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NEURONAL DIFFERENTIATION OF NEURO 2A CELLS BY LACTACYSTIN AND ITS PARTIAL INHIBITION BY THE PROTEIN PHOSPHATASE INHIBITORS CALYCULIN A AND OKADAIC ACID

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SUMMARY: Lactacystin (1.3 μM), a metabolite from an actinomycete, induced the formation of bipolar projections at both sides of the cell body of Neuro 2a cells 1 day after treatment and networks at and after 3 days and enhanced acetylcholinesterase activity (a marker of neuronal differentiation). Thus, the neuronal differentiation was characterized both morphologically and functionally. The experiments with various inhibitors of protein kinases and phosphatases revealed that the protein phosphatase inhibitors calyculin A (0.5 nM) and okadaic acid (0.6 nM) inhibit the formation of bipolar projections at 1 day, but does not inhibit the network formation at and after 3 days.

Lactacystin was isolated as a neuritogenesis inducer from a cultured broth of a soil actinomycete [1,2]. As reported preliminarily, it induces neurite outgrowth in Neuro 2a mouse neuroblastoma cells [1] and arrests cell cycle at both G0/G1 and G2 phases [3]. However, the function of neurites induced by lactacystin and the mechanism of induction of the neuritogenesis remain to be clarified. To examine the function, the effect of lactacystin on acetylcholinesterase activity in Neuro 2a cells was assayed.

From the studies of mode of action of the known neuritogenesis inducers, it has been found that some protein kinases are involved in neuritogenesis of some cell lines. Gangliosides, which induce neurite outgrowth, inhibit protein kinase C (PKC) [4]. H-7, a PKC inhibitor, induces morphological and functional differentiation in Neuro 2a cells [5-7]. The neuritogenesis induced by forskolin and dibutyryl cyclic AMP (Bt_2cAMP) in PC-12D pheochromocytoma cells is inhibited by pretreatment with H-89, a specific inhibitor of protein kinase A (PKA) [8]. Recently, Tsuji et al. [9] reported that ganglioside GQ1b-dependent neuritogenesis of the

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human neuroblastoma cell line GOTO is closely associated with GQ1b-dependent ecto-type protein phosphorylation. To assess if inhibitors of protein kinases and phosphatases are responsible for the neuritogenesis induced by lactacystin, we performed this study, and found that the protein phosphatase inhibitors calyculin A and okadaic acid inhibit the formation of bipolar projections at the both side of the cell body 1 day after treatment.

The present paper deals with the neuronal differentiation of Neuro 2a cells by lactacystin and the effect of various inhibitors of protein kinases and phosphatases on neuritogenesis.

MATERIALS AND METHODS

Materials: Lactacystin and herbimycin A were prepared as described previously [1, 10, respectively]. The protein tyrosine phosphatase inhibitor dephostatin was a generous gift from Professor K. Umezawa (Keio University, Japan). 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was a generous gift from Asahi Chemical Industry Co. Ltd., Japan. Other chemicals were commercially obtained : acetylcholinesterase, Bt_2cAMP, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calyculin A and okadaic acid from Wako Pure Chemical (Osaka, Japan), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), KN-62 and CK1-7 from Seikagaku Co. (Tokyo, Japan), and ganglioside GM1 and 5,6-dichlorobenzimidazole riboside (DBR) from Sigma Chemical. Compounds were dissolved in minimal amount of methanol or dimethyl sulfoxide required for solubilization. No more than 0.4% solvent was present in any assay.

Cell cultures: Neuro 2a cells were purchased from Dainippon Pharmaceutical (Osaka, Japan). They were grown at 37°C in Eagle's minimum essential medium with Hank's salt (MEM-H) containing 10% fetal bovine serum (FBS) and 1% non-essential amino acids. Cells were subcultured twice a week. The viability of cells was determined by trypan blue exclusion.

Assay for acetylcholinesterase: Acetylcholinesterase activity was assayed as described by Doctor et al. [11].

Effect of inhibitors on neuritogenesis: Neuro 2a cells were plated at a density of 1 x 10^4 cells per cm^2 and grown for 24 h in MEM-H with 10% FBS prior to any treatment. In the relevant experiments, H-89 and inhibitors were added 30 min or 1 h before addition of Bt_2cAMP or lactacystin. The number of cells with neurites were counted, and their lengths were measured 24 h after addition of Bt_2cAMP or lactacystin. In the experiment of TPA treatment, after incubation for 24 h in media containing H-7 or lactacystin, the media were replaced with fresh one containing TPA, and the number of cells with neurites and their lengths were examined after 2 h.

