Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4335–4339, June 1990
Cell Biology

Inhibition of protein kinase C induces differentiation in Neuro-2a cells

(neuritogenesis/neuroblastoma cells)

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Communicated by Severo Ochoa, February 28, 1990

ABSTRACT 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H7), a potent inhibitor of protein kinase C, induced neuritogenesis in Neuro-2a cells, whereas *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA 1004), which inhibits more efficiently cAMP- and cGMP-dependent protein kinases, did not. The effect, noticeable after 3 hr, was maximum (13-fold increase at 500 μM H7) between 1 and 3 days and was maintained over 2 months. In controls, 90% of the cells were undifferentiated, whereas after 3 hr with 500 μM H7 only 25% of the cells remained undifferentiated. DNA synthesis decreased as the number of differentiated cells increased. Differentiation is also functional since acetylcholinesterase activity increased ≈7-fold after 48 hr with 500 μM H7. Phorbol 12-myristate 13-acetate, a specific activator of protein kinase C, prevented or reversed the induction of neuritogenesis and the inhibition of DNA synthesis by H7. There is a good correlation between the level of protein kinase C and the percentage of differentiated cells. The results indicate that protein kinase C may play a key role in the control of differentiation of neural cells. Some possible clinical implications are briefly discussed.

The molecular regulation of cellular growth and differentiation is one of the fundamental problems of cell biology. It was shown some years ago that addition of gangliosides induces the differentiation of neuroblastoma cell lines with concomitant sprouting and extension of neurites (1–3). However, the underlying mechanism of ganglioside-modulated neuritogenesis remained unknown (4, 5). It has been reported by others that gangliosides are inhibitors of protein kinase C (PKC) (6–8). It therefore appeared possible that the two findings might be related and that gangliosides might stimulate neuritogenesis by inhibiting PKC. To test this hypothesis we have studied the effect of 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), a potent inhibitor of PKC (9, 10), on differentiation in Neuro-2a cells. We find that H7 induces neuritogenesis and that this effect is prevented and reversed by phorbol 12-myristate 13-acetate (PMA), an activator of PKC (11). It is further shown that there is a good correlation between the level of the enzyme and the percentage of differentiated cells. The results indicate that PKC may play an important role in the control of differentiation of neural cells.

MATERIALS AND METHODS

Cell Cultures. The clonal line Neuro-2a, C1300 mouse neuroblastoma, was obtained from the American Type Culture Collection. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 international units of penicillin per ml, and 100 μg of streptomycin per ml. Stock cells were grown in Falcon 250-ml plastic tissue flasks at 37°C and were subcultured twice weekly. The viability of cells was determined by trypan blue exclusion.

Incubations with H7. H7 was dissolved (34 mM) in dimethyl sulfoxide and added after 24 hr of subculture. The same volume of dimethyl sulfoxide was added to controls. Medium was changed and fresh H7 was added every second day. Viabilities were 93%, 86%, 87%, and 84% for 0, 17, 85, and 500 μM H7, respectively.

Experiments with PMA and Colchicine. PMA was dissolved in ethanol. For the experiments of prevention of neuritogenesis cells were seeded at 200,000 per ml in Falcon 50-ml plastic tissue culture flasks and incubated at 37°C. After 24 hr of subculture PMA was added. After an additional 3 hr, 500 μM H7 was added and cells were photographed 3 hr later. For reversal by PMA of neuritogenesis induced by H7, PMA was added 24 hr after H7. Experiments with colchicine were carried out exactly as with PMA using 16 ng of colchicine per ml.

Quantification of Neuritogenesis. Several randomly chosen fields of the cultures were photographed in a phase-contrast light microscope. The number of neurites on each cell was counted and their lengths were measured.

