Hepatoma cell migration through a mesothelial cell monolayer is inhibited by cyclic AMP-elevating agents via a Rho-dependent pathway

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Abstract 1-Oleoyl lysophosphatidic acid (LPA) induces transmonolayer migration (in vitro invasion) of rat ascites hepatoma MM1 cells and their morphological changes leading to the migration. We have previously shown that an LPA analog, palmitoyl cyclic phosphatidic acid (Pal-cPA), suppresses transmonolayer migration of MM1 cells by rapidly increasing the intracellular cyclic AMP (cAMP) concentration. We report here that various cAMP-elevating agents, including dibutyryl cAMP, forskolin, cholera toxin and 3-isobutyl-1-methylxanthine, consistently inhibited LPA-induced transmonolayer migration of MM1 cells. Moreover, pull-down assays for GTP-bound, active RhoA demonstrated that the blockage by cAMP-elevating agents of morphological changes leading to the migration was probably mediated through inhibiting RhoA activation.

Key words: cAMP; Rho; Fibronectin; Motility; LPA

1. Introduction

Metastasis is a multistep process that includes detachment of cancer cells from a primary site, invasion into surrounding tissues, spread via the circulation, extravasion and proliferation in distant organs [1,2]. Thus, the ability to invade into surrounding tissues is a prominent phenotype of cancer cells.

Rat ascites hepatoma (AH130) cells implanted into the peritoneal cavity of a Donryu rat invade the peritoneum extensively. The mesothelial cell monolayer covering the peritoneum is the first barrier in this process. To evaluate peritoneal dissemination, we have established an in vitro model system in which a primary cultured mesothelial cell monolayer is penetrated by overlaid AH130 cells (transmonolayer migration) [3]. This in vitro system is considered to be a good model for the first step of cancerous peritonitis. In fact, the invasive capacity of AH130 cells determined with this in vitro system correlated well with their invasiveness in vivo in the peritoneal cavity [4,5]. Using this system, we have already clarified that rat ascites hepatoma (MM1, a highly invasive clone of AH130) cells require fetal calf serum (FCS) for the transmonolayer migration [6]. 1-Oleoyl lysophosphatidic acid (LPA) could completely substitute for the serum in inducing the transmonolayer migration [7]. The activity of LPA is not specific to MM1 cells, but is also observed with mouse melanoma B16, human pancreatic adenocarcinoma PSN-1 and human lung cancer OC-10 cells. A low molecular weight GTPase, RhoA, plays a pivotal role in this LPA-induced transmonolayer migration, as shown by the fact that *Clostridium botulinum* C3 exoenzyme [8], which specifically ADP-ribosylates and inhibits Rho, strongly suppressed the transmonolayer migration [9,10]. The introduction of dominant active RhoA cDNA into MM1 cells cancelled the FCS or LPA requirement of MM1 cells for transmonolayer migration [10]. We have reported that LPA induced polymerization of actin [11] and increased phosphorylation of myosin light chain through the Rho-ROCK (Rho-associated kinase) cascade [12]; both of these processes are essential for pseudopodia formation and generation of the motile force in transmonolayer migration.

LPA is a potent inducer of transmonolayer migration of a variety of tumor cells. Palmitoyl cyclic phosphatidic acid (Pal-cPA), a synthetic derivative of PHYLPA [13] (a novel lysophosphatidic acid composed of cyclic phosphate and cyclopropane-containing hexadecanoic acid, first isolated from myxoamebae of *Physarum polycephalum*) was tested for its activity against LPA, because Pal-cPA was reported to inhibit the cell growth of human fibroblasts which LPA stimulated. We have demonstrated that Pal-cPA suppresses LPA-induced transmonolayer migration [11]. Furthermore, we showed that Pal-cPA inhibited the LPA-induced actin polymerization that is essential for morphological changes leading to transmonolayer migration, possibly through elevating the intracellular cyclic AMP (cAMP) concentration in MM1 cells [11].

