ABSTRACT The glycogen body (GB) is at the dorsal area of the lumbosacral spinal cord in birds and is composed of uniform cells that are characterized by high-glycogen storage. Previous morphological and embryological examinations suggest that the GB is derived from the neuroepithelium and contains many blood vessels and a few nerve fibers. However, the function of the GB and role of the glycogen are unknown. Mammalian astrocytes are major sites for glycogen stores in the central nervous system. The metabolic features of astrocytes have been defined by using cultured cells. As a first step toward investigating the function of GB, we established primary culture of chicken GB cells and telencephalon astrocytes. The cultured GB cells maintained high glycogen content and glial fibrillary acidic protein (GFAP) in the cytoplasm.

(Key words: astrocyte, glycogen metabolism, neurotransmitter, adrenergic receptor, brain)

INTRODUCTION

The glycogen body (GB) is an ovaloid gelatinous mass located in the lumbosacral region of the spinal cord in birds and is composed of uniform GB cells (Watterson, 1949). The GB cells have a very high concentration of glycogen and contain glial fibrillary acid protein (GFAP; Jankaskova et al., 1988). Developmentally, the GB appears as a periodic acid Shiff (PAS)-positive cell cluster at about 7 to 8 d of incubation and increases in volume as elaborated large stores of glycogen after 15 d of incubation (Louis, 1959; Daniel, 1972). Moreover, the PAS-positive cells arise from neuroepithelium that comprises the ependyma and roof plate of the avian lumbosacral spinal cord (Louis, 1993). Thus, it is recognized that the GB cells are derived from a glial cell lineage and classified as specialized astrogial cells.

The brain in a resting state has the highest oxygen consumption of the body, excluding the heart and kidneys. Glucose is always supplied to the brain as its largest energy reserves along with oxygen because the rate of glucose consumption has a syntropic relationship with the rate of oxygen consumption and blood flow. In the brain, a small fraction of the glucose is transformed into glycogen, which is predominantly localized in the astrocytes (Peters et al., 1976). The glycogen in the brain is only an energy reservoir.

Cell culture methods for astrocytes in rodents are established, and many scientists have studied glycogen metabolism and the function of astrocytes by cultured cells in vivo and in vitro. With astrocyte cultures, it has been reported that the glycogen content is changed by a variety of neurotransmitters such as serotonin, histamine, vasointestinal peptides, adenosine, and noradrenalin and some hormones such as insulin, methionine sulfoximine, and glucagon (Swanson et al., 1990; Hevor and Delorme, 1991; Sorg and Magistretti, 1991, Dringen and Hamprecht, 1992). In particular, a number of neuromodulators decrease the glycogen content of astrocytes (Magistretti et al., 1983; Pearce et al., 1988; Sorg and Magistretti, 1991,

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Abbreviation Key: cAMP = cyclic adenosine monophosphate; CL = clonidine; DMEM = Dulbecco’s modified Eagle’s medium; FCS = fetal calf serum; GB = glycogen body; GFAP = glial fibrillary acidic protein; ISO = isoproterenol; MET = methoxamine; NA = noradrenaline; PAS = periodic acid Shiff; Tc = telencephalon.
1992). Some of the glycogenolytic effects occur through adrenergic receptors. In fact, the cultured rat astrocytes express α- and β-adrenergic receptors, the former being found in some cells (Subbarao and Hertz, 1990) but the latter found in almost all astrocytes (Harden and McCarthy, 1982; Hertz et al., 1984; Hansson and Ronnback, 1989).

The GB cells contain several enzymes for glycogen metabolism. It has been reported that the GB lacks glucose-6-phosphatase but contains relatively high levels of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Benzo et al., 1975). However, the glycogen content of the GB in vivo is not altered by a variety of enzymes or hormones such as insulin, glucagon, or adrenocorticotropic hormone or by starvation (Smith and Geiger, 1961; Snedecor et al., 1963; Martha et al., 1970; George et al., 1972). Thus, it is suggested that the glycogenolytic activity of GB cells is very low (Lervold et al., 1970; George et al., 1972). The GB cells contain several enzymes for glycogen metabolism and it is reasonable to assume that GB cells respond to some neurotransmitters and that the glycogen content is modulated by autonomic innervation. In vitro analyses should offer some useful information about the characteristics of GB cells, as in mammal astrocytes. In the present study, we established the culture method of GB cells and studied the effect of adrenergic agonist on the glycogen content in the primary astrocyte culture of GB and telencephalon (Tc).

