Androgens Increase Intracellular Calcium Concentration and Inositol 1,4,5-Trisphosphate and Diacylglycerol Formation via a Pertussis Toxin-sensitive G-protein*

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Bone is a target tissue of androgens, but the mechanisms by which they act on bone are still unclear. This study examines the early (5–60 s) effects of 1 µm testosterone on cytosolic free Ca²⁺ concentration ([Ca²⁺]) and inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG) formation in confluent male rat osteoblasts. 10 µm to 100 µm testosterone increased [Ca²⁺]ᵢ within 5 s via Ca²⁺ influx as shown by the effects of EGTA and the Ca²⁺ channel blockers nifedipine and verapamil and via Ca²⁺ mobilization from the endoplasmic reticulum as shown by the effects of thapsigargin and neomycin. 10 µm to 100 µm testosterone increased InsP₃ and DAG formation within 10 s. Testosterone immobilized on bovine serum albumin (testosterone (O-carboxymethyl)oxime/bovine serum albumin) and its derivative, (O-carboxymethyl)oxime, rapidly increased [Ca²⁺]ᵢ and InsP₃ and DAG formation and were full agonists, although they were less potent than the free steroid. Cyproterone acetate, a nuclear antagonist, did not block the increase in [Ca²⁺]ᵢ and InsP₃ and DAG formation induced by testosterone. Finally, neomycin and pertussis toxin totally abolished the effects of testosterone on InsP₃ and DAG. These results suggest that male rat osteoblasts bear nongenomic unconventional cell-surface receptors for testosterone that belong to the class of the membrane receptors coupled to a phospholipase C via a pertussis toxin-sensitive G-protein.

The sex steroids (androgens and estrogens) are major regulators of bone metabolism in males and females, respectively (1). Both hormones interact with growth hormone in the control of adolescent growth spurt (2). In males, hypogonadism is associated with bone loss, which is stabilized by testosterone administration (3). Recently, androgen receptors have been isolated in various cell types, such as hepatocytes (5), granulosa cells (6), oocytes (7, 8), spermatocytes (9), uterine smooth muscle cells (11, 12), and osteoblasts (13), suggesting the possibility of an alternative, nongenomic mechanism of action, presumably at receptors on the cell surface.

No information is available about the early effects of androgens on intracellular calcium and on the turnover of membrane phosphoinositides, except one work showing an increase in intracellular calcium levels via Ca²⁺ influx through L-type channels in a prostatic cell line (LNCaP) (14).

We examined the early (5–60 s) effects of androgens on intracellular calcium concentration and phospholipid metabolism of male rat osteoblasts. Testosterone covalently bound to high molecular weight moecules (7, 9), which did not enter the cell, were also used to examine the possible involvement of a plasma membrane receptor.

EXPERIMENTAL PROCEDURES

Animals and Materials—Testosterone, 5α-dihydrotestosterone, dehydroepiandrosterone, testosterone 3-(O-carboxymethyl)oxime (T-CMO), testosterone 3-(O-carboxymethyl)oxime/bovine serum albumin (T-CMO/BSA) (26 mol of testosterone/mol of BSA), and all chemicals were from Sigma. 1,6-(17β-3-methoxyestradiol-1,3,4,5,6-trien-17-yl)-amino(hexyl)-1H-pyrrrole-2,5-dione (U-73122) and 1,6-(17β-3-methoxyestradiol-1,3,4,5,6-trien-17-yl)-amino(hexyl)-2,5-pyrroolidine-2,5-dione (U-73343) were from Bioimol Research Laboratory (Plymouth, MA) and Tebu (Le Pernay en Yvelines, France). α-Minimal essential medium (without phenol red) and fetal calf serum (FCS) were from Eurobio (Paris). Pico-fluor 40 was from Packard (Rungis, France). Econfluor was from New England Nuclear (Paris, France). Dowex 1-X8 was from Serva (Heidelberg, Germany), and silica gel TLC plates (DC-Fertigplatten and DC-Plastikfolien Kieselgel 60) were from Merck (Darmstadt, Germany).

