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Cell aggregation on agar as an indicator for cell-matrix adhesion: effects of opioids

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Abstract The slow aggregation assay is generally used to study the functionality of cell–cell adhesion complexes. Single cells are seeded on a semisolid agar substrate in a 96-well plate and the cells spontaneously aggregate. We used HEK FLAG-MOP cells that stably overexpress the mu opioid receptor and the mu-opioid-receptor-selective agonists DAMGO and morphine to study whether other factors than functionality of cell–cell adhesions complexes can contribute to changes in the pattern of slow aggregation on agar. HEK FLAG-MOP cells formed small compact aggregates. In the presence of DAMGO and morphine, larger and fewer aggregates were formed in comparison to the vehicle control. These aggregates were localized in the center of the agar surface, whereas in the vehicle control they were dispersed over the substrate. However, in suspension culture on a Gyrotory shaker, no stimulation of aggregation was observed by DAMGO and morphine, showing that opioids do not affect affinity. A dissociation experiment revealed that HEK FLAG-MOP aggregates formed in the absence or presence of opioids are resistant to de-adhesion. We demonstrated that the larger aggregates are neither the result of cell growth stimulation by DAMGO and morphine. Since manipulations of the substrate such as increasing the agar concentration or mixing agar with agarose induced the same changes in the pattern of slow aggregation as treatment with opioids, we suggest that cell–substrate adhesion may be involved in opioid-stimulated aggregation.

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Introduction

In the slow aggregation assay (SAA), as described by Boterberg et al. (2001), a single-cell suspension is seeded on a semisolid agar substrate in a 96-well plate. The cells spontaneously aggregate. The SAA is generally used to study the functionality of cell–cell adhesion complexes (Boterberg et al. 2001). Inactivation of cell–cell adhesion complexes by genomic mutations in the αE-catenin gene (*CTNNA1*) like in HCT-8 colon cancer cells (Vermeulen et al. 1999) or functional deficiencies in the E-cadherin/catenin complex like in the MCF-7/6 cell line (Bracke et al. 1991) results in decreased cell aggregation, whereas the formation of larger aggregates of human MCF-7/6 breast carcinoma cells by compounds like tamoxifen is explained by the activation of cell–cell adhesion complexes (Bracke et al. 1994).

However, an aggregate is the net result of input of cells by cell–cell adhesion and cell growth and output of cells by de-adhesion. Drugs that change the pattern of slow aggregation can therefore interfere with cell–cell adhesion as well as with cell growth or de-adhesion. Apart from the type and the functionality of homotypical cell–cell adhesion complexes, which determine affinity, cell–cell adhesion depends on avidity, which is defined as the probability that cells adhere to each other.

We used HEK FLAG-MOP cells that stably overexpress the human mu opioid receptor (MOP) and the MOP-selective agonists DAMGO and morphine to evaluate cell aggregation in the SAA. We found that larger aggregates were formed in the presence of the opioids than in the

vehicle control. Surprisingly, our experiments provided evidence to accept that the formation of larger aggregates in the presence of opioids was not due to increased affinity. We investigated what other factors could be responsible for the observed changes in the pattern of slow aggregation.

Material and Methods

Cell culture. The HEK FLAG-MOP cell line was a kind gift of G. Milligan (Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, Scotland, UK). It has been generated as described by Parenty et al. (2008). The cells constitutively express human MOP and can be induced to express hemagglutinin (HA)-CXCR1 chemokine receptor. MOP is fused to a FLAG-tag at its N terminus and to enhanced yellow fluorescent protein at its C terminus, as described in McVey et al. (2001).

HEK FLAG-MOP cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and L-glutamine and without pyruvate (Invitrogen, Carlsbad, CA) supplemented with 10% tetracyline-free fetal bovine serum (Greiner Bio-One, Kremsmuenster, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and 2.5 μg/ml Fungizone (Bristol-Myers Squibb, New York, NY) in a humidified atmosphere of 10% CO_2 in air at 37°C. Tetracycline-free serum was used to repress expression of HA-CXCR1. To ensure stable expression of the MOP construct, selection pressure was applied by supplementing the culture medium with 1 mg/ml G418 every five passages.

Radioligand binding experiment. Radioligand binding studies were performed on attached cells as described by Marie et al. (2003). Briefly, HEK FLAG-MOP cells were seeded in 24-well plates. Before binding studies, cells were rinsed twice with DMEM/20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, followed by two rinses with phosphate-buffered saline (PBS)/0.2% (*w*/*v*) bovine serum albumin (BSA). Cells were then incubated for 30 min at 37°C with increasing concentrations of [^{3}H]diprenorphine (PerkinElmer, Boston, MA; 0.05–5 nM) in the presence (non-specific binding) or in the absence (total binding) of 20 μM DAMGO (Sigma-Aldrich, St Louis, MO) to specifically target MOP in a final volume of 300 μl of 50 mM Tris–HCl, 1% (*w*/*v*) BSA, pH 7.4. Cells were washed twice with PBS/0.2% (*w*/*v*) BSA, followed by a wash step with PBS before harvesting. For measuring radioactivity, cells were harvested in 200 μl of 1 M NaOH, incubated for 30 min at 37°C, and neutralized with 200 μl of 1 M HCl. Of the samples, 200 μl were placed into vials containing 3 ml of a scintillation mixture (Insta-Gel Plus®, PerkinElmer). Vials were counted for radioactivity in a scintillation counter (1500 Tri-Carb® Liquid Scintillation Analyzer, PerkinElmer). Each determination was carried out in duplicate. To determine protein concentration, cells were harvested in 100 μl of 1 M NaOH, incubated for 30 min at 37°C, and diluted with 100 μl Aqua Dest. Protein concentration was measured according to the Lowry method (Bio-Rad, Hercules, CA). Scatchard analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) to calculate K_d and B_{max} values.

