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Chapter

MODULATION OF CYCLIC AMP PRODUCTION IN FIBROBLASTS ATTACHED TO SUBSTRATA WITH DIFFERENT SURFACE CHEMISTRIES

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ABSTRACT

The interactions of cells with biomaterials have been widely studied. However, little is known about the influence of the properties and chemistry of the substratum on the activation of one of the major signaling cascades, the cyclic adenosine 3',5'-monophosphate (cAMP) pathway, in adhering cells. The second messenger cAMP plays a major role in modulating cell morphology, enabling cells to survive, proliferate and differentiate. Our previous study showed that murine Swiss 3T3 fibroblasts loosely attached to a hydrophilic cellulose membrane have a high amount of intracellular cAMP. By contrast, cells well spread on tissue culture polystyrene (PS) contained low concentrations of cAMP. But the surface properties of the cellulose membrane are heterogeneous in terms of rugosity, porosity and chemical composition.

Hence, to study the impact of surface chemistry on Swiss 3T3 fibroblast behaviors, we have prepared self-assembled monolayers (SAMs) on glass from alkylsilanes to obtain model surfaces with a variety of terminating functional groups, such as carboxylic acid (COOH), amine (NH_2), poly(ethylene glycol) (PEG) and methyl (CH_3). Our results revealed that the cAMP production was significantly lower in cells attached to COOH- and NH_2-terminated SAMs than in cells on PEG and CH_3 substrata. Spread cells attached to COOH- and NH_2-terminated SAMs could organize their cytoskeleton, phosphorylate

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the Tyr^{397} of focal adhesion kinase (FAK) and activate RhoA. By contrast, cells on PEG and CH_3 substrata remained rounded up with few punctuate focal adhesion complexes, while FAK phosphorylation on Tyr^{397} and RhoA activation were partly inhibited. Indeed, an increase in intracellular cAMP severely impaired the formation of focal adhesion complexes and decreased the phosphorylation of Tyr^{397} in FAK. We also found that the phosphorylation of ERK1/2 was also significantly greater in cells attached to COOH and NH_2 substrata than in cells adhering to PEG-terminated SAM. We conclude that monitoring cAMP may contribute to a better understanding of the complex phenomenon of cell-material interactions.

Keywords: *signal transduction, alkylsilane, focal adhesion, kinases*

INTRODUCTION

Several studies have demonstrated that cell-biomaterial interactions depend on surface properties such as roughness topography, wettability, charge, chemistry and surface energy [1,2]. These characteristics influence the conformation, orientation and quantities of adsorbed adhesion proteins such as vitronectin or fibronectin [3,4]. The adsorbed proteins onto the substratum profoundly affect integrin-receptor binding and subsequent cell adhesive events especially the focal adhesion formation [4,5]. Focal adhesion contacts are flat and elongated structures often located at cell periphery [6,7]. They anchor bundles of actin stress fibers through a plaque made up of many different proteins such as cell membrane integrin receptor, vinculin and phosphotyrosine proteins [8].

Nevertheless, few studies have examined the influence of the substratum surface properties on the activation of early biochemical events, such as the cyclic adenosine 3',5'-monophosphate (cAMP) pathway [9,10]. The second messenger cAMP is involved in a wide range of cell functions, including cell proliferation and motility [11], but the cAMP pathway is especially important in cell adhesion, cytoskeletal structure and focal contact formation [12,13]. An increase in intracellular cAMP induced by β adrenergic agonists or by forskolin, a direct activator of adenylyl cyclase, the enzyme producing cAMP from ATP causes marked morphological changes with a loss of focal adhesion and the fragmentation of actin stress fibers in adherent cells [14,15]. An increase in intracellular cAMP can also severely impair the formation of focal adhesion complexes and decrease the phosphorylation of the tyrosine in focal adhesion kinase (FAK) in several cell types inhibiting the spreading of the cells [11,12,16,17]. FAK plays a key role in mediating integrin signal transduction and becomes autophosphorylated on Tyr^{397} after integrin stimulation [16,17]. Phosphorylated Tyr^{397} represents a binding site for the Src-homology 2-domain of Src family kinases [18,19] The cAMP pathway is also a negative modulator of RhoA synthesis. This small GTPase is involved in the activation of integrins by promoting avidity modulation, a process known as inside-out signaling [20-22]. The phosphorylation of the Ser^{188} of RhoA by cAMP-dependent protein kinase (PKA) is a central event in mediating the cellular effects of cAMP [23]. The translocation of phosphorylated RhoA from the membrane to the cytosol by its binding to guanine nucleotide dissociation inhibitor (GDI) terminates RhoA signaling [23]. Cyclic AMP can also inhibit the signaling of RAS/RAF/MAPK/extracellular signal-regulated kinase

(ERK) [24]. ERK phosphorylates many substrates, regulating such cell functions as gene expression, cell morphology, proliferation, differentiation and cell death [24].