RESULTS AND DISCUSSION

Differentiation of Neuro 2a neuroblastoma cells by lactacystin: Figure 1 shows morphological changes of Neuro 2a cells induced by lactacystin. When Neuro 2a cells were cultured in MEM-H containing 4 or 10% FBS, the shape was almost round, and cells with a long neurite(s) were scarcely observed at 1-day, although some cells have a short neurite(s) (mean length : 25.1 μm) [Figure 1 (A, B and C)]. On the other hand, lactacystin (1.3 μM) induced the formation of relatively bold bipolar projections at 1-day (Figure 1D). Then, the bipolar projections disappeared at 2-day (Figure 1E) and in further incubation for 1 or more days, a marked morphological change was induced to form networks (Figure 1 F), which has been characterized with a neurite-like structure by experiments using scanning and transmission electron microscopes [1]. The lactacystin-induced neurite-like processes have a solid structure of varicosities and

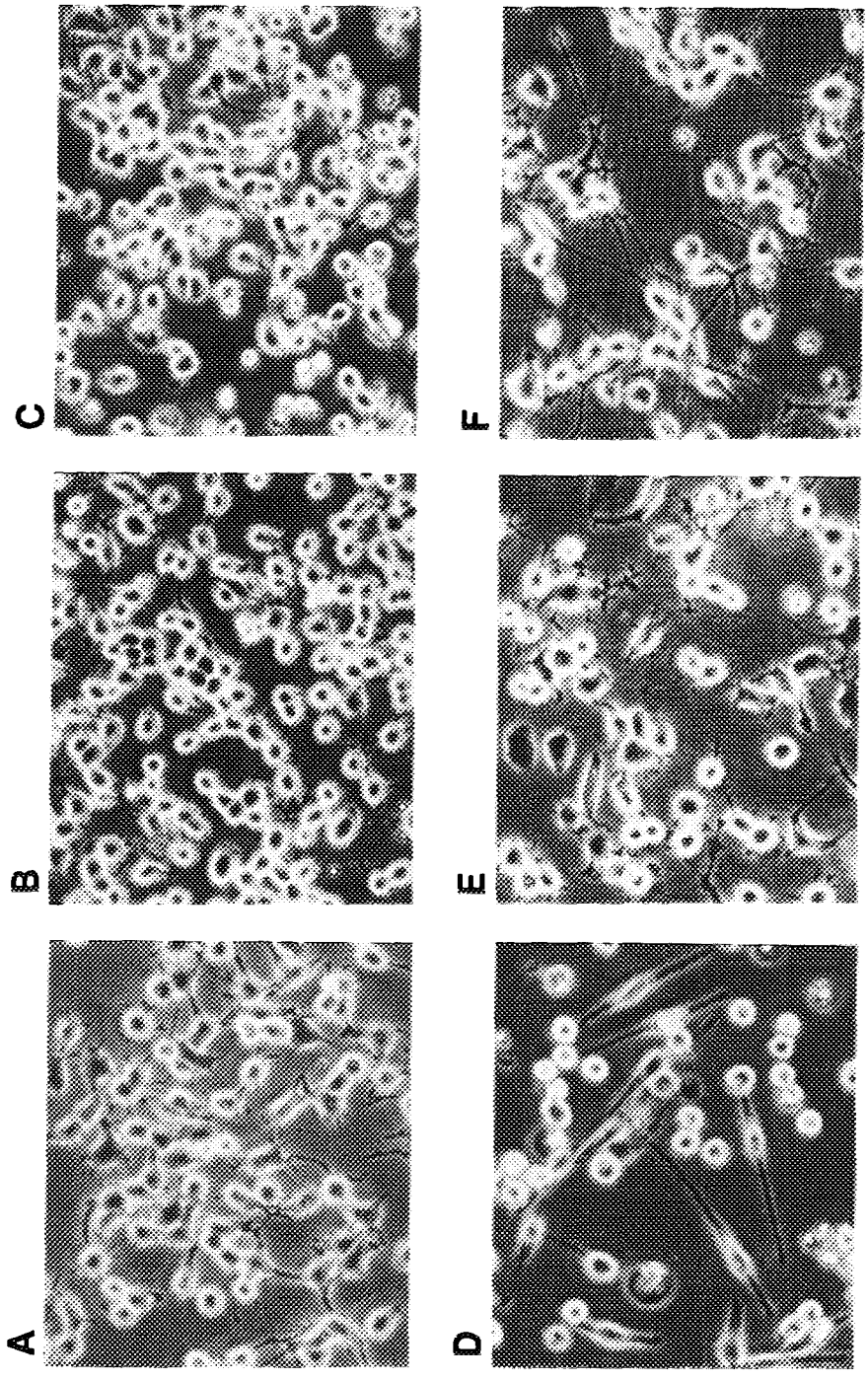

Figure 1. Photomicrographs of lactacystin-induced morphological changes in Neuro 2a cells. Cells were incubated in the absence (A, B and C) or presence (D, E and F) of 1.3 μM lactacystin for 1 (A and D), 2 (B and E) or 4 days (C and F), and photographed using a phase contrast microscope.

