Steroid hormones exert genotropic actions through members of the nuclear receptor family. Here, we have demonstrated genotropic actions of testosterone that are independent of intracellular androgen receptors (iAR). Through plasma membrane androgen receptors (mAR), testosterone induces a rapid rise in the intracellular free Ca\(^{2+}\) concentration of iAR-free murine RAW 264.7 macrophages. This nongenomic testosterone signaling, which is independent of both iAR and estrogen receptors, does not in itself activate either of the mitogen-activated protein kinase (MAPK) families ERK1/2, p38, and JNK/SAPK, the stably and transiently transfected c-fos promoter, or NO production. In the context of lipopolysaccharide (LPS) signaling, however, testosterone attenuates LPS activation of the c-fos promoter and NO production, which is abolished by the intracellular Ca\(^{2+}\) chelator BAPTA. Testosterone also attenuates the LPS activation of p38 but not that of ERK1/2 and JNK/SAPK, and this attenuation is abrogated by BAPTA. Moreover, the p38 inhibitor, SB 203580, largely reduces LPS activation of the c-fos promoter and NO production, and the remaining levels are no longer regulated by testosterone. This study is the first to provide information on genotropic actions of mAR-mediated nongenomic testosterone Ca\(^{2+}\) signaling by cross-talk with the LPS signaling pathway through p38 MAPK with impact on cell function.

Steroid hormones play a key role as signaling molecules in the development, differentiation, and physiology of multicellular organisms. According to the traditional view, steroids exclusively exert their effects on cells through members of the intracellular nuclear receptor family (1). The classic genotropic mode of action of these ligand-inducible transcription factors is to bind as homo- or heterodimers to specific response elements in target gene promoters, causing subsequent activation or repression of transcription (2). Previous studies (3, 4) indicate that hormone-activated nuclear receptors are also able to interact with other transcription factors on target gene promoters without direct binding to DNA. Recent findings (5, 6) also indicate that nuclear steroid receptors are able to mediate steroid-induced activation of other signaling molecules, such as the mitogen-activated protein kinase (MAPK)1-familyp ERK1/2 by transcription-independent mechanisms.

Attention has recently been paid to a totally different mode of steroid action, i.e. action through cell surface receptors (7–12). These receptors in the plasma membranes of cells, though mostly not yet defined in molecular terms, mediate nongenomic actions that have been described for different steroids, including the brassinosteroids of the plant Arabidopsis thaliana (13, 14). The latter is remarkable in that the plant genomes do not seem to encode any transcription factors of the nuclear receptor family (15). Nongenomic actions of steroids through surface receptors often become evident as rapid rises in the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) (16–20). Nonetheless, the Ca\(^{2+}\) signals, though steroid-specific, generally exhibit low amplitude and short duration so that their consequences for cell functioning are not immediately obvious. Hence, the nongenomic steroid actions are often disregarded as meaningless.

Testosterone has been described as exerting both genotropic and nongenomic actions. Genotropic actions are mediated through the classic intracellular androgen receptor (iAR), which is a 110-kDa protein with domains for androgen binding, DNA binding, and transactivation (21, 22). The ligand-activated iAR is not only able to act on transcription but has also been recently reported to exert nongenomic actions such as activation of ERK1/2 and p21-activated kinases (23–25). By contrast, nongenomic actions of testosterone through membrane androgen receptors (mAR) on cell surfaces become evident as intracellular Ca\(^{2+}\) signaling, which varies with cell type. In murine T-cells, for example, mAR mediate ligand-induced Ca\(^{2+}\) influx through non-voltage-gated, Ni\(^{2+}\)-blockable Ca\(^{2+}\) channels (26, 27). In rat osteoblasts, testosterone induces both the influx of extracellular Ca\(^{2+}\) via voltage-gated Ca\(^{2+}\) channels and Ca\(^{2+}\) release from intracellular stores through G-protein-coupled receptors activating phospholipase C via a pertussis toxin-sensitive G-protein (28). Murine macrophages of the cell line IC-21 respond to testosterone with predominantly intracellular Ca\(^{2+}\) mobilization mediated through G-protein-coupled receptors for testosterone (29, 30). However, the functional importance of testosterone-induced nongenomic Ca\(^{2+}\) signaling, in particular with regard to gene expression and cell function, is not yet understood.
To get information for possible genotropic effects of non-genomic testosterone Ca\(^{2+}\)/H11001 signaling, we have chosen as a model system mouse RAW 264.7 macrophages transiently or stably transfected with a c-fos promoter linked to human secreted alkaline phosphatase (SEAP) as a reporter, because the immediate early gene c-fos is known to be Ca\(^{2+}\)/H11001-sensitive (31, 32). Here, we have demonstrated that testosterone-induced nongenomic Ca\(^{2+}\)/H11001 signaling through mAR cannot directly activate the c-fos promoter. However, nongenomic testosterone signaling is able to exert genotropic actions in context with the LPS signaling pathway through p38 MAPK with impact on cell function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—The macrophage cell line RAW 264.7 (ATCC no. TIB-71) was grown in phenol red-free, low endotoxin Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) supplemented with 5% heat-inactivated, low endotoxin FCS (PAA Laboratories, Colbe, Germany). The mouse c-fos promoter from -520 to +109 was cloned into the EcoRI site of pSEAP2-Basic (CLONTECH, Heidelberg, Germany) and either transiently or stably transfected into RAW 264.7 cells labeled with testosterone-BSA-FITC (T-BSA-FITC) and BSA-FITC. C, confocal laser-scanning microscopy of RAW-fos13 cells labeled with T-BSA-FITC or ConA-rhodamine (ConA-Rh). Bars represent 10 \(\mu\)m.
ARD1, 5'TGTAAGG-3' cloned into pCR2.1 TOPO TA cloning vector (Invitrogen), and sectaki(acetoxymethyl)ester (Sigma). The Ca2+-incubated in IMDM with 5% stripped FCS for 18 h before adding 1