Our previous study showed that more cAMP is produced when cells are loosely attached to a hydrophilic cellulose membrane [10]. These cells have a disorganized actin cytoskeleton and subnormal amounts of RhoA in the cell membrane. By contrast, cells spread on tissue culture polystyrene (PS) contain low concentrations of cAMP. Cellulose membrane is a polymeric material which possesses a large degree of surface heterogeneity with regard to the type and distribution of functional groups, surface roughness and porosity. Hence, it is difficult to analyze the influence of the surface composition of these materials on the observed results.

We have therefore used model substrata, self-assembled monolayers (SAMs) of alkyl silanes, to clarify the impact of surface properties like the chemical composition of materials on signal transduction. SAMs have specific terminal functions, such as COOH, NH_2, poly(ethylene glycol) (PEG) and CH_3. Water contact angle measurements have revealed that SAMs terminated with -CH_3 produced hydrophobic surfaces, while those with -NH_2 and -COOH prepared moderately wettable surfaces and those with -PEG created wettable surfaces [25]. We analyzed the effect of these functional groups on the response of Swiss 3T3 fibroblasts by measuring cAMP production, FAK Tyr^{397} phosphorylation and the state of RhoA activation. Total and phosphorylated ERK1/2 MAPK (pERK1/2) were also measured.

MATERIALS AND METHODS

Preparation of Substrata

Glass treatment. Glass microscope coverslips (Superior-Marienfeld, Germany) were cleaned by immersion in freshly prepared piranha solution (3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2) for 15 min at room temperature. They were then rinsed exhaustively with distilled water (10 x 6 min) and dried.

Sample preparation. The cleaned glass coverslips were coated with SAMs terminating in $COOCH_3$, NH_2, PEG or CH_3, as described previously [25,26]. Briefly, each of these monolayers was prepared by one-step procedures using ethanolic 1% (v/v) 10-carbomethoxydecyldimethylchlorosilane, 1% (v/v) 3-aminopropyldimethylethoxysilane, 1% (v/v) 2-methoxypolyethyleneoxypropyltrimethoxysilane and 5% (v/v) octadecyldimethyl-chlorosilane in hexane (Gelest, Tullytown, USA), overnight at room temperature. The coverslips were rinsed with ethanol, washed with distilled water and dried in air. Carboxylic acid (COOH) groups were generated from $COOCH_3$ by heating the $COOCH_3$-terminated monolayers for 30 min at 100°C in acidified water pH 2.5. They were then rinsed with water, dried for 10 min at 110°C and stored in a vacuum dessicator.

Cell Experiments

Cell culture. Swiss 3T3 mouse fibroblast cells (CCL-92™, ATCC, Manassas USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Burlington, Canada) supplemented with 10% foetal bovine serum (FBS, Sigma, Oakville, Canada) and 1% antibiotic-antimycotic solution (Sigma). Cells were removed by trypsinization (Invitrogen), resuspended in DMEM with 10% FBS and seeded on SAMs at a density of 2 x 10^4 cells/cm^2. Cultures were incubated for 45 min or 120 min at 37 °C in a humidified 5% CO_2 atmosphere.

cAMP measurements. Adherent Swiss 3T3 fibroblasts were incubated with ^{3}H-adenine triphosphate (ATP) (5 µCi/mL) for 2h, removed from the coverslip by trypsinization, suspended in DMEM with 10% FBS and seeded on SAMs and PS at a density of 2 x 10^4 cells/cm^2. The cells were incubated for 45 min, rinsed two times with PBS pH 7.4 and the ^{3}H cAMP in the attached cells was extracted using trichloroacetic acid. The broken cell suspension was centrifuged at 4 000 rpm for 5 min and the intracellular ^{3}H cAMP was separated on a Dowex AG50x8-Alumina column and counted in a scintillation counter. Assays were carried out in triplicate and the results obtained were referred to the number of attached cells determined by counting the non-adherent cells.

