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Integrin Stimulation Favors Uptake of Macro--molecules by Cardiomyocytes *In Vitro*

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Key Words

Cardiomyocytes • Integrins • Dysferlin • Membrane • Wounding • Patch repair • RGD

Abstract

Previously, our research group showed that integrin stimulation induces release of cardiac troponin I from viable neonatal rat ventricular cardiomyocytes (NRCMs), but would it also stimulate uptake of exogenous macromolecules? For this purpose, beating NRCMs were incubated without or with an RGD motif-containing peptide (GRGDS) to stimulate integrins in the presence of Texas Red-conjugated ovalbumin (OTR; 45 kDa) or dextran (DTR; 70 kDa). After incubation periods of 8, 16 and 24 hours endocytosis of red label was quantified by fluorescence microscopy. Uptake of OTR and DTR by NRCMs was intensified by GRGDS treatment (p for trend <0.001 and 0.019, respectively) and increased with duration of incubation (p<0.001 for both). The GRGDS-induced uptake of OTR by NRCMs correlated positively with OTR concentration (p<0.001). Experiments with pharmacological inhibitors of endocytosis indicated that in the absence of GRGDS, NRCMs take up OTR by the clathrin-mediated pathway of endocytosis while the GRGDS-dependent OTR uptake occurs by macropinocytosis. Cultures of NRCMs that were stretched cyclically showed ≈4-fold increased uptake of OTR compared to stationary NRCM cultures. Immunofluorescence microscopy revealed that the dysferlin-positive plasma membrane (PM) areas in beating GRGDS-treated NRCMs were ≈3-fold larger than in contracting NRCMs incubated with vehicle (p<0.001). However, in non-beating NRCMs exposure to GRGDS did not induce larger dysferlin-positive PM areas, nor did it stimulate uptake of OTR. After inhibition of dysferlin expression by short hairpin RNA-mediated RNA interference, OTR uptake by contracting NRCMs could no longer be stimulated via GRGDS treatment. We conclude that in NRCMs, stimulation of integrins by RGD motif-containing peptides or stretch cause uptake of labeled macromolecules. The latter process appears to depend on the contractile behavior of the NRCMs and on the PM repair protein dysferlin, probably because of its role in macropinocytosis.

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Introduction

The plasma membrane (PM) is a biological barrier between the extracellular and intracellular environment and is essential to the maintenance of cell integrity. Damage to this barrier leads to cell dysfunction or death. PM disruptions, however, commonly occur under physiological conditions in a wide variety of mechanically active mammalian tissues. Especially contractile cells such as smooth muscle cells [1], skeletal muscle cells [1] and cardiomyocytes endure frequent PM damage [2]. These cells hence possess an active repair mechanism for PM resealing. Membrane repair is mediated by rapid Ca^{2+}-triggered exocytosis of various intracellular vesicles, such as lysosomes and enlargeosomes, which create a membrane patch that reseals the PM [3, 4]. Recent findings suggest a crucial role for dysferlin in this repair process, possibly as a Ca^{2+} sensor that triggers vesicle fusion [5, 6].

Mechanical stretch causes integrin stimulation of cardiomyocytes and plays an important role in the development of myocardial hypertrophy [7, 8]. The mechanical forces exerted on cells are sensed by integrins, mechanotransducer proteins linking the extracellular matrix (ECM) to the intracellular cytoskeleton [9]. The RGD motif present in fibronectin and in several other ECM proteins is a ligand of integrins [10]. Oligopeptides containing this motif can be used to simulate myocardial mechanical stretch *in vitro*.

Previously, our research group demonstrated that integrin stimulation of neonatal rat ventricular cardiomyocytes (NRCMs) using an RGD motif-containing pentapeptide (i.e. NH2-Gly-Arg-Gly-Asp-Ser-COOH, abbreviated by GRGDS) resulted in (i) a rise in the intracellular concentrations of NO and Ca^{2+} [11], (ii) cardiomyocyte hypertrophy [12] and (iii) release of cTnI from viable NRCMs [13]. Moreover, Kaye et al. [14] have shown that release of basic fibroblast growth factor (bFGF) from electrically stimulated ventricular cardiomyocytes of adult rats was accompanied by uptake of 10-kilodalton (kDa) fluorescein-labeled dextran molecules from the culture medium.

In the present study, NRCM cultures were used to show that also integrin stimulation promotes uptake of extracellular macromolecules (i.e. Texas Red-conjugated ovalbumin [OTR] and Texas Red-labeled dextran [DTR]) by cardiomyocytes. In addition, we investigated whether this uptake is dependent on (i) the extracellular concentrations of the RGD motif-containing pentapeptide, (ii) the extracellular concentrations of the macromolecule used and (iii) the duration of the incubation with the macromolecules. In a separate series of experiments, NRCMs were subjected to cyclic stretch to test its impact on the uptake of OTR. The effect of cardiomyocyte contraction on the endocytosis of macromolecules was studied using NRCM cultures treated with blebbistatin, a cell-permeable inhibitor of class II myosins. To investigate the possible involvement of dysferlin in integrin stimulation-induced uptake of macromolecules by NRCMs, the size of PM-associated dysferlin-enriched patches was determined under various conditions. A lentivirus vector directing the synthesis of dysferlin-specific short hairpin RNA (shRNA) molecules was used to more directly study the role of dysferlin in the endocytosis of macromolecules. Finally, several different small-molecule inhibitors of endocytosis were applied to obtain information about the specific pathway(s) involved in the endocytosis of OTR by GRGDS-treated NRCMs and by their unstimulated counterparts [15].

Materials and Methods

Cell culture

Primary cultures of NRCMs were prepared from the ventricles of 2-day-old Wistar rats as described previously [16]. Briefly, ventricles were minced and cells were isolated enzymatically using collagenase type I (CLS, Worthington, Lakewood, NJ, USA) at 37°C. The cell suspension was centrifuged and the cell pellet was resuspended in Ham's F10 medium containing 10% fetal bovine serum (FBS), 10% horse serum (HS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Breda, the Netherlands). The cells were seeded in 6-cm-∅ Primaria culture dishes (Becton Dickinson, Etten-Leur, the Netherlands), and after 45 min non-adherent cells representing the cardiomyocytes were collected and plated onto rat-tail collagen type I (Sigma-Aldrich, Zwijndrecht, the Netherlands)-coated glass coverslips (∅ 25 mm) in wells (∅ 35 mm) of six-well plates (Costar, Corning Life Sciences, Amsterdam, the Netherlands) at a density of 6×10^5 cells per well. The NRCMs were incubated in a humidified incubator at 37°C and 5% CO2 with so-called growth medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and Ham's F10 medium containing 5% HS, penicillin (100 U/mL) and streptomycin (100 µg/mL) for 24 h. The next 24 h the cultures were incubated in the same medium supplemented with 5-bromo-2-deoxyuridine (BrdU, 100 µmol/L; Sigma-Aldrich) to inhibit proliferation of cardiac fibroblasts. Subsequently, the cells were maintained in growth medium without BrdU for 24 h. This typically gave rise to cultures of spontaneously beating cardiomyocytes with a confluency of 60-70%, which were used for the experiments described below. The investigations had the approval of the Animal Experiments Committee of the LUMC, according to Dutch law.

