G-protein-coupled glucocorticoid receptors on the pituitary cell membrane

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Summary

Rapid, nongenomic actions of glucocorticoids (GCs) have been well documented, but information about putative membrane receptors that mediate them is scarce. We used fluorescence correlation spectroscopy to search for membrane GC-binding on the mouse pituitary cell line AtT-20. A slowly diffusing fraction (τ_3; diffusion constant 3×10^{-10} cm^2 s^{-1}) of fluorescein-labeled dexamethasone on the cell membrane corresponds to fluorescein-dexamethasone binding. Preincubation experiments were performed to test binding specificity: a 500-fold excess of unlabeled dexamethasone abolished subsequent fluorescein-dexamethasone membrane binding from 58±2 (control) to 8±1 (% of τ_3, mean ± s.e.m.), the natural ligand corticosterone prevented it partially (29±2), while the sex steroids estradiol (56±4) and progesterone (50±4) and the GC-receptor antagonist RU486 (56±2) had no effect. Preincubation with pertussis toxin resulted in disappearance of the slowest diffusion component (11±4) suggesting association of the receptor with a G-protein. Varying the concentration of fluorescein-dexamethasone showed that membrane binding is highly cooperative with an apparent K_d of 180 nM and B_{max} of 230 nM. Taken together, these results demonstrate high-affinity GC-binding on the cell membrane of AtT-20 cells with characteristics distinct from intracellular binding.

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Introduction

During the last decade, rapid steroid effects, i.e. effects occurring on a timescale of seconds to minutes, have been recognized as physiologically relevant and widespread through all classes of steroid hormones (Norman et al., 2004; Losel and Wehling, 2003). These effects cannot be explained by interaction with the well-characterized intracellular steroid receptors acting as ligand-activated transcription factors (Losel et al., 2003). Steroids have repeatedly been shown to act independently of gene transcription (nongenomically) on several pathways that are typically used by membrane receptors, such as the activation of phosphoinosite 3-kinase (PI3 kinase) (Simoncini et al., 2000) or protein kinase C (PKC) (Winter et al., 2004); they mediate vasorelaxation, cell lysis and insulin release, and inhibit apoptosis [Losel and Wehling (Losel and Wehling, 2003) and the references therein]. For membrane steroid receptors by contrast, relatively few data are available. In some instances the 'classical' intracellular steroid receptors can somehow be attached to the cell membrane and are then able to elicit rapid steroid responses. So is the estrogen receptor α (ERα) translocated to the plasma membrane upon stimulation with estradiol to form a protein complex together with Shc and the IGF-1 receptor (Kousteni et al., 2003; Song et al., 2004). Recently, it has been shown that the DNA-binding domain of ERα is responsible for the interaction of the receptor with PI3 kinase (Chambliss et al., 2004).

Additionally, a distinct class of membrane steroid receptors, unrelated to their intracellular counterparts, exist: the first unique vertebrate membrane steroid receptor, a progestin receptor that belongs to a new family of G-protein-coupled receptors, has been cloned and characterized recently (Zhu et al., 2003a; Zhu et al., 2003b).

Glucocorticoids (GCs) are, like cortisol or corticosterone (the main glucocorticoids of humans and rodents, respectively), a class of steroid hormones produced in the adrenal gland upon stimulation by the pituitary hormone adrenocorticotropin (ACTH). They are of vital importance in stress- and immune reactions as well as for metabolism, and affect virtually every cell in the body. GC blood levels are thus tightly regulated. By means of a feedback mechanism, GCs inhibit ACTH synthesis (Muglia et al., 2000) and release (Hinz and Hirschelmann, 2000; Antoni et al., 1992; Loechner et al., 1999). This latter effect occurs in vivo on a timescale of minutes (Hinz and Hirschelmann, 2000) and can be considered a physiological, nongenomic steroid effect.

The ACTH-producing mouse pituitary cell line AtT-20 is used as a model system for the fast-feedback inhibition of ACTH-release by GCs (Antoni et al., 1992; Loechner et al.,

1999). Dexamethasone, a potent synthetic GC, inhibits ACTH-release in this cell line in less than 10 minutes (Loechner et al., 1999). The existence of a membrane glucocorticoid receptor (mGR) was suggested as the result of binding studies of isolated AtT-20 cell membranes with radiolabeled ligands (Harrison et al., 1979; Spindler et al., 1991), and confocal microscopy with antibodies directed against the intracellular glucocorticoid receptor (iGR) on lymphoma cells (Gametchu, 1987) and blood leukocytes (Bartholome et al., 2004). To our knowledge, data on affinity and binding characteristics of these membrane GC-binding sites have, until now, never been published.

The aim of our study was to find specific binding of a GC ligand to the cell membrane of living AtT-20 cells. We decided to use fluorescence correlation spectroscopy (FCS) (Magde et al., 1972; Maiti et al., 1997) for this purpose. FCS measurements are non-invasive and can be carried out under equilibrium conditions in aqueous solution. Fluctuations in signal-intensity are recorded when fluorescence-labeled molecules diffuse through the excitation volume, and are quantified by autocorrelation analysis. Thus, faster and slower diffusion times can be assigned to unbound and bound ligand states, respectively. FCS has been used to investigate bacterial motor proteins (Cluzel et al., 2000), p53 latency (Yakovleva et al., 2001), active transport in plastid tubules (Köhler et al., 2000), amyloid-β-protein aggregates in cerebrospinal fluid (Pitschke et al., 1998) and ultrasensitive pathogen detection (Oehlenschlager et al., 1996; Korn et al., 2003), and to study diffusion behavior of and ligand binding to membrane receptors on single cells (Briddon et al., 2004; Patel et al., 2002; Rigler et al., 1999; Zhong et al., 2001; Pramanik et al., 2001; Pick et al., 2003; Licht et al., 2003; Schwille et al., 1999a; Schwille et al., 1999b).