TGTTATCTAGC-3' and 5'-GAACGTCTGGAGATTCTGATGATTGG-3' described recently (19, 29). We used the following primer pairs: ARS1, GGCTGGAGATTCTGATGATTGG-3'.

5'-CTCCAGGAGCAGGTCATAGAGG-3' and 5'-GCCGTGAGATTCTGATGATTGG-3' and 5'-AGCAGGAGAGCTAGCCAGG-3' and 5'-ATCTTTGCTGCCT-3' and 5'-CTCCAGGAGCAGGTCATAGAGG-3' and 5'-GCCGTGAGATTCTGATGATTGG-3'.

Nongenomic Testosterone Signaling

FIG. 3. Testosterone-induced rise in [Ca2+]i of RAW-fos13 cells. A, testosterone (T) elicits an immediate Ca2+ spike, whereas 1-DeHT is ineffective. B, cells stimulated with 5μM DHT or 5μM DHT, cells preincubated with cyproterone or raloxifene for 1 h before testosterone application. D, cells incubated with IC/182,780 or tamoxifen for 1 h before addition of testosterone. E, cells stimulated with T-BSA or BSA. F, cells pretreated with BAPTA/AM for 10 min before testosterone addition. Arrows indicate addition of reagents to RAW-fos13 cells. Representative experiments are shown, and the results were verified in at least four independent experiments.

phospho-p38 MAPK (Thr-180/Tyr-182), and rabbit anti-phospho-JNK/SAPK (Thr-183/Tyr-185) (New England Biolabs, Frankfurt/Main, Germany) before ECL Western blot detection (Amersham Biosciences). Stripped blots were reprobed with the corresponding anti-total kinase protein antibodies (New England Biolabs), and the relative activation of MAPKs was densitometrically evaluated using the software, Quan-tiscan (Biosoft, Cambridge, UK).

Assay for c-fos Promoter Activity—For reporter gene assays, 2.5 × 106 RAW 264.7 cells were cultured in 24-well plates overnight and then transiently transfected with the c-fos promoter, SEAP, and the control SV40 SEAP plasmids. LPS and steroid stimulation was performed 24 h post-transfection. For experiments with c-fos promoter stably transfected cells, 5 × 105 of RAW-fos13 were cultured in 24-well plates overnight followed by stimulation with LPS and steroids. Just before stimulation, 100 μl of the supernatants were collected from each well (RLU0h) and another 100 μl after 3 h of stimulation (RLU3h). LPS and steroids were added in a volume of 100 μl of medium. SEAP activity was determined using a Phospha-Light kit (Tropix, Weiterstadt, Germany) with a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) as described previously (34). The relative activity of the c-fos promoter was calculated by the equation, RLU3h−RLU0h = 0.9 × RLU3h−RLU0h normalized to controls.

NO Determination—NO was measured as nitrite using the Griess reagent as described previously (34).