DNA, RNA, and Protein Synthesis. One hundred thousand cells in 1 ml were grown in Nunc plates at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hr, the cells were incubated with H7 for 3 hr; then 5 μCi of [^{3}H]thymidine (5 Ci/mmol, 1 mCi/ml; 1 Ci = 37 GBq), [^{3}H]uridine (26 Ci/mmol, 1 mCi/ml), or [^{3}H]leucine (49 Ci/mmol, 1 mCi/ml) in 250 μl of Eagle's MEM was added and cells were incubated for 1 hr. Cells were washed twice with 1 ml of cold phosphate-buffered saline (PBS: 1.5 mM potassium phosphate/0.8 mM sodium phosphate/137 mM NaCl/2.7 mM KCl, pH 7.4) and trichloroacetic acid (TCA)-soluble material was removed by incubation (twice) with 1 ml of 10% TCA for 15 min at 4°C. After extraction with 1 ml of ethanol, the remaining material was solubilized in 500 μl of 0.5 M NaOH/0.4% deoxycholate for 90 min at 37°C, and 400-μl portions were taken to assay radioactivity. Protein was measured by the bicinchoninic acid protein assay reagent (Pierce).

Acetylcholinesterase Activity. Activity was determined by measuring the rate of hydrolysis of acetylthiocholine colorimetrically (12).

Immunofluorescence Staining of Tubulin, Microtubule-Associated Proteins (MAPs), and PKC. Experiments were carried out essentially as described (13). Cells were seeded at 200,000 per ml; after 24 hr, 500 μM H7 was added, followed by incubation for 24 hr. After extraction with acetone, the cells were immersed in anti-tubulin (1:50), anti-MAPs (1:100), or anti-PKC (1:200). Anti-tubulin was obtained by injecting

Abbreviations: PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; MAP, microtubule-associated protein; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; HA 1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide.

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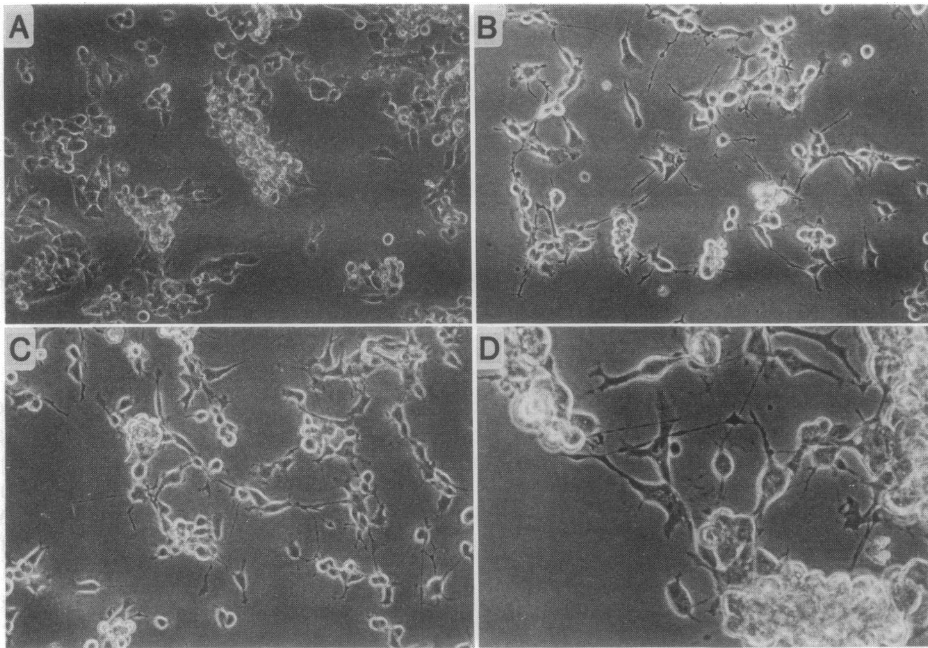

FIG. 1. Induction of neuritogenesis in Neuro-2a cells by H7. Cells were incubated in the absence (*A*) or presence of 500 μM H7 for 1 (*B*), 3 (*C*), or 9 (*D*) days and photographed using a phase-contrast light microscope. (*A–C*, ×53; *D*, ×106.)

rabbits with tubulin isolated from rat brain. Anti-MAPs was from ICN, and anti-PKC was from Amersham. Tubulin or MAPs were visualized by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG. For PKC (TRITC)-conjugated anti-mouse IgG was used.

Quantification of PKC by Immunoblotting. Cells were seeded at 200,000 per ml and 24 hr later H7 was added to reach 17, 85, and 500 μM. After 24 hr cells were washed with PBS, scraped in PBS containing 25 μg of aprotinin per ml, and collected by centrifugation. The pellet was resuspended in 62.5 mM Tris·HCl (pH 6.8) containing 10% glycerol, 5% 2-mercaptoethanol, and 2.3% SDS, and samples containing 3, 5, 10, and 20 × 10^5 cells were subjected to SDS/polyacrylamide electrophoresis using 10% gels. Immunoblotting and quantification of PKC were carried out as described (14) using a monoclonal anti-PKC (Amersham) that recognizes α- and β-isozymes.

