Testosterone induces Ca^{2+} influx via non-genomic surface receptors in activated T cells

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Abstract Using the Fura-2 method we investigated a possible direct action of testosterone on cytosolic free calcium of splenic T cells isolated from female C57BL/10 mice. Testosterone at physiological concentrations of 1–10 nM induces an increase in $[Ca^{2+}]_i$ within seconds, which is due to Ca^{2+} influx and not to Ca^{2+} release from intracellular stores. In contrast, estradiol induces both Ca^{2+} influx and Ca^{2+} release. The testosterone-induced Ca^{2+} influx is mediated by Ni^{2+}-blockable channels and is not inhibited by cyproterone, a blocker of the classical androgen receptor. Ca^{2+} influx can also be induced by testosterone conjugated to BSA which is impermeable to the plasma membrane. These data indicate a novel mode of direct action of testosterone on T cells which is not mediated through the classical androgen receptor response, but through unconventional plasma membrane receptors.

Key words: Testosterone; Ca^{2+} influx; T cell; Androgen receptor; Membrane receptor

1. Introduction

Testosterone is known to increase susceptibility to many infectious diseases including a wide variety of parasite infections [1,2], among these murine malaria [3,4]. At low circulating testosterone concentrations, C57BL/10 mice are normally resistant to *Plasmodium chabaudi* malaria, i.e. they are capable of self-healing blood stage infections of this parasite [3,5]. However, elevated serum testosterone concentrations cause a conversion from resistance to susceptibility, i.e. self-healing is prevented and the infections take a lethal course [3,5]. Concomitantly, splenic T cells change from a resistance-promoting phenotype to a susceptibility-mediating phenotype. These testosterone-induced functional changes in T cells become evident upon activation in response to *P. chabaudi* infections [6].

Androgens modulate gene expression by way of the classical nuclear androgen receptor (for reviews, see e.g. [7,8]). This is a protein of approximately 110 kDa and contains several domains for androgen binding, transactivation, DNA binding, nuclear localization and dimerization (for reviews, see e.g. [7,9]). In addition to the classical androgen receptor, unconventional non-genomic cell surface receptors for testosterone have been recently described in typical testosterone target cells, i.e. rat osteoblasts [10]. These receptors belong to that class of membrane receptors coupled to phospholipase C via a pertussis toxin-sensitive G-protein. Binding of testosterone to these surface receptors causes Ca^{2+} influx and an increased formation of inositol 1,4,5-trisphosphate and diacylglycerol.

The interactions of testosterone with T cells have remained elusive to date. Currently, the view predominates that testosterone is not able to act directly on T cells. The available information indicates that T cells do not contain classical androgen receptors (for reviews, see e.g. [1,2]). This study, however, provides first evidence that testosterone is able to act through non-genomic unconventional plasma membrane receptors, thus triggering Ca^{2+} influx in activated T cells.

2. Materials and methods

2.1. Isolation of activated T cells

Female mice of the inbred strain C57BL/10 aged 9–12 weeks were challenged with 10^6 *P. chabaudi*-infected erythrocytes for 7 days to activate splenic T cells. Spleens were aseptically removed and total nucleated spleen cells were isolated as detailed previously [6]. The activated T cells were then prepared using the nylon-wool procedure [11] resulting in 94% $Thy1.2^+$ T cells as examined by FACScan analysis.

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

Isolated T cells 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. Then, T cells (10^7/ml) were loaded with 3 μM Fura-2/acetoxymethylester (Amersham, Les Ulis, France) for 20 min in the same buffer at 37°C, diluted with 7 ml of HEPES buffer, and reincubated for 20 min at 37°C. Cells were rapidly pelleted at $400 \times g$ for 10 min and the pellet was resuspended in 2 ml of HEPES buffer. The Ca^{2+} measurements were performed with 1 ml of this cell suspension in a Hitachi F-2000 spectrofluorometer at a constant temperature of 37°C. Reagents were added directly to the cuvette under continuous stirring. Testosterone, testosterone 3-(*O*-carboxymethyl)oxime/BSA, estradiol and neomycin were from Sigma (St. Quentin, Fallavier, France), cyproterone was kindly provided by Schering (Berlin, Germany). Hormones were dissolved in ethanol. The final concentration of ethanol in the cuvette was 0.01%, which never affected $[Ca^{2+}]_i$ (cf. also [10]). 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 using the equation $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times Sf380/Sb380$ as previously described [12]. The dissociation constant for the Fura-2-Ca^{2+} complex was taken as 224 nM [12]. The values for R_{max} and R_{min} were calculated from measurements using 25 μM digitonin and 4 mM EGTA and enough Tris base to raise the pH to 8.3 or higher [10,12].

