Plasma Membrane Origin of the Steroidogenic Pool of Cholesterol Used in Hormone-induced Acute Steroid Formation in Leydig Cells*

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Hormone-sensitive acute steroid biosynthesis requires trafficking of cholesterol from intracellular sources to the inner mitochondrial membrane. The precise location of the intracellular cholesterol and its transport mechanism are uncertain. Perfringolysin O, produced by Clostridium perfringens, binds cholesterol. Its fourth domain (D4) retains cholesterol-binding properties but not cytotoxicity. We transfected steroidogenic MA-10 cells of mouse Leydig cell tumors with the mCherry-D4 plasmid. Tagged D4 with fluorescent proteins enabled us to track cholesterol. The staining was primarily localized to the inner leaflet of the plasma membrane and was partially released upon treatment with dibutyryl-cAMP (Bt2cAMP), a cAMP analog. Inhibitors of cholesterol import into mitochondria blocked steroidogenesis and prevented release of D4 (and presumably cholesterol) from the plasma membrane. We conclude that the bulk of the steroidogenic pool of cholesterol, mobilized by Bt2cAMP for acute steroidogenesis, originates from the plasma membrane. Treatment of the cells with steroid metabolites, 22(R)-hydroxycholesterol and pregnenolone, also reduced D4 release from the plasma membrane, perhaps evidence for a feedback effect of elevated steroid formation on cholesterol release. Interestingly, D4 staining was localized to endosomes during Bt2cAMP stimulation suggesting that these organelles are on the route of cholesterol trafficking from the plasma membrane to mitochondria. Finally, D4 was expressed in primary rat Leydig cells with a lentivirus and was released from the plasma membrane following Bt2cAMP treatment. We conclude that the plasma membrane is the source of cholesterol for steroidogenesis in these cells as well as in MA-10 cells.

Cholesterol is an important component of cell membranes, with unique structural and physical properties (1). The relative amount of cholesterol varies drastically between membranes in a cell, and this apparent critical balance is maintained by vesicular and non-vesicular trafficking; the latter is currently an intense area of research (2, 3). Cholesterol trafficking has been implicated in a number of diseases, including cardiovascular and brain diseases, cancer, and several rare monogenic diseases (4, 5).

All vertebrate steroid hormones are derived from the enzymatic metabolism of cholesterol (6, 7). In steroidogenic cells, such as those in the gonads and adrenal cortex, steroid biosynthesis is initiated by circulating pituitary peptide hormones. These hormones bind to their cognate receptors on the plasma membrane (8) and enhance the synthesis of cAMP (9). cAMP acts to promote cholesterol transport from intracellular stores to the mitochondria, where steroidogenesis is initiated (10). When the pool of free cholesterol dedicated to steroid biosynthesis reaches the mitochondria, it is inserted into the outer mitochondrial membrane (OMM)1 where it is segregated from the structural cholesterol until it is imported to the inner mitochondrial membrane (IMM) via an 800-kDa protein complex known as the steroidogenic metabolon. This complex includes the following mitochondrial proteins: translocator protein (TSPO; 18 kDa); the voltage-dependent anion channel (VDAC); the ATPase family AAA domain-containing protein 3 (ATAD3); and the cytochrome P450 side chain cleavage enzyme (CYP11A1) (11). This latter enzyme cleaves cholesterol to pregnenolone, which is the precursor of all cellular steroids (12). Steroidogenic acute regulatory protein (STAR) acts on this complex to accelerate cholesterol transfer from the OMM to the IMM (9, 11, 12).

Despite this detailed understanding of cholesterol transport and metabolism in steroid biosynthesis, it is not yet certain

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1 The abbreviations used are: OMM, outer mitochondrial membrane; 22(R)-HC, 22(R)-hydroxycholesterol; 22S-HC, 22(S)-hydroxycholesterol; CRAC, cholesterol recognition amino acid consensus sequence; CYP11A1, cytochrome P450 side chain cleavage enzyme; D4, domain 4 of the 9toxin; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; LD, lipid droplet; MJCD, methyl-β-cyclohexadrin; RIA, radioimmunoassay; STAR, steroidogenic acute regulatory protein; TSPO, translocator protein; U18666A, 3β;[2-(diethy lamino)ethoxy]androst-5-en-17-one; VDAC, voltage-dependent anion channel; ANOVA, analysis of variance; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EGFP, enhanced GFP.

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what the source of cholesterol is, particularly during acute steroidogenesis. A series of in vivo and in vitro studies performed in the 1970s led to the conclusion that lipid droplets (LDs), which contain esterified cholesterol, are the source of the steroidogenic pool of cholesterol. Early evidence came from an ultrastructural study that showed a decrease in the volume of LDs in adrenocortical cells after exposure to stimulatory hormones (13). Subsequently, the testes of adult male mice treated in vivo with human chorionic gonadotropin (hCG) were shown to have fewer LDs 1 day after treatment than the untreated mice (14). In another study, active transport of LD along microtubules in Y-1 mouse adrenocortical tumor cells was observed with non-perturbational imaging. The results also suggested an interaction between mitochondria and LD, consistent with cholesterol delivery from LDs to mitochondria (15). Other evidence for the importance of LD came from knock-out studies of the vimentin gene in mice, which codes for a LD-associated intermediate filament. Gene knock-outs disrupted steroidogenesis in adrenal but not testicular tissue (16), consistent with the known slower response of testes to steroid synthesis-inducing hormones compared with adrenal cortex. It was also demonstrated that hormones regulate the enzyme cholesterol ester hydrolase in LDs, affecting de-esterification of esterified cholesterol and increasing the pool of free cholesterol for steroid formation (17). Although LDs could provide a constant supply of cholesterol to sustain steroidogenesis, the kinetics of this process does not suggest that it is important in the acute response of these tissues to hormones.

The first evidence that the plasma membrane may provide the free cholesterol for steroidogenesis in the mitochondria came from studies by Freeman and co-workers (18–22). A series of metabolic labeling studies of Leydig and adrenal cell lines with radiolabeled acetate and cholesterol led them to suggest the importance of the plasma membrane. Among the cell membranes, the plasma membrane has the highest concentrations of cholesterol, with the next highest concentrations observed in endosomal recycling compartments and the Golgi apparatus (23). It is surprising that mitochondria, the site where steroid synthesis is initiated, and the endoplasmic reticulum (ER), where cholesterol is synthesized de novo, have lower concentrations of cholesterol than other cell membranes (23, 24). Because the cholesterol concentrations in cell membranes vary so widely, most researchers believe that vesicular trafficking using the secretory machinery is essential in cholesterol homeostasis (25, 26). However, recently there has been increased interest in non-vesicular cholesterol trafficking, especially in dynamic inter-organelle interactions (27). During these interactions, organelles come into close contact but do not fuse. Instead, they form microdomains rich in cholesterol that allow rapid communication between the organelles (28).

