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ALTERATION BY v-Ki-ras IN NaF, CHOLERA TOXIN AND FORSKOLIN-INDUCED ADENYLATE CYCLASE ACTIVATION IN NIH/3T3 FIBROBLAST CELLS

Lisa Konishi-Imamura[1)], Makoto Noda[2)], and Yasuyuki Nomura[1)]

1) Department of Pharmacology, Research Institute for WAKAN-YAKU (Oriental Medicine), Toyama Medical and Pharmaceutical University, 2630 sugitani, Toyama 930-01, Japan

2) Laboratory of Molecular Oncology, Institute of Physical and Chemical Research, 3-1-1 Takano-dai, Tanitabe, Tsukuba, Ibaragi 305, Japan

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It has been suggested that ras proteins are involved in the transmembrane signaling mechanism and they share structural features with GTP-binding proteins. To identify the role of ras oncogene and it's products in the coupling mechanism of GTP-binding proteins to adenylate cyclase, we examined effect of NaF, cholera toxin and forskolin in normal and v-Ki-ras transformed NIH/3T3 fibroblast cells. In transformants, adenylate cyclase activity was markedly enhanced by NaF and cholera toxin, in contrast to normal cells. It is suggested that ras oncogene proteins plays enhancing role in coupling of GTP-binding proteins to adenylate cyclase.

The ras gene family has been found in the genomes of a wide variety of eukaryotes (1). The yeast RAS product p21 of the ras family can positively regulate adenylate cyclase activity (2). It is also clarified that human ras p21 is structurally related to guanine nucleotide-binding protein (G-protein) (3). The function of ras oncogene and p21, however, has never been characterized. Like PC12 cells (4), Ki-MSV carrying v-Ki-ras induced marked morphological changes of the

Reprint request to Dr. Y. Nomura at the present address: Department of Pharmacology, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12-jyo Nishi 6-chyome, Kita-ku,,Sapporo 060, Japan.

Abbreviations: MSV, murine sarcoma virus; AC, adenylate cyclase; IBMX, isobutylmetylxanthine; CTX, cholera toxin; TBS, Tris-buffered saline; Gs, stimulaty GTP-binding protein.

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host cells, NIH/3T3 fibroblasts. This changes might be reflected in the alternation of the transmembrane signalling systems.

To clarify the regulatory role of ras and/or its product in the membrane AC system, we examined effects of several useful probes acting on adenylate cyclase(AC) activity in normal and v-Ki-ras transformed NIH/3T3 cells: such as forskolin, a direct activator of catalytic subunit (5) and NaF, a selective activator of AC by dissociating active -subunit in the stimulatory G-protein (Gs) (6). The present results indicate that v-Ki-ras transformed NIH/3T3 fibroblast cells (DT cells) could possess different sensitivity to NaF, cholera toxin and forskolin in AC system compared with that in normal NIH/3T3 cells.

Materials and methods

Materials

Normal and the v-Ki-ras transformed NIH/3T3 fibroblast cells (DT cells) were used. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) in addition to 0.015% glutamine and 4% fetal bovine serum at 37°C in a humidified atmosphere of 10% CO_2-air.

CTX (cholera toxin) treatment of the cells.

CTX (100 ng/ml) was added into the culture medium and incubated for 4 hours at 37°C in humidified atmosphere of 10% CO_2-air.

Adenylate cyclase (AC) activity

Cells were collected and washed twice with phosphate buffered saline (PBS; 20 mM Tris-HCl, 135 mM NaCl, 2.7 mM KCl, 8.0 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4 (pH 7.5)), then homogenized in 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 2.4 mM EGTA and 8 mM dithiothreitol. The total volume of the reaction mixture of 400 µl contained 50 mM Tris-HCl buffer (pH 7.7), 0.4 mM ATP (contained [^{3}H] ATP 0.5 uCi), 0.4 mM cAMP, 1 mM isobutylmethylxanthine (IBMX), 15 mM creatine phosphate, 25 U creatine phosphokinase, 10 µM GTP, 2 mM dithiothreitol, 0.6 mM EGTA, 1 mM $CaCl_2$, 5 mM $MgCl_2$, test drug or vehicle and membrane suspensions (approximately 1 mg protein). The mixture was incubated at 37°C for 10 min and terminated with adding 400 µl of 1 mM cAMP and boiling for 3 min. Subsequently, the reaction mixture was centrifuged for 3 min and 600 µl of the supernatant was applied to the column (Dowex 50w-X4, H^+ form, 200-400 mesh, 2.7 x 0.5 cm). The initial 0.5 ml effluent and the 1.0 ml effluent with distilled water were discarded and the next 1.5 ml effluent were applied to the 1.5 g Alumina column. The 1.5 ml effluent and the 1.0 ml effluent with 50 mM Tris-HCl buffer (pH 7.7) were discarded. The final 3.0 ml effluent buffer were collected and the radioactivity was measured by a liquid scintillation spectrometer. Protein was determined by the method of Lowry et. al. (7) with bovine serum albumin as a standard.