RESULTS

Induction of Neuritogenesis in Neuro-2a Cells by H7. As shown in Fig. 1 addition of H7 to Neuro-2a cells induced marked neuritogenesis within 1 day. In fact, neuritogenesis was noticeable in 3 hr after addition of H7. To quantify the effect we counted the number of neurites per cell at different times after addition of H7. As shown in Table 1, only 10% of control cells exhibited neurites, whereas after 1 day of treatment this level rose to 18%, 62%, and 84% for 17, 85, and 500 μM H7, respectively. The number of cells with 1 neurite reached a maximum (around 25–30%), which was not increased further with time or dose of H7; however, the number of cells with three or more neurites increased with the amount of H7—i.e., after 1 day they represented 1%, 2.1%, 15.3%, and 31% for 0, 17, 85, and 500 μM H7, respectively. The total number of neurites per 100 cells was increased by 2-, 9-, and

Table 1. Induction of neuritogenesis in Neuro-2a cells by H7

H7, μM	Time	Neurites per cell,* %					Mean length, μm	Cells counted
		0	1	2	3	>3		
0	†	90	7	2.0	0.6	0.4	23 ± 8	2800
17	3 hr	83	13	4.4	0.5	0	42 ± 10	190
17	1–3 days	82	13	3.8	1.6	0.5	62 ± 15	780
17	9 days	63	25	8.0	3.2	0.6	NM	320
85	3 hr	63	21	11.0	3.2	1.4	51 ± 15	220
85	1–3 days	38	24	23.0	8.8	6.5	61 ± 19	430
85	9 days	64	23	7.6	3.0	2.0	NM	400
500	3 hr	25	23	24.0	14.5	12.8	49 ± 18	250
500	1–3 days	16	24	28.0	16.0	15.0	61 ± 21	1280
500	9 days	35	29	22.0	10.0	5.0	NM	390

NM, not measured.
*Percentage of cells with the indicated number of neurites.
†There is no significant change with time in the number of neurites in control cells; therefore the mean for all times is given.

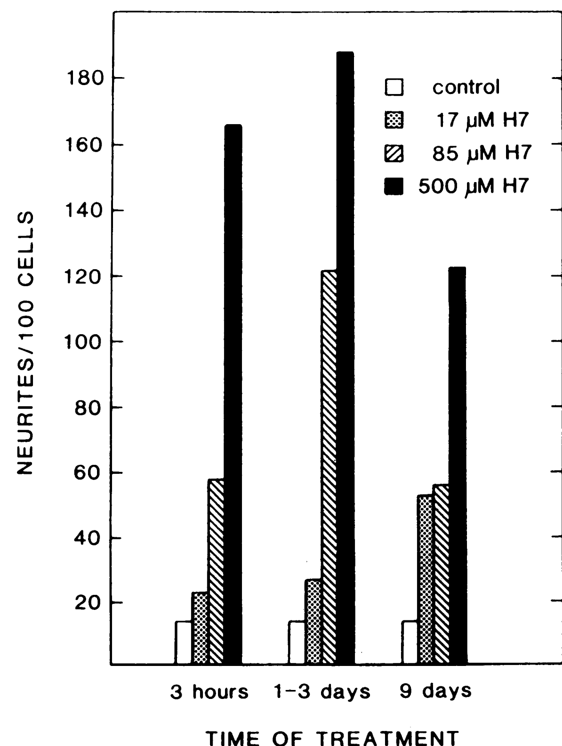

FIG. 2. Quantification of neurites induced by H7. Neuro-2a cells were incubated with the indicated amounts of H7. At the indicated times several randomly chosen fields were photographed and the number of neurites per cell was counted.

