Inhibition of high-affinity γ -aminobutyric acid uptake in primary astrocyte cultures by phorbol esters and phospholipase C

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The effects of phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC), on high-affinity Na^+ -dependent γ -aminobutyric acid (GABA) uptake were investigated in primary cultures of neurons and glial cells from rat brain cortex. Incubation of glial cells with PMA led to concentration- and time-dependent decreases in the GABA transport in glial cells. This effect could be completely suppressed by addition of the PKC inhibitor H7. The PMA effects could be mimicked by oleoylacetlyglycerol, the diacylglycerol kinase inhibitor R59022 and exogenous phospholipase C. Treatment with PMA did not affect GABA transport in neuronal cells.

INTRODUCTION

High-affinity Na^+ -dependent uptake of γ -aminobutyric acid (GABA) by neural tissue is thought to represent re-uptake by nerve endings and to be a major means of termination of their action (Iversen, 1971). Several lines of evidence suggest that glial cells of the central nervous system play an important role in the modulation of neural excitability via control of the levels of neuroactive substances in the extracellular milieu of neurons (Henn & Hamberger, 1971; Henn *et al.*, 1974; Schrier & Thompson, 1974; Henn, 1976; Hertz, 1979). Moreover, transport systems similar to those found in presynaptic terminals for several amino acids, including GABA, have been identified in glial cells (Schousboe *et al.*, 1977; Hertz *et al.*, 1978; Larsson *et al.*, 1981). Although the transport of GABA in neural cells has been studied in depth, by using synaptosomes or membrane vesicles derived from the plasma membrane (Levi & Raiteri, 1973; Martin, 1973; Kanner, 1978; Pastuszko *et al.*, 1981, 1982; Wood & Sidhu, 1987; Keynan & Kanner, 1988; Mabcjesh & Kanner, 1989; Shank *et al.*, 1990), cultured cells (Schousboe *et al.*, 1978; Balcar *et al.*, 1979; Schousboe, 1981) and in synaptic vesicles (Kish *et al.*, 1989; Hell *et al.*, 1990), the molecular basis of the transport and co-transport process, as well as its regulation, are in general not well known.

Protein kinase C (PKC) is found in high concentrations in the central nervous system (Nishizuka, 1986), and more recent evidence indicates that the phosphoinositide/PKC stimulus-response system is present in astrocytes (Neary *et al.*, 1988; Gustavsson & Hansson, 1990). The natural activator of PKC is diacylglycerol (DAG), which results from phosphatidylinositol breakdown by phospholipase C (Berridge, 1984). Phorbol esters, which bind specifically to PKC (Castagna *et al.*, 1982), are also potent activators of the enzyme (Nishizuka, 1984; Brasseur *et al.*, 1985). Through the use of specific activators, a wide spectrum of functional systems have been demonstrated to be sensitive to PKC activation, including Na^+ -dependent amino acid transport (Mohrmann *et al.*, 1986; Friedlander & Amiel, 1989). Moreover, in our laboratory an activation of the high-affinity uptake of glutamate by PKC in glial cells has been demonstrated (M. Casado, F. Zafra, C. Aragón & C. Giménez, unpublished work).

In this paper we report the effect of PKC activation on Na^+ -dependent uptake of GABA in primary cultures of glial cells from rat brain cortex. We show that phorbol esters and DAG analogues depressed the Na^+ -coupled GABA uptake in glial cells.

EXPERIMENTAL

Materials

[U- ^{14}C]GABA (233 Ci/mol) was obtained from New England Nuclear, Boston, MA, U.S.A. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco, Paisley, Scotland, U.K. Fetal-calf serum was from Biological Industries, Kibbutz Beth Haemek, Israel. Papain, BSA and phospholipase C from *Bacillus cereus* were from Boehringer, Mannheim, Germany. Phorbol 12-myristate 13-acetate (PMA), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), 4 α -phorbol 12,13-didecanoate (PDD), 1-(5-isoquinolinylnsulphonyl)-2-methylpiperazine (H7), amiloride, progesterone, sodium selenite, insulin, transferrin and putrescine were purchased from Sigma, St. Louis, MO, U.S.A. The DAG kinase inhibitor R59022 was from Calbiochem, San Diego, CA, U.S.A. Dishes and multiwell plates for tissue cultures were from Costar, Cambridge, MA, U.S.A. Monoclonal anti-[mouse glial fibrillary acidic protein (GFAP)] antiserum was from The Radiochemical Centre, Amersham, Bucks., U.K. Anti-M β 6-tubulin antiserum was a gift from Dr. J. Avila (Centro de Biología Molecular). All other reagents were of the highest purity available.