This is relevant here as several studies implicate organelle plasticity and inter-organelle associations in steroidogenesis (29). These include electron microscopic and biochemical studies that reveal increased incidence of ER-mitochondria interactions under hormone exposure (30, 31). We recently found that, in MA-10 mouse tumor Leydig cells, hormonal stimulation was followed by increased apposition of the ER and the OMM, known as mitochondrion-associated membranes (32). In addition to the mitochondrion-associated membranes, plasma membrane-associated microdomains involving the ER and mitochondria are also of current interest, as they are involved in signaling pathways, calcium influx, lipid synthesis, and most importantly, nonvesicular cholesterol trafficking (33–37). In a study using ultra-thin electron microscopy images, workers noted that plasma membrane-ER associations were 10 times more prevalent than ER-mitochondria interactions (38, 39).

The plasma membrane also interacts with other organelles in non-vesicular cholesterol trafficking. Maxfield and co-workers (40) observed that dehydroergosterol, an analog of cholesterol, trafficked from the plasma membrane to the endosome recycling compartments with the very short half-time of 2.5 min in TRVb-1 cells, a modified CHO cell line. It was even more rapidly transferred from the plasma membrane to LDs in macrophage foam cells, in which the half-time was 1.5 min (41).

Despite the improved understanding on how cells handle cholesterol trafficking and the knowledge available on cholesterol metabolism to steroid products, there is still a gap in our knowledge on the origin of free cholesterol used for acute steroid synthesis. To identify and visualize cholesterol used for steroid formation, we employed perfringolysin O (Θ toxin), a cytolsin produced by Clostridium perfringens that has the ability to bind cholesterol with high affinity (42, 43). Domain 4 of the Θ toxin (D4) is the C-terminal fragment and displays the same cholesterol binding affinity of the full protein, but it does not exert cytotoxicity (43). Tagging D4 with fluorescent proteins enabled us to track cholesterol movement in living cells. Here, we report on a study in which we used the D4 probe in Leydig cells treated with hormones or cAMP and examined the ability of the cells to form steroids. We also used a variety of steroidogenesis and cholesterol trafficking inhibitors to examine the specificity of the process. We conclude that cholesterol from the plasma membrane supplies hormone-induced acute steroidogenesis.

Results

Tracking Free Cholesterol Movement in MA-10 Mouse Tumor Leydig Cells Transfected with mCherry-D4—Free cholesterol was tracked during steroidogenesis in MA-10 cells by transfecting them with the mCherry-D4 plasmid and visualizing the fluorescent D4 protein with scanning confocal microscopy. The results are shown in Fig. 1A. In untreated cells, the fluorescent protein was predominantly bound to the plasma membrane, and there were few aggregates inside the cell. When the MA-10 cells were treated with Bt2cAMP, fluorescent intensity at the plasma membrane began to decline within 30 min, continued to decline over the next 30 min, and remained constant and low after that, although there was minimal change in untreated control cells (Fig. 1, A and B). Also, more fluorescent aggregates were seen in the treated than in the untreated cells. Acute stimulation of the cells by Bt2cAMP was confirmed by the measurement of progesterone, which increased during the 2-h treatment time. The accumulation of progesterone was unaffected by expression of mCherry-D4 (Fig. 1C).

We assessed the binding of mCherry-D4 to cholesterol in MA-10 cells by depleting the plasma membranes of cholesterol, which can be accomplished by treating cells with methyl-β-
cyclodextrin (MβCD). The data in Fig. 1D show that, following MβCD treatment, mCherry-D4 was no longer bound to the plasma membrane but formed aggregates in the cell as already observed (44). Upon depletion of cholesterol in the plasma membrane, mCherry-D4 loses its affinity to bind to it, confirming its specific binding to cholesterol-rich membranes.
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We next probed the effects of arresting cholesterol trafficking on the release of mCherry-D4 from the plasma membrane and on steroidogenesis. MA-10 cells were treated with U18666A, an inhibitor of cholesterol transfer from the plasma membrane to intracellular membranes (45). When cells were pre-treated with U18666A and Bt$_2$cAMP was added 4 h later, mCherry-D4 was retained in the plasma membrane, as evidenced by persistent high fluorescence 2 h later (Fig. 1E). We conclude that in the presence of the inhibitor, the cAMP analog was prevented from its expected action, the release of cholesterol from the plasma membrane. Furthermore, this cholesterol source appears to be important in steroidogenesis, as treatment with U18666A inhibited progesterone production in the presence of Bt$_2$cAMP (Fig. 1F), but did not affect basal steroid production in control cells.

Intracellular Cholesterol Distribution in mCherry-D4 Expressing MA-10 Cells—Considering the ability of D4 to bind cholesterol-rich membranes in live cells raised the question of whether the protein had an influence on cholesterol distribution in these cells. To examine this, MA-10 cells transfected with mCherry-D4 were stained with filipin, which binds to non-esterified cholesterol throughout the cell and compared with cells not expressing mCherry-D4 (Fig. 2, A and B). No change in cholesterol distribution was noticed throughout the cells, and the fluorescence intensity at the plasma membrane, where mCherry-D4 predominantly bound, remained unchanged (Fig. 2E). To study the effect of D4 in cholesterol distribution during hormone-induced steroidogenesis, MA-10 cells transfected with mCherry-D4 were treated with Bt$_2$cAMP and then fixed and stained with filipin (Fig. 2, C and D). Cholesterol distribution remained unchanged between control and mCherry-D4-expressing cells (Fig. 2, C and D). However, a significant drop in the filipin fluorescence intensity was noticed in the plasma membrane of Bt$_2$cAMP-treated cells when compared with control cells (Fig. 2, C–E).