Table 2. Prevention by PMA of induction of neuritogenesis by H7

PMA, nM	H7, μM	Neurites per cell,* %					Neurites per 100 cells	Mean length, μm	Cells counted
		0	1	2	3	>3			
0	0	90	7	2.0	0.6	0.4	14	23 ± 8	2800
0	500	25	23	24.0	14.5	12.8	166	49 ± 18	250
2.5	500	77	12	6.9	3.4	0.7	39	30 ± 11	1320
5	500	79	11	7.0	2.8	0.5	35	25 ± 9	1600
25	500	81	10	6.6	2.7	0.8	34	23 ± 9	1610

After incubation for 3 hr with the indicated amount of PMA, H7 was added and incubation was continued for 3 hr. At this time cells were photographed and neurites were counted and measured.
*Percentage of cells with the indicated number of neurites.

13-fold after 1 day with 17, 85, and 500 μM H7, respectively (Fig. 2). The effect was exactly the same after 1, 2, or 3 days of incubation, and therefore the results at these times are presented together in Table 1 and Fig. 2.

The length of neurites is also increased by H7. As shown in Table 1, the mean length for controls was 23 μm, whereas after 1 day in the presence of any concentration of H7 tested it increased 3-fold.

H7 is a selective, but not absolutely specific, inhibitor of PKC. In fact, cAMP- and cGMP-dependent kinases are also inhibited (15). Therefore we also tested the effect of *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA 1004), another inhibitor with differential kinase specificities, which inhibits cAMP- and cGMP-dependent protein kinases with K_i values ≈ 20 and 30 times lower than that of PKC (15). It was found that HA 1004 did not induce differentiation even at the highest levels tested (500 μM).

Prevention and Reversal by PMA of Induction of Neuritogenesis by H7. Incubation with 2.5 nM PMA prevented by 80–90% the induction of neuritogenesis by 500 μM H7. Addition of 10 times more PMA had no further effect (Table 2). When 250 nM PMA was added, prevention of neuritogenesis was complete but most cells were dead after 1 day. Also, the neurites remaining were shorter than in the absence of PMA, similar to those of control cells (Table 2).

The neuritogenesis induced by H7 is reversed by PMA. Cells incubated with 500 μM H7 for 1 day exhibited a larger number of neurites (Table 1). When 25 nM PMA was added to these cells most neurites were lost in ≈2 hr.

Effect of H7 on DNA, RNA, and Protein Synthesis. The effect of a 3-hr incubation with H7 on DNA, RNA, and protein synthesis is shown in Fig. 3. There was a slight inhibition of protein and RNA synthesis, whereas the inhibition of DNA synthesis was more marked. However, when it is expressed per undifferentiated cell (Fig. 4), DNA synthesis is only slightly (20%) inhibited. To test whether inhibition of DNA synthesis by H7 is prevented by preincubation with phorbol esters, 2.5 nM PMA was added 3 hr before addition of 500 μM H7. Under these conditions, DNA synthesis, determined 3 hr later, was 94% of control, whereas in the presence of H7 or PMA alone it was 29% and 109%, respectively (Table 3).

Induction of Acetylcholinesterase by H7. To assess whether differentiation induced by H7 is functional as well as morphological, we measured the activity of acetylcholinesterase. As shown in Fig. 5, incubation of Neuro-2a cells with 500 μM H7 increased acetylcholinesterase activity 2.7-, 5.8-, and 7.3-fold after 3, 24, and 48 hr, respectively.

Correlation Between PKC Levels and Differentiation. To confirm the link between PKC and differentiation of Neuro-2a cells we determined by immunoblotting the level of the enzyme in cells cultured for 24 hr in the absence or presence of H7. The amount of PKC decreased to 79%, 55%, and 41% of control for 17, 85, and 500 μM H7. As shown in Fig. 6, there is a good correlation between the amount of PKC and the percentage of differentiated cells.

DISCUSSION

Our results show that H7 induces marked neuritogenesis in Neuro-2a cells, whereas HA 1004 does not. Moreover, PMA, a specific activator of PKC (11), prevents or reverses this effect. It thus appears that PKC is involved in the control of differentiation in neuroblastoma cells.

