Nontranscriptional modulation of intracellular Ca^{2+} signaling by ligand stimulated thyroid hormone receptor

Nuttawut Saelim,[1] Linu M. John,[2] Jun Wu,[1] Jeong Soon Park,[1] Yidong Bai,[1] Patricia Camacho,[2] and James D. Lechleiter[1]

[1]Department of Cellular and Structural Biology and [2]Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229

Thyroid hormone 3,5,3′-tri-iodothyronine (T_3) binds and activates thyroid hormone receptors (TRs). Here, we present evidence for a nontranscriptional regulation of Ca^{2+} signaling by T_3-bound TRs. Treatment of *Xenopus* thyroid hormone receptor beta subtype A1 ($xTR_\beta A1$) expressing oocytes with T_3 for 10 min increased inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} wave periodicity. Coexpression of $TR_\beta A1$ with retinoid X receptor did not enhance regulation. Deletion of the DNA binding domain and the nuclear localization signal of the $TR_\beta A1$ eliminated transcriptional activity but did not affect the ability to regulate Ca^{2+} signaling. T_3-bound $TR_\beta A1$ regulation of Ca^{2+} signaling could be inhibited by ruthenium red treatment, suggesting that mitochondrial Ca^{2+} uptake was required for the mechanism of action. Both $xTR_\beta A1$ and the homologous shortened form of rat $TR_\alpha 1$ ($rTR_\alpha \Delta F1$) localized to the mitochondria and increased O_2 consumption, whereas the full-length rat $TR_\alpha 1$ did neither. Furthermore, only T_3-bound $xTR_\beta A1$ and $rTR_\alpha \Delta F1$ affected Ca^{2+} wave activity. We conclude that T_3-bound mitochondrial targeted TRs acutely modulate IP_3-mediated Ca^{2+} signaling by increasing mitochondrial metabolism independently of transcriptional activity.

Introduction

Thyroid hormones are lipophilic ligands composed of two iodinated tyrosine residues that regulate cellular differentiation and development, cardiac function, and basal metabolism (Abbaticchio et al., 1981; Oppenheimer et al., 1987, 1994; Nagai et al., 1989; Kawahara et al., 1991; Soboll, 1993a; Ichikawa and Hashizume, 1995). Thyroid receptors (TRs) are classified as steroid hormone receptors and have genomic effects similar to other nuclear receptors such as glucocorticoid, estrogen, and androgen receptors. Two separate genes encode thyroid hormone receptors α (TR_α) and β (TR_β). Alternative splicing or the use of different promoters generates multiple isoforms including the α ($TR_\alpha 1$, $TR_\alpha 2$) and β ($TR_\beta 1$, $TR_\beta 2$) subtypes (Lazar, 1993). Thyroid hormones have been shown to increase the number of mitochondria and to induce the expression of mitochondrial proteins encoded by both nuclear and mitochondrial genes (Das and Harris, 1991; Soboll, 1993a; Iglesias et al., 1995; Wrutniak et al., 1995; Meehan and Kennedy, 1997; Schonfeld et al., 1997). Isolated mitochondria from hyperthyroid cells exhibit enhanced substrate cycling and increased oxygen (O_2) consumption (Soboll, 1993b). Thyroid hormone also affects the mitochondrial membrane potential ($\Delta\Psi$) through the expression of mitochondrial proteins (Soboll, 1993a,b). Collectively, these long-term effects take days or weeks to manifest and are thought to be mediated by nuclear and mitochondrial transcriptional regulation.

Increasing evidence suggests that thyroid hormone exerts nontranscriptional effects on mitochondrial metabolism. Initial studies demonstrated that treatment of cells with 3,5,3′-triiodothyronine (T_3) results in a rapid increase in O_2 consumption and ATP production in rat liver mitochondria (Sterling, 1980). These effects persisted in the presence of protein synthesis inhibitors, suggesting that the mechanism of action was non-

Correspondence to James D. Lechleiter: lechleiter@uthscsa.edu

N. Saelim's current address is: Dept. of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Naresuan University, Pitsanulok, Thailand, 65000.

L.M. John's current address is: Pfizer, Inc., CVMD Biology, Groton, CT 06340.

Abbreviations used in this paper: ANT, adenine nucleotide translocator; DBD, DNA binding domain; $\Delta\Psi$, mitochondrial membrane potential; IP_3, inositol 1,4,5-trisphosphate; MBS, modified barth's solution; O_2, oxygen; pBOX, three amino acid sequence within the DNA binding domain that recognizes specific DNA binding sequences; RA, 9-cis retinoic acid; $rTR_\alpha 1$, rat thyroid hormone receptor alpha subtype 1; $rTR_\alpha \Delta F1$, shortened form of rat $TR_\alpha 1$; Ru_{360}, ruthenium 360; RXR, retinoid X receptor; SEAP, secreted placental alkaline phosphatase; T_3, 3,5,3′-tri-iodothyronine; TMRE, tetramethylrhodamine ethyl ester; TR, thyroid receptor; TRE, thyroid hormone response element; $xTR_\beta A1$, *Xenopus* thyroid hormone receptor beta subtype A1.

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transcriptional. Sterling and coworkers (Sterling, 1980; Sterling and Brenner, 1995) additionally demonstrated that exposure of mitochondria to T_3, isolated from rat hepatocytes, increased both ATP production and O_2 consumption. Acute exposure of isolated mitochondria to thyroid hormone has also been reported to increase ΔpH and to increase mitochondrial Ca^{2+} efflux (Sterling et al., 1980; Crespo-Armas and Mowbray, 1987; Soboll, 1993a). Mitochondrial localization of TRs was originally reported by Sterling and coworkers (Sterling, 1991). Later, Ardail et al. (1993) identified two high affinity T_3 binding proteins in rat liver mitochondria. Wrutniak et al. (1995) and Casas et al. (1999) reported the presence of a high affinity ($\sim$43 kD) T_3 binding protein in rat liver mitochondrial matrix extracts, which was identified as an NH_2 terminus shortened form of rat $TR_\alpha 1$ ($rTR_\alpha \Delta F1$). The full-length form of the rat thyroid hormone receptor alpha subtype 1 ($rTR_\alpha 1$) is predominantly localized to the nucleus where it binds to DNA response elements and regulates transcriptional events (Wrutniak et al., 1995). Wrutniak (Wrutniak et al., 1995) suggested that the mitochondrial form of the rTR may be involved in mitochondrial transcriptional activity.

Intracellular Ca^{2+} signaling has been intimately linked to mitochondrial metabolism. Several dehydrogenases within the citric acid cycle are Ca^{2+} dependent (McCormack and Denton, 1989). Ca^{2+} uptake into the mitochondria is a passive process driven by the mitochondrial $\Delta\Psi$ and occurs via the Ca^{2+} uniporter. Because of the low Ca^{2+} affinity of the uniporter, high cytosolic Ca^{2+} concentrations are required to cause significant mitochondrial Ca^{2+} uptake. Under physiological conditions, these concentrations only occur near an open ion channel pore. Consequently, close physical proximity between the ER and mitochondria is required for significant mitochondrial Ca^{2+} uptake (Rizzuto et al., 1998, 1999). Work from our laboratory also demonstrated that mitochondrial Ca^{2+} uptake itself modulated inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+} release (Jouaville et al., 1995). Subsequently, Hajnoczky et al. (1995) demonstrated that IP_3-mediated Ca^{2+} oscillations efficiently stimulated mitochondrial metabolism. The local Ca^{2+} signaling between the ER and mitochondria has now been supported by many other investigators (Simpson and Russell, 1996; Hajnoczky et al., 1999; Szalai et al., 2000). Control of mitochondrial metabolism by matrix Ca^{2+} appears to be a fundamental mechanism whereby cells meet their energy requirements.