Visualization of the distributions of focal adhesions. After incubation for 45 or 120 min, the cells attached to SAMs and PS with or without 1µM 8-Bromo-cAMP, an analog of cAMP (Sigma), or 1µM forskolin (Sigma), were fixed by incubation in 3% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 15 min and permeabilized for 5 min with 0.5% (v/v) Triton X-100 in PBS. Non-specific binding sites were blocked by incubating them in PBS containing 1% (v/v) bovine serum albumin (BSA, Sigma) for 30 min. Cells were immunostained by incubating them with mouse monoclonal antibodies raised against vinculin (Sigma, diluted 1:50). Primary antibody binding was visualized by incubation with a fluorescein (FITC)-conjugated anti-mouse IgG antibodies (Sigma, diluted 1:200). All antibodies were diluted in PBS containing 0.1% (v/v) BSA and cells were incubated with antibodies for 30 min at room temperature. Filamentous actin (F-Actin) was stained by incubation with BODIPY-phalloidin (Molecular Probes, Oregon, USA, diluted 1:200) for 30 min at room temperature. The coverslips were washed, mounted on glass slides and examined under an epifluorescence microscope (Eclipse TE2000-S, Nikon, Mississauga, Canada) equipped with a 60X oil immersion objective and a Retiga 1300R camera (Nikon).

Western blot analysis of the phosphorylated FAK on Tyr^{397}. Swiss 3T3 cells (2 x 10^4/cm^2) in DMEM with 10% FBS were seeded on COOH-, NH_2-, PEG-, CH_3 -terminated SAMs or PS with or without 1µM 8-Bromo-cAMP and incubated for 45 min at 37°C in a 5% CO_2 atmosphere. The cells attached to SAMs were lysed at 4°C in 1 mL of 50 mM Tris-HCl, pH 7.4 containing 10% glycerol (v/v) and protease inhibitors (0.1 mM phenylmethylsulfonylfluoride, 10 µg/mL aprotinin and 10 µg/mL leupeptin, Sigma). The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using a Transblot Semi-Dry electrophoretic transfer cell (Hoefer TE70, Amersham Pharmacia, Piscataway, USA). The nitrocellulose membranes were stained with Ponceau red (Sigma) to confirm transfer efficiency and then incubated overnight in a 5% (w/v) solution of non-fat dried milk in PBS Tween 20 0.1% (v/v). The nitrocellulose membranes were washed three times with PBS containing Tween 20 0.1% (v/v) and incubated for 120 min at room temperature with a primary mouse antibody against FAK phosphorylated on Tyr^{397} (Chemicon, Temecula, USA, diluted 1:1 000). The membranes were washed three times with

0.1% (v/v) PBS Tween 20 and bound specific antibody was revealed by incubation with a peroxidase-conjugated anti-mouse second antibody (Sigma, diluted 1:10 000). Immunoreactive bands were visualized by chemiluminescent detection (ECL, Roche Diagnostics, Penzberg, Germany) and exposure to X-Ray film (Kodak, Germany).

Detection of GTP-bound RhoA. Cell extracts (500 μg) were prepared as described above and incubated with glutathione S-transferase fusion protein (GST-Rhotekin) solution (Pierce Biotechnology, Rockford). The N-terminal part of Rhotekin binds to GTP-bound RhoA *in vitro*, but not to Rac1 or cdc42 [27]. Activated GTP-bound RhoA was solubilized, resolved by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad Laboratories, Mississauga, Canada). The blotted membrane was probed with antisera against RhoA and immunoreactive bands were visualized using a peroxidase-conjugated mouse Ig antibody followed by the ECL reaction and exposure to X-Ray film.

Detection of the phosphorylated ERK1/2. Swiss 3T3 cells in DMEM 10% FBS were seeded on SAMs as described above. The cells attached to SAMs were then lysed at 4°C with a cell lysis kit (Bio-Rad Laboratories). pERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}) was detected using a BioPlex phospho-ERK1/2 MAPK assay kit (BioRad Laboratories) according to the manufacturer's instruction. Briefly, 50 μL of each cell extract was adjusted to a protein concentration of 200-300 μg/mL and placed in a 96-well filter plate containing bead-conjugated antibody against pERK1/2. The plate was incubated overnight on a platform shaker at 300 rpm at room temperature, washed three time with wash buffer, and the antigen-antibody complexes were visualized with a fluorescently labelled antibody raised against pERK1/2. The ERK1/2 total protein was assayed using the BioPlex assay kit (BioRad Laboratories).