Cell cultures

Primary cultures of glial cells from rat brain cortex were prepared from 1-day-old Wistar rats essentially as described by Clarke *et al.* (1984). Cerebral cortices were removed and cleaned of meninges and blood vessels, dissociated mechanically by passing sequentially through 20G1 and 23G1 sterile needles, and collected by centrifugation at 800 *g* for 5 min. Cells were re-suspended in DMEM containing 10% (v/v) fetal-calf serum and non-essential amino acids mixture, plated on 24-well tissue-culture plates and maintained in a humid atmosphere of air/ CO_2 (19:1) at 37 °C. The medium was changed twice a week. The cells

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PDD, 4 α -phorbol 12,13-didecanoate; H7, 1-(5-isoquinolinylnsulphonyl)-2-methylpiperazine; PBS, phosphate-buffered saline; GABA, γ -aminobutyric acid; GFAP, glial fibrillary acidic protein.

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reached confluency at 10–12 days in culture. The experiments were performed after the cells were maintained for 16–19 days in culture.

Primary cultures of neuronal cells from rat brain cortex were prepared essentially as described by Ahmed *et al.* (1983). Timed 17-day-pregnant Wistar rats were killed by cervical dislocation, and the fetuses were removed and kept in PBS medium (0.9 mM-CaCl₂, 2.68 mM-KCl, 1.47 mM-KH₂PO₄, 0.49 mM-MgCl₂, 136.89 mM-NaCl, 7.37 mM-Na₂HPO₄, pH 7.4) at 37 °C. Cerebral cortices (from 12–16 fetuses) were cleaned of meninges and blood vessels, and enzymically dissociated in PBS containing 0.4 mg of papain/ml and 6 mM-glucose. Dissociated cells were collected by centrifugation at 800 *g* for 5 min in the presence of 1% (w/v) BSA. The cell pellet was resuspended in DMEM supplemented with 10% fetal-calf serum. Under these conditions, over 95% of the cells remain viable, on the basis of Trypan Blue exclusion. The cells were plated at 4×10^5 cells/cm² on tissue-culture dishes pretreated overnight with poly-L-lysine (10 μ M) and for 2 h with laminin (4 μ g/ml). The medium was replaced after 2 h by a serum-free medium, i.e. DMEM supplemented with 0.5 μ g of bovine insulin/ml, 10 μ g of human transferrin/ml, 2 nM-progesterone, 10 μ M-putrescine and 3 mM-sodium selenite, and changed again every 3 days. The experiments were performed after the cells were maintained for 6 days in culture.

Immunofluorescence staining

For GFAP staining of the glial culture, cells were fixed with methanol at –20 °C for 10 min, the coverslips were incubated with monoclonal anti-GFAP antibodies for 2 h at room temperature, washed with PBS and incubated (1 h) with fluorescein-conjugated sheep anti-mouse antibodies (1:40 in PBS). For M β 6-tubulin antiserum staining of the neuronal culture, first antibody was a rabbit anti-M β 6-tubulin, and the second was a goat anti-rabbit rhodamine-conjugated IgG (1:40 in PBS). Coverslips were mounted in glycerol/PBS (9:1, v/v) and examined with a Nikon Fluophot HFX fluorescence microscope.

Transport assays

All solutions used in the uptake experiments were prepared with distilled deionized water and filtered through Millipore filters (0.45 μ m pore size) to avoid possible bacterial contamination. Osmolarity of all solutions was kept constant during the uptake experiments. Growth medium was removed by aspiration and, after washing in PBS at 37 °C, cells were maintained for 30 min in 200 μ l of PBS containing 6 mM-glucose and the indicated additions. Uptake was started by adding 50 μ l of PBS containing 6 mM-glucose and radioactive GABA (2 μ Ci). Cells were incubated for the indicated time, and then the experiments were terminated by washing the cells with 3×500 μ l of ice-cold PBS. Cells were solubilized with 200 μ l of 0.2 M-NaOH. Samples (125 μ l) were placed directly in micro-vials and their radioactivity was measured in a liquid-scintillation counter (LKB 1219 Rackbeta). All incubations were carried out in triplicate. Each experiment was repeated at least three times with different cell cultures. For estimating statistical differences, the data were compared by Student's *t* test; differences at the 0.05 level were considered to be statistically significant.