Macromolecule uptake experiments

Three days after cell isolation, the growth medium was replaced by a 1:1 mixture of DMEM and Ham's F10 medium containing 2.5% HS, penicillin (100 U/mL) and streptomycin (100 µg/mL). The incubation experiments were started by adding OTR (45 kDa; Invitrogen) or DTR (70 kDa; Invitrogen) in the concentration range 0.01 - 0.5 µmol/L. These incubations were done in the absence or presence of various concentrations (100, 200 or 300 µg/mL) of the RGD motif-containing pentapeptide (GRGDS; Sigma-Aldrich) to stimulate integrins [10]. When indicated, the cell-permeable myosin class II inhibitor blebbistatin (Sigma-Aldrich) was added to the NRCM cultures at a final concentration of 10 µmol/L to stop cellular beating [17]. Incubations lasted 8, 16 or 24 h. The reasons to select the combination of OTR and DTR for the macromolecule uptake experiments were twofold. Firstly, by selection of two macromolecules with different molecular weights the influence of cargo size on uptake efficiency could be investigated. Secondly, by studying the uptake of a Texas Red-labeled polysaccharide (i.e. the glucan dextran) as well as a Texas Red-conjugated polypeptide (i.e. the protein ovalbumin) possible differences in the uptake of different types of macromolecules could be uncovered, while the use of the same label, i.e. Texas Red, allows quantification of uptake with equal sensitivity.

Cell stretch

For experiments with stretched NRCMs, these cells were cultured in stretch plates with flexible collagen type I-coated surfaces (BioFlex culture plates BF-3001C, FlexCell International Corporation, Dunn Labortechnik, Asbach, Germany) and placed in a stretch device (FX-4000T Flexcell Tension Plus System, FlexCell International Corporation) leading to biaxial stretch of all cells attached to the flexible bottom. Stretch was applied cyclically at 0.5 Hz for 24 h in a humidified incubator with 5% CO2 at 37°C [18].

Quantification of TexasRed-labeled macromolecules in NRCMs

At the end of the incubation period with labeled macromolecules, the medium in the NRCM cultures was removed and the cells were washed twice with phosphate-buffered saline (PBS) containing 1% FBS. The NRCMs were then fixed in 4% buffered formaldehyde (Klinipath, Duiven, the Netherlands) for 30 min, and washed twice with PBS + 1% FBS. Next, the coverslips with cells were mounted on microscope slides using Vectashield (Vector Laboratories, Burlingame, CA, USA). Cells cultured in stretch plates were processed in the same manner except that after the final wash step, they were overlaid with a drop of Vectashield and with a glass coverslip. Following uptake of TexasRed-labeled macromolecules, photographs of the NRCMs were taken using a fluorescence microscope (Nikon Eclipse, Nikon Europe, Badhoevedorp, the Netherlands) equipped with a 100× oil-immersion objective and a digital camera (Nikon DXM 1200). Per cell culture, 10-50 fields were photographed. Images were analyzed quantitatively using the color intensity measurement routine of the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) on all photographed cells available.

Immunocytology

NRCM cultures were rinsed three times with ice-cold PBS + 1% FBS, fixed in 4% buffered formaldehyde for 30 min, washed twice with ice-cold PBS + 1% FBS, incubated with ice-cold PBS + 0.1% Triton X-100 (Sigma-Aldrich) for 15 min, washed twice with ice-cold PBS + 1% FBS, and incubated overnight with primary antibody (100× diluted in PBS) at 4°C. After washing twice with PBS + 1% FBS, the cells were incubated with secondary antibody (200× diluted) for 60 min. Following two wash steps with PBS + 1% FBS, the cultures were incubated with 10 µg/mL Hoechst 33342 (Invitrogen) in PBS + 1% FBS for 8 min. After washing twice with PBS + 1% FBS, the coverslips with cells were mounted on microscope slides using Vectashield. To detect sarcomeric α-actinin, we used a mouse monoclonal antibody (clone: EA53; A7811; Sigma-Aldrich). Goat polyclonal antibodies raised against a peptide mapping within an internal region of human dysferlin were purchased from Santa Cruz (sc-16635; Santa Cruz, CA, USA). Fluorescein isothiocyanate-conjugated donkey anti-goat IgG was from Santa Cruz (sc-2024) and Alexa568-coupled rabbit anti-mouse IgG was from Invitrogen (A11061).

Since dysferlin is a type II transmembrane protein with a very short cytoplasmic tail [19], the epitope recognized by the dysferlin-specific goat polyclonal antibodies should only be available for antibody binding in cells with a disrupted PM.

The NRCMs in the stretch plates were fixed and stained using the same methods. However, as the cells in the stretch plates were not on coverslips instead of mounting them on a microscope slide, they were overlaid with a drop of Vectashield and with a glass coverslip.

Images were produced using the same fluorescence microscope, camera and off-line image analysis software as mentioned above, but now using the area measurement routine. The photographs of the dysferlin-stained NRCMs were acquired with a short exposure time to selectively record the signals associated with dysferlin-positive PM areas, the so-called patches.

Endocytosis

Chlorpromazine (Sigma-Aldrich) was used to inhibit clathrin-mediated endocytosis, as has been demonstrated in a rat alveolar type II epithelial cell line [20]. Filipin (Sigma-Aldrich) was used to inhibit caveolin-dependent endocytosis, a mechanism shown to be involved in the uptake of 155-kDa and 500-kDa dextrans by cultured primary bovine aortic endothelial cells [21]. Wortmannin (Sigma-Aldrich) was used to inhibit macropinocytosis, a Ca^{2+}-dependent uptake mechanism of 70-kDa dextran and small (∅ 20 nm) latex beads [22]. To determine the effects of the inhibitors on RGD-independent and RGD-dependent uptake of OTR, we always applied, per dose of inhibitor, two NRCM cultures with the particular dose of inhibitor, one without GRGDS and one with GRGDS. After 24 h of incubation the OTR fluorescence of the two cultures was x and (x+y), respectively. Two other cultures of NRCMs were incubated without inhibitor, one without GRGDS and one with GRGDS. After 24 h of incubation the OTR fluorescence of the two cultures was X and (X+Y), respectively. The average OTR fluorescence of the control culture without GRGDS (=X) was

set equal to 100%. The inhibiting effect of the dose of inhibitor on RGD-independent OTR fluorescence is equal to (X-x). The inhibiting effect of the dose of inhibitor on RGD-dependent OTR fluorescence is equal to (Y-y).

Downregulation of dysferlin expression

Lentiviral vector shuttle constructs encoding shRNAs targeting the human (TRCN0000000965) and mouse (TRCN0000111756) dysferlin genes were obtained from the Mission Library (Sigma-Aldrich). Both these shRNAs (which will be subsequently designated shRNA965 and shRNA756) contained a sense strand that perfectly matched with the coding sequence of the rat dysferlin gene and efficiently inhibited its expression as assessed by western blotting. As negative control, a lentiviral vector shuttle plasmid directing the synthesis of an enhanced green fluorescent protein (eGFP) gene-specific shRNA (SCH005, Sigma-Aldrich) was used. To allow for the easy assessment of transduction efficiencies, the puromycin N-acetyl transferase-coding sequence in these constructs was replaced by that of the green fluorescent protein of Renilla reniformis (hrGFPI). To this end, plasmid pLV.hPGK.hrGFPI (nucleotide sequence available upon request) was digested with the restriction endonucleases SpeI and KpnI and the resulting 1.1-kilobase (kb) DNA fragment containing the hrGFPI-coding sequence was combined with the 6.2-kb SpeI×KpnI fragment of each of the lentiviral vector shuttle constructs from the Mission library using T4 DNA ligase. Ligation mixtures were introduced in chemocompetent GeneHogs cells (Invitrogen) and large stocks of the correct plasmids were prepared with the aid of the JETSTAR 2.0. Plasmid Maxiprep Kit (Genomed, Löhne, Germany).

