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# Inhibition of Vanadate-Induced Astrocytic Stress Fiber Formation by C3 ADP-Ribosyltransferase

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Mechanisms of vanadate-induced actin reorganization were examined in cultured astrocytes. Treatment of protoplasmic astrocytes with 0.5 mM dibutyryl cAMP (DBcAMP) caused the disappearance of stress fibers (SFs) and focal adhesions (FAs) accompanied with cellular stellation. A subsequent addition of 1 mM orthovanadate ($VO_4^{3-}$) reorganized SFs and FAs in DBcAMP-treated cells. The newly formed FAs had increased phosphotyrosine levels. $VO_4^{3-}$ reorganized SFs and FAs in stellate astrocytes induced by 5 $\mu$M cytochalasin B, 50 $\mu$M ML-9 and 20 $\mu$M W-7. Cytoplasmic microinjection of 20 $\mu$g/ml C3 ADP-ribosyltransferase of *C. botulinum*, which inactivates rho proteins, caused disappearance of SFs. The effect of C3 enzyme on SFs was not reversed by a subsequent addition of $VO_4^{3-}$. These results suggest that rho proteins are involved in vanadate-induced reorganization of cytoskeletal actin. 

Cytoskeletal actin reorganization underlies several essential cellular responses induced by extracellular stimuli, such as mitosis, adhesion, cytosis and cell aggregation. In the studies on cytoskeletal actin organization in fibroblasts, it has been shown that increase in protein tyrosine phosphorylation is accompanied with formations of stress fibers (SFs) and focal adhesions (FAs) (1,2). Activation of tyrosine kinase-linked growth factor receptors, i.e. PDGF and EGF receptors, induced SFs (3,4). And SF formation was inhibited by tyrosine kinase inhibitors (1,5–7). From these observations, protein tyrosine phosphorylation is proposed to be involved in signal transduction processes leading to cytoskeletal actin organization. Vanadate shows growth factor-like actions in several cellular responses (8), and some of its responses are related to its action as a protein tyrosine phosphatase inhibitor (9). Vanadate stimulates SF formation in Swiss 3T3 and MDCK cells (7,10), but the site of its action is unclear. Recent studies showed an involvement of rho proteins, ras-like small GTP binding proteins, in SF and FA formations (3,11,12). C3 ADP-ribosyltransferase of *C. botulinum* (C3 enzyme) is a useful tool to investigate rho protein-mediated cellular responses, because it specifically inactivates rho protein (13). In order to investigate functional relations between rho proteins and possible action sites of vanadate, effect of C3 enzyme was examined in cultured astrocytes as a model system, where cytoskeletal actin organization is regulated by various extracellular stimuli (14–17).

## MATERIALS AND METHODS

*Cell culture.* Astrocytes were prepared from cerebrum of 1–2 day old Wistar rats as described previously (17). Cells were seeded on culture dishes and grown in Eagle's minimum essential medium (MEM)/10% fetal calf serum for 10–20 days. The monolayer cells were trypsinized and plated on glass coverslips coated with 10 $\mu$g/ml poly-L-lysine. Cells were cultured for 2–3 days on the coverslips. At this stage, about 95% of cells were glial fibrillary acidic protein positive.

*Fluorescence staining for F-actin, vinculin and phosphotyrosine.* After the culture medium was replaced by serum-free MEM containing 20 mM HEPES/NaOH pH 7.4 and 0.1% BSA, drugs were applied to the serum free-MEM. Cells were fixed by 3% paraformaldehyde and premeabilized by 0.1% Triton X-100. For F-actin staining, fixed cells were incubated with rhodamine-phalloidin (Molecular Probe Inc. Eugene) for 60 min. For double staining of vinculin and phosphotyrosine, cells were first incubated with mouse monoclonal anti-vinculin antibody (FB11, Biohit, Helsinki) and rabbit polyclonal anti-phosphotyrosine antibody (Transduction Lab. Kentucky). Subsequently, cells were incubated with FITC- and texas red-conjugated secondary antibodies.

