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Modulation of Adhesion-Dependent cAMP Signaling by Echistatin and Alendronate

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We measured intracellular cAMP levels in cells during attachment and spreading on different extracellular matrix (ECM) proteins. Increases in cAMP were observed within minutes when cells attached to fibronectin, vitronectin, and a synthetic RGD-containing fibronectin peptide (Petite 2000), but not when they adhered to another integrin $\alpha v\beta 3$ ligand, echistatin. Because echistatin also inhibits bone resorption, we measured the effects of adding another osteoporosis inhibitor, alendronate, in this system. Alendronate inhibited the cAMP increase induced by ligands that primarily utilize integrin $\alpha v\beta 3$ (vitronectin, Peptite 2000), but not by fibronectin which can also use integrin $\alpha 5\beta 1$. These results show that cell adhesion to ECM can increase intracellular cAMP levels and raise the possibility that inhibitors of osteoporosis may act, in part, by preventing activation of this pathway by integrins.

Extracellular matrix (ECM) plays a critical role during tissue morphogenesis by regulating cell growth and differentiation within local microenvironments [1,2]. The effects of ECM on cell function are mediated through binding of different cell surface integrin receptors. More than 20 different types of integrins have been identified which recognize distinct amino acid sequences that are found within ECM molecules and other adhesive ligands. The first integrin recognition sequence identified, Arg-Gly-Asp (RGD), is found within fibronectin (FN) and vitronectin (VN) as well as in "disintegrins", such as echistatin, that are naturally found in snake venom [3–5]. Their specificity for different integrin subtypes is determined by differences in the amino acid residues surrounding the RGD sequence. For example, the placement of RGD within intact FN supports binding to integrin $\alpha 5\beta 1$ (FN receptor) as well as integrin $\alpha v\beta 3$ (VN receptor) whereas the RGD context in VN is not recognized by the former [6–8]. The RGD sequence in echistatin similarly exhibits much higher affinity for integrin $\alpha v\beta 3$ compared with integrin $\alpha 5\beta 1$ or other integrin subtypes [9–11]. Small changes in the conformation of short synthetic peptides containing the original RGD-sequence from FN also can alter their binding specificity (e.g., enhance binding the VN receptor without changing FN receptor binding) [12,13]. In addition to mediating cell adhesion to ECM, cell surface integrins also are capable of activating intracellular biochemical signaling pathways inside the cell. For example, adhesion to ECM or clustering of specific integrin receptors has been shown to induce protein tyrosine phosphorylation, stimulate inositol lipid metabolism, activate G proteins, and enhance ion exchange across the cell surface [14–16]. ECM binding and integrin engagement also result in transmission of mechanical stresses across the cell surface that produce changes in cytoskeletal organization and associated alterations in cell shape [17–19].

While integrin signaling has been studied in many cell types, little is known about the effects of ECM binding on cAMP-dependent signaling mechanisms which appear to play a critical role in regulation of adhesion, cell shape and cytoskeletal organization in anchorage-dependent cells. In certain cell types, sustaining intracellular cAMP at high levels has been shown to suppress focal adhesion formation and F-actin assembly and to inhibit cell spreading, motility, and contraction [20–24]. On the other hand, increasing cAMP levels enhances attachment of adhesion-defective Chinese hamster ovary cells [25]. Importantly, mechanical stimulation of cells can increase cAMP production [26–28] and thus, cAMP levels might be expected to rise during cell spreading on ECM given that this process is mediated by transfer of mechanical stresses across the cell surface

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[17,18,29]. Understanding the relation between adhesion and control of this second messenger system could therefore have important implications for cell shape control as well as mechanosensation. Thus, the present study was initiated to explore whether cell adhesion to ECM alters intracellular cAMP levels. In these experiments, we utilized bovine capillary endothelial (CE) cells that have already been well characterized in terms of other integrin signaling behavior [30–33].