To further examine D4’s effect on cholesterol distribution, we measured cholesterol levels in subcellular organelles involved in steroidogenesis. For this we first isolated mitochondria, plasma membrane, and endosomes from MA-10 control and mCherry-D4-transfected cells. The purity of the organelle fractions was confirmed by immunoblot analysis (Fig. 2F). Rab5, an endosome marker, VDAC1, a mitochondrial marker, PMCA1 (plasma membrane Ca$^{2+}$-ATPase 1), a plasma membrane marker, and $\beta$-actin, a cytoplasmic marker were all found to be enriched in their respective organelle fractions indicating the purity and efficiency of the isolation procedure used (Fig. 2F). Isolated fractions were processed for cholesterol content measurement by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results obtained revealed an overall similarity between control and mCherry-D4-expressing MA-10 cell organelle fraction cholesterol content (Fig. 2G).

The plasma membrane, known to be rich in cholesterol, had the highest content of cholesterol, followed by endosomes and then the mitochondria and the cytoplasm that had the least amount of cholesterol as expected (Fig. 2G). No change in cholesterol levels were noticed between control and mCherry-D4-treated fractions, suggesting that mCherry-D4 expression in the MA-10 cells does not alter cholesterol distribution in plasma membrane, mitochondria, and endosomes.

Cholesterol Trafficking in MA-10 Cells upon Treatment with Steroidogenic Inhibitors—The next question examined was whether the cholesterol originating from plasma membrane was targeted to mitochondria. The mCherry-D4-transfected MA-10 cells were treated with a variety of steroidogenic inhibitors, specifically with a cholesterol recognition/interaction amino acid consensus CRAC domain ligand (46), erastin (11), aminoglutethimide (11), and a START domain of the steroidogenic acute regulatory STAR protein ligand (47). They are inhibitors of TSPO, VDAC, CYP11A1, and STAR proteins, respectively, and play important roles in cholesterol transport into mitochondria and steroid biosynthesis.

When aminoglutethimide was added to the MA-10 cells in the presence of 1 mM Bt$_2$cAMP to inhibit CYP11A1 (Fig. 3, A and E), the rapid decline in mCherry-D4 fluorescence that occurred at the plasma membrane with Bt$_2$cAMP alone was partially prevented indicating that cholesterol was retained in the plasma membrane, with a maximal significant effect at 60 min. In addition, Fig. 3E shows that retention of cholesterol in the plasma membrane in the presence of the inhibitor effectively blocked Bt$_2$cAMP-induced steroid formation. Similar results were observed with erastin, an inhibitor of the OMM protein VDAC, which is involved in cholesterol translocation to the IMM for steroidogenesis (Fig. 3B).

The drug ligand (N-[2-(4-ethyl-5-[2-oxo-2-(4-toluidino)ethyl]-sulfanyl)-4H-1,2,4-triazol-3-y1]ethyl]-4-methylbenzamide) binding to the cholesterol recognition amino acid consensus sequence (CRAC) domain of TSPO, an inhibitor of steroidogenesis (46), reduced the Bt$_2$cAMP-induced movement of mCherry-D4 from the plasma membrane to inside the cell in a dose-dependent manner (Fig. 1D). The half-maximal inhibitory concentration (IC$_{50}$) and maximal inhibitory concentration (IC$_{100}$) of the CRAC domain ligand have been shown to be 1 and 10 $\mu$M, respectively (Fig. 3, D and E) (46), and these concentrations were used here. We used the CRAC domain ligand in combination with Bt$_2$cAMP to block steroidogenesis while inducing the movement of cholesterol to the mitochondria. A similar experiment was conducted with the START domain ligand (16-[4-(difluoromethoxy)benzylidene]androst-5-ene-3,17-diol) of the STAR protein (Fig. 3, C and E). The mCherry-D4 fluorescence intensity at the plasma membrane was unaffected by the steroidogenic inhibitors alone, but the inhibitors did partially prevent the release of cholesterol from the plasma membrane, consistent with the proposal that cholesterol in the plasma membrane is trafficked to the mitochondria and used in steroid biosynthesis upon acute stimulation of MA-10 cells by Bt$_2$cAMP.

Cholesterol Trafficking in the Presence of Steroids in MA-10 Cells—To determine cholesterol trafficking in the presence of endogenous steroids, MA-10 cells expressing mCherry-D4 protein were incubated with 22R-HC, a membrane-permeable metabolic intermediate. When 22R-HC enters the mitochondria, MA-10 cells readily made progesterone, up to 1000 ng/mg protein (Fig. 4C), levels that are similar to MA-10 cells stimulated with 1 mM Bt$_2$cAMP (Fig. 4, A, C and D). The steroid...
intermediate added to MA-10 cells did not affect mCherry-D4 labeling at the plasma membrane, indicating that intracellular progesterone production does not induce cholesterol movement from the plasma membrane (Fig. 4A). However, when 22R-HC and Bt2cAMP were applied in combination to induce both steroid formation and cholesterol movement from the plasma membrane, mCherry-D4 labeling of the plasma membrane remained unaffected, even after 2 h of combined 22R-HC and Bt2cAMP treatment (Fig. 4A). These results could be explained by the existence of a feedback signal generated by the synthesized progesterone that blocks cholesterol mobilization from the plasma membrane. When MA-10 cells were treated with 22S-HC, an inactive isomer of 22R-HC, we observed, as expected, no stimulation of progesterone production (Fig. 4C). However, when the inactive isomer was combined with Bt2cAMP, progesterone accumulated to about 700 ng/mg protein (Fig. 4D). Thus, in contrast to the active isomer 22R-HC, MA-10 cells treated with 22S-HC and Bt2cAMP readily trafficked cholesterol from the plasma membrane, as evidenced by the decreased fluorescence, which was close to the background.
FIGURE 3. Effect of inhibitors of the proteins associated with the steroidogenic metabolon on cholesterol trafficking from the plasma membrane in MA-10 cells transfected with mCherry-D4. A–D, time course of mCherry-D4 fluorescence associated with the plasma membrane in the presence of various inhibitors in the presence or absence of Bt₂cAMP. A, aminoglutethimide (AMG), an inhibitor of CYP11A1. B, erastin, an inhibitor of VDAC. C, two concentrations of the START domain ligand, an inhibitor of the STAR protein. D, two concentrations of the CRAC domain ligand, an inhibitor of TSPO. E, progesterone production 2 h after exposure of the cells to various inhibitors. Data represent means ± S.D. of at least three independent experiments performed in triplicate; two-way ANOVA followed by Bonferroni’s post hoc test (*) was used to calculate statistical significance; *, p < 0.05; **, #, p < 0.01; ***, p < 0.001. CRAC, cholesterol recognition amino acid consensus sequence; CYP11A1, cytochrome P450 side chain cleavage enzyme; STAR, steroidogenic acute regulatory protein; TSPO, translocator protein; VDAC, voltage-dependent anion channel.
signal within 30 min of treatment (Fig. 4B). We interpret these results as strongly indicative that, in the absence of endogenous steroid synthesis, the feedback signal is not activated in response to Bt2cAMP, and cholesterol is trafficked to the mitochondria for steroid formation.