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*Microinjection of C3 enzyme into astrocytes.* C3 ADP-ribosyltransferase (C3 enzyme, Wako Pure Chem., Osaka) was dissolved at 20 $\mu$g/ml in a solution containing 120 mM KCl, 10 mM Tris/HCl pH 7.4 and 0.5 mg/ml mouse IgG as a marker of injected cells. C3 enzyme was injected into astrocytes by a microglass capillary at a constant air pressure. Through the injection procedure, injection solution was continuously released from the capillary at a flow rate of 1.7 ± 0.6 pL/sec (determination by $^3H_2O$ as a tracer). After drug treatments, cells were fixed and double-stained by rhodamine-phalloidin and FITC-conjugated anti-mouse IgG. F-actin organization of injected cells, which are distinguished from non-injected ones by a bright FITC fluorescence, was observed. Cell morphology was observed under a phase-contrast microscopy and cells having two or more processes longer than twice length of the cell body were defined as "stellate cells".

*[$^{32}$P]ADP ribosylation of astrocytic lysate.* Astrocytic lysate was incubated at 37°C for 60 min with 1 $\mu$M [$^{32}$P]NAD (NEN), 0.3 mM GTP, 10 mM thymidine and 1 $\mu$g/ml C3 enzyme in 50 mM Tris/HCl pH 7.4. The incubation was terminated by 20% TCA and the acid precipitated proteins were applied to SDS-PAGE. The gel was subjected to radioautography.

## RESULTS

Protoplasmic astrocytes had SFs (Fig.1A) and dots of vinculin staining at the marginal regions of cytoplasms (Fig. 1B arrows), which indicates that FAs were formed. In serum-free MEM, treatment with dibutyryl cAMP (DBcAMP) induced morphological changes with cytoplasmic retraction and process formation, i.e., stellation in astrocytes. Accompanied with the astrocytic stellation, SFs and FAs disappeared (Fig. 1D,E). $VO_4^{3-}$ (1 mM) rapidly induced cytoplasmic expansion together with reorganization of SFs and FAs (Fig. 1G,H). Most of protoplasmic astrocytes showed a faint phosphotyrosine staining (Fig. 1C). $VO_4^{3-}$ (1 mM) increased phosphotyrosine staining on FAs of protoplasmic astrocytes (data not shown). DBcAMP-induced stellate astrocytes had no dot of the phosphotyrosine staining (Fig. 1F). Accompanied with the SF formation, $VO_4^{3-}$ increased phosphotyrosine staining on the newly formed FAs (Fig. 1H,I). PMA (100 nM), a protein kinase C activator, PDGF (30 ng/ml) and EGF (30 ng/ml), did not stimulate SF formation in DBcAMP-induced stellate astrocytes (data not shown). Cytochalasin B (5 $\mu$M), ML-9 (50 $\mu$M) and W-7 (20 $\mu$M) induced astrocytic stellation within 60 min. In the stellate astrocytes induced by these agents, $VO_4^{3-}$ stimulated formations of SFs and FAs, which had phosphotyrosine immunoreactivity (summarized in Table 1). Most of protoplasmic astrocytes (91%) had SFs and protoplasmic morphology after microinjection of a marker protein ("control injection" in Table 2). Injection of C3 enzyme (20 $\mu$g/ml) induced astrocytic stellation and disruption of SFs within 60 min. A subsequent addition of $VO_4^{3-}$ failed to reverse the astrocytic stellation and also to reorganize SFs in the C3 enzyme-injected cells (Fig. 2 and Table 2). Even in the presence of 1 mM $VO_4^{3-}$, astrocytic stellation was induced by C3 enzyme within 60 min. When astrocytic cell lysate was incubated with C3 enzyme, only a 21–25 Kda protein was ribosylated by [$^{32}$P]NAD (data not shown).

TABLE 1
Effects of $VO_4^{3-}$ on Stellate Astrocytes Induced by Various Drugs

| | Stress Fiber | Focal adhesion | Tyrosine phosphorylation |
|---|---|---|---|
| DBcAMP (1 mM) | + | + | + |
| Cytochalasin B (5 $\mu$M) | + | + | + |
| ML-9 (50 $\mu$M) | + | + | + |
| W-7 (20 $\mu$M) | + | + | + |
| C3 enzyme (20 $\mu$g/ml) | − | not determined | |

Prior to examine the effect of $VO_4^{3-}$, astrocytic stellation was induced by treatments with drugs listed in the table for 60 min. Subsequently, $VO_4^{3-}$ (1 mM) was added to the drug-containing MEM. Cells were incubated for 60 min and then, cell morphology, actin organization and phosphorylation on FAs were observed. Drugs listed in the table have following pharmacological profiles; Cytochalasin B:actin depolymerizing agent, ML-9:myosin light chain kinase inhibitor, W-7:calmodulin inhibitor, +:observed, −:not observed.

