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Testosterone Signaling through Internalizable Surface Receptors in Androgen Receptor-free Macrophages

W. Peter M. Benten,* Michèle Lieberherr,† Olaf Stamm,* Christian Wrehlke,* Zhiyong Guo,* and Frank Wunderlich*‡

*Division of Molecular Parasitology and Centre of Biological-Medical Research, Heinrich-Heine-University, 40225 Duesseldorf, Germany; and †Centre National de la Recherche Scientifique, Unité Propre de Recherche 1524, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas, France

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Testosterone acts on cells through intracellular transcription-regulating androgen receptors (ARs). Here, we show that mouse IC-21 macrophages lack the classical AR yet exhibit specific nongenomic responses to testosterone. These manifest themselves as testosterone-induced rapid increase in intracellular free [Ca^{2+}], which is due to release of Ca^{2+} from intracellular Ca^{2+} stores. This Ca^{2+} mobilization is also inducible by plasma membrane-impermeable testosterone-BSA. It is not affected by the AR blockers cyproterone and flutamide, whereas it is completely inhibited by the phospholipase C inhibitor U-73122 and pertussis toxin. Binding sites for testosterone are detectable on the surface of intact IC-21 cells, which become selectively internalized independent on caveolae and clathrin-coated vesicles upon agonist stimulation. Internalization is dependent on temperature, ATP, cytoskeletal elements, phospholipase C, and G-proteins. Collectively, our data provide evidence for the existence of G-protein-coupled, agonist-sequestrable receptors for testosterone in plasma membranes, which initiate a transcription-independent signaling pathway of testosterone.

INTRODUCTION

Steroid hormones act on target cells through their cognate receptors belonging to the intracellular steroid receptor superfamily (reviewed by Evans, 1988; Beato, 1989; Jensen, 1996). These are hormone-regulated transcription factors eliciting either induction or repression of specific genes (reviewed by Kumar and Tindall, 1998). Evidence, however, is accumulating that steroids can also cause nongenomic responses of cells, i.e., responses not mediated through classical nuclear receptors but rather responses initiated at the plasma membrane, presumably through unconventional surface receptors (reviewed by Brann *et al.*, 1995; Wehling, 1997; Grazzini *et al.*, 1998; Nadal *et al.*, 1998; Nemere and Farach-Carson, 1998).

Testosterone, for example, is a steroid hormone that has been described to exert both genomic effects and, recently, also nongenomic effects. Genomic responses of testosterone are mediated through intracellular androgen receptors (ARs), which are 110-kDa proteins with domains for androgen binding, nuclear localization, dimerization, DNA binding, and transactivation (reviewed by Zhou *et al.*, 1994; Quigley *et al.*, 1995). The nongenomic effects are assumed to be mediated through unconventional receptors in plasma membranes. In rat osteoblasts, these membrane receptors have been recently shown to belong to the class of membrane receptors coupled to phospholipase C via a pertussis toxin-sensitive G-protein, which, after binding of testosterone, mediate a rapid increase in intracellular free [Ca^{2+}] ([Ca^{2+}]$_i$) and inositol 1,4,5-trisphosphate formation (Lieberherr and Grosse, 1994).

However, unconventional testosterone receptors in plasma membranes can be suspected to be classical intracellular ARs tightly associated with the plasma membrane. This view is not unlikely, because ARs, in contrast to most other steroid receptors, are reported to be localized in the cytoplasm and not in nuclei (Simental *et al.*, 1991; Zhou *et al.*, 1994). Also, rat osteoblasts are known to be typical testosterone target cells containing intracellular AR (Colvard *et al.*, 1989). Here, however, we provide evidence for functional testosterone receptors in plasma membranes of macrophages, which lack classical intracellular AR and which respond to plasma membrane-impermeable testosterone nongenomically with intracellular Ca^{2+} mobilization.

MATERIALS AND METHODS

IC-21 Cells

The SV40-transformed peritoneal macrophage cell line IC-21 (ATCC no. TIB-186) generated from a C57BL/6 mouse was obtained from

‡ Corresponding author. E-mail address: frank.wunderlich@uni-duesseldorf.de.

the American Type Culture Collection (Manassas, VA). The cells were grown in Iscove's modified Dulbecco's medium (IMDM) and L-glutamine (Life Technologies, Eggenstein, Germany) supplemented with 10% FCS, 50 μM β-mercaptoethanol, and 3.024 g of $NaHCO_3$ at 37°C, 5% CO_2, and 96% humidity, subcultured once per week, and incubated for 24 h in serum-free medium before use.

RNA Isolation

RNA was isolated from IC-21 cells and testes removed from C57BL/10 mice using the GTC-CsCl method (Sambrook *et al.*, 1989)

Reverse Transcription (RT)-PCR

The PCRs were carried out with the RNA PCR kit from Perkin Elmer (Weiterstadt, Germany). The initial random-primed RT was performed with 1 μg of total RNA in a Minicycler (MJ Research, Biozym, Oldendorf, Germany) and AmpliTaq DNA Polymerase (Perkin Elmer), and four different oligonucleotide primer pairs were used for PCR amplification of the AR. The primer pair AR-P1 (5′-GACCTTGGATGGAGAACTACTCCG-3′) and AR-M1 (5′-GGTTGGTTGTTGTCATGTCCGGC-3′) spanned 511 nucleotides (nt) of the DNA-binding domain of AR. The carboxyl terminus of the AR was probed with three different primer pairs: AR-P2 (5′-ACGTCCTGGAAGCCATTGAGCC-3′) and AR-M2 (5′-CTTGGTGAGCTGGTAGAAGCGC-3′), as well as the sense primers AR-P3 (5′-GAATGTCAGCCTATCTTTCTTAACG-3′) and AR-P4 (5′-TCCTTTGCTGCCTTGTTATCTAGC-3′) together with the antisense primer AR-M3 (5′-TGCCTCATCCTCACACACTGGC-3′). As a control for the integrity of the RNA isolated from IC-21 cells, the low abundant mRNA of the *mzfm* gene was amplified by RT-PCR using the primer pairs mzfm-P1 (5′-GGCTTAACACCCGAGAGTTCC-3′) and mzfm-M1 (5′-TTATCCTGAGCTGACTGAGGG-3′) as well as mzfm-P2 (5′-GGGTCTATCGCCTGCATCAAGG-3′) and mzfm-M2 (5′-TCCTCACTCTCATGGCTCGG-3′) (Wrehlke *et al.*, 1997, 1999). The AR-P1 and AR-M1 were subjected to 32 cycles at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 1 min. The other primer pairs were used in 32 cycles at 95°C for 1 min, at 60°C for 1 min, and at 72°C for 1 min. PCR fragments were separated in 2% Tris borate-EDTA gels, eluted, and cloned into the *pMOSBlue* T vector (Amersham, Braunschweig, Germany).

DNA Sequencing

Clones were sequenced with Thermo Sequenase fluorescent-labeled sequencing kit (Amersham) and analyzed with the LICOR sequencer (MWG, Ebersberg, Germany)

Western Blotting

Proteins were separated in 8% SDS polyacrylamide slab gels (Laemmli, 1970) and blotted onto nitrocellulose membranes (0.45 μm pore size; Schleicher & Schuell, Dassel, Germany) with a Biometra (Göttingen, Germany) semidry blot cell. Membranes were incubated with the anti-AR antibody AR (N-20) (Santa Cruz Biotechnology, Heidelberg, Germany) at a concentration of 0.1 μg/ml diluted in 10 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, and 0.05% Tween (TST) at 23°C for 1 h, washed three times with TST for 10 min, and incubated at 23°C for 1 h with HRP-conjugated goat-anti-rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology) diluted 1:50,000 in TST. Antibody detection was performed by the enhanced chemiluminescence plus Western blotting detection system (Amersham).

