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Source: Zoological Science, 14(5) : 809-815

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.809

ZOOLOGICAL SCIENCE 14: 809–815 (1997)

A Protein Kinase Inhibitor H-7 Induces Process Extrusion in Fetal Rat Thyroid C-Cells *in vitro*

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ABSTRACT—Calcitonin-producing cells (C-cells) are endocrine cells derived from the neural crest. We examined the effects of three types of protein kinase inhibitors on the induction of neuronal phenotypes in the rat thyroid C-cells *in vitro*. In a primary culture of 16-day-old fetal rat thyroid glands, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7, 25-75 μM) induced both process extrusion and expression of highly polysialylated neural cell adhesion molecule (NCAM) in the C-cells. These effects of H-7 were completely prevented by okadaic acid, a potent protein phosphatase inhibitor. In contrast to H-7, selective inhibitors for cyclic nucleotide-dependent protein kinases such as *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004, 25-200 μM) and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89, 0.25-20 μM) failed to induce process extrusion or the expression of highly polysialylated NCAM in fetal rat C-cells. In cultured C-cells of adult origin, H-7 failed to induce marked process elongation or the expression of highly polysialylated NCAM. These results suggest that the morphological plasticity of the fetal C-cells depends upon the degree of phosphorylation of some proteins, and that the plasticity of adult C-cells are more restricted than that of fetal origin.

INTRODUCTION

A wide variety of cells share embryonic origin in the neural crest, which appears transiently in the embryonic life of vertebrates. Neural crest cells differentiate along their various lineages, with narrowing of their developmental options, and finally become diverse functional cells, including pigment cells, Schwann cells, endocrine cells, and sensory and autonomic neurons (Anderson, 1989; Le Douarin, 1982).

The well-known endocrine derivatives of the neural crest are adrenal medullary chromaffin cells (Le Douarin and Teillet, 1974) and thyroid calcitonin-producing cells (C-cells) (Le Douarin *et al.*, 1974; Pearse and Polak, 1971). It is well-known that the chromaffin cells possess various neuronal properties. They are directly innervated by sympathetic nerves and have the potential to transdifferentiate into neurons in response to environmental stimuli, such as nerve growth factor and basic fibroblast growth factor (Stemple *et al.*, 1988; Unsicker *et al.*, 1978). The neuronal features of the chromaffin cells and PC-12 rat pheochromocytoma cells have extensively been studied. Although several lines of evidence show that thyroid C-cells derived from common progenitor neural crest cells with serotonergic neurons in the myenteric plexus (Bernd *et al.*, 1979; Le Douarin, 1982), the neuronal properties of C-cells have yet to be fully investigated.

Our previous studies (Nishiyama and Fujii, 1992) showed that thyroid C-cells of fetal rats extruded processes on a laminin substratum in a primary culture, irrespective of the addition of nerve growth factor or basic fibroblast growth factor. These results suggested that C-cells, which do not display neuronal morphology *in vivo*, also retained the potential to extrude neurite-like processes *in vitro* by the morphoregulatory mechanism which differs from that in the chromaffin cells.

In the present study, we report that a protein kinase inhibitor H-7 promotes process outgrowth and expression of highly polysialylated neural cell adhesion molecule (high PSA NCAM) in cultured rat thyroid C-cells of fetal origin.

MATERIALS AND METHODS

Cell culture

Thyroid glands were excised from 16-day-old (E16) fetuses, and 9-week-old male Wistar rats (Charles River Japan). The glands were enzymatically dissociated into single cells (Nishiyama and Fujii, 1988) and were plated on glass coverslips (14 mm in diameter) in a 24-well multi-dish plate (Falcon, No. 3047) at a density of 2×10^5 cells/cm^2. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, No.430-1600) supplemented with 5% heat-inactivated fetal

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calf serum (Irvine), 50 units/ml penicillin (GIBCO) and 50 μg/ml streptomycin (GIBCO). The cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

Protein kinase inhibitors

1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from SEIKAGAKU Corp., Japan. H-7 and HA1004 were dissolved in distilled water at 10 mM. H-89 was dissolved in dimethyl sulfoxide at 10 mM. Stock solutions were stored at 4°C in the dark until use.