Isolation and Cell Culture—Osteoblasts were isolated from parietal bones of 2-day-old male rats by sequential enzymatic digestion (15). These cells had the following osteoblast characteristics: high alkaline phosphatase activity, high type I collagen synthesis, a cAMP response to parathyroid hormone, and an osteocalcin response to 1,25-dihydroxyvitamin D₃. Osteoblasts were grown on rectangular glass coverslips (16) for 4 days or in Petri dishes (25 cm²) in phenol red-free α-minimal essential medium with 10% FCS. Cells were then incubated for 72 h in phenol red-free medium containing 1% heat-inactivated FCS and then transferred to serum-free medium 24 h before use.

Calcium Measurement and Experimental Protocol—The cells were washed three times with Hanks' Heps, pH 7.4 (137 mM NaCl, 4.41 mM KH₂PO₄, 0.442 mM Na,HPO₄, 0.885 mM MgSO₄, 27.7 mM glucose, 1.25 mM CaCl₂, 20 mM Hepes, 1 mg/ml BSA), and loaded with 1 µm Fura-2/AM for 40 min in the same buffer at room temperature. The glass coverslip carrying the cells was inserted into a cuvette containing 2.5 ml of Hanks' Heps, pH 7.4. The cuvette was placed in a thermostatted (37°C) Hitachi F-2000 spectrofluorometer. Drugs and reagents were added directly to the cuvette under continuous stirring.

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The abbreviations used are: T-CMO, testosterone 3-(O-carboxymethyl)oxime; BSA, bovine serum albumin; FCS, fetal calf serum; [Ca²⁺]ᵢ, intracellular calcium concentration; DAG, diacylglycerol; PA, phosphatidic acid; MAG, monoacylglycerol; InsP₃, inositol monophosphate; InsP₄, inositol bisphosphate; InsP₅, inositol 1,4,5-trisphosphate; InsP₆, inositol tetrakisphosphate; PTX, pertussis toxin.
Fig. 1. Effects of 1 nM testosterone, 10 nM T-CMO, and 10 nM T-CMO-BSA on intracellular calcium concentration in confluent osteoblasts. This experiment is representative of at least five similar glass coverslips for 25 different cultures.

Fig. 2. Dose-dependent effects of androgens on [Ca\(^{2+}\)], in confluent osteoblasts. Intracellular Ca\(^{2+}\) concentrations were determined at 20 s. Values are the means ± S.E. (\(n = 25\); five coverslips for each of the 25 different cultures) for each steroid and are significantly different from the basal level, *\(p < 0.001\).

The Fura-2 fluorescence response to intracellular calcium concentration ([Ca\(^{2+}\)]) was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. (17). The dissociation constant for the Fura-2-Ca\(^{2+}\) complex was taken as 224 nm (17). The values for \(R_{\infty}\) and \(R_{imin}\) were calculated from measurements using 25 μM digitonin and 4 μM EGTA and enough Tris base to raise the pH to 8.3 or higher. Each measurement on Fura-2-loaded cells was followed by a parallel experiment under the same conditions with non-Fura-2-loaded cells.

Table I
Blockade of androgen-induced changes in intracellular calcium

| [Ca\(^{2+}\)] | T-CMO (100 nM) | T-CMO/BSA (100 nM) |
|--------------|----------------|-------------------|
| EGTA (2 mM)  | 73 ± 2 123 ± 4 \(^a\) | 110 ± 2 \(^b\) 108 ± 2 \(^b\) |
| Nifedipine (1 μM) | 98 ± 2 145 ± 2 \(^a\) | 125 ± 3 \(^b\) 124 ± 2 \(^b\) |
| Verapamil (1 μM) | 85 ± 2 138 ± 4 \(^a\) | 119 ± 4 \(^d\) 118 ± 7 \(^d\) |

\(^{a}\) Testosterone.
\(^{b}\) Values significantly different from EGTA-pretreated cells (\(p < 0.001\)).
\(^{c}\) Values significantly different from nifedipine-pretreated cells (\(p < 0.001\)).
\(^{d}\) Values significantly different from verapamil-treated cells (\(p < 0.001\)).

androgen and to release Ca\(^{2+}\) from the associated stores (20, 21).