Two candidates for feedback regulation of cholesterol trafficking are progesterone and pregnenolone. In MA-10 cells expressing mCherry-D4 were treated with these steroids in the presence and absence of Bt2cAMP (Fig. 4, E and F), pregnenolone and to some extent progesterone blocked cholesterol trafficking. This effect was apparent even 2 h after treatment. It may be that the steroids trigger a feedback signal that inhibits the movement of cholesterol from the plasma membrane.
Identification of Other Organelles Involved in Cholesterol Trafficking—In addition to its binding on the plasma membrane, mCherry-D4 was also detected within cells. When steroidogenesis was enhanced with Bt$_2$cAMP, there was an increase in this accumulation. We wished to determine whether mCherry-D4 formed aggregates in the cell or localized to another organelle; the latter should be detectable by assessing increased free cholesterol in these organelles. The mitochondria, LD, lysosomes, and ER in live MA-10 cells were identified using their respective fluorescent dyes. However, early endosomes, late endosomes, and Golgi apparatus were labeled using specific plasmids transduced into MA-10 cells using insect baculovirus (BacMam 2.0, Thermo Fisher Scientific). Thus organelles in MA-10 cells were screened for co-localization with mCherry-D4 upon Bt$_2$cAMP stimulation. An increase in co-localization with early and late endosome markers Rab5a-GFP and Rab7a-GFP (CellLight® Late Endosomes-GFP, BacMam 2.0, respectively), were detected 60 min after Bt$_2$cAMP treatment, suggesting that these endosomes might be part of the route by which cholesterol is trafficked from the plasma membrane to the mitochondria (Fig. 5, A and B).

To study the role of endosomes in steroid biosynthesis, NPC-2, previously shown to be involved in cholesterol trafficking between endosomes and mitochondria, was knocked down by transfecting MA-10 cells with specific siRNA at 50 and 75 nM concentrations (Fig. 5C) (48). The reduction of NPC-2 levels was accompanied by an increase in progesterone production at basal productions (Fig. 5D), but no change in Bt$_2$cAMP stimulated MA-10 cells (Fig. 5E). These data suggest that NPC-2 may play a negative role in cholesterol trafficking.
during basal steroidogenesis but does not play a role during acute steroidogenesis.

To further analyze the role of endosomes in steroidogenesis, endosomes from MA-10 control and Bt2cAMP-treated cells were isolated, and their enrichment was verified by immunoblot analysis using anti-Rab5 antibody. Cholesterol extracted from the samples was analyzed by LC-MS/MS. There was no change in cholesterol distribution in the whole cell homogenate samples, and in endosome fractions slight but not significant increases in cholesterol levels were noticed in control and treated groups (data not shown).

Identification of the Plasma Membrane Leaflet from Which Cholesterol Is Mobilized for Steroidogenesis—The plasma membrane includes two leaflets, an inner and outer. We wanted to know the origin of the leaflet from which the free cholesterol was made available for steroidogenesis. Untreated and Bt2cAMP-treated cells were fixed with paraformaldehyde and incubated with recombinant EGFP-D4 protein. In intact cells, the exogenous recombinant EGFP-D4 was bound to the outer leaflet of the plasma membrane. Where a decrease in the inner leaflet binding mCherry-D4 was noticed, no change in the binding of recombinant EGFP-D4 was observed. Scale bar, 10 μm.

Tracking Free Cholesterol Movement in Primary Rat Leydig Cells Transfected with mCherry-D4—Another Leydig tumor cell line, rat R2C cells, was transfected with mCherry-D4 plasmid. These cells produce high levels of steroids in a constitutive, hormone-, and cAMP-independent manner. Cholesterol trafficking was measured in these cells, and the results are presented in Fig. 7. It was noted that the overall fluorescence intensity of mCherry-D4 at the plasma membrane in control R2C cell line was lower uniformly than the control MA-10 cells (Fig. 7A). In contrast to MA-10 cells, these cells do not respond to Bt2cAMP (Fig. 7, A and B). There were no major changes in the fluorescence intensity at the plasma membrane, despite some movement of mCherry-D4 aggregates following Bt2cAMP treatment (Fig. 7B). This may be due to high levels of constitutive pregnenolone formation in these cells. Progesterone production measured in R2C cells revealed mCherry-D4 transfection did not have an effect in steroid biosynthesis in both control and 2-h Bt2cAMP-treated cells (Fig. 7C).
were isolated, in control (uninfected) cells 18 h after incubation, and 18 h post-infection. After 18 h in culture, the control cells had less testosterone than just after isolation, probably due to loss of steroidogenic capacity in culture (Fig. 8C). However, there was no change in testosterone levels at 18 h in the control and infected cells, suggesting that the lentivirus containing mCherry-D4 did not affect steroid biosynthesis (Fig. 8C).

**Discussion**

All vertebrate steroid biosynthesis is initiated at the mitochondria (49). The substrate, cholesterol, must move from its intracellular location to the cholesterol-poor IMM, where it will be converted to pregnenolone by the CYP11A1 enzyme. Cholesterol transport is incompletely understood, partly due to its hydrophobicity, but is believed to entail a network of cellular signals, lipid transfer systems, and protein interactions that operate between and within organelle membranes (4). In-depth studies of hormone-induced acute steroidogenesis have shown that a multiprotein, 800-kDa complex is formed at the mitochondria that transports cholesterol from the OMM to the IMM (11). However, the mechanisms of cholesterol transport from its intracellular sources to the mitochondria are not clear.