RESULTS

Testosterone-unresponsiveness of MAPK and c-fos—Previous studies (5, 23, 24) have shown that steroid hormones, including testosterone, are able to activate ERK1/2 in different cell types. We therefore first investigated the possible direct effects of testosterone on ERK1/2 and the two other MAPK families, p38 and JNK/SAPK, in RAW 264.7 macrophages. However, testosterone at the physiological concentration of 10 nM is not able to activate any of the three MAPK families at different time points over 3 h as detected by specific anti-MAPK antibodies used in Western blotting (Fig. 1A). The reason for this testosterone-unresponsiveness of the three MAPK families is not an inherent failure of the cells, because anisomycin (35, 36) stimulates phosphorylation of all three kinases (Fig. 1A; compare also Fig. 4).

We then studied a possible direct effect of testosterone on activation of the c-fos promoter, which is used here only as a molecular marker for early genotropic signaling because the
immediate early gene c-fos is inducible by a wide variety of stimuli (37, 38). RAW 264.7 cells were transiently transfected with a c-fos promoter linked to a SEAP reporter gene and then stimulated with 10 nm testosterone for 3 h. However, this stimulation is not sufficient to induce any significant response of the c-fos promoter in comparison with non-stimulated control cells (Fig. 1B). In addition, testosterone is not able to induce any change in c-fos promoter activity in the cell clone RAW-fos13, which was derived from RAW 264.7 through a stable transfection with the same reporter construct (see "Experimental Procedures") (Fig. 1C). This unresponsiveness is not due to a possible defect in the ability of the transfected c-fos promoter construct to be stimulated, because LPS causes a significant induction in both transiently and stably transfected cells (Fig. 1, B and C).

Testosterone-induced \(\text{Ca}^{2+}\) Signaling through mAR—The non-inducibility of MAPK and c-fos promoter activity by testosterone may be due to the absence of functionally active iAR and/or mAR, which normally mediate the actions of testosterone on cells. We have therefore examined the occurrence of mAR and iAR in RAW 264.7 and RAW-fos13 cells. These cells do not express any significant amounts of iAR as proven by RT-PCR. When primer pairs flanking a region of the DNA-binding domain or three different regions of the steroid-binding domain were used, all four predicted PCR products could be amplified using RNA from mouse testes. Expression of iAR could not be detected with RNA from RAW 264.7 or RAW-fos13 cells, although the quality of the RNA and the cDNA from these cell lines was proven by amplification of the intracellular estrogen receptor a using two different primer pairs (Fig. 2A). These data are consistent with our previous results in IC-21 macrophages (29, 30).

For detection of mAR, the plasma membrane-impermeable testosterone-BSA-FITC conjugate was used to label the cells for 1 min, resulting in an increase in cellular fluorescence as analyzed by flow cytometry (Fig. 2B). No binding was detectable with BSA-FITC (Fig. 2B). The fluorescence of the bound testosterone-BSA-FITC was localized exclusively on the cell surface, as revealed by confocal laser-scanning microscopy (Fig. 2C). The surface binding of testosterone-BSA-FITC was also corroborated by colocalization with the red fluorescent cell surface marker, ConA-rhodamine (Fig. 2C). Moreover, the mAR on RAW cells are functionally active. This becomes evident as a face marker, ConA-rhodamine (Fig. 2). Further, the mAR on RAW cells could not be blocked by iAR blockers, cyproterone, or raloxifene, tamoxifen, or ICI 182,780, which are inhibitors of the intracellular estrogen receptor i through which testosterone may be due to the absence of functionally active iAR and/or mAR, which normally mediate the actions of testosterone on cells. We have therefore examined the occurrence of mAR and iAR in RAW 264.7 and RAW-fos13 cells. These cells do not express any significant amounts of iAR as proven by RT-PCR. When primer pairs flanking a region of the DNA-binding domain or three different regions of the steroid-binding domain were used, all four predicted PCR products could be amplified using RNA from mouse testes. Expression of iAR could not be detected with RNA from RAW 264.7 or RAW-fos13 cells, although the quality of the RNA and the cDNA from these cell lines was proven by amplification of the intracellular estrogen receptor a using two different primer pairs (Fig. 2A). These data are consistent with our previous results in IC-21 macrophages (29, 30).

