Ouabain-Induced Cell Proliferation in Cultured Rat Astrocytes

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ABSTRACT—Ouabain markedly stimulated not only [^{3}H]thymidine incorporation but also [^{3}H]uridine incorporation into astrocytes. The effects were observed at 36–48 hr and 12–72 hr after addition of ouabain, respectively. The dose-response curves were both bell-shaped types with a peak at 10^{-3} M for thymidine incorporation and 2×10^{-3} M for uridine incorporation. Ouabain increased cell number as determined by an assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and by a method using a hemocytometer. Low concentration of external K^+ mimicked the effect of ouabain in stimulating [^{3}H]-thymidine incorporation, and high concentration of external K^+ blocked the effect of ouabain. In contrast to astrocytes, ouabain did not stimulate [^{3}H]thymidine incorporation into C6 glioma and fibroblast cells. The effect of ouabain on [^{3}H]thymidine incorporation in astrocytes was dependent on external Ca^{2+}, and it was blocked by cycloheximide. These findings indicate that prolonged Na^+,K^+-ATPase inhibition causes cell proliferation in cultured astrocytes in cell-specific and Ca^{2+}-dependent manners.

Keywords: Ouabain, Na^+,K^+-ATPase, Cell proliferation, Thymidine incorporation, Astrocyte

Previous studies show that ouabain-like factors that may serve as in vivo regulators of the Na^+,K^+-ATPase activity are present in animal brain tissues (1, 2). Ouabain-like factors are considered to have an important role in hypertension and natriuresis (3–6). Consistent with the role of ouabain-like factors, exogenous ouabain acted centrally to cause sympathoexcitation and hypertension (7–10). In addition, injection of ouabain into the specific regions of rat brain produced a necrotic lesion (11–13). These in vivo effects may be due to ouabain-induced inhibition of the Na^+,K^+-ATPase in the brain, although the exact mechanism is not known. Since the Na^+,K^+-ATPase is localized not only in neurons but also in glial cells, the possibility must be considered that glial cells as well as neurons are involved in the central effects of ouabain-like factors or exogenous ouabain.

We have previously demonstrated that α_1- and α_2-isoforms of the Na^+,K^+-ATPase are present in cultured rat astrocytes, and the former isoform is selectively induced by an activation of insulin-like growth factor-I (IGF-I) receptors (14). Furthermore, we found that ouabain (1 mM for 24 hr) blocked the mitogenic effect of IGF-I in astrocytes (15), suggesting the involvement of the α_1-isoform in the mitogenic action of the growth factor in astrocytes. During the study, we unexpectedly observed that prolonged exposure of astrocytes to ouabain (1 mM for more than 36 hr) markedly stimulated [^{3}H]-thymidine incorporation into the DNA fraction. That is, Na^+,K^+-ATPase inhibition for a restricted time blocks IGF-I-induced cell proliferation, while inhibition for a longer time stimulates [^{3}H]thymidine incorporation in cultured astroyctes. This paper characterizes the ouabain-induced cell proliferation in cultured rat astrocytes.

MATERIALS AND METHODS

Materials

Cycloheximide and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Thymidine and [^{3}H]uridine were obtained from Amersham Japan (Tokyo). Fetal calf serum (FCS) was from Hazleton Biologics (Lenexa, KS, USA). All other chemicals used were of the highest purity commercially available.

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Cell cultures

Relatively pure cultures of polygonal astroglial cells were prepared from cerebral cortices of 1-day-old Sprague-Dawley rats (Japan SLC, Inc., Shizuoka) as previously reported (16). In brief, cells were cultured in Eagle minimum essential medium (MEM) containing 10% FCS and 2 mM L-glutamine at 37℃. After the cells became confluent (10–20 days), the secondary cultures (seeded at 2×10^4 cells per well) were grown in complete medium until subconfluence (usually 14–20 days). The subconfluent astrocytes used here consisted of more than 95% flat polygonal astrocytes (type-1 astrocytes), as confirmed by phase contrast microscopy and positive immunostaining with anti-glial fibrillary acidic protein antibody (14, 16), and it did not contain neurons as assessed by negative immunostaining with Na^+,K^--ATPase α_3-isoform antibody.

Fibroblasts were cultured from the meninges of the same neonatal rats used for the culturing of astrocytes as reported previously (17). Rat C6 glioma cells were grown in MEM containing 10% FCS and 2 mM L-glutamine as reported previously (18).