In planning this study, we recognized the utility of a probe that would permit us to track intracellular cholesterol movement within cells. The θ-toxin protein from *C. perfringens* seemed a likely candidate, as it can bind up to 30 mol % or more of cholesterol-containing membranes (50), and domain 4 of the protein retains the binding affinity without toxicity. The major advantages of using D4 over other fluorescent probes available to track cholesterol movement are that it does not damage the membranes to which it binds (Fig. 2E) and also does not disturb the intracellular distribution of cholesterol (Fig. 2G) (50–52). In addition, live-cell imaging is possible, which is not the case with filipin, another candidate probe. The binding affinity of D4 to membranes is low, so small changes in the cholesterol concentrations could be detected. Finally, it binds specifically to membrane cholesterol, avoiding the background signal that can be present with probes that bind indiscriminately to cellular cholesterol (43). In HeLa cells, D4 tagged with fluorescent proteins bound to both the outer and inner leaflet of the plasma membrane (42, 44). In another study with CHO cells, recombinant GFP-D4 successfully bound the outer leaflet of the plasma membrane, but the expressed mCherry-D4 in the cell could not bind the inner leaflet because of the low levels of cholesterol present in the membrane. To circumvent this, the workers employed a mutant mCherry-D4D434S (mCherry-D4H) that binds to the plasma membrane with higher affinity than the wild-type protein (51, 53). Hence, both D4 and D4H could be excellent tools to study cholesterol trafficking. For this study,
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we transfected MA-10 cells with mCherry-D4, permitting us to track cholesterol.

Given that the plasma membrane has a greater concentration of cholesterol than any other organelle (Fig. 2G), we expected D4 to readily bind it, as well as any other organelle with a membrane that contained greater than 30 mol % of cholesterol. Indeed, confocal images of mCherry-D4-transfected cells revealed fluorescence predominantly at the plasma membrane with a few aggregates in the cell (Fig. 1A). After initiating steroidogenesis with Bt2cAMP, there was a dramatic and significant decline in fluorescence intensity associated with the plasma membrane (Fig. 1, A and B). This could have been due either to release of cholesterol below 30 mol % or to a negative effect of Bt2cAMP on D4 binding. To distinguish between these possibilities, we used MβCD to expunge cholesterol from the membranes of the cells (54), which it did without binding to the membrane. Release of mCherry-D4 from the plasma membrane with MβCD alone led us to conclude that the removal of cholesterol prevented mCherry-D4 from binding (Fig. 1D).

Expression of mCherry-D4 did not alter Bt2cAMP-stimulated acute progesterone synthesis (Fig. 1C). Nevertheless, the ability of D4 to efficiently bind cholesterol-rich membranes raised the question whether D4 can influence the intracellular cholesterol distribution. Filipin staining analysis revealed no change in intracellular cholesterol distribution between control and mCherry-D4-expressing cells (Fig. 2, A–D). However, a significant drop in filipin fluorescence intensity at plasma membrane in Bt2cAMP-treated cells was seen, substantiating our finding that cholesterol for steroidogenesis is mobilized from the plasma membrane (Fig. 2E). In agreement with the filipin staining analysis, the subcellular distribution of cholesterol analyzed by mass spectrometry also did not reveal any change in cholesterol distribution in mitochondria, plasma membrane, and endosomes (Fig. 2G), suggesting that D4 does not affect intracellular cholesterol distribution during steroid biosynthesis.

To ensure that Bt2cAMP did not negatively affect D4 binding, we employed the inhibitor U18666A, which efficiently blocks cholesterol trafficking and interferes in cholesterol synthesis in the cell (55). In the presence of U18666A, cholesterol was trapped at the plasma membrane even after treatment with Bt2cAMP, and steroid formation was inhibited. This is evidence that the presence of Bt2cAMP did not have a negative influence on mCherry-D4 bound to the plasma membrane and strengthens our conclusion that cholesterol release from the plasma membrane can be attributed to reduced cholesterol in the membrane during steroidogenesis. Thus, we believe that, during Bt2cAMP-induced acute steroidogenesis in MA-10 cells, a pool of free cholesterol is trafficked from the plasma membrane.

In addition to inducing cholesterol transport, hormone stimulation affects a multiprotein complex called the transduceosome at the OMM (12) where the mitochondrial proteins are part of the steriodogenic metabolon, described earlier. The transduceosome includes the hormonally induced cytoplasmic protein STAR, the integral outer mitochondrial membrane proteins TSPO (56) and VDAC (57), and the IMM-associated protein CYP11A1 (7). Several studies show that interrupting the functionality of these proteins is detrimental to cholesterol transport to the IMM and, in turn, steroidogenesis. We asked whether the pool of cholesterol mobilized from the plasma membrane was, in fact, trafficked to the mitochondria after hormone stimulation. mCherry-D4-transfected MA-10 cells were treated with aminoglutethimide, a CRAC domain of the TSPO ligand, erastin, and a START domain of the STAR protein ligand. These are inhibitors of CYP11A1, TSPO, VDAC, and STAR proteins, respectively (Fig. 3, A–D). These inhibitors drastically increased the time required for the Bt2cAMP-induced movement of mCherry-D4 from the plasma membrane (Fig. 3, A–D), indicating that when there is a congestion of cholesterol at the OMM, it acts as a signal that prevents the mobilization of free cholesterol from the plasma membrane. By increasing the concentration of the STAR and TSPO ligands, the time of mCherry-D4 release was also delayed, further substantiating our conclusion (Fig. 3, B and D).

We also examined the kinetics of mCherry-D4 labeling of the plasma membrane of MA-10 cells exogenously supplied with the endogenous steroid substrate 22R-HC. This treatment stimulated steroid synthesis and maintained the mCherry-D4 labeling of the plasma membrane. Even when Bt2cAMP was supplied with 22R-HC, there was minimal movement of cholesterol from the plasma membrane. This may be evidence of a feedback signal that blocked the transfer of cholesterol from the plasma membrane. 22R-HC actively binds CYP11A1 and is rapidly converted to pregnenolone, so the congestion of cholesterol at the OMM might trigger a feedback signal similar to that observed with aminoglutethimide, which is an inhibitor of CYP11A1. To further probe the possible feedback mechanism, MA-10 cells were treated with 22S-HC, an inactive stereoisomer of 22R-HC (58). This isomer has the ability to bind CYP11A1 but does not undergo side chain cleavage to be converted to steroid products. In MA-10 cells treated with 22S-HC in the presence of Bt2cAMP, there was rapid displacement of cholesterol from the plasma membrane, even though there was some inhibition of steroid formation. We interpret these data to mean that it is the production of steroids and not the presence of hydroxycholesterols that induced the feedback mechanism. Indeed, the presence of progesterone has been previously shown to block cholesterol mobilization from late endosomal/lysosomal compartments in human fibroblast and monocyte cells (59, 60). So we next evaluated the release of mCherry-D4 from the plasma membrane in the presence of both steroids and Bt2cAMP. The release of mCherry-D4 was slightly retarded in the presence of progesterone, but D4 was clearly retained at the plasma membrane when pregnenolone was supplied (Fig. 4F). Therefore, it may be that pregnenolone acts in the feedback mechanism.

