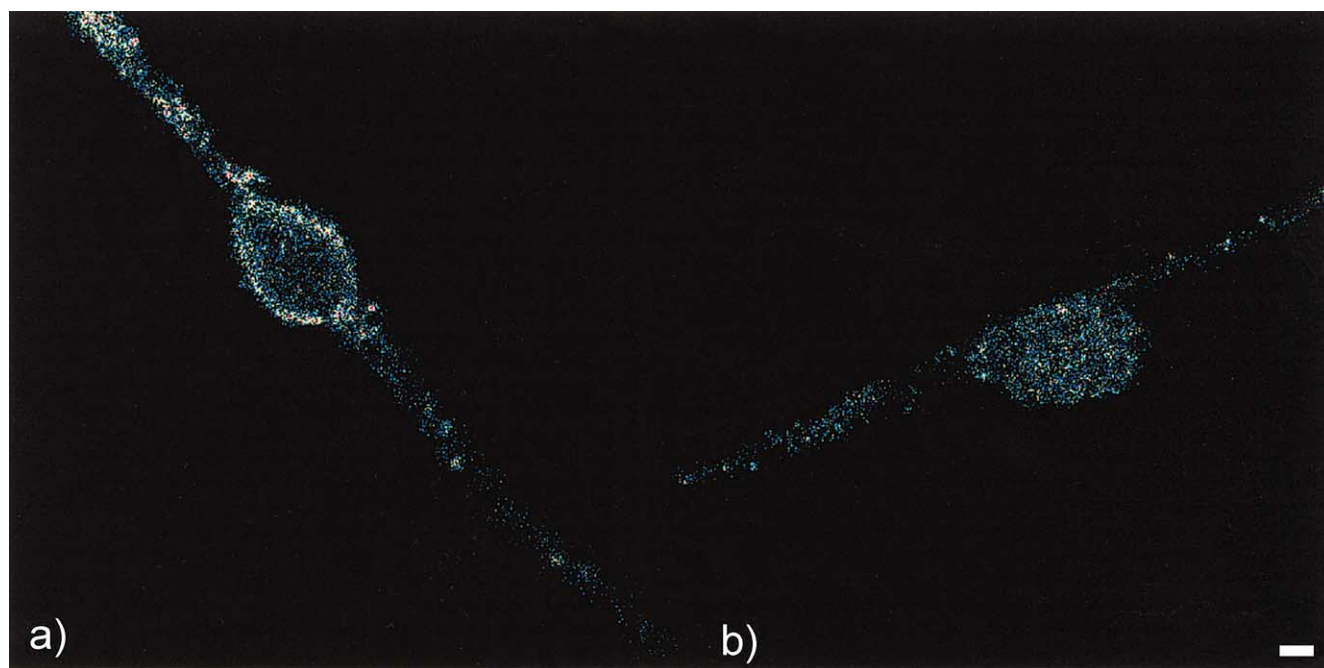


Fig. 2. Confocal images showing the  $\beta_{2/3}$  subunit containing GABA<sub>A</sub> receptors distribution in granule cells permeabilized with 0.1% Triton X-100 for 5 min before incubating in the primary antibody. (a) Control cell, and (b) cell incubated with genistein 200  $\mu$ M for 20 min. Bar=1  $\mu$ m.