ERK1/2 phosphorylation. HEK FLAG-MOP cells were serum-starved overnight in serum-free culture medium. Cells were pre-treated with vehicle (serum-free culture medium) or naloxone for 1 h at 37°C prior to exposure to respectively DAMGO or DAMGO and naloxone (Sigma-Aldrich) for the indicated times at 37°C. Cells were then rinsed once in ice-cold PBS containing the phosphatase inhibitors NaF (1 mM), sodium vanadate (1 mM), and $Na_4P_2O_7$ (2.5 mg/ml) and lysed with lysis buffer consisting of 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10 mM sodium phosphate pH 7.2, 1% sodium deoxycholate, 0.15 M sodium chloride, and the protease and phosphatase inhibitors sodium vanadate (1 mM), NaF (1 mM), phenylmethylsulfonyl fluoride (2 mM), $Na_4P_2O_7$ (2.5 mg/ml), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cell lysates were centrifuged at 20,800×*g* for 10 min at 4°C and protein concentration of the supernatants was measured. Samples were prepared at equal protein concentration by boiling them in Laemmli sample buffer and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by Western blotting (WB) on Hybond-ECL Nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Non-specific binding sites were blocked with 4% BSA in PBS containing 0.2% Tween-20 (BSA blocking buffer) for 30 min. Blots were then incubated with Phospho-p44/42 MAP kinase antibody (Cell Signaling Technology, Beverly, MA), 1:1,000 diluted in BSA blocking buffer, overnight at 4°C. After four washes in BSA blocking buffer, the blot was incubated with ECL™ anti-rabbit immunoglobulin G, horseradish-peroxidase-linked whole antibody (from donkey; GE Healthcare) for 60 min at room temperature, followed by six wash steps in PBS containing 0.2% Tween-20. Bands were visualized using ECL chemiluminescent Western blotting kit (GE Healthcare) and exposure to Amersham Hyperfilm™ ECL (GE Healthcare). To determine total extracellular signal-regulated kinase 1/2 (ERK1/2) levels, membranes were stripped using *Re-Blot Plus* Mild Antibody Stripping Solution (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Total ERK1/2 was visualized following immunostaining with p44/42 MAP kinase antibody (Cell Signaling Technology), 1:1,000 diluted in milk blocking buffer consisting of 5% non-fat milk in PBS containing 0.5%

Tween-20. Immunostaining was performed as described above, but BSA blocking buffer was replaced by milk blocking buffer. Band intensities were determined by densitometric analysis using scanner (Epson Perfection 4490 Photo, Epson, Nagano, Japan) and Quantity One software (Bio-Rad). Phosphorylation level of p-ERK1/2 was normalized to total ERK1/2.

Slow aggregation assay. Ninety-six-well plates were coated with semisolid agar medium consisting of 100 mg agar (Bacto™ Agar, BD Biosciences, Franklin Lakes, NJ) in 15 ml sterile Ringer's salt solution (Ringer B. Braun, Melsungen, Germany) that was boiled three times during 10 s to sterilize the solution. In some experiments, agar was replaced by agarose (Invitrogen) or a mixture of agar and agarose to coat the plates. After gelification, a single-cell suspension consisting of 20,000 HEK FLAG-MOP cells in 200 µl medium (culture medium containing 5% serum) was seeded onto the semisolid agar. Test compounds were added to the medium and cultures were incubated for 48 h in a humidified atmosphere of 10% CO_2 in air at 37°C. Aggregate formation was evaluated by phase-contrast microscopy (Leica DMI3000 B, Leica, Solms, Germany) or stereomicroscopy (Wild Heerbrugg Typ 374590, Leica), equipped with a camera (Leica DFC420 C, Leica). The size of the aggregates was determined by measuring their area on pictures using Quantity One software (Bio-Rad).

Suspension culture. Six milliliters of a single-cell suspension containing 300,000 HEK FLAG-MOP cells per milliliter of culture medium without or with DAMGO or morphine (Morphine.HCl, Sterop, Brussels, Belgium) were transferred to a 50-ml Erlenmeyer flask. The cells were kept in suspension for 48 h on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 70 rpm at 37°C. The flasks were gassed with a mixture of 10% CO_2 in air. Multicellular spheroid formation was evaluated under a phase-contrast microscope or stereomicroscope equipped with a camera.

Dissociation experiment. Aggregates were evaluated by stereomicroscopy before and after they were passed 30 times through Pasteur pipettes with an inner diameter of 1.5 mm.

Sulforhodamine B assay. Twenty thousand HEK FLAG-MOP cells were seeded in the wells of a 96-well plate in 100 µl culture medium. After 1 d, 100 µl culture medium was added without or with DAMGO, morphine, or 5-fluorouracil (5-FU). After 1, 2, 3, and 6 d of treatment, cellular protein content was determined with a sulforhodamine B assay. Briefly, cells were fixed by addition of 50 µl 50% trichloroacetic acid (Sigma-Aldrich) to the medium. After 1 h of incubation at 4°C, cells were rinsed with water, dried, and stained with 100 µl sulforhodamine B (0.4% in 1% acetic acid; Sigma-Aldrich) for 30 min at room temperature. Unbound dye was washed with 1% glacial acetic acid. After four wash steps, cells were dried and protein-bound dye was dissolved in 200 µl 10 mM Tris buffer, pH 10.5. Optical density of solubilized dye was determined at 490 nm with an enzyme-linked immunosorbent assay reader (Vmax Microplate Reader, Molecular Devices, Palo Alto, CA). Six replicate wells were tested for each condition.

Statistical analysis. Statistical analysis was performed with S-Plus® 8.0 software (Tibco, Palo Alto, CA).