The second messenger cAMP also exerts dramatic effects on the cytoskeletal architecture. Elevation of the cAMP concentration in a variety of cell types induces loss of actin stress fibers and focal adhesions, rounding of cells, and in some cases detachment from the underlying substratum [14–16].

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Although the mechanism(s) eliciting the effects of cAMP appear to be complex, recent studies point toward a role for this cyclic nucleotide in the down-regulation of RhoA [17–21].

Taken together, the above findings suggest that cAMP-elevating agents may universally inhibit LPA-induced transmonolayer migration as well as morphological changes, possibly through inhibiting RhoA activity.

In the present study, we examined the effects of various cAMP-elevating agents on LPA-induced transmonolayer migration and morphological changes in MM1 cells. Moreover, RhoA activation was assessed as GTP-bound RhoA levels using the Rhotekin-binding assay [22].

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA, fraction V), 1-oleoyl lysophosphatidic acid (LPA), fibronectin (FN) from bovine plasma, dibutyryl cyclic AMP (dbcAMP), a membrane-permeable cAMP analog, forskolin (an adenylate cyclase activator), cholera toxin (a stimulator of heterotrimeric G protein Gs) were purchased from Sigma (St. Louis, MO, USA). LPA was dissolved in PBS supplemented with 0.1% BSA. A chemiluminescence detection kit (ECL) was purchased from Amersham Pharmacia Biotech, UK. C3 exoenzyme was kindly supplied by Dr. R. Komagome (Hokkaido University, Sapporo, Japan). Fetal calf serum (FCS) and 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase, was from WAKO (Tokyo, Japan).

2.2. Cells and cell culture

The MM1 cell line, which is a highly invasive clone isolated from parental rat ascites hepatoma AH130 cells, was cultured in suspension in MEM containing 2-fold concentrated amino acids and vitamins (modified MEM) (Nissui, Tokyo, Japan) supplemented with 10% FCS. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. Transmonolayer migration (in vitro invasion) assay

The assay procedure to measure the in vitro invasive capacity of tumor cells was essentially the same as described in our previous report [3]. MM1 cells (2×10^5 cells) were seeded over a rat mesothelial cell monolayer (MCL) and cultured in medium containing the materials to be tested. Invasion experiments were started by the addition of 25 μM LPA. Twenty hours later, the supernatant was removed and the resultant monolayer was fixed in situ with 10% formalin. The number of penetrated single tumor cells and tumor cell colonies (collectively called invasion foci) was counted under a phase-contrast microscope. The invasive capacity was expressed as the number of invasion foci per cm^2.

2.4. Morphological observation of MM1 cells

Morphological observation of MM1 cells was performed on an MCL as described above and on an FN-coated dish. A dish of 35 mm diameter was coated with 10 μg of FN at room temperature for 3 h. An FN-coated dish was blocked with modified MEM supplemented with 1.5% BSA, and then MM1 cells were observed on an FN-coated dish in the presence of 25 μM LPA in modified MEM with or without 1.25 mM dbcAMP at 37°C under a phase-contrast microscope (Olympus IX70, Tokyo, Japan). To observe the effects of RhoA inactivation, MM1 cells were pretreated with 10 μg/ml of C3 exoenzyme for 24 h.

2.5. Pull-down assay for GTP-bound RhoA

MM1 cells were washed twice with modified MEM and incubated in fresh modified MEM without serum for 3 h; 25 μM LPA was added and the cell suspension was centrifuged at the indicated time after LPA addition, and then cell pellet was lysed in 50 mM Tris–HCl buffer, pH 7.5, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were clarified by centrifugation at $13\,000\times g$ at 4°C for 10 min, and were incubated for 45 min at 4°C with glutathione beads (Amersham Pharmacia Biotech, UK) coupled with bacterially expressed GST–RBD (Rho-binding domain of Rhotekin, which associates only with GTP-bound, active RhoA) fusion protein (provided by Dr. M. Schwartz, Scripps Research Institute, USA). The beads were washed four times with 50 mM Tris–HCl buffer, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF. Activated RhoA bound to beads or total RhoA in cell extracts was detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology, CA, USA).