MATERIALS AND METHODS

Experimental system. Bacteriological plastic dishes (60 mm, Falcon) were coated with a saturating density (30 μg/dish) of FN (Cappel), a synthetic RGD-containing FN peptide (Peptite 2000; Telios Pharmaceuticals), VN (Sigma), or echistatin (kindly provided by Merck) using a carbonate buffer technique [30]. To mediate cell attachment independent of integrins, dishes were coated with a high concentration (100 μg/dish) of thrombin, a ligand for metabolic cell surface receptors on CE cells. Non-specific attachment sites were blocked with 1% bovine serum albumin (BSA) in DMEM for 1hr at 37°C prior to use. Quiescent, serum-deprived (0.5% calf-serum for 48 hr) CE cell monolayers were dissociated into single cells by brief exposure to trypsin-EDTA, washed in 1% BSA/DMEM, and resuspended in chemically defined medium (DMEM, 10 μg/ml high density lipoprotein, and 5 μg/ml transferrin) without any exogenous peptide growth factors, as previously described [30]. Cell suspensions were incubated with the phosphodiesterase inhibitor, 3-isobutylmethylxanthine (IBMX; 1 mM), for 15 min at 37°C prior to plating as well as during subsequent incubations. Approximately 5×10^5 CE cells were either plated onto dishes coated with adhesive ligands or maintained in suspension on non-adhesive (BSA-coated) dishes at 37°C for the indicated times. In studies with alendronate (kindly provided by Merck), drug was added to the IBMX-treated cell suspensions for 15 min prior to plating in the presence of drug.

Quantitation of cellular cAMP. At the indicated times, adherent cells on dishes and suspended cells that were collected by centrifugation were washed in ice-cold PBS. cAMP was extracted by addition of 0.8 ml of 95% ethanol/5% 0.1N HCl for 2 hr at 4°C. Cellular proteins remaining on the dishes or in the cell pellets were dissolved in 0.1N NaOH and collected. The supernatants were concentrated in a Speed-Vac for 2 hr and redissolved in cAMP assay buffer (0.05 M acetate buffer). Total intracellular cAMP was measured using a commercial ^{125}I radioimmunoassay kit (Amersham), however, similar results were also obtained using an ELISA-based method (Advanced Magnetics). Intracellular cAMP levels measured in different samples were normalized on an equal protein basis, as determined within acid-neutralized samples using a BCA protein microassay (Pierce). The amount of protein that was precoated on the different dishes was measured and subtracted from the total protein values (obtained with adherent cells) prior to normalizing the different samples.

RESULTS AND DISCUSSION

To determine whether cell adhesion to ECM alters cAMP signaling, we compared intracellular cAMP levels measured in CE cells plated on FN-coated dishes with those in cells held in suspension (Fig. 1). Cell plating on FN resulted in approximately a 6 fold increase in total cellular cAMP (relative to time zero) which was detectable within 15 to 30 min after binding, before significant cell spreading was observed. While cAMP levels subsequently decreased, they were maintained at an elevated level (approximately 4 fold higher than time zero) for at least 2 hr of culture. In contrast, intracellular cAMP levels did not significantly increase in cells maintained in suspension for similar times (Fig. 1). The increased production of this chemical second messenger observed in response to adhesion most likely resulted from activation of adenylate cyclase since all of these studies were carried out in the presence of the phosphodiesterase inhibitor, IBMX [34].

The ability to induce an increase in intracellular cAMP was not limited to FN. Similar increases in cAMP levels were observed in cells that were allowed to attach and spread for 45 min on dishes coated with FN, VN, or a synthetic peptide that contains the RGD-cell binding site from FN (Peptide 2000) (Fig. 2). Importantly, this increase in second messenger levels did not appear to be a non-specific effect of cell adhesion per se since no increase in cAMP resulted when cells attached to dishes coated with thrombin (i.e., a ligand that mediates binding to cell surface metabolic receptors, rather than integrins) (Fig. 2). While intact FN and short peptides containing the RGD-cell binding site from FN can bind to both integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ [12], antibody inhibition studies show that the specific configuration of the RGD sequence within immobilized Peptite 2000 preferentially mediates cell attachment through integrin $\alpha v\beta 3$ [35]. In this manner, Peptite 2000 appears to more closely mimic VN which utilizes only αv-containing integrins [4].

Interestingly, while CE cells also attached well and spread on dishes coated with another

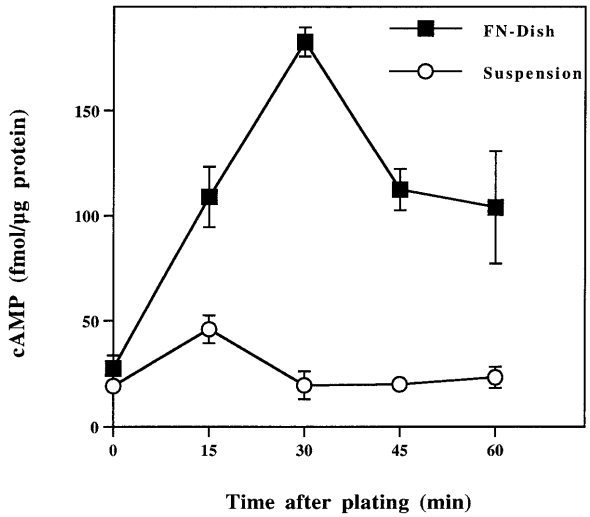

FIG. 1. Time course of the increase in cAMP induced by adhesion to FN. cAMP levels were measured in cells that were pretreated with 1 mM IBMX prior to plating on bacteriological dishes coated with FN or being placed in suspension on BSA-coated dishes. Data presented were normalized on an equal protein basis by quantitating protein levels within the same samples used for cAMP analysis; error bars indicate S.E.M..