3. Results

Using the Fura-2 method, testosterone at the physiological concentration of 1 nM was found to induce an increase in $[Ca^{2+}]_i$ of activated T cells (Fig. 1A). This increase was in the range between 10 and 25 nM Ca^{2+} and occurred either

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; BSA, bovine serum albumin; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid

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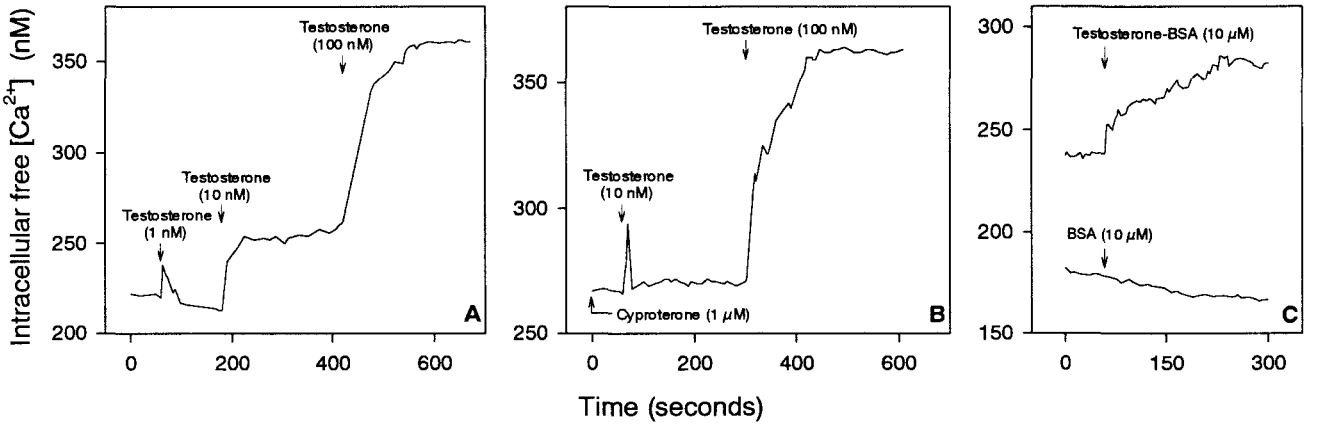

Fig. 1. Action of testosterone on cytosolic free calcium concentration in T cells. A: Direct effects of various physiological concentrations of testosterone; arrows indicate the addition of testosterone to the same T cell suspension. B: Effects of testosterone on T cells pretreated for 35 min with 1 μM cyproterone, a nuclear androgen receptor blocker. C: Effects of testosterone 3-(*O*-carboxymethyl)oxime BSA (testosterone-BSA), and BSA alone, as control, on intracellular calcium.

as a spike or as a prolonged elevation of $[Ca^{2+}]_i$. Higher testosterone concentrations of 10 nM or 100 nM normally induced higher increases in $[Ca^{2+}]$, ranging between about 10 and 60 nM or about 20 and 120 nM, respectively. A spike or prolonged elevation could be recorded up to about 40 nM Ca^{2+}, while there was always a prolonged elevation at Ca^{2+} concentrations exceeding 50 nM. The reason why there is either a spike or a prolonged elevation is not yet known. Testosterone added to the same cell suspension for a second or third time induced additional increases in $[Ca^{2+}]_i$ occurring always as a prolonged elevation (Fig. 1A). When T cells were first incubated with excess cyproterone, a blocker of the classical androgen receptor, before adding testosterone, the testosterone-induced increase in $[Ca^{2+}]_i$ was not prevented (Fig. 1B). Testosterone conjugated to BSA, which is impermeable for the plasma membrane, also induced an increase in $[Ca^{2+}]_i$ in activated T cells, whereas BSA alone had absolutely no effect on $[Ca^{2+}]_i$ (Fig. 1C).