Results

Characterization of the HEK FLAG-MOP cell line. HEK FLAG-MOP cells stably overexpress human MOP as evidenced by a radioligand binding experiment. HEK FLAG-MOP cells were incubated with increasing concentrations of [^{3}H]diprenorphine, a non-selective opioid, to measure total binding. Non-specific binding was detected by measuring [^{3}H]diprenorphine binding in the presence of a saturating concentration of the MOP-selective agonist DAMGO. Under these conditions, [^{3}H]diprenorphine can only bind to non-specific sites since MOP is occupied by DAMGO. Non-specific binding was lower than total binding at all concentrations, showing that MOP is present in HEK FLAG-MOP cells and binds opioids (Fig. 1*A*). Specific binding of [^{3}H]diprenorphine to MOP was obtained after subtraction of non-specific binding from total binding (Fig. 1*A*). Scatchard analysis revealed the presence of 775 fmol of MOP binding sites per milligram protein (B_{max}) in HEK FLAG-MOP cells and a dissociation constant (K_d) of 0.69 nM. Since opioids have been shown to activate ERK1//2 (Gutstein et al. 1997) and since we wanted to examine whether the exogenous MOP responds to opioids, ERK1/2 phosphorylation was studied in response to the MOP-selective agonist DAMGO. DAMGO induced within minutes a transient increase in ERK1/2 phosphorylation, which peaked at 5 min of incubation and could be blocked by the opioid receptor antagonist naloxone (Fig. 1*B*). This shows that DAMGO-stimulated ERK1/2 phosphorylation is MOP-dependent. Moreover, exposure of HEK FLAG-MOP cells to various concentrations of DAMGO for 5 min revealed a concentration-dependent stimulation of ERK1/2 phosphorylation (Fig. 1*C*).

Opioids change the pattern of slow aggregation (size, number, and position of the aggregates) of HEK FLAG-MOP cells on agar. HEK FLAG-MOP cells were tested in the SAA. As observed for MCF-7/6 cells by S. Vermeulen in our laboratory (personal communication), HEK FLAG-MOP cells spontaneously aggregated in a multistep process

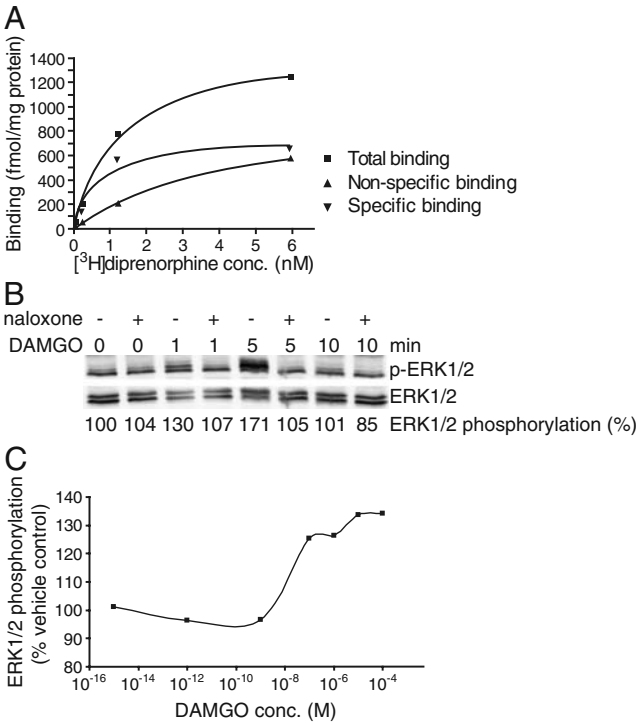

Figure 1. Characterization of the HEK FLAG-MOP cell line. (*A*) Ligand binding activity of the mu opioid receptor in HEK FLAG-MOP cells. HEK FLAG-MOP cells were incubated with increasing concentrations of [^{3}H]diprenorphine in the absence (total binding) or presence (non-specific binding) of 20 μM DAMGO. Specific binding of [^{3}H]diprenorphine to MOP was obtained after subtraction of non-specific binding from total binding. (*B*) ERK1/2 phosphorylation in HEK FLAG-MOP cells in response to DAMGO treatment. Following serum starvation overnight, HEK FLAG-MOP cells were pre-treated with vehicle or 10 μM naloxone for 1 h, prior to exposure to respectively 1 μM DAMGO or 1 μM DAMGO+10 μM naloxone for the indicated times. After opioid exposure, cell lysates were prepared and ERK1/2 activation was determined by SDS-PAGE, followed by Western blotting and immunostaining using anti-phospho-ERK1/2 antibody (p-ERK1/2). After stripping, membranes were re-probed with anti-ERK1/2 antibody (ERK1/2), showing equal protein loading. Representative immunoblots are shown. For quantification, optical densities, measured by densitometric analysis, of p-ERK1/2 immunoreactive bands were normalized to the ERK1/2 values. (*C*) Concentration-dependent stimulation of ERK1/2 phosphorylation in HEK FLAG-MOP cells in response to DAMGO treatment. HEK FLAG-MOP cells were exposed to various concentrations of DAMGO, ranging from 10^{-15} to 10^{-4} M, or vehicle for 5 min at 37°C. Cell lysates were prepared and separated by SDS-PAGE, followed by WB. WB analysis was performed with p-ERK1/2 antibody and with anti-ERK1/2 antibody after stripping. Band intensities were determined by densitometric analysis and band intensities of p-ERK1/2 were normalized to ERK1/2 band intensities. Results show ERK1/2 phosphorylation level expressed as a percent of the vehicle control.