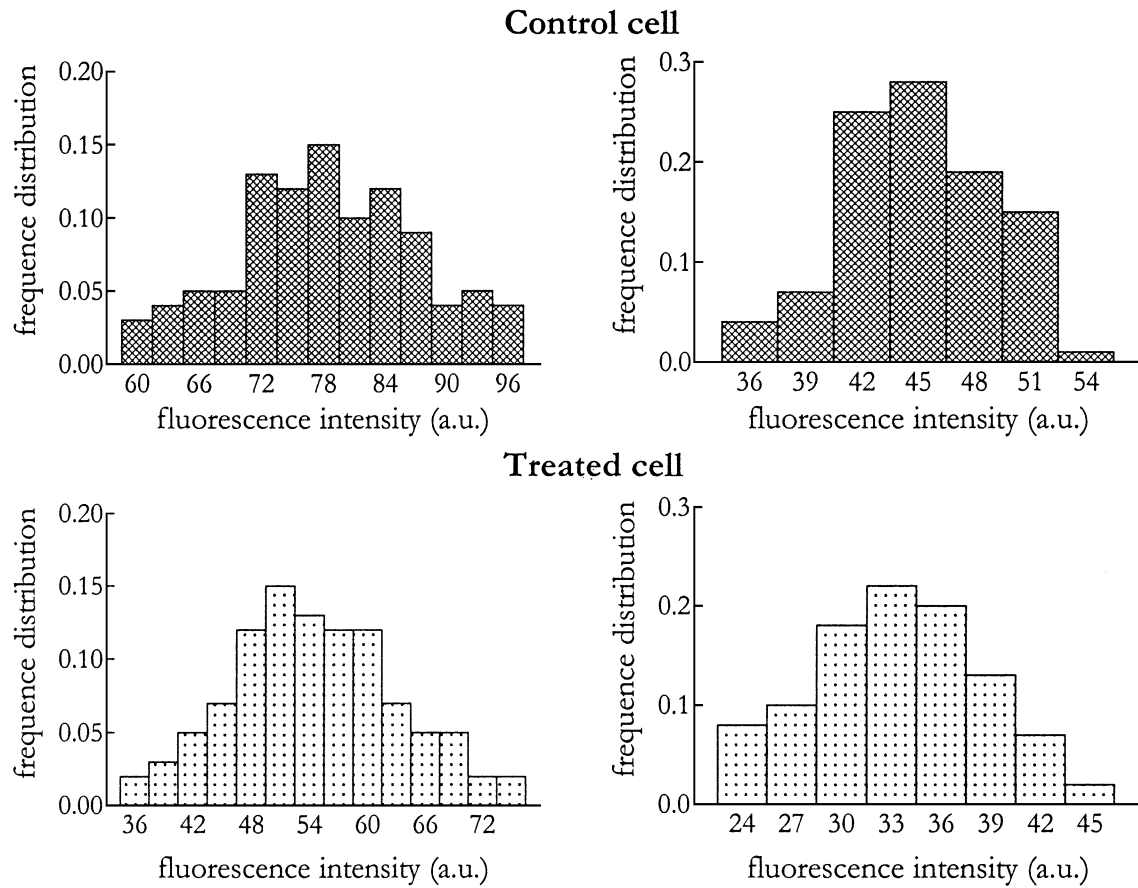


Fig. 3. Histograms of fluorescence intensity (a.u.) obtained from the cells shown in Fig. 1. Fluorescence values obtained from the control cell and the cell treated with genistein 200  $\mu$ M for 60 min for the cell body membrane (left) and for the dendrites (right).

stock solution, was added to the basal Eagle's medium to obtain the final concentration.

Some cells were incubated at 25 °C with 200  $\mu$ M daidzein for 30 min. Daidzein was dissolved in DMSO and added to the basal Eagle's medium.

Other cells were incubated at 25 °C with 810 nM phorbol 12-myristate 13-acetate (PMA), or 810 nM phorbol 12-mono-myristate (PMM), or 810 nM PMA plus 200  $\mu$ M genistein, for 40 min each. PMA and PMM too were dissolved in DMSO and added to the basal Eagle's medium.

After, these treated cells were processed for immunofluorescence.

Controls were incubated in DMSO for 20, 40 and 60 min, at a concentration of either 1% or 2%, to make up for the vehicle used in the treated cells. Of course the incubation time was equal to the one used for the treated cells in the various instances.

The control cells were then incubated with both monoclonal anti- $\beta_{2/3}$  subunits and secondary antibody.