It is generally accepted that cellular membranes are asymmetric bimolecular leaflets composed predominantly of phospholipids, varying concentrations of cholesterol, other minor species of lipids, and a plethora of proteins (1, 61, 62). Because of its fast flip-flop rate, the cholesterol distribution across the two leaflets of the plasma membrane has been quite challenging to determine, with experiments suggesting either an outer or inner leaflet enrichment (23, 63). A recent study, based on a large scale molecular dynamics simulation, showed an asym-
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**A**

- Cholesterol
- Progesterone
- Pregnenolone

**B**

dbcAMP

**C**

dbcAMP + Inhibitors

**D**

dbcAMP + 22R-HC (or) Pregnenolone

**FIGURE 9. Proposed model for cholesterol movement from plasma membrane to the mitochondria in Leydig cells during acute steroid formation.** A under basal conditions, cholesterol is enriched in both leaflets of the plasma membrane, whereas the mitochondrial membranes have little cholesterol. **B,** after 30–45 min of acute stimulation with Bt$_2$cAMP (dbcAMP), cholesterol is trafficked from the plasma membrane to the mitochondria, where a transduceosome and metabolon complex translocate cholesterol from the OMM into the IMM and enzymatically convert free cholesterol to pregnenolone. Pregnenolone further undergoes multiple enzymatic reactions and is converted to progesterone. C, inhibition of the protein components in the transduceosome and the metabolon initiates a negative feedback signal that obstructs the cholesterol release from the inner leaflet of the plasma membrane. **D,** presence of pregnenolone, progesterone, and the steroid intermediate 22R-HC in the system initiates a similar negative feedback signal, inhibiting the trafficking of cholesterol from the plasma membrane to the mitochondria. 22R-HC, 22(R)-hydroxycholesterol; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

metrical distribution of cholesterol, in which the outer leaflet of the plasma membrane included 54% of the total cholesterol with a slightly higher cholesterol content than the inner leaflet (comprising 46%) (62).

Compared with the inner leaflet, the outer leaflet is more rigid, with a higher concentration of sphingolipids that tightly interact with cholesterol through strong van der Waal’s forces, forming rafts. In contrast, the inner leaflet has fewer sphingolipids and more glycerolipids, and cholesterol binds less efficiently due to weak van der Waal’s forces. This may make it easier for the cholesterol to be mobilized from the inner leaflet. In our study, when the outer plasma membrane leaflet was labeled with recombinant EGFP-D4 and cells were subjected to Bt$_2$cAMP treatment, no change in the labeling occurred (Fig. 6, **A** and **B**). This can be explained if the cholesterol for steroidogenesis comes from the inner leaflet.

The first suggestion that the plasma membrane was the source for the steroidogenic pool of cholesterol was made by Freeman (19). Based on these results, it was also suggested that cholesterol moves from the plasma membrane passing through endosome compartments during steroidogenesis in MA-10 cells (22). Our screening of various organelles with the appropriate respective fluorescent markers is in agreement with this suggestion; mCherry-D4 co-localized with a late endosome marker, and the co-localization increased upon Bt$_2$cAMP stimulation (Fig. 5B), suggesting that the late endosome might be part of the route for cholesterol trafficking from the plasma membrane to mitochondria. However, in Bt$_2$cAMP-treated endosome-rich isolates, only a slight increase in cholesterol levels was noted when compared with control (Fig. 5D). Although this finding may not add further evidence to the data generated by confocal microscopy, it is likely that the sensitivity of the mCherry-D4 is such that when cholesterol concentrations cross the threshold of 35 mol %, the probe would be able to bind the membrane (42), and small increases in the concentrations of cholesterol may not be observed as significant when studied by mass spectrometry. Furthermore, knocking down NPC-2, a protein previously shown to be involved in the exit of cholesterol from the endosome/lysosome compartment to mitochondria (48), did not have an effect on Bt$_2$cAMP-induced steroidogenesis, although it had a negative effect in basal conditions, suggesting that NPC-2 may not play a role in the hormone-induced transport of the steroidogenic pool of cholesterol between endosome compartment and mitochondria (Fig. 5, D and E).

In contrast to the hormone-inducible MA-10 cells, the rat tumor R2C Leydig cells constitutively synthesize high levels of pregnenolone and progesterone (64). Interestingly, mCherry-D4 localized at the plasma membrane in R2C cells even though they were constitutively making steroids. Because these cells do not respond to hormone or cAMP stimulation, no release of mCherry-D4 from the plasma membrane was observed, suggesting that a different source organelle supplies cholesterol for steroid biosynthesis in R2C cells.

Even though MA-10 and R2C cell lines appear to be excellent models for studying steroid biosynthesis, the differences in cholesterol trafficking between these tumor Leydig cell lines led to concerns that tumorigenic transition of these cell lines altered the source of cholesterol utilized in steroidogenesis. To investigate this possibility, primary rat Leydig cells were isolated and infected with lentiviruses containing mCherry-D4 for efficient gene transfer. Even though the efficiency of the infection was ~95%, only a few cells had labeling at the plasma membrane. This trend was similar to that in MA-10 cells, in which chol-
Cholesterol movement at the plasma membrane was apparent 60–90 min after stimulation of steroid biosynthesis by Bt2cAMP. Thus, we conclude that the plasma membrane is the source of cholesterol used in steroid biosynthesis in primary rat Leydig cells.

Free cholesterol is considered to be the main source for steroid hormone production in rodent testes (5). This is in contrast to rodent adrenal gland and ovary, where an important reservoir of cholesterol for steroidogenesis is present in cholesterol esters stored in the LDs. The results presented herein show that the plasma membrane is one of the richest sources of free cholesterol available for binding to the mCherry-D4 probe under basal conditions (Fig. 9A). During acute stimulation of steroid biosynthesis by cAMP, MA-10 cells and primary Leydig cells mobilize this free cholesterol that is transported to the mitochondria, where the transduceosome complex translocates the cholesterol to the IMM for conversion to pregnenolone by CYP11A1, and then is subject to further metabolism to progesterone and other steroids in the ER (Fig. 9B). Inhibiting one of the transduceosome proteins may create a feedback signal instructing the trafficking system to stop sending cholesterol to the already congested mitochondria, slowing down steroidogenesis (Fig. 9C). Moreover, when a large amount of pregnenolone is added to the system, it may initiate another feedback signal that inhibits the movement of cholesterol from the plasma membrane (Fig. 9D). It seems that these feedback mechanisms serve as protective mechanisms to avoid cholesterol-induced toxicity at the OMM and excessive steroid production, which could be detrimental to the body. Although pregnenolone was proposed to be the messenger carrying the feedback, its sensor at the plasma membrane remains to be identified. Insights into cholesterol trafficking pathways will increase the understanding of steroid-related diseases and help the development of drugs affecting cholesterol trafficking and metabolism.