Assay of PKC activity

Cells were scraped with a rubber blade and homogenized by sonication in 6 vol. of 0.25 M-sucrose/20 mM-Tris/HCl, pH 7.5. The homogenate was centrifuged at 100 000 *g* for 1 h at 4 °C and the supernatant was kept. Phosphorylation of lysine-rich histone was conducted as previously described (Kikkawa *et al.*, 1983). Final assay conditions were 625 mM-magnesium acetate, 50 μ g of phosphatidylserine/ml, 25 μ g of OAG/ml, 62.5 μ M-CaCl₂,

250 μ g of H-1 histone/ml, 12.5 μ M-[γ -³²P]ATP (0.45 μ Ci), 25 mM-Tris/HCl, pH 7.5, and 10 μ g of protein from supernatants in a final volume of 50 μ l. After incubation at 30 °C for 5 min, the reaction was terminated by placing a 40 μ l sample on a 2 cm $\times$ 2 cm Whatman 3 MM paper simultaneously with 100 μ l of 25% trichloroacetic acid. Papers were then washed three times in a solution of 20 mM-sodium pyrophosphate and 10% trichloroacetic acid. Non-specific activity was determined by performing the experiment in a medium without phosphatidylserine, OAG and CaCl₂, and supplemented with 624 μ M-EGTA. This non-specific activity was subtracted from total activity to yield PKC-specific phosphorylation.

Protein determination

The protein concentration was determined by the method of Bradford (1976).

RESULTS

The cellular homogeneity of the primary glial and neuronal cell-culture populations that we have selected for the present study has been previously established by the criteria of immunofluorescent staining of specific neuronal and glial cell markers, i.e. anti-GFAP for glial cells and anti-M β 6-tubulin for neurons (Fung-Chow & Goldman, 1984; Sullivan & Cleveland, 1986). The cultures of glial cells were >95% GFAP-positive, and neuronal cultures were >90% anti-M β 6-tubulin-positive for neuronal cells. PKC activities of 15.7 ± 0.06 and 41.0 ± 0.1 fmol of P_i/mg of protein were found in glial and neuronal cells respectively at the time the experiments were performed.

Fig. 1 is a time course showing uptake of GABA in glial and neuronal cells at a concentration (4 μ M) which serves as substrate for the high-affinity GABA-transport system, with little involvement of the low-affinity components. The experiments were performed by measuring the initial rates of GABA transport corrected for non-saturable diffusion (non-specific transport). To make this correction, we used the experimental corrections described previously for other preparations (Kontro & Oja, 1978; Abelle *et al.*, 1983; Zafra & Giménez, 1989). The uptake

Fig. 1. Time course of GABA uptake in neurons (a) and glial cells (b)

The uptake of GABA was carried out in the presence of 4 μ M-[U-¹⁴C]GABA and in the presence of Na⁺ (●) or choline (○). Values represent means $\pm$ S.E.M. of three different duplicate experiments.

Fig. 2. Effects of PMA on Na^+ -dependent GABA uptake in neurons and glial cells

Neurons (●) or glial cells (○) were incubated in serum containing DMEM at 37 °C in the presence of 100 nM-PMA. Transport activity was measured for 10 min in PBS with glucose (6 mM) in the presence of 4 μM -[U- ^{14}C]GABA. Values represent means $\pm$ S.E.M. of three duplicate experiments.

Fig. 3. Effects of increasing concentrations of PMA on Na^+ -dependent GABA uptake in neurons (a) and glial cells (b)

Cells were incubated for 30 min at 37 °C with PMA at the indicated concentrations. Transport activity was measured in the presence of 4 μM -[U- ^{14}C]GABA. Values represent means $\pm$ S.E.M. of three duplicate experiments.

was almost completely Na^+ -dependent and increased linearly with time up to approx. 10 min. The effect of PMA on Na^+ -dependent GABA uptake in glial cells is shown in Fig. 2. Incubation of cells with PMA decreased GABA uptake in a time-dependent manner until approx. 1 h. Exposure for additional time did not result in any further significant effect of PMA. A dose-response relationship for the PMA effect on the high-affinity GABA transport in glial cells (Fig. 3) demonstrates that uptake decreased with PMA concentration, reaching a plateau at approx. 0.1 μM -PMA. In contrast, no effect was detected in neurons in the range of PMA concentrations used.