Cell viability

The number of surviving cells was measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and by the previously reported hemocytometer method (14).

DNA and RNA syntheses

DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation as reported previously (14). In brief, the cells in 24-well plates were preincubated in FCS-free MEM for 24 hr and incubated with ouabain for 48 hr in FCS-free MEM. [^{3}H]Thymidine (18.5 kBq/well) was added to the cultures for the last 24 hr of incubation. Then, the cells were washed and treated with trichloroacetic acid. The trichloroacetic acid-insoluble fraction was dissolved with 1 N NaOH for the radioactivity and protein assays. RNA synthesis was determined by measuring [^{3}H]uridine incorporation: the method was the same as that for [^{3}H]thymidine incorporation except that [^{3}H]-thymidine was replaced with [^{3}H]uridine. In the time course experiments, [^{3}H]thymidine and [^{3}H]uridine were added for the last 3 hr of incubation. The incorporation (radioactivity per protein) was expressed as a percentage of the control. Controls in [^{3}H]thymidine and [^{3}H]uridine incorporation were about 15×10^4 and 20×10^4 dpm/mg protein, respectively.

RESULTS

Stimulation by ouabain of astrocyte proliferation

Figure 1 shows the effect of ouabain on [^{3}H]thymidine and [^{3}H]uridine incorporation into DNA and RNA in the absence of FCS. Ouabain stimulated [^{3}H]thymidine incorporation into DNA at concentrations of 10^{-4} M to 10^{-3} M, and the effect was reduced progressively at con-

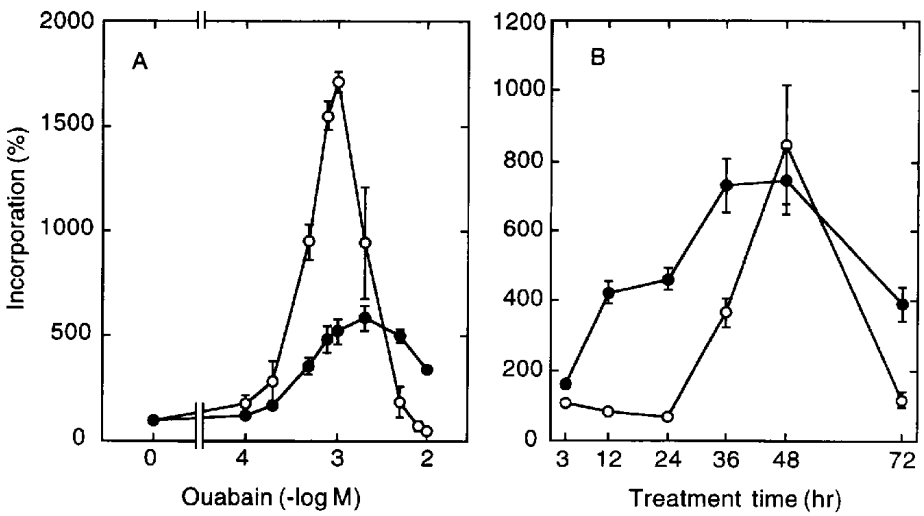

Fig. 1. Effect of ouabain on [^{3}H]thymidine and [^{3}H]uridine incorporation into cultured rat astrocytes. A: The cells were incubated with ouabain at the indicated concentrations for 48 hr in FCS-free MEM. [^{3}H]Thymidine (○) and [^{3}H]uridine (●) were added 24 hr after ouabain. Results shown as percentage of the value without ouabain are the means±S.E.M. of 4–12 wells. B: The cells were incubated with 1 mM ouabain for the indicated times in FCS-free MEM. [^{3}H]Thymidine (○) and [^{3}H]uridine (●) were added in the last 3 hr of incubation. Results shown as percentage of the value at 3 hr incubation without ouabain are means±S.E.M. of 10–12 wells.