Xenopus laevis oocytes do not express detectable levels of endogenous TRs (Banker et al., 1991; Kawahara et al., 1991; Eliceiri and Brown, 1994). Induction of TR expression in *Xenopus laevis* occurs during the embryonic stages of development (Yaoita and Brown, 1990; Banker et al., 1991; Kawahara et al., 1991; Eliceiri and Brown, 1994). Consequently, *Xenopus* oocytes offer a unique model system to study the effects of thyroid hormones and their receptors on intracellular Ca^{2+} signaling and mitochondrial metabolism.

We present evidence demonstrating that thyroid hormone-activated TRs acutely regulate mitochondrial metabolism and, thereby, Ca^{2+} wave activity. Only expression of the NH_2 terminus–truncated forms of TR that target the mitochondria were effective at stimulating mitochondria. Transcriptionally inactive TRs were fully capable of modulating Ca^{2+} wave activity. These observations suggest an acute nontranscriptional pathway for modulation of intracellular Ca^{2+} signaling via thyroid hormone receptor-stimulated mitochondrial metabolism.

Results

T_3-stimulated $TR_\beta A1$s modulate IP_3-mediated Ca^{2+} wave activity

Acute nongenomic effects of thyroid hormones occur within minutes of ligand treatment (Hummerich and Soboll, 1989). To examine the importance of TRs on the nongenomic modulation of intracellular Ca^{2+} signaling, stage VI *Xenopus* oocytes were injected with mRNA encoding the *Xenopus* thyroid hormone receptor betz subtype A1 ($xTR_\beta A1$) as described previously (Camacho and Lechleiter, 2000). Expression of $xTR_\beta A1$ was confirmed by Western blot analysis, 2–3 d after mRNA injection (Fig. 1). The Ca^{2+} indicator dye was injected into oocytes 30–45 min before confocal imaging. When oocytes were injected with IP_3, we observed repetitive Ca^{2+} wave activity with interwave periods of 6.62 $\pm$ 0.20 s (n = 70; Fig. 1). When $xTR_\beta A1$ expressing oocytes were treated with T_3 10 min before IP_3 injection, the Ca^{2+} wave periodicity increased significantly to 8.40 $\pm$ 0.30 s (Fig. 1, a and c; n = 24, $P < 0.0001$ ANOVA single factor). Treatment of oocytes with T_3 by itself did not induce Ca^{2+} release and no detectable changes in basal intracellular Ca^{2+} concentrations were observed. Application of T_3 ligand to nonexpressing control oocytes, had no effect on the

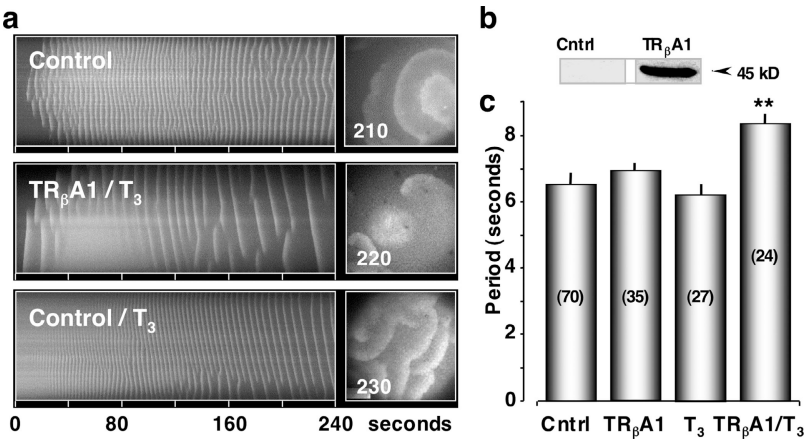

Figure 1. **T_3-bound $TR_\beta A1$ increases IP_3-induced Ca^{2+} wave period.** (a) Spatial-temporal stacks of IP_3 ($\sim$300 nM)-induced Ca^{2+} wave activity in a representative control (water injected) oocyte, a T_3-treated (100 nM) oocyte expressing $TR_\beta A1$ and a T_3 (100 nM) treated oocyte. Each image is 745 $\times$ 745 μm. (b) Western blot showing expression of $TR_\beta A1$. Protein extracts from all groups were collected and loaded at 0.5 oocytes per lane onto 10% SDS-PAGE. The membrane was probed with a monoclonal mouse anti–human TRs antibody (MA1-215) and labeled with an HRP-conjugated secondary antibody. (c) Histogram of average interwave period for each group of oocytes. *n* values in parentheses represent the total number of oocytes pooled from at least two frogs. Error bars correspond to the mean $\pm$ SEM. The asterisks (**) denote a statistic significant difference (ANOVA single factor, $P < 0.0001$).

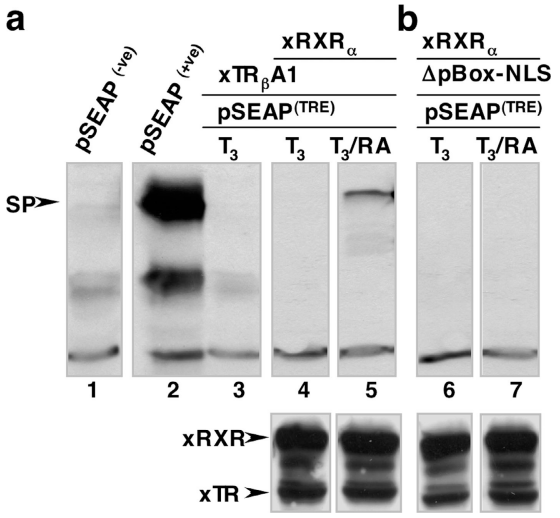

Figure 2. **Transcriptional activity of $TR_\beta A1$ requires $xRXR_\alpha$ and both cognate ligands.** Transcriptional activity was monitored with the TRE-reporter vector, $pSEAP^{(TRE)}$. (a) Lanes 1 and 2 are negative ($pSEAP^{(-ve)}$) and positive ($pSEAP^{(+ve)}$) vector controls. Oocytes expressing $TR_\beta A1$ or $TR_\beta A1$ plus $xRXR_\alpha$ were incubated with 100 nM T_3 (lanes 3–5) plus 100 nM RA (lane 5) for 3 d. Cytosolic extracts from each group of oocytes was prepared and loaded onto a 10% SDS-PAGE at 2.5 oocytes equivalents per lane. SEAP was detected with the polyclonal rabbit anti–human SEAP antibody and an HRP-conjugated secondary antibody. The SP labeled arrow indicates SEAP immunoreactivity, which was present only in oocytes expressing $TR_\beta A1$ and $xRXR_\alpha$ exposed to both T_3 and RA. (b) Transcriptional activity of $TR_\beta A1$ requires the pBOX within the DBD and the NLS. Oocytes expressing $xTR_\beta A1\Delta$pBox-NLS and $xRXR_\alpha$ show no SEAP immunoreactivity when incubated with T_3 (lane 6) or T_3 plus RA (lane 7). Western blot analysis shows that $xRXR_\alpha$, $TR_\beta A1$, and $xTR_\beta A1\Delta$pBox-NLS are expressed at comparable levels (Western blots below lanes 4–7). $TR_\beta A1$ and $xTR_\beta A1\Delta$pBox-NLS were detected with the monoclonal mouse anti–human TRs antibody (MA1-215). $xRXR_\alpha$ was detected with a polyclonal rabbit anti–human RXR antibody (Sc-774).

Ca^{2+} interwave period (6.38 ± 0.34 s, $n = 27$, Fig. 1, a and c). Similarly, $xTR_\beta A1$ expressing oocytes without T_3 treatment exhibited no change in Ca^{2+} wave periodicity (6.97 ± 0.24 s, $n = 35$). Peak Ca^{2+} wave amplitudes (ΔF/F) for $xTR_\beta A1$-expressing oocytes exposed to T_3 (0.69 ± 0.04, $n = 29$) was also significantly higher than that of control oocytes exposed to T_3 (0.55 ± 0.04, $n = 20$; $P < 0.05$, t test). We conclude from these data that T_3-stimulated $xTR_\beta A1$ acutely modulates IP_3-mediated Ca^{2+} wave activity and that both thyroid hormone and receptor expression are required for these effects.