Possible sources of the testosterone-induced $[Ca^{2+}]_i$ increase are the release of Ca^{2+} from intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} through the plasma membrane. These possibilities were examined using three independent approaches, i.e. reducing the concentration of extracellular Ca^{2+} with EGTA, blocking the release of Ca^{2+} from intracellular stores through inhibition of phospholipase C via binding to phosphoinositides with neomycin [13], and attenuating Ca^{2+} influx with the Ca^{2+} channel inhibitor Ni^{2+} [14–16]. When extracellular Ca^{2+} was removed by EGTA, testosterone induced only – if at all – a very small release of Ca^{2+} from intracellular Ca^{2+} stores, even if the testosterone concentration was as high as 10 μM (Fig. 2A). Blockade of phospholipase C by neomycin did not prevent the testosterone-induced rise in $[Ca^{2+}]_i$ (Fig. 2B). These data indicate that the testosterone-induced increase in $[Ca^{2+}]_i$ does not result from a release of Ca^{2+} from intracellular Ca^{2+} stores, such as the endoplasmic reticulum, but rather signals influx of extracellular Ca^{2+} into cells. This influx is not due to diffusion, but is channel-mediated. The specific Ca^{2+} channel blocker Ni^{2+} inhibited the testosterone-induced Ca^{2+} influx (Fig. 2C).

Further evidence for the specificity of the testosterone effect

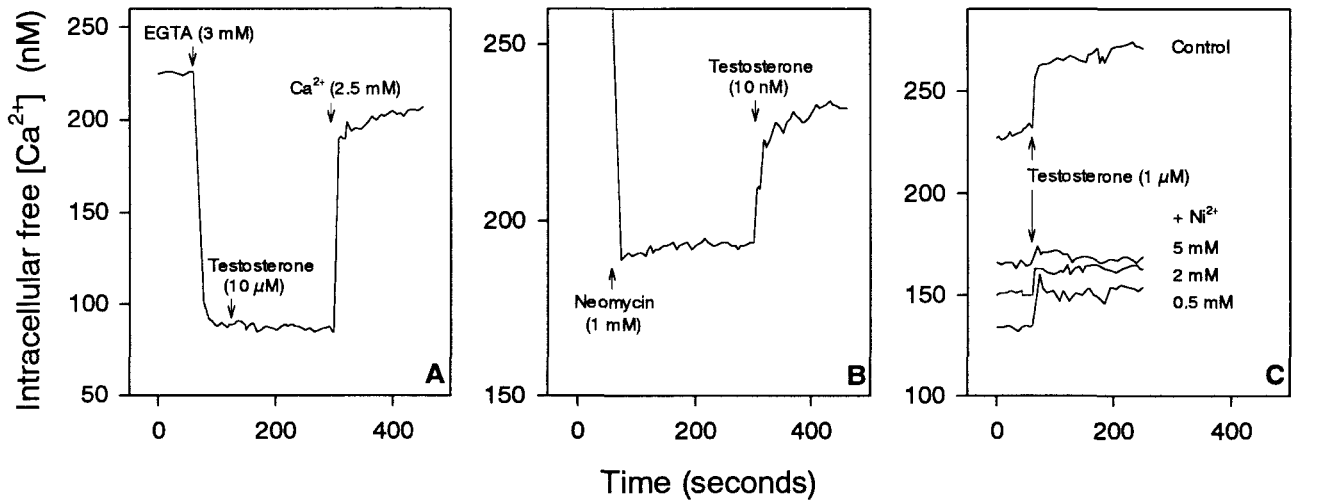

Fig. 2. Effects of different substances on intracellular calcium response to testosterone. A: T cells were incubated for 1 min with 3 mM EGTA, an extracellular Ca^{2+} chelator, before adding 10 μM testosterone. B: T cells were incubated for 3 min with 1 mM neomycin, an indirect inhibitor of phospholipase C via binding to phosphoinositides, before adding 10 nM testosterone. C: T cells were incubated for 5 min with various concentrations of Ni^{2+}, a specific Ca^{2+} channel blocker, before adding 1 μM testosterone.