In the case of studying GC membrane-binding, a possible interference of ligand, binding to the iGR, had to be excluded when measuring on whole cells. In a preliminary methodological study we used a 'scanning' procedure through the cell to measure membrane-binding as opposed to intracellular diffusion and/or binding of fluorescein-labeled dexamethasone (F-dexa) and found a characteristic, slowly diffusing component that was present exclusively on the cell membrane (Maier et al., 2002). Based on this model, the data presented here demonstrate specific and high-affinity binding of F-dexa on the membrane of AtT-20 cells.

Materials and Methods

Materials

AtT-20 cells (CCL-89) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cell culture media and reagents were from Gibco BRL (Paisley, UK). F-dexa was obtained from Molecular Probes (Leiden, The Netherlands). All other chemicals were from Sigma (St Louis, MI). All steroids were purchased in a water-soluble form (estradiol as glucuronide, dexamethasone and corticosterone as cyclodextrin complex, and progesterone as pure substance); stock solutions were prepared directly in binding buffer. Dexamethasone was also purchased as pure substance, which was dissolved in ethanol and diluted to final concentrations with binding buffer. Mifepristone (RU486) was dissolved in dimethyl sulfoxide (DMSO) and diluted to final concentrations in binding buffer (less than 0.1% DMSO). Pertussis toxin (PTX) was purchased from Sigma (P-2980) as 0.2 mg/ml stock solution and diluted to final concentrations with binding buffer. LabTek chambered coverglass (8 chambers) was from NalgeNunc (Naperville, IL).

Cell culture

Cells were maintained at 37°C, 6% CO_2, 95% relative humidity in 75 cm^2 cell culture flasks in HAM's F10 medium (supplemented with 12.5% horse serum, 2.5% fetal calf serum and antibiotics). The medium was changed twice weekly and cells were subcultured every 4 weeks. For the binding experiments only cells with a passage number up to five were used.

Incubation

Cells were transferred to poly-D-lysine coated LabTek chambered coverglass on the day of experiments and allowed to attach for 1 hour before washing them gently two times with phosphate buffered saline and replacing the culture medium with non-fluorescent binding buffer (containing 145 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 0.8 mM $CaCl_2$, 25 mM HEPES and 22 mM glucose). AtT-20 cells showed a very low uncorrelated autofluorescence of approximately 0.3 kHz. The viability of the cells was >90% after 3 hours of incubation at room temperature in the binding buffer (as assessed by Trypan Blue staining). The time required for measurements did not exceed 1.5 h.

To generate the binding curve, F-dexa was added in varying concentrations and cells were incubated for 1 hour at 37°C; measurements were carried out immediately afterwards at 20°C. Pure F-dexa was used for the low-concentration range (up to 80 nM); for the higher concentrations (240 and 540 nM) 80 nM F-dexa was mixed with unlabeled dexamethasone. Measurements using 100, 120, 140 and 160 nM were done with mixtures as well as with pure F-dexa solutions. For each concentration five cells from at least two independent incubation experiments were measured, and measurements were taken in triplicates.

The specificity of F-dexa binding was tested in a 3-hour preincubation experiment, with the unlabeled steroids dexamethasone, corticosterone, estradiol and progesterone at a 500-fold excess (60 μM) at 37°C, followed by a 1-hour incubation with 120 nM F-dexa. For each steroid, at least eight cells from three independent incubation experiments were measured in triplicates. Preincubation experiments with dexamethasone were done with both cyclodextrin-complexed and pure substance, and since the effects did not differ between the two groups, resulting data were pooled together. Preincubation with binding buffer alone was used as control in each experiment.

Preincubation with the GC-receptor antagonist RU486 (60 μM) and PTX (1 μg/ml) were performed in the same way as described for the specificity experiments; heat-inactivated PTX (1 μg/ml) served as negative control for the latter experiments. For each substance, at least seven cells from three independent experiments were measured in triplicates.

Fluorescence correlation spectroscopy

FCS measurements were carried out on a Confocor spectrofluorimeter (Carl Zeiss-Evotec, Jena, Germany) equipped with an Ar-laser, a water immersion objective (C-Apochromat 63×/1.2 W Korr), an avalanche photodiode (SPCM-CD 3017) and a hardware autocorrelator (ALV 5000, ALV, Langen, Germany).

Samples were excited with the 488-nm Ar-laser-line attenuated by optical density filters, the fluorescence signal was detected through a dichroic mirror with bandpass filters. The pinhole diameter was set to 45 μm, resulting in a confocal volume element of 0.17 μm in the radial and 2.4 μm in the axial dimension as determined by calibration using rhodamine 6G.

The confocal volume was positioned in the cells using an *x*-*y* stage

with 1 μm resolution, whereas the correct focus positioning on the cell membrane was ascertained by a scanning procedure (Maier et al., 2002). Briefly, the focus position was moved in 1-μm steps from outside the cell across the cell membrane towards the glass surface and measurements (15 seconds correlator scaling and 30 seconds measurement) were taken at each position. Measurements at the membrane position (typically 7-9 μm from the coverglass) were taken in triplicates and the correct positioning was re-evaluated at the end of each measurement series.