(Fig. 2*A*). First, the cells clustered. These clusters then fused and compacted to small aggregates. Upon contact with each other, aggregates fused to larger aggregates. As such, HEK FLAG-MOP cells formed small compact aggregates (Fig. 2*B*). However, in the presence of DAMGO and morphine, larger and fewer aggregates were formed. These aggregates were localized in the center of the agar surface, whereas in the vehicle control they were dispersed over the semisolid agar substrate. Quantitative image analysis of the aggregates revealed that in opioid-treated cultures significantly more aggregates exhibiting an area larger than 0.05 μm^2 were formed versus the vehicle control (Fig. 2*C*) and that significantly less aggregates were formed upon opioid treatment compared to the vehicle control (Fig. 2*D*), confirming our macroscopical observations. The changes in the pattern of slow aggregation were not observed when HEK FLAG-MOP cells were treated with the combination of DAMGO or morphine and the opioid receptor antagonist naloxone, indicating that DAMGO and morphine exert their effect via MOP (Fig. 2*E*).

Opioid-stimulated aggregation is not observed in suspension cultures on a Gyrotory shaker. An alternative assay to study cell aggregation is multicellular spheroid formation in suspension. HEK FLAG-MOP cells were cultured for 48 h in suspension on a Gyrotory shaker in the absence or presence of DAMGO or morphine. No differences were observed in the sizes of the multicellular spheroids of the vehicle control and DAMGO- or morphine-treated cultures, whereas HEK FLAG-MOP cells that were simultaneously tested in the SAA formed larger aggregates in the presence of the opioids (Fig. 3).

HEK FLAG-MOP aggregates are resistant to de-adhesion. Opioids may inhibit de-adhesion, resulting in larger aggregates as compared to the vehicle control. HEK FLAG-MOP aggregates were formed in the SAA in the absence or presence of DAMGO or morphine. After 48 h of incubation, aggregates were subjected to pipetting. Through application of shearing fluid forces, de-adhesion could be studied. Neither vehicle control nor opioid-treated aggregates dissociated (Fig. 4).

*Stimulation of cell growth cannot explain the larger aggregates.*To investigate whether the larger aggregates result from cell growth stimulation by opioids, a sulforhodamine B colorimetric assay was performed. HEK FLAG-MOP cells were cultured in 96-well plates in the absence or presence of DAMGO or morphine. After 1, 2, 3, and 6 d of treatment, cells were stained with sulforhodamine B to determine cellular protein content. In contrast to the growth inhibitor 5-FU, no opioid had an effect on cell growth that differed significantly from untreated cultures (Fig. 5*A*). Moreover, in the presence of the growth inhibitor 5-FU, DAMGO and morphine still stimulated aggregation of HEK FLAG-MOP cells in the SAA (Fig. 5*B*).

Cell–substrate adhesion as underlying mechanism for the changes in the pattern of slow aggregation on agar induced by opioids. Indications for a role of the substrate came from