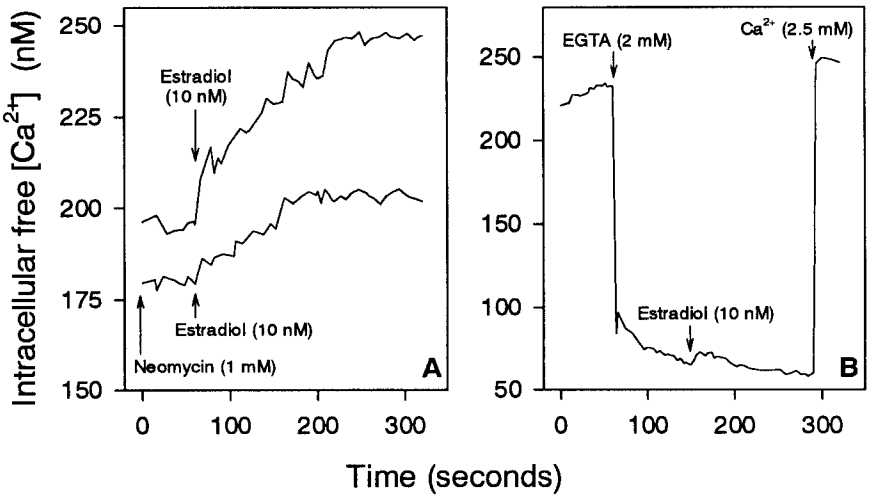

Fig. 3. Action of 17β-estradiol on cytosolic free calcium concentration in T cells. A: Direct effect of estradiol on T cells incubated or not for 3 min with 1 mM neomycin, an indirect inhibitor of phospholipase C before adding 10 nM estradiol. B: T cells were incubated with 2 mM EGTA for 1 min before adding 10 nM estradiol.

was obtained by testing the effect of estradiol on $[Ca^{2+}]_i$ of activated T cells. Estradiol also caused an increase in $[Ca^{2+}]_i$ as shown in Fig. 3A. However, this effect differed from the testosterone effect, since estradiol caused both Ca^{2+} influx and release from intracellular Ca^{2+} stores (Fig. 3A,B). When all the extracellular Ca^{2+} was complexed by EGTA, estradiol still induced a rise in $[Ca^{2+}]_i$ due to Ca^{2+} release from intracellular stores. This rise was about 10 nM at both 0.1 nM and 10 nM estradiol, and could be increased to 22 nM Ca^{2+} by 10 μM estradiol, in contrast to 10 μM testosterone (Fig. 2A).

The testosterone-induced changes in $[Ca^{2+}]_i$ obviously have consequences for the responsiveness of T cells to antigenic stimulation. When T cells were stimulated in vitro using the monoclonal anti-CD3 antibody 145-2011 (Pharmingen, San Diego, CA, USA) directed to the 25 kDa ε chain of the T cell receptor-associated CD3 complex, there was a rise in $[Ca^{2+}]_i$, and after reaching the maximum, $[Ca^{2+}]_i$ declined somewhat before reaching a plateau as illustrated in Fig. 4A, in accordance with previous findings (see e.g. [14]). Incubation of T cells with testosterone induced a small transient increase in $[Ca^{2+}]_i$, and the subsequent activation by anti-CD3 was perturbed (Fig. 4B). This became evident as an impaired increase in $[Ca^{2+}]_i$, lower maximal levels of $[Ca^{2+}]_i$, and an elevated sustained $[Ca^{2+}]_i$ plateau, when compared to T cell activation without testosterone. Fig. 4C shows that testosterone is still able to evoke an increase of $[Ca^{2+}]_i$ after anti-CD3 stimulation of T cells.

4. Discussion

This study provides first evidence for a direct, specific effect of testosterone on activated T cells. This direct effect is not mediated through the classical nuclear androgen receptor response, but obviously through unconventional non-genomic surface testosterone receptors. This is in accordance with previous studies which have been unable to detect classical androgen receptors in T cells [1,17–21]. The direct effect of testosterone on T cells in vitro manifests itself as an increase in $[Ca^{2+}]_i$. This increase is due to an influx of Ca^{2+} and can be induced even at concentrations as low as 1 nM. The Ca^{2+}