Cell Labeling—The action of androgens on the generation of inositol phosphates was measured in cells incubated for 72 h with or without myo-[2-3H]inositol (10 μCi/ml) in phenol red-free medium containing 1% heat-inactivated FCS. Its action on the formation of diacylglycerol (DAG), phosphatidic acid (PA), and monosodium glycerol (MAG) was assessed in cells incubated with [3H]arachidonic acid (0.25 μCi/ml) for the last 24 h of the 72-h incubation in phenol red-free medium with 1% heat-inactivated FCS. The labeled cells were washed five times with serum-free medium and incubated at 37 °C in fresh medium without heat-inactivated FCS for 4 h; ethanol solvent (0.01%) or androgens (0.1 pm) to 10 μM) were then added for 5–120 s.

Extraction and Determination of Inositol Phosphates—For inositol phosphates, the reaction was stopped by removing the medium and adding ice-cold trichloroacetic acid (final concentration of 5%). Trichloroacetic acid was removed with diethyl ether. The final extract was neutralized before either being used for quantitative determination of inositol 1,4,5-trisphosphate (radiocompetition; Kit TRK 1000, Amer sham) or applied to an anion-exchange column. The 2'-H-labeled extracts were applied to 1-m columns of Dowex 1-X8 (100–200 mesh, formic acid). Free inositol and inositol phosphates were eluted sequentially with the following: 1) water (free inositol); 2) 5 mM disodium borate, 60 mM sodium formate (glycerophosphoinositol); 3) 0.1 M formic acid, 0.2 M ammonium formate (inositol phosphate (InsP)); 4) 0.1 M formic acid, 0.4 M ammonium formate (inositol bisphosphate (InsP2)); 5) 0.1 M formic acid, 0.8 M ammonium formate (inositol trisphosphate (InsP3)); 6) 0.1 M formic acid, 1.2 M ammonium formate (inositol tetrakisphosphate (InsP4)). The Dowex column was calibrated with tritiated inositol phosphate standards. 1.2 M ammonium formate gave a good separation of InP4 from InP3, InP2 and higher inositol phosphates were eluted with 1.5 M ammonium formate. Radioactivity was counted in Picofluor 40 in a scintillation counter.

Lipid Extraction and Chromatography—For lipids, the reaction was stopped by removing the medium and adding cold methanol. The lipids were extracted according to Bligh and Dyer (23) with a final amount of 2 ml of methanol, 2 ml of chloroform, and 1.6 ml of aqueous salt solution (0.74% KCl, 0.04% CaCl2, 0.034% MgCl2). The first chloroform extract was removed, and the remaining methanol/water phase was acidified with 10 mM HCl (final concentration). Phospholipids were extracted at acid pH for the next two steps, except for neutral lipids (DAG and MAG), for which acidification was omitted. The chloroform phases were combined and dried in a rotary evaporator and dissolved in 200 μl of chloroform/methanol (2:1, v/v), and an aliquot was taken for thin-layer chromatography.