Results and discussion

To identity the relationship between Gs and AC, we examined effects of NaF on AC activity. The response in AC activity to different

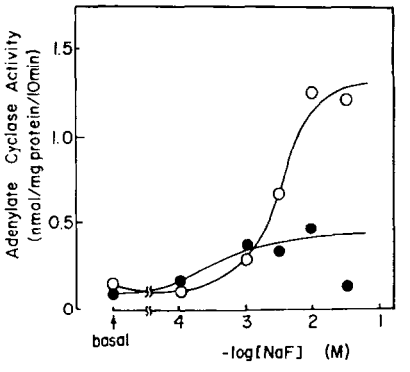

Fig. 1. Effect of NaF on adenylate cyclase in membranes prepared from normal NIH/3T3 cells(●) and transformed DT cells(○). NaF was added into the assay mixture and then adenylate cyclase activity was measured for 10 min at 37 °C.

concentrations was compared between NIH/3T3 and DT cells (Fig.1). In transformed cells, maximal response to NaF in AC was 2.7-fold higher than that in normal ones, suggesting that v-Ki-ras transformation induces changes in the NaF-sensitivity or the Gs content in membranes.

The preincubation of cells with 100 ng/ml CTX for four hours, significantly increased AC activities in both types of cells (Table 1). This result is consistent with a finding reported by others (11). Also, it is worthy to note that AC activity was markedly enhanced in DT cells, but not in normal cells. Therefore, the transformation of v-Ki-ras in NIH/3T3 fibroblasts seems to result in enhancement in Gs association with AC.

Table 1. Effect of cholera toxin on adenylate cyclase in normal NIH/3T3 cells and transformed DT cells

	Adenylate cyclase activity (pmol/mg protein 10 min.)		activaion by CTX
	Control	CTX treatment	
NIH/3T3 cells	263.5 ± 22.9	966.6 ± 94.5**	3.67
DT cells	163.1 ± 17.7	1699.0 ± 42.4***	10.42

Cells were incubated with cholera toxin for 4 hours at 37°C and then adenylate cyclase activity was measured. Each value shows the mean ± S.E. of 5 experiments. ** $p<0.01$, *** $p<0.001$ vs control.

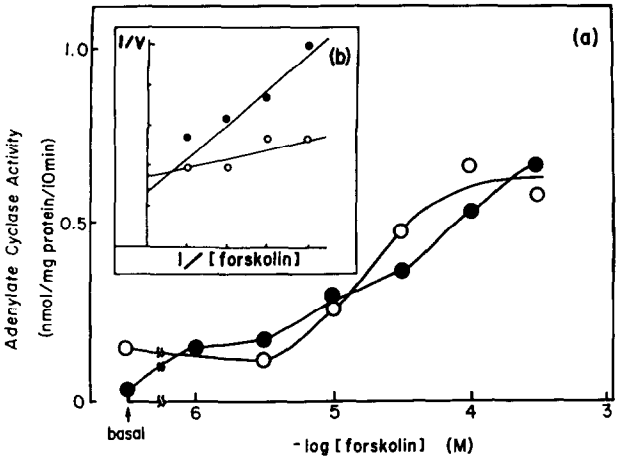

Fig. 2(a). Effect of forskolin on adenylate cyclase of membranes prepared from normal NIH/3T3 cells(●) and transformed DT cells(○). Forskolin was added into the assay mixture and then adenylate cyclase activity was measured for 10 min at 37°C. Values are means of triplicate determinations.
Fig. 2(b). Double-reciprocal plots for forskolin stimulation on adenylate cyclase of membranes prepared from normal NIH/3T3 cells (●) and transformed DT cells (○). AC activities were mesasured as described in legend of Fig. 2(a). Values are means of triplicate determinations.

Fig.2(a) shows the concentration-activation curves of forskolin on AC activity. Forskolin at the range of concentration from 12.5 - 50 μM more steeply elevated AC in transformants than in normal cells, although the maximal AC activation (0.65 nmol [^{3}H]cyclic AMP formed / mg protein / 10 min) was almost equal in both types of cells. A double-reciprocal plot of forskolin activation (Fig.2(b)) clearly shows higher affinity of forskolin to the AC system in DT cells than in normal ones. Regarding the mechanism of forskolin activation on AC, the following two possibilities have been proposed: 1) direct activation of AC (6) and/or 2) stimulating the interaction of Gs with AC (8-10). the present results suggests that p21 proteins encoded by v-Ki-ras genes and/or other physiological mechanisms changed by the infection could produced on enhancement of forskolin action in the binding to AC and/or the interaction of Gs and AC.

It has been demonstrated that the transformation by v-Ki-ras infection induced changes in the cAMP metabolism (12). In membranes of S49 cyc$^-$, a mutant which lacks Gs, however, the addition of E. coli-

derived ras oncogene product p21 did not exert the regulatory action on AC activity (13). Furthermore, ras encoded proteins did not directly affect AC in NIH/3T3 cells. In contrast, recent reports (16,17) suggest that ras oncogene product p21 is coupled to some receptors to activate phospholipase C causing phosphoinositide hydrolysis in NIH/3T3 fibroblasts. v-Ki-ras oncogene is presumably encoded into the protein, since p21 has been demonstrated to be formed by the v-Ki-ras transformation in DT cells presently used (18) as well as in thyroid epithelial cells (19). It is unknown, however, whether or not the content and/or function of G-proteins (Gs, Gi, etc.) can be modified by the transformation. It is of interest that viral ras p21 can act as the G-protein alpha-subunit and doesn't modulate the coupling of G-proteins to AC (19).

Further studies are required to clarify whether the changes in sensitivity of AC activation to NaF, cholera toxin and forskolin are mediated v-Ki-ras oncogene or its products.

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