Effects of protein kinase inhibitors and protein phosphatase inhibitor on the morphology of cultured C-cells

After the cells were cultured for 24 hr, protein kinase inhibitor H-7 (25-75 μM), HA1004 (25-200 μM) or H-89 (0.25-20 μM) was added to the culture medium. Equivalent volumes of vehicle, less than 0.1% of total volume, were added to the medium as control. After incubated for 24 hr, the cells were fixed and C-cell morphology was observed by immunocytochemistry using anti-calcitonin antiserum (see below). To test the antagonizing effect of a protein phosphatase inhibitor okadaic acid (LC Services Corp.), a mixture of okadaic acid (30 nM) and H-7 (50 μM) in dimethyl sulfoxide was added to the culture medium, and C-cell morphology was observed. At least five independent experiments were performed for each age.

Indirect immunofluorescence methods

All the procedures described below were performed at room temperature. The primary cultures of rat thyroid cells were fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr. After being washed with phosphate-buffered saline (PBS, pH 7.4), the cells were blocked with 1% BSA (Sigma Chemical Co., Fraction V) for 30 min and further incubated for 1hr with mouse monoclonal antibody (MAb) 12E3 (1:5,000, IgM), which recognizes the polysialic acid portion of high PSA NCAM (Seki and Arai, 1991; Sato *et al.*, 1995). To detect intracellular calcitonin of the C-cells, the cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min, and blocked with BSA. The permeabilized cells were incubated with rabbit anti-human calcitonin (1:400, ICN) for 1hr. After being washed with PBS, the cells were incubated for 30 min with a mixture of rhodamin-conjugated goat anti-rabbit IgG antibody (1:100, Cappel)