Statistics

All statistical computations were performed with GraphPad Instat®3.00 software (GraphPad Software Inc., San Diego, USA). The Student Newman Keuls multiple comparison test (ANOVA) or a Student *t*-test was applied. Values were considered significantly different if $p < 0.05$.

RESULTS

Production of cAMP in Swiss 3T3 Fibroblasts Attached to the SAMs

We analyzed the impact of the initial cell-SAM interactions on signal transduction by monitoring the intracellular concentration of cAMP. The cAMP produced after incubation for 45 min was referred to that of cells attached to PS. Swiss 3T3 fibroblasts attached to COOH and NH_2 contained significantly less cAMP than cells on PEG or CH_3 ($p < 0.05$, Fig. 1).

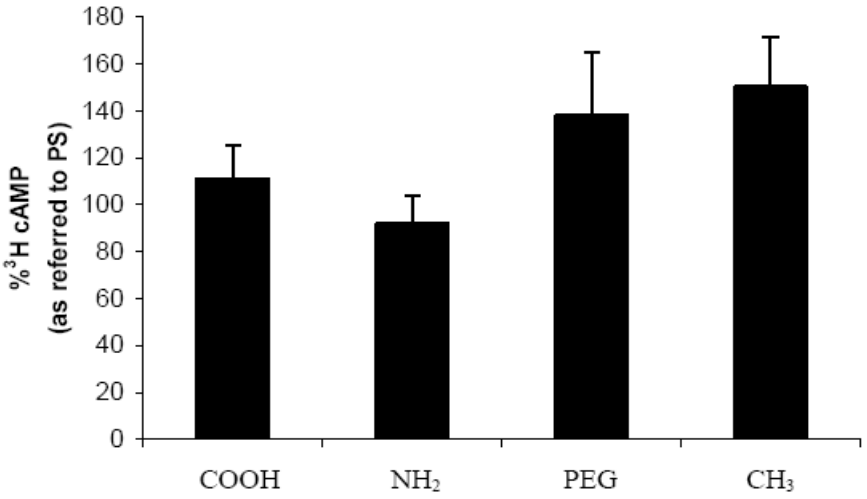

Fig. 1. Intracellular cAMP of Swiss 3T3 cells attached to SAMs. The cAMP in cells attached to SAMs was measured as described in *Materials and Methods*. The results are referred to the cAMP in cells attached to PS. Results are the means ± SD of triplicate measurements of a single experiment. Another independent experiment gave similar results.

Organization of the Focal Adhesions in Swiss 3T3 Fibroblasts Attached to SAMs

Focal adhesion complexes in adherent cells incubated for 45 min or 120 min (Fig. 2a) were assessed by immunostaining with antibodies against vinculin. F-Actin was also visualized using BODIPY-conjugated phalloidin. Most of the cells attached to COOH substratum had spread little after incubation for 45 min, but they contained some focal adhesion complexes and few actin stress fibers. On NH_2, the spread cells contained some focal adhesion plaques at the cell periphery and longitudinal actin stress fibers. Swiss 3T3 fibroblasts plated on PEG and CH_3 for 45 min had few focal adhesion complexes and very poorly organized thin stress fibers (Fig. 2a). The cells attached to COOH and NH_2 and incubated for 120 min were fully spread with many bundles of actin stress fibers anchored to the plasma membrane at sites of extended focal adhesion contacts, as demonstrated by intense vinculin clusters (Fig. 2a). By contrast, the cells attached to PEG and CH_3 contained only small focal adhesion complexes at the cell periphery. Most of these cells also remained rounded up and contained few thin stress fibers.

Controls using PS with or without 8-Bromo-cAMP or forskolin (Fig. 2b) revealed that fibroblasts attached to PS after incubation for 45 min at 37°C were well spread. They contained short linear focal adhesion plaques at the cell periphery and longitudinal actin stress fibers. In contrast, few focal adhesions and some actin stress fibers were observed on PS in the presence of cAMP analog. Few focal adhesion contacts or actin stress fibers were observed on PS in the presence of forskolin.