growth cones, parallel array of microtubules and intermediate filaments, and numerous electron density granules in the growth cones. The above neuritogenesis by lactacystin was dose-dependent. At higher concentrations than 5.2 or 10.4 μM, lactacystin exhibited some cytotoxicity. This type of morphological change differs from those induced by Bt_2cAMP, H-7 and gangliosides.

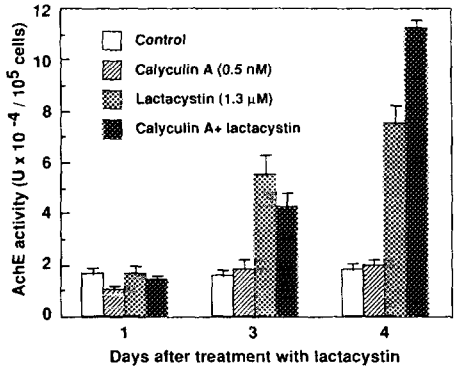

Figure 2. Effect of calyculin A on lactacystin-induced acetylcholinesterase (AChE) activity in Neuro 2a cells . AChE activity was determined using 10^5 cells incubated with 1.3 μM lactacystin for the indicated time. Values were expressed as the mean ± S.D. of three cultures.

Differentiation of neuroblastoma cells is characterized by the acquisition of functional neuronal properties such as an excitable membrane and the expression of high levels of specific neuronal enzymes, e.g. acetylcholinesterase [12]. As shown in Figure 2, acetylcholinesterase activity was increased with time by lactacystin. When the cells were treated with 1.3 μM lactacystin, the activities at 3- and 4-day were enhanced to 3.5 and 4.2 times, respectively, compared with control (without lactacystin). The effect was dose-dependent in 0.6~2.6 μM (data not shown). Thus, the differentiation of Neuro 2a neuroblastoma cells by lactacystin was characterized both morphologically and functionally.

Effect of various inhibitors of protein kinases and phosphatases: Chijiwa et al. [8] reported that H-89, an inhibitor of PKA, inhibits Bt_2cAMP-induced neuritogenesis in PC-12D pheochromocytoma cells. Minana et al. [7] reported that TPA, a PKC activator, reverses the induction of neuritogenesis by H-7, a PKC inhibitor. So, we investigated the effect of H-89 or TPA on neuritogenesis induced by Bt_2cAMP or H-7, respectively, and lactacystin in Neuro 2a cells. Bt_2cAMP (1 mM) induced the formation of 4 or more neurites per cell and the formation was inhibited by addition of H-89 (5 μM). These results are similar to those in PC-12D cells reported by Chijiwa et al. [8]. However, the formation of bipolar projections 24 h after addition of lactacystin (Figure 1E) was not affected by H-89 treatment, and also the network formation by lactacystin at and after 3-day (Figure 1F) was not affected (data not shown). Although the neuritogenesis induced by H-7 (500 μM) disappeared 2 h after treatment with TPA (5 nM), as reported by Minana et al. [7], the bipolar projections and networks induced by lactacystin did not disappear (data not shown). The above findings suggest that PKA and PKC are not involved directly in the induction of neuritogenesis by lactacystin, and that the mechanism of neuritogenesis induction by lactacystin differs from those by Bt_2cAMP and H-7.

Then, we investigated the effects of various inhibitors of protein kinases on lactacystin-induced neuritogenesis. KN-62 (20 μM), a Ca^{2+}/CaM kinase II inhibitor, inhibited the neuritogenesis induced by ganglioside GM1 (365 μM) in Neuro 2a cells as reported by Higashi and Kudo [13]. However, it did not inhibit the neuritogenesis induced by lactacystin (data not

Table 1. Effects of pretreatment of various protein phosphatase inhibitors on lactacystin-induced bipolar projections 1 day and network formation 4 days after treatment in Neuro 2a cells