Determination of $[Ca^{2+}]_i$

IC-21 cells in IMDM were grown on poly-L-lysine-coated glass coverslips until confluence. Then they were washed twice with 20 mM HEPES buffer, pH 7.2, supplemented with 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM Na_2HPO_4, and 1 mg/ml glucose before they were loaded with 1 μM Fura-2/AM (Amersham, Les Ulis, France) in the same HEPES buffer at room temperature for 30 min. The Ca^{2+} measurements were performed in a Hitachi (Mountain View, CA) F-2000 spectrofluorometer at 37°C. Reagents were added directly to the cuvette under continuous stirring. Testosterone, testosterone 3-(*O*-carboxymethyl)oxime-BSA (testosterone-BSA), estradiol, flutamide, and pertussis toxin were from Sigma (St. Quentin, Fallavier, France); 1-dehydrotestosterone was from Steraloids (Wilton, NH). Testosterone-BSA contained <0.1% free testosterone. Cyproterone was kindly provided by Schering (Berlin, Germany); U-73122 [1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl)-1H-pyrrole-2,5-dione] and U-73343 [1-(6-((17β-3-metoxystra-1,3,5(10)-trien-17-yl)-amino)-hexyl-2,5-pyrrolidine-2,5-dione] were from Biomol Reseach Laboratory (Plymouth, MA). Hormones were dissolved in ethanol. The final concentration of ethanol in the cuvette never exceeded 0.01%, which did not affect $[Ca^{2+}]_i$ (cf. Lieberherr and Grosse, 1994). The Fura-2 fluorescence was measured at 340 nm (calcium-bound Fura-2) and 380 nm (free Fura-2) for excitation and 510 nm for emission. The $[Ca^{2+}]_i$ was computed from the ratio of 340:380 nm fluorescence values as desribed previously (Grynkiewicz *et al.*, 1985).

Labeling with Testosterone-BSA-FITC

IC-21 cells (5×10^6 cells/ml) in IMDM were allowed to adhere onto poly-L-lysine-coated glass coverslips overnight, washed twice with PBS^+ (140 mM NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH 7.2), and then incubated at room temperature for 5 s up to 1 h with 100 μl of 1.5×10^{-5} M testosterone-BSA-FITC (Sigma, Deisenhofen, Germany). Only BSA-FITC and BSA were used in the corresponding control experiments. After two washings with PBS^+, cells were fixed with 0.5% paraformaldehyde for 30 min. Coverslips were briefly rinsed with PBS^+ and mounted on slides in a 1:1 (vol/vol) mixture of glycerol and Vectashield (Vector Laboratories, Burlingame, CA) containing 2% (wt/vol) 1,4-diazabicyclo-[2.2.2]octane (Merck, Darmstadt, Germany).

Confocal Laser Scanning Microscopy

The specimens were analyzed with a Leica TCS NT confocal laser scanning microscope (CLSM), version 1.5.451 (Leica Lasertechnik, Heidelberg, Germany). FITC fluorescence was excited by a 488-nm argon laser line, and Cy3 and TRITC fluorescence was excited by a 568-nm krypton laser line, respectively. Z-series optical sections were taken at 0.5-μm intervals (Benten *et al.*, 1998, 1999) and evaluated using Adobe Photoshop 5.0 for Windows (Adobe Systems, Mountain View, CA) and CorelDRAW 8 for windows (Corel, Ottawa, Ontario, Canada).

Flow Cytometry

IC-21 cells (10^7 cells/ml) were suspended in PBS^+, and aliquots of 150 μl were centrifuged. The cell pellets were labeled with testosterone-BSA-FITC, BSA-FITC, and the anti-AR antibody AR (N-20) (2 μg/ml) for 5 s up to 1 h as described above. Labeling with rabbit antiserum to sex hormone-binding globulin (SHBG) (batch 64.5; a gift from W. Rosner, St. Luke's-Roosevelt Hospital Center, New York, NY) was performed with dilutions between 1:60,000 and 1:60 for 1 h. Anti-rabbit IgG (whole molecule) FITC conjugate (working dilution, 1:320; Sigma) was used as secondary antibody for 45 min as described previously (Benten *et al.*, 1991). Cells were analyzed in a FACScan (Becton Dickinson, Sunnyvale, CA) with a sample size of 10,000 cells gated on the basis of forward and side scatter. The data were stored and processed using the FACScan software as described previously (Benten *et al.*, 1991).

Internalization of Testosterone-BSA-FITC

Intact IC-21 cells were incubated at room temperature or 37°C for 15 min or 1 h with testosterone-BSA-FITC (1.5×10^{-5} M), BSA-FITC

(1.5×10^{-5} M), concanavalin A (Con A)-rhodamine (1:50; Vector), or a rat anti-mouse F4/80 antibody (2 μg/ml; a gift from H. Mossmann, Max-Planck-Institut for Immunobiology, Freiburg, Germany) as first antibody, Biotin-SP-conjugated AffiniPure mouse anti-rat IgG (heavy and light chain; 1:500; Jackson ImmunoResearch, West Grove, PA) as secondary antibody, and streptavidin-fluorescein (6 $\mu g/10^7$ cells; Amersham). Colocalization was performed with LysoTracker Red DND-99 (10 μM; Molecular Probes, Göttingen, Germany), the anti-clathrin antibody heavy chain (N-19) (2 μg/ml; Santa Cruz Biotechnology), and a secondary donkey anti-goat-Cy3 antibody (1:200; a gift from P. Traub, Max-Planck-Institut for Cell Biology, Ladenburg, Germany) or with the anti-caveolin antibody caveolin-1 (N-20) (2 μg/ml; Santa Cruz Biotechnology) and as secondary antibody TRITC-conjugated AffiniPure goat anti-rabbit IgG (heavy and light chain; 1:80; Jackson ImmunoResearch). The samples were fixed, embedded, and analyzed by CLSM as described above.

Perturbation of Internalization

Intact IC-21 cells were preincubated at different temperatures or at 37°C with different substances for varying periods before incubation with testosterone-BSA-FITC (1.5×10^{-5} M, if not otherwise stated). The substances were NaN_3 (Merck), pertussis toxin (Sigma), U-73122, U-73343, and cytochalasin B and nocodazole (Sigma). The samples were fixed and analyzed by flow cytometry and CLSM as described above.

RESULTS

Absence of Intracellular AR

Different techniques were used to examine the presence of classical intracellular AR in mouse macrophages of the cell line IC-21. Both intact cells and permeabilized cells were investigated by flow cytometry using the anti-AR antibody AR (N-20), which is directed against an epitope corresponding to the amino acids 2–21 mapping at the amino terminus of the AR. Incubation of intact IC-21 cells with this antibody did not result in any significant labeling of the cells (Figure 1A). After permeabilization of IC-21 cells, incubation with AR (N-20) resulted in a slight increase in fluorescence intensity. However, this fluorescence was not AR specific, because it could not be competitively displaced by an AR (N-20)-specific blocking peptide (Figure 1A). Also, the anti-AR antibody AR (N-20) did not detect AR in IC-21 cells in Western blots, although this antibody reacted with the AR band at 110 kDa in AR-expressing human prostate cancer LNCaP cells (Figure 1B) (Taplin *et al.*, 1995). Moreover, RT-PCR was used to detect AR mRNA in IC-21 cells and in mouse testes as a control. Using primers spanning the DNA-binding domain and three different regions from the carboxyl terminus of the AR, RT-PCR revealed the expected bands in testes but not in IC-21 macrophages (Figure 1C). DNA sequencing confirmed that the PCR fragments derived from testes RNA contained the predicted regions of the AR. Moreover, the RNA isolated from IC-21 cells was intact, because the low abundant mRNA of the single-copy gene *mzfm* (Wrehlke *et al.*, 1997, 1999) could be amplified by the same RT-PCR procedure using two different primer pairs (Figure 1C).