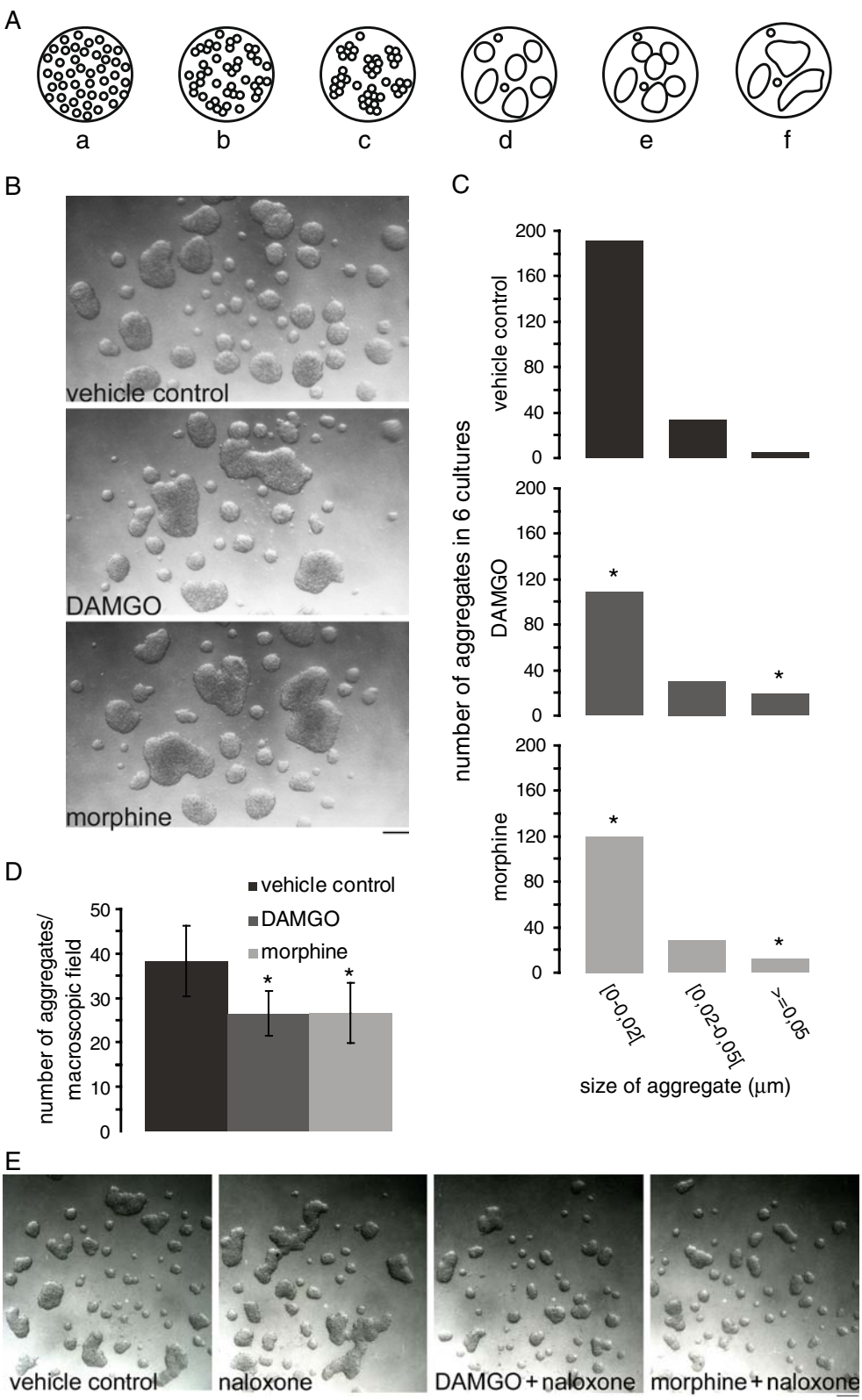

Figure 2. Opioids change the pattern of slow aggregation of HEK FLAG-MOP cells. (*A*) Schematic showing the multi-step aggregation process as occurring in the slow aggregation assay (SAA). Single cells (*a*) first cluster (*b*). These clusters then fuse (*c*) and compact to small aggregates (*d*). In the last step, aggregates can fuse (*e*) to form larger aggregates (*f*). (*B*) HEK FLAG-MOP cells were tested in the SAA in the absence (vehicle control) or presence of 1 μM DAMGO or 1 μM morphine. The cultures were photographed under a stereomicroscope after 48 h. *Scale bar* 200 μm. (*C*) Aggregates were classified according to their size, determined by measuring their area on photographs. *Bars* represent the sum of aggregates in six cultures exhibiting an area as indicated. *Asterisks*, significantly different from the vehicle control ($p<0.05$, chi-square test). (*D*) The number of aggregates was scored by counting the number of aggregates in a stereomicroscopic field. *Bars* represent mean from six cultures; *error bars* represent standard deviation. *Asterisks*, significantly different from the vehicle control ($p<0.05$, unpaired *t* test). (*E*) HEK FLAG-MOP cells were tested in the SAA in the absence or presence of 1 μM DAMGO or 1 μM morphine. To block MOP signaling, the medium was supplemented with the opioid receptor antagonist naloxone (10 μM). After 48 h, cultures were photographed under a stereomicroscope. *Scale bar* 200 μm.

the observations that the pattern of slow aggregation was changed in the same way as opioids do by increasing the agar concentration to coat the 96-well plates (Fig. 6*A*). Similarly, by mixing up agar with pure agarose, a natural component of agar, larger but fewer aggregates were formed in the center of the agar–agarose matrix with increasing agarose concentration (Fig. 6*B*). Slow aggregation on pure agarose substrate even resulted in the

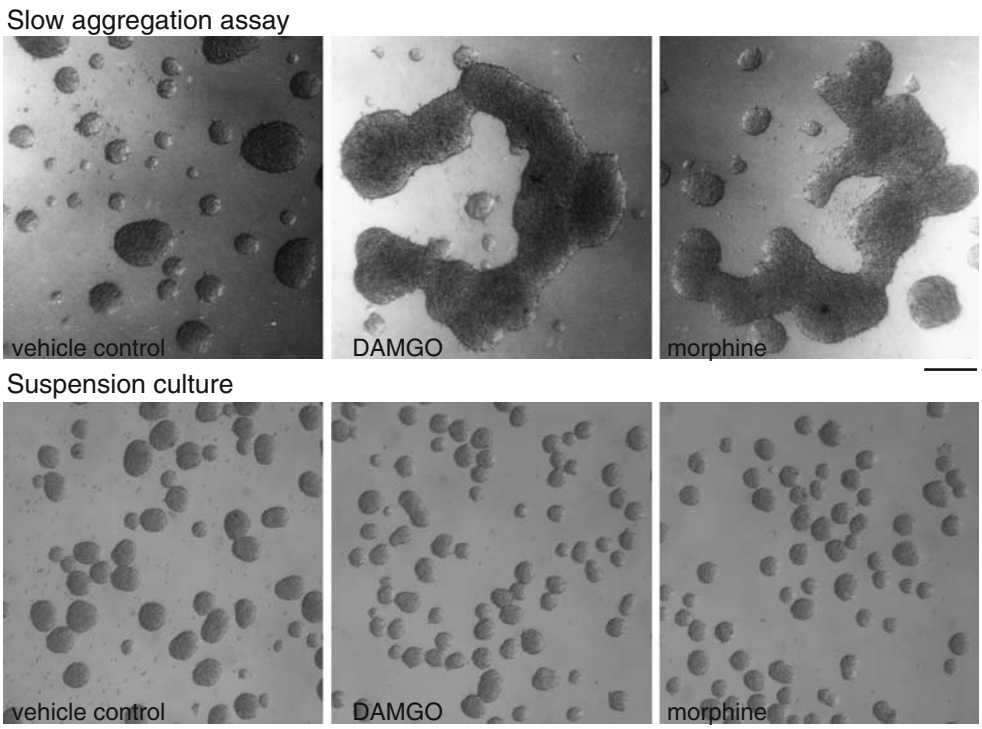

Figure 3. Opioids do not change the size of HEK FLAG-MOP multicellular spheroids formed in suspension culture on a Gyrotory shaker. HEK FLAG-MOP cells were simultaneously tested in the slow aggregation assay and in suspension culture. For aggregation in suspension culture, HEK FLAG-MOP cells were kept on a Gyrotory shaker. The cells were tested in the absence (vehicle control) or presence of 1 μM DAMGO or 1 μM morphine. After 48 h of incubation, the cultures were photographed. *Scale bars* 200 μm.

formation of a single large aggregate in the center of the substrate. Moreover, on these modified coatings, the effect of opioids was enhanced (Fig. 6*B*).