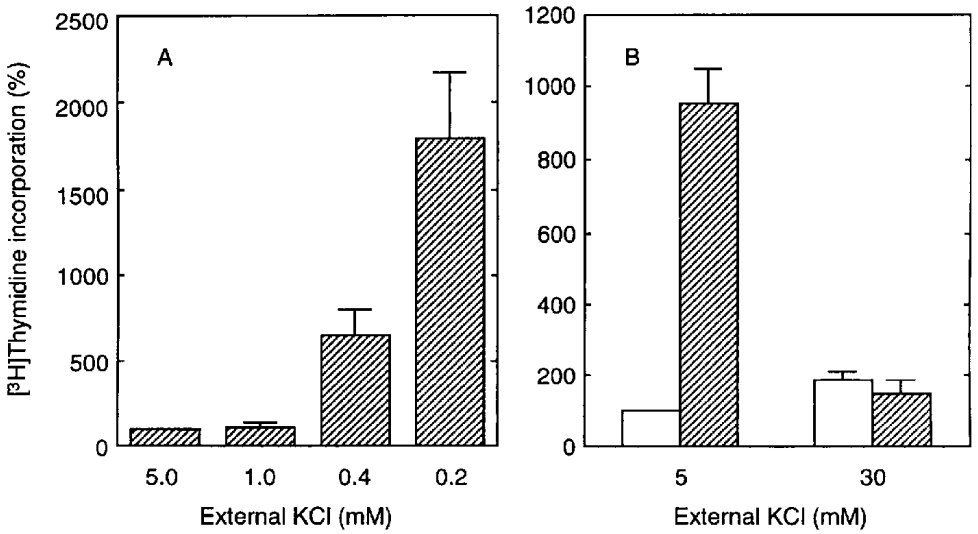

Fig. 2. Effect of extracellular K^- on [^{3}H]thymidine incorporation into cultured astrocytes. A: The cells were incubated in FCS-free MEM containing KCl at the indicated concentrations for 48 hr. Results shown as percentage of the value at 5 mM KCl are means ± S.E.M. of 8–9 wells. B: The cells were incubated in the absence (open column) or presence (hatched column) of 0.5 mM ouabain for 48 hr in FCS-free MEM containing the indicated concentrations of KCl. Results shown as percentage of the value at 5 mM KCl without ouabain are means ± S.E.M. of 8 wells.

centrations higher than 2×10^{-3} M: the apparent inhibitory effect of ouabain was observed at the concentration of 10^{-2} M. The effect of ouabain on RNA synthesis was slightly different from that of ouabain on DNA synthesis with regards to the dose-response curve (Fig. 1A) and time course (Fig. 1B). In addition, the effect of ouabain at 10^{-2} M was less in the case of [^{3}H]uridine incorporation than in that of [^{3}H]thymidine incorporation. The effect of ouabain on [^{3}H]thymidine incorporation into DNA was antagonized by a high concentration of external K^+ and mimicked by a low concentration of external K^+ (Fig. 2). MTT assay and direct cell counting using a hemocytometer showed that ouabain treatment caused an increase in cell number; The dose-response curve was similar to that of ouabain-induced stimulation of [^{3}H]thymidine incorporation into DNA (Fig. 3), but the degree of stimulation of [^{3}H]thymidine incorporation into DNA was much higher than that of the increase in cell number.

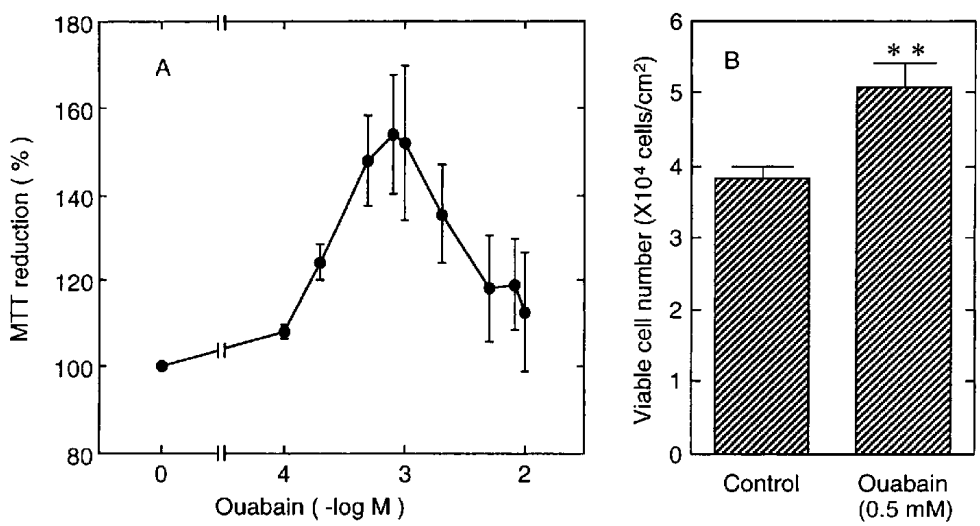

Fig. 3. Effect of ouabain on cell viability of cultured astrocytes. The cells were incubated with ouabain at the indicated concentrations for 48 hr, and MTT assay (A) and cell number (B) counting were carried out. Results are means ± S.E.M. of 5–8 wells. $^{**}P < 0.01$, compared with control (Student's *t*-test).