The signal response of F-dexa in solution was determined to be linear for up to 200 particles (corresponding to 1.6 μM F-dexa) in the focus; a negative control of pure fluorescein-dye incubated with the cells revealed unrestricted diffusion in all cellular compartments.

Data evaluation

A normalized autocorrelation function $G(\tau)$ describes fluctuations with time around the mean measured intensity F, where angular brackets represent the ensemble average:

$$G(\tau) = 1 + \langle \delta F(t) \cdot \delta F(t+\tau) \rangle / \langle F \rangle^2 .$$

$G(\tau)$ depends on the measurement geometry and on the diffusion properties of the fluorescent species. For a single diffusing species in a Gaussian confocal volume it has the following analytical form:

$$G(\tau) = 1 + 1 / [N(1+\tau/\tau_D)\,(1+\tau/(z/r)^2\tau_D)^{0.5}] ,$$

where N is the particle number and τ_D the characteristic diffusion time for the fluorescent molecule moving through the confocal volume with an axial (z) to radial (r) dimension. The confocal volume was determined by measurements of the correlation time for the fluorescent dye rhodamine 6G with the known diffusion coefficient D of 2.8×10^{-6} cm^2 s^{-1} employing the relationship $D=r^2/4\tau_D$. Experimental autocorrelation functions were evaluated using a two or three component model including the triplet state with the triplet fraction F_T and the triplet lifetime τ_T. The analytical formula for the two component model is given by:

$$G(\tau) = \frac{1}{N'} [(1-Y)g_{D_1}(\tau) + Yg_{D_2}(\tau)]\,[F_T\, e^{\tau/\tau_T} + (1-F_T)] + 1$$

$$\text{with} \quad N' = N(1-F_T) \quad \text{and} \quad g(\tau) = \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau}{(z/r)^2\tau_D^2}\right)^{-0.5} .$$

An analogous expression is valid for the three component model, which according to preliminary work (Maier et al., 2002) is adequate to fit the data obtained from scanning whole cells. Confocal volume calibrations using rhodamine 6G were carried out on each experimental day as described above and used as an input for the fits, which yielded a diffusion time τ_1 corresponding to free F-dexa in solution and two further cell-related diffusion times τ_2 and τ_3 as well as the corresponding relative amount of each component. The software FCS Access® (Evotec) and Origin® (Microcal) was used for nonlinear least squares fits.

Probing of membrane fluidity

The lipophilic membrane marker 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) was from Molecular Probes. To label cells with DiO they were incubated with 10 μM DiO (in binding buffer) for 20 minutes. After this time, DiO was replaced with binding buffer containing either 60 μM dexamethasone (DEXA), 1 μg/ml PTX (PTX) or binding buffer alone (control, CO). Cells were then incubated for 1 hour before FCS measurements were performed. Autocorrelation curves were best fitted to the two-component model with component τ_1 corresponding to the diffusion time of DiO in solution and component τ_2 representing DiO diffusion on the cell membrane derived from the fitting procedure.

In total, 22 (27 for control) autocorrelation curves taken at the membrane position from four individual cells were evaluated for each experimental subgroup. Statistical evaluation was performed using SPSS 12.0 statistical software. Diffusion times and percent fraction of the slower diffusion component from the CO, DEXA and PTX subgroups were compared by one-way ANOVA followed by Dunnet-t-test (two-sided, for comparison of either treatment group with CO) as post-hoc statistics, when appropriate. $P<0.05$ was set as level of statistical significance. Data are presented as mean ± s.e.m.

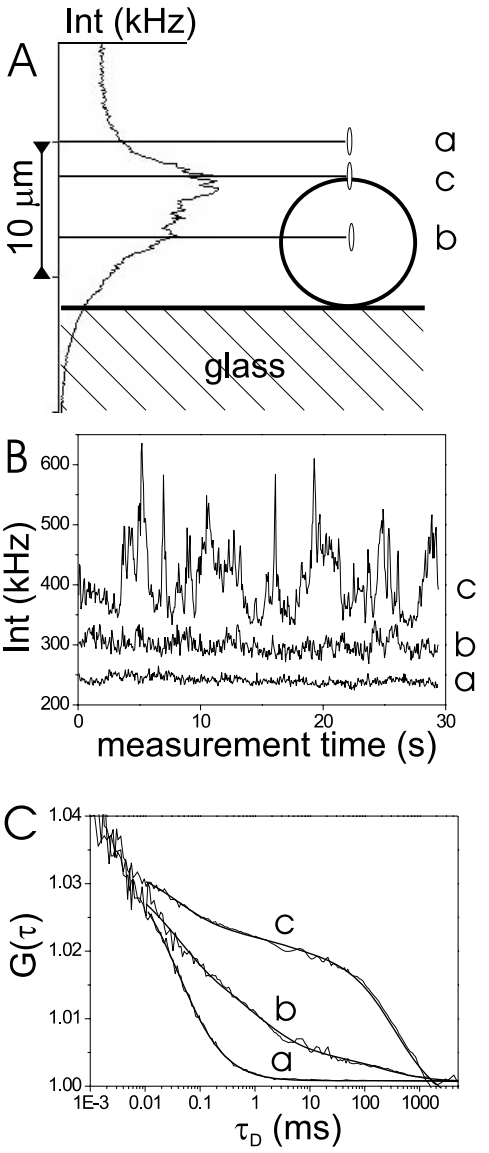

Fig. 1. FCS analysis of F-dexa in a single AtT-20 cell. (A) The confocal volume was positioned above the center of a cell and a z-intensity scan was performed towards the glass surface. Representative positions, (a) outside of the cell (in the solution), (b) inside the cytoplasm and (c) at the cell membrane, are indicated by horizontal lines. (B) Intensity fluctuations were recorded for 30 seconds and are shown for the three representative positions together with their normalized autocorrelation curves (C). Data were fitted with the three component model.