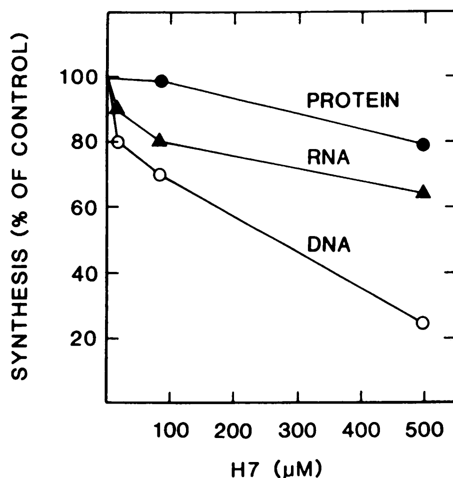

FIG. 3. Effect of H7 on DNA, RNA, and protein synthesis. Experiments were carried out in the absence or presence of the indicated amounts of H7. Values are the mean of triplicate samples from two different experiments. Standard deviations were always <5%.

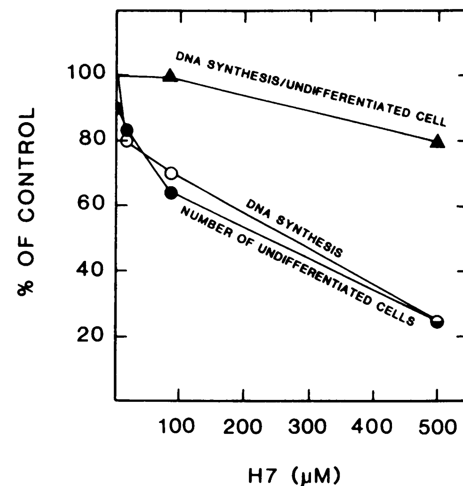

FIG. 4. Effect of H7 on DNA synthesis per undifferentiated cell. Experiments of DNA synthesis were the same as shown in Fig. 3. DNA synthesis per undifferentiated cell was calculated taking into account the percentage of undifferentiated cells after 3 hr of incubation with H7 (Table 1).

Table 3. Prevention by PMA of inhibition by H7 of cell proliferation and DNA synthesis

PMA, nM	H7, μM	Viability, %	Cell proliferation, cells per ml (×10⁻³)	DNA synthesis, cpm per 10⁵ cells
0	0	93	434	3720 ± 320
0	500	84	278	1080 ± 110
2.5	0	92	448	4050 ± 360
2.5	500	92	445	3490 ± 220

For assay of viability and cell proliferation, cells were seeded at 200,000 per ml, and after 24 hr PMA was added. H7 was added 3 hr later and cells were counted 24 hr later. For DNA synthesis cells were seeded at 100,000 per ml and DNA synthesis was determined 3 hr after addition of H7. Values are the mean ± SD of triplicate samples from three different experiments.

The induction of neuritogenesis depends on the dose (Fig. 2); it is very fast, with a 12-fold increase in the number of neurites per cell after only 3 hr at 500 μM H7, reaching a maximum between 1 and 3 days. Cells retain the neurites for >2 months provided H7 is added every 2 days. If differentiated cells are deprived of H7 or if it is not added after 2 days, the newly developed neurites are lost after 3–4 days, indicating that the effect is reversible and also that H7 is metabolized or degraded under the culture conditions used.

It is remarkable that doses as high as 0.5 mM H7 are almost devoid of toxicity; at this concentration viability was 83%, whereas for controls it was 93%.

As expected for true neurites, those induced by H7 contain microtubules, as revealed by the positive staining by immunofluorescence using antibodies against tubulin and MAPs (Fig. 7). Further, it is known that formation of microtubules is necessary for the extension of neurites (16). We found, in addition, that colchicine also prevented and reversed the neuritogenesis induced by H7 (not shown).

As shown, DNA synthesis per cell is strongly inhibited (Fig. 3) and, at all doses tested, the decrease of DNA synthesis parallels the decrease in the number of undifferentiated cells (Fig. 4). This result is in good agreement with the inhibition of cell proliferation produced by H7 (Table 3). Therefore, DNA synthesis per undifferentiated cell is not significantly affected by H7, and the inhibition of total DNA synthesis is due to the induction of differentiation and the concomitant inhibition of proliferation.