Vesicular stomatitis virus

G protein-pseudotyped self-inactivating human immunodeficiency virus type I vectors (SIN-LVs) were produced in 293T cells as described previously [23]. The gene transfer activity of the vector stocks was determined by end-point titration on HeLa indicator cells using flow cytometric analysis of hrGFPI expression as read-out. The titers of the SIN-LV preparations are thus expressed in HeLa cell-transducing units (HTUs) per ml.

NRCMs were transduced with SIN-LV particles at a multiplicity of infection (MOI) of 10 HTUs per cell. The next day, the cultures were washed three times with PBS and provided with fresh growth medium. At 7 days post-transduction, the cells were used for OTR uptake experiments.

Statistics

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed by Student´s t -test and by one-way analysis of variance (ANOVA) followed by Bonferroni´s *post hoc* test. Non-parametric tests used were the Kruskal-Wallis test to quantify effects of trend, and Spearman's r_s to quantify effects of correlation. Differences were regarded as statistically significant if $p<0.05$. SPSS16 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

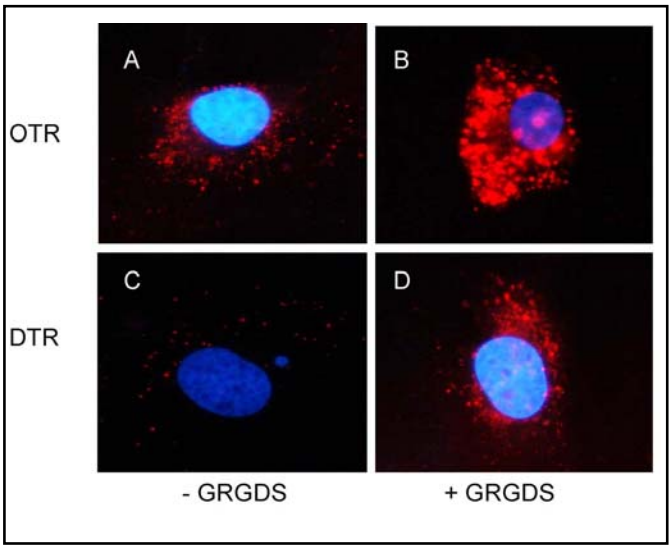

Fig. 1. Uptake of OTR (0.1 µmol/L) (A, B) or DTR (0.3 µmol/L) (C, D) by NRCMs incubated with these TexasRed-labeled macromolecules for 24 h in the absence (A, C) or presence (B, D) of 300 µg/mL of GRGDS.

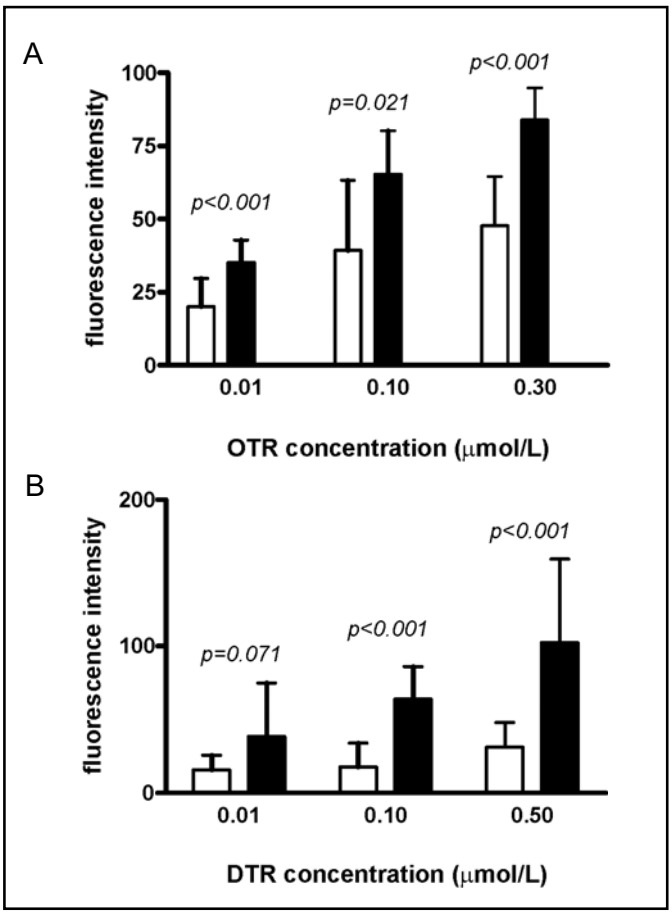

Fig. 2. Uptake of OTR (A) or DTR (B) by NRCMs incubated with different concentrations of these TexasRed-labeled macromolecules for 24 h in the absence (white) or presence (black) of 300 µg/mL of GRGDS. Number of data per bar: 8-10 for panel A en 10 for panel B. Indicated are mean values and SD.

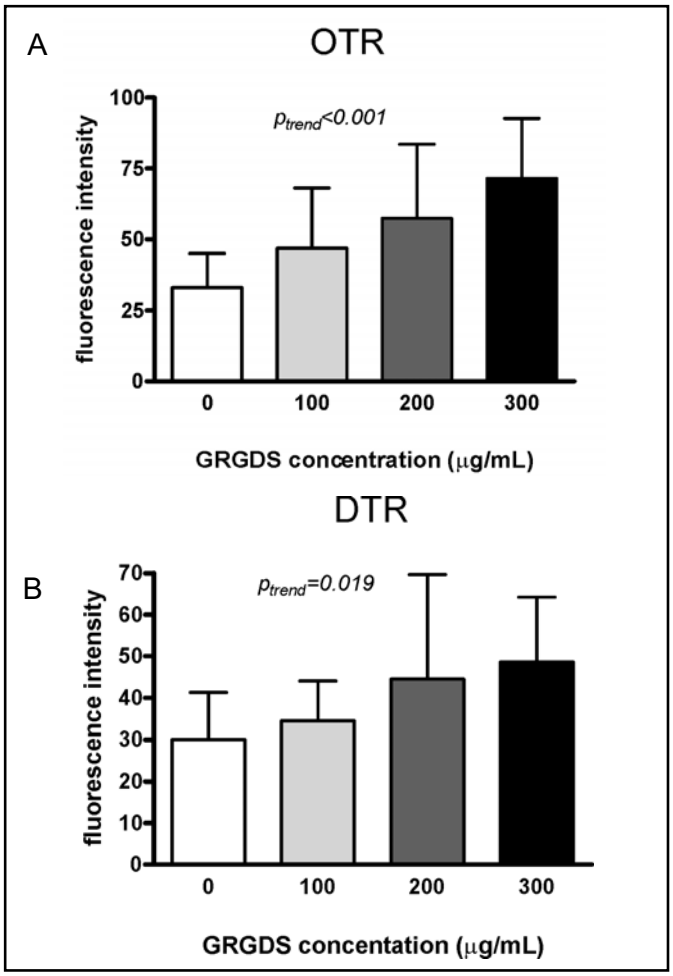

Fig. 3. Uptake of OTR (0.3 μmol/L) (A) or DTR (0.3 μmol/L) (B) by NRCMs incubated with these TexasRed-labeled macromolecules for 24 h in the absence of GRGDS (left bars in both panels) and in the presence of 100, 200 or 300 μg/mL of the RGD motif-containing pentapeptide. Number of data per bar: 24, 8, 8 and 8 moving from left to right in both panels. Indicated are mean values and SD.

