NEURITE FORMATION IN NG108-15 NEUROBLASTOMA × GLIOMA HYBRID CELLS BY KS-505a, A POTENT INHIBITOR OF BRAIN CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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(Received for publication March 18, 1993)

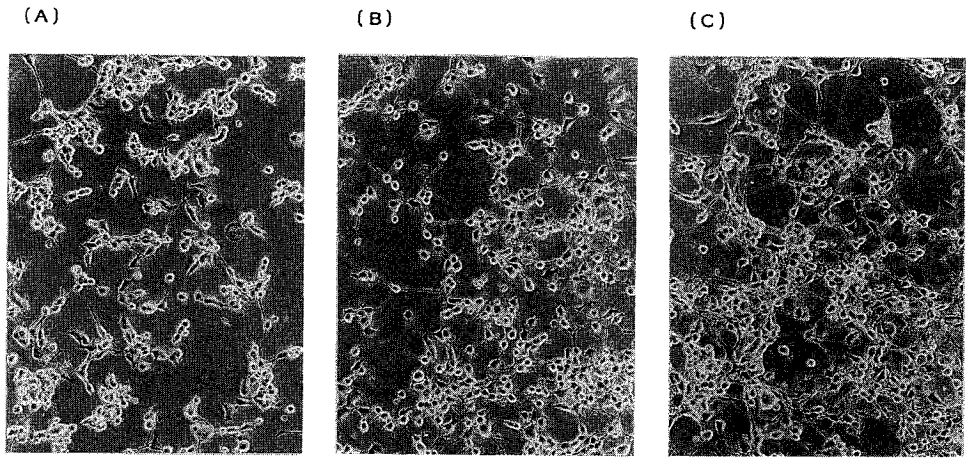
There are several papers showing a positive relationship between neurite formation and intracellular cyclic AMP level[1~5]. Griseolic acid[1], which is an inhibitor of cyclic nucleotide phosphodiesterase, and lactacystin[2] stimulate neurite formation and elevate an intracellular concentration of cyclic AMP in neuroblastoma cell line Neuro 2A. Cyclic AMP analogs can replace nerve growth factor (NGF) in promoting long-term survival and neurite outgrowth in cultures of rat neonatal sympathetic and embryonic sensory neurons[3]. Further, NG108-15 cells, a hybrid cell line of neuronal origin, differentiate morphologically, physiologically and biochemically, and exhibit an increase in many properties characteristic of mature neuronal cell lines when the cells are treated with *N*,2'-dibutyryladenosine 3',5'-cyclic monophosphate (dibutyryl cAMP)[4,5].

A compound KS-505a, whose structure has been found to be quite novel, was isolated from the culture broth of *Streptomyces argenteolus*[6]. The compound inhibits brain Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase with an IC_{50} value (the concentration causing 50% inhibition) of 0.065 μM, but not heart calmodulin-dependent and -independent cyclic nucleotide phosphodiesterase, and protein kinase C[6]. In the present study, we examined the effect of KS-505a on the morphology and the intracellular concentration of cyclic AMP in NG108-15 cells.

NG108-15 cells, which are generous gift of Professor H. HIGASHIDA, Cancer Research Institute of Kanazawa University, were cultured on 150 cm^2 flask in DULBECCO's modified EAGLE's media (DMEM) containing 10% horse serum, 100 μM hypoxanthine, 16 μM deoxythymidine, and 1 μM aminopterin at 37°C in a humidified atmosphere of 90% air and 10% CO_2 as described[7]. PC12 cells were grown on 150 cm^2 flask in DMEM supplemented with 7.5% fetal calf serum, 7.5% horse serum, 100 U/ml benzylpenicillin sodium, and 100 μg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 95% air and 5% CO_2 as described[8]. Neuro 2A cells were cultured as described[1]. After cells were cultured for 72 hours, photomicrographs of randomly chosen fields were taken. For the measurement of cyclic AMP production, the cells were seeded onto 24-well cluster plates. The confluent cell monolayer was washed twice with LOCKE's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose, and 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4) and was treated with each drug in LOCKE's solution at 37°C for the different period of time. After incubation, 2 ml of chilled 2.5% trichloroacetic acid solution was added to the cell monolayer after medium removal. The cell was frozen, thawed, and scraped. After agitating on a Vortex mixer, the suspension was immediately centrifuged at 800 × g for 10 minutes. The supernatants were then extracted three times with water-saturated diethylether and lyophilized. Each lyophilized sample was dissolved in water and cyclic AMP was measured using a radioimmunoassay kit (Yamasa, Japan).