### 2.3. Immunolabeling

Cells were fixed in 3.7% paraformaldehyde in PBS buffer (0.01 M, pH 7.4) for 5 min at room temperature,

washed three times for 5 min each with the same buffer and incubated overnight at 4 °C with the mouse monoclonal antibody against  $\beta_{2/3}$  subunits of the GABA<sub>A</sub> receptor (clone bd17), concentration 1.5  $\mu$ g/ml in PBS+IgG 11.4 mg/ml (dilution 1:4). Cells were washed in PBS three times for 10 min each, incubated for 2 h at 25 °C with anti-mouse secondary antibody conjugated with Cy3 (dilution 1:400),

Table 1

Intensity of fluorescence due to GABA<sub>A</sub> receptors containing  $\beta_{2/3}$  subunit in granule cells cell body membranes and dendrites

Treatments	Cell body membrane		Dendrites	
	Mean $\pm$ S.D.	n	Mean $\pm$ S.D.	n
controls	100 $\pm$ 14	8	100 $\pm$ 14	7
genistein 100 $\mu$ M, 40 min	95 $\pm$ 8	5	102 $\pm$ 9	5
genistein 100 $\mu$ M, 60 min	48 $\pm$ 4*	5	78 $\pm$ 7*	5
genistein 200 $\mu$ M, 20 min	45 $\pm$ 6*	4	63 $\pm$ 22*	6
genistein 200 $\mu$ M, 40 min	71 $\pm$ 17*	6	71 $\pm$ 18*	7
genistein 200 $\mu$ M, 60 min	71 $\pm$ 11*	6	74 $\pm$ 10*	8
daidzein 200 $\mu$ M, 60 min	102 $\pm$ 18	4	117 $\pm$ 21	4

Data are expressed as average  $\pm$  S.D. and compared to the controls (taken as 100) and indicate the effects of genistein and its inactive analogue daidzein. n indicates the number of cells. Asterisks indicate treatments that are significantly different from untreated control cells ( $P \leq 0.05$ ).

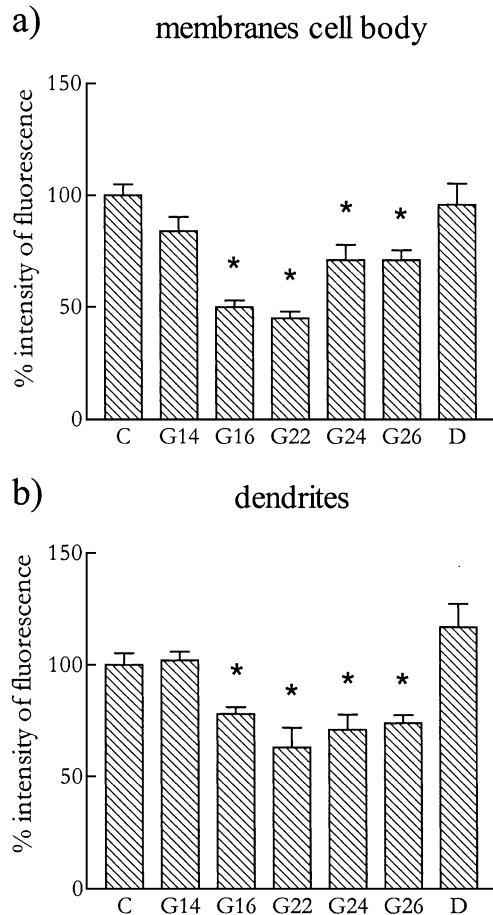


Fig. 4. Effect of genistein and daidzein on GABA<sub>A</sub> receptors distribution on cell body (a) and on the dendrites (b). Data are expressed as average  $\pm$  S.D. and compared to the controls (100%). Asterisks indicate treatments that are significantly different from untreated control cells ( $P \leq 0.05$ ). (C) control; (G14) 100  $\mu$ M genistein for 40 min; (G16) 100  $\mu$ M genistein for 60 min; (G22) 200  $\mu$ M genistein for 20 min; (G24) 200  $\mu$ M genistein for 40 min; (G26) 200  $\mu$ M genistein for 60 min; (D) 200  $\mu$ M daidzein for 60 min of incubation.

washed three times in PBS buffer for 10 min and mounted in glycerol/buffer (70:30).