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Attenuation by Testosterone of LPS-activated p38 MAPK—LPS stimulation of macrophages is known to activate numer-
FIG. 5. Effects of testosterone on LPS activation of c-fos promoter. RAW 264.7 macrophages were cultured in IMDM medium containing 5% stripped FCS for 18 h and then transiently transfected with c-fos promoter-SEAP (A) or SV40-SEAP (B) for 24 h before stimulation with 1 μg/ml LPS in combination with 10 nM testosterone or 1 nM E2 for 3 h. The c-fos promoter activity of each group was determined relative to the activity of the vehicle-treated control, which was set at 100% for the LPS group. The results shown are means ± S.E. of at least three independent experiments performed in triplicate. C, testosterone attenuation of the LPS-activated c-fos promoter. Cells were stimulated for 3 h with 1 μg/ml LPS in combination with 10 nM testosterone or 1 nM E2 for 3 h. The c-fos promoter activity of each group was determined relative to the activity of the vehicle-treated control, which was set at 100% for the LPS group. The results shown are means ± S.E. of at least three independent experiments performed in triplicate. D, cells were first preincubated with 10 μM BAPTA for 10 min or 50 μM SB 203580 (SB) for 30 min and then stimulated with 1 μg/ml LPS or LPS plus 10 nM testosterone for 3 h. Results are presented as in panel C.
Nongenomic Testosterone Signaling

203580, LPS stimulation of p38 is reduced by about 50%, and the remaining promoter activity is no longer regulated by testosterone (Fig. 5D). These results indicate that p38 is involved in the LPS-induced activation of the c-fos promoter and that testosterone attenuation of the LPS-stimulated c-fos promoter activity is achieved predominately, if not exclusively, by interference with p38 MAPK phosphorylation.

Down-regulation by Testosterone of LPS-stimulated NO Production through p38 MAPK—Activation of p38 MAPK is known to be important for induction of genes associated with macrophage activation manifesting, for example, as increased production of the key immune effector molecule NO (45). We therefore suspected that the testosterone-induced rise in [Ca^{2+}], could also influence NO production. Testosterone in itself is not able to affect NO production. However, testosterone caused a significant decrease of NO production in LPS-stimulated RAW-fos13 cells (Fig. 6A). This decrease was not sensitive to the iAR blocker, cyproterone (Fig. 6A). The suppressive effect of testosterone can be prevented by BAPTA under conditions blocking the testosterone-induced rise in [Ca^{2+}], (Fig. 6B). Moreover, LPS-induced NO production can be reduced by about 60% with 50 μM SB 203580 (Fig. 6B). This is consistent with previous reports that LPS-stimulated NO production of macrophages is mediated through p38 MAPK (46). When LPS-induced NO production is down-regulated by SB 203580, the remaining NO production is no longer regulated by testosterone (Fig. 6B). This indicates that testosterone exerts its attenuative effect on LPS-stimulated NO production largely through p38 MAPK.

DISCUSSION

Nongenomic actions of steroids on cells are obviously much more complex than anticipated to date. During recent years, a number of reports have appeared describing nongenomic steroid effects that are mediated through the classic intracellular steroid receptors. For instance, iER-mediated nongenomic E2 effects can activate members of the MAPK family such as ERK1/2 (5, 47–49) or endothelial NO synthase (50, 51) and can induce an increase of [Ca^{2+}] (47). Androgens have been found to activate ERK1/2 (52) and p21-activated kinases (25) by a nongenomic pathway via the classic iAR. Very recently, testosterone has been shown to activate the ERK signaling pathway nongenomically through either iAR or even iER in diverse cells such as osteoblasts, osteocytes, embryonic fibroblasts, and HeLa cells (24). By contrast, the present study has demonstrated that intracellular steroid receptors are not a necessary precondition to mediate nongenomic effects of steroids. Like the macrophage cell line IC-21 (29, 30), the murine RAW 264.7 macrophages investigated here do not express any significant amount of iAR. Nevertheless, in RAW 264.7 macrophages, testosterone is able to induce iAR-independent nongenomic actions, which can in turn even exert genotropic actions with impact on cell function.

Nongenomic testosterone signaling manifests itself as a rapid rise in [Ca^{2+}], in macrophages of the cell line RAW 264.7. Such rises have also been observed in other cell types (26–28, 53), and they are well known to be inducible by other steroids as well (10, 54). In RAW 264.7 cells, similar to IC-21 macrophages (29, 30), the testosterone-induced rise in [Ca^{2+}], is not prevented by the iAR blocker, cyproterone. We can also exclude the possible action of testosterone through the iER (24) because the Ca^{2+} response was not sensitive to the iER blockers, raloxifene, tamoxifen, and ICI 182,780. Rather, our data demonstrate that testosterone exerts its nongenomic iAR- and iER-independent effects through mAR. An increase in [Ca^{2+}], is also inducible with the plasma membrane-impermeable testosterone-BSA, and specific binding sites for testosterone can be localized on the surface of intact RAW-fos13 cells with testosterone-BSA conjugated to FITC. Though still unknown in molecular terms, the mAR mediate actions specific for different androgens; only testosterone and 5α-DHT induce about the same rise in [Ca^{2+}], whereas 5β-DHT and 1-DeHT are ineffective in evoking changes in [Ca^{2+}], of RAW-fos13 macrophages.