**MATERIALS AND METHODS**

**Chemicals**

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui Pharmaceutical Co. Ltd. Penicillin, streptomycin, fetal calf serum (FCS), trypsin, and calcium-magnesium-free Hank’s balanced salt solution were from Gibco. Noradrenaline (NA), methoxamine (MET), and clonidine (CL) were from Sigma Chemical Co. Isoproterenol (ISO) and rabbit anti-bovine GFAP were purchased from Wako Pure Chemical Industries Ltd., and goat anti-rabbit IgG-conjugated FITC was purchased Seikagaku Kogyo Co. Ltd.

**Cell Culture**

Astrocyte-rich primary cultures were prepared from the GB and Tc of White Leghorn chick embryos at 15 d of incubation. The GB and Tc were dissected out, freed of meninges, and washed in calcium-magnesium-free Hank’s balanced salt solution supplemented with 50 U penicillin and 50 μg streptomycin/mL. The Tc tissues were dissociated mechanically by passage through a steel mesh, and the GB was cut into small pieces. The partially disrupted tissues were suspended in 0.25% trypsin and incubated in a shaking water bath for 20 min (Tc) or 90 min (GB) at 37°C. After enzyme digestion, the tissues were further dissociated by repeated pipetting and were centrifuged at 400 × g for 5 min. The isolated cells were resuspended in 2 mL DMEM-FCS and seeded in 35-mm Petri dishes at a density of 7 to 8 × 10^5 (GB/dish) or 1 to 2 × 10^4 (Tc/dish). In all experiments, the cells were incubated in DMEM supplemented with 10% FCS, 50 U penicillin, and 50 μg streptomycin/mL at 37°C in a 5% CO₂ atmosphere with 95% humidity. The culture medium was renewed twice a week.

**Effect of Glucose Medium**

The cells were cultured in DMEM containing 0 mM, 10 mM, 25 mM, or 40 mM glucose for 14 d. The cultures were washed three times with ice-cold PBS and processed for glycogen assays and histochemical and ultrastructural observations as described below.

**Effect of Adrenergic Agonist**

The cells were cultured in DMEM containing 20 mM glucose. Confluent cell cultures (10 to 12 d) were preincubated in FCS-free DMEM, containing 20 mM glucose, for 2 h to maximize glycogen content. After preincubation, the cultures were incubated with fresh medium containing NA (100 μM), ISO (50 μM), CL (50 μM), or MET (50 μM) for various times. The reactions were stopped by washing the dishes by three times with ice-cold PBS. The cultures were processed for glycogen and histochemical assays.

**Glycogen Assay**

Cells were scraped from Petri dishes by adding 1 mL of 0.1 N NaOH, and the cell suspension was stored at −20°C until used for assay. After a sonication at room temperature, an 800-μL aliquot was used to assay glycogen content according to the modified procedure of Cummins et al. (1983). Briefly, the suspensions were heated to 80°C for 20 min to destroy the glucose. The samples were then cooled to room temperature and adjusted to pH 4.2 to 4.5 by addition of acetic acid. Thereafter, 5 μL of amyloglucosidase was added to hydrolyze glycogen to glucose in 30 min at room temperature. Subsequently, the samples were used for determination of glucose content with a mixture of mutarotase, glucose oxidase, peroxidase, 4-aminoantipyrine, and ascorbate oxidase (Miwa et al., 1972). The glucose levels were measured by ultraviolet spectrometer. A 10-μL aliquot was used to determine the protein content by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Each experiment was performed at least five times, with comparable results. Data were presented as glycogen content per pro-

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\[\text{7}\text{A 10-μL aliquot was used to determine the protein content by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Each experiment was performed at least five times, with comparable results. Data were presented as glycogen content per pro-}\]
tein (Figure 4) or percentage of glycogen content at 0 min (Figures 5 and 6). The differences were analyzed by Student’s t-test.