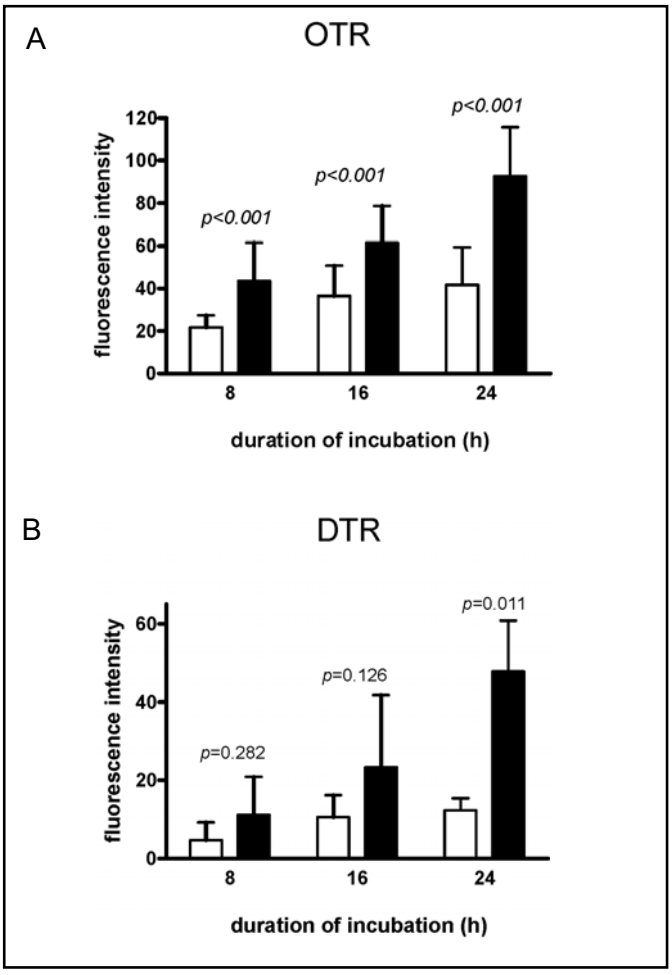

Fig. 4. Uptake of OTR (0.3 μmol/L) (A) or DTR (0.3 μmol/L) (B) by NRCMs incubated with these TexasRed-labeled macromolecules for 8, 16 or 24 h in the absence (white) or presence (black) of 300 μg/mL of GRGDS. Number of data per bar: 20 for panel A and 10 for panel B. Indicated are mean values and SD.

Results

Uptake of macromolecules: effect of macromolecule concentration

The uptake of both OTR and DTR by beating NRCMs was favored by integrin stimulation using the RGD motif-containing pentapeptide GRGDS (Fig. 1). The quantity of labeled macromolecules taken up in 24 h was dependent on their extracellular concentrations with, for OTR, a ptrend <0.001 and =0.006 in the presence and absence of GRGDS, respectively, and for DTR a ptrend =0.007 and =0.052 in the presence and absence of GRGDS, respectively. The positive effect of GRGDS treatment on OTR uptake was significant at all three protein doses tested (Fig. 2A). The effect of integrin stimulation on uptake of DTR only reached significance when the glucan was present at concentrations of 0.1 and 0.5 μmol/L (Fig. 2B).

Uptake of macromolecules: effect of GRGDS concentration

The effect of GRGDS on uptake of TexasRed-labeled macromolecules by beating NRCMs was strongly dependent on its concentration, being evident for OTR as well as for DTR (Fig. 3A and B). Uptake of OTR (0.3 μmol/L) after 24 h was 1.43-, 1.75- and 2.18-fold higher in the presence of 100, 200 and 300 μg/mL of the RGD motif-containing pentapeptide than in its absence (ptrend

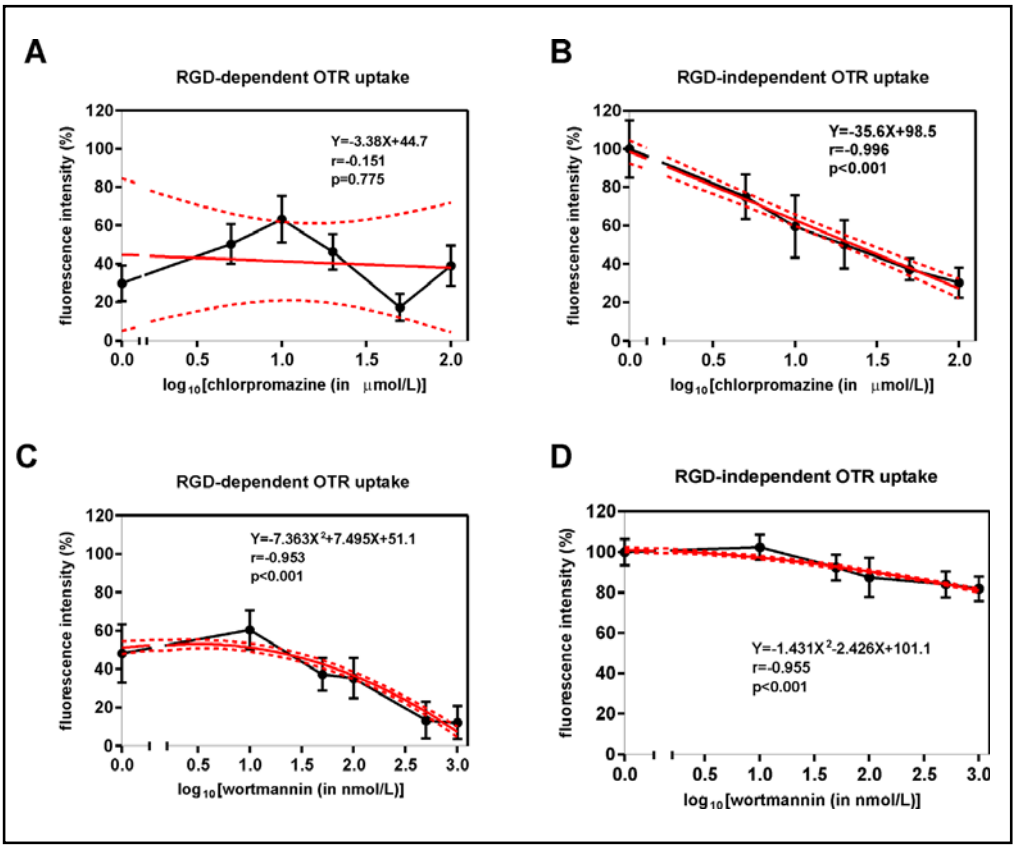

Fig. 5. RGD-dependent (A, C) and RGD-independent (B, D) uptake of OTR (0.3 µmol/L) by NRCMs incubated for 24 h with different concentrations of chlorpromazine (A, B) or wortmannin (C, D). Chlorpromazine, an inhibitor of clathrin-mediated endocytosis, inhibited RGD-independent OTR uptake, whereas wortmannin, an inhibitor of macropinocytosis, suppressed RGD-dependent OTR internalization. Indicated are regression lines or curves plus 95% confidence limits; data points represent mean values ± SEM. Number of data per point: 7-12 for panels A and B, and 8-14 for panels C and D. OTR-associated fluorescence intensities are normalized to the value determined in NRCMs incubated for 24 h without GRGDS and without inhibitors.