The phospholipids were separated in one dimension using methyl acetate/1-propanol/chloroform/methanol/KCl (0.25%; 25:25:25:10:9, by volume). This solvent system completely separated sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Neutral lipids were separated in one dimension using n-heptane/diethyl ether/acetic acid (75:25:4, by volume). This system completely separated free fatty acids, monosodium glycerol, diacylglycerol, oleic acid, and triacylglycerol (25). PA, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol standards were added as carriers for phospholipid separation, and monosodium glycerol, 1,2-DAG, 1,3-DAG, oleic acid, and triacylglycerol standards were added as carriers for neutral lipid.
cells were incubated for 3 min with 2 mM EGTA before adding 1 nM testosterone; cells were incubated for 3 min with 25 mM KCl, followed by 2 mM EGTA for 30 s before adding 1 nM testosterone. These experiments are representative of at least 25 similar cultures for each experimental case.

The developed TLC plates were analyzed with a Model LB 2820 TLC linear analyzer (Berthold, Munich, Germany). The 14C-labeled lipids of interest were scraped off and counted for radioactivity by liquid scintillation with Econofluor.

Results

Intracellular Calcium—The basal level of intracellular calcium concentration in confluent male rat osteoblasts was 140 ± 3 nM (mean ± S.E., n = 25).

Direct Effects of Androgens on Intracellular Calcium—Fig. 1 shows the transient increase in [Ca2+], induced by 1 nM testosterone; [Ca2+], dropped rapidly after 30 s, but remained higher than the basal level (19 ± 1%, mean ± S.E., n = 25, p < 0.001). T-CMO and T-CMO/BSA (Fig. 1) induced a smaller increase in [Ca2+]; the time course of the T-CMO/BSA effect was similar to that of testosterone or T-CMO. The concentration-dependent effects of testosterone were bell-shaped, with maximal activity at 1 nM (Fig. 2). Testosterone was more potent than either T-CMO and T-CMO/BSA, although these latter products were equipotent (Fig. 2). 5α-Dihydrotestosterone was as active as testosterone, while dehydroepiandrosterone had no effect (data not shown).

Blockade of Androgen-induced Changes in Intracellular Calcium Concentration—A small excess of EGTA (2 mM) was first used. EGTA caused a marked decrease in basal [Ca2+], 52 ± 2%, mean ± S.E., n = 25). The calcium entry blockers nifedipine and verapamil (1 μM) triggered rapid drops of 31 ± 2 and 41 ± 3%, respectively (mean ± S.E., n = 25). The steady-state level reached within 20 s was higher than that obtained with EGTA. 1 nM testosterone was added 1 min after EGTA or calcium entry blocker (Table 1). EGTA and calcium entry blockers not only diminished (30 ± 2%, means ± S.E., n = 25) the transient increase induced by testosterone, T-CMO, or T-CMO/BSA (Table 1), but totally abolished the sustained plateau phase (data only shown for EGTA) (Fig. 3A).

Effects of High K+ Buffer on Intracellular Calcium Response to Testosterone—The membrane depolarization with 25 mM KCl increased [Ca2+] in osteoblasts (Fig. 3B), and a further addition of 2 mM EGTA brought [Ca2+] back to the basal level. The plateau phase induced by 1 nM testosterone was abolished.
FIG. 4. Effects of thapsigargin on $[\text{Ca}^{2+}]_i$, response to testosterone. A, cells were incubated for 10 min with 1 μM thapsigargin before adding 1 nM testosterone. 1 μM thapsigargin caused a monotonic sustained increase in $[\text{Ca}^{2+}]_i$. Thapsigargin did not inhibit the sustained phase of $[\text{Ca}^{2+}]_i$ induced by testosterone. B, the traces show the changes in $[\text{Ca}^{2+}]_i$ induced by testosterone in cells treated with 1 μM thapsigargin in calcium-free medium. EGTA plus thapsigargin totally blocked the $[\text{Ca}^{2+}]_i$ increase induced by testosterone.

when the steroid was added 30 s after EGTA.