**Histochemical Assays**

Cultured cells were fixed for 1 h in 10% formalin in ethanol. To detect glycogen, the cells were rinsed with 66% ethanol and treated with PAS reaction. For cell identification, cells were incubated with primary antisera against GFAP for 18 h, rinsed with PBS, and incubated with FITC-conjugated goat anti-rabbit IgG for 2 h. Cells were observed under a confocal laser microscope.8

**Ultrastructural Observation**

Confluent cells were fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer for 2 h, followed by postfixation with 1% OsO4 in the same buffer for 1 h at 4°C. After dehydration with ethanol, the cell sheets were removed by resolving the dish in QY-2 and were embedded in Epok-812. Thin sections were cut and observed under transmission electron microscopy.9

**RESULTS**

**Cell Morphology in Culture**

Cultured GB cells were attached to the dish 1 d after seeding. The cells were flat or spindle-shaped with some processes and formed a cell sheet. After 2 to 3 d, cell islets appeared among the flat sheet of cells. The islet cells were round or polygonal, and aggregates protruded into the medium. Flat cells and islet cells were GFAP positive (Figure 1). The cytoplasm of the islet cells showed a strong reaction to glycogen, whereas the reaction of the flat cells varied from granules to droplets in the cytoplasm or only in the processes (Figure 2). At the periphery of the islets, intermediate cells between flat and islet cells were observed that had round cytoplasm with some processes and with medium PAS reactions. Under electron microscopy, the cytoplasm of the islet cells contained many glycogen granules and intermediate filaments, which were mainly around the nucleus and between the glyco-

**FIGURE 2.** Periodic acid Schiff reaction of cultured glycogen body cells cultured in Dulbecco’s Modified Eagle’s medium with 25 mM glucose. ×185.
GLYCOGENOLYSIS OF CULTURED GLYCOGEN BODY CELLS

Effect of Glucose Medium

At all glucose concentrations in the medium, GB cells grew and formed flat sheets and islet areas, as described above. Almost all flat cells in the medium with glucose were PAS positive, but some in the medium without glucose were PAS negative. The islet cells were strongly PAS positive at all concentrations. The glycogen levels in different glucose concentrations are shown in Figure 4. The glycogen levels in the GB and Tc cells were apparently higher in the medium with glucose than in the medium without glucose, but there were no significant differences among glucose concentrations (10, 25, and 40 mM). The GB cells contained more glycogen than Tc cells in any medium, and this difference increased as glucose in the medium was increased from 0 to 10 mM.

Effect of Adrenergic Agonists on Glycogenolysis

The time courses of the effects of NA, ISO, MET, and CL on glycogenolysis of GB cells and Tc cells are shown in Figures 5 and 6. The glycogen levels of Tc cell cultures were significantly lower than in control and gradually decreased over time in NA treatment. At 4 h of incubation, glycogen was about 62% of that in the control (at 0 h). The level of glycogen in ISO treatment cultures declined slightly over time. After 1 h of incubation, glycogen reached about 80%, but a significant difference from the control was observed 4 h after administration. The applications of MET as α1-adrenergic agonist and CL as α2-adrenergic agonist to a cell culture medium of Tc are shown in Figure 6. The change in glycogen levels by MET was not found in all periods. However, glycogenolysis by CL significantly decreased by about 10% after 2 h,
FIGURE 5. Effects of noradrenalin and isoproterenol on the glycogen contents of glycogen body cells (A) and telencephalon astrocytes (B). Values are shown as means ± standard deviation (n = 5), and asterisks indicate significant differences (P < 0.01) from control at the same time.

DISCUSSION

Under the present cell culture conditions, Tc-derived cells were GFAP positive and formed uniform flat sheets of cells. Thus, we identified cultured Tc cells as virtually one lineage of astrocyte. In the GB-derived cell cultures, islet-like cell masses were formed as well as flat sheets of cells from early-stage cultures. The islets were observed under all culture conditions and in all culture periods examined. The islet cells were spherical and multigonal in shape, had few processes, and showed strong immunoreactivity to GFAP. The ultrastructural features of the islet cells were similar to those in a previous report about GB cells in vivo (Smith and Geiger, 1961), suggesting that the islets of GB cells are similar to the GB in vivo. The cells forming flat cell sheets in GB cell cultures were similar to those in Tc cell cultures in their shape and immunoreactivity for GFAP. However, the PAS reaction of the flat cells was stronger than that of Tc cells. Because an intermediate type, between flat cells and islet cells, was detected at the periphery of the islets, we recognized that flat and islet
compared with control. After 3 h of incubation of the two α-adrenergic agonists in culture medium, glycogen levels were not changed significantly (data not shown).