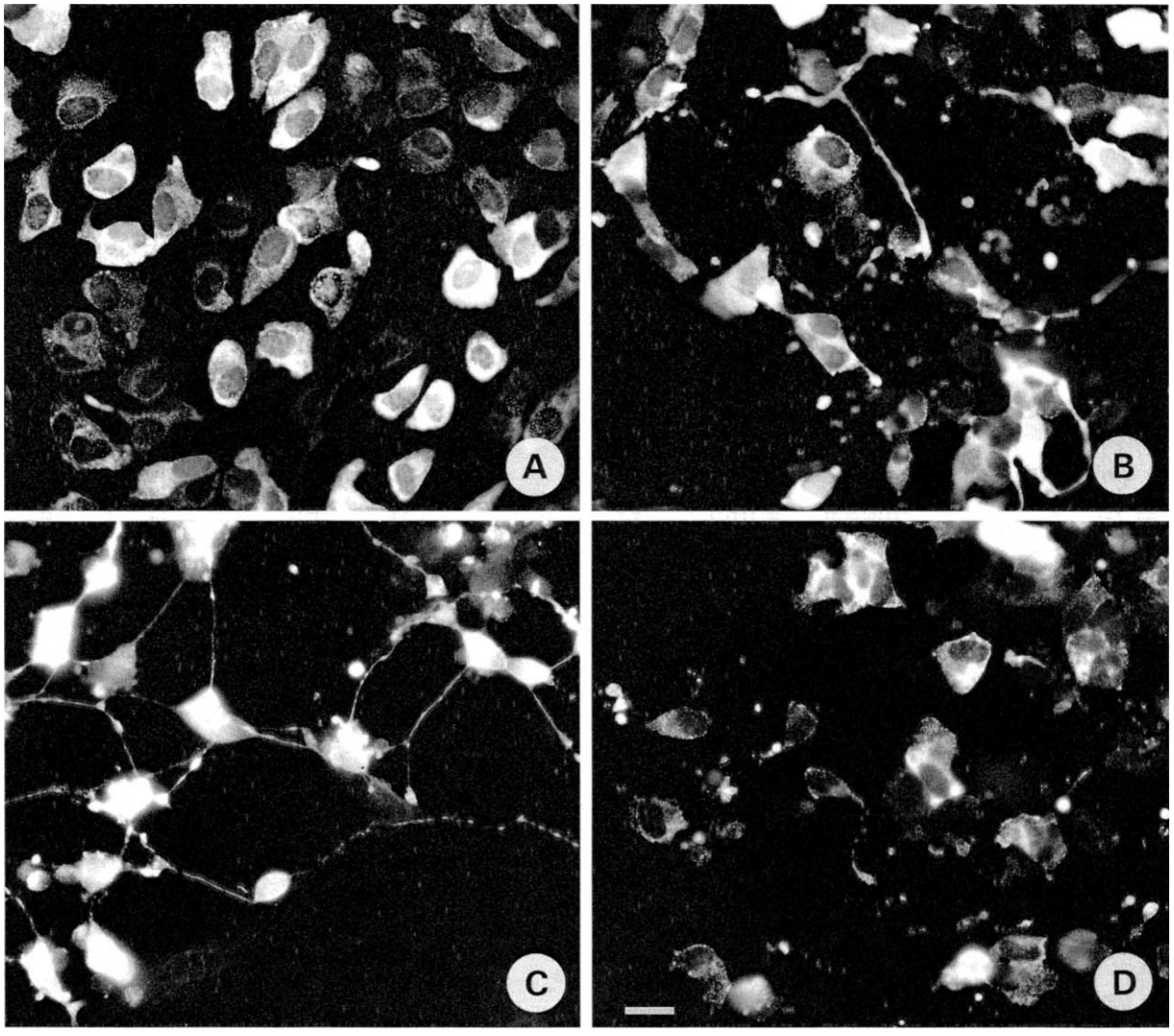

Fig. 1. Effects of H-7 and okadaic acid on C-cell morphology in the primary cultures of E16 rat thyroid glands. The primary cultures of E16 rat thyroid were treated with 0 (**A**), 25 (**B**) or 50 μM (**C**, **D**) H-7 in the absence (**A**-**C**) or presence (**D**) of 30 nM okadaic acid. After incubated for 24 hr, the cells were fixed and subjected to indirect immunofluorescence analysis using anti-calcitonin antiserum. Scale bar = 20 μm.

and fluorescein-conjugated goat anti-mouse IgM antibody (1:100, Cappel). After the removal of unbound antibodies with PBS, the specimens were embedded in glycerol-PBS (9:1) containing 0.1% *p*-phenylenediamine, and observed under a Olympus VANOX microscope equipped with fluorescein optics. As negative controls, BALB/c control ascites fluid (Cedarlane Lab.) and normal rabbit serum (GIBCO) were substituted for the primary antibodies.

RESULTS

Effects of protein kinase inhibitors and okadaic acid on the morphology of fetal rat C-cells

In the primary cultures of E16 rat thyroid glands, C-cells, which were identified as calcitonin-positive cells, were ovoid or polygonal in shape (Fig. 1A). The addition of H-7 to the primary cultures promoted process outgrowth from C-cells within 24 hr, concomitantly with an increased roundness of the cell bodies, depending on its concentrations (Fig. 1 B and C). The effective concentrations of H-7 were 25-75 μM. Higher concentrations of H-7 (100 μM <) did not induce process outgrowth. Substantial decrease in cell viability was caused by H-7 at the concentrations exceeding 150 μM. Withdrawing H-7 decreased the ratios of process-bearing cells to the control level within 48 hr, indicating the effect of H-7 is reversible (not shown).

In contrast, HA1004 (25-200 μM) or H-89 (0.25-20 μM) did not cause any noticeable changes in the morphology of fetal rat C-cells (not shown).

To confirm that H-7-induced changes in C-cell morphology depend upon a decreased level of phosphorylation of some proteins, okadaic acid, a potent inhibitor of protein phosphatases (Bialojan and Takai, 1988), was added to thyroid cell cultures, together with 50 μM H-7. Process extrusion in H-7-treated fetal C-cells was markedly inhibited by 30 nM okadaic acid (Fig. 1 compare D with C). Cell viability was not affected by 30 nM okadaic acid during the experimental period.

Effects of protein kinase inhibitors and okadaic acid on expression of high PSA NCAM in fetal rat C-cells

H-7 caused not only morphological changes, but also changes in the antigenic phenotype in the cultured C-cells of E16 rat fetuses. The addition of H-7 (25-75 μM) caused expression of intense immunoreactivity to MAb 12E3, which recognizes the polysialic acid portion in high PSA NCAM, on the surfaces of C-cells (Fig. 2). In the H-7-treated cultures, immunoreactivity for high PSA NCAM was detected in the entire surfaces of C-cells, while surrounding follicular cells and fibroblastic cells were negative to MAb 12E3 (Fig. 2 D,F). HA1004 or H-89 did not promote expression of immunoreactivity for high PSA NCAM in the C-cells.

H-7-induced expression of high PSA NCAM in fetal C-cells was completely inhibited by 30 nM okadaic acid (Fig. 3).