Testosterone-induced Ca^{2+} Mobilization

IC-21 cells were loaded with Fura-2 to determine the effect of testosterone on $[Ca^{2+}]_i$. Testosterone at the physiological concentration of 10 nM triggered an immediate spike in $[Ca^{2+}]_i$, which represented a Ca^{2+} increase by ~100 nM (Figure 2A). Such a spike was also induced when the cells were preincubated with the AR blockers cyproterone and flutamide in excess (Figure 2B). The Ca^{2+} increase may be due to influx of extracellular Ca^{2+} and/or release of Ca^{2+} from intracellular Ca^{2+} stores. To test this, extracellular Ca^{2+} was removed by EGTA before testosterone was added. Testosterone was still able to induce the Ca^{2+} spike (Figure 2C). However, the testosterone-induced Ca^{2+} spike was totally abolished by the direct phospholipase C inhibitor U-73122 but not by the inactive control compound U-73343 (Figure 2D). Also, the Ca^{2+} spike could be inhibited by pertussis toxin (Figure 2E). Mn^{2+} did not induce any quenching after testosterone treatment (Figure 2F).

However, when testosterone was added for a second or third time shortly after the first addition, it induced a prolonged elevation of $[Ca^{2+}]_i$ instead of a Ca^{2+} spike (Figure 3A). This prolonged elevation in $[Ca^{2+}]_i$ was due to both Ca^{2+} release and Ca^{2+} import, because after removal of extracellular Ca^{2+}, treatment with testosterone resulted only in a Ca^{2+} spike instead of a prolonged elevation (Figure 3B).

Testosterone coupled to BSA, which is not freely permeable through the cell membrane, had the same effects on $[Ca^{2+}]_i$ as free testosterone. It induced first a Ca^{2+} spike, whereas a second addition caused a prolonged elevation of $[Ca^{2+}]_i$ (Figure 3C). The Ca^{2+} spike was only due to Ca^{2+} release, whereas the prolonged elevation of $[Ca^{2+}]_i$ was due to both Ca^{2+} release and Ca^{2+} import (Figure 3, D and E). Moreover, pertussis toxin blocked the testosterone-BSA-induced mobilization of intracellular Ca^{2+} (Figure 3F).

The amount of released Ca^{2+} induced by the first addition of both testosterone and testosterone-BSA increased with increasing concentrations, reaching apparent saturation at ~10 nM testosterone and 100 nM testosterone-BSA, respectively (Figure 4A). Moreover, cells responded to a second addition of testosterone again with a Ca^{2+} spike when the period between first and second additions exceeded at least 10 min (Figure 4B).

The rapid nongenomic effects of testosterone on $[Ca^{2+}]_i$ of IC-21 cells were specific for testosterone. First, estradiol caused Ca^{2+} responses differing from those induced by testosterone (Figure 5). Thus, estradiol at a concentration of only 1 nM induced a Ca^{2+} spike of 100 nM Ca^{2+}, whereas the Ca^{2+} spike was halved to 50 nM when estradiol was added at 10 nM (Figure 5A). Moreover, a second addition of estradiol resulted in a second Ca^{2+} spike instead of a prolonged elevation. In addition, the estradiol-induced Ca^{2+} spikes were due to both Ca^{2+} release from intracellular stores and Ca^{2+} influx, because removal of extracellular Ca^{2+} by EGTA led to a shortened and reduced Ca^{2+} spike after estradiol treatment (Figure 5B). Second, 1-dehydrotestosterone, the structure of which is very similar to that of testosterone, did not induce any specific Ca^{2+} response of the cells (Figure 5C).

Surface Binding of Testosterone

Testosterone binding sites were identified on the surface of intact IC-21 cells. When cells were incubated with the impeded ligand testosterone-BSA coupled to FITC for 5 s, flow cytometry revealed an increase in fluorescence intensity (Figure 6A). Incubation with BSA-FITC alone or together with free testosterone did not result in any significant labeling in comparison with unlabeled control cells (cf. Figure 7A). CLSM detected the