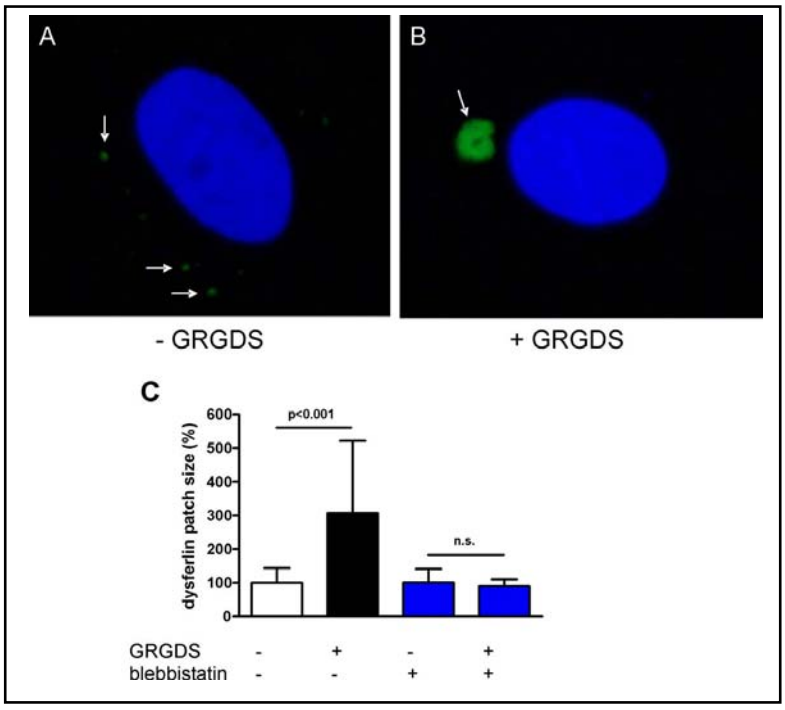

Fig. 6. Size of dysferlin-positive PM patches in NRCMs after incubation for 24 h without (A) or with (B) 300 µg/mL of GRGDS. (C) Size of dysferlin-positive PM patches after 24 h of incubation without or with 300 µg/mL of the RGD motif-containing pentapeptide, in the absence or presence of 10 µmol/L of the myosin class II inhibitor blebbistatin. Dysferlin patch sizes are normalized to the value determined in NRCMs incubated for 24 h without GRGDS and without blebbistatin. Number of data per bar: 100, 105, 7 and 7, moving from left to right. Indicated are mean values and SD.

<0.001). For DTR (0.3 µmol/L) the uptake by NRCMs was 1.15-, 1.47- and 1.63-fold higher in the presence of 100, 200 and 300 µg/mL of GRGDS than in its absence (ptrend <0.02).

Uptake of macromolecules: effect of incubation period

The quantity of TexasRed-labeled macromolecules taken up by beating NRCMs in the absence and pres-