Pretreatment with	Lactacystin (1.3μM)	Neurites / cell (%)							
		1 day				4 days			
		0	1	2	>2	0	1	2	>2
Control	-	77.6	5.5	0.7	16.2	80.7	2.4	1.0	15.9
	+	57.4	3.5	31.6	7.5	41.6	2.7	2.4	53.3
Calyculin A	-	81.8	2.4	0.8	15.0	79.8	3.3	0.8	16.1
(0.5 nM)	+	85.8	2.0	2.5	9.7	38.3	4.2	2.4	55.1
Okadaic acid	-	88.5	2.3	0.8	8.4	84.0	3.2	0	12.8
(0.6 nM)	+	80.9	5.3	2.6	11.2	42.2	2.1	0.5	55.2
Tautomycin	-	85.7	2.1	0.7	11.5	78.9	3.2	0.6	17.3
(39 nM)	+	60.3	5.1	27.9	6.7	41.0	4.0	2.9	52.1
Dephostatin	-	77.6	4.0	0.8	17.6	89.7	1.1	0	9.2
(5.1 μM)	+	57.2	3.5	32.2	7.1	43.9	3.8	1.9	50.4

After pretreatment of 0.5 nM calyculin A , 0.6 nM okadaic acid , 39 nM tautomycin or 5.1 μM dephostatin for 30 min , cells were incubated in the presence of 1.3 μM lactacystin. After 1 day or 4 days, randomly chosen fields were photographed using a phase contrast microscope, and the number of cells with neurites was counted.

shown). Ulloa et al. [14] reported that depletion of casein kinase II by antisense oligonucleoside prevents the neuritogenesis induced by serum starvation in Neuro 2a cells. We tested the effect of DBR, a casein kinase II inhibitor [15], on neuritogenesis induced by serum starvation and lactacystin. DBR (19.6 μM) inhibited the neuritogenesis induced by incubation in MEM-H with 1% FBS , but did not that induced by lactacystin (1.3 μM)(data not shown). Other protein kinase inhibitors, CKI-7 (50 μM)(casein kinase I inhibitor) [16] and herbimycin A (0.35 μM)(tyrosine kinase inhibitor) [17] did not inhibit the lactacystin-induced neuritogenesis (data not shown), although herbimycin A showed the tendency to delay somewhat the disappearance of bipolar projections at 2-day shown in Figure 1.

Next, we tested the effects of protein phosphatase inhibitors on neuritogenesis (Table 1). The protein phosphatase inhibitors calyculin A (0.5 nM) [18] and okadaic acid (0.6 nM) [19] inhibited the formation of bipolar projections at 1-day but did not affect the network formation at and after 3-day. Dephostatin, a protein tyrosine phosphatase inhibitor [20], did not affect lactacystin- induced neuritogenesis. Okadaic acid is potent active against type 2A (IC_{50}:0.07 nM) but less active against type 1 (IC_{50}:3.4 nM), while calyculin A is potent active against both type 1 and 2A (IC_{50}: 0.3 and 0.13 nM, respectively) [21]. This suggests that type 2A plays a more important role than type 1 in the formation of bipolar projections in Neuro 2a cells. The fact that calyculin A and okadaic acid inhibit the formation of bipolar projections at 1-day but did not the network formation at and after 3-day suggests that the mechanism of formation of bipolar projections differs from that of the network formation. The above findings also suggest that protein dephosphorylation is involved in the formation of bipolar projections, but it is dispensable to the network formation. Indeed, calyculin A did not inhibit significantly acetylcholinesterase activity in Neuro 2a cells induced by lactacystin but rather somewhat enhanced it at 4-day (Figure 2).

Our results demonstrate that lactacystin induces neuronal differentiation of Neuro 2a cells, and that the morphology and mechanism of neuritogenesis induced by lactacystin in the

cells are different from those induced by known neuritogenesis inducers, indicating that lactacystin is a new type of neuritogenesis inducer.

Recently, Fenteany *et al.* reported that *clasto*-lactacystin β-lactone also induces neuritogenesis in a Neuro 2a cells [22] and that lactacystin and the β-lactone are specific proteasome inhibitors, which covalently bind to the amino-terminal threonine on the β-subunit and inhibit three distinct peptidase activities of 20S proteasome (trypsin-like, chymotrypsin-like and peptidylglutamyl-peptide hydrolyzing activities) [23]. Because the cell cycle requires the ubiquitination and destruction of the mitotic cyclines by proteasome [24], our preceeding results [3] that lactacystin arrests cell cycle at both G1 and G2 phases coincide with the Fenteany's finding that it inhibits the peptidase activities of proteasome. Clarifying the relationship between the neuritegenesis and inhibition of proteasome (or cell cycle) induced by lactacystin is important in elucidation of mechanism for neuronal differentiation.

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