To test the possibility that the PMA-induced decrease in GABA uptake might be exerted through activation of Na^+/H^+

Table 1. Effect of PMA, PDD and H7 on Na^+ -dependent GABA uptake in glial cells

Cells were incubated in the presence or the absence of H7 (100 μM) for 45 min. Afterwards PMA or PDD was added (100 nM) or its solvent at equivalent concentration (ethanol; 0.2%, v/v) and incubated for 30 min at 37 °C before the transport assay started. The GABA-transport assay was carried out for 10 min in the presence of 4 μM -[U- ^{14}C]GABA. Data represent means $\pm$ S.E.M. of three different duplicate experiments. They were compared with the control value by Student's *t* test: **P* < 0.01.

Conditions	Na^+ -dependent GABA uptake (pmol/10 min per mg of protein)
Control	1050 $\pm$ 60
H7	1180 $\pm$ 30
PMA	650 $\pm$ 20*
PMA + H7	1030 $\pm$ 60
PDD	975 $\pm$ 60
PDD + H7	1060 $\pm$ 50

Table 2. Effect of OAG and DAG kinase inhibitor R59022 on Na^+ -dependent GABA uptake in glial cells

Cells were incubated in the presence of PMA (100 nM), OAG (50 $\mu\text{g}/\text{ml}$) or R59022 (10 μM) for 30 min at 37 °C before the transport assay started. The GABA-transport assay was carried out for 10 min in the presence of 4 μM -[U- ^{14}C]GABA. Data represent means $\pm$ S.E.M. of three different duplicate experiments. They were compared with the control value by using Student's *t* test: **P* < 0.01.

Conditions	Na^+ -dependent GABA uptake (pmol/10 min per mg of protein)
Control	1045 $\pm$ 52
H7	1020 $\pm$ 31
OAG	650 $\pm$ 15*
OAG + H7	1035 $\pm$ 40
R59022	680 $\pm$ 10*

exchange, a known effect of PKC activators in many cell types (Wiener *et al.*, 1986), the effect of PMA was also studied in the presence of amiloride, an inhibitor of Na^+/H^+ exchange. The results show that the presence of amiloride does not significantly influence by itself or in the presence of PMA the uptake of GABA (1010 $\pm$ 50 pmol/10 min per mg of protein in the controls versus 985 $\pm$ 30 pmol/10 min per mg of protein in the presence of 100 μM -amiloride).

The effect of preincubation with H7, an inhibitor of PKC, was examined in the presence or the absence of PMA and PDD, a permeant inactive phorbol ester. The results show that the presence of H7 itself slightly increased the uptake of GABA; however, it prevented the PMA-induced decrease in GABA uptake, whereas PDD was inactive (Table 1).

We also evaluated the effect of OAG, a DAG analogue, on GABA transport in glial cells in the presence or absence of H7 (Table 2). As shown in Fig. 4, OAG exerted its inhibition on GABA transport in a concentration-dependent manner. In Table 2 we also show that the presence of the DAG kinase inhibitor R59022 exerted similar effects to those of the DAG analogue.

The experiment presented in Fig. 5 shows that exogenous phospholipase C exerted a similar inhibitory effect on the GABA uptake, in a dose-dependent manner, to that observed in the presence of PMA or the DAG analogue. Maximal inhibition was achieved with 0.2 unit/ml after a 30 min treatment period.

Fig. 4. Effect of increasing concentrations of OAG on Na^+ -dependent GABA uptake in glial cells

Cells were incubated for 30 min at 37°C with the designated doses of OAG. Transport activity was measured in the presence of $4\text{ }\mu\text{M}$ -[^{14}C]GABA. Values represent means $\pm$ S.E.M. of three duplicate experiments.

Fig. 5. Effects of increasing concentrations of exogenous phospholipase C (*Bacillus cereus*) on Na^+ -dependent GABA uptake in glial cells

Cells were incubated for 30 min at 37°C with the indicated concentrations of phospholipase C. Transport activity was measured in the presence of $4\text{ }\mu\text{M}$ -[^{14}C]GABA in PBS with glucose (6 mM) for 10 min. Values represent means $\pm$ S.E.M. of three duplicate experiments.

Fig. 6. Effects of PMA on the kinetics of Na^+ -dependent GABA uptake in glial cells

Cells were incubated during 30 min at 37°C with PMA (100 nM; ○) or its solvent at equivalent concentration (0.2% ethanol; ●) before the transport assay started. Triplicate 1 min measurements were taken. The Figure is representative of four independent experiments (K_m and V_{max} values were determined from Lineweaver-Burk plots).