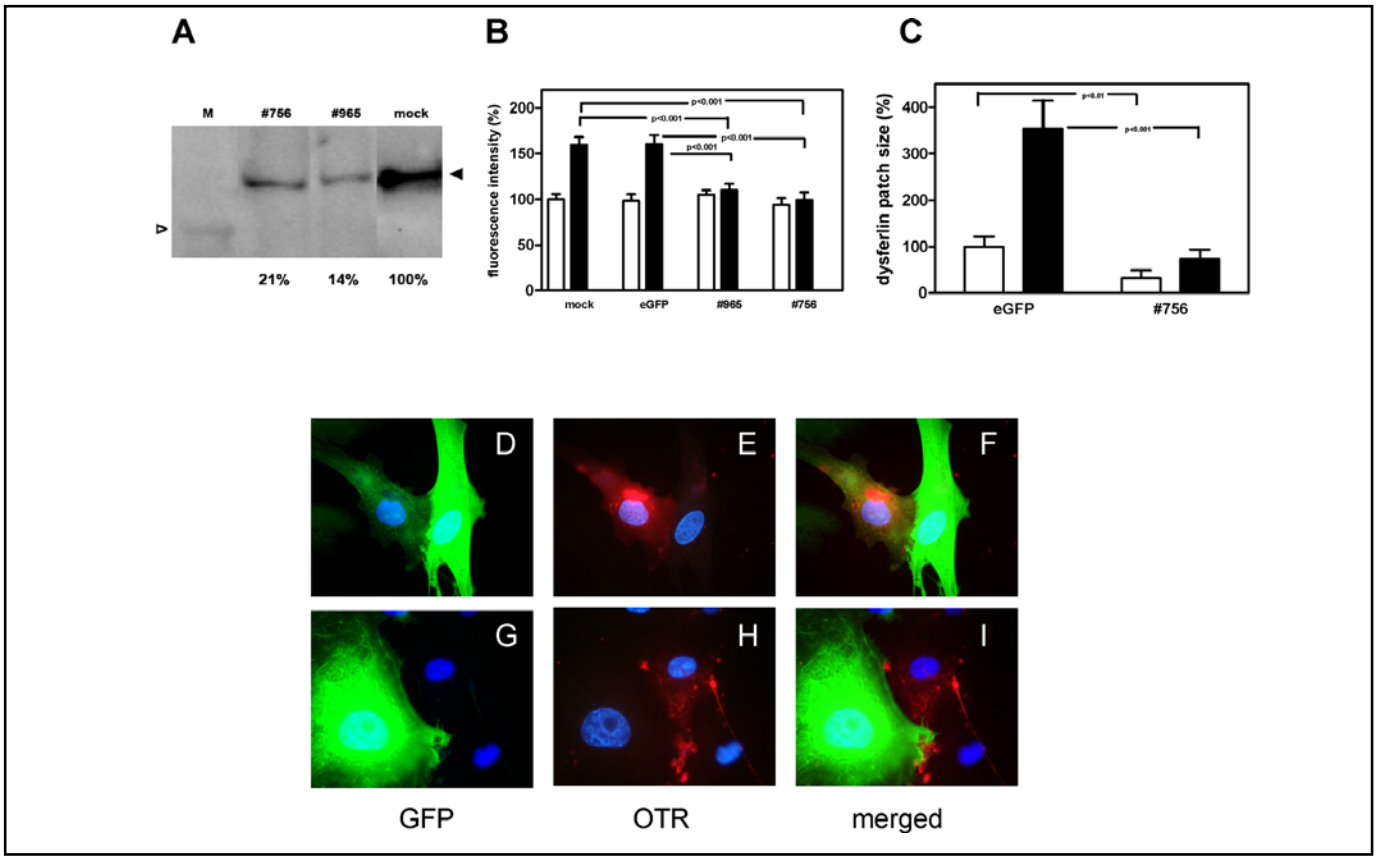

Fig. 7. Role of dysferlin in the uptake of OTR by NRCMs. Before the start of the uptake experiment, NRCMs were either mock-transduced (mock) or infected with bicistronic SIN-LVs encoding hrGFPI and a shRNA specific for either eGFP (eGFP) or for dysferlin (#965 and #756) at an MOI of 10 HTUs per cell. (A) Western blot analysis indicated that dysferlin levels in the NCRMs transduced with SIN-LV965 or SIN-LV756 were 4- to 5-fold lower than those in mock-transduced NRCMs. M, lane with marker proteins; ▷, 225 kDa marker protein; ◀, dysferlin (230 kDa). (B) Uptake of OTR (0.3 µmol/L) by NRCMs incubated for 24 h in the absence (white) or presence (black) of 300 µg/mL of GRGDS. OTR-associated fluorescence intensities are normalized to the value determined in mock-transduced NRCMs incubated for 24 h without GRGDS. Number of data per bar from left to right: 35, 43, 41, 39, 40, 27, 33 and 37, originating from 3 independent experiments. Indicated are mean values and SEM. (C) Dysferlin patch sizes in NRCMs transduced with the SIN-LV encoding an eGFP-specific shRNA (eGFP) and in NRCMs infected with SIN-LV756 at an MOI of 10 HTUs per cell. NRCMs had been incubated for 24 h in the absence (white) or presence (black) of 300 µg/mL of GRGDS. Dysferlin patch sizes are normalized to the value determined in NRCMs transduced with the SIN-LV encoding the eGFP-specific shRNA (eGFP) incubated for 24 h without GRGDS. Indicated are mean values and SEM. (D-I) NRCMs transduced with SIN-LV756 were incubated for 24 h with OTR (0.3 µmol/L) in the presence of 300 µg/mL of GRGDS. Comparison of the hrGFPI fluorescence (GFP, green; D and G) and the OTR-associated red fluorescence (E and H) revealed an inverse relationship between hrGFPI expression level (which is coupled to the shRNA756 expression level) and the intracellular OTR concentration. In other words, the lower the dysferlin expression, the lower the uptake of OTR during integrin stimulation. Panels F and I are merged images.

ence of GRGDS was strongly dependent on the duration of incubation, being evident for OTR as well as for DTR (Fig. 4A and B). Ptrend was <0.001 for OTR + GRGDS, 0.001 for OTR without GRGDS, <0.001 for DTR + GRGDS, and 0.149 for DTR without integrin stimulation.

Mechanism of OTR uptake

To elucidate the mechanism(s) involved in the uptake of macromolecules by NRCMs, the cells were treated with pharmacological inhibitors of three different endocytic pathways. Chlorpromazine was used to inhibit clathrin-mediated endocytosis, whereas macropinocytosis and caveolin-dependent endocytosis were blocked with the phosphoinositide 3-kinase inhibitor wortmannin and the cholesterol-binding antibiotic filipin, respectively. The uptake of OTR by NRCMs in the absence of GRGDS was suppressed by chlorpromazine (15 µmol/L) by 31.9% ($p<0.05$), but was not affected by filipin (4 µg/mL) or by

wortmannin (0.1 μmol/L). The dose-dependent effects of chlorpromazine and wortmannin on OTR uptake in the absence of integrin stimulation are presented in Figures 5B and D. These data suggest that unstimulated NRCMs take up OTR by clathrin-mediated endocytosis.

The uptake of OTR in the presence of GRGDS, thus the sum of RGD-independent and RGD-dependent OTR fluorescence, was not sensitive to treatment of the NRCMs with filipin (4 μg/mL) but it was suppressed by 69.1% ($p<0.01$) by chlorpromazine (15 μmol/L) and by 44.1% ($p<0.05$) by wortmannin (0.1 μmol/L). The integrin stimulation-dependent OTR uptake (=Y-y) was inhibited by wortmannin (EC50 = 276 nmol/L) but not by chlorpromazine (Fig. 5A and C). The GRGDS-independent uptake of OTR (=X-x) was inhibited by chlorpromazine (EC50 = 23 μmol/L) but hardly by wortmannin (Fig. 5B and D). These results imply the involvement of macropinocytosis in the GRGDS-dependent OTR uptake by beating NRCMs. Thus, during integrin stimulation of NRCMs with GRGDS, basal OTR uptake by clathrin-mediated endocytosis is augmented by macropinocytosis.

Role of dysferlin in uptake of macromolecules

To study the role of dysferlin in the uptake of macromolecules by NRCMs, cells that had been incubated with Texas Red-labeled macromolecules for 24 h in the absence and presence of GRGDS were stained with antibodies directed against an epitope located in the large endodomain of dysferlin. NRCMs that were stimulated with the RGD motif-containing pentapeptide showed approximately 3-fold larger dysferlin-positive PM patches than their untreated counterparts (Fig. 6A-C). This finding suggests that the PM of NRCMs is sufficiently disrupted following fixation of the cells with formalin to allow excess of the dysferlin-specific antibodies to their intracellularly located epitope. Our results are also in line with previous reports indicating an important role for dysferlin-enriched PM patches in the repair of PM injury [4-6].

Until now all our experiments were performed with cultures of contracting cardiomyocytes. As it has been argued that mechanical activity favors the occurrence of transient membrane disruptions [14], macromolecule uptake experiments in the presence and absence of GRGDS were performed in beating and in blebbistatin-treated (i.e. contraction-arrested) NRCMs. In non-beating NRCMs, the average size of dysferlin-positive PM patches did not differ between GRGDS-treated and unstimulated cells (Fig. 6C). Accordingly, in the absence of contractile activity the uptake of OTR was not enhanced by the presence of 300 μg/mL GRGDS (107.2 ± 48.6% and 94.5 ± 59.1% in the presence and absence of GRGDS, respectively; p=n.s.) and as high as uptake of OTR by beating NRCMs in the absence of GRGDS (100 ± 53.6%).