3. Results

3.1. Inhibition of transmonolayer migration by various cAMP-elevating agents

We have already reported that LPA induces the transmonolayer migration of MM1 cells through a mesothelial cell monolayer [7]. An LPA analogue, Pal-cPA, effectively inhibits the transmonolayer migration, with approximately 93.8% inhibition at the concentration of 25 μM [11]. Because Pal-cPA induced a rapid increase of the cAMP concentration in MM1 cells, this inhibition might be ascribed to an increased level of intracellular cAMP [11]. In the present study, we investigated the effects of various cAMP-elevating agents on the transmonolayer migration of MM1 cells. As shown in Table 1, all of the cAMP-elevating agents used in this study, i.e. dbcAMP, forskolin, cholera toxin and IBMX, strongly suppressed LPA-induced transmonolayer migration of MM1 cells. These results suggest that transmonolayer migration of MM1 cells is suppressed by increasing the intracellular cAMP concentration.

3.2. The effects of dbcAMP on the LPA-induced morphological changes leading to the transmonolayer migration of MM1 cells

We have reported that LPA induces transient polymerization of actin through RhoA activation and that this polymerization of actin is suppressed by Pal-cPA or dbcAMP [11]. We have also shown that cooperation of LPA with the interaction between FN and β1 integrin mediated the formation of focal adhesions necessary for the transmonolayer migration of MM1 cells [23].

When MM1 cells on an FN-coated dish were stimulated by LPA, they showed a unidirectionally extended fusiform shape with the nucleus localized in one end (equivalent to pseudopodia formation) and the fusiform-shaped cells migrated with the nuclear end as the moving front, as seen under a phase-contrast microscope (Fig. 1a). LPA induced the same morphological changes in MM1 cells that were seeded on an MCL (Fig. 1b). These observations suggest that the morphological changes to the fusiform shape are essential for transmonolayer migration of MM1 cells.

Table 1
cAMP-elevating agents inhibit the transmonolayer migration of MM1 cells

cAMP-elevating agents	Inhibition of transmonolayer migration in vitro (%)
dbcAMP (1.25 mM)	93.0
Cholera toxin (1 μg/ml)	86.1
Forskolin (10 μM)	97.1
IBMX (1 mM)	96.0

dbcAMP, dibutyryl cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine.