Effects of H-7 in adult rat C-cells

In order to compare the morphological plasticity of rat C-cells of adult origin with that of fetal origin, the effects of H-7 were tested in thyroid cultures of 9-week-old rats. In contrast to fetal C-cells, H-7 (25-75 μM) did not induce neurite-like process extrusion, but caused the outgrowth of short (<30 μm) and thick processes from some of the C-cells of adult origin (Fig. 4C). In this case, immunoreactivity for high PSA NCAM was not observed in the C-cells (Fig. 4D). A prolonged incubation period with H-7 up to 72 hr or higher concentrations of H-7 (100 μM) also failed to induce marked process elongation or expression of high PSA NCAM in the C-cells of adult origin.

DISCUSSION

The physiological role of the C-cells is to synthesize and to secrete calcitonin, a polypeptide hypocalcemic hormone (Care *et al.*, 1968). In the course of fetal development, C-cells differentiate from neural crest cells (Le Douarin *et al.*, 1974; Pearse and Polak, 1971). As predicted from their origin, C-cells possess several neuronal properties. They are capable of synthesizing serotonin from L-tryptophan, and contain it in their secretory granules (Bernd *et al.*, 1979). In many animal species including rats, C-cells are known to produce calcitonin gene-related peptide (Kameda, 1987), which is expressed preferentially in neurons as another product of the calcitonin gene (Sabate *et al.*, 1985). Our previous study revealed that thyroid C-cells exhibit immunoreactivity for tau (Nishiyama *et al.*, 1995), which is a family of neuronal microtubule-associated proteins (Tucker, 1990). Electrophysiological similarities between C-cells and neurons have also been demonstrated (Kawa, 1988; Sand *et al.*, 1981, 1986). Despite of these neuronal properties, C-cells *in vivo* do not display neuronal morphology. The present results have shown that H-7, a selective inhibitor for protein kinase C (Hidaka *et al.*, 1984), induces marked process extrusion in fetal rat thyroid C-cells *in vitro*. It suggests that thyroid C-cells, which *in vivo* contain a variety of neuronal markers, have potential to display morphological traits characteristic of neurons.

In contrast to H-7, HA1004 and H-89 failed to induce process extrusion in fetal rat C-cells *in vitro*. These inhibitors have different kinase specificities: HA1004 and H-89 are selective inhibitors of cyclic nucleotide-dependent protein kinases, while H-7 in addition inhibits protein kinase C (Hidaka *et al.*, 1984). Therefore it is plausible that inhibition of protein kinase C causes the extrusion of processes in the fetal rat C-cells. However, the kinase-inhibitory effects of these compounds are not absolutely specific. Further experiments are required to identify the kinase type participating in the morphological changes of C-cells.

As shown in Fig. 1D, the process-generating effects of H-7 on C-cells were counteracted by okadaic acid. Okadaic acid is a potent inhibitor of protein phosphatases 1 and 2A (Bialojan and Takai, 1988), and is known to stimulate protein phosphorylation in intact cells (Haystead *et al.*, 1989). Therefore these results are not contradictory to the hypothesis that the morphological changes in C-cells caused by H-7 are mediated by a decreased level of phosphorylation of some pro-