Characterization of Intracellular Organelle Responsible for Calcium Increase—Thapsigargin, which modifies calcium sequestration by the endoplasmic reticulum, was used at 100 nM as this concentration had the greatest effect on $[\text{Ca}^{2+}]_i$. Fig. 4A shows the $[\text{Ca}^{2+}]_i$ response to thapsigargin and testosterone. The rise in $[\text{Ca}^{2+}]_i$, induced by thapsigargin reached a peak within 90 s and then slowly decayed. Testosterone was added 10 min after thapsigargin. Pretreatment with thapsigargin totally abolished the increase, but not the plateau phase. The responses to T-CMO and T-CMO/BSA were comparable to those to testosterone (data not shown). However, when cells were pretreated with both EGTA and thapsigargin, the $[\text{Ca}^{2+}]_i$ response to testosterone (Fig. 4B), T-CMO, or T-CMO/BSA (data not shown) was totally abolished.

Cells were pretreated for 2 min with 2 mM neomycin (which inhibits phospholipase C via binding to phosphoinositides) (26), with 0.5–3 μM U-73122 (a phospholipase C inhibitor) (27), or with 0.5–3 μM U-73343 (a closed analog of U-73122, but inactive) (28) before adding 1 nM testosterone. As shown in Fig. 5A, neomycin totally blocked the increase response, while the plateau remained unchanged. U-73122 (1–3 μM) itself triggered transient dose-dependent (1–3 μM) increases in $[\text{Ca}^{2+}]_i$ (data not shown). Pretreatment with 2 μM U-73122 did not modify the plateau phase, but abolished the transient increase (Fig. 5A). U-73343 (0.5–3 μM) had no effect on the response to testosterone (data not shown).

Effects of Pertussis Toxin (PTX) on Intracellular Calcium Response to Testosterone—Osteoblasts were incubated for 24 h with 100 ng/ml PTX. Fura-2/AM loading and $[\text{Ca}^{2+}]_i$ measurements were carried out with 100 ng/ml PTX. PTX partially blocked the increase, while the plateau phase was unchanged. Pretreatment of the PTX-treated cells with neomycin totally abolished the remaining first increase, but did not modify the sustained response (Fig. 5B).

Effects of Cyproterone Acetate on Intracellular Calcium Response to Testosterone—Osteoblasts were incubated for 5, 10, or 45 min or 24 h with cyproterone acetate (10 nM and 1 μM), and 1 nM testosterone was then added. Preincubation with cyproterone acetate did not modify basal $[\text{Ca}^{2+}]_i$, and did not inhibit the effects of testosterone on intracellular calcium whatever the incubation time and the concentration of the nuclear antagonist (Table II).

Androgen Effects on Phospholipid Metabolism—Cultured male rat osteoblasts incubated with myo-[2-3H]inositol contained six radioactive peaks: free inositol, glycerophosphoinositol, InsP, InsP$_2$, InsP$_3$, and InsP$_4$. The percentages of radioactivity incorporated in each peak were 95–98% for free inositol, 1–2% for glycerophosphoinositol, 1–3% for InsP, 0.1–0.3% for InsP$_2$, 0.1–0.4% for InsP$_3$, and 0.2–0.4% for InsP$_4$. The basal concentration of inositol 1,4,5-trisphosphate, measured by radiocompetition, was 45.8 ± 1.6 pmol/10 μg of DNA ($n$ = 25) (29).

The formation of lipids was followed by measuring DAG, MAG, and PA. The percentage of radioactivity incorporated into each was 2–4% for DAG, 0.1–0.3% for MAG, and 1–3% for PA.

The action of testosterone (1 μM to 10 μM) on inositol phosphates and lipid formation was dose-dependent in a bell-shaped manner. The bell-shaped dose dependence was found for each inositol phosphate or lipid studied (data not shown, except for InsP$_3$ and DAG) (Fig. 6, A and B). Fig. 7 shows the response profiles of InsP$_4$, InsP$_3$, and InsP to 1 nM testosterone. The InsP$_4$, InsP$_3$, and InsP responses showed one stimulation
FIG. 5. Effects of different drugs on ([Ca\(^{2+}\)]\(_i\)) response to testosterone. A, cells were incubated for 2 min with either 2 mM neomycin or 2 \(\mu\)M U-73122 before adding 1 nM testosterone; B, cells were pretreated for 24 h with 100 ng/ml pertussis toxin or for 24 h with 100 ng/ml pertussis toxin, followed by 2 mM neomycin for 2 min before adding 1 nM testosterone. These traces are representative of at least five similar coverslips for each of the 25 cultures.