No changes in the pattern of slow aggregation were observed between the vehicle control and opioid-treated cultures when 24-well plates or 6-well plates were used to perform the SAA (data not shown). In contrast to 96-well plates, the agar surface in 24-well plates and 6-well plates is homogenously flat. So, in addition to its composition, the slope created by the meniscus of the agar substrate is critical for observing the opioid-induced changes in the slow aggregation pattern.

Discussion

In the slow aggregation assay, single cells are seeded on agar and spontaneously aggregate. This assay has generally been used to study the functionality of cell–cell adhesion complexes. However, an aggregate is the net result of input of cells by cell–cell adhesion and cell growth and output of cells by de-adhesion. Therefore, drugs that change the pattern of slow aggregation on agar can interfere with cell–cell adhesion as well as with cell growth and de-adhesion. Cell–cell adhesion depends on affinity and avidity. Affinity is defined here as the strength of adhesion and depends on

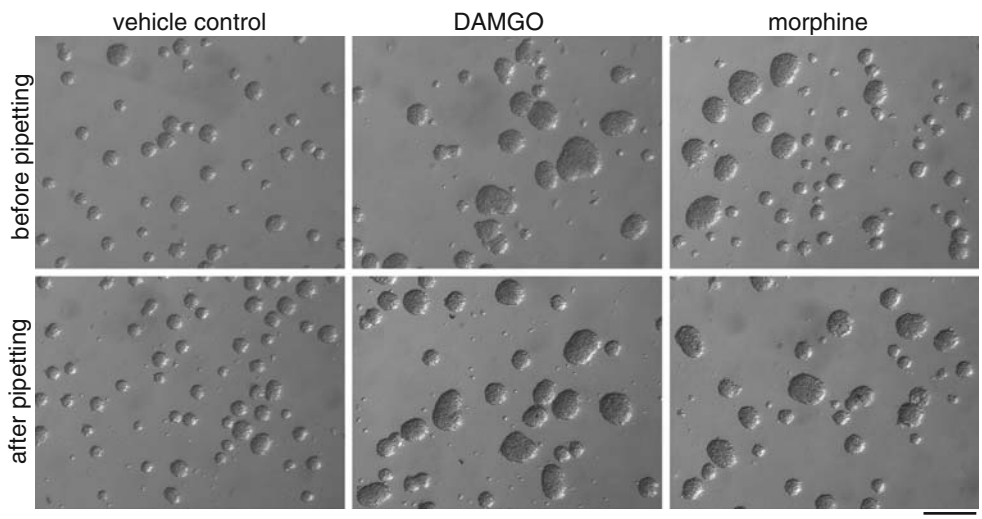

Figure 4. HEK FLAG-MOP aggregates are resistant to de-adhesion. HEK FLAG-MOP aggregates were formed in the SAA in the absence (vehicle control) or presence of 1 μM DAMGO or 1 μM morphine. After 48 h of incubation, aggregates were passed 30 times through Pasteur pipettes. Aggregates were evaluated under a stereomicroscope before and after pipetting. *Scale bar* 200 μm.

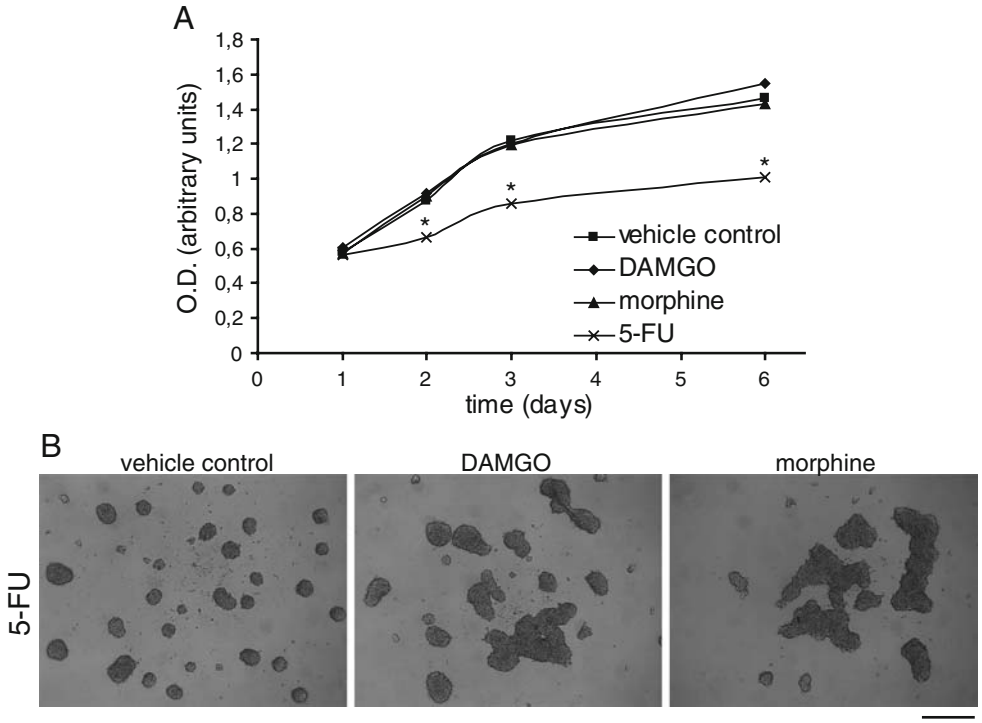

Figure 5. Opioids do not affect HEK FLAG-MOP cell growth. (*A*) HEK FLAG-MOP cells were seeded in 96-well plates. Next day, cells were left untreated (vehicle control) or were treated with 1 μM DAMGO or 1 μM morphine. 5-Fluorouracil (*5-FU*, 1 μg/ml) was used as a growth inhibitor. Cells were stained with sulforhodamine B after 1, 2, 3, and 6 d of treatment. Cellular protein content was measured as optical density (*OD*) at 490 nm. The graph shows mean OD±standard deviation of six cultures. *Asterisks*, significantly different from untreated cultures ($p<0.05$, unpaired *t* test). (*B*) A slow aggregation assay was performed with HEK FLAG-MOP cells in the absence (vehicle control) or presence of DAMGO (1 μM) or morphine (1 μM). To inhibit growth, 5-fluorouracil (1 μg/ml) was added to the medium. Cultures were photographed under a phase-contrast microscope after 48 h of incubation. *Scale bar* 200 μm.