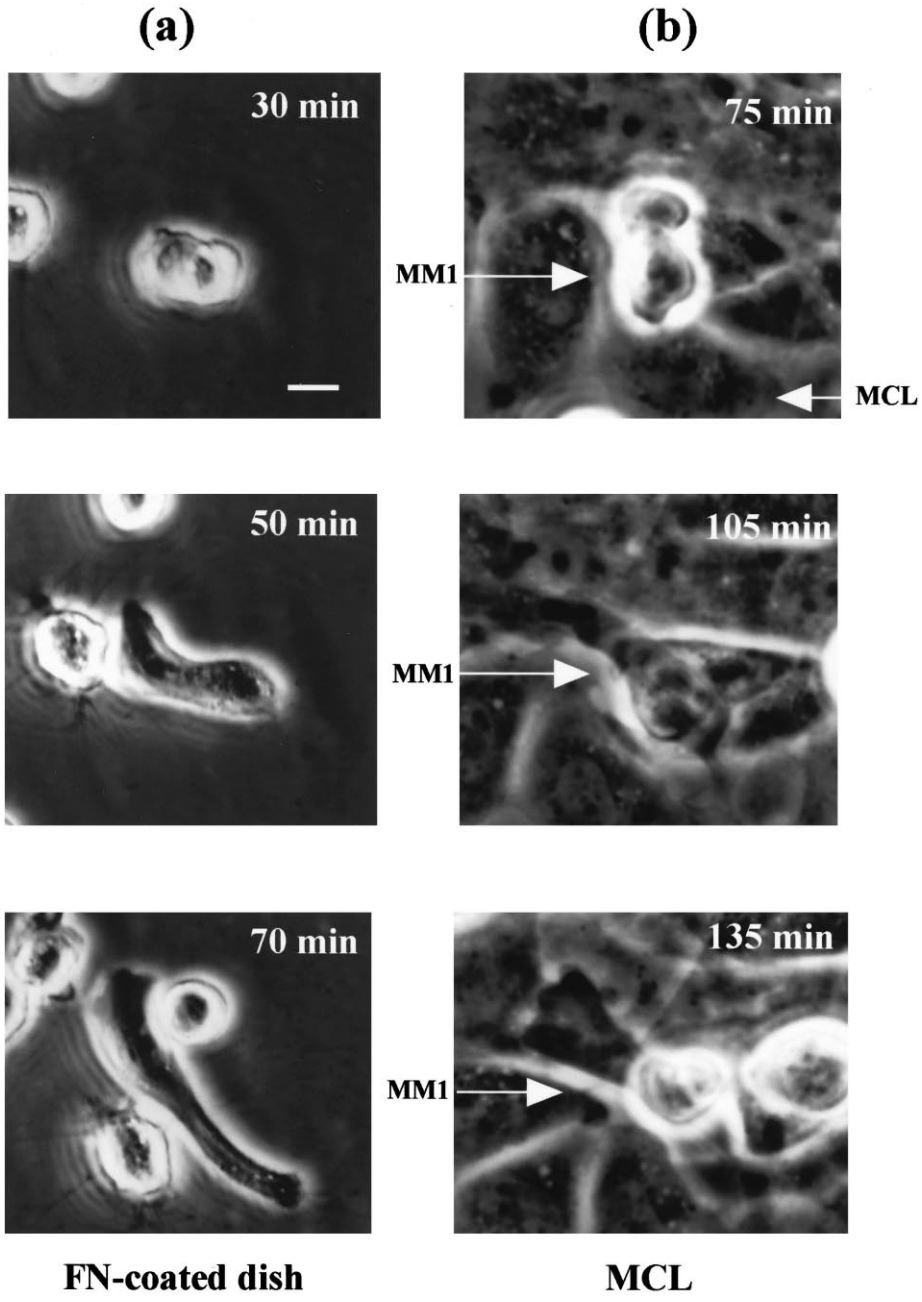

Fig. 1. Morphological changes of MM1 cells stimulated with LPA. MM1 cells (3×10^5) seeded on an FN-coated dish (a) or on a mesothelial cell monolayer (MCL) (b) were cultured with 25 μM LPA. Phase-contrast micrographs are shown at the indicated time points after LPA addition. Scale bar: 10 μm.

Since dbcAMP inhibited actin polymerization [11], the effects of dbcAMP on the LPA-induced morphological changes of MM1 cells were investigated. Fig. 2a shows the fusiform-shaped MM1 cells induced by LPA on an FN-coated dish. The morphological changes to the fusiform shape were remarkably suppressed by addition of dbcAMP (Fig. 2b). When MM1 cells that had been pretreated with C3 exoenzyme to inactivate RhoA GTPase were seeded on an FN-coated dish and stimulated by LPA, neither fusiform-shaped cells (Fig. 2c), nor the transmonolayer migration were observed, as described previously [9,10]. Thus, MM1 cells treated with dbcAMP and those treated with C3 exoenzyme showed a similar cell shape.