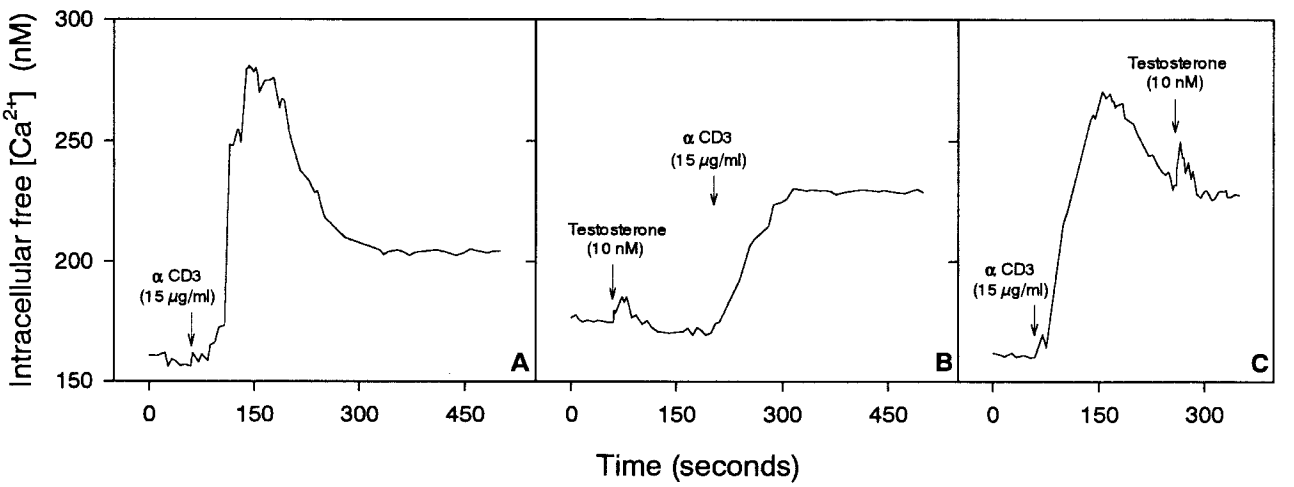

Fig. 4. Effects of testosterone and anti-CD3 on cytosolic free calcium concentration in T cells. A: Direct effect of 15 μg/ml anti-CD3. B: Effect of 15 μg/ml anti-CD3 on T cells pretreated with 10 nM testosterone for 2 min. C: Effect of 10 nM testosterone on T cells stimulated with 15 μg/ml anti-CD3 for 3 min.

influx is specific, i.e. it is not a simple diffusion phenomenon, but rather it is mediated by Ni^{2+}-blockable Ca^{2+} channels [14–16]. Moreover, the Ca^{2+} influx cannot be inhibited by classical androgen receptor blockers such as cyproterone. It is noteworthy that this Ca^{2+} influx is also inducible by testosterone conjugated to BSA, which is not permeable to the plasma membrane. This indicates that testosterone binds to unconventional non-genomic surface testosterone receptors and, thus, triggers an opening of Ca^{2+} channels in the plasma membrane. However, the mechanisms of the testosterone-induced increase of $[Ca^{2+}]_i$ in T cells are not identical to those recently identified in rat osteoblasts [10]. In the latter, the binding of testosterone to membrane-bound androgen receptors induces not only a Ca^{2+} influx, but also a Ca^{2+} release from intracellular stores via the inositol 1,4,5-trisphosphate pathway. Such a release from intracellular stores is obviously not inducible in T cells by testosterone, though activated T cells contain such Ca^{2+} stores which release Ca^{2+} upon induction by estradiol (cf. also [22,23]). In accordance with our findings, it has been recently shown that the channel-mediated Ca^{2+} influx in murine T cells is not necessarily coupled with an inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores [14,24]. Moreover, our data demonstrate that testosterone is able to affect directly the responsiveness of T cells to antigenic stimulation. Thus, in vitro activation of T cells by anti-CD3, at least the initial events associated with the increase in $[Ca^{2+}]_i$, is perturbed by testosterone. This effect of testosterone is not due to testosterone binding per se or to competition of testosterone with anti-CD3 binding sites on the T cell surface since, after T cells have been activated by anti-CD3, testosterone can still cause an increase in $[Ca^{2+}]_i$.

Previously, we have shown that in vivo testosterone induces functional changes in splenic T cells which acquire the capability to confer the testosterone-induced susceptibility to *P. chabaudi* malaria [6]. The present data suggest that the basically initial event for these functional changes is the binding of testosterone to unconventional surface receptors to T cells triggering Ca^{2+} influx. It may therefore essentially contribute to those mechanisms mediating testosterone-induced susceptibility to *P. chabaudi* malaria, which has been previously found to be also not mediated through the classical androgen receptor response [25,26].

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