the type of homotypical cell–cell adhesion complexes formed and their functionality. Avidity is considered here as the probability that cells adhere to each other. Both cell motility and the distance between cells can influence avidity. By increasing cell motility, contacts between cells are promoted and this may result in increased cell–cell adhesion. Cell motility can be active through migration over the substrate or passive, for instance by shaking the cultures. Cell–cell adhesion may also increase by decreasing the distance between cells. This can be achieved by seeding more cells per surface unit or by decreasing the surface that cells occupy.

In the slow aggregation assay, we observed that larger HEK FLAG-MOP aggregates were formed in the presence of DAMGO and morphine than in vehicle control cultures. In a dissociation experiment, HEK FLAG-MOP aggregates were resistant to pipetting, excluding the possibility that opioids interfere with de-adhesion. We could demonstrate that opioids neither modulate cell growth. In suspension culture on a Gyrotory shaker, HEK FLAG-MOP cells did not form larger multicellular spheroids in the presence of DAMGO and morphine, demonstrating that opioids do not affect affinity. We therefore suggest that opioids affect avidity. Moreover, in opioid-treated cultures, aggregates were observed in the center of the agar substrate, whereas in the vehicle control aggregates were dispersed over the surface. These observations show that in opioid-treated cultures the cells are occupying a smaller surface than in the vehicle control, which may stimulate cell–cell adhesion and therefore result in the formation of larger aggregates. This hypothesis is encouraged by the observation that the slope, created by the meniscus of the agar substrate, is critical for observing the opioid-stimulated aggregation. Opioid-treated cells may move down the slope towards the center of the substrate.

We further have evidence to accept that opioids interfere with cell–substrate adhesion since manipulations of the substrate induced the same changes in the pattern of slow aggregation as opioids. We suggest that opioids decrease cell–substrate adhesion, which may explain why opioid-treated cells move down the slope in contrast to vehicle controls. In the slow aggregation assay, all cells tend to move down the slope by gravity, but cell movement is normally resisted by cell–substrate adhesion. Decreased cell–substrate adhesion by opioids, however, may facilitate the cells to move down the slope. This increased cell motility may promote contacts between cells and decrease the surface occupied by the cells. Both may result in increased cell–cell adhesion and the formation of larger aggregates.