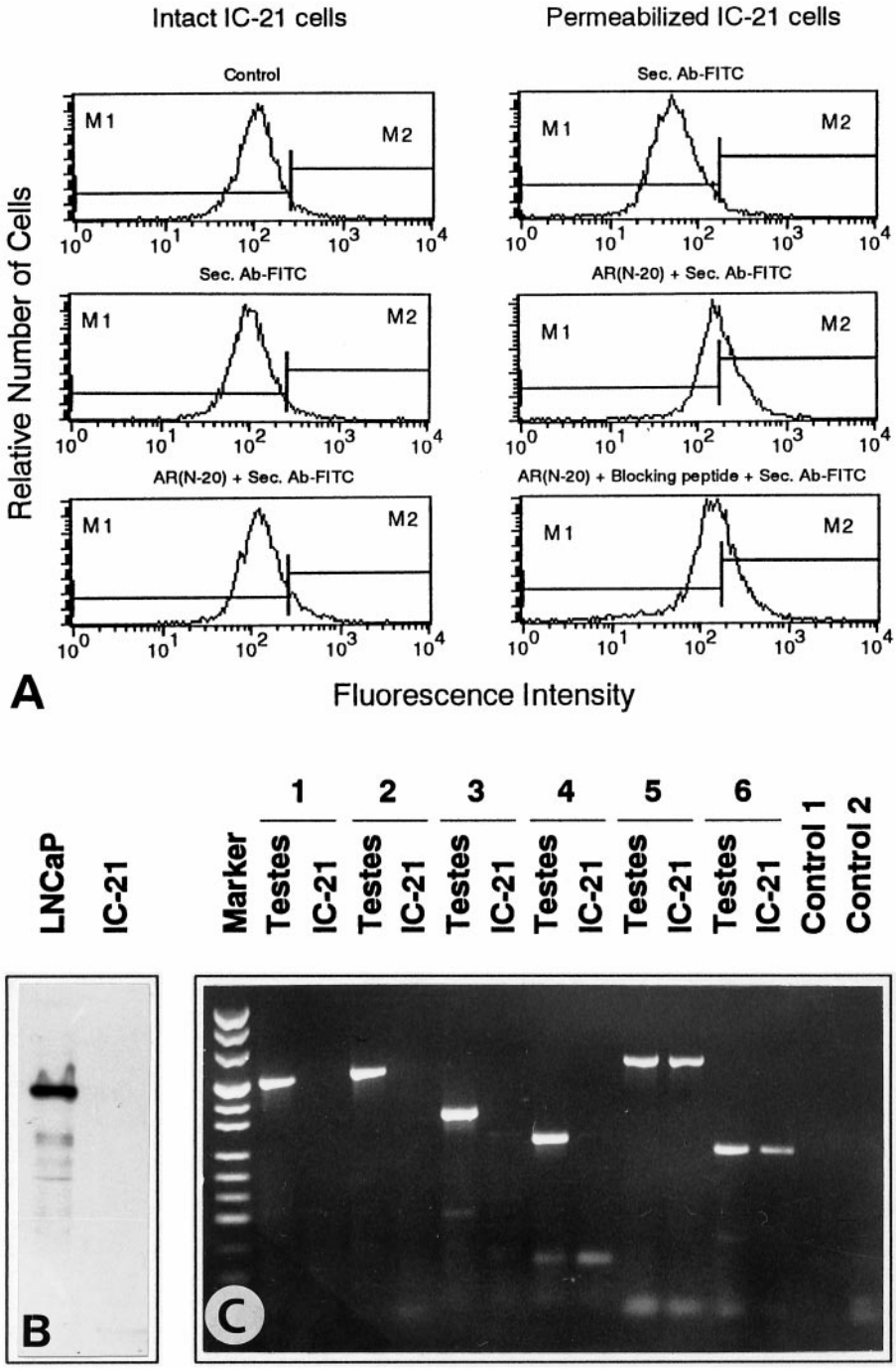

Figure 1. Absence of intracellular AR in IC-21 cells. (A) Flow cytometry of intact and permeabilized IC-21 cells incubated for 1 h with the anti-AR antibody AR (N-20) and secondary fluorescent antibody (Sec. Ab-FITC). In permeabilized cells, the blocking peptide AR (N-20)P cannot competitively displace the slight increase in fluorescence of AR (N-20). (B) IC-21 cells and LNCaP cells as a control were subjected to Western blotting using the anti-AR antibody AR (N-20) and the ECL detection system. Only LNCaP cells reveal the AR band at 110 kDa. (C) RT-PCR with RNA isolated from mouse testes and IC-21 cells with markers (pUC mix, MBI Fermentas) on the left. The primer pair AR-P1/AR-M1 (1) spanned a 511-nt region of the DNA-binding domain of AR. The primer pairs AR-P2/AR-M2 (2), AR-P3/AR-M3 (3), and AR-P4/AR-M3 (4) spanned regions of the carboxyl terminus of the AR with 560, 365, and 281 nt, respectively. The expected bands were only revealed in testes. The primer pairs mzfm-P1/mzfm-M1 (5) and mzfm-P2/mzfm-M2 (6) yielded bands of 640 and 253 nt, respectively, of the low abundant mRNA of the gene *mzfm* in both testes and IC-21 cells. Control 1, RT-PCR without primers; Control 2, PCR with the primer pair AR-P1/AR-M1 without RNA.

fluorescence of the bound testosterone-BSA-FITC exclusively on the surface of IC-21 cells (Figure 6B). The same labeling pattern on the surface showed the plasma membrane marker ConA-rhodamine (cf. Figure 7B). There is some evidence that the SHBG can bind to specific receptors on the plasma membranes, which are able to mediate rapid effects of testosterone and estradiol (Rosner *et al.*, 1998). However, the surface of IC-21 cells had not bound any significant amounts of SHBG, as identified by flow cytometry and CLSM using an anti-SHBG antibody (our unpublished data).

Selective Internalization of Testosterone Receptors

There is evidence that G-protein-coupled surface receptors can be sequestered (reviewed by Koenig and Edwardson, 1997). To identify such a possible sequestration of surface

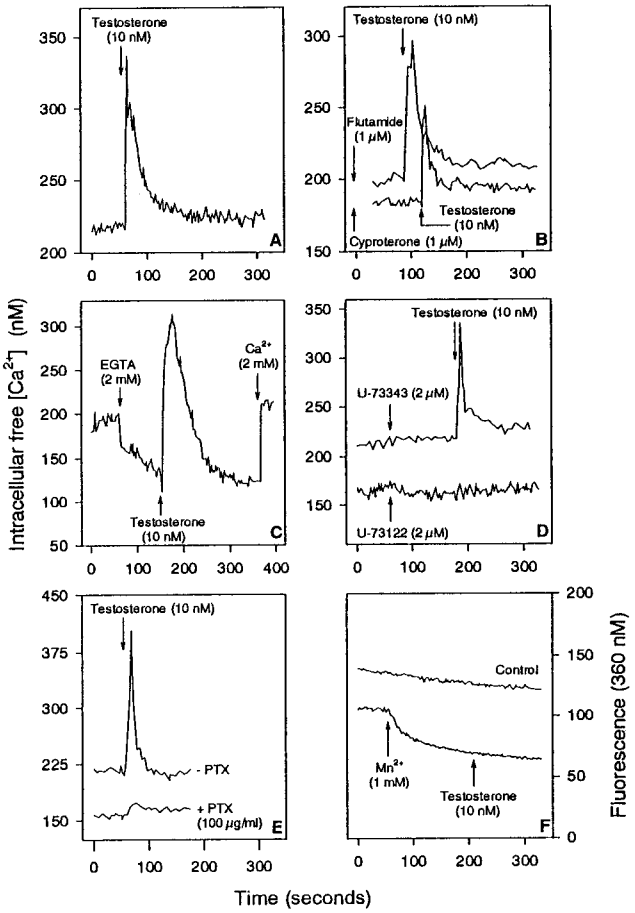

Figure 2. Testosterone-induced Ca^{2+} mobilization in IC-21 cells. (A) Testosterone causes an immediate Ca^{2+} spike. (B) Cells were treated with cyproterone for 30 min or flutamide for 60 min before adding testosterone. (C) Cells were incubated with EGTA for 90 s before addition of testosterone. (D) Cells were treated with the phospholipase C inhibitor U-73122 or with the inactive control compound U-73343 for 2 min before adding testosterone. (E) Cells were pretreated with pertussis toxin for 16 h (+ PTX) before addition of testosterone. (F) Cells were incubated with Mn^{2+} for 2 min before adding testosterone. Arrows indicate addition of substances to IC-21 cells.

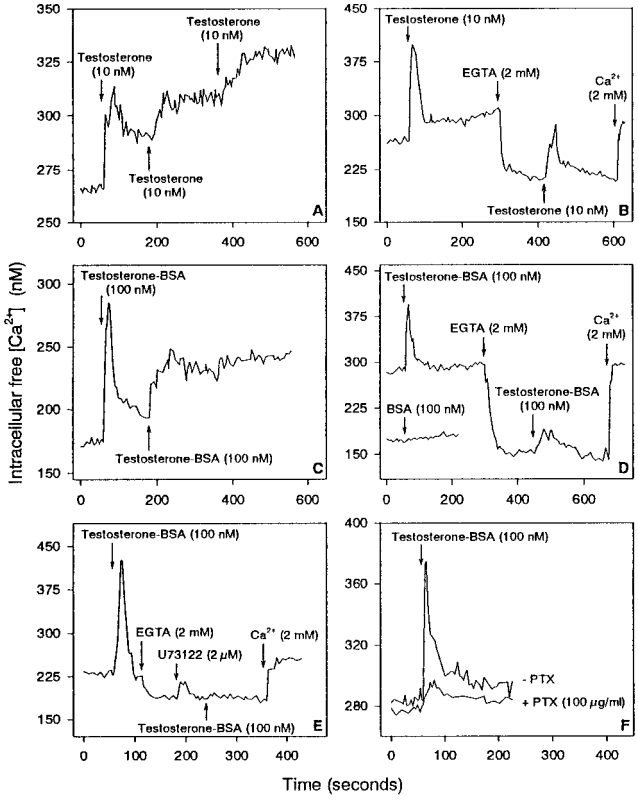

Figure 3. Ca^{2+} Responses of IC-21 cells to testosterone and testosterone-BSA. (A) A second or third addition of testosterone shortly after the first treatment induces a sustained increase in $[Ca^{2+}]_i$ rather than a Ca^{2+} spike. (B) Cells were treated for 4 min with testosterone and then for 2 min with EGTA before the second addition of testosterone that induced only a Ca^{2+} spike. (C) The first addition of testosterone-BSA induced a Ca^{2+} spike, whereas the second addition induced a sustained Ca^{2+} increase. (D) BSA alone had no effect on $[Ca^{2+}]_i$ (lower line). After incubation with testosterone-BSA for 2 min, cells were treated with EGTA before the second addition of testosterone-BSA. (E) After incubation of cells with testosterone-BSA, the cells were pretreated with both EGTA and U-73122 before the second addition of testosterone-BSA. (F) Cells were treated with pertussis toxin for 16 h (+ PTX) before adding testosterone-BSA. Arrows indicate addition of substances to IC-21 cells.