Transcriptional activity of $TR_\beta A1$ is undetectable in the absence of xRXR

Classically, activated thyroid hormone receptors heterodimerize to initiate transcription responses. Retinoid X receptor (RXR) is the most common dimerization partner that binds to the thyroid hormone response element (TRE; Leid et al., 1992; Bhat et al., 1994; Wong and Shi, 1995). To investigate the transcriptional activity of $xTR_\beta A1$, we coinjected oocytes with $xTR_\beta A1$ mRNA and a plasmid reporting vector containing a TRE system with two direct repeats (DR4) upstream of the secreted placental alkaline phosphatase (SEAP) gene (p-TRE-SEAP; CLONTECH Laboratories, Inc.). If the hormone receptor dimerizes and binds to the TRE enhancer, the oocyte expresses SEAP, which is secreted into the medium. mRNA-injected oocytes were continuously bathed in T_3 (100 nM) for 3 d and the presence of SEAP was subsequently quantified by Western blot analysis and used as a marker for transcriptional activity. Using this TRE-reporting system, we observed no transcriptional activity in oocytes expressing the $xTR_\beta A1$ protein by itself (Fig. 2 a, lane 3). However, when we coexpressed $xRXR_\alpha$ with $xTR_\beta A1$ and oocytes were incubated with T_3 (100 nM) and 9-cis retinoic acid (RA; 100 nM) for 3 d, SEAP expression was significantly increased (Fig. 2 a, lane 5). Note that $xTR_\beta A1/xRXR_\alpha$-mediated transcription requires both ligands, T_3 and RA (Fig. 2 a, lanes 4 and 5). These data indicate that stimulation of $xTR_\beta A1$ by T_3 does not initiate detectable transcription in *Xenopus* oocytes.

Acute modulation of Ca^{2+} signaling does not require heterodimerization with RXR

To test whether heterodimerization of $xTR_\beta A1$ with $xRXR_\alpha$ affects the acute modulation of Ca^{2+} activity, we coinjected oocytes with both $xRXR_\alpha$ and $xTR_\beta A1$ mRNA and confirmed protein expression levels using Western analysis 2–3 d after injection of mRNA (Fig. 3 b). Oocytes were loaded with Ca^{2+} indicator dye and confocally imaged. Oocytes coexpressing $xTR_\beta A1$ and

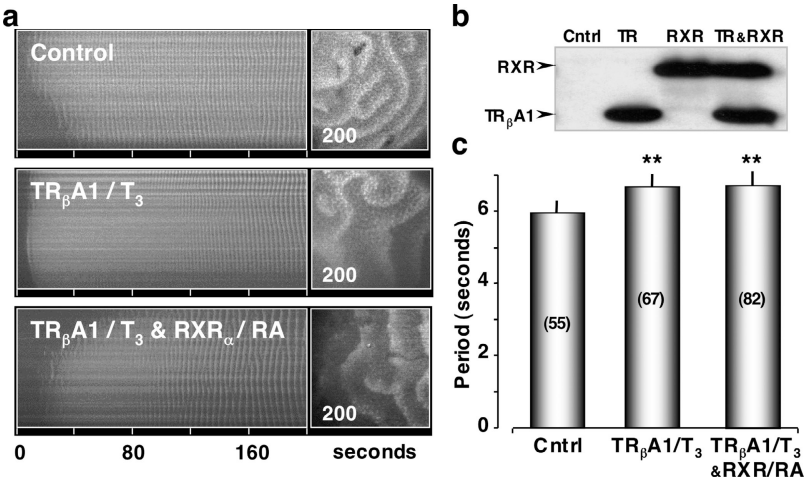

Figure 3. **Acute modulation of Ca^{2+} signaling does not require heterodimerization of $TR_\beta A1$ with $xRXR_\alpha$.** (a) Spatial-temporal stacks of IP_3-induced Ca^{2+} wave activity in control oocytes compared with oocytes expressing $TR_\beta A1$ or $TR_\beta A1$ with $xRXR_\alpha$. T_3 (100 nM) and RA (100 nM) were added as indicated 10–15 min before injection with IP_3 (~300 nM). Scale is the same as Fig. 1. (b) Western blots of oocytes expressing $TR_\beta A1$ and $xRXR_\alpha$. Primary and secondary antibodies were identical to those used in Figs. 1 and 2. (c). Histogram of average interwave period (second) of each group of oocytes. The asterisks (**) denote a statistic significance using ANOVA single factor ($P < 0.0001$). Values in parentheses represent the number of oocytes.

$xRXR_\alpha$ were initially exposed to both T_3 (100 nM) and RA (100 nM) 10 min before injection with IP_3 (~300 nM). The average Ca^{2+} interwave period for $xTR_\beta A1$-expressing oocytes was 6.58 ± 0.26 s ($n = 67$), whereas that of $xRXR_\alpha/xTR_\beta A1$ coexpressing oocytes was 6.72 ± 0.31 s ($n = 82$; Fig. 3, a and c). These values were not significantly different from each other ($P = 0.22$), but were both significantly larger than values in the control oocytes that exhibited an average Ca^{2+} interwave period of 5.90 ± 0.43 s ($n = 55$, ANOVA single factor, $P < 0.0001$; Fig. 3, a and c). We conclude that the $xRXR_\alpha$ coexpression does not affect the ability of T_3-bound $xTR_\beta A1$ to modulate Ca^{2+} signaling.

The DNA binding domain and NLS of $TR_\beta A1$ are not required for acute affects on Ca^{2+} signaling

The ability of T_3-bound $xTR_\beta A1$ to rapidly modulate Ca^{2+} activity suggested a nontranscriptional mechanism of action. Our strategy to test this hypothesis was to delete the DNA binding domain (DBD) and mutate the NLS from the thyroid hormone receptor and test whether the mutant receptors were (a) transcriptionally inactive and (b) still effective at modulating Ca^{2+} signaling. Oocytes were injected with the p-TRE-SEAP plasmid reporting vector. This reporting system requires heterodimerization of $xRXR_\alpha$ and $xTR_\beta A1$ to transactivate the reporter gene (Fig. 2 a). Consequently, test oocytes were coinjected with xRXR mRNA with mRNA encoding either wild-type $xTR_\beta A1$ (control), mutant $xTR_\beta A1$ lacking the NLS ($xTR_\beta A1$-ΔNLS), or the mutant lacking both the NLS and the pBOX ($xTR_\beta A1$ΔpBox-NLS). Once injected, oocytes were continuously bathed in T_3 (100 nM) and RA (100 nM) for 3 d. Expression levels of $xTR_\beta A1$ mutants and $xRXR_\alpha$ groups were comparable to $xTR_\beta A1$ and $xRXR_\alpha$ groups (Fig. 2 b, bottom). Using the expression of SEAP as a marker for transcriptional activity, we confirmed that oocytes expressing the $xTR_\beta A1$ mutants and $xRXR_\alpha$ proteins were transcriptionally inactive, whereas oocytes expressing wild-type $xTR_\beta A1$ and $xRXR_\alpha$ proteins exhibited strong transcriptional activity (Fig. 2, lanes 7 and 5).