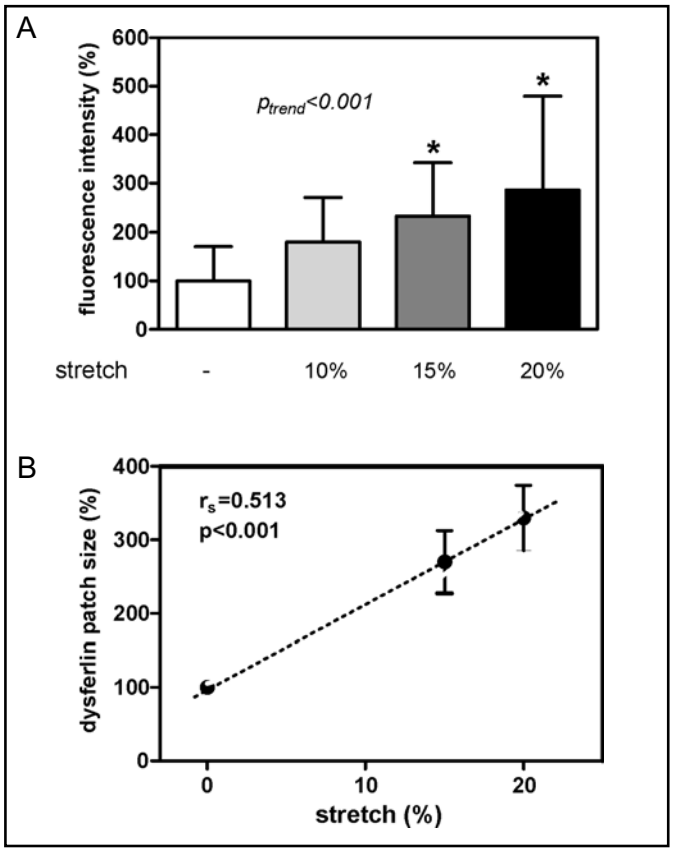

Fig. 8. (A) Uptake of OTR (0.3 μmol/L) by NRCMs incubated for 24 h in the absence (-) or presence of 10%, 15% or 20% stretch. OTR-associated fluorescence intensity is normalized to the value determined in NRCMs incubated for 24 h without stretch. Number of data per bar from left to right: 36, 10, 14 and 10. Indicated are mean values and SD. * $p<0.05$ vs. no stretch by Student t-test and Bonferroni correction. (B) Size of the dysferlin-positive PM patches in NRCMs after 24 h incubation without stretch or in the presence of 15% or 20% stretch. r_s=0.512 indicates a high level of significance ($p<0.001$). Dysferlin patch sizes are normalized to the value determined in NRCMs incubated for 24 h without stretch. Indicated are mean values ± SEM. The SEM of the values measured at 0% stretch is 7.2 and has disappeared within the size of the symbol. Number of data per point: 49 (no stretch), 27 (15% stretch) and 27 (20% stretch).

Effect of dysferlin level on OTR uptake

To study the effect of dysferlin level on OTR uptake, NRCMs were transduced with SIN-LVs directing the synthesis of dysferlin-specific shRNA965 or shRNA756 (SIN-LV965 and SIN-LV756) at an MOI of 10 HTUs per cell. Western blot analysis showed that this

led to 86% reduction of dysferlin protein for shRNA965 and to 79% reduction of dysferlin protein for shRNA756 (Fig. 7A). Contrary to NRCMs transduced with the SIN-LV encoding an eGFP-specific shRNA (negative control), NRCMs expressing the dysferlin-specific shRNAs displayed no extra OTR uptake upon GRGDS addition (160.5 ± 60.7% in control vs. 109.8 ± 36.5% and 99.3 ± 50.1% in shRNA965- and shRNA756-transfected cells respectively; both $p<0.001$ compared to control), while basal OTR uptake was unaffected (105.2 ± 29.0% and 94.0 ± 42.1% in cells transfected with shRNA965 and shRNA756, respectively, both being not significantly different from 100%) (Fig. 7B). SIN-LV756-transduced NRCMs displayed a reduction in dysferlin-positive PM patch size compared to the NRCMs transduced with the SIN-LV encoding an eGFP-specific shRNA both in the absence of GRGDS (32.6 vs. 100%) as well as in its presence (73.3 vs. 353.4%) (Fig. 7C). Apparently, the lack of dysferlin had no effect on the GRGDS-independent uptake of OTR by clathrin-mediated endocytosis, but completely prevented integrin stimulation-dependent OTR uptake by macropinocytosis. The transduction level of NRCMs with SIN-LV756 (reflected by the intensity of the hrGFPI-associated green fluorescence) was inversely correlated with cellular OTR uptake ($r=-0.70$, $p<0.005$), demonstrating the inhibitory effect of dysferlin downregulation on internalization of OTR. Figures 7D-I show that the cells with strong dysferlin downregulation being stained intensely green, demonstrated less OTR accumulation than the cells with slight dysferlin downregulation stained slightly green.

Uptake of macromolecules during stretch

NRCMs incubated in the presence of OTR (0.3 μmol/L) were either stretched for 24 h at amplitudes of 10, 15 and 20%, or left stationary in identical 6-well plates with collagen type I-coated silicone surfaces. The higher the stretch amplitude, the more OTR was taken up ($p_{trend}<0.001$) (Fig. 8A), and the larger was the dysferlin patch size ($r_s=0.513$, $p<0.001$) (Fig. 8B).

Discussion

The major findings of this study are that 1. stimulation of integrins in NRCMs by an RGD-motif containing pentapeptide leads to extra uptake of macromolecules, 2. uptake of macromolecules by NRCMs, either stimulated by GRGDS or not, is dependent on the extracellular concentrations of these macromolecules, and 3. on the duration of incubation with macromolecular cargo, 4. GRGDS-stimulated uptake of macromolecules by NRCMs is dependent on the extracellular concentrations of the pentapeptide, 5. cell stretch stimulates the uptake of macromolecules by NRCMs, 6. uptake of OTR by NRCMs in the absence of GRGDS occurs by clathrin-mediated endocytosis, whereas 7. macropinocytosis is involved in the uptake of OTR by NRCMs during integrin stimulation with RGD motif-containing pentapeptide, 8. dysferlin-positive PM patches in NRCMs incubated with GRGDS are ≈3-fold larger than those observed in unstimulated NRCMs incubated without GRGDS, whereas 9. contraction-arrested NRCMs do not show a GRGDS-dependent increase in dysferlin patch size, nor a GRGDS-dependent increase in OTR uptake, 10. downregulation of dysferlin expression by shRNA-mediated RNA interference has no effect on basal OTR uptake but 11. completely blocks the GRGDS-dependent OTR internalization.

Our research group demonstrated that integrin stimulation promotes the release of cTnI from NRCMs [13]. The present study indicates that integrin stimulation also facilitates the uptake of proteins (i.e. OTR) and other macromolecules (i.e. DTR) by cardiomyocytes. These findings may be explained by the theory of PM "wounding", which proposes that mechanical forces cause transient increases in PM permeability allowing macromolecules to both leave and enter the injured cells [2, 14, 24]. The uptake of macromolecules by cardiomyocytes is apparently not carried out by cargo-specific uptake systems, as we found more or less similar results for the uptake of the protein ovalbumin and the branched glucose polymer dextran.

Although the uptake of OTR and DTR by NRCMs increased with their extracellular concentration, it cannot be explained solely by simple diffusion given the stimulatory effect of GRGDS on the internalization process. In fact, basal OTR uptake was sensitive to chlorpromazine, suggesting that it occurs via clathrin-mediated endocytosis [20]. Conversely, the RGD-dependent OTR uptake was sensitive to wortmannin, indicating the involvement of macropinocytosis [22]. The observation that integrin stimulation triggers macropinocytosis is consistent with a previous report showing that human adenovirus serotype 2-induced macropinocytosis is integrin-dependent [25]. Apparently, macropinocytosis is also dysferlin-dependent, as lowering dysferlin levels by RNA interference inhibited RGD-dependent OTR uptake (Fig. 7B). While pharmacological inhibitors of endocytosis usually provide a good first indication of the specific pathway(s) involved

in the uptake of a particular compound by cells, the results obtained with these drugs should be interpreted with caution due to possible pleiotropic effects and lack of specificity [26]. Accordingly, definitive proof for the role of clathrin-mediated endocytosis and macropinocytosis in the uptake of extracellular macromolecules by NRCMs awaits future studies involving the use of electron microscopy, dominant-negative versions of proteins with well-defined roles in endocytosis and/or RNA interference of genes engaged in specific endocytic pathways.