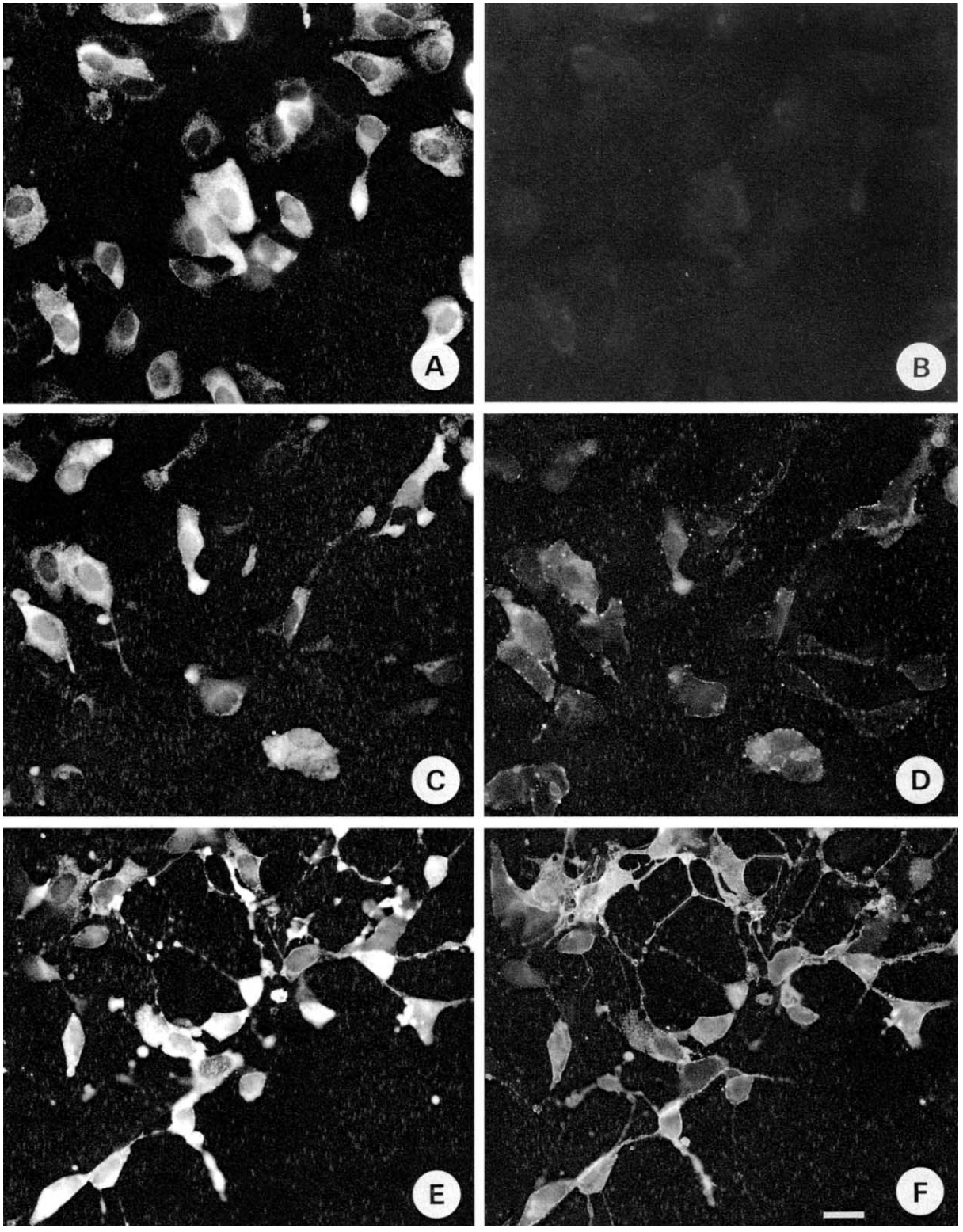

Fig. 2. Immunofluorescence analysis of highly polysialylated NCAM in the primary cultures of E16 rat thyroid glands. The primary cultures of E16 rat thyroid were maintained with 0 (**A**, **B**), 25 (**C**, **D**) or 50 μM (**E**, **F**) H-7 for 24 hr. The cells were examined by double immunofluorescence procedures using anti-calcitonin antiserum (**A**, **C**, **E**) and anti-highly polysialylated NCAM antibody (**B**, **D**, **F**). All the calcitonin-immunoreactive cells in **C** and **E**, but none in **A**, exhibit immunoreactivity for highly polysialylated NCAM. Scale bar = 20 μm.