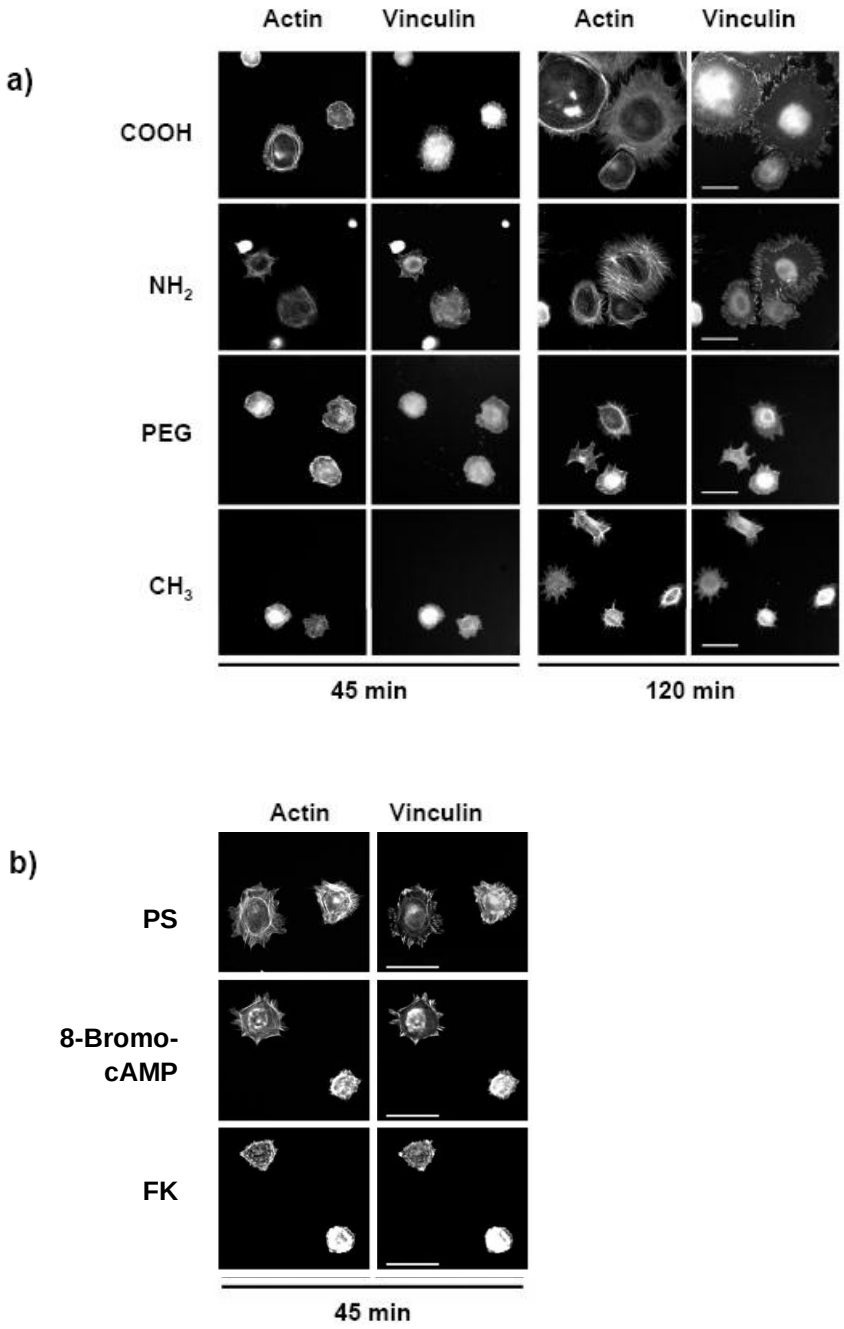

Fig. 2. Cytoskeletal organization were visualized by immunostaining of vinculin and labelling of actin in Swiss 3T3 cells attached to SAMs after incubation for 45 min or 120 min (a). Fibroblasts were also seeded on PS in the presence of 1μM 8-Bromo-cAMP or 1μM forskolin (FK) and incubated for 45 min (b). The results shown are representative of at least two other experiments. Bar = 50 μm.

Western Blot Analysis of FAK Phosphorylation

The influence of the SAMs on FAK phosphorylation on Tyr^{397} was analyzed by immunoblotting (Fig. 3a) of the proteins in cell extracts prepared from Swiss 3T3 fibroblasts attached to COOH, NH_2, PEG and CH_3 and incubated for 45 min at 37°C. FAK Tyr^{397} phosphorylation was higher in cells on COOH and NH_2 substrata than in cells on PEG and CH_3. The same immunoblot reprobed with antibodies against actin is shown in figure 3a. The various cell extracts contained similar amounts of actin.

Controls using PS with or without 8-Bromo-cAMP (Fig. 3b) revealed that fibroblasts attached to PS after incubation for 45 min contained higher amount of phosphorylated FAK on Tyr^{397} than those attached to PS in the presence of cAMP analog. Control immunoblot probed with antibodies against total FAK is shown in figure 3b. The various cell extracts contained similar amounts of FAK.