Subsequently, we tested whether the transcriptionally inactive $xTR_\beta A1$ mutants could still acutely regulate Ca^{2+} signaling. Oocytes were injected with $xTR_\beta A1$ mRNA or its mutants and protein expression levels were confirmed using Western analysis 2–3 d after injection (Fig. 4 c). Oocytes expressing $xTR_\beta A1$ or the mutants were exposed to T_3 (100 nM) 10 min before injection with IP_3 (~300 nM). Ca^{2+} activity was confocally imaged, as described above. The average Ca^{2+} interwave period for the control group (water-injected oocytes) was 6.6 ± 0.20 s ($n = 70$), which was significantly shorter than that in the $xTR_\beta A1$ expressing oocytes (8.40 ± 0.30 s, $n = 40$; ANOVA single factor, $P < 0.0001$; Fig. 4 b, d; Fig. 1. More importantly, regulation of the Ca^{2+} wave period in oocytes expressing either the single mutant $xTR_\beta A1$-ΔNLS (9.6 ± 0.48 s, $n = 24$) or the double mutant, $xTR_\beta A1$ΔpBox-NLS (8.4 ± 0.28 s; $n = 24$) was indistinguishable from oocytes expressing wild-type $xTR_\beta A1$ (Fig. 4, b and d). We conclude from these data that neither the pBOX nor the NLS of $TR_\beta A1$ is required for acute regulation of Ca^{2+} signaling.

T_3-bound $TR_\beta A1$ appears to regulate Ca^{2+} signaling by increasing mitochondrial respiration

We reported previously that pyruvate/malate-energized mitochondria increase the amplitude and interwave period of IP_3-induced Ca^{2+} waves in *Xenopus* oocytes (Jouaville et al., 1995). These effects on Ca^{2+} wave activity were similar to those observed in $TR_\beta A1$ overexpressing oocytes with acute T_3 incubation (Fig. 1). Sterling and colleagues (Sterling, 1980) initially reported that T_3 increases mitochondrial metabolism, particularly oxidative phosphorylation, in less than 30 min. Consequently, we hypothesized that the regulation of Ca^{2+} signaling by T_3-activated $xTR_\beta A1$ was mediated by its acute modulation of mitochondrial metabolism, which, in turn, increased mitochondrial Ca^{2+} uptake. Our strategy to test this hypothesis was threefold. First, we examined the effect of T_3 on $\Delta\Psi$ in $TR_\beta A1$ expressing oocytes using the potential sensitive dye tetramethylrhodamine ethyl ester (TMRE). Oocytes were bathed in 200 nM TMRE for 5 min before imaging fluorescence with two-

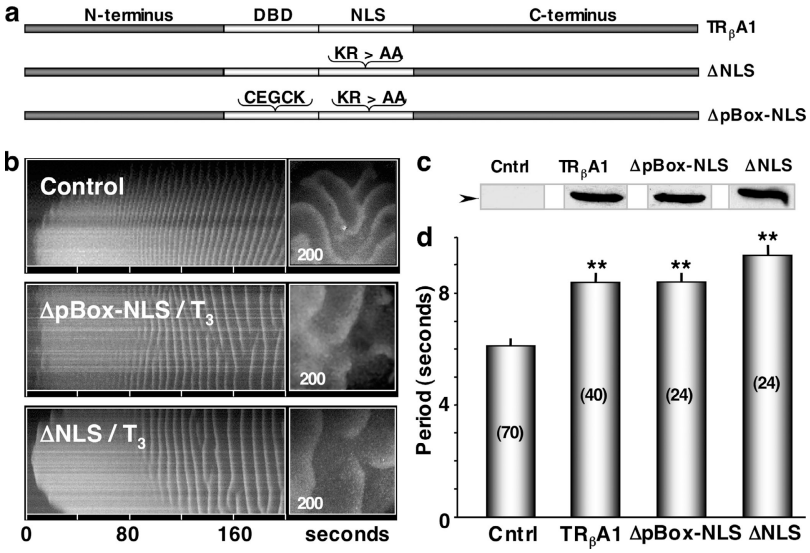

Figure 4. **The pBOX and NLSs of $TR_\beta A1$ are not required for the acute regulation of Ca^{2+} signaling.** (a) Schematic figure depicting the position of the pBOX deletion in the DBD and the NLS modification within $TR_\beta A1$. (b) Spatial-temporal stack of IP_3-induced Ca^{2+} wave activity in control oocytes compared with oocytes expressing TR mutants ΔpBox-NLS and ΔNLS. Oocytes expressing the TR mutants were incubated with T_3 (100 nM) 10–15 min before IP_3 (~300 nM) injections. (c) Western blot analysis confirming comparable levels of protein expression for both wild-type and mutant $TR_\beta A1$. (d) Histogram of the average Ca^{2+} wave periods for each group of oocytes (*n* values are in parentheses). Statistic significance over control oocytes is indicated by the asterisks (**; ANOVA single factor, $P < 0.0001$).

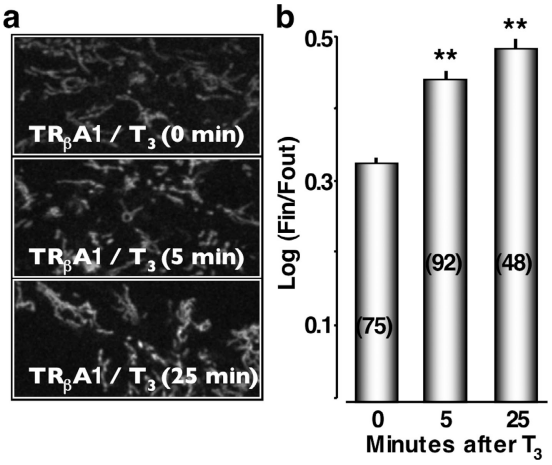

Figure 5. **T_3 stimulation of oocytes expressing $TR_\beta A1$ increases the $\Delta\Psi$.** (a) Images of mitochondria labeled with the potential sensitive dye TMRE. The oocytes are expressing $TR_\beta A1$ and have been exposed to T_3 for the indicated amount of time. Images are 50 × 100 μm. (b) Histogram of the log of mitochondrial TMRE fluorescence (F_{mito}) divided by the cytosolic fluorescence (F_{cyto}) at the indicated times of T_3 exposure. Values in parentheses refers to the number of mitochondrion analyzed. Statistical significance is indicated by the asterisks (**; ANOVA single factor, $P < 0.001$).

photon excitation (800 nm). $\Delta\Psi$ was estimated by monitoring $\text{Log}(F_{mito}/F_{cyto})$ where F_{mito} is the fluorescence intensity of individual mitochondria and F_{cyto} is the cytosolic fluorescence (Farkas et al., 1989). We found that T_3 significantly increased $\Delta\Psi$ from a resting value of 0.33 ± 0.01 ($n = 75$) to 0.44 ± 0.01 ($n = 92$, $P < 0.0001$) at 5 min and to 0.48 ± 0.02 ($n = 48$, $P < 0.0001$) at 25 min (Fig. 5). These data suggest that T_3-bound $TR_\beta A1$ regulates Ca^{2+} signaling by increasing $\Delta\Psi$. Second, we injected a subgroup of the $TR_\beta A1$-expressing oocytes with ruthenium 360 (Ru_{360}; Calbiochem, ~1 μM final concentration), a polycation that inhibits the electrogenic mitochondrial Ca^{2+} uniporter (Ying et al., 1991) ~60 min before IP_3 injection and Ca^{2+} imaging. A control group of $TR_\beta A1$-expressing oocytes were injected with buffer only. We found that Ru_{360} treatment completely inhibited the affect of T_3-bound $TR_\beta A1$ on Ca^{2+} wave activity (Fig. 6). Untreated $TR_\beta A1$ expressing oocytes exhibited the expected increase in wave periodicity (7.65 ± 0.4 s, $n = 11$) when preexposed to T_3 for 10 min. However, the average wave period of Ru_{360}-treated $TR_\beta A1$ expressing oocytes was only 5.75 ± 0.22 s ($n = 13$) when preexposed to T_3. The Ru_{360}-treated average was nearly identical to untreated control oocytes (5.59 ± 0.04 s, $n = 4$) as well as Ru_{360}-treated nonexpressing oocytes (5.53 ± 0.4 s, $n = 3$). These data are consistent with the hypothesis that T_3-bound $TR_\beta A1$ regulates Ca^{2+} signaling by increasing mitochondrial Ca^{2+} uptake via an increase in $\Delta\Psi$.