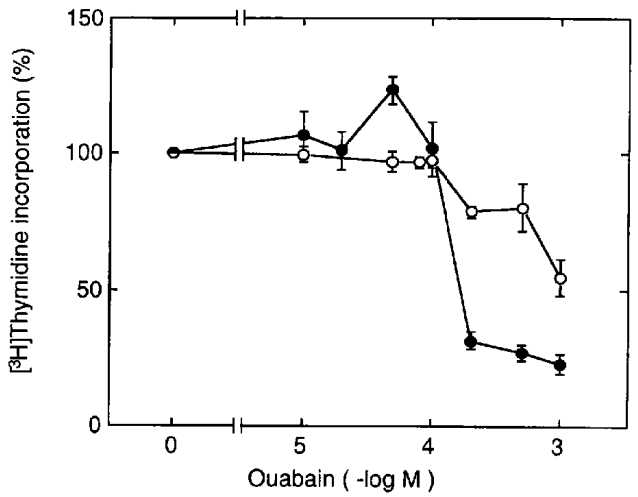

Fig. 4. Effect of ouabain on [^{3}H]thymidine incorporation into C6 glioma and fibroblast cells. Cultured C6 glioma (○) and rat fibroblast (●) cells were incubated with ouabain at the indicated concentrations in FCS-free MEM for 48 hr. Results shown as percentage of the value without ouabain are means±S.E.M. of 5–8 wells.

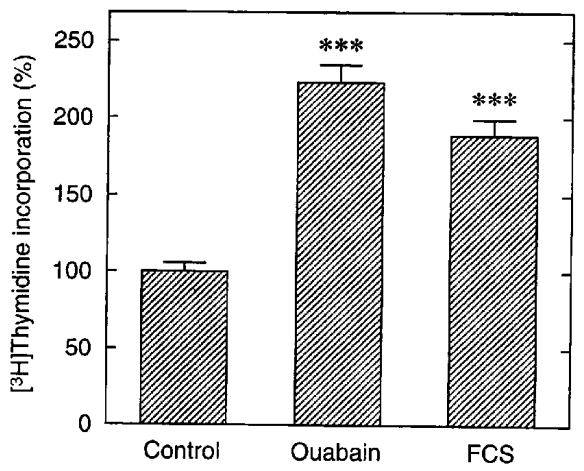

Fig. 6. Effect of ouabain on DNA synthesis in astrocytes preloaded with [^{3}H]thymidine. The cells were incubated with [^{3}H]thymidine for 1 hr in FCS-free MEM and washed. The preloaded cells were incubated with 1 mM ouabain or 10% serum for 48 hr. Results (shown as percentage of the value without ouabain and serum) are means± S.E.M. of 8 wells. ***$P<0.001$, compared with control (Student's *t*-test).

Cell specificity for ouabain-stimualted DNA synthesis

The effect of ouabain was cell-specific: ouabain inhibited [^{3}H]thymidine incorporation into DNA in C6 glioma and fibroblast cells (Fig. 4), although FCS stimulated the incorporation in these cells (data not shown).

Involvement of the transport process in ouabain-stimulated [^{3}H]thymidine incorporation

FCS alone stimulated [^{3}H]thymidine incorporation into DNA, but the degree was less than that of ouabain stimulation (Fig. 5). Ouabain stimulated [^{3}H]thymidine incorporation into DNA even in the presence of FCS (Fig. 5). The effects of ouabain and FCS on [^{3}H]thymidine

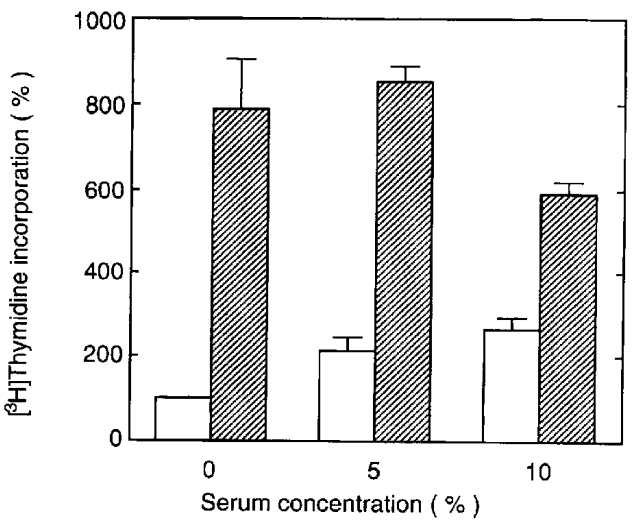

Fig. 5. Effects of ouabain and serum on [^{3}H]thymidine incorporation into astrocytes. The cells were incubated with (hatched) or without (open) 1 mM ouabain in MEM containing the indicated concentrations of FCS. Results shown as percentage of the value without ouabain and serum are means±S.E.M. of 8 wells.