In additional experiments, granules were permeabilized with 0.1% Triton X-100 for 5 min before incubating in the primary antibody.

#### 2.4. Image acquisition

The granule cells were imaged using a confocal laser scanning microscope Nikon PCM2000 (Nikon Instr., Florence, Italy), described in detail elsewhere [7]. Cy3 was excited at 543 nm using a He–Ne laser. Fluorescence was collected with a selective emission filter at 590 nm. This filter is HQ series by Chroma and is centered on 590 nm with a bandwidth of 60 nm. We used an oil immersion 100 $\times$  objective ( $N_A=1.3$ ) coupled to a 50  $\mu$ m confocal pinhole condition [8].

EZ2000 (Coord, Amsterdam, NL) software was used for acquisition, storage and visualization.

For each sample, we divided the whole cell (thickness=8–10  $\mu$ m) into 20 optical sections.

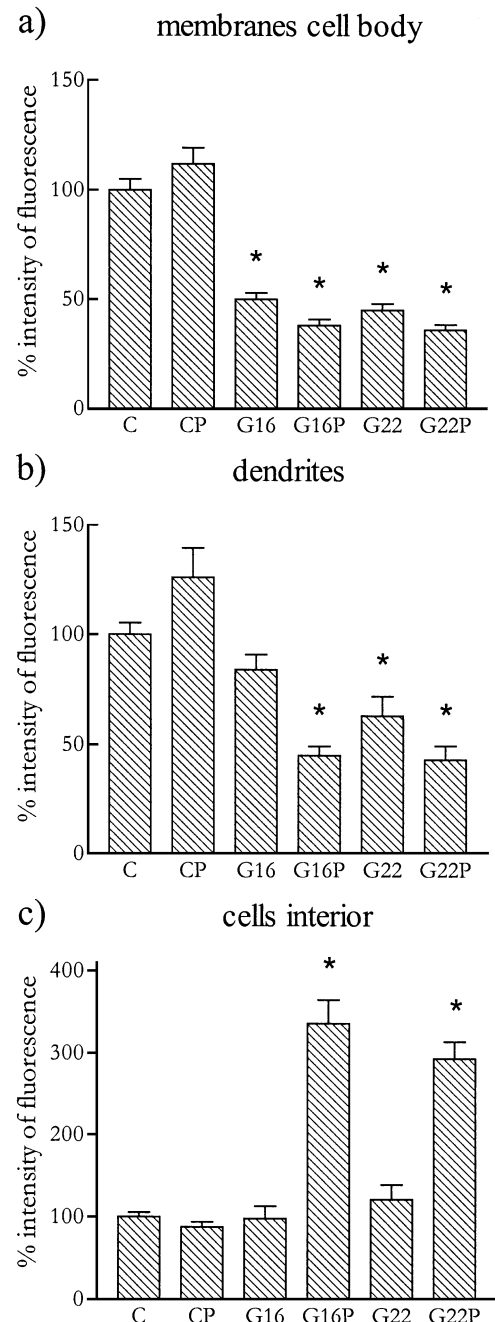


Fig. 5. Comparison of GABA<sub>A</sub> receptor distribution on cell body (a), on the dendrites (b) and on the cell interior (c), between not permeabilized [controls (C), 100  $\mu$ M genistein for 60 min (G16), 200  $\mu$ M genistein for 20 min (G22)], and permeabilized granule cells [controls (CP), 100  $\mu$ M genistein for 60 min (G16P), 200  $\mu$ M genistein for 20 min (G22P)]. Data are expressed as average  $\pm$  S.D. and compared to the controls (100%). Asterisks indicate treatments that are significantly different from untreated control cells ( $P \leq 0.05$ ).