RGD-containing peptide, echistatin, no increase in cAMP could be detected (Fig. 2). Echistatin, a member of the disintegrin family of proteins, has been previously shown to competitively inhibit integrin $\alpha v\beta 3$ binding with great efficiency ($IC_{50} = 10^{-9}M$) when added in a soluble form [36]. In osteoclasts, echistatin binding produces an increase in intracellular calcium and thus, it mimics the effects of binding physiological integrin ligands [37]. In contrast, the present results show that echistatin fails to induce cAMP synthesis when it binds to cell surface integrins, even though other integrin $\alpha v\beta 3$ ligands (e.g., VN, Peptite 2000) produce this effect. On the other hand, our results are consistent with the recent finding that other snake venom disintegrins produce integrin signaling responses (e.g., protein tyrosine phosphorylation patterns) than differ from those produced by physiological integrin ligands in platelets [5,38].

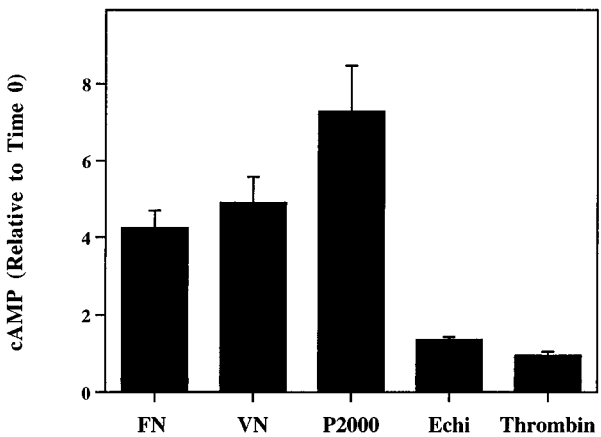

FIG. 2. Various adhesive molecules differed in their ability to induce cAMP accumulation. cAMP levels were determined in suspended CE cells before plating (Time 0) and in cellc 45 min after plating in defined medium on dishes coated with fibronectin (FN), vitronectin (VN), Peptite 2000 (P2000) or echistatin (Echi). cAMP levels were analyzed 2 hr after plating in cells attached to thrombin-coated dishes because cells attached more slowly on these dishes. Results are expressed as fold increase relative to the cAMP value measured at time 0 for each condition.

Our findings with echistatin suggested that some of its reported effects on cell behavior could be mediated, in part, through changes in integrin-dependent cAMP signaling. One of the most potent effects of echistatin is its ability to prevent osteoclast-mediated bone resorption, apparently by interfering with integrin $\alpha v\beta 3$ binding [10,39,40]. If changes in cAMP signaling mediate the effects of this inhibitor of bone resorption, then it is possible that other drugs that inhibit osteoporosis could use a similar mechanism. Alendronate is a bisphosphonate derivative that also inhibits osteoclast-mediated bone resorption [41]. Alendronate is currently in clinical use for treatment of osteoporosis, however, its mechanism of action is unknown. To begin to explore the possibility that integrin-dependent cAMP signaling could represent a common target for both osteoporosis inhibitors, we tested alendronate in our in vitro system. We found that when CE cells were plated on dishes coated with Peptite 2000 in the presence of 10–150 uM alendronate, dose-dependent inhibition of the adhesion-induced increase in cAMP was observed (Fig. 3), even though there was no effect on cell attachment or spreading (not shown). Half-maximal inhibition of the cAMP response was observed at approximately 50 uM, with doses greater than 100 uM producing saturating effects. Similar doses of this drug have been shown to effectively inhibit bone resorption by osteoclasts in vitro and in vivo [41]. Interestingly, 100 uM alendronate inhibited the cAMP increase produced in response to cell binding to VN as well as Peptite 2000, but not to FN (Fig. 4). Furthermore, alendronate did not have any additional inhibitory effect on cAMP levels in cells plated on echistatin-coated dishes (Fig. 4), nor did it interfere with the increase in cAMP produced by forskolin treatment in suspended cells (not shown). Thus, this drug did not appear to produce a generalized decrease in adenylate cyclase activity, rather it prevented the increase in cAMP specifically produced in response to binding of integrin $\alpha v\beta 3$ ligands (i.e., VN, Peptite 2000). FN apparently can circumvent this inhibition based on its ability to bind other integrin subtypes (e.g., $\alpha 5\beta 1$) as well [4,7].