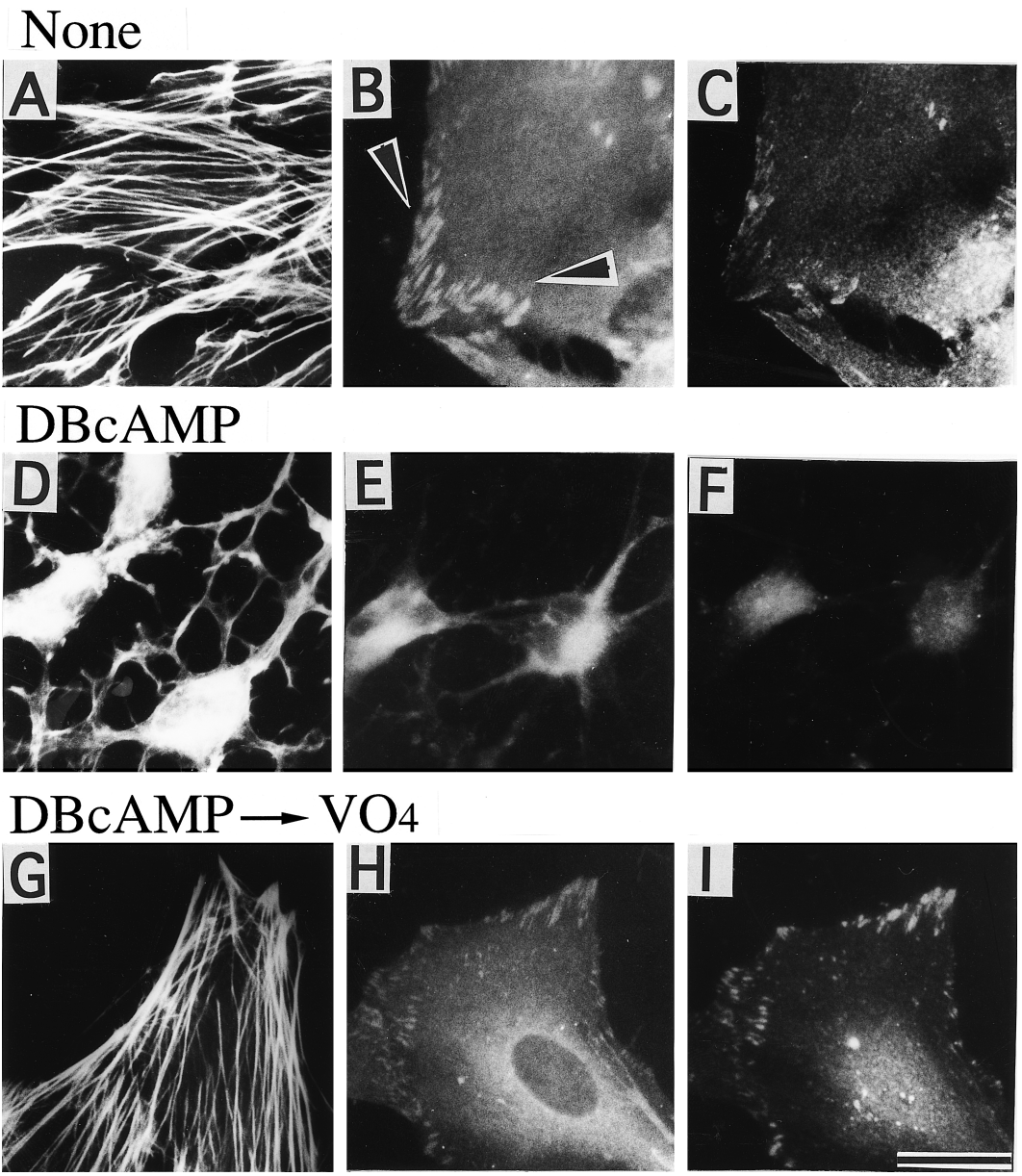


**FIG. 1.** Effects of DBcAMP and $VO_4^{3-}$ on stress fiber, focal adhesion formation and tyrosine phosphorylation of cultured astrocytes. To induce stellation, protoplasmic astrocytes were treated with 0.5 mM DBcAMP for 60 min. Then, 1 mM $VO_4^{3-}$ was added to the DBcAMP-containing MEM. After 60 min, astrocytes were fixed and cytoskeletal actin **(A,D,G),** vinculin **(B,E,H)** and phosphotyrosine **(C,F,I)** were stained as described in "MATERIALS and METHODS". Stainings for vinculin and phosphotyrosine were observed at the same field. In phosphotyrosine stainings, all photographs were exposed and developed in a same condition to make semiquantitative comparisions of relative fluorescence intensity. **(A,B,C)** protoplasmic astrocytes, **(D,E,F)** DBcAMP-induced stellate cells, **(G,H,I)** DBcAMP-stellate cells 60 min after $VO_4$ addition. Bar = 25 $\mu$m.

## DISCUSSION

Vanadate, a phosphotyrosine phosphatase inhibitor, is shown to induce SF formation in Swiss 3T3 and MDCK cells (7,10). In the present study, $VO_4^{3-}$ reorganized SFs and FAs in stellate

TABLE 2
Effect of $VO_4^{3-}$ on Cell Morphology and Stress Fiber Formation of Microinjected Astrocytes

| | Cell Numbers | | |
|---|---|---|---|
| | Total | Stress Fiber (+) | Stellate Shape |
| No addition | | | |
| Control Injection | 191 | 175 (91.6%) | 15 (8.3%) |
| C3 enzyme | 280 | 3 (1.1%) | 252 (90.0%) |
| $VO_4^{3-}$ addition | | | |
| Control Injection | 209 | 183 (87.6%) | 26 (12.4%) |
| C3 enzyme | 258 | 4 (1.6 %) | 228 (88.3%) |

After C3 enzyme was injected with an injection marker, astrocytes were incubated in serum-free MEM for 60 min. Then, some of injected cells were fixed (No addition). The remaining cells were further treated 1 mM $VO_4^{3-}$ for 60 min and fixed ($VO_4^{3-}$ addition). Cell morphology and actin organization of the injected cells were observed as described in "MATERIALS and METHODS". In "control injection", only a marker was injected. In each condition, 191–280 cells were microinjected (Total cells). And cell numbers of SF possessing- and of stellate shape ones are presented with their percentages to total injected cells in the parentheses.