To perform analysis, we selected for each sample, among the optical section, its middle plane. Then we performed the analysis of the fluorescence of this optical section due to the presence of GABA<sub>A</sub> receptors either on cell body membrane, cell body interior and on the dendrites. We speak of “dendrites” and not of “dendrites membrane” because we could not separate the two membranes and the cytoplasm between them; in fact in our experimental conditions, the optical resolution of the microscope (0.3  $\mu\text{m}$ ) was very similar to the diameter of dendrites.

### 2.5. Image analysis

For fluorescence intensity measurements, we used the method described in a previous work [5]. Regions of interest (ROIs) definition has been made according to Fig. 1a. The number of pixel for averaging was set at a constant and the intensity averaged value was linearly corrected for the PMTs gain parameter. We defined the ROI value considering the  $x$ – $y$  parameters of our system and the sampling (typical: 0.068–0.068  $\mu\text{m}/\text{pixel}$ ) performed during the scanning.

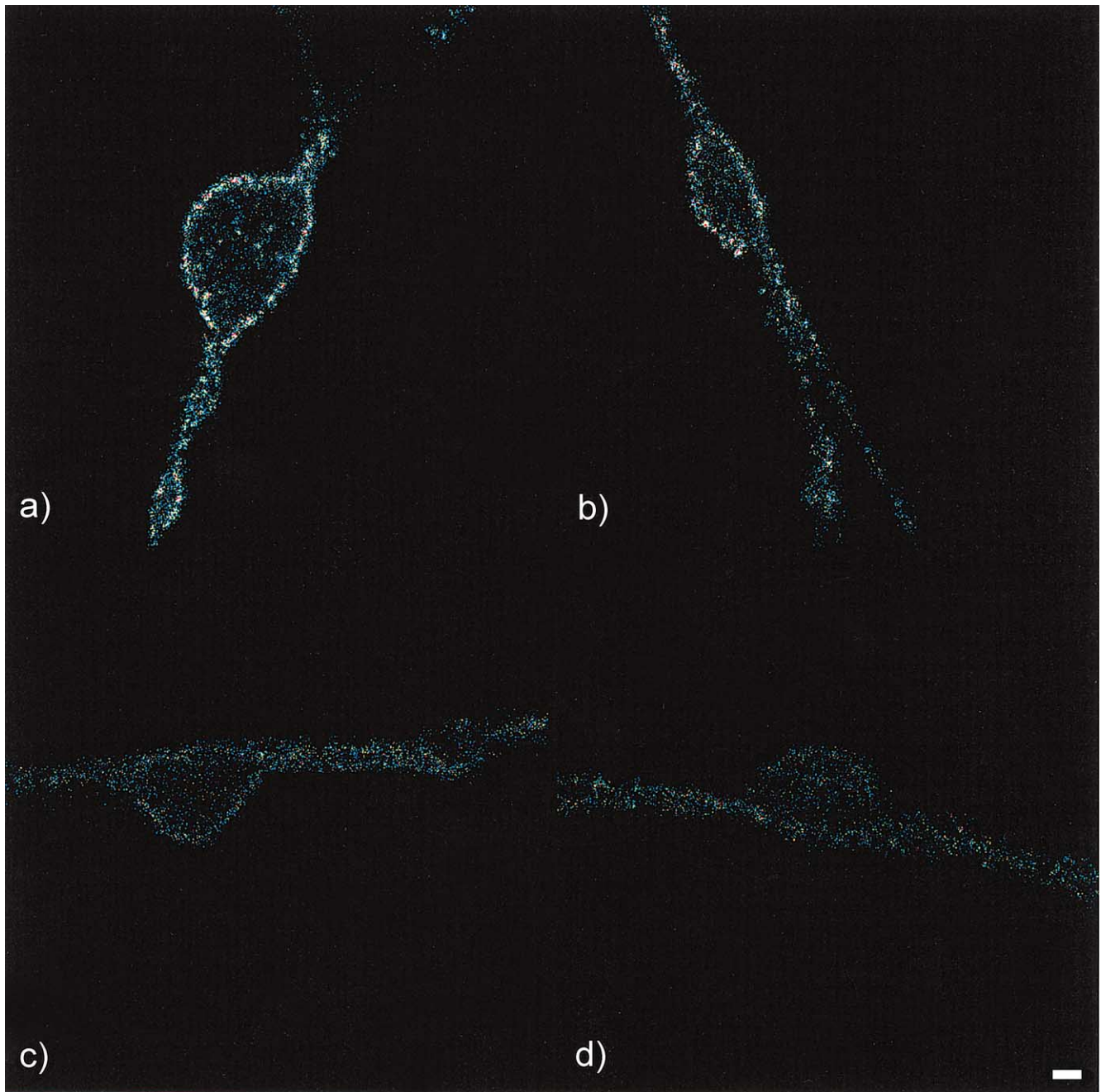


Fig. 6. GABA<sub>A</sub> receptors distribution on granule cells: not treated (a), incubated in 810 nM PMM for 40 min (b), incubated in 810 nM PMA for 40 min (c), incubated in 810 nM PMA plus 200  $\mu\text{M}$  genistein for 40 min (d). Bar=1  $\mu\text{m}$ .

Thus, intensity values are related to the average among the averaged values for each sample. Background noise measured was less than 2% of the signal.

Data were plotted and statistical analysis performed using an unpaired Student's *t*-test (GraphPad Prism version 2.01, Graph-Pad software). The criterion for significance was set at  $P \leq 0.05$ .

## 2.6. Chemicals

Cy<sup>TM</sup> 3-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) was obtained from Jackson ImmunoResearch Laboratories. Anti-GABA<sub>A</sub> receptor  $\beta_{2/3}$ -chain (clone bd17) was purchased from Boehringer Mannheim, Germany. Phosphate buffer, goat IgG, PMA, PMM and the other chemicals were from Sigma, St. Louis, MO, USA.