Protein release from vital cardiomyocytes is a well-known phenomenon. There is abundant literature about release of cardiac troponins in patients without acute coronary syndrome [27-29]. Transient disruptions of PM integrity are now known to occur in a variety of mechanically active tissues under physiological conditions. These tissues include skin, gut, aorta, skeletal muscle and heart [30]. Approximately 25% of the cardiomyocytes examined in the normal rat heart had suffered constitutive non-lethal PM disruptions, as demonstrated by the intracellular presence of serum albumin [2]. Myocardial overloading, such as occurs during cardiac pressure or volume overload, increases this frequency of cardiomyocyte membrane wounding [1, 2, 14], probably mediated by integrins that transmit the mechanical force across the PM [31].

The present study shows that uptake of OTR and DTR by NRCMs was intensified by integrin stimulation. Several mechanisms modulating endocytosis play a role in the uptake of extracellular macromolecules by cardiomyocytes, including the activation of stress-induced ion channels, mechanotransduction via integrin binding to ECM components, and internal cytoskeletal tension [7, 32-34]. Macromolecule uptake by myocytes has also been described in terms of contraction-induced resealable sarcolemmal membrane disruptions ("wounding") that have been observed in skeletal muscle as well as in isolated perfused rat hearts [1, 2]. Altered transient sarcolemmal permeability sufficient to release bFGF was inferred from the increased uptake of fluorescein-labeled dextran by ventricular cardiomyocytes of adult rats after pacing [14]. Uptake of fluorescein-labeled dextran by paced cardiomyocytes was 3- to 50-fold higher than by unstimulated control cells depending on the specific preparation of cardiomyocytes and batch of fluorescently labeled dextran used [14]. Along the same line, treatment with the β-adrenergic agonist isoproterenol stimulated the release of bFGF from beating adult rat hearts and was accompanied by an increased cardiac uptake of serum albumin from the circulation [2]. To determine whether membrane depolarization, mechanical activity, or both, were required to facilitate fluorescein-labeled dextran uptake, Kaye and co-workers [14] used the L-type Ca^{2+} channel antagonist verapamil and the non-competitive inhibitor of class II myosin 2,3-butanedione monoxime (BDM) to block both Ca^{2+} currents and myofibrillar contractions or to exclusively interfere with myofibrillar contractions, respectively. In the presence of 10 μmol/L of verapamil uptake of fluorescein-labeled dextran by cardiomyocytes exposed for 48 h to continuous uniform electric field stimulation was below that observed in quiescent control cells. Moreover, addition of BDM to a final concentration of 10 mmol/L caused uptake of fluorescein-labeled dextran by paced cardiomyocytes and by quiescent control cells to be equally low [14]. These data were interpreted as evidence that mechanical activity favors the occurrence of transient PM disruptions and that these lesions facilitate the uptake of extracellular macromolecules by cardiomyocytes. Our finding that upon incubation with GRGDS non-beating NRCMs showed no significant increase in OTR uptake or dysferlin patch size is consistent with this idea (Fig. 6C). Our observation that the average size of the dysferlin-positive PM patches is ≈ 3-fold larger in GRGDS-treated beating NRCMs than in untreated beating NRCMs (Fig. 6A-C), suggests that integrin stimulation results in extra membrane wounding and its subsequent repair by a dysferlin-dependent PM resealing mechanism. Alternatively, dysferlin may play an active role in GRGDS-stimulated macropinocytosis.

The RGD sequence is a ligand for integrins [10], and can be used to simulate myocardial mechanical stretch in vitro. Indeed, integrin stimulation and mechanical stretch of NRCMs had very similar effects on OTR uptake (Fig. 3A and 8A) and on the average size of dysferlin-positive PM patches (Fig. 6C and 8B). Stretch of NRCMs showed a stretch-dependent increase of OTR uptake and corresponding increase in dysferlin patch size (Fig. 8A and B). These observations indicate that spontaneously contractile NRCMs undergoing integrin stimulation by incubation with RGD-motif containing pentapeptide are a valuable model to study integrin-mediated processes such as occur during stretch of these cells *in vitro* and probably also in overload-induced hypertrophic and postinfarct remodeling *in vivo* [35]. However, the model depends on contractile activity as in non-beating NRCMs stimulation with GRGDS had no effect on OTR uptake nor on dysferlin patch size.

One of the responses of the PM of NRCMs to integrin stimulation is an increase in cellular permeability to macromolecules associated with an increase in dysferlin-positive PM areas. Dysferlin was the first identified member of a muscle-specific repair complex that permitted rapid sealing of PM disruptions induced by mechanical stress. In normal myofibers, dysferlin is predominantly localized in the PM and in subsarcolemmal vesicles [4]. According to recent findings, membrane disruption causes influx of extracellular Ca^{2+} ions into muscle fibers. The resulting high intracellular Ca^{2+} concentration triggers the aggregation of dysferlin-carrying repair vesicles and the migration of these vesicles toward the site of injury [36], where they fuse with each other and with the PM. Dysferlin is thought to facilitate vesicle docking and fusion with the PM in this repair process, resulting in a membrane patch across the site of membrane disruption and sealing of the disrupted PM. The understanding of dysferlin and its role in membrane repair, signaling, muscle physiology, and pathophysiology has greatly improved in studies in patients and experimental animals with muscular dystrophy [36, 37]. However, further studies should elucidate the active role of dysferlin in the GRGDS-stimulated macropinocytosis of NRCMs.

Conclusion: In NRCMs, stimulation of integrins by a RGD motif-containing pentapeptide causes uptake of macromolecules through macropinocytosis. Dysferlin may have a role in this uptake process as reducing the intracellular levels of this membrane repair protein 4- to 5-fold through RNA interference prohibited GRGDS-induced uptake of OTR. Contractile activity is a crucial factor in the RGD-dependent uptake of OTR which may argue for the existence of a functional link between the repair of PM damage and macropinocytosis.

Abbreviations

ANOVA (One-way analysis of variance); a.u. (Arbitrary units); bFGF (Basic fibroblast growth factor); BrdU (5-bromo-2-deoxyuridine); cTnI (Cardiac troponin I); DMEM (Dulbecco's modified Eagle's medium); DTR (Texas Red-conjugated dextran); ECM (Extracellular matrix), FAK (Focal adhesion kinase); FBS (Fetal bovine serum); GRGDS (NH2-Gly-Arg-Gly-Asp-Ser-COOH (RGD-containing pentapeptide)); hrGFPI (Renilla reniformis green fluorescent protein version I); HS (Horse serum); HTU (HeLa cell-transducing unit); Kb (Kilobase); kDa (Kilodalton); MOI (Multiplicity of infection); NRCM (Neonatal rat ventricular cardiomyocyte); n.s. (Non-significant); OTR (Texas Red-conjugated ovalbumin); PBS (Phosphate-buffered saline); PM (Plasma membrane); rs (Spearman's rank correlation coefficient); RGD motif (The amino acid sequence NH2-Arg-Gly-Asp-COOH); SD (Standard deviation); SEM (Standard error of the mean); shRNA (Short hairpin RNA (for RNA interference experiments)); SIN-LV (Self-inactivating human immunodeficiency virus type I vector).

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