Results

Affinity of F-dexa–binding

To determine the localization of F-dexa, AtT-20 cells were scanned by moving the laser focus along the *z*-direction through the cell. In Fig. 1 a fluorescence intensity profile of a *z*-scan (Fig. 1A) is shown together with recorded fluorescence fluctuations (Fig. 1B) and corresponding autocorrelation curves (Fig. 1C) for three distinct focus positions. Examination of the Brownian motion of free F-dexa in the buffer medium above the cell surface showed a homogeneous distribution of the intensity fluctuations. When the focus was positioned at the upper cell membrane, an increase and broadening of the fluctuation peaks was observed together with a large shift of the autocorrelation function towards longer diffusion times. These characteristics were significantly reduced when the focus was positioned completely inside the cell. Analysis of the autocorrelation curves yielded consistently the following three diffusion coefficients: D_1=1.8×10^{-6} cm^2 s^{-1}, D_2=2×10^{-8} cm^2 s^{-1}, and D_3=3×10^{-10} cm^2 s^{-1}.

Increasing the concentration of F-dexa in the buffer-medium led to an increased proportion of the slowest component. In Fig. 2 the intensity fluctuation and autocorrelation curves are displayed for low, medium and high concentrations of F-dexa. Increase and broadening of the fluctuation peaks corresponded well with the increase of the slowest component in the autocorrelation curves.

A quantitative study of F-dexa–binding on the cell membrane was based on the dependence of the autocorrelation data on the concentration of F-dexa. To obtain a binding

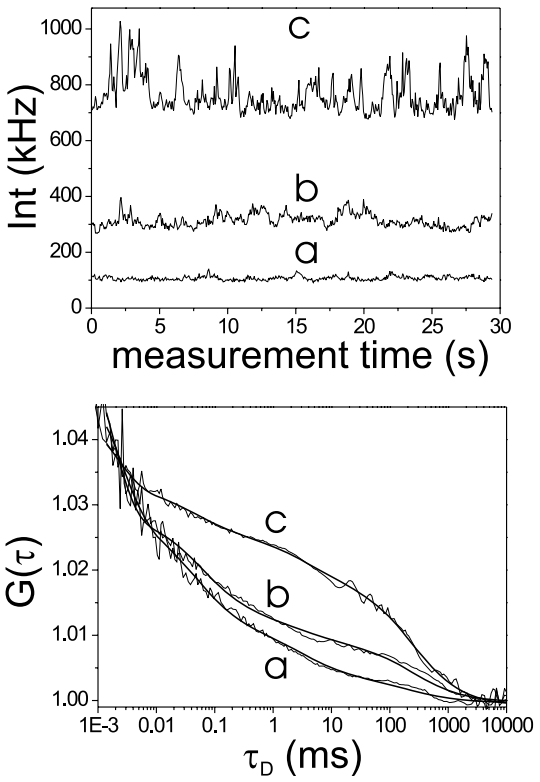

Fig. 2. F-dexa–binding to the membrane of single AtT-20 cells quantified by FCS. Intensity fluctuations and the measured and calculated corresponding autocorrelation curves G(τ) at the cell membrane position for representative F-dexa concentrations of (a) 25, (b) 80 and (c)120 nM. Data were fitted with the three component model, with the fixed diffusion times of τ_1=0.05, τ_2=3.6 and τ_3=255 ms. The resulting fractions were f_1=0.61, f_2=0.26, and f_3=0.13 for 25 nM, f_1=0.55, f_2=0.16, and f_3=0.30 for 80 nM, and f_1=0.23, f_2=0.21, and f_3=0.56 for 120 nM.