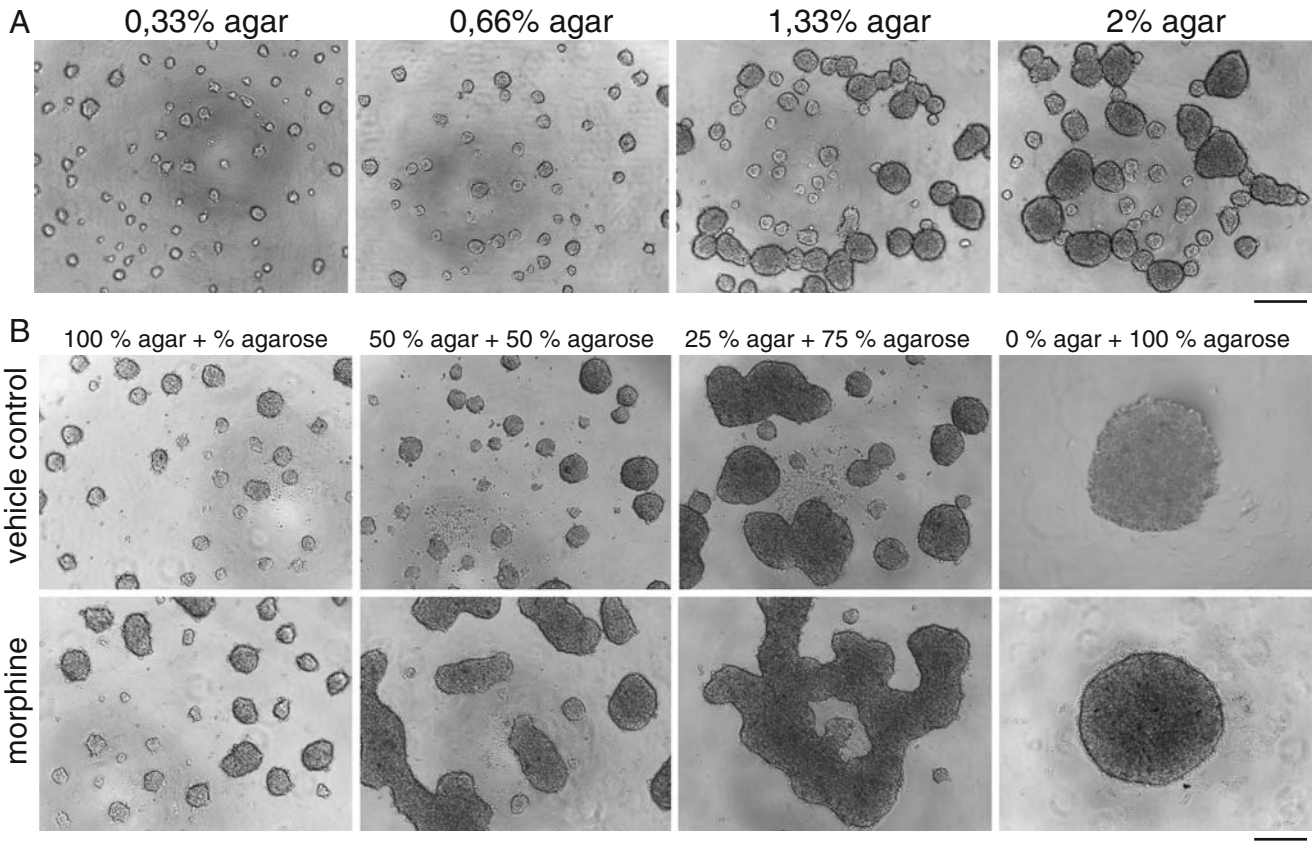

Figure 6. Manipulations of the substrate, like opioids, change the pattern of slow aggregation of HEK FLAG-MOP cells. (*A*) Different concentrations of agar (*w*/*v* percent) were used to coat the wells of a 96-well plate. A slow aggregation assay was performed on these coatings with HEK FLAG-MOP cells in the absence of opioids. Pictures of the cultures were taken after 48 h under a phase-contrast microscope. *Scale bar* 200 μm. (*B*) The composition of the agar was changed by mixing agar with pure agarose. A slow aggregation assay was performed with HEK FLAG-MOP cells in the absence (vehicle control) or presence of morphine (1 μM). Cultures were photographed after 48 h under a phase-contrast microscope. *Scale bar* 200 μm.

So far, we could not reveal which molecules are involved in the cell–substrate adhesion complex formed between HEK FLAG-MOP cells and the semisolid agar substrate. Since agar is a polygalactose, galactose-binding lectins might directly be involved. However, on pure agarose, one of the natural components of agar, one large aggregate is formed in the center of the substrate, suggesting that HEK FLAG-MOP cells do not adhere to agarose. Apart from agarose, agar contains agaropectin. In contrast to agarose, agaropectin is heavily modified with acidic side groups, such as sulfate and pyruvate, making agaropectin likely to interact with proteins (Lahaye and Rochas 1991).

Alternatively, cell–substrate adhesion may be the result of coating of the semisolid agar substrate with serum components such as fibronectin from the culture medium.

The slow aggregation assay has generally been used to study affinity by testing the functionality of cell–cell adhesion complexes. Although the same changes in the pattern of slow aggregation on agar could be observed when MCF-7/6 cells were treated with the phytoestrogen 8-prenylnaringenin (8-PN) as we observed with DAMGO and morphine in HEK FLAG-MOP cells, Rong et al. (2001) could demonstrate that 8-PN-stimulated aggregation was due to increased affinity. In contrast to our results, they indeed observed that multicellular spheroids formed in Gyrotory shakers were larger in the presence of 8-PN. Boterberg et al. (2000) could demonstrate internalization of the E-cadherin/catenin complex and, thus, decreased affinity, as the underlying mechanism for the appearance of smaller MCF-7/AZ aggregates after treatment with conditioned medium from human skin squamous carcinoma cells COLO 16. Van Marck et al. (2005) showed that aggregation of BLM cells after P-cadherin transduction was the result of increased affinity.

We suggest that the SAA might also be used to evaluate cell aggregation as a result of altered cell–substrate adhesion. How altered cell–substrate adhesion contribute to changes in cell–cell adhesion is still unclear. Canonici et al. (2007) demonstrated re-localization of α_v integrin from cell–cell contacts to focal contact sites in HT29-D4 cells on

IGF-I stimulation, showing a possible mechanism for linking cell–substrate adhesion to cell–cell adhesion. Interestingly, IGF-I was also shown to inhibit spontaneous aggregation of HT29-D4 cells in the slow aggregation assay.

Cell aggregation can be studied using other aggregation assays than the slow aggregation assay on agar. For suspension cultures, a single-cell suspension is transferred to an Erlenmeyer that is incubated on a Gyrotory shaker. Cell aggregation is evaluated after 24 h or more; hence, this assay is also termed slow. In the fast aggregation assay, as described by Boterberg et al. (2001), cells are allowed to aggregate on BSA-coated plates that are placed on a Gyrotory shaker for 30 min. Because of the short incubation period, potential interference with cell growth is excluded and it allows us to test drugs that may be cytotoxic on the long run. This assay is also recommended to evaluate the initial steps of cell aggregation. In order not to alter cell–cell adhesion characteristics during the assay, a smooth detachment procedure must be applied here, with the risk not to obtain a single-cell suspension. In the slow aggregation assays, more aggressive detachment procedures, yielding a perfect single-cell suspension, can be used because the longer incubation times allow the turnover of cell–cell adhesion molecules. Unlike the slow aggregation assay on agar, these alternative aggregation assays will not reveal avidity changes because avidity is determined here by the shaking of the cultures. Moreover, because of the shaking, cell–substrate adhesion is unlikely to interfere and hence the effects of opioids were not observed in suspension cultures on a Gyrotory shaker.

Conclusions

We suggest that other factors than functionality of cell–cell adhesion complexes may be responsible for changes in the pattern of slow aggregation on agar. We used HEK FLAG-MOP cells that stably overexpress the mu opioid receptor and the MOP-selective agonists DAMGO and morphine to evaluate cell aggregation in the slow aggregation assay. Larger and fewer HEK FLAG-MOP aggregates were formed in the presence of DAMGO and morphine than in the vehicle control. Moreover, these aggregates were localized in the center of the semisolid agar substrate, whereas in the vehicle control they were dispersed over the surface. The larger aggregates did not result from enhanced affinity by opioids; neither did opioids inhibit de-adhesion. We demonstrated that the formation of larger aggregates was not due to cell growth stimulation by opioids. Our experiments provided evidence to accept that opioids affect cell–substrate adhesion. We suggest that opioids decrease cell–substrate adhesion, which may result in more cell movement. Increased cell motility may in turn promote contacts between cells and decrease the distance between cells, which might increase cell–cell adhesion and result in the formation of larger aggregates.

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