Third, we directly test whether thyroid hormone receptor together with T_3 stimulates mitochondrial respiration. *Xenopus* oocytes were injected with $TR_\beta A1$ mRNA or water and incubated for 3 d. The rate of O_2 consumption was measured as an indicator of respiration. 200 oocytes in each group were loaded into a 2-ml O_2 probe chamber filled with modified barth's solution (MBS) solution. After 15 min of stabilization, the medium was exchanged with fresh MBS and O_2 consumption was monitored for 30 min. The medium was exchanged a third time with MBS containing 100 nM T_3 and O_2 consumption was followed for another 30 min (Fig. 7, a and b). After this protocol, the rate of O_2 consumption in water-injected oocytes after T_3 exposure was 0.42 ± 0.25 nmol/min ($n = 8$). In contrast, the rate of O_2 consumption in $TR_\beta A1$-injected oocytes after T_3 exposure was significantly increased to 1.68 ± 0.52 nmol/min ($n = 4$, $P < 0.05$). These data support the hypothesis that a T_3 / $TR_\beta A1$-mediated increase in mitochondrial respiration was responsible for the modulation of IP_3-mediated Ca^{2+} wave activity.

TRs targeted to the mitochondria are required for a T_3-stimulated increase in respiration and the regulation of Ca^{2+} signaling

T_3 treatment has previously been reported to increase mitochondrial metabolism (Sterling et al., 1980; Soboll, 1993a).

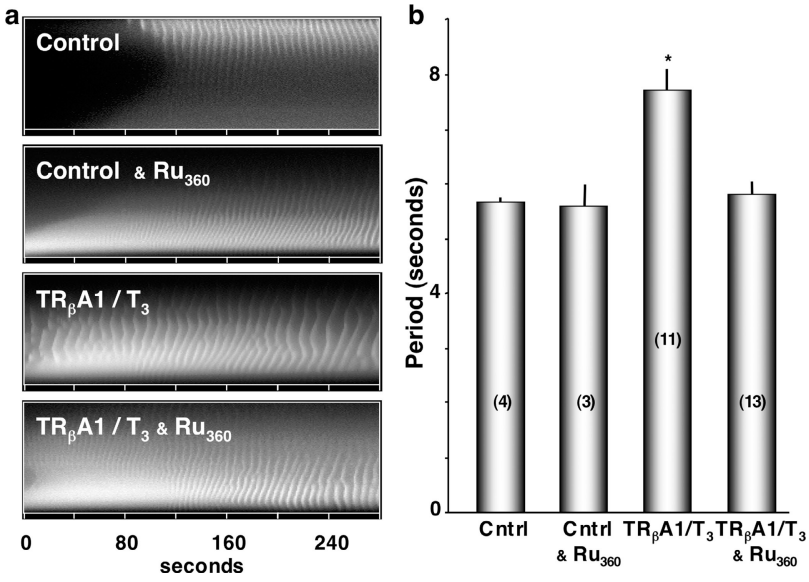

Figure 6. **Ru_{360} blocks T_3-bound $TR_\beta A1$ increases in IP_3-induced Ca^{2+} wave period.** (a) Spatial-temporal stacks of the effect of Ru360 treatment on Ca^{2+} wave activity in control oocytes compared with oocytes expressing $TR_\beta A1$as labeled. (b) Histogram of average interwave period (seconds) of each group of oocytes shown in a. The asterisk (*) denotes a statistic significance using ANOVA single factor ($P < 0.01$). Values in parentheses represent the number of oocytes.

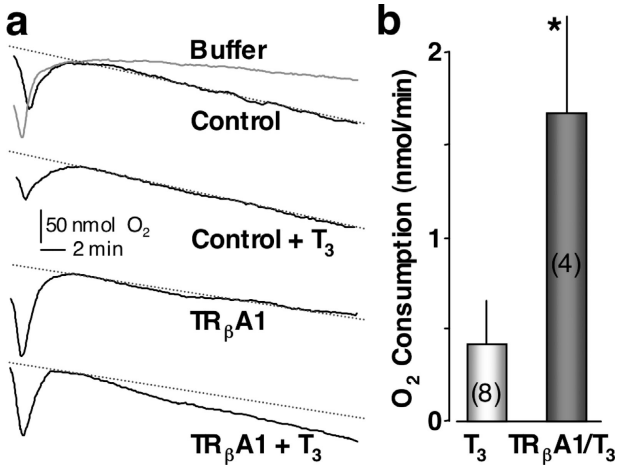

Figure 7. **T_3 stimulation of oocytes expressing $TR_\beta A1$ increases O_2 consumption.** (a) Plots of O_2 levels in oocytes as labeled ($n = 200$ oocytes per group). (b) Histogram represents average change of O_2 consumption rates (before and after T_3 exposure) in control and $xTR_\beta A1$ groups. Statistical significance is indicated by the asterisk (*; *t* test, $P < 0.05$).

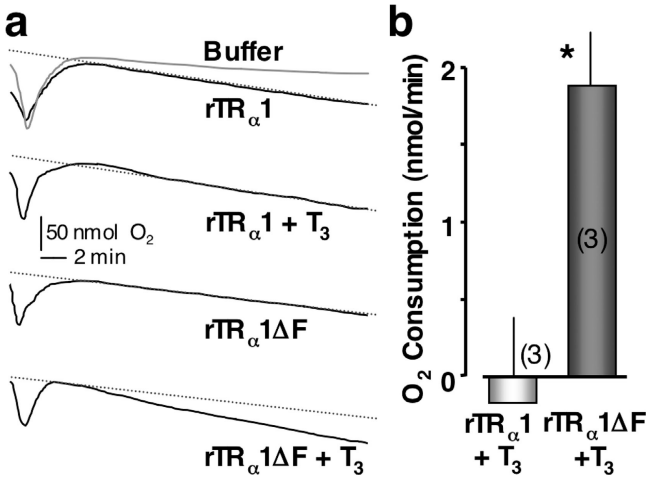

Figure 9. **NH_2-terminal truncated rat $TR_\alpha 1$ ($rTR_\alpha 1\Delta F$) stimulates O_2 consumption.** (a) Plots of O_2 levels for oocytes expressing full-length $rTR_\alpha 1$ with and without T_3 compared with oocytes expressing the NH_2-terminal truncated $rTR_\alpha 1\Delta F$ with or without T_3. Protocols used were identical to those described in Fig. 7. (b) Histogram of the average change of the O_2 consumption rates after T_3 exposure in $rTR_\alpha 1$ versus $rTR_\alpha 1\Delta F$ groups. The asterisk (*) indicates statistical significance (*t* test, $P < 0.05$).