The initial rates of GABA uptake, measured over 1 min, showed saturation kinetics both in the controls and after 30 min pretreatment with PMA (100 nM). Further kinetic analysis of the data by Lineweaver-Burk plots shows that PMA affected the K_m , but not the V_{max} , of GABA transport (K_m value of $21.82 \pm 1.38\text{ }\mu\text{M}$ in the controls versus $34.84 \pm 0.94\text{ }\mu\text{M}$ in the presence of PMA, and V_{max} value of $715.9 \pm 38\text{ pmol/min per mg of protein}$ in the controls versus $724.0 \pm 51\text{ pmol/min per mg}$ in the presence of PMA) (Fig. 6). The kinetic features found in the controls confirmed that we are indeed dealing with the high-affinity transport system of GABA.

DISCUSSION

Considering the results reported herein, it is possible to establish that the inhibition of the high-affinity transport system for GABA exerted by phorbol esters in glial cells was actually mediated by the activation of PKC. Firstly, inhibition of the Na^+ -dependent GABA uptake is obtained in the presence of the phorbol ester PMA, a tumour-promoter agent known to stimulate PKC (Castagna *et al.*, 1982; Nishizuka, 1986). On the basis of the data in the literature, it is assumed that the actions of PMA at low doses are mediated by PKC. The effect of PMA on Na^+ -dependent GABA uptake was independent of the Na^+/H^+ exchange system, as shown by the studies in the presence of amiloride. The possibility that PMA alters Na^+ gradients by a direct ionophoretic action was excluded on the basis of measurements of membrane permeability to $^{22}\text{Na}^+$ in the presence of PMA (results not shown). Moreover, the ability of phorbol esters to inhibit GABA uptake in glial cells is time- and dose-dependent, and correlates with the degree of specificity of the phorbol esters to stimulate PKC (Mudd & Raizada, 1990). On the other hand, the concentrations of phorbol esters are consistent with their potency in binding to and promoting PKC-stimulated events in neuronal cells and other tissues, and would imply that the effects of phorbol esters on glial GABA uptake are mediated through the activation of PKC.

The inhibitory effect observed on GABA transport can be achieved by OAG, a permeant DAG analogue which has been reported to stimulate PKC in several cell types (Nishizuka, 1984). Moreover, increased levels of endogenous DAG by inhibition of DAG kinase with R59022 mimic the action observed with PMA. The inhibition of GABA uptake in glial cells observed in the presence of PMA was reversed by H7, an inhibitor of PKC (Matsui *et al.*, 1986). On the other hand, it has been reported that phospholipase C generates DAG from membrane phospholipids when added exogenously to a variety of cell types (Blackmore *et al.*, 1986; Farese *et al.*, 1988). This would result in an increase in intracellular free Ca^{2+} and activation of PKC through the second messengers inositol trisphosphate and DAG respectively.

In the overall process of GABA-mediated neurotransmission, the high-affinity transport system for GABA plays a crucial step, as a competition is established between neurons and the surrounding glial cells for the uptake of GABA. In this respect, in our hands the Na^+ -dependent high-affinity uptake of GABA in neurons and glial cells responds differently to PKC activation.

Although it has been demonstrated that both neurons and glial cells contain immunoreactive PKC (Mudd & Raizada, 1990) and that they also have PKC molecules capable of binding phorbol esters (Raizada *et al.*, 1987; Gardiner & de Bellerocche, 1990), it is worth noting the lack of effect of PMA on neuronal GABA transport. A similar differential effect of PMA actions on neurons and glial cells has been shown by Mudd & Raizada (1990) when studying the action of phorbol esters on insulin binding, and moreover we have found a similar differential effect on the transport of glutamate in neurons and glial cells

(M. Casado, F. Zafra, C. Aragón & C. Giménez, unpublished work). As we and others have found an appreciable PKC activity in our cultures of neurons and glial cells, the failure of PMA to alter GABA transport in neurons cannot be attributed to a lack of PKC, nor to a non-functional PKC. PKC activation in neurons has been implicated in the regulation of GABA release and other functions (Nichols *et al.*, 1987; Gardiner & de Belleruche, 1990). The dissimilar effects of PMA on different activities in neurons and glial cells may be due to the different isotypes of PKC that are present in both neurons and glial cells, or to the presence of a smaller pool of PKC available for translocation in neurones, as has been suggested (Mudd & Raizada, 1990).

When the surface area of glial cells and their close proximity to neuronal processes are considered, glial GABA uptake appears to be of great importance in the process of inactivation of GABA. A differential regulation of the GABA-transport system in neurons and glial cells could represent a cellular device allowing neurons an adequate rate of firing in situations where enhanced neuronal GABA neurotransmission may be of benefit.

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