astrocytes treated with DBcAMP and the other agents (Fig. 1 and Table 1). Recent studies showed that rho proteins are involved in SF and FA formations (3,11,12). Rho proteins are specifically inactivated by C3 enzyme of *C. botulinum* (13). As has been reported in fibroblasts (11,12), injection of C3 enzyme induced stellation of astrocytes. The C3 enzyme-induced stellation was neither prevented nor reversed by $VO_4^{3-}$ (Fig. 2). This result indicates that rho proteins are required for $VO_4^{3-}$ to reveal its action on actin organization. As well as SF formation, vanadate caused neurite retraction of PC12 cells (18) and aggregation of platelets (19,20), which are also rho protein-mediated responses (21,22). While functional relations between rho proteins and tyrosine phosphorylation have not been fully understood, the present results on the action of vanadate suggest two possible mechanisms. One is that activity of rho proteins is up-regulated by $VO_4^{3-}$. By analogy of the insulin-like action in hepatocytes (23), phosphorylation of receptor tyrosine kinase is considered to underlie the action of $VO_4^{3-}$. However, different from Swiss 3T3 cells, astrocytes did not form SFs in response to PDGF and EGF. It indicates that intracellular signals to these growth factor do not lead actin reorganization in astrocytes. We previously found that endothelins caused SF formation in cultured astrocytes (17). Including endothelins, protein tyrosine phosphorylation is induced by several ligands having trimeric GTP binding protein-linked receptors (24,25), where negative regulation of protein tyrosine phosphatase activity is suggested to be involved in part (26–28). $VO_4^{3-}$ may share the same action site with the endothelin-induced mechanisms in the up-stream of rho proteins. The other possible mechanism is that $VO_4^{3-}$ acts in down-stream of rho proteins, where $VO_4^{3-}$ does not directly activate their effectors but cooperates with the constitutive rho signals in the effector systems. Since $Ca^{2+}$ sensitization of smooth muscle contraction and neurite retraction of N1E 115 cells are mediated by rho proteins (21,29,30), phosphorylation of myosin light chain is suggested to be an effector system. Agreed with the previous study (15), ML-9, a myosin light chain kinase inhibitor, and W-7 caused astrocytic stellation and eliminated SFs. However, the finding that $VO_4^{3-}$ reversed the effects of ML-9 and W-7 indicates no involvement of myosin light chain kinase. Ridley and Hall (5) suggested that rho proteins reorganized SFs through tyrosine phosphorylation on FAs. PMA activates a novel tyrosine kinase which phosphorylates FA-associated proteins (31). However, PMA did not reorganized SFs in DBcAMP-induced stellate astrocytes. In addition, neither PMA nor $VO_4^{3-}$ prevented the effect of C3 enzyme. These observations suggest that tyrosine phosphorylation of the FA-associated proteins is insuf-