Our data suggest that the acute effects of T_3 on mitochondrial metabolism are likely to be mediated by T_3-activated thyroid hormone receptors. A truncated form of rat $TR_\alpha 1$ ($rTR_\alpha 1\Delta F$) has been shown to localize to mitochondria matrix (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Furthermore, the NH_2 terminus of the *Xenopus* $TR_\beta A1$ that we used throughout this work has a high homology to the NH_2 terminus of $rTR_\alpha 1\Delta F$ (Fig. 8 a). Our strategy in this experiment was to test whether mitochondrial targeting of TRs was necessary to modulate Ca^{2+} signaling. First, we examined the cellular targeting of $xTR_\beta A1$, $rTR_\alpha 1$, and $rTR_\alpha 1\Delta F$ by injecting *Xenopus* oocytes with their respective mRNAs. After 3 d of expression, mitochondria were isolated by centrifugation. Whole oocyte extract (minus mitochondria) and mitochondrial extract from each group were subjected to immunoprecipitation using a TR antibody (MA1-215; Affinity BioReagents, Inc.). The immunocomplexes (TRs/MA1-215) were loaded onto a 10% SDS-PAGE gel for Western blot analysis. As shown in Fig. 8 c, only $xTR_\beta A1$ and $rTR_\alpha 1\Delta F$ were detected in the mitochondria extracts. Full-length $rTR_\alpha 1$ did not localize to mitochondria. These results are consistent with previous reports (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Our next step was to compare the rate of O_2 consumption for oocytes expressing either $rTR_\alpha 1$ or $rTR_\alpha 1\Delta F$ (Fig. 9). Consistent with its mitochondrial targeting, the rate of O_2 consumption in $rTR_\alpha 1\Delta F$ expressing oocytes after T_3 exposure was significantly increased 1.88 ± 0.35 nmol/min ($n = 3$, $P < 0.05$). In contrast, the rate of O_2 consumption in oocytes expressing the full-length $rTR_\alpha 1$ was not significantly affected by T_3 exposure (-0.16 ± 0.55, $n = 3$). We conclude that mitochondrial targeting of TRs is required for a T_3 mediated increase in mitochondrial respiration.

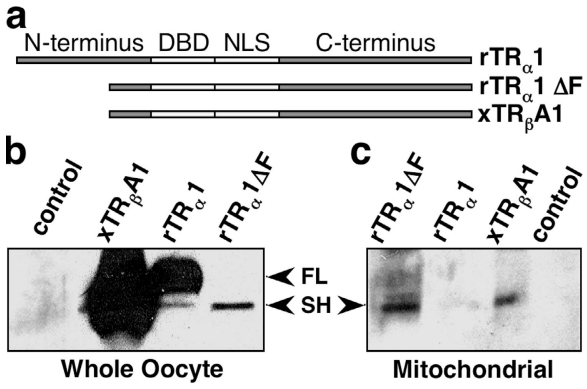

Figure 8. ***Xenopus* $TR_\beta A1$ and NH_2-terminal truncated rat $TR_\alpha 1$ ($rTR_\alpha 1\Delta F$) localize to mitochondria.** (a) Schematic diagram of TRs showing that $rTR_\alpha 1\Delta F$ and $xTR_\beta A1$ have a similar NH_2 terminus. (b and c) Western blots of $TR_\alpha 1$, $rTR_\alpha 1\Delta F$, and $xTR_\beta A1$ expression in whole oocytes and mitochondrial extracts respectively. FL, full-length receptor; SH, shortened form of the receptor. Extracts were prepared from 300 oocytes in each group. All oocytes were exposed to 100 nM T_3 for at least 15 min before organelle extraction. TRs were immunoprecipitated with a monoclonal mouse anti–human TRs antibody (MA1-215), captured with immobilized protein G, concentrated, and loaded onto a 10% SDS-PAGE. An HRP-conjugated secondary antibody was used for visualization.

Finally, we tested whether targeting of TRs to mitochondria was required to regulate Ca^{2+} signaling. As before, oocytes were injected with either full-length $rTR_\alpha 1$ or NH_2 terminus–truncated $rTR_\alpha 1\Delta F$ mRNAs. Protein expression levels were measured 2–3 d after injection (Fig. 10 b). Ca^{2+} activity was confocally imaged 10 min after treatment with T_3 (100 nM). We found that the average Ca^{2+} interwave period for $rTR_\alpha 1\Delta F$-injected oocytes was 8.8 ± 0.26 s ($n = 24$), which was significantly higher (ANOVA single factor, $P < 0.01$) than full-length $rTR_\alpha 1$-expressing oocytes (7.9 ± 0.38 s, $n = 22$) and the water-injected control group (7.2 ± 0.24 s; $n = 30$; Fig. 10, a and c). Together, these data strongly indicate that the regulation of Ca^{2+} signaling by T_3-activated TRs requires their localization within mitochondria.

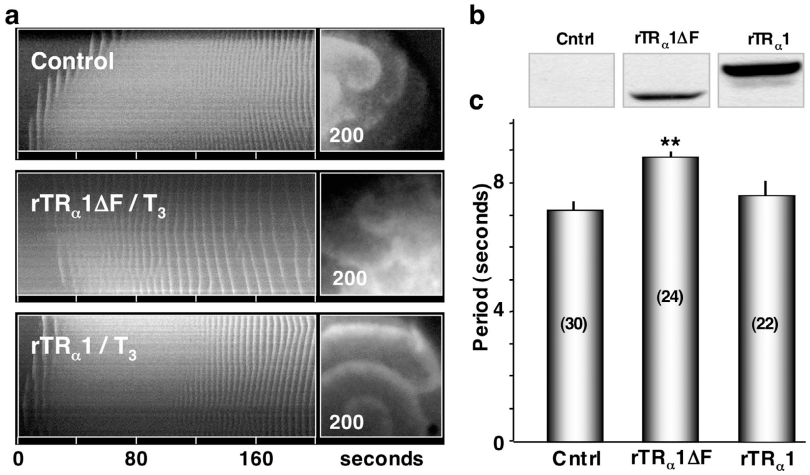

Figure 10. **The truncated rTR$_\alpha$1ΔF regulates intracellular Ca^{2+} release.** (a) Spatio-temporal stacks of IP$_3$-induced Ca^{2+} wave activity in control oocytes compared with oocytes expressing rTR$_\alpha$1ΔF or rTR$_\alpha$1ΔF. TR expressing oocytes were treated with 100 nM T$_3$ 10–15 min before IP$_3$ (~300 nM) injections and confocal imaging. (b) Western blots of rTR$_\alpha$1 and rTR$_\alpha$1ΔF expression levels in experimental oocytes. (c) Histogram of the average inter-wave periods for each group (*n* values in parentheses). Note that rTR$_\alpha$1ΔF has significantly longer periods even though its expression levels are lower than those of full-length rTR$_\alpha$1. The asterisks (**) indicate statistical significance with $P < 0.01$ using ANOVA single factor.

Discussion

In this work, we report that the acute exposure of oocytes expressing mitochondrially targeted TR to T$_3$ regulates IP$_3$-mediated Ca^{2+} wave activity. We observed a T$_3$-bound TR induced increase in the Ca^{2+} wave period and amplitude. These changes in Ca^{2+} activity were similar to those observed in *Xenopus* oocytes when mitochondria were energized with respiratory chain substrates (Jouaville et al., 1995). In that report, the modulation of IP$_3$-mediated Ca^{2+} release was due to an increase in mitochondrial Ca^{2+} uptake via an increase in the $\Delta\Psi$. Our current work is consistent with this model because we could inhibit the effects of T$_3$-bound TRs by inhibiting mitochondrial Ca^{2+} uptake with Ru$_{360}$. We also directly demonstrated that T$_3$ exposure increased $\Delta\Psi$ in oocytes expressing TRs. An increase in $\Delta\Psi$ could be attributed to either a direct effect on electron transport or to a decrease in proton leak (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994; Gunter et al., 1998). The application of T$_3$ to mitochondria has been reported to decrease proton leak in several preparations (Crespo-Armas and Mowbray, 1987; Soboll, 1993a). However, we found that T$_3$ exposure increases O$_2$ consumption in TR-expressing oocytes. An increase in the rate of O$_2$ consumption is not consistent with a decrease in proton leak. Together, our data favors the conclusion that T$_3$-bound TR regulates Ca^{2+} activity by increasing $\Delta\Psi$ via an increase in proton pumping by the respiratory chain.