In GB cell culture, as significant differences in glycogen levels between control and adrenergic agonist-treated cultures were not observed after 1 or 2 h of incubation, we remeasured the glycogen content 10, 20, and 30 min after adrenergic agonist application. The effects of NA and ISO on GB cell culture are shown in Figure 5. The glycogen levels in NA treatment decreased, but the duration of the effect was shorter than in Tc. The glycogen level rapidly decreased at 10 min and reached 70% of the control level. After 10 min, the levels slightly increased and showed significant differences even 30 min after administration. ISO-induced glycogenolysis was not observed in GB cells at any incubation time examined. As shown in Figure 6, the glycogen levels reached by addition of MET did not differ when compared with the control. We observed a significant difference only 20 min after the addition of CL (Figure 6).
cells were the same GB cells at different phases under present culture conditions.

It has been reported that the glycogen contents of cultured rat astrocytes are changed by the glucose concentration in the medium (Dringen and Hamprecht, 1992). The glycogen level of mouse astrocytes is low in the glucose-deprived medium but increases with the glucose concentration in the medium, reaching a plateau of over 10 mM glucose in the medium (Cummins et al., 1983). The present histochemical and glycogen assays showed that the glycogen contents of the Tc and GB cells were changed by glucose concentration in the same pattern as mouse astrocytes. The glycogen level of Tc astrocytes was similar to that of the mouse. Although the GB cells contained a high level of glycogen even in glucose-free medium, the increasing ratio of glycogen levels from 0 to 10 mM glucose in the medium was higher than that of the Tc. Glycogen synthase and glycogen phosphorylase are the rate-limiting enzymes in the regulation of glycogen metabolism in glycogen-storing tissues, and both enzymes have active and inactive forms in vivo. The glucose in the medium is one of the elements that activates the glycogen phosphorylase in astrocytoma cells (Passonneau and Crites, 1976). In GB cells, glycogen phosphorylase is present, but most of it remains in an inactive form in vivo (Benzo et al., 1975). Glycogen metabolism of GB is different from that in the Tc astrocytes.

The present study showed that the effects of adrenergic agonists on glycogenolysis were different between GB cells and Tc astrocytes in the chicken. NA, which is an \( \alpha \) and \( \beta \) adrenergic agonist, and ISO, which is a \( \beta \) adrenergic agonist, decreased the glycogen content of the Tc astrocytes in a time-dependent manner. After 2 h of incubation, the glycogen levels decreased about 40% for NA and about 20% for ISO. In the experiments using MET as an \( \alpha \)-1 agonist and CL as an \( \alpha \)-2 agonist, the Tc cells reacted to CL at one time examined, i.e., when the glycogen levels were slightly decreased. This action of NA in chicken astrocytes is in agreement with previous reports that the glycogen content of mouse astrocytes is mediated predominantly by a \( \beta \) adrenergic receptor and only slightly by an \( \alpha \)-2 adrenergic receptor (Pearce et al., 1988; Sorg and Magistretti, 1991; Subbarao and Hertz, 1990, 1991). The present results with Tc cells suggest that chick astrocytes have similar characteristics to the mouse astrocytes in their glycogenesis and glycogenolysis.

Although, NA stimulated the glycogenolysis significantly in GB cells, ISO had no effect. The glycogen levels suddenly decreased about 20% to 10 min after administration of NA and gradually increased until 2 h. The glycogenolytic reactions to the \( \alpha \)-1 and \( \alpha \)-2 adrenergic receptors were similar to those of Tc astrocytes. Significant reactions were observed 20 min after CL administration. However, the loss of glycogen by CL was lower than that by NA. These results suggest that the glycogenolysis of GB cells is partially affected by the \( \alpha \) adrenergic receptors but not by the \( \beta \) adrenergic receptor.

Previously published data have suggested that NA increases cyclic adenosine monophosphate (cAMP) formation mediated by \( \beta \)-adrenergic receptors and that the cAMP leads to the conversion from inactive phosphorylase to active phosphorylase to stimulate glycogen hydrolysis (Passonneau and Crites, 1976; Quach et al., 1978; Magistretti, 1983; Sorg and Magistretti, 1991, 1992). As well as cAMP formation, NA also stimulates calcium formation (Salm and McCarthy, 1990), lactate formation, and phospholipase D activity (Quach et al., 1978; Pearce et al., 1985, 1988; Northam and Mobjley, 1987; Subbarao and Hertz, 1990, 1991; Sorg and Magistretti, 1991; Bruna et al., 1995; Ronald et al., 1996) in cultured mouse astrocytes. It is possible that the action of NA on GB cell glycogenolysis is not a direct effect of the \( \alpha \)-adrenergic receptor, but, rather, it travels via another metabolic pathway, for example, via lactate.

REFERENCES