To corroborate that the above effects are due to inhibition of PKC we carried out experiments preincubating the cells

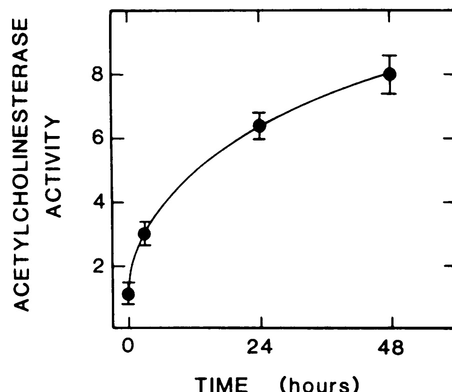

FIG. 5. Effect of H7 on acetylcholinesterase activity. Cells were seeded at 100,000 per ml and acetylcholinesterase activity was determined using 10⁵ cells after the indicated times of incubation with 500 μM H7. Activity is expressed as μmol of acetylthiocholine hydrolyzed per min per 10⁹ cells. Values are the mean ± SD of duplicate samples from two experiments.

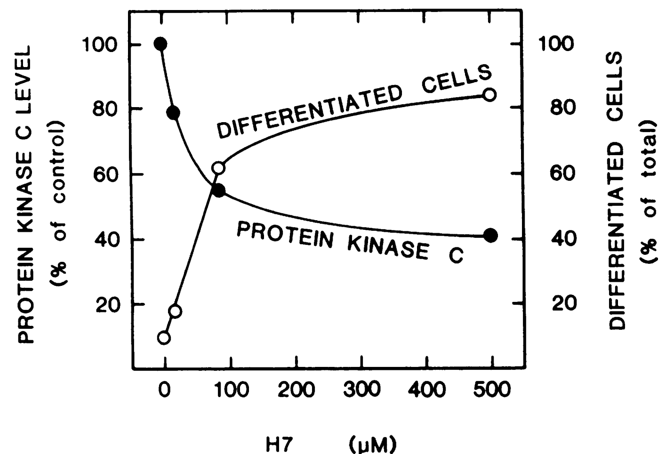

FIG. 6. Correlation between PKC levels and the percentage of differentiated cells. Cells were cultured in the absence or presence of the indicated concentrations of H7. PKC was quantified by immunoblotting. To measure differentiation, several randomly chosen fields were photographed and the number of differentiated and undifferentiated cells was counted.

with the specific activator of PKC, PMA, before adding H7. PMA prevented (and reversed) induction of neuritogenesis (Table 2) and the inhibition of DNA synthesis (Table 3). Also, the inhibition by H7 of cell proliferation is abolished by preincubation with PMA. All of this provides confirmation that induction of differentiation by H7, with concomitant neuritogenesis and inhibition of DNA synthesis, is actually due to inhibition of PKC. Moreover, incubation with H7 decreases the cellular content of PKC and there is a good correlation between the level of the enzyme and the percentage of differentiated cells (Fig. 6). The reduced content of PKC after treatment with H7 was confirmed by immunofluorescence (Fig. 8).

Differentiation of neuroblastoma cells is ultimately characterized by the acquisition of functional neuronal properties such as an excitable membrane and the expression of high levels of specific neuronal enzymes (17). As shown in Fig. 5, after incubation with 500 μM H7, acetylcholinesterase activity increased ≈7-fold.

Although gangliosides have been shown to trigger differentiation of neuroblastoma cells (1–3), the underlying molecular mechanism remains unknown (4, 5). Since gangliosides do inhibit PKC (6–8) and we now show that differentiation of neuroblastoma cells is promoted by H7, the effect of gangliosides may be in fact due to their inhibition of PKC.

Based on their neural regenerative effect, gangliosides have been used successfully in animal models for the treatment of certain neurological disorders, such as diabetic neuropathy (18–20), and for acceleration of functional recovery after central nervous system damage (21–23). Recently the use of gangliosides has been extended to human treatment. Our findings suggest that these therapeutic effects might be obtained with other PKC inhibitors, thus advocating the development of new, specific, and more potent compounds for the treatment of some neuropathies.

A potential therapeutic effect of inhibitors of PKC would be in the treatment of neuroblastoma. A child's neuroblastoma behaves as a highly malignant tumor, resistant to irradiation and chemotherapy (24); however, it has the highest rate of spontaneous regression (7% of all cases) (24, 25) and is capable of spontaneously differentiating or maturing to a benign ganglioneuroma (26). Inhibitors of PKC might induce differentiation and stop tumor growth.