Experimental Procedures

Cell Culture—MA-10 cells from mouse Leydig cell tumor (kindly provided by Dr. M. Ascoli, University of Iowa, Iowa City) and R2C cells from rat Leydig cell tumor cells (American Type Culture Collection, Manassas, VA) were cultured with Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (Invitrogen) supplemented with 1% penicillin and 1% streptomycin and maintained at 37 °C. The media for MA-10 and R2C cells were supplemented with 5% fetal bovine serum (FBS) and 2.5% heat-inactivated horse serum, and the cultures were kept on growth medium overnight. The next day, cells were transfected with mCherry-D4 plasmid and incubated for another 24 h. Following the treatment period, medium was removed, and cells were washed three times with 1 × PBS and fixed with 4% paraformaldehyde for 30 min. The cells were then stained for cholesterol using 50 μg/ml filipin III (Sigma) for 1 h, washed, and visualized under scanning laser confocal microscope.

Isolation of Organelles—MA-10 cells were grown in 875-cm² Falcon® rectangular straight neck cell culture multiflasks to 80% confluence, washed with PBS, and trypsinized. Isolation of cytoplasm, mitochondria, and plasma membranes was carried out following a well established methodology with minor modifications (65). In brief, cells were homogenized with a Teflon pestle and an overhead stirrer by using 6–7 strokes at 3000 rpm. Cell integrity was assessed using trypan blue staining and visualized under microscope until 90% cell damage had been attained. Homogenates were centrifuged at 800 × g for 5 min at 4 °C. The collected supernatants were centrifuged twice at 10,000 × g for 10 min. The pellets obtained contained crude mitochondria isolates, which were further centrifuged at 10,000 × g for 10 min to obtain the mitochondrial fraction. To confirm the enrichment of mitochondria, immunoblot analysis was performed using an anti-VDAC1 antibody. The supernatant was centrifuged at 25,000 × g for 20 min at 4 °C. The obtained supernatant contained the microsomes and the cytoplasm fractions, which were further subjected to ultracentrifugation at 95,000 × g for 2 h and 30 min at 4 °C to obtain the isolated cytoplasm. The purity of the fraction was confirmed by immunoblot analysis using anti-β-actin. The pellet from the 25,000 × g centrifugation step contained primarily plasma membrane and further mitochondrial contaminants. The pellet was resuspended and layered onto 38, 43, and 53% sucrose density gradients and ultracentrifuged at 4 °C from which the plasma membrane fraction was separated from the mitochondrial contamination. The purity of the plasma membrane fraction was confirmed by immunoblot analysis using an anti-PMCA1 antibody.

To isolate endosomes, MA-10 cell homogenates were subjected to discontinuous density gradient centrifugation using 8, 35, and 42% sucrose density gradients and ultracentrifuged at 210,000 × g for 1.5 h at 4 °C as described previously (66). The purity of the endosome fraction was confirmed with immunoblot analysis with an anti-Rab5 antibody.

Immunoblot Analysis—Proteins from subcellular organelle fractions were extracted using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). Proteins were quantified using the Bradford assay (Bio-Rad). 15 μg of protein each from whole cell homogenates, cytoplasm, endosomes, mitochondria, and plasma membranes were separated in a Novex NuPAGE BisTris 4–12% (w/v) precast gel (Invitrogen), transferred to polyvinylidene fluoride (PVDF) membranes for Blot analysis with an anti-Rab5 antibody. Purity of the endosome fraction was confirmed with immunoblot analysis with an anti-PMCA1 antibody.
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standard Western blotting, and blocked for 90 min at room temperature in blocking buffer (20 nM Trizma (Tris base), 100 mM NaCl, 1% Tween 20, 5% skim milk). Membranes were incubated overnight at 4 ºC with anti-Rab5 (1:2000, Abcam), anti-VDAC1 (1:5000, Abcam), anti-PMCA1 (1:1000, Abcam), and anti-ß-actin (1:1000, Abcam), followed by appropriate secondary horse-radish peroxidase (HRP)-conjugated antibodies (1:1000, Cell Signaling Technology). Proteins of interest were visualized using the chemiluminescence kit from Amersham Biosciences and a FUJI image reader LAS4000 (FUJIFILM) for capturing images.

Cholesterol Analysis using AB Sciex Triple-TOF Mass Spectrometer—For the purpose of quantification of cholesterol from subcellular organelle fractions, d<sub>2</sub>-cholesterol was utilized as an internal standard. Samples were extracted using 2×1 ml of methyl tert-butyl ether. Aliquots were combined in 10×13-mm test tubes dried in a Thermo SpeedVac<sup>TM</sup> for 20 min, re-dissolved in a 500-µl aliquot of methyl tert-butyl ether, vortexed, and dried again for ~10 min. Samples were resuspended in 200 µl of 50% (aqueous) methanol and vortexed. The contents were transferred into autosampler vials and were stored at −20 ºC until ready for LC-MS/MS analysis. All measurements were made by AB Sciex 5600+ triple-TOF mass spectrometer coupled with a Shimadzu Nexera XR UHPLC system. A binary mobile phase consisting of (a) water with 0.1% formic acid and (b) acetonitrile with 0.1% formic acid and acid was utilized. A 2-µl aliquot of sample was injected into liquid chromatograph. Analytes were chromatographically resolved by isocratic elution (90% (B) at 350 ml/min) with an Agilent Eclipse Plus C-8 analytical column (50×2.1-mm inner diameter×1.8-µm particle). The total run time was 10 min. The mass spectrometer was operated in a positive HESI mode with a vaporization temperature of 550 ºC and a spray voltage of 5.5 kV. For quantification purposes, a calibration curve was run along with the samples. After data acquisition, the intensity of each ion was integrated MultiQuant<sup>TM</sup> software.