3.3. Inhibition of RhoA activation by cAMP-elevating agents

Since dbcAMP inhibited actin polymerization [11] and morphological changes to the fusiform shape (Fig. 2b), it was suggested that dbcAMP might inactivate RhoA. Therefore, we measured the intracellular levels of the GTP-bound, active form of RhoA by the pull-down assay system [22]. The Rho-binding domain of Rhotekin [24], which is known to associate only with the GTP-bound form of RhoA, was fused to glutathione S-transferase, the resultant fusion protein was incubated with cell extracts, and the bound RhoA was detected by Western blotting. As shown in Fig. 3a, the level of GTP-bound RhoA was elevated transiently after the addition of LPA and reached the peak level at 15 min after LPA addition,

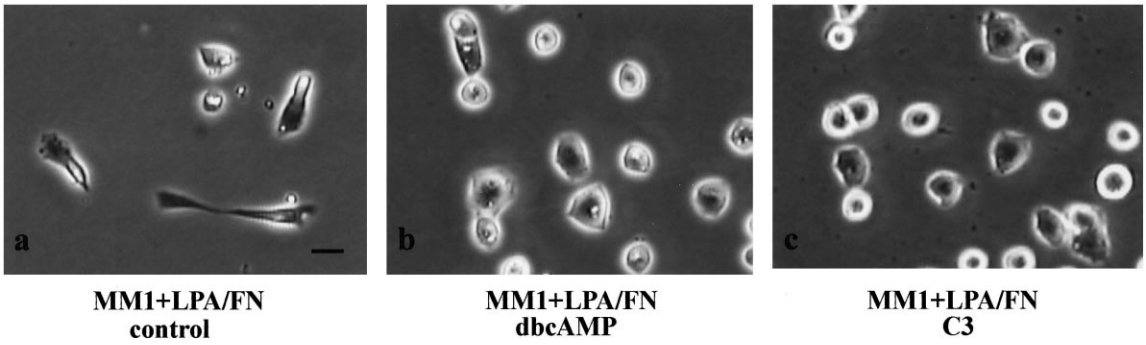

Fig. 2. Suppression of LPA-induced morphological changes by treatment with dbcAMP or C3. MM1 cells on an FN-coated dish were stimulated with 25 μM LPA (a). MM1 cells on an FN-coated dish were treated with 1.25 mM dbcAMP in the presence of 25 μM LPA (b). MM1 cells were pretreated with 10 μg/ml of C3 exoenzyme for 24 h, then washed with modified MEM, and incubated on an FN-coated dish in the presence of 25 μM LPA (c). Phase-contrast images of the cells are shown. Scale bar: 10 μm.

whereas dbcAMP and other cAMP-elevating agents decreased the level of active RhoA (Fig. 3b). Since the total amount of RhoA in each lysate was almost constant, cAMP-elevating agents inhibited RhoA activation.

4. Discussion

It is important for understanding the molecular mechanisms of cancer invasion and metastasis to analyze the signal transduction pathways leading to cell migration. Using our in vitro invasion model, we have already reported that a low molecular weight GTPase, RhoA, plays an important role in LPA-induced transmonolayer migration [9,10].

LPA induces the transmonolayer migration of MM1 cells, while Pal-cPA, an analogue of LPA, suppresses it [11]. Pal-cPA also suppresses the transmonolayer migration of mouse melanoma cells (B16), human pancreatic adenocarcinoma cells (PSN-1), and human lung cancer cells (OC-10), which require LPA for transmonolayer migration. Transmonolayer migration of human fibrosarcoma cells (HT-1080), which do not require LPA for transmonolayer migration, was also suppressed by Pal-cPA. We have previously suggested that this suppression may be due to the elevation of intracellular cAMP concentration rather than to the competition for LPA receptor [11].

The mechanisms through which cAMP regulates various cellular functions still remain controversial. Tanaka et al. demonstrated that cAMP suppresses in vitro invasion of HT-1080 cells using Boyden chamber assays [25]. They suggested that this suppression of in vitro invasion is due to the inhibition of type IV collagenolytic activity resulting from increased levels of the mRNAs and proteins of TIMPs.

Laundanna et al. have shown that cAMP abrogates GDP/GTP exchange of RhoA, resulting in the inhibition of RhoA function [18]. More recently, Busca et al. have demonstrated that the cAMP-induced differentiation of melanoma cells is mediated by the inhibition of RhoA through the suppression of Rac [19]. These investigators suggested that cAMP regulates RhoA function by a mechanism that acts upstream of RhoA.