Our results indicate that PKC plays an important role in the control of differentiation and proliferation of neural cells;

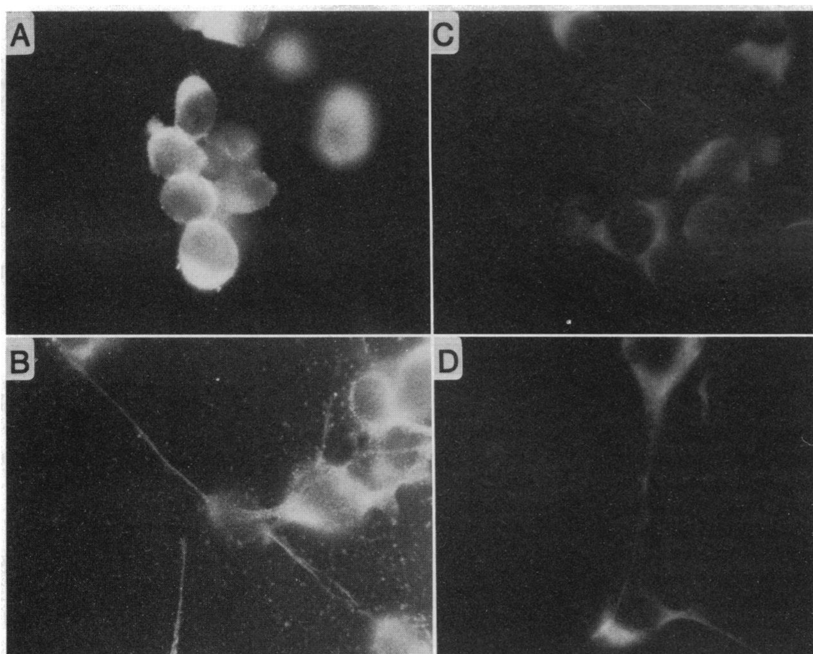

FIG. 7. Immunofluorescence staining of tubulin and MAPs in Neuro-2a cells. Tubulin (*A* and *B*) and MAPs (*C* and *D*) in Neuro-2a cells were visualized by immunofluorescence. (*A* and *C*) Control cells. (*B* and *D*) Cells incubated with 500 μM H7 for 24 hr. (×212.)

therefore agents that modify its activity might be of importance in the treatment of certain neuropathies and tumors, especially neuroblastomas.

During the revision of this manuscript it has been shown that H7 also induces differentiation and neuritogenesis in PC12 cells (27). It has also been reported that neurite outgrowth induced by the phorbol ester PMA in neuroblastoma cells is associated with a down-regulation of PKC (28) and that the induction of differentiation of Neuro-2a cells *in vitro* by agents such as the ganglioside GM1 or 8-bromoadenosine 3′,5′-cyclic monosphosphate is associated with down-regulation of the mRNA for PKC (29). These results, together with those reported in this paper, clearly demonstrate that PKC plays an essential role in the control of neural differentiation.

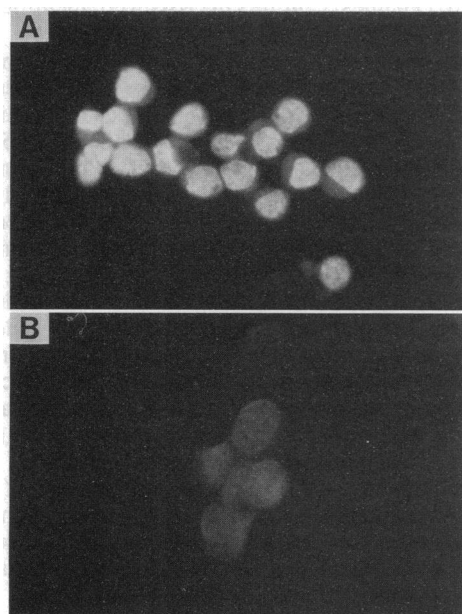

FIG. 8. Immunofluorescence staining of PKC in Neuro-2a cells. PKC in Neuro-2a cells cultured for 24 hr in the absence (*A*) or the presence of 500 μM H7 (*B*) was visualized by immunofluorescence. (×170.)

This research was supported in part by Glaxo España, Fondo de Investigaciones Sanitarias, Institució Valenciana d'Estudis i Investigació, and Instituto de Investigaciones Citológicas-Kansas University Medical Center International Cytology Program.

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