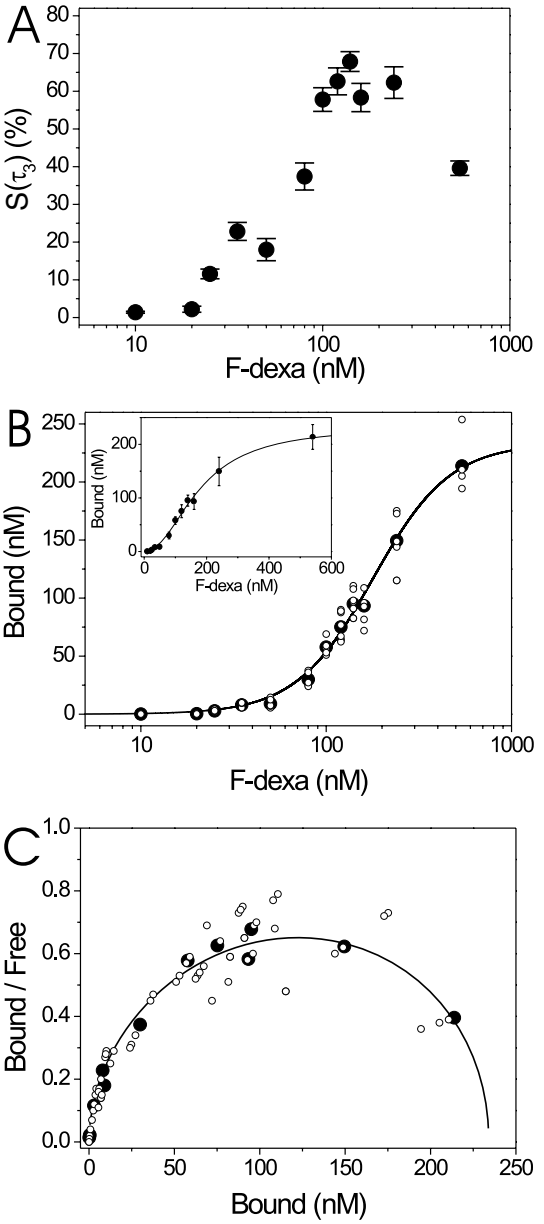

Fig. 3. F-dexa–binding isotherm. (A) Membrane-bound F-dexa S(τ_3) as a function of the F-dexa concentration in the binding medium for the average of measurements for each particular concentration (± s.d.). (B) Calculated molar concentrations of bound F-dexa for individual measurements (○) and for the average of measurements (●) for each particular concentration. Data were fitted to a Hill equation with variable slope (solid line), which yields a B_{max} of 230±10 nM, an apparent K_d of 180±10 nM and a Hill coefficient of 2.1±0.2. Insert shows the average of measurements (●) for each particular concentration (± s.d.) on a linear F-dexa concentration scale. (C) Scatchard plot of transformed data (individual measurements (○) and for the average of measurements (●) together with the transformed nonlinear fit (solid line).

isotherm over a suitably broad range of concentrations, it was necessary to mix labeled with unlabeled dexamethasone in the high concentration range (see Materials and Methods). In the intermediate range (100-160 nM), measurements were performed with pure F-dexa solutions as well as with mixtures to exclude a possible bias caused by this procedure.

Fig. 3A shows the average values of the relative statistical weights $S(\tau_3)$ of the slowly diffusing component (τ_3) as a function of the concentration of F-dexa on a logarithmic scale. With the assumption that τ_3 is the specific signature of the membrane-bound ligand, $S(\tau_3)$ can be equaled to the bound fraction. Fig. 3A can thus be read as a binding curve reflecting the simultaneous presence of free and bound ligand; the magnitude of the bound fraction necessarily decreases at high ligand concentrations once the binding sites are saturated. The molar concentrations of membrane-bound ligands were calculated from the bound fraction $S(\tau_3)$ and the F-dexa concentrations, and are shown in Fig. 3B for both the individual measurements and for the average of measurements for each particular concentration on a log scale. The average data are shown in the insert of Fig. 3B on a linear concentration scale; this representation clearly demonstrates a sigmoidal shape of the binding curve, which obviously cannot be fitted by a hyperbolic function, suggesting cooperativity. Evaluation of the binding curve was performed by nonlinear fitting, based on the Hill equation with a variable slope (Cantor and Schimmel, 1980) yielding a Hill constant of 2.1±0.2, an apparent K_d value for the interacting sites of 180±10 nM and a maximum concentration of bound ligand B_{max} of 230±10 nM; the best fits are shown in Fig. 3B and its insert. The data were used to generate a Scatchard plot (Fig. 3C), whose pronounced concave shape is again characteristic of positive cooperativity. The transformed regression curve of Fig. 3B may be used to visualize the saturation value B_{max} of 230 nM in the Scatchard plot.

Specificity of F-dexa–binding

The specificity of F-dexa–binding was assessed by preincubation for 3 hours in 500-fold excess of unlabeled dexamethasone, the natural ligand corticosterone, the sex steroids estradiol (which does not bind to the iGR) and progesterone (which binds with low affinity to the iGR), followed by an 1-hour incubation with F-dexa. Results are the mean of at least eight cells from three independent experiments.

Representative intensity fluctuations and corresponding autocorrelation curves for the various steroid hormones are given in Fig. 4. Preincubation with unlabeled dexamethasone reduced F-dexa membrane-binding from 58±2% (fraction of membrane-bound F-dexa in the control, mean ± s.e.m.) to 8±1%. Corticosterone, the natural ligand of the rodent GC receptor, reduced subsequent F-dexa–binding to 29±2%, whereas progesterone (50±4%) and estradiol (56±4%) were unable to prevent F-dexa–binding on the cell membrane. An overview of the results is given in Fig. 5.