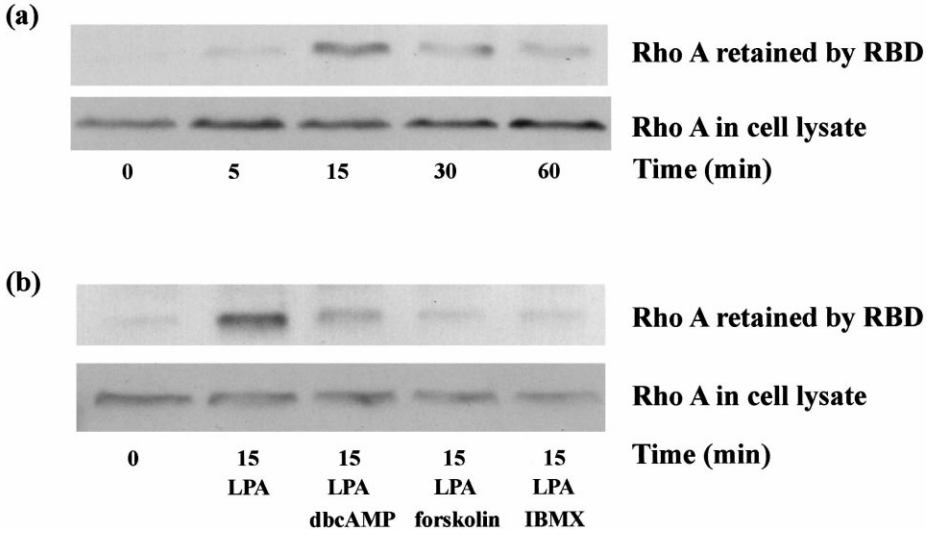

Fig. 3. Activation of cellular RhoA in response to stimulation with LPA. MM1 cells were incubated in suspension in the absence of serum for 3 h, and subsequently cultured in the presence of 25 μM LPA alone (a) or 25 μM LPA plus various cAMP-elevating agents (1.25 mM dbcAMP, 10 μM forskolin or 1 mM IBMX) (b). Cell lysates were prepared from MM1 cells treated with LPA for the indicated periods. The lysates were then incubated with GST–RBD beads. After extensive washing, the bound protein and total RhoA in the cell lysate were analyzed by Western blotting with a monoclonal anti-RhoA antibody.

Recently, Schwarz et al. have developed a new method for determination of the level of activated RhoA [22], the utilizing Rho-binding domain of Rhotekin [24]. Using this method, O'Connor et al. found a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration [26]. We also demonstrated, employing this method, that cAMP-elevating agents suppressed RhoA activation in transmonolayer migration of MM1 cells (Fig. 3b). Pal-cPA also suppressed RhoA activation (data not shown).

Lang et al. reported that RhoA is phosphorylated on Ser-188 by PKA, which acts downstream of cAMP, and that this phosphorylation of RhoA increases its interaction with guanine nucleotide dissociation inhibitor (GDI) even in its GTP-bound state. They suggested that the translocation of phosphorylated RhoA GTP from membrane to cytosol by its binding to GDI terminates RhoA signaling by preventing its interaction with downstream effectors, independently of its cycling from GTP to GDP [20]. It remains to be clarified whether RhoA is phosphorylated by PKA in MM1 cells treated with cAMP-elevating agents. Further studies of this subject will be useful for understanding the molecular mechanisms of cancer invasion and metastasis. In conclusion, we showed here that cAMP-elevating agents blocked morphological changes leading to transmonolayer migration, probably through inhibiting RhoA activation.