testosterone receptors, IC-21 cells were incubated with testosterone-BSA-FITC between 5 s and 1 h and analyzed by flow cytometry and CLSM. Flow cytometry revealed an increased labeling with progressive incubation periods (Figure 6A). When incubation lasted for 5 s or 1 min, >80% of the cells were labeled with testosterone-BSA-FITC. After 5 min, however, the percentage of labeled cells was increased to >95%, and the fluorescence intensity of the cells was higher. Thereafter, the number of fluorescent cells remained about the same, whereas the fluorescence intensity of the cells still increased with progressing incubation times, reaching a maximum after ~1 h. Obviously, cells bound increasing amounts of testosterone-BSA-FITC with progressing incubation times. In parallel with the increase in fluorescence intensity, CLSM revealed an increasing punctate fluorescence inside cells (Figure 6B). Whereas the fluorescence was exclusively localized on the cell surface after 5 s and 1 min, punctate weak fluorescence emerged after 5 min inside cells at their periphery, besides surface fluorescence. After 15 min and 1 h, the punctate fluorescence was increased in intensity and was distributed throughout the whole cytoplasm inside cells.

The internalization of testosterone binding sites was selective. BSA alone or BSA-FITC did not induce any sequestration (Figure 7A). Also, when cells were incubated with free testosterone together with BSA-FITC for 15 min, there was no sequestration, although sequestration was observed when cells were incubated in parallel with testosterone-BSA-FITC (Figure 7, A and B). Moreover, internalization occurred neither with surface-bound ConA-rhodamine nor

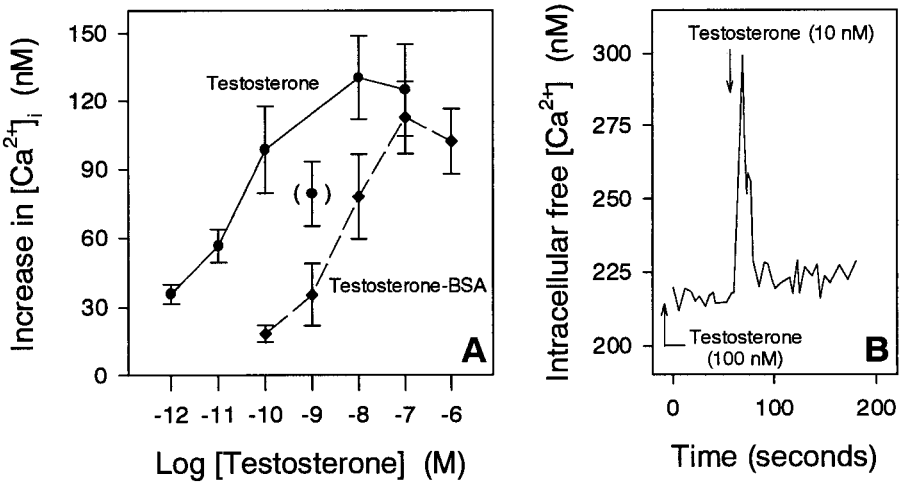

Figure 4. Effect of different concentrations of testosterone, testosterone-BSA, and testosterone pretreatment on $[Ca^{2+}]_i$ of IC-21 cells. (A) Increase in $[Ca^{2+}]_i$ with increasing concentrations of testosterone and testosterone-BSA, respectively. (B) Cells were pretreated with testosterone for 60 min before adding testosterone for a second time.

with the macrophage specific surface marker F4/80 identified by a rat monoclonal antibody against F4/80. Even if the surface labeling of IC-21 cells was performed in the presence of testosterone, there was no internalization of ConA-rhodamine and F4/80 (Figure 7B).

Gross Characteristics of Receptor Internalization

The sequestrated testosterone-BSA-FITC was not contained in acidic vesicles. The latter were identified by CLSM using LysoTracker Red DND-99. The vesicles stained with LysoTracker Red DND-99 did not colocalize with the green punctate fluorescence of testosterone-BSA-FITC (Figure 8A). Also, the sequestrated testosterone binding sites did colocalize neither with clathrin as detected by anti-clathrin antibodies (Figure 8B) nor with caveolin as monitored by anti-caveolin antibodies (Figure 8C).

To determine the effect of various parameters on receptor internalization, intact IC-21 cells were incubated with testosterone-BSA-FITC for 15 min, and subsequently fluorescence intensity was analyzed by flow cytometry, and fluorescence localization was analyzed by CLSM. Figure 9A shows that internalization of testosterone-BSA-FITC could be competitively reduced by testosterone but not by the structurally similar compound 1-dehydrotestosterone. Moreover, internalization of surface-bound testosterone-BSA-FITC was largely inhibited at temperatures below ~16°C, whereas temperature did not affect binding of testosterone-BSA-FITC to the cell surface (Figure 9B). Depletion of ATP by sodium azide resulted in a decrease of fluorescence intensity by

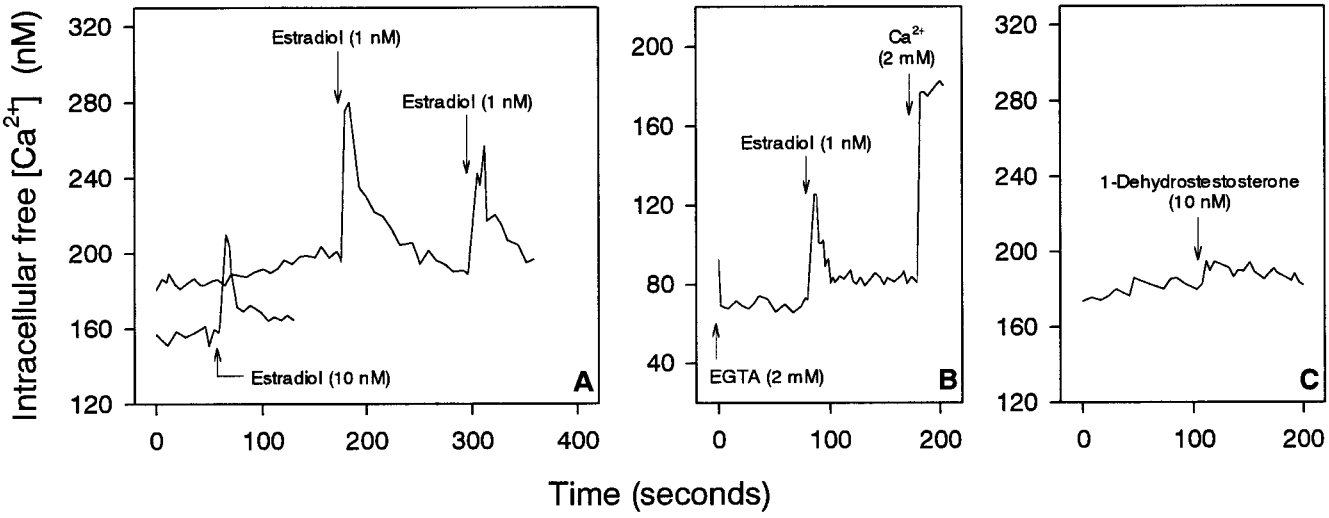

Figure 5. Effect of estradiol and 1-dehydrotestosterone on $[Ca^{2+}]_i$ of IC-21 cells. (A) Treatment of cells with 1 nM estradiol resulted in a higher increase in $[Ca^{2+}]_i$ than 10 nM estradiol. A second addition of estradiol shortly after the first addition again induced a Ca^{2+} spike. (B) Cells were incubated with EGTA for 1 min before adding estradiol. (C) Addition of 1-dehydrotestosterone had no effect on $[Ca^{2+}]_i$. Arrows indicate the addition of substances to IC-21 cells.