Characterization of F-dexa binding

The relation of the membrane-binding sites to the iGR was

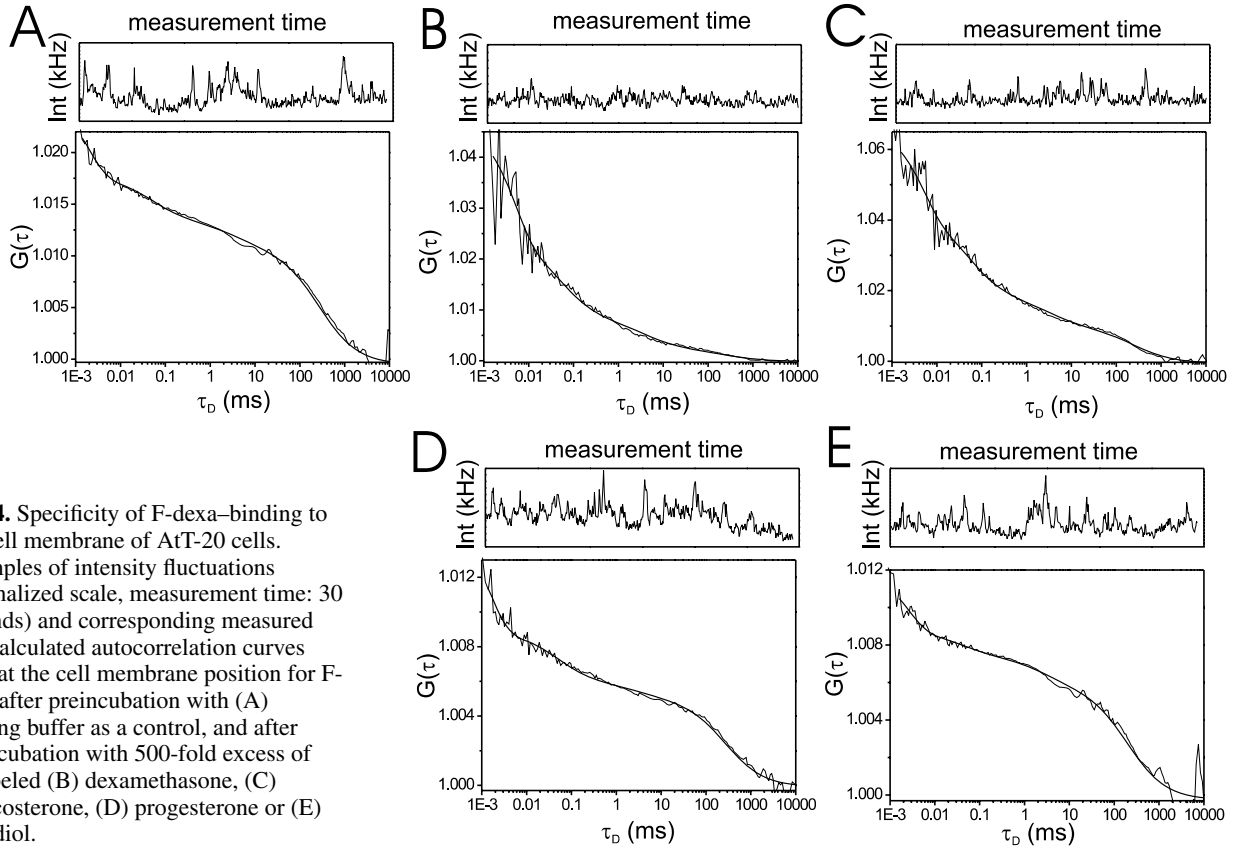

Fig. 4. Specificity of F-dexa–binding to the cell membrane of AtT-20 cells. Examples of intensity fluctuations (normalized scale, measurement time: 30 seconds) and corresponding measured and calculated autocorrelation curves G(τ) at the cell membrane position for F-dexa after preincubation with (A) binding buffer as a control, and after preincubation with 500-fold excess of unlabeled (B) dexamethasone, (C) corticosterone, (D) progesterone or (E) estradiol.

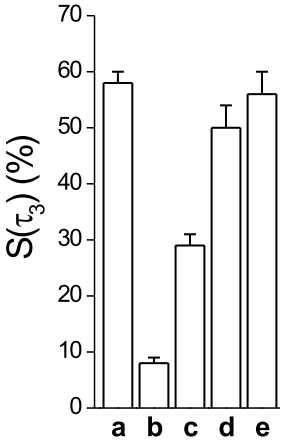

Fig. 5. Overview of specificity experiments. Fraction S(τ_3) (mean ± s.e.m.) of at least eight cells from three independent experiments are shown. Subsequent F-dexa binding is reduced from the control value 58±2% (a) by dexamethasone (8±1%) (b) and corticosterone (29±2%) (c), but unaltered by progesterone (50±4%) (d) and estradiol (56±4%) (e).

assessed by preincubation with the iGR antagonist RU486; a 500-fold excess of RU486 did not alter subsequent F-dexa–binding (56±2 vs 58±2%, control).

The possible interaction with a Gi protein was assessed by preincubation with PTX (1 μg/ml). After 3 hours of PTX treatment, a subsequent incubation with F-dexa still led to enrichment of F-dexa in the cell membrane, but the τ_3 component was reduced to the same extent as in the preincubation experiments with a 500-fold excess of unlabeled dexamethasone (see above): 11±4 vs 58±2% (control). Instead, the τ_2 component (the component which is normally seen predominantly in the cytosol) showed a maximum on the position of the cell membrane. By contrast, heat-inactivated PTX was unable to alter subsequent F-dexa–binding (data not shown). Representative intensity fluctuations and corresponding autocorrelation curves, and an overview of the results are given in Figs 6 and 7, respectively.