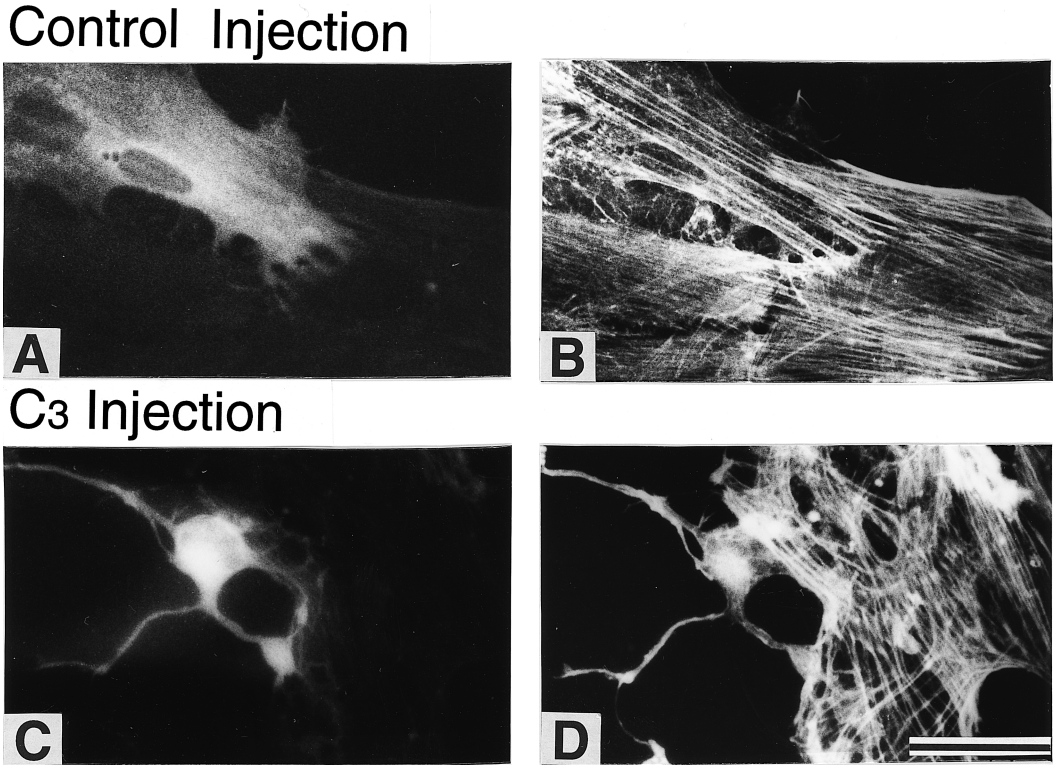


**FIG. 2.** Effect of $VO_4^{3-}$ on C3 enzyme-induced stellate astrocytes. **(A,B) Control Injection:** Only mouse IgG (0.5 mg/ml) was injected into cytoplasms of protoplasmic astrocytes as an injection marker. **(C,D)C3 Enzyme Injection:** C3 enzyme (20 $\mu$g/ml) was injected with mouse IgG into protoplasmic astrocytes. The control and C3 enzyme injected cells were incubated in serum-free MEM for 60 min, where stellation by C3 enzyme was induced. Then, cells were further treated with 1 mM $Na_3VO_4$ for 60 min and stained F-actin and the marker protein. A typical result was presented. **(A,C)** Staining for an injection marker. **(B,D)** F-actin staining at the same fields of **(A)** and **(C),** respectively. Bar = 50 $\mu$m.

ficient to induce astrocytic actin reorganization. Besides tyrosine phosphorylation on FAs, $VO_4^{3-}$ may affect another effector systems of rho proteins required for SF formation.

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