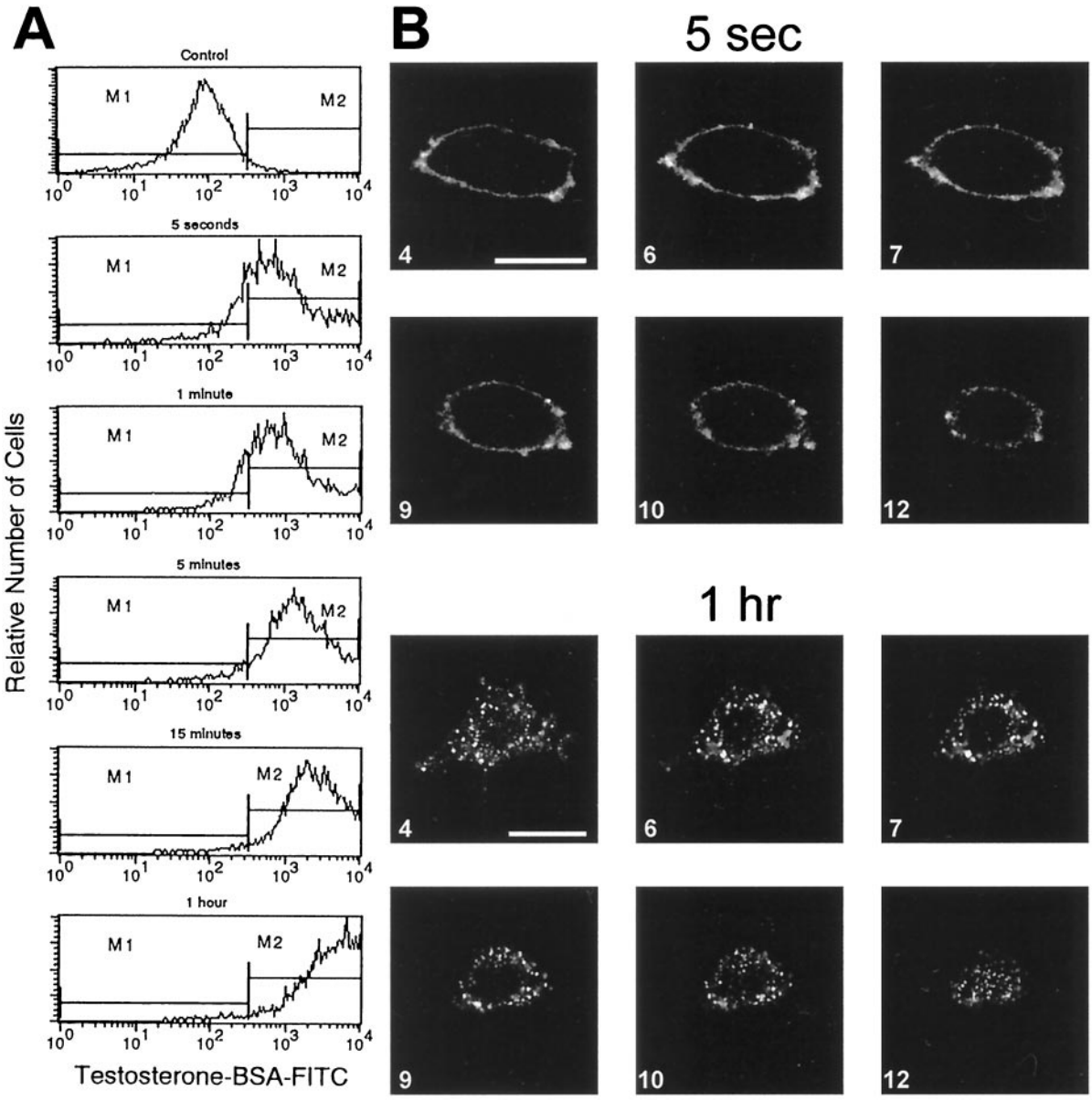

Figure 6. Surface binding sites of testosterone and their internalization in intact IC-21 cells. (A) Cells were incubated with testosterone-BSA-FITC for various periods between 5 s and 1 h and then analyzed by flow cytometry. (B) CLSM of cells incubated with testosterone-BSA-FITC for 5 s and 1 h. Optical slices of 0.5 μm. Bars, 10 μm.

~40% (Table 1). This fluorescence was localized almost exclusively on the cell surface. Internalization of surface-bound testosterone-BSA-FITC also could be abolished by preincubation with pertussis toxin (Table 1). The phospholipase C inhibitor U-73122 but not the inactive compound U-73343 also blocked internalization, because preincubation with 2 μM U-73122 for 2 min resulted in complete surface localization of testosterone-BSA-FITC, whereas controls have internalized testosterone-BSA-FITC, as revealed by the ~10% higher fluorescence intensity (Table 1). Finally, internalization obviously involved cytoskeletal elements. Both the tubulin blocker nocodazole and the microfilament blocker cytochalasin B inhibited internalization but not surface binding of testosterone-BSA-FITC (Table 1). Control cells revealed higher fluorescence intensities by ~25% because of internalization of surface-bound testosterone-BSA-FITC.

DISCUSSION

This study provides evidence for the presence of functional receptors for testosterone on the surface of the intracellular AR-free macrophages of the cell line IC-21. Using the im-