Effects on membrane fluidity

Incorporation of DiO in the cell membrane resulted in a characteristic diffusion behavior at the membrane position (supplementary material Fig. S1a). Autocorrelation curves were best fitted with a two-component model, with the first (τ_1) component representing diffusion of free DiO in solution and the second (τ_2) component diffusion of DiO in the cell membrane. A two component fit with τ_1 fixed resulted in a diffusion time of τ_2=2.23±0.2×10^{-3} seconds (mean±s.e.m.) corresponding to a diffusion constant of 3.7×10^{-8} cm^2 s^{-1}. Treatment with 60 μM DEXA (the highest concentration used in the competition experiments) or 1 μg/ml PTX did not have any influence on the diffusion of DiO on the cell membrane: diffusion times were 2.10±0.1×10^{-3} seconds (DEXA) and 1.78±0.2×10^{-3} seconds (PTX), respectively (P=0.2, one-way ANOVA).

The fraction of the τ_2 component on the cell membrane was significantly different between groups (P<0.0001, one-way ANOVA). DEXA treatment led to a significant reduction of the τ_2 fraction (22.0±1.2%, DEXA vs 33.5±1.2%, CO; P<0.0001, Dunnet-T). By contrast, PTX did not change the τ_2 fraction (36.9±1.7%, PTX vs 33.5±1.2%, CO; P=0.15, Dunnet-T). Representative autocorrelation curves together with their two-component fits are shown in supplementary material, Fig. S1.

Discussion

The data presented here show for the first time specific, high-affinity membrane-binding of a GC ligand to whole pituitary cells. Applying the highly sensitive FCS technique to the mouse pituitary cell line AtT-20, we were able to discriminate the diffusion behavior of F-dexa inside the cell, on the cell membrane and outside the cell in solution. The small measuring volume of FCS makes it possible to measure steroid membrane-binding on whole cells without interference of intracellular receptor-binding. Moreover, FCS allows to study ligand-binding without any manipulation of the cells. This is particularly important when considering that most membrane steroid-receptors seem to be very sensitive to changes of cell-culture conditions when studied in vitro (Watson et al., 2002). Another advantage is the possibility to directly discriminate, by autocorrelation of the diffusion behavior of the fluorescently-labeled ligand, between specific binding and

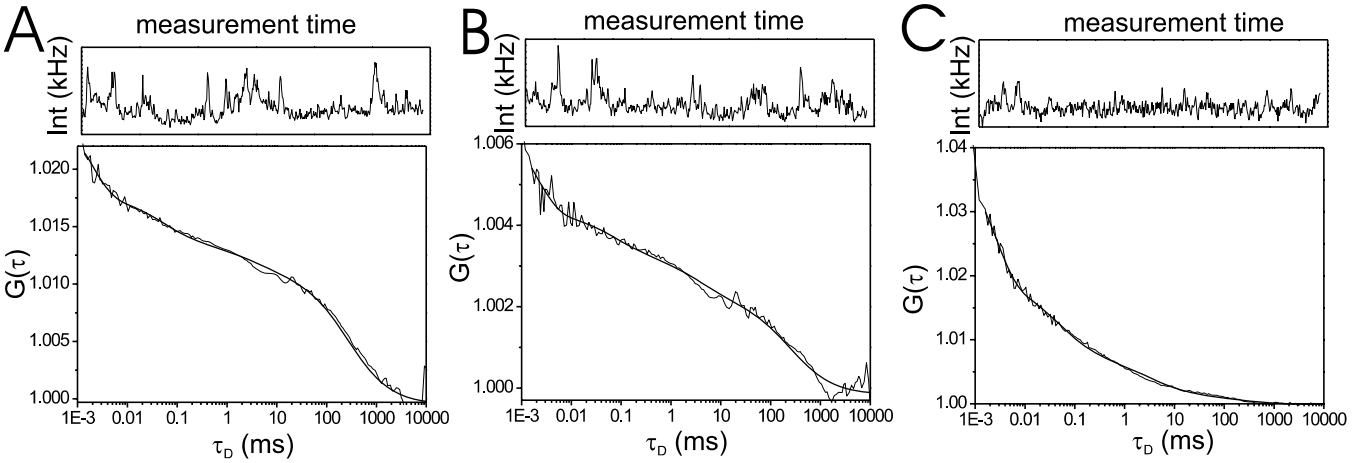

Fig. 6. Characterization of F-dexa–binding to the cell membrane of AtT-20 cells. Examples of intensity fluctuations (normalized scale, measurement time: 30 seconds) and measured and calculated corresponding autocorrelation curves G(τ) at the cell-membrane-position for F-dexa after preincubation with (A) binding buffer as a control, and after preincubation with (B) RU486 or (C) PTX.

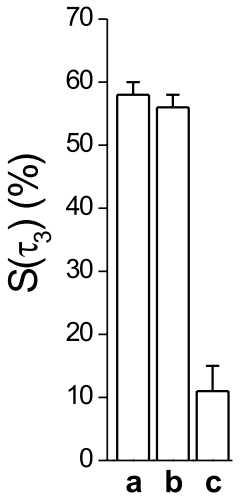

Fig. 7. Overview of characterization experiments. Fraction $S(\tau_3)$ (mean ± s.e.m.) of at least seven cells from three independent experiments are shown. Subsequent F-dexa–binding is not reduced from the control value 58±2% (a) by RU486 (56±2%) (b) but is almost abolished by PTX (11±4%) (c).

unspecific attachment to the cell membrane. Thus, even a high level of unspecific binding owing to the lipophilic nature of steroid hormones does not hamper detection of specific binding to the cell membrane, as would be the case when using radioligands (Watson and Gametchu, 2001).