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References

[1] Fidler, I.J., Gersten, D.M. and Hart, I.R. (1978) Adv. Cancer Res. 28, 149–250.
[2] Liotta, L.A., Rao, C.N. and Barsky, S.H. (1983) Lab. Invest. 49, 636–649.
[3] Akedo, H., Shinkai, K., Mukai, M., Mori, Y., Tateishi, R., Tanaka, K., Yamamoto, R. and Morishita, T. (1986) Cancer Res. 46, 2416–2422.
[4] Mukai, M., Shinkai, K., Tateishi, R., Mori, Y. and Akedo, H. (1987) Cancer Res. 47, 2167–2171.
[5] Akedo, H., Shinkai, K., Mukai, M. and Komatsu, K. (1989) Invasion Metastasis 9, 134–148.
[6] Imamura, F., Horai, T., Mukai, M., Shinkai, K. and Akedo, H. (1991) Jpn. J. Cancer Res. 82, 493–496.
[7] Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M. and Akedo, H. (1993) Biochem. Biophys. Res. Commun. 193, 497–503.
[8] Sekine, A., Fujiwara, M. and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605.
[9] Imamura, F., Shinkai, K., Mukai, M., Yoshioka, K., Komagome, R., Iwasaki, T. and Akedo, H. (1996) Int. J. Cancer 65, 627–632.
[10] Yoshioka, K., Matsumura, F., Akedo, H. and Itoh, K. (1998) J. Biol. Chem. 273, 5146–5154.
[11] Mukai, M., Imamura, F., Ayaki, M., Shinkai, K., Iwasaki, T., Murakami-Murofushi, K., Murofushi, H., Kobayashi, S., Yamamoto, T., Nakamura, H. and Akedo, H. (1999) Int. J. Cancer 81, 918–922.
[12] Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T. and Narumiya, S. (1999) Nat. Med. 5, 221–225.
[13] Murakami-Murofushi, K., Shioda, M., Kaji, K., Yoshida, S. and Murofushi, H. (1992) J. Biol. Chem. 267, 21512–21517.
[14] Lamb, N.J.C., Fernandez, A., Conti, M.A., Adelstein, R., Glass, B.B., Welch, W.J. and Feramisco, J.R. (1988) J. Cell Biol. 106, 1955–1971.
[15] Lampugnani, M.G., Giorgi, M., Gaboli, M., Dejana, E. and Marchisio, P.C. (1990) Lab. Invest. 63, 521–531.
[16] Glass, W.F. and Kreisberg, J.I. (1993) J. Cell. Physiol. 157, 296–306.
[17] Kreisberg, J.I., Ghosh-Choudhury, N., Radnik, R.A. and Schwartz, M.A. (1997) Am. J. Physiol. 273, F283–F288.
[18] Laudanna, C., Campbell, J.J. and Butcher, E.C. (1997) J. Biol. Chem. 272, 24141–24144.
[19] Busca, R., Bertolotto, C., Abbe, P., Englaro, W., Ishizaki, T., Narumiya, S., Boquet, P., Ortonne, J.-P. and Ballotti, R. (1998) Mol. Biol. Cell 9, 1367–1378.
[20] Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M. and Bertoglio, J. (1996) EMBO J. 15, 510–519.
[21] Dong, J.-M., Leung, T., Manser, E. and Lim, L. (1998) J. Biol. Chem. 273, 22554–22562.
[22] Ren, X.D., Kiosses, W.B. and Schwartz, M.A. (1999) EMBO J. 18, 578–585.
[23] Ayaki, M., Mukai, M., Imamura, F., Iwasaki, T., Mammoto, T., Shinkai, K., Nakamura, H. and Akedo, H. (2000) Biochim. Biophys. Acta 1495, 40–50.
[24] Reid, T., Furuyashiki, T., Ishizaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P. and Narumiya, S. (1996) J. Biol. Chem. 271, 13556–13560.
[25] Tanaka, K., Iwamoto, Y., Ito, Y., Ishibashi, T., Nakabeppu, Y., Sekiguchi, M. and Sugioka, Y. (1995) Cancer Res. 55, 2927–2935.
[26] O'Connor, K.L., Nguyen, B.-K. and Mercurio, A.M. (2000) J. Cell Biol. 148, 253–258.