Effects of Cyproterone Acetate on DAG Response to Androgens—Osteoblasts were incubated for 45 min with cyproterone acetate (10 nM and 1 \(\mu\)M), and 1 nM testosterone was then added. Cyproterone acetate did not modify the level of incorporated radioactivity and did not inhibit the testosterone effects on DAG formation (data not shown).

Effects of Neomycin and Pertussis Toxin on InsP\(_3\) and DAG Responses to Testosterone—Osteoblasts were preincubated for 1 or 5 min with neomycin (2 mM); 1 nM testosterone was then added. Neomycin, whatever the incubation time, inhibited the increasing effect of testosterone on InsP\(_3\) and DAG (data not shown).

Osteoblasts were preincubated with 100 ng/ml PTX for 24 h. Incubations with 1 nM testosterone were carried out with PTX. PTX totally abolished the increasing effect of testosterone on InsP\(_3\) and DAG (data not shown).

Specificity of Testosterone Effects on Intracellular Calcium—1 pm to 100 nm 17\(\beta\)-estradiol and 1 pm to 100 nm progesterone had no effect on ([Ca\(^{2+}\)]\(_i\)) in male osteoblasts. 1 pm to 100 nm testosterone had no effect on ([Ca\(^{2+}\)]\(_i\)) in female osteoblasts, which were isolated from parietal bones of 2-day-old female rats and cultured in the same medium as described above (\(\alpha\)-minimal essential medium without phenol red).

DISCUSSION

This is, to our knowledge, the first study showing very rapid (5-60 s) effects of testosterone on cytosolic free calcium and membrane phospholipid metabolism in male rat osteoblasts. These effects are produced by physiological concentrations as low as 10 pm and are bell-shaped, with a maximum at 1 nm. This bell-shaped dose-dependent action can be compared to that found for calcitriol, the hormonally active form of vitamin D, in different cell types (11, 13).

Testosterone triggers a transient increase in ([Ca\(^{2+}\)]\(_i\)), followed by a sustained plateau phase. Testosterone modulates ([Ca\(^{2+}\)]\(_i\)), via two mechanisms: Ca\(^{2+}\) influx from the extracellular milieu and Ca\(^{2+}\) mobilization from the endoplasmic reticulum. On the one hand, EGTA, nifedipine (dihydropyridine-type blocker), and verapamil (phenylalkylamine-type blocker) decrease the increase response to testosterone by ~30% and totally abolish the sustained response. The testosterone-induced increase in ([Ca\(^{2+}\)]\(_i\)) implicates K+-dependent channels as shown by depolarizing the membrane. These results show that calcium influx occurs via voltage-gated calcium channels, which have been described in these cells (30). They are in agreement with what has been observed in LNCaP cells, in which androgens increase intracellular calcium levels in 1-2 min (14). On the other hand, thapsigargin (which modifies calcium sequestration by the endoplasmic reticulum) and phospholipase C inhibitors (indirect as neomycin or direct as U-73122) partially or totally block the increase without effect on the sustained plateau phase. Here is the first evidence that androgens trigger the release of calcium from the endoplasmic reticulum, which is mediated through phosphoinositide breakdown.