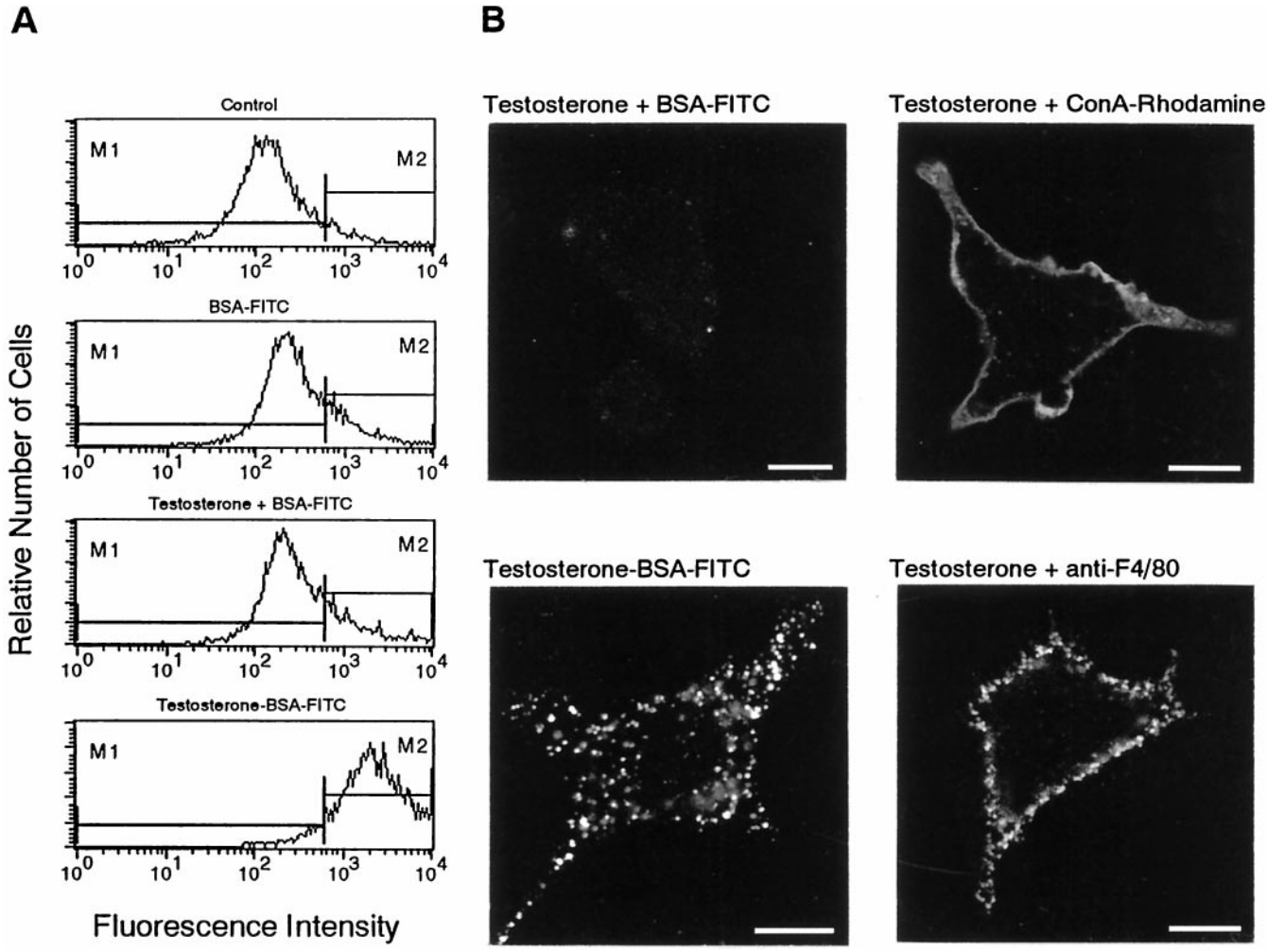

Figure 7. Selective internalization of surface binding sites of testosterone. (A) Flow cytometry of IC-21 cells treated with the indicated substances for 15 min. (B) CLSM of cells incubated for 15 min with the indicated substances and the corresponding FITC-labeled secondary antibody. Note the difference between the smooth uniform surface labeling with Con A-rhodamine and the granular surface fluorescence of the F4/80 antigens. Bars, 10 μm.

peded ligand testosterone-BSA-FITC, which is not freely permeable to the plasma membrane, CLSM localizes testosterone binding sites on plasma membranes of intact IC-21 cells. These membrane receptors for testosterone cannot be identical with classical intracellular ARs, because ARs are not expressed in IC-21 cells. ARs were detectable by neither flow cytometry nor Western blotting using the anti-AR antibody AR (N-20) directed against the amino terminus of AR or at the mRNA level by RT-PCR using primers probing the DNA-binding domain and three different regions of the carboxyl terminus of the AR. Moreover, the AR blockers cyproterone and flutamide were not able to inhibit binding of testosterone to IC-21 cells. In accordance, previous studies also could not identify intracellular AR in macrophages of the cell line RAW 264.7 (Benten *et al*., 1999) and in macrophages of different tissues (Gulshan *et al*., 1990; Frazier-Jessen and Kovacs, 1995; Miller *et al*., 1996), although the presence of AR was reported in immature monocytic cells (Danel *et al*., 1985; Cutolo *et al*., 1993). The reason for this discrepancy is unknown, but the expression of AR in monocytes and macrophages may be developmentally regulated, as it is postulated, for example, to occur in T cells (Kovacs and Olsen, 1987; Viselli *et al*., 1995; Benten *et al*., 1999).

The testosterone receptors on the surface of IC-21 cells are functionally coupled to intracellular Ca^{2+} homeostasis. Indeed, binding of the plasma membrane-impermeable testosterone-BSA induces a rapid increase in $[Ca^{2+}]_i$. This increase, which is also induced by the physiological concentration of 10 nM testosterone, occurs within seconds and is predominantly due to the release of Ca^{2+} from intracellular Ca^{2+} stores. However, external Ca^{2+} also contributes to the increase in $[Ca^{2+}]_i$, which is imported through Ca^{2+} channels, becoming particularly evident upon a second stimulus with testosterone or testosterone-BSA. Moreover, the specificity of membrane testosterone receptors is further corroborated by our findings 1) that the testosterone-induced Ca^{2+} release is saturable, 2) that 1-dehy-

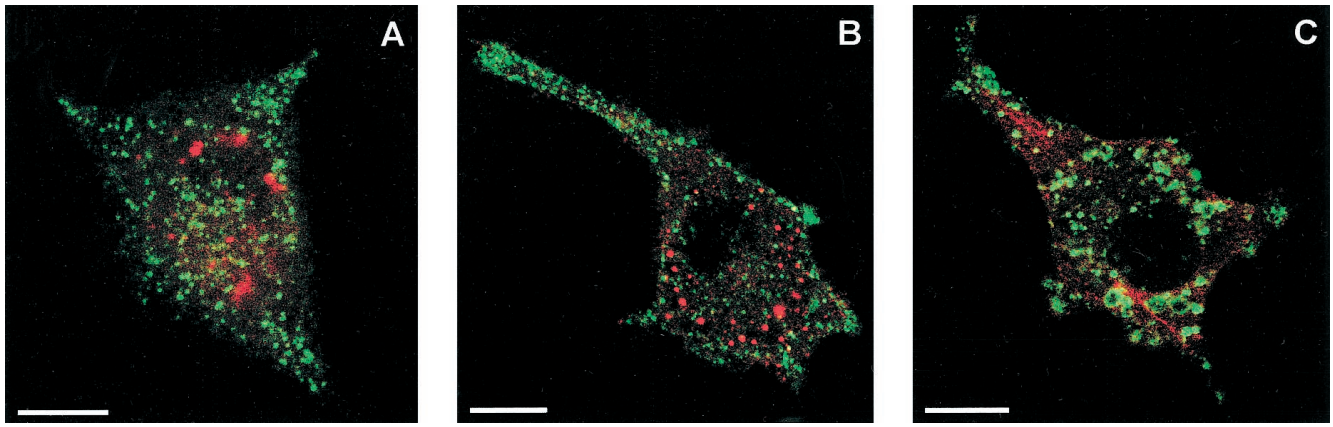

Figure 8. CLSM colocalization of the sequestered surface binding sites of testosterone. (A) IC-21 cells were incubated in parallel with testosterone-BSA-FITC and LysoTracker Red DND-99 at 37°C for 1 h. Testosterone-BSA-FITC did not colocalize with acidic vesicles stained with LysoTracker Red DND-99. (B) Testosterone-BSA-FITC was not sequestrated within clathrin-coated vesicles as detected by anti-clathrin antibodies and the Cy3-labeled corresponding secondary antibody. (C) Internalized punctate fluorescence of testosterone-BSA-FITC was not labeled with an anti-caveolin antibody and its corresponding secondary TRITC-conjugated antibody. Bars, 10 μm.

drotestosterone, which is very similar in structure to testosterone, is largely inactive to induce specific Ca^{2+} release, and 3) that estradiol evokes Ca^{2+}-responses differing from those of testosterone. Moreover, our data reveal that the membrane testosterone receptors not only are functionally coupled with Ca^{2+} channels in the plasma membrane but also belong to that class of membrane receptors that are coupled to phospholipase C via a pertussis toxin-sensitive G-protein, because Ca^{2+} release can be blocked by both pertussis toxin and the phospholipase C inhibitor U-73122 but not by the inactive compound U-73343.