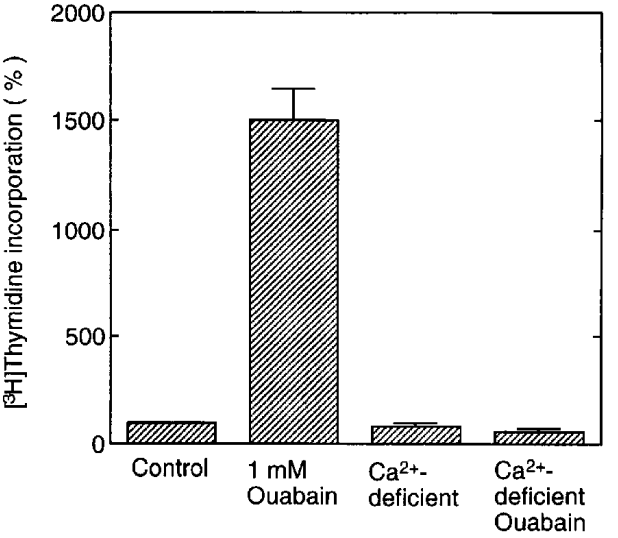

Fig. 7. Effect of extracellular Ca^{2+} on ouabain-stimulated [^{3}H]-thymidine incorporation into astrocytes. The cells were incubated with 1 mM ouabain in the presence and absence of extracellular Ca^{2+} in FCS-free MEM for 48 hr. Results are means±S.E.M. of 18 wells.

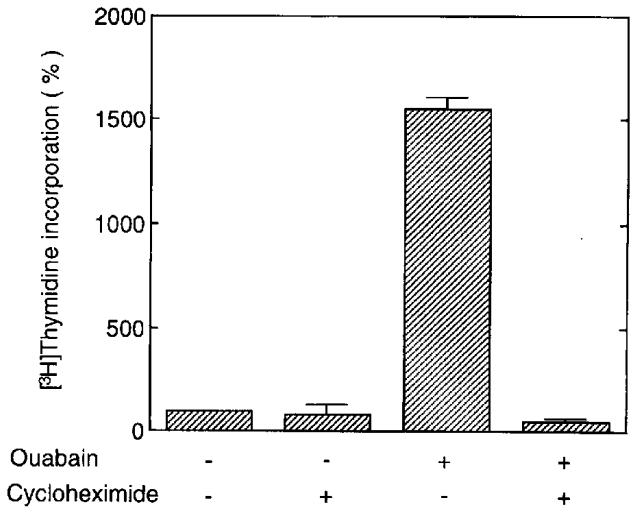

Fig. 8. Effect of cycloheximide on ouabain-stimulated [^{3}H]thymidine incorporation into astrocytes. The cells were incubated with 1 mM ouabain in the absence and presence of 1 μM cycloheximide in FCS-free MEM for 48 hr. Results are means±S.E.M. of 12–13 wells.

incorporation into DNA were examined under conditions that would eliminate the uptake process: the cells were preloaded with [^{3}H]thymidine, washed, and treated with ouabain or FCS (Fig. 6). Under the conditions, ouabain and FCS stimulated [^{3}H]thymidine incorporation into DNA to the similar degree.

Mechanism for the effect of ouabain

The effect of ouabain on [^{3}H]thymidine incorporation into DNA was not observed in Ca^{2+}-free medium (Fig. 7). The protein synthesis inhibitor cycloheximide, which alone did not affect cell viability (14), blocked ouabain-induced stimulation of [^{3}H]thymidine incorporation into DNA (Fig. 8).