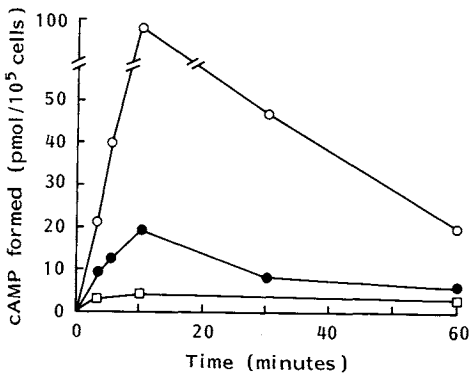
After subculture of NG108-15 cells for 1 day, the cells were continued to culture for 3 days in the absence or presence of KS-505a or dibutyryl cyclic AMP. The control NG108-15 cells cultured without drugs exhibited minimal neurite extension as shown in Fig. 1A. When NG108-15 cells were treated with KS-505a at concentrations ranging from 50 to 500 μM, the cells extended many neurites from the cell body in a dose-dependent manner; the neuritegenic effect was evident at 90 μM of KS-505a as shown in Fig. 1B. At concentrations higher than 1 mM, KS-505a showed some cytotoxicity such as cell retraction and release from the substratum. On the other hand, as shown in Fig. 1C, dibutyryl cAMP at 1 mM also induced differentiation of NG108-15 cells; this is consistent with the previous observation[4,5]. The other neuroblastoma cell line, Neuro 2A, were also affected by KS-505a as did

Fig. 1. Cell morphology of NG108-15 cells treated with KS-505a and dibutyryl cAMP.



After subculture of NG108-15 cells for 1 day, the cells were continued to culture for 3 days in the absence (control, A) or presence of KS-505a at 90 μM (B) or dibutyryl cyclic AMP at 1 mM (C).

Fig. 2. Effect of KS-505a and prostaglandin E_1 on the intracellular cAMP level in NG108-15 cells.



For the measurement of cyclic AMP production, the NG108-15 cells were seeded onto 24-well cluster plates. The confluent cell monolayer was washed twice with LOCKE's solution and was then treated with prostaglandin E_1 at 1 μM (○), KS-505a at 90 μM (●), or LOCKE's solution (□) at 37°C for the indicated time. After the incubation, intracellular cyclic AMP was measured using a radioimmunoassay kit (Yamasa, Japan) as described in the text. All the experiments were performed in triplicate.

NGF108-15 cells in a similar concentration range (data not shown). For further experiments, we selected PC12 rat pheochromocytoma because of the cells form neurite upon treatment with dibutyryl cAMP[9]. No morphological change, however, was caused by KS-505a at concentrations up to 500 μM in PC12 cells. Although it is uncertain whether this property of KS-505a is related to the specificity for brain Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase, KS-505a indeed shows a selectivity in neurite-promoting activity between the two cell lines. In any case, further studies are needed to clarify this point.

We next examined the effect of KS-505a on the intracellular cyclic AMP level of NG108-15 cells since a similar morphological changes were also observed with prostaglandin E_1 at .1 μM, which is known to elevate the cyclic AMP level[10]. When NG108-15 cells were treated with KS-505a at 90 μM, the intracellular cyclic AMP level increased and reached a maximum of 20 pmol/10^5 cells 10 minutes after treatment, and decreased gradually to the basal level in 60 minutes. As a positive control, prostaglandin E_1 at 1 μM also caused a transient increase in intracellular cyclic AMP in NG108-15 cells. The extent of the increase in intracellular cyclic AMP level by prostaglandin E_1 is, however, much greater than that by KS-505a. But KS-505a did not affect the intracellular cyclic AMP level in PC12 cells; this is consistent with the properties of inability of neurite promotion in PC12 cells.

The simplest interpretation of our present results would be a model in which KS-505a inhibits a brain-type phosphodiesterase and then increases in intracellular cyclic AMP level. Neurite formation would subsequently be induced by an increased cyclic AMP level. KS-505a is a novel chemical entity and may be a useful tool to understand the neuritegenesis of nerve cells. It may possess neuroprotective properties that is useful in the treatment of diseases involving the dysfunction of

nervous system due to the deficiency of neurotrofic factors.

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