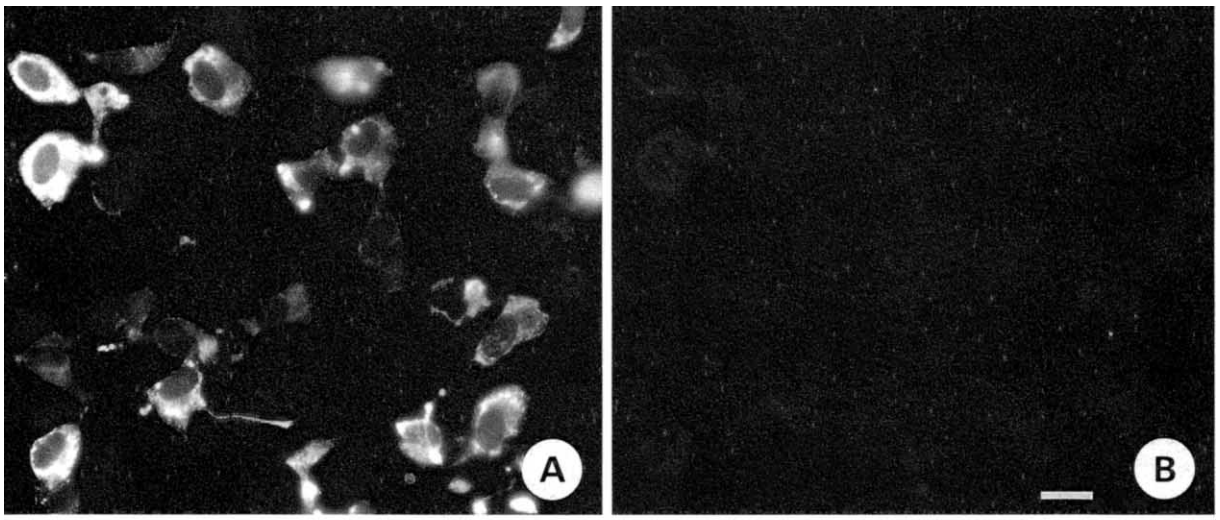

Fig. 3. Effects of okadaic acid on H-7-induced expression of highly polysialylated NCAM in E16 C-cells. The primary cultures of E16 rat thyroid were treated with 50 μM H-7 and 30 nM okadaic acid for 24 hr. The cells were examined by double immunofluorescence procedures using anti-calcitonin antiserum (**A**) and anti-highly polysialylated NCAM antibody (**B**). No immunoreactivity for highly polysialylated NCAM was observed. Scale bar = 20 μm.