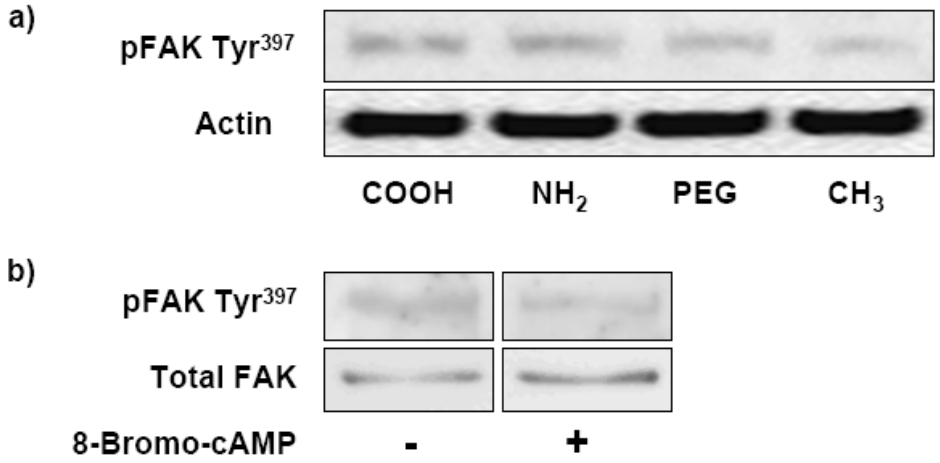

Fig. 3. FAK phosphorylation in Swiss 3T3 cells adhering to COOH-, NH_2-, PEG- and CH_3-terminated SAMs after incubation for 45 min (a). FAK phosphorylation in fibroblasts seeded on PS in the presence of 1µM 8-Bromo-cAMP (b). Proteins (50 µg) in cell extracts were resolved by SDS PAGE and immunoblotted with monoclonal antibody against FAK phosphorylated on Tyr^{397}, a monoclonal antibody against actin (a) or a monoclonal antibody against FAK (b), for normalization. The blots shown are representative of two other experiments.

Western Blot Analysis of GTP-Bound RhoA

GTP-bound RhoA was precipitated from extracts (Fig. 4) of Swiss 3T3 fibroblasts attached to COOH, NH_2, PEG and CH_3 and incubated for 45 min at 37°C. The results revealed a major band characteristic of RhoA about 23 kDa [28]. The extracts from Swiss 3T3 cells attached to COOH and NH_2 contained more GTP-bound RhoA than did extracts of

cells attached to PEG and CH_3. Control immunoblot probed with antibodies against total RhoA revealed similar amounts of RhoA in all cell extracts (Fig 4).

Fig. 4. Activation state of RhoA in Swiss 3T3 cells on SAMs. Western blot analysis of GTP-bound RhoA Immunoprecipitated by GST-Rhotekin and total RhoA in cell extracts prepared from Swiss 3T3 cells attached to COOH-, NH_2-, PEG- and CH_3- terminated SAMs. The blots shown are representative of two other experiments showing similar results.

The Phosphorylated State of ERK1/2 in Cell Extracts

pERK1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187}) was detected in extracts of Swiss 3T3 fibroblasts attached to SAMs after incubation for 45 min at 37°C (Fig 5). As the results for cells on PEG and CH_3 had been similar throughout the study, only the pERK1/2 content of cells on PEG was assessed and not that of cells on CH_3. Each result is expressed relative to the total ERK1/2 content of cells on COOH (11564 ± 2572 fluorescence arbitrary units), NH_2 (13187 ± 1626) or PEG (20342 ± 2387). There was significantly more pERK1/2 in cells on COOH and NH_2 than in cells on PEG ($p < 0.05$).

DISCUSSION

The surface properties of substrata control the morphology and behavior of cells that adhere to them [29,30]. Cell adhesion and spreading have been studied on SAMs, well-defined model surfaces with specific terminal groups giving them a low rugosity and controllable wettability [29,31]. Although protein adsorption is the dominant factor regulating cell adhesion [32-34], the way cells perceive information through signal transduction on different substrata is not well understood. The interplay between signals involving cAMP and the cytoskeleton may be especially important in cells attached to biomaterials. The second messenger cAMP can modulate cell morphology regulating cell behaviors [11-15]. We have previously shown that the catalytic activity of adenylyl cyclase, the enzyme that produces cAMP from ATP, is modulated by the attachment of Swiss 3T3 fibroblasts to a cellulose membrane [35]. In this study, we have therefore used SAMs [25,36,37] with a variety of terminating functional groups (COOH, NH_2, PEG and CH_3) to study the cAMP production generated by cell-substratum interactions.