Affinity data were obtained by incubation with different concentrations of F-dexa that resulted in an average K_d of 180 nM and a B_{max} of 230 nM, indicating a binding affinity significantly lower than that of the iGR, which was determined using radioligands to be in the range of 5 nM or less (Schaaf and Cidlowski, 2003 and the references therein). Indeed, the nongenomic GC effects described so far all occur at higher concentrations than those necessary to saturate iGR binding. This also includes in vivo data, such as the vasorelaxing effect of dexamethasone in mice where the plasma concentrations for maximal effect were in the range of 100 nM (Hafezi-Moghadam et al., 2002). GCs are used therapeutically in life-threatening conditions such as status asthmaticus, severe episodes of multiple sclerosis or systemic autoimmune diseases, and leukaemia. Initially, very high doses, resulting in GC plasma concentrations of several 100 nM, are used that are necessary for therapeutical effect. It has been postulated that at very high concentrations, GCs might be able to alter physico-chemical properties of cell membranes (the so-called 'unspecific nongenomic effects' of GCs) and thus inhibit cation cycling across the cell membrane (Buttgereit et al., 2000). The highest concentration of dexamethasone used in this study, 60 μM, competed with DiO for membrane incorporation but was unable to alter membrane fluidity. It is therefore highly unlikely that an unspecific nongenomic effect could cause the changes in diffusion behavior of F-dexa observed in our work.

Most remarkably, the binding curve that we were able to obtain with FCS revealed positive cooperativity of the binding process. Thus, ligand binding to the membrane binding sites enhances further binding. In other words, once a certain threshold in the concentration of GCs is reached, small changes in the GC concentration can elicit pronounced effects. Physiologically, GC plasma levels undergo diurnal variations, and the body can react very precisely to sudden alterations of cortisol (or corticosterone, for rodents) concentrations. It is not known, which GC concentrations are reached locally at the pituitary gland but – under the assumption that the membrane binding sites described here are related to the feedback inhibition of ACTH release – a membrane receptor with cooperative binding properties would be well suited to mediate rapid adaptations of the system required in situations of acute stress. Basal regulation might be mediated by GC-inhibition of ACTH synthesis *via* genomic pathways.

Specificity of dexamethasone binding was shown by preincubation experiments with various steroids. Not only unlabeled dexamethasone, but also the natural ligand corticosterone were able to prevent subsequent F-dexa–binding, whereas the sex steroids estradiol and progesterone had no influence. Thus, F-dexa binds to GC-specific binding sites on the cell membrane of the corticotrope cell line AtT-20.

Like the affinity of binding, the binding characteristics were also clearly distinct from the iGR. The iGR antagonist RU486 was unable to prevent F-dexa membrane-binding, as was progesterone, which is known to bind – albeit with low affinity – to the iGR (which is expressed in the AtT-20 cell line in high abundance). Distinct membrane binding sites – possibly related to opioid-like receptors – have been found in the amphibian brain (Evans et al., 2000). By contrast, the mGR found on lymphoma cells and peripheral blood leukocytes was detected using an antibody against iGR (Gametchu, 1987; Bartholome et al., 2004), indicating structural resemblance of these binding sites with the iGR. With the data presented here, no direct conclusion on the structure of the binding sites can be drawn, because attachment to the cell membrane would certainly alter the binding characteristics of the iGR as well. However, it seems reasonable that distinct binding characteristics should be associated with distinct function.

Another remarkable feature of the observed binding is the small diffusion constant (3×10^{-10} cm^2 s^{-1}) obtained for the bound ligand. Measurements of ligand binding to cell membranes typically result in diffusion constants that are one order of magnitude higher (e.g. Briddon et al., 2004; Rigler et al., 1999). Possible interpretations of a smaller diffusion constant are that the binding sites form huge clusters or, alternatively, that they are not freely diffusing in the cell membrane but are more closely attached to, e.g. the cytoskeleton. This interpretation is supported by the fact that preincubation with PTX abolished the τ_3 component in the autocorrelation function on the membrane position while F-dexa enrichment was still apparent. Pertussis toxin decouples G-proteins from the receptor through ADP ribosylation of the Gα subunit. Since F-dexa can still bind to the receptor (the ligand is enriched in the cell membrane) it can be concluded that upon G-protein decoupling also clustering and/or cytoskeleton attachment of the receptor is disrupted. Like dexamethasone, PTX was unable to change membrane fluidity at the concentration used in our experiments.

Redistribution of the actin cytoskeleton plays a central role in ACTH-release (Mains et al., 1999) and has also been shown to be involved in rapid steroid effects. The steroid hormone testosterone has been described to increase PSA secretion from a prostate cell line through binding to a membrane receptor and modifying the actin cytoskeleton (Papakonstanti et al., 2003). Testosterone is also able to act on GnRH release through a membrane receptor and this membrane AR-signaling has been shown to be coupled to a Gi protein (Shakil et al., 2002). The estrogen receptor α has been shown to interact with the

cytoskeletal protein p130Cas (which is involved in the linkage of the actin cytoskeleton to the extracellular matrix) resulting in Erk1/2 MAPK activation (Cabodi et al., 2004).

In summary, this study demonstrates for the first time specific, positive cooperative and G-protein-coupled membrane glucocorticoid receptors, distinct from their intracellular counterparts and well suited to mediate acute HPA-axis regulation.

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