The G-protein-coupled receptors for testosterone (GPCRT) in IC-21 cells exhibit a novel peculiarity, i.e., agonist-triggered sequestration. Indeed, this sequestration manifests itself as internalized punctate fluorescence of testosterone-BSA-FITC. The internalization begins a short while 1) after binding of testosterone-BSA-FITC to the surface of IC-21 cells and 2) after Ca^{2+} mobilization by testosterone. The latter, therefore, may be a precondition for ligand-induced internalization of the GPCRT. This view is also supported by the fact that the phospholipase C inhibitor U-73122 and pertussis toxin inhibit both Ca^{2+} mobilization and internalization of surface-bound testosterone-BSA-FITC. Our data reveal that the internalization process is not a simple fluid endocytosis or a constitutive endocytotic pathway of IC-21 cells but rather is ligand specific. Thus, internalization of surface-bound testosterone-BSA-FITC is competitively inhibited by testosterone but not by 1-dehydrotestosterone. In addition, GPCRT internalization is selective; i.e., only distinct plasma membrane domains are internalized excluding surface markers such as F4/80 and Con A-rhodamine. GPCRT internalization is consistent with other findings showing that a wide variety of G-protein coupled receptors (GPCRs), e.g., the prototypic β_2-andrenergic receptor and angiotensin II type 1A receptor, become sequestrated after ligand binding (von Zastrow and Kobilka, 1992; Moore *et al.*, 1995; Koenig and Edwardson, 1997), which is considered important for regulation of signaling,

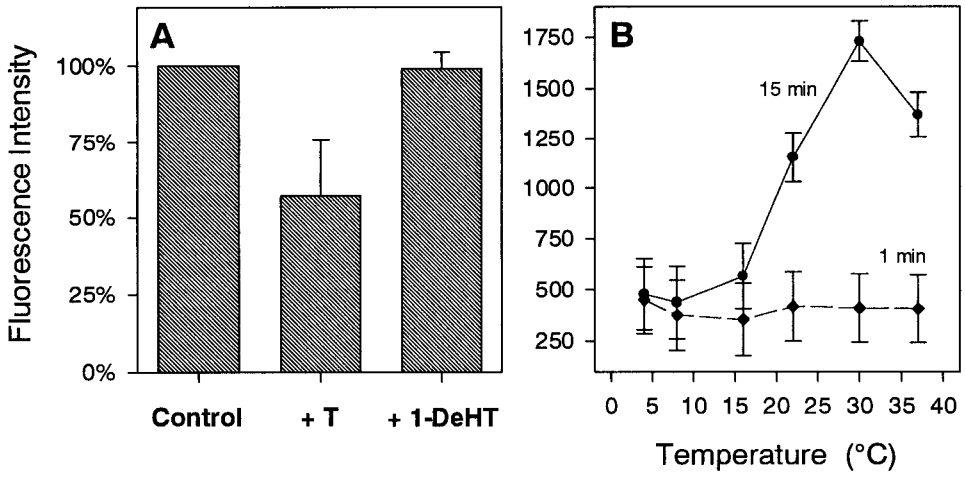

Figure 9. Flow cytometry of the internalization of surface-bound testosterone-BSA-FITC in IC-21 cells. (A) Cells were incubated for 15 min with testosterone-BSA-FITC (10^{-6} M) in the absence (Control) or in the presence of a 10-fold excess of unlabeled testosterone (+ T) or 1-dehydrotestosterone (+ 1-DeHT). Values normalized to controls are given as means ± SD from five different experiments. (B) Cells were equilibrated for 30 min at the indicated temperatures and then incubated with 1.5×10^{-5} M testosterone-BSA-FITC for 1 or 15 min at the same temperatures. Values represent means ± SD from at least two different experiments.

Table 1. Effect of various substances on sequestration of surface-bound testosterone-BSA-FITC

Substance	Fluorescence intensity (%)	Surface-bound fluorescence	Internalized fluorescence
Control	100	+	+
NaN_3 (0.04%, 30 min)	62.9 ± 6.5	+	−
Pertussis toxin (500 ng/ml, 16 h)	70.4 ± 0.5	+	−
U-73122 (2 μM, 2 min)	89.2 ± 5.8	+	−
U-73343 (2 μM, 2 min)	101.8 ± 5.4	+	+
Nocodazole (2.5 μg/ml, 15 min)	72.0 ± 5.5	+	−
Cytochalasin B (10 μg/ml, 15 min)	72.2 ± 5.4	+	−

Intact IC-21 cells (1.5×10^6) were incubated with the different substances at the indicated concentrations for the indicated periods before testosterone-BSA-FITC was added for 15 min. Thereafter, cells were fixed, and fluorescence intensity was evaluated by flow cytometry and fluorescence localization by CLSM. Values normalized to controls are given as means ± SD from at least two different experiments.

recycling, down-regulation, and responsiveness of the GPCRs (Yu *et al*., 1993; Pippig *et al*., 1995; Koenig and Edwardson, 1997).

In general, GPCRs internalize via the clathrin-coated vesicle-mediated endocytotic pathway (Doxsey *et al*., 1987; Robinson *et al*., 1996; Zhang *et al*., 1996), although entry also may be mediated via caveolae (Chun *et al*., 1994; Kiss and Geuze, 1997). However, our data suggest that GPCRT internalization does not proceed along such pathways. Indeed, the punctate fluorescence of internalized testosterone-BSA-FITC in IC-21 cells is associated with neither clathrin nor caveolin nor acidic vesicles. Obviously, the ligand-triggered entry of GPCRT into IC-21 cells is mediated by a clathrin- and caveolin-independent internalization pathway (cf. Roettger *et al*., 1995; Robinson *et al*., 1996). On the other hand, the internalization process of GPCRT in IC-21 cells resembles that observed for numerous other GPRCs insofar as this process is critically dependent on temperature, ATP, and cytoskeletal elements (von Zastrow and Kobilka, 1994; Roettger *et al*., 1995; Morrison *et al*., 1996; Koenig and Edwardson, 1997; Koenig *et al*., 1997).

Testosterone signaling through testosterone surface receptors has also been described in rat osteoblasts (Lieberherr and Grosse, 1994) and murine T cells (Benten *et al*., 1997, 1999). Howerver, there exist differences in comparison with IC-21 cells. For instance, plasma membranes of rat osteoblasts also possess GPCRT, but these do not become sequestrated upon agonist stimulation and mediate both Ca^{2+} import of external Ca^{2+} via voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular Ca^{2+} stores (Lieberherr and Grosse, 1994). In T cells, the membrane testosterone receptors are not sequestrable, and they mediate only ligand-induced Ca^{2+} import through non–voltage-gated, Ni^{2+}-blockable Ca^{2+} channels (Benten *et al*., 1997, 1999). At present it is too premature to discriminate whether the membrane testosterone receptors in all these different cell types are different or identical but coupled to different signaling pathways dependent on the cell type. In this context, there is also information available that not all cells possess membrane testosterone receptors. For instance, hepatocytes do not respond to testosterone with Ca^{2+} mobilization, although the cells are able to mobilize Ca^{2+} in response to progesterone and estradiol (Sanchez-Bueno *et al*., 1991).

Collectively, our data unequivocally show the presence of functional unconventional GPCRT in plasma membranes of IC-21 cells, which do not mediate the classical genomic AR response but rather initiate novel nongenomic testosterone signaling pathways involving Ca^{2+} as one of several other possible intracellular mediators. Signal integration into cell functioning and the physiological significance remain to be determined. In particular, it remains elusive whether the testosterone-induced increase in $[Ca^{2+}]_i$ per se modulates secondarily expression of specific genes, for example, through Ca^{2+}-responsive promotor elements, negative Ca^{2+}-responsive promotor elements, and/or Ca^{2+}-modulatable transcription factors such as NF-AT, NF-κB, and c-Jun N-terminal kinase (Negulescu *et al*., 1994; Dolmetsch *et al*., 1997).

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