17\(\beta\)-Estradiol and progesterone, used from 1 pm to 100 nm, have no effect on intracellular calcium, leading to a specific action of androgens in male rat osteoblasts. Moreover, 1 pm to 100 nm testosterone does not elicit any increase in ([Ca\(^{2+}\)]\(_i\)) in female rat osteoblasts, while 17\(\beta\)-estradiol increases ([Ca\(^{2+}\)]\(_i\)) in
these cells at concentrations as low as 1 pm (31). This suggests that the rapid effects of androgens and estrogens in rat osteoblasts are sex-dependent instead of due to the absence or presence of an aromatic A ring. In addition, the rapidity of the androgen effect excludes any possibility of a further conversion of testosterone to estradiol.

No information is available on the rapid effect of testosterone on the rapid turnover of membrane phospholipids. This work shows for the first time that testosterone induces a concomitant

TABLE II

Effects of androgens on intracellular calcium concentration in
cyproterone acetate-treated cells

Osteoblasts were incubated with the nuclear antagonist for 45 min before the addition of 1 nm testosterone (T). Data are the intracellular Ca²⁺ concentrations 20 s after the steroid addition. Values are the means ± S.E. (n = 15; three coverslips in 15 different cultures).

|                      | Basal level (T = 0 nm) | Stimulated level (T = 1 nm) |
|----------------------|------------------------|----------------------------|
| Cyproterone acetate  |                        |                            |
| 0                    | 142 ± 3                | 238 ± 3                    |
| 10 nm                | 139 ± 6                | 245 ± 6                    |
| 1 µm                 | 145 ± 6                | 240 ± 3                    |

rapid (within 10 s) increase in the cellular content of inositol 1,4,5-trisphosphate and DAG formation in male rat osteoblasts. The concomitant increase in InsP₃ and DAG formation plus the inhibition of the two products formed by neomycin and U-73122 provide further support for the activation of the phospholipase C linked to phosphatidylinositol 4,5-bisphosphate. The response of the osteoblasts to testosterone seems to follow the pattern described for agonist-stimulated phosphoinositide turnover in general (32, 33). There are also significant increases in InsP₁, InsP₂, and InsP formation. In addition to the increase in DAG formation, this study also documents rises in MAG and PA levels in response to testosterone. The maximal formation of DAG and MAG preceding that of PA may indicate the sequential action of a specific phospholipase C and DAG kinase.

The data point to direct interactions of androgen-specific membrane steroid recognition moieties. First, androgens immobilized by covalent linkage to BSA, which do not enter the cell, also increase [Ca²⁺] via calcium influx and calcium mobilization and InsP₃ and DAG formation. The effects of T-CMO/BSA are due to covalently bound steroid and not to free steroid or to T-CMO in T-CMO/BSA. Second, direct or indirect inhibitors of phospholipase C totally block the rapid responses induced by testosterone. Third, cyproterone acetate, which com-

Fig. 6. Concentration dependence of testosterone-induced accumulation of inositol 1,4,5-trisphosphate (A) and diacylglycerol (B) as function of time. Cell incubation, extraction, and InsP₃ quantification (mass detection) and DAG extraction and separation were as described under "Experimental Procedures." Changes in InsP₃ and DAG concentration are expressed as a percentage of the control value (ethanol-treated cells) at each time. Data are means ± S.E. (n = 25; *, p < 0.001).
the activation of phosphoinositide-phospholipase C: one class of G-protein-mediated responses is pertussis-sensitive, and the other is insensitive (34). Precubination of the osteoblasts with PTX totally abolishes InsP₃ and DAG formation. The toxin seems to uncouple the androgen nongenomic receptor from its G-protein by blocking the signal transduction that activates the phospholipase C.

Taken together, these data suggest that male rat osteoblasts display membrane nongenomic androgen receptors that belong to a class of membrane receptors linked to intracellular effector coupled to phospholipase C via a pertussis toxin-sensitive G-protein. In conclusion, these findings may open up entirely new areas of investigation in the field of bone metabolism since these rapid changes may represent a mechanism whereby osteoblasts integrate different input signals, whereas the genomic nuclear pathway regulates the long-term osteoblast adaption to the needs of bone turnover.

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