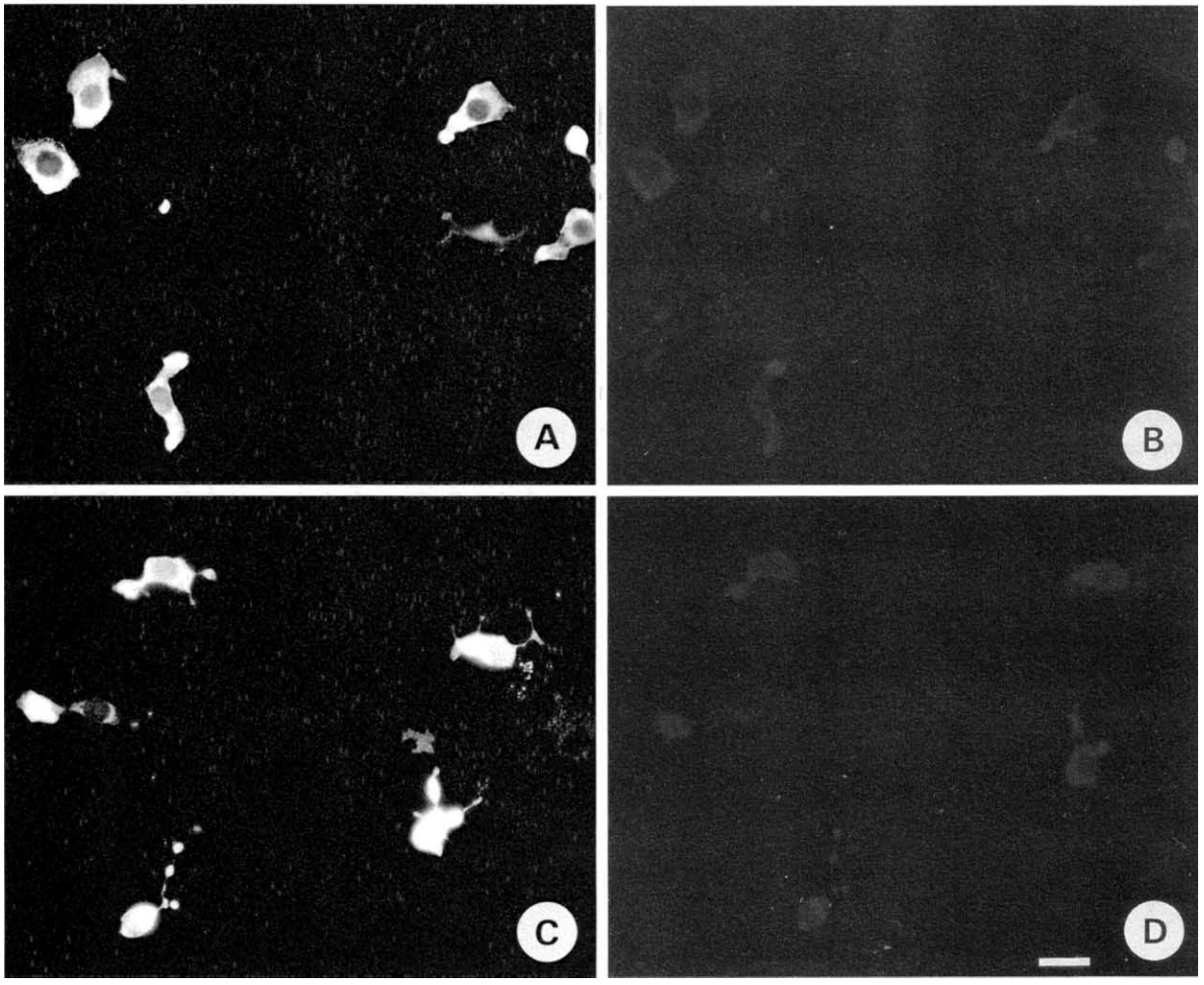

Fig. 4. Effects of H-7 on C-cell morphology in the primary cultures of 9-week-old rat thyroid glands. The primary cultures of 9-week-old rat thyroid were maintained with (**C**, **D**) or without (**A**, **B**) 50 μM H-7 for 24 hr, and subjected to double immunofluorescence procedures using anti-calcitonin antiserum (**A**, **C**) and anti-highly polysialylated NCAM antibody (**B**, **D**). H-7 induced less marked effects on the morphology of C-cells of adult origin than those of fetal origin, and failed to induce the expression of highly polysialylated NCAM (compare with Fig. 2). Scale bar = 20 μm.

teins in the cells.

The morphological changes of C-cells induced by H-7 must be accompanied with changes in cytoskeletal dynamics. As to the cytoskeletal components, we have demonstrated that microtubules (Nishiyama and Fujii, 1992), microtubule-associated protein tau (Nishiyama *et al.*, 1995), and cytokeratin (Nishiyama *et al.*, in preparation) are contained in the thyroid C-cells. It is well established that the function of these cytoskeletal molecules is influenced by the degree of phosphorylation of these molecules (Lindwall and Cole, 1984; Yano *et al.*, 1991; Yeagle *et al.*, 1990). The effects of H-7 and/or okadaic acid on the extent of phosphorylation of these cytoskeletal proteins in the C-cells are now being investigated in our laboratories.

In thyroid primary cultures, H-7 induced marked morphological changes in C-cells of fetal origin, but not in those of adult origin. These results suggest that developmental potential is more limited in adult C-cells than in fetal C-cells. Although there may be various differences between fetal and adult C-cells, we take note of high PSA NCAM on the cell surfaces. The present results showed that H-7 induced the expression of high PSA NCAM in C-cells of fetal origin but not in those of adult origin. Although NCAM has calcium-independent homophilic binding capability in the polypeptide portion, the polysialic acid moiety of NCAM reduces the binding capability of several cell adhesion molecules, including NCAM itself (Acheson *et al.*, 1991; Hoffman and Edelman, 1983; Landmesser *et al.*, 1990; Sadoul *et al.*, 1983). These effects of polysialic acid are thought to be due to the hindrance of membrane opposition by charge perturbation or by the large excluded volume of saccharide chains (Edelman, 1983; Rutishauser *et al.*, 1985). Therefore surface expression of high PSA NCAM results in increased plasticity in cell-to-cell and/or cell-to-substrate adhesion, and allows the cells to migrate or extend processes. Indeed, in the central nervous system, high PSA NCAM expression is specifically found in postmitotic neurons that extend neurites or migrate in the course of neural development (Seki and Arai, 1993). Furthermore, *in vitro* study by Doherty *et al.* (1990) have indicated that polysialic acid can reduce cell adhesion and promote neurite outgrowth. If this is also the case in C-cells, surface expression of high PSA NCAM caused by H-7 through an unknown pathway may allow cell surface movement and consequently allow process extrusion from the C-cells caused by simultaneous changes in cytoskeletal dynamics.

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(Received April 11, 1997 / Accepted July 3, 1997)