## 3. Results

From the beginning, we observed that untreated granule cells did not exhibit autofluorescence either on dendrites or on cell body. We started our experiments by examining GABA<sub>A</sub> receptors distribution on the cell surface: we stained the granules with anti-GABA<sub>A</sub> receptor  $\beta_{2/3}$ -chain (clone bd17) and then with anti-mouse secondary antibody conjugated with Cy3.

In the cells used as controls, fluorescence was concentrated on the membrane of the cell body and on the dendrites. The intensity of fluorescence, *i*, measured in arbitrary units (a.u.) by the method described in Materials and Methods, was  $i = 76 \pm 10$  (number of cells:  $n = 8$ ) for the membrane of the cell body and  $i = 43 \pm 6$  ( $n = 7$ ) for the dendrites. A weak fluorescence was also present inside the cell body:  $i = 8 \pm 1$  ( $n = 5$ ). Typical fluorescence in control cells is shown in Figs. 1a and 6a.

Control cells, permeabilized with 0.1% Triton X-100 for 5 min before incubating in the primary antibody, did not present a significant increase of the fluorescence in the cell body ( $i = 7 \pm 1$ ,  $n = 4$ ) (see Fig. 2a).

As shown in Fig. 1b, 60 min treatment with 200  $\mu$ M genistein caused a significant decrease of the fluorescence. In Fig. 3 we report, as an example, the histograms of the fluorescence intensity for the control cell of Fig. 1a and for the treated cell of Fig. 1b.

Overall results of the effect of genistein on the  $\beta_{2/3}$  subunit-related fluorescence on granule cells are summarized in Table 1, where the data are expressed as percentages of the controls (taken as 100).

As shown in this table, 40-min treatments with 100  $\mu$ M genistein did not influence the distribution of the GABA<sub>A</sub> receptors on the membrane of the cell body or on the dendrites. Longer treatments (60 min) with 100  $\mu$ M genistein caused a significant decrease of the fluorescence on the membrane of the cell body, and also on the dendrites (Fig. 4a and b). Two hundred micromolars genistein applied for

20, 40 and 60 min causes a significant decrease of the fluorescence both on the membrane of the cell body and the dendrites (Fig. 1b).