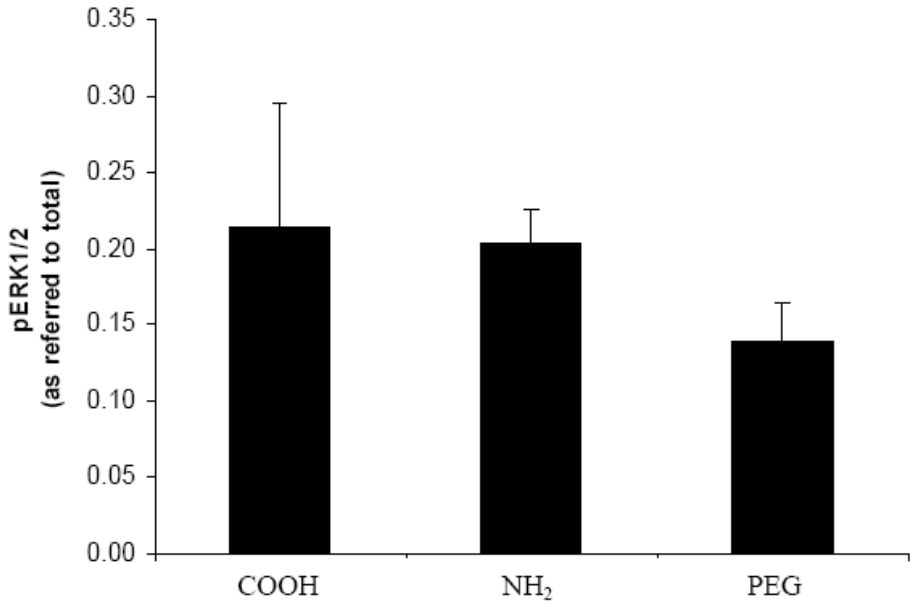

Fig. 5. Phosphorylation of ERK1/2 in Swiss 3T3 cells on SAMs. Swiss 3T3 fibroblasts in DMEM plus 10% FBS (2 x 10^4cells/cm²) were seeded for 45 min at 37°C in 5% CO_2 on SAMs. The total and phosphorylated ERK1/2 in cell extracts were quantified as described in *Materials and Methods*. Results are the means ± SD of six measurements from two independent experiments.

Cells attached to moderately wettable surfaces (COOH and NH_2) and incubated for 45 min can spread and organize their cytoskeleton and contain less cAMP than round cells on highly hydrophilic (PEG) or hydrophobic (CH_3) substrata. Several studies have shown that moderately wettable surfaces support the formation of focal adhesions, while hydrophobic and hydrophilic surfaces inhibit the formation of these structures [38-40]. Although COOH, NH_2 and CH_3 substrata all adsorb vitronectin from FBS [25], only COOH- and NH_2-terminated SAMs support the formation of focal adhesions. Indeed, the conformations of adsorbed proteins onto CH_3 substratum are changed, and this inhibits the adhesion of cells via integrins [41,42]. In contrast, PEG-terminated SAM does not adsorb adhesive proteins [25]. We have recently shown that a lack of binding between adhesive proteins and the integrins activates the cAMP pathway [9]. Here, we showed that cells on PEG- and CH_3-terminated SAMs contained a higher amount of cAMP and lacked well-developed focal adhesions and actin polymerization, indicating impaired adhesive protein-integrin binding, even after incubation for 120 min. An increase in intracellular cAMP can also prevent the formation of focal adhesion [13,14]. Indeed, we showed in this study that Swiss 3T3 fibroblasts attached to PS in the presence of cAMP analog or activator of the adenylyl cyclase contained only few focal adhesion plaques and some actin stress fibers.

We have also analyzed the phosphorylation state of FAK Tyr^{397}. FAK plays a major role in integrin-mediated signal transduction and is autophosphorylated on Tyr^{397} when cells bind to material surfaces [43-45]. Our results reveal that fibroblasts attached to COOH and NH_2 substrata contain similar quantities of phosphorylated FAK on Tyr^{397}, while the phosphorylation of this site is partly inhibited in cells on PEG- and CH_3-terminated SAMs.