The nongenomic testosterone Ca^{2+} signaling through mAR is not able in itself to induce genotropic actions in terms of activation of the stably and transiently transfected promoter of the Ca^{2+}-inducible gene c-fos in RAW 264.7 cells. Nevertheless, the nongenomic testosterone signaling has the potency to in-

![Fig. 6. Influence of testosterone on LPS-induced NO production.](image-url)

A. RAW-fos13 cells were cultured in IMDM medium containing 5% stripped FCS for 18 h and then stimulated for 24 h with 10 nM testosterone (T) and 1 μg/ml LPS. The cells were preincubated with 1 μM cyproterone (Cyp) for 1 h before addition of testosterone and LPS. N.D., not detectable. The results shown are means ± S.E. from three independent experiments performed in triplicate. B. RAW 264.7 cells were preincubated with 10 μM BAPTA for 10 min or 50 μM SB 203580 (SB) for 30 min before the addition of 1 μg/ml LPS or LPS plus 10 nM testosterone. NO₂⁻ was determined 24 h later as in panel A.
duce genotoxic actions. This becomes evident in the context of co-stimulation of the LPS signaling pathway. Thus, LPS stimulates c-fos promoter activation, and this stimulation is attenuated by testosterone-induced Ca2+ signaling. This attenuation can be abolished by BAPTA.

The nongenomic testosterone Ca2+ signaling not only exerts genotoxic actions in context with LPS signaling but also has a specific impact on cell function. This manifests itself as a testosterone-induced attenuation of LPS-stimulated NO production of RAW 264.7 macrophages. In accordance, a recent report (55) also has shown testosterone-induced attenuation of LPS-stimulated NO production. Our data demonstrate that nongenomic testosterone Ca2+ signaling is the reason for testosterone-attenuated LPS-activated NO production, because the latter can be abolished by BAPTA.

The testosterone-induced attenuation of LPS-induced c-fos promoter activation and NO production may be thought to reflect only a simple uniform dampening of all LPS signaling parameters in macrophages through the increased free Ca2+ ions. However, this attenuation is specific, which has to be deduced from our results. Thus, LPS activates all three MAPK families, ERK1/2, JNK/SAPK, and p38, in accordance with previous reports (39, 41). Testosterone, however, selectively down-regulates only the LPS-induced activation of p38, not that of ERK1/2 and JNK/SAPK. This p38 down-regulation is indeed due to nongenomic testosterone Ca2+ signaling. When the free Ca2+ ions induced by testosterone are captured by BAPTA, p38 is again fully able to be stimulated by LPS. The central role of p38 MAPK is further substantiated by our finding that the p38 inhibitor, SB 203580, diminishes the LPS-induced activation of c-fos promoter activation and NO production and abolishes the responsiveness to testosterone of the remaining LPS effect. Collectively, our results indicate that there is a cross-talk of the testosterone-induced nongenomic Ca2+ signaling with the LPS signaling pathway. The testosterone-induced rise in [Ca2+]i, attenuates the LPS-induced activation of p38, which further down-stream attenuates both the LPS-activated c-fos promoter and NO production.

The nongenomic iAR-independent testosterone signaling we have observed here in RAW 264.7 macrophages is a novel paradigm for a nongenomic steroid effect with impact on mRNA expression and cell functioning, independent of the cognate intracellular steroid receptor. This nongenomic signaling is presumably of importance with respect to the long known immunosuppressive activities of testosterone that are not yet explainable by the classic iAR response. In particular, the testosterone-induced nongenomic decrease in NO production of macrophages is compatible with our previous findings that testosterone dramatically diminishes the ability of mice to eliminate blood stages of Plasmodium chabaudi malaria (56, 57). Furthermore, testosterone not only prevents the development of protective immunity against P. chabaudi infections but also impairs the efficacy of protective vaccination against P. chabaudi malaria (58). These immunosuppressive effects of testosterone are mediated neither through iAR nor, after aromatization of testosterone to E2, through iER (59, 60).
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