It is important to stress that the fluorescence, at this genistein concentration, tends to be lower for the 20-min treatment and to increase for the 40- and 60-min treatments (see Fig. 4a and b).

The effect of genistein is most probably due to a blockade of PTKs since 200  $\mu$ M daidzein, an inactive genistein analogue, did not affect GABA<sub>A</sub> receptors even with a 60 min incubation ( $i = 102 \pm 18\%$ ,  $n = 4$ , plasma membranes of the cell body;  $i = 117 \pm 21\%$ ,  $n = 4$ , dendrites) (see Fig. 4). In all these cells, which were not permeabilized, there was no difference in cytoplasmic fluorescence between controls and treated cells.

Since the most significant decrease of the fluorescence was with 100  $\mu$ M genistein for 60 min and 200  $\mu$ M genistein for 20 min, we permeabilized some cells with 0.1% Triton X-100 for 5 min before incubating with the primary antibody and studied the effects of such genistein treatments (Fig. 2a and b). Fig. 5 shows that the results were

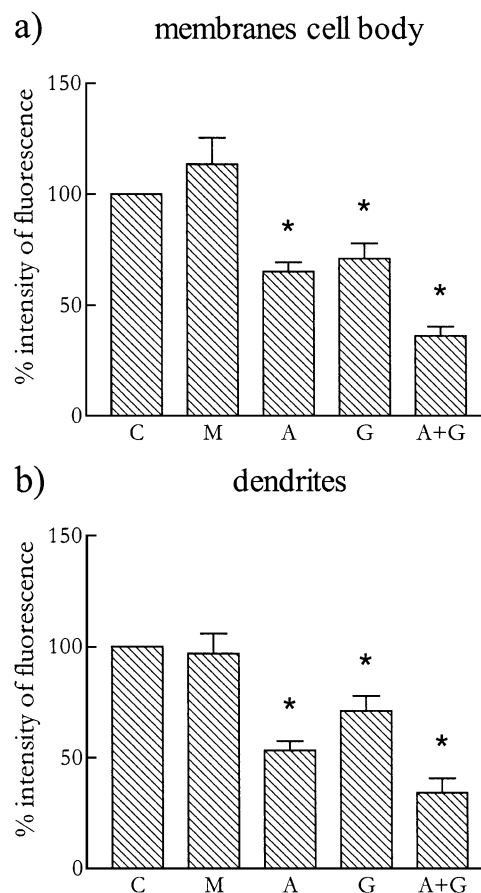


Fig. 7. GABA<sub>A</sub> receptors distribution on cell body (a) and on the dendrites (b) of granule cells: not treated (C), incubated in 810 nM PMM for 40 min (M), incubated in 810 nM PMA for 40 min (A), incubated in 200  $\mu$ M genistein for 40 min (G) and incubated in 810 nM PMA plus 200  $\mu$ M genistein for 40 min (A+G).



similar to those with cells that were not permeabilized in the case of the plasma membranes of the cell body and dendrites. However, the intensity of fluorescence increased significantly in the cell bodies' interior:  $336 \pm 37\%$  for 100  $\mu\text{M}$  genistein and  $293 \pm 32\%$  for 200  $\mu\text{M}$  genistein (Fig. 5c).

Finally, we used PMA, a PKC activator, and observed that a 40-min incubation at 810 nM causes a significant decrease of fluorescence ( $38 \pm 8\%$ ) (Fig. 6a and c). This decrease is higher ( $63 \pm 7\%$ ) when incubating with 810 nM PMA plus 200  $\mu\text{M}$  genistein for 40 min (Fig. 6d).

This suggests that the effects of the two substances are additive. The effect of PMA is due to an activation of PKCs since 810 nM PMM, an inactive PMA analogue, did not affect GABA<sub>A</sub> receptors with a 40-min incubation (see Fig. 6b). All results are summarized in Fig. 7a and b.