Using MC3T3-E1 preosteoblast-like cells on fibronectin-coated SAMs, Keselowski *et al.* [46] showed that FAK Tyr^{397} is most highly phosphorylated in cells on fibronectin-coated NH_2 substrata, closely followed by COOH substratum. In contrast, the FAK Tyr^{397} of cells on fibronectin-coated CH_3-terminated SAM is minimally phosphorylated [46]. Here, we showed that the presence of an analog of cAMP drastically reduced the phosphorylation of FAK on Tyr^{397} in Swiss 3T3 attached to PS after incubation for 45 min. Padmanabhan *et al.* [16] have also demonstrated that treating cells with the cAMP analog dibutyryl cAMP decreases FAK phosphophorylation in astrocytes. Thus, cAMP production in cells attached to PEG and CH_3-terminated SAM might be involved in the decreased FAK phosphorylation.

The Rho family of small GTPases, which includes Rho, Rac, and cdc42, plays also a role in the formation of focal adhesions and their maintenance [47]. We have directly measured the amount of GTP-bound RhoA, the activated form of RhoA, in Swiss 3T3 cells attached to the COOH- and NH_2-terminated SAMs. These cells contained more GTP-bound RhoA than do cells on PEG- and CH_3-terminated SAMs. McClary *et al.* [48] have shown that the membranes of spread cells attached to COOH-terminated SAM contained a higher concentration of RhoA than do cells on CH_3-terminated SAM. The cAMP-dependent PKA mediates the phosphorylation of RhoA in cytotoxic T lymphocytes, leading to an inhibition of RhoA [23]. Furthermore, increased cAMP production decreases the amount of RhoA in the membranes of Swiss 3T3 fibroblasts on a hydrophilic cellulosic membrane [10]. This effect is mediated through the PKA since an inhibitor of the kinase (PKI) can restore RhoA at the cell membrane [10]. In addition, Swiss 3T3 fibroblasts attached to PS in the presence of forskolin or an analog of cAMP contained a low amount of GTP-RhoA in comparaison to PS without treatment (data not shown). Therefore, the cAMP production in Swiss 3T3 fibroblasts attached to both hydrophilic PEG and hydrophobic CH_3 substrata may mediate the inhibition of RhoA.

GTP-bound RhoA, which plays a critical role in the spreading of the Swiss 3T3 fibroblasts, is also involved in the activation of the ERK1/2 MAPK pathway [49]. This pathway is activated by the binding of growth factors to the tyrosine kinase receptors. This leads to the activation of the small G-protein RAS. Then, c-RAF, MEK and ERK1/2 are activated in a cascade of phosphorylation events [49].

Cyclic AMP inhibits the ERK pathway in several cell types, including adipocytes, fibroblasts [49,50] and smooth muscle cells [51]. The cell-permeable cAMP analog 8-Chloro-cAMP also blocks the ERK pathway in fibroblasts [21,49,52], showing that there is crosstalk between these pathways. Our results indicate that ERK1/2 is more highly phosphorylated in cells on COOH and NH_2 substrata than in cells on a PEG-terminated SAM. It is generally agreed that the inactivation of ERK1/2 by the cAMP pathway is due to inhibition of c-RAF, but the precise mechanism is not clear. This crosstalk may imply that there are other intracellular targets, since cAMP still inhibits ERK, even when c-RAF signaling is restored [49,52].

CONCLUSION

Few studies have investigated the influence of surfaces bearing different terminating groups on the early biochemical events generated by cell-substratum interactions. A better

knowledge of these phenomena is required for the development of new biomaterials that produce specific cell responses. This study of the early steps of cell-material adhesions (Table 1) highlights variations in cAMP produced by Swiss 3T3 fibroblasts on SAMs with different surface groups and may be associated with states of FAK, RhoA and ERK1/2 activation. Thus, monitoring the early intracellular concentration of cAMP may indicate the quality of the cell-substratum interactions controlling the subsequent signal transduction and therefore cell behavior.

TABLE 1. Cytoskeletal organization and signal transduction of Swiss 3T3 cells on SAMs.

Surface Properties	*Functional End Groups*	*cAMP Production*	*Cytoskeleton Organization*	*FAK Phosphorylation on Tyr^{397}*	*GTP-Bound RhoA*	*ERK1/2 Phosphorylation*
Moderate Wettable	COOH	+/-	++	+++	+++	++
Moderate Wettable	NH_2	+/-	++	+++	++	++
Hydrophilic	PEG	++	+/-	++	+	+
Hydrophobic	CH_3	++	+/-	+	+	NA

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