Application of thyroid hormones to mitochondria has long been known to increase metabolism (Sterling, 1980). Mitochondria were also known to be target organelles of T$_3$ accumulation in cells (Sterling et al., 1984; Morel et al., 1996). However, a mitochondrial hormone receptor that mediated these effects has never been conclusively identified. Sterling (1986, 1991) initially suggested that the adenine nucleotide translocator (ANT) bound to T$_3$ with high affinity. Romani et al. (1996) also suggested that thyroid hormone had its specific mitochondrial target site at the matrix side of ANT. They found that bongkrekic acid, a membrane-permeant inhibitor of ANT, blocked a thyroid hormone-induced release of Mg^{2+} from mitochondria. On the other hand, Wrutniak and coworkers (Wrutniak-Cabello et al., 2001) found no evidence demonstrating a direct interaction between ANT and T$_3$. Our data indicate that ANT alone is not the thyroid hormone receptor that mediates the regulation of mitochondrial metabolism. Rather, our data reveal that a mitochondrial targeted TR is a required element of acute thyroid hormone regulation of metabolism. The use of *Xenopus* oocytes in these experiments was crucial in this determination because oocytes do not express endogenous TRs (Yaoita and Brown, 1990; Kawahara et al., 1991). The ubiquitous expression of endogenous TRs would have hidden this finding in earlier studies.

The ability of specific thyroid hormone receptors to target mitochondria has been demonstrated by other investigators. A truncated form of rat TR$_\alpha$1 (rTR$_\alpha$1ΔF) and not its full-length form, localized to the matrix of mitochondria (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Our work corroborated these reports and further demonstrated that the xTR$_\beta$A1, which is highly similar to rTR$_\alpha$1ΔF, targeted the mitochondria. Casas and coworkers (Casas et al., 1999) reported that mitochondrial activity was stimulated by overexpression of p43 (mitochondria-targeted, truncated-TR$_\alpha$), which in turn, stimulated mitochondrial genome transcription of some enzyme units that played a role in the respiratory chain. The p43 protein had the same affinity to T$_3$ as the full-length TR$_\alpha$ to bind to the D-loop of two mt-TREs in the mitochondria, leading to mitochondrial protein synthesis (Casas et al., 1999). Their data suggested that p43 bound to mt-TREs as a homodimer because no RXR-isoform in the mitochondrial extract was detected (Casas et al., 1999). Hadzic suggested that the NH$_2$ terminus of TRs plays a role in TR-homodimerization in mitochondria (Hadzic et al., 1998). Together, these studies demonstrated that mitochondrial-targeted TRs could regulate mitochondrial metabolism by initiating transcription. However, our results cannot be accounted for by this mechanism of action. Specifically, transcriptionally inactive TR mutants modulated Ca^{2+} wave activity with the same efficacy as the wild type, xTR$_\beta$A1. We confirmed that xRXR$_\alpha$ was required for xTR$_\beta$A1 to transactivate a reporter gene in our system, but more importantly, the presence of xRXR did not affect the ability of xTR$_\beta$A1 to modulate Ca^{2+} activity. Thus, we concluded that the mechanism by which T$_3$-activated TRs regulate Ca^{2+} signaling cannot be attributed to transcription.

Nongenomic effects of various steroid receptors have been reported for mineralocorticoids (Moura and Worcel, 1984; Zhou and Bubien, 2001), glucocorticoids (Borski, 2000; Borski et al., 2002), gonadal steroids (Pietras and Szego, 1975; Wasserman et al., 1980; Lieberherr and Grosse, 1994; Guo et al., 2002a,b; Minshall et al., 2002), vitamin D3 (Sergeev and Rhoten, 1995), and thyroid hormone (Hummerich and Soboll, 1989; Davis and Davis, 1996, 2002; Rojas et al., 2003). Most of these studies proposed the presence of specific membrane-bound receptors for nongenomic effects; however, specific receptors were not cloned or identified. For thyroid hormones in particular, Davis and Davis (2002) suggested that the mechanism of the nongenomic effects of thyroid hormone may not require TRs, and could involve actions of the hormone itself on signal transduction pathway via specific G protein–coupled protein. Recent work by Scanlan et al. (2004) identified an endogenous, rapid-acting derivative of thyroid hormone that is a potent agonist of the G protein-coupled trace amine receptor (TAR1). Activation of TAR1 increased cAMP production, which in turn, would active protein kinase A and phosphorylation of multiple proteins in cells. Our results do not exclude a potential role of second messenger systems in the mechanism of action of T_3 on mitochondria. Rather, they demonstrate that classic TRs, those that have long been known to regulate gene transcription, will also acutely regulate mitochondrial activity when bound with T_3. Stimulation is dependent on mitochondrial targeting of the TR, but not on its ability to initiate transcription. Together, these observations reveal a nontranscriptional pathway for modulation of intracellular Ca^{2+} signaling via T_3/TR-stimulated mitochondrial metabolism.

The discovery of T_3/TR-regulated Ca^{2+} signaling is potentially important for several reasons. First, any process that acutely regulates intracellular Ca^{2+} release will impact the multitude of Ca^{2+}-sensitive cellular processes ranging from contractility and secretion to proteolysis and cell death. Second, the ability of a steroid hormone to increase proton pumping provides a rapid method to increase metabolism in response to short-term energy requirements; for example, during increased neuronal activity or during a transient increase in muscle activity. Third, and potentially more importantly, a rapid increase in mitochondrial Ca^{2+} uptake could protect cells under conditions of stress. Mitochondria have long been recognized for their capacity to sequester large Ca^{2+} concentrations under pathological conditions (Gunter et al., 1994). The ability to transiently remove Ca^{2+} from the cytosol could be used to minimize tissue damage after stroke in neuronal tissue or to reduce the instability of cardiac cells after periods of hypoxia. Clearly, the identification of a mitochondrial receptor for thyroid hormone-induced increases in metabolism offers a new pharmacological target from which it will be possible to regulate a broad range of physiological and pathological processes.

Materials and methods

Expression vector construction

The coding fragments of rat $TR_\alpha1$ cDNA, *Xenopus* RXR_α (a gift from R.M. Evans, The Howard Hughes Medical Institute, Chevy Chase, MD, and The Salk Institute for Biological Studies, La Jolla, CA) were amplified by PCR with a forward primer 5′-acgtggatccatggaacagaagccaagcaaggtg-3′ (for $rTR_\alpha1$), 5′-acgtggatccatgagttcagcagccatggacaccaaacat-3′ (for $xRXR_\alpha$) containing a BamHI site, and a reverse primer, 5′-atcgaagcttttagacttcctgatcctcaaagacctc-3′ (for $rTR_\alpha1$), 5′-atcgaagcttttaagtcatttggtgaggggcttccagcat-3′ (for $xRXR_\alpha$) containing a HinDIII site. The PCR products were then subcloned into the *Xenopus* oocyte expression vector, pGEM-HeNot between the BamHI and HinDIII sites (Camacho and Lechleiter, 1995). All restriction enzymes were purchased from Life Technologies. The truncated form of rTR_α· ($rTR_\alpha1\Delta F$) was generated by PCR at the second ORF of $rTR_\alpha1$ cDNA using a forward primer, 5′-acgtggatccatgtcagggtatatccctagttacc-3′ containing a BamHI site, and a reverse primer, 5′-atcgaagcttttagacttcctgatcctcaaagacctc-3′ containing a HinDIII site. The PCR product was placed into the pGEM-HeNot vector between the BamHI and HinDIII sites.