#### 4. Discussion

The “run-down” phenomenon of GABA<sub>A</sub> receptors has been described in neurons in culture when studied in the “whole-cell” configuration in the absence of ATP in the pipette medium [1–3]. In our hands, it was present in rat cerebellar granule cells and it was not dependent on agonist application [3]. This event may be the manifestation, in the particular condition of the whole-cell configuration in the absence of ATP, of a dynamic equilibrium, in the intact cell, between receptors exocytosis and their endocytosis/recycling [9]. In particular, in the case of our granule cell cultures in the absence of intracellular ATP, one may have a predominance of ligand-independent endocytosis [9].

The present results confirm, by a different approach, what we have found in previous experiments involving whole-cell studies of GABA-activated chloride currents. In that case, we found a “run-down” of GABA-activated chloride currents in rat granule cells by treatment with PTK inhibitors [4]; here we find a decrease of the membrane expression of GABA<sub>A</sub> receptor-associated  $\beta_{2/3}$  subunits. This effect was found in both the dendritic and cell body membranes. An important parallel effect was the increase of  $\beta_{2/3}$  subunits inside the neuronal cell bodies of permeabilized granules. The effect was not there when we used an analogue of genistein devoid of inhibitory activity on PTK, daidzein. We suggest that the mechanism of these effects is an impairment of membrane delivery of newly synthesized receptors via a block of vesicles exocytosis. It has in fact been demonstrated that PTK activity is involved in exocytotic accumulation of receptors from receptor-bearing vesicles to the plasma membrane [10,11].

The summation of this effect with that of a potent PKC activator, such as PMA, can be explained by taking into account that PKC activity plays a role in the opposite event, receptor removal from plasma membranes via endocytosis [12,13].

In other words, our results show that the two phenomena proceed independently. Under normal steady state, recep-

tors' exocytosis and endocytosis balance each other. Obviously, when exocytosis is slowed and endocytosis is accelerated, there is a strong decline of surface expression of GABA<sub>A</sub> receptors (compare Fig. 6d vs. Fig. 6a). An obvious consequence of this line of reasoning is that in the condition of the run-down of the GABA<sub>A</sub> receptors, we found electrophysiologically in granule cells in the whole-cell configuration in the absence of ATP, the overriding effect is the impairment of exocytosis by hypofunction of PTK. Evidently, PKC activity under these circumstances is a minor player; otherwise, its hypofunction in the absence of ATP would have compensated the effect due to the reduced activity of PTK.

We have previously demonstrated that genistein causes a decrease in surface expression of recombinant GABA<sub>A</sub> receptors in *Xenopus* oocytes [5]. The impairment of expression of plasma membrane receptors by decreased protein tyrosine phosphorylation applies also to NMDA receptors [14].

These results underline the existence of a dynamic equilibrium between GABA<sub>A</sub> receptors added to and removed from the neuronal plasma membrane. This balance allows a continuous regulation of the receptors' expression according to the demands of the function of the cells. In turn, these effects are probably mediated by second messengers calling into play appropriate protein kinases and/or phosphatases.

As a final remark, we used in the present experiments the “classical” approach of visualizing GABA<sub>A</sub> receptors via subunit specific primary antibodies and fluorescent secondary antibodies. Recent reports in the literature have shown that a new and alternative way is that of using receptor subunits–green fluorescent protein (GFP) chimeras to study the fate of ionotropic receptors in live cells [15,16]. This approach has been applied for instance to GABA<sub>A</sub> receptors using  $\gamma_2$ L-GFP chimeras [16] by injection of the corresponding DNA into hippocampal neurons in culture. In our view, its application to small cells such as the cerebellar granules may pose serious technical problems. However, in principle, this approach, as long as the corresponding receptors maintain the biochemical, physiological and pharmacological characteristics of the native ones [16], is certainly an extremely interesting alternative, especially in studies of GABA<sub>A</sub> receptors trafficking.

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