Taken together, these results show that cell attachment to various immobilized ECM proteins that mediate binding to different cell surface integrin receptors results in a rapid increase in intracellular cAMP levels. However, while the RGD-containing snake venom protein, echistatin,

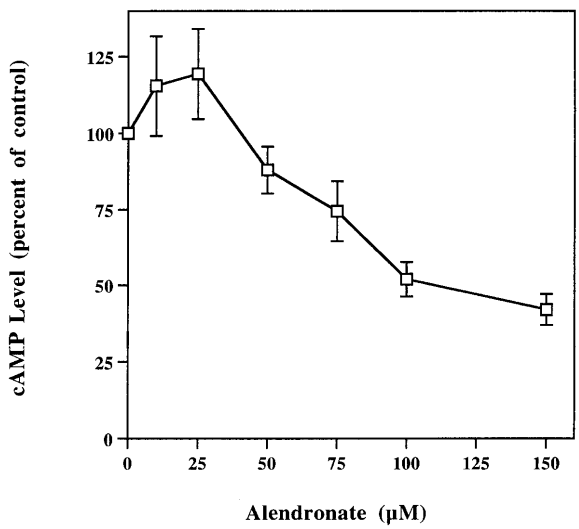

FIG. 3. Dose-dependent inhibition of the adhesion-induced increase in cAMP by alendronate. Suspended CE cells were incubated with the indicated concentrations of alendronate for 15 min and then plated on dishes coated with the RGD-peptide, Peptite 2000, in the continued presence of drug. cAMP was extracted and measured within cells 45 min after plating and normalized on an equal protein basis. Data are presented as the percent of cAMP level measured in cells cultured in parallel in the absence of alendronate (control).

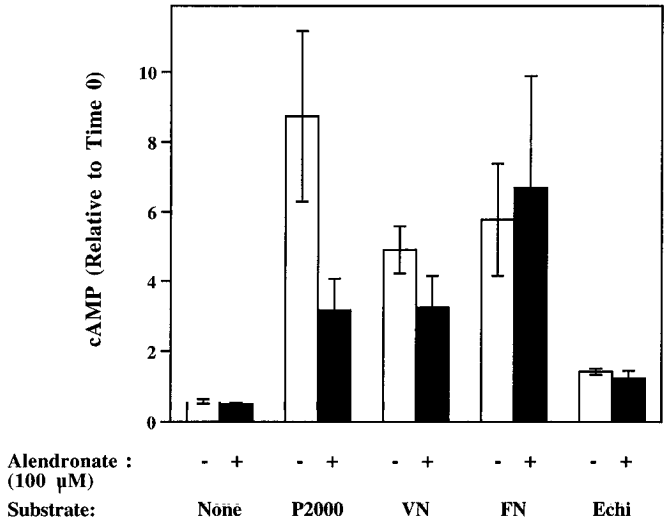

FIG. 4. Effects of alendronate on the increase in cAMP induced in response to cell adhesion to different RGD-containing proteins. Suspended cells were incubated in the presence (+) or absence (−) of a saturating concentration of alendronate (100uM) for 15 min prior to being placed in suspension (Substrate: none) or plated on dishes coated with Peptite 2000 (P2000), vitronectin (VN), fibronectin (FN), or echistatin (Echi) in the same medium (i.e., with or without drug). cAMP levels were measured 45 min after plating and normalized on an equal protein basis. Data are presented relative to cAMP levels measured in suspended cells at the time of plating (Time 0).

also binds integrin $\alpha v\beta 3$ with high affinity [10,11] and mediates CE cell attachment and spreading, it failed to promote this rise in cAMP. Furthermore, another inhibitor of osteoporosis, alendronate, acted in a analogous manner by preventing normally functional $\alpha v\beta 3$ integrin ligands (i.e., VN and Peptite 2000) from producing their characteristic cAMP response. Importantly, alendronate's effects on cAMP signaling did not result from inhibition of adhesion or from a generalized suppression of cAMP production. Rather, its inhibitory effects were specifically limited to cells adherent to adhesive ligands that primarily utilize integrin $\alpha V\beta 3$. Thus, these results raise the possibility that different inhibitors of osteoporosis share a common end-point: inhibition of integrin $\alpha V\beta 3$ signaling, and particularly suppression of its effects on intracellular cAMP.

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