Xenopus $TR_\beta A1$ was amplified by PCR with primers 5′-gctaggatccatggaagggtatatacccagctacttgg-3′ and 5′-atcgaagcttctagtcctcaaacacttccaagaacagtggggg-3′ and subcloned into vector pGEM-HeNot between the BamHI and HinDIII sites to create pGEM-HeNot-$xTR_\beta A1$. *Xenopus* mutant $xTR_\beta A1$-ΔNLS had its NLS removed by modifying the sequence from KR to AA. $xTR_\beta A1\Delta$pBox-NLS had the same NLS modification as well as the pBOX deletion of CEGCK within the DBD. Both mutants were generated by QuikChange site-directed mutagenesis (Stratagene) using pGEM-HeNot-$xTR_\beta A1$ as a template. The forward primer for $xTR_\beta A1$NLS was 5′-ggttttggatgacaacgcagctttggcaaaaagaaagc-3′ and the reverse complement primer was 5′-gctttcttttttgccaaagctgcgttgtcatccaaaacc-3′. For the double mutation, pGEM-HeNot-$xTR_\beta A1$NLS was used as a template. The forward primer for $xTR_\beta A1\Delta$pBox-NLS was 5′-gggtatcattatagatgtatcaccggctttttttagaagaactattcag-3′ and the reverse compliment primer was 5′-ctgaatagttcttctaaaaaagccggtgatacatctataatgataccc-3′. All mutations were confirmed by nucleotide sequencing (UTHSCSA DNA core facility).

In vitro transcriptions and oocyte protocols

Synthetic mRNA was prepared as described previously (Camacho and Lechleiter, 1995). In brief, the pGEM-HeNot vector containing cDNA template was linearized by a NotI restriction enzyme. From the linearized templates, mRNA was generated using the T7 promoter (MEGAscript; Ambion). Cap analogue, $m^7G(5')ppp(5'')$ (Ambion) was added to the reaction. The mRNA products were quantified by 1% agarose gel and spectrophotometry. RNase-free synthetic RNAs were resuspended at a concentration of 1.5–2.0 μg/μl and stored in aliquots of 3 μl at –80°C.

Stage VI oocytes were obtained from adult female *Xenopus laevis*. After defolliculation, oocytes were incubated in MBS (in mM: 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 0.82 $MgSO_4$, 2.40 $NaHCO_3$, 10 Hepes, pH 7.5) at 18°C. mRNA was injected into the oocytes by a 50-nL bolus using a positive pressure injector (Nanoject; Drummond Scientific Co.). Control oocytes were injected with diethyl pyrocarbonate–treated water. Oocytes were incubated at 18°C for 2–3 d to allow full expression of proteins in MBS supplemented with antibiotics streptomycin, penicillin, and fungizone (GIBCO-BRL). Media was changed daily. Unhealthy oocytes were discarded daily.

Imaging acquisition and analysis

Ca^{2+} wave activity was imaged as described previously (Camacho and Lechleiter, 1995). In brief, oocytes were injected with 50 nl of a fluorescent Ca^{2+} sensitive dye (0.25 mM, Oregon green BAPTA2-cell impermeant; Molecular Probes) and incubated for 30–60 min before the experiment. Images were acquired with a confocal laser-scanning microscope (model PCM2000; Nikon) attached to an inverted microscope (model TE200; Nikon) at the rate of 1.5 images/s. We used a 10× 0.45 NA objective (UVFLUOR; Nikon). Each group of mRNA-injected oocytes was randomly assigned into two subgroups, one was exposed to 100 nM of T_3 for 10 min and the other was untreated with T_3. Ca^{2+} wave activity was initiated by injecting a 50-nl bolus of 6 μM IP_3. The Ca^{2+} waves were analyzed with ANALYZE software (The Mayo Foundation, Rochester, MN). Statistical significance was calculated by either one-factor ANOVA or a *t* test as indicated.

$\Delta\Psi$ was estimated as described previously (Lin and Lechleiter, 2002). In brief, 200 nM TMRE (Molecular Probes) was added to the bath and images were acquired with a 60× 1.4 NA objective on the Nikon PCM2000 custom adapted for two-photon imaging. TMRE was excited at 800 nm using a Ti-sapphire Coherent Mira 900 Laser pumped with a 5W Verdi laser (Coherent Inc.). Laser intensity was attenuated with a neutral-density filter wheel such that no detectable photobleaching of TMRE was observed.

Transcriptional activity assay

The transcriptional activity of TR and mutants were confirmed by using a reporting vector with the thyroid response element (TRE) as a cis-acting en-

hancer for the SEAP gene (Mercury Pathway Profiling SEAP System2; CLONTECH Laboratories, Inc.). The negative control vector (pSEAP$^{(-ve)}$) lacks the enhancer element, but contains a promoter and SEAP reporter gene. Oocytes in each group were injected with mRNA (0.5 μg) and vector (0.5 μg) as designated, and incubated in 1 ml MBS with 100 nM T_3, and/or RA for 3 d. Media was collected and replaced every 24 h for 3 d. Collected media from each group was pooled, concentrated (Amicon ultra 10000 MWCO; Millipore) to 40 μl and run on a 10% SDS-PAGE. Oocyte cytosolic extract from each group was prepared and loaded onto 10% SDS-PAGE at amounts equivalent to 2.5 oocytes per lane. SEAP was detected with polyclonal rabbit anti–human SEAP antibody (Zymed Laboratories, Inc.). HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc.) was used and visualized by chemiluminescence (PerkinElmer).

Western blot analysis

Oocytes were washed twice times in homogenization buffer (in mM: 15 Tris-HCl, 140 NaCl, 250 sucrose, 1% Triton X-100, Complete protease inhibitor cocktail) at a concentration of 40 μl/oocyte. Washed oocytes were homogenized and centrifuged at 4,500 *g* for 15 min at 4°C. The supernatant was collected and loaded at 0.5 oocytes per lane onto 10% SDS-PAGE. TRs and mutants were detected with monoclonal mouse anti–human TRs antibody (MA1-215; Affinity BioReagents, Inc.). $xRXR_\alpha$ was detected with polyclonal rabbit anti–human RXRs antibody (Sc-774; Santa Cruz Biotechnology, Inc.). HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc.) was used and visualized by chemiluminescence (PerkinElmer).

Cytosolic and mitochondrial extract preparations

300 oocytes in each group (water, $xTR_\beta A1$, $rTR_\alpha 1$, $rTR_\alpha 1\Delta F$) were allowed to express for 3 d and then treated with 100 nM T_3 for 15 min at RT. Oocytes were washed twice times with buffer A (in mM: 190 sorbitol, 1 $CaCl_2$, 10 TES, pH 7.4) and resuspended in buffer A at a final volume of 500 μl. Oocytes were sequentially homogenized with a hand-held homogenizer and centrifuged at 1,000 *g* for 5 min at 4°C. The supernatant was transferred to new tube and centrifuged at 14,000 *g* for 15 min at 4°C. Supernatant and pellet were collected separately. The pellet, which contained mitochondria, was washed several times with buffer B (in mM: 195 sorbitol, 5 EDTA, 5 TES, pH 7.4) and spun at 1,000 *g* for 5 min at 4°C to eliminate contaminants. The mitochondrial portion was finally obtained by centrifugation at 14,000 *g* for 15 min at 4°C. Mitochondria in each group were washed twice by resuspending in buffer B, centrifuged again at 14,000 *g* for 15 min at 4°C and lysed in the presence of 1% Triton X-100. The cytosolic fraction was centrifuged at 100,000 *g* for 15 min at 4°C to eliminate contaminating membranes.

O_2 consumption assay

A biological O_2 monitor (model 5300; YSI Inc.) was used to measure O_2 consumption. 200 oocytes in each group were loaded into a 2-ml O_2 probe chamber avoiding contact of the oocytes with the O_2 probe. 1.5 ml of MBS was added to the chamber and the system was allowed to stabilize for 15 min. The medium was subsequently exchanged with 1.25 ml of fresh MBS solution and O_2 consumption was monitored for 30 min. The media was exchanged again with MBS containing 100 nM T_3 and O_2 consumption was followed for the next 30 min. The slope of O_2 levels was calculated before and after the addition of T_3.

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