DISCUSSION

The present study shows that ouabain stimulates [^{3}H]-thymidine incorporation into DNA with an increase in cell number in cultured astrocytes (Figs. 1 and 3). Although the effects were observed at the relatively high concentrations of ouabain, the stimulation of DNA synthesis was blocked by excess external K^+ and mimicked by low external K^+ (Fig. 2). We have shown that there are two isoforms (α_1 and α_2) of the Na^+,K^+-ATPase in cultured astroyctes, and the major isoform α_1 is insensitive to ouabain inhibition (14). These observations suggest that the effect of ouabain is due to its binding to the α_1-isoform of the Na^+,K^+-ATPase in the plasma membranes, and the reduced enzyme activity is responsible for the stimulation of DNA synthesis. The bell-shaped dose-response curve had two obvious components: DNA synthesis increased in response to lower concentrations of ouabain, while it gradually decreased in response to higher concentrations of the agent, probably due to a toxic effect (Fig. 1). Pettmann et al. (19) reported that gangliosides stimulated astrocyte DNA synthesis with a dose-response curve similar to that of ouabain. They found that the GM1-induced progressive reduction in cell-subsratum adhesion and the cells could be readily washed off the culture wells. However, it was unlikely that the inhibitory effect of ouabain on DNA synthesis might be due to removal of the cells, since ouabain at 10 mM did not decrease protein content in astrocytes (data not shown).

Ouabain also stimulated RNA synthesis in astrocytes, but the effect on RNA synthesis was a little different from that on DNA synthesis. The difference in dose-response curve (Fig. 1A) suggests that RNA synthesis is more resistant to ouabain toxicity than DNA synthesis, but the details are not known. It appears that the difference in time course between DNA and RNA syntheses (Fig. 1B) reflects the cell cycle: RNA synthesis starts at the G1 phase, while DNA synthesis occurs at the S phase.

The present study showed that the stimulation of [^{3}H]-thymidine incorporation into DNA was accompanied with an increase in cell number in astrocytes (Fig. 3). This suggests that treatment of astrocytes with ouabain causes cell proliferation. However, the degree of the stimulation was much higher than that of the increase in cell number (Figs. 1 and 3). The large disparity in the results between these two assays may be explained by the possible contribution of the [^{3}H]thymidine uptake process to [^{3}H]thymidine incorporation, in view of the previous observation that ouabain has direct membrane effects (20). In this experiment, astrocytes were incubated with [^{3}H]thymidine before exposure to ouabain to eliminate the effect on the [^{3}H]thymidine-uptake process. We found that ouabain caused only a twofold stimulation of [^{3}H]thymidine incorporation into DNA in the preloaded astrocytes (Fig. 6). The finding suggests that ouabain increases not only DNA synthesis but also thymidine uptake into the cells. We observed the stimulatory effect by ouabain on DNA synthesis in astrocytes and the inhibitory effect in C6 glioma and fibroblast cells (Fig. 4). The similar stimulation was reported in embryonic neural retina cells (21), but not in other cells (22, 23). A slight contamination with micorglia in the astrocyte preparation was considered, but it is not known whether ouabain affects microglial proliferation. These observations suggest that the effect of ouabain on DNA synthesis is specific for some types of cells. The cell-specificity may be explained by differences in the Na^+,K^+-ATPase heterogeneity among cells, but the details are not known.

The mechanism for ouabain-induced cell proliferation is not known. Rayson (24, 25) reported that Ca^{2+} plays an important role in ouabain-induced up-regulation of Na^+,K^+-ATPase in rat outer medullary tubule preparation. We found that ouabain-stimulated DNA synthesis in astrocytes was dependent on Ca^{2+} (Fig. 7) and blocked by the protein synthesis inhibitor cycloheximide (Fig. 8). In a separate experiment, we have found that ouabain increases the levels of the Na^+,K^+-ATPase α_1 and β_1 mRNAs and proteins in cultured astrocytes (unpublished). These findings imply that the enzyme may be a candidate for the newly synthesized protein responsible for ouabain-induced cell proliferation.

Ouabain injected into specific brain regions stimulates the release of neurotransmitters (26–28). This effect may be involved in ouabain-induced brain damage (11–13, 29) or ouabain-induced sympathetic response (7, 8), but the details are not known. The present finding that ouabain has biphasic effects on astrocyte proliferation depending on the concentration implies that astrocytes may play a role in the in vivo effects of ouabain. It should be noted that the biphasic effect of ouabain on DNA synthesis was similar to that of ganglioside in cultured astrocytes (19, 30), which had a protective effect against ischemic brain damage (31, 32). Further studies are required to clarify the possible role of astrocytes in the in vivo effects of ouabain.

Acknowledgments

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