Co-localization Analysis—To assess the localization of mCherry-D4 protein to the Golgi apparatus, early endosomes, or late endosomes, mCherry-D4-transfected MA-10 cells were transfected with the appropriate green fluorescent protein (GFP), using BacMam 2.0 (Thermo Fisher Scientific) at 100 virus particles per cell for 24 h at 37 ºC. The GFPs used were as follows: CellLight Golgi-GFP; CellLight early Endosomes-GFP; or CellLight Late Endosomes-GFP (Thermo Fisher Scientific). To study the localization of the mCherry-D4 probe to mitochondria, mCherry-D4-transfected cells were treated with 100 nM MitoTracker Green fluorescent mitochondrial stain (Green FM; Cell Signaling Technology) for 30 min at 37 ºC. The cells were washed three times with PBS and visualized. To detect localization of the mCherry-D4 probe to LDs, mCherry-D4-transfected cells were treated with 2 µg/ml boron-dipyromethene (BODIPY 493/503; Thermo Fisher Scientific), incubated for 30 min at 37 ºC, washed three times with PBS, and then visualized. mCherry-D4 probe in lysosomes was detected by labeling lysosomes with 70 nM LysoSensor Green DND-189 (Thermo Fisher Scientific), incubated for 30 min at 37 ºC, and washed three times with PBS before visualization. Localization of the mCherry-D4 probe to the ER was assessed by incubating cells with 250 nM ER-Tracker<sup>TM</sup> Blue-White DPX dye for 45 min at 37 ºC, washed three times with PBS, and visualized under confocal microscope.

Small Interfering RNA Transfection Analysis—MA-10 cells were plated onto 6-well plates at a concentration of 3×10<sup>5</sup> cells/well and immediately transfected using ON-TARGET<sup>plus</sup> mouse NPC2 siRNA 50 and 75 nM (Dharmacon) using 4 µl of jetPRIME<sup>®</sup> reagent (Polyplus-transfection) and 200 µl of jetPRIME<sup>®</sup> buffer based on the protocol specified by the manufacturer. A scrambled siRNA (ON-TARGET<sup>plus</sup> non-targeting siRNA; 40 nM) from Dharmacon was used as a transfection control. 72 h post-transfection, medium was collected to analyze progesterone production using RIA before and after Bt<sub>c</sub>CAMP treatments. Cells were subjected to protein extraction using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific). Proteins were quantified using Bradford assay. 20 µg of protein each from control, mock, scrambled, and siRNA-transfected samples were subjected to immunoblot analysis.

Cell Treatments—The cell treatments were as follows: 1 mM Bt<sub>c</sub>CAMP (Sigma); 20 µM 22R-RC (Sigma); 20 µM 22S-RC (Sigma); 20 µM progesterone; 20 µM pregnenolone; 1 or 10 µM TSPO CRAC domain ligand (N-[2-(4-ethyl-5-[2-oxo-2-(4-toluidino)ethyl]sulfanyl-4H-1,2,4-triazol-3-yl]ethyl]1-methylbenzamide) (46); 100 µM START domain of STAR protein ligand (16-[4-(difluoromethoxy)benzylidene]androst-5-ene-3,17-diol) (47); 0.67 mM DL-aminogluthetemide (Sigma), a CYP11A1 inhibitor, or 100 µM erastin (Sigma), an inhibitor of VDAC. Chemicals were supplied in serum-free media with or without Bt<sub>c</sub>CAMP for 2 h.

Cholesterol was removed from internal sources by treating MA-10 cells with 10 mM M<sub>B</sub>CD (Sigma), for 30 min at 37 ºC. Cholesterol trafficking was arrested by treating MA-10 cells with 7 µM U18666A (Sigma) for 4 h and re-induced by treating them with Bt<sub>c</sub>CAMP.

Steroid Measurement—MA-10 cells and R2C cells were plated onto 96-well plates (2×10<sup>4</sup> cells/well). Twenty four hours after incubation, the old cell culture media were replaced with fresh media for control cells, and the cells for treatments were transfected with mCherry-D4 plasmid and incubated for another 24 h, after which the media were replaced with serum-free media. At the end of the treatments, the culture media were collected, and progesterone production was measured by RIA with progesterone antiserum (MP Biomedicals) and [1,2,6,7-<sup>3</sup>H]progesterone (specific activity, 94.1 Ci/mmol; PerkinElmer Life Sciences) using the manufacturer’s recommended conditions. Progesterone production was normalized to the amount of protein in each well. The RIA data were analyzed with Prism 4.02 from GraphPad.

To assess steroidogenesis in the primary rat Leydig cells, cells were plated into wells of a 96-well plate (1×10<sup>5</sup> cells/well). Testosterone was measured 2 and 20 h after plating or, for the cells with lentiviral infection, 20 h after plating. Steroidogenesis was induced with 1 mM Bt<sub>c</sub>CAMP for 2 h, when the culture medium was collected, and testosterone was measured by RIA with testosterone antiserum (MP Biomedicals) and radiolabeled testosterone (1,2,6,7-<sup>3</sup>H; specific activity, 83.4 Ci/mmol;
PerkinElmer Life Sciences) according to the manufacturer’s instructions. Recombinant EGFP-D4 Protein Labeling—MA-10 cells were seeded in medium containing serum. Twenty four hours after incubation, cells were washed with PBS and then incubated for 2 h in fresh media without serum, either with or without 1 mM Ba2+-cAMP. Cells were then washed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and incubated in 5 µg/ml EGFP-D4 in binding buffer (0.1% BSA/PBS) for 30 min at room temperature. Recombinant EGFP-D4 was prepared as described previously (42). The cells were washed three times again with PBS and visualized using a scanning confocal microscope.

Lentiviral Vector Packaging Using 293FT Cells—The D4 cDNA coding sequence was subcloned into the HIV-based lentiviral expression vector, pLVX-mCherry-C1 (Clontech), at the EcoRI and BamHI sites using the following primers: 5’-gaattcgtacagAAGCTTaaggg-3’ and 5’-ggatccGCGGGTT- TAAACCTCGAG-3’. Three microgram of the pLVX-mCherry-D4 vector was then co-transfected with 9 µg of ViraPower lentiviral packaging mix in the presence of Lipo-mix. Images were taken 48 h post-transfection. The cells were washed three times with PBS, and incubated in 5 µg/ml EGFP-D4 in binding buffer (0.1% BSA/PBS) for 30 min at room temperature. Recombinant EGFP-D4 was prepared as described previously (42). The cells were washed three times again with PBS and visualized using a scanning confocal microscope.

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